ENCYCLOPEDIA OF BIOCHEMISTRY

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By
SANADA CHATTERJEE

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DISCOVERY PUBLISHING HOUSE PVT. LTD.
NEW DELHI-110 002

PREFACE

It is always an ultimate outlook to give students the best information in a good package. So I took up the job of writing a series of books in chemistry starting from a very introductory level to this advance level of biochemistry.

Biochemistry, to express something about it for me would be difficult in one word, it should be one's part of life; the reactions of protein, amino-acids the DNA RNA double helix pattern. The nynhydrin reactions are all the wonders of biochemistry - man's anato-biochemical linkages.

Students who want to become doctors or medical practitioners must be a good biochemist first because biochemistry is the theory behind and the medicine is the practical application.

The effects and side effects of the particular medicine whether it is allergic or non-allergic to the patients are well understood by the doctors but are taught in biochemistry.

The chemical functions of the different human organs and systems; the kidney, the respiratory system, the blood, the chemical functions of hormones vitamins, the liver etc are all well covered by this book.

The subject of this book is therefore is deeply intended for those upcoming doctors who keep themselves for the real service of the mankind.

The object of this book is not only meant for the doctors but it is the last bangle of that long garland which has started all along from the introduction chapter of The Architecture of Chemistry, The Man's scientific attitude.

Biochemistry also comes in use in the field of agriculture the biochemical configuration of the soil. The biochemical configuration of the plants to be cultivated, the application of the pesticides, and the relation of the pesticides with the human population are well studied under biochemistry.

The Agricultural Scientist, the Pathological Scientist, Forensic Scientist, is definitely inter-joined with each other by a single buffer the Biochemistry.

This book is dedicated to those promising scientific readers and interested students of biochemistry and advanced chemical science, who wants to make a fruitful attempt in the field of modern scientific world.

The author will be great full and surely thankful to see the best utilization of this book in any of the field of application.

Sananda Chatterjee

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Chapter 1

General Review

SECTION 1.1—ORIGIN OF LIFE

SUBSECTION 1.1A—MOLECULAR LOGIC OF LIVING MATTERS

Marco Polo describes a bridge, stone by stone
"But which is the stone that supports the arch?" Kublai khan asks
"This bridge is not supported by one stone or another, "– Marco Polo answers
But by the line of the arch that they form"
Kublai khan remains silent, reflecti`ng. Then he adds "why do you speak
To me of the stones? It is only the arch that matters to me"
Polo answers "without stone there is no arch"

—*Italo Calvino*

ORIGIN OF LIFE

Molecular Logic of Living Matters

When biochemists set out to tackle a problem, our first step is commonly grind the intricate fabric of sells and tissues into a pulp (a homogenate). This is a significant action, representing a drastic reduction in the level of organization. It allows us to treat living matter as a mixture of chemical and encourages us to isolate and also purify individual constituents. Every student of biochemistry is warned not to misuse clean thinking on dirty enzymes! To be sure, something is sacrificed by this destructive procedure–not only life itself, but all the special order that impresses any one who inspects a photomicrograph.

But never mind: biochemists still cherish the premise that nothing irretrievable is lost by homogenization; and that given the macromolecules, all the essentials are present and accounted for. We know quite well that this cannot be true, but the focus on the molecules defines that layer of knowledge that we designate as biochemistry molecular biology, and undergirds our professional identity.

Let us for the present set aside the levels of order lost to the tissue grinder, and celebrate the astonishing achievements that grew from the meticulous examination of life' s fragments. All the activities of living things are carried out by molecules, and are thus ultimately rooted in molecular structures and interactions. Without subscribing to the lop-sided view that, therefore, all of biology is molecular biology, it must still be said that one cannot reflect usefully on the phenomenon of life without taking account of its material basis. There can be no arch without stones. Besides the molecular 1evel provides an excellent introduction to the exploration of biological order in general. Regularity, purpose, and complexity pervade the field. We can observe individual molecules coming together to perform novel and emergent functions, each of them a whole larger than the sum of its parts. We can discern generalizations that apply to all organisms on earth; they qualify locally as laws of biology , and assure us that all life is of one kind. We also find variations on all but the most basic themes; these report the roles of mutation and selection, constraints and contingency, in the genesis of living order. And we can ponder the deep question, what might we expect to find, if and when we see life beyond the solar system.

Biochemists and molecular biologists revel in the details of their subject; the key to finding order in the profusion is the concept *of function.* "Living organisms are composed of lifeless molecules the late Albert Lehninger's proclaims on the opening page of his classic textbook, but those molecules are special. The molecules of life differ from those encountered in the inorganic world" not in their chemical qualities, but in their biological ones: with, few exceptions such as waste products each perform a job in the service of the organism as a whole. The notion of function is meaningless when applied to the constituents of clay or of petroleum for those molecules are the products of physical and chemical forces alone but function becomes crucial when we ask why leaves are green and blood is red. Function implies purpose, and therefore, order. "The molecules of which living things are composed conform to all the familiar laws of chemistry, bur they also interact with each other in accordance with another set of principles, which we shall refer to collectively as the *molecular logic of living matter*. In this chapter we will be walking in Lehninger's trail most of its factual content is drawn from the text book of biochemistry and also from the students of biochemistry to the general reader by underscoring the principles and omitting the details; some of the latter will be found in Boyce Rensberger's useful book (3), which is specifically addressed to a lay audience.

For the purposes of this chapter, we may think of a cell as an intricate and sophisticated chemical factory (Fig. 1.1). Matter, energy and infor-mation enter the cell from the environment while waste products and heat are discharged. The object of the entire exercise is to replicate the chemical composition and organization of the original cell, making two cells grow where there was one before. Even in the

simplest cells, this calls for the collaborative interactions of many thousands of molecules large and small, and requires hundreds of concurrent chemical reactions. These breakdown foodstuffs extract energy manufacture precursors. Assemble constituents note and execute generic instructions and keep all this frantic activity coordinated. The term "metabolism" designates the sum total of all these chemical processes derived from the Greek word for exchange." Biochemistry, then, is the study of the chemical basis of all biological activity. My purpose here is of necessity much more modest: to introduce the reader to terms, discoveries and ideas that are indispensable for closer reflection on the nature of life.
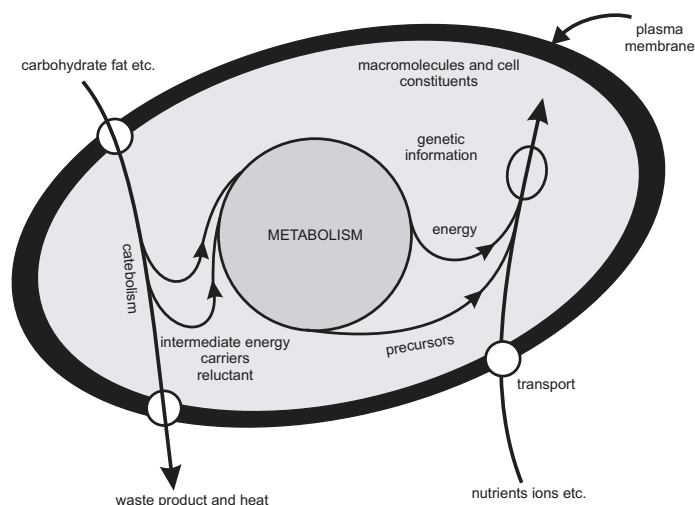
Fig. 1.1 : Showing the cell as a chemical factory

A beginner, on first encountering the profusion of chemical reactions that take place in every cell is apt to suspect that any reaction that can possibly go, does go. Closer inspection do correct the false impression: in reality, cellular metabolism is highly selective and quite purposeful Each reaction is mediated by a particular enzyme whose function is enable that reaction to proceed at a high rate, often with extreme specificity and with minimal formation of useless by-products. Step by simple step, the cell's complement of enzymes breaks down foodstuffs turns them into metabolites and then into cell constituents, and harnesses the energy of some reactions drive others (Fig. 1.2). Enzymes select the channels through which matter and energy flow. They can be studied as single molecules and often are, but they derive meaning from being parts of a larger whole, the metabolic web.

How enzymes perform theircatalyt1c feats, greater by many orders of magnitude than those of inorganic catalysts, has long been one of the central questions in biochemistry. The heart of the matter is the specific, intimate, and tight binding of the substrate (or substrates) to the enzyme Proteins (and virtually all enzymes are proteins) are not commonly entails changes in the configuration of both substrate and enzyme, inducing stresses and strains that contribute to the mechanism of catalysis. Besides, the catalytic site supplies chemically active groups in the form of amino add side-chains that actually participate in the reaction. The catalytic site is tailored, as it were to its particular task, linking its structure to its function.

The genome of *E. coli* encodes approximately 4.000 proteins that of yeast 6.000; it takes 50.000 proteins or more to make a man. What do they all do? Many proteins are enzymes but by no means all. Some proteins serve as the building blocks of structural scaffolding. Some make tracks for the movement of organelles itself mediated by motor proteins. Proteins act as receptors for signals from within the cell or from the outer world; the)' transport nutrients, waste products and viruses across membranes. Proteins also commonly modulate the activities of other proteins, or of genes. The general principle is that except for the storage and transmission of generic information and the construction of compartments, almost alt that cells do is done by proteins.

The explanation for the function of proteins is not chemical so much as physical. Amino add chains can fold into a variety of shapes globular and fibrous, each determined by the sequence of the amino acids that make up the protein in question. As they fold, each generates a unique contour with its own pattern of structural features: rods and hinges, platforms and channels, holes and crevices. Moreover proteins arc flexible and dynamic constructs that commonly change shape when they interact with legends or with each other. The range of stable configurations that amino acid chains can assume is wider than that of other classes of macromolecules, nucleic acids in particular; and their flexibility permits all sorts of mechanical actions demanded of molecular machines.

Proteins, as catalyses and structural elements are part of biochemical tradition; more recently we have come to see many of them as mechanical devices char rely on energized motion to perform their tasks. Even enzymes can be profitably looked at from this point of view: with the growing catalogue of enzyme structures has come the recognition that active sites and their elements commonly undergo rearrangement as part of the catalytic cycle and its regulation. Other proteins are there to bring about overt movement, either of molecules or of larger objects. Transport carriers reorient the binding site from one membrane surface to the other, and back again; sometimes the mechanical cycle is coupled to an energy source, turning the carrier into a pump. Students of eukaryotic cells are finding ever more motor proteins that trans locate vesicles, chromosomes, or elements of the cytoskeleton from one place to another. The most familiar example is myosin whose cyclic change of conformations underlies muscle contraction and some instances of cell motility. And bear in mind ribosome and polymerizes that transcribe and replicate genetic information: energized movements are central to their operations. As we unravel the molecular workings of life, the cell presents itself an assemblage of tiny machines; mundane mechanical engineering looms as large as the subtle flow of the energy and information.

Few generalizations in biology get by without qualification, and that applies to the status of proteins as the tools for all tasks: surprisingly, some tasks fan to ribonucleic acids. RNAs make up almost two

thirds of the ribosome's mass and playa catalytic role in linking up the amino acids during protein synthesis. They contribute to the structure and function of several less familiar organelles, such as the particles involved in the translocations of proteins across membranes.. Most remarkably, RNA alone sometimes serves as a catalyst. Catalytic RNAs or ribozymes participate chiefly in the manipulation of RNA, and they do not challenge the predominance of proteins in the catalysis line. But their discovery in the eighties overthrew the universal consensus that all enzymes must be proteins, and it suggested an altogether novel line of enquiry to the students of origin of life.

The composition of living cells is grossly different from that of their environment. That is possible because each cell is enclosed by a membrane; extremely thin and flexible, that is essentially impermeable to molecules large and small Biological membranes are composed of phospholipids (or in Archæa, of the related ether-lipids), that form closed bilayered structures spontaneously (Fig. 1.2). The oily core of the bilayer excludes water-soluble molecules, and thus imposes a barrier to the diffusion of most substances; water itself, oxygen and other gases are among the exceptions. Membranes are as essential to life as genes and proteins: "To stay alive you have to be able to hold out against equilibrium, maintain imbalance, bank against entropy, and you can only transact this business with membranes ill our kind of world".

Membrane spanning
protein

Phospholipid bilayer
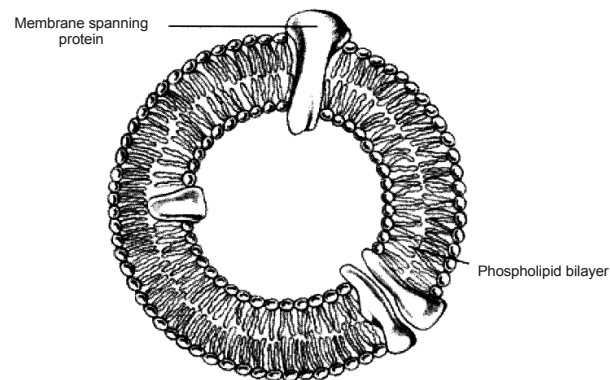
**Fig. 1.2 : The Architecture of biological membrane. A phospholipid bilayer
makes up the basic structure.**

The corollary is that cells require special means to transport nutrients in and 'waste products out. With very few exceptions, this is accomplished with the aid of proteins that span the membrane, linking the aqueous phases inside and out. Transport proteins are akin to enzymes in recognizing and binding particular substrates but most of them do not catalyze any chemical reaction, they merely translocate the substrate from one side to the other by shifting the orientation of the binding site from one surface

to the other. In some cases this reorientation is linked to an. energy source, allowing the transport protein to "pump" its substrate against a concentration gradient, others merely facilitate downhill diffusion. The plasma membrane of a bacterial cell may contain as many as a hundred different transport catalysts, each Dore or less specific for a particular substrate.

Transport proteins carry matter; receptors deal in information. Bacteria cells sense the presence of nutrients in the medium by virtue of specialized proteins that span the plasma membranes When a potential nutrient binds to the receipt of protein, the news is carried across the membrane by a. conformational change; this, in turn activate an enzyme or an enzyme cascade. What passes across the membrane is neither matter nor energy, but a signal: "now", or sometimes "here."

Bacteria, as a rule feature but a single membrane, the plasma membrane that defines the cell; but in eukaryotic cells, intercellular membranes are a conspicuous feature. Each intercellular membrane enclose a defined space whose composition and function is more or less distinct and which communicates cytoplasmic fluid by means of specialized transport catalysts. These compartments are also dynamic, fusing with one another and detaching a controlled and functional manner.

Traffic across membranes is not confined to small nutrients and waste products. In a bacterial cell many constituents are situated external to the plasma membrane: the cell wall an array of enzymes proteins that serve as receptors for environmental signals, flagella and they must meanwhile maintain the integrity of their bounding membrane. The role of membranes, like that of the sea in human history, is dual; they separate compartments while channeling the flow of matter and of information. When one thinks about membranes, one stands on the border between molecules and cells.

> The man behind the microscope
> Has this advice for you
> Never ask what some thing is
> Just ask, what does it Do?
>
> —*Hilaire Belloc*

SUB-SECTION 1.1B—WHY BIOCHEMISTRY IS INCLUDED IN MEDAL CURRICULUM?

Biochemistry as it is said is the basic subject which discusses the chemical constitution of the human body.

The chemical constitution of the body that means the different function of different organs are dealt in biochemistry.

The action of vitamins, enzymes co-enzymes, hormones, carbohydrates etc are all preprogrammed by biochemistry. The function of Kidney, Liver, the respiratory system (digestive system is a total biochemical programmed) work on complicated chemical reactions. These chemical reactions are all preprogrammed as said earlier any disturbance in them can cause a fatal disaster. In human metabolism. To set right these disturbances—"diseases" as they are commonly called, some correcting agents called "medicines" are admixture.

So in order to give or admixture these medicines doctors had to know biochemistry very well other

wise a big incorrigible problem can occur which could be more fatal. Some patients can be allergic to may drugs the study of these problems is carried in the advances medical biochemistry.

The hypo and hyper activity of these drugs precisely can cause what kind of metabolic disorder can only be understood in biochemistry.

We can take a very common example, a very common disease the diabetes the inconsiderable increase of sugar level in blood; to decrease this sugar level increment in blood insulin is administered.

Now this problem is entirely a disorder in biochemical system, so to handle this problem a medical practitioner must first examine the blood sugar level and then start admixture insulin. Here comes the use of biochemistry……… Now what is the normal blood sugar level? What is the abnormal level are all well answered in biochemistry.

Apart from this the different pathological tests are all been developed in biochemistry, without which a medical practitioner would not be able to diagnose the problem of the patient.

Biochemistry also deals with the mechanism of drugs, which makes a doctor well updated to treat a patient.

The steroids, the cholesterol' the action and mechanism are also well interpreted by biochemistry to the doctors.

So in the end it can be translated that biochemistry must become a useful tool for those doctors who can offer a good treatment to the patients.

SUB-SECTION 1.1C—CELLULAR STRUCTURE AND FUNCTION IN CHEMICAL TERMS

In this section we discuss the cell, the smallest unit of the human organism capable of reproducing itself. An understanding of cellular structure and function forms the basis for understanding malignant growth and anticancer therapy because (1) all malignancies are composed of abnormal cells, and (2) chemotherapy attacks cancer on the cellular level. In Section 5 the cell life-cycle, i.e., the various phases through which a cell passes during the process of cell division, will be reviewed. A knowledge of the life-cycle of the cell helps us to better understand how chemotherapeutic agents work.

> Introduction

Because cells are so different, there is no *typical* cell, but for purposes of illustration we can construct a hypothetical *composite* cell having all the component structures observed in many cells. For our purposes, we need only study the following cell parts and "organelles" shown in Figure 2a cell membrane, cytoplasm, nucleus, nucleolus, nuclear membrane, ribosomes, centrioles, and endoplasmic reticulum.

> Functional Anatomy of the Cell

Separating the internal structures of the cell from the intercellular fluid is the *cell membrane.* Also called the plasma or cytoplasmic membrane, it is a very thin, flexible, elastic membrane composed of a lipid (fat) bilayer with protein molecules embedded within the bilayer. Passing through the lipid layers are channels, which allow the controlled passage of chemicals into and out of the cell. Proteins embedded in the membrane perform

> The Cell Membrane

these specialized transport functions. These special transport structures give the cell membrane its *selective permeability*. One that is s*electively permeable* permits only certain sizes and types of molecules to pass through it, while excluding others. Because of its selective permeability, the cytoplasmic membrane regulates the amount and type of material entering and leaving the cell, thus helping to maintain the cell's homeostasis.
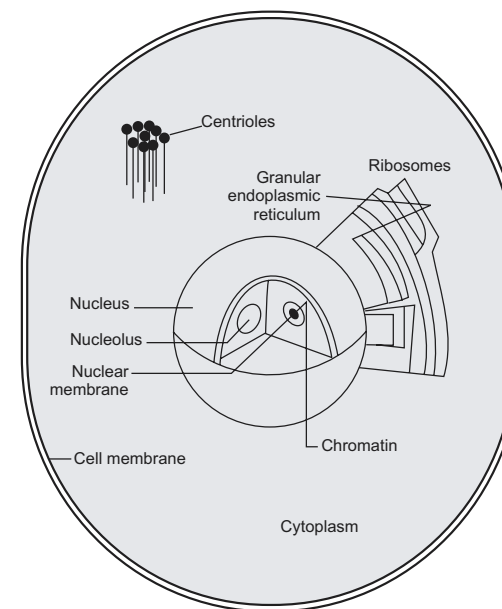
Fig. 1.2A : Diagrammatic representation of the living cell illustrating the major organelles.

Homeostasis is the condition whereby the internal and external environments of a cell remain relatively constant and in balance. Any disruption of homeostasis can damage the cell and cause it to malfunction. An understanding of *homeostasis* has helped develop cellular poisons (anticancer agents) capable of disrupting homeostasis of cells and killing them. Because the conditions of homeostasis differ slightly from one type of cell to another, it is possible to exercise some selectivity in the types of cells affected by a poison but not identical molecules or ions and there is competition for the transport

> Homeostasis

between these molecules. Thus, if one molecule is present in high concentration, it may competitively inhibit the transport of a second molecule. For example, folic acid is actively transported in some cells and this process can be inhibited competitively by methotrexate, a folic acid analog.

The *cytoplasm* surrounds the nucleus and consists of the cytosol, or fluid portion, and the various cellular organelles suspended in it. Most intermediary metabolism takes place in the cytosol, which represents about 55 per cent of total cell volume. In the cytosol, one finds thousands of enzymes that catalyze reactions involving (1) energy substrate production (gluconeogenesis and glycolysis); (2) biosynthesis of sugars, fatty acids, nucleotides, and amino acids; and (3) different cytoskeletal proteins, which give a structural framework to the cell. Within the cytoplasm are various organelles that perform specific functions:

> The Cytoplasm

Diffusion is the process by which molecules or ions spread from an area of high concentration to an area of low concentration until the concentration is uniform throughout, whereupon a state of equilibrium is said to exist. Most chemotherapeutic agents enter the cell through the cell membrane by *diffusion.* Also called *passive transport,* diffusion requires no expenditure of energy while transporting a substance across the cell membrane. Molecules and ions in body fluids are constantly in motion and they are thus available for diffusion through membranes. Diffusion can move substances either into or out of a cell—oxygen diffuses into cells while carbon dioxide diffuses out of cells is the process by which molecules or ions are moved (carried) across the cell membrane, sometimes in a direction opposite to that of diffusion. It is a process that allows a cell to maintain a state of non equilibrium at the cost of using energy in the transport process. Most chemotherapeutic agents enter the cell by active transport. Active transport systems act on *groups* of similar but not identical molecules or ions and there is competition for the transport between these molecules. Thus, if one molecule is present in high concentration, it may competitively inhibit the transport of a second molecule. For example, folic acid is actively transported in some cells and this process can be inhibited competitively by methotrexate, a folic acid analog.

> Diffusion

> Active transport

The Cytoplasm

The *cytoplasm* surrounds the nucleus and consists of the cytosol, or fluid portion, and the various cellular organelles suspended in it. Most intermediary metabolism takes place in the cytosol, which represents about 55% of total cell volume. In the cytosol, one finds thousands of enzymes that catalyze reactions involving (1) energy substrate production (gluconeogenesis and glycolysis); (2) biosynthesis of sugars, fatty acids, nucleotides, and amino acids; and (3) different cytoskeletal proteins, which give a structural framework to the cell. Within the cytoplasm are various organelles that perform specific functions:

Within the cytoplasm are various organelles that perform specific functions are shown in the table 1.1.

> The Nucleus

Table 1.1 : Showing the different functions of cell

Organelle	% Cell Volume	Function
Free ribosomes	<1%	Protein synthesis
Endoplasmic retitulum (with membrane-bound ribosomes) and Golgi apparatus	9%	Synthesis of membrane is lipid and proteins to be secreted intracellular transport of macromelecules
Mitochondria (about 1,700/cell)	22%	ATP (energy) production
Lysosomes (about 300/cell)	1%	Digestive enzyme packets to degrade foreign macro-molecules
Peroxisomes (about 400/cell)	1%	Enzyme packets involved in oxidative reactions

The *nucleus* is usually located near the center of the cell. Relatively large and often spherical in shape, the nucleus is the control center of the cell. Separating the nucleus from the cytoplasm is the clear nuclear membrane, whose porous nature permits most substances to move back and forth between the nucleus and the cytoplasm. Unlike the wide variety of organelles found in the cytoplasm, the only prominent structural feature in the *nucleus* is the nucleolus, or "little nucleus," which is a minute body composed primarily of proteins and RNA. The nucleolus functions in the processing of the RNA that moves to the ribosomes in the cytoplasm. The nucleus of a cell contains the hereditary material DNA. The DNA in the nucleus is tightly coiled around specialized proteins called histones. The double-helical DNA strands packed about the histone proteins are called chromatin fibers (DNA plus histone proteins). The bulk of DNA, wrapped around histone proteins, is called a nucleosome. It is attached to other nucleosomes by a short "linker" section of DNA. The chromatin is organized into massive, short, rod-like bodies called chromosomes. The normal human cell contains 46 chromosomes. This is the diploid state. In contrast, the sperm and egg each contain only 23 chromosomes (haploid). Cancer cells may be aneuploid (unusual number of chromosomes) or tetraploid (twice the normal number of chromosomes) egg each contain only 23 chromosomes (haploid). Cancer cells may be aneuploid (unusual number of chromosomes) or tetraploid (twice the normal number of chromosomes).

Nucleic acids are huge organic molecules, so named because they were first discovered in the nuclei of cells. The two main kinds of nucleic acids are DNA and RNA.

> Nucleic Acids

Deoxyribonucleic acid is a very long helical structure that associates with a complementary strand via hydrogen-bonding reactions. The single strands are made up of an unbranched linear polymer composed of millions of nucleotides (a nucleic acid base, a phosphate, and the sugar deoxyribose) (Figure 1.3). The sequence of nucleotide bases represents the genetic code, which carries the cells' hereditary information. A gene is a section of DNA that codes for a single cellular trait or function. One chromosome may contain more than 20,000 genes. The four *bases* in DNA are adenine, thymine, guanine, and cytosine (Figure 1.4). Each genetic unit in the DNA code is composed of three nucleotides in a specific

> DNA

order. This is called a codon. When a strand of DNA acts as a template for the synthesis of RNA (transcription), there is a "start" codon and a "stop" codon at the beginning and the end of the sequence that codes for the RNA that carries the message to the ribosome, where the protein is formed. Some portions of the DNA code for an amino acid sequence in the protein (exons), and other portions of the DNA strand do not (introns). The non-coding portions of the RNA message are removed when the RNA is processed in the nucleus before it migrates to the ribosome. The whole DNA molecule consists of two strands—two chains of nucleotides—that wind around each other in the form of a double helix. This is illustrated in Figure 1.5. The two strands are held together by hydrogen bonding between the bases. There is a very specific order in the bonding process. Adenine bonds only to thymine, and guanine bonds only to cytosine. This is called *complementary base pairing,* as illustrated in Figure 1.6. Complementary base pairing makes it possible for the DNA molecule to engage in self-replication.
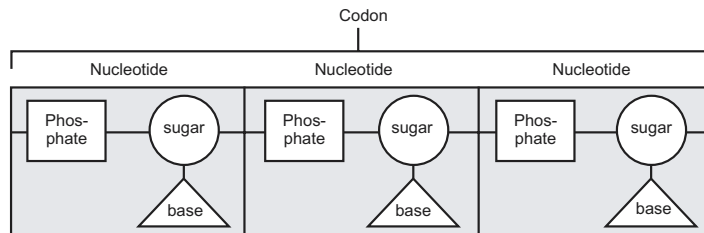
Fig. 1.3 : Linear organization of nucleotides of the DNA molecule.
A "codon" consistsof three sequential nucleotides

Coded information stored in DNA directs the production of RNA (transcription), which in turn directs the chemical synthesis of proteins (translation) (see Figure 7). *Enzymes* are proteins that act as catalysts to induce or speed up chemical reactions inside or outside the cell. The unique characteristic of a catalyst is that it remains unchanged by the chemical reaction it engenders. Enzymes govern all of a cell's metabolic reactions, and the enzymes themselves are proteins synthesized under the control of DNA. Some antineoplastic agents act by interfering with the activity of enzymes involved in the synthesis of DNA.

Ribonucleic acid (RNA) is part of the messenger system through which DNA controls protein production within the cell. There are at least three different kinds of RNA: messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA).

| RNA |

mRNA carries the genetic message for the gene to the ribosome. The nucleotide sequence of mRNA specifies the amino acid sequence in the protein to be synthesized at the ribosomes. tRNA transfers the amino acid from the enzyme that recognizes it to the ribosome where it is added to the growing peptide chain, and rRNA makes up part of the organelle on which protein synthesis takes place. The RNA molecule is also a linear sequence of nucleotides, but there are three ways in which it differs from DNA. First, the sugar-phosphate backbone of RNA uses ribose instead of deoxyribose. Second, RNA

does not contain the base thymine, but in its place RNA contains the closely related base *uracil.* And third, RNA is usually single stranded, whereas DNA is usually double stranded.
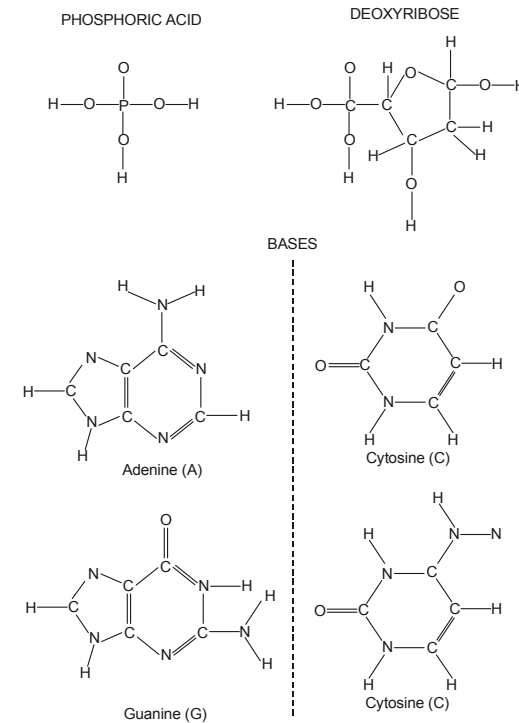
Fig. 1.4 : Showing the Constituents of DNA

The nucleus contains both DNA and free nucleotide bases (adenine, thymine, cytosine, and guanine) that are not bound to the DNA. In dividing cells, the DNA component is replicated during S phase, which normally lasts about 12 hours. In order to synthesize new DNA, the two opposing strands of DNA separate. As the two strands unwind, the nucleotide bases that were once paired together (ie, adenine with thymine and cytosine with guanine) now become exposed to the nuclear environment. This process can be inhibited by some cancer

| DNA Synthesis |

drugs. For example, alkylating agents such as cyclophosphamide and nitrogen mustards form covalent crosslinks between the two strands of DNA so that they cannot unwind and separate for replication. If unperturbed, the enzyme DNA polymerase matches free (unbound) nucleotides with the exposed nucleotide bases along one strand of DNA to form a complementary copy. The antimetabolite cytosine arabinoside is known to inhibit this enzyme. As noted previously, the chemical structure of the bases requires that adenine pair with thymine and cytosine with guanine. When the DNA component is doubled, replication ends and the two new double stranded DNA molecules coil up to form helix just like the parent molecule. The cell now has twice the normal amount of DNA. Thus, each strand of the parent DNA molecule serves as the template for its own replication (Figure 8). The two resulting DNA molecules each have one old DNA strand and one new DNA strand. At this point, S phase ends and
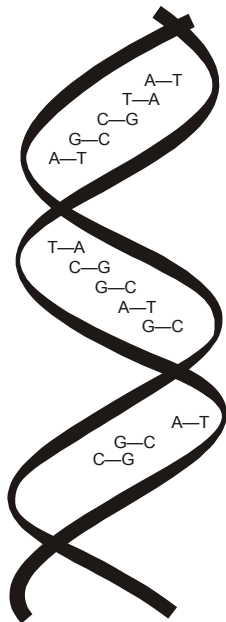
Fig. 1.5 : The DNA double helix. Note that adenine always pairs with thymine, while ganine always pairs with cytosine
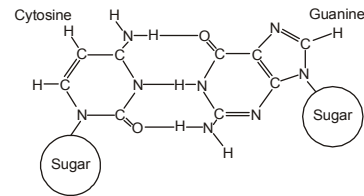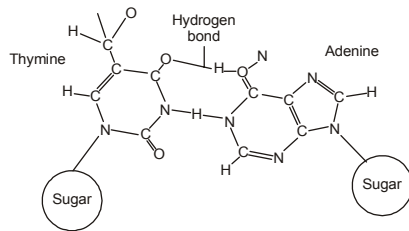
Fig. 1.6 : Showing the Complementary base pairing. The bases are united by hydrogen bonds, holding the double helix together
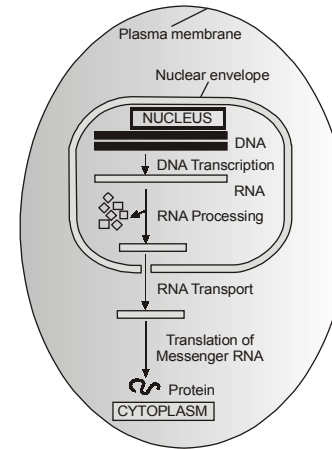
Fig. 1.7 : A schematic view of the expanded process of protein synthesis ("DNA—RNA—protein") in eukaryotes. Because of the nuclear envelope, RNA processing and RNA transport steps are interposed between transcription and the translation of mesenger RNA into protein

the cell moves into the G2 phase of division. Because of the way the enzyme DNA synthetase works, it cannot complete the DNA chain all the way to the end. So with each cell division, a few bases are lost at each end of the chromosome. The bases lost make up the telomere, which is a special section of DNA at the end that protects the DNA. This "end replication problem" leads to unstable chromosomes after 50 to 100 doublings, and this is thought to be a biologic clock that accounts for cell aging. Germ cells and stem cells have an enzyme, telomerase, which completes the telomere. Cancer cells also express this enzyme, which explains their immortality.

Fig. 1.8 : showing DNA replication

Proteins are composed of long chains of amino acids linked to each other by peptide bonds. Each protein has a unique three-dimensional structure designed to perform a specific function. It is the sequence of amino acids in a protein that determines the details of the three-dimensional structure.

Proteins typically contain hundreds of amino acids and the chain has two distinct ends: an amino terminus and a carboxyl terminus. The functions performed by these molecules include all the functions necessary for life: enzymatic reactions, active transport, structural functions as for bone and muscle, etc. Any errors in the sequence of amino acids in a protein (a mutation) will cause an error in the three-dimensional structure and will cause a malfunction of that protein. For example, a single incorrect amino acid in hemoglobin results in sickle cell hemoglobin and a serious defect in red blood cell function, leading to anemia. When new proteins or enzymes are needed, DNA begins unraveling at a specific point along the chain. Unraveling occurs at the specific point that bears the code for the particular enzyme that is needed. Only a specific number of sites are exposed. As in DNA synthesis, the exposed nucleotides along the sugar-phosphate backbone are acted upon by an enzyme, RNA synthetase, to pair the free nucleotides in the nucleus with the nucleotide backbone. The result is that mRNA is formed (see Figure 9). This process is called transcription.

—A—U— (mRNA)
—A—U—
—C—G—
—G—C—
—A—U—
—G—C—
—C—G—
—G—C—
—A—U—   → To Cytoplasm
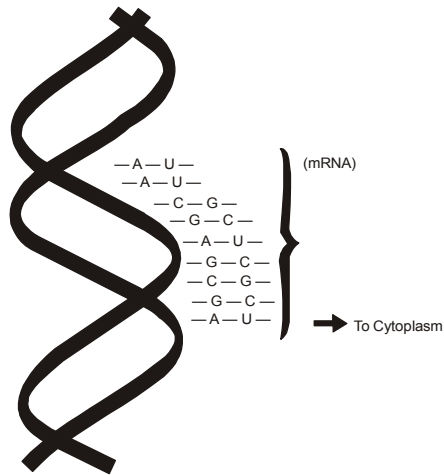
Fig. 1.9. Messenger RNA (mRNA) synthesis.
(Note that uracil pairs with adenine on the DNA strand.)

An example of an anticancer drug that inhibits RNA transcription is actinomycin D (dactinomycin). The mRNA is processed in the nucleus and then goes to the cytoplasm through small holes in the nuclear membrane. The mRNA migrates to the ribosome in the endoplasmic reticulum. Figure 10 is a highly schematic picture of protein synthesis. The ribosome moves along the strand of mRNA in assembly-line fashion; amino acids are "delivered" by the carrier molecule, tRNA, and are attached one

by one until the polypeptide chain is complete. Each codon, or sequence of three bases, such as adenine-adenine-thymine, "spells out" a particular amino acid. Using the same process of complementary base pairing used in the nucleus, tRNA molecules "know" exactly where to deliver amino acids along the mRNA molecule, thus insuring that the polypeptide chain of the protein molecule will be synthesized according to the DNA "blueprint." Figure 11 is an overview showing how DNA controls cellular homeostasis.

Free ribosome about to attach to mRNA   Completed chain

Ribosome
Growing polypeptide chain
mRNA
tRNA
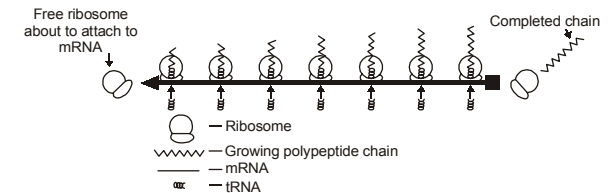
Fig. 1.10 : The ribosomal "protein factory". mRNA moves along the ribosome and amino acids attach one at a time until the chain is synthesized

DNA
RNA synthesis
Protein synthesis
Cell structure   Enzymes
Cell function
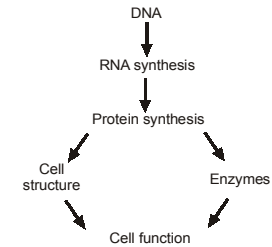
Fig. 1.11 : Overview: the role of DNA in homeostasis

2

Chapter

Chemistry of Living Matters

SECTION 2.1—CARBOHYDRATES AND ITS NNOMENCLATURE

Carbohydrates, as the name implies believed to consist solely of the elements carbon and water in a ration 1:1 but our recent understanding is that a number of other atoms and functional groups can be present in a carbohydrate and the original formula of $C_xH_{2x}O_x$ is no long is no longer rigidly adhered to. Carbohydrates are traditionally considered to three broad groups :

1. Monosaccharide
2. Oligosaccharides
3. Polysaccharide

With monosaccharide being the simplest one, which cannot be hydrolyzed into smaller ones and from which the two other groups are obtained by formation of glycosidic linkages. This simple classification system defines oligosaccharides as simple polymers of monosachharides containing between two and 10 monosachharides unites or residues. Polysaccharides are higher molecular weight polymers of monosachharides which contain in excess of 10 residues, whilst the division between oligosaccharides and polysaccharides is arbitrarily set at 10residues, nature has made the division much easier to observe since carbohydrates containing between five and 15 residues rarely exist in nature while only a few consist of 25 to 75 residues. The majority of naturally occurring polysaccharides contain 80 to 100 residues although a few negative cellulose comprising a series of polymers with an average molecular weight distribution equivalent to 3000 residues. Such weight but a range of macromolecules having a distribution of molecular weights. This phenomenon is known as mocroheterogneity.

> Nomenclature

The members of these groups of compounds are denoted by the term – *ose*, the hydroxy – aldehyde (%CHO group) being called *aldoses* and the hydroxy ketones (%CO) are called *ketoses*. Whilst the number of carbon atoms in the compound is indicated by prefixing the great numerical to the syllable – ose. Thus glycolic aldehyde is an aldo – triose; where as dihydroxy – acetone is a keto – triose. The necessity for this system of nomenclature is apparent when dealing with the higher members of the series, each of which can exist, in several (glyceric aldehyde) 4 possible aldo – ketoses, 8 – possible aldopentoses and 16 possible aldo – hexoses. There are also heptoses, octoses, nonoses and decose with 7, 8, 9 and 10 atoms.

Aldotriose   keto triose
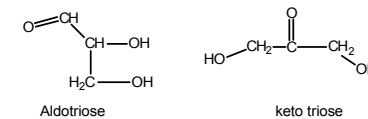
The existence of the large number of isomers in the example given above is due to the difference in space arrangement or con-figuration of the atoms, which is discussed in a later part of this chapter.

SUB-SECTION 2.1A—CLASSIFICATION WITH EXAMPLES

A. Monosaccharides (simple sugars)

1. Hexoses

a. ***Glucose:*** honey, fruits, corn syrup, sweet grapes, sweet corn; hydrolysis of starch and cane sugar. Physiologically the most important sugar; the "sugar" carried by the blood and the principal one used for tissues.

> Classification of Carbohydrates

b. ***Fructose:*** honey, ripe fruits, some vegetables; hydrolysis of sucrose insulin. Can be changed to glucose in the liver and intestine and is an intermediate metabolite in glycogen breakdown.

c. ***Galactose:*** not found free in nature; digestive end product of lactose hydrolysis. Can be changed to glucose in the liver; synthesized in body to make lactose and is a constituent of glycolipids.

d. ***Mannose:*** found in legumes; hydrolysis of plant mannosans and gums. A constituent of polysaccharide of albumins, globulins, and mucoids.

2. Pentoses

a. ***Arabinose:*** derived from gum arabic plus plum and cherry gums; not found free in nature. Has no known physiologic function in man; used in metabolism studies of bacteria.

b. ***Ribose:*** derived from nucleic acid of meats and seafoods. Structural element of nucleic acids, ATP, and coenzymes (NAD and FAD).

c. ***Ribulose:*** formed in metabolic processes. Intermediate in direct oxidative pathway of glucose breakdown.

      d. Xylose: wood gums, corncobs, and peanut shells; not found free in nature. Very poorly digested and has no known physiologic function; used medicinally as a diabetic food.

B. Oligosaccharides (2-10 sugar units)

  1. Disaccharides

    a. Sucrose: cane and beet sugar, maple syrup, molasses, and sorghum. Hydrolyzed to glucose and fructose; a non-reducing sugar.

    b. Maltose: malted products and germinating cereals; an intermediate product of starch digestion. Hydrolyzed to two molecules of glucose; a reducing sugar; does not occur free in tissues.

    c. Lactose: milk and milk products; formed in the body from glucose nature. Hydrolyzed to glucose and galactose; may occur in urine during pregnancy; a reducing sugar.

  2. Trisaccharides

    a. Raffinose: cottonseed meal, molasses, sugar beets and stems. Only partially digestible but can be hydrolyzed by enzymes of intestinal bacteria to glucose, fructose, and galactose.

    b. Melizitose: honey, poplars, and conifers. Composed of one fructose unit and two glucose units.

C. Polysaccharides (more than 10 sugar units)

  1. Digestible

    a. Glycogen: meat products and seafoods; polysaccharides of the animal body, often called animal starch; storage form of carbohydrates in body, mainly in liver and muscle.

    b. Starch: cereal grains, unripe fruits, vegetables, legumes, and tubers. Most important food source of carbohydrates; storage form of carbohydrates in plants. Composed chiefly of amylose and amylopectin; hydrolyzed to glucose.

    c. Dextrin: toasted bread, intermediate product of starch digestion. Formed in course of hydrolytic breakdown of starch.

  2. Partially Digestible

    a. Inulin: tubers and roots of dahlias, artichokes, dandelions, onions, and garlic. Hydrolizable to fructose; used in physiologic investigation for determination of glomerular filtration rate.

    b. Mannosan: legumes and plant gums. Hydrolyzable to mannose but digestion incomplete; further splitting by bacteria may occur in large bowel.

  3. Indigestible

    a. Cellulose: skins of fruits, outercoverings of seeds, plus stalks and leaves of vegetables. Not subject to attack of digestive enzymes in man, thus an important source of "bulk" in diet; may be partially split to glucose by bacterial action in large bowel.

    b. Hemicellulose and pectin: woody fibers and leaves. Less polymerized than cellulose; may be digested to some extent by microbial enzymes, yielding xylose.

Indigestible is another name for indigestable polysaccharides is dietary fiber which is comprised of 2 groups:

    c. Insoluble dietary fibers (cellulose, lignin, and cutin) which are the most abundant organic compounds in the world. They help prevent constipation, colon cancer, and diverticulosis, but not hypercholesterolemia.

    d. Soluble dietary fibers (hemicellulose, pectins, gums, and algal polysaccharides) which are useful in decreasing serum cholesterol and in regulating blood glucose levels.

SUB-SECTION 2B—CHEMICAL STRUCTURES OF CARBOHYDRATES IN PYRANOSE AND FURANOSE FORM

Pyranose is a collective term for carbohydrates which have a chemical structure that includes a six-membered ring consisting of five carbons and one oxygen. The pyranose ring is formed by the reaction of the C-5 alcohol group of a sugar with its C-1 aldehyde forming an intramolecular hemiacetal. The name derives from its similarity to the oxygen heterocycle pyran.

    Furanose ring is a cyclic hemiacetal of an aldopentose or a cyclic hemiketal of a ketohexose.

    A furanose ring structure consists of four carbon and one oxygen atom with the anomeric carbon to the right of the oxygen. The highest numbered chiral carbon (typically to the left of the oxygen in a Haworth projection) determines whether or not the structure has a D-configuration or L-configuration. In an L-configuration furanose, the substituent on the highest numbered chiral carbon is pointed downwards out of the plane, and in a D-configuration furanose, the highest numbered chiral carbon is facing upwards.

    The furanose ring will have either alpha or beta configuration, depending on which direction the anomeric hydroxy group is pointing. In a D-configuration furanose, alpha configuration has the hydroxy pointing down, and beta has the hydroxy pointing up. It is the opposite in an L-configuration furanose. Typically, the anomeric carbon undergoes mutarotation in solution, and the alpha-beta configuration switches constantly—thus it is in an equilibrium state.

    The number of monosaccharides known in the neighbourhood of fifty, of which 10 occur in nature and the remaining are synthetic. The existence of such a large number of comnpounds is due to the presence of presence of asymmetric carbon atoms within the molecules. Aldohexoses, for example which includes glucose, a sugar of great theoretical and practical interest, contain no less than four asymmetric atoms, each of which may be present in either the *d* and *l* – configuration. It has already been shown that how rapidly stereo isomerides increases with each additional asymmetrical atom.

    A list of best monocaccharides is given below in detail in the table.

    An explanation is required as to the way in which the optical isomerides are named. In accordance with a suggestion of Fischer, the structure of the sugar, where ever possible, referred back to that of

Monosaccharides

Name	Formula	Chemical name
Biose	$CH_2OH.CHO$	Glycolic aldehyde
Trioses	1. $CH_2OH.CHOH.CHO$	Glyceric aldehyde
	2. $CH_2OH.CO. CH_2OH$	Dihydroxy acetone
Tetrose	$CH_2OH.(CHOH)_2.CHO$	Erithrose
Pentose	$CH_2OH(CHOH)_3CHO$	Binose, xylose, ribose
Methyl pentose	$CH_3(CHOH)_4CHO$	Rhamnose
Hexose	1.$CH_2OH(CHOH)CHO$	Glucose, gulose, talose mannose
	2. $CH_2OH(CHOH)_4CHO$	Fructose , sorbose
Heptose	$CH_2OH(CHOH)_3CO CH_2OH$	Monoheptose, glucoheptose, galaheptose
Octose	$C_8H_{16}O_8C_9H_{18}O_9$	
Nonose	$C_8H_{16}O_8C_9H_{18}O_9$	

the active glucoses. Any other monosaccharide standing in close structural relationship to one or other of the glucose is generally labeled with the corresponding letter d and l – irrespective of the actual sign of its rotation. Ordinarily fructose, or example, which is lævorotatory, but termed as d – fructose, owing to its spatial relationship to d – fructose. Naturally occurring arbinose, which is dexorotatory, is termed as l – arbinose to indicate its relationship to l – glucose. This is illustrated in the following formulæ, in which the terminal aldehydic or ketonic group is written uppermost links blinding the H and OH addenda are assumed to be inclined from the chain of central carbon atoms towards the observer. According to the usual convention a CHOH group having the hydroxyl on the right of the formula employed.

d-fructose   d-glucose   l-glucose   l-arbinose
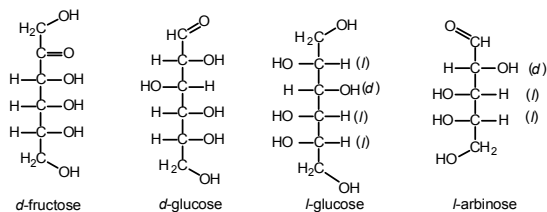
In some cases the actual sign of the rotation given by a compound is indicated in the following pages by the use of the sign + and %, eg d(%) fructose for ordinary fructose. In addition, the prefix dl – indicates a racemic and mesoform.

Wohl's work on glycerose now enables the family relationship to be carried back to the d and l glyceroses from which all other sugars can be derived by extending the molecule on the side of the

aldehyde group by reaction with. The nomenclature fortunately remains unchanged as d – glycerose is d – rotatory and is genetically related to d – glucose. Hence it will be seen that the family of a sugar is determined in each case by the spatial arrangement of the CHOH group adjustment to the terminal CH_2OH, no matter what the disposition of the rest of the molecule may be. If this group has a dexo configuration, as indicated by writing it with OH to the right the sugar is classified as belonging to the d – family. The same system is applied to other derivatives of monosaccharides.

d glecerose   L glecerose
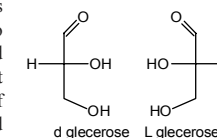
The monosaccharides possess strong reducing properties, causing the separation of the silver form ammonia – silver nitrate solution and precipitating cuprous oxide from an alkaline solution of copper sulphate. They thus resembles aldehydes, moreover, like aldehydes and ketones, they form cyanohydrines with hydrocyanic acid, oximes with hydroxylamine, and phenylhydrazones with phenylhydrazones. The phenylhydrazones are as rule very soluble in water but by the further action of phenylhydrazine, they are converted into a yellow insoluble compound called osazones. The osazones are nearly insoluble in water, and readily separate from a solution of monosaccharide. They have, more over a definite melting point, and seen under microscope possess a characteristic appearance.

General Properties
or
Monosaccharides

The reaction is therefore regarded as the most important process in detecting and identifying certain sugars. The monosaccharides are readily oxidized. The aldoses yield mono and di basic acids containing the same number of carbon atoms; the ketones break up into acids with a fewer carbon atoms. They all yield oxalic acid when warmed with strong nitric acid, with strong hydrochloric acid they form levulinic acid. Finally they undergo alcoholic fermentations with yeast. These reactions are going to be discussed under glucose.

GLUCOSE

Introdcution

Glucose a monosaccharide (or simple sugar), is an important carbohydrate in biology. The living cell uses it as a source of energy and metabolic intermediate. Glucose is one of the main products of photosynthesis and starts cellular respiration in both prokaryotes and eukaryotes. The name comes from the Greek word *glykys* (āëõêýò), which means "sweet", plus the suffix "-ose" which denotes a sugar.

Two stereoisomers of the aldohexose sugars are known as glucose, only one of which (D-glucose) is biologically active. This
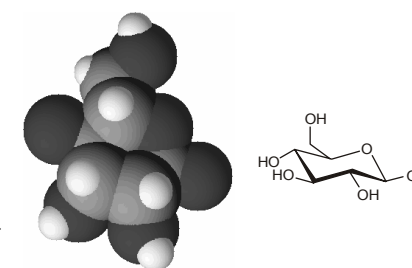
form (D-glucose) is often referred to as **dextrose monohydrate**, or, especially in the food industry, simply **dextrose** (from *dextrorotatory glucose*. The mirror-image of the molecule, L-glucose, cannot be metabolized by cells in the biochemical process known as glycolysis. Glucose is commonly available in the form of a white substance or as a solid crystal. It can also be commonly found as an aqueous solution.

Synthesis

In small quantities pure glucose is most readily obtained from cane sugar. Cane sugar is dissolved in 80% alcohol and a little strong hydrochloric acid added. On gently warming the mixture, the cane sugar is hydrolysed, and breaks up into glucose and fructose

$$C_{12}H_{22}O_{11} \quad + \quad H_2O \quad \rightarrow \quad C_6H_{12}O_6 + C_6H_{12}O_6$$
Cane sugar   Glucose Fructose
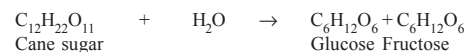
Glucose, being less soluble in alcohol separates out as crystals.

Glucose is manufactured by boiling starch with dilute sulphuric acid; the starch is hydrolysed and converted into glucose. The liquid neutralized with soda ash and filtered and filtrate decolourised by filtration through animal charcoal. (See fig 12)

The solution is evaporated to the requisite consistency in vacuum pans. The product solidifies on cooling and forms an amorphous looking mass, which always contains dextrin.

The Vacuum Pan (See Fig. 12)

It consists of an iron pan which is heated by vertical steam coils placed in the lower part of the vessel; two or three pans are connected so that the steam rising from the evaporation of the liquid in the first pan is utilized for heating the next and its vapour passes on to the third. Between the pans a small cylindrical vessel is interposed, which serves to collect any juice which "primes" or collects and carried over during boiling. The evaporation is continued until the liquid is so far concentrated that it shows "grains" or commences to crystallize. It is then run out and cooled and the un crystallized portion or molasses separate in a centrifugal extractor.

Pure glucose dissolves in 1.2 parts of water. It crystallizes from aqueous solution with 1 molecule of water, the crystal melts at 66°C, and whilst from alcohol the anhydrous compound separates, melting at 146°C. Glucose is dexorotatory in aqueous solution, when freshly dissolved in water it shows a specific rotation of +110.2°, which falls rapidly, and then gradually more and more slowly until after the lapse of about 6 hours it reaches a constant value of + 52.5°. This change of rotatory behaviour is called *muta rotation*, and this is due to structural changes which occur in solution. Many other sugars
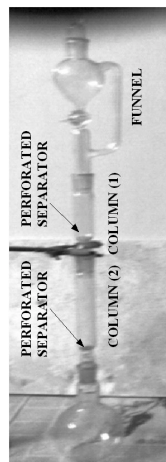
Fig. 2.1 : Showing the picture of the column filter with animal charcoal used in Author's lab

Properties of Glucose

under go muta rotation. Another variety of glucose obtained by crystallization from alcohol has a specific rotation of + 19°, a solution of which on standing rises to + 52.5°. The two sugars are called a and b glucose (see constitution) and the value + 52.5°C is the specific rotatory power of an equilibrium mixture. In genral the a - sugars are characterized by a *fall* and the b - sugars a *rise* in the initial value of the rotation.
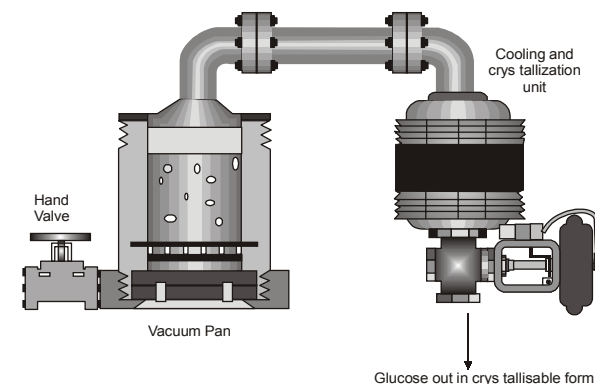
Fig. 2.2 : Showing the Manufacturing process of Glucose from starch

When lime or baryta solution is added to a solution of glucose and then alcohol, glucose, glucosates of calcium or barium are precipitated. These compounds are soluble in water and are decomposed by carbon dioxide into the original sugar and the carbonate of the metal. Calcium glucostate has the formula $C_6H_{12}O_6CaO$

Some Detail Physical Properties of Glucose

Molecular Formula	$= C_6H_{12}O_6$
Formula Weight	= 180.15588
Composition	= C(40.00%) H(6.71%) O(53.29%)
Melting point	= 146°C
Molar Refractivity	= 37.25cm³
Molar Volume	= 104.0 cm³
Parachor	= 312.7 cm³
Index of Refraction	= 1.635

Surface Tension	= 81.7 dyne/cm
Density	= 1.732 g/cm^3
Polarizability	= 14.76 10^{-24}cm^3
Monoisotopic Mass	= 180.063388 Da
Nominal Mass	= 180 Da
Average Mass	= 180.1559 Da

Structure of Glucose

Glucose forms a phenoacetyl derivative with acetic anhydride and therefore contains a hydroxyl groups. Each hydroxyl group probably attached to the different carbon atom, seeing that the arrangement of attachment of 2 hydroxyl groups to the same carbon atom would form a very unstable arrangement. The only point which is left uncertain chain. This point is determined by the reduction of glucose to sorbitol, and the conversion of the latter by means of hydriodic into normal secondary hexyl iodide.

It has also been shown that glucose combines with hydrocyanic acid and forms cyanhydrin which, on hydrolysis, yields an acid, By the reduction of this acid with hydriodic acid, normal heptylic acid is produced. These changes are represented by the following formulæ.

glucose   glucose cyanohydrin   hydroxy heptic acid   heptylic acid
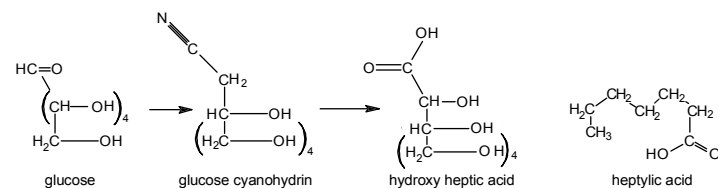
The formula of glucose would therefore appear to be a penta hydroxy aldehyde

$$CH_2(OH).CH(OH). CH(OH). CH(OH). CH(OH).CHO$$

The formula does not, however strictly confirm to its behaviour for a study of the two methyl glucosides has revealed the fact that both glucose and the glucosides exist in cyclic form. Since the terminal (aldehyde) group of glucose is involved in the ring formation, this carbon atom becomes asymmetric, and in this way gives rise to the stereoisomers α and β - glucose and α and β glucosides. Further investigations has shown that the rings are 6 membered, with oxygen atom linked to the first and fifth group were present, it can presumably isomerise easily to an open chain structure. The a and b ring structure and the open chain formula for glucose are as follows.

It is convenient to be able to refer to the different carbon atoms by means of numbers, which are indicated on the right hand side. The two ring structures differ only in the disposition of the groups attached to No. 1 carbon atom. The configuration of the other four asymmetric carbon atoms (no 2, 3,
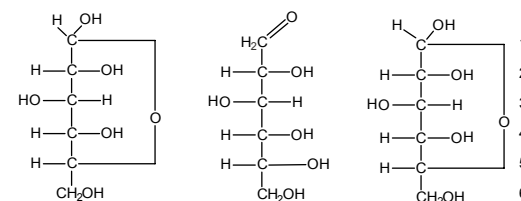
4, and 6) has been accurately determined, and is here indicated by means of projection formulæ (see chap organic mechanism). Although these projection formulæ are quite satisfactory for open chain compounds, some confusion is at to arise whenever the chain is closed to a ring, so that Howorth has adopted the use of perspective formulæ, which reveal the ring as a hexagon the groups attached to the carbon atoms being shown either above or below the plane of the hexagon as the case may be.

α- glucopyranose   β- glucopyranose
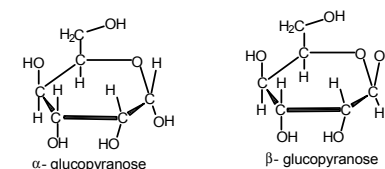
These are called pyranose forms. Other rings called furanose rings are formed with only four carbon atoms and one of oxygen. These terms are derived from *pyron* and *furon*.

Since every asymmetric atom gives rise to two stereoisomers every additional one of them must produce two more form each of these. Thus the aldopentoses with three asymmetric carbon atoms exist in eight active form, whilst the aldo hexoses with 4 asymmetric atoms give rise to 2 × 8 or 16, all of which are known, the most important of these are *d* – glucose, *d* – galactose and *d* – mannose.

Chemical Properties

In considering simple sugars or monosaccharides, it is useful to discuss in detain the chemical properties of the glucose. The fact is that it may be isolated from the acid – catalyzed hydrolysis of starch, suggests that the carbon atoms in glucose are attached to each other by carbon – carbon bonds rather than more labile ether bonds (although the existence) of the later is addition to the carbon – carbon bonds not excluded).

Quantitative Acylation of glucose (or equivalent benzoylation) suggests (in the equation below) that there are five hydroxy groups per molecule.

$$Na^+OCOCH_3^{\%}$$
$$(i) \ C_6H_7O(OH)_5 + 5(CH_3CO)_2O \rightarrow C_6H_2O(OCOCH_3)_3 + 5CH_3CO_2H$$
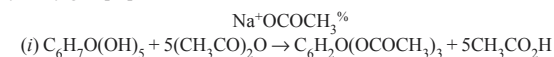
Treatment of glucose with anhydrous methanol in presence of hydrogen chloride replaces only one

hydroxy group with dilute aqueous acid, so that the product assumed be acetal.

$$C_6H_{11}O_8(OH) + CH_3OH \; D \; C_6H_{11}O_5(OCH_3) + H_2O$$

All the hydroxy group in the sugar can be methylated by the treatment with methyl iodide and silver hydroxide. Of the methyl group the acetyl methyl is readily removed by the hydrolysis, others are not because they are linkages.

Glucose   + 5CH$_3$I + 5AgOH   →   Pentamethyl glucose   →   tetramethyl glucose
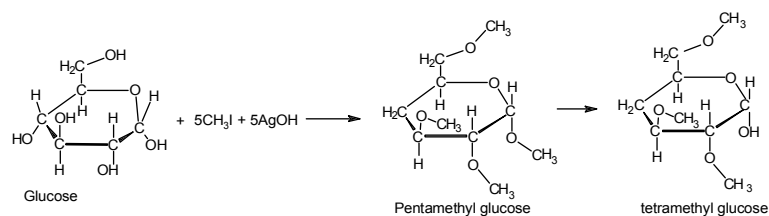
These results show that four of the five hydroxy groups are alcohol groups, while one is a hemiacetal group.

The presence of a carbonyl group (or equivalent) in glucose is demonstrable by treatment with hydroxylamine. The anticipated oxime is produced. However treatment of monosaccharide with phenylhydrazine produce an osazone reaction similar to that observed with benzoin. Oxidation occurs only at the hydroxy group adjustment to the original carboxyl group, so that a phenyl osazone result.

Glucose + phenylhydrazine → Glucose phenyl osazone + aniline + NH$_3$ ammonia + 2H$_2$O water
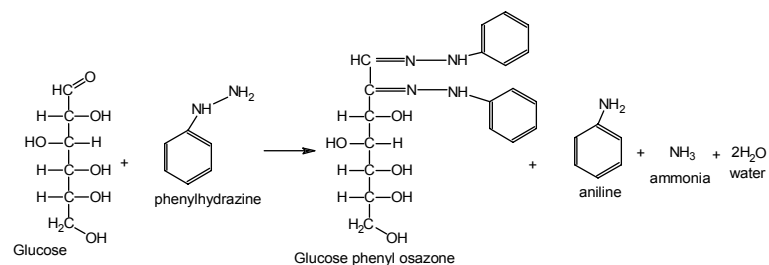
Both aldoses and ketoses are readily oxidized by Fehling's solution and Benedict's reagents these reagents are important for the determination of free available carboxyl group. Both consist of alkaline complexes of cupric ion. Fehling's solution is a mixture of citrate cupric ion and sodium carbonate. These reagents are reduced to cuprous oxide by α - hydroxy aldehydes and α - hydroxyketones. A positive test is indicated by complete disappearance of colour from the solution and the formation of a coral to red – brown precipitate.
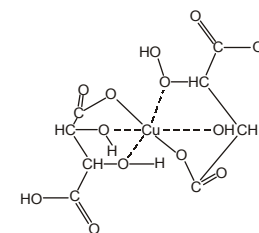
One possible tatatocupriccomplex

Bromine and dilute acid are reagents useful for oxidation of aldehyde groups of aldoses. The products are termed aldonic acids. Oxidation of a aldoses with boiling concentrated nitric acid attack both the aldehyde group and the primary alcohol group to give a dicarboxylic acid formerly called saccharic acid, but now given the generic name glyceric acid. The aldehydic acids produced by oxidation of the primary alcohol group are called glycuronic acid. This oxidation involves procedures where the other groups susceptible to oxidation are suitable protected and then the protecting substituents are removed later.

$$RCO + Br_2 + H_2O \rightarrow RCO_2H + 2HBr$$
$$HCCH_2(CHOH)_nCHO + 6HNO_3 \rightarrow HOCO(CHOH)_nCO_2H + 6NO_2 + 4H_2O$$

Saccharic acid   ←   Gluconic acid   ←   Glucose   →   Glucuronic acid
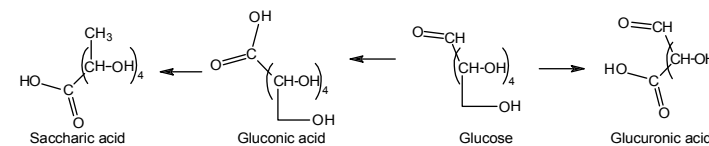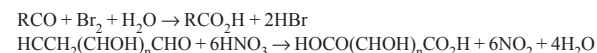
Like similar aldehydes and ketones monosaccharides react with hydrogen producing cyanohydrines. This fact provides a tool effectual in the structural analysis of mono-saccharides as well as a means of synthesis of aldoses of one more carbon atom per molecule than the original aldose.

Interconversions: Heinrich Killani was first to apply cyanohydrin formation to the synthesis of higher aldoses. The reactions of his method (Killani synthesis) are summerised in outline. The new asymmetric center marked with an asterisk makes possible two product dia stereoisomers, which are formed in unequal amounts and differ in configuration at only one carbon atom (the 2 – position). These are called *epimers*.[1]

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[1] In chemistry, an **epimer** is a stereoisomer of another compound that has a different configuration at only one of several stereogeniccenters. Stereoisomers include enantiomers and diastereomers, both which contain a stereogenic center (excluding geometric isomers, which is a class of diastereomers).
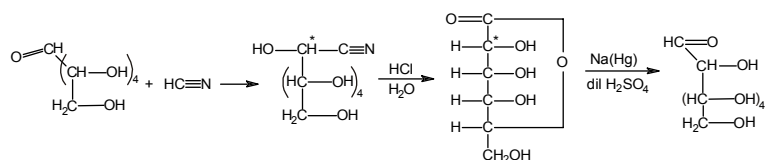
Vigorous reduction of the seven carbon lactone (I) with hydriodic acid results in the formation of heptanoic acid, This demonstrates the straight – chain nature of the carbon skeleton of glucose, as well as aldehyde (as opposed to keto) structure,
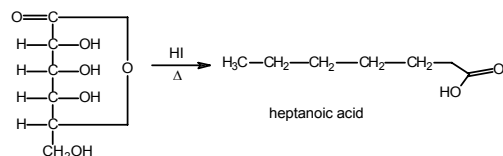
Degradation of aldoses to aldoses with one less carbon atom can be accomplished by either, in retrograde order of the Killani synthesis. This method is summerised in equation; the second degradation is the Ruff degradation.
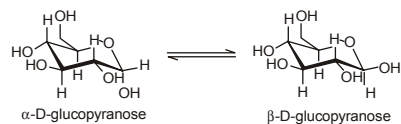
For example, the sugars α-glucose and β-glucose are epimers. In α-glucose, the -OH group on the first (anomeric) carbon is in the direction opposite the methylene group (in the axial position). In β-glucose, the -OH group is oriented in the same direction as the methylene group (in the equatorial position). These two molecules are both epimers and anomers.
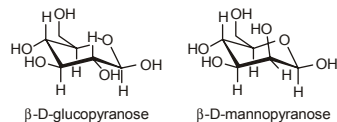
In this case, β-D-glucopyranose and β-D-mannopyranose are epimers because they differ only in the stereochemistry at the 2 position. The hydroxyl group in β-D-glucopyranose is equatorial (in the "plane" of the ring) while in β-D-mannopyranose the 2 hydroxyl group is axial (up from the "plane" of the ring). These two molecules are epimers but not anomers.In chemical nomenclature, one of the epimeric pairs is given the prefix **epi-** for example in quinine and *epi-quinine*. When the pairs are enantiomers, the prefix becomes **ent**.
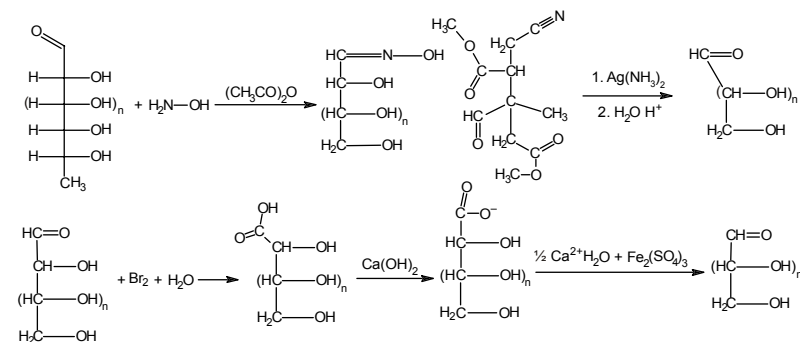
Conversion of aldose to a 2 ketose involves formation of the 1.2 – dicarboxyl compounds either by osazone synthesis and hydrolysis or by oxidation of the 2 – hydroxy group with hydrogen peroxide and ferric sulphate. The aldehyde group can be reduced preferentially with powdered zinc and acetic acid.

The reverse, conversion of a ketose to an aldose, is more equivocal. The first step is reduction of the ketose to the two corresponding stereo – isomeric polyols. Either of the two primary alcohols groups of a molecule of each polyol is then oxidized to each polyol is then oxidized to yield a lactone mixture. The lactone are then reduced to aldoses. Either the mixture of intermediate lactones or that of final aldoses must be separated by fractional crystallization.

possible separation
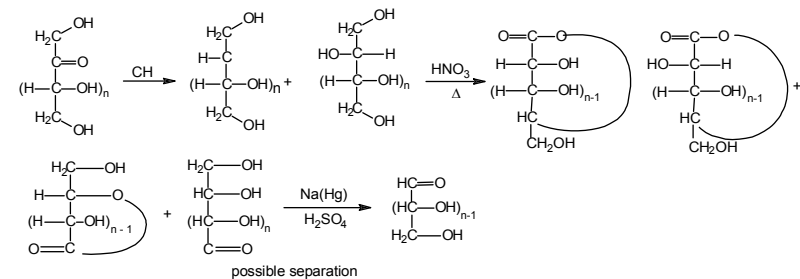
Conversion of an aldose to its epimer at C_2 is readily accomplished. In practice, epimerization depends upon formation of equilibrium between the two epimeric aldonic acid in boiling pyridine. Consequently the same equilibrium results by starting with either epimer.
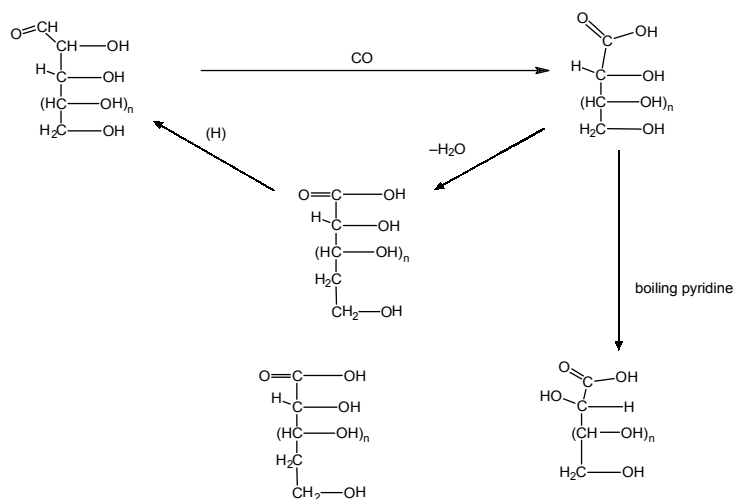
Another method of epimerization introduces the ketoses into the equilibrium. Treatment with dilute alkali operates through enedol forms to give the equilibrium mixture shown in the equation below.
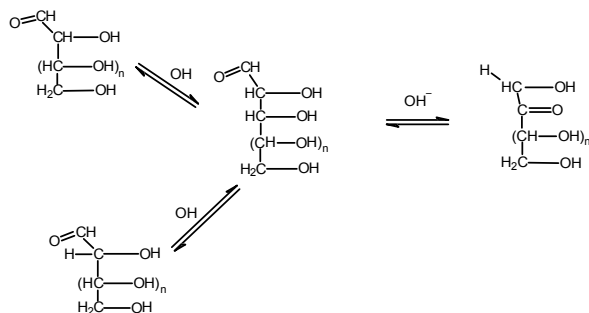
Osazone and the Conversion of Aldoses And Ketoses

As aldehydes or ketones the glucose also reacts with hydrazines and hydroxylamine. Phenyl – hydrazine $C_6H_5NHNH_2$ has provided of the greatest value in the separation identification and interconversion of

the various other monosaccharides, without the aid of this reagents the brilliant researches of Fischer in the sugar group would hardly have been possible.

When I mol of phenylhydrazine reacts with I mol of an aldose or ketoses, the first product is a normal hydrazone.

$$CH_2OH[CH.OH]_4.CHO{:}O + H_2N - NHC_6H_5 \rightarrow CH_2.OH\,[CH.OH]_4\,NH - N\,C_6H_5 + H_2O$$
Glucose phenyl hydrazone
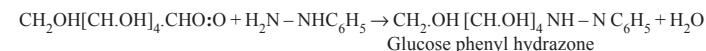
On warming with excess of phenylhydrazine, however, the hydrazone first formed is oxidized in such way that the CHOH group adjacent to the original aldehydic or ketonic group is converted into a CO group. The latter than combines with more phenyl – hydrazine to give a di – hydrazone containing the group I. These compounds are termed as osazones (see chemical properties).

Prior to Fischer's researches one of the greatest barriers to a wider knowledge of the monosachharides lay in the difficulty of spreading mixtures of these sugars by crystallization, owing to their high solubility in water and tendency to form syrups. The value depends on the fact that they are sparingly soluble and easily separable by crystallization, and in addition, from their characteristic melting – points and crystalline forms as seen under a microscope it is possible to identify.
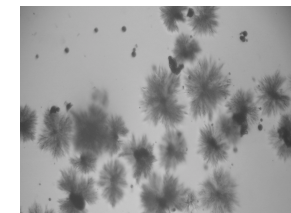
Fig. 2.2 : Showing the Osazones under microscope

The recognition and isolation of ketoses is a matter of some difficulty owing to their lack characteristic reactions, with secondary hydrazine, however, the ketoses give phenyl – methyl osazones by which they may be identified. Aldodses usually react with this base to form colourless hydrazones, which in all cases are readily distinguished or separated from the highly coloured osazones.

Osazones like hydrazones, are hydrolyzed on being heated with hydrochloric acid, when phenyl hydrazine is regenerated, The sugar originally employed, however, is not regained, as the group

$C(:N.NH.C_6H_6) - CH(:N.NH.C_6H_5)$ is converted into the group $- CO - CHO$. The compound so formed is thus and oxidation product of the original sugar, and is termed as *osone*

$CH_2OH.(CHOH)_3CO.CHO$ Glucosone.
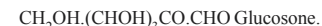
On mild reduction of this compound with zinc dust and dilute acetic acid, the aldehydic group remaining unchanged. In this case, therefore the sugar finally obtained is fructose in place of the glucose used as starting material.

In these reactions we have a general method of transforming an aldose into a ketose, according to the scheme.

$$\text{Aldose} \xrightarrow[\text{hydrazine}]{\text{Phenyl}} \text{Osazone} \xrightarrow{\text{hydrolysis}} \text{Osone} \xrightarrow{\text{rediction}} \text{Ketose}$$
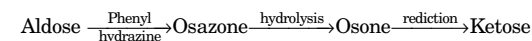
Further Structural Details of the Glucose Molecule

The data given by the idea that glucose is a mixture of tautomers involving equilibrium between free hydroxyaldehyde (2, 3, 4, 5 6 – pentahydroxyhexanal) and one or more internal hemiacetal forms.

Although there must be small amount of free aldehyde present, the principal substances in a solution of glucose are the hemiacetals and we must now enquire into their number and their structure. Experimentally, two hemiacetals α and β termed anomers are obtainable by special procedures. Thus, when glucose is crystallized from solution at room temperature, the a anomers is obtained. It has a specific rotation. + 111 when initially dissolved in water, but the rotation gradually falls to + 52.5.Crystallization of glucose at about 100 results in the β - anomers. The rotation of the solution of the β - anomers = + 19. The change in rotation with time, which is catalyzed by both acids and bases is called mutarotation and is caused by equilibrium mixture of the two containing 63% of β and 37% of an β anomers.

The mutarotation of glucose and the existence of two crystalline forms are accountable on the basis of the formation of cyclic hemiacetals (with introduction of an additional asymmetric center at carbon I) we have noted may times before that both five and six membered ring are readily formed and therefore sugar chemists had to understate a study of the ring size in sugars.
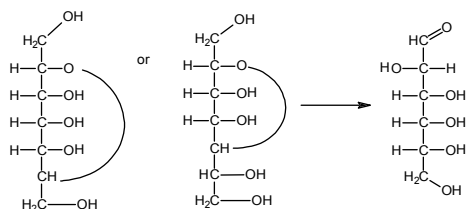
To ascertain the structure of the cyclic form of the glucose, the molecular configuration must be secured so that the molecule cannot change structure during a chemical reaction utilized form analysis. This apparently is achieved by complete methylation of glucose withy methyl sulphate and sodium hydroxide.
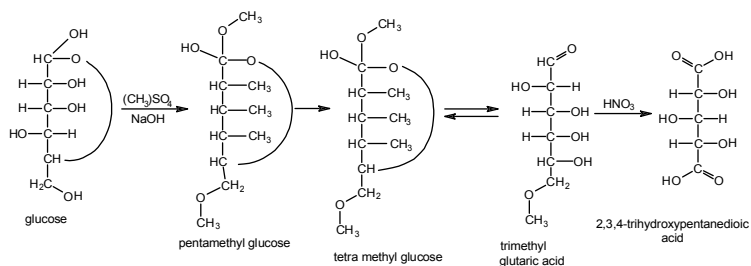
Hydrolysis of phentamethyl glucose in acid then frees the aldehyde group and the hydroxy group involved in the acetal linkage. Oxidation of the tetramethyl glucose occurs at the aldehyde group and the free hydroxy group. Upon oxidation the tetramethyl glucose forms a trimethoxy glutaric acid. These reactions are summerised in the above equation. Those sugar

or sugar derivatives having a five membered ring lead to dimethoxy succcinic acids by the Haworth Procedure. Since the six membered ring is structurally related to pyran a six membered acetal ring in a sugar is called a pyranose ring. A furanose ring because of its relation to furan.
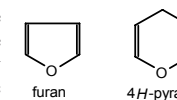
Structural analysis of the aldoses and the ketoses follows a pattern much like that outline d for glucose.

Cyclic Formulas For Glucose

After this work determining the relative configurations of the aldohesoses and aldopentoses. Emil Fischer devises a projection formation to represent the open(aldehydo) forms of these monosaccharides. In the Fischer projection, the carbon chain is represented vertically and the bonds from each carbon atom to its neighbouring carbon atoms are thought of a lying behind the carbon atom under consideration. The hydrogen atoms and hydroxy groups are considered to project up in front of the carbon atom to which they are attached. Thus the configurationally formula, II, represents a molecule in which all hydroxy groups are on the right side and all hydrogen atoms on the left side and the middle of the carbon chain loops upon towards the observer III the top carbon atom is the I position.
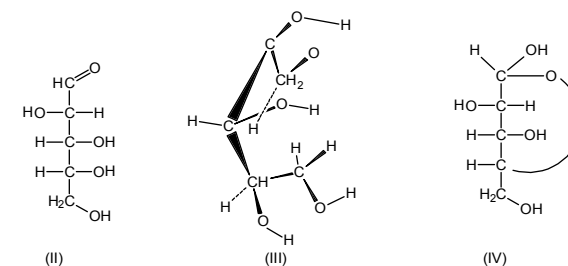
Although the Fischer projection serves well enough for the open forms of the monosaccharides it is afterward when used to represent cyclic forms such as IV, The long bent " bond" between the oxygen atom and one of the carbon atoms does not represent the situation well; well hence it use is misleading in a formula intending to be a spatial representation of a molecule. To correct this, Haworth devised a modified projection formula which represent carbohydrates in conventionalized cyclic forms. The ring oxygen is usually placed at the upper right, with the carbon atoms placed in clockwise order of numbering after the oxygen atom. Hydroxy group placed to the right if Fischer projection of α - D fructofuranose and of the anomeric β - D fructofuranose.

α D fructofuranose
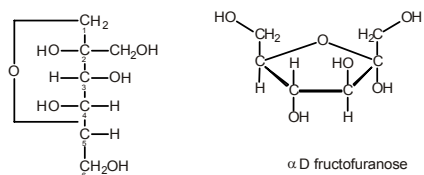
In the oligosaccharides and polysaccharides the virtues of the Haworth projections are even more striking since, the links between units as well as the rings oxygens must be distorted in Fischer projections, where as Haworth projections cope well with this feature.

Sometimes the Haworth projection is inverted or reversed so as to avoid other obstacles to clear formulation. The projection is a picture of the ring with the top always farthest from the viewer.

Compare the formulas for β - D fructofuranose below with the given above.

β-D-Fructofuranose

Inverted

reversed

rotated
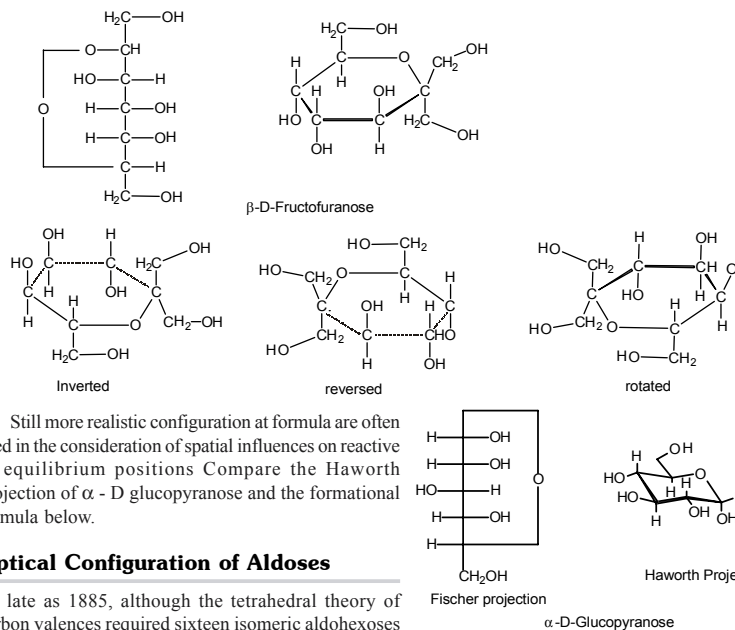
Still more realistic configuration at formula are often used in the consideration of spatial influences on reactive of equilibrium positions Compare the Haworth projection of α - D glucopyranose and the formational formula below.

Optical Configuration of Aldoses

As late as 1885, although the tetrahedral theory of carbon valences required sixteen isomeric aldohexoses

Fischer projection

Haworth Projection

α-D-Glucopyranose

and eight aldopentoses, and although several of the required isomers were recognized, the configurations of the monosaccharides remained unknown. It was the great structural chemist, Emil Fischer, who basing much of his work on the studies of Killani, Wöhl and Ruff, solved the problem required.

The point of departure is the establishment of epimeric relationship and relationships among the aldoses via Killani synthesis and the Wöhl and Ruff degradation. These relationships are given in the table. Corresponding to the epimeric and series relationships are the configuration relationship of aldoses shown in the fig 15. In this figure the molecular configuration are indicated by abbreviated Fisher projections using triangles to represent the orientation of hydroxy groups relative to the backbone of the molecule the orientation of hydroxy groups relative to the back of the molecule, which is the vertical line connecting the circle and triangle. Since the optically opposite series, but in minor image position, the configurational relationships of only one series need be considered. The D – series aldoses are aldoses discussed in the analysis. These are the aldoses that can be built up D – glyceraldehydes by successive Killani synthesis and, therefore, have the lowest asymmetric carbon in the Fisher convention attached to hydroxy on the right hydrogen on the left.

Table : Showing the Table of Killani Synthesis series

Triose	Tetroses	Pentoses	Hexoses
D-Glyceraldehyde	D-threose	D-lyxose	D-galactose
			D-talose
		D-xylose	D-idose
			D-gulose
	D-erythrose	D-arabinose	D-glucose
			D-mannose
		D-ribose	D-allose
			D-altrose

It should be apparent at first glance the whereas D – glyceraldehydes can be assigned by convention the configuration, the relationship between the other numbered formulæ and the aldoses listed in the table above are by no means self – evident. Tetrose 2 might be either D – threose or D – erithrose. To ascertain the configurational identity of the various aldoses. Fisher resorted to oxidations leading to glyceric (saccharic)acids. These yield structurally symmetrical compound of which a certain number should be meso acids. For example oxidation of ribose and xylose by nutric acid yields optically inactive hence, these two aldopentoses must be 4 and 6 (not necessary in order. To check this the carbonyl groups can be indicated by another sign like X and the configuration of the molecule checked for a plane of symmetry.

Lyoxose and arbinose, which yield optically active glyceric acids, are 5 and 7 (not necessarily in order). Tallose and galactose are epimers derivable from lyxose glucose and mannose epimers derivable from arbinose. Of these hexode only galactose, yield a meso glyceric acid hence galactose is 14 and

Tallose 15. Lyxose must by 7, arbinose 5 Xylose, the epimer of lyxode is 6 and ribose, the epimer of lyxose is 6 and ribose, the epimer of arbinose, is 4. Another inactive acid is produced by allose. Hence, allose is 8. Altrose which yields an optically active acid and is the epimer of allose is 9.
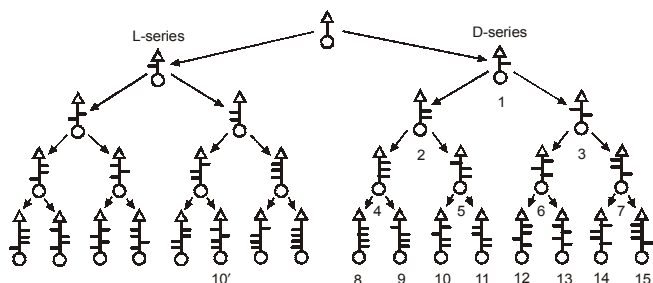
Fig. 2.4 : The Configurational Relationship of Aldoses

To establish the configuration of glucose recourse was made to Killani synthesis to the aldoheptoses. Oxidation of the two aldoheptoses yields one optically active glyceric acid and one meso acid. Consideration of formula 10 and 11, the epimeric hexoses related to arbinose, shows that the result substance which produces this result must have the configuration 10. Hence the glucose has the configuration 10. Mannose the epimer of glucose, which yields two optically active heptaglyceric acid is 11.

Upon oxidation, glucose gives glyceric acid specifically named v saccharic acid. Glucose yields enantimorph L – saccharic acid. Since D – glucose cannot belong to the L – series, this means that when the aldehyde group and the primary alcohol group lose their identify by transformation to carboxyl groups, the same result unuses as when L – glucose is oxidized. To see the relationship, one rotates a model of glucose 180°C so that its aldehyde group coincides with the primary alcohol group of L - glucose 10 and its primary alcohol group coincides with the aldehyde group of L glucose. The formula which fits this description is 12. The remaining hexode iodose is 13.

Occasionally one wishes to indicate the direction of optional rotation od an optically active rotation of an optically active material. This is done by placing (+) or (%) between the series indication and the remainder of the name, Thus D (+) glucose D(%) ribose D(+) glyceraldehydes and D(%) tartaric acid are examples.

Analysis of Glucose

Glucose gives the following series of reactions during analysis; caustic alkalies added to a solution of glucose and warmed, produce a brown solution.

Exp: Add a few drops of sodium hydroxide solution to a dilute solution of glucose, and warm gently. The colour of this liquid turns from yellow to brown.

Glucose reduces an Ammonical – Silver oxide solution to metallic silver which gets deposited on heating.

Exp: Add a few drops of Tollen's Reagent (see A chemical Analyser's Guide) glucose solution and heat the mixture in a water bath, for few minutes the metallic silver is deposited

The following reaction which is given by all soluble carbohydrate is Molisch's Test

Exp: Add 1 ml of alcoholic a - naphthol solution (Molisch's reagent) (see A chemical Analyser's Guide)to a solution of glucose, then 1 ml of conc. Sulphuric acid is allowed to run down by the side of the tube so that a junction of two layers are formed which slowly turns blue

An alkaline copper sulphate solution is reduced and cupric oxide is precipitated.

Exp: Add a few drops of copper sulphate solution to a solution of glucose and add a few drops of sodium hydroxide when the solution is heated the colour changes from blue to red and a red precipitate of cuprous oxide is produced

Quantitative Test of Glucose

The above reaction is utilized for the quantitative estimation of glucose as well as other sugars. A standard solution of copper sulphate is prepared by dissolving 34.64 gm in 500ml (.434mol/L) A second solution is made by dissolving 60 gms of sodium hydroxide in 500ml water. This solution when mixed and run into a flask and boiled in a measured amount, and the sugar solution is added gradually from a burette into the hot solution mixture until all the cuprous oxide is precipitated. The quantity of sugar solution taken is a measure of the amount of glucose present 10cc of the alkaline copper solution corresponds to 0.05 gm of glucose.

Use

We can speculate on the reasons why glucose, and not another monosaccharide such as fructose (Fru), is so widely used in evolution, the ecosystem, and metabolism. Glucose can form from formaldehyde under abiotic conditions, so it may well have been available to primitive biochemical systems. Probably more important to advanced life is the low tendency of glucose, by comparison to other hexose sugars, to non-specifically react with the amino groups of proteins. This reaction (glycation) reduces or destroys the function of many enzymes. The low rate of glycation is due to glucose's preference for the less reactive cyclic isomer. Nevertheless, many of the long-term complications of diabetes (e.g., blindness, kidney failure, and peripheral neuropathy) are probably due to the glycation of proteins or lipids. In contrast, enzyme-regulated addition of glucose to proteins by glycosylation is often essential to their function.

As an energy source

Glucose is a ubiquitous fuel in biology. It is used as an energy source in most organisms, from bacteria to humans. Use of glucose may be by either aerobic or anaerobic respiration (fermentation). Carbohydrates are the human body's key source of energy, through aerobic respiration, providing approximately 3.75 kilocalories (16 kilojoules) of food energy per gram.[4] Breakdown of carbohydrates

(e.g. starch) yields mono- and disaccharides, most of which is glucose. Through glycolysis and later in the reactions of the Citric acid cycle (TCAC), glucose is oxidized to eventually form CO_2 and water, yielding energy, mostly in the form of ATP. The insulin reaction, and other mechanisms, regulate the concentration of glucose in the blood. A high fasting blood sugar level is an indication of prediabetic and diabetic conditions.Glucose is a primary source of energy for the brain, and hence its availability influences psychological processes. When glucose is low, psychological processes requiring mental effort (e.g., self-control) are impaired.

FRUCTOSE

Introduction

Fructose (also **levulose** or **laevulose**) is a simple reducing sugar (monosaccharide) found in many foods and is one of the three most important blood sugars along with glucose and galactose. Honey, tree fruits, berries, melons, and some root vegetables, such as beets, sweet potatoes, parsnips, and onions, contain fructose, usually in combination with sucrose and glucose. Fructose is also derived from the digestion of sucrose, a disaccharide consisting of glucose and fructose that is broken down by glycoside hydrolase enzymes during digestion. Fructose is the sweetest naturally occurring sugar, estimated to be twice as sweet as sucrose. Fructose is often recommended for, and consumed by, people with diabetes mellitus or hyperglycemia, because it has a very low glycemic index (GI) relative to cane sugar (sucrose). However, this benefit is tempered by concern that fructose may have an adverse effect on plasma lipid and uric acid levels, and the resulting higher blood levels of fructose can be damaging to proteins (see below). The low GI is due to the unique and lengthy metabolic pathway of fructose, which involves phosphorylation and a multi-step enzymatic process in the liver. See health effects and glycation for further information.

Synthesis

Fructose can be obtained from cane sugar by hydrolysis with sulphuric acid in dilute state. The acid id removed by the precipitation with barium carbonate and the filtrate is concentrated. Milk of lime is then added, when the lime compound or the calcium fructosate (corresponding to calcium glucosate which is only slightly soluble separates out and is filtered and washed. The calcium compound is then suspended in water and decomposed by carbon dioxide. The solution is again filtered from calcium carbonate and evaporated. On introducing a crystal of fructose into the syrup, the later crystallizes.

Fructose is also prepared from inulin which turns to fructose inulin which turns to fructose on hydrolysis with sulphuric acid. After removing the acid the liquid is evaporated preferably under reduced pressure. When a syrup is left which solidifies if a crystal of the solid is added.
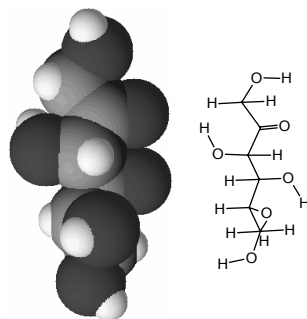
Physical Properties

Molecular Formula	= $C_6H_{12}O_6$
Formula Weight	= 180.15588
Composition	= C(40.00%) H(6.71%) O(53.29%)
Melting point	= D-fructose: 103°C
Molar Refractivity	= 37.42 cm^3
Molar Volume	= 113.3 cm^3
Parachor	= 351.7 cm^3
Index of Refraction	= 1.574
Surface Tension	= 92.6 dyne/cm
Density	= 1.589 g/cm^3
Polarizability	= 14.83 10^{-24}cm^3
Monoisotopic Mass	= 180.063388 Da
Nominal Mass	= 180 Da
Average Mass	= 180.1559 Da
Log P	= -1.63

Chemical Properties and Constitution

Fructose is more soluble than glucose but crystallizes from rhombic prisms which melt at 103°C. it has a sweet taste and gives many of the reactions of glucose. Although fructose is not an aldehyde but a ketone, it nevertheless reduces alkaline copper solution. This is due to the presence of the easily oxidisable group – CO_2 – $CH_2(OH)$ with phenylhydrazine, fructose yields an osazone, which is identical with glucosazone. Fructose also undergoes fermentation with yeast, through less readily than glucose, glucose being first removed when a solution of the two sugars is fermented. Like glucose fructose undergoes mutarotation in solution the specific rotation rising from –135.5 to –92.0°

Fructose forms a pentacetyl derivative like glucose. On reduction it is converted into a mixture of sorbitol and mannitol, since an additional asymmetric combination has been introduced. On oxidation it does not like glucose form an acid with same number of carbon atom but breaks up into formic acid and trihydroxybutaric acid. This decomposition points to the presence of a ketone group in the molecule, which is further confirmed by the following reactions.

fructose   Cyanohydrin of fructose   hydroxy acid
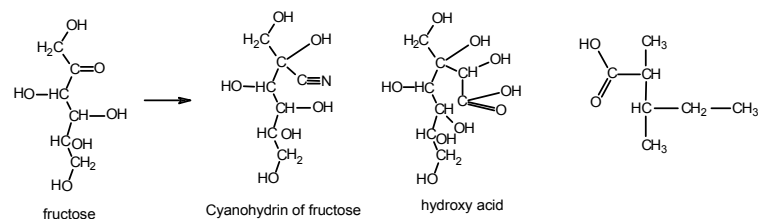
The above formula for fructose agrees moreover, with the synthesis of inactive fructose from the mixture glyceric aldehyde and dihydroacetone and with the production of the same osazone as that obtained from glucose, a reaction in which the two end carbon atoms of the chain are involved.

phenylhydrazine   fructose phenylhydrazone

fructose

phenylhydrazine

fructose phenylhydrazone

(5Z)-5,6-bis(2-phenylhydrazinylidene)hexane-1,2,3,4-tetrol

Phenyl glucosazone
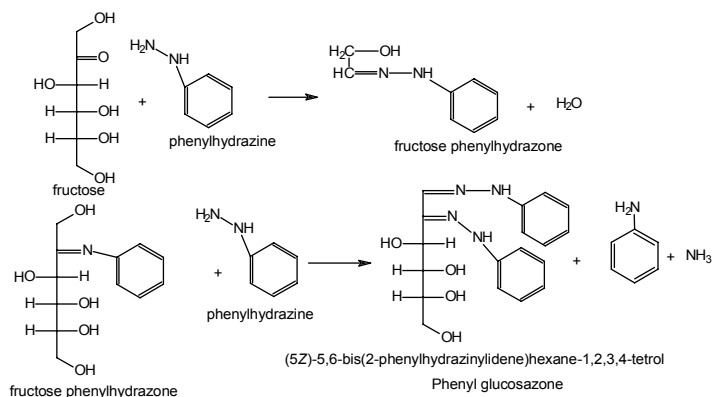
Although fructose in many of its reactions a ketone its exists like glucose in a and stereoisomeric pyranose forms and also in γ or furanose rings

Keto fiormula fructose   α-D-Fructopyranose   β-D-Fructopyranose   α-D-Fructofuranose
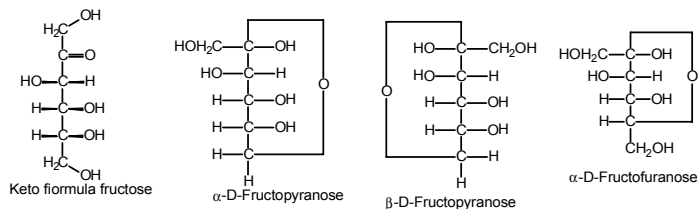
The perspective formulæ of α and β fructopyranose are γ - fructose is a constituent of cane sugar but when liberated from its union with glucose passes into the pyranose form.

α-D-Fructofuranose   β-D-Fructopyranose
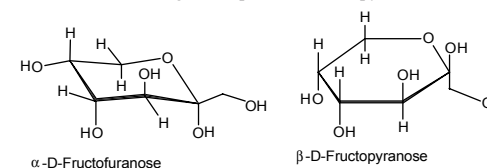
Uses

Fructose is often recommended for diabetics due to its glycemic index being significantly lower than both glucose, sucrose and starches. The medical profession thinks fructose is better for diabetics than sugar, but every cell in the body can metabolize glucose. However, all fructose must be metabolized in the liver. The livers of the rats on the high fructose diet looked like the livers of alcoholics, plugged with fat and cirrhotic This is not entirely true as certain other tissues do use fructose directly, notably the cells of the intestine, and sperm cells (for which fructose is the main energy source).

Fructose is a reducing sugar, as are all monosaccharides. The spontaneous addition of single sugar molecules to proteins, known as glycation, is a significant cause of damage in diabetics. Fructose appears to be as dangerous as glucose in this regard and so does not seem to be a better answer for diabetes for this reason alone. This may be an important contribution to senescence and many age-related chronic diseases. Fructose is used as a substitute for sucrose (composed of one unit each of fructose and glucose linked together with a relatively weak glycosidic bond) because it is less expensive and has little effect on measured blood glucose levels. Often, fructose is consumed as high fructose corn syrup, which is corn syrup (glucose) that has been enzymatically treated by the enzyme glucose isomerase. This enzyme converts a portion of the glucose into fructose thus making it sweeter. This is done to such a degree as to yield corn syrup with an equivalent sweetness to sucrose by weight. While most carbohydrates have around the same amount of calories, fructose is sweeter and manufacturers can use less of it to get the same result. The free fructose present in fruits, their juice, and honey is responsible for the greater sweetness of these natural sugar sources. Some studies point to fructose as key factors in hyperactivity and tooth decay in children.

GALACTOSE

Galactose (Gal) (also called **brain sugar** is a type of sugar which is less sweet than glucose and not very water-soluble. It is considered a nutritive sweetener because it has food energy. Galactan is a polymer of the sugar galactose. It is found in hemicellulose and can be converted to galactose by hydrolysis.
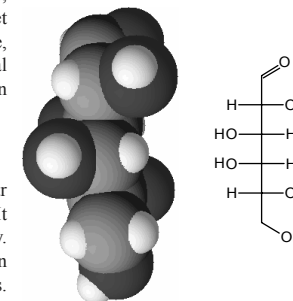
It is found in dairy products, in sugar beets and other gums and mucilages. It is also synthesized by the body, where it forms part of glycolipids and glycoproteins in several tissues.

Relationship to Lactose

Galactose is a monosaccharide constituent, together with glucose, of the disaccharide lactose. The hydrolysis of lactose to glucose and galactose is catalyzed by the enzyme lactase, a β-galactosidase. In the human body, glucose is changed into galactose in order to enable the mammary glands to secrete lactose. Galactose and glucose are produced by hydrolysis of lactose by β-galactosidase. This enzyme is produced by the *lac* operon in *Escherichia coli* (*E. coli*).

Clinical Significance

Two studies have suggested a possible link between galactose in milk and ovarian cancer. Other studies show no correlation, even in the presence of defective galactose metabolism. More recently, pooled analysis done by the Harvard School of Public Health showed no specific correlation between lactose containing foods and ovarian cancer, and showed statistically insignificant increases in risk for consumption of lactose at >30 g/d. More research is necessary to ascertain possible risks. There are some ongoing studies which suggest that galactose may have a role in treatment of focal segmental glomerulosclerosis (a kidney disease resulting in kidney failure and proteinuria). This effect is likely to be a result of binding of galactose to FSGS factor.

Structure and Isomerism

The first and last –OH groups point the same way and the second and third –OH groups point the other way. D-Galactose has the same configuration at its penultimate carbon as D-glyceraldehyde. Galactose is a diastereomer of glucose.

Metabolic Disorders

There are 3 important disorders involving galactose:

Name	Enzyme	Description
Galactosemia	Galactokinase	Causes cataracts and mental retardation. If a galactose-free diet starts sufficiently early, the cataracts will regress without complications however neurological damage is permanent
UDPgalactose-4-epimerase deficiency	UDPgalactose-4-epimerase	Is extremely rare (only 2 reported cases). It causes nerve deafness
Galactose-1-phosphate uridyl transferase deficiency	Galactose-1-phosphate uridyl transferase	Is the most problematic, as galactose-free diets do not have considerable long-term effects

MANNOSE

Mannose is a sugar monomer of the hexose series of carbohydrates.

Formation

Mannose can be formed by the oxidation of mannitol.

It can also be formed from D-glucose in the Lobry-de Bruyn-van Ekenstein transformation

Etymology

The root of both "mannose" and "manitol" is manna, which the Bible records as the food supplied to the Israelites during their journey through the Sinai Peninsula. Manna is a sweet secretion of several trees and shrubs, such as *Fraxinus ornus*.

Cranberry juice

D-Mannose, which appears in some fruits including cranberry, has been postulated to prevent the adhesion of bacteria to the epithelium of the urinary tract and Urinary bladder through a mechanism presumed to be competitive in nature with the polysaccharide coating of the cystic epithelium. While no studies have objectively verified this mechanism, anecdotal reports from patients show some improvement in symptoms.

Configuration

The fact that D-mannose has the same configuration at its penultimate carbon as D-glyceraldehyde is unsurprising as that is what defines the dextro classification. However, mannose differs from D-glucose by inversion of the C2 chiral centre. This apparently simple change leads to the drastically different chemistry of the two hexoses, as it does the remaining six hexoses.

Metabolism

Mannose enters the carbohydrate metabolism stream in two steps:

- First it undergoes phosphorylation to mannose-6-phosphate by hexokinase.
- Then, it is converted to fructose-6-phosphate by mannose phosphate isomerase.

Mannose is present in numerous glycoconjugates including N-linked glycosylation of proteins. C-mannosylation is also abundant and can be found in collage-like regions. Mannose is a C2 epimer of glucose and displays a $^2C_4$ pucker in the solution ring form.
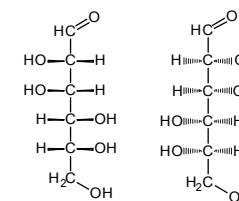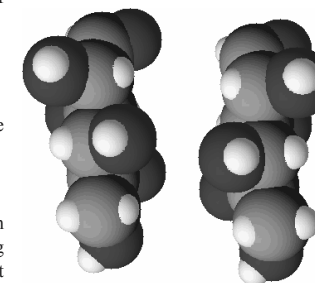
Pentoses

ARBINOSE

Arabinose is an aldopentose — a monosaccharide containing five carbon atoms, and including an aldehyde (CHO) functional group. It has chemical formula $C_5H_{10}O_5$ and a molar mass of 150.13 g/mol.For biosynthetic reasons, saccharides are almost always more abundant in nature as the "D" form, or structurally analogous to D-(+)-glyceraldehyde. However, L-arabinose is in fact more common than D-arabinose in nature and is found in nature as a component of biopolymers such as hemicellulose and pectin. The L-arabinose operon is a very important operon in molecular biology and bioengineering. A classic method for the organic synthesis of arabinose from glucose is the Wohl degradation.
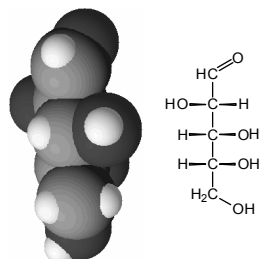
RIBOSE

Ribose, primarily seen as **D-ribose**, is an aldopentose — a monosaccharide containing five carbon atoms, and including an aldehyde functional group in its linear form. It has the chemical formula $C_5H_{10}O_5$, and was discovered in 1905 by Phoebus Levene.As a component of the RNA that is used for genetic transcription, ribose is critical to living creatures. It is related to deoxyribose, which is a component of DNA. It is also a component of ATP, NADH, and several other chemicals that are critical to metabolism.Refer to the article on deoxyribose for more information on both sugars, how they relate to each other, and how they relate to genetic material.
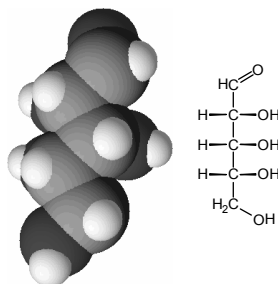
Physical Properties

Molecular Formula	= $C_5H_{10}O_5$
Formula Weight	= 150.1299
Composition	= C(40.00%) H(6.71%) O(53.29%)
Melting Point	= 99 °C
Molar Refractivity	= 31.41 cm^3
Molar Volume	= 99.5 cm^3
Parachor	= 299.0 cm^3
Index of Refraction	= 1.543

Surface Tension	= 81.4 dyne/cm
Density	= 1.508 g/cm^3
Polarizability	= 12.45 $10^{-24}cm^3$
Monoisotopic Mass	= 150.052823 Da
Nominal Mass	= 150 Da
Average Mass	= 150.1299 Da

RIBULOSE

Ribulose is a ketopentose — a monosaccharide containing five carbon atoms, and including a ketone functional group. It has chemical formula $C_5H_{10}O_5$. Two enantiomers are possible, D-ribulose (D-erythro-pentulose) and L-ribulose (L-erythro-pentulose). D-Ribulose is the diastereomer of D-xylulose.Ribulose sugars are composed in the pentose phosphate pathway. They are important in the formation of many bioactive substances. For example, D-ribulose is an intermediate in the fungal pathway for D-arabitol production. Also, as the 1,5-bisphosphate, D-ribulose combines with carbon dioxide at the start of the photosynthetic process in green plants (carbon dioxide trap).
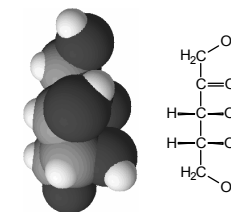
A synthetic form of ribulose known as sucroribulose is found in many brands of artificial sweeteners.

XYLOSE

Xylose or wood sugar is an aldopentose — a monosaccharide containing five carbon atoms and including an aldehyde functional group. It has chemical formula $C_5H_{10}O_5$. Xylose is found in the embryos of most edible plants.

In animal medicine, xylose is used to test for malabsorption by administering to the patient in water after fasting. If xylose is detected in blood and/or urine within the next few hours, it has been absorbed by the intestines.
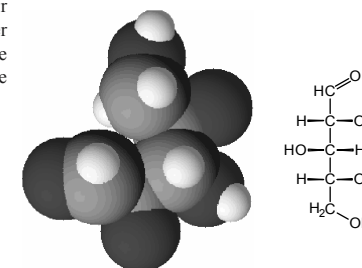
Physcial Properties

Molecular Formula	= $C_5H_{10}O_5$
Formula Weight	= 150.1299
Composition	= C(40.00%) H(6.71%) O(53.29%)
Melting Point	=144-145 °C
Molar Refractivity	= 31.41 cm^3
Molar Volume	= 99.5 cm^3
Parachor	= 299.0 cm^3
Index of Refraction	= 1.543
Surface Tension	= 81.4 dyne/cm
Density	= 1.508 g/cm^3
Polarizability	= 12.45 $10^{-24}cm^3$
Monoisotopic Mass	= 150.052823 Da
Nominal Mass	= 150 Da
Average Mass	= 150.1299 Da

Oligosaccharides

Disaccharides

SUCROSE

Sucrose (common name: **table sugar**, also called **saccharose**) is a disaccharide (glucose + fructose) with the molecular formula $C_{12}H_{22}O_{11}$. Its systematic name is α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside (ending in "oside", because it's not a reducing sugar). It is best known for its role in human nutrition and is formed by plants but not by other organisms such as animals.
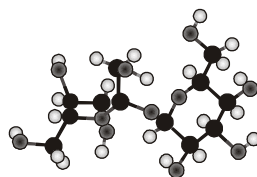
Synthesis of Sucrose

The manufacture of cane sugar is based on gradual removal of fibre, non-sugars, water, colouring matter and non crystallisable sugars.

(a) Fibre is removed by crushing and milling;

(b) Non-sugars are removed by clarification;
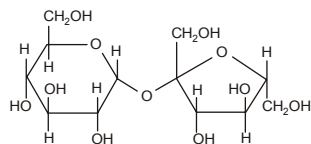
(c) Water is removed by evaporation and centrifuging;

(d) Colouring matter is removed by bleaching *or* absorption by bone charcoal *or* activated carbon;

(e) Non-crystallisable sugars are eliminated during crystallisation. White sugar is produced either in two steps *or* in one step.

(a) In two steps method, first raw *or* un-refined crystalline sugar is manufactured, and then white sugar is manufactured by refining raw sugar.

(b) In one step process in which chrification is done by sulphitation *or* double carbonation method. In this process the syrup is bleached by sulphur dioxide and the thin film *of* molasses on the crystals is finally removed by double centrifuging.

Extraction of the Juice or Separation of the Fibre from the Juice (Fig. 17)
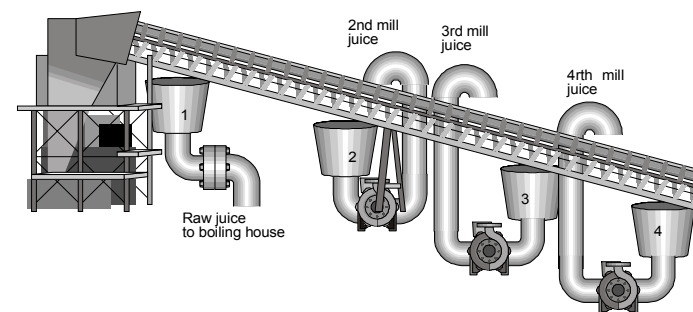
Fig. 2.4 : Showing the Pressing of Sugar Cane

The process of extraction consists of

(a) Cutting of the stalk and leaves.

(b) Cutting of the canes by means of sharp rapidly revolving knives (550-600 r.p.m.) fitted on a horizontal shaft 2" inches apart. The canes move on a carrier under the knives which make diagonal cuts, converting the canes into chips in which the soft portion is exposed to the maximum.

(c) The chips are then brought to the extractor consisting of one two- rolled toothed crusher and four three-rolled grooved mills. (In India, the crusher and the mills are mostly made of grey cast iron; in other places it is made *of* cast steel)

The three rollers of the mill are placed at the crusher of a triangle; only the top roller of the crusher or of the mill moves, the bottom rollers in the milling train remain stationery during operation. A

pressure of 3 to 6 tons per sq. in. is maintained on each of the top roller. There are intermediate carriers between the crusher and the first mill and between the two subsequent mills to carry the bagasse to the next mill or out of the milling train. The crusher and the first mill are provided with vibrating screens and arrangement for transferring the juice to the raw juice tank. The 2nd, 3rd and 4th mills are provided with containers to hold unstrained juice which is used for combined imbibition process (Fig17).

When the chips are fed into the crusher, the chips during their passage through the rollers are pressed to extract about 50 % of the juice in the cane. The bagasse (the fibre) containing 50% of the juice is fed into the first mill. The juice from these two are strained on the strainer below and disposed as mentioned .above. The bagasse still containing some juice pass successively through -the second, third and fourth mills and then to the storage or to the kiln in which it is fed for steam raising. Even in recent times, most of the Indian mills used bagasse as fuel, but the present tendency is to replace it by coal or coke.

Compound Imbibition Process

After the crusher, the baggasse has to be, in all cases, moistened for efficient pressing. Had it been done by water the sugar extracted would have been less; hence the maximum amount of sugar is pressed out by combined imbibition process. In this process the juice from the second mill is sprayed all the baggasse as it passes from' the crusher and the first 'milt. The juice from the third mill is sprayed between first and second mill, juice from the fourth mill is sprayed between the second and thud mills, while the baggasse passing from the third to the fourth mill is sprayed with hot water. About 25 % of the weight of the cane is used in the imbibition process.

Clarification

Why clarification is done?

Cane juice contains

78 -86 % water

10% -20 % sucrose

0.5- 1 % organic matter

0.5- 2.5 % reducing sugar

0.3- 0.77% ash

Besides these the cane juice contains finely divided pith and fibre and also some colloidal matters. These substances together with gums, pectin's, proteins and other non-sugars definitely hamper crystallisation of sucrose. Some of these are also responsible for the colouring of the crystallized sugar.

The process of clarification by which the above impurities are eliminated is, therefore, an important step in the production of maximum quantity of white crystalline sugar. In simple defecation by lime the impurities are not completely eliminated; hence by crystallising the syrup (the concentrated form of juice) a coloured crystalline sugar known as raw sugar is obtained. This raw sugar is then converted into white crystalline sugar by a refining process.

In most of the countries, white sugar is produced by the above-mentioned process. In India"Java and some other parts of the world white sucrose is produced by one step. In these places clarification

is done either by sulphitation or carbonation process. In the sulphitation and sometimes in the carbonation process also the concentrated juice is. treated with sulphur dioxide. In carbonation process, more. efficient clarification is done by double carbonation process. Hence these processes are known as double sulphitation and double carbonation processes.   .

Lime Defecation Process: The cane juice is acid having pH from 5.1 to 5.7. This acidity helps the inversion of sucrose to reducing non-crystallisable sugars. For this reason the juice after extraction should be made alkaline without delay. If acidity is removed by liming, ie. by adding adequate amount of milk of lime, then insol1.ible salts of organic acids such as oxalic and' propionic are precipitated. Lime reacts with phosphoric' acid or insoluble, phosphates to form precipitate of. tricalcium phosphate Now 'when the alkalinity is increased, gums, pectins, and proteins, etc. which are in colloidalform are precipitated due to coagulation in presence of alkali. These colloidal matters are also precipitated by being entrained in the precipitates of phos-phates etc. Along with colloidal matter finely divided pith and fibre and also clay are precipitated.

The raw cane juice is heated in juice heaters to 60°C and then limed to a pH of about 8. The mixing is helped by stirring. The limed juice is then passed through brass tubular heater, where it is heated to boiling. The hot limed juice is then pumped into settling tank for subsidization of the scum and the mud. By heating the protein matter is coagulated and settling of the precipitates is accelerated. In the settling tank the hot limed juice separates into three layers." The dear juice in the middle lies between the top la.yer of scum (consisting of fine fibre particles) and the bottom layer of mud (consisting of the heavy precipitates. The scum is skimmed of and the clear juice is allowed to drain off through a pipe which is gradually lowered to a level above the mud layer. The clear juice is sent to the boiling house for evaporation to syrup. The mud and the scum are filtered in a filter press. The cakes are washed during filtering (the wash water is sent back to the clarifier), and then dried. These tilter cakes contain sucrose and phosphate. Hence, the added cakes are used as manure after rotting for a year. This removes the sucrose which attracts white ants.

Clarification by Sulphitation : To prepare white sugar by One step clarifica-tion is done in India by passing sulphur dioxide gas through limed juice. The production of white is further helped by bleaching the syrup by sulphur dioxide. Better clarification takes place due to two causes:

(1) By adding more lime, more collidal matters and non-sugars are precipitated, because increased alkalinity increases the coagulation of colloids;

(2) By addition of precipitates of calcium sulphite formed by the action of sulphur dioxide on lime as well as on the ttricalcium sucrate ($C_{11}H_{22}O_{11}$, $3ao.-3so_2 = 3CaSO_3—C_{12}H_{22}O_{11}$) there takes place the precipitation of more colloidal matter by entrainment. The heavy precipitate of calcium sulphite also brings about quick and better subsidization. Sulphur dioxide also helps to bleach the juice.

Sulphur dioxide is passed from the bottom of a tank (with conical bottom) fitted with perforated baffles (at different levels) down which heated (temp. 140°F) limed cane juice comes down in a shower. The gas is passed until the pH of the resultant solution is '7 (neutral). The discharge from the sulphitation tank is heatf;d almost to boiling point in a second juice heater. The amount of lime added is more than that added in simple defecation process (2-4 lbs of lime per ton of cane). From the second

heater the hot fluid containing suspended precipitates is sent to the settling tank, where, after subsidization, the discharge separates into dear juice and mud. The subsequent operations have been already mentioned.

Clarification by Carbonation: In India a *few* factories have substituted the sulphitation process by double carbonation process. In this process, the limed juice is treated with carbon dioxide gas to precipitate the excess of lime as $CaCO_3$. This $CaCO_3$ subsequcntly acts like $CaSO_3$ in the sulphitation process. CaCOs is formed by the action of CO: on $Ca(OH)_2$ as well as on $C_{12}H_{22}O_{11}$ 3CaO (tricalcium sucrate). In this process much more lime is added than in sulphitation process. (1-1.5 p.c. of lime on the weight of cane). This amount of lime works out to be 16-24 Ibs of lime per ton of ca.ne. Due to the large alkalinity prodliced by such excessive amount of lime, the removal of non-sugar and colouring matter become more complete. Moreover, due to the formation of much larger quantity of insoluble precipitate in the juice, the subsidization becomes quicker and clarification becomes more effective. Due to removal of solu.ble impurities more completely the resultant syrup becomes more mobile and more sucrose crystallises cut. Due to the mobility of the syrup the movement of the syrup up and down the calendria in the striking pan becomes easier. This ensures uniform heating and better crystallization. Had sulphur dioxide been used for the CO_2, very large amount of valuable sulphur would have been necessary. Hence, the tenuency in Indian mills is to take to carbonation process. In carbonation process, the excessive amount of lime precipitates the gums, pectins, etc. much more completely when liming takes place below 60°C. But when the alkalinity is neutraliscd by CO_2 ,these-precipitates pass into solution. Also these precipitates become soluble near boiling point of the juice. Hence, instead of single carbonation, double carbonation is adopted. (Described below).

First, cane juice is heated to 55°C and limed by adding milk of lime to the heated juice. Temperature during liming is not allowed to rise above 55°C. Keeping the limed juice at this temperature in ajacketed tank carbon dioxide obtained by heating lime stone in a kiln, is pumped.through the limed juice until the alkalinity is represented by 0.05 p.c. of lime.

The juice along with the precipitate is filtered en-masse (without settling). The juice is then raised to boiling point, saturated with CO_2, again boiled to break the bicarbonate formed, and then filtered en-masse. The filter cake is washed; washings may be added to the juice or sent back for clarification. The juice is sent to the boiling house to make syrup.

Evaporation to make Syrup (Fig. 17)
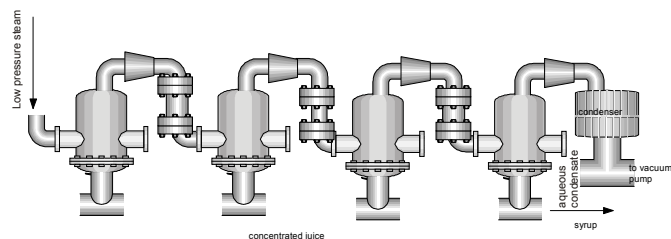
Fig. 2.5 : Showing the Quadruple Effect Vacuum Evaporator

Crystalline sugar is obtained from clarified cane juice in two steps. First, the juke is concentrated to syrup containing 50 *%* water; next the syrup is concentrated to massecuite A, containing 9-11 % water. Crystalline sugar—coloured or white-is obtained from the massecuite by removing the molasses.

Before the introduction of the present day method of evaporation under vacuum, the juice was concentrated in open cauldron 'heated by solid fuels. In India, 'Gur' (crystalline sugar mixed viith molasses) is still made by this process. Formerly, crystalline sugar was manufactured from 'gur; by removing the molasses by an indigenous process. By this process much sucrose is lost as caramel, and portion of sucrose is converted into glucose'. Even concentra-tion by steam coils under atmospheric pressure produces loss. Hence, the present-day method of evaporation at a lower temperature uno.er reduced pressure has been adopted.

The clarified juice obtained from any of the above methods is concentrated into the thick syrup by concentrating in multiple-effect evaporators. Usually quadruple-effect or tripple-effect evaporators are used.

An evaporator, is a tall cylindrical vessel made of riveted mild steel plates or cast in sections from cast iron. The top is bottle-necked and the bottom is spheroidal. The evaporators are placed vertically and in a battery of three or four. The cylinder is divided into three chambers by fitting two parallel plates a few feet arart at the lower part of the cylinder; the chambers above the upper plate and below the lower plate are the juice chambers, and the chamber between the two plates form the steam chamber or calendria.

'Several narrow vertical pipes join the upper plate to the lower plate (the pipes open in, the two juice chambers). A few wider pipes also vertically connect the upper and the lower pipe. The juice circulates inside the tubes and the steam circulates outside the tubes. There is a steam inlet pipe to each of the calendrias. The bottle-neck is closed at the top, but is fitted with a vapour outlet pipe at one side. The outlet pipe of the first evaporator is joined to the calcndria of the second evaporator, and so on up to the last calendria. Arrester baffle plates are fitted to the neck to prevent any juice or syrup to pass through the vapour outlet pipe. A narrow pipe known as "Ammonia pipe' (by analogy with beet juice evaporators) joins the calendria to the main body to remove air and other uncondensible gases accumulated in the calendria of the body, and ultimately delivers these gases to the vacuum and condensers attached to the tast body.

Heating of the juice in the first evaporator is done by passing steam exhaust or line through the calendria. The juice submerges the calendria, and during heating the juice which is admitted from the bottom by a feed pipe circulates up through the narrow tubes, and down through the wider tubes. In this way by rapid circulation and heating at a vacuum, the water is rapidly evaporated. The vapour from the first evaporator enters into the calcndria of the second evaporator. This stem, although of the lower temperature, helps rapid evaporation of the partially thickened syrup which passed from the first evaporator to the second by the opening of the valve of the bottom feed pipe: being actuated by difference of pressure. The low temperature steam helps rapid evaporation because of a higher vacuum maintained in the second evaporator. In quadruple-effect evaporators, the vacuum main-tained in the 3rd body is greater than in the second, and that maintained in the last body is greater than in the 3rd. The vacuum pump together with a condenser is attached to the vapour outlet pipe of the 4th evaporator.

This pump gradually reduces the pressure from the first to the last body. The vacuum and corresponding temperature maintained in each evaporator along with the steam temperature is as follows:

	Evaporators			
	I	II	III	IV
Temp of Heating Steam	125°C	99°C	86°C	76°C
Temp of boiling Juice	99°C	86°C	76°C	60°C
Vacuum maintained (in cm)	2.744	30.48	50.8	62.03

From the data mentioned above, it is found that boiling of the juice take place from 210°F to 140°F in the last evaporator. This means that most of the water has been removed at comparatively lower temperature and in absence of air, to ensure least loss of sugar by evaporation. The evaporation and subsequent transfer of fluid is so adjusted as to keep the level of the liquid in the evaporators above the level of the calendria.

From the last evaporator the pale yellow thick syrup (50-66 % solid matter) is discharged into the syrup tank. From this tank it is pumped to the top of the sulphitation tank wherefrom it is allowed to percolate down the cylinder over the perforated baffle plates, in a shower, to meet an ascending stream of sulphur dioxide.

The sulphur dioxide bleaches the syrup and reacts with residual calcium ions. This process is omitted when manufacture of raw sugar is aimed at. This process is, therefore, followed by the Indian factories a few factories where clarification is done by carbonation, bleaching by sulphur dioxide may be omitted if double carbonation has been properly carried out.

Crystallization (Fig. 19)

The syrup is next treated for forming crystalline sugar. This is done by removing most of the water by heating in a single-effect vacuum evaporator known as pan. The vacuum pan is either fitted with calendria or steam coils. The calendria fitted pan is similar to the evaporator used for boiling the juice. But it is larger in diameter and the tubes of its calendria are wider. It is fitted with vacuum gauge and a condenser, a thermometer, a sampling stick and safety device to prevent the boiling mass getting into the vapour) outlet pipe and outlet pipe at the bottom to dump the massecuite. There is the usual feed pipe at the bottom.

The crystallization takes place in two steps, viz.

(a) Formation of seed crystals 'by graining;

(b) Allowing the seed crystals to grow into fully developed crystals.

(a) The syrup is introduced into the pan until the steam chest is under it. Then by heating under vacuum the syrup is concentrated until, it is supersaturated with sucrose (83.5-84..5 %.sucrose at 80°C). 1hen the operator adopts either waiting method or shock meth9d to arrive at the graining point, i.e., induce the formation of seed crystals by breaking the supersaturation. In the waiting method the
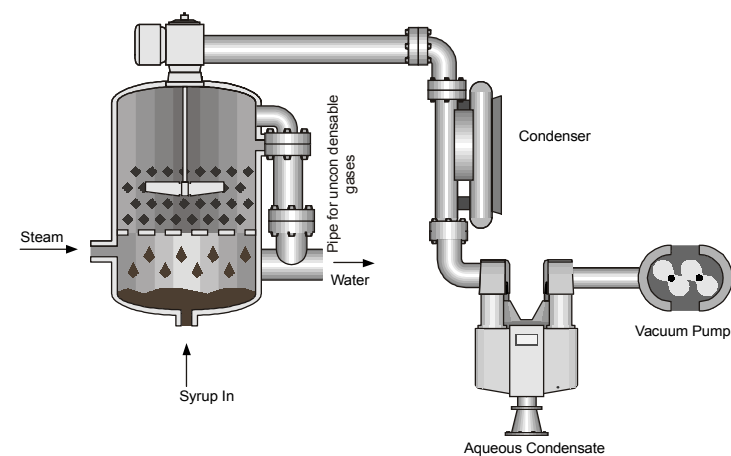
Fig. 2.6 : Showing the Single Effect Vacuum Evaporators

operator gradually cools down to break the supersaturation. In the shock method which is usually adopted, the supersaturation is broken by sudden chilling which is, done either by increasing the vacuum or by decreasing the rate of steam flow or by suddenly introducing large' amount of cold syrup'. Circulation. of the syrup is necessary for producing proper seed crystal.

(b) After. graining point is reached more and more syrup is intr.oduced in instalments. By proper circulation of the very thick syrup up and down the tubes of the calendria which is helped by injecting dry steam through, the bottom even, boiling is carried out at high vacuum (28.5 t inches) to force out sucrose out of the concentrated syrup which is deposited as crystals on the nuclei formed at the grainfng point. As moree and more syrup is introduced the crystats already formed grow more and more, until a semi-solid of mass fills up the pan. When this point is reached the flow of syrup is stopped and Concentratyion continued up to 92° - 93° Brix (9 to 11 % water) at a temperature of 65°C and vacuum of 28.7 inches. The semi-solid mass con-sisting of sugar crystals arid molasses-called massecute A is removed through the bottom outlet in crystalliser tanks. During the formation of crystals in the pan, false grains are formed which hamper the growth of crystals. False grains consist of minute crystals formed between the proper crystals. The object of the operator is to allow the grains to grow and to prevent the formation of fresh grains.

Physical Properties

Molecular Formula	$= C_{12}H_{22}O_{11}$
Formula Weight	= 342.29648
Composition	= C(42.11%) H(6.48%) O(51.42%)
Melting Point	=186 °C
Solubility in water	211.5 g/100 ml (20 °C)
Molar Refractivity	= 71.04 cm^3
Molar Volume	= 189.2 cm^3
Parachor	= 583.9 cm^3
Index of Refraction	= 1.674
Surface Tension	= 90.6 dyne/cm
Density	= 1.808 g/cm^3
Polarizability	= 28.16 10^{-24}cm^3
Monoisotopic Mass	= 342.116212 Da
Nominal Mass	= 342 Da
Average Mass	= 342.2965 Da
Log P	= -1.82

Chemical Properties

Pure sucrose is most often prepared as a fine, white, odorless crystalline powder with a pleasing, sweet taste; the common table sugar. Large crystals are sometimes precipitated from water solutions of sucrose onto a string (or other nucleation surface) to form rock candy, a confection. Like other carbohydrates, sucrose has a hydrogen to oxygen ratio of 2:1. It consists of two monosaccharides, α-glucose and fructose, joined by a glycosidic bond between carbon atom 1 of the glucose unit and carbon atom 2 of the fructose unit. What is notable about sucrose is that unlike most polysaccharides, the glycosidic bond is formed between the reducing ends of both glucose and fructose, and not between the reducing end of one and the nonreducing end of the other. The effect of this inhibits further bonding to other saccharide units. Since it contains no free anomeric carbon atom, it is classified as a nonreducing sugar. Sucrose melts and decomposes at 186°C to form caramel, and when combusted produces carbon, carbon dioxide, and water. Water breaks down sucrose by hydrolysis, however the process is so gradual that it could sit in solution for years with negligible change. If the enzyme sucrase is added however, the reaction will proceed rapidly. Reacting sucrose with sulfuric acid dehydrates the sucrose and forms the element carbon, as demonstrated in the following equation:

$$C_{12}H_{22}O_{11} + H_2SO_4 \text{ catalyst} \rightarrow 12 \text{ C} + 11 \text{ H}_2O$$

Sugar as a macronutrient

In mammals, sucrose is very readily digested in the stomach into its component sugars, by acidic hydrolysis. This step is performed by a glycoside hydrolase, which catalyzes the hydrolysis of sucrose to the monosaccharides glucose and fructose. Glucose and fructose are rapidly absorbed into the bloodstream in the small intestine. Undigested sucrose passing into the intestine is also broken down by sucrase or isomaltase glycoside hydrolases, which are located in the membrane of the microvilli lining the duodenum. These products are also transferred rapidly into the bloodstream.

Sucrose is digested by the enzyme invertase in bacteria and some animals. Acidic hydrolysis can be used in laboratories to achieve the hydrolysis of sucrose into glucose and fructose.

Use in human nutrition

Sucrose is an easily assimilated macronutrient that provides a quick source of energy to the body, provoking a rapid rise in blood glucose upon ingestion. However, pure sucrose is not normally part of a human diet balanced for good nutrition, although it may be included sparingly to make certain foods more palatable. Overconsumption of sucrose has been linked with some adverse health effects. The most common is dental caries or tooth decay, in which oral bacteria convert sugars (including sucrose) from food into acids that attack tooth enamel. Sucrose, as a pure carbohydrate, has an energy content of 3.94 kilocalories per gram (or 17 kilojoules per gram). When a large amount of foods that contain a high percentage of sucrose is consumed, beneficial nutrients can be displaced from the diet, which can contribute to an increased risk for chronic disease. It has been suggested that sucrose-containing drinks may be linked to the development of obesity and insulin resistance.[1] However, most soft drinks in the USA are now made with high-fructose corn syrup, not sucrose. The rapidity with which sucrose raises blood glucose can cause problems for people suffering from defects in glucose metabolism, such as persons with hypoglycemia or diabetes mellitus. Sucrose can contribute to development of the metabolic syndrome. In an experiment with rats that were fed a diet one-third of which was sucrose, the sucrose first elevated blood levels of triglycerides, which induced visceral fat and ultimately resulted in insulin resistance. Another study found that rats fed sucrose-rich diets developed high triglycerides, hyperglycemia, and insulin resistance.[4] Extension of average lifespan can be achieved by good diet, exercise and avoidance of hazards such as smoking and excessive eating of sugar-containing foods.

MALTOSE

Maltose, or malt sugar, is a disaccharide formed from two units of glucose joined with an α(1→4) linkage. It is the second member of an important biochemical series of glucose chains. The addition of another glucose unit yields maltotriose; further additions will produce dextrins (also called maltodextrins) and eventually starch.
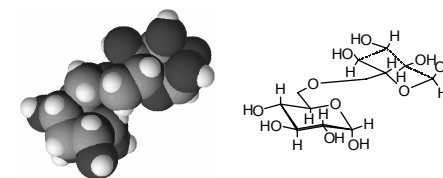
Maltose can be broken down into two glucose molecules by hydrolysis. In living organisms, the enzyme maltase can achieve this very rapidly. In the laboratory, heating with a strong acid for several minutes will produce the same result.

The production of maltose from germinating cereals, such as barley, is an important part of the brewing process. When barley is malted, it is brought into a condition in which the concentration of maltose-producing amylases has been maximized. Mashing is the process by which these amylases convert the cereal's starches into maltose. Metabolism of maltose by yeast during fermentation then leads to the production of ethanol and carbon dioxide

Physical Properties

Molecular Formula	= $C_{12}H_{22}O_{11}$
Formula Weight	= 342.29648
Composition	= C(42.11%) H(6.48%) O(51.42%)
Melting Point	= 102-103 °C
Molar Refractivity	= 71.13 cm^3
Molar Volume	= 190.8 cm^3
Parachor	= 579.5 cm^3
Index of Refraction	= 1.668
Surface Tension	= 85.0 dyne/cm
Density	= 1.793 g/cm^3
Polarizability	= 28.20 $10^{-24}cm^3$
Monoisotopic Mass	= 342.116212 Da
Nominal Mass	= 342 Da
Average Mass	= 342.2965 Da

LACTOSE
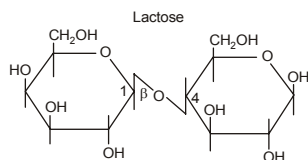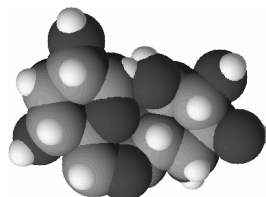
Lactose

Lactose (also referred to as *milk sugar*) is a sugar which is found most notably in milk. Lactose makes up around 2–8% of milk (by weight). The name comes from the Latin word for milk, plus the -ose ending used to name sugars. Its systematic name is β-D-galactopyranosyl-(1↔4)α-D-glucopyranose.

Chemistry

Lactose is a disaccharide that consists of β-D-galactose and β-D-glucose molecules bonded through a β1-4 glycosidic linkage.

Solubility

Lactose has a solubility of 1 in 4.63 measured %w/v. This translates to 0.216 g of lactose dissolving readily in 1 mL of water.

The solubility of lactose in water is 18.9049 g at 25°C, 25.1484 g at 40°C and 37.2149 g at 60°C per 100 g solution. Its solubility in ethanol is 0.0111 g at 40°C and 0.0270 g at 60°C per 100 g solution.

Digestion of lactose

Infant mammals are fed on milk by their mothers. To digest it an enzyme called lactase (β-D-galactosidase) is secreted by the intestinal villi, and this enzyme cleaves the molecule into its two subunits glucose and galactose for absorption.Since lactose occurs mostly in milk, in most mammals the production of lactase gradually decreases with maturity.Many people with ancestry in Europe, the Middle East, India, or parts of East Africa, maintain normal lactase production into adulthood. In many of these cultures, mammals such as cattle, goats, and sheep are milked for food. Hence, it was in these regions that genes for lifelong lactase production first evolved.

Trisaccharides

RAFFINOSE

Raffinose is a trisaccharide composed of galactose, fructose, and glucose. It can be found in beans, cabbage, brussels sprouts, broccoli, asparagus, other vegetables, and whole grains. Raffinose can be hydrolyzed to D-galactose and sucrose by the enzyme α-galactosidase (α-GAL), an enzyme not found in humans. α-GAL also hydrolyzes other α-galactosides such as stachyose, verbascose, and galactinol, if present. The enzyme does not cleave β-linked galactose, as in lactose.The raffinose family of oligosaccharides (RFOs) are alpha-galactosyl derivatives of sucrose, and the most common are the trisaccharide raffinose, the tetrasaccharide stachyose, and the pentasaccharide verbascose. RFOs are almost ubiquitous in the plant kingdom, being found in a large variety of seeds from many different families, and they rank second only to sucrose in abundance as soluble carbohydrates.
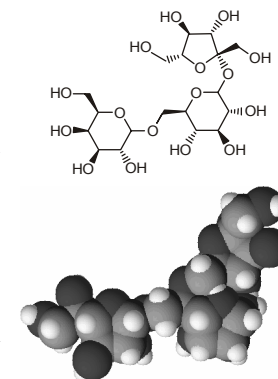
Humans and other monogastric animals (pigs and poultry) do not possess the α-GAL enzyme to break down RFOs and these oligosaccharides pass undigested through the stomach and upper intestine. In the lower intestine, they are fermented by gas-producing bacteria which do posses the α-GAL enzyme and make carbon dioxide, methane, and/or hydrogen — leading to the flatulence commonly associated with eating beans and other vegetables. α-GAL is present in digestive aids such as the product Beano.

Procedures concerning cryopreservation have utilized raffinose to provide hypertonicity for cell desiccation prior to freezing. Either raffinose or sucrose is used as a base substance for sucralose.

MELEZITOSE

Melezitose, also spelled **melicitose**, is a nonreducing trisaccharide sugar that is produced from lice such as *Cinara pilicornis* by an enzyme reaction. This is beneficial to the insects, as it reduces the stress of osmosis by reducing their own water potential. The melezitose is part of the honeydew of the honey produced by bees, and acts as an attractant for ants. This is useful to the lice as they have a symbiotic relationship with ants. Melezitose can be partially hydrolyzed to glucose and turanose the latter of which is an isomer of sucrose
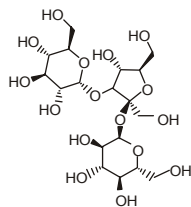
Polysachharide

Digestible

GLYCOGEN

Glycogen is a polysaccharide of glucose (Glc) which functions as the primary short term energy storage in animal cells. It is made primarily by the liver and the muscles, but can also be made by the brain, uterus, and the vagina. Glycogen is the analogue of starch, a less branched glucose polymer in plants, and is commonly referred to as **animal starch**, having a similar structure to amylopectin. Glycogen is found in the form of granules in the cytosol in many cell types, and plays an important role in the glucose cycle. Glycogen forms an energy reserve that can be quickly mobilized to meet a sudden need for glucose, but one that is less compact than the energy reserves of triglycerides (fat). In the liver hepatocytes, glycogen can compose up to 8% of the fresh weight (100–120 g in an adult) soon after a meal. Only the glycogen stored in the liver can be made accessible to other organs. In the muscles, glycogen is found in a much lower concentration (1% of the muscle mass), but the total amount exceeds that in liver. Small amounts of glycogen are found in the kidneys, and even smaller amounts in certain glial cells in the brain and white blood cells. The uterus also stores glycogen during pregnancy to nourish the embryo.
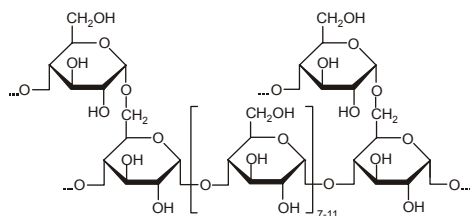
Synthesis

Glycogen synthesis differs from glycogen breakdown. Unlike breakdown, synthesis is endergonic, meaning that glycogen is not synthesized without the input of energy. Energy for glycogen synthesis comes from UTP, which reacts with glucose-1-phosphate, forming UDP-glucose, in reaction catalysed by UDP-glucose pyrophosphorylase. Glycogen is synthesized from monomers of UDP-glucose by the enzyme Glycogen synthase, which progressively lengthens the glycogen chain. As glycogen synthase can only lengthen an existing chain, the protein glycogenin is needed to initiate the synthesis of glycogen.

Breakdown

Glycogen is cleaved from the nonreducing ends of the chain by the enzyme glycogen phosphorylase to produce monomers of glucose-1-phosphate that is then converted to Glucose 6-phosphate. A special debranching enzyme is needed to remove the alpha(1-6) branches in branched glycogen and reshape the chain into linear polymer. The G6P monomers produced have three possible fates:

- G6P can continue on the glycolysis pathway and be used as fuel.
- G6P can enter the pentose phosphate pathway via the enzyme Glucose-6-phosphate dehydrogenase to produce NADPH and 5-carbon sugars.
- In the liver and kidney, G6P can be dephosphorylated back to Glucose by the enzyme Glucose 6-phosphatase. This is the final step in the gluconeogenesis pathway.

Structure and Biochemistry

Glycogen is a highly branched polymer that is better described as a dendrimer of about 60,000 glucose residues and has a molecular weight between 10^6 and 10^7 daltons (~4.8 million) Most of Glc units are linked by α-1,4 glycosidic bonds, approximately 1 in 12 Glc residues also makes -1,6 glycosidic bond with a **second Glc**, which results in the creation of a branch. Glycogen does not possess a reducing end: the 'reducing end' glucose residue is not free but is covalently bound to a protein termed glycogenin as a beta-linkage to a surface tyrosine residue. Glycogenin is a glycosyltransferase and occurs as a dimer in the core of glycogen. The glycogen granules contain both glycogen and the enzymes of glycogen synthesis (glycogenesis) and degradation (glycogenolysis). The enzymes are nested between the outer branches of the glycogen molecules and act on the non-reducing ends. Therefore, the many non-reducing end-branches of glycogen facilitate its rapid synthesis and catabolism.

Function and Regulation of Liver Glycogen

As a meal containing carbohydrates is eaten and digested, blood glucose levels rise, and the pancreas secretes insulin. Glucose from the portal vein enters the liver cells (hepatocytes). Insulin acts on the hepatocytes to stimulate the action of several enzymes, including glycogen synthase. Glucose molecules are added to the chains of glycogen as long as both insulin and glucose remain plentiful. In this postprandial or "fed" state, the liver takes in more glucose from the blood than it releases. After a meal has been digested and glucose levels begin to fall, insulin secretion is reduced, and glycogen synthesis stops. About four hours after a mealglycogen begins to be broken down and converted again to glucose. Glycogen phosphorylase is the primary enzyme of glycogen breakdown. For the next 8–12 hours,
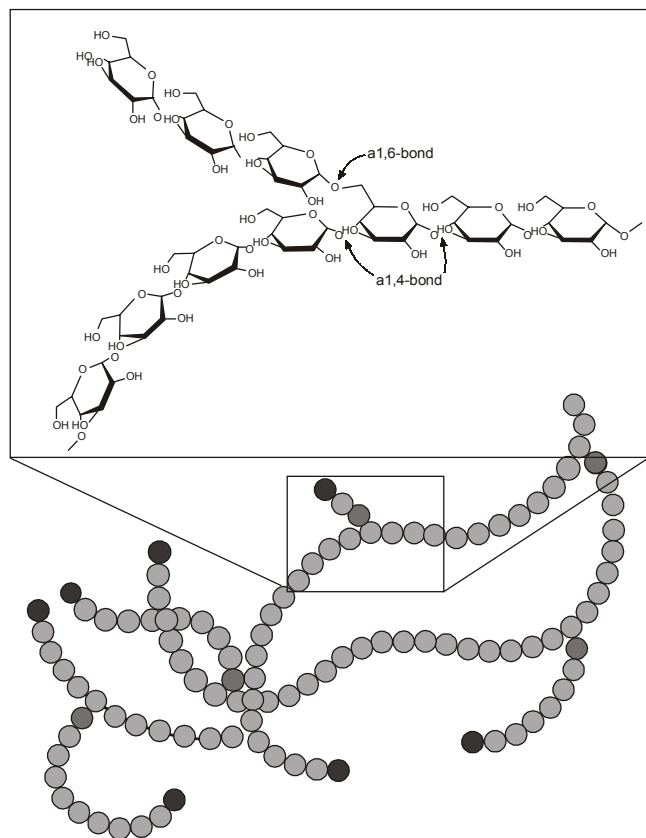
glucose derived from liver glycogen will be the primary source of blood glucose to be used by the rest of the body for fuel. Glucagon is another hormone produced by the pancreas, which in many respects serves as a counter-signal to insulin. When the blood sugar begins to fall below normal, glucagon is secreted in increasing amounts. It stimulates glycogen breakdown into glucose even when insulin levels are abnormally high.

In muscle and other cells

Muscle cell glycogen appears to function as an immediate reserve source of available glucose for muscle cells. Other cells that contain small amounts use it locally as well. Muscle cells lack glucose-6-phosphatase enzyme, so they lack the ability to pass glucose into the blood, so the glycogen they store internally is destined for internal use and is not shared with other cells, unlike liver cells.

Glycogen debt and endurance exercise

Due to the body's inability to hold more than around 2,000 kcal of glycogenlong-distance athletes such as marathon runners, cross-country skiers, and cyclists go into glycogen debt, where almost all of the athlete's glycogen stores are depleted after long periods of exertion without enough energy consumption. This phenomenon is referred to as "hitting the wall" or "bonking". In marathon runners it normally happens around the 20 mile (32 km) point of a marathon, where around 100 kcal are spent per mile depending on the size of the runner and the race course. However, it can be delayed by a carbohydrate loading before the task. When experiencing glycogen debt, athletes often experience extreme fatigue to the point that it is difficult to move.

Disorders of glycogen metabolism

The most common disease in which glycogen metabolism becomes abnormal is diabetes, in which, because of abnormal amounts of insulin, liver glycogen can be abnormally accumulated or depleted. Restoration of normal glucose metabolism usually normalizes glycogen metabolism as well. In hypoglycemia caused by excessive insulin, liver glycogen levels are high, but the high insulin level prevents the glycogenolysis necessary to maintain normal blood sugar levels. Glucagon is a common treatment for this type of hypoglycemia. Various inborn errors of metabolism are caused by deficiencies of enzymes necessary for glycogen synthesis or breakdown. These are collectively referred to as glycogen storage diseases.

STARCH

Introduction

These are both complex carbohydrate polymers of glucose (chemical formula of glucose $C_6H_{12}O_6$), making starch a glucose polymer as well, as seen by the chemical formula for starch, regardless of the ratio of amylose:amylopectin. The word is derived from Middle English *sterchen*, meaning to stiffen, which is appropriate since it can be used as a thickening agent when dissolved in water and heated.

Synthesis

The starch is located in the crown and glutinous regions of the endosperm. Large, round granules are loosely held in the crown region, associated with a relatively small amount of protein. The separation of
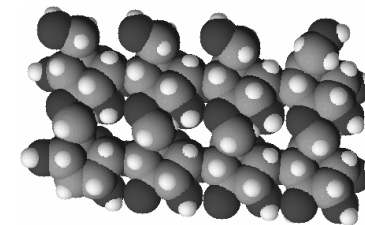
these granules in the milling process offers no serious problem. Smaller, polygonally shaped granules are tightly held in a water- insoluble protein matrix in the glutinous region. The hydration and disintegration
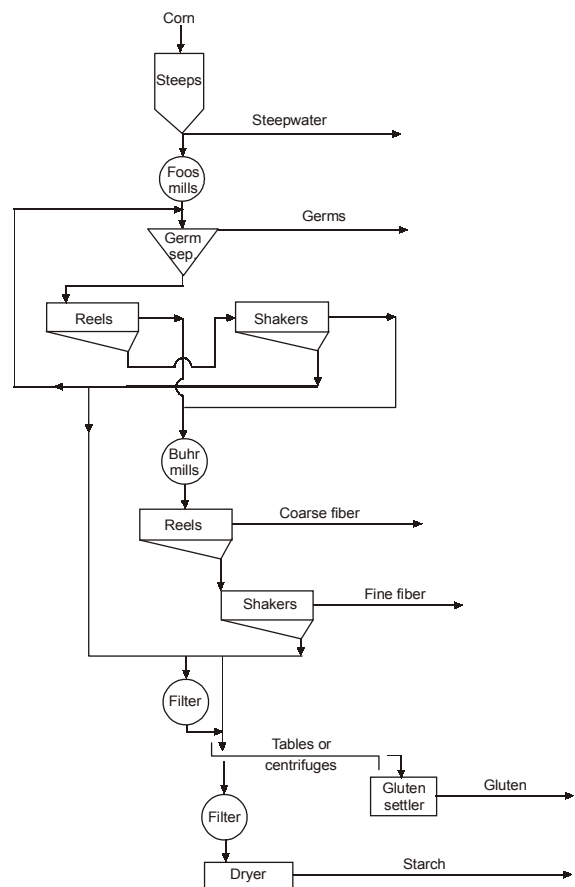
Fig. 2.7 : Showing the Industrial Prepartion of Starch

of this protein in order to loosen the starch, the separation of the starch from the finely dispersed protein, and the accomplishment of these ends without a serious deteriora-tion of the more fragile and less resistant granules in the crown region constitute the chief problems in milling. The steps of the *wet milling process* are indicated diagram-matically in Figure 20.

In order to facilitate the separation of the constituents of the grain, the corn is hy-drated by soaking *(steeping)* in water at about 50°C for 35-45 hours. Usually, about 0.15 – 0.20% of sulfur dioxide is added to the water (pH about 4.0) in order to control biological activity (19) and to facilitate the disintegration of the protein structures. The moisture content of the corn increases during steeping to about 45%. The steeped corn is passed to coarse grinding mills (such as the Foos mill) called degenni-nators, which loosen the hulls and separate the tough, rubbery germs with very little dis-integration of these constituents. The grist, as a water slurry with a density corre-sponding to 8-10.5°Be. at 90-95°F., is fed continuously to V-shaped tanks. Here the germs tend to float because of their high oil content and are removed from the surface by overflowing into a weir box. The heavier part of the grist is continuously removed from the bottom of the tank.

The coarse portion of the degerminated grist is finely ground in buhrstone mills. Hulls and fiber are removed by pas-sage over a series of metal screens and finally over a series of screens made of bolting silk. The res1ilting slurry contains only finely dispersed protein, starch, and some water-soluble material. The density is adjusted to betwem 6 and 12°Be., the sulphur dioxide concentration to between 0.06 and 0.08%, and the temperature to about 85-90 of. Adjustment of density by concentration is usually required in mills with table feed liquors of the order of 10 to 12° Be. Concentration may be affected by passing a portion of the liquors from the shakers over filters and then adding the filter cake to the balance of the unfiltered slurry.

Starch is *sedimented* from the gluten (water-insoluble proteins, zein and zeanin, together with minor amounts of corn fiber, endosperm lipides, and unsepa-rated starch) either by gravity, by the use of centrifuges, or by a combination of both methods. In separation by gravity, the slurry is allowed to run down inclined troughs called tables. These are about 2 ft. wide and 120 ft. long. For feed rates of 1.5-2.0 gaL/minute of mill liquor, the slope of these tables is about 3>16 in.jft. Variables af-fecting the separation are: (1) rate of feed to the tables, (2) slope of the table com-pared-to its dimensions, (3) density of the slurry, (4) temperature, and (5) pH and ionic strength of the liquor (12). Starch sediments on the tables, while the gluten passes off the lower end. Gluten particles which are not sufficiently hydrated and which contain a relatively large number of starch granules will obviously settle with the starch and increase the protein content of the latter. A smaller number of starch granules may not cause the gluten to sediment, but this starch will increase the carbo-hydrate content of the gluten fraction and result in a reduced yield of starch. Centri-fuges of the Merco (bowl) type may be used to separate the starch, or these centrifuges may be used after tabling (38) in order further to purify either the starch or the gluten fractions. Starch is washed from the tables by high-pressure water jets. After filtration, the starch is washed with water and dried. American (drum-type) and Oliver (disk-type) filters are usually used in these operations followed by drying either in kilns, rotary dryers, or preferably endless belt dryers of the type manufactured by Proctor and Schwartz

The wet milling of corn employs the countercurrent principle throughout in the usn of water. Aside from the water which enters the system as moisture in the corn grain, the balance, making a total of

10-12 gal. per bushel of corn processed, is added in the final washing of the starch before filtration. This wash water is then used to flush the starch from the tables and is used in turn in various cycles of the milling process, until as gluten overflow water from the tables it contains about 1-2 grams of dissolved ma-terial per 100 ml. After sedimentation of the gluten in tanks called gluten settlers, the water is finally used in the steeping operation. After steeping and before final de-watering, the pH of the starch is usually adjusted to a range between 5 and 7. The water is removed from the corn and evaporated to recover the extracted corn solids. Steep water may contain as much as 8 grams of nonvolatile solids per 100 ml. and has proved to be a practical commercial source of meso-inositol, which is of interest as a member of the vitamin-B complex. The various by-product con-stituents of the grain such as the germs, the hulls, and fiber are in turn washed free of adhering starch by the process waters in the system and are then further processed

Physical Properties

Molecular Formula	$= C_{48}H_{76}O_{38}$
Formula Weight	= 1261.09424
Composition	= C(45.72%) H(6.07%) O(48.21%)
Molar Refractivity	$= 262.69\ cm^3$
Molar Volume	$= 774.8\ cm^3$
Parachor	$= 2243.7\ cm^3$
Index of Refraction	= 1.593
Surface Tension	= 70.3 dyne/cm
Density	$= 1.627\ g/cm^3$
Polarizability	$= 104.14\ 10^{-24} cm^3$
Monoisotopic Mass	= 1260.401458 Da
Nominal Mass	= 1260 Da
Average Mass	= 1261.0942 Da

Chemical Properties

Most varieties of starch contain two types of polymers which differ from each other in molecular weight and in chemical structure. The linear polymer, **amylose,** consists of 200-1000 glucopyranose units joined to each other through a-l,4-glucosidic linkages, whereas the branched or ramified polymer, **amylo-pectin,** is made up of 1500 or more glucopyranose units (6,10,15,20) (see Fig. 139). In addition to the normal or predominating a-l,4 linkages, an anomalous a-l,6linkage is present in the ramified structure at the origin or point of branching in a ratio of about 1 :25.

The linear polymer and the longer branches of the nonlinear polymer show a pro-nounced tendency to orient and associate with other linear members. This property is characteristic of many thread-like molecules which contain a large number of hy-droxyl or other hydrogen-bond-forming groups distributed along their chain length. Starch molecules are highly associated in the granule.
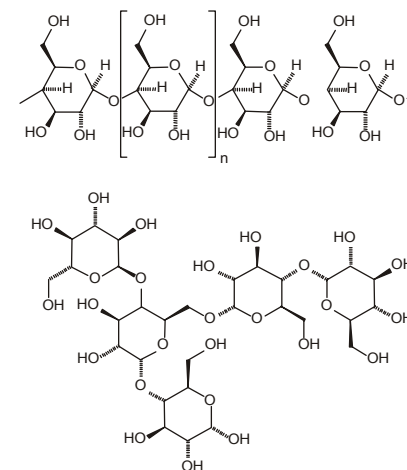
When starch is dispersed in water, the sol may be fractionated into its polymeric components by the addition of polar substances, particularly the higher alcohols. The amylose forms an insoluble complex with butyl alcohol which may be sedimented by centrifugation. The amount of amylose obtained varies with the origin of the starch. Starches from so-called waxy grains, such as waxy corn and sorghum, contain no amylose; in contrast, the starch from certain varieties of peas contains 75% by weight of amylose. The common industrial starches contain about 15-30% by weight of amylose.

The chemical property of starch which is at present of greatest industrial im-portance is the comparative ease with which the plymeric bond is hydrolyzed by both enzymes and acids. When starch is gelatinized by heating in water or when it is dissolved by suitable solvating agent~, for example, sodium hydroxide, followed by neutralization, its glucoside linkages (the polymeric bonds) are readily hydrolyzed by **amylases.** These enzymes are obtained from a large number of sources such as bac-teria, fungi, vegetables, and animals. The amylases present in these preparations are grouped into three broad classifications or types since the course of the hydrolysis varies in a characteristic manner with the type of amylase employed.

α-*Amylase* splits the high-polymer starch molecules into molecules of progressively smaller size. Eventually, sugar molecules are produced. Characteristic of this reac-tion is the rapid decrease in

viscosity of the starch medium with very little production of reducing sugars. Accordingly, the reaction is used industrially to produce low-vis-cosity sizes and adhesives from native starch. *{3-Amylase* splits the disaccharide *maltose* directly from starch molecules. Amylose molecules are almost quantitatively hy-drolyzed to maltose in this manner. Amylopectin molecules are hydrolyzed 50-60%. The residue which remains ("beta-amylase limit. dextrin") is of comparatively high molecular weight and still contains all of the anomalous glucoside linkages which were present in the original starch molecules. A third type of amylase, called *amyloglucosi-dase*, splits starch molecules of both structures directly to the monosaccharide, D-glucose (dextrose). The saccharifying reactions are used by fermentation industries employing starch as substrate. In as much as both the maltose- and glucose-producing enzymes are believed to act only on the non-reducing terminal sugar groups of the starch polymer, it follows that one of the principal functions of a-amylase, as it occurs normally in nature in conjunction with saccharifying enzymes, is to facilitate the action of the latter by creat-ing more terminal groups per unit weight. Thus, the complete hydrolysis of starch to sugars is accelerated.

Although amylases of anyone class act on starch in qualitatively the same manner, members of a class, depending on source, may show quantitative differences in their mode of action. The relatively complex chemistry involved in the action of amylases has been summarized by Kerr and Gehman.

All starches are hydrolyzed by acid to D-glucose, as in the commercial production at high temperatures of dextrose *(q.v.)* and starch sirups (mixtures consisting of dextrose, maltose, and higher polysaccharides generally called dextrins). In common with the action of enzymes, acid at first causes a reduction in paste viscosity. In contrast to enzymes, however, acid readily pene-trates at least the amorphous regions of the native un swollen granule, so that hydrol-ysis may take place even at temperatures materially lower than those required for gelatinization. This fact is utilized in the manufacture of modified, "thin-boiling" starches (18). When these acid-modified starches are gelatinized, the consistency of their pastes at a given concentration is less than that of the native starch. The intermediate product of acid degradation is known as **amylodextrin.**

The alcohol groups in starch may be oxidized to form carbonyl or carboxyl groups. The course of oxidation varies with the oxidant and conditions employed. Specific oxidants such as nitrogen dioxide act principally to convert primary alcoholic groups to carboxyls, whereas others such as periodate act on the two adjacent secondary hydroxyl groups, converting them first into ketone groups and subsequently into alde-hydes with a rupture of the bond between carbons 2 and 3. Supplementary oxidants such as bromine water may then convert them further to diacids. Less specific agents used industrially, such as hypochlorite, very likely create many carboxyl groups along the starch molecule, thus increasing its solubility in water particularly in neutralized or dilute alkaline solution. The alcohol groups in starch react with aldehydes, under acidic conditions, by a condensation reaction, in which cross-linkages are very likely established between two adjacent starch molecules through the carbonyl carbon of the aldehyde. The end prod-uct is completely nondispersible in water. Starch also reacts to form a product which is nondispersible in water with agents such as epichlorohydrin, dibasic acids or their acid chlorides, potassium pyroantimonate, and allyl bromide. Where the reaction involves the uniting of two starch molecules, it may be viewed as a poly-merization. Starch, or more particularly the linear polymer of starch, reacts charac-teristically with iodine to form a blue-colored complex. The iodine test is used in food analysis as a test for unconverted starch.

The aldehyde group in the starch molecule is reducible. Polyhydric alcohols such as sorbitol are formed by hydrogenation (involving also hydrolysis) and hydrogenolysis

Tests

Iodine solution is used to test for Starch. A bluish-black color indicates the presence of iodine in the starch solution. It is thought that the iodine fits inside the coils of amylose. A 0.3% w/w solution is the standard concentration for a dilute starch indicator solution. It is made by adding 4 grams of soluble starch to 1 litre of heated water; the solution is cooled before use (starch-iodine complex becomes unstable at temperatures above 35 °C). This complex is often used in redox titrations: in presence of an oxidizing agent the solution turns blue, in the presence of reducing agent, the blue color disappears because triiodide (I_3^-) ions break up into three iodide ions, disassembling the complex.

Under the microscope, starch grains show a distinctive Maltese cross effect (also known as 'extinction cross' and birefringence) under polarized light.

Uses

Starch in food

In terms of human nutrition, starch is by far the most consumed polysaccharide in the human diet. It constitutes more than half of the carbohydrates even in many affluent diets, and much more in poorer diets. Traditional staple foods such as cereals, roots and tubers are the main source of dietary starch.

Starch (in particular cornstarch) is used in cooking for thickening foods such as sauces. In industry, it is used in the manufacturing of adhesives, paper, textiles and as a mold in the manufacture of sweets such as wine gums and jelly beans. It is a white powder, and depending on the source, may be tasteless and odorless.

Fruit, seeds, rhizomes or tubers of plants and is the major source of energy in these food items. The major resources for starch production and consumption worldwide are rice, wheat, corn, and potatoes. Cooked foods containing starches include boiled rice, various forms of bread and noodles (including pasta).

As an additive for food processing, arrowroot and tapioca are commonly used as well. Commonly used starches around the world are: arracacha, buckwheat, banana, barley, cassava, kudzu, oca, sago, sorghum, regular household potatoes, sweet potato, taro and yams. Edible beans, such as favas, lentils and peas, are also rich in starch.

When a starch is pre-cooked, it can then be used to thicken cold foods. This is referred to as a pregelatinized starch. Otherwise starch requires heat to thicken, or "gelatinize." The actual temperature depends on the type of starch.

A modified food starch undergoes one or more chemical modifications, which allow it to function properly under high heat and/or shear frequently encountered during food processing. Food starches are typically used as thickeners and stabilizers in foods such as puddings, custards, soups, sauces, gravies, pie fillings, and salad dressings, but have many other uses.

Resistant starch is starch that escapes digestion in the small intestine of healthy individuals.

Plants use starch as a way to store excess glucose, and thus also use starch as food during mitochondrial oxidative phosphorylation.

Commercial applications

Papermaking is the largest non-food application for starches globally, consuming millions of metric tons annually. In a typical sheet of copy paper for instance, the starch content may be as high as 8%. Both chemically modified and unmodified starches are used in papermaking. In the wet part of the papermaking process, generally called the "wet-end", starches are chemically modified to contain a cationic or positive charge bound to the starch polymer, and are utilized to associate with the anionic or negatively charged paper fibers and inorganic fillers. Starch also helps get out cleaning stains from dirty washing.

These cationic starches impart the necessary strength properties for the paper web to be formed in the papermaking process (wet strength), and to provide strength to the final paper sheet (dry strength). In the dry end of the papermaking process the paper web is rewetted with a solution of starch paste that has been chemically, or enzymatically depolymerized. The starch paste solutions are applied to the paper web by means of various mechanical presses (size press). The dry end starches impart additional strength to the paper web and additionally provide water hold out or "size" for superior printing properties.

Corrugating glues are the next largest consumer of non-food starches globally. These glues are used in the production of corrugated fiberboard (sometimes called corrugated cardboard), and generally contain a mixture of chemically modified and unmodified starches that have been partially gelatinized to form an opaque paste. This paste is applied to the flute tips of the interior fluted paper to glue the fluted paper to the outside paper in the construction of cardboard boxes. This is then dried under high heat, which provides the box board strength and rigidity.

Another large non-food starch application is in the construction industry where starch is used in the or wall board manufacturing process. Chemically modified or unmodified starches are added to the rock mud containing primarily gypsum. Top and bottom heavyweight sheets of paper are applied to the mud formulation and the process is allowed to heat and cure to form the eventual rigid wall board. The starches act as a glue for the cured gypsum rock with the paper covering and also provide rigidity to the board.

Clothing starch or **laundry starch:** is a liquid that is prepared by mixing a vegetable starch in water (earlier preparations also had to be boiled), and is used in the laundering of clothes. Starch was widely used in Europe in the 16th and 17th centuries to stiffen the wide collars and ruffs of fine linen which surrounded the necks of the well-to-do. During the 19th century and early 20th century, it was stylish to stiffen the collars and sleeves of men's shirts and the ruffles of girls' petticoats by applying starch to them as the clean clothes were being ironed.

Aside from the smooth, crisp edges it gave to clothing, it served practical purposes as well. Dirt and sweat from a person's neck and wrists would stick to the starch rather than fibers of the clothing, and would easily wash away along with the starch. After each laundering, the starch would be reapplied.

Starch is also used to make some packing peanuts, and some dropped ceiling tiles.

Printing industry - in the printing industry food grade starch is used in the manufacture of anti-set-off spray powder used to separate printed sheets of paper to avoid wet ink being set off. Starch is also used in the manufacture of glues for book-binding.

Hydrogen production - Starch can be used to produce Hydrogen.

Oil exploration - starch is used as to adjust the viscosity of drilling fluid which is used to lubricate the drill head in (mineral) oil extraction.

Use as a mold: Gummed sweets such as jelly beans and wine gums are not manufactured using a mold in the conventional sense. A tray is filled with starch and leveled. A positive mold is then pressed into the starch leaving an impression of 1000 or so jelly beans. The mix is then poured into the impressions and then put into a stove to set. This method greatly reduces the number of molds that must be manufactured.

Starch can be modified by addition of some chemical forms to be a hard glue for paper work , some of those forms are Borax , Soda Ash , which mixed with the starch solution at 50-70C to gain a very good adhesive, Sodium Silicate can be added to reinforce this formula.

DEXTRIN

Dextrins are a group of low-molecular-weight carbohydrates produced by the hydrolysis of starch. Dextrins are mixtures of linear α-(1,4)-linked D-glucose polymers starting with an α-(1,6) bond. Because branched amylopectin and glycogen also contain α-(1,6) bonds, which α-amylase cannot hydrolyze in humans, the digest resulting from this action contains a mixture of dextrins. They have the same general formula as carbohydrates but are of shorter chain length. Industrial production is, in general, performed by acidic hydrolysis of potato starch. Dextrins are water-soluble, white to slightly yellow solids that are optically active. Under analysis, dextrins can be detected with iodine solution, giving a red coloration.
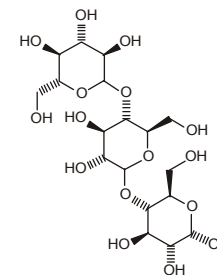
The cyclical dextrins are known as cyclodextrins. They are formed by enzymatic degradation of starch by certain bacteria, for example, Bacillus macerans. Cyclodextrins have toroidal structures formed by 6-8 glucose residues.

Dextrins find widespread use in industry, due to their non-toxicity and their low price. They are used as water-soluble glues, as thickening agents in food processing, and as binding agent in pharmaceuticals. In pyrotechnics, they are added to fire formulas, allowing them to solidify as pellets or "stars." Cyclodextrins find additional use in analytical chemistry as a matrix for the separation of hydrophobic substances, and as excipients in pharmaceutical formulations. Not all forms of dextrin are digestible, and indigestible dextrin is sometimes used in fiber supplements.

For example, **maltodextrin** either can be moderately sweet or have hardly any flavor at all. Maltodextrin is a polysaccharide that is used as a food additive. It is produced from starch and is usually found as a creamy-white hygroscopic powder. Maltodextrin is easily digestible, being absorbed as rapidly as glucose.

Maltodextrin can be derived from any starch. In the US, this starch is usually rice, corn or potato; elsewhere, such as in Europe, it is commonly wheat. This is important for coeliacs, since the wheat-derived maltodextrin can contain traces of gluten. There have been recent reports of coeliac reaction to maltodextrin in the United States. This might be a consequence of the shift of corn to ethanol production and its replacement with wheat in the formulation.

Foods containing maltodextrin may contain traces of amino acids, including glutamic acid as a manufacturing by-product. The amino acids traces would be too small to have any dietary significance.

Partly Digestible

INULIN

Inulins are a group of naturally occurring polysaccharides (several simple sugars linked together) produced by many types of plants.[1] They belong to a class of fibers known as fructans. Inulin is used by some plants as a means of storing energy and is typically found in roots or rhizomes. Most plants which synthesize and store inulin do not store other materials such as starch.

Biochemistry

Inulins are polymers mainly comprised of fructose units and typically have a terminal glucose. The fructose units in inulins are joined by a beta-(2-1) glycosidic bond. Plant inulins generally contain between 20 to several thousand fructose units. Smaller compounds are called fructooligosaccharides, the simplest of these is 1-ketose, which has 2 fructose units and 1 glucose unit.

Inulins are named in the following manner, where **n** is the number of fructose residues and **py** is the abbreviation for pyranosyl:

- Inulins with a terminal glucose are known as *alpha-D-glucopyranosyl-[beta-D-fructofuranosyl](n-1)-D-fructofuranosides*, abbreviated as **GpyFn**.
- Inulins without glucose are *beta-D-fructopyranosyl-[D-fructofuranosyl](n-1)-D-fructofuranosides*, abbreviated as **FpyFn**.

Hydrolysis of inulins may yield fructooligosaccharides, which are oligomers with a degree of polymerization (DP) of <= 10.

Uses

Processed foods

Inulin is used increasingly in foods because it has unusual nutritional characteristics. It ranges from completely bland to subtly sweet and can be used to replace sugar, fat, and flour. This is particularly advantageous because inulin contains a third to a quarter of the food energy of sugar or other carbohydrates and a sixth to a ninth of the food energy of fat. It also increases calcium absorption[2] and possibly magnesium absorption,[3] while promoting intestinal bacteria. Nutritionally, it is considered a form of soluble fiber, and it is important to note that consuming large quantities (particularly for

sensitive and/or unaccustomed individuals) can lead to gas and bloating. Inulin has a minimal impact on blood sugar, and—in sharp contrast to fructose—is not insulemic and does not raise triglycerides, making it generally considered suitable for diabetics and potentially helpful in managing blood sugar-related illnesses.

Industrial use

Nonhydrolyzed inulin can also be directly converted to ethanol in a simultaneous saccharification and fermentation process which may have great potential for converting crops high in inulin into ethanol for fuel.

Medical

Inulin is used to help measure kidney function by determining the Glomerular filtration rate (**GFR**). GFR is the volume of fluid filtered from the renal (kidney) glomerular capillaries into the Bowman's capsule per unit time.

Calculation of Glomerular Filtration Rate (GFR)

Inulin is uniquely treated by nephrons in that it is completely filtered at the glomerulus but neither secreted nor reabsorbed by the tubules. This property of inulin allows the clearance of inulin to be used clinically as a highly accurate measure of Glomerular filtration rate (GFR)—the percentage of plasma from the afferent arteriole that is filtered into Bowman's capsule.

It is useful to contrast the properties of inulin with those of para-aminohippuric acid (PAH). PAH is completely filtered from plasma at the glomerulus and not reabsorbed by the tubules, in a manner identical to inulin. PAH is different from inulin in that the fraction of PAH that bypasses the glomerulus and enters the nephron's tubular cells (via the Peritubular capillaries) is completely secreted. Renal Clearance of PAH is thus useful in calculation of renal plasma flow (RPF), which empirically is (1-Hematocrit) times renal blood flow. Of note, the clearance of PAH is reflective only of RPF to portions of the kidney that deal with urine formation, and thus underestimates actually RPF by about 10%.[7]

The measurement of GFR by inulin is still considered the gold-standard. Practically, however, it has now been largely replaced by other, simpler measures that are approximations of GFR. These measures, which involve clearance of such substrates as EDTA and creatinine, have had their utility confirmed in large cohorts of patients with chronic kidney disease.

Health effects

Inulin is indigestible by the human enzymes ptyalin and amylase, which are adapted to digest starch. As a result, inulin passes through much of the digestive system intact. It is only in the colon that bacteria metabolise inulin, with the release of significant quantities of carbon dioxide, hydrogen and/or methane. Inulin-containing foods can be rather gassy, particularly for those unaccustomed to inulin, and these foods should be consumed in moderation at first.

There are two types of dietary fiber, soluble and insoluble. Insoluble fiber increases the movement of materials through the digestive system and increases stool bulk; it is especially helpful for those suffering from constipation or stool irregularity. Soluble fiber dissolves in water to form a gelatinous material. Some soluble fibres may help lower blood cholesterol and glucose levels. Inulin is considered

a soluble fiber. Because normal digestion does not break inulin down into monosaccharides, it does not elevate blood sugar levels and may therefore be helpful in the management of diabetes. Inulin also stimulates the growth of bacteria in the gut.[4] Inulin passes through the stomach and duodenum undigested and is highly available to the gut bacterial flora. This contrasts with proprietary probiotic formulations based on Lactic acid bacteria (LAB) in which the bacteria have to survive very challenging conditions through the gastrointestinal tract before they are able to colonize the gut.

Some traditional diets contain up to 20g per day of inulin or fructo-oligosaccharides. Many foods naturally high in inulin or fructo-oligosaccharides, such as chicory, garlic, and leek, have been seen as "stimulants of good health" for centuries. Inulin is also used in medical tests to measure the total amount of extracellular volume and determine the function of the kidneys.

Inulin is generally recognized as safe (GRAS) by the FDA. Contrary to the health benefits as mentioned above, allergic reactions to inulin in foods have been reported in a letter[11] to the New England Journal of Medicine. Between about 30-40% of the population suffers from fructose malabsorption. Since inulin is a fructan, it is problematic for people with fructose malabsorption. It is recommended that fructan intake for people with fructose malabsorption be kept to less than 0.5 grams/serving.

Natural sources of inulin

Plants that contain high concentrations of inulin include:

- Elecampane (*Inula helenium*)
- Dandelion (*Taraxacum officinale*)
- Wild Yam (*Dioscorea* spp.)
- Jerusalem artichokes (*Helianthus tuberosus*)
- Chicory (*Cichorium intybus*)
- Jicama (*Pachyrhizus erosus*)
- Burdock (*Arctium lappa*)
- Onion (*Allium cepa*)
- Garlic (*Allium sativum*)
- Agave (*Agave* spp.)
- Yacon (*Smallanthus sonchifolius* spp.)

Indigestible

CELLULOSE

History

Cellulose was discovered in 1838 by the French chemist Anselme Payen, who isolated it from plant matter and determined its chemical formula. Cellulose was used to produce the first successful thermoplastic polymer, celluloid, by Hyatt Manufacturing Company
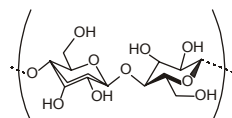
in 1870. Hermann Staudinger determined the polymer structure of cellulose in 1920. The compound was first chemically synthesized (without the use of any biologically-derived enzymes) in 1992, by Kobayashi and Shoda.

Cellulose is an organic compound with the formula $(C_6H_{10}O_5)_n$, a polysaccharide consisting of a linear chain of several hundred to over ten thousand $\beta(1\rightarrow4)$ linked D-glucose units.
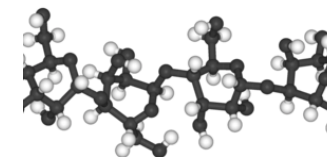
Cellulose is the structural component of the primary cell wall of green plants, many forms of algae and the oomycetes. Some species of bacteria secrete it to form biofilms. Cellulose is the most common organic compound on Earth. About 33 percent of all plant matter is cellulose (the cellulose content of cotton is 90 percent and that of wood is 50 percent).

For industrial use, cellulose is mainly obtained from wood pulp and cotton. It is mainly used to produce cardboard and paper; to a smaller extent it is converted into a wide variety of derivative products such as cellophane and rayon.

Some animals, particularly ruminants and termites, can digest cellulose with the help of symbiotic micro-organisms that live in their guts. Cellulose is not digestible by humans and is often referred to as 'dietary fiber' or 'roughage', acting as a hydrophilic bulking agent for feces.

Structure and properties

Cellulose is taste- and odorless, hydrophilic, not soluble in water or most organic solvents, chiral and biodegradable.

Cellulose is derived from D-glucose units, which condense through $\beta(1\rightarrow4)$-glycosidic bonds. This linkage motif contrasts with that for $\alpha(1\rightarrow4)$-glycosidic bonds present in starch, glycogen, and other carbohydrates. Cellulose is a straight chain polymer: unlike starch, no coiling occurs, and the molecule adopts an extended and rather stiff rod-like conformation. The multiple hydroxyl groups on the glucose residues from one chain form hydrogen bonds with oxygen molecules on another chain, holding the chains firmly together side-by-side and forming *microfibrils* with high tensile strength. This strength is important in cell walls, where they are meshed into a carbohydrate *matrix*, conferring rigidity to plant cells.

Compared to starch, cellulose is also much more crystalline. Whereas starch undergoes a crystalline to amorphous transition when heated beyond 60-70 °C in water (as in cooking), cellulose requires a temperature of 320 °C and pressure of 25 MPa to become amorphous in water.

Chemically, cellulose can be broken down into its glucose units by treating it with concentrated acids at high temperature.

Many properties of cellulose depend on its degree of polymerization or chain length, the number of glucose units that make up one polymer molecule. Cellulose from wood pulp has typical chain lengths between 300 and 1700 units; cotton and other plant fibers as well as bacterial celluloses have chain lengths ranging from 800 to 10,000 units. Molecules with very small chain length resulting from the

break down of cellulose are known as cellodextrins; in contrast to long-chain cellulose, cellodextrins are typically soluble in water and organic solvents.

Plant-derived cellulose is usually contaminated with hemicellulose, lignin, pectin and other substances, while microbial cellulose is quite pure, has a much higher water content, and consists of long chains.

Assaying cellulose

Given a cellulose-containing material, the portion that does not dissolve in a 17.5% solution of sodium hydroxide at 20 °C is α *cellulose*, which is true cellulose. Acidification of the extract precipitates β *cellulose*. The portion that dissolves in base but does not precipitate with acid is $\tilde{a}$ *cellulose*.

Cellulose can be assayed using a method described by Updegraff in 1969, where the fiber is dissolved in acetic and nitric acid, and allowed to react with anthrone in sulfuric acid. The resulting coloured compound is assayed spectrophotometrically at a wavelength of approximately 635 nm.

In addition, cellulose is represented by the difference between acid detergent fiber (ADF) and acid detergent lignin(ADL).

Biosynthesis
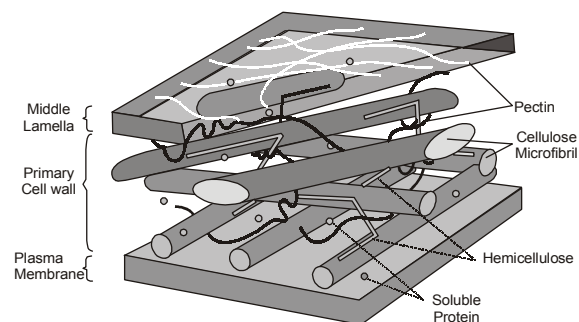
Fig. 2.7 : Location and arrangement of cellulose microfibrils in the plant cell wall.

In vascular plants cellulose is synthesized at the plasma membrane by rosette terminal complexes (RTC's). The RTC's are hexameric protein structures, approximately 25 nm in diameter, that contain the cellulose synthase enzymes that synthesise the individual cellulose chains. Each RTC floats in the cell's plasma membrane and "spins" a microfibril into the cell wall.

The RTC's contain at least three different cellulose syntheses, encoded by *CesA* genes, in an unknown stoichiometry. Separate sets of *CesA* genes are involved in primary and secondary cell wall biosynthesis. Cellulose synthase utilizes UDP-D-glucose precursors to generate microcrystalline cellulose.

Cellulose synthesis requires chain initiation and elongation, and the two processes are separate. *CesA* glucosyltransferase initiates cellulose polymerization using a steroid primer, 'sitosterol-beta-glucoside' and UDP-glucose. A cellulase may function to cleave the primer from the mature chain.

Breakdown (cellulolysis)

Cellulolysis is the process of breaking down cellulose into smaller polysaccharides called cellodextrins or completely into glucose units; this is a hydrolysis reaction. Because cellulose molecules bind strongly to each other, cellulolysis is relatively difficult compared to the break down of other polysaccharides.[11]

Mammals do not have the ability to break down cellulose directly. Some ruminants like cows and sheep contain certain symbiotic anaerobic bacteria (like *Cellulomonas*) in the flora of the gut wall, and these bacteria produce enzymes to break down cellulose; the break down products are then used by the mammal. Similarly, lower termites contain in their hindguts certain flagellate protozoa which produce such enzymes; higher termites contain bacteria for the job. Fungi, which in nature are responsible for recycling of nutrients, are also able to break down cellulose.

The enzymes utilized to cleave the glycosidic linkage in cellulose are glycoside hydrolases including endo-acting cellulases and exo-acting glucosidases. Such enzymes are usually secreted as part of multienzyme complexes that may include dockerins and cellulose binding modules; these complexes are in some cases referred to as cellulosomes.

Hemicellulose

Hemicellulose is a polysaccharide related to cellulose that comprises ca. 20% of the biomass of most plants. In contrast to cellulose, hemicellulose is derived from several sugars in addition to glucose, including especially xylose but also mannose, galactose, rhamnose, and arabinose. Hemicellulose consists of shorter chains - around 200 sugar units as opposed to 7,000 - 15,000 glucose molecules in the average cellulose polymer. Furthermore, hemicellulose is branched, whereas cellulose is unbranched.

Commercial products

Cellulose is the major constituent of paper and cardboard and of textiles made from cotton, linen, and other plant fibers.

Cellulose can be converted into cellophane, a thin transparent film, and into rayon, an important fiber that has been used for textiles since the beginning of the 20th century. Both cellophane and rayon are known as "regenerated cellulose fibers"; they are identical to cellulose in chemical structure and are usually made from viscose, a viscous solution made from cellulose. A more recent and environmentally friendly method to produce rayon is the Lyocell process. Cellulose is used in the laboratory as the stationary phase for thin layer chromatography. It is the raw material in the manufacture of nitrocellulose (cellulose nitrate) which was historically used in smokeless gunpowder and as the base material for celluloid used for photographic and movie films until the mid 1930s. Cellulose is used to make hydrophilic and highly absorbent sponges and water-soluble adhesives and binders such as methyl cellulose and carboxymethyl cellulose which are used in wallpaper paste. Microcrystalline cellulose (E460i) and powdered cellulose (E460ii) are used as an inactive filler in tablets and as thickeners and stabilizers in processed foods.

Cellulose source and energy crops

The major combustible component of non-food energy crops is cellulose, with lignin second. Non-food energy crops are more efficient than edible energy crops (which have a large starch component), but still compete with food crops for agricultural land and water resources.[6] Typical non-food energy crops include industrial hemp, switchgrass, *Miscanthus*, *Salix* (willow), and *Populus* (poplar) species.

Some bacteria can convert cellulose into ethanol which can then be used as a fuel; see cellulosic ethanol.

Nitrocellulose (also: **cellulose nitrate**, **flash paper**) is a highly flammable compound formed by nitrating cellulose through exposure to nitric acid or another powerful nitrating agent. When used as a propellant or low-order explosive, it is also known as **guncotton**.

Guncotton and gunpowder

Various types of smokeless powder, consisting primarily of nitrocellulose

Henri Braconnot discovered in 1832 that nitric acid, when combined with starch or wood fibers, would produce a lightweight combustible explosive material which he named *xyloïdine*. A few years later in 1838 another French chemist Théophile-Jules Pelouze (teacher of Ascanio Sobrero and Alfred Nobel) treated paper and cardboard in the same way. He obtained a similar material he called *nitramidine*. Both of these substances were highly unstable, and were not practical explosives.

However, Christian Friedrich Schönbein, a German-Swiss chemist, discovered a more practical solution around 1846. As he was working in the kitchen of his home in Basle, he spilled a bottle of concentrated nitric acid on the kitchen table. He reached for the nearest cloth, a cotton apron, and wiped it up. He hung the apron on the stove door to dry, and as soon as it was dry there was a flash as the apron exploded. His preparation method was the first to be widely imitated — one part of fine cotton wool to be immersed in fifteen parts of an equal blend of sulfuric and nitric acids. After two minutes the cotton was removed and washed in cold water to set the esterification level and remove all acid residue. It was then slowly dried at a temperature below 100°C. Schönbein collaborated with the Frankfurt professor Rudolf Böttger, who had discovered the process independently in the same year. By a strange coincidence there was even a third chemist, the Braunschweig professor F. J. Otto, who had also produced guncotton in 1846 and was the first to publish the process, much to the disenchantment of Schönbein and Böttger. (Itzehoer Wochenblatt, 29 October 1846, columns 1626 f.)

The process uses the nitric acid to convert the cellulose into cellulose nitrite and water:

$$2HNO_3 + C_6H_{10}O_5 \rightarrow C_6H_8(NO_2)_2O_5 + 2H_2O$$
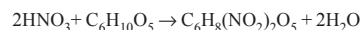
The sulfuric acid is present to prevent the water produced in the reaction from diluting the concentrated nitric acid.

The power of guncotton made it suitable for blasting. As a projectile driver, it has around six times the gas generation of an equal volume of black powder and produces less smoke and less heating. However the sensitivity of the material during production led the British, Prussians and French to discontinue manufacture within a year.

Jules Verne viewed the development of guncotton with optimism. He referred to the substance several times in his novels. His adventurers carried firearms employing this substance. Most notably, in his *From the Earth to the Moon*, guncotton was used to launch a projectile into space.

Further research indicated that the key was the very careful preparation of the cotton: unless it was very well cleaned and dried, it was likely to explode spontaneously. The British, led by Frederick Augustus Abel, also developed a much lengthier manufacturing process at the Waltham Abbey Royal Gunpowder Mills, patented in 1865, with the washing and drying times each extended to 48 hours and repeated eight times over. The acid mixture was also changed to two parts sulfuric acid to one part nitric.

Guncotton remained useful only for limited applications. For firearms, a more stable and slower burning mixture would be needed. Guncotton-like preparations were eventually prepared for this role, known at the time as smokeless powder.

Guncotton, dissolved at approximately 25% in acetone, forms a lacquer used in preliminary stages of wood finishing to develop a hard finish with a deep luster. It is normally the first coat applied, sanded, and followed by other coatings that bond to it.

Nitrate film

Nitrocellulose was used as the first flexible film base, beginning with Eastman Kodak products in August, 1889. Camphor is used as plasticizer for nitrocellulose film. It was used until 1933 for X-ray films (where its flammability hazard was most acute) and for motion picture film until 1951. It was replaced by safety film with an acetate base. German WW2 newsreel film (circa 1940) believed to be nitrocellulose, made by AGFA The use of nitrocellulose film for motion pictures led to a widespread requirement for fireproof projection rooms with wall coverings made of asbestos. Famously, the US Navy shot a training film for projectionists which included footage of a controlled ignition of a reel of nitrate film which continued to burn even when fully submerged in water. Due to public safety precautions, the London Underground forbade transport of nitrate films on its system until well past the introduction of safety film. A cinema fire caused by ignition of nitrocellulose film stock (foreshadowed by an earlier small fire) was a central plot element in the Italian film *Cinema Paradiso*. Today nitrate film projection is usually highly regulated and requires extensive precautionary measures including extra projectionist health and safety training. Additionally, projectors certified to run nitrate films have many containment strategies in effect. Among them, this includes the chambering of both the feed and takeup reels in thick metal covers with small slits to allow the film to run through. Furthermore, the projector is modified to accommodate several fire extinguishers with nozzles all aimed directly at the film gate; the extinguishers automatically trigger if a piece of flammable fabric placed near the gate starts to burn. While this triggering would likely damage or destroy a significant portion of the projection components, it would prevent a devastating fire which almost certainly would cause far greater damage.

It was discovered decades later that nitrocellulose gradually decomposes, releasing nitric acid which further catalyzes the decomposition (usually into a still-flammable powder or goo). Low temperatures can delay these reactions indefinitely. It is estimated that the great majority of films produced during the early twentieth century were lost forever either through this accelerating, self-

catalyzed disintegration or studio warehouse fires. Salvaging old films is a major problem for film archivists (see film preservation).

Nitrocellulose film base manufactured by Kodak can be identified by the presence of the word *Nitrate* in dark letters between the perforations. Acetate film manufactured during the era when nitrate films were still in use was marked *Safety* or *Safety Film* between the perforations dark letters. Letters in white or light colors are print-through from the negative.

Color negative film was never manufactured with a nitrate base, nor were 8 mm or 16 mm motion picture film stocks.

Other uses

Depending on the manufacturing process, nitrocellulose is esterified to varying degrees. Table tennis balls, guitar picks and some photographic films have a fairly low esterification level and burn comparatively slowly with some charred residue. See celluloid.

In professional wrestling, nitrocellulose, in the form of a small piece of flash paper, is used to execute an illegal (and dangerous) move called a **Fireball**. The wrestler sets the piece of quick-burning paper alight (using a concealed lighter) and throws it at the opponent, giving the impression of a supernatural ball of fire emerging from their hand.

Nitrated cotton: Used as liftcharge for indoor fireworks, for hand flashers and magicians

Nitrated yarn: Used as fuse for indoor fireworks and to make things fall down on command

Nitrated paper: Mainly used by magicians to make paper disapear in a flash, but also indoor fireworks as comets

Nitrated cellulose: Ice fountains, indoor fireworks, making smokeless gunpowder, celluloid, paints

Collodion is a solution of nitrocellulose in ether or acetone, sometimes with the addition of alcohols. Its generic name is pyroxylin solution. It is toxic and highly flammable. As the solvent evaporates, it dries to a celluloid-like film. It was discovered about 1846 by the French chemist and writer Louis Ménard. n 1851, the Englishman Frederick Scott Archer discovered that collodion could be used as an alternative to albumen on glass plates. This also reduced the exposure time when making the image. This became known as the wet plate Collodion or wet collodion method. Collodion was also grainless and colorless, and allowed for one of the first high quality duplication processes, also known as negatives. This process also produced positives, the Ambrotype and the Ferrotype (aka Tintypes).

Celloidin is a pure type of pyroxylin used to embed specimens which will be examined under a microscope.

Uses

- Collodion is widely used to glue electrodes to the head for electroencephalography.
- Pyroxylin with added pigments is used as a nitrocellulose lacquer.
- It was also added to nitroglycerine to stabilise it as blasting gelatine.
- Collodion is also used in theatrical makeup for various effects, such as simulating old-age wrinkles or scars.

- Similarly, it was sometimes used in boxing and other applications to cover up cuts. However, it has been generally replaced by the less toxic Liquid Bandage, which includes pyroxylin as an ingredient.
- Collodion also finds use in the cleaning of optics such as telescope mirrors. The collodion is applied to the surface of the optic, usually in two or more layers. Sometimes a piece of thin cloth is applied between the layers, to hold the collodion together for easy removal. After the collodion dries and forms a solid sheet covering the optic, it is carefully peeled away taking contamination with it.
- Collodion was also used by pathologist Thomas Stoltz Harvey to preserve Einstein's brain in 1955.

SECTION 2.2—ISOMERISM

- The simplest aldosugar (aldose) is glyceraldehyde and the simplest ketosugar (ketose) is dihydroxyacetone.

Aldoses

Definition

Carbohydrates are defined as organic polyhydroxyaldehydes or polyhydroxyketones or compounds which produce them on hydrolysis.

Functions of carbohydrates

They are important due to their multiple roles in all forms of life:

- They serve as energy stores and fuels. Energy from the sun captured by plants during photosynthesis is stored in the form of carbohydrates. Breakdown of carbohydrates provides the energy that maintains animal life. Glucose is main source of energy for the brain.
- They participate in the structure of cell membranes.
- Pentose sugars (ribose and deoxyribose) form the structure of nucleic acids.
- Carbohydrates play important roles in recognition between cell types or recognition of cellular structures by other molecules. Recognition events are important in normal cell growth, fertilization and transformation of cells.
- Carbohydrates form the structure of glycoproteins and glycolipids.

Classification of Carbohydrates

They are classified according to the "number of structural units" into:

· Monosaccharides
· Disaccharides

CHO
2 H—C—OH
3 CH₂OH
D-Glyceraldehyde

1 CHO
2 H—C—OH
3 H—C—OH
4 CH₂OH
D-Erythrose

CHO
HO—C—H
H—C—OH
CH₂OH
D-Threose

CHO
H—C—OH
H—C—OH
H—C—OH
CH₂OH
D-Ribose

CHO
HO—C—H
H—C—OH
H—C—OH
CH₂OH
D-Arabinose

CHO
H—C—OH
HO—C—H
H—C—OH
CH₂OH
D-Xylose

CHO
HO—C—H
HO—C—H
H—C—OH
CH₂OH
D-Lyxose

CHO / CHO
D-Allose   D-Altrose

CHO / CHO
D-Glucose D-Mannose

CHO / CHO
D-Gulose   D-Idose

CHO / CHO
D-Guluctose D-Tolose

CH₂OH
C = O
CH₂OH
Dihydroxyacetone

CH₂OH
C = O
HOCH
CH₂OH
D-Xylulose

CH₂OH
C = O
HCOH
HCOH
CH₂OH
D-Ribulose

CH₂OH
C = O
HOCH
HCOH
HCOH
CH₂OH
D-Fructose

CH₂OH
C = O
HOCH
HCOH
HCOH
HCOH
CH₂OH
D-Sedoheptulose

· Oligosaccharides and
· Polysaccharides

Monosaccharides

• They are carbohydrates that cannot be hydrolysed into simpler units.
  General formula is (C.H2O)n.

• The smallest ones, for which n = 3, are glyceraldehyde and dihydroxyacetone. They are trioses.
• Glyceraldehyde is also an aldose because it contains an aldehyde group; whereas dihydroxyacetone is a ketose because it contains a keto group, (the suffix- ose means sugar)

Classification of Monosaccharides

They are classified according to :

1. Number of carbon atoms in the molecule: They may be subdivided into, trioses, tetroses, pentoses, hexoses, heptoses or octoses.

General formula	Aldosugars	Ketosugrs
• Trioses $(C_3H_6O_3)$	Glyceraldehyde	Dihydroxyacetone
• Tetrases $(C_4H_8O_4)$	Erythorse	Ertythrulose
• Pentoses $(C_5H_{10}O_5)$	Ribose	Ribulose
• Hexoses $(C_6H_{12}O_6)$	Glucose	Fructose

2. Type of functional group as aldosugars and ketosugars.
   • Examples of aldoses are glyceraldehyde, erythrose, ribose, glucose and galactose.
   • Examples of ketoses are dihydroxyacetone, Erythrulose, Ribulose, Fructose.

General formula	Aldosugars	Ketosugrs
• Trioses $(C_3H_6O_3)$	Glyceraldehyde	Dihydroxyacetone
• Tetrases $(C_4H_8O_4)$	Erythorse	Ertythrulose
• Pentoses $(C_5H_{10}O_5)$	Ribose	Ribulose
• Hexoses $(C_6H_{12}O_6)$	Glucose	Fructose

Diasaccharides

• These carbohydrates yield two molecules of monosaccharides when hydrolysed.

MALTOSE ⟶ GLUCOSE + GLUCOSE

SUCROSE ⟶ GLUCOSE + FRUCTOSE

LACTOSE ⟶ GLUCOSE + GALACTOSE
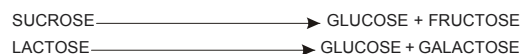
Oligosaccharides

- They yield three to ten molecules of monosaccharides on hydrolysis, example is Maltotriose which is a trisaccharide containing three glucose residues.

Polysaccharides

- They yield more than ten molecules of monosaccharides on hydrolysis. They may be either linear or branched polymers and may contain hundreds or even thousands of monosaccharide units . Examples are starch, glycogen, dextran.

Properties of Monosaccharides In Reference to Glucose

D-Glucose

Projects right for the D isomer
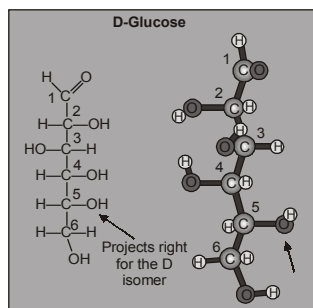
D-glucose
- Glucose is an aldohexose sugar.
- Common names include dextrose, grape sugar, blood sugar.
- Most important sugar in out diet.
- Most abundant organic compound found in nature.
- Level in blood can be as high as 0.1%.
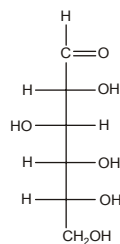
1. All monosaccharide are crystals and dissolve in water.

2. All have a Sweet taste.

3. Monosaccharides contain asymmetric carbon atom (which is a carbon atom attached to four different chemical groups) except dihydroxyacetone.

Due to presence of asymmetric carbon atom, monosaccharides have two main physical properties which are:

- Optical Isomerism or Stereoisomerism
- Optical Activity

4. Cyclic structure of Monosaccharides

5. Mutarotation

Asymmetric Carbon

Asymmetric carbon

4 asymmetric carbons

Asymmetric carbon atiom

D-Glucose

Dihydroxyacetone

Projects right for the D isomer
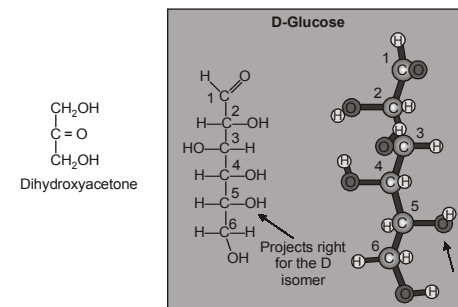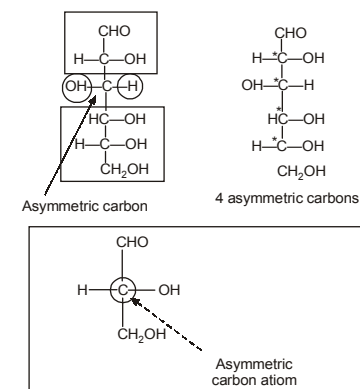
SUBSECTION 2.2B—OPTICAL ISOMERISM OR STEREOISOMERISM

- Optical isomerism or stereoisomerism is the ability of substances to be present in more than one form. It is due to the presence of asymmetric carbon atom. These forms are named optical isomers.

- So optical isomers or stereoisomers or isomers are compounds that have same structural formula but differ in their configuration around the asymmetric carbon atom.

- A substance containing more than one asymmetric carbon atom can exist in a number of isomers equal to 2n, where n equal to the number of asymmetric carbon atoms e.g Glucose has 4 asymmetric carbon atoms, so the number of optical isomers equal to 24 =2x2x2x2 = 16.

Enantiomers

- Enantiomers are a type of isomers designated as "D" and "L" isomers that are mirror images of each other and differ in the configuration around the asymmetric carbon before the last (sub-terminal) e.g D and L glyceradehyde, D and L glucose.

D-glyceraldehyde   L-glyceraldehyde

L-Glucose   D-Glucose
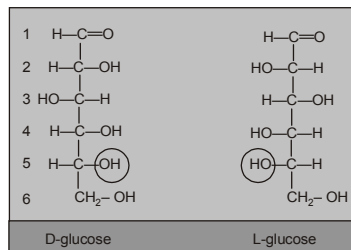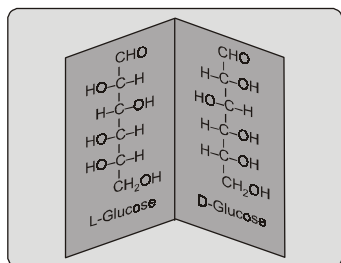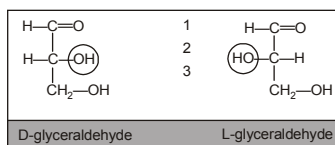
D-glucose   L-glucose

- In D isomer (D-glucose) the OH group attached to the asymmetric carbon (sub-terminal C5) is towards the right while in L isomer (L-glucose) OH group is on the left.

"The majority of the sugars in humans are D-sugars".

Galctose

C-4 epimers

Glucose

C-2 epimers

Mannose

Isomers

Fructose
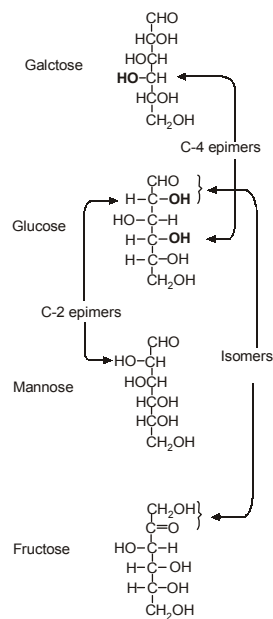
Optical Activity
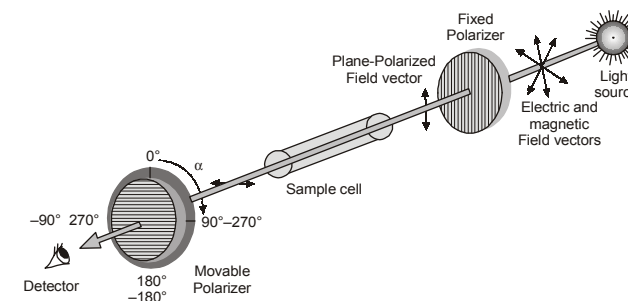
Fig. 2.7 : Showing the Polarimeter arrangement for optical isomerism experiment

- It is the ability of an organic compound containing one or more asymmetric carbon atom to rotate a beam of polarized light either to the right or to the left.
- If the rotation is to the right it is called dextrorotatory (+ or d) and if it is to the left it is known as levorotatory (- or l).
- Polarized light is the light which vibrates in only one direction. It is obtained by the passage of ordinary light through a prism.

SUB-SECTION 2.2C EPIMERS

- Epimers are sterioisomers that differ in the position of (OH) group at only one of their asymmetric carbon atom other than before the last (sub-terminal) carbon atom. e.g
- D-Glucose and D-Galactose are C4 epimers.
- D-Glucose and D-Mannose are C2 epimers.

SUB-SECTION 2.2D—MUTAROTATION AND SPECIFIC ROTATION

- The α- and β- anomers of carbohydrates are typically stable solids.

Mutarotation: interconversion of α- and α- anomers

- However, in aqueous solution, they quickly equilibrate to an equilibrium mixture of the two forms.
- For example, in aqueous solution, glucose exists as a mixture of 36% α- and 64% β- (>99% of the pyranose forms exist in solution).
- The equilibration occurs via the ring opening of the cyclic sugar at the anomeric center with the acyclic form as the intermediate.

α-D-glucose   D-glucose   β-D-glucose

Or, in a simplified form with all the "extra" hydroxy groups removed:

ring-opening of the   Conformational change   Ring-closure to form
cyclic hemi-acetal   of the carbonyl group   the cyclic hemi-acetal
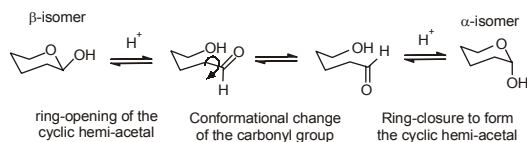
Chemists use polarimeters (see fig 22) to investigate the influence of compounds (in the sample cell) on plane polarized light. Samples composed only of achiral molecules (e.g. water or hexane), have no effect on the polarized light beam. However, if a single enantiomer is examined (all sample molecules being right-handed, or all being left-handed), the plane of polarization is rotated in either a clockwise (positive) or counter-clockwise (negative) direction, and the analyzer must be turned an appropriate matching angle, α, if full light intensity is to reach the detector. In the above illustration, the sample has rotated the polarization plane clockwise by +90º, and the analyzer has been turned this amount to permit maximum light transmission.

The observed rotations (α) of enantiomers are opposite in direction. One enantiomer will rotate polarized light in a clockwise direction, termed **dextrorotatory** or (+), and its mirror-image partner in a counter-clockwise manner, termed **levorotatory** or (–). The prefixes dextro and levo come from the Latin *dexter*, meaning right, and *laevus*, for left, and are abbreviated *d* and *l* respectively. If equal quantities of each enantiomer are examined , using the same sample cell, then the magnitude of the rotations will be the same, with one being positive and the other negative. To be absolutely certain whether an observed rotation is positive or negative it is often necessary to make a second measurement using a different amount or concentration of the sample. In the above illustration, for example, α might be –90º or +270º rather than +90º. If the sample concentration is reduced by 10%, then the positive rotation would change to +81º (or +243º) while the negative rotation would change to –81º, and the correct α would be identified unambiguously.

Since it is not always possible to obtain or use samples of exactly the same size, the observed rotation is usually corrected to compensate for variations in sample quantity and cell length. Thus it is common practice to convert the observed rotation, α, to a **specific rotation, [α]**, by the following formula:

Specific Rotation   =   where l = cell length in dm, c = concentration in g/ml
D is the 589 nm light from a sodium lamp

Compounds that rotate the plane of polarized light are termed **optically active**. Each enantiomer of a stereoisomeric pair is optically active and has an equal but opposite-in-sign specific rotation. Specific

rotations are useful in that they are experimentally determined constants that characterize and identify pure enantiomers. For example, the lactic acid and carvone enantiomers discussed earlier have the following specific rotations.

Carvone from caraway: $[\alpha]_D = +62.5º$ this isomer may be referred to as (+)-carvone or *d*-carvone

Carvone from spearmint: $[\alpha]_D = -62.5º$ this isomer may be referred to as (–)-carvone or *l*-carvone

Lactic acid from muscle tissue: $[\alpha]_D = +2.5º$ this isomer may be referred to as (+)-lactic acid or *d*-lactic acid

Lactic acid from sour milk: $[\alpha]_D = -2.5º$ this isomer may be referred to as (–)-lactic acid or *l*-lactic acid

A 50:50 mixture of enantiomers has no observable optical activity. Such mixtures are called **racemates** or racemic modifications, and are designated (±). When chiral compounds are created from achiral compounds, the products are racemic unless a single enantiomer of a chiral co-reactant or catalyst is involved in the reaction. The addition of HBr to either cis- or trans-2-butene is an example of racemic product formation (the chiral center is colored red in the following equation).

$$CH_3CH=CHCH_3 + HBr (\pm) CH_3CH_2CHBrCH_3$$

Chiral organic compounds isolated from living organisms are usually optically active, indicating that one of the enantiomers predominates (often it is the only isomer present). This is a result of the action of chiral catalysts we call enzymes, and reflects the inherently chiral nature of life itself. Chiral synthetic compounds, on the other hand, are commonly racemates, unless they have been prepared from enantiomerically pure starting materials.

There are two ways in which the condition of a chiral substance may be changed:

1. A racemate may be separated into its component enantiomers. This process is called resolution.

2. A pure enantiomer may be transformed into its racemate. This process is called racemization.

SECTION 2.3 GLYCOSIDIC LINKAGES

SUBSECTION 2.3A DEOXY SUGARS

Deoxy sugars are sugars that have had a hydroxyl group replaced with a hydrogen.

Comparison of the chemical structure of ribose (top) and deoxyribose (bottom)

Deoxyribose, also known as **D-Deoxyribose** and **2-deoxyribose**, is an aldopentose — a monosaccharide containing five carbon atoms, and including an aldehyde functional group in its linear structure. It is a deoxy sugar derived from the pentose sugar ribose by the replacement of the hydroxyl group at the 2 position with hydrogen, leading to the net loss of an oxygen atom.
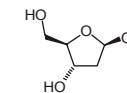
Replacement of the hydroxyl group also shifts the conformation of the ring from C3'-endo to C2'-endo. It has a chemical formula of $C_5H_{10}O_4$; it was discovered in 1929 by Phoebus Levene.

Ribose forms a five-member ring composed of four carbon atoms and one oxygen. Hydroxyl groups are attached to three of the carbons. The other carbon and a hydroxyl group are attached to one of the carbon atoms adjacent to the oxygen. In deoxyribose, the carbon furthest from the attached carbon is stripped of the oxygen atom in what would be a hydroxyl group in ribose. Due to the common C3' and C4' stereochemistry of D-ribose and D-arabinose, D-2-deoxyribose is also D-2-deoxyarabinose.
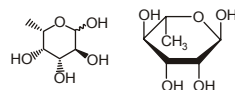
Deoxyribofuranose is an alternative name for the ring structure of deoxyribose. This alternative name merely refers to the fact that deoxyribose has a five membered ring consisting of four carbons and an oxygen and is more a structural description than a name

Ribose and 2-deoxyribose derivatives have an important role in biology. Among the most important derivatives are those with phosphate groups attached at the 5 position. Mono-, di-, and triphosphate forms are important, as well as 3-5 cyclic monophosphates. There are also important diphosphate dimers called coenzymes that purines and pyrimidines form an important class of compounds with ribose and deoxyribose. When these purine and pyrimidine derivatives are coupled to a ribose sugar, they are called nucleosides. In these compounds, the convention is to put a 2 (pronounced "prime") after the carbon numbers of the sugar, so that in nucleoside derivatives a name might include, for instance, the term "52 -monophosphate", meaning that the phosphate group is attached to the fifth carbon of the sugar, and not to the base. The bases are attached to the 12 ribose carbon in the common nucleosides. Phosphorylated nucleosides are called nucleotides.

| Biological importance of deoxyribose |

One of the common bases is adenine (a purine derivative); coupled to ribose it is called adenosine; coupled to deoxyribose it is called deoxyadenosine. The 52 -triphosphate derivative of adenosine, commonly called ATP, for adenosine triphosphate, is an important energy transport molecule in cells.

See *Nucleic acid nomenclature* for a diagram showing the numbered positions in a 52 -2-Deoxyribose and ribose nucleotides are often found in unbranched 52 -32 polymers. In these structures, the 32 carbon of one monomer unit is linked to a phosphate that is attached to the 52 carbon of the next unit, and so on. These polymer chains often contain many millions of monomer units. Since long polymers have physical properties distinctly different from those of small molecules, they are called macromolecules. The sugar-phosphate-sugar chain is called the backbone of the polymer. One end of the backbone has a free 52 phosphate, and the other end has a free 32 OH group. The backbone structure is independent of which particular bases are attached to the individual sugars.

Genetic material in earthly life often contains poly 52 -32 , 22 -deoxyribose nucleotides, in structures called chromosomes, where each monomer is one of the nucleotides deoxy- adenine, thymine, guanine or cytosine. This material is commonly called deoxyribonucleic acid, or simply DNA for short.

DNA in chromosomes forms very long helical structures containing two molecules with the backbones running in opposite directions on the outside of the helix and held together by hydrogen bonds between

complementary nucleotide bases lying between the helical backbones. The lack of the 22 hydroxyl group in DNA appears to allow the backbone the flexibility to assume the full conformation of the long double-helix, which involves not only the basic helix, but additional coiling necessary to fit these very long molecules into the very small volume of a cell nucleus.

In contrast, very similar molecules, containing ribose instead of deoxyribose, and known generically as RNA, are known to form only relatively *short* double-helical complementary base paired structures. These are well known, for instance, in ribosomal RNA molecules and in transfer RNA (tRNA), where so-called *hairpin* structures from palindromic sequences within one molecule.

Deoxyribopyranodr 75%   0.7%   Deoxyribofuranose 25%
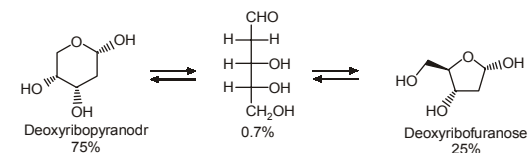
Fucose is a hexose deoxy sugar with the chemical formula $C_6H_{12}O_5$. It is found on *N*-linked glycans on the mammalian, insect and plant cell surface, and is the fundamental sub-unit of the fucoidan polysaccharide. Alpha1→3 linked core fucose is a suspected carbohydrate antigen for IgE-mediated allergy.

Two structural features distinguish fucose from other six-carbon sugars present in mammals: the lack of a hydroxyl group on the carbon at the 6-position (C-6) and the L-configuration. It is equivalent to 6-deoxy-L-galactose.

In the fucose-containing glycan structures, fucosylated glycans, fucose can exist as a terminal modification or serve as an attachment point for adding other sugars.[2] In human *N*-linked glycans, fucose is most commonly linked α-1,6 to the reducing terminal beta-*N*-acetlyglucosamine. However, fucose at the non-reducing termini linked α-1,2 to galactose forms the H antigen, the substructure of the A and B blood group antigens.

Fucose is metabolized by an enzyme called alpha-fucosidase. Fucose is abundant in human breast milk and certain mushrooms. It has been shown to influence brain development, act as immune modulator by inhibiting tumour growth and its spread and enhancer of cellular communication. It also guards against respiratory tract infections and inhibits allergic reactions. High concentrations of fucose are found at the junctions of nerves, as well as in the kidneys, testes, and in the outer layer of skin.

Rhamnose is a naturally-occurring deoxy sugar. It can be classified as either a methyl-pentose or a 6-deoxy-hexose. Rhamnose occurs in nature in its L-form as L-rhamnose (6-deoxy-L-mannose). This is unusual, since most of the naturally-occurring sugars are in D-form. Exceptions are the methyl pentoses L-fucose and L-rhamnose and the pentose L-arabinose. L-Rhamnose can be isolated from Buckthorn (Rhamnus) and poison sumac. It is also found as a glycoside in a variety of other plants. Rhamnose is a component of the outer cell membrane of acid-fast bacteria in the Mycobacterium genus, which includes the organsism that causes tuberculosis.
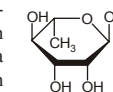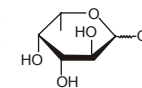
Amino Sugars

In chemistry, an **amino sugar** contains an amine group in place of a hydroxyl group. Derivatives of amine containing sugars, such as N-acetylglucosamine and sialic acid, while not formally containing an amine, are also considered amino sugars.

Aminoglycosides are a class of antimicrobial compounds that inhibit bacterial protein synthesis. These compounds frequently contain amino sugars (but in some cases contain aminocyclitols).

Common examples of amino sugars include:
- Galactosamine
- Glucosamine
- Sialic acid
- N-Acetylglucosamine

Galactosamine is a hexosamine derived from galactose with the molecular formula $C_6H_{13}NO_5$. This amino sugar is a constituent of some glycoprotein hormones such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Other sugar constituents of FSH and LH include glucosamine, galactose and glucose.Galactosamine is a hepatotoxic, or liver-damaging, agent that is sometimes used in animal models of liver failure.

Glucosamine was first identified in 1876 by Dr. Georg Ledderhose, but the stereochemistry was not fully defined until 1939 by the work of Walter Haworth.[1] D-Glucosamine is made naturally in the form of glucosamine-6-phosphate, and is the biochemical precursor of all nitrogen-containing sugars.[2] Specifically, glucosamine-6-phosphate is synthesized from fructose-6-phosphate and glutamine[3] as the first step of the hexosamine biosynthesis pathway.[4] The end-product of this pathway is UDP-N-acetylglucosamine (UDP-GlcNAc), which is then used for making glycosaminoglycans, proteoglycans, and glycolipids. As the formation of glucosamine-6-phosphate is the first step for the synthesis of these products, glucosamine may be important in regulating their production. However, the way that the hexosamine biosynthesis pathway is actually regulated, and whether this could be involved in contributing to human disease, remains unclear.

Glucosamine ($C_6H_{13}NO_5$) is an amino sugar and a prominent precursor in the biochemical synthesis of glycosylated proteins and lipids. A type of glucosamine forms chitosan and chitin, which composes the exoskeletons of crustaceans and other arthropods, cell walls in fungi and many higher organisms. Glucosamine is one of the most abundant monosaccharides. It is produced commercially by the hydrolysis of crustacean exoskeletons or, less commonly and more expensive to the consumer, by fermentation of a grain such as corn or wheat.
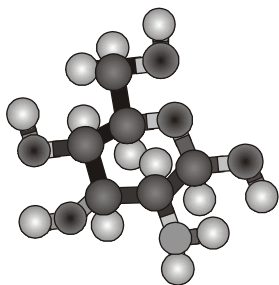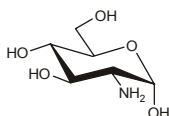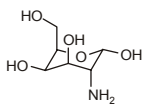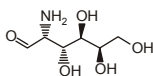
Glucosamine is commonly used as a treatment for osteoarthritis, although its acceptance as a medical therapy varies.

Sialic acid The term "sialic" (from the Greek salia (salia) 'saliva') was first introduced by Swedish biochemist, Gunnar Blix, in 1952

Sialic acid is a generic term for the N- or O-substituted derivatives of neuraminic acid, a monosaccharide with a nine-carbon backbone. It is also the name for the most common member of this group, N-acetylneuraminic acid (Neu5Ac or NANA).sialic acids are found widely distributed in animal tissues and in bacteria, especially in glycoproteins and gangliosides. The amino group bears either an acetyl or a glycolyl group. The hydroxyl substituents may vary considerably: acetyl, lactyl, methyl, sulphate, and phosphate groups have been found. Sialic acid-rich glycoproteins bind selectin in humans and other organisms. Cancer cells that can metastasize often have a lot ofsialic acid-rich glycoproteins. This helps these late-stage cancer cells enter the blood stream.sialic acid also plays an important role in Human Influenza infections. The influenza viruses (orthomyxoviridae) have Hemagglutin Activity (HA) glycoproteins on their surface that bind tosialic acids found on the surface of human erythrocytes and on the cell membranes of the upper respiratory tract. This is the basis of heme-agglutination when viruses are mixed with blood cells, and entry of the virus into cells of the upper respiratory tract. Sialic acid-rich oligosaccharides on the glycoconjugates found on surface membranes help keep water at the surface of cells. Thesialic acid-rich regions contribute to creating a negative charge on the cells' surface. Since water is a polar molecule with partial positive charges on both hydrogen atoms, it is attracted to cell surfaces and membranes. This also contributes to cellular fluid uptake.sialic acid can "hide" mannose antigens on the surface of host cells or bacteria from mannose-binding lectin. This prevents activation of complement.
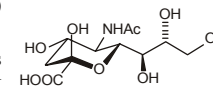
N-Acetylglucosamine (N-Acetyl-D-Glucosamine, or GlcNAc, or NAG) is a monosaccharide derivative of glucose. Chemically it is an amide between glucosamine and acetic acid. It has a molecular formula of $C_8H_{15}NO_6$, a molar mass of 221.21 g/mol, and it is significant in several biological systems. It is part of a biopolymer in the bacterial cell wall, built from alternating units of GlcNAc and N-acetylmuramic acid (MurNAc), cross-linked with oligopeptides at the lactic acid residue of MurNAc. This layered structure is called peptidoglycan. GlcNAc is the monomeric unit of the polymer chitin, which forms the outer coverings of insects and crustaceans. GlcNAc is also of note in neurotransmission, where it is thought to be an atypical neurotransmitter functioning in nociceptive (pain) pathways. Polymerized with glucuronic acid it forms hyaluronan. It has been proposed as a treatment for autoimmune diseases.
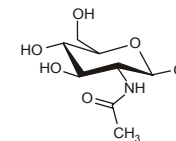
SUB-SECTION—2.3B

Homopolysaccharides are polymers composed of a single type of sugar monomer. For example, cellulose is an unbranched homopolysacchaide comprised of glucose monomers connected via beta-glycosidic linkages; glycogen is a branched form, where the glucose monomers are joined by alpha-glycosidic linkages

Glycosaminoglycans (GAGs) or **mucopolysaccharides** are long unbranched polysaccharides consisting of a repeating disaccharide unit.

Members of the glycosaminoglycan family vary in the type of hexosamine, hexose or hexuronic acid unit they contain (e.g. glucuronic acid, iduronic acid, galactose, galactosamine, glucosamine).

Classification

They also vary in the geometry of the glycosidic linkage. Examples of GAGs include:

Name	Hexuronic acid/ Hexose	Hexosamine	Linkage geometry between predominant monomeric units	Unique features
Chondroitin sulphate	GlcUA or GlcUA(2S)	GalNAc or GalNAc(4S) or GalNAc(6S) or GalNAc(4S,6S)	-4GlcUAβ1-3GalNAcβ1-	Most prevalent GAG
Dermatan sulphate	GlcUA or IdoUA or IdoUA(2S)	GalNAc or GalNAc(4S) or GalNAc(6S) or GalNAc(4S,6S)	-4IdoUAβ1-3GalNAcβ1-	Distinguished from chondroitin sulphate by the presence of iduronic acid, although some hexuronic acid monosaccharides may be glucuronic acid.
Keratan sulphate	Gal or Gal(6S)	GlcNAc or GlcNAc(6S)	-3Gal(6S)β1-4GlcNAc(6S)β1-	Keratan sulphate type II may be fucosylated.
Heparin	GlcUA or IdoUA(2S)	GlcNAc or GlcNS or GlcNAc(6S) or GlcNS(6S)	-4IdoUA(2S)β1-4GlcNS(6S)β1-	Highest negative charge density of any known biological molecule
Heparan sulphate	GlcUA or IdoUA or IdoUA(2S)	GlcNAc or GlcNS or GlcNAc(6S) or GlcNS(6S)	-4GlcUAβ1-4GlcNAcβ1-	Highly similar in structure to heparin, however heparan sulphates disaccharide units are organised into distinct sulphated and non-sulphated domains.
Hyaluronan	GlcUA	GlcNAc	-4GlcUAβ1-3GlcNAcβ1-	The only GAG that is exclusively non-sulphated

Abbreviations
- **GlcUA** = β-D-glucuronic acid
- **GlcUA(2S)** = 2-O-sulfo-β-D-glucuronic acid
- **IdoUA** = α-L-iduronic acid
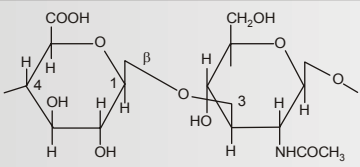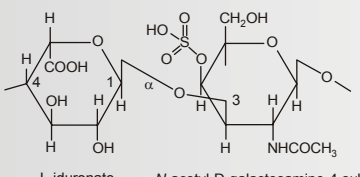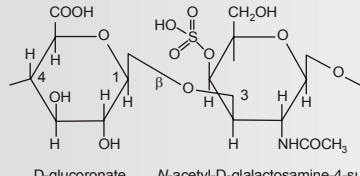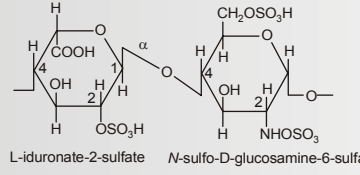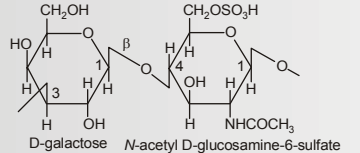
- **IdoUA(2S)** = 2-O-sulfo-α-L-iduronic acid
- **Gal** = β-D-galactose
- **Gal(6S)** = 6-O-sulfo-β-D-galactose
- **GalNAc** = β-D-N-acetylgalactosamine
- **GalNAc(4S)** = β-D-N-acetylgalactosamine-4-O-sulphate
- **GalNAc(6S)** = β-D-N-acetylgalactosamine-6-O-sulphate
- **GalNAc(4S,6S)** = β-D-N-acetylgalactosamine-4-O, 6-O-sulphate
- **GlcNAc** = α-D-N-acetylglucosamine
- **GlcNS** = α-D-N-sulfoglucosamine
- **GlcNS(6S)** = α-D-N-sulfoglucosamine-6-O-sulphate

SUB-SECTION 2.3C—GLYCOSAMINOGLYCANS

Glycosaminoglycans (GAGs) are a family of sulphated sugar chains that play important roles in neuronal communication by binding to different proteins. Subtle variations in **stereochemistry**, **length**, and **patterns of sulfation** differ between and within GAG families. This structural diversity creates an enormous number of protein binding motifs. However, it also has hampered efforts to define the structural and functional properties of GAGs.

We are developing two general methods for the synthesis of glycosaminoglycans. Chemical and chemoenzymatic approaches will allow us to synthesize sugar chains with precise control over length, stereochemistry, and sulfation patterns. Synthetic access to defined GAG structures will allow us to understand their biological roles in the brain. For example, we will address which GAG structures are important for learning and memory and how changes in sulfation pattern impact aging and neurodegenerative disease.

These molecules are long unbranched polysaccharides containing a repeating disaccharide unit. The disaccharide units contain either of two modified sugars, *N*-acetylgalactosamine (GalNAc) or *N*-acetylglucosamine (GlcNAc), and a uronic acid such as glucuronate or iduronate. GAGs are highly negatively charged molecules, with extended conformation that imparts high viscosity to the solution. GAGs are located primarily on the surface of cells or in the extracellular matrix (ECM). Along with the high viscosity of GAGs comes low compressibility, which makes these molecules ideal for a lubricating fluid in the joints. At the same time, their rigidity provides structural integrity to cells and provides passageways between cells, allowing for cell migration. The specific GAGs of physiological significance are hyaluronic acid, dermatan sulphate, chondroitin sulphate, heparin, heparan sulphate, and keratan sulphate. Although each of these GAGs has a predominant disaccharide component (see Table below), heterogeneity does exist in the sugars present in the make-up of any given class of GAG. Hyaluronic is unique among the GAGs in that it does not contain any sulphate and is not found covalently attached to proteins as a proteoglycan. It is, however, a component of non-covalently formed complexes with proteoglycans in the ECM. Hyaluronic acid polymers are very large (with molecular weights of 100,000 - 10,000,000) and can displace a large volume of water. This property makes them excellent lubricators and shock absorbers.

Structure	Description
D-glucuronate *N*-acetyl-D-glucosamine	**Hyaluronates:** composed of D-glucuronate + GlcNAc linkage is $\beta(1, 3)$
L-iduronate *N*-acetyl-D-galactosamine-4-sulfate	**Dermatan sulphates:** composed of L-iduronate (many are sulphated)+ GalNAc-4-sulphate linkages is $\alpha(1, 3)$
D-glucoronate *N*-acetyl-D-glalactosamine-4-sulfate	**Chondroitin 4- and 6-sulphates :** composed of D-glucuronate and GalNAc-4- or 6-sulphate linkage is $\beta(1, 3)$ (the figure contains GalNAc 4-sulphate)
L-iduronate-2-sulfate *N*-sulfo-D-glucosamine-6-sulfate	**Heparin and Heparan sulphates:** composed of iduronate-2-sulphate (D-glucuronate-2-sulphate) and *N*-sulfo-D-glucosamine-6-sulphate linkage is $\alpha(1, 4)$ (heparans have less sulphate than heparins)
D-galactose *N*-acetyl D-glucosamine-6-sulfate	**Keratan sulphates:** composed of galactose + GlcNAc-6-sulphate linkage is $\beta(1, 4)$

GAG	Localization	Comments
Hyaluronate	synovial fluid, vitreous humor, ECM of loose connective tissue	large polymers, shock absorbing
Chondroitin sulphate	cartilage, bone, heart valves	most abundant GAG
Heparan sulphate	basement membranes, components of cell surfaces	contains higher acetylated glucosamine than heparin
Heparin	component of intracellular granules of mast cells lining the arteries of the lungs, liver and skin	more sulphated than heparan sulphates
Dermatan sulphate	skin, blood vessels, heart valves	
Keratan sulphate	cornea, bone, cartilage aggregated with chondroitin sulphates	

Proteoglycans

The majority of GAGs in the body are linked to core proteins, forming proteoglycans (also called **mucopolysaccharides**). The GAGs extend perpendicularly from the core in a brush-like structure. The linkage of GAGs to the protein core involves a specific trisaccharide composed of two galactose residues and a xylose residue (GAG-GalGalXyl-O-CH_2-protein). The trisaccharide linker is coupled to the protein core through an *O*-glycosidic bond to a S residue in the protein. Some forms of keratan sulphates are linked to the protein core through an *N*-asparaginyl bond. The protein cores of proteoglycans are rich in S and T residues, which allows multiple GAG attachments.
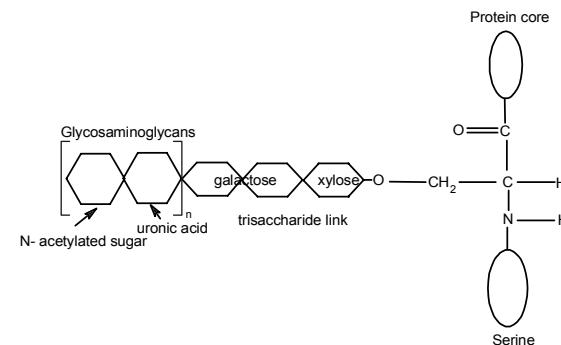
Fig. 2.7 : Structure of the GAG linkage to protein in proteoglycans

Clinical Significance

Proteoglycans and GAGs perform numerous vital functions within the body, some of which still remain to be studied. One well-defined function of the GAG heparin is its role in preventing coagulation of the blood. Heparin is abundant in granules of mast cells that line blood vessels. The release of heparin from these granules, in response to injury, and its subsequent entry into the serum leads to an inhibition of blood clotting, in the following manner. Free heparin complexes with and activates antithrombin III, which in turn inhibits all the serine proteases of the coagulation cascade. This phenomenon has been clinically exploited in the use of heparin injection for anti-coagulation therapies. Several genetically inherited diseases, for example the lysosomal storage diseases, result from defects in the lysosomal enzymes responsible for the metabolism of complex membrane-associated GAGs. These specific diseases, termed **mucopolysaccharidoses (MPS)** (in reference to the earlier term, mucopolysaccharide, used for glycosaminoglycans) lead to an accumulation of GAGs within lysosomes of affected cells. There are at least 14 known types of lysosomal storage diseases that affect GAG catabolism; some of the more commonly encountered examples are indicated in the Table below. All of these disorders, excepting Hunter's syndrome (X-linked), are inherited in an autosomal recessive manner. To see a diagram of the locations of the enzyme defects in GAG degradation go to the Mucopolysaccharidoses page.

Type: Syndrome	Enzyme Defect	Affected GAG	Symptoms
Hurler MPSIH (MPS1H)	α-L-iduronidase	dermatan sulphate, heparan sulphate	corneal clouding, dystosis multiplex, organomegaly, heart disease, dwarfism, mental retardation; early mortality
Scheie MPSIS (MPS1S)	α-L-iduronidase	dermatan sulphate, heparan sulphate	corneal clouding; aortic valve disease; joint stiffening; normal intelligence and life span
Hurler-Scheie MPSIHS (MPS1HS)	α-L-iduronidase	dermatan sulphate, heparan sulphate	intermediate between I H and I S
Hunter MPSII (MPS2)	L-iduronate-2-sulfatase	dermatan sulphate, heparan sulphate	mild and severe forms, only X-linked MPS, dystosis multiplex, organomegaly, facial and physical deformities, no corneal clouding, mental retardation, death before 15 except in mild form then survival to 20 - 60
Sanfilippo A MPSIIIA (MPS3A)	heparan N-sulfatase	heparan sulphate	profound mental deterioration, hyperactivity, skin, brain, lungs, heart and skeletal muscle are affected in all 4 types of MPS-III

Type: Syndrome	Enzyme Defect	Affected GAG	Symptoms
Sanfilippo B MPSIIIB (MPS3B)	α-N-acetyl-D-glucosaminidase	heparan sulphate	phenotype similar to III A
Sanfilippo C MPSIIIC (MPS3C)	acetylCoA:α-glucosaminide-acetyltransferase	heparan sulphate	phenotype similar to III A
Sanfilippo D MPSIIID (MPS3D)	N-acetylglucosamine-6-sulfatase	heparan sulphate	phenotype similar to III A
Morquio A MPSIVA (MPS4A)	galactose-6-sulfatase	keratan sulphate, chondroitin 6-sulphate	corneal clouding, odontoid hypoplasia, aortic valve disease, distinctive skeletal abnormalities
Morquio B MPSIVB (MPS4B)	β-galactosidase	keratan sulphate	severity of disease similar to IV A
MPS V, a designation no longer used			
Maroteaux-Lamy MPSVI (MPS6)	arylsulfatase B also called N-acetylgalac-tosamine-4-sulfatase	dermatan sulphate	3 distinct forms from mild to severe, aortic valve disease, dystosis multiplex, normal intelligence, corneal clouding, coarse facial features
Sly MPSVII (MPS7)	β-glucuronidase	heparan sulphate, dermatan sulphate, chondroitin 4-, 6-sulphates	hepatosplenomegaly, dystosis multiplex, wide spectrum of severity, hydrops fetalis
MPS VIII, a designation no longer used			

Blood Group Polysaccharides

The so – called blood group polysaccharides are present in erythrocytes; saliva, gastric mucin, cystic fluids, and other body secretions, when combined with proteins, they constitute the A, B O (H), Rh and other antigens of the erythrocytes and differentiate the blood groups or types, when red cells containing a specific type polysaccharide antigen are mixed with specific antibodies of serum, agglutinated by antibodies (isoagglutinins)found in type O or B blood group A serum agglutination characteristic of cells and sera, it is possible to determine whether or not a given blood is of the proper type for transfusion into a particular patient so that agglutination and disastrous result will not follow

Blood type	Serum Isoagglutinins	Agglutination of Red cells			
		A	B	AB	O
A	β	–	+	+	–
B	α	+	–	+	–
AB	None	–	–	–	–
O(H)	α and β	+	+	+	–

The various blood group polysaccharides are generally polymers of D – glucosomanine or D – glactsamine and simple sugar. Both D – galactosamine and D – glucosoamine may be present. Blood group polysaccharides A, B and O (H) from ovarian cyst fluid yield D – galactosamine , D – glucosoamine D – glactose L – fructose, and sialic acid (N – acetyleneuraminic acid). The specific polysaccharisdes from hog gastric mucin are made up to D – galactose N – acetyl – D – glucosomanine and L – fucose

Bacterial Polysaccharides

Complex carbohydrate substances have been isolated from many different species of bacteria. A number of these carbohydrates have been found to be responsible for the specificity of immune reaction. They are also referred as specific polysaccharides.

While the hydrolytic products of the different polysaccharides vary, the following substances have been obtained from the group as a whole; uronic acids especially glucouronic acids and glactouronic; monosachharides, such as glucose, galactose arbinose mannose rhamnose, inosotol, amino sugar, notably glucosomanine galactosamine and acetic acid. The amino sugars present are N – acetylated.

Heidelberger and Avery first demonstrated the presence of specific bacterial polysaccharides by their work on penemococcus types II and III. Where the polysaccharides are found in the bacterial capsules. Type II polysaccharides are compounds consisting D – glucose, L – rhamnose (40%) and D – glucouronic acid units. While type III polysaccharides contains only D – glucose and D – glucouronic acid groups. By careful hydrolysis of type III polysaccharide and aldobiuronic acid units, 3 – O b - D

Glucouronic acid   Glucose   Glucouronic acid
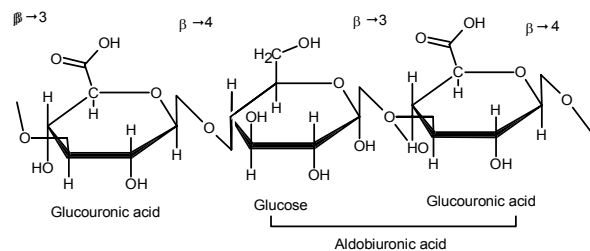
Aldobiuronic acid

– glucopyranosyluronic acid. The structure of the polysaccharide is a linear chain with alternative glucose and glucouronic acid groups. It may be considered chain polymer of the above aldobiuronic acid.

When the type III polysaccharide molecule is hydrolyzed at the 1, 4 linkages an aldobiuronic acid with D – glucose linked to D – glucouronic acid by 1 " 3 linkage is obtained, 3 – O - b D – glucopyranosyl – O – glucopyranosyluronic acid is obtained, 3 – O - b - D – Glucopyranosyl – O – glucopyranosyluronic acid. If however the 1, 3 linkages are hydrolyzed, leaving the glucuronic acid and glucose joined by 1 – 4 bonds, a different aldobiuronic acid is formed cello biuronic acid 4 – O - b - D – glucopyranosyluronic acid – D – glucopyranose.

The polysaccharide type I penemococcus is composed of D – glactouronic acid (28%) and N – acetyl – D – glucosamine, while type VIII polysaccharide is made up of glucose and D – glucose and D – glucouronic acid (7: 2), and type XIV polysaccharide has D – galactose and N – acetyl – D – glucosoamine as constituent units. Only the type III polysaccharide structure has been definitely established.

The immunologically active polysaccharides to contain D – glactose, D mannose D – arbinose inositol.

The so called V_1 antigen of *Salmonella typhosa* and other bacterial series appears to be largely a polymer of an N – acetyl aminohexuronic acid possibly of N – acetyl – D – glucosaminuronic acid

Dextran is a complex, branched glucan (polysaccharide) made of many glucose molecules joined into chains of varying lengths (from 10 to 150 kilodaltons), used as an antithrombotic (anti-platelet), and to reduce blood viscosity.

The straight chain consists of α-1,6 glycosidic linkages between glucose molecules, while branches begin from α-1,4 linkages (and in some cases, α-1,2 and α-1,3 linkages as well). (For information on the numbering of carbon atoms in glucose, see the glucose article.) Dextran is synthesized from sucrose by certain lactic-acid bacteria, the best-known being *Leuconostoc mesenteroides* and *Streptococcus mutans*. Dental plaque is rich in dextrans. Dextran is also formed by the lactic acid bacterium *Lactobacillus brevis* to create the crystals of tibicos, or water kefir fermented beverage which supposedly has some health benefits.
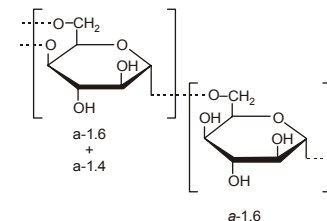
Microsurgery uses

These agents are used commonly by microsurgeons to decrease vascular thrombosis. The antithrombotic effect of dextran is mediated through its binding of erythrocytes, platelets, and vascular endothelium, increasing their electronegativity and thus reducing erythrocyte aggregation and platelet adhesiveness. Dextrans also reduce factor VIII-Ag Von Willebrand factor, thereby decreasing platelet function. Clots formed after administration of dextrans are more easily lysed due to an altered thrombus structure

(more evenly distributed platelets with coarser fibrin). By inhibiting α-2 antiplasmin, dextran serves as a plasminogen activator and therefore possesses thrombolytic features.

Outside from these features, larger dextrans, which do not pass out of the vessels, are potent osmotic agents, and thus have been used urgently to treat hypovolemia. The hemodilution caused by volume expansion with dextran use improves blood flow, thus further improving patency of microanastomoses and reducing thrombosis. Still, no difference has been detected in antithrombotic effectiveness in comparison of intraarterial and intravenous administration of dextran.

Dextrans are available in multiple molecular weights ranging from 10,000 Da to 150,000 Da. The larger dextrans are excreted poorly from the kidney and therefore remain in the blood for as long as weeks until they are metabolized. Subsequently, they have prolonged antithrombic and colloidal effects. In this family, dextran-40 (MW: 40,000 Da), has been the most popular member for anticoagulation therapy. Close to 70% of dextran-40 is excreted in urine within the first 24 hours after intravenous infusion while the remaining 30% will be retained for several more days.

Other medical uses

- It is used in some eye drops as a lubricant, and in certain intravenous fluids to solubilise other factors, e.g. iron (=iron dextran).
- Dextran in intravenous solution provides an osmotically neutral fluid that once in the body is digested by cells into glucose and free water. It is occasionally used to replace lost blood in emergency situations, when replacement blood is not available, but must be used with caution as it does not provide necessary electrolytes and can cause hyponatremia or other electrolyte disturbances.
- It also increases blood sugar levels.

Laboratory uses

- Dextran is used in the osmotic stress technique for applying osmotic pressure to biological molecules.
- It is also used in some size-exclusion chromatography matrices; an example is Sephadex.
- Dextran has also been used in bead form to aid in bioreactor applications.
- Dextran has been used in immobilization in biosensors.
- Dextran preferentially binds to early endosomes; fluorescently-labelled dextran can be used to visualize these endosomes under a fluorescent microscope.
- Dextran can be used as a stabilising coating to protect metal nanoparticles from oxidation and improve biocompatibility.

Side effects

Although there are relatively few side-effects associated with dextran use, these side-effects can be very serious. These include anaphylaxis, volume overload, pulmonary edema, cerebral edema, or platelet dysfunction. An uncommon but significant complication of dextran osmotic effect is acute renal failure.

The pathogenesis of this renal failure is the subject of many debates with direct toxic effect on tubules and glomerulus versus intraluminal hyperviscosity being some of the proposed mechanisms. Patients with history of diabetes mellitus, renal insufficiency, or vascular disorders are most at risk. Brooks and others recommend the avoidance of dextran therapy in patients with chronic renal insufficiency and CrCl<40 cc per minute.

Alginic acid (**algin**, **alginate**) is a viscous gum that is abundant in the cell walls of brown algae.

Structure

It is a linear copolymer with homopolymeric blocks of (1-4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) residues, respectively, covalently linked together in different sequences or blocks.

The monomers can appear in homopolymeric blocks of consecutive G-residues (G-blocks), consecutive M-residues (M-blocks), alternating M and G-residues (MG-blocks), or randomly organized blocks.

Forms

Commercial varieties of alginate are extracted from seaweed, including the giant kelp *Macrocystis pyrifera*, *Ascophyllum nodosum*, and various types of *Laminaria*. It is also produced by two bacterial genera *Pseudomonas* and *Azotobacter*, which played a major role in the unravelling of its biosynthesis pathway. Bacterial alginates are useful for the production of micro- or nanostructures suitable for medical applications.[1]

Alginate absorbs water quickly, which makes it useful as an additive in dehydrated products such as slimming aids, and in the manufacture of paper and textiles. It is also used for waterproofing and fireproofing fabrics, as a gelling agent, for thickening drinks, ice cream and cosmetics, and as a detoxifier that can absorb poisonous metals from the blood. Alginate is also produced by certain bacteria, notably Azotobacter species.

Alginate ranges from white to yellowish-brown, and takes filamentous, grainy, granular, and powdered forms.

Uses

Alginate is used in various pharmaceutical preparations such as Gaviscon, Bisodol, and Asilone. Alginate is used extensively as a mold-making material in dentistry, prosthetics, lifecasting, and textiles. It is also used in the food industry, for thickening soups and jellies. Calcium alginate is used in different types of medical products, including burn dressings that promote healing and can be removed with less pain than conventional dressings.

Also, due to alginate's biocompatibility and simple gelation with divalent cations such as Ca^{2+}, it is widely used for cell immobilization and encapsulation.

Alginic acid (alginato) is also used in culinary arts, most notably in the "Esferificación" (Sphereification) techniques of Ferrαn Adriα of El Bulli in Roses, Girona, where natural juices of fruits and vegetables are encapsulated in bubbles that "explode" on the tongue when consumed. One of the

most famous examples of this use of alginic acid was when Ferran Adrià used alginic acid to make apple caviar.[2]

Due to its ability to absorb water quickly, alginate can be changed through a lyophilization process to a new structure that has the ability to expand. It is used in the weight loss industry as an appetite suppressant. A new type of diet using alginate is the CM3-Alginate Diet.

Chitin ($C_8H_{13}O_5N$)_n is a long-chain polymer of a N-acetylglucosamine, a derivative of glucose, and is found in many places throughout the natural world. It is the main component of the cell walls of fungi, the exoskeletons of arthropods, such as crustaceans (like the crab, lobster and shrimp) and the insects, including ants, beetles and butterflies, the radula of mollusks and the beaks of the cephalopods, including squid and octopi. Chitin has also proven useful for several medical and industrial purposes. Chitin is a biological substance which may be compared to the polysaccharide cellulose and to the protein keratin. Although keratin is a protein, and not a carbohydrate like chitin, both keratin and chitin have similar structural functions. Chemistry, physical properties and biological function Chitin is a polysaccharide; it is synthesized from units of N-acetylglucosamine (more completely, N-acetyl-D-glucos-2-amine). These units form covalent β-1,4 linkages (similar to the linkages between glucose units forming cellulose). Chitin may therefore be described as cellulose with one hydroxyl group on each monomer substituted with an acetylamine group. This allows for increased hydrogen bonding between adjacent polymers, giving the chitin-polymer matrix increased strength. In its unmodified form, chitin is translucent, pliable, resilient and quite tough. In arthropods, however, it is often modified, becoming embedded in a hardened proteinaceous matrix, which forms much of the exoskeleton. In its pure form it is leathery, but when encrusted in calcium carbonate it becomes much harder. The difference between the unmodified and modified forms can be seen by comparing the body wall of a caterpillar (unmodified) to a beetle (modified).Chitin is one of many naturally occurring polymers. Its breakdown may be catalyzed by enzymes called chitinases, secreted by microorganisms such as bacteria and fungi, and produced by some plants. Some of these microorganisms have receptors to simple sugars from the decomposition of chitin. If chitin is detected, they then produce enzymes to digest it by cleaving the glycosidic bonds in order to convert it to simple sugars and ammonia. Chemically, chitin is closely related to chitosan (a more water-soluble derivative of chitin). It is also closely related to cellulose in that it is a long unbranched chain of glucose derivatives. Both materials contribute structure and strength, protecting the organism.

Hyaluronan (also called hyaluronic acid or hyaluronate) is a non-sulphated glycosaminoglycan distributed widely throughout connective, epithelial, and neural tissues. It is one of the chief components of the extracellular matrix, contributes significantly to cell proliferation and migration, and may also be involved in the progression of some malignant tumors. The average 70 kg (154 lbs) person has roughly 15 grams of
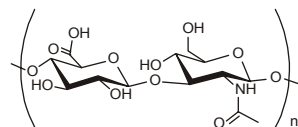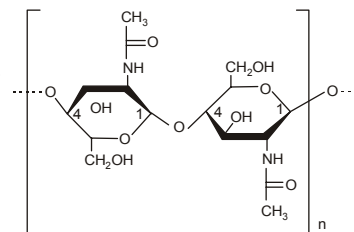
hyaluronan in his body, one-third of which is turned over (degraded and synthesised) every day. Hyaluronic acid is also a component of the group A streptococcal extracellular capsule, and is believed to play a role in virulence

Functions

Until the late 1970s, hyaluronan was described as an "ectoplasm" molecule, an ubiquitous high energy carbohydrate polymer that is part of the extracellular matrix. For example, hyaluronan is a major component of the synovial fluid and was found to increase the viscosity of the fluid. Along with lubricin, it is one of the fluid's main lubricating components.

Hyaluronan is an important component of articular cartilage, where it is present as a coat around each cell (chondrocyte). When aggrecan monomers bind to hyaluronan in the presence of link protein, large highly negatively-charged aggregates form. These aggregates imbibe water and are responsible for the resilience of cartilage (its resistance to compression). The molecular weight (size) of hyaluronan in cartilage decreases with age, but the amount increases.

Hyaluronan is also a major component of skin, where it is involved in tissue repair. When skin is excessively exposed to UVB rays, it becomes inflamed (sunburn) and the cells in the dermis stop producing as much hyaluronan, and increase the rate of its degradation. Hyaluronan degradation products also accumulate in the skin after UV exposure.

While it is abundant in extracellular matrices, hyaluronan also contributes to tissue hydrodynamics, movement and proliferation of cells, and participates in a number of cell surface receptor interactions, notably those including its primary receptor, CD44. Upregulation of CD44 itself is widely accepted as a marker of cell activation in lymphocytes. Hyaluronan's contribution to tumor growth may be due to its interaction with CD44. Receptor CD44 participates in cell adhesion interactions required by tumor cells.

Although hyaluronan binds to receptor CD44, there is evidence that hyaluronan degradation products transduce their inflammatory signal through Toll-like receptor 2 (TLR2), TLR4 or both TLR2, and TLR4 in macrophages and dendritic cells. TLR and hyaluronan play a role in innate immunity.

High concentrations of hyaluronan in the brains of young rats, and reduced concentrations in the brains of adult rats suggest that hyaluronan plays an important role in brain development.

Structure

The chemical structure of hyaluronan was determined in the 1950s in the laboratory of Karl Meyer. Hyaluronan is a polymer of disaccharides, themselves composed of D-glucuronic acid and D-N-acetylglucosamine, linked together via alternating β-1,4 and β-1,3 glycosidic bonds. Hyaluronan can be 25,000 disaccharide repeats in length. Polymers of hyaluronan can range in size from 5,000 to 20,000,000 Da *in vivo*. The average molecular weight in human synovial fluid is 3"4 million Da, and hyaluronan purified from human umbilical cord is 3,140,000 Da.

Hyaluronan is energetically stable in part because of the stereochemistry of its component disaccharides. Bulky groups on each sugar molecule are in sterically favored positions, whereas the smaller hydrogens assume the less-favorable axial positions.

Biological Synthesis

Hyaluronan is synthesized by a class of integral membrane proteins called hyaluronan synthases, of which vertebrates have three types: HAS1, HAS2, and HAS3. These enzymes lengthen hyaluronan by repeatedly adding glucuronic acid and N-acetylglucosamine to the nascent polysaccharide as it is extruded through the cell membrane into the extracellular space.

Hyaluronan synthesis (HAS) has been shown to be inhibited by 4-Methylumbelliferone (hymecromone, heparvit), a 7-Hydroxy-4-methylcoumarin derivative. This selective inhibition (without inhibiting other Glycosaminoglycans) may prove useful in preventing metastasis of malignant tumor cells.

Degradation

Hyaluronan is degraded by a family of enzymes called hyaluronidases. In humans, there are at least seven types of hyaluronidase-like enzymes, several of which are tumor suppressors. The degradation products of hyaluronan, the oligosaccharides and very low-molecular-weight hyaluronan, exhibit pro-angiogenic properties. In addition, recent studies showed that hyaluronan fragments, not the native high-molecular mass of hyaluronan, can induce inflammatory responses in macrophages and dendritic cells in tissue injury and in skin transplant rejection.

Medical applications

Hyaluronan is naturally found in many tissues of the body, such as skin, cartilage, and the vitreous humour. It is therefore well suited to biomedical applications targeting these tissues. The first hyaluronan biomedical product, Healon, was developed in the 1970s and 1980s by Pharmacia, and is approved for use in eye surgery (i.e., corneal transplantation, cataract surgery, glaucoma surgery and surgery to repair retinal detachment). Other biomedical companies also produce brands of hyaluronan for ophthalmic surgery.

Hyaluronan is also used to treat osteoarthritis of the knee. Such treatments, called *viscosupplementation*, are administered as a course of injections into the knee joint and are believed to supplement the viscosity of the joint fluid, thereby lubricating the joint, cushioning the joint, and producing an analgesic effect. It has also been suggested that hyaluronan has positive biochemical effects on cartilage cells. However, some placebo controlled studies have cast doubt on the efficacy of hyaluronan injections, and hyaluronan is recommended primarily as a last alternative to surgery. Oral use of hyaluronan has been lately suggested, although its effectiveness needs to be demonstrated. At present, there are some preliminary clinical studies that suggest that oral administration of Hyaluronan has a positive effect on osteoarthritis, but it remains to be seen if there is any real benefit to the treatment.

Due to its high biocompatibility and its common presence in the extracellular matrix of tissues, hyaluronan is gaining popularity as a biomaterial scaffold in tissue engineering research.

In some cancers, hyaluronan levels correlate well with malignancy and poor prognosis. Hyaluronan is thus often used as a tumor marker for prostate and breast cancer. It may also be used to monitor the progression of the disease.

Hyaluronan may also be used postoperatively to induce tissue healing, notably after cataract surgery [19]. Current models of wound healing propose that larger polymers of hyaluronic acid appear in the

early stages of healing to physically make room for white blood cells, which mediate the immune response.

Hyaluronan has also been used in the synthesis of biological scaffolds for wound healing applications. These scaffolds typically have proteins such as fibronectin attached to the hyaluronan to facilitate cell migration into the wound. This is particularly important for individuals with diabetes who suffer from chronic wounds.

In 2007, the EMEA extended its approval of Hylan GF-20 as a treatment for ankle and shoulder osteoarthritis pain.

Heparin, a highly-sulphated glycosaminoglycan, is widely used as an injectable anticoagulant and has the highest negative charge density of any known biological molecule. It can also be used to form an inner anticoagulant surface on various experimental and medical devices such as test tubes and renal dialysis machines. Pharmaceutical grade heparin is derived from mucosal tissues of slaughtered meat animals such as porcine (pig) intestine or bovine (cow) lung.
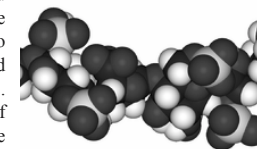
Although used principally in medicine for anticoagulation, the true physiological role in the body remains unclear, because blood anti-coagulation is achieved mostly by endothelial cell-derived heparan sulphate proteoglycans. Heparin is usually stored within the secretory granules of mast cells and released only into the vasculature at sites of tissue injury. It has been proposed that, rather than anticoagulation, the main purpose of heparin is in a defensive mechanism at sites of tissue injury against invading bacteria and other foreign materials.[4] In addition, it is preserved across a number of widely different species, including some invertebrates which lack a similar blood coagulation system. Heparin structure
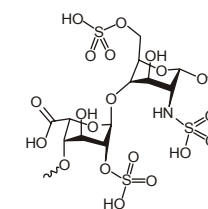
Native heparin is a polymer with a molecular weight ranging from 3 kDa to 50 kDa, although the average molecular weight of most commercial heparin preparations is in the range of 12 kDa to 15 kDa. Heparin is a member of the glycosaminoglycan family of carbohydrates (which includes the closely-related molecule heparan sulphate) and consists of a variably-sulphated repeating disaccharide unit. The main disaccharide units that occur in heparin are shown below. The most common disaccharide unit is composed of a 2-O-sulphated iduronic acid and 6-O-sulphated, N-sulphated glucosamine, IdoA(2S)-GlcNS(6S). For example, this makes up 85% of heparins from beef lung and about 75% of those from porcine intestinal mucosa. Not shown below are the rare disaccharides containing a 3-O-sulphated glucosamine (GlcNS(3S,6S)) or a free amine group (GlcNH$_3^+$). Under physiological conditions, the ester and amide sulphate groups are deprotonated and attract positively-charged counterions to form a heparin salt. It is in this form that heparin is usually administered as an anticoagulant.

1 unit of heparin (the "Howell Unit") is an amount approximately equivalent to 0.002 mg of pure heparin, which is the quantity required to keep 1 mL of cat's blood fluid for 24 hours at 0°C.

Three-dimensional structure

The three-dimensional structure of heparin is complicated by the fact that iduronic acid may be present in either of two low-energy conformations when internally positioned within an oligosaccharide. The conformational equilibrium being influenced by sulfation state of adjacent glucosamine sugars. Nevertheless, the solution structure of a heparin dodecasacchride composed solely of six GlcNS(6S)-IdoA(2S) repeat units has been determined using a combination of NMR spectroscopy and molecular modeling techniques. Two models were constructed, one in which all IdoA(2S) were in the $^2S_0$ conformation (**A** and **B** below), and one in which they are in the $^1C_4$ conformation (**C** and **D** below). However there is no evidence to suggest that changes between these conformations occur in a concerted fashion. These models correspond to the protein data bank code 1HPN.

A B C D
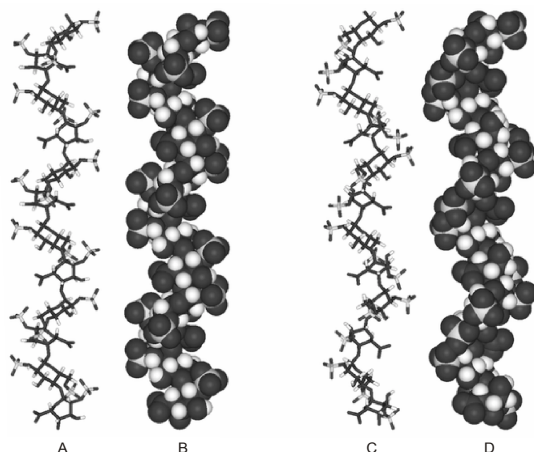
In the image above:

- **A** = 1HPN (all IdoA(2S) residues in $^2S_0$ conformation) Jmol viewer
- **B** = van der Waals radius space filling model of *A*
- **C** = 1HPN (all IdoA(2S) residues in $^1C_4$ conformation) Jmol viewer
- **D** = van der Waals radius space filling model of *C*

In these models, heparin adopts a helical conformation, the rotation of which places clusters of sulphate groups at regular intervals of about 17 angstroms (1.7 nm) on either side of the helical axis.

Medical use

Heparin is a naturally-occurring anticoagulant produced by basophils and mast cells. Heparin acts as an anticoagulant, preventing the formation of clots and extension of existing clots within the blood. While heparin does not break down clots that have already formed (unlike tissue plasminogen activator), it allows the body's natural clot lysis mechanisms to work normally to break down clots that have already formed. Heparin is used for anticoagulation for the following conditions:

- Acute coronary syndrome, e.g., NSTEMI
- Atrial fibrillation
- Deep-vein thrombosis and pulmonary embolism
- Cardiopulmonary bypass for heart surgery.

Heparin and its derivatives (enoxaparin, dalteparin, and so forth) are effective at preventing deep-vein thromboses and pulmonary emboli in patients at risk, but there is no evidence that they are effective at preventing death. Current guidelines recommend aspirin and leg stockings instead.

Heparin binds to the enzyme inhibitor antithrombin (AT) causing a conformational change that results in its activation through an increase in the flexibility of its reactive site loop. The activated AT then inactivates thrombin and other proteases involved in blood clotting, most notably factor Xa. The rate of inactivation of these proteases by AT can increase by up to 1000-fold due to the binding of heparin.

AT binds to a specific pentasaccharide sulfation sequence contained within the heparin polymer

GlcNAc/NS(6S)-GlcA-GlcNS(3S,6S)-IdoA(2S)-GlcNS(6S)

The conformational change in AT on heparin-binding mediates its inhibition of factor Xa. For thrombin inhibition however, thrombin must also bind to the heparin polymer at a site proximal to the pentasaccharide. The highly-negative charge density of heparin contributes to its very strong electrostatic interaction with thrombin. The formation of a ternary complex between AT, thrombin, and heparin results in the inactivation of thrombin. For this reason heparin's activity against thrombin is size-dependent, the ternary complex requiring at least 18 saccharide units for efficient formation. In contrast anti factor Xa activity only requires the pentasaccharide binding site.

GlcNS(6S) GlcA GlcNS(3S, 6S) IdoA (2S) GlcNS(6S) OMe
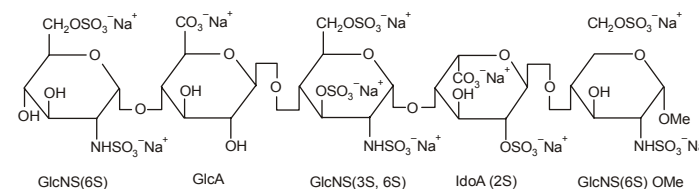
Fig. 2.8 : Chemical structure of fondaparinux

This size difference has led to the development of low-molecular-weight heparins (LMWHs) and more recently to fondaparinux as pharmaceutical anticoagulants. Low-molecular-weight heparins and

fondaparinux target anti-factor Xa activity rather than anti-thrombin (IIa) activity, with the aim of facilitating a more subtle regulation of coagulation and an improved therapeutic index. The chemical structure of fondaparinux is shown to the left. It is a synthetic pentasaccharide, whose chemical structure is almost identical to the AT binding pentasaccharide sequence that can be found within polymeric heparin and heparan sulphate.

With LMWH and fondaparinux, there is a reduced risk of osteoporosis and heparin-induced thrombocytopenia (HIT). Monitoring of the APTT is also not required and indeed does not reflect the anticoagulant effect, as APTT is insensitive to alterations in factor Xa.

Danaparoid, a mixture of heparan sulphate, dermatan sulphate, and chondroitin sulphate can be used as an anticoagulant in patients who have developed HIT. Because danaparoid does not contain heparin or heparin fragments, cross-reactivity of danaparoid with heparin-induced antibodies is reported as less than 10%.

The effects of heparin are measured in the lab by the partial thromboplastin time (aPTT), (the time it takes the blood plasma to clot).

Administration

Details of administration are available in clinical practice guidelines by the American College of Chest Physicians:

- Non-weight-based heparin dose adjustment
- Weight-based-heparin dose adjustment

Heparin is given parenterally, as it is degraded when taken by mouth. It can be injected intravenously or subcutaneously (under the skin). Intramuscular injections (into muscle) are avoided because of the potential for forming hematomas.

Because of its short biologic half-life of approximately one hour, heparin must be given frequently or as a continuous infusion. However, the use of low molecular weight heparin (LMWH) has allowed once daily dosing, thus not requiring a continuous infusion of the drug. If long-term anticoagulation is required, heparin is often used only to commence anticoagulation therapy until the oral anticoagulant warfarin takes effect.

Adverse reactions

A serious side-effect of heparin is heparin-induced thrombocytopenia (HIT syndrome). HITS is caused by an immunological reaction that makes platelets a target of immunological response, resulting in the degradation of platelets. This is what causes thrombocytopenia. This condition is usually reversed on discontinuation, and can generally be avoided with the use of synthetic heparins. There is also a benign form of thrombocytopenia associated with early heparin use, which resolves without stopping heparin.

Two nonhemorrhagic side effects of heparin treatment. The first is elevation of serum aminotransferase levels, which has been reported in as many as 80% of patients receiving heparin. This abnormality is not associated with liver dysfunction, and it disappears after the drug is discontinued. The other complication is hyperkalemia, which occurs in 5 to 10% of patients receiving heparin, and is the result of heparin-induced aldosterone suppression. The hyperkalemia can appear within a few days after the onset of heparin therapy.

Rarer side-effects include alopecia and osteoporosis with chronic use.

As with many drugs, overdoses of heparin can be fatal. In September 2006, heparin received worldwide publicity when 3 prematurely-born infants died after they were mistakenly given overdoses of heparin at an Indianapolis hospital.

Treatment of overdose

In case of overdose, protamine sulphate (1 mg per 100 Units of Heparin that had been given over 4 hours) can be given to counteract the action of heparin.

History

Heparin is one of the oldest drugs currently still in widespread clinical use. Its discovery in 1916 predates the establishment of the United States Food and Drug Administration, although it did not enter clinical trials until 1935. It was originally isolated from canine liver cells, hence its name (*hepar* or "ηπαρ" is Greek for "liver"). Heparin's discovery can be attributed to the research activities of two men, Jay McLean and William Henry Howell.

In 1916, McLean, a second-year medical student at Johns Hopkins University, was working under the guidance of Howell investigating pro-coagulant preparations, when he isolated a fat-soluble phosphatide anti-coagulant. It was Howell who coined the term *heparin* for this type of fat-soluble anticoagulant in 1918. In the early 1920s, Howell isolated a water-soluble polysaccharide anticoagulant, which was also termed *heparin*, although it was distinct from the phosphatide preparations previously isolated. It is probable that the work of McLean changed the focus of the Howell group to look for anticoagulants, which eventually led to the polysaccharide discovery.

Between 1933 and 1936, Connaught Medical Research Laboratories, then a part of the University of Toronto, perfected a technique for producing safe, non-toxic heparin that could be administered to patients in a salt solution. The first human trials of heparin began in May 1935, and, by 1937, it was clear that Connaught's heparin was a safe, easily-available, and effective blood anticoagulant. Prior to 1933, heparin was available, but in small amounts, and was extremely expensive, toxic, and, as a consequence, of no medical value.

Novel drug development opportunities for heparin

As detailed in the table below, there is a great deal of potential for the development of heparin-like structures as drugs to treat a wide range of diseases, in addition to their current use as anticoagulants.

Disease states sensitive to heparin	Heparins effect in experimental models	Clinical status
1	2	3
Adult respiratory distress syndrome	Reduces cell activation and accumulation in airways, neutralizes mediators and cytotoxic cell products, and improves lung function in animal models	Controlled clinical trials

1	2	3
Allergic encephalomyelitis	Effective in animal models	—
Allergic rhinitis	Effects as for adult respiratory distress syndrome, although no specific nasal model has been tested	Controlled clinical trial
Arthritis	Inhibits cell accumulation, collagen destruction and angiogenesis	Anecdotal report
Asthma	As for adult respiratory distress syndrome, however it has also been shown to improve lung function in experimental models	Controlled clinical trials
Cancer	Inhibits tumour growth, metastasis and angiogenesis, and increases survival time in animal models	Several anecdotal reports
Delayed type hypersensitivity reactions	Effective in animal models	—
Inflammatory bowel disease	Inhibits inflammatory cell transport in general. No specific model tested	Controlled clinical trials
Interstitial cystitis	Effective in a human experimental model of interstitial cystitis	Related molecule now used clinically
Transplant rejection	Prolongs allograph survival in animal models	

—indicates no information available

As a result of heparin's effect on such a wide variety of disease states a number of drugs are indeed in development whose molecular structures are identical or similar to those found within parts of the polymeric heparin chain.

