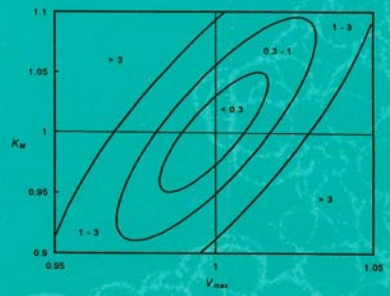
COMPREHENSIVE ENZYME KINETICS

VLADIMIR LESKOVAC



Comprehensive Enzyme Kinetics

This page intentionally left blank

Comprehensive Enzyme Kinetics

Vladimir Leskovac
University of Novi Sad

KLUWER ACADEMIC PUBLISHERS
NEW YORK, BOSTON, DORDRECHT, LONDON, MOSCOW

eBook ISBN: 0-306-48390-4
Print ISBN: 0-306-46712-7

Created in the United States of America

Visit Kluwer Online at: http://kluweronline.com
and Kluwer's eBookstore at: http://ebooks.kluweronline.com

Preface

Welcome to your study of enzyme kinetics, the subject that underlies all enzymology, which in turn underlies all aspects of biochemistry. This text will give you an introduction to a wide range of topics that constitute the modern enzyme kinetics. This textbook is directed at graduate students in biochemistry, chemistry, and life sciences, for advanced courses in enzyme kinetics, enzymology, and enzyme chemistry. For this reason, the whole book is organized in a systematic and scholarly fashion. It is unlikely that the student will be expected to cover everything in the text, but in a later career she or he may find it an invaluable reference for topics that are needed in practice. The concepts, definitions and detailed algebra of enzyme kinetics are laid out in accurate detail. For that reason, this textbook can also serve as a handbook for enzyme kinetics for research workers in the field. The research worker will find it a useful source, which can be used for solving the daily experimental problems in the laboratory.

The preparation of the manuscript for this book was under the constant surveillance of W. Wallace Cleland, Professor of Chemical Science at the University of Wisconsin in Madison, and one of the founders of modern enzyme kinetics. Without his help and advice, this book would not be possible. Several versions of the manuscript were constantly corrected and improved by Svetlana Trivić, Professor of Biochemistry at the University of Novi Sad. The final version of the manuscript was corrected by Dexter B. Northrop, Professor of Biochemistry at the University of Wisconsin in Madison, by Richard L. Schowen, Professor of Chemistry and Biochemistry at the University of Kansas in Lawrence, and by Bryce V. Plapp, Professor of Biochemistry at the University of Iowa in Iowa City.

The writing and the technical preparation of this book was supported by able computer engineers in Novi Sad, who provided the software, the hardware, and the maintenance of our computer facilities. The Faculty of Technology at the University of Novi Sad provided the logistics and the financial support for the preparation of the manuscript. Also, I owe my thanks to the staff of the Kluwer Academic/Plenum Publishers, for their constant help and patience during the preparation of this book. Alden Bookset was responsible for an excellent typesetting of this book.

Finally, as the former disciple of Professor Gerhard Pfleiderer, I dedicate this book to him, in recognition of his germinal and indelible contributions to the field of enzyme structure and function.

Vladimir Leskovac

This page intentionally left blank

Contents

This page intentionally left blank

Chapter 1

Introduction

This textbook for advanced courses in enzyme chemistry and enzyme kinetics covers the field of steady-state enzyme kinetics from the basic principles inherent in the Michaelis–Menten equation to the expressions that describe the multi-substrate enzyme reactions. The purpose of this book is to provide a simple but comprehensive framework for the study of enzymes with the aid of kinetic studies of enzyme-catalyzed reactions. The aim of enzyme kinetics is twofold: to study the *kinetic mechanism* of enzyme reactions, and to study the *chemical mechanism of action* of enzymes.

By the kinetic mechanism of enzyme reactions, we understand: (a) the kinetic mechanism, which is a qualitative description of the order of substrate combination and product release from the enzyme, and (b) the determination of rate-limiting steps from quantitative analysis of the kinetic mechanism.

By chemical mechanism of action of enzyme, we understand: (a) the identification of any intermediates, (b) the identification of any groups on the enzyme acting as acid–base catalysts, (c) the roles of any cofactors, and (d) the nature of the transition state for the chemical reaction catalyzed by enzyme.

A variety of kinetic experiments is used to deduce this information. The algebraic form of the rate equation as a function of substrate concentrations limits the kinetic mechanism, while inhibition patterns for products or dead-end inhibitors versus the various substrates pin it down, and often help to determine the rate-limiting step. Isotope exchange and partitioning studies complete the analysis of kinetic mechanism. The chemical mechanism is deduced by studying the pH variation of the kinetic parameters, which identifies the acid–base catalysts, and necessary protonation states of the substrate for binding and catalysis, and by certain kinetic isotope effect studies.

Since all enzymes are proteins, a logical starting point for the discussion of enzymes is to consider the general features of protein structure. Features of particular interest to enzymology are considered briefly, but this topic is treated more completely in the references cited at the end of this chapter.

1.1 ENZYME STRUCTURE

Enzymes are macromolecular protein catalysts, consisting of linear condensed polymers of α-amino acids joined in amide linkages. Each enzyme has a genetically determined and unique primary sequence, and it folds in three dimensions into a precise orientation (Lodish *et al.,* 1999).

The *primary* structure of a protein is specified by the order in which the amino acids are linked together through the peptide bonds. The most important feature of this structure is the peptide linkage shown in Fig. 1.

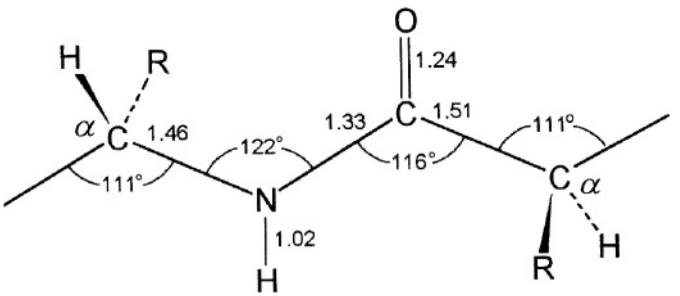


Figure 1. The planar and trans peptide bonds with standard distances in Ångstroms.

Because of resonance, which gives the N–C bond a partially double bond character, the peptide bond is planar. Furthermore, the α-carbons are almost always trans. These two features of the peptide bond play a dominant role in determining protein structure. The other covalent linkage of importance is the disulfide bond that joins different parts of the protein chain in enzymes secreted outside the cells. Special note should also be made of an imino acid proline, which creates a very rigid peptide bond.

The final structure of a protein is determined by the above factors and optimization of noncovalent interactions involving the peptide backbone and the amino acid side chains. The large variety of amino acid side chains provides the possibility of several different types of non-covalent interactions, such as van der Waals, π-electron stacking, hydrogen bonding, and electrostatic. The main types of amino acid side chains and their functions are summarized in Table 1.

To gain further insight into the nature of protein structure, noncovalent interactions are considered in more detail. Potentially, the largest amounts of energy are available from electrostatic interactions. For example, the energy of interaction between two univalent charges is e^2/dr, where e is the charge of an electron, d, the dielectric constant, and r, the distance between the charges. In water ($d = 80$) for charge separation of a few Ångstroms, the energy is few kilocalories per mole. The correct dielectric constants to use when considering the protein interior is not obvious, but the values are probably somewhere between that of water and organic solvents ($d = 10$). Ion pairs are more stable in non-aqueous media, since the ions can approach each other closely. In water, they are surrounded by hydration shells, and the neutral forms are relatively more stable. Thus, for example, the His–Cys pairs exist in enzymes as ion pairs, while in water it would be neutral–neutral.

While the static aspects of electrostatic interactions can be readily formulated, the dynamics are more difficult to ascertain (Hammes, 1982). A few kinetic studies for ion-pair formation have been carried out, and the rate constants appear to be those of diffusion-controlled reactions. For reactions between small molecules with univalent charges of opposite sign, k_f, the rate constant for

Table 1. Amino acid side chains and their functions

Side chain group	Amino acid	Functions and interactions
Hydrocarbon	Alanine, leucine, valine, isoleucine	van der Waals
Aromatic	Phenylalanine, tyrosine, tryptophan	van der Waals, π-electron stacking
Carboxyl	Aspartic acid, glutamic acid	Electrostatic, hydrogen bonding, acid–base catalysis
Amino	Lysine, arginine	Electrostatic, hydrogen bonding, acid–base catalysis
Imidazole	Histidine	Electrostatic, hydrogen bonding, acid–base catalysis, van der Waals, π-electron stacking
Hydroxyl	Serine, threonine, tyrosine	Hydrogen bonding, acid–base catalysis
Amide	Asparagine, glutamine	Hydrogen bonding
Sulfhydryl	Cysteine	Hydrogen bonding, electrostatic, acid–base catalysis

association is approximately $10^{10}\ M^{-1}\ s^{-1}$, while k_d, the rate constant for dissociation is approximately $10^{10}\ s^{-1}$.

Another type of electrostatic interactions of great importance in proteins is the hydrogen bond. Some typical hydrogen bonds and lengths are illustrated in Table 2.

Table 2. Some typical hydrogen bonds and bond lengths

Hydrogen bond	Bond length (Å)
OH ··· O	2.7
OH ··· N	2.9
NH ··· O	3.0
NH ··· N	3.1

In non-hydrogen bonding solvents, typical enthalpies of formation are few kilocalories per hydrogen bond per mole. The rate constants for making and breaking of single hydrogen bonds in water are $\geq 10^8\ s^{-1}$. When the pK_a's of the heteroatoms are matched, the strength of the hydrogen bond is much greater and the distance shorter. These low barrier hydrogen bonds play an important role in enzyme catalysis in a number of cases (Cleland & Kreevoy, 1994).

The next type of non-covalent interactions is the hydrophobic interactions; hydrophobic interactions are usually rather loosely defined to describe what happens when hydrocarbons are put into water. Actually, two types of interactions can be distinguished: (a) hydrocarbons interact very weakly with

each other due to dispersion forces, and (b) planar π-electron systems tend to stack on top of each other. Both of these interactions are very short range. The dynamics of hydrophobic interactions have been studied in model systems by measuring the rates of water solvation and dissolvation of hydrophobic molecules. The individual rate constants have been found to be $\geq 10^8\ s^{-1}$, ensuring that such interactions are clearly very rapid.

While the noncovalent interactions in proteins are generally very weak on individual basis, hundreds of these interactions exist in a single protein molecule so that the total energy involved is large. The hydrophobic interactions can be thought of as the dominant, rather nonspecific forces, leading to the establishment of a protein structure, with hydrogen bonding and electrostatic forces being most important in generating specificity.

A delicate balance between these noncovalent interactions is achieved in the native protein structure. Since the rate constants for forming and breaking noncovalent interactions are quite large, this would lead us to expect a floppy

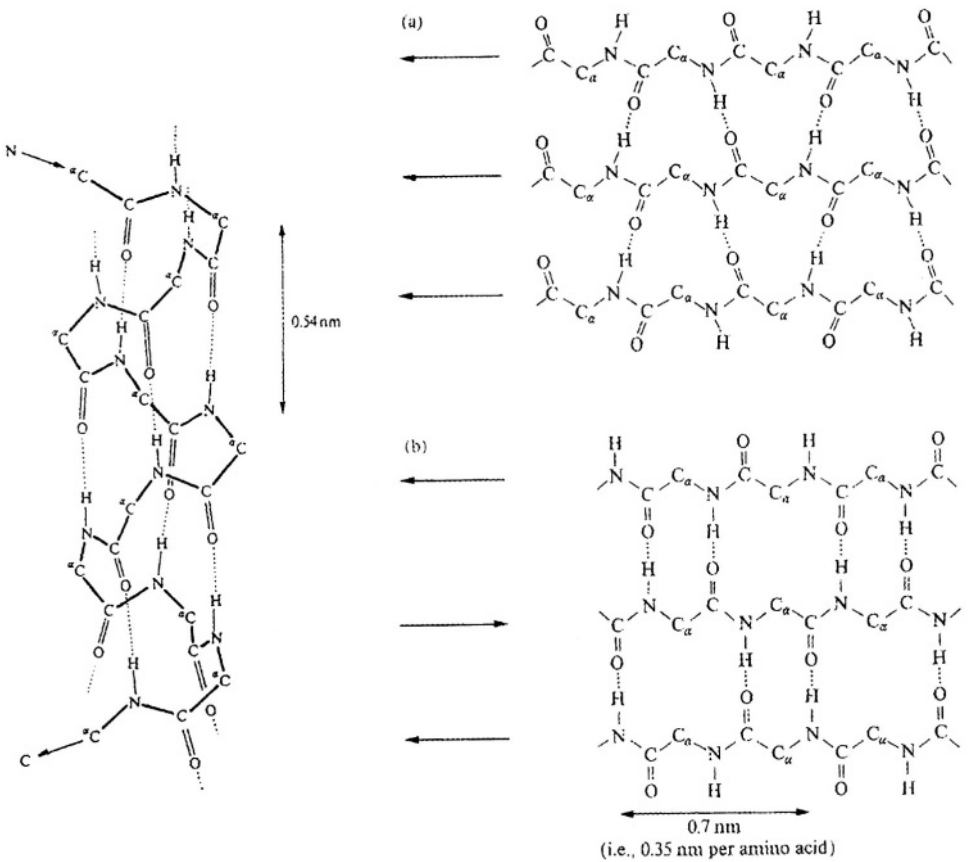


Figure 2. Secondary structure of proteins. Left figure shows the α-helix structure. Right figure shows (a) the parallel and (b) the antiparallel β-pleated sheet structure.

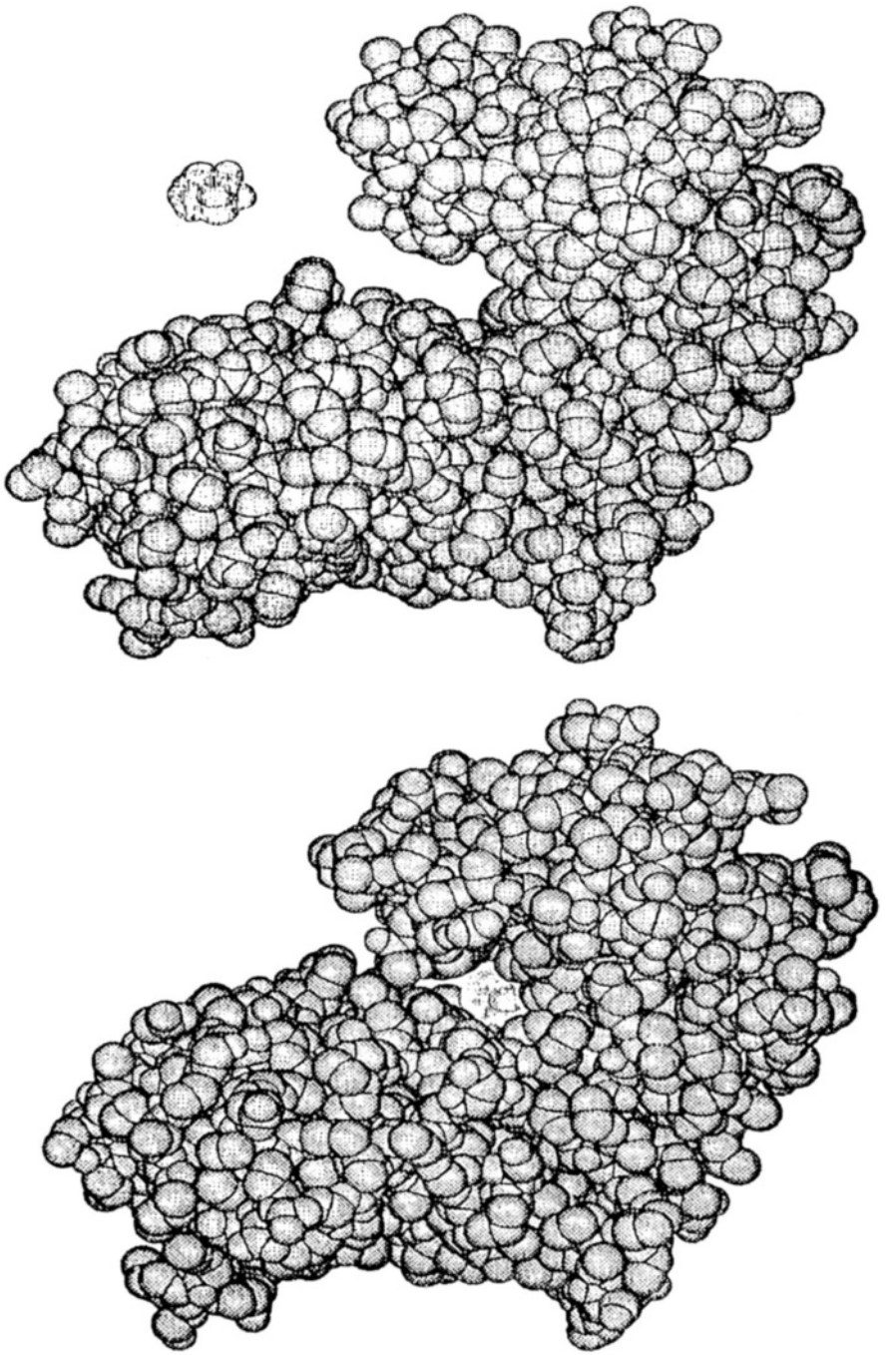


Figure 3. Three-dimensional structure of yeast hexokinase. Top figure shows the molecule of enzyme and a molecule of D-glucose. The bottom figure shows the Michaelis complex enzyme–glucose. Upon binding of glucose, the enzyme closes around substrate (Courtesy of Dr. Thomas A. Steitz).

and rapidly changing structure. However, this is not the case because a very important factor has not been discussed so far, namely, the *cooperativity,* which coordinates the many weak noncovalent interactions. Elegant theories of cooperative processes in proteins have been developed but not considered here (Poland & Scheraga, 1970). The essence of cooperativity in proteins is simple: when a single noncovalent interaction occurs, a second occurs more readily than the first, a third more readily than the second, etc. In terms of protein structure, the cooperative nature of the noncovalent structure restricts protein to a very limited number of structures which are thermodynamically more stable than the others (Brändén & Tooze, 1999). Thus, the protein polypeptide chain forms predominantly two main types of local structures in proteins, that are termed the secondary structures: the *α-helix* and the *β-pleated sheet* structure (Cantor & Schimmel, 1980).

The α-helix is a spring-like structure with residues three spaces apart on chain joined by a hydrogen bond between a peptide oxygen and a nitrogen proton. A right-handed α-helical structure is shown schematically in Fig. 2. Note that the hydrogen bonds are approximately parallel to the axis of the helix; 3.6 amino acids are in each turn of the α-helix.

Another important secondary structure is the β-pleated sheet, which is again stabilized by hydrogen bonds between peptide linkages. A parallel β-pleated sheet forms when the chains are aligned in the same direction; when the chains alternate in direction, an antiparallel β-pleated sheet is formed (Fig. 2).

The tertiary structure of a protein is the complete three-dimensional structure of the polypeptide chain. If multiple polypeptide chains are present, the arrangement of their polypeptide chains with respect to each other is the quaternary structure; in such cases, enzymes are polymers composed of two or more subunits (Table 3). In order to illustrate the tertiary structure of a protein, the three-dimensional structure of the glycolytic enzyme hexokinase is shown in Fig. 3 with the aid of a space-filling model. Hexokinase catalyzes the phosphorylation of D-glucose with ATP. The groove in the middle of the structure is where the substrates bind (Cantor & Schimmel, 1980).

1.2 THE ACTIVE SITE

The binding of substrate to an enzyme is a necessary feature of all enzymatic reactions. However, most enzymes other than hydrolytic ones catalyze bisubstrate reactions, with two substrates and one or more products of reaction. True monosubstrate reactions are rare and trisubstrate reactions are less numerous than the bisubstrate reactions (IUBMB, 1992). One substrate is always a molecule of a metabolite, while the other reactant may be another metabolite, a molecule of water, a metal ion, etc.; in many cases, however, the other reactant is a coenzyme (Zubay, 1988; Walsh, 1998). In addition to substrates, an enzyme may bind specifically the molecules of inhibitors, activators, allosteric activators or inhibitors, etc., at or near the active site, as well as at regions distant from the same.

Table 3. Molecular weight of yeast enzymes, their substrates and coenzymes

Enzyme		Subunits	Substrate		Coenzyme	
Invertase	270.000	1	Sucrose	342	—	
Hexokinase	102.000	2	D-Glucose	180	ATP	509
Alcohol dehydrogenase	144.000	4	Ethanol	46	NAD^+	663

There is a large difference in size between the enzymes and their substrates and coenzymes, usually 2–3 orders of magnitude. The enzymes are large protein molecules, while substrates, coenzymes, and effectors are usually low molecular organic substances, or metal ions. Protons are the most important effectors in enzyme catalysis (Table 3).

Coenzymes play a specific role in enzyme catalysis in a sense that they enable the enzyme to catalyze reactions that are outside the chemical reactivity of protein's amino acids alone. Table 4 lists the structures of principal coenzymes (Zubay, 1988; Walsh, 1998).

The noncovalent interactions involved in the stabilization of the enzyme–substrate complex are the same as those involved in the stabilization of the protein structure. Many interactions are involved, but the strength of the interaction between enzyme and substrate varies considerably, although the equilibrium dissociation constants generally fall into the micromolar and millimolar range. In all cases studied so far, very specific binding sites for substrates are found on enzymes; these are called the *active sites.*

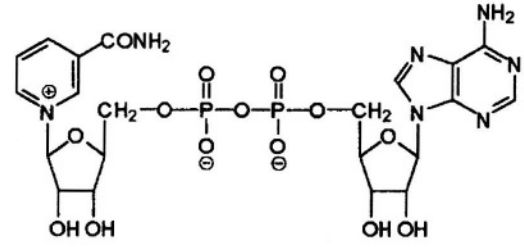
Since the difference in size between an enzyme and its substrates is large, the active site is a relatively small, topologically defined region on an enzyme responsible for the binding of substrates, coenzymes, metal ions, and protons that directly participate in the chemical transformation catalyzed by an enzyme. Active site need not be part of the same protein subunit of a multi-subunit enzyme complex. The active site is generally a pocket or groove on the surface of an enzyme molecule, with a structure very closely complementary to the substrate: hydrogen bonds form between the substrate and protein backbone or side chains, nonpolar parts of the substrate fit with nonpolar parts of the protein and favorable interactions occur between electrically charged parts of the molecules.

The space-filling model of the free enzyme and an enzyme–substrate complex is shown for hexokinase in Fig. 3. Note that the conformation of hexokinase changes markedly upon binding of its substrate glucose.

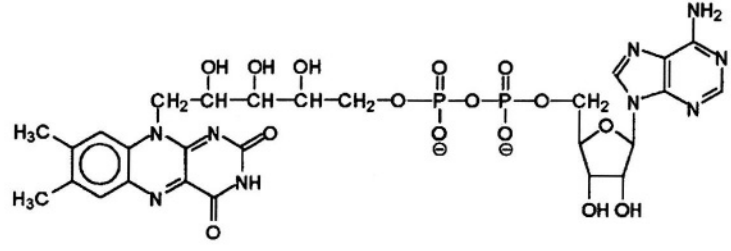
All enzyme-catalyzed reactions are taking place while the reactants are adsorbed on the surface of enzyme in the active site. Therefore, the concept of the active site is probably the most important kinetic and mechanistic concept in enzymology and enzyme kinetics. This concept provides a fundamental difference between the common chemical and the enzymatic reactions; chemical reactions usually proceed via collision complexes, whereas enzymatic reactions proceed via adsorption complexes (Fersht, 1999).

Table 4. The structures of principal coenzymes

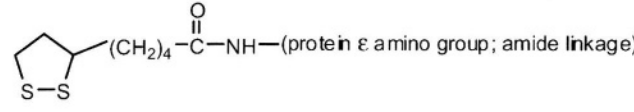
Nicotinamide adenine dinucleotide



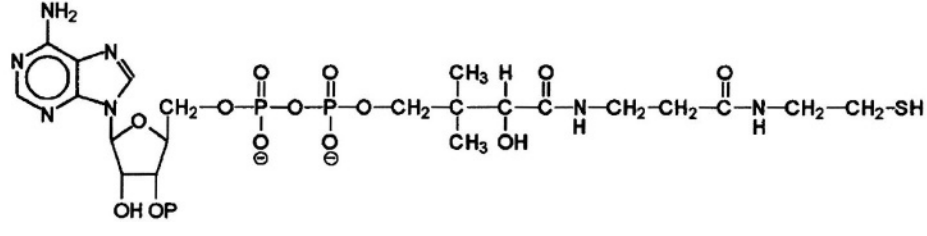
Flavine adenine dinucleotide



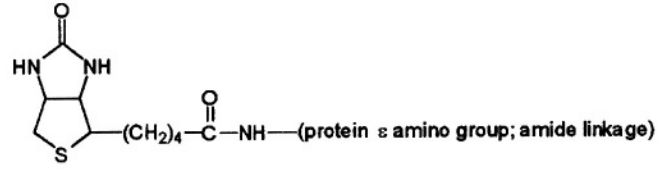
Lipoic acid



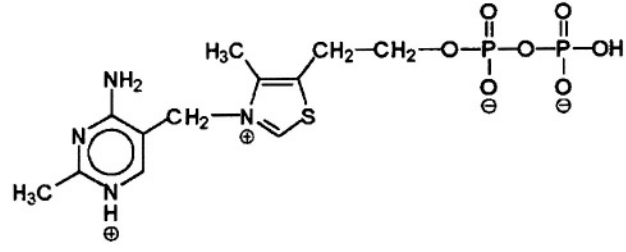
Coenzyme A



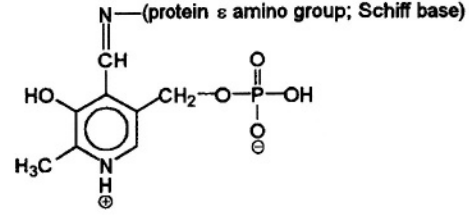
Biotin



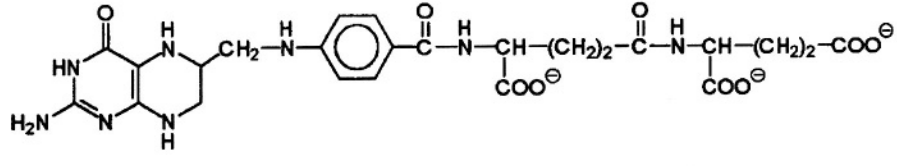
Thiamine diphosphate



Pyridoxal phosphate



Tetrahydrofolic acid



REFERENCES

Brändén, C.-I. & Tooze, J. (1999) *Introduction to protein structure*, 2nd ed., Garland Publishing, Hamden, CT.

Cantor, C.R. & Schimmel, P.R. (1980) *Biophysical chemistry*, Parts I, II and III, Freeman, San Francisco.

Cleland, W.W. & Kreevoy, M.M. (1994) *Science 264*, 1887–1890.

Fersht, A. (1999) *Structure and mechanism in protein science,* W.H. Freeman, New York.

Hammes, G.G. (1982) *Enzyme catalysis and regulation*, Academic Press, New York.

IUBMB (1992) *Enzyme Nomenclature*, International Union of Biochemistry and Molecular Biology, Academic Press, London.

Lodish, H., Darnell, J., Beck, A., Zipursky, L., Mitsudira, P., & Baltimore, D. (1999) *Molecular cell biology*, W.H. Freeman, New York.

Poland, D. & Scheraga, H.A. (1970) *Theory of helix–coil transitions in biopolymers*, Academic Press, New York.

Price, N.C. (1996) *Proteins Labfax*, Bios Scientific Publishers, Oxford.

Walsh, C. (1998) *Enzymatic reactions and mechanisms*, W.H. Freeman, San Francisco.

Zubay, G. (1988) *Biochemistry*, 2nd ed., MacMillan, New York.

Chapter 2

Chemical Kinetics

Chemical kinetics is a branch of physical chemistry devoted to the laws governing chemical processes in time. Thus, chemical kinetics deals mainly with how to find the dependence of the rate of chemical reactions on the concentration of the reactants by using the fundamental postulates of physical chemistry (Panchenkov & Lebedev, 1976; Hammes, 1978; Moore & Pearson, 1982; Fierke & Hammes, 1995; Atkins & de Paula, 2002). Enzyme kinetics is the chemical kinetics of enzyme-catalyzed reactions.

2.1 MOLECULARITY AND ORDER OF REACTION

A chemical reaction can be classified according to its *molecularity* or according to its *order*. The molecularity defines the number of molecules that are altered in the reaction. With the aid of the activated complex, it is possible to obtain a more precise definition of the molecularity of reaction: molecularity of reaction is equal to the number of molecules in the activated complex. It is clear that the molecularity of reaction must be an integer and, in fact, it was found that it is 1, 2, or seldom 3 (Jencks, 1969).

Thus, the reactions are given as follows:

$$\begin{array}{ll} A \rightarrow P & \text{unimolecular} \\ A + B \rightarrow P & \text{bimolecular} \\ A + B + C \rightarrow P & \text{termolecular} \end{array} \qquad (2.1)$$

Reactions of higher molecularity are extremely rare, if they occur at all.

The *order of reaction* is a mathematical description of the kinetics of reaction and defines how many concentration terms must be multiplied together to get an expression for the rate of reaction, that is, to obtain a *rate law*.

Rate laws of chemical reactions have a great practical value, as they provide a concise expression for the progress of reaction and, also, serve for the calculation of time and the yield of reaction. Very often, the rate laws provide an insight into the mechanism of chemical reactions.

For a simple reaction that consists of a single step, or for each step in a complex reaction, the order is usually the same as the molecularity. However, many chemical reactions consist of sequences of unimolecular and bimolecular steps and the molecularity of the complete reaction need not be the same as its order. Complex chemical reactions often have no meaningful order, as the rate often cannot be expressed as a product of concentration terms. This is almost universal in enzyme kinetics, and even the simplest enzyme-catalyzed reactions

do not have simple orders. Nevertheless, the concept of order is important for understanding enzyme kinetics, because the individual steps in enzyme-catalyzed reactions nearly always do have simple orders, usually being first or second order (Jencks, 1969). It is important to remember that the order of reaction refers to the experimental rate equation, whereas the molecularity refers to the theoretical mechanism.

2.1.1 First-order Reactions

For a first-order irreversible reaction, $A \xrightarrow{k} P$, the rate law is

$$v = \frac{dP}{dt} = -\frac{dA}{dt} = kA \tag{2.2}$$

where A and P are the concentrations of reactant and product, respectively, at any time t, and k is the *first-order rate constant* (dimension: s^{-1}).

The first two equality signs in Eq. (2.2) show that the rate (v) is defined in terms of appearance of product or disappearance of substrate (Plowman, 1972; Cornish-Bowden, 1995). The next equality specifies that this is a first-order reaction, because it states that the rate is proportional to the concentration of reactant A. Finally, it is clear that $A_0 = A + P$.

Equation (2.2) can be readily integrated by separating the two variables P and t.

$$\int \frac{dP}{A_0 - P} = \int k\,dt \tag{2.3}$$

Therefore,

$$-\ln(A_0 - P) = kt + \alpha \quad \text{where } \alpha = -\ln A_0 \tag{2.4}$$

Thus,

$$\ln\left(\frac{A_0 - P}{A_0}\right) = -kt \tag{2.5}$$

or

$$\ln\left(\frac{A_0}{A_0 - P}\right) = kt \tag{2.6}$$

Equation (2.5) can be also rearranged to give

$$P = A_0(1 - e^{-kt}) \tag{2.7}$$

$$A = A_0 e^{-kt} \tag{2.8}$$

It is important to note that the constant of integration α was included in this derivation, evaluated and found to be non-zero; when the kinetic equations are integrated, the constants of integration are rarely found to be zero.

2.1.2 Second-order Reactions

The commonest type of bimolecular reactions has the form: $A + B \xrightarrow{k} P + Q$ (Plowman, 1972; Cornish-Bowden, 1995). The rate law for this irreversible reaction is given by

$$v = \frac{dP}{dt} = kAB = k(A_0 - P)(B_0 - P) \tag{2.9}$$

in which k is now a *second-order rate constant* (dimension: $M^{-1}\ s^{-1}$). Again, integration is achieved by separating the two variables P and t:

$$\int \frac{dP}{(A_0 - P)(B_0 - P)} = \int k\,dt \tag{2.10}$$

$$\int \frac{dP}{A_0 - P} - \int \frac{dP}{B_0 - P} = \int (B_0 - A_0)k\,dt \tag{2.11}$$

Hence,

$$-\ln(A_0 - P) + \ln(B_0 - P) = (B_0 - A_0)kt + \alpha \tag{2.12}$$

where the constant of integration $\alpha = \ln(B_0/A_0)$.

Thus, we have

$$\ln\left[\frac{A_0(B_0 - P)}{B_0(A_0 - P)}\right] = (B_0 - A_0)kt \tag{2.13}$$

2.1.3 Pseudo-first-order Reactions

A special case arises when A_0 is very small compared with B_0; then, P must be also very small compared with B_0 at all times. So $(B_0 - A_0)$ and $(B_0 - P)$ can both be approximated with B_0, which simplifies Eq. (2.13) into

$$\ln\left(\frac{A_0 - P}{A_0}\right) = -B_0kt \tag{2.14}$$

Equation (2.14) is exactly of the same shape as Eq. (2.5) for a first-order reaction. This type of reaction is known as *pseudo-first-order reaction,* and kB_0 is a *pseudo-first-order rate constant* (dimension: s^{-1}).

2.1.4 Third-order Reactions

Termolecular reactions, such as $A + B + C \rightarrow P$ do not usually consist of a single trimolecular step, and consequently are not usually third order. Instead, the reaction is likely to proceed via two or more *elementary steps,* such as $A + B \rightarrow X$, followed by $X + C \rightarrow P$. If one step in such a reaction is much slower than the others, the rate of the complete reaction is equal to the rate of the slow

step, which is accordingly known as the *rate-limiting* step. If there is no clearly defined rate-limiting step, the rate equation is likely to be complex and to have no integral order (Moore, 1962; Jencks, 1969).

2.1.5 Zero-order Reactions

Some reactions are observed to be of *zero order,* that is, the rate is found to be constant, independent of the concentration of reactants. If a reaction is of zero order with respect to only one reactant, this may simply mean that the reactant enters the reaction after the rate-limiting step. However, some reactions are of zero order overall, that is, independent of all reactant concentrations; such reactions are invariably catalyzed reactions and occur if every reactant is present in such a large excess that the full potential of the catalyst is realized. Zero-order kinetics occur in enzyme-catalyzed reactions when the substrate concentrations are saturating.

2.2 DETERMINATION OF THE ORDER OF REACTION

The simplest way to determine the order of reaction is to measure the rate v at different concentrations of A of the reactant. Then, a plot of $\log v$ against $\log A$ gives a straight line with the slope equal to the order of reaction. If there are more reactants, it is useful to know the order with respect to each reactant. This can be found by altering the concentration of each reactant separately, keeping the other concentrations constant; then, the slope of the line will be equal to the order with respect to the variable reactant. For example, if the reaction is second-order in A and first-order in B:

$$v = kA^2B \tag{2.15}$$

then

$$\log v = \log k + 2\log A + \log B \tag{2.16}$$

Hence, a plot of $\log v$ against $\log A$ (with B held constant) will have a slope of 2, and a plot of $\log v$ against $\log B$ (with A held constant) will have a slope of 1. It is important to realize that if the rates are determined from the slopes of the progress curve (i.e., a plot of concentration against time), the concentration of all reactants will change. Therefore, if valid results are to be obtained, the constant reactant must be in large excess at the start of the reaction, so that the changes in its concentration are insignificant.

2.3 DETERMINATION OF RATE CONSTANTS

Once the order of reaction is determined, one can proceed with the determination of the rate constants of reaction (Moore, 1962; Jencks, 1969). First-order rate constants are determined from the linear plot of Eq. (2.6), and second-order rate constants from the plot of Eq. (2.13) (Fig. 1).

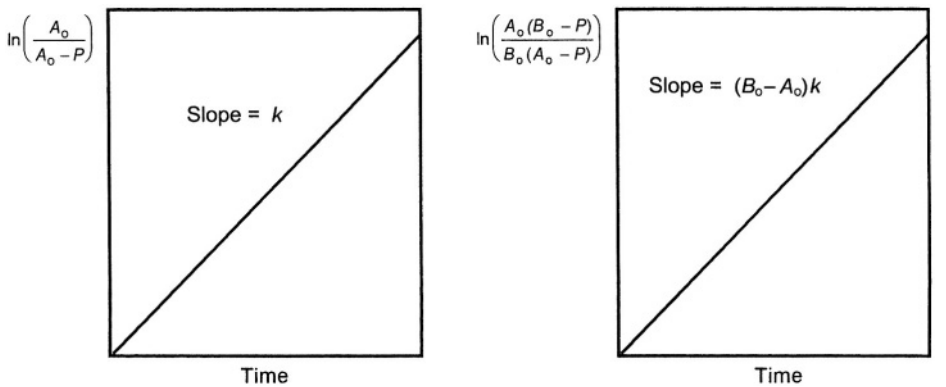


Figure 1. Graphical determination of the first-order (left) and the second-order rate constant (right), according to Eqs. (2.6) and (2.13).

Many reactions are first order in each reactant, and in these cases it is often possible to carry out the reaction under pseudo-first-order conditions overall, by keeping every reactant except one in large excess. Thus, in many practical situations, the problem of determining a rate constant can be reduced to the problem of determining a first-order rate constant.

Guggenheim (1926) pointed out a major objection to plots in Fig. 1, that depend heavily on an accurate determination of A_0 or P_∞. Therefore, he proposed an alternative, but hazardous, method for the determination of pseudo-first-order rate constants for the reaction:

$$\mathrm{A} + \mathrm{B} \xrightarrow{k} \mathrm{P} \tag{2.17}$$

where B_0 is very large compared to A_0, and remains nearly constant during the reaction. The concentration of the ensuing product P is measured at small and equal time intervals during the course of reaction; thus P_t represents the concentration of P at time t, $P_{t+\Delta}$ the concentration of P at time $(t + \Delta)$, and Δ an arbitrarily chosen small time interval.

A simple algebraic derivation, similar to the one shown in Section 2.1, will afford the *Guggenheim equation*:

$$\ln(P_{t+\Delta} - P_t) = \ln \alpha P_\infty - kB_0 t \tag{2.18}$$

where

$$\alpha = 1 - e^{-kB_0\Delta} \tag{2.19}$$

Figure 2 shows the graphical presentation of Eq. (2.18). Thus, in order to produce the Guggenheim plot, one has to measure the concentrations of the product at equal time intervals along the reaction progress curve and plot the data according to Eq. (2.18).

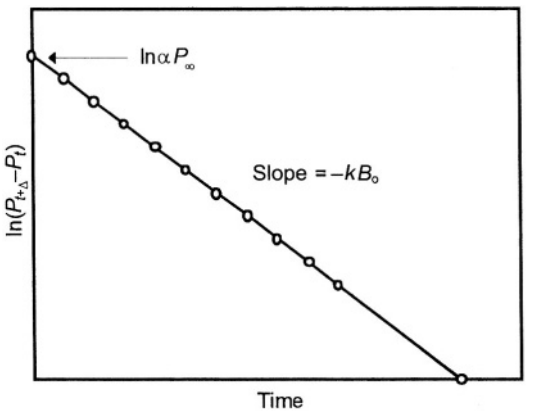


Figure 2. The Guggenheim plot (Eq. (2.18)).

This plot has the major advantage that it does not require an estimate of P_∞. The Guggenheim plot is hazardous, as it is insensitive to deviations from the first-order kinetics, that is, it can give an apparently good straight line even if the first-order kinetics are not accurately obeyed. One can only have confidence in a reaction being truly first order if the points fit equally well along the straight line of a first-order plot all the way to at least 95% of the complete reaction.

Another test for the first-order behavior is the determination of successive halftimes. The halftime ($t_{1/2}$) is the time taken for completion of half the remaining reaction from any starting point along the reaction path. If the halftimes

$$t_{1/2} = \frac{\ln 2}{k} \tag{2.20}$$

calculated from various starting points along the reaction record remain constant, the reaction is likely to be the first order (Jencks, 1969; Gutfreund, 1972). Nevertheless, the Guggenheim plot should not be used to determine the order of reaction, which should be established independently (Swinbourne, 1960).

2.4 REVERSIBLE REACTIONS

Many reactions are fully reversible, and therefore, the reverse reaction must be introduced in the net rate equation (Plowman, 1972; Cornish-Bowden, 1995):

$$\mathrm{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{P} \tag{2.21}$$

In this case,

$$v = \frac{\mathrm{d}P}{\mathrm{d}t} = k_1(A_0 - P) - k_2P = k_1A_0 - (k_1 + k_2)P \tag{2.22}$$

This differential equation can be solved in the same way as Eq. (2.2):

$$\int \frac{dP}{k_1A_0 - (k_1 + k_2)P} = \int dt \tag{2.23}$$

$$\frac{\ln[k_1A_0 - (k_1 + k_2)P]}{-(k_1 + k_2)} = t + \alpha \tag{2.24}$$

Setting $P = 0$, when $t = 0$, gives a constant of integration, $\alpha = -\ln(k_1A_0)/(k_1 + k_2)$, and so,

$$\ln\left[\frac{k_1A_0 - (k_1 + k_2)P}{k_1A_0}\right] = -(k_1 + k_2)t \tag{2.25}$$

Taking exponentials of both sides, we obtain

$$\frac{k_1A_0 - (k_1 + k_2)P}{k_1A_0} = e^{-X} \tag{2.26}$$

where $X = (k_1 + k_2)t$.

Equation (2.26) can be rearranged to show the change in the concentration of P with respect to time:

$$P = \frac{k_1A_0(1 - e^{-X})}{k_1 + k_2} = P_\infty(1 - e^{-X}) \tag{2.27}$$

where $P_\infty = k_1A_0/(k_1 + k_2)$ is the value of P after infinite time, that is, at equilibrium. This value follows from the fact that the exponential term approaches zero as the time interval becomes large.

Rate constants for reversible reactions cannot be determined as described for irreversible reactions. In order to determine the magnitude of rate constants in reaction (2.21), one must rearrange Eq. (2.27), as follows:

$$P_\infty - P = P_\infty e^{-X} \tag{2.28}$$

or, in the logarithmic form:

$$\ln(P_\infty - P) = \ln P_\infty - (k_1 + k_2)t \tag{2.29}$$

Thus, a plot of $\ln(P_\infty - P)$ versus t gives a straight line of slope $-(k_1 + k_2)$. As k_1/k_2 is equal to the equilibrium constant, which can be estimated independently, the values of the individual rate constants k_1 and k_2 can be calculated from the two combinations.

The differential method of Guggenheim is also applicable to reversible reactions. For example, reaction (2.21) can be treated with the Guggenheim method to obtain a Guggenheim equation for a reversible monomolecular reaction:

$$\ln(P_{t+\Delta} - P_t) = \ln P_\infty + \ln\left(1 - e^{-\{k_1+k_2\}\Delta}\right) - (k_1 + k_2)t \tag{2.30}$$

2.5 CONSECUTIVE REACTIONS

In any sequence involving two or more reactions, in order to derive the rate law, separate kinetic equations must be written for each step in the mechanism:

$$\mathrm{A} + \mathrm{B} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{C} \xrightarrow{k_3} \mathrm{D} \tag{2.31}$$

The mechanistic steps of reaction (2.31) have three kinetic equations that serve to define the reaction (Mahler & Cordes, 1966; Plowman, 1972):

$$\frac{dA}{dt} = \frac{dB}{dt} = k_2C - k_1AB \tag{2.32}$$

$$\frac{dC}{dt} = k_1AB - (k_2 + k_3)C \tag{2.33}$$

$$\frac{dD}{dt} = k_3C \tag{2.34}$$

plus the mass conservation equations, such as

$$A + B + C + D = A_0 + B_0 \text{ or } A - B = A_0 - B_0 \tag{2.35}$$

If all the first-order and pseudo-first-order rate constants, k_1A, k_1B, k_2, and k_3 are of the same order of magnitude, the explicit solution is not possible and the computer is the simplest means to arrive at a solution (Fig. 3).

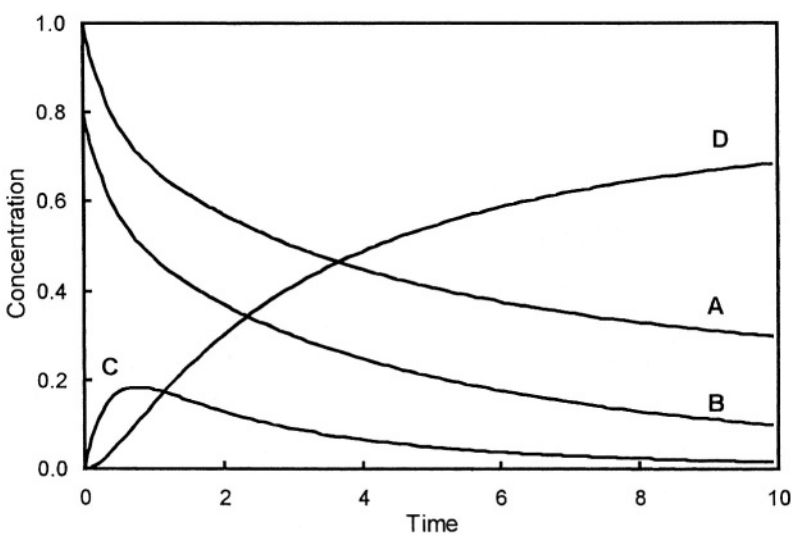


Figure 3. Progress curve for reaction $\mathrm{A} + \mathrm{B} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{C} \xrightarrow{k_3} \mathrm{D}$, with $A = k_1 = k_2 = k_3 = 1$, and $B = 0.8$. Note that, $k_1A \cong k_2B \cong k_2 \cong k_3$.

Although the explicit solution is not possible, several limiting cases provide solutions that are capable of simple interpretation. If k_3 is much smaller than k_2, the rate of the product formation, d*D*/d*t,* is governed by the unimolecular breakdown of C, which in turn is formed rapidly and reversibly from A plus B; kinetically the reaction is first order in either reactant:

$$\frac{dD}{dt} = \left(\frac{k_1 k_3}{k_2}\right) AB \tag{2.36}$$

If $k_3 >> k_1 \cong k_2$, the intermediate C is converted to D as rapidly as it is formed and the reaction is again first order in either reactant, despite the difference in the rate-limiting step:

$$\frac{dD}{dt} = k_1 AB \tag{2.37}$$

A special case arises when $dC/dt \approx 0$, which is called a steady state or a stationary state. This condition is of special importance in enzyme kinetics, and it is treated in detail in Chapter 3.

2.6 INFLUENCE OF TEMPERATURE ON RATE CONSTANTS

2.6.1 Arrhenius Equation

From the earliest studies of reaction rates, it has become evident that they are profoundly influenced by temperature. Harcourt (1867) established a well-known rule of thumb that the rates of many reactions approximately doubled for each 10° rise in temperature. The early studies of van't Hoff (1884) and Arrhenius (1889) form the starting point for all modern theories of the temperature dependence of equilibrium and rate constants. Van't Hoff and Arrhenius attempted to find quantitative relationships between the temperature and equilibrium and rate constants by comparing kinetic observations with the known properties of these constants.

Any equilibrium constant *K* varies with the absolute temperature *T* in accordance with the *van't Hoff law*:

$$\frac{d \ln K}{dT} = \frac{\Delta H^0}{RT^2} \tag{2.38}$$

where *R* is the gas constant and ΔH^0 is the standard enthalpy change in the reaction. Arrhenius has shown that an empirical relationship, an *Arrhenius equation,* is also valid over a moderate range of temperature

$$\frac{d \ln k}{dT} = \frac{E_a}{RT^2} \tag{2.39}$$

where *k* is the rate constant of reaction, and E_a the *activation energy.*

Integration with respect to T gives

$$\ln k = \ln A - \frac{E_a}{RT} \tag{2.40}$$

where ln A is the constant of integration. This form of Arrhenius equation is the most convenient for graphical presentation, as it shows that a plot of ln k against 1/T is a straight line of slope $-E_a/R$; an Arrhenius plot is only approximately linear (Fig. 4).

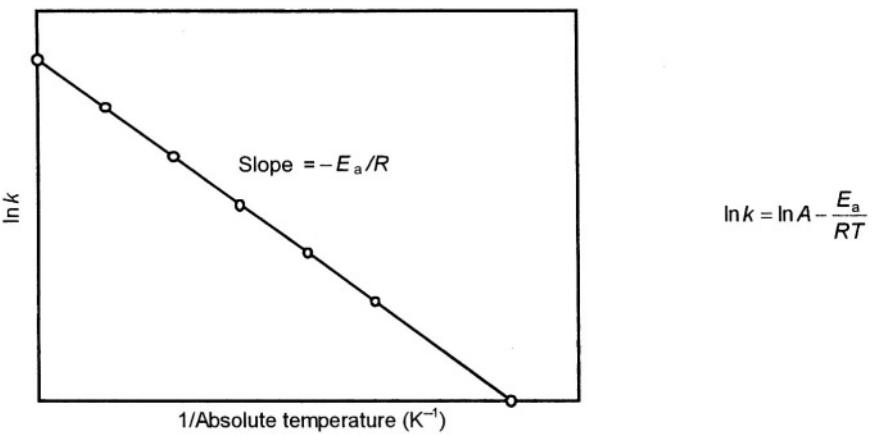


Figure 4. Graphical presentation of the Arrhenius equation (2.39).

In order to assess properly the meaning of the activation energy, Eq. (2.40) must be written in the exponential form:

$$k = Ae^{-E_a/RT} \tag{2.41}$$

The exponential term is often called a Boltzmann term, because, according to Boltzmann's theory, the distribution of energies among molecules, the number of molecules in a mixture that have an energy in excess of E_a, is proportional to $e^{-E_a/RT}$. We can, therefore, interpret the Arrhenius equation to mean that molecules can take part in a reaction only if their energy exceeds some threshold value, the activation energy. In this interpretation, the constant A is equal to the frequency of collisions of molecules, Z, at least for bimolecular reactions.

However, in addition to colliding with sufficient energy, molecules must also be oriented correctly if they are to react. The factor P is then taken into account, in order to measure the probability that the correct orientation will be adopted spontaneously, so that Eq. (2.41) must be modified into

$$k = PZe^{-E_a/RT} \tag{2.42}$$

The factor P is a measure of the probability that the correct orientation for the reaction is adopted.

2.6.2 Transition-State Theory

Equation (2.42) is now in accordance with modern theories of reaction rates, but for most purposes, it is better to approach the same problem from a different point of view, the *transition-state theory,* which is derived largely from the work of Eyring (1935). Transition-state theory is a theoretical description of absolute reaction rates in terms of the rate-limiting formation of an activated complex during the course of a reaction.

The transition state of a unimolecular reaction occurs at the peak in the reaction coordinate diagram, in which the energy of the reactants is plotted as the reaction proceeds (Fig. 5). In the transition state, chemical bonds are in the process of being made and broken. In contrast, intermediates, whose bonds are fully formed, occupy the troughs in the diagram. A simple way of deriving the rate law of reaction is to consider that the transition state and the ground state are in thermodynamic equilibrium, so that the concentration of the transition state may be calculated from the difference in their energies. The overall reaction rate is then obtained by multiplying the concentration of the transition state by the rate constant for its decomposition.

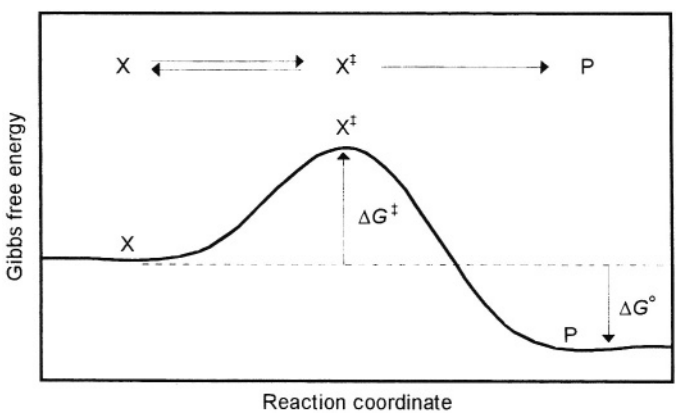


Figure 5. Reaction profile for a monomolecular reaction according to transition-state theory. $\Delta G^{\ddagger}$ is the Gibbs free energy of activation, while ΔG° is the change in Gibbs free energy for the overall reaction.

Transition-state theory can also be expressed in thermodynamic terms (Panchenkov & Lebedev, 1976; Moore & Pearson, 1982; Atkins & de Paula, 2002). Suppose that the difference in the Gibbs free energy between the transition state, $\Delta X^{\ddagger}$, and the ground state, X, is equal $\Delta G^{\ddagger}$. Then, according to the equilibrium thermodynamics, it is derived that

$$[X^{\ddagger}] = [X]e^{-\Delta G^{\ddagger}/RT} \tag{2.43}$$

The frequency at which the transition state decomposes is the same as the vibrational frequency ν of the bond that is breaking. This frequency is obtained

from the equivalence of the energies of an excited oscillator calculated from quantum theory ($E = h\nu$) and classical physics ($E = k_B T$), that is,

$$\nu = \frac{k_B T}{h} \tag{2.44}$$

where k_B and h are the Boltzmann and the Planck's constant, respectively.

The rate of transformation of X is thus given by

$$-\frac{d[X]}{dt} = \nu[X^{\ddagger}] = [X]\left(\frac{k_B T}{h}\right)\left(e^{-\Delta G^{\ddagger}/RT}\right) \tag{2.45}$$

The first-order rate constant for the decomposition of X is given by

$$k = \left(\frac{k_B T}{h}\right)\left(e^{-\Delta G^{\ddagger}/RT}\right) \tag{2.46}$$

A more rigorous approach includes a factor known as the transmission coefficient, but it is generally close to unity and so may be ignored (Panchenkov & Lebedev, 1976).

The free energy of activation, $\Delta G^{\ddagger}$, may be separated into enthalpic and entropic terms by using another relationship from equilibrium thermodynamics:

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \tag{2.47}$$

where $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the enthalpy and entropy of activation, respectively. Now, the rate constant becomes

$$k = \left(\frac{k_B T}{h}\right)\left(e^{(\Delta S^{\ddagger}/R)-(\Delta H^{\ddagger}/RT)}\right) \tag{2.48}$$

Taking logarithms, we obtain

$$\ln k = \ln\left(\frac{k_B T}{h}\right) + \frac{\Delta S^{\ddagger}}{R} - \frac{\Delta H^{\ddagger}}{RT} \tag{2.49}$$

and differentiating

$$\frac{d \ln k}{dT} = \frac{\Delta H^{\ddagger} + RT}{RT^2} \tag{2.50}$$

Comparing this equation with the Arrhenius equation (2.39), one can see that the activation energy E_a is equal $\Delta H^{\ddagger} + RT$, and is not strictly independent of temperature. For this reason, the Arrhenius plot looks linear in the accessible temperature range, but is actually curved and asymptotically approaches infinity at high temperatures. The apparent intercept thus varies with the temperature range of the data, so accurate conclusions about entropy of activation cannot be made.

Dividing Eq. (2.48) by T and taking natural logarithms, we obtain

$$\ln\left(\frac{k}{T}\right) = \ln\left(\frac{k_B}{h}\right) + \left(\frac{\Delta S^{\ddagger}}{R}\right) - \left(\frac{\Delta H^{\ddagger}}{RT}\right) \tag{2.51}$$

Equation (2.51) is a linear relationship, because one assumes that both $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are not the functions of T (Fig. 6).

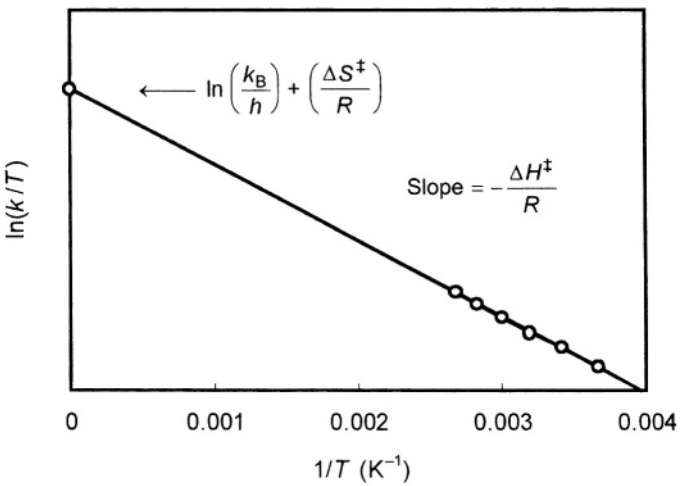


Figure 6. The Eyring plot: graphical presentation of Eq. (2.51). The data points are shown in the temperature range 0–100°C; $[\ln(k_B/h) = 23.76]$.

Thus, one can in practice, avoid the slight source of error arising from the curvature of the Arrhenius equation by simply plotting $\ln(RT/k)$ against $1/T$ [or $\ln(k/T)$ against $1/T$], instead of the usual Arrhenius plot of $\ln(k)$ against $1/T$. From the graph in Figure 6, one can easily determine $\Delta H^{\ddagger}$, the enthalpy of activation, and $\Delta S^{\ddagger}$, the entropy of activation. The Gibbs free energy of activation, $\Delta G^{\ddagger}$, can be obtained from the rate constant at any temperature by use of Eq. (2.46), or from Eq. (2.47) at a constant temperature. The Eyring plot in Fig. 6 is much superior to the Arrhenius plot, and one should discourage the use of the latter in favor of the former (Cleland & Northrop, 1999).

The reader should be aware of the fact that the much more rigorous treatment of the transition-state thermodynamics of the unimolecular and bimolecular reactions is found in textbooks of physical chemistry (Panchenkov & Lebedev, 1976; Atkins & de Paula, 2002).

2.7 PROPERTIES OF WATER

All biological processes are either directly or indirectly under the influence of some of the characteristic properties of water. The special properties of water are due to the fact that it retains a relatively ordered structure as a liquid (Zubay, 1988).

The molecule of water is composed of two hydrogen atoms covalently bonded to an oxygen atom with tetrahedral (sp^3) electron orbital hybridization. As a result, two lobes of the oxygen sp^3 orbital contain pairs of unshared electrons, giving rise to a dipole in the molecule as a whole (Fig. 7). This in turn is related to the structure of individual water molecules which results in strong dipolar

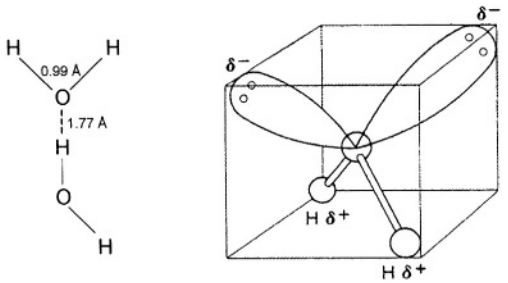


Figure 7. Properties of water molecules. Left: hydrogen bonding in water and in ice. Right: Dipolar properties of water molecule.

interactions. A water molecule interacts with another as shown in Fig. 7, forming an extensive network of hydrogen bonds, in liquid water as well as in ice.

These properties of water are essential for understanding chemical reactions in biological systems. Water represents the major part of the body weight in most living organisms.

2.8 IONIZATION OF ACIDS

Most enzymatic reactions *in vivo* and *in vitro* take place in aqueous solutions, in an environment with a high and practically constant concentration of water molecules (55.5 M) (Streitwieser & Heathcock, 1998). One important property of water is its self-ionization:

$$H_2O + H_2O \underset{}{\overset{K}{\rightleftharpoons}} H_3O^+ + OH^- \tag{2.52}$$

In absolutely pure water, the total concentration of H_3O^+ and OH^- is very low, only 10^{-7} mol/liter. The ion product of the self-dissociation constant, K_W, is defined as

$$K_W = [H_3O^+][OH^-] = 1.0 \times 10^{-14}\ \text{mol}^2\ \text{liter}^{-2}\ (\text{or}\ \text{M}^2) \tag{2.53}$$

This is not a normal equilibrium constant, which includes the concentration of reactants and products. For water, the concentration is 55.5 mol/liter. The equilibrium constant for dissociation, from Eq. (2.52), is therefore

$$K = \frac{(10^{-7})(10^{-7})}{(55.5)(55.5)} = 3.25 \times 10^{-18} \tag{2.54}$$

Note that K is unitless. The relationship between K and K_W is

$$K_W = K(55.5\ \text{M})^2 \tag{2.55}$$

In an aqueous environment, $[H^+]$ varies from about 1 M to about 10^{-14} M. This enormous range of concentrations was reduced by Sørensen (1909) to more manageable proportions by the use of a logarithmic scale:

$$\mathrm{pH} = -\log[\mathrm{H}^+] \tag{2.56}$$

From Eq. (2.53), it follows that the sum of logarithms of the concentrations of H^+ ions and OH^- ions in water is a constant value:

$$\log[\mathrm{H}^+] + \log[\mathrm{OH}^-] = -14 \tag{2.57}$$

2.8.1 Titration of Monobasic Acids

Out of many available definitions of acids and bases, perhaps the best and most accurate was given by Brönsted (1925): "An acid is a species having a tendency to lose a proton, and a base is a species having a tendency to add on a proton."

Strong monobasic acids are fully dissociated in aqueous solutions; thus, hydrochloric acid is fully dissociated into a chloride anion and a proton:

$$\mathrm{HCl} \rightarrow \mathrm{H}^+ + \mathrm{Cl}^- \tag{2.58}$$

This reaction is highly endothermic because a bond is broken and charges are separated; both of these processes require energy. In aqueous solutions, both ions are further solvated by water molecules, such as

$$\mathrm{HCl(aq)} \rightleftharpoons \mathrm{H}^+(\mathrm{aq}) + \mathrm{Cl}^-(\mathrm{aq}) \tag{2.59}$$

The solvation bonds that are formed with the molecules of water are sufficiently strong in the aggregate to compensate for H–Cl bond strength and the electrostatic energy required to separate negative from positive charges.

Therefore, the equilibrium can also be described as a proton transfer from an acid to a base:

$$\mathrm{HCl} + \mathrm{H_2O} \rightleftharpoons \mathrm{H_3O}^+ + \mathrm{Cl}^- \tag{2.60}$$

The equilibrium constant for aqueous HCl is large, about 10^7, and the equilibrium position lies far to the right. We speak of HCl as a strong acid and Cl^- as a weak base; more properly, we should speak of HCl as a strong acid relative to $\mathrm{H_3O}^+$ and Cl^- as a weak base relative to $\mathrm{H_2O}$ (Streitwieser & Heathcock, 1998).

In general, the acidity of an acid HA in water is defined as the equilibrium constant:

$$\mathrm{HA(aq)} \overset{K_a}{\rightleftharpoons} \mathrm{H}^+(\mathrm{aq}) + \mathrm{A}^-(\mathrm{aq}) \tag{2.61}$$

$$K_a = \frac{[\mathrm{H}^+][\mathrm{A}^-]}{[\mathrm{HA}]} \tag{2.62}$$

Note that K_a has the units of mol/liter (or M).

Acidity equilibrium constants vary over a wide range. Acids with $K_a > 1$ are referred to as strong acids; acids with $K_a < 10^{-4}$ are weak acids. The acidity equilibrium constant is usually defined as an *acid dissociation constant* and

expressed as an exponent of 10 in order to accommodate this large range of possible values. The pK_a is defined as

$$pK_a = -\log K_a \tag{2.63}$$

Thus, pK_a of HCl is about –7; the smaller or more negative the pK_a, the stronger is the acid. Taking the logarithm of both sides in Eq. (2.62) and substituting pH for $-\log[H^+]$ and pK_a for $-\log K_a$, we obtain the *Henderson–Hasselbalch equation*:

$$pH = pK_a + \log\left(\frac{[A^-]}{[HA]}\right) \tag{2.64}$$

From this equation, one can easily calculate the pK_a by plotting $\log([A^-]/[HA])$ versus pH; a straight line is obtained with an intercept on the ordinate equal to K_a (Henderson, 1908; Hasselbalch, 1916).

The molecule of water may act as a base, with pK_a of –1.7, but also as an acid with pK_a of 15.7. These values are half of 55 M, that is when half of the water is H_3O^+ or OH^-.

2.8.2 Titration of Polybasic Acids

Dibasic acids, polybasic acids, and proteins, with two or more ionizable groups, give more complex titration curves than the simple monobasic acids (Schulz, 1994). Consider the titration curve of a simple amino acid which has no charge in its side chain. In aqueous solution, a monoamino, monocarboxylic acid is distributed between three species, namely the fully protonated acid, the zwitterion, and the fully dissociated base:

$$[A_T] = [H_2A^+] + [HA^0] + [A^-] \tag{2.65}$$

The superscript in Eq. (2.65) indicates the net charge of each species of the acid. The protons dissociate from the acidic groups according to the following equilibria:

$$H_2A^+ \rightleftharpoons HA^0 + H^+ \quad K_1 = \frac{[HA^0][H^+]}{[H_2A^+]} \tag{2.66}$$

$$HA^0 \rightleftharpoons A^- + H^+ \quad K_2 = \frac{[A^-][H^+]}{[HA^0]} \tag{2.67}$$

From Eqs. (2.66) and (2.67), the following relationships are obtained:

$$[H_2A^+] = \frac{[HA^0][H^+]}{K_1} = \frac{[A^-][H^+]^2}{K_1K_2} \tag{2.68}$$

$$[HA^0] = \frac{K_1[H_2A^+]}{[H^+]} = \frac{[A^-][H^+]}{K_2} \tag{2.69}$$

$$[A^-] = \frac{K_2[HA^0]}{[H^+]} = \frac{K_1K_2[H_2A^+]}{[H^+]^2} \tag{2.70}$$

Thus, Eq. (2.65) can be expressed in terms of $[A_T]$ and any one of the species.

$$[A_T] = [H_2A^+]\left(1 + \frac{K_1}{[H^+]} + \frac{K_1K_2}{[H^+]^2}\right) = [H_2A^+]\phi^+ \tag{2.71}$$

$$[A_T] = [HA^0]\left(1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}\right) = [HA^0]\phi^0 \tag{2.72}$$

$$[A_T] = [A^-]\left(1 + \frac{[H^+]}{K_2} + \frac{[H^+]^2}{K_1K_2}\right) = [A^-]\phi^- \tag{2.73}$$

The expressions containing the concentration of protons are abbreviated by ϕ, and are called the Michaelis pH functions (Michaelis, 1922). The Michaelis pH functions show directly the relative concentration of each acidic group in the mixture, since

$$\frac{1}{\phi^+} = \frac{[H_2A^+]}{[A_T]}; \quad \frac{1}{\phi^0} = \frac{[HA^0]}{[A_T]}; \quad \frac{1}{\phi^-} = \frac{[A^-]}{[A_T]}; \tag{2.74}$$

A typical amino acid, without a charge in its side chain, has a $pK_a \approx 2$ for the carboxylic group, and $pK_a \approx 9$ for the α-amino group. Now, with the aid of Eqs. (2.74), a titration curve for a typical amino acid can be constructed, assuming that $pK_1 = 2$, and $pK_2 = 9$ (Fig. 8). Figure 8 shows that it is convenient to express the Michaelis ϕ functions in the reciprocal form; from this, it is evident that the zwitterion form predominates in the pH region between pK_1 and pK_2.

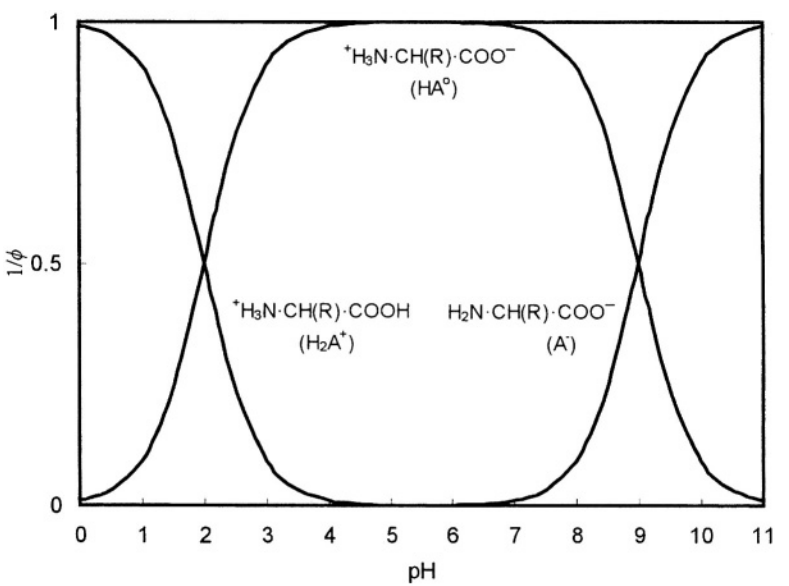


Figure 8. Change of the Michaelis ϕ functions with pH for an amino acid with $pK_a = 2$ for a carboxylic group, and $pK_a = 9$ for the α-amino group.

2.9 DIMENSIONS AND DIMENSIONAL ANALYSIS

Dimensions are the currency of science. Therefore, it is of utmost importance, especially in enzyme kinetics, to take care of dimensions and to perform the dimensional analysis of kinetic equations and expressions in order to avoid the algebraic mistakes and to check the derivation of rate equations.

The dimensions are referring to the three major properties of the universe: the matter, the time, and the energy. Earlier, a c.g.s. system of units (centimeter, gram, and second) was in general use, but now, another system of units has been adopted in many parts of the world, known as SI (*System International d'Unites)* (Table 1).

In this international system of units, the seven fundamental units are established: length = meter (m), mass = kilogram (kg), time = second (s), electrical current = ampere (A), temperature = degree Kelvin (°K), luminosity = candelas (cd), and the quantity of matter = mole (mole). Many traditional units, such as calories (cal) or kilocalories (kcal) and centimeters (cm), are still in common use among chemists.

In chemistry, the quantity of matter is usually expressed in moles (mole) and the concentration of matter is usually expressed in mole/liter (M). Reaction rates are expressed in mole/liter/second (Ms^{-1}). The first-order rate constants have the dimension of $time^{-1}$ (s^{-1}) and the second-order rate constants have the dimension of $concentration^{-1} \times time^{-1}$ ($M^{-1}\ s^{-1}$); zero-order rate constants have the dimension of $concentration^{-1} \times time^{-1}$ ($M^{-1}\ s^{-1}$).

Most kinetic expressions and kinetic equations are presented in the literature, as well as in this book, without dimensions; the dimensions of kinetic parameters,

Table 1. Useful dimensions in the c.g.s. and in the SI system

Dimensions	c.g.s. units	SI units	Practical units
Length	cm	m	$\mu = 10^{-6}$ m; Å $= 10^{-10}$ m
Area	cm^2	m^2	
Volume	cm^3	m^3	
Mass	g	kg	
Time	sec	sec	
Velocity	cm sec^{-1}	m sec^{-1}	
Force	g cm sec^{-2} (dyne)	kg m sec^{-2} (newton)	newton $= 10^5$ dyne
Work, Energy	g cm^2 sec^{-2} (erg)	kg m^2 sec^{-2} (joule)	calorie = 4.186 joules
Power	g cm^2 sec^{-3}	kg m^2 sec^{-3}	watt = joules sec^{-1}
Density	g cm^{-3}	kg m^{-3}	
Pressure	g cm^{-1} sec^{-2} (dyne cm^{-2})	kg m^{-1} sec^{-2} (newton m^{-2})	Atmosphere $= 10^6$ dyne cm^{-2}
Electric current	$\sqrt{\text{erg cm}}$ sec^{-1}	Ampere (A)	A = coulomb sec^{-1}
Electric charge	$\sqrt{\text{erg cm}}$	A sec = coulomb	
Electric potential	$\sqrt{\text{erg cm}^{-1}}$	volt = joules $coulomb^{-1}$	
Electrical energy	erg		(e.s.u.)^2 cm^{-1}

The second is abbreviated as s.

rate constants, etc., are usually not shown in kinetic expressions. Therefore, it is of an utmost importance to perform a dimensional analysis with each and every kinetic expression and kinetic equation, and especially check the derivation of kinetic expressions by dimensional analysis. Thus, the dimensional analysis provides one of the simplest and most important techniques in enzyme kinetics for testing algebraic mistakes and checking the results.

Attention must be paid to the rules of multiplication, division, addition, and subtraction of values containing various dimensions; these rules are to be found in the elementary algebraic calculus.

In addition to the ordinary dimensions of quantity, concentration, volume, time, and temperature, the kinetic expressions in enzyme kinetics may contain various natural constants, and it is always useful to keep in mind the definitions, magnitudes, and the dimensions of principal natural constants. Table 2 lists the major natural constants that are of practical use in enzyme kinetics.

Table 2. Useful natural constants in enzyme kinetics

Description	Dimensions	Symbols
Avogadro's number	$N = 6.022 \times 10^{23}$	N
Faraday	$1\ F = 96.485\ C\ mol^{-1}$ (coulomb per mole) $= 23\ kcal\ mol^{-1}\ V^{-1}$	F
Coulomb	$1\ C = 1\ A\ s$ (ampere second) $= 6.23 \times 10^{18}$ electronic charges	C
The Boltzmann constant	$k_B = R/N = 1.3807 \times 10^{-23}\ J\ deg^{-1}$	k_B
The Planck constant	$h = 66252 \times 10^{-34}\ J\ s$	h
The gas constant	$R = Nk_B = 8.3144\ J\ deg^{-1}\ mol^{-1}$ $= 1.987\ cal\ deg^{-1}\ mol^{-1}$	R
Unit of temperature	$0°C = 273.16°K$ °C = Celsius; °K = Kelvin	K

REFERENCES

Books

Atkins, P. & de Paula, J. (2002) *Physical chemistry,* 7th ed., Oxford University Press, Oxford.

Cornish-Bowden, A. (1995) *Fundamentals of enzyme kinetics,* revised edition, Portland Press, London.

Gutfreund, H. (1972) *Enzymes: physical principles,* Wiley-Interscience, London.

Hammes, G.G. (1978) *Principals of chemical kinetics,* Academic Press, New York.

Jencks, W.P. (1969) *Catalysis in chemistry and enzymology,* McGraw-Hill, New York [Also available as *Catalysis in chemistry and enzymology* (1986) Dover Publications, New York].

Mahler, H.R. & Cordes, E.H. (1966) *Biological chemistry,* Harper & Row, New York.

Michaelis, L. (1922) *Die Wasserstoffionenkonzentration,* Springer-Verlag, Berlin.

Moore, W.J. (1962) *Physical chemistry,* Prentice-Hall, New York.

Moore, W.J. & Pearson, R.J. (1982) *Kinetics and mechanism,* 3rd ed., Wiley, New York.

Panchenkov, V. & Lebedev, A. (1976) *Chemical kinetics,* Mir, Moscow.

Plowman, K.N. (1972) *Enzyme kinetics,* McGraw-Hill Book Company, New York.

Schulz, A.G. (1994) *Enzyme kinetics,* Cambridge University Press, Cambridge.
Streitwieser, A. & Heathcock, C.H. (1998) *Introduction to organic chemistry,* 4th ed., Prentice Hall, New York.
Van't Hoff, J. H. (1884) *Etudes de Dynamiques Chimique,* Muller, Amsterdam.
Zubay, G. (1988) *Biochemistry,* 2nd ed., McMillan, New York.

Review articles

Cleland, W.W. & Northrop, D.B. (1999) *Methods Enzymol. 308,* 3–27.
Fierke, C.A. & Hammes, G.G (1995) *Methods Enzymol. 249*, 3–37.

Specific references

Arrhenius, S. (1889) *Z. Physik. Chem. 4,* 226–248.
Brönsted, J.N. (1925) *Rec. Trav, Chim. Pays-Bas 42,* 718–728.
Eyring, H. (1935) *J. Phys. Chem. 3,* 107–115.
Eyring, H. (1935) *Chem. Rev. 17,* 65–90.
Guggenheim, E.A. (1926) *Philos. Mag. Ser. VII 2,* 538–543.
Harcourt, A.V. (1867) *J. Chem. Soc. 20,* 460–492.
Hasselbalch, H. (1916) *Biochem. Z. 78,* 112–120.
Henderson, L.J. (1908) *Am. J. Physiol. 21,* 169–180.
Sørensen, A. (1909) *C. R. Trav. Lab. Carlsberg 8,* 1–168.
Swinbourne, E.S. (1960) *J. Chem. Soc.,* 2371–2372.

Chapter 3

Kinetics of Monosubstrate Reactions

The earliest studies of the rates of enzyme-catalyzed reactions appeared in the scientific literature in the latter part of the nineteenth century (Wurz, 1880; O'Sullivan & Thompson, 1890; Fischer, 1894; Brown, 1882, 1902; Henri, 1902, 1903). At that time, no enzyme was available in a pure form, methods of assay were primitive, and the use of buffers to control pH had not been introduced. Moreover, it was customary to follow the course of the reaction over a period of time, in contrast to the usual modern practice of measuring initial reaction rates at various different initial substrate concentrations, which gives results that are much easier to interpret.

3.1 WORK OF MICHAELIS AND MENTEN

Many of the early studies were conducted with enzymes from fermentation, particularly invertase, which catalyzes the hydrolysis of sucrose to monosaccharides D-glucose and D-fructose. With the introduction of the concept of hydrogen ion concentration, expressed by the logarithmic scale of pH (Sørensen, 1909), Michaelis and Menten (1913) realized the necessity for carrying out definitive experiments with invertase. They controlled the pH of the reaction medium by using acetate buffer, allowed for the mutarotation of the product and measured initial reaction rates at different substrate concentrations. Michaelis and Menten described their experiments by a simple kinetic law which afforded a foundation for a subsequent rapid development of numerous kinetic models for enzyme-catalyzed reactions. Although the contribution of previous workers, especially Henri (1902, 1903), was substantial, Michaelis and Menten are regarded as the founders of modern enzyme kinetics due to the definitive nature of their experiments and the viability of their kinetic theory.

Michaelis and Menten made a fortunate choice to study the enzymatic hydrolysis of sucrose:

$$\text{Sucrose} + \text{Water} \xrightarrow{\textit{Invertase}} \text{D-Glucose} + \text{D-Fructose} \qquad (3.1)$$

Since the concentration of water in this reaction is virtually constant (55.5 M), an essentially bimolecular chemical reaction can be treated kinetically as a monomolecular, which simplifies considerably the kinetic treatment.

Kinetic work of Michaelis and Menten was conducted by measuring the influence of increasing concentrations of substrate, or increasing concentrations of enzyme, on initial rates of reaction, with the following experimental protocol:

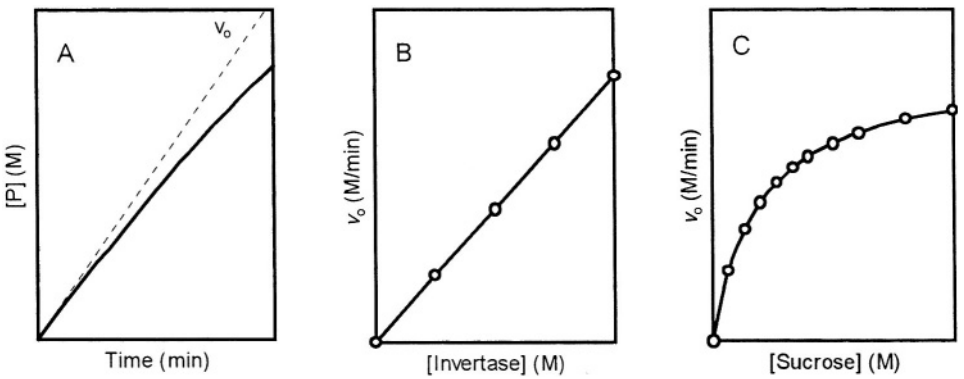


Figure 1. (A) Time course of reaction; v_o is the initial rate of reaction, [P] is the concentration of product. (B) Influence of enzyme concentration on initial reaction rate. (C) Influence of substrate concentration on initial reaction rate.

(a) Determination of the initial rate of reaction from changes in substrate or product concentration with time (Fig. 1A).
(b) Determination of the influence of increasing concentrations of enzyme on initial rates of reaction (Fig. 1B).
(c) Determination of the influence of increasing initial concentrations of substrate on initial rates of reaction (Fig. 1C).

With most enzymatic reactions which are carried out *in vitro,* the concentration of enzyme is much lower than the concentration of substrates, due to the high catalytic activity of enzymes. Therefore, with the progress of enzymatic reaction its rate gradually decreases due to depletion of substrate; for this reason, it is always necessary to determine an *initial rate of reaction,* which is equal to the tangent to reaction progress curve shown in Fig. 1A. Figure 1B shows that the initial rate of reaction responds linearly to increasing concentrations of enzyme, provided the substrate concentration is much larger than the concentration of enzyme, and Fig. 1C shows that the initial rate of reaction responds nonlinearly to increasing concentrations of substrate, provided the similar conditions are fulfilled.

Invertase proved to be a true catalyst, as it was not destroyed in the reaction and it was still active after catalyzing the hydrolysis of 100,000 times its weight of sucrose. The thermal stability of the enzyme was much greater in the presence of its substrate, sucrose, than in its absence; this striking fact indicated that the invertase enters into combination with the sugar, which protects the enzyme from inactivation.

Thus, the enzyme has a kinetic behavior consistent with a role of a recycling catalyst, which led Michaelis and Menten to propose the following kinetic mechanism of action for invertase (Fig. 2).

A single molecule of enzyme (E) combines reversibly with a single molecule of substrate (A) to form an enzyme-substrate complex (X), which is transformed

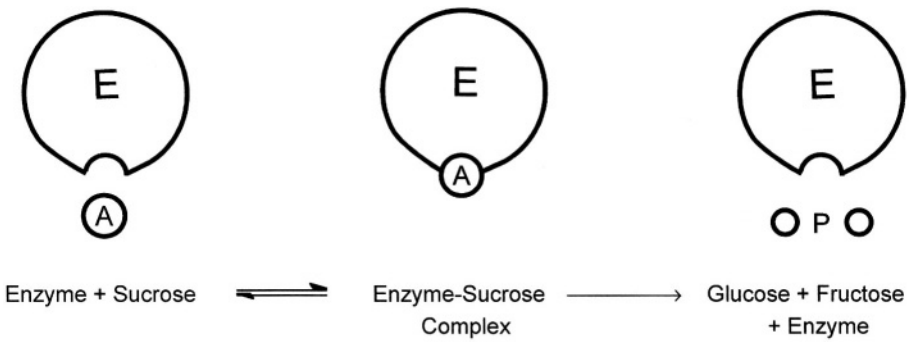


Figure 2. The mechanism of enzymatic hydrolysis of sucrose.

irreversibly to free enzyme and the products of reaction (P_1, P_2); kinetically, this is a monomolecular chemical reaction in which the enzyme plays a role of a recycling catalyst (Mahler & Cordes, 1966):

$$\mathrm{E} + \mathrm{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{X} \xrightarrow{k_3} \mathrm{P_1} + \mathrm{P_2} + \mathrm{E} \tag{3.2}$$

Michaelis and Menten imposed two restrictions on the enzymatic reaction by dividing it into a fast reversible and a slow irreversible part. The reversible part, $\mathrm{E} + \mathrm{A} \rightleftharpoons \mathrm{X}$, is much faster than the irreversible one, $\mathrm{X} \rightarrow \mathrm{P_1} + \mathrm{P_2} + \mathrm{E}$, because

$$k_3 << k_2 \tag{3.3}$$

Thus, the substrate is not "sticky", that is, it dissociates from the collision complex faster than it reacts to give products. For this reason, the enzyme–substrate complex is in a thermodynamic equilibrium with the free enzyme and the free substrate

$$K_\mathrm{A} = \frac{k_2}{k_1} = \frac{E \cdot A}{X} \tag{3.4}$$

where *E, A,* and *X* are the concentrations of free enzyme, free substrate and enzyme–substrate complex, respectively.

The total enzyme (E_0) and the total substrate (A_0) are divided between free and complex bound forms:

$$E_0 = E + X \qquad A_0 = A + X \tag{3.5}$$

From Eq. (3.4), it follows that

$$X = \frac{(E_0 - X)A_0}{K_\mathrm{A}} \quad \text{or} \quad X = \frac{E_0 A_0}{K_\mathrm{A} + A_0} \tag{3.6}$$

The irreversible part of reaction is a slow monomolecular process with a rate constant k_3; thus, the initial rate (v_0) of the overall reaction is equal:

$$v_0 = \frac{k_3 E_0 A_0}{K_A + A_0} \tag{3.7}$$

After substituting the constant value

$$k_3 E_0 = V_{max} \tag{3.8}$$

we obtain the kinetic law for reaction (3.2):

$$v_0 = \frac{V_{max} A_0}{K_A + A_0} \tag{3.9}$$

Equation (3.9) is the fundamental equation of enzyme kinetics, generally known as the *Michaelis–Menten equation,* the constant value K_A as the *Michaelis constant* and the constant value V_{max} as the *maximal velocity* of reaction.

An equation equivalent to the Michaelis-Menten rate law has been derived earlier by Henri (1902,1903); indeed, Michaelis and Menten in their original publication in 1913 honored the contribution of Henri. Therefore, in appreciation of the work of Henri, it is often also referred to as the *Henri–Michaelis–Menten equation.*

3.2 STEADY-STATE APPROXIMATION

The formulation of Michaelis and Menten, which treats the first step of enzyme catalysis as an equilibrium (Eq. (3.4)), makes unwarranted and unnecessary assumptions about the rate constants (Eq. (3.3)). Indeed, as we now know, the rate constants k_1, k_2, and k_3 can vary within very wide limits in many, if not the most, enzymatic reactions.

Briggs and Haldane (1925) examined a general mechanism for reaction (3.2), allowing all rate constants to vary within wide limits; they derived a kinetic law for this case, assuming that the steady state is achieved shortly after the beginning of reaction and that the concentration of enzyme–substrate complex remained nearly constant over a prolonged period of time. It is important to note that this *steady-state assumption* has become the central dogma underlying the derivation of most of the rate equations and most of the rate laws in enzyme kinetics.

The derivation of the rate law starts by writing the four kinetic equations for the reaction scheme (3.2), which describe the change of concentrations for substrate, free enzyme, enzyme–substrate complex, and the product with time.

$$-\frac{dA}{dt} = k_1 AE - k_2 X \tag{3.10}$$

$$\frac{dX}{dt} = k_1 AE - (k_2 + k_3)X \tag{3.11}$$

$$\frac{dE}{dt} = (k_2 + k_3)X - k_1 AE \tag{3.12}$$

$$\frac{dP}{dt} = k_3 X \tag{3.13}$$

plus the mass conservation equation:

$$E_0 = E + X \tag{3.14}$$

In the most general case, when there exists no restriction with respect to the relative magnitude of rate constants k_1, k_2, and k_3, the relative concentration of E and A, and the particular time t for which we want to establish the rate law, the explicit solution is not possible. The four differential Eqs. (3.10)–(3.13) plus the conservation Eq. (3.14) provide us with four unknowns and they can only be integrated with the help of a computer. A typical solution, obtained with a KINSIM computer program (Barshop *et al.,* 1983), is shown in Fig. 3.

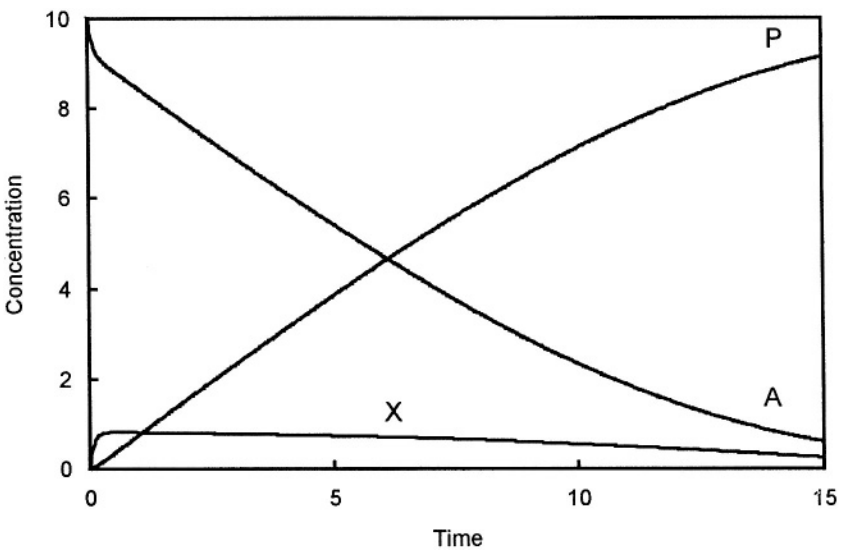


Figure 3. Progress curve for the enzyme-catalyzed reaction $A + E \rightleftharpoons X \longrightarrow E + P$, assuming that $k_1 = k_2 = k_3$, and $A_0 = 10E_0$.

Figure 3 shows that the concentration of enzyme–substrate complex is nearly constant over a prolonged period of time, that is,

$$\frac{dX}{dt} \approx 0 \tag{3.15}$$

As long as this condition prevails, the steady-state approximation can be applied to reaction (3.2). Equation (3.11) now becomes

$$k_1AE \approx (k_2 + k_3)X \tag{3.16}$$

and permits us to express X in terms of A and E_0, by substituting E in Eq. (3.14):

$$X = \frac{k_1AE_0}{k_1A + k_2 + k_3} \tag{3.17}$$

Substituting X in Eq. (3.13), we obtain

$$v_0 = -\frac{dA}{dt} = \frac{dP}{dt} = k_3 X = \frac{k_1 k_3 A E_0}{k_1 A + k_2 + k_3} \tag{3.18}$$

Since $A_0 \approx A$, this reduces to the fundamental Michaelis–Menten equation:

$$v_0 = \frac{V_{max} A_0}{K_A + A_0}$$

where

$$V_{max} = k_3 E_0 \quad \text{and} \quad K_A = \frac{k_2 + k_3}{k_1} \tag{3.19}$$

It is important to note that the derivation of the rate law in the Briggs–Haldane mechanism gives the same result as in the Michaelis–Menten mechanism, namely the fundamental Michaelis–Menten equation (3.9). However, in the former case, the Michaelis constant K_A is increased by a factor k_3/k_1, compared with the latter case; V_{max} constant has the same meaning in both mechanisms.

The earlier recommendation of the Enzyme Commission of the International Union of Biochemistry was that the K_S should be applied for the Michaelis–Menten mechanism and K_M for the Briggs–Haldane mechanism (Enzyme Nomenclature, 1973); in this case, $K_M = K_S + k_3/k_1$. This practice must be discouraged because it leads to cumbersome and ambiguous expressions in multisubstrate reactions.

3.3 REVERSIBLE MECHANISM WITH ONE CENTRAL COMPLEX

All enzymatic reactions are in principle reversible, in a sense that significant amounts of both substrates and products exist in the equilibrium mixture (Alberty, 1959; Cleland, 1970; Plowman, 1972). Therefore, it is evident that both Michaelis–Menten and Briggs–Haldane mechanisms are incomplete, and that allowance must be made for the reverse reaction:

$$\mathrm{E} + \mathrm{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{X} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{P} + \mathrm{E} \tag{3.20}$$

The steady-state assumption is now expressed by

$$\frac{dX}{dt} = k_1(E_0 - X)A + k_4(E_0 - X)P - (k_2 + k_3)X = 0 \tag{3.21}$$

Rearranging, we obtain

$$X = \frac{k_1 E_0 A + k_4 E_0 P}{k_1 A + k_2 + k_3 + k_4 P} \tag{3.22}$$

The net rate of formation of product P is the difference between the forward and reverse reactions:

$$v_0 = \frac{dP}{dt} = k_3X - k_4(E_0 - X)P \tag{3.23}$$

Substitution for X in this expression gives the rate law for reaction (3.20):

$$v_0 = \frac{(k_1k_3A - k_2k_4P)E_0}{k_1A + k_2 + k_3 + k_4P} \tag{3.24}$$

Let us define the constant values:

$$V_1(\text{or } \overrightarrow{V_{max}}) = k_3E_0 \quad V_2(\text{or } \overleftarrow{V_{max}}) = k_2E_0 \tag{3.25}$$

$$K_A = \frac{k_2 + k_3}{k_1} \quad K_P = \frac{k_2 + k_3}{k_4} \tag{3.26}$$

where V_1 and V_2 represent the maximal rates in the forward and reverse directions, and K_A and K_P the respective Michaelis constants. If the constant values are substituted from Eqs. (3.25) and (3.26), the rate law (3.24) obtains the form of the Michaelis–Menten equation:

$$v_0 = \frac{\left(\frac{V_1}{K_A}\right)A - \left(\frac{V_2}{K_P}\right)P}{1 + \frac{A}{K_A} + \frac{P}{K_P}} \tag{3.27}$$

This equation is the general reversible form of the Michaelis–Menten equation. If the enzymatic reaction is completely reversible, it can be started by mixing the substrate with the enzyme, or vice versa, by mixing the product with the enzyme. In the former case, $P = 0$, and Eq. (3.27) reduces to the Michaelis–Menten equation in the forward direction:

$$v_0 = \frac{V_1A_0}{K_A + A_0} \tag{3.28}$$

In the latter case, $A = 0$, and Eq. (3.27) reduces to the Michaelis–Menten equation in the reverse direction:

$$v_0 = \frac{V_2P_0}{K_P + P_0} \tag{3.29}$$

The values of individual rate constants can also be calculated from the kinetic constants K_A, K_P, V_1 and V_2, provided E_0 is known:

$$k_1 = \frac{V_1 + V_2}{K_AE_0} \quad k_2 = \frac{V_2}{E_0} \quad k_3 = \frac{V_1}{E_0} \quad k_4 = \frac{V_1 + V_2}{K_PE_0} \tag{3.30}$$

3.4 REVERSIBLE MECHANISM WITH TWOCENTRAL COMPLEXES

The mechanism (3.20) is incomplete in a sense that it allows the formation of an enzyme–substrate complex, but not of the enzyme–product complex; a more realistic case is a reversible mechanism in which the conversion of A into P

and the release of P from the enzyme are treated as distinct reactions (Alberty, 1959; Cleland, 1970; Plowman, 1972). Thus, the following mechanism for enzyme-catalyzed monosubstrate reactions is more realistic:

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} X \underset{k_4}{\overset{k_3}{\rightleftharpoons}} Y \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E + P \tag{3.31}$$

The steady-state rate equations can be derived by setting the rates of change of both intermediates concentrations to zero:

$$\frac{dX}{dt} = k_1A(E_0 - X - Y) - (k_2 + k_3)X + k_4Y = 0 \tag{3.32}$$

$$\frac{dY}{dt} = k_6P(E_0 - X - Y) - (k_4 + k_5)Y + k_3X = 0 \tag{3.33}$$

and solving the resulting equations for X and Y:

$$X = \frac{k_1(k_4 + k_5)E_0A + k_4k_6E_0P}{k_2k_4 + k_2k_5 + k_3k_5 + k_1(k_3 + k_4 + k_5)A + k_6(k_2 + k_3 + k_4)P} \tag{3.34}$$

$$Y = \frac{k_6(k_2 + k_3)E_0P + k_1k_3E_0A}{k_2k_4 + k_2k_5 + k_3k_5 + k_1(k_3 + k_4 + k_5)A + k_6(k_2 + k_3 + k_4)P} \tag{3.35}$$

The net rate is the difference between the forward and reverse rates for any step:

$$v_0 = k_3X - k_4Y = \frac{(k_1k_3k_5A - k_2k_4k_6P)E_0}{k_2k_4 + k_2k_5 + k_3k_5 + k_1(k_3 + k_4 + k_5)A + k_6(k_2 + k_3 + k_4)P} \tag{3.36}$$

Equation (3.36) is of the same form as Eq. (3.24), but the definitions of kinetic parameters are more complex:

$$\begin{aligned} V_1 &= \frac{k_3k_5E_0}{k_3 + k_4 + k_5} \qquad K_A = \frac{k_2k_4 + k_2k_5 + k_3k_5}{k_1(k_3 + k_4 + k_5)} \\ V_2 &= \frac{k_2k_4E_0}{k_2 + k_3 + k_4} \qquad K_P = \frac{k_2k_4 + k_2k_5 + k_3k_5}{k_6(k_2 + k_3 + k_4)} \end{aligned} \tag{3.37}$$

If the constant values are substituted from Eq. (3.37), Eq. (3.36) obtains again the form of a Michaelis–Menten equation, identical with Eq. (3.27):

$$v_0 = \frac{\left(\frac{V_1}{K_A}\right)A - \left(\frac{V_2}{K_P}\right)P}{1 + \frac{A}{K_A} + \frac{P}{K_P}}$$

3.5 EQUILIBRIUM CONSTANT AND THE HALDANE RELATIONSHIP

At equilibrium, both reversible reactions (3.20) and (3.31) come to a dynamic standstill, although the reaction has not stopped. An equilibrium constant for the overall process $A \leftrightarrow P$, for reaction (3.20), is equal to

$$K_{eq} = \frac{k_1 k_3}{k_2 k_4} \tag{3.38}$$

and for reaction (3.31) equal to

$$K_{eq} = \frac{k_1 k_3 k_5}{k_2 k_4 k_6} \tag{3.39}$$

Using Eqs. (3.38) and (3.39), one can cast the general reversible forms of the Michaelis–Menten equations ((3.24) and (3.36)) into another useful form:

$$v_0 = \frac{V_1 V_2 \left(A - \frac{P}{K_{eq}}\right)}{K_A V_2 + V_2 A + \frac{V_1}{K_{eq}} P} \tag{3.40}$$

Equation (3.40) is quite general and describes the effect of reactant concentration on rate, not only at the start of the reaction in the complete absence of P (in the forward direction) or A (in the reverse direction), but also at any time during the approach to equilibrium (Alberty, 1959; Cleland, 1963, 1977).

If we substitute the values for rate constants into the equations for equilibrium constants ((3.38) and (3.39)), we obtain

$$K_{eq} = \frac{V_1 K_P}{V_2 K_A} \tag{3.41}$$

Equation (3.41) is known as the *Haldane relationship* (Haldane, 1930). It is a very important relationship, because it states that the kinetic parameters of every reversible enzymatic reaction are not independent of one another and are limited by the thermodynamic equilibrium constant of the overall reaction.

The Haldane relationship can be used to eliminate K_{eq} partly or completely from Eq. (3.40). For example, this equation is best written for consideration of the forward reaction as

$$v_0 = \frac{V_1 \left(A - \frac{P}{K_{eq}}\right)}{K_A \left(1 + \frac{P}{K_P}\right) + A} \tag{3.42}$$

In this form, both the contribution of the reverse reaction near the equilibrium point and the product inhibition caused by P are obvious. Thus, only the four kinetic

constants K_A, K_P, V_1, V_2 and the equilibrium constant, K_{eq}, are necessary to describe the kinetic behavior in such a steady-state system (Cleland, 1982).

3.6 RATE EQUATIONS IN COEFFICIENT FORM

Let us compare the rate laws for the two reversible Michaelis–Menten mechanisms ((3.20) and (3.31)), that is, Eq. (3.24) with Eq. (3.36). We can rewrite both equations in a more general form, using the kinetic coefficients in a manner of Cleland (1963):

$$v_0 = \frac{(\text{numerator}_1)A - (\text{numerator}_2)P}{(\text{constant}) + (\text{coef A})A + (\text{coef P})P} \tag{3.43}$$

The use of coefficients may be of only marginal help in making Eqs. (3.24) and (3.36) less formidable; however, this nomenclature is of immense help in more complex cases, particularly with multisubstrate reactions.

Also, one can see that, in both cases, the catalytic constants can be cast in a more general form:

$$V_1 = \frac{\text{numerator}_1}{\text{coefA}} \tag{3.44}$$

$$V_2 = \frac{\text{numerator}_2}{\text{coefP}} \tag{3.45}$$

$$K_A = \frac{\text{constant}}{\text{coefA}} \tag{3.46}$$

$$K_P = \frac{\text{constant}}{\text{coefP}} \tag{3.47}$$

$$K_{eq} = \frac{\text{numerator}_1}{\text{numerator}_2} \tag{3.48}$$

Relationships similar to Eqs. (3.44)–(3.48) are applicable not only to mechanisms (3.20) and (3.31) but also to any other monosubstrate or multisubstrate mechanism. Therefore, with the help of coefficients from an appropriate rate law, one can write the equations for all kinetic constants in terms of rate constants for almost any kinetic mechanism.

3.7 ENZYME DISTRIBUTION EQUATIONS

Reversible Michaelis–Menten mechanisms provide us with another useful general relationships, the *enzyme distribution equations.* Since many enzymatic reactions are fully reversible, immediately after mixing the enzyme with the substrate, or vice versa, after mixing the substrate with the enzyme, the steady-state concentration of all forms of enzyme is established. In the reversible mechanism with one central complex, the enzyme is divided among two forms:

$$\frac{E}{E_0} + \frac{X}{E_0} = 1 \tag{3.49}$$

and in the reversible mechanism with two central complexes, the enzyme is divided between three forms:

$$\frac{E}{E_0} + \frac{X}{E_0} + \frac{Y}{E_0} = 1 \tag{3.50}$$

Now, we can cast the enzyme distribution equations in terms of all individual rate constants for both kinetic mechanisms. In the reversible mechanism with one central complex, the enzyme distribution equations are

$$\begin{aligned} \frac{E}{E_0} &= \frac{k_2 + k_3}{\text{denominator}} \\ \frac{X}{E_0} &= \frac{k_1A + k_4P}{\text{denominator}} \end{aligned} \tag{3.51}$$

The denominator value is from the appropriate rate law, Eq. (3.24).

In the reversible mechanism with two central complexes, the enzyme distribution equations are

$$\begin{aligned} \frac{E}{E_0} &= \frac{k_2k_4 + k_2k_5 + k_3k_5}{\text{denominator}} \\ \frac{X}{E_0} &= \frac{k_1k_4A + k_1k_5A + k_4k_6P}{\text{denominator}} \\ \frac{Y}{E_0} &= \frac{k_1k_3A + k_2k_6P + k_3k_6P}{\text{denominator}} \end{aligned} \tag{3.52}$$

In this case, the denominator value should be taken from the rate law appropriate to this mechanism (Eq. 3.36).

The distribution equations describe the distribution of enzyme among various possible forms. These distribution equations when multiplied by E_0 give the steady-state concentrations of various enzyme forms. The distribution equations are very complex with bisubstrate and trisubstrate reactions. They have some inherent interest by themselves, however, and are useful in deriving rate equations for reactions in the presence of dead-end inhibitors (Chapter 11), and equations for rates of isotopic exchange (Chapter 16).

3.8 HYPERBOLIC NATURE OF THE MICHAELIS–MENTEN EQUATION

Figure 4 shows the graphical presentation of the Michaelis–Menten equation (rate law (3.9)).

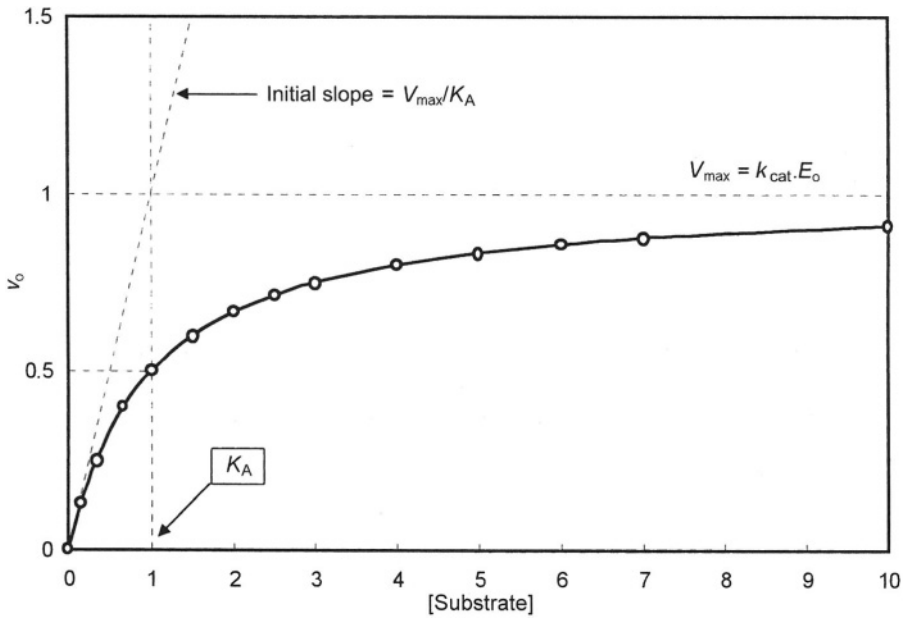


Figure 4. Initial reaction rates, v_o, plotted against increasing substrate concentrations for a reaction obeying the Michaelis–Menten kinetics, and assuming that $V_{max} = K_A = 1$.

A graph of v_o plotted against A is a section of a *right rectangular hyperbola,* with all mathematical properties of this geometric function (Cornish-Bowden, 1976). The usual expression for a rectangular hyperbola with x and y axes as asymptotes is

$$xy = a \tag{3.53}$$

The hyperbolas encountered in mathematics always have two limbs, whereas the plot of v_o against A appears to have only one. However, if we rearrange the Michaelis–Menten equation, we shall obtain

$$(A + K_A)(v_o - V_{max}) = -V_{max}K_A \tag{3.54}$$

which clearly shows that Eq. (3.9) has a hyperbolic nature with a horizontal asymptote approaching $v_o = V_{max}$, and a vertical asymptote approaching $A = -K_A$. As the vertical asymptote occurs at a negative value of A, it becomes clear why the curve appears to have only one limb: the whole of the negative limb cannot be observed experimentally.

3.9 SIGNIFICANCE OF KINETIC PARAMETERS

Michaelis–Menten equations for the monosubstrate reactions (Eqs. (3.9) and (3.27)) in the forward direction ($A \rightarrow P$), have four fundamental *kinetic constants* or *steady-state kinetic constants*:

V_{max}, the maximal velocity of reaction,
k_{cat}, the catalytic constant,
K_A, the Michaelis constant, and
k_{cat}/K_A, the specificity constant.

Table 1 gives the kinetic significance of these constants in various kinetic mechanisms described in Sections 3.1–3.4, followed by a brief description of the physical meaning of kinetic constants.

Table 1. Significance of kinetic parameters in various kinetic mechanisms

Mechanism	V_{max}	K_A	$\frac{k_{cat}}{K_A}$
Michaelis–Menten	k_3E_0	$\frac{k_2}{k_1}$	$\frac{k_1k_3}{k_2}$
Briggs–Haldane	k_3E_0	$\frac{k_2+k_3}{k_1}$	$\frac{k_1k_3}{k_2+k_3}$
Reversible with one central complex	k_3E_0	$\frac{k_2+k_3}{k_1}$	$\frac{k_1k_3}{k_2+k_3}$
Reversible with two central complexes	$\frac{k_3k_5E_0}{k_3+k_4+k_5}$	$\frac{k_2k_4+k_2k_5+k_3k_5}{k_1(k_3+k_4+k_5)}$	$\frac{k_1k_3k_5}{k_2k_4+k_2k_5+k_3k_5}$

The maximal velocity of reaction (V_{max}), dimension [concentration time^{-1}]. One can easily appreciate from Fig. 4 that the maximal velocity of reaction is obtained when all the enzyme is bound in the enzyme–substrate complex, that is, when the enzyme is saturated with the substrate ($A_0 >> K_A$). All kinetic expressions for V_{max} in Table 1 contain the concentration term for enzyme; therefore, the calculation of V_{max} in enzyme kinetics does not require the knowledge of enzyme concentration.

The catalytic constant (k_{cat}), dimension [time^{-1}]. Catalytic constant is obtained by dividing the maximal rate of reaction with the concentration of enzyme:

$$k_{cat} = \frac{V_{max}}{E_0} \tag{3.55}$$

which requires the knowledge of enzyme concentration. If the enzyme has two or more active sites per molecule, k_{cat} is usually called the *turnover number*:

$$\textit{Turnover number} = \frac{V_{max}}{[\text{enzyme active sites}]} \tag{3.56}$$

The turnover number represents the maximal number of substrate molecules converted to products per active site per unit time, or the number of times the molecule of enzyme "turns over" per unit time; the dimension is again [time^{-1}].

In the simple Michaelis–Menten mechanism in which there is only one enzyme–substrate complex and all binding steps are fast, k_{cat} is the first-order rate constant for the chemical conversion of the EA complex to products. For more complicated mechanisms, k_{cat} is a function of several first- and second-order rate constants, and it cannot be assigned to any particular process except when simplifying features occur (Table 1); k_{cat} cannot be greater than any first-order rate constant in the forward direction and thus sets the *lower* limit on the chemical rate constants. As a rule, the k_{cat} is the first-order rate constant which refers to the properties and reactions of enzyme–substrate, enzyme–product, and enzyme–intermediate complexes.

The Michaelis constant (K_A), dimension [concentration]. One can easily appreciate from Fig. 4 that the value of the Michaelis constant is obtained when $v_0 = V_{max}/2$. K_A is equal to dissociation constant of the enzyme–substrate complex only in the simple Michaelis–Menten mechanism, and in all other cases, it is a complex function of several rate constants (Table 1). However, K_A may be treated for some purposes as an *apparent* dissociation constant; for example, the concentration of free enzyme in solution may be calculated from the relationship:

$$K_A = \frac{[E][A]}{\sum [EA]} \tag{3.57}$$

where $\Sigma[EA]$ is the sum of all the bound enzyme species. Therefore, as a rule, the K_A is an apparent dissociation constant that may be treated as the overall dissociation constant of all enzyme-bound species (Fersht, 1999).

The specificity constant (k_{cat}/K_A), dimension [concentration^{-1} time^{-1}]. The importance of k_{cat}/K_A is that it relates the reaction rate to the concentration of free, rather than total, enzyme. At low substrate concentrations, when $K_A \gg A$, the Michaelis–Menten equation reduces to

$$v_0 = \left(\frac{k_{cat}}{K_A}\right) E_0 A \tag{3.58}$$

At low substrate concentrations, the enzyme is largely unbound and $E \approx E_0$; therefore, k_{cat}/K_A is an apparent second-order rate constant, which is not a true microscopic rate constant except in the extreme case in which the rate-limiting step in the reaction is the encounter of enzyme and substrate. Only in the Briggs–Haldane mechanism, when k_3 is much greater than k_2, k_{cat}/K_A is equal to k_1, the rate constant for the association of enzyme and substrate. Recently, Northrop (1999) raised a serious objection to this classical definition of the specificity constant, and pointed out that k_{cat}/K_A actually provides a measure of the rate of capture of substrate by free enzyme into a productive complex or complexes destined to go on to form products and complete a turnover at some later time.

The value of k_{cat}/K_A cannot be greater than that of any second-order rate constant on the forward reaction pathway; it thus sets a *lower* limit on the rate constant for the association of enzyme and substrate. As a rule, the k_{cat}/K_A is an apparent second-order rate constant that refers to the properties and reactions of free enzyme and free substrate.

Table 2. Kinetics of pepsin action on synthetic substrates of the type Carbobenzoxy-His-Phe-X-OMe at pH 4.0 and 37°C

Substrate	k_{cat} (s^{-1})	K_A (mM)	k_{cat}/K_A ($M^{-1}\ s^{-1}$)
Z-His-Phe-Gly-OMe	0.0021	1.6	1.3
Z-His-Phe-Ala-OMe	0.0037	1.8	2.1
Z-His-Phe-Leu-OMe	0.0052	0.5	10
Z-His-Phe-Met-OMe	0.014	0.9	16
Z-His-Phe-Phe-OMe	0.17	0.33	520
Z-His-Phe-Tyr-OMe	0.17	0.29	590
Z-His-Phe-Trp-OMe	0.51	0.2	2550

Adopted from the data of Inouye and Fruton (1967), and Trout and Fruton (1969).

Thus, the specificity constant gives us a direct measure of the catalytic efficiency at substrate concentrations that are significantly below the saturating levels. It is especially useful in distinguishing the specificity of enzyme for different substrates, particularly if they are structurally closely related (Table 2).

3.10 GRAPHICAL PRESENTATION OF DATA

Michaelis–Menten equation is a segment of a rectangular hyperbola showing a *saturation kinetics* (Fig. 4). From the direct plot of v_0 versus [substrate] in Fig. 4, it is difficult to determine the kinetic parameters V_{max} and K_A with precision, and a linear transformation of the same would provide better means for the graphical estimation of V_{max} and K_A.

The Michaelis–Menten equation can be cast in several linear transformations; in practice, the most popular are the following:

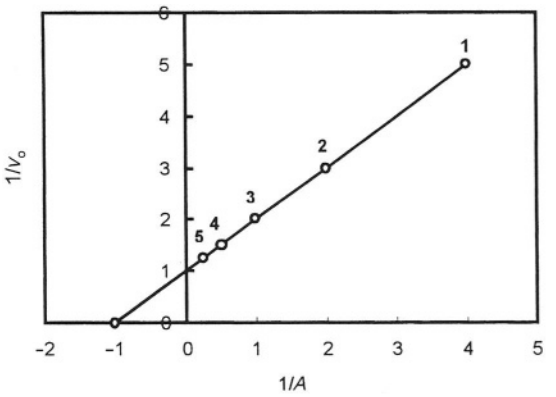
Lineweaver – Burk (1935)
$$\frac{1}{v_0} = \frac{1}{V_{max}} + \left(\frac{K_A}{V_{max}}\right)\frac{1}{A} \tag{3.59}$$

Hanes (1932)
$$\frac{A}{v_0} = \frac{K_A}{V_{max}} + \left(\frac{1}{V_{max}}\right)A \tag{3.60}$$

Eadie (1942) and Hofstee (1952)
$$v_0 = V_{max} - K_A\left(\frac{v_0}{A}\right) \tag{3.61}$$

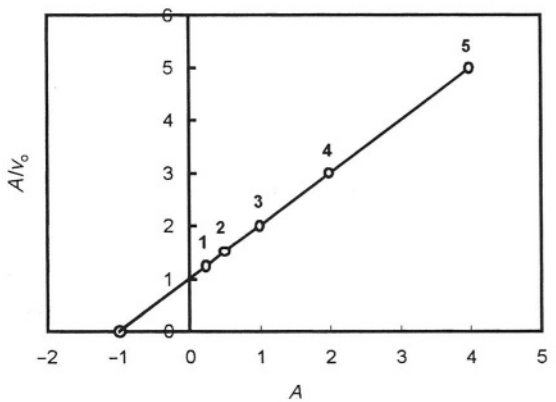
Table 3. Properties of the Michaelis–Menten equation, with $K_A = 1$ and $V_{max} = 1$

Data point	1	2	3	4	5	—
A	0.25	0.5	1	2	4	∞
v_0	0.2	0.333	0.5	0.666	0.8	1.0
$1/A$	4	2	1	0.5	0.25	0
$1/v_0$	5	3	2	1.5	1.25	1.0
A/v_0	1.25	1.5	2	3	5	∞



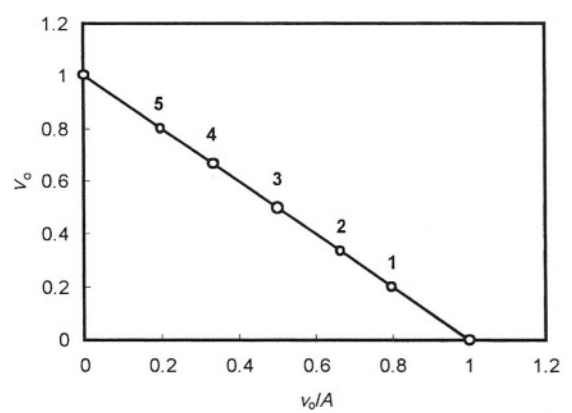
Lineweaver–Burk plot

Intercept on abscissa: $-1/K_A$
Intercept on ordinate: $1/V_{max}$
Slope: K_A/V_{max}



Hanes plot

Intercept on abscissa: $-K_A$
Intercept on ordinate: K_A/V_{max}
Slope: $1/V_{max}$



Eadie–Hofstee plot

Intercept on abscissa: V_{max}/K_A
Intercept on ordinate: V_{max}
Slope: $-K_A$

Figure 5. Linear transformations of the Michaelis–Menten equation (Eqs. (3.59)–(3.61)).

Let us take a numerical example from Table 3, in order to produce the graphical presentation of the three linear transformations of the Michaelis–Menten equation (Eqs. (3.59)–(3.61)).

Figure 5 shows the graphical presentation of Eqs. (3.59)–(3.61), using the numerical data from Table 3.

Before the advent of computer technology and computational methods, the linear transformations of the Michaelis–Menten equation were extensively used for the calculation of kinetic parameters (Allison & Purich, 1979); with the aid of a linear transformation of rectangular hyperbola, one can calculate with precision the asymptotes (V_{max} and K_A) by linear regression (Fig. 5). The merits of various transformations were estimated with respect to the statistical bias inherent in most linear transformations of the Michaelis–Menten equation (Wilkinson, 1961; Johanson & Lumry, 1961; Johanson & Faunt, 1992; Straume & Johnson, 1992; Ritchie & Prvan, 1996). The detailed statistical treatment of initial rate data, however, is presented in Chapter 18.

In the common laboratory practice, by far the most widely used is the *Lineweaver–Burk plot,* usually known as the *double reciprocal plot*; however, the statistical analysis indicated that it is also by far the worst plot (Dowd & Riggs, 1965). Inspection of Fig. 5 indicates that, in this plot, the weight of experimental values obtained at low concentrations of substrate is overemphasized, and those are the values which are associated normally with the largest experimental error. The Lineweaver–Burk plot has the advantage that it separates the variables. The *Eadie–Hofstee plot* has the disadvantage that v_o, usually regarded as a dependent variable, appears in both coordinates; on the other hand, this plot best reveals the presence of exceptionally bad measurements, with large experimental errors (Augustinsson, 1948). On balance, the *Hanes plot* is the most satisfactory of the three (Hultin, 1967; Fromm, 1975; Rudolph & Fromm, 1979).

An another useful way to examine the experimental data is a plot of log [A] versus v_o (Michaelis & Menten, 1913). With the aid of such a plot, one can examine the initial velocities of reaction in a very broad range of substrate concentrations and, although nonlinear, the plot becomes almost linear if the substrate concentrations are approximately $0.3–3K_A$.

It is sometimes claimed that the spread of computational methods has made plotting methods obsolete. This claim must be definitely discarded. There are good reasons why plotting methods are essential, not only for publishing the experimental results but also for their analysis. The human eye is much less easily deceived than any computer program currently available and is capable of recognizing unexpected behavior even if nothing currently available is described in the literature (Cornish-Bowden, 1995). Further, in the common laboratory practice, the experimental data are usually inspected by plotting methods in the first place. The plotting methods are used very easily at the laboratory bench while an experiment is still in progress, so that one has an immediate visual idea of the likely parameter values and of the design needed for defining them precisely.

For definitive work, it is unwise to use any plot, linear or otherwise, for estimating the parameters. Instead, a suitable statistical procedure supported by a computer program must be used, as outlined in Chapter 18.

3.11 DIMENSIONAL ANALYSIS

At this point, it is appropriate to introduce the important subject of dimensional analysis of the double reciprocal plots, a subject that was mentioned in the preceding chapter (Section 2.9). In the scientific and technical literature, the presentation of double reciprocal plots falls into two main categories (Fig. 6).

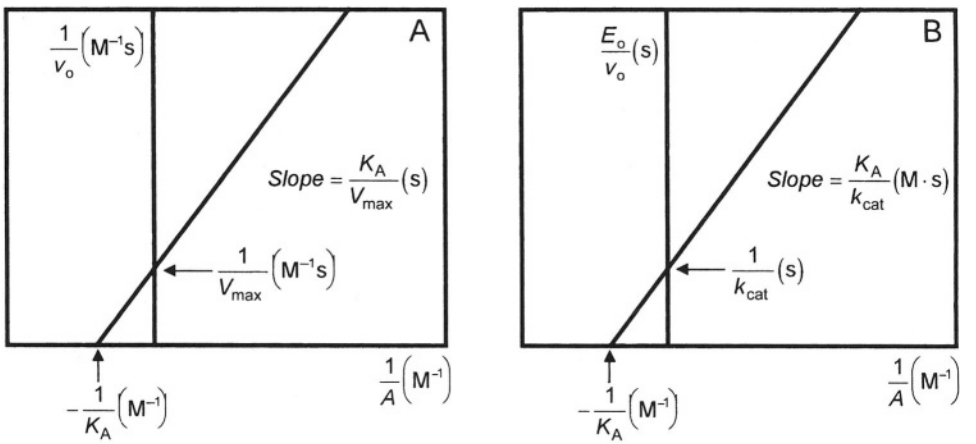


Figure 6. Dimensional analysis of double reciprocal plots.

In the first case (A), the reciprocal value of the initial rate of reaction (v_o, dimension: mol liter^{-1} s^{-1}) is shown as the function of the reciprocal substrate concentration *(A,* dimension: mol liter^{-1}). The slope of this plot is equal to K_A/V_{max} and has a dimension of time (s). The advantage of this presentation is that the knowledge of the actual concentration of enzyme (E_o) is not required.

In the second case (B), the reciprocal value of the apparent catalytic constant ($k_{cat}^{APP} = v_o/E_o$, dimension: s^{-1}) is plotted against the reciprocal substrate concentration. In this case, the slope of the plot is equal K_A/k_{cat} (remember that k_{cat}/K_A is the specificity constant with dimensions M^{-1} s^{-1}).

The first plot (A) is easily transformed into the second plot (B), simply by dividing all initial rates of reaction, v_o, with the concentration of enzyme, E_o. Therefore, the difference between the two presentations appears trivial. However, this is not the case, because both presentations appear in the literature; a failure to appreciate the difference can produce serious inconveniences with bisubstrate and trisubstrate reactions and especially with the interpretation of replots of slopes and intercepts on ordinate, because of the difference in dimensions (IUBMB, 1992).

REFERENCES

Books

Cornish-Bowden, A. (1976) *Principles of enzyme kinetics,* Butterworths, London.
Cornish-Bowden, A. (1995) *Fundamentals of enzyme kinetics,* revised edition, Portland Press, London.

Enzyme Nomenclature (1973) Elsevier Publishing Company, Amsterdam.
Fersht, A. (1999) *Structure and mechanism in protein science,* W.H. Freeman, New York.
Fromm, H.J. (1975) *Initial rate enzyme kinetics,* Springer-Verlag, Berlin.
Haldane, J.B.C. (1930) *Enzymes,* Longmans & Green, London.
Henri, V. (1903) Lois *Generales de l'Action des Diastases,* Hermann, Paris.
IUBMB (1992) *Enzyme Nomenclature,* International union of biochemistry and molecular biology, Academic Press, London.
Mahler, H.R. & Cordes, E.H. (1966) *Biological chemistry,* Harper & Row, New York.
Plowman, K.M. (1972) *Enzyme kinetics,* McGraw-Hill, New York.

Review articles

Alberty, R.A. (1959) *The Enzymes,* 2nd ed., Vol. 1, 130–150.
Allison, R.D. & Purich, D.L. (1979) *Methods Enzymol. 63,* 3–15.
Cleland, W.W. (1970) *The Enzymes,* 3rd ed., Vol. 2, 1–67.
Cleland, W.W. (1977) *Adv. Enzymol. Rel. Areas Mol. Biol. 45,* 273–387.
Cleland, W.W. (1982) *Methods Enzymol. 87,* 366–370.
Johnson, M.L. & Faunt, L.M. (1992) *Methods Enzymol. 210,* 1–37.
Northrop, D.B. (1999) *Adv. Enzymol. 72,* 12–40.
Rudolph, F.B. & Fromm, H.J. (1979) *Methods Enzymol. 63,*138–159.
Straume, M. & Johnson, M.L (1992) *Methods Enzymol. 210,* 87–105.

Specific references

Augustinsson, K.-B. (1948) *Acta Physiol. Scand. 15,* Suppl. 52.
Barshop, B.A., Wrenn, R.F., & Frieden, C. (1983) *Anal. Biochem. 130,* 134–145.
Briggs, G.E. & Haldane, J.B.S. (1925) *Biochem. J. 19,* 338–339.
Brown, A.J. (1882) *J. Chem. Soc. Trans. 61,* 369–385.
Brown, A.J. (1902) *J. Chem. Soc. Trans. 81,* 371–388.
Cleland, W.W. (1963) *Biochim. Biophys. Acta 67,* 104–137.
Dowd, J.E. & Riggs, D.S. (1965) *J. Biol. Chem. 240,* 863-869.
Eadie, G.S. (1942) *J. Biol. Chem. 146,* 85–93.
Fischer, E. (1894) *Ber. Dtsch. Chem. Ges. 27,* 2985-2993.
Hanes, C.S. (1932) *Biochem. J. 26,*1406-1421.
Henri, V. (1902) *R. Hebd, Seances Acad. Sci. 135,* 916–919.
Hofstee, B.H.J. (1952) *J. Biol. Chem. 199,* 357–364.
Hultin, E. (1967) *Acta Chem. Scand. 21,* 1575–1590.
Inouye, A. & Fruton, J.S. (1967) *Biochemistry 6,* 1765–1777.
Johanson, G. & Lumry, R. (1961) *C. R. Trav. Lab. Carlsberg 32,* 185–214.
Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc. 56,* 658–666.
Michaelis, L. & Menten, M.L. (1913) *Biochem. Z. 49,* 333–369.
O'Sullivan, C. & Thompson, F.W. (1890) *J. Chem. Soc. Trans. 57,* 834–931.
Ritchie, R.J. & Prvan, T. (1996) *J. Theor. Biol. 178,* 239–254.
Sørensen, S.P.L. (1909) *C. R. Trav. Lab. Carlsberg 8,* 1–168.
Trout, G.E. & Fruton, J.S (1969) *Biochemistry 8,* 4183–4190.
Wilkinson, G.N. (1961) *Biochem. J. 80,* 324–332.
Wurz, A. (1880) C. *R. Hebd. Seances Acad. Sci. 91,* 787–791.

This page intentionally left blank

Chapter 4

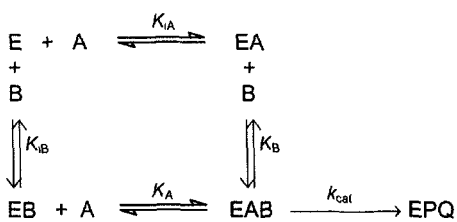
Derivation of Rate Equations

The derivation of rate equations for simple monosubstrate reactions was described in Chapter 3. For bisubstrate reactions, the derivation is usually much more complex, and requires the application of a special mathematical apparatus and special mathematical procedures.

4.1 VELOCITY EQUATIONS FOR RAPID EQUILIBRIUM SYSTEMS

Velocity equations can be obtained easily, even for seemingly complex systems, if rapid equilibrium conditions prevail. No derivation is really necessary. In fact, the velocity equation for any rapid equilibrium system can be written directly from an inspection of the equilibria between enzyme species (Briggs & Haldane, 1925; Fromm, 1975; Wong, 1975; Fromm, 1979).

Let us illustrate this statement with the following example, a rapid equilibrium bireactant system:



(4.1)

We start by writing the velocity dependence equation:

$$v_o = k_{cat}[EAB] \tag{4.2}$$

and by writing the sum of all forms of the enzyme:

$$[E_o] = [E] + [EA] + [EB] + [EAB] \tag{4.3}$$

Dividing Eq. (4.2) by Eq. (4.3), we obtain

$$\frac{v_o}{[E_o]} = \frac{k_{cat}[EAB]}{[E] + [EA] + [EB] + [EAB]} \tag{4.4}$$

Now all we need to do is express the concentration of each species in terms of [E]. If we substitute $k_{cat}[E_o]$ with V_{max}, we shall obtain

$$v_0 = \frac{V_{max}\left(\frac{[EAB]}{[E]}\right)}{1 + \frac{[EA]}{[E]} + \frac{[EB]}{[E]} + \frac{[EAB]}{[E]}} \tag{4.5}$$

After substituting the concentrations of various forms of enzyme with kinetic constants, we obtain

$$v_0 = \frac{V_{max}\left(\frac{[A][B]}{K_{iA}K_B}\right)}{1 + \frac{[A]}{K_{iA}} + \frac{[B]}{K_{iB}} + \frac{[A][B]}{K_{iA}K_B}} \tag{4.6}$$

Also, after rearranging, we obtain a usual velocity equation for the Rapid Equilibrium Random bisubstrate mechanism:

$$v_0 = \frac{V_{max}AB}{K_{iA}K_B + AK_B + BK_A + AB} \tag{4.7}$$

4.2 NET RATE CONSTANT METHOD

In 1975, Cleland introduced the net rate constant method to simplify the treatment of simple enzyme kinetic mechanisms that *do not involve branched pathways* (Cleland, 1975). This method can be successfully applied to obtain rate laws for isotope exchange. Since the net rate constant method allows one to obtain V_{max}/K_M and K_M in terms of the individual rate constants, this method has the greatest value for the characterization of isotope effects on V_{max}/K_M and K_M (Huang, 1979; Purich & Allison, 2000).

4.2.1 Uni Uni Monosubstrate Mechanism

Let us start to illustrate the method with the simplest Uni Uni monosubstrate mechanism:

$$E \underset{k_2}{\overset{k_1 A}{\rightleftharpoons}} EA \xrightarrow{k_3} EP \xrightarrow{k_4} E + P \tag{4.8}$$

To start with, reaction (4.8) is first converted to a series of unidirectional rate constants (indicated by primes). The steady-state flux through each step is given by

$$E \xrightarrow{k_1'} EA \xrightarrow{k_3'} EP \xrightarrow{k_4'} E + P \tag{4.9}$$

where each step has an associated net rate constant k_i'. Since the mechanism is a linear series, the rate for the overall reaction is the sum of the inverse of the net rate constants through the individual steps.

Thus, the rate equation is

$$\frac{v_0}{E_0} = \frac{1}{\frac{1}{k_1'} + \frac{1}{k_3'} + \frac{1}{k_4'}} \tag{4.10}$$

First, one has to express the net rate constants in terms of the actual rate constants. For any irreversible step, the net rate constant and the actual rate constant is identical; thus,

$$k_3' = k_3 \quad \text{and} \quad k_4' = k_4 \tag{4.11}$$

Moving leftward, we express k_1' as the real forward rate constant, k_1, multiplied by the factor that relates the fraction of EA reacting in the forward direction as opposed to the fraction that returns to E. Note that all bimolecular rate constants must be transformed into monomolecular, by multiplying by the concentration of substrate or product. Thus, we obtain

$$k_1' = \frac{k_1 A k_3}{k_2 + k_3} \tag{4.12}$$

Now, returning to Eq. (4.10) and substituting the k_i' terms, we obtain

Equation (4.13) can be written in the Michaelis–Menten fashion as

$$\frac{v_0}{E_0} = \frac{\left(\frac{k_3 k_4}{k_3 + k_4}\right) A}{\frac{k_4(k_2 + k_3)}{k_1(k_3 + k_4)} + A} \tag{4.14}$$

Equation (4.14) shows the composition of kinetic constants:

$$V_{\max} = \frac{k_3 k_4 E_0}{k_3 + k_4} \quad \text{and} \quad K_A = \frac{k_4(k_2 + k_3)}{k_1(k_3 + k_4)} \tag{4.15}$$

When the goal is to obtain $V_{\max}$ or $V_{\max}/K_A$, the following procedure avoids the need to derive the entire rate equation.

First. $V_{\max}$ is obtained at saturating A concentrations, such that k_1' will be infinite and the $(1/k_1')$ term drops out, so that $V_{\max}$ becomes

$$V_{\max} = \frac{E_0}{\frac{1}{k_3'} + \frac{1}{k_4'}} = \frac{k_3 k_4 E_0}{k_3 + k_4}$$

Second. $V_{\max}/K_A$ is obtained as A is extrapolated to near zero. In this case, the k_1' term becomes the smallest net rate constant, and

$$\frac{V_{max}}{K_A} = k_1' E_0 = \frac{k_1 k_3 A E_0}{k_2 + k_3}$$

4.2.2 Ordered Uni Bi Mechanism

The above process will be repeated with a more complex Ordered Uni Bi mechanism:

$$E \underset{k_2}{\overset{k_1 A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EPQ \underset{k_6 P}{\overset{k_5}{\rightleftharpoons}} EQ \xrightarrow{k_7} E + Q \tag{4.16}$$

First, reaction (4.16) is converted into a series of unidirectional or net rate constants. The steady-state flux through each step remains the same as above, and we obtain

$$E \xrightarrow{k_1'} EA \xrightarrow{k_3'} EPQ \xrightarrow{k_5'} EQ \xrightarrow{k_7'} E + Q \tag{4.17}$$

Because the mechanism is again a linear series, the rate for the overall reaction is the sum of the inverse of the net rate constants through the individual steps:

$$\frac{v_0}{E_0} = \frac{1}{\frac{1}{k_1'} + \frac{1}{k_3'} + \frac{1}{k_5'} + \frac{1}{k_7'}} \tag{4.18}$$

where the net rate constants must now be expressed in terms of the actual rate constants, bearing in mind that all bimolecular rate constants must be transformed into the monomolecular rate constants by multiplying with the concentration of substrate or product.

We start with the only irreversible step in the mechanism in reaction (4.16); for that irreversible step, the net rate constant and the real rate constant are identical:

$$k_7' = k_7 \tag{4.19}$$

Moving from right to the left, we express k_5' as the real forward rate constant multiplied by a factor that relates the fraction of EQ reacting in the forward direction as opposed to the fraction that returns to EPQ. In this case, since $k_7' = k_7$, we obtain

$$k_5' = k_5 \left(\frac{k_7}{k_7 + k_6 P} \right) \tag{4.20}$$

Again, moving from right to the left, we obtain

$$k_3' = k_3 \left(\frac{k_5'}{k_4 + k_5'} \right) \tag{4.21}$$

Then, replacing k_5' from Eq. (4.20), we obtain

$$k_3' = \frac{\left(\dfrac{k_3k_5k_7}{k_7 + k_6P}\right)}{\left(k_4 + \dfrac{k_5k_7}{k_7 + k_6P}\right)} = \frac{k_3k_5k_7}{k_4k_7 + k_5k_7 + k_4k_6P} \tag{4.22}$$

Also, again we deal with the leftwardmost term k_1':

$$k_1' = \frac{k_1Ak_3'}{k_2 + k_3'} \tag{4.23}$$

which upon substituting k_3' from Eq. (4.22), gives

$$k_1' = \frac{k_1k_3k_5k_7A}{k_2k_4k_7 + k_2k_5k_7 + k_3k_5k_7 + k_2k_4k_6P} \tag{4.24}$$

Now, we shall return to Eq. (4.18) and substitute all the k_i' terms:

$$\begin{aligned} v_0 &= \frac{E_0}{\dfrac{1}{k_1'} + \dfrac{1}{k_3'} + \dfrac{1}{k_5'} + \dfrac{1}{k_7'}} \\ v_0 &= \frac{k_1k_3k_5k_7E_0A}{k_7(k_2k_4 + k_2k_5 + k_3k_5) + k_2k_4k_6P} \\ &\quad + k_1(k_3k_7 + k_4k_7 + k_5k_7 + k_3k_5)A + k_1k_6(k_3 + k_4)AP \end{aligned} \tag{4.25}$$

4.3 METHOD OF KING AND ALTMAN

In principle, the steady-state rate equation for *any* enzyme mechanism can be derived in the same way as that for the simple reversible monosubstrate Michaelis–Menten mechanism. What we need is to write down expressions for the rates of change of concentrations of all of the intermediates, set them equal to zero and solve the simultaneous equations that result, as described in Chapter 3 (Sections 3.1–3.4). In practice, however, this method is extremely laborious and liable to error for all but the simplest monosubstrate mechanisms (Fromm, 1975; Wong, 1975; Chou, 1990).

In 1956, King and Altman have described a schematic method that is simple to apply to any mechanism that consists of a series of reactions between different forms of one enzyme. For the application of this method, it is necessary that *all enzyme species in the mechanism are connected by reversible reactions.* It is not applicable to non-enzymatic reactions or to reactions that contain non-enzymatic steps.

The method of King and Altman rendered an invaluable service to enzymology because, with its help, the rate laws for many major reaction mechanisms in enzyme kinetics were developed. It is not necessary to understand the theory of the King-Altman method in order to apply it in practice, and indeed the theory is considerably more difficult than the practice. Therefore, we shall describe in the following sections the derivation of rate laws for several simple mechanisms

with the aid of the King–Altman method, with the emphasis on the practical application of the method, rather than its mathematical background.

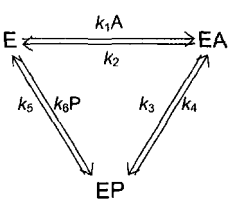
4.3.1 Uni Uni Monosubstrate Mechanism

In Chapter 3 (Section 3.4), we have derived the rate law for the reversible Michaelis–Menten mechanism with two central complexes (Reaction (3.31)) with the aid of the steady-state approximation. We shall proceed now with the derivation of the same rate law with the aid of the King–Altman method. In order to apply the King-Altman method efficiently to a specific mechanism, a rigorous procedure must be strictly followed (Fromm, 1975; Wong, 1975; Purich & Allison, 2000).

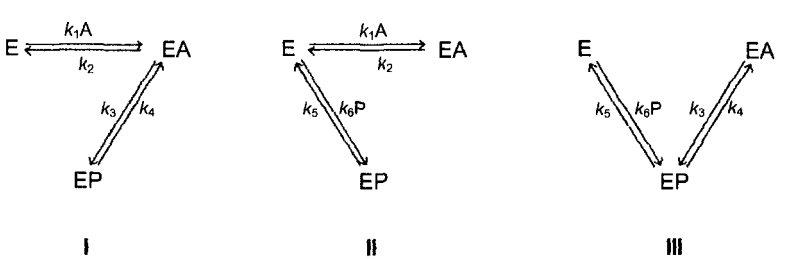
The *first step* in the King–Altman method is to write the mechanism of enzymatic reaction in the usual form (Reaction (3.31)):

$$\mathrm{E} \underset{k_2}{\overset{k_1\mathrm{A}}{\rightleftharpoons}} \mathrm{EA} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{EP} \underset{k_6\mathrm{P}}{\overset{k_5}{\rightleftharpoons}} \mathrm{E}$$

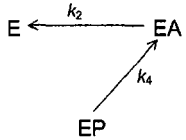
The *second step* is to draw a *master pattern* which represents the mechanism by a reaction which shows all of the enzyme species and the reactions between them. The master pattern must contain only closed loops, without loose ends. All of the reactions must be treated as first-order reactions; for example, the second-order rate constant k_1 is replaced with the pseudo-first-order rate constant k_1A, by including the concentration of substrate A.



In the *third step,* it is necessary to find all *partial patterns* from the master pattern. Every partial pattern: (1) consists only of lines from the master pattern, (2) connects every enzyme species, and (3) contains no closed loops. Each partial pattern will contain one line fewer than the number of enzyme species.



For *each* partial pattern and for *each* enzyme species, the product of the rate constants in the pattern leading to that species is written down. For example, for E, the first pattern gives a *product* k_2k_4, from



The arrowheads are drawn so that from any starting point, the arrows lead to the appropriate species, with only *one* arrow from each *other* species.

Since there are three enzyme species and three partial patterns, we shall have nine product expressions:

I		II		III	
E	k_2k_4	E	k_2k_5	E	k_3k_5
EA	k_1k_4A	EA	k_1k_5A	EA	k_4k_6P
EP	k_1k_3A	EP	k_2k_6P	EP	k_3k_6P

The fraction of each enzyme species in the steady-state mixture, the enzyme distribution equation, is the sum of all its products obtained from all partial patterns, divided by the sum of all products:

$$\frac{[\mathrm{E}]}{[\mathrm{E}_0]} = \frac{k_2k_4 + k_2k_5 + k_3k_5}{\text{denominator}} \tag{4.26}$$

$$\frac{[\mathrm{EA}]}{[\mathrm{E}_0]} = \frac{k_1k_4A + k_1k_5A + k_4k_6P}{\text{denominator}} \tag{4.27}$$

$$\frac{[\mathrm{EP}]}{[\mathrm{E}_0]} = \frac{k_2k_6P + k_3k_6P + k_1k_3A}{\text{denominator}} \tag{4.28}$$

Denominator is equal to the sum of all, in this case, nine products:

$$\text{Denominator} = (k_2k_5 + k_2k_4 + k_3k_5) + k_1(k_3 + k_4 + k_5)A + k_6(k_2 + k_3 + k_4)P \tag{4.29}$$

The initial rate of reaction (v_0) is equal $-\mathrm{d}A/\mathrm{d}t$ or $\mathrm{d}P/\mathrm{d}t$. If we consider the product, there is only one step that generates P (EA → EP), and only one step that consumes P (EP → EA). Therefore,

$$v_0 = \frac{\mathrm{d}P}{\mathrm{d}t} = k_3[\mathrm{EA}] - k_4[\mathrm{EP}] = \frac{(k_1k_3k_5A - k_2k_4k_6P)E_0}{\text{denominator}}$$

This equation is identical with Eq. (3.36) obtained previously with the initial velocity treatment.

A comparison of the derivation procedure for Eq. (3.36) described in Chapter 3 (Section 3.4) with the derivation procedure of the King–Altman method, clearly shows the advantage of the latter method. With increased complexity of mechanisms, the advantage of the King–Altman method increases dramatically.

4.3.2 Ordered Bi Bi Mechanism

Let us consider a more complicated mechanism, an Ordered Bi Bi mechanism which is very common with bisubstrate enzymes. In this mechanism, an enzyme reacts with two substrates in an ordered fashion affording two products, which are also released in an ordered fashion,

$$\underset{1}{\mathrm{E}} \underset{k_2}{\overset{k_1\mathrm{A}}{\rightleftharpoons}} \underset{2}{\mathrm{EA}} \underset{k_4}{\overset{k_3\mathrm{B}}{\rightleftharpoons}} \underset{3}{(\mathrm{EAB} == \mathrm{EPQ})} \underset{k_6\mathrm{P}}{\overset{k_5}{\rightleftharpoons}} \underset{4}{\mathrm{EQ}} \underset{k_8\mathrm{Q}}{\overset{k_7}{\rightleftharpoons}} \underset{1}{\mathrm{E}} \qquad (4.30)$$

Let us proceed with the derivation of a rate law for this mechanism with the aid of the King-Altman method. First, let us draw a master pattern as a closed loop, showing all enzyme forms and the reaction between them (Fig. 1).

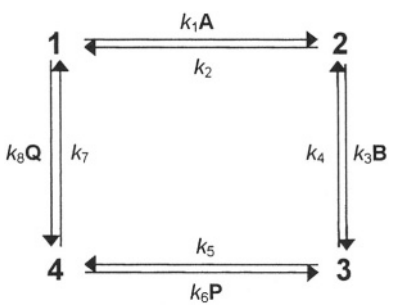
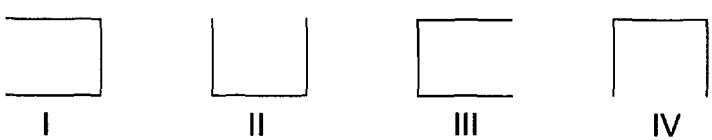


Figure 1. The King–Altman master pattern for an Ordered Bi Bi mechanism.

Second, let us draw all partial patterns from the master pattern. Since the mechanism has only four enzyme forms E, EA, (EAB==EPQ) and EQ, which are connected in a single closed loop, there will be four partial patterns:



Write all product expressions for each partial pattern and for each enzyme species, keeping in mind the rules outlined in Section 4.3.1. Since here are four partial patterns and four enzyme forms, there will be 16 product expressions

(for the sake of simplicity, we shall abbreviate the rate constants with their subscripts, such as $k_1 = 1$, $k_2 = 2$, etc.).