Drug molecule	Effect of new drug compared to heparin	Biological activities
Heparin tetrasaccharide	Non-anticoagulant, non-immunogenic, orally active	Anti-allergic
Pentosan polysulphate	Plant derived, little anticoagulant activity, Anti-inflammatory, orally active	Anti-inflammatory, anti-adhesive, anti-metastatic
Phosphomanno-pentanose sulphate inflammatory	Potent inhibitor of heparanase activity	Anti-metastatic, anti-angiogenic, anti-
Selectively chemically O-desulphated heparin	Lacks anticoagulant activity	Anti-inflammatory, anti-allergic, anti-adhesive

De-polymerisation techniques

Either chemical or enzymatic de-polymerisation techniques or a combination of the two underlie the vast majority of analyses carried out on the structure and function of heparin and heparan sulphate (HS).

Enzymatic

The enzymes traditionally used to digest heparin or HS are naturally produced by the soil bacterium Pedobacter heparinus (formerly named Flavobacterium heparinum). This bacterium is capable of utilizing either heparin or HS as its sole carbon and nitrogen source. In order to do this it produces a range of enzymes such as lyases, glucuronidases, sulfoesterases and sulfamidases. It is the lyases that have mainly been used in heparin/HS studies. The bacterium produces three lyases, heparinases I (EC 4.2.2.7), II (no EC number assigned) and III (EC 4.2.2.8) and each has distinct substrate specificities as detailed below.

Heparinase enzyme	Substrate specificity
Heparinase I	GlcNS(±6S)-IdoA(2S)
Heparinase II	GlcNS/Ac(±6S)-IdoA(±2S) GlcNS/Ac(±6S)-GlcA
Heparinase III	GlcNS/Ac(±6S)-GlcA/IdoA (with a preference for GlcA)

The lyases cleave heparin/HS by a beta elimination mechanism. This action generates an unsaturated double bond between C4 and C5 of the uronate residueThe C4-C5 unsaturated uronate is termed ÄUA or UA. It is a sensitive UV chromaphore (max absorption at 232nm) and allows the rate of an enzyme digest to be followed as well as providing a convenient method for detecting the fragments produced by enzyme digestion.

Chemical

Nitrous acid can be used to chemically de-polymerise heparin/HS. Nitrous acid can be used at pH 1.5 or at a higher pH of 4. Under both conditions nitrous acid effects deaminative cleavage of the chain.
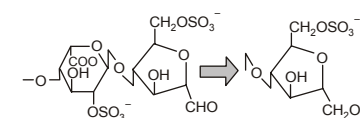
IdoA(2S)-aMan: The anhydromannose can be reduced to an anhydromannitol

At both 'high' (4) and 'low' (1.5) pH, deaminative cleavage occurs between GlcNS-GlcA and GlcNS-IdoA, all be it at a slower rate at the higher pH. The deamination reaction, and therefore chain cleavage, is regardless of O-sulfation carried by either monosaccharide unit.

At low pH deaminative cleavage results in the release of inorganic SO_4, and the conversion of

GlcNS into anhydromannose (aMan). Low pH nitrous acid treatment is an excellent method to distinguish N-sulphated polysaccharides such as heparin and HS from non N-sulphated polysacchrides such as chondroitin sulphate and dermatan sulphate; chondroitin sulphate and dermatan sulphate being un-susceptible to nitrous acid cleavage.

Evolutionary conservation

In addition to the bovine and porcine tissue from which pharmaceutical-grade heparin is commonly extracted, heparin has also been extracted and characterized from the following species:

1. Turkey.
2. Whale.
3. Dromedary camel.
4. Mouse.
5. Humans.
6. Lobster.
7. Fresh water mussel.
8. Clam.
9. Shrimp.
10. Mangrove crab.
11. Sand dollar.

The biological activity of heparin within species 6–11 is unclear and further supports the idea that the main physiological role of heparin is not anticoagulation. These species do not possess any blood coagulation system similar to that present within the species listed 1–5. The above list also demonstrates how heparin has been highly evolutionarily conserved with molecules of a similar structure being produced by a broad range of organisms belonging to many different phyla.

Other uses/information

- Heparin gel (topical) may sometimes be used to treat sports injuries. It is known that the diprotonated form of histamine binds site specifically to heparin.[43] The release of histamine from mast cells at a site of tissue injury contributes to an inflammatory response. The rationale behind the use of such topical gels may be to block the activity of released histamine, and so help to reduce inflammation.

- Heparin gains the capacity to initiate angiogenesis when its copper salt is formed. Copper-free molecules are non-angiogenic. In contrast heparin may inhibit angiogenesis when it is administered in the presence of corticosteroids. This anti-angiogenic effect is independent of heparins anticoagulant activity.

- Test tubes, Vacutainers, and capillary tubes that use the lithium salt of heparin (lithium heparin) as an anticoagulant are usually marked with green stickers and green tops. Heparin has the advantage over EDTA of not affecting levels of most ions. However, it has been shown that the

levels of ionized calcium may be decreased if the concentration of heparin in the blood specimen is too high. Heparin can interfere with some immunoassays, however. As lithium heparin is usually used, a person's lithium levels cannot be obtained from these tubes; for this purpose, royal-blue-topped Vacutainers containing sodium heparin are used.

- Heparin-coated blood oxygenators are available for use in heart-lung machines. Among other things, these specialized oxygenators are thought to improve overall biocompatibility and host homeostasis by providing characteristics similar to native endothelium.

- The DNA binding sites on RNA polymerase can be occupied by heparin, preventing the polymerase binding to promoter DNA. This property is exploited in a range of molecular biological assays.

- Common diagnostic procedures require PCR amplification of a patient's DNA, which is easily extracted from white blood cells treated with heparin. This poses a potential problem, since heparin may be extracted along with the DNA, and it has been found to interfere with the PCR reaction at levels as low as 0.002 U in a 50 iL reaction mixture.

- Immobilized heparin can be used as an affinity ligand in protein purification. The format of immobilized heparin can vary widely from coated plastic surfaces for diagnostic purposes to chromatography resin. Most types of immobilized heparin can be used in three ways. The first of which is to use heparin to select out specific coagulation factors or other types of heparin-binding proteins from a complex mixture of non-heparin-binding proteins. Specific proteins can then be selectively dissociated from heparin with the use of differing salt concentrations or by use of a salt gradient. The second use is to use heparin as a high-capacity cation exchanger. This use takes advantage of heparin's high number of anionic sulphate groups. These groups will capture common cations such as Na^+ or Ca^{2+} in solution. The third use for immobilized heparin is group-specific purification of RNA and DNA binding proteins such as transcription factors and/or virus coat proteins. This methodology takes advantage of heparin's similar properties to RNA and DNA i.e. negatively charged sugar molecule.

- Heparin does not break up fibrin, it only prevents conversion of fibrinogen to fibrin. Only thrombolytics can break up a clot.

SUB-SECTION 2.3D—CARBOHYDRATES IN CELL WALLS OF BACTERIA

Bacterial cell walls

Schematic of typical gram-positive cell wall showing arrangement of N-Acetylglucosamine and N-Acetlymuramic acid

Around the outside of the cell membrane is the bacterial cell wall. Bacterial cell walls are made of peptidoglycan (also called murein), which is made from polysaccharide chains cross-linked by unusual peptides containing D-amino acids. Bacterial cell walls are different from the cell walls of plants and fungi which are made of cellulose and chitin, respectively. The cell wall of bacteria is also distinct from that of Archaea, which do not contain peptidoglycan. The cell wall is essential to the survival of many bacteria. The antibiotic penicillin is able to kill bacteria by inhibiting a step in the synthesis of peptidoglycan.
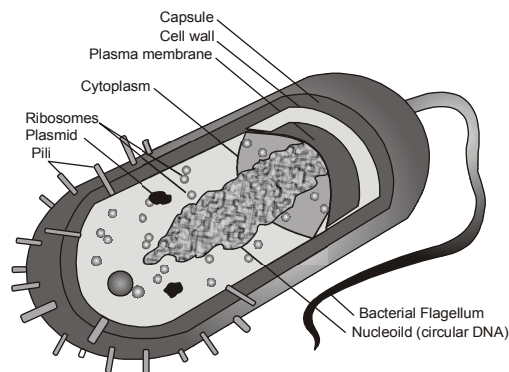
Fig. 2.9 : Diagram of a typical gram-negative bacterium, with the thin cell wall sandwiched
between the red outer membrane and the thin green plasma membrane

There are broadly speaking two different types of cell wall in bacteria, called Gram-positive and Gram-negative. The names originate from the reaction of cells to the Gram stain, a test long-employed for the classification of bacterial species.
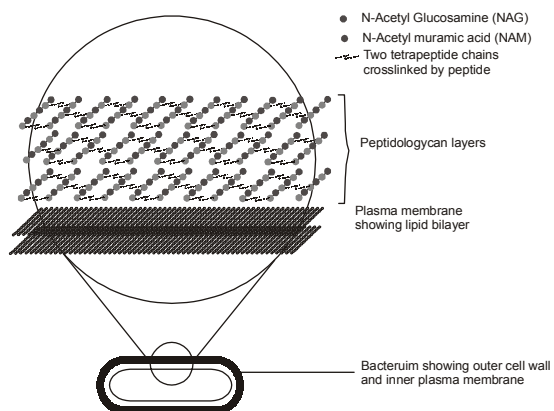
Fig. 2.10 : Showing the Simplified Schematic of cell wall in a gram positive bacterium

Gram-positive bacteria possess a thick cell wall containing many layers of peptidoglycan and teichoic acids. In contrast, Gram-negative bacteria have a relatively thin cell wall consisting of a few layers of peptidoglycan surrounded by a second lipid membrane containing lipopolysaccharides and lipoproteins. Most bacteria have the Gram-negative cell wall and only the Firmicutes and Actinobacteria (previously known as the low G+C and high G+C Gram-positive bacteria, respectively) have the alternative Gram-positive arrangement. These differences in structure can produce differences in antibiotic susceptibility, for instance vancomycin can kill only Gram-positive bacteria and is ineffective against Gram-negative pathogens, such as *Haemophilus influenzae* or *Pseudomonas aeruginosa*.

Archaeal cell walls

Although not truly unique, the cell walls of Archaea are unusual. Whereas peptidoglycan is a standard component of all bacterial cell walls, all archaeal cell walls lack peptidoglycan, with the exception of one group of methanogens. In that group, the peptidoglycan is a modified form very different from the kind found in bacteria There are four types of cell wall currently known among the Archaea.

One type of archaeal cell wall is that composed of pseudopeptidoglycan (also called pseudomurein). This type of wall is found in some methanogens, such as *Methanobacterium* and *Methanothermus*. While the overall structure of archaeal *pseudo*peptidoglycan superficially resembles that of bacterial peptidoglycan, there are a number of significant chemical differences. Like the peptidoglycan found in bacterial cell walls, pseudopeptidoglycan consists of polymer chains of glycan cross-linked by short peptide connections. However, unlike peptidoglycan, the sugar N-acetylmuramic acid is replaced by N-acetyltalosaminuronic acid, and the two sugars are bonded with a β,1-3 glycosidic linkage instead of β,1-4. Additionally, the cross-linking peptides are L-amino acids rather than D-amino acids as they are in bacteria.

A second type of archaeal cell wall is found in *Methanosarcina* and *Halococcus*. This type of cell wall is composed entirely of a thick layer of polysaccharides, which may be sulphated in the case of *Halococcus*. Structure in this type of wall is complex and as yet is not fully investigated.

A third type of wall among the Archaea consists of glycoprotein, and occurs in the hyperthermophiles, *Halobacterium*, and some methanogens. In *Halobacterium*, the proteins in the wall have a high content of acidic amino acids, giving the wall an overall negative charge. The result is an unstable structure that is stabilized by the presence of large quantities of positive sodium ions that neutralize the charge. Consequently, *Halobacterium* thrives only under conditions with high salinity.

In other Archaea, such as *Methanomicrobium* and *Desulfurococcus*, the wall may be composed only of surface-layer proteins, known as an *S-layer*. S-layers are common in bacteria, where they serve as either the sole cell-wall component or an outer layer in conjunction with polysaccharides. Most Archaea are Gram-negative, though at least one Gram-positive member is known.

**SUB-SECTION 2.3E—THE INTERPRETATION OF THE CHEMICAL
REACTION OF CARBOHYDRATES**

1. Reaction with hydrazine from hydrazines and osazones react with other monosaccharides and other carbohydrates containing a free sugar group to form hydrazones and osazones. The reactions of glucose with phenyl hydrazine may be used for illustration as commonly give they are:

D-Glucose

phenylhydrazine

D-Glucose phenyl hydrazine

(1E)-3,4,5,6,7-pentahydroxy-1-(2-phenylhydrazinylidene)
heptan-2-one intermediate product

phenylhydrazine

(5Z)-5,6-bis(2-phenylhydrazinylidene)hexane-1,2,3,4-tetrol

D-Glucose phenyl osazone
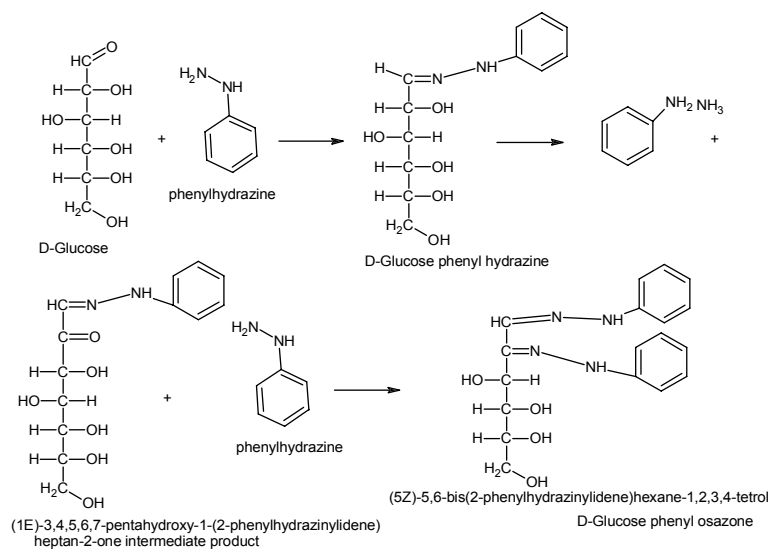
However, it appears that although the above sequence of reactions represents the result the conversation of the phenyl hydrazones to the phenyl osazones actually involves a more complex mechanism because phenyl hydrazine is reducing rather than an oxidizing agent. Weygand proposed that osazones formation may involve an Amadori Rearrangement.

phenylhydrazine

Amadori
rearrangement

phenyl osazone

+ H₂O
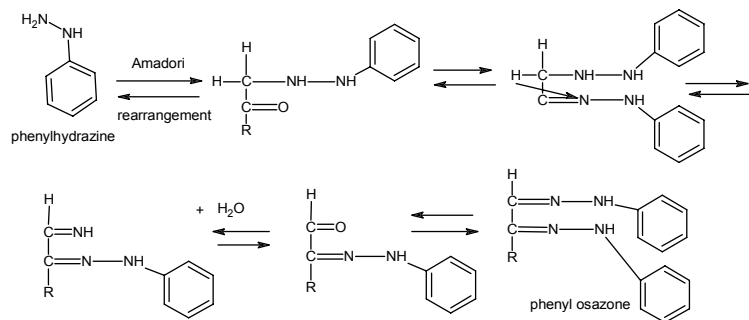
Fructose and other ketoses react similarly to form the hydrazones and osazones.

D -Fructose phenylhydrazone

D -Fructose phenylosazone
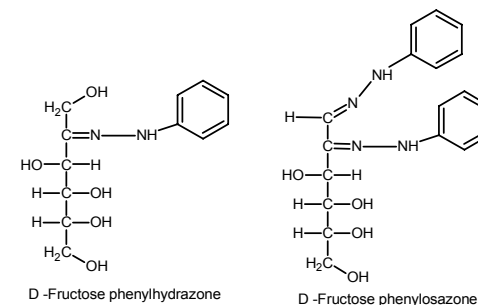
With few exceptions the hydrazones are soluble and difficult to isolate. One the other hand, the osazones are relatively insoluble and crystallize in a beautiful; and characteristic form for different sugars (see fig 13 for glucose) . It must be noted that the osazones of glucose fructose and mannose are identical and by this fact Fischer demonstrated that glucose, fructose and mannose are identical in the lower four carbon atoms. Similar relations were also found in the case of other groups of sugars, and thus the osazones become of prime importance in helping to determine the structural configurations of the sugars.

Osazones when treated with strong hydrochloric acid.

osazone

+ 2H₂O $\xrightarrow{HCl}$

Osone

+ 2
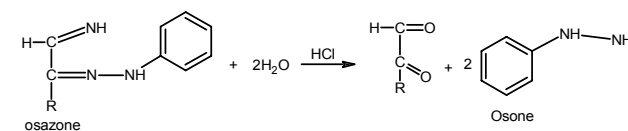
When osones are treated with zinc and acetic acid, the aldehyde group is preferentially reduced to form the corresponding ketoses.

Osone

+ 2H
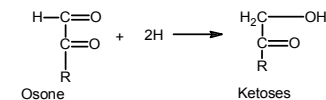
Ketoses

These reactions provide a method of converting an aldose into a ketose for example, the conversion of glucose to fructose.

Reaction with Hydroxylamine to form oximes hydroxylamine condenses with aldoses and ketoses as it does with ordinary aldehydes and ketoses, to form oximes.

D - Glucose + H₂N—OH → D - Glucose oxime
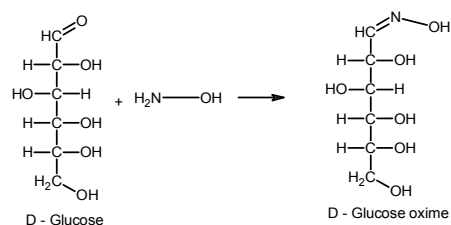
Whole developed a method based upon use of the sugar oxime, of shortening the sugar chain one carbon atom at a time. In this way a hexone may be degraded to pentose, a pentose to a tetrose etc, In order to do so the oxime is treated with acetic anhydride, which removed a molecule of water and converts the oxime to a cyanohydrine or nitrile ad also acetates the hydroxyl groups. The cyanohydrine then treated with ammonical silver nitrate solution (Tollen's Reagent) which removes the hydrogen cyanide to form an acetylated sugar with one less carbon atom than the original, which can be decomposed into sugar. The process is shown in outline form as follows the acetyl group has been omitted.

D - Glucose → D - Glucose Oxime → D - Gluconitrile   D - Arbinose
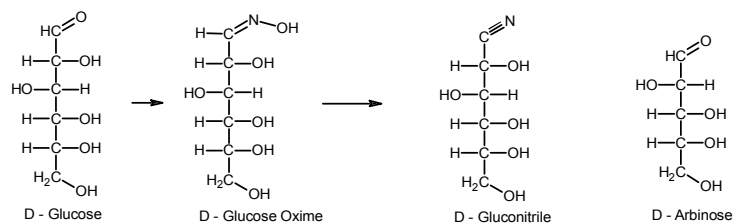
Reduction to form sugar alcohols both aldoses and ketoses may be reduced to the corresponding polyhydroxy alcohols. This may be accomplished with sodium amalgam or, better electrolytically or by hydrogen under high pressure in the presence of a catalyst. The alcohols formed from glucose mannose, and fructose is:

D - Glucose   D - Mannose   D - Fructose

D - Sorbitol   D - Mannitol   D - Mannitol   D - Sorbitol
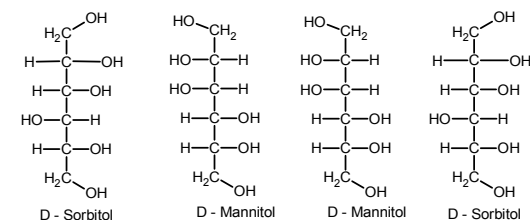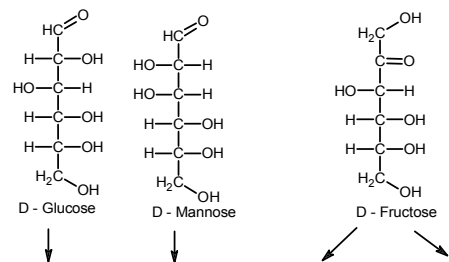
Each aldoses yield the corresponding alcohol upon reduction while a ketose form to alcohols.

Because of the appearance of a new asymmetry carbon atoms in the process. It is interesting to note that reduction of the ketohexose L – sorbose forms D – sorbitol and L – iditol

D - Sorbitol   L - Sorbose   L - Iditol
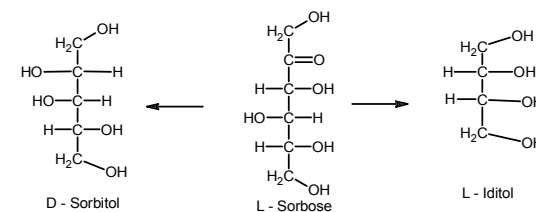
If the above formula of D Sorbitol is rotated longitudinally through the plane of the molecule 180° it will appear, the conventional formula of D – Sorbitol.

Reduction of glyceric aldehyde and dihydroxyacetone forms the trihydroxy alcohol glycerol.

Erythritol is the reduction product of the aldotetrose erithrose. Ribitol is the alcohol derived from aldopentose ribose.

Reduction of galactose produces dulcitol

Glycerol   Erithrititol   Ribitol   Dulcitol
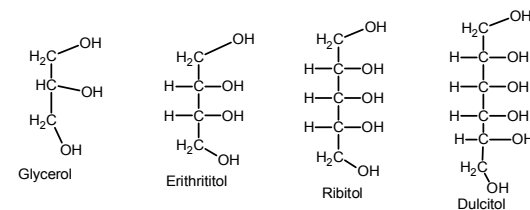
Each of these sugar alcohols is derived by the reduction of both D and L corresponding sugar, and the alcohols are not prefixed by the either D or L. They are optically inactive, though Erythritol, Ribitol and

dulcitol possesses asymmetric carbon atoms. This is due to the fact that the molecules are symmetrical and are internally compensated relative to polarized light just as meso- tartaric acid is .

All the sugar alcohols are well crystallized compounds, soluble in water and alcohol, and they have a sweet taste.

A number of the sugar alcohols may be oxidized to the corresponding ketoses by approximate bacteria in the presence of oxygen. For example D Sorbitol is oxidized as follows by *Acetobactor Suboxidans:*

D - Sorbitol   L sorbose   L sorbose
Longitudinal rotation
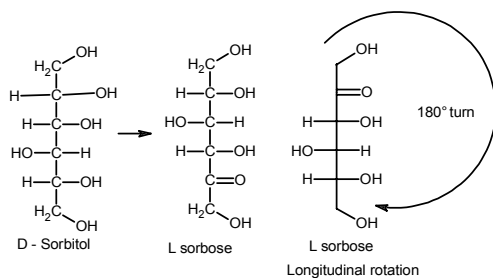
Oxidation occurs on the fifth carbon atom of D – Sorbitol to form a ketone belonging to the l – series, L Arbose. This reaction is used to produce large quantities of L Sorbose for the synthesis of ascorbic acid, or the vitamin C.

Dihydroxyacetone may be prepared similarly by oxidation of glycerol.

glycerol   1,3-dihydroxypropan-2-one
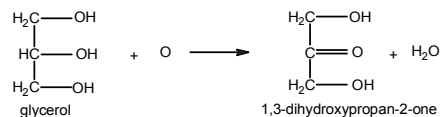
Oxidation to produce sugar acids, oxidized under the proper conditions, the aldoses may form monobasic aldonic acids, or dibasic saccharic acids, or monobasic uronic acid containing the aldehyde group.

Oxidation of a aldoses with bromine water converts the aldehyde group to carboxyl group and thereby forms the corresponding acid which water to form hypobromous acid which acts as the oxidizing agent.

$$Br_2 + HOH \rightleftharpoons HOBr + Br$$
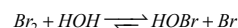
Oxidation of glucose forms glyconic acid:

D - Glucose   D - Gluconic acid
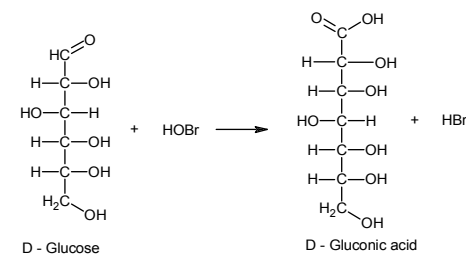
Similarly mannose, galactose, and arbinose give mannoic galactonic and arbonic acids, respectively. Other aldoses form the corresponding aldonic acid. Ketoses are not readily oxidized by bromine water.

So it is said that the oxidation is avoided in the normal metabolic state the aerated waters are one of the source of the oxidants by taking anti oxidants

Aldoses are readily oxidized to aldonic acids by alkaline iodine solution but ketoses are not oxidized. The reactions may be represented.

$$I_2 + 2NaoH \rightarrow NaOI + NaI + H_2O$$

$$R - CHO + NaOI + NaOH \rightarrow R - COONa + NaI + H_2O$$
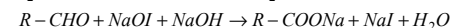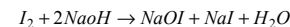
Willstätter and Schudel developed a method for the quantitative determination of aldoses based upon oxidation.

When gluconic acid is heated water is readily lost and it forms a mixture of gamma (g) and delta (d) lactones.

> So it is said that the oxidation is avoided in the normal metabolic state the aerated waters are one of the source of the oxidants by taking anti oxidants

γ - Gluconolactose   Gluconic acid   δ- Gluconolactose
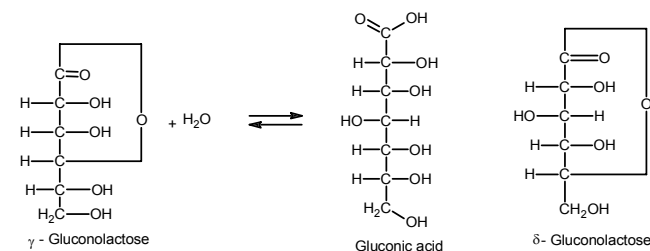
In aqueous solutions equilibrium is established between gluconic acid its two lactones. Other sugar acids containing five or more carbon atoms similarly form two lactones. Acids with four carbon atoms can form only the gamma lactones.

Sugar acids lactones may be reduced to the corresponding sugars by treatment with sodium amalgam in the presence of dilute sulphuric acid.

When the calcium salts of aldonic acids are oxidized with hydrogen peroxide in the presence of Fe^{+++} ions used as catalyst, carbon dioxide is split out and sugars of one less carbon are formed.

D - Gluconic acid   D - Arbinose
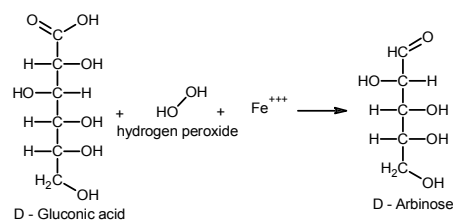
A sugar may be oxidized to the corresponding aldonic acid, and this then converting to the sugar with one less carbon atom.

Calcium gluconate is often administered as a source of calcium. Solutions of it are given intravenously to raise the blood calcium.

Saccharic or Aldaric acids Oxidation of aldoses with nitric acid under the proper conditions converts both aldehyde and primary alcohol groups to carboxyl forming dibasic sugar acids, the saccharic or Aldaric acids.

D - Gluconosaccharic acid   D - Mannosaccharic acid   Mucic Acid
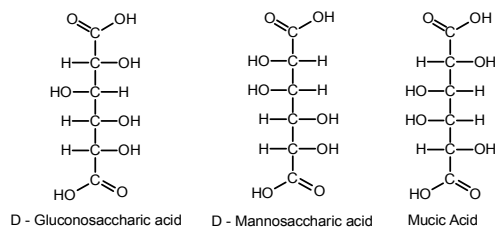
The saccharic acid nomenclature for these dibasic sugar acids has been used exclusively until recently. According to this system, the acids are named by adding to saccharic a prefix indicating the sugar from which the acids is derived – for example glucosaccharic (also called simply saccharic). Similarly "arbosaccharic" xylosaccharic.

The more recent designation of the dibasic sugar acids as aldaric acids appears preferable because the names are in many cases much shorter than those based upon the saccharic nomenclature. According to the aldaric acid system, the name of the acid from a sugar is the name of the sugar with the ending

 " – aric replacing " – ose for example " glucaricsw" "xylaric" " arbaric" "threaric" "hexaric" (form hexose) "pentaric" from pentose.

The aldaric acid form more complex mixtures of lactose than do the aldaric acid, due to the presence of carboxyl groups.

The acid salts of the alderic acids are often used in the identification of sugars because of their low solubility in water.

Galactaric (galactosaccharic) or mucic acid, produced by oxidation of glucose is relatively insoluble in water and well crystallized and its format is the free and combined states.

The aldaric acids have been of much value in proving the configurations of the aldoses sugars.

Uronic acids are produced when an aldoses is oxidized in such a way that the primary alcohol group is converted to carboxyl without oxidation of the aldehyde or sugar group is protected by conversion to a glucoside or the oxidized by oxygen with an activated platinum carbon catalyst.

1,2,0 Isopropyledene D - glucose   1,2,0 Isopropyledene glucuronic acid   Glucouronic acid
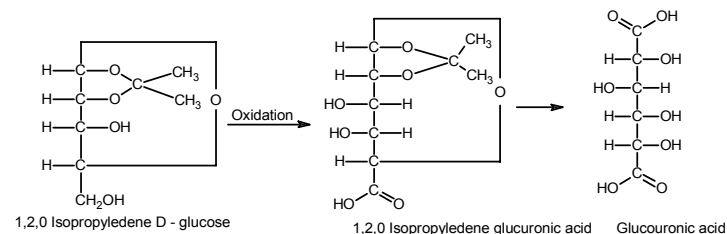
The isopropylidene group may be removed as acetone by carful acid hydrolysis to yield D – Glucuronic acid.

D - Glucouronic acid   D - Mannuronic acid   D - Galactouronic acid
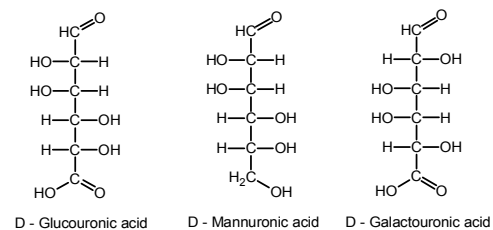
All three of these uronic acids are natural products.

D – Glucouronic acid occurs in combined in plant materials. It is also a constituent of chondoitin mucoitin sulphuric acid sulphuric acids of glycoproteins. Glucouronic acid is formed in the animal body in the process of detoxifying substances such as borneol camphor and benzoic acid; the Glucouronic acid may be prepared in the laboratory by feeding borneol to a dog and hydrolyzing the borneol compound of Glucouronic acid which is excreted in the urine.

D – Galactouronic acid is widely distributed as a constituent of pectin and many plant gums and mucilage. It may me conveniently prepared from pectin.

D – Mannouronic acid occurs as a polymer anhydride in alginic acid and to form the pentose D - xylose.

D - Glucouronic acid → D - Xylose + CO_2
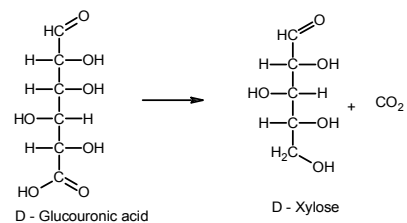
It is probable that at least some of the pentose of plants is formed form from hexoses through uronic acids in this way Decarboxylation of D – Galactouronic acid form acid forms L – arbinose.

D - Glucouronic acid   L - arbinose + CO_2
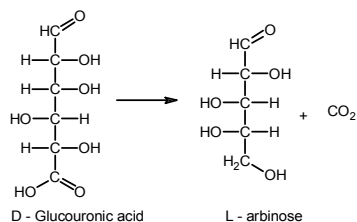
In this connection it is interesting to note that xylose is generally associated with D – xylose is generally associated with D – glucose in plants, while L – arbinose and D – galactose is associated.

Sugar acid
3,4,5,6,7-pentahydroxyheptanoic acid

benzene-1,2-diamine

2,3-dihydro-1*H*-benzimidazol-5-yl(1,2-dihydroquinoxalin-2-yl)methanol
Sugar acid benzimidiazoles

+ $2H_2O$
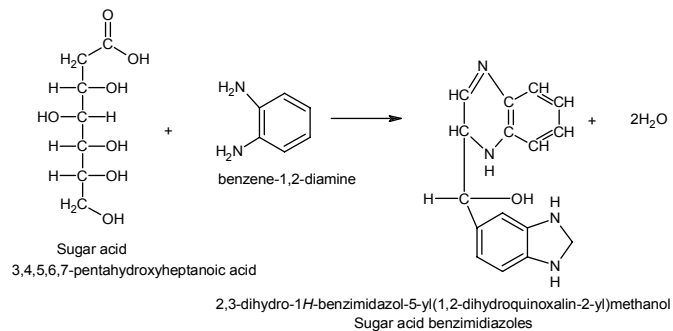
Formation of benzimidiazoles forms sugar acids, Aldonic, aldaric (saccharic) uronic and saccharic acids all react with o – phenyl enediamine to form benzimidiazoles, which are useful derivatives for the identification of these acids and the sugars form which they are derived.

The reactions are carried out in the presence of hot acid.

Action of alkalies upon sugars, where sugar behaves like weak acids and form salts high alkalinities. The pK values (pH at which the acid is half converted to salt.) of four common sugars are given below.

Sugar	pK_1	pk_2
Glucose	12.9	13.85
Fructose	11.68	13.24
Sucrose	12.60	13.52
Lactose	11.92	13.44

Monosaccharides, both aldoses and ketoses and compound carbohydrates containing a free sugar tatutomerise and form the enol salt in alkaline solution.

D - glucose   hept-1-ene-1,2,3,4,5,6,7-heptol
1,2 enidiol

+ Na—OH
sodium hydroxide

sodium salt of enidiol + H_2O

1,3,4,5,6,7-hexahydroxyheptan-2-one   hept-1-ene-1,2,3,4,5,6,7-heptol

+ Na—OH
sodium hydroxide

Sodium 2,3,4,5,6,7-
hexahydroxyhept-1-en-1-olate
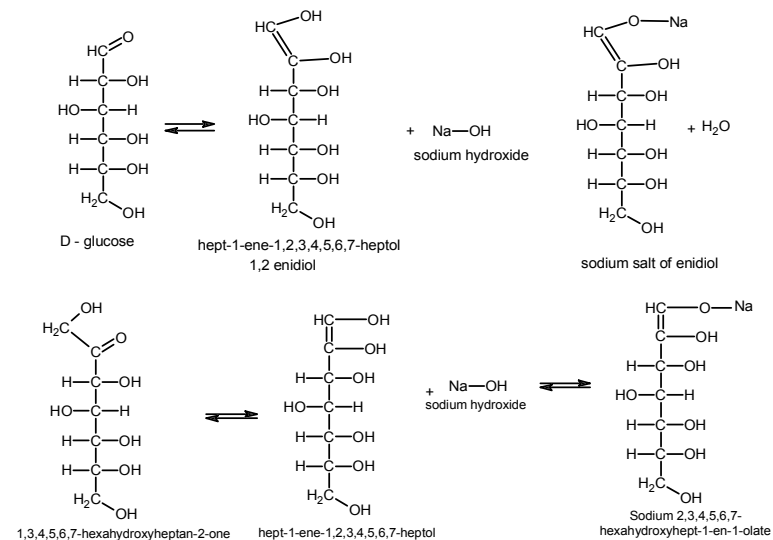
The enol forms of the sugar are enediols because two hydroxyl groups are attached to the double – bonded carbon system. It is uncertain which enolic hydroxyl first will react to form the salt.

It will be noted that glucose, mannose and fructose from the same enediols and enediols salts forms, When a solution of any one of these sugars is allowed to stand in dilute alkali (0.05N) for same time and then acidified a mixture of all these sugars is obtained. This is due to the fact that they all give the same enediols salts, and when this is decomposed to the free enediols by acidified with acetic acid, the enediols tatutomerise into all three sugar as follows:

D - Mannose hex-1-ene-1,2,3,4,5,6-hexol 2,3,4,5,6-pentahydroxyhexanal

D - Fructose
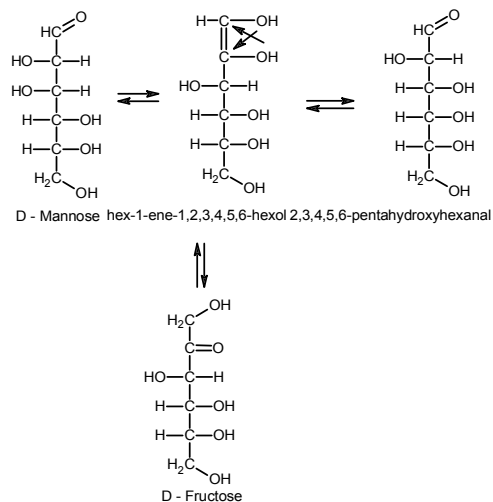
This inter conversion of related sugar by the action of dilute alkali is referred to as the **Lobry de Bruyn – Von Ekenstein Equation**; it has been used for the synthesis of certain sugars.

When sugars with free groups are treated with stronger alkali (0.5N and stronger) enolization occurs to form not only 1 – 2 but, also 2 – 3 and 3 – 4 enediols. The 2 – 3 and 3 – 4 enidiols are apparently produced from the ketose sugars present in the mixture. An outline of the probable process in gen below, glucose being used as the initial sugar.

In strong alkali the enediols forms are very unstable and reactive. The enediols break at the double bond forming a complex mixture of products For example, a – 1 – 2 enediol decomposes to give formaldehyde and pentose; a 2 – 3 enediol gives glycolic aldehyde CH$_2$OH . CHO and a tetrose; and a 3 – 4 enediol form glyceric aldehyde which is a triose. However, since aldoses forms by scission of the parent enediol also enolize and rearrange and therefore an extremely complex sugar is produced.

D - Glucose   1 - 2 Enediol   D - Fructose   2 - 3 Enediol

3 keto glucose   3 - 4 Enediol
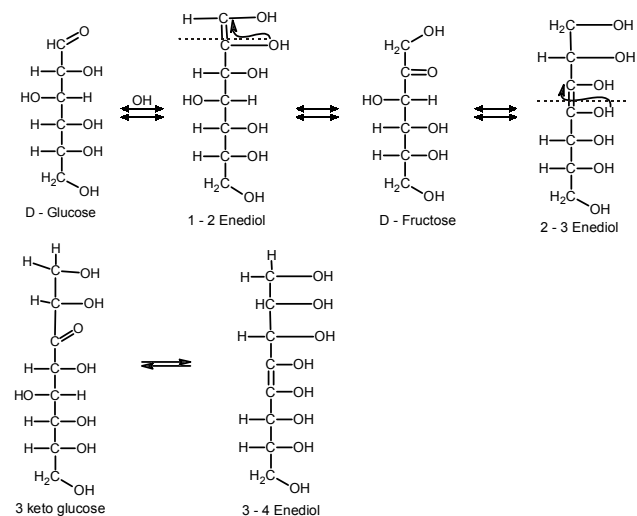
In the presence of oxygen or other oxidizing agents such as Cu^{++} (Fehling's Solution) the acids corresponding to the various scission products of the alkaline mixture are obtained. For, example, the following acids have been isolated from an oxidized alkaline sugar solution and identified as : formic acid D and L glyceric acid four trihydroxybutyric acid, eight tetrahydroxyvaleric acid, and eight pentahydroxyhexoic acids.

Oxidation of the enediols involves rupture of the double bond with formation of shorter – chain acids.

D - Glucose enediol   formic acid

D - Arbonic acid
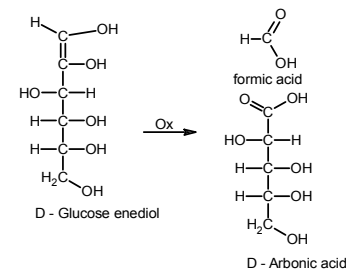
When strongly alkaline solutions of sugars are permitted to stand in the absence of an oxidizing agent, complex mixture of saccharic acid is formed as a result of intra – molecular oxidation – reduction and arrangement. The formulas of a number of these that have been identified are.

3-hydroxybutanoic acid
D - L Lactic

3,5,6-trihydroxyhexanoic acid

2,4,5,6-tetrahydroxy-
2-(hydroxymethyl)hexanoic acid

5-(hydroxymethyl)hexane-1,2,3,5,6-pentol   5-(hydroxymethyl)hexane-1,2,3,5,6-pentol
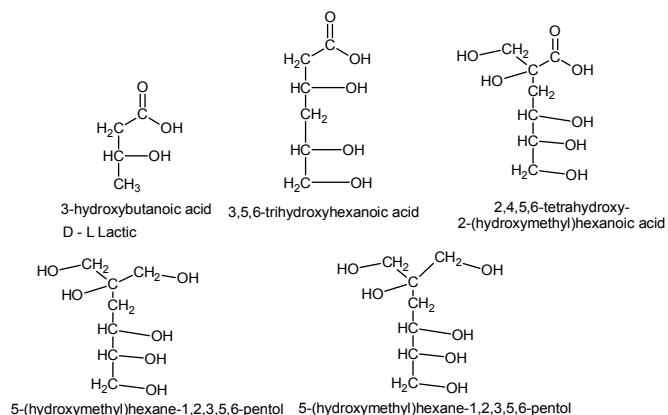
Lactic acid is apparently formed glyceric aldehyde by intermolecular oxidation – reduction involving the first and third carbon atoms. It is generally formed in rather large amounts.

Lime water at ordinary temperature causes formaldehyde to polymerise to a sweet $C_6H_{12}O_6$, which Fischer called "formose" Fischer formose to be a complex mixture, Fischer and Tafel oxidized glycerol to form a mixture dihydroxyacetone and glyceric aldehyde treated the mixture with dilute alkali and obtained a sweet syrup, which is called "α – acrose" D – L Fructose was identified as constituent of α - acrose.

propan-2-one   propan-2-ol

D - L Fructose
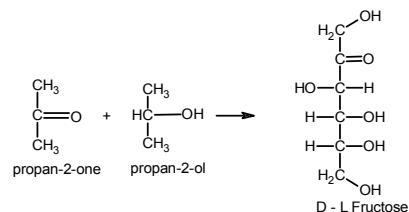
Three molecules of glycolic aldehyde $CH_2OH – CHO$ have also been found to polymerise to α – acrose when treated with dilute alkali.

The action of alkali upon formaldehyde and the lower sugars produces a very complex mixture of sugars.

Carbohydrates such as sucrose, which do not contain a free sugar group, are not enolized by alkali are therefore stable in alkaline solution.

When sugars containing free sugar group is heated with alkali, the solutions turn yellow to reddish brown as result of the formation of the complex resinous substances.

This is known as Moor's Test; the sugar enediols exist in the cis and trans configuration about the double bond. Topper has obtained evidence that the enzyme phosphoglucose isomerise acts stereo specifically upon H or C – 2 glucose – 6 – phosphate to form this cis – 1, 2 – enediol while the enzyme phosphomannose isomeriase acts upon the H on C – 2 of Mannose – 6 – phosphate.

2,3,4,5-tetrahydroxy-6-oxohexyl
dihydrogen phosphate
D-Glucose-6-PO₄ chain form

2,3,4,5,6,7-hexahydroxyhept-6-en-1-yl
dihydrogen phosphate
D-Glucose-6-PO₄ chain form cis 1,2 enediol
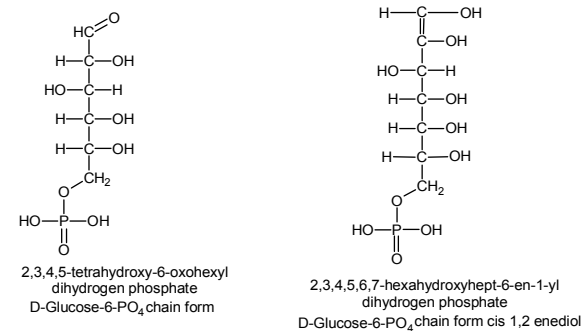
Phosphoglucose isomerise will not acts upon D – mannose 6 – PO_4, and phosphomannose isomerise will not act upon D – glucose 6 – PO_4

Reducing action of sugars in alkaline solution, determinations of sugars. All the sugars which contain the free sugar in alkaline solution, there the enediol forms are very reactive and also are readily oxidized by oxygen and other oxidizing agents which are very strong. This means that these sugars in alkaline solution are very strong reducing agents. As a consequence, they readily reduce oxidizing ions such as Ag^{++}, Hg^{++}, Bi^{+++} Cu^{++} and $Fe(CN)_6^{\circ}$ and the sugar are oxidized to complex mixtures of acids. This reducing action of sugars in alkaline solution is utilized for both the qualitative and the quantitative determination of sugars. Reagents containing Cu^{++} are commonly used; these are generally alkaline solutions of cupric sulphate containing sodium potassium tatrate (Rochelle) Sodium citrate. Sodium hydroxide is used as the alkali in the reagents, such as Fehling's Reagent but weaker alkalies, such as Sodium carbonate and sodium bicarbonate, are used in the more recent reagents like Benedict, Folin and Shaffer and Hatmann.

Citrate and Rochelle salt prevent the preparation of the cupric ions in the form of cupric hydroxide or carbonate by forming soluble, slightly dissociated complex with the Cu^{++} ions. These complexes

dissociate sufficiently to provide a continuous supply of readily available Cu^{++} ions for oxidation but at concentrations which prevent the solubility products of cupric hydroxide and carbonate being exceeded.

The alkali of the sugar reagents enolize the sugars and thereby cause them to be strong reducing agents.

When a reducing sugar is heated with one of alkaline copper reagents the process occur accordingly.

Sugar + Alkali $\longrightarrow$ enediols, reducing agents
+
Cu^{++} Copper Complex of tartrate and citrate

$Cu_2O \leftarrow CuOH \leftarrow OH^- + Cu^+ + $ mixture of sugar acids
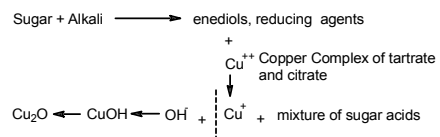
The Cu^{++} ions take electrons from the enediols and oxidize them to sugar acids and are in turn reduced to cuprous ions Cu^{++}. The cuprous ions combine with hydroxyl ions to form yellow cuprous hydroxide, which upon heating is converted to red cuprous oxide Cu_2O

$$2CuOH \rightarrow Cu_2O + H_2O$$
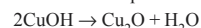
The appearance of a yellow – to – red precipitate indicates reduction and the quantity of sugar present can be roughly determined from the amount of precipitate. In quantitative estimation the amount of copper reduced is obtained by iodometric titration or colorimetric methods, and from this the amount of sugar is calculated.*

Alkaline ferricyanide ($K_3Fe(CN)_6$) solutions are also used in the quantitative determination of sugars the most common reagent being that of Hagendrom and Jenson. These are stronger oxidizing solutions than the copper reagents, which are more liable to oxidize non – sugars present. Their action involves oxidation of the sugar enediols by the ferricyanide, ion $Fe(CN)_6^°$, which is reduced to the ferricyanide ion. The amount of ferricyanide reduced is easily determined by iodine titration and from this the quantity of sugar is determined.

Polysaccharides such as glycogen as starch are generally quantitatively determined by preliminary hydrolysis with acid to the constituent monosaccharide, which are then estimated by one of the above reduction method.

An interesting determination of glucose is based upon its quantitative oxidation to D – glucono -δ - lactone is converted to D – gluconic acid, which is then titrated with alkali

Hydrogen peroxide also is formed in the oxidation with is used as the basis of a very sensitive colorimetric method for glucose estimation.

$$\text{Glucose} + O_2 \xrightarrow[oxidase]{glucose} \text{gluconolactone} + \text{Hydrogen Peroxide}$$
$$\Downarrow + H_2O$$
$$\text{D - Gluconicacid}$$
$$\text{Hydrogen Peroxide} + \text{o-dianicidine} \xrightarrow{peroxidase} \text{yellow compound}$$
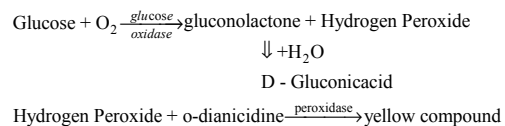
_____
*Now a days the Follin U method of sugar estimation is used in the modern pathological labs

A very useful method for the colorimetric determination of carbohydrates is based upon the reaction of anthrone with a carbohydrate in the presence of strong hydrochloric acid.

The carbohydrate is decomposed by the acid to give a substance, probably a furfural derivative, which reacts with anthrone to form a blue compound. The reaction is given by both free and combined sugar and is quite sensitive.
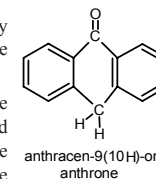
In general the Monosaccharides are relatively stable to that hot dilute hydrochloric acid though the ketoses are apparently decomposed by prolonged action. When the concentration of acid is increased to several moralities, the Monosaccharide molecules are decomposed. Pentoses yield the cyclic aldehyde furfural, as shown in the reaction equation below.

anthracen-9(10H)-one anthrone

D - ribose

hydroxymethylfurfural
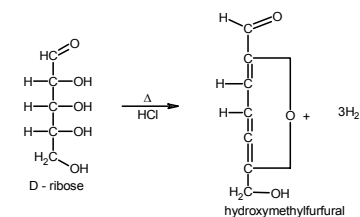
This reaction is used for the quantitative determination of Pentoses compound carbohydrates containing Pentoses (pentosan etc).12% hydrochloric acid has been found the most satisfactory acid for decomposition. Furfural forms, with phloroglucinol, a relatively insoluble compound, furfural – phoroglucide, which can be used in the estimation of the furfural formed in the reaction as a measure of the Pentoses present. The furfural can also be determined by use of its colour reaction with aniline acetate or by a titrimetric reaction.
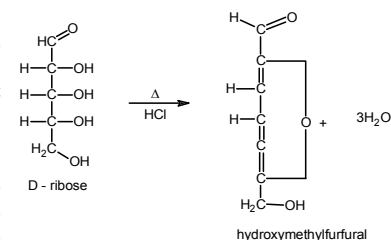
Hexoses are decomposed by hot strong acid to give hydroxymethylfurfural which decomposes into levulinic acid and other products.

Considerable brown or black resinous substances are also formed. When uronic acids such as Glucouronic acid and Galactouronic acids are heated with acid, they loose carbon dioxide and form the corresponding pentose which in turn is converted to furfural.

D - ribose

hydroxymethylfurfural

4-oxopentanoic acid
Levulinic acid

D -Glucouronic acid

D - xylose
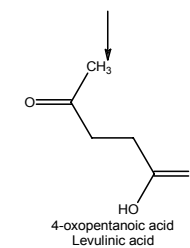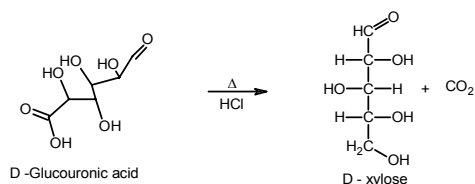
The yield of carbon dioxide is quantitative, and uronic acids are determined by estimating the amount of carbon dioxide liberated in the above reaction. The yield of furfural from the pentose in this reaction is not quantitative.

Reactions of Sugars due to hydroxyl group Most of the chemical properties of the sugars previously discussed were concerned directly or indirectly with the aldehyde or ketone groups, though a few reactions involving hydroxyl groups were considered. The hydroxyl groups of sugars have the properties of ordinary alcoholic groups but, because of the number present, may give rise to derivatives which are impossible with monohydroxy alcohols.

1. Formation of glucosides (glycosides). Production of the glucosides of the simple sugars has been referred to in a previous section. In general, the ring forms of the simple sugars react with alcohols in the presence of hydrogen chloride as catalyst to form the glucosides. Only the hydroxyl on carbon 1 (glucosidic hydroxyl) of the sugar reacts under these conditions. Generally a mixture of the *a-* and ,B-glucosides is obtained, and when the reaction is carried out at elevated temperatures, the glucosides contain the pyranose ring. Reaction at room or lower temperatures also may produce glucosides with the furanose ring.

α from of sugar

β from of sugar

α-Glucoside
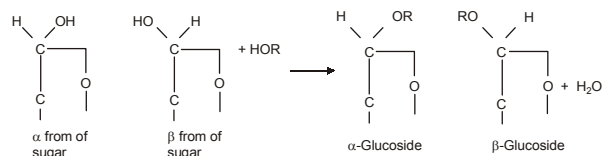
β-Glucoside

The pyranose glucosides are called "pyranosides" whereas the furanose the glucosides are "furanosides" The formula and names are below will show:

or

or

α-Methyl-D-glucofuranoside

β-Methyl-D-glucofuranoside

or

α-Ethyl-D-galactopyranoside

β-Ethyl-D-galactopyranoside

or

α-Methyl-D-fructofuranoside

β-Methyl-D-fructofuranoside
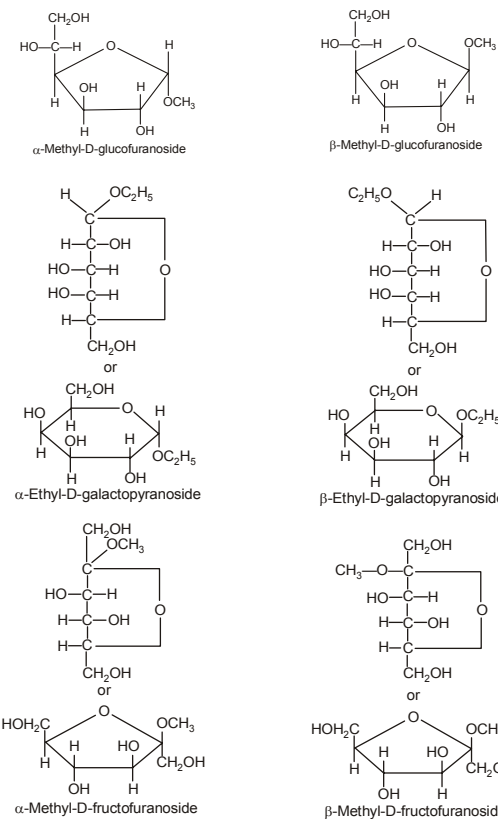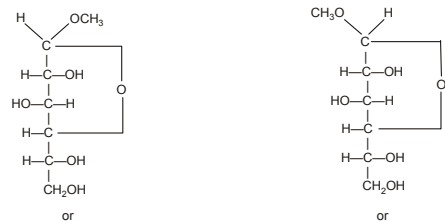
When the α and β forms of a free sugar or a glucoside are prepared, the problem of determining which shall be designated as α and which as β arises.

Boeseken found that polyhydroxy compounds having two hydroxyl groups on adjacent carbon atoms and on the same side of the chain form complexes with boric acid in solution which ionize more than boric acid alone and consequently increase the electrical conductivity. For example, α-D-glucose increases the conductivity of boric acid solutions, but the conductivity falls as the sugar mutarotates

and passes into the β isomer. The same thing is true with α-D-galactose. According to these findings the glucosidic hydroxyls of a-D-glucose and a-D-galactose lie on the same side of the carbon chain as the hydroxyls on carbon 2, while the hydroxyls are in the trans position for the β forms.

The configurations about carbon 1 of the α and β anomers of various sugars have been established by other methods, especially from the rotations of the dialdehydes obtained in the periodate oxidation of glycosides. In general, in the D-series the hydroxyl of the a monomer lies to the right in Fischer's projection ring formulas and below the ring in Haworth's ring formulas. In the L-series the hydroxyl of the a-anomer lies to the left in the Fischer formulas and above the ring in the Haworth formulas.

In the case of a sugar which exists as both α and β anomeric forms, belongs to the v-series, and mutarotates in solution, the a form of the sugar usually is the one which mutarotates to a value less positive than the initial rotation.

Isbell has pointed out that, when the glucosidic hydroxyl of a sugar lies in the same direction as the ring oxygen, the sugar is oxidized by bromine water more slowly than when the hydroxyl is directed away from the ring oxygen. That is, the β forms of glucose and galactose are more readily oxidized than the α forms. Isbell also proposed a general rule for differentiating the α and β pairs of a sugar, or its glucosides, or other derivatives, based upon optical rotation: "When the oxygen ring lies to the right, as in D-glucose, the more dextrorotatory member of the α-β pair shall be designated α, and the less dextrorotatory member β when the oxygen ring lies to the left, as in L-glucose, the more levorotatory member shall be designated α, and the less levorotatory β. This rule is applicable to the furanoses as well as to the pyranoses and to the derivatives of both."

The glucosides do not reduce alkaline copper solutions, because the sugar group is combined. For the same reason, they are resistant to the action of alkali. They may be hydrolyzed to the constituent reducing sugars by boil-ing with dilute mineral acids. α-Glucosides are hydrolyzed by maltase, an enzyme from yeast, while β - glucosides are hydrolyzed by the enzyme emul-sin, from bitter almonds. Enzyme hydrolysis thus affords a method of distinguishing between the two forms.

Many glucosides occur in the roots, bark, and fruit, and frequently the leaves of various plants. Glucosides are usually well-crystallized, colorless, bitter solids, soluble in water and alcohol. A number of the natural glucosides are important in medicine or otherwise. The groups attached to the sugars in the natural glucosides are frequently quite complex, but the union is al-ways through condensation of an alcoholic or phenolic hydroxyl with the glucosidic hydroxyl of the sugar.

Table below lists a number of the natural glucosides, with their hydrolytic products and source.

The group attached to the sugar in a glucoside is often referred to as the "aglucone" or "aglycone"; thus mandelonitrile is the aglucone of amygdalin, and digitogenin is the aglucone of digitonin.

Glucoside	Hydrolytic Products	Source
Arbutin	Glucose + Hydroquinone	Arbutus The European species are called Strawberry Trees
Phlorizin	Glucose + Phloretin	Rosewood Bark
Amygdalin	2 Glucose + D - mandelonitrile	Seeds of bitter almonds
Digitonin	4 Galactose + xylose + digitogenin ($C_{27}H_{44}O_5$)	Leaves of Foxglove
Saponin	Sugar + Sapogenin	Soapwort
Indican	Glucose + Indoxyl	Leaves of Indigofera

2. Formation of ethers. The hydrogen of hyoroxyl groups of sugars and of carbohydrates in general may be replaced by alkyl groups to form ethers. The methylated sugars which have already been discussed in some detail are methyl ethers of the sugars. Ethylated sugars, or ethyl ethers of the sugars, may be prepared by reactions analogous to those for the prepara-tion of the methylated sugars.

3. Formation of esters. The hydroxyl groups of the sugars may be esterified to give esters such as the sugar acetates, propionates, stearates, and benzoates. This is generally accomplished by treating the sugar with the appropriate acid anhydride or chloride under the proper conditions.

For example, when glucose is treated with acetic anhydride, pentaacetyl glucose or glucose pentaacetate is formed. By varying the conditions the α or β form may be obtained as the chief product:

In the case of sugar esters rigid systematic nomenclature requires that the acyl groups be designated" -O-acyl" as in "penta-O-acetyl-α-D -glucopyranose."

The acetyl group on the first carbon is readily hydrolyzed off, leaving tetraacetyl glucose with a free sugar group. All the acetyl groups may be removed by mild alkaline hydrolysis to re-form the sugar. The sugar ace-tates and other esters are used especially in the preparation of other sugar derivatives. They are generally insoluble in water and soluble in organic solvents.

While sugars form various inorganic esters such as sulphates and nitrates, the sugar phosphates are outstanding in biologic importance. The breakdown or metabolism of glucose and other sugars by animal tissues and by yeast and other microorganisms involves a succession of the phosphates of sugars and sugar derivatives. Nucleoproteins of cell nuclei also contain sugar phos-phates in combination. Sugar phosphates have been found as intermediate products in the carbohydrate metabolism of plants. Many of these sub-stances have been isolated and identified, and a number have been synthe-sized. Diagrams below gives the structures of phosphates of glucose and fructose which are of biologic importance.

It will be observed that, whereas glucose phosphates have pyranose rings, the phosphates of fructose are of the furanose type.

α-D-Glucopyranosyl-l-phosphate
Glucose-l-phosphate (Cori ester)

or

D-Glucopyranosyl-6-phosphate
Glucose-6-phosphate (Robison ester)

or

D-Fructofuranosyl-6-phosphate
Fructose-6-phosphate (Neuberg ester)

or

D-Fructofuranosyl-1,6-diphosphate
Fructose diphosphate (Harden-Young ester)

or
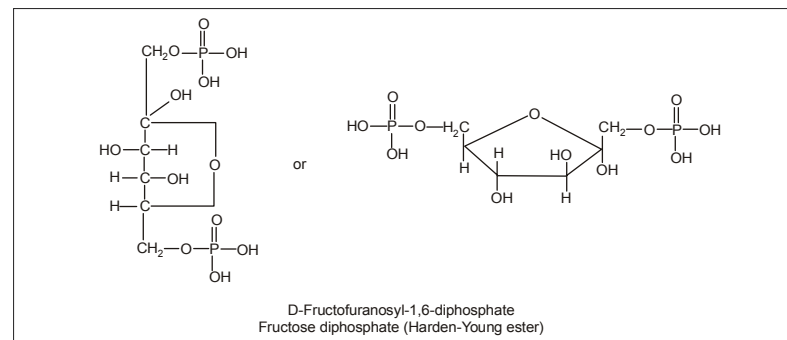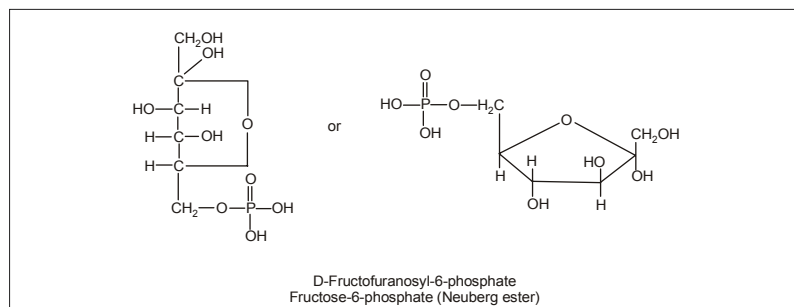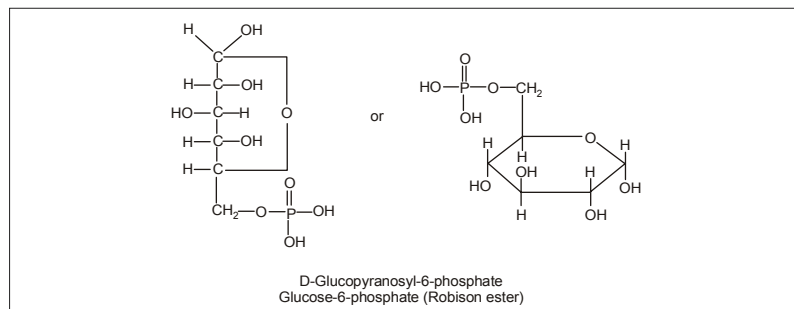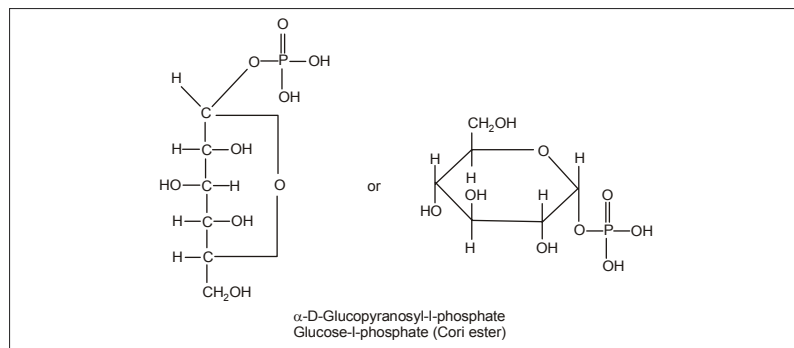
SECTION 2.4—LIPIDS

The lipids are a large and diverse group of naturally occurring organic compounds that are related by their solubility in nonpolar organic solvents (e.g. ether, chloroform, acetone & benzene) and general insolubility in water. There is great structural variety among the lipids, as will be demonstrated in the following sections. You may click on a topic listed below, or proceed page by page.

Fatty Acids

Soaps and Detergents

Fats and Oils

Waxes

Phospholipids

Eicosonoids

Terpenes

Steroids

Lipid Soluble Vitamins

Biosynthetic Pathways

Fats, Oils, Waxes & Phospholipids

1. Fatty Acids

The common feature of these lipids is that they are all esters of moderate to long chain fatty acids. Acid or base-cayalyzed hydrolysis yields the component fatty acid, some examples of which are given in the following table, together with the alcohol component of the lipid. These long-chain carboxylic acids are

generally referred to by their common names, which in most cases reflect their sources. Natural fatty acids may be saturated or unsaturated, and as the following data indicate, the saturated acids have higher melting points than unsaturated acids of corresponding size. The double bonds in the unsaturated compounds listed on the right are all cis (or Z).

Fatty Acids

Saturated Formula	Common Name	Melting Point
$CH_3(CH_2)_{10}CO_2H$	lauric acid	45ºC
$CH_3(CH_2)_{12}CO_2H$	myristic acid	55 ºC
$CH_3(CH_2)_{14}CO_2H$	palmitic acid	63 ºC
$CH_3(CH_2)_{16}CO_2H$	stearic acid	69 ºC
$CH_3(CH_2)_{18}CO_2H$	arachidic acid	76 ºC

Unsaturated Formula	Common Name	Melting Point
$CH_3(CH_2)_5CH=CH(CH_2)_7CO_2H$	palmitoleic acid	0 ºC
$CH_3(CH_2)_7CH=CH(CH_2)_7CO_2H$	oleic acid	13 ºC
$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7CO_2H$	linoleic acid	-5 ºC
$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7CO_2H$	linolenic acid	-11 ºC
$CH_3(CH_2)_4(CH=CHCH_2)_4(CH_2)_2CO_2H$	arachidonic acid	-49 ºC

The higher melting points of the saturated fatty acids reflect the uniform rod-like shape of their molecules. The cis-double bond(s) in the unsaturated fatty acids introduce a kink in their shape, which makes it more difficut to pack their molecules together in a stable repeating array or crystalline lattice. The trans-double bond isomer of oleic acid, known as elaidic acid, has a linear shape and a melting point of 45 ºC (32 ºC higher than its cis isomer). The shapes of stearic and oleic acids are displayed in the models below. **You may examine Chime models of these compounds by clicking on the desired model picture**.
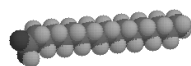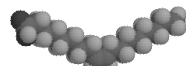
stearic acid   **oleic acid**

Two polyunsaturated fatty acids, linoleic and linolenic, are designated "essential" because their absence in the human diet has been associated with health problems, such as scaley skin, stunted growth and increased dehydration. These acids are also precursors to the prostaglandins, a family of physiologically potent lipids present in minute amounts in most body tissues.

Because of their enhanced acidity, carboxylic acids react with bases to form ionic salts, as shown in the following equations. In the case of alkali metal hydroxides and simple amines (or ammonia) the resulting salts have pronounced ionic character and are usually soluble in water. Heavy metals such as silver, mercury and lead form salts having more covalent character (3rd example), and the water solubility is reduced, especially for acids composed of four or more carbon atoms.

$$RCO_2H + NaHCO_3 \rightarrow RCO_2^{(-)}Na^{(+)} + CO_2 + H_2O$$
$$RCO_2H + (CH_3)_3N: \rightarrow RCO_2^{(-)}(CH_3)_3NH^{(+)}$$
$$RCO_2H + AgOH \rightarrow RCO_2^{\bar{a}(-)}Ag^{\bar{a}(+)} + H_2O$$
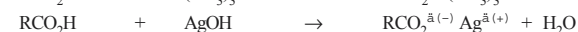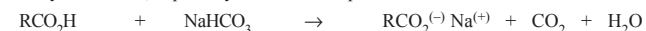
2. Soaps and Detergents

Carboxylic acids and salts having alkyl chains longer than eight carbons exhibit unusual behavior in water due to the presence of both hydrophilic (CO_2) and hydrophobic (alkyl) regions in the same molecule. Such molecules are termed **amphiphilic** (Gk. amphi = both) or **amphipathic**. Fatty acids made up of ten or more carbon atoms are nearly insoluble in water, and because of their lower

density, float on the surface when mixed with water. Unlike paraffin or other alkanes, which tend to puddle on the waters surface, these fatty acids spread evenly over an extended water surface, eventially forming a monomolecular layer in which the polar carboxyl groups are hydrogen bonded at the water interface, and the hydrocarbon chains are aligned together away from the water. This behavior is illustrated in the diagram on the right. Substances that accumulate at water surfaces and change the surface properties are called **surfactants**.Alkali metal salts of fatty acids are more soluble in water than

a soap
sodium stearate

an anionic detergent
sodium p-dodecylbenzenesulfonate

an cationic detergent
hexadecyltrimethylammonium chloride

a nonionic detergent
di(ethylene glycol) dodecyl ether

Nonpolar Hydrocarbon "Tail"   Polar "Head Group"
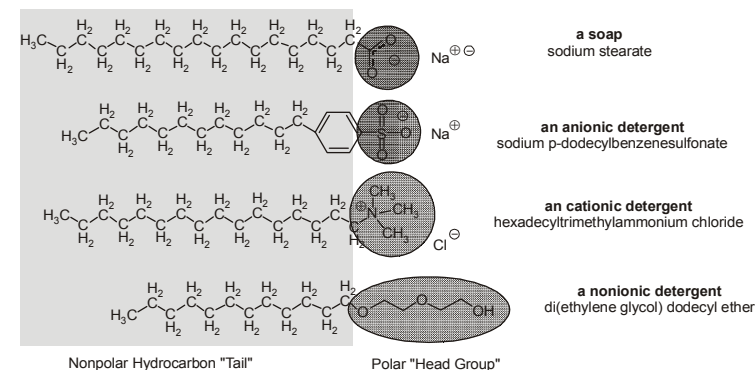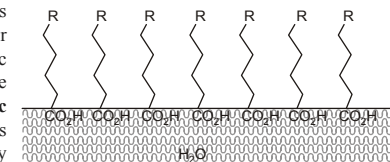
the acids themselves, and the amphiphilic character of these substances also make them strong surfactants. The most common examples of such compounds are soaps and detergents, four of which are shown below. Note that each of these molecules has a nonpolar hydrocarbon chain, the "tail", and a polar (often ionic) "head group". The use of such compounds as cleaning agents is facilitated by their surfactant character, which lowers the surface tension of water, allowing it to penetrate and wet a variety of materials.
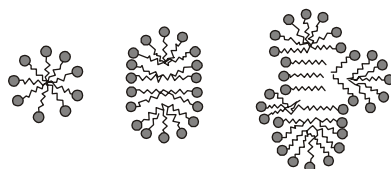
Micelle assemblies of amphiphiles

Very small amounts of these surfactants dissolve in water to give a random dispersion of solute molecules. However, when the concentration is increased an interesting change occurs. The surfactant molecules reversibly assemble into polymolecular aggregates called **micelles**. By gathering the hydrophobic chains together in the center of the micelle, disruption of the hydrogen bonded structure of liquid water is minimized, and the polar head groups extend into the surrounding water where they participate in hydrogen bonding. These micelles are often spherical in shape, but may also assume cylindrical and branched forms, as illustrated on the right. Here the polar head group is designated by a blue circle, and the nonpolar tail is a zig-zag black line. An animated display of micelle formation is presented below. Notice the brownish material in the center of the three-dimensional drawing on the left. This illustrates a second important factor contributing to the use of these amphiphiles as cleaning agents. Micelles are able to encapsulate nonpolar substances such as grease within their hydrophobic center, and thus solubilize it so it is removed with the wash water. Since the micelles of anionic amphiphiles have a negatively charged surface, they repel one another and the nonpolar dirt is effectively emulsified. To summarize, **the presence of a soap or a detergent in water facilitates the wetting of all parts of the object to be cleaned, and removes water-insoluble dirt by incorporation in micelles**.

Random distribution of amphiphiles in water
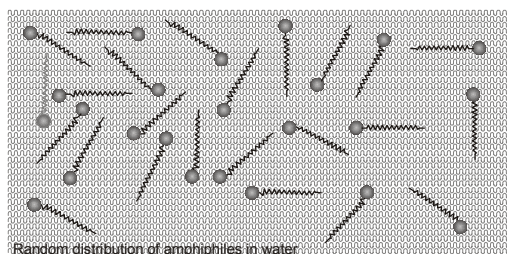
The oldest amphiphilic cleaning agent known to humans is soap. Soap is manufactured by the base-catalyzed hydrolysis (saponification) of animal fat (see below). Before sodium hydroxide was commercially available, a boiling solution of potassium carbonate leached from wood ashes was used.

Soft potassium soaps were then converted to the harder sodium soaps by washing with salt solution. The importance of soap to human civilization is documented by history, but some problems associated with its use have been recognized. One of these is caused by the weak acidity (pK_a ca. 4.9) of the fatty acids. Solutions of alkali metal soaps are slightly alkaline (pH 8 to 9) due to hydrolysis. If the pH of a soap solution is lowered by acidic contaminants, insoluble fatty acids precipitate and form a scum. A second problem is caused by the presence of calcium and magnesium salts in the water supply (hard water). These divalent cations cause aggregation of the micelles, which then deposit as a dirty scum.

These problems have been alleviated by the development of synthetic amphiphiles called detergents (or syndets). By using a much stronger acid for the polar head group, water solutions of the amphiphile are less sensitive to pH changes. Also the sulfonate functions used for virtually all anionic detergents confer greater solubility on micelles incorporating the alkaline earth cations found in hard water. Variations on the amphiphile theme have led to the development of other classes, such as the cationic and nonionic detergents shown above. Cationic detergents often exhibit germicidal properties, and their ability to change surface pH has made them useful as fabric softners and hair conditioners. These versatile chemical "tools" have dramatically transformed the household and personal care cleaning product markets over the past fifty years.

3. Fats and Oils

The triesters of fatty acids with glycerol (1,2,3-trihydroxypropane) compose the class of lipids known as fats and oils. These **triglycerides** (or triacylglycerols) are found in both plants and animals, and compose one of the major food groups of our diet. Triglycerides that are solid or semisolid at room temperature are classified as fats, and occur predominantly in animals. Those triglycerides that are liquid are called oils and originate chiefly in plants, although triglycerides from fish are also largely oils. Some examples of the composition of triglycerides from various sources are given in the following table.

Source	Saturated Acids (%)				Unsaturated Acids (%)			
	C_{10} & less	C_{12} lauric	C_{14} myristic	C_{16} palmitic	C_{18} stearic	C_{18} oleic	C_{18} linoleic	C_{18} unsaturated
Animal Fats								
butter	15	2	11	30	9	27	4	1
lard	-	-	1	27	15	48	6	2
human fat	-	1	3	25	8	46	10	3
herring oil	-	-	7	12	1	2	20	52
Plant Oils								
coconut	-	50	18	8	2	6	1	-
corn	-	-	1	10	3	50	34	-
olive	-	-	-	7	2	85	5	-
palm	-	-	2	41	5	43	7	-
peanut	-	-	-	8	3	56	26	7
safflower	-	-	-	3	3	19	76	-

As might be expected from the properties of the fatty acids, fats have a predominance of saturated fatty acids, and oils are composed largely of unsaturated acids. Thus, the melting points of triglycerides reflect their composition, as shown by the following examples. Natural mixed triglycerides have somewhat lower melting points, the melting point of lard being near 30 ºC, whereas olive oil melts near -6 ºC. Since fats are valued over oils by some Northern European and North American populations, vegetable oils are extensively converted to solid triglycerides (e.g. Crisco) by partial hydrogenation of their unsaturated components. Some of the remaining double bonds are isomerized (to trans) in this operation. These saturated and trans-fatty acid glycerides in the diet have been linked to long-term health issues such as atherosclerosis.

$$H_2C-OCO(CH_2)_{10}CH_3$$
$$HC-OCO(CH_2)_{10}CH_3$$
$$H_2C-OCO(CH_2)_{10}CH_3$$
trilaurin
mp 45°C

$$H_2C-OCO(CH_2)_{16}CH_3$$
$$HC-OCO(CH_2)_{16}CH_3$$
$$H_2C-OCO(CH_2)_{16}CH_3$$
Tristearin
mp 71°C

$$H_2C-OCO(CH_2)_7CH\overset{cis}{=}CH(CH_2)_7CH_3$$
$$HC-OCO(CH_2)_7CH\overset{cis}{=}CH(CH_2)_7CH_3$$
$$H_2C-OCO(CH_2)_7CH\overset{cis}{=}CH(CH_2)_7CH_3$$
triolein
mp-4°C
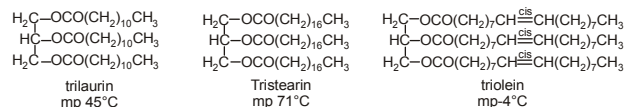
Triglycerides having three identical acyl chains, such as tristearin and triolein (above), are called "simple", while those composed of different acyl chains are called "mixed". If the acyl chains at the end hydroxyl groups (1 & 3) of glycerol are different, the center carbon becomes a chiral center and enantiomeric configurations must be recognized.

The hydrogenation of vegetable oils to produce semisolid products has had unintended consequences. Although the hydrogenation imparts desirable features such as spreadability, texture, "mouth feel," and increased shelf life to naturally liquid vegetable oils, it introduces some serious health problems. These occur when the cis-double bonds in the fatty acid chains are not completely saturated in the hydrogenation process. The catalysts used to effect the addition of hydrogen isomerize the remaining double bonds to their trans configuration. These unnatural **trans-fats** appear to to be associated with increased heart disease, cancer, diabetes and obesity, as well as immune response and reproductive problems.

4. Waxes

Waxes are esters of fatty acids with long chain monohydric alcohols (one hydroxyl group). Natural waxes are often mixtures of such esters, and may also contain hydrocarbons. The formulas for three well known waxes are given below, with the carboxylic acid moiety colored red and the alcohol colored blue.

spermaceti	beeswax	carnuba wax
$CH_3(CH_2)_{14}CO_2^-$	$CH_3(CH_2)_{30}CO_2^-$	$CH_3(CH_2)_{24}CO_2^-$
$(CH_2)_{15}CH_3$	$(CH_2)_{33}CH_3$	$(CH_2)_{29}CH_3$

Waxes are widely distributed in nature. The leaves and fruits of many plants have waxy coatings, which may protect them from dehydration and small predators. The feathers of birds and the fur of some animals have similar coatings which serve as a water repellent. Carnuba wax is valued for its toughness and water resistance.

5. Phospholipids

Phospholipids are the main constituents of cell membranes. They resemble the triglycerides in being ester or amide derivatives of glycerol or sphingosine with fatty acids and phosphoric acid. The phosphate moiety of the resulting phosphatidic acid is further esterified with ethanolamine, choline or serine in the phospholipid itself. The following diagram shows the structures of some of these components. Clicking on the diagram will change it to display structures for two representative phospholipids. Note that the fatty acid components (R & R') may be saturated or unsaturated.

Phospholipid Components
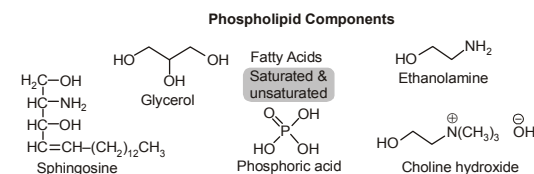
As ionic amphiphiles, phospholipids aggregate or self-assemble when mixed with water, but in a different manner than the soaps and detergents. Because of the two pendant alkyl chains present in phospholipids and the unusual mixed charges in their head groups, micelle formation is unfavorable relative to a bilayer structure. If a phospholipid is smeared over a small hole in a thin piece of plastic immersed in water, a stable planar bilayer of phospholipid molecules is created at the hole. As shown in the following diagram, the polar head groups on the faces of the bilayer contact water, and the hydrophobic alkyl chains form a nonpolar interior. The phospholipid molecules can move about in their half the bilayer, but there is a significant energy barrier preventing migration to the other side of the bilayer.
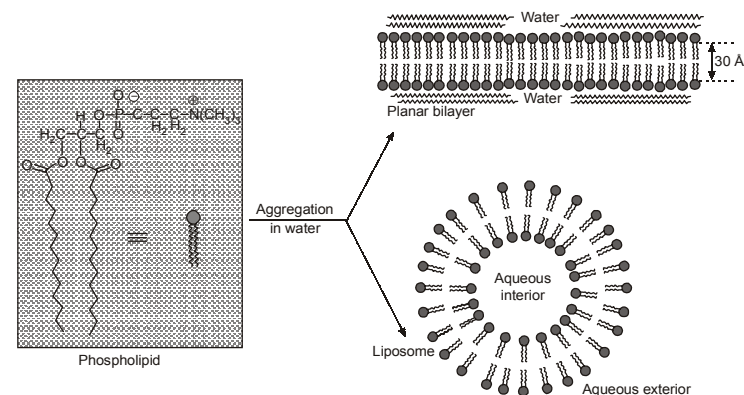
This bilayer membrane structure is also found in aggregate structures called **liposomes**. Liposomes are microscopic vesicles consisting of an aqueous core enclosed in one or more phospholipid layers. They are formed when phospholipids are vigorously mixed with water. Unlike micelles, liposomes have both aqueous interiors and exteriors.

A cell may be considered a very complex liposome. The bilayer membrane that separates the interior of a cell from the surrounding fluids is largely composed of phospholipids, but it incorporates many other components, such as cholesterol, that contribute to its structural integrity. Protein channels that permit the transport of various kinds of chemical species in and out of the cell are also important components of cell membranes. The interior of a cell contains a variety of structures (organelles) that conduct chemical operations vital to the cells existence. Molecules bonded to the surfaces of cells serve to identify specific cells and facilitate interaction with external chemical entities. The sphingomyelins are also membrane lipids. They are the major component of the myelin sheath surrounding nerve fibers. Multiple Sclerosis is a devastating disease in which the myelin sheath is lost, causing eventual paralysis. Prostaglandins Thromboxanes & Leukotrienes. The members of this group of structurally related natural hormones have an extraordinary range of biological effects. They can lower gastric secretions, stimulate uterine contractions, lower blood pressure, influence blood clotting and induce asthma-like allergic responses. Because their genesis in body tissues is tied to the metabolism of the essential fatty acid arachadonic acid (5,8,11,14-eicosatetraenoic acid) they are classified as **eicosanoids**. Many properties of the common drug asprin result from its effect on the cascade of reactions associated with these hormones.

The metabolic pathways by which arachidonic acid is converted to the various eicosanoids are complex and will not be discussed here. A rough outline of some of the transformations that take place is provided below. It is helpful to view arachadonic acid in the coiled conformation shown in the shaded box.
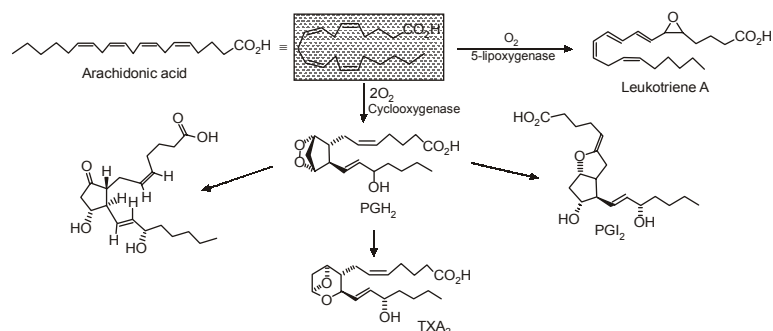
Leukotriene A is a precursor to other leukotriene derivatives by epoxide opening reactions. The prostaglandins are given systematic names that reflect their structure. The initially formed peroxide PGH_2 is a common intermediate to other prostaglandins, as well as thromboxanes such as TXA_2.

Terpenes

Compounds classified as terpenes constitute what is arguably the largest and most diverse class of natural products. A majority of these compounds are found only in plants, but some of the larger and more complex terpenes (e.g. squalene & lanosterol) occur in animals. Terpenes incorporating most of the common functional groups are known, so this does not provide a useful means of classification. Instead, the number and structural organization of carbons is a definitive characteristic. Terpenes may be considered to be made up of isoprene (more accurately isopentane) units, an empirical feature known as the **isoprene rule**. Because of this, terpenes usually have 5n carbon atoms (n is an integer), and are subdivided as follows:

Classification	Isoprene Units	Carbon Atoms
monoterpenes	2	C_{10}
sesquiterpenes	3	C_{15}
diterpenes	4	C_{20}
sesterterpenes	5	C_{25}
triterpenes	6	C_{30}

Isoprene itself, a C_5H_8 gaseous hydrocarbon, is emitted by the leaves of various plants as a natural byproduct of plant metabolism. Next to methane it is the most common volatile organic compound found in the armosphere. Examples of C_{10} and higher terpenes, representing the four most common classes are shown in the following diagram. The initial display is of monoterpenes; larger terpenes will be shown by clicking the "Toggle Structures" button under the diagram. Most terpenes may be structurally dissected into isopentane segments. To see how this is done click directly on the structures in the diagram.
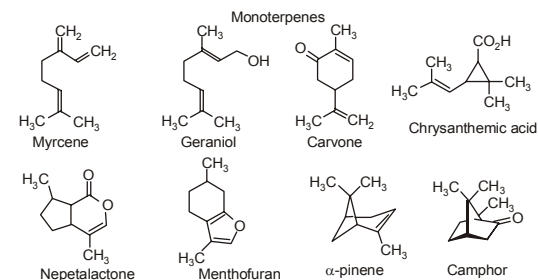
The isopentane units in most of these terpenes are easy to discern, and are defined by the shaded areas. In the case of the monoterpene camphor, the units overlap to such a degree it is easier to distinguish them by coloring the carbon chains. This is also done for alpha-pinene. In the case of the

triterpene lanosterol we see an interesting deviation from the isoprene rule. This thirty carbon compound is clearly a terpene, and four of the six isopentane units can be identified. However, the ten carbons in center of the molecule cannot be disected in this manner. Evidence exists that the two methyl groups circled in magenta and light blue have moved from their original isoprenoid locations (marked by small circles of the same color) to their present location. This rearrangement is described in the biosynthesis section. Similar alkyl group rearrangements account for other terpenes that do not strictly follow the isoprene rule.Polymeric isoprenoid hydrocarbons have also been identified. Rubber is undoubtedly the best known and most widely used compound of this kind. It occurs as a colloidal suspension called latex in a number of plants, ranging from the dandelion to the rubber tree (*Hevea brasiliensis*). Rubber is a polyene, and exhibits all the expected reactions of the C=C function. Bromine, hydrogen chloride and hydrogen all add with a stoichiometry of one molar equivalent per isoprene unit. Ozonolysis of rubber generates a mixture of levulinic acid ($CH_3COCH_2CH_2CO_2H$) and the corresponding aldehyde. Pyrolysis of rubber produces the diene isoprene along with other products.
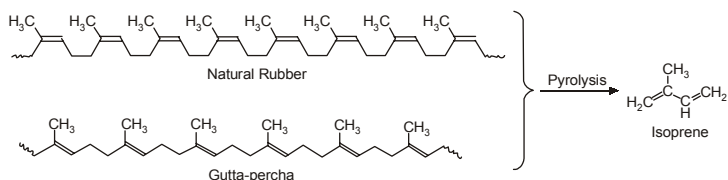
The double bonds in rubber all have a **Z**-configuration, which causes this macromolecule to adopt a kinked or coiled conformation. This is reflected in the physical properties of rubber. Despite its high molecular weight (about one million), crude latex rubber is a soft, sticky, elastic substance. Chemical modification of this material is normal for commercial applications. Gutta-percha (structure above) is a naturally occuring **E**-isomer of rubber. Here the hydrocarbon chains adopt a uniform zig-zag or rod like conformation, which produces a more rigid and tough substance. Uses of gutta-percha include electrical insulation and the covering of golf balls.. Steroids
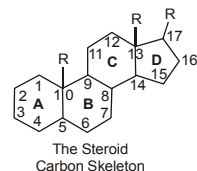
The important class of lipids called **steroids** are actually metabolic derivatives of terpenes, but they are customarily treated as a separate group. Steroids may be recognized by their tetracyclic skeleton, consisting of three fused six-membered and one five-membered ring, as shown in the diagram to the right. The four rings are designated A, B, C & D as noted, and the peculiar numbering of the ring carbon atoms (shown in red) is the result of an earlier misassignment of the structure. The substituents designated by R are often alkyl groups, but may also have functionality. The R group at the A:B ring fusion is most commonly methyl or hydrogen, that at the C:D fusion is usually methyl. The substituent at C-17 varies considerably, and is usually larger than methyl if it is not a functional group. The most common locations of functional groups are C-3, C-4, C-7, C-11, C-12 & C-17. Ring A is sometimes aromatic. Since a number of tetracyclic triterpenes also have this tetracyclic structure, it cannot be considered a unique identifier.

Steroids are widely distributed in animals, where they are associated with a number of physiological processes. Examples of some important steroids are shown in the following diagram. Different kinds of steroids will be displayed by clicking the "Toggle Structures" button under the diagram. Norethindrone is a synthetic steroid, all the other examples occur naturally. A common strategy in pharmaceutical chemistry is to take a natural compound, having certain desired biological properties together with undesired side effects, and to modify its structure to enhance the desired characteristics and diminish the undesired. This is sometimes accomplished by trial and error.The generic steroid structure drawn above has seven chiral stereocenters (carbons 5, 8, 9, 10, 13, 14 & 17), which means that it may have as many as 128 stereoisomers. With the exception of C-5, natural steroids generally have a single common configuration. This is shown in the last of the toggled displays, along with the preferred conformations of the rings.

Typical Animal Steroids
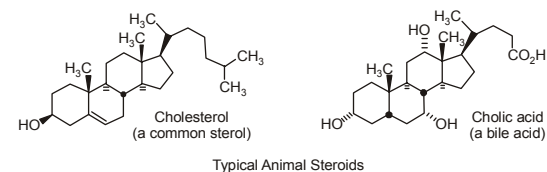
Chemical studies of the steroids were very important to our present understanding of the configurations and conformations of six-membered rings. Substituent groups at different sites on the tetracyclic skeleton will have axial or equatorial orientations that are fixed because of the rigid structure of the trans-fused rings. This fixed orientation influences chemical reactivity, largely due to the greater steric hindrance of axial groups versus their equatorial isomers. Thus an equatorial hydroxyl group is
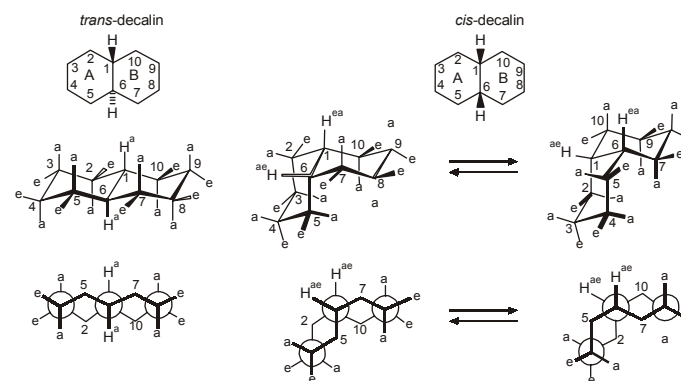
esterified more rapidly than its axial isomer. It is instructive to examine a simple bicyclic system as a model for the fused rings of the steroid molecule. Decalin, short for decahydronaphthalene, exists as cis and trans isomers at the ring fusion carbon atoms. Planar representations of these isomers are drawn at the top of the following diagram, with corresponding conformational formulas displayed underneath. The numbering shown for the ring carbons follows IUPAC rules, and is different from the unusual numbering used for steroids. For purposes of discussion, the left ring is labeled A (colored blue) and the right ring B (colored red). In the conformational drawings the ring fusion and the angular hydrogens are black.

The trans-isomer is the easiest to describe because the fusion of the A & B rings creates a rigid, roughly planar, structure made up of two chair conformations. Each chair is fused to the other by equatorial bonds, leaving the angular hydrogens (H^a) axial to both rings. Note that the bonds directed above the plane of the two rings alternate from axial to equatorial and back if we proceed around the rings from C-1 to C-10 in numerical order. The bonds directed below the rings also alternate in a complementary fashion.

Conformational descriptions of cis- decalin are complicated by the fact that two energetically equivalent fusions of chair cyclohexanes are possible, and are in rapid equilibrium as the rings flip from one chair conformation to the other. In each of these all chair conformations the rings are fused by one axial and one equatorial bond, and the overall structure is bent at the ring fusion. In the conformer on the left, the red ring (B) is attached to the blue ring (A) by an axial bond to C-1 and an equatorial bond to C-6 (these terms refer to ring A substituents). In the conformer on the right, the carbon bond to C-1 is equatorial and the bond to C-6 is axial. Each of the angular hydrogens (H^{ae} or H^{ea}) is oriented axial to one of the rings and equatorial to the other. This relationship reverses when double ring flipping converts one cis-conformer into the other. Cis-decalin is less stable than trans-decalin by about 2.7 kcal/mol (from heats of combustion and heats of isomerization data). This is due to steric crowding (hindrance) of the axial hydrogens in the concave region of both cis-conformers, as may be seen in the Chime display activated by the following button. This difference is roughly three times the energy of a gauche butane conformer relative to its anti conformer. Indeed three gauche butane interactions may be identified in each of the cis-decalin conformations, as will be displayed by clicking on the above conformational diagram. These gauche interactions are also shown in the Chime model. Steroids in which
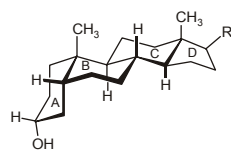
rings A and B are fused cis, such as the example on the right, do not have the same conformational mobility exhibited by cis-decalin. The fusion of ring C to ring B in a trans configuration prevents ring B from undergoing a conformational flip to another chair form. If this were to occur, ring C would have to be attached to ring B by two adjacent axial bonds directed 180º apart. This is too great a distance to be bridged by the four carbon atoms making up ring C. Consequently, the steroid molecule is locked in the all chair conformation shown here. Of course, all these steroids and decalins may have one or more six-membered rings in a boat conformation. However the high energy of boat conformers relative to chairs would make such structures minor components in the overall ensemble of conformations available to these molecules.

Lipid Soluble Vitamins

The essential dietary substances called **vitamins** are commonly classified as "water soluble" or "fat soluble". Water soluble vitamins, such as vitamin C, are rapidly eliminated from the body and their dietary levels need to be relatively high. The recommended daily allotment (RDA) of vitamin C is 100 mg, and amounts as large as 2 to 3 g are taken by many people without adverse effects. The lipid soluble vitamins, shown in the diagram below, are not as easily eliminated and may accumulate to toxic levels if consumed in large quantity. The RDA for these vitamins are:

Vitamin A	800 ìg (upper limit ca. 3000 ìg)
Vitamin D	5 to 10 ìg (upper limit ca. 2000 ìg)
Vitamin E	15 mg (upper limit ca. 1 g)
Vitamin K	110 ìg (upper limit not specified)

From this data it is clear that vitamins A and D, while essential to good health in proper amounts, can be very toxic. Vitamin D, for example, is used as a rat poison, and in equal weight is more than 100 times as poisonous as sodium cyanide.

Lipid soluble Vitamins

Vitamin A $C_{20}H_{30}O$ Part of the visual pigment

Vitamin E $C_{29}H_{50}O_2$ an antioxidant

Vitamin D$_2$ $C_{28}H_{44}O$ Calcium metabolism and bone growth

Vitamin K$_1$ $C_{31}H_{46}O_2$ a blood clotting factor
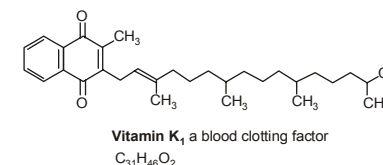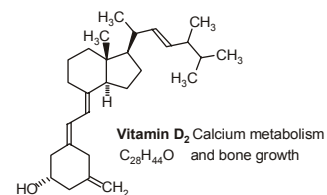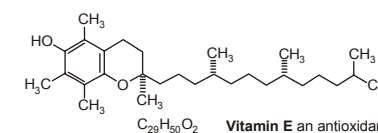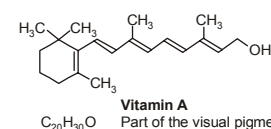
From the structures shown here, it should be clear that these compounds have more than a solubility connection with lipids. Vitamins A is a terpene, and vitamins E and K have long terpene chains attached to an aromatic moiety. The structure of vitamin D can be described as a steroid in which ring B is cut open and the remaining three rings remain unchanged. The precursors of vitamins A and D have been identified as the tetraterpene beta-carotene and the steroid ergosterol, respectively.

Biosynthetic Pathways

The complex organic compounds found in living organisms on this planet originate from photosynthesis, an endothermic reductive condensation of carbon dioxide requiring light energy and the pigment chlorophyll.

$$x\ CO_2\ +\ x\ H_2O\ +\ energy \rightarrow C_xH_{2x}O_x\ +\ x\ O_2$$

The products of photosynthesis are a class of compounds called carbohydrates, the most common and important of which is glucose ($C_6H_{12}O_6$). Subsequent reactions effect an oxidative cleavage of glucose to pyruvic acid (CH_3COCO_2H), and this in turn is transformed to the two-carbon building block, acetate. The multitude of lipid structures described here are constructed from acetate by enzymatic reactions that in many respects correspond to reactions used by chemists for laboratory syntheses of similar compounds. However, an important restriction is that the reagents and conditions must be compatible with the aqueous medium and moderate temperatures found in living cells. Consequently, the condensation, alkylation, oxidation and reduction reactions that accomplish the biosynthesis of lipids will not make use of the very strong bases, alkyl halides, chromate oxidants or metal hydride reducing agents that are employed in laboratory work.

1. Condensations

Claisen condensation of ethyl acetate (or other acetate esters) forms an acetoacetate ester, as illustrated by the top equation in the following diagram. Reduction, dehydration and further reduction of this product would yield an ester of butyric acid, the overall effect being the elongation of the acetate starting material by two carbons. In principle, repetition of this sequence would lead to longer chain acids, made up of an even number of carbon atoms. Since most of the common natural fatty acids have even numbers of carbon atoms, this is an attractive hypothesis for their biosynthesis.

Nature's solution to carrying out a Claisen-like condensation in a living cell is shown in the bottom equation of the diagram. Thioesters are more reactive as acceptor reactants than are ordinary esters, and preliminary conversion of acetate to malonate increases the donor reactivity of this species. The thiol portion of the thioester is usually a protein of some kind, with efficient acetyl transport occuring by way of acetyl coenzyme A. Depending on the enzymes involved, the condensation product may be reduced and then further elongated so as to produce fatty acids (as shown), or elongated by further condensations to polyketone intermediates that are precursors to a variety of natural phenolic compounds. Click on the diagram to see examples of polyketone condensations.

Claisen condensation

R = small alkyl group

Thioester condensation

R = Coenzyme A or a carrier protein

NAD ⊕
NADP⊕

NADH
NADPH
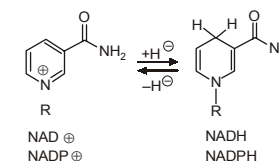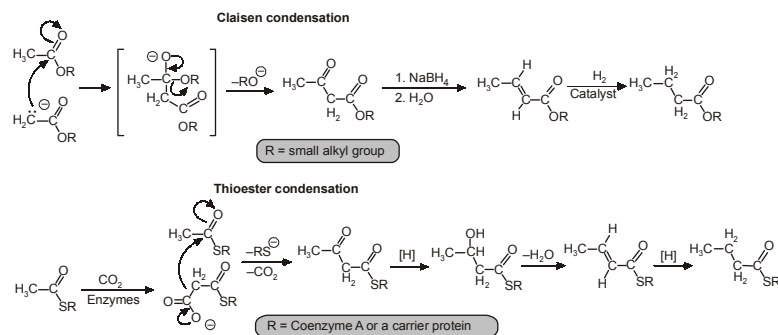
The reduction steps (designated by [H] in the equations) and the intervening dehydrations needed for fatty acid synthesis require unique coenzymes and phosphorylating reagents. The pyridine ring of nicotinamide adenine dinucleotide (NAD) and its 2'-phosphate derivative (NADP) function as hydride acceptors, and the corresponding reduced species (NADH & NADPH) as a hydride donors. Partial structures for these important redox reagents are shown on the right. Full structures may be seen by clicking on the partial formulas.

As noted earlier, the hydroxyl group is a poor anionic leaving group (hydroxide anion is a strong base). Phosphorylation converts a hydroxyl group into a phosphate (PO_4) or pyrophosphate (P_2O_7) ester, making it a much better leaving group (the pK_as at pH near 7 are 7.2 and 6.6 respectively). The chief biological phosphorylation reagents are phosphate derivatives of adenosine (a ribose compound). The strongest of these is the triphosphate ATP, with the diphosphate and monophosphate being less powerful. Formulas for these compounds may be seen by Clicking Here

The overall process of fatty acid synthesis is summarized for palmitic acid, $CH_3(CH_2)_{14}CO_2H$, in the following equation:

8 CH_3CO-CoA + 14 NADPH + 14 $H^{(+)}$ + 7 ATP + H_2O → $CH_3(CH_2)_{14}CO_2H$ + 8 CoA + 14 $NADP^{(+)}$ + 7 ADP + 7 $H_2PO_4^{(-)}$

2. Alkylations

The branched chain and cyclic structures of the terpenes and steroids are constructed by sequential

Pyrophosphate intermediates in terpene synthesis

Enantiotopic CO groups

R-(–) mevalonic acid

Pyrophosphate
$PPO = -O-PO_3-O-PO_3-OH$

Dimethylallyl pyrophosphate

Isopentenyl pyrophosphate

Geranyl Pyrophosphate
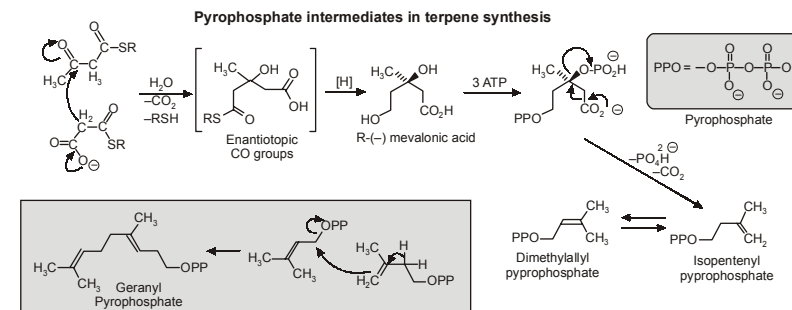
alkylation reactions of unsaturated isopentyl pyrophosphate units. As depicted in the following diagram, these 5-carbon reactants are made from three acetate units by way of an aldol-like addition of a malonate intermediate to acetoacetate. Selective hydrolysis and reduction gives a key intermediate called **mevalonic acid**. Phosphorylation and elimination of mevalonic acid then generate isopenenyl pyrophosphate, which is in equilibrium with its double bond isomer, dimethylallyl pyrophosphate. The allylic pyrophosphate group in the latter compound is reactive in enzymatically catalyzed alkylation reactions, such as the one drawn in the green box. This provides support for the empirical isoprene rule.

The simplest fashion in which isopentane units combine is termed "head-to-tail". This is the combination displayed in the green box, and these terms are further defined in the upper equation that will appear above on clicking the "Toggle Examples" button. Non head-to-tail coupling of isopentane units is also observed, as in the chrysanthemic acid construction shown in the second equation. A second click on the diagram displays the series of cation-like cyclizations and rearrangements that have been identified in the biosynthesis of the triterpene lanosterol. Lanosterol is a precursor in the biosynthesis of steroids. This takes place by metabolic removal of three methyl groups and degradation of the side chain.

3. An Alternative Isoprenoid Synthesis

For many years, the mevalonic acid route to isopentenyl pyrophosphate was considered an exclusive biosynthetic pathway. Recently, an alternative reaction sequence, starting from pyruvic acid and glyceraldehyde-3-phosphate, has been identified (bottom equations in the following diagram). By labeling selective carbon atoms (colored red) these distinct paths are easily distinguished. The new, **DXP** (1-deoxyxylulose-5-phosphate) path is widespread in microorganisms and chloroplast terpenes. The rearrangement to 2-methylerythritol-4-phosphate is an extraordinary transformation.

Classification of Lipids
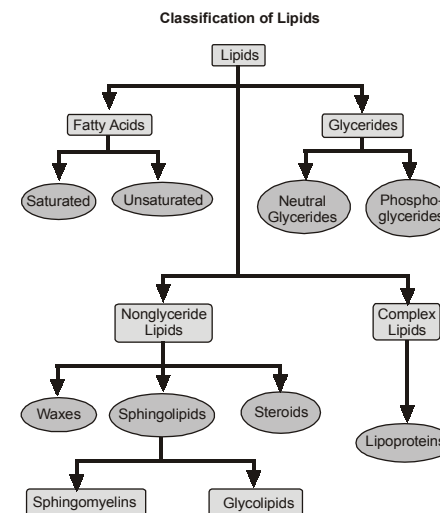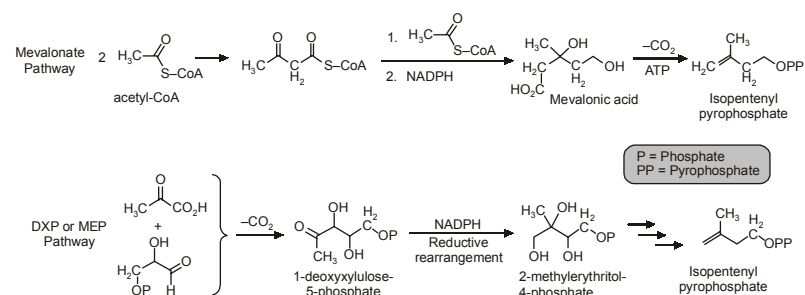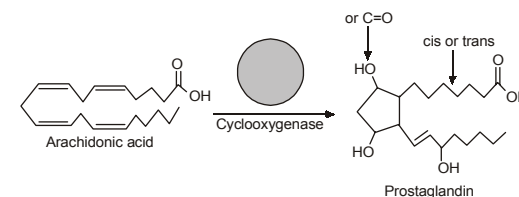
Prostaglandins

Prostalandins are hormones.
They transmit signals within cells and between cells.
Local activity only.
All cells make prostaglandins.
Different classes made in different tissues.
Action is determined by class produced.
Many different functions.

Blood Clotting
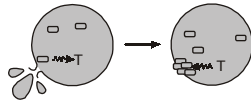
Thromboxin A (T) causes platelet aggregation → clot.

Prostacyclin (PGI2) inhibits aggregation → no clot.

Prostaglandins

Inflammatory Response and Aspirin

Arachidonic acid

Cyclooxygenase

Prostaglandin

Asprin

Archidonic acid

Cyclooxygenase

X

Enzyme is acetylated ⟶ inactivation
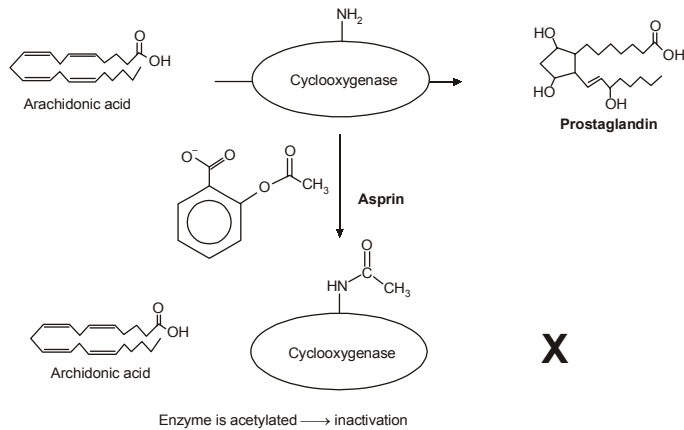
But ... Prostaglndins also
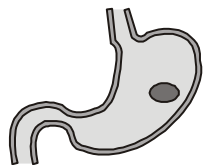
— inhibit stomach acid production

— increse mucous production

So trade headache for ulcers!

Sphinogomyelin

Fatty acid

Receptors

Axon Terminal

Myelin sheath

Cell body
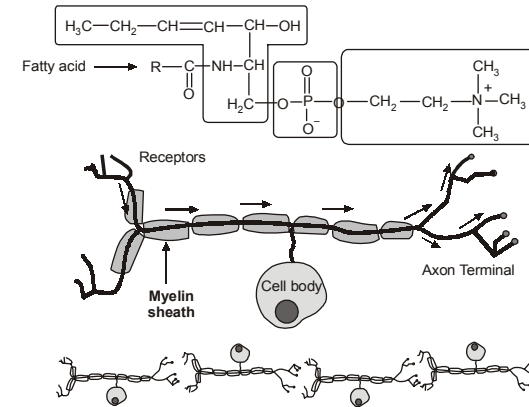
Myelin increases the speed of nerve impulse conduction & protects axon.

Development continues in infant ...

reason for less coordination and delayed responses.

Disorders

Niemann-Pick Disease:

No Sphingomyelinase (breaks sphingomyelin down)

accumulates incells (brain, bone marrow, liver)

Lack of motor skills,muscle strength and tone

Lose vision and hearing

Death within a few years

Multiple Sclerosis:

Believed to be anautoimmune response

Antibodies attack myelin around nerves

Different levels of severity

Lipoproteins

Chylomicron
triglycerides
from intestine to cells
for use

VLDL
triglycerides
from liver to cells for
storage

LDL
Cholesterol
to cells for use
Most cholesterol

HDL
Cholesterol
from cells to liver
for disposal

Cholesterol Regulation

Low cholesterol in cell
increased receptor production

Lysosome

ER

LDL absorbed by cells
Cholesterol turns receptor
generation off

ER

Cell has plenty of stored cholestrol
LDL stays in blood

ER

Arteriosclerosis

Normal
Artery

High
LDL

LDL loadges
into artery wall lining

LDL enters
lining

Cholesterol release
and oxidized

White blood cells
respond

White blood cells
consume cholesterol
forming plaque,
narrow artery

Plaque can rupture
through
artery wall lining

Causing clots
artery blockage

**SUB-SECTION 2.4—A CLASSIFICATION OF LIPIDS
(CHEMICAL STRUCTURE OF SIMPLE LIPIDS)**

Triacylglycerols: Nearly all the commercially important fats and oils of animal and plant origin consist almost exclusively of the simple lipid class triacylglycerols (termed "triglycerides" in the older literature). They consist of a glycerol moiety with each hydroxyl group esterified to a fatty acid. In nature, they

are synthesised by enzyme systems, which determine that a centre of asymmetry is created about carbon-2 of the glycerol backbone, so they exist in enantiomeric forms, i.e. with different fatty acids in each position.

Fischer projection of a
triacyl-*sn*-glycerol

1,2-dihexadecanoyl-3-(9z-octadeceryl)-*sn*-glycerol
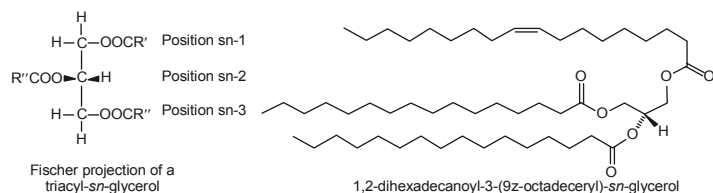
A stereospecific numbering system has been recommended to describe these forms. In a Fischer projection of a natural L-glycerol derivative, the secondary hydroxyl group is shown to the left of C-2; the carbon atom above this then becomes C-1 and that below is C-3. The prefix "*sn*" is placed before the stem name of the compound, when the stereochemistry is defined. Their primary biological function is to serve as a store of energy. As an example, the single molecular species 1,2-dihexadecanoyl-3-(9Z-octadecenoyl)-*sn*-glycerol is illustrated.

Nearly all the commercially important fats and oils of animal and plant origin consist almost exclusively of the simple lipid class - **triacylglycerols**. This includes all the vegetable oils, such as those from corn (maize), olive, palm, and sunflower, and animal fats, such as tallow, lard and butter, as well as commercial products such as margarines. Most of these are depots fats (from adipose tissue) or milk fats, where their main function may be as a store of energy, but some triacylglycerols (e.g. those of plasma or liver) may have a more dynamic role. Similarly, seed oils serve as a source of energy and structural fatty acids for the developing embryo.

In chemical terms, triacylglycerols consist of the trihydric alcohol glycerol esterified, almost invariably, with long-chain fatty acids. When the two primary hydroxyl groups are esterified with different fatty acids, the resulting triacylglycerol can be asymmetric and thus can display "optical activity", although this is usually too low to be measured. The conventional D/L or R/S systems could designate such enantiomers without ambiguity with simple molecules, but problems arise in application to the complex mixtures of triacylglycerols found in nature. Such problems can be avoided if the stereochemistry of triacylglycerols and other glycerolipids is described by the "stereospecific numbering" (*sn*) system as recommended by a IUPAC-IUB commission (see also our web page on Nomenclature).

Fischer projection of a
triacyl-*sn*-glycerol

1,2-dihexadecanoyl-3-(9z-octadeceryl)-*sn*-glycerol
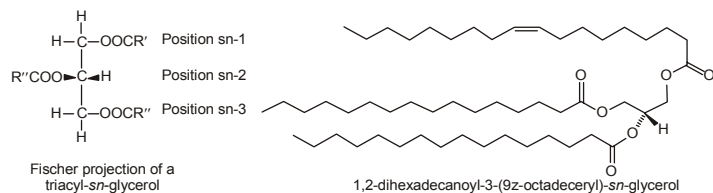
In a Fischer projection of a natural L-glycerol derivative as shown above, the secondary hydroxyl group is shown to the left of C-2; the carbon atom above this then becomes C-1 while that below becomes C-3, and the prefix *sn* is placed before the stem name of the compound. The term "triacyl-*sn*-glycerol" should then be used to designate the molecule rather than "triglyceride". The former is technically more accurate and is essential for the stereospecific numbering system, while the latter term may only be familiar now to older readers. When the detailed stereochemistry is not specified, the primary hydroxy groups are often termed the á- and á'-positions and the secondary, the â-position. As an example, the single molecular species 1,2-dihexadecanoyl-3-(9Z-octadecenoyl)-*sn*-glycerol is illustrated.

Differences in the distributions of fatty acids among the positions of the glycerol moiety in triacylglycerols from natural fats and oils were first demonstrated systematically by enzymatic hydrolysis procedures, specifically pancreatic lipase hydrolysis for the analysis of the fatty acids of position *sn*-2, before complex stereospecific hydrolysis procedures were developed that permitted the complete positional distributions of the fatty acids to be determined. Because of this historical development of the analytical procedures, there has been a tendency to assume that the composition of fatty acids esterified to the sole secondary hydroxyl group must have greater importance than those of the two primary positions. It is certainly true that the composition of position *sn*-2 is of great importance when triacylglycerols are consumed and digested by animals, since 2-monoacyl-*sn*-glycerols are then formed which can be absorbed by the intestines and utilised as such. On the other hand, the results of stereospecific analyses have shown that the compositions of all three positions in certain fats can be distinctive and can highlight important aspects of the biosynthetic processes.

The discussion that follows centres on the fatty acid compositions and the stereospecific distribution of fatty acids within triacyl-*sn*-glycerols with a few selected examples. Note that all data are presented as mol% not weight %. The second document here dealing with triacylglycerols (Triacylglycerols. Part 2) discusses the biochemistry of triacylglycerols and methods of analysis.

2. Triacylglycerols from Seed Oils

While seed and fruit oils within the plant kingdom as a whole can contain a vast range of different fatty acids, those that are most widely for food purposes tend to have relatively simple compositions in which C18 unsaturated fatty acids, especially oleate and linoleate are predominant. Thus, olive oils contains over 70% of oleic acid, while safflower oil can contain a similar proportion of linoleate, which is also the major component of maize (corn) oil and soybean oils. Linseed oil is used mainly for industrial purposes as it contains about 60% á-linolenic acid, which is not suitable for food purposes as it oxidizes too readily. Soybean oil, with 3 to 7% linolenic acid, is often hydrogenated before it is used as a food ingredient. Rapeseed oil is available in two forms, i.e. with high or low erucic acid contents. The former has many industrial uses, but is banned from foods because of fears about the safety of erucic acid for human consumption. Castor oil, with mainly industrial applications, contains up to 90% ricinoleic acid (see our web pages on hydroxy fatty acids). The so-called 'tropical oils', such as palm oil, contain higher amounts of saturated fatty acids than most other commercial oils. Palm kernel and coconut oils are rich sources of medium-chain fatty acids.

A considerable amount of data has been obtained by means of pancreatic lipase hydrolysis on the composition of position *sn*-2 of the triacyl-*sn*-glycerols of seed oils, and this has been enhanced in

recent years by stereospecific analyses. The author has of necessity been selective in presenting results, and some representative analyses are listed in Table 1.

Table 2.1 : Positional distributions of fatty acids (mol %) in triacyl-*sn*-glycerols of seed oils

Oil	Position	Fatty acid					
		16:0	18:0	18:1	18:2	18:3	C$_{20}$-C$_{24}$
Peanut	TG	9	3	58	23		7
	1	14	5	59	19		4
	2	2	tr	59	39		1
	3	11	5	57	10		15
Rapeseed*	TG	3	2	26	17	10	43
	1	4	2	23	11		53
	2	1		37	36	6	6
	3	4	3	17	4	20	70
Soyabean	TG	9	4	24	54	8	
	1	14	6	23	48	9	
	2	1	tr	22	70	7	
	3	13	6	28	45	9	
Linseed	TG	6	4	16	17	57	
	1	10	6	15	16	53	
	2	2	1	16	21	60	
	3	6	4	17	13	59	
Maize (corn)	TG	11	2	29	57	1	
	1	1	3	28	50	1	
	2	18	tr	27	70	1	
	3	2	3	31	52	1	
Olive	TG	10	2	76	10	1	
	1	13	3	72	10	1	
	2	1		83	14	1	
	3	17	4	74	5		
Cacao butter	TG	24	35	36	3	tr	1
	1	34	50	12	1	1	1
	2	2	2	87	9		
	3	37	53	9	tr		2
Palm	TG	48	4	36	10		
	1	60	3	27	9		
	2	13	tr	68	18		
	3	72	8	14	3		

tr = trace (<0.5%).   * High erucic acid rapeseed oil.   TG = intact triacylglycerols
Data from - Brockerhoff, H. and Yurkowski, M., *J. Lipid Res.*, 7, 62-64 (1966). Christie, W.W. *et al., Lipids*, 68, 695-701 (1991).

As was well known from studies involving hydrolysis with pancreatic lipase, position sn-2 of the triacylglycerols of seed oils is greatly enriched in polyunsaturated fatty acids (specifically linoleic and linolenic acids), while saturated fatty acids are concentrated in the primary positions, and monoenoic acids are relatively evenly distributed. There are exceptions to these rules and in cacao butter, for example, oleic acid is present largely in position sn-2. Minor differences only in the distributions of saturated and monoenoic fatty acids between positions sn-1 and sn-3 have been observed and too few samples have been analysed for definitive comment. Longer-chain fatty acids (C20-C24) are apparently concentrated in the primary positions with some small preference for position sn-3.

In those seed oils containing unusual fatty acids that have been subjected to stereospecific analysis, an allenic estolide was found entirely in position *sn*-3 in the triacyl-*sn*-glycerols of *Sapium sebiferum*, acetic acid appeared to be linked entirely to position *sn*-3 in *Euonymus verrucosus*, and much of the coriolic acid was in position *sn*-3 in *Monnina emarginata*.

In summary, seed oils containing the usual range of saturated and unsaturated fatty acids tend to have the polyunsaturated components in position *sn*-2, but relatively little difference between the primary positions; less-common fatty acids tend to be concentrated in position *sn*-3.

3. Triacylglycerols from Animal Fats—Adipose Tissue

Much of the triacylglycerols in animal tissues, including some of the commercially important fats such as lard or tallow, are contained within various adipose tissue sites, where they serve mainly as an energy store. The subcutaneous fats also help to insulate animals, while fat stores in fish and marine animals help to maintain buoyancy. Fatty acids are synthesised *de novo* in tissues (saturated and monoenoic acids), but the composition of the triacylglycerols also reflects the diet. In terrestrial animals, the composition tends to be quite simple, with C$_{16}$ (mainly 16:0) and C$_{18}$ fatty acids predominating. All the essential fatty acids, such as linoleic and á-linolenic acid, must come from the diet of course. Ruminant animals, such as the cow and sheep, have relatively saturated fats because the dietary unsaturated fatty acids are subjected to biohydrogenation in the rumen, a process that also generates ***trans*** fatty acids as by-products. These animals also tend to have relatively higher concentrations of odd- and branched-chain fatty acids from the rumen microflora. Non-ruminant herbivores, such as the horse, can have appreciable amounts of linolenic acid from grass in their adipose tissue, while that of marine mammals is characterized by high concentrations of long-chain mono- and polyenoic fatty acids because of their diet of fish.

Marked differences have been observed in the distributions of fatty acids among the three positions of the glycerol moiety in most species examined. There are also appreciable inter-species differences. A few representative results are listed in Table 2.

Data from – Christie, W.W. *et al.*, *Lipids*, **6**, 854-856 (1971); Brockerhoff, H. *et al., Biochim. Biophys. Acta*, **116**, 67-72 (1966); Christie, W.W. and Moore, J.H. *J. Sci. Food. Agric.*, **22**, 120-124(1971), *Biochim. Biophys. Acta*, **210**, 46-56 (1970), *J. Sci. Food. Agric.*, **23**, 73-77 (1972).

For most species, saturated fatty acids are found predominantly in position *sn*-1, although appreciable amounts of oleic acid are usually present also. Position *sn*-2 tends to contain mainly unsaturated fatty acids, especially linoleic acid, although some of the shorter-chain fatty acids also accumulate here in

Table 2.2 : Positional distributions of fatty acids (mol %)
in triacyl-*sn*-glycerols of animal depot fats

Species	Position	Fatty acid						
		14:0	16:0	16:1	18:0	18:1	18:2	18:3
Human	TG	5	24	7	8	46	7	1
	1	4	42	3	15	27	6	1
	2	6	10	12	2	55	4	2
	3	4	19	6	6	57	11	1
Cattle	TG	5	27	6	17	33	5	1
	1	4	41	6	17	20	4	1
	2	9	17	6	9	41	5	1
	3	1	22	6	24	37	5	1
Sheep[a]	TG	3	22	2	35	36	2	
	1	1	35	2	47	4	-	
	2	4	14	2	15	52	5	
	3	3	16	1	42	26	2	
Pig	TG	2	27	3	13	45	9	
	1	1	10	2	30	51	6	
	2	4	72	5	2	13	3	
	3	-	tr	2	7	70	18	
Rat	TG	2	23	5	6	35	26	1
	1	2	32	5	9	32	15	1
	2	1	10	4	1	37	45	1
	3	2	27	5	7	37	17	1
Rabbit	TG	3	28	9	3	29	20	4
	1	3	34	9	6	25	14	2
	2	6	25	12	1	26	23	5
	3	1	24	7	3	35	22	5
Chicken	TG	1	30	6	6	45	11	1
	1	1	47	7	8	31	5	1
	2	tr	13	5	6	55	19	1
	3	1	31	7	3	49	8	1

[a] Results are listed for *cis*-18:1 isomers only; *trans*-18:1 was present in positions *sn*-1, *sn*-2 and *sn*-3 as 5, 2 and 6 %, respectively.
tr = trace (<0.5%).   TG = intact triacylglycerols

some instances. There is some preference for the longer-chain fatty acids to be located in position *sn*-3. The main exception to these rules is in the pig and related species where it has been known for many years that palmitic acid can comprise more than 70% of the fatty acids in position *sn*-2. However, it is less well known though perhaps of equal interest that most of the stearic acid (75% of that in the tissue) is in position *sn*-1, while position *sn*-3 contains more than 70% of oleic acid. Although the absolute fatty acid compositions of adipose tissue at various sites in an animal can vary somewhat (subcutaneous fats tend to contain more unsaturated fatty acids than internal depot fats), the proportionate distributions of each fatty acid among the three positions does not vary significantly in any of these tissues in either in the pig or the sheep.

A variety of dietary factors can influence the fatty acid compositions and thence the structures of depot fat triacylglycerols, not least the composition of the diet. Few systematic studies of the relationship between triacylglycerol composition and structure have been performed, and in the example below the pig was the experimental animal. An example of the kind of result obtained is shown in Figure 26.
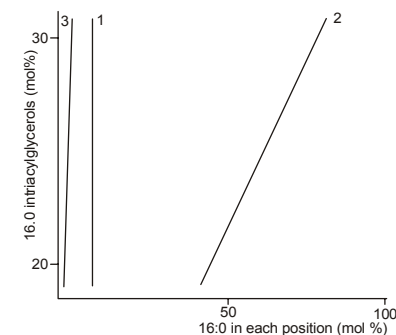
Fig. 2.10 : Variation of the composition of palmitic acid in position *sn*-1, *sn*-2 and *sn*-3 of the triacylglycerols of pig adipose tissue with changes in overall composition (Christie, W.W. and Moore, J.H. *Lipids*, 5, 921-928 (1970)).

As the proportion of palmitic acid in the triacylglycerols varied between 18 and 33%, the amount in position *sn*-1 remained constant at about 10%, that in position *sn*-3 increased linearly but relatively slowly from 2 to 4%, while that in position *sn*-2 was affected most and increased rapidly from less than 50% to more than 70%. Over the range of compositions studied, the amounts of all of the fatty acids varied in a characteristic manner that was linear in each of the three positions.

4. Triacylglycerols from Animal Fats – Milk Fat

Milk fats are the only animal fat designed by nature to serve as a food. The range of fatty acids is more extensive than in adipose tissue with often a higher proportion of short- and medium-chain fatty acids, which are not used as such for structural purposes but provide a rapid source of energy. Ruminants,

such as the cow, have a range of saturated fatty acids from butyric upwards (indeed even acetate has been found in esterified form), and there are relatively low amounts only of polyunsaturated fatty acids because of biohydrogenation in the rumen. In other species, the compositions are less extreme. In human milk, for example, 12:0 and 14:0 fatty acids are more abundant than in other tissues, but the linoleic acid concentration varies between 10 and 20% depending on diet.

Severe technical problems were encountered in the stereospecific analysis of milk triacyl-*sn*-glycerols from ruminants, because of the presence of short-chain fatty acids, which give rise to difficulties in the isolation of the required partially hydrolysed intermediates. Breckenridge, Kuksis *et al*. (see Table 3) overcame the problem by isolating fractions enriched in either long-chain or short-chain components by means of molecular distillation or thin-layer chromatography, and subjecting these separately to stereospecific analysis, recombining the results at the end of the procedure. These showed unequivocally that cows' milk is one of the most asymmetric of animal fats, containing all the butyric acid and most of the hexanoic acid in position *sn*-3. Some representative results for the cow and other species are listed in Table 3.

Table 2.3 : The composition of the fatty acids esterified to each position of the triacyl-*sn*-glycerols in the milk fats of various species

Species	Position	Fatty acid											
		4:0	6:0	8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3
Cow	TG	12	5	2	4	4	11	24	2	7	24	3	
	1	-	1	1	2	5	10	34	2	10	30	2	
	2	-	1	1	3	6	18	32	4	10	19	4	
	3	35	13	4	6	1	6	5	1	1	23	2	
Human	TG				tr	3	26	27	6	7	36	11	1
	1				tr	1	3	16	4	15	46	11	tr
	2				tr	2	7	58	5	3	13	7	1
	3				1	6	7	6	8	2	50	15	1
Rat	TG			6	19	14	12	21	2	3	13	10	1
	1			3	10	10	10	20	2	5	24	14	1
	2			6	20	16	18	29	2	1	3	5	1
	3			10	26	15	9	13	2	2	12	12	1
Pig	TG						4	32	9	5	39	10	1
	1						2	22	7	7	50	11	1
	2						7	58	11	1	15	8	1
	3						4	15	10	6	52	12	2

tr = trace (< 0.5 %).   TG = intact triacylglycerols.

Data from - Christie, W.W. and Moore, J.H. *Biochim. Biophys. Acta*, **210**, 46-56 (1970); Christie, W.W. *J. Dairy Res.*, **52**, 219-222 (1985); Christie, W.W. and Clapperton, J.L. *J. Soc. Dairy Technol.*, **35**, 22-24 (1982); Breckenridge, W.C. e*t al., Canad. J. Biochem.*, **47**, 761-769 (1969).

The pig and sheep have probably been most studied. With the latter in the liver triacylglycerols, for example, the stearic acid is distributed approximately equally between the three positions, and the oleic acid is present in high concentrations in positions *sn*-2 and *sn*-3, thus differing from the depot fats (Table 2). The distinctive feature of the sheep plasma triacylglycerols is a high concentration of palmitic acid in position *sn*-2, and in this they resemble those of the lymph, from which they are derived biosynthetically. In most other tissues of the sheep, the triacylglycerols resemble those of adipose tissue. However, in adrenal glands from which adhering adipose tissue had been carefully removed, triacylglycerols containing a high proportion of palmitic acid in position *sn*-2 were again found, and with long-chain polyunsaturated fatty acids (not listed) in positions *sn*-2 and *sn*-3. It is possible that triacylglycerols of this kind are more widespread than has been thought and that further research will reveal more examples.

Other distinctive triacylglycerols of animal origin are found in the tissues of the chicken. In the plasma triacylglycerols, for example, palmitic acid comprises over 70% of the fatty acids of position *sn*-1 with relatively small amounts in positions *sn*-2 and *sn*-3, while oleic acid comprised 60% of the fatty acids in position *sn*-1 and more than 70% of that in position *sn*-3. Virtually identical structures were found in the ovarian follicles and in the egg, suggesting a common biosynthetic origin. It is important to recognize that lipid structural analyses can rarely be used to prove the existence of particular biosynthetic pathways, but they can provide valuable pointers to the biochemist to potentially productive experimental approaches.

5. Triacylglycerols from Animal Fats – Other Tissues

The fatty acid compositions of triacyl-sn-glycerols animal tissues other than adipose tissue tend to resemble those of the latter, but the stereospecific distributions can differ. Adrenal tissues can contain longer-chain polyunsaturated fatty acids (10% or more). Such data are often of most value to the biochemist. Some representative analyses are listed in Table 4.

The pig and sheep have probably been most studied. With the latter in the liver triacylglycerols, for example, the stearic acid is distributed approximately equally between the three positions, and the oleic acid is present in high concentrations in positions *sn*-2 and *sn*-3, thus differing from the depot fats (Table 2). The distinctive feature of the sheep plasma triacylglycerols is a high concentration of palmitic acid in position *sn*-2, and in this they resemble those of the lymph, from which they are derived biosynthetically. In most other tissues of the sheep, the triacylglycerols resemble those of adipose tissue. However, in adrenal glands from which adhering adipose tissue had been carefully removed, triacylglycerols containing a high proportion of palmitic acid in position *sn*-2 were again found, and with long-chain polyunsaturated fatty acids (not listed) in positions *sn*-2 and *sn*-3. It is possible that triacylglycerols of this kind are more widespread than has been thought and that further research will reveal more examples.

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Table 2.4 : Positional distributions of fatty acids (mol %) in triacyl-*sn*-glycerols from animal tissues other than depot fats and milk

Source	Position	Fatty acid				
		16:0	16:1	18:0	18:1	18:2
Sheep liver[a]	TG	26	3	16	43	5
	1	47	2	17	21	1
	2	14	3	18	55	8
	3	16	3	13	54	5
Sheep plasma	TG	28	2	22	31	2
	1	34	2	33	21	2
	2	38	3	6	33	1
	3	11	2	28	40	2
Sheep adrenals	TG	23	2	16	32	3
	1	33	2	31	28	1
	2	41	4	4	26	3
	3	4	1	15	48	4
Chicken plasma	TG	29	5	5	50	11
	1	74	6	4	13	1
	2	4	3	2	60	31
	3	8	5	8	76	2
Chicken egg	TG	29	5	7	49	10
	1	71	5	4	17	2
	2	4	3	3	63	26
	3	12	6	14	67	1

[a] sheep tissues contain appreciable amounts of minor components not listed here (e.g. odd-and branched-chain, etc.).

TG = intact triacylglycerols.

Data from - Christie, W.W. and Noble, R.C. *J. Sci. Food. Agric.*, **35**, 617-624 (1984); Christie, W.W. and Moore, J.H. *J. Sci. Food. Agric.*, **22**, 120-124 (1971), *Comp. Biochem. Physiol.*, **41B**, 287-295 (1972), *Biochim. Biophys. Acta*, **218**, 83-88 (1970).

important to recognize that lipid structural analyses can rarely be used to prove the existence of particular biosynthetic pathways, but they can provide valuable pointers to the biochemist to potentially productive experimental approaches.

6. Triacylglycerols from Fish Oils

The fatty acid compositions of triacylglycerols of fish oils reflect their diet and usually comprise high concentrations of long chain monoenoic and polyunsaturated fatty acids, especially those of the *omega*-3 biosynthetic family. The triacyl-*sn*-glycerols in depot fats from a number of fish and other marine animals have been subjected to stereospecific analysis, and some typical results are listed in Table.

Table 2.5 : Positional distributions of fatty acids (mol %) in triacyl-*sn*-glycerols of fish oils

Species	Position	Fatty acid										
		14:0	16:0	16:1	18:0	18:1	18:2	20:1	22:1	20:5	22:5	22:6
Herring	TG	7	12	9	1	11	2	17	23	9	2	5
	1	6	12	13	1	16	3	25	14	3	1	1
	2	10	17	10	1	10	3	6	5	18	3	13
	3	4	7	5	1	8	1	20	50	4	1	1
Mackerel	TG	6	14	7	2	17	2	11	16	9	2	9
	1	6	15	11	3	21	2	8	18	5	1	2
	2	10	21	6	1	9	1	5	5	12	3	20
	3	2	5	4	2	21	2	19	24	10	1	5
Skate	TG	2	13	8	2	23	1	13	9	7	3	18
	1	2	19	12	5	30	1	12	8	4	1	5
	2	3	15	7	1	9	1	8	5	6	7	37
	3	1	6	6	1	28	2	19	11	11	2	11
Cod	TG	6	13	13	3	20	2	12	6	12	2	9
	1	6	15	14	6	28	2	12	6	2	1	1
	2	8	16	12	1	9	2	7	5	12	3	20
	3	4	7	14	1	23	2	17	7	13	1	6

TG = intact triacylglycerols.

Data from Brockerhoff, H., Hoyle, R.J., Hwang ,P.C. and Litchfeld, C. *Lipids*, **3**, 24-29 (1968).

Myristic, palmitic and palmitoleic acids are preferentially esterified to positions *sn*-1 and *sn*-3. Oleic and longer-chain monoenoic fatty acids are located mainly in the primary positions, with a tendency for a higher proportion to be in position *sn*-3 as the chain-length increases. Polyunsaturated fatty acids are in greatest concentration in position *sn*-2 with substantial amounts also being found in position *sn*-3. There are significant differences in triacylglycerol structure between those of fish and of marine animals that consume fish.

7. Triacylglycerols of Bacteria and Yeast

Triacylglycerols are fairly widespread in eukaryotic organisms such as yeasts, moulds and fungi, but among the prokaryotes only species from the actinomycetes group, including some of the human mycobacterial pathogens, accumulate triacylglycerols to a significant extent. They occur as cytoplasmic inclusions or lipid droplets within the organisms. As in eukaryotes, triacylglycerols appear to function as a reserve of fatty acyl groups. Those from a few species only have been subjected to stereospecific analyses, and the fatty acid compositions of each are very different, so no general conclusions can be drawn. Three sets of data are listed in Table below.

Table 2.6 : Positional distributions of fatty acids (mol %) in triacyl-*sn*-glycerols of bacteria and yeast

Species	Position	Fatty acid							
		14:0	16:0	16:1	17:0	18:0	18:1	18:2	Other
M. smegmatis	TG	3	24	10	1	10	29		22
	1	1	8	9	tr	7	60		10
	2	7	57	13	2	6	9		4
	3	1	7	7	tr	16	18		51
R. opacus	TG	4	26	10	13	3	22	-	22
	1	12	18	7	12	6	24	-	20
	2	11	59	tr	18	tr	tr	-	12
	3	-	-	21	7	5	42	-	34
L. lipoferus	TG	3	15	8		4	62	8	
	1	3	14	8		4	61	10	
	2	-	1	2		-	88	9	
	3	6	29	13		9	37	6	

tr = trace (<0.5%).   TG = intact triacylglycerols.   Other = branched, odd- or longer-chain.
Data from - Walker, R.W. *et al. Lipids*, **5**, 684-691 (1970); Wältermann, M. *et al. Microbiology*, **146**, 1143-1149 (2000); Haley, J.E. and Jack, R.C. *Lipids*, **9**, 679-681 (1974).

In the triacyl-*sn*-glycerols of *Mycobacterium smegmatis*, oleic acid is the main component of position *sn*-1, palmitic acid is the main component of position *sn*-2, while C_{18} and longer-chain fatty acids are the principal constituents of position *sn*-3; indeed 90% of the 24:0 is in position *sn*-3. Although the fatty acid composition of *Rhodococcus opacus* is very different from this, the distribution of fatty acids is somewhat similar in that saturated and shorter-chain fatty acids are concentrated in position *sn*-2. In the triacylglycerols of the yeast *Lipomyces lipoferus*, oleic acid is the main fatty acid in positions *sn*-1 and *sn*-2, especially, while much of the palmitic, palmitoleic and stearic acids are found in position *sn*-3.

A further document on this site dealing with triacylglycerols (Triacylglycerols. Part 2) discusses the biochemistry and metabolism of triacylglycerols.

CH₂—OOCR′
R″COO–CH
CH₂OH
1,2-/2,3-diacylglycerol
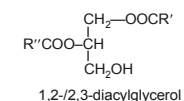
Diacylglycerols (less accurately termed "diglycerides") and monoacylglycerols (monoglycerides) contain two moles and one mole of fatty acids per mole of glycerol, respectively, and exist in various isomeric forms. They are sometimes termed collectively "partial glycerides". Although they are rarely present at greater than trace levels in fresh animal and plant tissues, synthetic materials have importance in commerce. However, 1,2-diacyl-*sn*-glycerols are key intermediates in the biosynthesis of triacylglycerols and other lipids, and they are vital cellular messengers, generated on hydrolysis of phosphatidylinositol and related lipids by a specific phospholipase C **Diacylglycerols as Components of Oils and Fats.**

Diacylglycerols (or "diglycerides") are esters of the trihydric alcohol glycerol in which two of the hydroxyl groups are esterified with long-chain fatty acids. They can exist in three stereochemical forms (see our web document on Triacylglycerols for a discussion of nomenclature).

CH₂OOCR   CH₂OH   CH₂OOCR
R′COO—C—H   R′COO—C—H   HO—C—H
CH₂OH   CH₂OOCR   CH₂OOOR′
sn-1,2-diacylglycerol   *sn*-2,3-diacylglycerol   *sn*-1,3-diacylglycerol
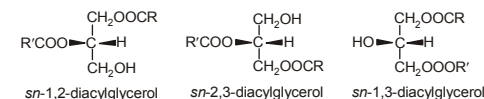
A racemic mixture of *sn*-1,2- and 2,3-diacylglycerols are sometimes termed á,â-diacylglycerols, while *sn*-1,3-diacylglycerols may be designated á,á′-diacylglycerols.

á,â-Diacylglycerols are formed as intermediates in the hydrolysis of triacylglycerols by pancreatic lipase and other hydrolytic enzymes in animal tissues, and they are generated in seed oils by the action of plant lipases. They are important technologically in commercial seed oils, as small amounts can have a profound influence on the physical properties.

Recently, edible oils consisting of 80% 1,3-diacylglycerols have been marketed in Japan as nutritional supplements. It is claimed that they are metabolized in a different way from triacylglycerols with beneficial nutritional effects. The 1(3)-monoacylglycerols formed when they are digested are absorbed into tissues relatively poorly, apparently limiting the accumulation of fats in body tissues.

It should be noted that it is easy to generate diacylglycerols artefactually on storing and extracting tissues if inappropriate methods are used. Often, attempts are made to analyse 1,2-/2,3- and 1,3-diacylglycerols separately, but the data may not be meaningful as acyl migration occurs rapidly until an equilibrium mixture is formed that contains about 67% of the 1,3-isomer. Diacylglycerols will isomerize slowly on standing in inert solvents or in the dry state even at low temperatures.

Diacylglycerols can be recovered from tissues with minimal isomerization, if this is necessary, by extracting the tissues with non-alcoholic solvents such as diethyl ether or chloroform, taking care not to heat extracts at any stage. When pure positional isomers are required, it is necessary to chromatograph

the partial glycerides on TLC plates coated with silica gel G impregnated with boric acid at a level of 10% of the adsorbent), using a solvent system of chloroform (alcohol-free)-acetone (96:4, v/v).

Routine determination of molecular species of diacylglycerols in oils and fats can be accomplished by various chromatographic methods of which high-temperature GC seems most appropriate, since information on the composition as well as the absolute amount is obtained in this way.

2. *sn*-1,2-Diacylglycerols in Tissues – Biological Importance

sn-1,2-Diacylglycerols tend to be minor components of most tissues in quantitative terms, but they are very important in animal tissues, as they function as second messengers in many cellular processes, modulating vital biochemical mechanisms. They arise by several mechanisms, For example, they are formed as intermediates both in the biosynthesis and catabolism of triacyl-*sn*-glycerols and in the biosynthesis of certain phospholipids. Thus, sn-1,2-diacylglycerol is a key intermediate in the formation of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. In this instance, phosphatidate phosphatase is an important enzyme, which is present mainly in the endoplasmic reticulum of the cell, and converts phosphatidic acid to sn-1,2-diacylglycerol. The reverse reaction in which phosphatidic acid is produced by the action of a diacylglycerol kinase is also of great biological importance.

$$CH_2OOCR \qquad\qquad CH_2OOCR$$
$$CHOOCR' \quad \overset{1}{\underset{2}{\rightleftharpoons}} \quad CHOOCR'$$
$$CH_2OPO_3H \qquad\qquad CH_2OH$$

Phosphatidic acid   *sn*-1, 2-diacylglycerol

1, phosphatidate phosphatase
2, diacylglycerol kinase
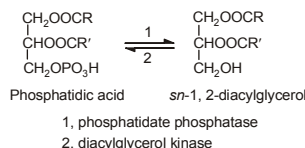
Most of the precursor phosphatidic acid is generated via the Kennedy pathway, but a second mechanism involves the action of a specific phospholipase D on phosphatidylcholine. The latter can also be a precursor for diacylglycerol production via the action of phospholipase C, or by an exchange reaction with ceramide in the biosynthesis of sphingomyelin (a related pathway may be important in some pathogenic fungi). These reactions occur more slowly but are of longer duration.

More significantly in relation to their signalling function, *sn*-1,2-diacylglycerols are formed along with the important signalling molecules, the water-soluble inositol phosphates, by the action of the enzyme phospholipase C (a family of eleven related enzymes in four sub-families exist) on phosphatidylinositol and the polyphosphoinositides and especially phosphatidylinositol-4,5-bisphosphate (see the web pages on these lipids for further information). These enzymes are activated by agonists at receptors on the cell surface, of which over one hundred types have been identified. The response is immediate if short-lived.

Phosphatidylinositol   *sn*-1, 2-diacylglycerol
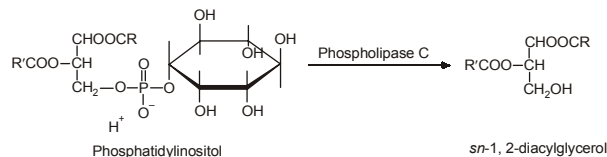
The fatty acid compositions of the diacylglycerols formed by these various routes then reflect the composition of the parent phospholipids. In particular, those derived from phosphatidylinositols are highly enriched in molecular species containing stearic in position *sn*-1 and arachidonic acid in position *sn*-2. There is evidence that the diacylglycerols must contain polyunsaturated fatty acids to fulfil their function as messengers optimally.

Diacylglycerols accumulate transiently in membranes, where they bind via strong hydrophobic interactions to specific proteins, and then cause changes in the physical properties of the bilayer. As their polar head group is small, they tend to form inverted micellar structures. In practice, this means that they introduce small areas of unstable negative curvature in membranes that facilitate membrane fission or fusion. By exposing small areas of the apolar regions of neighbouring lipids, they improve the hydrophobic interactions with proteins within these membrane regions thereby affecting their activities. The fusion of biological membranes is of great importance for the proper functioning of cells, and diacylglycerols in membranes are able to facilitate this process partly via their specific physical properties and partly through activation of certain proteins.

It appears that the formation of diacylglycerols may be initiated at the plasma membrane, but subsequently the reaction at internal membranes becomes more important. In particular, there is a significant production of diacylglycerols at the nucleus in response to stimuli. It is thought that diacylglycerols produced for purposes other than signalling are segregated spatially with the cell.

An especially important function of *sn*-1,2-diacylglycerols, and especially those derived from phosphatidylinositol, is that they affect vital processes in cell physiology by activating members of the protein kinase C family of enzymes, often acting in concert with the soluble phosphoinositides. The *sn*-1,2-configuration is essential for this activity. Diacylglycerols appear to function then by increasing the concentrations of calcium ions in the cell, which stimulate the translocation of the various iso-enzymes of protein kinase C to the inner face of the plasma membrane. By binding in a 1:1 ratio to the enzymes at a highly conserved cysteine-rich C1 domain, which consists of a sequence of 50 amino acids with a characteristic motif, they also increase their hydrophobicity and thus enhance their affinity for membrane lipids, especially phosphatidylserine, 4 to 10 molecules of which appear to be essential cofactors. This in turn increases the kinase activities of the enzymes by modifying their phosphorylation state.

The protein kinase C enzymes are involved in both short- and long-term modifications of normal cellular physiology with more than a hundred substrates identified to date. Of particular importance is the finding that the tumour-promoting phorbol esters mimic the activity of diacylglycerols and activate the same enzymes. Diacylglycerols therefore have a key role in the pathophysiology of cancer and other disease states. In addition, the identification of non-kinase receptors of *sn*-1,2-diacylglycerols, many but not all of which have the conserved C1 domain, has revealed new and strategic functions in regulating cellular responses and in cytoskeletal remodelling.

Diacylglycerols also bind to protein kinase D, a cytosolic serine-threonine kinase that in turn binds to the trans-Golgi membrane network and regulates transport of proteins to the cell surface. Protein transport is blocked in the absence of diacylglycerols.

In addition, *sn*-1,2-diacylglycerols can serve as precursors for biosynthesis of phosphatidic acid via the action of diacylglycerol kinase (as illustrated above) and of 2-arachidonylglycerol, both of

which also have second messenger functions. In particular in mammals, there is a family of at least ten diacylglycerol kinase isoenzymes, which are structurally related to the sphingosine kinase, each of which may have slightly different properties and functions, which may be segregated in distinct cellular organelles and activated by different means. Some are cytosolic, some are associated with membranes and some are located within the nucleus. In the brain different isoenzymes are expressed in different types of neuron, some of which have several isoenzymes. These enzymes have a negative effect on diacylglycerol signalling by reducing its concentration in cells, but they are believed to generate phosphatidic acid with specific signalling functions, rather than simply to serve as a precursor of other lipids.

In insects, although lipids are stored in the form of triacylglycerols in fat bodies, they are transported in hemolymph (the insect equivalent of plasma) in the form of *sn*-1,2-diacylglycerols bound to the lipoprotein lipophorin to tissues where they are required as a source of energy.

2-monoacylglycerol
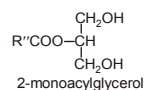
2-Monoacyl-*sn*-glycerols are formed as intermediates or end-products of the enzymatic hydrolysis of triacylglycerols; these and other positional isomers are powerful surfactants. 2-Arachidonoylglycerol has important biological properties (as an endocannabinoid).

Acyl migration occurs rapidly in partial glycerides at room temperature, but especially on heating, in alcoholic solvents or in the presence of acid or base, so special procedures are required for their isolation or analysis if the stereochemistry is to be retained. Synthetic 1-/3-monoacylglycerols are important in commerce as surfactants. (More..).
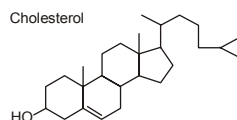
Cholesterol

Sterols and sterol esters: Cholesterol is by far the most common member of a group of steroids in animal tissues; it has a tetracyclic ring system with a double bond in one of the rings and one free hydroxyl group. It is found both in the free state, where it has an essential role in maintaining membrane fluidity, and in esterified form, i.e. as cholesterol esters. Other sterols are present in free and esterified form in animal tissues, but at trace levels only.

In plants, cholesterol is rarely present in other than small amounts, but such phytosterols as sitosterol, stigmasterol, avenasterol, campesterol and brassicasterol, and their fatty acid esters are usually found, and they perform a similar function.

Plant Sterols

Plants contain a rather different range of sterols from animals. Like cholesterol, to which they are related structurally and biosynthetically, plant sterols form a group of triterpenes with a tetracyclic

5α-cholestan-3β-ol
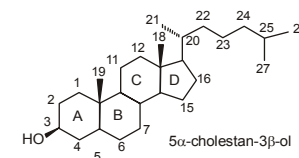
cyclopenta[*a*]phenanthrene structure and a side chain at carbon 17. The four rings (A, B, C, D) have *trans* ring junctions, and the side chain and two methyl groups (C-18 and C-19) are at an angle to the rings above the plane with â stereochemistry (as for the hydroxyl group on C-3 also). The basic sterol from which other sterol structures are defined is 5á-cholesten-3â-ol.

The phytosterols (as opposed to zoosterols) include campesterol, â-sitosterol, stigmasterol and Ä5 - avenasterol, some of which are illustrated below.

Campesterol   Sito sterol

Brassicasterol   Stigmasterol   avena sterol
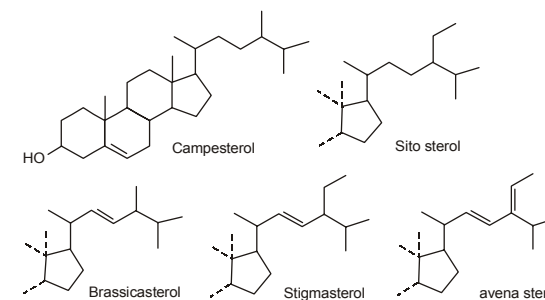
These more common plant sterols have a double bond in position 5, and a definitive feature – a one- or two-carbon substituent with variable stereochemistry in the side chain at C-24, which is preserved during subsequent metabolism. Occasionally, there is a double bond in this chain that can be of the *cis* or *trans* configuration. Phytosterols can be further classified on a structural or biosynthetic basis as 4-desmethyl sterols (i.e. with no substituent on carbon-4), 4á-monomethyl sterols and 4,4-dimethyl sterols. In addition, the 4-desmethyl sterols may be subdivided into Ä5-sterols, Ä7-sterols and Ä5,7-sterols depending on the position of the double bonds in the B ring. As the name suggests, brassicasterols are found mainly in the brassica family of plants. Phytostanols (fully saturated) are normally present at trace levels only in plants, but they are relatively abundant in cereal grains.

Many different sterols may be present in plant species (over 200 have been characterized), and the amounts and relative proportions of all are dependent on the plant species. As a rough generality, a typical plant sterol mixture would be 70% sitosterol, 20% stigmasterol and 5% campesterol. Table 1 contains data from some representative commercial seed oils. Cholesterol is usually a minor component only of plant sterols, but it is unwise to generalize too much as it can be the main sterol component of

red algae and of some families of higher plants (Solanaceae, Liliaceae, Scrophylariaceae), for example. It can also be a significant constituent sterol of chloroplasts, shoots, pollen and leaf surface lipids in other plant families. Yeasts and fungi tend to contain ergosterol as their main sterol (see below).

Table 2.6 : Sterol composition in some seed oils of commercial importance (mg/Kg).

	Corn oil	cottonseed	olive	palm	rapeseed	safflower	soybean	sunflower
cholesterol	-	-	-	26	-	-	-	-
campesterol	2691	170	28	358	1530	452	720	313
stigmasterol	702	42	14	204	-	313	720	313
β-sitosterol	7722	3961	1310	1894	3549	1809	1908	2352
Δ5-avenasterol	468	85	29	51	122	35	108	156
Δ7-stigmastenol	117	-	58	25	306	696	108	588
Δ7-avenasterol	-	-	-	-	-	104	36	156
brassicasterol	-	-	-	-	612	-	-	-
other	-	-	-	-	-	69	-	39

Data from Gunstone, F.D., Harwood, J.L. and Padley, F.B. *The Lipid Handbook (Second Edition)* (Chapman & Hall, London) (1994).

Cycloartenol
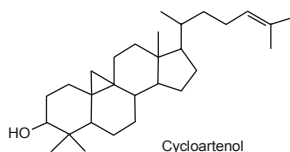
The biosynthetic route to plant sterols resembles that to cholesterol in many aspects in that it follows an isoprenoid biosynthetic pathway with isopentenyl pyrophosphate, derived primarily from mevalonate, as the key building block in the cytoplasm (but not plastids) at least. However, in photosynthetic organisms (as opposed to yeast and fungi), it differs in that the important intermediate in the route from squalene is **cycloartenol** rather than lanosterol. Then, the enzyme sterol methyltransferase 1 is of special importance in that it converts cycloartenol to 24-methylene cycloartenol, as the first step in introducing the methyl group onto C-24. There are more than thirty enzyme-catalysed steps in the overall process, each associated with membranes. The 4,4-dimethyl- and 4á-methylsterols are part of the biosynthetic pathway, but are only minor if ubiquitous sterol components of plants.

In addition, an alternative pathway for the biosynthesis of isopentenyl pyrophosphate and dimethylallyl pyrophosphate, the isoprene units, which does not use mevalonic acid as a precursor has been found in plant chloroplasts, algae, cyanobacteria, eubacteria, and some parasites, but not in animals. This route is variously termed the 'non-mevalonate', '1-deoxy-D-xylulose-5-phosphate' (DOXP) or better the '2*C*-methyl-D-erythritol 4-phosphate (MEP)' pathway, since the last compound is presumed to be the

first committed intermediate in sterol biosynthesis by this route. In the first step, pyruvate and glyceraldehyde phosphate are combined to form deoxyxylose phosphate, which is in turn converted to 2C-methyl-D-erythritol 4-phosphate. The pathway then proceeds via various erythritol intermediates until isopentenyl pyrophosphate and dimethylallyl pyrophosphate are formed, when sterol biosynthesis thereafter continues via squalene and cycloartenol. There is evidence that some of the isoprene units are exchanged between the cytoplasm and plastids.
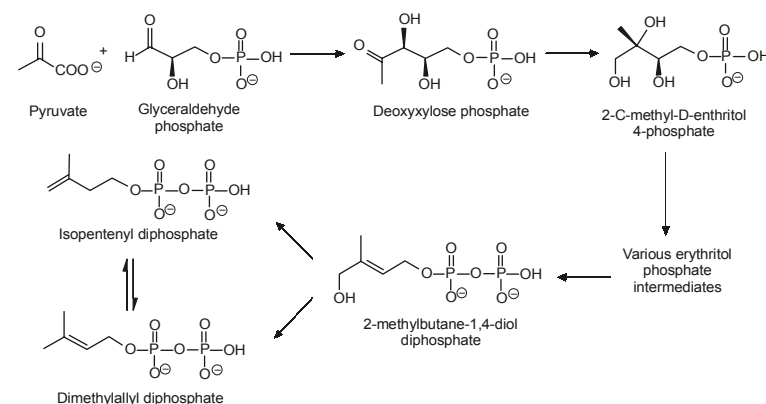
Like cholesterol, plant sterols are amphiphilic and are vital constituents of all membranes, and especially of the plasma membrane, the mitochondrial outer membrane and the endoplasmic reticulum. The three-dimensional structure of the plant sterols is such that there are planar surfaces at both the top and the bottom of the molecules, which permit multiple hydrophobic interactions between the rigid sterol and the other components of membranes. Indeed, they must govern the physical properties of membranes to an appreciable extent. It is believed that sitosterol and 24-methylcholesterol are able to regulate membrane fluidity and permeability in plant membranes by restricting the mobility of fatty acyl chains in a similar manner to cholesterol in mammalian cells. They may be involved in how plant membranes adapt to changes in temperature. Stigmasterol has much less effect on lipid ordering and no effect on the permeability of membranes. In the plasma membrane, plant sterols associate with the glycosphingolipids such as glucosylceramide in raft-like sub-domains, analogous to those in animal cells.

Plant sterols can modulate the activity of membrane-bound enzymes. Thus, stigmasterol and cholesterol regulate the activity of the Na$^+$/K$^+$-ATPase in plant cells, probably in a manner analogous to that of cholesterol in animal cells. Stigmasterol may be required specifically for cell differentiation and proliferation. Certain sterols, such as campesterol in *Arabidopsis thaliana*, in minute amounts are precursors of oxidized steroids that act as growth hormones and are collectively named **brassinosteroids**,

which have crucial importance for growth and development. Cholesterol is a precursor for the biosynthesis of some steroidal saponins and alkaloids, as well as of other steroids including the ecdysteroids (insect moulting hormones).

Substantial amounts of phytosterols are available as by-products of the refining of vegetable oils and of tall oil from the wood pulp industry. There is increasing interest in such commercial sources of plant sterols to be added as "nutraceuticals" to margarines and other foods, as it appears that they can inhibit the uptake of cholesterol from the diet and thereby reduce the levels of this in the plasma low-density lipoproteins. Hydrogenated phytosterols or "**stanols**" are also used for this purpose. In addition, dietary supplements of plant sterols have been reported to have anti-cancer effects. The actual absorption of dietary plant sterols and stanols in humans is low (0.02-3.5%) compared to cholesterol (35-70%). In some rare cases, increased levels of plant sterols in plasma serve as markers for an inherited lipid storage disease (phytosterolemia). There is evidence that while plant sterols can substitute for cholesterol in maintaining membrane function in mammalian cells, they can exert harmful effects by disrupting cholesterol homeostasis.

Phytosterols can be subjected to autoxidation in a similar manner to that of cholesterol in animals, resulting in ring products such as hydroxy-, keto-, epoxy- and triol-derivatives. In addition, enzymic reactions can oxidize the side chain. These oxy-phytosterols can enter the food chain and, although they are not efficiently absorbed, they have been detected in human plasma and have the potential to exert a variety of biological effects.

2. Sterol Esters

Sterol esters are present in plant tissues, but as minor components relative to the free sterols other than in waxes. Usually the sterol components of sterol esters are similar to the free sterols, although there may be relatively less of stigmasterol. The fatty acid components tend to resemble those of the other plant tissue lipids, but there can be significant differences on occasion. Sterol esters are presumed to serve as inert storage forms of sterols, as they are often enriched in the intermediates of sterol biosynthesis and can accumulate in lipid droplets within the cells. However, they have been found in membranes, especially in microsomes and mitochondrial preparations, although their function here is uncertain. They may also have a role in transport within cells and between tissues, as they have been found in the form of soluble lipoprotein complexes.

Biosynthesis of sterol esters in *Arabidopsis thaliana* is known to occur in the endoplasmic reticulum and involves transfer of a fatty acyl group to the sterol from position *sn*-2 of phosphatidylethanolamine. However, other enzymes may exist but have yet to be characterized. In yeasts, two sterol ester synthases have been identified that utilize CoA esters of fatty acids as substrate. Little appears to be known of the catabolism of sterol esters in plants, but specific sterol ester hydrolases have been characterized from yeasts.

Certain distinctive phytosterol esters occur in the aleurone cells of cereal grains, including *trans*-hydroxycinnamate, ferulate (4-hydroxy-3-methoxy cinnamate) and *p*-coumarate esters. Similarly, rice bran oil is a rich source of esters of ferulic acid and a mixture of sterols and triterpenols, termed 'ã-orizanol'', and an example of one of these compounds is illustrated below. This is sold as a health food

supplement, because of claimed beneficial effects, including cholesterol-lowering and antioxidant activities. It is also reputed to enhance muscle growth and sports performance. However, none of these effects have been confirmed by rigorous clinical testing.

Campesteryl ferulate
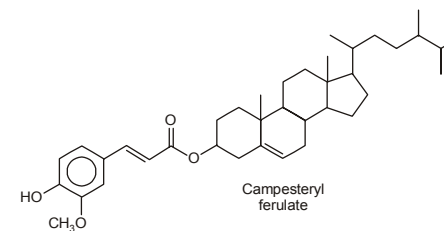
3. Sterol Glycosides

Leaf and other tissue in plants contain a range of **sterol glycosides** and **acyl sterol glycosides**, and typical examples (glucosides of â-sitosterol) are illustrated below.

Sterol glucoside   Acyl sterol glucoside
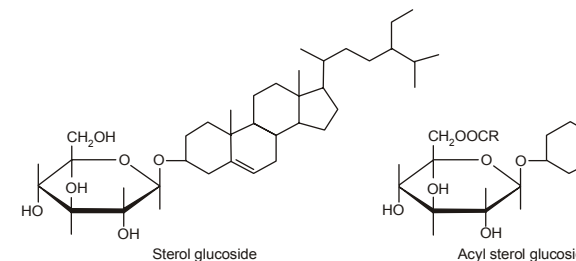
Most of the common plant sterols occur in this form, and the carbohydrate moieties (glucose, xylose, arabinose) can vary also with the plant species. The carbohydrate moiety can sometimes be quite complex with up to five hexose units linked in a linear fashion. Similarly, the nature of the fatty acid component in the acyl sterol glycosides can vary as well as the hydroxyl group to which they are linked, although it is usually position 6. In potato tubers, for example, the 6'-palmitoyl-â-D-glucoside of â-sitosterol is the major species, while the corresponding linoleate derivative predominates in soybeans. Usually, the acyl sterol glycosides are present at concentrations that are two to tenfold greater than those of the non-acylated forms.

Plant, animal, fungal and most bacterial steryl glycosides have a â-glycosidic linkage, but in a few bacterial species there is an á-linkage.

Sterol glycosides and acyl sterol glycosides may be involved in the adaptation of plant membranes to low temperatures. Biosynthesis involves reaction of free sterols with uridine diphosphoglucose

(UDP-glucose) in the presence of a UDP-glucose:sterol glucosyltransferase in plant membranes, probably the plasma membrane. Sitosterol-â-D-glucoside in the plasma membrane is the primer molecule for cellulose synthesis in plants and it is required for the initiation of glucan polymerization. The sterol is eventually removed from the polymer by a specific cellulase enzyme.

Sterol glycosides have only rarely been reported from organisms other than plants, although cholesteryl glucoside has been found in a few animal tissues. The gram-negative bacterium *Helicobacter pylori*, one of the most common human pathogens, is an important exception in that it contains appreciable amounts of unique cholesterol glucosides, including cholesteryl-6-*O*-acyl-á-D-glucopyranoside, in its cell walls. Other than this species, cholesteryl glucosides are rather rare in bacteria. It is known that the cholesterol of these lipids is obtained from an animal host and is not synthesised by bacteria.

In addition, a number of species of monocotyledons contain complex steroidal **saponins**, which consist of a furostanol- or spirostanol-based aglycone and an oligosaccharide chain of two to five hexose or pentose moieties attached to the 3-hydroxyl group of the sterol. Further conjugates of this type include other triterpenes with saccharide units linked at various positions

4. Sterols in Yeasts and Fungi
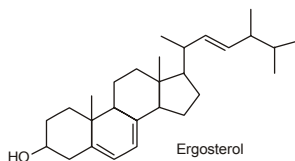
Ergosterol

Yeasts and fungi, together with microalgae and protozoa, can contain an enormous range of different sterols. **Ergosterol** ((22E)-ergosta-5,7,22-trien-3â-ol) is the main sterol in fungi and yeasts, and is accompanied by other sterols not normally abundant in higher plants including zymosterol (5á-cholesta-8,24-dien-3â-ol). Like cholesterol and in contrast to the plant sterols, it is synthesised via lanosterol as the key intermediate. Some antifungal drugs are targeted against ergosterol biosynthesis. Under some conditions, especially those that retard growth, a high proportion of the sterols in yeasts can be in esterified form.

Many mutants defective in ergosterol biosynthesis have been isolated, and these have yielded a great deal of information on the features of the sterol molecule required for its structural role in membranes of yeast and fungi. Ergosterol stabilizes the liquid-ordered phase in the same manner as cholesterol, and also forms rafts with sphingolipids, whereas lanosterol does not. It is also evident that ergosterol has a multiplicity of functions in the regulation of yeast growth.

Ergosterol esters are synthesised in yeast by enzymes (ARE1 and ARE2) related to ACAT-1 and ACAT-2 that perform this function in animals.

Protozoans also synthesise many different sterols. For example, some species of *Acanthamoeba* and *Naegleria* produce both lanosterol and cycloartenol, but only latter is used for synthesis of other sterols, especially ergosterol. In some other protozoan species, sterol biosynthesis occurs via lanosterol.

5. Bacterial Sterols

It has long been recognised that some bacteria take up cholesterol and other sterols from host animals for use as membrane constituents. Indeed, an external source of sterols is required for growth in species of *Mycoplasma*. In addition, there have been a number of reports of biosynthesis of sterols by various bacterial species, although a high proportion of these appear now to have been discounted because of fungal contamination. In particular, the possibility of sterol biosynthesis in cyanobacteria has been controversial, and molecular biology studies have yet to detect the presence of the required enzyme squalene epoxide cyclase.

That said, there is good evidence that a few species of prokaryotes at least have the capacity to synthesise sterols *de novo*. Among the eubacteria certain methylotrophs (*Methylobacterium* and *Methylosphaera* species) produce mono- and dimethyl sterols, including lanosterol. Similarly, some soil bacteria produce 4-desmethylsterols. It has now been established from gene sequence studies that certain bacteria contain enzymes of the sterol biosynthesis pathway, but as these have no obvious evolutionary link it seems probable that they were acquired via lateral transfer from eukaryotes. Of course, hopanoids take the place of sterols in many more species of bacteria.

6. Analysis

In the analysis of animal and plant sterols, a sterol fraction is first isolated from lipid extracts by thin-layer or column chromatography, following hydrolysis if necessary. Individual components can then be determined by gas chromatography in the presence of an internal standard (e.g. epicoprostanol or betulin), often after conversion to trimethylsilyl ether derivatives to give sharper peaks. Mass spectrometry may be required for identification of individual components. Analysis of the minor oxysterols that may be found in plasma or foods is a rather specialized task, because they tend to be present at rather low levels and there is a danger of further oxidation or side reactions during the analytical process. Rigorous attention to detail is necessary for meaningful results.

Sterol esters are trans-methylated for GC analysis of the fatty acid components, although the reaction may again be much slower than with glycerolipids. Intact sterol esters are best analysed by reversed-phase HPLC. Analysis of sterol glycosides is a more specialized endeavour that can be more concerned with carbohydrate than with lipid chemistry.

Hopanoids are related lipids produced by some bacterial species.

Waxes: In their most common form, wax esters consist of fatty acids esterified to long-chain alcohols with similar chain-lengths. The latter tend to be saturated or have one double bond only. Such compounds are found in animal, plant and microbial tissues and they have a variety of functions, such as acting as energy stores, waterproofing and lubrication.
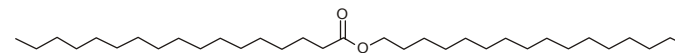
In some tissues, such as skin, avian preen glands or plant leaf surfaces, the wax components can be much more complicated in their structures and compositions. They can contain aliphatic diols, free alcohols, hydrocarbons (e.g. squalene), aldehydes and ketones. (More..).

α-toopherol
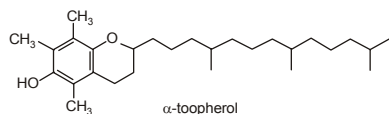
Tocopherols are substituted benzopyranols (methyl tocols) that occur in vegetable oils. Different forms (á-, â-, ã- and ä-) are recognized according to the number or position of methyl groups on the aromatic ring. á-Tocopherol (with the greatest Vitamin E activity) illustrated is an important natural antioxidant. Tocotrienols have similar ring structures but with three double bonds in the aliphatic chain. Tocopherols and Tocotrienols.

Structure, Composition, Biology and Analysis

1. Structure and Biosynthesis

Tocopherols constitute a series of related benzopyranols (or methyl tocols) that occur in plant tissues and vegetable oils and are powerful lipid-soluble antioxidants. In the tocopherols, the C_{16} side chain is saturated, and in the **tocotrienols** it contains three *trans* double bonds. Together, these two groups are termed the **tocochromanols.** In essence, the tocopherols have a 20-carbon phytyl tail (including the pyranol ring), and the tocotrienols a 20-carbon geranylgeranyl tail with double bonds at the 3', 7' and 11' positions, attached to the benzene ring. The side-chain methyl groups have *R,R,R* stereochemistry. The four main constituents of the two classes are termed - *alpha* (5,7,8-trimethyl), *beta* (5,8-dimethyl), *gamma* (7,8-dimethyl) and *delta* (8-methyl).

	R'	R''
alpha-tocophenol	—CH₃	—CH₃
beta-tocophenol	—CH₃	—H
gamma-tocophenol	—H	—CH₃
delta-tocophenol	—H	—H

Tocotrienols
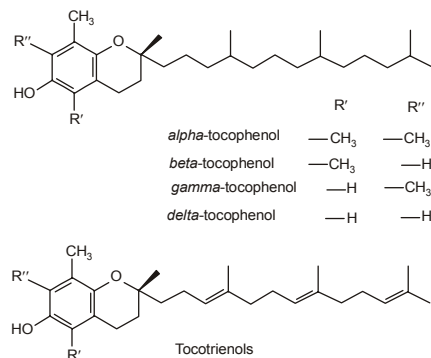
These compounds are only synthesised by plants and other oxygenic, photosynthetic organisms, but they are essential components of the diet of animals, and collectively they are termed 'vitamin E' (the individual tocopherols are 'vitamers'). In plants, there is a great range of tocochromanol contents

and compositions, and photosynthetic plant tissues contain from 10 to 50 ìg tocochromanols per g fresh weight. á-Tocopherol is often the main tocochromanol in leaves. Seed oils are a major source for the human diet and the compositions of tocopherols in some unrefined oils are listed in **Table**. Sunflower and olive oils are good sources of á-tocopherol and palm oil of the tocotrienols. In general, tocotrienols tend to be more abundant in seeds of monocots, such as wheat, rice and barley.

Table 2.7 : Tocopherol and tocotrienol contents (mg/Kg) in some seed oils

	α-T*	α-T	γ-T	δ-T	α-TT*	β-TT	γ-TT	δ-TT
palm	89	-	18	-	128	-	323	72
soybean	100	8	1021	421	-	-	-	-
maize	282	54	1034	54	49	8	161	6
sunflower	670	27	11	1	-	-	-	-
rapeseed	202	65	490	9	-	-	-	-

* Abbreviations: T, tocopherol; TT, tocotrienol

Data from: Gunstone, F.D., Harwood, J.L. and Padley, F.B. *The Lipid Handbook (Second Edition)* (Chapman & Hall, London) (1994).

The mechanism of biosynthesis of tocopherols is well understood, and involves coupling of phytyl diphosphate with homogentisic acid (2,5-dihydroxyphenylacetic acid), followed by cyclization and methylation reactions.

tyrosine

p-hydroxy-pyruvic acid

Homogentisic acid

2-methyl-6-phytyl-1,4-benzoquinol

2,3-dimethyl-5-phytyl-1, 4-benzoquinol

γ-tocopherol

α-tocpherol
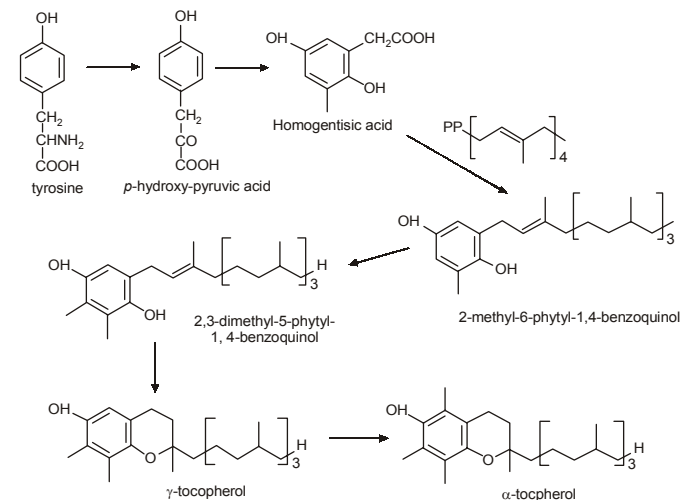
The plant chloroplast is the site of biosynthesis, and the aromatic amino acid tyrosine can be considered the basic precursor. This is oxidized to *p*-hydroxypyruvic acid, which in the first committed step is converted to homogentisic acid by the enzyme *p*-hydroxyphenylpyruvate dioxygenase. Homogentisic acid is condensed with phytyl diphosphate in a reaction catalysed by a prenyl transferase to yield 2-methyl-6-phytyl-plastoquinol, which is first methylated to form 2,3-dimethyl-5-phytyl-1,4-benzoquinol and then converted by the enzyme tocopherol cyclase to ã-tocopherol. A further methylation reaction produces á-tocopherol, while modifications to the pathway produce â- and ä-tocopherols and plastoquinones. Tocotrienols result from a similar series of reactions but with geranylgeranyl diphosphate as substrate in the condensation step.

Fish contain an unusual tocopherol that has been termed marine-derived á-tocomonoenol (a related isomer has been found in palm oil). It is found together with á-tocopherol in a wide range of marine fish species and appears to be a more efficient scavenger of free radicals at low temperatures.

Marine-derived α-tocomonoenol
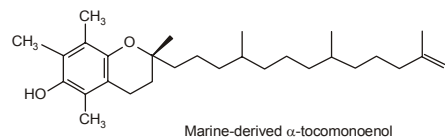
á-Tocopheryl phosphate has recently been detected at low levels in liver and adipose tissue, and it is possible that it may be a ubiquitous constituent of animal and plant tissues.

á-Tocopherol is a minor but ubiquitous component of the lipid constituents of animal cell membranes, with estimates ranging from one molecule of tocopherol to from 100 to 1000 molecules of phospholipid, depending on the membrane. The hydrophobic tail lies within the membrane, as might be expected, and the polar head group is orientated towards the surface but below the level of the phosphate moieties of the phospholipids. There may be some limited hydrogen bonding between the hydroxyl groups and phosphate depending on the degree of hydration of the membrane. On the other hand, there is a strong affinity of a-tocopherol for polyunsaturated fatty acids, where the chromanol unit may interact with the double bonds, suggesting that tocopherol is located deep within the membrane.

During the refining of vegetable oils, much of the natural tocopherols are lost or destroyed. Most commercial vitamin E is therefore prepared by chemical synthesis with trimethylhydroquinone and phytyl bromide as the precursors. The resulting product is a mixture of eight stereoisomers (from *R,R,R* to *S,S,S* methyl groups) of á-tocopherol, but it still has appreciable vitamin E activity. It is usually administered as the acetate derivative. Tocopherols are not usually regarded as effective antioxidants in the polyunsaturated seed oils of commerce, and at higher concentrations can even act as pro-oxidants, although the reasons for this are not understood. They are required in the developing seed (see below).

2. Tocopherols Metabolism in Animals

In animals, all tocopherols are absorbed to a similar extent in the intestines and are transported to the liver in chylomicrons mainly, but á-tocopherol is preferentially utilized and re-exported. This process is

mediated by a specific tocopherol-binding protein in the liver that has a marked affinity for á-tocopherol, transferring it to the plasma lipoproteins (mainly the LDL and HDL in humans) for transport to other tissues (together with lesser amounts of ã-tocopherol). The "á-tocopherol salvage pathway" results in a 20- to 30-fold enrichment of á-tocopherol in plasma (average concentration 22-28 ìM) relative to the other tocopherols. Transfer of tocopherols from the lipoproteins to peripheral tissues is promoted by the enzyme lipoprotein lipase. Concentrations of tocopherols can vary appreciably amongst tissues, with most in adipose tissue and adrenals, less in kidney, heart and liver, and least in the erythrocytes. Dietary tocotrienols are also absorbed in the intestines but less efficiently.

The process of conservation of one specific tocopherol appears to determine the relative vitamin E activities of the tocopherols and tocotrienols *in vivo*, rather than their individual potencies as antioxidants as measured in model systems *in vitro*. Only á-tocopherol (including synthetic material) or natural mixtures containing this can be sold under the label 'Vitamin E'. However, the tocotrienols are more potent antioxidants, *in vitro* at least, while ã-tocopherol (which is relatively abundant in skin) has some specific biological properties that are distinct from those of á-tocopherol.

Most of the tocochromanols other than á-tocopherol, together with any excess of the latter, are metabolized. The unwanted surplus may be excreted in the urine in the form of the so-called 'Simon metabolites', á-tocopheronic acid and á-tocopheronolactone, after oxidative cleavage of much of the phytyl tail. However, these are normally in the form of conjugates as sulphate or glucuronidate esters.

α-tocophenonic acid   α-tocopheronolactone
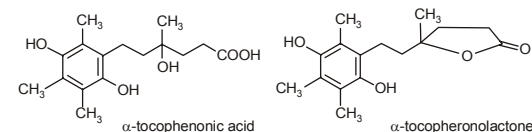
The first step in catabolism is ù-hydroxylation by cytochrome P450 (CYP4F2) at the 13' carbon to form a 13'-hydroxychromanol, followed by stepwise â-oxidation to cut off two or three carbon moieties from the phytyl chain in each cycle. Various carboxychromanol intermediates have been identified for all of the tocopherols together with sulphated forms of these in human cell cultures *in vitro*.

3. Tocopherols as Antioxidants

Although the syndrome associated with a lack of vitamin E in the diet of animals has been well known for decades, the mode of action and specific location of tocopherols in cell membranes are not clearly understood. Several theories have been proposed to explain their functions. Many argue that their primary task is to act as antioxidants to prevent free radical damage to unsaturated lipids or other membrane constituents and thence to tissues, while others now suggest that this may be secondary to more important biological functions (see below). That said there is no doubt that tocopherols are powerful antioxidants *in vitro* and surely have some such function *in vivo*. They are certainly extremely useful as antioxidants in non-biological systems, including foods, cosmetics, pharmaceutical preparations and so forth.

Because of their lipophilic character, tocopherols are located in the membranes or with storage lipids where that are immediately available to interact with lipid hydroperoxides. They react rapidly in a

non-enzymic manner unlike many other cellular antioxidants, which are dependent on enzymes, to scavenging lipid peroxyl radicals, i.e. the chain-carrying species that propagate lipid peroxidation. In model systems *in vitro*, all the tocopherols (á > ã > â > ä) and tocotrienols are good antioxidants, with the tocotrienols being the most potent.

In general, the oxidation of lipids is known to proceed by a chain process mediated by a free radical, in which the lipid peroxyl radical serves as a chain carrier. In the initial step of chain propagation, a hydrogen atom is abstracted from the target lipid by the peroxyl radical as shown—

$$LOO° + LH \rightarrow LOOH + L° \tag{1}$$
$$L° + O_2 \rightarrow LOO° \tag{2}$$

—where LH is a lipid, LOO is the lipid peroxyl radical and LOOH is the lipid hydroperoxide.

The main function of á-tocopherol is to scavenge the lipid peroxyl radical before it is able to react with the lipid substrate as–

$$LOO° + TOH \rightarrow LOOH + TOO° \tag{3}$$

—where TOH is tocopherol and TOO* is the tocopheroxyl radical

It thus prevents propagation of the chain reaction. The potency of an antioxidant is determined by the relative rates of reactions (1) and (2). Studies of the relative rates of chain propagation to chain inhibition by á-tocopherol in model systems have demonstrated that á-tocopherol is able to scavenge peroxyl radicals much more rapidly than the peroxyl radical can react with a lipid substrate.

In biological systems, oxidant radicals can spring from a number of sources, including singlet oxygen, alkoxyl radicals, superoxide, peroxynitrite, nitrogen dioxide and ozone. á-Tocopherol is most efficient at providing protection against peroxyl radicals in a membrane environment.

When a tocopheroxyl radical is formed, it is stabilized by delocalisation of the unpaired electron about the fully substituted chromanol ring system rendering it relatively unreactive. This also explains the high first order rate constant for hydrogen transfer from á-tocopherol to peroxyl radicals. Reaction of the tocopheroxyl radical with a lipid peroxyl radical, as illustrated, yields 8á-substituted tocopherones, which are readily hydrolysed to 8á-hydroxy tocopherones that rearrange spontaneously to form

To copheroxyl radical   8 α-alkyldioxytocopherone   tocopherol quinone

tocopherol hydroquinone
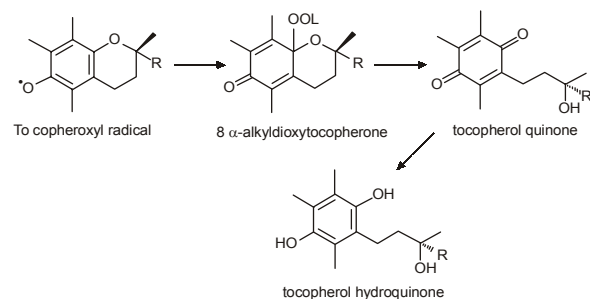
á-tocopherol quinones. In an alternative pathway, the tocopheroxyl radical reacts with the lipid peroxyl radical to form epoxy-8á-hydroperoxytocopherones, which hydrolyse and rearrange to epoxyquinones. Tocopherol dimers and trimers may also be formed as minor products.

In plant and animal tissues, tocopherols can be regenerated from the tocopheroxyl radicals in a redox cycle mediated by a number of endogenous antioxidants, including vitamins A and C and coenzyme Q, and this must greatly extend their biological potency. Vitamin C (ascorbate) may be especially important in aqueous systems, although it may also act at the surface of membranes.

Suggestions that dietary supplements of vitamin E may reduce the rate of oxidation of lipids in low-density lipoproteins and thence the incidence or severity of atherosclerosis now appear to be unfounded, although benefits in some conditions have been claimed. Indeed, there are suggestions that excessive vitamin E supplementation may even be harmful. A recent study has suggested that relatively high doses of natural á-tocopherol over a long period are required to demonstrate a significant reduction in the levels of F_2 isoprostanes in the urine, which are considered to be the most reliable marker for oxidative stress *in vivo*. This subject is highly contentious and I prefer to leave it to the clinical experts.

In plants, tocopherols are most abundant in the membranes of the chloroplasts, where they were long believed to be the most important antioxidants, limiting the damage from photosynthesis-derived reactive oxygen species during conditions of oxidative stress, including high-intensity light stress. However, recent studies seem to suggest that they are just one of a number of different components that are involved in photo-protection. Certainly, any tocochromanol peroxy radicals formed must be converted back to the original compounds by the concerted action of other plant antioxidants. On the other hand, there is no doubt that tocopherols are essential for the control of non-enzymatic lipid peroxidation during seed dormancy and germination of seedlings. In their absence, elevated levels of malondialdehyde and phytoprostanes are formed, and there can be inappropriate activation of plant defense responses.

4. Other Biological Functions of Tocopherols

With the discovery that the antioxidant effects of various tocopherols and tocotrienols have little relation to their vitamin E activities has come the realization that they have many other functions in tissues, most of which are specific to á-tocopherol. Indeed, it has even been suggested that these are so important that tocopherol may be protected from functioning as an antioxidant in tissues *in vivo* through a network of cellular antioxidant defences. Only when other antioxidants are exhausted are the tocopherols utilized. However, there is no experimental proof of this hypothesis, although it is certainly true that most other vitamins are essential cofactors for specific enzymes or transcription factors.

á-Tocopherol is believed to be a gene regulator, causing up-regulation of mRNA or protein synthesis that could be the result of effects on gene transcription, mRNA stability, protein translation, protein stability and post-translational events. â-Tocopherol has no such properties. Effects have also been observed on genes connected with tocopherol catabolism, lipid uptake, collagen synthesis, cellular adhesion, inflammation and cell signalling. Vitamin E modulates the activity of several enzymes involved in signal transduction, perhaps through influencing protein-membrane interactions. For example, á-tocopherol inhibits the enzyme protein kinase C, which in turn reduces the release of reactive oxygen

species in various ways and has effects on gene expression. It may also have secondary roles in stabilizing the structure of membranes, in regulating haem biosynthesis, in modulating the immune response, and as a participant in electron transport chains. Some non-antioxidant effects of ã-tocopherol in tissues in relation to reactive nitrogen oxide species have been observed, but the specificity of these is not yet certain.

Tocotrienols have been shown to have neuroprotective effects, to inhibit cholesterol synthesis and to reduce the growth of breast cancer cells *in vitro*. These properties are largely distinct from those of the tocopherols.

The biological function á-tocopheryl phosphate is not known, but it has been suggested that that it may be a storage or transport form or it could be involved in cellular signalling. Synthetic phosphate derivatives of ã-tocopherol and á-tocopheryl succinate are known to have potent anti-cancer properties.

In plants, there is evidence that tocopherols also play a part in intracellular signalling in that they regulate the amounts of **jasmonic acid** in leaves and so influence plant development and stress responses. Thus, by controlling the degree of lipid peroxidation in chloroplasts, they limit the accumulation of lipid hydroperoxides required for synthesis of jasmonic acid, which in turn regulates the expression of genes that affect a number of stress conditions. In addition, tocopherols are required for the development of the cell walls in phloem transfer cells under cold conditions.

5. Analysis

Tocopherols can be analysed by gas chromatography, both with flame-ionization and mass spectrometric detection, but the methods that are usually recommended involve high-performance liquid chromatography with fluorescence detection. Related methods are used for ubiquinones and the isoprenoid alcohols.

Free (unesterified) fatty acids are minor constituents of living tissues but are of biological importance as precursors of lipids as an energy source and as cellular messengers. **Occurrence and Biochemistry**

Free or unesterified fatty acids are ubiquitous if minor components of all living tissues. In animals, much of the dietary lipid is hydrolysed to free acids before it is absorbed and utilized for lipid synthesis. Intact lipids in tissues can be hydrolysed to free acids by a variety of lipolytic enzymes (*e.g.* lipoprotein lipase, hormone-sensitive lipase, phospholipase A), before being metabolized in various ways including oxidation, desaturation, elongation or re-esterification. As free acids can interact with a wide range of enzyme systems in both specific and non-specific ways, they must be rapidly sequestered in tissues by various means to ensure that their activities are closely regulated.

Monomeric fatty acids in the free state have very low solubilities in aqueous media. In serum, they are transported between tissues bound to the protein albumin, which has up to six strong binding sites and a large number of weak binding sites where non-polar interactions are possible between the fatty acid hydrocarbon chains and uncharged amino acid side chains. In this way, the concentration of a long-

chain fatty acid in serum can be increased by as much as 500 times above its normal maximum. However, the bound fatty acids can diffuse into the aqueous phase, where they are rapidly taken up into the outer leaflet of the plasma membrane by non-enzymatic mechanisms. It is then possible that fatty acids can then cross the membrane simply and rapidly by a biophysical process, i.e. by 'flip-flop'. On the other hand, there is also evidence that specific transporter proteins may be involved in part to activate by formation of acyl-coA prior to further esterification, but also to ensure vectorial transport so that specific fatty acids are directed towards particular purposes. Certainly within the cell, a family of fatty acid binding or transport proteins has essential functions in fatty acid trafficking pathways and in fatty acid activation. It appears that cells have several overlapping mechanisms that ensure sufficient uptake and directed intracellular movement of the fatty acids required for their physiological functions.
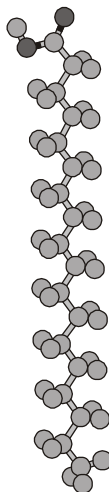
Apart from their obvious role as a source of energy (see our web page on acylcarnitines, for example), unesterified fatty acids can act as second messengers required for the translation of external signals, as they can be produced rapidly as a consequence of the binding of specific agonists to plasma membrane receptors. In this way, they can substitute for the second messengers of the inositide pathways. Fatty acids are effective also in operating at specific intracellular locations reversibly to amplify or otherwise modify signals. For example, they influence the activities of protein kinases, phospholipases, G-proteins, adenylate and guanylate cyclases, and many other metabolic processes. Part of the action of fatty acids may occur indirectly via metabolism of arachidonic acid to eicosanoids. On the other hand, there is much evidence that fatty acids *per se* are messengers that mediate the responses of the cell to extracellular signals. Many of these reactions are specific to particular fatty acids. For example, polyunsaturated fatty acids, including docosahexaenoic and arachidonic acids, bind to the retinoid X receptor and induce activation. Some related processes appear to occur in plants.

In addition in animal tissues, long-chain polyunsaturated fatty acids are involved in regulating gene expression, mainly targeting genes that encode proteins with roles in fatty acid transport or metabolism. In this respect, (n-3) fatty acids are more potent than (n-6) fatty acids. Straight-chain saturated and monoenoic fatty acids do not appear to be involved in the process, but surprisingly poly-methyl-branched fatty acids, such as phytanic and pristanic, may have a function. In some circumstances, both the free acids *per se* and their coenzyme A esters may be involved.

The mechanisms by which modulation of gene transcription occurs are only partially resolved, and this is the subject of considerable research effort, especially with respect to the family of transcription factors, i.e. peroxisome proliferator-activated receptors (PPARs), in the nuclei of cells. However, it is believed that fatty acid-binding-proteins are intimately involved and in effect act as nutrient sensors. They bind long-chain fatty acids with high affinity in the cytoplasm and transport them to nuclei, which they enter via the nuclear pores, where they are able to form complexes with nuclear receptors enabling them to regulate receptor activation.

The effects can be highly specific, different fatty acids binding to or activating different types of PPAR, although the PPARá and hepatocyte nuclear factor 4á (HNF4á) are especially important. In particular, polyunsaturated fatty acids may exert beneficial effects by up-regulating the expression of genes encoding enzymes involved in oxidation of fatty acids, while at the same time down-regulating genes for enzymes involved in lipid synthesis. They also influence glucose metabolism. As a result,

unesterified fatty acids may mitigate the undesirable symptoms of the metabolic syndrome and may even reduce the risk of heart disease. In contrast, abnormal PPAR activation can be a factor in the lipotoxicity observed with obesity, insulin resistance, type 2 diabetes and hyperlipidemia.

Similarly, in bacteria, it has been demonstrated that the bacterial fatty acid transport and trafficking system leads to fatty acid-responsive regulation of gene expression.

However, some of the mediator effects appear to be independent of PPARs and are characterized by involvement with cell surface receptors instead. Thus, multiple G-protein-coupled receptors for free fatty acids have been identified that function on the cell surface and have important roles in nutrition regulation. Some of these are activated by short-chain and others by medium- and long-chain free fatty acids. The former are expressed preferentially in pancreatic â-cells and mediate insulin secretion. In certain intestinal cells, there are believed to be such 'fatty acid sensing molecules', which recognise the presence of free long-chain acids in digesta. These stimulate the release of the hormone cholecystokinin, which in turn causes the gall-bladder to contract and release bile to aid digestion.

Free fatty acids have potent antimicrobial, antiviral and antifungal properties, and they exert such effects in some living systems, especially the skin and mucosa of the lung. As they are powerful detergents and will inhibit very many enzymes systems in a non-specific manner, it is not clear whether the biocidal properties are also non-specific. Unsaturated fatty acids seem to have the greatest effects, but this may be because they can insert more readily into membranes.

2. Analysis

Accurate measurement of free fatty acids concentrations in plasma and tissues can be a useful measure of metabolic status. Unfortunately, it is very easy to generate free acids artefactually by faulty storage or extraction. Lipases can continue to function slowly in some tissues, even at –20°C, and the process will accelerate if tissues are allowed to thaw prior to extraction. In one important series of experiments, it was shown that if animal tissues were pulverized and extracted at –70°C, very low levels only of free fatty acids were detected in comparison to more conventional procedures [Kramer, J.K.G. and Hulan, H.W., *J. Lipid Res*., **19**, 103-106 (1978)]. The high concentrations reported occasionally in the literature are obviously impossible in living tissues and are due to inappropriate sample handling.

Following extraction of lipids from tissues by a suitable procedure, a free fatty acid fraction can be isolated by thin-layer chromatography or by solid-phase extraction chromatography on a bonded amine phase. This can be methylated and analysed by gas chromatography with an internal standard.

Glycerophospholipids

Phosphatidic acid or 1,2-diacyl-*sn*-glycerol-3-phosphate is found in trace amounts only in tissues under normal circumstances, but it has great metabolic importance as a biosynthetic precursor of all other glycerolipids. It is strongly acidic and is usually isolated as a mixed salt. One specific isomer is illustrated as an example.

Lysophosphatidic acid with one mole of fatty acid per mole of lipid (in position *sn*-1) is a marker for ovarian cancer, and is a key cellular messenger.

1-hexadecanoyl, 2-(9Z, 12Z-octadecadienoyl)-*sn*-glycero-3-phosphate
(phosphatidic acid)
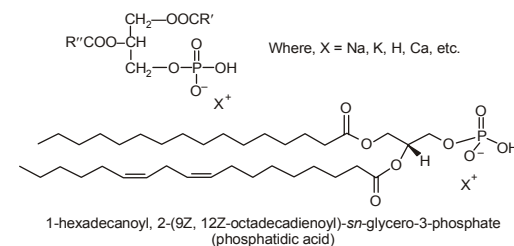
Phosphatidic Acid—Occurrence and Biosynthesis

Phosphatidic acid is not an abundant lipid constituent of any living organism to my knowledge, but it is extremely important as an intermediate in the biosynthesis of triacylglycerols and phospholipids. Indeed, it is often over-estimated in tissues as it can arise by inadvertent enzymatic hydrolysis during inappropriate storage or extraction conditions during analysis. The molecule is acidic and bears a negative charge so requires a counter ion.

1-hexadecanoyl, 2-(9Z, 12Z-octadecadienoyl-*sn*-glycero-3-phosphate

The main biosynthetic route in plant and animal tissues involves sequential acylation of á-glycerophosphate, derived from catabolism of glucose, by acyl-coA derivatives of fatty acids as illustrated. Specific acyltransferases catalyse first the acylation of position *sn*-1 to form lysophosphatidic acid (1-acyl-*sn*-glycerol-3-phosphate) and then of position *sn*-2 to yield phosphatidic acid.

In mammals, the glycerol-3-phosphate acyltransferase that catalyses the first step exists in four isoforms, two in mitochondria (designated GPAT1 and 2) and two in the endoplasmic reticulum (GPAT3 and 4). The activity in the endoplasmic reticulum predominates in adipose tissue, but the mitochondrial forms are responsible for half the activity in liver. All are membrane-bound enzymes, which are believed

to span the membranes, but many questions remain regarding the regulation and function of the different isoforms. Similarly, at least two acyl-CoA:lysophosphatidic acid acyltransferases (LPAAT1 and LPAAT2) are known that catalyse the second step. Human LPAAT1 showed higher activity with 14:0-, 16:0- and 18:2-CoAs, while LPAAT2 prefers 20:4-CoA.

Under some conditions, phosphatidic acid can be generated from 1,2-diacyl-*sn*-glycerols by the action of diacylglycerol kinases (see our webpage on diacylglycerols). However, a more important route in quantitative terms is via hydrolysis of other phospholipids, but especially phosphatidylcholine, by the enzyme phospholipase D (or by a family or related enzymes of this kind). This enzyme is present in most animal cell types, and its activity is regulated by phosphatidylinositol-4,5-bisphosphate and protein kinase C.

The subsequent steps in the utilization of phosphatidic acid in the biosynthesis of triacylglycerols and phospholipids are described in separate documents in this section of the website. In brief, hydrolysis of phosphatidic acid by the enzyme phosphatidate phosphatase is the source of *sn*-1,2-diacylglycerols (DAG), which are the precursors for the biosynthesis of triacylglycerols (TAG), phosphatidylcholine, (PC) and phosphatidylethanolamine (PE) via the so-called Kennedy pathway (also of monogalactosyldiacylglycerols in plants). Via reaction with cytidine triphosphate, phosphatidic acid is the precursor of cytidine diphosphate diacylglycerol, which is the key intermediate in the synthesis of phosphatidylglycerol (PG), and thence of cardiolipin, and of phosphatidylinositol (PI) and phosphatidylserine (PS). Depending on the organism and other factors, phosphatidylserine can be a precursor for phosphatidylethanolamine. Similarly, the latter can give rise to phosphatidylcholine by way of mono- and dimethylphosphatidylethanolamine intermediates.

The fatty acid composition of phosphatidic acid can resemble that of the eventual products, though in many instances this can be much altered by re-modelling after synthesis via deacylation-reacylation reactions.

2. Phosphatidic Acid - Biological Functions

The phosphatidic acid generated by the action of phospholipase D and by diacylglycerol kinases may have signalling functions as a second messenger, although it is not certain whether all the activities suggested by studies *in vitro* operate *in vivo*. Nonetheless, phosphatidic acid has been implicated in many aspects of animal cell biochemistry and physiology, including cell proliferation and differentiation, cell transformation, tumor progression and survival signalling. It appears to regulate some membrane trafficking events, and it is involved in activation of the enzyme NADPH oxidase, which operates as part of the defense mechanism against infection and tissue damage during inflammation. It may have a role in promoting phospholipase A_2 activity, and it appears to function in vesicle formation and transport within the cell. By binding to targeted proteins, including protein kinases, protein phosphatases and G-proteins, it may increase or inhibit their activities. For example in yeast, phosphatidic acid on the endoplasmic reticulum binds directly to a specific transcriptional repressor to keep it inactive outside the nucleus; when the lipid precursor inositol is added, this phosphatidic acid is rapidly depleted, releasing the transcriptional factor so that it can be translocated to the nucleus where it is able to repression target genes. The overall effect is a mechanism to control phospholipid synthesis. In addition, the murine phosphatidylinositol 4-phosphate 5-kinase does not appear to function unless phosphatidic acid is bound.

In relation to signalling activities, it should be noted that phosphatidic acid can be metabolized to *sn*-1,2-diacylglycerols or to lysophosphatidic acid (see next section), both of which have distinctive signalling functions in their own right. Conversely, both of these compounds can be in effect be de-activated by conversion back to phosphatidic acid.

In many cell types, vesicle trafficking, secretion and endocytosis may also require phosphatidic acid derived by the action of phospholipase D. For example, a specific form of this enzyme located on the outer surface of mitochondria acts upon cardiolipin to promote mitochondrial fusion.

Some of these effects may be explained simply by the physical properties of phosphatidic acid, which has a propensity to form a hexagonal II phase, especially in the presence of calcium ions. Thus, hydrolysis of phosphatidylcholine, a cylindrical, non-fusogenic lipid, converts it into cone-shaped, fusogenic phosphatidic acid, which promotes negative membrane curvature. It can effect membrane fusion in model systems, probably because of its ability to form non-bilayer phases. Also of relevance in this context is its overall negative charge, and it is not always clear whether some of the observed biological effects are specific to phosphatidic acid or simply to negatively charged phospholipids in general. However, it has been demonstrated that the positively charged lysine and arginine residues on proteins can bind with some specificity to phosphatidic acid through hydrogen bonding with the phosphate group, thus distinguishing it from other phospholipids.

Phosphatidic acid in plants. Phospholipase D activity and the phosphatidic acid produced may be even more significant in plants. They have long been recognized as of importance during germination

and senescence, and they appear to have a role in response to stress damage and pathogen attack. A high content of phosphatidic acid induced by phospholipase D action during wounding or senescence brings about a loss of the membrane bilayer phase, as a consequence of the conical shape of this phospholipid in comparison to the cylindrical shape of structural phospholipids. As a result, cells lose their viability. The phosphatidic acid generated in this way is broken down further by phosphatases, acyl-hydrolases and lipoxygenases into fatty acids and other small molecules, which are subsequently absorbed and recycled. In addition, phosphatidic acid is important in the response to other forms of stress, including osmotic stress (salinity or drought), cold, and oxidation, although much remains to be learned of the mechanism by which it exerts its effects.

Phosphatidic acid is of considerable importance in cellular signalling in plants, for example in promoting pollen-tube growth, decreasing peroxide-induced cell death, and mediating the signalling processes that lead to responses to the plant hormone abscisic acid. Thus in the 'model' plant *Arabidopsis*, which contains twelve distinct members of the phospholipase D family, phosphatidic acid generated by the action of the enzymes interacts with a protein phosphatase to signal the closure of stomata promoted by abscisic acid; it interacts also with a further enzyme to mediate the inhibition of stomatal opening effected by abscisic acid. Together these reactions constitute a signalling pathway that regulates water loss from plants.

3. Lysophosphatidic Acid

Lysophosphatidic acid or 1-acyl-*sn*-glycerol-3-phosphate differs from phosphatidic acid in having only one mole of fatty acid per mole of lipid. As such, it is the simplest possible glycerophospholipid. Molecular species with both saturated and unsaturated fatty acid constituents occur in different tissues. Although it is present at very low levels only in animal tissues, it is extremely important biologically, influencing many biochemical processes. These activities seem to be shared by the 1-alkyl- and alkenyl-ether forms. It is also important as the biosynthetic precursor of phosphatidic acid.

In particular, lysophosphatidic acid is an intercellular lipid mediator with growth factor-like activities, and is rapidly produced and released from activated platelets to influence target cells. However, a more important source is the activity of a specific lysophospholipase D ('autotaxin'), part of the blood-clotting process, on lysophosphatidylcholine, which yields lysophosphatidic acid in an albumin-bound form. This is more abundant in serum (1-5 ìM) than in plasma, where it accounts for much of the biological activity.

Lysophosphatidylcholine → Phospholipase D ('autotaxin') → Lysophosphatidic acid

It is now established that it is produced by a wide variety of cell types, both by the action of autotaxin and by that of a phosphatidic acid-selective phospholipase A_1, i.e. to produce a *sn*-2-acyl-

lysophosphatidic acid in this instance, and almost certainly also by the action of a lysophospholipase A_2. A surprising recent finding is that the activity of the phosphatidic acid-selective lysophospholipase A_1 is essential for normal hair growth in humans.

Most mammalian cells express receptors for lysophosphatidic acid, and lysophosphatidic acid may initiate signalling in the cells in which it is produced, as well as affecting neighbouring cells. In the last few years, the characterization of cloned lysophosphatidic acid receptors in combination with strategies of molecular genetics has allowed determination of both signalling and biological effects that are dependent on receptor mechanisms. At least seven G protein-coupled receptors that are specific for lysophosphatidic acid have now been identified. Experimental activation of these receptors has shown that a range of downstream signalling cascades mediate lysophosphatidic acid signalling. These include activation of protein kinases, adenyl cyclase and phospholipase C, release of arachidonic acid, and much more. There is evidence that lysophosphatidic acid is involved in cell survival in some circumstances, and in programmed cell death in others. In some instances, molecular species with specific fatty acid components may be involved.

There is particular interest in the activity of lysophosphatidic acid in various disease states, where intervention in its metabolism has the potential for beneficial health effects. For example, a finding that lysophosphatidic acid is markedly elevated in the plasma of ovarian cancer patients, compared to healthy controls may be especially significant. In particular, elevated plasma levels were found in patients in the first stage of ovarian cancer, suggesting that it may represent a useful marker for the early detection of the disease. Lysophosphatidic acid is believed to stimulate DNA synthesis and the proliferation of ovarian cancer cells, and it may induce cell migration. Therefore, it is a target of the pharmaceutical industry for cancer therapy.

In addition, lysophosphatidic acid generated by the action of a lysophospholipase D is believed to play an important role in reproductive biology. Under certain conditions, it can become athero- and thrombogenic and might aggravate cardiovascular disease. This may be especially important in cancer patients. Lysophosphatidic acid has also been found in saliva in significant amounts, and it has been suggested that it is involved in wound healing in the upper digestive organs such as the mouth, pharynx, and esophagus. It has similar effects when applied topically to skin wounds, probably by stimulating proliferation of new cells to seal the wound. There is also evidence that the lipid is involved in brain development and vascular remodelling.

Catabolic deactivation of lysophosphatidic acid is accomplished by dephosphorylation to monoacylglycerol by a family of three lipid phosphate phosphatases, which also dephosphorylate sphingosine-1-phosphate, phosphatidic acid and ceramide 1-phosphate in a non-specific manner. It can be converted back to phosphatidic acid by a membrane-bound *O*-acyltransferase (MBOAT2) specific for lysophosphatidic acid (and lysophosphatidylethanolamine) with a preference for oleoyl-CoA as substrate.

Other lysophospholipids and especially the sphingolipid analogue, sphingosine-1-phosphate, show a related range of activities.

4. Cyclic Phosphatidic Acid

Cyclic phosphatidic acid

Cyclic phosphatidic acid (sometimes termed 'cyclic lysophosphatidic acid') was isolated originally from a slime mould, but has now been detecte of cyclic phosphatidic acidd in a wide range of organisms including humans, especially in the brain but also bound to albumin in serum (at a concentration of 10^{-7}M, or a tenth that of lysophosphatidic acid). It has a cyclic phosphate at the *sn*-2 and *sn*-3 positions of the glycerol carbons, and this structure is absolutely necessary for its biological activity. It is most abundant in tissues subject to injury. In human serum, the main molecular species contains palmitic acid.

While cyclic phosphatidic acid may have some similar signalling functions to lysophosphatidic acid *per se* in that it binds to some of the same receptors, it also has some quite distinct activities in animal tissues. For example, cyclic phosphatidic acid is known to be a specific inhibitor of DNA polymerase *alpha*. It has an appreciable effect on the inhibition of cancer cell invasion and metastasis, a finding that is currently attracting great pharmacological interest. In addition, it inhibits the platelet aggregation induced by lysophosphatidic acid.

Studies of the biosynthesis of cyclic phosphatidic acid in fetal bovine serum suggest that it is the product of an enzyme related to the human enzyme autotaxin, a serum lysophospholipase D that produces lysophosphatidic acid. This enzyme appears to produce cyclic phosphatidic acid in serum by an intramolecular transphosphatidylation reaction. However, it can also be formed artefactually by the addition of strong acid to serum.

5. Pyrophosphatidic Acid

Pyrophosphatidic acid or *sn*-1,2-diacylglycero-3-pyrophosphate is an unusual and little known phospholipid that was first identified as a minor component in yeasts, and is also know to be present in mushrooms and higher plants as a product of the enzyme phosphatidic acid kinase.

diacyl-*sn*-glycerol-3-pyrophosphate

It is rapidly metabolized back to phosphatidic acid by a specific phosphatase and thence to diacylglycerols, and it may have a function in the phospholipase C and D signalling cascades in plants. Pyrophosphatidic acid is barely detectable in non-stimulated plant cells but its concentration increases very rapidly in response to stress situations, including osmotic stress and attack by pathogens. Such findings add to the belief that it is an important signalling molecule in plants under stress. In yeasts, it may have a role in the regulation of the synthesis and metabolism of phospholipids, especially phosphatidylserine.

6. Analysis

Phosphatidic acid and related lipids are not the easiest to analyse. On adsorption chromatography, retention times tend to be variable and may be dependent to some extent on the nature of the cations associated with the acidic lipids. However, two-dimensional TLC can give good results. Phosphatidic acid, lysobisphosphatidic acid and pyrophosphatidic acid are never easy to distinguish, and the best hope for success appears to lie with modern liquid chromatography-mass spectrometric methods.

Phosphatidylglycerol

Phosphatidylglycerol or 1,2-diacyl-*sn*-glycerol-3-phosphoryl-1'-*sn*-glycerol tends to be a trace constituent of most tissues, but it is often the main component of some bacterial membranes. It has important functions in lung surfactant, where its physical properties are significant, and in plant chloroplasts, where it appears to have an essential role in photosynthesis. Also, it is the biosynthetic precursor of cardiolipin. In some bacterial species, the 3'-hydroxyl of the phosphatidylglycerol moiety is linked to an amino acid (lysine, ornithine or alanine) to form an *O*-aminoacylphosphatidylglycerol or 'lipoamino acid'.

Phosphatidylglycerol in Bacteria and Plants

Phosphatidylglycerol is a ubiquitous lipid that can be the main component of some bacterial membranes, and it is found also in membranes of plants and animals where it appears to perform specific functions. The charge on the phosphate group means that it is an anionic lipid at neutral pH. The dihexadecanoyl species is illustrated as an example.

1,2-dihexadecanoyl-*sn*-glycero-3-phospho-(1'-sn-glycerol)
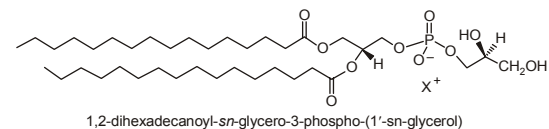
Phosphatidylglycerol is found in almost all bacterial types. For example, *Escherichia coli,* a widely studied organism, has up to 20% of phosphatidylglycerol in its membranes (phosphatidylethanolamine makes up much of the rest with a little cardiolipin). In many bacteria, the diacyl form of the lipid predominates, but in others the alkylacyl- and alkenylacyl forms are more abundant. Phosphatidylglycerol is synthesised only in mitochondria of non-photosynthetic eukaryotes, and it is used as the precursor for cardiolipin, which is located in the inner mitochondrial membrane and is required for proper functioning of the enzymes involved in oxidative phosphorylation.

There is conflicting evidence on as to whether *E. coli* has an absolute requirement for phosphatidylglycerol in its membranes. For example, studies with mutants deficient in

phosphatidylglycerol have suggested that its absence results in defective DNA replication and a lack of a necessary modification to the main cellular lipoprotein or proteolipid, leading to membrane welding and eventually cell death. However, others have concluded from similar experiments that phosphatidylglycerol and cardiolipin are entirely dispensable and can be substituted by other anionic phospholipids such as phosphatidic acid. There is evidence that in some bacterial membranes, phosphatidylglycerol may be segregated into distinct domains, which differ in lipid and protein composition and degree of order from other regions.

In cyanobacteria and plants, which are able to carry out aerobic photosynthesis, phosphatidylglycerol is found in all cellular membranes, but it appears to be especially important in the thylakoid membrane, which surrounds the chloroplast, and where it is the only phospholipid comprising up to 10% of the total lipids with a high proportion (up to 70%) in the outer monolayer (much of the remaining lipid is glycosyldiacylglycerols). While sulfoquinovosyldiacylglycerol can substitute for phosphatidylglycerol to a certain extent, especially under conditions of phosphate deficiency and presumably to maintain a required level of anionic lipids, a minimum level of phosphatidylglycerol appears to be essential for photosynthesis and growth.

In the photosynthetic membranes of leaf tissue of higher plants, the phosphatidylglycerol is unique in that it contains a high proportion of *trans*-3-hexadecenoic acid, which is located exclusively in position *sn*-2 (**Table**). This fatty acid is not found in other lipids of the thylakoid membrane. The rate of its synthesis in leaves deprived of light is greatly reduced (with accumulation of the precursor palmitic acid).

Table. Composition (mol %) of fatty acids in positions *sn*-1 and *sn*-2 of phosphatidylglycerol from leaves of *Arabidopsis thaliana*.

Position	Fatty acids					
	16:0	*trans*-3-16:1	18:0	18:1	18:2	18:3(n-3)
sn-1	22	-	trace	9	13	55
sn-2	43	41	trace	1	8	8

Data from: Browse, J., Warwick, N., Somerville, C.R. and Slack, C.R. *Biochem. J.*, **235**, 25-31 (1986).

It is interesting to note that saturated and monoenoic fatty acids are concentrated in position *sn*-2 and polyunsaturated in position *sn*-1, the opposite of that found for most animal phospholipids other than phosphatidylglycerol. This is because phosphatidylglycerol is synthesised in chloroplasts via the so-called "prokaryotic" pathway only (see below and in our web-pages on mono- and digalactosyldiacylglycerols for further discussion of this phenomenon). In some plant species, position *sn*-2 of the thylakoid phosphatidylglycerol is occupied exclusively with C_{16} fatty acids giving a rather distinctive molecular species distribution.

While the role of phosphatidylglycerol in the photosynthetic apparatus of higher plants is still unclear, it is known that in cyanobacteria, it is essential for the oligomerization of photosystems I and II. Analysis of the crystal structure of the photosystem I of cyanobacteria has shown that it contains three molecules of phosphatidylglycerol and one of monogalactosylglycerol as integral components,

while phosphatidylglycerol is one of 14 lipid molecules bound to the photosystem II complex. This phospholipid also appears to be required for crystallization and polymerization of the light-harvesting complex II in pea chloroplasts, where it may be the 'glue' that binds the individual protein components. A report that *trans*-3-hexadecenoic acid in phosphatidylglycerol is essential for the latter process has been questioned.

Disaturated molecular species of phosphatidylglycerol in plants are believed to be an important factor in sensitivity to chilling, and experiments with genetic modifications to increase the degree of unsaturation of this lipid have produced plants with a greater resistance to cold. However, there are discrepancies between the results of different experimental approaches, and other factors are certainly involved.

In cyanobacteria in addition to its role in photosynthesis, phosphatidylglycerol is intimately involved in the processes of cell fission and division. Here its propensity to form non-bilayer structures in the presence of calcium ions may be important, aided by its ability to bind to specific proteins. There may be parallels with the division of plastids in higher plants.

A fully acylated phosphatidylglycerol, termed **bis-phosphatidic acid** or phosphatidyldiacylglycerol, and plasmalogen analogues have been found in bacteria. While this structure has on occasion been ascribed in error to other lipids in developing seeds or brain, it can indeed be formed in animal tissues (see below). Two other unusual phosphatidylglycerol derivatives based on an archaeol backbone, i.e. phosphatidylglycerol sulfate and phosphatidylglycerol phosphate methyl ester, are unique constituents of the primitive organisms, the Haloarchaea. They are important constituents of bacteriorhodopsin, a retinal-containing integral membrane protein of the cytoplasmic membrane, which forms two-dimensional crystalline patches known as the purple membrane. The complex lipoamino acids and acylphosphatidylglycerols are discussed below.

2. Phosphatidylglycerol in Animal Tissues

Phosphatidylglycerol is present at a level of 1-2% in most animal tissues, but it can be the second most abundant phospholipid in lung surfactant at up to 11% of the total (in a few species, it is replaced by another acidic lipid, phosphatidylinositol). It is well established that the concentration of phosphatidylglycerol increases during foetal development, coincident with the formation of stable lamellar phases, but its precise function is a matter of conjecture. For example, it may aid the spreading of dipalmitoyl-phosphatidylcholine, which is presumed to be the main functional component of lung surfactant. The fatty acid composition of lung tissue from several species is listed in Table.

In each, the content of saturated fatty acids is high while that of the polyunsaturated components is relatively low in comparison to phospholipids in other tissues. It has also been shown that lung phosphatidylglycerol in many animals contains a high proportion of disaturated molecular species, although this does not appear to be true of human lung surfactant. It seems that the acidic head-group is more important to surfactant function than the precise molecular species composition.

The lung aside, phosphatidylglycerol may be present in animal tissues merely as a precursor for diphosphatidylglycerol (cardiolipin). As an example of another tissue, the positional distribution of fatty

Table 2.10 : Fatty acid composition (weight % of the total) in lung phosphatidylglycerol from various species

Fatty acid	Species			
	pig	cow	rabbit	guinea pig
16:0	27	34	29	37
16:1	2	1	3	6
18:0	21	15	19	18
18:1	34	37	27	24
18:2	7	3	8	5
18:3	1	2	1	2
20:3	1	1	1	trace
20:4(n-6)	3	3	4	3
22:4(n-6)	1	1	3	2
22:5	1	1	3	1
22:6(n-3)	1	1	1	1

Data from: Okano, G. and Akino, T. *Lipids*, 14, 541-546 (1979).

acids in rat liver phosphatidylglycerol is listed in **Table**. Like cardiolipin, there is a very high proportion of linoleate, much of which is concentrated in position *sn*-1.

Table 2.11 : Positional distribution of fatty acids in phosphatidylglycerol from rat liver

Position	Fatty acid					
	16:0	18:0	18:1	18:2	20:4	22:6
sn-1	7	3	3	81		
sn-2	3	1	34	50	2	1

Data from: Wood, R. and Harlow, R.D. *Arch. Biochem. Biophys.*, **135**, 272-281 (1969).

3. Biosynthesis of Phosphatidylglycerol

In animal, plant and microbial tissues, phosphatidylglycerol is formed from phosphatidic acid by a sequence of enzymatic reactions that proceeds via the intermediate, cytidine diphosphate diacylglycerol (CDP-diacylglycerol), which is rarely detected as a normal component of tissues amounting to only 0.05% or so of the total phospholipids. Biosynthesis proceeds by condensation of phosphatidic acid and cytidine triphosphate with elimination of pyrophosphate via the action of an enzyme phosphatidate cytidyltransferase (or CDP-synthase). The same liponucleotide is an important intermediate in the biosynthesis of phosphatidylinositol, but rather different routes are taken to phosphatidylcholine and phosphatidylethanolamine.

CDP-diacylglycerol reacts with glycerol-3-phosphate via phosphatidylglycerophosphate synthase to form 3-*sn*-phosphatidyl-1'-*sn*-glycerol 3'-phosphoric acid, with release of cytidine monophosphate (CMP). Finally, phosphatidylglycerol is formed by the action of one of two phosphatases. As biosynthesis is via glycerol-3-phosphate, the second glycerol moiety is attached at position *sn*-1 to the phosphate group. However, there are other minor biosynthetic routes to phosphatidylglycerol, e.g. by phospholipase D-catalysed catabolism of diphosphatidylglycerol (cardiolipin) or by glycerolysis of other phospholipids (also catalysed by phospholipase D), which can change the stereochemistry in part (in effect, racemization).
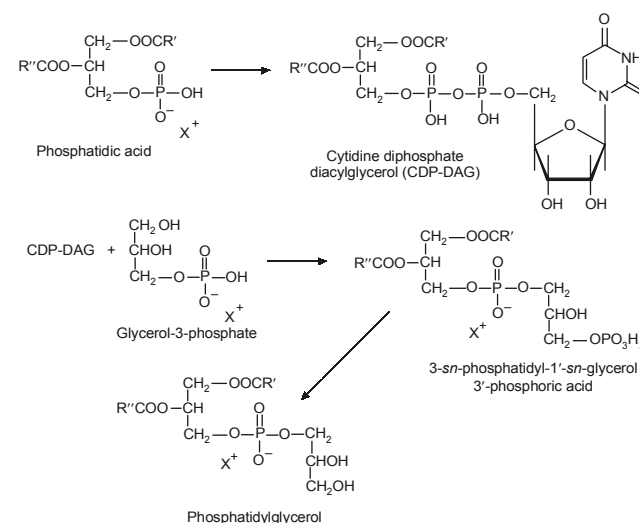
In animal tissues, the eventual fatty acid composition is attained by the process of remodelling known as the Lands' cycle (see the webpage on phosphatidylcholine, for example). The first step, is hydrolysis by a phospholipase A_2 to lysophosphatidylglycerol, followed by reacylation by means of an acyl-CoA:lysophosphatidylglycerol acyltransferase. The human form of the latter, designated LPGAT1, has been characterized and found to have a preference for 16:0-, 18:0-, and 18:1-CoAs as donors.

In cyanobacteria, a disaturated molecular species of phosphatidylglycerol is synthesised first, and the fatty acid in position *sn*-1 is subsequently desaturated by specific acyl-lipid desaturases. That in position *sn*-2 is not affected. In higher plants, phosphatidylglycerol is synthesised in three cellular compartments, plastids, endoplasmic reticulum and mitochondria. In the plastids, the selectivity of the acyltransferases is such that the initial molecular species formed contains oleic acid in position *sn*-1 and

palmitic acid in position *sn*-2. Some of the palmitate in position *sn*-2 is desaturated to the *trans*-3 isomer, while the oleate in position *sn*-1 is desaturated to 18:2 and 18:3 fatty acids. In the endoplasmic reticulum in contrast, the initial molecular species contain palmitic and oleic acids in position *sn*-1 and oleic acid in position *sn*-2. The oleate in both positions, but not the palmitate, is further desaturated by acyl-lipid desaturases until the final fatty acid compositions are attained. These details of the biosynthetic processes that occur in mitochondria have still to be determined.

Phosphatidylglycerol is the biosynthetic precursor of cardiolipin, lysobisphosphatidic acid and many glycophospholipids, as well as bacterial proteolipids, lipoteichoic acids and the complex lipoamino acids (the last are discussed below).

4. Acylphosphatidylglycerol

Acylphosphatidylglycerol or (1,2-diacyl-*sn*-glycero-3-phospho-(3'-acyl)-1'-*sn*-glycerol) was first isolated as a minor component of the phospholipids of the bacterium *Salmonella typhimurium*, and it has since been found in a number of prokaryotic species, including Escherichia coli. In particular, it is a characteristic component of the membranes of Corynebacteria and is especially abundant in those species that lack mycolic acids. *C. amycolatum*, for example, contained 20-29% of this lipid, with mainly C_{14} to C_{18} saturated and monoenoic fatty acid components; the fatty acid on the head group glycerol was mainly oleate. It has also been found in parasitic protozoa, such as *Trichomonas vaginalis* and *T. foetus*. The only report of its occurrence in plants is from oats (*Avena sativa*), which are also known to contain *N*-acylphosphatidylethanolamine in appreciable amounts.

Acylphosphatidyglycerol
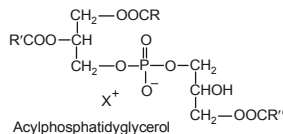
Acylphosphatidylglycerol is formed *in vitro* in experiments designed to study the biosynthesis of lysobisphosphatidic acid in animal cells, and in this instance the fatty acid on the glycerol head group is presumed to be in the *sn*-2' position. It is not clear whether it occurs naturally in animal tissues.

***Bis*-phosphatidic acid** or phosphatidyldiacylglycerol (fully acylated phosphatidylglycerol) can be produced as a minor component of animal cells by trans-phosphatidylation of phosphatidylcholine with diacylglycerol, catalysed by the enzyme phospholipase D, a possible mechanism for removing excess messenger diacylglycerol. In this instance, the stereochemistry of the glycerol is presumably different from that in normal phosphatidylglycerol, i.e. the phosphate will be attached to the *sn*-3/*sn*-3' positions. The *bis*-phosphatidic acid found in lysosomes is related to lysobisphosphatidic acid.

5. Complex Lipoamino Acids

In some Gram-positive bacterial species, the 3'-hydroxyl of the phosphatidylglycerol moiety may be esterified to an amino acid (lysine, ornithine or alanine) to form an *O*-aminoacylphosphatidylglycerol, or it can be linked to another fatty acid. The former have been termed **lipoamino acids**, though it might

be better to call them "complex lipoamino acids" to distinguish them from those consisting simply of a fatty acid linked to an amino acid (see our web page on simple amides).

Alanylphosphatid ylglycerol

Lysylphosphatidylglycerol
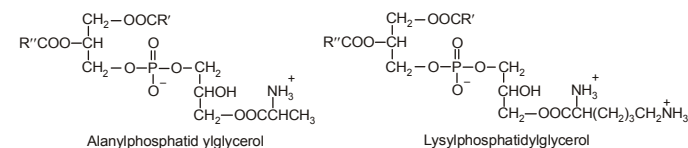
There is evidence that the function of lysylphosphatidylglycerol and other lipoamino acids in the membranes of pathogenic bacteria is to protect them from antimicrobial cationic polypeptides produced by plants and animals. Membranes containing this lipid are much less permeable than those containing phosphatidylglycerol *per se*, especially when exposed to acidic conditions.

The betaine lipids, together with phosphatidylserine, phosphatidylthreonine, and related lipids discussed elsewhere, could also be termed complex lipoamino acids.

6. Analysis

Phosphatidylglycerol is not the easiest phospholipid to analyse. It tends to elute close to phosphatidic acid in many chromatographic systems, but it can usually be resolved by two-dimensional thin-layer chromatography. Electrospray mass spectrometry under negative ionization conditions appears to be well suited to determination of molecular species composition. Similarly, modern mass spectrometric methods seem to be suited to the analysis of the complex lipoamino acids.

Cardiolipin (diphosphatidylglycerol or more precisely 1,3-*bis*(*sn*-3'-phosphatidyl)-*sn*-glycerol) is a unique phospholipid with in essence a dimeric structure, having four acyl groups and potentially carrying two negative charges (and is thus an acidic lipid). It is an important constituent of mitochondrial lipids especially, so heart muscle is a rich source. Amongst other functions, it plays a key role in modifying the activities of the enzymes concerned with oxidative phosphorylation. (More...).

Cardiolipin

Lysobisphosphatidic acid
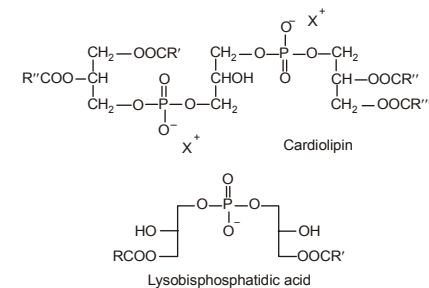
Lysobisphosphatidic acid or bis(monoacylglycerol)phosphate is an interesting lipid as its stereochemical configuration differs from that of all other animal glycero-phospholipids in that the phosphodiester moiety is linked to positions *sn*-1 and *sn*-1' of glycerol, rather than to position *sn*-3, to which the fatty acids are esterified (some think that position *sn*-2 is more likely for the latter). It is usually a rather minor component of animal tissues, but is enriched in the lysosomes of liver and appears to be a marker for this organelle. The glycerophosphate backbone is particularly stable, presumably because of the unusual stereochemistry.

Phosphatidic acid is not an abundant lipid constituent of any living organism to my knowledge, but it is extremely important as an intermediate in the biosynthesis of triacylglycerols and phospholipids. Indeed, it is often over-estimated in tissues as it can arise by inadvertent enzymatic hydrolysis during inappropriate storage or extraction conditions during analysis. The molecule is acidic and bears a negative charge so requires a counter ion.

1-hexadecanoyl, 2-(9Z, 12Z-octadecadienoyl-*sn*-glycero-3-phosphate

The main biosynthetic route in plant and animal tissues involves sequential acylation of á-glycerophosphate, derived from catabolism of glucose, by acyl-coA derivatives of fatty acids as illustrated. Specific acyltransferases catalyse first the acylation of position *sn*-1 to form lysophosphatidic acid (1-acyl-*sn*-glycerol-3-phosphate) and then of position *sn*-2 to yield phosphatidic acid.

In mammals, the glycerol-3-phosphate acyltransferase that catalyses the first step exists in four isoforms, two in mitochondria (designated GPAT1 and 2) and two in the endoplasmic reticulum (GPAT3 and 4). The activity in the endoplasmic reticulum predominates in adipose tissue, but the mitochondrial forms are responsible for half the activity in liver. All are membrane-bound enzymes, which are believed to span the membranes, but many questions remain regarding the regulation and function of the different isoforms. Similarly, at least two acyl-CoA:lysophosphatidic acid acyltransferases (LPAAT1 and LPAAT2) are known that catalyse the second step. Human LPAAT1 showed higher activity with 14:0-, 16:0- and 18:2-CoAs, while LPAAT2 prefers 20:4-CoA.

Under some conditions, phosphatidic acid can be generated from 1,2-diacyl-*sn*-glycerols by the

action of diacylglycerol kinases (see our webpage on diacylglycerols). However, a more important route in quantitative terms is via hydrolysis of other phospholipids, but especially phosphatidylcholine, by the enzyme phospholipase D (or by a family or related enzymes of this kind). This enzyme is present in most animal cell types, and its activity is regulated by phosphatidylinositol-4,5-bisphosphate and protein kinase C.

The subsequent steps in the utilization of phosphatidic acid in the biosynthesis of triacylglycerols and phospholipids are described in separate documents in this section of the website. In brief, hydrolysis of phosphatidic acid by the enzyme phosphatidate phosphatase is the source of *sn*-1,2-diacylglycerols (DAG), which are the precursors for the biosynthesis of triacylglycerols (TAG), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) via the so-called Kennedy pathway (also of monogalactosyldiacylglycerols in plants). Via reaction with cytidine triphosphate, phosphatidic acid is the precursor of cytidine diphosphate diacylglycerol, which is the key intermediate in the synthesis of phosphatidylglycerol (PG), and thence of cardiolipin, and of phosphatidylinositol (PI) and phosphatidylserine (PS). Depending on the organism and other factors, phosphatidylserine can be a precursor for phosphatidylethanolamine. Similarly, the latter can give rise to phosphatidylcholine by way of mono- and dimethylphosphatidylethanolamine intermediates.

The fatty acid composition of phosphatidic acid can resemble that of the eventual products, though in many instances this can be much altered by re-modelling after synthesis via deacylation-reacylation reactions.

2. Phosphatidic Acid - Biological Functions

The phosphatidic acid generated by the action of phospholipase D and by diacylglycerol kinases may have signalling functions as a second messenger, although it is not certain whether all the activities suggested by studies *in vitro* operate *in vivo*. Nonetheless, phosphatidic acid has been implicated in many aspects of animal cell biochemistry and physiology, including cell proliferation and differentiation, cell transformation, tumor progression and survival signalling. It appears to regulate some membrane trafficking events, and it is involved in activation of the enzyme NADPH oxidase, which operates as part of the defense mechanism against infection and tissue damage during inflammation. It may have a role in promoting phospholipase A_2 activity, and it appears to function in vesicle formation and transport within the cell. By binding to targeted proteins, including protein kinases, protein phosphatases and G-proteins, it may increase or inhibit their activities. For example in yeast, phosphatidic acid on the endoplasmic reticulum binds directly to a specific transcriptional repressor to keep it inactive outside the nucleus; when the lipid precursor inositol is added, this phosphatidic acid is rapidly depleted, releasing the transcriptional factor so that it can be translocated to the nucleus where it is able to repression target genes. The overall effect is a mechanism to control phospholipid synthesis. In addition, the murine phosphatidylinositol 4-phosphate 5-kinase does not appear to function unless phosphatidic acid is bound.

In relation to signalling activities, it should be noted that phosphatidic acid can be metabolized to *sn*-1,2-diacylglycerols or to lysophosphatidic acid (see next section), both of which have distinctive signalling functions in their own right. Conversely, both of these compounds can be in effect be de-activated by conversion back to phosphatidic acid.

In many cell types, vesicle trafficking, secretion and endocytosis may also require phosphatidic acid derived by the action of phospholipase D. For example, a specific form of this enzyme located on the outer surface of mitochondria acts upon cardiolipin to promote mitochondrial fusion.

Some of these effects may be explained simply by the physical properties of phosphatidic acid, which has a propensity to form a hexagonal II phase, especially in the presence of calcium ions. Thus, hydrolysis of phosphatidylcholine, a cylindrical, non-fusogenic lipid, converts it into cone-shaped, fusogenic phosphatidic acid, which promotes negative membrane curvature. It can effect membrane fusion in model systems, probably because of its ability to form non-bilayer phases. Also of relevance in this context is its overall negative charge, and it is not always clear whether some of the observed biological effects are specific to phosphatidic acid or simply to negatively charged phospholipids in general. However, it has been demonstrated that the positively charged lysine and arginine residues on proteins can bind with some specificity to phosphatidic acid through hydrogen bonding with the phosphate group, thus distinguishing it from other phospholipids.

Phosphatidic acid in plants. Phospholipase D activity and the phosphatidic acid produced may be even more significant in plants. They have long been recognized as of importance during germination and senescence, and they appear to have a role in response to stress damage and pathogen attack. A high content of phosphatidic acid induced by phospholipase D action during wounding or senescence brings about a loss of the membrane bilayer phase, as a consequence of the conical shape of this phospholipid in comparison to the cylindrical shape of structural phospholipids. As a result, cells lose

their viability. The phosphatidic acid generated in this way is broken down further by phosphatases, acyl-hydrolases and lipoxygenases into fatty acids and other small molecules, which are subsequently absorbed and recycled. In addition, phosphatidic acid is important in the response to other forms of stress, including osmotic stress (salinity or drought), cold, and oxidation, although much remains to be learned of the mechanism by which it exerts its effects.

Phosphatidic acid is of considerable importance in cellular signalling in plants, for example in promoting pollen-tube growth, decreasing peroxide-induced cell death, and mediating the signalling processes that lead to responses to the plant hormone abscisic acid. Thus in the 'model' plant *Arabidopsis*, which contains twelve distinct members of the phospholipase D family, phosphatidic acid generated by the action of the enzymes interacts with a protein phosphatase to signal the closure of stomata promoted by abscisic acid; it interacts also with a further enzyme to mediate the inhibition of stomatal opening effected by abscisic acid. Together these reactions constitute a signalling pathway that regulates water loss from plants.

3. Lysophosphatidic Acid

Lysophosphatidic acid or 1-acyl-*sn*-glycerol-3-phosphate differs from phosphatidic acid in having only one mole of fatty acid per mole of lipid. As such, it is the simplest possible glycerophospholipid. Molecular species with both saturated and unsaturated fatty acid constituents occur in different tissues. Although it is present at very low levels only in animal tissues, it is extremely important biologically, influencing many biochemical processes. These activities seem to be shared by the 1-alkyl- and alkenyl-ether forms. It is also important as the biosynthetic precursor of phosphatidic acid.

Lysophosphatidic acid

In particular, lysophosphatidic acid is an intercellular lipid mediator with growth factor-like activities, and is rapidly produced and released from activated platelets to influence target cells. However, a more important source is the activity of a specific lysophospholipase D ('autotaxin'), part of the blood-clotting process, on lysophosphatidylcholine, which yields lysophosphatidic acid in an albumin-bound form. This is more abundant in serum (1-5 ìM) than in plasma, where it accounts for much of the biological activity.

Lysophosphatidylcholine — Phospholipase D ('autotaxin') → Lysophosphatidic acid

It is now established that it is produced by a wide variety of cell types, both by the action of autotoxin and by that of a phosphatidic acid-selective phospholipase A_1, i.e. to produce a *sn*-2-acyl-lysophosphatidic acid in this instance, and almost certainly also by the action of a lysophospholipase A_2. A surprising recent finding is that the activity of the phosphatidic acid-selective lysophospholipase A_1 is essential for normal hair growth in humans.

Most mammalian cells express receptors for lysophosphatidic acid, and lysophosphatidic acid may initiate signalling in the cells in which it is produced, as well as affecting neighbouring cells. In the last few years, the characterization of cloned lysophosphatidic acid receptors in combination with strategies of molecular genetics has allowed determination of both signalling and biological effects that are dependent on receptor mechanisms. At least seven G protein-coupled receptors that are specific for lysophosphatidic acid have now been identified. Experimental activation of these receptors has shown that a range of downstream signalling cascades mediate lysophosphatidic acid signalling. These include activation of protein kinases, adenyl cyclase and phospholipase C, release of arachidonic acid, and much more. There is evidence that lysophosphatidic acid is involved in cell survival in some circumstances, and in programmed cell death in others. In some instances, molecular species with specific fatty acid components may be involved.

There is particular interest in the activity of lysophosphatidic acid in various disease states, where intervention in its metabolism has the potential for beneficial health effects. For example, a finding that lysophosphatidic acid is markedly elevated in the plasma of ovarian cancer patients, compared to healthy controls may be especially significant. In particular, elevated plasma levels were found in patients in the first stage of ovarian cancer, suggesting that it may represent a useful marker for the early detection of the disease. Lysophosphatidic acid is believed to stimulate DNA synthesis and the proliferation of ovarian cancer cells, and it may induce cell migration. Therefore, it is a target of the pharmaceutical industry for cancer therapy.

In addition, lysophosphatidic acid generated by the action of a lysophospholipase D is believed to play an important role in reproductive biology. Under certain conditions, it can become athero- and thrombogenic and might aggravate cardiovascular disease. This may be especially important in cancer patients. Lysophosphatidic acid has also been found in saliva in significant amounts, and it has been suggested that it is involved in wound healing in the upper digestive organs such as the mouth, pharynx, and esophagus. It has similar effects when applied topically to skin wounds, probably by stimulating proliferation of new cells to seal the wound. There is also evidence that the lipid is involved in brain development and vascular remodelling.

Catabolic deactivation of lysophosphatidic acid is accomplished by dephosphorylation to monoacylglycerol by a family of three lipid phosphate phosphatases, which also dephosphorylate sphingosine-1-phosphate, phosphatidic acid and ceramide 1-phosphate in a non-specific manner. It can be converted back to phosphatidic acid by a membrane-bound *O*-acyltransferase (MBOAT2) specific for lysophosphatidic acid (and lysophosphatidylethanolamine) with a preference for oleoyl-CoA as substrate.

Other lysophospholipids and especially the sphingolipid analogue, sphingosine-1-phosphate, show a related range of activities.

4. Cyclic Phosphatidic Acid

Cyclic phosphatidic acid (sometimes termed 'cyclic lysophosphatidic acid') was isolated originally from a slime mould, but has now been detecte of cyclic phosphatidic acidd in a wide range of organisms including humans, especially

Cyclic phosphatidic acid

in the brain but also bound to albumin in serum (at a concentration of 10^{-7}M, or a tenth that of lysophosphatidic acid). It has a cyclic phosphate at the *sn*-2 and *sn*-3 positions of the glycerol carbons, and this structure is absolutely necessary for its biological activity. It is most abundant in tissues subject to injury. In human serum, the main molecular species contains palmitic acid.

While cyclic phosphatidic acid may have some similar signalling functions to lysophosphatidic acid *per se* in that it binds to some of the same receptors, it also has some quite distinct activities in animal tissues. For example, cyclic phosphatidic acid is known to be a specific inhibitor of DNA polymerase *alpha*. It has an appreciable effect on the inhibition of cancer cell invasion and metastasis, a finding that is currently attracting great pharmacological interest. In addition, it inhibits the platelet aggregation induced by lysophosphatidic acid.

Studies of the biosynthesis of cyclic phosphatidic acid in fetal bovine serum suggest that it is the product of an enzyme related to the human enzyme autotaxin, a serum lysophospholipase D that produces lysophosphatidic acid. This enzyme appears to produce cyclic phosphatidic acid in serum by an intramolecular transphosphatidylation reaction. However, it can also be formed artefactually by the addition of strong acid to serum.

5. Pyrophosphatidic Acid

Pyrophosphatidic acid or *sn*-1,2-diacylglycero-3-pyrophosphate is an unusual and little known phospholipid that was first identified as a minor component in yeasts, and is also know to be present in mushrooms and higher plants as a product of the enzyme phosphatidic acid kinase.

diacyl-*sn*-glycerol-3-pyrophosphate

It is rapidly metabolized back to phosphatidic acid by a specific phosphatase and thence to diacylglycerols, and it may have a function in the phospholipase C and D signalling cascades in plants. Pyrophosphatidic acid is barely detectable in non-stimulated plant cells but its concentration increases very rapidly in response to stress situations, including osmotic stress and attack by pathogens. Such findings add to the belief that it is an important signalling molecule in plants under stress. In yeasts, it may have a role in the regulation of the synthesis and metabolism of phospholipids, especially phosphatidylserine.

6. Analysis

Phosphatidic acid and related lipids are not the easiest to analyse. On adsorption chromatography, retention times tend to be variable and may be dependent to some extent on the nature of the cations associated with the acidic lipids. However, two-dimensional TLC can give good results. Phosphatidic acid, lysobisphosphatidic acid and pyrophosphatidic acid are never easy to distinguish, and the best hope for success appears to lie with modern liquid chromatography-mass spectrometric methods.

phosphatidylcholine

Phosphatidylcholine or 1,2-diacyl-*sn*-glycerol-3-phosphorylcholine (or "lecithin", although the term is now used more frequently for the mixed phospholipid by-products of seed oil refining) is usually the most abundant lipid in the membranes of animal tissues, and it is often a major lipid component of plant membranes, but only rarely of bacteria. With the other choline-containing phospholipid, sphingomyelin, it is a key structural component and constitutes much of the lipid in the external monolayer of the plasmamembrane of animal cells especially. **Phosphatidylcholine – Structure and Occurrence**

Phosphatidylcholine (once given the trivial name 'lecithin') is usually the most abundant phospholipid in animal and plants, often amounting to almost 50% of the total, and as such it is obviously the key building block of membrane bilayers. In particular, it makes up a very high proportion of the outer leaflet of the plasma membrane. Phosphatidylcholine is also the principal phospholipid circulating in plasma, where it is an integral component of the lipoproteins, especially the HDL. On the other hand, it is less often found in bacterial membranes, perhaps 10% of species. It is a neutral or zwitterionic phospholipid over a pH range from strongly acid to strongly alkaline. In animal tissues, some of its membrane functions appear to be shared with the structurally related sphingolipid - sphingomyelin – although the latter has many unique properties of its own.

1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine
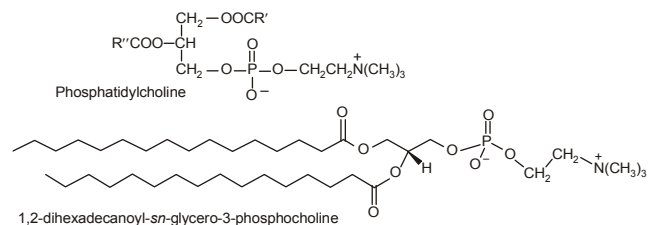
In animal tissues, phosphatidylcholine tends to exist in mainly in the diacyl form, but small proportions (in comparison to phosphatidylethanolamine and phosphatidylserine) of alkylacyl and alkenylacyl forms may also be present. Data for the compositions of these various forms from bovine heart muscle are listed in our web pages on Ether lipids. As a generalization, animal phosphatidylcholine tends to contain lower proportions of arachidonic and docosahexaenoic acids and more of the C_{18} unsaturated fatty acids than the other zwitterionic phospholipid, phosphatidylethanolamine. The saturated fatty acids are most abundant in position *sn*-1, while the polyunsaturated components are concentrated in position *sn*-2. Indeed, C_{20} and C_{22} polyenoic acids are exclusively in position *sn*-2. Dietary factors obviously influence fatty acid compositions, but in comparing animal species, it would be expected that the

structure of the phosphatidylcholine in the same metabolically active tissue would be somewhat similar in terms of the relative distributions of fatty acids between the two positions. **Table** lists some representative data.

Table 2.13 : Positional distribution of fatty acids in the phosphatidylcholine of some animal tissues

Position	Fatty acid						
	16:0	16:1	18:0	18:1	18:2	20:4	22:6
Rat liver [1]							
sn-1	23	1	65	7	1	trace	
sn-2	6	1	4	13	23	39	7
Rat heart [2]							
sn-1	30	2	47	9	11	-	-
sn-2	10	1	3	17	20	33	9
Rat lung [3]							
sn-1	72	4	15	7	3	-	-
sn-2	54	7	2	12	11	10	1
Human plasma [4]							
sn-1	59	2	24	7	4	trace	-
sn-2	3	1	1	26	32	18	5
Human erythrocytes [4]							
sn-1	66	1	22	7	2	-	-
sn-2	5	1	1	35	30	16	4
Bovine brain (gray matter) [5]							
sn-1	38	5	32	21	1	-	-
sn-2	33	4	trace	48	1	9	4
Chicken egg [6]							
sn-1	61	1	27	9	1	-	-
sn-2	2	1	trace	52	33	7	4

1, Wood, R. and Harlow, R.D. *Arch. Biochem. Biophys.*, **131**, 495-501 (1969); 2, Kuksis, A. *et al. J. Lipid Res.*, **10**, 25-32 (1969); 3, Kuksis, A. *et al. Can. J. Physiol. Pharm.*, **46**, 511-524 (1968); 4, Marai, L. and Kuksis, A. *J. Lipid Res.*, **10**, 141-152 (1969); 5, Yabuuchi, H. and O'Brien, J.S. J. Lipid Res., 9, 65-67 (1968); 6, Kuksis, A. and Marai, L. *Lipids*, **2**, 217-224 (1967).

There are some exceptions to the rule. The phosphatidylcholine in some organs contains relatively high proportions of disaturated molecular species. For example, it is well known that lung phosphatidylcholine in most if not all animal species studied to date contains a high proportion (50% or more) of dipalmitoylphosphatidylcholine. It appears that this is the main surface-active component, providing alveolar stability by decreasing the surface tension at the alveolar surface to a very low level. Also, the internal lipids of the animal cell nucleus (after the external membrane has been removed) contain a high proportion of disaturated phosphatidylcholine, amounting to 10% of the volume indeed. This is synthesised entirely within the nucleus, unlike phosphatidylinositol for example, and in contrast to other cellular lipids its composition cannot be changed by extreme dietary manipulation. It has been suggested that it may have a role in stabilizing or regulating the structure of the chromatin, as well as being a source of diacylglycerols with a signalling function.

The positional distributions of fatty acids in phosphatidylcholine in representative plants and yeast are listed in **Table**. In the leaves of the model plant *Arabidopsis thaliana*, saturated fatty acids are concentrated in position *sn*-1, but monoenoic fatty acids are distributed approximately equally between the two positions, and there is a preponderance of di- and triunsaturated fatty acids in position *sn*-2. The same is true for soybean 'lecithin'. The pattern is somewhat similar for the yeast *Lipomyces lipoferus*, except that much of the 16:1 is in position *sn*-1 in this instance.

Table 2.15 : Composition of fatty acids (mol %) in positions *sn*-1 and sn-2 in the phosphatidylcholine from plants and yeast

Position	Fatty acid					
	16:0	16:1	18:0	18:1	18:2	18:3
Arabidosis thaliana (leaves) [1]						
sn-1	42		4	5	23	26
sn-2	1		trace	5	47	47
Soybean 'lecithin' [2]						
sn-1	24		9	14	47	4
sn-2	5		1	13	75	6
Lipomyces lipoferus [3]						
sn-1	24	18	trace	37	16	4
sn-2	4	5	trace	39	31	19

1, Browse, J., Warwick, N., Somerville, C.R. and Slack, C.R. *Biochem. J.*, **235**, 25-31 (1986).

2, Blank, M.L., Nutter, L.J. and Privett, O.S. *Lipids*, **1**, 132-135 (1966).

2, Haley, J.E. and Jack, R.C. *Lipids*, **9**, 679-681 (1974).

2. Phosphatidylcholine – Biosynthesis and Biological Function

There are several mechanisms for the biosynthesis of phosphatidylcholine in animals, plants and micro-organisms. Choline itself is not synthesised as such by animal cells and is an essential nutrient. It must

be obtained from dietary sources or by degradation of existing choline-containing lipids, for example those produced by the second pathway described below. Once taken up into cells, choline is immediately phosphorylated by a choline kinase in the cytoplasm of the cell to phosphocholine, which is reacted with cytidine triphosphate (CTP) to form cytidine diphosphocholine. The membrane-bound enzyme CDP-choline:1,2-diacylglycerol cholinephosphotransferase in the endoplasmic reticulum catalyses the reaction of the last compound with *sn*-1,2-diacylglycerols to form phosphatidylcholine. This is the main pathway for the synthesis of phosphatidylcholine in animals and plants, and it is analogous to the biosynthesis of phosphatidylethanolamine.
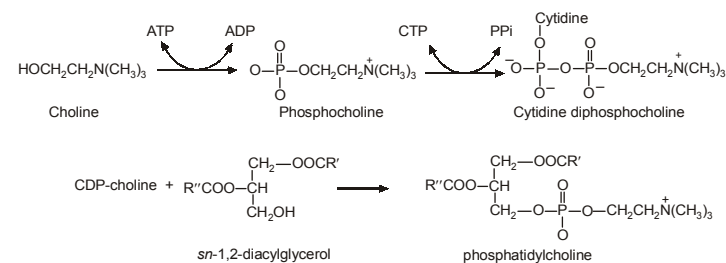
The discovery of the importance of this pathway depended a little on serendipity in that in experiments in the lab of Professor Eugene Kennedy, samples of adenosine triphosphate (ATP) contained some cytidine triphosphate (CTP) as an impurity. However, luck is of little value without receptive minds, and Kennedy and co-workers demonstrated that the impurity was an important metabolite that was essential for the formation of phosphatidylcholine.

The above reaction, together with the biosynthetic mechanism for phosphatidylethanolamine, is significantly different from that for phosphatidylglycerol, phosphatidylinositol and cardiolipin. Both make use of nucleotides, but with the latter, the nucleotide is covalently linked directly to the lipid intermediate, i.e. cytidine diphosphate diacylglycerol.

The source of the *sn*-1,2-diacylglycerol precursor, which is also a key intermediate in the formation of phosphatidylethanolamine and phosphatidylserine, and of triacylglycerols, is phosphatidic acid. In this instance, the important enzyme is phosphatidic acid phosphatase (or 'phosphatidate phosphatase' or 'lipid phosphate phosphatase'), which is present mainly in the endoplasmic reticulum of the cell.
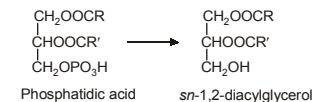
This enzyme is also important for the production of diacylglycerols as essential intermediates in the biosynthesis of triacylglycerols and of phosphatidylethanolamine. In mammals, much of the activity responsible for phospholipid biosynthesis resides in the endoplasmic reticulum (phosphatidic acid

phosphatase-2 (or PAP2)), but in addition three related cytoplasmic proteins, termed lipin-1, lipin-2, and lipin-3 may also have a role (see our webpage on triacylglycerol biosynthesis).

The second pathway for biosynthesis of phosphatidylcholine involves sequential methylation of phosphatidylethanolamine, with *S*-adenosylmethionine as the source of methyl groups, with mono- and dimethyl-phosphatidylethanolamine as intermediates and catalysed by the enzyme phosphatidylethanolamine *N*-methyltransferase. A single enzyme (~20 Kda) catalyses all three reactions and is located mainly in the endoplasmic reticulum where it spans the membrane. This is a major pathway in the liver, but not in other animal tissues or in general in higher organisms. It may be the main route to phosphatidylcholine in those bacterial species that produce this lipid and in yeasts.
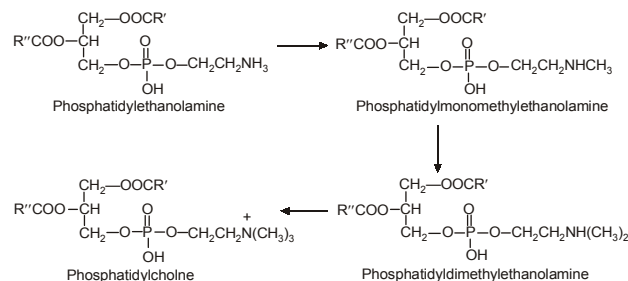
This liver enzyme is especially important when choline is deficient in the diet. A by-product of the biosynthesis of phosphatidylcholine from phosphatidylethanolamine is the conversion of *S*-adenosylmethionine to *S*-adenosylhomocysteine, which is hydrolysed in the liver to adenosine and homocysteine. It is noteworthy that elevated plasma homocysteine is a risk factor for cardiovascular disease and myocardial infarction.

Phosphatidylcholine biosynthesis by both pathways in the liver is necessary for normal secretion of the plasma lipoproteins (VLDL and HDL), and it is relevant to a number of human physiological conditions.

In one bacterial species, a third pathway for phosphatidylcholine biosynthesis that is choline-dependent, has been found in which the lipid is formed in one step via condensation of choline directly with CDP-diacylglycerol. The yeast *Saccharomyces cerevisiae* is able to reacylate endogenously generated glycerophosphocholine with acyl-CoA in the microsomal membranes, first to lysophosphatidylcholine and then to phosphatidylcholine.

While phosphatidylcholine is a major lipid in yeasts, recent work suggests that it is not essential if suitable alternative growth substrates are available, unlike higher organisms where perturbation of phosphatidylcholine synthesis can lead to inhibition of growth or even cell death. Enhanced synthesis of phosphatidylcholine appears to occur in cancer cells and solid tumours, and this may prove to be a target for therapeutic agents.

Whatever the mechanism of biosynthesis in tissues, it is apparent that the fatty acid compositions and positional distributions on the glycerol moiety are determined post synthesis by extensive re-modelling involving hydrolysis (phospholipase A_2 mainly) and re-acylation, a process that is sometimes termed the '**Lands' Cycle**' after its discoverer W.E.M. (Bill) Lands. The re-acylation step is catalysed by a membrane-bound coenzyme A-dependent lysophosphatidylcholine acyltransferase (MBOAT5 also designated 'LPCAT3'), which has been located chiefly within the endoplasmic reticulum, though also in mitochondria and the plasma membrane, in organs such as the liver, adipose tissue and pancreas. This incorporates linoleoyl and arachidonoyl chains specifically into lysophosphatidylcholine (see below). Polyunsaturated fatty acids introduced by this route can then be transferred to 1-alkyl and 1-alkenyl phospholipids by CoA-independent transacylases. A second such enzyme LPCAT4 is known and has a clear preference for 18:1-CoA. Similarly, the highly saturated molecular species of phosphatidylcholine found in the nucleus are formed from species with a more conventional composition by remodelling, presumably by acyltransferases with somewhat different specificity. These and further related enzymes are involved in remodelling of all other phospholipids.

In plants, fatty acids esterified to phosphatidylcholine can serve as substrates for desaturases, and this means that the fatty acid composition changes also after the initial synthetic process. The process is further complicated in plants in that biosynthesis or partial synthesis (via lysophosphatidylcholine) occurs in different organelles, such as the endoplasmic reticulum, plastids and mitochondria, from different fatty acid pools or with differing specificities.

Because of the generally cylindrical shape of the molecule, phosphatidylcholine spontaneously organizes into bilayers, so it is ideally suited to serve as the bulk structural element of biological membranes. Such properties are essential to act as a balance to those lipids that do not form bilayers or that form specific micro-domains such as rafts.

In addition to its function as a membrane constituent, phosphatidylcholine may have a role in signalling via the generation of diacylglycerols, especially in the nucleus. Although the pool of the precursor is so great in many tissues that turnover is not easily measured, the presence of phospholipases C and D specific for phosphatidylcholine, which are activated by a number of agonists, suggests such a function especially in the cell nucleus. Diacylglycerols formed in this way would be much more saturated than those derived from phosphatidylinositol, and would not be expected to be as active. The plasmalogen form of phosphatidylcholine may also have a signalling function, as thrombin treatment of endothelial cells activates a selective hydrolysis (phospholipase A_2) of molecular species containing arachidonic acid in the *sn*-2 position, releasing this fatty acid for eicosanoid production. The diacyl form of phosphatidylcholine may have a related function in signal transduction in other tissues. In addition, it is known that the enzyme 3-hydroxybutyrate dehydrogenase requires to be bound to phosphatidylcholine before it can function optimally.

Phosphatidylcholine is the biosynthetic precursor of sphingomyelin and as such must have some influence on the many metabolic pathways that constitute the sphingomyelin cycle. It is also a precursor for phosphatidic acid, lysophosphatidylcholine and platelet-activating factor, each with important signalling functions, and of phosphatidylserine.

On catabolism of choline-containing lipids, much of the choline is re-used for phosphatidylcholine biosynthesis, often after being returned to the liver. Some is oxidized in the kidney and liver to betaine,

which serves as a donor of methyl groups for S-adenosylmethionine production. A proportion is used in nervous tissues for production of acetylcholine, which is a neurotransmitter of importance to learning, memory and sleep. Some choline is lost through excretion of phosphatidylcholine in the bile.

3. Lysophosphatidylcholine

Lysophosphatidylcholine, with one mole of fatty acid per mole of lipid in position *sn*-1, is found in small amounts in most tissues. It is formed by hydrolysis of phosphatidylcholine by the enzyme phospholipase A_2, as part of the de-acylation/re-acylation cycle that controls its overall molecular species composition. It can also be formed inadvertently during extraction of lipids from tissues if the phospholipase is activated by careless handling. There is also a phospholipase A_1, which is able to cleave the *sn*-1 ester bond. As part of the Lands cycle (see above), it can be converted back to phosphatidylcholine by specific acyl transferases.

Lysophosphatidylcholine
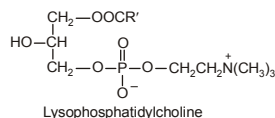
In plasma of animal species, appreciable amounts of lysophosphatidylcholine are formed by a specific enzyme system, lecithin:cholesterol acyltransferase (LCAT), which is secreted from the liver. The enzyme catalyses the transfer of the fatty acids of position *sn*-2 of phosphatidylcholine to the free cholesterol in plasma, with formation of cholesterol esters and of course of lysophosphatidylcholine. Identification of a highly specific phospholipase A_2 in peroxisomes that generates 2-arachidonoyl lysophosphatidylcholine suggests that this may be of relevance to eicosanoid generation and signalling.

Lysophosphatidylcholine has pro-inflammatory properties *in vitro* and it is known to be a pathological component of oxidized lipoproteins (LDL) in plasma and of atherosclerotic lesions. Recently, it has been found to have some functions in cell signalling, and specific receptors (coupled to G proteins) have been identified. It activates the specific phospholipase C that releases diacylglycerols and inositol triphosphate with resultant increases in intracellular Ca^{2+} and activation of protein kinase C. It also activates the mitogen-activated protein kinase in certain cell types.

Stearoyl lysophosphatidylcholine has been shown to be protective against lethal sepsis in experimental animals by various mechanisms, including stimulation of neutrophils to eliminate invading pathogens through a peroxide-dependent reaction.

4. Platelet-Activating Factor

Platelet-activating factor (PAF) or 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine is an ether analogue of phosphatidylcholine that is biologically active. This important lipid has its own webpage.

5. Analysis

Analysis of phosphatidylcholine presents no particular problems. It is readily isolated by thin-layer or high-performance liquid chromatography methods. Determination of the dipalmitoyl species in lung

surfactant is a more demanding task, but specific methods have been published. Phospholipase A_2 from snake venom is used in methods to determine the position of fatty acids on the glycerol moiety. Modern mass spectrometry methodology has greatly simplified the task of molecular species analysis.

Lysophosphatidylcholine, which contains only one fatty acid moiety in each molecule, generally in position *sn*-1, is sometimes present as a minor component of tissues. It is a powerful surfactant and

Lysophosphatidylcholine
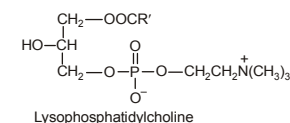
is more soluble in water than most other lipids.

Phosphatidylethanolamine
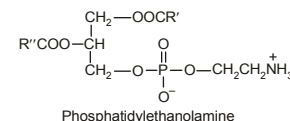
Phosphatidylethanolamine (once given the trivial name "cephalin") is usually the second most abundant phospholipid class in animal and plant tissues, and can be the major lipid class in microorganisms. As part of an important cellular process, the amine group can be methylated enzymically to yield first phosphatidyl-*N*-monomethylethanolamine and then phosphatidyl-*N,N*-dimethylethanolamine, but these never accumulate in significant amounts; the eventual product is phosphatidylcholine.

***N*-Acyl-phosphatidylethanolamine** is a minor component of some plant tissues, especially cereals, and it is occasionally found in animal tissues, where it is the precursor of some biologically active amides. Lysophosphatidylethanolamine contains only one mole of fatty acid per mole of lipid.

Phosphatidylserine is a weakly acidic lipid that is present in most tissues of animals and plants and is also found in microorganisms. It is located entirely on the inner monolayer surface of the plasma membrane and other cellular membranes. Phosphatidylserine is an essential cofactor for the activation of protein kinase C, and it is involved in many other biological processes, including blood coagulation and apoptosis (programmed cell death). (More...).

Phosphatidylserine
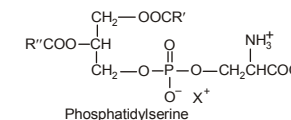
N-Acylphosphatidylserine has been detected in some animal tissues.

Phosphatidylinositol
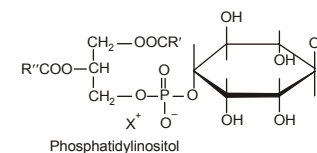
Phosphatidylinositol, containing the optically inactive form of inositol, *myo*-inositol, is a common constituent of animal, plant and microbial lipids. In animal tissues especially, it may be accompanied by small amounts of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate ('poly-phosphoinositides'). These compounds have a rapid rate of metabolism in animal cells, and are converted to metabolites such as diacylglycerols and inositol phosphates, which are important in regulating vital processes. For example, diacylglycerols regulate the activity of a group of enzymes known as protein kinase C, which in turn control many key cellular functions, including differentiation, proliferation, metabolism and apoptosis. In addition, phosphatidylinositol is the primary source of the arachidonate used for eicosanoid synthesis in animals, and it is known to be the anchor that can link a variety of proteins to the external leaflet of the plasma membrane via a glycosyl bridge (glycosyl-phosphatidylinositol(GPI)-anchored proteins) (see our web pages on phosphatidylinositol).

Phosphonolipids are lipids with a phosphonic acid moiety esterified to glycerol, i.e. with a carbon-phosphorus bond that is not easily hydrolysed by chemical reagents. Phosphonylethanolamine, for example, is found mainly in marine invertebrates and in protozoa. A ceramide analogue is often found in the same organisms (see below).

Phosphonylethanolamine

Plasmanylethanolamine

Plasmanylethanolamine
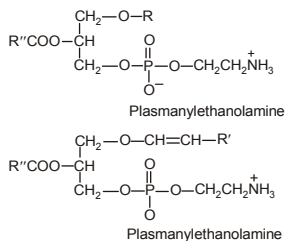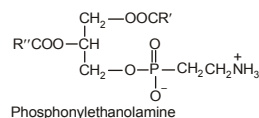
Ether lipids: Many glycerolipids, but mainly phospholipids, and those of animal and microbial origin especially, contain aliphatic residues linked either by an ether bond or a vinyl ether bond to position 1 of *L*-glycerol. When a lipid contains a vinyl ether bond, the generic term "plasmalogen" is often used. They can be abundant in the phospholipids of animals and microorganisms, and especially in the phosphatidylethanolamine fraction. In this instance, it has been recommended that they should be termed "plasmanylethanolamine" and "plasmenylethanolamine", respectively.

On hydrolysis of glycerolipids containing an alkyl ether bond, 1-alkylglycerols are released that can be isolated for analysis. Similarly, when plasmalogens are hydrolysed under basic conditions, 1-alkenylglycerols are released. Aldehydes are formed on acidic hydrolysis. With both groups of compound, the aliphatic residues generally have a chain-length of 16 or 18, and they are saturated or may contain one additional double bond, that is remote from the ether linkage.

1-Alkyl-2,3-diacyl-*sn*-glycerols, analogues of triacylglycerols, tend to be present in trace amounts

only in animal tissues, but can be major constituents of certain fish oils. Related compounds containing a 1-alk-1'-enyl moiety ('neutral plasmalogens') are occasionally present also.

'Platelet-activating factor' or 1-alkyl-2-acetyl-*sn*-glycerophosphorylcholine is an ether-containing phospholipid, that is presently being studied intensively because it can exert profound biological effects at minute concentrations. For example, it effects aggregation of platelets at concentrations as low as 10^{-11} M, and it induces a hypertensive response at very low levels. Also, it is a mediator of inflammation and has messenger functions.

Platelet-activating factor
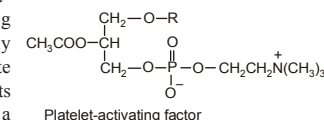
Glycoglycerolipids

In plants, especially the photosynthetic tissues, a substantial proportion of the lipids consists of 1,2-diacyl-*sn*-glycerols joined by a glycosidic linkage at position *sn*-3 to a carbohydrate moiety. The main components are the **mono-** and **digalactosyldiacylglycerols**, but related compounds have been found with up to four galactose units, or in which one or more of these is replaced by glucose moieties. It is clear that these have an important role in photosynthesis, but many of the details have still to be worked out.

Monogalactosyldiacylglycerol   Digalactosyldiacylglycerol

1,2-di-(9Z,12Z,15Z)-octadecatrienoyl-3-O-β-D-galactosyl-*sn*-glycerol
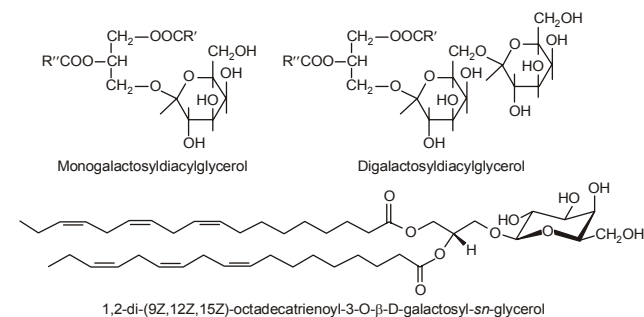
In addition, a 6-*O*-acyl-monogalactosyldiacylglycerol is occasionally a component of plant tissues. See our web pages dealing with plant galactolipids.

A related unique plant glycolipid is **sulfoquinovosyldiacylglycerol** or the "plant sulfolipid". It contains a sulfonic acid residue linked by a carbon-sulfur bond to the 6-deoxyglucose moiety of a monoglycosyldiacylglycerol and is found exclusively in the chloroplasts.

Monogalactosyldiacylglycerols are not solely plant lipids as they have been found in small amounts in brain and nervous tissue in some animal species. A range of complex glyceroglycolipids have also been characterized from intestinal tract and lung tissue. They exist in both diacyl and alkyl acyl forms.

Such compounds are destroyed by some of the methods used in the isolation of glycosphingolipids, so they may be more widespread than has been thought.

A complex glyco-glycero-sulfolipid, termed **seminolipid**, of which the main component is 1-*O*-hexadecyl-2-*O*-hexadecanoyl-3-*O*- (3'-sulfo-â-D-galactopyranosyl) -*sn*-glycerol, is the principal glycolipid in testis and sperm. See our web pages on animal glycosyldiacylglycerols.

A further range of highly complex glycolipids occur in bacteria and other micro-organisms. These include acylated sugars that do not contain glycerol.

Sphingomyelin and Glycosphingolipids

Sphingolipids consist of long-chain bases, linked by an amide bond to a fatty acid and via the terminal hydroxyl group to complex carbohydrate or phosphorus-containing moieties.

Sulfoquinovosyldiacylgycerol
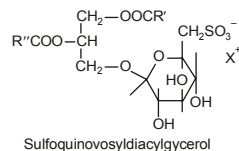
Long-chain bases (sphingoids or sphingoid bases) are the characteristic structural unit of sphingolipids. They are long-chain (12 to 22 carbon atoms) aliphatic amines, containing two or three hydroxyl groups, and often a distinctive *trans*-double bond in position 4. The commonest or most abundant of these in animal tissues is **sphingosine**, ((2*S*,3*R*,4*E*)-2-amino-4-octadecen-1,3-diol) (illustrated). More than 60 long-chain bases have been found in animals, plants and microorganisms, and many of these may occur in a single tissue, but almost always as part of a complex lipid as opposed to in the free form. The aliphatic chains can be saturated, monounsaturated and diunsaturated, with double bonds of either the *cis* or *trans* configuration, and they may sometimes have methyl substituents. In addition, saturated and monoenoic straight- and branched-chain trihydroxy bases are found. The aliphatic moiety is similar to that of a fatty acid in its physical properties.

$$CH_3(CH_2)_{13}.CHOH{>}CHOH.CHNH_2.CH_2OH$$
phytosphingosine
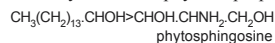
Phytosphingosine ((2*S*,3*S*,4*R*)-2-amino-octadecanetriol) is the most common long-chain base of plant origin.

For shorthand purposes, a nomenclature similar to that for fatty acids can be used, i.e. the chain-length and number of double bonds are denoted in the same manner with the prefix "d" or "t" to designate di- and trihydroxy bases respectively. Thus, sphingosine is d18:1 and phytosphingosine is t18:0. (More...).

R.CHOH.CH.CH₂OH
|
NHOC,R'
ceramide
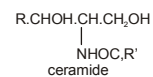
Ceramides contain fatty acids linked by an amide bond to the amine group of a long-chain base.

In general, they are present at low levels only in tissues, but they are key intermediates in the biosynthesis of the complex sphingolipids. In addition, they have important functions in cellular signalling, and especially in the regulation of apoptosis, and cell differentiation, transformation and proliferation.

Unusual ceramides have been located in the epidermis of the pig and humans; the fatty acids linked to the sphingoid base consist of C_{30} and C_{32} (ù-hydroxylated components, with predominantly the essential fatty acid, linoleic acid, esterified to the terminal hydroxyl group. They are believed to have a special role in preventing the loss of moisture through the skin.

Sphingomyelin is a sphingophospholipid and consists of a ceramide unit linked at position 1 to phosphorylcholine; it is found as a major component of the complex lipids of all animal tissues but not of plants or micro-organisms.

N-(octadecanoyl)-sphing-4-enine-1-phosphocholine
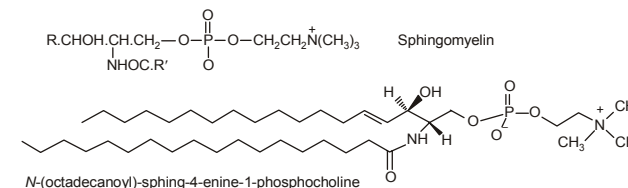
It resembles phosphatidylcholine in many of its physical properties, and can apparently substitute in part for this in membranes although it also has its own unique role. For example, it is a major constituent of the plasma membrane of cells, where it is concentrated together with sphingoglycolipids and cholesterol in tightly organized sub-domains termed 'rafts'. Sphingosine tends to be the most abundant long-chain base constituent, and it is usually accompanied by sphinganine and C_{20} homologues. Sphingomyelin is a precursor for a number of sphingolipid metabolites that have important functions in cellular signalling, including sphingosine-1-phosphate (see below), as part of the 'sphingomyelin cycle'. A correct balance between the various metabolites is vital for good health. Niemann-Pick disease is a rare lipid storage disorder that results from of a deficiency in the enzyme responsible for the degradation of sphingomyelin. (More...).

Ceramide phosphorylethanolamine
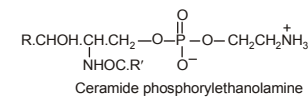
Ceramide phosphorylethanolamine is found in the lipids of insects and some fresh water invertebrates; the phosphonolipid analogue, ceramide 2-aminoethylphosphonic acid, has been detected in sea anemones and protozoa. Ceramide phosphorylinositol is also found in some organisms, and like phosphatidylinositol, it can be an anchor unit for oligosaccharide-linked proteins in membranes. (More...).

Neutral glycosylceramides: The most widespread glycosphingolipids are the **monoglycosylceramides** (or cerebrosides), and they consist of a basic ceramide unit linked by a glycosidic bond at carbon 1 of the long-chain base to glucose or galactose. They were first found in

brain lipids, where the principal form is galactosylceramide, but they are now known to be ubiquitous constituents of animal tissues. Glucosylceramide is also found in animal tissues, and especially in skin, where it functions as part of the water permeability barrier. It is the biosynthetic precursor of lactosylceramide, and thence of the complex oligoglycolipids and gangliosides. In addition, glucosylceramide is found in plants, where the main long-chain base is phytosphingosine.
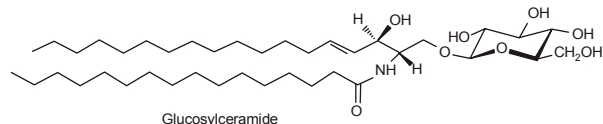
Glucosylceramide

O-Acyl-glycosylceramides have been detected in small amounts in some tissues, as have cerebrosides with monosaccharides such as xylose, mannose and fucose.

Di-, tri- and tetraglycosylceramides (oligoglycosylceramides) are present also in most animal tissues at low levels. The most common diglycosyl form is **lactosylceramide**, and it can be accompanied by related compounds containing further galactose or galactosamine residues. Tri- and tetraglycosylceramides with a terminal galactosamine residue are sometimes termed "globosides", while glycolipids containing fucose are known as "fucolipids". Lactosylceramide is the biosynthetic precursor of most of these with further monosaccharide residues being added to the end of the carbohydrate chain (up to as many as twenty). They are an important element of the immune response system. For example some glycolipids are involved in the antigenicity of blood group determinants, while others bind to specific toxins or bacteria. As the complex glycosyl moiety is considered to be of primary importance in this respect, it has received most attention from investigators. However, certain of these lipids have been found on occasion to have distinctive long-chain base and fatty acid compositions, which enhance their biological activity. Some glycolipids accumulate in persons suffering from rare disease syndromes, characterized by deficiencies in specific enzyme systems related to glycolipid metabolism.

Sulphate esters of galactosylceramide and lactosylceramide (**sulfoglycosphingolipids** - often referred to as "sulfatides" or "lipid sulphates"), with the sulphate group linked to position 3 of the galactosyl moiety, are major components of brain lipids and they are found in trace amounts in other tissues. (More...).

Complex plant sphingolipids, **phytoglycosphingolipids**, containing glucosamine, glucuronic acid and mannose linked to the ceramide via phosphorylinositol, were isolated and characterized from seeds initially, but related compounds are also known to be present in other plant tissues and in fungi.

Gangliosides are highly complex oligoglycosylceramides, which contain one or more sialic acid

Ceramide(1←1)Glu(4←1)Gal(4←1)GalNAc(3←1)Gal

Ganglioside G$_{M1}$
$$\begin{bmatrix} 3 \\ \downarrow \\ 2 \end{bmatrix}$$
NANA
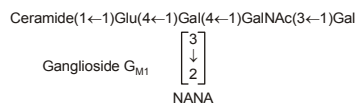
groups (*N*-acyl, especially acetyl, derivatives of neuraminic acid, abbreviated to "NANA") in addition to glucose, galactose and galactosamine.

The polar and ionic nature of these lipids renders them soluble in water (contrary to some definitions of a lipid). They were first found in the ganglion cells of the central nervous system, hence the name, but are now known to be present in most animal tissues. The long-chain base and fatty acid components of gangliosides can vary markedly between tissues and species, and they are presumably related in some way to function. Gangliosides have been shown to control growth and differentiation of cells, and they have important roles in the immune defense systems. They act as receptors for a number of tissue metabolites and in this way may regulate cell signalling. Also, they bind specifically to various bacterial toxins, such as those from botulinum, tetanus and cholera. A number of unpleasant lipidoses have been identified involving storage of excessive amounts of gangliosides in tissues, the most important of which is Tay-Sachs disease.

Sphingosine-1-phosphate is one of the simplest sphingolipids structurally. It is present at low levels only in animal tissues, but it is a pivotal lipid in many cellular signalling pathways (together with ceramide and ceramide-1-phosphate). For example, within cells, sphingosine-1-phosphate promotes cellular division (mitosis), while in the blood it may play a critical role in platelet aggregation and thrombosis. (More...).

Sphingosine-1-phosphate
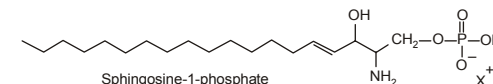
The fatty acids of sphingolipids: Although structures of fatty acids are discussed elsewhere, it is worth noting that the acyl groups of ceramides are very different from those in the glycerolipids. They tend to consist of long-chain (C_{16} up to C_{26} but occasionally longer) odd- and even-numbered saturated or monoenoic fatty acids and related 2-D-hydroxy fatty acids, both in plant and animal tissues. Linoleic acid may be present at low levels in sphingolipids from animal tissues, but polyunsaturated compounds are rarely found (although their presence is often reported in error).

Fatty Acids

The common fatty acids of plant tissues are C_{16} and C_{18} straight-chain compounds with zero to three double bonds of a *cis* (or *Z*) configuration. Such fatty acids are also abundant in animal tissues, together with other even numbered components with a somewhat wider range of chain-lengths and up to six *cis* double bonds separated by methylene groups (methylene-interrupted). The systematic and trivial names of those fatty acids encountered most often, together with their shorthand designations, are listed in the table.

$$HOOC\ (CH_2)_{14}CH_3$$
palmitic acid

The most abundant saturated fatty acid in nature is **hexadecanoic** or palmitic acid. It can also be designated a "16:0" fatty acid, the first numerals denoting the number of carbon atoms in the aliphatic chain and the second, after the colon, denoting the number of double bonds. All the even-numbered

saturated fatty acids from C_2 to C_{30} have been found innature, but only the C_{14} to C_{18} homologues are likely to be encountered in appreciable concentrations in glycerolipids, other than in a restricted range of commercial fats and oils.

Table 2.16 : The common fatty acids of animal and plant origin

Systematic name	Trivial name	Shorthand
Saturated fatty acids		
ethanoic	acetic	2:0
butanoic	butyric	4:0
hexanoic	caproic	6:0
octanoic	caprylic	8:0
decanoic	capric	10:0
dodecanoic	lauric	12:0
tetradecanoic	myristic	14:0
hexadecanoic	palmitic	16:0
octadecanoic	stearic	18:0
eicosanoic	arachidic	20:0
docosanoic	behenic	22:0
Monoenoic fatty acids		
cis-9-hexadecenoic	palmitoleic	16:1(n-7)
cis-6-octadecenoic	petroselinic	18:1(n-12)
cis-9-octadecenoic	oleic	18:1(n-9)
cis-11-octadecenoic	*cis-vaccenic*	18:1(n-7)
cis-13-docosenoic	erucic	22:1(n-9)
cis-15-tetracosenoic	nervonic	24:1(n-9)
Polyunsaturated fatty acids*		
9,12-octadecadienoic	linoleic	18:2(n-6)
6,9,12-octadecatrienoic	γ-linolenic	18:3(n-6)
9,12,15-octadecatrienoic	α-linolenic	18:3(n-3)
5,8,11,14-eicosatetraenoic	arachidonic	20:4(n-6)
5,8,11,14,17-eicosapentaenoic	EPA	20:5(n-3)
4,7,10,13,16,19-docosahexaenoic	DHA	22:6(n-3)

* all the double bonds are of the *cis* configuration

Oleic or ***cis*-9-octadecenoic** acid, the most abundant monoenoic fatty acid in nature, is designated as "18:1", or more precisely as "18:1(*n*-9)", to indicate that the last double bond is 9 carbon atoms from the terminal methyl group.

Oleic acid
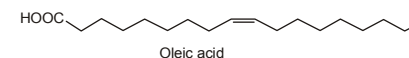
The latter form of the nomenclature is of special value to biochemists. Similarly, the most abundant *cis* monoenoic acids fall into the same range of chain-lengths, i.e. 16:1(*n*-7) and 18:1(*n*-9), though 20:1 and 22:1 are abundant in fish. Fatty acids with double bonds of the *trans* (or *E*) configuration are found occasionally in natural lipids, or are formed during food processing (hydrogenation) and so enter the food chain, but they tend to be minor components only of animal tissue lipids, other than of ruminants. Their suitability for human nutrition is currently a controversial subject.

The C_{18} polyunsaturated fatty acids, **linoleic** or *cis*-9,*cis*-12-octadecadienoic acid (18:2(*n*-6)) and **á-linolenic** or *cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid (18:3(*n*-3)), are major components of most plant lipids, including many of the commercially important vegetable oils.

Linoleic acid

α-linolenic acid
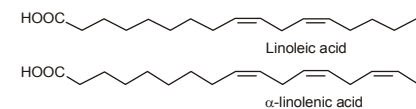
They are essential fatty acids in that they cannot be synthesised in animal tissues. On the other hand, as linoleic acid is almost always present in foods, it tends to be relatively abundant in animal tissues. In turn, these fatty acids are the biosynthetic precursors in animal systems of C_{20} and C_{22} polyunsaturated fatty acids, with three to six double bonds, via sequential desaturation and chain-elongation steps (desaturases in animal tissues can only insert a double bond on the carboxyl side of an existing double bond). Those fatty acids derived from linoleic acid, especially arachidonic acid (20:4(*n*-6)), are important constituents of the membrane phospholipids in mammalian tissues, and are also the precursors of the prostaglandins and other eicosanoids. In fish, linolenic acid is the more important essential fatty acid, and polyunsaturated fatty acids of the (*n*-3) series, especially eicosapentaenoic acid (20:5(n-3) or EPA) and docosahexaenoic acid (22:6(n-3) or DHA), are found in greater abundance.

20:4(n-6)

20:5(n-3)
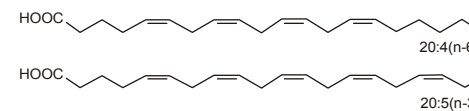
Many other fatty acids that are important for nutrition and health do of course exist in nature, and at present there is great interest in á-linolenic acid (18:3(*n*-6)), the active constituent of evening primrose oil -

γ-linolenic acid
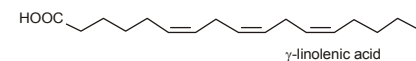
- and in conjugated linoleic acid (mainly, 9-*cis*,11-*trans*-octadecadienoate) or 'CLA', a natural constituent of dairy products, that is claimed to have remarkable health-giving properties.

9-*cis*,11-trans-octadecadienoic acid
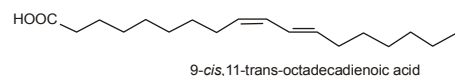
Branched-chain fatty acids are synthesised by many microorganisms (most often with an *iso*- or an *anteiso*-methyl branch) and they are synthesised to a limited extent in higher organisms. They can also enter animal tissues via the diet, especially those of ruminants.

iso-

anteiso-
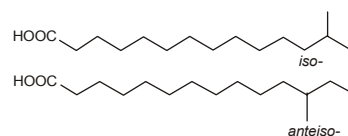
Phytanic acid, 3,7,11,15-tetramethylhexadecanoic acid, is a metabolite of phytol and is found in animal tissues, but generally at low levels only.

Fatty acids with many other substituent groups are found in certain plants and microorganisms, and they may be encountered in animal tissues, which they enter via the food chain. These substituents include acetylenic and conjugated double bonds, allenic groups, cyclopropane, cyclopropene, cyclopentene and furan rings, and hydroxy-, epoxy- and keto-groups. For example, 2-hydroxy fatty acids are synthesised in animal and plant tissues, and are often major constituents of the sphingolipids. **12-Hydroxy-octadec-9-enoic** or 'ricinoleic' acid is the main constituent of castor oil.

Ricinoleic acid
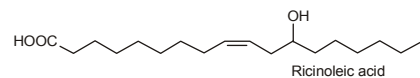
Eicosanoids and Related Lipids

The term eicosanoid is used to embrace biologically active lipid mediators (C20 fatty acids and their metabolites), including prostaglandins, thromboxanes, leukotrienes and other oxygenated derivatives, which exert their effects at very low concentrations. They are produced primarily by three classes of enzymes, cyclooxygenases (COX-1 and COX 2), lipoxygenases (LOX) and cytochrome P450 epoxygenase. The key precursor fatty acids are 8c,11c,14c-eicosatrienoic (dihomo-?-linolenic or 20:3(n-6)), 5c,8c,11c,14c-eicosatetraenoic (arachidonic or 20:4(n-6)) and 5c,8c,11c,14c,17c-eicosapentaenoic (20:5(n-3) or EPA) acids (see our web page on 'polyunsaturated fatty acids'). More recently docosanoids (resolvins and protectins) derived from 4c,7c,10c,13c,16c,19c-docosahexaenoic acid (22:6(n 3) or DHA) have been described. Other eicosanoids are produced by non-enzymic means (isoprostanes).

Those derived from arachidonic acid appear to be of special importance and have been most studied. The prostaglandins and thromboxanes have cyclic structures, generated by cyclo-oxygenase enzymes, and are involved in the processes of inflammation. The hydroxy-eicosatetraenoic acids are

generated by lipoxygenases, and of these the 5-lipoxygenase is especially important as it produces the first intermediate in the biosynthesis of leukotrienes. The resolvins and protectins have anti-inflammatory properties.

Prostagland in (PGE$_2$)

Leukotriene (LTC$_4$)

Thromboxane (TXA$_2$)

5-hydroxy-eico satetraenoic acid (5-HETE)

Lipoxin (LXA$_4$)
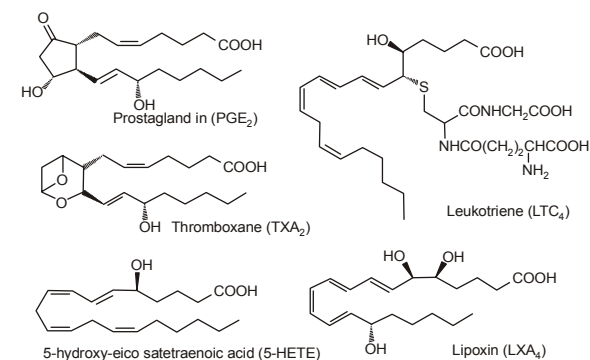
Plant products, such as the jasmonates and other oxylipins derived from 9c,12c,15c-octadecatrienoic (?-linolenic or 18:3(n-3)) are also generated by the action of lipoxygenases. They are involved in responses to physical damage by animals or insects, stress and attack by pathogens. There are obvious structural similarities between the jasmonates and prostanoids. Our introductory page on eicosanoids will lead you to further information.

7-*iso*-jasmonic acid
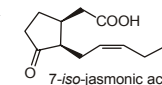
Of course, many more lipids occur in nature than can be described in this document. I have not touched on proteolipids and lipoproteins here, for example, but there is information on these and other lipids elsewhere on this website. New lipids continue to be found, and no doubt many remain to be discovered.

SUB-SECTION 2.4B—NOMENCLATURE OF SATURATED AND UNSATURATED FATTY ACIDS

Fatty acids are merely carboxylic acids with long hydrocarbon chains. The hydrocarbon chain length may vary from 10-30 carbons (most usual is 12-18). The non-polar hydrocarbon alkane chain is an important counter balance to the polar acid functional group. In acids with only a few carbons, the acid functional group dominates and gives the whole molecule a polar character. However, in fatty acids, the non-polar hydrocarbon chain gives the molecule a non- polar character.

The most common fatty acids are listed. Note that there are two groups of fatty acids—saturated and unsaturated. Recall that the term **unsaturated** refers to the presence of one or more double bonds between carbons as in alkenes. A **saturated fatty acid** has all bonding positions between carbons occupied by hydrogens.

Acid Name	Structure	MeltPoint
SATURATED		
Lauric	$CH_3(CH_2)_{10}COOH$	+44
Palmitic	$CH_3(CH_2)_{14}COOH$	+63
Stearic	$CH_3(CH_2)_{16}COOH$	+70
UNSATURATED		
Oleic	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	+16
Linoleic	$CH_3(CH_2)_4(CH=CHCH_2)_2(CH_2)_6COOH$	-5
Linolenic	$CH_3CH_2(CH=CHCH_2)_3(CH_2)_6COOH$	-11
Arachi-donic	$CH_3(CH_2)_4(CH=CHCH_2)_4(CH_2)_2COOH$	-50

The melting points for the saturated fatty acids follow the boiling point principle observed previously. Melting point principle: **as the molecular weight increases, the melting point increases.** This observed in the series lauric (C12), palmitic (C16), stearic (C18).

Room temperature is 25ºC, Lauric acid which melts at 44º is still a solid, while arachidonic acid has long since melted at -50º, so it is a liquid at room temperature.

Stearic acid   Linoleic Acid

Melting point = +70
Solid

Melting point = –5
liquid
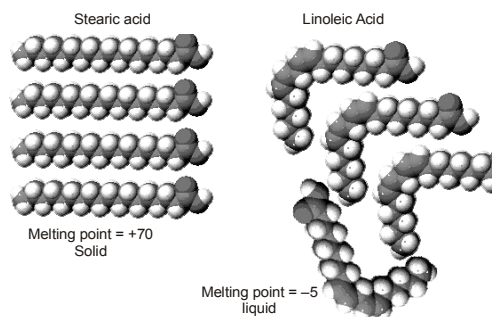
Fig. 2.10 : Showing the Fatty Acids

-Melting Points of Saturated vs. Unsaturated Fatty Acids

Note that as a group, the **unsaturated fatty acids have lower melting points than the saturated fatty acids**.

The reason for this phenomenon can be found by a careful consideration of molecular geometries. The tetrahedral bond angles on carbon results in a molecular geometry for saturated fatty acids that is relatively linear although with zigzags.

This molecular structure allows many fatty acid molecules to be rather closely "stacked" together. As a result, close intermolecular interactions result in relatively high melting points.

On the other hand, the introduction of one or more double bonds in the hydrocarbon chain in unsaturated fatty acids results in one or more "bends" in the molecule. The geometry of the double bond is almost always a cis configuration in natural fatty acids. These molecules do not "stack" very well. The intermolecular interactions are much weaker than saturated molecules. As a result, the melting points are much lower for unsaturated fatty acids.

Saturated vs. Unsaturated Fatty Acids in Fats and Oils:

Examine the table on the left, if you want the most unsaturated fatty acids in your diet, which is the healthiest, which fat or oil should you use the most? Answer = olive oil.

Which fat or oil contains the most saturated fatty acids? Answer = beef fat.

General Principle:

Vegetable oils contain more unsaturated fatty acids.

Animal fats contain more saturated fats.

Saturated Fatty Acids

They have commonly straight chains and even carbon number (4-30). They have the general formula: $CH_3(CH_2)nCOOH$

They are named from from the saturated hydrocarbon with the same number of carbon atoms, the final -e is changed to **-oic**. For example, the fatty acid with 18 carbon atoms is correctly termed octadecanoic acid but it has also a trivial name (as several common fatty acids), i.e. stearic acid. This compound may be defined also 18:0.

Table 2.15 : Below, is found a list of the most common saturated fatty acids.

Systematic name	Trivial name	Shorthand designation	Molecular wt.	Melting point (°C)
1	2	3	4	5
butanoic	butyric	4:0	88.1	-7.9
pentanoic	valeric	5:0		
hexanoic	caproic	6:0	116.1	-3.4
octanoic	caprylic	8:0	144.2	16.7
nonanoic	pelargonic	9:0	158.2	12.5
decanoic	capric	10:0	172.3	31.6
dodecanoic	lauric	12:0	200.3	44.2

1	2	3	4	5
tetradecanoic	myristic	14:0	228.4	53.9
hexadecanoic	palmitic	16:0	256.4	63.1
heptadecanoic	margaric (daturic)	17:0	270.4	61.3
octadecanoic	stearic	18:0	284.4	69.6
eicosanoic	arachidic	20:0	312.5	75.3
docosanoic	behenic	22:0	340.5	79.9
tetracosanoic	lignoceric	24:0	368.6	84.2
hexacosanoic	cerotic	26:0	396.7	88
heptacosanoic	carboceric	27:0	410.7	
octacosanoic	montanic	28:0	424.8	
triacontanoic	melissic	30:0	452.9	
dotriacontanoic	lacceroic	32:0	481	
tritriacontanoic	ceromelissic (psyllic)	33:0	495	
tetratriacontanoic	geddic	34:0	509.1	
pentatriacontanoic	ceroplastic	35:0	523.1	

Solution Properties

Normal fatty acids exhibit appreciable solubility in water compared to the corresponding hydrocarbons due to the presence of the polar carboxyl group. The first members of the saturated fatty acid series are miscible with water in all proportions at room temperature.

The solubility behavior of the fatty acids in organic solvents is of considerable theoretical and industrial importance. Solubility data for the most common saturated fatty acids are given in the table below (in grams per liter at 20°C).

On the basis of solubility data, it can be concluded that the normal saturated fatty acids are generally more soluble in chloroform and less soluble in acetonitrile than in any of the organic solvents investigated.

Up to 6 (or 4) carbon atoms, organic acids are considered "short-chain organic acids", they have substantial solubility in water. Furthermore, they do not behave physiologically like other fatty acids since they are more rapidly digested and absorbed in the intestinal tract

Solubility in water at 20°C (in grams acid per liter)

Carbon number	Solubility
2	infinite
4	infinite
6	9.7
8	0.7
10	0.15
12	0.055
14	0.02
16	0.007
18	0.003

and have unique properties in regulating sodium and water absorption through the mucosal epithelium.

Carbon number	Chloroform	Benzene	Cyclohexane	Acetone	Ethanol 95%	Acetic acid	Methanol	Acetonitrile
10	3260	3980	3420	4070	4400	5670	5100	660
12	830	936	680	605	912	818	1200	76
14	325	292	215	159	189	102	173	18
16	151	73	65	53.8	49.3	21.4	37	4
18	60	24.6	24	15.4	11.3	1.2	1	<1

Biochemically, they are more closely related to carbohydrates than to fats.

From 8 (or 6) to 10 (or 12) carbon atoms, fatty acids are said to have a medium chain. Physiological studies have shown that ingestion of triglycerides containing these medium-chain fatty acids may result, as for short-chain fatty acids, in increased energy expenditure via faster satiety. Thus, they facilitate weight control when included in the diet as a replacement for long-chain triglycerides Fatty acids which have 14 (or 12) and more carbon atoms are considered as long-chain fatty acids. Fatty acids with 4 to 12 carbon atoms are found mainly in milk fats (mainly butyric acid in cow and decanoic acid in sheep) but those with 10 and 12 carbon atoms are found also in certain seed oils such as coconut and other kernel fats of the palm family.

- **Butyric acid** (4:0) is the lowest member of the acetic acid series found in natural fats. It occurs (2 to 4%) as a component of milk fats. It gives a rancid odor to butter when triglycerides are hydrolyzed and is present in fermentation products of carbohydrates. This fatty acid has peculiar physiological properties in causing growth arrest and apoptosis in various cell types (*Urbano A et al., Leukemia 1998, 12, 930*). It was tested in the therapy of solid tumors or leukemia (*Kasukabe T et al., Br J Cancer 1997, 75, 850*).

- **Valeric acid** (5:0) has been identified in petroleum distillates and in oxidation products of oils and fats and fermentation of carbohydrates. It has a putrid odor.

- **Caproic acid** (6:0) occurs in milk fats to the extent of about 2%. It was first isolated from butter in 1816 by Chevreul. It has a characteristic odor of goats, hence its name (from the Latin *caper*, goat). Caproic acid is present as glucose ester in leaf trichomes of *Datura metel*.

- **Caprylic acid** (8:0) is widely distributed in animal and vegetable fats but rarely exceeding 8% of the total fatty acids, except in the seed oils of two Lythraceae, *Cuphea hookerina* and *C. painteri*, which contain about 70% caprylic acid (*Miller RW et al., JAOCS 1964, 41, 279*). It occurs to an extent of 1 to 4% in milk fats, and 6 to 8% in coconut and palm oils.

- **Pelargonic acid** (9:0) is the first example of the occurrence of an odd-numbered carbon fatty acid in natural products. It occurs in secretion of sebaceous glands and in essential oil of *Pelargonium roseum* from which it derives its name. It is also a primary product of oxidative fission of oleic acid.

- **Capric acid** (10:0) occurs as a minor component in the same fats that contain caprylic acid but also in the head oil of the sperm whale, and in wool and hair fats. It is a major constituent of elm seed oil (over 60% in *Ulmus americana* and over 70% in *Zelkova serrata)* but is absent in other Ulmaceae (*Apanthe, Morus*) (*Badami RC et al., Prog Lipid Res 1981, 19, 119*). Similarly, it was discovered that the seed oil of a Lythraceae, *Cuphea llavea*, contained about 80% of this acid (*Earle FR et al., JAOCS 1960, 37, 440*).

- **Lauric acid** (12:0) is one of the three most widely distributed saturated fatty acids found in nature (14:0, 16:0, and 18:0). It occurs extensively in *Lauraceae* seeds (*Laurus nobilis*) where it was discovered (*Marsson T Ann 1842, 41, 329*). It is dominant in cinnamon oil (80-90%), coconut oil (40-60% as trilaurin) and is found also in *Cuphea* species (*Umbelliferae*) whose production was initiated in Germany. The recent uses of lauric acid are in the manufacture of flavourings, cocoa butter, margarine, alkyd resins, soaps, shampoos and other surface active agents, including special lubricants. Lauric acid as monoglyceride is known to the pharmaceutical industry for its good antimicrobial properties. It may play a role in combating lipid-coated RNA and DNA viruses. The major sources of lauric acid for human food are palm kernel, coconut and palm.

- **Myristic acid** (14:0) is present in major amounts in seeds of the family *Myristicaceae* (nutmeg oil - or oil of mace - from *Myristica fragrans* contains about 60-70% of trimyristin) where it was first discovered (*Playfair L Ann 1841, 37, 152*). Nutmeg is found in Moluccas and spice islands of Indonesia. Coconut and palm kernel are also convenient sources of 14:0 (trimyristine) which may be isolated in a pure form by distillation. It is also present in milk fats (8-12%) and in the head oil of the sperm whale (15%).

- **Palmitic acid** (16:0) is the commonest saturated fatty acids in plant and animal lipids.
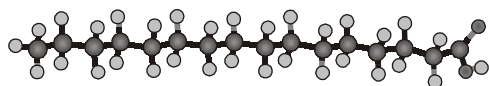
It was purified first by **Chevreul** in his researches on butter and tallow, but was first surely characterized by Fremy E (*Ann 1840, 36, 44*), who prepared it in pure form from palm oil, from which he named it. Despite its wide distribution, it is generally not present in fats in very large proportions. It usually forms less than 5% of the total fatty acids, sometimes as much as 10% in common vegetal oils (peanut, soybean, corn, coconut) and in marine-animal oils. Lard, tallow, cocoa butter palm oil contain 25 to 40% of this component.

- **Stearic acid** (18:0) was described by **Chevreul** (1823) in the course of his researches on fats. It is the highest molecular weight saturated fatty acid occurring abundantly in fats and oils. It occurs in small quantities in seed and marine oils. Milk fats (5-15%), lard (10%), tallow (15-30%), cocoa and shea butters ((30-35%) are the richest sources of stearic acid. It is the principal constituent of hydrogenated fats and oils (about 90%).

The longer chains are less frequent, they can be found in uncommon seed oils (C20-24 in *Leguminoseae* and *Sapindaceae*), in palm oil (C20-C32)(*Puah CW et al., Lipids 2006, 41, 305*), in

waxes (C24-30) and in some sphingolipids (C20-24). Long-chain saturated fatty acids (from C24 to C28) are produced by microalgae and it was estimated that diatoms contribute from 30 to 80% of these components in sandy sediments (*Volkman JK et al., Org Geochem 1998, 29, 1163*). These long-chain fatty acids derive from higher plant waxes and are more abundant in deep than in surface sediments (*Rieley G et al., Org Geochem 1991, 17, 901; Muri G et al., Org Geochem 2004, 35, 1083*).

- **Arachidic acid** (20:0) occurs in appreciable quantities in groundnut (*Arachis hypogea)* oil (3%) where it was discovered in 1854 by Gössmann A (*Ann Chemie 1854, 89, 1*). Larger amounts are found in seeds of *Sapindaceae* (up to 20%). It is also found in the depot fat of some animals and in milk fats.

- **Behenic acid** (22:0) was first reported as a constituent of ben (behen) oil (seeds of *Moringa oleifera*). Except for the seed oils of the *Crucifereae* (between 0.5 and 3.4%), this fatty chain does not occur in the principal oils. Large amounts are found in hydrogenated animal and vegetal oils (8-57%).

- **Lignoceric acid** (24:0) is present at trace levels in plant oils except in groundnut oil (about 1%) and notably in a Leguminous seed oil (*Adenanthera pavonina*) where it may amount to about 25%.It is the principal fatty acid present in carnauba wax (30% of the normal fatty acids). A major source is rice-wax bran (about 40%). Without double bonds or other functional groups, these fatty acids are nearly chemically inert and thus can be subjected to drastic chemical conditions (temperature, oxidation).

Saturated fatty acids were shown to be the major constituents of **adipocere** (similar to "adipocire" studied by Chevreul), the white and soap-like decomposition product which forms due to the post-mortem conversion of body adipose tissue. Immediately following death, triglycerides are hydrolyzed into free fatty acids and glycerol. The free fatty acids (mainly myristic, palmitic, and stearic acids) present in characteristic relations are formed by hydrogenation of triglyceride components under suitable environmental conditions. During that conversion process a number of byproducts may be formed, such as hydroxy or keto fatty acid derivatives. The occurrence of salts of these saturated fatty acids has been suggested as resulting from reaction with the surrounding mineral environment.

Saturated fatty acids with straight chain have been found in a number of sediments ranging in age from Precambrian to Recent. In most sediments, fatty acids with even-carbon chain are more abundant than those with odd-carbon chain. All fatty acids from C8 to C28 have been found in sediments. Experiments suggest that normal paraffins in petroleum may be produced from normal fatty acids of longer chain lengths by decarboxylation or other chemical reactions.

Branched-chain Fatty Acids

1. Branched chain fatty acids are found most frequently with an unsubstituted carbon chain (some branched polyunsaturated fatty acids are found in sponges) but may have one or several branched methyl groups :

Mono or multibranched chain fatty acids

2. Branched chain fatty acids (mono- branched) may have also a **methoxy** or a **hydroxy** substitution, they are found in exotic animals or bacteria :

Branched methoxy fatty acids and Branched hydroxy fatty acids (Mycolic acids)

Mono- and multibranched fatty acids As for hydrocarbons, they have usually either an iso-structure (methyl group at the penultimate carbon atom) or a anteiso-structure (methyl group on the third carbon from the end). Examples: 14-methyl pentadecanoic acid (isopalmitic) is of the iso-series and 13-methyl pentadecanoic acid is the corresponding anteiso-acid.

1. Monomethyl branched fatty acids are found in vegetal, animal, and microbial lipids but in small concentrations. In animals, some classic examples of these compounds include the 2- and 4-monomethylated fatty acids from the uropygial gland of ducks In vegetals, 14-methyl-16:0 has been identified in *Ginkgo biloba (Hierro MTG et al., J. Am Oil Chem Soc, 1996, 73, 575)* and was found to be characteristic of pine seed oil (up to 1%). This fatty acid was found exclusively in *Pinaceae* (genera *Pinus, Abies, Cedrus, Picea ...*). The unsaturated 11-methyloctadec-12-enoic and 12-methyloctadec-10-enoic acids were identified in the seed oil of *Byrsocarpus coccineus* (Connaraceae) (*Spencer GF et al., Lipids 1979, 14, 72*). A *trans*-monounsaturated branched-chain fatty acid, 8-methyl-*trans*-6-nonenoic acid, is characteristic of specific compounds, the capsaicinoids, found in fruits of the genus Capsicum (Solanaceae). These plants (bell pepper, chili pepper) are among the oldest cultivated plants, their pungent fruits being used as spices for over 6000 years. Capsaicinoids are synthesized by an enzymatic condensation of vanillylamine and a medium chain branched acid. More than 20 compounds, different only in the fatty acid structures, have been described.The sponges contain also large quantities of C14 up to C30 fatty acids with branch as well as odd-chains (*Carballeira N et al., Lipids 1989, 24, 229*). As an example, a new structure (20-methyl-26:0) has been elucidated in the sponge *Verongia aerophoba* from the Canary islands (*Nechev J et al., Eur J Lipid Sci Technol 2002, 104, 800*) while some monomethyl polyunsaturated fatty acids were described in different marine sponges (24-methyl-5,9-pentacosadienoic acid or 2-methoxy-13-methyl-6-tetradecenoic acid) (*Caballeira NM et al., J Nat Prod 2001, 64, 620*) . Similarly, two new 2-methyl branched monoenoic very long chain fatty acids (2-methyl-24:1 n-7 and 2-methyl-26:1 n-9) were described in a marine sponge *Halichondria panicea* (*Imbs AB et al., Chem Phys Lipids 2004, 129, 173*).It must be noticed that the similarity between the composition of the midchain branching pattern of fatty acids in some sponges and in bacteria suggests the presence of bacteria in these sponges. For *Calcarea*, the constant and prominent occurrence of iso- and anteiso-fatty acids (>40% of the total fatty acids in most species) suggests an origin of these compounds from the sponge cells rather than from bacterial lipids (*Schreiber A et al., Chem Phys Lipids 2006, 143, 29*). Another indication for a sponge cell origin of these compounds is the major presence (7-15 % of the total) of anteiso-nonadecanoic acid (16-methyloctadecanoic acid), an exotic compound that has not yet been reported as a major fatty acid in bacteria. It has been found that *Caenorhabditis elegans* is able to synthesized iso-C15 and iso-C17 and that these branched-chain fatty acids are essential for the animal growth and development (*Kniazeva M et al., PLoS Biol 2004, 2, E257*).

These results suggest that these fatty acids may play a potentially important role in other eukaryotes.

A new unsaturated methyl-branched fatty acid, 9-methyl-16:1(n-6) and the uncommon 11-methyl-18:1(n-6) were found in the lipid extract of a new strain of bacterium *Vibrio alginolyticus* associated with the alga *Cladophora coelothrix (Carballeira NM et al., Lipids, 1997, 32, 1271)*. Another novel methyl-branched fatty acid, 10-methyl-18:1(n-9), was found in the lipid extract of the marine fungus *Microsphaeropis olivacea (Yu CM et al., Can. J. Chem., 1996, 74, 730)*.

Branched methyl-substituted fatty acids of bacterial origin are commonly found in lake or marine sediments, decreasing rapidly with depth (*Matsuda H et al., Geochim Cosmochim 1977, 41, 777*). Long-chain monomethyl-branched anteiso acids were also identified in settlings particles and surface sediments from freshwater lakes where they may be useful molecular markers for lake acidity (*Fukushima K et al., Org Geochem 2005, 36, 311*). 10-methyl octadecanoic acid (**tuberculostearic acid**) is present in the phosphatidylinositol moiety of lipoarabinomannans found mainly in *Mycobacterium tuberculosis* but also in other bacteria in the genus *Mycobacterium*. The detection of this fatty acid in cerebrospinal fluid was proposed as a possibility for rapid and specific diagnosis of tuberculous meningitis. The presence of this fatty acid in sputum lipids was successively utilized for the diagnosis of tuberculous pneumonia (*Larsson L et al., J Clin Microbiol 1987, 25, 893*).

10-methyl nonadecanoic acid (**phytomonic acid**) is also found in *Mycobacterium*.

In animals, 17-methyl-6-octadecenoic and 17-methyl-7-octadecenoic acids were identified in the Australian mollusk *Siphonaria denticulata (Carballeira NM et al. J Nat Prod 2001, 64, 1426)*.

An unusual complex and polyunsaturated fatty acid substituted with one hydroxyl and one aldehyde group has been described as a new polyene pigment, **laetiporic acid**, in the wood-rotting basidiomycete *Laetiporus sulphureus (Weber RW et al., Tetrahedron lett 2004, 45, 1075)*. This orange pigment, with an UV-visible spectrum similar to that of carotenoids, bears an unprecedented decaene skeleton as part of its chromophore.
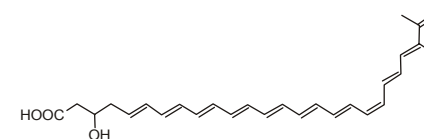
2 - Multimethyl branched acids are found mainly in bacteria.

Several dimethylated fatty acids (14 or 16 carbon atoms) with the first methyl substituent at carbon 2 or 4 have been isolated from a halophilic *Bacillus* species (*Carballeira NM et al., J Nat Prod 2001, 64, 256*).

Di- and tri-methylated fatty acids with 15 to 18 carbon atoms have been isolated from environmental subsurface sediments (*Hedrick DB et al., Lipids 2008, 43, 843*). These fatty acids may be of value for the knowledge of biomass and of the metabolic status of the viable microbial community.

Multimethyl branched acids are abundant in cell wall lipids of *Mycobacteria*, each methyl group being on even carbon atoms (2,4,6,8...from the methyl end). Thus, forming waxes and glycolipids (mycosides), several multibranched fatty acids are commonly found - **mycoceranic** (2,4,6-trimethyloctacosanoic) and **mycolipenic** (2,4,6-trimethyl-trans-2-tetracosenoic) acids- **mycocerosic acid** (2,4,6,8-tetramethyl C32 fatty acid)

- **phthioceranic acids** which are hepta or octamethyl fatty acids, some of them being also hydroxylated (hydroxyphthioceranic acid).

$$CH_3(CH_2)_{19} \left[\begin{array}{c} CH\text{-}CH_2 \\ | \\ CH_3 \end{array} \right]_3 \begin{array}{c} CH\text{-}COOH \\ | \\ CH_3 \end{array}$$

$$CH_3(CH_2)_{15}-\left[CH-CH_2\right]-CH-COOH$$
$$CH_3 \quad\big]_6 \quad CH_3$$

$$CH_3(CH_2)_{14}-\left[CH-CH_2\right]-CH-COOH$$
$$CH_3 \quad\big]_7 \quad CH_3$$
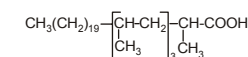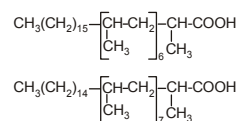
Branched acids but with shorter chains are also found in the depot fats of ruminant animals, in sebum and animal waxes (wool-wax). Ruminants produced a huge variety of C10-C18 acids with one to four branched methyl groups, they were detected in lamb adipose tissue. Multimethyl branched acids are also dominant in the uropygial waxes of the birds preen gland. Curiously, they were also identified in the lipid depot in the head of marine animals, location involved in their echo-locating abilities.

Unusual branched fatty acids have been isolated as minor components from the glycolipids (GL) fraction of freshwater sponges (*Dembitsky VM et al., Chem Phys Lipids 2003, 123, 117*).It is possible that these neo acids could be of cyanobacterial origin. An example of one of them with the longest carbon chain is shown below.
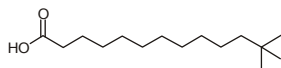
Others have isoprenoid structures, thus coming from the diterpene phytol derived from chlorophyll and not by the *de novo* pathway. Among them, two are found in marine organisms, in geological sediments but, one of them (**phytanic acid** or 3,7,11,15-tetramethyl hexadecanoic acid) is present in human diet or in animal tissues where it may be derived from chlorophyll in plant extracts.

Two very unusual phytyl esters were obtained from the extract of the hornwort *Megaceros flagellaris (Bryophyte, Anthocerothae)*. The fatty acid moiety comprises 3,7,11,15-tetramethyl-16:1 or 3,7,11,15-tetramethyl-16:0, which is esterified to the corresponding tetramethyl unsaturated (16:1) alcohol (Phytanic acid derives from the corresponding alcohol, phytol, and is oxidized into **pristanic acid**.

Pristanic acid   Phytanic acid
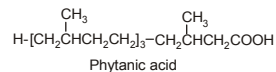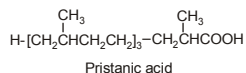
Pristanic acid was first isolated from butter (*Hansen RP et al., Biochemical Journal 1964, 93, 225*). The name of the substance is derived from pristane (2,6,10,14-tetramethylpentadecane), the corresponding hydrocarbon which was isolated from shark (*pristis* in Latin). It is also found in the lipids from many sources such as sponges, crustacea, milk fats, animal depot fat but also in petroleum samples.

Phytanic acid characterizes a precise human pathology, the Refsum's syndrome. This inherited neurological disorder (*Refsum S, Acta Psychiat Scand Suppl 1946, 38, 9*) is characterized by a accumulation of phytanic acid, normal metabolite of phytol, in blood and tissues (*Klenk E et al., Hoppe Seyler's Z Physiol Chem 1963, 333, 133*). The disorder was later related to deficiency in the a-oxidation pathway in the liver (*Herndon JH et al., J Clin Invest 1969, 48, 1017*; review in *: Mukherji M et al.,*

Prog Lipid Res 2003, 42, 359-376). Both phytanic acid and pristanic acid have been shown to activate the peroxisome proliferator-activated receptor* (PPAR) in a concentration-dependent manner Freshwater sponges contain polymethyl branched fatty acids such as 4,8,12-trimethyltridecanoic, phytanic and pristanic acids. These acids may have chemotaxonomical significance for both marine and freshwater sponges (*review in : Dembitsky VM et al., Chem Phys Lipids 2003, 123, 117*). The isoprenoic 4,8,12-trimethyltridecanoic was found to be always present in the marine calcareous sponges (Calcarea) but in minor amounts (*Schreiber A et al., Chem Phys Lipids 2006, 143, 29*), this acid being presumed to be derived from phytol, a degradation product of chlorophyll. Phytanic acid present in immature (recent) sediments is thought to derive from phytol, a supposition backed by stereochemical studies

Few sources, including sponges, contain branched polyunsaturated fatty acids (*Rezanka T, Prog Lipid Res 1989, 28, 147*). As an example, freshwater *Demospongia* (*Spongillidae*) were shown to contain di-, tri-, and tetramethyl substituted dienoic, tetraenoic, and hexaenoic fatty acids (*Rezanka T et al., J Nat Prod 2002, 65, 709*). A review of these rare polyenoic fatty acids was released by Dembitsky VM et.

Isoprenoid fatty acids

Some isoprene derivatives (sesquiterpenes) synthesized by invertebrates from farnesoic acid have important endocrinological functions (juvenile hormones) such as molting, reproduction and metamorphosis. Addition of isoprene acids to proteins (prenylation), discovered in 1984 (*Schmidt RA et al., J Biol Chem 1984, 259, 10175*), is an important post-translational modification of proteins which has been recognized as a key physiological process for facilitating cellular protein-protein interactions and membrane-associated protein trafficking. Protein prenylation occurs by the covalent addition of two types of isoprenoids, farnesyl pyrophosphate (a 15-carbon sesquiterpene) or geranylgeranyl pyrophosphate (a 20-carbon diterpene), to cysteine residues at or near the terminal carboxyl group . The largest family of prenylated proteins are the intracellular GTP-binding proteins that transduce extracellular signals into intracellular changes via downstream effectors Some isoprenoid fatty acids with conjugated double bonds are known. In this group, the most interesting is **retinoic acid** which derives from retinol but is synthetized ultimately from b-carotene (provitamin A). It has important functions in cell regulation.

β-retinoic acid (*all*-trans isomer)
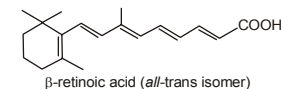
Abscissic acid, a methylated derivative of retinoic acid, plays a variety of roles in plant physiology.It is ubiquitous in plants, including algae.

Branched methoxy fatty acids

Iso or anteiso a-methoxylated C15-C16 fatty acids were reported in phospholipids of a Caribean sponge.

Thus, 2-methoxy-13-methyltetradecanoic acid, 2-methoxy-14-methylpentadecanoic acid, and 2-methoxy-13-methyl pentadecanoic acid were identified by gas-liquid chromatography and mass spectrometry.

It has been suggested that these compounds have originated from a novel bacteria in symbiosis with the sponge.

2-methoxy-14-methylpentadecanoic acid
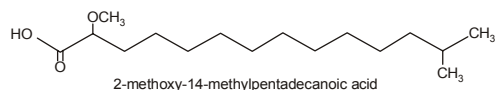
SECTION 2.5—ESSENTIAL FATTY ACIDS

SUB-SECTION 2.5A(ω—3 FATTY ACIDS OR OMEGA-3 FATTY ACIDS)

n–3 fatty acids (popularly referred to as **ù-3 fatty acids** or **omega-3 fatty acids**) are a family of unsaturated fatty acids that have in common a carbon–carbon double bond in the *n*–3 position; that is, the third bond from the methyl end of the fatty acid. Important nutritionally essential *n*–3 fatty acids are: á-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). The human body cannot synthesize *n*–3 fatty acids *de novo*, but it can form 20- and 22-carbon unsaturated *n*–3 fatty acids from the eighteen-carbon *n*–3 fatty acid, á-linolenic acid. These conversions occur competitively with *n*–6 fatty acids, which are essential closely related chemical analogues that are derived from linoleic acid. Both the *n*–3 á-linolenic acid and *n*–6 linoleic acid are essential nutrients which must be obtained from food. Synthesis of the longer *n*–3 fatty acids from linolenic acid within the body is competitively slowed by the *n*–6 analogues. Thus accumulation of long-chain *n*–3 fatty acids in tissues is more effective when they are obtained directly from food or when competing amounts of *n*–6 analogs do not greatly exceed the amounts of *n*–3.

Chemical structure of alpha-linolenic acid (ALA), an essential *n*–3 fatty acid, (18:3Ä9c,12c,15c, which means a chain of 18 carbons with 3 double bonds on carbons numbered 9, 12 and 15). Although chemists count from the carbonyl carbon (blue numbering), physiologists count from the *n* (ù) carbon (red numbering). Note that from the *n* end (diagram right), the first double bond appears as the third carbon-carbon bond (line segment), hence the name "*n*–3". This is explained by the fact that the *n* end is almost never changed during physiologic transformations in the human body, as it is more stable energetically, and other carbohydrates compounds can be synthetized from the other carbonyl end, for example in glycrids, or from double bonds in the middle of the chain.

The term *n*–3 (also called **ù–3** or **omega-3**) signifies that the first double bond exists as the **third** carbon-carbon bond from the terminal methyl end (*n*) of the carbon chain.

n–3 fatty acids which are important in human nutrition are: á-linolenic acid (18:3, *n*–3; ALA), eicosapentaenoic acid (20:5, *n*–3; EPA), and docosahexaenoic acid (22:6, *n*–3; DHA). These three polyunsaturates have either 3, 5 or 6 double bonds in a carbon chain of 18, 20 or 22 carbon atoms, respectively. All double bonds are in the *cis*-configuration, i.e. the two hydrogen atoms are on the same side of the double bond.

Most naturally-produced fatty acids (created or transformed in animalia or plant cells with an even

number of carbon in chains) are in *cis*-configuration where they are more easily transformable. The *trans*-configuration results in much more stable chains that is very difficult to further break or transform, forming longer chains that aggregate in tissues and lacking the necessary hydrophilic properties. This *trans*-configuration can be the result of the transformation in alkaline solutions, or of the action of some bacterias that are shortening the carbonic chains. Natural transforms in vegetal or animal cells more rarely affect the last *n*"3 group itself. However, *n*"3 compounds are still more fragile than *n*"6 because the last double bond is geometrically and electrically more exposed, notably in the natural *cis* configuration.

List of *n*-3 fatty acids

This table lists several different names for the most common *n*–3 fatty acids found in nature.

Common name	Lipid name	Chemical name
	16:3 (n–3)	all-cis-7,10,13-hexadecatrienoic acid
α-Linolenic acid (ALA)	18:3 (n–3)	all-cis-9,12,15-octadecatrienoic acid
Stearidonic acid (STD)	18:4 (n–3)	all-cis-6,9,12,15-octadecatetraenoic acid
Eicosatrienoic acid (ETE)	20:3 (n–3)	all-cis-11,14,17-eicosatrienoic acid
Eicosatetraenoic acid (ETA)	20:4 (n–3)	all-cis-8,11,14,17-eicosatetraenoic acid
Eicosapentaenoic acid (EPA)	20:5 (n–3)	all-cis-5,8,11,14,17-eicosapentaenoic acid
Docosapentaenoic acid (DPA), Clupanodonic acid	22:5 (n–3) acid	all-cis-7,10,13,16,19-docosapentaenoic
Docosahexaenoic acid (DHA)	22:6 (n–3)	all-cis-4,7,10,13,16,19-docosahexaenoic acid
Tetracosapentaenoic acid	24:5 (n–3)	all-cis-9,12,15,18,21-docosahexaenoic acid
Tetracosahexaenoic acid (Nisinic acid)	24:6 (n–3)	all-cis-6,9,12,15,18,21-tetracosenoic acid

Biological significances

The biological effects of the n*–3 are largely mediated by their interactions with the* n*"6 fatty acids; see Essential fatty acid interactions for detail.*

A 1992 article by biochemist William E.M. Lands provides an overview of the research into *n*"3 fatty acids, and is the basis of this section.

The 'essential' fatty acids were given their name when researchers found that they were essential to normal growth in young children and animals. (Note that the modern definition of 'essential' is more strict.) A small amount of *n*–3 in the diet (~1% of total calories) enabled normal growth, and increasing the amount had little to no additional effect on growth.

Likewise, researchers found that *n*–6 fatty acids (such as ã-linolenic acid and arachidonic acid) play a similar role in normal growth. However, they also found that *n*–6 was "better" at supporting dermal integrity, renal function, and parturition. These preliminary findings led researchers to concentrate their studies on *n*–6, and it was only in recent decades that *n*–3 has become of interest.

In 1963 it was discovered that the *n*–6 arachidonic acid was converted by the body into pro-inflammatory agents called prostaglandins. By 1979 more of what are now known as eicosanoids were discovered: thromboxanes, prostacyclins and the leukotrienes. The eicosanoids, which have important biological functions, typically have a short active lifetime in the body, starting with synthesis from fatty acids and ending with metabolism by enzymes. However, if the rate of synthesis exceeds the rate of metabolism, the excess eicosanoids may have deleterious effects. Researchers found that *n*–3 is also converted into eicosanoids, but at a much slower rate. Eicosanoids made from *n*–3 fats often have opposing functions to those made from *n*–6 fats (ie, anti-inflammatory rather than inflammatory). If both *n*–3 and *n*–6 are present, they will "compete" to be transformed, so the ratio of *n*–3 : *n*–6 directly affects the type of eicosanoids that are produced.

This competition was recognized as important when it was found that thromboxane is a factor in the clumping of platelets, which leads to thrombosis. The leukotrienes were similarly found to be important in immune/inflammatory-system response, and therefore relevant to arthritis, lupus, and asthma. These discoveries led to greater interest in finding ways to control the synthesis of *n*–6 eicosanoids. The simplest way would be by consuming more *n*–3 and fewer *n*–6 fatty acids.

Health benefits On September 8, 2004, the U.S. Food and Drug Administration gave "qualified health claim" status to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) *n*–3 fatty acids, stating that "supportive but not conclusive research shows that consumption of EPA and DHA [*n*–3] fatty acids may reduce the risk of coronary heart disease. This updated and modified their health risk advice letter of 2001 (see below).

People with certain circulatory problems, such as varicose veins, benefit from fish oil. Fish oil stimulates blood circulation, increases the breakdown of fibrin, a compound involved in clot and scar formation, and additionally has been shown to reduce blood pressure. There is strong scientific evidence that *n*–3 fatty acids significantly reduce blood triglyceride levels and regular intake reduces the risk of secondary and primary heart attack.

Some benefits have been reported in conditions such as rheumatoid arthritis and cardiac arrhythmias.

There is a promising preliminary evidence that *n*-3 fatty acids supplementation might be helpful in cases of depression and anxiety. Studies report highly significant improvement from *n*-3 fatty acids supplementation alone and in conjunction with medication.

Some research suggests that fish oil intake may reduce the risk of ischemic and thrombotic stroke. However, very large amounts may actually increase the risk of hemorrhagic stroke (see below). Lower amounts are not related to this risk, 3 grams of total EPA/DHA daily are considered safe with no increased risk of bleeding involved and many studies used substantially higher doses without major side effects (for example: 4.4 grams EPA/2.2 grams DHA in 2003 study).

Several studies report possible anti-cancer effects of *n*–3 fatty acids (particularly breast, colon and prostate cancer). No clear conclusion can be drawn at this time, however.

A 2006 report in the Journal of the American Medical Association concluded that their review of literature covering cohorts from many countries with a wide variety of demographic characteristics demonstrating a link between *n*–3 fatty acids and cancer prevention gave mixed results. This is similar to the findings of a review by the British Medical Journal of studies up to February 2002 that failed to find clear effects of long and shorter chain *n*–3 fats on total mortality, combined cardiovascular events and cancer.

In 1999, the GISSI-Prevenzione Investigators reported in the Lancet, the results of major clinical study in 11,324 patients with a recent myocardial infarction. Treatment 1 gram per day of *n*–3 fatty acids reduced the occurrence of death, cardiovascular death and sudden cardiac death by 20%, 30% and 45% respectively.[32] These beneficial effects were seen already from three months onwards.

In April 2006, a team led by Lee Hooper at the University of East Anglia in Norwich, UK, published a review of almost 100 separate studies into *n*–3 fatty acids, found in abundance in oily fish. It concluded that they do not have a significant protective effect against cardiovascular disease. This meta-analysis was controversial and stands in stark contrast with two different reviews also performed in 2006 by the American Journal of Clinical Nutrition and a second JAMA review that both indicated decreases in total mortality and cardiovascular incidents (i.e. myocardial infarctions) associated with the regular consumption of fish and fish oil supplements. In addition *n*–3 has shown to aid in other mental disorders such as aggression and ADHD (Attention-deficit hyperactivity disorder).

Several studies published in 2007 have been more positive. In the March 2007 edition of the journal *Atherosclerosis*, 81 Japanese men with unhealthy blood sugar levels were randomly assigned to receive 1800 mg daily of eicosapentaenoic acid (EPA — an *n*–3 essential fatty acid from fish oil) with the other half being a control group. The thickness of the carotid arteries and certain measures of blood flow were measured before and after supplementation. This went on for approximately two years. A total of 60 patients (30 in the EPA group and 30 in the control group) completed the study. Those given the EPA had a statistically significant decrease in the thickness of the carotid arteries along with improvement in blood flow. The authors indicated that this was the first demonstration that administration of purified EPA improves the thickness of carotid arteries along with improving blood flow in patients with unhealthy blood sugar levels.

In another study published in the American Journal of Health System Pharmacy March 2007, patients with high triglycerides and poor coronary artery health were given 4 grams a day of a combination of EPA and DHA along with some monounsaturated fatty acids. Those patients with very unhealthy triglyceride levels (above 500 mg/dl) reduced their triglycerides on average 45% and their VLDL cholesterol by more than 50%. VLDL is a bad type of cholesterol and elevated triglycerides can also be deleterious for cardiovascular health.

There was another study published on the benefits of EPA in *The Lancet* in March 2007. This study involved over 18,000 patients with unhealthy cholesterol levels. The patients were randomly assigned to receive either 1,800 mg a day of EPA with a statin drug or a statin drug alone. The trial went on for a total of five years. It was found at the end of the study those patients in the EPA group had superior cardiovascular function. Non-fatal coronary events were also significantly reduced in the EPA group. The authors concluded that EPA is a promising treatment for prevention of major coronary events, especially non-fatal coronary events.

Another study regarding fish oil was published in the *Journal of Nutrition* in April 2007. Sixty four healthy Danish infants from nine to twelve months of age received either cow's milk or infant formula alone or with fish oil. It was found that those infants supplemented with fish oil had improvement in immune function maturation with no apparent reduction in immune activation.

There was yet another study on *n*–3 fatty acids published in the April 2007 *Journal of Neuroscience*. A group of mice were genetically modified to develop accumulation of amyloid and tau proteins in the brain similar to that seen in people with poor memory. The mice were divided into four groups with one group receiving a typical American diet (with high ratio of *n*–6 to *n*–3 fatty acids being 10 to 1). The other three groups were given food with a balanced 1 to 1 *n*–6 to *n*–3 ratio and two additional groups supplemented with DHA plus long chain *n*–6 fatty acids. After three months of feeding, all the DHA supplemented groups were noted to have a lower accumulation of beta amyloid and tau protein. Some research suggests that these abnormal proteins may contribute to the development of memory loss in later years.

There is also a study published regarding *n*e3 supplementation in children with learning and behavioral problems. This study was published in the April 2007 edition of the *Journal of the Developmental and Behavioral Pediatrics* , where 132 children, between the ages of seven to twelve years old, with poor learning, participated in a randomized, placebo-controlled, double-blinded interventional trial. A total of 104 children completed the trial. For the first fifteen weeks of this study, the children were given polyunsaturated fatty acids (*n*e3 and *n*e6, 3000 mg a day), polyunsaturated fatty acids plus multi-vitamins and minerals or placebo. After fifteen weeks, all groups crossed over to the polyunsaturated fatty acids (PUFA) plus vitamins and mineral supplement. Parents were asked to rate their children's condition after fifteen and thirty weeks. After thirty weeks, parental ratings of behavior improved significantly in nine out of fourteen scales. The lead author of the study, Dr. Sinn, indicated the present study is the largest PUFA trial to date with children falling in the poor learning and focus range. The results support those of other studies that have found improvement in poor developmental health with essential fatty acid supplementation.

Research in 2005 and 2006 has suggested that the in-vitro anti-inflammatory activity of *n*e3 acids translates into clinical benefits. Cohorts of neck pain patients and of rheumatoid arthritis sufferers have demonstrated benefits comparable to those receiving standard NSAIDs. Those who follow a Mediterranean-style diet tend to have less heart disease, higher HDL ("good") cholesterol levels and higher proportions of *n*–3 in tissue highly unsaturated fatty acids. Similar to those who follow a Mediterranean diet, Arctic-dwelling Inuit - who consume high amounts of *n*e3 fatty acids from fatty fish - also tend to have higher proportions of *n*–3, increased HDL cholesterol and decreased triglycerides (fatty material that circulates in the blood) and less heart disease. Eating walnuts (the ratio of *n*–3 to *n*–6 is circa 1:4 respectively) was reported to lower total cholesterol by 4% relative to controls when people also ate 27% less cholesterol.

A study examining whether omega-3 exerts neuroprotective action in Parkinson's disease found that it did, using an experimental model, exhibit a protective effect (much like it did for Alzheimer's disease as well). The scientists exposed mice to either a control or a high omega-3 diet from two to twelve months of age and then treated them with a neurotoxin commonly used as an experimental

model for Parkinson's. The scientists found that high doses of omega-3 given to the experimental group completely prevented the neurotoxin-induced decrease of dopamine that ordinarily occurs. Since Parkinson's is a disease caused by disruption of the dopamine system, this protective effect exhibited could show promise for future research in the prevention of Parkinson's disease.

A study carried out involving 465 women showed serum levels of eicosapentaenoic acid is inversely related to the levels of anti-oxidized-LDL antibodies. Oxidative modification of LDL is thought to play an important role in the development of atherosclerosis.

In 2008 a German study suggested that Omega-3 fatty acids have a positive effect on atopic dermatitis.

Health risks

In a letter published October 31, 2000, the United States Food and Drug Administration Center for Food Safety and Applied Nutrition, Office of Nutritional Products, Labeling, and Dietary Supplements noted that known or suspected risks of EPA and DHA *n*–3 fatty acids may include the possibility of:

- Increased bleeding if overused (normally over 3 grams per day) by a patient who is also taking aspirin or warfarin. However, this is disputed.
- Hemorrhagic stroke (only in case of very large doses).
- Oxidation of *n*–3 fatty acids forming biologically active oxidation products.
- Reduced glycemic control among diabetics.
- Suppression of immune and inflammation responses, and consequently, decreased resistance to infections and increased susceptibility to opportunistic bacteria.
- An increase in concentration of LDL cholesterol in some individuals.

Subsequent advices from the FDA and national counterparts have permitted health claims associated with heart health.

Cardiac riskPersons with congestive heart failure, chronic recurrent angina or evidence that their heart is receiving insufficient blood flow are advised to talk to their doctor before taking *n*–3 fatty acids. There have been concerns if such persons take *n*–3 fatty acids or eating foods that contain them in substantial amounts In a recent large study, *n*–3 fatty acids on top of standard heart failure therapy produced a small but statistically significant benefit in terms of mortality and hospitalization.

In congestive heart failure, cells that are only barely receiving enough blood flow become electrically hyperexcitable. This, in turn, can lead to increased risk of irregular heartbeats, which, in turn, can cause sudden cardiac death. *n*–3 fatty acids seem to stabilize the rhythm of the heart by effectively preventing these hyperexcitable cells from functioning, thereby reducing the likelihood of irregular heartbeats and sudden cardiac death. For most people, this is obviously beneficial and would account for most of the large reduction in the likelihood of sudden cardiac death. Nevertheless, for people with congestive heart failure, the heart is barely pumping blood well enough to keep them alive. In these patients, *n*–3 fatty acids may eliminate enough of these few pumping cells that the heart would no longer be able to pump sufficient blood to live, causing an increased risk of cardiac death.

Dietary sources and Daily valuesAs macronutrients, fats are not assigned recommended daily allowances. Macronutrients have AI (Acceptable Intake) and AMDR (Acceptable Macronutrient Distribution Range) instead of RDAs. The AI for *n*–3 is 1.6 grams/day for men and 1.1 grams/day for women while the AMDR is 0.6% to 1.2% of total energy.

"A growing body of literature suggests that higher intakes of á-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) may afford some degree of protection against coronary heart disease. Because the physiological potency of EPA and DHA is much greater than that for á-linolenic acid, it is not possible to estimate one AMDR for all *n*–3 fatty acids. Approximately 10 percent of the AMDR can be consumed as EPA and/or DHA." There was insufficient evidence as of 2005 to set a UL (upper tolerable limit) for *n*–3 fatty acids.

A perceived risk of fish oil *n*–3 supplementation has been heavy metal poisoning by the body's accumulation of traces of heavy metals, in particular mercury, lead, nickel, arsenic and cadmium as well as other contaminants (PCBs, furans, dioxins), which potentially might be found especially in less-refined fish oil supplements. An independent test in 2006 of 44 fish oils on the US market found that all of the products passed safety standards for potential contaminants. The FDA recommends that total dietary intake of *n*–3 fatty acids from fish not exceed 3 grams per day, of which no more than 2 grams per day are from nutritional supplements.

Historically, the Council for Responsible Nutrition (CRN) and the World Health Organization (WHO) have published acceptable standards regarding contaminants in fish oil. The most stringent current standard is the International Fish Oils Standard (IFOS). Fish oils that typically make this highest grade are those that are molecularly distilled under vacuum, and have virtually no measurable level of contaminants (measured parts per billion and parts per trillion).

n–3 supplementation in food has been a significant recent trend in food fortification, with global food companies launching n–3 fortified bread, mayonnaise, pizza, yogurt, orange juice, children's pasta, milk, eggs, confections and infant formula.

Fish

The most widely available source of EPA and DHA is cold water oily fish such as salmon, herring, mackerel, anchovies and sardines. Oils from these fish have a profile of around seven times as much n–3 as n–6. Other oily fish such as tuna also contain n–3 in somewhat lesser amounts. Consumers of oily fish should be aware of the potential presence of heavy metals and fat-soluble pollutants like PCBs and dioxins which may accumulate up the food chain. Some supplement manufacturers remove heavy metals and other contaminants from the oil through various means, such as molecular distillation (see above), which increases purity, potency and safety. In addition, some companies use distillation processes to enhance the DHA to EPA ratio of the fish oil.

Even some forms of fish oil may not be optimally digestible. Of four studies that compare bioavailability of the triglyceride form of fish oil vs. the ester form, two have concluded that the natural triglyceride form is better, and the other two studies did not find a significant difference. No studies have shown the ester form to be superior although it is cheaper to manufacture.

Although fish is a dietary source of n–3 fatty acids, fish do not synthesize them; they obtain them from the algae in their diet.

Flax seeds produce linseed oil, which has a very high n–3 content

Six times richer than most fish oils in n–3, Flax (aka linseed) (Linum usitatissimum) and its oil are perhaps the most widely available botanical source of n–3. Flaxseed oil consists of approximately 55% ALA (alpha-linolenic acid). Flax, like chia, contains approximately three times as much n–3 as n–6.

15 grams of flaxseed oil provides ca. 8 grams of ALA, which is converted in the body to EPA and then DHA at an efficiency of 2-15% and 2-5%, respectively.

Botanical sources of n–3 fatty acids

Table 2.16 : n–3 content as the percentage of ALA in the seed oil

Common Name	Alternative name	Linnaean Name	% n–3
Chia	chia sage	Salvia hispanica	64
Kiwifruit	Chinese gooseberry	Actinidia chinensis	62
Perilla	shiso	Perilla frutescens	58
Flax	linseed	Linum usitatissimum	55
Lingonberry	Cowberry	Vaccinium vitis-idaea	49
Camelina	Gold-of-pleasure	Camelina sativa	36
Purslane	Portulaca	Portulaca oleracea	35
Black Raspberry		Rubus occidentalis	33

Table 2.17: n–3 content as the percentage of ALA in the whole food.

Common name	Linnaean name	% n–3
Flaxseed	Linum usitatissimum	18.1
Butternuts	Juglans cinerea	8.7
Walnuts	Juglans regia	6.3
Pecan nuts	Carya illinoinensis	0.6
Hazel nuts	Corylus avellana	0.1

Eggs

Eggs produced by chickens fed a diet of greens and insects produce higher levels of n–3 fatty acids (mostly ALA) than chickens fed corn or soybeans. In addition to feeding chickens insects and greens, fish oils may be added to their diet to increase the amount of fatty acid concentrations in eggs.

Meat

The n–6 to n–3 ratio of grass-fed beef is about 2:1, making it a more useful source of n–3 than grain-fed beef, which usually has a ratio of 4:1.

Commercially available lamb is almost always grass-fed, and subsequently higher in n–3 than other common meat sources.

The omega-3 content of chicken meat may be enhanced by increasing the animals' dietary intake of grains such as flax, chia, and canola.

Other sources

Milk and cheese from grass-fed cows may also be good sources of n–3. One UK study showed that half a pint of milk provides 10% of the recommended daily intake (RDI) of ALA, while a piece of organic cheese the size of a matchbox may provide up to 88%".

Krill, which are small, shrimp-like zooplankton, also contain the n–33 fatty acids EPA and DHA. One advantage of extracting n–33 fatty acids from krill, as opposed to sources higher in the food chain, is that krill contain fewer heavy metals and PCBs harmful to humans. However, in comparison to higher animals, they also contain fewer n–33 fatty acids per gram.

The microalgae Crypthecodinium cohnii and Schizochytrium are rich sources of DHA (22:6 n–33) and can be produced commercially in bioreactors. Oil from brown algae (kelp) is a source of EPA. Walnuts are one of few nuts that contain appreciable n–33 fat, with approximately a 1:4 ratio of n–33 to n–36.[48] Acai palm fruit also contains n–33 fatty acids.

Omega 3 is also found in softgels in pharmacies and nowadays it is also found in combination with omega 6, omega 9 and shark liver oil.

Some vegetables, too, contain a noteworthy amount of n-3, including strawberries and broccoli.

The n-36 to n-33 ratio

Clinical studies indicate that the ingested ratio of n–36 to n–33 (especially Linoleic vs Alpha Linolenic) fatty acids is important to maintaining cardiovascular health. However, two studies, published in 2005 and 2007, found no such correlations in humans.

Both n–33 and n–36 fatty acids are essential, i.e. humans must consume them in the diet. n–33 and n–36 compete for the same metabolic enzymes, thus the n–36:n–33 ratio will significantly influence the ratio of the ensuing eicosanoids (hormones), (e.g. prostaglandins, leukotrienes, thromboxanes etc.), and will alter the body's metabolic functionGenerally, grass-fed animals accumulate more n–33 than do grain-fed animals which accumulate relatively more n–36. Metabolites of n–36 are significantly more inflammatory (esp. arachidonic acid) than those of n–33. This necessitates that n–33 and n–36 be consumed in a balanced proportion; healthy ratios of n–36:n–33 range from 1:1 to 4:1. Studies suggest that the evolutionary human diet, rich in game animals, seafood and other sources of n–33, may have provided such a ratio.

Typical Western diets provide ratios of between 10:1 and 30:1 - i.e., dramatically skewed toward n–36. Here are the ratios of n–36 to n–33 fatty acids in some common oils: canola 2:1, soybean 7:1, olive 3-13:1, sunflower (no n–33), flax 1:3, cottonseed (almost no n–33), peanut (no n–33), grapeseed oil (almost no n–33) and corn oil 46 to 1 ratio of n–36 to n–33. It should be noted that olive, peanut and canola oils consist of approximately 80% monounsaturated fatty acids, (i.e. neither n–36 nor n–33) meaning that they contain relatively small amounts of n–33 and n–36 fatty acids. Consequently, the n–36 to n–33 ratios for these oils (i.e. olive, canola and peanut oils) are not as significant as they are for corn, soybean and sunflower oils.

Conversion efficiency of ALA to EPA and DHA

It has been reported that conversion of ALA to EPA and further to DHA in humans is limited, but varies with individuals. Women have higher ALA conversion efficiency than men, probably due to the lower rate of utilization of dietary ALA for beta-oxidation. This suggests that biological engineering of ALA conversion efficiency is possible. In the online book of The Benefits of Omega 3 Fatty Acids found in Seal Oil, as Opposed to Fish and Flaxseed Oils, Dr. Ho listed the several factors that inhibit the ALA conversion, which again indicate that the efficiency of ALA conversion could be adjusted by altering one's dietary habits, such as rebalancing the ratio of n–33 and n–36 fatty acid intake, restraining direct alcohol consumptions, and supplementing vitamins and minerals. However, Goyens et al. argues that it is the absolute amount of ALA, rather than the ratio of n–33 and n–36 fatty acids, which affects the conversion.

SUB-SECTION 2.5B—STRUCTURE AND FUNCTION OF PROSTAGLANDINS

History and name

The name *prostaglandin* derives from the prostate gland. When prostaglandin was first isolated from seminal fluid in 1935 by the Swedish physiologist Ulf von Euler, and independently by M.W. Goldblatt,[2] it was believed to be part of the prostatic secretions (in actuality prostaglandins are produced by the seminal vesicles); it was later shown that many other tissues secrete prostaglandins for various functions.

In 1971, it was determined that aspirin-like drugs could inhibit the synthesis of prostaglandins. The biochemists Sune K. Bergström, Bengt I. Samuelsson and John R. Vane jointly received the 1982 Nobel Prize in Physiology or Medicine for their research on prostaglandins.

A **prostaglandin** is any member of a group of lipid compounds that are derived enzymatically from fatty acids and have important functions in the animal body. Every prostaglandin contains 20 carbon atoms, including a 5-carbon ring. They are mediators and have a variety of strong physiological effects; although they are technically hormones, they are rarely classified as such.

The prostaglandins together with the thromboxanes and prostacyclins form the prostanoid class of fatty acid derivatives; the prostanoid class is a subclass of eicosanoids.

Biosynthesis

Prostaglandins are found in virtually all tissues and organs. They are produced by all nucleated cells except lymphocytes. They are autocrine and paracrine lipid mediators that act upon platelets, endothelium, uterine and mast cells, among others. They are synthesized in the cell from the essential fatty acids (EFAs).

An intermediate is created by phospholipase-A_2, then passed into one of either the cyclooxygenase pathway or the lipoxygenase pathway to form either prostaglandin and thromboxane or leukotriene. The cyclooxygenase pathway produces thromboxane, prostacyclin and prostaglandin D, E and F. The lipoxygenase pathway is active in leukocytes and in macrophages and synthesizes leukotrienes.
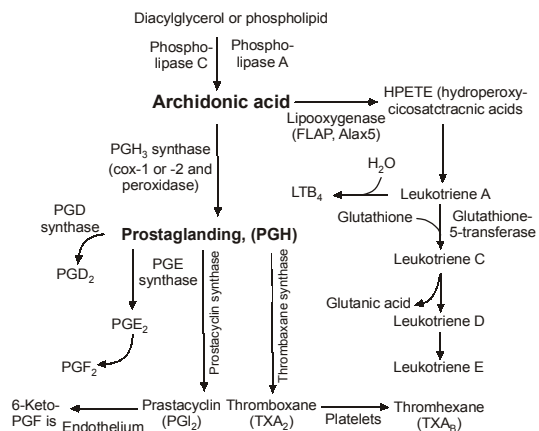
Fig. 2.28 : Biosynthesis of eicosanoids. (series-2)

Name	EFA Type	Series
Gamma-linolenic acid (GLA) via DGLA	ω-6	series-1
Arachidonic acid (AA)	ω-6	series-2
Eicosapentaenoic acid (EPA)	ω-3	series-3

Release of prostaglandins from the cell

Prostaglandins were originally believed to leave the cells via passive diffusion because of their high lipophilicity. The discovery of the prostaglandin transporter (PGT, SLCO2A1), which mediates the cellular uptake of prostaglandin, demonstrated that diffusion cannot explain the penetration of prostaglandin through the cellular membrane. The release of prostaglandin has now also been shown to be mediated by a specific transporter, namely the multidrug resistance protein 4 (MRP4, ABCC4), a member of the ATP-binding cassette transporter superfamily. Whether MRP4 is the only transporter releasing prostaglandins from the cells is still unclear.

Cyclooxygenases

Prostaglandins are produced following the sequential oxidation of AA, DGLA or EPA by cyclooxygenases (COX-1 and COX-2) and terminal prostaglandin synthases. The classic dogma is as follows:

- COX-1 is responsible for the baseline levels of prostaglandins.
- COX-2 produces prostaglandins through stimulation.

However, while COX-1 and COX-2 are both located in the blood vessels, stomach and the kidneys, prostaglandin levels are increased by COX-2 in scenarios of inflammation. A third form of COX, termed COX-3 is thought to exist in the brain and may be associated with relief of Headaches when on NSAID therapy.

Prostaglandin E synthase

Prostaglandin E_2 (PGE_2) is generated from the action of prostaglandin E synthases on prostaglandin H_2 (PGH_2). Several prostaglandin E synthases have been identified. To date, microsomal prostaglandin E synthase-1 emerges as a key enzyme in the formation of PGE_2.

Other terminal prostaglandin synthases

Terminal prostaglandin synthases have been identified that are responsible for the formation of other prostaglandins. For example, hematopoietic and lipocalin prostaglandin D synthases (hPGDS and lPGDS) are responsible for the formation of PGD_2 from PGH_2. Similarly, prostacyclin (PGI_2) synthase (PGIS) converts PGH_2 into PGI_2. A thromboxane synthase (TxAS) has also been identified. Prostaglandin F synthase (PGFS) catalyzes the formation of $9\alpha,11\beta$-$PGF_{2\alpha,\beta}$ from PGD_2 and $PGF_{2\alpha}$ from PGH_2 in the presence of NADPH. This enzyme has recently been crystallyzed in complex with PGD_2[4] and bimatoprost (a synthetic analogue of $PGF_{2\alpha}$).

Function

There are currently nine known prostaglandin receptors on various cell types. Prostaglandins ligate a subfamily of cell surface seven-transmembrane receptors, G-protein-coupled receptors. These receptors are termed DP1-2, EP1-4, FP, IP, and TP, corresponding to the receptor that ligates the corresponding prostaglandin (e.g., DP1-2 receptors bind to PGD2).

These varied receptors mean that Prostaglandins thus act on a variety of cells, and have a wide variety of actions:

- cause constriction or dilation in vascular smooth muscle cells
- cause aggregation or disaggregation of platelets
- sensitize spinal neurons to pain
- decrease intraocular pressure
- regulate inflammatory mediation
- regulate calcium movement
- control hormone regulation
- control cell growth

Prostaglandins are potent but have a short half-life before being inactivated and excreted. Therefore, they exert only a paracrine (locally active) or autocrine (acting on the same cell from which it is synthesized) function.

Types

Following is a comparison of the prostaglandin types Prostaglandin I_2 (PGI_2), Prostaglandin E_2 (PGE_2) and Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).

Type	Receptor	Function
PGI_2	IP	• vasodilation
		• inhibit platelet aggregation
		• bronchodilatation
PGE_2	EP_1	• bronchoconstriction
		• GI tract smooth muscle contraction
	EP_2	• bronchodilatation
		• GI tract smooth muscle relaxation
		• vasodilatation
	EP_3	• ↓ gastric acid secretion
		• ↑ gastric mucus secretion
		• uterus contraction (when pregnant)
		• GI tract smooth muscle contraction
		• lipolysis inhibition
		• ↑ autonomic neurotransmitters
	Unspecified	• hyperalgesia
		• pyrogenic
$PGF_{2\alpha}$	FP	• uterus contraction
		• bronchoconstriction

Role in pharmacology

Inhibition

Examples of prostaglandin antagonists are:
- NSAIDs (inhibit cyclooxygenase)
- Corticosteroids (inhibit phospholipase A2 production)
- COX-2 selective inhibitors or coxibs

However, both NSAIDs and Coxibs can raise the risk of myocardial infarction.

Clinical uses

Synthetic prostaglandins are used:
- To induce childbirth (parturition) or abortion (PGE_2 or PGF_2, with or without mifepristone, a progesterone antagonist);

- To prevent closure of patent ductus arteriosus in newborns with particular cyanotic heart defects (PGE1)
- To prevent and treat peptic ulcers (PGE)
- As a vasodilator in severe Raynaud's phenomenon or ischemia of a limb
- In pulmonary hypertension
- In treatment of glaucoma (as in bimatoprost ophthalmic solution, a synthetic prostamide analog with ocular hypotensive activity)
- To treat erectile dysfunction or in penile rehabilitation following surgery (PGE1 as alprostadil).
- To treat egg binding in small birds.

SUB-SECTION 2.5C—LEUCOTRIENS AND THROMBOXANES

The name *leukotriene*, introduced by Swedish biochemist Bengt Samuelsson in 1979, comes from the words *leukocyte* and *triene* (indicating the compound's three conjugated double bonds). What would be later named leukotriene C, "slow reaction smooth muscle-stimulating substance" (SRS) was originally described between 1938 and 1940 by Feldberg and Kellaway. The researchers isolated SRS from lung tissue after a prolonged period following exposure to snake venom and histamine.

Leukotrienes are commercially available to the research community. Leukotrienes are naturally produced eicosanoid lipid mediators, which may be responsible for the effects of an inflammatory response. Leukotrienes use both autocrine signalling and paracrine signalling to regulate the body's response. Leukotrienes are produced in the body from arachidonic acid by the enzyme 5-lipoxygenase. Their production by the body is part of a complex response that usually includes the production of histamine.

Biochemistry and Synthesis

Leukotrienes are synthesized in the cell from arachidonic acid by 5-lipoxygenase. The catalytic mechanism involves the insertion of an oxygen moiety at a specific position in the arachidonic acid backbone. The lipoxygenase pathway is active in leukocytes, including mast cells, eosinophils, neutrophils, monocytes and basophils. When such cells are activated, arachidonic acid is liberated from cell membrane phospholipids by phospholipase A2, and donated by the 5-lipoxygenase activating protein (FLAP) to 5-lipoxygenase.

5-lipoxygenase (5-LO) uses FLAP to convert arachidonic acid into 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which spontaneously reduces to 5-hydroxyeicosatetraenoic acid (5-HETE). The enzyme 5-LO acts again on 5-HETE to convert it into leukotriene A_4 (LTA_4), an unstable epoxide.

In cells equipped with LTA_4 hydrolase, such as neutrophils and monocytes, LTA_4 is converted to the dihydroxy acid leukotriene LTB_4, which is a powerful chemoattractant for neutrophils acting at BLT_1 and BLT_2 receptors on the plasma membrane of these cells.

In cells that express LTC_4 synthase, such as mast cells and eosinophils, LTA_4 is conjugated with the tripeptide glutathione to form the first of the cysteinyl-leukotrienes, LTC_4. Outside the cell, LTC_4

can be converted by ubiquitous enzymes to form successively LTD_4 and LTE_4, which retain biological activity.

The cysteinyl-leukotrienes act at their cell-surface receptors CysLT1 and CysLT2 on target cells to contract bronchial and vascular smooth muscle, to increase permeability of small blood vessels, to enhance secretion of mucus in the airway and gut, and to recruit leukocytes to sites of inflammation.

Both LTB4 and the cysteinyl-leukotrienes (LTC_4, LTD_4, LTE_4) are partly degraded in local tissues, and ultimately become inactive metabolites in the liver.

Function

Leukotrienes act principally on a subfamily of G protein coupled receptors. They may also act upon peroxisome proliferator-activated receptors. Leukotrienes are involved in asthmatic and allergic reactions and act to sustain inflammatory reactions; several leukotriene receptor antagonists (e.g. montelukast and zafirlukast) are used to treat asthma. Recent research points to a role of 5-lipoxygenase in cardiovascular and neuropsychiatric illnesses.

Leukotrienes are very important agents in the inflammatory response. Some such as LTB4 have a chemotactic effect on migrating neutrophils, and as such help to bring the necessary cells to the tissue. Leukotrienes also have a powerful effect in bronchoconstriction, they also increase vascular permeability.

Leukotrienes in asthma

Leukotrienes assist in the pathophysiology of asthma, causing or potentiating the following symptoms:

- airflow obstruction
- increased secretion of mucus
- mucosal accumulation
- bronchoconstriction
- infiltration of inflammatory cells in the airway wall

Role of cysteinyl leukotrienes

Cysteinyl leukotriene receptors **CysLT1** and **CysLT2** are present on mast cells, eosinophil and endothelial cells. During cysteinyl leukotriene interaction, they can stimulate proinflammatory activities such as endothelial cell adherence and chemokine production by mast cells. As well as mediating inflammation, they induce asthma and other inflammatory disorders, thereby reducing the airflow to the alveoli. In excess, the cysteinyl leukotrienes can induce anaphylactic shock.

Zileuton blocks 5-lipoxygenase inhibiting the synthetic pathway of leukotriene metabolism. Zileuton affects the LTB4 pathway, montelukast doesn't.

Thromboxane is a member of the family of lipids known as eicosanoids. The two major thromboxanes are thromboxane A2 and thromboxane B2.

Thromboxane is named for its role in clot formation (thrombosis).

Production

Enzymes and substrates associated with thromboxane and prostacyclin synthesis. It is produced in platelets by thromboxane-A synthase from the endoperoxides produced by the cyclooxygenase (COX) enzyme from arachidonic acid.

Mechanism

Thromboxane acts by binding to any of the thromboxane receptors, G-protein coupled receptors coupled to the G protein G_q

Functions

Thromboxane is a vasoconstrictor and a potent hypertensive agent, and it facilitates platelet aggregation.

It is in homeostatic balance in the circulatory system with prostacyclin, a related compound. The mechanism of secretion of thromboxanes from platelets is still unclear.

Role of A2 in platelet aggregation

Thromboxane A_2 (TXA_2), produced by activated platelets, has prothrombotic properties, stimulating activation of new platelets as well as increasing platelet aggregation.

Platelet aggregation is achieved by mediating expression of the glycoprotein complex GP IIb/IIIa in the cell membrane of platelets. Circulating fibrinogen binds these receptors on adjacent platelets, further strengthening the clot.

Pathology

It is believed that the vasoconstriction caused by thromboxanes plays a role in Prinzmetal's angina.

Suppression

The widely used drug aspirin acts by inhibiting the ability of the COX enzyme to synthesize the precursors of thromboxane within platelets. Low-dose, long-term aspirin use irreversibly blocks the formation of thromboxane A_2 in platelets, producing an inhibitory effect on platelet aggregation. This anticoagulant property makes aspirin useful for reducing the incidence of heart attacks.[2] 40 mg of aspirin a day is able to inhibit a large proportion of maximum thromboxane A_2 release provoked acutely, with the prostaglandin I2 synthesis being little affected; however, higher doses of aspirin are required to attain further inhibition. One side effect of this is that people who regularly take aspirin will suffer from excessive bleeding whenever the skin is perforated.

SECTION 2.6—CLASSIFICATION AND AND STRUCTURE OF PHOSPHOLIPIDS

SUB-SECTION 2.6—A SURFACTANT GLYCOLIPIDS

These are carbohydrate-attached lipids. Their role is to provide energy and also serve as markers for cellular recognition.

They occur where a carbohydrate chain is associated with phospholipids on the exoplasmic surface of the cell membrane. The carbohydrates are found on the outer surface of all eukaryotic cell membranes.

They extend from the phospholipid bilayer into the aqueous environment outside the cell where it acts as a recognition site for specific chemicals as well as helping to maintain the stability of the membrane and attaching cells to one another to form tissues.
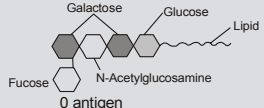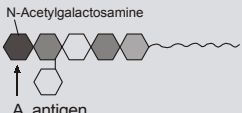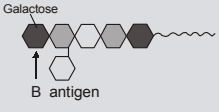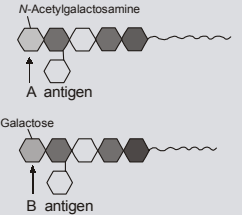
Types of glycolipids

The following is an incomplete listing of glycolipid types.

- Glyceroglycolipids
  – Galactolipids
  – Sulpholipids (SQDG)
- Glycosphingolipids
  – Cerebrosides
    - Galactocerebrosides
    - Glucocerebrosides
    - Glucobicaranateoets
  – Gangliosides (the most complex animal glycolipids; contain negatively chardged oligosacchrides with one or more sialic acid residues; more than 40 different ganglidosides have been identified; they are most adundant in cells)
  – Globosides
  – Sulfatides
  – Glycophosphosphingolipids (complex glycophospholipids from fungi, including yeasts, and in plants, where they were originally called "phytoglycolipidds" by dCdarter, et al., may comprise as complicated a ddof compounds as the negadtively charged gandgliosides in animals. The head group of a glycolipid is composed of sugars.

glycolipids are membrane components composed of lipids that are covalently bonded to monosaccharides or polysaccharides. One type of glycolipid found in human red blood cells is involved in the ABO blood type antigens. The table below shows the relationship between the ABO blood type, the RBC glycolipid and antibodies to the glycolipids in the blood plasma.

Glycolipids are present ubiquitously in cell surface membranes. The most common glycolipids in mammals are a class of sphingolipids, glycosphingolipids (GSLs). Major GSLs including gangliosides are derived from glucosylceramide (GlcCer) that is synthesized by a specific glycosyltransferase, ceramide glucosyltransferase (GlcT-1, UGCG). Galactosylceramide (GalCer) formed by ceramide galactosyltransferase (CGT) is also present as a dominant glycolipid in myelin sheath. The class of glyceroglycolipids is found in particular tissues: galactosylalkylacylglyceride (seminolipid) in the testis, and phosphatidyglucose in human cord blood cells. Glucosylated cholesterol is found in mammalian cells.

blood type	glycolipid	antibodies in blood
O	RBC of O type blood have this glycolipids in their RBCs plasma membranes.	**anti A and anti B** Individuals with type O blood have antibodies against the type A and type B antigens
A	Individuals with type A blood have this type of glycolipid in their RBCs plasma membranes. The A type glycolipid has the same carbohydrate composition as does the type O antigen with the addition of a additional N-Acetylgalactosamine which is the A type antigenic determinant.	**anti B** individuals of type A blood have antibodies against the type B determinant
B	Individuals with type B blood have the above kind of glycolipid in their RBC plasma membrane. The B type glycolipid has the same carbohydrate composition as does the type O antigen with the addition of an additional galactose which is the B type antigenic determinant.	**anti A** Individuals of type B blood have antibodies against the type A antigenic determinant
AB	Individuals with type AB blood have both of the above glycolipids in their RBC plasma membranes	**neither A or B** Individuals with they AB blood have antibodies against neither the the A nor B antigens

Table 2.17 : Roles of monoglycosylated lipids in mammals: almost all lipids are glycosylated

Glycolipid	Distribution	Synthetic enzyme	Role
GlcCer	ubiquitous	UGCG (human, mouse, rat)	precursor for GSLs biosynthesis, negative regulator for ceramide, axonal elongation of neuron, multidrug resistance
GalCer	myelin, kidney,	CGT (human, mouse, rat)	insulative function of myelin
ÉøGalCer FucCer	unknown colon cancer, lung cancer	unknown unknown	ligand for NKT cell unknown
Cholesteryl glucoside	human skin fibroblast	unknown	heat shock response
Galactosyldiacyl glycerol	sperm (seminolipid), intestine, the nervous system	CGT (the same as GalCer synthase)	sperm development
phosphatidylglucoside	cord blood cell, HL60	unknown	unknown

NKT: natural Killer T

Phosphatidylinositol is not included.

Table 2.18 : Gene targeted mice: glycolipid functions at the whole animal level

Gene disrupted	Phenotype Biochemistry	General
GlcCer synthase (UGCG)	loss of all GSLs except GalCer	embryonic lethality at E7.5, enhancemant of apoptosis in ectodermal layer
GalCer synthase (CGT)	loss of GalCer and appearance of GlcCer in myelin, loss of seminolipid in sperm	ataxia, loss of insulative function of myelin, sterility in male
Sulfatide synthase (CST)	loss of sulfatide in myelin and of sulfated seminolipid in sperm	similarity to CGT KO mouse
GM2/GD2 (GalNAc-T) synthase	loss of higher gangliosides (only GM3, GD3 and GT3)	normal development, defect in nerve regeneration
GD3 synthase	loss of higher gangliosides (only monosialo gangliosides)	normal development defect in nerve regeneration
GM2/GD3 double KO	loss of gangliosides except GM3	normal development, lethality to sound stress

UDCG:UDP-Glc Ceramide Glucosyltransferase

CGT: Ceramide Galactosyltransferase

CST: Cerebroside Sulfotransferase

Recent cell biological studies show that GSLs in cell membranes are preferentially distributed into lipid domains, so-called rafts (Fig.28,). Raft-like lipid microdomains can be isolated biochemically as a low density Triton X-100 insoluble fraction. The fraction isolated contains sphingolipids (GSLs and sphingomyelin) and cholesterol. Importantly, the glycolipid-enriched membrane domain also includes proteins related to signal transduction such as src-family kinases. The lipid domains are suggested to play roles in cell-cell adhesion and receptor-mediated signal transduction. A glycolipid-enriched lipid micodomain is also involved in target for host pathogens (Vibrio cholera, O157, HIY) and their toxin bindings (Fig. 29).
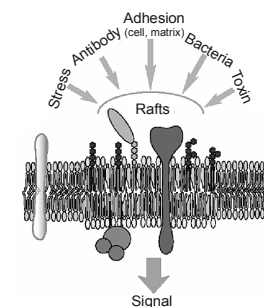
Fig. 2.29 : Showing the Diagramatic representation of Biological action of GSL

A cell line deficient in an entire group of GSLs was established from B16 mouse melanoma. This mutant cell line is defective in a GlcCer synthase activity. *In vitro* studies on glycolipid function using the mutant cells show that the glycolipid biosynthesis is not essential for cell survival and proliferation, at least in the melanoma cells. Sphingomyelin as the sole sphingolipid on the plasma membrane can act as a substitute. Similarly, GSLs are not a critical component for membrane domain formation. These results from *in vitro* experiments indicate that GSLs apparently do not have house-keeping functions when studied at the single cell level. Thus, GSL research is now focusing on multicellular systems or whole animals. Up to now, however, there is no case report of human genetic disease associated with glycolipid synthetic enzyme. Therefore, the physiological functions of GSLs remain unclear in humans.

To solve the riddle of glycolipid functions, a method to eliminate a gene of glycolipid glycosyltransferase in mice has been developed using a homologous recombination system and several knockout mice have been generated (Table II). Surprisingly, mutant mice deficient in complex gangliosides are born normally, showing no apparent abnormalities. Even a double knockout mouse of GD3 synthase and GM2/GD2 synthase, expressing only GM3 ganglioside, results in normal development. Studies with these mutant mice prove that complex gangliosides are not involved in cellular differentiation but rather in the maintenance (homeostasis) and regeneration of nervous system tissues (see "Glyco Word" by Furukawa). Very recently, Kawai et al. have shown that the double mutant is highly sensitive to sound stimulus and displays a sudden death phenotype. In 1999, a mutant mouse for GlcCer synthase (UGCG) was generated. Since the mutant mouse is deficient in all GSLs except for GalCer, it gives us valuable information on the general functions of GSLs. The mice die at around embryonic day 7.5, indicating clear evidence of the essential role of GSL biosynthesis during multicellular development. The mutant mice exhibit an elevated apoptosis in the ectoderm. However, it is difficult to explain clearly why ablation of the UGCG gene causes the enhancement of apoptotic cell death because UGCG has at least two distinct functions one of which is a negative regulatory factor for intracellular ceramide

content through ceramide glycosylation. The other is a positive regulatory role of the glycolipid, GlcCer in cell growth. Moreover, it is not evident if there is any relationship between the embryonic lethality and glycolipid functions in raft-like membrane microdomains. GalCer knockout mouse was first generated in 1996. In contrast to the UGCG mutant, the CGT knockout mouse is born normally and exhibits no obvious abnormalities in myelin structure, although GalCer and sulfatide, both enriched in myelin, are lacking. However, the mouse shows severe generalized tremor and mild ataxia, proving that the GSLs play a role in the insulative function of myelin. The same mouse shows that CGT is also essential for sperm development. As shown above, glycolipid research is now regarded as a potent and promising research target of the post-genomic era.

SECTION 2.7—CHOLESTEROL

Cholesterol is a lipid like, waxy alcohol found in the cell membranes and transported in the blood plasma of all animals. It is an essential component of mammalian cell membranes where it is required to establish proper membrane permeability and fluidity. Cholesterol is the principal sterol synthesized by animals while smaller quantities are synthesized in other eukaryotes such as plants and fungi. In contrast, cholesterol is almost completely absent among prokaryotes such as bacteria.[2] Cholesterol is classified as a sterol (a contraction of steroid and alcohol).

While minimum levels of cholesterol are essential for life, excess levels in the circulation are associated with atherosclerosis. Cholesterol is synthesized by virtually all the body cells but significant quantities can also be absorbed from the diet.

The name cholesterol originates from the Greek *chole-* (bile) and *stereos* (solid), and the chemical suffix *-ol* for an alcohol, as researchers first identified cholesterol in solid form in gallstones by François Poulletier de la Salle in 1769. However, it is only in 1815 that chemist Eugène Chevreul named the compound "cholesterine".

Physiology and Function Cholesterol is required to build and maintain cell membranes; it regulates membrane fluidity over a wide range of temperatures. The liver produces about 1 gram of cholesterol per day through the bile, which contains cholesterol. The hydroxyl group on cholesterol interacts with the polar head groups of the membrane phospho- and sphingolipids, while the bulky steroid and the hydrocarbon chain is embedded in the membrane, alongside the nonpolar fatty acid chains of the other lipids. Some research indicates that cholesterol may act as an antioxidant.[4] Cholesterol also aids in the manufacture of bile (which is stored in the gallbladder and helps digest fats), and is also important for the metabolism of fat soluble vitamins, including vitamins A, D, E and K. It is the major precursor for the synthesis of vitamin D and of the various steroid hormones (which include cortisol and aldosterone in the adrenal glands, and the sex hormones progesterone, the various estrogens, testosterone, and derivatives). It is the base structure for steroids. It also covers nerves in the form of myelin. This helps to conduct nerve impulses.

Recently, cholesterol has also been implicated in cell signaling processes, where it has been suggested that it assists in the formation of lipid rafts in the plasma membrane. It also reduces the permeability of the plasma membrane to hydrogen ions (protons) and sodium ions.

Cholesterol is essential for the structure and function of invaginated caveolae and clathrin-coated pits, including the caveolae-dependent endocytosis and clathrin-dependent endocytosis. The role of cholesterol in caveolae-dependent and clathrin-dependent endocytosis can be investigated by using methyl beta cyclodextrin (MâCD) to remove cholesterol from the plasma membrane.

Synthesis and absorption Most of the cholesterol in the body is synthesized by the body and some has dietary origin. Cholesterol is more abundant in tissues which either synthesize more or have more abundant densely-packed membranes, for example, the liver, spinal cord and brain. It plays a central role in many biochemical processes, such as the composition of cell membranes and the synthesis of steroid hormones.

Cholesterol is required in the membrane of mammalian cells for normal cellular function, and is either synthesized in the endoplasmic reticulum, or derived from the diet, in which case it is delivered by the bloodstream in low-density lipoproteins. These are taken into the cell by LDL receptor-mediated endocytosis in clathrin-coated pits, and then hydrolysed in lysosomes.

Cholesterol is primarily synthesized from acetyl CoA through the HMG-CoA reductase pathway in many cells and tissues. About 20-25% of total daily production (~1 g/day) occurs in the liver; other sites of higher synthesis rates include the intestines, adrenal glands and reproductive organs. For a person of about 150 pounds (68 kg), typical total body content is about 35 g, typical daily internal production is about 1 g and typical daily dietary intake is 200-300 mg in the United States and societies with similar dietary patterns. Of the cholesterol input to the intestines via bile production, 92-97% is reabsorbed in the intestines and recycled via enterohepatic circulation.

Konrad Bloch and Feodor Lynen shared the Nobel Prize in Physiology or Medicine in 1964 for their discoveries concerning the mechanism and regulation of the cholesterol and fatty acid metabolism.

Plasma transport Since cholesterol is insoluble in blood, it is transported in the circulatory system within lipoproteins, complex spherical particles which have an exterior composed mainly of water-soluble proteins; fats and cholesterol are carried internally. There is a large range of lipoproteins within blood, generally called, from larger to smaller size: chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). The cholesterol within all the various lipoproteins is identical.

Cholesterol is minimally soluble in water; it cannot dissolve and travel in the water-based bloodstream. Instead, it is transported in the bloodstream by lipoproteins-protein "molecular-suitcases" that are water-dispersible and carry cholesterol and triglycerides internally. The apolipoproteins forming the surface of the given lipoprotein particle determine from what cells cholesterol will be removed and to where it will be supplied.

Cholesterol is transported towards peripheral tissues by the lipoproteins chylomicrons, very low density lipoproteins (VLDL) and low-density lipoproteins (LDL). Large numbers of small dense LDL (sdLDL) particles are strongly associated with the presence of atheromatous disease within the arteries. For this reason, LDL is referred to as "bad cholesterol".

On the other hand, high-density lipoprotein (HDL) particles transport cholesterol back to the liver for excretion in a process known as reverse cholesterol transport (RCT).[7] Having large numbers of

large HDL particles correlates with better health outcomes.[8] In contrast, having small numbers of large HDL particles is independently associated with atheromatous disease progression within the arteries.

Homeostasis Biosynthesis of cholesterol is directly regulated by the cholesterol levels present, though the homeostatic mechanisms involved are only partly understood. A higher intake from food leads to a net decrease in endogenous production, while lower intake from food has the opposite effect. The main regulatory mechanism is the sensing of intracellular cholesterol in the endoplasmic reticulum by the protein SREBP (Sterol Regulatory Element Binding Protein 1 and 2).[9] In the presence of cholesterol, SREBP is bound to two other proteins: SCAP (SREBP-cleavage activating protein) and Insig1. When cholesterol levels fall, Insig-1 dissociates from the SREBP-SCAP complex, allowing the complex to migrate to the Golgi apparatus, where SREBP is cleaved by S1P and S2P (site-1 and -2 protease), two enzymes that are activated by SCAP when cholesterol levels are low. The cleaved SREBP then migrates to the nucleus and acts as a transcription factor to bind to the SRE (sterol regulatory element) of a number of genes to stimulate their transcription. Among the genes transcribed are the LDL receptor and HMG-CoA reductase. The former scavenges circulating LDL from the bloodstream, whereas HMG-CoA reductase leads to an increase of endogenous production of cholesterol.[10]

A large part of this signaling pathway was clarified by Dr. Michael S. Brown and Dr. Joseph L. Goldstein in the 1970s. In 1985, they received the Nobel Prize in Physiology or Medicine for their work. Their subsequent work shows how the SREBP pathway regulates expression of many genes that control lipid formation and metabolism and body fuel allocation.

Metabolism and excretion: Cholesterol is oxidized by the liver into a variety of bile acids. These in turn are conjugated with glycine, taurine, glucuronic acid, or sulfate. A mixture of conjugated and non-conjugated bile acids along with cholesterol itself is excreted from the liver into the bile. Approximately 95% of the bile acids are reabsorbed from the intestines and the remainder lost in the feces.[11] The excretion and reabsorption of bile acids forms the basis of the enterohepatic circulation which is essential for the digestion and absorption of dietary fats. Under certain circumstances, when more concentrated, as in the gallbladder, cholesterol crystallises and is the major constituent of most gallstones, although lecithin and bilirubin gallstones also occur less frequently.

Dietary sources: Animal fats are complex mixtures of triglycerides, with lesser amounts of phospholipids and cholesterol. Consequently all foods containing animal fat contain cholesterol to varying extents. Cholesterol is not present in plant based food sources unless it has been added during the foods preparation.[13] However plant products such as flax seeds and peanuts contain healthy cholesterol-like compounds called phytosterols, which are suggested to help lower serum cholesterol levels. Major dietary sources of cholesterol include cheese, egg yolks, beef, pork, poultry, and shrimp. Human breast milk also contains significant quantities of cholesterol.[15]

Dietary cholesterol plays a smaller role in blood cholesterol levels in comparison to fat intake. A number of measures can be taken to reduce blood cholesterol levels through changes in lifestyle, one of which is a change in diet. Trans and saturated fats are significant contributors to elevated cholesterol levels in the blood stream. Avoiding animal products may decrease the cholesterol levels in the body not through dietary cholesterol reduction alone, but primarily through a reduced saturated fat intake. Those

wishing to reduce their cholesterol through a change in diet should aim to consume less than 7% of their daily calories from saturated fat and less than 200mg of cholesterol per day.

Clinical significance (Hypercholesterolemia)

According to the lipid hypothesis, abnormally high cholesterol levels (hypercholesterolemia), or, more correctly, higher concentrations of LDL and lower concentrations of functional HDL are strongly associated with cardiovascular disease because these promote atheroma development in arteries (atherosclerosis). This disease process leads to myocardial infarction (heart attack), stroke and peripheral vascular disease. Since higher blood LDL, especially higher LDL particle concentrations and smaller LDL particle size, contribute to this process more than the cholesterol content of the LDL particles,[17] LDL particles are often termed "bad cholesterol" because they have been linked to atheroma formation. On the other hand, high concentrations of functional HDL, which can remove cholesterol from cells and atheroma, offer protection and are sometimes referred to colloquially as "good cholesterol". These balances are mostly genetically determined but can be changed by body build, medications, food choices and other factors.

Conditions with elevated concentrations of oxidized LDL particles, especially "small dense LDL" (sdLDL) particles, are associated with atheroma formation in the walls of arteries, a condition known as atherosclerosis, which is the principal cause of coronary heart disease and other forms of cardiovascular disease. In contrast, HDL particles (especially large HDL) have been identified as a mechanism by which cholesterol and inflammatory mediators can be removed from atheroma. Increased concentrations of HDL correlate with lower rates of atheroma progressions and even regression. A 2007 study pooling data on almost 900,000 subjects in 61 cohorts demonstrated that blood total cholesterol levels have an exponential effect on cardiovascular and total mortality, with the association more pronounced in younger subjects. Still, because cardiovascular disease is relatively rare in the younger population, the impact of high cholesterol on health is still larger in older people.

Elevated levels of the lipoprotein fractions, LDL, IDL and VLDL are regarded as atherogenic (prone to cause atherosclerosis). Levels of these fractions, rather than the total cholesterol level, correlate with the extent and progress of atherosclerosis. Conversely, the total cholesterol can be within normal limits, yet be made up primarily of small LDL and small HDL particles, under which conditions atheroma growth rates would still be high. In contrast, however, if LDL particle number is low (mostly large particles) and a large percentage of the HDL particles are large, then atheroma growth rates are usually low, even negative, for any given total cholesterol concentration Recently, a post-hoc analysis of the IDEAL and the EPIC prospective studies found an association between high levels of HDL cholesterol (adjusted for apolipoprotein A-I and apolipoprotein B) and increased risk of cardiovascular disease, casting doubt on the cardioprotective role of "good cholesterol".

Multiple human trials utilizing HMG-CoA reductase inhibitors, known as statins, have repeatedly confirmed that changing lipoprotein transport patterns from unhealthy to healthier patterns significantly lowers cardiovascular disease event rates, even for people with cholesterol values currently considered low for adults. As a result, people with a history of cardiovascular disease may derive benefit from statins irrespective of their cholesterol levels,[22] and in men without cardiovascular disease there is

benefit from lowering abnormally high cholesterol levels ("primary prevention"). Primary prevention in women is practiced only by extension of the findings in studies on men, since in women, none of the large statin trials has shown a reduction in overall mortality or in cardiovascular end points.

The 1987 report of National Cholesterol Education Program, Adult Treatment Panels suggest the total blood cholesterol level should be: < 200 mg/dL normal blood cholesterol, 200-239 mg/dL borderline-high, > 240 mg/dL high cholesterol. The American Heart Association provides a similar set of guidelines for total (fasting) blood cholesterol levels and risk for heart disease:

Level mg/dL	Level mmol/L	Interpretation
< 200	< 5.0	Desirable level corresponding to lower risk for heart disease
200-240	5.2-6.2	Borderline high risk
> 240	> 6.2	High risk

However, as today's testing methods determine LDL ("bad") and HDL ("good") cholesterol separately, this simplistic view has become somewhat outdated. The desirable LDL level is considered to be less than 100 mg/dL (2.6 mmol/L), although a newer target of < 70 mg/dL can be considered in higher risk individuals based on some of the above-mentioned trials. A ratio of total cholesterol to HDL-another useful measure-of far less than 5:1 is thought to be healthier. Of note, typical LDL values for children before fatty streaks begin to develop is 35 mg/dL.

Most testing methods for LDL do not actually measure LDL in their blood, much less particle size. For cost reasons, LDL values have long been estimated using the Friedewald formula: [total cholesterol] ? [total HDL] ? 20% of the triglyceride value = estimated LDL. The basis of this is that Total cholesterol is defined as the sum of HDL, LDL, and VLDL. Ordinarily just the total, HDL, and triglycerides are actually measured. The VLDL is estimated as one-fifth of the triglycerides. It is important to fast for at least eight hours before the blood test because the triglyceride level varies significantly with food intake.

Given the well-recognized role of cholesterol in cardiovascular disease, it is surprising that some studies have shown an inverse correlation between cholesterol levels and mortality in subjects over 50 years of age-an 11% increase overall and 14% increase in CVD mortality per 1 mg/dL per year drop in cholesterol levels. In the Framingham Heart Study, the researchers attributed this phenomenon to the fact that people with severe chronic diseases or cancer tend to have below-normal cholesterol levels. This explanation is not supported by the Vorarlberg Health Monitoring and Promotion Programme, in which men of all ages and women over 50 with very low cholesterol were increasingly likely to die of cancer, liver diseases, and mental diseases. This result indicates that the low cholesterol effect occurs even among younger respondents, contradicting the previous assessment among cohorts of older people that this is a proxy or marker for frailty occurring with age.

A small group of scientists, united in The International Network of Cholesterol Skeptics, continues to question the link between cholesterol and atherosclerosis. However, the vast majority of doctors and medical scientists accepts the link as fact.

Hypocholesterolemia Abnormally low levels of cholesterol are termed hypocholesterolemia. Research into the causes of this state is relatively limited, but some studies suggest a link with depression, cancer and cerebral hemorrhage. Generally, the low cholesterol levels seem to be a consequence of an underlying illness, rather than a cause.

SUB-SECTION 2.7A—STEROID HORMONES

The steroid hormones are all derived from cholesterol. Moreover, with the exception of vitamin D, they all contain the same cyclopentanophenanthrene ring and atomic numbering system as cholesterol. The conversion of C27 cholesterol to the 18-, 19-, and 21-carbon steroid hormones (designated by the nomenclature C with a subscript number indicating the number of carbon atoms, e.g. C19 for androstanes) involves the rate-limiting, irreversible cleavage of a 6-carbon residue from cholesterol, producing pregnenolone (C21) plus isocaproaldehyde. Common names of the steroid hormones are widely recognized, but systematic nomenclature is gaining acceptance and familiarity with both nomenclatures is increasingly important. Steroids with 21 carbon atoms are known systematically as pregnanes, whereas those containing 19 and 18 carbon atoms are known as androstanes and estranes, respectively. The important mammalian steroid hormones are shown below along with the structure of the precursor, pregneolone. Retinoic acid and vitamin D are not derived from pregnenolone, but from vitamin A and cholesterol respectively.

Pregnenolone: produced directly from cholesterol, the precusor molecule for all C18, C19 and C21 steroids.
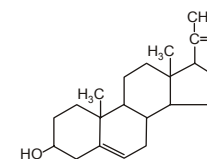
Progesterone: a progestin, produced directly from pregnenolone and secreted from the corpus luteum, responsible for changes associated with luteral phase of the menstrual cycle, differentiation factor for mammary glands.
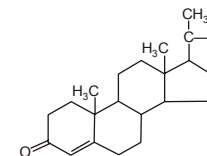
Aldosterone: the principal mineralocorticoid, produced from progesterone in the zona glomerulosa of adrenal cortex, raises blood pressure and fluid volume, increases Na+ uptake.
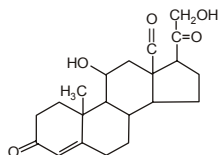
Testosterone: an androgen, male sex hormone synthesized in the testes, responsible for secondary male sex characteristics, produced from progesterone.
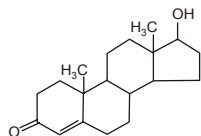
Estradiol: an estrogen, principal female sex hormone, produced in the ovary, responsible for secondary female sex characteristics.
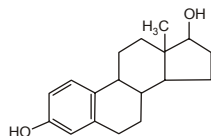
Cortisol: dominant glucocorticoid in humans, synthesized from progesterone in the zona fasciculata of the adrenal cortex, involved in stress adaptation, elevates blood pressure and Na+ uptake, numerous effects on the immune system.
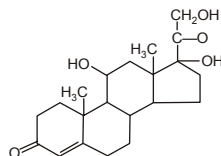
All the steroid hormones exert their action by passing through the plasma membrane and binding to intracellular receptors. The mechanism of action of the thyroid hormones is similar; they interact with intracellular receptors. Both the steroid and thyroid hormone-receptor complexes exert their action by binding to specific nucleotide sequences in the DNA of responsive genes. These DNA sequences are identified as hormone response elements, HREs. The interaction of steroid-receptor complexes with DNA leads to altered rates of transcription of the associated genes.

Steroid Hormone Biosynthesis Reactions

The particular steroid hormone class synthesized by a given cell type depends upon its complement of peptide hormone receptors, its response to peptide hormone stimulation and its genetically expressed complement of enzymes. The following indicates which peptide hormone is responsible for stimulating the synthesis of which steroid hormone:

Luteinizing Hormone (LH): progesterone and testosterone

Adrenocorticotropic hormone (ACTH): cortisol

Follicle Stimulating Hormone (FSH): estradiol

Angiotensin II/III: aldosterone

The first reaction in converting cholesterol to C_{18}, C_{19} and C_{21} steroids involves the cleavage of a 6-carbon group from cholesterol and is the principal committing, regulated, and rate-limiting step in steroid biosynthesis. The enzyme system that catalyzes the cleavage reaction is known as P450-linked side chain cleaving enzyme (P450ssc)or desmolase, and is found in the mitochondria of steroid-producing cells, but not in significant quantities in other cells.

Mitochondrial desmolase is a complex enzyme system consisting of cytochrome P450, and adrenadoxin (a P450 reductant). The activity of each of these components is increased by 2 principal cAMP- and PKA-dependent processes. First, cAMP stimulates PKA, leading to the phosphorylation of a cholesteryl-ester esterase and generating increased concentrations of cholesterol, the substrate for desmolase. Second, long-term regulation is effected at the level the gene for desmolase. This gene contains a cAMP regulatory element (CRE) that binds cAMP and increases the level of desmolase RNA transcription, thereby leading to increased levels of the enzyme. Finally, cholesterol is a negative feedback regulator of HMG CoA reductase activity (see regulation of cholesterol synthesis). Thus, when cytosolic cholesterol is depleted, *de novo* cholesterol synthesis is stimulated by freeing HMG CoA reductase of its feedback constraints. Subsequent to desmolase activity, pregnenolone moves to the cytosol, where further processing depends on the cell (tissue) under consideration.

The various hydroxylases involved in the synthesis of the steroid hormones have a nomenclature that indicates the site of hydroxylation (e.g. 17á-hydroxylase introduces a hydroxyl group to carbon 17). These hydroxylase enzymes are members of the cytochrome P450 class of enzymes and as such also have a nomenclature indicative of the site of hydroxylation in addition to being identified as P450 class enzymes (e.g. the 17á-hydroxylase is also identified as P450c17). The officially preferred nomenclature for the cytochrome P450 class of enzymes is to use the prefix **CYP.** Thus, 17á-hydroxylase should be identified as CYP17A1. There are currently 57 identified CYP genes in the human genome.

Steroids of the Adrenal Cortex

The adrenal cortex is responsible for production of 3 major classes of steroid hormones: glucocorticoids, which regulate carbohydrate metabolism; mineralocorticoids, which regulate the body levels of sodium and potassium; and androgens, whose actions are similar to that of steroids produced by the male gonads. Adrenal insufficiency is known as Addison disease, and in the absence of steroid hormone replacement therapy can rapidly cause death (in 1 - 2 weeks).

The adrenal cortex is composed of 3 main tissue regions: zona glomerulosa, zona fasciculata, and zona reticularis. Although the pathway to pregnenolone synthesis is the same in all zones of the cortex, the zones are histologically and enzymatically distinct, with the exact steroid hormone product dependent on the enzymes present in the cells of each zone. Many of the enzymes of adrenal steroid hormone synthesis are of the class called cytochrome P450 enzymes. These enzymes all have a common nomenclature and a standardized nomenclature. The standardized nomenclature for the P450 class of enzymes is to use the abbreviation CYP. For example the P450ssc enzyme (also called 20,22 desmolase or cholesterol desmolase) is identified as CYP11A1. In order for cholesterol to be converted to pregnenolone in the adrenal cortex it must be transported into the mitochondria where CYP11A1 resides. This transport process is mediated by steroidogenic acute regulatory protein (StAR). This transport process is the rate-limiting step in steroidogenesis.
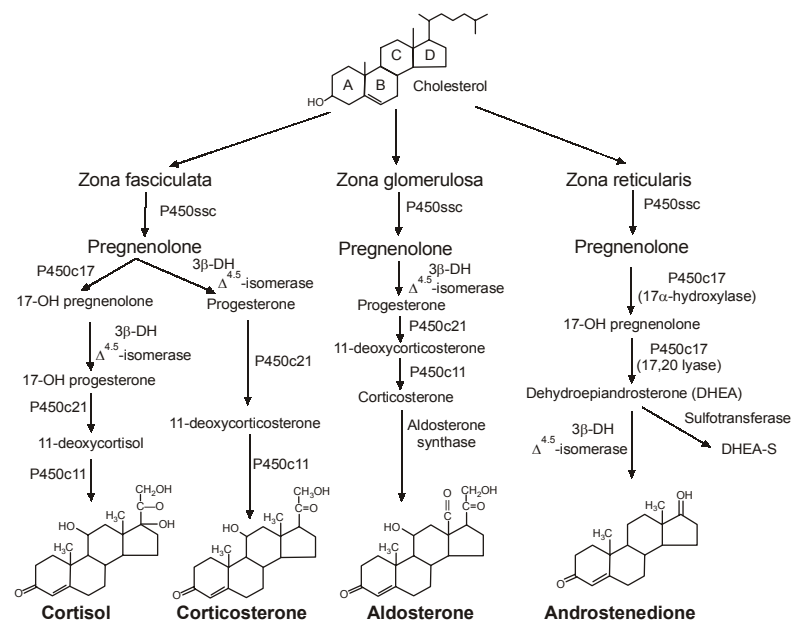
Fig. 2.30 : Steroid genesis

Synthesis of the various adrenal steroid hormones from cholesterol. Only the terminal hormone structures are included. 3â-DH and Ä^{4,5}-isomerase are the two activities of 3â-hydroxysteroid

dehydrogenase type 1 (gene symbol HSD3B2), P450c11 is 11â-hydroxylase (CYP11B1), P450c17 is CYP17A1. CYP17A1 is a single microsomal enzyme that has two steroid biosynthetic activities: 17á-hydroxylase which converts pregnenolone to 17-hydroxypregnenolone (17-OH pregnenolone) and 17,20-lyase which converts 17-OH pregnenolone to DHEA. P450c21 is 21-hydroxylase (CYP21A2, also identified as CYP21 or CYP21B). Aldosterone synthase is also known as 18á-hydroxylase (CYP11B2). The gene symbol for sulfotransferase is SULT2A1. Place your mouse over structure names to see chemical structures. Click here for a larger format picture.

Conversion of prenenolone to progesterone requires the two enzyme activities of HSD3B2: the 3â-hydroxysteroid dehydrogenase and Ä^{4,5}-isomerase activities. Zona glomerulosa cells lack the P450c17 that converts pregnenolone and progesterone to their C_{17} hydroxylated analogs. Thus, the pathways to the glucocorticoids (deoxycortisol and cortisol) and the androgens [dehydroepiandosterone (DHEA) and androstenedione] are blocked in these cells. Zona glomerulosa cells are unique in the adrenal cortex in containing the enzyme responsible for converting corticosterone to aldosterone, the principal and most potent mineralocorticoid. This enzyme is P450c18 (or 18á-hydroxylase, CYP11B2), also called aldosterone synthase. The result is that the zona glomerulosa is mainly responsible for the conversion of cholesterol to the weak mineralocorticoid, corticosterone and the principal mineralocorticoid, aldosterone.

Cells of the zona fasciculata and zona reticularis lack aldosterone synthase (P450c18) that converts corticosterone to aldosterone, and thus these tissues produce only the weak mineralocorticoid corticosterone. However, both these zones do contain the P450c17 missing in zona glomerulosa and thus produce the major glucocorticoid, cortisol. Zona fasciculata and zona reticularis cells also contain P450c17, whose 17,20-lyase activity is responsible for producing the androgens, dehydroepiandosterone (DHEA) and androstenedione. Thus, fasciculata and reticularis cells can make corticosteroids and the adrenal androgens, but not aldosterone.

As noted earlier, P450ssc is a mitochondrial activity. Its product, pregnenolone, moves to the cytosol, where it is converted either to androgens or to 11-deoxycortisol and 11-deoxycorticosterone by enzymes of the endoplasmic reticulum. The latter 2 compounds then re-enter the mitochondrion, where the enzymes are located for tissue-specific conversion to glucocorticoids or mineralocorticoids, respectively.

Regulation of Adrenal Steroid Synthesis

Adrenocorticotropic hormone (ACTH), of the hypothalamus, regulates the hormone production of the zona fasciculata and zona reticularis. ACTH receptors in the plasma membrane of the cells of these tissues activate adenylate cyclase with production of the second messenger, cAMP. The effect of ACTH on the production of cortisol is particularly important, with the result that a classic feedback loop is prominent in regulating the circulating levels of corticotropin releasing hormone (CRH), ACTH, and cortisol.

Mineralocorticoid secretion from the zona glomerulosa is stimulated by an entirely different mechanism. Angiotensins II and III, derived from the action of the kidney protease renin on liver-derived angiotensinogen, stimulate zona glomerulosa cells by binding a plasma membrane receptor

coupled to phospholipase C. Thus, angiotensin II and III binding to their receptor leads to the activation of PKC and elevated intracellular Ca^{2+} levels. These events lead to increased P450ssc activity and increased production of aldosterone. In the kidney, aldosterone regulates sodium retention by stimulating gene expression of mRNA for the Na^+/K^+-ATPase responsible for the reaccumulation of sodium from the urine.

The interplay between renin from the kidney and plasma angiotensinogen is important in regulating plasma aldosterone levels, sodium and potassium levels, and ultimately blood pressure. Among the drugs most widely employed used to lower blood pressure are the angiotensin converting enzyme (ACE) inhibitors. These compounds are potent competitive inhibitors of the enzyme that converts angiotensin I to the physiologically active angiotensins II and III. This feedback loop is closed by potassium, which is a potent stimulator of aldosterone secretion. Changes in plasma potassium of as little as 0.1 millimolar can cause wide fluctuations (+/- 50%) in plasma levels of aldosterone. Potassium increases aldosterone secretion by depolarizing the plasma membrane of zona glomerulosa cells and opening a voltage-gated calcium channel, with a resultant increase in cytoplasmic calcium and the stimulation of calcium-dependent processes.

Although fasciculata and reticularis cells each have the capability of synthesizing androgens and glucocorticoids, the main pathway normally followed is that leading to glucocorticoid production. However, when genetic defects occur in the 3 enzyme complexes leading to glucocorticoid production, large amounts of the most important androgen, dehydroepiandrosterone (DHEA), are produced. These lead to hirsutism and other masculinizing changes in secondary sex characteristics.

Functions of the Adrenal Steroid Hormones

Glucocorticoids: The glucocorticoids are a class of hormones so called because they are primarily responsible for modulating the metabolism of carbohydrates. Cortisol is the most important naturally occurring glucocorticoid. As indicated in the Figure above, cortisol is synthesized in the zona fasciculata of the adrenal cortex. When released to the circulation, cortisol is almost entirely bound to protein. A small portion is bound to albumin with more than 70% being bound by a specific glycosylated á-globulin called transcortin or corticosteroid-binding globulin (CBG). Between 5% and 10% of circulating cortisol is free and biologically active. Glucocorticoid function is exerted following cellular uptake and interaction with intracellular receptors as discussed below. Cortisol inhibits uptake and utilization of glucose resulting in elevations in blood glucose levels. The effect of cortisol on blood glucose levels is further enhanced through the increased breakdown of skeletal muscle protein and adipose tissue triglycerides which provides enegry and substrates for gluconeogenesis. Glucocorticoids also increase the synthesis of gluconeogenic enzymes. The increased rate of protein metabolism leads to increased urinary nitrogen excretion and the induction of urea cycle enzymes.

In addition to the metabolic effects of the glucocorticoids, these hormones are immunosuppressive and anti-inflammatory. Hence, the use of related drugs such as prednisone, in the acute treatment of inflammatory disorders. The anti-inflammatory activity of the glucocorticoids is exerted, in part, through inhibition of phospholipase A_2 (PLA_2) activity with a consequent reduction in the release of arachidonic acid from membrane phospholipids. Arachidonic acid serves as the precursor for the synthesis of various eicosanoids. Glucocorticoids also inhibit vitamin D-mediated intestinal calcium uptake, retard the rate of wound healing, and interfere with the rate of linear growth.

Mineralocorticoids: The major circulating mineralocorticoid is aldosterone. Deoxycorticosterone (DOC) exhibits some mineralocorticoid action but only about 3% of that of aldosterone. As the name of this class of hormones implies, the mineralocorticoids control the excretion of electrolytes. This occurs primarily through actions on the kidneys but also in the colon and sweat glands. The principle effect of aldosterone is to enhance sodium reabsorption in the cortical collecting duct of the kidneys. However, the action of aldosterone is exerted on sweat glands, stomach, and salivary glands to the same effect, i.e. sodium reabsorption. This action is accompanied by the retention of chloride and water resulting in the expansion of extracellular volume. Aldosterone also enhances the excretion of potassium and hydrogen ions from the medullary collecting duct of the kidneys.

Androgens: The androgens, androstenedione and DHEA, circulate bound primarily to sex hormone-binding globulin (SHBG). Although some of the circulating androgen is metabolized in the liver, the majority of interconversion occurs in the gonads (as described below), skin, and adipose tissue. DHEA is rapidly converted to the sulfated form, DHEA-S, in the liver and adrenal cortex. The primary biologically active metabolites of the androgens are testosterone and dihydrotestosterone which function by binding intracellular receptors, thereby effecting changes in gene expression and thereby, resulting in the manifestation of the secondary sex characteristics.

Clinical Significance of Adrenal SteroidogenesisDefective synthesis of the steroid hormones produced by the adrenal cortex can have profound effects on human development and homeostasis. In 1855 Thomas Addison identified the significance of the "suprarenal capsules" when he reported on the case of a patient who presented with chronic adrenal insufficiency resulting from progressive lesions of the adrenal glands caused by tubersclerosis. Addison disease thus represents a disorder characterized by adrenal insufficiency. In addition to diseases that result from the total absence of adrenocortical function, there are syndromes that result from hypersecretion of adrenocortical hormones. In 1932 Harvey Cushing reported on several cases of adrenocortical hyperplasia that were the result of basophilic adenomas of the anterior pituitary. As with Addison disease, disorders that manifest with adrenocortical hyperplasia are referred to as Cushing syndrome. Despite the characterizations of adrenal insufficiency and adrenal hyperplasia, there remained uncertainty about the relationship between adrenocortical hyperfunction and virilism (premature development of male secondary sex characteristics). In 1942 this confusion was resolved by Fuller Albright when he delineated the differences between children with Cushing syndrome and those with adrenogenital syndromes which are more commonly referred to as congenital adrenal hyperplasias (CAH).The CAH are a group of inherited disorders that result from loss-of-function mutations in one of several genes involved in adrenal steroid hormone synthesis. In the virilizing forms of CAH the mutations result in impairment of cortisol production and the consequent accumulation of steroid intermediates proximal to the defective enzyme. All forms of CAH are inherited in an autosomal recessive manner. There are two common and at least three rare forms of CAH that result in virilization. The common forms are caused by defects in either CYP21A2 (21-hydroxylase, also identified as just CYP21 or CYP21B) or CYP11B1 (11â-hydroxylase). The majority of CAH cases (90-95%) are the result of defects in CYP21A2 with a frequency of between 1 in 5,000 and 1 in 15,000. Three rare forms of virilizing CAH result from either defects in 3â-hydroxysteroid dehydrogenase (HSD3B2), placental aromatase or P450-oxidoreductase (POR). An additional CAH is caused by mutations that affect either the 17á-hydroxylase, 17,20-lyase or both activities encoded in the CYP17A1

gene. In individuals harboring CYP17A1 mutations that result in severe loss of enzyme activity there is absent sex steroid hormone production accompanied by hypertension resulting from mineralocorticoid excess.

Gonadal Steroid Hormones

Although many steroids are produced by the testes and the ovaries, the two most important are testosterone and estradiol. These compounds are under tight biosynthetic control, with short and long negative feedback loops that regulate the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) by the pituitary and gonadotropin releasing hormone (GnRH) by the hypothalamus. Low levels of circulating sex hormone reduce feedback inhibition on GnRH synthesis (the long loop), leading to elevated FSH and LH. The latter peptide hormones bind to gonadal tissue and stimulate P450ssc
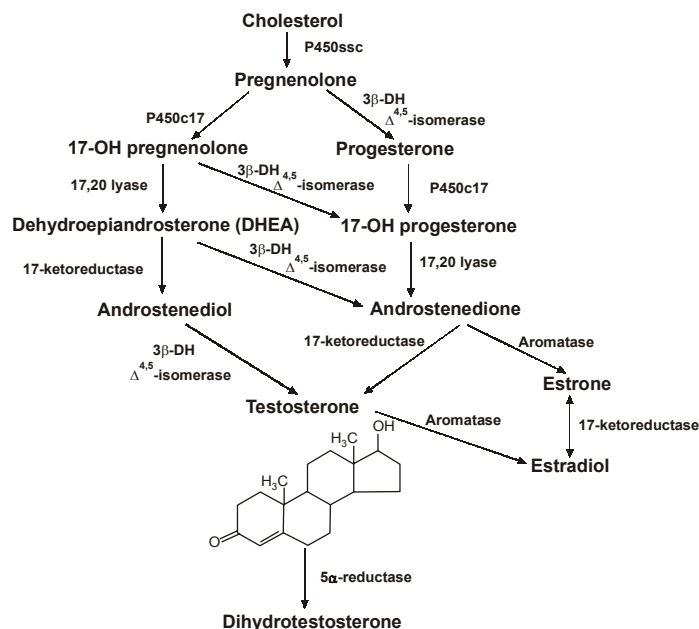
Fig. 2.31 : Synthesis of the male sex hormones in Leydig cells of the testis. P450SSC, 3â-DH, and P450c17 are the same enzymes as those needed for adrenal steroid hormone synthesis.

activity, resulting in sex hormone production via cAMP and PKA mediated pathways. The roles of cAMP and PKA in gonadal tissue are the same as that described for glucocorticoid production in the adrenals, but in this case adenylate cyclase activation is coupled to the binding of LH to plasma membrane receptors. The biosynthetic pathway to sex hormones in male and female gonadal tissue includes the production of the androgens, androstenedione and dehydroepiandrosterone. Testes and ovaries contain an additional enzyme, a 17â-hydroxysteroid dehydrogenase, that enables androgens to be converted to testosterone. In males, LH binds to Leydig cells, stimulating production of the principal Leydig cell hormone, testosterone. Testosterone is secreted to the plasma and also carried to Sertoli cells by androgen binding protein (ABP). In Sertoli cells the Ä4 double bond of testosterone is reduced, producing dihydrotestosterone. Testosterone and dihydrotestosterone are carried in the plasma, and delivered to target tissue, by a specific gonadal-steroid binding globulin (GBG). In a number of target tissues, testosterone can be converted to dihydrotestosterone (DHT). DHT is the most potent of the male steroid hormones, with an activity that is 10 times that of testosterone. Because of its relatively lower potency, testosterone is sometimes considered to be a prohormone.

17,20-lyase is the same activity of CYP17A1 described above for adrenal hormone synthesis. Aromatase (also called estrogen synthetase) is CYP19A1. 17-ketoreductase is also called 17â-hydroxysteroid dehydrogenase type 3 (gene symbol HSD17B3). The full name for 5á-reductase is 5á-reductase type 2 (gene symbol SRD5A2). Place your mouse over structure names to see chemical structures.

Testosterone is also produced by Sertoli cells but in these cells it is regulated by FSH, again acting through a cAMP- and PKA-regulatory pathway. In addition, FSH stimulates Sertoli cells to secrete androgen-binding protein (ABP), which transports testosterone and DHT from Leydig cells to sites of spermatogenesis. There, testosterone acts to stimulate protein synthesis and sperm development.
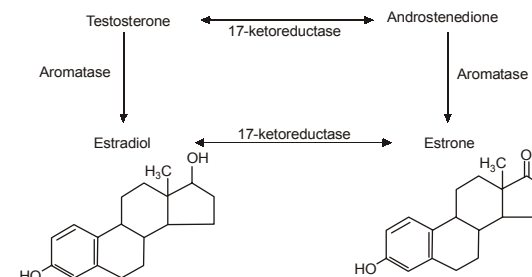
Fig. 2.33 : Synthesis of the major female sex hormones in the ovary

In females, LH binds to thecal cells of the ovary, where it stimulates the synthesis of androstenedione and testosterone by the usual cAMP- and PKA-regulated pathway. An additional enzyme complex known as aromatase is responsible for the final conversion of the latter 2 molecules into the estrogens. Aromatase is a complex endoplasmic reticulum enzyme found in the ovary and in numerous other

tissues in both males and females. Its action involves hydroxylations and dehydrations that culminate in aromatization of the A ring of the androgens.

Synthesis of testosterone and androstenedione from cholesterol occurs by the same pathways as indicated for synthesis of the male sex hormones. Aromatase (also called estrogen synthetase) is CYP19A1.

Aromatase activity is also found in granulosa cells, but in these cells the activity is stimulated by FSH. Normally, thecal cell androgens produced in response to LH diffuse to granulosa cells, where granulosa cell aromatase converts these androgens to estrogens. As granulosa cells mature they develop competent large numbers of LH receptors in the plasma membrane and become increasingly responsive to LH, increasing the quantity of estrogen produced from these cells. Granulosa cell estrogens are largely, if not all, secreted into follicular fluid. Thecal cell estrogens are secreted largely into the circulation, where they are delivered to target tissue by the same globulin (GBG) used to transport testosterone.

Steroid Hormone Receptors

The receptors to which steroid hormones bind are ligand-activated proteins that regulate transcription of selected genes. Unlike peptide hormone receptors, that span the plasma membrane and bind ligand outside the cell, steroid hormone receptors are found in the cytosol and the nucleus. The steroid hormone receptors belong to the steroid and thyroid hormone receptor super-family of proteins, that includes receptors for steroid hormones, thyroid hormones, vitamin D and vitamin A (retinoic acid).

When these receptors bind ligand they undergo a conformational change that renders them activated to recognize and bind to specific nucleotide sequences. These specific nucleotide sequences in the DNA are referred to as hormone-response elements (HREs). When ligand-receptor complexes interact with DNA they alter the transcriptional level (responses can be either activating or repressing) of the associated gene. Thus, the steroid-thyroid family of receptors all have three distinct domains: a ligand-binding domain, a DNA-binding domain and a transcriptional regulatory domain. Although there is the commonly observed effect of altered transcriptional activity in response to hormone-receptor interaction, there are family member-specific effects with ligand-receptor interaction. Binding of thyroid hormone to its receptor results in release of the receptor from DNA. Several receptors are induced to interact with other transcriptional mediators in response to ligand binding. Binding of glucocorticoid leads to translocation of the ligand-receptor complex from the cytosol to the nucleus.

The receptors for the retinoids (vitamin A and its derivatives) are identified as RARs (for retinoic acid, RA receptors) and exist in at least three subtypes, RARá, RARâ and RARã. In addition, there is another family of nuclear receptors termed the retinoid X receptors (RXRs) that represents a second class of retinoid-responsive transcription factors. The RXRs have been shown to enhance the DNA-binding activity of RARs and the thyroid hormone receptors (TRs). There are also three distinct RXRs (á, â and ã). The major difference between the RARs and RXRs is that the former exhibit highest affinity for all-*trans*-retinoic acid (all-*trans*-RA) and the latter for 9-*cis*-RA.

Additional super-family members are the peroxisome proliferator-activated receptors (PPARs). These receptors were originally discovered as proteins activated by agents that stimulate proliferation of peroxisomes in rat liver. An intracellular lipid-binding protein identified as aP2 is expressed exclusively

in differentiated adipocytes. An adipocyte-specific enhancer of the aP2 gene is a target for peroxisome proliferators, fatty acids and 9-*cis*-RA. Subsequent to these observations it was found that there is an adipocyte-specific PPAR family identified as PPARã. The PPARã proteins form heterodimers with RXRs to activate adipocyte-specific enhancers such as the one in the aP2 gene.

Recent evidence has demonstrated a role for PPARã proteins in the etiology of type 2 diabetes. A relatively new class of drugs used to increase the sensitivity of the body to insulin are the thiazolidinedione drugs. These compounds bind to and alter the function of PPARã. Mutations in the gene for PPARã have been correlated with insulin resistance. It is still not completely clear how impaired PPARã signaling can affect the sensitivity of the body to insulin or indeed if the observed mutations are a direct or indirect cause of the symptoms of insulin resistance.

SUB-SECTION 2.7B—BILE ACIDS

Bile acids (also known as bile salts) are steroid acids found predominantly in the bile of mammals. In humans, taurocholic acid and glycocholic acid (derivatives of cholic acid) represent approximately eighty percent of all bile acids. The two major bile acids are cholic acid, and chenodeoxycholic acid. They, their glycine and taurine conjugates, and their 7-alpha-dehydroxylated derivatives (deoxycholic acid and lithocholic acid) are all found in human intestinal bile. An increase in bile flow is exhibited with an increased secretion of bile acids. Bile acid's main function is to facilitate the formation of micelles, which promotes dietary fat processing.

Production and distribution

Bile acids are produced in the liver by the oxidation of cholesterol. They are conjugated with taurine or the amino acid glycine, or with a sulfate or a glucuronide, and are then stored in the gallbladder. Bile acid also serves the purpose of breaking down fats. Upon eating a meal containing fat, the contents of the gallbladder are secreted into the intestine. In humans, the rate limiting step is the addition of a hydroxyl group on position 7 of the steroid nucleus by the enzyme cholesterol 7 alpha-hydroxylase. Bile acids serve multiple functions, which include: eliminating cholesterol from the body; driving the flow of bile to eliminate catabolites from the liver; emulsifying lipids and fat soluble vitamins in the intestine; and aiding in the reduction of the bacteria flora found in the small intestine and biliary tract.

The term *bile acid* refers to the unconjugated form. *Bile salt* refers to the conjugated form. Bile salts are more efficient at emulsifying fats because at intestinal pH, bile salts are more electrically charged than bile acids.

Synthesis of bile acids is a major consumer of cholesterol in most species (other than humans). The body produces about 800 mg of cholesterol per day and about half of that is used for bile acid synthesis. In total about 20-30 grams of bile acids are secreted into the intestine daily; about 90% of excreted bile acids are reabsorbed (by active transport in the ileum) and recycled. This is referred to as the enterohepatic circulation. Bile is also used to break down fat goblets into tiny droplets. Bile from slaughtered animals can be mixed to use as soap.

Types

Bile salts constitute a large family of molecules, composed of a steroid structure with four rings, a five or eight carbon side-chain terminating in a carboxylic acid, and the presence and orientation of different numbers of hydroxyl groups. The four rings are labeled from left to right (as commonly drawn) A, B, C, and D, with the D-ring being smaller by one carbon than the other three. The hydroxyl groups have a choice of being in 2 positions, either up (or out) termed beta (often drawn by convention as a solid line), or down, termed alpha (seen as a dashed line in drawings). All bile acids have a hydroxyl group on position 3, which was derived from the parent molecule, cholesterol. In cholesterol, the 4 steroid rings are flat and the position of the 3-hydroxyl is beta.

In many species, the initial step in the formation of a bile acid is the addition of a 7-alpha hydroxyl group. Subsequently, in the conversion from cholesterol to a bile acid, the junction between the first two steroid rings (A and B) is altered, making the molecule bent, and in this process, the 3-hydroxyl is converted to the alpha orientation. Thus, the default simplest bile acid (of 24 carbons) has two hydroxyl groups at positions 3-alpha and 7-alpha. The chemical name for this compound is 3-alpha,7-alpha-dihydroxy-5-beta-cholan-24-oic acid, or as it is commonly known, chenodeoxycholic acid. This bile acid was first isolated from the domestic goose, from which the "cheno" portion of the name was derived.

Another bile acid, cholic acid (with 3 hydroxyl groups) had already been described, so the discovery of chenodeoxcholic acid (with 2 hydroxyl groups) made the new bile acid a "deoxycholic acid" in that it had one less hydroxyl group than cholic acid. The 5-beta portion of the name denotes the orientation of the junction between rings A and B of the steroid nucleus (in this case, they are bent). The term "cholan" denotes a particular steroid structure of 24 carbons, and the "24-oic acid" indicates that the carboxylic acid is found at position 24, which happens to be at the end of the side-chain. Chenodeoxycholic acid is made by many species, and is quite a functional bile acid. Its chief drawback lies in the ability of intestinal bacteria to remove the 7-alpha hydroxyl group, a process termed dehydroxylation. The resulting bile acid has only a 3-alpha hydroxyl group and is termed lithocholic acid (litho = stone). It is poorly water-soluble and rather toxic to cells. Bile acids formed by synthesis in the liver are termed "primary" bile acids, and those made by bacteria are termed "secondary" bile acids. As a result, chenodeoxycholic acid is a primary bile acid, and lithocholic acid is a secondary bile acid.

To avoid the problems associated with the production of lithocholic acid, most species add a third hydroxyl group to chenodeoxycholic acid. In this manner, the subsequent removal of the 7-alpha hydroxyl group by intestinal bacteria will result in a less toxic, still functional dihydroxy bile acid. Over the course of vertebrate evolution, a number of positions have been chosen for placement of the third hydroxyl group. Initially, the 16-alpha position was favored, particularly in birds. Later, this position was superseded by a large number of species selecting position 12-alpha. Primates (including humans) utilize 12-alpha for their third hydroxyl group position. The resulting primary bile acid in humans is 3-alpha,7-alpha,12-alpha-trihydroxy-5-beta-cholan-24-oic acid, or as it is commonly called, cholic acid.

In the intestine, cholic acid is dehydroxylated to form the dihydroxy bile acid deoxycholic acid. In many vertebrate orders still subject to speciation, new species are discarding 12-alpha hydroxylation in

favor of a hydroxy group on position 23 of the side-chain. It should be noted that vertebrate families and species exist that have experimented with and utilize just about every position imaginable on the steroid nucleus and side-chain.

The principal bile acids are:

  I. Cholic acid

 II. Chenodeoxycholic acid

III. Glycocholic acid

IV. Taurocholic acid

In humans, the most important bile acids are cholic acid, deoxycholic acid, and chenodeoxycholic acid, Prior to secretion by the liver, they are conjugated with either the amino acid glycine or taurine. Conjugation increases their water solubility, preventing passive re-absorption once secreted into the small intestine. As a result, the concentration of bile acids in the small intestine can stay high enough to form micelles and solubilize lipids. The term "critical micellar concentration" refers to both an intrinsic property of the bile acid itself and amount of bile acid necessary to function in the spontaneous and dynamic formation of micelles.

Regulation

As surfactants or detergents, bile acids are potentially toxic to cells and their levels are tightly regulated. They function directly as signaling molecules in the liver and the intestines by activating a nuclear hormone receptor known as FXR also known by its gene name *NR1H4*. This results in inhibition of bile acid synthesis in the liver when bile acid levels are too high. Emerging evidence associates FXR activation with alterations in triglyceride metabolism, glucose metabolism and liver growth.

Clinical significance

Since bile acids are made from endogenous cholesterol, the enterohepatic circulation of bile acids may be disrupted as a way to lower cholesterol. This is the mechanism of action behind bile acid sequestrants. Bile acid sequestrants bind bile acids in the gut, preventing their reabsorption. In so doing, more endogenous cholesterol is shunted into the production of bile acids, thereby lowering cholesterol levels. The sequestered bile acids are then excreted in the feces.

Cholic acid is a bile acid, a white crystalline substance insoluble in water (soluble in alcohol and acetic acid), with a melting point of 200-201 °C. Salts of cholic acid are called **cholates**. Cholic acid, along with chenodeoxycholic acid, is one of two major bile acids produced by the liver where it is synthesized from cholesterol. Of the two major bile acids, cholate derivatives represent approximately eighty percent of all bile acids. These derivatives are made from cholyl-CoA which forms a conjugate with either glycine, or taurine, yielding glycocholic and taurocholic acid respectively.
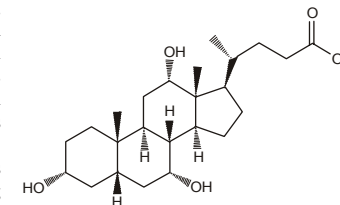
Cholic acid and chenodeoxycholic acid are the most important human bile acids. Some other mammals synthesize predominantly deoxycholic acid.

Chenodeoxycholic acid (also known as **chenodesoxycholic acid**) is a bile acid. It occurs as a white crystalline substance insoluble in water but soluble in alcohol and acetic acid, with melting point at 165-167 °C. Salts of this carboxylic acid are called **chenodeoxycholates**. Chenodeoxycholic acid is one of the 4 main organic acids produced by the liver.
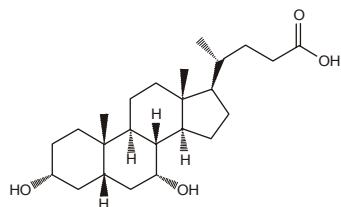
Chenodeoxycholic acid is synthesized in the liver from cholesterol.

This compound, when altered by bacteria in the colon, will result in conversion to its secondary bile acid known as lithocholic acid. Both of these bile acids, in addition to the others, can be conjugated to taurine or glycine. Conjugation, a function carried out by the liver will result in a lowered pKa and therefore, the compounds will remain ionized. These ionized compounds will stay in the gastrointestinal tract until reaching the ileum where they will be reabsorbed. The purpose of this conjugation is to keep the bile acids in the tract until the end to facilitate lipid digestion all the way to the ileum.

In cases where bacteria overgrow in the small intestine, often due to a blind loop in the intestine retaining chyme in one place, the bacteria will de-conjugate the bile acids and therefore impede fat digestion and absorption. This can lead to steatorrhea.

Chenodeoxycholic acid and cholic acid are the most important human bile acids. Some other mammals synthesize predominantly deoxycholic acid.

Potential applications
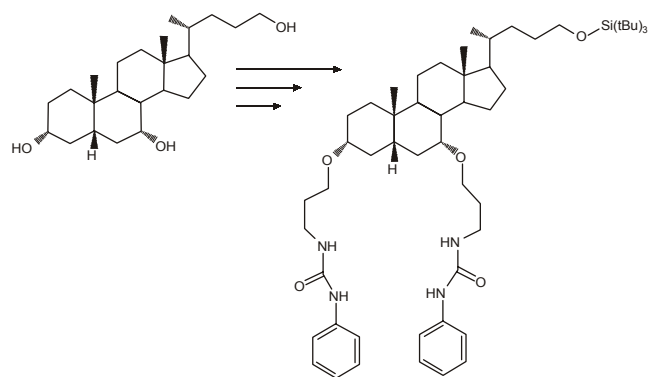
The Australian biotechnology company Giaconda has developed a treatment for Hepatitis C infection that combines chenodeoxycholic acid with bezafibrate.

In supramolecular chemistry molecular tweezers based on a **chenodeoxycholic acid** scaffold is a urea receptor [1] which can contain anions in its binding pocket in order of affinity: $H_2PO_4^-$ (dihydrogen phosphate) > Cl^- > Br^- > I^- reflecting their basicities (tetrabutylammonium counter ion).

Glycocholic acid, or **cholylglycine**, is a crystalline bile acid involved in the emulsification of fats. It occurs as a sodium salt in the bile of mammals. It is a conjugate of cholic acid with glycine. Its anion is called **glycocholate**.
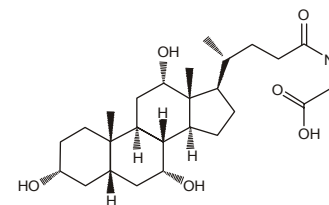
Taurocholic acid, known also as **cholaic acid**, **cholyltaurine**, or **acidum cholatauricum**, is a deliquescent yellowish crystalline bile acid involved in the emulsification of fats. It occurs as a sodium salt in the bile of mammals. It is a conjugate of cholic acid with taurine. In medical use, it is administered as a cholagogue and choleretic. Hydrolysis of taurocholic acid yields taurine, a nonessential amino acid. For commercial use, taurocholic acid is manufactured from cattle bile, a byproduct of the meat-processing industry.
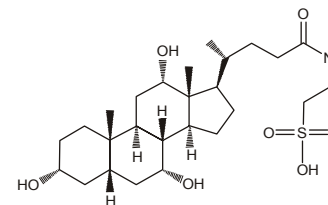
SUB-SECTION 2.8A—SAPONIFICATION NUMBER, IODINE NUMBER, ACID NUMBER, ACETYL NUMBER, POLENSKY NUMBER, AND R.M NUMBER

Saponification value (or "saponification number", also referred to as "sap" in short) represents the number of milligrams of potassium hydroxide or sodium hydroxide required to saponify 1g of fat under the conditions specified. It is a measure of the average molecular weight (or chain length) of all the fatty acids present. As most of the mass of a fat/triester is in the 3 fatty acids, it allows for comparison of the average fatty acid chain length.

If more moles of base are required to saponify N grams of fat then there are more moles of the fat the chain lengths are relatively small, given the following relation:

Number of moles = mass of oil/relative atomic mass

Handmade soapmakers who aim for bar soap use NaOH sap values which are derived from the

saponification value calculated by laboratories (KOH sap value). To convert KOH values to NaOH values, divide the KOH values by the ratio of the molecular weights of KOH and NaOH (1.403).

Standard methods for analysis are for example: ASTM D 94 (for petroleum) and DIN 51559.

The calculated saponification value is not applicable to fats and oils containing high amounts of unsaponifiable material, free fatty acids (>0.1%), or mono- and diacylglycerols (>0.1%).

The **iodine value**[1] (or "iodine adsorption value" or "iodine number" or "iodine index") in chemistry is the mass of iodine in grams that is consumed by 100 grams of a chemical substance. An iodine solution is yellow/brown in color and any chemical group in the substance that reacts with iodine will make the color disappear at a precise concentration. The amount of iodine solution thus required to keep the solution yellow/brown is a measure of the amount of iodine sensitive reactive groups.

One application of the iodine number is the determination of the amount of unsaturation contained in fatty acids. This unsaturation is in the form of double bonds which react with iodine compounds. The higher the iodine number, the more unsaturated fatty acid bonds are present in a fat. [1] In a typical procedure the acid is treated with an excess of the **Hanus solution** which is a solution of **iodobromine** (BrI). Unreacted iodobromine is reacted with potassium iodide which converts it to iodine. The iodine concentration is then determined by titration with sodium thiosulfate.

Standard methods for analysis are for example ASTM D5768-02(2006) and DIN 53241.

For a simple analysis, 0.2 grams of the fat is mixed with $20cm^3$ Wij's solution and $10cm^3$ 1,1,1-trichloroethane. It is then left in the dark for 30mins. Next, $15cm^3$ of 10% potassium iodide solution and $10cm^3$ of deionized water is added. This is then titrated against 0.1M sodium thiosulphate solution. $1cm^3$ of 0.1M sodium thiosulfate solution = 0.01269g of iodine. The difference between a control titration and the titration with the fat present multiplied by this factor gives the mass of iodine absorbed by the oil.

In chemistry, **acid value** (or "neutralization number" or "acid number" or "acidity") is the mass of potassium hydroxide (KOH) in milligrams that is required to neutralize one gram of chemical substance. The acid number is a measure of the amount of carboxylic acid groups in a chemical compound, such as a fatty acid, or in a mixture of compounds. In a typical procedure, a known amount of sample dissolved in organic solvent is titrated with a solution of potassium hydroxide with known concentration and with phenolphthalein as a color indicator.

The acid number is used to quantify the amount of acid present, for example in a sample of biodiesel. It is the quantity of base, expressed in milligrams of potassium hydroxide, which is required to neutralize the acidic constituents in 1 g of sample.

$$AN = \left(V_{eq} - b_{eq}\right) N \frac{56.1}{W_{oil}}$$

V_{eq} is the amount of titrant (ml) consumed by the crude oil sample and 1ml spiking solution at the equivalent point, b_{eq} is the amount of titrant (ml) consumed by 1 ml spiking solution at the equivalent point, and 56.1 is the molecular weight of KOH.

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[1]For the determination of the iodine number see appendix of this book also consult book A chemical Analyser's Guide by the same author.

The molarity concentration of titrant (N) is calculated as such:

$$N = \frac{1000 W_{KHP}}{204.23 V_{eq}}$$

In which W_{KHP} is the amount (g) of KHP in 50 ml of KHP standard solution, V_{eq} is the amount of titrant (ml) consumed by 50 ml KHP standard solution at the equivalent point, and 204.23 is the molecular weight of KHP.

There are standard methods for determining the acid number, such as ASTM D 974 and DIN 51558 (for mineral oils, biodiesel), or specifically for Biodiesel using the European Standard EN 14104 and ASTM D664 are both widely utilised worldwide. Acid number (mg KOH/g oil) for biodiesel should to be lower than 0.50 mgKOH/g in both EN 14214 and ASTM D6751 standard fuels. This is since the FFA produced may corrode automotive parts and these limits protect vehicle engines and fuel tanks.

As oil-fats rancidify, triglycerides are converted into fatty acids and glycerol, causing an increase in acid number. A similar observation is observed with Biodiesel aging through analogus oxidation processes and when subjected to prolonged high temperatures (ester thermolysis) or through exposure to acids or bases (acid/base ester hydrolysis).

Acetyl Number Number of milligrams of potassium hydroxide required to neutralize the acetic acid set free from 1 gram of acetylated compound when the latter is subjected to hydrolysis.

The **Polenske value** (also known as the **Polenske number**) is a value determined when examining fat. The Polenske value is an indicator of how much volatile fatty acid can be extracted from fat through saponification. It is equal to the number of mililiters of 0.1 normal hydroxide solution necessary for the neutralization of the water-insoluble volatile fatty acids distilled and filtered from 5 grams of a given saponified fat. (The hydroxide solution used in such a titration is typically made from sodium hydroxide, potassium hydroxide, or barium hydroxide.)

The value is named for the chemist who developed it, Eduard Polenske.

The Reichert value and Kirschner value are related numbers based on similar tests.

SECTION 2.9—PROTEINS*

The term protein has derived from the Greek word "protos" meaning primary and no doubt well chosen. It represents an enormous group of complex nitrogenous compounds, mebers of which occur in all animal and plant protoplasm.

There is greater diversity in the chemical composition of proteins than in that of any other group of biologically important compounds.

The Protein content of tissues varies widely, asn shown in the table below: -

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* See the chapter of Compounds of Biochemical Interest 'The Architecture of Chemistry An Organic Approach by same author" before reading this section.

Source	Protein content in percentage
Fresh mammalian muscle (striated† and unstriated)	18 – 20%
Blood Plasma	6.5 – 7.5%
Brain	8%
Egg Yolk	15%
Egg White	12%
Cow Milk	3.3%
Cheese	14 – 49%
Lettuce	1.2%
Asperegus	1.8%
Cabbage	1.6%
Potatoes	2%
Beets	1.6%
Cereal Grains	10 – 15%
Navy Beens	22%
Soya Beens	**37%**
Pea Nuts	26%
Almonts	21%
Fruits	0.4 – 1.5%
Fish	25%
Meat	21%
Beef	28%

Plant readilysynthesize proteins from simple substances such as nitrogen compounds, Animals, however cannot do this therefore are directly or indirectly dependant on plants for their protein supply. Althouigh animals utilize food carbohydrate and lipids to a small extent as structural components of protoplasm; their chief utilization is as sources of energy for operating the anatomical mechanism. On the otyehr hand the main function of food proteins is to provide the major organic structuires of the protoplasmic machine itself, although excess is utilized as asource of energy.

Much of the regulation and integration of physiological process uin the body is also accomplished by hormones which are secreated by endochine glands. Several proteins, such as insulin of the pancreas and the hormones of the anterior pituitary glands.

The chemical process in volved in the digestion of the food and also in the utilization or metabiolism of foods in animal tissue are in general catalized and directed by substances known "enzymes". The chemical processes of plants are similarly under the control of enzymes. Manyenzymes have been isolated purified and analyzed, without exception, they have all been found to be protein in character.

Certain deseases are caused by some critical agents commonly known as " virus" they are formation of complicated protein structures.

It should be noted that all the proteins no matter what has the basic composition C, O, H, N and generally S and sometimes P. Elements I, Fe, Cu, Zn are also occationaly present. The approximate average elementary composition of protein is as follows.

The average composition chart has been shown in the table below:

Element	Percent
Carbon	50
Hydrogen	7
Oxygen	23
Nitrogen	16
Sulphur	0-3
Phosphorous	0-3

SUB-SECTION 2.9A—CLASSIFICATION OF PROTEINS

Proteins cannot be classified purely on chemical basis at the present time because of deficiencies of knowledge of their composition and structure, and they must be classified on the basis of both physical properties of most of the values for classification purposes are those of solubility and heat coagulability. The classification given below is based upon the recommendatyion of a joint comette of the Americal Society of Biological Chemists.

According to this system main groups are :

1. Simple Proteins
2. Conjugated Proteins
3. Derived Proteins

And each of thease main groups are subdivided into classes.

Simple Protein

Albumin (Latin: albus, white) refers generally to any protein with water solubility, which is moderately soluble in concentrated salt solutions, and experiences heat coagulation (protein denaturation). Substances containing albumin, such as egg white, are called *albuminoids*. It has a molecular weight of about 65,000 and consists of 584 amino acids and contains no carbohydrate.

Types

Serum albumin

The most well-known type of albumin is the serum albumin in the blood.

Serum albumin is the most abundant blood plasma protein and is produced in the liver and forms a large proportion of all plasma protein. The human version is human serum albumin, and it normally constitutes about 70% of human plasma protein.

Serum albumins are important in regulating blood volume by maintaining the oncotic pressure (also known as colloid osmotic pressure) of the blood compartment. They also serve as carriers for molecules of low water solubility, including lipid soluble hormones, bile salts, unconjugated bilirubin, free fatty acids (apoprotein), calcium, iron (transferrin), and some drugs like warfarin, phenobutazone, clofibrate & phenytoin. Competition between drugs for albumin binding sites may cause drug interaction by increasing the free fraction of one of the drugs, thereby affecting potency.

Specific types include:

- human serum albumin
- bovine serum albumin (cattle serum albumin) or BSA, often used in medical and molecular biology labs.

Low albumin (hypoalbuminaemia) may be caused by liver disease, nephrotic syndrome, burns, protein-losing enteropathy, malabsorption, malnutrition, late pregnancy, artefact, genetic variations and malignancy.

High albumin is almost always caused by dehydration. In some cases of retinol (Vitamin A) deficiency the albumin level can become raised to High-normal values(ex: 4.9 g/dL). This is because retinol causes cells to swell with water (this is also the reason too much Vitamin A is toxic). In lab experiments it has been shown that All-trans retinoic acid down regulates human albumin production

Normal range of human serum albumin in adults (> 3 y.o.) is 3.5 to 5 g/dL. For children less than three years of age, the normal range is broader, 2.5-5.5 g/dL.

Other types

Other types include the storage protein ovalbumin in egg white, and different storage albumins in the seeds of some plants.

- Note that the protein 'albumin' is spelled with an "i", while "albumen" with an "e", is the white of an egg which contains (among other things) several dozen types of albumin (with an 'i'), mostly ovalbumin.

Albumin Measurement

Plasma albumin is a component of the Liver Function Tests (LFTs) but may be ordered separately. Albumin can be measured in Serum (yellow-top tube), plain tube with no additives red-top tube) or heparin plasma (green-top tube). The reference interval is 36 - 52 g/L. (note upper limit increased from 47 g/L on the 15th June 2007) One of the method used is Bromocresol green on a Roche Modular or Olympus AU2700 analyser.

Alpha 1-Antitrypsin or **α_1-antitrypsin** (**A1AT**) is a glycoprotein and generally known as **serum trypsin inhibitor**. The correct name, however, is alpha-1 proteinase inhibitor (A1PI) because it is a serine protease inhibitor (serpin), inhibiting a wide variety of proteases.[1] It protects tissues from

enzymes of inflammatory cells, especially elastase, and has a reference range in blood of 1.5 - 3.5 gram/liter, but the concentration can rise manyfold upon acute inflammation.[2] In its absence, elastase is free to break down elastin, which contributes to the elasticity of the lungs, resulting in respiratory complications such as emphysema, or COPD (chronic obstructive pulmonary disease) in adults and cirrhosis in adults or children.

Function

A1AT is a 52-kDa serpin (serine protease inhibitor), and, in medicine, it is considered the most prominent serpin; the terms *$\alpha1$-antitrypsin* and *protease inhibitor* (P_i) are often used interchangeably.

Most serpins inactivate enzymes by binding to them covalently, requiring very high levels to perform their function. In the acute phase reaction, a further elevation is required to "limit" the damage caused by activated neutrophil granulocytes and their enzyme elastase, which breaks down the connective tissue fiber elastin.

Like all serine protease inhibitors, A1AT has a characteristic secondary structure of beta sheets and alpha helices. Mutations in these areas can lead to non-functional proteins that can polymerise and accumulate in the liver (infantile hepatic cirrhosis).
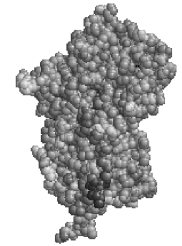
Role in disease

Disorders of the enzyme include alpha 1-antitrypsin deficiency, a hereditary disorder in which lack of alpha 1-antitrypsin leads to a chronic uninhibited tissue breakdown. This causes the subsequent degradation especially of lung tissue and to the manifestation of pulmonary emphysema.[3] Evidence has shown that cigarette smoke can lead to oxidation of methionine 358 of α_1-antitrypsin, a residue essential for binding elastase; this is the mechanism by which cigarette smoking (or second-hand smoke) can lead to emphysema. Because A1AT is created in the liver, certain mutations in the DNA code for the enzyme can cause misfolding and impaired secretion of the enzyme, which can lead to liver cirrhosis.

A remarkable form of P_i, termed $P_{i\text{Pittsburgh}}$, functions as an antithrombin (a related serpin), due to a mutation (Met358Arg). One patient with this abnormality has been described; he died of a lethal bleeding diathesis.

Nomenclature

The protein was called "antitrypsin" because of its ability to covalently bind and irreversibly inactivate the enzyme trypsin in vitro. Trypsin, a type of peptidase, is a digestive enzyme active in the duodenum and elsewhere.

The term *alpha-1* refers to the enzyme's behavior on protein electrophoresis. On electrophoresis, the protein component of the blood is separated by electric current. There are several *clusters*, the first being albumin, the second being the *alpha*, the third *beta* and the fourth *gamma* (immunoglobulins). The non-albumin proteins are referred to as globulins.

The *alpha* region can be further divided into two sub-regions, termed "1" and "2". Alpha 1-antitrypsin is the main enzyme of the alpha-globulin 1 region.

Another name used is *alpha-1 proteinase inhibitor* (á$_1$-PI).

Genetics

The gene is located on the long arm of the fourteenth chromosome (14q32.1).

Over 80 different versions of á$_1$-antitrypsin have been described in various populations. North-Western Europeans are most at risk for carrying a mutant form of A1AT.

Biochemical Properties

A1AT is a single-chain glycoprotein consisting of 394 amino acids in the mature form. The three N-linked glycosylations sites are mainly equipped with so-called diantennary N-glycans. However, one particular site shows a considerable amount of heterogeneity since tri- and even tetraantennary N-glycans can be attached to the Asparagine 107 (ExPASy amino acid nomenclature). These glycans carry different amounts of negatively-charged sialic acids, this causes the heterogeneity observed on normal A1AT when analysed by isoelectric focussing. In addition, the fucosylated triantennary N-glycans were shown to have the fucose as part of a so-called Sialyl Lewis x epitope, which could confer this protein particular protein-cell recognition properties. The single cysteine residue of A1AT in position 256 (ExPASy nomenclature) is found to be covalently linked to a free single cysteine by a disulfide bridge.

Analysis

As protein electrophoresis is imprecise, A1AT is analysed by electrofocusing (isoelectric focusing analysis), where the protein is passed along a pH gradient.

Normal A1AT is termed *M*, as it is neutral and does not run very far. Other variants are less functional, and are termed A-L and N-Z, dependent on whether they run proximal or distal to the M band. The presence of deviant bands on electrofocusing can signify the presence of alpha 1-antitrypsin deficiency.

As every person has two copies of the A1AT gene, a heterozygote with two different copies of the gene may have two different bands showing on electrofocusing, although heterozygote with one null mutant that abolishes expression of the gene will only show one band.

In blood test results, the electrofocusing results are notated as in P_iMM, where P_i stands for protease inhibitor and "MM" is the banding pattern of that patient.

Alpha 1-antitrypsin levels in the blood depend on the genotype. Some mutant forms fail to fold properly and are, thus, targeted for destruction in the proteasome, whereas others have a tendency to polymerise, being retained in the endoplasmic reticulum. The serum levels of some of the common genotypes are:

- PiMM: 100% (normal)
- PiMS: 80% of normal serum level of A1AT

- PiSS: 60% of normal serum level of A1AT
- PiMZ: 60% of normal serum level of A1AT
- PiSZ: 40% of normal serum level of A1AT
- PiZZ: 10-15% (severe alpha 1-antitrypsin deficiency)
- PiZ is caused by a glutamate to lysine mutation at position 342
- PiS is caused by a glutamate to valine mutation at position 264

Other rarer forms have been described; in all there are over 80 variants.

Therapeutic use

Recombinant alpha 1-antitrypsin is not yet commercially available, but is under investigation as a therapy for alpha 1-antitrypsin deficiency. Therapeutic concentrates are prepared from the blood plasma of blood donors.

Globulin is one of the two types of serum proteins, the other being albumin. This generic term encompasses a heterogeneous series of families of proteins, with larger molecules and less soluble in pure water than albumin, which migrate less than albumin during serum electrophoresis. The normal range in blood is 2 to 3.5 g/dl.

It is sometimes used synonymously with globular protein. However, albumin is also a globular protein, but not a globulin. All other serum globular proteins are globulins.

Protein electrophoresis is used to categorize globulins into the following four categories:

- Alpha 1 globulins
- Alpha 2 globulins
- Beta globulins
- Gamma globulins (one group of gamma globulins are immunoglobulins, that function as antibodies)

Alpha Globulins are a group of globular proteins in plasma, which are highly mobile in alkaline or electrically charged solutions. They inhibit certain blood protease and inhibitor activity.

Alpha 1 globulins

á$_1$-antitrypsin

Alpha 2 globulins

- Haptoglobin
- á$_2$-macroglobulin
- Ceruloplasmin
- Thyroxine-binding globulin

Haptoglobin (abbreviated as **Hp**) is a protein in the blood plasma that binds free hemoglobin released from erythrocytes with high affinity and thereby inhibits its oxidative activity. The haptoglobin-

hemoglobin complex will then be removed by the reticuloendothelial system (mostly the spleen). In clinical settings, the haptoglobin assay is used to screen for and monitor intravascular hemolytic anemia.

Clinical significance

Since the reticuloendothelial system will remove the haptoglobin-hemoglobin complex from the body, haptoglobin levels will be decreased in hemolytic anemias. In the process of binding hemoglobin, haptoglobin sequesters the iron within hemoglobin, preventing iron-utilizing bacteria from benefitting from hemolysis. It is theorized that because of this, haptoglobin has evolved into an acute phase protein.

Test order protocol

Haptoglobin is ordered whenever a patient exhibits symptoms of anemia, such as pallor, fatigue, shortness of breath along with physical signs of hemolysis, such as jaundice or dark-colored urine. The test is also commonly ordered as a hemolytic anemia battery which also includes a reticulocyte count and a peripheral blood smear. It can also be ordered along with a Direct Antiglobulin Test when a patient is suspected of having a transfusion reaction or symptoms of autoimmune hemolytic anemia. Finally, it may be ordered in conjunction with a bilirubin.

Results interpretation

A decrease in haptoglobin can support a diagnosis of hemolytic anemia, especially when correlated with a decreased RBC count, Hemoglobin, and Hematocrit, and also an increased reticulocyte count.

If the reticulocyte count is increased, but the haptoglobin level is normal, this may indicate that cellular destruction is occurring in the spleen and liver, which may indicate a drug induced hemolysis, or a red cell dysplasia. The spleen and liver recognize an error in the red cells (either Drug coating the red cell membrane, or a dysfunctional red cell membrane), and destroy the cell. This type of destruction does not release hemoglobin into the peripheral blood, so the haptoglobin cannot bind to it. Thus, the haptoglobin will stay normal.

If there are symptoms of anemia but both the reticulocyte count and the haptoglobin level are normal, the anemia is most likely not due to hemolysis, but instead some other error in cellular production, such as aplastic anemia

Haptoglobin levels which are decreased but do not accompany signs of anemia may indicate liver damage, as the liver is not producing enough haptoglobin to begin with.

As haptoglobin is indeed an acute phase protein, any inflammatory process (infection, extreme stress, burns, major crush injury, allergy, etc) may increase the levels of plasma haptoglobin.

Structure

Haptoglobin is produced mostly by hepatocytes but also by other tissues: e.g. skin, lung, and kidney. According to Trayburn and Woods (2004) several studies have shown that the Haptoglobin gene is expressed in murine and human adipose tissue. Haptoglobin, in its simplest form, consists of two á- and two â-chains, connected by disulfide bridges. The chains originate from a common precursor protein which is proteolytically cleaved during protein synthesis.

Hp exists in two allelic forms in the human population, so called *Hp1* and *Hp2*; the latter one having arisen due to the partial duplication of *Hp1* gene. Three phenotypes of Hp, therefore are found in humans: Hp1-1, Hp2-1, and Hp2-2. Hp of different phenotypes have been shown to bind hemoglobin with different affinities, with Hp2-2 being the weakest binder.

alpha-2-Macroglobulin, also known as **á$_2$-macroglobulin** and abbreviated as **á2M** and **A2M**, is a large plasma protein found in the blood. It is produced by the liver, and is a major component of the alpha-2 band in protein electrophoresis.

Structure

Alpha-2-macroglobulin is composed of four identical subunits bound together by -S-S- bonds.

Function

Alpha-2-macroglobulin is able to inactivate an enormous variety of proteinases (including serine-, cysteine-, aspartic- and metalloproteinases).

Alpha-2-macroglobulin has in its structure a 35 aminoacid "bait" region. Proteinases binding and cleaving the bait region become bound to á2M. The proteinase-á2M complex is recognised by macrophage receptors and cleared from the system.

It functions as an inhibitor of coagulation by inhibiting thrombin.

It functions as an inhibitor of fibrinolysis by inhibiting plasmin and kallikrein
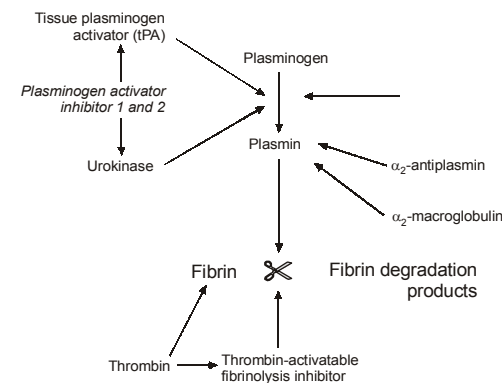
Fig. 2.34 : Fibrinolysis (simplified). Blue arrows denote stimulation, and red arrows inhibition

Disease

Alpha-2-macroglobulin levels are increased in nephrotic syndrome, a condition wherein the kidneys start to leak out some of the smaller blood proteins. Because of its size, á2-macroglobulin is retained in

the bloodstream. Increased production of all proteins means á2-macroglobulin concentration increases. This increase has little adverse effect on the health, but is used as a diagnostic clue. Longstanding chronic renal failure can lead to amyloid by *alpha-2-macroglobulin* (*see main article: amyloid*).

A common variant (29.5%) (polymorphism) of á2-macroglobulin leads to increased risk of Alzheimer's disease,[2][3] although the mechanism is unknown.

á-2-macroglobulin binds to and removes the active forms of the gelatinase (MMP-2 and MMP-9) from the circulation via scavenger receptors on the phagocytes.

Ceruloplasmin (or **caeruloplasmin**) is officially known as **ferroxidase** or **iron(II):oxygen oxidoreductase**. It is the major copper-carrying protein in the blood, and in addition plays a role in iron metabolism. It was first described in 1948.

Function: It is an enzyme (EC 1.16.3.1) synthesized in the liver containing 6 atoms of copper in its structure. Ceruloplasmin carries 90% of the copper in our plasma. The other 10% is carried by albumin, albumin may be confused at times to have a greater importance as a copper carrier because it binds copper less tightly than ceruloplasmin. Ceruloplasmin exhibits a copper-dependent oxidase activity, which is associated with possible oxidation of Fe^{2+} (ferrous iron) into Fe^{3+} (ferric iron), therefore assisting in its transport in the plasma in association with transferrin, which can only carry iron in the ferric state. The molecular weight of human ceruloplasmin is reported as 151kDa.

Pathology

Like any other plasma protein, levels drop in patients with hepatic disease due to reduced synthesizing capabilities.

- Mechanisms of low ceruplasmin levels:
  - Gene expression genetically low: aceruloplasminemia
  - Copper levels are low in general:
- Malnutrition/trace metal deficiency in the food source
  - Copper does not cross the intestinal barrier due to ATP7A deficiency in Menkes disease
  - Delivery of copper into the lumen of the ER-Golgi network is absent in hepatocyte due to absent ATP7B in Wilson's disease.

Copper availability doesn't affect the translation of the nascent protein. However, the apoenzyme without copper is unstable. Apoceruloplasmin is largely degraded intracellularly in the hepatocyte and the small amount that is released has a short circulation half life of 5 hours as compared to the 5.5 days for the holo-ceruloplasmin.

Mutations in the ceruloplasmin gene can lead to the rare genetic human disease aceruloplasminemia, characterized by iron overload in the brain, liver, pancreas, and retina.

Interpretation

Reference ranges for blood tests, comparing blood content of ceruloplasmin (shown in light blue) with other constituents.

Decreased levels

Lower-than-normal ceruloplasmin levels may indicate:

- Menkes disease (Menke's kinky hair syndrome) (very rare)
- Wilson's disease (a rare copper storage disease)
- Overdose of Vitamin C
- Copper deficiency
- Aceruloplasminemia

Elevated levels

Greater-than-normal ceruloplasmin levels may indicate:

- pregnancy
- lymphoma
- acute and chronic inflammation (it is an acute-phase reactant)
- rheumatoid arthritis
- Alzheimer's Disease

Thyroxine-binding globulin (TBG) binds Thyroid hormone in circulation. It is one of three proteins (along with transthyretin and albumin) responsible for carrying the thyroid hormones thyroxine (T4) and 3,5,3'-triiodothyronine (T3) in the bloodstream. Of these three proteins, TBG has the highest affinity for T4 and T3, but is present in the lowest concentration. Despite its low concentration, TBG carries the majority of T4 in serum. Due to the very low serum concentration of T4 & T3, TBG is rarely more than 25% saturated with its ligand. Unlike transthyretin and albumin, TBG has a single binding site for T4/T3. TBG is synthesized primarily in the liver as a 54 kDa protein. Genomically, TBG is a serpin, although it has no inhibitory function like many other members of this class of proteins.

Role in Diagnosis

TBG tests are sometimes used in finding the reason for elevated or diminished levels of thyroid hormone. This is done by measuring resin binding to labeled thyroid hormone, which only happens when the labeled thyroid hormone is free.

The patient's serum is mixed with the labeled thyroid hormone; then, the resin is added to the whole mixture, to measure the amount of free labeled thyroid hormone. So, for instance, if the patient is truly hypothyroid, and TBG levels are normal, then there are many sites open for binding on the TBG, since the total thyroid hormone level is low. Therefore, when the labeled hormone is added, it will mostly bind to the TBG, leaving little of it left for binding to the resin. Conversely, if the patient is truly hyperthyroid, and TBG levels are normal, the patient's endogenous hormone will saturate the TBG binding sites more, leaving less room for the labeled hormone, which allows greater binding to the resin.

In the situations described above, TBG testing is not very useful. However, total thyroid hormone levels point to hypothyroidism or hyperthyroidism, without the expected symptoms, the utility of TBG

testing becomes more evident, since TBG production can be modified by other factors such as estrogen, corticosteroids, or liver failure. If, for example, the TBG level is high, which can occur when estrogen levels are high, the TBG will bind more thyroid hormone, decreasing the free hormone available in the blood, which leads to stimulation of TSH, and the production of more thyroid hormone. In this case, the total thyroid hormone level will be high. However, when labeled hormone is added, since TBG is so high, it will bind to the TBG, leaving little free labeled hormone for uptake into the resin. Conversely, in the presence of corticosteroids, which lower TBG levels, the total thyroid hormone (bound and free) in the blood will be low. However, when the labeled hormone is added, since so little TBG is available in the blood, only a small portion of it will bind, leaving plenty available for uptake by the resin.

Beta globulins are a group of globular proteins in plasma that are more mobile in alkaline or electrically charged solutions than gamma globulins, but less mobile than alpha globulins.

Examples of beta globulins include:

- beta-2 microglobulin
- plasminogen
- angiostatins
- properdin
- sex hormone binding globulin
- transferrin

â$_2$ microglobulin also known as **B2M** is a component of MHC class I molecules, which are present on almost all cells of the body (red blood cells are a notable exception).[1][2]

â$_2$ microglobulin lies lateral to the á$_3$ chain on the cell surface. Unlike á$_3$, â$_2$ has no transmembrane region. Directly above â$_2$ (i.e. away from the cell) lies the á$_1$ chain, which itself is lateral to the á$_2$.

â$_2$ microglobulin associates not only with the alpha chain of MHC class I molecules, but also with class I-like molecules such as CD1 and Qa.

Clinical significance

In patients on long-term hemodialysis, it can aggregate into amyloid fibers that deposit in joint spaces, a disease known as dialysis-related amyloidosis.

Mice models deficient for the **â$_2$ microglobulin** gene have been engineered. These mice demonstrate that **â$_2$ microglobulin** is necessary for cell surface expression of MHC class I and stability of the peptide binding groove. In fact, in the absence of **â$_2$ microglobulin**, very limited amounts of MHC class I (classical and non-classical) molecules can be detected on the surface. In the absence of MHC class I, CD8 T cells cannot develop. (CD8 T cells are a subset of T cells involved in the development of acquired immunity.)
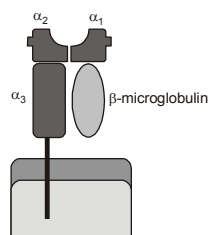
Fig. 2.35 : Schematic representation of MHC class I

Levels of beta-2 microglobulin can be elevated in multiple myeloma and lymphoma.

Plasmin also known as is an important enzyme (EC 3.4.21.7) present in blood that degrades many blood plasma proteins, most notable, fibrin clots. The degradation of fibrin is termed fibrinolysis. In humans, the plasmin protein is encoded by the *PLG* gene.

Function

Plasminogen (PLG) is a circulating zymogen that is converted to the active enzyme plasmin by cleavage of the peptide bond between Arg-560 and Val-561, which is mediated by urokinase and tissue plasminogen activator. The main function of plasmin is to dissolve fibrin blood clots. Plasmin, like trypsin, belongs to the family of serine proteases.

It is a serine protease that is released as **plasminogen** from the liver into the circulation and activated by tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), thrombin, fibrin, and factor XII (Hageman factor). It is inactivated by alpha 2-antiplasmin, a serine protease inhibitor (serpin).

Apart from fibrinolysis, plasmin proteolyses proteins in various other systems: It activates collagenases, some mediators of the complement system and weakens the wall of the Graafian follicle (leading to ovulation). It cleaves fibrin, fibronectin, thrombospondin, laminin, and von Willebrand factor.

Apart from fibrinolysis, plasminogen is shown to play important role in wound healing, liver repair as well as the mantainance of liver homeostasis.

Angiostatin is a naturally occurring protein found in several animal species, including humans. It is an endogenous angiogenesis inhibitor (i.e., it blocks the growth of new blood vessels), and it is currently undergoing clinical trials for its use in anticancer therapy.

Structure

Angiostatin is a 38 kDa fragment of a larger protein, plasmin (itself a fragment of plasminogen) enclosing three to five contiguous Kringle modules. Each module contains two small beta sheets and three disulfide bonds.

Generation

Angiostatin is produced, for example, by autoproteolytic cleavage of plasminogen, involving extracellular disulfide bond reduction by phosphoglycerate kinase. Furthermore angiostatin can be cleaved from plasminogen by different metalloproteinases (MMPs), elastase, prostata-specific antigen (PSA), 13 KD serine protease, or 24KD endopeptidase.

Biological activity

Angiostatin is known to bind many proteins, especially to angiomotin and endothelial cell surface ATP synthase but also integrins, annexin II, C-met receptor, NG2-proteoglycans, tissue-type plasminogen activator, chondroitin sulfate proteoglycans, and CD26. Additionally, smaller fragments of angiostatin may bind several other proteins. There is still considerable uncertainty on its mechanism of action, but it seems to involve inhibition of endothelial cell migration, proliferation and induction of apoptosis. It

has been proposed that angiostatin activity is related, among other things, to the coupling of its mechanical and redox properties.

Properdin or factor P is a globulin protein found in the blood serum of higher animals. In the complement system, an innate-immunity series of proenzymes dissolved in the circulation, it is also called "Factor P".

Function

It is known that it participates in some specific immune responses. It plays a part in tissue inflammation as well as the engulfing of pathogens by phagocytes. In addition it is known to help to neutralize some viruses.

As a component of the alternative pathway for complement activation (otherwise known as the "properdin pathway"), it complexes with another protein, C3b, to stabilize the alternative C3 convertase (C3bBb) that then cleaves more C3.
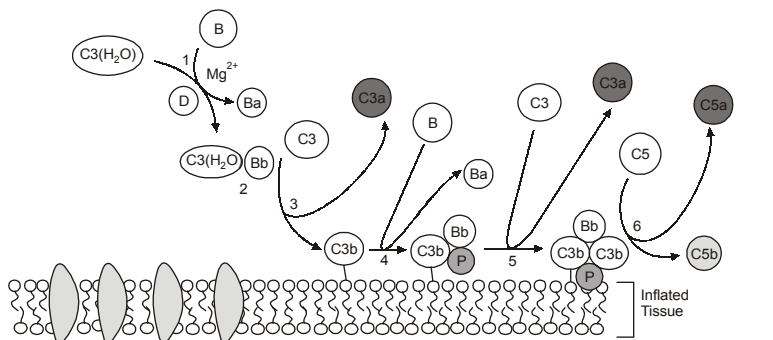
Fig. 2.36 : Alternative pathway. Properdin is the "P" in the blue circles.

The alternative pathway is not dependent on antibodies. This branch of the complement system is activated by IgA immune complexes and bacterial endotoxins, polysaccharides, and cell walls, and results in producing anaphylatoxins, opsonins, chemotactic factors, and the membrane attack complex, all of which help fight pathogens.

Sex hormone-binding globulin (**SHBG**) is a glycoprotein that binds to sex hormones, specifically testosterone and estradiol. Other steroid hormones such as progesterone, cortisol, and other corticosteroids are bound by transcortin.
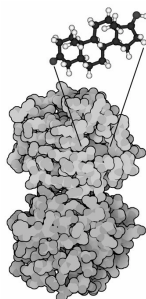
Transport of sex hormones

Testosterone and estradiol circulate in the bloodstream, bound mostly to SHBG and to some degree bound to serum albumin. Only a small fraction is unbound, or "free," and thus biologically active and able to enter a cell and activate its receptor. The SHBG inhibits the function of these hormones. Thus bioavailability of sex hormones is influenced by the level of SHBG.

SHBG production

SHBG is produced by the liver cells and is released into the bloodstream. Other sites that produce SHBG are the brain, uterus, and placenta and vagina. In addition SHBG is produced by the testes; testes-produced SHBG is also called androgen-binding protein. The gene for SHBG is located on chromosome 17.

Control

SHBG levels appear to be controlled by a delicate balance of enhancing and inhibiting factors. Its level is decreased by high levels of insulin and insulin-like growth factor 1 (IGF-1). Also, high androgen levels decrease SHBG, while high estrogen and thyroxine levels increase it.

However, recent evidence suggests that it is the liver's production of fats that reduces SHBG levels, not any direct effect of insulin and specific genetic mechanisms have been found that do this.

Conditions with high or low levels

Conditions with low SHBG include polycystic ovary syndrome, diabetes, and hypothyroidism. Conditions with high SHBG include pregnancy, hyperthyroidism, and anorexia nervosa. There has recently been research to link high SHBG levels with breast and testicular cancer as well.

Measurement of sex hormones

When determining levels of circulating estradiol or testosterone, either a total measurement could be done that includes the "free" and the bound fractions, or only the "free" hormone could be measured. A free androgen index expresses the ratio of testosterone to the sex hormone binding globulin and can be used to summarise the activity of free testosterone.

The total testosterone is likely the most accurate measurement of testosterone levels and should always be measured at 8 o'clock in the morning. Sex hormone binding globulin can be measured separate from the total fraction of testosterone.

Transferrin is a blood plasma protein for iron ion delivery which in humans is encoded by the *TF* gene.[1] Transferrin is a glycoprotein, which binds iron very tightly but reversibly. Although iron bound to transferrin is less than 0.1% (4 mg) of the total body iron, dynamically it is the most important iron pool, with the highest rate of turnover (25 mg/24 h). Transferrin has a molecular weight of around 80 kiloDaltons and contains 2 specific high affinity Fe(III) binding sites. The affinity of transferrin for Fe(III) is extremely high (10^{23} M^{-1} at pH 7.4) but decreases progressively with decreasing pH below neutrality.

When not bound to iron, it is known as "apo-transferrin" (see also apoprotein).

Transport mechanism

When a transferrin protein loaded with iron encounters a transferrin receptor on the surface of a cell (importantly, to erythroid precursors in the bone marrow), it binds to it and is consequently transported into the cell in a vesicle. The pH of the vesicle is reduced by hydrogen ion pumps (H^+ ATPases), causing transferrin to release its iron ions. The receptor (with its ligand, transferrin, bound) is then transported through the endocytic cycle back to the cell surface, ready for another round of iron uptake. Each transferrin molecule has the ability to carry two iron ions in the ferric form (Fe^{3+}).

The gene coding for transferrin in humans is located in chromosome band 3q21. Research on king snakes by Dessauer and Zwiefel in 1981 revealed that the inheritance of transferrin is a codominant trait.

Medical professionals may check serum transferrin level in iron deficiency, hemochromatosis and other iron overload disorders.

Immune system

Transferrin is also associated with the innate immune system. Transferrin is found in the mucosa and binds iron, thus creating an environment low in free iron, where few bacteria are able to survive. The levels of transferrin decreases in inflammation, seeming contradictory to its function

A decrease in the amount of transferrin would result in hemosiderin in the liver.

Other effects

The metal binding properties of transferrin have a great influence on the biochemistry of plutonium in humans. Transferrin has a bacteriocidal effect on bacteria, in that it makes Fe^{3+} unavailable to the bacteria.

Pathology

A deficiency is associated with atransferrinemia.

Gamma globulins, or Ig's, are a class of proteins in the blood, identified by their position after serum protein electrophoresis. The most significant gamma globulins are immunoglobulins.

Injections

Gamma globulin injections are usually given in an attempt to temporarily boost a patient's immunity against disease. Injections are most commonly used on patients who have been exposed to hepatitis A or measles, or to make a kidney donor and recipient compatible regardless of blood type of tissue match. Injections are also used to boost immunity in patients who cannot produce gamma globulins naturally because of an immune deficiency, such as X-linked agammaglobulinemia and hyper IgM syndrome. Such injections are less common in modern medical practice than they were previously, and injections of gamma globulin previously recommended for travelers have largely been replaced by the use of hepatitis A vaccine.

Gamma globulin infusions are also used to treat immunological diseases, such as idiopathic thrombocytopenia purpura (ITP), a disease in which the platelets are being attacked by antibodies,

leading to seriously low platelet counts. Gamma globulin apparently causes the spleen to ignore the antibody-tagged platelets, thus allowing them to survive and function.

Gamma globulin injections also provide substantial benefit to many suffering from Chronic Fatigue Syndrome, also known as Chronic Fatigue and Immune Dysfunction Syndrome; Myalgic Encephalitis; Chronic Epsteinn-Barr; Chronic Mono.

Another theory on how gamma globulin administration works in autoimmune disease is by overloading the mechanisms which degrade gamma globulins. Overloading the degradation mechanism causes the harmful gamma globulins to have a much shorter halflife in sera.

Pathology

An excess is known as hypergammaglobulinemia.

A disease of gamma globulins is called a "gammopathy" (for example, in monoclonal gammopathy of undetermined significance.)

Disease treatments

• Kawasaki disease

Kidney Transplant: Intravenous Gamma globulin was FDA approved in 2004 to reduce antibodies in a patient with kidney failure to allow that person to accept a kidney from a donor who has a different blood type, (ABO incompatible) or is an unacceptable tissue match. Dr. Stanley Jordan at Cedars-Sinai Medical Center in Los Angeles pioneered this treatment.

Glutelins are soluble in dilute acids or bases, detergents, chaotropic or reducing agents. They are generally prolamin-like proteins in certain grass seeds. Glutenin is the most common glutelin as it is found in wheat and is responsible from some of the refined baking properties in bread wheat. The glutelins of barley and rye have also been identified.

Typically there are HMW and LMW glutelins in these species, they crosslink with themselves and other proteins during baking via disulfide bonds. A HMW glutelin (glutenin) of the grass tribe *Triticeae* can be sensitizing agents for coeliac disease in individuals who possess the HLA-DQ8 class II antigen receptor gene. (Not yet characterized to the epitope level)

Prolamins are a group of plant storage proteins having a high proline content and found in the seeds of cereal grains: wheat (gliadin), barley (hordein), rye (secalin), corn (zein) and as a minor protein, avenin in oats. They are characterised by a high glutamine and proline content and are generally soluble only in strong alcohol solutions. Some prolamins, notably gliadin, and similar proteins found in the tribe Triticeae (see Triticeae glutens) may induce coeliac disease in genetically predisposed individuals.

Scleroproteins are one of the two main classes of protein tertiary structure (the other being globular proteins).

They are also called fibrous proteins.

Characteristics

They form long protein filaments, rod- or wire-like shapes. They are usually inert structural or storage proteins. They are generally water-insoluble and are found as an aggregate due to hydrophobic R

groups that stick out of the molecule. The amino acid sequences they are made from often have limited residues with repeats. These can form unusual secondary structures, e.g. collagen triple helix. The structures often contain 'cross-links' between chains, for example cys-cys disulfide bonds between keratin chains.

Globular proteins tend to denature more easily than fibrous proteins.

Functions

They usually play a role which is protective or supportive.

They are usually used to construct connective tissues, tendons, bone matrix and muscle fiber.

Attempts at artificial synthesis have been made.

Examples

Examples of include keratins, collagens and elastins.

Another example is fibroin.

Conjugated Proteins

A **nucleoprotein** is any protein which is structurally associated with nucleic acid (either DNA or RNA). The prototypical example is any of the histone class of proteins, which are identifiable on strands of chromatin. Telomerase, a RNP (RNA/protein complex), and Protamines are also nucleoproteins.
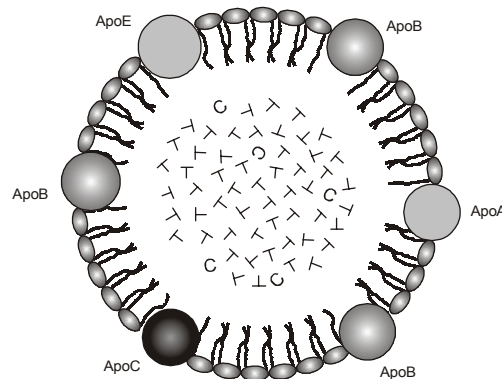
Fig. 2.37 : Chylomicron structure

Phosphoproteins are a group of proteins which are chemically bonded to a substance containing phosphoric acid (see phosphorylation for more). The category of organic molecules that includes Fc receptors, Ulks, Calcineurins, K chips, and urocortins.

A **lipoprotein** is a biochemical assembly that contains both proteins and lipids. The lipids or their derivatives may be covalently or non-covalently bound to the proteins. Many enzymes, transporters, structural proteins, antigens, adhesins and toxins are lipoproteins. Examples include the high density and low density lipoproteins which enable fats to be carried in the blood stream, the transmembrane proteins of the mitochondrion and the chloroplast, and bacterial lipoproteins.

Function

The function of lipoprotein particles is to transport lipids (fats) around the body in the aqueous blood, in which they would not normally dissolve.

All cells use and rely on fats and, for all animal cells, cholesterol as building blocks to create the multiple membranes which cells use to both control internal water content, internal water soluble elements and to organize their internal structure and protein enzymatic systems.

The protein particles have hydrophilic groups aimed outward so as to attract water molecules; this makes them soluble in the salt water based blood pool. Triglyceride-fats and cholesterol are carried internally, shielded from the water by the protein particle.

The interaction of the proteins forming the surface of the particles with (a) enzymes in the blood, (b) with each other and (c) with specific proteins on the surfaces of cells, determine whether triglycerides and cholesterol will be added to or removed from the lipoprotein transport particles.

Regarding atheroma development and progression vs. regression, the key issue has always been cholesterol transport patterns, not cholesterol concentration itself.

Transmembrane lipoproteins

The lipids are often an essential part of the complex, even if they seem to have no catalytic activity themselves. To isolate transmembrane lipoproteins from their associated membranes, detergents are often needed.

Classification

By density

General categories of lipoproteins, listed in order from larger and less dense (more fat than protein) to smaller and denser (more protein, less fat):

- Chylomicrons - carry triacylglycerol (fat) from the intestines to the liver, skeletal muscle, and to adipose tissue.
- Very low density lipoproteins (VLDL) - carry (newly synthesised) triacylglycerol from the liver to adipose tissue.
- Intermediate density lipoproteins (IDL) - are intermediate between VLDL and LDL. They are not usually detectable in the blood.
- Low density lipoproteins (LDL) - carry cholesterol from the liver to cells of the body. Sometimes referred to as the "bad cholesterol" lipoprotein.

- High density lipoproteins (HDL) - collects cholesterol from the body's tissues, and brings it back to the liver. Sometimes referred to as the "good cholesterol" lipoprotein.

Density (g/mL)	Class	Diameter (nm)	% protein	% choles-terol	% phospho-lipid	% triacyl-glycerol
>1.063	HDL	5-15	33	30	29	8
1.019-1.063	LDL	18-28	25	50	21	4
1.006-1.019	IDL	25-50	18	29	22	31
0.95-1.006	VLDL	30-80	10	22	18	50
<0.95	Chylomicrons	100-1000	<2	8	7	84

Alpha and beta

It is also possible to classify lipoproteins as "alpha" and "beta", akin to the classification of proteins in serum protein electrophoresis. This terminology is sometimes used in describing lipid disorders such as Abetalipoproteinemia.

Lipoprotein(a)

Lipoprotein(a) - Lp(a), Cardiology diagnostic tests

     < 14 mg/dL : Normal

     14-19 mg/dL : ?

     > 19 mg/dL : High risk

     How to lower: aerobic exercise, niacin, aspirin, guggulipid.

Metabolism

The handling of lipoproteins in the body is referred to as lipoprotein metabolism. It is divided into two pathways, exogenous and endogenous, depending in large part on whether the lipoproteins in question are composed chiefly of dietary (exogenous) lipids or whether they originated in the liver (endogenous).

Exogenous pathway

Epithelial cells lining the small intestine readily absorb lipids from the diet. These lipids, including triglycerides, phospholipids, and cholesterol, are assembled with apolipoprotein B-48 into chylomicrons. These nascent chylomicrons are secreted from the intestinal epithelial cells into the lymphatic circulation in a process that depends heavily on apolipoprotein B-48. As they circulate through the lymphatic vessels, nascent chylomicrons bypass the liver circulation and are drained via the thoracic duct into the bloodstream.

In the bloodstream, HDL particles donate apolipoprotein C-II and apolipoprotein E to the nascent chylomicron; the chylomicron is now considered mature. Via apolipoprotein C-II, mature chylomicrons activate lipoprotein lipase (LPL), an enzyme on endothelial cells lining the blood vessels. LPL catalyzes

a hydrolysis reaction that ultimately releases glycerol and fatty acids from the chylomicrons. Glycerol and fatty acids can be absorbed in peripheral tissues, especially adipose and muscle, for energy and storage.

The hydrolyzed chylomicrons are now considered chylomicron remnants. The chylomicron remnants continue circulating until they interact via apolipoprotein E with chylomicron remnant receptors, found chiefly in the liver. This interaction causes the endocytosis of the chylomicron remnants, which are subsequently hydrolyzed within lysosomes. Lysosomal hydrolysis releases glycerol and fatty acids into the cell, which can be used for energy or stored for later use.

Endogenous pathway

The liver is another important source of lipoproteins, principally VLDL. Triacylglycerol and cholesterol are assembled with apolipoprotein B-100 to form VLDL particles. Nascent VLDL particles are released into the bloodstream via a process that depends upon apolipoprotein B-100.

As in chylomicron metabolism, the apolipoprotein C-II and apolipoprotein E of VLDL particles are acquired from HDL particles. Once loaded with apolipoproteins C-II and E, the nascent VLDL particle is considered mature.

Again like chylomicrons, VLDL particles circulate and encounter LPL expressed on endothelial cells. Apolipoprotein C-II activates LPL, causing hydrolysis of the VLDL particle and the release of glycerol and fatty acids. These products can be absorbed from the blood by peripheral tissues, principally adipose and muscle. The hydrolyzed VLDL particles are now called VLDL remnants or intermediate density lipoproteins (IDLs). VLDL remnants can circulate and, via an interaction between apolipoprotein E and the remnant receptor, be absorbed by the liver, or they can be further hydrolyzed by hepatic lipase.

Hydrolysis by hepatic lipase releases glycerol and fatty acids, leaving behind IDL remnants, called low density lipoproteins (LDL), which contain relatively high cholesterol content. LDL circulates and is absorbed by the liver and peripheral cells. Binding of LDL to its target tissue occurs through an interaction between the LDL receptor and apolipoprotein B-100 or E on the LDL particle. Absorption occurs through endocytosis, and the internalized LDL particles are hydrolyzed within lysosomes, releasing lipids, chiefly cholesterol.

Lipoprotein(a) (also called Lp(a)) is a lipoprotein subclass. Studies have identified Lp(a) as a putative risk factor for atherosclerotic diseases such as coronary heart disease and stroke.

Lipoprotein(a) was dicovered in 1963 by Kåre Berg and the human gene encoding this protein was cloned in 1987.

Structure

Lipoprotein(a) [Lp(a)] consists of an LDL-like particle and the specific apolipoprotein(a) [apo(a)], which is covalently bound to the apoB of the LDL like particle. Lp(a) plasma concentrations are highly heritable and mainly controlled by the apolipoprotein(a) gene [LPA] located on chromosome 6q26-27. Apo(a) proteins vary in size due to a size polymorphism [KIV-2 VNTR], which is caused by a variable number of so called kringle IV repeats in the LPA gene. This size variation at the gene level is expressed

on the protein level as well, resulting in apo(a) proteins with 10 to > 50 kringle IV repeats (each of the variable kringle IV consists of 114 amino acids). These variable apo(a) sizes are known as "apo(a) isoforms". There is a general inverse correlation between the size of the apo(a)isoform and the Lp(a) plasma concentration which is caused by a variable rate of degradation before the apo(a) protein has matured for Lp(a) assembly. Apo(a) is expressed by the liver cells (hepatocytes), and the assembly of apo(a) and LDL particles seems to take place at the outer hepatocyte surface. The half-life of Lp(a) in the circulation is about 3 to 4 days.

Catabolism and clearance

The mechanism and sites of Lp(a) catabolism are largely unknown. Uptake via the LDL receptor is not a major pathway of Lp(a) metabolism. The kidney has been identified as playing a role in Lp(a) clearance from plasma.

Populations

Lp(a) concentrations vary over one thousandfold between individuals, from < 0.2 to > 200 mg/dL. This range of concentrations is observed in all populations studied so far. The mean and median concentrations between different world populations show distinct particularities, the main being the two- to threefold higher Lp(a) plasma concentration of populations of African descent compared to Asian, Oceanic, or European populations. The general inverse correlation between apo(a) isoform size and Lp(a)plasma concentration is observed in all populations, however, mean Lp(a) associated with certain apo(a) isoforms varies between populations.

Function

The physiological function of Lp(a)/apo(a) is still unknown. A function within the coagulation system seems plausible, given the aspect of the high homology between apo(a) and plasminogen. In fact, the LPA gene derives from a duplication of the plasminogen gene.

Other functions have been related to recruitment of inflammatory cells through interaction with Mac-1 integrin, angiogenesis, and wound healing.

However, individuals without Lp(a) or with very low Lp(a) levels seem to be healthy. Thus plasma Lp(a) is certainly not vital, at least under normal environmental conditions. Since apo(a)/Lp(a) derived rather recently in mammalian evolution - only old world monkeys and humans have been shown to harbour Lp(a) - its function might not be vital but just evolutionary advantageous under certain environmental conditions, e.g. in case of exposure to certain infectious diseases.

Pathology

Lipoprotein's structure is similar to plasminogen and tPA (tissue plasminogen activator) and it competes with plasminogen for its binding site, leading to reduced fibrinolysis. Also because Lp(a) stimulates secretion of PAI-1 it leads to thrombogenesis. In addition, because of LDL cholesterol content, Lp-a contributes to atherosclerosis.

Lipoprotein(a) and Disease

High Lp(a) in blood is a risk factor for coronary heart disease (CHD), cerebrovascular disease (CVD), atherosclerosis, thrombosis, and stroke. Lp-a concentrations may be affected by disease states, but are

only slightly affected by diet, exercise, and other environmental factors. Commonly prescribed lipid-reducing drugs have little or no effect on Lp(a) concentration. Niacin (nicotinic acid) and aspirin are two relatively safe, easily available and inexpensive drugs known to significantly reduce the levels of Lp(a) in some individuals with high Lp(a); they should be used under the supervision of a qualified physician.

High Lp(a) predicts risk of early atherosclerosis similar to high LDL, but in advanced atherosclerosis, Lp(a) is an independent risk factor not dependent on LDL. Lp(a) then indicates a coagulant risk of plaque thrombosis. Apo(a) contains domains that are very similar to plasminogen (PLG). Lp(a) accumulates in the vessel wall and inhibits binding of PLG to the cell surface, reducing plasmin generation which increases clotting. This inhibition of PLG by Lp(a) also promotes proliferation of smooth muscle cells. These unique features of Lp(a) suggest Lp(a) causes generation of clots and atherosclerosis.

Metalloprotein is a generic term for a protein that contains a metal ion cofactor. Metalloproteins have many different functions in cells, such as enzymes, transport and storage proteins, and signal transduction proteins. Indeed, about one quarter to one third of all proteins require metals to carry out their functions. The metal ion is usually coordinated by nitrogen, oxygen or sulfur atoms belonging to amino acids in the polypeptide chain and/or a macrocyclic ligand incorporated into the protein. The presence of the metal ion allows metalloenzymes to perform functions such as redox reactions that cannot easily be performed by the limited set of functional groups found in amino acids.

Storage and Transport Metalloproteins

Oxygen carriers

Hemoglobin, which is the principal oxygen carrier in humans has four sub-units in which the iron(II) ion is coordinated by the planar, macrocyclic ligand protoporhyrin IX (PIX) and the imidazole nitrogen atom of a histidine residue. The sixth coordination site contains a water molecule or a dioxygen molecule. myoglobin has only one such unit. The active site is located in an hydrophobic pocket. This is important as, without it, the iron(II) would be irreveribly oxidised to iron(III). The equilibrium constant for the formation of HbO_2 is such that oxygen is taken up or released depending on the partial pressure of oxygen in the lungs or in muscle. In hemoglobin the four sub-units show a cooperativity effect which allows for easy oxygen transfer from hemoglobin to myoglobin.

In both hemoglobin and myoglobin it is sometimes incorrectly stated that the oxygenated species contains iron(III). It is now known that the diamagnetic nature of these species is due to the fact that the iron(II) is in the low-spin state. In oxyhemoglobin the iron atom is located in the plane of the porphyrin ring, but in the paramagnetic deoxyhemoglobin the iron atom lies above the plane of the ring. The change in spin state is a cooperative effect of higher crystal field splitting and smaller ionic radius of Fe^{2+} in the oxy- moiety.

Hemerythrin is another iron-containing oxygen carrier. The oxygen binding site is a binuclear iron center. The iron atoms are coordinated to the protein through the carboxylate side chains of a glutamate and aspartate and five histidine residues. The uptake of O_2 by hemerythrin is accompanied by two-electron oxidation of the reduced binuclear center to produce bound peroxide (OOH-). The mechanism of oxygen uptake and release have been worked out in detail.

Hemocyanins carry oxygen in the blood of most molluscs, and some arthropods such as the horseshoe crab. They are second only to hemoglobin in biological popularity of use in oxygen transport. On oxygenation the two copper(I) atoms at the active site are oxidised to copper(II) and the dioxygen molecules is reduced to peroxide, O_2^{2-}

Cytochromes

Oxidation and reduction are reactions are not common in organic chemistry as few organic molecules can act as oxidizing or reducing agents. Iron(II), on the other hand, can easily be oxidized to iron(III). This functionality is used in cytochromes which function as electron-tranfer vectors. The iron atom in most cytochromes is contained in a heme group. The differences between those cytochromes lies in the different side-chains. For instance Cytochrome a has a heme a prosthetic group and cytochrome b has a heme b prosthetic group. These differences result in different Fe^{2+}/Fe^{3+} redox potentials such that various cytochromes are involved in the mitochondrial electron transport chain.

Cytochrome P450 enzymes perform the function of inserting an oxygen atom into a C—H bond, an oxidation reaction.
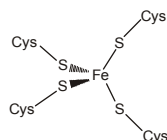
rubredoxin active site.

Rubredoxin

Rubredoxin is an electron-carrier found in sulfur-metabolizing bacteria and archaea. The active site contains an iron ion which is coordinated by the sulphur atoms of four cysteine residues forming an almost regular tetrahedron. Rubredoxins perform one-electron transfer processes. The oxidation state of the iron atom changes between the +2 and +3 states. In both oxidation states the metal is high spin, which helps to minimize structural changes.

Iron Storage and Transfer

Iron is stored as iron(III) in ferritin. The exact nature of the binding site has not yet been determined. The iron appears to be present as an hydrolysis product such as FeO(OH). Iron is transported by transferrin whose binding site structure is also as yet unknown.

The human body has no mechanism for iron excretion. This can lead to iron-overload problems in patients treated with blood transfusions, as, for instance, with â-thallasemia.

Ceruloplasmin

Cceruloplasmin is the major copper-carrying protein in the blood. Ceruloplasmin exhibits oxidase activity, which is associated with possible oxidation of Fe^{2+} (ferrous iron) into Fe^{3+} (ferric iron), therefore

assisting in its transport in the plasma in association with transferrin, which can only carry iron in the ferric state.

Metalloenzymes

Metalloenzymes all have one feature in common, namely, that the metal ion is bound to the protein with one coordination site free. As with all enzymes, the shape of the active site is crucial. The metal ion is usually located in a pocket whose shape fits the substrate. The metal ion catalyzes reactions which are difficult to achieve in organic chemistry.

Carbonic anhydrase
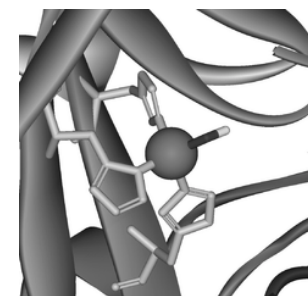
Fig. 2.39 : Active site of carbonic anhydrase. The three coordinating histidine residues are shown in pink, hydroxide in red and white, and the zinc in purple.

$$CO_2 + H_2O \rightleftharpoons H_2CO_3$$
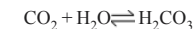
This reaction is very slow in tha absence of a catalyst, but quite fast in the presence of the hydroxide ion

$$CO_2 + OH^- \rightleftharpoons HCO_3^-$$

A reaction similar to this is almost instantaneaous with carbonic anhydrase. The structure of the active site in carbonic anhydrases is well-known from a number of crystal structures. It consists of a zinc ion coordinated by three imidazole nitrogen atoms from three histidine units. The fourth coordination site is occupied by a water molecule. The coordination sphere of the zinc ion is approximately tetrahedral. The positively charged zinc ion polarizes the coordinated water molecule and nucleophilic attack by the negatively charged hydoxide portion on carbon dioxide (carbonic anhydride) proceeds rapidly. The catalytic cycle produces the bicarbonate ion and the hydrogen ion as the equilibrium

$$H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$
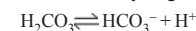
favours dissociation of carbonic acid at biological pH values.

Vitamin B12-dependent enzymes

Vitamin B12 catalyzes the tranfer of methyl (-CH$_3$) groups between two molecules, which involves the breaking of C-C bonds, a process that is energetically expensive in organic reactions. The metal ion lowers the activation energy for the process by forming a transient Co-CH$_3$ bond. The structure of the coenzyme was famously determined by Dorothy Hodgkin and co-workers, for which she received a Nobel prize.[12] It constists of a cobalt(II) ion coordinated by four nitrogen atons of a corrin rings and a fifth nitrogen atom from an imidazole group. In the resting state there is a Co-C ó bond with the 5' carbon atom of adenosine.[13] This is a naturally occurring organometallic compound, which explains its function in trans-methylation reactions, such as the reaction carried out by methionine synthase.

Nitrogenase (nitrogen fixation)

The fixation of atmospheric nitrogen is a very energy-intensive process, as it involves breaking the very stable triple bond between the nitrogen atoms. The enzyme nitrogenase is one of the few enzymes that can catalyze the process. The enzyme occurs in certain bacteria. There are three components to its action: a molybdenum atom at the active site, Iron-sulfur clusters which are involved in transporting the electrons needed to reduce the nitrogen and an abundant energy source. The energy is provided by a symbiotic relationship between the bacteria and a host plant, often a legume. The relationship is symbiotic because the plant supplies the energy by photosynthesis and benefits by obtaining the fixed nitrogen. The reaction may be written symbollically as

$$N_2 + 16\ \text{MgATP} + 8e^- \rightarrow 2NH_3 + 16\ \text{MgADP} + 16\ P_i + H_2$$

where P_i stands for inorganic phosphate. The precise structure of the active site has been difficult to determine. It appears to contain a MoFe_7S_8 cluster which is able to bind the dinitrogen molecule and, presumably, enable the reduction process to begin. The electrons are transported by the associated "P" cluster, which contains two cubical Fe_4S_4 clusters joined by sulphur bridges.

SuperOxide Dismutase

The superoxide ion, O_2^- is generated in biological systems by reduction of molecular oxygen. It has an unpaired electron, so it behaves as a free radical. It is a powerful oxidising agent. These properties render the superoxide ion very toxic and are deployed to advantage by phagocytes to kill invading micro organisms. Otherwise, the superoxide ion must be destroyed before it does unwanted damage in a cell. The superoxide dismutase enzymes perform this function very efficiently.

The formal oxidation state of the oxygen atoms is ½. In solutions at neutral pH, the superoxide ion disproportionates to molecular oxygen and hydrogen peroxide.

$$2\ O_2^- + 2\ H^+ \rightarrow O_2 + H_2O_2$$
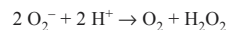
In biology this type of reaction is call a dismutation reaction. It involves both oxidation and reduction of superoxide ions. The superoxide dismutase group of enzymes, abbreviated as SOD, increase the rate of reaction to near the diffusion limited rate. The key to the action of these enzymes is a metal ion with variable oxidation state which can act as either an oxidizing agent or as a reducing agent.

$$\text{Oxidation: } M^{(n+1)+} + O_2^- \rightarrow M^{n+} + O_2$$

$$\text{Reduction: } M^{n+} + O_2^- + 2H^+ \rightarrow M^{(n+1)+} + H_2O_2.$$
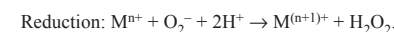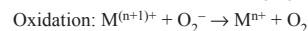
In human SOD the active metal is copper, as Cu^{2+} or Cu^+, coordinated tetrahedrally by four histidine residues. This enzyme also contains zinc ions. Other isozymes may contain iron, manganese or nickel. Ni-SOD is particularly interesting as it involves nickel(III), an unusual oxidation state for this elelement. The active site Ni geometry cycles from square planar Ni(II), with thiolate (Cys2 and Cys6) and backbone nitrogen (His1 and Cys2) ligands, to square pyramidal Ni(III) with an added axial His1 side chain ligand.

Chlorophyll

Chlorophyll plays a crucial role in photosynthesis. In contains a magnesium enclosed in a chlorin ring. However, the magnesium ion is not directly involved in the photosynthetic function and can be replaced by other divalent ions with a little loss of activity. Rather, the photon is absorbed by the chlorin ring, whose electronic structure is well-adapted for its purpose

Initially the absorption of a photon causes an electron to be excited into a singlet state of the Q band. This is the reason why chlorophyll is green. The excited state undergoes an intersystem crossing from the singlet state to a triplet state in which there are two electrons with parallel spin. This species is, effectively, a free radical, and is very reactive and allows an electron to be transferred to acceptors which are adjacent to the chlorophyll in the chloroplast. In the process chlorophyll is oxydised. Later in the phosynthetic cycle it reduced by accepting an electron and the final oxidation product is molecular oxygen whose atoms come from water molecules.

Signal-transduction metalloproteins

Calmodulin

Calmodulin is an example of a signal-transduction protein. It is a small protein which contains four EF-hand motifs, each of which can bind a Ca^{2+} ion.

In an EF-hand loop the calcium ion is coordinated in a pentagonal bipyramidal configuration. Six Glutamic acid and Aspartic acid residues involved in the binding are in positions 1, 3, 5, 7, 9 of the polypeptide chain. At position 12 there is a glutamate or aspartate ligand which behaves as a (bidentate ligand), providing two oxygen atoms. The ninth residue in the loop is necessarily glycine due to the conformational requirements of the backbone. The coordination sphere of the calcium ion contains only carboxylate oxygen atoms and no nitrogen atoms. This is consistent with the hard nature of the calcium ion.

The protein has two approximately symmetrical domains, separated by a flexible "hinge" region. Binding of calcium causes a conformational change to occur in the protein. Calmodulin participates in an intracellular signalling system by acting as a diffusible second messenger to the initial stimuli.

Transcription factors

Many transcription factors contain a structure known as a zinc finger, this is a structural module where a region of protein folds around a zinc ion. The zinc does not directly contact the DNA that these

proteins bind to, instead the cofactor is essential for the stability of the tightly-folded protein chain. In these proteins the zinc ion is usually coordinated by pairs of cysteine and histidine side chains.

Other metalloenzymes

Ion	Examples of enzymes containing this ion
Magnesium	Glucose 6-phosphatase Hexokinase DNA polymerase
Vanadium	vanabins
Manganese	arginase
Iron	Catalase Hydrogenase IRE-BP Aconitase
Nickel Hydrogenase	Urease
Copper Plastocyanin Laccase	Cytochrome oxidase
Zinc	Alcohol dehydrogenase carboxypeptidase Aminopeptidase
Molybdenum	Nitrate reductase
Selenium	Glutathione peroxidase
various	Metallothionein Phosphatase

Derived proteins

This class of proteins as the name depicts includes those substances formed from simple and conjugated proteins. It is the least well defined of the protein groups. Derived proteins are subdivided into primary derived proteins and secondary derived proteins.

Primary Derived proteins These proteins derivative are formed by process which cause only slight changes in the protein molecule and its properties. There is a little or no division of peptide bonds. The primary derived proteins are synonymous with denatured proteins.

Proteans The proteans are insoluble products formed by the incident action of water, very dilute acids and are particularly formed from globulins in being insoluble in dilute salt solutions. In general thsy have the physical characteristic of the naturally occurring glutelins.

Examples: Myosan from myosin; edystan from edestin and fibrin from fibrinogen

Metaproteins The Metaptroteins are formed by further action of acids and alkaliess upon proteins. They are generally soluble in very dilute acids and alkalies but sinsoluble in neutral solvents. Examples include acid and alkali metaproteins such as acid alkali albuminates.

Coagulated proteins The coagulated proteins are insoluble products formed by the action of heat or alcohol upon natural protein. Similar substances may also be formed by action of ultra violet light X-Ray, very high pressure, and mechanical shaking upon protein solutions at the iso – electric pH Examples are cooked egg albumin, cooked meal and other proteins and alcohols precipitated proteins.

Secondary Derived Protein These are also called secondary derived proteins, they are formed in the progressive hydrolytic division of the peptide union of protein molecules. They represent a gereat complexity of molecules of different sizes and amino acid composition. They are roughly grouped into proteoses, peptones and peptides, according to relative average molecular complexity. Each group is composed of many different substances.

Thrombin (activated **Factor II [IIa]**) is a coagulation protein that has many effects in the coagulation cascade. It is a serine protease (EC 3.4.21.5) that converts soluble fibrinogen into insoluble strands of fibrin, as well as catalyzing many other coagulation-related reactions.

Genetics

The prothrombin gene is located on the eleventh chromosome (11p11-q12). The molecular weight of prothrombin is approximately 72000 $gmol^{-1}$; in contrast, the molecular weight of thrombin is 36000 $gmol^{-1}$. Once activated, the catalytic domain of prothrombin is released from prothrombin fragment 1.2. There are an estimated 30 people in the world that have been diagnosed with the congenital form of Factor II deficiency (Degen, 1995)[3], which should not be confused with a mutation of prothrombin. The prothrombin gene mutation is called **Factor II mutation**. Factor II mutation is congenital.[4] The Factor II mutated gene is not usually accompanied by other factor mutations (i.e. the most common is Factor V Leiden). The gene may be inherited **heterozygous**, or much more rarely, **homozygous**, and is not related to gender or blood type. Homozygous mutations increase the risk of thrombosis more than heterozygous mutations, but the relative increased risk is not well documented. Other potential risks for thrombosis, such as **oral contraceptives** may be additive. The previously reported relationship of inflammatory bowel disease (i.e. Crohn's disease or Ulcerative Colitis) and prothrombin mutation or Factor V Leiden mutation have been contradicted by research.

Achoring of bovine prothrombin to the membrane through its Gla domain

Physiology and Generation

Thrombin is produced by the enzymatic cleavage of two sites on prothrombin by activated Factor X (Xa). The activity of factor Xa is greatly enhanced by binding to activated Factor V (Va), termed the
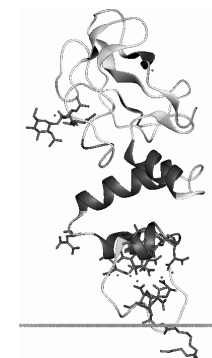
prothrombinase complex. Prothrombin is produced in the liver and is post-translationally modified in a vitamin K-dependent reaction that converts ten glutamic acids on prothrombin to gamma-carboxyglutamic acid (Gla). In the presence of calcium, the Gla residues promote the binding of thrombin to phospholipid bilayers (see the picture). Deficiency of vitamin K or administration of the anticoagulant warfarin inhibits the production of gamma-carboxyglutamic acid residues, slowing the activation of the coagulation cascade.

In human beings the level of prothrombin in the blood stream increases after birth and typically peaks on the 8th day after which the prothrombin level lowers to normal levels.[1]

Action

Thrombin converts fibrinogen to an active form that assembles into fibrin. Thrombin also activates factor XI, factor V, and factor VIII. This positive feedback accelerates the production of thrombin.

Factor XIII is also activated by thrombin. Factor XIIIa is a transglutaminase that catalyzes the formation of covalent bonds between lysine and glutamine residues in fibrin. The covalent bonds increase the stability of the fibrin clot.

Platelets

In addition to its activity in the coagulation cascades, thrombin also promotes platelet activation, via activation of protease-activated receptors on the platelet.

Negative feedback

Thrombin bound to thrombomodulin activates protein C, an inhibitor of the coagulation cascade. The activation of protein C is greatly enhanced following the binding of thrombin to thrombomodulin, an integral membrane protein expressed by endothelial cells. Activated protein C inactivates factors Va and VIIIa. Binding of activated protein C to protein S leads to a modest increase in its activity.

Role in disease

Activation of prothrombin is crucial in physiological and pathological coagulation. Various rare diseases involving prothrombin have been described (e.g., hypoprothrombinemia). Anti-thrombin antibodies in autoimmune disease may be a factor in the formation of the lupus anticoagulant also known as (antiphospholipid syndrome). Hyperprothrombinemia can be caused by a mutation at 20210a.

Thrombin, a potent vasoconstrictor and mitogen, is implicated as a major factor in vasospasm following subarachnoid hemorrhage. Blood from a ruptured cerebral aneurysm clots around a cerebral artery, releasing thrombin. This can induce an acute and prolonged narrowing of the blood vessel, potentially resulting in cerebral ischemia and infarction (stroke).

Biotechnology

Due to its high proteolytic specificity, thrombin is a valuable biochemical tool. The thrombin cleavage site (Leu-Val-Pro-Arg-Gly-Ser) is commonly included in linker regions of recombinant fusion protein constructs. Following purification of the fusion protein, thrombin can be used to selectively cleave

between the Arginine and Glycine residues of the cleavage site, effectively removing the purification tag from the protein of interest with a high degree of specificity.

Pharmacology

Prothrombin complex concentrate and fresh frozen plasma are prothrombin-rich coagulation factor preparations that can be used to correct deficiencies (usually due to medication) of prothrombin. Indications include intractable bleeding due to warfarin.

Manipulation of prothrombin is central to the mode of action of most anticoagulants. Warfarin and related drugs inhibit vitamin K-dependent carboxylation of several coagulation factors, including prothrombin. Heparin increases the affinity of antithrombin to thrombin (as well as factor Xa). The direct thrombin inhibitors, a newer class of medication, directly inhibit thrombin by binding to its active

Proteoses and Albuminoses

Proteoses are hydrolytic products of proteins which are soluble in water, are non coagulated by heat and are precipitated from their solutions by saturation with ammonium sulphate.

*Peptide*s (from the Greek *πεπτίδια*, "small digestibles") are short polymers `formed from the linking, in a defined order, of` á-amino acids. The link between one amino acid residue and the next is known as an amide bond or a peptide bond.

Proteins are **polypeptide** molecules (or consist of multiple polypeptide subunits). The distinction is that peptides are short and polypeptides/proteins are long. There are several different conventions to determine these, all of which have caveats and nuances.

Conventions

One convention is that those peptide chains that are short enough to be made synthetically from the constituent amino acids are called peptides rather than proteins. However, with the advent of better synthetic techniques, peptides as long as hundreds of amino acids can be made, including full proteins like ubiquitin. Native chemical ligation has given access to even longer proteins, so this convention seems to be outdated.

Another convention places an informal dividing line at approximately 50 amino acids in length (some people claim shorter lengths). However, this definition is somewhat arbitrary. Long peptides, such as the amyloid beta peptide linked to Alzheimer's disease, can be considered proteins; and small proteins, such as insulin, can be considered peptides.

Peptide classes

Here are the major classes of peptides, according to how they are produced:

Ribosomal peptides

Are synthesized by translation of mRNA. They are often subjected to proteolysis to generate the mature form. These function, typically in higher organisms, as hormones and signaling molecules. Some organisms produce peptides as antibiotics, such as microcins. Since they are translated, the

amino acid residues involved are restricted to those utilized by the ribosome. However, these peptides frequently have posttranslational modifications, such as phosphorylation, hydroxylation, sulfonation, palmitylation, glycosylation and disulfide formation. In general, they are linear, although lariat structures have been observed. More exotic manipulations do occur, such as racemization of L-amino acids to D-amino acids in platypus venom.

Nonribosomal peptides

These peptides are assembled by enzymes that are specific to each peptide, rather than by the ribosome. The most common non-ribosomal peptide is glutathione, which is a component of the antioxidant defenses of most aerobic organisms.[4] Other nonribosomal peptides are most common in unicellular organisms, plants, and fungi and are synthesized by modular enzyme complexes called *nonribosomal peptide synthetases*. These complexes are often laid out in a similar fashion, and they can contain many different modules to perform a diverse set of chemical manipulations on the developing product. These peptides are often cyclic and can have highly-complex cyclic structures, although linear nonribosomal peptides are also common. Since the system is closely related to the machinery for building fatty acids and polyketides, hybrid compounds are often found. Oxazoles, thiazoles often indicate that the compound was synthesized in this fashion.

Peptones

Are derived from animal milk or meat digested by proteolytic digestion. In addition to containing small peptides, the resulting spray-dried material includes fats, metals, salts, vitamins and many other biological compounds. Peptone is used in nutrient media for growing bacteria and fungi.

Peptide Fragments

Refer to fragments of proteins that are used to identify or quantify the source protein. Often these are the products of enzymatic degradation performed in the laboratory on a controlled sample, but can also be forensic or paleontological samples which have been degraded by natural effects.

Peptides in molecular biology

Peptides have received prominence in molecular biology in recent times for several reasons. The first and most important is that peptides allow the creation of *peptide antibodies* in animals without the need to purify the protein of interest.[12] This involves synthesizing antigenic peptides of sections of the protein of interest. These will then be used to make antibodies in a rabbit or mouse against the protein.

Another reason is that peptides have become instrumental in mass spectrometry, allowing the identification of proteins of interest based on peptide masses and sequence. In this case the peptides are most often generated by in-gel digestion after electrophoretic separation of the proteins.

Peptides have recently been used in the study of protein structure and function. For example, synthetic peptides can be used as probes to see where protein-peptide interactions occur.

Inhibitory peptides are also used in clinical research to examine the effects of peptides on the inhibition of cancer proteins and other diseases.

Well-known peptide families in humans

The peptide families in this section are all ribosomal peptides, usually with hormonal activity. All of these peptides are synthesized by cells as longer "propeptides" or "proproteins" and truncated prior to exiting the cell. They are released into the bloodstream where they perform their signalling functions.

The Tachykinin peptides
- Substance P
- Kassinin
- Neurokinin A
- Eledoisin
- Neurokinin B

Vasoactive intestinal peptides
- VIP (*Vasoactive Intestinal Peptide*; PHM27)
- PACAP *Pituitary Adenylate Cyclase Activating Peptide*
- Peptide PHI 27 (*Peptide Histidine Isoleucine 27*)
- GHRH 1-24 (*Growth Hormone Releasing Hormone 1-24*)
- Glucagon
- Secretin

Pancreatic polypeptide-related peptides
- NPY
- PYY (*Peptide YY)*
- APP (*Avian Pancreatic Polypeptide)*
- PPY *Pancreatic PolYpeptide*

Opioid peptides
- Proopiomelanocortin (POMC) peptides
- Enkephalin pentapeptides
- Prodynorphin peptides

Calcitonin peptides
- Calcitonin
- Amylin
- AGG01

Other peptides
- B-type Natriuretic Peptide (BNP) - produced in myocardium & useful in medical diagnosis

Notes on terminology
- A *polypeptide* is a single linear chain of amino acids.

• A *protein* is one or more polypeptides more than about 50 amino acids long.
• An *oligopeptide* or (simply) a *peptide* is a polypeptide less than 30-50 amino acids long.
• A *dipeptide* has two amino acids.
• A *tripeptide* has three amino acids.
• A *tetrapeptide* has four amino acids.
• A *pentapeptide* has five amino acids.
• An *octapeptide* has eight amino acids (e.g., angiotensin II).
• A *nonapeptide* has nine amino acids (e.g., oxytocin).
• A *decapeptide* has ten amino acids (e.g., gonadotropin-releasing hormone & angotensin I).
• A *neuropeptide* is a peptide that is active in association with neural tissue.
• A *peptide hormone* is a peptide that acts as a hormone.

SUB-SECTION 2.9B—STRUCTURE OF PROTEIN

Protein Structure

The primary structure of a segment of a polypeptide chain or of a protein is the amino-acid sequence of the polypeptide chain(s), without regard to spatial arrangement (apart from configuration at the alpha-carbon atom). This definition does not include the positions of disulphide bonds, and is, therefore, not identical with "covalent structure" (IUPAC-IUB, 1970). The commonly occurring amino acids are of 20 different kinds which contain the same dipolar ion group $H_3N^+.CH.COO^-$. They all have in common a central carbon atom to which are attached a hydrogen atom, an amino group (NH_2) and a carboxyl group (COOH). The central carbon atom is called the C_{alpha}-atom and is a chiral centre. All amino acids found in proteins encoded by the genome have the L-configuration at this chiral centre.

This configuration can be remembered as the **CORN** law. Imagine looking along the $H-C_{alpha}$ bond with the H atom closest to you.

When read clockwise, the groups attached to the C_{alpha} spell the word CORN (Richardson, 1981). There are 20 side chains found in proteins encoded by the genetic machinery of the cell. The side chains confer important properties on a protein such as the ability to bind ligands and catalyse biochemical reactions. They also direct the folding of the nascent polypeptide and
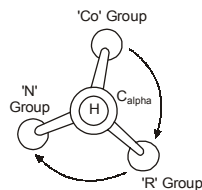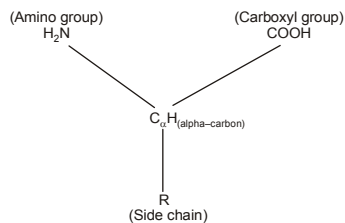
Fig. 2.40 : Showing the C_{alpha} bond

stabilise its final conformation. In molecular graphics, atoms can be represented in different ways. For expedience, molecules are often displayed only as lines or vectors between the atoms bonded together covalently. An elegant representation is the ball-and-stick type in which atoms are drawn as coloured spheres and their bonds as rod-like connections. Another useful display is the space-filling representation in which a surface is drawn around the atoms to indicate their van der Waals radii. This surface can be drawn as a series of dots or as a solid entity (Lesk, 1991). Amino acids in proteins(or polypeptides) are joined together by peptide bonds. The sequence of R-groups along the chain is called the **primary structure**.
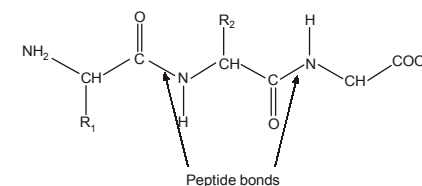
Peptide bonds

The Peptide bond

Pauling *et al.* (1951) analysed the geometry and dimensions of the peptide bonds in the crystal structures of molecules containing either one or a few peptide bonds. Their results are summarised in the diagram below where the consensus bond lengths are shown in Angstrom units. Bond angles in degrees are also shown for the peptide N and C atoms. It should be noted that the C-N bond length of the peptide is 10% shorter than that found in usual C-N amine bonds (Schulz and Schirmer, 1990; Creighton, 1993).
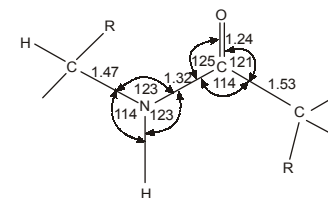
This is because the peptide bond has some double bond character (40%) due to resonance which occurs with amides. The two canonical structures are:
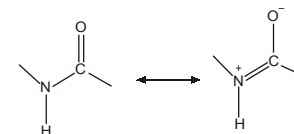
As a consequence of this resonance all peptide bonds in protein structures are found to be almost planar, i.e. atoms $C_{alpha}(i)$, $C(i)$, $O(i)$, $N(i+1)$ and $C_{alpha}(i+1)$ are approximately co-planar. This rigidity of the peptide bond reduces the degrees of freedom of the polypeptide during folding. The peptide bond nearly always has the *trans* configuration since it is more favourable than *cis*, which is sometimes found to occur with proline (Pro) residues (Schulz and Schirmer, 1990).

trans   cis   Proline
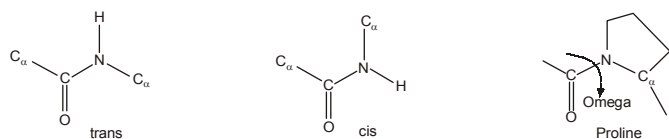
As can be seen from the previous page, steric hindrance between the functional groups attached to the C_{alpha} atoms will be greater in the *cis* configuration. However, for proline residues, the cyclic nature of the side chain means that both *cis* and *trans* configurations have more equivalent energies. Thus proline is found in the *cis* configuration more frequently than the other amino acids.

Properties of amino acids

The sequence and properties of side chains determine all that is unique about a particular protein, including its biological function and its specific three-dimensional structure.

Histidine (His) is the only side chain that titrates near physiological pH, making it especially useful for enzymatic reactions.
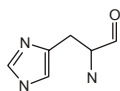
Lysine (Lys) and arginine (Arg) are normally positively charged and aspartate (Asp) and glutamate (Glu) are negatively charged. These charges are very seldom buried in protein interiors except when they are serving some special purpose, as in the activity and activation of chymotrypsin (Blow et al., 1969; Wright, 1973).

Lys   Arg

Asp   Glu
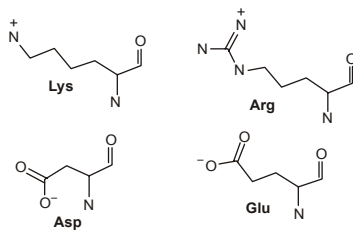
Asparagine (Asn) and glutamine (Gln) have interesting hydrogen-bonding properties, since they resemble the backbone peptides. The hydrophobic residues provide a very strong driving force for folding, through the indirect effect of their ceasing to disrupt the water structure once they are buried (Kauzmann, 1959). They also, however, affect the structure in a highly specific manner because their varied sizes and shapes fit together in very efficient packing (Lee and Richards, 1971).

Asn   **Gln**
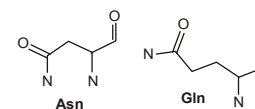
Proline has stronger stereochemical constraints than any other residue, with only one instead of two variable backbone angles, and it lacks the normal backbone NH for hydrogen bonding. It is both disruptive to regular secondary structure and also good at forming turns in the polypeptide chain, so that in spite of its hydrophobicity it is usually found at the edge of the protein (Richardson, 1981).

Glycine (Gly) has three different unique capabilities. As the smallest side group (only a hydrogen), it is often found where main chains approach each other very closely. In addition Gly can assume conformations normally restricted by close contacts of the beta-carbon and finally it is more flexible than other residues, thus contributing to parts of the backbone that need to move or hinge (Richardson, 1981).

Serine (Ser) and threonine (Thr) carry aliphatic hydroxyl groups capable of forming hydrogen bonds with suitable donor or acceptor groups, such as the imino nitrogen or the carbonyl oxygen of the main polypeptide chain. Serine reacts with phosphate by an ester bond, forms part of the catalytic site of many hydrolytic enzymes (Dickerson and Geis, 1969) and contributes to the lining of ion channels. Serine, threonine, and asparagine are also the binding sites of carbohydrates that are attached to the surface of many proteins. Carbohydrates bound to serine and threonine form O-glycosidic bonds and those linked to asparagine form N-glycosidic bonds (Perutz, 1992).

Ser   Thr
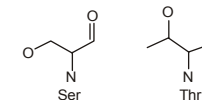
Cysteine (Cys) carries the highly reactive sulphydryl group. This does not ionise at physiological pH nor form hydrogen bonds of significant strength, but two cysteines placed some distance apart

along a polypeptide chain, or forming part of different chains, can be joined by oxidation to form the disulphide bridge of cystine which plays an important part in stabilizing protein structures. Disulphide bonds increase the conformational stability mainly by constraining the unfolded conformations of the protein and thereby decreasing their conformational entropy (Pace, 1990). Cysteines also bind zinc, copper, and iron ions. The sulphur atom in methionine is unreactive and generally serves no function other than imposing a special configuration on the aliphatic sidechain, but in cytochrome c it forms the link between the protein and the heme iron (Olson, 1992).

Protein structure determination

In terms of the accuracy of protein structure determinations, all of the bond lengths are invariant. Bond angles are also essentially invariant, except perhaps for T, the backbone N-C_{alpha}-C angle. The alpha-carbon is tetrahedral, which would give 110°, but there are indications from accurately refined protein structures (Deisenhofer and Steigemann, 1975; Watenpaugh *et al.*, 1979) that can sometimes stretch to larger values in order to accommodate other strains in the structure. The dihedral angle at the peptide is very close to 180° (producing a trans, planar peptide with the neighbouring alpha-carbons and the N, H, C, and O between them all lying in one plane). The remaining dihedral angles are the source of essentially all the interesting variability in protein conformation. The backbone dihedral angles are and in sequence order on either side of the alpha-carbon, so that is the dihedral angle around the N-C_{alpha} bond and around the C_{alpha}-C bond. The side chain dihedral angles are $_1$, $_2$, etc. An extremely useful device for studying protein conformation is the Ramachandran plot (Ramachandran *et al.*, 1963) which plots and . The values of and that are possible are constrained geometrically due to steric clashes between non-neighboring atoms. The permitted values of and are indicated on a two-dimensional map of the - plane (Branden and Tooze, 1991).

Fig. 2.41 : Showing the Alpha helix

Secondary Structure

The secondary structure of a segment of polypeptide chain is the local spatial arrangement of its main-chain atoms without regard to the conformation of its side chains or to its relationship with other segments (IUPAC-IUB, 1970). There are three common secondary structures in proteins, namely **alpha helices, beta sheets** and **turns**. That which cannot be classified as one of the standard three classes is usually grouped into a category called "other" or "random coil". This last designation is

unfortunate as no portion of protein three dimensional structure is truly random and it is not a coil either. Regular secondary structure conformations in segments of a polypeptide chain occur when all the ϕ bond angles in that polypeptide segment are equal to each other, and all the ψ bond angles are equal. The rotational angles for ϕ and ψ bonds for common regular secondary structures are shown in the table below.

Parameters of regular secondary structures. n is the number of residues per helical turn, p is the helical pitch, and A is the atoms in H-bonded loop

Structure	ϕ	ψ	n	p(Å)	A	H-bond (CO, HN)
Right-handed alpha helix[3.613helix]	-57	-47	3.6	5.4	13	i,i+2
310-helix	-74	-4	3.0	6.0	10	i,i+3
pi-helix	-57	-70	4.4	5.0	16	i,i+4
Parallel beta strand	-119	113	2.0	6.4		
Antiparallel beta strand	-139	135	2.0	6.8		

The alpha-helix and beta-structure conformations for polypeptide chains are generally the most thermodynamically stable of the regular secondary structures. However, particular amino acid sequences of a primary structure in a protein may support regular conformations of the polypeptide chain other than alpha-helical or beta-structure. Thus, whereas alpha-helical or beta-structure are found most commonly, the actual conformation is dependent on the particular physical properties generated by the sequence present in the polypeptide chain and the solution conditions in which the protein is dissolved. In addition, in most proteins there are significant regions of unordered structure in which the ϕ and ψ angles are not equal. A large proportion of (85%) of helices are distorted in some way i.e. radius of curvature greater than 90Å and deviation of axis from straight line is equal to or greater than 0.25Å. These may be due to a number of reasons:

- CO groups form hydrogen bonds with NH groups 3 residues along the chain forming a 3_{10} helix. A substantial amount of all 3_{10} helices occur at the ends of alpha-helices. They are called 3_{10} because there are 3 residues per turn and 10 atoms enclosed in a ring formed by each hydrogen bond. Dipoles are not aligned as in a normal right-handed alpha-helix.

- Packing of buried helices against other secondary structural elements in the core of a protein can lead to distortions since the side chains are on the surface of helices.

- Proline residues induce distortions of around 20deg in the direction of a helix. This is because proline cannot form a regular alpha-helix due to steric hindrance arising from its cyclic sidechain which blocks the main chain NH group. Proline causes 2 hydrogen bonds in the helix to be broken. Helices containing proline are usually long because shorter helices would be destabilized.

- Exposed helices are often bent away from the solvent. This is because the exposed C=O groups tend to point towards solvent to maximize their hydrogen bonding capacity i.e tend to form hydrogen-bonds to solvent as well as N-H groups. This gives rise to a bend in the helix axis.

- The pi helix is an extremely rare secondary structural element in proteins. Like the 3_{10} helix, one turn of pi helix is sometimes found at the ends of regular alpha helices. The infrequency of this particular form of secondary structure stems from the following properties: (i) the phi and psi angles of the pure pi helix (-57.1, -69.7) lie at the very edge of an allowed minimum energy region of the Ramachandran map, (ii) the pi helix requires that the angle tau (N-C_{alpha}-C') be larger (114.9) than the standard tetrahedral angle of 109.5degrees, (iii) the large radius of the pi helix means the polypeptide backbone is no longer in van der Waals contact across the helical axis forming an axial hole too small for solvent water to fill, and (iv) side chains are more staggered than the ideal 3_{10} helix but not as well as the alpha helix (Low and Grenville-Wells, 1953; Schulz and Schirmer, 1990).
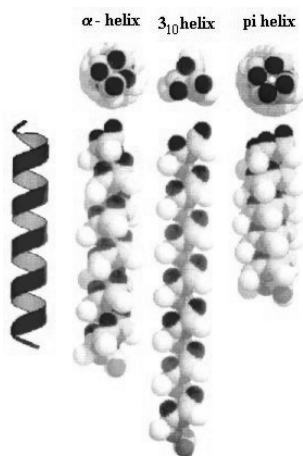
Fig. 2.42 : Showing the Alpha helix Beta Sheets and pi helix

Besides the alpha-helix, beta-sheets are another major structural element in globular proteins containing 20-28% of all residues (Kabsch and Sander, 1983; Creighton, 1993). The basic unit of a beta-sheet is a beta strand (which can be thought of as a helix with n=2 residues/turn) with approximate backbone dihedral angles phi = -120 and psi = +120 producing a translation of 3.2 to 3.4 Å / residue for residues in antiparallel and parallel strands, respectively. The beta strand is then like the alpha-helix, a repeating secondary structure. However, since there are no intra-segment hydrogen bonds and van der Waals interactions between atoms of neighbouring residues are not significant due to the extended nature of the chain, this extended conformation is only stable as part of a beta-sheet where contributions from hydrogen bonds and van der Waals interactions between aligned strands exert a stabilizing influence.

The beta-sheet is sometimes called the beta pleated sheet since sequential neighbouring C_{alpha} atoms are alternately above and below the plane of the sheet giving a pleated appearance. beta-sheets are found in two forms designated as "Antiparallel" or "Parallel" based on the relative directions of two interacting beta strand (as shown below).
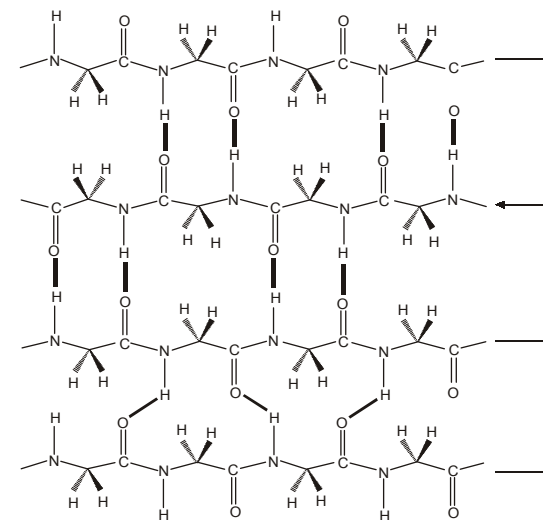
Hydrogen bond patterns in beta-sheets. A four-stranded beta-sheet is drawn schematically which contains three **antiparallel** and one **parallel** strand. Hydrogen bonds are indicated with red lines (antiparallel strands) and blue lines (parallel strands) connecting the hydrogen and receptor oxygen.

Like alpha-helices, beta-sheets have the potential for amphiphilicity with one face polar and the other apolar. However, unlike alpha-helices which are composed of residues from a continuous polypeptide segment (i.e., hydrogen bonds between CO of residue I and NH of residue I+3), beta-sheets are formed from strands that are very often from distant portions of the polypeptide sequence. Hydrogen bonds in beta-sheets are on average 0.1 Å shorter than those found in alpha-helices (Baker

and Hubbard, 1984). The classical beta-sheets originally proposed are planar but most sheets observed in globular proteins are twisted (0 to 30 degrees per residue).

Antiparallel beta-sheets are more often twisted than parallel sheets. Another irregularity found in antiparallel beta-sheets is the hydrogen-bonding of two residues from one strand with one residue from another called a beta bulge (as shown above). Bulges are most often found in antiparallel sheets with ~5% of bulges occurring in parallel strands (Richardson, 1981).

Turns are the third of the three "classical" secondary structures that serve to reverse the direction of the polypeptide chain. They are located primarily on the protein surface and accordingly contain polar and charged residues. Antibody recognition, phosphorylation, glycosylation, and hydroxylation sites are found frequently at or adjacent to turns. Turns were first recognised from a theoretical conformational analysis by Venkatachalam (1968). He considered what conformations were available to a system of three linked peptide units (or four successive residues) that could be stabilised by a backbone hydrogen bond between the CO of residue n and the NH of residue n+3. He found three general types, one of which (type III) actually has repeating Turns are the third of the three "classical" secondary structures that serve to reverse the direction of the polypeptide chain. They are located primarily on the protein surface and accordingly contain polar and charged residues. Antibody recognition, phosphorylation, glycosylation, and hydroxylation sites are found frequently at or adjacent to turns. Turns were first recognised from a theoretical conformational analysis by Venkatachalam (1968). He considered what conformations were available to a system of three linked peptide units (or four successive residues) that could be stabilised by a backbone hydrogen bond between the CO of residue n and the NH of residue n+3. He found three general types, one of which (type III) actually has repeating , values of -60deg, -30deg and is identical with the 3_{10}-helix. The three types each contain a hydrogen bond between the carbonyl oxygen of residue i and the amide nitrogen of i+3. These three types of turns are designated I, II, and III. Many have speculated on the role of this type of secondary structure in globular proteins. Turns may be viewed as a weak link in the polypeptide chain, allowing the other secondary structures (helix and sheet) to determine the conformational outcome. In contrast (based on the recent experimental finding of "turn-like" structures in short peptides in aqueous solutions (Dyson et al., 1988), turns are considered to be structure-nucleating segments, formed early in the folding process. Type I turns occur 2-3 times more frequently than type II. There are position dependent amino acid preferences for residues in turn conformations. Type I can tolerate all residues in position i to i+3 with the exception of Pro at position i+2. Proline is favoured at position i+1 and Gly is favoured at i+3 in type I and type II turns. The polar sidechains of Asn, Asp, Ser, and Cys often populate position i where they can hydrogen bond to the backbone NH of residue i+2.
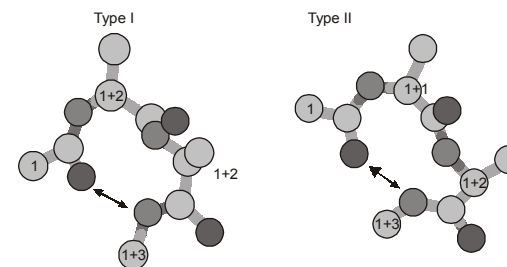
Supersecondary Structure

Rao and Rossmann (1973) observed that there were structural components comprising a few alpha-helices or beta-strands which were frequently repeated within structures, called "supersecondary structures" (being intermediate to secondary and tertiary structure) and suggested that these structures might be due to convergence. A variety of recurring structures were subsequently recognised such as the "Greek key" (Richardson, 1977). Some of these structural motifs can be associated with a particular function whilst others have no specific biological function alone but are part of larger structural and functional assemblies. The first comprehensive attempt to classify proteins on the basis of structural comparisons was due to Levitt and Chothia (1976) who used four major classifications:

- proteins containing mostly alpha-helix;
- proteins containing mostly beta-sheet;
- proteins that contain alpha-helices and beta-strands in an irregular sequence, and
- alpha/beta proteins with alternate segments of alpha-helices and beta-strands.

The simplest motif with a specific function consists of two alpha-helices joined by a loop region. Two such motifs are (i) a motif specific for DNA binding and (ii) a motif specific for calcium binding and is present in parvalbumin, calmodulin, troponin-C, and other proteins that bind calcium and thereby regulate cellular activities.

Tertiary structure

The tertiary structure of a protein molecule, or of a subunit of a protein molecule, is the arrangement of all its atoms in space, without regard to its relationship with neighbouring molecules or subunits (IUPAC-IUB, 1970). As an example of a protein's tertiary structure, the structure of lysozyme is shown below.

Quaternary structure

The quaternary structure of a protein molecule is the arrangement of its subunits in space and the ensemble of its intersubunit contacts and interactions, without regard to the internal geometry of the subunits (IUPAC-IUB, 1970). The subunits in a quaternary structure must be in noncovalent association.

Haemoglobin contains four polypeptide chains (alpha$_2$b$_2$) held together noncovalently in a specific conformation as required for its function.

Protein Stability

To be biologically active, proteins must adopt specific folded three-dimensional, tertiary structures. Yet the genetic information for the protein specifies only the primary structure, that is the linear sequence of amino acids in the polypeptide backbone. Many purified proteins can spontaneously refold *in vitro* after being completely unfolded, so the three-dimensional structure must be determined by the primary structure (Anfinsen, 1973). Different conformations of a protein differ only in the angle of rotation about the bonds of the backbone and amino acid side-chains. It may, therefore, appear surprising that a protein folds into a single unique conformation from all the possible rotational conformations available around single bonds in the primary structure of a protein. The question arises as to when a protein folds up to its native conformation, does this structure represent a local or a global energy minimum? When a protein folds it samples a number of conformations. Does the structure which results from the folding depend on its stability or on the energy barriers encountered by the polypeptide? The polypeptide whilst folding may become trapped in the local energy well and cannot fold to the global energy minimum (kinetic hypothesis of protein folding, Wetlaufer, 1973; Wetlaufer and Ristow, 1973).

The native structure of the protein may correspond to a metastable state with a very long lifetime. If proteins are only metastable, their structures must be grossly different from the most stable one. The polypeptide may adopt a structure corresponding to the global minimum. This means the final structure does not depend on the size of the energy barriers (thermodynamic hypothesis of protein folding, Epstein et al., 1963). Anfinson's work on ribonuclease provided some evidence for the thermodynamic hypothesis (Haber and Anfinsen, 1962). The initial stages of folding were considered to be nearly random. However, if the rest of the folding pathway was a random search, it would not be feasible for any protein to try out all of its conformations on a practical time scale. For example, if each residue of a 100 residue polypeptide had only three conformations, the total number of conformations would be $3^{100} = 5 \times 10^{47}$. Since conformational changes occur on the timescale of 10^{-13} seconds, the time required by the 100 residue protein to search all conformations would be $5 \times 10^{47} \times 10^{-13} \approx 10^{37}$ years. Nevertheless, proteins are observed to fold in 10^{-1} - 10^3 seconds both *in-vivo* and *in-vitro* (Creighton, 1993). The conclusion, therefore, was that proteins do not fold by sampling all possible conformations randomly until the one with the lowest free energy is encountered.

This led to the idea of a biased random search resulting in faster folding since a proportion of the conformations would be sterically disallowed. A framework model proposed (Baldwin, 1989; Kim and Baldwin, 1990) that the folded structure was formed by packing together pre-existing individual elements of secondary structure which had significant stability in the unfolded protein. Another mechanism postulated the unfolded polypeptide chain to undergo rapid hydrophobic collapse under refolding conditions (Dill, 1985), perhaps to something approximating the molten globule state. Simply constraining the polypeptide chain to be compact might greatly increase the probability of the final folded conformation being encountered (Gregoret and Cohen, 1991). It became evident through some experiments that the equilibrium of unfolding of proteins does not always follow a simple two-state model in which only the native and fully unfolded states are significantly populated (Wong and Tanford, 1973). An intermediate

compact structure known as the molten globule which is different from the native structure and whose formation is determined mainly by non-specific interactions of amino acid residues with their environment was presented. Specific interactions could direct the folding pathway by stabilizing folded conformations. The best studied example is bovine pancreatic trypsin inhibitor (Creighton, 1978). For this protein it was shown that formation of a disulphide bridge stabilizes secondary structure elements, and the protein refolds by a specific pathway of disulphide bond formation and rearrangement (Directed folding model). Since noncovalent forces act on the primary structure to cause a protein to fold into a unique conformational structure and then stabilize the native structure against denaturation processes, it is of importance to understand the properties of these forces.

Non-covalent Forces

Non-covalent forces are weak forces of bonding strength of 1-7kcal mol^{-1} (4-29kJ mol^{-1}) as compared to the strength of covalent bonds which have a bonding strength of at least 50 kcal mol^{-1}. The non-covalent bonding forces are just higher than the average kinetic energy of molecules at 37°C (0.6 kcal mol^{-1}). Apart from their involvement in the stabilization of molecules, they contribute to the ability of molecules to undergo changes in conformation and interact with each other. Since a major part of this project (Friedli, 1996) involves a study of interactions, an explanation of the forces involved is crucial for an understanding of the mechanisms at the molecular level. The major forces and interactions are:

- Ion - Ion forces, for example those involving; NaCl or MgSO$_4$;
- Ion - Dipole forces, for example those between NaCl and H$_2$O;
- van der Waals interactions including;
    1. Dipole - Dipole, such as the hydrogen bond;
    2. Dipole - Induced Dipole, for example: H$_2$O + I$_2$ and
    3. Dispersion forces including Induced Dipole - Induced Dipole, for example between two aliphatic hydrogens.
- Electrostatic interaction or salt bridge between charged residues and
- Hydrophobic forces between nonpolar residues.

Ion - dipole attractions depend on:

- the distance between the ion and the dipole thus the closer the ion and dipole are, the stronger the attraction;
- the charge on the ion - the greater the ion charge, the stronger the attraction, and
- the magnitude of the dipole - the greater the magnitude of the dipole, the stronger the attraction.

Water is an excellent example of a polar molecule, a molecule with positive and negative electrical poles. Hydrogen bonding is a special form of dipole - dipole attraction. When H is attached to a very electronegative atom X, the interaction between other molecules and the H - X bond dipole is significantly greater than expected for ordinary dipole - dipole attraction. This interaction is called hydrogen bonding because it occurs only when H is part of one or both of the interacting dipoles. The electronegativities of N (3.0), O (3.5), and F (4.0) are among the highest of all of the elements, while that of H (2.1) is considerably less (Bohinski, 1987).

A polar water molecule can induce a dipole in non-polar O_2. For the dipole to be induced depends on the atom's or molecule's polarizability. As the molecular mass of a molecule increases, either there is an increase in the number of valence electrons or the valence electrons are less tightly held. Therefore, the ease of polarization of the valence electron cloud generally increases with mass. Since a dipole is more readily induced as the polarizability increases, the strengths of dipole - induced dipole interactions generally increase with mass. Also, since the solubility of substances such as CO_2 or O_2 depends on the strength of the dipole - induced dipole interaction, the solubility of non-polar substances in polar solvents generally increases with mass (Kotz and Purcell, 1991).

The weakest of all intermolecular forces is between two induced dipoles. Such forces are often called London forces or dispersion forces. When atoms or molecules approach each other, each experiences the electric field provided by the other. This electric field distorts the charge distribution (Webster, 1990). The attraction of a molecule to its own distorted charge distribution creates an attractive force between the molecules even when they are a long distance apart. This force acts to bind the approaching atoms or molecules. Fritz London (1930) gave an approximate result for the attractive energy resulting from the interaction between two induced dipoles. The interaction energy, E^{London}/J, which is attractive for all inter-atomic, or inter-molecular distances, R/m, is expressed by the formula:

$$E^{london} = -3/2 \left\{ I_A I_B l (I_A + I_B) \right\} \left[(\alpha_A \alpha_B)/(4\pi\varepsilon_0)^2 R^6 \right]$$

where I_A/J and I_B/J are the first ionization energies for A and B, and $(\alpha_A/4\pi\varepsilon_0)/m^3$ and $(a_B/4\pi\varepsilon_0)/m^3$ are their polarizability volumes. E^{London} is called the London dispersion energy. The London energy will be larger in magnitude as R decreases but the key feature is that it creates an attraction even at large R values. For this reason it is usually classified as a long-range interaction. The London dispersion force, however, is only one component of van der Waals forces. A second component, the John Lennard-Jones potential (LJ potential), U(R)/J, is defined by:

$$U(R) = 4\varepsilon \left[(\sigma/R)^{12} - (\sigma/R)^6 \right]$$

The parameter ε represents the binding energy of a van der Waals molecule at its equilibrium geometry. The parameter σ is the value of R, the inter-atomic or inter-molecular distance, when U(R) = 0, other than at R = ∞. From equation (2), the term R^{-6}, approximates the London dispersion energy which is attractive and this is counteracted by a repulsive term having an R^{-12} dependence. An alternative expression which approximates the energy, $U(R)/kJmol^{-1}$, of van der Waals molecule is :

$$U(R) = b \exp(-aR) - d/R^6$$

The van der Waals force is of great importance in biopolymer structure. This force has an attractive term dependent on the 6th. power of the distance between two interacting atoms and a repulsive term dependent on the 12th. power of the distance between them. The attractive component is due to the induction of complementary partial charges or dipoles in the electron density of adjacent atoms when the electron orbitals of the two atoms approach to a close distance whereas the repulsive component of the van der Waals force predominates at closer distances, when the electro orbitals of the adjacent atoms begin to overlap. This type of repulsion is commonly called steric hindrance. The distance of

maximum favourable interaction between two atoms is known as the van der Waals contact distance, which is equal to the sum of the van der Waals radii for the two atoms. While a van der Waals - London dispersion interaction between any two atoms in a protein is usually less than 1 kcal mol^{-1}, the total number of these weak interactions in a protein molecule is in the thousands. Thus the sum of the attractive and repulsive van der Waals - London dispersion forces are extremely important to protein folding and stability. The van der Waals contact distances of 2.8-4.1 Å are longer than hydrogen-bond distances of 2.6–3.1 Å, and at least twice as long as normal covalent bond distances of 1.0–1.6 Å between C, H, N, and O atoms. Although the latter bonds are shorter than the van der Waals contact distance, a repulsive van der Waals force must be overcome in forming hydrogen bonds and covalent bonds between atoms.