Enzyme species	Pattern I	Pattern II	Pattern III	Pattern IV
E	P.2.4.6	B.3.5.7	2.5.7	2.4.7
EA	AP.1.4.6	PQ.4.6.8	A.1.5.7	A.1.4.7
EAB = EPQ	ABP.1.3.6	BPQ.3.6.8	PQ.2.6.8	AB.1.3.7
EQ	AB.1.3.5	BQ.3.5.8	Q.2.5.8	Q.2.4.8

After all 16 rate constants were successfully identified, one can easily sort them out in the form of enzyme distribution equations:

$$\frac{[E]}{[E_0]} = \frac{P.2.4.6 + B.3.5.7 + 2.5.7 + 2.4.7}{\text{denominator}}$$

$$\frac{[EA]}{[E_0]} = \frac{AP.1.4.6 + PQ.4.6.8 + A.1.5.7 + A.1.4.7}{\text{denominator}}$$

$$\frac{[EAB]}{[E_0]} = \frac{ABP.1.3.6 + BPQ.3.6.8 + PQ.2.6.8 + AB.1.3.7}{\text{denominator}}$$

$$\frac{[EQ]}{[E_0]} = \frac{AB.1.3.5 + BQ.3.5.8 + Q.2.5.8 + Q.2.4.8}{\text{denominator}} \tag{4.31}$$

The next step is to sort out all product expressions in a coefficient form, in order to make further operations easier. In doing so, we shall revert to the usual notation for rate constants:

$$\begin{aligned}
&\text{Constant} = k_2k_7(k_4 + k_5) && (\text{Coef PQ})PQ = k_6k_8(k_2 + k_4)PQ \\
&(\text{Coef A})A = k_1k_7(k_4 + k_5)A && (\text{Coef AP})AP = k_1k_4k_6AP \\
&(\text{Coef B})B = k_3k_5k_7B && (\text{Coef BQ})BQ = k_3k_5k_8BQ \\
&(\text{Coef P})P = k_2k_4k_6P && (\text{Coef ABP})ABP = k_1k_3k_6ABP \\
&(\text{Coef Q})Q = k_2k_8(k_4 + k_5)Q && (\text{Coef BPQ})BPQ = k_3k_6k_8BPQ \\
&(\text{Coef AB})AB = k_1k_3(k_5 + k_7)AB
\end{aligned} \tag{4.32}$$

The net steady-state velocity is then the net rate given by the difference between the forward and reverse velocities of any step. If we choose EA, there is one step that generates EA **(E+A → EA)** and one step that consumes EA **(EA → A+E)**, so we have

$$v_0 = k_1[E][A] - k_2[EA] \tag{4.33}$$

From Figure 1, it follows that the net rate that generates EA is equal $k_4k_6k_8PQ$ and the net rate that consumes EA is equal $k_3k_5k_7B$. Now, the rate law for reaction (4.33) can be written in the following form:

$$v_0 = \left[k_1[\mathrm{E_0}][\mathrm{A}]\left(\frac{k_3k_5k_7B}{\text{denominator}}\right)\right] - \left[k_2[\mathrm{E_0}]\left(\frac{k_4k_6k_8PQ}{\text{denominator}}\right)\right] \tag{4.34}$$

The denominator in this equation is a sum of all rate constants in Eq. (4.31). Consequently, one can write the rate Eq. (4.34) in terms of rate constants, and then proceed further by grouping the rate constants in a coefficient form:

$$\begin{aligned} v_0 = {} & \frac{(\text{Numerator}_1AB - \text{Numerator}_2PQ)E_0}{\text{Constant} + (\text{CoefA})A + (\text{CoefB})B + (\text{CoefAB})AB + (\text{CoefP})P} \\ & + (\text{CoefQ})Q + (\text{CoefPQ})PQ + (\text{CoefAP})AP \\ & + (\text{CoefBQ})BQ + (\text{CoefABP})ABP + (\text{CoefBPQ})BPQ \end{aligned} \tag{4.35}$$

The numerator terms in this equation have the values:

$$\text{Numerator}_1 = k_1k_3k_5k_7 \quad \text{Numerator}_2 = k_2k_4k_6k_8 \tag{4.36}$$

Now, one can proceed one step further by grouping the rate constants in the form of Michaelis parameters:

$$\begin{aligned} K_\mathrm{A} &= \frac{\text{CoefB}}{\text{CoefAB}} \quad K_\mathrm{iA} = \frac{\text{Constant}}{\text{CoefA}} = \frac{\text{CoefP}}{\text{CoefAP}} \quad V_1 = \frac{\text{Numerator}_1}{\text{CoefAB}} \\ K_\mathrm{B} &= \frac{\text{CoefA}}{\text{CoefAB}} \quad K_\mathrm{iQ} = \frac{\text{Constant}}{\text{CoefQ}} = \frac{\text{CoefB}}{\text{CoefBQ}} \quad V_2 = \frac{\text{Numerator}_2}{\text{CoefPQ}} \\ K_\mathrm{P} &= \frac{\text{CoefQ}}{\text{CoefPQ}} \quad K_\mathrm{iP} = \frac{\text{CoefAB}}{\text{CoefABP}} \quad K_\mathrm{eq} = \frac{\text{Numerator}_1}{\text{Numerator}_2} \\ K_\mathrm{Q} &= \frac{\text{CoefP}}{\text{CoefPQ}} \quad K_\mathrm{iB} = \frac{\text{CoefPQ}}{\text{CoefBPQ}} \end{aligned} \tag{4.37}$$

4.3.3 Transformation of the Velocity Equation

Equation (4.35) is the rate law for the Ordered Bi Bi mechanism written in terms of all rate constants in the mechanism. It is necessary now, for practical purposes, to rewrite the rate law in terms of kinetic constants. The process is shown by Eq. (4.38) (Schultz, 1994).

$$\begin{aligned} \frac{v_0}{E_0} = {} & \frac{\left(\frac{\text{numerator}_1}{\text{coefAB}}\frac{\text{numerator}_2}{\text{coefPQ}}\right)AB - \left(\frac{\text{numerator}_2}{\text{coefAB}}\frac{\text{numerator}_2}{\text{coefPQ}}\frac{\text{numerator}_1}{\text{numerator}_1}\right)PQ}{\left(\frac{\text{constant}}{\text{coefAB}}\frac{\text{numerator}_2}{\text{coefPQ}}\frac{\text{coefA}}{\text{coefA}}\right) + \left(\frac{\text{coefA}}{\text{coefAB}}\frac{\text{numerator}_2}{\text{coefPQ}}\right)A} \\ & + \left(\frac{\text{coefB}}{\text{coefAB}}\frac{\text{numerator}_2}{\text{coefPQ}}\right)B + \left(\frac{\text{coefAB}}{\text{coefAB}}\frac{\text{numerator}_2}{\text{coefPQ}}\right)AB \\ & + \left(\frac{\text{coefP}}{\text{coefAB}}\frac{\text{numerator}_2}{\text{coefPQ}}\frac{\text{numerator}_1}{\text{numerator}_1}\right)P \end{aligned}$$

$$+\left(\frac{\text{coefQ}}{\text{coefAB}}\frac{\text{numerator}_2}{\text{coefPQ}}\frac{\text{numerator}_1}{\text{numerator}_1}\right)Q$$

$$+\left(\frac{\text{coefPQ}}{\text{coefAB}}\frac{\text{numerator}_2}{\text{coefPQ}}\frac{\text{numerator}_1}{\text{numerator}_1}\right)PQ$$

$$+\left(\frac{\text{coefAP}}{\text{coefAB}}\frac{\text{numerator}_2}{\text{coefPQ}}\frac{\text{numerator}_1}{\text{numerator}_1}\frac{\text{coefP}}{\text{coefP}}\right)AP$$

$$+\left(\frac{\text{coefBQ}}{\text{coefAB}}\frac{\text{numerator}_2}{\text{coefPQ}}\frac{\text{coefB}}{\text{coefB}}\right)BQ+\left(\frac{\text{coefABP}}{\text{coefAB}}\frac{\text{numerator}_2}{\text{coefPQ}}\right)ABP$$

$$+\left(\frac{\text{coefBPQ}}{\text{coefAB}}\frac{\text{numerator}_2}{\text{coefPQ}}\frac{\text{numerator}_1}{\text{numerator}_1}\right)BPQ \qquad (4.38)$$

With the foregoing definition of kinetic constants, Eq. (4.38) can be reformulated into the kinetic form, and thus written by using the kinetic parameters only:

$$v_o=\frac{V_1V_2\left(AB-\dfrac{PQ}{K_{eq}}\right)}{K_{iA}K_BV_2+K_BV_2A+K_AV_2B+V_2AB+\dfrac{K_QV_1}{K_{eq}}P+\dfrac{K_PV_1}{K_{eq}}Q+\dfrac{V_1}{K_{eq}}PQ}$$

$$+\frac{K_QV_1}{K_{iA}K_{eq}}AP+\frac{K_AV_2}{K_{iQ}}BQ+\frac{V_2}{K_{iP}}ABP+\frac{V_1}{K_{iB}K_{eq}}BPQ \qquad (4.39)$$

This equation is quite general and describes the effect of reactant concentrations on rate, not only at the start of reaction, in the complete absence of P and Q, but at any time during the approach to equilibrium.

4.3.4 Transformation of Distribution Equations

Enzyme distribution equations for the Ordered Bi Bi system (Eq. (4.31)) are written in terms of kinetic constants and substrate concentrations. They all have the same denominators as the rate equation and thus, if they are multiplied by the same factors given in Eq. (4.38), used to convert the rate equation into kinetic constant form, their denominators will also be expressed in terms of kinetic constants. The terms in the numerators of these equations represent entire denominator terms, which are easily expressed in terms of kinetic constants, except when some denominator terms are split between the numerators of two or more equations. There are two split denominator terms in Eqs. (4.31). The rate constants multiplying *AB* and *PQ* in the numerator of the **[EAB]/[E$_o$]** distribution are only parts of (coefAB) and (coefPQ), respectively; the other part of (coefAB) comes from the **[EQ]/[E$_o$]** distribution, and the other part of (coefPQ) comes from the **[EA]/[E$_o$]** distribution.

In such cases the numerators can be expressed in terms of kinetic constants only if certain rate constants can be expressed in terms of kinetic constants. This process, which is analogous to the transformation of the rate Eq. (4.38), is shown

for the transformation of a split numerator term in the $[EAB]/[E_o]$ distribution for the (coefAB) term.

$$k_1k_3k_7AB = \frac{k_1k_3k_7\ (\text{numerator}_2)}{(\text{coefAB})(\text{coefPQ})}AB = \frac{k_1k_3k_7V_2}{k_1k_3(k_5+k_7)}AB = \frac{V_2AB}{1+k_5/k_7} \tag{4.40}$$

$$\frac{1}{k_5}+\frac{1}{k_7}=\frac{E_0}{V_1} \qquad k_7=\frac{V_2K_{iQ}}{K_QE_0} \tag{4.41}$$

$$k_1k_3k_7AB = \left(V_2-\frac{V_1K_Q}{K_{iQ}}\right)AB \tag{4.42}$$

In Eq. (4.42), the numerator term $k_1k_3k_7$ is transformed into the kinetic constant form. Transformations (4.40)–(4.42) are possible only because the rate constants k_5 and k_7 can be expressed in terms of corresponding kinetic constants (Eqs. (4.41)) (Section 9.2). If this is not the case, the corresponding distribution equation cannot be calculated; several examples for such a limitation of the method are found in Chapter 9 and in Chapter 12.

4.3.5 Calculation of Haldane Relationships

In order to calculate the Haldane relationship for the Ordered Bi Bi mechanism, the concentration of one pair of reactants must be set to zero (Cleland, 1982). If the concentrations of A and B is negligible, it is expected that the general rate Eq. (4.39) reduces to the rate equation for the reverse reaction in the presence of P and Q, and absence of A and B:

$$v_o = \frac{-V_2PQ}{K_{iQ}K_P+K_PQ+K_QP+PQ} \tag{4.43}$$

On the other hand, when $A = B = 0$, the general rate Eq. (4.39) becomes

$$v_0 = \frac{-V_1V_2\dfrac{PQ}{K_{eq}}}{K_{iA}K_BV_2+\dfrac{K_QV_1}{K_{eq}}P+\dfrac{K_PV_1}{K_{eq}}Q+\dfrac{V_1}{K_{eq}}PQ} \tag{4.44}$$

Multiplying the numerator and denominator with K_{eq}/V_1, we obtain

$$v_0 = \frac{-V_2PQ}{\dfrac{K_{eq}K_{iA}K_BV_2}{V_1}+K_PQ+K_QP+PQ} \tag{4.45}$$

Equations (4.43) and (4.45) must be identical. Therefore,

$$\frac{K_{eq}K_{iA}K_BV_2}{V_1} = K_{iQ}K_P \tag{4.46}$$

Equation (4.46) can be rearranged in the form of a Haldane relationship:

$$K_{eq} = \frac{V_1K_{iQ}K_P}{V_2K_{iA}K_B} \tag{4.47}$$

The other Haldane relationship for the Ordered Bi Bi mechanism is

$$K_{eq} = \left(\frac{V_1}{V_2}\right)^2 \frac{K_{iP}K_Q}{K_{iB}K_A} \tag{4.48}$$

4.3.6 King–Altman Patterns

The first task in the application of the King–Altman method is to write down the correct mechanism. The second task is to write all possible patterns from the mechanism. This is the difficult part, because some patterns may be easily

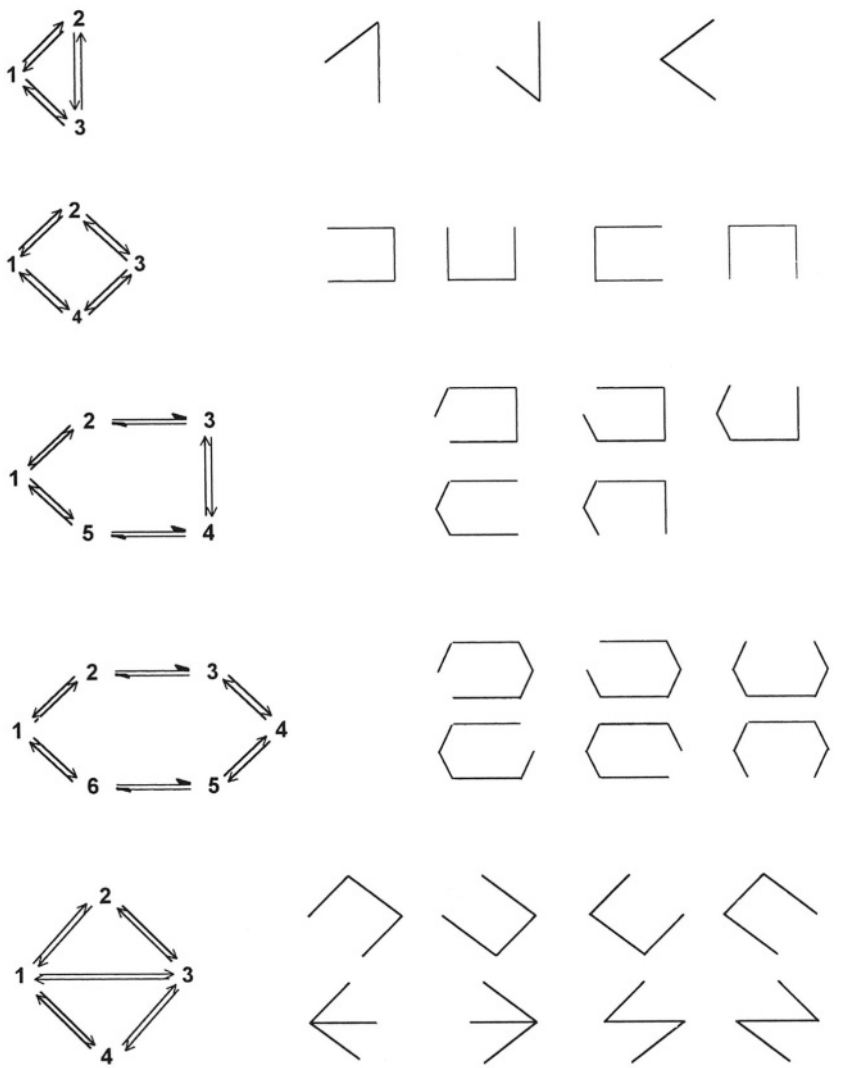


Figure 2. The King–Altman patterns for major bisubstrate and trisubstrate mechanisms.

omitted or overlooked. Figure 2 and Table 1 show the King–Altman patterns for the major types of bisubstrate and trisubstrate mechanisms.

Table 1. Assignment of King–Altman patterns shown in Fig. 2 for major steady-state mechanisms

Triangle
Uni Uni (Section 4.3)
Ordered Uni Bi (Section 9.4)
Theorell-Chance (Section (9.3)
Square
Ordered Bi Bi with one central complex (Section 9.2)
Ping Pong Bi Bi (Section 9.5)
Pentagon
Ordered Ter Bi (Section 12.3)
Ordered Bi Bi with two central complex (Section 9.2)
Hexagon
Ordered Ter Ter (Section 12.4)
Bi Bi Uni Uni Ping Pong (Section 12.4)
Bi Uni Uni Bi Ping Pong (Section 12.4)
Hexa Uni Ping Pong (Section 12.4)
A crossed square
Steady-State Random Bi Uni (Section 9.6)

The last example in Figure 2 clearly shows why, in complex cases, the finding of partial patterns from the master pattern is the most difficult part of the King–Altman method. Often, especially in complex mechanisms, it is not easy to write down all possible King–Altman patterns, and errors are a common occurrence.

The patterns containing one less line than the basic figure represent all the ways any one enzyme species can be formed from the others by path not involving closed loops. The total number of interconversion patterns is given by

$$\text{number of patterns with } (n-1) \text{ lines} = \frac{m!}{(n-1)!(m-n+1)!} \tag{4.49}$$

where n is the number of enzyme species or the number of corners in the basic figure and m the number of lines in the basic figure.

Let us take the last example in Table 1, the Steady-State Random Bi Uni mechanism. The number of enzyme species or corners in the basic figure is four and the number of lines is five. Thus,

$$\text{number of patterns with } (n-1) \text{ lines} = \frac{5!}{(4-1)!(5-4+1)!} = \frac{120}{6.2} = 10$$

However, two King-Altman patterns contain closed loops:

and, therefore, the total number of patterns must be diminished by two, which bring us to 10–2 = 8 King–Altman patterns shown in Fig. 2.

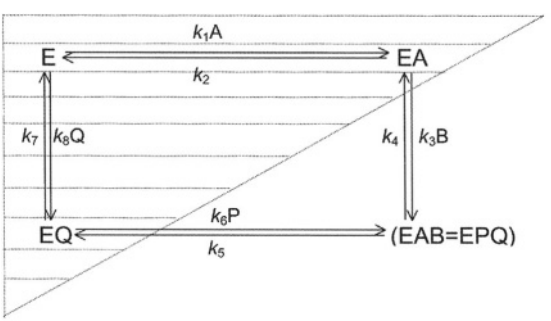
4.4 THE METHOD OF CHA

The King–Altman method is convenient and simple to apply to any of the simpler enzyme mechanisms. However, complex mechanisms often require very large number of patterns to be found. The derivation is then very laborious, and liable to errors on account of overlooking patterns or writing down incorrect terms. Therefore, the King–Altman method is not practical for analyzing complex mechanisms with six or more enzyme forms. In general, for complex mechanisms, it is better to search for means of simplifying the procedure.

In this respect, very efficient modifications of the King–Altman method have been described by Wong and Hanes (1962), Volkenstein and Goldstein (1966), Gulbinsky and Cleland (1968), Fromm (1975) and others. A special attention deserves the method of Cha (1968) which is a very useful and a very widespread simplification of the King–Altman method (Topham & Brockelhurst, 1992).

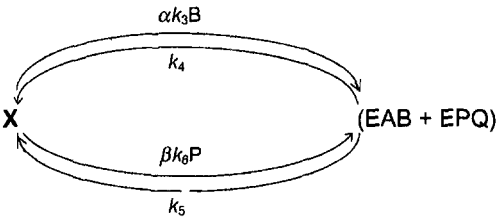
Cha (1968) has described a method for analyzing mechanisms that contain steps in equilibrium that is much simpler than the complete King–Altman analysis because each group of enzyme forms at equilibrium can be treated as a single species. Thus, the method of Cha provides for a condensation of King–Altman patterns and shortens considerably the procedure of derivation.

In order to illustrate the Cha method, let us consider the Ordered Bi Bi system that contains the rapid equilibrium segments (Reaction (4.50)).



(4.50)

The shaded area represents the rapid equilibrium segments. The binding and dissociation of the first substrate and the last product are both significantly faster than the other steps in the above reaction. The King–Altman master pattern can now be condensed into only two lines connecting X and the central complexes (EAB + EPQ), where [X] = [E] + [EA] + [EQ] (Reaction 4.51).



(4.51)

Thus, the King–Altman master pattern has now only two corners instead of four and consequently only two partial patterns. However, only a proportion of X will react now in one direction and an another proportion of X in the other direction. The symbols α and β now represent the fractional concentrations and stand for the relative proportion of the rapid equilibrium segment, X, that actually is involved in the given reaction. That is, of all the species comprising X, only EA will react with B to yield EAB and, in the opposite direction, only EQ will react with P to produce EPQ.

Now, one can begin the implementation of the Cha method by starting with the rapid equilibrium treatment first. Using the rapid equilibrium treatment of the (EQ ↔ E ↔ EA) segment, we shall obtain

$$\alpha = \frac{[EA]}{[X]} = \frac{\dfrac{A}{K_A}}{1 + \dfrac{A}{K_A} + \dfrac{Q}{K_Q}} = \frac{\dfrac{k_1}{k_2}A}{1 + \dfrac{k_1}{k_2}A + \dfrac{k_8}{k_7}Q} = \frac{k_1 k_7 A}{k_2 k_7 + k_1 k_7 A + k_2 k_8 Q} \tag{4.52}$$

$$\beta = \frac{[EQ]}{[X]} = \frac{\dfrac{Q}{K_Q}}{1 + \dfrac{A}{K_A} + \dfrac{Q}{K_Q}} = \frac{\dfrac{k_8}{k_7}Q}{1 + \dfrac{k_1}{k_2}A + \dfrac{k_8}{k_7}Q} = \frac{k_2 k_8 Q}{k_2 k_7 + k_1 k_7 A + k_2 k_8 Q} \tag{4.53}$$

Now, we can leave the rapid equilibrium treatment and proceed with the King–Altman treatment. The rate constants αk_3 and βk_6 in reaction (4.51) now represent the effective or apparent rate constants. The enzyme distribution equations are now:

$$\frac{[X]}{[E_o]} = \frac{k_4 + k_5}{\text{denominator}} \quad \text{and} \quad \frac{[EAB + EPQ]}{[E_o]} = \frac{\alpha k_3 B + \beta k_6 P}{\text{denominator}} \tag{4.54}$$

where denominator is the sum of both numerators. The velocity equation is obtained from the steady-state equation:

$$v_o = k_3[EA][B] - k_4[EAB + EPQ] = \alpha k_3[X][B] - k_4[EAB + EPQ] \tag{4.55}$$

After the substitution of [EA] from Eq. (4.52) and [EAB+EPQ] from Eq. (4.54) into the above equation, we obtain the rate equation containing only the rate constants and the concentration of reactants:

$$\frac{v_0}{[E_0]} = \frac{\alpha k_3 k_5 B - \beta k_4 k_6 P}{k_4 + k_5 + \alpha k_3 B + \beta k_6 P} \tag{4.56}$$

After the substitution of fractional concentrations of EA and EQ, α and β, into the above equation, we obtain

$$\frac{v_0}{[E_0]} = \frac{k_1 k_3 k_5 k_7 AB - k_2 k_4 k_6 k_8 PQ}{k_2 k_7 (k_4 + k_5) + k_1 k_7 (k_4 + k_5) A + k_2 k_8 (k_4 + k_5) Q + k_1 k_3 k_7 AB + k_2 k_6 k_8 PQ} \tag{4.57}$$

Equation (4.57) can also be transformed into a usual form containing only the kinetic constants instead of the rate constants:

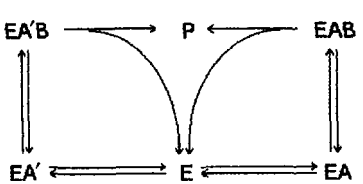
$$v_0 = \frac{V_1 V_2 \left(AB - \dfrac{PQ}{K_{eq}} \right)}{V_2 K_{iA} K_B + V_2 K_B A + \dfrac{V_1 K_P}{K_{eq}} Q + V_2 AB + \dfrac{V_1}{K_{eq}} PQ} \tag{4.58}$$

The velocity equation has now the same form as that for the total Rapid Equilibrium Ordered Bi Bi mechanism (Eq. (8.12)), except that the constants associated with B and P are the Michaelis constants and the constants associated with A and Q are the dissociation constants.

4.5 THE SYSTEMATIC APPROACH

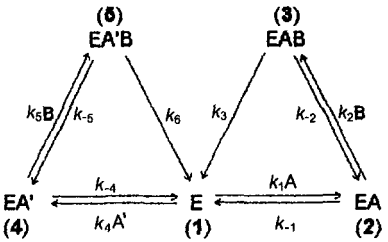
The systematic approach for deriving rate equations was first devised by Fromm (1975) based on certain concepts advanced by Volkenstein and Goldstein (1966); Volkenstein and Goldstein (1966) have applied the theory of graphs to the derivation of rate equations. The procedure to be described here is a modified method that includes the contribution from the above-mentioned workers and from Wong and Hanes (1962); its underlying principles, however, are more akin to the graphic method of King and Altman (1956).

Consider an example of the Ordered Bi Bi mechanism, in which an alternative substrate, A′, for the leading substrate, A, is present, and both substrates afford the same product P in an irreversible step (Purich & Allison, 2000).



(4.59)

The first task is to draw a correct graph for the enzyme mechanism in reaction (4.59).



(4.60)

Each enzyme-containing species is assigned a node number. For each node, a node value is written, which is simply a summation of all branch values (rate constants and concentration factors) leading away from the node.

$$\begin{aligned}(1) &= k_1A + k_4A' \\ (2) &= k_{-1} + k_2B \\ (3) &= k_{-2} + k_3 \\ (4) &= k_{-4} + k_5B \\ (5) &= k_{-5} + k_6\end{aligned} \tag{4.61}$$

The determinant for a given enzyme species is equal to the *noncycling terms* generated by multiplying together all the node values, excluding its own. Thus,

$$\begin{aligned}(E) &= (2)(3)(4)(5) = (k_{-1} + k_2B)(k_{-2} + k_3)(k_{-4} + k_5B)(k_{-5} + k_6) \\ (EA) &= (1)(3)(4)(5) = (k_1A + k_4A')(k_{-2} + k_3)(k_{-4} + k_5B)(k_{-5} + k_6) \\ (EAB) &= (1)(2)(4)(5) = (k_1A + k_4A')(k_{-1} + k_2B)(k_{-4} + k_5B)(k_{-5} + k_6)\end{aligned} \tag{4.62}$$

The *cyclic terms* are those that:

(a) contain the products of reversible steps, in this case k_1Ak_{-1}, k_2Bk_{-2}, $k_4A'k_{-4}$, and k_5Bk_{-5}, and
(b) are the products of closed loops; in this case, $k_4A'k_5Bk_6$, constituting the (1)(4)(5) loop and $k_1Ak_2Bk_3$, constituting the (1)(2)(3) loop.

Thus, the main task of the systematic approach is to remove the cycling terms in the process of expansion. We shall exercise the procedure for removing the cycling terms with an example of the central complex EAB.

From Eq. (4.62), it follows that

$$\begin{aligned}(EAB) &= (1)(2)(4)(5) = (k_1A + k_4A')(k_{-1} + k_2B)(k_{-4} + k_5B)(k_{-5} + k_6) \\ &= (\boxed{k_1Ak_{-1}} + k_4A'k_{-1} + k_1Ak_2B + k_4A'k_2B) \\ &\quad \times (k_{-4}k_{-5} + \boxed{k_5Bk_{-5}} + k_{-4}k_6 + k_5Bk_6)\end{aligned} \tag{4.63}$$

The cyclic terms—terms that contain the products of reversible steps are readily identified by their subscripts differing only in positive or negative signs. Thus, in the first expansion, the terms k_1Ak_{-1} and k_5Bk_{-5} are removed.

In the second expansion of Eq. (4.64) we obtain

$$\begin{aligned}(EAB) = &(\underline{\underline{k_4A'k_{-1}}} + k_1Ak_2B + \underline{\underline{k_4A'k_2B}}) \cdot (\underline{\underline{k_{-4}k_{-5}}}) \\ &+(\underline{\underline{k_4A'k_{-1}}} + k_1Ak_2B + \underline{\underline{k_4A'k_2B}}) \cdot (\underline{\underline{k_{-4}k_6}}) \\ &+(\underline{\underline{k_4A'k_{-1}}} + k_1Ak_2B + \underline{\underline{k_4A'k_2B}}) \cdot (\underline{\underline{k_5Bk_6}})\end{aligned} \tag{4.64}$$

In the first and the second row in Eq. (4.64), the products for the first and the last term afford the terms containing a cyclic term k_4k_{-4}, and are to be deleted to obtain the correct expression. In the last row in the above equation, the products of the first and the last term afford the terms containing the loop term $k_4A'k_5Bk_6$, and must be also deleted to obtain the correct expression.

In this way, we have deleted the cyclic terms, and thus obtain

$$\begin{aligned}(EAB) &= (k_1Ak_2B)(k_{-4}k_{-5}) + (k_1Ak_2B)(k_{-4}k_6) + (k_1Ak_2B)(k_5Bk_6) \\ &= k_1k_2k_{-4}(k_{-5} + k_6)AB + k_1k_2k_5k_6AB^2\end{aligned} \tag{4.65}$$

This procedure is simple and straightforward, and serves to remove the cyclic terms, both the reversible steps and the loop terms.

When all the loops have been removed, the resulting expression is expanded to obtain the desired determinant. Thus,

$$\begin{aligned}(EAB) &= k_1k_2k_{-4}(k_{-5} + k_6)AB + k_1k_2k_5k_6AB^2 \\ (EA) &= k_1k_{-4}(k_{-2} + k_3)(k_{-5} + k_6)A + k_1k_5k_6(k_{-2} + k_3)AB \\ (E) &= k_{-1}k_{-4}(k_{-2} + k_3)(k_{-5} + k_6) + [k_2k_3k_{-4}(k_{-5} + k_6) + k_{-1}k_5k_6(k_{-2} + k_3)]B \\ &\quad + k_2k_3k_5k_6B^2\end{aligned} \tag{4.66}$$

The determinants EA' and $EA'B$ can be obtained by the same approach, and the complete rate equation is expressed, as usual, in terms of rate constants. For this example, if the common product P is measured, we obtain

$$\frac{v_0}{E_0} = \frac{k_3(EAB) + k_6(EA'B)}{(E) + (EA) + (EAB) + (EA') + (EA'B)} \tag{4.67}$$

The above example clearly illustrates that the systematic approach has some advantages over the King–Altman method, in a sense that it does away with pattern drawing. The systematic approach, described above, can be expanded and made more efficient by using several simple graphic rules described by Fromm (1975).

4.6 COMPARISON OF DIFFERENT METHODS

For relatively simple mechanisms, all the diagrammatic and systematic procedures are quite convenient. The King–Altman method is best suited for single-loop mechanisms, but becomes laborious for more complex cases with five or more enzyme forms because of the work involved in the calculation and drawing of valid patterns. With multiple reaction schemes involving four to five

enzyme species, the systematic approach requires the least effort, especially when irreversible steps are present, since it does away with pattern drawing. When the number of enzyme forms reaches six or more in a mechanism with several alternate pathways, all the manual methods become tedious owing to the sheer number of terms involved.

Although there are several efficient methods for deriving steady-state rate equations, one has to be proficient at only one of them since, for a given reaction scheme, they all lead to the same equation.

4.6.1 Derivation of Rate Equations by Computer

The derivation of a rate equation, whether by the method of King and Altman or in any other way, is a purely mechanical process. As such, it is ideal for computer implementation, and a number of computer programs for deriving rate equations have been described (Rhoads & Pring, 1968; Hurst, 1964,1969; Fisher & Schultz, 1969; Rudolph & Fromm, 1971; Kinderlerer & Ainsworth, 1976; Cornish-Bowden, 1977; Fromm, 1979; Lam, 1981; Ishikawa *et al.,* 1988; Runyan & Gunn, 1989; Varon *et al.,* 1997). In almost all cases, the investigators have written computer programs, many of which require computer programming expertise, in order to obtain rate equations for complex kinetic mechanisms (Runyan & Gunn, 1989).

Recently, however, Fromm has described a computer-assisted procedures for deriving steady-state rate equations using only commercial, and widely available mathematical programs (Fromm & Fromm, 1999); in this way, one can derive even complex rate equations without a special programming expertise. Thus, with the advent of computer technology, the derivation of kinetic equations has passed away from manual algebraic manipulations to more rapid and much more reliable computers.

REFERENCES

Books

Fromm, H.J. (1975) *Initial rate enzyme kinetics,* Springer-Verlag, Berlin.
Lam, C.F. (1981) *Techniques for the analysis and modeling of enzyme kinetics mechanisms,* Research Studies Press, Chichester.
Purich, D.L. & Allison, R.D. (2000) *Handbook of biochemical kinetics,* Academic Press, San Diego.
Schultz, A.R, (1994) *Enzyme kinetics,* Cambridge University Press, Cambridge.
Wong, J.T. (1975) *Kinetics of enzyme mechanisms,* Academic Press, London.

Review articles

Cleland, W.W. (1982) *Methods Enzymol. 87*, 366–370.
Fromm, H.J. (1979) *Methods Enzymol. 63*, 84–103.
Huang, C.Y. (1979) *Methods Enzymol. 63*, 54–84.
Runyan, K.R. & Gunn, R.B. (1989) *Methods Enzymol. 171*, 164–190.

Specific references

Briggs, G.E. & Haldane, J.B.S. (1925) *Biochem. J. 19*, 338–339.
Cha, S. (1968) *J. Biol. Chem. 243*, 820–825.

Chou, K.-C. (1990) *Biophys. Chem. 35*, 1–24.
Cleland, W.W. (1975) *Biochemistry 14*, 3220–3224.
Cornish-Bowden, A. (1977) *Biochem. J. 165*, 55–59.
Fisher, D.D. & Schultz, A.R. (1969) *Math. Biosci. 4*, 189–200.
Gulbinsky, J.S. & Cleland, W.W. (1968) *Biochemistry 7*, 566–575.
Fromm, S.J. & Fromm, H.J. (1999) *Biochem. Biophys. Res. Comm. 265*, 448–452.
Hurst, R.O. (1967) *Can. J. Biochem. 45*, 2015–2039.
Hurst, R.O. (1969) *Can. J. Biochem. 47*, 941–944.
Ishikawa, H., Maeda, T., Kikita, H., & Miyatake, K. (1988) *Biochem. J. 251*, 175–181.
Kinderlerer, J. & Ainsworth, S. (1976) *Int. J. Biomed. Comput. 7*, 1–20.
King, E.L. & Altman, C. (1956) *J. Phys. Chem. 60*, 1375–1378.
Rhoads, D.G. & Pring, M. (1968) *J. Theor. Biol. 20*, 294–313.
Rudolph, F.B. & Fromm, H.J. (1971) *Arch. Biochem. Biophys. 147*, 515–526.
Topham, C.M. & Brockelhurst, K. (1992) *Biochem. J. 282*, 261–265.
Varon. R., Garcia-Sevilla, F., Garcia-Moreno, M,, Garcia-Canovas, F., Peyro, R., & Duggleby, R.G. (1997) *Comput. Appl. Biosci. 13*, 159–167.
Volkenstein, M.V. & Goldstein, B.N. (1966) *Biochim. Biophys. Acta 115*, 471– 485.
Wong, J.T. & Hanes, C.S. (1962) *Can. J. Biochem. Physiol. 40*, 763–804.

This page intentionally left blank

Chapter 5

Linear Inhibition

The catalytic activity of enzymes is inhibited by many hundreds of natural compounds and by many thousands of artificially synthesized chemical compounds. *Inhibitors* are chemical compounds that reduce the rate of enzyme-catalyzed reactions when they are added to the reaction mixture. Inhibition can arise in a wide variety of ways, and there are many different types of inhibitors.

5.1 CLASSIFICATION OF INHIBITORS

The first major division of inhibitors is into two large groups of compounds: irreversible and reversible inhibitors. *Irreversible inhibitors* are enzyme poisons, often chemically reactive compounds, which enter into chemical reactions with enzymes, forming irreversibly covalent bonds with the enzyme and reducing its activity to zero. An enzyme inactivated by an irreversible inhibitor cannot be reactivated by dialysis or a similar mild physical procedure. Many enzymes are poisoned by trace amounts of heavy metal ions; this type of inhibition can be, in principle, reversed by dialysis against chelators such as EDTA or histidine.

A much more important class of inhibitors for the study of enzyme kinetics is that of *reversible inhibitors.* Reversible inhibitors usually form noncovalent complexes with various enzyme forms and thus lower the amount of enzyme available for participation in the normal reaction sequence. Reversible inhibitors can be removed by dialysis, and the catalytic activity of enzyme restored completely.

All reversible inhibitors form dynamic complexes with the enzyme that have different catalytic properties from those of the free enzyme. Reversible inhibitors are divided, according to their influence on the kinetic behavior and on kinetic parameters of the enzymatic reaction, into three major types: (1) *competitive,* (2) *noncompetitive,* and (3) *uncompetitive.*

It is important to note that the competitive inhibition will take place in enzyme reactions with one or more substrates or products, whereas the noncompetitive and uncompetitive inhibition will take place almost only in reactions with two or more substrates or products (Cleland, 1963; Dixon & Webb, 1979; Fromm, 1979, 1995).

Further division of reversible inhibitors is made according to their influence on the form of rate equations; thus, we can make a difference between the *linear* and a *nonlinear inhibition.* In Chapter 5, we shall describe the linear inhibition and in Chapter 6 the main forms of the nonlinear inhibition. Thus, we could distinguish the various types of inhibition still further by referring to competitive inhibition as linear, hyperbolic, or parabolic inhibition. Even more complex forms are possible (Cleland, 1970).

5.2 COMPETITIVE INHIBITION

The simplest explanation for the competitive inhibition is that the inhibitor binds to the same site on the enzyme as the substrate, forming an abortive, nonproductive complex; inhibitor and substrate are mutually exclusive (Fromm, 1979, 1995). In other words, the substrate and inhibitor compete for the same site, so that only one enzyme–inhibitor complex is possible:

$$\begin{array}{ccccc} E + A & \overset{K_A}{\rightleftharpoons} & EA & \xrightarrow{k_{cat}} & P + E \\ + & & & & \\ I & & & & \\ \Updownarrow K_i & & & & \\ EI & & & & \end{array} \qquad (5.1)$$

where $V_1 = k_{cat}E_o$. In a simple competitive inhibition, EI is a dead-end complex, as it can break down only by dissociating back into E and I. Therefore, the concentration of enzyme–inhibitor complex is given by

$$K_i = \frac{[E][I]}{[EI]} \qquad (5.2)$$

where K_i is a true equilibrium constant which is termed the *inhibition constant*; it has a dimension of [concentration].

In this model, we must recognize that the total enzyme E_o is divided between three forms: free enzyme E, enzyme-substrate complex EA, and the enzyme–inhibitor complex EI. Keeping this in mind, a velocity equation in the presence of a competitive inhibitor can be easily derived from either rapid equilibrium or steady-state assumptions by an algebraic procedure described for monosubstrate reactions (Sections 3.1 and 3.2):

$$v_o = \frac{V_1 A}{K_A\left(1 + \dfrac{I}{K_i}\right) + A} \qquad (5.3)$$

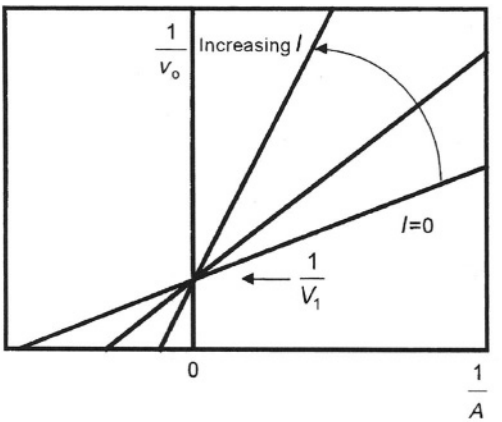
where I is the free inhibitor concentration and V_1 and K_A have their usual meanings.

The best-known example for the competitive inhibition shown in reaction (5.1) is the inhibition of succinate dehydrogenase by malonate, a compound structurally closely related to succinate (Price & Stewens, 1999).

Figure 1 shows the graphical presentation of Eq. (5.3), in three different linear forms: the Lineaweaver–Burk plot, the Hanes plot, and the Dixon plot.

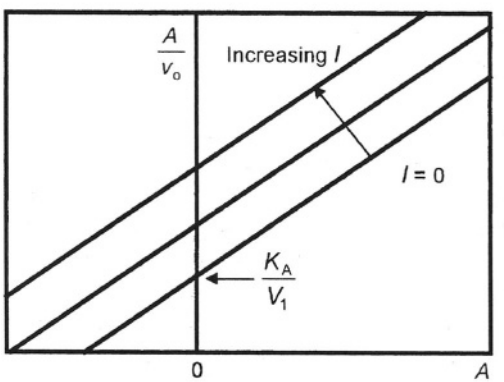
Equation (5.3) has a form of the Michaelis–Menten equation where the "apparent" kinetic constants are given by

$$V_1^{APP} = V_1 \quad K_A^{APP} = K_A\left(1 + \frac{I}{K_i}\right) \quad \left(\frac{V_1}{K_A}\right)^{APP} = \frac{\left(\dfrac{V_1}{K_A}\right)}{\left(1 + \dfrac{I}{K_i}\right)} \qquad (5.4)$$



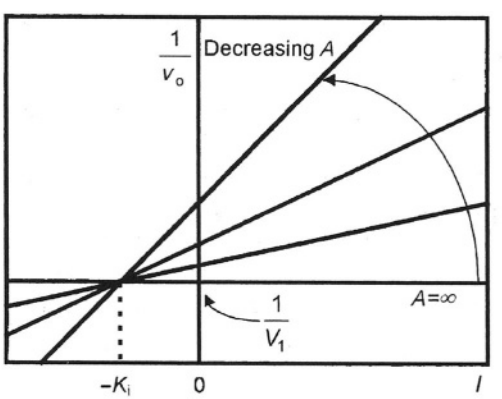
Lineweaver-Burk plot

$$\frac{1}{v_o} = \frac{1}{V_1} + \frac{K_A}{V_1}\left(1 + \frac{I}{K_i}\right)\frac{1}{A}$$



Hanes plot

$$\frac{A}{v_o} = \frac{K_A}{V_1}\left(1 + \frac{I}{K_i}\right) + \left(\frac{1}{V_1}\right)A$$



Dixon plot

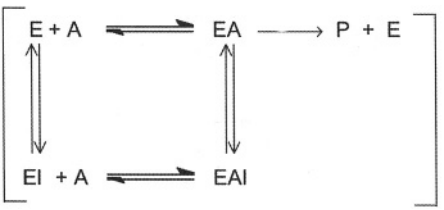
$$\frac{1}{v_o} = \frac{1}{V_1}\left(1 + \frac{K_A}{A}\right) + \frac{1}{V_1 K_i}\left(\frac{K_A}{A}\right)I$$

Figure 1. Competitive inhibition. Linear transformations of the rate Eq. (5.3).

Hence, the effect of a competitive inhibitor is to increase the apparent value of the Michaelis constant (K_A) by the factor ($1+I/K_i$), to reduce that of the specificity constant (V_1/K_A) by the same factor, and to leave V_1 unchanged.

5.3 NONCOMPETITIVE INHIBITION

In the simplest monosubstrate case, a noncompetitive inhibitor would have no effect on substrate binding and vice versa. The inhibitor and the substrate bind reversibly, randomly, and independently at different sites. The substrate binds to E and EI, and the inhibitor binds to E and EA; however, the resulting EAI complex is inactive. The binding of one ligand has no effect on the dissociation constant of the other; therefore, both binding reactions $E + A \rightleftharpoons EA$ and $EI + A \rightleftharpoons EAI$ have the same dissociation constant. The kinetic model for this type of inhibition would be

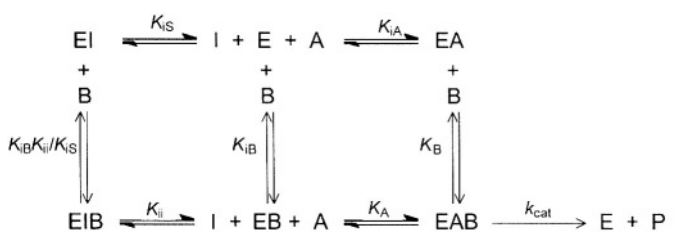


(5.5)

However, this case is extremely rare in nature. An example is the noncompetitive inhibition of phenyllactate versus an amide substrate for carboxypeptidase. In this case, the initial collision complex of substrate and enzyme has an interaction with the terminal carboxyl and the arginine on the enzyme, as well as with the rest of the polypeptide chain, but the aromatic group of the terminal amino acid is not in the specificity pocket. For it to seat itself requires twisting of the amide bond, which is the rate limiting and energy requiring step of the reaction. Thus, phenyllactate can slip into this pocket and prevent proper seating of the substrate. With an ester substrate, where rotation of the ester bond is not hindered, the collision complex has the specificity pocket filled, and phenyllactate is a competitive inhibitor (Auld & Holmquist, 1974).

This kinetic model is a usual description of a noncompetitive inhibition in biochemical textbooks, usually without specification that it is a very rare model.

As a rule, a noncompetitive inhibition occurs only if there are more than one substrate or product (Todhunter, 1979; Fromm, 1995). For example, a noncompetitive inhibition will take place in a random bisubstrate reaction, when an inhibitor competes with one substrate while the other substrate is varied. Thus, the equilibria shown below describe a Rapid Equilibrium Random bisubstrate system in which an inhibitor competes with A but allows B to bind.



(5.6)

where $V_1 = k_{cat}E_0$.

The general rate equation is

$$v_o = \frac{V_1\left(\frac{AB}{K_{iA}K_B}\right)}{1+\frac{A}{K_{iA}}+\frac{B}{K_{iB}}+\frac{I}{K_{is}}+\frac{AB}{K_{iA}K_B}+\frac{IB}{K_{ii}K_{iB}}} \tag{5.7}$$

$$v_o = \frac{V_1AB}{K_{iA}K_B\left(1+\frac{I}{K_{is}}\right)+AK_B+\frac{K_{iA}K_B}{K_{iB}}\left(1+\frac{I}{K_{ii}}\right)B+AB} \tag{5.8}$$

where $K_{iA}K_B = K_AK_{iB}$.

The general rate equation can be rearranged to show either A or B as the varied ligand.

$$\frac{1}{v_o} = \frac{1}{V_1}\left(1+\frac{K_B}{B}\right)+\frac{K_A}{V_1}\left[1+\frac{K_{iB}}{B}\left(1+\frac{I}{K_{is}}\right)+\frac{I}{K_{ii}}\right]\frac{1}{A} \tag{5.9}$$

$$\frac{1}{v_o} = \frac{1}{V_1}\left[1+\frac{K_A}{A}\left(1+\frac{I}{K_{ii}}\right)\right]+\frac{K_B}{V_1}\left[1+\frac{K_{iA}}{A}\left(1+\frac{I}{K_{is}}\right)\right]\frac{1}{B} \tag{5.10}$$

In this case, I will be a competitive inhibitor with respect to A, and a noncompetitive inhibitor with respect to B (Fig. 2).

From the primary plot of $1/v_o$ versus $1/B$ in Fig. 2 one can estimate the value of $K_{iB}K_{ii}/K_{is}$, and from the secondary Dixon plots, the inhibition constants K_{is} and K_{ii}. The crossover point in the primary plot can be above or below the axis, depending on the ratio K_{iA}/K_A.

From Eq. (5.10) one can see that, in the noncompetitive case, the apparent kinetic constants are given by

$$V_1^{APP} = \frac{V_1}{1+\frac{K_A}{A}\left(1+\frac{I}{K_{ii}}\right)} \tag{5.11}$$

$$\left(\frac{V_1}{K_B}\right)^{APP} = \frac{V_1}{K_B}\left[\frac{1}{1+(K_{iA}/A)(1+I/K_{is})}\right] \tag{5.12}$$

$$(K_B)^{APP} = K_B\left[\frac{1+\frac{K_{iA}}{A}\left(1+\frac{I}{K_{is}}\right)}{1+\frac{K_A}{A}\left(1+\frac{I}{K_{ij}}\right)}\right] \tag{5.13}$$

In contrast to the competitive case, the effect of a noncompetitive inhibitor is to decrease the apparent value of the maximal velocity, V_1, by a factor proportional to $(1+I/K_{ii})$, to decrease the specificity constant, V_1/K_B, by a factor proportional to $(1+I/K_{is})$, and to change the Michaelis constant, K_B, by a factor proportional to $(1+I/K_{is})/(1+I/K_{ii})$.

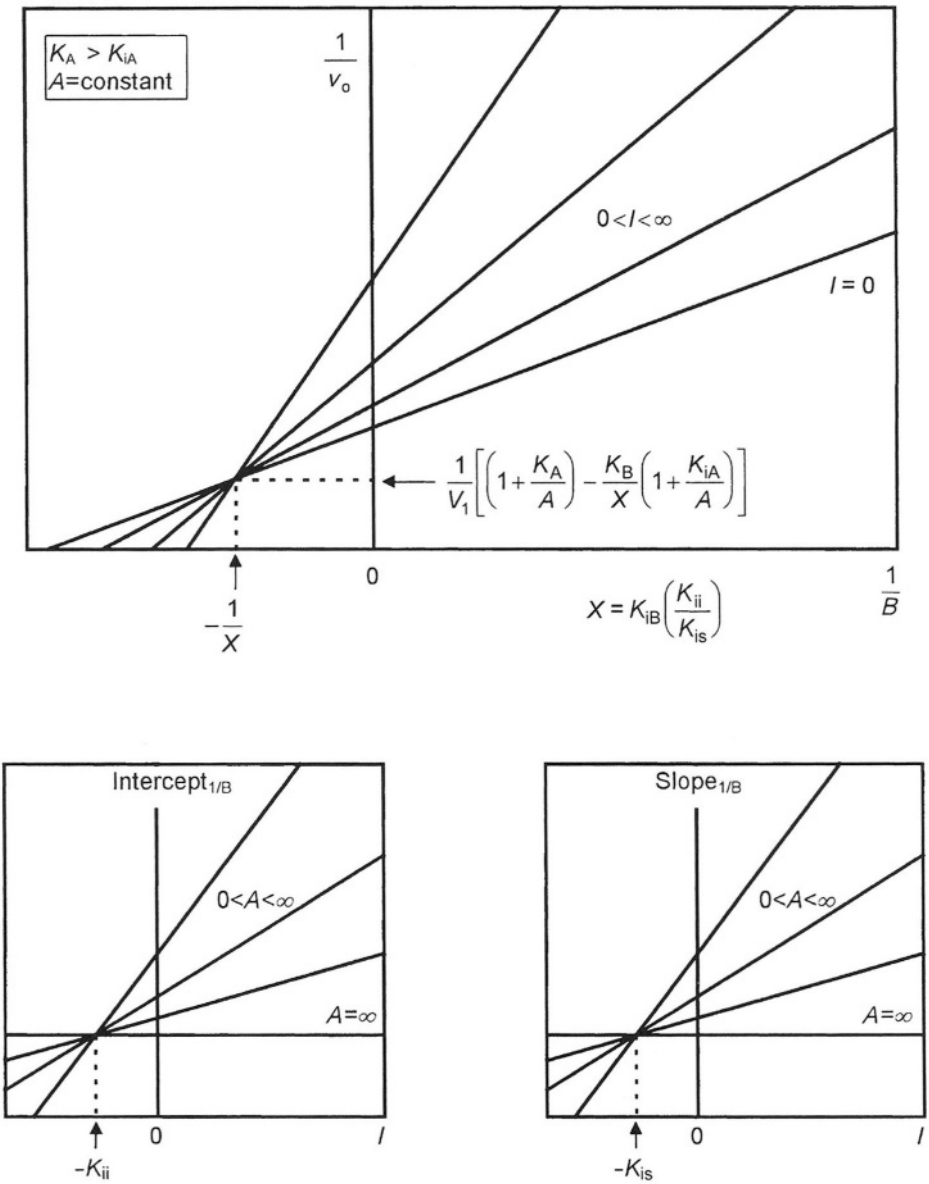
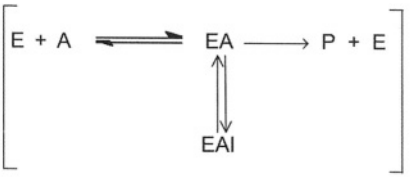


Figure 2. Noncompetitive inhibition. Rapid Equilibrium Random bisubstrate system with an inhibitor noncompetitive with B. Graphical presentation of Eq. (5.10), with A as a constant and B as a variable substrate.

$$\frac{1}{v_0} = \frac{1}{V_1}\left[1+\frac{K_A}{A}\left(1+\frac{I}{K_{ii}}\right)\right] + \frac{K_B}{V_1}\left[1+\frac{K_{iA}}{A}\left(1+\frac{I}{K_{is}}\right)\right]\frac{1}{B}$$

5.4 UNCOMPETITIVE INHIBITION

In the simplest monosubstrate case, an uncompetitive inhibitor would bind reversibly to the enzyme-substrate complex yielding an inactive EAI complex; the inhibitor does not bind to the free enzyme. The kinetic model for this type of inhibition would be

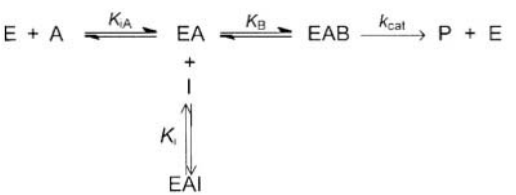


(5.14)

However, this case is not a common one. A very rare example is the reaction catalyzed by isocitrate dehydrogenase. In this reaction α-ketoglutarate shows a strong uncompetitive substrate inhibition versus NADPH or CO_2, which does not result from combination with the enzyme–NADP complex. Presumably, it occurs by imine formation with a lysine in the central complex. The closure of the active site exposes this lysine, which must have a low enough pK_a to form an imine at neutral pH. In support of this model, oxalyl-glycine, a mimic of α-ketoglutarate that binds with equal affinity, does not show the effect (Grissom & Cleland, 1988).

This kinetic model is the usual description of an uncompetitive inhibition in biochemical textbooks, again usually without specifying that it is a rare case.

As a rule, an uncompetitive inhibition occurs only if there are more than one substrate or product (Huang, 1990). For example, an uncompetitive inhibition will take place in a Rapid Equilibrium Ordered bisubstrate reaction, when an inhibitor competes with B while A is the variable substrate. Thus, the equilibria shown below describe an ordered bisubstrate system in which an inhibitor competes with B but does not bind to free enzyme.



(5.15)

The general rate equation is

$$v_0 = \frac{V_1\left(\frac{AB}{K_{iA}K_B}\right)}{1+\frac{A}{K_{iA}}+\frac{AB}{K_{iA}K_B}+\frac{IA}{K_{iA}K_i}} = \frac{V_1AB}{K_{iA}K_B+K_B\left(1+\frac{I}{K_i}\right)A+AB} \qquad (5.16)$$

where $V_1 = k_{cat}E_0$. The general rate equation can be rearranged to show either A or B as the varied ligand:

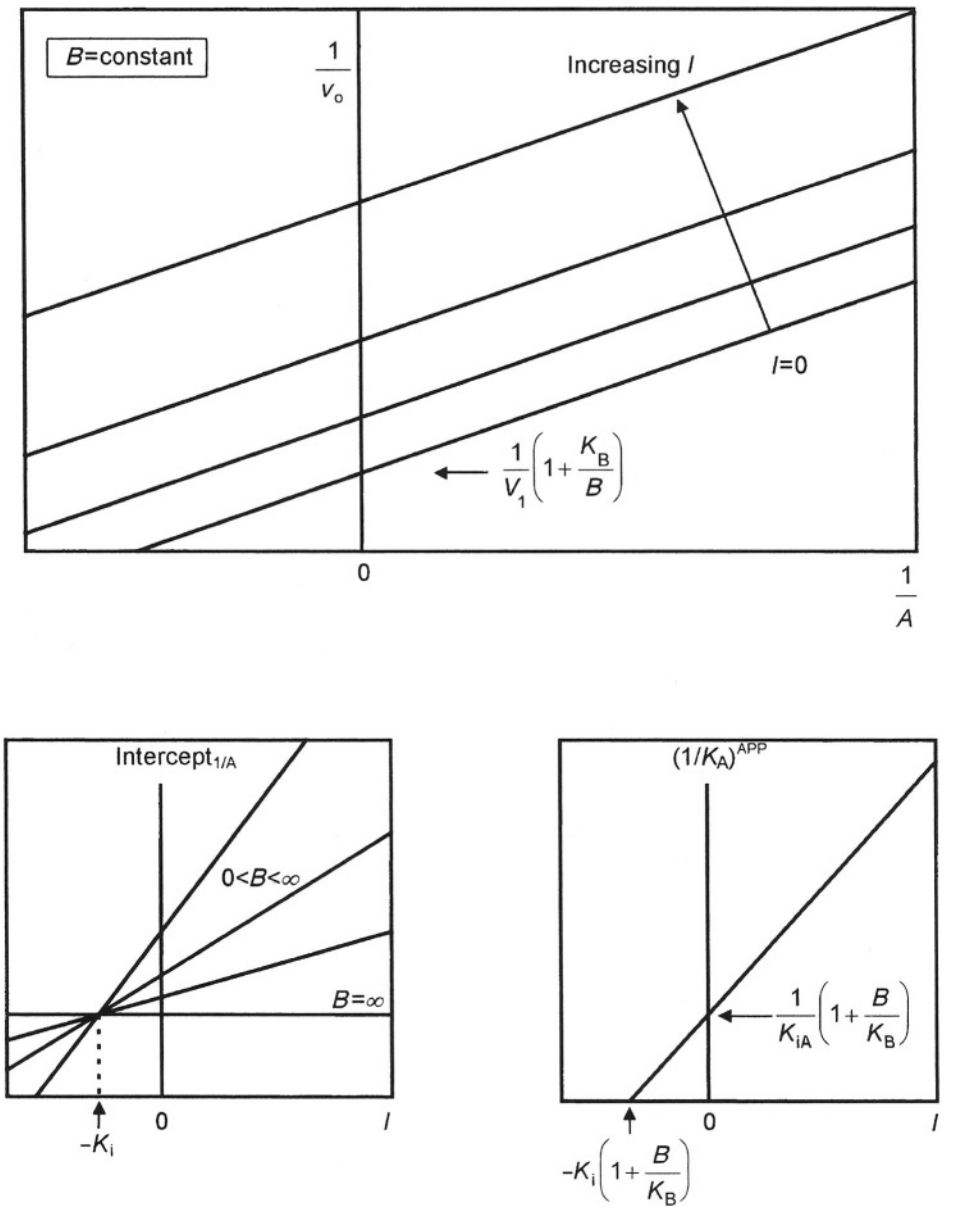


Figure 3. Uncompetitive inhibition. Rapid Equilibrium Ordered bisubstrate system with an inhibitor uncompetitive with A. Graphical presentation of Eq. (5.17), with B as a constant and A as a variable substrate.

$$\frac{1}{v_0} = \frac{1}{V_1}\left[1 + \frac{K_B}{B}\left(1 + \frac{I}{K_i}\right)\right] + \left(\frac{K_{iA}K_B}{V_1 B}\right)\frac{1}{A}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left[1 + \frac{K_B}{B}\left(1 + \frac{I}{K_i}\right)\right] + \left(\frac{K_{iA}K_B}{V_1 B}\right)\frac{1}{A} \tag{5.17}$$

$$\frac{1}{v_0} = \frac{1}{V_1} + \frac{K_B}{V_1}\left(1 + \frac{K_{iA}}{A} + \frac{I}{K_i}\right)\frac{1}{B} \tag{5.18}$$

In this case, I will be an uncompetitive inhibitor with respect to A and a competitive inhibitor with respect to B (Fig. 3).

From Eq. (5.17) one can see that, in the uncompetitive case, the apparent kinetic constants are given by

$$V_1^{APP} = \frac{V_1}{1 + \dfrac{K_B}{B}\left(1 + \dfrac{I}{K_i}\right)} \tag{5.19}$$

$$\left(\frac{V_1}{K_{iA}}\right)^{APP} = \frac{V_1}{K_{iA}}\left(\frac{B}{K_B}\right) \tag{5.20}$$

$$(K_{iA})^{APP} = K_{iA}\left[\frac{\dfrac{K_B}{B}}{1 + \dfrac{K_B}{B}\left(1 + \dfrac{I}{K_i}\right)}\right] \tag{5.21}$$

One can see from Eqs. (5.19) –(5.21) that an uncompetitive inhibition is characterized by equal effects on V_1 and K_{iA} but no effect on V_1/K_{iA}.

5.5 CHARACTERISTICS OF LINEAR INHIBITION

In the preceding sections, we have shown that all the rate equations, in the presence of a competitive, noncompetitive, or an uncompetitive inhibitor, have a form of a Michaelis–Menten equation, and can be linearized in the Lineweaver–Burk manner, in the fashion of Hanes, or in the form of Dixon plots:

Competitive: Equation (5.3); linear forms in Fig. 1.
Noncompetitive: Equation (5.7); linear form in Fig. 2 (Eq. (5.10)).
Uncompetitive: Equation (5.16); linear form in Fig. 3 (Eq. (5.17)).

All types of inhibition that have been described in preceding sections are examples of *linear inhibition,* because for all of them $(1/V_1)^{APP}$ and $(K_A/V_1)^{APP}$ display a simple linear dependence on the inhibitor concentration. Linear inhibition is also a *complete inhibition,* because the velocity approaches zero if the inhibitor concentration is high enough.

In the Lineweaver–Burk plot for *competitive inhibition* in Fig. 1, an intercept on ordinate is a constant value $(1/V_1)$, independent of the concentration of substrate or inhibitor. However, one can use the primary graph of $1/v_0$ versus $1/A$ to create the secondary plot, by plotting the slopes from the primary plots versus I. A replot of slopes versus I gives

$$\text{Slope} = \frac{K_A}{V_1} + \left(\frac{K_A}{V_1 K_i}\right) I \tag{5.22}$$

From Eq. (5.22), one can easily determine K_A and K_i, since V_1 is known from the intercepts.

In a *noncompetitive inhibition*, shown in Fig. 2, one can use the primary plot of $1/v_o$ versus $1/B$ to create the secondary graph, by plotting the slopes or intercepts from the primary plots versus I. A replot of slopes or intercepts versus I, at a constant concentration of A, gives

$$\text{Intercept}_{1/B} = \frac{1}{V_1}\left(1 + \frac{K_A}{A}\right) + \left(\frac{K_A}{V_1 A K_{ii}}\right) I \tag{5.23}$$

$$\text{Slope}_{1/B} = \frac{K_B}{V_1}\left(1 + \frac{K_{iA}}{A}\right) + \left(\frac{K_{iA} K_B}{V_1 A K_{is}}\right) I \tag{5.24}$$

From the linear plot of $\text{Intercept}_{1/B}$ versus I, one obtains the value of $K_{ii}(1+A/K_A)$ as an intercept on abscissa; similarly, from the linear plot of $\text{Slope}_{1/B}$ versus I, the value of $K_{is}(1+A/K_{iA})$ is obtained as an intercept on abscissa (Fig. 4).

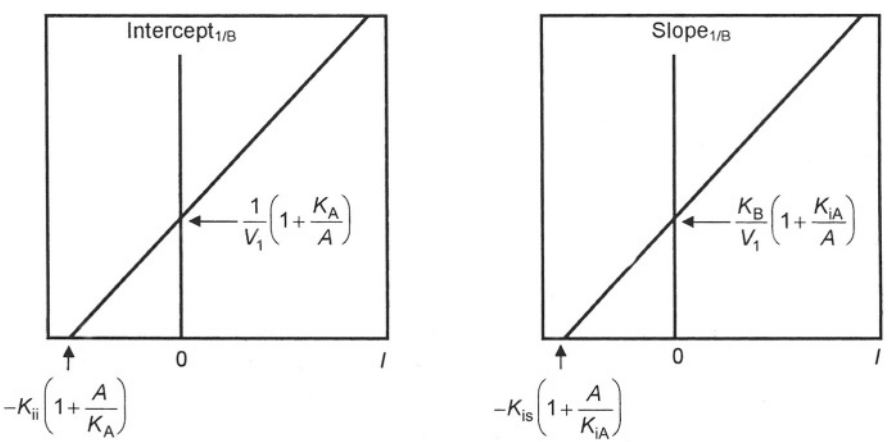


Figure 4. Noncompetitive inhibition. Graphical presentation of the intercept (Eq. (5.23)) and the slope function (Eq. (5.24)).

If both I and A are the variables, the Dixon plots in Fig. 2 are obtained. The inhibition constant K_{is} the dissociation constant of I from the enzyme–inhibitor complex, while the inhibition constant K_{ii} is the dissociation constant of I from the enzyme–substrate–inhibitor complex. Thus, a noncompetitive inhibitor has different effects on V and V/K, with two different inhibitory terms.

In an *uncompetitive inhibition,* shown in Fig. 3, one can use the primary plot of $1/v_o$ versus $1/A$ to create the secondary graph, by plotting the intercepts from the primary plots versus I. A replot of intercepts versus I, at a constant concentration of B, gives

$$\text{Intercept}_{1/A} = \frac{1}{V_1}\left(1+\frac{K_B}{B}\right) + \left(\frac{K_B}{V_1 B K_i}\right) I \qquad (5.25)$$

with $K_i(1+B/K_B)$ as an intercept on abscissal. Thus, in a similar way, from the linear plot of $\text{Intercept}_{1/A}$ versus I, one obtains the value of $K_i(1+B/K_B)$ as an intercept on abscissa. If both I and B are the variables, the Dixon plots in Fig. 3 are obtained. The inhibition constant K_i is the dissociation constant of I from the enzyme—substrate–inhibitor complex.

The properties of linear inhibitors are summarized in Table 1, where it can be seen that the effects of inhibitors on V_1^{APP}, K_A^{APP} and $(V_1/K_A)^{APP}$ are simple, regular and easy to remember.

Table 1. Influence of linear inhibitors on catalytic constants

Types of inhibition	V_{max}^{APP}	K_M^{APP}	$\left(\frac{V_{max}}{K_M}\right)^{APP}$	Equations
Competitive	V_{max}	$K_M\left(1+\frac{I}{K_i}\right)$	$\frac{\left(\frac{V_{max}}{K_M}\right)}{\left(1+\frac{I}{K_i}\right)}$	(5.4)
Noncompetitive	$\frac{(V_{max})}{\left(1+\frac{I}{K_{ii}}\right)}$	$K_M\frac{\left(1+\frac{I}{K_{is}}\right)}{\left(1+\frac{I}{K_{ii}}\right)}$	$\frac{\left(\frac{V_{max}}{K_M}\right)}{\left(1+\frac{I}{K_{is}}\right)}$	(5.11)—(5.13)
Uncompetitive	$\frac{(V_{max})}{\left(1+\frac{I}{K_i}\right)}$	$\frac{(K_M)}{\left(1+\frac{I}{K_i}\right)}$	$\frac{V_{max}}{K_M}$	(5.19)—(5.21)

The graphical presentation of rate equations shown in Figs. 1–3 is usually referred to as *Dixon plots,* according to Malcolm Dixon (Dixon, 1953).

5.6 DEAD-END INHIBITION IN STEADY-STATE BISUBSTRATE SYSTEMS

The kinetics of substrates, products, and alternate products define the form of the rate equation, and are certainly necessary to deduce the kinetic mechanism. However, they are often not sufficient to do this unequivocally and other kinetic approaches are necessary, especially when reaction can be studied in only one direction. One of the most useful approaches in such cases involves the use of dead-end inhibitors (Cleland, 1970, 1979, 1990).

Dead-end inhibitor is a compound that reacts with one or more enzyme forms to yield a complex that cannot participate in the reaction. These nonreacting

molecules compete with substrates for vacant positions in the active site and produce very specific inhibition effects, in addition to providing evidence concerning the geometry of the enzyme active site.

The velocity equation in the presence of a dead-end inhibitor can be derived in the usual manner by the King–Altman method. However, if we know the velocity equation for the uninhibited reaction, then we can easily write the new velocity equation as modified by the inhibitor, without going through an entire derivation. The effect of a dead-end inhibitor is to multiply certain terms in the denominator of the uninhibited velocity equation by the factor F ($F = 1 + I/K_i$), the *fractional concentration of an inhibitor.* The terms multiplied by F, are those representing the enzyme form, or enzyme forms, combining with the inhibitor. Then, the K_i represents the dissociation constant of the specific enzyme form-inhibitor complex.

In rapid equilibrium systems, the relative concentration of any particular enzyme form is given by a single denominator term in the velocity equation. For example, in the rapid equilibrium ordered bireactant system (Section 8.2), the velocity Eq. (8.2) is

$$v_0 = \frac{V_1 AB}{K_{iA}K_B + K_B A + AB}$$

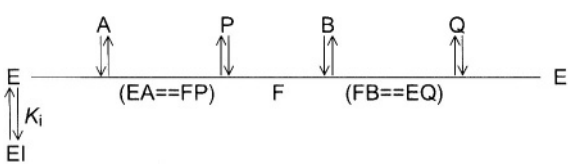
Here, $K_{iA}K_B$ represents free enzyme, $K_B A$ represents the EA complex and AB represents the EAB complex.

However, in steady-state systems, the relative concentration of a particular enzyme form may be expressed by several denominator terms, and, often, a particular denominator term represents more than one enzyme form. Generally, in steady-state systems, the denominator terms multiplied by the factor F are those which appear in the numerator of the distribution equation for the enzyme form combining with the inhibitor. If a distribution equation in terms of kinetic constants cannot be written for the enzyme form combining with the inhibitor, than K_i cannot be calculated kinetically.

To see how velocity equations are affected by a dead-end inhibitor, let us illustrate the above rule with several examples.

5.6.1 Dead-end Inhibition in a Ping Pong Bi Bi System

Consider a Steady-State Ping Pong Bi Bi system in which an inhibitor, I, combines in a dead-end fashion with the free enzyme, E.



(5.26)

The velocity equation for the forward reaction, derived from steady-state assumptions, and in the absence of any products of reaction, is (Eq. (9.59))

$$v_0 = \frac{V_1 AB}{K_B A + K_A B + AB}$$

The distribution equation for E/E_o, in the absence of P and Q, is (Eq. (9.58))

$$\frac{E}{E_o} = \frac{K_A B}{K_B A + K_A B + AB}$$

The I combines with E. Thus, in the presence of I, the $K_A B$ term in the denominator of the velocity equation is multiplied by the factor $(1+I/K_i)$:

$$v_o = \frac{V_1 AB}{K_B A + K_A B\left(1 + \frac{I}{K_i}\right) + AB} \tag{5.27}$$

The two reciprocal forms of Eq. (5.27) are

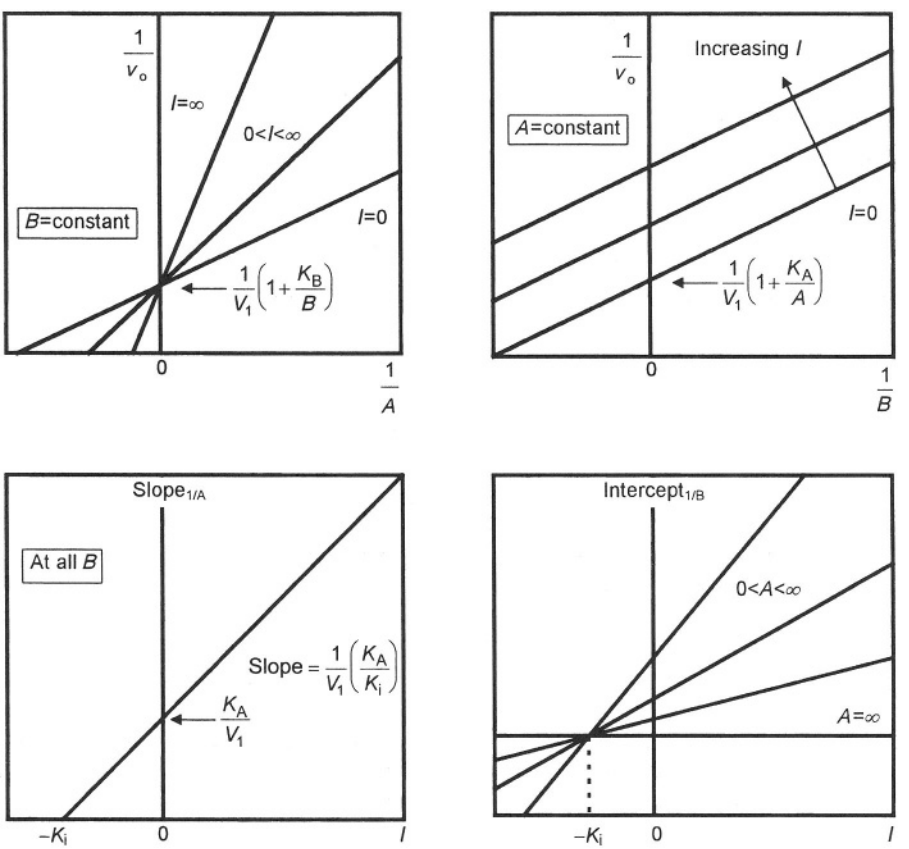


Figure 5. Dead-end inhibition in a Ping Pong Bi Bi system, when an inhibitor binds to the free enzyme, producing an EI complex. Graphical presentation of Eqs. (5.28) and (5.29).

$$\frac{1}{v_o} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \frac{K_A}{V_1}\left(1 + \frac{I}{K_i}\right)\frac{1}{A} \qquad \frac{1}{v_o} = \frac{1}{V_1}\left[1 + \frac{K_A}{A}\left(1 + \frac{I}{K_i}\right)\right] + \left(\frac{K_B}{V_1}\right)\frac{1}{B}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \frac{K_A}{V_1}\left(1 + \frac{I}{K_i}\right)\frac{1}{A} \tag{5.28}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left[1 + \frac{K_A}{A}\left(1 + \frac{I}{K_i}\right)\right] + \left(\frac{K_B}{V_1}\right)\frac{1}{B} \tag{5.29}$$

Figure 5 shows the graphical presentation of Eqs. (5.28) and (5.29).

Thus, I is a competitive inhibitor with respect to A if B is constant, but I becomes an uncompetitive inhibitor with respect to B, if A is constant.

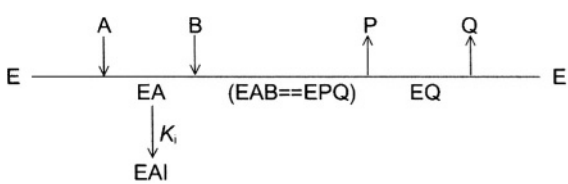
It is unlikely that an inhibitor would combine with either of the two central complexes, since all ligand-binding sites are occupied. If such a combination did occur, K_i could not be determined kinetically.

5.6.2 Dead-end Inhibition in a Rapid Equilibrium Ordered Bisubstrate System

In Section 5.4, a case of a dead-end inhibition in a Rapid Equilibrium Ordered bisubstrate system was described. One can compare this system with the following example.

5.6.3 Dead-end Inhibition in a Steady-State Ordered Bi Bi System

In this system, a frequent case is when a dead-end inhibitor combines with EA



(5.30)

In an Ordered Bi Bi system, and in the absence of products, the distribution equation for EA, is (Eq. (9.13)):

$$\frac{EA}{E_0} = \frac{V_2 K_B A}{\text{denominator of velocity equation}}$$

Thus, the K_BA term in the denominator represents the EA complex, and we must multiply the K_BA term in the rate Eq. (9.15) (Chapter 9) with $(1+I/K_i)$. Accordingly, in the absence of products, the rate equation in the presence of I becomes

$$v_0 = \frac{V_1 AB}{K_{iA}K_B + K_B A\left(1 + \frac{I}{K_i}\right) + K_A B + AB} \tag{5.31}$$

or, in the reciprocal forms:

$$\frac{1}{v_o} = \frac{1}{V_1}\left[1 + \frac{K_B}{B}\left(1 + \frac{I}{K_i}\right)\right] + \frac{K_A}{V_1}\left(1 + \frac{K_{iA}K_B}{K_A B}\right)\frac{1}{A} \tag{5.32}$$

$$\frac{1}{v_o} = \frac{1}{V_1}\left(1 + \frac{K_A}{A}\right) + \frac{K_B}{V_1}\left(1 + \frac{K_{iA}}{A} + \frac{I}{K_i}\right)\frac{1}{B} \tag{5.33}$$

Figure 6 shows the graphical presentation of Eqs. (5.32) and (5.33).