The fundamental law of electrostatics namely Coulomb's law, expresses the inverse square law of force between two electric charges q_1 q_2 separated by a distance R in a vacuum in the form:

$$F = q1 \; q2/(4\pi\varepsilon_0 R^2)$$

where ε_0 is the permittivity of free space (vacuum). If q_1 and q_2 have the same sign, the force is a repulsion; if they are of opposite sign, the force is an attraction. In the presence of a material medium surrounding both charges, the force is reduced by a factor $\varepsilon_x = \varepsilon/\varepsilon_0$, the relative permittivity (or dielectric constant) of the medium. The work done in bringing two charges together from infinite separation to a distance R in a medium of permittivity ε is, therefore, given by:

$$\Delta W = \int_{\infty}^{R} F dR = q1q2/(4\pi\varepsilon R) = E$$

and measures the electrical free energy of the system relative to that at infinite separation. Electrostatic interactions between charged groups are of importance to particular protein structures, in the binding of charged ligands and substrates to proteins, interaction between basic and acidic proteins (Friedli, PhD thesis chapter 8), and repulsion between charges of the same sign as between SWP and sodium alginate (Friedli *et al.*, 1995). The strength of the electrostatic force (E) is directly dependent on the charge (q) for each ion, and is inversely dependent on the dielectric constant (ε) of the solvent and the distance between the charges (R). Water has a high dielectric constant ($\varepsilon = 80$), and ionic charge interactions in water are relatively weak in comparison to electrostatic interactions in the interior of a protein, where the dielectric constant ($\varepsilon = 2$-40) is approximately a factor of 1 : 40 to 1 : 2 that of water. Consequently, the strength of an electrostatic interaction in the interior of a protein, where the dielectric constant is low, may be of significant energy. However, most charged groups of proteins are on the surface of the protein where they do not strongly interact with other charged groups from the protein or other biopolymers due to the high dielectric constant of the water solvent, but are stabilized by hydrogen bonding and polar interactions to the water. Electrostatic interactions in water are less than those in other solvents because of water's high dielectric constant, which results from the tendency of the large dipoles of water molecules to align with any electric field. The dielectric constant of pure water at 25°C is 78.5 and it decreases at higher temperatures because thermal motion overcomes the orienting effects of the water dipoles (Creighton, 1993). This effect of temperature explains some of the findings in (Friedli, PhD thesis, chapter 5). When small diffusible ions such as Na^+ and Cl^- are present in water, the apparent dielectric constant of the solution increases because the ions tend to

concentrate in the vicinity of charges of the opposite sign. Since the present project considered biopolymer interactions, most of the experiments were performed with water in order to avoid interference from salts.

The strong inclination of water molecules to form hydrogen bonds with each other influences their interactions with non-polar molecules that are incapable of forming hydrogen bonds (e.g., alkanes, hydrocarbons inert atoms etc.). When water molecules come in contact with such a molecule they are faced with an apparent dilemma that whichever way the water molecules face, it would appear that one or more of the four charges per molecule (ST2 model of water, named after Stillinger and Rahman, 1974) will have to point towards the inert solute molecule and thus be lost to hydrogen bond formation. Clearly the best configuration would have the least number of tetrahedral charges pointing towards the unaccommodating species so that the other charges can point towards the water phase and, therefore, participate in hydrogen bond attachments much as before. There are many options to salvage lost hydrogen bonds. If the non-polar solute molecule is not too large, it is possible for water molecules to pack around it without giving up any of their hydrogen-bonding sites, thus forming clathrate 'cages' around a dissolved non-polar solute molecule. Such structures are not rigid but labile, and their hydrogen bonds are not stronger than in pure water, but the water molecules forming these cages are more ordered than in the bulk liquid.

It is also clear that the sizes and shapes of non-polar solute molecules are fairly critical in determining the water structure adopted around them. This is often referred to as hydrophobic solvation or hydrophobic hydration. At present there is no simple theory of such solute-solvent interactions. However, both theoretical and experimental studies indicate that the re-orientation, or re-structuring, of water around non-polar solutes or surfaces is entropically very unfavourable, since it disrupts the existing water structure and imposes a new and more ordered structure on the surrounding water molecules. This immiscibility of inert substances with water, and the mainly entropic nature of this incompatibility is known as the hydrophobic effect (Kauzmann, 1959; Tanford, 1980), and such substances, e.g., hydrocarbons and fluorocarbons, are known as hydrophobic substances. Similarly, hydrophobic surfaces are not 'wetted' by water but when water comes into contact with such surfaces it rolls up into small lenses and subtends a large contact angle on them.

Closely related to the hydrophobic effect is the hydrophobic interaction, which describes the unusually strong attraction between hydrophobic molecules and surfaces in water which are often stronger than their attraction in free space. For example, the van der Waals interaction energy between two contacting methane molecules in free space is -2.5×10^{-21} J, while in water it is -14×10^{-21} J. Because of its strength it was originally believed that some sort of 'hydrophobic bond' was responsible for this interaction. But at present it is known that there is no bond associated with this mainly entropic phenomenon, which arises primarily from the rearrangements of hydrogen bond configurations in the overlapping solvation zones as two hydrophobic species come together, and which is also much of a longer range than any typical bond. To date there have been very few direct measurements of the hydrophobic interaction between dissolved non-polar molecules, mainly because they are so insoluble. Tucker *et al*. (1981) reported values of -8.4 and -11.3 kJ mol^{-1} for the free energies of dimerization of benzene-benzene and cyclohexane-cyclohexane, respectively, and Ben Naim et al. (1973) deduced a value of about -8.5kJ mol^{-1} for two methane molecules. There is as yet no satisfactory theory of the

hydrophobic interaction, though a number of promising theoretical approaches have been proposed (Dashevsky and Sarkisov, 1974; Pratt and Chandler, 1977; Marcelja *et al*., 1977; Pangali *et al*., 1979; Nicholson and Parsonage, 1982). Israelachvili and Pashley (1982) measured the hydrophobic force law between two macroscopic curved surfaces in water and found that in the range 0-10nm, the force decayed exponentially with distance with a decay length of about 1nm. Based on these findings Israelachvili and Pashley proposed that for small solute molecules, the hydrophobic free energy of dimerization increased in proportion with their diameter s according to:

DG (hydrophobic pair potential) $\approx -20\ \sigma$ kJ mol^{-1}

where σ is in nanometres.

The hydrophobic interaction plays a central role in;

- many surface phenomena;
- molecular self-assembly;
- micelle formation;
- biological membrane structure;
- determining the conformations of proteins, and
- in protein-protein interaction and gelation.

While there is no phenomenon actually known as the hydrophilic effect or the hydrophilic interaction, such effects can be recognised in the propensity of certain molecules and groups to be water soluble and to repel each other strongly in water, in contrast to the strong attraction exhibited by hydrophobic groups. Hydrophilic (i.e., water-loving) groups prefer to be in contact with water rather than with each other, and they are often hygroscopic (taking up water from vapour). Strongly hydrated ions and zwitterions are hydrophilic; but some uncharged and even non-polar molecules can be hydrophilic if they have the right geometry and if they contain electronegative atoms capable of associating with the hydrogen bond network in water, for example, the O atoms in alcohols and polyethylene oxide and the N atoms in amines. It is also important to note that a polar group is not necessarily hydrophilic and that a non-polar group is also not always hydrophobic. While hydrophobic molecules tend to increase the ordering of water molecules around them, hydrophilic molecules are believed to have a disordering effect. Certain highly polar molecules are so effective in altering or disrupting the local water structure that when dissolved in water they can have a drastic effect on other solute molecules. For example, when urea, $(NH_2)_2C=O$, is dissolved in water it can cause proteins to unfold. Such non-ionic but highly potent compounds are commonly referred to as chaotropic agents, a term that was coined to convey the idea that their disruption of the local water structure leads to chaos. It appears, therefore, that the hydrophilic and hydrophobic interactions, unlike electrostatic and dispersion interactions are inter-dependent and therefore not additive. Indeed, one would not expect them to be independent of each other, since both ultimately rely on the structure of the water hydrogen bonds adopted around dissolved groups. Because some of the systems studied in the present project were dispersions instead of solutions it is appropriate that a discussion on their interactions should involve long-range forces like the electric double-layer forces, van der Waals forces and steric polymer interactions.

As mentioned earlier, the van der Waals force between similar particles in a medium is always attractive, so that if only van der Waals forces were operating, we might expect all dissolved particles

to stick together (coagulate) immediately and precipitate out of solution as a mass of solid material. This normally does not happen, because particles suspended in water and any liquid of high dielectric constant are usually charged and can be prevented from coalescing by repulsive electrostatic forces. Other repulsive forces that can prevent coalescence are solvation and steric forces. The charging of a surface can come about by the ionisation or dissociation of surface groups (e.g., the dissociation of protons from surface carboxylic groups [$-COOH \rightarrow -COO^- + H^+$], which leaves behind a negatively charged surface). The surface charge is balanced by an equal but oppositely charged region of counterions, some of which are bound, usually transiently, to the surface within the so-called Stern or Helmholtz layer, while others form an atmosphere of ions in rapid thermal motion close to the surface, known as the diffuse electric double layer

The electric potential in the solution falls off exponentially with distance from the surface.

$$\psi = \psi_0 \exp(-kx)$$

where ψ_0 is the potential at the potential determining surface and ψ the potential at a distance x from the surface in the electrolyte solution. The quantity k is called the Debye-Hückel parameter. The quantity 1/k is referred to as the thickness of the double layer (Sennett and Olivier, 1965; Shaw, 1986; Ross and Morrison, 1988; Everett, 1989; Hunter, 1993; Atkins, 1994). As two charged surfaces come together, their double layers overlap and as a rough approximation, the electrical potentials arising from the two surfaces are additive. This implies an increase in the electrical contribution to the free energy of the system. A dispersion represents a state of higher free energy than that corresponding to the material in bulk. Passage to a state of lower free energy will, therefore, tend to occur spontaneously unless there is a substantial energy barrier preventing the elimination of the dispersed state (Everett, 1989). In the presence of such a barrier the system will be metastable and may remain in that state for a long time. On the other hand, if conditions are adjusted so that the energy barrier becomes negligibly small, or disappears altogether, then the dispersion becomes unstable and aggregate. The energy necessary to carry the system over the energy barrier comes from Brownian motion of the particles, which results from the random bombardment of the surface of the particles by molecules of the medium. Instability will ensue if the ratio of the energy barrier height to kT is reduced. This may arise in various ways. In principle, if the absolute height remained constant, then instability could be induced by an increase in temperature. The barrier height is also influenced by concentration and ionic strength (Hunter, 1993; Israelachvili, 1995). The double layer repulsion depends on the ionic strength of the medium as follows:

- the curves may show a high repulsive barrier at low ionic strength;
- a so-called secondary minimum at intermediate ionic strength, and
- a negligibly small barrier, or none at all, at higher ionic strengths.

The secondary minimum is of the order of a few kT, and that is where weakly bound aggregates (flocs) form. Although the aggregates formed at the secondary minimum are sufficiently stable and cannot be completely dissociated by Brownian motion, they may disintegrate under externally applied hydrodynamic forces, such as vigorous stirring. If the particles are able to overcome the primary maximum (energy barrier) and enter the primary minimum, then there is no turning back, the aggregates become irreversible. These are some of the situations encountered in the project as the proteins interact with each other or with other proteins.

Covalent Forces

When two or more atoms come together to form a molecule, as when two hydrogen atoms and one oxygen atom combine to form a water molecule, the forces that tightly bind the atoms together within the molecule are called covalent forces, and the inter-atomic bonds formed are called covalent bonds. In a covalent bond electrons are shared between two or more atoms so that the discrete nature of the atoms is lost. Depending on the position an atom (or element) occupies in the periodic table, it can participate in a certain number of covalent bonds with other atoms. This number or stoichiometry is known as the atomic valency. A further characteristic of covalent bonds is their directionality, that is, they are directed or oriented at well-defined angles relative to each other. Covalent forces are short range, that is, they operate over very short distances of the order of inter-atomic separations (0.1 - 0.2 nm) and tend to decrease in strength with increasing bond length. There are two types of covalent bonds in proteins, the peptide bond and the disulphide bond.

Disulphide bonds occur between the sulphurs of two cysteine side chains. They predominantly occur in extracellular proteins and are part of the primary structure. Inside the cell the sulphydryl is maintained in a reduced state by glutathione, but extracellularly, in the presence of oxygen, thiols are unstable relative to S-S bridges (Fahey et al., 1977). Although the disulphides are part of the primary structure, it has been shown that some native S-S bridges are only formed once the secondary and even tertiary structure of the protein has been achieved (Creighton, 1978). In extracellular proteins of known sequence, which contain disulphides, there is rarely more than one free -SH group. Ovalbumin, with one S-S and four cysteines, was the only extracellular exception found (Nisbet et al., 1981). Sulphydryls are very reactive in an extracellular environment and readily oxidise to form disulphides. Consequently if a cysteine is external this may lead to disastrous polymerisation or make folding more complex. When the disulphide conformation of all the proteins in the Protein Data Bank (PDB) were examined in detail, it was found that they could be grouped into two major categories (Richardson, 1981):
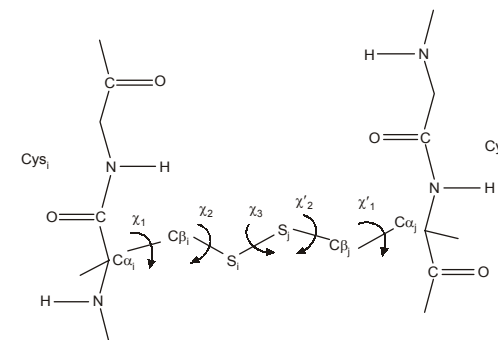
- The left-handed spiral conformation, with

$$x_1 = -60°, x_2 = -90°, x_3 = -90°, x_2^1 = -90°, x_1^1 = -60° \text{ and}$$

- The right-handed hook conformation, with

$$x_1 = -60°, \ x_2 = +120°, x_3 = +90°, x_2^1 = -50°, x_1^1 = -60°$$

A detailed analysis was done on the number of residues between half-cystines (Thornton, 1981) and it was found that the most frequent separation is 10 to 14 residues. The shortest connection found was two residues and connections longer than 150 residues are rare. A study was carried out to find out how disulphides fit into the three-dimensional structure of a protein (Sternberg and Thornton, 1976). The disulphides were divided into two groups: local disulphides, with half-cystines separated by less than 45 residues and non-local disulphides with a separation greater than 45 residues. Thirty-three out of the 34 local disulphides investigated had one of the structures shown in Figure 1.7. The most common being the coil-coil (cc), followed by c-bb-c. No helix-helix (aa) local disulphides were observed, although (aa) disulphides were found between non-local half-cystines.
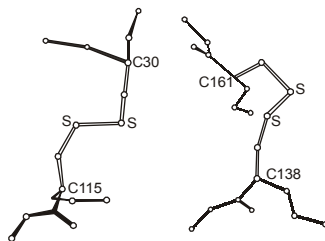
A left-handed spiral disulphide from hen egg white lysozyme (left) and a right-handed hook disulphide from carboxypeptidase A (right). [Richardson, 1981]
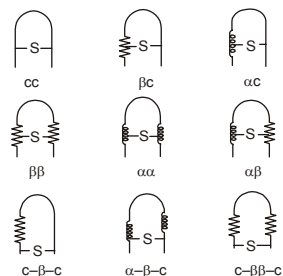
Local disulphide "single loop" topologies. c=coil, beta=strand, alpha=helix. (Thornton, 1981)

In summary,

- local disulphides are preferred where possible;
- disulphides between the amino and carboxyl termini are also favourable, even though they are non-local;
- secondary structure (alpha-helix and beta-sheet) often prevents formation of local disulphides
- disulphides are rarely found between cystines separated by more than 150 residues or between cystines in different domains;
- Half-cystines are predominantly in coiled regions in contrast to cysteines, which are preferentially helical, and
- local disulphides tend to be right handed whilst non-local disulphides are left-handed and adopt the left-handed spiral structure.

Disulphide bond formation was found to be important in the present study. Exposure of cysteines in BSA and beta-lactoglobulin through heating led to aggregation and gel. Glutenin polymerises through inter-disulphide bond formation. At room temperature, soluble wheat protein (SWP) was made to gel through reduction of its disulphide bonds. As the disulphide bond in proteins are reduced, the protein unfolds or denatures.

Protein Denaturation

Protein denaturation has been defined in several ways, for example as a change in solubility (Mirsky, 1941) or by simultaneous changes in chemical, physical and biological properties (Neurath et al., 1944; Langmuir, 1938) under some standard reference set of conditions (Timasheff and Gibbs, 1957). These changes in physical, and to a lesser extent chemical properties are manifestations of configurational changes taking place in the polypeptide chains. The denaturation process presumably involves an unfolding or at least an alteration in the nature of the folded structure (Foster & Samsa, 1951). Most denaturation changes consist of changes in secondary bonds: ion-dipole, hydrogen and Van der Waals, and in the rotational positions about single bonds which are controlled by the secondary bond structure (Lumry and Eyring, 1954). The term denaturation denotes the response of the native protein to heat, acid, alkali, and a variety of other chemical and physical agents which cause marked changes in the protein structure. Rice et al. (1958) suggested denaturation to mean a class of reactions which lead to changes in the structure of the macromolecule with no change in molecular weight. Timasheff and Gibbs (1957), pointed out that the approaches used to define the concept of denaturation can be classified into two types:

- molecular, in terms of actual structural changes taking place on the molecule, and
- operational, in terms of changes in measurable properties.

The operational approach to denaturation has the advantage of being purely phenomenological, but it cannot lead to a precise definition, since the concept of property, even restricted to measurable property, is itself without finite boundary. On the other hand, the molecular point of view, although still involves a few assumptions despite the important advances in investigations of protein structure, will allow us to reach a definition which is very convenient, owing to its general validity. Whatever it is,

changes can be quantitatively described by comparison with the native state. This seems simple, although in fact difficulties arise in attempting to obtain an accurate quantitative description of the native state since it is not a state that is static and fixed. Moreover, physico-chemical measurements are ordinarily not made in situ, but with extracted proteins, and it cannot be excluded that extractions and purification treatments do, sometimes, modify, to a certain extent, the structure of the protein molecules. Nevertheless, in practice, the state of the molecules prepared by the mildest extraction method is conventionally assumed to be the native state. Following these preliminary remarks, denaturation may be defined in very general terms as any modification of the secondary, tertiary or quaternary structure of the protein molecule, excluding any breaking of covalent bond (Joly, 1965). The degree to which the three-dimensional structure of a protein may differ from the native state may vary from a change in a single noncovalent bond or side-chain orientation to the case where almost no atom exist in the same spatial relationship to others except for the constraints of the primary structure.

The special importance attached to an understanding of the denaturation process is due to the fact that denaturation is usually a prerequisite for gelation. Since this project investigationed the elucidation of the gelation process it was considered that the control of denaturation could lead to a specific gelation properties. Evidence suggests that the various individual non-covalent bonds do not act independently but that there is a cooperative action of particular groups of 'bonds' or contacts in stabilizing various segments of the structure or even the total conformation (Tanford, 1968). Thus conformational transitions are found to pass through a few intermediate states or take place by an all-or-none type of mechanism between two states with no intermediates occurring in substantial concentration (Creighton, 1994).

The denaturation process can be achieved by any one of the following methods: increasing temperature, changing pH, using denaturants (i.e. urea, guanidine hydrochloride, beta-mercaptoethanol, dithiothreitol), inorganic salts (i.e lithium bromide, potassium thiocyanate, sodium iodide), organic solvents and (i.e. formamide, dimethylformamide, dichloro- and trichloroacetic acids and their salts), detergents (i.e. sodium dodecyl sulphate), high pressure and ultrasonic homogenisation.

The temperatures at which various proteins unfold vary enormously. Most proteins unfold at elevated temperatures, and some unfold at very low temperatures. Many proteins unfold at temperatures only a few degrees higher than those at which they function. Others are stable to much higher temperatures such as the gluten proteins. The driving force for denaturation is the increase in entropy that accompanies the transition of a single conformation into an ensemble of random ones. With increasing temperature the contribution of this entropy increases and becomes more significant, and at some temperature it overcomes the energy effect (the protein is heat denatured). It is interesting to consider possible intermediate structures. The early unlocking of the tertiary structure deletes a large number of the bonds holding the structure together but increases the randomness only insignificantly. The later stages of denaturation lead to larger increases in entropy. Thus, the intermediate states are relatively unstable, and heat denaturation is often an all-or-none phenomenon. The unfolding of the protein exposes the buried non-polar amino acid residues. Their intermolecular clustering leads to aggregation of the denatured protein. Consequently, heat denaturation is essentially irreversible.

In chemical denaturation the secondary bonds holding the protein segments together are disrupted by some chemicals capable of forming equally strong or stronger bonds with the groups holding the

conformation together. For disrupting the hydrogen bonds, urea or guanidine hydrochloride are used. At high concentrations of these substances (e.g, 8M urea or 5M guanidine hydrochloride) many proteins adopt a highly unfolded conformation in solution. Proteins of multiple subunits are likely to be separated into their constituent polypeptide chains. Other proteins aggregate upon denaturation in urea or guanidine hydrochloride which is frequently due to the formation of disulphide bridges between sulphydryl groups made accessible by the unfolding of the polypeptide chains. Such reactions may be inhibited by the addition of iodoacetate (Friedli, PhD thesis, chapter 3 and 5). Under these conditions, the denatured molecules remain in solution and may revert into native molecules if the denaturing agent is slowly dialysed away. Powerful detergents like SDS disrupt both hydrophobic and hydrogen bonds and effectively solvate the denatured molecule. beta-Mercaptoethanol and dithiothreitol (DTT) disrupt disulphide bonds and can be used in conjunction with urea or SDS to fully solubilise protein molecules.

Protein-Protein Interactions

Protein-protein interactions occur widely. These can either involve specific binding or non-specific interactions. Numerous examples of specific binding can be observed in biological systems:

- where proteins are directed to the correct compartments of cells by binding to other proteins;
- protein messengers bind to protein receptors on the outer surface of cell membranes to send signals between cells;
- proteins form structural connections between cells;
- some inhibitors of enzymes are proteins;
- proteins are modified and degraded by enzymes;
- interaction between different protein subunits are the basis of allosteric changes in multimers, and
- protein-protein interactions underlie very large-scale movements in organisms, such as muscle contraction.

However only a small selection of molecules are involved in specific binding (Zuckerkandl,1975) which is a property of individual proteins. The only category of proteins which has specialised in creating binding sites for practically all kinds of molecules are the immunoglobulins. In addition the substrates of serine proteases are proteins, therefore, the complex formation between these enzymes and their substrates can provide useful information on protein-protein interactions.

Protein-protein interactions are generally favoured under conditions which reduce the net charge on the molecules, i.e. pH values near the isoelectric point. High ionic strength tends to reduce electrostatic repulsion between proteins due to the shielding of ionizable groups by mobile ions. Protein-protein association involves the specific complementary recognition of two macromolecules to form a stable assembly (Jones and Thornton, 1995). Fundamental to the stabilization of protein association is the hydrophobic interaction (Chothia and Janin, 1975). The term hydrophobic interaction is used to describe the gain in free energy which occurs when non-polar residues of proteins associate in an aqueous environment (Kauzmann, 1959). The process of folding and protein-protein aggregation reduces the surface area in contact with water. When the protein-solvent interaction is attractive, the protein can reduce its total energy by surrounding itself with solvent molecules, conversely, when the interaction is

repulsive, the solvent is excluded (Tanaka, 1981). The aggregation of protein subunits buries the hydrophobic residues of the proteins, and hence minimizes the number of thermodynamically unfavourable solute-solvent interactions as found when SWP is hydrated in distilled water at room temperature (Friedli, PhD thesis, chapter 3).

Most of the proteins in food systems are denatured to varying degrees depending upon the type of processing used. The functionality required dictates the type and concentrations of ingredients and their environment. Protein-protein or protein-polysaccharide interactions under these circumstances are not specific, like in biological systems, but rather, depend mainly on physico-chemical forces. Proteins spontaneously aggregate when hydrated, therefore, molecular interactions are best studied in dilute systems. As their concentrations increase, their behaviour is better explained with colloid chemistry. Physical functions associated with proteins in a food system typically includes hydration and water binding which affect viscosity and gelation; modification of surface and interfacial activity which control emulsification and foaming ability and chemical reactivity leading to altered states of cohesion/adhesion and a potential for texturization (Fligner and Mangino, 1991).

A particular functional property may be a manifestation of a specific component of the food protein used or due to interactions involving the biopolymers in the system. Schoen, (1977) pointed out that one major objective of protein functionality research, as it relates to foods, is to understand how proteins interact with each other and with other components in mixed systems. Protein interactions under certain circumstances lead to gelation.

Gelation

Gels may be defined by their ability to immobilize liquid, macromolecular structure, textural or rheological properties (Kinsella, 1976). The Collins English Dictionary defines a gel as a semi-rigid jelly-like colloid in which liquid is dispersed in a solid (Latin, gelare, meaning 'to freeze'). Bungenberg de Jong (1949), defined a gel as a system of solid character in which the colloidal particles somehow constitute a coherent structure. Hermans (1949), defined a gel with three propositions : (a) they are coherent colloid systems of at least two components; (b) they exhibit mechanical properties characteristic of a solid; (c) both the dispersed component and the dispersion medium extend themselves continuously throughout the whole system. Flory (1974), classified gels based on their structure into four types as follows :

- Well-ordered lamellar structures, including gel mesophases;
- Covalent polymeric network ; completely disordered;
- Polymer networks formed through physical aggregation;
- predominantly disordered, but with regions of local order, and
- Particulate, disordered structures.

An example of the first type is a phospholipid with well-ordered lamellar structure as in biological membranes. Gels of the second type are completely disordered with structural units covalently linked to one another. The units are either bi-functional or poly-functional with a network which pervades the entire system giving it an infinite molecular weight for example elastin and condensation polymers. Gels of the third type are usually primary macromolecules bound together via crystalline domains or

multi-stranded helices where the network is caused by physical aggregation of chains, previously disordered, but with regions of local order. The orientation of the ordered regions are mutually uncorrelated and their locations may likewise be independent. Chains between the ordered domains are random. Whereas, the network in the second type of gels are covalently linked making them irreversible, the strands of the third type gels are linked by non-covalent forces and can under certain conditions be reversible. An example of this type of gel is gelatin, where triple helices like those in native collagen are formed and these appear to be further aggregated to form small crystalline domains at higher concentrations (Flory and Garrett, 1958). At low concentrations the triple helices appear to be individually dispersed without aggregation as crystallites (Flory and Garrett, 1958; Peniche-Covas et al., 1974; Eagland et al., 1974). Only a small fraction of the gelatin may be involved in the ordered domains. Gels formed by aggregation of proteins are of the fourth type, and they usually occur under conditions of partial denaturation. The proteins may be fibrillar or globular. Although specific interactions as in clotting of fibrin and antibody-antigen interactions may be responsible for the formation of gel-like aggregates, the aggregation of other proteins to a gel may be non-specific (Goldberg, 1952; Flory, 1974).

Biopolymer gels differ from synthetic polymer gels in a number of ways:

- They contain large amounts of solvent (usually water) than their synthetic counter-part
- The point covalent cross-links (e.g, vulcanised rubber) in synthetic polymer networks are replaced in biopolymer networks by a combination of many physical interactions which occur between sizeable regions of the biopolymer (Clark *et al.*, 1990).
- Elasticity of synthetic polymer networks is entropic in origin. In these networks, strands between cross-links can assume numerous conformations due to Brownian motion. Deformations of the network lowers the entropy of the strands and this is responsible for the resistance against permanent deformation (Walstra *et al.*, 1991). The elastic properties of such networks can be described by the Theory of Rubber Elasticity (Treolar, 1975), in which the shear modulus, G is expressed as: $(G = N_e R_g T / V_{mol})$ where N_e is the number of Elastically Active Network Chains (EANC) (Dobson and Gordon, 1965) per monomer and V_{mol} is the molar volume of a monomer. The elastic properties of biopolymer networks are caused by enthalpic rather than entropic changes.

The mechanical spectrum (frequency-dependence of the dynamic moduli, G' and G'') for a gel shows the storage modulus (G') to be greater than the loss modulus (G''), and both moduli are independent of frequency (i.e. rate of deformation). whereas, the dynamic viscosity decreases steeply with increasing frequency, and the slope of log h* vs log w approaches -1 when G' and G'' are constant.

And on a shorter timescale the behaviour of non-covalently cross-linked gels approximates to that expected for a permanent network (Morris, 1983).

Tanaka (1981), defined a gel as a form of matter intermediate between a solid and a liquid which consists of polymers, or long chain molecules, cross-linked to create a tangled network immersed in a liquid medium. The liquid prevents the polymer network from collapsing into a compact mass, whilst the network prevents the liquid from flowing away. Tanaka identified three forces which interact to either expand or shrink polymer networks. The forces are; rubber elasticity, the polymer-polymer

affinity and the hydrogen-ion pressure. The sum of these three forces was called the osmotic pressure of the gel, because it determines whether the gel tends to take up fluid or to expel it. The rubber elasticity is the elasticity of the individual polymer strands (i.e. the resistance the strands offer to either stretching or compression). The polymer-polymer affinity is the interaction between the polymer strands and the solvent. Such interactions can be either attractive or repulsive, depending on the electrical properties of the molecules. The hydrogen-ion pressure, is associated with the ionization of the polymer network, which releases an abundance of positively charged hydrogen ions (H^+) into the gel fluid. The hydrogen-ion give rise to pressure in the gel. A strand can be represented by a chain of rigid, jointed segments, each of which is in constant thermal motion. If the chain is stretched almost taut, the random movements of the segments give rise to a tension that pulls the ends of the chain inwards. If the chain is compressed into a compact ball, the force is directed outward. At an intermediate length of the chain the average force is zero. The rubber elasticity is proportional to the absolute temperature because thermal agitation is the ultimate root of the force. The polymer-polymer affinity decides if two polymers will aggregate and thereby exclude the solvent from the space between them or if there is a greater force of attraction between the polymer and the solvent than a polymer and another polymer.

A morphological classification of gels was proposed by Russo (1987) as :

- Fishnet gels; where cross-links, whether reversible or covalent, provide the 'strong points' of the structure and are separated by flexible strands which provide the elasticity.

- Lattice gels; where the division of the structure into cross-links and strands is inappropriate, i.e. the mechanical distinction between cross-links and strands is obscure, but nonetheless, a space-filling structure exits.

- Transient gels; a gel in which the structure is not permanent or due to high concentrations cause the polymers to overlap leading to temporary 'cross-links' due to chain entanglement.

Under this system of clasification, gelatin gels would be considered fishnet gels, globular protein network gels as lattice gels and yogurt as a transient gel. Aqueous solutions of biopolymers can often be converted to gels by thermal or chemical means. Clark (1986) classified thermal set gels into heat setting and cold setting. An example of a heat set gel is the aggregation of thermally unfolded globular proteins (Barbu and Joly, 1953; Clark *et al.*, 1981a; Clark *et al.*, 1981b; Tombs, 1970;). The cross-linking of gelatin, a fibrous protein and various types of polysaccharide via disorder-to-order transition induced by cooling, is typical of the cold-setting process (Eagland *et al.*, 1974; Peniche-Covas *et al.*, 1974; Morris *et al.*, 1980; Rees and Welsh, 1977).

Burchard and Ross-Murphy (1988), proposed a phenomenological definition, stating that : all gels possess at least one property which can stand as the operational definition of a gel. They possess a plateau in the real part of the complex modulus extending over an appreciable window of frequencies - i.e. they are, or can be coaxed under appropriate conditions to be, viscoelastic solids.

Almdal *et al.*, (1993) also suggested a phenomenological definition of a gel based on two criteria :

- A gel is a soft, solid or solid-like material of two or more components, one of which is a liquid present in substantial quantity.

- Solid-like gels are characterised by the absence of an equilibrium modulus, by a storage modulus,

G', which exhibits a pronounced plateau extending to times at least of the order of seconds, and a loss modulus, G'', which is considerably smaller than the storage modulus in the plateau region.

Morris (1985), characterised modes of gelation based on two different classes of materials, i.e. structural or storage. Storage materials are those which, even after extraction and purification, possess highly ordered structures (i.e. globular proteins). To gel aqueous solutions or dispersions it is usually necessary to first heat the samples in order to partially disrupt the ordered structure. Cooling leads to gelation which involves the formation of new intermolecular network. The original ordered structure cannot be recovered and such heat-set mechanisms are generally irreversible. Structural polymers are normally extracted from plant or animal cell walls (i.e. gelatin, alginate, pectin and carrageenans). Gelation occurs through a disorder-order transition which is reversible.

Most of the theories discussed so far are related to single component gels. The present project was carried out on mixed binary systems, therefore, a brief discussion on two component gels proposed by Morris (1985) would be appropriate. In a two component gel, the polymer that forms the network structure was labelled as 'active' and the other which is merely contained within the network structure as 'non-active'. A gel is called type I if it is made up of both active and non-active polymers, and type II, if both polymers are active. Complex formation between the active and non-active polymers in type I gels is unlikely. The non-active polymers will tend to concentrate the active polymers promoting intra- and inter-molecular interactions between them. The network structure in type II gels will depend on the relative values of the two active components and their degree of phase separation prior to gelation. The network formed were classified as:

- Interpenetrating networks, consisting of two independent network structures which interact only through mutual entanglements.

- Phase separated networks formed from the incompatability of the active polymers resulting in composites with upper and lower bound moduli.

- Coupled networks which form through inter-molecular interactions between the active polymers.

If conditions necessary for the gelation of the two active polymers differ, then different network structures could be produced by manipulating those conditions, for example gelling one polymer on heating and the other on cooling (Kasapis, 1994).

Gelation of globular proteins

Several factors affect gelation, such as protein type, protein concentration, temperature, ionic strength, type of ion and pH (Mulvihill and Kinsella, 1987). Probably the two most important factors in gelation are the protein concentration and heating temperature. If either, or both the temperature (Dunkerley and Hayes, 1980) and protein concentration (Ross-Murphy, 1991) are too low, gelation will not occur. Once these factors are above their critical values, gel strength increases and gelation time decreases with increasing temperature (Schmidt and Illingworth, 1978; Dunkerley and Hayes, 1980; Dunkerley and Zadow, 1984) and concentration (Ross-Murphy, 1991; Plock *et al.*, 1992). Ferry (1948) suggested a two-step mechanism of gelation which involves: (1) an initiation step involving unfolding or dissociation of the protein molecules, followed by (2) an aggregation step in which association or aggregation

reactions occur, resulting in gel formation under appropriate conditions. For the formation of a highly ordered gel, it is essential that the aggregation step proceeds at a slower rate than the unfolding step (Hermansson, 1978, 1979). Richardson and Ross-Murphy (1981) concluded from their gelation experiments, using 9% BSA solutions at pH 6.5 and at various temperatures, that the unfolding of BSA is rate-determining at temperatures below 57°C and that aggregation is rate-determining above that temperature. This finding is consistent with the views of Ferry (1948) that unfolding precedes aggregation and provides the driving force for protein-protein interaction.

There are a number of ways in which two or more proteins can interact with each other which will affect the properties of the gel formed. After mixing, proteins may be qualitatively considered incompatible, semicompatible, or compatible, depending on whether two immiscible phases are formed, partial mixing takes place at the molecular level, or a single thermodynamically stable phase is formed (Manson and Sperling, 1976). Composite or multicomponent gels are produced from mixtures of two or more gelling agents, or a single gelling and nongelling components. A second protein capable of gel formation may act as a non-gelling component if it is present in the mixture at a concentration below its critical concentration for gel formation (Ziegler and Foegeding, 1990). The second protein component may behave like a filler, interspersed throughout the primary gel network. The gel may be single phase with the filler remaining soluble (Figure1.9A), or two phase, where thermodynamic incompatibility causes phase separation to occur, with the filler existing as dispersed particles of liquid or as a secondary gel network (Figure 1.9B). The single phase system was labelled by Tolstoguzov (1986) as type I and the
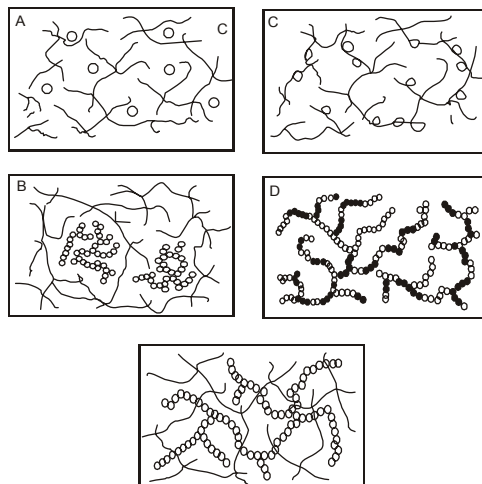
Fig. 2.43 : Types of mixed gels (Ziegler and Foegeding, 1990)

phase separated system as type II filled gel. In Figure 1.9C, the non-gelling component associates with the primary network in a random fashion via nonspecific interactions which may reduce the flexibility of the primary network chains and add rigidity to the gel. The two protein components may co-polymerise to form a single, heterogenous network (Figure 1.5D). An example of this type is the polymerisation which occurs in BSA-ovalbumin gels (Clark *et al*., 1982). Figure 1.9E is an example of the interpenetrating polymer network where both components form separate continuous network throughout the system.

Protein-Polysaccharide Interactions

Protein-polysaccharide interactions in food systems often plays a role in determining the functional properties of these systems (Stainsby, 1980). Understanding the mechanisms involved in the interactions between proteins and polysaccharides and the way in which these interactions are affected during processing is important when these components are added into foods to improve their functional properties (Ledward, 1979; Stainsby, 1980). Although there is evidence to indicate that the major forces responsible for these interactions are electrostatic in nature (Imeson et al., 1977), other interactions such as hydrogen, hydrophobic or covalent bonds may also be significant in the stabilization of the interaction.

Proteins and polysaccharide combinations have been used to stabilize emulsions (Dickinson and Euston, 1991; Dickinson and Galazka, 1991) through electroststic interactions and when covalently linked (Kato et al., 1990; Dickinson and Galazka, 1991; Dickinson and Galazka, 1992). The formation of soluble protein-polysaccharide complex in the pH range that would lead to protein precipitation has been utilised in the preparation of fruit flavoured milk beverages in which fruity flavour is best expressed at pH 4.5-5.0. CMC has been found to be particularly effective in keeping milk proteins in solution. In addition to holding milk proteins in solution, excess polysaccharide may resolubilize a precipitated complex at low pH. This behaviour is explained as a result of two reactions (Hidalgo and Hansen, 1969): a primary ionic reaction leading to the formation of insoluble complex, followed by a 'peptization' reaction, involving redistribution of protein molecules on the polysaccharide, giving rise to increased hydration and thus solubilization. The protein-polysaccharide interaction also inhibits precipitation of some water soluble proteins following denaturation

SUB-SECTION 2.9D—PHYSICAL AND CHEMICAL PROPERTIES OF PROTEINS

Taste. Pure proteins are generally tasteless, though the predominant taste of protein hydrolysates (proteoses, peptones, peptides, amino acids) is' bitter.

Odor. Pure proteins are odorless. When heated, they turn brown and char and give off the odor of burning feathers or hair.

Molecular weights of proteins. As previously indicated, protein molecules are exceedingly complex in structure and are very large. Because of the size of their molecules, proteins belong to the so-called colloidal state of matter.

The most important method for the determination of protein molecular weights is based upon the ultracentrifuge. The student should review that discussion at this time.

The minimal molecular weight of a protein may be calculated from the content of some characteristic component, if this is known. For example, horse hemoglobin contains 0.335 per cent iron. At least one iron atom must be present per molecule of hemoglobin. Since an atom of iron represents 55.85 units of weight and this is 0.335 per cent of the total molecular weight of hemoglobin (if only one Fe is present), the minimal molecular weight of hemoglobin is given by the expression: .

0.335 per cent: 100 per cent: : 55.85 : mol. wt.

mol. wt. = 16,671

Actually the molecular weight of hemoglobin is four times the minimal molecular weight as calculated on the assumption of one iron atom per mole-cule, which means that there are four iron atoms in each hemoglobin mole-cule, the true molecular weight is $4 \times 16,671 = 66,684$.

The amino acid content of a protein may also be used for calculating minimal molecular weights. The methods of analysis for tyrosine, cystine, and tryptophan give quite accurate results, and determination of these acids in proteins may be used for the calculation of minimal molecular weights. Minimal molecular weights are of value in checking on actual molecular weights determined by the ultracentrifuge or other physical methods. In some instances, by calculating the minimal molecular weight for several con-stituents, it is possible to calculate the actual molecular weight.

The molecular weights of proteins also may be obtained from osmotic pressure measurements and light scattering

Table below gives the molecular weights of a number of proteins, most of which were obtained by the ultra centrifugal method.

Table 2.20 : Some Physical Properties of Proteins

Protein	S_{20}*	D_{20} (cm²/sec)@	f/f_0#	V_{20} ml/g$	M_2%	IpH approximate
1	2	3	4	5	6	7
Actomyosin	12.0	0.30		(0.75)	3,900,000	
Adrenocorticotropic hormone (sheep)	2.1	10.5		(0.75)	20,000	
Albumin (serum, horse)	4.46	6.1	1.27	0.748	70,000	
Albumin (serum, human)	4.6	6.1	1.28	0.733	69,000	4.7
Aldolase	7.3	4.63	1.31	0.74	147,000	
β-Amylase (sweet potato)	8.9	5.77		(0.749)	152,000	
Carbonic anhydrase	2.8	9.0		0.749	152,000	
Carboxyhemoglobin (cow)	4.6			0.749		
Catalase	11.3	4.1	1.25	0.73	250,000	
Chymotrypsinogen	2.54	9.5	1.19	0.72	23,200	9.5
Cytochrome c (horse heart)	1.9	10.1	1.29	0.707	15,600	10.65

1	2	3	4	5	6	7
Diphtheria toxin	4.6	6.0	1.22	0.736	74,000	
Enolase	5.59	8.08	1.01	0.735	63,700	
Fibrinogen (cow)	7.9	2.02	2.34	0.706	330,000	
Fumarase	8.51	4.05		(0.745)	176,000	6.4
γ-Globulin (man)	7.1	3.84		(0.745)	176,000	6.4
Growth hormone (pituitary)	3.60	7.15	1.31	0.76	49,000	6.85
Hemoglobin (man)	4.46	6.9	1.16	(0.749)	63,000	6.7
Hexokinase	3.1	2.9	2.37	0.740	96,600	
Insulin (monomer)	1.6	15.0		0.749	12,000	
Lactablbumin (cow)	1.9	10.6		0.751	17,400	5.12
b-Lactoglobulin	3.12	7.3	1.26	0.7514	41,500	5.19
Lysozyme (chicken)	2.11	10.2-11.2	1.14-1.21	0.722	17,200	11.0
Myoglobin	2.04	11.3	1.11	0.741	16,900	7.0
Myosin	7.2	0.87	4.0	0.74	829,000-880,000	6.2-6.6
Ovalbumin	3.55	7.76	1.16	0.749	44,000	4.6
Pepsin	3.3	9.0	1.08	0.750	35,500	<1.1
Peroxidase	3.48	7.05	1.36	0.699	39,800	
Phosphorylase (rabbit muscle)	13.7	3.2-3.8		(0.74)	340,000-400,000	5.8
Pyruvic oxidase	40.4	0.91	1.51	(0.74)	4,000,000	
Urease	18.6	3.46	1.19	0.73	480,000	5.0-5.1
Virus (rabbit papilloma)	280.0	0.51	1.65	0.756	47,100,000	
Virus (tobacco mosaic, ordinary)	174.0	0.3	2.9	0.727	59,000,000	
Zein	1.9	4.0	2.7	0.776	50,000	

*S_{20} = sedimentation constant in Svedberg units reduced to water at 20° C.

@D_{20} = diffusion constant in units of 1×10^{-7} reduced to water at 20°C.

#f/f_0 = frictional ratio; the rtio of determined molar frictional constant, f, to the calculated molar frictional constnt, f_0, for nonsolvated spherical molecules of the same mass.

$$V_{20}$ = partial specific volume of the protein at 20°C. The partial specific volume of a protein represents the volume increment when 1 g of dried protein is added to a very large volume of water (solvent). Values in brackets in the table are assumed values based upon determinations on closely related proteins. One gram of proteinin solution generally occupies a volume of 0.70-0.75 ml, representing a protein density of 1.33-1.43 in water solution.

%M_8 = molecular weight by sedimenttion velocity and diffusion measurements.

A number of the naturally occurring proteins designated by a definite name really represent complex mixtures of different. proteins with molecules of widely different sizes. The crude material isolated from milk and known as "casein" is shown by the ultracentrifuge to be a complex mixture of mole-cules

varying in molecular weight from 75,000 to 375,000. If the casein is exhaustively purified according to Hammarsten's method, a fraction of mo-lecular weight 375,000 is obtained. Yon Hippel and Waugh (33) found that soluble casein at pH 12 and 0° C exists as monomeric units, *a-* and /3-casein monomers, with molecular weights in the range of 15,000 to 25,000. As the temperature is raised and the pH decreased to 7, these monomers polymerize into larger aggregates. The molecules of gelatin vary in mass from 10,000 to 100,000. Myoglobin of muscle apparently is composed of 17,000, 34,000, and 68,000 molecular weight fractions. The globulin fraction of blood serum is complex.

The molecular weights of proteins cannot be determined with a high degree of accuracy in most cases, and the values reported are more or less approximate.

Proteins as ampholytes. Isoelectric pH values of proteins. The properties of proteins as electrolytes are determined by the ionizable groups present. Since each open peptide chain contains only one free a-amino group and one free a-carboxyl group, those groups contribute relatively little to these properties. However, a number of the constituent amino acids contain ionizable groups nQt involved in peptide bond formation. These groups in-clude the f-amino group of lysine, the guanidine group of arginine, the imid-azole group of histidine, the ,a-carboxyl of aspartic acid, the 'Y-carboxyl of glutamic acid, the phenolic hydroxyl of tyrosine, and the sulfhydryl group of cysteine. The extra carboxyl groups of aspartic and glutamic acids occur both free and combined with ammonia as the amide group (asparagine and glutamine). The acidic properties of phosphoproteins such as casein are partly due to the phosphoric acid groups present the amino acids in acid solutions exist as cations

Fig. 2.43 : Dissociation curve of a standard gelatin preparation in hydrochloric acid or sodium hydroxide solution, as obtained from pH measurements at 300 C. The curve was drawn to fit the experimental points. (From Hitchcock, D. I.: *J.* Gen. *Physiol.,* 15, 125, 1932)

which dissociate to give H+ ions in stages to which acid dissociation constants K 1, K 2, K 3, etc., may be assigned. The zwitterion form of the amino acid is an intermediate stage in the process. Proteins in a similar manner exist as complex cations in acid solution and when titrated with alkali show successive overlapping stages of H^+ ion dissociation with the formation of zwitterions, and finally protein anions. Although the processes of protein dissociation represent many ioniza-ble groups, numbers of which may be functioning simultaneously, the general process may be represented as follows, in which the numbers of charges and H+ ions involved are not indicated:

$$\text{protein}^+ \rightleftarrows H^+ + \text{protein} \rightleftarrows H^+ + \text{protein}$$
$$\text{cation} \qquad\qquad \text{Zwitterion} \qquad\qquad \text{Anion}$$

Because protein molecules contain numbers of groups that interact reversi-bly with H+ ion~ over a wide pH range, their solutions have excellent buffer action, and much of the buffer capacity of body tissues is due to the protein buffers.

The dissociation curves, or titration curves, of proteins corresponding to the above equilibria with H+ ions may be determined, and an example is given in Figure 43 These curves extend over a wide range of pH and do not show the sharp breaks characteristic of the curves of monobasic weak acids. This is due to the large number of groups which ionize successively and simultaneously throughout most of the pH range.The nature of the ionizing groups in proteins may be determined from the titration curves under various conditions, and the heats of ionization, *AH,* of the amino acids and simple peptides. Table 8.8 compiled from Cohn and Edsall gives the *pK* values of the ionizable groups of proteins.

The numbers of different ionizable groups in various proteins have been estimated. For example, in the case of ,B-lactoglobulin with a molecular weight of 40,000, there are 4 imidazolium groups (histidine), 27 e-ammonium groups (lysine), 7 guanidinium groups (arginine), 30 ,B-:carboxyl groups (as-partic acid), 59 'Y-carboxyl groups (glutamic acid), and 30 amide groups (asparagine and glutamine). Since the amide groups are formed from some of the carboxyls of aspartic and glutamic acids, the total of the free carboxyl groups is 30 + 59 – 30 = 59.

The isoelectric pH of a protein is the pH at which the protein does not migrate in an electric field. At this pH the protein exists as the zwitterion form in which the total positive charges equal the total negative, and the net charge is zero, though the total charge may be high. The isoionic point of a protein is the pH at which the number of H+ ions dissociated from the protein is equal to the number taken up from the solution by the protein. The isoionic and isoelectric pH values are the same when the protein does not combine with ions other than the H+ ion. In general, in the presence of salts the anions and cations of the salt are likely to associate to somewhat different degrees with the protein charges and change appreciably the migra-tion in an electric field and the isoelectric pH; consequently, accurate iso-electric pH values can be given for proteins only under specified conditions of salt concentration and ionic strength.

The isoelectric pH of a protein is of great importance in relation to the physical and chemical properties of the protein. In general, the properties of a protein are minimal at the isoelectric pH. At this pH the net charge on the protein is least and the electrical conductivity is least. Also, at this pH the osmotic pressure, swelling capacity, viscosity, and solubility are minimum. Proteins exist as cations on

the acid side and as anions on the alkaline side of the isoelectric pH.

A number of methods are used for the determination of the isoelectric pH values of proteins. These methods are generally based upon determination of minima in properties, such as the pH at which electrical migration is least or the pH of minimum solubility.

The migration of particles in a solution between electrodes is referred to as "electrophoresis." Tiselius (35) has developed a very useful electropho-retic apparatus for studying the migration of proteins and other colloids. The solution to be examined is well buffered and is placed in a special square U-tube provided with compartments and with electrodes attached to the ends. Upon passage of a. current, the charged protein particles migrate toward the electrode of opposite charge. If the buffer solution is alkaline to the isoelectric pH of the protein, the particles migrate toward the positive electrode, while they migrate toward the negative electrode when the buffer solution is acid to the isoelectric pH of the protein. Migration of the particles ceases or is minimal when the pH of the buffer solution is the same as the isoelectric pH of the protein. The moving boundaries of the migrating pro-tein particles are observed by photographing them through a special optical system. If the protein particles under observation possess the same isoelectric pH and are of the same size and shape, they migrate at the same speed and a sharp boundary is obtained. On the other hand, if a mixture of proteins in which the components have different isoelectric pH values, molecular sizes, etc., is observed in the apparatus, each molecular speCIes will migrate at a different rate and become concentrated in different compartments of the cell, from which they can be removed. The Tiselius apparatus is of much impor-tance in the separation and purification of proteins, and in the determination 6f isoelectric pH values. See discussion in Chapter 15.

The isoelectric pH values of a number of proteins are given in Table 8.9. Since the IpH value varies with the ionic strength of the solution, the values given are only approximate for different ionic strengths. The values of the table are largely for an ionic strength of 0.1 and range all the way from around 1 for pepsin, with many acidic groups, to 11 for lysozyme, with many basic groups. The protamin salmin, containing much basic arginine, has an IpH of around 12. Table gives the distribution of a number of groups in several proteins which determine the isoelectric pH values and various chem-ical properties.

Proteins act as buffers on both sides of the isoelectric pH. The isoelectric pH values of most of the body proteins are below the pH of blood and tissue fluids and cells in which the proteins are found. This means that the body proteins are generally held at a pH alkaline to their isoelectric points and exist to a considerable extent as negative ions or salts with the cations Na^+, K^+, Ca^{++}, and Mg^{++}. In other words, the body proteins exist largely as buffers composed of protein- /H . protein. It is of interest that, in general, the imidazolium groups of histidine and the a-ammonium groups are respon-sible for the buffer action of proteins at physiologic pH. It will be seen from Table 8.9 that these are the only groups in proteins dissociating appreciably at physiologic pH.

Crystallization of proteins. Many of the proteins have been obtained in crystalline condition. Vegetable proteins in general are easy to crystallize. Among the animal proteins some hemoglobins crystallize very readily, while proteins such as serum albumin and ovalbumin are difficult to crystallize.

Many of the enzyme proteins have been crystallized. Among these are urease, pepsin, trypsin, and catalase.

Table 2.22 : Distribution of Groups in Some Proteins
Values are numbers of groups per 1000,000 grams of protein

Protein	Indole	Hydroxyl	Phenoxyl	Amide	–SH	Csteine/2 cystine–S–	Cations	Free anions	Total ionic
Aldolase	11.3	132.4	29.3	64.7	0	9.3	129.0	86.0	215.0
Casein (whole)	5.9	101.1	34.8	114.3	–	2.8	99.7	91.2	190.9
β-Casein	3.2	107.8	17.7	114.3	0	0	84.1	80.5	164.6
Chymotrypsinogen	27.3	193.9	14.9	109.4	10.8	27.5	76.7	23.0	99.7
Collagen	0	158.3	5.5	47.0	0	0	91.7	77.2	168.9
Edestin	7.2	92.4	23.5	126.8	4.2	7.2	128.6	104.2	232.8
Fibrinogen (human)	16.2	118.5	30.4	106.4	3.3	51.7	134.9	90.8	215.7
Gliadin	2.9	64.4	17.7	321.0	—	21	31.95	0	31.95
Insulin	0	67.3	69.0	99.5	0	104	68.4	78.5	146.9
β-Lactoglobulin	9.4	82.1	20.l4	76.5	9.1	19.1	104.3	141.5	245.8
Myoglobin (horse)	11.5	71.4	13.3	47.1	0	0	173.6	126.5	300.1
Myosin (rabbit)	3.9	84.1	18.8	85.7	0	11.7	139.2	131.5	270.7
Salmin	0	87.0	0	0	0	0	490	0	490
Serum albumin (human)	0.98	70.6	26.0	63.0	5.8	46.7	142.7	120.2	262.9

The crystallization of proteins often may be expedited by the addition of a salt such as ammonium sulfate or sodium chloride and adjustment toward the isoelectric pH. The addition of definite amounts of alcohol or acetone is occasionally advantageous. The added substances and adjustment to the isoelectric pH decrease the solubility of the protein. The protein is also least dissociated at the isoelectric pH and may crystallize best in this condition. However, some proteins crystallize best in the form of protein salt.

The relative ease of crystallization of proteins as compared with poly-saccharides is due to the high polarity *or* the protein molecules, giving rise to strong directional fields of force which orient the molecules and promote crystal formation.

Viscosity of protein solutions. The viscosity of protein solutions varies widely with the kind of protein and concentration. The viscosity of a protein in solution is closely related to molecular shape, long molecules giving higher viscosity than those more nearly globular in form. Viscosity measurements are of value in calculating the dimensions (axial lengths) of protein mole-cules. For a given shape large protein molecules give higher viscosities than small ones. Viscosity is least at the isoelectric pH, which means that protein ions orient in solution to increase frictional resistance to flow more than do neutral molecules.

Hydration of proteins. While proteins, as well as many other sub-stances, combine with and hold a certain amount of water through the action of poorly defined adsorptive forces of attraction, the peptide chains consti-tuting protein molecules contain many polar groups (-NH2, -COOH, -OH, -Co:-

, -NH-, etc.) which tend to combine with water and be-come hydrated. The nitrogen and oxygen containing groups of proteins con-tain unshared electron pairs, and these unite with the hydrogen of water to form more or less loose complexes of the type:

Since the water molecules combined with protein polar groups also possess unshared electron pairs, they may combine with more water to form aggre-gates attached to the polar groups such as:

Ionized acidic groups of proteins possess more attraction for water than the non-ionized groups because of the attraction of the negative charge the hydrogen of water:

Also, the positively charged ions of proteins coordinate readily with elec-1S of the oxygen of water:

accordingly, proteins hydrate to a greater extent when in the form of ions protein salts) than they do as undissociated molecules (in the isoelectric Idition) .

The water complexes with proteins are dissociable:

When electrolytes, sugars, alcohols, and other substances which form compl-exes with water are added to protein solutions, there is competition for the titer, and the degree of hydration of the protein is decreased.

In general, the combination of proteins with water is, within limits, de-pendent upon the concentration of the protein solution, the pH of the solu-tion in relation to the isoelectric pH of the protein, the presence of other dissolved substances which combine with water or form complexes with the protein, and the temperature of the solution.

That proteins do combine with water has been proved by observations showing changes in light absorption, dielectric values, protein density, etc., f proteins treated with varying quantities of water.

Since the water complexes of proteins are dissociable, it is very difficult to determine the degree of hydration with accuracy, since the method used nay markedly affect the dissociation equilibrium.

Some dozen different procedures have been applied to the problem, among which determination of the freezing-point depression and vapor-pressure effects of crystalloids added; 0 .protein solutions may be mentioned. In applying these methods, the assumption is made that the water not bound by protein constitutes the solvent for the crystalloid added, and the lowering of the freezing point or vapor pressure beyond that calculated if all water present acted as solvent is taken as an index of bound water. Another method, the dilatometric method, is based upon the volume changes of protein-water systems when cooled through a wide range of temperature as compared with an equal weight of water alone. In each case the volume progressively decreases, but the effect is greater in the protein – water mixture. The difference is utilized in calculating bound water. The values for bound water obtained by different methods vary over such a wide range that the validity of such determina-tions is extremely doubtful. For example, results on 1 per cent gelatin solu-tions by different methods indicate from 1 to 7 g of water to be bound per gram of gelatin.

Despite the fact that the combination of proteins with water represents reversible equilibria, some of the water is held with great tenacity, and the removal of the last traces of water from proteins by ordinary drying pro-cedures is very difficult and often incomplete.

The swelling of proteins when placed in aqueous solutions is definitely associated with hydration of the molecules, though a number of additional processes may be involved. The swelling of protein gels is discussed in the chapter dealing with the Donnan membrane equilibrium and is intimately related to osmotic effects within the gel induced by the Donnan membrane phenomenon.

The combination of living tissues with water is largely determined by hydration of the tissue proteins. A certain degree of tissue hydration is essen-tial for normal function. Severe dehydration of tissue proteins such as that caused by water deprivation leads to serious derangements of function.

Solubility and salting out of proteins. The solubility of a protein depends upon the proportions and distribution of polar hydrophilic groups and nonpolar hydrophobic groups in the molecule and the resulting protein dipole moment. The ionic polar groups of protein molecules interact electrostatically both within the same molecule and with surrounding molecules, tending to form aggregates and opposing solubility. This inter-action between protein-charged groups is decreased in pure water with a high dielectric constant; that is, the degree of interaction is inversely proportional to the dielectric constant of the solvent. The polar water molecules interact with the polar groups of proteins, tending to increase solubility. In the case of albumins, pseudoglobulins, and other water-soluble proteins the protein-water interactions promoting solubility pre-dominate over the protein-protein interactions opposing solubility, and the proteins are water-soluble.

The addition of an organic solvent, such as acetone or alcohol, to a solution of protein in water decreases the dielectric constant of the solvent and also displaces some of the water molecules associated with the protein and decreases the concentration of water present in the solution. These effects tend to decrease the solubility of the protein, and the addition of such solvents is often utilized in the precipitation of proteins from solu-tion. This is generally done at low temperatures to avoid protein denaturation.

When small amounts of salt are added to a protein dissolved in pure water, the activity coefficient of the protein is decreased and its solubility is increased. Likewise, proteins, such as euglobulins, which are insoluble in pure water become soluble in the presence of small amounts of salts. This

phenomenon, often called "salting in," is due to the forces of attraction between the protein ions and the salt ions. At low salt concentrations the increase in the logarithm of protein solubility, *S,* is proportional to the ionic strength of the solvent.

At high concentrations of very soluble saits such as ammonium sulfate, sodium sulfate, and phosphate buffers, proteins are "salted out" of solution. This salting out is due to a decrease in the activity of water, which diminishes the solubilizing interactions between water and the polar protein groups. The solubility, *S,* of many proteins at high salt con-centrations decreases logarithmically as the salt concentration increases:

$$\log S = \beta - K_s \omega$$

where *S* = the protein solubility in the salt solution, ω = the ionic strength of the salt solution, β = the solubility of the protein in pure water (generally hypothetical and obtained by extrapolation of the solubility curve to $\omega = 0$), and *K.* = the salting-out constant. The values of *K.* vary with protein and salt. For example, the *K.* values for horse hemoglobin and $MgSO_4$, $(NH_4)_2SO_4$, and Na_2SO_4 are 0.33,0.71, and 0.76, respec-tively; while the *K.* values for fibrinogen and NaCl and $(NH_4)_2SO_4$ are 1.07 and 1.46.

The relation of solubility of several proteins to ionic strength is shown in Fig. 44 taken from Cohn and Edsall. It will be observed from this figure that salting out may be used elrectivelyin the separation of proteins from solution.

In general, precipitation of a protein from solution is most complete (its solubility is least) at or near its isoelectric pH, as would be expected, because the protein is least soluble at this pH. However, there are some instances in which the addition of much salt causes the precipitation of a protein salt which separates best at a pH other than the isoelectric pH. For example, Sorenson found that the solubility of horse carboxyhemo-globin shows minimum solubilities in concentrated ammonium sulfate at pH 6.6.

Fig. 2.44 : Showing the Solubility of Proteins in ammonium sulphate

(nearthe isoelectric pH) and also at pH 5.4. The precipitate at pH 5.4 was combined with much sulfate and considered to be carboxyhemoglobin sulphate.

The effect of temperature upon the solubility of proteins in strong salt solutions is highly variable. Horse carboxyhemoglobin is about ten times more soluble at 0° than at 25° C in strong phosphate solution. Vegetable seed globulins are more soluble in salt solutions at higher than at lower temperatures. The solubility of egg albumin in salt solutions shows little variation with temperature.

The precipitation of proteins by salting out is one of the most used and valuable methods for the isolation and purification of proteins.

Precipitation of proteins .with positive and negative ions. Com-bination with dyes. Proteins may be precipitated from solution by a variety of positive and negative ions. Such precipitations are of importance in the isolation of proteins, in the deproteinization of blood and other bio-logic fluids and extracts for analysis, and in the preparation of useful protein derivatives.

The positive ions most commonly used for protein precipitation are those of heavy metals, such as Zn^{++}, Cd^{++}, Hg^{++}, Fe^{+++}, Cu^{++}, and Pb^{++}. These ions precipitate proteins from solutions alkaline to the protein isoelectric pH, because at this pH the protein is dissociated as protein- which combines with the positive metal ion to give an insoluble precipitate of metal pro-teinate. The metal ions may be removed from the metal proteinate by acidi-fication (protein- converted to protein+) or by precipitation of the metal by hydrogen sulfide or other agent. Metal protein precipitates are often dis-solved by the addition of strong alkali. It is evident from the above facts that the pH of the protein solution (within a certain range) is of primary importance for the most complete precipitation of proteins by heavy metal ions.

The positive ions of organic bases combine with proteins on the alkaline side of the protein isoelectric pH, and some of these combinations are insol-uble. Proteins of the protamin class, which are very basic in character, readily form insoluble compounds with various ordinary proteins. A notable example is the combination of protamin with insulin to form protamin insu-linate (protamin-insulin), which is widely used in the treatment of diabetes mellitus. The isoelectric pH of insulin is about 5.3, while the isoelectric pH of protamin is high on the alkaline side (9.7-12.4). When insulin and pro-tamin are mixed in solution at pH 7, the insulin is alkaline to its isoelectric pH and exists as insulin-, while the protamin is acid to its isoelectric pH and is present as protamin+. Accordingly, the positive and negative ions combine to form protamin insulinate. When this material is injected for the treat-ment of diabetes, it is gradually decomposed to yield insulin slowly to the body, thereby more nearly simulating pancreatic secretion of insulin than when insulin is injected alone.

Negative ions combine with proteins when in the form of protein+ (pH of the protein solution is acid to the protein isoelectric pH) to form protein salts. Several of these salts are insoluble and provide valuable methods for the precipitation of proteins from solution. Among the more common protein precipitants involving negative ion precipitation are tungstic acid, phospho-tungstic acid, trichloracetic acid, picric acid, tannic acid, ferrocyanic acid, and sulfosalicylic acid. When the above agents are added to protein solutions at the proper pH, precipitates of protein tungstate, phosphotungstate, pic-rate, tannate, etc., are formed. These precipitates are generally dissolved, and the protein salts are decomposed upon the addition of alkali (forms protein-). Tungstic acid, phosphotungstic acid, trichloracetic acid, and picric acid are commonly used for the preparation of protein-free ffitrates of blood and other biologic materials preliminary to analysis. Hides are converted to leather by treatment with tannic acid, which reacts with skin proteins to form insoluble protein tannates.

Rawlins and Schmidt (37) have shown that acid dyes (dissociate as acids → dye⁻-), such as Biebrich scarlet and naphthylamine brown, com-bine in definite proportions with proteins acid to the isoelectric pH (pro-tein+), the amount of dye combining being a function of pH and protein+ concentration. Similarly, basic dyes (base → dye⁺), such as methylene blue, induline scarlet, and safranine y, combine with proteins on the alkaline side of the isoelectric pH (protein~). Since the protein-dye combinations are ften highly insoluble, it is readily possible to determine the amount of dye)mbined with a given weight of protein. The above workers were able to lot dye-protein titration curves and to calculate the equivalent combining eights of proteins from their results.

The combination of proteins with dyes is of much importance in the stain-19 of tissues -and microorganisms for microscopic examination. The bac-~ricidal action of various dyes used in the treatment of infections such as Driflavin is probably related to combination of the dye with cellular proteins f the infecting organism rendering them incapable of normal function.

Precipitation of proteins by specific antibody proteins. The injec-tion of a foreign protein into an animal generally causes the appearance of so-called specific immune bodies or antibodies against the protein injected. 'or example, if a rabbit is given a series of injections with egg albumin at frequent intervals, its blood serum will develop the property of forming a precipitate with very minute amounts of egg albumin but not with other proteins. The injection of egg albumin causes the production of antibody proteins in the rabbit that specifically precipitate the egg albumin (antigen). The antibody proteins are present in the γ-globulin fraction of blood serum nd represent normal serum proteins which have been altered as a result of the presence of the injected protein (antigen).

The precipitation of proteins by their specific immune sera antibody pro-teins (precipitin reaction) affords a highly sensitive and specific method for the detection of proteins.

Reaction of proteins with nitrous acid. Since proteins generally con-ain some free amino groups, they react with nitrous acid to liberate nitro-en, with replacement of the amino group by a hydroxyl group, as is the ase in the reaction of amino acids with nitrous acid. Treatment of proteins with nitrous acid accordingly destroys the free amino groups, and produces deaminized proteins.

Acylation of proteins. Acid groups R-CO- may be introduced into he free hydroxyl and amino groups of proteins by treatment with acid chlo-ides and anhydrides. Acetylated, 'benzoylated, and benzenesulfonated pro-eins may be readily prepared. Ketene, CH2=C=O, is a valuable reagent or the acetylation of proteins. It reacts with amino and hydroxyl groups .s follows:

$R - NH_2 + CH_2 = C = O \rightarrow R - NH - CO\ CH_3$

$R - OH + CH_2 = C = O \rightarrow R - O - CO - CH_2$

By controlling the conditions it is possible to acetylate the amino groups }f proteins with ketene without acetylating the hydroxyl groups.

The acylation of proteins is of importance in studies of the influence of 'fee amino and hydroxyl groups upon the physiologic properties of the pro-,ein. For example, acetylation of only the amino groups of insulin reduces ts physiologic activity by about 20 per cent, while acetylation of both amino md tyrosine hydroxyl groups abolishes its activity, indicating that free phenolic hydroxyl groups are much more important to the function of insu-lin than are free amino groups.

Slphufhydryl-disulphide exchange reactions of pep tides and proteins. Much evidence has accumulated (38) that peptides and proteins containing intramolecular disulfide linkages form aggregates linked by intermolecular disulphide bonds, - S – S - These aggregates are formed in .a chain reaction initiated by a substance containing a free sulphuhydryl group, -SH, such as glutathione or another protein or peptide. The process may be illustrated diagrammatically as follows:

peptide chain

R-SH reacts with the peptide chain A, containing an intramolecular -&-S- group, to form B, containing a free -SH group. B then reacts

with another A to produce C, containing two A groups (also R-&-S-),

and since C contains a free -SH group, it in turn may react with A to form an aggregate with three A groups, etc. The denaturation of proteins appears often to involve such aggregations. The process appears to be involved in the formation of cross – S – S – linkages when fibrinogen is aggregated into fibrin, and there is some evidence to indicate its operation in the formation of cross linkages in the mitotic apparatus of cell division.

Cleavage of -*S-S*- groups of proteins. Since the -SH groups formed by the reduction of – S – S – groups in proteins are unstable and may reoxidize to – S – S – compounds, other methods which give more stable cleavage products are desirable when proteins are to be split into sim-pler pep tides for studies on amino acid sequence and other properties.

As indicated previously, Sanger split the – S – S – linkages of insulin to yield chain peptides by oxidation with performic acid, which breaks the -&-S- bond to form cysteic acid groups. If we designate a protein con-taining an -&-S- group by R-S-&- R', the reaction may be expressed as follows:

R- S- S- R' + 6H – CO₃H ↑ R- SO₃H + R'- SO₃H + 6H–COO⁻ + 4H⁺

where R–SO₃H and R'-SO₃H = peptides with a terminal cysteic acid group. Oxidation by performic acid has the disadvantage especially of disrupting tryptophan groups in the protein.

Swan has found that the – S – S – group in proteins is split by sulfite in the presence of cupric ions to form two thiosulfate groups (S-sulfocysteic acid groups). The reaction appears to take place in stages in which Curt is reduced to Cu⁺⁺

$$3R - S - S - R + 3SO_3^- \rightarrow 3R - S - SO_3 + 3RS^-$$

$$3RS^- + 2CU^{++} + SO_3^- \rightarrow R - S - SO_3^- + 2R - S - Cu$$

$$2RS - Cu + 4Cu^{++} + 2SO_3^- \rightarrow 2R - S - SO_3^- + 2Cu^{++}$$

over-all reaction is:

$$3R - S - S - R + 6SO_3^- + 6Cu^{++} \rightarrow 6R - S - SO_3^- + 6Cu^{++}$$
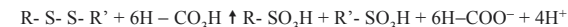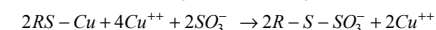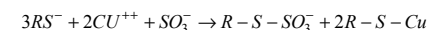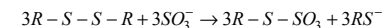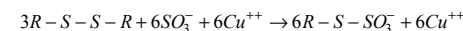
method of – S – S – cleavage has been applied to trypsinogen and chymotrypsinogen by and to wool keratin.

Cystine may exist in proteins as a component of a single peptide chain or two peptide chains linked by the – S – S – linkage:

The -S-S- linkages of proteins may be reduced to two –SH groups *by* treatment with reducing agents such as sodium cyanide, cysteine, thio-ycolic acid, and glutathione. Reduction of structures such as A forms a olecule of free cysteine and leaves a cysteine molecule in the peptide chain. Reduction of a structure such as B breaks the cross – S – S – linkage between the peptide chains, separating them, and leaving cysteine molecules resent in each chain.

The presence of certain – S – S – groups is necessary for the full activity f insulin. Their reduction to -SH groups results in the loss of about 50 percent of the insulin activity. .

Oxidation of -SH groups of proteins. 80me proteins contain free -SH groups which may be readily oxidized by agents such as ferricyanide, cystine, tetrathionate, porphyrindin, and Iodine. These oxidations apparently consist in the oxidation of two -8H groups to an – S – S – group presumably such an oxidation may involve two -8H groups in the same peptide chain, or in different peptide chains which would cause linkage of ;he chains and formation of a different and more complex protein structure.

The above oxidizing agents may be used for the quantitative estimation, of free -SH groups in proteins. The – S – S – linkages of proteins may be determined by reduction of the protein with thioglycolic acid, which reduces – S – S – to -SH groups which are then estimated. The – S – S – groups may be obtained from the difference in free -8H groups in the protein before and after reduction.

The determination of free -8H groups in proteins is of importance studying their relation to – properties of the protein (insulin, activity, for example) and other agencies. In general, the denaturation of a protein causes an in-crease in the number of free -SH groups.

Halogenation of proteins. When proteins are treated with alkaline iodine solution (NaOI), iodine is incorporated into the protein molecule. Hydrolysis of iodized protein yields diiodotyrosine, indicating that iodine substitutes into the tyrosine ring. More drastic methods of treatment lead to halogenation of the indole and imidazole groups of tryptophan and histidine. When proteins are treated with alkaline bromine solution (NaOBr), the cystine groups are oxidatively destroyed.

Iodine of iodides taken by mouth is utilized by the body in forming the iodized protein thyroglobulin, the precursor of thyroxine, the hormone of the thyroid gland. When thyroglobulin is hydrolyzed, it yields the iodine-containing compounds diiodotyrosine and thyroxin. Harington and Rivers (38) found thyroxin in the hydrolysis products of casein which had been treated with iodine. Practically all iodized proteins have some thyroid activity (increase metabolic rate).

Reaction of proteins with formaldehyde. Formaldehyde combines with proteins to change their properties markedly. Egg albumin becomes incoagulable by heat when treated with formaldehyde. Formaldehyde con-verts casein, soybean proteins, and other proteins into inert hard horny masses which are produced commercially as plastics (artificial ivories) in large quantities. Formaldehyde reacts with the proteins of tissues and hardens them when they are preserved in its solutions.

The chemical reactions involved in the action of formaldehyde upon pro-teins are obscure. Undoubtedly one of the reactions is combination of for-maldehyde with various amino groups in a manner siplllar to its reaction with the amino groups of amino acids.

Color reactions of proteins and amino acids. Because of their peptide structure and the presence of different amino acid groups in their molecules, proteins react with a variety of agents to form colored products. Several of these color reactions of proteins are of importance in the qualita-tive detection and quantitative estimation of proteins and of their constitu-ent amino acids.

(a) The ninhydrin reaction. The reaction of ninhydrin with amino acids has been discussed in detail on page 289, which should be con-sulted for the mechanism of reaction. Ninhydrin reacts with free a-amino acids and with proteins, proteoses, pep tones, and pep tides to give a blue color. The test is both the most general and one of the most delicate reactions known for the qualitative detection of proteins and their hydrolytic prod-ucts. It may be used to show the presence of amino acids in urine and in deproteinized body fluids. Apparently all amino acids of protein hydrolysis give the ninhydrin reaction. The colors given by different amino acids vary in shade and depth. The ninhydrin reaction is of value in detecting the end point of protein hydrolysis, at which time the color value is constant.

When urea is heated to about 180° C it de-composes to form biuret

Biuret Test: The Biuret Test is a general test for proteins. When a protein reacts with copper(II) sulphate (blue), the positive test is the formation of a violet colored complex.
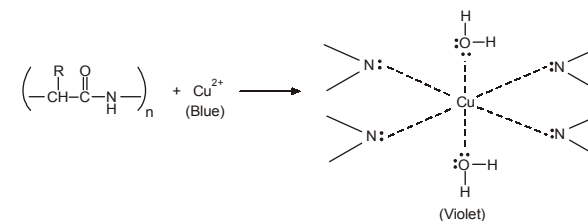
In this reaction an alkaline solution of biuret is treated with very dilute copper sulphate a violet colour is obtained. This reaction is given by substances containing two – $CONH_2$ groups joined either directly or through a carbon or nitrogen atom, Compounds containing $-CH_2NH_2 - C(OH)NH_2$ and – $CSNH_2$ in place of – $CONH_2$ groups give test. Peptide structyres are found in proteins and their derivatives which contain
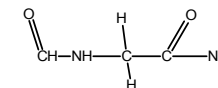
linkage also give the biuret test. Of the amino acids, hystidine gives a positive test. Dipeptides do not give this test, two or more peptide linkages being required.

When protein solutions are made strongly alkaline with sodium or potassium hydroxide and very little copper sulphate solution is added, apurplish or pinkish violet colour is obtained, this colour depends upon the complexity of the protein.

The biuret test is apparently used to the coordination of cupric ions with the unshared electrons pair of peptide nitrogen and trhe oxygen of water to form a coloured co-ordination complex, which may be represented in the diagram below:
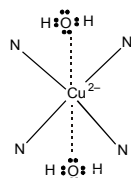
The presence of magnesium sulphate in the solution to be tested interferes with the biuret reaction because of the precipitation of magenisum hydroxide, Large amounts of ammonium salits also interfere with the test, but this may be minimized by the addition of a large excess of alkali. The biuret reaction is extensively used as a delicate test for presence of proteins in biologic materials. It has been converted into an excellent method for the quatitative determination of proteins in blood serum and other fuids.

The buiret reaction is extensively used as a delicate test for the presence of proteins in biological material. It has been converted into an excellent method for the quantitative dermination of proteins in blood serum and other fluids.

The Xanthotropic Reaction Also kwon as yellow protein reaction, which includes the addition of nitric acid in concentrated condition produces an initial white precipitate that turns yellow on heating; the colour turns orange, when the solution is made alkaline. Insoluble protein are turned yellow or orange on the surface.

The yellow stains upon the skin caused by nitric acid are the result of the xanthotropic reaction
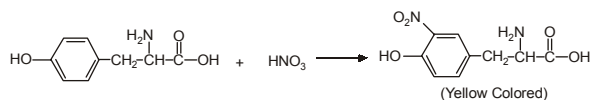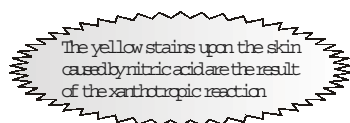
The xanthotropic reaction is due to nitration of phenyl rings present in tyrosine, phenyl amine and truptophan to give yellow nitro – substitution products which proteins give the xanthotripic reaction.

Millon's reagent is a composite mixture of mercurous nitriles and nitric acid. When it is added to protein solution, a white ppt is produced, which turns red upon heating. Secondary rpteoses and peptones give only red solution.

Insoluble proteins turn red when they are suspended in water and heated with the reagent. The reaction is not specific for proteins, since it is given by phenols in general. Since tyrosine is the only

phenolic amino acid in proteins a positive test indicates the presence of tyrosine. Solutions which are alkaline must be neutralized with acid (not HCl) before testing otherwise the mecury of the reagent is precipitated. Chloride interfere with the reaction probably because the nitric acid forms free chloride from them which destroyes the coloured compound. The reaction is dependant upon the formation of a coloured mercury compound with the hydrooxyphenyl group.

The Hopkin's Cole or glyoxalic reaction for tryptophan The reagent, which may be prepared by reduction of oxalic acid with sodium amalgam or magnesium powder.

The glyoxalic acid
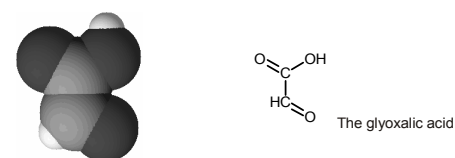
When a solution of tryptophan or protein which contains tryptophan is mixed with the reagent and the mixture is layered over concentrated sulphuric acid, a violet ring appears at the junction of the two liquids, the ring is the characteristics of indole ring, and other compounds than tryptophan hive the test. The nature of the coloured products fromed in the test is unknown. Chlorates nitrates and excess chlorides prevent the reaction A number of aldehydes other than glyoxalic acid give similar colour reaction with truptophan. Formaldehyde used similarly gives violet colour appears.

The Nitroprusside Reaction Proteins which contains free –SN groups (cystene) give a reddish colour with sodium nitroprusside, $Na_2Fe(CN)_5NO.2H_2O$ in ammonical solution. Many proteins which give negatibve test react positive after heat coagulation or denaturization by other means, indicating the liberation of free – SH groups. The cystene – S – S groups in protein may be reduced to –SH groups by reducing agents such as NaCN, after which they give the nitroprusside reaction.

Follin's Reaction Follin's amino acids reagent in sodium 1 – 2 napthoquinone – 4 sulphonate. This reagent gives deep red colour with proteins in alkaline medium.

The Sullivan Reaction This reaction involves a colourimetric method for the quantitative determination of cysteine and cystine. (After reduction of cysteine in protein hydrolysates. The method is based upon the red colour produced when cysteine is treated with sodiuk 1 – 2 naptholquinone – 4 – sulphonate in alkaline solution in the presence of sodium dithionate $Na_2S_2O_4$. The method has been widely used in the quantitative determination of proteins.

Sullivan's procedure provides a valuable colour test for cysteine, but experience of his quantitative methods in this laboratory has not been satisfactory.Thus it was found: (1) The depth of red colour is variable andt far from proportional to the amount of cysteine or cystine present. Rimington [1929] had already noted this poor proportionality and advised having the unknown within 15 % of the standard in estimation. (2) Even when compared with a standard of nearly the same colour, the results for the unknown are often irregular. (3) The presence of relatively considerable amounts of other amino-acids diminishes the amount of red colour due to cysteine or cystine, and the yellow colour due to the other amino-acids interferes when a pure cysteine or cystine standard is used for comparison. The diminution

in red colour was found to be attributable in part to the buffering effects of the amino-acids present upon the pH of the solution'.Two directions in which the accuracy of the method might be improved suggested themselves: (1) to maintain the same PE in standard and assay, and (2) to swamp both standard and assay with a considerable amount of aminoacid. It was found possible to affect both objects by adding glycine with appropriate amounts of alkali, and by this means, two of the most serious drawbacks to Sullivan's procedure (unreliability and lack of proportionality) were overcome. It was found that the reaction between the cystine and cyanide was the double decomposition:

RSSR + NaCN :- RSNa + RSCN
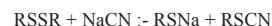
discovered by Pulewka and Winzer [1928]. Under conditions that may be varied considerably, this reaction proceeds so nearly to completion that any deviation from it cannot be detected colorimetrically.

Sakaguchi Reaction Is a terst for arginine either free or combined in proteins. The test consists in treating the sample solution with á – naphthol and sodium hypochlorite, with slow development of a intemse red colour. Various substances which contain the guanidine group give the reaction, but since arginione is the only amino acid in proteins which is the only amino acid in proteins which contains this group the test is specific for arginine when applied to protein free agrinine is stated to give a positive test in concentration of 0.0004 mg per ml. The reaction is modified and adopted to the quantitative determination for arginine.

Unoxidised sulphur test This test isto prove the presence of sulphur containing amino acids. The protein or amino acid is boiled with atrong alkali to split out sulphur in the form of N_2 detected by the addition of lead acetate, which causes the formation of brown or black lead sulphide.

SECTION 2.9E—GLOBULAR AND FIBROUS PROTEIN

Globular proteins, or **spheroproteins** are one of the two main protein classes, comprising "globe"-like proteins that are more or less soluble in aqueous solutions (where they form colloidal solutions). This main characteristic helps distinguishing them from fibrous proteins (the other class), which are practically insoluble.

The term globin can refer more specifically to proteins including the globin fold.

Globular Structure and Solubility

The term globular protein is quite old (dating probably from the 19th century) and is now somewhat archaic given the hundreds of thousands of proteins and more elegant and descriptive structural motif vocabulary. The globular nature of these proteins can be determined without the means of modern techniques, but only by using ultracentrifuges or dynamic light scattering techniques.

The spherical structure is induced by the protein's tertiary structure. The molecule's apolar (hydrophobic) amino acids are bounded towards the molecule's interior whereas polar (hydrophilic) amino acids are bound outwards, allowing dipole-dipole interactions with the solvent, which explains the molecule's solubility.

A wide range of roles in the organism

Unlike fibrous proteins which only play a structural function, globular proteins can act as:
- Enzymes, by catalyzing organic reactions taking place in the organism in mild conditions and with a great specificity. Different esterases fulfill this role.
- Messengers, by transmitting messages to regulate biological processes. This function is done by hormones, i.e. insulin etc.
- Transporters of other molecules through membranes
- Stocks of amino acids.
- Regulatory roles are also performed by globular proteins rather than fibrous proteins.

Members

Among the most known globular proteins is Hemoglobin, a member of the globin protein family. Other globular proteins are the immunoglobulins (IgA, IgD, IgE, IgG and IgM), and alpha, beta and gamma globulins. See protein electrophoresis for more information on the different globulins. Nearly all enzymes with major metabolic functions are globular in shape, as well as many signal transduction proteins.

Fibrous proteins, also called **scleroproteins**, are long filamentous protein molecules

Fibrous proteins are only found in animals.

Fibrous proteins form 'rod' or 'wire' -like shapes and are usually inert structural or storage proteins. They are generally water-insoluble. Fibrous proteins are usually used to construct connective tissues, tendons, bone matrix and muscle fiber.

Examples of fibrous proteins include keratins, collagens and elastins.
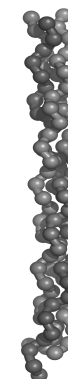
SECTION 2.10—AMINO ACIDS

In chemistry, an amino acid is a molecule containing both amine and carboxyl functional groups. These molecules are particularly important in biochemistry, where this term refers to alpha-amino acids with the general formula $H_2NCHRCOOH$, where R is an organic substituent.[1] In the alpha amino acids, the amino and carboxylate groups are attached to the same carbon, which is called the á-carbon. The various alpha amino acids differ in which side chain (R group) is attached to their alpha carbon. They can vary in size from just a hydrogen atom in glycine through a methyl group in alanine to a large heterocyclic group in tryptophan.

Amino acids are critical to life, and have a variety of roles in metabolism. One particularly important function is as the building blocks of proteins, which are linear chains of amino acids. Amino acids are also important in many other biological molecules, such as forming parts of coenzymes, as in S-adenosylmethionine, or as precursors for the biosynthesis of molecules such as heme. Due to this central role in biochemistry, amino acids are very important in nutrition.

The amino acids are commonly used in food technology and industry. For example, monosodium glutamate is a common flavor enhancer that gives foods the taste called *umami*. Beyond the amino acids that are found in all forms of life, amino acids are also used in industry, with the production of biodegradable plastics, drugs and chiral catalysts being particularly important applications.

Overview Alpha-amino acids are the building blocks of proteins. Amino acids combine in a condensation reaction, that is, through dehydration synthesis, that releases water and the new "amino acid residue" that is held together by a peptide bond. Proteins are defined by their unique sequence of amino acid residues; this sequence is the primary structure of the protein. Just as the letters of the alphabet can be combined to form an almost endless variety of words, amino acids can be linked in varying sequences to form a vast variety of proteins. Twenty standard amino acids are used by cells in protein biosynthesis, and these are specified by the general genetic code. These 20 amino acids are biosynthesized from other molecules, but organisms differ in which ones they can synthesize and which ones must be provided in their diet. The ones that cannot be synthesized by an organism are called *essential amino acids.*

History The first few amino acids were discovered in the early 1800s. In 1806, French chemist, Louis-Nicolas Vauquelin, isolated a compound in asparagus that proved to be the amino acid, asparagine. In 1812, William Hyde Wollaston found a substance in urine that he identified as a cystic oxide, and was later named cystine. And in 1820, another French chemist, Henri Braconnot, discovered the first two natural amino acids, glycine and leucine

General Structure

In the structure shown at the top of the page, R represents a side chain specific to each amino acid. The carbon atom next to the carbonyl group is called the á-carbon and amino acids with a side chain bonded to this carbon are referred to as *alpha amino acids*. These are the most common form found in nature. In the alpha amino acids, the á-carbon is a chiral carbon atom (with the exception of glycine).[3] In amino acids that have a carbon chain attached to the á-carbon, as in lysine on the right, the carbons are labeled in order as á, â, ã, ä, and so on. In some amino acids, the amine group is attached to the â or ã-carbon, and these are therefore referred to as *beta* or *gamma amino acids*.

Fig. 2.46 : Lysine with the carbon atoms in the side chain labeled

Amino acids are usually classified by the properties of their side chain into four groups. The side chain can make them behave like a weak acid, a weak base, a hydrophile if they are polar, and hydrophobe if they are nonpolar. The chemical structures of the 20 standard amino acids, along with their chemical properties, are catalogued in the list of standard amino acids.

The phrase "branched-chain amino acids" or BCAA is sometimes used to refer to the amino acids having aliphatic side chains that are non-linear; these are leucine, isoleucine, and valine. Proline is the

only proteinogenic amino acid whose side group links to the á-amino group and, thus, is also the only proteinogenic amino acid containing a secondary amine at this position. Proline has sometimes been termed an imino acid, but this is not correct in the current nomenclature.

Isomerism

Of the standard á-amino acids, all but glycine can exist in either of two optical isomers. While L-amino acids represent the vast majority of amino acids found in proteins, D-amino acids are found in some proteins produced by exotic sea-dwelling organisms, such as cone snails. They are also abundant components of the peptidoglycan cell walls of bacteria. and D-serine may act as a neurotransmitter in the brain.[8] The L
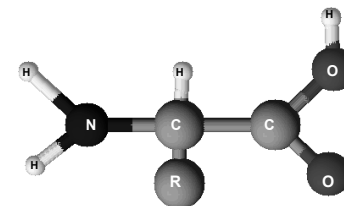
Fig. 2.47A. Showing the general structure of Amino acid

and D convention for amino acid configuration refers not to the optical activity of the amino acid itself, but rather to the optical activity of the isomer of glyceraldehyde from which that amino acid can theoretically be synthesized (D-glyceraldehyde is dextrorotary; L-glyceraldehyde is levorotary). Alternatively, the *(S)* and *(R)* designators are used to indicate the absolute stereochemistry. Almost all of the amino acids in proteins are *(S)* at the á carbon, with cysteine being *(R)* and glycine nonchiral. Cysteine is unusual since it has a sulfur atom at the first position in its side-chain, which has a larger atomic mass than the groups attached to the á-carbon in the other standard amino acids, thus the *(R)* instead of *(S)*.
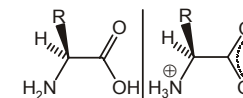
An amino acid in its (1) unionized and (2) zwitterionic forms

Zwitterions

As amino acids have both an amine and a carboxylic acid functional group and are therefore both acid and base at the same time. At a certain compound-specific pH known as the isoelectric point, the number of protonated ammonium groups with a positive charge and deprotonated carboxylate groups with a negative charge are equal, resulting in a net neutral charge These ions are known as a *zwitterion,* which comes from the German word *Zwitter* meaning "hybrid". Amino acids are zwitterions in solid phase and in polar solutions such as water and depending on the pH, but not in the gas phase. Zwitterions have minimal solubility at their isolectric point and amino acids are often isolated by precipitation from water after adjusting the pH to their isolectric point.

Occurrence and Functions in Biochemistry
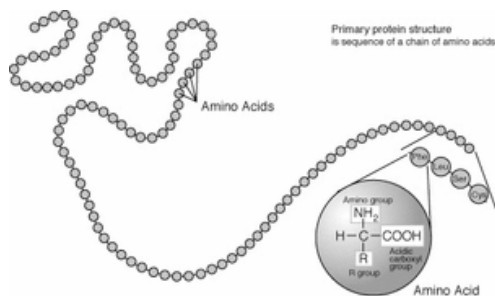
Fig. 2.47 : A polypeptide is a chain of amino acids

Standard amino acids

Amino acids are the basic structural building units of proteins. They form short polymer chains called peptides or longer chains called either polypeptides or proteins. These polymers are linear and unbranched. The process of making proteins is called *translation* and involves the step-by-step addition of amino acids to a growing protein chain by a ribozyme that is called a ribosome.[13] The order in which the amino acids are added is read through the genetic code from an mRNA template, which is a RNA copy of one of the organism's genes. Twenty amino acids are encoded by the standard genetic code and are called proteinogenic or *standard amino acids*.
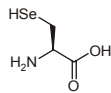
The amino acid selenocysteine

Non-standard Amino Acids

Aside from the twenty standard amino acids, there are a vast number of "non-standard" amino acids. Two of these can be specified by the genetic code, but are rather rare in proteins. Selenocysteine is incorporated into some proteins at a UGA codon, which is normally a stop codon. Pyrrolysine is used by some methanogenic archaea in enzymes that they use to produce methane. It is coded for with the codon UAG. Other non-standard amino acids found in proteins are formed by post-translational modification, which is modification after translation in protein synthesis. These modifications are often essential for the function or regulation of a protein; for example, the carboxylation of glutamate allows for better binding of calcium cations, and the hydroxylation of proline is critical for maintaining connective

tissues. Such modifications can also determine the localization of the protein, e.g., the addition of long hydrophobic groups can cause a protein to bind to a phospholipid membrane.
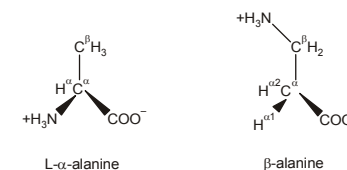
Fig. 2.48 : α-alanine and its β-alanine isomer

Examples of nonstandard amino acids that are not found in proteins include lanthionine, 2-aminoisobutyric acid, dehydroalanine and the neurotransmitter gamma-aminobutyric acid. Nonstandard amino acids often occur as intermediates in the metabolic pathways for standard amino acids — for example ornithine and citrulline occur in the urea cycle, part of amino acid catabolism (see below). A rare exception to the dominance of á-amino acids in biology is the â-amino acid beta alanine (3-aminopropanoic acid), which is used in plants and microorganisms in the synthesis of pantothenic acid (vitamin B5), a component of coenzyme A.

*In human nutrition*When taken up into the body in the diet, the 20 standard amino acids are either used to synthesize proteins and other biomolecules or oxidized to urea and carbon dioxide as a source of energy. The oxidation pathway starts with the removal of the amino group by a transaminase, the amino group is then fed into the urea cycle. The other product of transamidation is a keto acid that enters the citric acid cycle. Glucogenic amino acids can also be converted into glucose, through gluconeogenesis.

Of the 20 standard amino acids, 8 are called essential amino acids because the human body cannot synthesize them from other compounds at the level needed for normal growth, so they must be obtained from food. However, the situation is quite complicated since cysteine, taurine, tyrosine, histidine and arginine are semiessential amino acids in children, because the metabolic pathways that synthesize these amino acids are not fully developed. The amounts required also depend on the age and health of the individual, so it is hard to make general statements about the dietary requirement for some amino acids.

Essential	Nonessential		Tryptophan	Glycine*
Isoleucine	Alanine		Valine	Proline*
Leucine	Asparagine			Serine*
Lysine	Aspartate			Tyrosine*
Methionine	Cysteine*			Arginine*
Phenylalanine	Glutamate			Histidine*
Threonine	Glutamine*		(*) Essential only in certain cases.	

Several common mnemonics have evolved for remembering the ten amino acids often described as essential. PVT TIM HALL ("Private Tim Hall") uses the first letter of each of these amino acids.[29] Another mnemonic that frequently occurs in student practice materials beneath "AH TV TILL Past Midnight", is "These ten valuable amino acids have long preserved life in man".

Non-protein Functions

In humans, non-protein amino acids also have important roles as metabolic intermediates, such as in the biosynthesis of the neurotransmitter gamma-aminobutyric acid. Many amino acids are used to synthesize other molecules, for example:

- Tryptophan is a precursor of the neurotransmitter serotonin.
- Glycine is a precursor of porphyrins such as heme.
- Arginine is a precursor of nitric oxide.
- Ornithine and S-adenosylmethionine are precursors of polyamines.
- Aspartate, glycine and glutamine are precursors of nucleotides.

However, not all of the functions of other abundant non-standard amino acids are known, for example taurine is a major amino acid in muscle and brain tissues, but although many functions have been proposed, its precise role in the body has not been determined.

Some non-standard amino acids are used as defenses against herbivores in plants. For example canavanine is an analogue of arginine that is found in many legumes, and in particularly large amounts in *Canavalia gladiata* (sword bean). This amino acid protects the plants from predators such as insects and can cause illness in people if some types of legumes are eaten without processing. The non-protein amino acid mimosine is found in other species of legume, particularly *Leucaena leucocephala*. This compound is an analogue of tyrosine and can poison animals that graze on these plants.

Uses in Technology

Amino acids are used for a variety of applications in industry. The major use for these compounds is as an additive to animal feed, since many of the bulk components of these products, such as soybeans, either have low levels or lack some of the essential amino acids: lysine, methionine, threonine, and tryptophan are most important in the production of these feeds. The food industry is also a major consumer of amino acids, particularly glutamic acid, which is used as a flavor enhancer, and Aspartame (aspartyl-phenylalanine-1-methyl ester) as a low-calorie artificial sweetener. The remaining production of amino acids is used in the synthesis of drugs and cosmetics.

Amino acid derivative	Pharmaceutical application
5-HTP (5-hydroxytryptophan)	Experimental treatment for depression.
L-DOPA (L-dihydroxyphenylalanine)	Treatment for Parkinsonism.
Eflornithine	Drug that inhibits ornithine decarboxylase and is used in the treatment of sleeping sickness.

Chiral Catalysts

In the chemical industry, amino acids are important as low-cost feedstocks in chiral synthesis. Here, these compounds are used in chiral pool synthesis as enantiomerically-pure building blocks that can be assembled into the desired chiral product. Alternatively, amino acids can be used to create chiral catalysts, such as by incorporating a ruthenium atom into proline to produce a catalyst that can carry out asymmetric hydrogenation reactions.

Biodegradable Plastics

Amino acids are under development as components of a range of biodegradable polymers. These materials have applications as environmentally-friendly packaging and in medicine in drug delivery and the construction of prosthetic implants. These polymers include polypeptides, polyamides, polyesters, polysulfides and polyurethanes with amino acids either forming part of their main chains, or bonded as side chains to modify the physical properties and reactivities of the polymers. An interesting example of such materials is polyaspartate, a water-soluble biodegradable polymer that may have applications in disposable diapers and agriculture. Due to its solubility and ability to chelate metal ions, polyaspartate is also being used as a biodegradeable anti-scaling agent and a corrosion inhibitor. In addition, the aromatic amino acid tyrosine is being developed as a possible replacement for toxic phenols such as bisphenol A in the manufacture of polycarbonates.

Reactions: As amino acids have both a primary amine group and a primary carboxyl group, these chemicals can undergo most of the reactions associated with these functional groups. These include nucleophilic addition, amide bond formation and imine formation for the amine group and esterification, amide bond formation and decarboxylation for the carboxylic acid group. The multiple side chains of amino acids can also undergo chemical reactions. The types of these reactions are determined by the groups on these side chains and are discussed in the articles dealing with each specific type of amino acid.
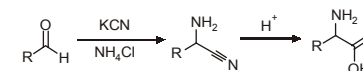
The Strecker Amino Acid Synthesis

Chemical synthesis Several methods exist to synthesize amino acids. One of the oldest uses the Hell-Volhard-Zelinsky halogenation to introduce a bromine atom on the á-carbon. Nucleophilic substitution of the bromine with ammonia then yields the amino acid. Alternatively, the Strecker amino acid synthesis involves the treatment of an aldehyde with potassium cyanide and ammonia, this produces an á-amino nitrile as an intermediate. Hydrolysis of the nitrile in acid then yields a á-amino acid. Using ammonia or ammonium salts in this reaction gives unsubstituted amino acids, while substituting primary and secondary amines will yield substituted amino acids. Likewise, using ketones, instead of aldehydes, gives á,á-disubstituted amino acids. The classical synthesis gives racemic mixtures of á-amino acids as products, but several alternative procedures using asymmetric auxiliaries or asymmetric catalysts have been developed.

Peptide Bond Formation

As both the amine and carboxylic acid groups of amino acids can react to form amide bonds, one amino acid molecule can react with another and become joined through an amide linkage. This polymerization of amino acids is what creates proteins. This condensation reaction yields the newly formed peptide bond and a molecule of water. In cells, this reaction does not occur directly; instead the amino acid is first activated by attachment to a transfer RNA molecule through an ester bond. This aminoacyl-tRNA is produced in an ATP-dependent reaction carried out by an aminoacyl tRNA synthetase. This aminoacyl-tRNA is then a substrate for the ribosome, which catalyzes the attack of the amino group of the elongating protein chain on the ester bond. As a result of this mechanism, all proteins made by ribosomes are synthesized starting at their N-terminus and moving towards their C-terminus.
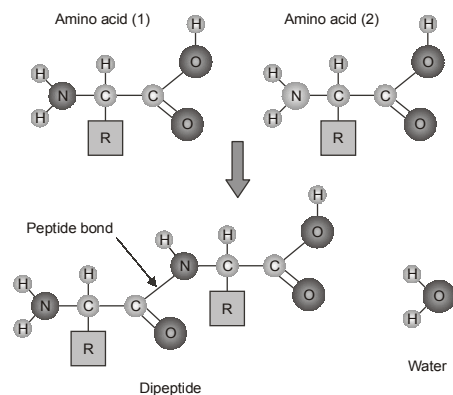
Fig. 2.49 : The condensation of two amino acids to form a peptide bond

However, not all peptide bonds are formed in this way. In a few cases, peptides are synthesized by specific enzymes. For example, the tripeptide glutathione is an essential part of the defenses of cells against oxidative stress. This peptide is synthesized in two steps from free amino acids. In the first step gamma-glutamylcysteine synthetase condenses cysteine and glutamic acid through a peptide bond formed between the side-chain carboxyl of the glutamate (the gamma carbon of this side chain) and the amino group of the cysteine. This dipeptide is then condensed with glycine by glutathione synthetase to form glutathione.

In chemistry, peptides are synthesized by a variety of reactions. One of the most used in solid-phase peptide synthesis, which uses the aromatic oxime derivatives of amino acids as activated units. These are added in sequence onto the growing peptide chain, which is attached to a solid resin support. The ability to easily synthesize vast numbers of different peptides by varying the types and order of

amino acids (using combinatorial chemistry) has made peptide synthesis particularly important in creating libraries of peptides for use in drug discovery through high-throughput screening.

Biosynthesis and catabolism In plants, nitrogen is first assimilated into organic compounds in the form of glutamate, formed from alpha-ketoglutarate and ammonia in the mitochondrion. In order to form other amino acids, the plant uses transaminases to move the amino group to another alpha-keto carboxylic acid. For example, aspartate aminotransferase converts glutamate and oxaloacetate to alpha-ketoglutarate and aspartate. Other organisms use transaminases for amino acid synthesis too. Transaminases are also involved in breaking down amino acids. Degrading an amino acid often involves moving its amino group to alpha-ketoglutarate, forming glutamate. In many vertebrates, the amino group is then removed through the urea cycle and is excreted in the form of urea. However, amino acid degradation can produce uric acid or ammonia instead. For example, serine dehydratase converts serine to pyruvate and ammonia.

Nonstandard amino acids are usually formed through modifications to standard amino acids. For example, homocysteine is formed through the transsulfuration pathway or by the demethylation of methionine via the intermediate metabolite S-adenosyl methionine, while hydroxyproline is made by a posttranslational modification of proline.

Microorganisms and plants can synthesize many uncommon amino acids. For example, some microbes make 2-aminoisobutyric acid and lanthionine, which is a sulfide-bridged alanine dimer. Both these amino acids are found in peptidic lantibiotics such as alamethicin. While in plants, 1-aminocyclopropane-1-carboxylic acid is a small disubstituted cyclic amino acid that is a key intermediate in the production of the plant hormone ethylene.

Hydrophilic and Hydrophobic Amino Acids

Depending on the polarity of the side chain, amino acids vary in their hydrophilic or hydrophobic character. These properties are important in protein structure and protein-protein interactions. The importance of the physical properties of the side chains comes from the influence this has on the amino acid residues' interactions with other structures, both within a single protein and between proteins. The distribution of hydrophilic and hydrophobic amino acids determines the tertiary structure of the protein, and their physical location on the outside structure of the proteins influences their quaternary structure.

For example, soluble proteins have surfaces rich with polar amino acids like serine and threonine, while integral membrane proteins tend to have outer ring of hydrophobic amino acids that anchors them into the lipid bilayer, and proteins anchored to the membrane have a hydrophobic end that locks into the membrane. Similarly, proteins that have to bind to positively-charged molecules have surfaces rich with negatively charged amino acids like glutamate and aspartate, while proteins binding to negatively-charged molecules have surfaces rich with positively charged chains like lysine and arginine. Recently a new scale of hydrophobicity based on the free energy of hydrophobic association has been proposed.

Hydrophilic and hydrophobic interactions of the proteins do not have to rely only on the sidechains of amino acids themselves. By various posttranslational modifications other chains can be attached to the proteins, forming hydrophobic lipoproteins, or hydrophilic glycoproteins.

Table of standard amino acid abbreviations and side chain properties

List of standard amino acids

Amino Acid	3-Letter	1-Letter	Side chain polarity	Side chain charge (pH 7)	Hydropathy index
Alanine	Ala	A	nonpolar	neutral	1.8
Arginine	Arg	R	polar	positive	-4.5
Asparagine	Asn	N	polar	neutral	-3.5
Aspartic acid	Asp	D	polar	negative	-3.5
Cysteine	Cys	C	nonpolar	neutral	2.5
Glutamic acid	Glu	E	polar	negative	-3.5
Glutamine	Gln	Q	polar	neutral	-3.5
Glycine	Gly	G	nonpolar	neutral	-0.4
Histidine	His	H	polar	positive	-3.2
Isoleucine	Ile	I	nonpolar	neutral	4.5
Leucine	Leu	L	nonpolar	neutral	3.8
Lysine	Lys	K	polar	positive	-3.9
Methionine	Met	M	nonpolar	neutral	1.9
Phenylalanine	Phe	F	nonpolar	neutral	2.8
Proline	Pro	P	nonpolar	neutral	-1.6
Serine	Ser	S	polar	neutral	-0.8
Threonine	Thr	T	polar	neutral	-0.7
Tryptophan	Trp	W	nonpolar	neutral	-0.9
Tyrosine	Tyr	Y	polar	neutral	-1.3
Valine	Val	V	nonpolar	neutral	4.2

In addition to the specific amino acid codes, placeholders were used historically in cases where chemical or crystallographic analysis of a peptide or protein could not conclusively determine the identity of a residue.

Ambiguous Amino Acids	3-Letter	1-Letter
Asparagine or aspartic acid	Asx	B
Glutamine or glutamic acid	Glx	Z
Leucine or Isoleucine	Xle	J
Unspecified or unknown amino acid	Xaa	X

Unk is sometimes used instead of Xaa, but is less standard.

Alanine (abbreviated as Ala or A) is an á-amino acid with the chemical formula $CH_3CH(NH_2)COOH$. The L-isomer is one of the 20 proteinogenic amino acids, i.e. the building blocks of proteins. Its codons are GCU, GCC, GCA, and GCG. It is classified as a nonpolar amino acid. L-alanine is second only to leucine, accounting for 7.8% of the primary structure in a sample of 1,150 proteins.[2] D-alanine occurs in bacterial cell walls and in some peptide antibiotics.

Structure The á-carbon atom of alanine is bound with a methyl group($-CH_3$), making it one of the simplest á-amino acids with respect to molecular structure and also resulting in alanine being classified as an aliphatic amino acid. The methyl group of alanine is non-reactive and is thus almost never directly involved in protein function.
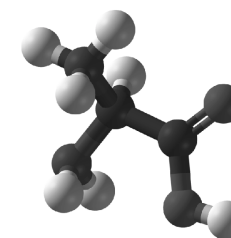
Dietary Sources

Alanine is a nonessential amino acid, meaning it can be manufactured by the human body, and does not need to be obtained directly through the diet. Alanine is found in a wide variety of foods, but is particularly concentrated in meats.
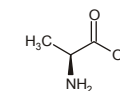
Good sources of alanine include:

- Animal sources: meat, seafood, caseinate, dairy products, eggs, fish, gelatin, lactalbumin
- Vegetarian sources: beans, nuts, seeds, soy, whey, brewer's yeast, brown rice bran, corn, legumes, whole grains.

Biosynthesis Alanine can be manufactured in the body from pyruvate and branched chain amino acids such as valine, leucine, and isoleucine.

Alanine is most commonly produced by reductive amination of pyruvate. Because transamination reactions are readily reversible and pyruvate pervasive, alanine can be easily formed and thus has close links to metabolic pathways such as glycolysis, gluconeogenesis, and the citric acid cycle. It also arises together with lactate and generates glucose from protein via the alanine cycle.

Chemical Synthesis

Racemic alanine can be prepared via the condensation of acetaldehyde with ammonium chloride in the presence of potassium cyanide by the Strecker reaction.

Physiological function As a carrier of ammonia and of the carbon skeleton of pyruvate in alanine cycle

Alanine plays a key role in glucose-alanine cycle between tissues and liver. In muscle and other tissues that degrade amino acids for fuel, amino groups are collected in the form of glutamate by transamination. Glutamate can then transfer its amino group through the action of alanine aminotransferase to pyruvate, a product of muscle glycolysis, forming alanine and alpha-ketoglutarate. The alanine formed is passed into the blood and transported to the liver. A reverse of the alanine aminotransferase

reaction takes place in liver. Pyruvate regenerated forms glucose through gluconeogenesis, which returns to muscle through the circulation system. Glutamate in the liver enters mitochondria and degrades into ammonium ion through the action of glutamate dehydrogenase, which in turn participate in the urea cycle to form urea.[4]

Glucose-alanine cycle enables pyruvate and glutamate to be removed from muscle and find their ways to liver. Glucose is able to be regenerated from pyruvate and returned to muscle. The energetic burden of gluconeogenesis is thus imposed on the liver instead of the muscle. All available ATP in muscle is devoted to muscle contraction.

Link to Hypertension

An international study led by Imperial College London found a correlation between high levels of alanine and higher blood pressure, energy intake, cholesterol levels, and body mass index.

Chemical Properties

Free Radical Stability

The deamination of an alanine molecule produces a stable alkyl free radical, $CH_3C^\bullet HCOO^-$. Deamination can be induced in solid or aqueous alanine by radiation.

This property of alanine is used in dosimetric measurements in radiotherapy. When normal alanine is irradiated, the radiation causes certain alanine molecules to become free radicals, and, as these radicals are stable, the free radical content can later be measured in order to find out how much radiation the alanine was exposed to. In this way, one can be assured that complex radiotherapy treatment plans will deliver the intended pattern of radiation dose.

Alliin is a sulfoxide that is a natural constituent of fresh garlic. It is a derivative of the amino acid cysteine. When fresh garlic is chopped or crushed, the enzyme alliinase converts alliin into allicin which is primarily responsible for the aroma of fresh garlic. Garlic has been used since antiquity as a therapeutic remedy for oxygen toxicity, and when this was investigated, garlic did indeed show strong antioxidant and hydroxyl radical scavenging properties, possibly owing to the alliin contained within.

When the effect of alliin is observed on blood cells in vitro, a noted increase in the engulfing capacity of phagocyting cells is seen.

Arginine (abbreviated as **Arg** or **R**)[1] is an á-amino acid. The L-form is one of the 20 most common natural amino acids. Its codons are CGU, CGC, CGA, CGG, AGA, and AGG. In mammals, arginine is classified as a semiessential or conditionally essential amino acid, depending on the developmental stage and health status of the individual. Infants are unable to meet their requirements and thus arginine is nutritionally essential for infants. Arginine was first isolated from a lupin seedling extract in 1886 by the Swiss chemist Ernst Schultze.
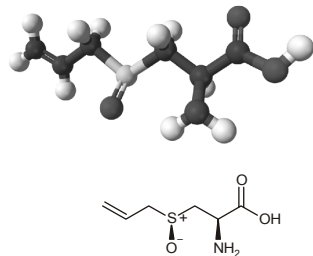
Structure

Arginine consists of a 4-carbon aliphatic straight chain, the distal end of which is capped by a complex guanidinium group. With a pK_a of 12.48, the guanidinium group is positively charged in neutral, acidic and even most basic environments, and thus imparts basic chemical properties to arginine. Because of the conjugation between the double bond and the nitrogen lone pairs, the positive charge is de-localized, enabling the formation of multiple H-bonds.
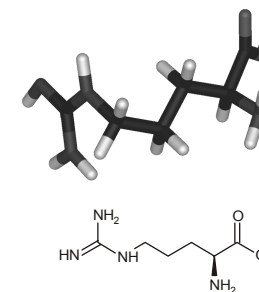
Sources

Dietary Sources

Arginine is a conditionally essential amino acid, meaning most of the time it can be manufactured by the human body, and does not need to be obtained directly through the diet. The biosynthetic pathway however does not produce sufficient arginine, and some must still be consumed through diet. Individuals who have poor nutrition or certain physical conditions may be advised to increase their intake of foods containing arginine. Arginine is found in a wide variety of foods, including

- **Animal sources**: dairy products (e.g. cottage cheese, ricotta, milk, yogurt, whey protein drinks), beef, pork (e.g. bacon, ham), poultry (e.g. chicken and turkey light meat), wild game (e.g. pheasant, quail), seafood (e.g. halibut, lobster, salmon, shrimp, snails, tuna in water)
- **Vegetable sources**: wheat germ and flour, buckwheat, granola, oatmeal, nuts (coconut, pecans, cashews, walnuts, almonds, Brazil nuts, hazelnuts, pinenuts, peanuts), seeds (pumpkin, sesame, sunflower), chick peas, cooked soybeans

Biosynthesis

Arginine is synthesized from citrulline by the sequential action of the cytosolic enzymes argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL). This is energetically costly, as the synthesis of each molecule of argininosuccinate requires hydrolysis of adenosine triphosphate (ATP) to adenosine monophosphate (AMP); *i.e.*, two ATP equivalents.

Citrulline can be derived from multiple sources:

- from arginine via nitric oxide synthase (NOS)
- from ornithine via catabolism of proline or glutamine/glutamate
- from asymmetric dimethylarginine (ADMA) via DDAH

The pathways linking arginine, glutamine, and proline are bidirectional. Thus, the net utilization or production of these amino acids is highly dependent on cell type and developmental stage.

On a whole-body basis, synthesis of arginine occurs principally via the intestinal–renal axis, wherein epithelial cells of the small intestine, which produce citrulline primarily from glutamine and glutamate, collaborate with the proximal tubule cells of the kidney, which extract citrulline from the circulation

and convert it to arginine, which is returned to the circulation. Consequently, impairment of small bowel or renal function can reduce endogenous arginine synthesis, thereby increasing the dietary requirement.

Synthesis of arginine from citrulline also occurs at a low level in many other cells, and cellular capacity for arginine synthesis can be markedly increased under circumstances that also induce iNOS. Thus, citrulline, a coproduct of the NOS-catalyzed reaction, can be recycled to arginine in a pathway known as the citrulline-NO or arginine-citrulline pathway. This is demonstrated by the fact that in many cell types, citrulline can substitute for arginine to some degree in supporting NO synthesis. However, recycling is not quantitative because citrulline accumulates along with nitrate and nitrite, the stable end-products of NO, in NO-producing cells.

Function

Arginine plays an important role in cell division, the healing of wounds, removing ammonia from the body, immune function, and the release of hormones. Arginine, taken in combination with proanthocyanidins[4] or yohimbine[5], has also been used as a treatment for erectile dysfunction.

The benefits and functions attributed to oral ingestion of L-arginine include:
- Precursor for the synthesis of nitric oxide (NO)
- Stimulation of the release of growth hormone.
- Improves immune function
- Reduces healing time of injuries (particularly bone)
- Quickens repair time of damaged tissue
- Reduces risk of heart disease
- Increases muscle mass
- Reduces adipose tissue body fat
- Helps improve insulin sensitivity
- Helps decrease blood pressure
- Alleviates male infertility, improving sperm production and motility
- Increases circulation throughout the body, including the sex organs

In proteins

The geometry, charge distribution and ability to form multiple H-bonds make arginine ideal for binding negatively charged groups. For this reason arginine prefers to be on the outside of the proteins where it can interact with the polar environment. Incorporated in proteins, arginine can also be converted to citrulline by PAD enzymes. In addition, arginine can be methylated by protein methyltransferases.

As a precursor

Arginine is the immediate precursor of NO, urea, ornithine and agmatine; is necessary for the synthesis of creatine; and can also be used for the synthesis of polyamines (mainly through ornithine and to a

lesser degree through agmatine), citrulline, and glutamate. For being a precursor of NO, (relaxes blood vessels), arginine is used in many conditions where vasodilation is required. The presence of asymmetric dimethylarginine (ADMA), a close relative, inhibits the nitric oxide reaction; therefore, ADMA is considered a marker for vascular disease, just as L-arginine is considered a sign of a healthy endothelium.

Treatment of herpes simplex virus

A low ratio of arginine to lysine may be of benefit in the treatment of herpes simplex virus. For more information, refer to Herpes - Treatment.

Possible increases in risk of death from heart disease

A clinical trial found that patients taking an L-arginine supplement following a heart attack didn't improve in their vascular tone or their hearts' ability to pump. In fact, more patients died who were taking L-arginine than placebo and the study was stopped early with the recommendation the supplement not be used by heart attack patients. The supplement still is widely marketed.

Lung inflammation and asthma

The Mayo Clinic web page on L-arginine reports that inhalation of L-arginine can increase lung inflammation and worsen asthma. No primary source for this observation is cited, making it difficult to judge the validity and clinical significance of this effect.

Growth hormone

Arginine increases the production of growth hormone. Reports of its effects on male muscular development are not clearly proven.

Asparagine (abbreviated as **Asn** or **N**; **Asx** or **B** represent either asparagine or aspartic acid) is one of the 20 most common natural amino acids on Earth. It has carboxamide as the side chain's functional group. It is not an essential amino acid. Its codons are AAU and AAC.

A reaction between asparagine and reducing sugars or reactive carbonyls produces acrylamide (acrylic amide) in food when heated to sufficient temperature. These products occur in baked goods such as french fries, potato chips, and roasted coffee.

History Asparagine was first isolated in 1806 from asparagus juice, in which it is abundant — hence its name — becoming the first amino acid to be isolated. The characteristic smell observed in the urine of individuals after their consumption of asparagus is attributed to various metabolic byproducts of asparagine.

Structural function in proteins Since the asparagine side chain can form hydrogen bond interactions with the peptide backbone, asparagine residues are often found near the beginning and the end of alpha-helices, and in turn motifs in beta sheets. Its role can be thought
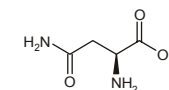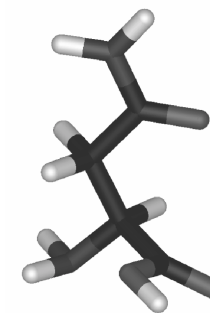
as "capping" the hydrogen bond interactions which would otherwise be satisfied by the polypeptide backbone. Glutamines, with an extra methylene group, have more conformational entropy and thus are less useful in this regard.

Asparagine also provides key sites for N-linked glycosylation, modification of the protein chain with the addition of carbohydrate chains.

Dietary Sources Asparagine is not an essential amino acid, which means that it can be synthesized from central metabolic pathway intermediates in humans and is not required in the diet. Asparagine is found in:

- **Animal sources**: dairy, whey, beef, poultry, eggs, fish, lactalbumin, seafood
- **Vegetarian sources**: asparagus, potatoes, legumes, nuts, seeds, soy, whole grains

Biosynthesis The precursor to asparagine is oxaloacetate. Oxaloacetate is converted to aspartate using a transaminase enzyme. The enzyme transfers the amino group from glutamate to oxaloacetate producing á-ketoglutarate and aspartate. The enzyme asparagine synthetase produces asparagine, AMP, glutamate, and pyrophosphate from aspartate, glutamine, and ATP. In the asparagine synthetase reaction, ATP is used to activate aspartate, forming â-aspartyl-AMP. Glutamine donates an ammonium group which reacts with â-aspartyl-AMP to form asparagine and free AMP.
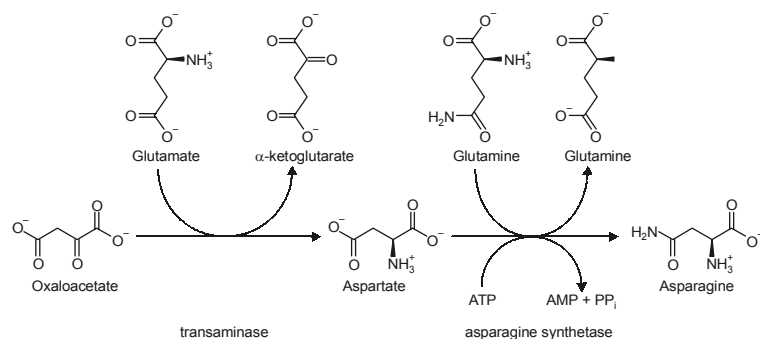
Fig. 2.50 : The biosynthesis of asparagine from oxaloacetate

Degradation

Aspartate is a glucogenic amino acid. L-asparaginase hydrolyzes the amide group to form aspartate and ammonium. A transaminase converts the aspartate to oxaloacetate which can then be metabolized in the citric acid cycle or gluconeogenesis.

Aspartic acid (abbreviated as **Asp** or **D**; **Asx** or **B** represent either aspartic acid or asparagine)[1] is an á-amino acid with the chemical formula $HO_2CCH(NH_2)CH_2CO_2H$. The carboxylate anion of aspartic

acid is known as **aspartate**. The L-isomer of aspartate is one of the 20 proteinogenic amino acids, i.e., the building blocks of proteins. Its codons are GAU and GAC.

Aspartic acid is, together with glutamic acid, classified as an acidic amino acid with a pKa of 4.0. Aspartic acid is pervasive in biosynthesis. As with all amino acids, the location of acid protons depends on the pH of the solution and the crystallization conditions.
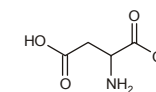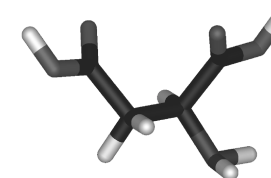
Role in biosynthesis of amino acids

Aspartic acid is non-essential in mammals, being produced from oxaloacetate by transamination. In plants and microorganisms, aspartic acid is the precursor to several amino acids, including four that are essential: methionine, threonine, isoleucine, and lysine. The conversion of aspartic acid to these other amino acids begins with reduction of aspartic acid to its "semialdehyde," $HO_2CCH(NH_2)CH_2CHO$.[2] Asparagine is derived from aspartic acid via transamidation:

$HO_2CCH(NH_2)CH_2CO_2H + GC(O)NH_2$ $HO_2CCH(NH_2)CH_2CONH_2 + GC(O)OH$
(where $GC(O)NH_2$ and $GC(O)OH$ are glutamine and glutamic acid, respectively)
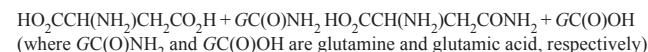
Other biochemical roles

Aspartate is also a metabolite in the urea cycle and participates in gluconeogenesis. It carries reducing equivalents in the malate-aspartate shuttle, which utilizes the ready interconversion of aspartate and oxaloacetate, which is the oxidized (dehydrogenated) derivative of malic acid. Aspartic acid donates one nitrogen atom in the biosynthesis of inositol, the precursor to the purine bases.

Neurotransmitter

Aspartate (the conjugate base of aspartic acid) stimulates NMDA receptors, though not as strongly as the amino acid neurotransmitter glutamate does. It serves as an excitatory neurotransmitter in the brain and is an excitotoxin.

As a neurotransmitter, aspartic acid may provide resistance to fatigue, and, thus, leads to endurance, although the evidence to support this idea is not strong.

Dietary Sources

Aspartic acid is not an essential amino acid, which means that it can be synthesized from central metabolic pathway intermediates in humans. Aspartic acid is found in:

- **Animal sources**: luncheon meats, sausage meat, wild game
- **Vegetable sources**: sprouting seeds, oat flakes, avocado, asparagus.
- **Dietary supplements**

Chemical Synthesis

Racemic aspartic acid can be synthesized from diethyl sodium phthalimidomalonate, $(C_6H_4(CO)_2NC(CO_2Et)_2)$.

β-peptides consist of β amino acids, which have their amino group bonded to the β carbon rather than the β carbon as in the 20 standard biological amino acids. The only commonly naturally occurring β amino acid is β-alanine; although it is used as a component of larger bioactive molecules, β-peptides in general do not appear in nature. For this reason β-peptide-based antibiotics are being explored as ways of evading antibiotic resistance. Pioneering studies in this field were published in 1996 by the group of Dieter Seebach and that of Gellman

L-α-alanine

β-alanine
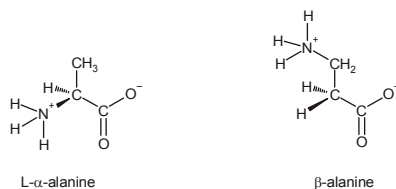
Chemical Structure and Synthesis

In á amino acids (molecule at left), both the carboxylic acid group (red) and the amino group (blue) are bonded to the same carbon, termed the á carbon ($C^á$) because it is one atom away from the carboxylate group. In â amino acids, the amino group is bonded to the â carbon ($C^â$), which is found in most of the 20 standard amino acids. Only glycine lacks a â carbon, which means that there is no á-glycine molecule.

The chemical synthesis of â amino acids can be challenging, especially given the diversity of functional groups bonded to the â carbon and the necessity of maintaining chirality. In the alanine molecule shown, the â carbon is achiral; however, larger amino acids have a chiral $C^â$ atom. A number of synthesis mechanisms have been introduced to efficiently form â amino acids and their derivatives notably those based on the Arndt-Eistert synthesis. Two main types of â-peptides exist: those with the

α-peptide

β³-peptide
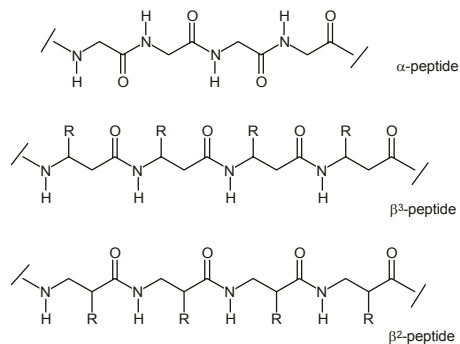
β²-peptide

organic residue (R) next to the amine are called â³-peptides and those with position next to the carbonyl group are called â²-peptides.

Secondary Structure

Because the backbones of â-peptides are longer than those of peptides that consist of á-amino acids, â-peptides form different secondary structures. The alkyl substituents at both the and â positions in a a â amino acid favor a gauche conformation about the bond between the á-carbon and â-carbon. This also affects the thermodynamic stability of the structure.

Many types of helix structures consisting of â-peptides have been reported. These conformation types are distinguished by the number of atoms in the hydrogen-bonded ring that is formed in solution; 8-helix, 10-helix, 12-helix, 14-helix, and 10/12-helix have been reported. Generally speaking, â-peptides form a more stable helix than á-peptides

The â-peptide *zwit-1F* with a fully described quaternary structure is called a **â-protein** because it has many characteristics of an actual protein. Eight 12-helix units self-assemble in water to a superstructure with a hydrophobic inner core.

Clinical potential

â-peptides are stable against proteolytic degradation in vitro and in vivo, an important advantage over natural peptides in the preparation of peptide-based drugs. â-peptides have been used to mimic natural peptide-based antibiotics such as magainins, which are extremely powerful but difficult to use as drugs because they are degraded by proteolytic enzymes in the body.

Citrulline is an á-amino acid. Its name is derived from *citrullus*, the Latin word for watermelon, from which it was first isolated in 1930. It has the idealized formula $H_2NC(O)NH(CH_2)_3CH(NH_2)CO_2H$. It is a key intermediate in the urea cycle, the pathway by which mammals excrete ammonia.
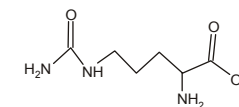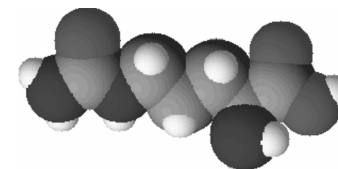
Biosynthesis

Citrulline is made from ornithine and carbamoyl phosphate in one of the central reactions in the urea cycle. It is also produced from arginine as a by-product of the reaction catalyzed by NOS family (NOS; EC 1.14.13.39). Arginine is first oxidized into N-hydroxyl-arginine, which is then further oxidized to citrulline concomitant with release of nitric oxide.

Function

Although citrulline is not coded for by DNA directly, several proteins are known to contain citrulline as a result of a posttranslational modification. These citrulline residues are generated by a family of

enzymes called peptidylarginine deiminases (PADs), which convert arginine into citrulline in a process called citrullination or deimination. Proteins that normally contain citrulline residues include myelin basic protein (MBP), filaggrin, and several histone proteins, whereas other proteins, such as fibrin and vimentin are susceptible to citrullination during cell death and tissue inflammation.

Patients with rheumatoid arthritis often have detectable antibodies against proteins containing citrulline. Although the origin of this immune response is not known, detection of antibodies reactive with citrulline (anti-citrullinated protein antibodies) containing proteins or peptides is now becoming an important help in the diagnosis of rheumatoid arthritis.

In recent studies, citrulline has been found to relax blood vessels.

Sources

Citrulline in the form of citrulline malate is sold as a performance-enhancing athletic dietary supplement which is said to reduce muscle fatigue.

The rind of watermelon (*Citrullus lanatus*) is a good natural source of citrulline.

Cystine is the amino acid dimer formed when a pair of cysteine molecules are joined by a disulfide bond. It is described by the formula $(SCH_2CH(NH_2)CO_2H)_2$. It is a colorless solid, and melts at 247-249 °C. It was discovered in 1810 by William Hyde Wollaston but was not recognized as a component of proteins until it was isolated from the horn of a cow in 1899. Through formation of disulfide bonds within and between protein molecules, cystine is a significant determinant of the tertiary structure of most proteins. Disulfide bonding, along with hydrogen bonding and hydrophobic interactions is partially responsible for the formation of the gluten matrix in bread. Human hair contains approximately 5% cystine by mass.
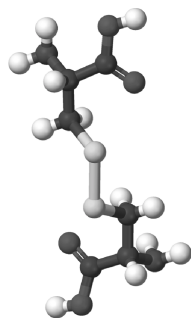
Properties

The disulfide link is readily reduced to give the corresponding thiol, cysteine. This reaction is typically effected with thiols such as mercaptoethanol or dithiothreitol.

$$(SCH_2CH(NH_2)CO_2H)_2 + 2\ RSH \rightarrow 2\ HSCH_2CH(NH_2)CO_2H + RSSR$$
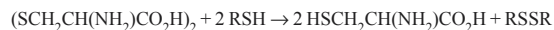
Nutritional Sources

Supplemental N-acetyl cysteine is claimed to be a source of cystine, but the dose of this supplement is limited by side effects. One of the richest nutritional sources of cystine in the diet is undenatured whey proteins from milk The disulfide-bonded cystine is not digested or significantly hydrolized by the stomach, but is transported by the blood stream to the tissues of the body. Here, within the cells of the body, the weak disulfide bond is cleaved to give cysteine, from which glutathione can be synthesized.

In Animal Feed

Disulfide bonds can be broken at temperatures above about 150 °C, especially at low moisture levels (below about 20%).

Side Effects

Nutritional sources of cystine are virtually free of the toxic side effects associated with the single molecule of cysteine, N-acetyl cysteine. The greatest dietary source of cystine is bio-active, unpasteurized or low-heat pasteurized undenatured whey proteins.

Djenkolic acid (or sometimes **jengkolic acid**) is a sulfur-containing non-protein amino acid naturally found in djenkol beans of the South-East Asian legumes jengkol (*Archidendron jiringa*). This compound consists of two cysteine radicals connected by a methylene group between the sulfurs, or it is 2-amino-3-[(2-amino-3-hydroxy-3-oxopropyl) sulfanylmethyl sulfanyl] propanoic acid. It is toxic to humans, especially causing nephrotoxicity.
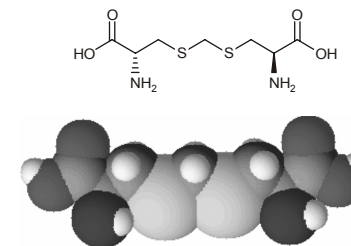
Toxicity

The toxicity of djenkolic acid in humans arises from its poor solubility under acidic conditions after consumption of the jenkol bean. The amino acid precipitates into crystals which cause mechanical irritation of the renal tubules and urinary tract, resulting in symptoms such as abdominal discomfort, loin pains, severe colic, nausea, vomiting, dysuria, gross hematuria, and oliguria, occurring 2 to 6 hours after the beans were ingested. Urine analysis of patients reveals erythrocytes, epithelial cells, protein, and the needle-like crystals of djenkolic acid. Urolithiasis can also happen, with djenkolic acid as the nucleus. In young children it has also been reported to produce painful swelling of the genitalia.

Treatment for this toxicity requires hydration to increase urine flow and alkalinization of urine by sodium bicarbonate. Furthermore, this poisoning can be prevented when consuming djenkol beans by boiling them beforehand, since djenkolic acid is removed from the beans.

Discovery and Synthesis

Djenkolic acid was first isolated by Van Veen and Hyman from the urine of the natives of Java who had eaten the djenkol bean and were suffering from poisoning. They then succeeded in isolating the djenkolic acid crystals from the djenkol beans treated with $Ba(OH)_2$ at 30°C for a prolonged period of time.

Djenkolic acid was later reported to be found as much as 20 grams in every kilogram of dry djenkol beans, and it has also been reported to be found to a lesser extent in the seeds of other leguminous plants such as *Leucaena esculenta* (2.2 g/kg) and *Pithecolobium ondulatum* (2.8 g/kg).

Du Vigneaud and Patterson managed to synthesize djenkolic acid by the condensation of methylene chloride with 2 moles of L-cysteine in liquid ammonia and to show that their synthetic compound was identical with the naturally occurring djenkolic acid. Later on, Armstrong and du Vigneaud prepared

djenkolic acid by the direct combination of 1 mole of formaldehyde with 2 moles of L-cysteine in a strongly acid solution.

Gamma-aminobutyric acid (GABA) is the chief inhibitory neurotransmitter in the mammalian central nervous system. It plays an important role in regulating neuronal excitability throughout the nervous system. In humans, GABA is also directly responsible for the regulation of muscle tone. In insect species GABA acts only on excitatory nerve receptors.

While technically an amino acid, GABA is rarely referred to as such in the scientific or medical communities, because the term "amino acid," used without a qualifier, refers to the alpha amino acids, which GABA is not, nor is it incorporated into proteins. In spastic diplegia in humans, GABA absorption by some nerves becomes damaged, which leads to hypertonia of the muscles signaled by those nerves.
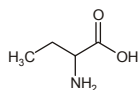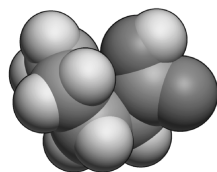
Function as Neurotransmitter

In vertebrates, GABA acts at inhibitory synapses in the brain by binding to specific transmembrane receptors in the plasma membrane of both pre- and postsynaptic neuronal processes. This binding causes the opening of ion channels to allow the flow of either negatively charged chloride ions into the cell or positively charged potassium ions out of the cell. This action results in a negative change in the transmembrane potential, usually causing hyperpolarization. Three general classes of GABA receptor are known: $GABA_A$ and $GABA_C$ ionotropic receptors, which are ion channels themselves, and $GABA_B$ metabotropic receptors, which are G protein-coupled receptors that open ion channels via intermediaries (G proteins).

Neurons that produce GABA as their output are called GABAergic neurons, and have chiefly inhibitory action at receptors in the adult vertebrate. Medium Spiny Cells are a typical example of inhibitory CNS GABAergic cells. In contrast, GABA exhibits excitatory actions in insects, mediating muscle activation at synapses between nerves and muscle cells, and also the stimulation of certain glands.

Whether GABA is excitatory or inhibitory depends on the direction (into or out of the cell) and magnitude of the ionic currents controlled by the $GABA_A$ receptor. When net positive ionic current is directed into the cell, GABA is excitatory, when the net positive current is directed out of the cell, GABA is inhibitory. A developmental switch in the molecular machinery controlling the polarity of this current is responsible for the changes in the functional role of GABA between the neonatal and adult stages. That is to say, GABA's role changes from excitatory to inhibitory as the brain develops into adulthood.

Development

In hippocampus and neocortex of the mammalian brain, GABA has primarily excitatory effects early in development, and is in fact the major excitatory neurotransmitter in many regions of the brain before the maturation of glutamate synapses

In the developmental stages preceding the formation of synaptic contacts, GABA is synthesized by neurons and acts both as an autocrine (acting on the same cell) paracrine (acting on nearby cells) signalling mediator.

GABA regulates the proliferation of neural progenitor cells the migration and differentiation the elongation of neurites and the formation of synapses.

GABA also regulates the growth of embryonic and neural stem cells. GABA can inûuence the development of neural progenitor cells via brain-derived neurotrophic factor (BDNF) expression. GABA activates the $GABA_A$ receptor, causing cell cycle arrest in the S-phase, limiting growth.

Structure and Conformation

GABA is found mostly as a zwitterion, that is, with the carboxyl group deprotonated and the amino group protonated. Its conformation depends on its environment. In the gas phase, a highly folded conformation is strongly favored because of the electrostatic attraction between the two functional groups. The stabilization is about 50 kcal/mol, according to quantum chemistry calculations. In the solid state, a more extended conformation is found, with a trans conformation at the amino end and a gauche conformation at the carboxyl end. This is due to the packing interactions with the neighboring molecules. In solution, five different conformations, some folded and some extended are found as a result of solvation effects. The conformational flexibility of GABA is important for its biological function, as it has been found to bind to different receptors with different conformations. Many GABA analogues with pharmaceutical applications have more rigid structures in order to control the binding better.

History

Gamma-aminobutyric acid was first synthesized in 1883, and was first known only as a plant and microbe metabolic product. In 1950, however, GABA was discovered to be an integral part of the mammalian central nervous system.

Synthesis

Organisms synthesize GABA from glutamate using the enzyme L-glutamic acid decarboxylase and pyridoxal phosphate (which is the active form of B6) as a cofactor. This process converts the principal excitatory neurotransmitter (glutamate) into the principal inhibitory one (GABA).

Pharmacology

Drugs that act as agonists of GABA receptors (known as GABA analogues or *GABAergic* drugs) or increase the available amount of GABA typically have relaxing, anti-anxiety and anti-convulsive effects. Many of the substances below are known to cause anterograde amnesia and retrograde amnesia.

It has been suggested that orally administered GABA increases the amount of Human Growth Hormone, but this is questionable since it is unknown whether GABA can pass the blood-brain barrier. However, orally administered GABA does have effects outside of the central nervous system (e.g. decreased muscle tone).

Drugs that affect GABA receptors:

- alcohol (ethanol)
- avermectins—doramectin, selamectin, ivermectin
- barbiturates
- bicucullines - GABA antagonist
- benzodiazepines
- baclofen
- baicalin and baicalein from skullcap scutellaria lateriflora
- carbamazepines
- cyclopyrrolone derivatives such as zopiclone
- fluoroquinolones
- gabazine (SR-95531)
- gamma-Hydroxybutyric acid (GHB)
- gamma-amino-beta-hydroxybutyric acid
- imidazopyridine derivatives such as zolpidem
- kavalactones
- meprobamate
- muscimol
- manganese
- modafinil
- phenytoin
- picamilon
- picrotoxin
- progabide
- propofol
- phenibut
- pyrazolopyrimidine derivatives such as zaleplon
- thujone—GABA antagonist
- Valerian extract
- pregabalin (tradename Lyrica) - GABA analogue

Drugs that affect GABA in other ways:

- tiagabine—potentiates by inhibiting uptake into neurons and glia
- vigabatrin—potentiates by inhibiting GABA-T, preventing GABA breakdown
- valproate—potentiates by inhibiting GABA-T

- tetanospasmin—primary toxin of tetanus bacteria, blocks release of GABA
- hyperforin—inhibits the reuptake of GABA
- thujone-A main ingredient in absinthe
- theanine - an amino acid found in green tea and the basidiomycete mushroom, increases GABA production

Glutamic acid (abbreviated as **Glu** or **E**) is one of the 20 proteinogenic amino acids and its codons are GAA and GAG. It is a non-essential amino acid. The carboxylate anions and salts of glutamic acid are known as **glutamates**.

Chemistry

The side chain carboxylic acid functional group has pK_a of 4.1 and exists in its negatively charged deprotonated carboxylate form at physiological pH.
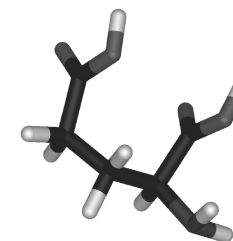
History

This compound was discovered in 1908 by the professor Kikunae Ikeda, who worked in the Imperial University of Tokyo. He loved seaweed which is used like spice in traditional Japanese food. He tried to find the root of this flavour. He discovered that the origin of this taste is glutamic acid. He isolated crystals of glutamic acid using a Kombu soup (one hundred grams of Kombu has nearly one gram of glutamic acid).
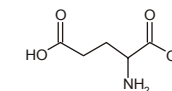
Moreover, he discovered that glutamate gave unique flavour to other foods. He called it "Umami" (meaning "yumminess" in Japanese). This distinctive flavor has brought glutamate the title of "the elusive fifth taste" to join the more traditional flavors, sweet, salty, sour, and bitter. [1]

Biosynthesis

Reactants	Products	Enzymes
Glutamine + H_2O	→ Glu + NH_3	GLS, GLS2
NAcGlu + H_2O	→ Glu + Acetate	(unknown)
α-ketoglutarate + NADPH + NH_4^+	→ Glu + $NADP^+$ + H_2O	GLUD1, GLUD2
α-ketoglutarate + α-amino acid	→ Glu + α-oxo acid	transaminase
1-pyrroline-5-carboxylate + NAD^+ + H_2O	→ Glu + NADH	ALDH4A1
N-formimino-L-glutamate + FH_4	→ Glu + 5-formimino-FH_4	FTCD

Function and Uses

Metabolism

Glutamate is a key molecule in cellular metabolism. In humans, dietary proteins are broken down by digestion into amino acids, which serves as metabolic fuel for other functional roles in the body. A key process in amino acid degradation is transamination, in which the amino group of an amino acid is transferred to an á-ketoacid, typically catalysed by a transaminase. The reaction can be generalised as such:

R_1-amino acid + R_2-á-ketoacid $\rightleftharpoons R_1$-á-ketoacid + R_2-amino acid
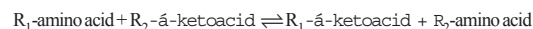
A very common á-ketoacid is á-ketoglutarate, an intermediate in the citric acid cycle. Transamination of á-ketoglutarate gives glutamate. The resulting á-ketoacid product is often a useful one as well, which can contribute as fuel or as a substrate for further metabolism processes. Examples are as follows:

Alanine + á-ketoglutarate $\rightleftharpoons$ pyruvate + glutamate

Aspartate + á-ketoglutarate $\rightleftharpoons$ oxaloacetate + glutamate

Both pyruvate and oxaloacetate are key components of cellular metabolism, contributing as substrates or intermediates in fundamental processes such as glycolysis, gluconeogenesis and also the citric acid cycle.

Glutamate also plays an important role in the body's disposal of excess or waste nitrogen. Glutamate undergoes deamination, an oxidative reaction catalysed by glutamate dehydrogenase, as follows:

glutamate + water + $NADP^+ \rightarrow$ á-ketoglutarate + NADPH + ammonia + H^+

Ammonia (as ammonium) is then excreted predominantly as urea, synthesised in the liver. Transamination can thus be linked to deamination, effectively allowing nitrogen from the amine groups of amino acids to be removed, via glutamate as an intermediate, and finally excreted from the body in the form of urea.

Neurotransmitter

Glutamate is the most abundant excitatory neurotransmitter in the mammalian nervous system. At chemical synapses, glutamate is stored in vesicles. Nerve impulses trigger release of glutamate from the pre-synaptic cell. In the opposing post-synaptic cell, glutamate receptors, such as the NMDA receptor, bind glutamate and are activated. Because of its role in synaptic plasticity, it is believed that glutamic acid is involved in cognitive functions like learning and memory in the brain.

Glutamate transporters are found in neuronal and glial membranes. They rapidly remove glutamate from the extracellular space. In brain injury or disease, they can work in reverse and excess glutamate can accumulate outside cells. This process causes calcium ions to enter cells via NMDA receptor channels, leading to neuronal damage and eventual cell death, and is called excitotoxicity. The mechanisms of cell death include

- Damage to mitochondria from excessively high intracellular Ca^{2+}

- Glu/Ca^{2+}-mediated promotion of transcription factors for pro-apoptotic genes, or downregulation of transcription factors for anti-apoptotic genes.

Excitotoxicity due to glutamate occurs as part of the ischemic cascade and is associated with stroke and diseases like amyotrophic lateral sclerosis, lathyrism, autism, some forms of mental retardation and Alzheimer's disease.

Glutamic acid has been implicated in epileptic seizures. Microinjection of glutamic acid into neurons produces spontaneous depolarisations around one second apart, and this firing pattern is similar to what is known as paroxysmal depolarizing shift in epileptic attacks. This change in the resting membrane potential at seizure foci could cause spontaneous opening of voltage-activated calcium channels, leading to glutamic acid release and further depolarization.

Experimental techniques to detect glutamate in intact cells include using a genetically-engineered nanosensor. The sensor is a fusion of a glutamate-binding protein and two fluorescent proteins. When glutamate binds, the fluorescence of the sensor under ultraviolet light changes by resonance between the two fluorophores. Introduction of the nanosensor into cells enables optical detection of the glutamate concentration. Synthetic analogs of glutamic acid that can be activated by ultraviolet light and two-photon excitation microscopy have also been described. This method of rapidly uncaging by photostimulation is useful for mapping the connections between neurons, and understanding synapse function.

Brain nonsynaptic glutamatergic signaling circuits

Extracellular glutamate in Drosophila brains has been found to regulate postsynaptic glutamate receptor clustering, via a process involving receptor desensitization. A gene expressed in glial cells actively transports glutamate into the extracellular space, while in the nucleus accumbens stimulating group II metabotropic glutamate receptors, this gene was found to reduce extracellular glutamate levels.[6] This raises the possibility that this extracellular glutamate plays an "endocrine-like" role as part of a larger homeostatic system.

GABA precursor

Glutamic acid also serves as the precursor for the synthesis of the inhibitory GABA in GABA-ergic neurons. This reaction is catalyzed by glutamic acid decarboxylase (GAD), which is most abundant in the cerebellum and pancreas.

Stiff-man syndrome is a neurologic disorder caused by anti-GAD antibodies, leading to a decrease in GABA synthesis and therefore, impaired motor function such as muscle stiffness and spasm. Since the pancreas is also abundant for the enzyme GAD, a direct immunological destruction occurs in the pancreas and the patients will have diabetes mellitus.

Flavor enhancer

Free glutamic acid is present in a wide variety of foods, including cheese and soy sauce and is responsible for one of the five basic tastes of the human sense of taste (umami). Glutamic acid is often used as a food additive and flavour enhancer in the form of its sodium salt, monosodium glutamate (MSG).

Nutrient

All meats, poultry, fish, eggs, dairy products, as well as kombu are excellent sources of glutamic acid. Some protein-rich plant foods also serve as sources. Ninety-five percent of the dietary glutamate is metabolized by intestinal cells in a first pass.

Plant growth

Auxigro is a plant growth preparation that contains 30% glutamic acid.

Production

China-based Fufeng Group Limited is the largest producer of glutamic acid in the world, with capacity increasing to 300,000 tons at the end of 2006 from 180,000 tons during 2006, putting them at 25%–30% of the Chinese market. Meihua is the second largest Chinese producer. Together, the top five producers have roughly 50% share in China. Chinese demand is roughly 1.1 million tons per year, while global demand, including China, is 1.7 million tons per year.

Pharmacology

The drug phencyclidine (more commonly known as PCP) antagonizes glutamic acid non-competitively at the NMDA receptor. For the same reasons, sub-anaesthetic doses of Ketamine have strong dissociative and hallucinogenic effects. Glutamate does not easily pass the blood brain barrier, but instead this transport is mediated by a high affinity transport system. It can also be converted into glutamine.

Role in sickle-cell disease

A point mutation (valine in place of glutamic acid at position 6) in the â-globin chain of hemoglobin forms HbS. This variant of hemoglobin can cause sickle-cell anemia, where the abnormal hemoglobin are prone to polymerization when deoxygenated, thus distorting the erythrocyte which are removed by the spleen or cause microvascular obstruction (ischemic crises). This trait and disease is common in areas with a high prevalence of *Plasmodium falciparum* (one of three Plasmodium species that causes malaria).

Glycine (abbreviated as **Gly** or **G**) is the organic compound with the formula NH_2CH_2COOH. It is the smallest of the 20 amino acids commonly found in proteins, coded by codons GGU, GGC, GGA and GGG. Glycine is unique among the proteinogenic amino acids in that it is not chiral. Most proteins contain only small quantities of glycine. A notable exception is collagen, which contains about 35% glycine.[3] In its solid, i.e., crystallized, form, glycine is a free-flowing, sweet-tasting crystalline material.

Production

Glycine is manufactured industrially, either by treating chloroacetic acid with ammonia:

$$ClCH_2COOH + NH_3 \rightarrow H_2NCH_2COOH + HCl$$
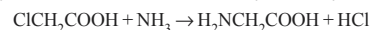
or via the Strecker amino acid synthesis.

There are two producers of glycine in the United States. Chattem Chemicals, Inc., purchased by

Sun Pharmaceutical, Mumbai, India and GEO Specialty Chemicals, Inc., who purchased the glycine and naphthalene sulfonate production facilities of Dow/Hampshire Chemical Corp.

Biosynthesis

Glycine is not essential to the human diet, since it is biosynthesized in the body from the amino acid serine, which is in turn derived from 3-phosphoglycerate. In most organisms, the enzyme Serine hydroxymethyltransferase catalyses this transformation by removing one carbon atom; pyridoxal phosphate is also necessary:

Serine + tetrahydrofolate $\rightarrow$ Glycine + N^5,N^{10}-Methylene tetrahydrofolate + H_2O
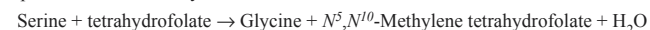
In the liver of vertebrates, glycine synthesis is catalyzed by glycine synthase (also called glycine cleavage enzyme). This conversion is readily reversible:

$CO_2 + NH_4^+ + N^5,N^{10}$-Methylene tetrahydrofolate + NADH + H$^+$ $\rightarrow$ Glycine + tetrahydrofolate + NAD^+

Degradation

Glycine is degraded via three pathways. The predominant pathway in animals involves the catalysis of glycine cleavage enzyme, the same enzyme also involved in the biosynthesis of glycine. The degradation pathway is the reverse of this synthetic pathway:

Glycine + tetrahydrofolate + NAD$^+$ $\rightarrow$ CO$_2$ + NH$_4^+$ + N^5, N^{10}-Methylene tetrahydrofolate + NADH + H$^+$
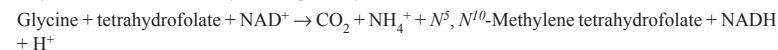
In the second pathway, glycine is degraded in two steps. The first step is the reverse of glycine biosynthesis from serine with serine hydroxymethyl transferase. Serine is then converted to pyruvate by serine dehydratase.

In the third pathway of glycine degradation, glycine is converted to glyoxylate by D-amino acid oxidase. Glycoxylate is then oxidized by hepatic lactate dehydrogenase to oxalate in an NAD$^+$-dependent reaction.

Physiological function

As a biosynthetic intermediate

Glycine is a building block to numerous natural products. In higher eukaryotes, D-Aminolevulinic acid, the key precursor to porphyrins, is biosynthesized from glycine and succinyl-CoA. Glycine provides the central C_2N subunit of all purines.

As a neurotransmitter

Glycine is an inhibitory neurotransmitter in the central nervous system, especially in the spinal cord, brainstem, and retina. When glycine receptors are activated, chloride enters the neuron via ionotropic receptors, causing an Inhibitory postsynaptic potential (IPSP). Strychnine is a strong antagonist at ionotropic glycine receptors, whereas bicuculline is a weak one. Glycine is a required co-agonist along

with glutamate for NMDA receptors. In contrast to the inhibitory role of glycine in the spinal cord, this behaviour is facilitated at the (NMDA) glutaminergic receptors which are excitatory. The LD_{50} of glycine is 7930 mg/kg in rats (oral), and it usually causes death by hyperexcitability.

As a potential antipsychotic

Dr. Daniel Javitt a clinical researcher had studied people who were addicted to PCP ("angel dust") and Ketamine ("special K") (Javitt, DC, Negative Schizophrenic Symptomatology and the Phencyclidine (PCP) Model of Schizophrenia, Hillside Journal of Psychiatry 1987 9:12-35. Their brains had been damaged by the use of this drug. In studies, it was found that their glutamate receptors had been damaged. Since use of PCP and ketamine creates psychosis similar to schizophrenia, it was hypothesized that giving glycine to people with schizophrenia would potentially reduce their psychotic symptoms. In a controlled study people with schizophrenia who were given glycine had their symptoms reduced in a measurable sense, primarily in the area of negative and cognitive symptoms when used as an adjunct to current antipsychotics. There have been some psychiatrists who have used it out of study as a primary antipsychotic with benefits on positive as well as negative and cognitive symptoms. Glycine's primary drawback is its required use in powdered format. However, as an NMDA receptor modulator, it is part of a class of antipsychotics in study that do not cause tardive dyskinesia or diabetes, the current long term side effects of dopaminergic antipsychotics as well as not creating extrapyramidal side effects (movement disorders), weight gain or sedation. These medications along with other new classes of medications in study may eventually replace the current antipsychotics which, from Thorazine to Abilify, have all been based on the dopamine hypothesis and in depleting the levels of dopamine create tardive dykinesia and other Parkinsonian movement disorders and potentially tardive psychosis which is still in study. Glycine, is part of a promising new class of treatment for schizophrenia that may promote a full recovery without debilitating physical side effects. Research continues on glycine and other NMDA receptors modulates, which are currently in Phase II FDA Study. Although other NMDA receptor modulates in study have shown promise as primary antipsychotics, in the specific controlled studies glycine is used as an adjunct antipsychotic and although available should only be taken under the supervision of a psychiatrist.

Industrial uses

Glycine is used as a sweetener/taste enhancer, buffering agent in antiperspirants based on zirconium/aluminum chlorohydrate (ZACH) salts complexed with glycine (aminoethanoic acid), which buffers ZACH salts without hindering performance, reabsorbable amino acid, chemical intermediate, metal complexing agent, and dietary supplement as well as in certain pharmaceuticals.

Antidumping tariffs

Glycine imported from China to the United States has been subject to antidumping duties since March, 1995.

In 2007, a United States manufacturer of Glycine, GEO Specialty Chemicals, Inc. filed petitions requesting that antidumping duties also be imposed on Glycine imported from Japan, the Republic of Korea, and India. On September 7, 2007 the Department of Commerce announced its affirmative

preliminary determinations in the antidumping duty investigations on imports of glycine from Japan and the Republic of Korea (Korea). On October 29, 2007 the Department of Commerce announced its affirmative preliminary determination in the antidumping duty investigation on imports of glycine from India.

Presence in the interstellar medium

In 1994 a team of astronomers at the University of Illinois, led by Lewis Snyder, claimed that they had found the glycine molecule in space. It turned out that, with further analysis, this claim could not be confirmed. Nine years later, in 2003, Yi-Jehng Kuan from National Taiwan Normal University and Steve Charnley claimed that they detected interstellar glycine toward three sources in the interstellar medium.[13] They claimed to have identified 27 spectral lines of glycine utilizing a radio telescope. According to computer simulations and lab-based experiments, glycine was probably formed when ices containing simple organic molecules were exposed to ultraviolet light.

In October 2004, Snyder and collaborators reinvestigated the glycine claim in Kuan *et al.* (2003). In a rigorous attempt to confirm the detection, Snyder showed that glycine was not detected in any of the three claimed sources.

In 2008, the glycine like molecule amino acetonitrile was discovered in the Large Molecule Heimat, a giant gas cloud near the galactic center in the constellation Sagittarius by the Max Planck Institute for Radio Astronomy

Should the glycine claim be substantiated, the finding would not prove that life exists outside the Earth, but certainly makes that possibility more plausible by showing that amino acids can be formed in the interstellar medium.

Histidine is one of the 20 standard amino acids present in proteins. In the nutritional sense, in humans, histidine is considered an essential amino acid, but mostly only in children. Its codons are CAU and CAC. Histidine was first isolated by German physician Albrecht Kossel in 1896.
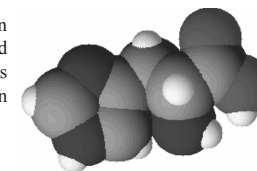
Chemical Properties

The imidazole side chains and the relatively neutral pKa of histidine (ca 6.0) mean that relatively small shifts in cellular pH will change its charge. For this reason, this amino acid side chain finds its way into considerable use as a coordinating ligand in metalloproteins, and also as a catalytic site in certain enzymes. The imidazole side chain has two nitrogens with different properties: One is bound to hydrogen and donates its lone pair to the aromatic ring and as such is slightly acidic, whereas the other one donates only one electron to the ring so it has a free lone pair and is basic. These properties are exploited in different ways in proteins. In catalytic triads, the basic nitrogen of histidine is used to abstract a proton from serine, threonine or cysteine to activate it as a nucleophile. In a histidine proton shuttle, histidine is used to quickly shuttle protons, it can do this by abstracting a proton with its basic
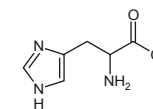
nitrogen to make a positively-charged intermediate and then use another molecule, a buffer, to extract the proton from its acidic nitrogen. In carbonic anhydrases, a histidine proton shuttle is utilized to rapidly shuttle protons away from a zinc-bound water molecule to quickly regenerate the active form of the enzyme.

Histidine can be aromatic. When it is deprotonated, and uncharged, it is not aromatic. It no longer obeys Hückel's rule because 8 electrons are in the ring system (an extra two from the deprotonated nitrogen). Histidine obeys Hückel's rule when it is protonated, so then is aromatic. It can form stacking interactions, but is complicated by the positive charge. It doesn't absorb at 280nm in either state, but does in the lower UV range more than some amino acids.

Metabolism

The amino acid is a precursor for histamine and carnosine biosynthesis.
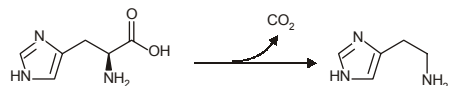
Conversion of **histidine** to histamine by histidine decarboxylase

The enzyme histidine ammonia-lyase converts histidine into ammonia and urocanic acid. A deficiency in this enzyme is present in the rare metabolic disorder histidinemia.

Supplementation

Supplementation of Histidine has been shown to cause rapid zinc excretion in rats with an excretion rate 3 to 6 times normal. Some people take histidine in an attempt to raise their histamine levels. Histamine is released under conditions of higher osmolality (dehydration/salt). Raising histamine may be more effective by adapting the body to a higher osmolality by eating sodium without greatly increasing water intake and taking supplements that help the body adapt to those conditions (zinc/taurine/B1/B12). Histidine supplementation likely tricks the body into believing it is producing less histamine and assumes it is well or overhydrated and is better off without the nutrients used to maintain cellular hydration in a hyperosmotic state. Histidine supplementation however is inadvisable because one of the hallmarks of today's chronic diseases (diabetes, Alzheimer's, Parkinson's) is zinc depletion.

The histidine bound heme group of succinate dehydrogenase, an electron carrier in the mitochondrial electron transfer chain. The large semi-transparent sphere indicates the location of the iron ion. From PDB 1YQ3.
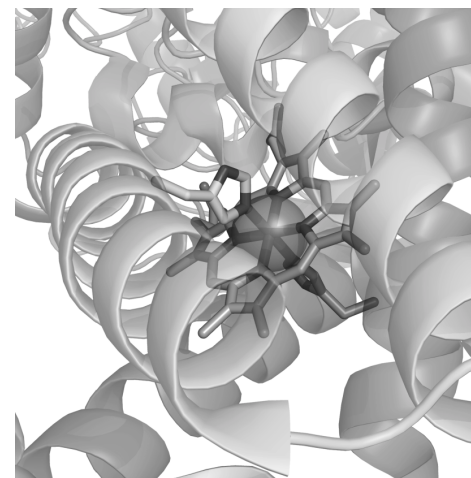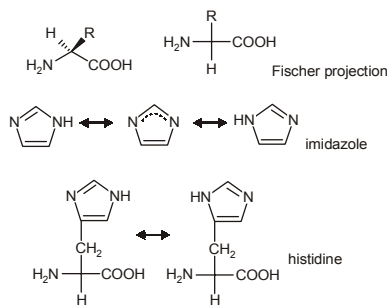
Fig. 2.48 : The histidine bound heme group of succinate dehydrogenase, an electron carrier in the mitochondrial electron transfer chain. The large semi-transparent sphere indicates the location of the iron ion. From PDB 1YQ3

4-Hydroxyproline, or hydroxyproline ($C_5H_9O_3N$), is an uncommon amino acid, abbreviated as **HYP.**

Structure

Hydroxyproline differs from proline by the presence of a hydroxyl (OH) group attached to the C (gamma) atom.

Other hydroxyprolines also exist in nature, notably 2,3-cis-3,4-trans-3,4-dihydroxyproline which occurs in diatom cell walls[1] and is postulated to have a role in silica deposition. Hydroxyproline is also found in the walls of oomycetes, fungus-like protists related to diatoms.
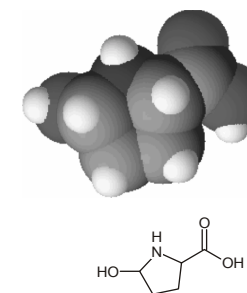
Production and Function

Hydroxyproline is produced by hydroxylation of the amino acid proline by the enzyme prolyl hydroxylase following protein synthesis (as a post-translational modification). The enzyme catalysed reaction takes place in the lumen of the endoplasmic reticulum.

Hydroxyproline is a major component of the protein collagen. Hydroxyproline and proline play key roles for collagen stability. They permit the sharp twisting of the collagen helix. In the canonical collagen Xaa-Yaa-Gly triad (where Xaa and Yaa are any amino acid), a proline occupying the Yaa position is hydroxylated to give a Xaa-Hyp-Gly sequence. This modification of the proline residue increases the stability of the collagen triple helix. It was initially proposed that the stabilization was due to water molecules forming a hydrogen bonding network linking the prolyl hydroxyl groups and the main-chain carbonyl groups. It was subsequently shown that the increase in stability is primarily through stereoelectronic effects and that hydration of the hydroxyproline residues provides little or no additional stability.

Hydroxyproline is found in few proteins other than collagen. The only other mammalian protein which includes hydroxyproline is elastin. For this reason, hydroxyproline content has been used as an indicator to determine collagen and/or gelatin amount.

Clinical Significance

Proline hydroxylation requires ascorbic acid. The most obvious, first effects (gum and hair problems) of absence of ascorbic acid in humans come from the resulting defect in hydroxylation of proline residues of collagen, with reduced stability of the collagen molecule causing scurvy.

Isoleucine is an á-amino acid with the chemical formula $HO_2CCH(NH_2)CH(CH_3)CH_2CH_3$. It is an essential amino acid, which means that humans cannot synthesize it, so it must be part of our diet. Its codons are AUU, AUC and AUA.

With a hydrocarbon side chain, isoleucine is classified as a hydrophobic amino acid. Together with threonine, isoleucine is one of two common amino acids that have a chiral side chain. Four stereoisomers of isoleucine are possible, including two possible diastereomers of L-isoleucine. However, isoleucine present in nature exists in one enantiomeric form, (2*S*,3*S*)-2-amino-3-methylpentanoic acid.

Biosynthesis

As an essential amino acid, isoleucine is not synthesized in animals, hence it must be ingested, usually as a component of proteins. In plants and microorganisms, it is synthesized via several steps, starting from pyruvic acid and alpha-ketoglutarate. Enzymes involved in this biosynthesis include:

1. Acetolactate synthase (also known as acetohydroxy acid synthase)
2. Acetohydroxy acid isomeroreductase
3. Dihydroxyacid dehydratase
4. Valine aminotransferase

Isomers of Isoleucine
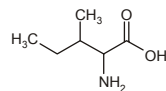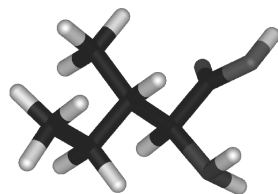
Forms of Isoleucine							
Common name	isoleucine	D-isoleucine	L-isoleucine	DL-isoleucine	allo-D-isoleucine	allo-L-isoleucine	allo-DL-isoleucine
Synonyms:		(R)-Isoleucine	L(+)-Isoleucine	(R*,R*)-isoleucine		alloisoleucine	
PubChem:	CID 791	CID 94206	CID 6306	CID 76551			
EINECS number:	207-139-8	206-269-2	200-798-2		216-143-9	216-142-3	221-464-2
CAS number:	443-79-8	319-78-8	73-32-5		1509-35-9	1509-34-8	3107-04-8

Synthesis

Isoleucine can be synthesized in a multistep procedure starting from 2-bromobutane and diethylmalonate. Synthetic isoleucine was originally reported in 1905.

Leucine (abbreviated as **Leu** or **L**) is an á-amino acid with the chemical formula $HO_2CCH(NH_2)CH_2CH(CH_3)_2$. It is an essential amino acid, which means that humans cannot synthesise it. Its codons are UUA, UUG, CUU, CUC, CUA, and CUG. With a hydrocarbon side chain, leucine is classified as a hydrophobic amino acid. It has an isobutyl R group. Leucine is a major component of the sub units in ferritin, astacin and other 'buffer' proteins.

Biosynthesis

As an essential amino acid, leucine is not synthesized in animals, hence it must be ingested, usually as a component of proteins. It is synthesized in plants and microorganisms via several steps starting from pyruvic acid. The initial part of the pathway also leads to valine. The intermediate á-ketovalerate is converted to á-isopropylmalate and then â-isopropylmalate, which is dehydrogenated to á-ketoisocaproate, which in the final step undergoes reductive amination. Enzymes involved in a typical leucine biosynthesis include

- Acetolactate synthase,
- Acetohydroxy acid isomeroreductase,
- Dihydroxyacid dehydratase,
- α-Isopropylmalate synthase,
- α-Isopropylmalate isomerase,
- Leucine aminotransferase.
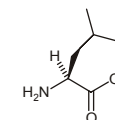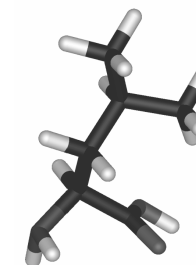
Biological Uses

As a dietary supplement, leucine has been found to slow the degradation of muscle tissue by increasing

the synthesis of muscle proteins. Leucine is utilized in the liver, adipose tissue, and muscle tissue. In adipose and muscle tissue, leucine is used in the formation of sterols, and the combined usage of leucine in these two tissues is seven times greater than its use in the liver.

Chemical Properties

Racemic leucine had been subjected to circularly polarized synchrotron radiation in order to better understand the origin of biomolecular asymmetry. An enantiomeric enhancement of 2.6 % had been induced, indicating a photochemical origin of biomolecules' homochirality.

Food additive

As a food additive L-Leucine has E number **E641** and is classified as a flavour enhancer

Lysine (abbreviated as **Lys** or **K**) is an á-amino acid with the chemical formula $HO_2CCH(NH_2)(CH_2)_4NH_2$. This amino acid is an essential amino acid, which means that humans cannot synthesize it. Its codons are AAA and AAG.

Lysine is a base, as are arginine and histidine. The á-amino group often participates in hydrogen bonding and as a general base in catalysis. Common posttranslational modifications include methylation of the á-amino group, giving methyl-, dimethyl-, and trimethyllysine. The latter occurs in calmodulin. Other posttranslational modifications at lysine residues include acetylation and ubiquitination. Collagen contains hydroxylysine which is derived from lysine by lysyl hydroxylase. *O*-Glycosylation of lysine residues in the endoplasmic reticulum or Golgi apparatus is used to mark certain proteins for secretion from the cell.
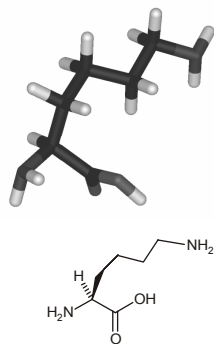
Biosynthesis

As an essential amino acid, lysine is not synthesized in animals, hence it must be ingested as lysine or lysine-containing proteins. In plants and microorganisms, it is synthesized from aspartic acid, which is first converted to â-aspartyl-semialdehyde. Cyclization gives dihydropicolinate, which is reduced to $Ä^1$-piperidine-2,6-dicarboxylate. Ring-opening of this heterocycle gives a series of derivatives of pimelic acid, ultimately affording lysine. Enzymes involved in this biosynthesis include:

1. Aspartokinase
2. â-aspartate semialdehyde dehydrogenase
3. Dihydropicolinate synthase
4. $Ä^1$-piperidine-2,6-dicarboxylate dehydrogenase
5. *N*-succinyl-2-amino-6ketopimelate synthase
6. Succinyl diaminopimelate aminotransferase
7. Succinyl diaminopimelate desuccinylase

8. Diaminopimelate epimerase
9. Diaminopimelate decarboxylase

Metabolism

Lysine is metabolised in mammals to give acetyl-CoA, via an initial transamination with á-ketoglutarate. The bacterial degradation of lysine yields cadaverine by decarboxylation.

Synthesis

Synthetic, racemic lysine has long been known. A practical synthesis starts from caprolactam.

Dietary sources

The human nutritional requirement is 1–1.5 g daily. It is the limiting amino acid (the essential amino acid found in the smallest quantity in the particular foodstuff) in all cereal grains, but is plentiful in all pulses (legumes). Plants that contain significant amounts of lysine include:

- Buffalo Gourd (10,130–33,000 ppm) in seed
- Berro, Watercress (1,340–26,800 ppm) in herb.
- Soybean (24,290–26,560 ppm) in seed.
- Carob, Locust Bean, St.John's-Bread (26,320 ppm) in seed;
- Common Bean (Black Bean, Dwarf Bean, Field Bean, Flageolet Bean, French Bean, Garden Bean, Green Bean, Haricot, Haricot Bean, Haricot Vert, Kidney Bean, Navy Bean, Pop Bean, Popping Bean, Snap Bean, String Bean, Wax Bean) (2,390–25,700 ppm) in sprout seedling;
- Ben Nut, Benzolive Tree, Jacinto (Sp.), Moringa (aka Drumstick Tree, Horseradish Tree, Ben Oil Tree), West Indian Ben (5,370–25,165 ppm) in shoot.
- Lentil (7,120–23,735 ppm) in sprout seedling.
- Asparagus Pea, Winged Bean (aka Goa Bean) (21,360–23,304 ppm) in seed.
- Fat Hen (3,540–22,550 ppm) in seed.
- Lentil (19,570–22,035 ppm) in seed.
- White Lupin (19,330–21,585 ppm) in seed.
- Black Caraway, Black Cumin, Fennel-Flower, Nutmeg-Flower, Roman Coriander (16,200–20,700 ppm) in seed.
- Spinach (1,740–20,664 ppm).
- Amaranth, Quinoa
- Kasha

Good sources of lysine are foods rich in protein including meat (specifically red meat, lamb, pork, and poultry), cheese (particularly Parmesan), certain fish (such as cod and sardines), and eggs.

Properties

L-Lysine is a necessary building block for all protein in the body. L-Lysine plays a major role in calcium absorption; building muscle protein; recovering from surgery or sports injuries; and the body's production of hormones, enzymes, and antibodies.

Modifications

Lysine can be modified through acetylation, methylation, ubiquitination, sumoylation, neddylation, biotinylation and carboxylation which tends to modify the function of the protein of which the modified lysine residue(s) are a part.

Clinical Significance

It has been suggested that lysine may be beneficial for those with herpes simplex infections. However, more research is needed to fully substantiate this claim. For more information, refer to Herpes simplex - Lysine.

There are Lysine conjugates that show promise in the treatment of cancer, by causing cancerous cells to destroy themselves when the drug is combined with the use of phototherapy, while leaving non-cancerous cells unharmed.

In popular culture

The 1993 film *Jurassic Park*, which is based on the 1990 Michael Crichton novel *Jurassic Park*, features dinosaurs that were genetically altered so they could not produce lysine. This was supposed to prevent the cloned dinosaurs from leaving the park, forcing them to depend on lysine supplements provided by the park's veterinary staff. Most vertebrates cannot produce lysine by default (it is an essential amino acid).

The 2002 Max Tundra single "Lysine" (featuring Becky Jacobs) is a tribute to the advantages of lysine.

Linus Pauling believed people with existing atherosclerotic plaque can benefit from vitamin c and the amino acid lysine. Pauling filmed a video lecture in which he recommended that heart patients take between 2,000 and 6,000 mg of lysine daily with their vitamin C (more if serum Lp(a) is elevated).

In the sitcom The Big Bang Theory, the episode The Friendship Algorithm (air date 1/19/2009), it is revealed that Sheldon's favorite amino acid is Lysine. Although Raj had thought it was l-glutamine.

Methionine(abbreviated as **Met** or **M**) is an á-amino acid with the chemical formula $HO_2CCH(NH_2)CH_2CH_2SCH_3$. This Essential amino acid is classified as nonpolar. Together with cysteine, methionine is one of two sulfur-containing proteinogenic amino acids. Its derivative S-adenosyl methionine (SAM) serves as a methyl donor. Methionine is an intermediate in the biosynthesis of cysteine, carnitine, taurine, lecithin,
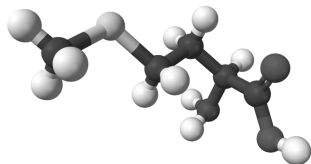
phosphatidylcholine, and other phospholipids. Improper conversion of methionine can lead to atherosclerosis. Methionine is one of only two amino acids encoded by a single codon (AUG) in the standard genetic code (tryptophan, encoded by UGG, is the other). The codon AUG is also the "Start" message for a ribosome that signals the initiation of protein translation from mRNA. As a consequence, methionine is incorporated into the N-terminal position of all proteins in eukaryotes and archaea during translation, although it is usually removed by post-translational modification.
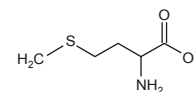
Biosynthesis
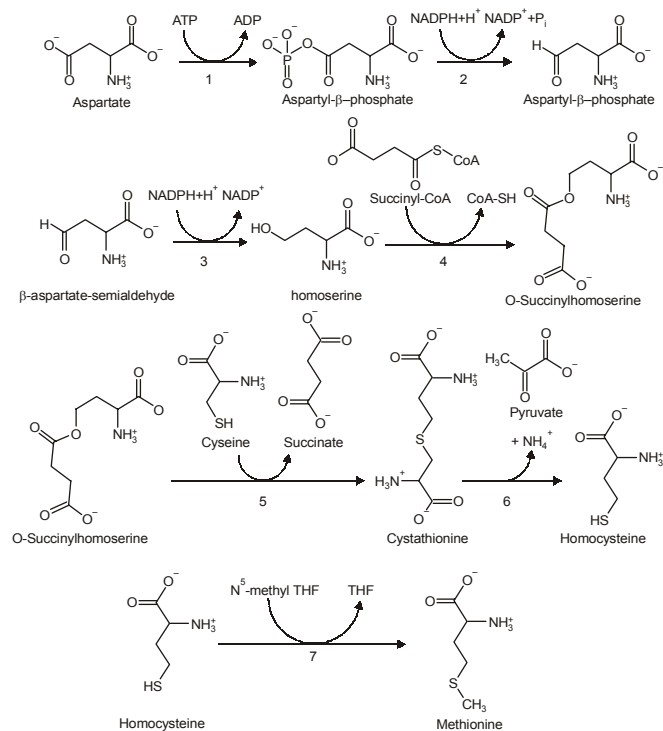
As an essential amino acid, methionine is not synthesized in humans, hence we must ingest methionine or methionine-containing proteins. In plants and microorganisms, methionine is synthesized via a pathway that uses both aspartic acid and cysteine. First, aspartic acid is converted via â-aspartyl-semialdehyde into homoserine, introducing the pair of contiguous methylene groups. Homoserine converts to *O*-succinyl homoserine, which then reacts with cysteine to produce cystathionine, which is cleaved to yield homocysteine. Subsequent methylation of the thiol group by folates affords methionine. Both cystathionine-ã-synthase and cystathionine-â-lyase require Pyridoxyl-5'-phosphate as a cofactor, whereas homocysteine methyltransferase requires Vitamin B12 as a cofactor.

Enzymes involved in methionine biosynthesis:

1. aspartokinase
2. â-aspartate semialdehyde dehydrogenase
3. homoserine dehydrogenase
4. homoserine acyltransferase
5. cystathionine-ã-synthase
6. cystathionine-â-lyase
7. methionine synthase (in mammals, this step is performed by homocysteine methyltransferase)

Other biochemical pathways

Although mammals cannot synthesize methionine, they can still utilize it in a variety of biochemical pathways:

Methionine is converted to S-adenosylmethionine (SAM) by (1) methionine adenosyltransferase. SAM serves as a methyl-donor in many (2) methyltransferase reactions and is converted to S-adenosylhomocysteine (SAH). (3) adenosylhomocysteinase converts SAH to homocysteine.

There are two fates of homocysteine:

- Methionine can be regenerated from homocysteine via (4) methionine synthase. It can also be remethylated using glycine betaine (NNN-trimethyl glycine) to methionine via the enzyme Betaine-homocysteine methyltransferase (E.C.2.1.1.5, BHMT). BHMT makes up to 1.5% of all the soluble protein of the liver, and recent evidence suggests that it may have a greater influence on methionine and homocysteine homeostasis than methionine synthase.

- Homocysteine can be converted to cysteine. (5) Cystathionine-â-synthase (a PLP-dependent enzyme) combines homocysteine and serine to produce cystathionine. Instead of degrading cystathionine via cystathionine-â-lyase, as in the biosynthetic pathway, cystathionine is broken down to cysteine and á-ketobutyrate via (6) cystathionine-ã-lyase. (7) á-ketoacid dehydrogenase converts á-ketobutyrate to propionyl-CoA, which is metabolized to succinyl-CoA in a three-step process (see propionyl-CoA for pathway).
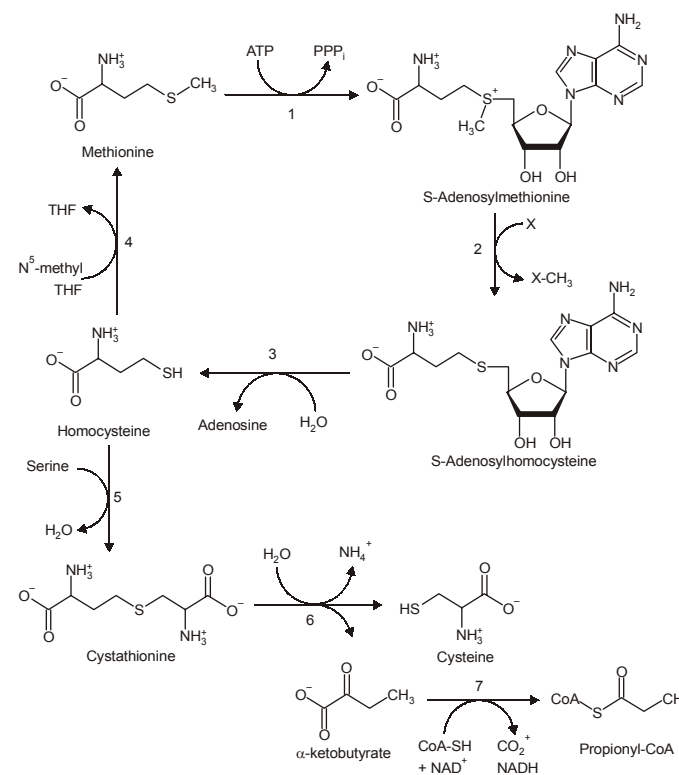
Synthesis

Racemic methionine can be synthesized from diethyl sodium phthalimidomalonate by alkylation with chloroethylmethylsulfide (ClCH$_2$CH$_2$SCH$_3$) followed by hydrolysis and decarboxylation.

Dietary aspects

High levels of methionine can be found in sesame seeds, Brazil nuts, fish, meats, and some other plant seeds. Most fruits and vegetables contain very little of it; however, some have significant amounts,

such as spinach, potatoes, and boiled corn. Most legumes, though high in protein, are also low in methionine. DL-methionine is sometimes added as an ingredient to pet foods. Methionine, cysteine, and soy protein heated in a small amount of water creates a meat-like aroma.

Ornithine is an amino acid which plays a role in the urea cycle.

Role in urea cycle

L-Ornithine is one of the products of the action of the enzyme arginase on L-arginine, creating urea. Therefore, ornithine is a central part of the urea cycle, which allows for the disposal of excess nitrogen.

Ornithine is not an amino acid coded for by DNA, and, in that sense, is not involved in protein synthesis. However, in mammalian non-hepatic tissues, the main use of the urea cycle is in arginine biosynthesis, so as an intermediate in metabolic processes, ornithine is quite important.

Lactamization

Ornithine lactamization
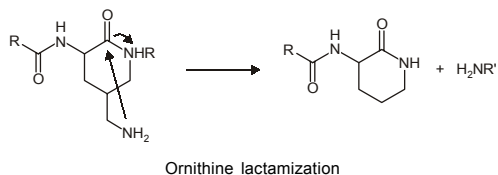
It is believed to not be a part of genetic code because polypeptides containing unprotected ornithines undergo spontaneous lactamization. This proved to be a problem when ornithine was artificially incorporated in 21st amino acid systems.

Phenylalanine (abbreviated as **Phe** or **F**) is an á-amino acid with the formula $HO_2CCH(NH_2)CH_2C_6H_5$, which is found naturally in the breast milk of mammals and manufactured for food and drink products and are also sold as nutritional supplements for their reputed analgesic and antidepressant effects. Phenylalanine is structurally closely related to dopamine, epinepherine (adrenaline) and tyrosine.

This essential amino acid is classified as nonpolar because of the hydrophobic nature of the benzyl side chain. The codons for L-phenylalanine are UUU and UUC. It is a white, powdery solid. L-Phenylalanine (LPA) is an electrically-neutral amino acid, one of the twenty common amino acids used to biochemically form proteins, coded for by DNA.
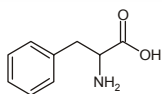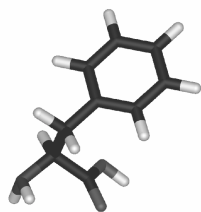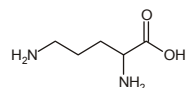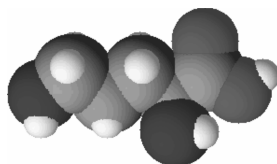
The most important consideration for the layman is to know that Phenylalanine has a laxative effect, it also gives you gas for extended periods. It is used in chewing gum such as Wrigleys Extra Fusion - a warning is displayed on the packet in very small text.

Other biological roles

L-phenylalanine can also be converted into L-tyrosine, another one of the DNA-encoded amino acids. L-tyrosine in turn is converted into L-DOPA, which is further converted into dopamine, norepinephrine (noradrenaline), and epinephrine (adrenaline). The latter three are known as the catecholamines.

Phenylalanine uses the same active transport channel as tryptophan to cross the blood-brain barrier, and, in large quantities, interferes with the production of serotonin.
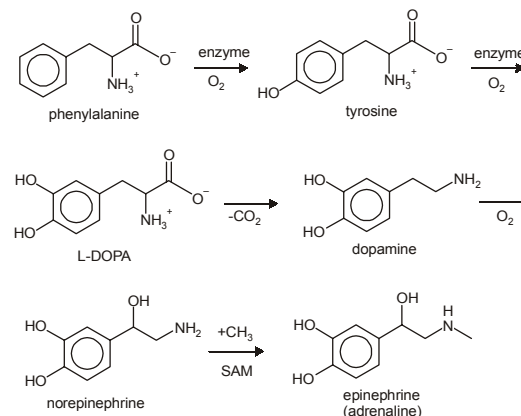
Lignan is derived from phenylalanine and from tyrosine. Phenylalanine is converted to cinnamic acid by the enzyme phenylalanine ammonia lyase.

Phenylketonuria

The genetic disorder phenylketonuria (PKU) is the inability to metabolize phenylalanine. Individuals with this disorder are known as "phenylketonurics" and must abstain from consumption of too much phenylalanine. This dietary restriction also applies to pregnant women with hyperphenylalanine (high levels of phenylalanine in blood) because they do not properly metabolize the amino acid phenylalanine. Persons suffering from PKU must monitor their intake of protein to control the buildup of phenylalanine as their bodies convert protein into its component amino acids.

A non food source of phenylalanine is the artificial sweetener aspartame. This compound, sold under the trade names "Equal" and "NutraSweet", is metabolized by the body into several chemical

byproducts including phenylalanine. The breakdown problems phenylketonurics have with protein and the attendant build up of phenylalanine in the body also occurs with the ingestion of aspartame, although to a lesser degree. Accordingly, all products in Australia, the U.S. and Canada that contain aspartame must be labeled: "Phenylketonurics: Contains phenylalanine." In the UK, foods containing aspartame must carry ingredients panels that refer to the presence of "aspartame or E951" [3] and they must be labeled with a warning "Contains a source of phenylalanine." These warnings are specifically placed to aid individuals who suffer from PKU so that they can avoid such foods.

In Non-Humans

Geneticists have recently sequenced the genome of macaques. Their investigations have found "some instances where the normal form of the macaque protein looks like the diseased human protein" including markers for PKU.

D- and DL-phenylalanine

The unnatural stereoisomer D-phenylalanine (DPA) can be produced by conventional organic synthesis, either as a single enantiomer or as a component of the racemic mixture. It does not participate in protein biosynthesis although it is found in proteins in small amounts - particularly aged proteins and food proteins that have been processed. The biological functions of D-amino acids remain unclear although some, such as D-phenylalanine, may have pharmacological activity.

DL-Phenylalanine (DLPA) is marketed as a nutritional supplement for its supposed analgesic and antidepressant activities. The reputed analgesic activity of DL-phenylalanine may be explained by the possible blockage by D-phenylalanine of enkephalin degradation by the enzyme carboxypeptidase A. The mechanism of DL-phenylalanine's supposed antidepressant activity may be accounted for by the precursor role of L-phenylalanine in the synthesis of the neurotransmitters, norepinephrine and dopamine. Elevated brain levels of norepinephrine and dopamine are thought to have an antidepressant effect] Following ingestion, D-Phenylalanine is absorbed from the small intestine and transported to the liver via the portal circulation. A small amount of D-phenylalanine appears to be converted to L-phenylalanine. D-Phenylalanine is distributed to the various tissues of the body via the systemic circulation. It appears to cross the blood-brain barrier less efficiently than L-phenylalanine, and so a small amount of an ingested dose of D-phenylalanine is not absorbed but excreted in the urine.

History

The genetic codon for phenylalanine was first discovered by J. Heinrich Matthaei and Marshall W. Nirenberg in 1961. They showed that by using *m*-RNA to insert multiple uracil repeats into the bacterium *E. coli*, the bacterium produced a new protein consisting solely of repeated phenylalanine amino acids. This discovery lead to the determination of the relationship between RNA and amino acids, which was foundational to the understanding of the Genetic Code.

Phenylalanine is contained in many foodstuffs and byproducts. Many products are advertised as containing this ingredient including the soft drink 'lilt', 'Diet 7-Up', 'Tango', '5-Hour Energy', 'Caffeine Free Diet Pepsi', 'Stride' Gum, 'Red Bull Sugar free', 'Fisherman's Friend', 'Diet Coca-Cola' and others

Proline (abbreviated as **Pro** or **P**) is an á-amino acid, one of the twenty DNA-encoded amino acids. Its codons are CCU, CCC, CCA, and CCG. It is not an essential amino acid, which means that humans can synthesize it. It is unique among the 20 protein-forming amino acids because the á-amino group is secondary.
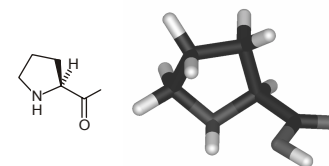
History

Hermann Emil Fischer discovered Proline between 1899 and 1908.

Biosynthesis

Proline is biosynthetically derived from the amino acid L-glutamate and its immediate precursor is the imino acid (*S*)-1-pyrroline-5-carboxylate (P5C). Enzymes involved in a typical biosynthesis include:

1. Glutamate kinase (ATP-dependent)
2. Glutamate dehydrogenase (requires NADH or NADPH)
3. Pyrroline-5-carboxylate reductase (requires NADH or NADPH)

Structural properties

The distinctive cyclic structure of proline's side chain locks its ö backbone dihedral angle at approximately -75°, giving proline an exceptional conformational rigidity compared to other amino acids. Hence, proline loses less conformational entropy upon folding, which may account for its higher prevalence in the proteins of thermophilic organisms. Proline acts as a structural disruptor in the middle of regular secondary structure elements such as alpha helices and beta sheets; however, proline is commonly found as the first residue of an alpha helix and also in the edge strands of beta sheets. Proline is also commonly found in turns, which may account for the curious fact that proline is usually solvent-exposed, despite having a completely aliphatic side chain. Because proline lacks a hydrogen on the amide group, it cannot act as a hydrogen bond donor, only as a hydrogen bond acceptor.

Multiple prolines and/or hydroxyprolines in a row can create a polyproline helix, the predominant secondary structure in collagen. The hydroxylation of proline by prolyl hydroxylase (or other additions of electron-withdrawing substituents such as fluorine) increases the conformational stability of collagen significantly. Hence, the hydroxylation of proline is a critical biochemical process for maintaining the connective tissue of higher organisms. Severe diseases such as scurvy can result from defects in this hydroxylation, e.g., mutations in the enzyme prolyl hydroxylase or lack of the necessary ascorbate (vitamin C) cofactor.

Sequences of proline and 2-aminoisobutyric acid (Aib) also form a helical turn structure.

In 2006, scientists at ASU discovered that solutions of TiO_2 illuminated with ultraviolet radiation can serve as an extremely cost-effective and accurate protein cleavage catalyst. The TiO_2 catalyst preferentially and rapidly cleaves protein at sites where proline is present, while taking much longer to degrade the protein from its endpoints.

Cis-trans isomerization

Peptide bonds to proline, and to other *N*-substituted amino acids (such as sarcosine), are able to populate both the *cis* and *trans* isomers. Most peptide bonds overwhelmingly adopt the *trans* isomer (typically 99.9% under unstrained conditions), chiefly because the amide hydrogen (*trans* isomer) offers less steric repulsion to the preceding C^α atom than does the following C^α atom (*cis* isomer). By contrast, the *cis* and *trans* isomers of the X-Pro peptide bond (where X represents any amino acid) both experience steric clashes with the neighboring substitution and are nearly equal energetically. Hence, the fraction of X-Pro peptide bonds in the *cis* isomer under unstrained conditions ranges from 10-40%; the fraction depends slightly on the preceding amino acid, with aromatic residues favoring the *cis* isomer slightly.

From a kinetic standpoint, *Cis-trans* proline isomerization is a very slow process that can impede the progress of protein folding by trapping one or more proline molecules crucial for folding in the non-native isomer, especially when the native protein requires the *cis* isomer. This is because proline residues are exclusively synthesized in the ribosome as the *trans* isomer form. All organisms possess prolyl isomerase enzymes to catalyze this isomerization, and some bacteria have specialized prolyl isomerases associated with the ribosome. However, not all prolines are essential for folding, and protein folding may proceed at a normal rate despite having non-native conformers of many X-Pro peptide bonds.

Uses

Proline and its derivatives are often used as asymmetric catalysts in organic reactions. The CBS reduction and proline catalysed aldol condensation are prominent examples.

L-Proline is an osmoprotectant and therefore is used in many pharmaceutical, biotechnological applications.

Specialities

Proline is one of the two amino acids that do not follow along with the typical Ramachandran plot, along with glycine. Due to the ring formation connected to the Beta-carbon, the ø and ö angles about the peptide bond have less allowable degrees of rotation.

Pyrrolysine is a naturally occurring, genetically coded amino acid used by some methanogenic archaea in enzymes that are part of their methane-producing metabolism.

This lysine derivative is encoded by the UAG codon (normally the 'amber' stop codon), possibly modified by the presence of a specific downstream sequence, named PYLIS, which forms a stem-loop in the mRNA, forcing the incorporation of pyrrolysine instead of terminating translation. It is also of interest to note that UAG appears to be used much less often than other stop codons and whenever it is found in
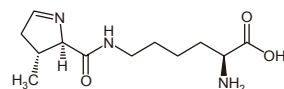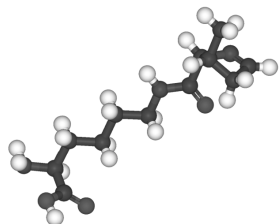
an open reading frame it is always followed by one or more of the other two stop codons shortly after.

Near a methyltransferase gene cluster of *Methanosarcina barkeri* is the *pylT* gene, which encodes an unusual transfer RNA (tRNA) with a CUA anticodon. The adjacent *pylS* gene encodes a class II aminoacyl-tRNA synthetase that charges the *pylT*-derived tRNA with pyrrolysine. The operon containing *pylT* and *pylS* are also found in the genomes of other sequenced members of the *Methanosarcinaceae* family. Homologs of *pylS* and *pylT* are found in a Gram-positive bacterium, *Desulfitobacterium hafniense*, although the function of these putative genes in this organism is unknown. It was initially shown that *pylT* encoded tRNA (CUA) can be charged with lysine by PylS. Recently, it has been shown that the tRNA(CUA) can be charged with lysine *in vitro* by the concerted action of the *M. barkeri* Class I and Class II Lysyl-tRNA synthetases. Charging a tRNA(CUA) with lysine was originally hypothesized to be the first step in translating UAG amber codons as pyrrolysine in certain methanogens. The current model based on *in vitro* and *in vivo* data favors direct charging of pyrrolysine on to the tRNA(CUA) by the protein product of the *pylS* gene. This makes pyrrolysine the 22nd genetically encoded natural amino acid. The mechanism of encoding makes it the 21st natural directly encoded amino acid.

The joint nomenclature committee of the IUPAC/IUBMB has officially recommended the three-letter symbol **Pyl** and the one-letter symbol **O** for pyrrolysine.

Selenocysteine is an amino acid that is present in several enzymes (for example glutathione peroxidases, tetraiodothyronine 5' deiodinases, thioredoxin reductases, formate dehydrogenases, glycine reductases and some hydrogenases).

 Nomenclature

The joint nomenclature committee of the IUPAC/IUBMB has officially recommended the three-letter symbol **Sec** and the one-letter symbol **U** for selenocysteine.

Structure

Selenocysteine has a structure similar to cysteine, but with an atom of selenium taking the place of the usual sulfur. Proteins that contain one or more selenocysteine residues are called selenoproteins.

Biology

Unlike other amino acids present in biological proteins, Selenocysteine is not coded for directly in the genetic code. Instead, it is encoded in a special way by a UGA codon, which is normally a stop codon. The UGA codon is made to encode selenocysteine by the presence of a SECIS element (SElenoCysteine Insertion Sequence) in the mRNA. The SECIS element is defined by characteristic nucleotide sequences and secondary structure base-pairing patterns. In bacteria, the SECIS element is located immediately following the UGA codon within the reading frame for the selenoprotein. In archaea and in eukaryotes, the SECIS element is in the 3' untranslated region (3' UTR) of the mRNA, and can direct multiple UGA codons to encode selenocysteine residues. When cells are grown in the absence of selenium, translation of selenoproteins terminates at the UGA codon, resulting in a truncated, nonfunctional enzyme.
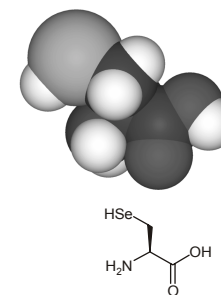
Like the other amino acids used by cells, selenocysteine has a specialized tRNA. The primary and secondary structure of selenocysteine tRNA, tRNA(Sec), differ from those of standard tRNAs in several respects, most notably in having an 8-base (bacteria) or 10-base (eukaryotes) pair acceptor stem, a long variable region arm, and substitutions at several well-conserved base positions. The selenocysteine tRNAs are initially charged with serine by seryl-tRNA ligase, but the resulting Ser-tRNA(Sec) is not used for translation because it is not recognised by the normal translation factor (EF-Tu in bacteria, EF1-alpha in eukaryotes). Rather, the tRNA-bound seryl residue is converted to a selenothiomise-residue by the pyridoxal phosphate-containing enzyme selenocysteine synthase. Finally, the resulting Sec-tRNA(Sec) is specifically bound to an alternative translational elongation factor (SelB or mSelB) which delivers it in a targeted manner to the ribosomes translating mRNAs for selenoproteins. The specificity of this delivery mechanism is brought about by the presence of an extra protein domain (in bacterial SelB) or an extra subunit (SBP-5 for eukaryotic mSelB) which bind to the corresponding RNA secondary structures formed by the SecIS elements in selenoprotein mRNAs. The SecIS elements of bacterial selenoproteins (as far as analysed) are located within the coding sequences immediately following the UGA codons for selenocysteine, those of Eukarya and Archaea are located in the 3' UTR of the respective mRNAs. In addition, at least one case has been described for an archaeal selenoprotein mRNA containing its SecIS in the 5' UTR.

Serine (abbreviated as **Ser** or **S**) is an organic compound with the formula $HO_2CCH(NH_2)CH_2OH$.

Occurrence

It is one of the 20 naturally occurring proteinogenic amino acids. Its codons are UCU, UCC, UCA, UCG, AGU and AGC. Only the L-stereoisomer appears naturally in proteins. It is not essential to the human diet, since it is synthesized in the body from other metabolites, including glycine. Serine was first obtained from silk protein, a particularly rich source, in 1865. Its name is derived from the Latin for silk, *sericum*. Serine's structure was established in 1902. By virtue of the hydroxyl group, serine is classified as *a polar amino acid*.
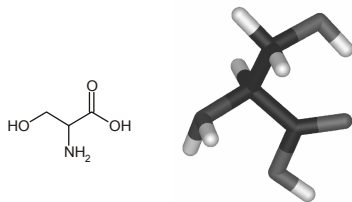
Biosynthesis

The biosynthesis of serine starts with the oxidation of 3-phosphoglycerate to 3-phosphohydroxypyruvate and NADH. Reductive amination of this ketone followed by hydrolysis gives serine. Serine hydroxymethyltransferase catalyzes the reversible, simultaneous conversions of L-serine to glycine (retro-aldol cleavage) and 5,6,7,8-tetrahydrofolate to 5,10-methylenetetrahydrofolate (hydrolysis).

Chemical synthesis

Racemic serine can be prepared from methyl acrylate via several steps. It is also naturally produced when UV light illuminates simple ices such as a combination of water, methanol, hydrogen cyanide, and ammonia, suggesting that it may be easily produced in cold regions of space.

Metabolic Function

Serine is important in metabolism in that it participates in the biosynthesis of purines and pyrimidines. It is also the precursor to several amino acids, including glycine, cysteine, and, in bacteria, tryptophan. It is also the precursor to numerous of other metabolites, including sphingolipids. Serine is also a precursor to folate, which is the principal donor of one carbon fragments in biosynthesis.

Structural role

Serine plays an important role in the catalytic function of many enzymes. It has been shown to occur in the active sites of chymotrypsin, trypsin, and many other enzymes. The so-called nerve gases and many substances used in insecticides have been shown to act by combining with a residue of serine in the active site of acetylcholine esterase, inhibiting the enzyme completely. The unmetabolized acetylcholine cannot be recycled into the nerve for signaling. This results in depletion of acetylcholine at the neuromuscular junction, resulting in the inability to control muscles, which results in asphyxiation, and death.

As a constituent (residue) of proteins, its side chain can undergo O-linked glycosylation, which may be functionally related to diabetes. It is one of three amino acid residues that are commonly phosphorylated by kinases during cell signaling in eukaryotes. Phosphorylated serine residues are often referred to as phosphoserine. Serine proteases are a common type of protease.

Signaling

D-serine, synthesized by serine racemase from L-serine, serves as a neuronal signal by activating NMDA receptors in the brain.

Threonine (abbreviated as **Thr** or **T**) is an á-amino acid with the chemical formula $HO_2CCH(NH_2)CH(OH)CH_3$. Its codons are ACU, ACA, ACC, and ACG. This essential amino acid is classified as polar. Together with serine and tyrosine, threonine is one of three proteinogenic amino acids bearing an alcohol group.

The threonine residue is susceptible to numerous posttranslational modifications. The hydroxy side chain can undergo *O*-linked glycosylation. In addition, threonine residues undergo phosphorylation through the action of a threonine kinase. In its phosphorylated form, it can be referred to as phosphothreonine.
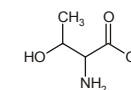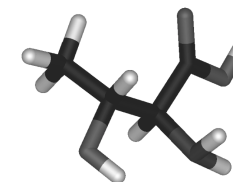
Allo-threonine

With two chiral centers, threonine can exist in four possible stereoisomers, or two possible diastereomers of L-threonine. However, the name L-threonine is used for one single enantiomer, (2*S*,3*R*)-2-amino-3-hydroxybutanoic acid. The second diastereomer (2*S*,3*S*), which is rarely present in nature, is called L-*allo*-threonine.

Biosynthesis

As an essential amino acid, threonine is not synthesized in humans, hence we must ingest threonine in the form of threonine-containing proteins. In plants and microorganisms, threonine is synthesized from aspartic acid via á-aspartyl-semialdehyde and homoserine. Homoserine undergoes *O*-phosphorylation; this phosphate ester undergoes hydrolysis concomitant with relocation of the OH group. Enzymes involved in a typical biosynthesis of threonine include:

1. aspartokinase
2. α-aspartate semialdehyde dehydrogenase
3. homoserine dehydrogenase
4. homoserine kinase
5. threonine synthase.
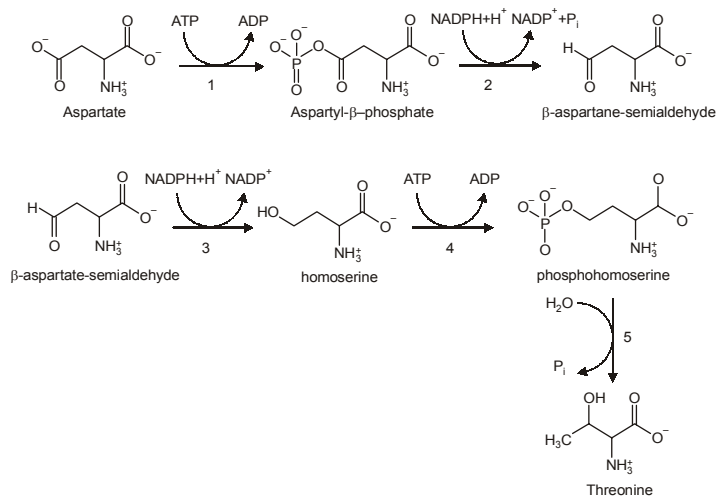
Threonine biosynthesis

Metabolism

Threonine is metabolized in two ways:

- It is converted to pyruvate via threonine dehydrogenase. An intermediate in this pathway can undergo thiolysis with CoA to produce Acetyl-CoA and glycine.

- In humans, it is converted to alpha-ketobutyrate in a less common pathway via the enzyme serine dehydratase, and thereby enters the pathway leading to succinyl-CoA.

Sources

Foods high in threonine include cottage cheese, poultry, fish, meat, lentils, and sesame seeds.

Racemic threonine can be prepared from crotonic acid by alpha-functionalization using mercury(II) acetate.

Tryptophan (abbreviated as **Trp** or **W**) is one of the 20 standard amino acids, as well as an essential amino acid in the human diet. It is encoded in the standard genetic code as the codon *UGG*. Only the L-stereoisomer of tryptophan is used in structural or enzyme proteins, but the D-stereoisomer is occasionally found in naturally produced peptides (for example, the marine venom peptide contryphan). The distinguishing structural characteristic of tryptophan is that it contains an indole functional group.

Isolation

The isolation of tryptophan was first reported by Sir Frederick Hopkins in 1901 through hydrolysis of casein. From 600 grams of crude casein one obtains 4-8 grams of tryptophan.

Biosynthesis and industrial production

Plants and microorganisms commonly synthesize tryptophan from shikimic acid or anthranilate.[5] The latter condenses with phosphoribosylpyrophosphate (PRPP), generating pyrophosphate as a by-product. After ring opening of the ribose moiety and following reductive decarboxylation, indole-3-glycerinephosphate is produced, which in turn is transformed into indole. In the last step, tryptophan synthase catalyzes the formation of tryptophan from indole and the amino acid, serine.

The industrial production of tryptophan is also biosynthetic and is based on the fermentation of serine and indole using either wild-type or genetically modified *E. coli*. The conversion is catalyzed by the enzyme tryptophan synthase.

Function

L-tryptophan

Indoleamine 2,3-dioxygenase

N'-formyl-kynurenine

tryptophan hydroxylase

formamidase

5-hydroxy-tryptophan

aromatic amine acid decarboxylase

kynurenine

5-hydroxy-tryptamine (5-HT)

serotonin

2-amino-3-(3-oxoprop-1-enyl)- fumaric acid

N-acetyl transferase

non-enzymatic cyclization

N-acetyl-5-HT

quinolinate

5-hydroxyindole-O-methyltransferase
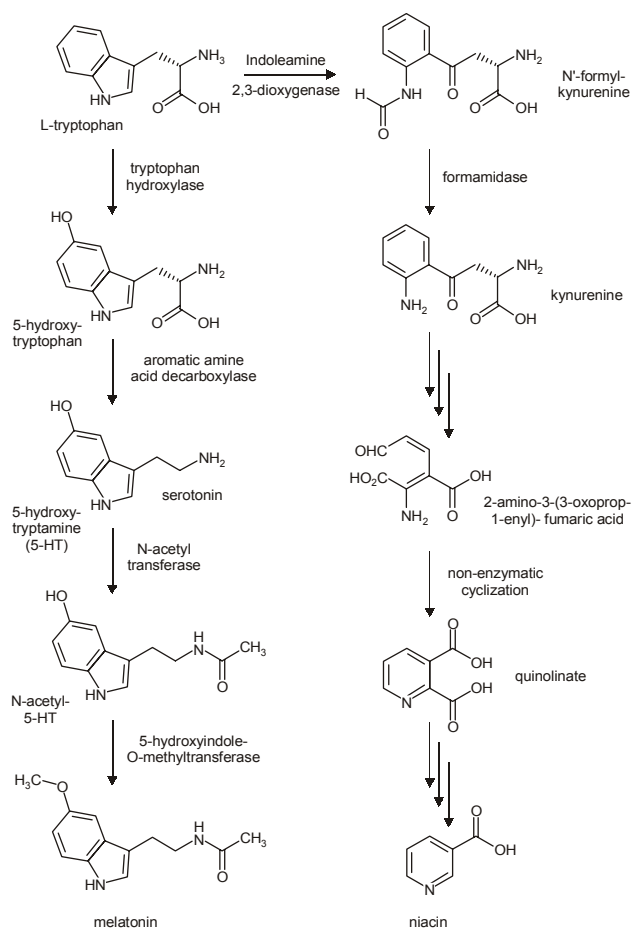
melatonin

niacin

Metabolism of L-tryptophan into serotonin and melatonin (left) and niacin (right). Transformed functional groups after each chemical reaction are highlighted in red.

For many organisms (including humans), tryptophan is an essential amino acid. This means that it cannot be synthesized by the organism and therefore must be part of its diet. Amino acids, including tryptophan, act as building blocks in protein biosynthesis. In addition, tryptophan functions as a biochemical precursor for the following compounds (see also figure to the right):

- Serotonin (a neurotransmitter), synthesized via tryptophan hydroxylase.[7][8] Serotonin, in turn, can be converted to melatonin (a neurohormone), via N-acetyltransferase and 5-hydroxyindole-O-methyltransferase activities.
- Niacin is synthesized from tryptophan via kynurenine and quinolinic acids as key biosynthetic intermediates.

The disorder Fructose Malabsorption causes improper absorption of tryptophan in the intestine, reduced levels of tryptophan in the blood and depression.

In bacteria that synthesize tryptophan, high cellular levels of this amino acid activate a repressor protein, which binds to the trp operon. Binding of this repressor to the tryptophan operon prevents transcription of downstream DNA that codes for the enzymes involved in the biosynthesis of tryptophan. So high levels of tryptophan prevent tryptophan synthesis through a negative feedback loop and, when the cell's tryptophan levels are reduced, transcription from the trp operon resumes. The genetic organisation of the trp operon thus permits tightly regulated and rapid responses to changes in the cell's internal and external tryptophan levels.

Dietary Sources

Tryptophan is a routine constituent of most protein-based foods or dietary proteins. It is particularly plentiful in chocolate, oats, bananas, durians, mangoes, dried dates, milk, yogurt, cottage cheese, red meat, eggs, fish, poultry, sesame, chickpeas, sunflower seeds, pumpkin seeds, spirulina, and peanuts.[14] It is found in turkey at a level typical of poultry in general.

Table 2.42 : Tryptophan (Trp) Content of Various Foods

Food	Protein [g/100 g of food]	Tryptophan [g/100 g of food]	Tryptophan/Protein [%]
1	2	3	4
egg, white, dried	81.10	1.00	1.23
spirulina, dried	57.47	0.93	1.62
cod, atlantic, dried	62.82	0.70	1.11
soybeans, raw	36.49	0.59	1.62
cheese, Parmesan	37.90	0.56	1.47
caribou	29.77	0.46	1.55
sesame seed	17.00	0.37	2.17

1	2	3	4
cheese, cheddar	24.90	0.32	1.29
sunflower seed	17.20	0.30	1.74
pork, chop	19.27	0.25	1.27
turkey	21.89	0.24	1.11
chicken	20.85	0.24	1.14
beef	20.13	0.23	1.12
salmon	19.84	0.22	1.12
lamb, chop	18.33	0.21	1.17
perch, Atlantic	18.62	0.21	1.12
egg	12.58	0.17	1.33
wheat flour, white	10.33	0.13	1.23
milk	3.22	0.08	2.34
rice, white	7.13	0.08	1.16
potatoes, russet	2.14	0.02	0.84
banana	1.03	0.01	0.87

Use as a dietary supplement

For some time, tryptophan has been available in health food stores as a dietary supplement, although it is common in dietary protein. Many people found tryptophan to be a safe and reasonably effective sleep aid, probably due to its ability to increase brain levels of serotonin (a calming neurotransmitter when present in moderate levels) and/or melatonin (a sleep-inducing hormone secreted by the pineal gland in response to darkness or low light levels).

Clinical research has shown mixed results with respect to tryptophan's effectiveness as a sleep aid, especially in normal patients and for a growing variety of other conditions typically associated with low serotonin levels or activity in the brain such as premenstrual dysphoric disorder and seasonal affective disorder. In particular, tryptophan has been showing considerable promise as an antidepressant alone, and as an "augmenter" of antidepressant drugs. However, the reliability of these clinical trials has been questioned.

Metabolites

5-Hydroxytryptophan (5-HTP), a metabolite of tryptophan, has been suggested as a treatment for epilepsy and depression, although clinical trials are regarded inconclusive and lacking.

5-HTP readily crosses the blood-brain barrier and in addition is rapidly decarboxylated to serotonin (5-hydroxytryptamine or 5-HT) and therefore may be useful for the treatment of depression. However serotonin has a relatively short half-life since it is rapidly metabolized by monoamine oxidase, and

therefore is likely to have limited efficacy. It is marketed in Europe for depression and other indications under the brand names Cincofarm and Tript-OH.

In the United States, 5-HTP does not require a prescription, as it is covered under the Dietary Supplement Act. However, since the quality of dietary supplements is not regulated by the U.S. Food and Drug Administration, the quality of dietary and nutritional supplements tends to vary, and there is no guarantee that the label accurately depicts what the bottle contains.

Turkey meat and drowsiness

One belief is that heavy consumption of turkey meat (as for example in a Thanksgiving or Christmas feast) results in drowsiness, which has been attributed to high levels of tryptophan contained in turkey. While turkey does contain high levels of tryptophan, the amount is comparable to that contained in most other meats. Furthermore, postprandial Thanksgiving sedation may have more to do with what is consumed along with the turkey, in particular carbohydrates and alcohol, rather than the turkey itself.

It has been demonstrated in both animal models and in humans that ingestion of a meal rich in carbohydrates triggers release of insulin. Insulin in turn stimulates the uptake of large neutral branched-chain amino acids (LNAA) but not tryptophan (trp) into muscle, increasing the ratio of trp to LNAA in the blood stream. The resulting increased ratio of tryptophan to large neutral amino acids in the blood reduces competition at the large neutral amino acid transporter resulting in the uptake of tryptophan across the blood-brain barrier into the central nervous system (CNS). Once inside the CNS, tryptophan is converted into serotonin in the raphe nuclei by the normal enzymatic pathway. The resultant serotonin is further metabolised into melatonin by the pineal gland. Hence, these data suggest that "feast-induced drowsiness," and in particular, the common post-Christmas and American post-Thanksgiving dinner drowsiness, may be the result of a heavy meal rich in carbohydrates which, via an indirect mechanism, increases the production of sleep-promoting melatonin in the brain.

Fluorescence

The fluorescence of a folded protein is a mixture of the fluorescence from individual aromatic residues. Most of the intrinsic fluorescence emissions of a folded protein are due to excitation of tryptophan residues, with some emissions due to tyrosine and phenylalanine; but be aware that di-sulfide bonds also have appreciable absorption in this wavelength range. Typically, tryptophan has a wavelength of maximum absorption of 280 nm and an emission peak that is solvatochromic, ranging from ca. 300 to 350 nm depending in the polarity of the local environment. Hence, protein fluorescence may be used as a diagnostic of the conformational state of a protein. Furthermore, tryptophan fluorescence is strongly influenced by the proximity of other residues (*i.e.*, nearby *protonated* groups such as Asp or Glu can cause quenching of Trp fluorescence). Also, energy transfer between tryptophan and the other fluorescent amino acids is possible, which would affect the analysis, especially in cases where the Förster acidic approach is taken. In addition, tryptophan is a relatively rare amino acid; many proteins contain only one or a few tryptophan residues. Therefore, tryptophan fluorescence can be a very sensitive measurement of the conformational state of individual tryptophan residues. The advantage compared to extrinsic probes is that the protein itself is not changed. The use of intrinsic fluorescence for the study of protein conformation is in practice limited to cases with few (or perhaps only one)

tryptophan residues, since each experiences a different local environment, which gives rise to different emission spectra.

Tyrosine (abbreviated as **Tyr** or **Y**) or **4-hydroxyphenylalanine**, is one of the 20 amino acids that are used by cells to synthesize proteins. This is a non-essential amino acid and it is found in casein. Tyrosine has a non-polar/hydrophobic side group (see table, amino acid). In fact, the word "tyrosine" is from the Greek *tyros*, meaning *cheese*, as it was first discovered in 1846 by German chemist Justus von Liebig in the protein casein from cheese.
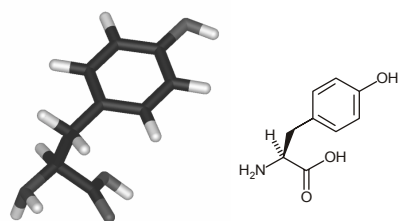
Functions

Aside from being a proteogenic amino acid, tyrosine has a special role by virtue of the phenol functionality. It occurs in proteins that are part of signal transduction processes. It functions as a receiver of phosphate groups that are transferred by way of protein kinases (so-called receptor tyrosine kinases). Phosphorylation of the hydroxyl group changes the activity of the target protein.

A tyrosine residue also plays an important role in photosynthesis. In chloroplasts (photosystem II), it acts as an electron donor in the reduction of oxidized chlorophyll. In this process, it undergoes deprotonation of its phenolic OH-group. This radical is subsequently reduced in the photosystem II by the four core manganese cluster.

Dietary Sources

Tyrosine, which can be synthesised in the body from phenylalanine, is also found in many high protein food products such as soy products, chicken, turkey, fish, peanuts, almonds, avocados, bananas, milk, cheese, yogurt, cottage cheese, lima beans, pumpkin seeds, and sesame seeds. Tyrosine can also be obtained through supplementation.

Biosynthesis
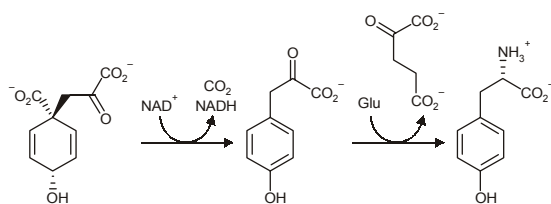
Plant biosynthesis of tyrosine from shikimic acid.

In plants and most microorganisms, **tyr** is produced via prephenate, an intermediate on the shikimate pathway. Prephenate is oxidatively decarboxylated with retention of the hydroxyl group to give *p*-hydroxyphenylpyruvate, which is transaminated using glutamate as the nitrogen source to give tyrosine and á-ketoglutarate.

Mammals synthesize tyrosine from the essential amino acid phenylalanine (**phe**), which is derived from food. The conversion of **phe** to **tyr** is catalyzed by the enzyme phenylalanine hydroxylase, a monooxygenase. This enzyme catalyzes the reaction causing the addition of an hydroxyl group to the end of the 6-carbon aromatic ring of phenylalanine, such that it becomes tyrosine.

Metabolism
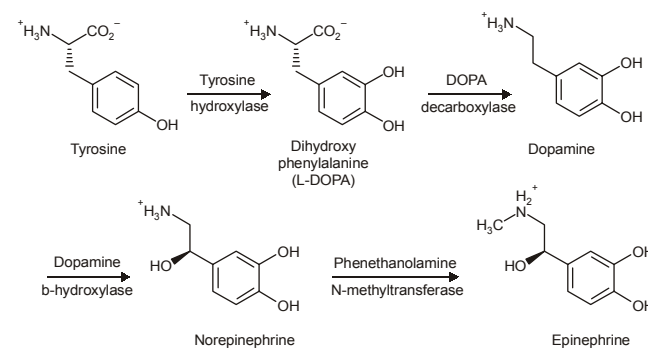
Catecholamine hormones produced from tyrosine metabolism.

Phosphorylation and sulphation

Some of the tyrosine residues can be *tagged* with a phosphate group (phosphorylated) by protein kinases. (In its phosphorylated state, it is referred to as **phosphotyrosine**). Tyrosine phosphorylation is considered to be one of the key steps in signal transduction and regulation of enzymatic activity. Phosphotyrosine can be detected through specific antibodies. Tyrosine residues may also be modified by the addition of a sulfate group, a process known as tyrosine sulfation. Tyrosine sulfation is catalyzed by tyrosylprotein sulfotransferase (TPST). Like the phosphotyrosine antibodies mentioned above, antibodies have recently been described that specifically detect sulfotyrosine.

Precursor to hormones

In the adrenal gland, tyrosine is converted to levodopa by the enzyme tyrosine hydroxylase (TH). TH is also the rate-limiting enzyme involved in the synthesis of the catecholamine hormones dopamine, norepinephrine (noradrenaline), and epinephrine.

The thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) in the colloid of the thyroid also are derived from tyrosine.

Precursor to alkaloids

In *Papaver somniferum*, the opium poppy, tyrosine is used to produce the alkaloid morphine.

Precursor to pigments

Tyrosine is also the precursor to the pigment *melanin*.

Degradation
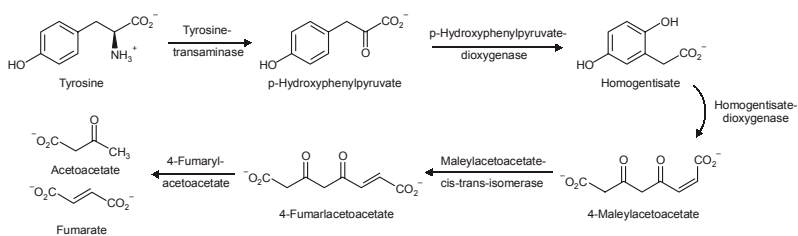
The decomposition of tyrosine to acetoacetate and fumarate. Two dioxygenases are necessary for the decomposition path. The end products can then enter into the citric acid cycle.

The decomposition of L-tyrosine (syn. *para*-hydroxyphenylalanine) begins with an á-ketoglutarate dependent transamination through the tyrosine transaminase to *para*-hydroxyphenylpyruvate. The positional description *para*, abbreviated *p*, mean that the hydroxyl group and side chain on the phenyl ring are across from each other (see the illustration below).

The next oxidation step catalyzes by *p*-hydroxylphenylpyruvate-dioxygenase and splitting off CO_2 homogentisate (2,5-dihydroxyphenyl-1-acetate). In order to split the aromatic ring of homogentisate, a further dioxygenase, homogentistate-oxygenase is required. Thereby, through the incorporation of a further O_2 molecule, maleylacetoacetate is created.

Fumarylacetate is created maleylacetoacetate-*cis-trans*-isomerase through rotation of the carboxyl group created from the hydroxyl group via oxidation. This *cis-trans*-isomerase contains glutathione as a coenzyme. Fumarylacetoacetate is finally split via fumarylacetoacetate-hydrolase through the addition of a water molecule.

Thereby fumarate (also a metabolite of the citric acid cycle) and acetoacetate (3-ketobutyroate) are liberated. Acetoacetate is a ketone body, which is activated with succinyl-CoA, and thereafter it can be converted into acetyl-CoA which in turn can be oxidized by the citric acid cycle or be used for fatty acid synthesis.

Ortho- and meta-tyrosine
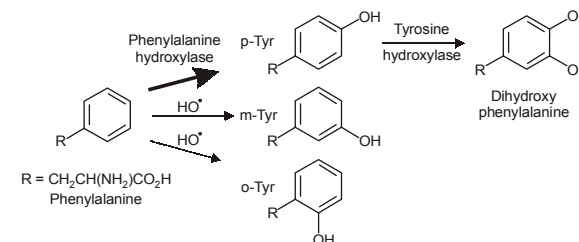
Enzymatic oxidation of tyrosine by phenylalanine hydroxylase (top) and non-enyzmatic oxidation by hydroxyl free radicals (middle and bottom).

Three isomers of tyrosine are known. In addition to common amino acid L-tyrosine which is the para isomer (*para*-tyr, *p*-tyr or 4-hydroxyphenylalanine) there are two additional regioisomers, namely *meta*-tyrosine (*m*-tyr or 3-hydroxyphenylalanine or **L-m-tyrosine**) and *ortho*-tyrosine (*o*-tyr or 2-hydroxyphenylalanine) which occur in nature. The *m*-tyr and *o*-tyr isomers, which are rare, arise through non-enzymatic free-radical hydroxylation of phenylalanine under conditions of oxidative stress.

m-Tyrosine and analogues (rare in nature and therefore available synthetically) have shown application in Parkinson's Disease, Alzheimer's disease and arthritis.

Medical use

Tyrosine is a precursor to neurotransmitters and increases plasma neurotransmitter levels (particularly dopamine and norepinephrine) but has little if any effect on mood. The effect on mood is more noticeable in humans subjected to stressful conditions (see below).

A number of studies have found tyrosine to be useful during conditions of stress, cold, fatigue, prolonged work and sleep deprivation, with reductions in stress hormone levels, reductions in stress-induced weight loss seen in animal trials, improvements in cognitive and physical performance seen in human trials. Because tyrosine hydroxylase is the rate limiting enzyme, however, effects are less significant than those of l-dopa.

Tyrosine does not seem to have any significant effect on mood, cognitive or physical performance in normal circumstances. A daily dosage supported in the literature is about 100 mg/kg for an adult. The usual dosage amounts to 500-1500 mg per day (dose suggested by most manufacturers; usually an equivalent to 1-3 capsules of pure tyrosine). It is not recommended to exceed 12000 mg (12 g) per day. In fact, too high doses result in reduced levels of dopamine. Tyrosine may decrease the absorption of other amino acids in high or chronic doses. It decreases absorption of l-dopa.

Drug Detoxification

Tyrosine appears to be a successful addition to conventional treatment for cocaine abuse and withdrawal.

It may be used in conjunction with other amino acids such as tryptophan. Some individuals using tyrosine have also reported successful withdrawal from caffeine and nicotine.

Valine (abbreviated as **Val** or **V**) is an á-amino acid with the chemical formula $HO_2CCH(NH_2)CH(CH_3)_2$. L-Valine is one of 20 proteogenic amino acids. Its codons are GUU, GUC, GUA, and GUG. This essential amino acid is classified as nonpolar. Along with leucine and isoleucine, valine is a branched-chain amino acid. It is named after the plant valerian. In sickle-cell disease, valine substitutes for the hydrophilic amino acid glutamic acid in hemoglobin. Because valine is hydrophobic, the hemoglobin does not fold correctly.
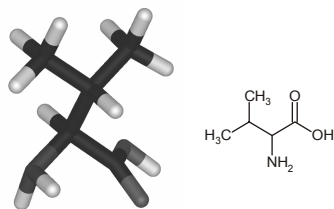
Biosynthesis

Valine is an essential amino acid, hence it must be ingested, usually as a component of proteins. It is synthesized in plants via several steps starting from pyruvic acid. The initial part of the pathway also leads to leucine. The intermediate á-ketovalerate undergoes reductive amination with glutamate. Enzymes involved in this biosynthesis include:

1. Acetolactate synthase (also known as acetohydroxy acid synthase)
2. Acetohydroxy acid isomeroreductase
3. Dihydroxyacid dehydratase
4. Valine aminotransferase

Synthesis

Racemic valine can be synthesized by bromination of isovaleric acid followed by amination of the á-bromo derivative

$$HO_2CCH_2CH(CH_3)_2 + Br_2 \rightarrow HO_2CCHBrCH(CH_3)_2 + HBr$$

$$HO_2CCHBrCH(CH_3)_2 + 2\,NH_3 \rightarrow HO_2CCH(NH_2)CH(CH_3)_2 + NH_4Br$$
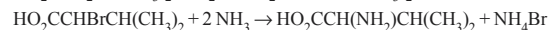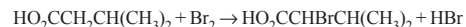
Dietary aspects

Nutritional sources of valine include cottage cheese, fish, poultry, peanuts, sesame seeds, and lentils.

SUB-SECTION 2.10—A CHEMICAL REACTIONS OF AMINO ACID

The amino acids in grenral give the reactions characteristic of the carboxyl groups. Some of these reactions take place with more difficulty or under more restricted consitions than in the case of ordinary organic acids because of participation of carboxyl group of amino acids in Zwiterion structure.$

$A **zwitterion** (first part pronounced "tsvitter", from German "*Zwitter*" — "hybrid," "hermaphrodite") is a chemical compound that carries a total net charge of 0, thus electrically neutral but carries formal positive and negative charges on

Salt Formation and tritration Both the acidic and the basic groups of amino acids form salts. However owing to the Zwitterion formation the titration of amino acid in aqueous solutions is impossible because no sharp endpoint is obtained. The carboxyl group can be titrated with alkali in alcoholic or Acetone medium. In which the end point of the indicatior is shifted more towards the alkaline pH. The basic groups of amiono acids may be titrated with glacial acetic acid solution with standard sulphuric hydrobromic or perchloric acid using as indicator such as crytal violet.

The titration curves of amino acids in aqueous may be run with the glass of hydrogen electrodes. Theoretical curves may be calculated by the Henderson – Hasselbalch equation* from pK values.

These differences all point to internal salt formation by a proton transfer from the acidic carboxyl function to the basic amino group. The resulting ammonium carboxylate structure, commonly referred to as a **zwitterion**, is also supported by the spectroscopic characteristics of alanine.

$$CH_3CH(NH_2)CO_2H \rightleftharpoons CH_3CH(NH_3)^{(+)}CO_2^{(-)}$$
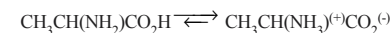
As expected from its ionic character, the alanine zwitterion is high melting, insoluble in nonpolar solvents and has the acid strength of a 1°-ammonium ion. To the right above is a Jmol display of an L-amino acid. The model will change to its zwitterionic form by clicking the appropriate button beneath

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different atoms. Some chemists restrict this term to refer to compounds with non-adjacent positive and negative charges.[1] This would exclude compounds such as *N*-oxides. Zwitterions are polar and are usually very water-soluble, but poorly soluble in most organic solvents.

Ampholytes are molecules that contain both acidic and basic groups (and are therefore amphoteric) and will exist mostly as zwitterions in a certain range of pH. The pH at which the average charge is zero is known as the molecule's isoelectric point.

Applications

Ampholytes are used to establish a stable pH gradient for **use** in isoelectric focusing.

Typical examples of zwitterions are:

- Most amino acids at physiological pH are for the most part zwitterionic.
- Used as buffering agents in Good's buffers:
  - The amino-sulfonic acid based MES, MOPS, HEPES, PIPES or CAPS
  - The amino-carboxylic acid (amino acid) based glycine, its derivatives bicine and tricine, and alanine
- Used as detergents:
  - CHAPSO
- Natural products like the alkaloids psilocybin and lysergic acid.
- Betaines

Less common examples of zwitterions are:

- Quinonoid zwitterions.
- Drugs such as Fexofenadine (Allegra) and Cephaloridine.
- decaphenylferrocene [(ç5-C5Ph5)2Fe] has been shown to have a zwitterionic linkage isomer [(ç5-C5Ph5)Fe+ (ç6-C6H5C5Ph4−)].

*See the page no. 184-185 of A Chemical Analser's Guide for more.

the display. Examples of a few specific amino acids may also be viewed in their favored neutral zwitterionic form. Note that in lysine the amine function farthest from the carboxyl group is more basic than the alpha-amine. Consequently, the positively charged ammonium moiety formed at the chain terminus is attracted to the negative carboxylate, resulting in a coiled conformation.

Decarboxylation When amino acids are heated, preferably in the presence of barium hydroxide or diphenylamine, carbon dioxide is last and amine is formed. The Decarboxylation of histidine may ve taken as an example.

Histidine (His)   Hysdamine
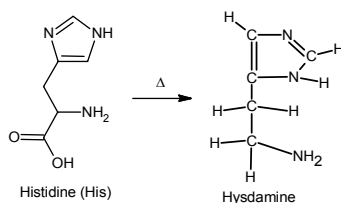
Decarboxylation of amino acids is caused also by dicarboxylase enzymes of bacteria. This occurs in the large intestine as relut of the action putrefactive organism. Some of the amines produced, such as histamine tryamine (from tyrosine) are pharmacologically active.

Amide formation amides cn be prepared by treatment of the amino acid esters with alcoholc or anhydrous ammonia.

Histidine (His)   hystamine
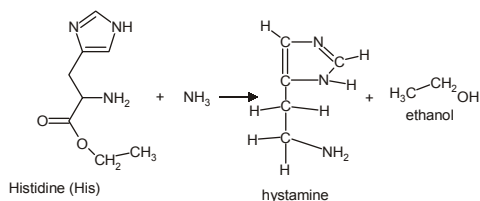
Methylation of the Amino acids, the amino groups of the amino acids may be exhaustively methylated by treatment with methyl iodie or dimethyl sulphate in alkaline solution. The methylation takes place in stages but the final product is a betaine of the aminop acid. The reaction may be represented.

The basicity of the amino group is greatly increased by methylation to the quaternary nitrogen stage of the betaine structure, and the betaines represent internal salt or Zwitterion structure. The betaines forms well –crystallized double salts with the chlorides of platinum gold, and mercury which are useful in their separation and purification. Mixtures of amino acids have been separated by methylation to betained followed by formation and separation of the betaine heavy metal double salts. Various betains occur in plants.
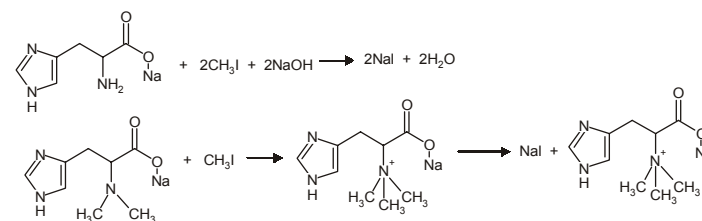
Reaction with Sanger's reagent A very important reaction of the amino groups of amino aciod is with the 1 – fluro-2-4- dinitrobenzene, FDNB also called Sanger's reagent. The reagent condenses with free amino groups in the cold in mild alkaline solution (bicarbonate of sodium or potassium).

amino acid   1-fluoro-2,4-dinitrobenzene   2-[(2,4-dinitrophenyl)amino]propanoic acid   hydrogen fluoride
               Sanger's
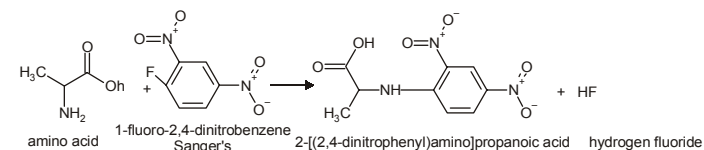
Most of the DNP amino acid are coloured ywllow and most are soluble in ether. The DNP bond is generally more stable in acid hydrolysis than peptide bonds. The importance of the reaction is largely due to the fact the terminal amino acid group in peptide or protein chainforms a DNP derivative which may be hydrolysis with acid to give the DNP amino acid. In this way it is possible to identify one of the terminal amino acid in a paptide or protein.

Reaction of Nitrous acid with amino acid Amino group of the acid reacts with nitrous acid to form the corresponding hydroxyl acids with liberation of nitrogen gas.

amino acid   Nitrous acid   2-hydroxypropanoic acid
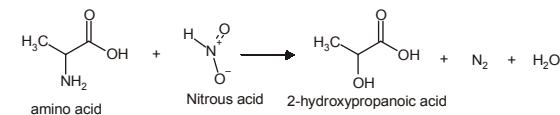
Since each amino group gives one molecule of nitrogen, which can be accurately measured. Van Slyke has utilized the treaction in an excellent method for the estimation of free amino group in the amino acid, peptides and proteins. As commonly carried out, the amino acid, protein or peptide solution is treated with sodium nitrite and acetic acid, which react to form nitrous acid in a mild acidic medium. Under these conditions the α – amino group react quantitatively in these with three to four minutes while the amino group of the lysine react more slowly.

The Van Slyke's amino – nitrogen method may be used conveniently to follow the rate of hydrolysis of proteins. Since for each peptide linkage hydrolysed a α – amino group is liberated. The amino nitrogen thus progressively increases until hydrolysis of the protein is complete.

Carboxylic Acid Esterification: Amino acids undergo most of the chemical reactions characteristic of each function, assuming the pH is adjusted to an appropriate value. Esterification of the carboxylic acid is usually conducted under acidic conditions, as shown in the two equations written below. Under such conditions, amine functions are converted to their ammonium salts and carboxyic acids are not dissociated. The first equation is a typical Fischer esterification involving methanol. The initial product is a stable ammonium salt. The amino ester formed by neutralization of this salt is unstable, due to acylation of the amine by the ester function. The second reaction illustrates benzylation of the two carboxylic acid functions of aspartic acid, using p-toluenesulfonic acid as an acid catalyst. Once the carboxyl function is esterified, zwitterionic species are no longer possible and the product behaves like any 1°-amine.
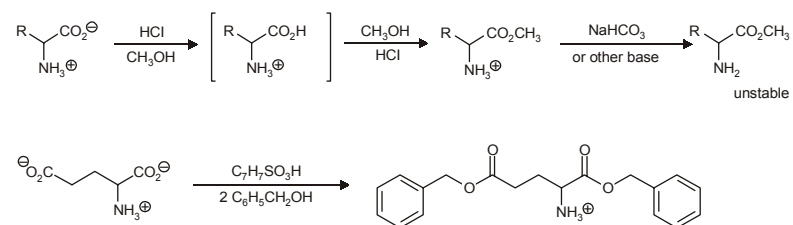
Amine Acylation: In order to convert the amine function of an amino acid into an amide, the pH of the solution must be raised to 10 or higher so that free amine nucleophiles are present in the reaction system. Carboxylic acids are all converted to carboxylate anions at such a high pH, and do not interfere with amine acylation reactions. The following two reactions are illustrative. In the first, an acid chloride serves as the acylating reagent. This is a good example of the superior nucleophilicity of nitrogen in acylation reactions, since water and hydroxide anion are also present as competing nucleophiles. A similar selectivity favoring amines was observed in the Hinsberg test. The second reaction employs an anhydride-like reagent for the acylation. This is a particularly useful procedure in peptide synthesis, thanks to the ease with which the t-butylcarbonyl (t-BOC) group can be removed at a later stage. Since amides are only weakly basic ($pK_a \sim$ -1), the resulting amino acid derivatives do not display zwitterionic character, and may be converted to a variety of carboxylic acid derivatives.
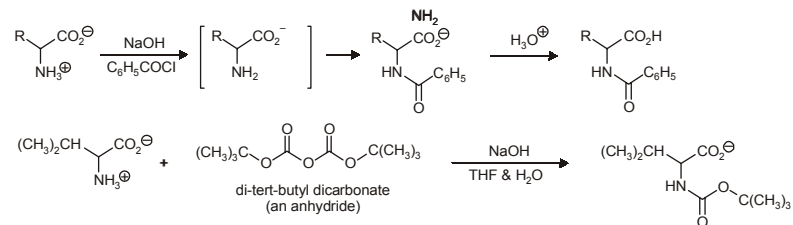
Specific Oxidatio: The mild oxidant iodine reacts selectively with certain amino acid side groups. These include the phenolic ring in tyrosine, and the heterocyclic rings in tryptophan and histidine, which all yield products of electrophilic iodination. In addition, the sulfur groups in cysteine and methionine are also oxidized by iodine. Quantitative measurent of iodine consumption has been used to determine the number of such residues in peptides. The basic functions in lysine and arginine are onium cations at pH less than 8, and are unreactive in that state. Cysteine is a thiol, and like most thiols it is oxidatively dimerized to a disulfide, which is sometimes listed as a distinct amino acid under the name cystine. Disulfide bonds of this kind are found in many peptides and proteins. For example, the two peptide chains that constitute insulin are held together by two disulfide links. Our hair consists of a fibrous protein called keratin, which contains an unusually large proportion of cysteine. In the manipulation called "permanent waving", disulfide bonds are first broken and then created after the hair has been reshaped. Treatment with dilute aqueous iodine oxidizes the methionine sulfur atom to a sulfoxide.

Cysteine-Cystine Interconversion
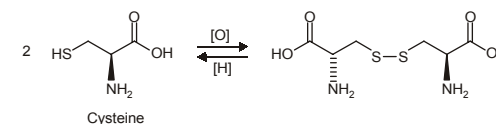
SUB-SECTION 2.10B—ISOELECTRIC POINT

The titration curve for alanine, shown below, demonstrates this relationship. At a pH lower than 2, both the carboxylate and amine functions are protonated, so the alanine molecule has a net positive charge. At a pH greater than 10, the amine exists as a neutral base and the carboxyl as its conjugate base, so the alanine molecule has a net negative charge. At intermediate pH's the zwitterion concentration increases, and at a characteristic pH, called the **isoelectric point (pI)**, the negatively and positively charged
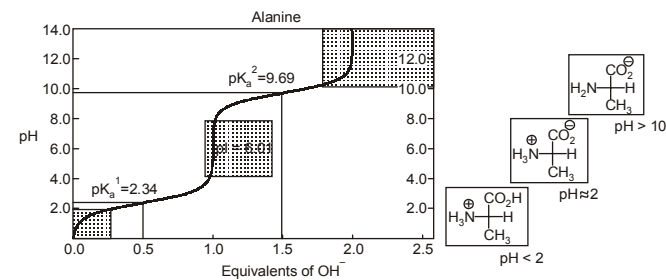
Fig. 2.49 : Showing the Titration Curve of amino acid

molecular species are present in equal concentration. This behavior is general for simple (difunctional) amino acids. Starting from a fully protonated state, the pK_a's of the acidic functions range from 1.8 to 2.4 for $-CO_2H$, and 8.8 to 9.7 for $-NH_3^{(+)}$. The isoelectric points range from 5.5 to 6.2. Titration curves show the neutralization of these acids by added base, and the change in pH during the titration.

The distribution of charged species in a sample can be shown experimentally by observing the movement of solute molecules in an electric field, using the technique of **electrophoresis**. For such experiments an ionic buffer solution is incorporated in a solid matrix layer, composed of paper or a crosslinked gelatin-like substance. A small amount of the amino acid, peptide or protein sample is placed near the center of the matrix strip and an electric potential is applied at the ends of the strip, as shown in the following diagram. The solid structure of the matrix retards the diffusion of the solute molecules, which will remain where they are inserted, unless acted upon by the electrostatic potential. In the example shown here, four different amino acids are examined simultaneously in a pH 6.00 buffered medium. To see the result of this experiment, click on the illustration. Note that the colors in the display are only a convenient reference, since these amino acids are colorless.
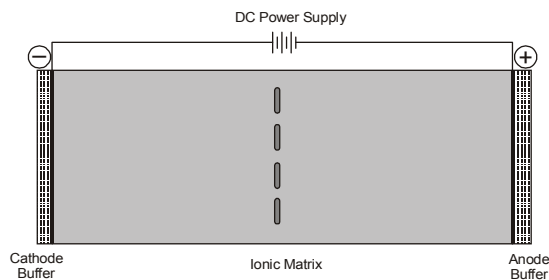
Fig. 2.50 : Showing the Electrophoresis

At pH 6.00 alanine and isoleucine exist on average as neutral zwitterionic molecules, and are not influenced by the electric field. Arginine is a basic amino acid. Both base functions exist as "onium" conjugate acids in the pH 6.00 matrix. The solute molecules of arginine therefore carry an excess positive charge, and they move toward the cathode. The two carboxyl functions in aspartic acid are both ionized at pH 6.00, and the negatively charged
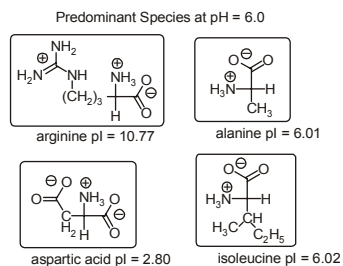
Fig. 2.51 : Showing the Structures for all these species

solute molecules move toward the anode in the electric field. Structures for all these species are shown in the fig 51.

pK_a Values of Polyfunctional Amino Acids

Amino Acid	$\alpha-CO_2H pK_a^1$	$\alpha-NH_3 pK_a^2$	Side Chain pK_a^3	pI
Arginine	2.1	9.0	12.5	10.8
Aspartic Acid	2.1	9.8	3.9	3.0
Cysteine	1.7	10.4	8.3	5.0
Glutamic Acid	2.2	9.7	4.3	3.2
Histidine	1.8	9.2	6.0	7.6
Lysine	2.2	9.0	10.5	9.8
Tyrosine	2.2	9.1	10.1	5.7

It should be clear that the result of this experiment is critically dependent on the pH of the matrix buffer. If we were to repeat the electrophoresis of these compounds at a pH of 3.00, the aspartic acid would remain at its point of origin, and the other amino acids would move toward the cathode. Ignoring
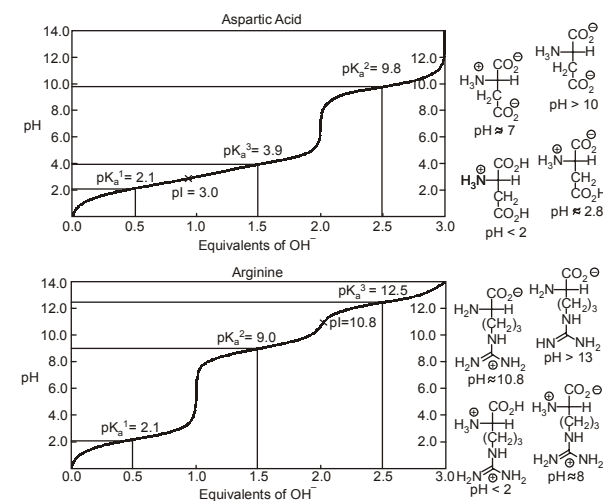
Fig. 2.52 : Showing the pK values of Aspartic acid and Agrinine

differences in molecular size and shape, the arginine would move twice as fast as the alanine and isoleucine because its solute molecules on average would carry a double positive charge.

As noted earlier, the titration curves of simple amino acids display two inflection points, one due to the strongly acidic carboxyl group (pK_a^1 = 1.8 to 2.4), and the other for the less acidic ammonium function (pK_a^2 = 8.8 to 9.7). For the 2°-amino acid proline, pK_a^2 is 10.6, reflecting the greater basicity of 2°-amines.

Some amino acids have additional acidic or basic functions in their side chains. These compounds are listed in the table on the right. A third pK_a, representing the acidity or basicity of the extra function, is listed in the fourth column of the table. The pI's of these amino acids (last column) are often very different from those noted above for the simpler members. As expected, such compounds display three inflection points in their titration curves, illustrated by the titrations of arginine and aspartic acid shown below. For each of these compounds four possible charged species are possible, one of which has no overall charge. Formulas for these species are written to the right of the titration curves, together with the pH at which each is expected to predominate. The very high pH required to remove the last acidic proton from arginine reflects the exceptionally high basicity of the guanidine moiety at the end of the side chain.

The Isoelectric Point

As defined above, the isoelectric point, **pI**, is the pH of an aqueous solution of an amino acid (or peptide) at which the molecules on average have no net charge. In other words, the positively charged groups are exactly balanced by the negatively charged groups. For simple amino acids such as alanine, the pI is an average of the pK_a's of the carboxyl (2.34) and ammonium (9.69) groups. Thus, the pI for alanine is calculated to be: (2.34 + 9.69)/2 = 6.02, the experimentally determined value. If additional acidic or basic groups are present as side-chain functions, the pI is the average of the pK_a's of the two most similar acids. To assist in determining similarity we define two classes of acids. The first consists of acids that are neutral in their protonated form (e.g. CO_2H & SH). The second includes acids that are positively charged in their protonated state (e.g. $-NH_3^+$). In the case of aspartic acid, the similar acids are the alpha-carboxyl function (pK_a = 2.1) and the side-chain carboxyl function (pK_a = 3.9), so pI = (2.1 + 3.9)/2 = 3.0. For arginine, the similar acids are the guanidinium species on the side-chain (pK_a = 12.5) and the alpha-ammonium function (pK_a = 9.0), so the calculated pI = (12.5 + 9.0)/2 = 10.75.

SUB-SECTION 2.10C—PH

Since amino acids, as well as peptides and proteins, incorporate both acidic and basic functional groups, the predominant molecular species present in an aqueous solution will depend on the pH of the solution. In order to determine the nature of the molecular and ionic species that are present in aqueous solutions at different pH's, we make use of the **Henderson-Hasselbach Equation**, written below. Here, the pK_a represents the acidity of a specific conjugate acid function (HA). When the pH of the solution equals pK_a, the concentrations of HA and $A^{(-)}$ must be equal (log 1 = 0).

$$\text{Henderson-Hasselbach Equation}: pK_a = pH + \log \frac{[HA]}{\left[A^-\right]}$$

SUB-SECTION 2.10C—ACID BASE PROPERTIES OF AMINO ACIDS

This subsection looks at what happens to amino acids as you change the pH by adding either acids or alkalis to their solutions. For simplicity, the page only looks at amino acids which contain a single $-NH_2$ group and a single $-COOH$ group.

Amino acids as zwitterions and Zwitterions in simple amino acid solutions

An amino acid has both a basic amine group and an acidic carboxylic acid group.
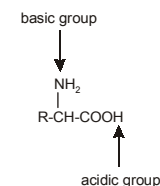
There is an internal transfer of a hydrogen ion from the $-COOH$ group to the $-NH_2$ group to leave an ion with both a negative charge and a positive charge.

This is called a *zwitterion*.
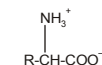
This is the form that amino acids exist in even in the solid state. If you dissolve the amino acid in water, a simple solution also contains this ion.

Adding an alkali to an amino acid solution If you increase the pH of a solution of an amino acid by adding hydroxide ions, the hydrogen ion is removed from the $-NH_3^+$ group.

Notice that this is now a negative ion -no longer a z witterion
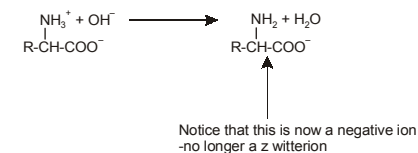
You could show that the amino acid now existed as a negative ion using *electrophoresis*.

In its simplest form, electrophoresis can just consist of a piece of moistened filter paper on a microscope slide with a crocodile clip at each end attached to a battery. A drop of amino acid solution is placed in the centre of the paper.

Although the amino acid solution is colourless, its position after a time can be found by spraying it with a solution of *ninhydrin*. If the paper is allowed to dry and then heated gently, the amino acid shows up as a coloured spot.

The amino acid would be found to travel towards the anode (the positive electrode).

Adding an acid to an amino acid solution If you decrease the pH by adding an acid to a solution of an amino acid, the -COO⁻ part of the zwitterion picks up a hydrogen ion.

$$NH_3^+ \quad\quad\quad\quad NH_3^+$$
$$R\text{-CH-COO}^- + H^+_{(aq)} \longrightarrow R\text{-CH-COOH}$$

Notice that this is now a negative ion
-no longer a z witterion
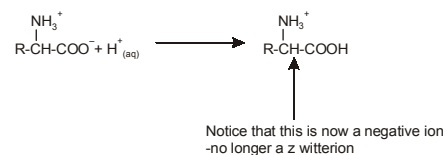
This time, during electrophoresis, the amino acid would move towards the cathode (the negative electrode).

Shifting the pH from one extreme to the other Suppose you start with the ion we've just produced under acidic conditions and slowly add alkali to it. That ion contains two acidic hydrogens - the one in the -COOH group and the one in the -NH₃⁺ group. The more acidic of these is the one in the -COOH group, and so that is removed first - and you get back to the zwitterion.

> You might have expected the isoelectric point to be at pH 7 - when the solution is neither acidic nor alkaline. In fact, the isoelectric point for many amino acids is about pH 6. Explaining why it isn't at pH 7 is quite long-winded and almost certainly beyond what you will need for UK A level (or its equivalent) purposes. If you are interested, the problem is discussed at the bottom of this subsection

$$NH_3+ \quad\quad\quad\quad NH_3+$$
$$R\text{-CH-COOH} + OH^- \longrightarrow R\text{-CH-COO}^- + H_2O$$
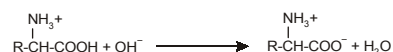
So when you have added just the right amount of alkali, the amino acid no longer has a net positive or negative charge. That means that it wouldn't move towards either the cathode or anode during electrophoresis. The pH at which this lack of movement during electrophoresis happens is known as the *isoelectric point* of the amino acid. This pH varies from amino acid to amino acid.

If you go on adding hydroxide ions, you will get the reaction we've already seen, in which a hydrogen ion is removed from the -NH₃⁺ group.

$$NH_3^+ \quad + \quad OH^- \quad\quad\quad\quad NH_2 + H_2O$$
$$R\text{-CH-COO}^- \quad\quad \longrightarrow \quad R\text{-CH-COO}^-$$
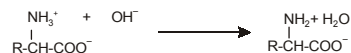
You can, of course, reverse the whole process by adding an acid to the ion we've just finished up with.

That ion contains two basic groups - the –NH₂ group and the –COO⁻ group. The -NH₂ group is the stronger base, and so picks up hydrogen ions first. That leads you back to the zwitterion again.

$$NH_2 \quad + \quad H^+_{(aq)} \quad\quad\quad\quad NH_3^+$$
$$R\text{-CH-COO}^- \quad\quad \longrightarrow \quad R\text{-CH-COO}^-$$
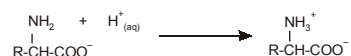
. . . and, of course, you can keep going by then adding a hydrogen ion to the –COO⁻ group.

$$NH_3^+ \quad\quad\quad\quad NH_3^+$$
$$R\text{-CH-COO}^- + H^+_{(aq)} \longrightarrow R\text{-CH-COOH}$$
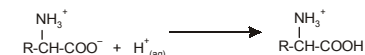
Why isn't the isoelectric point of an amino acid at pH 7?

When an amino acid dissolves in water, the situation is a little bit more complicated than we tend to pretend at this level. The zwitterion interacts with water molecules - acting as both an acid and a base.

As an acid:

$$NH_3^+ \quad\quad\quad\quad NH_2$$
$$R\text{-CH-COO}^- + H_2O \rightleftharpoons R\text{-CH-COO}^- + H_3O^+$$
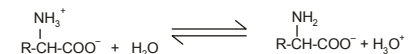
The –NH₃⁺ group is a weak acid and donates a hydrogen ion to a water molecule. Because it is only a weak acid, the position of equilibrium will lie to the left.

As a base:

$$NH_3^+ \quad\quad\quad\quad NH_2^+$$
$$R\text{-CH-COO}^- + H_2O \rightleftharpoons R\text{-CH-COOH} + OH^-$$
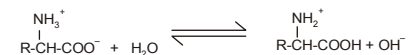
The –COO⁻ group is a weak base and takes a hydrogen ion from a water molecule. Again, the equilibrium lies to the left.

When you dissolve an amino acid in water, both of these reactions are happening.

But, the positions of the two equilibria aren't identical - they vary depending on the influence of the "R" group. In practice, for the simple amino acids we have been talking about, the position of the first equilibrium lies a bit further to the right than the second one. That means that there will be rather more of the negative ion from the amino acid in the solution than the positive one. In those circumstances, if you carried out electrophoresis on the unmodified solution, there would be a slight drift of amino acid towards the positive electrode (the anode). To stop that, you need to cut down the amount of the negative ion so that the concentrations of the two ions are identical. You can do that by adding a very small amount of acid to the solution, moving the position of the first equilibrium further to the left. Typically, the pH has to be lowered to about 6 to achieve this. For glycine, for example, the isoelectric point is pH 6.07; for alanine, 6.11; and for serine, 5.68.

SECTION 2.11—GEL FITRATION

Gel Filtration

Gel Fitration encompasses a variety of techniques that involve a mobile phase flowing through a bed of stationary phase. A mixture of solutes is resolved or separated as a result of such differences in "affinity" of the solutions for the mobile phase relative to the stationary phase. A solute that has more "affinity" for the mobile phase moves forward with the mobile phase whereas a solute with more "affinity" for

the stationary phase is retarded. Thus, solutes are separated into bands based on their distribution between phases.

Molecules of biological interest are separated or resolved by a variety of chromatography techniques including adsorption chromatography, ion-exchange chromatography, partition chromatography (paper or thin layer chromatography), gas-liquid chromatography, and molecular exclusion chromatography (molecular sieve or gel filtration).

Chromatography involves a "mobile" phase (liquid or gas) flowing over a "stationary" phase (solid or liquid; Figure 1). A mixture of solutes is separated based on the solute components' degree of affinity for the mobile and the stationary phases. If Component #1 has a stronger affinity for the stationary phase than Component #2, then #2 will migrate through the column more rapidly than #1.
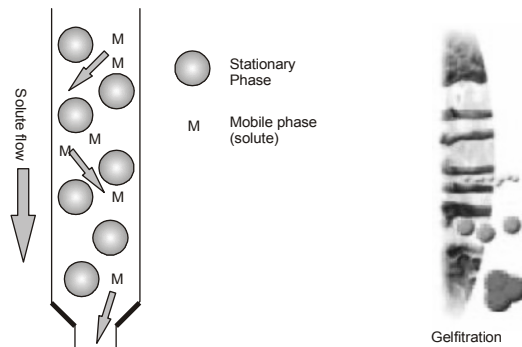
Fig. 2.53 and 2.53a. Showing the Gelfitration diagram

Nutshell overview of gel filtration

The most important factors to understand about gel filtration are:

- Gel filtration separates molecules based solely on their size.
- Larger molecules elute first from the column.
- Smaller molecules elute later from the column.
- Gel filtration chromatography is used primarily to purify proteins or other molecules of interest.
- Gel filtration chromatography can be used to estimate the size of unknown proteins.

Principles of gel filtration

In gel filtration or molecular exclusion process molecules in solution are separated by size as they pass through a column of cross-linked beads that form a three-dimensional network. These polymer beads

(frequently made of dextran, agarose, or acrylamide) comprise the stationary phase. The liquid phase is the solvent that is found both around the beads and in the pores of the stationary phase matrix.

As a sample passes through the column, there are two routes that molecules can take through the column, depending on their size and the size of the pores in the beads. Molecules that are larger than the pores will not enter the stationary phase, staying in the solution (mobile phase) surrounding the beads. Hence they elute first from the column. Smaller molecules will enter the pores in the beads and so move more slowly through the column (Figure 53 & 54). Molecules of intermediate size will enter the stationary phase to some extent, but will not spend as much time there as do the smaller molecules.

To summarize, larger molecules will elute from the column first, and the smallest molecules will elute last, with intermediate molecules strung out in between.

Putting gel filtration in terms that fit the general description of chromatography given above, the beads comprise the stationary phase and the solution is the mobile phase. Larger molecules have a greater affinity for the mobile phase than the stationary phase, so they migrate through the column more rapidly than smaller molecules, which have a greater affinity for the stationary phase.

As the samples elute from the bottom of the column, they are collected in tubes as fractions. Usually, fractions of a particular volume are collected. In most cases, however, the eluted fractions must be tested to determine both what fractions contain the samples (usually proteins) and how much of the sample (protein) is in the fraction. Several methods that are commonly used are: (1) spectrophotometric examination of the fractions; (2) SDS-PAGE of the fractions; and (3) assaying the fractions for a particular enzymatic activity.
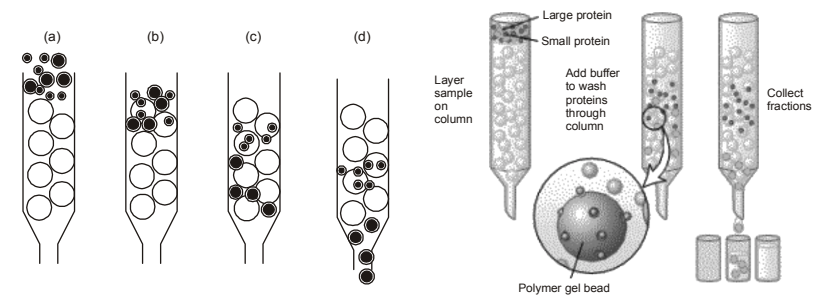
Fig. 2.54 and 2.54A. Showing the Protein Separation

Once the sample concentrations in the fractions have been determined, the concentrations can be graphed against the elution volume to create an elution profile (Figure 55). The first volume that elutes from the column before any fractions is called the void volume (V_0) of the column. Any proteins or other molecules that are too large to enter the pores of the beads will elute immediately after the void volume.
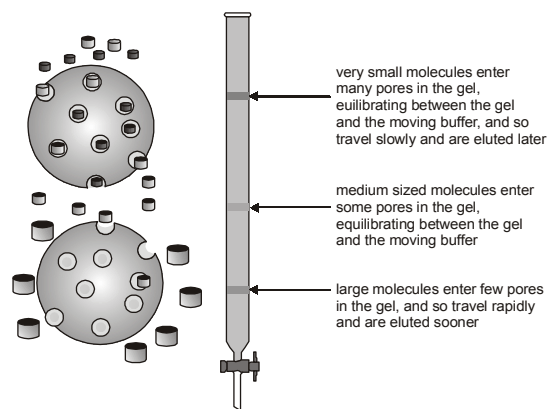
Fig. 2.53A. Showing the Details of Gel fitration in column

If the molecular weights of the proteins are already known, then these data can be used to create a standard curve to calculate the molecular weight of an unknown protein. The elution volume is plotted against the known molecular weights of the proteins on semilog graph paper (Figure 56) and a line derived. From this line and the elution volume of an unknown protein, the molecular weight of the unknown can be estimated.
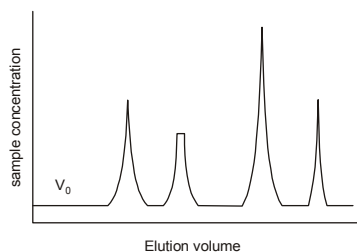
Fig. 2.55 : Elution profile from filtration chromatography. Plot of elution volume against concentration for 4 proteins. V_0 is the void volume of the column.

There are many types of gel filtration materials. The choice of matrix depends on the range of size of molecules to be separated and the goal of the separation. Different bead types have pores of different sizes. Consult a reference book such as those listed in the below for more details on factors to be considered in choosing a matrix.

The gel filtration material that will be used in the experiment below is called Sephadex G-75 and it will separated molecules with molecular weights from 3,000 to 70,000. Molecules with molecular weights larger than 70,000 will be excluded from entering the beads.

Notes on use of gel filtration

- The choice of matrix depends on the range of size of molecules to be separated and the goal of the separation. Different bead types have pores of different sizes. Consult a reference book such as those listed below for more details on factors to be considered in choosing a matrix.

- The matrix beads normally come in dry form and must be swollen before use. It is important not to use a magnetic stirrer when preparing the beads, or the beads can be fragmented. It takes several days to swell beads like the Sephadex that you will use today. One short cut, however, is to autoclave the solution. This causes the beads to swell more rapidly without damaging them.

- Never allow a gel filtration column to dry out. If it dries out, the column must be re-poured. It is crucial for good separation that the column be consistent from top to bottom.

Fig. 2.56 : Molecular weight vs. elution volume. Plot of molecular weight of proteins against their elution volume from a gel filtration column. Proteins are yeast alcohol dehydrogenase [150 kilodaltons(kD)], bovine serum albumin (66 kD), carbonic anhydrase (29 kD), and cytochrome c (12.4 kD).

In Part 1 of this experiment you will pour your own gel filtration column (G-100 fast flow). You will then calibrate your column using four separate molecules: Blue Dextran, yeast alcohol dehydrogenase, BSA, and cytochrome-c.

In Part 2, you will use your calibrated gel filtration column to determine the molecular weight of your selected protein.

Part I: Column Pouring and Calibration

Materials

1 chromatographic columns, 1.0 cm (diameter) X 20 cm (length)

Sephadex G-150 -superfine resin equilibrated with 0.1 M phosphate buffer, pH 7.0

0.1 M phosphate buffer, pH 7.0

standards: solution containing blue dextran yeast alcohol dehydrogenase, BSA, and cytochrome-c.

test tubes and rack, ring stand, clamps, pinch clamps

selected protein sample: 1 mg/mL sample of your selected protein

96-well plate

BCA reagents

Procedure

Label the column (G-150) with your names. Use the pinch clamp to close the effluent tubes of the column. Pour several mL phosphate buffer into the column. Gently open the pinch clamp to allow some of the buffer to flow out of the column. The object of this step is to eliminate air bubbles from the bottom of the column and the effluent tubes. Once the air bubbles are eliminated, close the pinch clamp. There should be several mL of buffer remaining in the column at this point. Gently swirl the Sephadex G-50 and pour into the column labeled "G-50". After the resin as settled, open the pinch clamp and allow the column to drip until the settled chromatographic bed reaches a height of about 18 cm. DO NOT ALLOW THE COLUMN TO RUN DRY!! Add phosphate buffer to the column to keep the fluid level above the resin bed.

Once an 18 cm resin bed has been established, close the column effluent tube with the pinch clamp. Measure the actual height of the resin bed. Remove most of the buffer from the top of the column. Carefully open the pinch clamp and allow the column to drip until the liquid level reaches the top if the resin bed. Immediately add 0.2 mL of the sample to the top of the resin by allowing the solution to gently run down the wall of the column. Place the effluent tube in the first test tube in the test tube rack (this will be fraction 1) and open the pinch clamp. Do not disturb the top of the resin! Allow the sample to enter the resin and then gently add a few drops of the phosphate buffer. Allow this buffer to penetrate the column and then gently add phosphate buffer to fill the column. Collect fractions until all the colored material has eluted from the column. Close the patch clamp. Collect 1.0 mL of effluent in each tube. After 1 mL has been collected in the first tube (fraction 1), switch to the second tube (fraction 2) and collect the next 1 mL, etc. Perform a BCA assay on selected (every other, every third) tube to determine the amount of protein present. Note that blue dextran is not a protein; it will not be detected by the BCA assay. However, the molecule has a blue color. The fraction containing the most intense blue color is the should be taken as the Ve for the blue dextran (which is also the Vo for the column).

Graphically represent the "elution profile" for your columns by plotting absorbance (y-axis) versus elution volume, mL (x-axis). You should have three series of data, one for each molecule/color present.

Problems

1. Describe and explain your results.
2. From your data, calculate vt (total column volume), vo (void volume), and ve (elution volume for each solute).

   vt = vo + vi+ vm

   where vm is the volume of the gel itself and vi is the volume included in the gel.

   Since vm is very small, it is assumed that

   vt = vi + vo.

3. Also be sure to include answers to the assigned problems.

Fig. 2.57 : Showing the Different photographs of gel Fitration Experiment

Fig. 2.57A and 57B Showing the Actual Gel Fitration colun in action

SUB-SECTION 2.11B—ELECTROPHORESIS

Electrophoresis is the best-known electrokinetic phenomenon. It was discovered by Reuss in 1807. He observed that clay particles dispersed in water migrate under influence of an applied electric field. There are detailed descriptions of Electrophoresis in many books on Colloid and Interface Science.[2][3][4][5][6] There is an IUPAC Technical Report prepared by a group of well known experts on the electrokinetic phenomena. Generally, electrophoresis is the motion of dispersed particles relative to a fluid under the influence of an electric field that is space uniform. Alternatively, similar motion in a space non-uniform electric field is called dielectrophoresis.
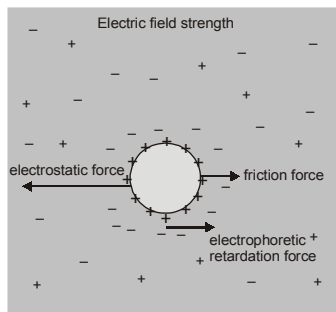
Fig. 2.58 and 58A: Showing the Pattern of the electrophoresis system (diagramatic representation)

Electrophoresis occurs because particles dispersed in a fluid almost always carry an electric surface charge. An electric field exerts electrostatic Coulomb force on the particles through these charges. Recent molecular dynamics simulations, though, suggest that surface charge is not always necessary for electrophoresis and that even neutral particles can show electrophoresis due to the specific molecular structure of water at the interface.

The electrostatic Coulomb force exerted on a surface charge is reduced by an opposing force which is electrostatic as well. According to double layer theory, all surface charges in fluids are screened by a diffuse layer. This diffuse layer has the same absolute charge value, but with opposite sign from the surface charge. The electric field induces force on the diffuse layer, as well as on the surface charge. The total value of this force equals to the first mentioned force, but it is oppositely directed. However, only part of this force is applied to the particle. It is actually applied to the ions in the diffuse layer. These ions are at some distance from the particle surface. They transfer part of this electrostatic force to the particle surface through viscous stress. This part of the force that is applied to the particle body is called **electrophoretic retardation force**.

There is one more electric force, which is associated with deviation of the double layer from spherical symmetry and surface conductivity due to the excess ions in the diffuse layer. This force is called the **electrophoretic relaxation force**.

All these forces are balanced with hydrodynamic friction, which affects all bodies moving in viscous fluids with low Reynolds number. The speed of this motion v is proportional to the electric field strength E if the field is not too strong. Using this assumption makes possible the introduction of electrophoretic mobility μ_e as coefficient of proportionality between particle speed and electric field strength:

$$\mu_e = \frac{\upsilon}{E}$$

Multiple theories were developed during 20th century for calculating this parameter. Ref. 2 provides an overview.

Theory
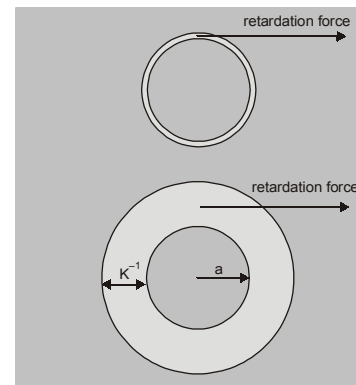
Fig. 2.59 : Showing the Force Oreintation of the protein molecules in the electrophoresis system

The most known and widely used theory of electrophoresis was developed by Smoluchowski in 1903

$$\mu_e = \frac{\varepsilon\varepsilon_0\varsigma}{\eta}$$

where ε is the dielectric constant of the dispersion medium, ε_0 is the permittivity of free space ($C^2\ N^{-1}\ m^{-2}$), η is dynamic viscosity of the dispersion medium (Pa s), and ς is zeta potential (i.e., the electrokinetic potential of the slipping plane in the double layer).

Smoluchowski theory is very powerful because it works for dispersed particles of any shape and any concentration, when it is valid. Unfortunately, it has limitations of its validity. It follows, for instance, from the fact that it does not include Debye length $ê^{-1}$. However, Debye length must be important for electrophoresis, as follows immediately from the Figure on the right. Increasing thickness of the DL leads to removing point of retardation force further from the particle surface. The thicker DL, the smaller retardation force must be.

Detailed theoretical analysis proved that Smoluchowski theory is valid only for sufficiently thin DL, when Debye length is much smaller than particle radius *a*:

$$êa >> 1$$

This model of "thin Double Layer" offers tremendous simplifications not only for electrophoresis theory but for many other electrokinetic theories. This model is valid for most aqueous systems because the Debye length is only a few nanometers there. It breaks only for nano-colloids in solution with ionic strength close to water

Smoluchowski theory also neglects contribution of surface conductivity. This is expressed in modern theory as condition of small Dukhin number

$$Du << 1$$

Creation of electrophoretic theory with wider range of validity was a purpose of many studies during 20th century.

One of the most known considers an opposite asymptotic case when Debye length is larger than particle radius:

$$êa < 1$$

It is called the "thick Double Layer" model. Corresponding electrophoretic theory was created by Huckel in 1924 [11]. It yields the following equation for electrophoretic mobility:

$$\mu_e = \frac{2\varepsilon\varepsilon_0\varsigma}{3\eta}$$

This model can be useful for some nano-colloids and non-polar fluids, where Debye length is much larger.

There are several analytical theories that incorporate surface conductivity and eliminate restriction of the small Dukhin number. Early pioneering work in that direction dates back to Overbeek and Booth.

Modern, rigorous theories that are valid for any Zeta potential and often any $êa$, stem mostly from the Ukrainian (Dukhin, Shilov and others) and Australian (O'Brien, White, Hunter and others) Schools.

Historically the first one was Dukhin-Semenikhin theory. Similar theory was created 10 years later by O'Brien and Hunter [15]. Assuming **thin Double Layer**, these theories would yield results that are very close to the numerical solution provided by O'Brien and White.

The Electrophoresis System: The Electrophoresis chamber and the complete unit

electrophoresis chamber is quite easy, inexpensive and fun. After you have made your chamber, try it out using the Colorful Electrophoresis experiments.

SAFETY: Remember to wear safety glasses at all times while constructing this chamber.

TIP: Leave the paper on each Plexiglas piece until you are ready to use it. Write the name of each piece of Plexiglas® on the paper covering the surface before beginning construction. Remove the paper as you work with each piece.

Fig. 2.60 and 60A : Showing the Electrophoresis chamber and the complete unit (this complete product is alsoavailable with tarson)$

Agarose gel electrophoresis is a method used in biochemistry and molecular biology to separate DNA, or RNA molecules by size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field (electrophoresis). Shorter molecules move faster and migrate farther than longer ones.

Applications

- Estimation of the size of DNA molecules following restriction enzyme digestion, e.g. in restriction mapping of cloned DNA.
- Analysis of PCR products, e.g. in molecular genetic diagnosis or genetic fingerprinting
- Separation of restricted genomic DNA prior to Southern transfer, or of RNA prior to Northern transfer.

$ if any student wants to make his or her's own electrophoresis unit (as it is very costly to purchase) consult the author's Architecture of Chemistry an instrumental approach.

The advantages are that the gel is easily poured, does not denature the samples. The samples can also be recovered. The disadvantages are that gels can melt during electrophoresis, the buffer can become exhausted, and different forms of genetic material may run in unpredictable forms. After the experiment is finished, the resulting gel can be stored in a plastic bag in refrigerator.

Factors affecting migration

The most important factor is the length of the DNA molecule, smaller molecules travel farther. But conformation of the DNA molecule is also a factor. To avoid this problem linear molecules are usually separated, usually DNA fragments from a restriction digest, linear DNA PCR products, or RNAs.

Increasing the agarose concentration of a gel reduces the migration speed and enables separation of smaller DNA molecules. The higher the voltage, the faster the DNA moves. But voltage is limited by the fact that it heats and ultimately causes the gel to melt. High voltages also decrease the resolution (above about 5 to 8 V/cm).

Conformations of a DNA plasmid that has not been cut with a restriction enzyme will move with different speeds (slowest to fastest): nicked or open circular, linearised, or supercoiled plasmid.

Visualisation: Ethidium Bromide (EtBr) and dyes

The most common dye used to make DNA or RNA bands visible for agarose gel electrophoresis is ethidium bromide, usually abbreviated as EtBr. It fluoresces under UV light when intercalated into DNA (or RNA). By running DNA through an EtBr-treated gel and visualizing it with UV light, any band containing more than ~20ng DNA becomes distinctly visible. EtBr is a known carcinogen, however, and safer alternatives are available.

SYBR Green I is another dsDNA stain, produced by Invitrogen. It is more expensive, but 25 times more sensitive, and possibly safer than EtBr, though there is no data addressing its mutagenicity or toxicity in humans.

SYBR Safe is a variant of SYBR Green that has been shown to have low enough levels of mutagenicity and toxicity to be deemed nonhazardous waste under U.S. Federal regulations.[3] It has similar sensitivity levels to EtBr, but, like SYBR Green, is significantly more expensive.

Since EtBr stained DNA is not visible in natural light, scientists mix DNA with negatively charged **loading buffers** before adding the mixture to the gel. Loading buffers are useful because they are visible in natural light (as opposed to UV light for EtBr stained DNA), and they co-sediment with DNA (meaning they move at the same speed as DNA of a certain length). Xylene cyanol and Bromophenol blue are common loading buffers; they run about the same speed as DNA fragments that are 5000 bp and 300 bp in length respectively, but the precise position varies with percentage of the gel. Other less frequently used progress markers are Cresol Red and Orange G which run at about 125 bp and 50 bp.

Percent agarose and resolution limits

Agarose gel electrophoresis can be used for the separation of DNA fragments ranging from 50 base pair to several megabases (millions of bases) using specialized apparatus. The distance between DNA bands of a given length is determined by the percent agarose in the gel. In general lower concentrations

of agarose are better for larger molecules because they result in greater separation between bands that are close in size. The disadvantage of higher concentrations is the long run times (sometimes days). Instead high percentage agarose gels should be run with a pulsed field electrophoresis (PFE), or field inversion electrophoresis.

Most agarose gels are made with between 0.7% (good separation or resolution of large 5–10kb DNA fragments) and 2% (good resolution for small 0.2–1kb fragments) agarose dissolved in electrophoresis buffer. Up to 3% can be used for separating very tiny fragments but a vertical polyacrylamide gel is more appropriate in this case. Low percentage gels are very weak and may break when you try to lift them. High percentage gels are often brittle and do not set evenly. 1% gels are common for many applications.
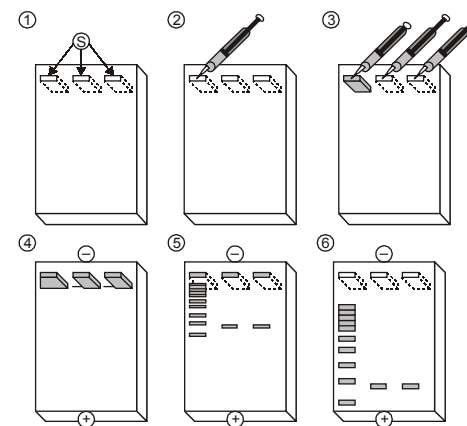
Fig. 2.61 : Showing the Diagramatic representation of the agros gel electrophoresis

Buffers

There are a number of buffers used for agarose electrophoresis. The most common being: tris acetate EDTA (TAE), Tris/Borate/EDTA (TBE) and Sodium borate (SB). TAE has the lowest buffering capacity but provides the best resolution for larger DNA. This means a lower voltage and more time, but a better product. SB is relatively new and is ineffective in resolving fragments larger than 5 kbp; However, with its low conductivity, a much higher voltage could be used (up to 35 V/cm), which means a shorter analysis time for routine electrophoresis. As low as one base pair size difference could be resolved in 3% agarose gel with an extremely low conductivity medium (1 mM Lithium borate).

Analysis

After electrophoresis the gel is illuminated with an ultraviolet lamp (usually by placing it on a light box, while using protective gear to limit exposure to ultraviolet radiation) to view the DNA bands. The ethidium bromide fluoresces reddish-orange in the presence of DNA. The DNA band can also be cut out of the gel, and can then be dissolved to retrieve the purified DNA. The gel can then be photographed usually with a digital or polaroid camera. Although the stained nucleic acid fluoresces reddish-orange, images are usually shown in black and white (see figures).

Gel electrophoresis research often takes advantage of software-based image analysis tools, such as ImageJ.

Materials and Method

Typically 10-30 ìl/sample of the DNA fragments to separate are obtained, as well as a mixture of DNA fragments (usually 10-20) of known size (after processing with DNA size markers either from a commercial source or prepared manually).

- Buffer solution, usually TBE buffer or TAE 1.0x, pH 8.0
- Agarose
- An ultraviolet-fluorescent dye, ethidium bromide, (5.25 mg/ml in H_2O). The stock solution be careful handling this. Alternative dyes may be used, such as SYBR Green.
- Nitrile rubber gloves. Latex gloves do not protect well from ethidium bromide
- A color marker dye containing a low molecular weight dye such as "bromophenol blue" (to enable tracking the progress of the electrophoresis) and glycerol (to make the DNA solution denser so it will sink into the wells of the gel).
- A gel rack
- A "comb"
- Power Supply
- UV lamp or UV lightbox or other method to visualize DNA in the gel

Preparation

There are several methods for preparing gels. A common example is shown here. Other methods might differ in the buffering system used, the sample size to be loaded, the total volume of the gel (typically thickness is kept to a constant amount while length and breadth are varied as needed). Most agarose gels used in modern biochemistry and molecular biology are prepared and run horizontally.

1. Make a 1% agarose solution in 100ml TAE, for typical DNA fragments (see figures). A solution of up to 2-4% can be used if you analyze small DNA molecules, and for large molecules, a solution as low as 0.7% can be used.

Wear gloves from here on, ethidium bromide is a mutagen
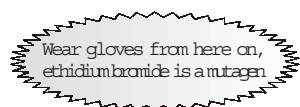
2. Carefully bring the solution just to the boil to dissolve the agarose, preferably in a microwave oven.
3. Let the solution cool down to about 60 °C at room temperature, or water bath. Stir or swirl the solution while cooling.
1. Add 5 µl ethidium bromide stock (10 mg/ml) per 100 ml gel solution for a final concentration of 0.5 ug/ml. Be very careful when handling the concentrated stock. Some researchers prefer not to add ethidium bromide to the gel itself, instead soaking the gel in an ethidium bromide solution after running.
2. Stir the solution to disperse the ethidium bromide, then pour it into the gel rack.
3. Insert the comb at one side of the gel, about 5-10 mm from the end of the gel.
4. When the gel has cooled down and become solid, carefully remove the comb. The holes that remain in the gel are the wells or slots.
5. Put the gel, together with the rack, into a tank with TAE. Ethidium bromide at the same concentration can be added to the buffer. The gel must be completely covered with TAE, with the slots at the end electrode that will have the negative current.

Procedure

After the gel has been prepared, use a micropipette to inject about 2.5 µl of stained DNA (a DNA ladder is also highly recommended). Close the lid of the electrophoresis chamber and apply current (typically 100 V for 30 minutes with 15 ml of gel). The colored dye in the DNA ladder and DNA samples acts as a "front wave" that runs faster than the DNA itself. When the "front wave" approaches the end of the gel, the current is stopped. The DNA is stained with ethidium bromide, and is then visible under ultraviolet light.

Fig. 2.62: Showing the method before the interpretation of the electrophoresis pattern

1. The agarose gel with three slots/wells (S).
2. Injection of DNA ladder (molecular weight markers) into the first slot.
3. DNA ladder injected. Injection of samples into the second and third slot.

4. A current is applied. The DNA moves toward the positive anode due to the negative charges on its phosphate backbone.

5. Small DNA strands move fast, large DNA strands move slowly through the gel. The DNA is not normally visible during this process, so the marker dye is added to the DNA to avoid the DNA being run entirely off the gel. The marker dye has a low molecular weight, and migrates faster than the DNA, so as long as the marker has not run past the end of the gel, the DNA will still be in the gel.

6. Add the color marker dye to the DNA ladder.
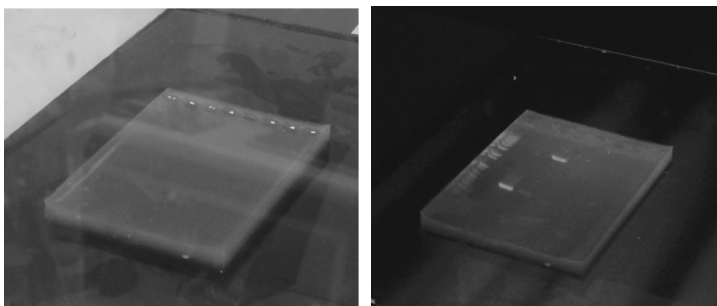
Fig. 2.63 and 63A: Showing the gel Slab after the experiment and under UV light
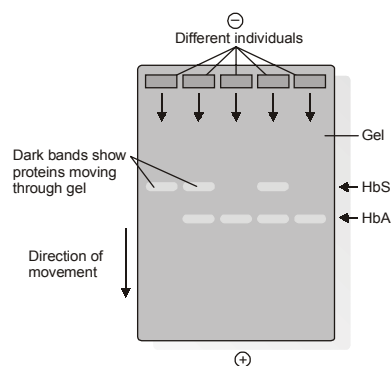
Fig. 2.63B: Showing the digaramatic interpretration of a gel slab after electrophoresis

SUB-SECTION 2.11C—ULTRACENTRIFUGATION

When you put a particle in a centrifugal field, it is acted upon by the centrifugal force, which is proportional to the molecular weight (M), to the square of the speed (angular velocity, rpm) of the rotor (w^2) and to the distance of the solution from the center of rotation (r): Cent. Force ~ Mw^2r. Acting in the opposite direction to particle motion is friction. This frictional force is proportional to the velocity of the particle (V) and to a coefficient of friction (f) that depends on the shape of the particle: Friction force ~ fV. The particle will accelerate until a velocity is achieved such that these two opposing forces are equal, after which the particle will continue to sediment, but at a constant velocity. Setting the 2 forces equal to each other we have: $Mw^2r = fV$, and so:

$$V \sim Mw^2r/f.$$

In general, this means that velocity increases as mass (molecular weight) increase, and velocity decreases as the cross-sectional size (diameter) increases (f increases with cross-sectional size).

V depends on the particular machine and on the speed of rotation. So you can compare results irregardless of machine and speed, you calculate the Svedberg constant or S value instead of V. $S = V/w^2r$. Macromolecules usually have S values betw een 1 and 100×10^{-13} sec. A value of 1×10^{-13} sec is called one Svedberg unit or S. Many molecules are known by their S values, for example 23 S ribosomal RNA. Its S value was known before its function or molecular weight, and so has become its name.

Fig. 2.64 : Showing the Ultracentrifuge

Molecules of different S values are usually separated by layering them on top of a dense solution (such as sucrose) and then running the centrifuge to force the molecules through the solution. The molecules of larger S value go farther because of eithe r larger molecular weight or less frictional drag or both. You stop the centrifuge when the different molecules have traveled different distances down the tube and then remove the solution by puncturing the bottom of the tube or pumping the solution off t he top. The invention of the ultracentrifuge (a super fast centrifuge) by Svedberg was what originally made the purification of macromolecules possible.

The **ultracentrifuge** is a centrifuge optimized for spinning a rotor at very high speeds, capable of generating acceleration as high as 1,000,000 g (9,800 km/s²). There are two kinds of ultracentrifuges, the preparative and the analytical ultracentrifuge. Both classes of instruments find important uses in molecular biology, biochemistry and polymer science. Theodor Svedberg invented the analytical ultracentrifuge in 1923, and won the Nobel Prize in Chemistry in 1926 for his research on colloids and proteins using the ultracentrifuge.

The vacuum ultracentrifuge was invented by Edward Greydon Pickels. It was his contribution of the vacuum which allowed a reduction in friction generated at high speeds. Vacuum systems also enabled the maintenance of constant temperature.

In 1946, Pickels cofounded Spinco (Specialized Instruments Corp.) and marketed an ultracentrifuge based on his design. Pickels, however, considered his design to be complicated and developed a more "foolproof" version. But even with the enhanced design, sales of the technology remained low, and Spinco almost went bankrupt. The company survived and was the first to commercially manufacture ultracentrifuges, in 1947. In 1949, Spinco introduced the Model L, the first preparative ultracentrifuge to reach a maximum speed of 40,000 rpm. In 1954, Beckman Instruments (now Beckman Coulter) purchased the company, forming the basis of its Spinco centrifuge division.

Analytical ultracentrifuge

In an analytical ultracentrifuge, a sample being spun can be monitored in real time through an optical detection system, using ultraviolet light absorption and/or interference optical refractive index sensitive system. This allows the operator to observe the evolution of the sample concentration versus the axis of rotation profile as a result of the applied centrifugal field. With modern instrumentation, these observations are electronically digitized and stored for further mathematical analysis. Two kinds of experiments are commonly performed on these instruments: sedimentation velocity experiments and sedimentation equilibrium experiments.

Sedimentation velocity experiments aim to interpret the entire time-course of sedimentation, and report on the shape and molar mass of the dissolved macromolecules, as well as their size-distribution. The size resolution of this method scales approximately with the square of the particle radii, and by adjusting the rotor speed of the experiment size-ranges from 100 Da to 10 GDa can be covered. Sedimentation velocity experiments can also be used to study reversible chemical equilibria between macromolecular species, by either monitoring the number and molar mass of macromolecular complexes, by gaining information about the complex composition from multi-signal analysis exploiting differences in each components spectroscopic signal, or by following the composition dependence of the sedimentation rates of the macromolecular system, as described in Gilbert-Jenkins theory.

Sedimentation equilibrium experiments are concerned only with the final steady-state of the experiment, where sedimentation is balanced by diffusion opposing the concentration gradients, resulting in a time-independent concentration profile. Sedimentation equilibrium distributions in the centrifugal field are characterized by Boltzmann distributions. This experiment is insensitive to the shape of the macromolecule, and directly reports on the molar mass of the macromolecules and, for chemically reacting mixtures, on chemical equilibrium constants.

The kinds of information that can be obtained from an analytical ultracentrifuge include the gross shape of macromolecules, the conformational changes in macromolecules, and size distributions of macromolecular samples. For macromolecules, such as proteins, that exist in chemical equilibrium with different non-covalent complexes, the number and subunit stoichiometry of the complexes and equilibrium constant constants can be studied.

Preparative Ultracentrifuge

Preparative ultracentrifuges are available with a wide variety of rotors suitable for a great range of experiments. Most rotors are designed to hold tubes that contain the samples. *Swinging bucket rotors* allow the tubes to hang on hinges so the tubes reorient to the horizontal as the rotor initially accelerates. *Fixed angle rotors* are made of a single block of metal and hold the tubes in cavities bored at a predetermined angle. *Zonal rotors* are designed to contain a large volume of sample in a single central cavity rather than in tubes. Some zonal rotors are capable of dynamic loading and unloading of samples while the rotor is spinning at high speed.

Preparative rotors are used in biology for pelleting of fine particulate fractions, such as cellular organelles (mitochondria, microsomes, ribosomes) and viruses. They can also be used for gradient separations, in which the tubes are filled from top to bottom with an increasing concentration of a dense substance in solution. Sucrose gradients are typically used for separation of cellular organelles. Gradients of caesium salts are used for separation of nucleic acids. After the sample has spun at high speed for sufficient time to produce the separation, the rotor is allowed to come to a smooth stop and the gradient is gently pumped out of each tube to isolate the separated components.

Hazards
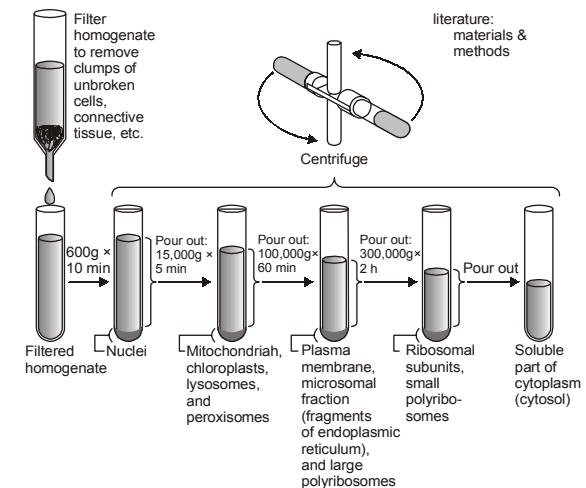
Fig. 2.65 : Showing the Different biological components getting separated by means of differencial centrifugation

The tremendous rotational kinetic energy of the rotor in an operating ultracentrifuge makes the catastrophic failure of a spinning rotor a serious concern. The stresses of routine use and harsh chemical solutions eventually cause rotors to deteriorate. Proper use of the instrument and rotors within recommended limits and careful maintenance of rotors to prevent corrosion and to detect deterioration are necessary to avoid this hazard.

Differential Centrifugation. Generally, the cellular homogenate is first filtered or centrifuged at relatively low speeds to remove unbroken cells. Then centrifugation of the homogenate at a slightly faster speed or for a longer duration will selectively pellet the nucleus—the largest organelle (usually 5–10 îm in diameter). A centrifugal force of 600 g (600 times the force of gravity) is necessary to sediment nuclei; this is generated by a typical centrifuge rotor operating at 500 revolutions per minute (rpm). The undeposited material (the supernatant) is next centrifuged at a higher speed (15,000g × 5 min), which deposits the mitochondria, chloroplasts, lysosomes, and peroxisomes. A subsequent centrifugation in the ultracentrifuge (100,000 g × 60 min) results in deposition of the plasma membrane, fragments of the endoplasmic reticulum, and large polyribosomes. A force of 100,000 g requires about 50,000 rpm in an ultracentrifuge; at this speed, the rotor chamber is kept in a high vacuum to reduce heating due to friction between air and the spinning rotor. The recovery of ribosomal subunits, small polyribosomes, and particles such as complexes of enzymes requires additional centrifugation at still higher speeds. Only the cytosol—the soluble aqueous portion of the cytoplasm—remains undeposited after centrifugation at 300,000 g for 2 hours.

Differential centrifugation schemes involve stepwise increases in the speed of centrifugation. At each step, more dense particles are separated from less dense particles, and the successive speed of centrifugation is increased until the target particle is pelleted out. The final supernatant is removed, the pellet is resuspended, and further study or purification can be done on it. The fractionation of rat liver is an example of how this process works:

An important thing to note is that there is cross contamination between the second and third pellets. Mitochondria show up in Pellet 3 and lysosomes show up in Pellet 2. This shows that the separations made by this technique aren't absolute purifications, but relative enrichments of organelles.
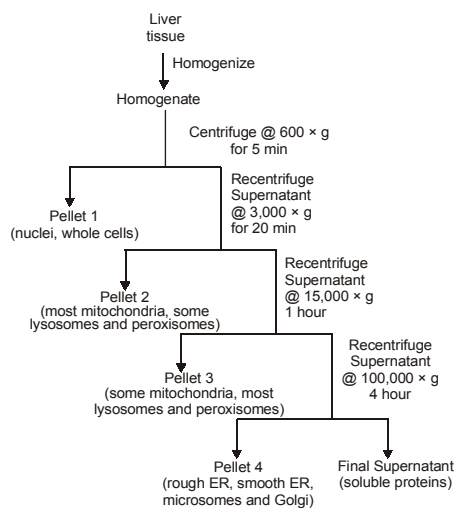
Fig. 2.66 : Showing the flow chart for the protein separation from liver tissue

SUB-SECTION 2.11D—NINHYDRIN REACTION

Ninhydrin (2,2-Dihydroxyindane-1,3-dione) is a chemical used to detect ammonia or primary and secondary amines. When reacting with these free amines, a deep blue or purple color known as Ruhemann's purple is evolved. Ninhydrin is most commonly used to detect fingerprints, as amines left over from peptides and proteins (terminal amines or lysine residues) sloughed off in fingerprints react with ninhydrin.

Uses

Ninhydrin can also be used to monitor deprotection in solid phase peptide synthesis (Kaiser Test).[2] When the growing peptide chain is deprotected, a ninhydrin test yields blue. If the next peptide residue is coupled then the test is colorless or yellow.

Ninhydrin is also used in amino acid analysis of proteins: Most of the amino acids are hydrolyzed and reacted with ninhydrin except proline; Also, certain amino acid chains are degraded. Therefore, separate analysis is required for identifying such amino acids that either react differently or don't react at all with ninhydrin. The rest of the amino acids are then quantified colorimetrically after separation by chromatography.

A solution suspected of containing the ammonium ion can be tested by ninhydrin by dotting it onto a solid support (such as silica gel); treatment with ninhydrin should result in a dramatic purple color if the solution contains this species. In the analysis of a chemical reaction by thin layer chromatography (TLC), the reagent can also be used. It will detect, on the TLC plate, virtually all amines, carbamates and also, after vigorous heating, amides.

Reactivity

The carbon atom of a carbonyl bears a partial positive charge, so the central carbon of a 1,2,3-tricarbonyl is less stable and more electrophilic than a simple ketone. In most compounds, a carbonyl is more stable than the dihydroxy (hydrate) form. However, ninhydrin is a stable hydrate of the central carbon because this form does not have the destabilizing effect of adjacent carbonyl partial-positive centers. Indane-1,2,3-trione reacts readily with nucleophiles.

Note that in order to generate the ninhydrin chromophore, the amine is condensed with a molecule of ninhydrin to give a Schiff base. Thus only ammonia and primary amines can proceed past this step. At this step, there must also be an alpha proton (H* in the diagram) for Schiff base transfer, so an amine adjacent to a tertiary carbon cannot be detected by the ninhydrin test. The reaction of ninhydrin with secondary amines gives an iminium salt, which is also coloured, and this is generally yellow-orange in color.

SUB-SECTION 2.11D—DETERMINATION FO PROTEIN STRUCTURE WITH NINHYDRIN

Spectrophotometric method is quantitative in contrast to previous ones (1, 3, 4), because exclusion of oxygen during the reactions prevents fading of the red and blue colors of hydrindantin. Control of the oxygen content and of the pH provides for a clear distinction between hydrindantin reactions and those

of ninhydrin. These controls are necessary, for, on the one hand, oxidation of hydrindantin yields 2 moles of ninhydrin, and, on the other hand, ninhydrin at certain pH values can form hydrin-dantin by way of o-carboxyphenylglyoxal. The probable structure of the red-colored derivative of hydrindantin is indanone-enediol and not, as Ruhemann (10) believed, the monovalent salt of intact hydrindantin. In the reaction of amino acids and hydrindantin, 1 mole of indanone-enediol is used up for each mole of Ruhemann's purple formed. The reaction with amines summarized in formulae I could be eit.her a simultaneous or a sequential condensation with indanone-enediol and ninhydrin. The alternatives will be considered in a separate ,paper on the order of reaction, which the present method has made possible.

Appartus—

1. Spectrophotometers. The Beckman model DU instrument, with quartz prism, and the Coleman clinical instrument, model 6, were used.

2. Cuvettes and reaction vessels. For the Beckman instrument silica for the measurement of ultraviolet absorption, Corex for visible light.

The length of the light path was 1 cm. For the Coleman instrument,

Hamilton vessels (11) were calibrated for length of the light path, which averaged 1.89 cm, and were used as reaction vessels as well.

Preparation of Hydrindantin- A solution of 0.5 gm. of ascorbic acid and 1 gm. of ninhydrin in 200 ml. of McIlvaine's buffer (0.1 M) at pH 3 washeated to 90° and the crystals allowed settling at room temperature. Recrystallization from hot acetone yielded 350 mg. of colorless anhydrous hydrindantin. The purity of the product was checked by elementary analysis and by its melting point. Preparation of Oxygen-Free Reaction Solutions. Since at room temperature water does not dissolve hydrindantin, it was dissolved in acetone in a concentration of 1 mg. per ml. 1 ml. was delivered to each Hamilton vessel and dried by passing a stream of nitrogen above, not in, the solution. 10 ml. of buffer solution, either alone as a control or containing another reagent under test, oxygen-free after a stream of nitrogen was passed through it for 5 minutes, were delivered to each vessel, which was lubricated and quickly closed so as to be air-tight. The gas was then removed by suction from a motor pump until the pressure was constant at about 2 mm. of Hg. Each vessel in the control and test group was again made air-tight and was immersed upright in a frame in a boiling, distilled water bath for a known time interval. Spectrophotometric Readings-The optical density was recorded at wave-lengths of 490 and 570 ml.c in the Coleman instrument. Sometimes the measurements were made as quickly as possible after removal from the boiling water bath, in order to record the optical density at a temperature close to 100". In such a case, it was found that three measurements could be taken comfortably in from 1 to 2 minutes. For the most part, they were taken at room temperature after cooling in a water bath, and again after passing air through the solutions for 3 minutes. Absorption Spectra of, Red and Blue Colors Derived from Hydrindantin Different from Ruhemann's Purple .Solutions of hydrindantineither in Sorensen's NaOH-borate buffer at pH 9.2 or in 0.4 N NaOH were prepared to be oxygen-free in cuvettes. The visible colors were constant for at least 48 hours, but ultraviolet absorption, without change in position of the maxima, slowly lessened until a constant value was reached at the end of 48 hours. The result is explained by hydrolysis of hydrindantin into diketohydrindol and ninhydrin, followed by irreversible transformation of ninhydrin into either o-carboxyphenylglyoxal or o-carboxymandelic

acid, depending on the pH; either process can be detected by change in the ultraviolet absorption spectrum of ninhydrin (see MacFadyen.The spectrum, Curve 1, Fig. 66, for the blue color is markedly different from the curve for Ruhemann's purple with respect to position and intensity of maxima. These differences invalidate Retinger's concept. Formation of Ruhemann's Purple from ol-Alanine and Hydrindantin Independent of NH_3 Pathway (Table below)-At pH 5.3 at lOO", and anaerobically, the intensity of purple color in the reaction of hydrindantin, 0.3 mM, with the amino acid was 5 times that for ammonium salts in the same concentration, 0.56 mM. Therefore, the concepts of Ruhemann (1) and Harding et al. (3, 4), necessitating an NH_3 pathway, are inadequate. The of the reaction.Hydrindantin Red Color Disappears As Ruhemann's Purple is Formed, Mole for Mole (Table I)-Difficulties of quantitative estimation of concentrations of Ruhemann's purple and the red color from hydrindantin, together in reaction mixtures, were obviated in the following ways.

Table 2.50 : Formation of Ruhemann's Purple from α-Alaninne and Hydrindantin with Disappearance of Hydrindantin Red Color

Reaction time in boiling water bath	Observed optical densities, units × 10³					Hydrindantin disappearance	Formation$ of Ruhemann's purpose
	a-Alanine			Present	Absent		
	P_{570}	D_{490}^{-O}	D_{490}^{+O}	R_{490}'	R_{490}''		
	a*	b	c	d = b − c	c	f = 687 (c-d) × 10⁻³	g = af,f₂×10⁻³ (average
min.						mM per l.	mM per l.
10	360	283$$	123	160	175	4.8-13.7	12.1
	360	290	122	168	180		
20	620	365	200	165	187	15.1-20.6	22.7
	640	363	199	164	194		
30	860	441	278	163	205	25.4–31.6	35.5
	880	440	281	159	200		
40	1010	465	324	141	212	39.2–52.9	46.4
	1000	498	338	160	218		
50	1150	492	370	122	232	74.8–77.0	63.5
	1280	541	418	123	234		
60	1290	546	434	112	230	77.0–81.0	76.4
	1360	566	450	116	228		

*For explanation of P_{570}, R_{490}, computation of the factor 687, and for f1 and f2 see the text.
$For $(NH_4)_2SO_4$ the results, in chronological order, were 2.2, 5.0, 5.7, 9.5, 14.3 and 15.7.
$$The third figure was estimated by interpolation.

Ruhemann's purple is decolorized at pH 5.3, even under anaerobic conditions, when its solutions are heated to boiling, but not significantly at room temperature. Therefore, estimates of the amount formed in a given time interval, in contrast to the amount present, required measurements of the intensity of the purple color remaining in solution of known amounts of the sodium salt of diketohydrindamine-diketohydrindyli(lcne (8) under the conditions of the cu-alaninc reaction with hydrindanbin including all reagents except oc-alanine. Such tests provided us with factors, fl, by which the optical density at X = 570 rnp observed at a given time interval of boiling was converted into the initial optical density before heating. Furthermore, while it is true that Ruhemann's purple obeys Beer's law (8), it was necessary to correct for spectrophotometric conditions of the Hamilton vessels and the Coleman instrument with respect to deviations from Beer's law, but not in the case of the red color. For this purpose the sodium salt of diketohydrindamine-diketohydrin-dylidene was dissolved in buffer at pH 9, and the optical densities at varied concentration determined in the Hamilton vessels in the Coleman instrument. From these results factors, fi, were computed which when multiplied by the optical density at X = 570 mp, in so far as it is referable only to Ruhemann's purple, yielded the concentration of the sodium salt in micromoles per liter.

Application of the factors, depending on a clear distinction between the purple color due to the sodium salt and the red color due to hydrindantin,was made by taking advantage of two facts. Whereas Ruhemann's purple is stable when oxygenated at pH 10 to 11 at room temperature, the red color is discharged in 3 minutes. Therefore, the optical density after oxygenation, measured at X = 570 m,u, D $72, when corrected in the usual manner for variation in length' of the light path and translucency from vessel to vessel, is representative of Ruhemann's purple and is denoted as P670, whereas the optical density difference due to oxygenation, measured at h = 490 rnp, DG: - D&$, when similarly corrected, is representative of the hydrindantin red color and is denoted by ROW.

The experiments were carried out with 0.5 mg. of cr-alanine (or 0.37 mg. of (NH&Sod) and 1 mg. of hydrindantin in 10 ml. of 0.1 M acetate buffer at pH 5.3. The solutions, in duplicate, were heated to boiling, anaerobically, for a given time interval of 10, 20, 30, 40, 50, or 60 minutes. Then they were cooled in ice water for 4 minutes and brought to room temperature at 27", about 25 minutes later. The optical densities in the absence of oxygen, D-O, were recorded. The vessels were opened, the pH of the solutions changed to 10 to 11 by adding about 0.02 ml. of 40 per cent NaOH, and the red color was discharged by bubbling air through the solutions for 3 minutes. Then the opt,ical densities, D+O, were recorded.The observed data were converted to micromolar concentrations (X) of substance responsible for the red or purple colors as follows: In the case of the red color, (X) = Rw/(E X 10m6 X 1 X a), where E is the molar absorption coefficient at X = 490 rnp and at pH 9.2, assuming complete hydrolysis of hydrindantin into diketohydrindol, 1 is the length of the light path in .cm., and CY is the ratio of optical density at pH 5.3 to that at pH 9.2. The numerical values were 1400, 1.89, and 0.55 respectively. The equation simplifies to (X) = 687R~~. In the case of the purple color,(X) = Psm X fl X f2, previously described. For the time intervals 10,20, 30, 40, 50, and 60 minutes the numerical values of fl were 1.31, 1.44, 1.58, 1.74, 1.91, and 2.10, respectively, and for f~ were 24.8, 25.2, 25.8, 26.5, 27.4, and 27.5, respectively.
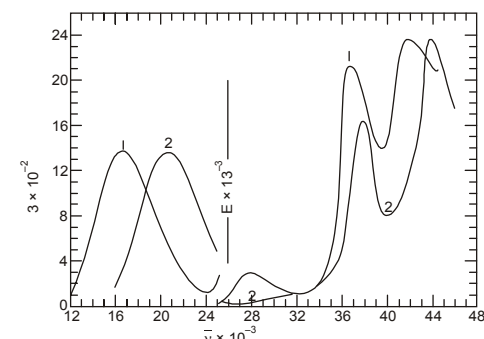
Fig. 2.67 : Absorption curves of the red and blue colors derived from hydrindantin. Curve 1, for the blue color in 0.5 N NaOH; Curve 2, for the red color at pH 9.2. The ordinates refer to the molar absorfition coefficients; the abscissae to the wave number per cm
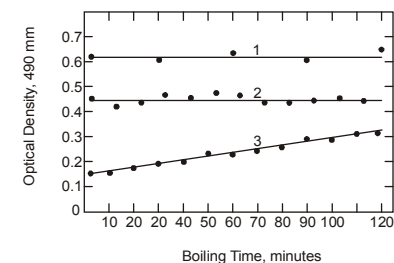
Fig. 2.68 : Hydrolysis of hydrindantin (0.31 mM) at pH 5.3 in boiling aqueous anaerobic solution with and without dimethyldihydroresorcinol. Curve 1, optical density at 100", with or without dimethyldihydroresorcinol; Curve 2, optical density after cooling to 25", in the presence of dimethyldihydroresorcinol; Curve 3, optical density after cooling to 25', without dimethyldihydroresorcinol

The disappearance of red color associated with formation of Ruhemann's purple was calculated from R&O, obtained when the reaction mixture contained cr-alanine, and from R$, obtained when all reagents were present except cr-alanine. The disappearance, in terms of micromoles of hydrindantin per liter, = 687(R" - R')490.In the case of a-alanine, the results show that the disappearance of hydrindantin red color is proportional to the formation of Ruhemann's purple, within the limits of error of the method.Red and Blue Colors from Hydrindczntin Due to Dilcetohydrindol (Fig. S)- Our claim

that the red color evolved from hydrindantin, as well as the undisputed blue color (l), is due to diketohydrindol rests on the following facts. (a) The blue color is reversibly changed into the red by acid-base titration under anaerobic conditions, pK' = 12.3 at 25'. When the blue color is formed, the other component of hydrindantin, namely ninhydrin, is changed into o-carboxymandelic acid (1) by irreversible internal oxidation-reduction. This change is complete in a few minutes, but the color change is quantitatively reversible for days. Therefore, the change from blue to red does not necessitate the reformation of hydrindantin; the claim (10) that the red color is due to the monovalent anion of hydrindantin is invalid. (b) The same play of colors with change in pH was observed by Hassall (12) in connection with the hydrolysis of acetoxyindan-dione to diketohydrindol, which was identified by adding ninhydrin to acidified solutions from which hydrindantin was obtained. (c) In acid solutions of hydrindantin dimethyldihydroresorcinol accelerates the formation of the red color, which attains a constant intensity for a given concentration of hydrindantin. The explanation offered is hydrolysis of hydrindantin into the red color and ninhydrin, accelerated by combinationwith the resorcinol (see Fig. 68). The resorcinol combines with ninhydrin.The compound, inert to oxygenation, can be detected spectrophoto-metrically in the solutions after oxygenation. (d) Above pH 7, there is no difference in red color caused by dimethyldihydroresorcinol or by cooling the solutions from 100" to room temperature. If a red alkaline solution is acidified to pH 5.3, anaerobically, the color fades as hydrindantin is precipitated. The data could be interpreted, as Ruhemann concluded (lo), to show that intact hydrindantin is responsible for the red colour. However, at a pH, temperature, and time interval (pH 10, 25°C, 24 hours) insuring complete irreversible transformation of ninhydrin to o-carboxyphenylglyoxal, acidification no longer causes reformation of hydrindantin, the fading of the red color being what would be expected from its titration curve, pK' = 5.2. In this case, addition of ninhydrin causes and is necessary for precipitation

(II)   Colored forms of indanone-enediol
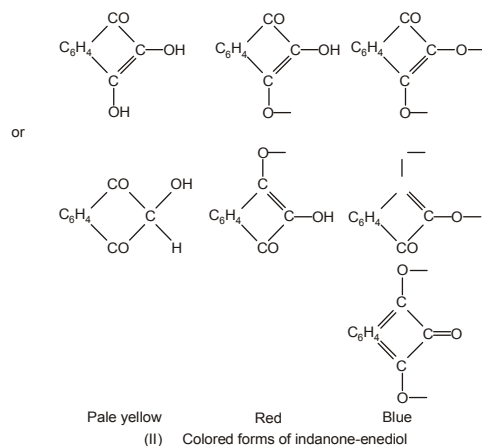
of hydrindantin. (e) By careful adjustment of the concentration of added hydrosulfite, the intensity of the red color from hydrindantin can be doubled. Having shown that the red and blue colors of hydrindantin solutions are derivatives of diketohydrindol readily convertible under anaerobic conditions one to the other and to diketohydrindol, simply by change in pH (pK' = 5.2 and 12.3), we conclude that the colors are due to the anions of diketohydrindol. Enolization of diketohydrindol would allow for two ionizable groups. Therefore, the red color is attributable to the monovalent anion, the blue color to the divalent anion. The ease of oxidation on exposure to air is consistent with the indene structure formable by enolization. The enediol formulae shown in II provide for resonance which would explain the difference in color and the chemical behavior of the substances.
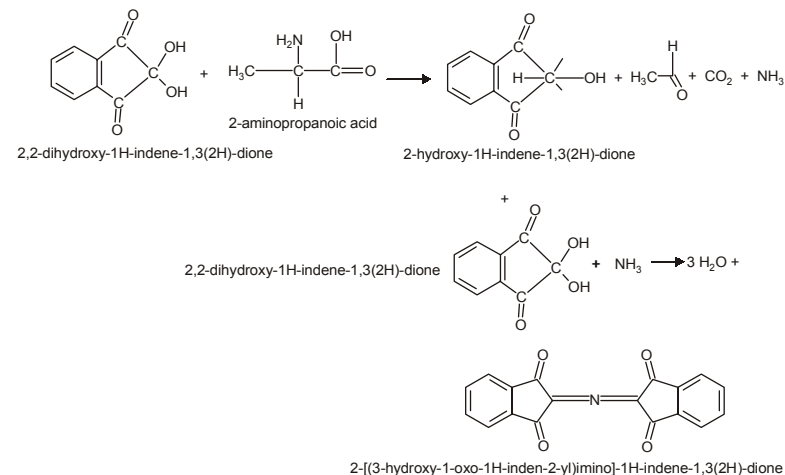
On reduction with hydrosulphite the red and blue colors disappear but careful oxygenation restores their original intensity. On the other hand, the effect of oxygenation is not reversible in the case of the blue color and is only reversible in the case of the red color if ninhydrin is present in, solution. Irreversibility is explained by oxidation of the red color to o-carboxyphenylglyoxal and of the blue color to o-carboxymandelic acid.

Mechanism of Reaction of a-Alanine with Hydrindantin-The disappearance of the red color in this reaction may now be reconsidered. It is ascribed to conversion of indanone-enediol into Ruhemann's purple, the anion of diketohydrindamine-diketohydrindylidene. The alternative, reduction of Ruhemann's purple which would become colorless while the enediol was oxidized to colorless ninhydrin, is untenable, because the chromogenic reaction of a-alanine is faster with 1 mole of hydrindantin than with 2 moles of ninhydrin ($C_9H_6O_4$). The non-enolic component of Ruhemann's purple must be supplied by ninhydrin,

also from hydrolysis of hydrindantin, for only 1 mole of indanone-enediol isused up for each mole of Ruhemann's purple formed. The details of the mechanism, summarized in formula, will be discussed in a paper on the order of the reactions of amines with hydrindantin and ninhydrin.

SECTION 2.12—SEPARATIONS OF AMINO ACIDS

Chromatography is a word used to encompass a range of techniques in which mixtures of pure substances are separated into the individual substances by using a mobile phase (usually a liquid or gas) to push the mixture along a stationary phase (usually a solid or liquid coated on a solid). Because the individual substances have different molecular structures, they interact differently with both the stationary and mobile phases, and consequently are "pushed" at different rates by the mobile phase.

SUB-SECTION 2.12A—PAPER CHROMATOGRAPHY*

A solution of the mixture to be separated is "spotted", usually from a micropipet, near one edge of a piece of filter paper, and the solvent is evaporated. Usually several sample and standard spots are placed along the edge. Then the chromatogram is "developed" by immersing that edge of the paper in a solvent that migrates through the paper as the mobile phase. The solvent often has two, three or four components, one of which is usually water. Development is normally done in a chamber that is saturated with solvent vapor. The water from the solvent, in particular, is adsorbed and tightly held on the paper fibers, so the sample components partition between the migrating mobile phase and the tightly-held water. If the bottom edge of the paper is immersed in the solvent, the process is "ascending" chromatography. Alternatively, the upper edge can be immersed in a trough of solvent and hung down for "descending" chromatography. Usually the process is stopped before the solvent front reaches the opposite edge of the paper, and the solvent is evaporated. The ratio of the distance moved by a particular component to the distance moved by the solvent front is the "R_f", or "retardation factor", which is characteristic of that component under the conditions used. After the separation, any strongly colored spots are visible on the "chromatogram". Colorless materials can be visualized by heating the paper in an oven so that substances (but not the paper) char and leave black spots. Sometimes the paper is first sprayed with a solution of sulfuric acid for better charring. Fluorescent materials can be visualized with ultraviolet light. Reagents specific for certain components may be sprayed on to make colored spots. Radioactive spots can be located with a detector, or the chromatogram can be pressed against X-ray film for minutes or hours to expose the film. Sample spots can be tentatively identified if they have the same R_f values as known standard spots.

Sometimes spots, once located, are cut out so that the material in the spot can be recovered. There are also instruments that (more or less accurately) quantitate the material in the spot by measuring light absorbance.

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* To know more about these instrumental techniques please see The Architecture of Chemistry An Instrumental Approach by the same author.

Variant: Circular chromatography: the sample is applied to the center of a circle of filter paper. Then the mobile phase is slowly dripped onto the spot and the chromatogram develops as a circle.

Variant: Electrochromatography: as the mobile phase flows downward, an electrical field is applied transversely (electrophoresis) to get a two-dimensional separation of ionized molecules.

Variant: Two-dimensional chromatography: One spot is applied near a corner of a sheet of paper, and is developed with one solvent system. Then a second solvent system is applied from another edge so that mixed spots that did not separate with the first system may separate with the second one. These sheets may be as large as 75 cm x 75 cm.

Lab reports or procedures will usually specify the solvent mixture (e. g., methyl isobutyl ketone:isopropyl alcohol:ethyl acetate:water:ammonium hydroxide (50:45:35:18:3), the paper used (often Whatman #3) and the means by which the spots are visualized.

SUB-SECTION 2.12B—THIN LAYER CHROMATOGRAPHY

Thin-Layer Chromatography (TLC) is a simple and inexpensive technique that is often used to judge the purity of a synthesized compound or to indicate the extent of progress of a chemical reaction. In this technique, a small quantity of a solution of the mixture to be analyzed is deposited as a small spot on a TLC plate, which consists of a thin layer of silica gel (SiO_2) or alumina (Al_2O_3) coated on a glass or plastic sheet. The plate constitutes the stationary phase. The sheet is then placed in a chamber containing a small amount of solvent, which is the mobile phase. The solvent gradually moves up the plate via capillary action, and it carries the deposited substances along with it at different rates. The desired result is that each component of the deposited mixture is moved a different distance up the plate by the solvent. The components then appear as a series of spots at different locations up the plate. Substances can be identified from their so-called R_f values. The R_f value for a substance is the ratio of the distance that the substance travels to the distance that the solvent travels up the plate. For example, an R_f value of 0.5 means that the spot corresponding to the substance travels exactly half as far as the solvent travels along the plate.

The Process of TLC. Performing a TLC analysis consists of a number of steps: preparing a
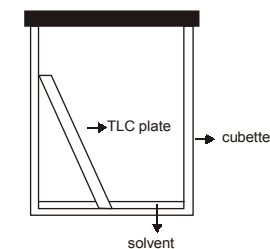
Fig. 2.69 : Showing the Diagram of the TLC set up for experment

Fig. 2.70 : Showing the Actual TLC set-up

spotting capillary; marking the TLC plate; spotting the TLC plate; developing the TLC plate; drying the plate; visualizing the substance spots, and measuring the R_f values. We consider these steps in turn.

Preparing a spotting capillary. Glass capillaries used for spotting TLC plates are commercially available. However, it is occasionally necessary to make your own capillaries. To accomplish this, light a Bunsen burner and adjust for a medium flame. Hold a melting point capillary in the flame until it just begins to soften, then quickly pull the two ends of the capillary in opposite directions. The central, soft part of the glass will elongate and thin down to a capillary with very small diameter. Break the two pieces apart at the center of the thin portion to obtain two TLC spotting capillaries.

Marking the TLC Plate. Obtain a silica gel TLC plate that is approximately 2 cm wide and 5 cm long. Mark the TLC plate as follows using a pencil (pencil must be used rather than pen because inks are moved by many developing solvents). First, LIGHTLY draw a straight line parallel to the short dimension of the plate, about 1 cm from one end of the plate. Don't gouge the silica gel or make a trough with the pencil. Second, LIGHTLY make two small marks perpendicular to this line to divide the line into thirds. This subdivided line will serve as a guide for placing the substance spots, and as a point from which to measure R_f values. Third, LIGHTLY draw a second line parallel to the first line and about 1 cm from the other end of the plate. When you develop the plate, you will allow the solvent front to rise to this second line.

Activating the TLC plate. Place your marked TLC plate in an oven at 50-60°C for 15-20 minutes to "activate" it. Activation involves driving off water molecules that bond to the polar sites on the plate.

Spotting the TLC plate. Place the narrow end of one of your capillaries into a vial containing a solution of the substance to be analyzed, and allow the solution to rise in the capillary; this will happen spontaneously. Once the capillary is loaded, hold it vertically just above the subdivided pencil line on the plate and centered in one of the three sections. Lower it until the narrow end of the capillary just touches the plate right on the pencil line. You will observe that some of the solution leaves the capillary and deposits on the plate. Leave the capillary in contact with the plate only briefly so that the spot is no larger than 1 mm in diameter, then raise the capillary. Allow the solvent to completely evaporate from the spot (if the solvent is water, evaporation will be slow; you can hasten it by putting the plate in the oven for 5 minutes). Then, if desired, make a second deposit on the same spot with the capillary. Again allow the solvent to completely evaporate.

Developing the TLC plate. Pour the desired developing solvent into a small wide-mouth glass or plastic bottle to a depth of about 4-5 mm. Using tweezers, pick up the TLC plate at the top, which is the end opposite where the subdivided pencil line is drawn. Place it carefully in the developing bottle so that it stands somewhat but not excessively tilted—that is, the bottom of the slide should be somewhat away from the wall of the bottle, while the top of the slide rests against the wall of the bottle. It is important that you not allow the TLC slide to tilt too much when in the developing bottle. If the slide is excessively tilted, solvent will not advance uniformly along the plate and development will not take place properly. Similarly, if the bottom of the slide is against the wall of the bottle, solvent will advance more rapidly up the edges of the slide than in the middle, causing the substances to be pushed toward the center of the slide as they move up. Leave the slide in the chamber until solvent has advanced to the top pencil line on the slide. Development normally requires at least 30 minutes. When the solvent front has advanced to the top pencil line, use the tweezers to withdraw the slide from the chamber.

Drying the Plate. Place the plate flat on a clean dry surface and allow the solvent to completely evaporate. If the solvent is not highly volatile, this can be facilitated by placing the slide on a flat surface in an oven at a temperature of 50-60 °C (higher temperatures will melt the plastic substrate material). When the plate is completely dry, it is ready for visualization.

Visualization of the TLC Plate. If the substances being separated are colored, the spots can be seen without any further effort. Using a pencil, draw a boundary around each spot that matches the shape of the spot. Many substances are colorless (white) and do not show up on the white silica gel unless steps are taken to make them visible. There are a number of techniques for doing this. *First* is the technique of iodination. The dry plate is placed in a chamber containing a few crystals of iodine. The iodine vapor in the chamber oxidizes the substances in the various spots, making them visible to the eye. Once the spots are visible, they may be outlined with a pencil before the iodine coloration fades. *Second* is the ninhydrin technique, which is particularly effective for visualizing amino acid spots. In this method, a solution containing 0.2% ninhydrin in ethanol is sprayed on the dry plate. Alternately, the plate can be dipped in ninhydrin solution. In contact with an amino acid, ninhydrin displays a purple coloration that is easily seen. It usually takes a few minutes for this color to develop, so after the plate is sprayed, it is allowed to sit for several minutes. Placing it in a 50 °C oven will hasten the appearance of the purple color. Once this appears, the spots may be circled in pencil to permanently mark their positions. *Third*, one may use TLC plates that have been loaded with a fluorescent substance that is uniformly distributed in the silica gel. Substances moving up the plate block this fluorescence at their locations. When the dried plate is viewed using a special UV light, the substance spots are visible for their lack of fluorescence on an otherwise uniformly fluorescing field. The spots may be outlined with pencil while being viewed in the light.

Note: Ninhydrin Stains the Skin Purple, too, Due to the Presence of Protein (Amino Acids!). To avoid this, Wear Gloves When Handling It.

Measurement of R_f. The distance between the 2 horizontal pencil lines is the distance of solvent advance. The distance from the bottom pencil line to the center of a substance spot is the distance of

$$R_f = \frac{b}{a}$$

Tartrazine
Ponoceau 4R
Yellow Orange S
Naphthol Red S
Fast Red E
Brillant Black BN

Erythrosin 2nd spot
Erythosin main spot
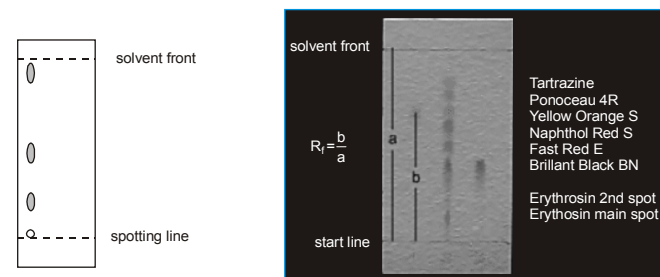
Fig. 2.71 and 71A Showing the TLC plate for interpretation

advance of the substance. The ratio of substance advance distance to solvent advance distance is the R_f value for the substance. The figure shows a developed plate for a mixture consisting of 4 components with Rf values of about 0.05, 0.2, 0.5, and 0.9.

TLC of Inks. Before attempting to apply TLC to the challenging problem of separating and identifying amino acids, it is advisable to learn and practice the technique by applying it to mixtures that are easily visualized and separated. Inks provide an ideal practice vehicle for TLC because they normally contain several colored components that separate nicely in common solvents such as ethanol, acetone, or chloroform. Spotting the plate is also easy: it may be done simply by making a VERY small mark on the plate with the tip of a pen, just above the pencil line drawn across the bottom edge of the plate. Inks are of different types and colors, of course. Some are washable (water-soluble), others are permanent. Different types require different solvents for development. Common sources of ink are ball point pens, felt-tip markers, and roller ball pens. Bottled ink is still available for people using fountain pens. Particularly good ink sources are bottled inks by Parker, Sheaffer, and Mont Blanc; Sharpie marker inks (black, red, blue, orange, brown, yellow, green); Marks-a-Lot Stay sharp markers; and Sanford calligraphy pens.You should select inks from at least 2 different sources available in the lab and should find a solvent or mixture of solvents that separates each ink into its component colors.

Experimental Procedure. You will be assigned two ink samples to examine. Obtain four TLC plates, four TLC spotting capillaries, and 3 developing tanks (snap-top plastic vials). Mark your TLC plates with a narrow pencil line about 1cm from the bottom. Prepare spotting solutions of your ink samples in 1-dram vials by diluting 1 drop of ink with 9 drops of ethanol (this is called a 1-to-10 dilution). Insert one end of a spotting capillary into the first ink solution. You should see the ink solution rise in the capillary. Withdraw the capillary when the liquid has risen to a height of 1-2 cm. Hold the capillary vertically and briefly touch the filled end to the pencil line, about 5 mm from the left edge of the TLC plate. Liquid should flow from the capillary to the plate to form a spot. THIS HAPPENS RAPIDLY. If possible, lift the capillary before the spot gets any bigger than 1 mm in diameter. Allow the ethanol to evaporate. Touch the capillary to a Kimwipe to empty it of ink solution. Use the other end of the same capillary to draw up your second ink solution, and spot the plate about 5 mm from the right edge. Discard the capillary in the broken glass receptacle. Allow the ethanol to evaporate from the plate.

Into your first developing tank, pour ethanol to a depth of about 5 mm. Using tweezers to hold the top end of the spotted plate, lower the plate into the tank. Allow the bottom of the plate to rest on the bottom of the tank, with the top of the plate leaning against a tank sidewall. NOTE: IT IS VERY IMPORTANT THAT THE START LINE AND SPOTS BE ABOVE THE LEVEL OF SOLVENT. Close the tank and monitor the movement of solvent up the plate. When solvent has advanced to the top pencil line, remove the plate from the tank, and allow all solvent to evaporate. Use a pencil to outline each observed spot on the plate, preserving the shape of the spot.

If ethanol did not effect a separation of the ink into components, try another solvent. If you found that the ink moved along with or close to the solvent front, try a less polar solvent. If you found that the ink did not move at all with ethanol, try a more polar solvent (e.g., methanol). Your goal is to cleanly separate each of your ink samples into its constituents, each constituent producing a single TLC spot. Be aware that some inks contain only one component!

TLC of Amino Acids. TLC of amino acids is more difficult than TLC of inks, because amino acids are colorless. Therefore, not only can you not monitor their progress up the plate, but you cannot see the spots with the naked eye once the plate is fully developed and dried. To see the spots, it is necessary to use either the ninhydrin or the black-light visualization techniques. Of course, the latter works only if you use fluorescent TLC plates, which are available in the lab. Until you see the spots, you will not know whether or not a chosen solvent system has been effective in moving an amino acid or in separating a mixture. Therefore the process of finding an effective solvent system can be long and painstaking. For this reason, we will specify the solvent system that you are to use. As points of general information, amino acids are quite polar and tend to move on silica gel plates with polar solvents. They have R_f values close to 1 when water or concentrated ammonia is used as the developing solvent, probably because of their high solubility in water. Diluting a polar solvent with a less polar one results in smaller R_f values, roughly in proportion to the amount of less polar solvent used, Thus, alanine, glycine, threonine, and proline all have R_f values of around 0.60 when developed with a 50/50 mixture of water and n-propanol, and around 0.40 when developed with a 30/70 mixture of concentrated NH_3 and n-propanol. The following procedure assumes the use of 50/50 water/n-propanol as the developing solvent, but you are free to try other polar/non-polar combinations.

Experimental Procedure. In the hood, prepare 10 mL of a mixture consisting of 50% 1-propanol and 50% water by volume, and pour about half of this into a clean developing tank. Make sure that the level of liquid in the tank is no higher than 5 mm, and close the lid. In a 1-dram vial, prepare a solution of about 0.001 g of your amino acid in 0.2 mL of water. Dissolve the acid, then draw some solution up in a spotting capillary and double-spot a properly marked and activated TLC plate. Allow the plate to dry for 5 minutes, then use tweezers to carefully lower the plate into the developing tank so that its bottom is submerged in the developing solvent. NOTE: IT IS VERY IMPORTANT THAT THE START LINE AND SPOTS BE ABOVE THE LEVEL OF SOLVENT. Close the lid, and allow the plate to develop until solvent has risen to the pencil line at the top of the plate. Remove the plate from the tank and place it in an oven at 50 ºC to dry. When the plate is dry, visualize it using ninhydrin spray or iodination. Circle the amino acid spots with pencil, and calculate R_f values. Compare the measured R_f values with the values posted for the amino acids.

On this basis, you can narrow down the identity of your amino acid. In combination with other data that you obtain, this information will help you unambiguously identify your amino acid. Suppose that your amino acid has an R_f value similar to that of, say, alanine. You should then prepare a small amount of alanine solution and spot it alongside your amino acid on a new TLC plate. Develop, dry, and visualize the plate to confirm that your amino acid indeed has exactly the same R_f value as alanine, and that the spot is the same shape and color.

Finally, it is very important to be observant of detail in doing TLC. In addition to the R_f value for a substance, the shape of the spot produced by a particular developing solvent and the shade of color produced by iodine or ninhydrin can be characteristic of the substance. Please note all of these things. For example, when alanine, glycine, threonine, and proline are spotted side-by-side on a plate and developed with 70% n-propanol/30% conc NH_3, the following observations can be seen in the table below:

Amino Acid	Solvent	Spot Color after Iodination	Spot Color with Ninhydrin	R_f Value	Spot Shape
alanine	30/70 conc NH_3/n-propanol	white on brown bkgrnd	purple		elongated oval
alanine	50/50 water/n-propanol	white on brown bkgrnd	purple	0.65	circle
glycine	30/70 conc NH_3/n-propanol	white on brown bkgrnd	pink	0.25	elongated oval
glycine	50/50 water/n-propanol	white on brown bkgrnd	pink	0.55	circle
threonine	30/70 conc NH_3/n-propanol	white on brown bkgrnd	purple		elongated oval
threonine	50/50 water/n-propanol	white on brown bkgrnd	purple	0.57	circle
proline	30/70 conc NH_3/n-propanol	dark brown on brown	yellow with pink border		elongated oval
proline	50/50 water/n-propanol	white on brown bkgrnd	yellow with pink border	0.65	circle

Problems in TLC

Over-large Spots. Sample spots made using TLC capillaries should be no larger than 1-2 mm in diameter, because component spots in the developed plate will be no smaller than, and will usually be larger than, the size of the initial spot. If the initial spot is larger than 2 mm in diameter, then components with similar R_f values may not be resolved because their spots will be so large that they will overlap considerably and may appear to be one large spot. Small initial spots, on the other hand, maximize the potential of complete separation of components.

Uneven Advance of Solvent Front. A common problem in TLC is uneven advance of solvent along the plate. Instead of a straight line, the solvent front may appear to bow either up or down in the center. Uneven advance of solvent leads to uneven advance of substance spots, and inaccurate R_f values result. A frequent cause of uneven solvent advance is the use of a developing chamber that does not have a flat bottom. Glass bottles usually have bottoms that curve upward from the edges to the center. If the bottom of the TLC plate is placed on this curved surface, the shape of the solvent advance line may mirror the shape of the container bottom. It is therefore important to use flat-bottomed developing tanks in TLC. A bowed solvent front may also result if too little developing solvent is placed in the chamber; if the plate is cut improperly, so that the sides are not exactly perpendicular to the bottom edge; and if the slide is excessively tilted in the chamber. Care in choosing and using a developing chamber is the best defense against curved solvent fronts.

Water is seldom used as a developing solvent because it has a tendency to produce a dramatically curved front. This may be due to its unusually high surface tension.

Streaking. Sometimes a substance will move along a TLC plate as a long streak, rather than as a single discrete spot. This is the result of spotting the plate with too much substance, more than the moving solvent can handle. The solvent moves as much substance as it can, but a substantial amount of substance is left behind. The substance is dragged along by the solvent leaving a trail of substance that may sometimes span the entire distance between the starting line and the solvent front. Streaking can be eliminated by systematically diluting the spotting solution until development and visualization show the substances moving as single spots, rather than elongated streaks.

SUB-SECTION 2.12C—HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Research with thin-layer and column chromatography showed that separations are much more effective when the stationary phase is a very thin layer on the surface of very small and very uniform spherical beads. However, resistance to flow of the mobile phase is very much higher, and in order to get a useful flow of a liquid mobile phase, e. g., 1 - 3 milliliters/minute, pressures of around 15 Mpa (about 2,000 psi) must be applied to the mobile phase. It is possible to apply such pressure from a cylinder of compressed gas, but most systems use a reciprocating piston pump or diaphragm pump with some means of damping

Fig. 2.45 : Showin the Analytical HPLC system

the pressure fluctuations from the piston. The sample is usually dissolved in the mobile phase before injection. Columns are typically 4.6 mm ID (6 mm OD) stainless steel tubing 250 mm long. A typical packing will have octadecylsilyl (C_{18}-Si-) (ODS) groups bonded to 5 μm silica beads. The packing is held inside the column by "frits", discs with pores about 0.5 μm in diameter.

Liquid-liquid chromatography began with samples dissolved in organic solvents and a stationary phase of water adsorbed on particles or fibers of the solid support. More generally, the stationary phase was more "polar" than the mobile phase. That is the so-called "normal phase" chromatography. But stationary phases such as ODS have been particularly useful for separating samples dissolved in water (and most HPLC is now done with bonded phases). Liquid chromatography with the stationary phase less polar than the mobile phase is called "reverse phase", but is now the common situation. The mobile phase is very often not just water but a mixture of water with methanol (CH_3OH) or acetonitrile (CH_3CN). "Solvent programming", a stepwise or continuous change (gradient elution) of the mobile phase composition, is used to speed up separations, like temperature programming in gas chromatography.

"Chiral" columns have been developed relatively recently to separate optical isomers. This separation is important because many pharmaceuticals are active in only one chiral form. For instance, natural Vitamin E is D-a-tocopherol, while half of synthetic Vitamin E is the less active L- isomer.

As in gas chromatography, a few microliters of the solution are measured into a "sample loop" on an "injector". When the injector is operated, the sample loop is suddenly switched into the flow of

mobile phase just before it reaches the column. The mobile phase leaving the column passes immediately into a "detector" which is used to determine the presence and concentration of a solute.

Because the pressure on the mobile phase drops rapidly as it leaves the column, bubbles may form from dissolved gases. Chromatographers "degas" the mobile phase before use by boiling it, ultrasonic treatment, bubbling helium through it to flush out other gases, or applying a vacuum.
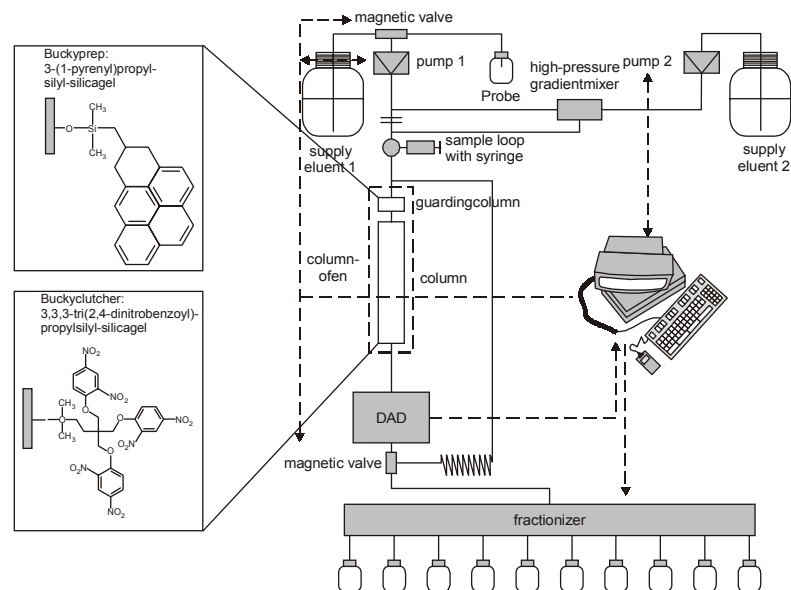
Fig. 2.72 : Showing the Diagramatic representation of the HPLC system

There are no relatively inexpensive HPLC detectors as sensitive and broadly useful as the flame ionization and electron capture detectors used in gas chromatography. A refractive index (RI) detector responds to most components, but is not very sensitive. An ultraviolet (UV) detector is quite sensitive for molecules which absorb ultraviolet light, and a variable wavelength UV detector can be set to the absorption maximum for a particular molecule of interest, or to a short wavelength where most molecules absorb. A diode array detector (DAD) disperses the transmitted light into a spectrum, providing an absorption spectrum of each component that absorbs ultraviolet light. Still more sensitive, and still less general, is the fluorescence detector which measures fluorescence emitted from components which

have absorbed ultraviolet light. There are various special-purpose detectors. Amperometric systems measure electron flow which oxidizes or reduces certain components (sugars, for instance), and polarimetric detectors, generally not very sensitive, detect components are optically active. Mass spectrometric detectors are now used, but were late to arrive because it was difficult to separate the mobile phase molecules so as to maintain adequate vacuum in the mass spectrometer.

Special case: Supercritical fluid chromatography (SFC). Liquids can vaporize, and gases can condense to the liquid phase. Both changes depend on the pressure and temperature. Every gas has a critical temperature above which it cannot be condensed at any pressure. The critical pressure is the pressure required at the critical temperature. If a liquid (such as a condensed gas) is warmed, under high pressure, to the critical temperature and beyond, strange things happen. The boundary between gas and liquid vanishes, and it is best not to ask physical chemists too many questions about the remaining phase, the "supercritical fluid". Whatever their exact nature, supercritical fluids can be very useful solvents for HPLC, which runs under high pressure anyway. The most commonly used of these fluids is carbon dioxide. (Supercritical fluids are also useful for extracting things from solids, and, when the solids are clothing, for dry cleaning.)Reports and procedures will specify the mobile phase composition, flow rate and perhaps the pressure; the column dimensions; the column packing (particle type and size; coating); the detector and its operating conditions (e. g., wavelengths for UV and fluorescence detectors), integrator settings, and, somewhere, retention times.There are some kinds of liquid chromatography that do not fit the partition or adsorption models very well. They are often done in simple vertical columns.

SECTION 2.12C—POLYACRYLAMIDE GEL ELECTROPHORESIS

Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by "wrapping around" the polypeptide backbone - and SDS binds to proteins fairly specifically in a mass ratio of 1.4:1. In so doing, SDS confers a **negative charge** to the polypeptide in proportion to its length - ie: the denatured polypeptides become "**rods" of negative charge cloud** with equal charge or charge densities per unit length. It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size: this is done with **2-mercaptoethanol or dithiothreitol**. In denaturing SDS-PAGE separations therefore, migration is determined not by intrinsic electrical charge of the polypeptide, but by **molecular weight**.

Determination of Molecular Weight

This is done by SDS-PAGE of proteins - or PAGE or agarose gel electrophoresis of nucleic acids - of known molecular weight along with the protein or nucleic acid to be characterised. A linear relationship exists between the logarithm of the molecular weight of an SDS-denatured polypeptide, or native nucleic acid, and its *Rf*. The Rf is calculated as the **ratio of the distance migrated by the molecule to that migrated by a marker dye-front**. A simple way of determining relative molecular weight by electrophoresis *(M*r) is to plot a standard curve of distance migrated vs. log10MW for known samples, and read off the log*Mr* of the sample after measuring distance migrated on the same gel.

Continuous and Discontinuous Buffer Systems

There are two types of buffer systems in electrophoresis, **continuous and discontinuous**. A **continuous** system has only a single separating gel and uses the same buffer in the tanks and the gel. In a **discontinuous** system, a non-restrictive large pore gel, called a stacking gel, is layered on top of a separating gel called a resolving gel. Each gel is made with a different buffer, and the tank buffers are different from the gel buffers. The resolution obtained in a discontinuous system is much greater than that obtained with a continuous system.*

Protocol

NB: Acrylamide Monomer is a Potent Cumulative Neurotoxin. Do not Mouth Pipette Acrylamide Solutions, and Wear Gloves when Handling Unpolymerised Solutions.

Assembling Gel Apparatus

Assemble two glass plates (one notched) with two side spacers, clamps, grease, etc. as shown by demonstrators. Stand assembly upright using clamps as supports, on glass plate. Pour some **pre-heated 1% agarose** onto glass plate, place assembly in pool of agarose: this seals the bottom of the assembly.

Resolving Gels

Gel concentration of 12.5% in 0.25 M Tris-HCl pH 8.8

	Volume Reagent: (ml: TO MAKE 30 ML)	Volume (ml: TO MAKE 10 ML)
40% Acrylamide stock*:	9.4	3.1
water (distilled)	12.3	3.8
1 M Tris-HCl pH 8.8	7.5	2.5
10% SDS	0.3	0.1
Peroxydisulphate 1%	0.5	0.5
TEMED (added last)	20 ul	20 ul

* = 19:1 - 38:1 w:w ratio of acrylamide to N,N'-methylene bis-acrylamide

Mix ingredients **GENTLY!** in the order shown above, ensuring no air bubbles form. Pour into glass plate assembly **CAREFULLY**. **Overlay gel with isopropanol** to ensure a flat surface and to exclude air. Wash off isopropanol with water after gel has set (+15 min).

Stacking Gels

Gel concentration of 4.5% in 0.125 M Tris-HCl pH 6.8

_____

 * (Read about this in Architecture of chemistry An instrumental approach textbook).

	Volume Reagent: (ml: TO MAKE 30 ML)	Volume (ml: TO MAKE 10 ML)
40% Acrylamide stock	1.7	1.1
water	10.8	7.1
1 M Tris-HCl pH 6.8	1.9	1.25
10% SDS	0.15	0.1
Peroxydisulphate 1%	0.5	0.5
TEMED (stir quickly)	20 ul	20 ul

Mix as before, then **pour onto top of set resolving gel**, insert comb, allow to set, remove comb, fill with electrophoresis buffer. Assemble top tank onto glass plate assembly. Fill with electrophoresis buffer.

Electrophoresis buffer

The final **TANK** buffer composition is **196mM glycine / 0.1% SDS / 50mM Tris-HCl pH 8.3**, made by diluting a **10x stock** solution. This goes in both top and bottom tanks.

Samples

Grind a little leaf material (eg. 2 grams) in a mortar. Centrifuge in an Eppendorf tube for 3 min. Take supernatant and **mix 100ul 1:1 (v:v) with SDS-PAGE disruption mix**: this is **125mM Tris-HCl pH 6.8 / 10% 2-mercaptoethanol / 10% SDS / 10% glycerol**, containing a little bromophenol blue. **BE CAREFUL WITH THIS AS IT SMELLS AWFUL and is poisonous to boot!!**

For liquid / purified samples, take eg. 100 ul and add 50 - 100 ul of disruption mix.

Heat sample Eppendorfs for 5 min at 95oC in a "float" in a waterbath. Layer samples under buffer on stacking gels. Connect up apparatus and electrophorese as shown.

Staining of Gels

1. Coomassie Brilliant Blue/Page-Blue 83

Make up stain: **0.2% CBB in 45:45:10 % methanol:water:acetic acid**. Cover gel with staining solution, seal in plastic box and leave overnight on shaker (RT) or for 2 to 3 hours at 37 c also with agitation. Destain with 25% 65% 10% methanol water acetic acid mix, with agitation.

2. Copper Chloride (0.3M CuCl2

Rinse gel in distilled water, immerse in copper chloride solution with agitation for about 20 minutes (RT), rinse with distilled water and immerse in sufficient fresh distilled water to cover the gel (this acts as the destaining step). Seal in a plastic box.

SECTION 2.13—CLASSIFICATION AND STRUCTURE OF IMMUNOGLOBINS AND PLASMA PROTEINS

Antibodies (also known as **immunoglobulins**, abbreviated **Ig**) are gamma globulin proteins that are found in blood or other bodily fluids of vertebrates, and are used by the immune system to identify and neutralize foreign objects, such as bacteria and viruses. They are typically made of basic structural units—each with two large heavy chains and two small light chains—to form, for example, monomers with one unit, dimers with two units or pentamers with five units. Antibodies are produced by a kind of white blood cell called a B cell. There are several different types of antibody heavy chains, and several different kinds of antibodies, which are grouped into different *isotypes* based on which heavy chain they possess. Five different antibody isotypes are known in mammals, which perform different roles, and help direct the appropriate immune response for each different type of foreign object they encounter.

Although the general structure of all antibodies is very similar, a small region at the tip of the protein is extremely variable, allowing millions of antibodies with slightly different tip structures to exist. This region is known as the hypervariable region. Each of these variants can bind to a different target, known as an antigen. This huge diversity of antibodies allows the immune system to recognize an equally wide diversity of antigens. The unique part of the antigen recognized by an antibody is called an epitope. These epitopes bind with their antibody in a highly specific interaction, called induced fit, that allows antibodies to identify and bind only their unique antigen in the midst of the millions of different molecules that make up an organism. Recognition of an antigen by an antibody *tags* it for attack by other parts of the immune system. Antibodies can also neutralize targets directly by, for example, binding to a part of a pathogen that it needs to cause an infection.

The large and diverse population of antibodies is generated by random combinations of a set of gene segments that encode different antigen binding sites (or *paratopes*), followed by random mutations in this area of the antibody gene, which create further diversity. Antibody genes also re-organize in a process called class switching that changes the base of the heavy chain to another, creating a different isotype of the antibody that retains the antigen specific variable region. This allows a single antibody to be used by several different parts of the immune system. Production of antibodies is the main function of the humoral immune system.

Antibody forms

Activated B cells differentiate into either antibody-producing cells called plasma cells that secrete soluble antibody or memory cells that survive in the body for years afterward in order to allow the immune system to remember an antigen and respond faster upon future exposures. Antibodies are, therefore, an essential product of the adaptive immune system that learns and remembers responses to invading pathogens. Antibodies occur in two forms: a soluble form secreted into the blood and other fluids in the body, and a membrane-bound form that is attached to the surface of a B cell.

Soluble antibodies that are secreted from an activated B cell (in its plasma cell form) bind to foreign substances and signal for their destruction by the rest of the immune system. They may also be called *free antibodies* (until they bind an antigen and become part of an *immune complex*) or *secreted antibodies*.

The membrane-bound form of an antibody may be called a *surface immunoglobulin* (sIg) or a *membrane immunoglobulin* (mIg). It is part of the *B cell receptor* (BCR), which allows a B cell to detect when a specific antigen is present in the body and triggers B cell activation. The BCR is composed of surface-bound IgD or IgM antibodies and associated Ig-á and Ig-â heterodimers, which are capable of signal transduction. A typical human B cell will have 50,000 to 100,000 antibodies bound to its surface.[9] Upon antigen binding, they cluster in large patches, which can exceed 1 micrometer in diameter, on lipid rafts that isolate the BCRs from most other cell signaling receptors. These patches may improve the efficiency of the cellular immune response. In humans, the cell surface is bare around the B cell receptors for several thousand Ångstroms, which further isolates the BCRs from competing influences.

Isotypes

Antibody isotypes of mammals

Name	Types	Description	Antibody Complexes
IgA	2	Found in mucosal areas, such as the gut, respiratory tract and urogenital tract, and prevents colonization by pathogens. Also found in saliva, tears, and breast milk.	Monomer IgD, IgE, IgG
IgD	1	Functions mainly as an antigen receptor on B cells that have not been exposed to antigens.[12] Its function is less defined than other isotypes.	Dimer IgA
IgE	1	Binds to allergens and triggers histamine release from mast cells and basophils, and is involved in allergy. Also protects against parasitic worms.	Pentamer IgM
IgG	4	In its four forms, provides the majority of antibody-based immunity against invading pathogens. The only antibody capable of crossing the placenta to give passive immunity to fetus.	
IgM	1	Expressed on the surface of B cells and in a secreted form with very high avidity. Eliminates pathogens in the early stages of B cell mediated (humoral) immunity before there is sufficient IgG.	

Antibodies can come in different varieties known as isotypes or classes. In placental mammals there are five antibody isotypes known as IgA, IgD, IgE,IgG and IgM. They are each named with an "Ig" prefix that stands for immunoglobulin, another name for antibody, and differ in their biological properties, functional locations and ability to deal with different antigens, as depicted in the table.

The antibody isotype of a B cell changes during cell development and activation. Immature B cells, which have never been exposed to an antigen, are known as naïve B cells and express only the IgM isotype in a cell surface bound form. B cells begin to express both IgM and IgD when they reach maturity—the co-expression of both these immunoglobulin isotypes renders the B cell 'mature' and ready to respond to antigen. B cell activation follows engagement of the cell bound antibody molecule with an antigen, causing the cell to divide and differentiate into an antibody producing cell called a plasma cell. In this activated form, the B cell starts to produce antibody in a secreted form rather than a membrane-bound form. Some daughter cells of the activated B cells undergo isotype switching, a mechanism that causes the production of antibodies to change from IgM or IgD to the other antibody isotypes, IgE, IgA or IgG, that have defined roles in the immune system.

Structure

Antibodies are heavy (~150kDa) globular plasma proteins that are also known as immunoglobulins. They have sugar chains added to some of their amino acid residues. In other words, antibodies are *glycoproteins*. The basic functional unit of each antibody is an immunoglobulin (Ig) monomer (containing only one Ig unit); secreted antibodies can also be dimeric with two Ig units as with IgA, tetrameric with four Ig units like teleost fish IgM, or pentameric with five Ig units, like mammalian IgM.Several immunoglobulin domains make up the two heavy chains (red and blue) and the two light chains (green and yellow) of an antibody. The immunoglobulin domains are composed of between 7 (IgC) and 9 (IgV) â-strands.

Immunoglobulin domains

The Ig monomer is a "Y"-shaped molecule that consists of four polypeptide chains; two identical *heavy chains* and two identical *light chains* connected by disulfide bonds. Each chain is composed of structural domains called Ig domains. These domains contain about 70-110 amino acids and are classified into different categories (for example, variable or IgV, and constant or IgC) according to their size and function. They have a characteristic immunoglobulin fold in which two beta sheets create a "sandwich" shape, held together by interactions between conserved cysteines and other charged amino acids.

Heavy chain

There are five types of mammalian Ig heavy chain denoted by the Greek letters: á, ä, å, ã, and ì.[3] The type of heavy chain present defines the *class* of antibody; these chains are found in IgA, IgD, IgE, IgG, and IgM antibodies, respectively. Distinct heavy chains differ in size and composition; á and ã contain approximately 450 amino acids, while ì and å have approximately 550 amino acids.

Each heavy chain has two regions, the *constant region* and the *variable region*. The constant region is identical in all antibodies of the same isotype, but differs in antibodies of different isotypes. Heavy chains ã, á and ä have a constant region composed of *three* tandem (in a line) Ig domains, and a hinge region for added flexibility; heavy chains ì and å have a constant region composed of *four* immunoglobulin domains. The variable region of the heavy chain differs in antibodies produced by different B cells, but is the same for all antibodies produced by a single B cell or B cell clone. The variable region of each heavy chain is approximately 110 amino acids long and is composed of a single Ig domain.
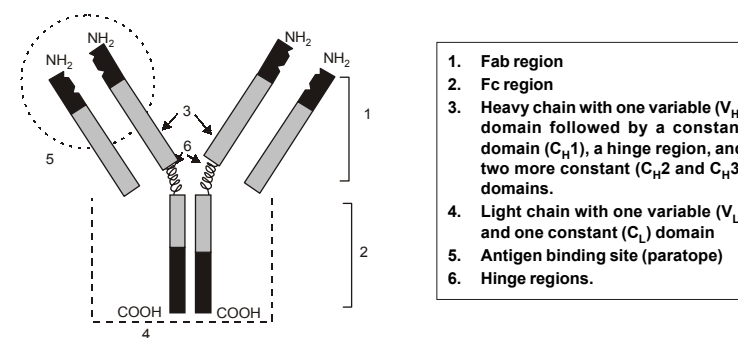
Fig. 2.73 : Showing the Immunoglobin Structure

1. **Fab region**
2. **Fc region**
3. **Heavy chain with one variable (V_H) domain followed by a constant domain (C_H1), a hinge region, and two more constant (C_H2 and C_H3) domains.**
4. **Light chain with one variable (V_L) and one constant (C_L) domain**
5. **Antigen binding site (paratope)**
6. **Hinge regions.**

Light chain

In mammals there are two types of light chain, which are called lambda (ë) and kappa (ê). A light chain has two successive domains: one constant domain and one variable domain. The approximate length of a light chain is 211 to 217 amino acids. Each antibody contains two light chains that are always identical; only one type of light chain, ê or ë, is present per antibody in mammals. Other types of light chains, such as the iota (é) chain, are found in lower vertebrates like Chondrichthyes and Teleostei.

Fab and Fc Regions

Some parts of an antibody have unique functions. The tips of the Y, for example, contain the site that bind antigen and, therefore, recognize specific foreign objects. This region of the antibody is called the *Fab (fragment, antigen binding) region*. It is composed of one constant and one variable domain from each heavy and light chain of the antibody. The paratope is shaped at the amino terminal end of the antibody monomer by the variable domains from the heavy and light chains.

The base of the Y plays a role in modulating immune cell activity. This region is called the *Fc (Fragment, crystallizable) region*, and is composed of two heavy chains that contribute two or three constant domains depending on the class of the antibody. By binding to specific proteins the Fc region ensures that each antibody generates an appropriate immune response for a given antigen. The Fc region also binds to various cell receptors, such as Fc receptors, and other immune molecules, such as complement proteins. By doing this, it mediates different physiological effects including opsonization, cell lysis, and degranulation of mast cells, basophils and eosinophils.

Function

Since antibodies exist freely in the bloodstream, they are said to be part of the humoral immune system. Circulating antibodies are produced by clonal B cells that specifically respond to only one antigen (an

example is a virus capsid protein fragment) . Antibodies contribute to immunity in three main ways: they can prevent pathogens from entering or damaging cells by binding to them; they can stimulate removal of a pathogen by macrophages and other cells by coating the pathogen; and they can trigger direct pathogen destruction by stimulating other immune responses such as the complement pathway.

Activation of complement

Antibodies that bind to surface antigens on, for example a bacterium, attract the first component of the complement cascade with their Fc region and initiate activation of the "classical" complement system. This results in the killing of bacteria in two ways. First, the binding of the antibody and complement molecules marks the microbe for ingestion by phagocytes in a process called opsonization; these phagocytes are attracted by certain complement molecules generated in the complement cascade. Secondly, some complement system components form a membrane attack complex to assist antibodies to kill the bacterium directly.

Activation of Effector Cells

To combat pathogens that replicate outside cells, antibodies bind to pathogens to link them together, causing them to agglutinate. Since an antibody has at least two paratopes it can bind more than one antigen by binding identical epitopes carried on the surfaces of these antigens. By coating the pathogen, antibodies stimulate effector functions against the pathogen in cells that recognize their Fc region.[6]

Those cells which recognize coated pathogens have Fc receptors which, as the name suggests, interacts with the Fc region of IgA, IgG, and IgE antibodies. The engagement of a particular antibody with the Fc receptor on a particular cell triggers an effector function of that cell; phagocytes

Fig. 2.74 : The secreted mammalian IgM has five Ig units. Each Ig unit (labeled 1) has two epitope binding Fab regions, so IgM is capable of binding up to 10 epitopes.

will phagocytose, mast cells and neutrophils will degranulate, natural killer cells will release cytokines and cytotoxic molecules; that will ultimately result in destruction of the invading microbe. The Fc receptors are isotype-specific, which gives greater flexibility to the immune system, invoking only the appropriate immune mechanisms for distinct pathogens.

Immunoglobulin Diversity

Virtually all microbes can trigger an antibody response. Successful recognition and eradication of many different types of microbes requires diversity among antibodies; their amino acid composition varies allowing them to interact with many different antigens. It has been estimated that humans generate

about 10 billion different antibodies, each capable of binding a distinct epitope of an antigen. Although a huge repertoire of different antibodies is generated in a single individual, the number of genes available to make these proteins is limited. Several complex genetic mechanisms have evolved that allow vertebrate B cells to generate a diverse pool of antibodies from a relatively small number of antibody genes.

Domain Variability
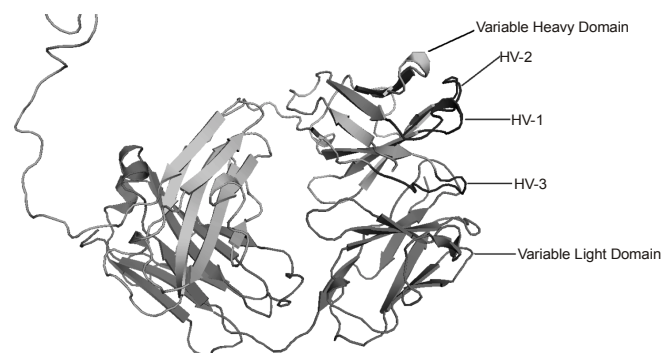
Fig. 2.75 : Showing the hypervariable regions of the heavy chain is shown in red, PDB 1IGT

The region (locus) of a chromosome that encodes an antibody is large and contains several distinct genes for each domain of the antibody—the locus containing heavy chain genes (IGH@) is found on chromosome 14, and the loci containing lambda and kappa light chain genes (IGL@ and IGK@) are found on chromosomes 22 and 2 in humans. One of these domains is called the variable domain, which is present in each heavy and light chain of every antibody, but can differ in different antibodies generated from distinct B cells. Differences, between the variable domains, are located on three loops known as hypervariable regions (HV-1, HV-2 and HV-3) or complementarity determining regions (CDR1, CDR2 and CDR3). CDRs are supported within the variable domains by conserved framework regions. The heavy chain locus contains about 65 different variable domain genes that all differ in their CDRs. Combining these genes with an array of genes for other domains of the antibody generates a large cavalry of antibodies with a high degree of variability. This combination is called V(D)J recombination discussed below.

V(D)J recombination

Somatic recombination of immunoglobulins, also known as *V(D)J recombination*, involves the generation of a unique immunoglobulin variable region. The variable region of each immunoglobulin heavy or light chain is encoded in several pieces—known as gene segments. These segments are called variable (V), diversity (D) and joining (J) segments. V, D and J segments are found in Ig heavy chains, but only V

and J segments are found in Ig light chains. Multiple copies of the V, D and J gene segments exist, and are tandemly arranged in the genomes of mammals. In the bone marrow, each developing B cell will assemble an immunoglobulin variable region by randomly selecting and combining one V, one D and one J gene segment (or one V and one J segment in the light chain). As there are multiple copies of each type of gene segment, and different combinations of gene segments can be used to generate each immunoglobulin variable region, this process generates a huge number of antibodies, each with different paratopes, and thus different antigen specificities.
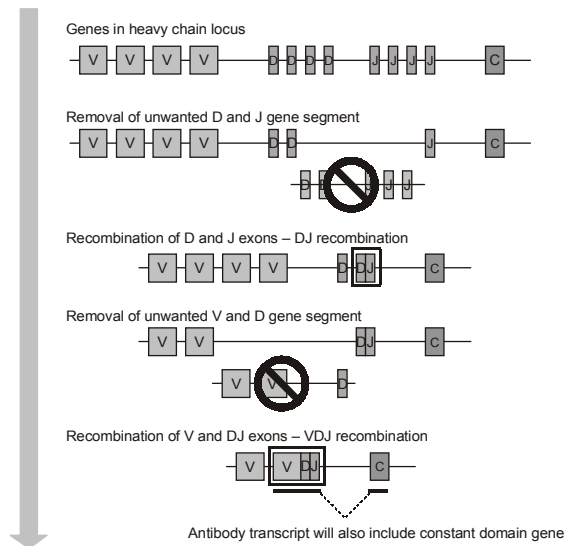
Fig. 2.76 : Showing the Simplistic overview of V(D)J recombination of immunoglobulin heavy chains

After a B cell produces a functional immunoglobulin gene during V(D)J recombination, it cannot express any other variable region (a process known as allelic exclusion) thus each B cell can produce antibodies containing only one kind of variable chain.

Somatic Hypermutation and Affinity Maturation

Following activation with antigen, B cells begin to proliferate rapidly. In these rapidly dividing cells, the genes encoding the variable domains of the heavy and light chains undergo a high rate of point mutation, by a process called *somatic hypermutation* (SHM). SHM results in approximately one nucleotide change

per variable gene, per cell division. As a consequence, any daughter B cells will acquire slight amino acid differences in the variable domains of their antibody chains. This serves to increase the diversity of the antibody pool and impacts the antibody's antigen-binding affinity. Some point mutations will result in the production of antibodies that have a weaker interaction (low affinity) with their antigen than the original antibody, and some mutations will generate antibodies with a stronger interaction (high affinity). B cells that express high affinity antibodies on their surface will receive a strong survival signal during interactions with other cells, whereas those with low affinity antibodies will not, and will die by apoptosis. Thus, B cells expressing antibodies with a higher affinity for the antigen will outcompete those with weaker affinities for function and survival. The process of generating antibodies with increased binding affinities is called *affinity maturation*. Affinity maturation occurs in mature B cells after V(D)J recombination, and is dependent on help from helper T cells.
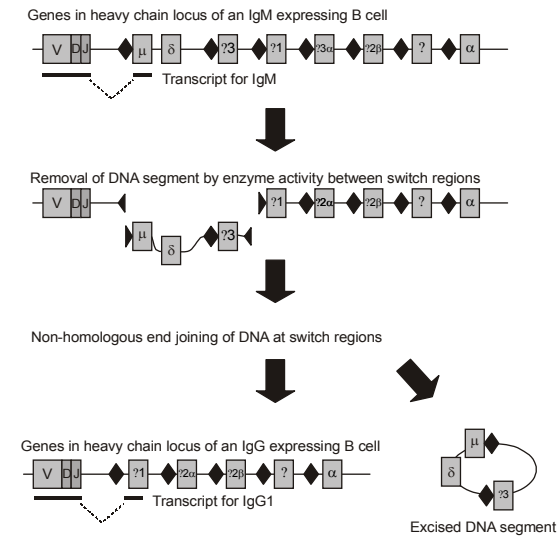
Fig. 2.77 : Showing the Mechanism of class switch recombination that allows isotype switching in activated B cells

Class switching

Isotype or class switching is a biological process occurring after activation of the B cell, which allows the cell to produce different classes of antibody (IgA, IgE, or IgG). The different classes of antibody,

and thus effector functions, are defined by the constant (C) regions of the immunoglobulin heavy chain. Initially, naïve B cells express only cell-surface IgM and IgD with identical antigen binding regions. Each isotype is adapted for a distinct function, therefore, after activation, an antibody with a IgG, IgA, or IgE effector function might be required to effectively eliminate an antigen. Class switching allows different daughter cells from the same activated B cell to produce antibodies of different isotypes. Only the constant region of the antibody heavy chain changes during class switching; the variable regions, and therefore antigen specificity, remain unchanged. Thus the progeny of a single B cell can produce antibodies, all specific for the same antigen, but with the ability to produce the effector function appropriate for each antigenic challenge. Class switching is triggered by cytokines; the isotype generated depends on which cytokines are present in the B cell environment. Class switching occurs in the heavy chain gene locus by a mechanism called class switch recombination (CSR). This mechanism relies on conserved nucleotide motifs, called *switch (S) regions*, found in DNA upstream of each constant region gene (except in the ä-chain). The DNA strand is broken by the activity of a series of enzymes at two selected S-regions. The variable domain exon is rejoined through a process called non-homologous end joining (NHEJ) to the desired constant region (ã, á or å). This process results in an immunoglobulin gene that encodes an antibody of a different isotype.

Medical Applications and Disease Diagnosis

Detection of particular antibodies is a very common form of medical diagnostics, and applications such as serology depend on these methods. For example, in biochemical assays for disease diagnosis,[36] a titer of antibodies directed against Epstein-Barr virus or Lyme disease is estimated from the blood. If those antibodies are not present, either the person is not infected, or the infection occurred a *very* long time ago, and the B cells generating these specific antibodies have naturally decayed. In clinical immunology, levels of individual classes of immunoglobulins are measured by nephelometry (or turbidimetry) to characterize the antibody profile of patient. Elevations in different classes of immunoglobulins are sometimes useful in determining the cause of liver damage in patients whom the diagnosis is unclear.[4] For example, elevated IgA indicates alcoholic cirrhosis, elevated IgM indicates viral hepatitis and primary biliary cirrhosis, while IgG is elevated in viral hepatitis, autoimmune hepatitis and cirrhosis. Autoimmune disorders can often be traced to antibodies that bind the body's own epitopes; many can be detected through blood tests. Antibodies directed against red blood cell surface antigens in immune mediated hemolytic anemia are detected with the Coombs test. The Coombs test is also used for antibody screening in blood transfusion preparation and also for antibody screening in antenatal women. Practically, several immunodiagnostic methods based on detection of complex antigen-antibody are used to diagnose infectious diseases, for example ELISA, immunofluorescence, Western blot, immunodiffusion, immunoelectrophoresis, and Magnetic immunoassay.

Disease therapy

"Targeted" monoclonal antibody therapy is employed to treat diseases such as rheumatoid arthritis, multiple sclerosis, psoriasis, and many forms of cancer including non-Hodgkin's lymphoma, colorectal cancer, head and neck cancer and breast cancer. Some immune deficiencies, such as X-linked agammaglobulinemia and hypogammaglobulinemia, result in partial or complete lack of antibodies.

These diseases are often treated by inducing a short term form of immunity called passive immunity. Passive immunity is achieved through the transfer of ready-made antibodies in the form of human or animal serum, pooled immunoglobulin or monoclonal antibodies, into the affected individual.

Prenatal therapy

Rho(D) Immune Globulin antibodies are specific for human Rhesus D (RhD) antigen, also known as Rhesus factor. These anti-RhD antibodies are known under several brand names, including RhoGAM, BayRHo-D, Gamulin Rh, HypRho-D, and WinRho SDF. Rhesus factor is an antigen found on red blood cells; individuals that are Rhesus-positive (Rh+) have this antigen on their red blood cells and individuals that are Rhesus-negative (Rh–) do not. During normal childbirth, delivery trauma or complications during pregnancy, blood from a fetus can enter the mother's system. In the case of an Rh-incompatible mother and child, consequential blood mixing may sensitize an Rh- mother to the Rh antigen on the blood cells of the Rh+ child, putting the remainder of the pregnancy, and any subsequent pregnancies, at risk for hemolytic disease of the newborn. Anti-RhD antibodies are administered as part of a prenatal treatment regimen to prevent sensitization that may occur when a Rhesus-negative mother has a Rhesus-positive fetus. Treatment of a mother with Anti-RhD antibodies prior to and immediately after trauma and delivery destroys Rh antigen in the mother's system from the fetus. Importantly, this occurs before the antigen can stimulate maternal B cells to "remember" Rh antigen by generating memory B cells. Therefore, her humoral immune system will not make anti-Rh antibodies, and will not attack the Rhesus antigens of the current or subsequent baby. Rho(D) Immune Globulin treatment prevents sensitization that can lead to Rh disease, but does not prevent or treat the underlying disease itself.

Blood proteins, also called *serum proteins and Plasma Proteins*, are proteins found in blood plasma. Serum total protein in blood is 7g/dl, which in total makes 7% of total body weight. They serve many different functions, including

- circulatory transport molecules for lipids, hormones, vitamins and metals
- enzymes, complement components, protease inhibitors, and kinin precursors
- regulation of acellular activity and functioning and in the immune system.

Separating serum proteins by electrophoresis is a valuable diagnostic tool as well as a way to monitor clinical progress.

Often mentioned blood proteins

Blood protein	Normal level	%	Function
Albumins	3.5-5.0 g/dl	60%	create osmotic pressure and transports other molecules
immunoglobulins	1.0-1.5 g/dl	37%	participate in immune system
Fibrinogens	0.2-0.45 g/dl	4%	blood coagulation
alpha 1-antitrypsin			neutralize trypsin that has leaked from the digestive system
Regulatory proteins		<1%	Regulation of gene expression

Other types of blood proteins include: Prealbumin Alpha 1 antitrypsin Alpha 1 acid glycoprotein Alpha 1 fetoprotein Haptoglobin Alpha 2 macroglobulin Ceruloplasmin Transferring C3/C4 Beta 2 microglobulin Beta lipoprotein Gamma globulin proteins C-reactive protein (CRP)

- alpha2-macroglobulin
- Other globulins, which are of three types- alpha, beta and gamma.
- Lipoproteins (chylomicrons, VLDL, LDL, HDL)
- Transferrin
- Prothrombin

All the plasma proteins are synthesized in liver except gamma globulins. We shall dicuss this topic in detain in the Hæmato-chemistry chapter.

60% of plasma proteins are made up of the protein albumin, which are major contributors to osmotic pressure of plasma which assists in the transport of lipids and steroid hormones. Globulins make up 35% of plasma proteins and are used in the transport of ions, hormones and lipids assisting in immune function. 4% is fibrinogen and this is essential in the clotting of blood and can be converted into insoluble fibrin. Regulatory proteins which make up less than 1% of plasma proteins are proteins such as enzymes, proenzymes and hormones. Current research regarding blood plasma proteins is centered on performing proteomics analyses of serum/plasma in the search for biomarkers. These efforts started with two-dimensional gel electrophoresisefforts in the 1970s and in more recent times this research has been performed using LC-tandem MS based proteomics.

SUB-SECTION 2.13B—HEAMOGLOBIN AND MYOGLOBIN

Hemoglobin (also spelled **haemoglobin** and abbreviated **Hb** or **Hgb**) is the iron-containing oxygen-transport metalloprotein in the red blood cells of vertebrates.

Structure

In mammals, the protein makes up about 97% of the red blood cell's dry content, and around 35% of the total content (including water). Hemoglobin transports oxygen from the lungs or gills to the rest of the body, such as to the muscles, where it releases the oxygen for cell use. It also has a variety of other roles of gas transport and effect-modulation which vary from species to species, and are quite diverse in some invertebrates.

Myoglobin is a single-chain globular protein of 153 amino acids, containing a heme (iron-containing porphyrin) prosthetic group in the center around which the remaining apoprotein folds. It has eight alpha helices and a hydrophobic core. It has a molecular weight of
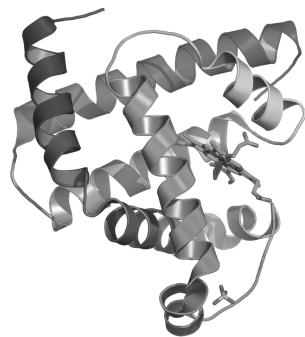
Fig. 2.78 : Showing the Myoglobin protein

16,700 daltons, and is the primary oxygen-carrying pigment of muscle tissues. Unlike the blood-borne hemoglobin, to which it is structurally related,[3] this protein does not exhibit cooperative binding of oxygen, since positive cooperativity is a property of multimeric/oligomeric proteins only. Instead, the binding of oxygen by myoglobin is unaffected by the oxygen pressure in the surrounding tissue. Myoglobin is often cited as having an "instant binding tenacity" to oxygen given its hyperbolic oxygen dissociation curve. High concentrations of myoglobin in muscle cells allow organisms to hold their breaths longer. In 1958, John Kendrew and associates successfully determined the structure of myoglobin by high-resolution X-ray crystallography. For this discovery, John Kendrew shared the 1962 Nobel Prize in chemistry with Max Perutz. The human version of this gene is MB. Despite being one of the most studied proteins in biology, its true physiological function is not yet conclusively established: mice genetically engineered to lack myoglobin are viable and show no obvious defects.

Meat Color

An X-ray diffraction image for the protein myoglobin.

Myoglobin forms pigments responsible for making meat red. The color that meat takes is partly determined by the charge of the iron atom in myoglobin and the oxygen attached to it. When meat is in its raw state, the iron atom is in the +2 oxidation state, and is bound to a dioxygen molecule (O_2). Meat cooked well done is brown because the iron atom is now in the +3 oxidation state. Having lost an electron, and is now coordinated by a water molecule. Under some conditions, meat can also remain pink all through cooking, despite being heated to high temperatures. If meat has been exposed to nitrites, it will remain pink because the iron atom is bound

Fig. 2.79 : Showing the X-Ray diffraction picture of the Myoglobin

to NO, nitric oxide (true of, e.g., corned beef or cured hams). Grilled meats can also take on a pink "smoke ring" that comes from the iron binding a molecule of carbon monoxide to give metmyoglobin. Raw meat packed in a carbon monoxide atmosphere also shows this same pink "smoke ring" due to the same coordination chemistry. Notably, the surface of the raw meat also displays the pink color, which is usually associated in consumers' minds with fresh meat. This artificially-induced pink color can persist in the meat for a very long time, reportedly up to one year. [8] Hormel and Cargill are both reported to use this meat-packing process, and meat treated this way has been in the consumer market since 2003. Myoglobin is found in Type I muscle, Type II A and Type II B, but most texts consider myoglobin not to be found in smooth muscle.

Role in Disease

Myoglobin is released from damaged muscle tissue (rhabdomyolysis), which has very high concentrations

of myoglobin. The released myoglobin is filtered by the kidneys but is toxic to the renal tubular epithelium and so may cause acute renal failure.

Myoglobin is a sensitive marker for muscle injury, making it a potential marker for heart attack in patients with chest pain. CK-MB and cTnT is used in combination with ECG, and the clinical signs to diagnose Acute Myocardial Infarction (AMI).

Structure and Bonding

Myoglobin contains a porphyrin ring with an iron center. There is a *proximal histidine* group attached directly to the iron center, and a *distal histidine* group on the opposite face, not bonded to the iron.

Many functional models of myoglobin have been studied. One of the most important is that of *picket fence porphyrin* by James Collman. This model was used to show the importance of the distal prosthetic group. It serves three functions:

1. To form hydrogen bonds with the dioxygen moiety, increasing the O_2 binding constant

2. To prevent the binding of carbon monoxide, whether from within or without the body. Carbon monoxide binds to iron in an end-on fashion, and is hindered by the presence of the distal histidine, which forces it into a bent conformation. CO binds to heme 23,000 times better than O_2, but only 200 times better in hemoglobin and myoglobin. Oxygen binds in a bent fashion, which can fit with the distal histidine.

3. To prevent irreversible dimerization of the oxymyoglobin with another deoxymyoglobin species

SUB-SECTION 2.13C—ROLE OF 2, 3 DPG

3-BPG is formed from 1,3-BPG by an enzyme called bisphosphoglycerate mutase. It is broken down by a phosphatase to form 3-phosphoglycerate. Its synthesis and breakdown are therefore a way around a step of glycolysis.
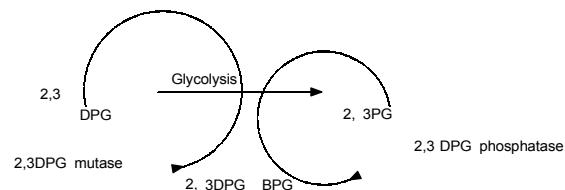
Effects of Binding

When 2,3-BPG binds deoxyhemoglobin, it acts to stabilize the low oxygen affinity state (T state) of the oxygen carrier. It fits neatly into the cavity of the deoxy- conformation, exploiting the molecular symmetry and positive polarity by forming salt bridges with lysine and histidine residues in the four subunits of hemoglobin. The R state, with oxygen bound to a heme group, has a different conformation and does not allow this interaction.

By selectively binding to deoxyhemoglobin, 2,3-BPG stabilizes the T state conformation, making it harder for oxygen to bind hemoglobin and more likely to be released to adjacent tissues. 2,3-BPG is part of a feedback loop that can help prevent tissue hypoxia in conditions where it is most likely to occur. Conditions of low tissue oxygen concentration such as high altitude (2,3-BPG levels are higher in those acclimated to high altitudes), airway obstruction, or congestive heart failure will tend to cause RBCs to generate more 2,3-BPG in their effort to generate energy by allowing more oxygen to be released in tissues deprived of oxygen. Ultimately, this mechanism increases oxygen release from RBCs under circumstances where it is needed most. This release is potentiated by the Bohr effect in tissues with high energetic demands.

Fetal hemoglobin

Interestingly, fetal hemoglobin (HbF) exhibits a low affinity for 2,3-BPG, resulting in a higher binding affinity for oxygen. This increased oxygen binding affinity relative to that of adult hemoglobin (HbA) is due to HbF having two á/ã dimers as opposed to the two á/â dimers of HbA. The positive histidine residues of HbA â-subunits that are essential for forming the 2,3-BPG binding pocket are replaced by serine residues in HbF ã-subunits.

SUB-SECTION 2.13D—THASSEMIA

The fundamental abnormality in thalassemia is impaired production of either the alpha or beta hemoglobin chain. Thalassemia is a difficult subject to explain, since the condition is not a single disorder, but a group of defects with similar clinical effects. More confusion comes from the fact that the clinical descriptions of thalassemia were coined before the molecular basis of the thalassemias were uncovered. As a result, the organizational structure is somewhat disorderly. Review of thalassemia is best approached by separately examining its genetic basis and clinical expression.

Genetic Classification of the Thalassemias

Thalassemia(1) includes disorders affecting the alpha hemoglobin chain genes and the beta hemoglobin chain gene (see Hemoglobin Overview for explanation of alpha and beta chains).

Alpha Thalassemia

Alpha thalassemia occurs when one or more of the four alpha chain genes fails to function. Alpha chain protein production, for practical purposes, is evenly divided among the four genes. With alpha thalassemia, the "failed" genes are almost invariably lost from the cell due to a genetic accident.

(i) The loss of one gene diminishes the production of the alpha protein only slightly. This condition is so close to normal that it can be detected only by specialized laboratory techniques that, until recently, were confined to research laboratories. A person with this condition is called a "silent carrier" because of the difficulty in detection.

(ii) The loss of two genes (two-gene deletion alpha thalassemia) produces a condition with small red blood cells, and at most a mild anemia. People with this condition look and feel normal. The condition can be detected by routine blood testing, however.

(iii) The loss of three alpha genes produces a serious hematological problem (three-gene deletion alpha thalassemia). Patients with this condition have a severe anemia, and often require blood transfusions to survive. The severe imbalance between the alpha chain production (now powered by one gene, instead of four) and beta chain production (which is normal) causes an accumulation of beta chains inside the red blood cells. Normally, beta chains pair only with alpha chains. With three-gene deletion alpha thalassemia, however, beta chains begin to associate in groups of four, producing an abnormal hemoglobin, called "hemoglobin H". The condition is called "hemoglobin H disease". Hemoglobin H has two problems. First it does not carry oxygen properly, making it functionally useless to the cell. Second, hemoglobin H protein damages the membrane that surrounds the red cell, accelerating cell destruction. The combination of the very low production of alpha chains and destruction of red cells in hemoglobin H disease produces a severe, life-threatening anemia. Untreated, most patients die in childhood or early adolescence.

(iv) The loss of all four alpha genes produces a condition that is incompatible with life. The gamma chains produced during fetal life associate in groups of four to form an abnormal hemoglobin called "hemoglobin Barts". Most people with four-gene deletion alpha thalassemia die *in utero* or shortly after birth. Rarely, four gene deletion alpha thalassemia has been detected *in utero*, usually in a family where the disorder occured in an earlier child. *In utero* blood transfusions have saved some of these children. These patients require life-long transfusions and other medical support.

Beta Thalassemia

The fact that there are only two genes for the beta chain of hemoglobin makes beta thalassemia a bit simpler to understand than alpha thalassemia (2). Unlike alpha thalassemia, beta thalassemia rarely arises from the complete loss of a beta globin gene. The beta globin gene is present, but produces little beta globin protein. The degree of suppression varies. Many causes of suppressed beta globin gene expression have been found. In some cases, the affected gene makes essentially no beta globin protein (beta-0-thalassemia). In other cases, the production of beta chain protein is lower than normal, but not zero (beta-(+)-thalassemia). The severity of beta thalassemia depends in part on the type of beta thalassemic genes that a person has inherited.

(i) one-gene beta thalassemia has one beta globin gene that is normal, and a second, affected gene with a variably reduced production of beta globin. The degree of imbalance with the alpha globin depends on the residual production capacity of the defective beta globin gene. Even when the affected gene produces no beta chain, the condition is mild since one beta gene functions normally. The red cells are small and a mild anemia may exist. People with the condition generally have no symptoms. The condition can be detected by a routine laboratory blood evaluation. (Note that in many ways, the one-gene beta thalassemia and the two-gene alpha thalassemia are very similar, from a clinical point of view. Each results in small red cells and a mild anemia).

(ii) two-gene beta thalassemia produces a severe anemia and a potentially life-threatening condition. The severity of the disorder depends in part on the combination of genes that have been

inherited: beta-0-thal/ beta-0-thal; beta-0-thal/ beta-(+)-thal; beta-(+)-thal/ beta-(+)-thal. The beta-(+)-thalassemia genes vary greatly in their ability to produce normal hemoglobin. Consequently, the clinical picture is more complex than might otherwise be the case for three genetic possibilities outlined.

Clinical Classification of the Thalassemias

Alphathalassemia

Alpha thalassemia has four manifestations, that correlate with the number of defective genes. Since the gene defect is almost invariably a loss of the gene, there are no "shades of function" to obscure the matter as occurs in beta thalassemia.

(i) Silent carrier state. This is the one-gene deletion alpha thalassemia condition. People with this condition are hematologically normal. They are detected only by sophisticated laboratory methods.

(ii) Mild alpha-thalassemia. These patients have lost two alpha globin genes. They have small red cells and a mild anemia. These people are usually asymptomatic. Often, physicians mistakenly diagnose people with mild alpha-thalassemia as having iron deficiency anemia. Iron therapy, of course, does not correct the anemia.

(iii) Hemoglobin H disease. These patients have lost three alpha globin genes. The result is a severe anemia, with small, misshapen red cells and red cell fragments. These patients typically have enlarged spleens. Bony abnormalities particularly involving the cheeks and forehead are often striking. The bone marrow works at an extraordinary pace in an attempt to compensate for the anemia. As a result, the marrow cavity within the bones is stuffed with red cell precursors. These cells gradually cause the bone to "mold" and flair out. Patients with hemoglobin H disease also develop large spleens. The spleen has blood forming cells, the same as the bone marrow. These cells become hyperactive and overexpand, just as those of the bone marrow. The result is a spleen that is often ten-times larger than normal. Patients with hemoglobin H disease often are small and appear malnourished, despite good food intake. This feature results from the tremendous amount of energy that goes into the production of new red cells at an extremely accelerated pace. The constant burning of energy by these patients mimics intense aerobic exercise; exercise that goes on for every minute of every day.

(iv) Hydrops fetalis. This condition results from the loss of all four alpha globin genes. The affected individual usually succumbs to the severe anemia and complications before birth.

Betathalassemia

(i) Thalassemia minor, or thalassemia trait. These terms are used interchangeably for people who have small red cells and mild (or no) anemia due to thalassemia. These patients are clinically well, and are usually only detected through routine blood testing. Physicians often mistakenly diagnose iron deficiency in people with thalassemia trait. Iron replacement does not correct the condition. The primary caution for people with beta-thalassemia trait involves the possible

problems that their children could inherit if their partner also has beta-thalassemia trait. These more severe forms of beta-thalassemia trait are outlined below.

(ii) Thalassemia intermedia. Thalassemia intermedia is a confusing concept. The most important fact to remember is that thalassemia intermedia is a description, and not a pathological or genetic diagnosis. Patients with thalassemia intermedia have significant anemia, but are able to survive without blood transfusions. The factors that go into the diagnosis are:

- The degree to which the patient tolerates the anemia.
- The threshold of the physician to transfuse patients with thalassemia.

With regard to the tolerance of the anemia, most patients with thalassemia have substantial symptoms with a Hb of much below 7 or 8 gm/dl. With hemoglobins of this level, excess energy consumption due to the profound hemolysis can produce small stature, poor weight gain, poor energy levels, and susceptibility to infection. Further, the extreme activity of the bone marrow produces bone deformities of the face and other areas, along with enlargement of the spleen. The long bones of the arms and legs are weak and fracture easily. Patients with this clinical condition usually do better with regular transfusions. The need for regular transfusions would then place them under the heading of thalassemia major (see below). On the other hand, some patients with marked thalassemia can maintain a hemoglobin of about 9 to 10 gm/dl. The exercise tolerance of these patients is significantly better. The question then becomes whether the accelerated bone marrow activity needed to maintain this level of hemoglobin causes unacceptable side-effects such as bone abnormalities or enlarged spleen. This is a judgment decision. A given patient at the critical borderline would be transfused by some physicians to prevent these problems, even if they are slight. The patient then would be *clinically* classified as having thalassemia major. Another physician might choose to avoid the complications of chronic transfusion. The same patient then would be *clinically* classified as thalassemia intermedia. The patient has thalassemia that is more severe than thalassemia trait, but not so severe as to require chronic transfusion as do the patients with thalassemia major. A patient can change status. The spleen is enlarged in these patients. The spleen plays a role in clearing damaged red cells from the blood stream. Since all of the red cells in patients with severe thalassemia have some degree of damage, clearance by the spleen accelerates the rate of cell loss. Therefore the bone marrow has to work harder to replace these cells. In some patients, removal of the spleen slows the rate of red cell destruction just enough, that they can manage without transfusion, and still not have the unacceptable side-effects. In this case, the patient converts *clinically* from thalassemia major to thalassemia intermedia.

(iii) Thalassemia major. This is the condition of severe thalassemia in which chronic blood transfusions are needed (3). In some patients the anemia is so severe, that death occurs without transfusions. Other patients could survive without transfusions, for a while, but would have terrible deformities. While transfusions are life-saving in patients with thalassemia major, transfusions ultimately produce iron overload. Chelation therapy, usually with the iron-binding agent, desferrioxamine (Desferal), is needed to prevent death from iron-mediated organ injury.

Relationship of the Genetic and Clinical Classifications of Thalassemia

The advent of modern molecular biology permits the genetic classification of thalassemias, outlined earlier in this document. A rough correlation exists between the clinical and genetic classifications. The relationship between genetics and clinical state is not absolute, however:

- thalassemia trait (minor)- normal beta gene/ thalassemia gene (beta zero or +)
- thalassemia intermedia- often two beta-(+)-genes
- thalassemia major- two beta-(+)-genes (where the plus is not substantial); beta-(+)-gene/ beta-0-gene; beta-0-gene/ beta-0-gene

SECTION 2.14—COLLAGEN

Collagen is the main protein of connective tissue in animals and the most abundant protein in mammals, making up about 25% to 35% of the whole-body protein content.

Uses

Collagen is one of the long, fibrous structural proteins whose functions are quite different from those of globular proteins such as enzymes. Tough bundles of collagen called *collagen fibers* are a major component of the extracellular matrix that supports most tissues and gives cells structure from the outside, but collagen is also found inside certain cells. Collagen has great tensile strength, and is the main component of fascia, cartilage, ligaments, tendons, bone and skin. Along with soft keratin, it is responsible for skin strength and elasticity, and its degradation leads to wrinkles that accompany aging. It strengthens blood vessels and plays a role in tissue development. It is present in the cornea and lens of the eye in crystalline form. It is also used in cosmetic surgery and burns surgery. Hydrolyzed collagen can play an important role in weight management. As a protein, it can be advantageously used for its satiating power.

Industrial uses

If collagen is partially hydrolyzed, the three tropocollagen strands separate into globular, random coils, producing gelatin, which is used in many foods, including flavoured gelatin desserts. Besides food, gelatin has been used in pharmaceutical, cosmetic, and photography industries. Collagen and gelatin are poor-quality protein since they do not contain all the essential amino acids that the human body requires— they are not complete proteins. Manufacturers of collagen-based dietary supplements claim that their products can improve skin and fingernail quality as well as joint health. However, mainstream scientific research has not shown any evidence to support these claims. Individuals with problems in these areas are more likely to be suffering from some other underlying condition rather than protein deficiency.

From the Greek for glue, *kolla*, the word collagen means "glue producer" and refers to the early process of boiling the skin and sinews of horses and other animals to obtain glue. Collagen adhesive was used by Egyptians about 4,000 years ago, and Native Americans used it in bows about 1,500 years ago. The oldest glue in the world, carbon-dated as more than 8,000 years old, was found to be collagen—

used as a protective lining on rope baskets and embroidered fabrics, and to hold utensils together; also in crisscross decorations on human skulls. Collagen normally converts to gelatin, but survived due to the dry conditions. Animal glues are thermoplastic, softening again upon reheating, and so they are still used in making musical instruments such as fine violins and guitars, which may have to be reopened for repairs—an application incompatible with tough, synthetic plastic adhesives, which are permanent. Animal sinews and skins, including leather, have been used to make useful articles for millennia.

Gelatin-resorcinol-formaldehyde glue (and with formaldehyde replaced by less-toxic pentanedial and ethanedial) has been used to repair experimental incisions in rabbit lungs.

Medical uses

Collagen has been widely used in cosmetic surgery, as a healing aid for burn patients for reconstruction of bone and a wide variety of dental, orthopedic and surgical purposes. Some points of interest are:

1. when used cosmetically, there is a chance of allergic reactions causing prolonged redness; however, this can be virtually eliminated by simple and inconspicuous patch testing prior to cosmetic use, and

2. most medical collagen is derived from young beef cattle (bovine) from certified BSE (Bovine spongiform encephalopathy) free animals. Most manufacturers use donor animals from either "closed herds", or from countries which have never had a reported case of BSE such as Australia, Brazil and New Zealand.

3. porcine (pig) tissue is also widely used for producing collagen sheet for a variety of surgical purposes.

4. alternatives using the patient's own fat, hyaluronic acid or polyacrylamide gel are readily available.

Collagens are widely employed in the construction of artificial skin substitutes used in the management of severe burns. These collagens may be derived from bovine, equine or porcine, and even human, sources and are sometimes used in combination with silicones, glycosaminoglycans, fibroblasts, growth factors and other substances.

Collagen is also sold commercially as a joint mobility supplement. This lacks supportive research as the proteins would just be broken down into its base amino acids during digestion, and could go to a variety of places besides the joints depending upon need and DNA orders.

Recently an alternative to animal-derived collagen has become available. Although expensive, this human collagen, derived from donor cadavers, placentas and aborted fetuses, may minimize the possibility of immune reactions.

Collagen is now being used as a main ingredient for some cosmetic makeup.

Conformation and Structure

Collagen structure is complex. Its conformation can be considered at the monomeric level (individual) collagen molecules and/or at its aggregate level, how the monomers are arranged i.e. their packing structure (fibrils, networks, etc. - see table below).

History and Background

The molecular and packing structures of collagen have eluded scientists for decades; the first evidence that it possess a regular structure at the molecular level was presented in the mid-1930s. Since that time many prominent scholars, including (but not limited to) Nobel laureate Crick, and Pauling, Rich, Yonath, Brodsky, Berman and Ramachandran concentrated on the conformation of the collagen monomoer. Several competing models although correctly dealing with the conformation of each individual peptide chain, gave way to the triple-helical "Madras" model which provided an essentially correct model of the molecules quaternary structure although this model still required some refinement. The packing structure of collagen has not been defined to the same degree outside of the fibrillar collagen types, although it has been long known to be hexagonal or quasi-hexagonal.As with its monomeric structure, several conflicting models alleged that either the packing arrangement of collagen molecules is 'sheet-like' or microfibrillar. Recently it was confirmed that the microfibrillar structure as described by Fraser, Miller, Wess (amongst others) was closest to the observed structure, although it over-simplified the topological progression of neighboring collagen molecules and hence did not predict the correct conformation of the discontinuous D-periodic pentameric arrangement termed simply: the microfibril.

Molecular Structure

The *tropocollagen* or "collagen molecule" is a subunit larger collagen aggregates such as fibrils. It is approximate 300 nm long and 1.5 nm in diameter, made up of thr polypeptide strands (called alpha peptides), each possessi the conformation of a left-handed helix (its name is not be confused with the commonly occurring alpha helix right-handed structure). These three left-handed helices twisted together into a right-handed coiled coil, a triple he or "super helix", a cooperative quaternary structure stabiliz by numerous hydrogen bonds. With type I collagen a possibly all fibrillar collagens if not all collagens, each trip helix associates into a right-handed super-super-coil tha referred to as the collagen microfibril. Each microfibril interdigitated with its neighboring microfibrils to a degr that might suggest that they are individually unstable althougn within collagen fibrils they are so well ordered as to be crystalline.

Fig. 2.79: Collagen Fibre

A distinctive feature of collagen is the regular arrangement of amino acids in each of the three chains of these collagen subunits. The sequence often follows the pattern Gly-Pro-Y or Gly-X-Hyp, where X and Y may be any of various other amino acid residues. Proline or hydroxyproline constitute about 1/6 of the total sequence. With Glycine accounting for the 1/3 of the sequence, this means that approximately half of the collagen sequence is not glycine or proline, a fact often missed due to the distraction of the unusual GXY character of collagen alpha-peptides. This kind of regular repetition and high glycine content is found in only a few other fibrous proteins, such as silk fibroin. 75-80% of silk

is (approximately) -Gly-Ala-Gly-Ala- with 10% serine—and elastin is rich in glycine, proline, and alanine (Ala), whose side group is a small, inert methyl group. Such high glycine and regular repetitions are never found in globular proteins save for very short sections of their sequence. Chemically-reactive side groups are not needed in structural proteins as they are in enzymes and transport proteins, however collagen is not quite just a structural protein. Due to its key role in the determination of cell phenotype, cell adhesion, tissue regulation and infrastructure, many sections of its non-proline rich regions have cell or matrix association regulation roles. The relatively high content of Proline and Hydroxyproline rings, with their geometrically constrained carboxyl and (secondary) amino groups, along with the rich abundance of glycine, accounts for the tendency of the individual polypeptide strands to form left-handed helices spontaneously, without any intrachain hydrogen bonding.

Because glycine is the smallest amino acid with no side-chain, it plays a unique role in fibrous structural proteins. In collagen, Gly is required at every third position because the assembly of the triple helix puts this residue at the interior (axis) of the helix, where there is no space for a larger side group than glycine's single hydrogen atom. For the same reason, the rings of the Pro and Hyp must point outward. These two amino acids help stabilize the triple helix—Hyp even more so than Pro—a lower concentration of them is required in animals such as fish, whose body temperatures are lower than most warm-blooded animals.

Fibrillar Structure

The tropocollagen subunits spontaneously self-assemble, with regularly staggered ends, into even larger arrays in the extracellular spaces of tissues. In the fibrillar collagens, the molecules are staggered from each other by about 67nm (a unit that is referred to as 'D' and changes depending upon the hydration state of the aggregate). Each D-period contains 4 and a fraction collagen molecules. This is because 300 nm divided by 67 nm does not give an integer (the length of the collagen molecule divided by the stagger distance D). Therefore in each D-period repeat of the microfibril, there is a part containing 5 molecules in cross-section – called the "overlap" and a part containing only 4 molecules. The triple-helices are also arranged in a hexagonal or quasi-hexagonal array in cross-section, in both the gap and overlap regions.

There is some covalent crosslinking within the triple helices, and a variable amount of covalent crosslinking between tropocollagen helices forming well organized aggregates (such as fibrils). Larger fibrillar bundles are formed with the aid of several different classes of proteins (including different collagen types), glycoproteins and proteoglycans to form the different types of mature tissues from alternate combinations of the same key players. Collagen's insolubility was a barrier to the study of monomeric collagen until it was found that tropocollagen from young animals can be extracted because it is not yet fully crosslinked. However, advances in microscopy techniques (Electron Microscopy - EM and Atomic Force Microscopy -AFM) and X-ray diffraction have enabled researchers to obtain increasingly detailed images of collagen structure *in situ*. These later advances are particularly important to better understanding the way in which collagen structure affects cell-cell and cell-matrix communication and how tissues are constructed in growth and repair, and changed in development and disease[28][29].

Collagen fibrils are collagen molecules packed into an organized overlapping bundle. Collagen fibers are bundles of fibrils.

Collagen fibrils / aggregates are arranged in different combinations and concentrations in various tissues to provide varying tissue properties. In bone, entire collagen triple helices lie in a parallel, staggered array. 40 nm gaps between the ends of the tropocollagen subunits probably serve as nucleation sites for the deposition of long, hard, fine crystals of the mineral component, which is (approximately) hydroxyapatite, $Ca_{10}(PO_4)_6 (OH)_2$ with some phosphate. It is in this way that certain kinds of cartilage turn into bone. Type I collagen gives bone its tensile strength.

Types and Associated Disorders

Collagen occurs in many places throughout the body. There are more then 28 types of collagen described in literature. Over 90% of the collagen in the body, however, are of type I, II, III, and IV.

- Collagen One - skin, tendon, vascular, ligature, organs, bone (main component of bone)
- Collagen Two - cartilage (main component of cartilage)
- Collagen Three - reticulate (main component of reticular fibers), commonly found alongside type I.
- Collagen Four - forms bases of cell basement membrane

Collagen diseases commonly arise from genetic defects that affect the biosynthesis, assembly, postranslational modification, secretion, or other processes in the normal production of collagen.

Type	Notes	Gene(s)	Disorders
1	2	3	4
I	This is the most abundant collagen of the human body. It is present in scar tissue, the end product when tissue heals by repair. It is found in tendons, skin, artery walls, the endomysium of myofibrils, fibrocartilage, and the organic part of bones and teeth	COL1A1, COL1A2	osteogenesis imperfecta, Ehlers-Danlos Syndrome
II	Hyaline cartilage, makes up 50% of all cartilage protein. Vitreous humour of the eye	COL2A1	Collagenopathy, types II and XI
III	This is the collagen of granulation tissue, and is produced quickly by young fibroblasts before the tougher type I collagen is synthesized. Reticular fiber. Also found in artery walls, skin, intestines and the uterus	COL3A1	Ehlers-Danlos Syndrome
IV	basal lamina; eye lens. Also serves as part of the filtration system in capillaries	COL4A1, COL4A2,	Alport syndrome

1	2	3	4
	and the glomeruli of nephron in the kidney	COL4A3, COL4A4, COL4A5, COL4A6	
V	most interstitial tissue, assoc. with type I, associated with placenta	COL5A1, COL5A2, COL5A3	Ehlers-Danlos syndrome (Classical)
VI	most interstitial tissue, assoc. with type I	COL6A1, COL6A2, COL6A3	Ulrich myopathy and Bethlem myopathy
VII	forms anchoring fibrils in dermal epidermal junctions	COL7A1	epidermolysis bullosa
VIII	some endothelial cells	COL8A1, COL8A2	—
IX	FACIT collagen, cartilage, assoc. with type II and XI fibrils	COL9A1, COL9A2, COL9A3	- EDM2 and EDM3
X	hypertrophic and mineralizing cartilage	COL10A1	—
XI	cartilage	COL11A1, COL11A2	Collagenopathy, types II and XI
XII	FACIT collagen, interacts with type I containing fibrils, decorin and glycosaminoglycans	COL12A1	—
XIII	transmembrane collagen, interacts with integrin a1b1, fibronectin and components of basement membranes like nidogen and perlecan.	COL13A1	—
XIV	FACIT collagen	COL14A1	—
XV	—	COL15A1	—
XVI	—	COL16A1	—
XVII	transmembrane collagen, also known as BP180, a 180 kDa protein	COL17A1	Bullous Pemphigoid and certain forms of junctional epidermolysis bullosa
XVIII	source of endostatin	COL18A1	—
XIX	FACIT collagen	COL19A1	—
XX	—	COL20A1	—

1	2	3	4
XXI	FACIT collagen	COL21A1	—
XXII	—	COL22A1	—
XXIII	MACIT collagen -	COL23A1	—
XXIV	—	COL24A1	—
XXV	—	COL25A1	—
XXVI	—	EMID2	—
XXVII	—	COL27A1	—
XXVIII	—	COL28A1	—
XXIX	epidermal collagen	COL29A1	Atopic Dermatitis

In addition to the above mentioned disorders, excessive deposition of collagen occurs in Scleroderma.

Staining

In histology, collagen is brightly eosinophilic (pink) in standard H&E slides. The dye methyl violet may be used to stain the collagen in tissue samples.

The dye methyl blue can also be used to stain collagen and immunohistochemical stains are available if required.

The best stain for use in differentiating collagen from other fibers is Masson's trichrome stain.

Synthesis

Amino acids

Collagen has an unusual amino acid composition and sequence:

- Glycine (Gly) is found at almost every third residue
- Proline (Pro) makes up about 9% of collagen
- Collagen contains two uncommon derivative amino acids not directly inserted during translation. These amino acids are found at specific locations relative to glycine and are modified post-translationally by different enzymes, both of which require vitamin C as a cofactor.
  — Hydroxyproline (Hyp), derived from proline.
  — Hydroxylysine, derived from lysine. Depending on the type of collagen, varying numbers of hydroxylysines have disaccharides attached to them.

Collagen I formation

Most collagen forms in a similar manner, but the following process is typical for type I:

1. Inside the cell

    1. Three peptide chains are formed (2 alpha-1 and 1 alpha-2 chain) in ribosomes along the Rough Endoplasmic Reticulum (RER). These peptide chains (known as preprocollagen) have registration peptides on each end; and a signal peptide is also attached to each
    2. Peptide chains are sent into the lumen of the RER
    3. Signal Peptides are cleaved inside the RER and the chains are now known as procollagen
    4. Hydroxylation of lysine and proline amino acids occurs inside the lumen. This process is dependent on Ascorbic Acid (Vitamin C) as a cofactor
    5. Glycosylation of specific hydroxylated amino acid occurs
    6. Triple helical structure is formed inside the RER
    7. Procollagen is shipped to the golgi apparatus, where it is packaged and secreted by exocytosis

2. Outside the cell

    1. Registration peptides are cleaved and tropocollagen is formed by procollagen peptidase.
    2. Multiple tropocollagen molecules form collagen fibrils, and multiple collagen fibrils form into collagen fibers
    3. Collagen is attached to cell membranes via several types of protein, including fibronectin and integrin.

Synthetic pathogenesis

Vitamin C deficiency causes scurvy, a serious and painful disease in which defective collagen prevents the formation of strong connective tissue. Gums deteriorate and bleed, with loss of teeth; skin discolors, and wounds do not heal. Prior to the eighteenth century, this condition was notorious among long duration military, particularly naval, expeditions during which participants were deprived of foods containing Vitamin C. In the human body, a malfunction of the immune system, called an autoimmune disease, results in an immune response in which healthy collagen fibers are systematically destroyed with inflammation of surrounding tissues. The resulting disease processes are called Lupus erythematosus, and rheumatoid arthritis, or collagen tissue disorders.

Many bacteria and viruses have virulence factors which destroy collagen or interfere with its production.

Keratins are a family of fibrous structural proteins; tough and insoluble, they form the hard but nonmineralized structures found in reptiles, birds, amphibians and mammals. They are rivaled as biological materials in toughness only by chitin.

There are various types of keratins within a single animal.
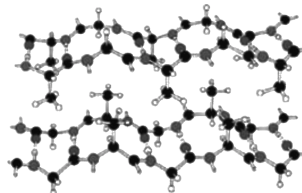
Variety of Animal Uses

Keratins are the main constituent of structures that grow from the skin:

• the *á-keratins* in the hair (including wool), horns, nails, claws and hooves of mammals the harder *â-keratins* found in nails and in the scales and claws of reptiles, their shells (chelonians, such as tortoise, turtle, terrapin), and in the feathers, beaks, claws of birds and quills of porcupines. (These keratins are formed primarily in beta sheets. However, beta sheets are also found in á-keratins.)

Arthropods such as crustaceans often have parts of their armor or exoskeleton made of keratin, sometimes in combination with chitin.

The baleen plates of filter-feeding whales are made of keratin.

They can be integrated in the chitinophosphatic material that makes up the shell and setae in many brachiopods.

Keratins are also found in the gastrointestinal tracts of many animals, including roundworms (which also have an outer layer made of keratin).

Although it is now difficult to be certain, the scales, claws, some protective armour and the beaks of dinosaurs would, almost certainly, have been composed of a type of keratin.

In Crossopterygian fish, the outer layer of cosmoid scales was keratin.

Cornification

It used to be thought that keratins were separable into 'hard' and 'soft,' or 'cytokeratins' and 'other keratins', but those designations are now understood to be incorrect. In 2006, a new nomenclature was adopted for describing keratins which takes this into account.

Keratins are intermediate filaments. Like all intermediate filaments, keratin proteins form filamentous polymers in a series of assembly steps beginning with dimerization; dimers assemble into tetramers and octamers and eventually, the current hypothesis holds, into unit-length-filaments (ULF) capable of annealing end-to-end into long filaments. During the process of epithelial differentiation, cells become cornified as keratin protein is incorporated into longer keratin intermediate filaments. Eventually the nucleus and cytoplasmic organelles disappear, metabolism ceases and cells undergo a programmed death as they become fully keratinized. In many other cells types, such as dermis, keratin functions in the cytoplasm to mechanically stabilize the cell against physical stress. It does this through connections to desmosomes, cell-cell junctional plaques, and hemidesmosomes, cell-basement membrane adhesive structures.

Cells in the epidermis contain a structural matrix of keratin which makes this outermost layer of the skin almost waterproof, and along with collagen and elastin, gives skin its strength. Rubbing and pressure cause keratin to proliferate with the formation of protective calluses — useful for athletes and on the fingertips of musicians who play stringed instruments. Keratinized epidermal cells are constantly shed and replaced.

These hard, integumentary structures are formed by intercellular cementing of fibers formed from the dead, cornified cells generated by specialized beds deep within the skin. Hair grows continuously and feathers moult and regenerate. The constituent proteins may be phylogenetically homologous but

differ somewhat in chemical structure and supermolecular organization. The evolutionary relationships are complex and only partially known. Multiple genes have been identified for the â-keratins in feathers, and this is probably characteristic of all keratins.

Molecular Biology and Biochemistry

The properties which make structural proteins like keratins useful depend on their supermolecular aggregation. These depend on the properties of the individual polypeptide strands, which depend in turn on their amino acid composition and sequence. The á-helix and â-sheet motifs, and disulfide bridges, are crucial to the conformations of globular, functional proteins like enzymes, many of which operate semi-independently, but they take on a completely dominant role in the architecture and aggregation of keratins.

Glycine and Alanine

Keratins contain a high proportion of the smallest of the 20 amino acids, glycine, whose "side group" is a single hydrogen atom; also the next smallest, alanine, with a small and noncharged methyl group. In the case of â-sheets, this allows sterically-unhindered hydrogen bonding between the amino and carboxyl groups of peptide bonds on adjacent protein chains, facilitating their close alignment and strong binding. Fibrous keratin molecules can twist around each other to form helical intermediate filaments.

Limited interior space is the reason why the triple helix of the (unrelated) structural protein collagen, found in skin, cartilage and bone, likewise has a high percentage of glycine. The connective tissue protein elastin also has a high percentage of both glycine and alanine. Silk fibroin, considered a â-keratin, can have these two as 75–80% of the total, with 10–15% serine, with the rest having bulky side groups. The chains are antiparallel, with an alternating C'!N orientation.[1] A preponderance of amino acids with small, nonreactive side groups is characteristic of structural proteins, for which H-bonded close packing is more important than chemical specificity.

Disulphide bridges

In addition to intra- and intermolecular hydrogen bonds, keratins have large amounts of the sulfur-containing amino acid cysteine, required for the disulfide bridges that confer additional strength and rigidity by permanent, thermally-stable crosslinking—a role sulfur bridges also play in vulcanized rubber. Human hair is approximately 14% cysteine. The pungent smells of burning hair and rubber are due to the sulfur compounds formed. Extensive disulfide bonding contributes to the insolubility of keratins, except in dissociating or reducing agents.

The more flexible and elastic keratins of hair have fewer interchain disulfide bridges than the keratins in mammalian fingernails, hooves and claws (homologous structures), which are harder and more like their analogs in other vertebrate classes. Hair and other á-keratins consist of á-helically-coiled single protein strands (with regular intra-chain H-bonding), which are then further twisted into superhelical ropes that may be further coiled. The â-keratins of reptiles and birds have â-pleated sheets twisted together, then stabilized and hardened by disulfide bridges.

Silk

The silk fibroins produced by insects and spiders are often classified as keratins, though it is unclear whether they are phylogenetically related to vertebrate keratins.

Silk found in insect pupae, and in spider webs and egg casings, also has twisted â-pleated sheets incorporated into fibers wound into larger supermolecular aggregates. The structure of the spinnerets on spiders' tails, and the contributions of their interior glands, provide remarkable control of fast extrusion. Spider silk is typically about 1 to 2 micrometres (µm) thick, compared with about 60 µm for human hair, and more for some mammals. (Hair, or fur, occurs only in mammals.) The biologically and commercially useful properties of silk fibers depend on the organization of multiple adjacent protein chains into hard, crystalline regions of varying size, alternating with flexible, amorphous regions where the chains are randomly coiled.[4] A somewhat analogous situation occurs with synthetic polymers such as nylon, developed as a silk substitute. Silk from the hornet cocoon contains doublets about 10 µm across, with cores and coating, and may be arranged in up to 10 layers; also in plaques of variable shape. Adult hornets also use silk as a glue, as do spiders.

Pairing

A (neutral-basic)	B (acidic)	Occurrence
keratin 1, keratin 2	keratin 9, keratin 10	stratum corneum, keratinocytes
keratin 3	keratin 12	cornea
keratin 4	keratin 13	stratified epithelium
keratin 5	keratin 14, keratin 15	stratified epithelium
keratin 6	keratin 16, keratin 17	squamous epithelium
keratin 7	keratin 19	ductal epithelia
keratin 8	keratin 18, keratin 20	simple epithelium

The entries KRT23, KRT24, KRT25, KRT26, KRT27, KRT28, KRT31, KRT32, KRT33, KRT33A, KRT34, KRT35, KRT36, KRT37, KRT38, KRT39, KRT40, KRT71, KRT72, KRT73, KRT74, KRT75, KRT76, KRT77, KRT78, KRT79, KRT8, KRT80, KRT81, KRT82, KRT83, KRT84, KRT85 and KRT86 have been used to describe keratins past 20.

Clinical Significance

Some infectious fungi, such as those which cause athlete's foot, ringworm or the *Batrachochytrium dendrobatidis* (Chytrid fungus) which is killing amphibians all over the world, feed on keratin.

Diseases caused by mutations in the keratin genes include

- Epidermolysis bullosa simplex
- Ichthyosis bullosa of Siemens
- Epidermolytic hyperkeratosis

- Steatocystoma multiplex
- Keratosis pharyngis

Elastin is a protein in connective tissue that is elastic and allows many tissues in the body to resume their shape after stretching or contracting. Elastin helps skin to return to its original position when it is poked or pinched. Elastin is also an important load-bearing tissue in the bodies of mammals and used in places where mechanical energy is required to be stored.

Composition

Elastin is primarily composed of the amino acids glycine, valine, alanine, and proline. It is a specialized protein with a molecular weight of 64 to 66 kDa, and an irregular or random coil conformation made up of 830 amino acids.

Elastin is made by linking many soluble tropoelastin protein molecules, in a reaction catalyzed by lysyl oxidase, to make a massive insoluble, durable cross-linked array. The amino acid responsible for these cross-links is lysine.

Desmosine and isodesmosine are both found in elastin.

Locations in Body

Elastin serves an important function in arteries and is particularly abundant in large elastic blood vessels such as the aorta. Elastin is also very important in the lungs, elastic ligaments, the skin, the bladder, elastic cartilage, and the intervertebral disc above the sacroiliac. It is present in all vertebrates above the jawless fish.

SECTION 2.15—PURINES

Purines

- Adenine = 6-amino purine
- Guanine = 2-amino-6-oxy purine

Adenine

Hypoxanthine
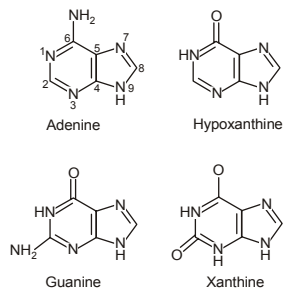
Guanine

Xanthine

- Hypoxanthine = 6-oxy purine
- Xanthine = 2,6-dioxy purine

Adenine and guanine are found in both DNA and RNA. Hypoxanthine and xanthine are not incorporated into the nucleic acids as they are being synthesized but are important intermediates in the synthesis and degradation of the purine nucleotides.

SUB-SECTION 2.15A—PYRIMIDINES

- Uracil = 2,4-dioxy pyrimidine
- Thymine = 2,4-dioxy-5-methyl pyrimidine
- Cytosine = 2-oxy-4-amino pyrimidine
- Orotic acid = 2,4-dioxy-6-carboxy pyrimidine

Cytosine

Uracil

Thymine

Orotic Acid
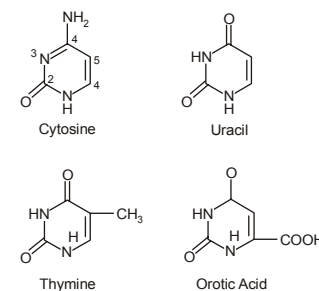
Cytosine is found in both DNA and RNA. Uracil is found only in RNA. Thymine is normally found in DNA. Sometimes tRNA will contain some thymine as well as uracil.

Nucleosides

If a sugar, either **ribose** or **2-deoxyribose**, is added to a nitrogen base, the resulting compound is called a **nucleoside**. Carbon 1 of the sugar is attached to nitrogen 9 of a purine base or to nitrogen 1 of a pyrimidine base. The names of **purine** nucleosides end in **-osine** and the names of **pyrimidine** nucleosides end in **-idine.** The convention is to number the ring atoms of the base normally and to use l', etc. to distinguish the ring atoms of the sugar. Unless otherwise specified, the sugar is assumed to be ribose. To indicate that the sugar is 2'-deoxyribose, a **d-** is placed before the name.

- Adenosine
- Guanosine
- Inosine - the base in inosine is **hypoxanthine**
- Uridine

- Thymidine
- Cytidine

Adenosine is a nucleoside composed of a molecule of adenine attached to a ribose sugar molecule (ribofuranose) moiety via a â-N$_9$-glycosidic bond.

Adenosine plays an important role in biochemical processes, such as energy transfer - as adenosine triphosphate (ATP) and adenosine diphosphate (ADP) - as well as in signal transduction as cyclic adenosine monophosphate, cAMP. It is also an inhibitory neurotransmitter, believed to play a role in promoting sleep and suppressing arousal, with levels increasing with each hour an organism is awake.

Pharmacological Effects

Adenosine is an endogenous purine nucleoside that modulates many physiologic processes. Cellular signaling by adenosine occurs through four known adenosine receptor subtypes (A1, A2A, A2B, and A3).

Extracellular adenosine concentrations from normal cells are approximately 300 nM; however, in response to cellular damage (e.g. in inflammatory or ischemic tissue), these concentrations are quickly elevated (600-1,200 nM). Thus, in regards to stress or injury, the function of adenosine is primarily that of cytoprotection preventing tissue damage during instances of hypoxia, ischemia, and seizure activity. Activation of A2A receptors produces a constellation of responses that in general can be classified as anti-inflammatory.

Adenosine Receptors

The different adenosine receptor subtypes (A1, A2A, A2B, and A3) are all seven transmembrane spanning G-protein coupled receptors. These four receptor subtypes are further classified based on their ability to either stimulate or inhibit adenylate cyclase activity. The A2A and A2B receptors couple to GÜs and mediate the stimulation of adenylate cyclase, while the A1 and A3 adenosine receptors couple to GÜi which inhibits adenylate cyclase activity. Additionally, A1 receptors couple to GÜo, which has been reported to mediate adenosine inhibition of Ca2+ conductance, whereas A2B and A3 receptors also couple to GÜq and stimulate phospholipase activity.

Anti-inflammatory Properties

Adenosine is a potent anti-inflammatory agent, acting at its four G-protein coupled receptors. Topical treatment of adenosine to foot wounds in diabetes mellitus has been shown in lab animals to drastically increase tissue repair and reconstruction. Topical administration of adenosine for use in wound healing deficiencies and diabetes mellitus in humans is currently under clinical investigation.

Action on the Heart

When administered intravenously, adenosine causes transient heart block in the AV node. This is mediated via the A1 receptor, inhibiting adenyl cyclase, reducing cAMP and so causing cell hyperpolarization by increasing outward K+ flux. It also causes endothelial dependent relaxation of smooth muscle as is found inside the artery walls. This causes dilatation of the "normal" segments of arteries, i.e. where the

endothelium is not separated from the tunica media by atherosclerotic plaque. This feature allows physicians to use adenosine to test for blockages in the coronary arteries, by exaggerating the difference between the normal and abnormal segments.

In individuals suspected of suffering from a supraventricular tachycardia (SVT), adenosine is used to help identify the rhythm. Certain SVTs can be successfully terminated with adenosine. This includes any re-entrant arrhythmias that require the AV node for the re-entry (e.g., AV reentrant tachycardia (AVRT), AV nodal reentrant tachycardia (AVNRT). In addition, atrial tachycardia can sometimes be terminated with adenosine.

Adenosine has an indirect effect on atrial tissue causing a shortening of the refractory period. When administered via a central lumen catheter, adenosine has been shown to initiate atrial fibrillation because of its effect on atrial tissue. In individuals with accessory pathways, the onset of atrial fibrillation can lead to a life threatening ventricular fibrillation.

Fast rhythms of the heart that are confined to the atria (e.g., atrial fibrillation, atrial flutter) or ventricles (e.g., monomorphic ventricular tachycardia) and do not involve the AV node as part of the re-entrant circuit are not typically converted by adenosine. However, the ventricular response rate is temporarily slowed with adenosine in such cases.

Because of the effects of adenosine on AV node-dependent SVTs, adenosine is considered a class V antiarrhythmic agent. When adenosine is used to cardiovert an abnormal rhythm, it is normal for the heart to enter ventricular asystole for a few seconds. This can be disconcerting to a normally conscious patient, and is associated with angina-like sensations in the chest.

Caffeine   Adenosine
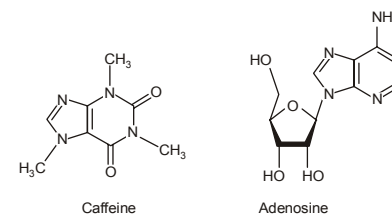
Caffeine's principal mode of action is as an antagonist of adenosine receptors in the brain. They are presented here side by side for comparison.

By nature of caffeine's purine structure it binds to some of the same receptors as adenosine. The pharmacological effects of adenosine may therefore be blunted in individuals who are taking large quantities of methylxanthines (e.g., caffeine, found in coffee and tea, or theobromine, as found in chocolate).

Action in CNS

Generalized, adenosine has an inhibitory effect in the central nervous system (CNS). Caffeine's stimulatory effects, on the other hand, are primarily (although not entirely) credited to its inhibition of

adenosine by binding to the same receptors, and therefore effectively blocking adenosine receptors in the CNS. This reduction in adenosine activity leads to increased activity of the neurotransmitters dopamine and glutamate.

Dosage

When given for the evaluation or treatment of an SVT, the initial dose is 6 mg, given as a fast IV/IO push. Due to adenosine's extremely short half-life, the IV line is started as proximal to the heart as possible, such as the antecubital fossa. The IV push is often followed with an immediate flush of 5-10ccs of saline. If this has no effect (e.g., no evidence of transient AV block), a 12mg dose can be given 1-2 minutes after the first dose. If the 12mg dose has no effect, a second 12mg dose can be administered 1-2 minutes after the previous dose. Some clinicians may prefer to administer a higher dose (typically 18 mg), rather than repeat a dose that apparently had no effect. When given to dilate the arteries, such as in a "stress test", the dosage is typically 0.14 mg/kg/min, administered for 4 or 6 minutes, depending on the protocol.

The recommended dose may be increased in patients on theophylline since methylxanthines prevent binding of adenosine at receptor sites. The dose is often decreased in patients on dipyridamole (Persantine) and diazepam (Valium) because adenosine potentiates the effects of these drugs. The recommended dose is also reduced by half in patients who are presenting Congestive Heart Failure, Myocardial Infarction, shock, hypoxia, and/or hepatic or renal insufficiency, and in elderly patients.

Drug Interactions

Dopamine may precipitate toxicity in the patient. Carbamazepine may increase heart block. Theophylline and caffeine (methylxanthines) competitively antagonize adenosine's effects; may require increased dose of adenosine.

Side Effects

Many individuals experience facial flushing, lightheadedness, asystole, diaphoresis, or nausea after administration of adenosine due to its vasodilatory effects. Metallic taste is a hallmark side effect of adenosine administration. These symptoms are transitory, usually lasting less than one minute. It is classically associated with a sense of "impending doom", more prosaically described as apprehension. This lasts a few seconds after administration intravenously.

Metabolism

When adenosine enters the circulation, it is broken down by adenosine deaminase, which is present in red cells and the vessel wall.

Dipyridamole, an inhibitor of adenosine deaminase, allows adenosine to accumulate in the blood stream. This causes an increase in coronary vasodilatation.

Guanosine is a nucleoside comprising guanine attached to a ribose (ribofuranose) ring via a â-N_9-glycosidic bond.

Guanosine can be phosphorylated to become GMP (guanosine monophosphate), cGMP (cyclic guanosine monophosphate), GDP (guanosine diphosphate) and GTP (guanosine triphosphate).

When guanine is attached to a deoxyribose ring, it is known as a deoxyguanosine.

Uridine is a molecule (known as a nucleoside) that is formed when uracil is attached to a ribose ring (also known as a ribofuranose) via a â-N_1-glycosidic bond.

If uracil is attached to a deoxyribose ring, it is known as a deoxyuridine.

Harvard researchers report that supplementation in rats with a combination of uridine and EPA/DHA omega-3 fatty acids has antidepressant activity equivalent to that of commonly prescribed antidepressant medications, such as Prozac and other SSRIs.

Dietary Sources of Uridine

Uridine is found is a variety of foods. Sugarcane extract is rich in nucleosides, especially uridine. Tomatoes also (about 500-1000 mg. of uridine per kilogram of dry matter). Brewer's yeast is also a good source of uridine, as yeast is high in RNA (ribonucleic acid), which after digestion is broken down into ribosyl pyrimidines (uridine and cytidine), which are absorbed intact . About 3 percent of yeast (dry weight) results in digestion uridine products. This assumes the usual 9% RNA content found in Brewer's yeast. Alternatively, drinking beer also results in increased plasma uridine. The ingestion of one liter of beer results in increased plasma uridine at a level that is comparable to those reached after ingestion of CDP-choline (citicoline) (as in, the increase is measured as a percent change relative to baseline plasma uridine). Alternative uridine/cytidine sources include other high RNA foods such as organ meats (liver, pancreas, etc) or broccoli. High RNA foods may result in high blood purine levels, which may increase uric acid production in humans, which may aggravate conditions such as gout. Because of this, it has been suggested that the RNA content of yeast products should be chemically reduced if these products are to be consumed in high amounts as a source of protein (50 grams or more per day). However, such processing is expensive, and as of today (2008), it seems that commonly available Brewer's yeast products are not RNA-reduced. Consumption of moderate amounts of yeast (5 grams per day) should provide enough uridine for improved health, while minimizing possible side effects such as increased uric acid

Cytidine is a nucleoside molecule that is formed when cytosine is attached to a ribose ring (also known as a ribofuranose) via a â-N_1-glycosidic bond. Cytidine is a component of RNA.

If cytosine is attached to a deoxyribose ring, it is known as a deoxycytidine.

Cytidine has been shown to exhibit "antidepressant-like effects in rats.".

Dietary Sources of Cytidine

Dietary sources of cytidine include foods with high RNA (ribonucleic acid) content, such as organ meats, Brewer's yeast, as well as pyrimide-rich foods such as beer. During digestion, RNA-rich foods are broken-down into ribosyl pyrimidines (cytidine and uridine), which are absorbed intact.[2] In humans, dietary cytidine is converted into uridine, which is probably the compound behind cytidine's metabolic effects

Thymine is one of the four bases in the nucleic acid of DNA that make up the letters ATGC. The others are adenine, guanine, and cytosine. Thymine (T)

Cytidine
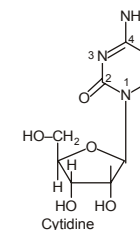
always pairs with adenine. Thymine is also known as **5-methyluracil,** a pyrimidine nucleobase. As the name suggests, thymine may be derived by methylation of uracil at the 5th carbon. In RNA, thymine is replaced with uracil in most cases. In DNA, thymine(T) binds to adenine (A) via two hydrogen bonds to assist in stabilizing the nucleic acid structures.

Thymine combined with deoxyribose creates the nucleoside deoxythymidine, which is synonymous with the term thymidine. Thymidine can be phosphorylated with one, two, or three phosphoric acid groups, creating, respectively, TMP, TDP, or TTP (thymidine mono-, di-, or triphosphate).

One of the common mutations of DNA involves two adjacent thymines or cytosine, which, in presence of ultraviolet light, may form thymine dimers, causing "kinks" in the DNA molecule that inhibit normal function.

Thymine could also be a target for actions of 5-fluorouracil (5-FU) in cancer treatment. 5-FU can be a metabolic analog of thymine (in DNA synthesis) or uracil (in RNA synthesis). Substitution of this analog inhibits DNA synthesis in actively-dividing cells.

Thymine bases are frequently oxidized to hydantoins over time after the death of an organism.

Nucleotides

Adding one or more phosphates to the sugar portion of a nucleoside results in a **nucleotide**. Generally, the phosphate is in ester linkage to carbon 5' of the sugar. If more than one phosphate is present, they are generally in acid anhydride linkages to each other. If such is the case, no position designation in the name is required. If the phosphate is in any other position, however, the position must be designated. For example, 3'-5' cAMP indicates that a phosphate is in ester linkage to both the 3' and 5' hydroxyl groups of an adenosine molecule and forms a cyclic structure. 2'-GMP would indicate that a phosphate is in ester linkage to the 2' hydroxyl group of a guanosine. Some representative names are:

- AMP = adenosine monophosphate = adenylic acid
- CDP = cytidine diphosphate
- dGTP = deoxy guanosine triphosphate
- dTTP = deoxy thymidine triphosphate (more commonly designated TTP)
- cAMP = 3'-5' cyclic adenosine monophosphate

Adenosine Monophosphate
AMP
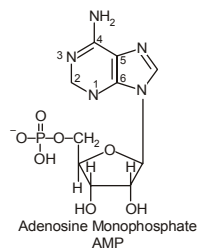
Polynucleotides

Nucleotides are joined together by 3'-5' phosphodiester bonds to form polynucleotides. Polymerization of ribonucleotides will produce an RNA while polymerization of deoxyribonucleotides leads to DNA.

Hydrolysis of Polynucleotides

Most, but not all, nucleic acids in the cell are associated with protein. Dietary nucleoprotein is degraded by pancreatic enzymes and tissue nucleoprotein by lysosomal enzymes. After dissociation of the protein and nucleic acid, the protein is metabolized like any other protein.

The nucleic acids are hydrolyzed randomly by **nucleases** to yield a mixture of polynucleotides. These are further cleaved by **phosphodiesterases** (exonucleases) to a mixture of the mononucleotides. The specificity of the pancreatic nucleotidases gives the 3'-nucleotides and that of the lysosomal nucleotidases gives the biologically important 5'-nucleotides.

The nucleotides are hydrolyzed by **nucleotidases** to give the nucleosides and P_i. This is probably the end product in the intestine with the nucleosides being the primary form absorbed. In at least some tissues, the nucleosides undergo phosphorolysis with **nucleoside phosphorylases** to yield the base and ribose 1-P (or deoxyribose 1-P). Since R 1-P and R 5-P are in equilibrium, the sugar phosphate can either be reincorporated into nucleotides or metabolized via the Hexose Monophosphate Pathway. The purine and pyrimidine bases released are either degraded or salvaged for reincorporation into nucleotides. There is significant turnover of all kinds of RNA as well as the nucleotide pool. DNA doesn't turnover but portions of the molecule are excised as part of a repair process.
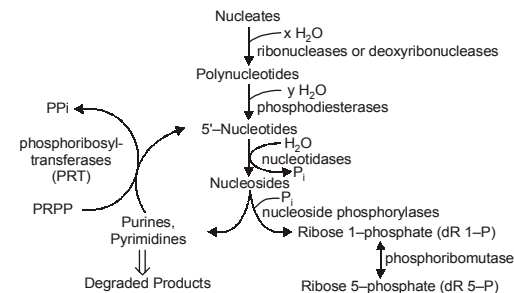
Purine and pyrimidines from tissue turnover which are not salvaged are catabolized and excreted. Little dietary purine is used and that which is absorbed is largely catabolized as well. Catabolism of purines and pyrimidines occurs in a less useful fashion than did the catabolism of amino acids in that we do not derive any significant amount of energy from the catabolism of purines and pyrimidines. Pyrimidine catabolism, however, does produce beta-alanine, and the endproduct of purine catabolism, which is uric acid in man, may serve as a scavenger of reactive oxygen species.

Purine Catabolism

The end product of purine catabolism in man is **uric acid**. Other mammals have the enzyme urate oxidase and excrete the more soluble allantoin as the end product. Man does not have this enzyme so urate is the end product for us. Uric acid is formed primarily in the liver and excreted by the kidney into the urine.

Urate   Allantoin
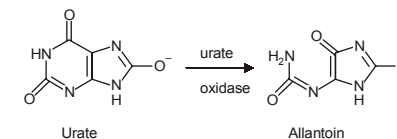
Nucleotides to Bases

Guanine nucleotides are hydrolyzed to the nucleoside **guanosine** which undergoes phosphorolysis to **guanine** and **ribose 1-P**. Man's intracellular nucleotidases are not very active toward AMP, however. Rather, **AMP is deaminated by the enzyme adenylate (AMP) deaminase to IMP**. In the catobilsm of purine nucleotides, IMP is further degraded by hydrolysis with nucleotidase to **inosine** and then phosphorolysis to **hypoxanthine**.
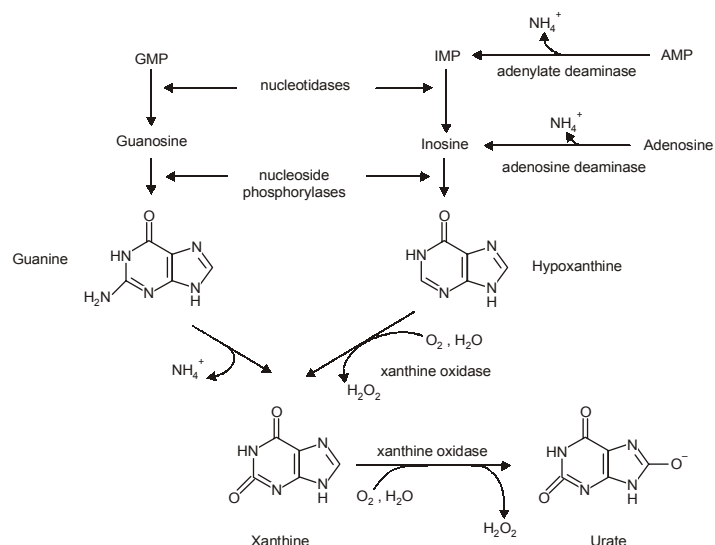
Adenosine does occur but usually arises from S-Adenosylmethionine during the course of transmethylation reactions. Adenosine is deaminated to inosine by an adenosine deaminase. Deficiencies in either **adenosine deaminase** or in the **purine nucleoside phosphorylase** lead to two different immunodeficiency diseases by mechanisms that are not clearly understood. With **adenosine deaminase deficiency**, both T and B-cell immunity is affected. The **phosphorylase deficiency** affects the T cells but B cells are normal. In September, 1990, a 4 year old girl was treated for adenosine deaminase deficiency by genetically engineering her cells to incorporate the gene. The treatment, so far, seems to be successful.

Whether or not **methylated** purines are catabolized depends upon the location of the methyl group. If the methyl is on an -NH$_2$, it is removed along with the -NH$_2$ and the core is metabolized in the usual fashion. If the methyl is on a ring nitrogen, the compound is excreted unchanged in the urine.

Bases to Uric Acid

Both adenine and guanine nucleotides converge at the common intermediate **xanthine**. Hypoxanthine, representing the original adenine, is oxidized to xanthine by the enzyme **xanthine oxidase**. Guanine is deaminated, with the amino group released as ammonia, to xanthine. If this process is occurring in tissues other than liver, most of the ammonia will be transported to the liver as glutamine for ultimate excretion as urea.

Xanthine, like hypoxanthine, is oxidized by oxygen and xanthine oxidase with the production of hydrogen peroxide. In man, the urate is excreted and the hydrogen peroxide is degraded by catalase. Xanthine oxidase is present in significant concentration only in liver and intestine. The pathway to the nucleosides, possibly to the free bases, is present in many tissues.

Gouts and Hyperuricemia

Both undissociated uric acid and the monosodium salt (primary form in blood) are only sparingly soluble. The limited solubility is not ordinarily a problem in urine unless the urine is very acid or has high [Ca^{2+}]. [Urate salts coprecipitate with calcium salts and can form stones in kidney or bladder.] A very high concentration of urate in the blood leads to a fairly common group of diseases referred to as gout. The incidence of gout in this country is about 3/1000.

Gout is a group of pathological conditions associated with markedly elevated levels of urate in the blood (3-7 mg/dl normal). **Hyperuricemia** is not always symptomatic, but, in certain individuals, something triggers the deposition of sodium urate crystals in joints and tissues. In addition to the extreme pain accompanying acute attacks, repeated attacks lead to destruction of tissues and severe arthritic-like malformations. The term gout should be restricted to hyperuricemia with the presence of these tophaceous deposits.

Urate in the blood could accumulate either through an overproduction and/or an underexcretion of uric acid. In gouts caused by an **overproduction** of uric acid, the defects are in the control mechanisms governing the production of - not uric acid itself - but of the nucleotide precursors. The **only major control of urate production that we know so far is the availability of substrates (nucleotides, nucleosides or free bases)**.

One approach to the treatment of gout is the drug **allopurinol**, an isomer of hypoxanthine.

Hypoxanthine   Allopurinol
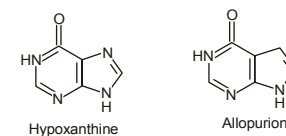
Allopurinol is a substrate for xanthine oxidase, but the product binds so tightly that the enzyme is now unable to oxidized its normal substrate. Uric acid production is diminished and xanthine and hypoxanthine levels in the blood rise. These are more soluble than urate and are less likely to deposit as crystals in the joints. Another approach is to stimulate the secretion of urate in the urine.

Summary

In summary, all, except ring-methylated, purines are deaminated (with the amino group contributing to the general ammonia pool) and the rings oxidized to uric acid for excretion. Since the purine ring is excreted intact, no energy benefit accrues to man from these carbons.

Pyrimidine Catabolism

In contrast to purines, pyrimidines undergo ring cleavage and the usual end products of catabolism are beta-amino acids plus ammonia and carbon dioxide. Pyrimidines from nucleic acids or the energy pool are acted upon by nucleotidases and pyrimidine nucleoside phosphorylase to yield the free bases. The 4-amino group of both cytosine and 5-methyl cytosine is released as ammonia.

Ring Cleavage

In order for the rings to be cleaved, they must first be **reduced by NADPH**. Atoms 2 and 3 of both rings are released as ammonia and carbon dioxide. The rest of the ring is left as a **beta-amino acid**. Beta-amino isobutyrate from thymine or 5-methyl cytosine is largely excreted. Beta-alanine from cytosine or uracil may either be excreted or incorporated into the brain and muscle dipeptides, **carnosine** (his-beta-ala) or **anserine** (methyl his-beta-ala).
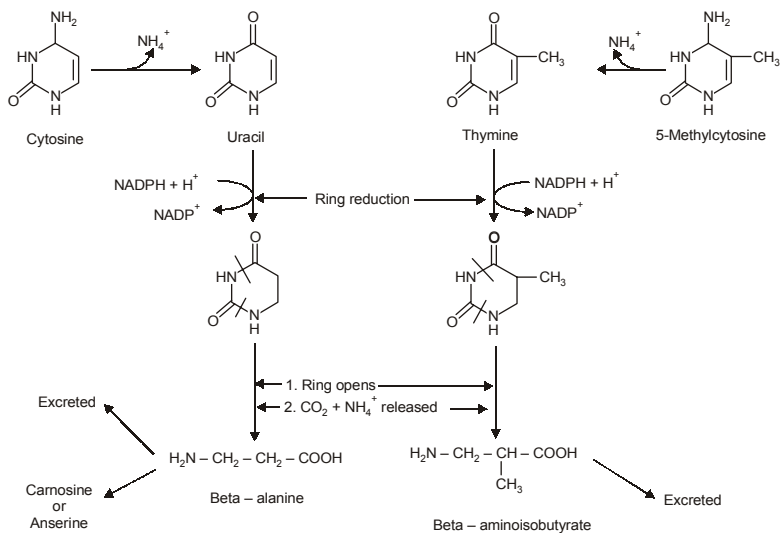
General Comments

Purine and pyrimidine bases which are not degraded are recycled - *i.e.* reincorporated into nucleotides. This recycling, however, is not sufficient to meet total body requirements and so some *de novo* synthesis is essential. There are definite tissue differences in the ability to carry out *de novo* synthesis. *De novo* synthesis of purines is most active in liver. Non-hepatic tissues generally have limited or even no *de novo* synthesis. Pyrimidine synthesis occurs in a variety of tissues. For purines, especially, non-hepatic tissues rely heavily on preformed bases - those salvaged from their own intracellular turnover supplemented by bases synthesized in the liver and delivered to tissues via the blood.

"Salvage" of purines is reasonable in most cells because xanthine oxidase, the key enzyme in taking the purines all of the way to uric acid, is significantly active only in liver and intestine. The bases generated by turnover in non-hepatic tissues are not readily degraded to uric acid in those tissues and, therefore, are available for salvage. The liver probably does less salvage but is very active in *de novo* synthesis - not so much for itself but to help supply the peripheral tissues.

De novo synthesis of both purine and pyrimidine nucleotides occurs from readily available components.

De Novo Synthesis of Purine Nucleotides
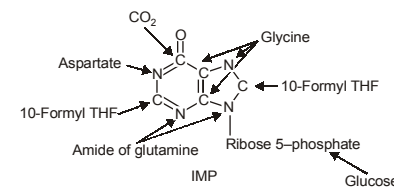
We use for purine nucleotides the entire glycine molecule (atoms 4, 5,7), the amino nitrogen of aspartate (atom 1), amide nitrogen of glutamine (atoms 3, 9), components of the folate-one-carbon pool (atoms 2, 8), carbon dioxide, ribose 5-P from glucose and a great deal of energy in the form of ATP. In de novo synthesis, IMP is the first nucleotide formed. It is then converted to either AMP or GMP.

PRPP

Since the purines are synthesized as the ribonucleotides, (not as the free bases) a necessary prerequisite is the synthesis of the activated form of ribose 5-phosphate. Ribose 5-phosphate reacts with ATP to form **5-Phosphoribosyl-1-pyrophosphate (PRPP)**.
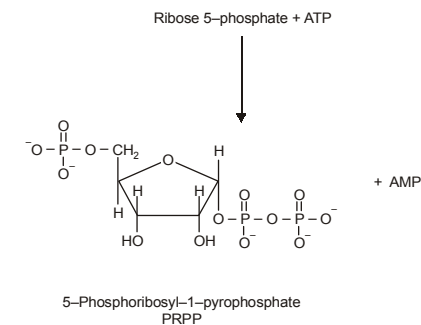
This reaction occurs in many tissues because PRPP has a number of roles - purine and pyrimidine nucleotide synthesis, salvage pathways, NAD and NADP formation. The enzyme is heavily controlled by a variety of compounds (di- and tri-phosphates, 2,3-DPG), presumably to try to match the synthesis of PRPP to a need for the products in which it ultimately appears.

Commitment Step

De novo purine nucleotide synthesis occurs actively in the cytosol of the liver where all of the necessary enzymes are present as a macro-molecular aggregate. The first step is a replacement of the pyrophosphate of PRPP by the amide group of glutamine. The product of this reaction is **5-Phosphoribosylamine**. The amine group that has been placed on carbon 1 of the sugar becomes nitrogen 9 of the ultimate purine ring. This is the commitment and rate-limiting step of the pathway.

Amide nitrogen of glutamine becomes N–9 of purine

5–Phosphoribosylamine
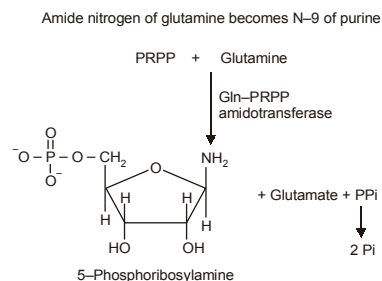
The enzyme is under tight allosteric control by feedback inhibition. Either **AMP, GMP, or IMP** alone will **inhibit the amidotransferase** while **AMP + GMP or AMP + IMP** together act **synergistically**. This is a fine control and probably the major factor in minute by minute regulation of the enzyme. The nucleotides inhibit the enzyme by causing the small active molecules to aggregate to larger inactive molecules.

[PRPP] also can play a role in regulating the rate. Normal intracellular concentrations of PRPP (which can and do fluctuate) are below the KM of the enzyme for PRPP so there is great potential for increasing the rate of the reaction by increasing the substrate concentration. The kinetics are sigmoidal. The enzyme is not particularly sensitive to changes in [Gln] (Kinetics are hyperbolic and [gln] approximates KM). Very **high [PRPP]** also **overcomes the normal nucleotide feedback inhibition** by causing the large, inactive aggregates to dissociate back to the small active molecules.

Glutamine–PRPP Amidotransferase
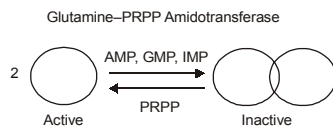
Purine *de novo* synthesis is a complex, energy-expensive pathway. It should be, and is, carefully controlled.

Formation of IMP

Once the commitment step has produced the 5-phosphoribosyl amine, the rest of the molecule is formed by a series of additions to make first the 5- and then the 6-membered ring. (Note: the numbers given to the atoms are those of the completed purine ring and names, etc. of the intermediate compounds are not given.) The whole glycine molecule, at the expense of ATP adds to the amino group to provide what will eventually be atoms 4, 5, and 7 of the purine ring (The amino group of 5-phosphoribosyl amine becomes nitrogen N of the purine ring.) One more atom is needed to complete the five-membered ring portion and that is supplied as 5, 10-Methenyl tetrahydrofolate.

Before ring closure occurs, however, the amide of glutamine adds to carbon 4 to start the six-membered ring portion (becomes nitrogen 3). This addition requires ATP. Another ATP is required to join carbon 8 and nitrogen 9 to form the five-membered ring.

The next step is the addition of carbon dioxide (as a carboxyl group) to form carbon 6 of the ring. The amine group of aspartate adds to the carboxyl group with a subsequent removal of fumarate. The amino group is now nitrogen 1 of the final ring. This process, which is typical for the use of the amino

Schematic Representation of Purine Nucleotide Synthesis

IMP
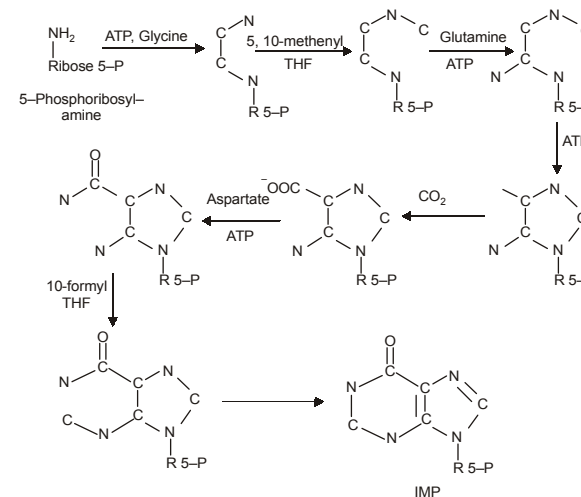
group of aspartate, requires ATP. The final atom of the purine ring, carbon 2, is supplied by 10-Formyl tetrahydrofolate. Ring closure produces the purine nucleotide, IMP.

Note that at least 4 ATPs are required in this part of the process. At no time do we have either a free base or a nucleotide.

Formation of AMP and GMP

IMP can then become **either** AMP or GMP. **GMP** formation requires that IMP be first oxidized to XMP using NAD. The oxygen at position 2 is substituted by the amide N of glutamine at the expense of ATP. Similarly, GTP provides the energy to convert IMP to **AMP**. The amino group is provided by aspartate in a mechanism similar to that used in forming nitrogen 1 of the ring. Removal of the carbons of aspartate as fumarate leaves the nitrigen behind as the 6-amino group of the adenine ring. The monophosphates are readily converted to the di- and tri-phosphates.

Conversion of IMP to either AMP or GMP
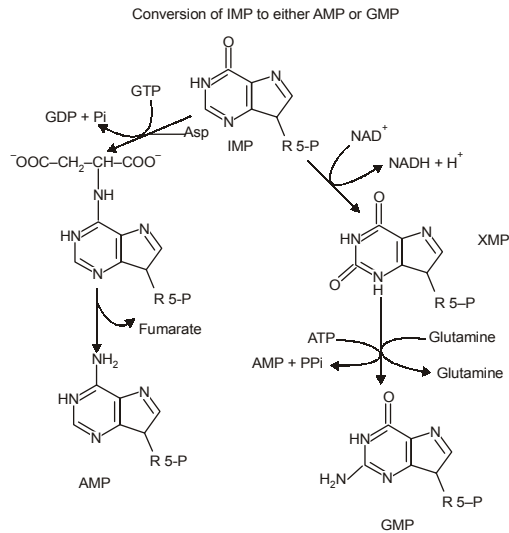
Control of De Novo *Synthesis*

Control of purine nucleotide synthesis has two phases. Control of the **synthesis as a whole** occurs at the amidotransferase step by nucleotide inhibition and/or [PRPP]. The second phase of control is involved with **maintaining an appropriate balance (not equality) between ATP and GTP**. Each one stimulates the synthesis of the other by providing the energy. Feedback inhibition also controls the

branched portion as GMP inhibits the conversion of IMP to XMP and AMP inhibits the conversion of IMP to adenylosuccinate.

○ Inhibitory   ○ Activating

GTP provides the energy for AMP synthesis
ATP provides the energy for GMP synthesis
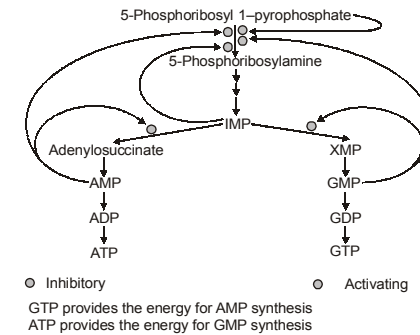
Possible Scenario

One could imagine the controls operating in such a way that if only one of the two nucleotides were required, there would be a partial inhibition of de novo synthesis because of high levels of the other and the IMP synthesized would be directed toward the synthesis of the required nucleotide. If both nucleotides were present in adequate amounts, their synergistic effect on the amidotransferase would result in almost complete inhibition of de novo synthesis.

De Novo Synthesis of Pyrimidine Nucleotides

Since pyrimidine molecules are simpler than purines, so is their synthesis simpler but is still from readily available components. Glutamine's amide nitrogen and carbon dioxide provide atoms 2 and 3 or the pyrimidine ring. They do so, however, after first being converted to carbamoyl phosphate. The other four atoms of the ring are supplied by aspartate. As is true with purine nucleotides, the sugar phosphate portion of the molecule is supplied by PRPP.

Generic pyrimidine base
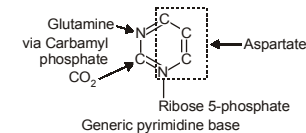
Carbamoyl Phosphate

Pyrimidine synthesis begins with **carbamoyl phosphate** synthesized in the cytosol of those tissues capable of making pyrimidines (highest in spleen, thymus, GItract and testes). This uses a different

enzyme than the one involved in urea synthesis. **Carbamoyl phosphate synthetase II (CPS II)** prefers glutamine to free ammonia and has no requirement for N-Acetylglutamate.

$$H_2N - CO - (CH_2)_2 - CH - COO^- + HCO_3^- + 2\,ATP$$

Glutamine

Carbamoyl phosphate synthetase II

+ Glutamate + 2 ADP + P$_i$

Carbamoyl phosphate
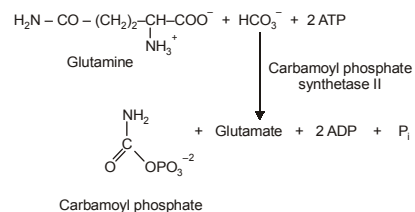
Formation of Orotic Acid

Carbamoyl phosphate condenses with aspartate in the presence of **aspartate transcarbamylase** to yield N-carbamylaspartate which is then converted to dihydroorotate.

In man, **CPSII, asp-transcarbamylase, and dihydroorotase activities** are part of a **multifunctional protein**.

Oxidation of the ring by a complex, poorly understood enzyme produces the free pyrimidine, orotic acid. This enzyme is located on the outer face of the inner mitochondrial membrane, in contrast to the other enzymes which are cytosolic. Note the contrast with purine synthesis in which a nucleotide is formed first while pyrimidines are first synthesized as the **free base**.

Carbamoyl phosphate + A3partate

Aspartate + transcarbamylase

P$_i$

N-Carbamoylaspartate

H$_2$O   Dihydroorotase +

Dihydroorotate

NADH + H$^+$ NAD$^+$

Orotic Acid

Dihydroorotate dehydrogenase
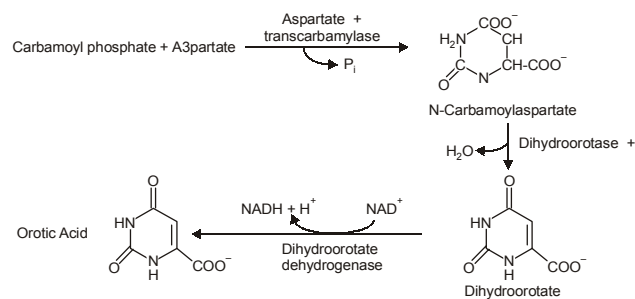
+ Part of the same multi–functional protein

Formation of the Nucleotides

Orotic acid is converted to its nucleotide with PRPP. **OMP** is then **converted sequentially** - not in a branched pathway - to the other pyrimidine nucleotides. Decarboxylation of OMP gives **UMP**. **O-PRT**

and OMP decarboxylase are also a **multifunctional protein**. After conversion of UMP to the triphosphate, the amide of glutamine is added, at the expense of ATP, to yield **CTP**.

Orotate + PRPP   O–PRT$^+$ OMP$^+$ decarboxylase

PP$_i$ CO$_2$

Ribose 5-P   Ribose 5-P

OMP   UMP

UMP ⟶ UDP ⟶ UTP

UTP   Glutamine   Glutamate   CTP

ATP   ADP + P$_i$

Ribose 5-P-P-P   Ribose 5-P-P-P

+ Part of the same multi–functional protein
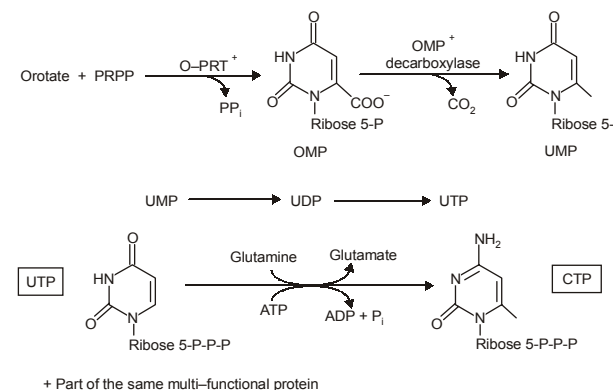
Control

The control of pyrimidine nucleotide synthesis in man is exerted primarily at the level of **cytoplasmic CPS II**. **UTP inhibits** the enzyme, competitively with ATP. **PRPP activates** it. Other secondary sites of control also exist (e.g. OMP decarboxylase is inhibited by UMP and CMP). These are probably not very important under normal circumstances.

In bacteria, aspartate transcarbamylase is the control enzyme. There is only one carbamoyl phosphate synthetase in bacteria since they do not have mitochondria. Carbamoyl phosphate, thus, participates in a branched pathway in these organisms that leads to either pyrimidine nucleotides or arginine.

Interconversion of Nucleotides

The monophosphates are the forms synthesized *de novo* although the triphosphates are the most commonly used forms. But, of course, the three forms are in equilibrium. There are several enzymes classified as **nucleoside monophosphate kinases** which catalyze the general reaction:(= represents a reversible reaction)

Base-monophosphate + ATP = Base-diphosphate + ADP

e.g. Adenylate kinase: AMP + ATP = 2 ADP

There is a different enzyme for GMP, one for pyrimidines and also enzymes that recognize the deoxy forms.

Similarly, the diphosphates are converted to the triphosphates by **nucleoside diphosphate kinase**:

BDP + ATP = BTP + ADP
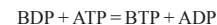
There may be only one nucleoside diphosphate kinase with broad specificity. One can legitimately speak of a pool of nucleotides in equilibrium with each other.

Salvage of Bases

Salvaging of purine and pyrimidine bases is an exceedingly important process for most tissues. There are two distinct pathways possible for salvaging the bases.

Salvaging Purines

The more important of the pathways for **salvaging purines** uses enzymes called **phosphoribosyltransferases (PRT)**:

PRTs catalyze the addition of ribose 5-phosphate to the base from PRPP to yield a nucleotide.:

Base + PRPP = Base-ribose-phosphate (BMP) + PPi

We gave already seen one example of this type of enzyme as a normal part of *de novo* synthesis of the pyrimidine nucleotides, - O-PRT.

As a salvage process though, we are dealing with purines. There are two enzymes, A-PRT and HG-PRT. **A-PRT** is not very important because we generate very little adenine. (Remember that the catabolism of adenine nucleotides and nucleosides is through inosine). **HG-PRT**, though, is exceptionally important and it is inhibited by both IMP and GMP. This enzyme salvages guanine directly and adenine indirectly. Remember that AMP is generated primarily from IMP, not from free adenine.

Lesch-Nyhan Syndrome

HG-PRT is deficient in the disease called **Lesch-Nyhan Syndrome**, a severe neurological disorder whose most blatant clinical manifestation is an uncontrollable self-mutilation. Lesch-Nyhan patients have very **high blood uric acid** levels because of an essentially **uncontrolled *de novo* synthesis**. (It can be as much as 20 times the normal rate). There is a significant increase in PRPP levels in various cells and an inability to maintain levels of IMP and GMP via salvage pathways. Both of these factors could lead to an increase in the activity of the amidotransferase.

Salvaging Pyrimidines

A second type of salvage pathway involves two steps and is the major pathway for the pyrimidines, uracil and thymine.

Base + Ribose 1-phosphate = Nucleoside + Pi (nucleoside phosphorylase)

Nucleoside + ATP - Nucleotide + ADP (nucleoside kinase - irreversible)

There is a uridine phosphorylase and kinase and a deoxythymidine phosphorylase and a thymidine kinase which can salvage some thymine in the presence of dR 1-P.

Formation of Deoxyribonucleotides

De novo synthesis and most of the salvage pathways involve the ribonucleotides. (Exception is the small amount of salvage of thymine indicated above.) Deoxyribonucleotides for DNA synthesis are formed from the ribonucleotide diphosphates (in mammals and *E. coli*).

A base diphosphate (BDP) is reduced at the 2' position of the ribose portion using the protein, **thioredoxin** and the enzyme **nucleoside diphosphate reductase**. Thioredoxin has two sulfhydryl groups which are oxidized to a disulfide bond during the process. In order to restore the thioredoxin to its reduced for so that it can be reused, **thioredoxin reductase and NADPH** are required.
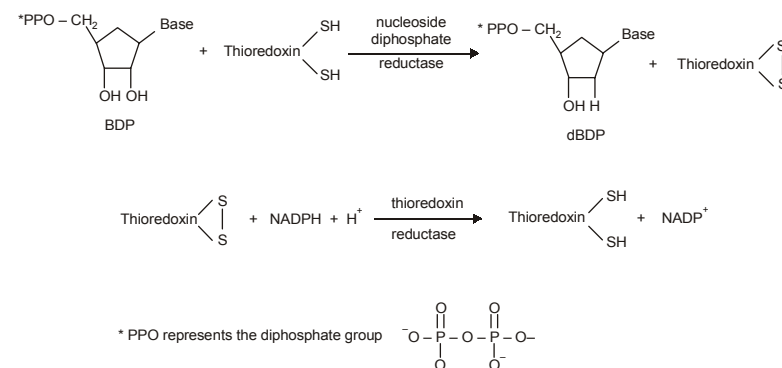
This system is very tightly controlled by a variety of allosteric effectors. dATP is a general inhibitor for all substrates and ATP an activator. Each substrate then has a specific positive effector (a BTP or dBTP). The result is a maintenance of an appropriate balance of the deoxynucleotides for DNA synthesis.

Synthesis of dTMP

DNA synthesis also requires dTMP (dTTP). This is not synthesized in the *de novo* pathway and salvage is not adequate to maintain the necessary amount. dTMP is generated from dUMP using the folate-dependent one-carbon pool.
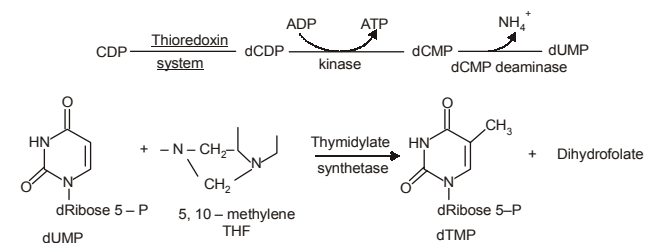
Dihydrofolate must be subsequently reduced to the tetrahydro form

Since the nucleoside diphosphate reductase is not very active toward UDP, CDP is reduced to dCDP which is converted to dCMP. This is then deaminated to form dUMP. In the presence of **5,10-Methylene tetrahydrofolate** and the enzyme **thymidylate synthetase**, the carbon group is both transferred to the pyrimidine ring and further reduced to a methyl group. The other product is **dihydrofolate** which is subsequently reduced to the tetrahydrofolate by dihydrofolate reductase.

Chemotherapeutic Agents

Thymidylate synthetase is particularly sensitive to availability of the folate one-carbon pool. Some of the cancer chemotherapeutic agents interfere with this process as well as with the steps in purine nucleotide synthesis involving the pool.

Cancer chemotherapeutic agents like **methotrexate** (4-amino, 10-methyl folic acid) and **aminopterin** (4-amino, folic acid) are structural analogs of folic acid and inhibit dihydrofolate reductase. This interferes with maintenance of the folate pool and thus of *de novo* synthesis of purine nucleotides and of dTMP synthesis. Such agents are highly toxic and administered under careful control.

SECTION 2.16—STRUCTURES AND PROPERTIES OF DNA IN DIFFERENT FORMS

Deoxyribonucleic acid (**DNA**) is a nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms and some viruses. The main role of DNA molecules is the long-term storage of information. DNA is often compared to a set of blueprints or a recipe, or a code, since it contains the instructions needed to construct other components of cells, such as proteins and RNA molecules. The DNA segments that carry this genetic information are called genes, but other DNA sequences have structural purposes, or are involved in regulating the use of this genetic information.

Chemically, DNA consists of two long polymers of simple units called nucleotides, with backbones made of sugars and phosphate groups joined by ester bonds. These two strands run in opposite directions to each other and are therefore anti-parallel. Attached to each sugar is one of four types of molecules called bases. It is the sequence of these four bases along the backbone that encodes information. This information is read using the genetic code, which specifies the sequence of the amino acids within proteins. The code is read by copying stretches of DNA into the related nucleic acid RNA, in a process called transcription.
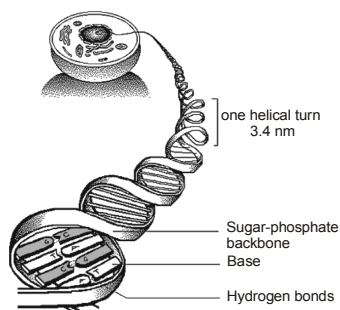
Fig. 2.79A. Showing the Digramatic representation of the structure of DNA

Within cells, DNA is organized into structures called chromosomes. These chromosomes are duplicated before cells divide, in a process called DNA replication. Eukaryotic organisms (animals, plants, fungi, and protists) store their DNA inside the cell nucleus, while in prokaryotes (bacteria and archae) it is found in the cell's cytoplasm. Within the chromosomes, chromatin proteins such as histones compact and organize DNA. These compact structures guide the interactions between DNA and other proteins, helping control which parts of the DNA are transcribed.

Properties
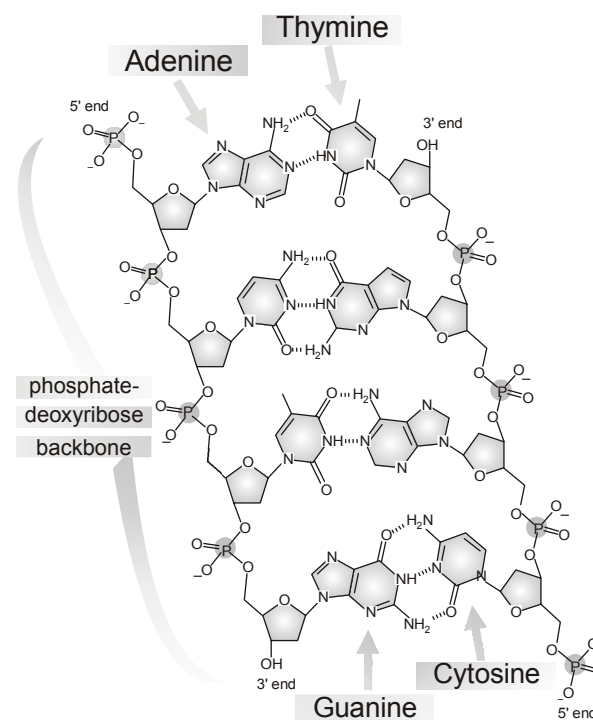
Fig. 2.80 : Showing The chemical structure of DNA. Hydrogen bonds are shown as dotted lines

DNA is a long polymer made from repeating units called nucleotides. The DNA chain is 22 to 26 Ångströms wide (2.2 to 2.6 nanometres), and one nucleotide unit is 3.3 Å (0.33 nm) long. Although each individual repeating unit is very small, DNA polymers can be very large molecules containing millions of nucleotides. For instance, the largest human chromosome, chromosome number 1, is approximately 220 million base pairs long.

In living organisms, DNA does not usually exist as a single molecule, but instead as a tightly-associated pair of molecules. These two long strands entwine like vines, in the shape of a double helix. The nucleotide repeats contain both the segment of the backbone of the molecule, which holds the chain together, and a base, which interacts with the other DNA strand in the helix. In general, a base linked to a sugar is called a nucleoside and a base linked to a sugar and one or more phosphate groups is called a nucleotide. If multiple nucleotides are linked together, as in DNA, this polymer is called a polynucleotide.

The backbone of the DNA strand is made from alternating phosphate and sugar residues.[8] The sugar in DNA is 2-deoxyribose, which is a pentose (five-carbon) sugar. The sugars are joined together by phosphate groups that form phosphodiester bonds between the third and fifth carbon atoms of adjacent sugar rings. These asymmetric bonds mean a strand of DNA has a direction. In a double helix the direction of the nucleotides in one strand is opposite to their direction in the other strand. This arrangement of DNA strands is called antiparallel. The asymmetric ends of DNA strands are referred to as the 52 (*five prime*) and 32 (*three prime*) ends, with the 5' end being that with a terminal phosphate group and the 3' end that with a terminal hydroxyl group. One of the major differences between DNA and RNA is the sugar, with 2-deoxyribose being replaced by the alternative pentose sugar ribose in RNA.

The DNA double helix is stabilized by hydrogen bonds between the bases attached to the two strands. The four bases found in DNA are adenine (abbreviated A), cytosine (C), guanine (G) and thymine (T). These four bases are attached to the sugar/phosphate to form the complete nucleotide, as shown for adenosine monophosphate.

These bases are classified into two types; adenine and guanine are fused five- and six-membered heterocyclic compounds called purines, while cytosine and thymine are six-membered rings called pyrimidines. A fifth pyrimidine base, called uracil (U), usually takes the place of thymine in RNA and differs from thymine by lacking a methyl group on its ring. Uracil is not usually found in DNA, occurring only as a breakdown product of cytosine.

Grooves

Normally, the double helix is a right-handed spiral. As the DNA strands wind around each other, they leave gaps between each set of phosphate backbones, revealing the sides of the bases inside. There are two of these grooves twisting around the surface of the double helix: one groove, the major groove, is 22 Å wide and the other, the minor groove, is 12 Å wide. The narrowness of the minor groove means that the edges of the bases are more accessible in the major groove. As a result, proteins like transcription factors that can bind to specific sequences in double-stranded DNA usually make contacts to the sides of the bases exposed in the major groove.[11] This situation varies in unusual conformations of DNA

within the cell *(see below)*, but the major and minor grooves are always named to reflect the differences in size that would be seen if the DNA is twisted back into the ordinary B form.
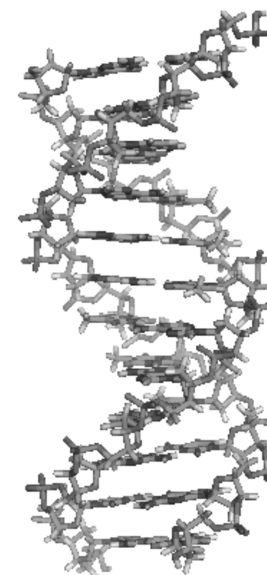
Fig. 81. Animation of the structure of a section of DNA. The bases lie horizontally between the two spiraling strands

Base pairing

Each type of base on one strand forms a bond with just one type of base on the other strand. This is called complementary base pairing. Here, purines form hydrogen bonds to pyrimidines, with A bonding only to T, and C bonding only to G. This arrangement of two nucleotides binding together across the double helix is called a base pair. As hydrogen bonds are not covalent, they can be broken and rejoined relatively easily. The two strands of DNA in a double helix can therefore be pulled apart like a zipper, either by a mechanical force or high temperature. As a result of this complementarity, all the information in the double-stranded sequence of a DNA helix is duplicated on each strand, which is vital in DNA replication. Indeed, this reversible and specific interaction between complementary base pairs is critical for all the functions of DNA in living organisms.
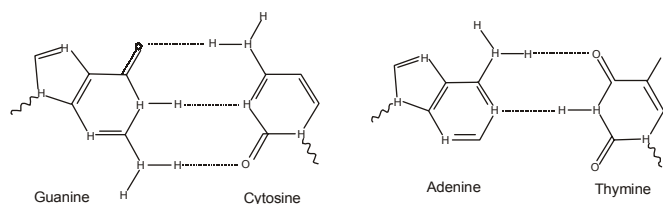
Fig. 2.82 : Top, a GC base pair with three hydrogen bonds. Bottom, an AT base pair with two hydrogen bonds. Non-covalent hydrogen bonds between the pairs are shown as dashed lines

The two types of base pairs form different numbers of hydrogen bonds, AT forming two hydrogen bonds, and GC forming three hydrogen bonds. DNA with high GC-content is more stable than DNA with low GC-content, but contrary to popular belief, this is not due to the extra hydrogen bond of a GC basepair but rather the contribution of stacking interactions (hydrogen bonding merely provides specificity of the pairing, not stability). As a result, it is both the percentage of GC base pairs and the overall length of a DNA double helix that determine the strength of the association between the two strands of DNA. Long DNA helices with a high GC content have stronger-interacting strands, while short helices with high AT content have weaker-interacting strands. In biology, parts of the DNA double helix that need to separate easily, such as the TATAAT Pribnow box in some promoters, tend to have a high AT content, making the strands easier to pull apart. In the laboratory, the strength of this interaction can be measured by finding the temperature required to break the hydrogen bonds, their melting temperature (also called T_m value). When all the base pairs in a DNA double helix melt, the strands separate and exist in solution as two entirely independent molecules. These single-stranded DNA molecules have no single common shape, but some conformations are more stable than others.

Sense and Antisense

A DNA sequence is called "sense" if its sequence is the same as that of a messenger RNA copy that is translated into protein. The sequence on the opposite strand is called the "antisense" sequence. Both sense and antisense sequences can exist on different parts of the same strand of DNA (i.e. both strands contain both sense and antisense sequences). In both prokaryotes and eukaryotes, antisense RNA sequences are produced, but the functions of these RNAs are not entirely clear. One proposal is that antisense RNAs are involved in regulating gene expression through RNA-RNA base pairing.

A few DNA sequences in prokaryotes and eukaryotes, and more in plasmids and viruses, blur the distinction between sense and antisense strands by having overlapping genes.[20] In these cases, some DNA sequences do double duty, encoding one protein when read along one strand, and a second protein when read in the opposite direction along the other strand. In bacteria, this overlap may be involved in the regulation of gene transcription, while in viruses, overlapping genes increase the amount of information that can be encoded within the small viral genome.

Supercoiling

DNA can be twisted like a rope in a process called DNA supercoiling. With DNA in its "relaxed" state, a strand usually circles the axis of the double helix once every 10.4 base pairs, but if the DNA is twisted the strands become more tightly or more loosely wound. If the DNA is twisted in the direction of the helix, this is positive supercoiling, and the bases are held more tightly together. If they are twisted in the opposite direction, this is negative supercoiling, and the bases come apart more easily. In nature, most DNA has slight negative supercoiling that is introduced by enzymes called topoisomerases.[24] These enzymes are also needed to relieve the twisting stresses introduced into DNA strands during processes such as transcription and DNA replication.
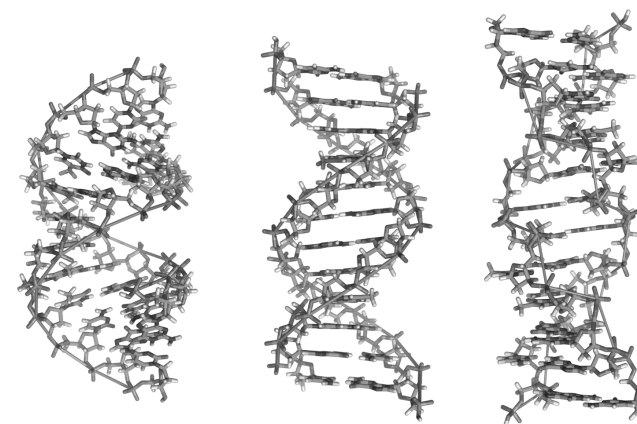
Fig. 2.83 : From left to right, the structures of A, B and Z DNA

Alternative Structures

DNA exists in many possible conformations. However, only A-DNA, B-DNA, and Z-DNA have been observed in organisms. Which conformation DNA adopts depends on the sequence of the DNA, the amount and direction of supercoiling, chemical modifications of the bases and also solution conditions, such as the concentration of metal ions and polyamines. Of these three conformations, the "B" form described above is most common under the conditions found in cells. The two alternative double-helical forms of DNA differ in their geometry and dimensions.

The A form is a wider right-handed spiral, with a shallow, wide minor groove and a narrower, deeper major groove. The A form occurs under non-physiological conditions in dehydrated samples of DNA, while in the cell it may be produced in hybrid pairings of DNA and RNA strands, as well as in

enzyme-DNA complexes. Segments of DNA where the bases have been chemically-modified by methylation may undergo a larger change in conformation and adopt the Z form. Here, the strands turn about the helical axis in a left-handed spiral, the opposite of the more common B form. These unusual structures can be recognized by specific Z-DNA binding proteins and may be involved in the regulation of transcription.[31]
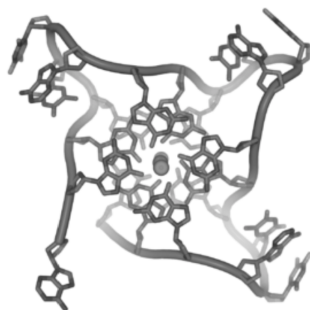
Fig. 2.84 : Structure of a DNA quadruplex formed by telomere repeats. The conformation of the DNA backbone diverges significantly from the typical helical structure

Quadruplex Structures

Further Information: G-quadruplex

At the ends of the linear chromosomes are specialized regions of DNA called telomeres. The main function of these regions is to allow the cell to replicate chromosome ends using the enzyme telomerase, as the enzymes that normally replicate DNA cannot copy the extreme 32 ends of chromosomes.[33] These specialized chromosome caps also help protect the DNA ends, and stop the DNA repair systems in the cell from treating them as damage to be corrected. In human cells, telomeres are usually lengths of single-stranded DNA containing several thousand repeats of a simple TTAGGG sequence.[35]

These guanine-rich sequences may stabilize chromosome ends by forming structures of stacked sets of four-base units, rather than the usual base pairs found in other DNA molecules. Here, four guanine bases form a flat plate and these flat four-base units then stack on top of each other, to form a stable *G-quadruplex* structure. These structures are stabilized by hydrogen bonding between the edges of the bases and chelation of a metal ion in the centre of each four-base unit.[37] Other structures can also be formed, with the central set of four bases coming from either a single strand folded around the bases, or several different parallel strands, each contributing one base to the central structure.

In addition to these stacked structures, telomeres also form large loop structures called telomere loops, or T-loops. Here, the single-stranded DNA curls around in a long circle stabilized by telomere-

binding proteins. At the very end of the T-loop, the single-stranded telomere DNA is held onto a region of double-stranded DNA by the telomere strand disrupting the double-helical DNA and base pairing to one of the two strands. This triple-stranded structure is called a displacement loop or D-loop.

Branched DNA

In DNA fraying occurs when non-complimentary regions exist at the end of an otherwise complimentary double-strand of DNA. However, branched DNA can occur if a third strand of DNA is introduced and contains adjoining regions able to hybridize with the frayed regions of the pre-existing double-strand. Although the simplest example of branched DNA involves only three strands of DNA, complexes involving additional strands and multiple branches are also possible.

Fig. 2.85 and 85A: A DNA structure with a single branching point

A DNA structure with multiple branches

Chemical Modifications

Structure of cytosine with and without the 5-methyl group. After deamination the 5-methylcytosine has the same structure as thymine.
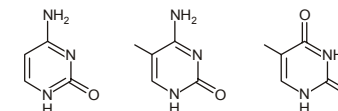
Base modifications

The expression of genes is influenced by how the DNA is packaged in chromosomes, in a structure called chromatin. Base modifications can be involved in packaging, with regions that have low or no gene expression usually containing high levels of methylation of cytosine bases. For example, cytosine methylation, produces 5-methylcytosine, which is important for X-chromosome inactivation.[40] The average level of methylation varies between organisms - the worm *Caenorhabditis elegans* lacks cytosine methylation, while vertebrates have higher levels, with up to 1% of their DNA containing 5-methylcytosine. Despite the importance of 5-methylcytosine, it can deaminate to leave a thymine base, methylated cytosines are therefore particularly prone to mutations. Other base modifications include adenine methylation in bacteria and the glycosylation of uracil to produce the "J-base" in kinetoplastids.

Damage

DNA can be damaged by many different sorts of mutagens, which change the DNA sequence. Mutagens include oxidizing agents, alkylating agents and also high-energy electromagnetic radiation such as ultraviolet light and X-rays. The type of DNA damage produced depends on the type of mutagen. For example, UV light can damage DNA by producing thymine dimers, which are cross-links between pyrimidine bases.[46] On the other hand, oxidants such as free radicals or hydrogen peroxide produce multiple forms of damage, including base modifications, particularly of guanosine, and double-strand breaks.[47] In each human cell, about 500 bases suffer oxidative damage per day.[48][49] Of these oxidative lesions, the most dangerous are double-strand breaks, as these are difficult to repair and can produce point mutations, insertions and deletions from the DNA sequence, as well as chromosomal translocations.[50]
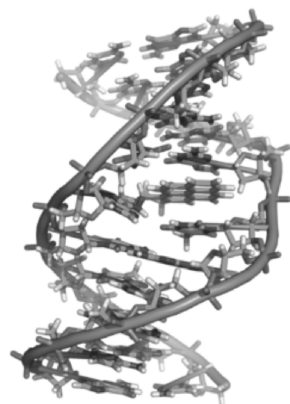
Fig. 2.86 : Benzopyrene, the major mutagen in tobacco smoke, in an adduct to DNA

Many mutagens fit into the space between two adjacent base pairs, this is called *intercalating*. Most intercalators are aromatic and planar molecules, and include Ethidium bromide, daunomycin, and doxorubicin. In order for an intercalator to fit between base pairs, the bases must separate, distorting the DNA strands by unwinding of the double helix. This inhibits both transcription and DNA replication, causing toxicity and mutations. As a result, DNA intercalators are often carcinogens, and benzopyrene diol epoxide, acridines, aflatoxin and ethidium bromide are well-known examples. Nevertheless, due to their ability to inhibit DNA transcription and replication, these toxins are also used in chemotherapy to inhibit rapidly-growing cancer cells.

Biological Functions

DNA usually occurs as linear chromosomes in eukaryotes, and circular chromosomes in prokaryotes. The set of chromosomes in a cell makes up its genome; the human genome has approximately 3 billion base pairs of DNA arranged into 46 chromosomes. The information carried by DNA is held in the sequence of pieces of DNA called genes. Transmission of genetic information in genes is achieved via complementary base pairing. For example, in transcription, when a cell uses the information in a gene, the DNA sequence is copied into a complementary RNA sequence through the attraction between DNA and the correct RNA nucleotides. Usually, this RNA copy is then used to make a matching protein sequence in a process called translation which depends on the same interaction between RNA nucleotides. Alternatively, a cell may simply copy its genetic information in a process called DNA replication. The details of these functions are covered in other articles; here we focus on the interactions between DNA and other molecules that mediate the function of the genome.

Genes and Genomes

Genomic DNA is located in the cell nucleus of eukaryotes, as well as small amounts in mitochondria and chloroplasts. In prokaryotes, the DNA is held within an irregularly shaped body in the cytoplasm called the nucleoid. The genetic information in a genome is held within genes, and the complete set of this information in an organism is called its genotype. A gene is a unit of heredity and is a region of DNA that influences a particular characteristic in an organism. Genes contain an open reading frame that can be transcribed, as well as regulatory sequences such as promoters and enhancers, which control the transcription of the open reading frame.
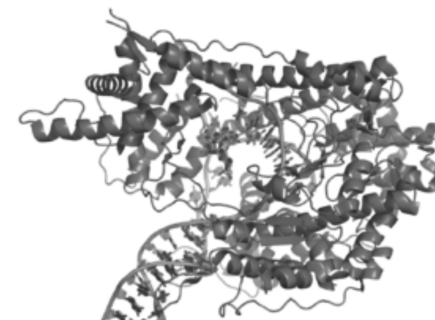
Fig. 2.87: T7 RNA polymerase (blue) producing a mRNA (green) from a DNA template (orange)

In many species, only a small fraction of the total sequence of the genome encodes protein. For example, only about 1.5% of the human genome consists of protein-coding exons, with over 50% of human DNA consisting of non-coding repetitive sequences. The reasons for the presence of so much non-coding DNA in eukaryotic genomes and the extraordinary differences in genome size, or *C-value*, among species represent a long-standing puzzle known as the "C-value enigma." However, DNA sequences that do not code protein may still encode functional non-coding RNA molecules, which are involved in the regulation of gene expression.

Some non-coding DNA sequences play structural roles in chromosomes. Telomeres and centromeres typically contain few genes, but are important for the function and stability of chromosomes. An abundant form of non-coding DNA in humans are pseudogenes, which are copies of genes that have been disabled by mutation. These sequences are usually just molecular fossils, although they can occasionally serve as raw genetic material for the creation of new genes through the process of gene duplication and divergence.

Transcription and Translation

A gene is a sequence of DNA that contains genetic information and can influence the phenotype of an organism. Within a gene, the sequence of bases along a DNA strand defines a messenger RNA sequence, which then defines one or more protein sequences. The relationship between the nucleotide sequences of genes and the amino-acid sequences of proteins is determined by the rules of translation, known collectively as the genetic code. The genetic code consists of three-letter 'words' called *codons* formed from a sequence of three nucleotides (e.g. ACT, CAG, TTT).

In transcription, the codons of a gene are copied into messenger RNA by RNA polymerase. This RNA copy is then decoded by a ribosome that reads the RNA sequence by base-pairing the messenger

RNA to transfer RNA, which carries amino acids. Since there are 4 bases in 3-letter combinations, there are 64 possible codons (4^3 combinations). These encode the twenty standard amino acids, giving most amino acids more than one possible codon. There are also three 'stop' or 'nonsense' codons signifying the end of the coding region; these are the TAA, TGA and TAG codons.
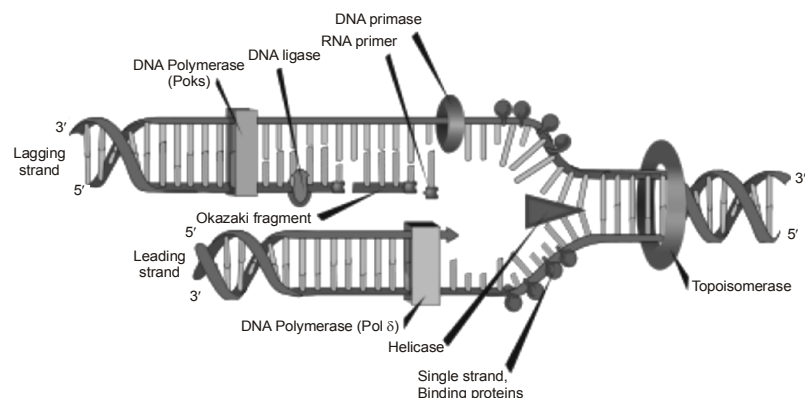
Fig. 2.88: DNA replication. The double helix is unwound by a helicase and topoisomerase. Next, one DNA polymerase produces the leading strand copy. Another DNA polymerase binds to the lagging strand. This enzyme makes discontinuous segments (called Okazaki fragments) before DNA ligase joins them together

Replication

Cell division is essential for an organism to grow, but when a cell divides it must replicate the DNA in its genome so that the two daughter cells have the same genetic information as their parent. The double-stranded structure of DNA provides a simple mechanism for DNA replication. Here, the two strands are separated and then each strand's complementary DNA sequence is recreated by an enzyme called DNA polymerase. This enzyme makes the complementary strand by finding the correct base through complementary base pairing, and bonding it onto the original strand. As DNA polymerases can only extend a DNA strand in a 52 to 32 direction, different mechanisms are used to copy the antiparallel strands of the double helix.[64] In this way, the base on the old strand dictates which base appears on the new strand, and the cell ends up with a perfect copy of its DNA.

Interactions with Proteins

All the functions of DNA depend on interactions with proteins. These protein interactions can be non-specific, or the protein can bind specifically to a single DNA sequence. Enzymes can also bind to DNA

and of these, the polymerases that copy the DNA base sequence in transcription and DNA replication are particularly important.

DNA-binding Proteins
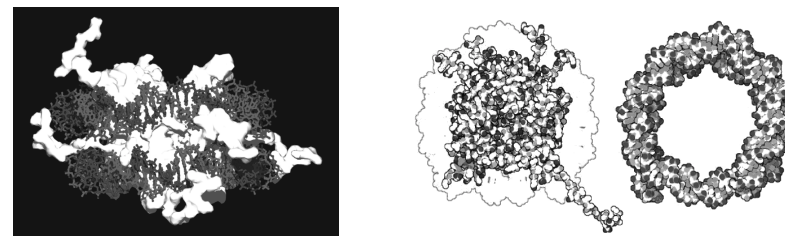
Fig. 2.89: AInteraction of DNA with histones (shown in white, top). These proteins' basic amino acids (below left, blue) bind to the acidic phosphate groups on DNA (below right, red).

Structural proteins that bind DNA are well-understood examples of non-specific DNA-protein interactions. Within chromosomes, DNA is held in complexes with structural proteins. These proteins organize the DNA into a compact structure called chromatin. In eukaryotes this structure involves DNA binding to a complex of small basic proteins called histones, while in prokaryotes multiple types of proteins are involved. The histones form a disk-shaped complex called a nucleosome, which contains two complete turns of double-stranded DNA wrapped around its surface. These non-specific interactions are formed through basic residues in the histones making ionic bonds to the acidic sugar-phosphate backbone of the DNA, and are therefore largely independent of the base sequence. Chemical modifications of these basic amino acid residues include methylation, phosphorylation and acetylation. These chemical changes alter the strength of the interaction between the DNA and the histones, making the DNA more or less accessible to transcription factors and changing the rate of transcription. Other non-specific DNA-binding proteins in chromatin include the high-mobility group proteins, which bind to bent or distorted DNA. These proteins are important in bending arrays of nucleosomes and arranging them into the larger structures that make up chromosomes.
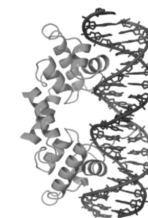
Fig. 2.90: The lambda repressor helix-turn-helix transcription factor bound to its DNA target

A distinct group of DNA-binding proteins are the DNA-binding proteins that specifically bind single-stranded DNA. In humans, replication protein A is the best-understood member of this family and is used in processes where the double helix is separated, including DNA replication, recombination and DNA repair. These binding proteins seem to stabilize single-stranded DNA and protect it from forming stem-loops or being degraded by nucleases.

In contrast, other proteins have evolved to bind to particular DNA sequences. The most intensively-studied of these are the various transcription factors, which are proteins that regulate transcription. Each transcription factor binds to one particular set of DNA sequences and activates or inhibits the transcription of genes that have these sequences close to their promoters. The transcription factors do this in two ways. Firstly, they can bind the RNA polymerase responsible for transcription, either directly or through other mediator proteins; this locates the polymerase at the promoter and allows it to begin transcription. Alternatively, transcription factors can bind enzymes that modify the histones at the promoter; this will change the accessibility of the DNA template to the polymerase.

As these DNA targets can occur throughout an organism's genome, changes in the activity of one type of transcription factor can affect thousands of genes. Consequently, these proteins are often the targets of the signal transduction processes that control responses to environmental changes or cellular differentiation and development. The specificity of these transcription factors' interactions with DNA come from the proteins making multiple contacts to the edges of the DNA bases, allowing them to "read" the DNA sequence. Most of these base-interactions are made in the major groove, where the bases are most accessible.

DNA-modifying Enzymes

Nucleases and Ligases

Nucleases are enzymes that cut DNA strands by catalyzing the hydrolysis of the phosphodiester bonds. Nucleases that hydrolyse nucleotides from the ends of DNA strands are called exonucleases, while endonucleases cut within strands. The most frequently-used nucleases in molecular biology are the restriction endonucleases, which cut DNA at specific sequences. For instance, the EcoRV enzyme shown to the left recognizes the 6-base sequence 5?-GAT|ATC-3? and makes a cut at the vertical line. In nature, these enzymes protect bacteria against phage infection by digesting

Fig. 2.91 : The restriction enzyme EcoRV (green) in a complex with its substrate DNA

the phage DNA when it enters the bacterial cell, acting as part of the restriction modification system. In technology, these sequence-specific nucleases are used in molecular cloning and DNA fingerprinting.

Enzymes called DNA ligases can rejoin cut or broken DNA strands. Ligases are particularly important in lagging strand DNA replication, as they join together the short segments of DNA produced at the replication fork into a complete copy of the DNA template. They are also used in DNA repair and genetic recombination.

Topoisomerases and helicases

Topoisomerases are enzymes with both nuclease and ligase activity. These proteins change the amount of supercoiling in DNA. Some of these enzyme work by cutting the DNA helix and allowing one section to rotate, thereby reducing its level of supercoiling; the enzyme then seals the DNA break. Other types of these enzymes are capable of cutting one DNA helix and then passing a second strand of DNA through this break, before rejoining the helix. Topoisomerases are required for many processes involving DNA, such as DNA replication and transcription.

Helicases are proteins that are a type of molecular motor. They use the chemical energy in nucleoside triphosphates, predominantly ATP, to break hydrogen bonds between bases and unwind the DNA double helix into single strands.These enzymes are essential for most processes where enzymes need to access the DNA bases.

Polymerases

Polymerases are enzymes that synthesize polynucleotide chains from nucleoside triphosphates. The sequence of their products are copies of existing polynucleotide chains - which are called templates. These enzymes function by adding nucleotides onto the 3′ hydroxyl group of the previous nucleotide in a DNA strand. Consequently, all polymerases work in a 5′ to 3′ direction. In the active site of these enzymes, the incoming nucleoside triphosphate base-pairs to the template: this allows polymerases to accurately synthesize the complementary strand of their template. Polymerases are classified according to the type of template that they use.

In DNA replication, a DNA-dependent DNA polymerase makes a copy of a DNA sequence. Accuracy is vital in this process, so many of these polymerases have a proofreading activity. Here, the polymerase recognizes the occasional mistakes in the synthesis reaction by the lack of base pairing between the mismatched nucleotides. If a mismatch is detected, a 3′ to 5′ exonuclease activity is activated and the incorrect base removed. In most organisms DNA polymerases function in a large complex called the replisome that contains multiple accessory subunits, such as the DNA clamp or helicases.

RNA-dependent DNA polymerases are a specialized class of polymerases that copy the sequence of an RNA strand into DNA. They include reverse transcriptase, which is a viral enzyme involved in the infection of cells by retroviruses, and telomerase, which is required for the replication of telomeres. Telomerase is an unusual polymerase because it contains its own RNA template as part of its structure.

Transcription is carried out by a DNA-dependent RNA polymerase that copies the sequence of a DNA strand into RNA. To begin transcribing a gene, the RNA polymerase binds to a sequence of DNA called a promoter and separates the DNA strands. It then copies the gene sequence into a messenger RNA transcript until it reaches a region of DNA called the terminator, where it halts and detaches from the DNA. As with human DNA-dependent DNA polymerases, RNA polymerase II, the enzyme that transcribes most of the genes in the human genome, operates as part of a large protein complex with multiple regulatory and accessory subunits.

Genetic Recombination

A DNA helix usually does not interact with other segments of DNA, and in human cells the different chromosomes even occupy separate areas in the nucleus called "chromosome territories".This physical separation of different chromosomes is important for the ability of DNA to function as a stable repository for information, as one of the few times chromosomes interact is during chromosomal crossover when they recombine. Chromosomal crossover is when two DNA helices break, swap a section and then rejoin.

Recombination allows chromosomes to exchange genetic information and produces new combinations of genes, which increases the efficiency of natural selection and can be important in the rapid evolution of new proteins. Genetic recombination can also be involved in DNA repair, particularly in the cell's response to double-strand breaks.

Fig. 2.92: A Structure of the Holiday junction intermediate in genetic recombination. The four separate DNA strands are coloured red, blue, green and yellow

Fig. 2.93 : Recombination involves the breakage and rejoining of two chromosomes (M and F) to produce two re-arranged chromosomes (C1 and C2).

The most common form of chromosomal crossover is homologous recombination, where the two chromosomes involved share very similar sequences. Non-homologous recombination can be damaging to cells, as it can produce chromosomal translocations and genetic abnormalities. The recombination reaction is catalyzed by enzymes known as recombinases, such as RAD51. The first step in recombination is a double-stranded break either caused by an endonuclease or damage to the DNA. A series of steps catalyzed in part by the recombinase then leads to joining of the two helices by at least one Holliday junction, in which a segment of a single strand in each helix is annealed to the complementary strand in the other helix. The Holliday junction is a tetrahedral junction structure that can be moved along the pair

of chromosomes, swapping one strand for another. The recombination reaction is then halted by cleavage of the junction and re-ligation of the released DNA.

Evolution

DNA contains the genetic information that allows all modern living things to function, grow and reproduce. However, it is unclear how long in the 4-billion-year history of life DNA has performed this function, as it has been proposed that the earliest forms of life may have used RNA as their genetic material. RNA may have acted as the central part of early cell metabolism as it can both transmit genetic information and carry out catalysis as part of ribozymes. This ancient RNA world where nucleic acid would have been used for both catalysis and genetics may have influenced the evolution of the current genetic code based on four nucleotide bases. This would occur since the number of unique bases in such an organism is a trade-off between a small number of bases increasing replication accuracy and a large number of bases increasing the catalytic efficiency of ribozymes.

Unfortunately, there is no direct evidence of ancient genetic systems, as recovery of DNA from most fossils is impossible. This is because DNA will survive in the environment for less than one million years and slowly degrades into short fragments in solution. Claims for older DNA have been made, most notably a report of the isolation of a viable bacterium from a salt crystal 250-million years old, but these claims are controversial.

Uses in Technology

Genetic Engineering

Methods have been developed to purify DNA from organisms, such as phenol-chloroform extraction and manipulate it in the laboratory, such as restriction digests and the polymerase chain reaction. Modern biology and biochemistry make intensive use of these techniques in recombinant DNA technology. Recombinant DNA is a man-made DNA sequence that has been assembled from other DNA sequences. They can be transformed into organisms in the form of plasmids or in the appropriate format, by using a viral vector. The genetically modified organisms produced can be used to produce products such as recombinant proteins, used in medical research, or be grown in agriculture.

Forensics

Forensic scientists can use DNA in blood, semen, skin, saliva or hair found at a crime scene to identify a matching DNA of an individual, such as a perpetrator. This process is called genetic fingerprinting, or more accurately, DNA profiling. In DNA profiling, the lengths of variable sections of repetitive DNA, such as short tandem repeats and minisatellites, are compared between people. This method is usually an extremely reliable technique for identifying a matching DNA. However, identification can be complicated if the scene is contaminated with DNA from several people. DNA profiling was developed in 1984 by British geneticist Sir Alec Jeffreys, and first used in forensic science to convict Colin Pitchfork in the 1988 Enderby murders case.

People convicted of certain types of crimes may be required to provide a sample of DNA for a database. This has helped investigators solve old cases where only a DNA sample was obtained from

the scene. DNA profiling can also be used to identify victims of mass casualty incidents. On the other hand, many convicted people have been released from prison on the basis of DNA techniques, which were not available when a crime had originally been committed.

Bioinformatics

Bioinformatics involves the manipulation, searching, and data mining of DNA sequence data. The development of techniques to store and search DNA sequences have led to widely-applied advances in computer science, especially string searching algorithms, machine learning and database theory.[111] String searching or matching algorithms, which find an occurrence of a sequence of letters inside a larger sequence of letters, were developed to search for specific sequences of nucleotides.[112] In other applications such as text editors, even simple algorithms for this problem usually suffice, but DNA sequences cause these algorithms to exhibit near-worst-case behaviour due to their small number of distinct characters. The related problem of sequence alignment aims to identify homologous sequences and locate the specific mutations that make them distinct. These techniques, especially multiple sequence alignment, are used in studying phylogenetic relationships and protein function.[113] Data sets representing entire genomes' worth of DNA sequences, such as those produced by the Human Genome Project, are difficult to use without annotations, which label the locations of genes and regulatory elements on each chromosome. Regions of DNA sequence that have the characteristic patterns associated with protein- or RNA-coding genes can be identified by gene finding algorithms, which allow researchers to predict the presence of particular gene products in an organism even before they have been isolated experimentally.

DNA Nanotechnology
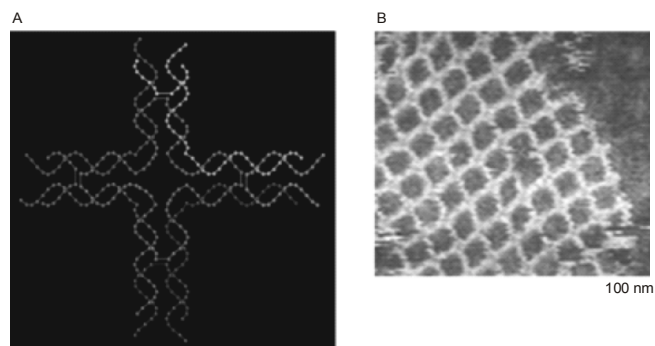
Fig. 2.94: The DNA structure at left (schematic shown) will self-assemble into the structure visualized by atomic force microscopy at right. DNA nanotechnology is the field which seeks to design nanoscale structures using the molecular recognition properties of DNA molecules. Image from Strong, 2004

DNA nanotechnology uses the unique molecular recognition properties of DNA and other nucleic acids to create self-assembling branched DNA complexes with useful properties. DNA is thus used as a structural material rather than as a carrier of biological information. This has led to the creation of two-dimensional periodic lattices (both tile-based as well as using the "DNA origami" method) as well as three-dimensional structures in the shapes of polyhedra. Nanomechanical devices and algorithmic self-assembly have also been demonstrated, and these DNA structures have been used to template the arrangement of other molecules such as gold nanoparticles and streptavidin proteins.

History and Anthropology

Because DNA collects mutations over time, which are then inherited, it contains historical information and by comparing DNA sequences, geneticists can infer the evolutionary history of organisms, their phylogeny.[115] This field of phylogenetics is a powerful tool in evolutionary biology. If DNA sequences within a species are compared, population geneticists can learn the history of particular populations. This can be used in studies ranging from ecological genetics to anthropology; for example, DNA evidence is being used to try to identify the Ten Lost Tribes of Israel.

DNA has also been used to look at modern family relationships, such as establishing family relationships between the descendants of Sally Hemings and Thomas Jefferson. This usage is closely related to the use of DNA in criminal investigations detailed above. Indeed, some criminal investigations have been solved when DNA from crime scenes has matched relatives of the guilty individual.

Primary DNA Molecular Structure

Basic Properties of Primary DNA Molecular Structure

Fundamental Properties of DNA

- Two antiparallel and complimentary strands of deoxyribonucleic acid
- Hydrophillic polar external sugar-phosphate backbone
- Hydrophobic core of bases: Adenine, Thymine, Guanine, Cytosine
- DNA has significant secondary structure.

Sugar-Phosphate Backbone

The basic structure of DNA can be divided into two portions: the external sugar-phosphate backbone, and the internal bases. The sugar-phosphate backbone, as its name implies, is the major structural component of the DNA molecule. The backbone is constructed from alternating ribose sugar and phosphate molecules which are highly polar. Because the backbone is polar, it is hydrophillic which means that it likes to be immersed in water.

Molecular Structure of DNA
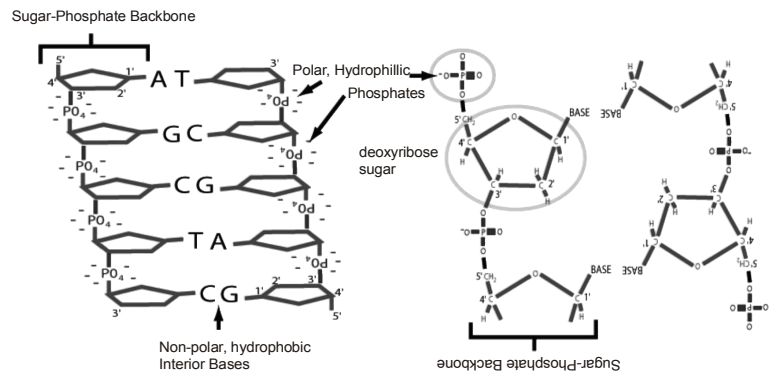
Fig. 2.95: Showing the Molecular structure of DNA

Complimentary Core of Bases

The interior portion of a DNA molecule is composed of a series of 4 nitrogenous bases: adenine (A), guanine (G), thymine (T), and cytosine (C). These bases are non-polar and therefore hyrdophobic (they don't like water). Inside a DNA molecule these bases pair up, A to T and C to G, forming hydrogen bonds that stabilize the DNA molecule. Because the interior bases pair up in this manner, we say the DNA double helix is complimentary. It is this sequence of bases inside the DNA double helix that we refer to as the genetic code.

Hydrostatic Interactions

Hydrostatic forces are very important to the molecular structure of DNA. Hydrostatic forces arise because of hydrogen bonding between the hydrogen and oxygen atoms in water. Polar molecules, because of thier charge, can interact with water without disrupting the ubiquitous latice of hydrogen bonds that the water molecules naturally form. This allows polar molecules to easily dissolve into water. Therefore we call them hydrophilic. Non-polar molecules, however, cannot form electrostatic bonds with the hydrogen and oxygen atoms in water. Non-polar molecules, to be immersed in water, must break potential electrostatic hydrogen bonds between water molecules. Breaking potential bonds represents a net increase in the free energy of the system of water molecules and this has a destabilizing effect the result of which is that non-polar molecules tend to get pushed out of water. Thus we call them hydrophobic. If you have ever seen a water and oil mixture shook up and then slowly settle apart, you have seen these forces in action. The desktop wave boxes are an example of this. Actually, you see these forces in action constantly; you wouldn't exist without them: your cells would lyse apart, your

proteins wouldn't fold properly, and your would die a horribly messy death if these hydrostatic forces quit working:)

The sugar-phosphate backbone of DNA is polar, and therefore hydrophillic; thus it likes to be proximal to water. The interior portion of DNA, the bases, are relatively non-polar and therefore hydrophobic. This duality has a very stabilizing effect on the overall structure of the DNA double helix: the hydrophobic core of the DNA molecule 'wants' to be hidden inside the sugar-phosphate backbone which acts to isolate it from the polar water molecules. Due to these hydrostatic forces there is a strong pressure gluing the two strands of DNA together.

Antiparallel—Direction and Polarity in DNA

DNA is Antiparallel
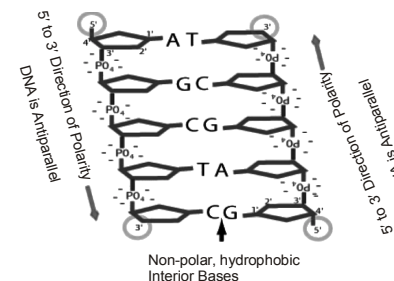
Fig. 2.97: Showing the Direction polarity of DNA

That DNA is antiparallel means that the two strands of DNA have opposite chemical polarity, or, stated another way, their sugar-phosphate backbones run in opposite directions. Direction in nucleic acids is specified by referring to the carbons of the ribose ring in the sugar-phosphate backbone of DNA. 5' specifies the the 5th carbon in the ribose ring, counting clockwise from the oxygen molecule, and 3' specifies the 3rd carbon in the ring. Direction of, and in reference to, DNA molecules is then specified relative to these carbons. For example, transcription, the act of transcribing DNA to RNA for eventual expression, always occurs in the 5' to 3' direction. Nucleic acid polymerization cannot occur in the opposite direction, 3' to 5', because of the difference in chemical properties between the 5' methyl group and the 3' ring-carbon with an attached hydroxyl group.

SUB-SECTION 2.16A—STRUCTURE AND PROPERTIES OF M-RNA, T-RNA, R-RNA AND HN-RNA

Messenger or mRNA is a copy of the information carried by a gene on the DNA. The role of mRNA is to move the information contained in DNA to the translation machinery.

mRNA is heterogeneous in size and sequence. It always has a 5 ' cap composed of a 5' to 5' triphosphate linkage between two modified nucleotides: a 7-methylguanosine and a 2 ' O-methyl purine. This cap serves to identify this RNA molecule as an mRNA to the translational machinery. In addition, most mRNA molecules contain a poly-Adenosine tail at the 3' end. Both the 5' cap and the 3' tail are added after the RNA is transcribed and contribute to the stability of the mRNA in the cell.
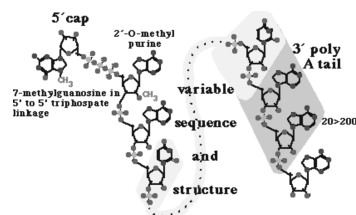
Fig. 2.98 : Showing the m-RNA and its operation

mRNA is not made directly in a eukaryotic cell. It is transcribed as heterogeneous nuclear RNA (hnRNA) in the nucleus. hnRNA contains introns and exons. The introns are removed by RNA splicing leaving the exons, which contain the information, joined together. In some cases, individual nucleotides can be added in the middle of the mRNA sequence by a process called RNA editing. In the figure the exons are represented as the region of variable sequence.

hnRNA and mRNA are never found free in the cell. Like DNA, they are bound by cations and proteins. These complexes are termed ribonucleoproteins or RNPs. The variability in sequence and structure means that no structure has been determined for an mRNA.

Messenger ribonucleic acid (**mRNA**) is a molecule of RNA encoding a chemical "blueprint" for a protein product. mRNA is transcribed from a DNA template, and carries coding information to the sites of protein synthesis: the ribosomes. Here, the nucleic acid polymer is translated into a polymer of amino acids: a protein. In mRNA as in DNA, genetic information is encoded in the sequence of nucleotides arranged into codons consisting of three bases each. Each codon encodes for a specific amino acid, except the stop codons that terminate protein synthesis. This process requires two other types of RNA: transfer RNA (tRNA) mediates recognition of the codon and provides the corresponding amino acid, while ribosomal RNA (rRNA) is the central component of the ribosome's protein manufacturing machinery.

Synthesis, processing, and function

The brief existence of an mRNA molecule begins with transcription and ultimately ends in degradation. During its life, an mRNA molecule may also be
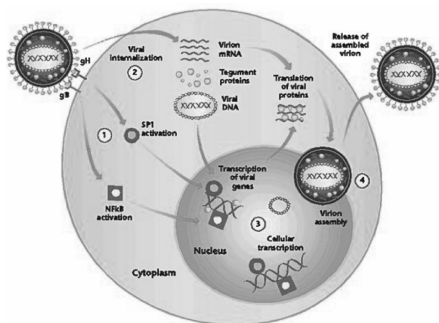
Fig. 2.99 : Showing the Life Cycle and Action of m-RNA

processed, edited, and transported prior to translation. Eukaryotic mRNA molecules often require extensive processing and transport, while prokaryotic molecules do not.

Transcription

During transcription, RNA polymerase makes a copy of a gene from the DNA to mRNA as needed. This process is similar in eukaryotes and prokaryotes. One notable difference, however, is that eukaryotic RNA polymerase associates with mRNA processing enzymes during transcription so that processing can proceed quickly after the start of transcription. The short-lived, unprocessed or partially processed, product is termed *pre-mRNA*; once completely processed, it is termed *mature mRNA*.

Eukaryotic pre-mRNA Processing

Processing of mRNA differs greatly among eukaryotes, bacteria and archea. Non-eukaryotic mRNA is essentially mature upon transcription and requires no processing, except in rare cases. Eukaryotic pre-mRNA, however, requires extensive processing.

5' cap Addition

A *5' cap* (also termed an RNA cap, an RNA 7-methylguanosine cap or an RNA m^7G cap) is a modified guanine nucleotide that has been added to the "front" or 5' end of a eukaryotic messenger RNA shortly after the start of transcription. The 5' cap consists of a terminal 7-methylguanosine residue which is linked through a 5'-5'-triphosphate bond to the first transcribed nucleotide. Its presence is critical for recognition by the ribosome and protection from RNases.

Cap addition is coupled to transcription, and occurs co-transcriptionally, such that each influences the other. Shortly after the start of transcription, the 5' end of the mRNA being synthesized is bound by a cap-synthesizing complex associated with RNA polymerase. This enzymatic complex catalyzes the chemical reactions that are required for mRNA capping. Synthesis proceeds as a multi-step biochemical reaction.

Splicing

Splicing is the process by which pre-mRNA is modified to remove certain stretches of non-coding sequences called introns; the stretches that remain include protein-coding sequences and are called exons. Sometimes pre-mRNA messages may be spliced in several different ways, allowing a single gene to encode multiple proteins. This process is called alternative splicing. Splicing is usually performed by an RNA-protein complex called the spliceosome, but some RNA molecules are also capable of catalyzing their own splicing.

Editing

In some instances, an mRNA will be edited, changing the nucleotide composition of that mRNA. An example in humans is the apolipoprotein B mRNA, which is edited in some tissues, but not others. The editing creates an early stop codon, which upon translation, produces a shorter protein.

Polyadenylation

Polyadenylation is the covalent linkage of a polyadenylyl moiety to a messenger RNA molecule. In eukaryotic organisms, most messenger RNA (mRNA) molecules are polyadenylated at the 3' end. The

poly(A) tail and the protein bound to it aid in protecting mRNA from degradation by exonucleases. Polyadenylation is also important for transcription termination, export of the mRNA from the nucleus, and translation. mRNA can also be polyadenylated in prokaryotic organisms, where poly(A) tails act to facilitate, rather than impede, exonucleolytic degradation.

Polyadenylation occurs during and immediately after transcription of DNA into RNA. After transcription has been terminated, the mRNA chain is cleaved through the action of an endonuclease complex associated with RNA polymerase. After the mRNA has been cleaved, around 250 adenosine residues are added to the free 3' end at the cleavage site. This reaction is catalyzed by polyadenylate polymerase. Just as in alternative splicing, there can be more than one polyadenylation variant of a mRNA.

Transport

Another difference between eukaryotes and prokaryotes is mRNA transport. Because eukaryotic transcription and translation is compartmentally separated, eukaryotic mRNAs must be exported from the nucleus to the cytoplasm. Mature mRNAs are recognized by their processed modifications and then exported through the nuclear pore. In neurons mRNA must be transported from the soma to the dendrites where local translation occurs in response to external stimuli. Many messages are marked with so-called "zip codes" which targets their transport to a specific location.

Translation

Because prokaryotic mRNA does not need to be processed or transported, translation by the ribosome can begin immediately after the end of transcription. Therefore, it can be said that prokaryotic translation is *coupled* to transcription and occurs *co-transcriptionally*.

Eukaryotic mRNA that has been processed and transported to the cytoplasm (i.e. mature mRNA) can then be translated by the ribosome. Translation may occur at ribosomes free-floating in the cytoplasm, or directed to the endoplasmic reticulum by the signal recognition particle. Therefore, unlike prokaryotes, eukaryotic translation *is not* directly coupled to transcription.

Structure

The structure of a typical human protein coding mRNA including the untranslated regions (UTRs)
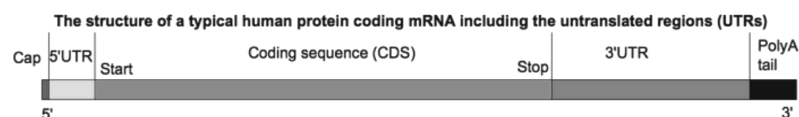
Fig. 2.100 : The structure of a mature eukaryotic mRNA. A fully processed mRNA includes a 5' cap, 5' UTR, coding region, 3' UTR, and poly(A) tail

5' Cap

The *5' cap* is a modified guanine nucleotide added to the "front" (5' end) of the pre-mRNA using a 5'-5'-triphosphate linkage. This modification is critical for recognition and proper attachment of mRNA to

the ribosome, as well as protection from 5' exonucleases. It may also be important for other essential processes, such as splicing and transport.

Coding Regions

Coding regions are composed of codons, which are decoded and translated into one (mostly eukaryotes) or several (mostly prokaryotes) proteins by the ribosome. Coding regions begin with the start codon and end with the a stop codons. Generally, the start codon is an AUG triplet and the stop codon is UAA, UAG, or UGA. The coding regions tend to be stabilised by internal base pairs, this impedes degradation.[3][4] In addition to being protein-coding, portions of coding regions may serve as regulatory sequences in the pre-mRNA as exonic splicing enhancers or exonic splicing silencers.

Untranslated Regions

Untranslated regions (UTRs) are sections of the mRNA before the start codon and after the stop codon that are not translated, termed the five prime untranslated region (5' UTR) and three prime untranslated region (3' UTR), respectively. These regions are transcribed with the coding region and thus are exonic as they are present in the mature mRNA. Several roles in gene expression have been attributed to the untranslated regions, including mRNA stability, mRNA localization, and translational efficiency. The ability of a UTR to perform these functions depends on the sequence of the UTR and can differ between mRNAs.

The stability of mRNAs may be controlled by the 5' UTR and/or 3' UTR due to varying affinity for RNA degrading enzymes called ribonucleases and for ancillary proteins that can promote or inhibit RNA degradation.

Translational efficiency, including sometimes the complete inhibition of translation, can be controlled by UTRs. Proteins that bind to either the 3' or 5' UTR may affect translation by influencing the ribosome's ability to bind to the mRNA. MicroRNAs bound to the 3' UTR also may affect translational efficiency or mRNA stability.

Cytoplasmic localization of mRNA is thought to be a function of the 3' UTR. Proteins that are needed in a particular region of the cell can actually be translated there; in such a case, the 3' UTR may contain sequences that allow the transcript to be localized to this region for translation.

Some of the elements contained in untranslated regions form a characteristic secondary structure when transcribed into RNA. These structural mRNA elements are involved in regulating the mRNA. Some, such as the SECIS element, are targets for proteins to bind. One class of mRNA element, the riboswitches, directly bind small molecules, changing their fold to modify levels of transcription or translation. In these cases, the mRNA regulates itself.

Poly(A) tail

The 3' poly(A) tail is a long sequence of adenine nucleotides (often several hundred) at the 3' end of the pre-mRNA. This tail promotes export from the nucleus and translation, and protects the mRNA from degradation.

Monocistronic versus Polycistronic mRNA

An mRNA molecule is said to be monocistronic when it contains the genetic information to translate only a single protein. This is the case for most of the eukaryotic mRNAs. On the other hand, polycistronic mRNA carries the information of several genes, which are translated into several proteins. These proteins usually have a related function and are grouped and regulated together in an operon. Most of the mRNA found in bacteria and archea are polycistronic. Dicistronic is the term used to describe a mRNA that encodes only two proteins.

mRNA circularization

In eukaryotes it is thought that mRNA molecules form circular structures due to an interaction between the cap binding complex and poly(A)-binding protein. Circularization is thought to promote recycling of ribosomes on the same message leading to efficient translation.

Degradation

Different mRNAs within the same cell have distinct lifetimes (stabilities). In bacterial cells, individual mRNAs can survive from seconds to more than an hour; in mammalian cells, mRNA lifetimes range from several minutes to days. The greater the stability of an mRNA, the more protein may be produced from that mRNA. The limited lifetime of mRNA enables a cell to alter protein synthesis rapidly in response to its changing needs. There are many mechanisms which lead to the destruction of a message, some are described below.

Prokaryotic mRNA degradation

In prokaryotes the lifetime of mRNA is generally much shorter than in eukaryotes. The regulation of mRNA degradation in prokaryotes is much simpler than in eukaryotes. Prokaryotes have numerous RNases which degrade messages rapidly regardless of the sequence of the mRNA. Alternatively, small RNA molecules (sRNA) of tens to hundreds of nucleotides long can recognize specific mRNAs and stimulate their degradation. Complementary sequences in the sRNA bind to the mRNA creating a double-stranded RNA molecule which is a substrate for certain classes of RNAses. It was recently shown that bacteria also have a sort of 5' cap consisting of a triphosphate on the 5' end. Removal of two of the phosphates leaves a 5' monophosphate causing the message to be destroyed by the exonuclease RNAse E.

Eukaryotic mRNA Turnover

Inside eukaryotic cells there is a balance between the processes of translation and mRNA decay. Messages which are being actively translated are bound by polysomes, the eukaryotic initiation factors eIF-4E and eIF-4G, and poly(A)-binding protein. eIF-4E and eIF-4G block the decapping enzyme (DCP2), and poly(A)-binding protein blocks the exosome complex, protecting the message. In nutrient-starvation conditions or during viral infection translation may be compromised and decay is stimulated. The balance between translation and decay is reflected in the size and abundance of the cytoplasmic structures known as P-bodies During rounds of translation the poly-A tail of the mRNA is shortened by exonucleases. This is thought to disrupt the circular structure of the message and destabilize the cap

binding complex. The message is then subject to degradation by either the exosome complex or the decapping complex. In this way inactive messages are destroyed quickly and active messages remain intact leading to selection of those messages which the cell needs at the present time. The mechanism by which translation stops and the message and is handed-off to decay complexes is not understood in detail.

AU-rich Element Decay

The presence of AU-rich elements in some mammalian mRNAs tends to destabilize those transcripts through the action of cellular proteins that bind these sequences. Rapid mRNA degradation via AU-rich elements is a critical mechanism for preventing the overproduction of potent cytokines such as tumor necrosis factor (TNF) and granulocyte-macrophage colony stimulating factor (GM-CSF). AU-rich elements also regulate oncogenic transcription factors like c-Jun and c-Fos. Binding of proteins which recognize AU-rich elements is thought to promote decay by both the exosome complex and decapping complex.

Nonsense mediated decay

Eukaryotic messages are subject to surveillance by nonsense mediated decay (NMD) which checks for the presence of premature stop codons (nonsense codons) in the message. These can arise via alternative splicing, V(D)J recombination in the adaptive immune system, mutations in DNA, transcription errors, leaky scanning by the ribosome causing a frame shift, and other causes. Detection of a premature stop codon results in decay by the decapping complex from the 5' end, the exosome complex from the 3' end, or endonucleolytic cleavage.

Small interfering RNA (siRNA)

In metazoans, small double-stranded RNA that is processed by Dicer is incorporated into a complex known as the RNA-induced silencing complex or RISC. This complex contains an endonuclease that cleaves the message leading to destruction of both fragments by exonucleases. siRNA is commonly used in laboratories to block the function of genes in cell culture. It is thought to be part of the innate immune system as a defense against double-stranded RNA viruses.

Micro RNA (miRNA)

Main article: microRNA

Micro RNA (miRNA) are small RNAs that are almost perfectly complementary to a sequence in a messenger RNA. Binding of the miRNA to the mRNA can lead to repression of translation of the message or removal of the 5' cap by the decapping complex. The method of action of miRNA is the subject of active research.

Other Decay Mechanisms

There are other ways which messages can be decayed including Non-stop decay, silencing by Piwi-interacting RNA, and surely other means.

Transfer RNA (abbreviated **tRNA**) is a small RNA (usually about 74-95 nucleotides) that transfers a specific amino acid to a growing polypeptide chain at the ribosomal site of protein synthesis during translation. It has a 3' terminal site for amino acid attachment. This covalent linkage is catalyzed by an aminoacyl tRNA synthetase. It also contains a three base region called the anticodon that can base pair to the corresponding three base codon region on mRNA. Each type of tRNA molecule can be attached to only one type of amino acid, but because the genetic code contains multiple codons that specify the same amino acid, tRNA molecules bearing different anticodons may also carry the same amino acid.

Fig. 2.101: Structure of tRNA. *CCA tail* **in orange,** *Acceptor stem* **in purple,** *D arm* **in red,** *Anticodon arm* **in blue with** *Anticodon* **in black,** *T arm* **in green**

Structure
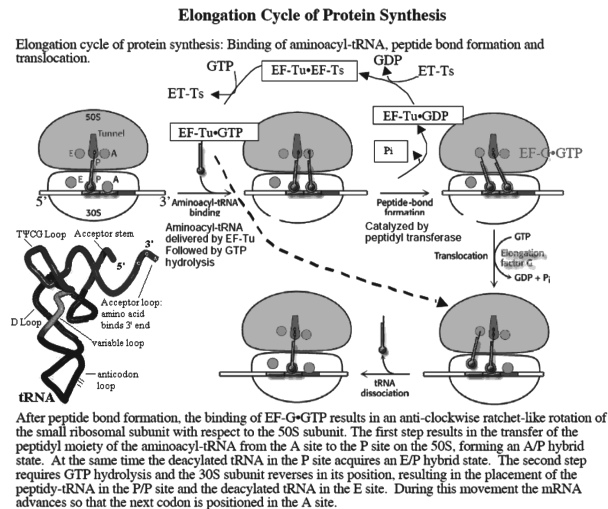
Fig. 2.101: Showing the Detail Action of t-RNA

tRNA has primary structure, secondary structure (usually visualized as the *cloverleaf structure*), and tertiary structure (all tRNAs have a similar L-shaped 3D structure that allows them to fit into the P and A sites of the ribosome).

1. The 5'-terminal phosphate group.

2. The acceptor stem is a 7-bp stem made by the base pairing of the 5'-terminal nucleotide with the 3'-terminal nucleotide (which contains the CCA 3'-terminal group used to attach the amino acid). The acceptor stem may contain non-Watson-Crick base pairs.

3. The CCA tail is a CCA sequence at the 3' end of the tRNA molecule. This sequence is important for the recognition of tRNA by enzymes critical in translation. In prokaryotes, the CCA sequence is transcribed. In eukaryotes, the CCA sequence is added during processing and therefore does not appear in the tRNA gene.

4. The D arm is a 4 bp stem ending in a loop that often contains dihydrouridine.

5. The anticodon arm is a 5-bp stem whose loop contains the anticodon.

6. The T arm is a 5 bp stem containing the sequence TØC where Ø is a pseudouridine.

7. Bases that have been modified, especially by methylation, occur in several positions outside the anticodon. The first anticodon base is sometimes modified to inosine (derived from adenine) or pseudouridine (derived from uracil).

Anticodon

An **anticodon** is a unit made up of three nucleotides that correspond to the three bases of the codon on the mRNA. Each tRNA contains a specific anticodon triplet sequence that can base-pair to one or more codons for an amino acid. For example, one codon for lysine is AAA; the anticodon of a lysine tRNA might be UUU. Some anticodons can pair with more than one codon due to a phenomenon known as wobble base pairing. Frequently, the first nucleotide of the anticodon is one of two not found on mRNA: inosine and pseudouridine, which can hydrogen bond to more than one base in the corresponding codon position. In the genetic code, it is common for a single amino acid to be specified by all four third-position possibilities; for example, the amino acid glycine is coded for by the codon sequences GGU, GGC, GGA, and GGG.

To provide a one-to-one correspondence between tRNA molecules and codons that specify amino acids, 61 tRNA molecules would be required per cell. However, many cells contain fewer than 61 types of tRNAs because the wobble base is capable of binding to several, though not necessarily all, of the codons that specify a particular amino acid.

Aminoacylation

Aminoacylation is the process of adding an aminoacyl group to a compound. It produces tRNA molecules with their CCA 3' ends covalently linked to an amino acid.

Each tRNA is aminoacylated (or *charged*) with a specific amino acid by an aminoacyl tRNA synthetase. There is normally a single aminoacyl tRNA synthetase for each amino acid, despite the fact that there can be more than one tRNA, and more than one anticodon, for an amino acid. Recognition of

the appropriate tRNA by the synthetases is not mediated solely by the anticodon, and the acceptor stem often plays a prominent role.

Reaction:

1. amino acid + ATP → aminoacyl-AMP + PPi

2. aminoacyl-AMP + tRNA → aminoacyl-tRNA + AMP

Binding to Ribosome

The ribosome has three binding sites for tRNA molecules: the A, P, and E sites. During translation the A site binds an incoming aminoacyl-tRNA as directed by the codon currently occupying this site. This codon specifies the next amino acid to be added to the growing peptide chain. The A site only works after the first aminoacyl-tRNA has attached to the P site. The P-site codon is occupied by peptidyl-tRNA that is a tRNA with multiple amino acids attached as a long chain. The P site is actually the first to bind to aminoacyl tRNA. This tRNA in the P site carries the chain of amino acids that has already been synthesized. The E site is occupied by the empty tRNA as it is about to exit the ribosome.

tRNA Genes

Organisms vary in the number of tRNA genes in their genome. The nematode worm *C. elegans*, a commonly used model organism in genetics studies, has 29,647 genes in its nuclear genome, of which 620 code for tRNA. The budding yeast *Saccharomyces cerevisiae* has 275 tRNA genes in its genome. In the human genome, which according to current estimates has about 27,161 genes in total, there are about 4,421 non-coding RNA genes, which include tRNA genes. There are 22 mitochondrial tRNA genes; 497 nuclear genes encoding cytoplasmic tRNA molecules and there are 324 tRNA-derived putative pseudogenes.

Cytoplasmic tRNA genes can be grouped into 49 families according to their anticodon features. These genes are found on all chromosomes, except 22 and Y chromosome. High clustering on 6p is observed (140 tRNA genes), as well on 1 chromosome.

tRNA molecules are transcribed (in eukaryotic cells) by RNA polymerase III, unlike messenger RNA which is transcribed by RNA polymerase II. pre-tRNAs contain introns; in bacteria these self-splice, whereas in eukaryotes and archaea they are removed by tRNA splicing endonuclease.

History

The existence of tRNA was first hypothesized by Francis Crick, based on the assumption that there must exist an adapter molecule capable of mediating the translation of the RNA alphabet into the protein alphabet. Significant research on structure was conducted in the early 1960s by Alex Rich and Don Caspar, two researchers in Boston, the Jacques Fresco group in Princeton University and a United Kingdom group at King's College London.[11] A later publication reported the primary structure in 1965 by Robert W. Holley. The secondary and tertiary structures were derived from X-ray crystallography studies reported independently in 1974 by American and British research groups headed, respectively, by Alexander Rich and Aaron Klug.

Ribosomal RNA (rRNA) is a component of the ribosomes, the protein synthetic factories in the cell. Eukaryotic ribosomes contain four different rRNA molecules: 18 s, 5.8 s, 28 s, and 5 s rRNA. Three of the rRNA molecules are synthesized in the nucleolus, and one is synthesized elsewhere. rRNA molecules are extremely abundant. They make up at least 80% of the RNA molecules found in a typical eukaryotic cell.

Synthesis of the three nucleolar rRNA molecules is unusual because they are made on one primary transcript that is chopped up into three mature rRNA molecules. These rRNA molecules and the 5 s rRNA combine with the ribosomal proteins in the nucleolus to form pre 40 s and pre 60 s ribosomal subunits. These pre-subunits are exported to the nucleus where they mature and assume their role in protein synthesis.
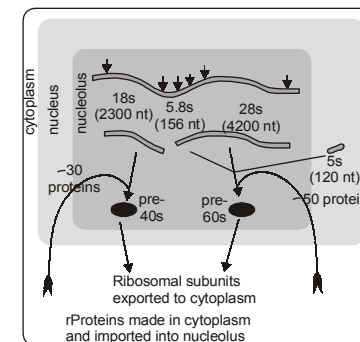
Fig. 2.102: Showing the action of r-RNA

The rRNA molecules have several roles in protein synthesis. First, the 28 s rRNA has a catalytic role, it forms part of the peptidyl transferrase activity of the 60 s subunit. Second, 18s rRNA has a recognition role, involved in correct positioning of the mRNA and the peptidyl tRNA. Finally, the rRNA molecules have a structural role. They fold into three-dimensional shapes that form the scaffold on which the ribosomal proteins assemble. The model on the fig 102 shows the three dimensional structure that the 5 s rRNA from the African frog, Xenopus laevis is thought to adopt.

Precursor mRNA (pre-mRNA), also termed **heterogeneous nuclear RNA (hnRNA)**, is an immature single strand of messenger ribonucleic acid (mRNA). pre-mRNA is synthesized from a DNA template in the cell nucleus by transcription.

Once pre-mRNA has been completely processed, it is termed "mature messenger RNA", "mature mRNA", or simply "mRNA".

Processing

Eukaryotic pre-mRNA exists only briefly before it is fully processed into mRNA. pre-mRNAs include two different types of segments, exons and introns. Exons are segments that are retained in the final mRNA, while introns are removed in a process called splicing, which is performed by the spliceosome (except for self-splicing introns).

Additional processing steps attach modifications to the 5' and 3' ends of the pre-mRNA. These include a 5' cap of 7-methylguanosine and a poly-A tail.

When a pre-mRNA strand has been properly processed to an mRNA sequence, it is exported out of the nucleus and eventually translated into a protein – a process accomplished in conjunction with ribosomes.