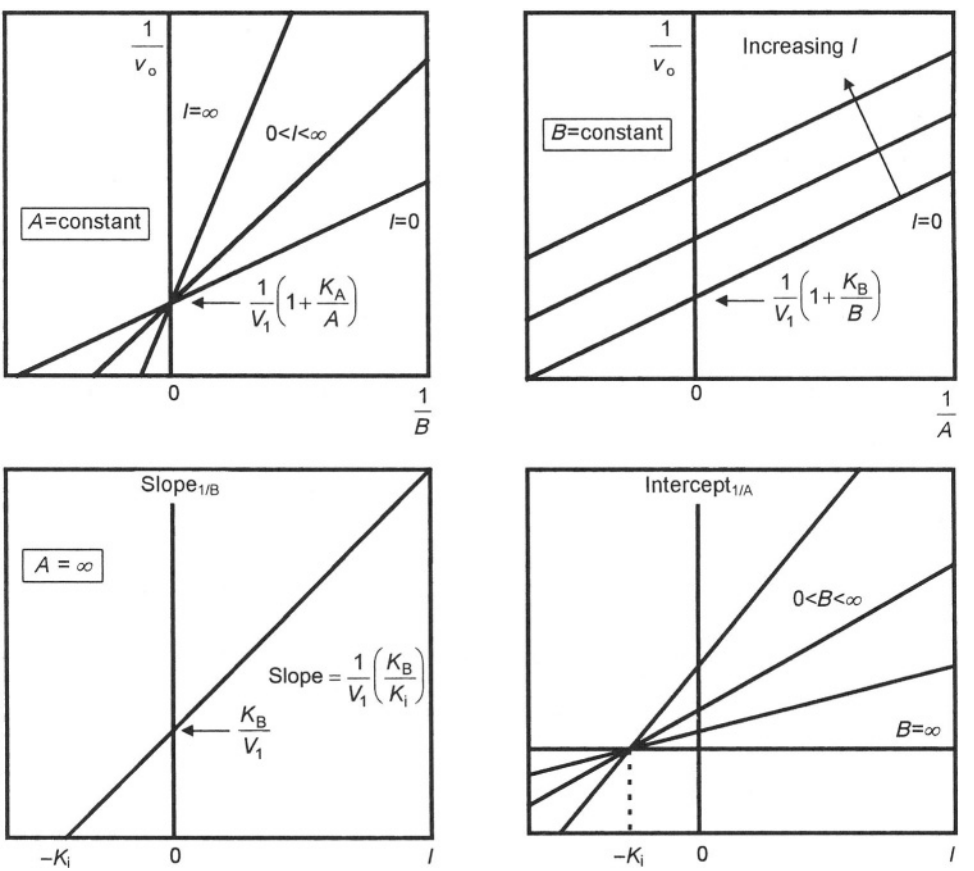


Figure 6. Dead-end inhibition in a Steady-State Ordered Bi Bi system, when an inhibitor binds to the EA complex, producing an EAI complex. Graphical presentation of Eqs. (5.33) and (5.32).

$$\frac{1}{v_o} = \frac{1}{V_1}\left(1 + \frac{K_A}{A}\right) + \frac{K_B}{V_1}\left(1 + \frac{K_{iA}}{A} + \frac{I}{K_i}\right)\frac{1}{B}$$

$$\frac{1}{v_o} = \frac{1}{V_1}\left[1 + \frac{K_B}{B}\left(1 + \frac{I}{K_i}\right)\right] + \frac{K_A}{V_1}\left(1 + \frac{K_B K_{iA}}{K_A B}\right)\frac{1}{A}$$

This time, I is a competitive inhibitor with respect to B if A is constant, but I becomes an uncompetitive inhibitor with respect to A, if B is constant. This is a situation exactly opposite to that in the Ping Pong Bi Bi system.

It is unlikely that an inhibitor would combine with either of the two central complexes, since all ligand-binding sites are occupied.

One of the most useful functions of dead-end inhibitors is in verifying ordered addition of substrates in cases where product inhibition studies cannot or do not give an unequivocal answer. This is one of the main reasons why the application of dead-end inhibitors gained the popularity in enzyme kinetics (Leskovac *et al.*, 1998, 1999). For this purpose, Fromm (1979) has developed a set of rules which are helpful in the identification of various mechanisms with the aid of dead-end inhibitors (Table 2).

Table 2. Use of dead-end competitive inhibitors for determining bisubstrate kinetic mechanisms (Fromm, 1979)[a]

Mechanism	Dead-end inhibitor competitive with substrate	$\frac{1}{A}$ plot	$\frac{1}{B}$ plot
Random Bi Bi and	A	Competitive	Noncompetitive
Random Bi Uni	B	Noncompetitive	Competitive
Ordered Bi Bi and	A	Competitive	Noncompetitive
Theorell–Chance	B	Uncompetitive	Competitive
Ping Pong Bi Bi	A	Competitive	Uncompetitive
	B	Uncompetitive	Competitive

[a]These entries apply only to inhibitors that confine themselves to a single site; if they cover the binding sites of both substrates, different patterns are obtained.

5.7 INHIBITION BY A MIXTURE OF TWO INHIBITORS

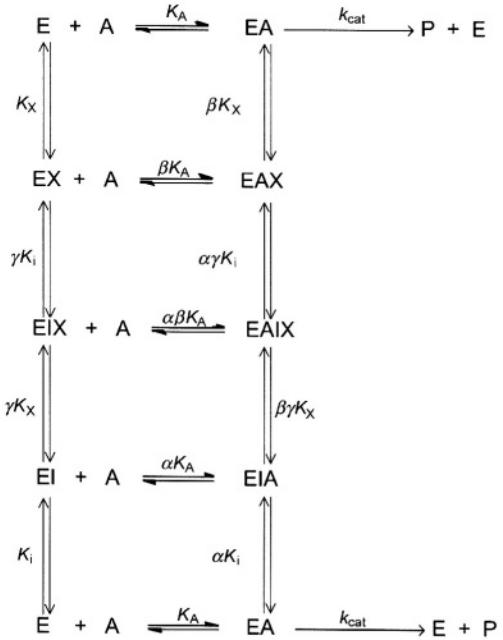
In this section, we shall examine the kinetics of monosubstrate enzymes that are capable of binding two different inhibitors. Although the monosubstrate enzyme reactions are not very realistic because they are relatively rare in nature, we shall, for the sake of simplicity, open this section with monosubstrate reactions.

If an enzyme is able to accommodate a molecule of substrate and a molecule of only one inhibitor at a time, but not the molecule of the other inhibitor, we have the case of two *exclusive* inhibitors; thus, the two inhibitors are mutually exclusive. If the enzyme can bind a substrate and both inhibitors simultaneously, we have the case of two *nonexclusive* inhibitors. We assume that the presence of either inhibitor prevents the catalytic reaction.

The terms: exclusive and nonexclusive binding, will be applied throughout this book not only to inhibitors, but also to ligands other then substrates.

5.7.1 The General Case

In the rapid equilibrium scheme (5.34), A is the substrate while I and X are the two nonexclusive inhibitors.



(5·34)

The interaction factors α, β, and γ represent, respectively, the effect of I on the binding of A, the effect of X on the binding of A, and the effect of I on the binding of X. The inhibitors could act in a pure, partial, or mixed type fashion, depending on the values of the interaction factors. We shall assume, for the sake of simplicity, that EAI, EAX, and EAXI complexes are catalytically inactive.

If the inhibitors are *purely noncompetitive,* they will have no effect on substrate binding, and we shall have $\alpha = \beta = 1$, but $\gamma \neq 0$.

The rate equation for this rapid equilibrium case is

$$v_0 = \frac{V_1 A}{K_A\left(1+\frac{I}{K_i}+\frac{X}{K_x}+\frac{IX}{\gamma K_i K_x}\right)+A\left(1+\frac{I}{K_i}+\frac{X}{K_x}+\frac{IX}{\gamma K_i K_x}\right)} \tag{5·35}$$

where $V_1 = k_{cat}E_0$.

If the I and X are two different *partial competitive inhibitors,* than the binding of I will change K_A by a factor α and the binding of X will change K_A by the factor β. If the inhibitors have no effect on the binding of each other, than $\gamma = 1$. In this case, the general rate equation is

$$v_0 = \frac{V_1\left(1+\frac{I}{\alpha K_i}+\frac{X}{\beta K_x}+\frac{IX}{\alpha\beta K_i K_x}\right)A}{K_A\left(1+\frac{I}{K_i}+\frac{X}{K_x}+\frac{IX}{K_i K_x}\right)+A\left(1+\frac{I}{\alpha K_i}+\frac{X}{\beta K_x}+\frac{IX}{\alpha\beta K_i K_x}\right)} \tag{5·36}$$

At any given substrate concentration, increasing I (in the absence of X) will decrease the initial velocity hyperbolically to a limit given by

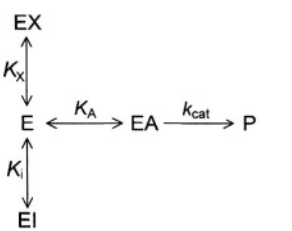
$$v_0 = \frac{V_1 A}{\alpha K_A + A} \tag{5.37}$$

Similarly, increasing X (in the absence of I) will decrease the initial velocity hyperbolically to a limit given by

$$v_0 = \frac{V_1 A}{\beta K_A + A} \tag{5.38}$$

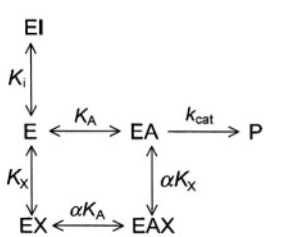
Table 3. Inhibition by a mixture of two exclusive inhibitors

Case 1. Pure competitive inhibition by two exclusive inhibitors



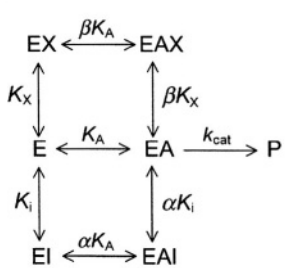
$$\frac{1}{v_0} = \frac{1}{V_1} + \frac{K_A}{V_1}\left(1 + \frac{I}{K_i} + \frac{X}{K_x}\right)\frac{1}{A}$$

Case 2. I is competitive and X is noncompetitive with respect to substrate



$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{X}{\alpha K_x}\right) + \frac{K_A}{V_1}\left(1 + \frac{I}{K_i} + \frac{X}{K_x}\right)\frac{1}{A}$$

Case 3. Competitive and noncompetitive inhibition by two mutually exclusive inhibitors



$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{I}{\alpha K_i} + \frac{X}{\beta K_x}\right) + \frac{K_A}{V_1}\left(1 + \frac{I}{K_i} + \frac{X}{K_x}\right)\frac{1}{A}$$

5.7.2 Inhibition by Two Exclusive Inhibitors

From the general case shown in reaction (5.34), several special cases can be derived (Segel, 1975).

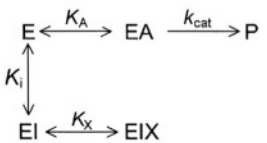
Let us first examine the three cases of inhibition with a mixture of two exclusive inhibitors, derived from reaction (5.34); remember that exclusive inhibitors prevent the binding of each other (Table 3).

5.7.3 Inhibition by Two Nonexclusive Inhibitors

Let us now examine the three cases of inhibition with a mixture of two non-exclusive inhibitors, derived again from the general scheme (5.34); Table 4 summarizes these cases. Note that, in contrast to the preceding case, in all the rate equations with two nonexclusive inhibitors, the cross term (*IX*) is always present.

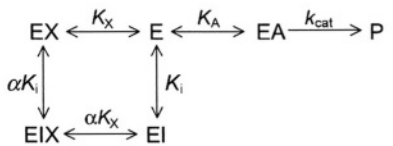
Table 4. Inhibition by a mixture of two nonexclusive inhibitors

Case 4. Ordered binding of two nonexclusive inhibitors



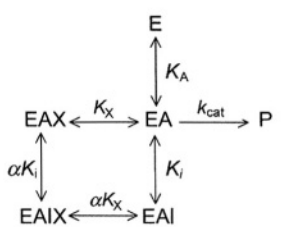
$$\frac{1}{v_0} = \frac{1}{V_1} + \frac{K_A}{V_1}\left(1 + \frac{I}{K_i} + \frac{IX}{K_i K_x}\right)\frac{1}{A}$$

Case 5. Pure competitive inhibition by two nonexclusive inhibitors



$$\frac{1}{v_0} = \frac{1}{V_1} + \frac{K_A}{V_1}\left(1 + \frac{I}{K_i} + \frac{X}{K_x} + \frac{IX}{\alpha K_i K_x}\right)\frac{1}{A}$$

Case 6. Uncompetitive inhibition by two nonexclusive inhibitors



$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{I}{K_i} + \frac{X}{K_x} + \frac{IX}{\alpha K_i K_x}\right) + \left(\frac{K_A}{V_1}\right)\frac{1}{A}$$

5.7.4 Graphical Analysis of Data

In monosubstrate and bisubstrate reactions with a mixture of two inhibitors, the two inhibitors may bind nonexclusively to the enzyme or may be mutually

exclusive. The principal kinetic question is how to differ these two types of inhibition; a graphical analysis may provide an answer.

Consider the pure noncompetitive inhibition by two nonexclusive inhibitors, depicted by Eq. (5.35). If there are two dead-end inhibitors I and X, the kinetic question is whether an EIX complex forms, and if so, whether the dissociation constant of I from EIX is the same as from EI (and similarly for X from EIX and EX). To answer this, the substrate concentration is held constant, the concentration of the two inhibitors is varied, and initial velocities are determined (Yonetani & Theorell, 1965; Yonetani, 1982).

The equation for this situation is

$$\frac{1}{v_i} = \frac{1}{v_o}\left(1 + \frac{I}{K_i} + \frac{X}{K_x} + \frac{IX}{\alpha K_i K_x}\right) \tag{5.39}$$

where v_o is an uninhibited rate, v_i is the inhibited rate, K_i and K_x are dissociation constants for EI and EX, and α is an interaction coefficient which tells the extent to which the binding of I and X is mutually exclusive. A plot of $1/v_i$ versus I at different X levels (including zero) gives lines that are parallel or that cross somewhere to the left of the vertical axis (Fig. 7).

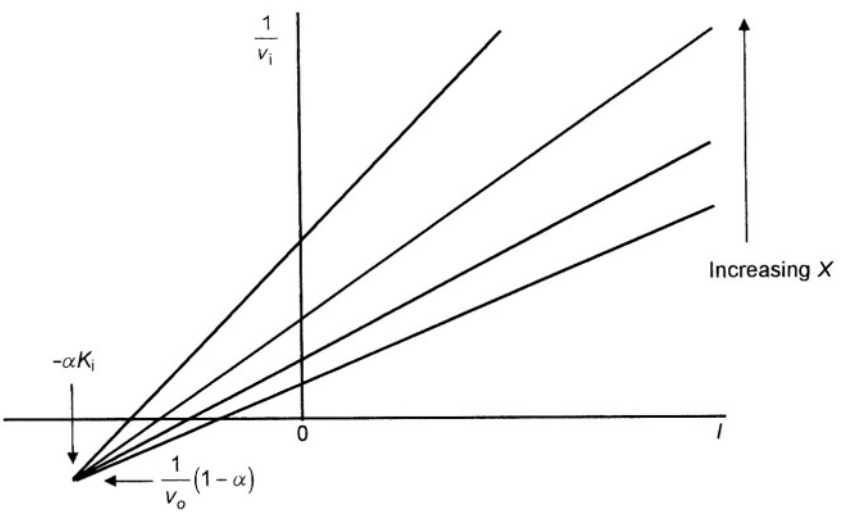


Figure 7. Dixon plot for the determination whether inhibitors are mutually exclusive. Graphical presentation of Eq. (5.39), when $\alpha > 1$ and [A] = constant.

The vertical intercepts of these plots will always vary with X (they represent the effect of X when $I = 0$), but the slopes will vary only if an EIX complex forms. If I can combine with EX as readily as with E, while X still inhibits the reaction in the absence of I, the apparent K_i for I is unchanged by the presence of X, since both E and EX are available for reaction. Thus, the vertical intercepts vary with X, but the horizontal intercept is constant and the pattern crosses on the horizontal axis.

If the presence of X raises the dissociation constant of I, but not to infinity, the pattern will now cross below the horizontal axis (as in Fig. 7). If the presence of X lowers the dissociation constant of I, the patterns will cross above the horizontal axis. If α is above 1, the presence of one inhibitor hinders but does not prevent binding of the other, and the lines cross below the horizontal axis. If $\alpha = 1$, binding is independent, and the pattern crosses on the horizontal axis. When α is less than one, binding is synergistic and the crossover point is above the axis. The coordinates of the crossover point are $I = -\alpha K_i$, and $1/v_i = (1/v_o)(1 - \alpha)$. One can calculate the value of αK_X from an analogous plot, simply by changing inhibitors on both axis. Finally, one has to determine K_i from separate competitive inhibition experiments in order to determine α.

If α is infinity, binding is exclusive, and the pattern is parallel (Fig. 8).

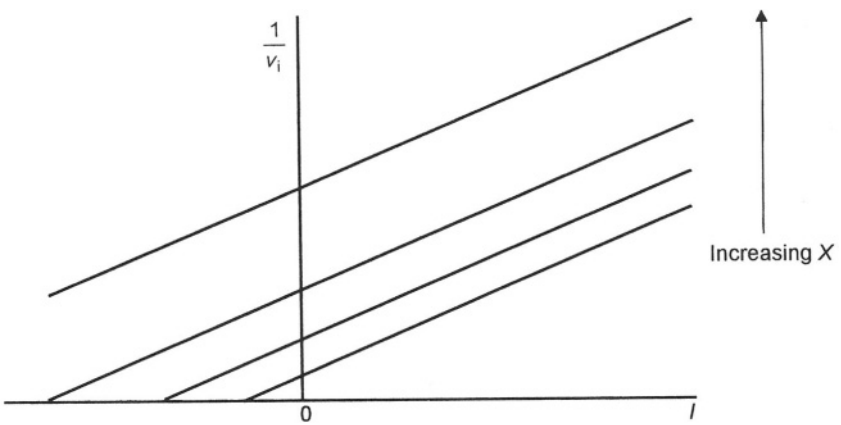


Figure 8. Dixon plot for the determination whether inhibitors are mutually exclusive. Graphical presentation of Eq. (5.39), when $\alpha > \infty$ and [A] = constant.

This method can be applied to any set of two inhibitors by holding the substrate concentrations constant and varying the two inhibitor concentrations. What we are really doing is testing for the presence of the cross-term in the rate Eq. (5.39). Thus, with isocitrate dehydrogenase, this method was applied with product inhibition levels of **$NADP^+$** and substrate inhibition levels of **α-ketoglutarate,** holding **CO_2** and NADPH constant. The pattern was parallel, showing that the complex **enzyme-$NADP^+$-α-ketoglutarate** was not present. This experiment showed that **α-ketoglutarate** combined with the central complex rather than with the **E-$NADP^+$** complex (Grissom & Cleland, 1988).

A popular version of the Dixon plot in Fig. 7 is the Yonetani–Theorell plot, in which the ratios of inhibited and uninhibited reaction rates are plotted against increasing concentrations of one inhibitor, in the presence of a constant concentration of the other. This method was applied for analysis of the competitive inhibition of liver alcohol dehydrogenase by ADP and ***o*-phenanthroline** (Yonetani & Theorell, 1964).

REFERENCES

Books

Dixon, M. & Webb, E.C. (1979) *Enzymes,* 3rd ed., Longmans & Green, New York.
Price, N. & Stewens, L. (1999) *Fundamentals of enzymology,* Oxford University Press, Oxford.
Segel, I.H. (1975) *Enzyme kinetics,* Wiley, New York.

Review articles

Cleland, W.W. (1970) *The Enzymes,* 3rd ed., Vol. II, 1–65.
Cleland, W.W. (1979) *Methods Enzymol. 63,* 500–513.
Cleland, W.W. (1990) *The Enzymes,* 3rd ed., Vol. 19, 119–130.
Fromm, H.J. (1979) *Methods Enzymol. 63,* 567–580.
Fromm, H.J. (1995) *Methods Enzymol. 259,* 123–143.
Todhunter, J.A. (1979) *Methods Enzymol. 63,* 383–511.
Yonetani, T. (1982) *Methods Enzymol. 87,* 500–509.

Specific references

Auld, D.S. & Holmquist, B. (1974) *Biochemistry 13,* 4355–4361.
Cleland, W.W. (1963) *Biochim. Biophys. Acta 67,* 105–137.
Cleland, W.W. (1963) *Biochim. Biophys. Acta 67,* 173–187.
Cleland, W.W. (1963) *Biochim. Biophys. Acta 67,* 188–196.
Dixon, M. (1953) *Biochem. J. 55,* 170–171.
Grissom, C.B. & Cleland, W.W. (1988) *Biochemistry 27,* 2934–2943.
Huang, C.Y. (1990) *Biochemistry 29,* 158–170.
Leskovac, V., Trivić, S., & Anderson, B.M. (1998) *Molec. Cell. Biochem. 178,* 219–227.
Leskovac, V., Trivić, S., & Anderson, B.M. (1999) *Europ. J. Biochem. 264,* 840–847.
Yonetani, T. & Theorell, H. (1964) *Arch. Biochem. Biophys. 106,* 243–250.

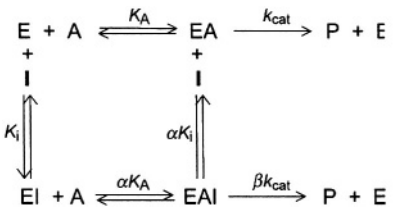
Chapter 6

Hyperbolic and Parabolic Inhibition

In Chapter 5, we have examined the properties of several simple linear types of inhibition systems. All these types have, in common, a dead-end EI or a non-productive EAI complex, or both. In order to introduce more complex cases of inhibition, we shall start with an analysis of nonlinear inhibition with less-demanding monosubstrate reactions (Cleland, 1970, 1977; Fromm, 1975).

6.1 HYPERBOLIC INHIBITION IN MONOSUBSTRATE REACTIONS

Inhibition of monosubstrate enzyme reactions with an inhibitor may be expressed in a more general way:



(6.1)

In reaction (6.1), we have admitted that the enzyme can form an EAI complex that can yield product with equal or less facility than can the EA complex. In this case, the apparent values of $(1/V_1)^{APP}$ and $(K_A/V_1)^{APP}$ functions will display a nonlinear dependence on the inhibitor concentration. Therefore, these types of inhibition are called *nonlinear inhibitions,* contrary to linear inhibitions described in Chapter 5.

In addition, we have admitted that EI complex may have different affinities for the substrate than the free enzyme, and that EA complex may have different affinities for the inhibitor than the free enzyme. A rate equation for this general case may be derived from the rapid equilibrium assumptions:

$$v_o = \frac{V_1 A}{K_A\left(\dfrac{1+\dfrac{I}{K_i}}{1+\dfrac{\beta I}{\alpha K_i}}\right) + A\left(\dfrac{1+\dfrac{I}{\alpha K_i}}{1+\dfrac{\beta I}{\alpha K_i}}\right)} \tag{6.2}$$

where $V_1 = k_{cat}E_o$.

Equation (6.2) is quite general, and it describes a number of different inhibition mechanisms, including the simple linear inhibition mechanisms described in Chapter 5. Thus, $\alpha = 1$ and $\beta = 0$ describe a simple linear noncompetitive inhibition (Section 5.3), and when $\alpha = \infty$ we have a simple linear competitive inhibition (Section 5.2).

Equation (6.2) can be rearranged in the Lineweaver-Burk manner:

$$\frac{1}{v_0} = \frac{1}{V_1}\left(\frac{\alpha K_i + I}{\alpha K_i + \beta I}\right) + \frac{\alpha K_A}{V_1}\left(\frac{K_i + I}{\alpha K_i + \beta I}\right)\frac{1}{A} \tag{6.3}$$

and presented graphically (Fig. 1).

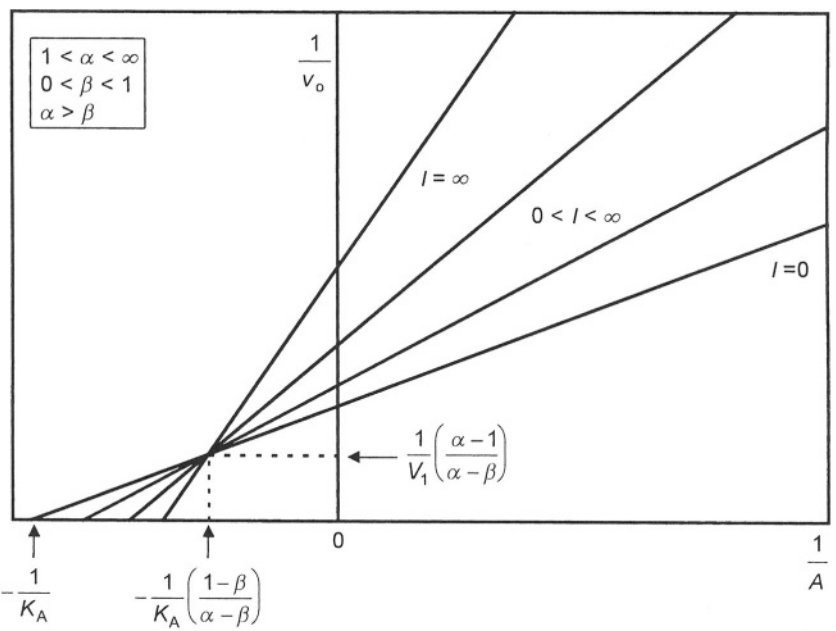


Figure 1. Nonlinear inhibition in a monosubstrate reaction. Graphical presentation of Eq. (6.3).

At infinitely high I, Eq. (6.2) reduces to

$$v_0 = \frac{\beta V_1 A}{\alpha K_A + A} \tag{6.4}$$

An infinitely high A will drive all the enzyme to a mixture of EA and EAI, and at infinitely high I all the enzyme will be converted to EI and EAI. Because EAI *can* form the product, the velocity cannot be driven to zero by increasing I (Fig. 1). This clearly shows that the nonlinear inhibition is *not a complete inhibition,* as the velocity does not approach zero even at higher concentrations of an inhibitor.

In the simple linear inhibition, $1/V_1^{APP}$ (intercept) and $(K_A/V_1)^{APP}$ (slope) functions display a simple linear dependence on the inhibitor concentration (Section 5.7). However, if both α and β are different from unity, as in the general Eq. (6.2), the slopes or intercepts become complex functions of I:

$$\text{Slope} = \frac{\alpha K_A}{V_1}\left(\frac{K_i + I}{\alpha K_i + \beta I}\right) \tag{6.5}$$

$$\text{Intercept} = \frac{1}{V_1}\left(\frac{\alpha K_i + I}{\alpha K_i + \beta I}\right) \tag{6.6}$$

Figure 2 shows a replot of slopes and intercepts from Fig. 1 as a function of increasing concentrations of an inhibitor.

In Fig. 2, the limiting value of slopes at infinite I is $\alpha K_A/\beta V_1$ (4.0) and for intercepts this value is $1/\beta V_1$ (2.0). In contrast to the simple linear inhibition, the replots of slopes and intercepts are hyperbolas; therefore, this type of inhibition is usually called a *hyperbolic* or *partial inhibition.* Thus, we could distinguish the various types of inhibition still further by referring to noncompetitive inhibition as linear or hyperbolic.

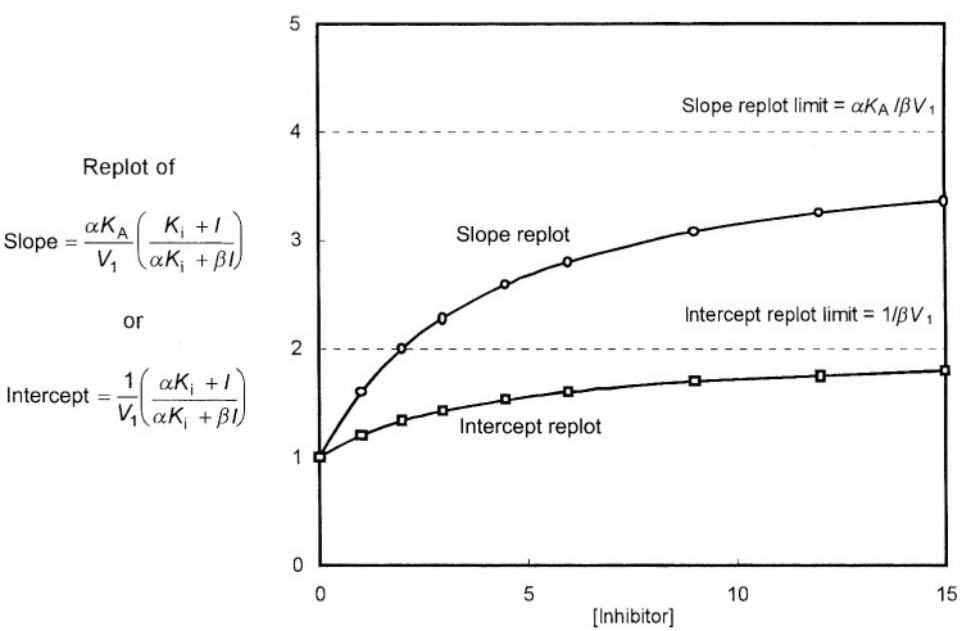


Figure 2. Hyperbolic noncompetitive inhibition in a monosubstrate reaction. Replot of slopes (Eq. (6.5)) and intercepts (Eq. (6.6)), assuming that $\alpha = 2$ and $\beta = 0.5$ ($V_1 = K_A = K_i = 1$).

6.2 DETERMINATION OF KINETIC CONSTANTS IN HYPERBOLIC INHIBITION

Since the replots of slopes or intercepts versus I are nonlinear, it is not possible to determine directly the values of kinetic constants from the data in Fig. 2; instead, it is necessary to apply a differential method to rate equations, in order to obtain a graphical solution (Cleland, 1967, 1979). By using the differential method, we are raising the horizontal axis in Fig. 2 ensuring that curves become hyperbola that start at the origin:

$$\Delta \text{ Intercept} = \frac{1}{V_1}\left(\frac{\alpha K_i + I}{\alpha K_i + \beta I}\right) - \frac{1}{V_1} \tag{6.7}$$

$$\Delta \text{ Slope} = \frac{\alpha K_A}{V_1}\left(\frac{K_i + I}{\alpha K_i + \beta I}\right) - \frac{K_A}{V_1} \tag{6.8}$$

$$\frac{1}{\Delta \text{ Intercept}} = V_1\left(\frac{\beta}{1-\beta}\right) + K_i V_1\left(\frac{\alpha}{1-\beta}\right)\frac{1}{I} \tag{6.9}$$

$$\frac{1}{\Delta \text{ Slope}} = \frac{V_1}{K_A}\left(\frac{\beta}{\alpha-\beta}\right) + \frac{K_i V_1}{K_A}\left(\frac{\alpha}{\alpha-\beta}\right)\frac{1}{I} \tag{6.10}$$

Thus, with Eqs. (6.9) and (6.10), we achieve a linearization of the slope and intercept function. Figure 3 shows the graphical presentation of Eqs. (6.9) and (6.10), from which we can calculate kinetic parameters α and β.

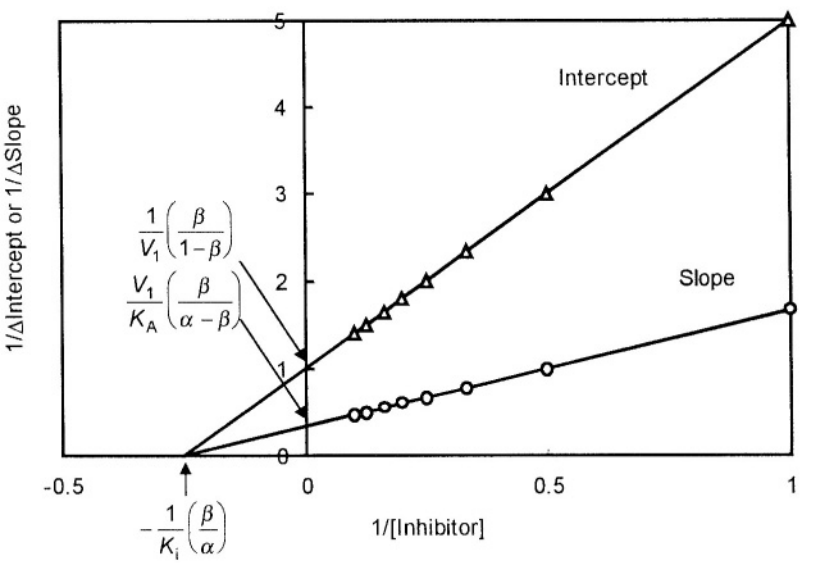


Figure 3. Determination of inhibition constants in hyperbolic inhibition by a differential method. Graphical presentation of Eqs. (6.8) and (6.9), assuming that $\alpha = 2$ and $\beta = 0.5$ ($V_1 = K_A = K_i = 1$).

6.3 VARIATIONS OF HYPERBOLIC INHIBITION IN MONOSUBSTRATE REACTIONS

As we have pointed out in Section 6.1, Eq. (6.2) is quite general and describes a number of different inhibition mechanisms, including many linear and nonlinear inhibitions. A difference between various types of nonlinear mechanisms is made on the basis of the relative magnitudes of α and β values (Segel, 1975).

Figure 4 shows the survey of different types of *nonlinear hyperbolic inhibition* mechanisms and their characteristics. A basic property of all nonlinear mechanisms, shown in Fig. 4, is that the double reciprocal plot of $1/v_o$ versus $1/A$, in the presence of different constant concentrations of an inhibitor is a *family of straight lines* with a common intersection point. This common intersection point is found either in the I, III, or in the IV quadrant, depending on the mechanism; only in Case 5 (hyperbolic uncompetitive type), the double reciprocal plot is a family of parallel straight lines without a common intersection point.

Thus, it is clear that the primary double reciprocal plots of $1/v_o$ versus $1/A$ represent a valuable general diagnostic tool for the estimation of the type of nonlinear inhibition mechanisms.

A rate equation for each nonlinear mechanism shown in Fig. 4 can be easily derived from the general rate Eq. (6.2), simply by inserting the proper values for kinetic constants α and β, and canceling out identical numerators and

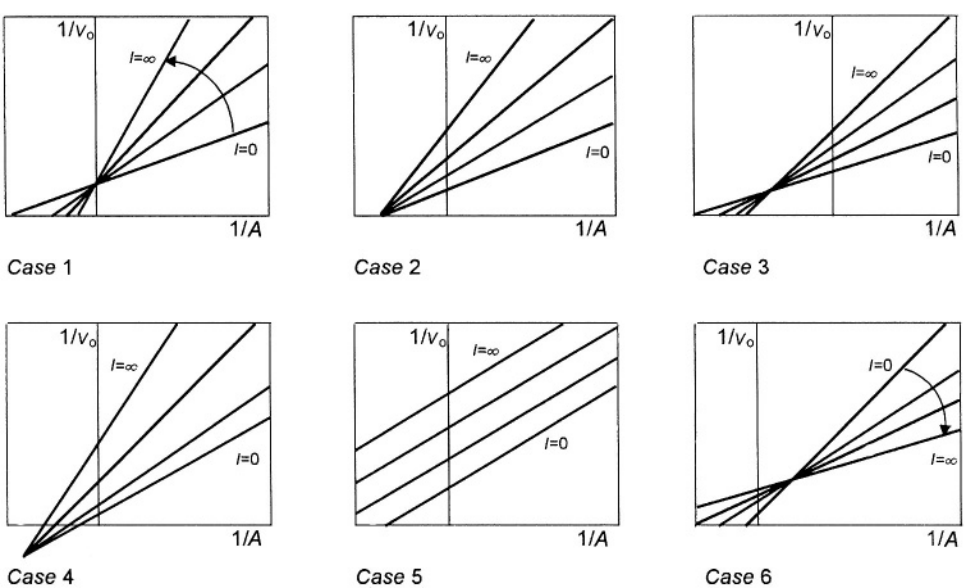


Figure 4. Different types of nonlinear hyperbolic inhibition mechanisms. In each case, the concentration of an inhibitor increases counterclockwise, except in Case 6.

denominators. Thus, we shall obtain the following rate equations for the partial competitive inhibition (Case 1), partial noncompetitive inhibition (Case 2), hyperbolic uncompetitive inhibition (Case 5), and the other three types of hyperbolic noncompetitive inhibitions (Cases 3, 4, and 6), respectively:

Partial competitive inhibition (Case 1)

$$v_0 = \frac{V_1 A}{K_A\left(\dfrac{1+\dfrac{I}{K_i}}{1+\dfrac{I}{\alpha K_i}}\right)+A} \tag{6.11}$$

$$v_0 = \frac{V_1 A}{K_A\left(\dfrac{1+\dfrac{I}{K_i}}{1+\dfrac{\beta I}{K_i}}\right)+A\left(\dfrac{1+\dfrac{I}{K_i}}{1+\dfrac{\beta I}{K_i}}\right)} \tag{6.12}$$

Hyperbolic uncompetitive inhibition (Case 5)

$$v_0 = \frac{V_1 A}{K_A + A\left(\dfrac{1+\dfrac{I}{\alpha K_i}}{1+\dfrac{I}{K_i}}\right)} \tag{6.13}$$

Hyperbolic noncompetitive inhibition (Cases 3, 4, and 6)

$$v_0 = \frac{V_1 A}{K_A\left(\dfrac{1+\dfrac{I}{K_i}}{1+\dfrac{\beta I}{\alpha K_i}}\right)+A\left(\dfrac{1+\dfrac{I}{\alpha K_i}}{1+\dfrac{\beta I}{\alpha K_i}}\right)}$$

Note that the rate equation for the hyperbolic noncompetitive inhibitions (Cases 3, 4, and 6) is identical with the general rate Eq. (6.2).

Table 1 lists the coordinates of intersection points in all mechanisms shown in Fig. 4. From the coordinates of intersection points, one can estimate the magnitudes of α and β, provided the kinetic constants V_1 and K_A are known. However, such estimates are often not sufficiently accurate and a more precise differential method must be applied.

Table 1. Coordinates of intersection points for the double reciprocal plots shown in Fig. 4

Mechanism	α	$\frac{\alpha}{\beta}$	β	Intersection point	Coordinates of intersection points
Case 1 Partial competitive	$\alpha>1$	$\alpha>\beta$	$\beta=1$	On ordinate	$\frac{1}{v_0}=\frac{1}{V_1}$
Case 2 Hyperbolic noncompetitive	$\alpha=1$	$\alpha>\beta$	$0<\beta<1$	On abscissa	$\frac{1}{A}=-\frac{1}{K_A}$
Case 3 Hyperbolic noncompetitive	$1<\alpha<\infty$	$\alpha>\beta$	$0<\beta<1$	In the IV quadrant	$\frac{1}{v_0}=\frac{1}{V_1}\left(\frac{\alpha-1}{\alpha-\beta}\right)$ $-\frac{1}{A}=\frac{1}{K_A}\left(\frac{1-\beta}{\alpha-\beta}\right)$
Case 4 Hyperbolic noncompetitive	$0<\alpha<1$	$\alpha>\beta$	$0<\beta<1$	In the III quadrant	$\frac{1}{v_0}=\frac{1}{V_1}\left(\frac{\alpha-1}{\alpha-\beta}\right)$ $-\frac{1}{A}=\frac{1}{K_A}\left(\frac{1-\beta}{\alpha-\beta}\right)$
Case 5 Hyperbolic uncompetitive	$0<\alpha<1$	$\alpha=\beta$	$0<\beta<1$	Without intersection point	
Case 6 Hyperbolic noncompetitive	$0<\alpha<1$	$\beta>\alpha$	$0<\beta<1$	In the I quadrant	$\frac{1}{v_0}=\frac{1}{V_1}\left(\frac{\alpha-1}{\alpha-\beta}\right)$ $\frac{1}{A}=\frac{1}{K_A}\left(\frac{\beta-1}{\alpha-\beta}\right)$

In Fig. 4, in each case, the slopes of lines increase with an increase in the concentration of an inhibitor, with the exception of the Case 6. However, this increase is not linearly related to the concentration of an inhibitor, but hyperbolically. In the same way, the intercepts on the ordinate in Fig. 4 always increase with an increase in the concentration of an inhibitor, with the exception of the competitive Case 1. However, similarly as with the replot of slopes, this increase is not linearly related to the concentration of an inhibitor, but hyperbolically.

Despite these nonlinearities of the secondary graphs, the limiting values can be calculated in each case by the differential method, as was described in Section 6.2. In each nonlinear hyperbolic mechanism shown in Fig. 4, the replots of primary graphs in the form of $1/\Delta$ Slope versus I will give a straight line, similar to the plot shown in Fig. 3 (with the exception of Case 5). In the same way, the replots of primary graphs in the form of $1/\Delta$ Intercept versus I will give a straight line, similar to the plot shown in Fig. 3 (with the exception of Case 1). Thus, in each case, one can calculate easily the kinetic parameters α and β by a differential method.

Hyperbolic noncompetitive inhibition, Case 6, represents a special example. In this case, the inhibitor not only decreases the rate constant for product

formation, but also markedly increases the affinity of the enzyme for the substrate. For example, at a fixed inhibitor concentration, the inhibitor acts as an activator below a certain fixed concentration of substrate and as an inhibitor above that concentration. Thus, in this case, there may be some doubt whether to call I an inhibitor or an activator.

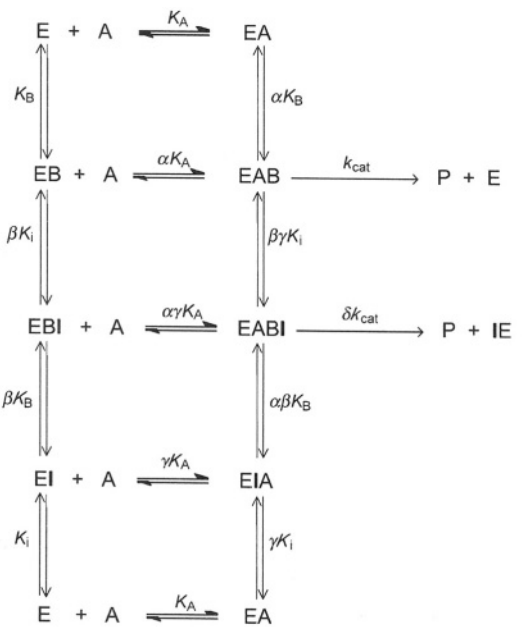
From the survey of different hyperbolic inhibition patterns outlined above, we may single out the following general conclusions:

(a) All hyperbolic inhibition mechanisms do not afford complete inhibition, since the velocity does not approach zero even at higher concentrations of an inhibitor (Fig. 4). The reason for this is that at infinitely high I all enzyme is driven to a mixture of EA and EAI, whereby both complexes are catalytically active and can form products.

(b) In each case, the slopes or intercepts from the primary plots of $1/v_o$ versus $1/A$ may be plotted against increasing concentrations of I. Such secondary plots will be always curved. Applying the differential method (outlined in Section 6.2), one can use the data from the secondary plots to calculate graphically all kinetic constants including the interaction factors α and β.

6.4 HYPERBOLIC INHIBITION IN BISUBSTRATE REACTIONS

Let us, now, depart from monosubstrate reactions and turn our attention to a much more realistic case of a hyperbolic inhibition in bisubstrate reactions (Segel, 1975; Dixon & Webb, 1979; Purich & Allison, 2000). In the rapid equilibrium reaction (6.14), A and B are the substrates while I is a nonexclusive inhibitor:



(6.14)

The interaction factors α, β, and γ represent, respectively, the effect of A on the binding of B, the effect of I on the binding of B, and the effect of I on the binding of A. The factor δ represents the effect of I on the catalytic activity of the EABI complex. The derivation of rate equation for reaction (6.14) would be extremely laborious from the steady-state assumptions. Therefore, the general velocity equation is derived from the rapid equilibrium assumptions:

$$v_o = \frac{V_1\left(\frac{AB}{\alpha K_A K_B} + \frac{\delta ABI}{\alpha\beta\gamma K_A K_B K_i}\right)}{1 + \frac{A}{K_A} + \frac{B}{K_B} + \frac{I}{K_i} + \frac{AB}{\alpha K_A K_B} + \frac{AI}{\gamma K_A K_i} + \frac{BI}{\beta K_B K_i} + \frac{ABI}{\alpha\beta\gamma K_A K_B K_i}} \tag{6.15}$$

where $V_1 = k_{cat}$. The velocity Eq. (6.15) is derived with an assumption that both complexes, EAB and EABI, are catalytically active and afford the products.

The rate equations in the reciprocal form, derived from Eq. (6.15), are extremely complex:

$$\frac{1}{v_o} = \frac{1}{V_1}\left[\frac{1 + \frac{\alpha K_B}{B}\left(1 + \frac{I}{\gamma K_i}\right) + \frac{I}{\beta\gamma K_i}}{1 + \frac{\delta I}{\beta\gamma K_i}}\right] + \frac{\alpha K_A}{V_1}\left[\frac{1 + \frac{K_B}{B}\left(1 + \frac{I}{K_i}\right) + \frac{I}{\beta K_i}}{1 + \frac{\delta I}{\beta\gamma K_i}}\right]\frac{1}{A} \tag{6.16}$$

$$\frac{1}{v_o} = \frac{1}{V_1}\left[\frac{1 + \frac{\alpha K_A}{A}\left(1 + \frac{I}{\beta K_i}\right) + \frac{I}{\beta\gamma K_i}}{1 + \frac{\delta I}{\beta\gamma K_i}}\right] + \frac{\alpha K_B}{V_1}\left[\frac{1 + \frac{K_A}{A}\left(1 + \frac{I}{K_i}\right) + \frac{I}{\gamma K_i}}{1 + \frac{\delta I}{\beta\gamma K_i}}\right]\frac{1}{B} \tag{6.17}$$

Similarly as for the nonlinear inhibition with a single substrate and a single inhibitor molecule (Section 6.1), the double reciprocal plots of $1/v_o$ versus $1/A$ or $1/v_o$ versus $1/B$, are a family of straight lines with a single intersection point to the left of the vertical axis. Also, the slope and intercept replots are hyperbolic.

In the general case, when both complexes EAB and EABI are catalytically active, the inhibitor constants are conveniently obtained from replots of $1/\Delta$ Slope and $1/\Delta$ Intercept versus $1/I$, obtained at saturating concentration of one constant substrate, while the other is varied. Under such conditions, the system behaves as a single substrate, hyperbolic noncompetitive inhibition system. In this case, the inhibition with respect to a varied ligand cannot be overcome by saturation with the nonvaried ligand.

For example, if the factors β, γ, and δ are different from unity, and A is saturating, the slopes and intercepts of the double reciprocal plot $1/v_o$ versus $1/B$ are given by the following expressions:

$$\text{Intercept}_{1/B} = \frac{1}{V_1}\left(\frac{1 + \frac{I}{\beta\gamma K_i}}{1 + \frac{\delta I}{\beta\gamma K_i}}\right) = \frac{1}{V_1}\left(\frac{\beta\gamma K_i + I}{\beta\gamma K_i + \delta I}\right) \tag{6.18}$$

$$\text{Slope}_{1/B} = \frac{\alpha K_B}{V_1}\left(\frac{1+\dfrac{I}{\gamma K_i}}{1+\dfrac{\delta I}{\beta\gamma K_i}}\right) = \frac{\alpha\beta K_B}{V_1}\left(\frac{\gamma K_i + I}{\beta\gamma K_i + \delta I}\right) \tag{6.19}$$

The system behaves in the same way as the hyperbolic noncompetitive type of inhibition (with a single substrate and a single inhibitor molecule), described in Section 6.1. If we create the appropriate $1/\Delta$ Intercept and $1/\Delta$ Slope functions, the plots of these functions versus $1/I$ are again straight lines, with a common intersection point on abscissa (Fig. 5). From Fig. 5, all three interaction factors, β, γ, and δ, can be calculated, however, only if the constant substrate A is saturating.

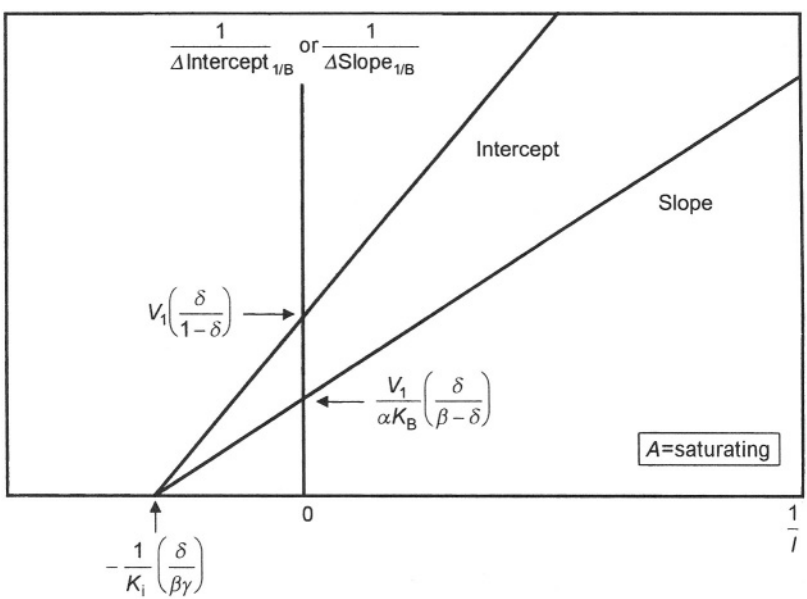


Figure 5. Hyperbolic inhibition in Rapid Equilibrium bisubstrate reactions. Determination of kinetic constants by a differential method with the aid of Eqs. (6.18) and (6.19).

However, Eq. (6.15) can be simplified in special cases. If the rate of transformation of both complexes, EAB and EABI, is equal, then $\delta = 1$. In this case, the numerator term reduces to an expression

$$V_1\left(\frac{AB}{\alpha K_A K_B}\right)\left(1+\frac{I}{\beta\gamma K_i}\right) \tag{6.20}$$

On the other hand, if the EABI complex is catalytically inactive, then $\delta = 0$. In this case, Eq. (6.15) can be rearranged into relatively simple linear forms to show either A or B as the varied ligand:

$$\frac{1}{v_0} = \frac{1}{V_1}\left[1 + \frac{\alpha K_B}{B}\left(1 + \frac{I}{\gamma K_i}\right) + \frac{I}{\beta\gamma K_i}\right] + \frac{\alpha K_A}{V_1}\left[1 + \frac{K_B}{B}\left(1 + \frac{I}{K_i}\right) + \frac{I}{\beta K_i}\right]\frac{1}{A} \quad (6.21)$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left[1 + \frac{\alpha K_A}{A}\left(1 + \frac{I}{\beta K_i}\right) + \frac{I}{\beta\gamma K_i}\right] + \frac{\alpha K_B}{V_1}\left[1 + \frac{K_A}{A}\left(1 + \frac{I}{K_i}\right) + \frac{I}{\gamma K_i}\right]\frac{1}{B} \quad (6.22)$$

Equations (6.21) and (6.22) are obtained directly from Eqs. (6.16) and (6.17), simply by omitting the denominator terms. It is clear that the interaction factors β and γ, and the K_i can be obtained from the slope and intercept replots obtained from the primary $1/v_0$ versus $1/A$ and $1/v_0$ versus $1/B$ data at different concentrations of I and a saturating nonvaried substrate.

When A is saturating, the slope of Eq. (6.22) is

$$\text{Slope}_{1/B} = \frac{\alpha K_B}{V_1} + \left(\frac{\alpha K_B}{\gamma K_i V_1}\right) I \quad (6.23)$$

When B is saturating, the slope of Eq. (6.21) is

$$\text{Slope}_{1/A} = \frac{\alpha K_A}{V_1} + \left(\frac{\alpha K_A}{\beta K_i V_1}\right) I \quad (6.24)$$

From Eqs. (6.23) and (6.24), the values of γK_i and βK_i are obtained directly. Similarly, from the corresponding replots of intercepts, the value of $\beta\gamma K_i$ can be obtained.

6.5 PARABOLIC INHIBITION IN MONOSUBSTRATE REACTIONS

In addition to hyperbolic inhibition, we may also encounter different parabolic types of inhibition. In such cases, the family of straight lines in primary plots $1/v_0$ versus $1/A$, at different fixed concentrations of an inhibitor, will have or will not have a common intersection point to the left of the vertical axis. However, in each case, the replot of $\text{Slope}_{1/A}$ and/or $\text{Intercept}_{1/A}$ function from the primary plots versus increasing I will be a nonlinear parabolic function (Rudolph, 1979; Fromm, 1995).

In this case, we shall revert again to monosubstrate reactions in order to obtain a simple kinetic expressions and to facilitate the explanation of parabolic patterns.

Parabolic types of inhibition may take several forms. They can be parabolic competitive, *S*-linear *I*-parabolic noncompetitive, *S*-parabolic *I*-linear noncompetitive, or *S*-parabolic *I*-parabolic noncompetitive. The general rate equations for these four types of inhibition are

$$v_0 = \frac{V_1 A}{K_A(1 + aI + bI^2) + A} \qquad \text{Parabolic competitive} \quad (6.25)$$

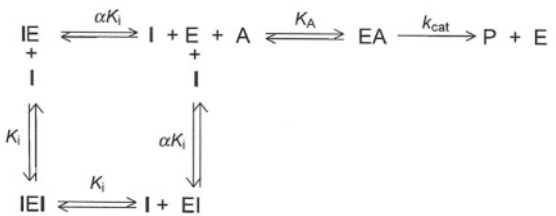
$$v_0 = \frac{V_1 A}{K_A(1 + aI) + A(1 + bI + cI^2)} \qquad S\text{-linear } I\text{-parabolic noncompetitive} \tag{6.26}$$

$$v_0 = \frac{V_1 A}{K_A(1 + aI + bI^2) + A(1 + cI)} \qquad S\text{-parabolic } I\text{-linear noncompetitive} \tag{6.27}$$

$$v_0 = \frac{V_1 A}{K_A(1 + aI + bI^2) + A(1 + cI + dI^2)} \qquad S\text{-parabolic } I\text{-parabolic noncompetitive} \tag{6.28}$$

6.5.1 Parabolic Competitive Inhibition

Consider the first case, a parabolic competitive inhibition. This case occurs in a monosubstrate reaction in which an enzyme can bind two molecules of the same competitive inhibitor, in a random manner at different sites (Yonetani, 1982). The binding of I at either site is sufficient to exclude the substrate.



(6.29)

The rate equation for this case, derived from rapid equilibrium assumptions, is:

$$v_0 = \frac{V_1 \cdot A}{K_A\left[1 + 2\left(\frac{I}{K_i}\right) + \frac{1}{\alpha}\left(\frac{I}{K_i}\right)^2\right] + A} \tag{6.30}$$

where $k_{cat} = V_1 E_0$.

The reciprocal form of Eq. (6.30) is:

$$\frac{1}{v_0} = \frac{1}{V_1} + \frac{K_A}{V_1}\left[1 + 2\left(\frac{I}{K_i}\right) + \frac{1}{\alpha}\left(\frac{I}{K_i}\right)^2\right]\frac{1}{A} \tag{6.31}$$

The binding of the first inhibitor molecule changes the intrinsic dissociation constant of the vacant site by a factor α. Equation (6.31) shows that in this type of inhibition inhibitor is competitive with substrate, but the slope function becomes parabolically related to I. Thus, this type of inhibition is a parabolic competitive (Fig. 6).

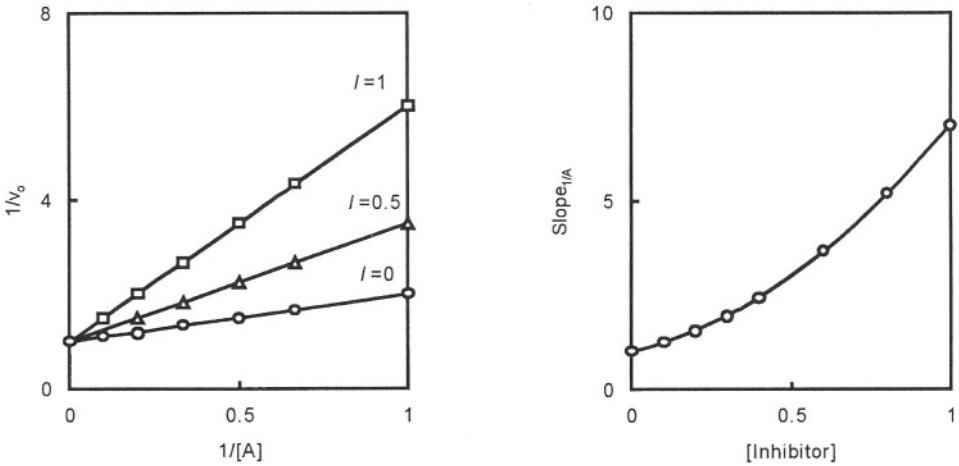


Figure 6. Parabolic inhibition: parabolic competitive type. Graphical presentation of Eq. (6.30), assuming that $\alpha = 0.5$ ($V_1 = K_A = K_i = 1$).

$$v_0 = \frac{V_1 A}{K_A(1 + aI + bI^2) + A}$$

In this case, the inhibition constants, K_i and αK_i, may be determined graphically from the linear form of the slope function:

$$\left(\frac{V_1 \cdot \text{Slope}_{1/A}}{K_A} - 1\right) \cdot \frac{1}{I} = \frac{2}{K_i} + \left(\frac{1}{\alpha K_i^2}\right) I \qquad (6.32)$$

6.5.2 *S*-Linear *I*-Parabolic Noncompetitive Inhibition

This case is more realistic, because it is very unlikely that all interaction factors, *a*, *b*, and *c*, will be identical (Wang *et al.*, 1970; Purich & Fromm, 1972). Figure 7 shows the primary double reciprocal plot and the secondary plot for this case.

The primary plot in Fig. 7 has a very characteristic look, because the family of straight lines does not have a common intersection point.

An example for the second (Eq. (6.26)) and the third case (Eq. (6.27)), is also described in Chapter 11 (Section 11.4). It occurs in a Steady-State Ordered Bi Bi system with a dead-end EAP-complex. In this system, the inhibition by the product P is *S*-parabolic *I*-linear noncompetitive when the substrate B is variable (and *A* constant), and the rate equation has the form of Eq. (6.27). The inhibition pattern becomes *S*-linear *I*-parabolic noncompetitive, when *A* is variable (and *B* constant), and the rate equation takes the form of Eq. (6.26).

Parabolic inhibition is always a complete inhibition, because a saturating concentration of I will always drive all the enzyme into an inactive form IEI.

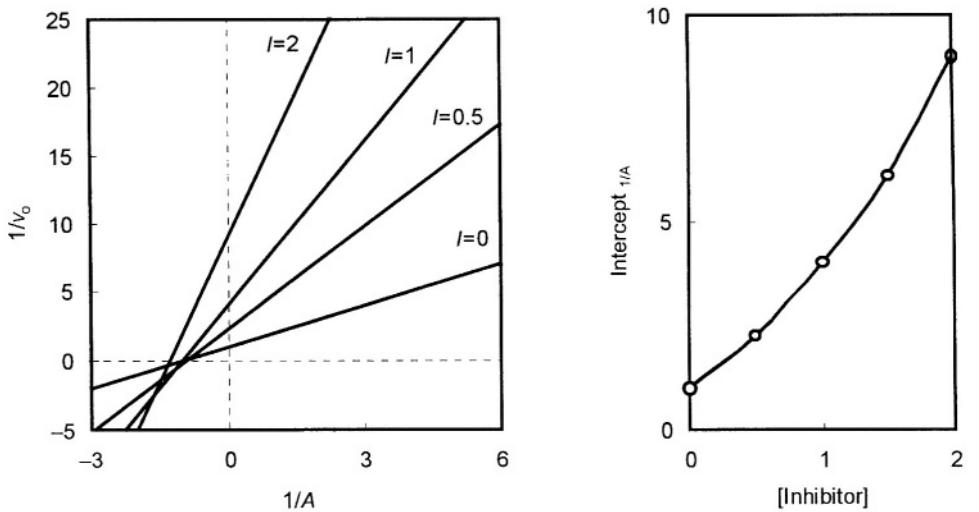


Figure 7. *S*-linear *I*-parabolic noncompetitive inhibition. The data are drawn according to Eq. (6.26), assuming that the interaction factors are not equal ($a = 3$; $b = 2$; $c = 1$) and that $V_1 = K_A = 1$.

$$v_0 = \frac{V_1 A}{K_A(1 + aI) + A(1 + bI + cI^2)}$$

6.6 NOMENCLATURE OF DOUBLE RECIPROCAL PLOTS IN THE PRESENCE OF INHIBITORS

In Chapters 5 and 6, we have examined different types of inhibition, both in monosubstrate and in bisubstrate reactions. All these types of inhibition can be classified into several kinetic forms. The *linear* types of inhibition, described in Chapter 5, are always complete inhibitions and their rate equations can be presented in a general form:

$$v_0 = \frac{V_1 A}{K_A(1 + aI) + A(1 + bI)} \tag{6.33}$$

The *hyperbolic* types of inhibition, described in Chapter 6, are always partial inhibitions, and their velocity equations can be presented in a general form:

$$v_0 = \frac{V_1 A}{K_A\left(\frac{1 + aI}{1 + bI}\right) + A\left(\frac{1 + cI}{1 + dI}\right)} \tag{6.34}$$

The *parabolic* types of inhibition, described in Section 6.5, are always complete inhibitions, and their velocity equations can be described in a general form by Eqs. (6.25)–(6.28).

The nomenclature of reciprocal plots in the foregoing chapters on enzyme inhibition is based on the usual (or common) nomenclature of enzyme inhibitors. Thus, the term competitive describes the systems yielding reciprocal plots that

intersect on ordinate. The term noncompetitive refers to systems yielding reciprocal plots that intersect above, on, or below the abscissa, Uncompetitive refers to systems yielding parallel reciprocal plots. Some people are using the term mixed-type inhibition for systems that yield reciprocal plots which do not intersect on either axis.

This nomenclature is not perfect and attempts were made to implement other terminologies (Rainer, 1969; Segel, 1975). However, the nomenclature proposed by Cleland (1963) appears to convey the most information about the true nature of inhibition in most cases. Thus, in Table 2, the common and Cleland's nomenclature for inhibitors are compared.

Table 2. Cleland's nomenclature of inhibition types

Common practice	Cleland's nomenclature	Equations	Figures
Pure competitive	Simple intersecting linear competitive	(5.3)	Figure 1 of Chapter 5
Noncompetitive	Simple intersecting (slope and intercept linear) noncompetitive	(5.10)	Figure 2 of Chapter 5
Uncompetitive	Linear uncompetitive	(5.17)	Figure 3 of Chapter 5
Partial noncompetitive	Simple intersecting hyperbolic noncompetitive	(6.3)	Table 1
Partial noncompetitive	Simple intersecting (slope and intercept hyperbolic) noncompetitive	(6.12)	Table 1 Case 2
Mixed type ($\beta \neq 0$)	Intersecting (slope and intercept hyperbolic) noncompetitive	(6.2)	Table 1 Case 3 Case 4
Mixed type ($\alpha = \beta$)	Hyperbolic uncompetitive	(6.13)	Table 1 Case 5
Parabolic	Parabolic competitive	(6.30)	Fig. 6
Parabolic	*S*-linear *I*-parabolic noncompetitive	(6.26)	Fig. 7

The nomenclature of Cleland is very versatile and can be applied to even more complex inhibition patterns that occur in double reciprocal plots. In addition, the nomenclature of Cleland is also applicable to double reciprocal plots for bisubstrate and trisubstrate reactions, in the absence and in the presence of the products of reaction, which makes this nomenclature even more versatile.

REFERENCES

Books

Dixon, M. & Webb, E.C. (1979) *Enzymes,* 3rd ed., Academic Press, New York.
Fromm, H.J. (1975) *Initial rate enzyme kinetics,* Springer-Verlag, Berlin.
Segel, I.H. (1975) *Enzyme kinetics,* Wiley, New York.
Purich, D.L. & Allison, R.D. (2000) *Handbook of biochemical kinetics,* Academic Press, San Diego.
Reiner, J. (1969) *Behavior of enzyme systems,* 2nd ed., Van Nostrand-Reinhold, New York.

Review articles

Cleland, W.W. (1967) *Adv. Enzymol. 29,* 1–32.
Cleland, W.W. (1970) *The Enzymes,* 3rd ed., Vol. II, 1–65.
Cleland, W.W. (1977) *Adv. Enzymol. Rel. Areas Mol. Biol. 45,* 273–387.
Cleland, W.W. (1979) *Methods Enzymol. 63,*103–138.
Fromm, H.J. (1995) *Methods Enzymol. 249,*123–143.
Rudolph, F.B. (1979) *Methods Enzymol. 63,* 3027–3040.
Yonetani, T. (1982) *Methods Enzymol. 87,* 600–609.

Specific references

Cleland, W.W. (1963) *Biochim. Biophys. Acta 67*, 104–138.
Cleland, W.W. (1963) *Biochim. Biophys. Acta 67,* 173–187.
Cleland, W.W. (1963) *Biochim. Biophys. Acta 67,* 188–196.
Purich, D.L. & Fromm, H.J. (1972) *Bioch. Biophys. Acta 268,* 1–10.
Wang, J.J., Tu, J.I., & Lo, F.M. (1970) *J. Biol. Chem. 246,* 3116–3120.

Chapter 7

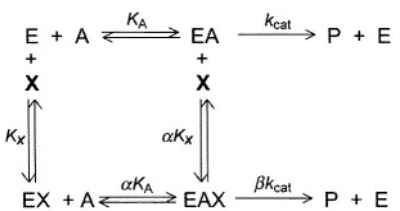
Enzyme Activation

In enzymology, *activators* are the molecules that increase the velocity of an enzyme-catalyzed reaction due to reversible binding to the enzyme. In *nonessential activation,* the reaction can occur in the absence of the activator, as well as in its presence. In an *essential activation,* the reaction will not take place in the absence of an activator (Reinhold, 1969; Segel, 1975; Dixon & Webb, 1979).

7.1 NONESSENTIAL ACTIVATION

Kinetically, the nonessential activation can be treated in the same manner as a general nonlinear inhibition; however, this time the changes are in the opposite direction: wherever we have had an inhibition, now we have an activation (Segel, 1975).

For a monosubstrate reaction, the kinetic model is analogous to a general model for a nonlinear hyperbolic inhibition, described in Chapter 6 (Section 6.1):



(7.1)

where $V_1 = k_{cat}E_0$, X is the activator molecule, and K_X an *activator dissociation constant,* with a dimension [concentration].

A rate equation for this general case may be derived from rapid equilibrium assumptions to obtain a Michaelis-Menten form:

$$v_0 = \frac{V_1 A}{K_A\left(\frac{1+\frac{X}{K_X}}{1+\frac{\beta X}{\alpha K_X}}\right) + A\left(\frac{1+\frac{X}{\alpha K_X}}{1+\frac{\beta X}{\alpha K_X}}\right)} \tag{7.2}$$

Equation (7.2) has the same form as Eq. (6.2) (Chapter 6) for the hyperbolic inhibition in monosubstrate reactions. In enzyme activation, however, contrary to inhibition, $\beta > 1$ and $\alpha < 1$.

Equation (7.2) can be rearranged in the Lineweaver–Burk manner:

$$\frac{1}{v_0} = \frac{1}{V_1}\left(\frac{\alpha K_X + X}{\alpha K_X + \beta X}\right) + \frac{\alpha K_A}{V_1}\left(\frac{K_X + X}{\alpha K_X + \beta X}\right)\frac{1}{A} \tag{7.3}$$

and presented graphically (Fig. 1).

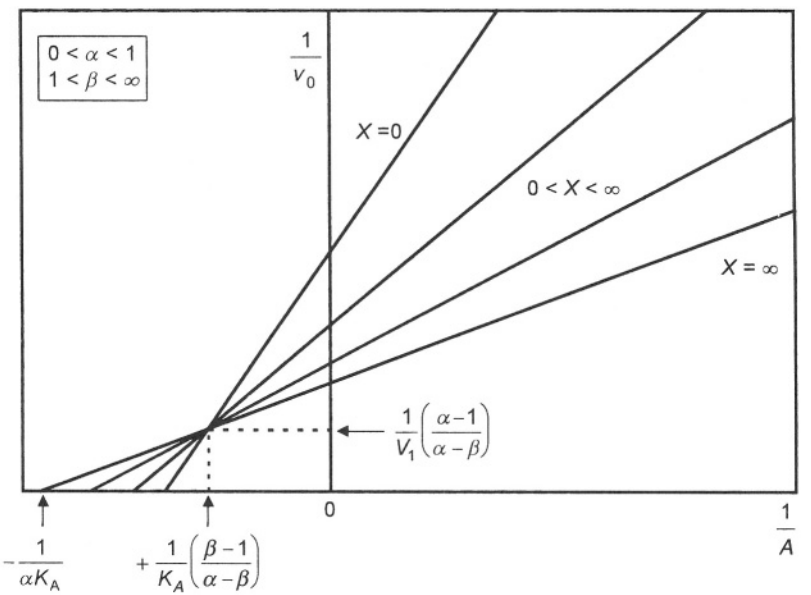


Figure 1. Nonessential activation. Graphical presentation of Eq. (7.3).

Compare Fig. 1 for the nonlinear inhibition of Chapter 6, and notice that the double reciprocal plot in both figures is a family of straight lines with a common intersection point; this intersection point has different coordinates in activation and inhibition systems.

Similarly to nonlinear inhibition, at infinitely high X, Eq. (7.2) reduces to

$$v_0 = \frac{\beta V_1 A}{\alpha K_A + A} \tag{7.4}$$

Thus, at infinitely high activator concentration, the apparent K_A is equal to αK_A and the apparent maximal velocity is equal to βV_1.

The slopes and intercepts for the plot shown in Fig. 1 are equal:

$$\text{Slope}_{1/A} = \frac{\alpha K_A}{V_1}\left(\frac{K_X + X}{\alpha K_X + \beta X}\right) \tag{7.5}$$

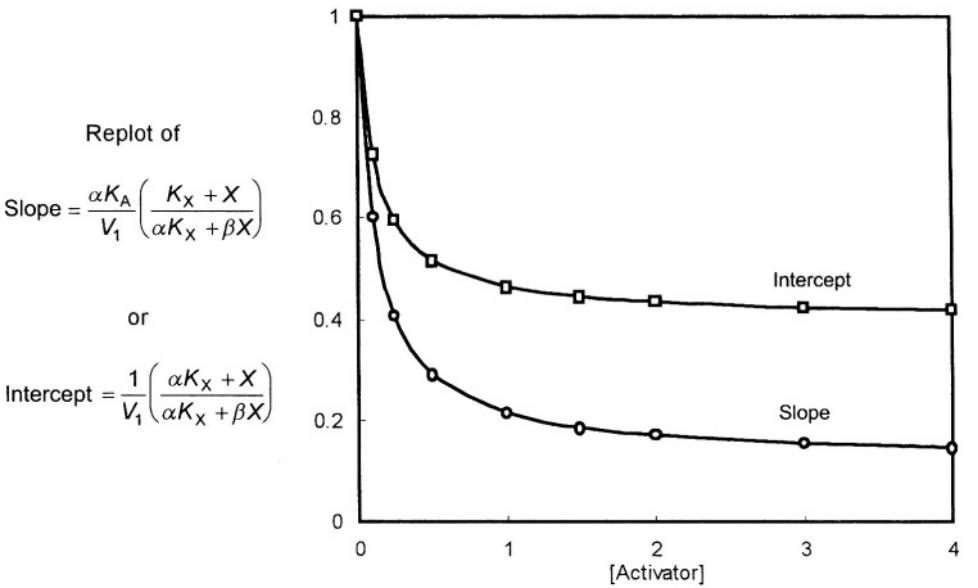


Figure 2. Replot of slopes and intercepts drawn according to the general rate Eq. (7.3). The data points are calculated assuming that $\alpha = 0.3$ and $\beta = 2.5$; $V_1 = K_A = K_X = 1$.