Chapter **3**

Enzymes

SECTION 3.1—INTRODUCTIONS

Enzyme comes from the Greek Words "Eos " and "Prozyme" meaning in and through life. To define *Enzyme mainly works as a chemical disintegrator and also catalyses many biochemical reactions in the human body*. Enzymes are found in leaving cells, they are comprised of long polypeptide chains with molecular weight from 10,000 to a minion or more. Another important characteristics is its specificity i.e a given enzyme can catalyze only one particular reaction and not other. Some examples are being

1. Redox (oxyreductase)
2. Transference of special radicals or groups (transferases)
3. Hydrolysis (proteolytic)
4. Removal from or addition to the substrate of specific chemical groups (lyases)
5. Iosmerizations (isomerases)
6. Combination or binding together substrate units (ligases).

The name of enzyme always end as *–in* or *–ase*

The following list will prove the above statement and also will indicate the definite role played in metabolism.

EC 2.7.3.2

enzyme commission

class (transferase)

sub-subclass (phosphotransferase with a nitrogenous acceptor)

this is the second one!

subclass (phosphotransferase)
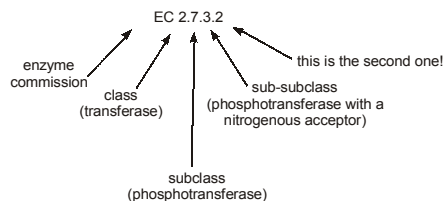
Enzymes are essential to many biochemical processes especially in the food and beverage, and also in the pharmaceutical industries.

Amylase	Starch hydrolysis
Carboxylase	Decomposes pyruvic acid
Cellulose	Converts cellulose to glucose
Cholinesterase, pepsin, rennin	Inactivates acetyl choline
Chymotrypsin	Hydrolyses Protein
Invertase	Converts sucrose to glucose and fructose
Lipase	Hydrolyses Fat
Maltase	Converts Maltose to glucose
Protease	Hydrolysis of peptide linkage
Ribonuclease	Decomposes RNA
Trypsin	Splits proteins into amino acids
Urease	Decomposes urea to NH_4 and CO_2
zymase	Converts sugar to alcohol and CO_2(fermentation)

SECTION 3.1B—CLASSIFICATIN OF ENZYMES

There are 3000 enzymes[$] known to us but only a few we use in the human metabolism and so we are going to discuss about them in this chapter.

Esterases (EC 3.1.1)*

Pancreatic lipase This enzyme is present in the pancreas and hydrolyses fat into fatty acids and glycerol, Lipase is found in the gastric juice.

(A) Liver Esterase: This enzyme is obviously present in the liver, hydrolyses esters into simple alcohols and is an ester – rather than a fat splitting enzyme.

(B) Ricinus Lipase: This enzyme is found in the seeds of the caster beans and is similar to pancreatic lipase.

(C) Chlorophyllase: This is an enzyme present in green plants and hydrolyses chlorophyll into phytol and ethyl chlorophyllide.

[$]If a student is interested in the full database of the enzyme then please contact author

*The **Enzyme Commission number** (**EC number**) is a numerical classification scheme for enzymes, based on the chemical reactions they catalyze. As a system of **enzyme nomenclature**, every EC number is associated with a recommended name for the respective enzyme. This is devised by IUBMB (International Union of Biochemistry and Molecular Biology)

Strictly speaking, EC numbers do not specify enzymes, but enzyme-catalyzed reactions. If different enzymes (for instance from different organisms) catalyze the same reaction, then they receive the same EC number. By contrast, UniProt identifiers uniquely specify a protein by its amino acid sequence.

(D) *Phosphatases:* These enzymes are present in various tissues and hydrolyses various esters of phosphoric acid. The phospytase of which they seem to be quite a number are divided into two groups the alkaline phospytase, with an optimum pH of form 8 to 10 found intestinal mucosa, kidneys, liver etc and acid phosphates, with an optimum pH of 4 to 5, present in blood serum etc.

(E) *Azolesterases:* These enzymes are named as such because they hydrolyze the nitrogen - alcohol esters (azote – nitrogen). The best known example is cholinesterase, the enzyme which hydrolyses acetylcholine.

Proteinases and Peptidases EC 3.4

(A) *Pepsin, EC = 3.4.23.1*: This enzyme is present in the stomach and hysdrolyses proteins to protons. Pepsin can also act on synthetic substrates much similar in composition than proteins. The enzymes have been crystallized.

(B) *Trpsin*: This enzyme is present in pancreas and hydrolyses proteins into peptides and amino acids. Life pepsin, Trypsin can act on substances much simpler chemically.

(C) *Erepsin*: This substance undoubtedly represents a mixture of enzymes of the peptidase variety.

(D) *Renisn*: This enzyme is also present in stomach reacts with the casein of the milk and converts it into paracasein, which reacts with calcium to form a milk clot.

(E) *Papain*: This enzyme is present in the juice of the melon tree and in the plant cells in general. It is similar to the Trypsin into action and has also been isolated in crystallized form (balls)

(F) *Cathepsin*: This enzyme is found in the various animal cells. There are many types of cathepsin, and they are believed to play important roles in synthesis and hydrolyse within the cell. They all activated by HCN, H_2S and –SH compounds. These activators are for the most part reducing substances. These activators are for the most part reducing substances. The papainases are inactivated by mild oxidation and reactivated by reduction.

(G) *Ficin:* This enzyme is found in the milk sap of the fig tree. It digests proteins at about pH 5. It is not activated by –SH compounds.

(H) *Aminopeptidase:* This is an enzyme present in the intestine and on polypeptides containing a free amino group.

The essential difference between hard I is the place of rupture in the peptide linkage

(I) *Carbooxydase:* This is an enzyme found in the pancreas and acts on carboyl group. It has been crystallized (anson).

(J) *Dipeptidase:* This enzyme, present in intestinal juice, this hydrolyses the dipeptides. Since both amino peptides and carboxyl peptides.

Amidiases EC 3.5.1

This acts upon the carbon – nitrogen linkage

(A) *Urease:* This is an enzyme which is present in leguminous plants (soy and jack beans) and converts urea into ammonia. It has been isolated in crystalline form.

(B) *Arginase:* This is an enzyme converts arginine into ornithine and urea.

(C) *Purine:* Amidiases, These are the representative of the group of enzymes present in liver. Deaminise purines. Adenase is an example

(D) *Phosphorylases:* These are enzymes which can decompose polysaccharides as well as bring about the synthesis of the polysaccharides. For example a phosphorylase obtained from muscle converts hexose – 1 phosphate into a polysaccharide. The reaction is a reversible one. An X – Ray diffraction pattern shows it to be similar in structure to starch obtained from plates. In contrast to this when heart or liver phosphorylase is used in place of the one obtained from muscle, the polysaccharide obtained resembles glycogen.

Carbohydrates EC 3.2.1

(A) *Sucrase:* This enzyme is present in animal and plant tissue and hydrolyses sucrose into glucose and fructose. Maltase and Lactase found with sucrose in the small intestine hydrolyse maltose and lactose respectively.

(B) *Amylase, Alpha amylase or diastase:* These enzymes are found in plant and animal tissues and hydrolyze starch and glycogen into maltose. The ptyalin found in saliva and the amylase present in pancreatic juice are example.

Oxidase

(A) *Dehyrogenes:* These are enzymes, found in many tissues (muscles for example) which bring about oxidation by the removal of hydrogen from substances for example succinic dehydrogenase converts succinic acid into fumaric acid.

(B) *Catalase:* These engyme is found in plant and animal tissue (liver example) and molecular oxygen.

(C) *Peroxydase:* These enzymes are present in may tissues (instance, the spleen and horse raddish, as they transfer peroxide oxygen to oxydisable substances.

SUB-SECTION 3.1A—APOENZYME, CO-ENZYME, HOLENZYME AND CO FACTORS

Coenzymes are small organic non-protein molecules that carry chemical groups between enzymes.[1] Coenzymes are sometimes referred to as *cosubstrates*. These molecules are substrates for enzymes and do not form a permanent part of the enzymes' structures. This distinguishes coenzymes from prosthetic groups, which are non-protein components that are bound tightly to enzymes - such as iron-sulfur centers, flavin or haem groups. Both coenzymes and prosthetic groups are types of the broader group of cofactors, which are any non-protein molecules (usually organic molecules or metal ions) that are required by an enzyme for its activity.

In metabolism, coenzymes are involved in both group-transfer reactions, for example coenzyme A and adenosine triphosphate (ATP), and redox reactions, such as coenzyme Q_{10} and nicotinamide adenine dinucleotide (NAD^+). Coenzymes are consumed and recycled continuously in metabolism, with one set of enzymes adding a chemical group to the coenzyme and another set removing it. For example,

enzymes such as ATP synthase continuously phosphorylate adenosine diphosphate (ADP), converting it into ATP, while enzymes such as kinases dephosphorylate the ATP and convert it back to ADP.

Coenzymes molecules are often vitamins or are made from vitamins. Many coenzymes contain the nucleotide adenosine as part of their structures, such as ATP, coenzume A and NAD+. This common structure may reflect a common evolutionary origin as part of ribozymes in an ancient RNA world.

Coenzymes as Metabolic Intermediates

$$NAD^+ + H^+ + 2e^- \longrightarrow NADH$$
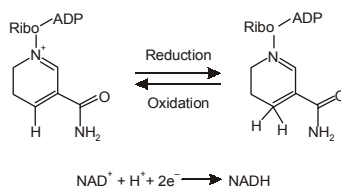
The redox reactions of nicotinamide adenine dinucleotide.

Metabolism involves a vast array of chemical reactions, but most fall under a few basic types of reactions that involve the transfer of functional groups. This common chemistry allows cells to use a small set of metabolic intermediates to carry chemical groups between different reactions.[4] These group-transfer intermediates are the coenzymes.

Each class of group-transfer reaction is carried out by a particular coenzyme, which is the substrate for a set of enzymes that produce it, and a set of enzymes that consume it. An example of this are the dehydrogenases that use nicotinamide adenine dinucleotide (NADH) as a cofactor. Here, hundreds of separate types of enzymes remove electrons from their substrates and reduce NAD+ to NADH. This reduced coenzyme is then a substrate for any of the reductases in the cell that need to reduce their substrates.

Coenzymes are therefore continuously recycled as part of metabolism. As an example, the total quantity of ATP in the human body is about 0.1 mole. This ATP is constantly being broken down into ADP, and then converted back into ATP. Thus, at any given time, the total amount of ATP + ADP remains fairly constant. The energy used by human cells requires the hydrolysis of 100 to 150 moles of ATP daily which is around 50 to 75 kg. Typically, a human will use up their body weight of ATP over the course of the day. This means that each ATP molecule is recycled 1000 to 1500 times daily.

Types

Acting as coenzymes in organisms is the major role of vitamins, although vitamins do have other functions in the body. Coenzymes are also commonly made from nucleotides: such as adenosine triphosphate, the biochemical carrier of phosphate groups, or coenzyme A, the coenzyme that carries acyl groups. Most coenzymes are found in a huge variety of species, and some are universal to all forms of life. An exception to this wide distribution is a group of unique coenzymes that evolved in methanogens, which are restricted to this group of archaea.

Vitamins and derivatives

Coenzyme	Vitamin	Additional component	Chemical group(s) transferred	Distribution
NAD+ and NADP+	Niacin (B₃)	ADP	Electrons	Bacteria, archaea and eukaryotes
Coenzyme A	Pantothenic acid (B5)	ADP	Acetyl group and other acyl groups	Bacteria, archaea and eukaryotes
Tetrahydrofolic acid	Folic acid (B9)	Glutamate residues	Methyl, formyl, methylene and formimino groups	Bacteria, archaea and eukaryotes
Menaquinone	Vitamin K	None	Carbonyl group and electrons	Bacteria, archaea and eukaryotes
Ascorbic acid	Vitamin C	None	Electrons	Bacteria, archaea and eukaryotes
Coenzyme F420	Riboflavin (B2)	Amino acids	Electrons	Methanogens and some bacteria

Non-vitamins

Coenzyme	Chemical group(s) transferred	Distribution
Adenosine triphosphate	Phosphate group	Bacteria, archaea and eukaryotes
S-Adenosyl methionine	Methyl group	Bacteria, archaea and eukaryotes
3'-Phosphoadenosine-5'-phosphosulfate	Sulfate group	Bacteria, archaea and eukaryotes
Coenzyme Q	Electrons	Bacteria, archaea and eukaryotes
Tetrahydrobiopterin	Oxygen atom and electrons	Bacteria, archaea and eukaryotes
Cytidine triphosphate	Diacylglycerols and lipid head groups	Bacteria, archaea and eukaryotes
Nucleotide sugars	Monosaccharides	Bacteria, archaea and eukaryotes
Glutathione	Electrons	Some bacteria and most eukaryotes
Coenzyme M	Methyl group	Methanogens
Coenzyme B	Electrons	Methanogens
Methanofuran	Formyl group	Methanogens
Tetrahydromethanopterin	Methyl group	Methanogens

Evolution

Coenzymes, such as ATP and NADH, are present in all known forms of life and form a core part of metabolism. Such universal conservation indicates that these molecules evolved very early in the development of living things. At least some of the current set of coenzymes may therefore have been present in the last universal ancestor, which lived about 4 billion years ago.

Coenzymes may have been present even earlier in the history of life on Earth. Interestingly, the nucleotide adenosine is present in coenzymes that catalyse many basic metabolic reactions such as methyl, acyl, and phosphoryl group transfer, as well as redox reactions. This ubiquitous chemical scaffold has therefore been proposed to be a remnant of the RNA world, with early ribozymes evolving to bind a restricted set of nucleotides and related compounds. Adenosine-based coenzymes are thought to have acted as interchangeable adaptors that allowed enzymes and ribozymes to bind new coenzymes through small modifications in existing adenosine-binding domains, which had originally evolved to bind a different cofactor. This process of adapting a pre-evolved structure for a novel use is referred to as *exaptation*.

History

The first coenzyme to be discovered was NAD^+, which was identified by Arthur Harden and William Youndin 1906. They noticed that adding boiled and filtered yeast extract greatly accelerated alcoholic fermentation in unboiled yeast extracts. They called the unidentified factor responsible for this effect a *coferment*. Through a long and difficult purification from yeast extracts, this heat-stable factor was identified as a nucleotide sugar phosphate by Hans von Euler-Chelpin. Other coenzymes were identified throughout the early 20th century, with ATP being isolated in 1929 by Karl Lohmann, and coenzyme A being discovered in 1945 by Fritz Albert Lipmann.

The functions of coenzymes were at first mysterious, but in 1936, Otto Heinrich Warburg identified the function of NAD^+ in hydride transfer. This discovery was followed in the early 1940s by the work of Herman Kalckar, who established the link between the oxidation of sugars and the generation of ATP. This confirmed the central role of ATP in energy transfer that had been proposed by Fritz Albert Lipmann in 1941. Later, in 1949, Morris Friedkin and Albert L. Lehninger proved that the coenzyme NAD^+ linked metabolic pathways such as the citric acid cycle and the synthesis of ATP.

SECTION 3.2—KINETICS OF ENZYME

SUB-SECTION 3.2A—REACTION VELOCITY

In most cases, an enzyme converts one chemical (the *substrate*), into another (the *product*). A graph of product concentration vs. time follows three phases as shown in the following graph.

At very early time points, the rate of product accumulation increases over time. Special techniques are needed to study the early kinetics of enzyme action, since this transient phase usually lasts less than a second (the figure greatly exaggerates the first phase).
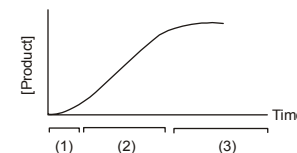
Fig. 3.1 : Showing the Enzyme reaction of enzyme

For an extended period of time, the product concentration increases linearly with time.

At later times, the substrate is depleted, so the curve starts to level off. Eventually the concentration of product reaches a plateau and doesn't change with time.

It is difficult to fit a curve to a graph of product as a function of time, even if you use a simplified model that ignores the transient phase and assumes that the reaction is irreversible. The model simply cannot be solved to an equation that expresses product concentration as a function of time. To fit these kind of data (called an *enzyme progress curve*) you need to use a program that can fit data to a model defined by differential equations or by an implicit equation. Prism cannot do this. For more details, see RG Duggleby, "Analysis of Enzyme Reaction Progress Curves by Nonlinear Regression", Methods in Enzymology, 249: page 60-, 1995.

Rather than fit the enzyme progress curve, most analyses of enzyme kinetics fit the initial velocity of the enzyme reaction as a function of substrate concentration. The velocity of the enzyme reaction is the slope of the linear phase, expressed as amount of product formed per time. If the initial transient phase is very short, you can simply measure product formed at a single time, and define the velocity to be the concentration divided by the time interval.

This chapter considers data collected only in the second phase. The terminology describing these phases can be confusing. The second phase is often called the "initial rate", ignoring the short transient phase that precedes it. It is also called "steady state", because the concentration of enzyme-substrate complex doesn't change. However, the concentration of product accumulates, so the system is not truly at steady state until, much later, the concentration of product truly doesn't change over time.

Enzyme velocity as a function of substrate concentration

If you measure enzyme velocity at many different concentrations of substrate, the graph generally looks like this:
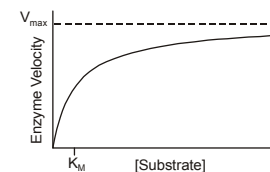
Fig. 3.2 : Showing the Enzyme Velocity

Enzyme velocity as a function of substrate concentration often follows the Michaelis-Menten equation:

$$\text{Velocity} = V = \frac{V_{max}[S]}{[S] + K_M}$$

Vmax is the limiting velocity as substrate concentrations get very large. Vmax (and V) are expressed in units of product formed per time. If you know the molar concentration of enzyme, you can divide the observed velocity by the concentration of enzyme sites in the assay, and express Vmax as units of moles of product formed per second per mole of enzyme sites. This is the *turnover number*, the number of molecules of substrate converted to product by one enzyme site per second. In defining enzyme concentration, distinguish the concentration of enzyme molecules and concentration of enzyme sites (if the enzyme is a dimer with two active sites, the molar concentration of sites is twice the molar concentration of enzyme).

SUB-SECTION 3.2B—MICHAELIS–MENTEN KINETICS

Michaelis–Menten kinetics (occasionally also referred to as **Michaelis–Menten–Henri kinetics**) approximately describes the kinetics of many enzymes. It is named after Leonor Michaelis and Maud Menten. This kinetic model is relevant to situations where very simple kinetics can be assumed, (i.e. there is no intermediate or product inhibition, and there is no allostericity or cooperativity). More complex models exist for the cases where the assumptions of Michaelis–Menten kinetics are not appropriate any more.

Fig. 3.3 : Michaelis-Menten Plot relating the reaction rate v_0 to the substrate concentration [S].

The Michaelis-Menten equation relates the initial reaction rate v_0 to the substrate concentration [S].

The Michaelis-Menten equation relates the initial reaction rate v_0 to the substrate concentration [S]. The corresponding graph is a hyperbolic function; the maximum rate is described as v_{max}.

$$v_0 = \frac{V_{max}[S]}{K_m + [S]}$$

The Michaelis–Menten equation describes the rates of irreversible reactions. A steady state solution for a chemical equilibrium modeled with Michaelis–Menten kinetics can be obtained with the Goldbeter–Koshland equation.

History

The modern relationship between substrate and enzyme concentration was proposed in 1903 by Victor Henri. A microscopic interpretation was thereafter proposed in 1913 by Leonor Michaelis and Maud

Menten, following earlier work by Archibald Vivian Hill. It postulated that enzyme (catalyst) and substrate (reactant) are in fast equilibrium with their complex, which then dissociates to yield product and free enzyme.

The current derivation, based on the quasi steady state approximation (that the concentrations of the intermediate complexes remain constant) was proposed by Briggs and Haldane.

Equation

The validity of the following derivation rests on the reaction scheme given below and two key assumptions: that the total enzyme concentration and the concentration of the intermediate complex do not change over time. The most convenient derivation of the Michaelis–Menten equation, described by Briggs and Haldane, is obtained as follows (Note that often the experimental parameter k_{cat} is used but in this simple case it is equal to the kinetic parameter k_2):

The enzymatic reaction is assumed to be irreversible, and the product does not bind to the enzyme.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\longrightarrow} ES + P \tag{1}$$
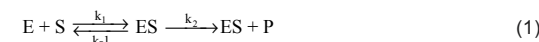
The first key assumption in this derivation is the quasi-steady-state assumption (or pseudo-steady-state hypothesis), namely that the concentration of the substrate-bound enzyme ([ES]) changes much more slowly than those of the product ([P]) and substrate ([S]). This allows us to set the rate of change of [ES] to zero and also write down the rate of product formation:

$$\frac{dES}{dt} = k_1[E][S] - [ES](k_{-1} + k_2) \overset{!}{=} 0 \tag{2}$$

$$\frac{d[P]}{dt} = k_2 \, [ES] \tag{3}$$

The second key assumption is that the total enzyme concentration ([E]) does not change over time, thus we can write the total concentration of enzyme $[E]_0$ as the sum of the free enzyme in solution [E] and that which is bound to the substrate [ES]:

$$[E_0] = [E] + [ES] \overset{!}{=} \text{Constant}$$

Substituting this into equation (2), we obtain an expression for [ES] which in turn we can use in equation (3) to find an expression for the rate of product formation:

$$0 = k_1[S](E_0[ES]) - [ES](k_{-1} + k_2)$$

$$k_1[S][E]_0 = k_1[S][ES] + [ES](k_{-1} + k_2)$$

$$[S][E]_0 = [S][ES] + [ES]\underbrace{\frac{(k_{-1} + k_2)}{k_1}}_{k_M}$$

$$[S][E]_0 = (k_M + [S])[ES]$$

$$[ES] = \frac{[S][E]_0}{k_M + [S]}$$

$$\frac{d[P]}{dt} = v_0 = k_2[ES] = k_2[ES] = k_2[E]_0 \frac{[S]}{k_M + [S]}$$

$$v_0 = \frac{v_{max}[S]}{k_M + [S]} \qquad (4)$$

$$\frac{1}{v_0} = \frac{k_M}{v_{max}} \cdot \frac{1}{S} + \frac{1}{v_{max}} \qquad (5)$$

Because the concentration of substrate changes as the reaction takes place, the initial reaction rate v_0 is used to simplify analysis, taking the initial concentration of substrate as [*S*]. The equation for the reaction rate (4) can also be rewritten in equation (5) which uses the inverse of v_0 and [*S*]. This makes it easier to determine the constants from measured data (a procedure that results in a Lineweaver–Burk plot or a Hanes–Woolf plot).
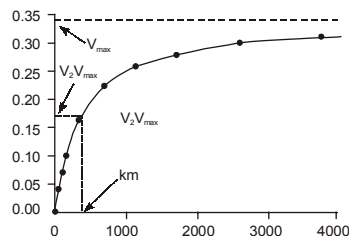
Fig. 3.4 : Saturation curve for an enzyme showing the relation between the concentration of substrate and rate

Equation (4) results in a so called saturation curve which can be observed in the graph on the right. Several interesting cases can be distinguished mathematically and graphically:

- If [*S*] is large compared to K_M then the term $[S]/k_m + [S] \approx$. Therefore, the rate of product formation is

$$\frac{d[P]}{dt} \gg v_{max} = k_2[E]_0$$

Thus the product formation rate only depends on the enzyme concentration, the equation resemebles a unimolecular reaction with a corresponding pseudo-first order rate constant k_2. Thus it only matters how fast the [ES] complex turns its bound substrate into product and not how often the enzyme and the substrate meet.

- If [*S*] = K_M then $[S]/k_m + [S] = [S]/2[S] = \frac{1}{2}$. Therefore, the rate of product formation is

$$\frac{d[P]}{dt} = 0.5.v_{max} = 0.5k_2[E]_0$$

- If [*S*] is small compared to K_M then the term $[S]/k_m + [S] \approx [S]/k_m = \frac{1}{2}$ and also very little ES complex is formed, thus $[E]_0 \approx [E]$. Therefore, the rate of product formation is

$$\frac{d[P]}{dt} \approx v_{max}[S]/k_m \approx \frac{k_2}{k_m}[E][S]$$

Thus the product formation rate depends on the enzyme concentration as well as on the substrate concentration, the equation resembles a bimolecular reaction with a corresponding pseudo-second order rate constant k_2/K_M. This constant is a measure of how efficiently an enzyme converts a substrate into product. The most efficient enzymes reach a k_2/K_M in the range of $10^8 - 10^{10}$ M^{-1} s^{-1} which is the diffusion limit. These enzymes are so efficient they effectively catalyze a reaction each time they encounter a substrate molecule and have thus reached an upper theoretical limit for efficiency, thus these enzymes have often be termed as *perfect enzymes*.

Determination of Constants
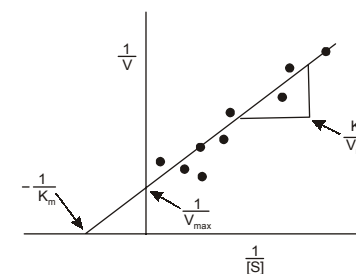
Fig. 3.5 : Lineweaver–Burk plot

To determine the maximum rate of an enzyme mediated reaction, a series of experiments is carried out with varying substrate concentration ([*S*]) and the initial rate of product formation is measured. 'Initial' here is taken to mean that the reaction rate is measured after a relatively short time period, during which complex builds up but the substrate concentration remains approximately constant and the quasi-steady-state assumption will hold. The measurements can then be plotted in a Lineweaver–Burk plot, plotting the inverse of substrate concentration against the inverse of the initial velocity

$$\frac{1}{v_0} = \frac{k_M + [S]}{v_{max} + [S]} = \frac{k_M}{v_{max}} \cdot \frac{1}{[S]} + \frac{1}{v_{max}}$$

The values of the desired constants K_M and V_{max} can be read directly off the plot. It should be noted that accurate values for K_M and V_{max} can only be determined by non-linear regression of Michaelis-Menten data[6]. The inverse plot while useful for visualization should never be the source of the actual value of the enzyme constant due to large insensitivity to errors inherent in all inverse plots. Should one be forced to derive a value from an inverse plot, the Hanes-Woolf plot is the most accurate.

Michaelis Constant K_M and its Significance

The reaction rate V is the number of reactions per second catalyzed per mole of the enzyme. The reaction rate increases with increasing substrate concentration $[S]$, asymptotically approaching the maximum rate V_{max}. There is therefore no clearly-defined substrate concentration at which the enzyme can be said to be saturated with substrate. A more appropriate measure to characterize an enzyme is the substrate concentration at which the reaction rate reaches half of its maximum value ($V_{max}/2$). This concentration can be shown to be equal to the Michaelis constant (K_M), see the section above for a derivation.

For enzymatic reactions which exhibit simple Michaelis–Menten kinetics, the Michaelis constant is defined as (this result comes directly from the derivation of the equation):

$$K_M = \frac{k_{-1} + k_2}{k_1}$$

In the most simple case, when product formation is the rate-limiting step (i.e. when $k_2 \ll k_{-1}$) the constant will just be equal to the dissociation constant (affinity for substrate) of the enzyme-substrate (ES) complex

$$K_M \approx \frac{k_{-1}}{k_1} = \frac{[E][S]}{[ES]} = K_d$$

However, often $k_2 \gg k_{-1}$, or k_2 and k_{-1} are comparable, in which case there will be significant contributions to K_M in addition to the affinity of the enzyme for the substrate.

Limitations

The first source of limitations for the Michaelis-Menten kinetics is that it is an approximation of the kinetics derived by the law of mass action. In particular, Michaelis-Menten kinetics is based on the quasi-steady state assumption that $[ES]$ does not change,

$$\frac{d[ES]}{dt} = 0,$$

which is only approximately true: the rate of change of the complex $[ES]$ is very small but non-zero. The quality of the approximation depends on the timescale separation present in the dynamics on the phase space, which controls the magnitude of the rate of change of $[ES]$. In particular, it has been shown[8] that this timescale separation is measured by a small, positive parameter,

$$\varepsilon = \frac{E_0}{S_0 + K_M},$$

where S_0 is the initial concentration of the substrate and S_0 and K_M have been defined above. The smaller is, the larger the timescale separation present in the system and the more accurate is the quasi-steady state approximation.

The second limitation is that Michaelis–Menten kinetics relies upon the law of mass action which is derived from the assumptions of free (Fickian) diffusion and thermodynamically-driven random collision. However, many biochemical or cellular processes deviate significantly from such conditions. For example, the cytoplasm inside a cell behaves more like a gel than a freely flowable or watery liquid, due to the very high concentration of protein (up to ~400 mg/mL) and other "solutes", which can severely limit molecular movements (e.g., diffusion or collision). This causes macromolecular crowding, which can alter reaction rates and dissociation constants.

For heterogeneous enzymatic reactions, such as those of membrane enzymes, molecular mobility of the enzyme or substrates can also be severely restricted, due to the immobilization or phase-separation of the reactants. For some homogeneous enzymatic reactions, the mobility of the enzyme or substrate may also be limited, such as the case of DNA polymerase where the enzyme moves along a chained substrate, rather than having a three-dimensional freedom. The limitation on molecular mobility (as well as other "non-ideal" conditions) demands modifications on the conventional mass-action laws, and Michaelis–Menten kinetics, to better reflect certain real world situations. Although it has been shown that the law of mass action can be valid in heterogeneous environments.
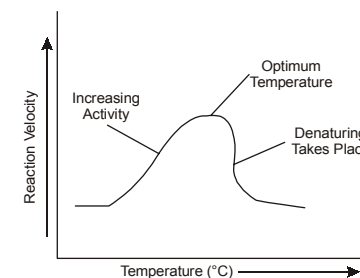
Fig. 3.6 : Showing the effect of temperature on enzyme activity

SUB-SECTION 3.2C—EFFECT OF TEMPERATURE

Like most chemical reactions, the rate of an enzyme-catalyzed reaction increases as the temperature is raised. A ten degree Centigrade rise in temperature will increase the activity of most enzymes by 50 to 100%. Variations in reaction temperature as small as 1 or 2 degrees may introduce changes of 10 to

20% in the results. In the case of enzymatic reactions, this is complicated by the fact that many enzymes are adversely affected by high temperatures. As shown in Figure 108 the reaction rate increases with temperature to a maximum level, then abruptly declines with further increase of temperature. Because most animal enzymes rapidly become denatured at temperatures above 40·C, most enzyme determinations are carried out somewhat below that temperature. Over a period of time, enzymes will be deactivated at even moderate temperatures. Storage of enzymes at 5·C or below is generally the most suitable. Some enzymes lose their activity when frozen.

SUB-SECTION 3.2D—EFFECT OF PH

Enzymes are affected by changes in pH. The most favorable pH value - the point where the enzyme is most active - is known as the optimum pH. This is graphically illustrated in Figure 109.

Extremely high or low pH values generally result in complete loss of activity for most enzymes. pH is also a factor in the stability of enzymes. As with activity, for each enzyme there is also a region of pH optimal stability. The optimum pH value will vary greatly from one enzyme to another, as Table below shows:
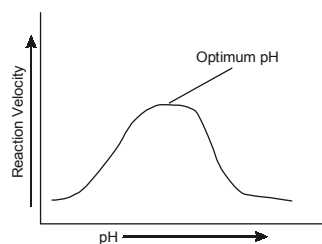
Fig. 3.7 : Showing the Effect of pH on the Enzyme Activity

Table 3.1 : pH for Optimum Activity

Enzyme	pH Optimum
Lipase (pancreas)	8.0
Lipase (stomach)	4.0 - 5.0
Lipase (castor oil)	4.7
Pepsin	1.5 - 1.6
Trypsin	7.8 - 8.7
Urease	7.0
Invertase	4.5
Maltase	6.1 - 6.8
Amylase (pancreas)	6.7 - 7.0
Amylase (malt)	4.6 - 5.2
Catalase	7.0

In addition to temperature and pH there are other factors, such as ionic strength, which can affect the enzymatic reaction. Each of these physical and chemical parameters must be considered and optimized in order for an enzymatic reaction to be accurate and reproducible.

SECTION 3.4—INHIBITORS OF ENZYME ACTION

SUB-SECTION 3.4A—COMPETITIVE INHIBITORS
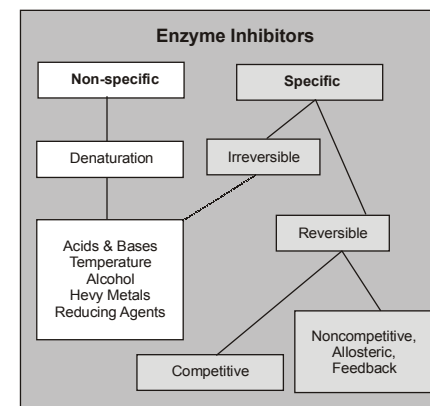
Fig. 3.7 : Showing the relation between Enzyme inhibitors

A competitive inhibitor is any compound which closely resembles the chemical structure and molecular geometry of the substrate. The inhibitor competes for the same active site as the substrate molecule. The inhibitor may interact with the enzyme at the active site, but no reaction takes place. The inhibitor is "stuck" on the enzyme and prevents any substrate molecules from reacting with the enzyme. However, a competitive inhibition is usually reversible if sufficient substrate molecules are available to ultimately displace the inhibitor. Therefore, the amount of enzyme inhibition depends upon the inhibitor concentration, substrate concentration, and the relative affinities of the inhibitor and substrate for the active site.

Example: Ethanol is metabolized in the body by oxidation to acetaldehyde, which is in turn further oxidized to acetic acid by aldehyde oxidase enzymes. Normally, the second reaction is rapid so that acetaldehyde does not accumulate in the body.

*A drug, **disulfiram (Antabuse) inhibits** the aldehyde oxidase which causes the accumulation of acetaldehyde with subsequent unpleasant side-effects of nausea and vomiting. This drug is sometimes used to help people overcome the drinking habit.*

Methanol poisoning occurs because methanol is oxidized to formaldehyde and formic acid which attack the optic nerve causing blindness. Ethanol is given as an antidote for methanol poisoning because ethanol competitively inhibits the oxidation of methanol. Ethanol is oxidized in preference to methanol and consequently, the oxidation of methanol is slowed down so that the toxic by-products do not have a chance to accumulate.

SUB-SECTION 3.4B—NON COMPETITIVE INHIBITORS

A noncompetitive inhibitor is a substance that interacts with the enyzme, but usually not at the active site. The noncompetitive inhibitor reacts either remote from or very close to the active site. The net effect of a non competitive inhibitor is to change the shape of the enzyme and thus the active site, so that the substrate can no longer interact with the enzyme to give a reaction. Non competitive inhibitors are usually reversible, but are not influenced by concentrations of the substrate as is the case for a reversible competive inhibitor. See the Fig. 111 .
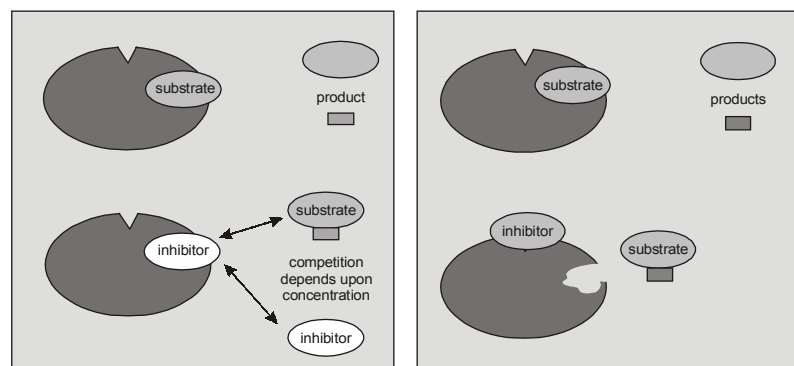
Fig. 3.8 : Showing the Copetitive Inhibitors   **Fig. 3.9 : Showing Non-Competitive Inhibitor**

SUB-SECTION 3.4C

Irreversible Inhibitors form strong covalent bonds with an enzyme. These inhibitors may act at, near, or remote from the active site. Consequently, they may not be displaced by the addition of excess substrate. In any case, the basic structure of the enzyme is modified to the degree that it ceases to work.

Since many enzymes contain sulfhydral (-SH), alcohol, or acid groups as part of their active sites, any chemical which can react with them acts as an irreversible inhibitor. Heavy metals such as Ag^+, Hg^{2+}, Pb^{2+} have strong affinities for -SH groups.

Nerve gases such as diisopropylfluorophosphate (DFP) inhibit the active site of acetylcholine esterase by reacting with the hydroxyl group of serine to make an ester.

Oxalic and citric acid inhibit blood clotting by forming complexes with calcium ions necessary for the enzyme metal ion activator.

Example: Chymotrpsin is an enzyme which hydrolyzes peptides at the carbonyl side of tyr or phe or trp (i.e. those that have an aromatic side chain. In the graphic on the left, the substrate and the irreversible inhibitor are shown in the active site pocket. In the case of the inhibitor the reaction starts in the same way as with the substrate, but the end result is that the inhibitor is covalently bonded to the histidine-57 in the active site and is not reversible.

SUB-SECTION 3.4D

Suicide inhibition, also known as suicide inactivation, is a form of irreversible enzyme inhibition that occurs when an enzyme binds a substrate analogue and forms an irreversible complex with it through a covalent bond during the "normal" catalysis reaction.

Examples

Some clinical examples of suicide inhibitors include:

- Penicillin, which inhibits transpeptidase from building bacterial cell walls.

- Sulbactam, which prohibits penicillin-resistant strains of bacteria from metabolizing penicillin.

- Allopurinol, which inhibits uric acid production by xanthine oxidase in the treatment of gout.

- AZT (zidovudine) and other chain-terminating nucleoside analogues used to inhibit HIV-1 reverse transcriptase in the treatment of HIV/AIDS.

- Eflornithine, one of the drugs used to treat sleeping sickness is a suicide inhibitor of ornithine decarboxylase.

SUB-SECTION 3.4E—MODE OF ACTION , ALLOSTERIC AND COVALENT REGULATION

SUB-SECTION 3.4F

Blood plasma contains many enzymes, which are classified into functional and non-functional plasma enzymes.

Differences between functional and non-functional plasma enzymes :

Sources of non-functional plasma enzymes

1. Increase in the rate of enzyme synthesis) e.g. bilirubin increases the rate of synthesis of alkaline phosphatase in obstructive liver diseases.

2. Obstruction of normal pathway e.g. obstruction of bile ducts increases alkaline phosphatase.

3. Increased permeability of cell membrane as in tissue hypoxia.

	Functional plasma enzymes	**Non-functional plasma enzymes**
Concentration in plasma	Present in plasma in higher concentrations in comparison to tissues	Normally, present in plasma in very low concentrations in comparison to tissues
Function	Have known functions	No known functions
The substrates	Their substrates are always present in the blood	Their substrates are absent from the blood
Site of synthesis	Liver	Different organs e.g. liver, heart, brain and skeletal muscles
Effect of diseases	Decrease in liver diseases	Different enzymes increase in different organ diseases
Examples	Clotting factors e.g. prothrombin, Lipoprotein lipase and pseudo- choline esterase	ALT, AST, CK, LDH, alkaline phosphatase, acid phosphatase and amylase,

4. Cell damage with the release of its content of enzymes into the blood e.g. myocardial infarction and viral hepatitis.

Medical importance of non-functional plasma enzymes

Measurement of non-functional plasma enzymes is important for:

1. Diagnosis of diseases as diseases of different organs cause elevation of different plasma enzymes.

2. Prognosis of the disease; we can follow up the effect of treatment by measuring plasma enzymes before and after treatment.

Examples of medically important non-functional plasma enzymes

1. Amylase and lipase enzymes increase in diseases of the pancreas as acute pancreatitis.

2. Creatine kinase (CK) enzyme increases in heart, brain and skeletal muscle diseases.

3. Lactate dehydrogenase (LDH) enzyme increases in heart, liver and blood diseases.

4. Alanine transaminase (ALT) enzyme, it is also called serum glutamic pyruvic transaminase (SGPT). It increases in liver and heart diseases.

5. Aspartate transaminase (AST) enzyme, it is also called serum glutamic oxalacetic transaminase (SGOT). It increases in liver and heart diseases.

6. Acid phosphatase enzyme increases in cancer prostate.

7. Alkaline phosphatase enzyme increases in obstructive liver diseases, bone diseases and hyperparathyroidism.

SUB-SECTION 3.5D—MEASUREMENTS OF ENZYME ACTIVITY AND INTERPRETATION OF UNITS

Amounts of enzymes can either be expressed as molar amounts, as with any other chemical, or measured in terms of activity, in enzyme units.

Enzyme activity = moles of substrate converted per unit time = rate × reaction volume. Enzyme activity is a measure of the quantity of active enzyme present and is thus dependent on conditions, *which should be specified*. The SI unit is the katal, $1\,katal = 1\,mol\,s^{-1}$, but this is an excessively large unit. A more practical and commonly-used value is 1 enzyme unit (EU) = $1\,\mu mol\,min^{-1}$ (μ = micro, x 10^{-6}). 1 U corresponds to 16.67 nanokatals.

The specific activity of an enzyme is another common unit. This is the activity of an enzyme per milligram of total protein (expressed in $\mu mol\,min^{-1}mg^{-1}$). Specific activity gives a measurement of the purity of the enzyme.

SECTION 3.6—ISOZYMES

Also known as **isoenzymes** are enzymes that differ in amino acid sequence but catalyze the same chemical reaction. These enzymes usually display different kinetic parameters (i.e. different K_M values), or different regulatory properties. The existence of isozymes permits the fine-tuning of metabolism to meet the particular needs of a given tissue or developmental stage (for example lactate dehydrogenase (LDH)). In biochemistry, isozymes (or isoenzymes) are isoforms (closely related variants) of enzymes. In many cases, they are coded for by homologous genes that have diverged over time. Although, strictly speaking, **allozymes** represent enzymes from different alleles of the same gene, and isozymes represent enzymes from different genes that process or catalyse the same reaction, the two words are usually used interchangeably.

Isozymes were first described by R. L. Hunter and Clement Markert (1957) who defined them as *different variants of the same enzyme having identical functions and present in the same individual*. This definition encompasses (1) enzyme variants that are the product of different genes and thus represent different loci (described as *isozymes*) and (2) enzymes that are the product of different alleles of the same gene (described as *allozymes*).Isozymes are usually the result of gene duplication, but can also arise from polyploidisation or nucleic acid hybridization. Over evolutionary time, if the function of the new variant remains *identical* to the original, then it is likely that one or the other will be lost as mutations accumulate, resulting in a pseudogene. However, if the mutations do not immediately prevent the enzyme from functioning, but instead modify either its function, or its pattern of gene expression, then the two variants may both be favoured by natural selection and become specialised to different functions. For example, they may be expressed at different stages of development or in different tissues. Allozymes may result from point mutations or from insertion-deletion (*indel*) events that affect the DNA coding sequence of the gene. As with any other new mutation, there are three things that may happen to a new allozyme:

1. It is most likely that the new allele will be non-functional — in which case it will probably result in low fitness and be removed from the population by natural selection.

2. Alternatively, if the amino acid residue that is changed is in a relatively unimportant part of the enzyme, for example a long way from the active site then the mutation may be selectively neutral and subject to genetic drift.

3. In rare cases the mutation may result in an enzyme that is more efficient, or one that can catalyse a slightly different chemical reaction, in which case the mutation may cause an increase in fitness, and be favoured by natural selection.

An example of an Isozyme

An example of an isozyme is glucokinase, a variant of hexokinase which is not inhibited by glucose 6-phosphate. Its different regulatory features and lower affinity for glucose (compared to other hexokinases), allows it to serve different functions in cells of specific organs, such as control of insulin release by the beta cells of the pancreas, or initiation of glycogen synthesis by liver cells. Both of these processes must only occur when glucose is abundant, or problems occur.

Distinguishing Isozymes

Isozymes (and allozymes) are variants of the same enzyme. Unless they are identical in terms of their biochemical properties, for example their substrates and enzyme kinetics, they may be distinguished by a biochemical assay. However, such differences are usually subtle (particularly between *allozymes* which are often neutral variants). This subtlety is to be expected, because two enzymes that differ significantly in their function are unlikely to have been identified as *isozymes*. Whilst isozymes may be almost identical in function, they may differ in other ways. In particular, amino acid substitutions that change the electric charge of the enzyme (such as replacing aspartic acid with glutamic acid) are simple to identify by gel electrophoresis, and this forms the basis for the use of isozymes as molecular markers. To identify isozymes, a crude protein extract is made by grinding animal or plant tissue with an extraction buffer, and the components of extract are separated according to their charge by gel electrophoresis. Historically, this has usually been done using gels made from potato starch, however, acrylamide gels provide better resolution, and cellulose acetate gels are now (as of 2005) the norm. All the proteins from the tissue are present in the gel, so that individual enzymes must be identified using an assay that links their function to a staining reaction. For example, detection can be based on the localised precipitation of soluble indicator dyes such as tetrazolium salts which become insoluble when they are reduced by cofactors such as NAD or NADP, which generated in zones of enzyme activity. This assay method requires that the enzymes are still functional after separation (native gel electrophoresis), and provides the greatest challenge to using isozymes as a laboratory technique.

Isozymes and Allozymes as Molecular Markers

Population genetics is essentially a study of the causes and effects of genetic variation within and between populations, and in the past isozymes have been amongst the most widely used Molecular markers for this purpose. Although they have now been largely superseded by more informative DNA-based approaches (such as direct DNA sequencing, single nucleotide polymorphisms and microsatellites), they are still amongst the quickest and cheapest marker systems to develop, and remain (as of 2005) an excellent choice for projects that only need to identify low levels of genetic variation, e.g. quantifying mating systems.

4

Chapter

Physical Aspects of Living Matters

SECTION 4.1—ISOTOPES[1]

Isotopes (Greek esos = "equal", tópos = "site, place") are any of the different types of atoms (nuclides) of the same chemical element, each having a different atomic mass (mass number). Isotopes of an element have nuclei with the same number of protons (the same atomic number) but different numbers of neutrons. Therefore, isotopes have different mass numbers, which give the total number of nucleons, the number of protons plus neutrons.

A nuclide is any particular atomic nucleus with a specific number of an atom Z and mass number A; it is equivalently an atomic nucleus with a specific number of protons and neutrons. Collectively, all the isotopes of all the elements form the set of *nuclides*. The distinction between the terms *isotope* and *nuclide* has somewhat blurred, and they are often used interchangeably. *Isotope* is better used when referring to several different nuclides of the same element; *nuclide* is more generic and is used when referencing only one nucleus or several nuclei of different elements. For example, it is more correct to say that an element such as fluorine consists of one stable nuclide rather than that it has one stable isotope.

Isotopes and nuclides are specified by the name of the particular element, implicitly giving the atomic number, followed by a hyphen and the mass number (e.g. helium-3, carbon-12, carbon-13, iodine-131 and uranium-238). In symbolic form, the number of nucleons is denoted as a superscripted prefix to the chemical symbol (e.g. ^{3}He, ^{12}C, ^{13}C, ^{131}I and ^{238}U).

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[1] For further reading please consult the chapter 24 radioactive chemistry of the architecture of chemistry an inorganic approach by the same author.

About 339 nuclides occur naturally on Earth, of which 256 (about 75%) are stable (or, to be careful, have never been observed to decay; this note is necessary because many "stable" isotopes are predicted to be radioactive with very long half-lives). Counting the radioactive nuclides not found in nature that have been created artificially, more than 3100 nuclides are currently known.

History

The term isotope was coined in 1913 by Margaret Todd, a Scottish doctor, during a conversation with Frederick Soddy (to whom she was distantly related by marriage). Soddy, a chemist at Glasgow University, explained that it appeared from his investigations as if several elements occupied each position in the periodic table. Hence Todd suggested the Greek term for "at the same place" as a suitable name. Soddy adopted the term and went on to win the Nobel Prize for Chemistry in 1921 for his work on radioactive substances. Soddy's use of the word isotope was initially with regard to radioactive (unstable) atoms. However, in 1913, as part of his exploration into the composition of canal rays, J. J. Thomson channeled a stream of ionized neon through a magnetic and an electric field and measured its deflection by placing a photographic plate in its path. Thomson observed two patches of light on the photographic plate (see image on right), which suggested two different parabolas of deflection. This was the first observation of different stable isotopes for an element. Thomson eventually concluded that some of the atoms in the neon gas were of higher mass than the rest.
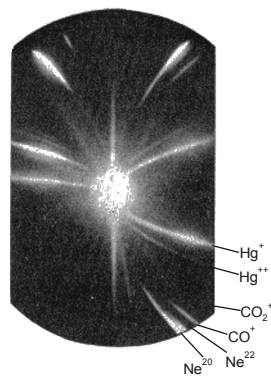
Fig. 4.1 : Showing in the bottom right corner of JJ Thomson's photographic plate are markings for the two isotopes of neon: neon-20 and neon-22

Variation in Properties between Isotopes Chemical and Atomic Properties

A neutral atom has the same number of electrons as protons. Thus, different isotopes of a given element all have the same number of protons and electrons and the same electronic structure, and because the chemical behavior of an atom is largely determined by its electronic structure, different isotopes exhibit nearly identical chemical behavior. The main exception to this is the kinetic isotope effect: due to their larger masses, heavier isotopes tend to react somewhat more slowly than lighter isotopes of the same element. This is most pronounced for protium (^{1}H) vis-à-vis deuterium (^{2}H), because deuterium has twice the mass of protium. The mass effect between deuterium and the relatively light protium also affects the behavior of their respective chemical bonds, by means of changing the center of gravity (reduced mass) of the atomic systems. However, for heavier elements, the absolute mass of nucleus relative to electrons is far more, and the relative mass difference between isotopes is much less, and thus the mass-difference effects on chemistry are usually negligible.
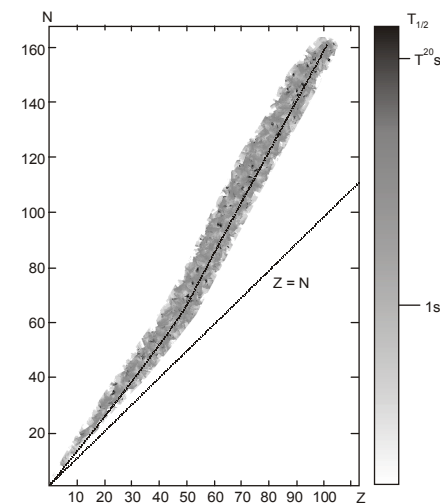
Fig. 4.2 : Isotope half lifes. Note that the darker more stable isotope region departs from the line of protons Z = neutrons N, as the element number Z becomes larger

Similarly, two molecules which differ only in the isotopic nature of their atoms (*isotopologues*) will have identical electronic structure and therefore almost indistinguishable physical and chemical properties (again with deuterium providing the primary exception to this rule). The vibrational modes of a molecule are determined by its shape and by the masses of its constituent atoms. Consequently, isotopologues will have different sets of vibrational modes. Since vibrational modes allow a molecule to absorb photons of corresponding energies, isotopologues have different optical properties in the infrared range.

Nuclear properties and stability

Atomic nuclei consist of protons and neutrons bound together by the strong nuclear force. Because protons are positively charged, they repel each other. Neutrons, which are electrically neutral, allow some separation between the positively charged protons, reducing the electrostatic repulsion. Neutrons also stabilize the nucleus because at short ranges they attract each other and protons equally by the strong nuclear force, and this extra binding force also offsets the electrical repulsion between protons. For this reason, one or more neutrons are necessary for two or more protons to be bound into a nucleus. As the number of protons increases, an increasing ratio of neutrons are needed to form a stable nucleus (see graph at right). For example, although the neutron:proton ratio of ^{3}He is 1:2, the neutron:proton ratio of ^{238}U is greater than 3:2.

Of the 80 elements with a stable isotope, the largest number of stable isotopes observed for any element is ten (for the element tin). Xenon is the only element which has nine stable isotopes. There is no element with exactly eight stable isotopes. See list of elements by nuclear stability for a complete list. Five elements have seven stable isotopes, eight have six stable isotopes, nine have five stable isotopes, nine have four stable isotopes, nine have three stable isotopes, 16 have two stable isotopes (counting Ta-180m as stable), and 26 elements have only a single stable isotope (of these, 19 are so-called mononuclidic elements, having a single primordial stable isotope which dominates and fixes the atomic weight of the natural element to high precision; 3 radioactive mononuclidic elements occur as well).[5]. In total, there are 256 nuclides which have not been observed to decay (see List of elements by nuclear stability). For the 80 elements which have one or more stable isotopes, the average number of stable isotopes is 256/80 = 3.20 isotopes per element.

Other effects besides the bulk ratio of protons and neutrons affect nuclear stability. For example, the extreme stability of helium-4 due to a double pairing of 2 protons and 2 neutrons prevents *any* nuclides containing five nucleons from existing for long enough to serve as platforms for building up of heavier elements during fusion formation in stars (see triple alpha process). A similar pairing pattern shows in the fact that the 256 known stable nuclides contain only five that have both an odd number of protons *and* an odd number of neutrons (odd-odd nuclei): ^{2}H, ^{6}Li, ^{10}B, ^{14}N, ^{180m}Ta (the last one was predicted to decay but this process was never observed). Also, four long-lived radioactive odd-odd nuclides (^{40}K, ^{50}V, ^{138}La, ^{176}Lu) occur naturally. Most odd-odd nuclides are highly unstable with respect to beta decay, because the decay products are even-even, and are therefore more strongly bound, due to nuclear pairing effects.

Although isotopes exhibit nearly identical electronic and chemical behavior, their nuclear behavior varies dramatically. Adding neutrons to isotopes can vary their nuclear spins and nuclear shapes, causing differences in neutron capture cross-sections and gamma spectroscopy and nuclear magnetic resonance properties.

Occurrence in Nature

Elements are composed of one or more naturally occurring isotopes, which are normally stable. Some elements have unstable (radioactive) isotopes, either because their decay is so slow that a fraction still remains since they were created (examples: uranium, potassium), or because they are continually created through cosmic radiation (tritium, carbon-14) or by decay from an isotope in the first category (radium, radon).

As discussed above, only 80 elements have any stable isotopes, and 26 of these have only one stable isotope. Thus, about two-thirds of stable elements occur naturally on Earth in multiple stable isotopes, with the largest number of stable isotopes for an element being ten, for tin (element number 50). There are about 94 elements found naturally on Earth (up to plutonium, element 94, inclusive), though some are detected only in very tiny amounts, such as plutonium-244. Scientists[1] estimate that the elements which occur naturally on Earth (some only as radioisotopes) occur as 339 isotopes (nuclides) in total. Only 256 of these naturally-occurring isotopes are stable in the sense of never having been observed to decay as of the present time. All the known stable isotopes occur naturally on Earth); the other 85 naturally-occurring isotopes are radioactive, but occur on Earth due to their relatively

long half-lives, or else due to other means of ongoing natural production. An additional ~ 2700 radioactive isotopes not found in nature have been created in nuclear reactors and in particle accelerators. Many short-lived isotopes not found naturally on Earth have also been observed by spectroscopic analysis, being naturally created in stars or supernovae. An example is aluminum-26, which is not naturally found on Earth, but which is found in abundance on an astronomical scale.

The tabulated atomic masses of elements are averages that account for the presence of multiple isotopes with different masses. A good example is chlorine, having the composition ^{35}Cl, 75.8%, and ^{37}Cl, 24.2%, giving an atomic mass of 35.5. Values like this confounded scientists before the discovery of isotopes, as most light element atomic masses are close to integer multiples of hydrogen.

According to generally accepted cosmology only isotopes of hydrogen and helium, and traces of some isotopes of lithium, beryllium and boron were created at the Big Bang, while all other isotopes were synthesized later, in stars and supernovae, and in interactions between energetic particles such as cosmic rays, and previously-produced isotopes. The most common isotope of hydrogen has no neutrons at all. (See nucleosynthesis for details of the various processes thought to be responsible for isotope production.) The respective abundances of isotopes on Earth result from the quantities formed by these processes, their spread through the galaxy, and the rates of decay for isotopes that are unstable. After the initial coalescence of the solar system, isotopes were redistributed according to mass, and the isotopic composition of elements varies slightly from planet to planet. This sometimes makes it possible to trace the origin of meteorites.

Atomic Mass of Isotopes

The atomic mass (M_r) of an isotope is determined mainly by its mass number (i.e. number of nucleons in its nucleus). Small corrections are due to the binding energy of the nucleus (see Mass defect), to slightly different masses of neutron and proton, and to the mass of electron shell of the atom. One should take into account that the mass number is an integer dimensionless quantity, whereas the atomic mass is a real number expressed in atomic mass units. But the difference between these quantities is less then 1% in any case.

The atomic masses of naturally occurring isotopes of an element determine the atomic weight of the element. When the element contains N isotopes, the equation below is applied for the atomic weight M:

$$M = M_1 \times \eta_1 + M_2 \times \eta_2 + ... + M_N \times \eta N,$$

where $M_1, M_2, ..., M_N$ are the atomic masses of each individual isotope, and $\eta_1, ..., \eta_N$ are the relative abundances of these isotopes.

Applications of Isotopes

Several applications exist that capitalize on properties of the various isotopes of a given element.

Use of chemical and biological properties

- Isotope analysis is the determination of isotopic signature, the relative abundances of isotopes of a given element in a particular sample. For biogenic substances in particular, significant

variations of isotopes of C, N and O can occur. Analysis of such variations has a wide range of applications, such as the detection of adulteration of food products.[6] The identification of certain meteorites as having originated on Mars is based in part upon the isotopic signature of trace gases contained in them.

- Another common application is isotopic labeling, the use of unusual isotopes as tracers or markers in chemical reactions. Normally, atoms of a given element are indistinguishable from each other. However, by using isotopes of different masses, they can be distinguished by mass spectrometry or infrared spectroscopy (see "Properties"). For example, in 'stable isotope labeling with amino acids in cell culture (SILAC)' stable isotopes are used to quantify proteins. If radioactive isotopes are used, they can be detected by the radiation they emit (this is called *radioisotopic labeling*).

- A technique similar to radioisotopic labelling is radiometric dating: using the known half-life of an unstable element, one can calculate the amount of time that has elapsed since a known level of isotope existed. The most widely known example is radiocarbon dating used to determine the age of carbonaceous materials.

- Isotopic substitution can be used to determine the mechanism of a reaction via the kinetic isotope effect.

Use of nuclear properties

- Several forms of spectroscopy rely on the unique nuclear properties of specific isotopes. For example, nuclear magnetic resonance (NMR) spectroscopy can be used only for isotopes with a nonzero nuclear spin. The most common isotopes used with NMR spectroscopy are ^{1}H, ^{2}D, ^{15}N, ^{13}C, and ^{31}P.

- Mössbauer spectroscopy also relies on the nuclear transitions of specific isotopes, such as ^{57}Fe.

- Radionuclides also have important uses. Nuclear power and nuclear weapons development require relatively large quantities of specific isotopes. The process of isotope separation represents a significant technological challenge, but more so with heavy elements such as uranium or plutonium, than with lighter elements such as hydrogen, lithium, carbon, nitrogen, and oxygen. The lighter elements are commonly separated by gas diffusion of their compounds such as CO and NO. Uranium isotopes have been separated in bulk by gas diffusion, gas centrifugation, laser ionization separation, and (in the Manhattan Project) by a type of production mass spectroscopy.

SUB-SECTION 4.1A—RADIO-ISOTOPES

In most cases, elements like to have an equal number of protons and neutrons because this makes them the most stable. Stable atoms have a binding energy that is strong enough to hold the protons and neutrons together. Even if an atom has an additional neutron or two it may remain stable. However, an additional neutron or two may upset the binding energy and cause the atom to become **unstable**. In an unstable atom, the nucleus changes by giving off a neutron to get back to a balanced state. As the

unstable nucleus changes, it gives off radiation and is said to be radioactive. Radioactive isotopes are often called **radioisotopes**.

SUB-SECTION 3.1B—IONIZING RADIATION

Ionizing radiation consists of subatomic particles or electromagnetic waves that are energetic enough to detach electrons from atoms or molecules, ionizing them. The occurrence of ionization depends on the energy of the impinging individual particles or waves, and not on their number. An intense flood of particles or waves will not cause ionization if these particles or waves do not carry enough energy to be ionizing. Roughly speaking, particles or photons with energies above a few electron volts (eV) are ionizing.

Examples of ionizing particles are energetic alpha particles, beta particles, and neutrons. The ability of electromagnetic waves (photons) to ionize an atom or molecule depends on their wavelength. Radiation on the short wavelength end of the electromagnetic spectrum - ultraviolet, x-rays, and gamma rays - is ionizing.

Ionizing radiation comes from radioactive materials, x-ray tubes, particle accelerators, and is present in the environment. It has many practical uses in medicine, research, construction, and other areas, but presents a health hazard if used improperly. Exposure to radiation causes microscopic damage to living tissue, resulting in skin burns and radiation sickness at high doses and cancer, tumors and genetic damage at low doses.

Types of Radiation

Alpha (α) radiation consists of Helium-4 (^{4}He) nuclei and is stopped by a sheet of paper. Beta (β) radiation, consisting of electrons, is halted by an aluminium plate. Gamma (γ) radiation, consisting of energetic photons, is eventually absorbed as it penetrates a dense material. Neutron (n) radiation consists of free neutrons which are blocked using light elements, like hydrogen, which slow and/or capture them.

Various types of ionizing radiation may be produced by radioactive decay, nuclear fission and nuclear fusion, extremely hot objects via blackbody radiation, and by particle accelerators.

In order for a particle to be ionizing, it must both have a high enough energy and interact with the atoms of a target. Photons interact strongly with charged particles, so photons of sufficiently high energy also are ionizing. The energy at which this begins to happen with photons (light) is in the ultraviolet region of the electromagnetic spectrum; sunburn is one of the effects of ionization. Charged particles such as electrons, positrons, and alpha particles also interact strongly with electrons of an atom or molecule. Neutrons, on the other hand, do not interact strongly with electrons, and so they cannot directly cause ionization by this mechanism. However, fast neutrons will interact with the protons in hydrogen (in the manner of a billiard ball hitting another, sending it away with all of the first ball's energy of motion), and this mechanism produces proton radiation (fast protons). These protons are ionizing because of their strong interaction with electrons in matter. A neutron can also interact with an atomic nucleus, depending on the nucleus and the neutron's velocity; these reactions happen with fast neutrons and slow neutrons, depending on the situation. Neutron interactions in this manner often

produce radioactive nuclei, which produce ionizing radiation when they decay, they then can produce chain reactions in the mass that is decaying, sometimes causing a larger effect of ionization.

In the picture at left, gamma rays are represented by wavy lines, charged particles and neutrons by straight lines. The little circles show where ionization processes occur.

An ionization event normally produces a positive atomic ion and an electron. High-energy beta particles may produce bremsstrahlung when passing through matter, or secondary electrons (δ-electrons); both can ionize in turn.

Unlike alpha or beta particles (see particle radiation), gamma rays do not ionize all along their path, but rather interact with matter in one of three ways: the photoelectric effect, the Compton effect, and pair production. By way of example, the figure shows Compton effect: two Compton scatterings that happen sequentially. In every scattering event, the gamma ray transfers energy to an electron, and it continues on its path in a different direction and with reduced energy.

In the same figure, the neutron collides with a proton of the target material, and then becomes a fast recoil proton that ionizes in turn. At the end of its path, the neutron is captured by a nucleus in an (n,γ)-reaction that leads to a neutron capture photon.

The negatively-charged electrons and positively charged ions created by ionizing radiation may cause damage in living tissue. If the dose is sufficient, the effect may be seen almost immediately, in the form of radiation poisoning. Lower doses may cause cancer or other long-term problems. The effect of the very low doses encountered in normal circumstances (from both natural and artificial sources, like cosmic rays, medical X-rays and nuclear power plants) is a subject of current debate. A 2005 report released by the National Research Council (the BEIR VII report, summarized in [3]) indicated that the overall cancer risk associated with background sources of radiation was relatively low.

Radioactive materials usually release alpha particles, which are the nuclei of helium, beta particles, which are quickly moving electrons or positrons, or gamma rays. Alpha and beta particles can often be stopped by a piece of paper or a sheet of aluminium, respectively. They cause most damage when they are emitted inside the human body. Gamma rays are less ionizing than either alpha or beta particles, and protection against gammas requires thicker shielding. The damage they produce is similar to that caused by X-rays, and include burns and also cancer, through mutations. Human biology resists germline mutation by either correcting the changes in the DNA or inducing apoptosis in the mutated cell.

Non-ionizing radiation is thought to be essentially harmless below the levels that cause heating. Ionizing radiation is dangerous in direct exposure, although the degree of danger is a subject of debate. Humans and animals can also be exposed to ionizing radiation internally: if radioactive isotopes are present in the environment, they may be taken into the body. For example, radioactive iodine is treated as normal iodine by the body and used by the thyroid; its accumulation there often leads to thyroid cancer. Some radioactive elements also bioaccumulate.

The units used to measure ionizing radiation are rather complex. The ionizing effects of radiation are measured by units of exposure:

- The coulomb per kilogram (C/kg) is the SI unit of ionizing radiation exposure, and measures the amount of radiation required to create 1 coulomb of charge of each polarity in 1 kilogram of matter.

Table 4.1 : Weighting factors W$_R$ for equivalent dose

Radiation	Energy	wR
x-rays, gamma rays, electrons, positrons, muons	1	
neutrons	< 10 keV	5
	10 keV - 100 keV	10
	100 keV - 2 MeV	20
	2 MeV - 20 MeV	10
	> 20 MeV	5
protons	> 2 MeV	2
alpha particles, fission fragments, heavy nuclei		20

Interaction of ionizing Radiation with Matter
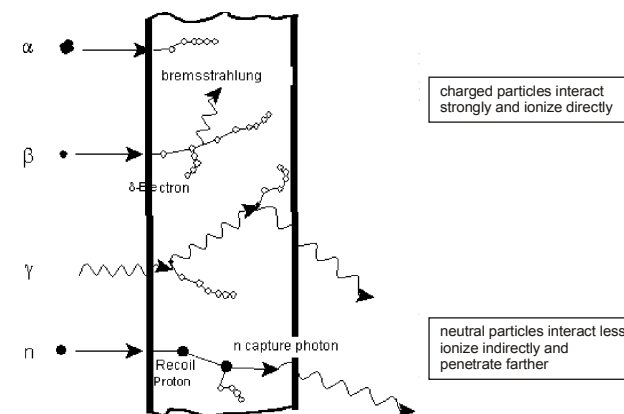
Fig. 4.2 : Showing the Ionization of radiation

- The roentgen (R) is an older traditional unit that is almost out of use, which represented the amount of radiation required to liberate 1 esu of charge of each polarity in 1 cubic centimeter of dry air. 1 Roentgen = 2.58×10^{-4} C/kg

However, the amount of damage done to matter (especially living tissue) by ionizing radiation is more closely related to the amount of energy deposited rather than the charge. This is called the absorbed dose.

- The gray (Gy), with units J/kg, is the SI unit of absorbed dose, which represents the amount of radiation required to deposit 1 joule of energy in 1 kilogram of any kind of matter.
- The rad (Roentgen absorbed dose), is the corresponding traditional unit which is 0.01 J deposited per kg. 100 rad = 1 Gy.

Equal doses of different types or energies of radiation cause different amounts of damage to living tissue. For example, 1 Gy of alpha radiation causes about 20 times as much damage as 1 Gy of x-rays. Therefore the equivalent dose was defined to give an approximate measure of the biological effect of radiation. It is calculated by multiplying the absorbed dose by a weighting factor W_R which is different for each type of radiation (see above table).

- The sievert (Sv) is the SI unit of equivalent dose. Although it has the same units as grays, J/kg, it measures something different. It is the dose of any type of radiation in Gy that has the same biological effect on a human as 1 Gy of x-rays or gamma radiation.
- The rem (Roentgen equivalent man) is the traditional unit of equivalent dose. 1 sievert = 100 rem. Because the rem is a relatively large unit, typical equivalent dose is measured in millirem (mrem), 10^{-3} rem, or in microsievert (μSv), 10^{-6} Sv. 1 mrem = 10 μSv.

For comparison, the 'background' dose of natural radiation received by a US citizen is around 3 mSv (300 mrem) per year. The lethal dose of radiation for a human is around 4 - 5 Sv (400 - 500 rem).

Uses

Ionizing radiation has many uses, such as to kill cancerous cells. However, although ionizing radiation has many applications, overuse can be hazardous to human health. For example, at one time, assistants in shoe shops used X-rays to check a child's shoe size, but this practice was halted when it was discovered that ionizing radiation was dangerous.

Technical uses of Ionizing Radiation

Since ionizing radiations can penetrate matter, they are used for a variety of measuring methods.

Radiography by means of gamma or X rays

This is a method used in industrial production. The piece to be radiographed is placed between the source and a photographic film in a cassette. After a certain exposure time, the film is developed and it shows internal defects of the material if there are any.

Gauges

Gauges use the exponential absorption law of gamma rays

- Level indicators: Source and detector are placed at opposite sides of a container, indicating the presence or absence of material in the horizontal radiation path. Beta or gamma sources are used, depending on the thickness and the density of the material to be measured. The method is used for containers of liquids or of grainy substances
- Thickness gauges: if the material is of constant density, the signal measured by the radiation detector depends on the thickness of the material. This is useful for continuous production, like of paper, rubber, etc.

Applications using ionization of gases by radiation

- To avoid the build-up of static electricity in production of paper, plastics, synthetic textiles, etc., a ribbon-shaped source of the alpha emitter ^{241}Am can be placed close to the material at the end of the production line. The source ionizes the air to remove electric charges on the material.
- Smoke detector: Two ionisation chambers are placed next to each other. Both contain a small source of ^{241}Am that gives rise to a small constant current. One is closed and serves for comparison, the other is open to ambient air; it has a gridded electrode. When smoke enters the open chamber, the current is disrupted as the smoke particles attach to the charged ions and restore them to a neutral electrical state. This reduces the current in the open chamber. When the current drops below a certain threshold, the alarm is triggered.
- Radioactive tracers for industry: Since radioactive isotopes behave, chemically, mostly like the inactive element, the behavior of a certain chemical substance can be followed by *tracing* the radioactivity. Examples:
  - Adding a gamma tracer to a gas or liquid in a closed system makes it possible to find a hole in a tube.
  - Adding a tracer to the surface of the component of a motor makes it possible to measure wear by measuring the activity of the lubricating oil.

Potential electricity generation through nanomaterials

Using layers of carbon nanotubes interlaced with gold and lithium hydride, has been shown to produce a current when the gold particles are hit by radiation, releasing electrons which can travel through the carbon nanotubes to the lithium hydride, and then to electrodes in order to generate electricity.

Biological and medical applications of ionizing radiation

In biology, radiation is mainly used for sterilization, and enhancing mutations. For example, mutations may be induced by radiation to produce new or improved species. A very promising field is the sterile insect technique, where male insects are sterilized and liberated in the chosen field, so that they have no descendants, and the population is reduced.

Radiation is also useful in sterilizing medical hardware or food. The advantage for medical hardware is that the object may be sealed in plastic before sterilization. For food, there are strict regulations to prevent the occurrence of induced radioactivity. The growth of a seedling may be enhanced by radiation, but excessive radiation will hinder growth.

Electrons, x rays, gamma rays or atomic ions may be used in radiation therapy to treat malignant tumors (cancer). Furthermore, just like in industrial application, x rays can also be used in radiography to create images of hard-to-image objects, such as inside one's body.

Tracer methods are used in nuclear medicine in a way analogous to the technical uses mentioned above.

SUB-SECTION 4.1C—RADIOIMMUNOASSAY

Radioimmunoassay (RIA) is a scientific method used to test antigens (for example, hormone levels in the blood) without the need to use a bioassay. It was developed by Rosalyn Yalow and Solomon Aaron Berson in the 1950s. In 1977, Rosalyn Sussman Yalow received the Nobel Prize in Medicine for the development of the RIA for insulin: the precise measurement of minute amounts of such a hormone was considered a breakthrough in endocrinology.

Although the RIA technique is extremely sensitive and extremely specific, it requires a sophisticated apparatus and is costly. It also requires special precautions, since radioactive substances are used. Therefore, today it has been largely supplanted by the ELISA method, where the antigen-antibody reaction is measured using colorometric signals instead of a radioactive signal.

To perform a radioimmunoassay, a known quantity of an antigen is made radioactive, frequently by labeling it with gamma-radioactive isotopes of iodine attached to tyrosine. This radiolabeled antigen is then mixed with a known amount of antibody for that antigen, and as a result, the two chemically bind to one another. Then, a sample of serum from a patient containing an unknown quantity of that same antigen is added. This causes the unlabeled (or "cold") antigen from the serum to compete with the radiolabeled antigen for antibody binding sites.

As the concentration of "cold" antigen is increased, more of it binds to the antibody, displacing the radiolabeled variant, and reducing the ratio of antibody-bound radiolabeled antigen to free radiolabeled antigen. The bound antigens are then separated from the unbound ones, and the radioactivity of the free antigen remaining in the supernatant is measured. A binding curve can then be plotted, and the exact amount of antigen in the patient's serum can be determined.

With this technique, separating bound from unbound antigen is crucial. Initially, the method of separation employed was the use of a second "anti-antibody" directed against the first for precipitation and centrifugation.

General Procedure for Performing a RIA Analysis

- Mix sample containing drug with fixed quantity of labeled drug and antibody
- Allow to equilibrate - incubate
- Separate drug bound to antibody from unbound drug
    - Charcoal adsorption of antibody (and bound drug)
    - Antibody - antibody binding precipitates bound drug
    - Antibody bonded to container
- Measure radioactivity associated with bound labeled drug
    - low drug concentration means more bound radioactivity and higher measurement
    - high drug concentration means less bound radioactivity and lower measurement
- Determine standard curve
    - Non-linear plot of radioactivity versus concentration
    - Logit-log concentration plot is linear

A Blank and Three Standard Samples
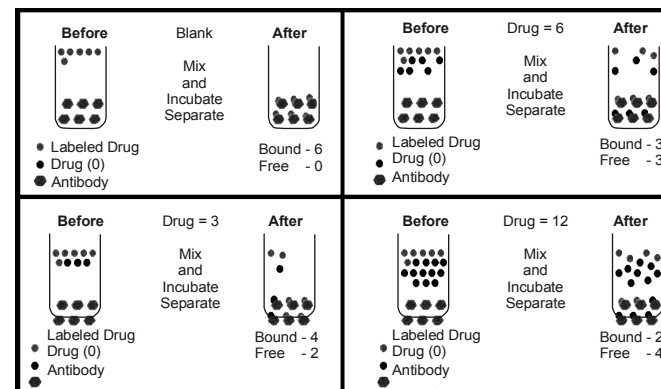
Fig. 4.4 : RIA before and after Incubation—Blank and Three Standard Samples

Table 4.4 : Total Bound and Free Drug Concentrations

Total [Drug]	Bound [Drug]	Free [Drug]
0	6	0
3	4	2
6	3	3
12	2	4
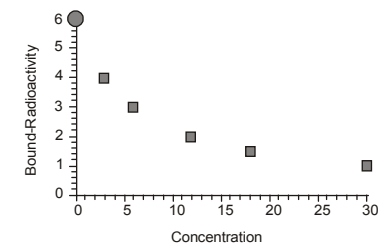
Fig. 4.5 : Plot of Bound versus Total Drug Concentration
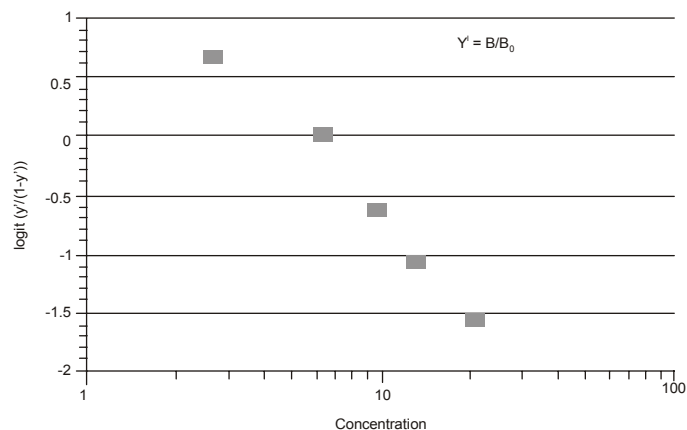
Fig. 4.6 : Logit versus Log Total C Plot

SECTION 4.2—COLLOID AND SECTION 4.2A—CRYSTALLOID[1]

A body that in solution can pass through a semipermeable membrane, as distinguished from a colloid, which cannot do so.

SUB-SECTION 4.2B—OSMOTIC PRESSURE

Generaly if two solutions of the same substance but of different concentrations be placed in contact with each other there will be a tendency on the one part of the solutions to equalize concentrations by movements of the solute from the more concentrated solution to the dilute one by diffiusion amd movement of solvent from the dilute solution to the concentrated one. This tendency proves the fact that the free energy of the solute in the concentrated solution is greater than that in the dilute one and hence there will be a tendency for equalization of concentration by movement of growth. The free energy of the solvent is greater in
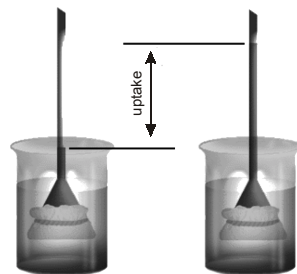
Fig. 4.7 : Showing the Experiment of Osmosis

[2] See chapter 20 Solution and solubility. The architecture of chem author.

the dilute solution and hence the solvent will also flow in the opposite direction. If, however, we do not allow the solute by the help of a semi-permeable membrane which allows the solvent molecules to pass through but stops movement of the solute (like animal bladder or copper ferricyanide paper membrane) the solvent will flow from the dilute solution to the more concentrated one and will tend to dilute it. This phenomenon is osmosis. The reason for this phenomenon of osmosis can be explained by the help of Le Chatelier's Principle because the solution (under strain) will tend to go back to the state of pure solvent in its attempt undergo the effect of the stain.

This osmosis experiment can be demonstrated simply by closing the broad end of a thistle funnel by an animal bladder or a parchment paper (see fig 119 and 119A). This is then filled up with a sugar solution and dipped in the beaker containing plain water, such that the level of the solution inside the funnel is equal to the water level inside the beaker, and outside the funnel. After about 4 to 5 hours the solution level inside the funnel will be seen raised up indicating the entrance of the water from the beaker.

Fig. 4.8 : Showing the Lab set up for Osmosis experiment

After 24 hours the level will not rise any further but halt at some height. This height will exert a pressure which is equal and opposite to what is called the osmotic pressure of the solution. The tendency on the part of the solution to get itself diluted it attracts the solvent towards itself. This attractive force per unit area is the *osmotic pressure*. This being balanced by the hydrostatic pressure set up in the funnel. This hydrostatic pressure is therefore the measure of the osmotic pressure in the funnel.

SUB-SECTION 4.2C—COLLOIDAL OSMOTIC PRESSURE

Oncotic pressure, or **colloid osmotic pressure**, is a form of osmotic pressure exerted by proteins in blood plasma that usually tends to pull water into the circulatory system.

Throughout the body, dissolved compounds have an osmotic pressure. Because large plasma proteins cannot easily cross through the capillary walls, their effect on the osmotic pressure of the capillary interiors will, to some extent, balance out the tendency for fluid to leak out of the capillaries. In other words, the oncotic pressure tends to pull fluid into the capillaries. In conditions where plasma proteins are reduced, e.g. from being lost in the urine (proteinuria) or from malnutrition, the result of low oncotic pressure can be excess fluid buildup in the tissues (edema).

Oncotic pressure is represented by the symbol ð in the Starling equation and elsewhere.

Used in Intravenous Therapy

Clinically, there are two types of fluids that are used for intravenous drips; crystalloids and colloids. Crystalloids are aqueous solutions of mineral salts or other water-soluble molecules. Colloids contain larger insoluble molecules, such as gelatin; blood itself is a colloid.

Colloids preserve a high *colloid osmotic pressure* in the blood, while, on the other hand, this parameter is decreased by crystalloids due to hemodilution. However, there is still controversy to the actual difference in efficacy by this difference. Another difference is that crystalloids generally are much cheaper than colloids.

Osmotic pressure and oncotic pressure can be *measured* by suitable instruments. They can also be *calculated* for an ideal solution by appropriate substitutions in the van't Hoff equation

Osmotic pressure = n x (c/M) x RT

where:
- n is the number of particles into which the substance dissociates (n = 1 for plasma proteins)
- c is the concentration in G/l
- M is the MW of the molecules
- c/M is thus the molar concentration of the substance
- R is the universal gas constant
- T is the absolute temperature (K)

As an example, if values are substituted in this equation for a typical plasma sample:
- T = 310K (ie temp of 37C)
- R = 0.082
- n = 1 (for plasma proteins as they do not dissociate)

and:
- Multiplying by 0.001 to convert from Osmoles to mOsmoles
- Multiplying by 760 to convert the result from atmospheres to mmHg
- Multiplying by 280 to convert the osmotic pressure per mOsm/kg to a value for plasma with an osmolality of 280 mOsm/kg

then:

Total plasma osmotic pressure = 1 x 0.082 x 310 x 0.001 x 760 x 280 = 5409 mmHg

For a plasma osmolality of 280 mOsm/kg at 37C, total osmotic pressure is about 5409mmHg (ie about 7.1 atmospheres!)

Each mOsm/kg of solute contributes about 19.32mmHg to the osmotic pressure

Now consider the case of plasma proteins alone and calculate the colloid osmotic (oncotic) pressure.

Using typical values for concentration & MW of the plasma proteins, the protein concentration is about 0.9 mOsmol/kg which predicts an oncotic pressure of 17.3 mmHg (ie 19.32 x 0.9). Measurement

in an oncometer shows the actual plasma oncotic pressure is about 25 mmHg which is equivalent to a plasma protein concentration of 1.3 mmol/kg.

SECTION 4.3—ACID BASES AND pH[3]

For thousands of years people have known that vinegar, lemon juice and many other foods taste sour. However, it was not until a few hundred years ago that it was discovered why these things taste sour - because they are all acids. The term acid, in fact, comes from the Latin term *acere*, which means "sour". While there are many slightly different definitions of acids and bases, in this lesson we will introduce the fundamentals of acid/base chemistry.

In the seventeenth century, the Irish writer and amateur chemist Robert Boyle first labeled substances as either acids or bases (he called bases alkalies) according to the following characteristics:
- **Acids** taste sour, are corrosive to metals, change litmus (a dye extracted from lichens) red, and become less acidic when mixed with bases.
- **Bases** feel slippery, change litmus blue, and become less basic when mixed with acids.

While Boyle and others tried to explain why acids and bases behave the way they do, the first reasonable definition of acids and bases would not be proposed until 200 years later.

In the late 1800s, the Swedish scientist Svante Arrhenius proposed that water can dissolve many compounds by separating them into their individual ions. Arrhenius suggested that **acids** are compounds that contain hydrogen and can dissolve in water to release hydrogen ions into solution. For example, hydrochloric acid (HCl) dissolves in water as follows:

$$HCl \xrightarrow{H_2O} H^+(aq) + Cl^-(aq)$$
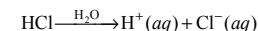
Arrhenius defined bases as substances that dissolve in water to release hydroxide ions (OH-) into solution. For example, a typical base according to the Arrhenius definition is sodium hydroxide (NaOH):

$$NaOH \xrightarrow{H_2O} Na^+(aq) + OH^-(aq)$$
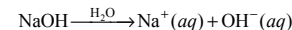
The Arrhenius definition of acids and bases explains a number of things. Arrhenius's theory explains why all acids have similar properties to each other (and, conversely, why all bases are similar): because all acids release H^+ into solution (and all bases release OH-). The Arrhenius definition also explains Boyle's observation that acids and bases counteract each other. This idea, that a base can make an acid weaker, and vice versa, is called neutralization.

Neutralization: As you can see from the equations, acids release H^+ into solution and bases release OH-. If we were to mix an acid and base together, the H^+ ion would combine with the OH- ion to make the molecule H_2O, or plain water:

$$H^+(aq) + OH^-(aq) \rightarrow H_2O$$

---

[3] To read further about this please consult chapter 18. The Architecture of Chemistry an Inorganic approach by the same author.

The neutralization reaction of an acid with a base will always produce water and a salt, as shown below:

Acid		**Base**		**Water**		**Salt**
HCl	+	NaOH	→	H_2O	+	NaCl
HBr	+	KOH	→	H_2O	+	KBr

Though Arrhenius helped explain the fundamentals of acid/base chemistry, unfortunately his theories have limits. For example, the Arrhenius definition does not explain why some substances, such as common baking soda ($NaHCO_3$), can act like a base even though they do not contain hydroxide ions.

In 1923, the Danish scientist Johannes Brønsted and the Englishman Thomas Lowry published independent yet similar papers that refined Arrhenius' theory. In Brønsted's words, "... acids and bases are substances that are capable of splitting off or taking up hydrogen ions, respectively." The Brønsted-Lowry definition broadened the Arrhenius concept of acids and bases.

The Brønsted-Lowry definition of acids is very similar to the Arrhenius definition, any substance that can donate a hydrogen ion is an acid (under the Brønsted definition, acids are often referred to as **proton** donors because an H^+ ion, hydrogen minus its electron, is simply a proton).

The Brønsted definition of **bases** is, however, quite different from the Arrhenius definition. The Brønsted base is defined as any substance that can accept a hydrogen ion. In essence, a base is the opposite of an acid. NaOH and KOH, as we saw above, would still be considered bases because they can accept an H^+ from an acid to form water. However, the Brønsted-Lowry definition also explains why substances that do not contain OH^- can act like bases. Baking soda ($NaHCO_3$), for example, acts like a base by accepting a hydrogen ion from an acid as illustrated below:

Acid		**Base**		**Salt**		
HCl	+	$NaHCO_3$	→	H_2CO_3	+	NaCl
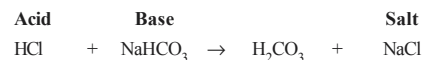
pH

Under the Brønsted-Lowry definition, both acids and bases are related to the concentration of hydrogen ions present. Acids increase the concentration of hydrogen ions, while bases decrease the concentration of hydrogen ions (by accepting them). The acidity or basicity of something therefore can be measured by its hydrogen ion concentration.

In 1909, the Danish biochemist Sören Sörensen invented the pH scale for measuring acidity. The pH scale is described by the formula:

pH = -log [H^+]-Note: concentration is commonly abbreviated by using square brackets, thus [H^+] = hydrogen ion concentration. When measuring pH, [H^+] is in units of moles of H^+ per liter of solution.

For example, a solution with [H^+] = 1 x 10^{-7} moles/liter has a pH equal to 7 (a simpler way to think about pH is that it equals the exponent on the H^+ concentration, ignoring the minus sign). The pH scale ranges from 0 to 14. Substances with a pH between 0 and less than 7 are acids (pH and [H^+] are inversely related - lower pH means higher [H^+]). Substances with a pH greater than 7 and up to 14 are bases (higher pH means lower [H^+]). Right in the middle, at pH = 7, are neutral substances, for

example, pure water. The relationship between [H^+] and pH is shown in the table below alongside some common examples of acids and bases in everyday life.

SECTION 4.3

In chemistry, the **Henderson–Hasselbalch** (often misspelled as *Henderson–Hasselbach*) **equation** describes the derivation of pH as a measure of acidity (using pK_a, the acid dissociation constant) in biological and chemical systems. The equation is also useful for estimating the pH of a buffer solution and finding the equilibrium pH in acid-base reactions (it is widely used to calculate isoelectric point of the proteins).

Two equivalent forms of the equation are

$$pH = pK_a + \log \frac{[A^-]}{[HA]}$$

and

$$pH = pK_a + \log \left(\frac{[base]}{[acid]} \right)$$

Here, pK_a is $-\log(K_a)$ where K_a is the acid dissociation constant, that is:

for the non-specific Brønsted acid-base reaction:

In these equations, A^- denotes the ionic form of the relevant acid. Bracketed quantities such as [base] and [acid] denote the molar concentration of the quantity enclosed.

In analogy to the above equations, the following equation is valid:

Where BH^+ denotes the salt of the corresponding base B.

SECTION 4.4—BUFFER

A **buffer solution** is an aqueous solution consisting of a mixture of a weak acid and its conjugate base or a weak base and its conjugate acid. It has the property that the pH of the solution changes very little when a small amount of acid or base is added to it. Buffer solutions are used as a means of keeping pH at a nearly constant value in a wide variety of chemical applications.

Theory

In a simple buffer solution there is an equilibrium between a weak acid, HA, and its conjugate base, A^-.

$$HA + H_2O \ H_3O^+ + A^-$$
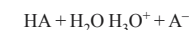
When hydrogen ions are added to the solution the equilibrium moves to the left, in accordance with Le Chatelier's principle, as there are hydrogen ions on the right-hand side of the equilibrium expression.

When hydroxide ions are added the equilibrium moves to the right as hydrogen ions are removed in the reaction $H^+ + OH^- \rightarrow H_2O$. Thus, some of the added reagent is consumed in shifting the equilibrium and the pH changes by less than it would do if the solution were not buffered.

The acid dissociation constant for a weak acid, HA, is defined as

$$K_a = \frac{[H^+][A^-]}{[HA]}$$

Simple manipulation with logarithms gives the Henderson-Hasselbalch equation, which describes pH in terms of pK_a

$$pH = pK_a + \log_{10}\frac{[A^-]}{[HA]}.$$

In this equation $[A^-]$ is the concentration of the conjugate base and $[HA]$ is the concentration of the acid. It follows that when the concentrations of acid and conjugate base are equal, often described as half-neutralization, $pH = pK_a$. In general, the pH of a buffer solution may be easily calculated, knowing the composition of the mixture, by means of an ICE table.

The same considerations apply to a mixture of a weak base, B and its conjugate acid BH^+.

$$B + H_2O \rightleftharpoons BH^+ + OH^-.$$
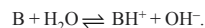
The pK_a value to be used is that of the acid conjugate to the base.

In general a buffer solution may be made up of more than one weak acid and its conjugate base; if the individual buffer regions overlap a wider buffer region is created by mixing the two buffering agents.

Buffer Capacity

Buffer capacity is a quantitative measure of the resistance of a buffer solution to pH change on addition of hydroxide ions. It can be defined as follows.

$$\text{buffer capacity} = \frac{dn}{d(pH)}$$

where dn is an infinitesimal amount of added base and d(pH) is the resulting infinitesimal change in pH. With this definition the buffer capacity can be expressed as

$$\frac{dn}{d(pH)} = 2.303\left(\frac{K_w}{[H^+]} + [H^+] + \frac{C_A K_a [H^+]}{\left(K_a + [H^+]\right)^2}\right)$$
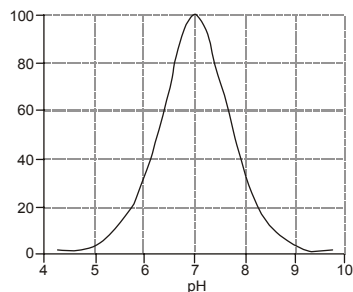
Fig. 4.10 : Showing the graph Buffer capacity for pK_a=7 as percentage of maximum

where K_w is the self-ionization constant of water and C_A is the analytical concentration of the acid, equal to $[HA]+[A^-]$. The term $K_w/[H^+]$ becomes significant at pH greater than about 11.5 and the second term becomes significant at pH less than about 2. Both these terms are properities of water and are independent of the weak acid. Considering the third term, it follows that

Buffer capacity of a weak acid reaches its maximum value when $pH = pK_a$

At $pH = pK_a \pm 1$ the buffer capacity falls to 33% of the maximum value. This is the approximate range within which buffering by a weak acid is effective. Note: at $pH = pK_a - 1$, The Henderson-Hasselbalch equation shows that the ratio $[HA]:[A^-]$ is 10:1.

Buffer capacity is directly proportional to the analytical concentration of the acid.

Applications

Their resistance to changes in pH makes buffer solutions very useful for chemical manufacturing and essential for many biochemical processes. The ideal buffer for a particular pH has a pK_a equal to that pH, since such a solution has maximum buffer capacity.

Buffer solutions are necessary to keep the correct pH for enzymes in many organisms to work. Many enzymes work only under very precise conditions; if the pH strays too far out of the margin, the enzymes slow or stop working and can denature, thus permanently disabling its catalytic activity. A buffer of carbonic acid (H_2CO_3) and bicarbonate (HCO_3^-) is present in blood plasma, to maintain a pH between 7.35 and 7.45.Industrially, buffer solutions are used in fermentation processes and in setting the correct conditions for dyes used in colouring fabrics. They are also used in chemical analysis and calibration of pH meters.Majority of biological samples that are used in research are made in buffers specially PBS (phosphate buffer saline) at pH 7.4.

Useful buffer mixtures

Components	pH range
HCl, sodium citrate	1 - 5
citric acid, sodium citrate	2.5 - 5.6
acetic acid, sodium acetate	3.7 - 5.6
Na_2HPO_4, NaH_2PO_4	6 - 9
Borax, sodium hydroxide	9.2 - 11

"Universal" buffer mixtures

By combining substances with pK_a values differing by only two or less and adjusting the pH a wide-range of buffers can be obtained. Citric acid is a useful component of a buffer mixture because it has three pK_a values, separated by less than two. The buffer range can be extended by adding other buffering agents. The following two-component mixtures have a buffer range of pH 3 to 8.

0.2M Na_2HPO_4 /mL	0.1M Citric Acid /mL	pH...
20.55	79.45	3.0
38.55	61.45	4.0
51.50	48.50	5.0
63.15	36.85	6.0
82.35	17.65	7.0
97.25	2.75	8.0

A mixture containing citric acid, potassium dihydrogen phosphate, boric acid, and diethyl barbituric acid can be made to cover the pH range 2.6 to 12.

Table 4.11 : Common buffer compounds used in biology

Common Name	pK_a at 25°C	Buffer Range	Temp Effect d pH/d T in (1/K) **	Mol. Weight	Full Compound Name
TAPS	8.43	7.7–9.1	–0.018	243.3	3-{[tris(hydroxymethyl) methyl] amino} propanesulfonic acid
Bicine	8.35	7.6–9.0	–0.018	163.2	N,N-bis(2-hydroxyethyl) glycine
Tris	8.06	7.5–9.0	–0.028	121.14	tris (hydroxymethyl) methylamine
Tricine	8.05	7.4–8.8	–0.021	179.2	N-tris (hydroxymethyl) methylglycine
HEPES	7.48	6.8–8.2	–0.014	238.3	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
TES	7.40	6.8–8.2	–0.020	229.20	2-{[tris(hydroxymethyl) methyl] amino} ethanesulfonic acid
MOPS	7.20	6.5–7.9	–0.015	209.3	3-(N-morpholino) propanesulfonic acid
PIPES	6.76	6.1–7.5	–0.008	302.4	piperazine-N,N2 -bis (2-ethanesulfonic acid)
Cacodylate	6.27	5.0–7.4		138.0	dimethylarsinic acid
MES	6.15	5.5–6.7	–0.011	195.2	2-(N-morpholino) ethanesulfonic

The kidneys and the lungs work together to help maintain a blood pH of 7.4 by affecting the components of the buffers in the blood. Therefore, to understand how these organs help control the pH of the blood, we must first discuss how buffers work in solution.

Acid-base buffers confer resistance to a change in the pH of a solution when hydrogen ions (protons) or hydroxide ions are added or removed. An acid-base buffer typically consists of a **weak acid**, and its **conjugate base** (salt) (see Equations 2-4 in the blue box, below). Buffers work because the concentrations of the weak acid and its salt are large compared to the amount of protons or hydroxide ions added or removed. When protons are added to the solution from an external source, some of the base component of the buffer is converted to the weak-acid component (thus using up most of the protons added); when hydroxide ions are added to the solution (or, equivalently, protons are removed from the solution; see Equations 8-9 in the blue box, below), protons are dissociated from some of the weak-acid molecules of the buffer, converting them to the base of the buffer (and thus replenishing most of the protons removed). However, the change in acid and base concentrations is small relative to the amounts of these species present in solution. Hence, the **ratio** of acid to base changes only slightly. Thus, the effect on the pH of the solution is small, within certain limitations on the amount of H^+ or OH^- added or removed.

The Carbonic-Acid-Bicarbonate Buffer in the Blood

By far the most important buffer for maintaining acid-base balance in the blood is the carbonic-acid-bicarbonate buffer. The simultaneous equilibrium reactions of interest are

$$H^+(aq) + HCO_3^-(aq) \rightleftharpoons H_2CO_3(aq) \rightleftharpoons H_2O(l) + CO_2(g) \qquad (1)$$
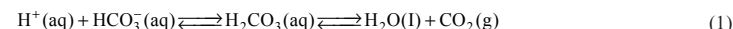
We are interested in the change in the pH of the blood; therefore, we want an expression for the concentration of H^+ in terms of an equilibrium constant (see blue box, below) and the concentrations of the other species in the reaction (HCO_3^-, H_2CO_3, and CO_2).

Recap of Fundamental Acid-Base Concepts

An **acid** is a chemical species that can donate a proton (H^+), and a **base** is a species that can accept (gain) a proton, according to the common Brønstead-Lowry definition. (A subset of the Brønstead-Lowry definition for aqueous solutions is the Arrhenius definition, which defines an acid as a proton producer and a base as a hydroxide (OH^-) producer.) Hence, the conjugate base of an acid is the species formed after the acid loses a proton; the base can then gain another proton to return to the acid. In solution, these two species (the acid and its conjugate base) exist in equilibrium.

Recall from this and earlier experiments in Chem 151 and 152 the definition of pH:

$$pH = -\log\left[H^+\right]_{,-2} \qquad (2)$$

where $[H^+]$ is the molar concentration of protons in aqueous solution. When an acid is placed in water, free protons are generated according to the general reaction shown in Equation 3. **Note**: HA and A^- are generic symbols for an acid and its deprotonated form, the conjugate base.

$$\begin{array}{ccccccc} \text{acid} & & & & & \text{conjugatedbase} & \\ HA & + & H_2O & \rightleftharpoons & H_3O^+ & + & A \\ & & \text{base} & & \text{conjugatedacid} & & \end{array} \qquad (3)$$
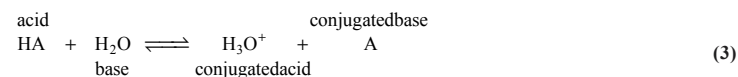
Equation 3 is useful because it clearly shows that HA is a Brønstead-Lowry acid (giving up a proton to become A⁻) and water acts as a base (accepting the proton released by HA). However, the nomenclature H_3O^+ is somewhat misleading, because the proton is actually solvated by many water molecules. Hence, the equilibrium is often written as Equation 4, **where H₂O is the base**:

$$HA \rightleftharpoons H^+ + A^- \tag{4}$$

The Law of Mass Action and Equilibrium Constants

Using the Law of Mass Action, which says that for a balanced chemical equation of the type

$$aA + bB \xrightleftharpoons{K} cC + dD \tag{5}$$
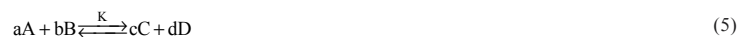
in which A, B, C, and D are chemical species and a, b, c, and d are their stoichiometric coefficients, a constant quantity, known as the **equilibrium constant** (K), can be found from the expression:

$$K = \frac{[C]^c [D]^d}{[A]^a [B]^b} \tag{6}$$

where the brackets indicate the concentrations of species A, B, C, and D **at equilibrium**.

Equilibrium Constant for an Acid-Base Reaction

Using the Law of Mass Action, we can also define an equilibrium constant for the acid dissociation equilibrium reaction in Equation 4. This equilibrium constant, known as K_a, is defined by Equation 7:

$$K_a = \frac{\left[H^+\right]\left[A^-\right]}{[HA]} \tag{7}$$

Equilibrium Constant for the Dissociation of Water

One of the simplest applications of the Law of Mass Action is the dissociation of water into H⁺ and OH⁻ (Equation 8).

$$H_2O(l) \rightleftharpoons H^+(aq) + OH^-(aq) \tag{8}$$

The equilibrium constant for this dissociation reaction, known as K_w, is given by

$$K_w = \left[H^+\right]\left[OH^-\right] \tag{9}$$

(H₂O is not included in the equilibrium-constant expression because it is a pure liquid.) Hence, we can see that increasing the OH⁻ concentration of an aqueous solution has the effect of decreasing the H⁺ concentration, because the product of these two concentrations must remain constant at a given temperature. Thus, in water, the equilibrium in Equation 8 underlies the equivalency of the Brønstead-Lowry definition of a base (an H⁺ acceptor) and the Arrhenius definition of a base (an OH⁻ producer).

To more clearly show the two equilibrium reactions in the carbonic-acid-bicarbonate buffer, Equation 1 is rewritten to show the direct involvement of water:

$$H_3O + (aq) + HCO_3^- (aq) \underset{}{\overset{K_1}{\rightleftharpoons}} H_2CO_3(aq) + H_2O(l) \underset{}{\overset{K_2}{\rightleftharpoons}} 2 H_2O(l) + CO_2(g) \tag{10}$$
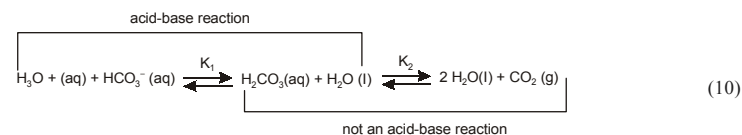
The equilibrium on the left is an acid-base reaction that is written in the reverse format from Equation 3. Carbonic acid (H_2CO_3) is the acid and water is the base. The conjugate base for H_2CO_3 is HCO_3^- (bicarbonate ion). (**Note:** To view the three-dimensional structure of HCO_3^-, consult the Table of Common Ions in the Periodic Properties tutorial from Chem 151.) Carbonic acid also dissociates rapidly to produce water and carbon dioxide, as shown in the equilibrium on the right of Equation 10. This second process is not an acid-base reaction, but it is important to the blood's buffering capacity, as we can see from Equation 11, below.

$$pH = pK - \log\left(\frac{[CO_2]}{HCO_3^-}\right) \tag{11}$$

The derivation for this equation is shown in the yellow box, below. Notice that Equation 11 is in a similar form to the **Henderson-Hasselbach equation** presented in the introduction to the Experiment (Equation 16 in the lab manual). Equation 11 does not meet the strict definition of a Henderson-Hasselbach equation, because this equation takes into account a non-acid-base reaction (*i.e.,* the dissociation of carbonic acid to carbon dioxide and water), and the ratio in parentheses is not the concentration ratio of the acid to the conjugate base. However, the relationship shown in Equation 11 is frequently referred to as the Henderson-Hasselbach equation for the buffer in physiological applications.

In Equation 11, pK is equal to the negative log of the equilibrium constant, K, for the buffer (Equation 12).

where K=K_a/K_2 (from Equation 10).

$$pK = -\log K \tag{12}$$

This quantity provides an indication of the degree to which HCO_3^- reacts with H⁺ (or with H_3O^+ as written in Equation 10) to form H_2CO_3, and subsequently to form CO_2 and H_2O. In the case of the carbonic-acid-bicarbonate buffer, pK=6.1 at normal body temperature.

Derivation of the pH Equation for the Carbonic-Acid-Bicarbonate Buffer

We may begin by defining the equilibrium constant, K_1, for the left-hand reaction in Equation 10, using the Law of Mass Action:

$$K_1 = \frac{[H_2CO_3]}{\left[H^+\right]\left[HCO_3^-\right]} \tag{13}$$

K_a (see Equation 9, above) is the equilibrium constant for the acid-base reaction that is the reverse of the left-hand reaction in Equation 10. It follows that the formula for K_a is

$$K_a = \frac{1}{K_1} = \frac{\left[H^+\right]\left[HCO_3^-\right]}{\left[H_2CO_3\right]} \tag{14}$$

The equilibrium constant, K_2, for the right-hand reaction in Equation 10 is also defined by the Law of Mass Action:

$$K_2 = \frac{\left[CO_2\right]}{\left[H_2CO_3\right]} \tag{15}$$

Because the two equilibrium reactions in Equation 10 occur simultaneously, Equations 14 and 15 can be treated as two simultaneous equations. Solving for the equilibrium concentration of carbonic acid gives

$$\left[H_2CO_3\right] = \frac{\left[H^+\right]\left[HCO_3^-\right]}{K_a} = \frac{\left[CO_2\right]}{K_2} \tag{16}$$

Rearranging Equation 16 allows us to solve for the equilibrium proton concentration in terms of the two equilibrium constants and the concentrations of the other species:

$$\left[H^+\right] = \left(\frac{K_a}{K_2}\right)\left(\frac{\left[CO_2\right]}{\left[HCO_3\right]}\right) \tag{17}$$

Because we are interested in the pH of the blood, we take the negative log of both sides of Equation 17:

$$-\log\left[H^+\right] = -\log(K) - \log\left(\frac{\left[CO_2\right]}{\left[HCO_3^-\right]}\right) \tag{18}$$

Recalling the definitions of pH and pK (Equations 2 and 12, above), Equation 18 can be rewritten using more conventional notation, to give the relation shown in Equation 11, which is reproduced below:

$$pH = pK - \log\left(\frac{\left[CO_2\right]}{\left[HCO_3^-\right]}\right)$$

As shown in Equation 11, the pH of the buffered solution (*i.e.,* the blood) is dependent only on the **ratio** of the amount of CO_2 present in the blood to the amount of HCO_3^- (bicarbonate ion) present in the blood (at a given temperature, so that **pK remains constant**). This ratio remains relatively constant, because the concentrations of both buffer components (HCO_3^- and CO_2) are very large, compared to the amount of H^+ added to the blood during normal activities and moderate exercise. When H^+ is added to the blood as a result of metabolic processes, the amount of HCO_3^- (relative to the amount of CO_2) decreases; however, the amount of the change is tiny compared to the amount of HCO_3^- present in the blood. This optimal buffering occurs when the pH is within approximately 1 pH unit from the pK value for the buffering system, *i.e.,* when the pH is between 5.1 and 7.1.

However, the normal blood pH of 7.4 is outside the optimal buffering range; therefore, the addition of protons to the blood due to strenuous exercise may be too great for the buffer alone to effectively control the pH of the blood. When this happens, other organs must help control the amounts of CO_2 and HCO_3^- in the blood. The lungs remove excess CO_2 from the blood (helping to raise the pH via shifts in the equilibria in Equation 10), and the kidneys remove excess HCO_3^- from the body (helping to lower the pH). The lungs' removal of CO_2 from the blood is somewhat impeded during exercise when the heart rate is very rapid; the blood is pumped through the capillaries very quickly, and so there is little time in the lungs for carbon dioxide to be exchanged for oxygen.

SECTION 4.5—BIOLOGICAL OXIDATION

SUB-SECTION 4.5A—INTRODUCTION

The oxidative degradation of carbohydrates, fats and amino acids at cellular level needs oxygen and any metabolism after complete oxidation forms CO_2 and H2O. Hence, any biological oxidation taking place at tissue level is associated with the uptake of oxygen and release of carbon dioxide and rapidly liberates energy. This biological oxidation accompanied by specific enzymes and coenzymes in a step wise fashion involves the union between hydrogen atoms with oxygen atom to form water. During the electron transport, the electrons are transferred from organic substrates to oxygen yielding energy in the generation bond energy in the form of Adenosine triphosphates (ATP) from Adenosine diphosphates (ADP).

ATP and ADP are known as high energy phosphates as the cleavage of phosphate bond in them yield energy and inorganic phosphate. This energy is utilized for the anabolic and catabolic processes. The oxidative phosphorylation enables the aerobic living organisms to capture a far greater proportion of available free energy of the oxidizing substrates inthe form of ATP. Oxidation involving phosphorylation is a very vital process and it is a continuous process and any disturbance of its function is incompatible with life.

Redox Couple

Always every *oxidation is accompanied by a reduction process*. All such reactions are termed as *oxidation-reduction reactions* and shortly referred as *redox.* These redox reactions are associated with movements of electron. The electron donor is called as reductant or reducing agent and the electron acceptor, the oxidant or oxidizing agent. The system which transfer its electron is changed into oxidant form while the system which accepts electrons gets converted to the reductant form.

Oxidation reduction system is simplified and shown below (Fig. 120)
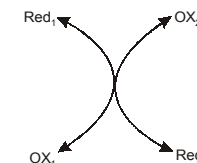
Fig. 4.15 : Oxidation-Reduction System

$$\text{Electron donor} \longrightarrow e^- + \text{electro acceptor}$$

A specific example is oxidation of ferrous iron to ferric iron indicates the removal of electron (e^-) from ferrous iron.

$$\underset{\text{(Ferrous ion)}}{Fe^{++}} \longrightarrow \underset{\text{(Ferric ion)}}{Fe^{+++}} + e^-$$

Since, the electron is not stable in the free form it gets attached to another molecule and thus every oxidation is followed by a reduction. Always these redox reactions are energy yielding. A direct transfer of electrons from substrate to the oxidant would liberate a sudden burst of energy and most of it will be wasted by dissipation. Normally when the electrons of hydrogen combine with oxygen results in explosion. In biological system this oxidation reduction process takes place smoothly without increasing the temperature because the transfer of hydrogen pairs

,,occurs in a step by step process till it reacts with oxygen. This permits the liberation of energy in small amounts so that it can be captured and saved.

Redox Potential

In oxidation and reduction reactions the free energy exchange is proportionate to the tendency of reactants to donate or to accept electrons. The affinity for the electron by the oxidant is called the electron affinity or redox potential. In biochemistry, the oxygen has the highest redox potential or electron affinity (E0) and therefore the electron pass from the systems of hydrogen donors which have lower potentials. It is usual to compare the redox potential of a system (E0) against the potential of the hydrogen electrode, which is at pH 0 designated as 0.0 volts. However, in a biological system it is normal to express the redox potential (E0) at pH 7.0 at which the electrode potential of hydrogen electrode is - 0.42volts. In the biological system, the enzymes concerned with this oxidation reduction processes are designated as oxidoreductases. Some of the redox potentials are given in the table below:

Some of the redox potential of special interest shown in the table

System	E_0 (volts)
H^+ / H_2	-0.42
Oxygen/water	$+0.82$
Cytochrome a Fe^{3+} / Fe^{2+}	$+0.29$
Cytochrome b Fe^{3+} / Fe^{2+}	$+0.08$
Cytochrome c Fe^{3+} / Fe^{2+}	$+0.22$
$NAD^+ / NADH + H +$	-0.32

A positive value for the standard reduction potential means that a compound in question preferentially is reduced when involved in a redox reaction with hydrogen. A negative value means that a compound in question is preferentially oxidized. However, listings of standard reduction potentials are always given in the form of a reduction reaction.

Electron Transport System
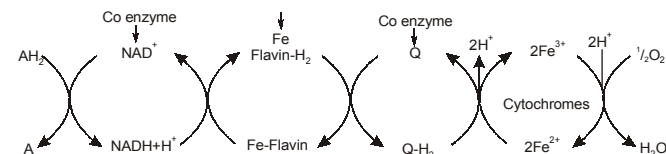
Fig. 4.20 : Showing the electron transport chain in the enzyme reaction system

The electron transport chain (figure 8.2) consists of series of proteins which tightly bound involves the passage of a pair of electrons from one chemical to the next, whereby each chemical in the sequence has less reduced energy than the previous. The electron transport chain oxidizes (i.e. "burns") the NADH+H + and FADH2 cofactors, using molecular oxygen as the final electron acceptor. In the electron transport chain electron carriers and hydrogen-electron acceptors are positioned alternatively to carry the function. There are three different regions in the electron transport chain, where energy is released. In each region there is a formation of one ATP. All these reactions and capturing of energy takes place in mitochondria.

Components of Mitochondria with Marker Enzymes

The histochemical and ultra centrifugation studies clearly established that the major site of cellular oxidation is mitochondria. These are sub cellular organelles and quite vary in size and shape. Ellipsoidal, spherical or rod shaped structures measuring about 0.5-5 μ in length and 0.1-0.6μ in width. Since, the energy released in the oxidation process is converted into chemical energy (ATP). Mitochondria otherwise called as **power house** of the cell. Hence the number of mitochondria in a cell depends on it's metabolic activity. All the reducing equivalence that can release energy during oxidation of carbohydrates, fatty acids and proteins are available in the mitochondria. In mitochondria, a series of catalysts referred as respiratory chain that collects these reducing equivalents and direct them towards oxygen to form water. The electron microscopic picture of the mitochondria shows a double membrane, an *outer and inner membrane which* consists of different specific enzymes. The folding of the inner membrane produces a number of partitions called *cristae* that extend into the matrix. The inner membrane encloses the matrix and it is very selective in its permeability. Inner membrane is highly complex in its structure and function. The space between the inner and the outer membrane is called as *Inter membrane space* which is surrounded by **matrix.** The mitochondria contains its own circular DNA and ribosomes. Some mitochondrial proteins are thus coded for and produced by the mitochondria itself. Other mitochondrial proteins are coded by nuclear DNA, synthesized by cytosolic ribosomes, and subsequently transported to the mitochondria. The structure of mitochondria (figure 122) and the location of various essential enzymes are given in the form of diagram.

The electron transport chain is initiated by the reaction of an organic metabolite (intermediate in metabolic reactions) with the coenzyme NAD+ (nicotinamide adenine dinucleotide is a coenzyme containing the B-vitamin, nicotinamide). This is an oxidation reaction where 2 hydrogen atoms (or 2

hydrogen ions and 2 electrons) are removed from the organic metabolite. (The organic metabolites are usually from the citric acid cycle and the oxidation of fatty acids—details in following pages). The reaction can be represented simply where M = any metabolite.

$$MH2 + NAD^+ \rightarrow NADH + H^+ + M: + energy$$
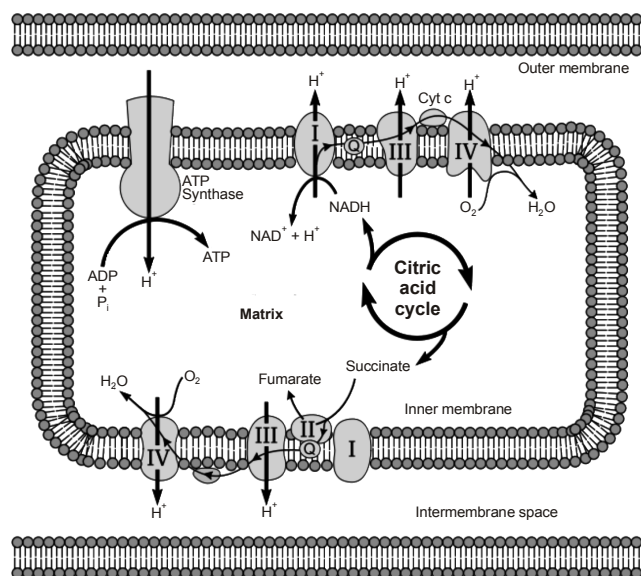
Fig. 4.21 : Showing the Enzymes used inside the Mitochondria

Complex I—*NADH dehydrogenase*, also called NADH coenzyme Q reductase located in the inner mitochondrial membrane and also contains non heme iron atoms. These dehydrogenase enzyme does not react with oxygen instead an electron carrier is interposed between the metabolite and next member in the chain. These enzymes consist of a protein part and a non protein part which is a coenzyme. The co enzyme NAD+ or NADP+ are utilized as the prime carriers of hydrogen.

Complex II—*Coenzyme Q* (Q for Quinone) or cytochrome c reductase is a Ubiquinone. It is in the inner membrane in the free form or protein bound form. Coenzyme Q occupies the position between metalloflavoproteins and cytochrome in the chain. At the point of coenzyme the H+ ion dissociate and go into solution, leaving the electrons to the cytochromes.

Complex III—Cytochrome c oxidase. Cytochromes are very similar to the structure of myoglobin or hemoglobin. The significant feature is the heme structure containing the iron (Fe) ions, initially in the +3 state andchanged to the +2 state by the addition of an electron. Cytochrome molecules accept only the electron from each hydrogen, not the entire atom. The several types of cytochromes hold electrons at slightly different energy levels. Electrons are passed along from one cytochrome to the next in the chain, losing energy as they go. Finally, the last cytochrome in the chain, cytochrome a3, passes two electrons to molecular oxygen. These cytochromes are proteins that carry a prosthetic group that has an embedded metal atom. The protein 'steals' the ability of the metal atom to accept and release electrons.

Complex IV—ATP synthase, also known as the F0 F1 particle has two components F0 and F1 (F - indicates the factor). F1 protruding into matrix from the inner membrane and F0 embedded and extend across the inner membrane. The protruding F1 is essential for the energy coupling to ATP molecule. Careful removal of this component (experimentally) leads to impairment in ATP production though the intact respiratory chain is present.

Reactions of Electron Transport

The electron acceptors in the electron transport chain include FMN, ubiquinone (coQ), and a group of closely related proteins called cytochromes. The figure 123 shows arrangement of the protein
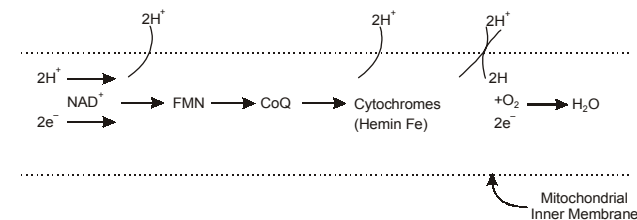
Fig. 4. : Showing the Simplified Electron Transport

Oxidative Phosphorylation in Electron Transport Chain consist of the

electron donors

NADH+H+

FADH2

The coupled oxidation/reduction reaction is

$$NADH + H^+ + 1/2O_2 \longrightarrow H_2O + NAD^+$$
$$FADH_2 + 1/2O_2 \longrightarrow H_2O + FAD$$
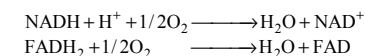
This coupled reactions yield free energy NADH+H+ yields 52 Kcal/mole as the electrons from NADH+H+ transfer to oxygen consist of three pumps yield 3 ATP molecule at 3 sites FADH2 yields 36

Kcal/mole as the electron from FADH2 transfer to oxygen there are two pumps yield 2 ATP molecule at 2 sites. The position at which the energy capture occurring as ATP are given in figure 124
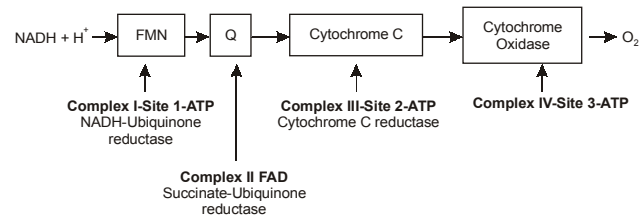
Fig. 4. : Arrangement of Proteins in Electron Transport Chain

Spontaneous flow of electrons through each of the respiratory chaincomplexes I, III, & IV is coupled to **ejection of H+** from the mitochondrial matrix to inner membrane space. The ejection of proton gradient is done through inner membrane protein, **ATPase that uses released energy to drive the synthesis of ATP from ADP.** The terminal acceptor of electrons is molecular oxygen and it is reduced to water. However not all the energy released are captured as high energy phosphate bond and liberated as heat. In warm blooded animals this heat is used for the maintenance of body temperature. The important *respiratory control of electron transport chain is the availability of ADP,* the substrate for the ATP Synthase.

The use of inhibitors gives much information about the electron transport chain. They are classified as (a) **inhibitors of respiratory chain,** (b) **inhibitors of oxidative phosphorylation**, and (c) **uncouplers of phosphorylation**.

(a) Inhibitors that arrest respiration are barbiturates like amobarbital, antibiotic like piericidin A , antimycin A and fish poison retinone. **The carbon monoxide and cyanide inhibit cytochrome oxidase** so that it cannot transport electrons to oxygen. This blocks the further passage of electrons through the chain, halting ATP production and life.

(b) Inhibitors of oxidative phosphorylation are oligomycin and atrctyloside.

(c) Uncouplers dissolve in the membrane, and function as **carriers for H+ or it can be an ionophores.** Uncouplers block oxidative phosphorylation by dissipating the H+ electro chemical gradient by an un coupling the essential linkage between electron transport and ATP synthesis. Un couplers are 2,4 dinitro phenol, dinitrocresol, pentacholorophenol.

Ionophores (ion carriers) are lipid soluble substance capable of carrying specific ions through the membrane. They slightly differ in their action from the uncouplers as they also transport cation other than H+ through the membrane. Valiomycin forms a lipid complex through which the K+ ion readily pass through. The ionophore gramicidin induces penetration to H+, K+ or Na+ and uncouples the oxidative phosphorylation.

Oxidative phosphorylation Hydrogens or their electrons, pass down the electron transport chain

in a series of redox reaction. The electrons entering the electron transport system have relatively high energy content. As they pass along the chain of electron acceptors, they lose much of their energy, some of which is used to pump the protons across the inner mitochondrial membrane. The flow of electrons in the electron transport is usually coupled tightly to the production of ATP with the help of the enzyme ATP synthetase, and it does not occur unless the phosphorylation of ADP can also proceed. This prevents a waste of energy, because high-energy electrons do not flow unless ATP can be produced. Because the phosphorylation of ADP to form ATP is coupled with the oxidation of electron transport components, this process of making ATP is referred to as oxidative phosphorylation. The electron transport and oxidative phosphorylation depends upon the availability of ADP and Pi and it is referred as acceptor control of respiration

Chemiosmotic theory Peter Mitchell got the Nobel prize in 1978 for his theory of chemiosmosis. **The chemiosmotic theory of Mitchell claims that oxidation of components in respiratory chain generates hydrogen ion and ejected across the inner membrane.** The electrochemical potential difference resulting from the asymmetric distribution of the hydrogen ion is used as the driving force (potential energy). This consist of a **chemical concentration gradient of protons across the membrane (pH gradient) also provides a charge gradient.** The inner mitochondrial membrane is impermeable to the passage of protons, which can flow back into the matrix of the mitochondrion only through special channels in the inner mitochondrial membrane. In these channels, the enzyme ATP synthetase is present. As the protons move down the energy gradient **(proton motive force = chemiosmotic energy)**, the energy releases is used by ATP synthetase to produce ATP. The chemiosmotic model explains that this electrochemical potential difference across the membrane is used to drive a membrane located ATP synthetase which couple the energy to ADP, to form ATP. Protons are pumped across the inner mitochondrial membrane by three electron transfer complexes, each associated with particular steps in the electron transport system. As electrons are transferred along the acceptors in the electron transport chain, sufficient energy is released at three points to convey protons across the inner mitochondrial membrane and ultimately to synthesize ATP.

Role of F0 – F 1 ATPase ATP synthetase or Fo F1 ATPase, has two major components, Fo and F1 (F for factor). **F1** consists of 5 polypeptides, with stoichiometry **a3, b3, g, d, e.** The F1 component resembles a doorknob protruding into the matrix from the inner membrane. It is attached to Fo by a stalk, which is embedded in the inner membrane and extend across it. Fo is a complex of integral membrane proteins. When F1 is carefully extracted (from inside out vesicles prepared) from the inner mitochondrial membrane, the vesicles still contain intact respiratory chains. However, since it no longer contain the F1 knobs, as confirmed by electron microscopy, they cannot make ATP. When a preparation of isolated F1 is added back to such depleted vesicles under appropriate conditions, to reconstitute the inner membrane structure, with F1 knobs, the capacity of the inner membrane vesicles to carry out energy coupling between electron transport and ATP formation is restored. This shows the precise arrangement of these Fo and F1 make the ATP synthetase to form complexes called respiratory assemblies. It is proposed that an irregularly shaped "**shaft**" linked to Fo was able to produce conformational changes as follows:

1. A **loose** conformation in which the active site can loosely bind ADP + Pi
2. A **tight** conformation in which substrates are tightly bound and ATP is formed

3. An **open** conformation that favours ATP release. As the protons move down the energy gradient, the energy releases is used by ATP synthetase to produce ATP.

High energy compounds The high energy compound is the ATP. The other high energy compounds include ADP,1,3-diphospho glycerate, phosphoenol pyruvate and also creatine phosphate.

The phosphate group of the high energy phosphate may transfer directly to another organic compound. For this reason the term **group transfer potential** is preferred by some high energy bond. However, the phosphorylated compound may or may not have high energy phosphate though the total energy content of the molecule is higher than a nonphosphorylated compound.

Storage form of high energy compounds They are called as **phosphogens** and help to store the high energy. The example for this is the creatine phosphate present in the vertebrate muscles, the reaction works in both directions it is a reversible reaction form ATP when ATP is required. When ATP is more, creatine reacts with ATP and forms the phosphocreatine.
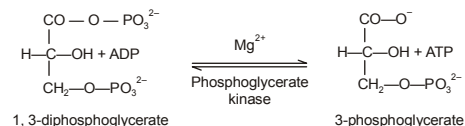
$$CO — O — PO_3^{2-} \qquad\qquad CO—O^-$$
$$| \qquad\qquad\qquad\qquad\quad |$$
$$H—C—OH + ADP \;\xrightleftharpoons[\text{Phosphoglycerate kinase}]{Mg^{2+}}\; H—C—OH + ATP$$
$$| \qquad\qquad\qquad\qquad\qquad |$$
$$CH_2—O—PO_3^{2-} \qquad\qquad CH_2—O—PO_3^{2-}$$

1, 3-diphosphoglycerate   3-phosphoglycerate

One of the phosphate groups undergoes hydrolysis to form the acid and a phosphate ion, giving off energy. This first energy producing reaction is coupled with the next endothermic reaction making ATP. The phosphate is transferred directly to an ADP to make ATP and this is catalysed by phosphoglycerate Kinase enzyme. Since one molecule of glucose yield 2 molecule of Glyceraldehyde 3-phosphate, 2 high energy ATP are produced for one molecule of glucose.

Role of Phosphoenol pyruvate Phosphoenol pyruvate, which is formed during breakdown of glucose to lactic acid, donates its phosphate group to ADP in a reaction catalyzed by Pyruvate kinase. One of the phosphate groups undergoes hydrolysis to form the acid and a phosphate ion, giving off energy. This first energy producing reaction is coupled with the next endothermic reaction making ATP. The phosphate can only exist as the high energy enol form. Thus, when the phosphate group is removed, the pyruvate can revert back to the stable, low-energy keto form and the surplus energy is released. Production of ATP in this reaction is controlled by pyruvate kinase.

$$CO —O^- \qquad\qquad CO —O^- \qquad\qquad CO —O^-$$
$$| \qquad\qquad\qquad\qquad | \qquad\qquad\qquad\qquad |$$
$$C — O — PO_3^{2-} \xrightarrow{\text{Pyruvate kinase}} C = OH \xrightarrow{\text{Spontaneously}} C = O$$
$$\| \qquad\qquad\qquad\qquad \| \qquad\qquad\qquad\qquad |$$
$$CH_2 \qquad ADP \quad ATP \qquad CH_2 \qquad\qquad\qquad CH_3$$

Phospho enol pyruvate   Enol pyruvate   Pyruvate
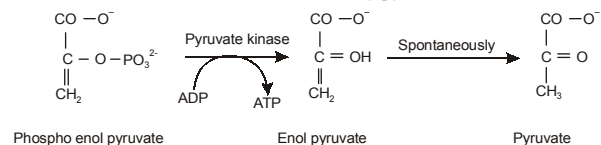
Actually, this reaction takes place in two steps. First the enolate form of pyruvate is formed, then the transfer of the phosphate group to ADP occur as second step. The keto pyruvic acid may reduced to lactic acid in the lack of oxygen. *Mitochondria is not involved* . Since one molecule of glucose yield 2 molecule of Glyceraldehyde 3 phosphate, 2 high energy ATP are produced for one molecule of glucose.

Phosphocreatine
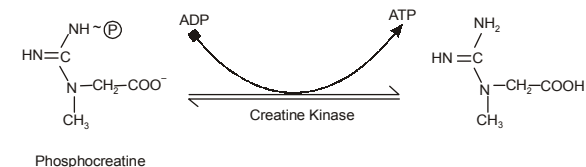
Phosphocreatine

Phosphocreatine is a phosphogen and it interacts with ADP to form ATP. When ATP is more creatine reacts with ATP and forms phosphocreatine. The enzyme involved is creatine kinase. This energy transfer from creatine phosphate to ADP helps to produce ATP molecule to provide energy during muscle contraction.

ATP as high energy compound ATP is the most widely distributed high-energy compound within the human body. Adenosine triphosphate (ATP) is a useful free-energy currency because the dephosphorylation reaction or hydrolysis, yield an **unusually largeamount ofenergy;** *i.e.,* it releases a large amount of free energy. "High energy" bonds are often represented by the **"~"** symbol (squiggle), with ~P representing a phosphate group with a high free energy on hydrolysis. The terminal phosphate group is then transferred by hydrolysis to another compound, a process called *phosphorylation*, producing ADP, phosphorylated new compound and energy. **Thus, the dephosphorylation reaction of ATP to ADP and inorganic phosphate is often coupled with non spontaneous reactions.** Generally, ATP is connected to another reaction—a process called *coupling* which means the two reactions occur at the same time and at the same place, usually utilizing the same enzyme complex. Release of phosphate from ATP is exothermic (a reaction that gives off heat) and this reaction is connected to an endothermic reaction (requires energy input in order to occur). The free energy yielded can be coupled to endothermic reaction and useful for the works such as:

Chemical work: ATP energy is consumed to synthesize macromolecules that make up the cell.

Transport work: ATP energy is utilized to pump substances across the plasma membrane.

Mechanical work: ATP provides energy to contract the muscles of the body.

Some time the phosphate group can be transferred to an acceptor molecule and such group transfer potential are associated with some high energy compound. Thus, ATP act as a common intermediate that serves as a vehicle for transfer of chemical energy.

Structure of ATP ATP is an abbreviation for *adenosine triphosphate*, a complex molecule that contains the nucleoside *adenosine*, ribose and a tail consisting of three phosphates.

Adenosine triphosphate

The bond is known as a "high-energy" bond and is depicted in the Equation above by a wavy line. The bond between the first and the second phosphate is also "high-energy" bond.

$$ATP + H_2O \longrightarrow ADP + Pi + energy\ released$$

ATP
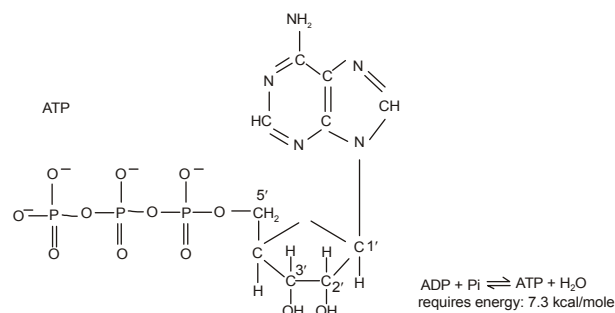
$$\Delta G^0 = 7,300 \text{ calories/mol} = -7.3 \text{ kcal/mol} = -30.5 \text{ kJ/mol } (\Delta G^0 \text{measured at } 37°C)$$

ATP is sometimes referred to as a "High Energy" compound. High energy in this case does **not** refer to total energy in compound, rather just **toenergyof hydrolysis. Thus ATPhasa larger negative** DG for hydrolysis. For biochemistry *High Energy* is defined in terms of ATP: if a compound's free energy for hydrolysis is equal to or greater than ATP's then it is "High Energy," if its free energy of hydrolysis is less than ATP's then it is not a "high energy" compound. Note that ATP has twohighenergy anhydride bonds (AMP ~**P ~P**). DG of ATP hydrolysis also depends on the local environments it varies with pH, divalent metal ion concentration, ionic strength and Consumption of ATP. An EATP of -7.3 kcal /mol requires ATP, ADP, and phosphate to be present at equal concentrations. In cells, however the concentration of ATP is often 5 to 10 times that of ADP. As a result, the free energy of ATP hydrolysis is about -12 kcal /mol. One must be clear that the bond energy generally meant by physical chemist is the energy required to break a covalent bond between two atoms. Since relatively a large amount of energy is required to break a covalent bond, the phosphate bond energy is totally a different one.

Phosphate bond energy specifically denotes the difference in the free energyof the reactants when phosphorylated compound undergoes hydrolysis.

Mono (ortho) phosphate cleavage and Pyrophosphate cleavage ATP may under go either an orthophosphate or pyrophosphatecleavage during it's utilization in biosynthetic pathways. In an ATP molecule, when the terminal phosphate is cleaved it is called as mono phosphate or ortho phosphate cleavage.
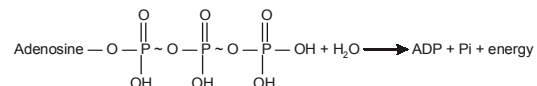
However, in many ATP utilizing reactions instead of one terminal phosphate two terminal phosphate groups are enzymatically hydrolyzed to give a pyro phosphate molecule and a large amount of energy which is greater than the mono phosphate or ortho phosphate cleavage.
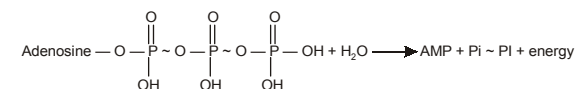
Pyrophosphate (PPi) is often the product of a reaction that needs a driving force. Its spontaneous hydrolysis, catalyzed by Pyrophosphatase enzyme, drives the reaction for which PPi is a substrate. The DG (free energy) for this pyrophosphate cleavage is 10.0 Kcal./mol and thus an extra thermodynamic push is given to certain enzymatic reaction which require more energy than that of a mono phosphate cleavage and assure the completeness of certain biosynthetic reactions.

SUB-SECTION 4.6B—GENERATION OF SUPER OXIDE FREE RADICALS

Superoxide is the anion O_2^-. It is important as the product of the one-electron reduction of dioxygen, which occurs widely in nature.[1] With one unpaired electron, the superoxide ion is a free radical, and, like dioxygen, it is paramagnetic.

Synthesis, Basic reactions and Structure

Superoxides are compounds in which the oxidation number of oxygen is –½. The O-O bond distance in O_2^- is 1.33 Å, vs. 1.21 Å in O_2 and 1.49 Å in O_2^{2-}.

The salts CsO_2, RbO_2, KO_2, and NaO_2 are prepared by the direct reaction of O_2 with the respective alkali metal.[2] The overall trend corresponds to a reduction in the bond order from 2 (O_2), to 1.5 (O_2^-), to 1 (O_2^{2-}).

The alkali salts of O_2^- are orange-yellow in color and quite stable, provided they are kept dry. Upon dissolution of these salts in water, however, the dissolved O_2^- undergoes disproportionation (dismutation) extremely rapidly:

$$2\,O_2^- + 2\,H_2O \rightarrow O_2 + H_2O_2 + 2\,OH^-$$
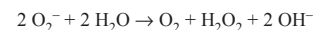
In this process $O_2^{''}$ acts as a strong Brønsted base, initially forming HO_2. The pKa of its conjugate acid, hydrogen superoxide (HO_2, also known as "hydroperoxyl" or "perhydroxy radical"), is 4.88 so that at neutral pH 7 the vast majority of superoxide is in the anionic form, O_2^-.

Salts also decompose in the solid state, but this process requires heating:

$$2NaO_2 \rightarrow Na_2O_2 + O_2$$
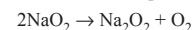
This reaction is the basis of the use of potassium superoxide as an oxygen source in chemical oxygen generators, such as those used on the space shuttle and on submarines.

Superoxide in Biology

Superoxide is biologically quite toxic and is deployed by the immune system to kill invading microorganisms. In phagocytes, superoxide is produced in large quantities by the enzyme NADPH oxidase for use in oxygen-dependent killing mechanisms of invading pathogens. Mutations in the gene coding for the NADPH oxidase cause an immunodeficiency syndrome called chronic granulomatous

disease, characterized by extreme susceptibility to infection. In turn, micro-organisms genetically engineered to lack superoxide dismutase (SOD), lose virulence. Superoxide is also deleteriously produced as a byproduct of mitochondrial respiration (most notably by Complex I and Complex III), as well as several other enzymes, for example xanthine oxidase.

Despite being chemically rather benign, superoxide is so toxic that intracellular levels above 1nM are lethal. The biological toxicity of superoxide is not entirely understood, but derives in part from its capacity to inactivate iron-sulfur cluster containing enzymes (which are critical in a wide variety of metabolic pathways), thereby liberating free iron in the cell, which can undergo Fenton chemistry and generate the highly reactive hydroxyl radical. In its HO_2 form (hydroperoxyl radical), superoxide can also initiate lipid peroxidation of polyunsaturated fatty acids. It also reacts with carbonyl compounds and halogenated carbons to create toxic peroxy radicals. Superoxide can also react with nitric oxide (NO) to form $ONOO^{..}$. Superoxide can also form tyrosine peroxides as a result of reaction with enzymes containing tyrosyl radicals (such as ribonucleotide reductase). Superoxide can also oxidize hemoglobin (forming the non-oxygen carrying met-hemoglobin), and possibly other low-potential heme proteins. Finally, superoxide can oxidize low potential thiols. As such, superoxide is one of the main causes of oxidative stress.

Because superoxide is toxic, nearly all organisms living in the presence of oxygen contain isoforms of the superoxide scavenging enzyme, superoxide dismutase, or SOD. SOD is an extremely efficient enzyme; it catalyzes the neutralization of superoxide nearly as fast as the two can diffuse together spontaneously in solution. Other proteins, which can be both oxidized and reduced by superoxide, have weak SOD-like activity (e.g. hemoglobin). Genetic inactivation ("knockout") of SOD produces deleterious phenotypes in organisms ranging from bacteria to mice and have provided important clues as to the mechanisms of toxicity of superoxide in vivo.

Yeast lacking both mitochondrial and cytosolic SOD grow very poorly in air, but quite well under anaerobic conditions. Absence of cytosolic SOD causes a dramatic increase in mutagenesis and genomic instability. Mice lacking mitochondrial SOD (MnSOD) die around 21 days after birth due to neurodegeneration, cardiomyopathy and lactic acidosis. Mice lacking cytosolic SOD (CuZnSOD) are viable but suffer from multiple pathologies, including reduced lifespan, liver cancer, muscle atrophy, cataracts, thymic involution, haemolytic anemia and a very rapid age-dependent decline in female fertility.

Superoxide may contribute to the pathogenesis of many diseases (the evidence is particularly strong for radiation poisoning and hyperoxic injury), and perhaps also to aging via the oxidative damage that it inflicts on cells. While the action of superoxide in the pathogenesis of some conditions is strong, for instance, mice and rats overexpressing CuZnSOD or MnSOD are more resistant to strokes and heart attacks, the role of superoxide in aging, must be regarded as unproven for now. In model organisms (yeast, the fruit fly Drosophila and mice), genetically knocking out CuZnSOD shortens lifespan and accelerates certain features of aging (cataracts, muscle atrophy, macular degeneration, thymic involution), but the converse, increasing the levels of CuZnSOD, does not seem (except perhaps in *Drosophila*), to consistently increase lifespan. The most widely accepted view is that oxidative damage (derived amongst other factors, from superoxide) is but one of several factors limiting lifespan.

SUB-SECTION 4.6C—CYTOCHROME 450

Cytochrome P450 proteins in humans are drug metabolizing enzymes and enzymes that are used to make cholesterol, steroids and other important lipids such as prostacyclins and thromboxane A2. These last two are metabolites of arachidonic acid. Mutations in cytochrome P450 genes or deficiencies of the enzymes are responsible for several human diseases. Induction of some P450s is a risk factor in several cancers since these enzymes can convert procarcinogens to carcinogens. P450 enzymes play a major role in drug interactions. The name cytochrome P450 derives from the fact that these proteins have a heme group, and an unusual spectrum. Mammalian cytochrome P450s are membrane bound. They were originally discovered in rat liver microsomes. Microsomes are turbid suspensions made by grinding up cells and isolating the membrane fraction that is still in suspension after the cell debris and mitochondria have been pelleted. These mixtures are very opaque to standard spectroscopy, because they scatter light so badly. The only way to measure a spectrum on turbid samples like these was to make a special instrument with the light detector very close to the cuvette, and to use dual beams and do difference spectroscopy. In this way all the interfering substances and the light scattering could be subtracted out. With this setup, microsomes treated with dithionite (reduced microsomes) and with carbon monoxide gas added to one cuvette only give a very strong absorption band at 450 nm, thus P450 (P is for pigment). This is called a reduced CO difference spectrum. The CO binds tightly to the ferrous heme, giving a difference between the absorbance of the two cuvettes. This spectrum was first observed in 1958. Other heme containing proteins don't absorb at 450 nm. The reason why cytochrome P450 absorbs in this range is the unusual ligand to the heme iron.
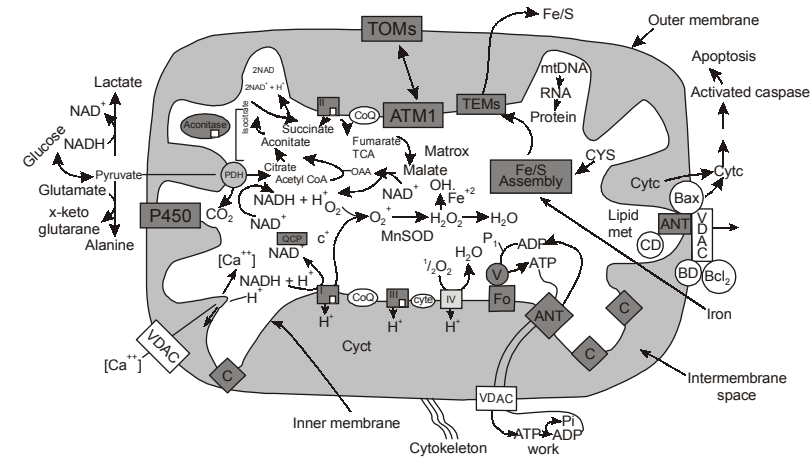
Fig. 4. : Showing the Mitochondrial Respiratiory chain action with p450 Cytochrome

Four ligands are provided by nitrogens on the heme ring. Above and below the plane of the heme, there is room for two more ligands, the 5th and 6th ligands. In cytochrome P450s, the 5th ligand is a thiolate anion, a sulphur with a negative charge, S(-). The sulfur comes from a conserved cysteine at the heme binding region of the active site.

X-ray crystal structures are now available for more than ten different bacterial P450s (CYPs 51 [Mycobacterium], 55A1, 101A1, 102A1, 107A1 [eryF], 111A1, 119A1, 121A1 [P450Mt2], 152, 175A1). These proteins are soluble as compared to the eukaryotic P450s that are membrane bound. The structures are similar and they probably form a good model for membrane bound versions, except for the membrane anchoring parts. Here is a picture of the first P450 crystallized. This is P450 cam from Pseudomonas putida, a bacterium that can use camphor as its sole carbon source. This bug is found growing in soil under camphor trees. The protein is shaped like a triangle with the heme buried deep inside. In this structure, there is no access channel for substrate or products, even water, to enter or leave the active site. Therefore, we must assume that the structure breathes when it functions, so a channel will be open at some point in the catalytic cycle. Half of the enzyme is rich in alpha-helix and the other half is beta-sheet or non-repetetive structure. The mammalian P450s are similar to this fold, but with an N-terminal membrane anchor. A cartoon of one possible view is given here. This model shows a single transmembrane segment, but membrane attachment is more complex than that. When the N-terminal sequence is removed the protein still sticks to membranes. The mammalian CYP2C5 protein has been crystallized after removal of the N-terminal anchor peptide and replacement of an internal hydrophobic sequence with a more water soluble sequence from a related enzyme. The X-ray structure has been solved (Williams PA, Cosme J, Sridhar V, Johnson EF, McRee DE. J Inorg Biochem 2000 Aug 31;81(3):183-90 Microsomal cytochrome P450 2C5: comparison to microbial P450s and unique features.) A brief description of the main features had appeared earlier (Arch. Biochem. Biophys. 369 Sept. 1, 24-29 1999). The structure is similar to the soluble bacterial enzymes, but there are significant differences. The phamaceutical industry is very interested in P450 crystal structures with drugs bound, so they can do improvement of drug design. Here we see a press release from May 3, 2001 about a research agreement between Astex and AstraZeneca to determine crystal structures of human P450s with AstraZeneca drugs bound in the active site. Look at the outlined sections. "Cytochrome P450 enzymes are the most prominent group of drug-metabolising enzymes in humans, and consequently are of great importance to the pharmaceutical industry Astex has now solved the crystal structures of CYP2C9 and CYP3A4, two of the most significant drug metabolising enzymes in humans. This structure can now be used to modify existing drugs to make them poorer substrates for 3A4 (or better substrates). Poorer P450 substrates would last longer in the body before elimination, which is desirable for the pharmaceutical industry.

P450s catalyze many types of reactions, but the one that is most important for us is hydroxylation. These enzymes are called mixed function oxidases or monooxygenases, because they incorporate one atom of molecular oxygen into the substrate and one atom into water. They differ from dioxygenases that incorporate both atoms of molecular oxygen into the substrate. Foreign chemicals or drugs are also called xenobiotics. Cytochrome P450s play an important role in xenobiotic metabolism, especially for lipophilic drugs. The metabolism of these compounds takes place in two phases. Phase I is chemical modification to add a functional group that can be used to attach a conjugate. The conjugate makes the

modified compound more water soluble so it can be excreted in the urine. Many P450s add a hydroxyl group in a Phase I step of drug metabolism. The hydroxyl then serves as the site for further modifications in Phase 2 drug metabolism. For cytochrome P450s to function, they also need a source of electrons. The addition of two electrons (reduction) to the heme iron makes the difficult chemistry of breaking the oxygen-oxygen bond possible. The electrons are donated by another protein that binds briefly to the P450 and passes an electron from a prosthetic group. This handoff of electrons between proteins is called an electron transfer chain, and it is similar to the electron transfers that go on in complexes I to IV of the electron transfer chain in mitochondria. (However, this is not the same electron transfer chain.)There are two different kinds of electron transfer chains for cytochrome P450s. These depend on the location of the enzyme in the cell. Some P450s are found in the mitochondrial inner membrane and some are found in the endoplasmic reticulum (ER). Both types of P450s are membrane bound proteins. The protein that donates electrons to P450s in the ER is called NADPH cytochrome P450 reductase. It is also membrane bound by an N-terminal tail that crosses the ER membrane once. The bulk of this protein is on the cytosolic side of the ER membrane. This protein has two domains that each contain one flavin. Two electrons are acquired from NADPH and migrate from FAD to FMN, then to the P450 heme iron.In the mitochondria, the electron transfer chain is a little longer.

Ferredoxin (called adrenodoxin in the adrenals, but exactly the same gene codes for both proteins) is the immediate donor of electrons to the P450s in mitochondria (CYP11A1, CYP11B1, CYP11B2, CYP24, CYP27A1, CYP27B1, CYP27C1). Ferredoxin has an iron sulfur cluster instead of a flavin, however, ferredoxin is reduced by ferredoxin reductase (or adrenodoxin reductase in the adrenals) that does contain a flavin. NADPH is the source of electrons that flow from ferredoxin reductase to ferredoxin and then to P450. A few P450s also can accept electrons from cytochrome b5. This is a small membrane bound heme containing protein that gets its reducing equivalents (electrons) from NADH.

The families of human P450s The P450 proteins are categorized into families and subfamilies by their sequence similarities. Sequences that are greater than 40% identical at the amino acid level belong to the same family. Sequences that are greater than 55% identical are in the same subfamily. There are now more than 2500 cytochrome P450 sequences known.**Humans have 18 families of cytochrome P450 genes and 43 subfamilies**CYP1 drug metabolism (3 subfamilies, 3 genes, 1 pseudogene)

CYP2 drug and steroid metabolism (13 subfamilies, 16 genes, 16 pseudogenes)

CYP3 drug metabolism (1 subfamily, 4 genes, 2 pseudogenes)

CYP4 arachidonic acid or fatty acid metabolism (5 subfamilies, 11 genes, 10 pseudogenes)

CYP5 Thromboxane A2 synthase (1 subfamily, 1 gene)

CYP7A bile acid biosynthesis 7-alpha hydroxylase of steroid nucleus (1subfamily member)

CYP7B brain specific form of 7-alpha hydroxylase (1 subfamily member)

CYP8A prostacyclin synthase (1 subfamily member)

CYP8B bile acid biosynthesis (1 subfamily member)

CYP11 steroid biosynthesis (2 subfamilies, 3 genes)

CYP17 steroid biosynthesis (1 subfamily, 1 gene) 17-alpha hydroxylase

CYP19 steroid biosynthesis (1 subfamily, 1 gene) aromatase forms estrogen

CYP20 Unknown function (1 subfamily, 1 gene)

CYP21 steroid biosynthesis (1 subfamily, 1 gene, 1 pseudogene)

CYP24 vitamin D degradation (1 subfamily, 1 gene)

CYP26A retinoic acid hydroxylase important in development (1 subfamily member)

CYP26B probable retinoic acid hydroxylase (1 subfamily member)

CYP26C probabvle retinoic acid hydroxylase (1 subfamily member)

CYP27A bile acid biosynthesis (1 subfamily member)

CYP27B Vitamin D3 1-alpha hydroxylase activates vitamin D3 (1 subfamily member)

CYP27C Unknown function (1 subfamily member)

CYP39 7 alpha hydroxylation of 24 hydroxy cholesterol (1 subfamily member)

CYP46 cholesterol 24-hydroxylase (1 subfamily member)

CYP51 cholesterol biosynthesis (1 subfamily, 1 gene, 3 pseudogenes) lanosterol 14-alpha demethylase

Humans have 57 sequenced CYP genes and 58 pseudogenes.

only full length functional genes are named below

1A1, 1A2, 1B1, 2A6, 2A7, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 2J2, 2R1, 2S1, 2U1, 2W1, 3A4, 3A5, 3A7, 3A43, 4A11, 4A22, 4B1, 4F2, 4F3, 4F8, 4F11, 4F12, 4F22, 4V2, 4X1, 4Z1 5A1, 7A1, 7B1, 8A1, 8B1, 11A1, 11B1, 11B2, 17, 19, 20, 21A2, 24, 26A1, 26B1, 26C1, 27A1, 27B1, 27C1, 39, 46, 51,

Detailed information on mouse, human, dog, cattle, rat and other species P450s can be found on my website http://drnelson.utmem.edu/CytochromeP450.html

A P450 name followed by P stands for a pseudogene. A pseudogene is a defective gene that does not produce a functional protein. There are several reasons why this happens, but in the end, the protein product is not made. Pseudogenes are relics of gene duplications where one of the copies has degenerated and lost its function.

Induction of P450 enzymes. P450 enzymes have a variety of gene regulatory mechanisms. Many of these genes can be turned on or induced by a chemical signal. The steroid hormones are under strict endocrine control. Their levels are tightly regulated. One example is the induction of steroid biosynthetic P450s by ACTH adrenocorticotropic hormone. ACTH stimulates production of cAMP that presumably activates a protein kinase that phosphorylates some unidentified protein, leading to an increase in gene transcription. Another type of P450 gene regulation is that shown by peroxisome proliferators like clofibrate. These drugs act through a binding protein called the PPAR or peroxisome proliferator activated receptor. When drug is bound to this protein it migrates to the nucleus, heterodimerizes with retinoid X receptor (RXR) and binds to specific DNA sequences in the regulatory region of genes that are needed for peroxisome generation. The CYP4A1 gene is turned on by this mechanism. Peroxisomes oxidize fatty acids and the 4A1 P450 is a known fatty acid hydroxylase.

The members of the CYP1 family are induced by aromatic hydrocarbons. The activation involves a specialized receptor called the Ah receptor. Ah stands for aryl hydrocarbon. This receptor protein binds the aromatic hydrocarbon, but it cannot reach the nucleus to activate gene transcription without another protein called arnt for Ah receptor nuclear translocator. These two proteins bind and together they then bind DNA and activate transcription. Other chemicals also induce P450s. Ethanol induces the CYP2E enzymes. Phenobarbital induces the rat CYP2B enzymes 40-50 fold, through a phenobarbital receptor called CAR. This receptor also dimerizes with RXR as seen above with the PPAR receptor. The heterodimer binds to a phenobarbital response element in the DNA to activate the gene. For details on these receptor mediated induction mechanisms see the review by Waxman (Archives Biochem. Biophys. 369, 11-23, 1999). The general feature that many P450 enzymes are inducible is probably related to P450's role in detoxification of foreign chemicals found in plants.

Noninvasive markers for measuring levels of P450 enzymes in humans P450 enzymes catalyze specific reactions that can be monitored by sampling the urine, blood or breath of patients given a noninvasive marker. Caffeine is a marker for CYP1A2. It is demethylated, and the rate at which it is demethylated is related to the amount of CYP1A2 in a person's liver. By administering caffeine and measuring the rate of demethylation, it is possible to estimate the level of CYP1A2 in a human. This can show if a person has been induced by exposure to polycyclic aromatic hydrocarbons (PAHs). There are a variety of non-invasive markers for different P450s. Assays of CYP1A enzymes from fish livers can also be used to monitor water pollution levels, since certain types of pollutants will induce the enzyme. This is also being done in soil using nematodes like C. elegans.

Functions of Human P450s and Diseases caused by Defects in P450s

The **CYP1 family** of P450s can hydroxylate estrogen (CYP1A2 and 1B1)and oxidize uroporphyrinogen to uroporphyrin (CYP1A2) in heme metabolism,but they may have additional undiscovered endogenous substrates. These enzymes are inducible by some polycyclic hydrocarbons, some of which are found in cigarette smoke and charred food. These enzymes are of interest, because in assays, they can activate compounds to carcinogens. High levels of CYP1A2 have been linked to an increased risk of colon cancer. Since the 1A2 enzyme can be induced by cigarette smoking, this links smoking with colon cancer.

The **CYP1B1** gene has been linked to primary congenital glaucoma. The normal substrate in mammals is not known, but it is speculated that this P450 may be required to eliminate a signaling molecule. Defects in the gene could lead to chronic high concentrations of the signaling molecule that lead to glaucoma. The molecule affected may be a steroid.As you can see from the table of human P450s, the 2 family is the largest family in humans. About one third of human P450s are in this family. Many of these proteins can hydroxylate steroids, and some of them are expressed in a sex specific manner. This would be expected for enzymes that only act on sex specific steroids. Some of these may also be drug metabolism enzymes that are defensive, to protect us from toxins in our food. Plants especially make many toxic components that are probably defensive for the plants. Since we eat almost anything, it is necessary to have a detoxification system coded in our genes. This idea has been called plant animal warfare on the chemical level.

CYP2B is inducible by barbiturates in rodents. It was one of the first P450s to be purified from mammals, but its role in humans is not understood.

CYP2C8 is known to catalyze the 6-alpha hydroxylation of taxol. This is a drug used in treating breast cancer.

CYP2C9 is one of two human P450s that has a known crystal structure. The other is CYP3A4 (still confidential). CYP2C9 structure was published in Nature this summer (Williams PA, Cosme J, Ward A, Angove HC, Matak Vinkovic D, Jhoti H. Crystal structure of human cytochrome P450 2C9 with bound warfarin.Nature. 2003 Jul 24;424, 464-468.)

CYP2C19 metabolizes omeprazole, a common ulcer medication.Polymorphisms in this gene cause a higher incidence of poor metabolizer phenotypes in Asians (23%) vs caucasians (3-5%).

 Drug metabolism differences caused by polymorphisms in P450s. A polymorphism is a difference in DNA sequence found at 1% or higher in a population. These differences in DNA sequence can lead to differences in drug metabolism, so they are important features of P450 genes in humans. CYP2C19 has a polymorphism that changes the enzyme's ability to metabolize mephenytoin (a marker drug). In Caucasians, the polymorphism for the poor metabolizer phenotype is only seen in 3% of the population. However, it is seen in 20% of the asian population. Because of this difference, it is important to be aware of a person's race when drugs are given that are metabolized differently by different populations. Some drugs that have a narrow range of effective dose before they become toxic might be overdosed in a poor metabolizer. Very recently, Roche has marketed a CYP450 DNA chip to detect major known polymorphisms in human CYP2D6 and CYP2C19. For about $400 you can test a person to see if they are a poor metabolizer, normal metabolizer or ultra metabolizer, for a large number of drugs. Since 1A2, 2C9, 2C19, 2D6 and 3A4 are responsible for oxidizing more than 90% of currently used drugs (2C9 paper above), this is a significant beginning to characterizing risk of adverse drug reactions in people. A cytochrome P450 allele website is available from Sweden at http://www.imm.ki.se/CYPalleles/

CYP2D6 is perhaps the best studied P450 with a drug metabolism polymorphism. This enzyme is responsible for more than 70 different drug oxidations. Since there may be no other way to clear these drugs from the system, poor metabolizers may be at severe risk for adverse drug reactions.

I heard a statistic at a meeting that adverse drug reactions are the number 4 cause of hospitalization in the US. There are at least 72 named alleles identified in CYP2D6.

CYP2D6 Substrates

- **Antiarrhythmics:** Flecainide, Mexiletine, Propafenone
- **Antidepressants:** Amitriptyline, Paroxetine, Venlafaxine, Fluoxetine (Prozac), Trazadone
- **Antipsychotics:** Clorpromazine, Haloperidol, Thoridazine
- **Beta-Blockers:** Labetalol, Timolol, Propanolol, Pindolol, Metoprolol
- **Analgesics:** Codeine, Fentanyl, Meperidine, Oxycodone, Propoxyphene

oxycodone is oxycontin, a favorite drug of abuse.

CYP2E1 is induced in alcoholics. There is a polymorphism associated with this gene that is more common in Chinese people. The mutation correlates with a 2-fold increased risk of nasopharyngeal cancer linked to smoking. This is the second P450 enzyme that may be related to smoking induced cancer (see 1A2 above).

The **CYP3A subfamily** is one of the most important drug metabolizing families in humans. The crystal structure of 3A4 is known, but confidential and heavily patented. CYP3A4 is "the most abundantly expressed P450 in human liver". (Arch. Biochem. Biophys. 369, 11-23 1999) The color of perfused liver is due to this protein. CYP3A4 is known to metabolize more than 120 different drugs. Some of these are well known and I give a list here of some of the recognizable ones.

CYP3A4 Substrates

- **Acetominophen** (Tylenol)
- **Codeine** (narcotic)
- **Cyclosporin A** (an immunosuppresant),
- **Diazepam** (Valium)
- **Erythromycin** (antibiotic)
- **Lidocaine** (anaesthetic),
- **Lovastatin** (HMGCoA reductase inhibitor, a cholesterol lowering drug),
- **Taxol** (cancer drug),
- **Warfarin** (anticoagulant).

Poisoning by acetominophen overdose is caused by P450 enzymes in the liver and kidney that convert acetominophen into a very toxic intermediate that can react with cellular macromolecules to damage cells and eventually kill them. This intermediate normally reacts with glutathione, a naturalantioxidant in cells. It is only when the glutathione is depleted that cell death can occur. That's why acetominophen overdoses don't have any serious symptoms until 3-4 days later. This problem is worse in alcoholics, since they have induced P450 enzymes that make more of the toxic intermediate.

There are common drugs given for special purposes that inhibit P450 enzymes. These include erythromycin (an antibiotic), ketoconazole, and itraconazole (both antifungals that inhibit the fungal CYP51 and unintentionally they also inhibit CYP3A4). If these drugs are given with other drugs that arenormally metabolized by P450 enzymes, the lifetime of these other drugs will be prolonged, and plasma levels will be increased, since they won't be cleared as fast. If these drugs affect heart rhythms or other critical systems, the result can be fatal. For example, inhibition of CYP3A4 in a patient taking warfarin can cause bleeding.

This is called a drug interaction. Drug interactions are one of the major causes of death in hospitalized patients. Another factor in drug dosage is interfering substances from food. Grapefruit juice contains a CYP3A4 inhibitor that causes about a 12 fold increase in some drug concentrations. And the effect lasts for several days. It is advisable to discourage your patients from drinking grapefruit juice while on medication metabolized by CYP3A4.Now we will leave the drug metabolizing enzymes behindand talk about P450s that are very specific in their reactions, just the opposite of CYP3A4.

These enzymes tend to be in families with one or two members and they have only one substrate. Most of these enzymes use steroids or steroid precursors as their substrates. **CYP5** is the thromboxane A2 synthase. Thromboxane A2 is a fatty acid in the arachidonic acid cascade. Arachidonic acid can be metabolized in two pathways, the linear pathway that leads to leukotrienes, and the cyclic pathway that leads to prostaglandins and thromboxanes. The first enzymes leading to cyclic products of arachidonic acid are cyclooxygenases 1 and 2. These enzymes are inhibited by aspirin and non-steroidal antiinflammatory drugs (NSAIDS). Aspirin acetylates a serine in the enzyme that blocks the binding of arachidonic acid. Current research shows that COX2 is inducible and is found to be induced in inflammation. COX1 is constitutive. This difference suggests that COX2 specific inhibitors would block inflammation while not interfering with the beneficial effects of COX1, such as maintaining the stomach lining. These drugs are now on the market. Mouse knockouts have been made, but the full analysis of these COX1 and COX2 knockouts is not finished yet. After this step the pathway branches. Two of the branches include cytochrome P450 reactions. One leads to thromboxane A2 (CYP5) and the other to prostacyclin (CYP8A1). Thromboxane A2 causes platelet aggregation and that is why aspirin prevents platelet aggregation. Prostacyclin acts in opposition to thromboxane A2. It is a vasodilator and an inhibitor of platelet aggregation. The acetylation of COX1 and COX2 in platelets is critical since the platelets have no nucleus and cannot resynthesize the inhibited enzymes.

CYP7A is the first and rate limiting step of bile acid synthesis. This pathway is the only means the body has of eliminating cholesterol in liver. CYP39 can substitute for it in brain. As we will see later, CYP51 is a key enzyme in cholesterol biosynthesis, so P450s are active at both ends of cholesterol metabolism. In 2002 patients were found with defects in this gene. They had elevated levels of cholesterol, decreased levels of bile acids and increased triglycerides, as a compensation for the reduced bile acids. John Kane et al. Journal of Clin. Invest. July 2002.

CYP7B a novel brain cytochrome P450, catalyzes the synthesis of neurosteroids 7-alpha hydroxy dehydroepiandrosterone and 7-alpha hydroxy pregnenolone Proc. Natl. Acad. Sci. USA 94, 4925-4930 (1997)**CYP8A** is prostacyclin synthase (prostaglandin I2). It is part of a regulatory component of hemostasis that opposes CYP5 that makes thromboxane A2. **CYP8B** is the 12-alpha hydroxylase needed in bile acid biosynthesis**CYP11A1** is the side chain cleavage enzyme that converts cholesterol to pregnenolone. This is the first step in steroid biosynthesis. Defects in this enzyme lead to a lack of glucocorticoids, feminization and hypertension. [mitochondrial]

CYP11B1 is the 11-beta hydroxylase enzyme that can act on 11-deoxycortisol to make cortisol or it can hydroxylate 11-deoxycorticosterone to make corticosterone. [mitochondrial] Defects in this gene lead to congenital adrenal hyperplasia.

CYP11B2 is aldosterone synthase that hydroxylates coricosterone at the 18 position. [mitochondrial] Defects in this gene lead to congenital hypoaldosteronism.

CYP17 is the 17 alpha hydroxylase and 17-20 lyase (two enzymes in one). A mutation in this gene is described in Nature Genetics 17, 201-205 (1997) that causes the loss of the 17-20 lyase activity

without affecting the 17 hydroxylase activity. This enzyme is required for production of testosterone and estrogen. Defects in this enzyme affect proper development at puberty.

CYP19 is aromatase that makes estrogen by aromatizing the A ring of the steroid nucleus. Lack of this enzyme causes a lack of estrogen and failure of women to develop at puberty. An interesting defect found in a male was an overactive CYP19 enzyme with about 50 times normal activity. This boy developed breasts at a young age.

CYP20 is a new P450 found only on chordates so far, it may be chordate specific and be involved in development. Nothing is known yet.

CYP21 is the C21 steroid hydroxylase. Defects in this gene cause congential adrenal hyperplasia due to lack of cortisol synthesis. Since cortisol is not made, the precursor 17 hydroxy progesterone builds up and this causes excessive androgen (testosterone) biosynthesis resulting in virilization.

CYP24 is a 25-hydroxyvitamin D(3) 24-hydroxylase used in the degradation or inactivation of vitamin D metabolites. [mitochondrial]

CYP26A1 is an all trans retinoic acid hydroxylase. It does not recognize 9-cis or 13-cis retinoic acid. CYP26A1 has been mutated in zebrafish and it causes a developmental defect. The human and mouse cDNAs have been cloned, but the effects of a mutation in mammals is not yet determined. Retinoic acid is known to be an important molecule in vertebrate development. It operates through several retinoic acid receptors. The hydroxylase may be a means of degrading the retinoic acid signal and thus turning off a developmental switch.

CYP26B1 is a recently discovered human P450. It metabolizes retinoic acid and its expression is induced by retinoic acid during development in chickens (and probably all vertebrates). (See Nelson, D.R. A second CYP26 P450 in humans and zebrafish: CYP26B1. Archives of Biochem. Biophys. 371, 345-347 1999 andGene Expr Patterns. 2003 Oct;3(5):621-7. Expression of the retinoic acid catabolising enzyme CYP26B1 in the chick embryo and its regulation by retinoic acid. Reijntjes S, Gale E, Maden M.)

CYP26C1 is only known from genomic DNA sequencing. The function is not known.

Use of a P450 for Gene Therapy in Cancer

I mentioned earlier that CYP1A2 can activate procarcinogens to carcinogens. The induction of this enzyme may be a cancer risk. The activation of a prodrug to an active form by a P450 mediated reaction has been exploited to fight cancer. A vector with a P450 gene on it (and a P450 reductase gene) can be injected into cancer tumors. Some of these cells take up the vector and express The P450 and its reductase. Then a non-toxic prodrug is administered that is converted by the P450 into a toxic compound that kills the cells. Since the cancer cells have cellular connections, the toxin gets shared around and the tumor dies. For a review on this approach to cancer therapy see Waxman DJ, Chen L, Hecht JE, Jounaidi Y Cytochrome P450-based cancer gene therapy: recent advances and future prospects. Drug Metab Rev 31,503-22 1999.

SECTION 4.7—ATP/ADP CYCLE

An **ATP synthase** (EC 3.6.3.14) is a general term for an enzyme that can synthesize adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate by using some form of energy. This energy is often in the form of protons moving down an electrochemical gradient, such as from the lumen into the stroma of chloroplasts or from the inter-membrane space into the matrix in mitochondria. The overall reaction sequence is:

$$ADP + P_i \rightarrow ATP$$
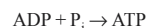
These enzymes are of crucial importance in almost all organisms, because ATP is the common "energy currency" of cells.

The antibiotic oligomycin inhibits the F_O unit of ATP synthase.
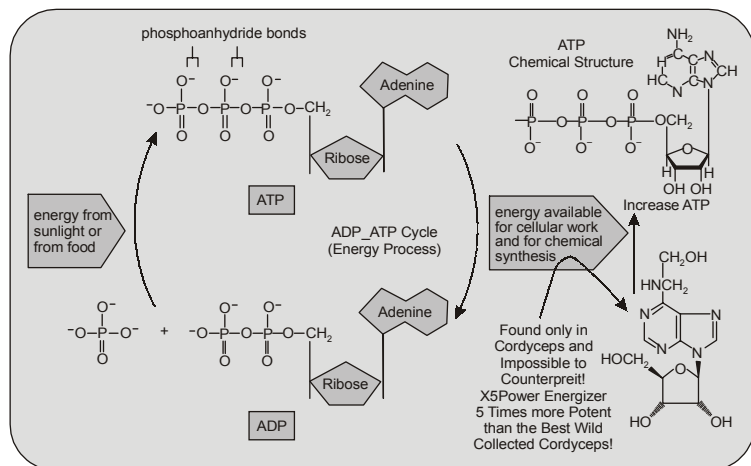
Fig. 4. : Showing the Complete ATP – ADP cycle

Structure and Nomenclature

In mitochondria, the F_1F_O ATP synthase has a long history of scientific study.

- the F_O portion is within the membrane.
- The F_1 portion of the ATP synthase is above the membrane.

The nomenclature of the enzyme suffers from a long history. The F_1 fraction derives it name from

the term "Fraction 1" and F_O (written as a subscript "O", not "zero") derives its name from being the oligomycin binding fraction.

Taking as an example the nomenclature of subunits in the bovine enzyme, many subunits have alphabet names:

- Greek letters: alpha, beta, gamma, delta, epsilon
- Roman letters: a, b, c, d, e, f, g, h

Others have more complex names:

- F_6 (from "Fraction 6")
- OSCP (the oligomycin sensitivity conferral protein), *ATP5O*
- A6L (named for the gene that codes for it in the mitochondrial genome)
- IF1 (inhibitory factor 1), *ATPIF1*

The F_1 particle is large and can be seen in the transmission electron microscope by negative staining.[1] These are particles of 9 nm diameter that pepper the inner mitochondrial membrane. They were originally called elementary particles and were thought to contain the entire respiratory apparatus of the mitochondrion, but through a long series of experiments, Ephraim Racker and his colleagues (who first isolated the F_1 particle in 1961) were able to show that this particle is correlated with ATPase activity in uncoupled mitochondria and with the ATPase activity in submitochondrial particles created by exposing mitochondria to ultrasound. This ATPase activity was further associated with the creation of ATP by a long series of experiments in many laboratories.

Binding change Mechanism

In the 1960s through the 1970s, Paul Boyer developed the binding change, or flip-flop, mechanism, which postulated that ATP synthesis is coupled with a conformational change in the ATP synthase
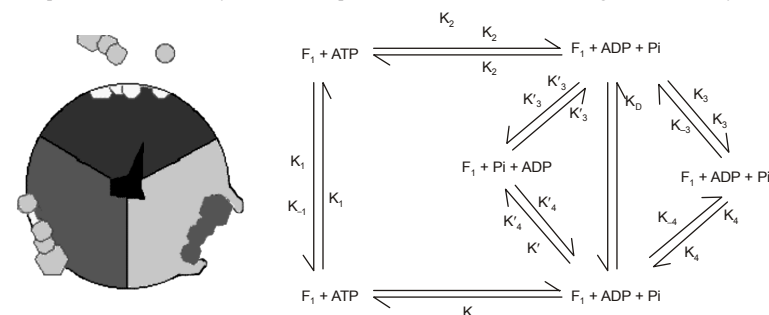
Fig. 4.22 and 4.22A: Showing The Mechanism of ATP synthase. ATP is shown in red, ADP and phosphate in pink and the rotating g subunit in black

generated by rotation of the gamma subunit. The research group of John E. Walker, then at the MRC Laboratory of Molecular Biology in Cambridge but now at the MRC Dunn Human Nutrition Unit (also in Cambridge) crystallized the F_1 catalytic-domain of ATP synthase. The structure, at the time the largest asymmetric protein structure known, indicated that Boyer's rotary-catalysis model was essentially correct. For elucidating this Boyer and Walker shared half of the 1997 Nobel Prize in Chemistry. Jens Christian Skou received the other half of the Chemistry prize that year "for the first discovery of an ion-transporting enzyme, Na+, K+ -ATPase"

The crystal structure of the F_1 showed alternating alpha and beta subunits (3 of each), arranged like segments of an orange around an asymmetrical gamma subunit. According to the current model of ATP synthesis (known as the alternating catalytic model), the proton-motive force across the inner mitochondrial membrane, generated by the electron transport chain, drives the passage of protons through the membrane via the F_O region of ATP synthase. A portion of the F_O (the ring of c-subunits) rotates as the protons pass through the membrane. The c-ring is tightly attached to the asymmetric central stalk (consisting primarily of the gamma subunit) which rotates within the $alpha_3 beta_3$ of F_1 causing the 3 catalytic nucleotide binding sites to go through a series of conformational changes that leads to ATP synthesis. The major F_1 subunits are prevented from rotating in sympathy with the central stalk rotor by a peripheral stalk that joins the $alpha_3 beta_3$ to the non-rotating portion of F_O. The structure of the intact ATP synthase is currently known at low-resolution from electron cryo-microscopy (cryo-EM) studies of the complex. The cryo-EM model of ATP synthase suggests that the peripheral stalk is a flexible structure that wraps around the complex as it joins F_1 to F_O. Under the right conditions, the enzyme reaction can also be carried out in reverse, with ATP hydrolysis driving proton pumping across the membrane.

The binding change mechanism involves the active site of a β subunit cycling between three states.[2] In the "open" state, ADP and phosphate enter the active site, in the diagram to the right this is shown in brown. The protein then closes up around the molecules and binds them loosely - the "loose" state (shown in red). The enzyme then undergoes another change in shape and forces these molecules together, with the active site in the resulting "tight" state (shown in pink) binding the newly-produced ATP molecule with very high affinity. Finally, the active site cycles back to the open state, releasing ATP and binding more ADP and phosphate, ready for the next cycle of ATP production.

Physiological Role

Like other enzymes, the activity of $F_1 F_O$ ATP synthase is reversible. Large enough quantities of ATP cause it to create a transmembrane proton gradient, this is used by fermenting bacteria which do not have an electron transport chain, and hydrolyze ATP to make a proton gradient, which they use for flagella and transport of nutrients into the cell.

In respiring bacteria under physiological conditions, ATP synthase generally runs in the opposite direction, creating ATP while using the protonmotive force created by the electron transport chain as a source of energy. The overall process of creating energy in this fashion is termed oxidative phosphorylation. The same process takes place in the mitochondria, where ATP synthase is located in the inner mitochondrial membrane (so that F_1-part sticks into mitochondrial matrix, where ATP synthesis takes place).

ATP Synthase in Different Organisms

Plant ATP Synthase

In plants ATP synthase is also present in chloroplasts ($CF_1 F_O$-ATP synthase). The enzyme is integrated into thylakoid membrane; the CF_1-part sticks into stroma, where dark reactions of photosynthesis (Also called the light-independent reactions or the Calvin cycle) and ATP synthesis take place. The overall structure and the catalytic mechanism of the chloroplast ATP synthase are almost the same as those of the mitochondrial enzyme. However, in chloroplasts the proton motive force is generated not by respiratory electron transport chain, but by primary photosynthetic proteins.

E. coli ATP Synthase

E. coli ATP synthase is the simplest known form of ATP synthase, with 8 different subunit types.

Yeast ATP Synthase

Yeast ATP synthase is one of the best-studied eukaryotic ATP synthases and five F_1, eight F_O subunits and seven associated proteins have been identified.[3] Most of these proteins have homologues in other eukaryotes.[4]

Human ATP Synthase

The following is a list of humans genes that encode components of ATP synthases:

- ATP5A1, ATP5AL1
- ATP5B, ATP5BL1
- ATP5C2, ATP5D, ATP5E, ATP5F1, ATP5G1, ATP5G2, ATP5G3, ATP5H, ATP5HP1, ATP5I, ATP5J, ATP5J2, ATP5L, ATP5L2, ATP5O, ATP5S
- ATP6, ATP6AP1, ATP6AP2
- ATPSBL1, ATPSBL2
- MT-ATP6, MT-ATP8

SECTION 4.8—SHUTTLES AND MECHANISM

Shuttles are systems of enzymes and transporters. The enzymes convert molecules into metabolites that are capable of crossing membranes via the transporters, a process that is frequently followed by reformation of the original molecule. The electrons of NADH produced in the cytoplasm must be transported into the mitochondria for conversion to ATP by the electron transport pathway. Because the NADH itself cannot cross the mitochondrial membrane, one important function of shuttle mechanisms is the transport of reducing equivalents across the mitochondrial membrane. Two separate methods are used for this purpose: the Glycerophosphate Shuttle Malate-Aspartate Shuttle

The glycolytic intermediate dihydroxyacetone phosphate can be converted to glycerol-3-phosphate by glycerol-3- phosphate dehydrogenase; this process also results inconversion of NADH to NAD. Glycerol-3-phosphate can then be converted back to dihydroxyacetone phosphateby flavoprotein dehydrogenase (a different glycerol-3-phosphate dehydrogenase); this second enzyme is an FAD-dependent enzyme located in the mitochondrial inner membrane. Like Complex II of the electron transport chain, flavoprotein dehydrogenase donates electrons directly to Coenzyme Q without pumping protons. The glycerophosphate shuttle is essentially **irreversible**, and therefore can be used under essentially all conditions. Because the electrons using the glycerophosphate shuttle enter the electron transport pathway at the level of Coenzyme Q, theelectrons can only be used to synthesize a maximum of **two ATP**, instead of the maximum of three ATP derived from NADH formed inside the mitochondria. The glycerophosphate shuttle is heavily used in insect flight muscle (a tissue heavily specialized for conversion of chemical energy to mechanical energy). Some mammalian tissues use the glycerophosphate shuttle also, but tend to prefer a more energy efficient shuttle system that uses malate and aspartate.
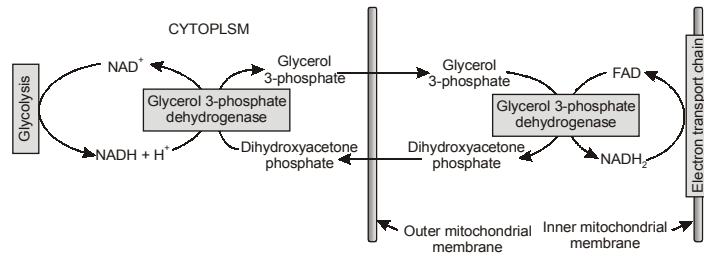
Fig. 4.23 : Glycerophosphate shuttle

Malate-Aspartate Shuttle

Mammalian tissues can use a shuttle system involving malate and aspartate to transport electrons across the mitochondrial inner membrane. Oxaloacetate in the cytoplasm is converted to malate by malate dehydrogenase, oxidizing NADH to NAD. The malate enters the mitochondria using an exchanger protein that must also transport a-ketoglutarate in the opposite direction. The malate is then oxidized to oxaloacetate by the mitochondrial malate dehydrogenase, resulting in formation of NADH, which can then enter the electron transport pathway. Return of the oxaloacetate to the cytoplasm requires a separate transporter, which exchanges aspartate for glutamate. (Note that this conserves the nitrogens present in these amino acids; oxaloacetate and a-ketoglutarate are the a-keto acid counterparts of aspartate and glutamate, respectively.) This separate exchanger is necessary to allow net movement of electrons from one side of the membrane to the other.Endogenous creatine, which is already in skeletal muscle, assists in recharging adenosine triphosphate (ATP), an energy-carrying molecule, by transferring a high-energy phosphate group to adenosine diphosphate (ADP).
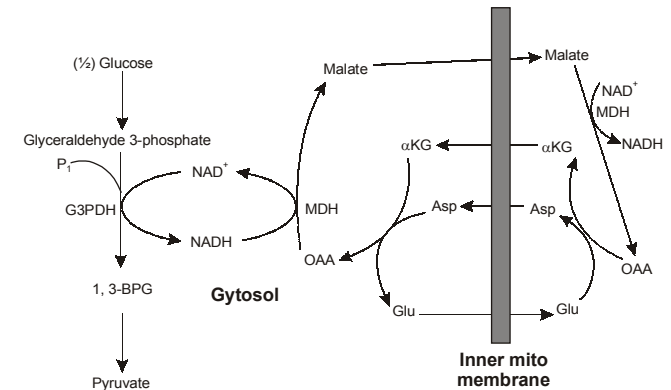
Fig. 4.24 : Showing the Malate-Aspartate Shuttle

Regeneration of Phosphocreatine at Rest
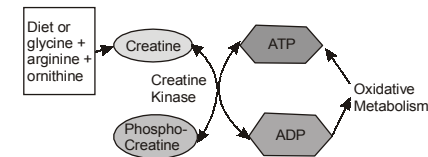
Fig. 4.25 : Showing the Creatine Phosphate Shuttle

Although there has been a considerable amount of research on the properties of creatine, there is no conclusive answer as to exactly how creatine supplements work. However, there are several proposed mechanisms:

1. Increased intramuscular creatine may help maintain a high ATP/ADP ratio
2. Creatine phosphate may buffer protons accumulating in the acidic environment of anaerobic exercise
3. Creatine may help energy transfer from the mitochondria to sites of ATP use in a process described as the creatine phosphate energy shuttle
4. Excess creatine in the muscle cell may osmotically draw water into the cell and stimulate protein synthesis
5. Exogenous creatine may be converted back into amino acids and serve as precursors for muscle protein synthesis

As described in subsequent sections, the mechanism of action for creatine may actually be a combination of these proposed mechanisms.

Citrate-pyruvate Shuttle

A related shuttle is used to move reducing equivalents out of the mitochondria. Citrate can exchange for malate. The citrate in the cytoplasm can act as a regulator of phosphofructokinase, or as a substrate for ATP-citrate lyase, an enzyme that reverses the citrate synthase reaction, and produces acetyl-CoA and oxaloacetate.(Note that ATP is required to generate the high-energy bond present in acetyl-CoA.) The oxaloacetate produced in the ATP-citrate lyase reaction can be converted tomalate. Both the oxaloacetate and malate can be used for a variety of reactions; for malate one additional reaction, catalyzed by malic enzyme, results in the formation of NADPH and pyruvate and carbon dioxide. The pyruvate produced by malic enzyme can return to the mitochondria to complete the cycle.

Note that pyruvate has a proton-dependent pump, and generally cannot leave the mitochondria directly.
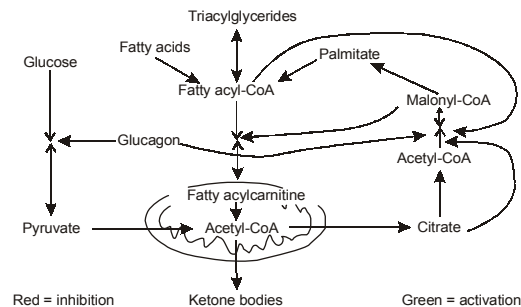
Fig. 4.26 : Showing the Citrate-pyruvate shuttle

The net reaction for this shuttle reveals that two ATP are being used to transport acetyl-CoA out of the mitochondria and to transfer electrons from NADH toNADPH. The citrate-pyruvate shuttle as drawn here is irreversible, because four of the enzymes (pyruvate carboxylase, citrate synthase, ATP-citrate lyase, and malic enzyme) and the pyruvate pump mediate irreversible processes. Note, however, that the citrate and malate are transported across the membrane by exchangers that allow movement in either direction, and that malate has several mechanisms for both entering and leaving the mitochondria. The shuttle mechanisms presented here are examples of commonly used processes. The discussion above does not include a complete list of the exchangers and pumps in the mitochondria membrane. In addition, note that malate is used in both the citrate-pyruvate and malate-aspartate shuttles; cells can use mixtures of theseprocesses to move a variety of carbon units or electrons from one side of the mitochondrial membrane to the other.

SECTION 4.9—TRANSPORT MECHANISMS ACROSS BIOMEMBRANE

SUB-SECTION 4.9A—ACTIVE TRANSPORT

Active transport is the pumping of molecules or ions through a membrane **against** their concentration gradient. It requires:

- a transmembrane protein (usually a complex of them) called a transporter and
- energy. The source of this energy is **ATP**.

The energy of ATP may be used directly or indirectly.

- **Direct Active Transport.** Some transporters bind ATP directly and use the energy of its hydrolysis to drive active transport.
- **Indirect Active Transport.** Other transporters use the energy already stored in the gradient of a directly-pumped **ion**. Direct active transport of the ion establishes a concentration gradient. When this is relieved by facilitated diffusion, the energy released can be harnessed to the pumping of some other ion or molecule.
- **Passive Transport**requires no energy from the cell. Examples include the diffusion of oxygen and carbon dioxide, osmosis of water, and facilitated diffusion.

SUB-SECTION 4.9B—DIRECT ACTIVE TRANSPORT

1. The Na^+/K^+ ATPase

The cytosol of animal cells contains a concentration of potassium ions (K^+) as much as 20 times higher than that in the extracellular fluid. Conversely, the extracellular fluid contains a concentration of sodium ions (Na^+) as much as 10 times greater than that within the cell.

These concentration gradients are established by the active transport of both ions. And, in fact, the same transporter, called the Na^+/K^+ ATPase, does both jobs. It uses the energy from the hydrolysis of ATP to

- actively transport 3 Na^+ ions out of the cell
- for each 2 K^+ ions pumped into the cell.

This accomplishes several vital functions:

- It helps establish a net charge across the plasma membrane with the interior of the cell being negatively charged with respect to the exterior. This **resting potential** prepares nerve and muscle cells for the propagation of action potentials leading to nerve impulses and muscle contraction.
- The accumulation of sodium ions outside of the cell draws water out of the cell and thus enables it to maintain osmotic balance (otherwise it would swell and burst from the inward diffusion of water).

Fig. 4.27 : Showing the passive transport

- The gradient of sodium ions is harnessed to provide the energy to run several types of indirect pumps.

The crucial roles of the Na+/K+ ATPase are reflected in the fact that almost one-third of all the energy generated by the mitochondria in animal cells is used just to run this pump.

Passive Transport

Requires no energy from the cell. Examples include the diffusion of oxygen and carbon dioxide, osmosis of water, and facilitated diffusion.

Symport Pumps

In this type of indirect active transport, the driving ion (Na^+) and the pumped molecule pass through the membrane pump in the **same** direction.

Examples:

- **The Na+/glucose transporter**. This transmembrane protein allows sodium ions and glucose to enter the cell together. The sodium ions flow **down** their concentration gradient while the glucose molecules are pumped **up** theirs. Later the sodium is pumped back out of the cell by the Na+/K+ ATPase.
- The Na+/glucose transporter is used to actively transport glucose out of the intestine and also out of the kidney tubules and back into the blood.
- All the **amino acids** can be actively transported, for example
  - out of the kidney tubules and into the blood [Example]
  - the reuptake of Glu from the synapse back into the presynaptic neuron

  by sodium-driven symport pumps.
- **The Na+/iodide transporter**. This symporter pumps iodide ions into the cells of the thyroid gland (for the manufacture of thyroxine) and also into the cells of the mammary gland (to supply the baby's need for iodide).
- The **permease** encoded by the lac operon of E. coli that transports lactose into the cell.

Antiport Pumps

In antiport pumps, the driving ion (again, usually sodium) diffuses through the pump in one direction providing the energy for the active transport of some other molecule or ion in the opposite direction.

Example: Ca^{2+} ions are pumped out of cells by sodium-driven antiport pumps.

Antiport pumps in the vacuole of some plants harness the outward facilitated diffusion of protons (themselves pumped into the vacuole by a H^+ ATPase) to the active inward transport of sodium ions. This sodium/proton antiport pump enables the plant to sequester sodium ions in its vacuole. Transgenic tomato plants that overexpress this sodium/proton antiport pump are able to thrive in saline soils too salty for conventional tomatoes. to the active inward transport of nitrate ions ($NO_3^{"}$).

Some Inherited ion-channel Diseases

A growing number of human diseases have been discovered to be caused by inherited mutations in genes encoding channels.

Some examples:

- **Chloride-channel** diseases
  - Cystic fibrosis
  - inherited tendency to kidney stones (caused by a different kind of chloride channel than the one involved in cystic fibrosis)
- **Potassium-channel** diseases
  - some inherited life-threatening defects in the heartbeat
  - a rare, inherited tendency to epileptic seizures in the newborn.
  - several types of inherited **deafness** [Discussion]
- **Sodium-channel** diseases
  - inherited tendency to certain types of muscle spasms
  - Liddle's syndrome. Inadequate sodium transport out of the kidneys, because of a mutant sodium channel, leads to elevated **osmotic pressure** of the blood and resulting hypertension (high blood pressure).

SUB-SECTION 4.9C—ENDOCYTOSIS

In endocytosis, the cell engulfs some of its extracellular fluid (**ECF**) including material dissolved or suspended in it. A portion of the plasma membrane is invaginated and pinched off forming a membrane-bounded **vesicle** called an **endosome**.

Phagocytosis

Phagocytosis ("cell eating"):

- results in the ingestion of particulate matter (e.g., bacteria) from the ECF.
- The endosome is so large that it is called a **phagosome** or **vacuole**.

• Phagocytosis occurs only in certain specialized cells (e.g., neutrophils, macrophages, the amoeba), and

• occurs sporadically.

This micrograph shows a guinea phagocyte ingesting polystyrene beads. Several beads are already enclosed in phagosomes while the others are in the process of being engulfed.

In due course, phagosomes deliver their contents to lysosomes. The membranes of the two organelles fuse. Once inside the lysosome, the contents of the phagosome, e.g. ingested bacteria, are destroyed by the degradative enzymes of the lysosome.
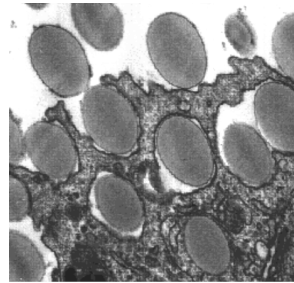
Fig. 4.29 : Showing the Phagocytosis

Games Parasites Play!

Phagocytic cells, like macrophages and neutrophils, are an early line of defense against invading bacteria. However, some bacteria have evolved mechanisms to avoid destruction even after they have been engulfed by phagocytes.

Two examples:

• **Salmonella enterica** is a bacterium that causes food poisoning in humans. Once engulfed by phagocytosis, it secretes a protein that prevents the fusion of its phagosome with a lysosome.

• **Mycobacteria** (e.g., the tubercle bacillus that causes tuberculosis) use a different trick.

  – When the phagosome is first pinched off from the plasma membrane, it is coated with a protein called "TACO" (for tryptophan-aspartate-containing coat protein).

  – This must be removed before the phagosome can fuse with a lysosome.

  – Mycobacteria taken into a phagosome are able, in some way, to keep the TACO coat from being removed.

  – Thus there is no fusion with lysosomes and the mycobacteria can continue to live in this protected intracellular location.

Pinocytosis

In pinocytosis ("cell drinking"), the drop engulfed is relatively small.

Pinocytosis

• occurs in almost all cells

• occurs continuously
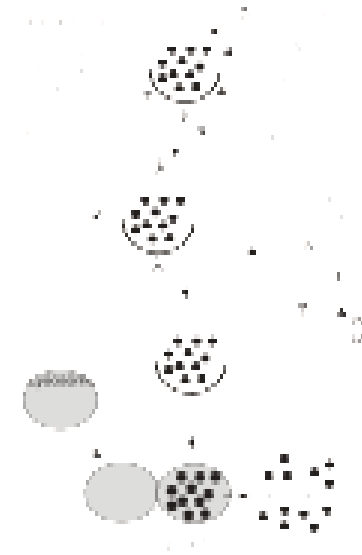
Fig. 4.31 : The diagram showing the Endocytosis in LDL

A cell sipping away at the ECF by pinocytosis acquires a representative sample of the molecules and ions dissolved in the ECF. But pinocytosis also provides a much more elegant method for cells to pick up critical components of the ECF that may be in scant supply.

Receptor-Mediated Endocytosis

Some of the integral membrane proteins that a cell displays at its surface are receptors for particular components of the ECF. For example, iron is transported in the blood complexed to a protein called **transferrin**. Cells have receptors for transferrin on their surface. When these receptors encounter a molecule of transferrin, they bind tightly to it. The complex of transferrin and its receptor is then engulfed by endocytosis. Ultimately, the iron is released into the cytosol. The strong **affinity** of the transferrin receptor for transferrin (its **ligand**) ensures that the cell will get all the iron it needs even if transferrin represents only a small fraction of the protein molecules present in the ECF. Receptor-mediated endocytosis is many thousand times more efficient than simple pinocytosis in enabling the cell to acquire the macromolecules it needs.

Another Example: the Low-Density Lipoprotein (LDL) Receptor

Cells take up cholesterol by receptor-mediated endocytosis. Cholesterol is an essential component of all cell membranes. Most cells can, as needed, either synthesize cholesterol or acquire it from the ECF. Human cells get much of their cholesterol from the liver and, if your diet is not strictly "100% cholesterol-free", by absorption from the intestine.

Cholesterol is a hydrophobic molecule and quite insoluble in water. Thus it cannot pass from the liver and/or the intestine to the cells simply dissolved in blood and ECF. Instead it is carried in tiny droplets of lipoprotein. The most abundant cholesterol carriers in humans are the **low-density lipoproteins** or **LDL**s.

LDL particles are spheres covered with a single layer of phospholipid molecules with their hydrophilic heads exposed to the watery fluid (e.g., blood) and their hydrophobic tails directed into the interior. Over a thousand molecules of cholesterol are bound to the hydrophobic interior of LDL particles. One molecule of a protein, called **apolipoprotein B-100** (Apo B-100) is exposed at the surface of each LDL particle.

The first step in acquiring LDL particles is for them to bind to **LDL receptors** exposed at the cell surface. These transmembrane proteins have a site that recognizes and binds to the apolipoprotein B-100 on the surface of the LDL. The portion of the plasma membrane with bound LDL is internalized by endocytosis. A drop in the pH (from ~7 to ~5) causes the LDL to separate from its receptor. The vesicle then pinches apart into two smaller vesicles: one containing free LDLs; the other containing now-empty receptors. The vesicle with the LDLs fuses with a lysosome to form a **secondary lysosome**. The enzymes of the lysosome then release free cholesterol into the cytosol. The vesicle with unoccupied receptors returns to and fuses with the plasma membrane, turning inside out as it does so (exocytosis). In this way the LDL receptors are returned to the cell surface for reuse.

People who inherit two defective (mutant) genes for the LDL receptor have receptors that function poorly or not at all. This creates excessively high levels of LDL in their blood and predisposes them to atherosclerosis and heart attacks. The ailment is called **familial** (because it is inherited) **hypercholesterolemia**.

Mutations in the **Apo B-100 gene** cause another form of inherited hypercholesterolemia.

Other small hydrophobic molecules are also transported in the blood while bound to soluble proteins:
- the retinoid vitamin A (retinol) bound to the retinol-binding protein
- the steroids
  - 25[OH] vitamin D_3 bound to the vitamin D binding protein
  - cortisol bound to the corticosteroid binding globulin
  - testosterone and estrogens bound to the sex hormone binding globulin

and there is growing evidence that, like cholesterol, they are taken into the cell by receptor-mediated endocytosis.

More Games Parasites Play

Some **intracellular** parasites exploit receptor-mediated endocytosis to sneak their way into their host cell.

They have evolved surface molecules that serve as decoy ligands for receptors on the target cell surface. Binding to these receptors tricks the cell into engulfing the parasite.

Some examples:

This is the organism that led to the discovery that genes are DNA

- **Epstein-Barr Virus** (**EBV**). This virus causes mononucleosis and is a contributing factor in the development of Burkitt's lymphoma, a cancer of B lymphocytes. It binds to a receptor present on the surface of B cells [Link].
- **Influenza virus**. The **hemagglutinin** on the surface of the virus binds to carbohydrate on the surface of the target cell tricking the cell into engulfing it [More].
- **Listeria monocytogenes**. This food-borne bacterium can be dangerous to people with defective immune systems as well as to pregnant women and their newborn babies. It has two kinds of surface molecules each a ligand for a different receptor on the target cell surface.
- **Streptococcus pneumoniae**. Epithelial cells like those in the nasopharynx have receptors that are responsible for transporting IgA and IgM antibodies from the blood to the cell surface. The pneumococcus exploits this receptor for a return trip into the cell.

Coming full Circle

Endocytosis removes portions of the plasma membrane and takes them inside the cell. To keep in balance, membrane must be returned to the plasma membrane. This occurs by exocytosis.

SECTION 4.10—TRANSPORT THROUGH CELL MEMBRANE

Importance

All cells acquire the molecules and ions they need from their surrounding **extracellular fluid** (**ECF**). There is an unceasing traffic of molecules and ions

- in and out of the cell through its plasma membrane
  - Examples: glucose, Na^+, Ca^{2+}
- In eukaryotic cells, there is also transport in and out of membrane-bounded intracellular compartments such as the nucleus, endoplasmic reticulum, and mitochondria.
  - Examples: proteins, mRNA, Ca^{2+}, ATP

Two problems to be considered

1. Relative concentrations

Molecules and ions move spontaneously down their concentration gradient (i.e., from a region of higher to a region of lower concentration) by **diffusion**.

Molecules and ions can be moved **against** their concentration gradient, but this process, called **active transport**, requires the expenditure of energy (usually from ATP).

2. Lipid bilayers are impermeable to most essential molecules and ions

The lipid bilayer is permeable to **water** molecules and a few other small, uncharged, molecules like oxygen (O_2) and carbon dioxide (CO_2). These diffuse freely in and out of the cell. The diffusion of water through the plasma membrane is of such importance to the cell that it is given a special name: **osmosis**.

Lipid bilayers are **not** permeable to:

- **ions** such as
    - K^+, Na^+, Ca^{2+} (called **cations** because when subjected to an electric field they migrate toward the cathode [the negatively-charged electrode])
    - Cl^-, HCO_3^- (called **anions** because they migrate toward the anode [the positively-charged electrode])
- small hydrophilic **molecules** like glucose
- **macromolecules** like proteins and RNA

This page will examine how ions and small molecules are transported across cell membranes. The transport of macromolecules through membranes is described in Endocytosis.

Solving these problems

Mechanisms by which cells solve the problem of transporting ions and small molecules across their membranes:

Facilitateddiffusion

Transmembrane proteins create a water-filled pore through which ions and some small hydrophilic molecules can pass by diffusion. The channels can be opened (or closed) according to the needs of the cell.

Activetransport

Transmembrane proteins, called transporters, use the energy of ATP to force ions or small molecules through the membrane **against** their concentration gradient.

Facilitated Diffusion of Ions

Facilitated diffusion of ions takes place through proteins, or assemblies of proteins, embedded in the plasma membrane. These transmembrane proteins form a water-filled channel through which the ion can pass **down** its concentration gradient.

The transmembrane channels that permit facilitated diffusion can be opened or closed. They are said to be **"gated"**.

Some types of gated ion channels:

- ligand-gated
- mechanically-gated
- voltage-gated
- light-gated

Ligand-gated ion channels

Many ion channels open or close in response to binding a small signaling molecule or **"ligand"**. Some ion channels are gated by extracellular ligands; some by intracellular ligands. In both cases, the ligand is **not** the substance that is transported when the channel opens.

External ligands

External ligands (shown here in green) bind to a site on the extracellular side of the channel.

Examples:

- **Acetylcholine** (**ACh**). The binding of the neurotransmitter acetylcholine at certain synapses opens channels that admit Na^+ and initiate a nerve impulse or muscle contraction.
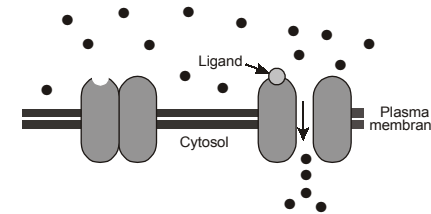
Fig. 4.31 : Showing diffusion through a ligand-gated channel

- **Gamma amino butyric acid** (**GABA**). Binding of GABA at certain synapses — designated $GABA_A$ — in the central nervous system admits Cl^- ions into the cell and inhibits the creation of a nerve impulse. [More]

Internal ligands

Internal ligands bind to a site on the channel protein exposed to the cytosol.

Examples:

- "Second messengers", like **cyclic AMP** (**cAMP**) and **cyclic GMP** (**cGMP**), regulate channels involved in the initiation of impulses in neurons responding to odors and light respectively.
- **ATP** is needed to open the channel that allows chloride (Cl^-) and bicarbonate (HCO_3^-) ions out of the cell. This channel is defective in patients with **cystic fibrosis**. Although the energy

liberated by the hydrolysis of ATP is needed to open the channel, this is **not** an example of active transport; the ions diffuse through the open channel following their concentration gradient.

Mechanically-gated ion channels

Examples:

- Sound waves bending the cilia-like projections on the hair cells of the inner ear open up ion channels leading to the creation of nerve impulses that the brain interprets as sound. [More]
- Mechanical deformation of the cells of stretch receptors opens ion channels leading to the creation of nerve impulses. [More]

Voltage-gated ion Channels

In so-called "excitable" cells like **neurons** and **muscle cells**, some channels open or close in response to changes in the charge (measured in volts) across the plasma membrane.

Example: As an impulse passes down a neuron, the reduction in the voltage opens sodium channels in the adjacent portion of the membrane. This allows the influx of Na^+ into the neuron and thus the continuation of the nerve impulse. [More]

Some 7000 sodium ions pass through each channel during the brief period (about 1 millisecond) that it remains open. This was learned by use of the patch clamp technique.

The Patch Clamp Technique

The properties of ion channels can be studied by means of the patch clamp technique.

- A very fine pipette (with an opening of about 0.5 μm) is pressed against the plasma membrane of
- either an intact cell or
- the plasma membrane can be pulled away from the cell and the preparation placed in a test solution of desired composition.
- Current flow through a single ion channel can then be measured.

Such measurements reveal that each channel is either fully open or fully closed; that is, facilitated diffusion through a single channel is "all-or-none".

This technique has provided so much valuable information about ion channels that its inventors, Erwin Neher and Bert Sakmann, were awarded a Nobel Prize in 1991.
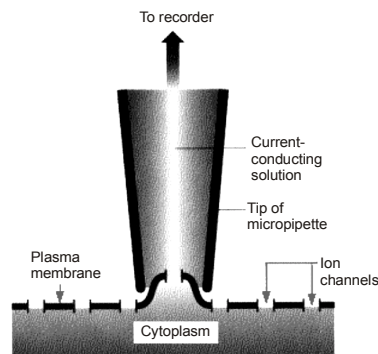
Fig. 4.32 : Showing the patch clamp technique

Facilitated Diffusion of Molecules

Some small, hydrophilic organic molecules, like sugars, can pass through cell membranes by facilitated diffusion.

Once again, the process requires transmembrane proteins. In some cases, these — like ion channels — form water-filled pores that enable the molecule to pass in (or out) of the membrane following its concentration gradient.

Example: **Maltoporin**. This homotrimer in the outer membrane of E. coli forms pores that allow the disaccharide maltose and a few related molecules to diffuse into the cell.

Another example: The plasma membrane of human red blood cells contain transmembrane proteins that permit the diffusion of glucose from the blood into the cell.

Note that in all cases of facilitated diffusion through channels, the channels are **selective**; that is, the structure of the protein admits only certain types of molecules through.

Whether all cases of facilitated diffusion of small molecules use channels is yet to be proven. Perhaps some molecules are passed through the membrane by a conformational change in the shape of the transmembrane protein when it binds the molecule to be transported.

In either case, the interaction between the molecule being transported and its transporter resembles in many ways the interaction between an enzyme and its substrate. Link to a discussion of the energetics of enzyme-substrate interactions.

Active Transport

Active transport is the pumping of molecules or ions through a membrane **against** their concentration gradient. It requires:

- a transmembrane protein (usually a complex of them) called a transporter and
- energy. The source of this energy is **ATP**.

The energy of ATP may be used directly or indirectly.

- **Direct Active Transport.** Some transporters bind ATP directly and use the energy of its hydrolysis to drive active transport.
- **Indirect Active Transport.** Other transporters use the energy already stored in the gradient of a directly-pumped **ion**. Direct active transport of the ion establishes a concentration gradient. When this is relieved by facilitated diffusion, the energy released can be harnessed to the pumping of some other ion or molecule.

Direct Active Transport

1. The Na⁺/K⁺ ATPase

The cytosol of animal cells contains a concentration of potassium ions (K^+) as much as 20 times higher than that in the extracellular fluid. Conversely, the extracellular fluid contains a concentration of sodium ions (Na^+) as much as 10 times greater than that within the cell.

These concentration gradients are established by the active transport of both ions. And, in fact, the same transporter, called the Na^+/K^+ ATPase, does both jobs. It uses the energy from the hydrolysis of ATP to

- actively transport 3 Na^+ ions out of the cell
- for each 2 K^+ ions pumped into the cell.

This accomplishes several vital functions:

- It helps establish a net charge across the plasma membrane with the interior of the cell being negatively charged with respect to the exterior. This **resting potential** prepares nerve and muscle cells for the propagation of action potentials leading to nerve impulses and muscle contraction.
- The accumulation of sodium ions outside of the cell draws water out of the cell and thus enables it to maintain osmotic balance (otherwise it would swell and burst from the inward diffusion of water).
- The gradient of sodium ions is harnessed to provide the energy to run several types of indirect pumps.

The crucial roles of the Na^+/K^+ ATPase are reflected in the fact that almost one-third of all the energy generated by the mitochondria in animal cells is used just to run this pump.

2. The H⁺/K⁺ ATPase

The parietal cells of your stomach use this pump to secrete gastric juice. These cells transport protons (H^+) from a concentration of about 4×10^{-8} M within the cell to a concentration of about 0.15 M in the gastric juice (giving it a pH close to 1). Small wonder that parietal cells are stuffed with mitochondria and uses huge amounts of energy as they carry out this three-million fold concentration of protons.

3. The Ca²⁺ ATPases

A Ca^{2+} ATPase is located in the **plasma membrane** of all eukaryotic cells. It uses the energy provided by one molecule of ATP to pump one Ca^{2+} ion out of the cell. The activity of these pumps helps to maintain the ~20,000-fold concentration gradient of Ca^{2+} between the cytosol (~ 100 nM) and the ECF (~ 20 mM).

In resting skeletal muscle, there is a much higher concentration of calcium ions (Ca^{2+}) in the sarcoplasmic reticulum than in the cytosol. Activation of the muscle fiber allows some of this Ca^{2+} to pass by facilitated diffusion into the cytosol where it triggers contraction. [Link to discussion].

After contraction, this Ca^{2+} is pumped back into the sarcoplasmic reticulum. This is done by another Ca^{2+} ATPase that uses the energy from each molecule of ATP to pump 2 Ca^{2+} ions.

Pumps **1. - 3.** are designated **P-type ion transporters** because they use the same basic mechanism: a conformational change in the proteins as they are reversibly phosphorylated by ATP. And all three pumps can be made to run backward. That is, if the pumped ions are allowed to diffuse back through the membrane complex, ATP can be synthesized from ADP and inorganic phosphate.

4. ABC Transporters

ABC ("ATP-Binding Cassette") transporters are transmembrane proteins that

- expose a ligand-binding domain at one surface and a
- ATP-binding domain at the other surface.

The ligand-binding domain is usually restricted to a single type of molecule.

The ATP bound to its domain provides the energy to pump the ligand across the membrane.

The human genome contains 48 genes for ABC transporters. Some examples:

- **CFTR** — the cystic fibrosis transmembrane conductance regulator
- **TAP**, the transporter associated with antigen processing. [Discussion]
- the transporter that liver cells use to pump the salts of bile acids out into the bile.
- ABC transporters that pump chemotherapeutic drugs out of cancer cells thus reducing their effectiveness.

ABC transporters must have evolved early in the history of life. The ATP-binding domains in archaea, eubacteria, and eukaryotes all share a homologous structure, the ATP-binding "cassette".

Indirect Active Transport

Indirect active transport uses the downhill flow of an ion to pump some other molecule or ion against its gradient. The driving ion is usually sodium (Na^+) with its gradient established by the Na^+/K^+ ATPase.

Symport Pumps

In this type of indirect active transport, the driving ion (Na^+) and the pumped molecule pass through the membrane pump in the **same** direction.

Examples:

The Na^+/glucose transporter. This transmembrane protein allows sodium ions and glucose to enter the cell together. The sodium ions flow **down** their concentration gradient while the glucose molecules are pumped **up** theirs. Later the sodium is pumped back out of the cell by the Na^+/K^+ ATPase.

The Na^+/glucose transporter is used to actively transport glucose out of the intestine and also out of the kidney tubules and back into the blood.

The energy relationships for these processes can be quantified.

All the **amino acids** can be actively transported, for example

out of the kidney tubules and into the blood [Example]

the reuptake of Glu from the synapse back into the presynaptic neuron

by sodium-driven symport pumps.

The Na^+/iodide transporter. This symporter pumps iodide ions into the cells of the thyroid gland (for the manufacture of thyroxine) and also into the cells of the mammary gland (to supply the baby's need for iodide).

The **permease** encoded by the lac operon of E. coli that transports lactose into the cell.

Antiport Pumps

In antiport pumps, the driving ion (again, usually sodium) diffuses through the pump in one direction providing the energy for the active transport of some other molecule or ion in the opposite direction.

Example: Ca^{2+} ions are pumped out of cells by sodium-driven antiport pumps [Link].

Antiport pumps in the vacuole of some plants harness the outward facilitated diffusion of protons (themselves pumped into the vacuole by a H^+ ATPase)

- to the active inward transport of sodium ions. This sodium/proton antiport pump enables the plant to sequester sodium ions in its vacuole. Transgenic tomato plants that overexpress this sodium/proton antiport pump are able to thrive in saline soils too salty for conventional tomatoes.
- to the active inward transport of nitrate ions (NO_3^-).

Some inherited ion-channel diseases

A growing number of human diseases have been discovered to be caused by inherited mutations in genes encoding channels.

Some examples:

- **Chloride-channel** diseases
  - Cystic fibrosis
  - inherited tendency to kidney stones (caused by a different kind of chloride channel than the one involved in cystic fibrosis)
- **Potassium-channel** diseases
  - some inherited life-threatening defects in the heartbeat
  - a rare, inherited tendency to epileptic seizures in the newborn.
  - several types of inherited **deafness**
- **Sodium-channel** diseases
  - inherited tendency to certain types of muscle spasms
  - Liddle's syndrome. Inadequate sodium transport out of the kidneys, because of a mutant sodium channel, leads to elevated **osmotic pressure** of the blood and resulting hypertension (high blood pressure).

Osmosis

Osmosis is a special term used for the diffusion of water through cell membranes.

Although water is a polar molecule, it is able to pass through the lipid bilayer of the plasma membrane. Transmembrane proteins that form hydrophilic channels accelerate the process, but even without these, water is still able to get through.

Water passes by diffusion from a region of higher to a region of lower concentration. Note that this refers to the concentration of water, NOT the concentration of any solutes present in the water.

Water is never transported actively; that is, it never moves against its concentration gradient. However, the concentration of water can be altered by the active transport of solutes and in this way the movement of water in and out of the cell can be controlled.

Example: the reabsorption of water from the kidney tubules back into the blood depends on the water following behind the active transport of Na^+. [Discussion]

Hypotonic Solutions

If the concentration of water in the medium surrounding a cell is greater than that of the cytosol, the medium is said to be **hypotonic**. Water enters the cell by osmosis.

A red blood cell placed in a hypotonic solution (e.g., pure water) bursts immediately ("hemolysis") from the influx of water.

Plant cells and bacterial cells avoid bursting in hypotonic surroundings by their strong cell walls. These allow the buildup of **turgor** within the cell. When the turgor pressure equals the osmotic pressure, osmosis ceases.

> How the kidneys of freshwater fishes and amphibians permit their owners to live in their hypotonic surroundings.

Isotonic Solutions

When red blood cells are placed in a 0.9% salt solution, they neither gain nor lose water by osmosis. Such a solution is said to be **isotonic**.

The extracellular fluid (ECF) of mammalian cells is isotonic to their cytoplasm. This balance must be actively maintained because of the large number of organic molecules dissolved in the cytosol but not present in the ECF. These organic molecules exert an osmotic effect that, if not compensated for, would cause the cell to take in so much water that it would swell and might even burst. This fate is avoided by pumping sodium ions out of the cell with the Na^+/K^+ ATPase.

Hypertonic Solutions

If red cells are placed in sea water (about 3% salt), they lose water by osmosis and the cells shrivel up. Sea water is **hypertonic** to their cytosol.

Similarly, if a plant tissue is placed in sea water, the cell contents shrink away from the rigid cell wall. This is called **plasmolysis**. [Link to a view of it.]

Sea water is also hypertonic to the ECF of most marine vertebrates. To avoid fatal dehydration, these animals (e.g., bony fishes like the cod) must

- continuously drink sea water and then
- desalt it by pumping ions out of their gills by active transport. (Marine reptiles — turtles and snakes — use special salt glands for the same purpose.)

How the kidneys of marine fishes are adapted for life in a hypertonic environment.

Osmosis is important A report in the 23 April 1998 issue of **The New England Journal of Medicine** tells of the life-threatening complications that can be caused by an ignorance of osmosis.

- Large volumes of a solution of 5% human albumin are injected into people undergoing a procedure called plasmapheresis.
- The albumin is dissolved in physiological saline (0.9% NaCl) and is therefore isotonic to human plasma (the large protein molecules of albumin have only a small osmotic effect).
- If 5% solutions are unavailable, pharmacists may substitute a proper dilution of a 25% albumin solution. Mixing 1 part of the 25% solution with 4 parts of diluent results in the correct 5% solution of albumin.
- **BUT**, in several cases, the diluent used was sterile water, not physiological saline.
- **SO**, the resulting solution was strongly hypotonic to human plasma.
- **The Result:** massive, life-threatening hemolysis in the patients.

SUB-SECTION 4.10A—ROLE OF G PROTEINS

G proteins, short for **guanine nucleotide-binding proteins**, are a family of proteins involved in second messenger cascades.

G proteins are so called because they function as "molecular switches," alternating between an inactive guanosine diphosphate (GDP) and active guanosine triphosphate (GTP) bound state, ultimately going on to regulate downstream cell processes.

G proteins were discovered when Alfred G. Gilman and Martin Rodbell investigated stimulation of cells by adrenaline. They found that when a hormone like adrenaline bound to a receptor, the receptor did not stimulate enzymes like adenylate cyclase directly. Instead, the receptor stimulated a G protein, which then stimulated the adenylate cyclase to produce a second messenger, cyclic AMP. For this discovery they won the 1994 Nobel Prize in Physiology or Medicine.

G proteins belong to the larger group of enzymes called GTPases.

Function

G proteins are important signal transducing molecules in cells. In fact, diseases such as diabetes, blindness, allergies, depression, cardiovascular defects and certain forms of cancer, among other pathologies, are thought to arise due to derangement of G protein signaling.

The human genomes encodes roughly 350 G protein-coupled receptors, which detect hormones, growth factors and other endogenous ligands. Approximately 150 of the GPCRs found in the human genome have unknown functions.

Types of G protein Signaling

G protein can refer to two distinct families of proteins. Heterotrimeric G proteins, sometimes referred to as the "large" G proteins that are activated by G protein-coupled receptors and made up of alpha (α),

beta (β), and gamma (γ) subunits. There are also *"small" G proteins* (20-25kDa) that belong to the Ras superfamily of small GTPases. These proteins are homologous to the alpha (α) subunit found in heterotrimers, and are in fact monomeric. However, they also bind GTP and GDP and are involved in signal transduction.

Heterotrimeric G Proteins

Heterotrimeric G proteins share a common mode of action, i.e., activation in response to a conformation change in the G-protein-coupled receptor, exchange of GTP for GDP and dissociation in order to activate further proteins in the signal transduction pathway. However, the specific mechanism differs between different types of G proteins.

Common Mechanism
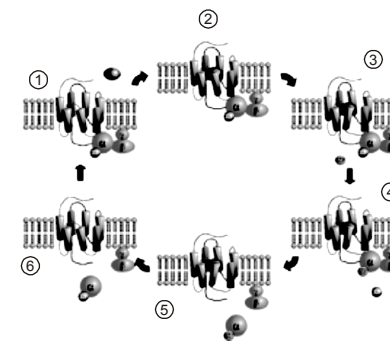
Fig. 4.25 : Activation cycle of G-proteins by G-protein-coupled receptors

Receptor-activated G proteins are bound to the inside surface of the cell membrane. They consist of the G_α and the tightly associated $G_{\beta\gamma}$ subunits. At the present time, four main families exist for G_α subunits: $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q/11}$, and $G_{\alpha 12/13}$. These groups differ primarily in effector recognition, but share a similar mechanism of activation.

Activation

When a ligand activates the G protein-coupled receptor, it induces a conformation change in the receptor (a change in shape) that allows the receptor to function as a guanine nucleotide exchange factor (GEF) that exchanges GTP in place of GDP on the G_α subunit. In the traditional view of heterotrimeric protein activation, this exchange triggers the dissociation of the G_α subunit, bound to GTP, from the $G_{\beta\gamma}$ dimer and the receptor. However, models that suggest molecular rearrangement, reorganization, and pre-complexing of effector molecules are beginning to be accepted. Both G_α-GTP and $G_{\beta\gamma}$ can then activate different *signaling cascades* (or *second messenger pathways*) and effector proteins, while the receptor is able to activate the next G protein.

Termination

The G_α subunit will eventually hydrolyze the attached GTP to GDP by its inherent enzymatic activity, allowing it to re-associate with $G_{\beta\gamma}$ and starting a new cycle. There do exist groups of proteins called RBMs that act as GTPase-activating proteins (GAPs), which are specific for G_α subunits, which act to accelerate hydrolysis and terminate the transduced signal. In some cases the effector itself may possess intrinsic GAP activity, which helps deactivate the pathway. This is true in the case of phospholipase C beta, which possesses GAP activity within its C-terminal region. This is an alternate form of regulation for the G_α subunit.

Specific mechanisms

- G_{as} stimulates the production of cAMP from ATP. This is accomplished by direct stimulation of the membrane-associated enzyme adenylate cyclase. cAMP acts as a second messenger that goes on to interact with and activate protein kinase A (PKA). PKA can then phosphorylate a myriad of downstream targets.
- G_{ai} inhibits the production of cAMP from ATP.
- $G_{aq/11}$ stimulates membrane-bound phospholipase C beta, which then cleaves PIP_2 (a minor membrane phosphoinositol) into two second messengers, IP3 and diacylglycerol (DAG).
- $G_{a12/13}$ are involved in Rho family GTPase signaling (through RhoGEF superfamily) and control cell cytoskeleton remodeling, thus regulating cell migration.
- G_{bg} sometimes also have active functions, e.g., coupling to L-type calcium channels.

Small GTPases

Small GTPases also bind GTP and GDP and are involved in signal transduction. These proteins are homologous to the alpha (α) subunit found in heterotrimers, but exist as monomers. They are small (20-kDa to 25-kDa) proteins that bind to guanosine triphosphate (GTP). This family of proteins is homologous to Ras GTPases and is also called the Ras superfamily GTPases.

Lipidation

In order to associate with the inner leaflet of the plasma membrane, many G proteins are covalently modified with lipid extensions, i.e., they are lipidated.

- Heterotrimeric G protein subunits may be myristolated, palmitoylated, or prenylated.
- Small G proteins may be prenylated.

SUB-SECTION 4.10B—CYCLIC ADENOSINE MONOPHOSPHATE

Cyclic adenosine monophosphate (**cAMP**, **cyclic AMP** or 3'-5'-cyclic adenosine monophosphate) is a second messenger that is important in many biological processes. cAMP is derived from adenosine triphosphate (ATP) and used for intracellular signal transduction in many different organisms.

Synthesis and Decomposition

cAMP is synthesised from ATP by adenylyl cyclase which is located at the cell membranes. Adenylyl cyclase is activated by a range of signaling molecules through the activation of adenylyl cyclase stimulatory G (G_s)-coupled receptors and inhibited by agonists of adenylyl cyclase inhibitory G (G_i)-protein coupled receptors. Liver adenylyl cyclase responds more strongly to glucagon, and muscle adenylyl cyclase responds more strongly to adrenaline.

cAMP decomposition into AMP is catalyzed by the enzyme phosphodiesterase.

Functions

cAMP is a second messenger, used for intracellular signal transduction, such as transferring the effects of hormones like glucagon and adrenaline, which cannot get through the cell membrane. Its purposes include the activation of protein kinases and regulating the effects of adrenaline and glucagon. It is also used to regulate the passage of Ca^{2+} through ion channels.

In humans

Epinephrine (adrenaline) binds its receptor, that associates with an heterotrimeric G protein. The G protein associates with adenylyl cyclase that converts ATP to cAMP, spreading the signal (more details...)

cAMP and its associated kinases function in several biochemical processes, including the regulation of glycogen, sugar, and lipid metabolism.

In humans, cyclic AMP works by activating protein kinase A (PKA, also known as cAMP-dependent protein kinase). This is normally inactive as a tetrameric holoenzyme, consisting of 2 catalytic and 2 regulatory units (C_2R_2), with the regulatory units blocking the catalytic centers of the catalytic units. Cyclic AMP binds to specific locations on the regulatory units of the protein kinase, and causes dissociation between the regulatory and catalytic subunits, thus activating the catalytic units and enabling them to phosphorylate substrate proteins.

The active subunits catalyze the transfer of phosphate from ATP to specific serine or threonine residues of protein substrates. The phosphorylated proteins may act directly on the cell's ion channels, or may become activated or inhibited enzymes. Protein kinase A can also phosphorylate specific proteins that bind to promoter regions of DNA, causing increased expression of specific genes. Not all protein kinases respond to cAMP: several types of protein kinases are not cAMP dependent, for example protein kinase C. Further effects depend on cAMP-dependent protein kinase, which vary based on the type of cell.

Chapter **5**

Metabolism

SECTION 5.1—AN OVER VIEW

Metabolism is the set of chemical reactions that occur in living organisms in order to maintain life. These processes allow organisms to grow and reproduce, maintain their structures, and respond to their environments. Metabolism is usually divided into two categories. Catabolism breaks down organic matter, for example to harvest energy in cellular respiration. Anabolism, on the other hand, uses energy to construct components of cells such as proteins and nucleic acids.

The chemical reactions of metabolism are organized into metabolic pathways, in which one chemical is transformed into another by a sequence of enzymes. Enzymes are crucial to metabolism because they allow organisms to drive desirable but thermodynamically unfavorable reactions by coupling them to favorable ones, and because they act as catalysts to allow these reactions to proceed quickly and efficiently. Enzymes also allow the regulation of metabolic pathways in response to changes in the cell's environment or signals from other cells.

The metabolism of an organism determines which substances it will find nutritious and which it will find poisonous. For example, some prokaryotes use hydrogen sulfide as a nutrient, yet this gas is poisonous to animals. The speed of metabolism, the metabolic rate, also influences how much food an organism will require.

A striking feature of metabolism is the similarity of the basic metabolic pathways between even vastly different species. For example, the set of carboxylic acids that are best known as the intermediates in the citric acid cycle are present in all organisms, being found in species as diverse as the unicellular bacteria *Escherichia coli* and huge multicellular organisms like elephants. These striking similarities in

metabolism are most likely the result of the high efficiency of these pathways, and of their early appearance in evolutionary history.

SUB-SECTION 5.1A—CONTROL OF METABOLIC PATHWAYS

In biochemistry, a metabolic pathway is a series of chemical reactions occurring within a cell. In each pathway, a principal chemical is modified by chemical reactions. Enzymes catalyze these reactions, and often require dietary minerals, vitamins and other cofactors in order to function properly. Because of the many chemicals that may be involved, pathways can be quite elaborate. In addition, many pathways can exist within a cell. This collection of pathways is called the metabolic network. Pathways are important to the maintenance of homeostasis within an organism.

Metabolism is a step by step modification of the initial molecule to shape it into another product. The result can be used in one of three ways.

- Stored by the cell.
- Be used immediately, as a metabolic product.
- Initiate another metabolic pathway, called a flux generating step.

A molecule called a substrate enters a metabolic pathway depending on the needs of the cell and the availability of the substrate. An increase in concentration of anabolic and catabolic end products would slow the metabolic rate for that particular pathway.

Each metabolic pathway is composed of a series of biochemical reactions that are connected by their intermediates: the reactants (or substrates) of one reaction are the products of the previous one, and so on. Metabolic pathways are usually considered in one direction (although all reactions are chemically reversible, conditions in the cell are such that it is thermodynamically more favorable for flux to be in one of the directions).

- Glycolysis was the first metabolic pathway discovered:
    1. As glucose enters a cell it is immediately phosphorylated by ATP to glucose 6-phosphate in the irreversible first step. This is to prevent the glucose leaving the cell.
    2. In times of excess lipid or protein energy sources glycolysis may run in reverse (gluconeogenesis) in order to produce glucose 6-phosphate for storage as glycogen or starch.
- Metabolic pathways are often regulated by feedback inhibition, or by a cycle where one of the products in the cycle starts the reaction again, such as the Krebs Cycle (see below).
- Anabolic and catabolic pathways in eukaryotes are separated by either compartmentation or by the use of different enzymes and cofactors.

Cellular Respiration

Several distinct but linked metabolic pathways are used by cells to transfer the energy released by breakdown of fuel molecules to ATP. These occur within all living organisms in some forms:

1. Glycolysis
2. Anaerobic respiration
3. Krebs cycle/Citric acid cycle
4. Oxidative phosphorylation

Other pathways occurring in (most or) all living organisms include:
- Fatty acid oxidation (β-oxidation)
- Gluconeogenesis
- HMG-CoA reductase pathway (isoprene prenylation chains, see cholesterol)
- Pentose phosphate pathway (hexose monophosphate shunt)
- Porphyrin synthesis (or heme synthesis) pathway
- Urea cycle

Creation of energetic compounds from non-living matter:
- Photosynthesis (plants, algae, cyanobacteria)
- Chemosynthesis (some bacteria)

SECTION 5.2—METABOLISM OF CARBOHYDRATE

The chief primary materials for carbohydrate metabolism supplied to the blood from the intestine are the monosaccharides from the digestion of food for the synthesis of tissue proteins in growth and maintenance. The materials absorbed into the portal blood pass to the liver. Where fructose and galactose are converted to glucose and glycogen and some of the amino acids are deaminized[*] to for keto acids which are also converted to these carbohydrate. Lactic and pyruvic acids which are also converted to these carbohydrates. Lactic and pyruvic acids which are also converted to glucose and glycogen. All the process by which the liver converts non-glucose substances into glucose constitutes the gluconeogenetic mechanism of the liver. Although the kidneys have some capacity for gluconeogenesis, it is very limited as compared with that of the liver. Gluconeogenesis is constantly taking place but at widely varying rates. It is increased on high protein diets when large amounts of amino acids are absorbed into the blood, and decreased on high carbohydrate diets. When there is an abundance of preformed glucose. It is increased during exercise, when large amount of lactic acid and pyruvic acids escape from the working muscles and there is need to keep up the blood glucose and replenish the muscle glycogen supply. Under these conditions the liver acts to recover and return to them sources of energy lost by the muscles. During starvation gluconeogenesis from the amino acids of tissue protein is the chief source of blood sugar and tissue glycogen. In diabetes the rate if gluconeogenesis from both food and tissue protein may be greatly increased, contributing to body emaciation.

The liver stores glucose as glycogen when the supplies of blood glucose and glucose precursors are liberal and reconverts this glycogen to glucose for addition to the blood when the blood sugar falls.

_____

*Deaminized, Deamination is the removal of an amine group from a molecule.

The liver thus acts as a glucostatic mechanism to maintain the blood glucose within normal physiologic limits. The conversion of glucose to glycogen by the liver (as well as other tissues) requires preliminary phosphorylation of the glucose, for which ATP is essential chemical energy in the form of ATP is required also for many other liver processes. Part of this ATP is provided through oxidation of fatty acids, and a partly by the oxidation of the glycogen. In this latter process the glycogen first undergoes breakdown or glycolysis through a long series of phosphyrelated intermediates of pyruvic acid. In this process some useful energy as ATP is stored up.

The pyruvic acid is then oxidized through the tricarboxylic or citric acid cycle to CO_2 and H_2O, with the formation of most of the ATP which is produced from glycogen, while both lactic and pyruvic acids are formed in the liver cells, essentially none escapes into the blood (in contrast with muscle.

This is due to the efficiency of reconversion to glycogen; in case oxidation of pyruvic acid cannot keep up with the rate of its formation in the glycolytic stage. Lactic acid is first converted to pyruvic acid is then converted to glycogen. These processes thus from lactic acid and pyruvic acid. It will be noted from the fig 136 that glycerol is formed in the glycolytic phase of glycogen breakdown in the liver. Fatty acids are synthesized from pyruvic acid, which upon combination with the glycerol from the glycerides of fat form the carbohydrates. The liver also synthesizes cholesterol from the pyruvic acid of glycogen breakdown. This synthesis proceeds through acetic acid (acetyl CoA) as an intermediate stage as also does the synthesis of fatty acids from the pyruvic acid. Acetic acid as acetyl coenzyme CoA formed from pyruvic acid is used by the liver in acetylating amines in the process of detoxication.

The chemical mechanism involved in liver gluconeogenesis from non glucose monosaccharides and amino acids will be discussed latter.

Carbohydrate metabolism in muscle is highly specialized and designed primarily for the production of ATP as a source of energy for the concentrated process. Muscle is limited essentially to blood glucose for its carbohydrate supply. In muscle, glucose is converted to glycogen through the same phosphorylating reactions involved in the formation of liver glycogen yields energy as ATP through glycolysis and oxidation of pyruvic acid in the tricarboxylic acid cycle just as Liver glycogen does.

Muscle also forms glycogen from lactic acid through pyruvic acid and reversal of the glycolytic reactions. A part of lactic acid is reconverted to glycogen, in the case when rigorous exercise the rate of production of pyruvic and lactic acid in muscle greatly exceeds the rate at which they can be oxidized or reconverted to glycogen, and much escapes into the blood stream. Any condition such as asphyxia which causes tissue anoxia or which limits the oxidative processes in tissue likewise causes the rapid breakdown of muscle glycogen and escape of these acids into the blood most of the acids are not picked up from the blood and utilized by the muscles and other peripheral tissues are transported to the liver and converted to glycogen, which is passed back into the blood as glucose when the blood sugar level falls. However, the conservation by the liver of lactic and pyruvic acids rise appreciable amounts may be lost in the urine.

While pyruvic acid is the primary product of glycolysis it is reversely convertible to lactic acid under the influence of the enzyme lactic acid and the blood of normal resting adults generally contains 10 mg of lactic acid and 1 mg of pyruvic acid per 100ml. the lactate – pyruvate ratio then is about 10:1 These values vary with exercise and disturbances in normal tissue oxidative process.
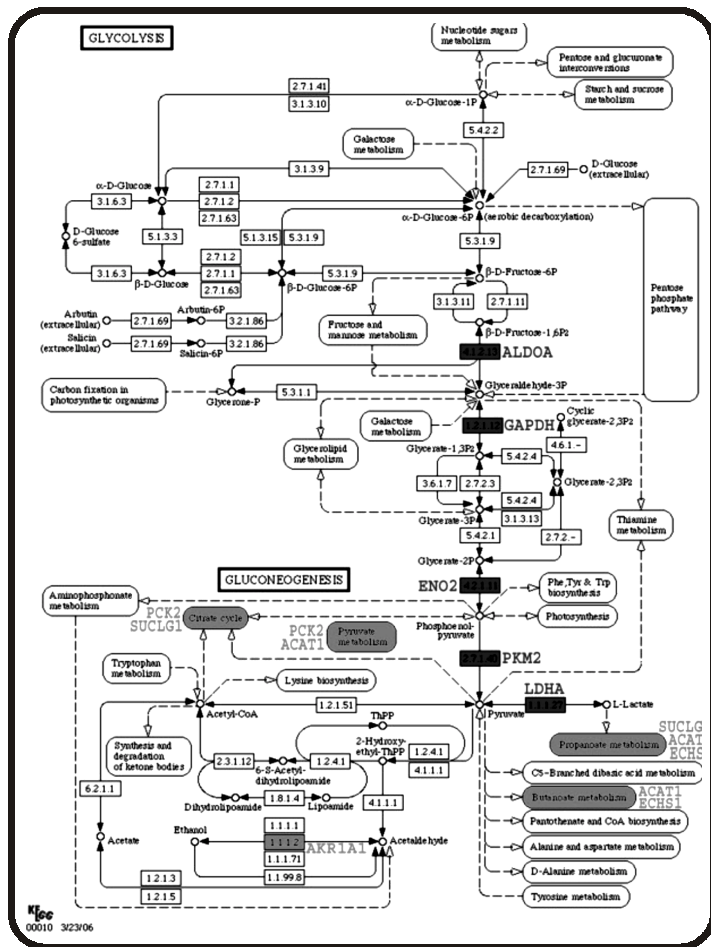
Fig. 4.40 : Showing the Carbohydrate Metabolism

Chemcial Mechanism involved in Carbohydrate Metabolism

The chemical reactions involved in carbohydrate metabolism in the body represent complex groups sequences and cycles of reactions which integrate at various points with the reactions concerned in the metabolism. So that the reactions involved in one stage of metabolism. For example the glycolytic breakdown of glucose or glycogen through many phosphorylated intermediates to pyruvic acid and the subsequent oxidation of this pyruvic acid through the sequence of reactions in the tricarboxylic acid section 5.6. Now we can see that the reactions of glycolysis must take place preliminary to the reactions of carbohydrate oxidation. The synthesis of fat from carbohydrate involves synthesis of fatty acids from pyruvic acids formed by glycolysis and combinations of these fatty acids with glycerol, which is also a product of glycolytic reactions. Another interesting aspect of carbohydrate metabolism is that the reactions involved in one phase of metabolism may represent essentially the reverse of the reaction concerned in a second phase of metabolism. For example, the process of glucogenesis from the pyruvic acid in liver consists chiefly in reversal of the glycolysis reactions by which the liver converts glucose to pyruvic acid. Thus it is seen that while we may differentiate carbohydrate metabolism into various phases such as glucogenesis glycolysis (breakdown of carbohydrate generally glycogen or glucose) glycogenesis (glycogen synthesis) glycogenolysis (breakdown of glycogen synthesis specially) carbohydrate oxidation, and fat synthesis from carbohydrate it is impossible to segregate into different distinct groups the chemical reactions concerned with these phases of carbohydrate metabolism.

The major types chemical processes involved in carbohydrate metabolism may be grouped as follows:

(i) The reversible process glucose $\rightleftharpoons$ glycogen

(ii) The process by which sugar such as fructose mannose, and galactose, are converted to glucose

(iii) The reversible process by which glucose is converted to pyruvic acid

(iv) The oxidation of pyruvic acid to CO_2 and H_2O in the citric acid cycle.

(v) Reduction of —CHOH groups to the conversion of carbohydrate carbon (–CHOH) to fatty acid carbon (–CH$_2$) and of amino acid carbon (–CH$_2$) to carbohydrate carbon (–CHOH).

Lactose Intolerance

Lactose intolerance is the inability to metabolize lactose, a sugar found in milk and other dairy products, because the required enzyme lactase is absent in the intestinal system or its availability is lowered. Some people also mention pasteurized dairy products as a cause (raw milk contains small amounts of lactase). It is estimated that 75% of adults show some decrease in lactase activity during adulthood worldwide. The frequency of decreased lactase activity ranges from nearly 5% in northern Europe to more than 90% in some Asian and African countries.

SUB-SECTION 5.2B—FATE OF GLUCOSE AFTER ABSORPTION

Glucose is primarily the carbohydrate supplied by the diet and utilized by the tissues, Other sugars, such as fructose, galactose, and mannose are convertible into glucose and glycogen and undergo metabolism largely through such conversion.

The interconversions of sugars and their conversion to glycogen are effected through enzymatic reactions of the sugar phosphates.

For orientation purposes, general relations of the sugars in metabolism are shown in Fig. 137.

Galactose

Galactose-I-P

Glucose ⟷ Glucose 6-P ⟷ Glucose I-P ⟷ Glycogen

Fructose ← Fructose

Fructose 1-6 dap

Manoose-6-P ⟷ Manoose

Glucosamine-6-P ⟷ Glucosamine

Glucosamine-1-P

⟷ reversible reaction

⇄ reversible reaction by separtate reaction
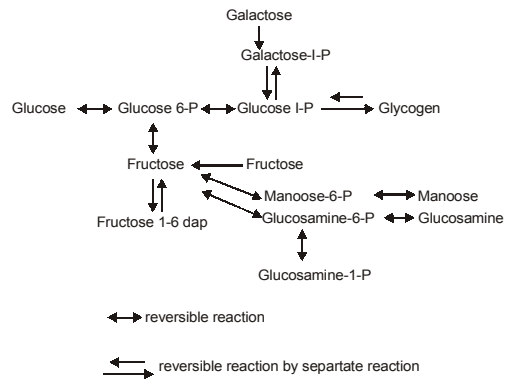
Fig. 5.42 : Showing the Conversion reaction of different Glucoses after absorption

The metabolism of sugars takes place through their phosphate to pyruvic (and lactic) acid in process called glycolysis which may proceed anaerobically. Pyruvic acid is then converted to acetyl CoA which is oxidized to carbon dioxide and water in the citric acid cycle. Discussion in this subsection will deal particularly with the metabolism of sugars relating to their conversion to sugar phosphates, and with the relation of these phosphates to each other after absorption.

The Hexokinase Reaction The first reaction involved in the metabolism of sugar in phosphorylation by ATP, which is catalysed by a Hexokinase enzyme.

$$\text{Sugar} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{Sugarphosphate} + \text{ATP}$$
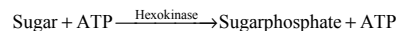
The Hexokinase reaction was first discovered in yeast. The enzyme catalyzes the phosphorylation of glucose mannose, fructose, glucosamine and 2 dexoxyglucose to the 6 – Phosphate. A glucokinase specific for glucose is present in animal tissues and bacteria. It forms glucose 6 – P Liver and muscle contain a fructokinase which forms fructose – 1 – P instead of the 6 – phosphate. Galactokinase present present in yeast and animal tissue forms galactose – 1 – P. Liver galactokinase also acts upon galactosamine.

The free energy liberated in the Hexokinase reaction is relatively large (about 4 K/cal for glucose)

$$\text{Glucose} + \text{ATP} \xrightleftharpoons{\text{Glucokinase}} \text{Glucose 6 P} + \text{ATP}$$
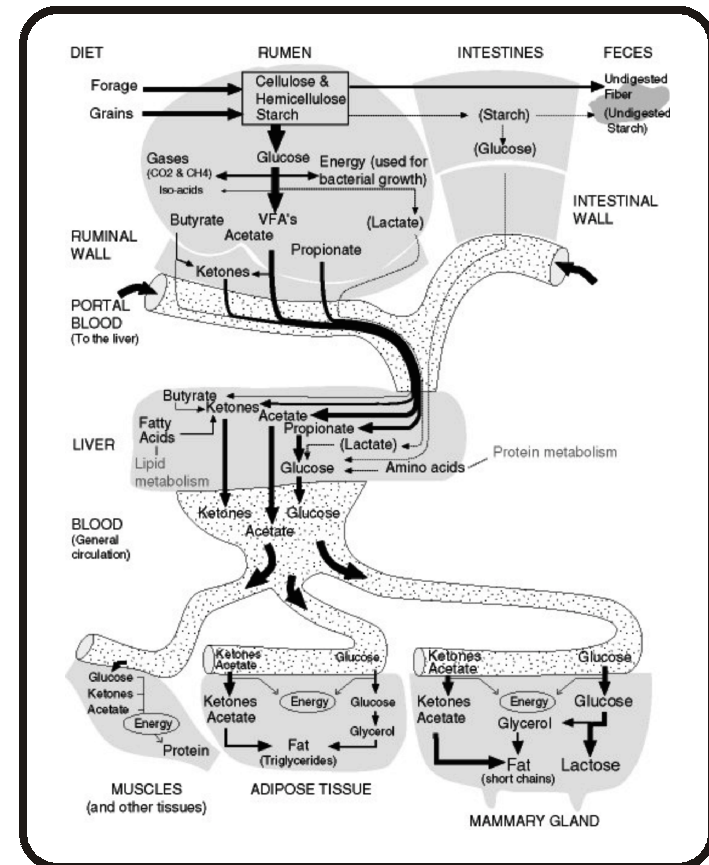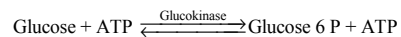
Fig. 4.45 : Showing the Total Absorption Diagram of the Carbohydrate after Absorption

Though the reaction is revesible it is slight and of no appreciable metabolic importance. Glucose – 6 – P in the liver is hydrolyzed to glucose and Pi by a specific enzyme glucose – 6 – phosphatase. While glucose 6 – P is formed in the muscle it is not converted to glucose because of the absence of glucose

– 6 – phosphatase. Hydrolysis of glucose – 6 – P in liver represents that final reaction is the formation of glucose from glycogen and other sugar phosphates. The enzyme is deficient in Von Gieke's disease which leads to massive accumulation of glycogen in liver.

Interconversion of Glucose-1-P Glucose-6-P Phosphoglucomutase

As pointed out glycogen is synthesized from glucose – 1 – P and broken down into glucose – 6 P in the Hexokinase reaction it is reversely converted to glucose – 1 – P by the phosphoglucomutase is a phosphoprotein and the phosphate group of the enzyme participate in the reaction.

$$\text{Glucose - 1 - P + Enzyme 1 P} \rightleftarrows \text{Glucose 1 - 6 dip + Enzyme}$$

$$\text{Glucose 1 - 6 dip + Enzyme} \rightleftarrows \text{Glucose - 6 - P + Enzyme P}$$
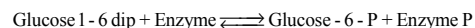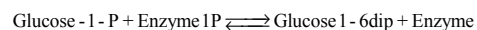
Muscle and yeast contain and enzyme glucose – 1 – Phosphate kinase, which catalyses the phosphorylation of glucose 1 – P to glucose – 1 – 6 – diP by ATP

Also muscle and bacteria contain glucose – 1 – phosphate transphophorylase which forms glucose – 1 – 6 diP by the relation.

$$\text{2 Glucose} - \text{IP} \rightleftarrows \text{Glucose} - 1 - 6 \text{diP} + \text{Glucose}$$

These processes supply the amounts of glucose-1-6 diP necessary for the phosphoglucomutase reaction. Glucose – 6 – P is reversely converted to fructose 6 – P by a phosphohexose isomerizes enzyme.

$$\text{Glucose} - 6 - P \rightleftarrows \text{Fructose} - 6 - P$$
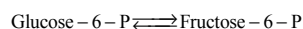
At equilibrium (30°C pH 8) there is about 30% fructose – 6 – P and 70% glucose – 6 – P. The reaction appears to proceed through an enol intermediate
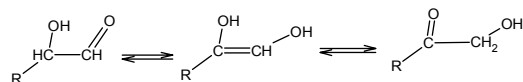
The phosphorylation of fructose – 6 – P to fructose – 1 – 6 disphosphate by ATP is catalysed by the enzyme phosphofructokinase, and resembles the hexokynase reaction.

$$\text{Frucose} - 6 - P + \text{ATP} \rightarrow \text{Fructose} - 1 - 6 \text{diP}$$

The reaction like the hexokynase reaction is inappreciably reversible, and fructose – 1 – 6 disphosphate is converted back to fructose – 6 – P may be through hydrolysis by a specific enzyme.

$$\text{Frucose} - 1 - 6 - \text{diP} + H_2O \rightarrow \text{Fructose} - 6 - P + \text{Pi}$$

It was proved that fructose – 6 – P may be phosphorylated to fructose – 1 – 6 diphosphate also by isosine triphosphate (ITP) uridine triphosphate (UTP)

Metabolism of fructose: Fructose is phosphorylated by ATP through the action of two different fructokinase. As previously indicated, glucose forms fructose – 6 – P.

$$\text{Fructose} + \text{ATP} \rightarrow \text{Fructose} - 6 - P + \text{ATP}$$

The fructose – 6 –P may then be converted to glucose – 1 – P through glucose, or to glucose – 1 – P through glucose – 6 – P, and then to glycogen. It may also be phosphorylated to fructose – 1 – 6 – dip, which undergoes conversion of pyruvic acid (by oxidation etc) to acetylCoA, which is oxidized to CO_2 and H_2O is the citric acid cycle. Other sugars are metabolized similarly by being converted meeting point in the metabolic stream of sugar.

Another fructokinase in animal tissues as previously indicated, with ATP phosphrylates fructose to fructose – 1 – P

$$\text{Fructose} + \text{ATP} \rightarrow \text{Fructose} - 1 - P$$
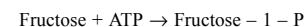
Fructose – 1 – P can be converted to fructose – 1 – 6 – Disphosphate as follows:

$$\text{Fructose} - 1 - P \xrightarrow{\text{Aldolase}} \text{Dihydroxoyacetone} - P + \text{Glyceraldehyde}$$

$$\text{Glyceraldehyde} - 3 - P + \text{Dihydroxyacetone} - P \xrightleftharpoons{\text{Aldolase}} \text{Fructose} - 1, \text{6 Diphosphate}$$
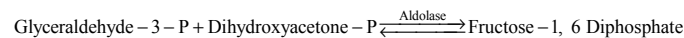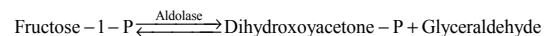
Metabolism of Mannose

Mannose enters the carbohydrate stream through phosphorylation in the hexokinase reaction and then conversion to fructose – 6 – P

$$\text{Manmose} + \text{ATP} \xrightarrow{\text{Glucokinase}} \text{Manmose} - 6P + \text{ADP}$$

$$\text{Manmose} - 6P \xrightleftharpoons[\text{Isomerase, Muscle}]{\text{Phosp hom annose}} \text{Fructose} - 6P$$
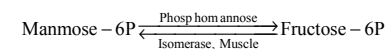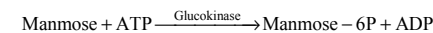
Metabolism of Galactose

Galactose enters the blood stream from the intestine after digestion of carbohydrate rather lactose. It is converted into glucose – P by the following reaction:

$$\text{Galactose} + \text{ATP} \xrightarrow{\text{Galactokinase}} \text{Galactose} - 1 - P + \text{ADP}$$

$$\text{Galactose} - 1 - P + \text{UDP} - \text{Glu cos e} \xrightleftharpoons[\text{Uridyltransferase}]{\text{Phosphogalactase}} \text{UDP} - \text{Galactase} + \text{Glucose} - 1P$$

$$\text{UDP} - \text{Galactase} \xleftarrow[\text{Epimerase}]{\text{UDP}-\text{Galactose}} \text{UDP} - \text{Glucose}$$

$$\text{Galactose} + \text{ATP} \rightarrow \text{Glucose} - 1 - P + \text{ADP}$$
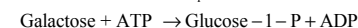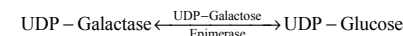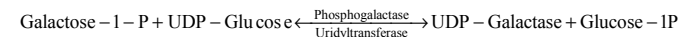
The Glucose 1 – P then may undergo the transformations. It has also indicated that congenital absence of the enzyme phosphogalactase uridyl transferase is the cause of the disease galactosemia in the children. This means that in galactosemia the conversion of the galactose – 1 – P to glucose – 1 – P definitely is executed.

Under these conversion of galactose – 1 – P accumulates in erythrocytes and other tissues, causing damage particularly to the liver brain, and optic lens. Treatment consists in feeding galactose (lactose free diets. UDP – galactose is required in forming tissue glycolipids and lactose in the adult female, the galactosemia this is formed form glucose through the epimerize reaction.

$$\text{UDPGlucose} \xleftrightarrow{\text{Epimerase}} \text{UDP} - \text{galactose}$$
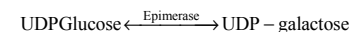
An alternate route for the metabolism of galactose is due to the presence of the enzyme uridine diphosphate galactose pyrophosphorylase, which catalyses the formation of UDP – galactose from galactose – 1 – P

$$Galactose - 1 - P + UTP \rightleftharpoons UDP + galactosePPi$$
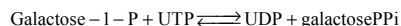
UDP – galactose pyrophosphorylase is present in low activity in infant liver but increases with age, so the galactosemic adult to metabolise appreciable quantities of galactose.

Summary

The Monosaccharaides produced by the action of oligosaccharides and disaccharides of the brush boders of the intestine cells by several types of transport mechanism. Fructose moves into the cell on a membrane but is not actively transported against a concentration gradient i.e. facilitated diffusion. Glucose and galactose are actively transported and this is accomplished by a sodium symport system see fig 138. This transport process ensures that the intracellular glucose concentration is maintained at a vascular side of the cell, thus ensuring that glucose will be transport passively into the bloodstream. The carrier – bound sodium ions and glucose are internalized along an electrochemical gradient that results from the low intracellular sodium concentration. On the luminal side, one molecule of glucose and two molecules of sodium bind to the membrane carrier. Presumably the binding of the sodium ions produces a conformational change such that glucose now binds with greater affinity to the carrier.
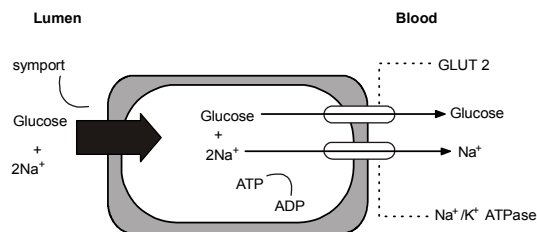
Fig. 4.44 : Showing the Glucose Symport

Inside the cell, the sodium ions are released from the carrier, and diminished affinity allows the glucose to dissociate. A Na^+/K^+ ATPase allows the sodium ions to be transported into the lateral intercellular spaces against a concentration gradient using the free energy of ATP hydrolysis. Since glucose transport does not involve ATP directly, it can be considered as secondary active transport. Glucose is transported from the mucosal cell into the intercellular space by a high-capacity glucose transporter (GLUT2)

SECTION 5.3—GLYCOLYTIC PATHWAYS

We can now start our consideration of the glycolytic pathway. This pathway is common to virtually all cells, both prokaryotic and eukaryotic cells. In eukaryotic cells the glycolysis takes place in the cystol.

This pathway can be thought of as consisting of three major stages; Stage 1 includes the conversion of glucose into fructose 1p – diphosphate through three intermediate processes first of all the phosphorylation then the isomerization and followed by a second phosphorylation reaction.

The strategy of these individual steps in glycolysis is to trap the glucose in the cell and then from a compound that can be readily changed into phosphorylated three carbon units

Stage 2 Includes the transformation of fructose – 1 – 6 diphosphate into two three carbon fragments. Thus resulting three carbon units which are readily interconvertable. In stage three ATP is produced when three carbon fragments are converted to pyruvic acid by oxidation. See fig 139 and 139A.

Glucose enters cell through specific transport proteins and has one principal fate, it is phosphorylated by ATP to form glucose 6 – phosphate. This step is notable for two reasons (1) glucose 6 – phosphate cannot diffuse through the membrane because of its negative charges and (2) the addition of the phosphoryl group begins to destabilize glucose, thus facilitating its furdoxyl group on carbon 6 of glucose is catalyzed by hexokynase.
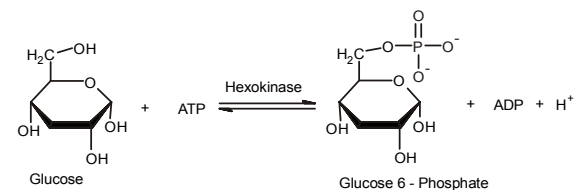
Phosphoryl transfer[§] is a fundamental reaction in biochemistry and is one that has been discussed. Kinases are enzymes that catalyze the transfer of a phosphoryl group from ATP to an acceptor. Hexokinase then catalyzes the transfer of a phosphoryl group from ATP to a variety of six carbon sugar (hexose) such as glucose and mannose, Hexokinase like adenylatekinase. And all other kinase requiring Mg^{2+} (or another divalent metal ion such as Mn^{2+}) for activity.

The divalent metal ions form complex with ATP. The result of X – Ray crystallographic study of the yeast hexokinase revealed that the binding of glucose induces a large conformational change on the enzyme, analogous to the conformational change under gone by NMP kinases on substrate binding. Hexokinase consists of two parts which move toward each other when glucose is bound see fig 139. On glucose binding one part rotates 12 degree angle with the respect of the other part, resulting in the movements of the polypeptide backbone of as much as 8Å. The cleft between the parts closes and the bond glucose becomes surrounded by protein, except of the hydroxyl group of carbon 6, which will accept the phosphoryl group ATP. The closing of the cleft in hexokinase is striking example of the role of induced fit in enzyme action[‡]

_____

[§]glucose
[‡]See Chapter 3

STAGE 1

Glucose

Hexokinase

ATP

ADP

Glucose-6-Phosphate

Phosphoglucose
isomerase

Fructose-6-Phosphate

Phosphofructokinase

ATP

ADP

Fructose-1,6-Diphosphate

STAGE 2

Triose phosphate
isomarase

Dihydroxycetone
phosphate

Glyceraldehyde
3-Phosphate

Fig. 4.45 : Showing the Glycolytic Pathway

STAGE 3

**Glyceraldehyde
3-Phosphate**

Glyceraldehyde
3-Phosphate
dehydrogenase

$P_i NAD^+$

NADH

1,3-Diphosphoglycerate

Phosphoglycerate
Kinase

ADP

ATP

3-Phosphoglycerate

Phosphoglycerate
Mutase

2-Phosphoglycerate

Enolase

H_2O

Phosphoglycerate

Pyruvate Kinase

ADP

ATP

Pyruvate
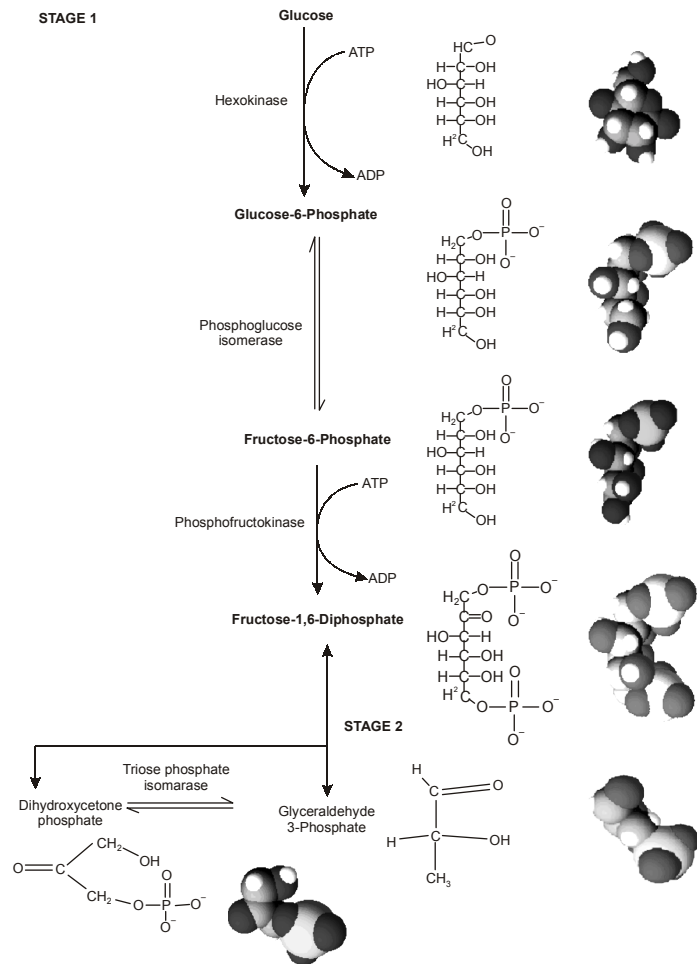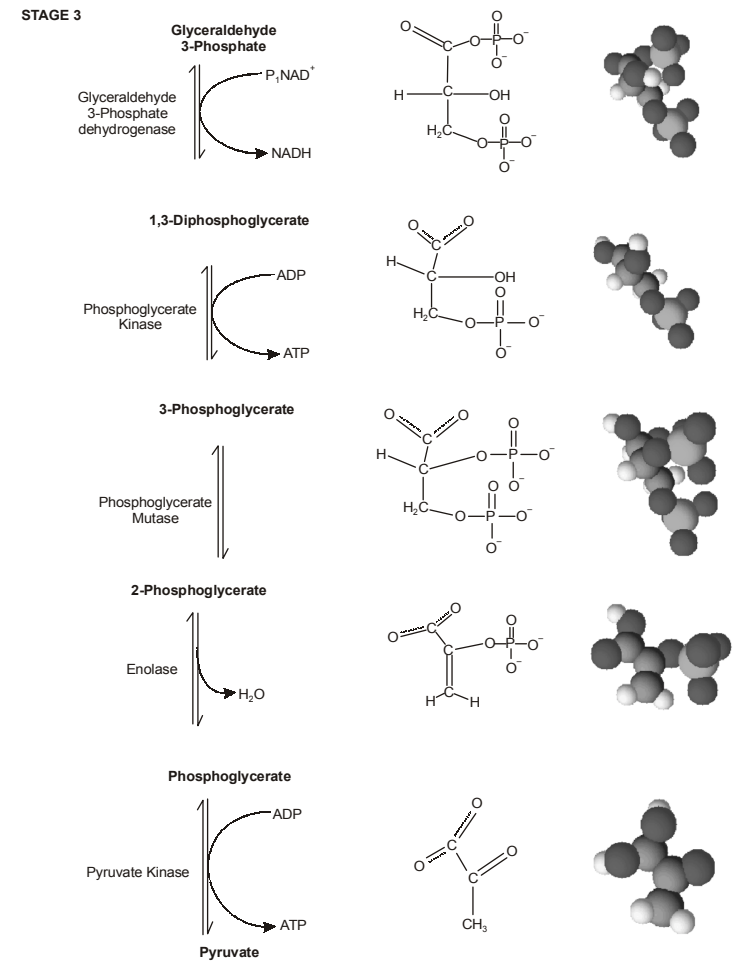
Fig. 4.45A : Showing the Glycolytic Pathway

The glucose included structural changes are significant in two respects. First the environment around the glucose become much more non-polar, which favours the donation of the terminal phosphoryl group of ATP. Secondly as noted the substrate induced conformational changes within the kinase enables it to discriminate against H_2O as a substrate. If hexokinase was rigid a molecule of water occupying the binding site for the $-CH_2OH$ of glucose would attract the phosphoryl group of ATP, forming ADP and P_1. In other words, a rigid kinase would necessarily also is an ATPase. It is interesting to note that other kinase taking part in glycolysis – pyruvate kinase, phosphoglycerate kinase and phosphofructokinase also contain clefts between parts that close when substrate is bound although the structures of these enzymes are different in other regards Substrate – introduced cleft closing is a general feature of kinase.

The formation of Fructose 1, 6 Diphosphate from Glucose 6 – Phosphate The next step in glycolysis is the isomerization of glucose – 6 – phosphate to fructose – 6 – phosphate. Remember that the open chain from of glucose has an aldehyde group at carbon 1, where as the open chain form the fructose has a keto group at carbon 2. Thus the isomerization of glucose 6 – phosphate to fructose – 6 – phosphate is a conversion of an aldose into a ketose. The reaction catalyzed by phosphoglucose isomerizes include addition steps because both glucose – 6 – phosphate pair is present primarily in the cyclic froms. The enzyme must first not open the six membered ring of glucose – 6 – phosphate catalyzes the isomerization, and then promote the formation of the five membered ring of fructose 6 – phosphate.

Glucose 6 - Phosphate   Fructose - 6 - Phosphate
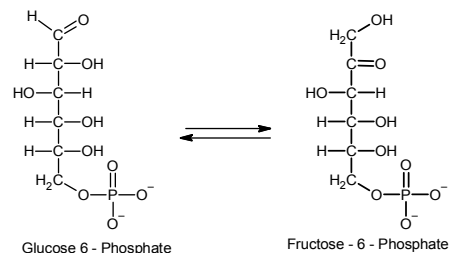
A second phosphorylation reaction follows the isomerization step. Fructose – 6 – phosphate is phosphorylated by ATP to fructose 1 – 6 diphosphate.

Fructose - 6 - Phosphate   Fructose - 1,6 Diphosphate
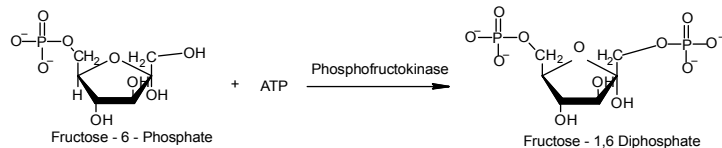
The reaction is catalyzed by the enzyme phosphofructokinase; it is allosteric[‡] in nature, that states the pace of glycolysis. As we all learn, this enzymes plays a central role in the integration very much of metabolism.

The Six – Carbon Sugar is cleaved into Two Three Carbon fragments by Aldose. The second stage of glycolysis begins with the splitting of fructose 1, 6 disphosphate into glyceraldehyde 3 – phosphate and dihydroxyacetone phosphate. The products of the remaining steps in glycolysis consists of three carbon units rather than six – carbon units.

Fructose - 6 - diphosphate   Dihydooxyacetone phosphate   Glyceraldehyde
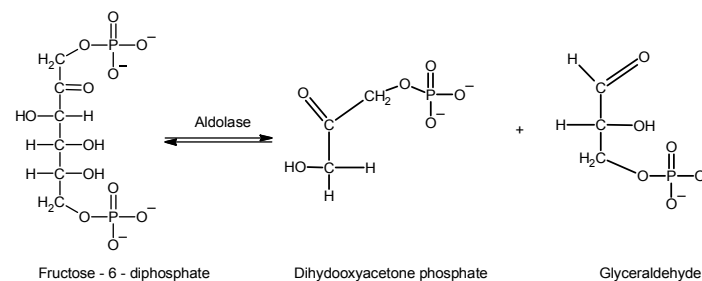
This reaction is catalyzed by aldolase. This enzyme derives its name from the nature of the reverse reaction, an aldol condensation. The reaction catalyzed by aldolase is readily reversible under intermolecular conditions.

The formation of ATP from 1, 3 Diphosphoglycerate

The final stage in glycolysis generation of ATP from the phosphorylated three carbon metabolites of glucose. Phosphoglycerate kinase catalyzes the transfer of the phosphoryl group from the acyl phosphate of 1, 3 diphosphoglycerate are the products.

1, 3 - Phosphoglycerate   3 - Phosphoglycerate
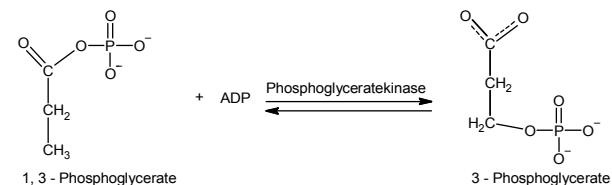
[‡] The term *allostery* comes from the Greek *allos*, "other", and *stereos*, "solid (object) Allosteric enzymes are enzymes that change their conformation upon binding of an effector. An allosteric enzyme is an oligomer whose biological activity is affected by *altering* the conformation(s) of its tertiary structure. Allosteric enzymes tend to have several subunits.

The formation of ATP in this manner is referred to as substrate – level phosphorylation because the phosphate donor 1, 3 DPG is a substrate with high phosphorous transfer potential we will clear this matter of the ATP formation, with that of ATP formed from ionic gradients in chapter 2.

Thus the outcomes of the reactions catalyzed by glyceraldehyde – 3 - phosphate dehydrogenese and phosphoglyceratekinase are:

1. Glyceraldehyde 3 – Phosphate and aldehyde is oxidized to 3 – phosphoglycerate a carboxylic acid.
2. NAD$^+$ is concomitantly§ reduced to NADH
3. ATP$^+$ is formed from P$_1$ and ADP at the enzyme expense of carbon oxidation energy.

Keep it in mind, that because of the actions of aldolase and triose phosphate isomerase, two molecules of ATP were generated. These ATP molecules of ATP consumed in the first stage of glycolysis.

The Generation of Additional ATP and the Formation of Pyruvate In the remaining steps of glycolysis 3 – phosphoglycerate is converted into pyruvate with the concomitant conversion of ADP to ATP.

3 - Phosphoglycerate   2 - Phosphoglycerate   Phosphoglycerate   Pyruvate
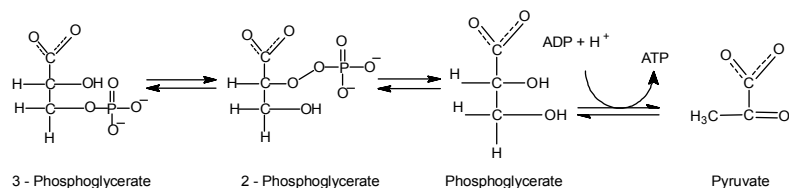
The first reaction is a rearrangement; the position of the phosphoryl group shifts in the conversion of 3 phosphoglycerate into 2 – phosphoglycerate, a reaction catalyzed BY PHSOPHOGLYCERATE MUTASE. In general, a mutase is an enzyme that catalyzes the intramolecular or shift of a chemical group, such as a phosphoryl group. The phosphoglycerate mutase reaction has an interesting mechanism; the phosphoryl group is not simply moved from one carbon to another.

This enzyme requires catalytic amounts of 2, 3 – diphosphoglycerate to maintain an active site hystidine residue in a phosphorylated form.

Enz – His – phosphate + 2,3 Diphosphoglycerate $\rightleftharpoons$ Enz – His – phosphate + 2 phosphoglycerate

Enz – His – phosphate + 3Phosphoglycerate $\rightleftharpoons$ Enz – His + 2,3 Diphosphoglycerate
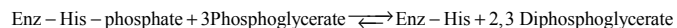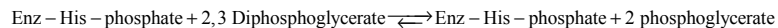
The sum of these reaction equation yields mutase reaction

$$3 - \text{Phosphoglycerate} \rightleftharpoons 2 - \text{Phosphoglycerate}$$
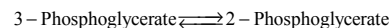
Examination of the first partial reaction reveals that the mutase functions as a phosphate i.e. converts 2, 3 disphosphoglycerate into 2, phosphoryl group remains linked to the enzyme. This phosphoryl group is then transferred to 3 – phosphoglycerate to reform 2, 3 diphosphoglycerate.

In the next reaction an enol is formed by the dehydration of 2 phosphoglycerate. Enolase catalyzes to formation of phosphoenolpyruvate (PEP). This dehydration distingusihly displays the transfer potential of the phosphoryl group. An enol phosphate has a high phosphoryl transfer potential where as the phosphate ester such as 2 – phosphoglycerate of an ordinary alcohol is low one. It is a fact that phosphoenol pyruvate has a very high phosphoryl transfer potential. The phosphoryl group traps the molecule in its unstable enol form, when the phosphoryl group has been donated to ATP the enol undergoes a conversion into the more stable ketone namely pyruvate.

phsophoenol pyruvate   Pyruvate (enol form)   Pyruvate (keto form)
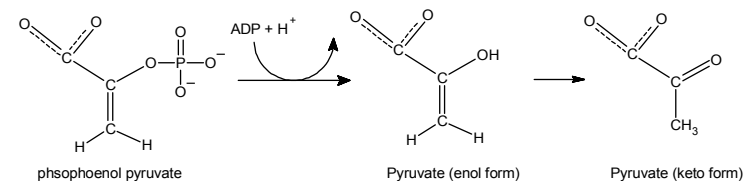
Thus, the high phosphoryl transfer potential of phosphoenolpyruvate arises primarily from the large driving force of the subsequent enol ketone conversion. Hence pyruvate is formed and ATP is generated concomitantly. The virtually irreversibly transfer of a phosphoryl group from the phosphoenolpyruvate to ADP is catalyzed by pyruvatekinase. Because the molecules of ATP used in forming fructose 1,6 diphosphate have already been regenerated the two molecules of ATP generated from phosphoenolpyruvate are " profit"

Enzyme	Reaction Type	ΔG° in kcal §mol^{-1}	ΔG° in kcal mol^{-1}
Hexokinase	Phosphoryl transfer	–4.0	–8.0
Phosphoglucose isomerase	Isomerization	+0.4	–0.6
Phosphofructokinase	Phosphoryl Transfer	–3.4	–5.3
Aldolase	Aldol Cleavage	+5.7	–0.3
Triose phosphate isomerase	Isomerization	+1.8	+0.6
Glyceraldehyde 3-phosphate Dehydrogenese	Phosphorylation coupled to oxidation	+1.5	–0.4
Phosphoglycerate kinase	Phosphoryl transfer	–4.5	+0.3
Phosphoglycerate mutase	Phosphoryl shift	+1.1	+0.2
Enolase	Dehydration	+0.4	–0.8
Pyruvate Kinase	Phosphoryl transfer	–7.5	–4.0

§Note ΔH, the normal free energy change has been calculated from ΔG, and known concentrations of reactants under typical physiologic conditions. Glycolysis can proceed only if the ΔG values of all reactions are negative. The small ΔG values of the above reactions indicate that the concentrations of metobolities in vivo in cells undergoing glycolysis are not precisely known.

SECTION 5.4—GLYCOGENESIS AND GLYCOGENOLYSIS

SUB-SECTION 5.4A—GLYCOGENESIS INTRODUCTION

Glycogen is found in many cell types in the body but only in high concentration in liver and muscle. A fed man weighing 70 kg will have about 1.6 kg liver containing about 100 g glycogen and 35 kg of muscle containing approximately 400 g glycogen. At a caloric value of 17 kJ/g, the stores of glycogen represent about 8500 kJ of fuel. Relative to the fat stores, this is a small reserve yet it has great functional significance. Glycogen is stored in the fed state and utilized during fasting and exercise. Synthesis *(glycogenesis)* and breakdown *(glycogenolysis)* occur by separate but related pathways (Fig. 140) and are controlled in an integrated fashion via allosteric and covalent mechanisms; hormonal control is very critical. The enzymes of glycogen metabolism are associated with the glycogen granules in cells.
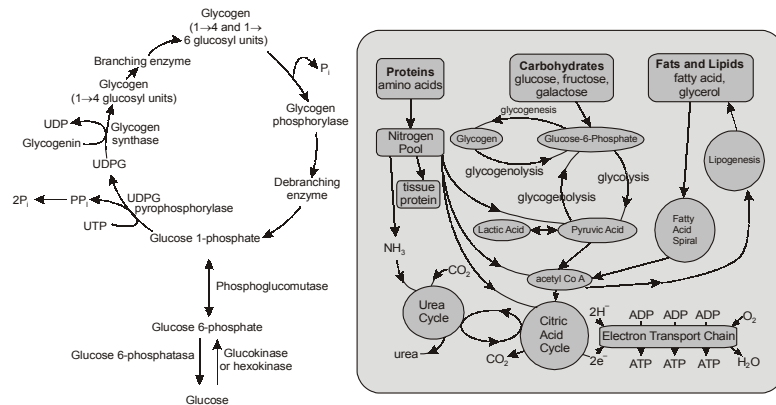
Fig. 4.45 : Showing the Glycogenesis and Glycogenolysis Process

SUB-SECTION 5.4B—GLYCOGENESIS

The key points about the storage of glycogen are:

1. A step unique to glycogenesis is the formation of UDP-glucose from G-I-P and the pyrimidine nucleotide, UTP

2. The glucose moieties added to form glycogen come directly from UDP-glucose

3. Each glycogen molecule contains a protein, *glycogen in.* The identification of this protein explained how the synthesis of the large glycogen molecule is initiated. Glycogenin has enzymatic activity catalyzing the addition of the first 4-8 glucose moieties to a tyrosine in glycogenin, using UDPG as the source of the glucoses.

4. Glycogen synthase transfers the glucose moiety of UDPG to the non-reducing end of the primer, giving a polymer with a-l,4-linkages

5. Glycogen is branched and the *branching enzyme* involved is a 4:6-transferase.

SUB-SECTION 5.4C—GLYCOGENOLYSIS

Key points about the breakdown of stored glycogen are:

1. *Glycogen phosphorylase* catalyses the interaction of inorganic phosphate (Pj) with terminal a-l:4-glycosidic bonds at the multiple non-reducing ends of glycogen to yield G-l-P

2. Phosphorylase contains the coenzyme pyridoxal phosphate.

3. The branching of glycogen means that there are many sites (ends) for phosphorolysis and this allows for rapid production of G-l-P, which is beneficial in both liver and muscle.

4. *Debranching enzyme* has two distinct catalytic sites and is important for complete utilization of glycogen. Glucoses are removed from a branch by phosphorylase action until there are only four glucoses attached. Then, a 4:4 *glucan transferase* transfers the trisaccharide attached to the glucose in a l,6-linkage to a different chain. This leaves a single glucose attached at the branch point and the a-l,6-linkage is hydrolyzed by *a-l:6-glucosidase* to yield free glucose

5. There are different end-products in liver compared with muscle. Muscle lacks *glucose 6-phosphatase (G-6-Pase)* so the end-products of increased muscle glycogenolysis will be pyruvate and lactate (following glycolysis); liver contains G-6-Pase which means that the end product is glucose

6. Clearly this is consistent with the role of glycogen in muscle, which is to supply that tissue with ATP and the role of glycogen in liver to maintain blood glucose levels.

It is essential to consider the control of the synthetic breakdown pathways together (see Fig. 140B)
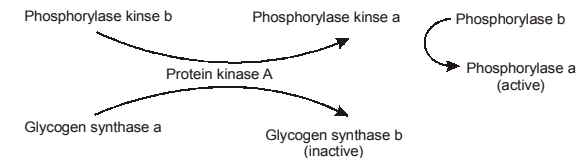
(b) The fed state: high insulin levels, low glucagon levels.

Phosphorylase kinase a   Phosphorylase kinse b (inactive)

Phosphoprotein phosphatase 1 (PP1G)

Glycogen synthase b   Glycogen synthase a (active)
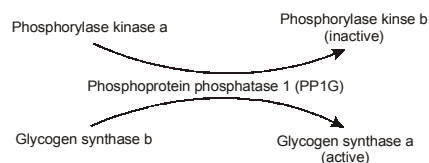
Result: glycogen synthesised

Fig. 5. : Showing the pathway breakdown

The control steps in glycogenesis and glycogenolysis are catalyzed by glycon synthase and glycogen phosphorylase, respectively. They are both subject to control by allosteric and covalent modification.

ATP and G – 6 – P levels high	Glycogen synthase active, phosphorylase inactive
Amp levels High	Glycogen synthase inactive, phosphorylase active

High ATP in muscle is an indication of high energy charge and is a signal that there is less need for glycogen breakdown. In contrast, increased AMP is a signal that ATP utilization is high.

Elevated G – 6 – P in both liver and muscle is associated with the fed state and increased availability or glucose (for storage).

Covalent Modification of Enzyme

Cyclic AMP dependant protein kinase Active	Glycogen Synthase Inactive, Phosphorylase Active
Phosphoprotein Phosphate Active	Glycogen Synthase Active, Phosphorylase Inactive

Hormonal Control Glucogen (liver) and adrenaline (muscle) action results in increased concentrated cyclic AMP within these cells and activation of PKA. This results in Activation of phosphorylase and inactivation of synthase.

Insulin* release in the fed state leads to the dephosphorylation of both phosphorylase and glycogen synthase. This results in inactivation of phosphorylase and activation of glycogen synthase. Phosphorylase kinase is also activated by increased concentrations of calcium. This is explained by the fact that phosphorylase kinase has four subunits αβγδ and the δ-subunit is the calcium binding polypeptide calmodulin**. Binding of calcium activities the enzymes leading finally
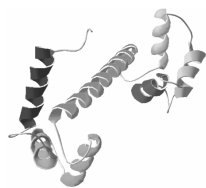
Fig. 5. : Calmodulin structure

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*We shall read more about this in the chapter of hormones and vitamins (chapter 12).

Calmodulin (CaM) (an abbreviation for Calcium Modulated protein) is a clcium-binding protein expressed in all eukaryotic cells. It can bind to and regulate a number of different protein targets, thereby affecting many differ cellular functions.

to increased phosphorylation of the a and the b - subunits of phosphorylase kinase (and thus to activation). Calcium is an important signal for muscle contraction. Activation of glycogen breakdown will lead to the increased ATP formation required to support this. Adrenaline action on the liver is mediated via a_1 adrenoreceptors leading to increased levels of inositoltrisphosphate and calcium.

SUB-SECTION 5.4D—CORI CYCLE

The Cori cycle, named after its discoverers, Carl Cori and Gerty Cori, refers to the metabolic pathway in which lactate produced by anaerobic glycolysis in the muscles moves to the liver and is converted to glucose, which then returns to the muscles and is converted back to lactate. Muscular activity requires energy, which is provided by the breakdown of glycogen in the muscles. The breakdown of glycogen generates ATP which is used by muscles as an energy source. Since throughout muscular activity ATP is used to supply energy, it needs to be constantly replenished. Whilst the supply of oxygen is sufficient, glucose-6-phosphate produced by the breakdown of glycogen can be aerobically oxidized, feeding pyruvate into the TCA cycle. However, when oxygen is in short supply - i.e. during intense muscular activity - energy must be released from G-6-P through anaerobic respiration. Anaerobic respiration through glycolysis alone causes a build-up of pyruvate, which is converted to lactate. In the Cori cycle, glycolysis continues when pyruvic acid is converted back to lactic acid. Pyruvic acid is reduced to lactic acid by lactate dehydrogenase, which oxidises the NADH created during glycolysis back to NAD+, transferring NADH's two electrons to pyruvic acid, creating lactic acid. This occurs in order to maintain a concentration of NAD+ that can be reduced during glycolysis, so that glycolysis can

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Function

CaM mediates processes such as inflammation, metabolism, apoptosis, muscle contraction, intracellular movement, short-term and long-term memory, nerve growth and the immune response. CaM is expressed in many cell types and can have different subcellular locations, including the cytoplasm, within organelles, or associated with the plasma or organelle membranes. Many of the proteins that CaM binds are unable to bind calcium themselves, and as such use CaM as a calcium sensor and signal transducer. CaM can also make use of the calcium stores in the endoplasmic reticulum, and the sarcoplasmic reticulum. CaM undergoes a conformational change upon binding to calcium, which enables it to bind to specific proteins for a specific response. CaM can bind up to four calcium ions, and can undergo post-translational modifications, such as phosphorylation, acetylation, methylation and proteolytic cleavage, each of which can potentially modulate its actions. Calmodulin can also bind to edema factor toxin from the anthrax bacteria.

Structure

Calmodulin is a small, acidic protein approximately 148 amino acids long (16706 Dalton) and, as such, is a favorite for testing protein simulation software. It contains four EF-hand "motifs", each of which binds a Ca^{2+} ion. The protein has two approximately symmetrical domains, separated by a flexible "hinge" region. Calcium participates in an intracellular signalling system by acting as a diffusible second messenger to the initial stimuli.

Mechanism

Calcium is bound via the use of the EF hand motif, which supplies an electronegative environment for ion coordination. After calcium binding, hydrophobic methyl groups from methionine residues become exposed on the protein via conformational change. This presents hydrophobic surfaces, which can in turn bind to Basic Amphiphilic Helices (BAA helices) on the target protein. These helices contain complementary hydrophobic regions. The flexibility of calmodulin's hinged region allows the molecule to "wrap around" its target. This property allows it to tightly bind to a wide range of different target proteins. The members are Calmodulin 1, 2 and 3.

continue. The lactic acid is then taken up by the liver in the next part of the cycle. The liver converts the lactic acid back to pyruvic acid and then to glucose through gluconeogenesis. The glucose then enters the blood and returns to the muscles to be used for energy if muscle activity has continued. If muscle activity has stopped by this time then the glucose is used to replenish the supplies of glycogen through glycogenesis. In the Cori cycle the gluconeogenic leg of the cycle is energy consuming. While there is a gain of 2 moles of ATP in the anaerobic glycolysis of glucose, there is a cost of 6 moles of ATP in the gluconeogenesis part of the cycle. The cost of the 4 moles of ATP means the cycle cannot be sustained continuously.
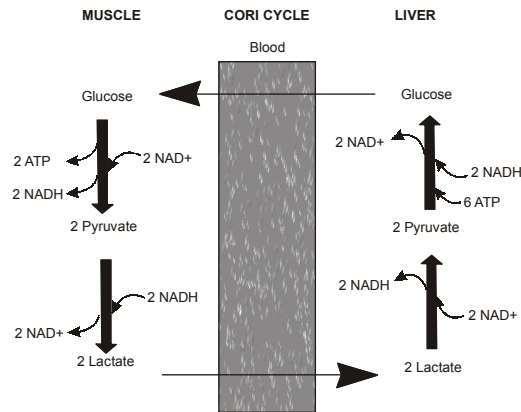
Fig. 5.4 : Showing the Cori Cycle

Significance

The cycle's importance is based on the prevention of lactic acidosis in the muscle under anaerobic conditions. However, normally before this happens the lactic acid is moved out of the muscles into the liver. The cycle is also important in producing ATP, an energy source, during muscle activity. The Cori cycle functions more efficiently when muscle activity has ceased because the oxygen debt can be made up so that the citric acid cycle and electron transport chain also work.

SUB-SECTION 5.4E—GLYCOGEN STORAGE DISEASE

Defects in enzymes of glycogenolysis usually result in a glycogen storage disease. Subjects with defective branching system in enzyme in liver have a tendency to develop hypoglycemia but the most severe form is seen in those defective livers G –B phase. Subjects with defective muscle phosphorylase cannot support the high level of physical activity.

SECTION 5.5—THE CITRIC ACID CYCLE/TRICARBOXYLIC ACID/KREB'S ACID

The citric acid cycle, also known as the tricarboxylic acid cycle (TCA cycle) or the Krebs cycle (or, rarely, the Szent-Györgyi–Krebs cycle), is a series of enzyme-catalysed chemical reactions of central importance in all living cells that use oxygen as part of cellular respiration. In eukaryotes, the citric acid cycle occurs in the matrix of the mitochondrion. The components and reactions of the citric acid cycle were established by seminal work from both Albert Szent-Györgyi and Hans Krebs.

In aerobic organisms, the citric acid cycle is part of a metabolic pathway involved in the chemical conversion of carbohydrates, fats and proteins into carbon dioxide and water to generate a form of usable energy. Other relevant reactions in the pathway include those in glycolysis and pyruvate oxidation before the citric acid cycle, and oxidative phosphorylation after it. In addition, it provides precursors for many compounds including some amino acids and is therefore functional even in cells performing fermentation.
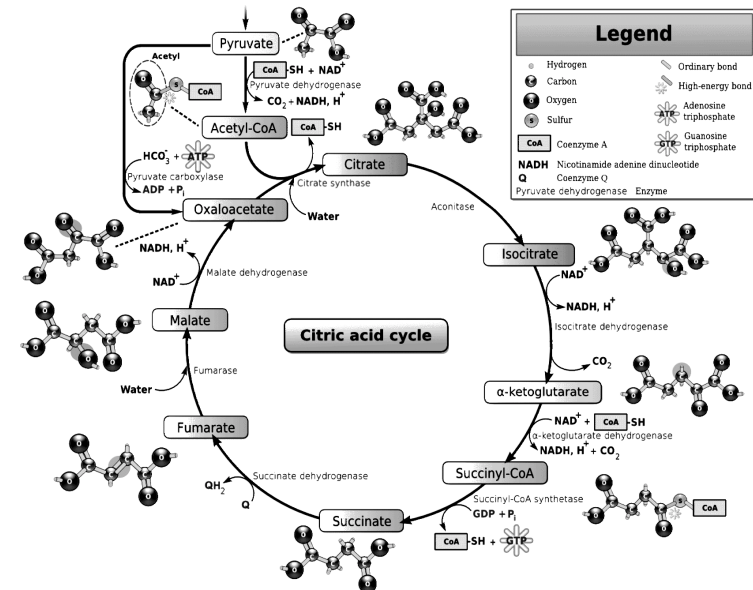
Fig. 5.6 : Showing the Citric Acid Cycle

Two carbons are oxidized to CO_2, and the energy from these reactions is transferred to other metabolic processes by GTP (or ATP), and as electrons in NADH and QH_2. The NADH generated in the TCA cycle may later donate its electrons in oxidative phosphorylation to drive ATP synthesis; $FADH_2$ is covalently attached to succinate dehydrogenase, an enzyme functioning both in the TCA cycle and the mitochondrial electron transport chain in oxidative phosphorylation. $FADH_2$ thereby facilitates transfer of electrons to coenzyme Q, which is the final electron acceptor of the reaction catalyzed by the Succinate:ubiquinone oxidoreductase complex, also acting as an intermediate in the electron transport chain.

The citric acid cycle is continuously supplied with new carbons in the form of acetyl-CoA, entering at step 1 below.

Substrates	Products	Enzyme	Reaction type	Comment
1. Oxaloacetate + Acetyl CoA + H_2O	Citrate + CoA-SH	Citrate synthase	Aldol condensation	rate limiting stage, extends the 4C oxaloacetate to a 6C molecule
2. Citrate	cis-Aconitate + H_2O	Aconitase	Dehydration	reversible isomerisation
3. cis-Aconitate + H_2O	Isocitrate		Hydration	generates NADH (equivalent of 2.5 ATP)
4. Isocitrate + NAD+	Oxalosuccinate + NADH + H +		Oxidation	irreversible stage, generates a 5C molecule
5. Oxalosuccinate	?-Ketoglutarate + CO_2	Isocitrate dehydrogenase	Decarboxylation	generates NADH (equivalent of 2.5 ATP), regenerates the 4C chain (CoA excluded) or ADP->ATP,[1]
6. Ketoglutarate + NAD+ + CoA-SH	Succinyl-CoA + NADH + H+ + CO_2	?-Ketoglutarate dehydrogenase	Oxidative decarboxylation	generates 1 ATP or equivalent
7. Succinyl-CoA + GDP + Pi	Succinate + CoA-SH + GTP	Succinyl-CoA synthetase	substrate level phosphorylation	uses FAD as a prosthetic group (FAD->FADH2 in the first step of the reaction) in the enzyme,[1]
8. Succinate + ubiquinone (Q)	Fumarate + ubiquinol (QH_2)	Succinate dehydrogenase	Oxidation	generates the equivalent of 1.5 ATP
9. Fumarate + H_2O	L-Malate	Fumarase	H2O addition (hydration)	
10. L-Malate+ NAD+	Oxaloacetate + NADH + H+	Malate dehydrogenase	Oxidation	generates NADH (equivalent of 2.5 ATP)

Mitochondria in animals including humans possess two succinyl-CoA synthetases, one that produces GTP from GDP, and another that produces ATP from ADP. Plants have the type that produces ATP (ADP-forming succinyl-CoA synthetase). Several of the enzymes in the cycle may be loosely-associated in a multienzyme protein complex within the mitochondrial matrix.

Products of the first turn of the cycle are: one GTP (or ATP), three NADH, one QH_2, two CO_2.

Because two acetyl-CoA molecules are produced from each glucose molecule, two cycles are required per glucose molecule. Therefore, at the end of all cycles, the products are: two GTP, six NADH, two QH_2, and four CO_2

Description

The sum of all reactions in the citric acid cycle is:

Reactants	Products
Acetyl-CoA + 3 NAD+ + Q + GDP + P_1 + 2 H_2O	$\rightarrow$ CoA-SH + 3 NADH + 3 H+ + QH_2 + GTP + 2 CO_2
Pyruvic acid + 4 NAD+ + Q + GDP + P_1 + 2 H_2O	$\rightarrow$ 4 NADH + 4 H+ + QH_2 + GTP + 3 CO_2
Glucose + 10 NAD+ + 2 Q + 2 ADP + 2 GDP + 4 P_1 + 2 H_1O	$\rightarrow$ 10 NADH + 10 H+ + 2 QH_2 + 2 ATP + 2 GTP + 6 CO_2

Combining the reactions occurring during the pyruvate oxidation with those occurring during the citric acid cycle, the following overall pyruvate oxidation reaction is obtained:

Combining the above reaction with the ones occurring in the course of glycolysis, the following overall glucose oxidation reaction (excluding reactions in the respiratory chain) is obtained:

(the above reactions are equilibrated if P_i represents the $H_2PO_4^-$ ion, ADP and GDP the ADP_2^- and GDP_2^- ions, respectively, and ATP and GTP the ATP_3^- and GTP_3^- ions, respectively).

Estimates for the total number of ATP obtained after complete oxidation of one glucose in glycolysis, citric acid cycle, and oxidative phosphorylation given in the literature range from 30-38 molecules of ATP. A recent assessment of the total ATP yield obtained in these distinct reaction cycles, taking into account updated proton-to-ATP ratios, has arrived at an estimate of 29.85 ATP per glucose molecule.

Regulation

Although pyruvate dehydrogenase is not technically a part of the citric acid cycle, its regulation is included here.

The regulation of the TCA cycle is largely determined by substrate availability and product inhibition. NADH, a product of all dehydrogenases in the TCA cycle with the exception of succinate dehydrogenase, inhibits pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase, and also citrate synthase. Acetyl-CoA inhibits pyruvate dehydrogenase, while succinyl-CoA inhibits succinyl-CoA synthetase and citrate synthase. When tested in vitro with TCA enzymes, ATP inhibits citrate synthase and α-ketoglutarate dehydrogenase; however, ATP levels do not change more than 10% in vivo between rest and vigorous exercise. There is no known allosteric mechanism that can account for

large changes in reaction rate from an allosteric effector whose concentration changes less than 10% [7].

Calcium is used as a regulator. It activates pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase. This increases the reaction rate of many of the steps in the cycle, and therefore increases flux throughout the pathway.

Citrate is used for feedback inhibition, as it inhibits phosphofructokinase, an enzyme involved in glycolysis that catalyses formation of fructose 1,6-bisphosphate, a precursor of pyruvate. This prevents a constant high rate of flux when there is an accumulation of citrate and a decrease in substrate for the enzyme.

Recent work has demonstrated an important link between intermediates of the citric acid cycle and the regulation of hypoxia inducible factors (HIF). HIF plays a role in the regulation of oxygen haemostasis, and is a transcription factor which targets angiogenesis, vascular remodeling, glucose utilization, iron transport and apoptosis. HIF is synthesized constitutively and hydroxylation of at least one of two critical proline residues mediates their interaction with the von Hippel Lindau E3 ubiquitin ligase complex which targets them for rapid degradation. This reaction is catalyzed by prolyl 4-hydroxylases. Fumarate and succinate have been identified as potent inhibitors of prolyl hydroxylases thus leading to the stabilization of HIF.

Major Metabolic Pathways Converging on the TCA Cycle

Several catabolic pathways converge on the TCA cycle. Reactions that form intermediates of the TCA cycle in order to replenish them (especially during the scarcity of the intermediates) are called anaplerotic reactions.

The citric acid cycle is the third step in carbohydrate catabolism (the breakdown of sugars). Glycolysis breaks glucose (a six-carbon-molecule) down into pyruvate (a three-carbon molecule). In eukaryotes, pyruvate moves into the mitochondria. It is converted into acetyl-CoA by decarboxylation and enters the citric acid cycle.

In protein catabolism, proteins are broken down by proteases into their constituent amino acids. The carbon backbone of these amino acids can become a source of energy by being converted to Acetyl-CoA and entering into the citric acid cycle.

In fat catabolism, triglycerides are hydrolyzed to break them into fatty acids and glycerol. In the liver the glycerol can be converted into glucose via dihydroxyacetone phosphate and glyceraldehyde-3-phosphate by way of gluconeogenesis. In many tissues, especially heart tissue, fatty acids are broken down through a process known as beta oxidation which results in acetyl-CoA which can be used in the citric acid cycle. Beta oxidation of fatty acids with an odd number of methylene groups produces propionyl CoA, which is then converted into succinyl-CoA and fed into the citric acid cycle.

The citric acid cycle is always followed by oxidative phosphorylation. This process extracts the energy (as electrons) from NADH and QH_2, oxidizing them to NAD^+ and Q, respectively, so that the cycle can continue. Whereas the citric acid cycle does not use oxygen, oxidative phosphorylation does.

The total energy gained from the complete breakdown of one molecule of glucose by glycolysis, the citric acid cycle and oxidative phosphorylation equals about 30 ATP molecules, in eukaryotes. The citric acid cycle is called an amphibolic pathway because it participates in both catabolism and anabolism.

A Simplified view of the Process

- The citric acid cycle begins with acetyl-CoA transferring its two-carbon acetyl group to the four-carbon acceptor compound (oxaloacetate) to form a six-carbon compound (citrate).

- The citrate then goes through a series of chemical transformations, losing first one, then a second carboxyl group as CO_2. The carbons lost as CO_2 originate from what was oxaloacetate, not directly from acetyl-CoA. The carbons donated by acetyl-CoA become part of the oxaloacetate carbon backbone after the first turn of the citric acid cycle. Loss of the acetyl-CoA-donated carbons as CO_2 requires several turns of the citric acid cycle. However, because of the role of the citric acid cycle in anabolism, they may not be lost since many TCA cycle intermediates are also used as precursors for the biosynthesis of other molecules.

- Most of the energy made available by the oxidative steps of the cycle is transferred as energy-rich electrons to NAD^+, forming NADH. For each acetyl group that enters the citric acid cycle, three molecules of NADH are produced.

- Electrons are also transferred to the electron acceptor Q, forming QH_2.

- At the end of each cycle, the four-carbon oxaloacetate has been regenerated, and the cycle continues.

SECTION 5.5A—PYRUVATE FORMATION FROM ACETYL COA

The formation of Acetyl CoA from carbohydrates is less direct than from fat. Recall that carbohydrates, especially glucose are processed by glycolysis into pyruvate. Under anaerobic conditions, the pyruvate is converted into lactic acid or ethanol, depending on the organism. Under aerobic conditions, the pyruvate is transported into mitochondria in exchange for OH^- by the pyruvate carrier an antiporter. In the mitochondrial matrix pyruvate is oxidative dehydrogenase complex to form acetyl coenzyme.

$$Pyruvate + CoA + NaD^+ \rightarrow AcetylCoA + CO_2 + NADH$$
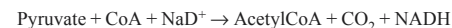
This irreversible reaction is the link between glycolysis and the citric acid cycle.*

The pyruvate dehydrogenase complex is a large, highly integrated complex of three kinds of enzyme. Pyruvate dehydrogenase is a member of a family of homologous complexes that includes the citric acid cycle enzyme α-ketoglutarate dehydrogenase.

The conversion of isocitrate into α-ketoglutarate is followed by a second oxidative decarboxylation reaction, the formation of succinyl CoA from α-ketoglutarate.

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* Note that in the preparation of the glucose derivative pyruvate for the citric acid cycle an oxidative decarboxylation takes place and high – transfer potential electrons in form of NADH are captured. Thus the pyruvate dehydrogenese reaction has many of the key features of the reactions of the citric acid itself.
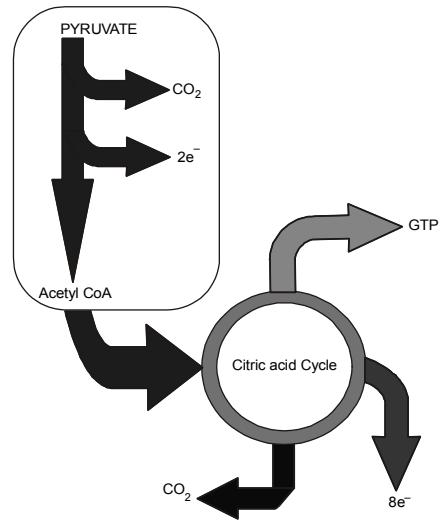
Fig. 5. : Showing the Linkage between Glycolysis and the citric acid cycle

The oxidative decarboxylation of α-ketoglutarate closely resembles that of pyruvate also an α-ketoacid.

$$Pyruvate + CoA + NAD^+ \rightarrow AcetylCoA + CO_2 + NADH$$

α - ketoglutarate   Succinyl Co enzyme
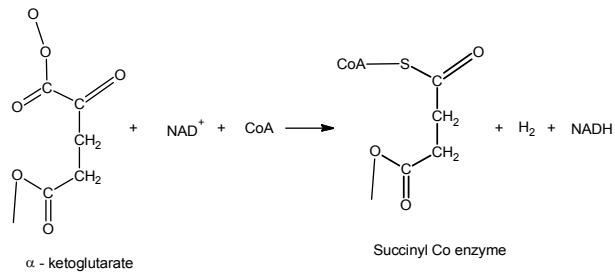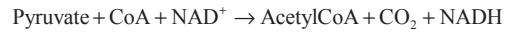
Both the reactions include the decarboxylation of an α - ketoacid and the subsequent formation of a high – transfer potential thio – ester linkage with CoA. The complex that catalyzes the oxidative decarboxylation of α - ketoglutarate is homologous to the pyruvate dehydrogenase complex and the reaction mechanism is entirely analogous. The α - ketoglutarate dehydrogenase component (E_2) and transsuccinase (E_1) are different form but homologous to the pyruvate dehydrogenase complex where as the dihydroliopyl dehydrogenase components (E_3) of the two complexes are identical. These complexes are giant, with molecular masses ranging from 4 to 10 million Daltons. As we will see their elaborate structures allow traveling from one active site to another, connected by thioethers to the core of the structure. The mechanism of the pyruvate dehydrogenase reaction is wonderful complex, more so than is suggested by its relatively simple stoichiometry. The reaction requires the participation of the three enzymes of the pyruvate dehydrogenase complex, each composed of several polypeptide chains and five enzymes. Thiamine pyrophosphate lipoic acid and FAD serve as catalytic co factors and CoA and NAD^+ are stoichiometric co factors.
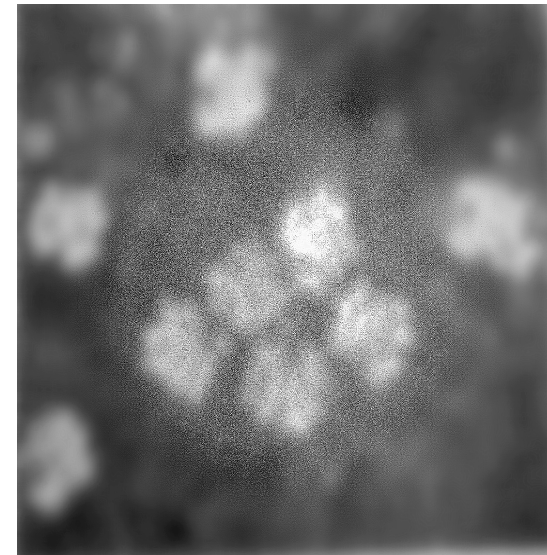
Fig. 5. : Showing the Microscopic Photograph of dehydrogenase Complex

The table of Pyruvate dehydrogenase complex of E Coli

Enzyme	Abbre-viation	No of Chain	Prosthetic group	Reaction Catalysis
Pyruvate dehydrogenase component	E_1	24	TPP	Oxidative decarboxylation of Pyruvate
Dihydroliopyl transacetylase	E_2	24	Lipoamide	Transfer of the acetyl group to CoA
Dihydroliopyl dehydrogenase	E_3	12	FAD	Regeneration of the oxidized form of Lipoamide

Lipoic Acid

Thyamine Pyrophopshate
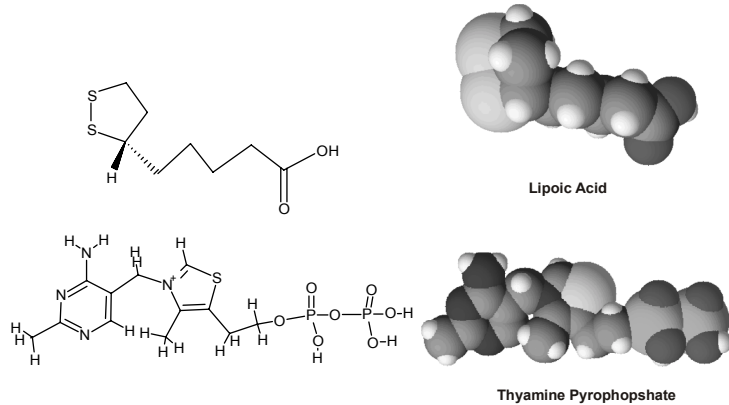
At least two additional enzyme regulate the activity if the complex. The conversion iof pyruvate into acetyl CoA consists of these three steps decarboxylation, oxidation and transfer of the resultant acetyl group to CoA.

Pyruvate   decarboxylation   oxidation   transfer of CoA   Acetyl CoA
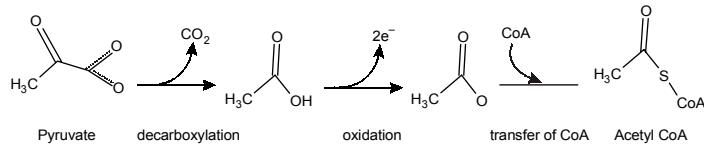
These steps must be coupled to preserve the free energy derived from the decarboxylation step to drive the formation of NADH and acetyl CoA. First pyruvate combines with TPP and is then decarboxylated.

Pyruvate   Addition Compound   resonance forms of hydroxyethyl TPP   hydroxyethyl TPP
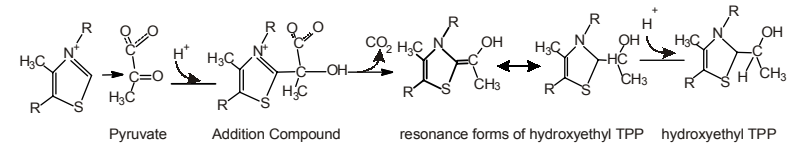
Equation showing the decarboxylation of E1 the pyruvate dehydrogenase component of pyruvate dehydrogenase complex.

This reaction is catalyzed by the pyruvate dehydrogenase component, is that the carbon atom between the nitrogen and sulphur atoms in the thiozole ring is much more acidic that most $=$ CH group, with a pKa value of 10. This centre ionizes to form a carbanion, which readily absorbs to the carbonyl group of pyruvate.

TPP   Carbanion TPP
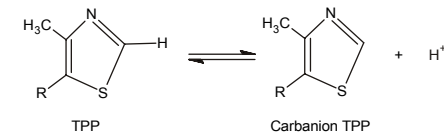
This addition is followed by the decarboxylation if pyruvate. The positively charged ring of the TPP acts as an electron sink that stabilizes the negative charge that is transferred to the ring as a part of decarboxylation. Protonation yields hydroxymethyl group attached to TPP is oxidized to form an acetyl group and concomitantly transferred to Lipoamide, a derivative of lipoic acid that is linked with the side chain of a residue by an amide linkage.

hydroxyethyl - TPP   Lipoamide   Carbanion TPP   Acetyllipoamide
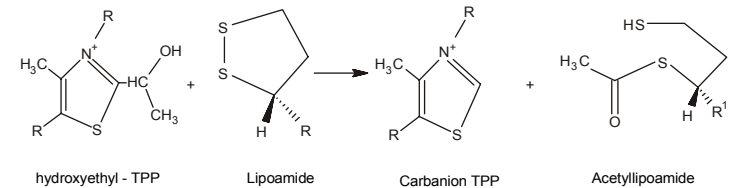
The oxidant in this reaction is the disulphide group of Lipoamide, which is reduced to its disulphydryl form. This reaction also catalyzed by the pyruvate dehydrogenase component E1 yields acetyllipoamide.

Third, the acetyl group is transferred from acetyllipoamide to CoA to form acetyl CoA. D. Dihydroliopyl transacetylase (E2) catalyzes; the energy reach thioester bond is preserved as the acetyl group is transferred to CoA.
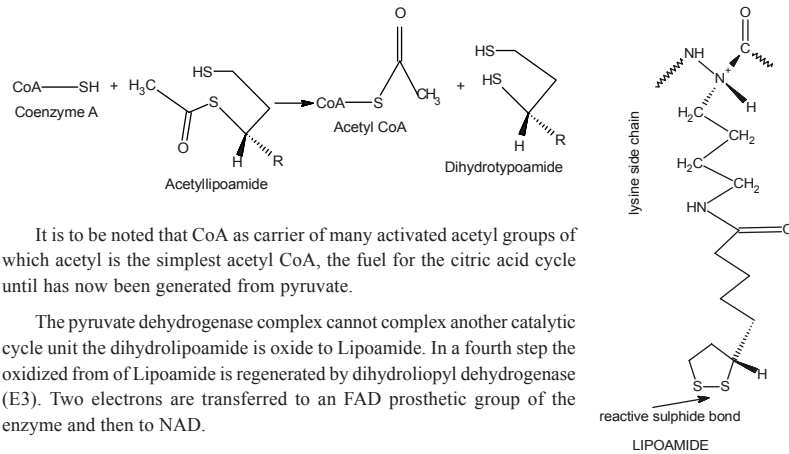
It is to be noted that CoA as carrier of many activated acetyl groups of which acetyl is the simplest acetyl CoA, the fuel for the citric acid cycle until has now been generated from pyruvate.

The pyruvate dehydrogenase complex cannot complex another catalytic cycle unit the dihydrolipoamide is oxide to Lipoamide. In a fourth step the oxidized from of Lipoamide is regenerated by dihydroliopyl dehydrogenase (E3). Two electrons are transferred to an FAD prosthetic group of the enzyme and then to NAD.
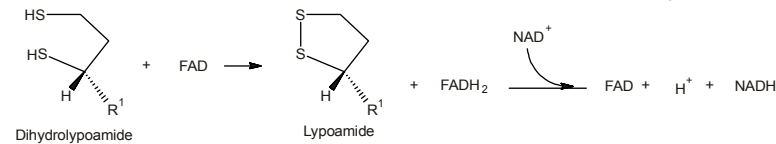
The electron transfer to FAD is unusual, because the common role for FAD is to receive electrons from NADH. The electron transfer potential of FAD is altered by its association with the enzyme and enables it to transfer electrons to NAD$^+$. Proteins tightly associated with FAD or flavin mono – nucleotide (FMN) are also called flavoproteins.

AS it has been previously stated that citric acid cycle begins with four carbon units, oxaloacetate, reacts with acetyl CoA and H$_2$O to yield citrate and CoA.
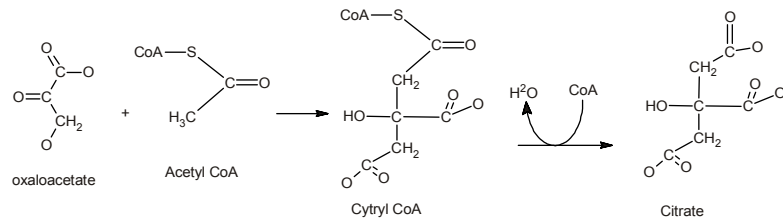
This reaction, which is aldol condensation followed by hydrolysis, is catalyzed by citrate synthase. Oxaloacetate first condenses with acetyl CoA to form citryl CoA, a high – energy thioester intermediate, drivers the overall reaction far in the direction of the synthesis of citrate. In essence the hydrolysis of the thioester powers the synthesis of the new molecule from two precursors. Because this reaction initiates the cycle, it is very important initiates the cycle, it is very important that side reactions are kept minimum.

Let us now briefly discuss how the citrate synthesis prevents wasteful processes such as the hydrolysis of acetyl CoA. Mammalian citrate synthase is a dimmer of identical 49 – kd subunits. Each active site is located in a clef between large and small domains of a subunit adjacent to the subunit interface. The result of the X – Ray crystallographic studies of citrate synthase and its complexes with several substrates and inhibitors revealed that the enzyme undergoes large conformational changes in the course of catalysis. Citrate synthase exhibits sequential, ordered kinetics: oxaloacetate binds first, followed by acetyl CoA. The reason for the ordered binding is that oxaloacetate induces a major structural rearrangement leading to the creation of binding site for acetyl CoA. The open form of the enzyme observed in the absence of ligands is converted into a closed form by the binding of oxaloacetate See Fig. 145.
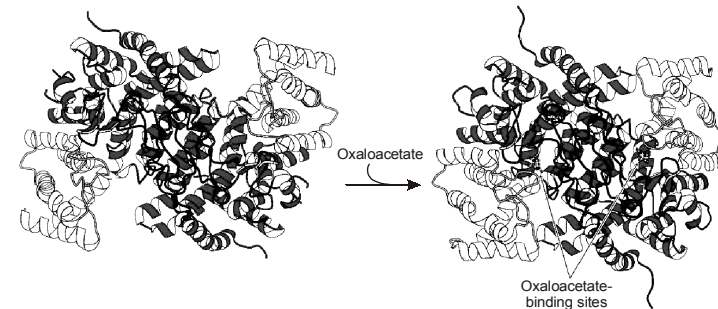
Fig. 5.5 : Showing the conformational changes in the Citrate Synthase in binding the Oxaloacetate

In each submit, the small domain rotates 19° relative to the large domain. Movements as large as 15Å are produced by the rotation of a helices elicited by quite small shift of side chains around bound oxaloacetate. This conformational transition is reminiscent of the cheft closure in hexokinase induced by the binding of glucose.

Citrate synthase catalyses the condensation reaction by bringing the substrates into close proximity orienting them and polarization of certain bonds. Two hystidine residues and an aspirate residue are important players.

Oxaloacetate

substrate complex

enol intermediate

His 320

His 274

Asp 375

Oxaloacetate

enol intermediate

citryl CoA Complex
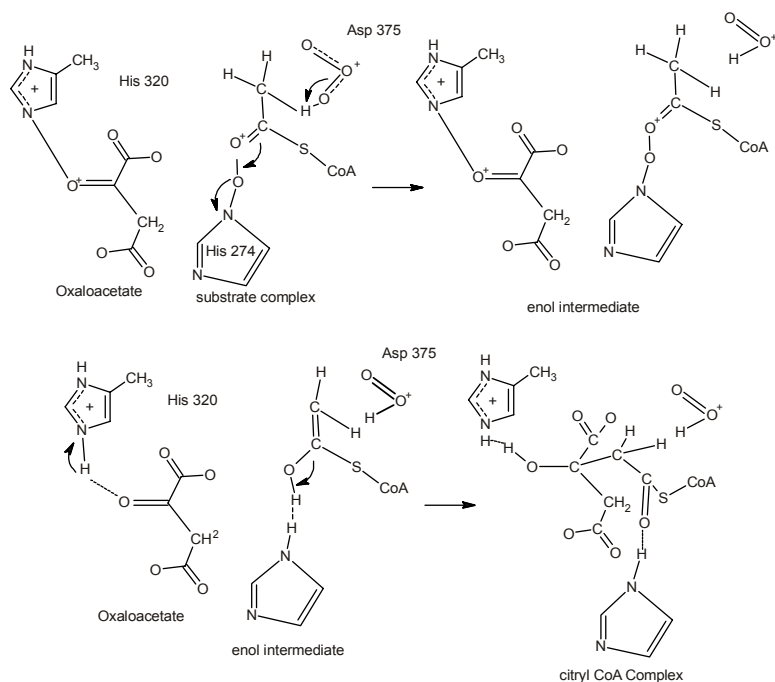
His 320

Asp 375

One of the hystidine residues (His 274) donates a proton to the carbonyl oxygen of acetyl CoA to promote the removal of a methyl proton by Asp 375. Oxaloacetate is activated by the transfer of a proton from His 320 to its carbonyl carbon atom. The concomitant attack of the enol of a carbon – carbon bond. The newly formed citryl CoA induces additional closed; His 274 participates again as a proton donor to hydrolyze the thioester, Coenzyme A leaves the enzyme then the citrate follows it and the enzyme returns to the initial open conformation.

We can now understand the wasteful hydrolysis of acetylation co –enzyme CoA is prevented. Citrate synthase is well suited to hydrolyze citryl CoA but not acetyl CoA. How is this discrimination accomplished? First acetyl CoA does not bind to the enzyme until oxaloacetate is bond and ready for condensation. Second, the catalytic residues crucial for the hydrolysis of the thioester linkage are not appropriately positioned until citryl CoA is formed. As with hexokinase and triose phosphate isomeriose induced fit prevents and undesirable side reaction.

The tertiary hydroxyl group is not properly located in the citrate molecule for the oxidative decarboxylation that follows. Thus, citrate isomerizes into isocitrate to enable the six carbon unit to undergo oxidative decarboxylation.

The isomerization of citrate is accomplished by a dehydration step followed by hydration step. The result is an inter change of a hydrogen atom and a hydroxyl group. The enzyme catalyzing both step is called aconitase because cis – aconitate is an intermediate.

citrate

cis - aconitate
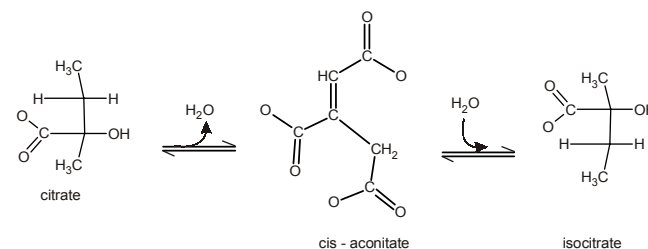
isocitrate

Aconitase is an iron – sulphur protein*, or non – hæme iron protein. It contains four iron atoms are complexed to four inorganic sulphides and three cystine sulphur atoms leaving one iron atoms available to bind citrate and then isocitrate through their carbohydrate and hydroxyl groups. This iron centre with in conjugation with other groups on the enzyme, facilitates the dehydration and rehydration reactions, we will consider the role of these iron sulphur cluster in the electron – transfer reactions of oxidative phosphorylation subsequently.

The oxidative decarboxylation of isocitrate is catalysed by isocitrate dehydrogenase.

$$\text{Isocitrate} + NAD^+ \rightarrow \alpha\text{-ketoglutarate} + CO_2 + NADH$$

Iron-sulphur proteins are proteins characterized by the presence of iron-sulphur clusters containing sulfide-linked di-, tri-, and tetrairon centers in variable oxidation states. Iron-sulphur clusters are found in a variety of metalloproteins, such as the ferredoxins, as well as NADH dehydrogenase, hydrogenases, Coenzyme Q - cytochrome c reductase, Succinate - coenzyme Q reductase and nitrogenase. Iron-sulphur clusters are best known for their role in the oxidation-reduction reactions of mitochondrial electron transport. Both Complex I and Complex II of oxidative phosphorylation have multiple Fe-S clusters. They have many other functions including catalysis as illustrated by aconitase, generation of radicals as illustrated by SAM-dependent enzymes, and as sulphur donors in the biosynthesis of lipoic

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* Proteins in which non-haem iron is coordinated with cysteine sulphur and usually also with inorganic sulphur. Divided into three major categories: rubredoxins; "simple iron-sulphur proteins", containing only iron-sulphur clusters; and "complex iron-sulphur proteins", containing additional active redox centres such as flavin, molybdenum or haem.In most iron-sulphur proteins, the clusters function as electron-transfer groups, but in others they have other functions, such as catalysis of hydratase/dehydratase reactions, maintenance of protein structure, or regulation of activity 1997, 69, 1281 IUPAC Compendium of Chemical Terminology.

acid and biotin. Additionally some Fe-S proteins regulate gene expression. Fe-S proteins are vulnerable to attack by biogenic nitric oxide.

Structural Motifs

In almost all Fe-S proteins, the Fe centers is tetrahedral and the thiolato sulphur centers, from cysteinyl residues, are terminal ligands. The sulfide groups are either two- or three-coordinated. Three distinct kinds Fe-S clusters with these features are most common.

2Fe-2S Clusters

The simplest polymetallic system, $[Fe_2S_2]$ cluster, is constituted by two iron ions bridged by two sulfide ions and coordinated by four cysteinyl ligands (in Fe2S2 ferredoxins) or by two cysteines and two histidines (in Rieske proteins). The oxidized proteins contain two Fe3+ ions, whereas the reduced proteins contain one Fe^{3+} and one Fe^{2+} ion. These species exist in two oxidation states, $(Fe^{III})_2$ and $Fe^{III}Fe^{II}$.
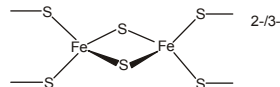
4Fe-4S Clusters

A common motif features a four iron ions and four sulfide ions placed at the vertices of a cubane-type structure. The Fe centers are typically further coordinated by cysteinyl ligands. The $[Fe_4S_4]$ electron-transfer proteins ($[Fe_4S_4]$ ferredoxins) may be further subdivided into low-potential (bacterial-type) and high-potential (HiPIP) ferredoxins. Low- and high-potential ferredoxins are related by the following redox scheme:
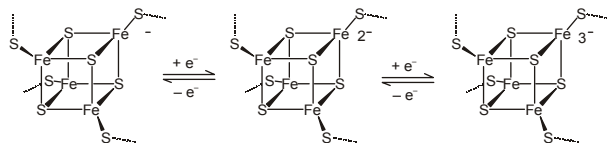
In HiPIP, the cluster shuttles between $[2Fe^{3+}, 2Fe^{2+}]$ $(Fe_4S_4^{2+})$ and $[3Fe^{3+}, Fe^{2+}]$ $(Fe_4S_4^{3+})$. The potentials for this redox couple range from 0.4 to 0.1 V. In the bacterial Fd's, the pair of oxidation states are $[Fe^{3+}, 3Fe^{2+}]$ $(Fe_4S_4^+)$ and $[2Fe^{3+}, 2Fe^{2+}]$ $(Fe_4S_4^{2+})$. The potentials for this redox couple range from –0.3 to –0.7 V. The two families of 4Fe-4S clusters share the $Fe_4S_4^{2+}$ oxidation state. The difference in the redox couples is attributed to the degree of hydrogen bonding, which strongly modified the basicity of the cysteinyl thiolate ligands. A further redox couple, which is still more reducing than the bacterial Fd's is implicated in the nitrogenase.

Some 4Fe-4S clusters bind substrates and are thus classified as enzymes. In *aconitase*, the Fe-S cluster binds *aconitate* at the one Fe centre that lacks a thiolate ligand. The cluster does not undergo redox, but serves as a *Lewis acid* catalyst to convert aconitate to *isocitrate*. In the radical-SAM enzymes, the cluster binds and reduces *S-adenosylmethionine* to generate a radical, which is involved in many biosyntheses

3Fe-4S Clusters

Proteins are also known to contain $[Fe_3S_4]$ centres, which feature one iron less than the more common $[Fe_4S_4]$ cores. Three sulfide ions bridge two iron ions each, while the fourth sulfide bridges three iron ions. Their formal oxidation states may vary from $[Fe_3S_4]^+$ (all-Fe^{3+} form) to $[Fe_3S_4]^{2-}$ (all-Fe^{2+} form). In a number of iron-sulphur proteins, the $[Fe_4S_4]$ cluster can be reversibly converted by oxidation and loss of one iron ion to a $[Fe_3S_4]$ cluster. E.g., the inactive form of aconitase possesses an $[Fe_3S_4]$ and is activated by addition of Fe^{2+} and reductant.

The intermediate in this reaction is oxaloacetate, an unstable β-ketoacid. While bound to the enzyme it loses CO_2 to form α - ketoglutarate.

isocitrate   Oxalosuccinate   ?? keto glutarate
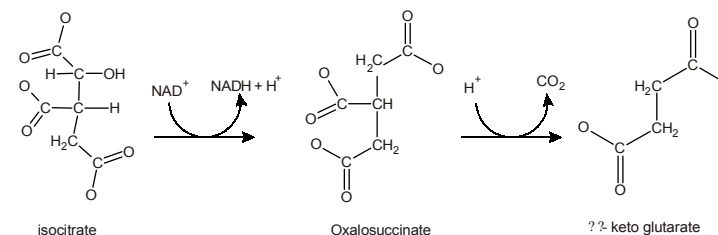
The rate of formation of α - ketoglutarate is important in determining the overall rate of the cycle. This oxidation generates the first high transfer potential electron carrier NADH in the cycle.

The net reaction of the citric acid cycle is

Acetyl CoA + $3NAD^+$ + FAD + GDP + P_1 + $2H_2O$ → $2CO_2$ + 3NADH + $FADH_2$ + GDP + $2H^+$ + CoA
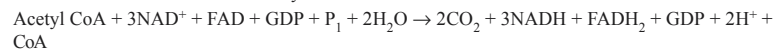
SECTION 5.7—GLUCONEOGENESIS

Gluconeogenesis (abbreviated GNG) is a metabolic pathway that results in the generation of glucose from non-carbohydrate carbon substrates such as pyruvate, lactate, glycerol, and glucogenic amino acids.

The vast majority of gluconeogenesis takes place in the liver and, to a smaller extent, in the cortex of kidneys. This process occurs during periods of fasting, starvation, or intense exercise and is highly endergonic. Gluconeogenesis is often associated with ketosis. Gluconeogenesis is also a target of therapy for type II diabetes, such as metformin, which inhibit glucose formation and stimulate glucose uptake by cells. This pathway is defined as the formation of glucose from non – carbohydrate sources. Gluconeogenesis is vital to normal brain function in the fasting state in that the brain receives its principal fuel, glucose, directly from the blood. Despite the high concentration of glycogen in the liver, its total content would be used up by about 16 – 24 hours of fasting. As a result glucose synthesis is vital. The liver is the principal site for gluconeogenesis although the kidney also has the pathway (and it is simulated in response to acidosis.

Most of the reactions of gluconeogenesis are catalyzed by the enzymes of the glycolytic sequence (Fig. 146).
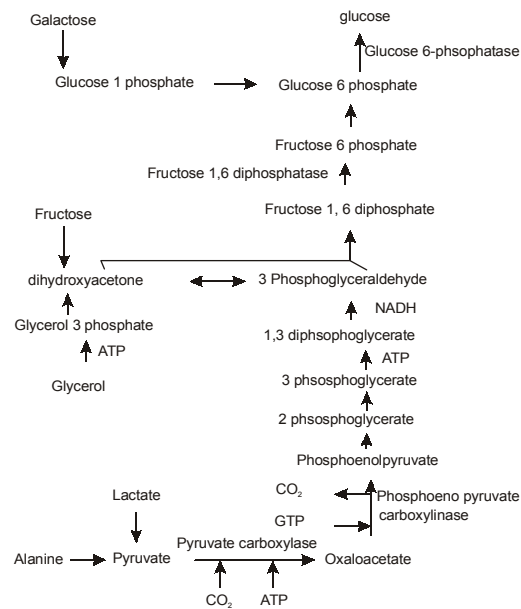
Fig. 5. : Showing the Glycolytic sequence in the Gluconeogenesis Pathway

Because glycolysis and gluconeogenesis are opposing pathways, there has to be control so that glucose formation or breakdown will occur in a physiologically sound fashion. The flux through the respective pathways governed by:

(a) Allosteric effectors

(b) Covalent modification of Enzymes

(c) Enzyme concentrations

Covalent Modification of enzymes is brought about (in main) by fluctuations in the ratio of insulin to glucagon in blood. Insulin is the principal modulator in the fed state. When gluconeogenesis should be active. Three steps in glycolysis are virtually irreversible and have to be passed in gluconeogenesis; three substrate cycles are involved where control can be imposed. (See fig 147).
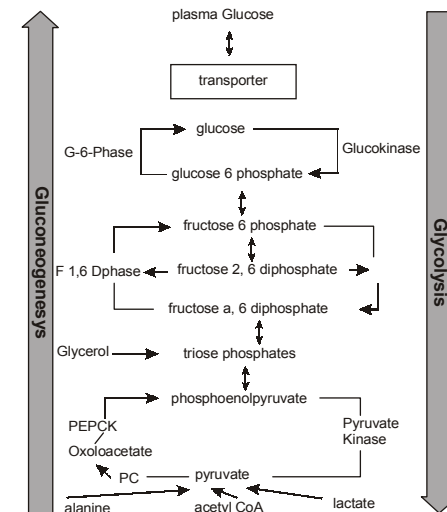
Fig. 5. : Showing the Comparison of Gluconeogenesis and Glycolysis Pathways

SUB-SECTION 5.7A—GLUCONEOGENESIS FROM LACTATE

Lactate is a predominate source of carbon atoms for glucose synthesis by gluconeogenesis. During anaerobic glycolysis in skeletal muscle, pyruvate is reduced to lactate by lactate dehydrogenase (LDH). This reaction serves two critical functions during anaerobic glycolysis. First, in the direction of lactate formation the LDH reaction requires NADH and yields NAD^+ which is then available for use by the glyceraldehyde-3-phosphate dehydrogenase reaction of glycolysis. These two reactions are, therefore, intimately coupled during anaerobic glycolysis. Secondly, the lactate produced by the LDH reaction is released to the blood stream and transported to the liver where it is converted to glucose. The glucose is then returned to the blood for use by muscle as an energy source and to replenish glycogen stores. This cycle is termed the Cori cycle. See Fig. 141.

SUB-SECTION 5.7B—GLUCOGENIC AMINO ACIDS[1]

All 20 of the amino acids, excepting leucine and lysine, can be degraded to TCA cycle intermediates as discussed in the metabolism of amino acids. This allows the carbon skeletons of the amino acids to be

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[1] If students are interested in the detail discussion of this subsection of this subsection An experimental study can be provided.

converted to that in oxaloacetate and subsequently into pyruvate. The pyruvate thus formed can be utilized by the gluconegenic pathway. When glycogen stores are depleted, in muscle during exertion and liver during fasting, catabolism of muscle proteins to amino acids contributes the major source of carbon for maintenance of blood glucose levels.

SECTION 5.8—THE PENTOSE PHOPSHATE PATHWAY

The pentose phosphate pathway (also called hexose monophopshate shunt) is an alternative pathway for the oxidative metabolism of G – P leading to the production of pentose phosphate and NADPH. It is important to distinguish this pathway from glycolysis in that it is a significant pathway of glucose metabolism out does not result in ATP synthesis. The entire set of reactions can be summarized as follows:

Reactants	Products	Enzyme	Description
Glucose 6-phosphate + NADP+	→ 6-phosphoglucono→ lactone + NADPH	glucose 6-phosphate dehydrogenase	Dehydrogenation. The hemiacetal hydroxyl group located on carbon 1 of glucose 6-phosphate is converted into a carbonyl group, generating a lactone, and, in the process, NADPH is generated.
6-phosphoglucono- → -lactone + H_2O	→ 6-phosphogluconate + H_+	6-phosphoglu- conolactonase	Hydrolysis
6-phosphogluconate + $NADP^+$	→ ribulose 5-phosphate + $NADPH + CO_2$	6-phosphogluconate dehydrogenase	Oxidative decarboxylation. $NADP^+$ is the electron acceptor, generating another molecule of NADPH, a CO_2, and ribulose 5-phosphate.

The overall reaction for this process is:

Glucose 6-phosphate + 2 $NADP^+$ + H_2O → ribulose 5-phosphate + 2 NADPH + 2 H^+ + CO_2
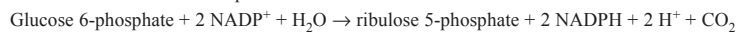
The pentose phosphate pathway's Nonoxidative phase:

Glucose-6-phosphate dehydrogenase is the rate-controlling enzyme of this pathway. It is allosterically stimulated by $NADP^+$. The ratio of $NADPH:NADP^+$ is normally about 100:1 in liver cytosol. This makes the cytosol a highly-reducing environment. Formation of $NADP^+$ by a NADPH-utilizing pathway, thus, stimulating production of more NADPH.

The importance of the pentose phosphate pathway is as follows.

• It produces ribose – 5 – phosphate, which is required for the biosynthesis of purine and pyrimidine nucleotides and therefore for RNA and DNA the numerous nucleotides that play roles in the intermediatory metabolism including ATP, UTP, CTP GTP – S – Adenosyl – metionine FAD, NAD and NADP.

Reactants	Products	Enzymes
ribulose 5-phosphate	→ ribose 5-phosphate	Ribulose 5-Phosphate Isomerase
ribulose 5-phosphate	→ xylulose 5-phosphate	Ribulose 5-Phosphate 3-Epimerase
xylulose 5-phosphate + ribose 5-phosphate	→ glyceraldehyde 3-phosphate + sedoheptulose 7-phosphate	transketolase
sedoheptulose 7-phosphate + glyceraldehyde 3-phosphate	→ erythrose 4-phosphate + fructose 6-phosphate	transaldolase
xylulose 5-phosphate + erythrose 4-phosphate	→ glyceraldehyde 3-phosphate + fructose 6-phosphate	transketolase
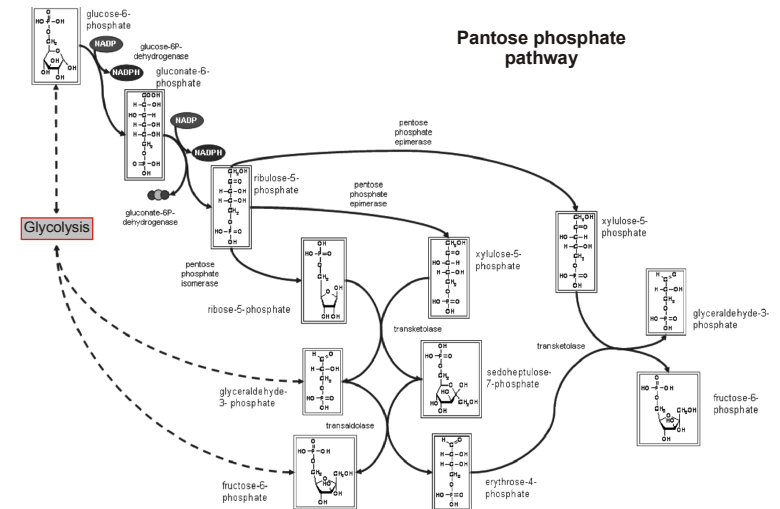
Fig. 5.8 : Showing the pentose phosphate pathway

• It produces reducing power in the form of NADPH which is required for the synthesis of fatty acids cholesterol and steroid hormones.

- In plants, the pathways are modified to participate in the formation of glucose from carbon dioxide in photosynthesis.

SECTION 5.9—GLUCURONIC ACID

Glucuronic acid (from Greek γëõêåñûò - "sweet") is a carboxylic acid. Its structure is similar to that of glucose. However, glucuronic acid's sixth carbon is oxidized to a carboxylic acid. Its formula is $C_6H_{10}O_7$.

The salts and esters of glucuronic acid are known as glucuronates; the anion $C_6H_9O_7-$ is the glucuronate ion.Glucuronic acid should not be confused with gluconic acid, a linear carboxylic acid resulting from the oxidation of a different carbon of glucose. Both glucuronic acid and gluconic acid are reported to be found in the fermented drink known as kombucha. Glucuronic acid has been attributed with the mildly-alcoholic effect that drinking the tea has for some.

SUB-SECTION 5.9—FUNCTIONS GLUCURONIDATION

Glucuronic acid is highly soluble in water. In the animal body, glucuronic acid is often linked to the xenobiotic metabolism of substances such as drugs, pollutants, bilirubin, androgens, estrogens, mineralocorticoids, glucocorticoids, fatty acid derivatives, retinoids, and bile acids. These linkages involve glycosidic bonds, and this linkage process is known as glucuronidation. Glucuronidation occurs mainly in the liver, although the enzyme responsible for its catalysis, UDP-glucuronyltransferase, has been found in all major body organs, e.g., heart, kidneys, adrenal gland, spleen, and thymus. UDP-glucuronic acid (glucuronic acid linked via a glycosidic bond to uridine diphosphate) is an intermediate in the process and is formed in the liver. One example is this N-glucuronidation of an aromatic amine, 4-aminobiphenyl, by UGT1A4 or UGT1A9 from human, rat, or mouse liver.
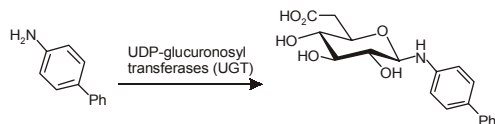
The substances resulting from glucuronidation are known as glucuronides (or glucuronosides) and are typically much more water-soluble than the non-glucuronic acid-containing substance from which they were originally synthesised. The human body uses glucuronidation to make a large variety of substances more water-soluble, and, in this way, allow for their subsequent elimination from the body upon urination. Hormones may also be glucuronidated to allow for easier transport around the body.
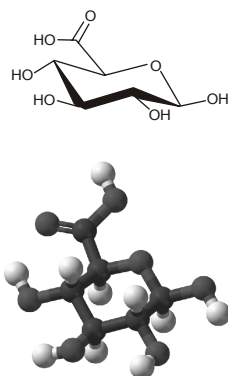
Pharmacologists also commonly link drugs to glucuronic acid to allow for easier drug delivery.

The conjugation of xenobiotic molecules with hydrophilic molecular species such as glucuronic acid is known as phase II metabolism.

SECTION 5.10—GLUCOSE TOLERANCE TEST

A glucose tolerance test in medical practice is the administration of glucose to determine how quickly it is cleared from the blood. The test is usually used to test for diabetes, insulin resistance, and sometimes reactive hypoglycemia. The glucose is most often given orally so the common test is technically an oral glucose tolerance test (OGTT). The test may be performed as part of a panel of tests, such as the comprehensive metabolic panel.

SUB-SECTION 5.10A—PROCEDURE FOR OGTT

The patient is instructed not to restrict carbohydrate intake in the days or weeks before the test. The test should not be done during an illness, as results may not reflect the patient's glucose metabolism when healthy. A full adult dose should not be given to a person weighing less than 43 kg (94 lb), or exaggerated glucoses may produce a false positive result. The patient should have been fasting for the previous 8-14 hours (water is allowed). Usually the OGTT is scheduled to begin in the morning (0700-0800) as glucose tolerance exhibits a diurnal rhythm with a significant decrease in the afternoon. A zero time (baseline) blood sample is drawn. The patient is then given a glucose solution to drink. The standard dose since the late 1970s has been 1.75 grams of glucose per kilogram of body weight, to a maximum dose of 75 g. It should be drunk within 5 minutes. Prior to 1975 a dose of 100 g was often used.

Blood is drawn at intervals for measurement of glucose (blood sugar), and sometimes insulin levels. The intervals and number of samples vary according to the purpose of the test. For simple diabetes screening, the most important sample is the 2 hour sample and the 0 and 2 hour samples may be the only ones collected. In research settings, samples may be taken on many different time schedules.

If renal glycosuria (sugar excreted in the urine despite normal levels in the blood) is suspected, urine samples may also be collected for testing along with the fasting and 2 hour blood tests.

SUB-SECTION 5.10B—INTERPRETATION OF OGTT RESULTS

Fasting plasma glucose should be below 6.1 mmol/l (110 mg/dl). Fasting levels between 6.1 and 7.0 mmol/l (110 and 126 mg/dl) are borderline ("impaired fasting glycaemia"), and fasting levels repeatedly at or above 7.0 mmol/l (126 mg/dl) are diagnostic of diabetes.

The 2 hour glucose level should be below 7.8 mmol/l (140 mg/dl). Levels between this and 11.1 mmol/l (200 mg/dl) indicate "impaired glucose tolerance." Glucose levels above 11.1 mmol/l (200 mg/dl) at 2 hours confirms a diagnosis of diabetes.

Table 5. : 1999 WHO Diabetes criteria—Interpretation of Oral Glucose Tolerance Test

Glucose levels	NORMAL		impaired fasting glycaemia (IFG)		impaired glucose tolerance (IGT)		Diabetes Mellitus (DM)	
Venous Plasma	Fasting	2hrs	Fasting	2hrs	Fasting	2hrs	Fasting	2hrs
(mmol/l)	<6.1	<7.8	> 6.1 & <7.0	<7.8	<7.0	>7.8	>7.0	>11.1
(mg/dl)	<110	<140	>110 & <126	<140	<126	>140	>126	>200

A standard 2 hour OGTT is sufficient to diagnose or exclude all forms of diabetes mellitus at all but the earliest stages of development. Longer tests have been used for a variety of other purposes, such as detecting reactive hypoglycemia or defining subsets of hypothalamic obesity. Insulin levels are sometimes measured to detect insulin resistance or deficiency.

The OGTT is of limited value in the diagnosis of reactive hypoglycemia, since (1) normal levels do not preclude the diagnosis, (2) abnormal levels do not prove that the patient's other symptoms are related to a demonstrated atypical OGTT, and (3) many people without symptoms of reactive hypoglycemia may have the late low glucoses that are said to be characteristic. Using a glucose tolerance in this context resembles use of a Rorschach test in that it is often used to support a diagnosis that the patient and doctor are already reaching agreement on based on other evidence, but it is inadequate by itself to confirm or refute the diagnosis (unlike its use for diabetes).

When the glucose is given intravenously it is termed an intravenous glucose tolerance test (IVGTT). This has been used in the investigation of early insulin secretion abnormalities in prediabetic states.

SUB-SECTION 5.10C—GLYCOSURIA

Glycosuria or glucosuria is the excretion of glucose into the urine. Ordinarily, urine contains no glucose because the kidneys are able to reclaim all of the filtered glucose back into the bloodstream. Glycosuria is nearly always caused by elevated blood glucose levels, most commonly due to untreated diabetes mellitus. Rarely, glycosuria is due to an intrinsic problem with glucose reabsorption within the kidneys themselves, a condition termed renal glycosuria.[1] Glycosuria leads to excessive water loss into the urine with resultant dehydration, a process called osmotic diuresis. Blood is filtered by millions of nephrons, the functional units that comprise the kidneys. In each nephron, blood flows from the arteriole into the glomerulus, a tuft of leaky capillaries. Bowman's capsule surrounds each glomerulus, and collects the filtrate that the glomerulus forms. The filtrate contains waste products (e.g. urea), electrolytes (e.g. sodium, potassium, chloride), amino acids, and glucose. The filtrate passes into the renal tubules of the kidney. In the first part of the renal tubule, the proximal tubule, glucose is reabsorbed from the filtrate, across the tubular epithelium and into the bloodstream. The proximal tubule can only reabsorb a limited amount of glucose. When the blood glucose level exceeds about 160 - 180 mg/dl (8.9 - 10 mmol/l), the proximal tubule becomes overwhelmed and begins to excrete glucose in the urine.

This point is called the renal threshold of glucose (RTG). Some people, especially children and pregnant women, may have a low RTG (less than ~7 mmol/L glucose in blood to have glucosuria).

If the RTG is so low that even normal blood glucose levels produce the condition, it is referred to as renal glycosuria. Glucose in urine can be identified by Benedict's qualitative test. Renal glycosuria, also known as renal glucosuria, is a rare condition in which the simple sugar glucose is excreted in the urine despite normal or low blood glucose levels. With normal kidney (renal) function, glucose is excreted in the urine only when there are abnormally elevated levels of glucose in the blood. However, in those with renal glycosuria, glucose is abnormally eliminated in the urine due to improper functioning of the renal tubules, which are primary components of nephrons, the filtering units of the kidneys.

SUB-SECTION 5.10D—GLYCOSYLATED HEAMOGLOBIN

Glycosylated Haemoglobin (GHb), the resultant of chronic hyperglycaemia, has hitherto been used as an index of long term glycaemic control in diabetics. Since it is a refiector of integrated long term glycaemic status, it can also be used as a screening test for the diagnosis of Diabetes Mellitus (DM). In this study of 1000 subjects, when GHb was compared to Oral Glucose Tolerance Test (OGTT) in the diagnosis of DM, GHb was found to have 93.98% sensitivity, 85.86% specificity and 93.14% efficiency in the diagnosis of DM. The predictive value of an elevated GHb is 98.2%. Hence it is concluded that GHb is a useful screening test for DM and that it may also be considered when criteria for the diagnosis of DM are discussed. Glycosylated Haemoglobin reflects retrospective time averaged glycaemic status of an individual and thus has became a useful tool in assessing the long term control of DM1,2,3. Being a reflector of chronic hyperglycaemia, an elevated GHb, can also be used as a screening test for the diagnosis of DM4,5. It has the added convenience that blood sample can be collected at any time of the day without any specific time interval in relation to the meal. Glycosylated Haemoglobin was determined in 1000 non-pregnant adults The OGTT was interpreted following the WHO criteria. Glycosylated Haemoglobin was estimated by modified Fluckinger and Winterhalter's method. The OGTT following WHO criteria, revealed that among the 1000 subjects studied, 797 had DM while 111 had Impaired Glucose Tolerance (IGT). The remaining 92 had normal glucose tolerance (Table-1). The mean GHb in those with normal glucose tolerance, IGT and Diabetes Mellitus was $6.9 \pm 1.05\%$, $8.4 \pm 2.50\%$ and $12.6 \pm 1.80\%$ respectively. Table 2 shows the 95% confidence limits of GHb in each of these categories. The +2SD figure for those with those with normal glucose tolerance and the -2SD figure for those with DM are both 9%,indicating this to be a clear cut-off point between the normal-and diabetic population in this study. The mean GHb value in those with IGT lies between those of the other two two categories. The GHb was elevated in 749 diabetics, 60 subjects with IGT and 13 subjects with normal glucose tolerance. It was normal in 48 diabetics, 51 subjects with IGT and 79 normal individuals. Further analysis was restricted to those who had either normal glucose tolerance or DM. Table 2 shows the GHb results in relation to the OGTT results in subjects who had either diabetic curve or normal glucose tolerance. Oral Glucose Tolerance Test was taken as the reference "Gold Standard" against which the validity of GHb determination in the diagnosis of Diabetes Mellitus was assessed. Based on the OGTT and GHb results, subjects could be placed into one of the following 4 wells depicted in Fig. 149. Thus,

Table 5.1 : Results of OGTT

n = 1000

Diagnostic category (WHO)	Number	Percentage
DM	797	79.7
IGT	111	11.1
Normal	92	9.2

Table 5.2 : GHb in different categories of Glucose Tolerance

n = 1000

Categories of Glucose Tolerance	No. of subjects	GHb% Mean + S.D.	GHb −2SD	GHb +2SD
Normal	92	6.9 ± 1.05	4.8	9.0*
IGT	111	8.4 ± 2.50	5.9	10.9
DM	797	12.6 ± 1.8	9.0*	16.2

*+2SD of GHb in those with normal glucose tolerance and -2SD of GHb in, those with DM are both 9%, including that a GHb level of 9% is the cut-off point between the, two categories.

Classification of subjected based on OGTT and GHb

		OGTT	
		Abnormal	Normal
GHb	Elevated	a	b
	Normal	c	d

Well "a" represents diabetics with elevated GHb

Well "b" represents normal subjects with elevated GHb

Well "c" represents diabetics with normal GHb, and

Well "d" represents normal subjects with normal GHb

In other words,

Well "a" represents True positivity

Well "b" represents False positivity

Well "c" represents False negativity

Well "d" represents True Negativity of GHb estimations in the diagnosis of DM in relation to OGTT

After so placing the results of GHb determination in relation to OGTT results, we proceeded to analyse the various attributes of GHb in the diagnosis of DM. The sensitivity of GHb in the diagnosis of DM, i.e. its ability to correctly identify those with DM as compared to OGTT is given by the formula:

$$\frac{a}{a+c} \times 100$$

The specificity of GHb in the diagnosis of DM, i.e. its ability to correctly classify those who do not have Diabetes, as compared to OGTT is given by the formula :

$$\frac{d}{b+d} \times 100$$

The predictive value of raised GHb in the diagnosis of DM represents the percentage of diabetics among all those who have raised GHb and is given by the formula :

$$\frac{a}{a+c} \times 100$$

By the predictive value of normal GHb is meant the percentage of true normals by OGTT among all those who have a normal GHb and this is given by the formula :

$$\frac{d}{c+d} \times 100$$

Having analysed the sensitivity, specificity and predictive value of GHb in the diagnosis of DM, we proceeded to analyse its overall efficiency as a diagnostic test for DM. The efficiency of a test represents its ability to bisect the test subjects into either normals or abnormals-i.e: in this instance place them either into well 'a' or well 'd' and is given by the formula

$$\frac{a+d}{N} \times 100$$

were N represents the total number of subjects tested.

Various tests for the diagnosis of DM exist. Glycosuria depends on the renal threshold for glucose so much that it is less sensitive in diagnosing diabetes. Since DM can be present despite a normal fasting blood sugar, it is also less sensitive. Further even when it is elevated, National Diabetes Data Group (NDDG)a requires it to be elevated on more than one occasion to be diagnostic. Unequivocal elevation of random or postprandial blood sugars are also required to be demonstrated on more than one occasion. When all these tests are inconclusive and yet the diagnosis of DM is in doubt, an OGTT is recommended. All these blood sugar tests have the inconvenience of having to collect the blood sample at a specified time or preparing the patient. Glycosylated Haemoglobin determination which reflects integrated glycaemic status of about 2-3 months is a useful screening test for the diagnosis of DM by the very nature of its evolution, viz. duration and degree of hyperglycaemia. In addition, it has the advantage that blood sample can be collected at any time without any specified time interval in relation to meal.

Saturated fatty acids : $\underset{\omega}{CH_3} - (CH_2)_n - \overset{3}{\underset{\beta}{C}H_2} - \overset{2}{\underset{\alpha}{C}H_2} - \overset{1}{C}OO^-$

Examples:	Butyrate	C_4
	Palmitate	C_{16}
	Stearate	C_{18}

Monounsaturated fatty acids: $CH_3-CH_2)_n - CH = CH - (CH_2)_n - COO-$

| Examples: | Palmitoleate | $C_{16: 1; 9}$ (or Δ^9) |
| | Oleate | $C_{18:1; 9}$ (or Δ^9) |

Polyunsaturated fatty acids :

Examples:	Linoleate	$C_{18:2; 9, 12}$
	a-Linolenate	$C_{18:3; 9, 12, 15}$
	Arachidonate	$C_{20: 4;5, 8, 11, 14}$

Triacylglycerols (Triglycerides):
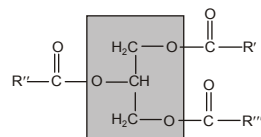
Fig 5.12: Showing the fatty acid and triglycerols structures. Two different nomenclatures are shown for fatty acids. In one, the carbonyl carbon is carbon–1 and the rest of the carbons follow from that, which the terminal methyl group carbon having the number corresponding to the number of carbons in the fatty acid. The other system uses Greek alphabets starting from the carbon following the carbonyl carbon. In that scheme, the terminal methyl group is referred as the omega (w) carbon. In the unsaturated fatty acids the number that follows colon (:) refers to the location of the double bonds. In most unsaturated fatty acids found in the body the bonds are *cis*

SECTION 5.11—METABOLISM OF LIPIDS

The major dilatory lipids are triglycerols (triglycerides as commonly called), which are esters of an alcohols the glycerol, and fatty acids. See fig 150 naturally occurring fats usually have different long chain fatty acids in all three ester positions. The fatty acid can be saturated e.g. palminate or stearate or unsaturated e.g. oleate other fatty acids are poly unsaturated. Fats are hydrophobic molecules.

SUB-SECTION 5.11A—DIGESTION AND ABSORPTION OF FATS

Dietary triglycerides are absorbed after undergoing partial hydrolysis with bile salts playing a key role. The main steps are listed:

1. Salivary and Lingual lipases preferentially hydrolyze triglycerols composed of short and medium chain fatty acids (as found in cow milk)

2. In the small intestine, bile salts emulsify fats

3. Pancreatic lipase and co – lipase are secreted from the pancreas. Co - Lipase and provides an anchor for the pancreatic lipase at the triglycerols – bile salt water interface. In the small intestine pancreatic lipase catalyses the hydrolysis of triglycerols, to monoacylglycerol by removing fatty acids from 1 and 3 positions.

4. Bile salt micelles are formed that contains triacylglecerols, mono – glycerides, fatty acids and fat soluble vitamins and these allows for the mucosal cells.

5. Fatty acids of less than 10 to 12 carbons go directly to the liver via the portal vein

6. Triacylglecerols are reformed intestinal mucosal cells form long chain fatty acids and mono – glycerides and are then incorporated into chlomicrons (see page 283 lipoproteins) which are lipoproteins involved in the delivery of fatty acids.

7. Cholesterol esters in the diet are hydrolyzed by the action of cholesteryl ester hydrolase. Unesterified cholesterol and cholesteryl esters are included in the fat micelles.

8. Phospholipids in the diet are hydrolyzed by the action of pancreatic phospholipase A, which removes the fatty acid at the carbon 2 – position leaving a lysophopsholipid, a powerful detergent. The fatty acids released and the lysophopsholipids are incorporated into micelles and transported into mucosal cells and appear chylomicrions.

SUB-SECTION 5.11B—MICHELLE FORMATION

A micelle is formed when a variety of molecules including soaps and detergents are added to water. The molecule may be a fatty acid, a salt of a fatty acid (soap), phospholipids, or other similar molecules. The molecule must have a strongly polar "head" and a non-polar hydrocarbon chain "tail". When this type of molecule is added to water, the non-polar tails of the molecules clump into the center of a ball like structure, called a micelle, because they are hydrophobic or "water hating". The polar head of the molecule presents itself for interaction with the water molecules on the outside of the micelle. The theoretical model shows 54 molecules of dodecylphosphocholine (DPC) and about 1200 H_2O molecules. Each lipid has a polar head group (phosphocholine) and a hydrophobic tail (dodecyl = C_{12}).

The fig 151 represents a cross section of a micelle. The gray spheres on the interior represent the long hydrocarbon chains of the dodecyl groups which are massed together because they are non-polar. The polar head groups of the phosphate are shown as red and orange spheres. The amine nitrogen is shown in blue surrounded by the gray methyl groups. The water molecules are represented as red and white spheres surrounding the outside of the micelle and penetrates all of the spaces in the head group region.

The hydrophobic tails are shown Spacefill. H2O is excluded from this entire interior volume. The hydrocarbon chains vary in their individual conformations (e.g. trans/gauche configuration at each carbon-carbon bond), but adapt so as to fill all of the interior space. A typical micelle in aqueous solution forms an aggregate with the hydrophilic "head" regions in contact with surrounding solvent, sequestering the hydrophobic single tail regions in the micelle centre. This phase is caused by the insufficient packing issues of single tailed lipids in a bilayer. The difficulty filling all the volume of the interior of a bilayer, while accommodating the area per head group forced on the molecule by the hydration of the lipid head group leads to the formation of the micelle. This type of micelle is known as a normal phase micelle (oil-in-water micelle). Inverse micelles have the headgroups at the centre with the tails extending out (water-in-oil micelle). Micelles are approximately spherical in shape. Other phases, including shapes such as ellipsoids, cylinders, and bilayers are also possible. The shape and size of a micelle is a function of the molecular geometry of its surfactant molecules and solution conditions such as surfactant concentration, temperature, pH, and ionic strength. The process of forming micellae is known as micellisation and forms part of the phase behaviour of many lipids according to their polymorphism. The ability of a soapy solution to act as a detergent has been recognised for centuries. However, it was only at the beginning of the twentieth century that the constitution of such solutions was scientifically studied. Pioneering work in this area was carried out by James William McBain at the University of Bristol. As early as 1913 he postulated the existence of "colloidal ions" to explain the good electrolytic conductivity of sodium palmitate solutions.* These highly mobile, spontaneously formed clusters came to be called micelles, a term borrowed from biology and popularized by G.S. Hartley in his classic book "Paraffin Chain Salts, A Study in Micelle Formation".* Individual surfactant molecules that are in the system but are not part of a micelle are called "monomers." Lipid micelles represent a molecular assembly in which the individual components are thermodynamically in equilibrium with monomers of the same species in the surrounding medium. In water, the hydrophilic "heads" of surfactant molecules are always in contact with the solvent, regardless of whether the surfactants exist as monomers or as part of a micelle. However, the lipophilic "tails" of surfactant molecules have less contact with water when they are part of a micelle – this being the basis for the energetic drive for micelle formation. In a micelle, the hydrophobic tails of several surfactant molecules assemble into an oil-like core the most stable form of which has no contact with water. By contrast, surfactant monomers are surrounded by water molecules that create a "cage" of molecules connected by hydrogen bonds. This water cage is similar to a clathrate and has an ice-like crystal structure and can be characterized according to the hydrophobic effect. The extent of lipid solubility is determined by the unfavorable entropy contribution due to the ordering of the water structure according to the hydrophobic effect. Micelles composed of ionic surfactants have an electrostatic attraction to the ions that surround them in solution, the latter known as counterions. Although the closest counterions partially mask a charged micelle (by up to 90%), the effects of micelle charge affect the structure of the surrounding solvent at appreciable distances from the micelle. Ionic micelles influence many properties of the mixture, including its electrical conductivity. Adding salts to a colloid containing micelles can decrease the strength of electrostatic interactions and lead to the formation of larger ionic micelles. This is more accurately seen from the point of view of an effective change in hydration of the system. Micelles only form when the concentration of surfactant is greater than the critical micelle concentration (CMC), and the temperature of the system is greater than the critical micelle temperature, or Krafft

temperature. The formation of micelles can be understood using thermodynamics: micelles can form spontaneously because of a balance between entropy and enthalpy. In water, the hydrophobic effect is the driving force for micelle formation, despite the fact that assembling surfactant molecules together reduces their entropy. At very low concentrations of the lipid, only monomers are present in true solution. As the concentration of the lipid is increased, a point is reached at which the unfavorable entropy considerations, derived from the hydrophobic end of the molecule, become dominant. At this point, the lipid hydrocarbon chains of a portion of the lipids must be sequestered away from the water. Therefore, the lipid starts to form micelles. Broadly speaking, above the CMC, the entropic penalty of
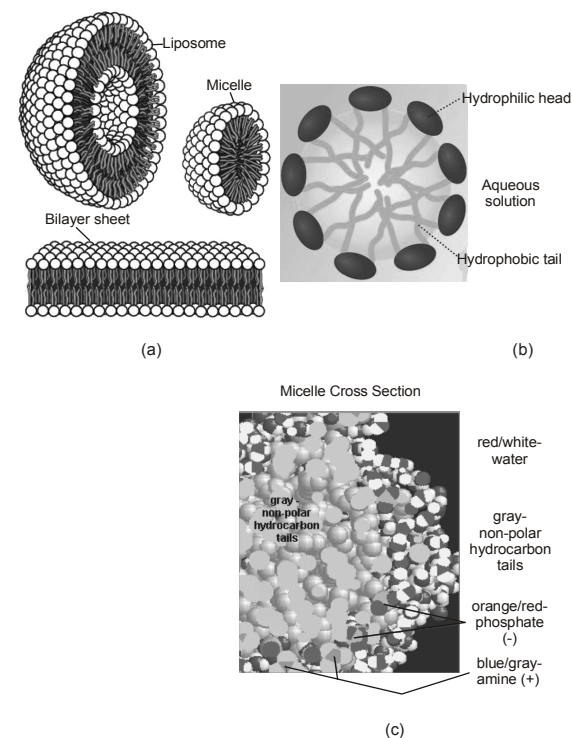
Fig. 5.12 : Scheme of an inverse micelle formed by Phospholipids in an Organic Solvent

assembling the surfactant molecules is less than the entropic penalty of caging the surfactant monomers with water molecules. Also important are enthalpic considerations, such as the electrostatic interactions that occur between the charged parts surfactants.

In a non-polar solvent, it is the exposure of the hydrophilic head groups to the surrounding solvent that is energetically unfavorable, giving rise to a water-in-oil system. In this case the hydrophilic groups are sequestered in the micelle core and the hydrophobic groups extend away from the centre. These inverse micelles are proportionally less likely to form on increasing headgroup charge, since hydrophilic sequestration would create highly unfavorable electrostatic interactions. When surfactants are present above the CMC (Critical micelle concentration), they can act as emulsifiers that will allow a compound that is normally insoluble (in the solvent being used) to dissolve. This occurs because the insoluble species can be incorporated into the micelle core, which is itself solubilized in the bulk solvent by virtue of the head groups' favorable interactions with solvent species. The most common example of this phenomenon is detergents, which clean poorly soluble lipophilic material (such as oils and waxes) that cannot be removed by water alone. Detergents also clean by lowering the surface tension of water, making it easier to remove material from a surface. The emulsifying property of surfactants is also the basis for emulsion polymerization. Micelle formation is essential for the absorption of fat-soluble vitamins and complicated lipids within the human body. Bile salts formed in the liver and secreted by the gall bladder allow micelles of fatty acids to form. This allows the absorption of complicated lipids (e.g., lecithin) and lipid soluble vitamins (A, D, E and K) within the micelle by the small intestine.

SUB-SECTION 5.11C—GASTRIC MUCOSA AND LIPID METABOLISM

The gastric mucosa is the mucous membrane layer of the stomach which contains the glands and the gastric pits. In men it is about 1 mm thick and its surface is smooth, soft, and velvety. It consists of epithelium, lamina propria, and the muscularis mucosae.

In its fresh state, it is of a pinkish tinge at the pyloric end and of a red or reddish-brown color over the rest of its surface. In infancy it is of a brighter hue, the vascular redness being more marked.

It is thin at the cardiac extremity, but thicker toward the pylorus. During the contracted state of the organ it is thrown into numerous plaits or rugae, which, for the most part, have a longitudinal direction, and are most marked toward the pyloric end of the stomach, and along the greater curvature. These folds are entirely obliterated when the organ becomes distended.

When examined with a lens, the inner surface of the mucous membrane presents a peculiar honeycomb appearance from being covered with funnel-like depressions or foveolae of a polygonal or hexagonal form, which vary from 0.12 to 0.25 mm. in diameter. These are the ducts of the gastric glands, and at the bottom of each may be seen one or more minute orifices, the openings of the gland tubes. Gastric glands are simple or branched tubular glands that emerge on the deeper part of the gastric foveola, inside the gastric areas and outlined by the folds of the mucosa.

There are three types of glands: cardiac glands (in the proximal part of the stomach), oxyntic glands (the dominating type of gland), and pyloric glands. The cardiac glands mainly contain mucus producing cells. The bottom part of the oxyntic glands is dominated by zymogen (chief) cells that

produce pepsinogen (an inactive precursor of the pepsin enzyme). Parietal cells, which secrete hydrochloric acid are scattered in the glands, with most of them in the middle part. The upper part of the glands consist of mucous neck cells; in this part the dividing cells are seen. The pyloric glands contain mucus-secreting cells. Several types of endocrine cells are found in all regions of the gastric mucosa. In the pyloric glands contain gastrin producing cells (G cells); this hormone stimulates acid production from the parietal cells. ECL (enterochromaffine-like) cells, found in the oxyntic glands release histamine, which also is a powerful stimulant of the acid secretion. The A cells produce glucagon, which mobilizes the hepatic glycogen, and the enterochromaffin cells that produce serotonin, which stimulates the contraction of the smooth muscles.

The surface of the mucous membrane is covered by a single layer of columnar epithelium . This epithelium commences very abruptly at the cardiac orifice, where there is a sudden transition from the stratified epithelium of the esophagus. The epithelial lining of the gland ducts is of the same character and is continuous with the general epithelial lining of the stomach.
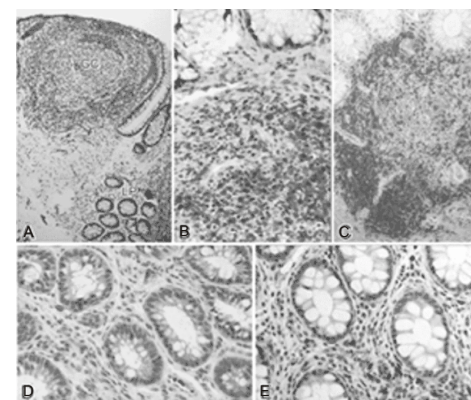
Fig. 5.15 : Showing the Mucosal Cells viewed under the phase contrast microscope in the Author's Lab with a magnification of ´1200 under H & E stain

SUB-SECTION 5.11D—ROLE OF BILE AND PANCREATIC SECRETION

Bile is a complex fluid containing water, electrolytes and a battery of organic molecules including bile acids, cholesterol, phospholipids and bilirubin that flows through the biliary tract into the small intestine. There are two fundamentally important functions of bile in all species:

- Bile contains bile acids, which are critical for digestion and absorption of fats and fat-soluble vitamins in the small intestine.

- Many waste products, including bilirubin, are eliminated from the body by secretion into bile and elimination in feces.

Adult humans produce 400 to 800 ml of bile daily, and other animals proportionately similar amounts. The secretion of bile can be considered to occur in two stages:

- Initially, hepatocytes secrete bile into canaliculi, from which it flows into bile ducts. This hepatic bile contains large quantities of bile acids, cholesterol and other organic molecules.
- As bile flows through the bile ducts it is modified by addition of a watery, bicarbonate-rich secretion from ductal epithelial cells.

In species with a gallbladder (man and most domestic animals except horses and rats), further modification of bile occurs in that organ. The gall bladder stores and concentrates bile during the fasting state. Typically, bile is concentrated five-fold in the gall bladder by absorption of water and small electrolytes - virtually all of the the organic molecules are retained.

Secretion into bile is a major route for eliminating cholesterol. Free cholesterol is virtually insoluble in aqueous solutions, but in bile, it is made soluble by bile acids and lipids like lethicin. Gallstones, most of which are composed predominantly of cholesterol, result from processes that allow cholesterol to precipitate from solution in bile.

Role of Bile Acids in Fat Digestion and Absorption

Bile acids are derivatives of cholesterol synthesized in the hepatocyte. Cholesterol, ingested as part of the diet or derived from hepatic synthesis is converted into the bile acids cholic and chenodeoxycholic acids, which are then conjugated to an amino acid (glycine or taurine) to yield the conjugated form that is actively secreted into cannaliculi.

Bile acids are facial amphipathic, that is, they contain both hydrophobic (lipid soluble) and polar (hydrophilic) faces. The cholesterol-derived portion of a bile acid has one face that is hydrophobic (that with methyl groups) and one that is hydrophilic (that with the hydroxyl groups); the amino acid conjugate is polar and hydrophilic.

Their amphipathic nature enables bile acids to carry out two important functions:

Glycocholic acid (amphipathic)
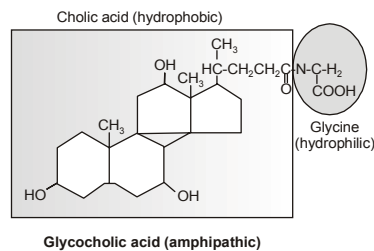
Fig. 5.16 : Showing the Structure of Cholic acid

- **Emulsification of lipid aggregates:** Bile acids have detergent action on particles of dietary fat which causes fat globules to break down or be emulsified into minute, microscopic droplets. Emulsification is not digestion per se, but is of importance because it greatly increases the surface area of fat, making it available for digestion by lipases, which cannot access the inside of lipid droplets.
- **Solubilization and transport of lipids in an aqueous environment:** Bile acids are lipid carriers and are able to solubilize many lipids by forming micelles - aggregates of lipids such as fatty acids, cholesterol and monoglycerides - that remain suspended in water. Bile acids are also critical for transport and absorption of the fat-soluble vitamins.

Role of Bile Acids in Cholesterol Homeostasis

Hepatic synthesis of bile acids accounts for the majority of cholesterol breakdown in the body. In humans, roughly 500 mg of cholesterol are converted to bile acids and eliminated in bile every day. This route for elimination of excess cholesterol is probably important in all animals, but particularly in situations of massive cholesterol ingestion.

Interestingly, it has recently been demonstrated that bile acids participate in cholesterol metabolism by functioning as hormones that alter the transcription of the rate-limiting enzyme in cholesterol biosynthesis.

Enterohepatic Recirculation

Large amounts of bile acids are secreted into the intestine every day, but only relatively small quantities are lost from the body. This is because approximately 95% of the bile acids delivered to the duodenum are absorbed back into blood within the ileum.

Venous blood from the ileum goes straight into the portal vein, and hence through the sinusoids of the liver. Hepatocytes extract bile acids very efficiently from sinusoidal blood, and little escapes the healthy liver into systemic circulation. Bile acids are then transported across the hepatocytes to be resecreted into canaliculi. The net effect of this enterohepatic recirculation is that each bile salt molecule is reused about 20 times, often two or three times during a single digestive phase.

It should be noted that liver disease can dramatically alter this pattern of recirculation - for instance, sick hepatocytes have decreased ability to extract bile acids from portal blood and damage to the canalicular system can result in escape of bile acids into the systemic circulation. Assay of systemic levels of bile acids is used clinically as a sensitive indicator of hepatic disease.

Pattern and Control of Bile Secretion

The flow of bile is lowest during fasting, and a majority of that is diverted into the gallbladder for concentration. When chyme from an ingested meal enters the small intestine, acid and partially digested fats and proteins stimulate secretion of cholecystokinin and secretin. As discussed previously, these enteric hormones have important effects on pancreatic exocrine secretion. They are both also important for secretion and flow of bile:

• **Cholecystokinin**: The name of this hormone describes its effect on the biliary system - cholecysto = gallbladder and kinin = movement. The most potent stimulus for release of cholecystokinin is the presence of fat in the duodenum. Once released, it stimulates contractions of the gallbladder and common bile duct, resulting in delivery of bile into the gut.

• **Secretin**: This hormone is secreted in response to acid in the duodenum. Its effect on the biliary system is very similar to what was seen in the pancreas - it simulates biliary duct cells to secrete bicarbonate and water, which expands the volume of bile and increases its flow out into the intestine.

The processes of gallbladder filling and emptying described here can be visualized using an imaging technique called scintography. This procedure is utilized as a diagnostic aid in certain types of hepatobiliary disease.

Pancreatic juice is composed of two secretory products critical to proper digestion: digestive enzymes and bicarbonate. The enzymes are synthesized and secreted from the exocrine acinar cells, whereas bicarbonate is secreted from the epithelial cells lining small pancreatic ducts.

The pancreas secretes a magnificent battery of enzymes that collectively have the capacity to reduce virtually all digestible macromolecules into forms that are capable of, or nearly capable of being absorbed. Three major groups of enzymes are critical to efficient digestion:

1. Proteases

Digestion of proteins is initiated by pepsin in the stomach, but the bulk of protein digestion is due to the pancreatic proteases. Several proteases are synthesized in the pancreas and secreted into the lumen of the small intestine. The two major pancreatic proteases are trypsin and chymotrypsin, which are synthesized and packaged into secretory vesicles as an the inactive proenzymes trypsinogen and chymotrypsinogen. As you might anticipate, proteases are rather dangerous enzymes to have in cells, and packaging of an inactive precursor is a way for the cells to safely handle these enzymes. The
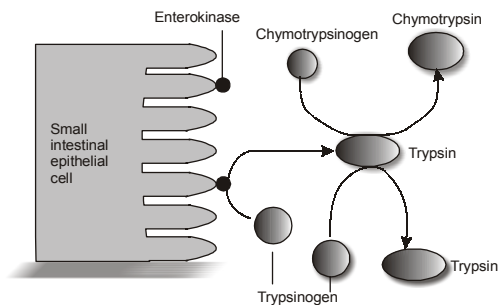
Fig. 5.17 : Showing the action of Pancreas during Digestion

secretory vesicles also contain a trypsin inhibitor which serves as an additional safeguard should some of the trypsinogen be activated to trypsin; following exocytosis this inhibitor is diluted out and becomes ineffective - the pin is out of the grenade. Once trypsinogen and chymotrypsinogen are released into the lumen of the small intestine, they must be converted into their active forms in order to digest proteins. Trypsinogen is activated by the enzyme enterokinase, which is embedded in the intestinal mucosa.

Once trypsin is formed it activates chymotrypsinogen, as well as additional molecules of trypsinogen. The net result is a rather explosive appearance of active protease once the pancreatic secretions reach the small intestine.

Trypsin and chymotrypsin digest proteins into peptides and peptides into smaller peptides, but they cannot digest proteins and peptides to single amino acids. Some of the other proteases from the pancreas, for instance carboxypeptidase, have that ability, but the final digestion of peptides into amino acids is largely the effect of peptidases on the surface of small intestinal epithelial cells. More on this later.

2. Pancreatic Lipase

A major component of dietary fat is triglyceride, or neutral lipid. A triglyceride molecule cannot be directly absorbed across the intestinal mucosa. Rather, it must first be digested into a 2-monoglyceride and two free fatty acids. The enzyme that performs this hydrolysis is pancreatic lipase, which is delivered into the lumen of the gut as a constituent of pancreatic juice.

Sufficient quantities of bile salts must also be present in the lumen of the intestine in order for lipase to efficiently digest dietary triglyceride and for the resulting fatty acids and monoglyceride to be absorbed. This means that normal digestion and absorption of dietary fat is critically dependent on secretions from both the pancreas and liver.
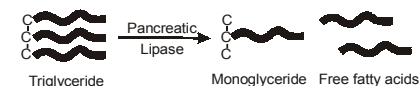
Pancreatic lipase has recently been in the limelight as a target for management of obesity. The drug orlistat (Xenical) is a pancreatic lipase inhibitor that interferes with digestion of triglyceride and thereby reduces absorption of dietary fat. Clinical trials support the contention that inhibiting lipase can lead to significant reductions in body weight in some patients.

3. Amylase

The major dietary carbohydrate for many species is starch, a storage form of glucose in plants. Amylase (technically alpha-amylase) is the enzyme that hydrolyses starch to maltose (a glucose-glucose disaccharide), as well as the trisaccharide maltotriose and small branchpoints fragments called limit dextrins. The major source of amylase in all species is pancreatic secretions, although amylase is also present in saliva of some animals, including humans.

Other Pancreatic Enzymes

In addition to the proteases, lipase and amylase, the pancreas produces a host of other digestive enzymes, including ribonuclease, deoxyribonuclease, gelatinase and elastase.

Bicarbonate and Water

Epithelial cells in pancreatic ducts are the source of the bicarbonate and water secreted by the pancreas. Bicarbonate is a base and critical to neutralizing the acid coming into the small intestine from the stomach. The mechanism underlying bicarbonate secretion is essentially the same as for acid secretion parietal cells and is dependent on the enzyme carbonic anhydrase. In pancreatic duct cells, the bicarbonate is secreted into the lumen of the duct and hence into pancreatic juice.

SECTION 5.12—TRANSPORT OF LIPIDS
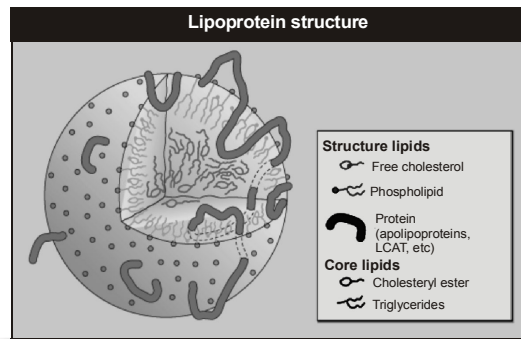
Fig. 5.18 : Showing the Structure of lipoprotein

Once lipids are disassembled in the intestinal lumen and mucosal cell (enterocyte) they are reassembled in the mucosal cell as chylomicrons (CM's) and very low density lipoproteins (VLDL's). These vehicles contain primarily nonpolar cholesterol esters and triglycerides in the core and polar cholesterol, protein, and phospholipids in their membranes. They are transported via the lymph and blood circulation to the liver, fat depots, and muscles. There the endothelial enzyme lipoprotein lipase removes the lipid contents.

Lipid carrying vehicles are also made by the liver primarily as very low density lipoproteins (VLDL) and these function to move lipids made by the body itself into tissues. On the other hand, high density lipoproteins (HDL), which are made in the intestines and liver, function primarily to reverse this process and transport lipids from tissue to liver hepatocytes.1 HDL's are of two types HDL3 and HDL2. HDL3

is an empty package composed of a bilayer lipid membrane plus proteins. Lysolecithin cholesterol acyl transferase (LCAT) and apoprotein A associated with HDL3 remove free cholesterol from the blood, esterify it and fill the HDL3 package.The LCAT enzyme uses the fatty acid in the number two position of lecithin to esterify to cholesterol. If this fatty acid is saturated, the process is inhibited if it is unsaturated, the process is enhanced. Thus, cholesterol blood clearing by HDL3 is linked to dietary intake of saturated and unsaturated fatty acids. High saturated triglycerides are often clinically associated with high blood cholesterol levels.As HDL3 swells with cholesterol ester, it becomes HDL2, which in the liver releases its cholesterol through the action of hepatic lipase. Released cholesterol is conjugated with the amino acids glycine (predominantly in most species) and taurine (predominantly in cats) to form bile salts which are then excreted in the bile into the small intestine.2,3 Some cholesterol is then reabsorbed via the enterohepatic circulation and some passes with the feces. The less reabsorbed, the lower the blood levels of cholesterol. A variety of complex factors influences the reuptake of bile cholesterol. For example, some of the beneficial effects of fiber and certain bowel microorganisms can be related to decreasing cholesterol uptake.4,5Characterization of lipid transport vehicles is based on physical density, size and ratios of constituents. Chylomicrons are the largest particles, the very low density lipoprotein (VLDL) is the next largest, the intermediate density lipoprotein (IDL) is the next largest, the low density lipoprotein (IDL) is the next largest, and then high density lipoproteins (HDL) are the smallest. In terms of their constituents, as the particle becomes smaller as it is hydrolyzed by
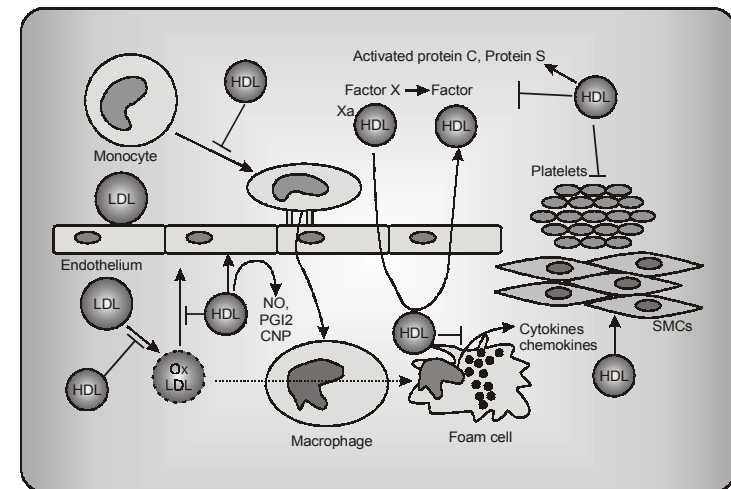
Fig. 5.20 : Showing the Action of Lipoprotein

lipoprotein lipase on capillary endothelial cells, its protein and cholesterol content becomes greater, triglyceride content becomes smaller and its density increases.6 Thus chylomicrons are laden with lipid but lean of protein, whereas high density lipoproteins contain smaller amounts of lipid and larger measures of protein.

Diagnostically the measure of these lipid carriers in the blood is important as indicators of risk particularly to cardiovascular disease. If there are high levels of LDL's, this would be unfavorable whereas high levels of HDL's would be favorable. High levels of LDL's mean that there is a large amount of circulating cholesterol which may have atherogenic potential. On the other hand, a high level of HDL's would mean that lipid stores are being mobilized from tissue and metabolized in the liver to be excreted in the bile.

SUB-SECTION 5.12A—CLASSIFICATION OF LIPOPROTEINS

There are five major lipoproteins, each of which has a different function (as will be described below).

Chylomicrons — Chylomicrons are very large particles that carry dietary lipid. They are associated with a variety of apolipoproteins, including A-I, A-II, A-IV, B-48, C-I, C-II, C-III, and E.
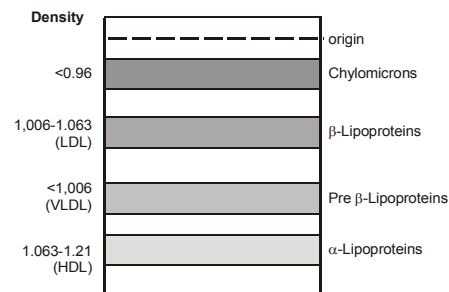
Fig. 5.21 : Showing the Plasma Lipoproteins

Very low density lipoprotein — Very low density lipoprotein (VLDL) carries endogenous triglycerides and to a lesser degree cholesterol. The major apolipoproteins associated with VLDL are B-100, C-I, C-II, C-III,andE.

Intermediate density lipoprotein — Intermediate density lipoprotein (IDL) carries cholesterol esters and triglycerides.It is associated with apolipoproteins B-100, C-III, and E.

Low density lipoprotein — Low density lipoprotein (LDL) carries cholesterol esters and is associated with apolipoprotein B-100.

High density lipoprotein — High density lipoprotein (HDL) also carries cholesterol esters. It is associated with apolipoproteins A-I, A-II, C-I, C-II, C-III, D, and E.

Apolipoproteins — The major function of the different apolipoproteins can be summarized as follows [1]. Understanding these functions is important clinically, because defects in apolipoprotein metabolism lead to abnormalities in lipid handling..The assembly and secretion of apolipoprotein B containing lipoproteins in the liver and intestines is dependent upon microsomal triglyceride transfer protein which transfers lipids to apolipoprotein B. In one study, apolipoprotein B and microsomal transfer protein genes were expressed in the human heart, strongly suggesting that the heart synthesizes and secretes apolipoprotein B containing lipoproteins. This may represent a pathway of "reverse triglyceride transport" by which the cardiac myocytes can unload surplus fatty acids not required for fuel.

- A-I — Structural protein for HDL; activator of lecithin-cholesterol acyltransferase (LCAT).
- A-II — Structural protein for HDL; activator of hepatic lipase.
- A-IV — Activator of lipoprotein lipase and LCAT.
- B-100 — Structural protein for VLDL, IDL, LDL, and Lp(a); ligand for the LDL receptor; required for assembly and secretion of VLDL.
- B-48 — Contains 48 percent of B-100; required for assembly and secretion of chylomicrons; does not bind to LDL receptor.
- C-I — Activator of LCAT.
- C-II — Essential cofactor for LPL.
- C-III — Interferes with apo-E mediated clearance of triglyceride-enriched lipoproteins by cellular receptors [3]; inhibits triglyceride hydrolysis by lipoprotein lipase and hepatic lipase [4].
- D — May be a cofactor for cholesteryl ester transfer protein.
- E — Ligand for hepatic chylomicron and VLDL remnant receptor, leading to clearance of these lipoproteins from the circulation; ligand for LDL receptor. There are three different apo E alleles in humans: E2, which has cysteine residues at positions 112 and 158; E3, which occurs in 60 to 80 percent of Caucasians and has cysteine at position 112 and arginine at position 158; and E4, which has arginine residues at positions 112 and 158 . These alleles encode for a combination of apo E isoforms that are inherited in a codominant fashion. Compared to apo E3, apo E2 has reduced affinity and apo E4 has enhanced affinity for the LDL (apo B/E) receptor. These isoforms are important clinically because apo E2 is associated with familial dysbetalipoproteinemia (due to less efficient clearance of VLDL and chylomicrons) and apo E4 is associated with an increased risk of hypercholesterolemia and coronary heart disease. (See "Primary disorders of LDL-cholesterol metabolism", section on Polygenic hypercholesterolemia, and see "Approach to the patient with hypertriglyceridemia", section on Familial dysbetalipoproteinemia).
- Apo(a) — Structural protein for Lp(a); inhibitor of plasminogen activation on Lp(a).

SUB-SECTION 5.12B—CHEMICAL STRUCTURE OF LIPOPROTEIN

The general structure of a lipoprotein includes, as depicted at right: a core consisting of a droplet of triacylglycerols and/or cholesteryl esters a surface monolayer of phospholipid, unesterified cholesterol and specific proteins (apolipoproteins, e.g., apoprotein B-100 in low density lipoprotein).

Lipoproteins differ in the ratio of protein to lipids, and in the particular apoproteins and lipids that they contain, as summarized in Table 12-6 p. 439. They are classified based on their density:

- chylomicron (largest; lowest in density due to high lipid/protein ratio; highest in triacylglycerols as % of weight)
- VLDL (very low density lipoprotein; 2nd highest in triacylglycerols as % of weight)
- IDL (intermediate density lipoprotein)
- LDL (low density lipoprotein, highest in cholesteryl esters as % of weight)
- HDL (high density lipoprotein, highest in density due to high protein/lipid ratio).

Apolipoprotein Structure: Amphipathic a-helices (polar along one surface of a helix and hydrophobic along the other side) are common structural motifs. One view is that these a-helices may float on the phospholipid surface of the lipoprotein. Some domains of apolipoproteins have roles in interaction of lipoproteins with cell surface receptors.

Apolipoprotein A-I (apoA-I) of human HDL, in the absence of lipid, is found consist of an N-terminal antiparallel 4-helix bundle and a C-terminal domain that is also a-helical, as depicted at right.

A truncated apoA-I, engineered to lack the first 43 amino acids, was earlier found to have a more open structure, with a horseshoe shape, shown below right. Lack of the first a-helix at the N-terminus may prevent stabilization of the 4-helix bundle. On interacting with lipid, the compact structure of the intact apolipoprotein A-I is assumed to open up into a structure resembling the horseshoe shape observed for the truncated protein.

In the open configuration, proline residues are observed to interrupt a-helical segments, providing curvature that would be appropriate for wrapping around a spherical or elliptical lipid micelle.

A strip of hydrophobic residues runs along one edge of the amphipathic a-helix. In the crystal, antiparallel dimers were found to be formed by association of these hydrophobic residues. At right is a view of such a dimer in cartoon display. At the far right the same view of the apoA-I dimer is displayed as spacefill, with hydrophobic residues colored magenta and polar residues cyan.

Apolipoprotein E (apoE), a constituent of several classes of lipoprotein also has an N-terminal domain that folds as a 4-helix bundle in the absence of lipid. Based in part on a low resolution structure determined in the presence of phospholipids, it has been proposed that interaction with lipids converts apoE to an a-helical hairpin that wraps around the lipid particles.
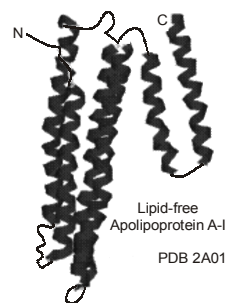
Fig. 5.21 : Showing the Structure of Apo lipoprotein
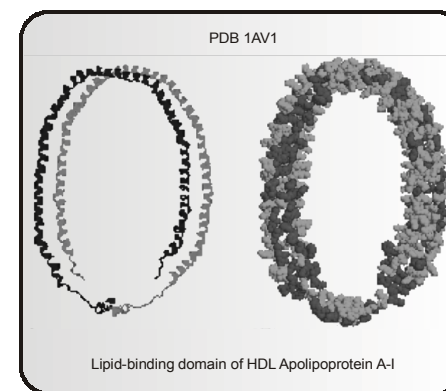
Fig. 5.22: Apolipoprotein A-I lacking residues 1-43, structure

There is special interest in the structure and stability of apolipoprotein E. In addition to being a constituent of various lipoproteins, e.g. VLDL and HDL, a variant of apolipoprotein E, designated apoE4, is implicated in Alzheimer's disease and other neurological conditions.

SECTION 5.13—METABOLISMS OF CHYLOMICRONS

Chylomicrons are large lipoprotein particles that transport dietary lipids from the intestines to other locations in the body. Chylomicrons are one of the 5 major groups of lipoproteins (chylomicrons, VLDL, IDL, LDL, HDL) which enable fats and cholesterol to move within the water based solution of the blood streamChylomicrons transport exogenous lipids to liver, adipose, cardiac and skeletal muscle tissue where their triglyceride components are unloaded by the activity of lipoprotein lipase. Consequently chylomicron remnants are left over which are taken up by the liver.

SUB-SECTION 5.13A—VLDL, LDL, HDL

Very low-density lipoprotein (VLDL) is a type of lipoprotein made by the liver. VLDL is one of the five major groups of lipoproteins (chylomicrons, VLDL, IDL, LDL, HDL) which enable fats and cholesterol to move within the water based solution of the blood stream. It is assembled in the liver from cholesterol and apolipoproteins. It is converted in the bloodstream to low-density lipoprotein (LDL). VLDL particles have a diameter of 30-80 nm. VLDL transports endogenous products where chylomicrons transport exogenous (dietary) products.VLDL transports endogenous triglycerides,

phospholipids, cholesterol and cholesteryl esters. It functions as the body's internal transport mechanism for lipids.

Nascent VLDL circulates in blood and picks up apolipoprotein C-II and apolipoprotein E donated from High-Density Lipoprotein (HDL). At this point, the nascent VLDL becomes a mature VLDL. Once in circulation, the VLDL will come in contact with lipoprotein lipase (LPL) in the capillary beds in the body (adipose, cardiac, and skeletal muscle). The LPL will remove triglycerides from the VLDL for storage or energy production.

The VLDL now meets back up with HDL where apoC-II is transferred back to the HDL (but keeps apoE). In addition to this, the HDL transfers cholesteryl esters to the VLDL in exchange for phospholipids and triglycerides (via cholesteryl ester transfer protein).

As more and more triglycerides are removed from the VLDL because of the action of the LPL enzyme, the composition of the molecule changes, and it becomes intermediate density lipoprotein (IDL).

50% of IDL are recognized by receptors in the liver cells (because of the apoB-100 and apoE they contain) and are endocytosed.

The other 50% of IDL lose their apoE. When their cholesterol content becomes greater than the triglyceride content, they become low-density lipoprotein (LDL), with the primary apolipoprotein being apoB-100. The LDL is taken into a cell via the LDL receptor (endocytosis) where the contents are either stored, used for cell membrane structure, or converted into other products (steroid hormones or bile acids)

VLDL levels have been correlated with accelerated rates of atherosclerosis, and are elevated in a number of diseases and metabolic states.

Low-density lipoprotein (LDL) is a type of lipoprotein that transports cholesterol and triglycerides from the liver to peripheral tissues. LDL is one of the five major groups of lipoproteins; these groups include chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein, and high-density lipoprotein (HDL). Like all lipoproteins, LDL enables fats and cholesterol to move within the water based solution of the blood stream. LDL also regulates cholesterol synthesis at these sites. It commonly appears in the medical setting as part of a cholesterol blood test, and since high levels of LDL cholesterol can signal medical problems like cardiovascular disease, it is sometimes called "bad cholesterol" (as opposed to high-density lipoprotein (HDL), which is frequently referred to as the "good cholesterol").

Each native LDL particle contains a single apolipoprotein B-100 molecule (Apo B-100, a protein with 4536 amino acid residues) that circulates the fatty acids, keeping them soluble in the aqueous environment. In addition, LDL has a highly-hydrophobic core consisting of polyunsaturated fatty acid known as linoleate and about 1500 esterified cholesterol molecules. This core is surrounded by a shell of phospholipids and unesterified cholesterol as well as a single copy of B-100 large protein (514 kD). LDL particles are approximately 22 nm in diameter and have a mass of about 3 million daltons, but since LDL particles contain a changing number of fatty acids, they actually have a mass and size distribution.

LDL particles vary in size and density, and studies have shown that a pattern that has more small dense LDL particles—called "Pattern B"—equates to a higher risk factor for coronary heart disease (CHD) than does a pattern with more of the larger and less dense LDL particles ("Pattern A"). This is because the smaller particles are more easily able to penetrate the endothelium. "Pattern I," meaning "intermediate," indicates that most LDL particles are very close in size to the normal gaps in the endothelium (26 nm).

The correspondence between Pattern B and CHD has been suggested by some in the medical community to be stronger than the correspondence between the LDL number measured in the standard lipid profile test. Tests to measure these LDL subtype patterns have been more expensive and not widely available, so the common lipid profile test has been used more commonly.

There has also been noted a correspondence between higher triglyceride levels and higher levels of smaller, denser LDL particles and alternately lower triglyceride levels and higher levels of the larger, less dense LDL.

With continued research, decreasing cost, greater availability and wider acceptance of other "lipoprotein subclass analysis" assay methods, including NMR spectroscopy, research studies have continued to show a stronger correlation between human clinically obvious cardiovascular event and quantitatively-measured particle concentrations.

When a cell requires cholesterol, it synthesizes the necessary LDL receptors, and inserts them into the plasma membrane. The LDL receptors diffuse freely until they associate with clathrin-coated pits. LDL particles in the blood stream bind to these extracellular LDL receptors. The clathrin-coated pits then form vesicles that are endocytosed into the cell.

After the clathrin coat is shed, the vesicles deliver the LDL and their receptors to early endosomes, onto late endosomes to lysosomes. Here the cholesterol esters in the LDL are hydrolysed. The LDL receptors are recycled back to the plasma membrane.

Because LDLs transport cholesterol to the arteries and can be retained there by arterial proteoglycans starting the formation of plaques, increased levels are associated with atherosclerosis, and thus heart attack, stroke, and peripheral vascular disease. For this reason, cholesterol inside LDL lipoproteins is often called "bad" cholesterol. This is a misnomer. The cholesterol transported on LDL is the same as cholesterol transported on other lipoprotein particles. The cholesterol itself is not "bad"; rather, it is how and where the cholesterol is being transported, and in what amounts over time, that causes adverse effects.

Increasing evidence has revealed that the concentration and size of the LDL particles more powerfully relates to the degree of atherosclerosis progression than the concentration of cholesterol contained within all the LDL particles. The healthiest pattern, though relatively rare, is to have small numbers of large LDL particles and no small particles. Having small LDL particles, though common, is an unhealthy pattern; high concentrations of small LDL particles (even though potentially carrying the same total cholesterol content as a low concentration of large particles) correlates with much faster growth of atheroma, progression of atherosclerosis and earlier and more severe cardiovascular disease events and death.

LDL is formed as VLDL lipoproteins lose triglyceride through the action of lipoprotein lipase (LPL) and become smaller and denser, containing a higher proportion of cholesterol.

A hereditary form of high LDL is familial hypercholesterolemia (FH). Increased LDL is termed hyperlipoproteinemia type II (after the dated Fredrickson classification).

LDL poses a risk for cardiovascular disease when it invades the endothelium and becomes oxidized, since the oxidized form is more easily retained by the proteoglycans. A complex set of biochemical reactions regulates the oxidation of LDL, chiefly stimulated by presence of free radicals in the endothelium. Nitric oxide down-regulates this oxidation process catalyzed by L-arginine. In a corresponding manner, when there are high levels of asymmetric dimethylarginine in the endothelium, production of nitric oxide is inhibited and more LDL oxidation occurs.

The mevalonate pathway serves as the basis for the biosynthesis of many molecules, including cholesterol. The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) is an essential component in the pathway.

The use of statins (HMG-CoA reductase inhibitors) is effective against high levels of LDL cholesterol. Statins inhibit the enzyme HMG-CoA reductase in the liver, which stimulates LDL receptors, resulting in an increased clearance of LDL.

Clofibrate is effective at lowering cholesterol levels, but has been associated with significantly increased cancer and stroke mortality, despite lowered cholesterol levels.

Torcetrapib was a drug developed to treat high cholesterol levels, but its development was halted when studies showed a 60% increase in deaths when used in conjunction with atorvastatin versus the statin alone.

Niacin (B_3), lowers LDL by selectively inhibiting hepatic diacyglycerol acyltransferase 2, reducing triglyceride synthesis and VLDL secretion through a receptor HM74 and HM74A or GPR109A.

Insulin induces HMG-CoA reductase activity, whereas glucagon downregulates it. While glucagon production is stimulated by dietary protein ingestion, insulin production is stimulated by dietary carbohydrate. The rise of insulin is, in general, determined by the unfolding of carbohydrates into glucose during the process of digestion. Glucagon levels are very low when insulin levels are high.

A ketogenic diet may have similar response to taking niacin (lowered LDL and increased HDL) through beta-hydroxybutyrate, a ketone body, coupling the niacin receptor (HM74A).

Lowering the blood lipid concentration of triglycerides helps lower the amount of LDL, because VLDL gets converted in the bloodstream into LDL.

Fructose, a component of sucrose as well as high-fructose corn syrup, upregulates hepatic VLDL synthesis.

Because LDL appears to be harmless until oxidized by free radicals, it is postulated that ingesting antioxidants and minimizing free radical exposure may reduce LDL's contribution to atherosclerosis, though results are not conclusive.

Chemical measures of lipid concentration have long been the most-used clinical measurement, not because they have the best correlation with individual outcome, but because these lab methods are less

expensive and more widely available. However, there is increasing evidence and recognition of the value of more sophisticated measurements. To be specific, LDL particle number (concentration), and to a lesser extent size, have shown much tighter correlation with atherosclerotic progression and cardiovascular events than is obtained using chemical measures of total LDL concentration contained within the particles. LDL cholesterol concentration can be low, yet LDL particle number high and cardiovascular events rates are high. Also, LDL cholesterol concentration can be relatively high, yet LDL particle number low and cardiovascular events are also low. If LDL particle concentration is tracked against event rates, many other statistical correlates of cardiovascular events, such as diabetes mellitus, obesity, and smoking, lose much of their additional predictive power.

The lipid profile does not measure LDL level directly but instead estimates it via the Friedewald equation using levels of other cholesterol such as HDL:

$$LDL-C \approx Total\ cholesterol - HDL-C - 0.20 * Total\ triglycerides$$

In mg/dl: LDL cholesterol = total cholesterol – HDL cholesterol – (0.20 × triglycerides)

In mmol/l: LDL cholesterol = total cholesterol – HDL cholesterol – (0.45 × triglycerides)

There are limitations to this method, most notably that samples must be obtained after a 12 to 14 h fast and that LDL-C cannot be calculated if plasma triglyceride is >4.52 mmol/L (400 mg/dL). Even at LDL-C levels 2.5 to 4.5 mmol/L, this formula is considered to be inaccurate. If both total cholesterol and triglyceride levels are elevated then a modified formula may be used

In mg/dl: LDL-C = Total-C – HDL-C – (0.16 × Trig)

This formula provides an approximation with fair accuracy for most people, assuming the blood was drawn after fasting for about 14 hours or longer. (However, the concentration of LDL particles, and to a lesser extent their size, has far tighter correlation with clinical outcome than the content of cholesterol with the LDL particles, even if the LDL-C estimation is about correct.)

In the USA, the American Heart Association, NIH, and NCEP provide a set of guidelines for fasting LDL-Cholesterol levels, estimated or measured, and risk for heart disease. As of 2003, these guidelines were:

Level mg/dL	Level mmol/L	Interpretation
<100	<2.6	Optimal LDL cholesterol, corresponding to reduced, but not zero, risk for heart disease
100 to 129	2.6 to 3.3	Near optimal LDL level
130 to 159	3.3 to 4.1	Borderline high LDL level
160 to 189	4.1 to 4.9	High LDL level
>190	>4.9	Very high LDL level, corresponding to highest increased risk of heart disease

These guidelines were based on a goal of presumably decreasing death rates from cardiovascular disease to less than 2% to 3% per year or less than 20% to 30% every 10 years. Note that 100 is not considered optimal; less than 100 is optimal, though it is unspecified how much less.

Over time, with more clinical research, these recommended levels keep being reduced because LDL reduction, including to abnormally low levels, has been the most effective strategy for reducing cardiovascular death rates in large double blind, randomized clinical trials; far more effective than coronary angioplasty/stenting or bypass surgery.

For instance, for people with known atherosclerosis diseases, the 2004 updated American Heart Association, NIH and NCEP recommendations are for LDL levels to be lowered to less than 70 mg/dL, unspecified how much lower. It has been estimated from the results of multiple human pharmacologic LDL lowering trials that LDL should be lowered to about 50 to reduce cardiovascular event rates to near zero. For reference, from longitudinal population studies following progression of atherosclerosis-related behaviors from early childhood into adult hood it has been discovered that the usual LDL in childhood, before the development of fatty streaks, is about 35 mg/dL. However, all the above values refer to chemical measures of lipid/cholesterol concentration within LDL, not LD Lipoprotein concentrations, probably not the better approach.

High-density lipoproteins (HDL) is one of the 5 major groups of lipoproteins (chylomicrons, VLDL, IDL, LDL, HDL) which enable lipids like cholesterol and triglycerides to be transported within the water based blood stream. In healthy individuals, about thirty percent of blood cholesterol is carried by HDL.

It is hypothesized that HDL can remove cholesterol from atheroma within arteries and transport it back to the liver for excretion or re-utilization—which is the main reason why HDL-bound cholesterol is sometimes called "**good cholesterol**", or HDL-C. A high level of HDL-C seems to protect against cardiovascular diseases, and low HDL cholesterol levels (less than 40 mg/dL) increase the risk for heart disease. When measuring cholesterol, any contained in HDL particles is considered as protection to the body's cardiovascular health, in contrast to "bad" LDL cholesterol. HDL are the smallest of the lipoprotein particles. They are the densest because they contain the highest proportion of protein. Their most abundant apolipoproteins are apo A-I and apo A-II. The liver synthesizes these lipoproteins as complexes of apolipoproteins and phospholipid, which resemble cholesterol-free flattened spherical lipoprotein particles. They are capable of picking up cholesterol, carried internally, from cells by interaction with the ATP Binding Cassette Transporter A1 (ABCA1). A plasma enzyme called lecithin-cholesterol acyltransferase (LCAT) converts the free cholesterol into cholesteryl ester (a more hydrophobic form of cholesterol) which is then sequestered into the core of the lipoprotein particle eventually making the newly synthesized HDL spherical. They increase in size as they circulate through the bloodstream and incorporate more cholesterol and phospholipid molecules from cells and other lipoproteins, for example by the interaction with the ABCG1 transporter and the phospholipid transport protein (PLTP).

HDL deliver their cholesterol mostly to the liver or steroidogenic organs such as adrenals, ovary and testes by direct and indirect pathways. The direct HDL removal pathways involve HDL receptors such as scavenger receptor BI (SR-BI) which mediate the selective uptake of cholesterol from HDL. In humans, the probably most relevant pathway is the indirect one, which is mediated by cholesteryl ester transfer protein (CETP). This protein exchanges triglycerides of VLDL against cholesteryl esters of HDL. As the result, VLDL are processed to LDL which are removed from the circulation by the LDL

receptor pathway. The triglycerides are not stable in HDL, but degraded by hepatic lipase so that finally small HDL particles are left which restart the uptake of cholesterol from cells.

The cholesterol delivered to the liver is excreted into the bile and hence intestine either directly or indirectly after conversion into bile acids. Delivery of HDL cholesterol to adrenals, ovaries and testes are important for the synthesis of steroid hormones.

Several steps in the metabolism of HDL can contribute to the transport of cholesterol from lipid laden macrophages of atherosclerotic arteries, termed foam cells to the liver for secretion into the bile. This pathway has been termed reverse cholesterol transport and is considered as the classical protective function of HDL towards atherosclerosis. However, HDL carries many lipid and protein species, many of which have very low concentrations but are biologically very active. For example, HDL and their protein and lipid constituents help to inhibit oxidation, inflammation, activation of the endothelium, coagulation or platelet aggregation. All these properties may contribute to the ability of HDL to protect from atherosclerosis, and it is not yet known what is most important. In the stress response, serum amyloid A, which is one of the acute phase proteins and an apolipoprotein, is under the stimulation of cytokines (IL-1, IL-6) and cortisol produced in the adrenal cortex and carried to the damaged tissue incorporated into HDL particles. At the inflammation site, it attracts and activates leukocytes. In chronic inflammations, its deposition in the tissues manifests itself as amyloidosis. It has been postulated that the concentration of large HDL particles more accurately reflects protective action, as opposed to the concentration of total HDL particles. This ratio of large HDL to total HDL particles varies widely and is only measured by more sophisticated lipoprotein assays using either electrophoresis (the original method developed in the 1970s), or newer NMR spectroscopy methods, developed in the 1990s. Men tend to have noticeably lower HDL levels, with smaller size and lower cholesterol content, than women. Men also have an increased incidence of atherosclerotic heart disease. Epidemiological studies have shown that high concentrations of HDL (over 60 mg/dL) have protective value against cardiovascular diseases such as ischemic stroke and myocardial infarction. Low concentrations of HDL (below 40 mg/dL for men, below 50 mg/dL for women) increase the risk for atherosclerotic diseases.Data from the landmark Framingham Heart Study showed that for a given level of LDL, the risk of heart disease increases 10-fold as the HDL varies from high to low. Conversely, for a fixed level of HDL, the risk increases 3-fold as LDL varies from low to high.

Even people with very low LDL levels are exposed to some increased risk if their HDL levels are not high enough.

The American Heart Association, NIH and NCEP provides a set of guidelines for male fasting HDL levels and risk for heart disease.

Level mg/dL	Level mmol/L	Interpretation
<40	<1.03	Low HDL cholesterol, heightened risk for heart disease, <50 is the value for women
40-59	1.03-1.52	Medium HDL level
>60	>1.55	High HDL level, optimal condition considered protective against heart disease

Many laboratories used a two-step method : chemical precipitation of lipoproteins containing apoprotein B, then calculating HDL as cholesterol remaining in the supernate but there are also direct methods. Labs use the routine dextran sulfate-Mg2+ precipitation method with ultracentrifugation/ dextran sulfate-Mg2+ precipitation as reference method. HPLC can be used Subfractions (HDL-2C, HDL-3C) can be measured and have clinical significance. A link has been shown between level of HDL and onset of dementia. Those with high HDL were less likely to have dementia. Low HDL-C in late-middle age has also been associated with memory loss.

SUB-SECTION 5.13B—DISORDERS OF LIPOPROTEIN METABOLISM

The most common lipoprotein disorders are hypercholesterolemia (type II hyperlipoproteinemia); hypertriglyceridemia (primarily types IV and V hyperlipoproteinemia); hypoalphalipoproteinemia; and high lipoprotein(a) (Lp[a]) levels. However, hypercholesterolemia and hypoalphalipoproteinemia may not be as prevalent among the elderly as among the general population because mortality risk is so high that patients with these disorders do not survive to old age.

The association between high serum cholesterol levels and coronary artery disease (CAD), whose prevalence and mortality rate increase with age, is well established. High levels of low-density lipoprotein cholesterol (LDL-C) and Lp(a) and a low level (< 35 mg/dL [< 0.91 mmol/L]) of high-density lipoprotein cholesterol (HDL-C) are significant independent positive risk factors for CAD and carotid artery atherosclerosis. A high level (>= 60 mg/dL [>= 1.55 mmol/L]) of HDL-C is a significant independent negative risk factor. However, the total cholesterol (TC)/HDL-C ratio is a more valuable measure of CAD risk than TC or LDL-C levels alone; the risk is higher in men when the ratio is > 6.4 and in women when the ratio is > 5.6.

For the elderly, the predictive value of high cholesterol levels in determining the risk of CAD is unclear, and the value of lowering high cholesterol levels—in terms of quality of life, morbidity, and mortality—is disputed. Some studies suggest that high cholesterol levels are an important risk factor for CAD in the elderly, others suggest that the risk decreases with age, and still others suggest a U-shaped relationship in which both high and low cholesterol levels are associated with an increased risk of morbidity and mortality. Some studies suggest sex differences: the mortality rate was lowest at a TC level of 215 mg/dL (5.55 mmol/L) for elderly men and 270 to 280 mg/dL (7.00 to 7.25 mmol/L) for elderly women.

There may also be a U-shaped relationship between cholesterol levels and death due to stroke. High cholesterol levels have been associated with increased risk of death due to nonhemorrhagic stroke, whereas low cholesterol levels have been associated with increased risk of death due to hemorrhagic stroke.

Studies of lipid-lowering treatments in the elderly, particularly primary prevention studies, have not consistently shown a significant reduction in the overall mortality rate. However, most studies have shown that for patients who have had a myocardial infarction (MI) or who have hemodynamically significant CAD, lowering lipid levels can stop or reverse disease progression and reduce the incidence of CAD events, CAD mortality, and all-cause mortality.

Lipoprotein disorders may be primary (usually familial and usually classified based on lipoprotein elevation pattern or secondary. However, genetic and secondary factors, such as various disorders and drugs, diet, obesity, physical activity, alcohol use, and cigarette smoking, are often interrelated.

The most common cause of secondary hypercholesterolemia is probably a diet high in saturated fat or cholesterol, whether or not a polygenic tendency for hypercholesterolemia exists. Covert hypothyroidism (with normal thyroxine and high thyroid-stimulating hormone levels) is a relatively common cause of high TC and LDL-C levels and is associated with an increased risk of premature MI.

The most common causes of secondary hypertriglyceridemia among the elderly are excessive alcohol intake, exogenous estrogen supplementation, poorly controlled diabetes, uremia, corticosteroid use, and β-blocker use. Isolated low HDL-C levels with normal triglyceride (TG) levels may result from smoking, androgenic steroid use, severe restriction of physical activity, or morbid obesity.

Generalized obesity may cause an increase in TG levels but not in cholesterol levels and, in persons > 60, may not contribute to risk of CAD. However, abdominal/upper body (male-pattern) obesity appears to be correlated with increased risk.

Age and sex have a major influence on lipid levels. For persons living in most industrialized countries, cholesterol and TG levels increase through middle age. In men, mean levels of TC increase until about age 50, then plateau, and then decrease starting at about age 70. In women, they increase more gradually up to age 65 to 69, then decrease. Starting at about age 55 to 60, women have higher TC levels than men. The age-related increase in TC, particularly in women, results primarily from an increase in LDL-C levels and much less from a small increase in very-low-density lipoprotein cholesterol (VLDL-C) levels.

TG levels progressively increase from birth through adulthood. The rate of increase is greater in men than in women. TG levels increase until age 55 in men and until about age 70 in women, then decrease, gradually in men.

In men, mean levels of HDL-C decrease at puberty, increase at about age 45, and then level off at age 50 to 59. These changes may be an effect of testosterone; generally, levels of plasma testosterone and HDL-C are positively correlated in adult men. After puberty, women have higher HDL-C levels than men despite a decrease after age 65.

Premenopausal women have lower LDL-C and higher HDL-C levels than men, partly because of endogenous estrogens. This difference may contribute to the lower CAD rates in premenopausal women. At menopause (whether natural or surgical), women lose this protection against CAD: LDL-C and Lp(a) levels increase and HDL-C levels decrease. Patients who have had an MI, a stroke, or other manifestations of significant atherosclerosis (eg, peripheral arterial disease, carotid artery stenosis) before age 60 should be screened for familial lipoprotein abnormalities. The Lp(a) level should be measured in patients who have had an MI or a stroke or who have CAD and in patients who have other major CAD risk factors, because a high Lp(a) level probably acts synergistically with other risk factors. If a lipoprotein abnormality is detected, the physician should try to determine whether the lipoprotein disorder is primary or secondary and assess the patient for other CAD risk factors (eg, smoking, hypertension, a high-fat diet, physical inactivity). If secondary causes can be ruled out, the disorder is usually one of the common familial hyperlipoproteinemias.

Screening and identification criteria for hypercholesterolemia are controversial. The National Cholesterol Education Program (NCEP) provides guidelines for identifying high TC and LDL-C levels and low and high HDL-C levels.

According to NCEP guidelines, no single cholesterol value should be used to classify a patient clinically, because values may vary from day to day. If the first screening test detects an abnormality, two subsequent tests are recommended. The NCEP does not give guidelines by age group; its guidelines are based on data from middle-aged persons and do not consider the increase in serum lipids that occurs with age. (As a result, 60% of persons > 65 would be classified as candidates for treatment.)

In contrast, the American College of Physicians guidelines recommend a single measurement of TC alone to identify patients who would benefit from lipid-lowering therapy. These guidelines note that evidence is insufficient to recommend or discourage the screening of men and women aged 65 to 75 for primary prevention, and screening is not recommended for persons > 75.

If elderly persons are screened, a full lipid profile should be obtained. For many elderly persons, the predominant reason for a high TC level is a high HDL-C level, not a high LDL-C level; therefore, their risk of CAD is decreased, not increased. Some persons have normal TC and TG levels but an HDL-C level below the 10th percentile and thus have a very high risk of CAD.

TG levels can be accurately measured only after a fast. If the TG level is < 400 mg/dL (< 4.52 mmol/L), the LDL-C component of the lipid profile can be estimated using the Friedewald equation: LDL-C = TC - [HDL-C + (TG/5)].

Basal lipoprotein measurements usually cannot be determined in the following situations: during a fever or major infection; within 4 weeks of an acute MI, a stroke, or major surgery; immediately after acute excessive alcohol intake; in diabetes mellitus that is severely out of control (fasting blood glucose level > 250 mg/dL (> 13.9 mmol/L), glycosylated hemoglobin > 9%); or during rapid weight loss.

Certain characteristic findings (eg, tendinous, tuberous, or palmar-planar xanthomas; arcus juvenilis corneae) are diagnostically useful (see Table 63-1). Obesity (with or without essential hypertension), glucose intolerance, and hyperuricemia may indicate primary hypertriglyceridemia or hypoalphalipoproteinemia.

Secondary lipoprotein disorders are common, even among patients with a well-defined primary lipoprotein disorder, and may exacerbate the expression of the primary disorder, particularly severe hypertriglyceridemia. Thus, when a primary lipoprotein disorder is first diagnosed, a physical examination should be performed, and a drug, occupational, family, dietary, and alcohol-intake history should be obtained. Levels of thyroxine, thyroid-stimulating hormone, blood urea nitrogen or creatinine, and fasting blood glucose should be measured, and urinalysis and liver function tests should be performed.

The NCEP guidelines recommend treatment of persons in the top quintile for TC levels (although in the elderly, use of TC levels alone can be misleading) and of persons with an LDL-C level >= 160 mg/dL (>= 4.14 mmol/L) or lower depending on the number of CAD risk factors and the presence of CAD or other atheromatous disease. Patients are considered at high risk if they have two or more CAD risk factors (age > 45 for men or > 55 for women, premature menopause without estrogen replacement therapy, a family history of CAD, current cigarette smoking, hypertension, low HDL-C levels, and diabetes). One risk factor is subtracted if HDL-C levels are >= 60 mg/dL (>= 1.55 mmol/L).

Therefore, NCEP recommendations for initiation of dietary or drug treatment are based on LDL-C levels . The goals of treatment are to lower TC and LDL-C levels to the recommended levels, to lower TG levels to < 500 mg/dL (< 5.65 mmol/L) and thus avoid pancreatitis, and to increase HDL-C levels to > 35 mg/dL (> 0.91 mmol/L), preferably to >= 40 mg/dL (>= 1.03 mmol/L). For postmenopausal women, a high TG level is an important independent risk factor for CAD; thus, lowering the level to < 250 mg/dL (< 2.82 mmol/L) is probably valuable.

Because the NCEP guidelines are not based on data from nor designed specifically for the elderly, several factors should be considered when deciding whether elderly patients with a lipoprotein abnormality should be treated:

- Decisions should not be based primarily on the patient's age. Many elderly patients are physiologically and mentally much younger than their chronologic age.
- Some benefits of treatment may be realized almost immediately: Significant lowering of LDL-C levels can lead to increased endothelial cell production of nitrous oxide (a potent vasodilator) and can reduce platelet aggregation; significant lowering of TG levels sharply decreases plasminogen activator inhibitor activity and increases fibrinolysis. Often, treatment stops progression of atherosclerotic lesions or induces regression within 1 to 2 years.
- If patients have other life-limiting conditions, aggressive lipid-lowering treatment may be inappropriate or less useful.
- Elderly patients who take many drugs may be less likely to comply with drug regimens, and their risk of drug-drug interactions is increased. Although lipid-lowering treatment can reduce long-term costs for some patients, elderly patients with a fixed income may have difficulty affording the better tolerated and more effective drugs.
- Strict lipid-lowering diets may be effective in the elderly but may lead to or exacerbate malnutrition, as may voluntary dietary restriction. Unappetizing low-fat diets can cause anorexia and eventually lead to nutritional deficiencies. The potential morbidity of protein-energy malnutrition probably outweighs that of moderate hypercholesterolemia.

Dietary treatment: Because the elderly have difficulty maintaining adequate caloric and protein intake and are at risk of malnutrition, a moderate approach to diet is generally recommended (see Table 63-4). Patients can be advised to trim fat from meat; to increase intake of fish, soybean products, and beans; to avoid fried foods; and to use monounsaturated fats (eg, olive oil). Consumption of foods rich in soluble fiber (eg, oat bran) may also lower lipid levels. Phytosterols, present in soybean products, and stanol esters, available as a margarine product, decrease cholesterol absorption.

A strict diet may be preferred for some patients. Aerobic exercise should be included as part of treatment because the benefits of diet without exercise may be limited. A low-saturated-fat, low-cholesterol diet should be tried for 6 months before drug therapy is instituted.

For patients with a high LDL-C level, the American Heart Association and NCEP recommend a two-step diet. High-risk patients should start with the step 1 diet. Patients who are already following the equivalent of a step 1 diet should start with the step 2 diet. Elderly persons may have difficulty consuming adequate calories and protein, so the diet must be modified cautiously. However, the step 1

and 2 dietary guidelines and the practical approach shown in Table 63-4 should be safe for most elderly persons. The full effects of dietary treatment at either step may not be achieved for 8 to 12 weeks; therefore, patients should advance to step 2 only if the therapeutic goal is not reached in 8 to 12 weeks. Weight loss helps lower TC, LDL-C, and TG levels but is at least as difficult for the elderly as for younger persons.

For patients with hypertriglyceridemia, a low HDL-C level, and an increased risk of CAD, dietary interventions are important. The major goal is to increase the HDL-C level, although lowering the TG level alone may be valuable for women > 50. When the TG level is > 1000 mg/dL (> 11.29 mmol/L), a sharp reduction in total fat intake (to < 30 g/day) helps prevent TG-induced pancreatitis. Other goals are to lose weight, because modest weight loss can markedly lower TG levels; to reduce alcohol intake to <= 3 drinks per week or, if TG levels are >= 500 mg/dL (>= 5.65 mmol/L), to discontinue alcohol; and to reduce the intake of total fat, saturated fat, and cholesterol using the step 2 diet.

For patients who have a TG level >= 1000 mg/dL (>= 11.29 mmol/L) with high levels of chylomicron and VLDL-TG or who have the much rarer primary hyperchylomicronemia, total fat intake should be restricted to 10 to 20% of total calories, primarily to prevent TG-induced pancreatitis. Also, the sharp decrease in VLDL-C levels and the increase in HDL-C levels that result may help prevent patients with high levels of chylomicron and VLDL-TG from developing severe premature CAD. Weight loss is crucial for obese patients. For patients with severe hypertriglyceridemia, estrogen is contraindicated.

For patients who have type III hyperlipoproteinemia and are overweight, the single most important strategy is weight loss. Patients should reduce intake not only of total fat, saturated fat, and cholesterol but also of dietary sugars.

For patients with hypoalphalipoproteinemia, the only consistently effective dietary strategy is weight loss, which may increase HDL-C levels. Other strategies to increase HDL-C levels include supplementation with fish oils rich in omega-3 fatty acids (4 to 12 g/day), smoking cessation (or at least reduction), and aerobic activity (three to five 30-minute periods per week). At least initially, exercise should be supervised by a physician. Intake of moderate amounts of alcohol can increase HDL-C levels, but only if hepatic synthetic function is normal.

Drug treatment: If hyperlipoproteinemia persists after secondary causes have been identified and treated when possible and after dietary treatment has been tried, drug treatment should be used. When a single drug is inadequate, two drugs may be required.

Bile acid-binding resins (eg, cholestyramine, colestipol) interrupt the normal enterohepatic circulation of bile acids and indirectly increase the liver's catabolism of LDL-C by stimulating hepatocytes to synthesize more LDL receptors. These drugs can reduce CAD risk.

Resins are effective and safe as first-line drugs to lower the LDL-C level in patients whose primary abnormality is a high LDL-C level and whose TG level is < 250 mg/dL (< 2.82 mmol/L). However, they may increase the TG level. If the TG level increases to > 300 mg/dL (> 3.38 mmol/L) during resin therapy, nicotinic acid or gemfibrozil (1200 mg/day) may be added, particularly if the patient has high LDL-C and TG levels; the resin and nicotinic acid act synergistically. Alternatively, an HMG-CoA reductase inhibitor (statin) can be used instead of the resin or resin combination therapy.

Resins should be started in small doses (8 to 10 g/day), particularly for the elderly. Many patients respond to the lowest dose, but the dose should be adjusted according to effect on LDL-C levels. Constipation, the most common adverse effect, can usually be avoided by increasing dietary fiber or by using stool softeners. Because resins are not systemically absorbed, they have essentially no systemic adverse effects (except for rare, mild, reversible changes in liver enzymes).

Resins can augment warfarin's effects; however, if a resin and warfarin are taken within a short time, the resin can also bind warfarin. Thus, resins should be used cautiously, if at all, with warfarin-like anticoagulants. Resins should not be given concurrently with exogenous thyroid hormones, sex hormones, prednisone, or digoxin, all of which may be bound in the intestine by resins. These drugs should be given at least 2 hours before the first resin dose of the day.

Resins are probably contraindicated as single-drug therapy in patients with a high LDL-C level and a TG level > 300 mg/dL and in those with severe hemorrhoids or a history of bowel resection or severe constipation; a statin may be used.

Nicotinic acid (niacin) inhibits secretion of VLDL from the liver, lowering VLDL-C and LDL-C levels. In patients with CAD, it reduces the incidence of recurrent MI and all-cause mortality. Nicotinic acid can be used as a first-line drug, but it is usually added to resin therapy if the resin does not lower LDL-C levels sufficiently. The initial dose is 100 mg bid or tid. The frequency of administration and total daily dose should be increased slowly at about weekly intervals, as necessary. Generally, an initial maintenance dosage of 1.5 to 2 g/day is required. Every 6 to 8 weeks, liver function tests and stool tests for occult blood should be performed, and blood glucose, uric acid, and LDL-C levels should be measured.

Adverse effects of nicotinic acid are common, bothersome, and often severe (see Table 63-6). Flushing may be ameliorated by taking aspirin 80 to 325 mg 30 to 60 minutes before taking nicotinic acid, but aspirin may cause gastrointestinal adverse effects in the elderly. Fast-release formulations of nicotinic acid are preferable because hepatotoxicity appears to be more common and more severe with slow-release formulations. If possible, nicotinic acid should not be used concurrently with a statin, because risk of myositis and hepatotoxicity is increased.

HMG-CoA reductase inhibitors (statins) block intracellular cholesterol biosynthesis and force cells to synthesize more LDL receptors, thus increasing the catabolism of LDL cholesterol. Statins include atorvastatin, cerivastatin, fluvastatin, lovastatin, pravastatin, and simvastatin. Statins are becoming the cholesterol-lowering drugs of choice because they are generally safe, have a relatively favorable adverse effect profile, and effectively lower TC levels (by 15 to 45%) and LDL-C levels (by 20 to 40%) and increase HDL-C levels (by 5 to 15%). Statins also effectively lower TG levels (by 10 to 35%). Lovastatin should be taken with food. The others do not have this restriction and are usually taken at bedtime.

Adverse effects are relatively rare and usually transient. Because liver enzyme (particularly transaminase) levels may increase, liver function tests should be performed before treatment, at 6 and 12 weeks after initiation of treatment or after elevation in dose, and at 6-month intervals thereafter. Increases in transaminase levels should be monitored until levels return to normal. Usually, a statin is not discontinued unless the liver enzyme elevations exceed three times the upper limit of normal.

A statin may be the drug of choice for patients with a high LDL-C level and a TG level > 300 mg/dL and for those with severe hemorrhoids or a history of bowel resection or severe constipation. Gemfibrozil may be added if the TG level remains > 300 mg/dL and the patient is at high risk of or has had an atherosclerotic event.

Statins can be used effectively with resins but probably should not be used with nicotinic acid because the risks of myopathy and hepatotoxicity are increased.

The concurrent use of a statin and cyclosporine commonly produces myopathy and may also produce rhabdomyolysis and myoglobinuria. This drug combination should thus be restricted to situations in which other lipid-lowering regimens are ineffective, and patients should be closely monitored.

Erythromycin and its derivatives should not be given concurrently with a statin because the risk of myopathy is increased.

Gemfibrozil, a fibric acid derivative, increases the hydrolysis of VLDL-TG and the synthesis of HDL-C and apolipoprotein A-I. Gemfibrozil lowers LDL-C and TG levels and reduces CAD morbidity and mortality rates in appropriate patients. It is the drug of choice for elderly patients with hypertriglyceridemia or hypoalphalipoproteinemia. Gemfibrozil should be considered for patients with a high TG level (> 300 mg/dL), a low HDL-C level (< 35 mg/dL [< 0.91 mmol/L]), and a moderately high LDL-C level (< 190 mg/dL [< 4.92 mmol/L]). Well tolerated by most patients, gemfibrozil rarely causes gastrointestinal upset or myopathy, although myopathy is more common when the drug is given to patients with impaired renal function. For such patients, especially if they are also receiving cyclosporine, the dose should be reduced.

Combined gemfibrozil-statin therapy may be necessary for patients at highest risk of CAD who have evidence of atherosclerosis. Such patients often have combined hyperlipidemia, usually with high TC, high TG, and low HDL-C levels, which are maintained despite significant dietary, weight, and exercise modification. When these patients are treated with gemfibrozil alone, TG levels can usually be normalized and HDL-C levels can often be increased to > 35 mg/dL (> 0.91 mmol/L), but TC and LDL-C levels may not be adequately lowered and often increase. Conversely, when these patients are treated with a statin alone, LDL-C levels can usually be normalized, but TG levels often remain high, and HDL-C levels often remain low (< 35 mg/dL).

Because myopathy, rhabdomyolysis, myoglobinuria, and renal injury have been reported in patients taking combined gemfibrozil-statin therapy, the following guidelines are recommended:

- It should be reserved for secondary prevention.
- It should not be used if patients have a very low creatinine clearance rate, because the risk of myopathy is increased.
- It should not be used concurrently with cyclosporine or nicotinic acid, because the risk of myopathy is increased.
- It can be used if patients are reliable and are well informed about the possibility of myopathy.
- Creatine kinase levels should be measured and liver function tests should be performed at baseline and every 6 to 8 weeks thereafter.

- Patients should take gemfibrozil 1.2 g/day po and the lowest starting dose of the statin. The statin dose should be adjusted to the lowest dose that will lower LDL-C levels to the target range.

Probucol appears to increase the rate of LDL-C catabolism, probably through pathways not mediated by LDL receptors. One hypothesis is that the antioxidant effect of probucol may reduce atherosclerosis by reducing the atherogenic effect of oxidized LDL-C. The effect of probucol on CAD risk has not been established.

Probucol is considered a second-line drug in the NCEP guidelines. Probucol usually lowers LDL-C levels by about 8 to 15%, but because it also lowers HDL-C levels by as much as 25%, its role in treating patients with a high LDL-C level is uncertain. Xanthoma regression has been reported as HDL-C levels decrease.

Generally, probucol is well tolerated; diarrhea is the most common adverse effect. Because the drug can prolong the QT interval, it is probably contraindicated in patients with ventricular irritability and an initially prolonged QT interval and in those taking other drugs that prolong the QT interval. The combination of probucol and a resin is effective; the reduction in the HDL-C level is less than that with probucol alone, and constipation is much less common than with a resin alone.

Fish oils containing omega-3 fatty acids are available over the counter but can also be obtained in reasonable amounts by eating a 3-oz (cooked) portion of oily fish (eg, herring, mackerel, salmon, shad, trout). Data about the long-term effectiveness and adverse effects of fish oil supplements are scarce; however, for short-term use, doses of <= 15 g/day appear to be safe and can lower TG levels, but they are not useful in lowering cholesterol levels. They may increase the hydrolysis of VLDL-TG. In patients with hypertriglyceridemia, fish oils may increase HDL-C levels by 10 to 15%. At much higher doses (usually > 50 g/day), they may be associated with thrombocytopenia and increased bleeding time; such doses are almost never used clinically. In rare cases when doses of > 20 g/day are used, platelet counts and bleeding time should be monitored. At such doses, fish oils may interfere with glucose control in patients with diabetes.

Estrogen replacement therapy: Postmenopausal women who receive unopposed estrogen replacement therapy have lower LDL-C levels (by 15 to 25%) and higher HDL-C levels (by 16 to 21%) than those who do not receive this therapy; as a result the LDL-C/HDL-C ratio is substantially decreased. Such therapy also lowers Lp(a) levels. Unopposed estrogen therapy appears to reduce the risk of cardiovascular death. The addition of a progestin to reduce the risk of endometrial hyperplasia and endometrial cancer may limit or eliminate the benefit observed with unopposed estrogen, and may even initially increase the risk of coronary artery disease.

Estrogen replacement therapy can be used alone or with other lipid-modifying treatments. Estrogen substantially elevates TG levels in women with preexisting hypertriglyceridemia, occasionally to levels that can cause lethal pancreatitis. Consequently, fasting TG levels should be measured before initiating estrogen replacement therapy (with or without a progestin). Estrogen replacement therapy is contraindicated in women with familial hypertriglyceridemia and a TG level > 300 mg/dL after modification of diet and alcohol intake.

Antioxidant therapy: The toxicity of LDL-C may be reduced by the use of antioxidants. According

to one hypothesis, atherosclerosis progresses because "toxic LDL" triggers the development of fatty streaks. Toxic LDL is formed when the lipid component of the lipoprotein is oxidized by endothelial cells and smooth muscle cells. Oxidation occurs via a free radical mechanism involving superoxide anions and hydrogen peroxide. The oxidized LDL functions as a chemotactic agent for monocytes, transforming them to macrophages, which stimulate cholesterol esterification and the formation of foam cells.

α-Tocopherol (vitamin E) inhibits the oxidation of LDL in vitro. In several studies, vitamin E consumption appeared to be strongly and inversely correlated with CAD risk. Vitamin E supplementation for short periods produced no benefit, but supplementation for at least 2 years was associated with a lower risk of CAD in men and women.

Vitamin A, β-carotenoid, may affect atherosclerosis by scavenging oxidizing free radicals. In observational studies, the CAD mortality rate appeared to be strongly and inversely correlated with dietary carotene intake.

Ascorbic acid (vitamin C) is considered a secondary antioxidant; it works synergistically with vitamin E, regenerating vitamin E from the vitamin E radical. Vitamin C may also enhance the transformation of cholesterol into bile acids.

Treatment of special conditions: Treating elderly patients with an isolated low HDL-C level (usually well below 35 mg/dL [0.91 mmol/L]), a TC level < 200 mg/dL (< 2.26 mmol/L), and a TG level < 250 mg/dL (< 2.82 mmol/L) is a challenge. If lifestyle changes (weight loss, increased aerobic exercise, cessation of cigarette smoking) do not increase the HDL-C level and particularly if the patient has had an atherosclerotic event or is at high risk because of primary hypoalphalipoproteinemia or other CAD risk factors, drug treatment is warranted. However, the best approach has not been established. Nicotinic
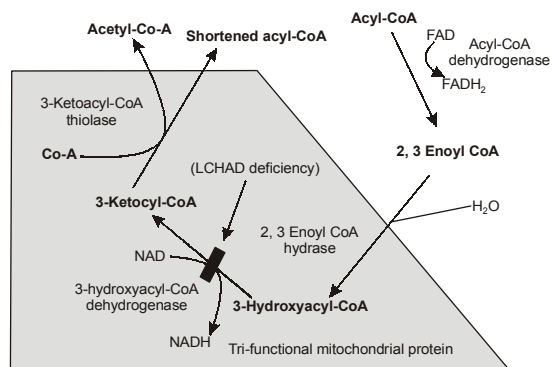
Fig. 5.25 : Showing the Beta Oxidation pathway

acid 1.5 to 6.0 g/day or gemfibrozil 1.2 g/day may be effective, particularly if the Lp(a) level is also high. If these drugs are ineffective or cannot be tolerated, a statin may be given to lower the TC level to < 160 mg/dL (< 4.14 mmol/L) or the LDL-C level to < 100 mg/dL (< 2.59 mmol/L); the HDL-C level usually does not change, but the TC/HDL-C ratio is usually substantially decreased.

High Lp(a) levels cannot be lowered by diet and can be lowered only modestly by nicotinic acid. Most other drugs that lower LDL-C levels do not substantially alter Lp(a) levels. Therefore, aggressive modification of other CAD risk factors, if present, is important.

SECTION 5.14—OXIDATION OF FATTY ACIDS

Oxidation of fatty acids occurs in the mitochondria. The transport of fatty acyl-CoA into the mitochondria is accomplished via an acyl-carnitine intermediate, which itself is generated by the action of carnitine palmitoyltransferase I (CPT I, also called carnitine acyltransferase I, CA I) an enzyme that resides in the outer mitochondrial membrane. The acyl-carnitine molecule then is transported into the mitochondria where carnitine palmitoyltransferase II (CPT II, also called carnitine acyltransferase II, CA II) catalyzes the regeneration of the fatty acyl-CoA molecule. The process of fatty acid oxidation is termed β-oxidation since it occurs through the sequential removal of 2-carbon units by oxidation at the β-carbon position of the fatty acyl-CoA molecule.

SUB-SECTION 5.14A—BETA OXIDATION

Beta oxidation is the process by which fatty acids, in the form of Acyl-CoA molecules, are broken down in mitochondria and/or in peroxisomes to generate Acetyl-CoA, the entry molecule for the Krebs cycle.

Free fatty acids can penetrate the plasma membrane due to their poor water solubility and high fat solubility. Once in the cytosol, a fatty acid reacts with ATP to give a fatty acyl adenylate, plus inorganic pyrophosphate. This reactive acyl adenylate then reacts with free coenzyme A to give a fatty acyl-CoA ester plus AMP.

Once inside the mitochondria, the β-oxidation of fatty acids occurs via four recurring steps:

Description	Equation of the reaction	Enzyme	End product
1	2	3	4
Oxidation by FAD: The first step is the oxidation of the fatty acid by Acyl-CoA-Dehydroge-nase. The enzyme catalyzes the formation of a double bond between the C-2 and C-3.		acyl CoA dehydrogenase	trans-β2-enoyl-CoA

1	2	3	4
Hydration: The next step is the hydration of the bond between C-2 and C-3. The reaction is stereospecific, forming only the L isomer.	trans-Δ²-Enoyl-CoA — Enoyl-CoA-Hydralase — L-3-Hydroxyacyl-CoA ($+H_2O$ / $-H_2O$)	enoyl CoA hydratase	L-β-hydroxyacyl CoA
Oxidation by NAD+: The third step is the oxidation of L-β-hydroxyacyl CoA by NAD^+. This converts the hydroxyl group into a keto group.	L-3-Hydroxyacyl-CoA — Hydroxyacyl-CoA-Dehydrogenase — 3-ketoacyl-CoA (NAD^+, $NADH + H^+$)	L-β-hydroxyacyl CoA dehydrogenase	β-ketoacyl CoA
Thiolysis: The final step is the cleavage of β-ketoacyl CoA by the thiol group of another molecule of CoA. The thiol is inserted between C-2 and C-3.	3-ketoacyl-CoA — Thiolase — Actyl-CoA + Acetyl-CoA (+ CoA-SH)	β-ketothiolase	An acetyl CoA molecule, and an acyl CoA molecule, which is two carbons shorter

Fatty acyl-CoA $CH_3(CH_2)_n - C_\beta - C_\alpha - C - SCoA$

acyl-CoA dehydrogenase (FAD → $FADH_2$)

trans-Δ₂-enoyl-CoA $CH_3(CH_2)_n - C = C - C - SCoA$

enoyl-CoA hydratase (H_2O)

3-L-hydroxyacyl-CoA $CH_3(CH_2)_n - C - CH_2 - C - SCoA$ (OH)

3-L-hydroxyacyl-CoA dehydrogenase (NAD^* → $NAD + H$)

β-ketoacyl-CoA $CH_3(CH_2)_n - C - CH_2 - C - SCoA$

β-ketothiolase (HSCOA)

$CH_3(CH_2)_n - C - SCoA$ + $CH_3 - C - SCoA$

Fatty acyl-CoA   Acetyl-CoA
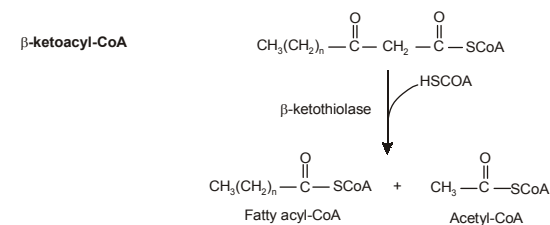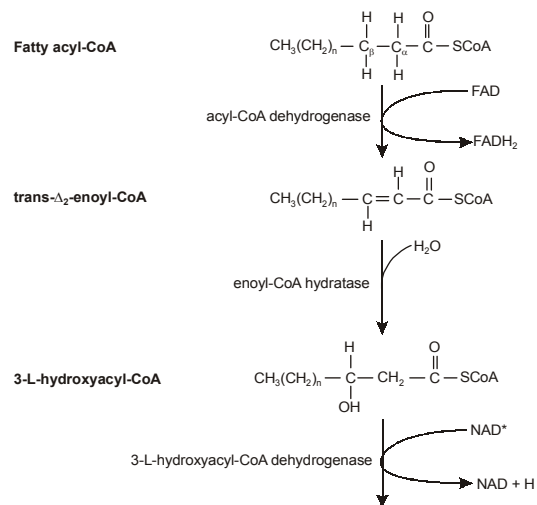
Fig. 5.22 : Showing the mechanism of Beta oxidation

This process continues until the entire chain is cleaved into acetyl CoA units. The final cycle produces two separate acetyl CoA's, instead of one acyl CoA and one acetyl CoA. For every cycle, the Acyl CoA unit is shortened by two carbon atoms. Concomitantly, one molecule of FADH2, NADH and acetyl CoA are formed.

SUB-SECTION 5.14B—β-OXIDATION OF UNSATURATED FATTY ACIDS

β-oxidation of unsaturated fatty acids poses a problem since the location of a cis bond can prevent the formation of a trans-Δ2 bond. These situations are handled by an additional two enzymes.

Whatever the conformation of the hydrocarbon chain, β-oxidation occurs normally until the acyl CoA (because of the presence of a double bond) is not an appropriate substrate for *acyl CoA dehydrogenase*, or *enoyl CoA hydratase*:

- If the acyl CoA contains a cis-Δ3 bond, then cis-Δ3-Enoyl CoA isomerase will convert the bond to a trans-Δ2 bond, which is a regular substrate.

- If the acyl CoA contains a cis-Δ4 double bond, then its dehydrogenation yields a 2,4-dienoyl intermediate, which is not a substrate for enoyl CoA hydratase. However, the enzyme 2,4 Dienoyl CoA reductase reduces the intermediate, using NADPH, into trans-Δ3-enoyl CoA. As in the above case, this compound is converted into a suitable intermediate by 3,2-Enoyl CoA isomerase.

To summarize:

- odd numbered double bonds are handled by the isomerase.

- even numbered double bonds by the reductase (which creates an odd numbered double bond) and the isomerase.

SUB-SECTION 5.14C—β-OXIDATION OF ODD-NUMBERED CHAINS

Fatty acids with an odd number of carbon are generally found in the lipids of plants and some marine organisms. Many ruminant animals form large amount of 3-carbon propionate during fermentation of carbohydrate in rumen.[1]

Chains with an odd-number of carbons are oxidized in the same manner as even-numbered chains, but the final products are propionyl-CoA and acetyl-CoA.

Propionyl-CoA is first carboxylated using a bicarbonate ion into D-stereoisomer of methylmalonyl-CoA, in a reaction that involves a biotin co-factor, ATP, and the enzyme propionyl-CoA carboxylase. The bicarbonate ion's carbon is added to the middle carbon of propionyl-CoA, forming a D-methylmalonyl-CoA. However, the D conformation is enzymatically converted into the L conformation by methylmalonyl-CoA epimerase, then it undergoes intramolecular rearrangement which is catalyzed by methylmalonyl-CoA mutase(requires coenzyme-B_{12} as it's coenzyme) to form succinyl-CoA. The succinyl-CoA formed can then enter the citric acid cycle.

Because it cannot be completely metabolized in the citric acid cycle, the products of its partial reaction must be removed in a process called cataplerosis. This allows regeneration of the citric acid cycle intermediates, possibly an important process in certain metabolic diseases.

Fatty acid oxidation also occurs in peroxisomes, when the fatty acid chains are too long to be handled by the mitochondria. However, the oxidation ceases at octanyl CoA. It is believed that very long chain (greater than C-22) fatty acids undergo initial oxidation in peroxisomes which is followed by mitochondrial oxidation.

One significant difference is that oxidation in peroxisomes is not coupled to ATP synthesis. Instead, the high-potential electrons are transferred to O_2, which yields H_2O_2. The enzyme catalase, found exclusively in peroxisomes, converts the hydrogen peroxide into water and oxygen.

Peroxisomal β-oxidation also requires enzymes specific to the peroxisome and to very long fatty acids. There are three key differences between the enzymes used for mitochondrial and peroxisomal β-oxidation:

1. β-oxidation in the peroxisome requires the use of a peroxisomal carnitine acyltransferase (instead of carnitine acyltransferase I and II used by the mitochondria) for transport of the activated acyl group into the peroxisome.
2. The first oxidation step in the peroxisome is catalyzed by the enzyme acyl CoA oxidase.
3. The β-ketothiolase used in peroxisomal β-oxidation has an altered substrate specificity, different from the mitochondrial β-ketothiolase.

Peroxisomal oxidation is induced by high fat diet and administration of hypolipidemic drugs like clofibrate.

The ATP yield for every oxidation cycle is 14 ATP (according to the P/O ratio), broken down as follows:

Source	ATP	Total
1 FADH$_2$	x 1.5 ATP	= 1.5 ATP (some sources say 2 ATP)
1 NADH	x 2.5 ATP	= 2.5 ATP (some sources say 3 ATP)
1 acetyl CoA	x 10 ATP	= 10 ATP (some sources say 12 ATP)
Total		**= 14 ATP**

For an even-numbered saturated fat (C_{2n}), n − 1 oxidations are necessary and the final process yields an additional acetyl CoA. In addition, two equivalents of ATP are lost during the activation of the fatty acid. Therefore, the total ATP yield can be stated as:

(n - 1) * 14 + 10 − 2 = total ATP

For instance, the ATP yield of palmitate (C_{16}, $n = 8$) is:

(8 - 1) * 14 + 10 − 2 = 106 ATP

Represented in table form:

Source	ATP	Total
7 FADH$_2$	x 1.5 ATP	= 10.5 ATP
7 NADH	x 2.5 ATP	= 17.5 ATP
8 acetyl CoA	x 10 ATP	= 80 ATP
Activation		= –2 ATP
Total		**= 106 ATP**

For sources that use the larger ATP production numbers described above, the total would be 129 ATP equivalents per palmitate.

Beta-oxidation of unsaturated fatty acids changes the ATP yield due to the requirement of two possible additional enzymes.

SUB-SECTION 5.14D—ROLE OF CARNITINE

Carnitine is a quaternary ammonium compound biosynthesized from the amino acids lysine and methionine. In living cells, it is required for the transport of fatty acids from the cytosol into the mitochondria during the breakdown of lipids (or fats) for the generation of metabolic energy. It is often sold as a nutritional supplement. Carnitine was originally found as a growth factor for mealworms and labeled vitamin Bt. Carnitine exists in two stereoisomers: its biologically active form is L-carnitine, while its enantiomer, D-carnitine, is biologically inactive. Carnitine transports long-chain acyl groups from fatty acids into the mitochondrial matrix, so that they can be broken down through β-oxidation to acetate to obtain usable energy via the citric acid cycle. In some organisms such as fungi, the acetate is used in the glyoxylate cycle for gluconeogenesis and formation of carbohydrates. Fatty acids must be activated before binding to the carnitine molecule to form acyl-carnitine. The free fatty acid in the cytosol is attached with a thioester bond to coenzyme A (CoA). This reaction is catalyzed by the enzyme fatty acyl-CoA synthetase and driven to completion by inorganic pyrophosphatase.

The acyl group on CoA can now be transferred to carnitine and the resulting acyl-carnitine transported into the mitochondrial matrix. This occurs via a series of similar steps:

1. Acyl-CoA is conjugated to carnitine by carnitine acyltransferase I (palmitoyltransferase) located on the outer mitochondrial membrane
2. Acyl-carnitine is shuttled inside by a carnitine-acylcarnitine translocase

CYTOSOL   Acyl-CoA from cytosol to   MATRIX
                 the mitochondrial matrix

Acyl-CoA
+ Carnitine

Acyl-CoA
+ Carnitine

Acyl-Carnitine

Acyl-Carnitine ──────→ Acyl-Carnitine ────── Acyl-Carnitine

Carnitine ←────────────── Carnitine

● CPTI (Carnitine Palmitoyl Transferase I)   Transiocase

● CPTI (Carnitine Palmitoyl Transferase I)
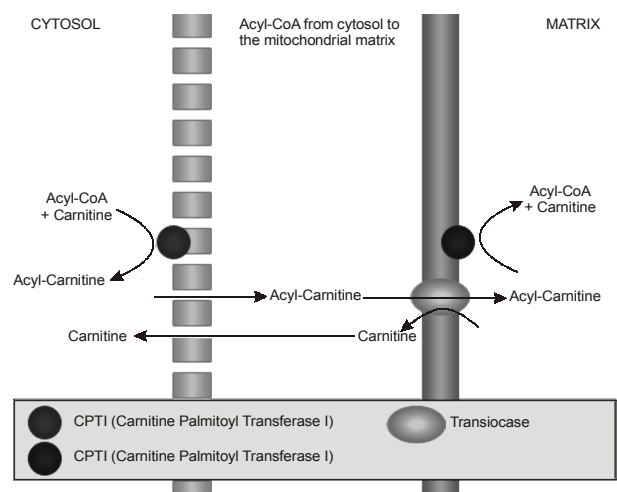
Fig. 5.25 : Showing the Carnitine involvement in Fatty Acid Metabolism

3. Acyl-carnitine is converted to acyl-CoA by carnitine acyltransferase II (palmitoyltransferase) located on the inner mitochondrial membrane. The liberated carnitine returns to the cytosol.

Human genetic disorders such as primary carnitine deficiency, carnitine palmitoyltransferase I deficiency, carnitine palmitoyltransferase II deficiency and carnitine-acylcarnitine translocase deficiency affect different steps of this process.

Carnitine acyltransferase I undergoes allosteric inhibition as a result of malonyl-CoA, an intermediate in fatty acid biosynthesis, in order to prevent futile cycling between β-oxidation and fatty acid synthesis.

SUB-SECTION 5.14E—FATTY ACID OXIDATION DISORDERS

Fatty Oxidation Disorders (FODs) are genetic metabolic deficiencies in which the body is unable to oxidize (breakdown) fatty acids to make energy because an enzyme is either missing or not working correctly. The main source of energy for the body is a sugar called glucose. Normally when the glucose runs out, fat is broken down into energy. However, that energy is not readily available to children and adults with an FOD.

Fatty acid transport and mitochondrial oxidation is a complex pathway that plays a major role in energy production during times of fasting and metabolic stress. Once free acids are released into the blood they are taken up by the liver and muscle cells and activated to coenzyme A esters. Then they are

transported into the mitochondria and oxidized in a cyclic fashion by four sequential reactions that are each catalyzed by one of multiple enzymes. The acyl-CoA dehydrogenases are chain-length specific enzymes. Deficiencies or abnormalities in these result in very long chain acyl-CoA dehydrogenase deficiency (VLCAD), long chain acyl CoA dehydrogenase deficiency (LCAD), medium chain acyl-CoA dehydrogenase deficiency (MCAD)*, and short chain acyl-CoA dehydrogenase deficiency (SCAD). Any illness may lead to a fasting state that can then lead to the depletion of glucose stores. Once this occurs, fatty acid metabolism becomes the dominant energy source. If there is an abnormality in fatty acid metabolism, then life-threatening episodes of metabolic decompensation can ensue. Relatively simple dietary management may avoid symptoms.

SUB-SECTION 5.14F—KETOSIS AND KETOGENESIS

During high rates of fatty acid oxidation, primarily in the liver, large amounts of acetyl-CoA are generated. These exceed the capacity of the TCA cycle, and one result is the synthesis of ketone bodies, or ketogenesis. The ketone bodies are **acetoacetate, β-hydroxybutyrate**, and **acetone**.

The formation of acetoacetyl-CoA occurs by condensation of two moles of acetyl-CoA through a reversal of the thiolase catalyzed reaction of fat oxidation. Acetoacetyl-CoA and an additional acetyl-CoA are converted to β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase, an enzyme found in large amounts only in the liver. HMG-CoA in the mitochondria is converted to acetoacetate by the action of HMG-CoA lyase. Acetoacetate can undergo spontaneous decarboxylation to acetone, or be enzymatically converted to β-hydroxybutyrate through the action of β-hydroxybutyrate dehydrogenase.

When the level of glycogen in the liver is high the production of β-hydroxybutyrate increases. When carbohydrate utilization is low or deficient, the level of oxaloacetate will also be low, resulting in a reduced flux through the TCA cycle. This in turn leads to increased release of ketone bodies from the liver for use as fuel by other tissues. In early stages of starvation, when the last remnants of fat are oxidized, heart and skeletal muscle will consume primarily ketone bodies to preserve glucose for use by the brain. Acetoacetate and β-hydroxybutyrate, in particular, also serve as major substrates for the biosynthesis of neonatal cerebral lipids.

Ketone bodies are utilized by extrahepatic tissues through the conversion of β-hydroxybutyrate to acetoacetate and of acetoacetate to acetoacetyl-CoA. The first step involves the reversal of the β-hydroxybutyrate dehydrogenase reaction, and the second involves the action (shown below) of acetoacetate:succinyl-CoA transferase, also called β-ketoacyl-CoA-transferase.

The fate of the products of fatty acid metabolism is determined by an individual's physiological status. Ketogenesis takes place primarily in the liver and may by affected by several factors:

1. Control in the release of free fatty acids from adipose tissue directly affects the level of ketogenesis in the liver. This is, of course, substrate-level regulation.

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* MCAD deficiency is the most common of the disorders with an incidence of 1/60000 to 1/10,000. Deficiency of VLCAD, LCAD, and SCAD is rare compared to MCAD.

2. Once fats enter the liver, they have two distinct fates. They may be activated to acyl-CoAs and oxidized, or esterified to glycerol in the production of triacylglycerols. If the liver has sufficient supplies of glycerol-3-phosphate, most of the fats will be turned to the production of triacylglycerols.

3. The generation of acetyl-CoA by oxidation of fats can be completely oxidized in the TCA cycle. Therefore, if the demand for ATP is high the fate of acetyl-CoA is likely to be further oxidation to CO_2.

4. The level of fat oxidation is regulated hormonally through phosphorylation of ACC, which may activate it (in response to glucagon) or inhibit it (in the case of insulin).
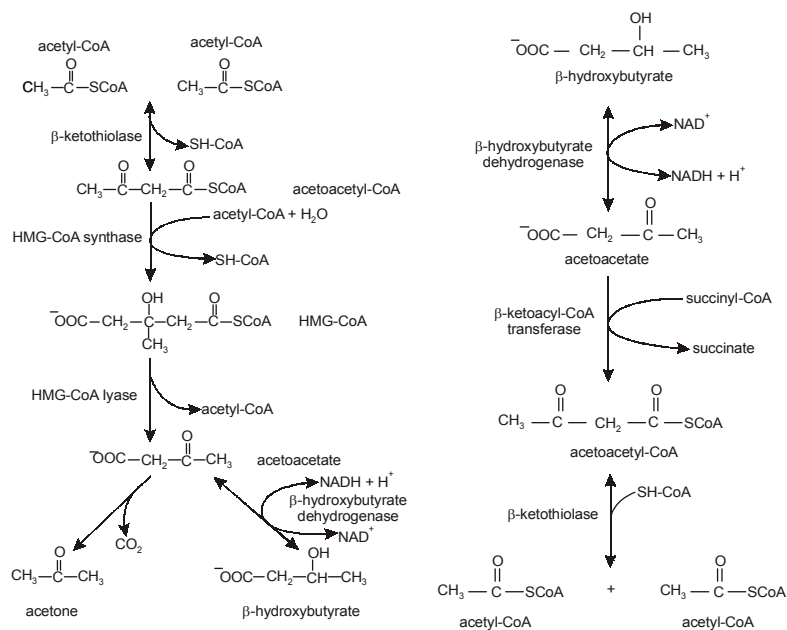
Fig. 5.28 : Showing the Process of Ketogenesis   **Fig. 5.28A: Showing the Utilization of Ketones**

SUB-SECTION 5.14G—FATTY LIVER

Fatty liver, also known as **fatty liver disease (FLD), steatorrhoeic hepatosis,** or **steatosis hepatitis**, is a reversible condition where large vacuoles of triglyceride fat accumulate in liver cells via the process of steatosis. Despite having multiple causes, fatty liver can be considered a single disease that occurs worldwide in those with excessive alcohol intake and those who are obese (with or without effects of insulin resistance). The condition is also associated with other diseases that influence fat metabolism[1]. Morphologically it is difficult to distinguish alcoholic FLD from non alcoholic FLD and both show micro-vesicular and macrovesicular fatty changes at different stages.

Fatty liver is commonly associated with alcohol or metabolic syndrome (diabetes, hypertension and dyslipidemia) but can also be due to any one of many causes[2][3]:

Metabolic

Abetalipoproteinemia, glycogen storage diseases, Weber-Christian disease, Wolman disease, acute fatty liver of pregnancy, lipodystrophy

Nutritional

Malnutrition, total parenteral nutrition, severe weight loss, refeeding syndrome, jejuno-ileal bypass, gastric bypass, jejunal diverticulosis with bacterial overgrowth

Drugs and Toxins

Amiodarone, methotrexate, diltiazem, highly active antiretroviral therapy, glucocorticoids, tamoxifen, environmental hepatotoxins (e.g. phosphorus, toxic mushroom)

Other

Inflammatory bowel disease, HIV, Hepatitis C especially genotype 3.

The treatment of fatty liver is related to the cause. It is important to remember that simple fatty liver may not require treatment. The benefit of weight loss, dietary fat restriction, and exercise in obese patients is inconsistent.

Reducing or eliminating alcohol use can improve fatty liver due to alcohol toxicity. Controlling blood sugar may reduce the severity of fatty liver in patients with diabetes. Ursodeoxycholic acid may improve liver function test results, but its effect on improving the underlying liver abnormality is unclear.

SUB-SECTION 5.14H—LIPOTROPIC FATORS

Our blood contains certain constituents known as lipotropic factors. In addition to playing important roles in the mobilization and utilization of dietary fats, lipotropic factors act as the body's natural emulsifiers, holding blood lipids in solution and resisting lipid deposition within the cardiovascular system. Sufficient lipotropic factors can also keep homocystein levels in check to support cardiovascular health, including the health of the arterial walls and blood lipids. The body is able to synthesize its own

lipotropic factors when given all the ingredients: choline, inositol, betaine, folic acid and B vitamins. These elements are key to the proper metabolism and elimination of homocysteine, a potent oxidant (something you don't want).

SUB-SECTION 5.15I—METABOLISM OF LIPIDS IN ADIPOSE TISSUE AND ITS INFLUENCE OF HORMONES

Adipose tissue is not just a static lump of fats it is in "Dynamic state" breakdown of fats and synthesis takes place all the time.

Triglycerides stores in the body is continually undergoing lypolysis and esterification. These two processes are not forward and reverse process of the same reaction. They are entirely different pathways involving different reactants and enzymes.

Many of the nutritional metabolic and hormonal factors regulate either of these two mechanisms i.e. esterification and lypolysis.

Resultant of these two processes determine the magnitude of free fatty acids (FFA) circulating in the blood. Triglycerides in the adipose tissue undergoes hydrolysis by a hormone TG – lipase enzyme to form free fatty acids and glycerol. Adipolytic lipase is triacyl glycerol (the main regulating enzyme) and the Diacyl glycerol lipase.

These lipases are distinct from lipoprotein lipase that catalyzes lipoprotein TG (present in chylomicrions and VLDL. The free fatty acids formed by lypolysis can be reconverted in the tissue to acyl CoA by Acyl – CoA synthase and re – esterified with a glycerol – P from TG.

Thus there is an continuous cycle of lypolysis and resterification within the tissue.

When the rate of re- esterification is less < than rate of lypolysis FFA accumulates and diffuses into the plasma where it raises the level of FFA ↑ in plasma
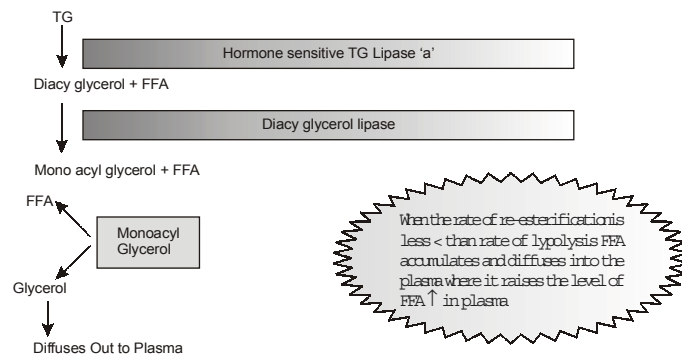
Fig. 5.28 : Showing the Conversion of TG to FFA

Under conditions of adequate nutritional intake or when utilization of glucose by the adipose tissue is increased then more α - glycerol – P will be available. Re-esterification will be greater than lypolysis as a result FFA level outflow decreases and plasma FFA is not mediated by decreasing the rate of lypolysis.

It proves that the effect is due to provision of α - glycerol – P from glycolysis, which enhances esterification.

In diabetes mellitus and in an starvation, availability of glucose in adipose tissue is grossly reduced, resulting to lack of α - glycerol – P. Thus rate of re – esterification is decreased. Lypolysis is greater than re – esterification, resulting to accumulation of FFA and increasing in plasma FFA level.

SUB-SECTION 5.14J—INFLUENCE OF HORMONES ON ADIPOSE TISSUE
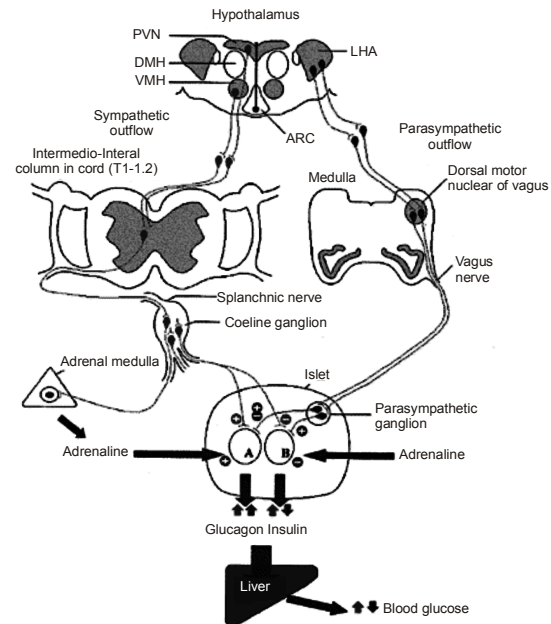
Fig. 5.29 : Showing the Total Fat Metabolism including the Thyroid Hormonal Influence

Rate of release of FFA from adipose tissue, is affected by many hormones which influence either the (a) rate of esterification (b) rate of lypolysis.

List of hormones that increase the rate of esterification

1. Insulin is the principal hormone

2. Prolactin – effective in large doses

Net result of insulin on adipose tissue, which results in fall of circulating FAA

(a) The above is brought about by decreasing the level of cyclic AMP level in the cells. This achieved by

(i) Inhibiting adenyl cyclase and

(ii) Increasing the phopshodiesterase activity.

Lowered cyclic AMP level in the cell inhibits the activity of hormone sensitive – TG lipase (conversion from 'b' → to 'a' does not occur. The action is mediated through C – AMP – dependant protein kinase, which is not activated. Thus it is not only decreases the release of free FA but also glycerol.

(b) Insulin also enhances the update of glucose in the adipose cells. Glucose oxidation provides α - glycerol – P through dihydroxyacetone – P enhancing esterification.

Increase uptake of glucose is activated by the insulin thereby causing the translocation of glucose 'transporters' from the Golgi apparatus to the plasma membrane.

There is also increase F.A synthesis as glucose is oxidized by HMP shunt pathway and provides NADPH

(c) Insulin also affects the enzyme by increasing the activity of Pyruvate dehydrogenase, acetyl CoA carboxylase and glycerol – P – acyl transferase, which reinforce the effects arising from increased glucose uptake on the enhancement of FA synthesis and also T.G synthesis.

(d) Insulin also been shown to inhibit Hormone sensitive TG lipase 'a' independent c – AMP pathway.

Effect of prolactorin is similar to insulin provided it is given in larger doses.

List of hormones that increase the rate of Lypolysis are given below:[2] [3]

1. Caticolamines – epinephrine and nor – epinephrine are principal hormones.

2. Glucagon

3. Growth hormone

4. Glucocorticoids

5. ACTH a and b MSH TSH and vasopressin.

---

[1] For an optional effect most of this lypolytic process require the presence of glucocorticoids (GC) and thyroid hormones in minimal amounts. On their own these hormones i.e. G and thyroid hormones do not increase the lypolysis markedly but act in a "facilitatory" or " permissive" capacity with other lypolytic endocrine hormones

[2] Hormones that act rapidly in promoting lypolysis are the catacholamines they stimulate the activity of adenyl cyclase and increase cyclic AMP level. Thyroid hormones in minimal activity is necessary of its full lypolytic activity.
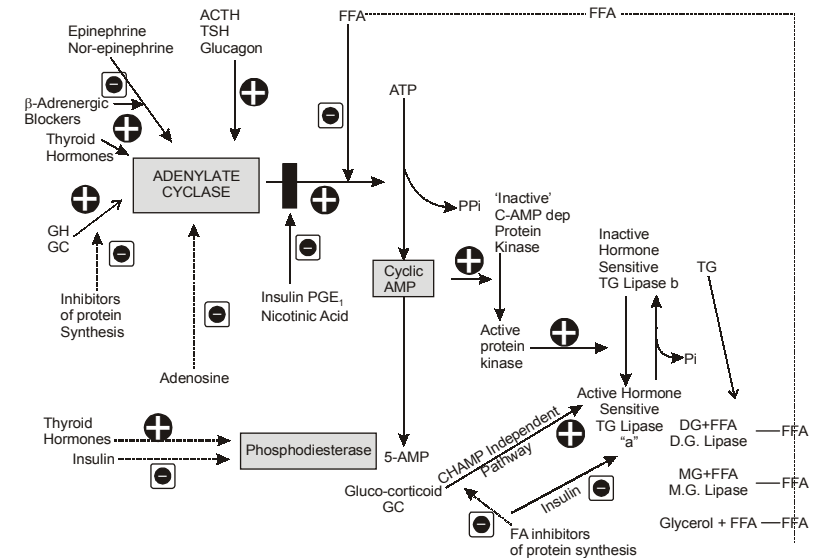
Fig. 5.32 : Showing the Influence of Hormones in the Adipose Tissue Metabolism

These hormones accelerate the release of FFA form adipose tissue and raise the plasma FFA level by increasing lyposis of TG.

Most of them act by activating adenyl cyclase, thus increasing the cyclic AMP level in cells. Cyclic AMP in turn converts 'inactive' cyclic AMP dep protein kinase to the 'active' from, which phosphorylates inactive hormones sensitive TG lipase 'b' to active from 'a' and brings about lypolysis.

Growth hormone in promoting lypolysis is slow. It is dependent on synthesis of proteins involved in the formation of C – AMP i.e. adenylate cyclase

Glucocorticoids are responsible for the plasma FFA increment. This achieved by

(i) Glucocorticoids depress uptake of glucose and there is less a - glycerol – P available. Thus decreases rate of esterification.

(ii) Stimulates synthesis of adenylate cyclase thus increasing C – AMP level in the cells.

(iii) Facilitates adipokinetic property of growth hormones and

(iv) Increases synthesis of new lipase protein by a c – AMP independent pathway.

SECTION 5.15—BIOSYNTHESIS OF FATTY ACIDS

Fatty acid biosynthesis is different from the β-oxidation degradation pathway.

1. Fatty acid biosynthesis occurs in the cytosol, β-oxidation occurs in the mitochondrial matrix.

2. All of the carbon atoms of fatty acids come from acetyl CoA.

3. Intermediates of fatty acid biosynthesis are covalently linked to an acyl carrier protein. In β-oxidation the fatty acids are attached to Coenzyme A.

4. In animals, the enzymes involved in fatty acid biosynthesis are contained in one long polypeptide chain, whereas the enzymes of β-oxidation are independent enzymes found in the matrix.

5. The oxidation/reduction reagents of fatty acid biosynthesis are NADP+/NADPH whereas the redox reagents of β-oxidation are NAD+/NADH and FAD/FADH2.

The Design Strategy for Fatty Acid Biosynthesis.

• Fatty acids are constructed by the addition of two carbon units derived from acetyl-CoA.
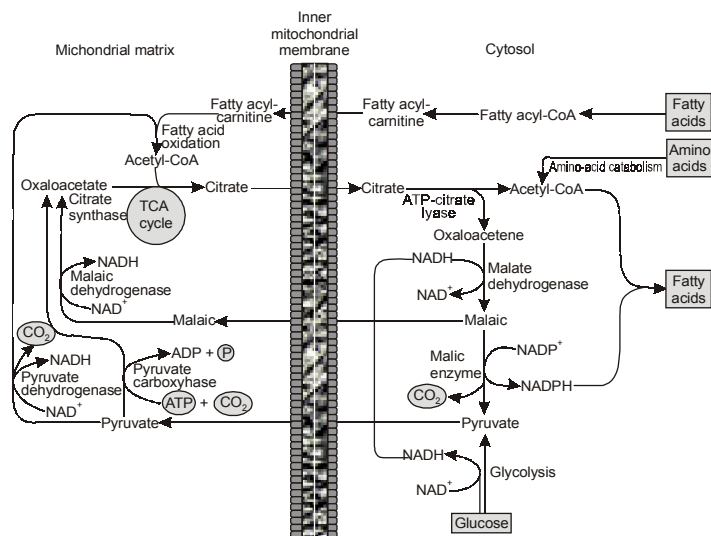
Fig. 5.40 : Showing the Biosynthesis of Fatty Acids

• The acetate units are activated by the formation of malonyl-CoA at the expense of ATP.

• The driving force for the addition of two carbon units to the growing chain is the decarboxylation of malonyl-CoA.

• The chain elongation stops at palmitoyl-CoA.

• Other enzymes add double bonds or additional carbon atom to the carbon chain.

Fatty acid biosynthesis in the cytosol requires a sufficient concentration of NADPH and acetyl-CoA.

NADPH is generated in the cytosol by the pentose phosphate pathway, and by the malic enzyme which oxidizes malate into pyruvate and CO2, generating NADPH. There are 3 principle ways of producing acetyl-CoA in the cytosol of the cell.

1. Amino acid degradation produces acetyl-CoA.

2. Fatty acid oxidation in the matrix of the mitochondria produces acetyl-CoA which is converted into citrate which is transported into the cytosol by the tricarboxylate transporter. ATP-citrate lyase convertes citrate in the cytosol into acetyl-CoA.

3. Glycolysis generates pyruvate which can be carboxylated in the mitochondria into oxaloacetate and then converted into citrate which is transported into the cytosol by the translocase. ATP-citrate lyase converts citrate in the cytosol into acetyl-CoA.

The acetyl-CoA formed by amino acid degradation is insufficient for fatty acid biosynthesis.

I. Acetyl-CoA Carboxylase (ACC)

Acetyl CoA molecules are the building blocks of fatty acid synthesis.

The acetyl CoA molecules need to be activated for fatty acid biosynthesis.
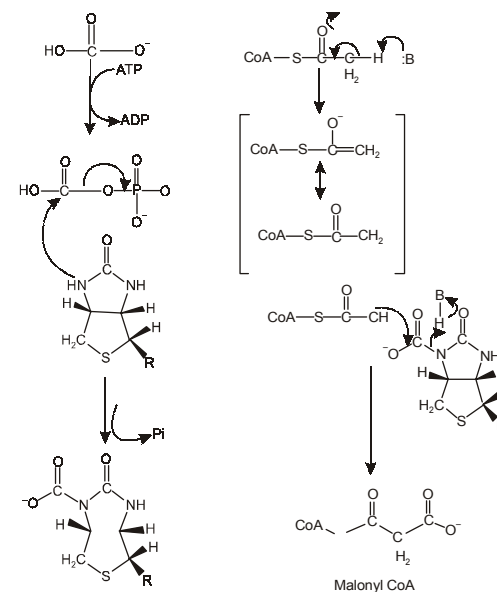
Malonyl CoA

Acetyl CoA is carboxylated to form malonyl CoA by acetyl CoA carboxylase which is a biotin containing enzyme.

The carboxylation reaction is irreversible and is the first committed step of fatty acid biosyntehsis.

The mechanism of this carboxylase is the same as pyruvate carboxylase and propionyl CoA carboxylase.

ATP is used to activate bicrbonate in the form of carboxyphosphate which leads to the carboxylation of biotin.

The activated CO_2 group is transferred to acetyl-CoA to form malonyl CoA.

Acetyl CoA carboxylase has three domains:

1. A biotin carboxyl group carrier protein.

2. Biotin carboxylase which adds CO_2 to biotin.

3. A transcarboxylase which transfers the CO_2 group from biotin to acetyl CoA to form malonyl CoA.
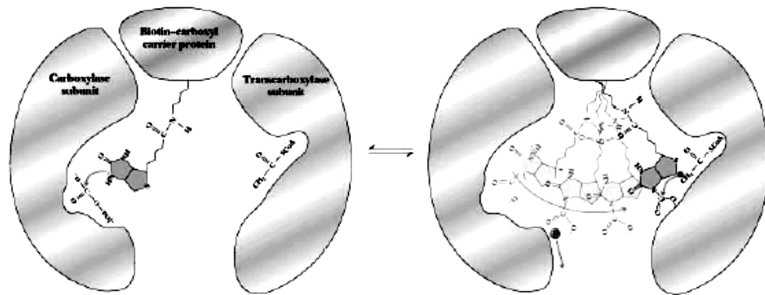
Fig. 5.37 : Showing the Biotin Carboxylase Transferase

Because this is the first committed step of fatty acid biosynthesis, this acetyl CoA carboxylase (ACC) is allosterically regulated. In animals, ACC is a filamentous polymer composed of 230 kD protomers. Each protomer contains the biotin carboxyl carrier protein, the carboxylase and the transcarboxylase domains as well as allosteric regulatory sites. The polymeric form of this enzyme is active, the individual protomers are not. The activity of ACC is dependent of the equilibrium between the two forms of this enzyme.

$$\text{Inactive protaimers} \rightleftharpoons \text{Active polymers}$$
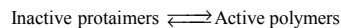
The final product of fatty acid biosynthesis is palmitoyl CoA. There is an allosteric binding site for palmitoyl-CoA which shifts the equilibrium toward the inactive protamers.
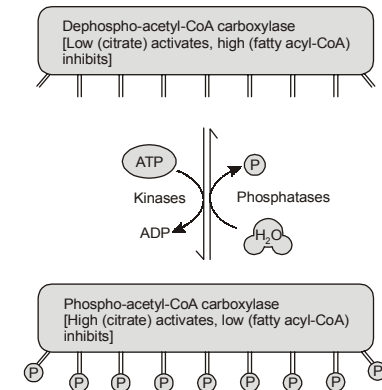
Fig. 5.39 : Showing the Formation of Phospho-acetyl carboxylase
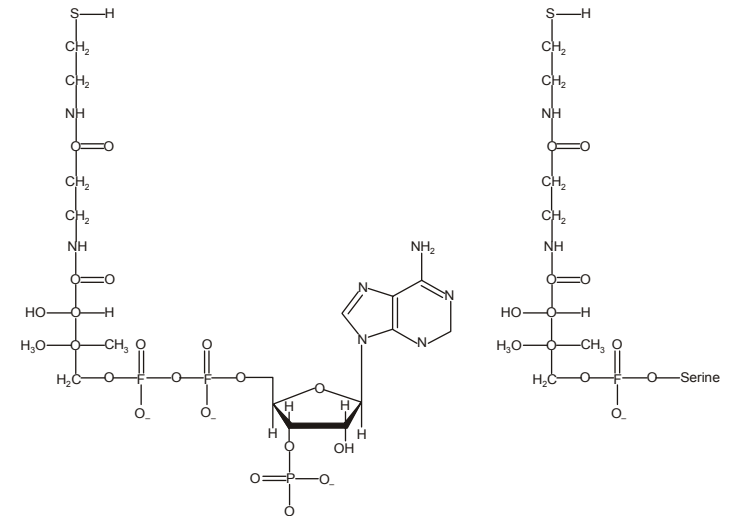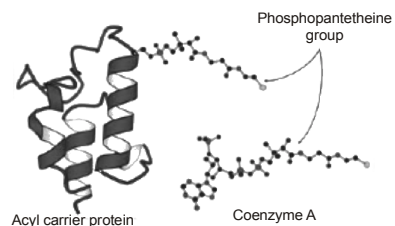
Fig. 5.40 : Showing the Acyl carrier Protein

Citrate which is a precursor for acetyl CoA formation in the cytosol is an allosteric activator of this enzyme. It binds to an allosteric binding site shifting the equilibrium towards the active polymers. The regulatory effects of citrate and palmitoyl CoA are modulated by the phosphorylation state of acetyl-CoA carboxylase. The animal ACC enzyme has 8-10 potential phosphorylation sites on each protomer. Some of these phosphorylation sites are regulatory others are silent and have no effect on enzyme activity. Acetyl CoA carboxylase is phosphorylated by protein kinases and dephosphorylated by protein phosphatases.

The unphosphorylated ACC binds citrate with high affinity and thus becomes fully active a very low citrate concentration. The phosphorylated ACC enzyme has a greatly reduced affinity for citrate, so high concentrations of citrate are required to activate the enzyme. Palmitoyl CoA binds preferably to the phosphorylated form of the enzyme, so when the ACC enzyme is phosphorylated it takes a small concentration of palmitoyl CoA to inactivate it. When the enzyme is dephosphorylated, the dephosphorylated enzyme has a low affinity for palmitoyl CoA and thus it takes a large concentration of palmitoyl CoA to inactivate it. The intermediates of fatty acid biosynthesis are not linked to Coenzyme A. Rather the intermediates of fatty acid biosynthesis are linked to an acyl carrier protein. The acyl carrier protein is very similar to Coenzyme A in that it contains a phosphopantetheine prosthetic group. Coenzyme contains the phosphopantetheine group attached to an adenosine nucleotide. In the acyl carrier protein, the phosphopantetheine group is attached to a serine residue.

SUB-SECTION 5.15A—ELONGATION OF FATTY ACIDS

Before fatty acid biosynthesis begins, fatty acid synthetase must be primed with acetyl CoA. The elongation phase of fatty acid biosynthesis begins with the formation of acetyl ACP and malonyl ACP.

Acetyl transacylase and malonyl transacylase catalyzes the reactions.

Acetyl CoA + ACP $\longrightarrow$ Acetyl ACP + CoA

Malonyl CoA + ACP $\longrightarrow$ Malonyl ACP + CoA
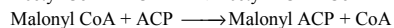
Acetyl transacylase will also catalyze the following reaction at a much slower rate:

Propionyl CoA + ACP $\longrightarrow$ Propionyl ACP + CoA

This reaction allows for the synthesis of fatty acid chains of odd length. Malonyl transacylase is specific for malonyl CoA.
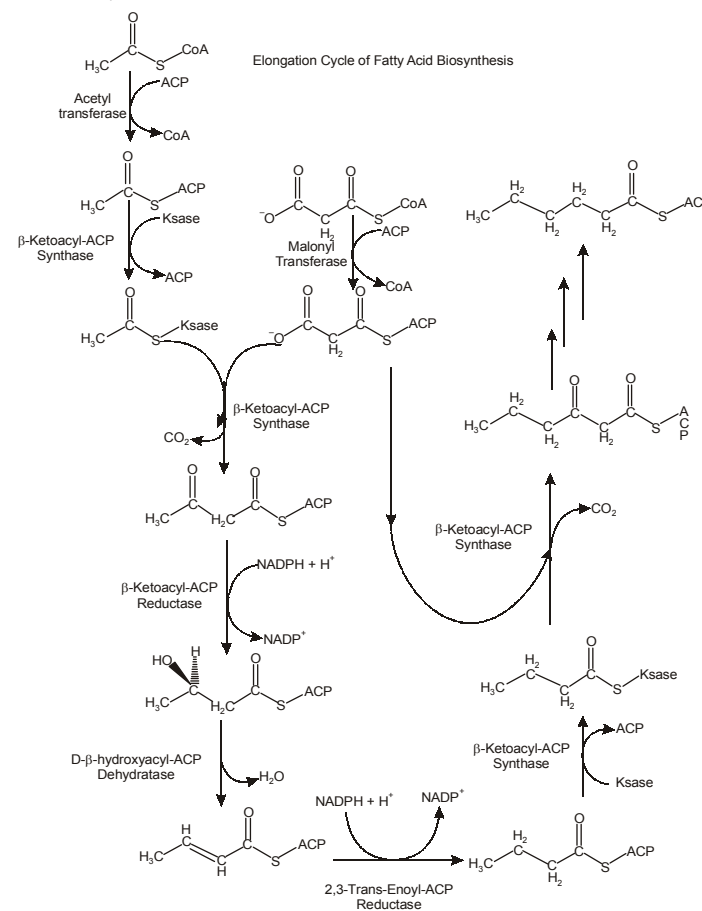
Fig. 5.41 : Showing the Elongation of Fatty Acid Biosynthesis

Once acetyl KSase and malonyl ACP have been formed, elongation can begin. First the acetyl group of acetyl ACP is transferred to a sulfhydryl residue of ketoacyl-ACP synthase also known as acyl-malonyl ACP condensing enzyme. The decarboxylation of malonyl ACP generates an enolate anion which is a good nucleophile that attacks the carbonyl of thioester of acetyl-S-KSase to form acetoacetyl ACP. The exergonic decarboxylation reaction drives the condensation reaction. The CO2 group added to acetyl CoA by acetyl CoA carboxylase is given up in this reaction. In effect this reaction is driven by ATP. ATP was used to activate bicarbonate in the acetyl CoA carboxylase reaction. The free energy was conserved in the malonyl CoA and released upon decarboxylation to drive the synthesis of acetoacetyl CoA. The next three reactions are the reverse of β-oxidation. First the ketone is reduced to the alcohol by the enzyme β-ketoacyl ACP reductase. This reaction differs from the reverse of the β-oxidation reaction in that the D-isomer rather than the L-isomer is formed and NADPH is the reducing agent rather than NADH. The alcohol is dehydrated D-β-hydroxyacyl ACP dehydratase to form crotonyl ACP which is reduced by enoyl-ACP reductase to form butyryl ACP. This step is also different than the mere reversal of β - oxidation in that NADPH is the reducing agent instead of $FADH_2$.

In the next round of fatty acid biosynthesis, butyryl ACP it transferred to the sulfhydryl group of β-ketoacyl-ACP synthase. The decarboxylation of a second molecule of malonyl CoA generates another nucleophile that attacks the carbonyl of the thioester of butyryl-S-KSase to form a C6-β-ketoacyl ACP which is then reduced the β-hydoxyacyl ACP, dehydrated to from the C6-.2-enoyl-ACP which is then reduced to form the C6-acyl-ACP which is then transferred to the sulfhydryl group of β-ketoacyl-ACP synthase, condensed with a third maloyl CoA ect. Ect. Ect., unitil palmitoyl ACP is formed. A thioesterase hydrolyzes the thioester bond of palmitoyl ACP to yield palmitate and ACP. The overall reaction is shown below:

Acetyl CoA + 7 Malonyl CoA + 14 NADPH + $14H^+$ → Palmitate + $7CO_2$ + 14 NADP+ + 8CoA + $6H_2O$
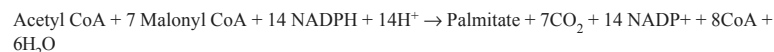
The formation of 7 malonyl CoA requires:

7 Acetyl CoA + 7 CO_2 + 7ATP → 7 Malonyl CoA + 7 ADP + 7 Pi + $7H^+$

Combining these two steps:

8 Acetyl CoA + 7 ATP + 14 NADPH + $7H^+$ → Palmitate + $14NADP^+$ + 7ADP + 7Pi + 8CoA + $6H_2O$

SUB-SECTION 5.15B—DETAIL ACTION OF BIOTIN

Biotin is the prosthetic group of certain enzymes that catalyze CO_2 transfer reaction (CO_2 fixation reaction). In biological system, biotin functions as the co – enzyme for the enzyme called Carboxylase which catalyzes the CO_2 fixation (carboxylation).

In this process biotin is first converted to carboxy biotin complex by reaction with HCO_3- and ATP "CO_2 biotin complex is the source of" active "CO_2 which transferred to the substrate, CO_2 becomes attached to the biotin co enzyme as above.

Example of carboxylation or CO_2 fixation reactions in biologic systems are given ahead and above.
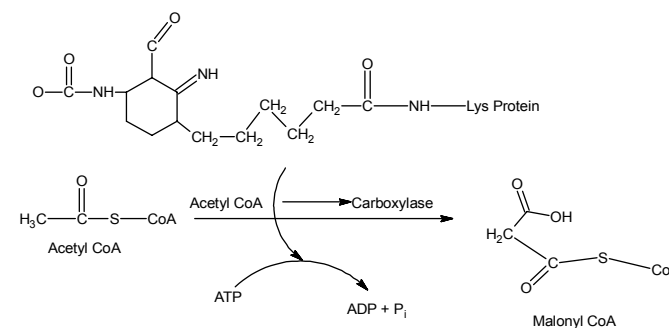
1. *Conversion of acetyl CoA to Matonyl CoA*: In the first step of extra de novo fatty acid synthesis, the acetyl CoA is converted to malonyl CoA, the reaction is catalyzed by the enzyme acetyl CoA carboxylase.
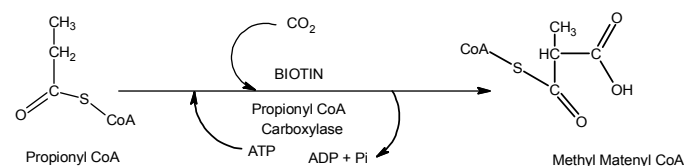
2. *Conversion of Propionyl CoA to Methyl Malonyl CoA*: The enzyme catalyzing the reaction is propionyl – CoA

3. *Conversion of Pyruvic Acid to Oxaloacetate*: The enzyme that catalyzes the reaction is pyruvate carboxylase. Other reactions where Biotin has been incriminated are

4. *Purine Synthesis:* Biotin has been made involved in the fixation of CO_2 for the formation of Carbon 6 of Purine nucleus. The above reaction is impaired in biotin deficient yeast cells, which suggests that Biotin plays an important role in Purine synthesis.

5. *Conversion of β - methyl crotonyl CoA to β - methyl gluconyl CoA*: In their conversion in leucine metabolism the reaction is catalyzed by the enzyme β- methyl crotonyl – CoA carboxylase.

6. *Other enzyme systems*: A number of other enzyme systems are reportedly influenced by Biotin. These include succinic acid dehydrogenase and decarboxylase and the deaminases of the amino acids aspartic acid, serine and threonine.

SUB-SECTION 5.15C—MULTIENZYME COMPLEX CONCEPT

In contrast to bacterial fatty acid biosynthesis, Eukaryotes fatty acid synthase is a multienzyme complexcontained in 2 different polypeptide chains. The α subunit is 213 kD, the β subunit is 203 kD. Animal fatty acid synthase complexes are dimers of αβ subunits. The separate activities of each dimer of αβ subunits are shown in the figure below. The α subunits contain the β-ketoacyl-ACP synthase (KSase) domain and the β-ketoacyl reductase domain. The β subunit contains the acetyl transferase domain, the malonyl transferase domain, the β-hydroxyacyl dehydrogenase domain and the enoyl reductase domain. The subunits are arranged in a head to tail fashion that allows the first domain of one subunit of fatty acid synthase to interact with the second and third domains of the other subunits.
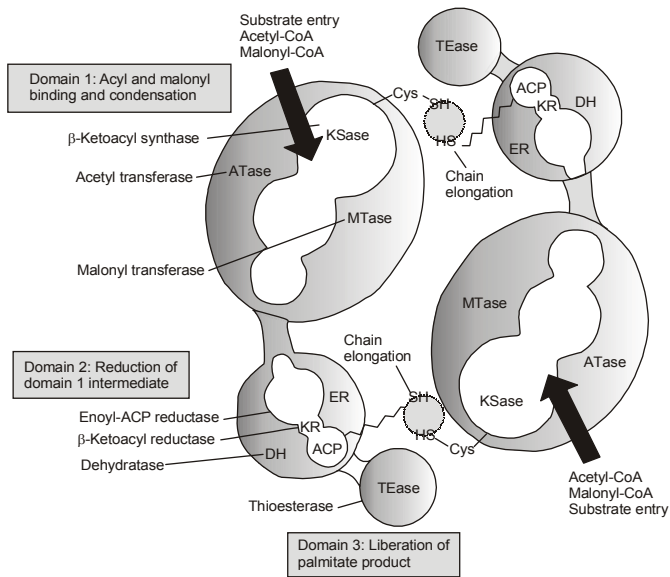
Fig. 5.45 : Showing the Multienzyme Complex Concept

The first step of the fatty acid synthase reaction is the formation of acetyl-O-enzyme intermediate between an acetyl group of acetyl CoA and an active site serine residue of the acetyl transferase domain as shown above on the left. In a similar manner, a malonyl-O-enzyme intermediate is formed between malonyl CoA and an active site serine residue of the malonyl transferase domain as shown above on the
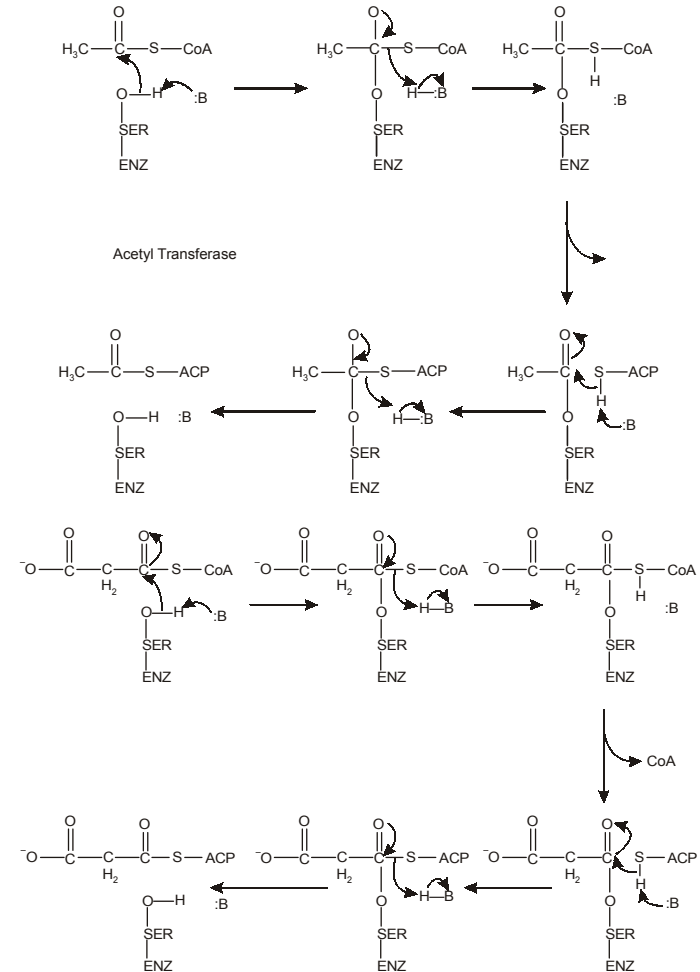
right. The next step is the transfer of the acetyl group to the sulfhydryl of the acyl carrier protein (ACP). This acyl group is then transferred one more time to a cysteine residue of β-ketoacyl-ACP synthase as shown below. This frees the acyl carrier protein to acquire the malonyl group from the malonyl transferase. The next step is the condensation reaction in which decarboxylation of the malonyl-ACP generates a highly reactive nucleophile that attacks the carbonyl of acetyl-S-KSase.
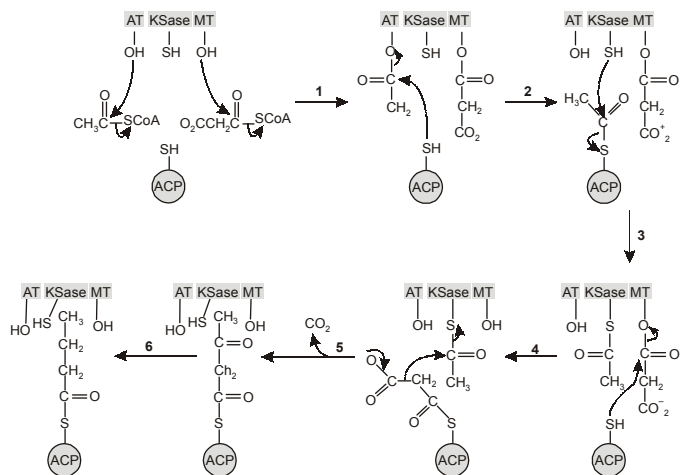
Fig. 5.46 : Showing the Reaction of Multiple Enzyme Complex Concept

The next three steps are the reduction of the carbonyl to the alcohol, the dehydration and the reduction of the alkene to form saturated butyryl-ACP. A second malonyl group is transferred from malonyl CoA to the active site serine of malonyl transferase. The butyryl-ACP is then transferred to the cysteine residue of KSase. This frees the acyl carrier protein to acquire the malonyl group from the malonyl transferase. The next step is the condensation reaction in which decarboxylation of the malonyl-ACP generates a highly reactive nucleophile that attacks the carbonyl of butyryl-S-KSase. This cycle continues until palmitoyl ACP is formed. The thioester bond is hydrolyzed by thioesterase to form palmitate.

  (i) Glucorticoids depress update of glucose and there is less α - glycerol – P available. Thus decreases rate of esterification.

 (ii) Stimulates synthesis of adenylate cyclase thus increasing C – AMP level in the cells.

(iii) Facilitates adipokinetic property of growth hormones and

(iv) Increases synthesis of new lipase protein by a c – AMP independent pathway.

SECTION 5.16—METABOLISM OF CHOLESTEROL

Cholesterol is oxidized by the liver into a variety of bile acids. These in turn are conjugated with glycine, taurine, glucuronic acid, or sulfate. A mixture of conjugated and non-conjugated bile acids along with cholesterol itself is excreted from the liver into the bile. Approximately 95% of the bile acids are reabsorbed from the intestines and the remainder lost in the feces. The excretion and reabsorption of bile acids forms the basis of the enterohepatic circulation which is essential for the digestion and absorption of dietary fats. Under certain circumstances, when more concentrated, as in the gallbladder, cholesterol crystallizes and is the major constituent of most gallstones, although lecithin and bilirubin gallstones also occur less frequently.

SUB-SECTION 5.16A—BIOSYNTHESIS OF CHOLESTEROL

Essentially all tissues form cholesterol, liver is the major site of cholesterol biosynthesis also other tissues are active in this regard e.g. adrenal cortex gonads within intestine are also most active.

Low order of synthesis – adipose tissue, muscle aorta and neutral tissues. Brain of new born synthesize cholesterol while the adult brain cannot synthesis cholesterol.

Efficiency of formation of cholesterol from labeled C^{14} acetate

Tissues	Efficiency of Cholesterol formation liver (100)
Liver	100
Adult Skin	90
Small Intestine	60
Kidney	4
Adult brain	0
New born Brain	185

Enzymes involved in the cholesterol biosynthesis are:

 1. Cytoplasmic particles "microsomes"

 2. Soluble fraction - crystal

'Active' acetate (acetyl CoA) is the starting material and principal precursor. The entire carbon – skeleton, all 27C of cholesterol in humans can be synthesis from the active acetate.

Cholesterol biosynthesis takes place in five groups of reactions. They are:

 1. Synthesis of Mevalnoate – a – 6 – C compound from Acetyl CoA.

 2. Formation of 'Iso – Pernoid units (C – 5) from Melvonate by successive phosphorylation and followed by loss of CO_2
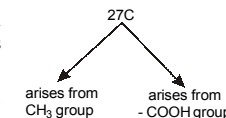
3. Formation of Squalene A 30 carbon aliphatic chain formed by condensation of six isoprenoid units*

4. Cyclization of Squalene to form Lanosterol

5. Conversion of Lanosterol to Cholesterol

Synthesis of Mevalnoate From Acetyl CoA

(a) Formation of HMG – CoA (β - OH methyl glutaryl CoA) HMG – CoA can be formed in the crystal from acetyl CoA in two steps catalyzed by the enzyme "Thiolase" and "HMG – CoA synthase"*

β -OH - β - methylglutaryl CoA (HMG - CoA)
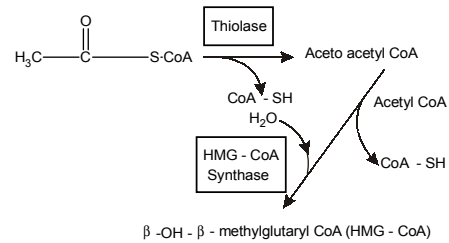
(b) In the next step, which is the "rate of limiting" step HMG CoA is converted to Mevalnoic acid (Mevalnoate) catalyzed by the enzyme HMG – CoA reductase.
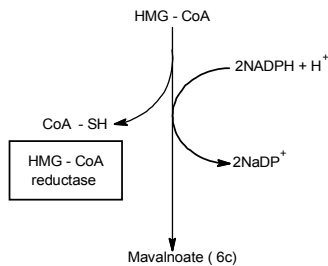
*The isoprenoid units are regarded as the building blocks of the steroids nucleus

*1. MG-CoA may also be produced as an intermediate in the metabolic degradation of ammonical L-Leucine.

2. There are two pools of HMG-CoA

(i) Mitochondial-concerned with Ketogenesis

(ii) Extramitochondiral (systolic) concerned with synthesis of Mevalnoate and isopernoid.

Characteristic of this Reaction

1. Most important and "rate limiting" step

2. Irreversible reaction

3. Enzyme contains - SH group

4. NADPH required as co –factor supplied by HMP – pathway

5. Enzyme activity not affected in Diabetic patients.

6. Diatory cholesterol and endogenously synthesized cholesterol inhibits this rate limiting step.

7. Hormones – Insulin and Thyroid increases the reductase activity Glucagon and glucocorticoids reduces the activity.
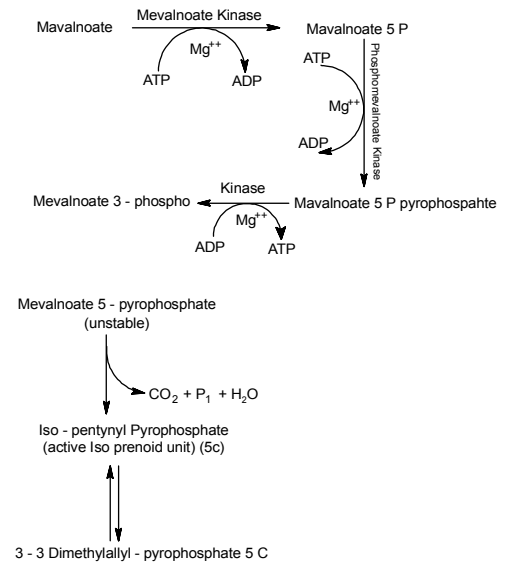
Formation of Isoprenoid Units

1. Mevalnoate is phosphorylated by ATP to form several 'active' phosphorylated intermediates.

2. Three such phosphorylated compounds are formed and it is followed by decarboxylation to form first "active" iso – prenoid unit Iso – pentenyl Pyrophosphate (5c). One of the intermediate pyrophosphorylated compound is "Mevalnoate – 3 – phosphor – 5 – Pyrophosphate" which is unstable

3. Iso – pentyl pyrophosphate under goes isomerization to form another 5 C iso – prenoid unit called " 3 – 3 Dimethyl allyl Pyrophosphate"

Formation of Squalene

(a) The Pyrophosphorylated isoprenoid units condense to form ultimately a 30 – carbon aliphatic chain called Squalene.

(b) The condensation occurs in three steps:

(i) One molecule of iso – pentenyl pyrophosphate first condenses with one molecule of 3, 3 dimethyl allyl prophopshate to form a 10 C compound called "generyl pyrophosphate" the reaction is catalyzed by the enzyme pyrophosphate synthase.

3 - 3 Dimethylallyl - pyrophosphate $\xrightarrow{\text{Isomerises}}$ Iso pentenyl Pyrophosphate

$\to PP_1$

Generyl Pyrophosphate Synthase

Farnesyl Pyrophosphate (15C)

2 molecules condensation (microsomal)

NADPH + H$^+$

Mg^{++} + Mn^{++}, Co^{++}

Squalene Synthase
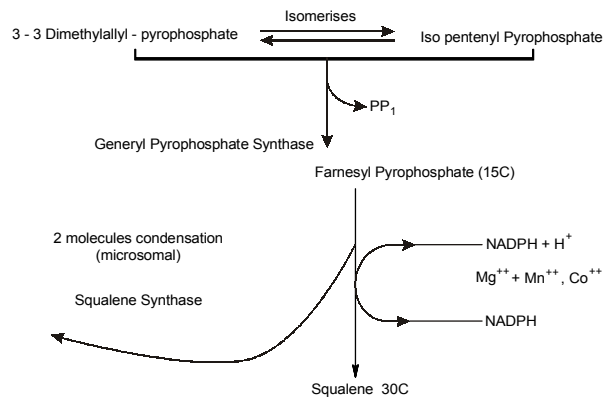
NADPH

Squalene 30C

Cyclization of Squalene to Lanosterol Formation

Generally this formation requires two steps to complete

(1) In the first step Squalene – 2 – 3 – expoxide is formed catalyzed by the enzyme Squalene mono – oxygenated which requires NADPH and molecular O_2

(2) In the next step an enzyme cyclase brings about the Cyclization of Squalene to form Lanosterol.

molecular O_2

Sqalene monoxygenase

Squalene $\longrightarrow$ Squalene 2, 3 expoxide

Cyclase
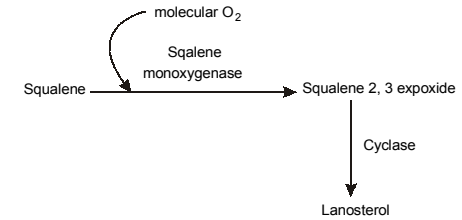
Lanosterol

Conversion of Lanosterol to Cholesterol

Main changes are as follows:

(1) Removal of three angular CH_3 group. This involves a series of reaction mechanism - demethylation is not properly known. CH_3 group at C14 is first eliminated

(2) Shift of double bond between C_8 and C_9 to C_5 and C_6

(3) Saturation of double bond in side chain

Therefore two possible pathways are been illustrated

Lanosterol

$\to CH_4$

14 Desmethyl lanosterol

$\to 2CH_3$

Zymosterol

$\Delta^{7, 24}$ —Cholestadienol

Cholesterol

Lanosterol

24, 25 dihydro lanosterol

α 4 methyl - Δ^8 Cholesterol

α 4 methyl - Δ^7 Cholesterol

Δ^7 Cholesterol
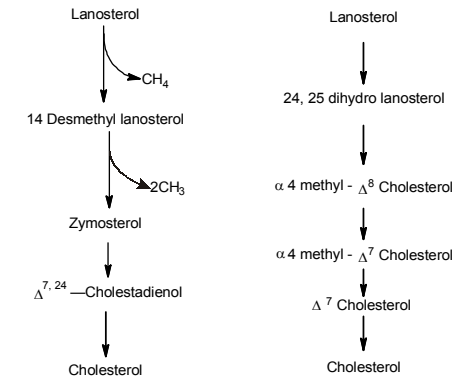
Cholesterol

SUB-SECTION 5.21A—CONTROL OF CHOLESTEROL BIOSYNTHESIS

1. The step in the biosynthesis of cholesterol to HMG CoA are reversible. The formation of Mevalnoate however in the next step is "irreversible" and is the "commited step".

2. The "rate limiting step in the biosyntheiss of cholesterol is the conversion of cholesterol is the conversion of HMG CoA to Mevalnoate by enzyme HMG CoA reductase*

3. Fast starving also inhibits the enzyme and activate HMG – CoAlyase to form ketone bodies.

4. A second control point appears to be at the Cyclization of sqwalene and conversion to Lanosterol, but details of the regulation at this step is not clear.

5. The feeding of cholesterol reduces the hepatic biosynthesis of cholesterol by reducing the activity of HMG – CoA reductase.

   In contrast feeding of diets high in fat or carbohydrates tend to increase hepatic cholesterol biosynthesis.

   Intestinal cholesterol biosynthesis does not respond to the feeding of high cholesterol diets

6. Role of cyclic AMP

   HMG – CoA reductase may exist in active and 'inactive' forms which is 'reversely' modified by phosphorylation dephosphorylation mechanisms which mechanisms which may be C – AMP dependant protein kinases Cyclic AMP inhibits cholesterol biosynthesis by converting HMF – CoA reductase to inactivate form.

SUB-SECTION 5.21B—CHOLESTEROL LOWERING DRUGS THEIR MECHANISM OF ACTION

Several drugs are known to block the formation of cholesterol are various stages in the biosynthetic pathway. Some may increase the catabolism/excretion of cholesterol also; many of the drugs have harmful side effects. The table below shows the drugs used cholesterol limiting.

Drugs	Mechanism of action
1	2
1. Aromatically substituted carboxylic acids— e.g. p-phenyl butyrate, p-bi-phenyl butyrate, p-bi-phenyl butyrate	• Inhibits acetate incorporation. Experiments in human volunteers-disappointing (Not used).
2. Triparanol compounds related to non-steroidal estrogens and estrogen antagonists	• Inhibits reduction of desmosterol. After widespread use-the drug was withdrawn due to side effects like—cataract, alopecia, change in hair colour, Leucopenia. (Not used)
3. Pro-adifen HCl	• Blocks pathway between mevalonate to squalene (Not used-due to side efects)

*Cholesterol itself inhibits the enzymes producing an effective product feedback inhibition controlling the synthesis.

1	2
4. Nicotinic acid— In large doses has hypocholesterolaemic effect.	• Reduces the flux of FFA by inhibiting adipose issue lipolysis, thereby inhibiting VLDL production in Liver. In larges does—may produce fatty Liver.
5. Oestrogen	• Lower cholesterol level and increases HDI.
6. Sitosterol	• Acts by blocking esterification of cholesterolin gut thus reducing cholesterol absorption. (Synthesis may be increased later on).
7. Dexitrothyroxine (cholaxin), and Neomycin	• Increases faecal excretion of cholesterol and bile acids.
8. Clofibrate (Atromid S) Gemfibrozil CPI B (Ethyl-p-cholorophenoxy isobutyrate)	• Acts by various ways— (i) inhibiting secretion of VLDL by liver, (ii) inhibiting hepatic cholesterol synthesis, (iii) probbly also increases faecal excretion, (iv) they facilitate hydrolysis of VLDL triacylglycerol by lipoprotein lipase (commonly used)
9. Certain Resins, e.g. • colestipol • cholestyramine (Questran)	• Prevent the reabsorption of bile salts by combining with them, increasing their faecal loss.
10. Probucol	• Increases catabolism of LDL by receptor—independant pathway.
11. Mevastatin • Lovostatin (Recent drugs—obtained from fungi)	• Reducess LDL cholesterol level—Few adverse effects (most commonly used)

SECTION 5.22—TRIGLYCERIDE AND PHOSPHOLIPID BIOSYNTHESIS

Fatty acids are stored for future use as triacylglycerols (TAGs) in all cells, but primarily in adipocytes of adipose tissue. TAGs constitute molecules of glycerol to which three fatty acids have been esterified. The fatty acids present in TAGs are predominantly saturated. The major building block for the synthesis of TAGs, in tissues other than adipose tissue, is glycerol. Adipocytes lack glycerol kinase, therefore, dihydroxyacetone phosphate (DHAP), produced during glycolysis, is the precursor for TAG synthesis in adipose tissue. This means that adipoctes must have glucose to oxidize in order to store fatty acids in the form of TAGs. DHAP can also serve as a backbone precursor for TAG synthesis in tissues other than adipose, but does so to a much lesser extent than glycerol.
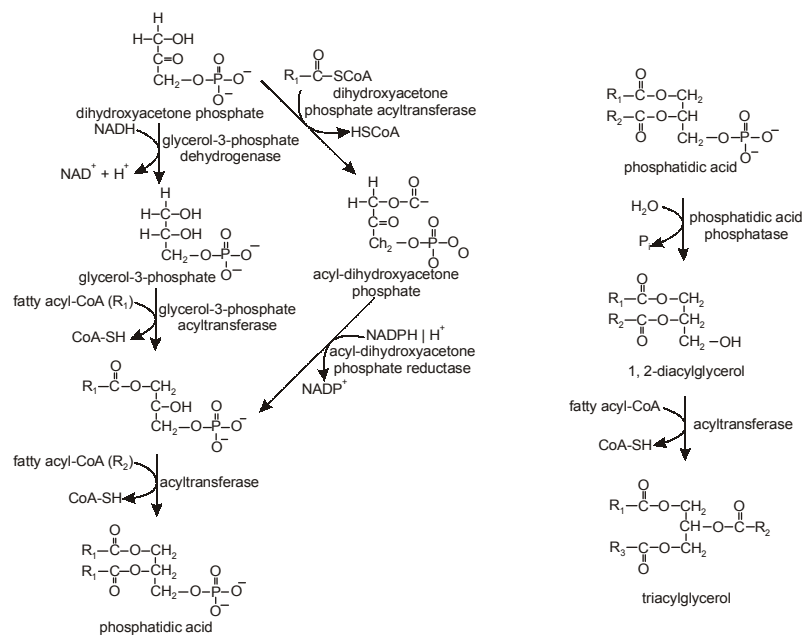
Phosphatidic acid Synthesis

Triglyceride Synthesis

The glycerol backbone of TAGs is activated by phosphorylation at the C-3 position by glycerol kinase. The utilization of DHAP for the backbone is carried out through either of two pathways depending upon whether the synthesis of triglycerides is carried out in the mitochondria and ER or the ER and the peroxisomes. In the former case the action of glycerol-3-phosphate dehydrogenase, a reaction that requires NADH (the same reaction as that used in the glycerol-phosphate shuttle), converts DHAP to glycerol-3-phosphate. Glycerol-3-phosphate acyltransferase then esterifies a fatty acid to glycerol-3-phosphate generating the monoacylglycerol phosphate structure called lysophosphatidic acid. The second reaction pathway utilizes the peroxisomal enzyme DHAP acyltransferase to fatty acylate DHAP to acyl-DHAP which is then reduced by the NADPH-requiring enzyme acyl-DHAP reductase. An interesting feature of the latter pathway is that DHAP acyltransferase is one of only a few enzymes that are targeted to the peroxisomes through the recognition of a peroxisome targeting sequence 2 (PTS2) motif in the enzyme. Most peroxisomal enzymes contain a PTS1 motif. For more information on peroxisome enzymes

The fatty acids incorporated into TAGs are activated to acyl-CoAs through the action of acyl-CoA synthetases. Two molecules of acyl-CoA are esterified to glycerol-3-phosphate to yield 1,2-diacylglycerol phosphate (commonly identified as phosphatidic acid). The phosphate is then removed, by phosphatidic acid phosphatase (PAP1), to yield 1,2-diacylglycerol, the substrate for addition of the third fatty acid. Intestinal monoacylglycerols, derived from the hydrolysis of dietary fats, can also serve as substrates for the synthesis of 1,2-diacylglycerols.

Recent studies have identified a critical role for the enzyme PAP1 in overall TAG and phospholipid homeostasis. In the yeast Saccharomyces cerevisiae, the PAP1 gene was identified as Smp2p and the encoded protein was shown to be the yeast ortholog of the mammalian protein called lipin-1. The fission yeast lipin-1 ortholog is identified as Ned1p. Lipin-1 is only one of four lipin proteins identified in mammals. The lipin-1 gene (symbol = LPN1) was originally identified in a mutant mouse called the fatty liver dystrophy (fld) mouse. The mutation causing this disorder was found to reside in the LPN1 gene. There are three lipin genes with the LPN1 gene encoding two isoforms derived through alternative splicing. These two lipin-1 isoforms are identified as lipin-1A and lipin-1B. Mutations in the LPN2 gene have recently been associated with Majeed syndrome which is characterized by chronic recurrent osteomyelitis, cutaneous inflammation, recurrent fever, and congenital dyserythropoietic anemia. In addition to the obvious role of lipin-1 in TAG synthesis, evidence indicates that the protein is also required for the development of mature adipocytes, coordination of peripheral tissue glucose and fatty acid storage and utilization, and serves as a transcriptional co-activator. The latter function has significance to diabetes as it has been shown that some of the effects of the thiazolidinedione (TZD) class of drugs used to treat the hyperglycemia associated with type 2 diabetes are exerted via the effects of lipin-1. Lipin-1 has been shown to interact with peroxisome proliferator-activated receptor-? [PPAR?] co-activator 1? (PGC-1?) and PPAR?. The interactions of lipin-1 with these other transcription factors leads to increased expression of fatty acid oxidizing genes such as carnitine palmitoyl transferase-1, acyl CoA oxidase, and medium-chain acylCoA dehydrogenase (MCAD).

Phospholipid Structures

Phospholipids are synthesized by esterification of an alcohol to the phosphate of phosphatidic acid (1,2-diacylglycerol 3-phosphate). Most phospholipids have a saturated fatty acid on C-1 and an unsaturated fatty acid on C-2 of the glycerol backbone. The most commonly added alcohols (serine, ethanolamine and choline) also contain nitrogen that may be positively charged, whereas, glycerol and inositol do not. The major classifications of phospholipids are:

Phosphatidylcholine (PC)

Phosphatidylethanolamine (PE)

	Phosphatidylserine (PS)
	Phosphatidylinositol (PI)
	Phosphatidylglycerol (PG)
	Diphosphatidylglycerol (DPG)
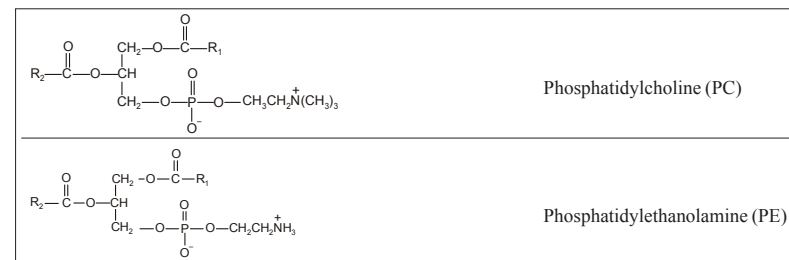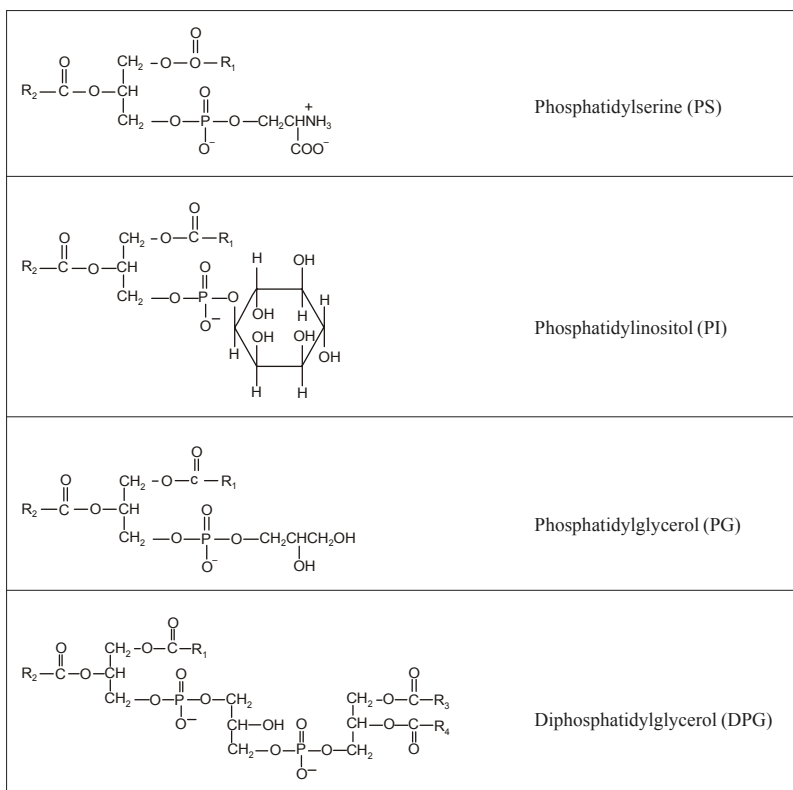
Phospholipid Synthesis

Phospholipids can be synthesized by two mechanisms. One utilizes a CDP-activated polar head group for attachment to the phosphate of phosphatidic acid. The other utilizes CDP-activated 1,2-diacylglycerol and an inactivated polar head group.

PC:This class of phospholipids is also called the lecithins. At physiological pH, phosphatidylcholines are neutral zwitterions. They contain primarily palmitic or stearic acid at carbon 1 and primarily oleic, linoleic or linolenic acid at carbon 2. The lecithin dipalmitoyllecithin is a component of lung

or pulmonary surfactant. It contains palmitate at both carbon 1 and 2 of glycerol and is the major (80%) phospholipid found in the extracellular lipid layer lining the pulmonary alveoli. Choline is activated first by phosphorylation and then by coupling to CDP prior to attachment to phosphatidic acid. PC is also synthesized by the addition of choline to CDP-activated 1,2-diacylglycerol. A third pathway to PC synthesis, involves the conversion of either PS or PE to PC. The conversion of PS to PC first requires decarboxylation of PS to yield PE; this then undergoes a series of three methylation reactions utilizing S-adenosylmethionine (SAM) as methyl group donor.

PE:These molecules are neutral zwitterions at physiological pH. They contain primarily palmitic or stearic acid on carbon 1 and a long chain unsaturated fatty acid (e.g. 18:2, 20:4 and 22:6) on carbon 2. Synthesis of PE can occur by two pathways. The first requires that ethanolamine be activated by phosphorylation and then by coupling to CDP. The ethanolamine is then transferred from CDP-ethanolamine to phosphatidic acid to yield PE. The second involves the decarboxylation of PS.

PS:Phosphatidylserines will carry a net charge of -1 at physiological pH and are composed of fatty acids similar to the phosphatidylethanolamines. The pathway for PS synthesis involves an exchange reaction of serine for ethanolamine in PE. This exchange occurs when PE is in the lipid bilayer of the a membrane. As indicated above, PS can serve as a source of PE through a decarboxylation reaction.

PI:These molecules contain almost exclusively stearic acid at carbon 1 and arachidonic acid at carbon 2. Phosphatidylinositols composed exclusively of non-phosphorylated inositol exhibit a net charge of -1 at physiological pH. These molecules exist in membranes with various levels of phosphate esterified to the hydroxyls of the inositol. Molecules with phosphorylated inositol are termed polyphosphoinositides. The polyphosphoinositides are important intracellular transducers of signals emanating from the plasma membrane. The synthesis of PI involves CDP-activated 1,2-diacylglycerol condensation with myo-inositol. PI subsequently undergoes a series of phosphorylations of the hydroxyls of inositol leading to the production of polyphosphoinositides. One polyphosphoinositide (phosphatidylinositol 4,5-bisphosphate, PIP_2) is a critically important membrane phospholipid involved in the transmission of signals for cell growth and differentiation from outside the cell to inside.

PG:Phosphatidylglycerols exhibit a net charge of -1 at physiological pH. These molecules are found in high concentration in mitochondrial membranes and as components of pulmonary surfactant. Phosphatidylglycerol also is a precursor for the synthesis of cardiolipin. PG is synthesized from CDP-diacylglycerol and glycerol-3-phosphate. The vital role of PG is to serve as the precursor for the synthesis of diphosphatidylglycerols (DPGs).

DPG:These molecules are very acidic, exhibiting a net charge of -2 at physiological pH. They are found primarily in the inner mitochondrial membrane and also as components of pulmonary

surfactant. One important class of diphosphatidylglycerols is the cardiolipins. These molecules are synthesized by the condensation of CDP-diacylglycerol with PG.

The fatty acid distribution at the C-1 and C-2 positions of glycerol within phospholipids is continually in flux, owing to phospholipid degradation and the continuous phospholipid remodeling that occurs while these molecules are in membranes. Phospholipid degradation results from the action of phospholipases. There are various phospholipases that exhibit substrate specificities for different positions in phospholipids.

In many cases the acyl group which was initially transferred to glycerol, by the action of the acyl transferases, is not the same acyl group present in the phospholipid when it resides within a membrane. The remodeling of acyl groups in phospholipids is the result of the action of phospholipase A1 (PLA$_1$) and phospholipase A2 (PLA$_2$).
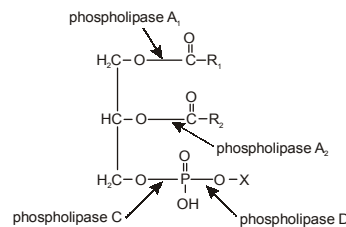
Sites of Action of the Phospholipases A1, A2, C and D

The products of these phospholipases are called lysophospholipids and can be substrates for acyl transferases utilizing different acyl-CoA groups. Lysophospholipids can also accept acyl groups from other phospholipids in an exchange reaction catalyzed by lysolecithin:lecithin acyltransferase (LLAT).

PLA2 is also an important enzyme, whose activity is responsible for the release of arachidonic acid from the C-2 position of membrane phospholipids. The released arachidonate is then a substrate for the synthesis of the eicosanoids. In fact there is not just a single PLA2 enzyme. At least 19 enzymes have been identified with PLA2activity. There are 10 isozymes that are in the secretory pathway and these PLA2 isozymes are abbreviated sPLA2. These secretory enzymes are low molecular weight proteins that are Ca2+-requiring and are involved in numerous processes including modification of eicosanoid generation, host defense, and inflammation. The cytosolic PLA2family (cPLA2) comprises three isozymes with cPLA2? being an essential component of the initiation of arachidonic acid metabolism. Like the sPLA2 enzymes, the cPLA2 enzymes are tightly regulated by Ca2+. In addition, this class of PLA2 enzyme is regulated by phosphorylation. There is an additional family of two PLA2 isozymes that are not dependent on Ca2+ for activity and they identified as iPLA2. This latter class of enzyme is involved primarily with the remodeling of phospholipids.

SECTION 5.23—METABOLISMS OF PROTEINS

SUB-SECTION 5.23A—DIATARY REQUIREMENTS

Proteins are broken down in the stomach during digestion by enzymes known as proteases into smallerpolypeptides to provide amino acids for the organism, including the essential amino acids that the organism cannot biosynthesize itself. Aside from their role in protein synthesis, amino acids are also important nutritional sources of nitrogen.

Proteins, like carbohydrates, contain 4 kilocalories per gram as opposed to lipids which contain 9 kilocalories and alcohols which contain 7 kilocalories. The liver, and to a much lesser extent the kidneys, can convert amino acids used by cells in protein biosynthesis into glucose by a process known asgluconeogenesis. The amino acids leucine and lysine are exceptions.

Dietary sources of protein include meats, eggs, nuts, grains, legumes, and dairy products such as milk and cheese.[1] Of the 20 amino acids used by humans in protein synthesis, 11 "nonessential" amino acids can be synthesized in sufficient quantities by the adult body, and are not required in the diet (though there are exceptions for some in special cases). The nine essential amino acids, plus arginine for the young[3], cannot be created by the body and must come from dietary sources.

Most animal sources and certain vegetable sources have the complete complement of all the essential amino acids in adequate proportions. However, it is not necessary to consume a single food source that contains all the essential amino acids, as long as all the essential amino acids are eventually present in the diet: see complete protein and protein combining.

Different proteins have different levels of biological availability (BA) to the human body. Many methods have been introduced to measure protein utilization and retention rates in humans. They include biological value, net protein utilization, and PDCAAS (Protein Digestibility Corrected Amino Acids Score) which was developed by the FDA as an improvement over the Protein Efficiency Ratio (PER) method. These methods examine which proteins are most efficiently used by the body. In general they conclude that animal complete proteins that contain all the essential amino acids such as milk, eggs, and meat, and the complete vegetable protein soy are of most value to the body.

Egg whites have been determined to have the standard biological value of 100 (though some sources may have biological values higher), which means that most of the absorbed nitrogen from egg white protein can be retained and used by the body. The biological value of plant protein sources is usually considerably lower than animal sources.[4] For example, corn has a BA of 70 while peanuts have a relatively low BA of 40.

According to the recently updated US/Canadian Dietary Reference Intake guidelines, women aged 19-70 need to consume 46 grams of protein per day, while men aged 19-70 need to consume 56 grams of protein per day to avoid a deficiency. The difference is because men's bodies generally have more muscle mass than those of women, or this may be attributed to weight difference by taking 0.8 g(of protein)/kg of lean body weight.

Because the body is continually breaking down protein from tissues, even adults who do not fall into the above categories need to include adequate protein in their diet every day. If enough energy is not taken in through diet, as in the process of starvation, the body will use protein from the muscle mass to meet its energy needs, leading to muscle wasting over time. If the body does not consume adequate protein in nutrition, then muscle will also waste as more vital cellular processes (e.g. respiration enzymes, blood cells) recycle muscle protein for their own requirements.

Other recommendations suggest 0.8 gram of protein per kilogram of lean bodyweight per day while other sources suggest that higher intakes of 1-1.4 grams of protein per kilogram of bodyweight for enhanced athletes or those with a large muscle mass.

How much protein needed in a person's daily diet is determined in large part by overall energy intake, as well as by the body's need for nitrogen and essential amino acids. Physical activity and exertion as well as enhanced muscular mass increase the need for protein. Requirements are also greater during childhood for growth and development, during pregnancy or when breast-feeding in order to nourish a baby, or when the body needs to recover from malnutrition or trauma or after an operation.

Protein deficiency is a serious cause of ill health and death in developing countries. Protein deficiency plays a part in the disease kwashiorkor. War, famine, overpopulation and other factors can increase rates of malnutrition and protein deficiency. Protein deficiency can lead to reduced intelligence or mental retardation, see deficiency in proteins, fats, carbohydrates.

In countries that suffer from widespread protein deficiency, food is generally full of plant fibers, which makes adequate energy and protein consumption very difficult. Symptoms of kwashiorkor include apathy, diarrhea, inactivity, failure to grow, flaky skin, fatty liver, and edema of the belly and legs. This edema is explained by the normal functioning of proteins in fluid balance and lipoprotein transport. [12]

Dr. Latham, director of the Program in International Nutrition at Cornell University claims that malnutrition is a frequent cause of death and disease in third world countries. Protein-energy malnutrition (PEM) affects 500 million people and kills 10 million annually. In severe cases white blood cell numbers decline and the ability of leukocytes to fight infection decreases.

Protein deficiency is relatively rare in developed countries but some people have difficulty getting sufficient protein due to poverty. Protein deficiency can also occur in developed countries in people who are dieting or crash dieting to lose weight, or in older adults, who may have a poor diet. Convalescent people recovering from surgery, trauma, or illness may become protein deficient if they do not increase their intake to support their increased needs.

The body is unable to store excess protein. Protein is digested into amino acids which enter the bloodstream. Excess amino acids are converted to other usable molecules by the liver in a process called deamination. Deamination converts nitrogen from the amino acid into ammonia which is converted by the liver into urea in the urea cycle. Excretion of urea is performed by the kidneys. These organs can normally cope with any extra workload but if kidney disease occurs, a decrease in protein will often be prescribed.

Many researchers think excessive intake of protein forces increased calcium excretion. If there is to be excessive intake of protein, it is thought that a regular intake of calcium would be able to stabilize, or even increase the uptake of calcium by the small intestine, which would be more beneficial in older women.

Specific proteins are often the cause of allergies and allergic reactions to certain foods. This is because the structure of each form of protein is slightly different; some may trigger a response from the immune system while others remain perfectly safe. Many people are allergic to casein, the protein in milk; gluten, the protein in wheat and other grains; the particular proteins found in peanuts; or those in shellfish or other sea foods.

The classic assay for protein concentration in food is the Kjeldahl method*. This test determines the total nitrogen in a sample. The only major component of most food which contains nitrogen, is protein (fat, carbohydrate and dietary fibre do not contain nitrogen). If the amount of nitrogen is multiplied by a factor depending on the kinds of protein expected in the food the total protein can be determined. On food labels the protein is given by the nitrogen multiplied by 6.25, because the average nitrogen content of proteins is about 16%. The Kjeldahl test is used because it is the method the AOAC International has adopted and is therefore used by many food standards agencies around the world.

The limitations of the Kjeldahl method were at the heart of the 2008 Chinese Milk Scandal in which the toxic chemical melamine was added to the milk to increase the measured "protein".

SUB-SECTION 5.23B—BIOLOGICAL VALUE

Biological value (BV) is a measure of the proportion of absorbed protein from a food which becomes incorporated into the proteins of the organism's body. It summarizes how readily the broken down protein can be used in protein synthesis in the cells of the organism. Proteins are the major source of nitrogen food, unlike carbohydrates and fats. This method assumes protein is the only source of nitrogen and measures the proportion of this nitrogen absorbed by the body which is then excreted. The remainder must have been incorporated into the proteins of the organisms body. A ratio of nitrogen incorporated into the body over nitrogen absorbed gives a measure of protein 'usability' - the BV.

Unlike some measures of protein usability, biological value does not take into account how readily the protein can be digested and absorbed (largely by the small intestine). This is reflected in the experimental methods used to determine BV.

BV, confusingly, uses two similar scales:

1. The true percentage utilization (usually shown with a percent symbol).
2. The percentage utilization relative to a readily utilizable protein source, often egg (usually shown as unit less).

These two values will be similar but not identical.

The BV of a food varies greatly, and depends on a wide variety of factors. In particular the BV value of a food varies depending on its preparation and the recent diet of the organism. This makes

*see page 2781 A chemical analyzer's guide.

reliable determination of BV difficult and of limited use - fasting prior to testing is universally required in order to make the values reliable.

BV is commonly used in nutrition science in many mammalian organisms, and is a relevant measure in humans. It is a popular guideline in body building in protein choice.

For accurate determination of BV

1. the test organism must only consume the protein or mixture of proteins of interest (the test diet).
2. the test diet must contain no non-protein sources of nitrogen.
3. the test diet must be of suitable content and quantity to avoid use of the protein primarily as an energy source.

These conditions mean the tests are typically carried out over the course of over one week with strict diet control. Fasting prior to testing helps produce consistency between subjects (it removes recent diet as a variable).

There are two scales on which BV is measured; percentage utilization and relative utilization. By convention percentage BV has a percent sign (%) suffix and relative BV has no unit.

Percentage utilization

Biological value is determined based on this formula.

$$BV = (N_r / N_a) * 100$$

Where:

N_a = nitrogen absorbed in proteins on the test diet

N_r = nitrogen incorporated into the body on the test diet

> However direct measurement of Nr is essentially impossible. It will typically be measured indirectly from nitrogen excretion in urine. Faecalexcretion of nitrogen must also be taken into account - this protein is not absorbed by the body and so not included in the calculation of BV.
>
> $$BV = ((N_i - N_{e(f)} - N_{e(u)} - N_b) / N_i - N_{e(f)}) * 100$$
>
> Where:

N_i = nitrogen intake in proteins on the test diet

$N_{e(f)}$ = nitrogen excreted in faeces whilst on the test diet

$N_{e(u)}$ = nitrogen excreted in urine whilst on the test diet

N_b = nitrogen excreted on a protein free diet

Note:

$N_r = N_i - N_{e(f)} - N_{e(u)} - N_b$

$N_a = N_i - N_{e(f)}$

This can take any value of 100 or less, including negative. A BV of 100% indicates complete utilization of a dietary protein, ie. 100% of the protein ingested and absorbed is incorporated into proteins into the body. Negative values are possible if excretion of nitrogen exceeds intake in proteins. All non-nitrogen containing diets have negative BV. The value of 100% is an absolute maximum, no more than 100% of the protein ingested can be utilized (in the equation above Ne(u), Ne(f) and Nb cannot go negative, setting 100% as the maximum BV).

Due to experimental limitations BV is often measured relative to an easily utilizable protein. Normally egg protein is assumed to be the most readily utilizable protein and given a BV of 100. For example:

Two tests of BV are carried out on the same person; one with the test protein source and one with a reference protein (egg protein).

$$relative\ BV = (BV_{(test)} / BV_{(egg)}) * 100$$

Where:

$BV_{(test)}$ = percentage BV of the test diet for that individual

$BV_{(egg)}$ = percentage BV of the reference (egg) diet for that individual

This is not restricted to values of less than 100. The percentage BV of egg protein is only 93.7% which allows other proteins with true percentage BV between 93.7% and 100% to take a relative BV of over 100. For example, whey protein* takes a relative BV of 104, while its percentage BV is under 100%.

The principal advantage of measuring BV relative to another protein diet is accuracy; it helps account for some of the metabolic variability between individuals. In a simplistic sense the egg diet is testing the maximum efficiency the individual can take up protein, the BV is then provided as a percentage taking this as the maximum.

Providing it is known which protein measurements were made relative to it is simple to convert from relative BV to percentage BV:

$$BV_{(percentage)} = (BV_{(relative)} / BV_{(reference)}) * 100$$

$$BV_{(relative)} = (BV_{(percentage)} / 100) * BV_{(reference)}$$

Where:

$BV_{(relative)}$ = relative BV of the test protein

$BV_{(reference)}$ = percentage BV of reference protein (typically egg: 93.7%).

$BV_{(percentage)}$ = percentage BV of the test protein

• Whey protein is a mixture of globular proteins isolated from whey, the liquid material created as a by-product of cheese production. Some preclinical studies in rodents have suggested that whey protein may influence glutathione production and possess anti-inflammatory or anti-cancer properties; however, human data are lacking. Whey protein is commonly marketed and ingested as a dietary supplement, and various health claims have been attributed to it in the alternative medicine community. Whey is a common allergen, and is responsible for a significant proportion of cow milk allergies.[4] In people without milk allergies, whey protein is generally considered safe.

While this conversion is simple it is not strictly valid due to the differences between the experimental methods. It is, however, suitable for use as a guideline.

The determination of BV is carefully designed to accurately measure some aspects of protein usage whilst eliminating variation from other aspects. When using the test (or considering BV values) care must be taken to ensure the variable of interest is quantified by BV. Factors which affect BV can be grouped into properties of the protein source and properties of the species or individual consuming the protein.

Three major properties of a protein source affect its BV:

- Amino acid composition, and the limiting amino acid, which is usually lysine
- Preparation (cooking)
- Vitamin and mineral content

Amino acid composition is the principal effect. All proteins are made up of combinations of the 21 biological amino acids. Some of these can be synthesised or converted in the body, whereas others cannot and must be ingested in the diet. These are known as essential amino acids (EAAs), of which there are 9 in humans. The number of EAAs varies according to species (see below).

EAAs missing from the diet prevent the synthesis of proteins that require them. If a protein source is missing critical EAAs, then its biological value will be low as the missing EAAs form a bottleneck in protein synthesis. For example, if a hypothetical muscle protein requiresphenylalanine (an essential amino acid), then this must be provided in the diet for the muscle protein to be produced. If the current protein source in the diet has no phenylalanine in it the muscle protein cannot be produced, giving a low usability and BV of the protein source.

In a related way if amino acids are missing from the protein source which are particularly slow or energy consuming to synthesise this can result in a low BV.

Methods of food preparation also have an impact on availability of amino acids in a food source. Some of food preparation may damage or destroy some EAAs, reducing the BV of the protein source.

Many vitamins and minerals are vital for the correct function of cells in the test organism. If critical minerals or vitamins are missing from the protein source this can result in a massively lowered BV. Many BV tests artificially add vitamins and minerals (for example in yeast extract) to prevent this.

Variations in BV under test conditions are dominated by the metabolism of the individuals or species being tested. In particular differences in the essential amino acids (EAAs) species to species has a significant impact, although even minor variations in amino acid metabolism individual to individual have a large effect.

The fine dependence on the individual's metabolism makes measurement of BV a vital tool in diagnosing some metabolic diseases.

The principal effect on BV in everyday life is the organism's current diet, although many other factors such as age, health, weight, sex, etc. all have an effect. In short any condition which can affect the organism's metabolism will vary the BV of a protein source.

In particular, whilst on a high protein diet the BV of all foods consumed is reduced - the limiting rate at which the amino acids may be incorporated into the body is not the availability of amino acids

but the rate of protein synthesis possible in cells. This is a major point of criticism of BV as a test; the test diet is artificially protein rich and may have unusual effects.