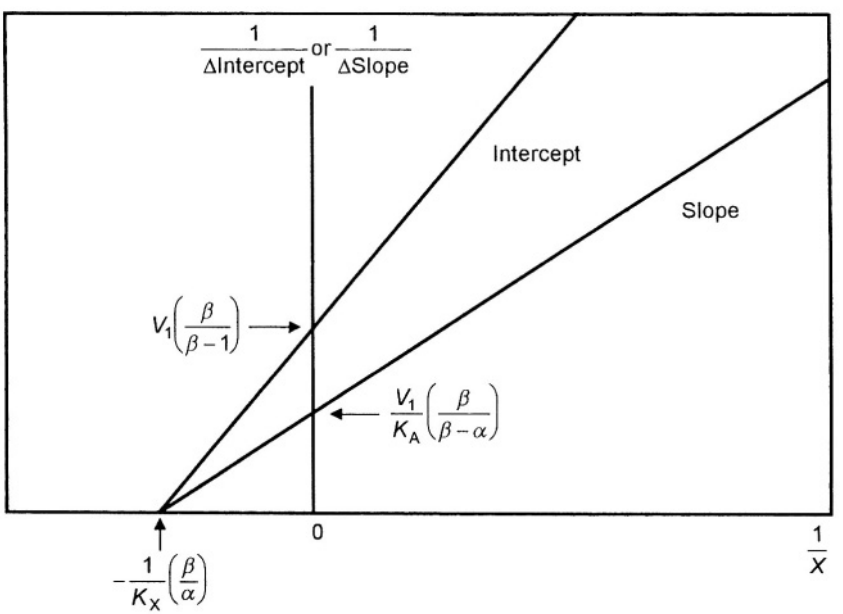


Figure 3. Nonessential activation. Analysis of Eqs. (7.5) and (7.6) by the differential method.

$$\text{Intercept}_{1/A} = \frac{1}{V_1}\left(\frac{\alpha K_X + X}{\alpha K_X + \beta X}\right) \tag{7.6}$$

Figure 2 shows a replot of slopes and intercepts from Fig.1 as a function of increasing concentrations of an activator. From Fig. 2, it is clear that the nonessential activation, in the general case, is a *nonlinear activation,* as the replots of slopes and intercepts are hyperbolas.

In this case, since both functions are nonlinear, it is possible to determine the kinetic constants α and β by the application of the differential method, similarly as described for the nonlinear inhibition in Chapter 6 (Section 6.2) (Fig. 3).

The nonessential activation, in the general case, is a hyperbolic nonlinear activation. Analogous to hyperbolic inhibition, we can derive a number of different activation mechanisms, by inserting different values for α and β into Eq. (7.2), as was described in Chapter 6 (Section 6.3), for different types of hyperbolic inhibitions. However, in activation processes, it is always $\beta > 1$.

7.2 ESSENTIAL ACTIVATION

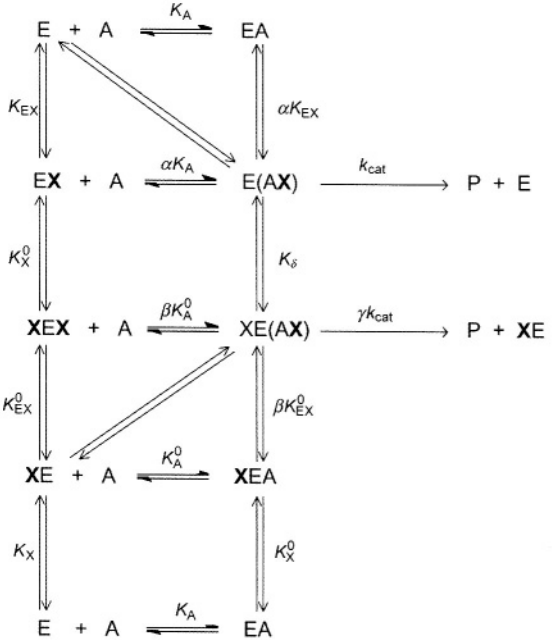
7.2.1 Activation by Substrates

According to our definition of essential activators in Section 7.1, the reaction will not take place in the absence of an activator. Thus, according to this definition, we may treat all the bisubstrate and trisubstrate reactions as essential activations in which both substrates in turn may be regarded as an activator for other substrates (Purich & Allison, 2000). This topic, however, is described in detail in Chapters 8 and 9 (bisubstrate reactions), and in Chapter 12 (trisubstrate reactions).

7.2.2 Complex Activation by Metal Ions

All enzymatic reactions involving ATP require Mg^{2+} ion as an activator. These types of reactions are very common in nature, especially with kinases. In such cases, the true substrate is $Mg \cdot ATP^{2-}$ complex, that is, a *substrate–activator complex,* and free ATP molecules are not the active substrates of enzymes. In addition to forming an active complex with substrate, metal ions may also combine with the enzyme at an additional specific *activation site*; this additional binding site may be *essential* or *nonessential.* Thus, the metal ions may be treated as true substrates of enzymes.

London and Steck (1969) have developed a general model, based on rapid equilibrium assumptions, for a monosubstrate enzyme that combines with substrate, activator, and a substrate–activator complex. The kinetic model for this type of activation is rather complex (Reaction (7.7)).



(7.7)

Additional equilibria:

$$\mathrm{E} + \mathrm{AX} \underset{}{\overset{K_{AX}}{\rightleftharpoons}} \mathrm{E(AX)}$$

$$\mathrm{XE} + \mathrm{AX} \overset{K_{AX}^0}{\rightleftharpoons} \mathrm{XE(AX)}$$

$$K_\delta = \frac{\beta K_A^0 K_X^0}{\alpha K_A}$$

The complex reaction (7.7) may be drawn in two dimensions as a cube, whereby the eight corners of the cube are represented by eight enzyme forms: E, EA, EX, E(AX), XEX, XE(AX), XE, and XEA.

The metal activator (X) not only combines with a free enzyme to form an enzyme–activator complex (XE), but also combines with EA to form E(AX), with an EX complex to form XEX, and with E(AX) complex to form XE(AX). If the activation is nonessential, both E(AX) and XE(AX) are catalytically active. Since AX $(\mathbf{Mg \cdot ATP^{2-}})$ is a true substrate of enzyme, EA and XEA are inactive. The general velocity equation may be derived from the rapid equilibrium assumptions, in the following form:

$$\frac{v_o}{E_o} = \frac{k_{cat}\left(\frac{[AX]}{K_{AX}}\right) + \gamma k_{cat}\left(\frac{[X][AX]}{K_X K_{AX}^0}\right)}{1 + \frac{[A]}{K_A} + \frac{[X]}{K_{EX}} + \frac{[AX]}{K_{AX}} + \frac{[X]}{K_X} + \frac{[A][X]}{K_A^0 K_X} + \frac{[X]^2}{K_X K_{EX}} + \frac{[X][AX]}{K_X K_{AX}^0}} \tag{7.8}$$

Equation (7.8) can be rearranged as:

$$\frac{v_0}{E_0} = \frac{k_{cat}\left(\frac{[AX]}{K_{AX}}\right) + \gamma k_{cat}\left(\frac{[X][AX]}{K_X K^o_{AX}}\right)}{\left(1 + \frac{[A]}{K_A} + \frac{[X]}{K_{EX}} + \frac{[AX]}{K_{AX}}\right) + \frac{1}{K_X}\left(1 + \frac{[A]}{K^o_A} + \frac{[X]}{K_{EX}} + \frac{[AX]}{K^o_{AX}}\right)\cdot[X]} \tag{7.9}$$

If the activation by a metal ion is *essential,* the first numerator term AX/K_{AX} is omitted from Eq. (7.9).

The situation shown in reaction (7.7) may be further complicated if the metal ion combines with the substrate; this is often the case in reactions catalyzed by kinases, where the metal ion (usually Mg^{2+}) combines with ATP, thus affording an additional equilibrium:

$$Mg^{2+} + ATP^{4-} \overset{K_0}{\rightleftharpoons} (Mg \cdot ATP)^{2-} \tag{7.10}$$

The model shown in reaction (7.7) is a general model, very similar to the model for an inhibition by a mixture of two inhibitors in monosubstrate reactions, described in Chapter 5 (Section 5.7). From the model in reaction (7.7), one can derive several special cases that comprise only the parts of the general model shown in the reaction (Morrison, 1979). The derivation of rate equations for enzyme activation is analogous to the derivation of rate equations for enzyme inhibition; the main difference is that the binding of activators increases, whereas the binding of inhibitors decreases the activity of enzymes. In the general model (Reaction (7.7)), that is, described by Eq. (7.8), the dependence of initial velocity, v_0, on the concentration of activator X does not provide a hyperbola; that is, the response of v_0 to increasing concentrations of X is sygmoid. Therefore, this model will not be discussed further in this chapter; the nonhyperbolic kinetic systems are discussed in Chapter 13.

London and Steck (1969) have extended their work with activators by describing a useful graphical method for analyzing enzyme activation, particularly by metal ions.

REFERENCES

Dixon, M. & Webb, E.C. (1979) *Enzymes,* 3rd ed., Academic Press, New York.
London, W.P. & Steck, T.L. (1969) *Biochemistry 8,* 1767–1777.
Morrison, J.F. (1979) *Methods Enzymol. 73,* 257–294.
Purich, D.L. & Allison R.D. (2000) *Handbook of biochemical kinetics,* Academic Press, San Diego.
Reinhold, J. (1969) *Behavior of enzyme systems, 2*nd ed., Van Nostrand-Reinhold, New York.
Segel, I.H. (1975) *Enzyme kinetics,* Wiley, New York.

Chapter 8

Kinetics of Rapid Equilibrium Bisubstrate Reactions

The steady-state kinetics of monosubstrate enzyme reactions has been described in Chapter 3. However, true monosubstrate reactions are quite rare in nature and are restricted only to some isomerases and epimerases. The majority of enzyme reactions are multisubstrate reactions, with two or three substrates and one, two, or three products of reaction (IUBMB, 1992).

8.1 NOMENCLATURE

In this chapter, the subject of *bisubstrate enzyme reactions* is introduced by examining the rapid equilibrium bireactant systems, with two substrates and two products of reaction. The main characteristics of this type of reactions is that both substrates, A and B, an both products of reaction, P and Q, are bound to enzyme very rapidly, and that all binding and dissociation steps are, in most cases, much faster than the chemical transformations of substrates to products or, vice versa, the transformation of products to substrates.

The velocity equations for bisubstrate and trisubstrate reactions are usually formidable if expressed in terms of individual rate constants, especially in the steady-state treatment. The resulting equations are almost useless until the rate constants are grouped into relatively simple kinetic constants that can be experimentally determined (Chapter 4). Various methods for grouping the individual rate constants have been developed by Alberty (1953), Dalziel (1957), Bloomfield *et al.* (1962), Wong & Hanes (1962), Cleland (1963), Mahler & Cordes (1966), Segel (1975), and others. The *nomenclature of Cleland* is now in general use.

In Cleland's nomenclature, *substrates* are designated by the letters A, B, and C in the order which they add to the enzyme, and *products* by the letters P, Q, and R in order in which they leave the enzyme. There are two types of enzyme forms: those which are *stable* on the timescale of several minutes or more, and those which are basically *enzyme–reactant complexes* and will dissociate on a timescale of seconds or milliseconds. Stable enzyme forms are designated E, F, and G. The stable forms can usually be isolated by chemical methods and latter shown to transfer the group they are carrying to one of the reactants.

Enzyme–reactant complexes which readily break down are called *transitory complexes* and consist of two types: those in which the active site is not completely filled, so that they can bind another reactant, and those in which the active site is completely filled with reactants, so that only dissociation of

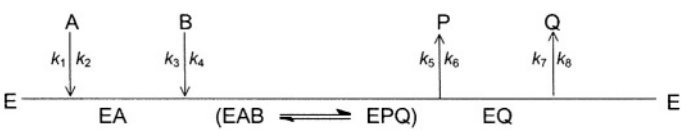
substrates or products is possible; the latter are called *central complexes.* Transitory complexes are designated by letters indicating their components such as EA, EAB, EQ, etc.

The number of kinetically important reactants in a given direction is the reactancy in this direction, and indicated by the syllables Uni, Bi, and Ter. Thus, a reaction with one substrate and two products is Uni Bi and is unireactant in the forward and bireactant in the reverse direction.

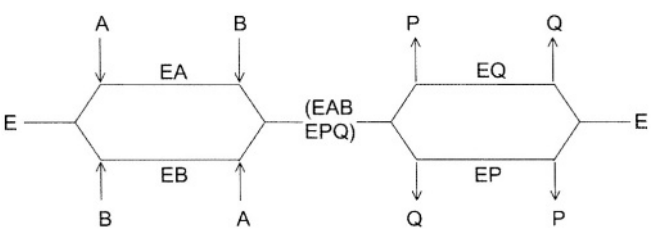
The Michaelis constants corresponding to various reactants are designated by *K,* with an identifying subscript: K_A, K_B, K_P, and K_Q; K_{iA}, K_{iB}, K_{iQ}, etc. are used for inhibition or dissociation constants, and other special constants may be defined as well, if need arises. All such constants have dimensions of [concentration]. The overall equilibrium constant of the chemical reaction is designated K_{eq}. The maximum velocities in forward and reverse directions are designated V_1 and V_2; these have dimensions of [concentration/unit time]. The expressions V_1/E_o and V_2/E_o are turnover numbers, and have dimensions $[\text{time}^{-1}]$.

All kinetic mechanisms can be presented in shorthand notations; the most popular is that proposed by Cleland (1963). However, the reader is warned that he or she will find other systems in the literature, as well as in this book, and will have to examine the definitions given for the kinetic constants in each case.

The shorthand notation of Cleland is illustrated with the example of an Ordered Bi Bi mechanism:



If there is no obligatory order of addition of substrates and dissociation of products, the reaction becomes Random Bi Bi:



In a similar way, all other mono and multisubstrate mechanisms may be presented in a shorthand notation.

Kinetic measurements with bisubstrate reactions are performed by measuring the initial reaction rates in the presence of increasing concentrations of substrate A, keeping the substrate B constant and repeating the experiment at several fixed concentrations of substrate B; thus, A represents a *variable substrate* and B a *constant substrate.* In the double reciprocal plot, the experimental data present a family of straight lines, with a common *intersection point* which is found on ordinate, on abscissa, in the III or in the IV quadrant. One can use the same set

of data by plotting B as a variable against A as a constant substrate, and obtain the same result. The double reciprocal plot with a family of straight lines is a *primary graph.* In the primary plot, each line has a *slope* and an *intercept on ordinate,* which are gradually increasing with decreasing concentrations of the constant substrate.

The *secondary graphs* are obtained by plotting the slopes or intercepts from the primary graph against reciprocal concentration of the constant substrate. The slopes and intersection points on abscissa or on ordinate in the secondary graphs usually afford simple kinetic expressions from which all kinetic parameters can be easily calculated.

In the literature, literally dozens of kinetic mechanisms have been proposed for bisubstrate enzymes (Alberty, 1958; Alberty & Hammes, 1958; Peller & Alberty, 1959; Wong & Hanes, 1962; Fromm, 1967; Dalziel, 1969; Hurst, 1969; Rudolph & Fromm, 1969, 1971, 1973). However, only those pathways that are either well documented, or seem to be a logical extension of established mechanisms, will be presented in this and the following chapters. Thus, we shall divide the rapid equilibrium bisubstrate reactions into the following major types, according to the type and number of enzyme–substrate or enzyme–product complexes that can form (Alberty, 1953; Cleland, 1970, 1977; Fromm, 1979; Engel, 1996; Purich & Allison, 2000):

(1) Ordered bisubstrate system,
(2) Random bisubstrate system,
(3) Random Bi Bi system with a dead-end EBQ complex, and
(4) Random Bi Bi system with dead-end complexes EBQ and EAP.

The theoretical basis and kinetic expressions for two substrate reactions were developed largely by Alberty (1953), Dalziel (1957), and Cleland (1963).

8.2 RAPID EQUILIBRIUM ORDERED SYSTEM

Rapid Equilibrium Ordered system occurs when the substrates A and B combine with the enzyme in an ordered manner, that is, when B can bind only to the EA complex and EB complex is not formed:

$$\mathrm{E} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{EA} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{EAB} \xrightarrow{k_5} \mathrm{P} + \mathrm{E} \qquad (8.1)$$

In order to obtain the rapid equilibrium conditions, the k_2 rate constant has to be much higher than k_5, while the k_4 constant can be the same size or smaller than k_5. Thus, K_{iA} is equal k_2/k_1, a true dissociation constant of the enzyme–substrate complex, K_A is zero, and K_B is equal $(k_4 + k_5)/k_3$. The velocity equation for this reaction is

$$v_0 = \frac{V_1 AB}{K_{iA}K_B + K_B A + AB} \qquad (8.2)$$

where $V_1 = k_5 E_0$.

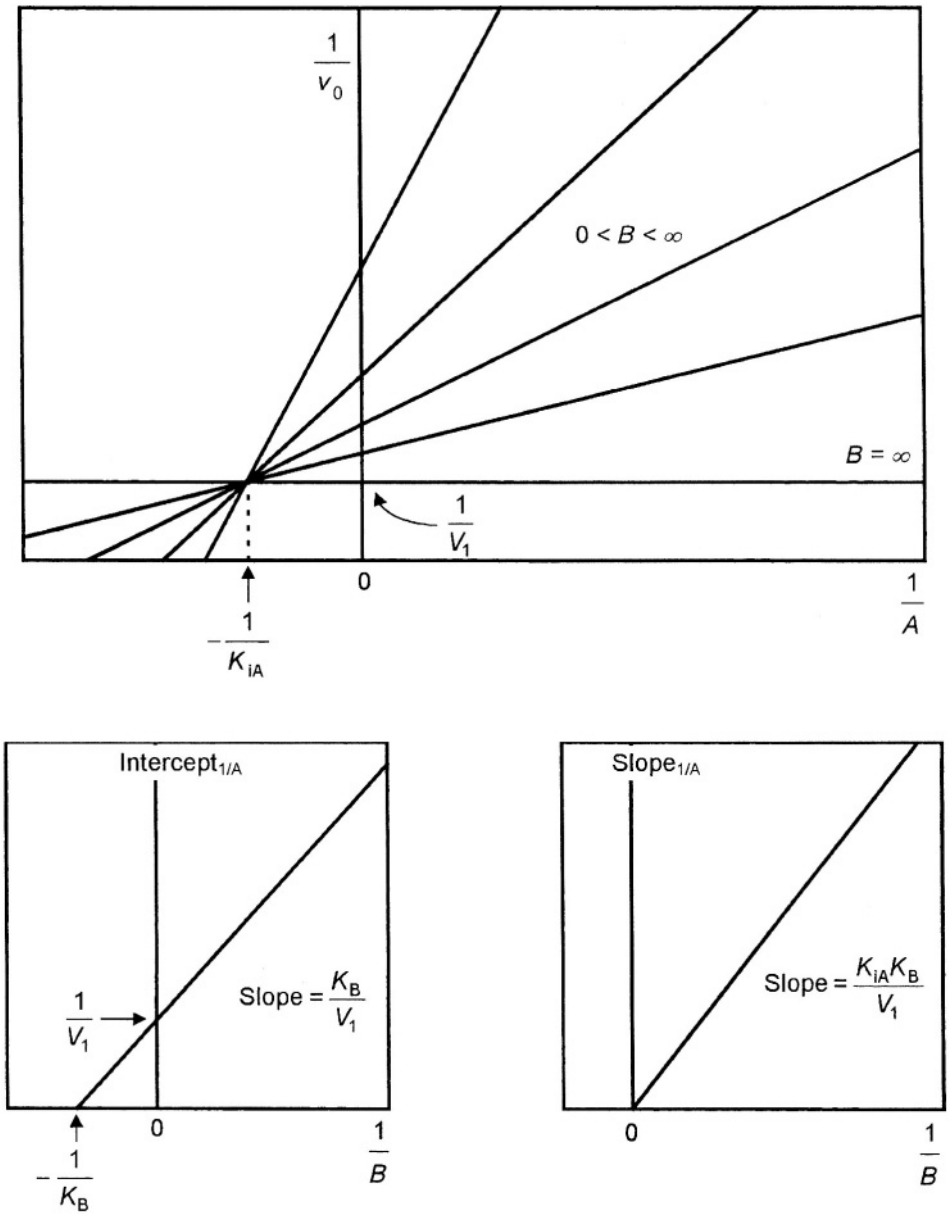


Figure 1. Rapid Equilibrium Ordered bisubstrate system. Graphical presentation of Eq. (8.4), with B as a constant and A as a variable substrate.

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \left(\frac{K_{iA}K_B}{V_1 B}\right)\frac{1}{A}$$

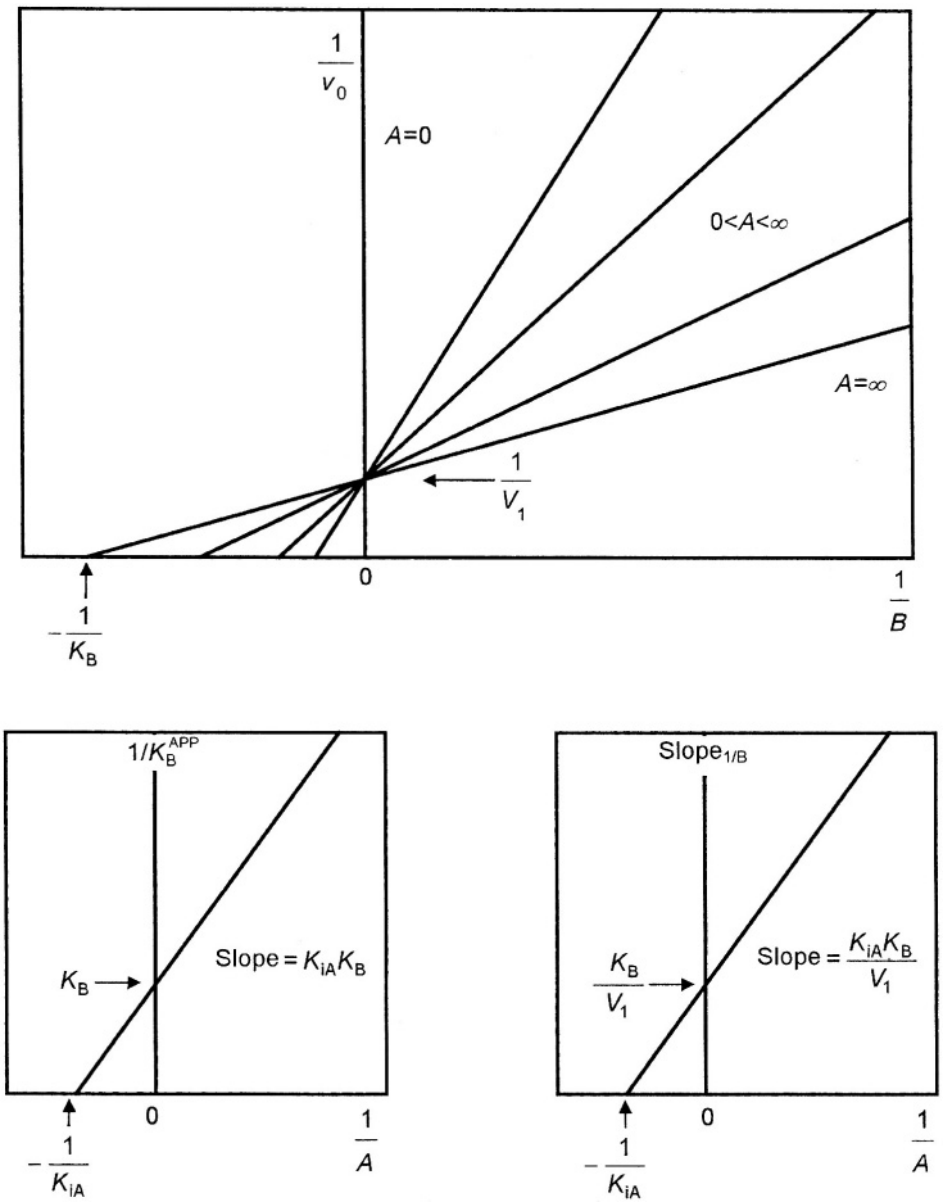


Figure 2. Rapid Equilibrium Ordered bisubstrate system. Graphical presentation of Eq. (8.5), with A as a constant and B as a variable substrate.

$$\frac{1}{v_0} = \frac{1}{V_1} + \frac{K_B}{V_1}\left(1 + \frac{K_{iA}}{A}\right)\frac{1}{B}$$

The reciprocal form of Eq. (8.2) is

$$\frac{V_1}{v_0} = 1 + \frac{K_B}{B} + \frac{K_{iA}K_B}{AB} \tag{8.3}$$

The separation of the variable from the constant substrate in Eq. (8.3) provides:

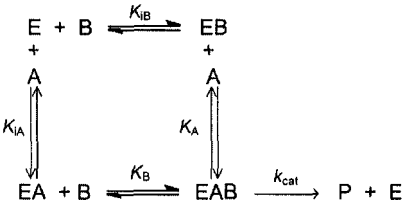
$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \left(\frac{K_{iA}K_B}{V_1 B}\right)\frac{1}{A} \tag{8.4}$$

$$\frac{1}{v_0} = \frac{1}{V_1} + \frac{K_B}{V_1}\left(1 + \frac{K_{iA}}{A}\right)\frac{1}{B} \tag{8.5}$$

Figures, 1 and 2 show the graphical presentation of Eqs. (8.4) and (8.5)

8.3 RAPID EQUILIBRIUM RANDOM SYSTEM

Rapid Equilibrium Random system, in the absence of products, occurs if the substrates A and B bind *randomly*. If the binding of one substrate changes the dissociation constant for the other substrate by the same factor α ($\alpha = K_A/K_{iA} = K_B/K_{iB}$), the system can be described by the equilibria shown below:



(8.6)

In the random case, in order to obtain the rapid equilibrium conditions, only the off rate constants of A and B from their binary complexes have to be much faster then k_{cat}. If $V_1 = k_{cat}E_0$, the velocity equation for reaction scheme (8.6) is

$$v_0 = \frac{V_1 AB}{K_{iA}K_B + K_B A + K_A B + AB} \tag{8.7}$$

or, in the reciprocal form:

$$\frac{V_1}{v_0} = 1 + \frac{K_A}{A} + \frac{K_B}{B} + \frac{K_{iA}K_B}{AB} \tag{8.8}$$

The same equation is obtained if one assumes that A and B dissociate from the EAB complex with rates similar or smaller than k_{cat}. In such cases, the Michaelis constants, K_A and K_B, are no longer dissociation constants, but are more complex expressions; K_{iA} and K_{iB} are still true dissociation constants of respective binary complexes.

Thus, any substrate that can add last can bind either in rapid equilibrium or in steady-state fashion without changing the form of the rate equation. Comparison of Eqs. (8.2) and (8.7), however, shows that a substrate that cannot add last will change the rate equation if it adds in rapid equilibrium.

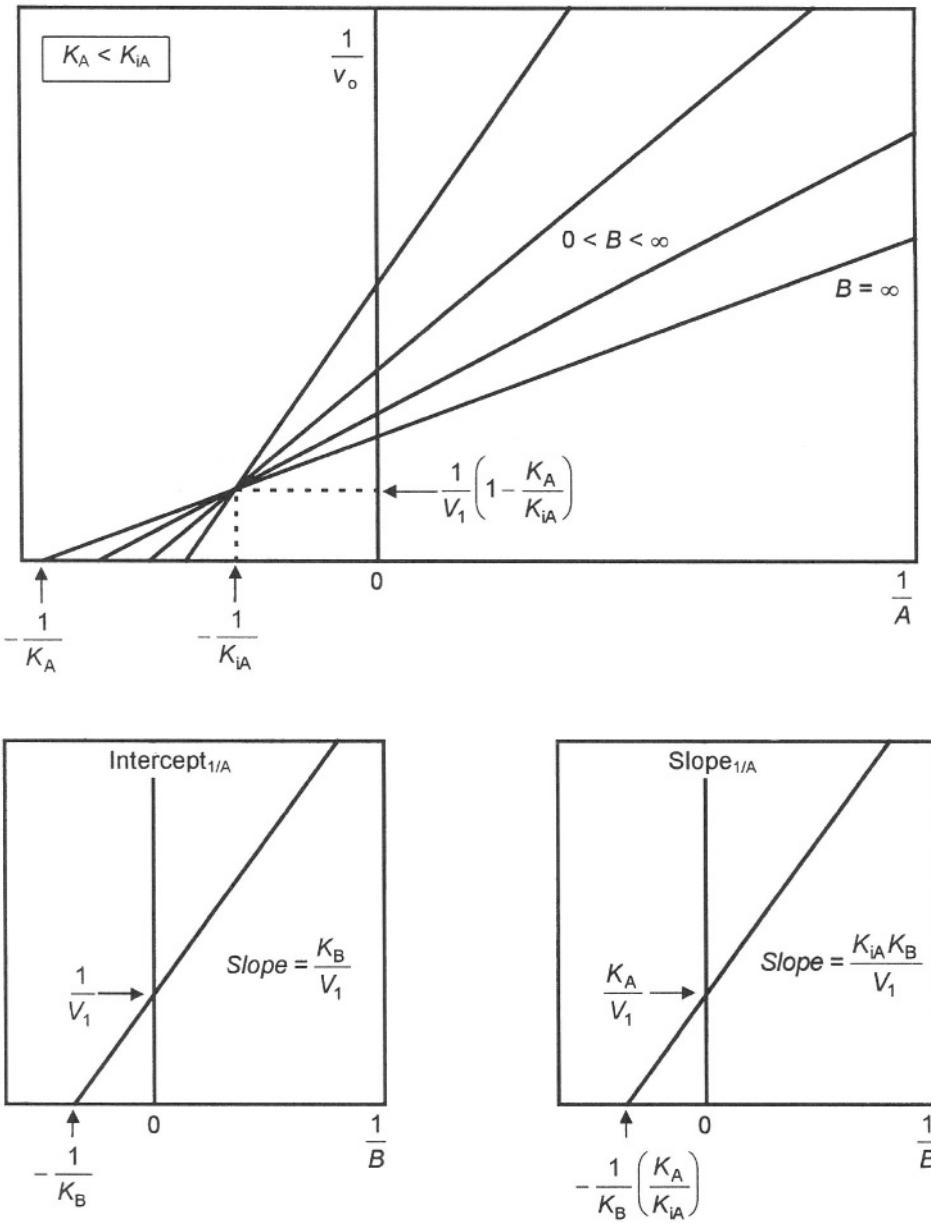


Figure 3. Rapid Equilibrium Random bisubstrate system. Graphical presentation of Eq. (8.9), with B as a constant and A as a variable substrate.

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \frac{K_A}{V_1}\left(1 + \frac{K_{iA}K_B}{BK_A}\right)\frac{1}{A}$$

Equation (8.8) may be rearranged into two linear forms:

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \frac{K_A}{V_1}\left(1 + \frac{K_{iA}K_B}{BK_A}\right)\frac{1}{A} \tag{8.9}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_A}{A}\right) + \frac{K_B}{V_1}\left(1 + \frac{K_{iA}}{A}\right)\frac{1}{B} \tag{8.10}$$

Figure 3 shows the graphical presentation of Eq. (8.9).

Equations (8.9) and (8.10) are completely symmetrical and, therefore, their graphical presentations are also symmetrical. For this reason, the graphical presentation of Eq. (8.10) is omitted, because it is not necessary to perform two separate experiments to construct the reciprocal plots for two varied substrates. One primary plot, for example, $1/v_0$ versus $1/A$, contains all the information necessary to calculate all kinetic constants.

If K_A/K_{iA} is less than unity, than the binding of one ligand increases the affinity of enzyme for the other ligand and, vice versa, if K_A/K_{iA} is larger than unity, than the binding of one ligand decreases the affinity for the other. The double reciprocal plot in Fig. 3 represents a family of straight lines with a common intersection point. It is clear that this point is in the IV quadrant, above abscissa, if $K_{iA}/K_A > 1$, and in the III quadrant, below abscissa, if $K_{iA}/K_A < 1$. If $K_{iA}/K_A = 1$, the plot intersects on abscissa, showing that one ligand has no effect on the binding of the other.

8.4 PRODUCT INHIBITION IN A RAPID EQUILIBRIUM ORDERED BI BI SYSTEM

Why product inhibition occurs. The products of reaction are formed at the active site of enzyme and are the substrates for the reverse reaction. Consequently, a product may act as an inhibitor by occupying the same site as the substrate from which it is derived. In the Rapid Equilibrium Random bisubstrate mechanism, most ligand dissociations are very rapid compared to the interconversion of EAB and EPQ. Thus, the levels of EP and EQ are essentially zero in the absence of added P and Q. In the presence of only one of the products, the reverse reaction can be neglected, as the concentration of the other product is essentially zero during the early part of the reaction. Nevertheless, the forward reaction will be inhibited because finite P (or Q) ties up some of the enzyme. The type of this *product inhibition* depends on the number and type of enzyme–product complexes that can form. Consequently, product inhibition studies can be very valuable in the diagnostics of kinetic mechanisms (Rudolph, 1979).

General rate equation. Let us examine the product inhibition in the Rapid Equilibrium Ordered Bi Bi system, in the presence of products P or Q. Both substrates and both products of reaction are binding in an ordered fashion.

$$\mathrm{E} \overset{K_{iA}}{\rightleftharpoons} \mathrm{EA} \overset{K_B}{\rightleftharpoons} \mathrm{EAB} \underset{xk_{cat}}{\overset{k_{cat}}{\rightleftharpoons}} \mathrm{EPQ} \overset{K_P}{\rightleftharpoons} \mathrm{EQ} \overset{K_{iQ}}{\rightleftharpoons} \mathrm{E} \tag{8.11}$$

The complete velocity equations for this system are

$$v_0 = \frac{V_1\left(\frac{AB}{K_{iA}K_B}\right) - V_2\left(\frac{PQ}{K_PK_{iQ}}\right)}{1 + \frac{A}{K_{iA}} + \frac{Q}{K_{iQ}} + \frac{AB}{K_{iA}K_B} + \frac{PQ}{K_PK_{iQ}}} = \frac{V_1V_2\left(AB - \frac{PQ}{K_{eq}}\right)}{V_2K_{iA}K_B + V_2K_BA + V_2AB + \frac{V_1K_P}{K_{eq}}Q + \frac{V_1}{K_{eq}}PQ} \tag{8.12}$$

Reaction scheme (8.11) describes a reversible reaction with two maximal velocities, one in the forward and the other in the reverse direction, both being the products of catalytic constants and the total concentration of enzyme, $V_1 = k_{cat}E_0$ and $V_2 = xk_{cat}E_0$. It is important to note that the interconversion of above two equations is achieved with the aid of the Haldane relationship (8.43).

Rate equation in the presence of product P. Since P does not bind to the free enzyme, it is clear that the initial velocity equation in the presence of substrates A and B and the product P, has the same form as Eq. (8.2).

Rate equation in the presence of product Q. In the presence of substrates A and B, and the product Q, Eq. (8.12) has the form

$$v_0 = \frac{V_1\left(\frac{AB}{K_{iA}K_B}\right)}{1 + \frac{A}{K_{iA}} + \frac{Q}{K_{iQ}} + \frac{AB}{K_{iA}K_B}} = \frac{V_1AB}{K_{iA}K_B\left(1 + \frac{Q}{K_{iQ}}\right) + K_BA + AB} \tag{8.13}$$

or, in the reciprocal forms:

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \frac{K_{iA}K_B}{V_1B}\left(1 + \frac{Q}{K_{iQ}}\right)\frac{1}{A} \tag{8.14}$$

$$\frac{1}{v_0} = \frac{1}{V_1} + \frac{K_B}{V_1}\left[1 + \frac{K_{iA}}{A}\left(1 + \frac{Q}{K_{iQ}}\right)\right]\frac{1}{B} \tag{8.15}$$

Equations (8.14) and (8.15) can be plotted in the form of the primary graphs as shown in Figs. 4 and 5.

In product inhibition studies, it is customary to form the primary graphs by plotting $1/v_0$ versus $1/A$, in the presence of constant concentrations of one of the products, Q or P, and a constant concentration of substrate B; by repeating the experiment at several fixed concentrations of the product, we shall obtain a primary graph, with A as a variable substrate, B as a constant substrate, and P or Q as the variable product of reaction.

An analogous primary graph is formed by plotting $1/v_0$ versus $1/B$ in the presence of constant A, and variable Q or P.

In product inhibition studies, the secondary graphs are formed by plotting the slopes or intercepts from the primary graphs against increasing concentrations of a product inhibitor (P or Q). Thus, the secondary plots will provide information that is missing from the primary plots.

In Fig. 4, the secondary plots are apparently identical. The reason for this is that the $\mathrm{Slope}_{1/A}$ function and the K_A^{APP} function have the same form, although different meanings and different dimensions:

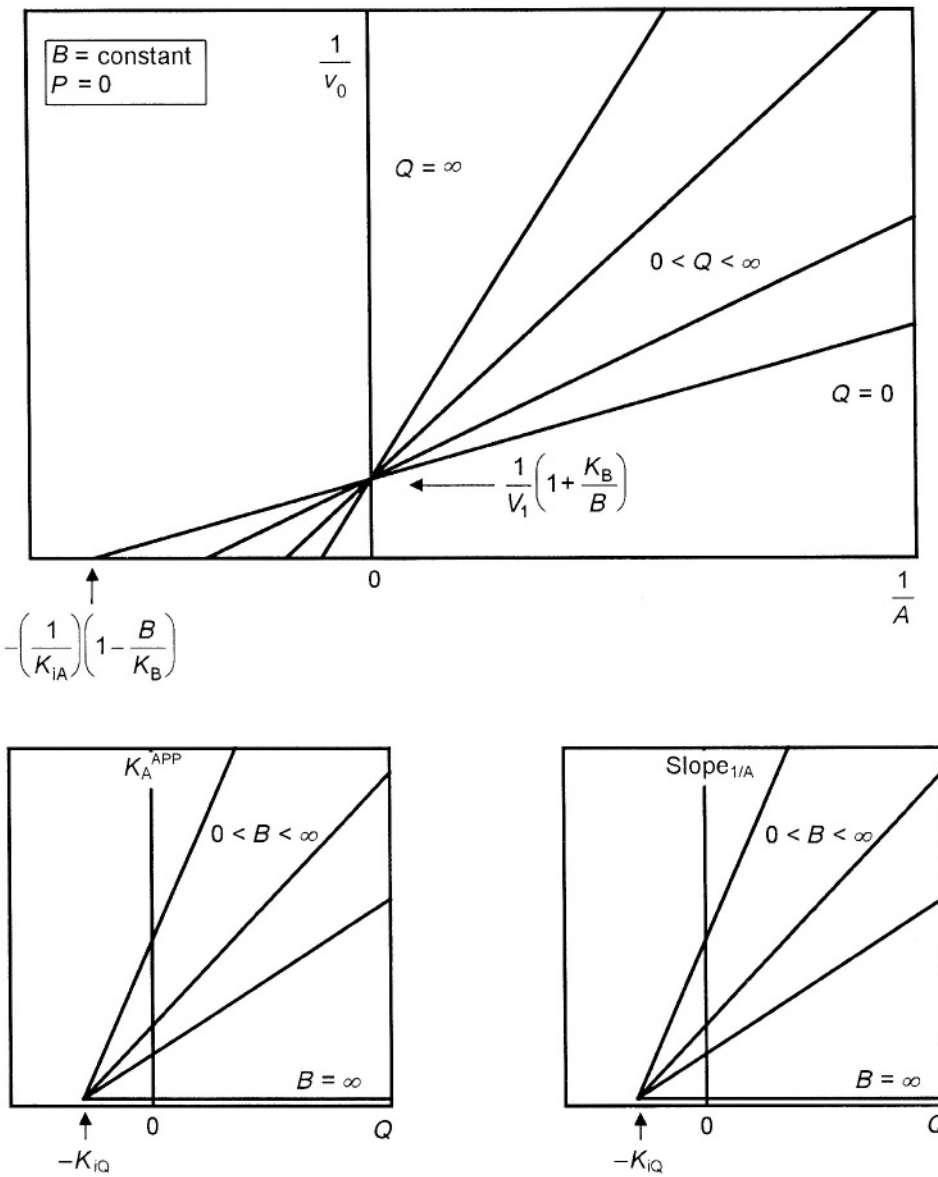


Figure 4. Product inhibition by Q in the Rapid Equilibrium Ordered Bi Bi system. Graphical presentation of Eq. (8.14), with B as a constant and A as a variable substrate.

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \frac{K_{iA}K_B}{V_1 B}\left(1 + \frac{Q}{K_{iQ}}\right)\frac{1}{A}$$

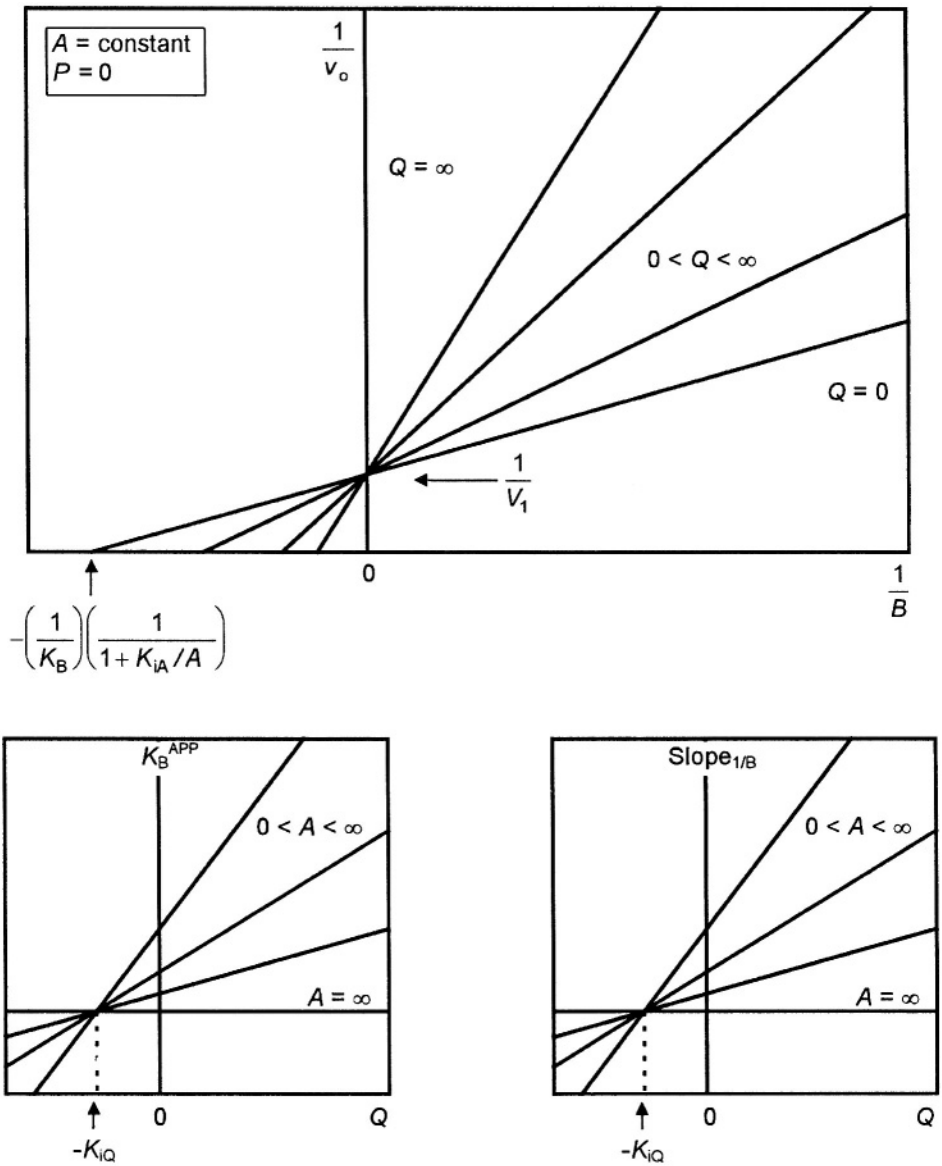


Figure 5. Product inhibition by Q in the Rapid Equilibrium Ordered Bi Bi system. Graphical presentation of Eq. (8.15), with A as a constant and B as a variable substrate.

$$\frac{1}{v_0} = \frac{1}{V_1} + \frac{K_B}{V_1}\left[1 + \frac{K_{iA}}{A}\left(1 + \frac{Q}{K_{iQ}}\right)\right]\frac{1}{B}$$

$$\text{Slope}_{1/A} = \frac{K_{iA}K_B}{V_1 B} + \left(\frac{K_{iA}K_B}{V_1 B}\right)\frac{Q}{K_{iQ}} \tag{8.16}$$

$$K_A^{APP} = \frac{K_{iA}K_B}{(B+K_a)} + \left(\frac{K_{iA}K_B}{B+K_B}\right)\frac{Q}{K_{iQ}} \tag{8.17}$$

Similarly, in Fig. 5, the secondary plots are also apparently identical. In this case, again the $\text{Slope}_{1/B}$ function and the K_B^{APP} function have the same form, although different meanings and different dimensions:

$$\text{Slope}_{1/B} = \frac{K_B}{V_1}\left(1 + \frac{K_{iA}}{A}\right) + \left(\frac{K_{iA}K_B}{V_1 A}\right)\frac{Q}{K_{iQ}} \tag{8.18}$$

$$K_B^{APP} = K_B\left(1 + \frac{K_{iA}}{A}\right) + \left(\frac{K_{iA}K_B}{A}\right)\frac{Q}{K_{iQ}} \tag{8.19}$$

Thus, any of the four secondary plots in Figs. 4 and 5 will provide the means for the graphical estimation of the inhibition constant K_{iQ}.

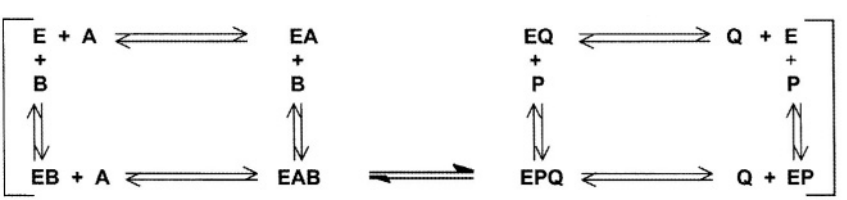
8.5 PRODUCT INHIBITION IN A RAPID EQUILIBRIUM RANDOM BI BI SYSTEM

In bisubstrate reactions, a very frequent case is the ability of enzyme to form the "wrong" ternary complexes, that is, to combine with a pair of substrates it is not supposed to bind together. In bisubstrate reactions, such ternary complexes are EBQ and EAP; these dead-end complexes are nonproductive and they can only dissociate back into their composite parts:

$$\begin{aligned} &EBQ \rightleftharpoons EB + Q; \quad EBQ \rightleftharpoons EQ + B; \\ &EAP \rightleftharpoons EA + P; \quad EAP \rightleftharpoons EP + A. \end{aligned} \tag{8.20}$$

The dead-end combinations EAQ and EBP are unlikely to form, because the A–Q and B–P pair of substrates are usually mutually exclusive; typical examples are the nucleotide coenzymes of dehydrogenases and kinases.

Consider a hypothetical Rapid Equilibrium Random Bi Bi system. Both products are present in the reaction mixture, A and B or P and Q can occupy the enzyme active site simultaneously, and all four ligands can bind by themselves; however, the dead-end complexes EAP and EBQ are not formed.



(8.21)

This hypothetical model is not valid. If the mechanism is random, one has to have at least one dead end complex—the one involving the two small reactants. Thus, if a portion of A is transferred in the reaction to B to produce P, one may expect to have an EBQ complex as the result of the definition of randomness. One may or may not have the EAP complex, depending on how serious the overlap between the reactants is (Fig. 6).

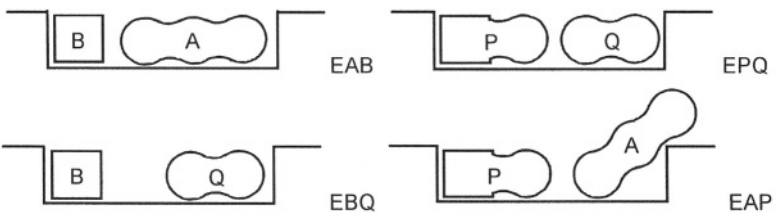


Figure 6. Formation of ternary complexes in bisubstrate reactions.

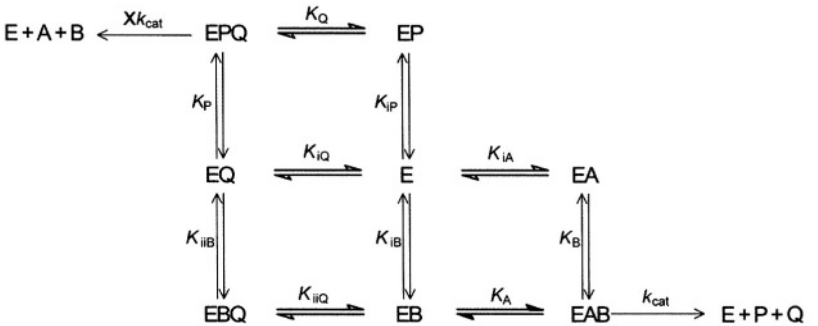
For example, in isocitrate dehydrogenase reaction, we shall see the formation of an enzyme–isocitrate–NADH complex because the extra hydrogen is not a problem (Grissom & Cleland, 1988). When the common group is larger, such as a phosphate group or bigger, one usually will not see the EAP complex, or the dissociation constants for this complex are elevated. Thus, in the hexokinase reaction

$$\underset{(B)}{\text{Sugar}} + \underset{(A)}{\text{ATP}} \rightleftharpoons \underset{(P)}{\text{Sugar-phosphate}} + \underset{(Q)}{\text{ADP}} \qquad (8.22)$$

a large phosphate group is transferred to the sugar to produce an even larger sugar-phosphate; two larger substrates, A and P, overlap seriously and cannot form the EAP complex. Note that Q is the "product" of A, and P is the "product" of B. However, the A–Q and B–P relationships are arbitrary in random systems as the result of the definition of randomness.

8.6 RAPID EQUILIBRIUM RANDOM BI BI SYSTEM WITH A DEAD-END EBQ COMPLEX

This is a very frequent case which takes place when A reacts with B, and a portion of substrate A is transferred to substrate B. In the presence of P, an EP complex can form. P excludes both A and B from the surface of enzyme; hence, there is no EAP and EBP complex. In the presence of Q, an EQ complex can form, but Q will not interfere with the binding of B; consequently, a dead-end complex EBQ can form.



(8.23)

In the presence of both substrates A and B and both products P and Q, the net velocity equations for the system shown in reaction (8.23) are

$$v_o = \frac{V_1\left(\frac{AB}{K_{iA}K_B}\right) - V_2\left(\frac{PQ}{K_PK_{iQ}}\right)}{1 + \frac{A}{K_{iA}} + \frac{B}{K_{iB}} + \frac{P}{K_{iP}} + \frac{Q}{K_{iQ}} + \frac{AB}{K_{iA}K_B} + \frac{PQ}{K_PK_{iQ}} + \frac{BQ}{K_{iB}K_{iiQ}}} \tag{8.24}$$

$$v_o = \frac{V_1V_2\left(AB - \frac{PQ}{K_{eq}}\right)}{V_2K_{iA}K_B + V_2K_BA + V_2K_AB + V_2AB + \frac{V_1K_P}{K_{eq}}Q + \frac{V_1K_Q}{K_{eq}}P + \frac{V_1}{K_{eq}}PQ + \frac{V_2K_A}{K_{iiQ}}BQ} \tag{8.25}$$

The maximal velocities, V_1 and V_2, have the usual meaning, and the interconversion of equations is achieved with the aid of the Haldane relationship (8.43). Note that, from reaction (8.23), $K_{iiQ}K_{iiB} = K_{iQ}K_{iB}$.

Product inhibition in the Rapid Equilibrium Random Bi Bi system with a dead-end EBQ complex takes place if, in addition to the presence of substrates A and B, also the product P, or alternatively the product Q, is also present.

Reaction of A *with* B *in the presence of product* Q. If both substrates A and B are present, plus the product Q, the velocity equation is

$$v_o = \frac{V_1\left(\frac{AB}{K_{iA}K_B}\right)}{1 + \frac{A}{K_{iA}} + \frac{B}{K_{iB}} + \frac{Q}{K_{iQ}} + \frac{AB}{K_{iA}K_B} + \frac{BQ}{K_{iB}K_{iiQ}}} \tag{8.26}$$

$$v_o = \frac{V_1AB}{K_{iA}K_B\left(1 + \frac{Q}{K_{iQ}}\right) + K_BA + \frac{K_{iA}K_B}{K_{iB}}\left(1 + \frac{Q}{K_{iiQ}}\right)B + AB} \tag{8.27}$$

In this case, the velocity equations, with each of the substrates A and B, as the varied ligands, are

$$v_0 = \frac{V_1 A}{K_A\left(1+\frac{K_{iB}}{B}+\frac{K_{iB}Q}{BK_{iQ}}+\frac{Q}{K_{iiQ}}\right)+A\left(1+\frac{K_B}{B}\right)} \tag{8.28}$$

$$v_0 = \frac{V_1 B}{K_B\left(1+\frac{K_{iA}}{A}+\frac{K_{iA}Q}{AK_{iQ}}\right)+B\left(1+\frac{K_A}{A}+\frac{K_A Q}{K_{iiQ}A}\right)} \tag{8.29}$$

The reciprocal forms of Eqs. (8.28) and (8.29), are

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1+\frac{K_B}{B}\right)+\frac{K_A}{V_1}\left[1+\frac{K_{iB}}{B}\left(1+\frac{Q}{K_{iQ}}\right)+\frac{Q}{K_{iiQ}}\right]\frac{1}{A} \tag{8.30}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left[1+\frac{K_A}{A}\left(1+\frac{Q}{K_{iiQ}}\right)\right]+\frac{K_B}{V_1}\left[1+\frac{K_{iA}}{A}\left(1+\frac{Q}{K_{iQ}}\right)\right]\frac{1}{B} \tag{8.31}$$

Equations (8.30) and (8.31) are quite complex. The former is showing a competitive while the latter the noncompetitive type of inhibition by the product Q.

Agraphical presentation of Eq. (8.30), a double reciprocal plot of $1/v_0$ versus $1/A$, will show a family of straight lines with a common intersection point on the ordinate if the concentration of substrate B is kept constant and the concentration of product Q is steadily increased. In this case, a replot of $\text{Slope}_{1/A}$ versus Q will provide the value of the dissociation constant of the EBQ complex (K_{iiQ}) (Fig. 7).

Contrary to that, a graphical presentation of Eq. (8.31) a reciprocal plot of $1/v_0$ versus $1/B$, shows a family of straight lines with a common intersection point in the IV quadrant if the concentration of substrate A is kept constant and the concentration of Q is steadily increased. In this case, only the replot of $\text{Intercept}_{1/B}$ versus Q will provide the value of the dissociation constant K_{iiQ} (Fig. 8).

Reaction of A *with* B *in the presence of product* P. If both substrates A and B are present, plus the product P, the velocity equation is

$$v_0 = \frac{V_1\left(\frac{AB}{K_{iA}K_B}\right)}{1+\frac{A}{K_{iA}}+\frac{B}{K_{iB}}+\frac{P}{K_{iP}}+\frac{AB}{K_{iA}K_B}} = \frac{V_1 AB}{K_{iA}K_B\left(1+\frac{P}{K_{iP}}\right)+AK_B+\frac{K_{iA}K_B}{K_{iB}}B+AB} \tag{8.32}$$

The reciprocal forms of Eq. (8.32) are

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1+\frac{K_B}{B}\right)+\frac{K_A}{V_1}\left[1+\frac{K_{iB}}{B}\left(1+\frac{P}{K_{iP}}\right)\right]\frac{1}{A} \tag{8.33}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1+\frac{K_A}{A}\right)+\frac{K_B}{V_1}\left[1+\frac{K_{iA}}{A}\left(1+\frac{P}{K_{iP}}\right)\right]\frac{1}{B} \tag{8.34}$$

These expressions predict competitive inhibition by P against either A or B unless the fixed substrate concentration is saturating.

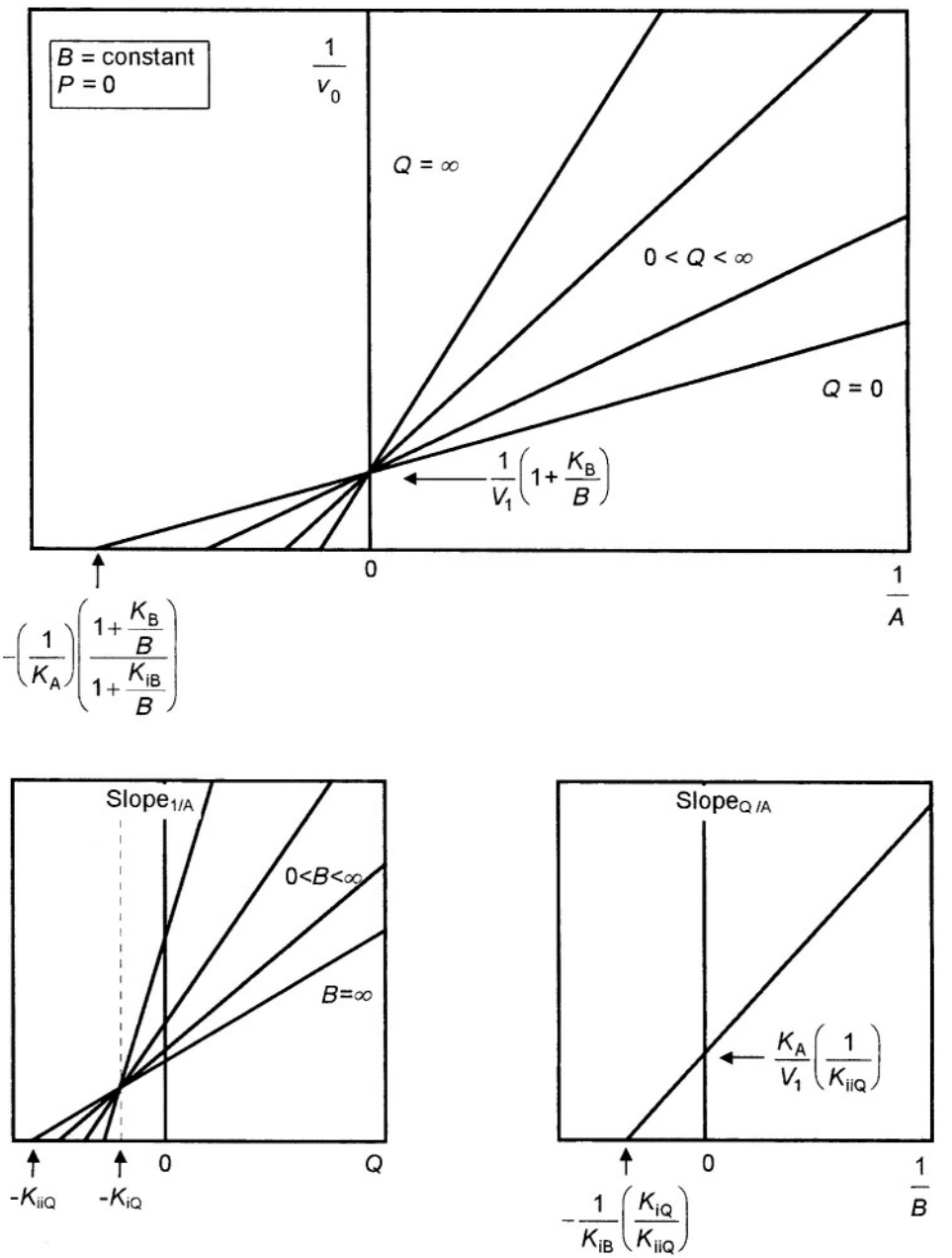


Figure 7. Product inhibition by Q in the Rapid Equilibrium Random Bi Bi system with a dead-end EBQ complex. Graphical presentation of Eq. (8.30) with B as a constant and A as a variable substrate.

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \frac{K_A}{V_1}\left[1 + \frac{K_{iB}}{B}\left(1 + \frac{Q}{K_{iQ}}\right) + \frac{Q}{K_{iiQ}}\right]\frac{1}{A}$$

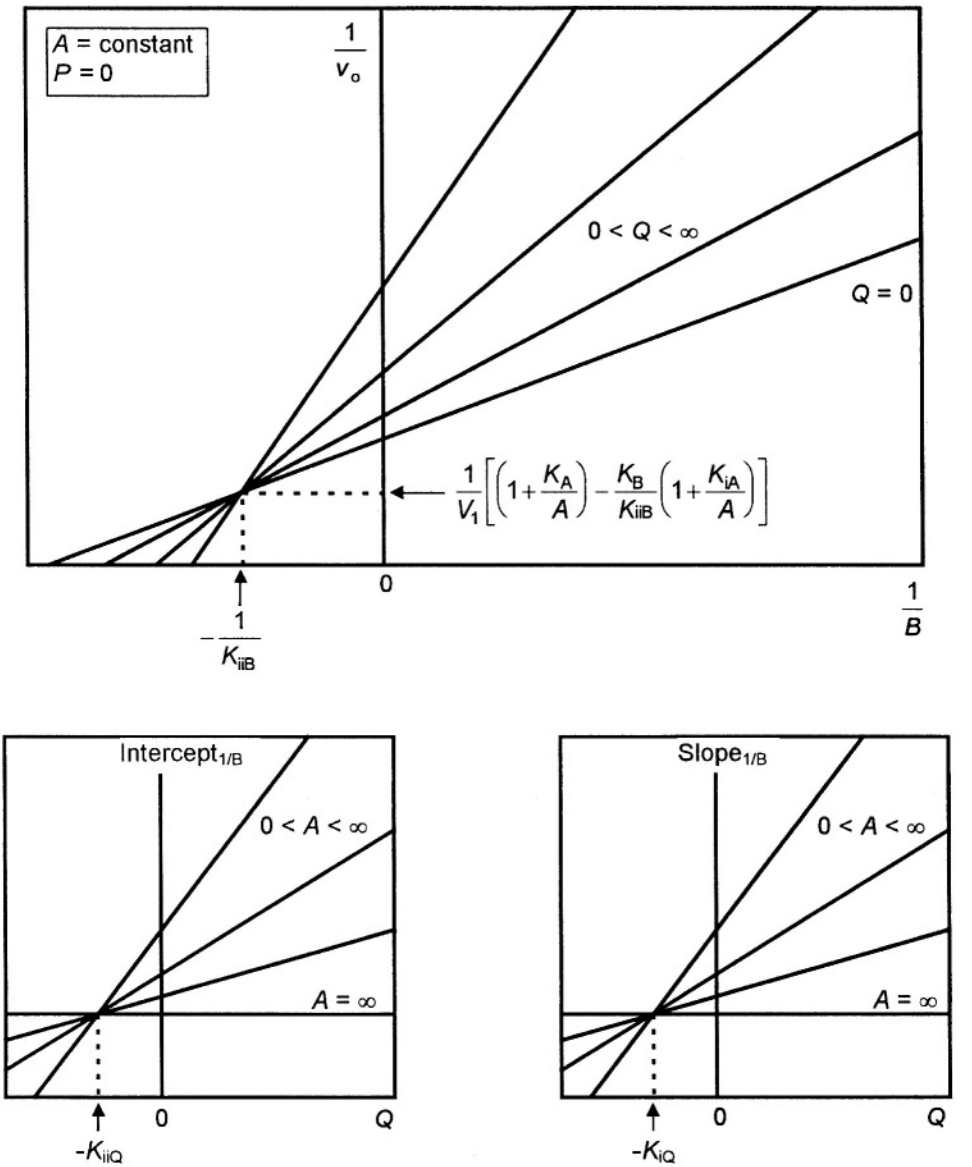
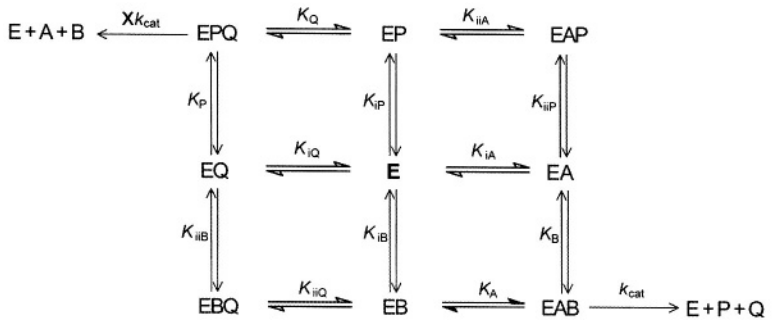


Figure 8. Product inhibition by Q in the Rapid Equilibrium Random Bi Bi system with a dead-end EBQ complex. Graphical presentation of Eq. (8.31), with A as a constant and B as a variable substrate.

$$\frac{1}{v_o} = \frac{1}{V_1}\left[1 + \frac{K_A}{A}\left(1 + \frac{Q}{K_{iiQ}}\right)\right] + \frac{K_B}{V_1}\left[1 + \frac{K_{iA}}{A}\left(1 + \frac{Q}{K_{iQ}}\right)\right]\frac{1}{B}$$

8.7 A RANDOM MODEL WITH TWO DEAD-END COMPLEXES

In the most general case, A and B or P and Q can occupy the enzyme simultaneously and all four ligands can bind by themselves. In addition, the dead-end complexes EAP and EBQ are formed. However, in this case, the EAP complex often forms with elevated dissociation constant, relative to those of the binary complexes, because of the overlap of the common pieces in reactants. The reaction will proceed in both directions when all four ligands are present.



(8.35)

The net velocity in the forward direction, $A + B \rightarrow P + Q$, is

$$v_{net} = v_{forward} - v_{reverse} = k_{cat}E_o - xk_{cat}E_o \tag{8.36}$$

The general rate equations, if all four substrates and products are present, and when interconverted with the Haldane relationship (8.43) are

$$v_o = \frac{V_1\left(\frac{AB}{K_{iA}K_B}\right) - V_2\left(\frac{PQ}{K_PK_{iQ}}\right)}{1 + \frac{A}{K_{iA}} + \frac{B}{K_{iB}} + \frac{P}{K_{iP}} + \frac{Q}{K_{iQ}} + \frac{AB}{K_{iA}K_B} + \frac{PQ}{K_PK_{iQ}} + \frac{BQ}{K_{iB}K_{iiQ}} + \frac{AP}{K_{iA}K_{iiP}}} \tag{8.37}$$

$$v_o = \frac{V_1V_2\left(AB - \frac{PQ}{K_{eq}}\right)}{V_2K_{iA}K_B + V_2K_BA + V_2K_AB + V_2AB + \frac{V_1K_Q}{K_{eq}}P + \frac{V_1K_P}{K_{eq}}Q + \frac{V_1}{K_{eq}}PQ + \frac{V_2K_A}{K_{iiQ}}BQ + \frac{V_2K_B}{K_{iiP}}AP} \tag{8.38}$$

In rapid equilibrium systems, all inhibition constants represent the true dissociation constants of respective enzyme complexes. The nomenclature of Cleland, described in Section 8.1, is sufficient to describe most kinetic constants. However, for those constants that are leading to the formation of dead-end complexes (K_{iiB}, K_{iiQ}, K_{iiA}, K_{iiP}), novel or extra descriptions are necessary (Cleland, 1967). Since all enzyme forms in reaction (8.35) are in the thermodynamic equilibrium, the novel constants are mutually related by the following relationships:

$$K_{iiA} = K_{iA}\left(\frac{K_{iiP}}{K_{iP}}\right); \quad K_{iiB} = K_{iB}\left(\frac{K_{iiQ}}{K_{iQ}}\right); \quad K_{iiQ} = K_{iQ}\left(\frac{K_{iiB}}{K_{iB}}\right); \quad K_{iiP} = K_{iP}\left(\frac{K_{iiA}}{K_{iA}}\right) \tag{8.39}$$

Thus, the last two denominator terms in Eq. (8.37) can be written in yet another form:

$$\frac{BQ}{K_{iiB}K_{iQ}} \text{ and } \frac{AP}{K_{iiA}K_{iP}} \tag{8.40}$$

The rate Eq. (8.37) has nine denominator terms, each one for one of the enzyme forms: EA, EB, EP, EQ, EAB, EPQ, EBQ, and EAP; the unity represents the free enzyme. The rate equations for the forward reaction, in the presence of product Q or product P, are now identical with similar equations derived for cases with single dead-end complexes.

Rate equation in the presence of product Q. In the presence of both substrates A and B, and a product Q, the rate equation is

$$v_0 = \frac{V_1\left(\frac{AB}{K_{iA}K_B}\right)}{1 + \frac{A}{K_{iA}} + \frac{B}{K_{iB}} + \frac{Q}{K_{iQ}} + \frac{AB}{K_{iA}K_B} + \frac{BQ}{K_{iB}K_{iiQ}}}$$

This equation is identical with Eq. (8.26).

Rate equation in the presence of product P. In the presence of both substrates A and B, and a product P, the rate equation is

$$v_0 = \frac{V_1\left(\frac{AB}{K_{iA}K_B}\right)}{1 + \frac{A}{K_{iA}} + \frac{B}{K_{iB}} + \frac{P}{K_{iP}} + \frac{AB}{K_{iA}K_B} + \frac{AP}{K_{iA}K_{iiP}}} \tag{8.41}$$

This equation is completely symmetrical to the preceding equation.

8.8 DIAGNOSTICS OF RAPID EQUILIBRIUM SYSTEMS

In rapid equilibrium systems, the Haldane relationship can be obtained directly from rate equations. In equilibrium, the rate equation for the Rapid Equilibrium Ordered Bi Bi system (Eq. (8.12)), becomes

$$v_0 = \frac{V_1\left(\frac{AB}{K_{iA}K_B}\right) - V_2\left(\frac{PQ}{K_PK_{iQ}}\right)}{\text{denominator}} \tag{8.42}$$

Thus, for the Rapid Equilibrium Ordered Bi Bi system, the Haldane relationship is

$$K_{eq} = \left(\frac{PQ}{AB}\right)_{eq} = \frac{V_1 K_{iQ} K_P}{V_2 K_{iA} K_B} \tag{8.43}$$

The Haldane relationship is identical for all rapid equilibrium random systems (Haldane, 1930; Cleland, 1982). Thus, from Eq. (8.37), one also obtains

$$K_{eq} = \frac{V_1 K_{iQ} K_P}{V_2 K_{iA} K_B}$$

The derivation of velocity equations for the rapid equilibrium systems is very simple indeed, as already pointed out in Chapter 4 (Section 4.1), because all forms of enzyme are in a rapid equilibrium with each other and the slowest process is the chemical reaction between the central complexes.

In the general case, the Rapid Equilibrium Random Bi Bi system with both dead-end complexes EAP and EBQ (Reaction (8.35)), the denominator of the rate equation has nine terms, each for one form of enzyme; the unity represents the free enzyme (Eq. (8.37)). From the general rate Eq. (8.37), one can write down directly the rate equations for all other possible combinations of the Rapid Equilibrium Random Bi Bi system.

For example, the rate equation for the Rapid Equilibrium Random Bi Bi system with a dead-end EBQ complex (Reaction (8.23)) is obtained by omitting from Eq. (8.38) the term that contains AP, because the EAP complex does not form, thus affording directly the corresponding rate Eq. (8.25). Similarly, from the general rate Eq. (8.38), one can also delete denominator terms corresponding to B, P, BQ and AP, and obtain directly the general rate equation for the Rapid Equilibrium Ordered Bi Bi system (Eq. (8.12)).

These properties of the rapid equilibrium systems illustrate a simple rule: the denominator of the velocity equation in each rapid equilibrium system contains as many terms as there are enzyme forms in the mechanism.

The primary graphs alone, the double reciprocal plots in the absence of products, can be quite valuable in the diagnostics of rapid equilibrium mechanisms. Thus, one can easily distinguish the ordered from the random rapid equilibrium system, even from the primary graphs in the absence of the products of reaction (Table 1).

Table 1. Types of initial velocity patterns in the primary double reciprocal graphs in rapid equilibrium systems in the absence of products

Ordered		Random	
$1/v_0$ versus $1/A$; B increasing	$1/v_0$ versus $1/B$; A increasing	$1/v_0$ versus $1/A$; B increasing	$1/v_0$ versus $1/B$; A increasing
Slope replot through origin Eq. (8.4) Fig. 1	Pattern crosses on vertical axes Eq. (8.5) Fig. 2	Intersecting Eq. (8.9) Fig. 3	Intersecting Eq. (8.10) —

Table 2. Product inhibition patterns in rapid equilibrium bisubstrate mechanisms (Plowman, 1972; Segel, 1975)

		Varied substrate					
		A			B		
Mechanism	Product inhibitor	Unsat. with B	Saturat. with B	Equation (Figure)	Unsat. with A	Saturat. with A	Equation (Figure)
Rapid Equilibrium Ordered Bi Bi (no dead-end complexes)	P						
	Q	Comp	——	8.14 (Fig. 4)	Comp	——	8.15 (Fig. 5)
Rapid Equilibrium Ordered Bi Bi (with dead-end EBQ)	P	——	——		——	——	
	Q	Comp	Comp		Noncom	——	
Rapid Equilibrium Random Bi Bi (with dead-end EBQ)	P	Comp	——	8.33	Comp	——	8.34
	Q	Comp	Comp	8.30 (Fig. 7)	Noncom	——	8.31 (Fig. 8)
Rapid Equilibrium Random Bi Bi (with dead-end EAP and EBQ)	P	Noncom	——	8.41	Comp	Comp	8.41
	Q	Comp	Comp	8.30	Noncom	——	8.31

Type of inhibition: Comp = competitive; Noncom = noncompetitive.

However, the primary double reciprocal plots of some rapid equilibrium systems are identical. In rapid equilibrium systems, in the presence of the products of reaction, the primary reciprocal plots are very characteristic and depend on the number and type of enzyme–substrate and enzyme–product complexes that can form. Therefore, in order to distinguish between different types, one must revert to product inhibition patterns that can easily distinguish between all types of rapid equilibrium bisubstrate systems (Plowman, 1972; Segel, 1975) (Table 2).

REFERENCES

Books

Engel, P.C. (1996) *Enzymology Labfax,* Bios Scientific Publishers, Oxford.
IUBMB (1992) *Enzyme nomenclature,* International Union of Biochemistry and Molecular Biology, Academic Press, London.
Haldane, J.B.C. (1930) *Enzymes,* Longmans and Green, London [Reprint available as: Haldane, B.S. (1965) *Enzymes,* MIT Press, Cambridge, MA.
Mahler, H.R. & Cordes, E.H. (1966) *Biological chemistry,* Harper & Row, New York.
Plowman, K.M. (1972) *Enzyme kinetics,* McGraw-Hill, New York.
Purich, D.L. & Allison, D.R. (2000) *Handbook of biochemical kinetics,* Academic Press, San Diego.
Segel, I.H. (1975) *Enzyme kinetics,* Wiley, New York.

Review articles

Cleland, W.W. (1967) *Annual Rev. Biochem. 36,* 77–112.
Cleland, W.W (1970) *The Enzymes,* 3rd ed., Vol. 2, 1–67.
Cleland, W.W. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 45,* 273–287.
Cleland, W.W. (1982) *Methods Enzymol. 87,* 366–370.
Fromm, H.J. (1979) *Methods Enzymol. 63,* 42–53.
Rudolph, F.B. (1979) *Methods Enzymol. 63,* 411–436.

Specific references

Alberty, R.A. (1953) *J. Am. Chem. Soc. 75,* 1928–1932.
Alberty, R.A. (1958) *J. Am. Chem. Soc. 80,* 1777–1782.
Alberty, R.A. & Hammes, G.G. (1958) *J. Phys. Chem. 62,* 154–160.
Bloomfield, V., Peller, L., & Alberty, R.A. (1962) *J. Am. Chem. Soc. 84,* 4367–4380.
Cleland, W.W. (1963) Biochim. *Biophys. Acta 67,* 104–137.
Dalziel, K. (1957) *Acta Chem. Scand. 11,* 1706–1723.
Dalziel, K. (1969) *Biochem. J. 114,* 547–556.
Fromm, H.J. (1967) *Biochim. Biophys. Acta 139,* 221–230.
Grissom, C.B. & Cleland, W.W. (1988) *Biochemistry 27,* 2934–2943.
Hurst, R.O. (1969) Can. *J. Biochem. 47,* 369–377.
Peller, L. & Alberty, R.A. (1959) *J. Am. Chem. Soc. 81,* 5907–5915.
Rudolph, F.B. & Fromm, H.J. (1969) *J. Biol. Chem. 244,* 3832–3842.
Rudolph, F.B, & Fromm, H.J. (1971) *Arch. Biochem. Biophys. 147,* 515–526.
Rudolph, F.B. & Fromm, H.J. (1973) *J. Theor. Biol. 39,* 363–371.
Wong, J.T. & Hanes, C.S. (1962) *Can. J. Biochem. Physiol. 40,* 763–804.

Chapter 9

Steady-State Kinetics of Bisubstrate Reactions

The derivation of velocity equations for multisubstrate reactions is a difficult task, compared to monosubstrate reactions. Velocity equations for nonrapid equilibrium multisubstrate systems can be derived algebraically with the aid of steady-state assumptions. However, as the number of enzyme species increases, the algebraic manipulations become increasingly more complicated. Velocity equations for many multisubstrate reactions cannot be derived profitably from rapid equilibrium assumptions. In bisubstrate systems, the isomerization of central complex, and subsequent product release steps may be so rapid that E, EA, EAB, EPQ, and EQ never attain equilibrium. The distribution of enzyme species will depend on the rate constants of all of the steps, including those involving product release. The transitory enzyme–product complexes may now represent a significant fraction of the total enzyme even in the absence of added products. For such cases, the derivation of velocity equations can be shortened considerably by using the method of King and Altman, and other methods described in Chapter 4.

The simple and elegant method of King and Altman allows the steady-state rate equations for mechanisms of considerable complexity to be written down in terms of the individual rate constants without going through complex algebraic expansions of large determinants. It was used to derive all of the rate equations discussed in this and in the next chapters.

9.1 NOMENCLATURE

The velocity equations for bisubstrate reactions are usually formidable if expressed in terms of individual rate constants. The resulting equations are almost useless until the rate constants are grouped into relatively simple kinetic constants that can be experimentally determined. Various methods for grouping the individual rate constants have been developed by Alberty (1953), Dalziel (1957), Bloomfield *et al.* (1962), Wong & Hanes (1962), Cleland (1963), Mahler & Cordes (1966), and others.

The *nomenclature of Cleland* is now in general use although the *nomenclature of Dalziel* has been often used in the older literature; other methods did not stand the test of time (Purich & Allison, 2000).

We shall compare the nomenclature of Cleland and the nomenclature of Dalziel with the example of an Ordered Bi Bi mechanism, described in

Section 9.2. The velocity equation for this mechanism, in the absence of products, can be written as

Cleland's nomenclature:

$$v_0 = \frac{V_1 AB}{K_{iA}K_B + K_B A + K_A B + AB} \tag{9.1}$$

Dalziel's nomenclature:

$$v_0 = \frac{S_1 S_2}{\phi_{12} + \phi_2 S_1 + \phi_1 S_2 + \phi_0 S_1 S_2} \tag{9.2}$$

or, in the reciprocal form:

$$\frac{1}{v_0} = \frac{1}{V_1} + \frac{K_A}{V_1 A} + \frac{K_B}{V_1 B} + \frac{K_{iA}K_B}{V_1 AB} \tag{9.3}$$

$$\frac{1}{v_0} = \phi_0 + \frac{\phi_1}{S_1} + \frac{\phi_2}{S_2} + \frac{\phi_{12}}{S_1 S_2} \tag{9.4}$$

In Cleland's nomenclature, the concentrations of two substrates are indicated as A and B, and in Dalziel's nomenclature as S_1 and S_2. In Cleland's nomenclature, the maximal velocity is V_1, the Michaelis constants K_A and K_B, and the inhibition constant K_{iA}. In Dalziel's nomenclature, kinetic factors have the following meaning:

$$\phi_0 = \frac{1}{V_1} \quad \phi_1 = \frac{K_A}{V_1} \quad \phi_2 = \frac{K_B}{V_1} \quad \phi_{12} = \frac{K_{iA}K_B}{V_1} \tag{9.5}$$

In this chapter, we shall deal only with the hyperbolic bisubstrate mechanisms that produce the linear primary double reciprocal plots of $1/v_0$ versus 1/ [substrate]. In doing so, we shall describe the following major types of steady-state bisubstrate reactions (Fromm, 1979):

(1) Ordered Bi Bi,
(2) Theorell–Chance,
(3) Ordered Bi Uni,
(4) Ordered Uni Bi,
(5) Ping Pong Bi Bi, and
(6) Steady–State Random

This nomenclature has been introduced by Cleland (1963), but other descriptions of bisubstrate mechanisms are also found in the biochemical literature. For example, a sequential addition in bisubstrate reactions, an Ordered Bi Bi mechanism is also called a *compulsory-order ternary-complex* mechanism whereas a Random Bi Bi mechanism is called a *random-order ternary-complex*

mechanism; the Ping Pong mechanisms are termed *substituted-enzyme* mechanisms (Cornish-Bowden, 1995).

The majority of steady-state rate equations described in this chapter, in its present form, have been derived by Cleland (Cleland, 1963, 1967, 1970, 1977).

9.2 ORDERED BI BI SYSTEM

An Ordered Bi Bi system is fairly common in many enzyme reactions found in the nature, particularly among pyridine nucleotide-dependent dehydrogenases (Walsh, 1998). In this mechanism, there are two substrates A and B, and two products of reaction, P and Q, which add to the enzyme in an ordered fashion.

For example, consider a reaction catalyzed by alcohol dehydrogenase. The substrate A (usually NAD^+) and the product Q (usually NADH) are the leading substrates; they are added first, and the other substrate B (alcohol), or the other product P (aldehyde), cannot be added until the leading substrate is bound. Thus, complexes EB and EP do not form:

$$\mathrm{E} \underset{k_2}{\overset{k_1\mathrm{A}}{\rightleftharpoons}} \mathrm{EA} \underset{k_4}{\overset{k_3\mathrm{B}}{\rightleftharpoons}} \mathrm{EAB} \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} \mathrm{EPQ} \underset{k_6\mathrm{P}}{\overset{k_5}{\rightleftharpoons}} \mathrm{EQ} \underset{k_8\mathrm{Q}}{\overset{k_7}{\rightleftharpoons}} \mathrm{E} \tag{9.6}$$

9.2.1 Rate Equations with a Single Central Complex

The rate equation for this mechanism in terms of rate constants, and when the interconversion of central complexes EAB and EPQ is not taken into account (rate constants k_9 and k_{10} are absent and EAB and EPQ are lumped into a single central complex), is

$$\begin{aligned} v_0 = \frac{(k_1k_3k_5k_7AB - k_2k_4k_6k_8PQ)E_0}{{} & k_2k_7(k_4+k_5) + k_1k_7(k_4+k_5)A + k_3k_5k_7B + k_1k_3(k_5+k_7)AB \\ & + k_2k_4k_6P + k_2k_8(k_4+k_5)Q + k_3k_5k_8BQ + k_1k_4k_5AP \\ & + k_6k_8(k_2+k_4)PQ + k_1k_3k_6ABP + k_3k_6k_8BPQ \end{aligned} \tag{9.7}$$

In terms of kinetic constants, the rate equation becomes

$$\begin{aligned} v_0 = \frac{V_1V_2\left(AB - \dfrac{PQ}{K_{\mathrm{eq}}}\right)}{{} & V_2K_{\mathrm{iA}}K_{\mathrm{B}} + V_2K_{\mathrm{B}}A + V_2K_{\mathrm{A}}B + \frac{V_1K_{\mathrm{Q}}}{K_{\mathrm{eq}}}P + \frac{V_1K_{\mathrm{P}}}{K_{\mathrm{eq}}}Q + V_2AB \\ & + \frac{V_1K_{\mathrm{Q}}}{K_{\mathrm{iA}}K_{\mathrm{eq}}}AP + \frac{V_1}{K_{\mathrm{eq}}}PQ + \frac{V_2K_{\mathrm{A}}}{K_{\mathrm{iQ}}}BQ + \frac{V_2}{K_{\mathrm{iP}}}ABP + \frac{V_1}{K_{\mathrm{iB}}K_{\mathrm{eq}}}BPQ \end{aligned} \tag{9.8}$$

The rate Eq. (9.8) is written in Cleland's nomenclature, with one inhibition constant defined for each reactant in the mechanism (K_{iA}, K_{iB}, K_{iQ}, K_{iP}). This rate equation was dervied in Chapter 4 with the aid of the King-Altman method (Eq. (4.39)).

Generally, for all steady-state bisubstrate mechanisms without alternate reaction sequences a complete rate equation can be expressed in terms of only two

maximum velocities, one Michaelis and one inhibition constant for each reactant, and the equilibrium constant for the whole reaction.

Definition of kinetic constants in terms of rate constants

$$
\begin{aligned}
K_A &= \frac{\text{coefB}}{\text{coefAB}} = \frac{k_5 k_7}{k_1(k_5 + k_7)} & K_{iA} &= \frac{\text{coefP}}{\text{coefAP}} = \frac{\text{const}}{\text{coefA}} = \frac{k_2}{k_1} \\
K_B &= \frac{\text{coefA}}{\text{coefAB}} = \frac{k_7(k_4 + k_5)}{k_3(k_5 + k_7)} & K_{iB} &= \frac{\text{coefPQ}}{\text{coefBPQ}} = \frac{k_2 + k_4}{k_3} \\
K_P &= \frac{\text{coefQ}}{\text{coefPQ}} = \frac{k_2(k_4 + k_5)}{k_6(k_2 + k_4)} & K_{iP} &= \frac{\text{coefAB}}{\text{coefABP}} = \frac{k_5 + k_7}{k_6} \\
K_Q &= \frac{\text{coefP}}{\text{coefPQ}} = \frac{k_2 k_4}{k_8(k_2 + k_4)} & K_{iQ} &= \frac{\text{coefB}}{\text{coefBQ}} = \frac{\text{const}}{\text{coefQ}} = \frac{k_7}{k_8} \\
\frac{V_1}{E_0} &= \frac{\text{numer}_1}{\text{coefAB}} = \frac{k_5 k_7}{k_5 + k_7} & \frac{V_2}{E_0} &= \frac{\text{numer}_2}{\text{coefPQ}} = \frac{k_2 k_4}{k_2 + k_4} \\
K_{eq} &= \frac{\text{numer}_1}{\text{numer}_2} = \frac{k_1 k_3 k_5 k_7}{k_2 k_4 k_6 k_8}
\end{aligned}
\tag{9.9}
$$

Definition of rate constants in terms of kinetic constants

$$
\begin{aligned}
k_1 &= \frac{V_1}{E_0 K_A} & k_2 &= \frac{V_1 K_{iA}}{E_0 K_A} \\
k_3 &= \frac{V_1}{E_0 K_B}\left(1 + \frac{k_4}{k_5}\right) & \frac{1}{k_4} &= \frac{E_0}{V_2} - \frac{1}{k_2} \\
\frac{1}{k_5} &= \frac{E_0}{V_1} - \frac{1}{k_7} & k_6 &= \frac{V_2}{E_0 K_P}\left(1 + \frac{k_5}{k_4}\right) \\
k_7 &= \frac{V_2 K_{iQ}}{E_0 K_Q} & k_8 &= \frac{V_2}{E_0 K_Q}
\end{aligned}
\tag{9.10}
$$

Haldane relationships

$$
K_{eq} = \frac{V_1 K_{iQ} K_P}{V_2 K_{iA} K_B} = \left(\frac{V_1}{V_2}\right)^2 \frac{K_Q K_{iP}}{K_A K_{iB}} \tag{9.11}
$$

The various kinetic constants for any bisubstrate or trisubstrate steady-state mechanism are related to the thermodynamic equilibrium constant by Haldane equations of the general type:

$$
K_{eq} = \left(\frac{V_1}{V_2}\right)^n \frac{K_{(P)} K_{(Q)} K_{(R)} \cdots}{K_{(A)} K_{(B)} K_{(C)} \cdots} \tag{9.12}
$$

where $K_{(A)}$ may be either K_A or K_{iA} and so forth. The equation includes either a Michaelis or inhibition constant for each reactant. Cleland (1982) further distinguished two types of Haldane relationships: thermodynamic and kinetic.

There are two Haldanes for Ordered Uni Bi mechanism, with n = 1, and two for Ordered Bi Bi, but the second one depends on the definition of the inhibition constants. Ping Pong mechanisms with two stable enzyme forms have four Haldanes, one each with $n = 0$ and $n = 2$, and two with $n = 1$. The Theorell-Chance mechanism with only six rate constants has 16 Haldanes with n equal to minus one (1), zero (4), one (6), two (4), and three (1), corresponding to 4, 3, 2, 1, and 0 inhibition constants, respectively. Ordered mechanisms that are of Ter reactancy in either direction have two Haldanes, with $n = 0$ and $n = 1$ (Cleland, 1982).

Distribution equations

$$\frac{E}{E_o} = \frac{K_{iA}K_B V_2 + \frac{K_Q V_1}{K_{eq}} P + K_A V_2 B}{\text{denominator of rate equation}}$$

$$\frac{EA}{E_o} = \frac{K_B V_2 A + \frac{K_Q V_1}{K_{iA}K_{eq}} AP + \frac{K_A V_2}{K_{iA}K_{eq}} PQ}{\text{denominator of rate equation}} \qquad (9.13)$$

$$\frac{(EAB + EPQ)}{E_o} = \frac{\left(V_2 - \frac{V_1 K_Q}{K_{iQ}}\right) AB + \left(\frac{V_1}{K_{eq}} - \frac{V_2 K_A}{K_{iA}K_{eq}}\right) PQ + \frac{V_2}{K_{iP}} ABP + \frac{V_1}{K_{iB}K_{eq}} BPQ}{\text{denominator of rate equation}}$$

$$\frac{EQ}{E_o} = \frac{\frac{K_P V_1}{K_{eq}} Q + \frac{K_Q V_1}{K_{iQ}} AB + \frac{K_A V_2}{K_{iQ}} BQ}{\text{denominator of rate equation}}$$

The distribution equations describe the distribution of the enzyme among the various possible forms. These distribution equations when multiplied by E_o give the steady-state concentrations of the various enzyme forms. They have some inherent interest by themselves, however, and are useful in deriving rate equations for reactions in the presence of dead-end inhibitors (Chapter 11), and equations for rates of isotopic exchange (Chapter 16).

Effects of isomerizations

EAB + EPQ isomerizes: The distribution equations are still valid, and k_1, k_2, k_7, and k_8 can be calculated.
EA isomerizes: Only E/E_o, EQ/E_o, k_7 and k_8 can be calculated.
EA and EQ isomerize: Only E/E_o can be calculated.

9.2.2 Rate Equations with Two Central Complexes

If the interconversion of central complexes is taken into account (rate constants k_9 and k_{10} are present), the definition of kinetic constants in terms of rate constants becomes more complex (Plapp, 1973):

$$K_A = \frac{k_5k_7k_9}{k_1(k_5k_7 + k_5k_9 + k_7k_9 + k_7k_{10})} \qquad K_{iA} = \frac{k_2}{k_1}$$

$$K_B = \frac{k_7(k_4k_5 + k_4k_{10} + k_5k_9)}{k_3(k_5k_7 + k_5k_9 + k_7k_9 + k_7k_{10})} \qquad K_{iB} = \frac{k_2k_4 + k_2k_9 + k_2k_{10} + k_4k_{10}}{k_3(k_9 + k_{10})}$$

$$K_P = \frac{k_2(k_4k_5 + k_4k_{10} + k_5k_9)}{k_6(k_2k_4 + k_2k_9 + k_2k_{10} + k_4k_{10})} \qquad K_{iP} = \frac{k_5k_7 + k_5k_9 + k_7k_9 + k_7k_{10}}{k_6(k_9 + k_{10})} \tag{9.14}$$

$$K_Q = \frac{k_2k_4k_{10}}{k_8(k_2k_4 + k_2k_9 + k_2k_{10} + k_4k_{10})} \qquad K_{iQ} = \frac{k_7}{k_8}$$

$$\frac{V_1}{E_0} = \frac{k_5k_7k_9}{k_5k_7 + k_5k_9 + k_7k_9 + k_7k_{10}} \qquad \frac{V_2}{E_0} = \frac{k_2k_4k_{10}}{k_2k_4 + k_2k_9 + k_2k_{10} + k_4k_{10}}$$

$$K_{eq} = \frac{k_1k_3k_5k_7k_9}{k_2k_4k_6k_8k_{10}}$$

In this case, the kinetic constants cannot be used to calculate rate constants k_3, k_4, k_5, and k_6; however, the calculation of k_1, k_2, k_7, and k_8 is still valid.

9.2.3 Rate Equations in the Absence of Products

In the absence of products P and Q, the velocity equation reduces to

$$v_0 = \frac{V_1AB}{K_{iA}K_B + K_BA + K_AB + AB} \tag{9.15}$$

or, in the reciprocal form:

$$\frac{V_1}{v_0} = 1 + \frac{K_A}{A} + \frac{K_B}{B} + \frac{K_{iA}K_B}{AB} \tag{9.16}$$

Note that Eqs. (9.15) and (9.16) are identical with the corresponding rate equations for the Rapid Equilibrium Random bisubstrate system (Chapter 8; Eqs. (8.7) and (8.8)).

Equation (9.16) can be rearranged into two linear forms, in the Lineweaver–Burk fashion:

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \frac{K_A}{V_1}\left(1 + \frac{K_{iA}K_B}{K_AB}\right)\frac{1}{A} \tag{9.17}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_A}{A}\right) + \frac{K_B}{V_1}\left(1 + \frac{K_{iA}}{A}\right)\frac{1}{B} \tag{9.18}$$

Figures 1 and 2 show the primary and the secondary plots for Eqs. (9.17) and (9.18).

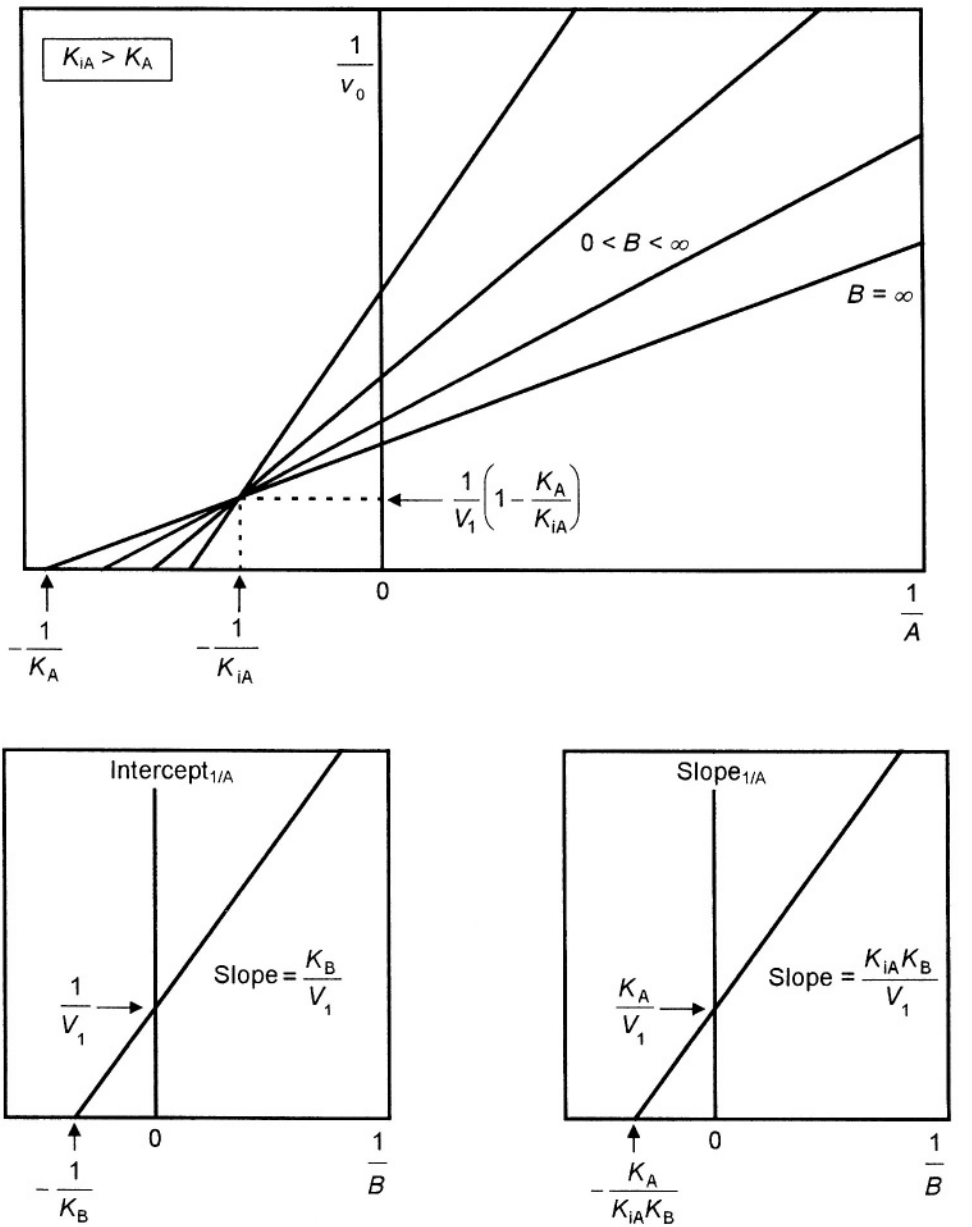


Figure 1. Ordered Bi Bi mechanism. Graphical presentation of Eq. (9.17), with B as a constant and A as a variable substrate.

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \frac{K_A}{V_1}\left(1 + \frac{K_{iA}K_B}{K_A B}\right)\frac{1}{A}$$

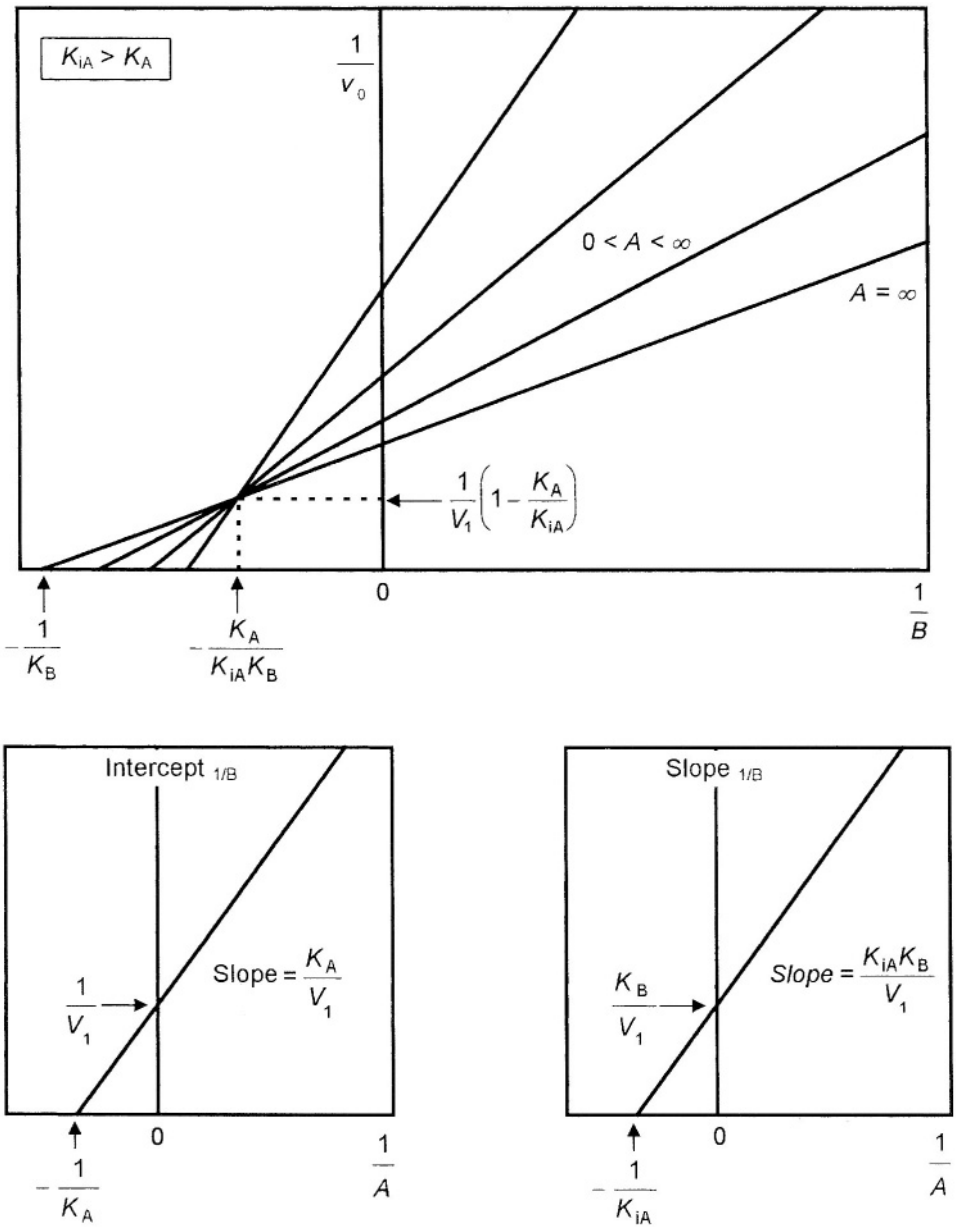


Figure 2. Ordered Bi Bi mechanism. Graphical presentation of Eq. (9.18), with A as a constant and B as a variable substrate.

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_A}{A}\right) + \frac{K_B}{V_1}\left(1 + \frac{K_{iA}}{A}\right)\frac{1}{B}$$

Figures 1 and 2 show that the various kinetic constants can all be determined from experimental data. Initial velocity analysis suffices for the Michaelis constants, maximum velocities, and the inhibition constants for reactants which yield non-central transitory complexes upon combination with the enzyme, while product inhibition experiments are necessary to determine the other inhibition constants.

In Fig. 1, the values of V_1 and K_B can be determined directly from the intercept replot. The value of K_A is determined from the ratio of the intercepts of the two replots, while the ratio of the slopes of the two replots is equal to K_{iA}. In Fig. 2, the values of V_1 and K_A can be determined directly from the intercept replot, while K_B comes from the ratio of intercepts of the two replots, and K_{iA} comes from the slope replot.

9.2.4 Product Inhibition in an Ordered Bi Bi Mechanism

Product inhibition in bisubstrate reactions normally takes place when, in addition to substrates, one or both products of reaction are present in the reaction mixture (Rudolph, 1979); the reasons why product inhibition occurs are described in Chapter 8 (Section 8.4).

Product inhibition by P. The rate equation for any product inhibition is obtained from the full rate equation for the entire mechanism (in this case, Eq. (9.8)), simply by setting the concentrations of all other products equal to zero. Thus, if A reacts with B in the presence of P, with $Q = 0$, the rate equation in the forward direction is

$$v_0 = \frac{V_1 AB}{K_{iA}K_B + K_B A + K_A B + \dfrac{K_{iA}K_B K_Q}{K_{iQ}K_P}P + AB + \dfrac{K_B K_Q}{K_{iQ}K_P}AP + \dfrac{1}{K_{iP}}ABP} \qquad (9.19)$$

Note that the expression $V_1/(V_2K_{eq})$ in the P and AP terms is replaced by an expression $(K_{iA}K_B)/(K_PK_{iQ})$ from the Haldane relationships (Eq. (9.11)).

When A is varied, the reciprocal form of Eq. (9.19) is

$$\frac{1}{v_0} = \frac{1}{V_1}\left[1 + \frac{K_B}{B}\left(1 + \frac{K_Q P}{K_{iQ}K_P}\right) + \frac{P}{K_{iP}}\right] + \frac{K_A}{V_1}\left[1 + \frac{K_{iA}K_B}{K_A B}\left(1 + \frac{K_Q P}{K_{iQ}K_P}\right)\right]\frac{1}{A} \qquad (9.20)$$

Figure 3 shows the graphical presentation of Eq. (9.20), the primary and the secondary plots drawn in the manner of Lineaweaver and Burk.

We can see that P will act as a noncompetitive inhibitor with respect to A at unsaturating B (from the primary plot), and that the inhibition cannot be overcome by saturation with B (from the secondary plot). At saturating B, P acts as an uncompetitive inhibitor with respect to A.

When B is varied, the equation becomes

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_A}{A} + \frac{P}{K_{iP}}\right) + \frac{K_B}{V_1}\left(1 + \frac{K_{iA}}{A}\right)\left(1 + \frac{K_Q P}{K_{iQ}K_P}\right)\frac{1}{B} \qquad (9.21)$$

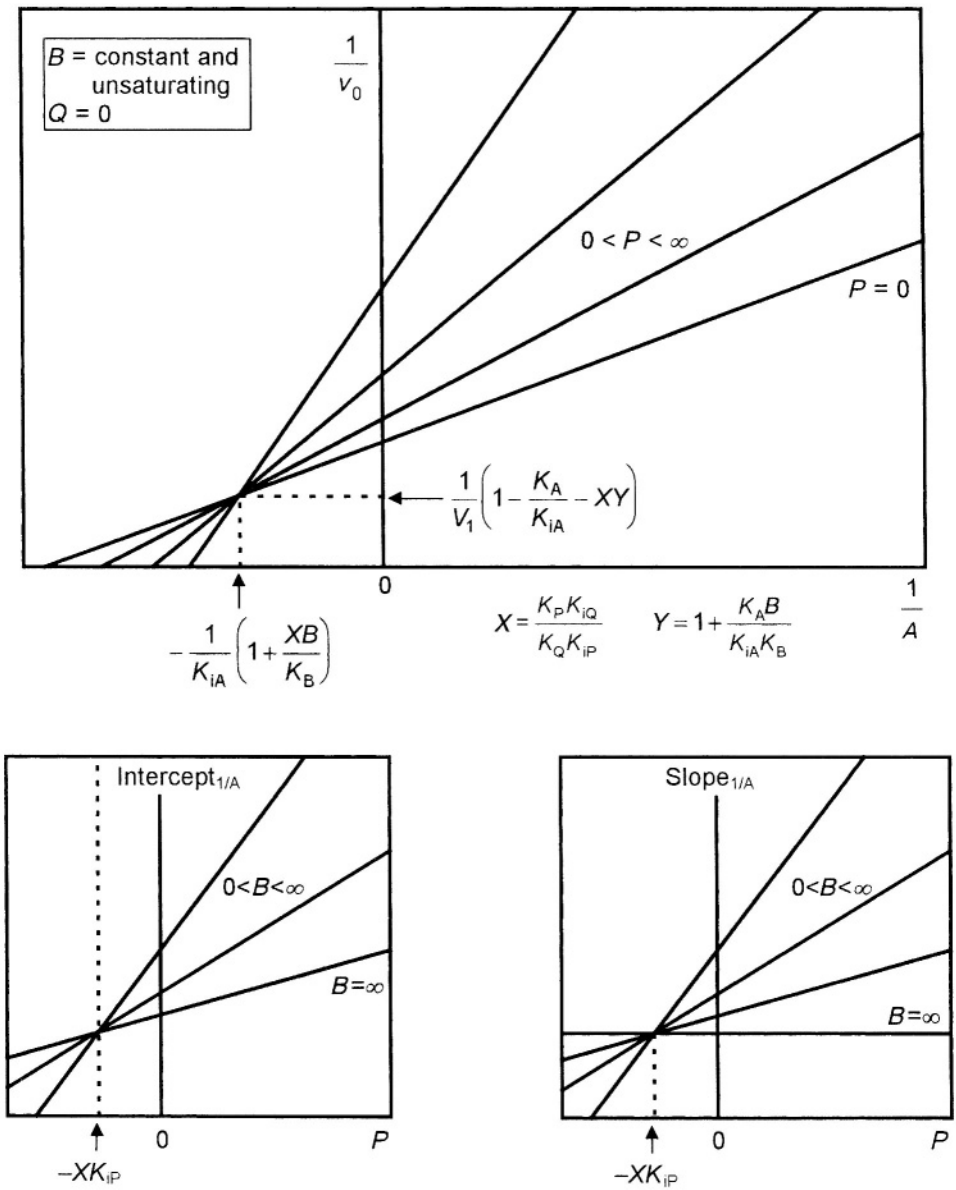


Figure 3. Product inhibition by P in an Ordered Bi Bi system. Graphical presentation of Eq. (9.20), with B as a constant and A as a variable substrate.

$$\frac{1}{v_0} = \frac{1}{V_1}\left[1 + \frac{K_B}{B}\left(1 + \frac{K_Q P}{K_{iQ}K_P}\right) + \frac{P}{K_{iP}}\right] + \frac{K_A}{V_1}\left[1 + \frac{K_{iA}K_B}{K_A B}\left(1 + \frac{K_Q P}{K_{iQ}K_P}\right)\right]\frac{1}{A}$$

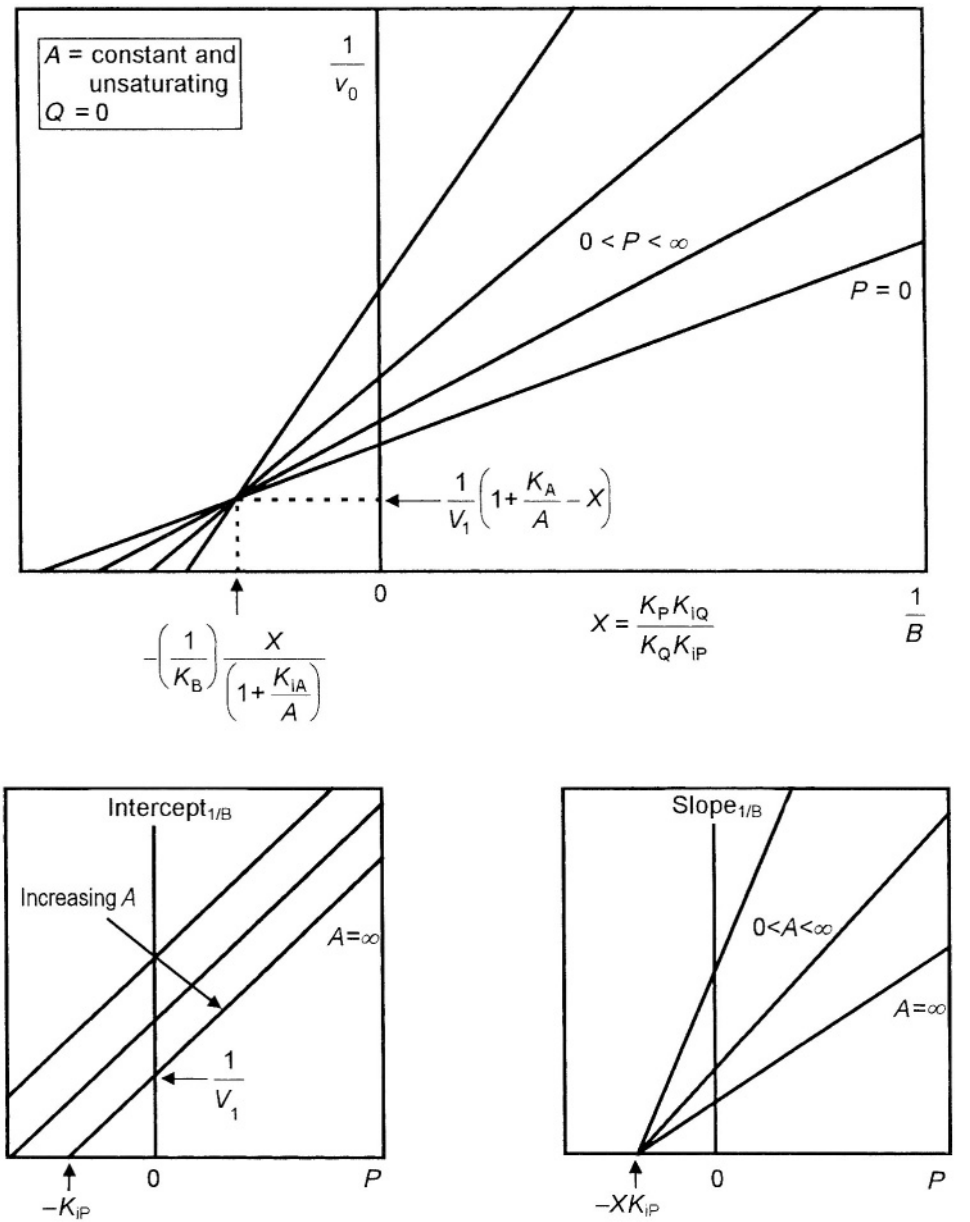


Figure 4. Product inhibition by P in the Ordered Bi Bi system. Graphical presentation of Eq. (9.21), with A as a constant and B as a variable substrate.

$$\frac{1}{v_o} = \frac{1}{V_1}\left(1 + \frac{K_A}{A} + \frac{P}{K_{iP}}\right) + \frac{K_B}{V_1}\left(1 + \frac{K_{iA}}{A}\right)\left(1 + \frac{K_Q P}{K_{iQ} K_P}\right)\frac{1}{B}$$

Figure 4 shows the graphical presentation of Eq. (9.21). P behaves as a noncompetitive inhibitor with respect to B at all concentrations of A. The intersection point may be above, on, or below the horizontal axis, depending on the ratio of $K_P K_{iQ}/K_Q K_{iP}$.

Product inhibition by Q. If A reacts with B in the presence of Q, with P = 0, the rate equation in the forward direction is

$$v_o = \frac{V_1 AB}{K_{iA}K_B + K_B A + K_A B + \dfrac{K_{iA}K_B}{K_{iQ}} Q + AB + \dfrac{K_A}{K_{iQ}} BQ} \tag{9.22}$$

Again, $V_1/(V_2 K_{eq})$ is replaced from the Haldane relationship.

When *A* is varied, the reciprocal form of Eq. (9.22) is

$$\frac{1}{v_o} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \frac{K_A}{V_1}\left(1 + \frac{K_{iA}K_B}{K_A B}\right)\left(1 + \frac{Q}{K_{iQ}}\right)\frac{1}{A} \tag{9.23}$$

Figure 5 shows the graphical presentation of Eq. (9.23). We see that Q acts as a competitive inhibitor with respect to A at all B concentrations.

When *B* is varied, the reciprocal form of Eq. (9.22) is

$$\frac{1}{v_o} = \frac{1}{V_1}\left[1 + \frac{K_A}{A}\left(1 + \frac{Q}{K_{iQ}}\right)\right] + \frac{K_B}{V_1}\left[1 + \frac{K_{iA}}{A}\left(1 + \frac{Q}{K_{iQ}}\right)\right]\frac{1}{B} \tag{9.24}$$

Figure 6 shows the graphical presentation of Eq. (9.24).

This time, Q acts as a noncompetitive inhibitor with respect to B at unsaturating A concentrations (from the primary plot). Saturation with A overcomes the inhibition (from the secondary plot). With a noncompetitive pattern in Fig. 6, one can define the apparent inhibition constants, $K_{iS} = K_{iQ}(1+K_{iA}/A)$ and $K_{ii} = K_{iQ}(1+K_A/A)$, making them useful for calculation of K_{iQ} from the observed values.

It is quite clear that the initial velocity studies, in the absence of products, are not sufficient for the determination of the order of addition of reactants in ordered sequences, while product inhibition experiments with either of the products provides this information unambiguously.

Once the inhibition constants K_{iB} and K_{iP} have been determined from the product inhibition studies, it is possible to calculate the dissociation constant of B from the EAB complex, and the dissociation constant of P from the EPQ complex. Ainslie and Cleland (1972) have shown that

$$\frac{k_4}{k_3} = \frac{K_A K_{iB} V_2}{K_{iA} V_1} \quad \text{and} \quad \frac{k_5}{k_6} = \frac{K_Q K_{iP} V_1}{K_{iQ} V_2} \tag{9.25}$$

Thus, in an Ordered Bi Bi mechanism, the addition of reactants in both directions is highly ordered. Therefore, it is possible to express all the rate constants in this mechanism in terms of Michaelis constants and inhibition constants. However, for real mechanisms when the interconversion of central complexes is included, one cannot calculate all the rate constants.

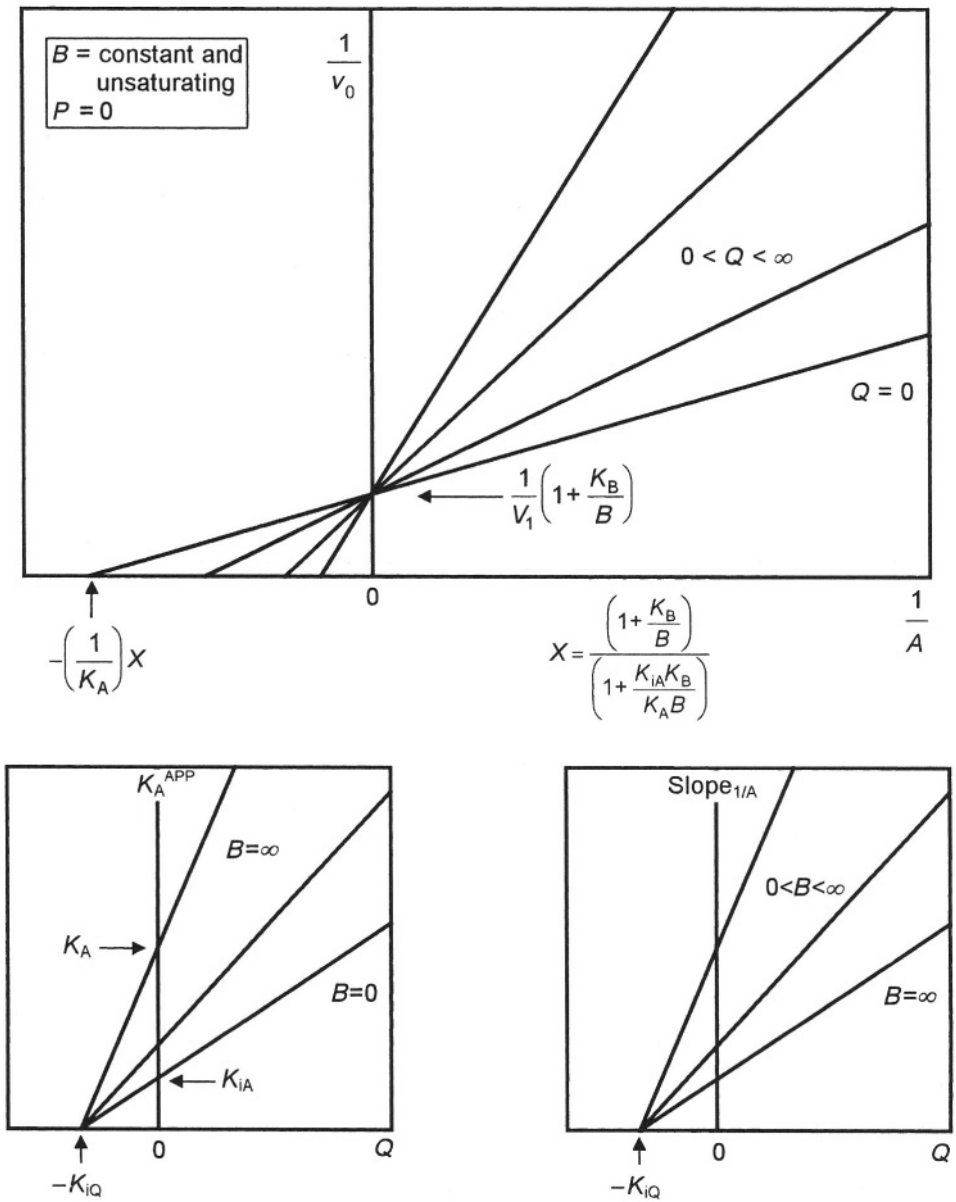


Figure 5. Product inhibition by Q in an Ordered Bi Bi system. Graphical presentation of Eq. (9.23) with B as a constant and A as a variable substrate.

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \frac{K_A}{V_1}\left(1 + \frac{K_{iA}K_B}{K_A B}\right)\left(1 + \frac{Q}{K_{iQ}}\right)\frac{1}{A}$$

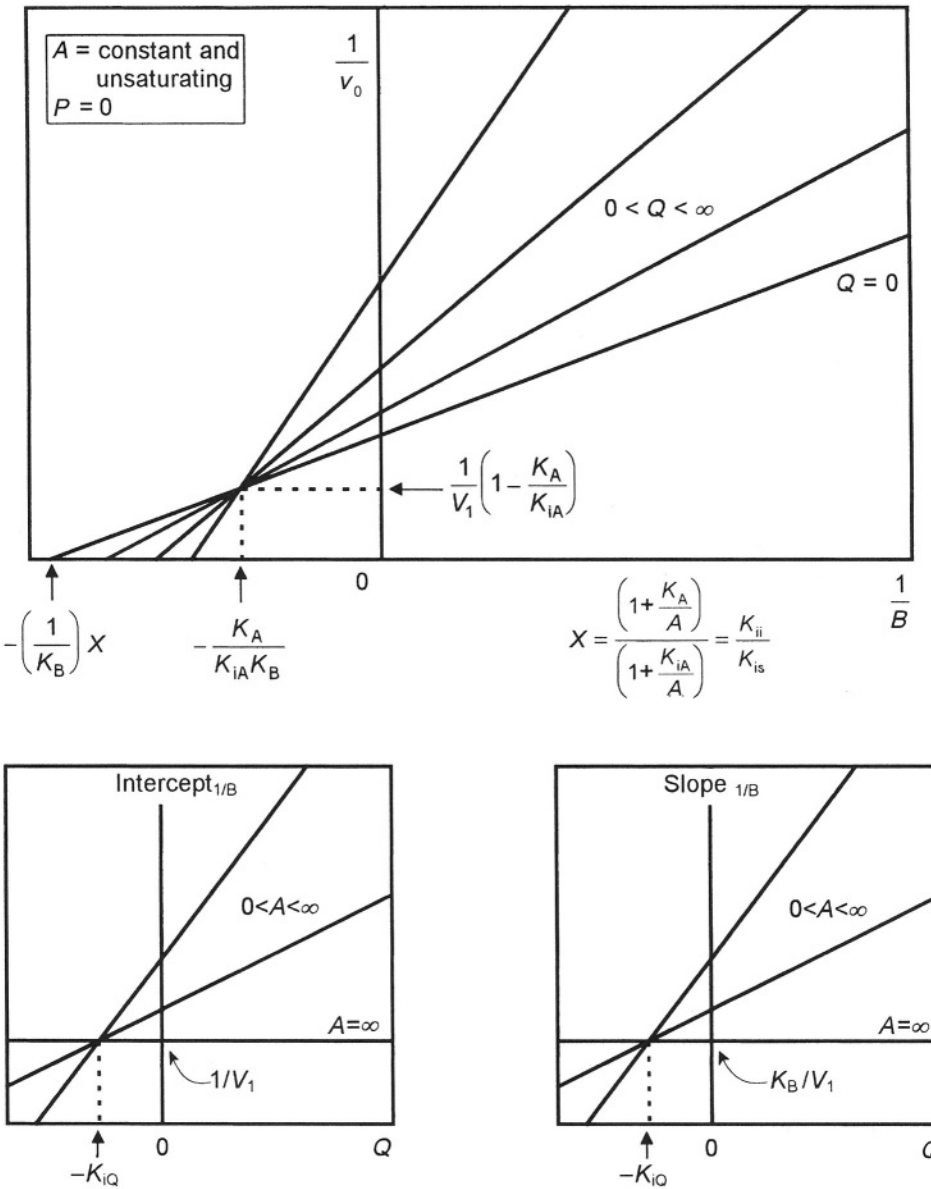


Figure 6. Product inhibition by Q an the Ordered Bi Bi system. Graphical presentation of Eq. (9.24), with A as a constant and B as a variable substrate.

$$\frac{1}{v_o} = \frac{1}{V_1}\left[1 + \frac{K_A}{A}\left(1 + \frac{Q}{K_{iQ}}\right)\right] + \frac{K_B}{V_1}\left[1 + \frac{K_{iA}}{A}\left(1 + \frac{Q}{K_{iQ}}\right)\right]\frac{1}{B}$$

9.2.5 Calculation of Intersection Points in an Ordered Bi Bi Mechanism

Calculation of the coordinates of intersection points in double reciprocal plots is very useful, because they usually represent a simple group of kinetic constants (Segel, 1975).

Consider an Ordered Bi Bi mechanism; the rate equation for this mechanism, when the concentration of A is varied, is given by Eq. (9.17):

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \frac{K_A}{V_1}\left(1 + \frac{K_{iA}K_B}{K_A B}\right)\frac{1}{A}$$

Figure 7 will be helpful for the calculation of coordinates for intersection points.

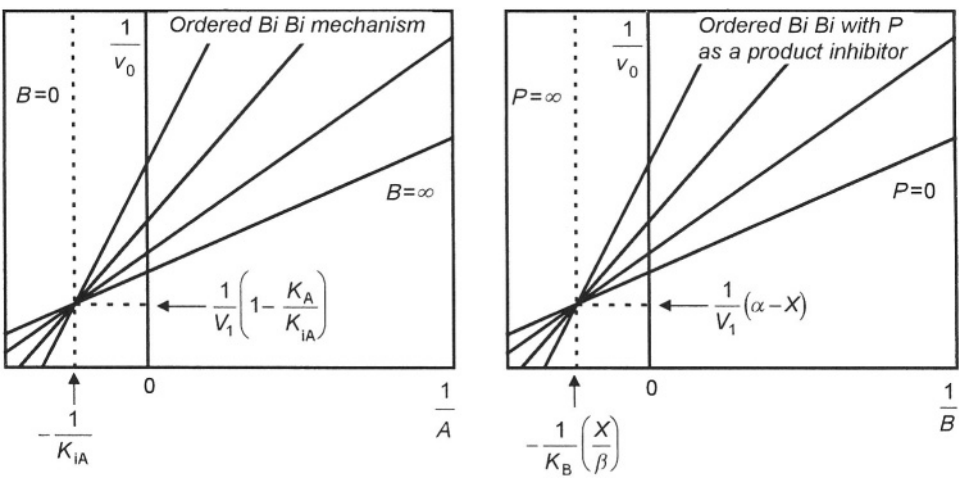


Figure 7. Calculation of the coordinates of intersection points.

This analysis is started by finding the coordinate of the crossover point on abscissa. Since $1/v_0$ is zero on abscissa, we can set $1/v_0 = 0$, and thus obtain

$$-\frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) = \frac{K_A}{V_1}\left(1 + \frac{K_{iA}K_B}{K_A B}\right)\frac{1}{A}$$

and

$$-A = K_A\left(\frac{K_A B + K_{iA}K_B}{K_A B + K_A K_B}\right)$$

If the concentration of B tends to zero ($B = 0$), the coordinate of an intersection point on abscissa tends to $-1/K_{iA}$ (Fig. 7). If the concentration of the substrate A is substituted by $-K_{iA}$ in the rate equation, we obtain

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \frac{K_A}{V_1}\left(1 + \frac{K_{iA}K_B}{K_A B}\right)\left(-\frac{1}{K_{iA}}\right)$$

It is clear from the above equation that, when B becomes saturating ($B = \infty$), the coordinate of the intersection point on the ordinate becomes

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 - \frac{K_A}{K_{iA}}\right)$$

Thus, the value of both coordinates is established.

9.2.6 Calculation of Intersection Points in an Ordered Bi Bi Mechanism with P as a Product Inhibitor

The rate equation for this mechanism is complex, given by Eq. (9.21):

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_A}{A} + \frac{P}{K_{iP}}\right) + \frac{K_B}{V_1}\left(1 + \frac{K_{iA}}{A}\right)\left(1 + \frac{K_Q P}{K_{iQ} K_P}\right)\frac{1}{B}$$

However, after substituting the complex values,

$$\alpha = 1 + \frac{K_A}{A} \quad \beta = 1 + \frac{K_{iA}}{A} \quad P_x = \frac{P}{K_{iP}} \quad X = \frac{K_P K_{iQ}}{K_Q K_{iP}}$$

the rate equation becomes much less formidable:

$$\frac{1}{v_0} = \frac{1}{V_1}(\alpha + P_x) + \frac{\beta K_B}{V_1}\left(1 + \frac{P_x}{X}\right)\frac{1}{B}$$

In order to find the coordinate of the intersection point on abscissa, we shall set $1/v_0 = 0$. Then,

$$-\frac{1}{V_1}(\alpha + P_x) = \frac{\beta K_B}{V_1}\left(1 + \frac{P_x}{X}\right)\frac{1}{B} \qquad \text{and} \qquad -B = \frac{\beta K_B}{X}\left(\frac{P_x + X}{P_x + \alpha}\right)$$

It is clear from the above equation that, when P becomes saturating ($P = \infty$), the coordinate on abscissa tends to $-X/\beta K_B$ (Fig. 7). If the concentration of the substrate B is substituted by $-X/\beta K_B$ in the rate equation, we obtain

$$\frac{1}{v_0} = \frac{1}{V_1}(\alpha + P_x) + \frac{\beta K_B}{V_1}\left(1 + \frac{P_x}{X}\right)\left(-\frac{X}{\beta K_B}\right)$$

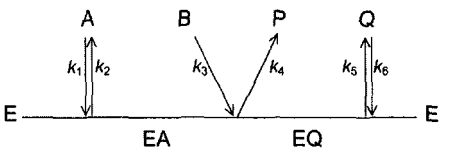
When the concentration of P_X tends to zero ($P_X = 0$), the terms in P_X cancel out, and the coordinate of the intersection point on the ordinate tends to

$$\frac{1}{v_0} = \frac{1}{V_1}(\alpha - X)$$

The simple procedure that is described above can be applied for the calculation of intersection points in all rate equations where the reciprocal plots cross at a single point.

9.3 THEORELL–CHANCE MECHANISM

Theorell–Chance mechanism is a simplified version of an Ordered Bi Bi mechanism where the steady-state level of central complexes is very low. Theorell and Chance (1951) have proposed an Ordered Bi Bi mechanism without the central ternary complexes for alcohol dehydrogenase from equine liver. The hit-and-run reaction sequence can be written:



(9.26)

The general rate equation for this mechanism, in the presence of both substrates and both products of reaction, in terms of rate constants, is

$$v_0 = \frac{(k_1k_3k_5AB - k_2k_4k_6PQ)E_0}{k_2k_5 + k_1k_5A + k_3k_5B + k_2k_4P + k_2k_6Q + k_1k_3AB + k_1k_4AP + k_3k_6BQ + k_4k_6PQ} \tag{9.27}$$

Compared to the Ordered Bi Bi, the equation for the Theorell–Chance mechanism lacks *ABP* and *BPQ* terms in the denominator. In terms of kinetic constants, the rate equation becomes

$$v_0 = \frac{V_1V_2\left(AB - \dfrac{PQ}{K_{eq}}\right)}{V_2K_{iA}K_B + V_2K_BA + V_2K_AB + \dfrac{V_1K_Q}{K_{eq}}P + \dfrac{V_1K_P}{K_{eq}}Q + V_2AB + \dfrac{V_1K_Q}{K_{iA}K_{eq}}AP + \dfrac{V_2K_A}{K_{iQ}}BQ + \dfrac{V_1}{K_{eq}}PQ} \tag{9.28}$$

Definition of rate constants in terms of kinetic constants

$$k_1 = \frac{V_1}{E_0K_A} \quad k_2 = \frac{V_1K_{iA}}{E_0K_A} \quad k_3 = \frac{V_1}{E_0K_B}$$
$$k_4 = \frac{V_2}{E_0K_P} \quad k_5 = \frac{V_2K_{iQ}}{E_0K_Q} \quad k_6 = \frac{V_2}{E_0K_Q} \tag{9.29}$$

Definition of kinetic constants in terms of rate constants

$$K_{iA} = \frac{\text{coefP}}{\text{coefAP}} = \frac{k_2}{k_1} \quad K_{iP} = \frac{\text{coefA}}{\text{coefAP}} = \frac{k_5}{k_4}$$
$$K_{iB} = \frac{\text{coefQ}}{\text{coefBQ}} = \frac{k_2}{k_3} \quad K_{iQ} = \frac{\text{coefB}}{\text{coefBQ}} = \frac{k_5}{k_6} \tag{9.30}$$

Distribution equations

$$\frac{E}{E_0} = \frac{K_{iA}K_BV_2 + \dfrac{K_QV_1}{K_{eq}}P + K_AV_2B}{\text{denominator of rate equation}}$$

$$\frac{EA}{E_0} = \frac{K_BV_2A + \dfrac{K_QV_1}{K_{iA}K_{eq}}AP + \dfrac{V_1}{K_{eq}}PQ}{\text{denominator of rate equation}} \qquad (9.31)$$

$$\frac{EQ}{E_0} = \frac{\dfrac{K_PV_1}{K_{eq}}Q + V_2AB + \dfrac{K_AV_2}{K_{iQ}}BQ}{\text{denominator of rate equation}}$$

Haldane relationships

$$K_{eq} = \frac{V_1K_{iP}K_Q}{V_2K_AK_{iB}} = \frac{V_1K_PK_{iQ}}{V_2K_AK_{iB}} = \frac{V_1K_PK_{iQ}}{V_2K_{iA}K_B} = \frac{V_1K_{iP}K_Q}{V_2K_{iA}K_B} \qquad (9.32)$$

There are 12 more Haldanes for the simple mechanism with six rate constants shown in reaction (9.26).

Effects of isomerizations

EA isomerizes: k_4, k_5 and k_6 may be calculated. In addition, the reciprocal of the rate constant for converting EA to EA* in the forward direction is equal $E_0/V_1 - 1/k_5$.

EA and EQ isomerize: No rate constant can be calculated; only E/E_0 may be calculated.

9.3.1 Rate Equation in the Absence of Products

In the absence of products, the rate equation in the forward direction is the same as that for the Ordered Bi Bi mechanism, given by Eq. (9.15):

$$v_0 = \frac{V_1AB}{K_{iA}K_B + K_BA + K_AB + AB}$$

Therefore, initial velocity studies alone will not distinguish between the two mechanisms; however, the missing *ABP* and *BPQ* denominator terms lead to different product inhibition patterns.

9.3.2 Product Inhibition

Product inhibition by P. If A reacts with B in the presence of P, with $Q = 0$, the complete velocity equation is obtained by replacing $V_1/(V_2K_{eq})$ by appropriate expressions from the Haldanes:

$$v_0 = \frac{V_1 AB}{K_{iA}K_B + K_B A + K_A B + AB + \dfrac{K_A K_{iB}}{K_{iP}} P + \dfrac{K_B}{K_{iP}} AP} \tag{9.33}$$

or, in the reciprocal forms:

$$\frac{1}{v_0} = \frac{1}{V_1}\left[1 + \frac{K_B}{B}\left(1 + \frac{P}{K_{iP}}\right)\right] + \frac{K_A}{V_1}\left[1 + \frac{K_{iB}}{B}\left(1 + \frac{P}{K_{iP}}\right)\right]\frac{1}{A} \tag{9.34}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_A}{A}\right) + \frac{K_B}{V_1}\left(1 + \frac{K_{iA}}{A}\right)\left(1 + \frac{P}{K_{iP}}\right)\frac{1}{B} \tag{9.35}$$

Product inhibition by Q. If A reacts with B in the presence of Q, with $P = 0$, the complete velocity equation is again obtained by replacing $V_1/(V_2K_{eq})$ by appropriate expressions from the Haldanes:

$$v_0 = \frac{V_1 AB}{K_{iA}K_B + K_B A + K_A B + AB + \dfrac{K_{iA}K_B}{K_{iQ}} Q + \dfrac{K_A}{K_{iQ}} BQ} \tag{9.36}$$

or, in the reciprocal forms:

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \frac{K_A}{V_1}\left(1 + \frac{K_{iB}}{B}\right)\left(1 + \frac{Q}{K_{iQ}}\right)\frac{1}{A} \tag{9.37}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left[1 + \frac{K_A}{A}\left(1 + \frac{Q}{K_{iQ}}\right)\right] + \frac{K_B}{V_1}\left[1 + \frac{K_{iA}}{A}\left(1 + \frac{Q}{K_{iQ}}\right)\right]\frac{1}{B} \tag{9.38}$$

The product inhibition patterns in the Theorell–Chance mechanism are different from those of the Ordered Bi Bi mechanism. Note that the product inhibition equations are symmetrical: Equations (9.34) and (9.38) are symmetrical and so are Eqs. (9.35) and (9.37). Thus, product inhibition studies only identify A–Q and B–P pairs and do not reveal the order of substrate addition and product release.

9.4 ORDERED UNI BI AND ORDERED BI UNI SYSTEMS

Ordered Uni Bi and Bi Uni systems are simplified versions of an Ordered Bi Bi mechanism (Cleland, 1963; Fromm, 1975). The reaction for both systems can be written:

$$E \underset{k_2}{\overset{k_1 A}{\rightleftharpoons}} (EA \rightleftharpoons EPQ) \underset{k_4 P}{\overset{k_3}{\rightleftharpoons}} EQ \underset{k_6 Q}{\overset{k_5}{\rightleftharpoons}} E \tag{9.39}$$

The general rate equation for both systems can be written as

$$v_0 = \frac{(k_1k_3k_5A - k_2k_4k_6PQ)E_0}{k_5(k_2 + k_3) + k_1(k_3 + k_5)A + k_2k_4P + k_6(k_2 + k_3)Q + k_1k_4AP + k_4k_6PQ} \tag{9.40}$$

In terms of kinetic constants, the rate equation becomes

$$v_0 = \frac{V_1V_2\left(A - \dfrac{PQ}{K_{eq}}\right)}{V_2K_A + V_2A + \dfrac{V_1K_Q}{K_{eq}}P + \dfrac{V_1K_P}{K_{eq}}Q + \dfrac{V_2}{K_{iP}}AP + \dfrac{V_1}{K_{eq}}PQ} \tag{9.41}$$

Definition of kinetic constants in terms of rate constants

$$\begin{aligned} K_A &= \frac{\text{const}}{\text{coefA}} & K_{iA} &= \frac{\text{coefP}}{\text{coefAP}} = \frac{k_2}{k_1} & \frac{V_1}{E_0} &= \frac{\text{numer}_1}{\text{coefA}} \\ K_Q &= \frac{\text{coefP}}{\text{coefPQ}} & K_{iQ} &= \frac{\text{const}}{\text{coefQ}} = \frac{k_5}{k_6} & \frac{V_2}{E_0} &= \frac{\text{numer}_2}{\text{coefPQ}} \\ K_P &= \frac{\text{coefQ}}{\text{coefPQ}} & K_P &= \frac{\text{coefA}}{\text{coefAP}} & K_{eq} &= \frac{\text{numer}_1}{\text{numer}_2} \end{aligned} \tag{9.42}$$

Definition of rate constants in terms of kinetic constants

$$\begin{aligned} k_1 &= \frac{V_2}{E_0K_{iA}} & k_2 &= \frac{V_2}{E_0} & \frac{1}{k_3} &= \frac{E_0}{V_1} - \frac{1}{k_5} \\ k_4 &= \frac{k_2 + k_3}{K_P} & k_5 &= \frac{V_2K_{iQ}}{E_0K_Q} & k_6 &= \frac{V_2}{E_0K_Q} \end{aligned} \tag{9.43}$$

Distribution equations

$$\begin{aligned} \frac{E}{E_0} &= \frac{K_AV_2 + \dfrac{K_QV_1}{K_{eq}}P}{\text{denominator of rate equation}} \\ \frac{EQ}{E_0} &= \frac{\dfrac{K_PV_1}{K_{eq}}Q + \dfrac{K_QV_1}{K_{iQ}}A}{\text{denominator of rate equation}} \\ \frac{(EA + EPQ)}{E_0} &= \frac{\left(V_2 - \dfrac{V_1K_Q}{K_{iQ}}\right)A + \dfrac{V_2}{K_{iP}}AP + \dfrac{V_1}{K_{eq}}PQ}{\text{denominator of rate equation}} \end{aligned} \tag{9.44}$$

Haldane relationships

$$K_{eq} = \frac{V_1K_{iQ}K_P}{V_2K_A} = \frac{V_1K_{iP}K_Q}{V_2K_{iA}} \tag{9.45}$$

Effects of isomerizations

The transitory complexes $EA \rightleftharpoons EPQ$ isomerize: The calculation of distribution equations is still valid, but the calculation of k_1, k_2, k_3, and k_4 is invalid. EQ isomerizes: All the rate constant calculations are invalid, and only E/E_0 can be calculated.

9.4.1 Ordered Uni Bi Mechanism

The Ordered Uni Bi reaction takes place in the forward direction:

$$A \longrightarrow P + Q$$

if the substrate A is split into two products, P and Q.

Rate equations in the absence of products. When both P and Q are zero, we obtain a usual Michaelis–Menten equation: $v_0 = V_1A/(K_A + A)$.

Product inhibition in the Uni Bi system. In the forward direction, $A \rightarrow P + Q$, if P is present and $Q = 0$, we obtain

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{P}{K_{iP}}\right) + \frac{K_A}{V_1}\left(1 + \frac{K_Q P}{K_{iQ}K_P}\right)\frac{1}{A} \tag{9.46}$$

If Q is present, and $P = 0$, we obtain

$$\frac{1}{v_0} = \frac{1}{V_1} + \frac{K_A}{V_1}\left(1 + \frac{Q}{K_{iQ}}\right)\frac{1}{A} \tag{9.47}$$

P and Q can be identified from the above product inhibition studies.

Note, however, that the product inhibition studies in the $A \rightarrow P + Q$ direction do not allow K_P and K_Q to be determined, but their ratio can be calculated from the Haldane relationship (Eq. (9.45)):

$$\frac{K_P}{K_Q} = \frac{K_A K_{iP}}{K_{iA} K_{iQ}} \tag{9.48}$$

9.4.2 Ordered Bi Uni Mechanism

The Ordered Bi Uni reaction takes place in the reverse direction:

$$P + Q \rightarrow A$$

when P and Q are condensed into a single product A.

Rate equation in the absence of product. If P reacts with Q, and $A = 0$, the rate equation in the reverse direction is

$$v_0 = \frac{V_2 PQ}{K_{iQ}K_P + K_Q P + K_P Q + PQ} \tag{9.49}$$

This equation is symmetrical with Eq. (9.15) for the Ordered Bi Bi system.

Product inhibition in the Bi Uni system. In the reverse direction, $P + Q \rightarrow A$, and in the presence of A, the writing of the velocity equation is complicated by the reaction in the $A \rightarrow P + Q$ direction. However, if one applies the isotope exchange method, the reaction $A \rightarrow P + Q$ can be made negligible. If A inhibits while one is still far from equilibrium, the reverse reaction is unimportant and one can measure product inhibition by A. It helps to keep either P or Q saturating to stay far from equilibrium. In this case, in the $P + Q \rightarrow A$ direction, the initial velocity equation is obtained by replacing V_2K_{eq}/V_1 from the Haldanes:

$$v_0 = \frac{V_2PQ}{K_PK_{iQ} + K_PQ + K_QP + PQ + \dfrac{K_PK_{iQ}}{K_A}A + \dfrac{K_Q}{K_{iA}}AP} \tag{9.50}$$

The reciprocal forms of Eq. (9.50) are

$$\frac{1}{v_0} = \frac{1}{V_2}\left[1 + \frac{K_Q}{Q}\left(1 + \frac{A}{K_{iA}}\right)\right] + \frac{K_P}{V_2}\left[1 + \frac{K_{iQ}}{Q}\left(1 + \frac{A}{K_A}\right)\right]\frac{1}{P} \tag{9.51}$$

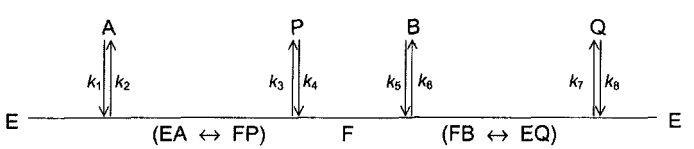
$$\frac{1}{v_0} = \frac{1}{V_2}\left(1 + \frac{K_P}{P}\right) + \frac{K_Q}{V_2}\left[1 + \frac{K_AK_{iP}}{K_{iA}P} + \frac{A}{K_{iA}}\left(1 + \frac{K_{iP}}{P}\right)\right]\frac{1}{Q} \tag{9.52}$$

P and Q can be identified from the above product inhibition studies. The product inhibition studies also allow the direct estimation of K_P and K_Q.

9.5 PING PONG BI BI MECHANISM

A bisubstrate mechanism in which the first product (P) is released before the second substrate (B) is bound, is called Ping Pong. Such a mechanism is typical for transaminases, where the amino acid substrate (A) is bound first, and the keto acid product (P) is released before the second substrate, keto acid (B), is added; the second product, amino acid (Q), leaves the enzyme last (Walsh, 1998).

The reaction sequence can be written as



(9.53)

where E = free enzyme; EA = the enzyme–substrate (amino $acid_1$) complex; FP = the amino enzyme–product (keto $acid_1$) complex; F = the amino enzyme; FB = the amino enzyme–substrate (keto $acid_2$) complex and EQ = the enzyme–product (amino $acid_2$) complex.

The general rate equation for this mechanism has no *ABP* and *BPQ* terms and no constant term in the denominator:

$$v_o = \frac{(k_1k_3k_5k_7AB - k_2k_4k_6k_8PQ)E_o}{k_1k_3(k_6 + k_7)A + k_5k_7(k_2 + k_3)B + k_1k_5(k_3 + k_7)AB + k_2k_4(k_6 + k_7)P + k_6k_8(k_2 + k_3)Q + k_1k_4(k_6 + k_7)AP + k_4k_8(k_2 + k_6)PQ + k_5k_8(k_2 + k_3)BQ} \tag{9.54}$$

In terms of kinetic constants, the rate equation becomes

$$v_o = \frac{V_1V_2\left(AB - \frac{PQ}{K_{eq}}\right)}{V_2K_BA + V_2K_AB + \frac{V_1K_Q}{K_{eq}}P + \frac{V_1K_P}{K_{eq}}Q + V_2AB + \frac{V_1K_Q}{K_{iA}K_{eq}}AP + \frac{V_1}{K_{eq}}PQ + \frac{V_2K_A}{K_{iQ}}BQ} \tag{9.55}$$

The rate Eq. (9.55) is identical with the rate equation for the Ordered Bi Bi mechanism (9.8), except that there is no *ABP, BPQ,* and the constant term in the denominator.