BV is designed to ignore variation in digestibility of a food - which in turn largely depends on the food preparation. For example compare raw soy beans and extracted soy bean protein. The raw soy beans, with tough cell walls protecting the protein, have a far lower digestibility than the purified, unprotected, soy bean protein extract. As a foodstuff far more protein can be absorbed from the extract than the raw beans, however the BV will be the same.

The exclusion of digestibility is a point of misunderstanding and leads to misrepresentation of the meaning of a high or low BV

BV provides a good measure of the usability of proteins in a diet and also plays a valuable role in detection of some metabolic diseases. BV is, however, a scientific variable determined under very strict and unnatural conditions. It is not a test designed to evaluate the usability of proteins whilst an organism is in everyday life - indeed the BV of a diet will vary greatly depending on age, weight, health, sex, recent diet, current metabolism, etc. of the organism. In addition BV of the same food varies significantly species to species. Given these limitations BV is still relevant to everyday diet to some extent. No matter the individual or their conditions a protein source with high BV, such as egg, will always be more easily used than a protein source with low BV.

There are many other major methods of determining how readily used a protein is, including:

- Net protein Utilization (NPU)
- Protein Efficiency Ratio (PER)
- Nitrogen Balance (NB)
- Protein digestibility (PD)
- Protein Digestibility Corrected Amino Acid Score (PDCAAS)

These all hold specific advantages and disadvantages over BV, although in the past BV has been held in high regard.

The Biological Value method is also used for analysis in animals such as cattle, poultry, and various laboratory animals such as rats. It was used by the poultry industry to determine which mixtures of feed were utilized most efficiently by developing chicken. Although the process remains the same, the biological values of particular proteins in humans differs from their biological values in animals due to physiological variations.

Common foodstuffs and their values: Note: this scale uses 100 as 100% of the nitrogen incorporated.

• Isolated Whey:	100
• Whole bean:	96
• Whole Soy Bean:	96
• Human milk:	95
• Chicken egg:	94

Note: These values use "whole egg" as a value of 100, so foodstuffs that provide even more nitrogen than whole eggs, can have a value of more that 100, 100, does not mean that 100% of the nitrogen in the food is incorporated into the body, and not excreted, as in in other.

• Soybean milk:	91
• Cow milk:	90
• Cheese:	84
• Rice:	83
• Defatted soy flour:	81
• Fish:	76
• Beef:	74.3
• Immature bean:	65
• Full-fat soy flour:	64
• Soybean curd (tofu):	64
• Whole wheat:	64
• White flour:	41

Common foodstuffs and their values:

Whey protein concentrate: 104

• Whole egg:	100
• Cow milk:	91
• Beef:	80
• Casein:	77
• Soy:	74
• Wheat gluten:	64

Note: These values use "whole egg" as a value of 100, so foodstuffs that provide even more nitrogen than whole eggs, can have a value of more that 100. 100, does not mean that 100% of the nitrogen in the food is incorporated into the body, and not excreted, as in in other charts.

Since the method measures only the amount that is retained in the body critics have pointed out what they perceive as a weakness of the biological value methodology. Critics have pointed to research that indicates that because whey protein isolate is digested so quickly it may in fact enter the bloodstream and be converted into carbohydrates through a process called gluconeogenesis much more rapidly than was previously thought possible, so while amino acid concentrations increased with whey it was discovered that oxidation rates also increased and a steady-state metabolism, a process where there is no change in overall protein balance, is created. They claim that when the human body consumes whey protein it is absorbed so rapidly that most of it is sent to the liver for oxidation. Hence they believe the reason so much is retained is that it is used for energy production not protein synthesis. This would bring into question whether the method defines which proteins are more biologically utilizable.

A further critique published in the Journal of Sports Science and Medicine states that the BV of a protein does not take into consideration several key factors that influence the digestion and interaction of protein with other foods before absorption, and that it only measures a proteins maximal potential quality and not its estimate at requirement levels. Also, the study by Poullain et al., which is often cited to demonstrate the superiority of whey protein hydrolysate by marketers, measured nitrogen balance in

rats after three days of starvation, which corresponds to a longer period in humans. The study found that whey protein hydrolysate led to better nitrogen retention and growth than the other proteins studied. However the study's flaw is in the BV method used, as starvation affects how well the body will store incoming protein (as does a very high caloric intake), leading to falsely elevated BV measures.

So, the BV of a protein is related to the amount of protein given. BV is measured at levels below the maintenance level. This means that as protein intake goes up, the BV of that protein goes down. For example, milk protein shows a BV near 100 at intakes of 0.2 g/kg. As protein intake increases to roughly maintenance levels, 0.5 g/kg, BV drops only around 70.[20] Pellet et al., concluded that "biological measures of protein quality conducted at suboptimal levels in either experimental animals or human subjects may overestimate protein value at maintenance levels." As a result, while BV may be important for rating proteins where intake is below requirements, it has little bearing on individuals with protein intakes far above requirements.

This flaw is supported by the FAO/WHO/UNU, who state that BV and NPU are measured when the protein content of the diet is clearly below that of requirement, deliberately done to maximize existing differences in quality as inadequate energy intake lowers the efficiency of protein utilization and in most N balance studies, calorie adequacy is ensured. And because no population derives all of its protein exclusively from a single food, the determination of BV of a single protein is of limited use for application to human protein requirements.

Another limitation of the use of Biological Value as a measure of protein quality is that proteins which are completely devoid of one essential amino acid (EAA) can still have a BV of up to 40. This is because of the ability of organisms to conserve and recycle EAAs as an adaptation of inadequate intake of the amino acid.

Lastly, the use of rats for the determination of protein quality is not ideal. Rats differ from humans in requirements of essential amino acids. This has led to a general criticism that experiments on rats lead to an over-estimation of the BV of high-quality proteins to man because human requirements of essential amino acids are much lower than those for rats (as rats grow at a much faster rate than humans). Also, because of their fur, rats are assumed to have relatively high requirements of sulphur-containing amino acids (methionine and cysteine).

As a result, the analytical method that is universally recognized by the FAO/WHO as well as the FDA, USDA, United Nations University (UNU) and the National Academy of Sciences when judging the quality of protein in the human is not PER or BV but the Protein Digestibility Corrected Amino Acid Score (PDCAAS), as it is viewed as accurately measuring the correct relative nutritional value of animal and vegetable sources of protein in the diet.

SECTION 5.24—DIGESTION OF PROTEIN

SUB-SECTION 5.24A—DIGESTION IN DIGESTIVE SYSTEM

There are no proteolytic enzymes present in the mouth. After mastication and chewing the bolus of food reaches stomach where it meets the gastric juice, then begins the digestion of food in stomach.

Gastric juice contains a number of proteolytic enzymes. They are 1. Pepsin, 2. Rennin, 3. Gastrisin, 4. Gelatenase.

Pepsin is a important proteolytic enzyme and is present in gastric juice of different species including the mammals. Pepsin is secreted as inactive zymogen form pepsinogen having a molecular weight 42, 500 approx. It is synthesized in 'chief cells' of stomach and 99% is poured gastric juice as pepsinogen. Remaining 1% is separated in the blood stream from where it is ultimately excreted in urine. The urinary pepsin is called Uropepsin.

Pepsinogen is hydrolyzed in stomach, with the help of HCl or pepsin itself (auto catalytically) to form the 'active pepsin having a molecular weight of (34,500). In the process of activation (i) an inactive peptide called 'pepsin inhibitor' and (ii) 5 smaller peptide are liberated.
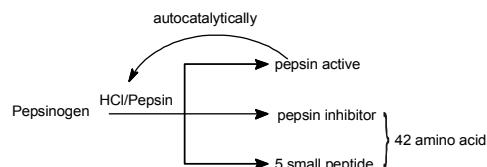
HCl maintains the gastric pH between 1 to 2 to ensure the proper pepsin activity. Optimum pH for pepsin is 1.6 to 2.5 and pepsin gets denaturized if the pH is greater than 5. Pepsin is protenase a non – specific endopeptidase and hydrolyses peptide bonds well inside the protein molecule and produced proteoses and peptones.

$$\text{Proteins} \xrightarrow{\text{Pepsin}} \text{Proteoses and Peptones}$$

It is particularly active on a peptide bond, which connects the – COOH group of an aromatic amino acid like Phe, Tyr and Tyrp with the amino group of either dicarboxylic or aromatic amino acid.

It can also hydrolyze the peptide bonds of
(i) COOH group of methionine and leucine
(ii) Leucine and glutamic acid
(iii) Glutamic acid and asparagines
(iv) Leucine valine and
(v) Valine and cystine

Pepsin cannot act on protein like terapenes, silfibrions mucoproteins mucoids and protamines.

$$\text{Casein (Soluble)} \xrightarrow[\text{Chymotrypsin}]{\text{Pepsin/Renin}} \text{Paracasein + Protease (whey protein)}$$

$$\text{Paracasein (Soluble) + Ca}^{++} \xrightarrow{\text{Spontaniousity}} \text{Calcium Paracaseinate (insolublecurd)}$$

Pepsin can also act on milk. It hydrolyzes the soluble phosphoprotein "casein" of milk to procedure "paracasein and proteose the latter is the whey protein. Paracasein is then precipitated as caparacaseinate which is further digested by pepsin to peptones.

Rennin not present in adult humans, nevertheless infants have little amount of rennin the optimum pH for its activity is 4 and the specificity for action of rennin is like pepsin, here rennin hydrolyses peptide bonds connected with L – aromatic amino acids. Like pepsin rennin also reacts with casein of milk to form paracasein which is immediately precipitated by Ca^{++}. Thus it is also coagulates milk pepsin.

Gastrisin is secreted in the gastric juice of humans as inactive zymogen form, which activated in the presence of HCl. The optimum activity of this enzyme is pH 3 to 4. It acts as a Protenase and requires an acidic medium for its activity.

After stomach the food stuff reaches Duodenum, the small intestine here it mixes with pancreatic juice. The pancreatic juice consists of several proteolytic enzymes which act on protein and partly digested food.

The main enzymes are:
1. Trypsin
2. Chymotrypsin
3. Carboxypeptidases
4. Elastases
5. Collagenases

Trypsin (EC 3.4.21.4) is a serine protease found in the digestive system of many vertebrates, where it hydrolyses proteins. Trypsin is produced in the pancreas as the inactive proenzyme trypsinogen. Trypsin predominantly cleaves peptide chains at thecarboxyl side of the amino acids lysine and arginine, except when either is followed byproline. It is used for numerous biotechnological processes. The process is commonly referred to as trypsin proteolysis or trypsinisation and proteins that have been digested/treated with trypsin are said to have been trypsinized.

Trypsin is secreted into the duodenum, where it acts to hydrolyse peptides into their smaller building blocks, namely amino acids (these peptides are the result of the enzyme pepsin breaking down the proteins in the stomach). This is necessary for the uptake of protein in the food as though peptides are smaller than proteins, they are still too big to be absorbed through the lining of the ileum. Trypsin catalyses the hydrolysis of peptide bonds.

The enzymatic mechanism is similar to other serine proteases. These enzymes contain acatalytic triad consisting of histidine-57, aspartate-102, and serine-195. These three residues form a charge relay which serves to make the active site serine nucleophilic. This is achieved by modifying the electrostatic environment of the serine. The enzymatic reaction that trypsins catalyze is thermodynamically favorable but requires significantactivation energy (it is "kinetically unfavorable"). In addition, trypsin contains an "oxyanion hole" formed by the backbone amide hydrogen atoms of Gly-193 and Ser-195 which serves to stabilize the developing negative charge on the carbonyl oxygen atom of the cleaved amide.

The aspartate residue (Asp 189) located in the catalytic pocket (S1) of trypsins is responsible for attracting and stabilizing positively-charged lysine and/or arginine, and is thus responsible for the specificity of the enzyme. This means that trypsin predominantly cleaves proteins at the carboxyl side (or "C-terminal side") of the amino acids lysine andarginine, except when either is followed

by proline. Trypsins are consideredendopeptidases, i.e., the cleavage occurs within the polypeptide chain rather than at the terminal amino acids located at the ends of polypeptides.

Trypsins have an optimal operating pH of about 8 and optimal operating temperature of about 37°C.

Trypsin is produced in the pancreas in the form of inactive zymogen, trypsinogen. When the pancreas is stimulated by cholecystokinin, it is then secreted into the small intestine. Once in the small intestine, the enzyme enteropeptidase activates it into trypsin by proteolytic cleavage. The resulting trypsins themselves activate more trypsinogens (autocatalysis), so only a small amount of enteropeptidase is necessary to start the reaction. This activation mechanism is common for most serine proteases, and serves to prevent autodigestion of the pancreas.

The activity of trypsins is not affected by the inhibitor tosyl phenylalanyl chloromethyl ketone TPCK, which deactivates chymotrypsin. This is important because, in some applications, like mass spectrometry, the specificity of cleavage is important.

Trypsin is available in high quantity in pancreases, and can be purified rather easily. Hence it has been used widely in various biotechnological processes.

In a tissue culture lab, trypsins are used to re-suspend cells adherent to the cell culture dish wall during the process of harvesting cells.

Trypsin can also be used to dissociate dissected cells (for example, prior to cell fixing and sorting).

Trypsins can be used to break down casein in breast milk. If trypsin is added to a solution of milk powder, the breakdown of casein will cause the milk to become translucent. The rate of reaction can be measured by using the amount of time it takes for the milk to turn translucent.

Trypsin is commonly used in biological research during proteomics experiments to digest proteins into peptides for mass spectrometry analysis, e.g. in-gel digestion. Trypsin is particularly suited for this, since it has a very well defined specificity, as it hydrolyzes only the peptide bonds in which the carbonyl group is contributed either by an Arg or Lys residue.

Trypsin can also be used to dissolve blood clots in its microbial form and treat inflammation in its pancreatic form.

Trypsin is used in baby food to pre-digest it. It can break down the protein molecules which helps the baby to digest it as its stomach is not strong enough to digest bigger protein molecules.

Trypsin and chymotrypsin, like most proleotytic enzymes, are synthesized as inactive zymogen precursors (trypsinogen and chymotrypsinogen) to prevent unwanted destruction of cellular proteins, and to regulate when and where enzyme activity occurs. The inactive zymogens are secreted into the duodenum, where they travel the small and large intestines prior to excretion. Zymogens also enter the bloodstream, where they can be detected in serum prior to excretion in urine. Zymogens are converted to the mature, active enzyme by proteolysis to split off a pro-peptide, either in a subcellular compartment or in an extracellular space where they are required for digestion.

Trypsin and chymotrypsin are structurally very similar, although they recognise different substrates. Trypsin acts on lysine and arginine residues, while chymotrypsin acts on large hydrophobic residues

such as tryptophan, tyrosine and phenylalanine, both with extraordinary catalytic efficiency. Both enzymes have a catalytic triad of serine, histidine and aspartate within the S1 binding pocket, although the hydrophobic nature of this pocket varies between the two, as do other structural interactions beyond the S1 pocket.

The human pancreas secretes three isoforms of trypsinogen: cationic (trypsinogen-1), anionic (trypsinogen-2) and mesotrypsinogen (trypsinogen-3). Cationic and anionic trypsins are the major isoforms responsible for digestive protein degradation, occurring in a ratio of 2:1, while mesotrypsinogen accounts for less than 5% of pancreatic secretions. Mesotrypsin is a specialised protease known for its resistance to trypsin inhibitors. It is thought to play a special role in the degradation of trypsin inhibitors, possibly to aid in the digestion of inhibitor-rich foods such as soybeans and lima beans. An alternatively spliced mesotrypsinogen in which the signal peptide is replaced with a different exon 1 is expressed in the human brain; the function of this brain trypsinogen is unknown.

There are two isoforms of pancreatic chymotrypsin, A and B, which are known to cleave proteins selectively at specific peptide bonds formed by the hydrophobic residues tryptophan, phenylalanine and tyrosine.

Chymotrypsin (bovine γ chymotrypsin: PDB 1AB9, EC 3.4.21.1) is a digestive enzyme that can perform proteolysis. Chymotrypsin cleaves peptides at the carboxyl side oftyrosine, tryptophan,
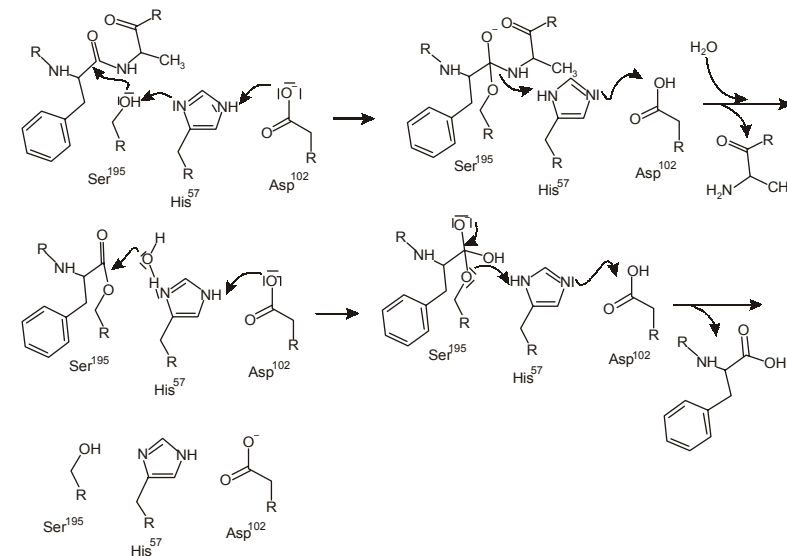
and phenylalanine because these three amino acids containaromatic rings, which fit into a 'hydrophobic pocket' in the enzyme. Over time, chymotrypsin also hydrolyzes other amide bonds, particularly those with leucine-donated carboxyls.

Chymotrypsin is synthesized in the pancreas by protein biosynthesis as a precursorcalled chymotrypsinogen that is enzymatically inactive. On cleavage by trypsin into two parts that are still connected via an S-S bond, cleaved chymotrypsinogen molecules can activate each other by removing two small peptides in a trans-proteolysis. The resulting molecule is active chymotrypsin, a three polypeptide molecule interconnected via disulfide bonds. n vivo, chymotrypsin is a proteolytic enzyme acting in the digestive systems of mammals and other organisms. It facilitates the cleavage of peptide bonds by ahydrolysis reaction, a process which albeit thermodynamically favourable, occurs extremely slowly in the absence of a catalyst. The main substrates of chymotrypsin include tryptophan, tyrosine, phenylalanine, leucine, and methionine, which are cleaved at the carboxyl terminal. Like many proteases, chymotrypsin will also hydrolyse ester bonds in vitro, a virtue that enabled the use of substrate analogs such as N-acetyl-L-phenylalanine p-nitrophenyl ester for enzyme assays.

Mechanism of Peptide Bond Cleavage in α-chymotrypsin

Chymotrypsin cleaves peptide bonds by attacking the unreactive carbonyl group with a powerful nucleophile, the serine 195 residue located in the active site of the enzyme, which briefly becomes covalently bonded to the substrate, forming an enzyme-substrate intermediate.

It was found that the reaction of chymotrypsin with its substrate takes place in two stages, an initial "burst" phase at the beginning of the reaction and a steady-state phase following Michaelis-Menten kinetics.It is also called "ping-pong" mechanism. The mode of action of chymotrypsin explains this as hydrolysis takes place in two steps. First acylation of the substrate to form an acyl-enzyme intermediate and then deacylation in order to return the enzyme to its original state.

Carboxypeptidase (EC number 3.4.16 - 3.4.18) is an enzyme that hydrolyzes the carboxy-terminal (C-terminal) end of a peptide bond. Carboxypeptidase is secreated from the pancreas and reaches the intestine Humans, animals, and plants contain several types of carboxypeptidases with diverse functions ranging from catabolism to protein maturation.

The first carboxypeptidases studied were those involved in the digestion of food (pancreatic carboxypeptidases A1, A2, and B). However, most of the known carboxypeptidases are not involved in catabolism; they help to mature proteins or regulate biological processes. For example, the biosynthesis of neuroendocrine peptides such as insulin requires a carboxypeptidase. Carboxypeptidases also function inblood clotting, growth factor production, wound healing, reproduction, and many other processes.

Carboxypeptidases are usually classified into one of several families based on their active site mechanism.

- Enzymes that use a metal in the active site are called "metallo-carboxypeptidases" (EC number 3.4.17).
- Other carboxypeptidases that use active site serine residues are called "serine carboxypeptidases" (EC number 3.4.16).
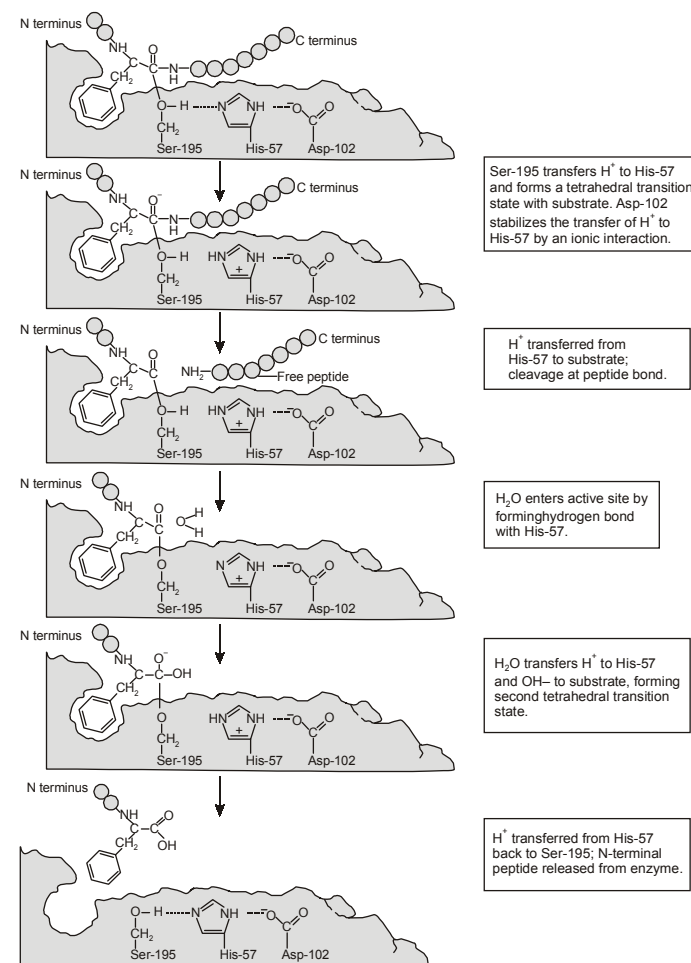
Fig. 5.45 : Showing the Catalytic reaction of Chymotrypsin

• Those that use an active site cysteine are called "cysteine carboxypeptidase" (or "thiol carboxypeptidases")(EC number 3.4.18).

These names do not refer to the selectivity of the amino acid that is cleaved.

Another classification system for carboxypeptidases refers to their substrate preference.

• In this classification system, carboxypeptidases that have a stronger preference for those amino acids containing aromatic or branchedhydrocarbon chains are called carboxypeptidase A (A for aromatic/aliphatic).

• Carboxypeptidases that cleave positively charged amino acids (arginine, lysine) are called carboxypeptidase B (B for basic).

A metallo-carboxypeptidase that cleaves a C-terminal glutamate from the peptide N-acetyl-L-aspartyl-L-glutamate is called "glutamate carboxypeptidase".

A serine carboxypeptidase that cleaves the C-terminal residue from peptides containing the sequence -Pro-Xaa (Pro is proline, Xaa is any amino acid on the C-terminus of a peptide) is called "prolyl carboxypeptidase".

Some, but not all, carboxypeptidases are initially produced in an inactive form; this precursor form is referred to as a procarboxypeptidase. In the case of pancreatic carboxypeptidase A, the inactive zymogen form, pro-carboxypeptidase A, is converted to its active form - carboxypeptidase A - by the enzyme enteropeptidase. This mechanism ensures that the cells wherein pro-carboxypeptidase A is produced are not themselves digested.

Trypsin inhibitors are chemicals that reduce the availability of trypsin, an enzyme essential to nutrition of many animals, includinghumans.

There are four commercial sources of trypsin inhibitors.

Source	Molecular weight	Inhibitatory power	Details
Serum (α1-antitrypsin)	52 kDa		Also known as serum trypsin inhibitor
Lima beans	8-10 kDa	2.2 times weight	There are six different lima bean inhibitors.
Bovinepancreas	6.5 kDa	2.5 times weight	Kunitz inhibitor is the best known pancreatic inhibitor. Chymotrypsin is also inhibited by this chemical, but less tightly. When extracted from lung tissue, this is known as aprotinin.
Ovomucoid	8-10 kDa	1.2 times weight	Ovomucoids are the glycoprotein protease inhibitors found in raw avian egg white. There are other protease inhibitors in ovomucoids as well.
Soybeans	20.7-22.3 kDa	1.2 times weight	Soybeans contain several inhibitors; the one in the chart is considered the primary one. All of them bind chymotrypsin to a lesser degree.

SUB-SECTION 5.24B—ABSORPTION OF AMINO ACIDS
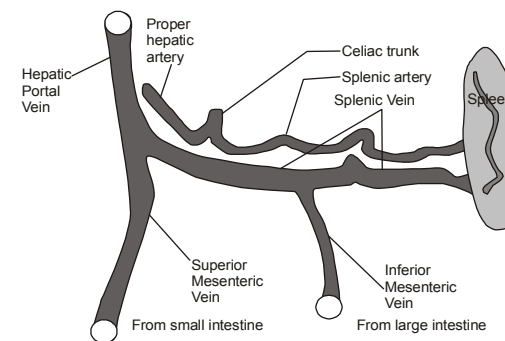
Fig. 5.46 : Showing the portal vein and the portal blood

Under normal circumstances, the dietary proteins are almost completely digested to their constituent amino acids. But some amounts of oligopeptides like tri and dipeptides may remain as such. The above products are rapidly absorbed. The amino acids are generally absorbed in between ileum and distant jejunum. Olegopeptides like di and tri peptides are absorbed from duodenum and proximal jejunum.

Amino acid and other products of digestions like di and tri peptides are carried by portal blood* to liver.

There is a marked rise of the amino acid in the portal blood after a protein meal. The rate of absorption of amino acids is different for e.g. The L – amino acids and the L – isomers are absorbed faster than the D – amino acids by the active transport process. The L – amino acids are actively transported across the intestine from mucosa to serosal surface.

Pyridoxal (P) (B6 – PO4) is probably involved in this process. In the case of D – amino acids, they are absorbed slowly and also by simple passive diffusion process.

Mechanism of L-amino Acid Absorption

This process is also called Ion – gradient hypothesis. Here amino acids are absorbed from small intestine by sodium (Na^+) dependant and energy is provided by ATP.

1. L – Amino acids and Na^+ combine with a common "carrier" protein molecule present on the outer or mucosal surface of the microvillus membrane to form a carrier amino acid Na^{++} complex

*the blood that is carried to the liver by portal vein from small intestine to liver.
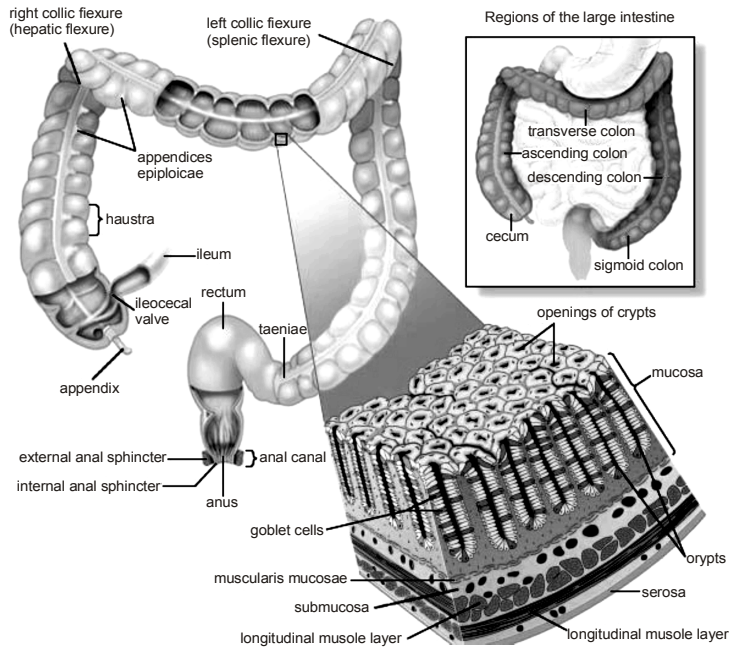
Fig. 5.47 : Showing the Mucosa and the Serosal surface

2. The complex passes to the inner or cytoplasmic surface of the same membrane. There it dissolved to generate the free amino acid and Na^{++}

3. Na+ is actively carried out through the cell membrane by a sodium pump mechanism with the help of transport ATPase to that intracellular Na$^+$ concentration is always maintained below.

4. Carrier protein molecule comes back to the brush border again.

5. The amino acid ultimately passes out through the serosal membrane of the cell by diffusion down an outward concentration gradiant of amino acid is taken by portal blood to the liver.

Evidences of "Active" Absorption

1. Rate and extent of absorption of L – amino acids is considerably higher than D – Isomers.

2. If Na$^+$ is replaced by lithium and/or K$^+$ in bathing fluid the rate of absorption is depressed and may be practically nil.

3. Inhibitors like dinitrophenol (DNP) or cyanide depress L – amino acid absorption. DNP acts an uncoupler in oxidative phosphorylation and thus interferes with the ATP formation.

Evidences for "Active"

4. Rates of absorption of different L – amino acid are different from each other and are independent for their diffusion and concentration gradients.

5. High concentration of one L – amino acid sometimes reduces the rate of absorption of some other L – amino acids indicating several L – amino acids may share common " carrier molecule and may complete with each other.

6. Basic and dicarboxylic acid are generated are also slowly absorbed than neutral amino acids.

L – Oligo peptides are also actively absorbed. Intramolecular peptides hydrolyze them into amino acids. This hydrolyses within the intestinal epithelial cells, is rapid enough to keep peptide concentration low in these cells. Transport mechanisms for L – peptides appear to be independent of L – amino acids.

Different classes of amino acids such as di–amino acids small neutral amino acids, imino acids and large neutral amino acids are believed to be absorbed by different 'carrier' protein molecules present in the microvillus membrane of intestinal cells

SUB-SECTION 5.25B—GAMMA—GLUTAMYL CYCLE

Meister has proposed that glutathione participates in an "active group" translocation of amino acids (except L – proline) into the cells of small intestine, kidneys, seminal vessels, epididymis and brain. He proposed a cyclic pathway in which the Glutathione is generated again, and it is called γ-**Glutamyl Cycle**. (see fig 170).
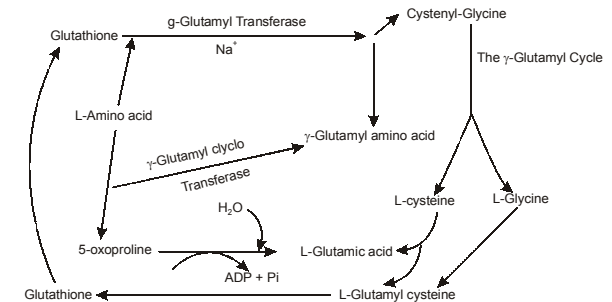
Fig. 5.48 : Showing the Gamma – Glutamyl Cycle.

Some individuals show sensitivity to dietary proteins. Proteins to be antigenic so that can stimulate an immunological response it should be relatively high molecule.

Normally proteins are digested in the G I tract to small peptides and amino acids. Digestions of protein to small peptides and amino acids stage destroy the antigenicity.

How dietary proteins can be antigenic is a puzzling feature? Hence it is suggested that in some individuals, there must be absorption of some "unhydrolyzed" protein probably by pinocytosis.

It has been shown that to be antigenic, the protein must be polypeptide containing 6 to 7 amino acids having mol wt of 828 – 928 Daltons and presence of glutinamine and proline in the protein molecule is a must.

SUB-SECTION 5.25C—FATE OF AMINO ACIDS AFTER

Absorption Amino Acids after absorption from intestine are carried out to liver by portal blood. They are taken up by liver cells to some extent and remaining enters the systemic circulation and diffuse throughout the body fluids and taken up by the tissue cells.

At the same time the tissue proteins both "structural" and functional proteins (including plasma) proteins are commonly undergoing disintegration undergoing to release amino acids which likewise enter the circulation. There is also a continuous flow of amino acids (except the essential amino acids). Amino acids from all the different sources get mixed up to constitute what is known as general amino acids pool of the body. Though amino acid pool has now anatomical reality, but represents the availability of the amino acid building units. No functional distinction can be drawn between the fate of the amino acids derived from the dietary sources and those derived from the tissue breakdown.

If the cells intake of amino acid is equal to its loss it is in a state of dynamic equilibrium and if the loss is greater the cells wastes and if the gain is greater the cells grows

All tissues including exocrine* and endocrine§ glands draw freely from the amino acids pool to synthesize the tissue proteins, enzymes and protein hormones. Amino acids is taken up by each cell according to its specific needs, to be built inside the cell structure and materials required.

If the cells intake of amino acid is equal to its loss it is in a state of dynamic equilibrium and if the loss is greater the cells wastes and if the gain is greater the cells grows. In man protein turnover involves the breakdown and resynthesis of 80 to 100 Gms of tissue protein per day about 1 of it occurring in liver. On average plasma proteins are completely replaced every 15 days. The pool is constantly undergoing depletion because

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* Exocrine glands are glands that secrete their products (hormones) into ducts (duct glands). They are the counterparts to endocrine glands, which secrete their products (hormones) directly into the bloodstream (ductless glands) or release hormones (paracrines) that affect only target cells nearby the release site.

§ Endocrine glands are glands of the endocrine system that secrete their products, hormones, directly into the blood rather than through a duct. The main endocrine glands include the pituitary gland, pancreas, ovaries, testes, thyroid gland, and adrenal glands.

1. Large deamination of presumably surplus amino acids takes place.
2. Amino acids and their derivatives e.g. urea creatinine are lost in the urine and the other excretions
3. Amino acids are commonly and continually building up into those proteins, which are not the part of the dynamic system. On the other hand, amino acid pool is being always re – established by amino acid derived from the following.
4. Reamination of certain non – nitrogenous residues.
5. Amination of appropriate fragments which are present in the common metabolic pool (and therefore derived from fat and carbohydrate breakdown)
6. Amino acid split off from dietary protein and absorbed from the intestine into the blood.

This state is called "continuing metabolism of the amino acids"

All amino acids occur in blood in varying concentrations and make a total of 30 to 50 mg/10ml in the post absorption period.

In terms of amino acid N2 it is 4 to 5mg/100ml.

Circadian changes in the Plasma Amino Acid Level.

The plasma levels of most amino acids do not remain constant though out 24 hours period but rather change by varying in circadian rhythm about a "mean value". This was first noted in amino acid tyrosine and latter on confirmed on the many other amino acids. In general, plasma amino acid levels are lowest in the early morning (4 am) and rise about 15 to 35% by noon to early afternoon.

Amino acids present at the highest mean concentration e.g. glycine, alanine, valine, serine etc, show most striking changes in level.

The exact physiological significance of the circadian changes occurring in plasma amino acid levels remains elucidated.

The amino acids are transferred and transported to tissues actively. Pyridoxal P (B6 – P) is one of the requirements for this active transport which has been discussed in the subsection 5.25e.

SUB-SECTION 5.25D—PROCESS OF TRANSAMINATION

It was first discovered by Brounstein and Kritzmann it is a process of combined Amination and deamination.

Transamination is a reversible reaction in which a - NH_2 group of one aminoacid is transferred to a keto acid resulting in formation of a new amino acid and a new keto acid.

The general process of Transamination may be represented as follows.

The process represents only and intermolecular transfer of NH2 group with the splitting out of NH3 also NH3 formation does not take place by transamination.

Donor amino acid (i) thus becomes a new keto acid (i) after losing the α - NH_2 group, and the recipient keto acid (II) after receiving the NH_2 group. Trans Amination reaction is

reversible reaction and catalyzed by enzymes. Transamination takes place in the liver, kidney heart and brain. But the enzyme is present in almost all the mammalian tissues and transamination can be carried out in all tissues to some extent. Transamination requires enzymes called transaminases, better known as aminotransferases.

L - amino acid (donor)   L - keto acid (recipient)   new keto acid   new amino acid
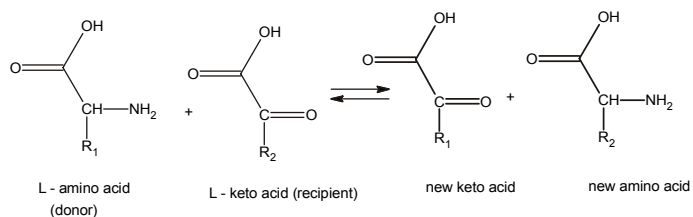
SUB-SECTION 5.25E—ROLE OF PYRIDOXAL PHOSPHATE

The CO – enzyme required for the transamination is the pyridoxal P (B6 –P). In the process of transamination, the amino acid reacts with the enzyme bond, pyridoxal – P to form an enzyme bond Schiff base complex, when then decomposes forming a second new amino acid and regenerates the pyridoxal – P. The reaction is shown below.

amino acid (donor)   pyridoxal - P   new amino acid

TRANSAMINASES

new keto acid   PYRODOXAMINE - P   new keto acid recipient
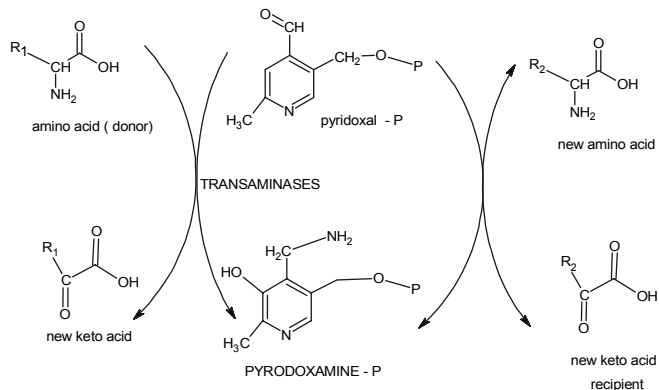
Limitations of Transamination

1. Group Transaminases and Specific Transaminases. (a) While most amino acids may act as Donor 1 the recipient keto acids may be either keto oxoglutartae, or oxaloacetate or pyruvate.

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2. It is to be noted that all are components of "TCA cycle" and hence there are common metabolities of cell and are easily available. The amino acids formed from these recipients ketoacid are respectively glutamic acid aspartic and alanine.

3. Specific transaminases. But there are two transaminases of clinical importance in the body in that they use specific amino acid and specipic keto acid.

These specific transaminases are (or Asparate amino transferase) Previously used to be called SGOT (Serum Glutamate Oxaloacetate Transaminases).

In this Aspartic acid is the donor amino acid and α - oxoglutarate is the recipient keto-acid. New amino acid formed is again always glutamic acid.

Asparate $+ \alpha$ – oxoglutarate $\rightleftarrows$ Oxalocetate + Glutamate
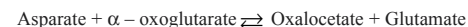
SUB-SECTION 5.25F—DEAMINATION

In the human body, deamination takes place primarily in the liver, however Glutamate is also deaminated in the kidneys. Deamination is the process by which amino acids are broken down when too much protein has been taken in. The amino group is removed from the amino acid and converted to ammonia. The rest of the amino acid is made up of mostly carbon and hydrogen, and is recycled or oxidized for energy. Ammonia is toxic to the human system, and enzymes convert it to urea or uric acid by addition of carbon dioxide molecules (which is not considered a deamination process) in the urea cycle‡, which also takes place in the liver. Urea and uric acid can safely diffuse into the blood and then be excreted in urine.

> Deamination is the removal of an amine group from a molecule

Deamination is a process by which N – of amino acid is removed as NH_3 and this is of two types (i) Oxidative (ii) Non – oxidative deamination.

(i) Oxidative Deamination

This type of deamination is mainly in the kidney and liver. The enzymes like D and L amino acid oxidases are present in the tissues which can act on the D and the L amino acid respectively and also can liberate oxidatively NH_3 form amino acids.

The essential difference between these two enzymes are shown in the table below.

D - amino acid oxidase	L - amino oxidase
1. Can action D - amino acids only	1. Can action L - amino acid
2. Can be readily extracted with water free from	2. Bound to tissue particles cannot be extracted with water
3. Contains FP(FAD)	3. Contains FP(FMN)
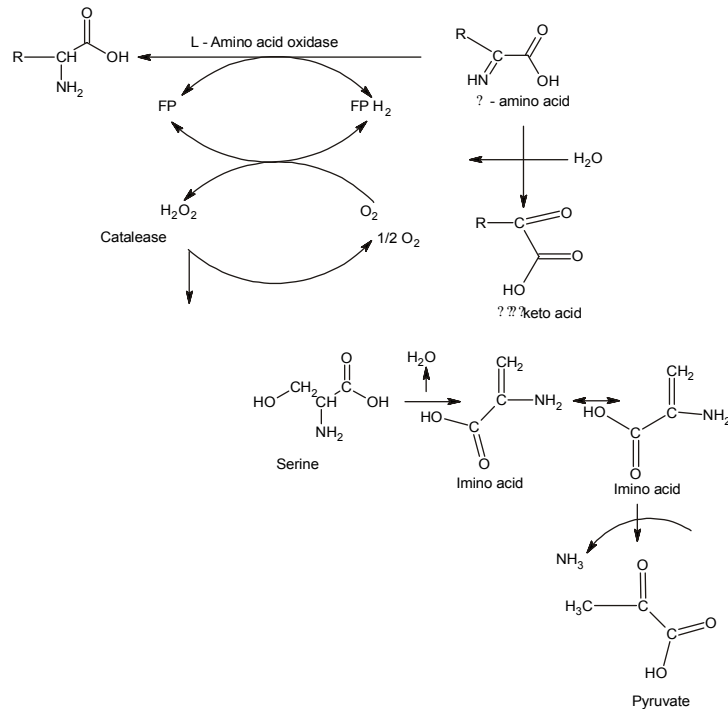
Non-Oxidative Deamination

There are certain amino acids which can be non - oxidatively deaminated by specific enzymes and can form NH_3

These reaction do contribute to NH_3 formation, but does not actively take part in the NH_3 formation. Only three types of non - oxidative deamination has been discussed.

Amino acid Dehydrate: The hydroxyl amino acids e.g. serine, threonine and homoserine are deaminated by specific enzymes called amino acid dehydrates which requires Pyridoxal - P - (B6 - P) co enzyme.

The enzyme catalyze a primary dehydration followed by spontaneous deamination. An example will make the things clear. Histidine is non -oxidatively deaminated by the enzyme histidase to form NH3 and urocanic acid.
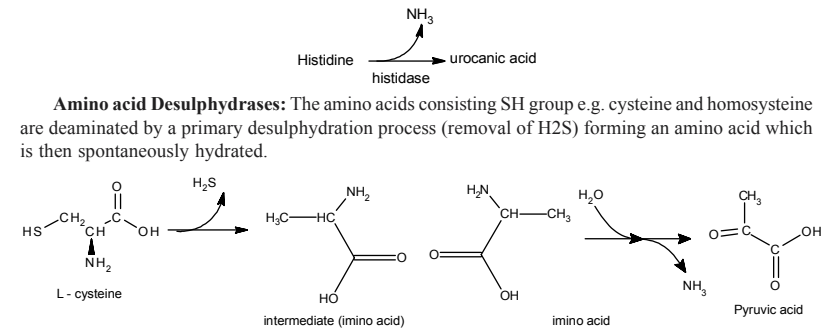
Amino acid Desulphydrases: The amino acids consisting SH group e.g. cysteine and homosysteine are deaminated by a primary desulphydration process (removal of H2S) forming an amino acid which is then spontaneously hydrated.

Transdeamination

It is to be noted that L – Glutamic acid is not deaminated by L – amino acid oxidase but by a specific enzyme called L – glutamate dehydrogenase.
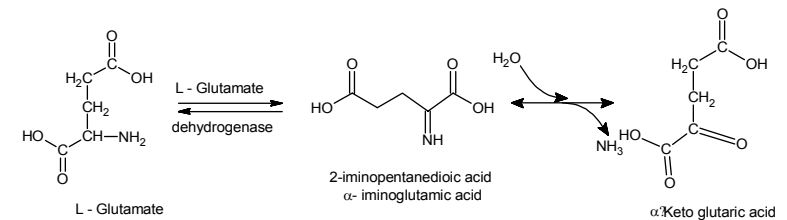
Characteristics of the enzyme L-Glutamate Dehydrogenase

1. The enzyme has four polypeptide chains
2. A Zn^+ containing metalloenzyme, one atom Zn^{++} present in each peptide chain.
3. It is widely distributed in tissues in humans and has high activity.
4. Specific for L – Glutamate.
5. It requires NAD^+ or $NADP^+$ as co – enzymes.
6. It is a regulated enzyme whose activity is affected by allosteric modifiers at ATP GTP NADH which inhibits the enzyme.
7. Certain hormones appear to influence the enzyme activity in vitro.

Reaction the enzyme L – Glutamate dehydrogenase the deamination of L – Glutamate to from α - Iminoglutaric acid, which on addition of a molecule of water forms ammonia and α - ketoglutarate.

It is to be noted that the reaction is reversible and the equibrium constant favours glutamate formation but the quick removal of NH_3 to form urea in urea cycle and α - ketoglutarate to TCA cycle favours an onward reaction of NH_3 formation.

SUB-SECTION 5.25G—TRANSMETHYLATION

Transmethylation is a biologically important organic chemical reaction in which a methyl group is transferred from one compound to another.

An example of transmethylation is the recovery of methionine from homocysteine. In order to sustain sufficient reaction rates during metabolic stress, this reaction requires adequate levels of vitamin B12 and folic acid. Methyl tetrahydrofolate delivers methyl groups to form the active methyl form of vitamin B12 that is required for methylation of homocysteine. Deficiencies of vitamin B12 or folic acid cause increased levels of circulatiing homocysteine. Elevated homocysteine is a risk factor for cardiovascular disease and is linked to the metabolic syndrome (insulin insensitivity).
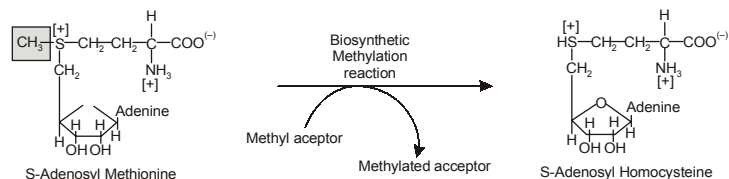
SUB-SECTION 5.25H—FORMATION OF CREATININE

The importance of creatine phosphate as a reservoir of high-energy phosphate readily convertible to A TP in muscles and other tissues has been pointed out. The synthesis of creatine involves the amino acids glycine, arginine, and methionine (as S-adenosylmethionine). Guanidoacetic acid is first formed by a transamidination reaction between glycine and arginine, in which the amidine group of arginine is transferred to glycine (163,164). The transamidinase enzyme is found in mammalian kidney and pancreas (165) but appears to be absent from such other tissues as liver heart. skeletal muscles spleen blood brain
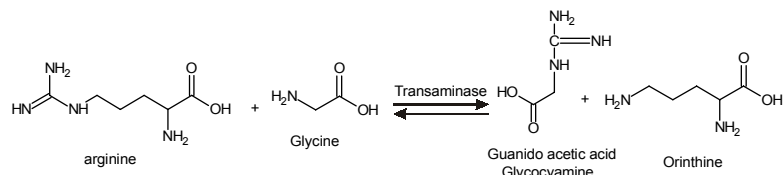
It is of interest that Walker (164) ohtained evidence that an amidine derivative of the transamidinase enzyme is an intermediate in the reaction.

The process may be represented as follows:
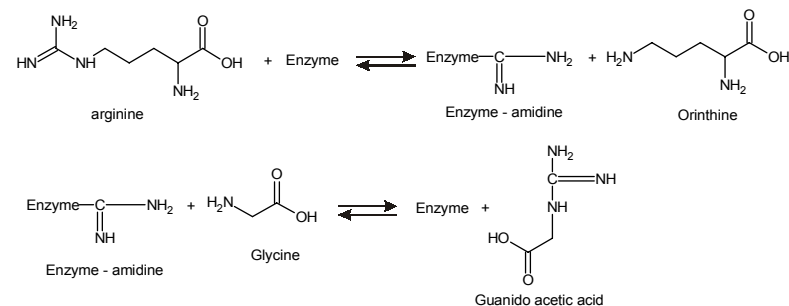
In the second stage of creatine synthesis methionine is converted to "active" methionine, S-adenosylmethionine, by A TP and the" methionine-activating" enzyme, and then guanidoacetic acid is methylated to creatine by S-adenosylmethionine and the enzyme" guanidoacetic methylpherase" The second stage takes place in the liver.
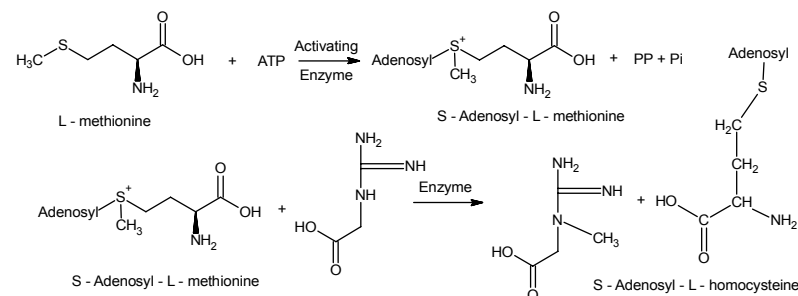
The methyl group of creatine is not transferred to other compounds, as is the methyl of S-adenosylmethionine and of betaine (later discussion). Also, the methyl group of creatine is not oxidized to formaldehyde or formate and, consequently, does not contribute .to the one-carbon pool. However, the methyl groups of creatine may be formed from the one-carbonpool (through methionine) .

Creatine occurs generally in the tissues of the body, though in uneven distribution. The highest concentrations are found in striated muscle, heart muscle, testes, liver, and kidneys, and somewhat lesser quantities in the brain. Very small amounts are present in blood, about 0.2 to 0.6 mg per cent in plasma and around 3 mg per cent in the cells. The muscles contain about 98 per cent of the total body creatine (ca. 0.5 per cent).

Most of the creatine in normal tissues (red cells excepted) occurs as the high-energy compound creatine phosphate.

The urine of normal adult persons contains only small amounts of creatine, but much creatinine, the anhydride of creatine. Creatinine apparently serves no useful function in the body but simply represents a waste product of creatine metabolism. Borsook and Dubnoff (166) showed that most of the creatinine formed in the body arises from the spontaneous decomposition of creatine phosphate according to the equation:

Creatinine Phosphate

creatinine

+ inorganic phosphate
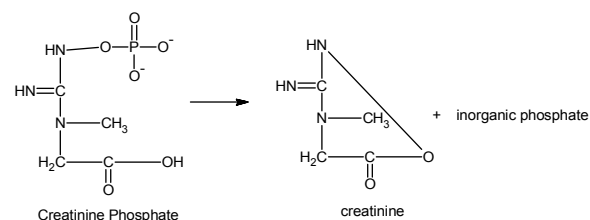
The blood plasma contains about 0.6 to 1.0 mg per cent of creatinine, while the concentration in the red cells has been found to be less, about 0.5 to 0.65 mg per cent. It appears that creatinine, unlike creatine, is distributed between the plasma and cells in proportion to their respective water con-tents, indicating free diffusibility. Baker and Miller (167) found 0.1 to 4.7 mg of creatinine p.er 100 g to be present in rat tissues, amounts proportional to the creatine present. Creatinine is present in sweat, in bile, and in all the gastrointestinal secretions.

Fate of ingested and injected creatine. The oral and parenteral administration of creatine into animals or adult human males is followed by the excretion of only a frac-tion in the urine. A single dose of creatine causes no increase in urinary creatinine, though extra creatinine is found in the urine after prolonged feeding of large amounts of creatine and continues to be excreted for several weeks after creatine ingestion is discontinued.

Chanutin and associates (168) gave large amounts of creatine to rats and mice and found that the muscle creatine increased sharply for about a day, after which it remained practically constant. Liver and kidney creatine also increased. Upon cessation of creatine feeding, the excess creatine rapidly disappeared from the tissues except the muscles, which retained excessive amounts for some time.

Bloch and Schoenheimer (119) showed that N15-labeled creatine fed to rats was deposited in the tissues, and the urinary creatinine excreted contained amounts of N15 indicating its origin from creatine. These workers also fed isotopic creatinine and found the prompt excretion of most of it in the urine. Also, no N15 was found in the body creatine, indicating that the conversion of creatine to creatinine is biologically irreversible.

Hoberman and associates (170) labeled the tissue creatine of a human subject on a creatine-free diet with N15 by feeding isotopic creatine for 38 days and then isotopic guanidoacetic acid for ten days. Nonisotopic creatine then was given for five days and the urine analyzed for 28 days. The rate of

turnover of endogenous creatine, as determined by the N15 concentration of excreted creatinine .(and creatine) after nonisotopic creatine was given, showed that 1.64 per cent of the tissue creatine (endogenous creatine) turned over per day. A balance between the amount of creatine retained in the body, and the amounts deposited in the tissues or excreted as extra creatinine in the urine could not be achieved. These workers suggest that the synthesis of endogenous creatine is retarded by the presence of exogenous creatine (administered). The amount of creatinine excreted per day as determined from its N15 content was directly proportional to the amount of creatine in the body.

Hoberman and associates (171) have shown, by the use of N15-labeled creatine, that the administration of the hormone methyltestosterone increases the rate of creatine synthesis in the body.

Bloch and Schoenheimer (169) gave isotopic creatinine (N15) to rats and found 75 per cent of the N15 in urinary creatinine. The remaining 25 per cent could not be accounted for in either tissues or urine.

Creatinuria. Creatine occurs only in small amounts in the urine of normal adults. It is present, along with creatinine, during the process of growth, and it ,decreases to very low values as maturity is approached. Creatinuria is found in fevers, starvation, on a carbohydrate-free diet, and in diabetes mellitus. Its presence may be the result of excessive tissue de-struction and liberation of creatine, or failure under these conditions to keep the creatine properly phosphorylated, permitting the free creatine to diffuse from the tissues into the blood.

Excessive amounts of creatine may be excreted in the urines of patients with muscular dystrophy or hyperthyroidism.
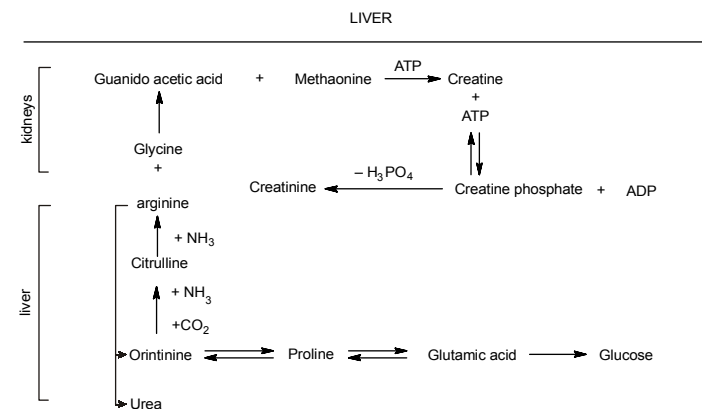
Constancy of creatinine—creatine excretion. Shaffer (177) showed that the daily excretion of creatinine by the adult male and of creatinine + creatine in subjects with physiologic creatinuria (such

as growing children) is remarkably constant. It is little affected by diet (excluding diets high.in creatine and creatinine), exercise, or urine volume. The creatinine (or creat-inine + creatine) excretion apparently is characteristic of a healthy indi-vidual and is proportional to size and particularly to muscle mass. The daily excretion of creatinine is sufficiently constant for a given individual under ordinary conditions that the accuracy of 24-hour urine collections may be checked by its determination.

Creatinine coefficient. The creatinine coefficient (or creatinine + creatine coefficient) represents the milligrams of creatinine (or creatinine + creatine) per kilogram of body weight excreted daily. The creatinine coefficient normally averages 20 to 26 for men and 14 to 22 for women.

The urine of premature infants and of normal infants during the first few days of life contains little creatine, and the creatinine coefficients are low.

Relations of Arginine and Creatine

Creatinuria and creatinine coefficients gradually increase during the first month of life:

McClugage and associates (173) found the daily creatinine excretion of obese persons to be low in relation to weight, but normal in relation to their ideal weight. Upon weight reduction through dietary control, the creatinine excretion remained constant. Underweight persons were found to have ab-normally low creatinine coefficients.

Hodgson and Lewis (174) consider the difference in creatinine coefficients of males and females to be related to the differences in muscular develop-ment rather than sex differences, since they found women with unusual mus-cular development to have creatinine coefficients comparable with those of males.

The relations between the metabolism of arginine and of creatine are sum-marized in the diagram above

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SECTION 5.26—FORMATIONS AND DISPOSAL OF AMMONIA

The ammonia in the system is generated by the deamination process of amino acids as the ammonia is harmful it is disposed of from the system by the formation of uric acid and urea.

SUB-SECTION 5.26A—THE UREA CYCLE

The **urea cycle** (also known as the **ornithine cycle**) is a cycle of biochemical reactions occurring in many animals that produces urea$(NH_2)_2CO$ from ammonia (NH_3). This cycle was the first metabolic cycle discovered (Hans Krebs and Kurt Henseleit, 1932). In mammals, the urea cycle takes place only in the liver.

Organisms that cannot easily and quickly remove ammonia usually have to convert it to some other substance, like urea or uric acid, which are much less toxic. Insufficiency of the urea cycle occurs in some genetic disorders (inborn errors of metabolism), and in liver failure. The result of liver failure is accumulation of nitrogenous waste, mainly ammonia, which leads to hepatic encephalopathy.
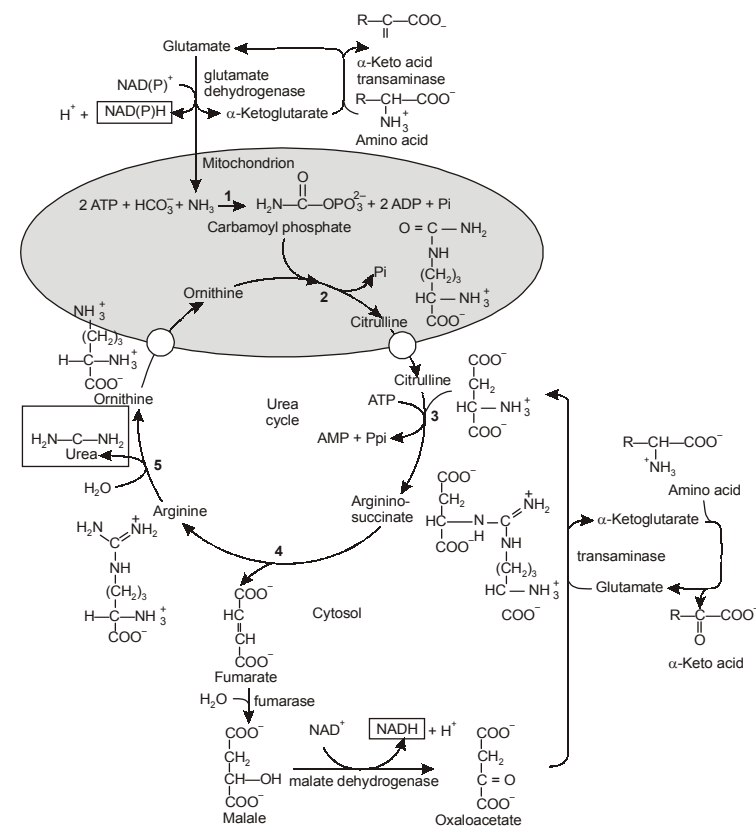
Fig. 5.48 : Showing the Urea Cycle

The urea cycle consists of five reactions - two mitochondrial and three cytosolic. The cycle converts two amino groups, one from NH_4^+ and one from Asp, and a carbon atom from HCO_3^-, to relatively nontoxic excretion product, urea, at the cost of four "high-energy" phosphate bonds (3 ATP hydrolyzed to 2 ADP and one AMP). Orn is the carrier of these carbon and nitrogen atoms.

Reactions of urea cycle:

| Step	Reactant	Product	Catalyzed by	Location
1.	$2ATP + HCO_3^- + NH_4^+$	carbamoyl phosphate + $2ADP + P_i$	CPS1	mitochondria
2.	carbamoyl phosphate + ornithine	citrulline + P_i	OTC	mitochondria
3.	citrulline + aspartate + ATP	argininosuccinate + $AMP + PP_i$	ASS	cytosol
4.	argininosuccinate	Arg + fumarate	ASL	cytosol
5	$Arg + H_2O$	ornithine + urea	ARG1	cytosol

Overall energy requirement:

- $NH_3 + CO_2 + Aspartate + 3 ATP + 2 H_2O \rightarrow urea + Fumarate + 2 ADP + 4 P_i + AMP$

Overall equation of the urea cycle:

- $2 NH_3 + CO_2 + 3 ATP + H_2O \rightarrow urea + 2 ADP + 4 P_i + AMP + 2H$

Note that reactions related to the urea cycle also cause the reduction of 2 NADH, so the urea cycle releases slightly more energy than it consumes. These NADH are produced in two ways:

- One NADH molecule is reduced by the enzyme glutamate dehydrogenase in the conversion of glutamate to ammonium and a-ketoglutarate.Glutamate is the non-toxic carrier of amine groups. This provides the ammonium ion used in the initial synthesis of carbamoyl phosphate.
- The fumarate released in the cytosol is converted to malate by cytosolic fumarase. This malate is then converted to oxaloacetate by cytosolic malate dehydrogenase, generating a reduced NADH in the cytosol. Oxaloacetate is one of the keto acids preferred bytransaminases, and so will be recycled to aspartate, maintained the flow of nitrogen into the urea cycle.

The two NADH produced can provide energy for the formation of 5 ATP, a net production of one high energy phosphate bond for the urea cycle. However, if gluconeogenesis is underway in the cytosol, the latter reducing equivalent is used to drive the reversal of the GAPDH step instead of generating ATP.

The fate of oxaloacetate is either to produce aspartate via oxidative deamination or to be converted to phosphoenol pyruvate, which is a substrate to glucose.

An excellent way to memorize the Urea Cycle is to remember the phrase "Ordinarily Careless Crappers Are Also Frivolous About Urination." The first letter of each word corresponds to the order in which reactants are combined to give products or intermediates that break apart as one progresses through the cycle.

N-Acetylglutamic Acid

The synthesis of carbamoyl phosphate and the urea cycle are dependent on the presence of NAcGlu, which allosterically activates CPS1. Synthesis of NAcGlu by NAGS, is stimulated by Arg - allosteric

stimulator of NAGS, and Glu - a product in the transamination reactions and one of NAGS's substrates, both of which are elevated when free amino acids are elevated. So, Arg is not only a substrate for the urea cycle reactions but also serves as an activator for the urea cycle.

Substrate Concentrations

The remaining enzymes of the cycle are controlled by the concentrations of their substrates. Thus, inherited deficiencies in the cycle enzymes other than ARG1 do not result in significant decrease in urea production (the total lack of any cycle enzyme results in death shortly after birth). Rather, the deficient enzyme's substrate builds up, increasing the rate of the deficient reaction to normal.

The anomalous substrate buildup is not without cost, however. The substrate concentrations become elevated all the way back up the cycle to NH_{4+}, resulting in hyperammonemia (elevated $[NH_4^+]_P$).

Although the root cause of NH_4^+ toxicity is not completely understood, a high $[NH_4^+]$ puts an enormous strain on the NH_4^+-clearing system, especially in the brain (symptoms of urea cycle enzyme deficiencies include mental retardation and lethargy). This clearing system involves GLUD1 and GLUL, which decrease the 2OG and Glu pools. The brain is most sensitive to the depletion of these pools. Depletion of 2OG decreases the rate of TCAC, whereas Glu is both a neurotransmitter and a precursor to GABA, another neurotransmitter.

SUB-SECTION 5.26B—THE DISORDERS OF UREA CYCLE

An **urea cycle disorder** or **urea cycle defect** is a genetic disorder caused by a deficiency of one of the enzymes in the urea cycle which is responsible for removing ammonia from the bloodstream. The urea cycle involves a series of biochemical steps in which nitrogen, a waste product ofprotein metabolism, is removed from the blood and converted to urea. Normally, the urea is transferred into the urine and removed from the body. In urea cycle disorders, the nitrogen accumulates in the form of ammonia, a highly toxic substance, and is not removed from the body.

Urea cycle disorders are included in the category of inborn errors of metabolism. There is no cure.

Inborn errors of metabolism are generally considered to be rare but represent a substantial cause of brain damage and death among newborns and infants. Because many cases of urea cycle disorders remain undiagnosed and/or infants born with the disorders die without a definitive diagnosis, the exact incidence of these cases is unknown and underestimated. It is believed that up to 20% of Sudden Infant Death Syndromecases may be attributed to an undiagnosed inborn error of metabolism such as urea cycle disorder. In April 2000, research experts at the Urea Cycle Consensus Conference estimated the incidence of the disorders at 1 in 10000 births. This represents a significant increase in case diagnosis in the last two years.

Children with very severe urea cycle disorders typically show symptoms after the first 24 hours of life. The baby may be irritable at first, followed by vomiting and increasing lethargy. Soon after, seizures, hypotonia (poor muscle tone), respiratory distress, and coma may occur. If untreated, the child will die. These symptoms are caused by rising ammonia levels in the blood. Acute neonatal symptoms are most frequently seen in, but not limited to, boys with OTC Deficiency.

Children with mild or moderate urea cycle enzyme deficiencies may not show symptoms until early childhood, or may be diagnosed subsequent to identification of the disorder in a more severely affected relative or through newborn screening. Early symptoms may includehyperactive behavior, sometimes accompanied by screaming and self-injurious behavior, and refusal to eat meat or other high-protein foods. Later symptoms may include frequent episodes of vomiting, especially following high-protein meals; lethargy and delirium; and finally, if the condition is undiagnosed and untreated, coma and death. Children with this disorder may be referred to child psychologists because of their behavior and eating problems. Childhood episodes of hyperammonemia (high ammonia levels in the blood) may be brought on by viral illnesses including chicken pox, high-protein meals, or even exhaustion. The condition is sometimes misdiagnosed as Reye's Syndrome. Childhood onset can be seen in both boys and girls.

Recently, the number of adult individuals being diagnosed with urea cycle disorders has increased at an alarming rate. Recent evidence has indicated that these individuals have survived undiagnosed to adulthood, probably due to less severe enzyme deficiencies. These individuals exhibit stroke-like symptoms, episodes of lethargy, and delirium. These adults are likely to be referred to neurologists or psychiatrists because of their psychiatric symptoms. However, without proper diagnosis and treatment, these individuals are at risk for permanent brain damage, coma, and death. Adult-onset symptoms have been observed following viral illnesses, childbirth, and use of valproic acid (an anti-epileptic drug).

There are six disorders of the urea cycle. Each is referred to by the initials of the missing enzyme.

Location	Abb.	Enyzme	Disorder	Measurements
Mitochondria	NAGS	N-Acetylglutamate synthetase	N-Acetylglutamate synthase deficiency	+Ammonia
Mitochondria	CPS1	Carbamoyl phosphate synthetase I	Carbamoyl phosphate synthetase I deficiency	+Ammonia
Mitochondria	OTC	Ornithine transcarbamylase	Ornithine transcarbamylase deficiency	+Ornithine, +Uracil, +Orotic acid
Cytosol	AS	Argininosuccinic acid synthetase	"AS deficiency" or citrullinemia	+Citrulline
Cytosol	AL	Argininosuccinase acid lyase	"AL deficiency" or argininosuccinic aciduria(ASA)	+Citrulline, +Argininosuccinic acid
Cytosol	AG	Arginase	"Arginase deficiency" or argininemia	+Arginine

Individuals with childhood or adult onset disease may have a partial enzyme deficiency. All of these disorders are transmitted genetically as autosomal recessive genes - each parent contributes a defective gene to the child, except for one of the defects, Ornithine Transcarbamylase Deficiency. This urea cycle disorder is acquired in one of three ways: as an X-linked trait from the mother, who may be an undiagnosed carrier; in some cases of female children, the disorder can also be inherited from the father's X-chromosome; and finally, OTC deficiency may be acquired as a "new" mutation occurring in

the fetus uniquely. Recent research has shown that some female carriers of the disease may become symptomatic with the disorder later in life, suffering high ammonia levels. Several undiagnosed women have died during childbirth as a result of high ammonia levels and on autopsy were determined to have been unknown carriers of the disorder.

The treatment of urea cycle disorders consists of balancing dietary protein intake in order that the body receive the essential amino acidsresponsible for cell growth and development, but not so much protein that excessive ammonia is formed. This protein restriction is used in conjunction with medications which provide alternative pathways for the removal of ammonia from the blood. These medications are usually given by way of tube feedings, either via gastrostomy tube (a tube surgically implanted in the stomach) or nasogastric tube through the nose into the stomach. The treatment may also include supplementation with special amino acid formulas developed specifically for urea cycle disorders, multiple vitamins and calcium supplements. Frequent blood tests are required to monitor the disorders and optimize treatment, and frequently hospitalizations are necessary to control the disorder.

At the most extreme end of the spectrum, a few liver transplants have been done successfully as a cure to the disorder. This treatment alternative must be carefully evaluated with medical professionals to determine if potential of success as compared to the potential for new medical concerns.
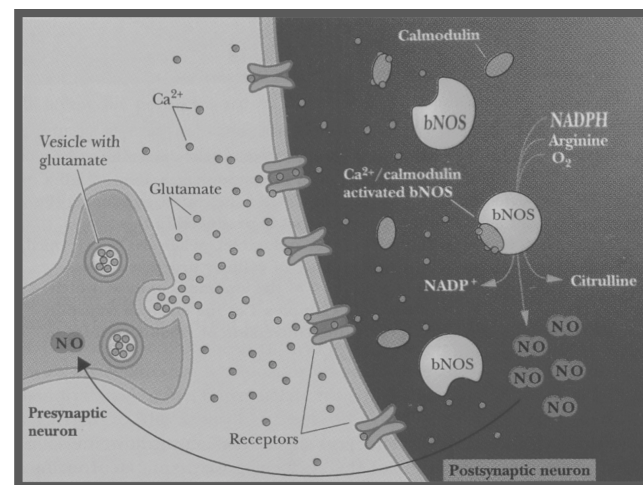
SUB-SECTION 5.26C—FORMATION OF NITRIC OXIDES (NOS)

Fig. 5.48 : Showing the formation of Nitric Oxide (NOS)

The activation of nitric oxide synthase in brain (bNOS). A neuron by a nerve centre impulse (the presynaptic neuron at left, releases glutamate into the synapse. Glutamate binds to a membrane receptor on the adjacent postsynaptic neuron. As a result of the binding, a channel opens in the receptor, allowing Ca^{2+} ions to flow into the cell and to bind to the calmodulin. The calcium – calmodulin complex then binds bNOS, thereby activating it so that it can catalyze the formation of nitric oxide. The nitric oxide thus formed can diffuse to other neurons to reinforce neural connections.

The small, free radical molecule nitric oxide (NO; N = O) has been identified as a major signal transduction molecule in vertebrates (animals). NO is derived from arginine in two steps catalyzed by nitric oxide synthase (NOS; EC 1.14.13.39). NOS catalyzes the net reaction:

$$\text{L-Arginine} + \text{n NADPH} + \text{mO}_2 = \text{Citrulline} + \text{Nitric oxide} + \text{n NADP}^+$$
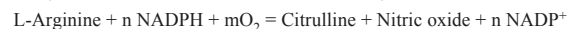
with the intermediate N-(omega)-Hydroxyarginine (C05933). The catalytic activity of *nitric oxide synthase* is related to the monooxygenase activity of *cytochrome P450*. This catalytic relationship becomes apparent when comparing the gene structure of both enzymes. Nitric oxide synthase is the larger of the two enzymes. Its C-terminal domain is identical to the smaller Cyt P450 protein.
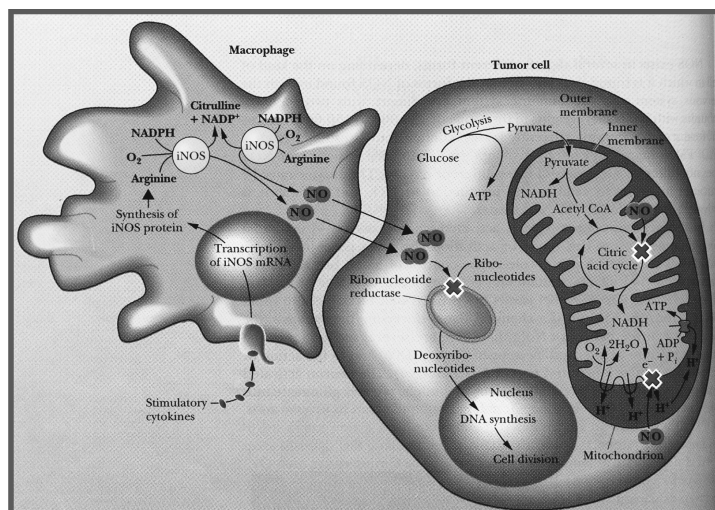
Fig. 5.48 : Showing the Role of Nitric acid in the immune system

There are three forms of nitric oxide synthase - a neuronal type called nNOS, an epithelial type called eNOS, and an inducible form called iNOS. The latter is only expressed under certain conditions

like immune system regulation by cytokines or pathological induction in the presence of endotoxins (bacterial lipopolysaccharide) and cytotoxins (which affect cytokine secretion). No production is a stress response and can lead to either tissue injury because of its radical chemistry, or be cytoprotective, protecting cells from damage by destroying pathogenic microorganisms first. For example, stomach ulcers have lately been associated with a bacterial infection. Helicobacter pylori (H.pylori) causes ulcers and gastric cancers. Nitric oxide and particularly its superoxide derivative peroxynitrite cause DNA damage in the bacteria.

The production of NO in the immune system takes place in macrophages (components of the immune system).. When stimulatory cytokines bind to macrophages, they set off a chain of events that leads to synthesis of NOS. The DNA that encodes the gene for NOS is activated, producing the messenger RNA for the enzyme which is then synthesized by the cell. An enzyme that is produced in this way is referred to as inducible. Consequently, this form of NOS is designated as iNOS (i for inducible). When iNOS is formed it diffuses to tumor cells close to the macrophage. There it interferes with a number of cellular processes, which occurs at high rates in rapidly growing cancer cells. One site of action is inhibition of the enzyme aconitase, which catalyzes one of the early steps of the citric acid cycle. Nitric oxide also interferes with complex I of the electron transport chain. Another site of action is inhibition of ribonucleotide reductase, preventing conversion of ribonucleotide to deoxyribonucleotides and thus interfering with DNA synthesis in the cancer cells.

Free radicals as antimicrobials: ONOO induces DNA damage through chemical modifications (mutations) while NO inhibits ribonucleotide reductase. Both DNA damage and reductase inhibition keep the cell in a state of energy costly nucleotide synthesis and repair mode. This leads eventually to cell death by energy depletion of bacterial cells.

Such defense mechanism, however, have their draw backs. Inducible NOS, which is expressed as an emergency mechanism to suppress tumor growth in gastric epithelia, breast tissue, and the brain, is linked to septic shock. Bacterial endotoxins (e.g. from H.pylori or E.coli infections) induce the iNOS gene, which in turn produces high levels of NO damaging pathogenic DNA and inhibiting respiration (inhibits metabolic energy production needed for cell division). The free radicals, however, cannot discriminate pathogenic DNA from host DNA and overstimulation of iNOS therefore induces cell and tissue damage, sometimes leading to a fatal development (septic shock) in the course of bacterial infections. This is a well known situation in hospitals affecting patients with an already suppressed immune system.

The neuronal and epithelial NOS isoforms are constitutively expressed and regulated by calcium concentration via calmodulin interaction. The calcium-calmodulin complex stabilizes the homodimer. Each monomer contains a reductase and oxygenase subunit containing FAD, NADPH & FMN or heme & tetrahydrobiopterine (B4H) cofactors, respectively. B4H is essential for NOS dimer formation. (H4biopterine is an important cofactor of aromatic amino acid hydroxylases.) Nitric oxide synthase is a membrane bound protein, anchored to the cytoplasmic side of endoplasmatic reticulum, Golgi, or plasma membrane by myristoylation or palmitoylation. The lipid anchored NOS are preferentially found

in cholesterol and glycolipid rich membrane domains. The compartmentalization of NOS appears to be crucial for its functionality by providing local NO levels.

NOS Regulation

The activity of eNOS and nNOS is controlled by tetrahydrobiopterin and Ca/CaM availability because these two cofactors are needed for the proper dimer formation of an active synthetase. The dependence on calmodulin has been used as a model to explain the role of glutamate in neurotoxicity in the central nervous system. Neurotoxicity is a mechanism of glutamate induced neuronal cell death. The immediate effect of glutamate on neurons is its role in activating glutamate receptor, namely to pharmacological subtypes known as NMDA Receptors (NMDA is a methylated derivative of aspartate). Glutamate receptors are selective for calcium ions. Thus, prolonged activation of glutamate receptors stimulates eNOS via Ca/CaM complex binding to the synthetase. The formation of NO is implicated in cell death as described above: DNA damage, suppressed mitochondrial respiration, leading to energy depletion. Neurons are particularly sensitive to impaired mitochondrial ATP synthesis capacity, because neurons depend almost exclusively on the oxidative degradation of glucose and ketone bodies. The formed ATP is used by ion selective pumps to maintain the proper ion gradients for action potential generation and neurotransmitter release of presynaptic membranes.

NO can only be synthesized, however, if the amino acid arginine is available. Neuronal NOS critically depends on this substrate, which is mainly synthesized in adjacent glial cells and is transported into neurons. Arginine uptake into neurons is controlled by non-NMDA glutamate receptors. This became evident when these receptors were blocked by arginine-uptake inhibitors such as L-lysine which functions as antagonist of these glutamate receptors. The physiological role of nNOS in mechanisms such as long term potentiation has been shown to involve retrograde transport (diffusion) of NO synthesized in post synaptic neurons across the synaptic cleft into synapses, where they stimulated guanyl cyclase.

Nitric Oxide and free Radical Biochemistry

Nitric oxide is a free radical molecule and its major effect is the activation of cytoplasmic, soluble guanyl cyclase (sGC; EC 4.6.1.2). This enzyme catalyzes the cyclization of GTP to cGMP + PPi. Cyclic GMP is a signaling molecule (similar to cAMP) by virtue of activating protein kinases.

Nitric oxide binds to the heme group of cyclase. Other protein targets are metallo enzymes, where NO binds to Fe-S clusters. Aconitase is inactivated by NO, as is complex IV, the cytochrome oxidase in the inner membrane of mitochondria. Thus NO as an inhibitory effect on oxidative phosphorylation by blocking the electron transport chain and controlling the levels of citrate in the Krebs cycle essentially blocking the oxidative degradation of acetyl-CoA.

NO is a short lived chemical transmitter, which is freely diffusible across membranes. The molecule possesses a small dipole moment because of the similar electro negativity of oxygen and nitrogen, making it essentially hydrophobic. Its reactivity is due to the unpaired electron in the outer valence orbital of its oxygen constituent. NO is almost non reactive as free radical as compared to other oxygen radicals. Indeed, NO decays within seconds after its synthesis if left unbound in solution because it reacts with either molecular oxygen or superoxide.

NO strongly interacts with molecular oxygen to form dinitro trioxide (N_2O_3), or with superoxide O_2^- to form peroxynitrite ($ONOO^-$). NO also binds to sulphydryl groups (SH) and unsaturated fatty acids. The reaction with superoxide can be diminished by superoxide dismutase (SOD) which removes O_2^-. to form hydrogen peroxide (H_2O_2). NO can be 'stored' by covalent interaction to glutathione to form S-nitroso-glutathion. Both H_2O_2 and S-nitrosoglutathion can have a stimulatory effect on guanine cyclase. Superoxide dismutase thereby prevents the loss of nitric oxide to peroxynitrite forming hydrogen peroxide instead and increasing the cyclase stimulatory capacity of the cell. See glutathione metabolism

NO can potentially be regenerated from $ONOO^-$ in two steps; a first reduction of peroxynitrite by cytochrome C oxidase to nitrite (NO_2), followed by a reduction of nitrite to NO by the enzyme nitrate reductase. The latter enzyme exists in two isoforms, a mitochondrial type and an endoplasmatic reticulum resident protein. Both receive their electrons needed for nitrite reduction to NO from either NADH or NADPH, and interact with flavoproteins (FAD prosthetic groups) and cytochromes (cytochrome c oxidase in mitochondrial membrane; cytochrome P450 in ER membrane).

Cytochrome C oxidase and nitrite reductase therefore reduce the concentration of highly reactive, secondary metabolites, and potentially contribute to NO signaling. The latter has only been shown in plant cells, where nitrate reductase reaction appears to be a considerable contributor to this signaling molecule.

Peroxynitrite, hydrogen peroxide, and dinitro trioxide all have been linked to cell death (apoptosis = programmed cell death) through protein nitration and increased mutagenesis. The latter is a consequence of DNA stand breakage and guanine nitration. For example, acute neural toxicity is linked to the overproduction of peroxynitrite, which inhibits respiratory enzymes and also damages DNA by covalent bond formation to DNA and removal of bases. Inhibitors of nitric oxide synthase and antioxidants are known to have neuroprotective properties because the limit the formation of highly reactive nitrogen containing radicals.

Antioxidants

The free radical chemistry in cells can be prevented or at least diminished by adding antioxidants or free radical scavengers, molecules which have a high affinity and strongly react with these free radicals. Antioxidants are either hydrophilic or hydrophobic. Hydrophilic antioxidants include glutathione peroxidase, Fe(II) chelators like the proteins ceruloplasmin and transferrin, and hydroxylated aromatic molecules like uric acid or ascorbate (vitamin C). Hydrophobic antioxidants include flavin-nucleotide or carotene containing proteins and vitamin E.

Melatonin too is a major physiological antioxidant (and hormone) by directly reacting with hydroxyl and peroxyl radicals, or by stimulating the expression of superoxide dismutase, glutathione peroxidase, or glutathione reductase. Melatonin has also been reported to inhibit nitric oxide synthetase.

Physiological Role of NO as Neurotransmitter

In epithelial cells, NO causes vascular dilatation by controlling smooth muscle contractility. In the central nervous system it affects synaptic transmission stimulating learning and memory capacity. Glutamate is produced and released by a synapse and activates the NMDA receptor subtype of glutamate

receptors. This leads to an influx of calcium ions which in turn bind to calmodulin, activating the neuronal NOS. NOS synthesizes NO depending on the availability of L-arginine, which is mainly supplied from extra-neuronal sites (mainly glial cells). NO not only activates the postsynaptic guanyl cyclases, but can diffuse across the synaptic cleft back into the synapse that originally released the glutamate. This retrograde transport of NO is thought to reinforce the capability of glutaminergic signaling. Such a prolonged reinforcement of synaptic stimulatory activity is known as long term potentiation and is implicated as a possible molecular mechanism promoting long term memory and learning.

In blood plasma NO induces platelet aggregation, an important factor in wound healing and blood coagulation. It has been shown that hemoglobin is a major transport vehicle for NO in blood.

SUB-SECTION 5.26D—THE METABOLISM OF PHENYLALANINE AND TYROSINE

Generally mammals are unable to synthesize Phenyl alanine and tyrosine, but some microorganisms like E coli, neurospora etc are all capable of producing Phenylalanine and tyrosine. The amino acids being derived from common precursor, prehenic acid. The fig 174 will demonstrate the metabolism of Phenyl alanine and tyrosine.
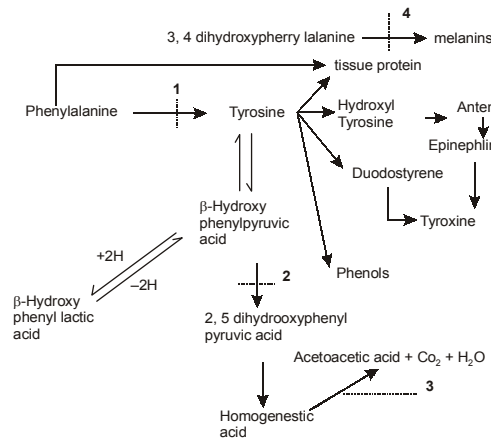
1. Blocked in Phenylketonurea (phenyl pyruvic aligophrenia
2. Blocked in tyrosinosis
3. Blocked in alcaptourea
4. Blocked in albinism

Fig. 5.46 : Showing the Metabolism of Tyrosine and Phenylalanine
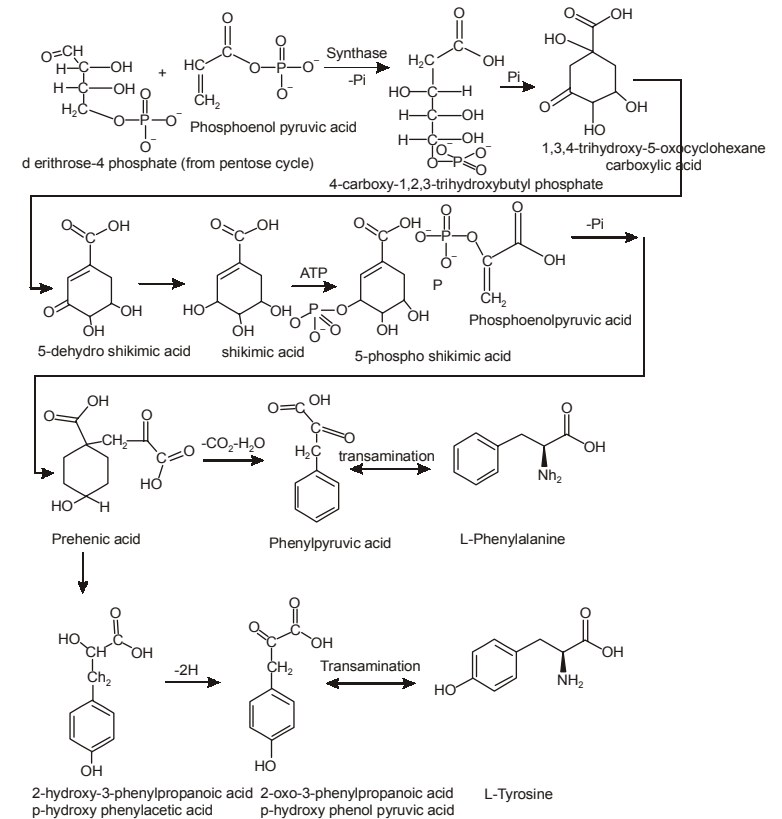
Fig. 5.47 : Showing the Tyrosine and Phenylalanine Biosynthesis

SUB-SECTION 5.26E—FORMATION OF MELANIN

Melanin (Greek ìÝëavl, melani black; is a class of compounds found in the plant, animal, and protista kingdoms, where it serves predominantly as a pigment. The class of pigments are derivatives of the

amino acid tyrosine. The most common form of biological melanin is eumelanin, a brown-black polymer of dihydroxindole, dihydroxyindole carboxylic acid, and their reduced forms. Another common form of melanin is pheomelanin, a red-brown polymer of benzothiazine units largely responsible for red hair and freckles. The presence of melanin in the archaea and bacteria kingdoms is an issue of ongoing debate amongst researchers in the field. The increased production of melanin in human skin is called melanogenesis. It is stimulated by the DNA damages that are caused by UVB-radiation, and it leads to a delayed development of a tan. This melanogenesis-based tan takes more time to develop, but it is long lasting.
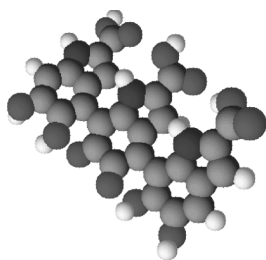
The photochemical properties of melanin make it an excellent photoprotectant. It absorbs harmful UV-radiation and transforms the energy into harmless amounts of heat through a process called "ultrafast internal conversion". This property enables melanin to dissipate more than 99.9% of the absorbed UV radiation as heat and it keeps the generation of free radicals at a minimum (see photoprotection). This prevents the indirect DNA damage which is responsible for the formation of malignant melanoma.

In humans, melanin is the primary determinant of human skin color and also found in hair, the pigmented tissue underlying the iris, the medulla and zona reticularis of the adrenal gland, the stria vascularis of the inner ear, and in pigment-bearing neurons within areas of the brain stem, such as the locus ceruleus and the substantia nigra.

Dermal melanin is produced by melanocytes, which are found in the stratum basale of the epidermis. Although human beings generally possess a similar concentration of melanocytes in their skin, the melanocytes in some individuals and ethnic groups more frequently or less frequently express the melanin-producing genes, thereby conferring a greater or lesser concentration of skin melanin. Some individual animals and humans have very little or no melanin in their bodies, a condition known as albinism.

Because melanin is an aggregate of smaller component molecules, there are a number of different types of melanin with differing proportions and bonding patterns of these component molecules. Both pheomelanin and eumelanin are found in human skin and hair, but eumelanin is the most abundant melanin in humans, as well as the form most likely to be deficient in albinism.

Eumelanin polymers have long been thought to comprise numerous cross-linked 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) polymers; recent research into the electrical properties of eumelanin, however, has indicated that it may consist of more basic oligomers adhering to one another by some other mechanism. Thus, the precise nature of eumelanin's molecular structure is once again the object of study. Eumelanin is found in hair and skin, and colors hair grey, black, yellow, and brown. In humans, it is more abundant in peoples with dark skin. There are two different types of eumelanin, which are distinguished from each other by their pattern of polymer bonds. The two types are black eumelanin and brown eumelanin, with black melanin being darker than brown. Black eumelanin

is in mostly non-Europeans and aged Europeans, while brown eumelanin is in mostly young Europeans. A small amount of black eumelanin in the absence of other pigments causes grey hair. A small amount of brown eumelanin in the absence of other pigments causes yellow (blond) color hair.

Pheomelanin is also found in hair and skin and is both in lighter skinned humans and darker skinned humans. In general women have more pheomelanin than men, and thus women's skin is generally redder than men's. Pheomelanin imparts a pink to red hue and, thus, is found in particularly large quantities in red hair. Pheomelanin is particularly concentrated in the lips, nipples, glans of the penis, and vagina.[4] Pheomelanin also may become carcinogenic when exposed to the ultraviolet rays of the sun. Chemically, pheomelanin differs from eumelanin in that its oligomer structure incorporates benzothiazine units which are produced instead of DHI and DHICA when the amino acid L-cysteine is present.

Neuromelanin is the dark pigment present in pigment bearing neurons of four deep brain nuclei: the substantia nigra (in Latin, literally "black substance") - Pars Compacta part, the locus ceruleus ("blue spot"), the dorsal motor nucleus of the vagus nerve (cranial nerve X), and the median raphe nucleus of the pons. Both the substantia nigra and locus ceruleus can be easily identified grossly at the time of autopsy due to their dark pigmentation. In humans, these nuclei are not pigmented at the time of birth, but develop pigmentation during maturation to adulthood. Although the functional nature of neuromelanin is unknown in the brain, it may be a byproduct of the synthesis of monoamine neurotransmitters for which the pigmented neurons are the only source. The loss of pigmented neurons from specific nuclei is seen in a variety of neurodegenerative diseases. In Parkinson's disease there is massive loss of dopamine producing pigmented neurons in the substantia nigra. A common finding in advanced Alzheimer's disease is almost complete loss of the norepinephrine producing pigmented neurons of the locus ceruleus. Neuromelanin has been detected in primates and in carnivores such as cats and dogs.

Biosynthetic Pathways

The first step of the biosynthetic pathway for both eumelanins and pheomelanins is catalysed by tyrosinase:

Tyrosine $\rightarrow\rightarrow$ DOPA $\rightarrow$ dopaquinone

Dopaquinone can combine with cysteine by two pathways to benzothiazines and pheomelanins

Dopaquinone + cysteine $\rightarrow$ 5-S-cysteinyldopa $\rightarrow$ benzothiazine intermediate $\rightarrow$ pheomelanin

Dopaquinone + cysteine $\rightarrow$ 2-S-cysteinyldopa $\rightarrow$ benzothiazine intermediate $\rightarrow$ pheomelanin
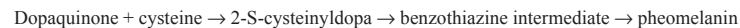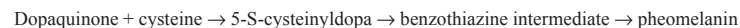
Alternatively, dopaquinone can be converted to leucodopachrome and follow two more pathways to the eumelanins

Dopaquinone $\rightarrow$ leucodopachrome $\rightarrow$ dopachrome $\rightarrow$ 5,6-dihydroxyindole-2-carboxylic acid $\rightarrow$ quinone $\rightarrow$ eumelanin

Dopaquinone $\rightarrow$ leucodopachrome $\rightarrow$ dopachrome $\rightarrow$ 5,6-dihydroxyindole $\rightarrow$ quinone $\rightarrow$ eumelanin

DOPA

Dopa quinone

5-hydroxy-2,3,5,6-tetrahydro-1H-indole-2-carboxylic acid

1H-indole-5,6-diol

1H-indole-5,6-dione
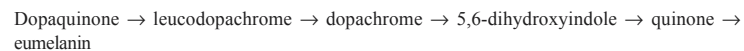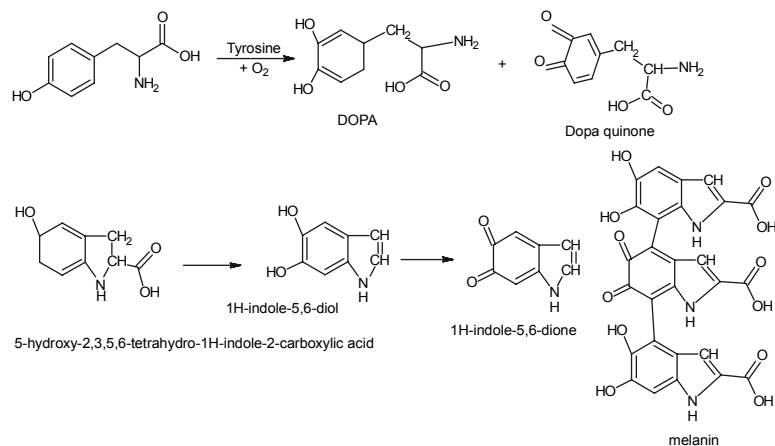
melanin

Microscopic Appearance

Under the microscope melanin is brown, non-refractile and finely granular with individual granules having a diameter of less than 800 nanometers. This differentiates melanin from common blood breakdown pigments which are larger, chunky and refractile and range in color from green to yellow or red-brown. In heavily pigmented lesions, dense aggregates of melanin can obscure histologic detail. A dilute solution of potassium permanganate is an effective melanin bleach.

Melanin Deficiency in Genetic Disorders and Disease States

Melanin deficiency has been connected for some time with various genetic abnormalities and disease states.

There are approximately ten different types of oculocutaneous albinism, which is mostly an autosomal recessive disorder. Certain ethnicities have higher incidences of different forms. For example, the most common type, called oculocutaneous albinism type 2 (OCA2), is especially frequent among people of black African descent. It is an autosomal recessive disorder characterized by a congenital reduction or absence of melanin pigment in the skin, hair and eyes. The estimated frequency of OCA2 among African-Americans is 1 in 10,000, which contrasts with a frequency of 1 in 36,000 in white Americans. In some African nations, the frequency of the disorder is even higher, ranging from 1 in 2,000 to 1 in 5,000. Another form of Albinism, the "yellow oculocutaneous albinism", appears to be more prevalent among the Amish, who are of primarily Swiss and German ancestry. People with this IB variant of the disorder commonly have white hair and skin at birth, but rapidly develop normal skin pigmentation in infancy.

Ocular albinism affects not only eye pigmentation, but visual acuity, as well. People with albinism typically test poorly, within the 20/60 to 20/400 range. Additionally, two forms of albinism, with approximately 1 in 2700 most prevalent among people of Puerto Rican origin, are associated with mortality beyond melanoma-related deaths.

Mortality also is increased in patients with Hermansky-Pudlak syndrome and Chediak-Higashi syndrome. Patients with Hermansky-Pudlak syndrome have a bleeding diathesis secondary to platelet dysfunction and also experience restrictive lung disease (pulmonary fibrosis), inflammatory bowel disease, cardiomyopathy, and renal disease. Patients with Chediak-Higashi syndrome are susceptible to infection and also can develop lymphofollicular malignancy.

The role that melanin deficiency plays in such disorders remains under study.

The connection between albinism and deafness has been well known, though poorly understood, for more than a century-and-a-half. In his 1859 treatise *On the Origin of Species*, Charles Darwin observed that "cats which are entirely white and have blue eyes are generally deaf". In humans, hypopigmentation and deafness occur together in the rare Waardenburg's syndrome, predominantly observed among the Hopi in North America. The incidence of albinism in Hopi Indians has been estimated as approximately 1 in 200 individuals. Interestingly, similar patterns of albinism and deafness have been found in other mammals, including dogs and rodents. However, a lack of melanin per se does not appear to be directly responsible for deafness associated with hypopigmentation, as most individuals lacking the enzymes required to synthesize melanin have normal auditory function. Instead the absence of melanocytes in the stria vascularis of the inner ear results in cochlear impairment, though why this is is not fully understood. It may be that melanin, the best sound absorbing material known, plays some protective function. Alternately, melanin may affect development, as Darwin suggests.

In Parkinson's disease, a disorder that affects neuromotor functioning, there is decreased neuromelanin in the substantia nigra as consequence of specific dropping out of dopaminergic pigmented neurons. This results in diminished dopamine synthesis. While no correlation between race and the level of neuromelanin in the substantia nigra has been reported, the significantly lower incidence of Parkinson's in blacks than in whites has "prompt[ed] some to suggest that cutaneous melanin might somehow serve to protect the neuromelanin in substantia nigra from external toxins.". Also see Nicolaus review article on the function of neuromelanins

In addition to melanin deficiency, the molecular weight of the melanin polymer may be decreased due to various factors such as oxidative stress, exposure to light, perturbation in its association with melanosomal matrix proteins, changes in pH or in local concentrations of metal ions. A decreased molecular weight or a decrease in the degree of polymerization of **ocular melanin** has been proposed to turn the normally anti-oxidant polymer into a pro-oxidant. In its pro-oxidant state, melanin has been suggested to be involved in the causation and progression of macular degeneration and melanoma.

Higher eumelanin levels also can be a disadvantage, however, beyond a higher disposition toward vitamin D deficiency. Dark skin is a complicating factor in the laser removal of port-wine stains. Effective in treating white skin, lasers generally are less successful in removing port-wine stains in people of Asian or African descent. Higher concentrations of melanin in darker-skinned individuals

simply diffuse and absorb the laser radiation, inhibiting light absorption by the targeted tissue. Melanin similarly can complicate laser treatment of other dermatological conditions in people with darker skin.

Freckles and moles are formed where there is a localized concentration of melanin in the skin. They are highly associated with pale skin.

Melanin and Human Adaptation

Melanocytes insert granules of melanin into specialized cellular vesicles called melanosomes. These are then transferred into the other skin cells of the human epidermis. The melanosomes in each recipient cell accumulate atop the cell nucleus, where they protect the nuclear DNA from mutations caused by the ionizing radiation of the sun's ultraviolet rays. People whose ancestors lived for long periods in the regions of the globe near the equator generally have larger quantities of eumelanin in their skins. This makes their skins brown or black and protects them against high levels of exposure to the sun, which more frequently results in melanomas in lighter skinned people.

With humans, exposure to sunlight stimulates the skin to produce vitamin D. Because high levels of cutaneous melanin act as a natural sun screen, dark skin can be a risk factor for vitamin D deficiency.

In the United Kingdom, which lies at a northern latitude, descendants of the Britons have white skin. When their skin is exposed to the meager sunlight, the scant amount of melanin their skin produces is unable to block the sunlight. Therefore, their bodies are able to make Vitamin D with the help of sunlight. Vitamin D, a vitamin found in fish oil, is necessary to prevent rickets, a bone disease caused by too little calcium.

In contrast, in Sub-Saharan Africa, which is near the equator, humans with a higher concentration of melanin absorb more intense sunlight to make Vitamin D. Africans visiting the United Kingdom during the Industrial Revolution developed symptoms of rickets, such as retarded growth, bowed legs, and fractures because sunlight at that latitude was insufficient for their melanin levels.

Fortunately, in 1930, Vitamin D was discovered and dispensed as a supplement to add to the diet. Now many common foods like milk and bread are Vitamin D fortified.

The most recent scientific evidence indicates that all humans evolved in Africa, then populated the rest of the world through successive radiations. It is most likely that the first people had relatively large numbers of eumelanin producing melanocytes and, accordingly, darker skin (as displayed by the indigenous people of Africa, today). As some of these original peoples migrated and settled in areas of Asia and Europe, the selective pressure for eumelanin production decreased in climates where radiation from the sun was less intense. Thus variations in genes involved in melanin production began to appear in the population, resulting in lighter hair and skin in humans residing at northern latitudes. Studies have been carried out to determine whether these changes were due to genetic drift or positive selection, perhaps driven by requirement for vitamin D. Of the two common gene variants known to be associated with pale human skin, does not appear to have undergone positive selection, while SLC24A5 has.

As with peoples who migrated northward, those with light skin who migrate southward acclimatize to the much stronger solar radiation. Most people's skin darkens when exposed to UV light, giving

them more protection when it is needed. This is the physiological purpose of sun tanning. Dark-skinned people, who produce more skin-protecting eumelanin, have a greater protection against sunburn and the development of melanoma, a potentially deadly form of skin cancer, as well as other health problems related to exposure to strong solar radiation, including the photodegradation of certain vitamins such as riboflavins, carotenoids, tocopherol, and folate.

Melanin in the eyes, in the iris and choroid, helps protect them from ultraviolet and high-frequency visible light; people with blue and gray eyes are more at risk for sun-related eye problems and for red-eye effect in photographs. Further, the ocular lens yellows with age, providing added protection. However, the lens also becomes more rigid with age, losing most of its accommodation — the ability to change shape to focus from far to near — a detriment due probably to protein crosslinking caused by UV exposure.

Recent research by J.D. Simon suggests that melanin may serve a protective role other than photoprotection. Melanin is able to effectively ligate metal ions through its carboxylate and phenolic hydroxyl groups, in many cases much more efficiently than the powerful chelating ligand ethylenediaminetetraacetate (EDTA). It may thus serve to sequester potentially toxic metal ions, protecting the rest of the cell. This hypothesis is supported by the fact that the loss of neuromelanin observed in Parkinson's disease is accompanied by an increase in iron levels in the brain.

Physical Properties and Technological Applications

"Melanin" is the name not only of the biological substance described in the article thus far, but of a quite different class of synthetic substances. This section uses the term "melanin" chiefly to refer to the synthetic substances.

Melanins, in the synthetic sense, are "rigid-backbone" conductive polymers composed of polyacetylene, polypyrrole, and polyaniline "Blacks" and their mixed copolymers. The simplest melanin is polyacetylene, and some fungal melanins are pure polyacetylene.

In 1963, DE Weiss and coworkers reported high electrical conductivity in a melanin, iodine-doped and oxidized polypyrrole "Black". They achieved the quite high conductivity of 1 Ohm/cm. A decade later, John McGinness, and coworkers reported a high conductivity "ON" state in a voltage-controlled solid-state threshold switch made with DOPA melanin [4]. Further, this material emitted a flash of light—electroluminescence—when it switched. Melanin also shows negative resistance, a classic property of electronically-active conductive polymers. Likewise, melanin is the best sound-absorbing material known due to strong electron-phonon coupling. This may be related to melanin's presence in the inner ear.

Melanin voltage-controlled switch, an "active" organic polymer electronic device from 1974. Now in the Smithsonian.

These early discoveries were "lost" until the recent emergence of such melanins in device applications, particularly electroluminescent displays. In 2000, the Nobel Prize in Chemistry was awarded to three scientists for their subsequent 1977 (re)discovery and development of such conductive organic polymers. In an essential reprise of the work by Weiss et al, these polymers were oxidized, iodine-

doped "polyacetylene black" melanins. There is no evidence the Nobel committee was aware of the almost identical prior report by Weiss of passive high conductivity in iodinated polypyrrole black or of switching and high electrical conductivity in DOPA melanin and related organic semiconductors. The melanin organic electronic device is now in the Smithsonian Institution's National Museum of American History's "Smithsonian Chips" collection of historic solid-state electronic devices.

Melanin influences neural activity and mediates the conduction of radiation, light, heat and kinetic energy. As such, it is the subject of intense interest in biotech research and development, most notably in organic electronics (sometimes called "plastic electronics") and nanotechnology, where dopants are used to dramatically boost melanin conductivity. Pyrrole black and acetylene black are the most commonly studied organic semiconductors.

Although synthetic melanin (commonly referred to as BSM, or "black synthetic matter") is made up of 3-6 oligomeric units linked together—the so-called "protomolecule"—there is no evidence that naturally occurring biopolymer (BCM, for "black cell matter") mimics this structure. However, since there is no reason to believe that natural melanin does not belong to the category of the polyarenes and polycationic polyenes, like pyrrol black and acetylene black, it is necessary to review all the chemical and biological analytic data gathered to date in the study of natural melanins (eumelanins, pheomelanins, allomelanins)."

Evidence exists in support of a highly cross-linked heteropolymer bound covalently to matrix scaffolding melanoproteins. It has been proposed that the ability of melanin to act as an antioxidant is directly proportional to its degree of polymerization or molecular weight. Suboptimal conditions for the effective polymerization of melanin monomers may lead to formation of lower-molecular-weight, pro-oxidant melanin that is has been implicated in the causation and progression of macular degeneration and melanoma. Signaling pathways that upregulate melanization in the retinal pigment epithelium (RPE) also may be implicated in the downregulation of rod outer segment phagocytosis by the RPE. This phenomenon has been attributed in part to foveal sparing in macular degeneration.

Melanin-based bias in Human Societies

Consequently, sampling in much of Asia and some remote localities is insufficient (see e.g. Japanese, Inuit and Tibetans which ought to be darker-skinned than represented here because mountain area).

When skin pigmentation as a characteristic of race is linked to social status or other human attributes, this phenomenon is known as racialism. Many people and societies overlay racialism with racist perceptions and systems which arbitrarily assign to groups of people a status of inherent superiority or inferiority, privilege or disadvantage based on skin color or racial classification. Apartheid-era South Africa is an example of a white supremacist society based on a system of stratification of power and privilege by skin color, as well as racial admixture. Similar examples can be found in Brazil's highly socially color-stratified society; and, in the U.S., segregation and institutional racism on the part of white-controlled institutions, and internal "color consciousness" on the part of members of some ethnic minorities.

SUB-SECTION 5.26F—THE FORMATION OF CATICOLAMINES

Catecholamines are chemical compounds derived from the amino acid tyrosine. Their name is derived from the fact that they contain catechol and amine moieties. Some of them are biogenic amines.

Catecholamines are water-soluble and are 50% bound to plasma proteins, so they circulate in the bloodstream. The most abundant catecholamines are epinephrine (adrenaline), norepinephrine (noradrenaline) and dopamine, all of which are produced from phenylalanine and tyrosine. Tyrosine is created from phenylalanine by hydroxylation by the enzyme phenylalanine hydroxylase. (Tyrosine is also ingested directly from dietary protein). It is then sent to catecholamine-secreting neurons. Here, many kinds of reactions convert it to dopamine, to norepinephrine, and eventually to epinephrine. Catecholamines are hormones that are released by the adrenal glands in situations of stress such as psychological stress or low blood sugar levels.

Location

Catecholamines are produced mainly by the chromaffin cells of the adrenal medulla and the postganglionic fibers of the sympathetic nervous system. Dopamine, which acts as a neurotransmitter in the central nervous system, is largely produced in neuronal cell bodies in two areas of the brainstem: the substantia nigra and the ventral tegmental area.

Synthesis

Dopamine is the first catecholamine to be synthesised from steps shown. Norepinephrine and epinephrine, in turn, are derived from further modifications of
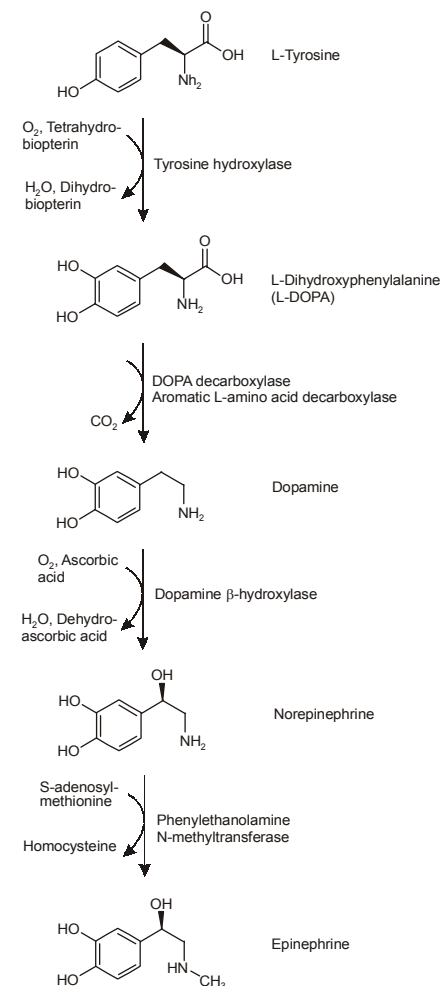
dopamine. It is important to note that the enzyme dopamine hydroxylase requires copper as a cofactor (not shown) and DOPA decarboxylase requires PLP (not shown).

Function Modality

Two catecholamines, norepinephrine and dopamine, act as neuromodulators in the central nervous system and as hormones in the blood circulation. The catecholamine norepinephrine is a neuromodulator of the peripheral sympathetic nervous system but is also present in the blood (mostly through "spillover" from the synapses of the sympathetic system).

High catecholamine levels in blood are associated with stress, which can be induced from psychological reactions or environmental stressors such as elevated sound levels, intense light, or low blood sugar levels.

Extremely high levels of catecholamines (also known as catecholamine toxicity) can occur in central nervous system trauma due to stimulation and/or damage of nuclei in the brainstem, in particular those nuclei affecting the sympathetic nervous system. In emergency medicine, this occurrence is widely known as catecholamine dump.

Extremely high levels of catecholamine can also be caused by neuroendocrine tumors in the adrenal medulla, a treatable condition known as pheochromocytoma.

High levels of catecholamines can also be caused by monoamine oxidase A deficiency. This is the gene responsible for degradation of these neuortransmitters and thus increases the circulation of them considerably. It occurs in the absence of pheochromocytoma, neuroendocrine tumors, and carcinoid syndrome, but it looks similar to carinoid syndrome such as facial flushing, aggression, and ADHD. Effects

Catecholamines cause general physiological changes that prepare the body for physical activity (fight-or-flight response). Some typical effects are increases in heart rate, blood pressure, blood glucose levels, and a general reaction of the sympathetic nervous system. Some drugs, like tolcapone (a central COMT-inhibitor), raise the levels of all the catecholamines.

Function in Plants

"They have been found in 44 plant families, but no essential metabolic function has been established for them. They are precursors of benzo[c]phenanthridine alkaloids, which are the active principal ingredients of many medicinal plant extracts. CAs has been implicated to have a possible protective role against insect predators, injuries, and nitrogen detoxification. They have been shown to promote plant tissue growth, somatic embryogenesis from in vitro cultures, and flowering. CAs inhibit indole-3-acetic acid oxidation and enhance ethylene biosynthesis. They have also been shown to enhance synergistically various effects of gibberellins."

Structure

Catecholamines have the distinct structure of a benzene ring with two hydroxyl groups, an intermediate ethyl chain, and a terminal amine group.

Degradation

They have a half-life of approximately a few minutes when circulating in the blood. Monoamine oxidase (MAO) is the main enzyme responsible for degradation of catecholamines. Amphetamines and MAOIs bind to MAO in order to inhibit its action of breaking down catecholamines. This is primarily the reason why the effects of amphetamines have a longer lifespan than those of cocaine and other substances. Amphetamines not only cause a release of dopamine, epinephrine, and norepinephrine into the blood stream but also suppress re-absorption.

SUB-SECTION 5.26G—THE SULPHUR CONTAINING AMINO ACIDS

Most of the sulphur of proteins is represented by the methionine and cysteine, present though in small amounts of cysteine the reduced product of cysteine may be present also. This is indicated by the presence of free –SH groups in a large number of enzyme and other proteins.

Demethylation of methyl methionine produces homosysteine, which may be remethylated to methionine. Cysteine is reversely convertible to cystine and homosysteine by redox reaction. The chemical reactions and relations between these amino acids can be shown as follows:

cystine   cysteine

Methionine   Homocystine
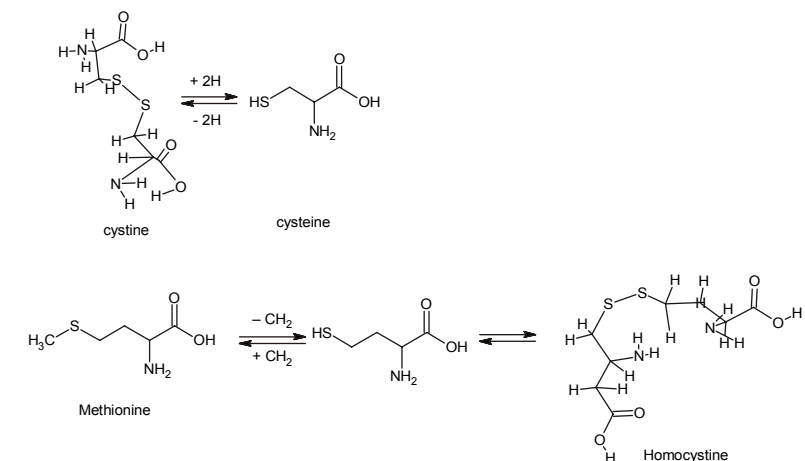
Cysteine is non enzymatically and reversibly oxidized to cystine by glutathionine present in cells.

$$cysteine + G-S-S-G \rightleftharpoons 2GSH + cystine$$
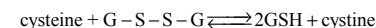
Cysteine cannot form Keto Acid

The methyl group of methionine is removed through S – adinosyl methionine in the formation of creatine and choline (A methyl group is added to homosysteine to form methionine) as pointed out in the discussion of one carbon methabolism.

Casteine cannot form keto acid

Since cysteine, cystine and homocysteines are interconvertable readily, processes which lead to the formation of one of either pair of these compounds may be considered as to form the other pair.

Methionine but not cysteine is an essential amino acid in the diet of all met by feeding the corresponding keto acid, a - keto - g - methylatiobutyric acid which is aminated to L – methionine through keto acid (and hydroxyl acid through the keto acid) to methionine therefore it cannot synthesize keto acid.

Since cysteine is not an essential amino acid, this indicates the formation of cysteine from methionine. The presence of cysteine in the diet reduces the methionine requirement.

Pathways of Methionine Metabolism

Methionine undergoes transamination in the liver with a - ketoglutoric acid to yield the keto acid α - keto - γ - methylthiobutaric acid

Methionine + α - Keto glutaric acid $\underset{\text{Pyridoxal Sulphate}}{\overset{\text{Transamination}}{\rightleftharpoons}}$ α- Keto - γ-methylthiobutyric acid + glutamic acid
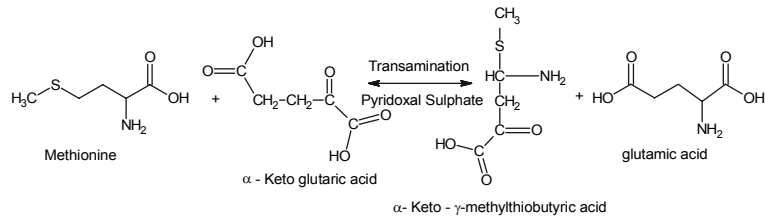
The main pathway of glutamic acid – methionine metabolism leads to cysteine and homoserine.

The processes involved in the pathway of methionine may be represented in the equation below.

It is to be noted that the reactions of methionine breakdown that only the sulphur of methionine is used in the synthesis of cysteine molecule being derived from serine. The reminder of the methionine molecule is converted to homoserine, which is breakdown through a - ketobutyric acid to propionic acid. The S of methionine goes the pathways of cysteine metabolism.

As indicated in the reaction pyridoxal phosphate is required in the formation of cystathionine and the liver preparations from pyridoxine (vitamin B6) deficient rats do not form cysteine from homocysteine and serine unless pyridoxal phosphate is added.

In patients with severe liver disease exhibiting foul smell in breadth, methyl mercaptan is found in the urine. It appears to be formed by liver enzyme acting upon the keto acid derived from methionine.

Serine → Cystathionine → Cysteine + Homosrine

Intermediate (i), Intermediate (ii), imino acid, α - ketobutyric acid, Glucose

a - Keto - g -hydroxybutyric acid

α - Keto - γ -methylthiobutyric acid + H_2O → methyl mercaptan + homoserine

α - ketobutyric acid → Transamination → α - Aminobutyric acid
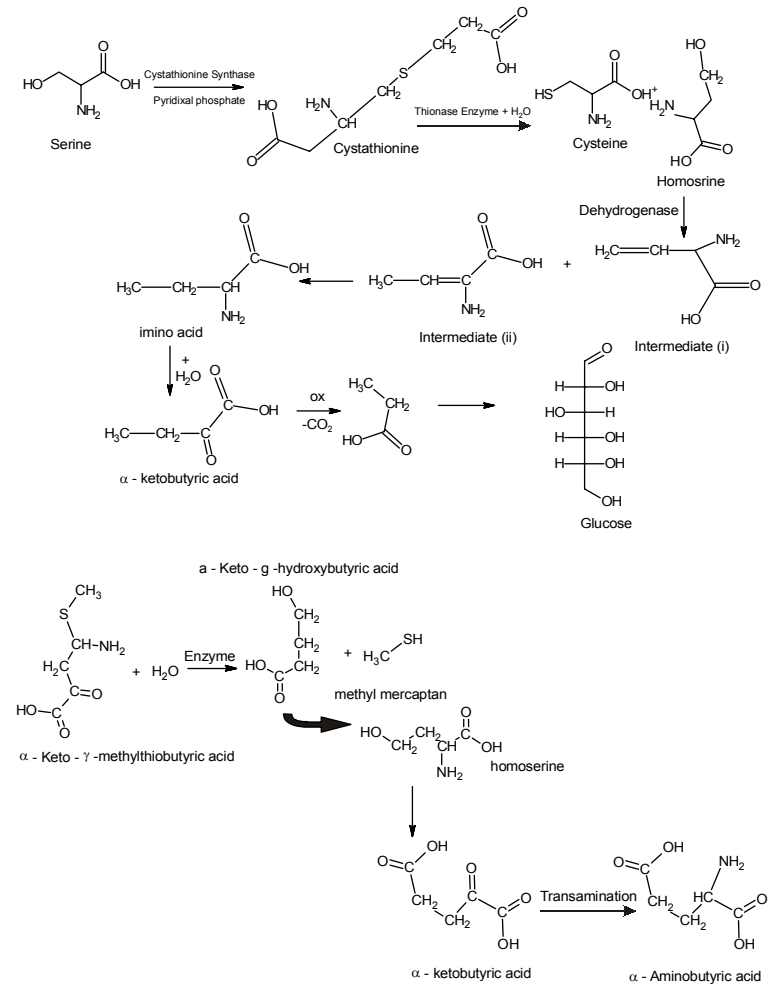
In the fanchoni syndrome* which is characterized by a low renal threshold for amino acids, amino butyric acid formed from α - ketobutyric acid and as indicated above is expected above, is excreted in the urine.

Homocysteine is convertible by a liver desuplydrase enzyme, thionase into - α - ketobutyric acid NH_3 and H_2S, most of the hydrogen sulphide formed into sulphate and a smaller portion to thiosulphate.

homocysteine   2-aminobut-3-enoic acid   2-aminobut-2-enoic acid   2-iminopropanoic acid   a - ketobutyric acid   propanoic acid   Glucose
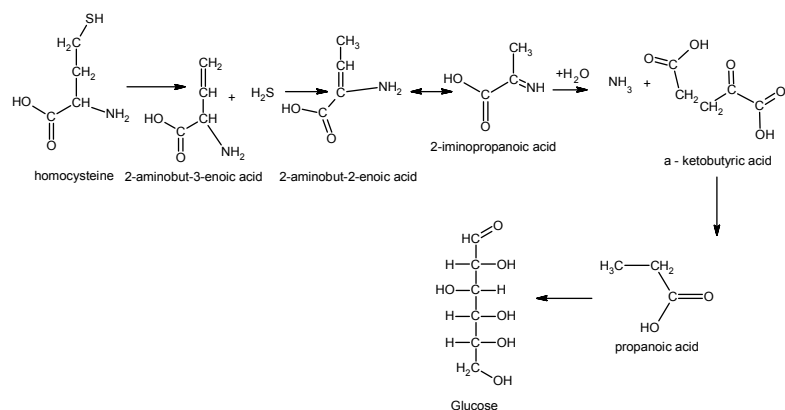
Metabolism of Cysteine and Cystine

Cysteine metabolism appears to proceed entirely through cysteine into which it is readily converted, when cysteine or methionine is given to the animals a large proportion of sulphur of amino acids is rapidly oxidized to sulphate and excreted in urine.

Urinary sulphur is derived almost entirely from the metabolism of the sulphur amino acids. It consists of (a) sulphur of inorganic sulphate sulphur of organic sulphide which is 15 to 20%. Oxidation of the sulphur, of the sulphur containing sulphuric acid to sulphate represents the final stage of sulphur oxidation in the body. Ethereal sulphates generally represent compounds formed in the detoxication of phenols, such as phenyl sulphuric acid, indicant, and skatoxysulphuric acid. A small portion of the organic sulphide is made up of unchanged sulphur containing sulphuric acid, and some may mercaptan sulphur, especially in certain liver diseases, but nature of most of this fraction of the fraction is still unknown. It is undoubtedly a highly complex mixture of substances.

The ethereal sulphate compounds are formed by reaction of "active sulphate" 3 – phosphoadenosine – 5' – phosphosulphate in with compounds.

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* Amino aciduria: Increase in excretion of amino acid in urine due to congenital defect in the protein metabolism of protein and the reabsorptive function of kidney are abnormal.

The formation of cysteine from methionine has been discussed. Since in this process only the S of methionine is converted to the S of cysteine the metabolism of cysteine involves only the S but not the carbon chain of the methionine.

Cysteine is metabolized in animals by several pathways. Cysteine is converted to the pyruvic acid, H2S and NH_3 by a desulphydrase enzyme found in liver, kidney and pancreases.

Cysteine   2-aminoprop-2-enoic acid   imino acid   pyruvic acid
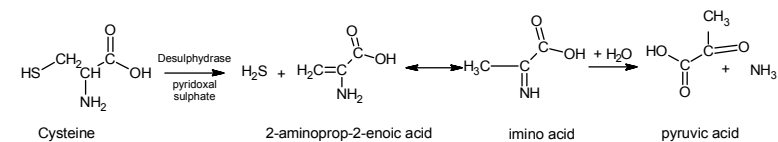
Cysteine transaminates with α - ketoglutoric acid to α - mercaptopyruvic acid (α - thiopyruvic acid)

cysteine   α - Keto glutaric acid   β - mercaptopyruvic acid   glutamic acid
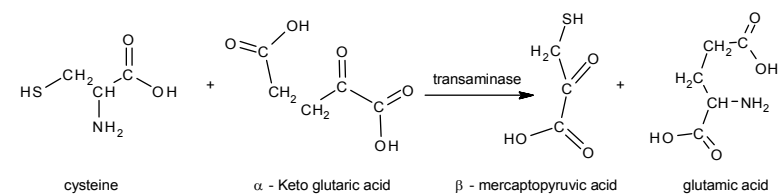
The S is converted to H_2S by reducing agents such as cysteine and glutathione

Cysteine   cystine
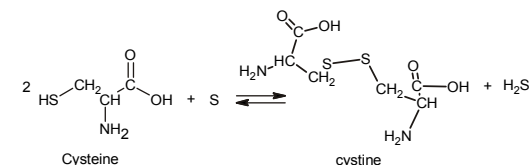
β - Mercaptopyruvic acid transfers sulphur to the cyanide ion to form sulphocyanate. The reaction is catalyzed by a liver transsulphurase enzyme.

? - mercaptopyruvic acid   Pyruvic acid
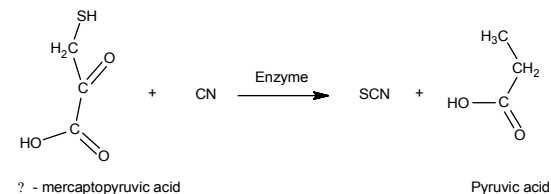
Thiosulphate is formed by reaction of β - mercaptopyruvic acid with sulphite through the action of transsulphurase enzyme found in kidney and liver.

β - mercaptopyruvic acid

Pyruvic acid
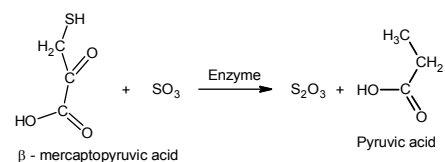
Sulphocyanate may also be formed through the action of a liver enzyme rhodanese from CN^- and S and form CN^- and $S_2O_3^-$

$$CN^- + S \longrightarrow SCN^-$$

$$CN^- + S_2O_3 \longrightarrow SCN^- + SO_3^-$$
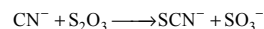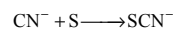
It appears that a major pathway of cysteine metabolism is through cysteine sulphinic acid. Cysteine is oxidized to cysteine sulphinic acid by a liver enzyme requiring ATP, TPN and Mg^{++}

Cysteine

cysteine sulphinic acid
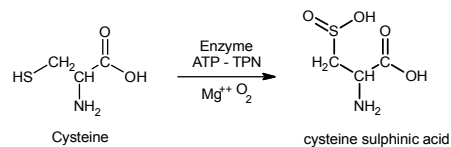
Cysteine sulphinic acid rapidly transaminates to form β - sulphinylpyruvic acid, which to form pyruvic acid is quite analogous to the decarboxylation of oxaloacetic acid to form pyruvic acid.

The SO_2 (sulphite) is oxidized to sulphate by a liver enzyme, sulphate oxidase which appears to require hypoxanthine and lipoic acid.

Taurine appears to be formed from cysteine sulphinic acid in the liver by two major pathways. One of these is by oxidation to systemic acid then decarboxylation of the cysteic acid.

Cysteine sulphinic acid

(2S)-2-amino-3-sulfopropanoic acid
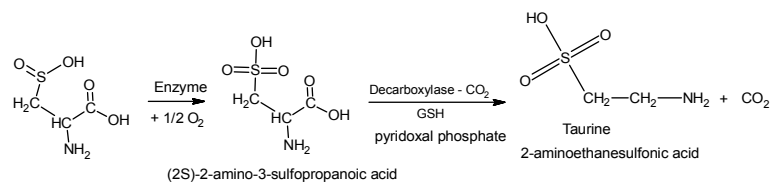
Taurine

2-aminoethanesulfonic acid

Taurine is conjugated with alcohol CoA in the liver to form taurocholic acid

$$Cholic\ acid + HS - CoA + ATP \xrightarrow[Mg^{++}]{Enzyme} Cholyl - S - CoA + AMP + PP$$

$$Taurine + Cholyl - S - CoA \xrightarrow{Enzyme} Taurocholic\ acid + HS - CoA$$
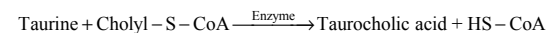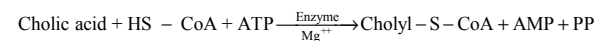
The role of cysteine in the formation of mercapturic acids for detoxification has been considered. It is also known that cysteine is a constituent of the important tripeptide glutathione.

SUB-SECTION 5.26H—METABOLISM OF TRYPTOPHAN

Tryptophan (abbreviated as Trp or W) is one of the 20 standard amino acids, as well as an essential amino acid in the human diet. It is encoded in the standard genetic code as the codon UGG. Only the L-stereoisomer of tryptophan is used in structural or enzyme proteins, but the D-stereoisomer is occasionally found in naturally produced peptides (for example, the marine venom peptide contryphan). The distinguishing structural characteristic of tryptophan is that it contains an indole functional group.

Biosynthesis and Industrial Production

Plants and microorganisms commonly synthesize tryptophan from shikimic acid or anthranilate. The latter condenses with phosphoribosylpyrophosphate (PRPP), generating pyrophosphate as a by-product. After ring opening of the ribose moiety and following reductive decarboxylation, indole-3-glycerinephosphate is produced, which in turn is transformed into indole. In the last step, tryptophan synthase catalyzes the formation of tryptophan from indole and the amino acid, serine.

Chorismate

Anthranilate

N-(4'-Phospho-ribosyl)-anthranilate

1-(o-Carboxyphenylamino)-1-desoxyribuose-5-phosphate

Indole-3-glycerinphosphate

Tryptophan

Indole

The industrial production of tryptophan is also biosynthetic and is based on the fermentation of serine and indole using either wild-type or genetically modified E. coli. The conversion is catalyzed by the enzyme tryptophan synthase.

Function

For many organisms (including humans), tryptophan is an essential amino acid. This means that it cannot be synthesized by the organism and therefore must be part of its diet. Amino acids, including tryptophan, act as building blocks in protein biosynthesis. In addition, tryptophan functions as a biochemical precursor for the following compounds (see also figure to the right):

L-tryptophan

Indoleamine
2,3-dioxygenase

N-formyl-kynurenine

tryptophan hydroxylase

formamidase

5-hydroxy-tryptophan

kynurenine

aromatic amino acid decarboxylase

5-hydroxy-tryptanine (5-HT)

serotonin

2-amino-3-(3-oxoprop-1-entl)-fumaric acid

N-acetyl transferase

non-enzymatic cycilization

N-acetyl-5-HT

quinolinate

5-hydroxyindole-O-methyltransferase
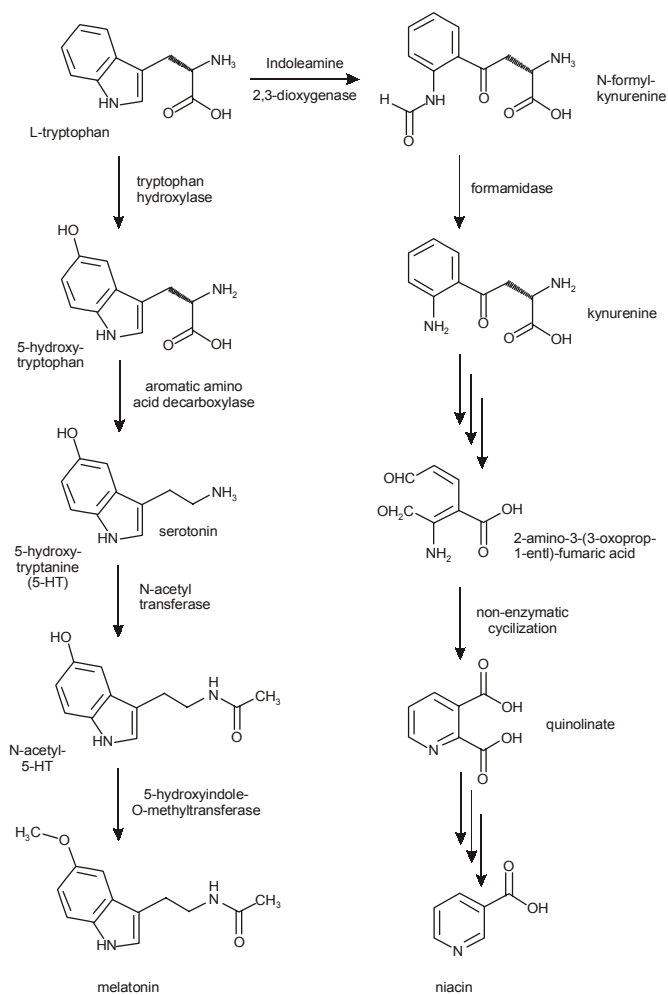
melatonin

niacin

- Serotonin (a neurotransmitter), synthesized via tryptophan hydroxylase. Serotonin, in turn, can be converted to melatonin (a neurohormone), via N-acetyltransferase and 5-hydroxyindole-O-methyltransferase activities.

- Niacin is synthesized from tryptophan via kynurenine and quinolinic acids as key biosynthetic intermediates.

- Auxin (a phytohormone) when sieve tube elements undergo apoptosis tryptophan is converted to auxins.

The disorders Fructose Malabsorption and Lactose intolerance causes improper absorption of tryptophan in the intestine, reduced levels of tryptophan in the blood and depression.

In bacteria that synthesize tryptophan, high cellular levels of this amino acid activate a repressor protein, which binds to the trp operon. Binding of this repressor to the tryptophan operon prevents transcription of downstream DNA that codes for the enzymes involved in the biosynthesis of tryptophan. So high levels of tryptophan prevent tryptophan synthesis through a negative feedback loop and, when the cell's tryptophan levels are reduced, transcription from the trp operon resumes. The genetic organisation of the trp operon thus permits tightly regulated and rapid responses to changes in the cell's internal and external tryptophan levels.

Dietary Sources

Tryptophan is a routine constituent of most protein-based foods or dietary proteins. It is particularly plentiful in chocolate, oats, durians, mangoes, dried dates, milk, yogurt, cottage cheese, red meat, eggs, fish, poultry, sesame, chickpeas, sunflower seeds, pumpkin seeds, spirulina, and peanuts. It is found in turkey at a level typical of poultry in general.

Table 5. : Tryptophan (Trp) Content of Various Foods

Food	Protein[g/ 100 g of food]	Tryptophan[g/ 100g of food]	Tryptophan/ Protein [%]
1	2	3	4
egg, white, dried	81.10	1.00	1.23
spirulina, dried	57.47	0.93	1.62
cod, atlantic, dried	62.82	0.70	1.11
soybeans, raw	36.49	0.59	1.62
cheese, Parmesan	37.90	0.56	1.47
caribou	29.77	0.46	1.55
sesame seed	17.00	0.37	2.17
cheese, cheddar	24.90	0.32	1.29
sunflower seed	17.20	0.30	1.74

1	2	3	4
pork, chop	19.27	0.25	1.27
turkey	21.89	0.24	1.11
chicken	20.85	0.24	1.14
beef	20.13	0.23	1.12
salmon	19.84	0.22	1.12
lamb, chop	18.33	0.21	1.17
perch, Atlantic	18.62	0.21	1.12
egg	12.58	0.17	1.33
wheat flour, white	10.33	0.13	1.23
milk	3.22	0.08	2.34
rice, white	7.13	0.08	1.16
potatoes, russet	2.14	0.02	0.84
banana	1.03	0.01	0.87

Use as a Dietary Supplement

For some time, tryptophan has been available in health food stores as a dietary supplement, although it is common in dietary protein. Many people found tryptophan to be a safe and reasonably effective sleep aid, probably due to its ability to increase brain levels of serotonin (a calming neurotransmitter when present in moderate levels) and/or melatonin (a sleep-inducing hormone secreted by the pineal gland in

response to darkness or low light levels). Some users of ecstasy will eat tryptophan-containing foods to shorten the 'come down' effect of having lower levels of serotonin than usual (due to an extra large release caused by the drug).Clinical research has shown mixed results with respect to tryptophan's effectiveness as a sleep aid, especially in normal patients and for a growing variety of other conditions typically associated with low serotonin levels or activity in the brain such as premenstrual dysphoric disorder and seasonal affective disorder. In particular, tryptophan has been showing considerable promise as an antidepressant alone, and as an "augmenter" of antidepressant drugs. However, the reliability of these clinical trials has been questioned. The keto acid and the hydroxy acid corresponding to tryptophan are utilized in place of tryptophan in the diet.

The following reaction reactions have been demonstrated between tryptophan, indole pyruvic acid indole acetic acid and tryptamine.

The daily urine excretion of indole acetic acid in man generally amounts to 5 to 18 mg but may reach 200mg per day in certain pathological conditions and after tryptophan administration.
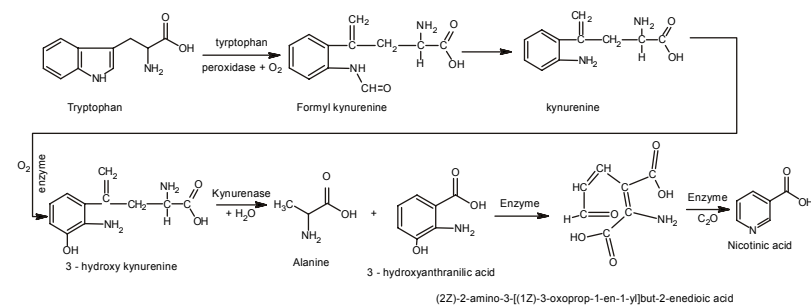
The open chain intermediate from 3 – hydroxyanthranilic acid apparently is also converted to quinolinic acid and picholinic acid.
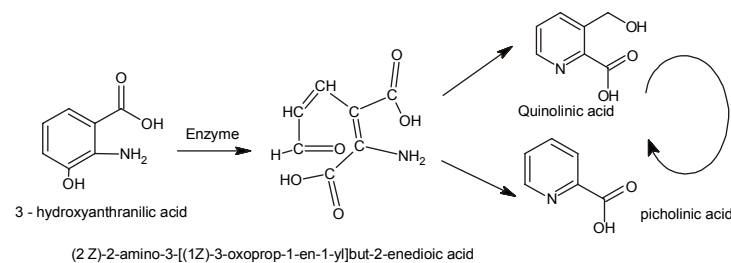
The vaso-constrictor substance* 5 – hydroxytryptamine or serotonin is present in the blood is present in the blood, particularly in the gastric mucosa, intestine brain, mast cells and blood platelets. Patients with malignant carcinoid excrete large amount of the serotonin metabolite 5 – hydroxy indole acetic acid in the urine. Such patients have been estimated to utilize as much as 60% of the tryptophan metabolize on the formation of serotonin as compared with 1% for the individual. Serotonin is considered to function as a neurohumoral$ agent. The formation of serotonin from tryptophan has been established by the use of L – tryptophan.

L - tryptophan   5 - hydroxy L tryptophan   5 - hydroxytryptamine

serotonin

monoamine oxidase

+ O_2 + H_2O

5 - hydroxy indole acetic acid   + NH_3 + H_2O_2
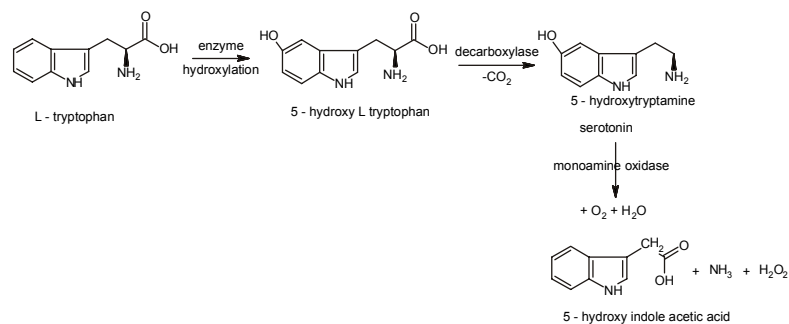
From the above reactions it will be seen that three carbon atoms of tryptophan form alanine which may form pyruvic acid and glucose and that carbon 2 of the pyrol ring yields formic acid; thus

kynurenine

kynureinase
pyridoxal phosphate

Anthranilic acid   Alanine

transamination

Anthraanylpyruvic acid   kynurenic acid   dehyderoxylation   quinoline-2-carboxylic acid

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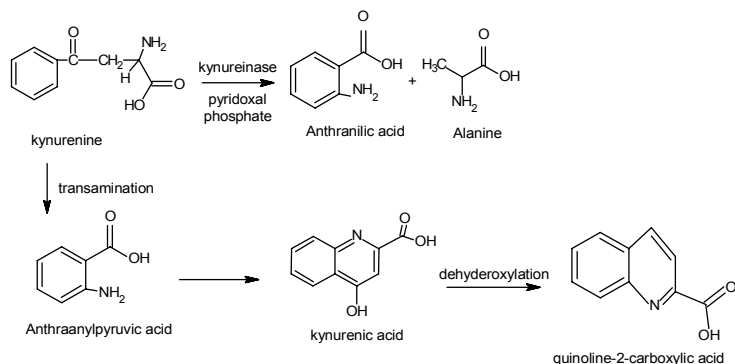
* The substances which constrict the cavity of the bold vessels
$ A substance liberated at the nerve ending that participates in the transmission of nerve impulse

tryptophan contributes to the one carbon pool. Also, the metabolism of tryptophan yields nicotinic acid which is converted to the vitamin niacin (nicotinamide) in the animal body.

Kynurenine transaminates to form the corresponding keto acid, which spontaneously forms kynurenic acid. Kynurenic acid is converted to anthranilic acid and to quinaldic acid by dehydroxylation.

3 – Hydroxykynurenine undergoes reactions analogous to those of Kynurenine. For example 3 – Hydroxykynurenine is converted to 3 – hydroxyxanthranilic acid by kynureninase, as shown above in the main line of tryptophan metabolism.

3 – Hydroxykynurenine, through the action of Kynurenine transaminases is converted to xanthuric acid, which then forms 8 – hdyroxyquinaldic acid. This process is quite analogous to the Kynurenine reaction.

3 - hydroxykynurenine   4-(2-amino-3-hydroxyphenyl)-2,4-dioxobutanoic acid

4,8-dihydroxyquinoline-2-carboxylic acid

xanthuric acid

dehydroxylation

8 - hydroxy quinaldic acid
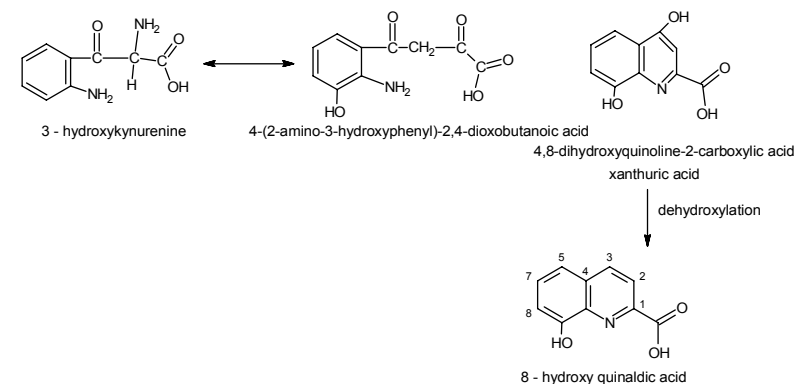
Bacterial Putrefaction

The action of bacteria upon tryptophan in the gut produces a large number of substances which are extracted as such or modified from (detoxified) in the urine or feces.
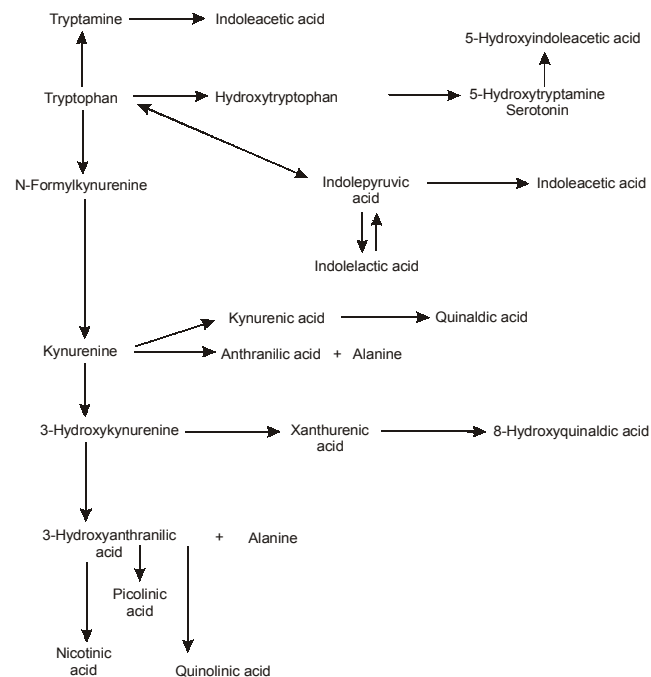
Fig. 5.48 : Showing the Tryptophan Metabolism

SUB-SECTION 5.26I—THE METABOLISM OF HISTIDINE

Histidine (abbreviated as His or H) is one of the 20 standard amino acids present in proteins. Nutritionally, Histidine is considered an essential amino acid in human infants. After reaching several years of age, humans
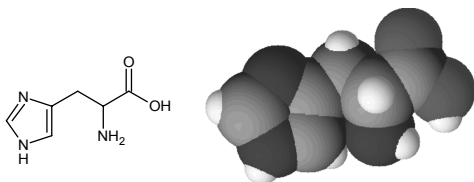
begin to synthesize it and it thus becomes a non-essential amino acid. Histidine was first isolated by German physician Albrecht Kossel in 1896.

Histidine synthesis includes the activity of imidiazoleglycerol phosphate, which is an intermediate is formed from ATP ribose 5 phosphate ATP and glutamine.
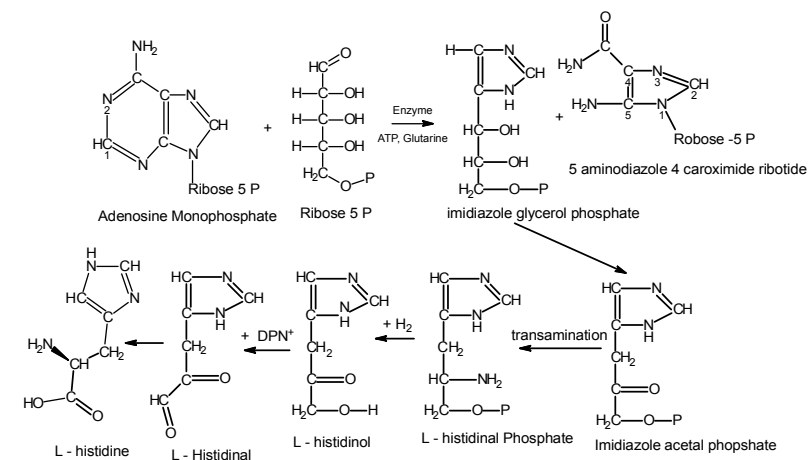
It appears that nitrogen 1 and carbon 2 of the purine ring of AMP become nitrogen 3 and carbon 2 of the imidoazole ring of histidine. It appears that nitrogen 1 and carbon 2 of the purine ring of AMP become nitrogen 3 and carbon 2 of the imidoazole ring of histidine and that the five carbon chain of histidine is derived from the ribose carbon. Nitrogen 1 of the imidoazole group of histidine apparently is derived from the amide group of glutamine.

As indicated before the adult human balances nitrogen without histidine in the diet. It has been suggested that microbial synthesis in the intestine might provide histidine for the human. However, it seems that nothing is known regarding such synthesis except that human liver apparently can incorporate formate carbon into position 2 of the imidoazole group.

Small amounts of methyl histidine, 1 – methyl histidine 3 – methyl histidine have been found in the urines of some animals.

SECTION 5.27—THE METABOLSIM OF INORGANIC ELEMENTS

It is observed that there are least 29 different types of element in our body. Organic components such as carbohydrates, proteins and lipids form about 90% of the solid matter and mainly consist of C, H O, N

The elements of body are divided into three major groups,(among them first two groups are being discussed) they are in the table below.

Group	Type	Name of the elements
Group 1	Nutritionally important minerals daily requirement > 100mg/day	Na, K, Cl, Ca, P, Mg and S
Group 2	Trace elements which are essential. The requirement is < 100mg/day	Cr, Co, Cu I, Fe, Mn, Mo, Se, Zn
Group 3	These are additional trace elements But their exact role is not known	Cd, Ni, Si, Sn, Vn

The total percentage requirement is given in the table below:

Element	Percentage
Oxygen	65.
Crbon	18.
Hydrogen	10.
Nitrogen	3.
Calcium	2.a
Phosphorus	1.1b
Potassium	0.35
Sulfur	0.25
Sodium	0.15
Chlorine	0.15
Magnesium	0.05
Iron	0.004
Manganese	0.00013
Copper	0.00015
Iodine	0.00004
Cobalt	c
Zinc	c

Others of more doubtful status

a Estimates varywidely.

b Percentage varies with that of calcium.

c Believed to be essential, but quantitative data are not yet at hnd.

IRON

MOST	DAILY REQUIREMENT
ESSENTIAL	MALE: 3.8gm
TRACE	FEMALE 2.3 gm
ELEMENT	

Dietary Source of Iron

Animal sources include, meat liver red marrow consist of 2.0 to 6.0 mg iron per 100 gm. Vegetable sources include 2.0 to 8.0gm /100gm The table below shows the iron content in different dietary sources

Food	Iron (in mg.) per 100 gm. fresh substance
Beans, dried	10.5
Egg yolk	8.6
Peas, dried	5.7
Wheat, entire grain	4.8
Oatmeal	3.1
Eggs	4.8
Beef	3.1
Prunes	3.0
Spinach	2.8
Beefsteak, medium fat	2.5
Cheese	2.0
Beans, string, fresh	1.3
Potatoes	1.1
White flour	1.0
Rice, polished	0.9
Beets	0.8
Carrots	0.6
Bananas	0.6
Turnips	0.5
Oranges	0.5
Tomatoes	0.4
Apples	0.3
Milk	0.2

Digestion of Iron

Iron absorption is affected by the form in which iron is presented to the digestive tract, and inorganic iron ions change oxidation state during the absorption process.

There are two major forms of dietary iron.

- Heme iron, found primarily in red meats, is the most easily absorbed form.
- Other forms of iron are bound to some other organic constituent of the food. Cooking tends to break these interactions and increase iron availability.

Some iron-rich foods are poor sources of the mineral because other compounds render it non-absorbable.

- The classic example is spinach. It contains iron, but it also contains considerable oxalate, which chelates it and renders it non-absorbable.
- Phytates, present in whole grains that have not been subjected to fermentation by yeast (for example, during bread making), have a similar effect.

Iron ions undergo two important changes of oxidation state during digestion and absorption.

The first change occurs in the stomach.

- Here iron (III) is reduced to iron (II).
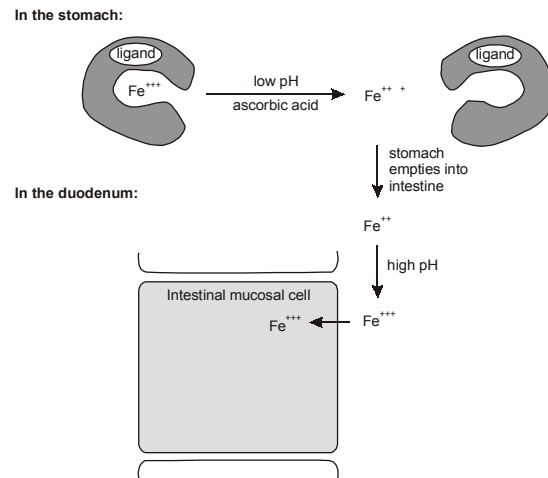
Fig. 5.48 : Showing the fate of iron after digestion

- This reduction is favored by the low pH. Reducing agents, such as ascorbic acid, assist this process.
- Reduction is important because iron (II) dissociates from ligands more easily than iron (III).

The second change occurs in the duodenum.

- The duodenum is bicarbonate-rich, and alkaline.
- In the alkaline environment
  - Heme is absorbed directly by the mucosal cells. Within the cells, the iron dissociates from it.
  - Free iron (II) ions are oxidized to iron (III), which is taken up by the mucosal cells in substantial amounts under all circumstances of nutritional iron status.

Iron Absorption

Despite the fact that iron is the second most abundant metal in the earth's crust, iron deficiency is the world's most common cause of anemia. When it comes to life, iron is more precious than gold. The body hoards the element so effectively that over millions of years of evolution, humans have developed no physiological means of iron excretion. Iron absorption is the sole mechanism by which iron stores are physiologically manipulated.

The average adult stores about 1 to 3 grams of iron in his or her body. An exquisite balance between dietary uptake and loss maintains this balance. About 1 mg of iron is lost each day through sloughing of cells from skin and mucosal surfaces, including the lining of the gastrointestinal tract (Cook et al., 1986). Menstration increases the average daily iron loss to about 2 mg per day in premenopausal female adults (Bothwell and Charlton, 1982). No physiologic mechanism of iron excretion exists. Consequently, absorption alone regulates body iron stores (McCance and Widdowson, 1938). The augmentation of body mass during neonatal and childhood growth spurts transiently boosts iron requirements (Gibson et al., 1988).
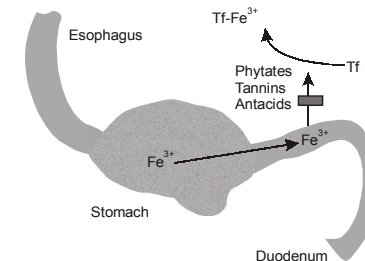
Figure 178. Iron absorption. Iron enters the stomach from the esophagus. Iron is oxidized to the Fe^{3+} state no matter its original form when taken in orally. Gastric acidity as well as solubilizing agents such as ascorbate prevent precipitation of the normally insoluble Fe^{3+}. Intestinal mucosal cells in the

duodenum and upper jejunum absorb the iron. The iron is coupled to transferrin (Tf) in the circulation which delivers it to the cells of the body. Phytates, tannins and antacids block iron absorption.

Iron absorption occurs predominantly in the duodenum and upper jejunum (Muir and Hopfer, 1985) (Figure 178). The mechanism of iron transport from the gut into the blood stream remains a mystery despite intensive investigation and a few tantalizing hits (see below). A feedback mechanism exists that enhances iron absorption in people who are iron deficient. In contrast, people with iron overload dampen iron absorption. The physical state of iron entering the duodenum greatly influences its absorption however. At physiological pH, ferrous iron (Fe^{2+}) is rapidly oxidized to the insoluble ferric (Fe^{3+}) form. Gastric acid lowers the pH in the proximal duodenum, enhancing the solubility and uptake of ferric iron (Table 1below). When gastric acid production is impaired (for instance by acid pump inhibitors such as the drug, prilosec), iron absorption is reduced substantially. Heme is absorbed by machinery completely different to that of inorganic iron. The process is more efficient and is independent of duodenal pH . Consequently meats are excellent nutrient sources of iron. In fact, blockade of heme catabolism in the intestine by a heme oxygenase inhibitor can produce iron deficiency (Kappas et al., 1993). The paucity of meats in the diets of many of the people in the world adds to the burden of iron deficiency. A number of dietary factors influence iron absorption. Ascorbate and citrate increase iron uptake in part by acting as weak chelators to help to solubilize the metal in the duodenum (Table 1) (Conrad and Umbreit, 1993). Iron is readily transferred from these compounds into the mucosal lining cells. Conversely, iron absorption is inhibited by plant phytates and tannins. These compounds also chelate iron, but prevent its uptake by the absorption machinery (see below). Phytates are prominent in wheat and some other cereals, while tannins are prevalent in (non-herbal) teas. Lead is a particularly pernicious element to iron metabolism (Goya, 1993). Lead is taken up by the iron absorption machinery, and secondarily blocks iron through competitive inhibition. Further, lead interferes with a number of important iron-dependent metabolic steps such as heme biosynthesis. This multifaced attack has particularly dire consequences in children, were lead not only produces anemia, but can impair cognitive development. Lead exists naturally at high levels in ground water and soil in some regions, and can clandestinely attack children's health. For this reason, most pediatricians in the U.S. routinely test for lead at an early age through a simple blood test. Immaturity of the gastrointestinal tract can exacerbate iron deficiency in newborns. The gastrointestinal tract does not achieve competency for iron absorption for several weeks after birth. The problem is even more severe for premature infants, who tend to be anemic for a variety of reasons. A substantial portion of iron stores in newborns are transferred from the mother late in pregnancy. Prematurity short circuits this process. Parenteral iron replacement is possible, but not often used because of the often delicate health of premature infants.

Table 5.8 : Factors That Influence Iron Absorption

Physical State (bioavailability)	heme > Fe^{2+} > Fe^{3+}
Inhibitors	phytates, tannins, soil clay, laundry starch, iron overload, antacids
Competitors	lead, cobalt, strontium, manganese, zinc
Facilitators	ascorbate, citrate, amino acids, iron deficiency

Transfusion becomes the default option in this circumstance. The mechanism by which iron enters the mucosal cells lining the upper gastrointestinal tract is unknown. Most cells in the rest of the body are believed to acquire iron from plasma transferrin (an iron-protein chelate), via specific transferrin receptors and receptor-mediated endocytosis (Klausner, et al, 1983). The hypothesis that apotransferrin (or an equivalent molecule) secreted by intestinal cells or present in bile chelates intestinal iron and facilitates its absorption(Huebers et al., 1983) is unsubstantiated. The transferrin gene is not expressed in intestinal cells. Later work indicated that transferrin found in the intestinal lumen is derived from plasma (Idzerda et al., 1986). Plasma transferrin entering bile is fully saturated with iron, obviating any intraluminal chelating function (Schumann et al., 1986). Furthermore, hypoxia, which greatly increases iron absorption, has no effect on intestinal transferrin levels (Simpson et al., 1986). Exogenous transferrin cannot donate iron to intestinal mucosal cells (Bezwoda et al., 1986), and the brush boarder membrane lacks transferrin receptors (Parmley et al., 1985) (although they are present on the basolateral surface of intestinal epithelial cells (Levin et al., 1984); (Banerjee et al., 1986). Lastly and perhaps most compellingly, humans and mice with hypotransferrinemia paradoxically absorb more dietary iron than normal. Although the erythron is iron deficient, these individuals develop hepatic iron overload

Mechanism of Iron Absorption

In searching for molecules involved in intestinal iron transport, Conrad and co-workers took the approach of characterizing proteins that bind iron [summarized in (Conrad and Umbreit, 1993)]. Their hypothesis of iron transport is based on identification of iron binding proteins at several key sites. They propose that mucins bind iron in the acid environment of the stomach, thereby maintaining it in solution for later uptake in the alkaline duodenum. According to their model, mucin-bound iron subsequently crosses the mucosal cell membrane in association with integrins. Once inside the cell, a cytoplasmic iron-binding protein, dubbed "mobilferrin", accepts the element, and shuttles it to the basolateral surface of the cell, where it is delivered to plasma. In this model mobilferrin could serve as a rheostat sensitive to plasma iron concentrations. Fully occupied mobilferrin would dampen mucosal iron uptake, and while the process would be enhanced by unsaturated mobilferrin (Conrad and Umbreit, 1993). This model has not gained universal acceptance however. A very different scheme of iron uptake has been proposed by investigators studying iron transport in yeast. Yeast face the problem of taking in iron from the environment, a process similar to that of intestinal mucosal cells. Dancis et al. used genetic selection to isolate Sacchromyces cerevisiae mutants with defective iron transport (Dancis et al., 1994); (Stearman et al., 1996). They constructed an expression plasmid in which an enzyme necessary for histidine biosynthesis was under the control of an iron-repressible promoter. The plasmid was introduced into a yeast histidine auxotroph (i.e. a strain of yeast that requires histidine to survive). Mutants were selected in the absence of histidine, in the presence of high levels of iron. Among the mutats they isolated, were cells with defective iron uptake. They discovered that membrane iron transport depends absolutely upon copper transport. In this model, ferric iron in yeast culture medium is reduced to its ferrous form by an externally oriented reductase (FRE1). The element is shuttled rapidly into the cell by a ferrous transporter, which appears to be coupled to an externally oriented copper-dependent oxidase (FET3) embedded in the cell membrane (De Silva et al., 1995); (Stearman et al., 1996). FET3 is strikingly homologous to the mammalian copper oxidase ceruloplasmin. The re-oxidation of ferrous to ferric iron
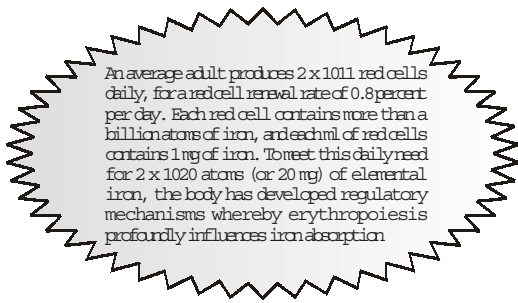
is apparently an obligatory step in the transport mechanism, although the coupling mechanism of oxidation and membrane transport is unclear. (De Silva et al., 1995); (Stearman et al., 1996); (Yuan et al., 1995). Although the genetic evidence for this scheme is compelling, the central component, the ferrous transporter itself, remains elusive. These investigators speculate that mammalian intestinal iron transport is analogous to the yeast iron uptake process (Harford et al., 1994). This assertion is supported by studies of copper-deficient swine, which show co-existing iron deficiency unresponsive to iron therapy

Genetic Insights into Mammalian Iron Absorption

Mouse genetics provides a different perspective on mammalian intestinal iron transport. Mouse breeders readily recognize pale animals, and have developed anemic stocks with various mutations. Intestinal mucosal iron transport is defective in two mutant strains. Microcytic (mk) mice and sex-linked anemia (sla) mice have severe iron deficiency due apparently to defects in iron uptake and release, respectively, from the intestinal cell (reviewed in [Bannerman, 1976].) Mice with the homozygous autosomal recessive mk mutation absorb iron poorly, have low serum iron levels, and lack stainable iron in intestinal mucosal cells. These findings are consistent with a defect in an apical iron transport molecule. Intriguingly, mk/mk mice are not rescued by parenteral iron replacement. Anemia develops in normal mice tranplanted with mk bone marrow, indicating that mk erythroid precursor cells also have a defect in red cell iron uptake. A common component to iron transport may therefore exist in intestinal cells and red cell precursors (Andrews, et al, 2000).

Mice that are homozygous or heterozygous for the sla mutation (sla/sla or sla/y) also have low serum iron levels. In contrast to mk mice, they have abnormal iron deposits within intestinal mucosal cells, suggesting that this X-linked defect impairs intracellular iron trafficking or basolateral export of iron to the plasma. The sla animals differ further from the mk mice by correction of anemia by parenteral iron. Based on studies of these mutants, distinct apical and basolateral iron transport systems possibly exist that function coordinately to transfer iron from intestinal lumen to plasma.

Whatever the mechanism of iron uptake, normally only about 10% of the elemental iron entering the duodenum is absorbed. However, this value increases markedly with iron deficiency (Finch, 1994). In contrast, iron overload reduces but does not eliminate absorption, reaffirming the fact that absorption is regulated by body iron stores. In addition, both anemia and hypoxia boost iron absorption. A portion of the iron that enters the mucosal cells is retained sequestered within ferritin. Intracellular intestinal iron is lost when epithelial cells are sloughed from the lining of the gastrointestinal tract. The

An average adult produces 2×10^{11} red cells daily, for a red cell renewal rate of 0.8 percent per day. Each red cell contains more than a billion atoms of iron, and each ml of red cells contains 1 mg of iron. To meet this daily need for 2×10^{20} atoms (or 20 mg) of elemental iron, the body has developed regulatory mechanisms whereby erythropoiesis profoundly influences iron absorption

remaining iron traverses the mucosal cells, to be coupled to transferrin for transport through the circulation.

Erythropoiesis and Iron AbsorptionApproximately 80% of total body iron is ultimately incorporated into red cell hemoglobin. An average adult produces 2×10^{11} red cells daily, for a red cell renewal rate of 0.8 percent per day. Each red cell contains more than a billion atoms of iron, and each ml of red cells contains 1 mg of iron. To meet this daily need for 2×10^{20} atoms (or 20 mg) of elemental iron, the body has developed regulatory mechanisms whereby erythropoiesis profoundly influences iron absorption. Plasma iron turnover (PIT) represents the mass turnover of transferrin-bound iron in the circulation, expressed as mg/kg/day (Huff et al., 1950). Accelerated erythropoiesis increases plasma iron turnover, which is associated with enhanced iron uptake from the gastrointestinal tract (Weintraub et al., 1965). The mechanism by which PIT alters iron absorption is unknown. A circulating factor related to erythropoiesis that modulates iron absorption has been hypothesized, but not identified (Beutler and Buttenweiser, 1960); (Finch, 1994). Several candidate factors have been excluded, including transferrin (Aron et al., 1985) and erythropoietin (Raja et al., 1986). Clinical manifestations of this apparent communication between the marrow and the intestine includes iron overload that develops in patients with severe thalassemia in the absence of transfusion. The accelerated (but ineffective) erythropoiesis in this condition substantially boosts iron absorption. In some cases, the coupling of increased PIT and increased gastrointestinal iron absorption is beneficial. In pregnancy, placental removal of iron raises the PIT. This process enhances gastrointestinal iron absorption thereby increasing the availability of the element to meet the needs of the growing and developing fetus. Competition studies suggest that several other heavy metals share the iron intestinal absorption pathway. These include lead, manganese, cobalt and zinc (Table 1). Enhanced iron absorption induced by iron deficiency also augments the uptake of these elements. As iron deficiency often coexists with lead intoxication, this interaction can produce particularly serious medical complications in children (Piomelli et al., 1987). Interestingly, copper absorption and metabolism appear to be handled mechanisms different to those of iron.

Iron Transport

Only a small proportion of total body iron daily enters or leaves the body's stores on a daily basis (Figure1). Consequently, intercellular iron transport, as a part of the iron reutilization process, is quantitatively more important that intestinal absorption. The greatest mass of iron is found in erythroid cells, which contain about 80% of the total body endowment. The reticuloendothelial system recycles a substantial amount of iron from effete red cells, approximating the amount used by the erythron for new hemoglobin production.

Transferrin

Of the approximate 3 grams of body iron in the adult male, approximately 3mg or 0.1% circulates in the plasma as an exchangeable pool (Table 1). Essentially all circulating plasma iron normally is bound to transferrin. This chelation serves three purposes: it renders iron soluble under physiologic conditions, it prevents iron-mediated free radical toxicity, and it facilitates transport into cells. Transferrin is the most important physiological source of iron for red cells (Ponka, 1997). The liver synthesizes transferrin and secretes it into the plasma. Transferrins are produced locally in the testes and CNS. These two
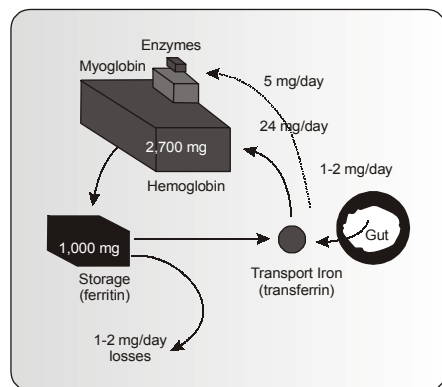
Fig. 5.50 : Showing the iron is assiduously conserved and recycled for use in heme and non-heme enzymes. About 1 to 2 mg of iron are lost each day to sloughing of skin and mucosal cells of the gastrointestinal and genitouretal tracts. This obligate iron loss is balanced by iron absorption from the gastrointestinal tract. Only a small fraction of the 4 grams of body iron circulate as part of transferrin at any given time. Body iron is most prominently represented in hemoglobin and in ferritin

sites are relatively inaccessible to proteins in the general circulation (blood: testis barrier, blood:brain barrier). The locally synthesized transferrin could play a role in iron metabolism in these tissues. Information on the function of transferrin produced in these localized sites is sparce, however. Plasma transferrin is an 80 kDa glycoprotein with homologous N-terminal and C-terminal iron-binding domains (reviewed in Huebers and Finch, 1987]. The molecule is related to several other proteins, including ovotransferrin in bird and reptile eggs (Williams et al., 1982), lactoferrin in extracellular secretions and neutrophil granules (Mazurier et al., 1983); (Metz-Boutigue et al., 1984) and melanotransferrin (p97), a protein produced by melanoma cells (Brown et al., 1982). Ovotransferrin may help protect the developing embryo in the semi-permeable egg by sequestering iron that microbes need to grow. Lactoferrin, in secretions such as milk and tears, might have a similar function. One recent report indicates that lactoferrin can act as a site-specific DNA binding protein, and could mediate transcriptional activation. Such a function is, however, at odds with its existence as an extracellular protein (He and Furmanski, 1995). X-ray crystal structures exist for human lactoferrin and rabbit transferrin (reviewed by [Baker and Lindley, 1992]. All members of the transferrin protein superfamily have similar polypeptide folding patterns. N-terminal and C-terminal domains are globular moieties of about 330 amino acids; each of these is divided into two sub-domains, with the iron- and anion-binding sites in the intersubdomain cleft. The binding cleft opens with iron release, and closes with iron binding. N- and C-terminal binding sites are highly similar.

Iron binding by Transferrin

The precise mechanics of iron loading onto transferrin as it leaves intestinal epithelial cells or reticuloendothelial cells is unknown. The copper-dependent ferroxidase, ceruloplasmin, may play a role. Compelling evidence indicates that the protein is involved in mobilizing tissue iron stores to produce diferric transferrin (Osaki and Johnson, 1969); (Osaki et al., 1971); (Yoshida et al., 1995); (Harris et al., 1995). Transferrin binds iron avidly with a dissociation constant of approximately 1022 M-1 (Aisen and Listowsky, 1980). Ferric iron couples to transferrin only in the company of an anion (usually carbonate) that serves as a bridging ligand between metal and protein, excluding water from two coordination sites (Aisen and Listowsky, 1980); (Harris and Aisen, 1989); (Shongwe et al., 1992). Without the anion cofactor, iron binding to transferrin is negligible. With it, ferric transferrin is resistant to all but the most potent chelators. The remaining four coordination sites are provided by the transferrin protein - a histidine nitrogen, an aspartic acid carboxylate oxygen, and two tyrosine phenolate oxygens (Bailey et al., 1988); (Anderson et al., 1989). Available evidence suggests that anion-binding takes place prior to iron-binding. Iron release from transferrin involves protonation of the carbonate anion, loosening the metal-protein bond.

Compartment	Iron	
	Iron (grams)	Percent of Total
Hemoglobin	2.7	66
Myoglobin	0.2	3
Heme Enzymes	0.008	0.1
Non-heme Enzymes	< 0.0001	—
Intracellular Storage (Ferritin)	1.0	30
Intracellular Labile Iron (Chelatable Iron)	0.07 (?)	1
Intercellular Transport (Transferrin)	0.003	0.1
Transferrin/Iron Physiology		

The sum of all iron binding sites on transferrin constitutes the total iron binding capacity (TIBC) of plasma. Under normal circumstances, about one-third of transferrin iron-binding pockets are filled. Consequently, with the exception of iron overload where all the transferrin binding sites are occupied, non-transferrin-bound iron in the circulation is virtually nonexistent. Distribution of plasma and tissue iron can be traced using 59Fe as a radioactive tag. The subject receives autologous transferrin loaded with radioactive iron that then can be monitored. Blood samples can be analyzed at timed intervals to determine the rate of loss of the radioactive label. Such ferrokinetic studies indicate that the normal half-life of iron in the circulation is about 75 minutes (Huff et al., 1950). The absolute amount of iron released from transferrin per unit time is the plasma iron turnover (PIT).

Such radioactive tracer studies indicate that at least eighty percent of the iron bound to circulating transferrin is delivered to the bone marrow and incorporated into newly formed erythrocytes (Jandl

and Katz, 1963); (Finch et al., 1982); Fig. 179). Other major sites of iron delivery include the liver, which is a primary depot for stored iron, and the spleen. Hepatic iron is found in both reticuloendothelial cells and hepatocytes. Reticuloendothelial cells acquire iron primarily by phagocytosis and breakdown of aging red cells These cells extract the iron from heme and return it to the circulation bound to transferrin. Hepatocytes take up iron by at least two different pathways. The first involves receptor-mediated endocytosis of transferrin. In addition, hepatocytes can take up ionic iron by a process independent of transferrin (Inman and Wesling-Resnick, 1993).

Ferrokinetics and the Bone Marrow

Given the preeminent role of the bone marrow in the clearance of labeled iron from the circulation, ferrokinetics provide a window on erythropoietic activity. Conditions that augment erythrocyte production increase the PIT. For example, hemolytic anemias such as hereditary spherocytosis and sickle cell disease induce rapid delivery of transferrin-bound iron to the marrow. In contrast, disorders that reduce red cell production prolong the PIT. This picture is seen, for example, with anemia due to Diamond Blackfan anemia. When erythrocytes are produced and released into the circulation in a normal fashion, the process of erythropoiesis is termed "effective". In patients with certain hemolytic anemias, however, the nascent red cells are so abnormal they are destroyed before leaving the marrow cavity. In this circumstance, the erythropoiesis is "ineffective", meaning simply that the erythropoietic precursors have failed to accomplish their primary task: the delivery of intact erythrocytes to the circulation. The ferrokinetic profiles such cases show rapid removal of iron from transferrin with a delayed entry of label into the pool of circulating red cell hemoglobin. ß+-thalassemia is an important example of this pattern of hemolytic anemia with ineffective erythropoiesis. In ß+-thalassemia, ineffective erythropoiesis is coupled with a markedly enhanced PIT.

Cellular Iron Uptake

Although transferrin was characterized fifty years ago (Laurell and Ingelman, 1947), its receptor eluded investigators until the early 1980s. In a quest to better understand the behavior of neoplastic cells, investigators prepared monoclonal antibodies against tumor cells. The target of these monoclonal antibodies later was found to be the cell surface transferrin receptor glycoprotein (Sutherland et al., 1980; Seligman et al., 1980). A broad body of literature now supports the concept that the iron-transferrin complex is internalized by receptor-mediated endocytosis. The general structure of the transferrin receptor is shown in Figure 180. This disulfide-linked homodimer has subunits containing 760 amino acids each (Kuhn et al., 1984); (Schneider et al., 1983); (Jing and Trowbridge, 1987). Oligosaccharides account for about 5% of the 90 kDa subunit molecular mass (Reckhow and Enns, 1988). Four glycosylation sites (three N-linked and one O-linked) line the protein (Hayes et al., 1992). Glycosylation-defective mutants have fewer disulfide bridges, bind transferrin less efficiently and are expressed less prominently on the surface expression than are normal receptors (Williams and Enns, 1993a); (Williams and Enns, 1993b). The transmembrane domain, between amino acids 62 and 89, functions as an internal signal peptide, as none exits at the N-terminal end (Zerial et al., 1986). A molecule of fatty acid (usually palmitate) covalently links each subunit to the internal edge of the transmembrane domain and could play a role in membrane localization. Interestingly, non-acylated

mutants mediate faster iron uptake than normal receptors (Alvarez et al., 1990) (Jing and Trowbridge, 1990). The transferrin binding regions of the protein are unidentified (Williams and Enns, 1993a); (Williams and Enns, 1993b). Efforts to crystallize transferrin receptor protein are underway.
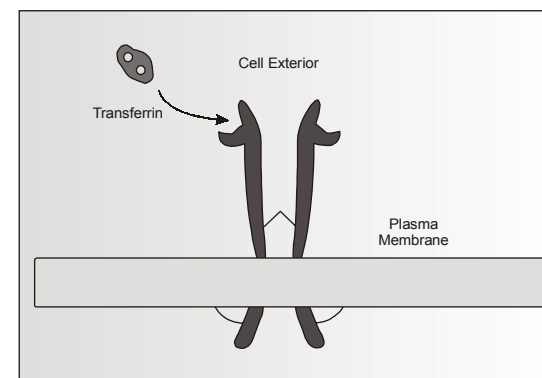
Fig. 5.50 : Schematic representation of the transferrin receptor. The molecule is a transmembrane homodimer linked by disulphide bonds. An acyl group attached to the cytoplasmic tail of the molecule anchors the assembly to the plasma membrane

Iron is taken into cells by receptor-mediated endocytosis of monoferric and diferric transferrin (Karin and Mintz, 1981); (Klausner et al., 1983); (Iacopetta and Morgan, 1983); (Fig. 181). Receptors on the outer face of the plasma membrane bind iron-loaded transferrin with a very high affinity. The C-terminal domain of transferrin appears to mediate receptor binding (Zak et al., 1994). Diferric transferrin binds with higher affinity than monoferric transferrin or apotransferrin (Huebers et al., 1984); (Young et al., 1984). The dissociation constant (Kd) for bound diferric transferrin ranges from 10-7 M to 10-9 M at physiologic pH, depending on the species and tissue assayed (Stein and Sussman, 1983); (Sawyer and Krantz, 1986). The Kd of monoferric transferrin is approximately 10-6 M. The concentration of circulating transferrin is about 25 mM. Therefore, cellular transferrin receptors ordinarily are fully saturated.

After binding to its receptor on the cell surface, transferrin is rapidly internalized by invagination of clathrin-coated pits with formation of endocytic vesicles (Figure 181). This process requires the short, 61 amino acid intracellular tail of the transferrin receptor molecule (Rothenberger et al., 1987); (Alvarez et al., 1990); (McGraw and Maxfield, 1990); (Girones et al., 1991); (Miller et al., 1991). Receptors with truncated N-terminal cytoplasmic domains do not recycle (Rothenberger et al., 1987). This portion of the molecule contains a conserved tyrosine-threonine-arginine-phenylalanine (YTRF) sequence which functions as a signal for endocytotic internalization (Collawn et al., 1993). Genetically engineered addition of a second YTRF sequence enhances receptor endocytosis (Collawn et al., 1993).
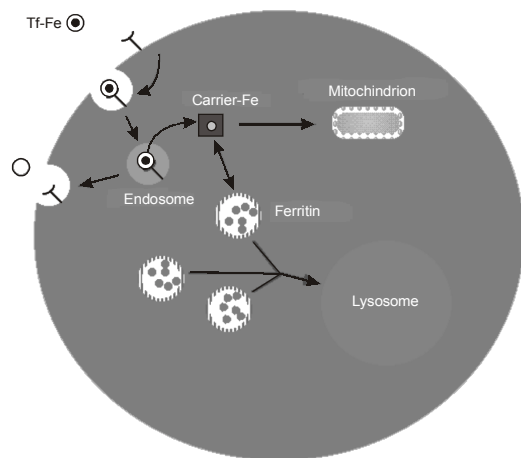
Fig. 5.51 : Receptor-mediated transferrin endocytosis. Ferro-transferrin binds to transferrin receptors on the external surface of the cell. The complex is internalized into an endosome, where the pH is lowered to about 5.5. Iron separates from the transferrin molecule, moving into the cell cytoplasm. Here, an iron transport molecule shuttles the iron to various points in the cell, including mitochondria and ferritin. Ferritin molecules accumulate excess iron. Lysosomes engulf aggregates of ferritin molecules in a process termed "autophagy"

A number of stimuli reversibly phosphorylate the serine residue adjacent to the YTRF sequence, at position 24 by the action of protein kinase C (Davis et al., 1986). The role of receptor phosphorylation is unclear. Despite removal of the phosphorylation site by site-directed mutagenesis, the transferrin receptor recycles normally (Rothenberger et al., 1987). An ATP-dependent proton pump lowers the pH of the endosome to about 5.5 (Van Renswoude et al., 1982); (Dautry-Varsat et al., 1983); (Paterson et al., 1984); (Yamashiro et al., 1984). The acidification of the endosome weakens the association between iron and transferrin. Even at pH 5.5, Fe^{3+} would not normally dissociate from transferrin in the several minutes between its endocytosis and the return of transferrin apoprotein to the cell surface (Ciechanover et al., 1983). A plasma membrane oxidoreductase reduces transferrin bound iron from the Fe^{3+} state to Fe^{2+}, directly or indirectly facilitating the removal of iron from the protein (Low et al., 1987); (Thorstensen and Romslo, 1988); (Nunez et al., 1990). Conformational changes in the transferrin receptor also play a role in iron release (Bali et al., 1991); (Sipe and Murphy, 1991).

Rather than entering lysosomes for degradation, as do ligands in other receptor-mediated endocytosis pathways, intact receptor-bound apotransferrin recycles to the cell surface, where neutral pH promotes detachment into the circulation (Zak and Aisen, 1990). Thus the preservation and re-use of transferrin

are accomplished by pH-dependent changes in the affinity of transferrin for its receptor (Van Renswoude et al., 1982); (Klausner et al., 1983); (Dautry-Varsat et al., 1983). Exported apotransferrin binds additional iron and undergoes further rounds of iron delivery to cells. The average transferrin molecule, with a half-life of eight days, may be used up to one hundred times for iron delivery (Harford et al., 1994).

Topologically, the cell exterior and the endosome interior are equivalent compartments. The primary role of the transferrin-transferrin receptor interaction is to bring iron into the vicinity of the cell surface, thereby increasing the likelihood of iron uptake. Following its release from transferrin within the endosome, iron must traverse the plasma membrane to enter the cytosol proper. The molecules effecting this transport have not been identified, but the process may be carrier-mediated (Egyed, 1988). Two anemic, mutant animals, the Belgrade rat (b/b) and the hemoglobin deficit mouse (hbd/hbd) appear to have lesions at or near this step. Their cells take up ferrotransferrin into endosomes, but fail to release iron into the cytoplasm (Garrick et al., 1987); (Garrick et al., 1993). The molecular basis of the defects in these animals have not been elucidated.

The endosomal transporter may reside on the plasma membrane of the cell prior to endocytosis (Pollack, 1992). If so, it should be oriented to transport iron directly into the cell, without the assistance of transferrin. Such non-transferrin-bound iron uptake activities have been characterized in tissue culture. This uptake system could function constitutively but inefficiently. Coupling the transferrin cycle to transport across the plasma membrane might augment iron uptake by creating an iron-rich environment for the transporter within the endosome. This same elusive transport molecule could also be involved in intestinal iron uptake. The phenotype of the mk/mk mouse (see above) suggests that red cell iron uptake and intestinal iron uptake share a common component which could be the 'endosomal' transporter.

Once inside the cell cytoplasm, iron appears to be bound by a low molecular weight carrier molecule, which may assist in delivery to various intracellular locations including mitochondria (for heme biosynthesis) and ferritin (for storage). The identity of the intracellular iron carrier molecule(s) remains unknown. The amount of iron in transit within the cell at any given time is minuscule and defies precise measurement. This minute pool of transit iron, which is believed to be in the Fe^{2+} oxidation state, is the biologically active form of the element. Metabolically inactive iron, stored in ferritin and hemosiderin, is in equilibrium with exchangeable iron bound to the low molecular weight carrier molecule (Figure 3).

Both prokaryotes and eukaryotes produce ferritin molecules for iron storage. Ferritins are complex twenty-four subunit heteropolymers of H (for heavy or heart) and L (for light or liver) protein subunits (Theil, 1987). L subunits are 19.7 kDa in mass, with isoelectric points of 4.5-5.0; H subunits are 21 kDa with isoelectric points of 5.0-5.7. The subunits of the ferritin molecule form a sphere with a central cavity in which up to 4500 atoms of crystalline iron is stored in the form of poly-iron-phosphate oxide (Theil, 1987). Eight channels through the sphere are lined by hydrophilic amino acid residues (along the three-fold axes of symmetry) and six more are lined by hydrophobic residues (along the four-fold axes; [Harrison et al., 1986].) Strong interspecies amino acid conservation exists in the residues that line the hydrophilic channels, while marked variation exists in those along the hydrophobic passages. Hydrophilic channels terminate with aspartic acid and glutamic acid residues , and are lined

by serine, histidine and cysteine residues (all of which potentially bind metal ligands). The evolutionary conservation of the hydrophilic channels suggests that they provide the route for iron entry and exit from the ferritin shell, but this contention remains unproved. Little is known about how iron is released from ferritin for use.

Although the two ferritin chains are highly homologous, only H ferritin has ferroxidase activity. A mechanism involving dioxygen converts ferrous to ferric iron, promoting incorporation into ferritin (Levi et al., 1988); (Lawson et al., 1991). The composition of ferritin shells varies from H-subunit homopolymers to L-subunit homopolymers, and includes all possible combinations between the two. Isoelectric focusing of ferritin from a particular tissue reveals multiple bands representing shells with different subunit compositions. These isoferritins, as they are called, show tissue specific variation (Drysdale, 1988). Ferritin from liver, for instance, is rich in L-subunits, as is that from the spleen. In contrast, the heart has ferritin rich in H-subunits. Increased H subunit content correlates with increased iron utilization, while increased L subunit content correlates with increased iron storage (Drysdale, 1988); (Theil, 1987). The H:L ratio rises with activation of heme synthesis or cell proliferation (Pattanapanyasat et al., 1987); (McClarty et al., 1990). Ferritin thus provides a flexible reserve of iron. Ferritin molecules aggregate over time to form clusters, which are engulfed by lysosomes and degraded (Iancu et al., 1977); (Bridges, 1987); Figure 3). The end-product of this process, hemosiderin, is an amorphous agglomerate of denatured protein and lipid interspersed with iron oxide molecules (reviewed by (Wixom et al., 1980). In cells overloaded with iron, lysosomes accumulate large amounts of hemosiderin which can be visualized by Prussian blue staining. Although the iron enmeshed in this insoluble compound constitutes an endstage product of cellular iron storage, it remains in equilibrium with soluble ferritin. Ferritin iron, in turn, is in equilibrium with iron complexed to low molecular weight carrier molecules. Therefore the introduction into the cell of an effective chelator captures iron from the low molecular weight "toxic iron" pool, draws iron out of ferritin, and eventually depletes iron from hemosiderin as well, though only very slowly. As might be expected, the bioavailability of hemosiderin iron is much lower than that of iron stored in ferritin.

Non-Transferrin-Bound Iron Uptake

Although compelling evidence exists that the transferrin cycle is important for iron acquisition by the erythron (Ponka and Schulman, 1993; Ponka, 1997)), other tissues can import iron by alternative mechanisms. Some patients and mutant mice that have little or no circulating transferrin (Heilmeyer, 1966); (Goya et al., 1972); (Bernstein, 1987); (Huggenvik et al., 1989). Despite severe hypochromic, microcytic anemia, non-erythroid tissues are grossly normal. While the red cells suffer from iron deficiency, serum iron levels (iron not bound to transferrin) are elevated, and excess iron is deposited in the liver. The iron-deprived bone marrow likely signals the gut to increase absorption, exacerbating tissue iron excess. Ponka and Schulman speculate that non-erythroid cells depend less on transferrin because their modest iron needs can be met by turnover of endogenous ferritin and heme iron. Red cells are more vulnerable because of greater iron use to form hemoglobin (Ponka and Schulman, 1993; Ponka, 1997). The transferrin cycle could serve primarily to enhance iron uptake by tissues with a great demand for the element. Iron overload produces fully saturated transferrin and non-transferrin bound iron circulating in a chelatable, low molecular weight form (Hershko et al., 1978); (Hershko and

Peto, 1978); (Craven et al., 1987); (Grootveld et al., 1989). This iron is weakly complexed to albumin, citrate, amino acids and sugars, and behaves differently from iron associated with transferrin. Non-hematopoietic tissues, particularly the liver, endocrine organs, kidneys and heart preferentially take up this iron.

Radiolabeled iron administered to mice with and without available transferrin binding capacity has quite different patterns of distribution (Craven et al., 1987). In normal animals, hematopoietic tissues are the prime sites of uptake. When free transferrin sites are absent, however, most iron is deposited in the liver and pancreas, indicating that these organs serve as iron reservoirs in the situation of iron overload. Notably, this pattern of distribution is similar to that seen in idiopathic hemochromatosis. These data support the idea that, while the transferrin pathway is important for meeting the needs of the erythron, it is not essential for iron uptake by all tissues. Kaplan and coworkers have studied iron incorporation from FeNH4 citrate (Sturrock et al., 1990); (Kaplan et al., 1991). Intriguingly, they find that transferrin-independent uptake increases in direct proportion to the concentration of this compound, similar to hepatic uptake of non-transferrin-bound iron in patients with saturated transferrin. They speculate that this is a protective alternative pathway that removes the toxic metal from the circulation. Other investigators have described similar uptake in HepG2 cells, and shown that it is reversible by addition of chelating compounds (Randell et al., 1994). A non-transferrin iron uptake mechanism with different properties has been described in K562 erythroleukemia cells (Inman and Wessling-Resnick, 1993). In the absence of ferric transferrin, iron uptake into K562 cells is sensitive to treatment with trypsin, suggesting that it requires a protein carrier. Higher ambient iron concentrations do not increase cellular iron uptake. As discussed above, this transport may be accomplished by the same machinery responsible for passage of iron out of transferrin cycle endosomes into the cytoplasm (Pollack, 1992). These two processes accomplish essentially the same task. The putative endosomal iron transporter must be oriented to transport iron from an endocytosed extracellular compartment into the cytoplasm. This transporter may exist on the cell surface prior to receptor-mediated endocytosis, with the capacity to transport iron to a modest extent. This activity is not restricted to erythroid cells. PHA-stimulated human peripheral lymphocytes have a similar transferrin-independent iron uptake mechanism (Hamazaki and Glass, 1992).

IODINE

MOST	DAILY REQUIREMENT	
ESSENTIAL	MALE: 0.05gm	
TRACE	FEMALE 0.05 gm	
	Infants 0-6 months	15 mg
ELEMENT	Infants 7-12 months	15 mg
	Children 1-6 years	6 mg
	School children 7-12 years	4 mg
	Adolescents and adults (12+ years)	2 mg

Dietary Source of Iodine

This element is an essential constituent of the body. It is estimated that a total 25 mg found in the human organism, 15 mg is in the thyroid. In this organ, a large percentage (but not all) of the iodine is in the combination in the organic molecule called thyroxine a hormone which will be discussed in the chapter 12.

An average person needs 0.05 mg per day. Since the ocean is rich in iodine, see foods, fish and oysters, are the food sources of this element. The table below shows the different sources of diet enriched in iodine.The iodine content of food depends on the iodine content of the soil in which it is grown. The iodine present in the upper crust of earth is leached by glaciation and repeated flooding and is carried to the sea. Sea water is, therefore, a rich source of iodine. The seaweed located near coral reefs has an inherent biologic capacity to concentrate iodine from the sea. The reef fish which thrive on seaweed are rich in iodine. Thus, a population consuming seaweed and reef fish has a high intake of iodine, as the case in Japan. The amount of iodine intake by the Japanese is in the range of 2-3 mg/day. In several areas of Asia, Africa, Latin America, and parts of Europe, iodine intake varies from 20 to 80 mg/day. In the United States and Canada and some parts of Europe, the intake is around 500 mg/day. The average iodine content of foods (fresh and dry basis) as reported by Koutras is given in Tables.

Average iodine content of foods (in μg/g)

Food	Fresh basis		Dry basis	
	Mean	Range	Mean	Range
Fish (fresh water)	30	17-40	116	68-194
Fish (marine)	832	163-3180	3715	471-4591
Shellfish	798	308-1300	3866	1292-4987
Meat	50	27-97	-	-
Milk	47	35-56	-	-
Eggs	93	-	-	-
Cereal grains	47	22-72	65	34-92
Fruits	18	10-29	154	62-277
Legumes	30	23-36	234	223-245
Vegetables	29	12-201	385	204-1636

Iodine Rich Foods List	ug	Portion
Haddock	up to 300	100
Cod	90+	100
Condensed milk	up to 70	100
Trifle	60	100
Eggs	50+	100
Mayonnaise	35	100
Jaffa cakes	32+	100
Cheddar cheese	40	100
Malt bread	29	100
Naan Bread	28	100
Pudding	26	100
Cheese cake	24	100
Sea kelp	High **	100
Seaweed	High **	100
Sea foods	High **	100

** high for the amounts consumed!

The RNI* for iodine is about 140ug daily for adults.

Iodine Absorption

At present, the only physiologic role known for iodine in the human body is in the synthesis of thyroid hormones by the thyroid gland. Therefore, the dietary requirement of iodine is determined by normal thyroxine (T4) production by the thyroid gland without stressing the thyroid iodide trapping mechanism or raising thyroid stimulating hormone (TSH) levels.

Iodine from the diet is absorbed throughout the gastrointestinal tract. Dietary iodine is converted into the iodide ion before it is absorbed. The iodide ion is bio-available and absorbed totally from food and water. This is not true for iodine within thyroid hormones ingested for therapeutic purposes. Iodine enters the circulation as plasma inorganic iodide, which is cleared from circulation by the thyroid and kidney. The iodide is used by the thyroid gland for synthesis of thyroid hormones, and the kidney excretes iodine with urine. The excretion of iodine in the urine is a good measure of iodine intake. In a normal population with no evidence of clinical iodine deficiency either in the form of endemic goitre or endemic cretinism, urinary iodine excretion reflects the average daily iodine requirement. Therefore, for determining the iodine requirements, the important indexes are serum T4 and TSH levels (indicating

normal thyroid status) and urinary iodine excretion. The simplified diagram of metabolic circuit of iodine is given in
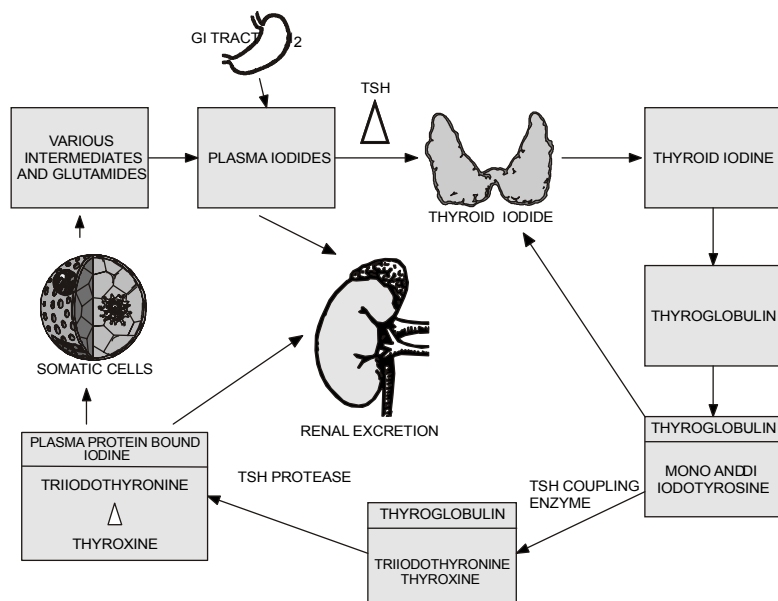

Fig. 5.52 : Simplified diagram of the metabolic circuit of iodine

All biologic actions of iodide are attributed to the thyroid hormones. The major thyroid hormone secreted by the thyroid gland is T_4 (tetra-iodo-thyronine). T_4 in circulation is taken up by the cells and is de-iodinated by the enzyme 5' prime-mono-de-iodinase in the cytoplasm to convert it into tri-iodo-thyronine (T_3), the active form of thyroid hormone. T_3 traverses to the nucleus and binds to the nuclear receptor. All the biologic actions of T_3 are mediated through the binding to the nuclear receptor, which controls the transcription of a particular gene to bring about the synthesis of a specific protein.

The physiologic actions of thyroid hormones can be categorised as growth and development and control of metabolic processes in the body. Thyroid hormones play a major role in the growth and development of brain and central nervous systems in humans from the 15th week of gestation to age 3 years. If iodine deficiency exists during this period and results in thyroid hormone deficiency, the

consequence is derangement in the development of brain and central nervous system. These derangements are irreversible, the most serious form being that of cretinism. The effect of iodine deficiency at different stages of life is given in Table below

The other physiologic role of thyroid hormone is to control several metabolic processes in the body. These include carbohydrate, fat, protein, vitamin, and mineral metabolism. For example, thyroid hormone increases energy production, increases lipolysis, and regulates neoglucogenesis, and glycolysis.

Table showing the Spectrum of Iodine Deficiency Disorders

Life stage Stage in life	Effects
Foetus	Abortions
	Stillbirths
	Congenital anomalies
	Increased perinatal mortality
	Increased infant mortality
	Neurological cretinism: mental deficiency, deaf mutism, spastic diplegia, and squint
	Myxedematous cretinism: mental deficiency and dwarfism
	Psychomotor defects
Neonate	Neonatal goitre
	Neonatal hypothyroidism
Child and Adolescent	Goitre
	Juvenile hypothyroidism
	Impaired mental function
	Retarded physical development
Adult	Goitre with its complications
	Hypothyroidism
	Impaired mental function
	Population at risk

Iodine deficiency affects all stages of human life, from the intra-uterine stage to old age, as shown in table above However, pregnant women, lactating women, women of reproductive age, and children younger than 3 years are considered to be at high risk. During foetal and neonatal growth and development, iodine deficiency leads to irreversible damage to the brain and central nervous system.

The iodine content of food varies with geographic location because there is a large variation in the iodine content of the inorganic world Table . Thus, the average iodine content of foods shown in Table can not be used universally for estimating iodine intake.

Recommended Intake

The daily intake of iodine recommended by the National Research Council of the US National Academy of Sciences in 1989 was 40 μg/day for young infants (0-6 months), 50 μg/day for older infants (6-12 months), 60-100 μg/day for children (1-10 years), and 150 μg/day for adolescents and adults (5). These values approximate 7.5 μg/kg/day for age 0-12 months, 5.4 μg/kg/day for age 1-10 years, and 2 μg/kg/day for adolescents and adults. These amounts are proposed to allow normal T4 production without stressing the thyroid iodide trapping mechanism or raising TSH levels.

Iodine Requirements in Infancy

The US recommendation of 40 μg/day for infants aged 0-6 months (or 8 μg/kg/day, 7 μg/100 kcal, or 50 μg/l milk) is probably derived from the observation that until the late 1960s the iodine content of human milk was approximately 50 μg/l and from the concept that nutrition of the human-milk-fed infant growing at a satisfactory rate has been the standard against which nutrition requirements have been set . However, more recent data indicate that the iodine content of human milk varies markedly as a function of the iodine intake of the population. For example, it ranges from 20 to 330 μg/l in Europe and from 30 to 490 μg/l in the United States. It is as low as 12 μg/l under conditions of severe iodine deficiency (6, 8). An average human-milk intake of 750 ml/day would give an intake of iodine of about 60 μg/day in Europe and 120 μg/day in the United States. The upper US value (490 μg/l) would provide 368 μg/day or 68 μg/kg/day for a 5-kg infant. Positive iodine balance in the young infant, which is required for the increasing iodine stores of the thyroid, is achieved only when the iodine intake is at least 15 μg/kg/day in full-term infants and 30 μg/kg/day in pre-term infants. The iodine requirement of pre-term infants is twice that of term infants because of a 50 percent lower retention of iodine by pre-term infants. This corresponds approximately to an iodine intake of 90 μg/day. (This is probably based on the assumption of average body weight of 6 kg for a child of 6 months, the mid-age of an infant.) This value is twofold higher than the US recommendations.

On the basis of these considerations, a revision is proposed for the earlier World Health Organization (WHO), United Nations Children's Fund (UNICEF), and International Council for the Control of Iodine Deficiency Disorders (ICCIDD) recommendations : an iodine intake of 90 μg/day from birth onwards is suggested. To reach this objective, and based on an intake of milk of about 150 ml/kg/day, the iodine content of formula milk should be increased from 50 to 100 μg/l for full-term infants and to 200 μg/l for pre-term infants.

Iodine content of the inorganic world

Location	Iodine content
Terrestrial air	1.0 μg/l
Marine air	100.0 μg/l
Terrestrial water	5.0 μg/l
Sea water	50.0 μg/l
Igneous rocks	500.0 μg/kg
Soils from igneous rocks	9000.0 μg/kg
Sedimentary rocks	1500.0 μg/kg
Soils from sedimentary rocks	4000.0 μg/kg
Metamorphic rocks	1600.0 μg/kg
Soils from the metamorphic rocks	5000.0 μg/kg

For a urine volume of about 4-6 dl/day from 0 to 3 years, the urinary concentration of iodine indicating iodine repletion should be in the range of 150-220 μg/l (1.18-1.73mmol/l) in infants aged 0-36 months. Such values have been observed in iodine-replete infants in Europe, Canada , and the United States. Under conditions of moderate iodine deficiency, as seen in Belgium, the average urinary iodine concentration is only 50-100 μg/l (0.39-0.79mmol/l) in this age group. It reaches a stable normal value of 180-220 μg/l (1.41-1.73mmol/l) only after several months of daily iodine supplementation with a physiologic dose of 90 μg/day .
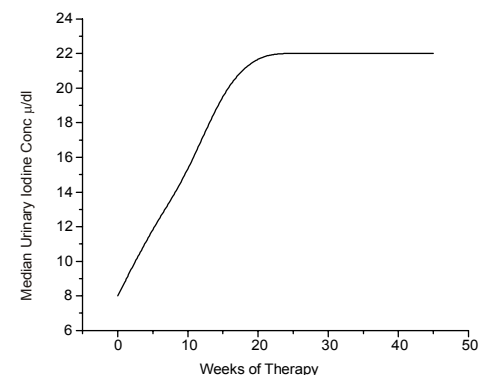
Fig. 5.45 : Changes over time for the median urinary concentration of iodine in healthy Belgian infants aged 6-36 months and supplemented with iodine at 90 μg/kg/day for 44 weeks

Each point represents 32-176 iodine determinations.

When the urinary iodine concentration in neonates and young infants is below a threshold of 50-60 μg/l (0.39-0.47mmol/l), corresponding to an intake of 25-35 μg/day, there is a sudden increase in the prevalence of neonatal serum TSH values in excess of 50 mU/ml, indicating sub-clinical hypothyroidism and eventually complicated by transient neonatal hypothyroidism. When the urinary iodine concentration is in the range of 10-20 μg/l (0.08-0.16mmol/l), as observed in severe endemic goitre regions, up to 10 percent of the neonates have overt severe hypothyroidism, with serum TSH levels above 100 mU/mL and serum T3 values below 30 μg/l (39 nmol/L) . Untreated, these infants progress to myxedematous endemic cretinism.

Thus, the iodine requirement of the young infant approximates 15 μg/kg/day (30 μg/kg/day in pre-term infants). Hyperthyrotropinemia (high levels of serum TSH), indicating sub-clinical hypothyroidism with the risk of brain damage, occurs when the iodine intake is about one-third of this value, and dramatic neonatal hypothyroidism resulting in endemic cretinism occurs when the intake is about one-tenth of this value.

Iodine Requirements in Children

The daily iodine need on a body weight basis decreases progressively with age. A study by Tovar and colleagues (16) correlating 24-hour thyroid radioiodine uptake and urinary iodine excretion in 9-13-year-old schoolchildren in rural Mexico suggested that an iodine intake in excess of 60 μg/day is associated with a 24-hour thyroidal radioiodine uptake below 30 percent. Lower excretion values are associated with higher uptake values. This would approximate 3 μg/kg/day in an average size 10-year-old child (approximate body weight of 20 kg), so that an intake of 60-100 μg/day for child of 1-10 years seems appropriate. These requirements are based on the body weight of Mexican children who participated in this study. The average body weight of a 10-year-old child, as per the Food and Agriculture Organization references, is 25 kg. Thus, the iodine requirement for a 1-10-year-old child would be 90-120 ig/day.

Iodine Requirements in Adults

Iodine at 150 μg/day for adolescents and adults is justified by the fact that it corresponds to the daily urinary excretion of iodine and to the iodine content of food in non-endemic areas (areas where iodine intake is adequate. It also provides the iodine intake necessary to maintain the plasma iodide level above the critical limit of 0.10 μg/dl, which is the average level likely to be associated with the onset of goitre. Moreover, this level of iodine intake is required to maintain the iodine stores of the thyroid above the critical threshold of 10 mg, below which an insufficient level of iodisation of thyroglobulin leads to disorders in thyroid hormone synthesis.

Data reflecting either iodine balance or its effect on thyroid physiology can help to define optimal iodine intake. In adults and adolescents in equilibrium with their nutritional environment, most dietary iodine eventually appears in the urine, so the urinary iodine concentration is a useful measure for assessing iodine intake. For this, casual samples are sufficient if enough are collected and if they accurately represent a community. A urinary iodine concentration of 100 μg/L corresponds to an intake of about 150 μg/day in the adult. Median urinary iodine concentrations below 100 μg/l in a population are associated with increases in median thyroid size and in serum TSH and thyroglobulin values. Correction of the iodine deficiency will bring all these measures back into the normal range. Recent data from the Thyro-Mobil project in Europe have confirmed these relations by showing that the largest thyroid sizes are associated with the lowest urinary iodine concentrations. Once a median urinary iodine excretion of about 100 μg/L is reached, the ratio of thyroid size to body size remains fairly constant. Moulopoulos et al reported that a urinary iodine excretion between 151 and 200 μg/g creatinine (1.18-1.57 mmol/g creatinine), corresponding to a concentration of about 200 μg/l (1.57 mmol/l), gave the lowest values for serum TSH in a non-goitrous population. Similar recent data from Australia show that the lowest serum TSH and thyroglobulin values were associated with urine containing 200-300 μg iodine/g creatinine (1.57-2.36mmol/g creatinine).

Other investigations followed serum TSH levels in subjects without thyroid glands who were given graded doses of T4 and found that euthyroidism established in adults with an average daily dose of 100 μg T4 would require at least 65 μg of iodine with maximal efficiency of iodine use by the thyroid. In practice such maximal efficiency is never obtained and therefore considerably more iodine is necessary.

Data from controlled observations associated a low urinary iodine concentration with a high goitre prevalence, high radioiodine uptake, and low thyroidal organic iodine content. Each of these measures reached a steady state once the urinary iodine excretion was 100 μg/l (0.78 mmol/l) or greater.

Iodine requirements in Pregnancy

The iodine requirement during pregnancy is increased to provide for the needs of the foetus and to compensate for the increased loss of iodine in the urine resulting from an increased renal clearance of iodine during pregnancy. These requirements have been derived from studies of thyroid function during pregnancy and in the neonate under conditions of moderate iodine deficiency. For example, in Belgium, where the iodine intake is estimated to be 50-70 μg/day, thyroid function during pregnancy is characterised by a progressive decrease of the serum concentrations of thyroid hormones and an increase in serum TSH and thyroglobulin. Thyroid volume progressively increases and is above the upper limit of normal in 10 percent of the women by the end of pregnancy. Serum TSH and thyroglobulin are still higher in the neonates than in the mothers. These abnormalities are prevented only when the mother receives a daily iodide supplementation of 161 μg/day during pregnancy (derived from 131 μg potassium iodide and 100 μg T4 given daily) . T4 with iodine was probably administered to the pregnant women to rapidly correct sub-clinical hypothyroidism, which would not have occurred if iodine had been administered alone. These data indicate that the iodine intake required to prevent the onset of sub-clinical hypothyroidism of mother and foetus during pregnancy, and thus to prevent the possible risk of brain damage of the foetus, is approximately 200 μg/day.

On the basis of the considerations reviewed above for the respective population groups to meet the daily iodine requirements, revisions of the current recommendations for daily iodine intake by WHO, UNICEF, and ICCIDD are proposed; these proposed revisions are presented in table below.

Proposed revision for daily iodine intake recommendations of 1996 by the World Health Organization, United Nations Children's Fund, and International Council for the Control of Iodine Deficiency Disorders

Population sub-groups	Total iodine intake μg/day	Iodine ig/kg/day
Infants (first 12 months)	90[a]	15.0
Children (1-6 years)	90	6.0
Schoolchildren (7-12 years)	120	4.0
Adults (12+ years)	150	2.0
Pregnant and lactating women	200	3.5

*Revised to 90 μg from the earlier recommendation of 50 μg.

Upper Limit of Iodine intake for Different Age Groups

An iodine excess also can be harmful to the thyroid of infants by inhibiting the process of synthesis and release of thyroid hormones (Wolff-Chaikoff effect). The threshold upper limit of iodine intake (the

intake beyond which thyroid function is inhibited) is not easy to define because it is affected by the level of iodine intake before exposure to iodine excess. Indeed, long-standing moderate iodine deficiency is accompanied by an accelerated trapping of iodide and by a decrease in the iodine stores within the thyroid. Under these conditions, the critical ratio between iodide and total iodine within the thyroid, which is the starting point of the Wolff-Chaikoff effect, is more easily reached during iodine depletion than under normal conditions. In addition, the neonatal thyroid is particularly sensitive to the Wolff-Chaikoff effect because the immature thyroid gland is unable to reduce the uptake of iodine from the plasma to compensate for increased iodine ingestion. For these reasons transient neonatal hypothyroidism or transient hyperTSHemia after iodine overload of the mother, especially after the use of povidone iodine, has been reported more frequently in European countries such as in Belgium, France, and Germany, which have prevailing moderate iodine deficiency.

Iodine intake in areas of moderate iodine deficiency

In a study in Belgium, iodine overload of mothers (cutaneous povidone iodine) increased the milk iodine concentration and increased iodine excretion in the term newborns (mean weight about 3 kg). Mean milk iodine concentrations of 18 and 128 µg/dl were associated with average infant urinary iodine excretion levels of 280 and 1840 µg/l (2.20-14.48 mmol/l), respectively. Estimated average iodine intakes would be 112 and 736 µg/day, or 37 and 245 µg/kg/day, respectively. The lower dose significantly increased the peak TSH response to exogenous thyroid releasing hormone but did not increase the (secretory) area under the TSH response curve. The larger dose increased both the peak response and secretory area as well as the baseline TSH concentration. Serum T4 concentrations were not altered, however. Thus, these infants had a mild and transient, compensated hypothyroid state. Non-contaminated mothers secreted milk containing 9.5 µg iodine/dl, and the mean urinary iodine concentration of their infants was 144 µg/l (1.13 mmol/l). These data indicate that modest iodine overloading of term infants in the neonatal period in an area of relative dietary iodine deficiency (Belgium) also can impair thyroid hormone formation.

Similarly, studies in France indicated that premature infants exposed to cutaneous povidone iodine or fluorescinated alcohol-iodine solutions and excreting iodine in urine in excess of 100 µg/day manifested decreased T4 and increased TSH concentrations in serum. The extent of these changes was more marked in premature infants with less than 34 weeks gestation than in those with 35-37 weeks gestation. The full-term infants were not affected. These studies suggest that in Europe the upper limit of iodine intake, which predisposes to blockage of thyroid secretion in premature infants (about 200 µg/day) is 2 to 3 times the average intake from human milk and about equivalent to the upper range of intake.

Iodine intake in areas of iodine sufficiency

Similar studies have not been conducted in the United States, where transient hypothyroidism is rarely seen perhaps because iodine intake is much higher. For example, urinary concentrations of 50 µg/dl and above in neonates, which can correspond to a Wolff-Chaikoff effect in Europe, are frequently seen in healthy neonates in North America.

The average iodine intake of infants in the United States in 1978, including infants fed whole cow milk, was estimated by the market-basket approach to be 576 µg/day (standard deviation [SD] 196);

that of toddlers was 728 µg/day (SD 315) and of adults was 952 µg/day (SD 589). The upper range for infants (968 µg/day) would provide a daily intake of 138 µg/kg for a 7-kg infant, and the upper range for toddlers (1358 µg/day) would provide a daily intake of 90 µg/kg for a 15-kg toddler.

Table below summarises the recommended dietary intake of iodine for age and approximate level of intake which appear not to impair thyroid function in the European studies of Delange in infants, in the loading studies of adults in the United States, or during ingestion of the highest estimates of dietary intake (just reviewed) in the United States. Except for the values for premature infants, these probably safe limits are 15-20 times more than the recommended intakes. These data refer to all sources of iodine intake. The average iodine content of infant formulas is approximately 5 µg/dl. The upper limit probably should be one that provides a daily iodine intake of no more than 100 µg/kg. For this limit and with the assumption that the total intake is from infant formula, with a daily intake of 150 ml/kg (100 kcal/kg), the upper limit of the iodine content of infant formula would be about 65 µg/dl. The current suggested upper limit of iodine in infant formulas of 75 µg/100 kcal (89µg/500 kJ or 50 µg/dl), therefore, seems reasonable.

Recommended dietary intakes of iodine and probable safe upper limits

Group	Recommended µg/kg/day	Upper limit[a] µg/kg/day
Premature infants	30	100
Infants 0-6 months	15	150
Infants 7-12 months	15	140
Children 1-6 years	6	50
School children 7-12 years	4	50
Adolescents and adults (12+ years)	2	30
Pregnancy and lactation	3.5	40

[a] Probably safe.

Excess iodine intake

Excess iodine intake is more difficult to define. Many people regularly ingest huge amounts of iodine - in the range 10-200 mg/day - without apparent adverse effects. Common sources are medicines (e.g., amiodarone contains 75 mg iodine per 200-mg capsule), foods (particularly dairy products), kelp (eaten in large amounts in Japan), and iodine-containing dyes (for radiologic procedures). Excess consumption of salt has never been documented to be responsible for excess iodine intake. Occasionally each of these may have significant thyroid effects, but generally they are tolerated without difficulty. Braverman et al. (35) showed that people without evidence of underlying thyroid disease almost always remain euthyroid in the face of large amounts of excess iodine and escape the acute inhibitory effects of excess intra-thyroidal iodide on the organification (i.e., attachment of ´oxidized iodine' species to throsyl residues in the thyroid gland for the synthesis of thyroid hormones) of iodide and on subsequent hormone synthesis (escape from or adaptation to the acute Wolff-Chaikoff effect). This adaptation

most likely involves a decrease in thyroid iodide trapping, perhaps corresponding to a decrease in the thyroid sodium-iodide transporter recently cloned. Some people, especially those with long-standing nodular goitre who live in iodine-deficient regions and are generally ages 40 years or older, may develop iodine-induced hyperthyroidism after ingestion of excess iodine in a short period of time.

Iodine Fortification

Iodine deficiency is present in almost all parts of the developed and developing world, and environmental iodine deficiency is the main cause of iodine deficiency disorders. Iodine is irregularly distributed over the earth's crust, resulting in acute deficiencies in areas such as mountainous regions and flood plains. The problem is aggravated by accelerated deforestation and soil erosion. Thus, the food grown in iodine-deficient regions can never provide enough iodine for the people and livestock living there. The iodine deficiency results from geologic rather than social and economic conditions. It cannot be eliminated by changing dietary habits or by eating specific kinds of foods but must be corrected by supplying iodine from external sources. It has, therefore, been a common practice to use common salt as a vehicle for iodine fortification for the past 75 years. Salt is consumed at approximately the same level throughout the year by the entire population of a region. Universal salt iodisation is now a widely accepted strategy for preventing and correcting iodine deficiency disorders.

There are areas where consumption of goitrogens in the staple diet (e.g., cassava) affects the proper utilisation of iodine by the thyroid gland. For example, in Congo, Africa, as a result of cassava diets there is an overload of thiocyanate. To overcome this problem, appropriate increases in salt iodisation are required to ensure the recommended dietary intake. The iodisation of salt is done either by spraying potassium iodate or potassium iodide in amounts that ensure a minimum of 150 µg iodine/day. Both of these forms of iodine are absorbed as iodide ions and are completely bio-available. Other methods of iodine prophylaxis are also used: iodised oil (capsule and injections), iodised water, iodised bread, iodised soya sauce, iodoform compounds used in dairy and poultry, and certain food additives.

Iodine loss occurs as a result of improper packaging, Humidity and moisture, and transport in open trucks and railway wagons exposed to sunlight. To compensate for these losses, higher levels of iodine are used during the production of iodised salt. Losses during the cooking process vary from 20 percent to 40 percent depending on the type of cooking used.

To ensure the consumption of recommended levels of iodine, the iodine content of salt at the production level should be monitored with proper quality assurance programmes. Regular evaluation of the urinary iodine excretion pattern in the population consuming iodised salt or exposed to other iodine prophylactic measures would help the adjusting of iodine intake.

Recommendations

Recommendations for future research

- elaborate the role of T_4 in brain development at the molecular level;
- investigate the relation between selenium and iodine deficiency, which has been reported in certain areas of Africa; and

- Investigate the possible interference of infections and other systemic illnesses with iodine or thyroid hormone use (such interference has not been reported on a population basis).

Recommendations for future actions

- establish quality assurance procedures at iodised salt production sites;
- track the progress of iodine deficiency disease elimination through the implementation of cyclic monitoring, which involves division of the country into five zones and carrying out the assessment in one zone each year; and
- Develop and validate quantitative testing kits for iodised salt.

COPPER

Essential	Daily requirement
Trace	Male 2 mg
Element	Female 2mg

Dietary Sources

Dietary sources of copper is shown in the table below:

Nuts	11.6mg
Legumes	9.0mg
Cereals	4.7 mg
Fruits	4.2 mg
Poultry	3.0 mg
Fish	2.5 mg
Green legumes	1.7 mg
Leafy veg	1.2 mg

Copper after Absorption

The claim has been made that copper plays some role in skin and hair pigmentation. It is said that the element accelerates the oxidation of dopa by dopa oxidase an enzyme present in the skin.

Some experiment have shown that anemia in the rat occurs due to copper deficiency, it is further accompanied by cytochrome oxidase activity of bone marrow it should be emphasized is one of the chief centres of hematopoiesis it would be seen that as if copper plays an important part in this reaction.

ZINC

Essential	Daily requirement
Trace	male 15 -20 mg
Element	female 15 – 20 mg

Dietary Source

Although we need zinc in only tiny amounts, the body makes use of it in many important ways. As with other trace elements, it is essential for the action of enzymes - the proteins that initiate vital chemical reactions in the body. It is present in the skin, eyes and bones, and in high concentrations in the liver and pancreas. Together with vitamin B6 (pyridoxine), it aids in cell formation. It has two other vital uses: aiding growth and sexual maturation, and keeping skin healthy. In the latter role, it has been found to have important healing properties in the treatment of wounds, burns and acne.

Zinc Rich Foods List	Milligrams	Portion
Oysters	25 +	100g
Shellfish	20	100g
Brewers Yeast	17	100g
Wheat Germ	17	100g
Wheat Bran	16	100g
All Bran cereal	6.8	100g
Pine Nuts	6.5	100g
Pecan Nuts	6.4	100g
Ok Sources of Zinc	Milligrams	Portion
Liver	6	100g
Cashew Nuts	5.7	100g
Parmesan Cheese	5.2	100g
Fish	3	100g
Eggs	2	100g

Pregnant and breast-feeding women must ensure they are getting adequate supplies of zinc from their diet and/or from supplements, as a lack of this mineral could lead to foetal abnormalities and stunted growth in their babies.

People with skin complaints, and particularly adolescents with acne, should consider supplementation, together with vitamins A, B2, B6 and E. Heavy drinkers, the elderly, convalescents, anyone with a diet high in processed foods and women on the pill MAY require extra zinc intake.

Zinc after Absorption

Only a small percentage of dietary zinc is absorbed ans the absorption occurs mainly from duodenum and ileum. It acts a binding factor which is secreated from pancreas, which forms complex with zinc and helps in its absorption.

Functions in the Body

1. Role in the Enzyme action
2. Role in the Vitamin a Absorption
3. Role in the insulin Secretion
4. Role in the Growth and Reproduction
5. Role in Would healing
6. Roile in Biosynthesis of Mononucleotides

SODIUM

Essential	Daily requirement
Primary	Male 1 - 3.5 gm
Element	Female - 1 - 3.5 gm
	Infants 0.1 gm - 0.5 gm
	Children 0.5 - 2.5gm

This is the most important and falls in the category of the principal elements. Sodium acts as an electrolyte which sis found in large concentration in extracellular fluid compartment.

Approximate body distribution of sodium is as follows:

	Total m/Mol	Conc m/Mol
Total body	3150	—
Intracellular	250	10
Extracellular	2900	140
Plama	400	140

The sodium is found in the body mainly associated with chloride or carbonate, NaCl or NaHCO3

Dietary Source

Sodium is widely distributed in food material the table below shows the sodium content in foodsGenerally all natural foods such as fruits and vegetables, are low in sodium. Whereas, all processed food products are often high in sodium, this is mainly due to the addition of salt as a preservative. Products like soy sauce, other sauces, powdered soups, marmite, gravy, mustard, oxo cubes and many pickled products are very high in sodium, however, they rarely contribute too much because they are usually eaten in small quantities!

| Sodium Rich Foods List	Milligrams	Portion
Anchovies (canned)	4000mg	100g
Cockles (boiled)	3500mg	100g
Olives (brine)	2200mg	100g
Bacon Rashers	2000mg	100g
Salami	1800mg	100g
Smoked Salmon	1800mg	100g
Prawns (boiled)	1600mg	100g
Savoury Rice	1400mg	100g
Feta Cheese	1400mg	100g
Danish Blue	1200mg	100g
Black Pudding (fried)	1200mg	100g
Some Smoked Fish	up to 1200mg	100g
Most processed foods / canned products	up to 1000+	100g
Processed Meats	up to 1000+	100g
Some Breakfast Cereals	up to 1000mg	100g
Most other Cheeses	up to 1000mg	100g
Bread	up to 800mg	100g
Pastries	up to 600mg	100g

Sodium Absorption

Sodium is widely absorbed via sodium pump situated in basal and lateral plams membrane of the intestinal and renal cells, Na pump actively transports sodium into extracelllar fluid.

Sodium Pump

Na+/K+-ATPase was discovered by Jens Christian Skou in 1957 while working as assistant professor at the Department of Physiology, University of Aarhus, Denmark. He published his work in 1957.[5]

In 1997, he received one-half of the Nobel Prize in Chemistry "for the first discovery of an ion-transporting enzyme, Na+, K+ -ATPase."
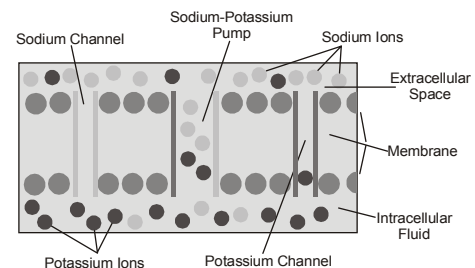
Fig. 5.55 : Showing the Sodium – Potassium Pump

Na^+/K^+-ATPase (also known as the Na^+/K^+ pump, sodium-potassium pump, or simply NAKA, for short) is an enzyme (EC 3.6.3.9) located in the plasma membrane (specifically an electrogenic transmembrane ATPase). It is found in the human cell and is found in all metazoa (animals).

Active transport is responsible for the well-established observation that cells contain relatively high concentrations of potassium ions but low concentrations of sodium ions. The mechanism responsible for this is the sodium-potassium pump which moves these two ions in opposite directions across the plasma membrane. This was investigated by following the passage of radioactively labeled ions across the plasma membrane of certain ones. It was found that the concentrations of sodium and potassium ions on the two other sides of the membrane are interdependent, suggesting that the same carrier transports both ions. It is now known that the carrier is an ATP-ase and that it pumps three sodium ions out of the cell for every two potassium ions pumped in.

The sodium-potassium pump was discovered in the 1950's by a Danish scientist, Jens Skou, who was awarded a Nobel Prize in 1997. It marked an important step forward in our understanding of how ions get into and out of cells, and it has a particular significance for excitable cells such as nervous cells, which depend on it for responding to stimuli and transmitting impulses.

(Advanced Biology - Michael Roberts, Michael Reiss, Grace Monger. 2000)

The Na^+/K^+–ATPase helps maintain resting potential, avail transport and regulate cellular volume. It also functions as signal transducer/integrator to regulate MAPK pathway, ROS as well as intracellular calcium.

In order to maintain the cell membrane potential, cells must keep a low concentration of sodium ions and high levels of potassium ions within the cell (intracellular). After the formation of an action potential, when the cell is repolarizing, that is within the cell it is becoming more and more negative as K^+ ions flood out, there is a stage where the membrane potential undershoots its resting membrane potential as K^+ channels take too long to close. This is called hyperpolarization. In order to restore the appropriate concentrations, the sodium-potassium pump pumps 3 sodium ions out by hydrolysing ATP and allows 2 potassium ions in through active transport. The carrier (pump) undergoes a conformational change in order to do this. The end result is that the resting membrane potential is restored and another

action potential can occur. However, one must not be mistaken as the Na$^+$/K$^+$ ATPase has no direct action in the formation of an action potential. The resting potential avails action potentials of nerves and muscles.

Export of sodium from the cell provides the driving force for several facilitated membrane transport proteins, which import glucose, amino acids and other nutrients into the cell.

Another important task of the Na$^+$-K$^+$ pump is to provide an Na$^+$ gradient that is used by certain carrier processes. In the gut, for example, sodium is transported out of the reabsorbing cell on the blood side via the Na$^+$-K$^+$ pump, whereas, on the reabsorbing side, the Na$^+$-Glucose symporter uses the created Na+ gradient as a source of energy to import both Na$^+$ and Glucose, which is far more efficient than simple diffusion. Similar processes are located in the renal tubular system.

One of the important functions of Na$^+$-K$^+$ pump is to maintain the volume of the cell. Inside the cell there are a large number of proteins and other organic compounds that cannot escape from the cell. Most, being negatively charged, collect around them a large number of positive ions. All these substances tend to cause the osmosis of water into the cell which, unless checked, can cause the cell to swell up and lyse. The Na$^+$-K$^+$ pump is a mechanism to prevent this. The pump transports 3 Na$^+$ ions out of the cell and in exchange takes 2 K$^+$ ions into the cell. As the membrane is far less permeable to Na$^+$ ions than K$^+$ ions the sodium ions have a tendency to stay there. This represents a continual net loss of ions out of the cell. The opposing osmotic tendency that results operates to drive the water molecules out of the cells. Furthermore, when the cell begins to swell, this automatically activates the Na$^+$-K$^+$ pump, which moves still more ions to the exterior.

Within the last decade, many independent labs have demonstrated that in addition to the classical ion transporting, this membrane protein can also relay extracellular ouabain binding signalling into the cell through regulation of protein tyrosine phosphorylation. The downstream signals through ouabain-triggered protein phosphorylation events include the activation of mitogen-activated protein kinase (MAPK) signal cascades, mitochondrial reactive oxygen species (ROS) production as well as activation of phospholipase C (PLC) and inositol triphosphate (IP3) receptor (IP3R) in different intracellular compartments.

Protein-protein interactions play very important role in Na$^+$-K$^+$ pump - mediated signal transduction. For example, Na$^+$-K$^+$ pump interacts directly with Src, a non-receptor tyrosine kinase, to form receptor complex. Na$^+$-K$^+$ pump also interacts with ankyrin, IP3R, PI3K, PLC-gamma and cofilin.

Mechanism

The pump, with bound ATP, binds 3 intracellular Na$^+$ ions.

- ATP is hydrolyzed, leading to phosphorylation of the pump at a highly conserved aspartate residue and subsequent release of ADP.

- A conformational change in the pump exposes the Na$^+$ ions to the outside. The phosphorylated form of the pump has a low affinity for Na$^+$ ions, so they are released.

- The pump binds 2 extracellular K$^+$ ions. This causes the dephosphorylation of the pump, reverting it to its previous conformational state, transporting the K$^+$ ions into the cell.
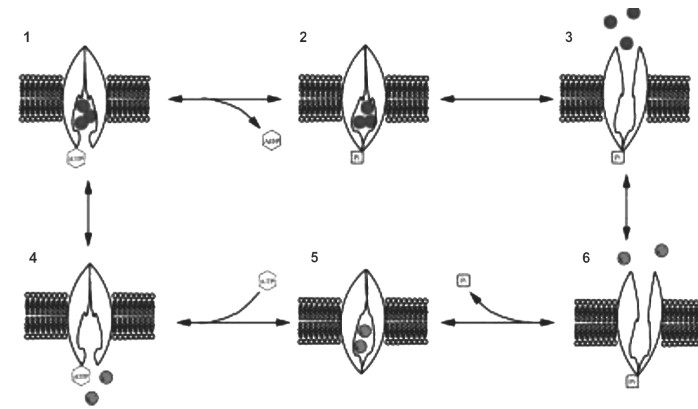
Fig. 5.60 : Showing the Mechanism of the Sodium Pump action

- The unphosphorylated form of the pump has a higher affinity for Na$^+$ ions than K$^+$ ions, so the two bound K+ ions are released. ATP binds, and the process starts again.

The Na$^+$/K$^+$-ATPase is upregulated by cAMP. Thus, substances causing an increase in cAMP upregulate the Na$^+$/K$^+$-ATPase. These include the ligands of the Gs-coupled GPCRs. In contrast, substances causing a decrease in cAMP downregulate the Na$^+$/K$^+$-ATPase. These include the ligands of the Gi-coupled GPCRs.

Note: Early studies indicated the opposite effect, but these were later found to be inaccurate due to additional complicating factors.

The Na$^+$-K$^+$-ATPase can be pharmacologically modified by administrating drugs exogenously.

For instance, Na$^+$-K$^+$-ATPase found in the membrane of heart cells is an important target of cardiac glycosides (for example digoxin and ouabain), inotropic drugs used to improve heart performance by increasing its force of contraction. Contraction of any muscle is dependent on a 100- to 10,000-times higher-than-resting intracellular Ca^{2+} concentration, which, as soon as it is put back again on its normal level by a carrier enzyme in the plasma membrane, and a calcium pump in sarcoplasmic reticulum, muscle relaxes.

Since this carrier enzyme (Na$^+$-Ca^{2+} translocator) uses the Na gradient generated by the Na$^+$-K$^+$ pump to remove Ca^{2+} from the intracellular space, slowing down the Na$^+$-K$^+$ pump results in a permanently-higher Ca^{2+} level in the muscle, which will eventually lead to stronger contractions.

Genes involed are stated below:

- Alpha: ATP1A1[1], ATP1A2[2], ATP1A3[3], ATP1A4[4]. #1 predominates in kidney. #2 is also known as "alpha(+)"
- Beta: ATP1B1[5], ATP1B2, ATP1B3[6], ATP1B4

Function of Soidum

1. *Fluid balance*: Sodium maintains crystalloid osmotic pressure of extra cellular fluids and helps in retaining water in ECF (extra cellular fluid)

2. *Neuro molecular Excitability*: Although other cations Na^+ is also involved in neuromolecular irritability which is given.

$$\text{Neuromolecular Irritability } \alpha = \frac{\left(K^+\right)\left(Na^+\right)}{\left(Ca^{++}\right)+\left(Mg^{++}\right)+\left(H^+\right)}$$

3. *Acid base balance*: Na^+ - H^+ exchange in renal tubule to acidify the urine.

4. *Maintanance of viscosity of the Blood:* The salts of Na with globulins are soluble and further Na^+ and K^+ both regulate in maintaining the degree of hydration of the plasma proteins.

5. *Role in resting membrane potential:* Plasma membrane has a poor Na^+ permeability and passive Na^+ in flow through it. Na^+ pump keeps Na^+ conc far higher outside than inside. This separation of charges is called polarization pf the membrane. It creates potential difference of 70 – 95 milivolt across the membrane and is called resting membrane potential.

6. *Role in Action Potential*: A local depolarization of nerve or muscle fibre is observed in stimulation. This rapidly increases its pemiability to Na^+ causing considerable transmembrane influx of Na^+ down its inward con – gradient.

We shall also discuss about the clinical problems arises due to sodium imbalance.

Hypernatremia or **hypernatraemia** is an electrolyte disturbance that is defined by an elevated sodium level in the blood. Hypernatremia is generally not caused by an excess of sodium, but rather by a relative deficit of free water in the body. For this reason, hypernatremia is often synonymous with the less precise term, dehydration. Water is lost from the body in a variety of ways, including perspiration, insensible losses from breathing, and in the feces and urine. If the amount of water ingested consistently falls below the amount of water lost, the serum sodium level will begin to rise, leading to hypernatremia. Rarely, hypernatremia can result from massive salt ingestion, such as may occur from drinking seawater. Ordinarily, even a small rise in the serum sodium concentration above the normal range results in a strong sensation of thirst, an increase in free water intake, and correction of the abnormality. Therefore, hypernatremia most often occurs in people such as infants, those with impaired mental status, or the elderly, who may have an intact thirst mechanism but are unable to ask for or obtain water.

Common causes of hypernatremia include:

- Inadequate intake of water, typically in elderly or otherwise disabled patients who are unable to take in water as their thirst dictates. This is the most common cause of hypernatremia.

- Inappropriate excretion of water, often in the urine, which can be due to medications like diuretics or lithium or can be due to a medical condition called diabetes insipidus
- Intake of a hypertonic fluid (a fluid with a higher concentration of solutes than the remainder of the body). This is relatively uncommon, though it can occur after a vigorous resuscitation where a patient receives a large volume of a concentrated sodium bicarbonate solution. Ingesting seawater also causes hypernatremia because seawater is hypertonic.
- Mineralcorticoid excess due to a disease state such as Conn's syndrome or Cushing's Syndrome
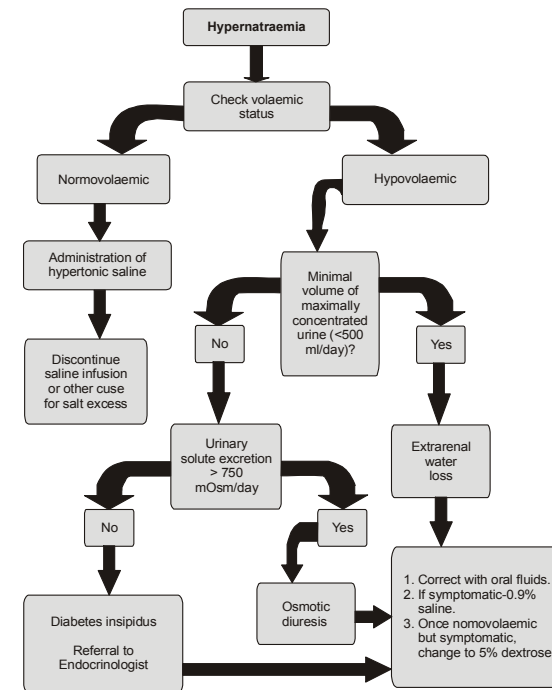
Symptoms

Fig. 5.60 : Showing the Flow chart for the Management of Hypernatremia

Clinical manifestations of hypernatremia can be subtle, consisting of lethargy, weakness, irritability, and edema. With more severe elevations of the sodium level, seizures and coma may occur.

Severe symptoms are usually due to acute elevation of the plasma sodium concentration to above 158 mEq/L (normal is typically about 135-145 mEq/L). Values above 180 mEq/L are associated with a high mortality rate, particularly in adults. However such high levels of sodium rarely occur without severe coexisting medical conditions.

Treatment

The cornerstone of treatment is administration of free water to correct the relative water deficit. Water can be replaced orally or intravenously. However, overly rapid correction of hypernatremia is potentially very dangerous. The body (in particular the brain) adapts to the higher sodium concentration. Rapidly lowering the sodium concentration with free water, once this adaptation has occurred, causes water to flow into brain cells and causes them to swell. This can lead to cerebral edema, potentially resulting in seizures, permanent brain damage, or death. Therefore, significant hypernatremia should be treated carefully by a physician or other medical professional with experience in treatment of electrolyte imbalances.

POTASSIUM

Essential	Daily Requirement
Primary	Male
Element	Female
	Children

Potassium is the major intracellular cation. It is widely distributed in the body fluids and tissues as follows:

Whole blood	200mg/dl
Plasma	20mg/dl
Cells	440mg/dl
Muscles tissue	250 - 400mg/dl

Dietary Source

Potassium rich foods	Potassium Content	Sodium content	RDA%*	Calories
1	2	3	4	5
Soya flour	1650mg	9mg	47%	450
Black treacle	1500mg	97mg	43%	260

1	2	3	4	5
Apricots ready-to-eat	1380mg	15mg	39%	160
Bran Wheat	1160mg	28mg	33%	200
Tomato Puree	1150mg	240mg	33%	70
Sultanas	1050mg	20mg	30%	275
Raisins	1020mg	60mg	30%	270
Potato chips (crisps UK)	1000mg	1000mg	29%	450
All Bran	1000mg	900mg	29%	260
Wheatgerm	950mg	5mg	27%	300
Figs	900mg	60mg	26%	100
Dried mixed fruit	880mg	48mg	25%	230
Bombay Mix	790mg	800mg	23%	500
Papadums	750mg	2400mg	22%	370
Currants	720mg	14mg	22%	270
Sultana Bran	660mg	700mg	19%	300
Seeds average	650mg	20mg	18%	500
Nuts average (unsalted)	600mg	300mg	17%	600
Baked Potato + skin	600mg	12mg	17%	130
Roast Potato	550mg	9mg	16%	160
Oven chips	530mg	50mg	15%	170
Bran Flakes	530mg	1000mg	15%	320
Gammon lean	520mg	2200mg	15%	170
Soya beans boiled	510mg	2mg	15%	140
Plantain boiled	500mg	4mg	14%	112
Raisin Splitz	500mg	10mg	14%	340
Weetos	500mg	300mg	14%	370
Crispbread	500mg	220mg	14%	320
Muesli low salt	450mg	390mg	13%	360
Sardines	430mg	650mg	12%	200
Pilchards	420mg	370mg	12%	125
Veal	420mg	110mg	12%	230
Wholemeal Pasta	400mg	130mg	11%	320
Banana	400mg	1mg	11%	96

Absorption of Potassium

As soon as it is absorbed potassium enters the cells. It is excreted in the urine. The amount of potassium excretion increases when there isan excessive dietary intake of sodium. Average nornmal human body contains 3.6 mols

Many functions of potassium and sodium are carried out in co-ordination with each other and are common. These function have already been described under sodium. Briefly

1. Influence of the muscular activity
2. Involved in acid – base balance
3. It has an important role in cardiac function.
4. Certain enzymes such as pyruvate kinase require K+ as a cofactor
5. Involved in neuromolecular irritability and nerve condition process

Function of Potassium

Potassium, the most abundant cation in the human body, regulates intracellular enzyme function and neuromuscular tissue excitability. Serum potassium is normally maintained within the narrow range of 3.5 to 5.5 mEq/L. The intracellular-extracellular potassium ratio (Ki/Ke) largely determines neuromuscular tissue excitability. Because only a small portion of potassium is extracellular, neuromuscular tissue excitability is markedly affected by small changes in extracellular potassium. Thus, the body has developed elaborate regulatory mechanisms to maintain potassium homeostasis. Because dietary potassium intake is sporadic and it cannot be rapidly excreted renally, short-term potassium homeostasis occurs via transcellular potassium shifts. Ultimately, long-term maintenance of potassium balance depends on renal excretion of ingested potassium. The illustrations in this chapter review normal transcellular potassium homeostasis as well as mechanisms of renal potassium excretion.

With an understanding of normal potassium balance, disorders of potassium metabolism can be grouped into those that are due to altered intake, altered excretion, and abnormal transcellular distribution.

The diagnostic algorithms that follow allow the reader to limit the potential causes of hyperkalemia and hypokalemia and to reach a diagnosis as efficiently as possible. Finally, clinical manifestations of disorders of potassium metabolism are reviewed, and treatment algorithms for hypokalemia and hyperkalemia are offered. Recently, the molecular defects responsible for a variety of diseases associated with disordered potassium metabolism have been discovered. Hypokalemia and Liddle's syndrome and hyperkalemia and pseudohypoaldosteronism type I result from mutations at different sites on the epithelial sodium channel in the distal tubules. The hypokalemia of Bartter's syndrome can be accounted for by two separate ion transporter defects in the thick ascending limb of Henle's loop. Gitelman's syndrome, a clinical variant of Bartter's syndrome, is caused by a mutation in an ion cotransporter in a completely different segment of the renal tubule. The genetic mutations responsible for hypokalemia in the syndrome of apparent mineralocorticoid excess and glucocorticoidremediable aldosteronism have recently been elucidated and are illustrated below.

Physiology of Potassium Balance : Distribution of Potassium

ECF 350 mEq (10%)	ICF 3150 mEq (90%)
Plasma 15 mEq (0.4%)	Musde 2650 mEq (76%)
Interstitial fluid 35 mEq (1%)	Liver 250 mEq (7%)
Bone 300 mEq (8.6%)	Erythrocytes 250 mEq (7%)
[K$^+$] = 3.5 – 5.0 mEq/L	[K+] = 140 -150 mEq/L
Urine 90-95 mEq/d	Urine 90-95 mEq/d
Stool 5-10 mEq/d	Stool 5-10mEq/d
Sweat < 5 mEq/d	Sweat < 5 mEq/d

External balance and distribution of potassium. The usual Western diet contains approximately 100 mEq of potassium per day. Under normal circumstances, renal excretion accounts for approximately 90% of daily potassium elimination, the remainder being excreted in stool and (a negligible amount) in sweat. About 90% of total body potassium is located in the intracellular fluid (ICF), the majority in muscle. Although the extracellular fluid (ECF) contains about 10% of total body potassium, less than 1% is located in the plasma. Thus, disorders of potassium metabolism can be classified as those that are due (1) to altered intake, (2) to altered elimination, or (3) to deranged transcellular potassium shifts.

Factors causing Transcellular Potassium Shifts

Factor	Δ Plasma K+
Acid-base status	
Metabolic acidosis	
Hyperchloremic acidosis	↑↑
Organic acidosis	↔
Respiratory acidosis	↓
Metabolic alkalosis	↑
Respiratory alkalosis	↑
Pancreatic hormones	
Insulin	↓↓
Glucagon	↑
Catecholamines	
β-Adrenergic	↓
α-Adrenergic	↑
Hyperosmolarity	↑
Aldosterone	↓, ↔
Exercise	↑

Extrarenal potassium homeostasis: insulin and catecholamines. Schematic representation of the cellular mechanisms by which insulin and _-adrenergic stimulation promote potassium uptake by extrarenal tissues. Insulin binding to its receptor results in hyperpolarization of cell membranes, which facilitates potassium uptake. After binding to its receptor, insulin also activates Na+-K+-ATPase pumps, resulting in cellular uptake of potassium. The second messenger that mediates this effect has not yet been identified. Catecholamines stimulate cellular potassium uptake via the _2 adrenergic receptor (_2R). The generation of cyclic adenosine monophosphate (3_, 5_ cAMP) activates Na+-K+-ATPase pumps, causing an influx of potassium in exchange for sodium [10]. By inhibiting the degradation of cyclic AMP, theophylline potentiates catecholaminestimulated potassium uptake, resulting in hypokalemia.
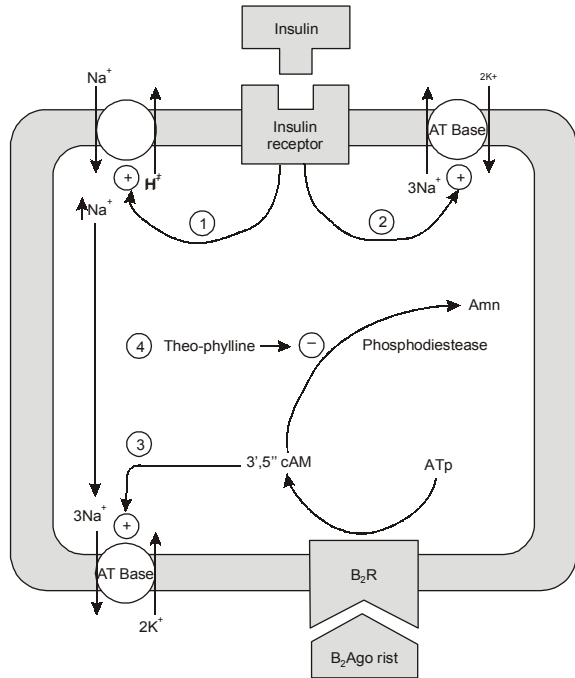

Fig. 5.60 : Showing the Disorder of Potassium Metabolism

Renal potassium handling. More than half of filtered potassium is passively reabsorbed by the end of the proximal convoluted tubule (PCT). Potassium is then added to tubular fluid in the descending limb of Henle's loop (see below). The major site of active potassium reabsorption is the thick ascending limb of the loop of Henle (TAL), so that, by the end of the distal convoluted tubule (DCT), only 10% to 15% of filtered potassium remains in the tubule lumen. Potassium is secreted mainly by the principal cells of the cortical collecting duct (CCD) and outer medullary collecting duct (OMCD). Potassium reabsorption occurs via the intercalated cells of the medullary collecting duct (MCD). Urinary potassium represents the difference between potassium secreted and potassium reabsorbed . During states of total body potassium depletion, potassium reabsorption is enhanced. Reabsorbed potassium initially enters the medullary interstitium, but then it is secreted into the pars recta (PR) and descending limb of the loop of Henle (TDL). The physiologic role of medullary potassium recycling may be to minimize potassium "backleak" out of the collecting tubule lumen or to enhance renal potassium secretion during states of excess total body potassium. The percentage of filtered potassium remaining in the tubule lumen is indicated in the corresponding nephron segment.
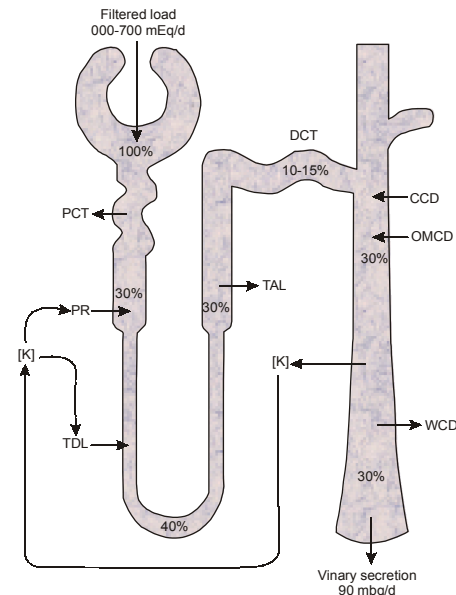

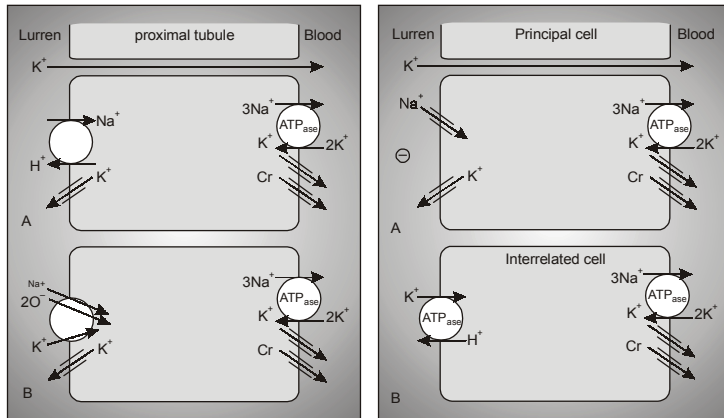**Fig. 5.61 : Showing the Renal Potassium Handling**

Fig. 5.62 : The Electrolyte Balance in the Water

Cellular mechanisms of renal potassium transport: proximal tubule and thick ascending limb. A, Proximal tubule potassium reabsorption is closely coupled to proximal sodium and water transport. Potassium is reabsorbed through both paracellular and cellular pathways. Proximal apical potassium channels are normally almost completely closed. The lumen of the proximal tubule is negative in the early proximal tubule and positive in late proximal tubule segments. Potassium transport is not specifically regulated in this portion of the nephron, but net potassium reabsorption is closely coupled to sodium and water reabsorption. B, In the thick ascending limb of Henle's loop, potassium reabsorption proceeds by electroneutral Na^+-K^+-$2Cl^-$ cotransport in the thick ascending limb, the low intracellular sodium and chloride concentrations providing the driving force for transport. In addition, the positive lumen potential allows some portion of luminal potassium to be reabsorbed via paracellular pathways. The apical potassium channel allows potassium recycling and provides substrate to the apical Na^+-K^+-$2Cl^-$ cotransporter. Loop diuretics act by competing for the Cl^- site on this carrier. Cellular mechanisms of renal potassium transport: cortical collecting tubule. A, Principal cells of the cortical collecting duct: apical sodium channels play a key role in potassium secretion by increasing the intracellular sodium available to Na^+-K^+-ATPase pumps and by creating a favorable electrical potential for potassium secretion. Basolateral Na^+-K^+-ATPase creates a favorable concentration gradient for passive diffusion of potassium from cell to lumen through potassium-selective channels. B, Intercalated cells. Under conditions of potassium depletion, the cortical collecting duct becomes a site for net potassium reabsorption. The H^+-K^+-ATPase pump is regulated by potassium intake. Decreases in total body potassium increase pump activity, resulting in enhanced potassium reabsorption. This pump may be partly responsible for the maintenance of metabolic alkalosis in conditions of potassium depletion.
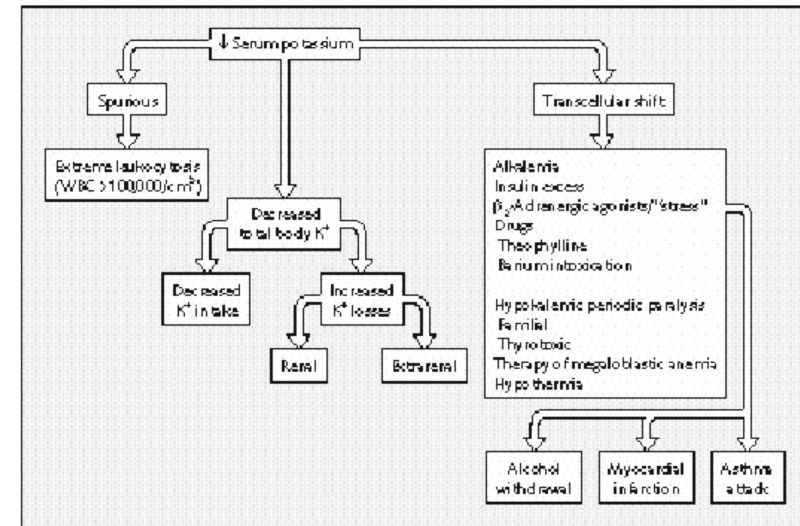
Fig. 5.63 : Showing the Diagnostic Approach of the Hyperkalemia

Overview of diagnostic approach to hypokalemia: Hypokalemia without total body potassium depletion. Hypokalemia can result from transcellular shifts of potassium into cells without total body potassium depletion or from decreases in total body potassium. Perhaps the most dramatic examples occur in catecholamine excess states, as after administration of _2adreneric receptor (_2AR) agonists or during "stress." It is important to note that, during some conditions (eg, ketoacidosis), transcellular shifts and potassium depletion exist simultaneously. Spurious hypokalemia results when blood specimens from leukemia patients are allowed to stand at room temperature; this results in leukocyte uptake of potassium from serum and artifactual hypokalemia. Patients with spurious hypokalemia do not have clinical manifestations of hypokalemia, as their in vivo serum potassium values are normal. Theophylline poisoning prevents cAMP breakdown (see Fig.187). Barium poisoning from the ingestion of soluble barium salts results in severe hypokalemia by blocking channels for exit of potassium from cells. Episodes of hypokalemic periodic paralysis can be precipitated by rest after exercise, carbohydrate meal, stress, or administration of insulin. Hypokalemic periodic paralysis can be inherited as an autosomal-dominant disease or acquired by patients with thyrotoxicosis, especially Chinese males. Therapy of megaloblastic anemia is associated with potassium uptake by newly formed cells, which is occasionally of sufficient magnitude to cause hypokalemia.

Diagnostic approach to hypokalemia: hypokalemia with total body potassium depletion secondary to extrarenal losses. In the absence of redistribution, measurement of urinary potassium is helpful in determining whether hypokalemia is due to renal or to extra renal potassium losses. The normal kidney responds to several (3 to 5) days of potassium depletion with appropriate renal potassium conservation. In the absence of severe polyuria, a "spot" urinary potassium concentration of less than 20 mEq/L indicates renal potassium conservation. In certain circumstances (eg, diuretics abuse), renal potassium losses may not be evident once the stimulus for renal potassium wasting is removed. In this circumstance, urinary potassium concentrations may be deceptively low despite renal potassiumlosses. Hypokalemia due to colonic villous adenoma or laxative abuse may be associated with metabolic acidosis, alkalosis, or no acid-base disturbance. Stool has a relatively high potassium content, and fecal potassium losses could exceed 100 mEq per day with severe diarrhea. Habitual ingestion of clay (pica), encountered in some parts of the rural southeastern United States, can result in potassium depletion by binding potassium in the gut, much as a cation exchange resin does. Inadequate dietary intake of potassium, like that associated ith anorexia or a "tea and toast" diet, can lead to hypokalemia, owing to delayed renal conservation of potassium; however, progressive potassium depletion does not occur unless intake is well below 15 mEq of potassium per day.

Diagnostic approach to hypokalemia: hypokalemia due to renal losses with normal acidbase status or metabolic acidosis. Hypokalemia is occasionally observed during the diuretic recovery phase of acute tubular necrosis (ATN) or after relief of acute obstructive uropathy, presumably secondary to increased delivery of sodium and water to the distal nephrons. Patients with acute monocytic and myelomonocytic leukemias occasionally excrete large amounts of lysozyme in their urine. Lysozyme appears to have a direct kaliuretic effect on the kidneys (by an undefined mechanism). Penicillin in large doses acts as a poorly reabsorbable anion, resulting in obligate renal potassium wasting. Mechanisms for renal potassium wasting associated with aminoglycosides and cisplatin are illdefined. Hypokalemia in type I renal tubular acidosis is due in part to secondary hyperaldosteronism, whereas type II renal tubular acidosis can result in a defect in potassium reabsorption in the proximal nephrons. Carbonic anhydrase inhibitors result in an acquired form of renal tubular acidosis. Ureterosigmoidostomy results in hypokalemia in 10% to 35% of patients, owing to the sigmoid colon's capacity for net potassium secretion. The osmotic dieresis associated with diabetic ketoacidosis. results in potassium depletion, although patients may initially present with a normal serum potassium value, owing to altered transcellular potassium distribution.

Hypokalemia and magnesium depletion. Hypokalemia and magnesium depletion can occur concurrently in a variety of clinical settings, including diuretic therapy, ketoacidosis, aminoglycoside therapy, and prolonged osmotic diuresis (as with poorly controlled diabetes mellitus). Hypokalemia is also a common finding in patients with congenital magnesium-losing kidney disease. The patient depicted was treated with cisplatin 2 months before presentation. Attempts at oral and intravenous potassium replacement of up to 80 mEq/day were unsuccessful in correcting the hypokalemia. Once serum magnesium was corrected, however, serum potassium quickly normalized.

CHLORINE

Essential	Daily Intake
Primary	100 – 200mMol
Element	

Chlorine is taken in diet as sodium chloride. Many vegetables and meats have small amount of chlorine in the form of chlorides. It is also available in the 'chloronated water which is normally supplied as a process of putrification of water for drinking purpose

Daily requirement and distribution

About 100 – 200mMol is taken in diet as sodiunm chloride

Distribution

Whole Blood	250 mg/dl
Plasma	375 mg/dl
CSF	440 mg/dl
Cells	190 mg/dl
Muscle	40 mg/dl

Absorption

It takes place in small intestines, the mechanism of chloride uptake is not clear, but it appears to depend upon the exchange process with the HCO_3^- whilst the accompanying sodium exchange for hydroxyl ion.

Extraction

1. Sweat 5mMol/day depends upon whether

2. Through Faces 5mMol/day

3. Renal 100 - 200mMol/day, 99% of the chlorine in the glomarular filtrate in reabsorbed by renal tubes mainly in proximal tubule (60 - 70%) and then in ascending loop of Hencle (20 - 25%) followed by the distatt tubule collecting duct (10 - 15%)

Regulation

Control of absorption and excretion of chloride appears to be similar that of sodium. Increase in clood volume decrease reabsorption of chloride and vice - versa. Plasma level of chloride carry with abd to a great extent depends upon the plasma concentration of HCO_3^-

$\downarrow$ Na associate with Cl^- $\downarrow$

$\uparrow$ Na associate with Cl^- $\uparrow$

$\uparrow$ HCO_3^- associate with Cl^- $\downarrow$

$\downarrow$ HCO_3^- associate with Cl^{-1} $\uparrow$

Functions

1. It is important in the production of HCL in gastric juice

2. It is important in chloride shift CO_2 that is derived as a end product from cellular metabolism, diffuses from tissues through the plasma and into RBC where the CO_2 conc is relatively low, within the erythrocyte the CO_2 is combined with the water to form H_2CO_3 by carbonic anhydrous. The acid then dissociates into a bicarbonate and H_+. The H_+ is buffered by Hb and the HCO_3^- diffuses from the red cell into the plams in exchange of Cl^-

The reverse reactions occur when the erythrocytes reach lungs where CO2 content exceeds that of the alveoli. Thus arterial and venus plams will differ slightly (2 - 3 mMol/L) in their constitution.

Clinical Importance

Spot urinary chloride is useful in classifying metabolic alterations into the saline responsive and saline non - responsive type.

Urine Cl < 10mMol/L - Saline responsive, metabolic alkalosis - vomiting, previous diuretic therapy chloride diarrhea igestion of alkali

Urine Cl . 20m/Mol/L - Saline unresponsive metabolic alkalosis mineralocorticoids. Excess Barter's Syndrome$ severe K+ deficiency, current diuretic therapy*. In certain conditions the urinary chloride concentration is useful tool in assessment of volume deplement, Hyperchloremis - Respiratory alkalosis and metabolic acidisis. Hyperchloremia may be associated with chloride loss, vomiting.

CALCIUM

Most	Daily req
Essential	Male: - 1000mg
Primary	Female: - 1000 - 1500mg
Element	Children : - 210 - 800 mg

Calcium is the most essential primary element mainly utilized for bone and teeth.

$ Bartter syndrome is a rare inherited defect in the thick ascending limb of the loop of Henle. It is characterized by low potassium levels (hypokalemia), decreased acidity of blood (alkalosis), and normal to low blood pressure. There are two types of Bartter syndrome: neonatal and classic. A closely associated disorder, Gitelman syndrome, is milder than both subtypes of Bartter syndrome.

* A diuretic therapy is the application of any drug that elevates the rate of urination and thus provides a means of forced diuresis.

Dietary Source

It is available in almost all food stuffs, the table below shows the foods containing calcium

Calcium intake Chart

Infants	Milligrams Per Day
0-5 months	210 mg
6-11 months	270 mg
Children	**Milligrams Per Day**
1-3 years	500 mg
4-8 years	800 mg
Males/Females	**Miligrams Per Day**
9-18 years	1,300 mg
19-50 years	1,000 mg
51-70 + years	1,200 mg
50 + years (women not on HRT)	1,500 mg
Pregnant and Lactating	**Miligrams Per Day**
< 18 years	1,300 mg
19 +	1,000 mg

Calcium-Rich Foods List	Milligrams	Portion
Hard Cheese	300-800	100g
Whitebait	800mg	100g (3.5oz)
Sardines	500mg	100g (3.5oz)
Tofu	500mg +	100g
Milk	300mg	1 glass (8 oz)
Anchovies	300mg	100g
Almonds	245mg	100g (3.5oz)
Milk Chocolate	220mg	100g
Fish Paste	200mg	100g
Most stuff made with milk	up to 200g	100g
Spinach	150mg	1 cupful
Yogurt	150mg	100g
Breads	up to 150	100g
broccoli	70mg	1 cupful

After Absorption

The total calcium of the body is 25 - 35 mol (100 - 170 gm). About 99% of its is found in the bones. It exista as carbonates or phosphate of calcium, about 0.5% is in soft tissue and 1% is in ECF(Eosinophil Chemotactic Factor). The normal level of claicum is 9 - 11mg/dl. The calcium in plasma is of 3 types namely, ionized calcium (diffusion) protein bound calcium and complexed calcium; it is probably complexed with organic acids.

About 40% of total calcium is in ionized form, Albumin is the major protein with which calcouim is bound. All the three forms if calcium imn plams remain in equilibrium with each other ionized calcium is physiologically active form of calcium.

About 40% of average daily intake of calcium is absorbed by the gut. The actual amount taken upon depends on:

1. Amount of ionized calcium Ca^{2+} available. Levels of Ca^{2+} in the intestinal lumen are reduced by dietary substance that form calcium Acid pH increases the ionization of calcium. Alkalinity promotes complex formation and diminished absorption.

2. Presence of the active metabolites of Vitamin D – 1 $25(OH)_2D_3$

Calcium is secreted into gut* as a normal constituent of bile and intestinal fluids. Fæcal output of calcium couldexceed intestinal absorption of situations, whre the dietr contains high levels of phylates or other sequestrating substances. Under normal circumstances the fæces are not an important excretion rout for calcium.

Action of Calcium in Kidney

Kindney filter about 250mMol of Ca^{++} every day, some 95% of which is reabsorbed by the tubules. The major portion of this filtered $Ca+2$ is taken up by proximal tubule without hormonal regulation. A fine adjustment to that amount reabsorbed occurs in distal tubules under the influence of PTH (PTH – uptake). Plasma level of ionized calcium conc is the principal regulator of PTH secretion by a simple negative feedback mechanism. A threshold level of magnesium is required for the PTH release. Hyper magnesemia inhibits PTH seretion. PTH secretion is also subject to negative feedback by the vitamin D metabolite 1, 25 $(OH)_2D_3$ PTH rapidly stimulates osteoclast activity, the increased bone resorption causing an increase in plams Ca^{+2} and PO_4 Vitamin D_3 plays a permissive role for thid effect.

PTH stimulates more slowly osteoblast activity PTH via c – AMP increases the distal nephron reap reabsorption of calcium and decreased that of PO_4 in the proximal tubule. In bonding so, PTH increases the tubular synthesis and excretion of c – AMP. PTH also stimulates the enzyme complex that converts 25 OHD_3 to $125(OH)_2D_3$ there by increasing calcium update form the gut.

Hypercalcemis stimulates calcitonin katacalin release while hypercalemia has inhibitory effect. Cacitonin strongly inhibits osteoblastic bone resorption. However the role of calcinations in calcium regulation is controvercial.

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* The gut flora consists of the microorganisms that normally live in the digestive tract of animals. The gut flora includes much of the human flora. The term "gut flora" is interchangeable with intestinal microflora and intestinal microbiota.

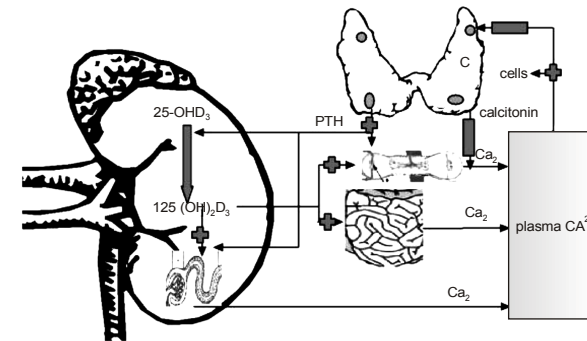Thyroid, ACTH and prostaglandins have some effect on calevel of plasma (see fig 191 And 192).
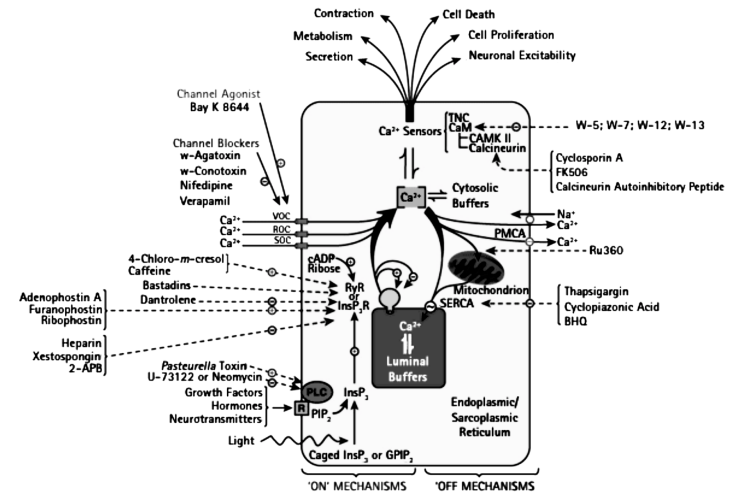

Fig. 5.60 : Showing the calcium metabolim in vital organs

Fig. 5.61 : Showing the General Scheme ofr calcium metabolism