Definition of kinetic constants in terms of rate constants

The definition of kinetic constants V_1, V_2, K_A, K_B, K_P, K_Q, K_{iA} and K_{iQ} is the same (in a coefficient form) as in the Ordered Bi Bi mechanism, but the definition of K_{iB} and K_{iP} has a new meaning.

$$\begin{aligned}
K_A &= \frac{\text{coefB}}{\text{coefAB}} = \frac{k_7(k_2 + k_3)}{k_1(k_3 + k_7)} & K_{iA} &= \frac{\text{coefP}}{\text{coefAP}} = \frac{k_2}{k_1} \\
K_B &= \frac{\text{coefA}}{\text{coefAB}} = \frac{k_3(k_6 + k_7)}{k_5(k_3 + k_7)} & K_{iB} &= \frac{\text{coefQ}}{\text{coefBQ}} = \frac{k_6}{k_5} \\
K_P &= \frac{\text{coefQ}}{\text{coefPQ}} = \frac{k_6(k_2 + k_3)}{k_4(k_2 + k_6)} & K_{iP} &= \frac{\text{coefA}}{\text{coefAP}} = \frac{k_3}{k_4} \\
K_Q &= \frac{\text{coefP}}{\text{coefPQ}} = \frac{k_2(k_6 + k_7)}{k_8(k_2 + k_6)} & K_{iQ} &= \frac{\text{coefB}}{\text{coefBQ}} = \frac{k_7}{k_8} \\
\frac{V_1}{E_o} &= \frac{\text{numer}_1}{\text{coefAB}} = \frac{k_3k_7}{k_3 + k_7} & \frac{V_2}{E_o} &= \frac{\text{numer}_2}{\text{coefPQ}} = \frac{k_2k_6}{k_2 + k_6} \\
K_{eq} &= \frac{\text{numer}_1}{\text{numer}_2} = \frac{k_1k_3k_5k_7}{k_2k_4k_6k_8}
\end{aligned} \tag{9.56}$$

Haldane relationships

$$K_{eq} = \frac{K_{iP}K_{iQ}}{K_{iA}K_{iB}} = \frac{V_1K_{iP}K_Q}{V_2K_{iA}K_B} = \frac{V_1K_PK_{iQ}}{V_2K_AK_{iB}} = \left(\frac{V_1}{V_2}\right)^2\frac{K_PK_Q}{K_AK_B} \tag{9.57}$$

Distribution equations

$$\frac{E}{E_0} = \frac{K_A V_2 B + \dfrac{K_Q V_1 P}{K_{eq}}}{\text{denominator of rate equation}}$$
$$\frac{F}{E_0} = \frac{K_B V_2 A + \dfrac{K_P V_1 Q}{K_{eq}}}{\text{denominator of rate equation}} \tag{9.58}$$

The fraction of the total enzyme present as EA + FP and FB + EQ cannot be calculated.

Rate constants. Individual rate constants cannot be calculated from the kinetic constants.

Effects of isomerizations. Isomerization of any of the transitory complexes, including central complexes, has no effect on the above distribution equations.

9.5.1 Rate Equations in the Absence of Products

Initial rate equation, in the absence of products P and Q, is given by

$$v_0 = \frac{V_1 AB}{K_B A + K_A B + AB} \tag{9.59}$$

or, in the reciprocal form:

$$\frac{V_1}{v_0} = 1 + \frac{K_A}{A} + \frac{K_B}{B} \tag{9.60}$$

If we separate the variable from the constant substrate, Eq. (9.60) becomes

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \left(\frac{K_A}{V_1}\right)\frac{1}{A} \tag{9.61}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_A}{A}\right) + \left(\frac{K_B}{V_1}\right)\frac{1}{B} \tag{9.62}$$

Figure 8 shows the graphical presentation of Eq. (9.61).

Equations (9.61) and (9.62) are completely symmetrical and, therefore, the primary plot $1/v_0$ versus $1/A$ (Fig. 8) is symmetrical with the primary plot $1/v_0$ versus $1/B$. While the system is completely symmetrical with respect to A and B, and also with respect to P and Q, there is generally no difficulty in identifying which substrate is A and which is B. Since we are usually aware of the chemical nature of reaction, we know that A must be the substrate with the group to be transferred and B the acceptor; similarly, P must be the product of A, while Q is the product of B plus the group transferred from A.

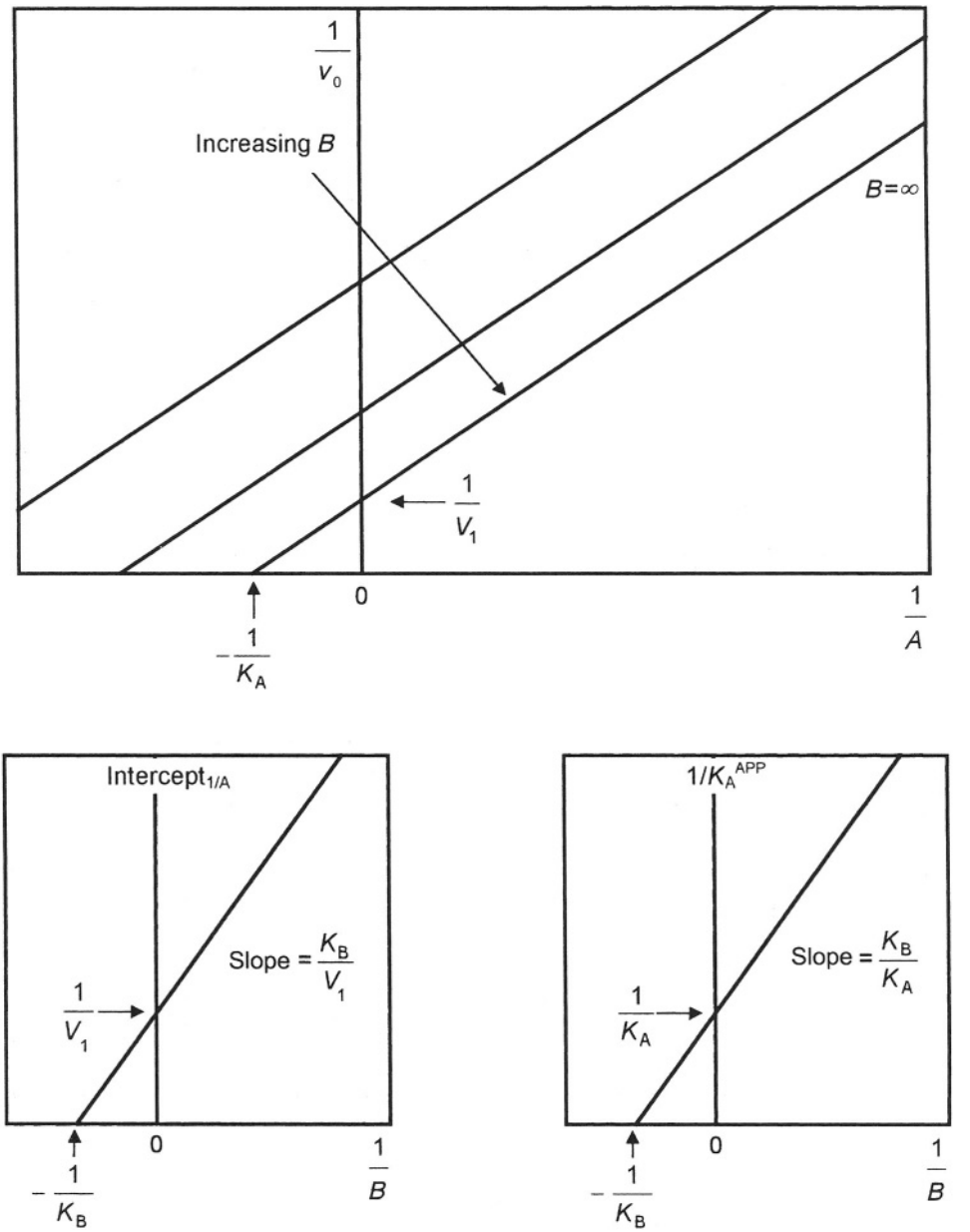


Figure 8. Ping Pong Bi Bi system. Graphical presentation of Eq. (9.61), with B as a constant and A as a variable substrate.

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \left(\frac{K_A}{V_1}\right)\frac{1}{A}$$

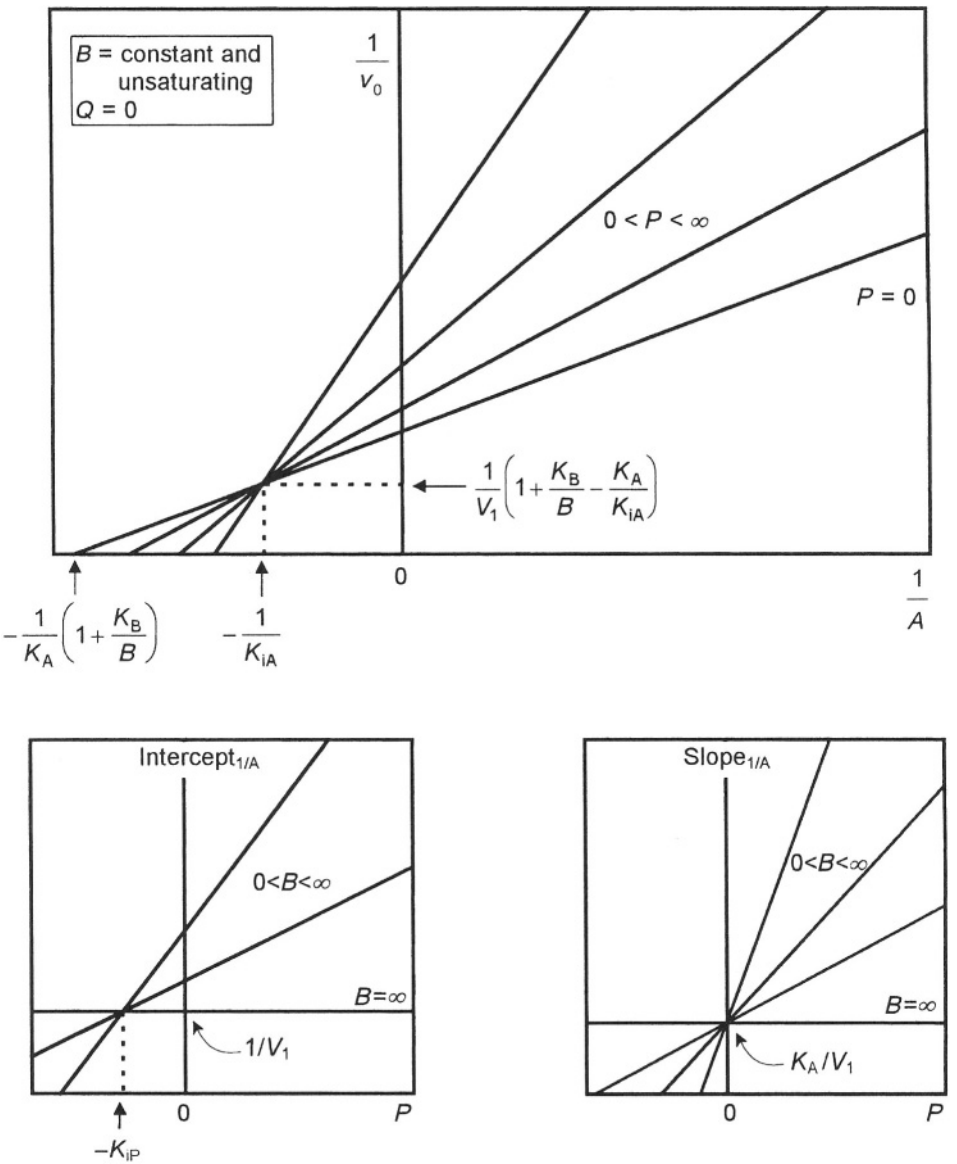


Figure 9. Product inhibition by P in the Ping Pong Bi Bi system. Graphical presentation of Eq. (9.64) with B as a constant and A as a variable substrate.

$$\frac{1}{v_0} = \frac{1}{V_1}\left[1 + \frac{K_B}{B}\left(1 + \frac{P}{K_{iP}}\right)\right] + \frac{K_A}{V_1}\left(1 + \frac{K_{iA}K_BP}{K_AK_{iP}B}\right)\frac{1}{A}$$

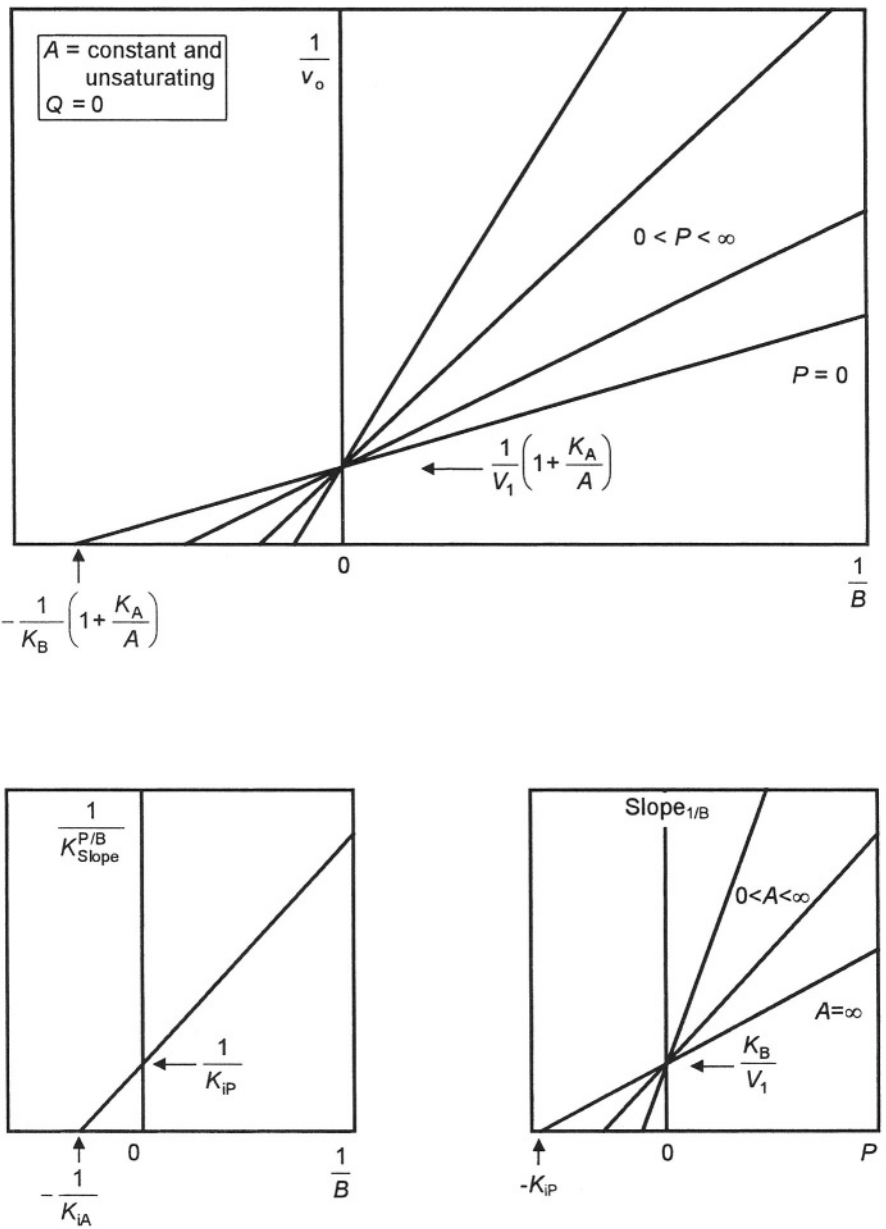


Figure 10. Product inhibition by P in a Ping Pong Bi Bi system. Graphical presentation of Eq. (9.65), with A as a constant and B as a variable substrate.

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_A}{A}\right) + \frac{K_B}{V_1}\left[1 + \frac{P}{K_{iP}}\left(1 + \frac{K_{iA}}{A}\right)\right]\frac{1}{B}$$

9.5.2 Product Inhibition in the Ping Pong Bi Bi System

Product inhibition by P. If A reacts with B, in the presence of product P, the rate equation, using the Haldanes to replace $V_1/(V_2K_{eq})$, is

$$v_0 = \frac{V_1AB}{K_BA + K_AB + AB + \dfrac{K_{iA}K_B}{K_{iP}}P + \dfrac{K_B}{K_{iP}}AP} \tag{9.63}$$

or, in the reciprocal forms:

$$\frac{1}{v_0} = \frac{1}{V_1}\left[1 + \frac{K_B}{B}\left(1 + \frac{P}{K_{iP}}\right)\right] + \frac{K_A}{V_1}\left(1 + \frac{K_{iA}K_BP}{K_AK_{iP}B}\right)\frac{1}{A} \tag{9.64}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_A}{A}\right) + \frac{K_B}{V_1}\left[1 + \frac{P}{K_{iP}}\left(1 + \frac{K_{iA}}{A}\right)\right]\frac{1}{B} \tag{9.65}$$

Figures 9 and 10 show the graphical presentation of Eqs. (9.64) and (9.65), the product inhibition by P. With a noncompetitive inhibition pattern in Fig. 9, one can define the apparent inhibition constants, $K_{ii} = K_{iP}(1+B/K_B)$ and $K_{is} = K_{iP}K_AB/(K_{iA}K_B)$, and with a competitive inhibition pattern in Fig. 10, the apparent inhibition constant $K_{is} = K_{iP}(1+K_{iA}/A)$, making them useful for calculation of K_{iP} from observed values.

Product inhibition by Q. If A reacts with B, in the presence of product Q, the rate equation is

$$v_0 = \frac{V_1AB}{K_BA + K_AB + AB + \dfrac{K_AK_{iB}}{K_{iQ}}Q + \dfrac{K_A}{K_{iQ}}BQ} \tag{9.66}$$

or, in the reciprocal forms:

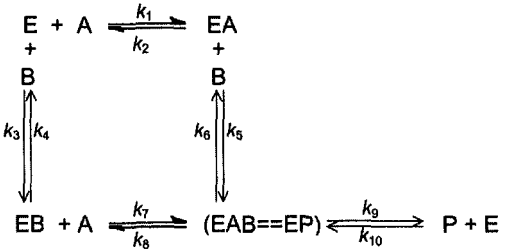
$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \frac{K_A}{V_1}\left[1 + \frac{Q}{K_{iQ}}\left(1 + \frac{K_{iB}}{B}\right)\right]\frac{1}{A} \tag{9.67}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left[1 + \frac{K_A}{A}\left(1 + \frac{Q}{K_{iQ}}\right)\right] + \frac{K_B}{V_1}\left(1 + \frac{K_{iB}K_AQ}{K_BK_{iQ}A}\right)\frac{1}{B} \tag{9.68}$$

Equations (9.64) and (9.68) are completely symmetrical; so are Eqs. (9.65) and (9.67). Therefore, there is no need to present graphically Eqs. (9.67) and (9.68), showing the product inhibition by Q.

9.6 STEADY-STATE RANDOM BI UNI MECHANISM

If the breakdown of the central complex in bisubstrate reactions is not the sole rate-limiting step, than the rate equation becomes quite complex. For example, consider the Steady-State Random Bi Uni system shown below:



(9.69)

The complete rate equation, obtained with the King–Altman method, and after grouping similar terms, is quite complex:

$$v_0 = \frac{(K_1AB + K_2A^2B + K_3AB^2 - K_4P - K_5AP - K_6BP)E_0}{\begin{pmatrix} K_7 + K_8A + K_9B + K_{10}AB + K_{11}A^2 + K_{12}B^2 + K_{13}A^2B \\ +K_{14}AB^2 + K_{15}P + K_{16}AP + K_{17}BP + K_{18}ABP \end{pmatrix}} \tag{9.70}$$

where K_1–K_{18} represent groups of rate constants:

$$\begin{aligned}
K_1 &= k_1k_4k_5k_9 + k_2k_3k_7k_9 & K_{10} &= k_1k_4k_5 + k_2k_3k_7 + k_1k_5k_8 \\
K_2 &= k_1k_5k_7k_9 & &\quad + k_3k_6k_7 + k_5k_7k_9 \\
K_3 &= k_3k_5k_7k_9 & K_{11} &= k_1k_6k_7 + k_1k_7k_9 \\
K_4 &= k_2k_4k_6k_{10} + k_2k_4k_8k_{10} & K_{12} &= k_3k_5k_8 + k_3k_5k_9 \\
K_5 &= k_2k_6k_7k_{10} & K_{13} &= k_1k_5k_7 \\
K_6 &= k_4k_5k_8k_{10} & K_{14} &= k_3k_5k_7 \\
K_7 &= k_2k_4k_6 + k_2k_4k_8 + k_2k_4k_9 & K_{15} &= k_2k_4k_{10} + k_2k_8k_{10} \\
K_8 &= k_1k_4k_6 + k_1k_4k_8 + k_1k_4k_9 & &\quad + k_4k_6k_{10} \\
&\quad + k_2k_6k_7 + k_2k_7k_9 & K_{16} &= k_2k_7k_{10} + k_6k_7k_{10} \\
K_9 &= k_2k_3k_6 + k_2k_3k_8 + k_2k_3k_9 & K_{17} &= k_4k_5k_{10} + k_5k_8k_{10} \\
&\quad + k_4k_5k_8 + k_4k_5k_9 & K_{18} &= k_5k_7k_{10}
\end{aligned} \tag{9.71}$$

Even in the absence of product P, the rate equation is complex, a rational polynomial of the order 2:2 with respect to both substrates. The reciprocal plots are nonlinear, but the departure from linearity may be very difficult to detect if both routes to EAB are about equally favorable.

This example clearly shows that completely randomized steady-state bisubstrate reactions will produce extremely complex rate equations which are, in most cases, unmanageable and almost useless for practical purposes. Thus, for example, the rate equation for an Ordered Bi Bi mechanism has 12 terms in the denominator (compare Eq. (9.8)). A completely Random Bi Bi mechanism yields an even more complicated rate equation with 37 new terms in the denominator. For this reason, and in such cases, we shall usually revert to simplifying assumptions, usually introducing the rapid equilibrium segments in the mechanism in order to reduce the rate equations to manageable forms.

9.7 ISOMERIZATION OF TRANSITORY COMPLEXES AND STABLE ENZYME FORMS

In mechanisms described in preceding sections, the isomerization of *transitory enzyme forms* may form a part of the reaction sequence without changing the rate equations. If isomerization of *stable enzyme forms* is a part of the reaction sequence, however, additional terms are present in the rate equations, and such mechanisms are designated "Iso Ordered", "Iso Random" and "Iso Ping Pong" to indicate the stable form isomerization. In Ping Pong mechanisms where several stable enzyme forms may occur, the mechanism can be designated "Di-Iso Ping Pong" or "Tri-Iso Ping Pong" if two or three stable enzyme forms isomerize during the reaction.

Isomerization of a transitory complex in any mechanism does not affect the form of the rate equation, but if a stable enzyme form isomerizes, additional terms occur in the denominator.

9.7.1 Isomerization of Transitory Complexes

The following reaction shows an isomerization of transitory EA and EQ complexes in the Ordered Bi Bi mechanism.

$$\begin{aligned} &\mathrm{E} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{EA} \underset{k_{ff}}{\overset{k_f}{\rightleftharpoons}} {}^{*}\mathrm{EA} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{EAB} \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} \mathrm{EPQ} \\ &\mathrm{EAB} \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} \mathrm{EPQ} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} {}^{*}\mathrm{EQ} \underset{k_r}{\overset{k_{rr}}{\rightleftharpoons}} \mathrm{EQ} \underset{k_8}{\overset{k_7}{\rightleftharpoons}} \mathrm{E} \end{aligned} \qquad (9.72)$$

The isomerization of a transitory complex does not affect the algebraic form of the velocity equation in the absence or in the presence of products, but the composition of some kinetic constants are changed by introducing the rate constants for isomerization: k_f and k_{ff} in the forward direction and k_r and k_{rr} in the reverse direction. The isomerization of transitory complexes is very common in many enzyme reactions, particularly among pyridine-dependent dehydrogenases.

Although isomerization of transitory forms does not alter the algebraic form of the rate equation, the existence of isomerization of non-central transitory complexes can often be inferred from kinetic data alone, or from comparison of kinetic data with the equilibrium constant (Chapter 10).

9.7.2 Isomerization of a Stable Enzyme Form

Isomerization of a stable enzyme form does not affect the algebraic form of the rate equation in the absence of products, but product inhibition patterns are modified so that the order of addition of substrates and release of products can not be determined by steady-state kinetic experiments. Rate constants for steps involving the isomerizing stable form or any central complex are not determinable, and steady-state distributions can be calculated only for non-isomerizing

stable forms and for non-central transitory forms, the rate constants for formation of which can be determined.

If a stable enzyme form isomerizes, new terms appear in the denominator of the velocity equation. The new denominator terms for several iso systems are summarized in Table 1.

Table 1. *New denominator terms in rate equations for the iso systems*

System	New denominator terms
Iso Uni Uni	AP
Iso Uni Bi	AQ and APQ
Iso Ordered Uni Bi	AQ and APQ
Iso Ordered Bi Bi	APQ, ABQ, and ABPQ
Iso Theorell–Chance Bi Bi	APQ and ABQ
Iso Tetra Uni Ping Pong (E isomerizes)	AQ, ABQ, and APQ
Uni Uni Iso Uni Uni Ping Pong (F isomerizes)	BP, ABP, and BPQ
Iso Tetra Uni Ping Pong Bi Bi (E and F isomerize)	AQ, ABQ, APQ, BP, ABP, and BPQ

Since the products are used one at a time in product inhibition studies, the extra denominator terms containing either P or Q (but not both) are the only ones that influence the inhibition patterns. A useful graphical procedure for characterizing isomerization mechanisms was developed by Darvey (Darvey, 1972; Rebholz & Northrop, 1995); the construction of a Darvey plot requires very accurate estimation of initial rates. A further graphical method for the characterization of isomerization mechanisms is the Foster–Niemann plot, a plot of an enzyme progress curve with respect to product inhibition. These plots are particularly useful with regard to enzymes with isomerization steps in which the product acts as a noncompetitive inhibitor (Foster & Niemann, 1953; Rebholz & Northrop, 1995).

The iso mechanisms, with isomerization of a stable enzyme form, are not very common in ordinary soluble enzyme systems. Well characterized examples of iso mechanisms are proline racemase, fumarase, and carbonic anhydrase (Rebholz & Northrop, 1995).

REFERENCES

Books

Cornish-Bowden, A. (1995) *Fundamentals of enzyme kinetics*, revised edition, Portland Press, London.
Fromm, H.J. (1975) *Initial rate enzyme kinetics*, Springer-Verlag, Berlin.
Mahler, H.R. & Cordes, E.H. (1966) *Biological chemistry*, Harper & Row, New York.
Purich, D.L. & Allison, D.R. (2000) *Handbook of biochemical kinetics*, Academic Press, San Diego.

Segel, I.H. (1975) *Enzyme kinetics,* Wiley, New York.
Walsh, C. (1998) *Enzymatic reaction mechanisms,* W.H. Freeman, New York.

Review articles

Cleland, W.W. (1967) *Ann. Rev. Biochem. 36*, 77–99.
Cleland, W.W. (1970) *The Enzymes,* 3rd ed., Vol. II, 1–65.
Cleland, W.W. (1977) *Adv. Enzymol. Rel. Areas Mol. Biol. 45*, 273–387.
Cleland, W.W. (1982) *Methods Enzymol. 87*, 366–370.
Fromm, H.J. (1979) *Methods Enzymol. 63*, 42–53.
Rebholz, K.L. & Northrop, D.B. (1995) Methods *Enzymol. 249*, 211–240.
Rudolph, F.B. (1979) *Methods Enzymol. 63*, 412–436.

Specific references

Ainslie, G.R. & Cleland, W.W. (1972) *J. Biol. Chem. 247*, 946–950.
Alberty, R.A. (1953) *J. Am. Chem. Soc. 75*, 1928–1940.
Bloomfield, V., Peller, L., & Alberty, R.A. (1962) *J. Am. Chem. Soc. 84*, 4367–4377.
Cleland, W.W. (1963) *Biochim. Biophys. Acta 67*,104–137.
Cleland, W.W. (1963) *Biochim. Biophys. Acta 67*, 173–187.
Cleland, W,W. (1963) *Biochim. Biophys. Acta 67*, 188–196.
Dalziel, K. (1957) *Acta Chem. Scand. 114*, 1706–1723.
Darvey, G. (1972) *Biochem. J. 128*, 383–393.
Foster, R.J. & Niemann, C. (1953) Proc. *Natl. Acad. Sci. U.SA.* 39, 999–1010.
Plapp, B.V. (1973) *Arch. Biochem. Biophys. 156*, 112–114.
Theorell, H. & Chance, B. (1951) *Acta Chem. Scand. 5*, 1127–1147.
Wong, J.T.-F. & Hanes, C.S. (1962) *Can. J. Physiol 40*, 763–804.

Chapter 10

Kinetic Analysis of Bisubstrate Mechanisms

One of the major reasons for carrying out kinetic studies on enzymes is to determine the kinetic mechanism. To achieve this, kinetic data are compared with the predicted kinetics of all possible mechanisms, and those which do not fit are eliminated. This may seem to be a formidable task, but the number of possible, or reasonably plausible mechanisms is not large; the possibilities can rapidly be narrowed and then tested, one by one, by kinetic techniques designed specifically to distinguish them. If all the techniques of enzyme kinetics are applied, one can in most cases deduce the actual kinetic mechanism with little or no ambiguity (Cleland, 1970, 1977; Dixon & Webb, 1979; Fromm, 1979).

In multisubstrate enzyme reactions, the kinetic mechanism can be predicted from effects of various compounds on the slope and on the intercept of reciprocal plots for a given varied substrate. An understanding of why patterns are observed for a given mechanism will help in the prediction of patterns for other mechanisms. To achieve this, the slope and intercept of a reciprocal plot must be measured to see how they are affected by the concentrations of various compounds. For this purpose, the kineticist may apply the following compounds:

(1) nonvaried substrates,
(2) varied substrates,
(3) normal products,
(4) alternate substrates,
(5) alternate products, and
(6) inhibitors, including dead-end inhibitors.

The principal methods that employ steady-state data for distinguishing kinetic mechanisms include the following:

(1) determining the initial velocity patterns,
(2) determining inhibition patterns, both by products and dead-end inhibitors,
(3) analysis of intersection points,
(4) checking the consistency of the experimentally determined kinetic constants with the Haldanes and other special relationships that are predicted between the constants, and
(5) isotope effects.

Intersection point analysis is much less dependable on the quality of experimental data than the first two criteria, and the Haldanes are really the same for

most mechanisms. However, it should be emphasized that, for a definitive work, a complete initial velocity experiment with a full matrix of substrate concentrations is important for assessing a correct mechanism and for a statistical evaluation of data (Chapter 18). Isotope effects are also very useful kinetic tools for distinguishing kinetic mechanisms (Chapters 16 and 17).

10.1 PREDICTION OF INITIAL VELOCITY PATTERNS

10.1.1 Initial Velocity Patterns

The complete analysis of the primary and the secondary double reciprocal plots is often not necessary to identify the mechanism; the mechanism can frequently be determined from the variation of apparent Michaelis constants and maximal rates with the concentration of the fixed substrate (Cleland, 1970, 1977).

The apparent Michaelis constant is the slope of a primary reciprocal plot (of $1/v_o$ versus 1/[substrate]) divided by the vertical intercept, and the apparent maximal velocity is the reciprocal of this intercept.

We shall distinguish three types of initial velocity patterns:

(1) intersecting,
(2) parallel, and
(3) equilibrium ordered.

The equilibrium ordered patterns vary depending on which substrate is varied, whereas the former two patterns do not.

For example, for the Ordered bisubstrate mechanism, the apparent Michaelis constants for A and B are

$$K_A \frac{\left(\frac{1+K_{iA}K_B}{K_AB}\right)}{\left(\frac{1+K_B}{B}\right)} \quad \text{and} \quad K_B \frac{\left(\frac{1+K_{iA}}{A}\right)}{\left(\frac{1+K_A}{A}\right)} \tag{10.1}$$

while for the Ping Pong bisubstrate mechanism, they are

$$\frac{K_A}{\left(1+\frac{K_B}{B}\right)} \quad \text{and} \quad \frac{K_B}{\left(1+\frac{K_A}{A}\right)} \tag{10.2}$$

For both types of mechanisms, the apparent V is

$$\frac{V_1}{\left(1+\frac{K_B}{B}\right)} \quad \text{and} \quad \frac{V_1}{\left(1+\frac{K_A}{A}\right)} \tag{10.3}$$

when A or B, respectively, is the variable substrate.

In the Ping Pong case, the apparent Michaelis constants increase to their limiting values as the concentration of the fixed (non-varied) substrate increases, and decrease to zero as the fixed substrate concentration goes to zero.

For the ordered and random mechanisms, as the concentration of the fixed substrate rises, the Michaelis constants may increase, decrease, or remain constant at their limiting values, depending on the ratio of K_{iA} and K_A. The intersecting patterns, which can intersect above, below, or on the axis, represent either ordered or random sequential mechanism, or, exceptionally, a Ping Pong ones with unstable F form or with a product present.

The ratio of apparent K to apparent V for the ordered case is

$$\frac{K_A}{V_1}\left(1+\frac{K_{iA}K_B}{K_A B}\right) \quad \text{or} \quad \frac{K_B}{V_1}\left(1+\frac{K_{iA}}{A}\right) \tag{10.4}$$

while for the Ping Pong case it is K_A/V_1 or K_B/V_1, respectively. Thus, if the ratio of apparent Michaelis constant to apparent maximum velocity remains constant as the concentration of the fixed substrate is changed, the mechanism is Ping Pong. Assignment of a Ping Pong mechanism should always be confirmed by isotopic exchange studies (Chapter 16).

10.1.2 Cleland's Rules

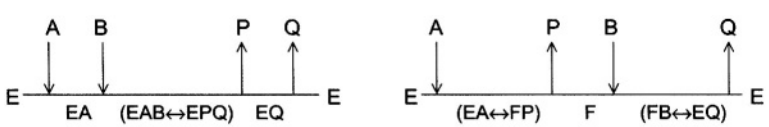
In 1963, Cleland has formulated a set of useful rules for the prediction of initial velocity patterns in the steady-state kinetics of enzyme-catalyzed reactions (Cleland, 1963, 1970).

The intercept rule. The intercepts of reciprocal plots will be affected by the changing *fixed* substrate unless (a) its combination with enzyme is at thermodynamic equilibrium, and (b) the *variable* substrate combines with the complex containing the changing *fixed* substrate in such a way that it traps the changing fixed substrate on the enzyme so it cannot leave.

The slope rule. The slopes of reciprocal plots will be affected by the changing *fixed* substrate whenever the points of combination of it and the *variable* substrate in the reaction sequence are connected by reversible steps. Saturation with substrate or release of products at zero concentration are considered irreversible steps. If no reversible connection exists, no slope effect is observed.

10.1.3 Slope and Intercept Rules in Bisubstrate Reactions

Let us illustrate the above rules with examples of an Ordered Bi Bi mechanism (Section 9.2) and the Ping Pong Bi Bi mechanism (Section 9.5):



The vertical intercept of a reciprocal plot is the reciprocal velocity when the variable substrate is at infinite concentration. Thus, to alter the intercept, a compound must alter the reaction rate when the variable substrate is saturating. This is normally always true for a second substrate, regardless of mechanism, since it is combining with an enzyme form other than that with which the variable

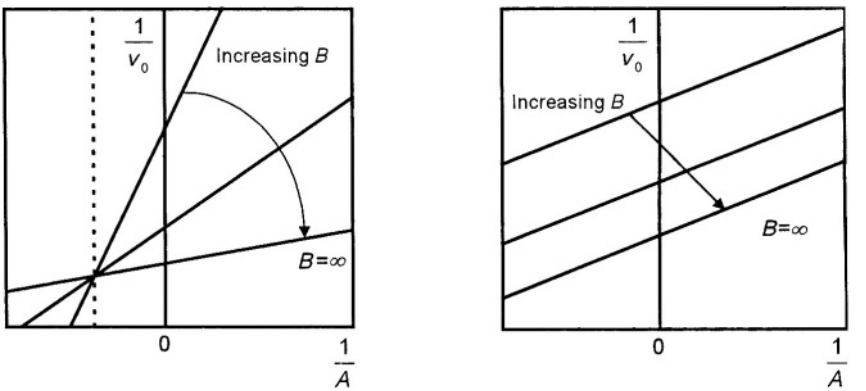


Figure 1. Comparison of double reciprocal plots for the Ordered bisubstrate (left) and the Ping Pong bisubstrate mechanism (right), with A as a variable, and B as a fixed substrate. The crossover point on the left panel can be above, on or below the axis.

substrate combines and will affect the rate even if the variable substrate is at infinite concentration. Thus, the two common initial velocity patterns, Ordered Bi Bi and Ping Pong Bi Bi, both show an effect on the intercepts by the substrate that is not a variable.

The intersecting pattern for an Ordered bisubstrate mechanism in Fig. 1 (left) will also be obtained with a Rapid Equilibrium bisubstrate mechanism; in each case, an intersecting point may be above, below, or on the axis.

The only exception is in the case of an equilibrium ordered mechanism (Section 8.2), where the saturation with B eliminates dependence on A by displacing the E+A $\longleftrightarrow$ EA equilibrium completely to the right as EA reacts with B (Chapter 8, Fig. 2). When B is varied, there is no intercept effect by A, and the lines cross on the vertical axis since no A, other than an amount stoichiometric with enzyme, is needed to give the full maximum velocity as long as B is saturating.

The interpretation of slope effects is more complex. The slope of a reciprocal plot is the reciprocal of the apparent first-order rate constant for reaction of substrate when it is at very low levels, that is, $v_o = (V_1/K_A)A$; the slope of the reciprocal plot is K_A/V_1. If A is the substrate we are considering, and it combines with the enzyme by the step E_X+A $\longleftrightarrow$ E_XA, it is clear that, in order to observe the most rapid net combination of E_X with A at low A levels, we want the concentration of E_X to be as high as possible. Thus, any factors which raise the level of E_X with respect to E_XA will lower the slope of the reciprocal plot.

Consider now the effect of a substrate other than the variable one (which we may suitably call the changing fixed substrate) in the Ordered Bi Bi mechanism (Fig. 1). A substrate combining with EA would obviously lower EA with respect to E; here, we need B to get to the central complex and prevent EA from dissociating.

Then, under what conditions would one not see a slope effect? This will happen whenever there is not a reversible connection between the points of addition in the reaction sequence of the variable and the changing fixed substrate. This is the case with Ping Pong mechanisms as long as the products are not present and the F form

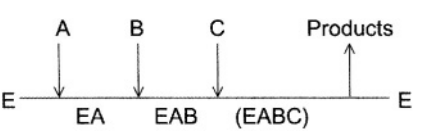
is stable (Fig. 1). In this case, one or more products are released after the addition of each substrate; in the absence of initially added products, these steps are irreversible. If A is the variable substrate, raising the B level will convert F to the second central complex and allow the reaction to be completed, but at low A, the rate at which this occurs is greater than the rate of combination of E and A, and the level of B does not matter.

Consider now the product inhibition by P in the Ping Pong Bi Bi mechanism (Fig. 1). If we add a finite amount of product P to the system initially, we see a different picture. P now continually combines with F and converts it back to EA; thus, the reaction is partly reversed. If we now raise the B level, keeping P constant, B will compete with P for F and by producing EQ from F prevent P from reversing the reaction. Here, we have a slope effect which produces an intersecting instead of a parallel pattern. The conversion of a parallel into an intersecting pattern by the addition of a fixed level of product is one diagnostic for a Ping Pong mechanism (Section 9.5). If F is unstable and can break down to alternate products, the level of B will then determine whether the final product Q is formed, and the initial velocity pattern is intersecting.

10.1.4 Slope and Intercept Rules in Trisubstrate Reactions

In bisubstrate reactions, Ping Pong mechanisms give the parallel initial velocity pattern and ordered mechanisms give the intersecting, or occasionally the equilibrium ordered pattern. When there are three substrates, however, this is no longer always true, and parallel patterns may occur with ordered mechanisms and intersecting ones with Ping Pong mechanisms.

Consider, for example, the ordered addition of three substrates:



Unless rapid equilibrium conditions apply, there will always be intercept effects here, and if *no* substrate is saturating the reaction sequence from E to EABC is reversible, so there will be slope effects.

Thus, the initial velocity pattern will be intersecting regardless of whether A and B, A and C, or B and C are the variable and changing fixed substrates. In each case, the third substrate would be held at constant concentration for the entire pattern. If substrate B is truly saturating, however, the reversible sequence is broken and the A–C initial velocity pattern becomes a parallel one. In practice, the slope effect becomes smaller and smaller as B is raised, but unless B is raised to over 100 times the Michaelis constant, a parallel pattern will not be seen. The A–B and B–C initial velocity patterns will always be intersecting, regardless of the level of the other substrate. The same pattern seen for the ordered mechanism is seen for one where A and B have to be added in that order, but C can be added randomly. Such mechanisms are known (Viola & Cleland, 1982).

While an ordered trisubstrate mechanism shows a parallel initial velocity pattern when substrate B is saturating, a completely random mechanism shows intersecting patterns at all times. If one substrate must be added first, but the other two can be added randomly (Section 12.3), parallel initial velocity pattern will be obtained when either B or C is saturating. This is easily understood if one remembers that the saturation with B leads to addition in order A, B, and C, while saturation with C causes the order to be A, C, and B, that is, saturation at the branch point diverts all reaction flux through one path or the other.

10.1.5 Alternative Substrates

Alternative substrate is any compound (other than the physiologically relevant one) that can serve as a substrate for a particular enzyme. Alternative substrates compete with the natural substrate for access to the enzyme's active site. Thus, if one is utilizing an assay that measures the production of the true substrate, then the presence of the alternative substrate will result in competitive inhibition relative to the true substrate (Purich & Allison, 2000). Alternative substrate is very useful for studying enzyme mechanisms since it presumably undergoes the same catalytic events as the normal substrate, yet its presence gives rise to alternative reaction pathways (Cleland, 1977). Wong and Hanes (1962) first pointed out that the effect of introducing alternative substrate into an enzyme system could be utilized to deduce the order of substrate addition.

The initial velocity equations for alternative substrates are derived in a usual manner; one example is given in Chapter 4 (Section 4.5).

Alternative substrates may be applied in two different ways. One approach is to determine the kinetic parameters for the normal substrate and the alternative substrate separately and compare the numerical values. Another approach is to evaluate the graphical patterns obtained in the presence of both substrate analogs. The latter approach can be carried out by measuring the rate of formation of either one single product, or the common product, or the summation of

Table 1. Invariant coefficients predicted for alternative substrates A′ and B′ (Huang, 1979)

	Alternative substrate	
	A′	B′
Ordered	None	$\frac{K_A}{V_1}$, K_{iA}
Theorell–Chance	None	$\frac{1}{V_1}$, $\frac{K_A}{V_1}$, K_{iA}
Rapid equilibrium random	$\frac{K_{iA}K_B}{K_A}$	K_{iA}
Ping Pong	$\frac{K_B}{V_1}$	$\frac{K_A}{V_1}$

two corresponding products (Huang, 1979). If the first method is applied, and by a judicious choice of an alternative substrate, one can easily differentiate several bisubstrate mechanisms by comparing the groups of kinetic constants (Table 1).

Like most kinetic tools, analysis with alternative substrates should be used in conjunction with other techniques.

10.2 PRODUCT INHIBITION

The advantages of having the full rate equation in hand, rather than just an initial velocity equation, are numerous. The entire equation describes the steady-state kinetic behavior of the system at any time and at any point, whether near or far from equilibrium. It easily yields simplified equations for situations where one or more reactants are at zero concentration, and thus saves the considerable time and effort necessary to derive separately a series of initial velocity and product inhibition equations. Since the product inhibition experiments are often the best means of distinguishing different mechanisms, this is one of the most important uses for the full equations (Fromm, 1975; Rudolph, 1979; Cooper & Rudolph, 1995).

The general rate equation for all Bi Bi mechanisms has always the same form:

$$v_0 = \frac{V_1 V_2 \left(AB - \dfrac{PQ}{K_{eq}} \right)}{\text{denominator of rate equation}}$$

Thus, the various Bi Bi mechanisms differ only by the composition of their denominators (Table 2).

It is exceedingly important to emphasize that, in rapid equilibrium systems, the relative concentration of any particular enzyme form is given by a *single denominator term* in the velocity equation. However, in steady-state systems, the relative concentration of a particular enzyme form may be expressed by several denominator terms and, often, a particular denominator term represents more than one enzyme form.

It is interesting to note that, in all Bi Bi mechanisms, the kinetic constant composition next to a given substrate concentration term is always identical in all mechanisms. Rapid Equilibrium Random Bi Bi mechanisms with dead-end complexes make an exception. Thus, if the dead-end complexes EBQ, or EAP + EBQ form, the kinetic constant composition next to the AP and BQ concentration terms, respectively, are

$$\frac{V_2 K_B}{K_{iiP}} \quad \text{and} \quad \frac{V_2 K_A}{K_{iiQ}}$$

where K_{iiP} and K_{iiQ} represent the dissociation constants of respective dead-end complexes (Table 2).

Table 2. Denominator terms in various Bi Bi mechanisms

		Rapid equilibrium mechanisms			Steady-state ordered mechanisms		
Concentration terms	Composition of denominator terms	Ordered Bi Bi	Random Bi Bi with dead-end EBQ	Random Bi Bi with dead-end EBQ and EAP	Ordered Bi Bi	Theorell–Chance	Ping Pong Bi Bi
Constant	$V_2K_{iA}K_B$	□	□	□	□	□	–
A	V_2K_B	□	□	□	□	□	□
B	V_2K_A	–	□	□	□	□	□
AB	V_2	□	□	□	□	□	□
P	$\frac{V_1K_Q}{K_{eq}}$	–	□	□	□	□	□
Q	$\frac{V_1K_P}{K_{eq}}$	□	□	□	□	□	□
AP	$\frac{V_1K_Q}{K_{iA}K_{eq}}$	–	–	©	□	□	□
PQ	$\frac{V_1}{K_{eq}}$	□	□	□	□	□	□
BQ	$\frac{V_2K_A}{K_{iQ}}$	–	®	®	□	□	□
ABP	$\frac{V_2}{K_{iP}}$	–	–	–	□	–	–
BPQ	$\frac{V_2}{K_{iB}K_{eq}}$	–	–	–	□	–	–
Equation		(8.12)	(8.25)	(8.38)	(9.8)	(9.28)	(9.55)

Different denominator terms: © $\frac{V_2K_B}{K_{iiP}}$ ® $\frac{V_2K_A}{K_{iiQ}}$.

The general rate equation for the Bi Uni mechanism has the form:

$$v_o = \frac{V_1 V_2 \left(AB - \dfrac{P}{K_{eq}} \right)}{\text{denominator of rate equation}}$$

Note, however, that the composition of denominator terms, in this respect, is *not identical in Bi Bi and Bi Uni mechanisms* (Section 9.4).

Some hyperbolic bisubstrate mechanisms can be easily distinguished by their primary double reciprocal plots in the absence of products, such as ordered from the Ping Pong mechanism. However, in many cases, the bisubstrate mechanisms cannot be distinguished in this way. Fortunately, in most cases, they can be clearly separated on the basis of their product inhibition patterns (Plowman, 1972) (Table 3).

The upper-part of Table 3 shows the rapid equilibrium mechanisms and the lower-part the steady-state mechanisms. At the end of the table is the Steady-State Random Bi Bi mechanism that is included for comparison. The steady-state random case in practice gives the same patterns as rapid equilibrium ones; one can usually tell the difference only by differential rates of isotopic exchange or the measurement of stickiness.

Initial velocity studies can place all the mechanisms listed in Table 3 into only three groups (Ping Pong, sequential, and equilibrium ordered), while all of these can be told apart by product inhibition analysis. This clearly illustrates the immense power of the product inhibition analysis.

10.2.1 Alternative Product Inhibition

Rate experiments that are typically carried out in the presence of different concentrations of an alternative product (or product analog) while using the normal substrates (Fromm, 1975; Rudolph, 1979). This approach is particularly useful when the normal product cannot be used because it is unstable, insoluble, or ineffective (Fromm & Zewe, 1962; Fromm, 1975).

10.3 ANALYSIS OF INTERSECTION POINTS

Intersection points in the double reciprocal plots of $1/v_o$ versus $1/A$ or $1/v_o$ versus $1/B$, in bisubstrate reactions, provide a criterion for an initial estimate of the reaction mechanism (Rudolph & Fromm, 1979). The choice of a mechanism listed in Table 4 could be made on the basis of the evaluation of the points of intersection in the double reciprocal plots in the forward and reverse directions.

The Ordered and Theorell–Chance mechanisms have certain constraints placed on their intersection points, but the Rapid Equilibrium Random mechanism has none, since only the equilibrium constant relates the forward and reverse reactions.

Table 3. Product inhibition patterns in bisubstrate mechanisms (Plowman, 1972; Fromm, 1975, 1979)

		Varied substrate							
		A				B			
Mechanism	Product inhibitor	Unsat. with B	Saturat. with B	Equation	Figure (Chapter)	Unsat. with A	Saturat. with A	Equation	Figure (Chapter)
Rapid Equilibrium	P	—	—			—	—		
Ordered Bi Bi (no dead-end complexes)	Q	C	—	(8.14)	4 (8)	C	—	(8.15)	5 (8)
Rapid Equilibrium	P	C	—	(8.33)		C	—	(8.34)	
Random Bi Bi (with dead-end EBQ)	Q	C	C	(8.30)	7 (8)	N	—	(8.31)	8 (8)
Rapid Equilibrium	P	N	—	(8.41)		C	C	(8.41)	
Random Bi Bi (with dead-end EAP and EBQ)	Q	C	C	(8.30)		N	—	(8.31)	8 (8)
Steady-State	P	N		(9.46)		—			
Ordered Uni Bi	Q	C		(9.47)		—			
Steady-State Ordered Bi Uni	P	C	C	(9.50)		N	—	(9.50)	
Steady-State	P	N	UC	(9.20)	3 (9)	N	N	(9.21)	4 (9)
Ordered Bi Bi	Q	C	C	(9.23)	5 (9)	N	—	(9.24)	6 (9)
Theorell—Chance	P	N	—	(9.34)		C	C	(9.35)	
	Q	C	C	(9.37)		N	—	(9.38)	
Ping Pong Bi Bi	P	N	—	(9.64)	9 (9)	C	C	(9.65)	
	Q	C	C	(9.67)		N	—	(9.68)	10 (9)
Steady-State	P	N	N			N	N		
Random Bi Bi	Q	N	N			N	N		

Types of inhibition: C = competitive; N = noncompetitive; UC = uncompetitive.

Table 4. Predicted points of convergence of double reciprocal plots for sequential bisubstrate mechanisms (Fromm, 1979, 1995)

	Intersection point of lines relative to X-axis				
			Reverse direction		
Mechanism	Forward direction		Above	On	Below
Ordered Bi Bi[a]	Above	+	+	+	+
or Iso-Ordered Bi Bi	On	+	+	N	N
	Below	+	+	N	N
Random Bi Bi	Above	+	+	+	+
	On	+	+	+	+
	Below	+	+	+	+
Theorell–Chance or	Above	+	N	N	+
Iso-Theorell–Chance	On	+	N	+	N
	Below	+	+	N	N

N indicates that intersection on that position is not possible for that particular mechanism.
[a]In an Ordered Bi Bi mechanism, it is possible for both crossover points to be on the axis, or at least appear so, if $k_2 = k_7$ and k_4, k_5 are both much greater than k_2 and k_7.

Intersection points in primary and secondary double reciprocal plots are very characteristic, with coordinates that are often simple expressions composed of kinetic constants. It is often possible to obtain the initial estimate of kinetic constants already from the inspection of intersection points in the primary plots. A simple method for the calculation of coordinates of intersection points was described in Chapter 9 (Section 9.2).

10.4 SPECIAL RELATIONSHIPS BETWEEN THE CONSTANTS

In the general rate equations for bisubstrate mechanisms, one can cast some kinetic constants and some useful groups of kinetic constants in terms of rate constants, in a very simple manner (Table 5).

Kinetic constants in Table 5 are shown for the forward reaction, $A+B \rightarrow P+Q$. An equivalent table may be drawn for the kinetic constants in the reverse direction $P + Q \rightarrow A + B$ (Engel, 1996).

Kinetic constants in Table 5 may be applied directly to calculate the thermodynamic equilibrium constant K_{eq} and compare with the same constant obtained by independent methods. Testing for consistency with Haldane relationships is theoretically a good method, but in practice involves the combination of large numbers of constants, each of which may be in error to some degree; the real use of Haldanes is to ensure that the experimental data are self-consistent.

The second possibility is to compare and evaluate the initial rate studies with two or more alternative substrates. For example, in the strictly Ordered Bi Bi mechanism, $k_1 = V_1/E_oK_A$, $k_8 = V_2/E_oK_Q$, $k_2 = V_1K_{iA}/E_oK_A$, and $k_7 = V_2K_{iQ}/E_oK_Q$

Table 5. The physical significance of kinetic coefficients for bisubstrate reactions

Mechanism	$\frac{E_0}{V_1}$	$\frac{E_0K_A}{V_1}$	$\frac{E_0K_B}{V_1}$	$\frac{E_0K_{iA}K_B}{V_1}$
Ordered Bi Bi with one central complex (Reaction (9.6))	$\frac{1}{k_5}+\frac{1}{k_7}$	$\frac{1}{k_1}$	$\frac{k_4+k_5}{k_3k_5}$	$\frac{k_2(k_4+k_5)}{k_1k_3k_5}$
Ordered Bi Bi with two central complexes (Section 9.2.2)	$\frac{1}{k_5}+\frac{1}{k_7}+\frac{\alpha}{k_9}$	$\frac{1}{k_1}$	$\frac{\alpha k_4+k_9}{k_3k_9}$	$\frac{k_2(\alpha k_4+k_9)}{k_1k_3k_9}$
Ordered Bi Bi with isomeric coenzyme complexes (Reaction (9.72))	$\frac{1}{k_5}+\frac{1}{k_7}+\frac{\alpha}{k_9}+\frac{1}{k_f}+\frac{1}{k_{rr}}+\frac{k_r}{k_7k_{rr}}$	$\frac{1}{k_1}\left(1+\frac{k_2}{k_f}\right)$	$\frac{\alpha k_4+k_9}{k_3k_9}\left(1+\frac{k_{ff}}{k_f}\right)$	$\frac{k_2k_{ff}(\alpha k_4+k_9)}{k_fk_1k_3k_9}$
Ping Pong Bi Bi (Reaction (9.53))	$\frac{1}{k_3}+\frac{1}{k_7}$	$\frac{k_2+k_3}{k_1k_3}$	$\frac{k_6+k_7}{k_5k_7}$	0

Definitions of k_f, k_{ff}, k_r, and k_{rr} constants are given in Chapter 9 (Scheme 9.72). $\alpha = 1 + k_{10}/k_5$.

(Eq. (9.10)). Because the maximum rate in both directions cannot exceed the rate of dissociation of the product Q or substrate A (usually a coenzyme), the "maximum rate relationships" exist between the forward and the reverse reactions (Dalziel, 1975),

$$V_2 \leqslant \frac{K_{iA}V_1}{K_A} \quad \text{and} \quad V_1 \leqslant \frac{K_{iQ}V_2}{K_Q} \tag{10.5}$$

From Eq. (10.5), it follows that

$$1 \leqslant \frac{K_{iA}V_1}{K_AV_2} \quad \text{and} \quad 1 \leqslant \frac{K_{iQ}V_2}{K_QV_1} \tag{10.6}$$

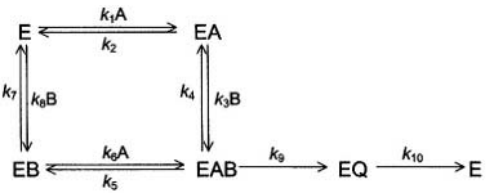
Thus, for a simple Ordered Bi Bi mechanism, the above ratios are always greater than unity. Values close to unity indicate that the dissociation of the product Q or a substrate A (usually the coenzymes with dehydrogenases) determines the maximum rate, that is, a Theorell–Chance mechanism. The values much greater than unity are inconsistent with a simple ordered mechanism, and suggest an isomerization of the enzyme–substrate complexes.

The specificity constant K_A/V_1 (or K_Q/V_2 in the reverse direction) should be independent of the nature of the second substrate B (or P) in a strictly ordered mechanism. If the specificity constants K_A/V_1 are strongly dependent on the nature of the other substrate, B, this usually signifies a significant dissociation of A from the EAB complex, that is, a partial randomization of the ordered mechanism.

The greatest value of above calculations is in discovering isomerizations of transitory complexes, for which this is really the only available method.

10.5 COMPLEX BISUBSTRATE MECHANISMS WITH A CENTRAL TERNARY COMPLEX

The order of addition of substrates in the Bi Bi mechanisms, with a central ternary complex, can be strictly ordered, completely random or partially random. We can employ reaction (10.7) in order to analyze most kinetic mechanisms that occur in bisubstrate systems:



(10.7)

In the absence of products of reaction, one can distinguish several different Bi Bi mechanisms which differ only by the magnitude of rate constants that characterize the binding of substrates (Cook & Cleland, 1981).

(1) *Rapid Equilibrium Ordered.* Rate constants k_5, k_6, k_7, and k_8 are not present; $k_2 >> V_1/E_0$.
(2) *Rapid Equilibrium Ordered with a dead-end EB complex.* Rate constants k_6 and k_5 are absent.
(3) *Steady-State Ordered.* Rate constants k_5, k_6, k_7, and k_8 are not present; k_2 less than or not much greater than V_1/E_0.
(4) *Rapid Equilibrium Random.* Rate constants k_2, k_4, k_5, and k_7 much greater than V_1/E_0. All substrates are released from the enzyme much faster than V_1/E_0.
(5) *Random.* Rate constants k_2 and k_7 are much greater than V_1/E_0; k_4 and k_5 are less than or not much greater than V_1/E_0. Both substrates are in rapid equilibrium with binary complexes, but sticky for the ternary ones.
(6) *Steady-State Random.* Rate constants k_2, k_4, k_5, and k_7 less than or not much larger than V_1/E_0.

Note that the case (5) is a subcase of (4). Cases (4)–(6) cannot be distinguished easily, because in each case the initial velocity patterns will be intersecting and look very much like the ordered mechanism. Only the equilibrium ordered mechanism will give different initial velocity patterns.

Thus, the above examples illustrate that, among the common bisubstrate mechanisms, simple correlations may be detected, in a sense that one mechanism can change into another when certain rate constants are changed.

10.5.1 Reduction of Steady-State Ordered to Rapid Equilibrium Ordered Bi Bi System

The Rapid Equilibrium Ordered Bi Bi system (Section 8.2) is a limiting case of the more realistic Steady-State Ordered Bi Bi system (Section 9.2). In bisubstrate mechanisms, the two approaches yield different velocity equations. As described

in Chapter 9, the steady-state velocity equation for an Ordered Bi Bi system contains both K_A and K_{iA} terms. When $K_{iA} >> K_A$, the system will appear to be rapid equilibrium and can be treated as such.

The Steady-State Ordered Bi Bi system reduces to a Rapid Equilibrium Ordered system when $k_2 \gg V_1/E_0$. In this case, K_A reduces to zero and the other kinetic constants reduce to dissociation constants. Let us write out again the definition of some kinetic constants in the Steady-State Ordered system (Eq. 9.9):

$$K_A = \frac{k_5 k_7}{k_1(k_5 + k_7)}; \quad K_{iA} = \frac{k_2}{k_1}; \quad \frac{V_1}{E_0} = \frac{k_5 k_7}{(k_5 + k_7)}.$$

We can see that the K_A term will drop out of the velocity equation without losing other terms when

$$K_{iA} \gg K_A \quad \text{or} \quad \frac{k_2}{k_1} \gg \frac{k_5 k_7}{k_1(k_5 + k_7)} \quad \text{or} \quad k_2 \gg \frac{k_5 k_7}{k_5 + k_7}$$

Thus, when the rate constant for the dissociation of A is greater than the maximal velocity in the forward direction ($k_2 >> V_1/E_0$), the K_A term is eliminated from the denominator of the velocity equation, but the K_{iA} term and other terms remain. Consequently, the velocity equation for the Steady-State Ordered Bi Bi system (Eq. (9.15)) reduces to the velocity equation for the Rapid Equilibrium Ordered Bi Bi system (Eq. (8.2)).

10.5.2 Reduction of Steady-State Ordered Bi Bi to Theorell–Chance

The Theorell–Chance mechanism is a special case of the Ordered Bi Bi mechanism in which the concentration of central complexes EAB and EPQ is essentially zero. The velocity equations are identical, except that the terms with ABP and BPQ are missing; thus, from the velocity equation (9.8) (Ordered Bi Bi), the terms with ABP and BPQ in the denominator are missing in Eq. (9.28) (Theorell–Chance). Thus, the Ordered Bi Bi mechanism reduces to Theorell–Chance when $K_{iP} = \infty$ and $K_{iB} = \infty$. Specifically, the Ordered Bi Bi mechanism reduces to Theorell–Chance when certain rate constants are very large compared to others, that is, when

$$k_5 \gg k_7, \; k_4 \gg k_3, \; k_4 \gg k_2, \text{and } k_5 \gg k_6.$$

10.5.3 Kinetic Importance of Central Complexes

Janson and Cleland (1974) discovered a simple way of evaluating the kinetic importance of the central complexes in Ordered Bi Bi systems, introducing the *ratio-R*.

$$R = \frac{\frac{1}{V_1}\left(1 - \frac{K_A}{K_{iA}}\right) + \frac{1}{V_2}\left(1 - \frac{K_Q}{K_{iQ}}\right)}{\frac{1}{V_1} + \frac{1}{V_2}} \tag{10.8}$$

The value of R varies from zero for a Theorell–Chance mechanism to 1.0 for a Rapid Equilibrium Ordered Bi Bi system. If V_1 and V_2 are unequal, R indicates the rate-limiting step in the slower direction only. In this case, R gives the fraction of the total enzyme present as the central complexes when both substrates for the slower direction are saturating. The ratio $R/(1-R)$ then gives the ratio of the central complexes to all other enzyme species present.

10.5.4 Partial Rapid Equilibrium Ordered Bi Bi System

In addition to common bisubstrate mechanisms, described in the preceding chapters, we can encounter more complex mechanisms that are combinations of rapid equilibrium and steady-state mechanisms. In other words, a common steady-state bisubstrate mechanism can contain rapid equilibrium segments and, vice versa, a rapid equilibrium mechanism can contain the steady-state segments. Such situations are common in bisubstrate mechanisms.

For example, in the Steady-State Ordered Bi Bi system, if the first ligand to add in either direction is in equilibrium with free enzyme and the respective complexes, but EAB and EPQ are not in equilibrium with EA and EQ, we shall obtain a Partial Rapid Equilibrium Ordered Bi Bi system.

The rate equation for this system was derived with the aid of the Cha method in Chapter 4 (Eq. (4.58)):

$$v_0 = \frac{V_1\left(\frac{AB}{K_{iA}K_B}\right) - V_2\left(\frac{PQ}{K_PK_{iQ}}\right)}{1 + \frac{A}{K_{iA}} + \frac{Q}{K_{iQ}} + \frac{AB}{K_{iA}K_B} + \frac{PQ}{K_PK_{iQ}}} \tag{10.9}$$

Note that Eq. (10.9) is identical with Eq. (4.58), if K_{eq} is eliminated from the latter equation with the aid of Haldane relationships. Equation (10.9) has the same form as that for the total Rapid Equilibrium Ordered Bi Bi system (Eq. (8.12)), except that the constants associated with B and P are the Michaelis constants, rather than dissociation constants.

What this shows is that the last substrate to add can do so either in steady-state or rapid equilibrium fashion without having an effect on the rate equation, while one that cannot add last will affect the rate equation if it adds in rapid equilibrium.

10.5.5 Random Bi Ordered Bi System

If the binding of substrates A and B is random and in a rapid equilibrium, the release of products P and Q is strictly ordered, and the concentration of products rapidly reaches the steady state, we obtain

(10.10)

where the shaded area represents the rapid equilibrium segments.

A complete velocity equation for this Random Bi Ordered Bi system is (Ganzhorn & Plapp, 1988)

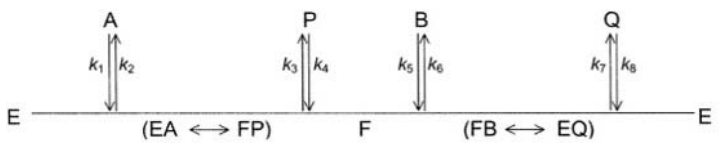
$$v_o = \frac{V_1 V_2 \left(AB - \dfrac{PQ}{K_{eq}} \right)}{V_2 K_{iA} K_B + V_2 K_B A + V_2 K_A B + \dfrac{V_1 K_Q}{K_{eq}} P + \dfrac{V_1 K_P}{K_{eq}} Q + V_2 AB + \dfrac{V_1 K_Q}{K_{iA} K_{eq}} AP + \dfrac{V_1 K_Q}{K_{iB} K_{eq}} BP + \dfrac{V_1}{K_{eq}} PQ + \dfrac{V_2}{K_{iP}} ABP} \qquad (10.11)$$

The expression is similar to that for the Steady-State Ordered Bi Bi mechanism (Eq. (9.8)), except that it has an additional term in BP, and terms in BQ and BPQ are missing.

10.6 COMPLEX PING PONG MECHANISMS

10.6.1 Partial Rapid Equilibrium Ping Pong Bi Bi System

If the substrate addition and product release steps in the Ping Pong Bi Bi mechanism are much faster than the interconversions of central complexes, then E, EA, and EQ are at equilibrium and also F, FB, and FP are at equilibrium.



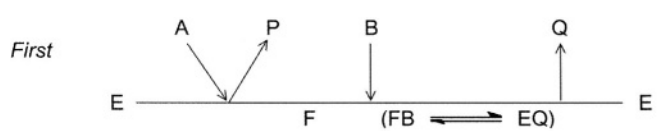
(10.12)

Thus, the pairs of rate constants, $k_1 \leftrightarrow k_2$, $k_3 \leftrightarrow k_4$, $k_5 \leftrightarrow k_6$, and $k_7 \leftrightarrow k_8$, all represent the rapid equilibrium segments in the mechanism.

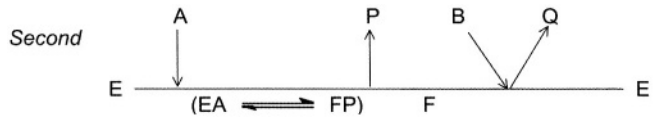
In this case, although the rapid equilibrium segments affect the composition of the kinetic constants in terms of rate constants, the final form of the velocity equation is unchanged. Thus, the reciprocal plots and product inhibition patterns are unchanged. This mechanism will occasionally appear in practice.

10.6.2 Hybrid Theorell–Chance Ping Pong Systems

There are two possible bisubstrate systems that combine the enzyme feature of the Ping Pong sequence with the hit-and-run feature of the Theorell–Chance mechanism. These are in fact the limiting cases of the common Ping Pong Bi Bi system, in which one of two central complexes has extremely short life. The reaction sequences are shown below:



(10.13)



(10.14)

In the absence of products, all initial velocity patterns are parallel, in the same way as in the Ping Pong Bi Bi mechanism. However, the product inhibition patterns are different, and may serve to distinguish between the different systems (Table 6).

Table 6. Product inhibition in hybrid Theorell–Chance Ping Pong systems

		A Varied		B Varied	
Case	Product inhibitor	Unsaturated with B	Saturated with B	Unsaturated with A	Saturated with A
First	P	Competitive	—	Competitive	—
	Q	Competitive	Competitive	Noncompetitive	—
Second	P	Noncompetitive	—	Competitive	Competitive
	Q	Competitive	—	Competitive	—

10.7 EXAMPLES OF ENZYME BISUBSTRATE MECHANISMS

Mechanism	Enzyme		References
Ordered Uni Bi	Adenylosuccinate lyase	(EC 4.3.2.2)	Bridger & Cohen, 1968
	Malylcoenzyme A lyase	(EC 4.1.3.24)	Hersh, 1974; Arps, 1990
Ordered Bi Bi	Lactate dehydrogenase	(EC 1.1.1.27)	Hakala *et al.*, 1956; Holbrook *et al.*, 1975; Oppenheimer & Handlon,1992
	Ribitol dehydrogenase	(EC 1.1.1.137)	Nordlie & Fromm, 1959
Ordered Bi Bi (Rapid equil.)	Creatine kinase	(EC 2.7.3.2)	Schimmerlik & Cleland, 1973
Random on-Ordered of[a]	Yeast alcohol dehydrogenase	(EC 1.1.1.1)	Leskovac *et al.*, 1999; Leskovac *et al.*, 2002
Partial Rapid Equil. Ordered	Galactotransferase	(EC 2.3.1.18)	Morrison & Ebner, 1971
Random Bi Bi	Adenylate kinase	(EC 2.7.4.3)	Rhoads & Lowenstein, 1968; Villafranca & Novak, 1992

(*continued*)

Mechanism	Enzyme		References
	Citrate synthase	(EC 4.1.3.7)	Matsuoka & Srere, 1973; Colman, 1990
	Hexokinase	(EC 2.7.1.1)	Ning *et al.*, 1969
	Phosphofructokinase	(EC 2.7.1.11)	Hanson *et al.*, 1973; Pilkis *et al.*, 1987
	Dihydrofolate reductase	(EC 1.5.1.3)	Penner & Frieden, 1987; Johnson & Benkovic, 1990
Ping Pong Bi Bi	Adenine phosphoribosyl transferase	(EC 2.4.2.7)	Hori & Henderson, 1966
	Glutamic-alanine transaminase	(EC 2.6.1.12)	Bulos & Handler, 1965; Cooper, 1985
	Glucose oxidase	(EC 1.1.3.4)	Gibson *et al.*, 1964; Bright & Porter, 1975
	Nucleosidediphosphate kinase	(EC 2.7.4.6)	Mourad & Parks, 1966; Garces & Cleland, 1969; Ray & Mathews, 1992
	Transcarboxylase	(EC 2.1.3.1)	Northrop, 1969; Knowles, 1989

(Adopted from: Fromm, 1979)
[a]Random with strongly preferred order of addition.

REFERENCES

Books

Dixon, M. & Webb, E.C. (1979) *Enzymes,* 3rd. ed., Academic Press, New York.
Engel, P.C. (1996) *Enzymology Labfax,* BIOS Scientific Publishers, Oxford.
Fromm, H.J. (1975) *Initial rate enzyme kinetics,* Springer-Verlag, Berlin.
Plowman, K.M. (1972) *Enzyme kinetics,* McGraw-Hill, New York.
Purich, D.A. & Allison, D.R. (2000) *Handbook of biochemical kinetics,* Academic Press, San Diego.

Review articles

Arps, P.J. (1990) *Methods Enzymol. 188,* 366–390.
Bright, H.J. & Porter, D.J.T. (1975) *The Enzymes,* 3rd ed. Vol. 12, 421–440.
Cleland, W.W. (1970) *The Enzymes,* 3rd ed., Vol. II, 1–76.
Cleland, W.W. (1977) *Adv. Enzymol. Rel. Areas Mol. Biol. 45,* 273–387.
Colman, R.F. (1990) *The Enzymes* 3rd ed., Vol. 19, 283–290.
Cooper, A.J.L. (1985) *Methods Enzymol. 113,* 69–80.
Cooper, B.F. & Rudolph, F.B. (1995) *Methods Enzymol. 249,* 188–211.
Dalziel, K. (1975) *The Enzymes,* 3rd ed., Vol. XI, 1–60.
Fromm, H.J. (1979) *Methods Enzymol. 63,* 42–53.
Fromm, H.J. (1995) *Methods Enzymol. 249,*123–143.
Holbrook, J.J., Liljas, A., Steindel, S.J., & Rossmann, M.G. (1975) *The Enzymes,* 3rd ed., Vol. XI, 191–293.
Huang, C.Y, (1979) *Methods Enzymol.* 63, 486–500.
Johnson, K.A. & Benkovic, S.J. (1990) *The Enzymes,* 3rd ed., Vol. 19, 159–190.
Knowles, J.R. (1989) Ann. *Rev. Biochem. 58,* 195–221.

Leskovac, V., Trivić, S., & Peričin (2002) *FEMS Yeast Research 2,* 481–496.
Oppenheimer, N.J. & Handlon, A.L. (1992) *The Enzymes,* 3rd ed., Vol. 20, 453–470.
Pilkis, S.J., Claus, T.H., Kountz, P.D., & El-Maghrabi, M.R. (1987) *The Enzymes,* 3rd ed., Vol. 18, 3–20.
Ray, N.B. & Mathews, C.K. (1992) *Curr. Top. Cell. Reg. 33,* 343–357.
Rudolph, F.B. & Fromm, H.J. (1979) *Methods Enzymol. 63,* 138–159.
Rudolph, F.B. (1979) *Methods Enzymol. 63,* 411–436.
Villafranca, J.J. & Novak, T. (1992) *The Enzymes,* 3rd ed., Vol. 20, 63–90.
Viola, A.A. & Cleland, W.W. (1982) *Methods Enzymol,* 87, 353–366.

Specific references

Bridger, W.A. & Cohen, L.H. (1968) *J. Biol. Chem. 243,* 644–650.
Bulos, L.B. & Handler, P. (1965) *J. Biol. Chem. 240,* 3283–3290.
Cleland, W.W. (1963) *Biochim. Biophys. Acta* 67, 104–137.
Cleland, W.W. (1963) *Biochim. Biophys. Acta* 67, 173–187.
Cleland, W.W. (1963) *Biochim. Biophys. Acta* 67, 188–196.
Cook, P.F. & Cleland, W.W. (1981) *Biochemistry 20,* 1797–1816.
Fromm, H.J. & Zewe, V. (1962) *J. Biol. Chem. 237,* 3027–3037.
Ganzhorn, A.J. & Plapp, B.V. (1988) *J. Biol. Chem. 263,* 5446–5454.
Garces, E. & Cleland, W.W. (1969) *Biochemistry 8,* 633–640.
Gibson, Q.H., Swoboda, B.E.P., & Massey, V. (1964) *J. Biol. Chem. 239,* 3927–3940.
Hakala, M.T., Glaid, A.J., & Schwert, G.W. (1956) *J. Biol. Chem. 221,* 191–200.
Hanson, R.L., Rudolph, F.B., & Lardy, H.A. (1973) *J. Biol. Chem. 248,* 7852–7859.
Hersh, L.B. (1974) *J. Biol. Chem. 249,* 5208–5212.
Hori, M. & Henderson, J.F. (1966) *J. Biol. Chem. 241,* 3404–3408.
Janson, C.A. & Cleland, W.W. (1974) *J. Biol. Chem. 249,* 2562–2566.
Leskovac, V., Trivić, S., & Anderson, B.M. (1999) *Europ. J. Biochem. 264,* 840–847.
Matsuoka, Y. & Srere, P.A. (1973) *J. Biol. Chem. 248,* 8022–8030.
Morrison, J.F. & Ebner, K.E. (1971) *J. Biol. Chem. 246,* 3977–3984.
Mourad, N. & Parks, R.E. (1966) *J. Biol. Chem. 241,* 271–278.
Ning, J., Purich, D.L., & Fromm, H.J. (1969) *J. Biol. Chem. 244,* 3840–3846.
Nordlie, R.C. & Fromm, H.J. (1959) *J. Biol. Chem. 243,* 2523–2530.
Northrop, D.B. (1969) *J. Biol. Chem. 244,* 5808–5819.
Penner, M.H. & Freiden, C. (1987) *J. Biol. Chem. 262,* 15908–15914.
Rhoads, D.G. & Lowenstein, J.M. (1968) *J. Biol. Chem. 243,* 3963–3972.
Schimmerlik, M.I. & Cleland, W.W. (1973) *J. Biol. Chem. 248,* 8418–8423.
Wong, C.S. & Hanes, J.T. (1962) *Can. J. Biochem. Physiol. 40,* 763–773.

This page intentionally left blank

Chapter 11

Substrate Inhibition and Mixed Dead-End and Product Inhibition

11.1 RATIONAL POLYNOMIALS

The rate equations for all completely reversible, nonequilibrium enzyme kinetic mechanisms, described in the preceding chapters, can be summarized by the following general expression:

$$v_0 = \frac{V_1 V_2 \left(ABC \cdots - \frac{PQR}{K_{eq}} \cdots \right)}{\text{denominator of rate equation}} \tag{11.1}$$

The general property of all above mechanisms is their adherence to the Michaelis–Menten kinetics. In the absence of products, the double reciprocal plots for all bisubstrate mechanisms represent a family of straight lines with a common intersection point, if one substrate is varied while the other substrate is held at different fixed concentrations. Similarly, the double reciprocal plots for all trisubstrate mechanisms represent a family of straight lines with a common intersection point, if one substrate is varied while the second substrate is held at different fixed concentrations, and the third substrate is held at a fixed concentration. This, however, is true only if each substrate adds just once; if one of them adds twice in sequential fashion, the reciprocal plots will be parabolic.

The rate equations for all these types of reactions fall into a class of mathematical expressions called *rational polynomials* of the order 1:1,

$$v_0 = \frac{\alpha_1 A}{\beta_0 + \beta_1 A} \tag{11.2}$$

In addition to rate equations that are described by a 1:1 rational polynomial, there are rate equations in enzyme kinetics which may be described by higher polynomials, such as a 1:2 rational polynomial:

$$v_0 = \frac{\alpha_1 A}{\beta_0 + \beta_1 A + \beta_2 (A)^2} \tag{11.3}$$

or, a 2:2 rational polynomial,

$$v_0 = \frac{\alpha_1 A + \alpha_2 (A)^2}{\beta_0 + \beta_1 A + \beta_2 (A)^2} \tag{11.4}$$

The rate equations may also be of a higher order (Schulz, 1994).

11.2 SUBSTRATE INHIBITION

At higher concentrations, the substrates will often act as dead-end inhibitors, particularly when a reaction is being studied in the nonphysiological direction; substrate inhibition does not normally occur at physiological substrate concentrations. To the kineticist, however, substrate inhibitions are one of the best diagnostic tools for studying mechanisms, and their importance cannot be overemphasized (Cleland, 1970, 1977, 1979; Fromm 1975).

11.2.1 Substrate Inhibition in Monosubstrate Reactions

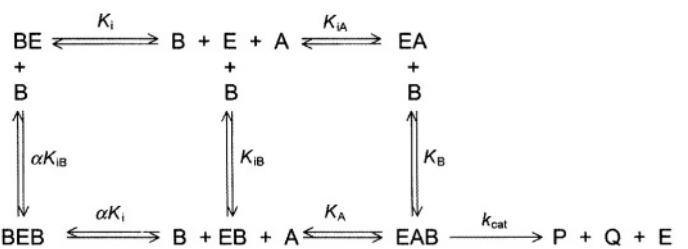
Monosubstrate enzyme reactions are rare in nature and, therefore, a substrate inhibition in monosubstrate reactions must be considered an extremely rare occurrence in the nature, although a Uni Bi mechanism does occur.

11.2.2 Substrate Inhibition in Bisubstrate Reactions

We shall turn now to much more realistic cases of bisubstrate reactions. The proper way to study a substrate inhibition in bisubstrate reactions is to vary a noninhibitory substrate, at differing high levels of the inhibitory one and see whether the slopes, intercepts, or both of reciprocal plots show the inhibitory effects (Cleland, 1979). These cases are then called competitive, uncompetitive, and noncompetitive substrate inhibition, respectively.

11.2.3 Substrate Inhibition in a Rapid Equilibrium Random Bisubstrate System

In rapid equilibrium random systems, one substrate may have an appreciable affinity for the other substrate's binding site, particularly when the two substrates are chemically similar. In such cases, a substrate inhibition by one of the substrates may take place. Let us examine the case when the binding of substrate B to the A site does not prevent B from binding to its own site, so that two dead-end complexes form, BE and BEB.



(11.5)

The velocity equation, in the absence of products, is

$$v_0 = \frac{V_1 AB}{K_{iA}K_B\left(1+\frac{B}{K_i}\right) + K_B A + K_A B\left(1+\frac{B}{\alpha K_i}\right) + AB} \tag{11.6}$$

where $V_1 = k_{cat}E_o$. One can see immediately that Eq. (11.6) has a form of a rational polynomial of the order 1:2 in B and of the order 1:1 in A. Equation (11.6) is not of the form of the Michaelis–Menten equation, by virtue of the term in B^2 and, therefore, this case might be treated as a nonlinear inhibition.

A direct plot of v_o versus [B] is not hyperbolic anymore, but has an unusual shape, characteristic for substrate inhibition (Fig. 1).

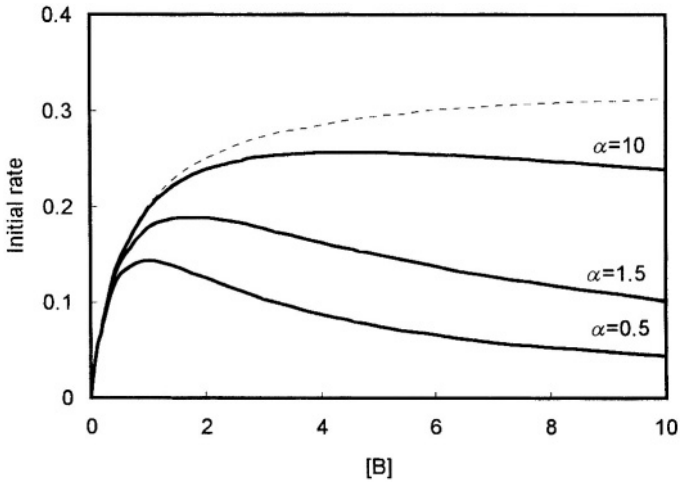


Figure 1. Substrate inhibition in a Rapid Equilibrium Random bisubstrate reaction. Rate equation (11.6) was drawn assuming that $A = V_1 = K_A = K_{iA} = K_B = K_i = 1$. Dotted line represents the uninhibited reaction ($\alpha = \infty$).

The reciprocal forms of the rate equation are

$$\frac{1}{v_o} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \frac{K_A}{V_1}\left(1 + \frac{K_{iA}K_B}{K_A B} + \frac{K_{iA}K_B}{K_A K_i} + \frac{B}{\alpha K_i}\right)\frac{1}{A} \qquad (11.7)$$

$$\frac{1}{v_o} = \frac{1}{V_1}\left(1 + \frac{K_A}{A} + \frac{K_{iA}K_B}{K_i A} + \frac{K_A B}{\alpha K_i A}\right) + \frac{K_B}{V_1}\left(1 + \frac{K_{iA}}{A}\right)\frac{1}{B} \qquad (11.8)$$

The primary graph of $1/v_o$ versus $1/B$, with A as a fixed substrate (Eq. (11.8)), *does not represent a family of straight lines* and, consequently, has no common intersection point (Fig. 2). The reciprocal plots approach straight lines at high values of $1/B$ (or low values of B). As the lines approach the vertical axis, they pass through a minimum and then bend upward. In this case, the primary graph has an unusual appearance which is very useful for the diagnostics of substrate inhibition. Since the primary reciprocal plot is not a family of straight lines and has no common intersection point, it is not possible to define definite values of either slopes or intercepts on the ordinate, for this case.

It is immediately clear from Figs, 1 and 2 that the substrate B acts as a strong inhibitor only at very high concentrations and when the ratio K_i/K_A is low.

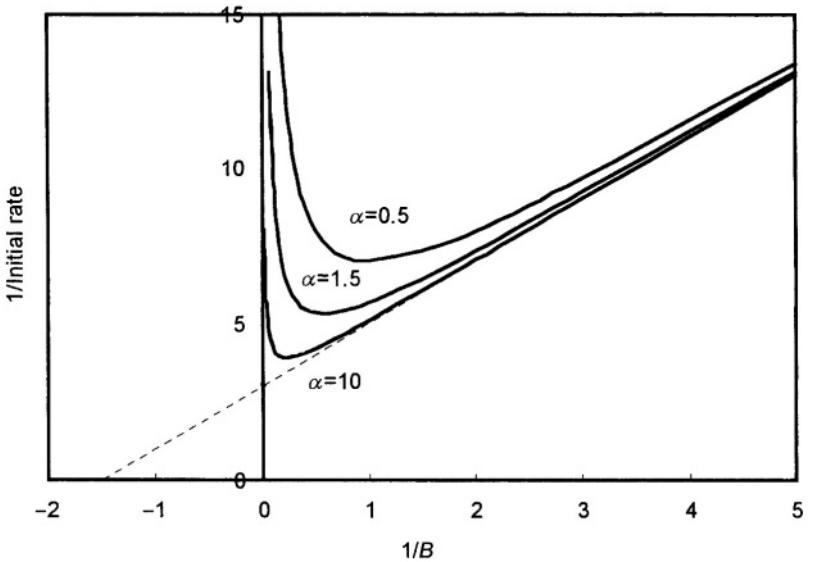


Figure 2. Substrate inhibition in a Rapid Equilibrium Random bisubstrate reaction. Rate equation (11.8) was drawn assuming that $A = V_1 = K_A = K_{iA} = K_B = K_i = 1$. Dotted line represents the uninhibited reaction ($\alpha = \infty$).

The proper way to study a substrate inhibition in bisubstrate reactions is to vary a noninhibitory substrate, at differing levels of the inhibitory one and see whether the slopes, intercepts, or both of reciprocal plots show the inhibitory effects (Rudolph, 1979; Cooper & Rudolph, 1995). Thus, the primary graph of $1/v_o$ versus $1/A$, with B as a fixed substrate (Eq. (11.7)), represents a family of straight lines which, in this case, has *no common intersection point.* Therefore, the primary graph has a quite unusual appearance; this characteristic appearance is again very useful in the diagnostics of substrate inhibition by substrate B (Fig. 3).

The secondary graph, the variation of $\text{Intercept}_{1/A}$ versus $1/B$ is, as usually, linear. However, the secondary plot of $\text{Slope}_{1/A}$ versus $1/B$ is a complex nonlinear function:

$$\text{Slope}_{1/A} = \frac{K_A}{V_1}\left(1 + \frac{B}{\alpha K_i}\right) + \frac{K_{iA}K_B}{V_1}\left(\frac{1}{K_i} + \frac{1}{B}\right) \qquad (11.9)$$

A plot of Eq. (11.9), $\text{Slope}_{1/A}$ versus $1/B$, will have the same appearance as the function shown in Fig. 2, when α increases.

At very low concentrations of B, Eq. (11.9) reduces to

$$\text{Slope}_{1/A} = \left(\frac{K_A}{V_1} + \frac{K_{iA}K_B}{V_1 K_i}\right) + \left(\frac{K_{iA}K_B}{V_1}\right)\frac{1}{B} \qquad (11.10)$$

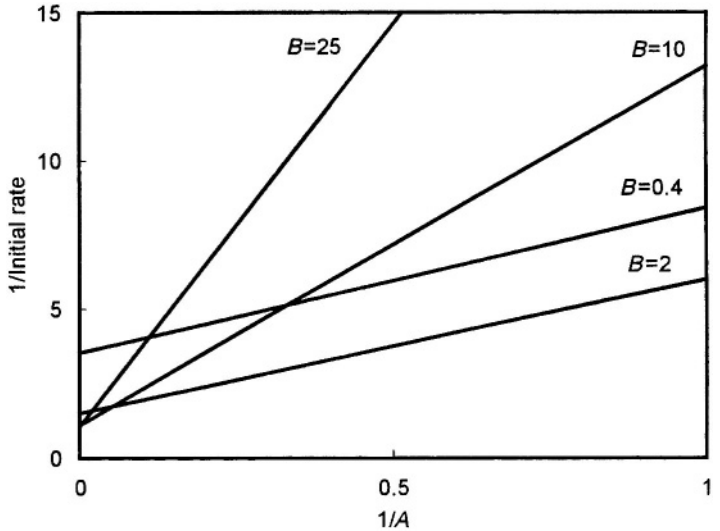
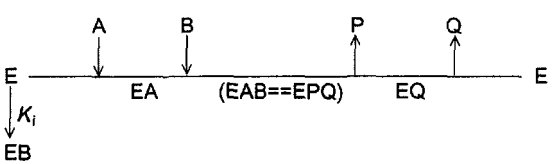


Figure 3. Substrate inhibition in a Rapid Equilibrium Random bisubstrate reaction. Rate equation (11.7) was drawn assuming that $V_1 = K_A = K_{iA} = K_B = K_i = \alpha = 1$.

It is obvious that, from Fig. 2, K_i cannot be calculated with precision. An approximate estimation of K_i can be obtained only with very low concentrations of B, in a situation when the secondary graph of $\mathbf{Slope}_{1/A}$ versus $1/B$ represents an almost linear function. However, as usual, the best method for obtaining the kinetic parameters is to fit the rate equation by nonlinear least square methods (Ratkowsky, 1983; Johnson & Faunt, 1992; Johnson, 1994; Watts, 1994).

11.2.4 Steady-State Ordered Bi Bi Mechanism with a Dead-End EB Complex

When the two substrates are structurally similar, B may react with the free enzyme to yield a dead-end EB complex:



(11.11)

The rate equation is obtained in the following manner. In the rate equation in the absence of products (Eq. (9.15)), those terms in the denominator representing free enzyme are multiplied by $(1+B/K_i)$, where K_i is the dissociation constant of the EB complex. The terms which represent the free enzyme are found in distribution equations for this system; in the absence of products, these terms are $K_{iA}K_B$ and K_AB (found in Eq. (9.13)). Thus, the velocity equation becomes

$$v_0 = \frac{V_1 AB}{K_{iA}K_B\left(1+\frac{B}{K_i}\right) + K_A B\left(1+\frac{B}{K_i}\right) + K_B A + AB} \tag{11.12}$$

One can see again that Eq. (11.12) has a form of a rational polynomial of the order 1:2 in B and of the order 1:1 in A. This equation is again not of the form of the Michaelis–Menten equation, by virtue of the term in B^2. The reciprocal forms of Eq. (11.12) are

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1+\frac{K_B}{B}\right) + \frac{K_A}{V_1}\left(1+\frac{K_{iA}K_B}{K_A B}\right)\left(1+\frac{B}{K_i}\right)\frac{1}{A} \tag{11.13}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1+\frac{K_A}{A}+\frac{K_{iA}K_B}{K_i A}+\frac{K_A B}{K_i A}\right) + \frac{K_B}{V_1}\left(1+\frac{K_{iA}}{A}\right)\frac{1}{B} \tag{11.14}$$

Equation (11.13) has a quite unusual form with respect to the slope function, that is, B is found both in the denominator and the numerator of the slope function. Nevertheless, the $1/v_o$ versus $1/A$ plots are still linear at all fixed B concentrations. However, this plot has an unusual look, because the lines apparently intersect on ordinate at high B; at low B, an apparent intersection point appears above, on, or below abscissa (Fig. 4).

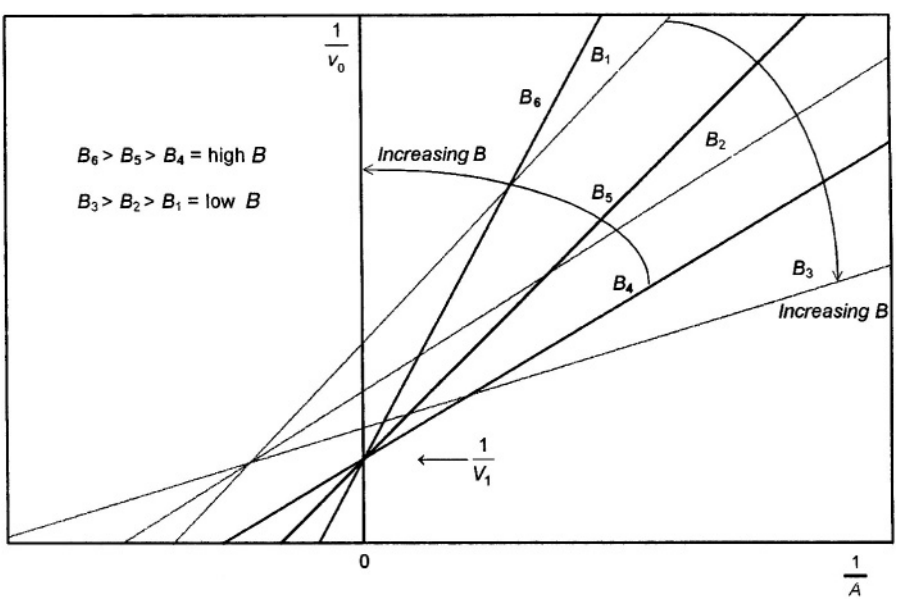


Figure 4. Competitive substrate inhibition in an Ordered Bi Bi system. B combines with E as well as with EA. Double reciprocal plots of Eq. (11.13) are shown at high and low fixed B concentrations.

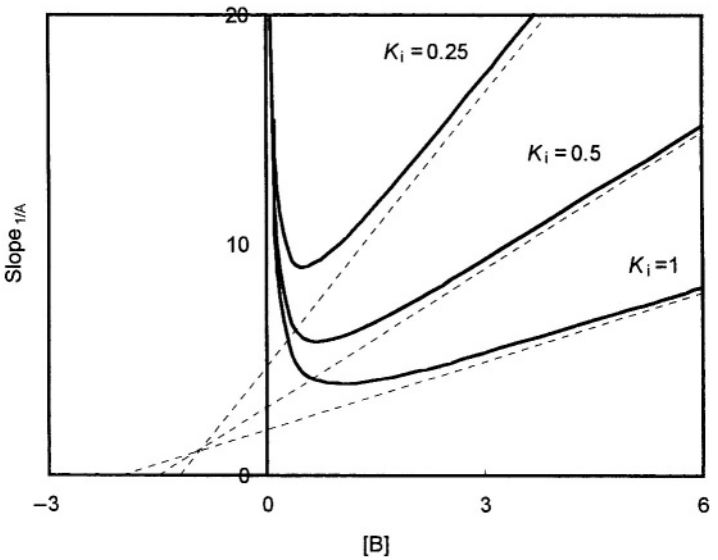


Figure 5. Replots of the $\text{Slope}_{1/A}$ function (from Fig. 4) versus [B]. The data points are calculated from the slope function (Eq. (11.15)), assuming that $V_1 = K_A = K_B = K_{iA} = 1$, and that K_i decreases. The dotted lines represent the asymptotes.

A replot of $\text{Intercept}_{1/A}$ from Fig. 4 versus $1/B$ gives a normal linear function. However, a replot of $\text{Slope}_{1/A}$ versus B,

$$\text{Slope}_{1/A} = \left(\frac{K_A}{V_1} + \frac{K_{iA}K_B}{V_1K_i}\right) + \left(\frac{K_A}{V_1K_i}\right)B + \left(\frac{K_{iA}K_B}{V_1}\right)\frac{1}{B} \qquad (11.15)$$

gives an unusual function, typical for substrate inhibition (Fig. 5).

Asymptotes in Fig. 5 are obtained by extrapolating the linear portions of the slope function at high B back to abscissa; the intersection points on abscissa are equal to $-(K_i + K_{iA}K_B/K_A)$. Thus, the values of intersection points depend on the relative ratio of K_i to the value of K_{iA}/K_A. However, extrapolating the linear portion of the curve is a bad way to get the asymptote and usually gives a line that is too close to the curve. The best way to calculate K_i is to fit Eq. (11.13) to experimental data by a suitable computer program.

The slope function in Eq. (11.15) has different forms at high compared to low concentrations of B, which explains the unusual shape of the slope function in Fig. 5.

At high B, the slope function approaches the value

$$\text{Slope}_{1/A} \approx \left(\frac{K_A}{V_1} + \frac{K_{iA}K_B}{V_1K_i}\right) + \left(\frac{K_A}{V_1K_i}\right)B \qquad (11.16)$$

and becomes linearly related to B.

At low concentrations of B, the slope function approaches the value

$$\text{Slope}_{1/A} \approx \left(\frac{K_A}{V_1} + \frac{K_{iA}K_B}{V_1K_i}\right) + \left(\frac{K_{iA}K_B}{V_1}\right)\frac{1}{B} \qquad (11.17)$$

and becomes inversely related to the concentration of B.

An entirely different situation takes place if B is the variable and A is a fixed substrate (Eq. (11.14)). In this case, the double reciprocal plots are not linear any more. As the fixed A increases, the minimum moves to higher B (lower $1/B$), until at saturating concentrations of A, there is no minimum and the plot is linear (Fig. 6). The asymptotes in Fig. 6 will have a common intersection point with coordinates: $1/V_1(1-K_A/K_{iA})$ on ordinate, and $-K_A/K_BK_{iA}$ on abscissa; therefore, if $K_A=K_{iA}$, the intersection point will be on abscissa, as in Fig. 6.

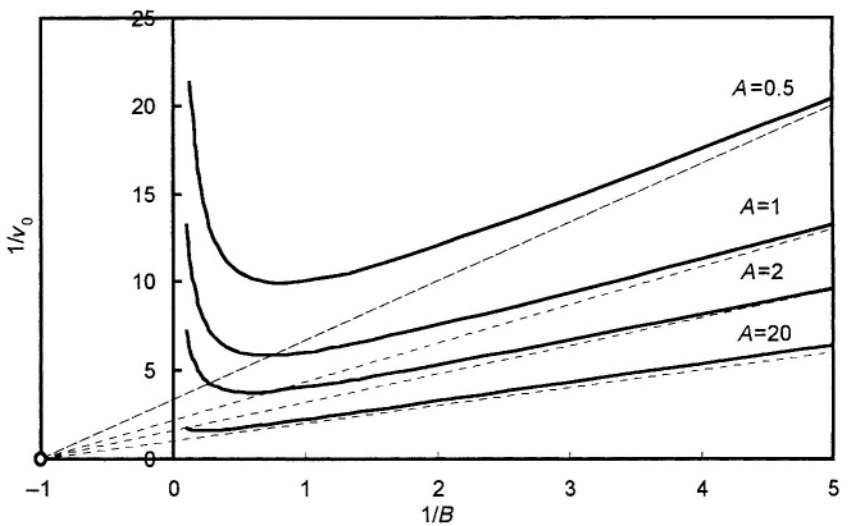
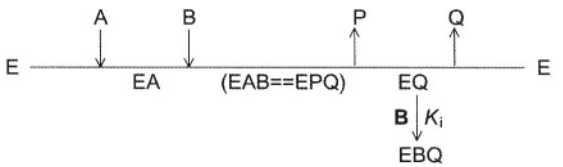


Figure 6. Substrate inhibition in an Ordered Bi Bi system. B combines with E, as well as with EA. Equation (11.14) was drawn assuming that $V_1 = K_A = K_B = K_{iA} = K_i = 1$. The dotted lines represent the asymptotes.

11.2.5 Steady-State Ordered Bi Bi Mechanism with a Dead-End EBQ Complex

A very frequent case in a Steady-State Ordered Bi Bi mechanism is the formation of a dead-end EBQ complex; this occurs when the second substrate to add can combine in dead-end fashion with the EQ as well as the EA complex, and EBQ complex can break down to give only EQ and B.



(11.18)

In a Steady-State Ordered Bi Bi system, there are no complete denominator terms that represent the EQ complex. The *AB* term represents both EQ and the central complexes (Eq. (9.13)). Consequently, a rate equation cannot be written for this case, and the dissociation constant for the EBQ complex cannot be determined. Nevertheless, uncompetitive substrate inhibition by B (dead-end complex EBQ) can be easily distinguished from the competitive substrate inhibition by B (dead-end complex EB). This is achieved simply by inspecting the reciprocal plots $1/v_0$ versus $1/A$, over a wide range of fixed B concentrations (Fig. 7).

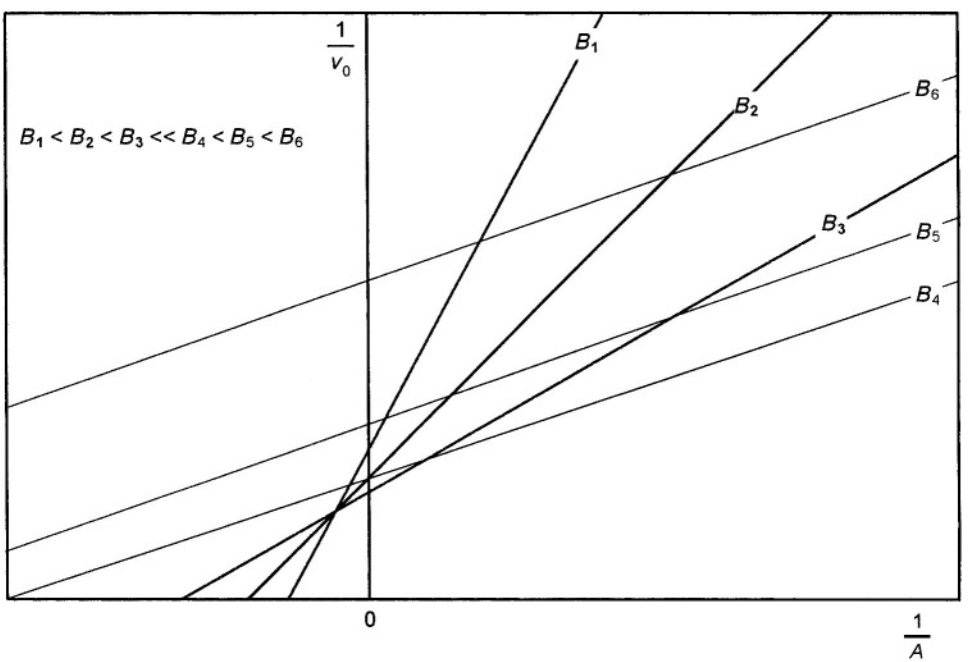
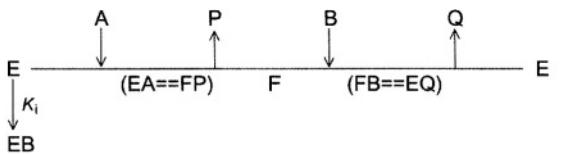


Figure 7. Uncompetitive substrate inhibition in an Ordered Bi Bi system. B combines with EQ in order to form a dead-end complex EBQ.

As B is increased, the $\text{Slope}_{1/A}$ decreases normally to a limit of K_A/V_1. However, the $\text{Intercept}_{1/A}$ decreases to a minimum and then increases again so that at high B the plots become parallel. This situation is very different from the situation with a dead-end EB complex (compare Fig. 4).

11.2.6 Substrate Inhibition in a Pine Pone Bi Bi Mechanism

Competitive substrate inhibition resulting from the combination of B with E (as well as B with F) is characteristic of a Ping Pong Bi Bi mechanism.



(11.19)

In this case, the $K_A B$ term in the denominator of the velocity equation in the absence of products represents E (compare Eq. (9.58)). Consequently, this term is multiplied by $(1+B/K_i)$:

$$v_o = \frac{V_1 AB}{K_B A + K_A B\left(1 + \frac{B}{K_i}\right) + AB} \tag{11.20}$$

The reciprocal forms of Eq. (11.20) are

$$\frac{1}{v_o} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \frac{K_A}{V_1}\left(1 + \frac{B}{K_i}\right)\frac{1}{A} \tag{11.21}$$

$$\frac{1}{v_o} = \frac{1}{V_1}\left[1 + \frac{K_A}{A}\left(1 + \frac{B}{K_i}\right)\right] + \left(\frac{K_B}{V_1}\right)\frac{1}{B} \tag{11.22}$$

A double reciprocal plot $1/v_o$ versus $1/A$, at various fixed concentrations of B, may appear similar to the pattern in Fig. 7, but this is a competitive substrate inhibition. However, in this case, at low fixed B concentrations, the plots may appear parallel, as long as $K_i > K_B$. As the fixed B increases, the $1/v_o$-axis intercept decreases, as usual, to the limit of $1/V_1$. The $\text{Slope}_{1/A}$ may appear constant for several low B concentrations, but eventually, as the fixed B increases, the slope also increases. In the secondary graphs, $\text{Intercept}_{1/A}$ is linearly related to $1/B$ and $\text{Slope}_{1/A}$ is linearly related to B.

When B is varied, with A as a fixed substrate (Eq. (11.22)), the primary plots appear parallel at high $1/B$ but pass through a minimum and bend up as they approach the $1/v_o$-axis.

11.2.7 Analysis of Substrate Inhibition Plots

Substrate inhibitions are usually analyzed graphically by making reciprocal plots of $1/v_o$ versus 1/[noninhibitory substrate], as is shown in Figs. 3, 4, and 7 (Cleland, 1970, 1977; Fromm, 1979). However, it is also useful to analyze data where the inhibitory substrate is the variable one. This is the case with patterns shown in Figs. 2 and 6. All linear substrate inhibition resulting from dead-end combinations of the substrate with some improper enzyme form will give the following equation, where V_1/K_A and K_i are the apparent constants:

$$v_0 = \frac{V_1 A}{K_A + A + \dfrac{A^2}{K_i}} \tag{11.23}$$

Note that Eq. (11.23) is equivalent to Eqs. (11.6), (11.12), and (11.20). If $1/v_0$ in Eq. (11.23) is plotted versus $1/A$, the slopes and intercepts of the asymptote determine the apparent K_A/V_1 and $1/V_1$. The minimum point in the reciprocal graph (such as in Fig. 2) has the coordinates

$$\frac{1}{A} = \frac{1}{\sqrt{K_A K_i}} \quad \text{and} \quad \frac{1}{v_0} = \frac{1}{V_1}\left(1 + 2\sqrt{\frac{K_A}{K_i}}\right) \tag{11.24}$$

It is instructive to compare Eq. (11.23) with Eqs. (11.6) (Rapid Equilibrium with dead-end BE and BEB), (11.12) (Ordered with dead-end EB), and (11.20) (Ping Pong with dead-end EB complex), and envisage the coordinates of the minimum point in corresponding double reciprocal plots.

In addition to double reciprocal plots, it is very instructive to show the substrate inhibition data by plotting v_0 versus log [A], thus producing bell-shaped curves with an apparent maximum value of v_0 at A_{max}. Figure 8 shows the plot of Eq. (11.23) in the logarithmic form.

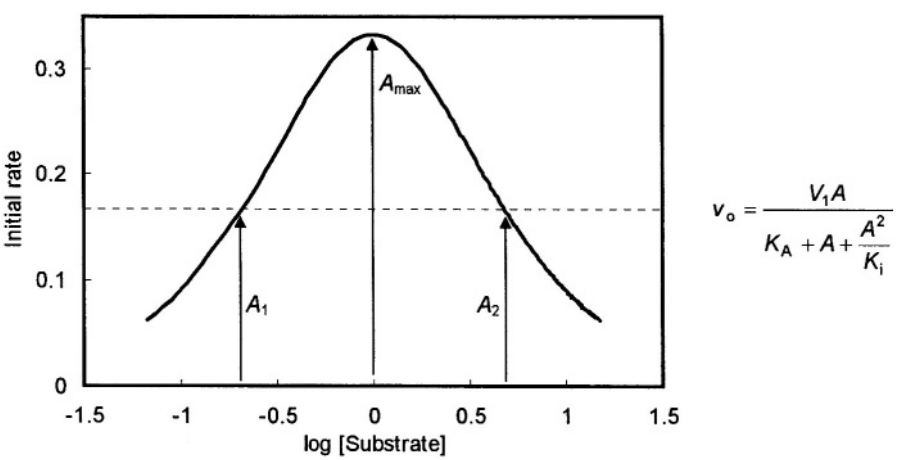


Figure 8. Substrate inhibition. Plot of v_0 versus log [A], drawn according to Eq. (11.23), assuming that $V_1 = K_A = K_i = 1$.

From such curves, the constants can be obtained as follows:

$$K_i = A_1 + A_2 - 4A_{max} \tag{11.25}$$

$$K_A = \frac{A_{max}^2}{K_i} \tag{11.26}$$

$$V_1 = A_{max}\left(1 + 2\sqrt{\frac{K_A}{K_i}}\right) \tag{11.27}$$

where A_1 and A_2 are values of A at the two points where the velocity is half of that at the maximum.

A useful graphical method for the estimation of kinetic parameters in substrate inhibition was described by Marmasse (1963). However, with substrate inhibition plots, even after a successful graphical analysis is completed, one should always fit the data to the appropriate equation with a computer program in order to estimate the kinetic constants.

11.3 NON-PRODUCTIVE BINDING

A frequent case in enzyme kinetics is the ability of enzymes to form numerous non-productive complexes with substrates, that do not break down (Fersht, 1999). If an enzyme molecule binds a single substrate molecule in a productive complex EA, but also another substrate molecule in a non-productive complex AE, we are referring to *non-productive binding* of this substrate. A kinetic model is analogous to that for a linear competitive inhibition (Section 5.2).

$$AE \overset{K_i}{\rightleftharpoons} A + E + A \overset{K_A}{\rightleftharpoons} EA \xrightarrow{k_{cat}} P + E \tag{11.28}$$

If $V_1 = k_{cat}E_o$, the rate equation is

$$v_o = \frac{V_1 A}{K_A\left(1 + \frac{A}{K_i}\right) + A} \tag{11.29}$$

or, in the reciprocal form

$$\frac{1}{v_o} = \frac{1}{V_1}\left(1 + \frac{K_A}{K_i}\right) + \left(\frac{K_A}{V_1}\right)\frac{1}{A} \tag{11.30}$$

Thus, the Michaelis–Menten equation is obeyed exactly for this mechanism and so the observed double reciprocal plot does not indicate whether the non-productive binding is significant or not. Since, in this case, the true V_{max} is decreased by a factor $(1+K_A/K_i)$, and V_1/K_A is not affected, it is always advisable to be aware of it when interpreting results for several substrates of an unspecific enzyme.

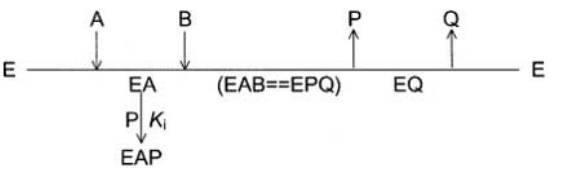
11.4 MIXED DEAD-END AND PRODUCT INHIBITION

Products usually bear some resemblance to the substrates. Consequently, we shall often find that products combine with some improper enzyme form to yield a dead-end complex (Plowman, 1972; Fromm, 1975). Formation of such complexes is a very frequent case, and it does not require nonphysiological substrates. It is common in the normal physiological direction of a reaction, since *in vivo* the

product concentration occasionally builds up sufficiently to cause product inhibition. Thus, it occasionally gets high enough to cause the dead-end inhibition, either, and evolution has had no way to cause dead-end inhibition to be eliminated completely. This is a reason why special attention should be paid to detection and identification of such complexes (Fromm, 1995; Purich & Allison, 2000).

11.4.1 Ordered Bi Bi System

In a Steady-State Ordered Bi Bi mechanism, A combines with the enzyme and induces the formation of the binding site for B. However, P, which bears a structural similarity to B, may bind instead of B.



(11.31)

When a product, P, combines with an another enzyme form, in addition to one it normally combines with as a substrate in the reverse direction, certain terms in the denominator of the rate equation are multiplied by the factor $(1+P/K_i)$, where K_i is the dissociation constant of the product from the dead-end complex.

If the denominator terms that are multiplied already contain a P term, the resulting rate equation will obtain a P^2 term, and the resulting inhibition will be parabolic.

In this case, the denominator terms that are multiplied by $(1+P/K_i)$ are those corresponding to the relative concentration of the EA complex. These terms are found in the numerator of the distribution equation for $[EA]/[E_o]$ for the Ordered Bi Bi mechanism (Chapter 9; Eq. (9.13)). In the presence of A, B, and P (and the absence of Q), the numerator term of the distribution equation for $[EA]/[E_o]$ contains an [A] term and an [A][P] term. Consequently, the rate equation in the presence of P (from Eq. (9.19)) is:

$$v_o = \frac{V_1 AB}{K_{iA}K_B + K_B\left(1+\frac{P}{K_i}\right)A + K_A B + AB + \frac{K_Q K_B K_{iA}}{K_{iQ}K_P}P + \frac{K_Q K_B}{K_{iQ}K_P}\left(1+\frac{P}{K_i}\right)AP + \frac{1}{K_{iP}}ABP} \tag{11.32}$$

or, in the reciprocal forms:

$$\frac{1}{v_o} = \frac{1}{V_1}\left[1+\frac{K_B}{B}\left(1+\frac{P}{K_i}\right)\left(1+\frac{K_Q P}{K_{iQ}K_P}\right)+\frac{P}{K_{iP}}\right]+\frac{K_A}{V_1}\left[1+\frac{K_{iA}K_B}{K_A B}\left(1+\frac{K_Q P}{K_{iQ}K_P}\right)\right]\frac{1}{A} \tag{11.33}$$

$$\frac{1}{v_o} = \frac{1}{V_1}\left(1+\frac{K_A}{A}+\frac{P}{K_{iP}}\right)+\frac{K_B}{V_1}\left(1+\frac{K_Q P}{K_{iQ}K_P}\right)\left(1+\frac{P}{K_i}+\frac{K_{iA}}{A}\right)\frac{1}{B} \tag{11.34}$$

Figure 9 shows the graphical presentation of Eq. (11.34), a plot of $1/v_0$ versus $1/B$, in the presence of increasing concentrations of P. The double reciprocal plot has an unusual appearance. Thus, the mixed product and dead-end inhibition by P can be distinguished from the normal product inhibition by P from the fact that the family of straight lines does not intersect at a common point.

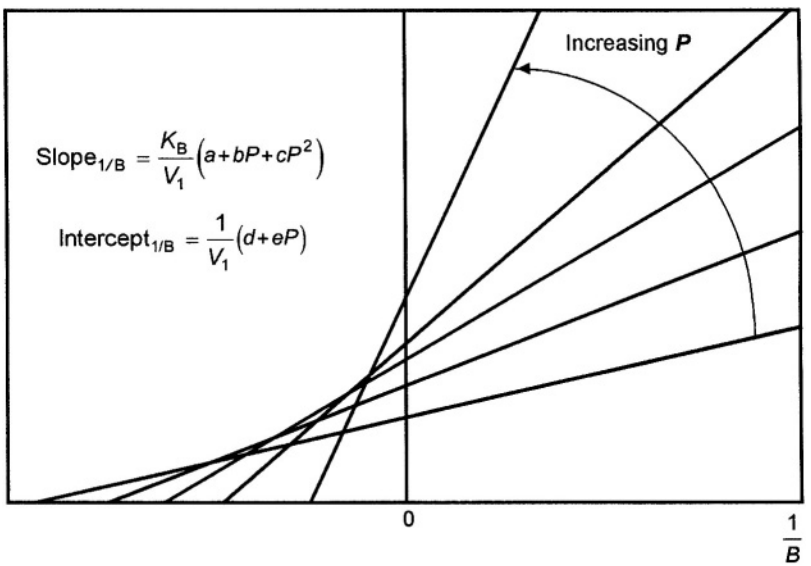


Figure 9. Mixed product and dead-end inhibition in an Ordered Bi Bi system. P reacts with EA as well as with EQ (Scheme (11.31)). The plot of $1/v_0$ versus $1/B$ is slope-parabolic, intercept-linear.

When A is varied, the resulting P^2 term appears in the $\text{Intercept}_{1/A}$ function, and when B is the varied substrate, the resulting P^2 term appears in the $\text{Slope}_{1/A}$ function. According to the nomenclature of Cleland (Chapter 6; Section 6.6), the system can be described as *S*-linear, *I*-parabolic (nonintersecting) noncompetitive inhibition when A is the varied substrate and B is unsaturating, changing to *I*-linear uncompetitive inhibition when B is saturating. When B is varied, we obtain an *S*-parabolic, *I*-linear noncompetitive pattern (Fig. 9).

The linear or parabolic nature of the replots is better seen if Eqs. (11.33) and (11.34) are regrouped. The slopes and intercepts of the secondary plots then have the general forms of Eqs. (11.35)–(11.38).

Straight line

$$\text{Slope}_{1/A} = \frac{K_A}{V_1}(a + bP) \tag{11.35}$$

$$\text{Intercept}_{1/B} = \frac{1}{V_1}(a + bP) \tag{11.36}$$

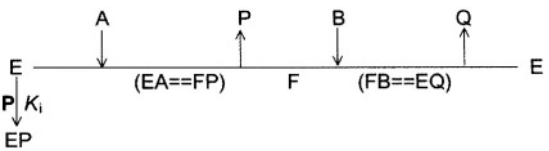
Parabola

$$\text{Intercept}_{1/A} = \frac{1}{V_1}(a + bP + cP^2) \tag{11.37}$$

$$\text{Slope}_{1/B} = \frac{K_B}{V_1}(a + bP + cP^2) \tag{11.38}$$

11.4.2 Ping Pong Bi Bi System

A frequent case in the Ping Pong Bi Bi mechanism is that the first product of reaction combines with the first stable enzyme form to produce a stable dead-end EP complex:



(11.39)

The situation shown in Eq. (11.39) is analogous to the dead-end inhibition by a nonreacting inhibitor, described in Chapter 6 (Section 6.5.1). The rate equation is derived in the usual manner; both B and P terms in the distribution equation are multiplied by $(1+P/K_i)$, to obtain

$$v_0 = \frac{V_1AB}{K_BA + K_AB\left(1 + \frac{P}{K_i}\right) + AB + \frac{K_B}{K_{iP}}AP + \left(\frac{K_{iA}K_B}{K_{iP}}P\right)\left(1 + \frac{P}{K_i}\right)} \tag{11.40}$$

or in the reciprocal forms

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B} + \frac{K_BP}{K_{iP}B}\right) + \frac{K_A}{V_1}\left(1 + \frac{P}{K_i}\right)\left(1 + \frac{K_{iA}K_BP}{K_AK_{iP}B}\right)\frac{1}{A} \tag{11.41}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_A}{A} + \frac{K_AP}{K_iA}\right) + \frac{K_B}{V_1}\left[1 + \frac{P}{K_{iP}} + \frac{K_{iA}P}{K_{iP}A}\left(1 + \frac{P}{K_i}\right)\right]\frac{1}{B} \tag{11.42}$$

The patterns are *S*-parabolic, *I*-linear (nonintersecting) noncompetitive for both substrates. Replots of $\text{Intercept}_{1/A}$ versus P, will permit K_{iP} to be determined, while replot of $\text{Intercept}_{1/B}$ versus P will allow K_i to be determined.

11.5 SUMMARY OF INHIBITION TYPES

In the preceding chapters, as well as in this chapter, we have encountered a number of different types of inhibitors. The inhibitors have been classified as competitive, uncompetitive, and noncompetitive. Further, the inhibition can be classified as linear, parabolic, and hyperbolic. Further types of inhibition, which

are specifically described in this chapter, are substrate inhibition, mixed dead-end and product inhibition, and a non-productive binding.

In the linear inhibition, the family of straight lines in the double reciprocal plot of $1/v_o$ versus $1/A$ (at different fixed concentrations of I) will always have a common intersection point. In the linear inhibition, the replot of $\text{Slope}_{1/A}$ and $\text{Intercept}_{1/A}$ from the double reciprocal plot, versus I, is a straight line; this type of inhibition was described in Chapter 5.

In the hyperbolic and parabolic types of inhibition, the family of straight lines in the double reciprocal plot of $1/v_o$ versus $1/A$ (at different fixed concentrations of I) will have a common intersection point only if the denominator $(1+I/K_i)$ terms are identical in slope and intercept terms. Usually this is not the case, that is, the K_i will be different in the denominator of the slope and intercept terms. There is not a crossover point if only slope or intercept is hyperbolic or parabolic and the other is linear.

In the hyperbolic inhibition, the replot of $\text{Slope}_{1/A}$ and/or $\text{Intercept}_{1/A}$ versus I is a hyperbola. In the parabolic inhibition, the replot of $\text{Slope}_{1/A}$ and/or $\text{Intercept}_{1/A}$ versus I will have a shape of a parabola. These types of inhibition are described in Chapter 6.

Table 1 summarizes the inhibition types in bisubstrate reactions which are encountered in this chapter.

Table 1. *Summary of inhibition types in bisubstrate reactions with substrate inhibitors or mixed substrate and dead-end inhibitors*

Inhibition type	Mechanism	Dead-end complex	Equation	Figures
Substrate inhibition	Rapid-Equilibrium Random bisubstrate	**B**EB	(11.6)	Fig. 1 Fig. 2
	Steady-State Ordered Bi Bi with dead-end EB complex	E**B**	(11.12)	Fig. 4 Fig. 6
	Steady-State Ordered Bi Bi with dead-end EBQ complex	E**B**Q	none	Fig. 7
	Ping Pong Bi Bi	E**B**	(11.20)	—
Mixed dead-end and product inhibition	Ordered Bi Bi	EA**P**	(11.32)	Fig. 9
	Ping Pong Bi Bi	E**P**	(11.40)	—

The graphical analysis of initial rate data, which is described extensively in this chapter, is useful, especially while the experiment is still in progress. However, it is important to emphasize that, for definitive results, one should always fit the data with appropriate rate equations for statistical analysis (Cleland, 1979).

REFERENCES

Books

Fromm, H.J. (1975) *Initial rate enzyme kinetics,* Springer-Verlag, Berlin.
Fersht, A. (1999) *Structure and mechanism in protein science,* W.H. Freeman, New York.
Plowman, K.M. (1972) *Enzyme kinetics,* McGraw-Hill, New York.
Purich, D.L. & Allison, D.R. (2000) *Handbook of biochemical kinetics,* Academic Press, San Diego.
Ratkowsky, D.A. (1983) *Nonliner regression modeling,* Marcel Dekker, New York.
Schultz, A.R. (1994) *Enzyme kinetics,* Cambridge University Press, Cambridge.

Review articles and specific references

Cleland, W.W. (1970) *The Enzymes,* 3rd ed., Vol. II, 1–65.
Cleland, W.W. (1977) *Adv. Enzymol. Rel. Areas Mol. Biol. 45,* 273 – 387.
Cleland, W.W. (1979) *Methods Enzymol. 63,* 103–138.
Cleland, W.W. (1979) *Methods Enzymol. 63,* 500–551.
Cooper, B.F. & Rudolph, F.B. (1995) *Methods Enzymol. 249,* 188–211.
Fromm, H.J. (1979) *Methods Enzymol. 63,* 467–486.
Fromm, H.J. (1995) *Methods Enzymol. 249,* 123–144.
Johnson, M.L. & Faunt, L.M. (1992) *Methods Enzymol. 210,* 1–37.
Johnson, M.L. (1994) *Methods Enzymol. 240,* 1–23.
Marmasse, C. (1963) *Biochim. Bioph. Acta 77,* 530–535.
Rudolph, F.B. (1979) *Methods Enzymol. 63,* 411–436.
Watts, D.G. (1994) *Methods Enzymol. 240,* 23–36.

This page intentionally left blank

Chapter 12

Trisubstrate Mechanisms

Three substrates can combine with an enzyme to produce two or three products of reaction, in a wide variety of ways. If all the binding steps are much faster than the catalytic step, which is the rate-limiting, then all forms of enzyme are in a *rapid equilibrium,* and all kinetic constants are true dissociation constants of respective enzyme–substrate complexes. If the catalytic step is not the slowest step in the kinetic mechanism, all forms of enzyme attain a *steady-state* concentration shortly after the mixing of enzyme with substrates. All kinetic mechanisms of steady-state reactions fall into only two major groups. Those in which all reactants must combine with the enzyme before reaction can take place and any product can be released, are called sequential. Mechanisms in which one or more products are released before all substrates have added are called Ping Pong (Cleland, 1963).

12.1 PREDICTION OF REACTION SEQUENCES IN TRISUBSTRATE MECHANISMS

When there are three substrates for an enzymatic reaction, the number of possible initial velocity patterns is large, and interpretation of the patterns is not as straightforward as when only two substrates are involved.

We shall start the analysis of trisubstrate mechanisms by examining the form of the rate equation, proceed with the mechanistic interpretation of the absence of various terms from the general rate equation, and conclude with experimental methods for establishing the presence or absence of such terms.

12.1.1 General Rate Equation in the Absence of Products

The general form of the rate equation for all trisubstrate mechanisms, in the absence of products and assuming linear reciprocal plots, is

$$v_0 = \frac{V_1 ABC}{\text{constant} + (\text{coefA})A + (\text{coefB})B + (\text{coefC})C + K_A BC + K_B AC + K_C AB + ABC} \tag{12.1}$$

or, in the reciprocal form

$$\frac{V_1}{v_0} = \frac{(\text{const})}{ABC} + \frac{(\text{coefA})}{BC} + \frac{(\text{coefB})}{AC} + \frac{(\text{coefC})}{AB} + \frac{K_A}{A} + \frac{K_B}{B} + \frac{K_C}{C} + 1 \tag{12.2}$$

It is useful to note the identity of terms in both equations, because both equations will be used for the graphical analysis of initial rate data in Section 12.1.3.

Since all trisubstrate mechanisms have the same general rate equation, the difference between them is found only in the presence or absence of individual terms in the general expression. We shall proceed with the mechanistic interpretation of the absence of various terms from the general rate equation according to Viola and Cleland (1982). Alternative kinetic methods for analysis of trisubstrate reactions were developed by Frieden (1959), Fromm (1967, 1975), Segel (1975), and Rudolh & Fromm (1979).

12.1.2 Mechanistic Interpretation of the Absence of Denominator Terms

No constant term

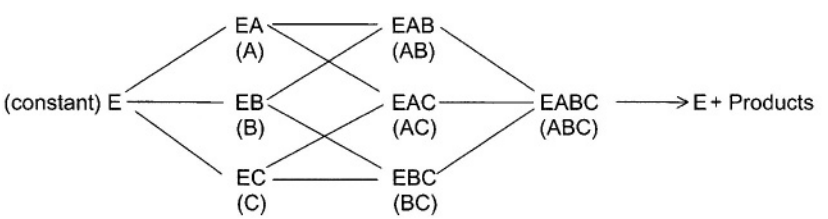
This means a Ping Pong type of mechanism. All Ping Pong types of mechanisms lack the constant term, as is evident from Tables 3 and 4; in addition to the constant term, the *A* and *B* terms are also missing. The Hexa Uni Ping Pong mechanism lacks not only the constant, but also the *A, B,* and *C* terms, and all initial velocity patterns *(A–B, B–C,* and *A–C)* are parallel.

Constant term present

Such mechanisms are sequential and proceed through an EABC complex. The *ABC* term is always present except in a Theorell–Chance type of mechanism in which the reaction of the third substrate to add results in such a rapid reaction and release of products, that *V* appears infinite.

In sequential mechanisms, a large variety of additional complex mechanisms may occur. Since the constant term is always present, the different sequential mechanisms will differ by the presence or absence of the *A, B, C, AB, AC,* or *BC* terms from the general velocity equation (12.1).

Several simple rules can be formulated for sequential trisubstrate mechanisms with the aid of reaction (12.3).



(12.3)

(1) It matters whether the first two reactants to add do so in steady-state fashion (k_{off} is less than, or does not exceed, V_1/E_o), or in rapid equilibrium ($k_{off} \gg V_1/E_o$), but it does not matter whether the last substrate to add does so in steady-state or rapid equilibrium fashion. Whether reactants add in

steady-state fashion can be determined by the isotope partition method (Rose *et al.,* 1974).

(2) Where a substrate adds in a steady-state fashion, it always has a finite Michaelis constant (i.e., for A the K_ABC term would be present). Conversely, the lack of K_ABC, K_BAC, or K_CAB term indicates a rapid equilibrium addition of the corresponding substrate when it adds in the first or the second position, and the absence of the corresponding complex in the reaction above (i.e., lack of K_ABC term means no EBC complex).

(3) The lack of an *A, B,* or *C* term indicates the absence of the corresponding binary complex in reaction (12.3), and thus an obligatory order of addition for at least one substrate. However, the presence of an *A, B,* or *C* term does not require that the corresponding binary complex form, if the mechanism is a steady state one.

With the aid of Table 1, one can identify the missing terms in principal sequential mechanisms.

12.1.3 Graphical Analysis of Initial Rate Data

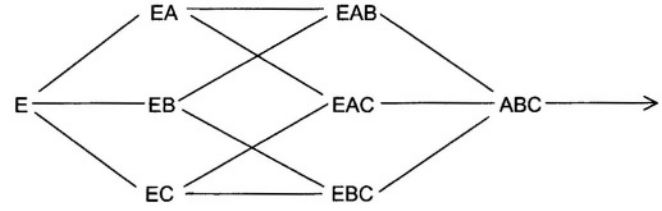
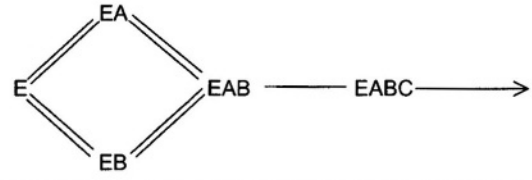
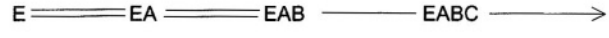
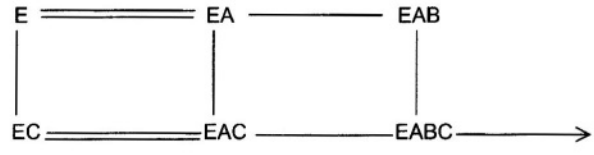
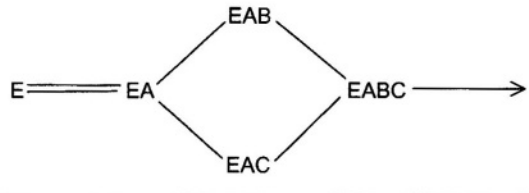
The usual procedure for the graphical analysis of initial rate data for trisubstrate reactions would be to treat each substrate as the *varied* substrate at different *fixed* concentrations of another substrate, maintaining a *constant* concentration of the third substrate. All such plots represent a family of straight lines with a common intersection point to the left of the $1/v_o$-axis. However, the main task of the graphical analysis would be to identify the terms which are missing from the velocity equation, and thus establish the general rate equation. The following rules are useful for this goal (Viola & Cleland, 1982).

First, when all three patterns (vary *A* and *B, B* and *C,* or *A* and *C,* at fixed nonsaturating levels of the third substrate) are intersecting, we have a sequential mechanism. For a Ping Pong mechanism, at least two patterns will be parallel.

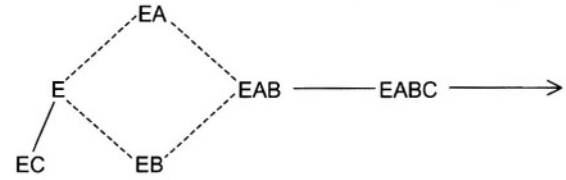
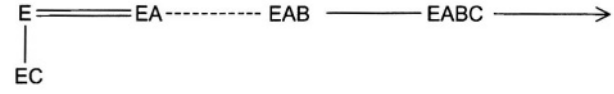
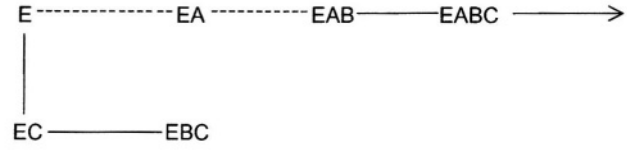
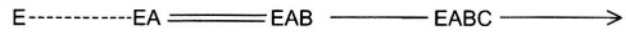
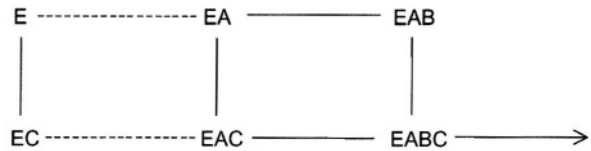
Second, if we are able to saturate with one substrate, we shall vary the other two substrates. If the resulting pattern is intersecting, we have a term present corresponding to the saturating substrate. If the pattern is parallel, the term is missing. For example, if we saturate with *B* and run the *A–C* pattern, an intersecting pattern shows that a *B* term is present, and a parallel pattern shows that it is absent. If the pattern crosses on the vertical axis when plotted against the reciprocal of one of the varied substrates, the Michaelis constant corresponding to the other varied substrate is zero. For example, if the *A–C* pattern crosses on the vertical axis when *C* is varied, then the K_ABC term is missing; if the crossover point is to the left of the vertical axis, the term is present.

Third, if we cannot saturate with a substrate (say, B), we shall vary the other two substrates at each of several levels of *B,* and plot the patterns as reciprocal plots versus one substrate (say, C) at a fixed levels of the other (A); then, we shall replot from each pattern the slopes and intercepts against the reciprocal

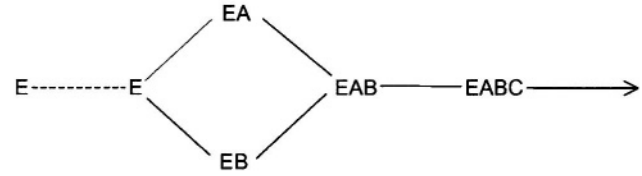
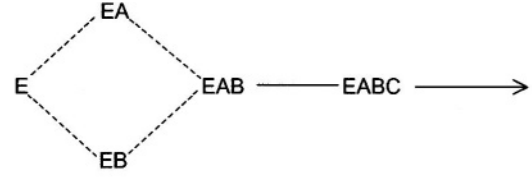
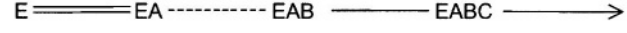
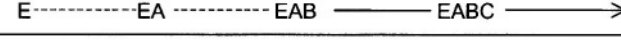
Table 1 Overview of sequential mechanisms (Viola & Cleland, 1982)

Terms missing	Examples	Mechanism
None	Adenylosuccinate synthetase (Rudolph & Fromm, 1969)	
	—	
B	Glutamate dehydrogenase (Rife & Cleland, 1980)	
	—	
B, C	Citrate cleavage enzyme (Plowman & Cleland, 1967)	

(*continued*)

Terms missing	Examples	Mechanism
K_ABC, K_BAC	—	
	—	
B, K_BAC	—	
	Galactosyl transferase (Morrison & Ebner, 1971)	
B, K_ABC	Inosine monophosphate dehydrogenase (Heyde *et al.*, 1976)	
	—	

(*continued*)

Terms missing	Examples	Mechanism
B, C, K_ABC	—	
C, K_BAC, K_ABC	—	
B, C, K_BAC	Glutamate dehydrogenase (Rife & Cleland, 1980)	
B, C, K_BAC, K_ABC	—	

Segments in the mechanism are: *Steady state* ═══; *Rapid equilibrium* – – –; *Steady-state or rapid equilibrium* ———

concentration of the other substrate (A) in the usual way. If the slope replots all go through the origin, two terms are missing (in this particular case, $K_C AB$ and A). If the intercept replots have no slope, two other terms are missing (in this particular case, $K_A BC$ and C). Each slope and intercept of a second replot corresponds to a single denominator term in the rate equation, and when the second replot goes through the origin, or has zero slope, we miss the corresponding term.

In order to illustrate this, we shall rearrange the general rate Eq. (12.2) in order to show the reciprocal plot of $1/v_o$ versus $1/C$:

$$\frac{1}{v_o} = \frac{1}{V_1}\left[1 + \frac{K_A}{A} + \frac{K_B}{B} + \frac{(\text{coefC})}{AB}\right] + \frac{1}{V_1}\left[K_C + \frac{(\text{coefA})}{B} + \frac{(\text{coefB})}{A} + \frac{(\text{const})}{AB}\right]\frac{1}{C} \tag{12.4}$$

A replot of slopes and intercepts from the above equation against $1/A$ gives the *first replot*:

$$\text{Slope}_{1/c} = \frac{1}{V_1}\left[K_C + \frac{(\text{coefA})}{B}\right] + \frac{1}{V_1}\left[(\text{coefB}) + \frac{(\text{const})}{B}\right]\frac{1}{A} \tag{12.5}$$

$$\text{Intercept}_{1/c} = \frac{1}{V_1}\left(1 + \frac{K_B}{B}\right) + \frac{1}{V_1}\left[K_A + \frac{(\text{coefC})}{B}\right]\frac{1}{A} \tag{12.6}$$

The replots of slopes and intercepts from Eq. (12.5) against $1/B$, gives the *second replot* (of slopes):

$$\text{Slope}_{(\text{Eq. (12.5)})} = \frac{1}{V_1}(\text{coefB}) + \frac{1}{V_1}(\text{const})\frac{1}{B} \tag{12.7}$$

$$\text{Intercept}_{(\text{Eq. (12.5)})} = \frac{1}{V_1}(K_C) + \frac{1}{V_1}(\text{coefA})\frac{1}{B} \tag{12.8}$$

In the same way, the replots of slopes and intercepts from Eq. (12.6) against $1/B$, gives the *second replot* (of intercepts):

$$\text{Slope}_{(\text{Eq. (12.6)})} = \frac{1}{V_1}(K_A) + \frac{1}{V_1}(\text{coefC})\frac{1}{B} \tag{12.9}$$

$$\text{Intercept}_{(\text{Eq. (12.6)})} = \frac{1}{V_1}(1) + \frac{1}{V_1}(K_B)\frac{1}{B} \tag{12.10}$$

Clearly, the slopes and intercepts of the secondary replots each correspond to the coefficient of one term from the denominator of the general rate equation divided by V_1. If any of these slopes or intercepts are zero, the corresponding term is absent from the rate equation.

The assignments of the terms in the rate equation to the slopes and intercepts of the secondary replots are given in Table 2.

Table 2 Identification of denominator terms by the analysis of first and second replots from initial reciprocal plots of $1/v_o$ versus $1/C$, in trisubstrate reactions

Primary plot ⟶	First replot ⟶	Second replot ⇒	Missing term
Intercepts of $1/v_o$ versus $1/C$	Intercept$_{1/C}$ versus $1/A$	Intercept versus $1/B$	ABC
Intercepts of $1/v_o$ versus $1/C$	Intercept$_{1/C}$ versus $1/A$	Slope versus $1/B$	K_BAC
Intercepts of $1/v_o$ versus $1/C$	Slope$_{1/C}$ versus $1/A$	Intercept versus $1/B$	K_ABC
Intercepts of $1/v_o$ versus $1/C$	Slope$_{1/C}$ versus $1/A$	Slope versus $1/B$	C
Slopes of $1/v_o$ versus $1/C$	Intercept$_{1/C}$ versus $1/A$	Intercept versus $1/B$	K_CAB
Slopes of $1/v_o$ versus $1/C$	Intercept$_{1/C}$ versus $1/A$	Slope versus $1/B$	A
Slopes of $1/v_o$ versus $1/C$	Slope$_{1/C}$ versus $1/A$	Intercept versus $1/B$	B
Slopes of $1/v_o$ versus $1/C$	Slope$_{1/C}$ versus $1/A$	Slope versus $1/B$	Constant

12.2 RATE EQUATIONS IN THE ABSENCE OF PRODUCTS

In this section, we shall review the rate equations for the major types of trisubstrate mechanisms, written in the absence of products (Cleland, 1963; Plowman, 1972; Fromm, 1975, 1979). All trisubstrate mechanisms in the rapid equilibrium category are relatively rare and the steady-state mechanisms are more common. However, the derivation of rate equations for rapid equilibrium mechanisms, in the absence of products, is less demanding, as it requires only the rapid equilibrium assumptions and, therefore, the resulting rate equations are relatively simple.

In Sections 12.2.1 and 12.2.2, we shall divide the rapid equilibrium trisubstrate mechanisms into the following major types:

Completely random
Strictly ordered
Random *A–B*, Ordered *C*
Ordered *A*, Random *B–C*, and
Random *A–C*, Ordered *B*.

Much more realistic are the steady-state trisubstrate mechanisms, that occur very frequently. In the "Ter Bi Mechanisms" Section, we shall develop the rate equations, in the absence of all products of reaction, for major Ter Bi mechanisms:

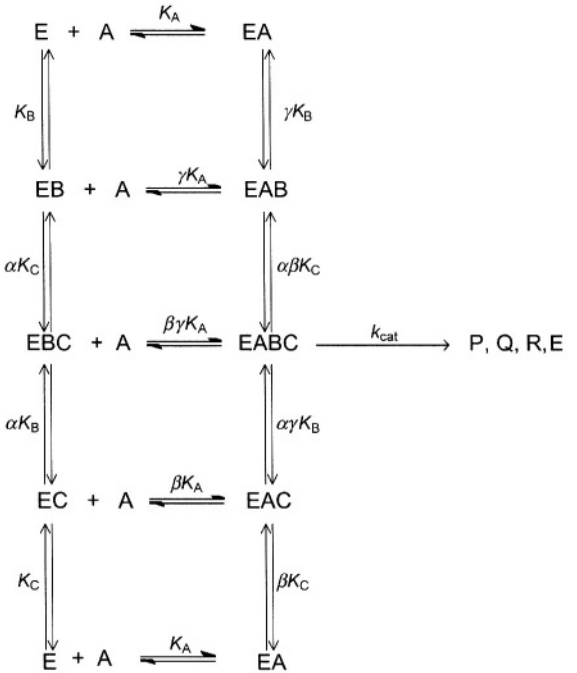
Ordered Ter Bi,
Ordered Bi Ter, and
Bi Uni Uni Uni Ping Pong Ter Bi

Finally, in the "Ter Ter Mechanisms" Section, we shall develop the rate equations, in the absence of all products of reaction, for major steady-state Ter Ter mechanisms:

Ordered Ter Ter
Bi Uni Uni Bi Ping Pong Ter Ter
Bi Bi Uni Uni Ping Pong Ter Ter, and
Hexa Uni Ping Pong Ter Ter.

12.2.1 Rapid Equilibrium Random Ter Ter System

If A, B, and C are the three substrates that yield three products, P, Q, and R, and all forms of enzyme are in a rapid equilibrium, the system can be designated as a Rapid Equilibrium Random Ter Ter, and the equilibria shown as



(12.11)

where $V_1 = k_{cat}E_0$.

The general rate equation for reaction (12.11) is

$$v_0 = \frac{V_1\left(\dfrac{ABC}{\alpha\beta\gamma K_A K_B K_C}\right)}{1 + \dfrac{A}{K_A} + \dfrac{B}{K_B} + \dfrac{C}{K_C} + \dfrac{AB}{\gamma K_A K_B} + \dfrac{AC}{\beta K_A K_C} + \dfrac{BC}{\alpha K_B K_C} + \dfrac{ABC}{\alpha\beta\gamma K_A K_B K_C}} \tag{12.12}$$

or, in the reciprocal form

$$\frac{V_1}{v_0} = \left(1 + \frac{\beta\gamma K_A}{A} + \frac{\alpha\gamma K_B}{B} + \frac{\alpha\beta K_C}{C}\right) + \alpha\beta\gamma\left(\frac{K_A K_B}{AB} + \frac{K_A K_C}{AC} + \frac{K_B K_C}{BC} + \frac{K_A K_B K_C}{ABC}\right) \tag{12.13}$$

The velocity equation can be rearranged to show any of the three substrates as the varied ligand at fixed concentrations of the other two ligands:

$$\frac{V_1}{v_0} = \left(1 + \frac{\alpha\gamma K_B}{B} + \frac{\alpha\beta K_C}{C} + \frac{\alpha\beta\gamma K_B K_C}{BC}\right) + \beta\gamma K_A\left(1 + \frac{\alpha K_B}{B} + \frac{\alpha K_C}{C} + \frac{\alpha K_B K_C}{BC}\right)\frac{1}{A} \tag{12.14}$$

$$\frac{V_1}{v_0} = \left(1 + \frac{\beta\gamma K_A}{A} + \frac{\alpha\beta K_C}{C} + \frac{\alpha\beta\gamma K_A K_C}{AC}\right) + \alpha\gamma K_B\left(1 + \frac{\beta K_A}{A} + \frac{\beta K_C}{C} + \frac{\beta K_A K_C}{AC}\right)\frac{1}{B} \tag{12.15}$$

$$\frac{V_1}{v_0} = \left(1 + \frac{\beta\gamma K_A}{A} + \frac{\alpha\gamma K_B}{B} + \frac{\alpha\beta\gamma K_A K_B}{AB}\right) + \alpha\beta K_C\left(1 + \frac{\gamma K_A}{A} + \frac{\gamma K_B}{B} + \frac{\gamma K_A K_B}{AB}\right)\frac{1}{C} \tag{12.16}$$

The family of straight lines in the double reciprocal plot of $1/v_0$ versus $1/A$ at any constant concentration of C and different fixed concentrations of B, will intersect to the left of the $1/v_0$-axis, and on, above or below the $1/A$-axis, depending on the values of interaction factors α, β, and γ. If $\alpha = \beta = \gamma = 1$, the reciprocal plots will intersect on the horizontal axis. The similar result is obtained with the double reciprocal plots of $1/v_0$ versus $1/B$ and $1/v_0$ versus $1/C$.

The usual procedure for the graphical analysis of initial rate data would be to treat each substrate as the varied substrate at different fixed concentrations of another substrate, maintaining a fixed concentration of the third substrate. All such plots represent a family of straight lines with a common intersection point to the left of the $1/v_0$-axis.

Let us illustrate this treatment with the aid of the above examples. If the substrate C is saturating, the rate Eq. (12.14) will reduce to

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{\alpha\gamma K_B}{B}\right) + \frac{\beta\gamma K_A}{V_1}\left(1 + \frac{\alpha K_B}{B}\right)\frac{1}{A} \tag{12.17}$$

Similarly, one can reduce the rate Eq. (12.15) by taking the saturating A, and reduce Eq. (12.16) by taking the saturating B. All resulting equations will be completely symmetrical and their graphical presentation will be completely symmetrical. From the primary double reciprocal plots of such equations and the corresponding replots of intercepts and slopes, one can determine graphically all kinetic constants and all interaction factors.

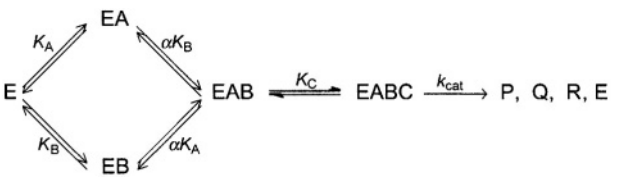
12.2.2 Ordered and Random-Ordered Rapid Equilibrium Trisubstrate Systems

Let us examine several ordered and random-ordered rapid equilibrium Ter Ter systems, in addition to the aforementioned total rapid equilibrium random system:

(a) *Strictly Ordered*

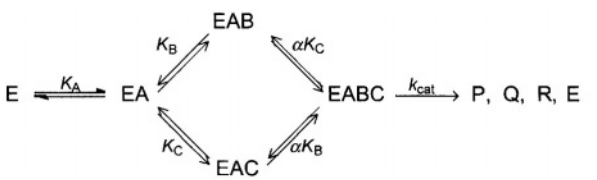
$$E \rightleftharpoons EA \rightleftharpoons EAB \rightleftharpoons EABC \longrightarrow P, Q, R, E \tag{12.18}$$

(b) *Random A–B, Ordered C*



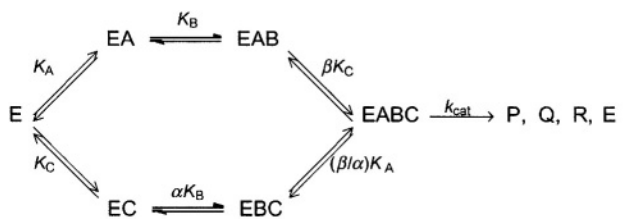
(12.19)

(c) *Ordered A, Random B–C*



(12.20)

(d) *Random A–C, Ordered B*



(12.21)

If we assume that $V_1 = k_{cat}E_o$, then the velocity equations for the above mechanisms, written in the reciprocal form, are

Strictly Ordered

$$\frac{V_1}{v_o} = \left(1 + \frac{K_C}{C} + \frac{K_B K_C}{BC} + \frac{K_A K_B K_C}{ABC}\right) \tag{12.22}$$

Random A–B, Ordered C

$$\frac{V_1}{v_o} = \left(1 + \frac{K_C}{C} + \frac{\alpha K_A K_C}{AC} + \frac{\alpha K_B K_C}{BC} + \frac{\alpha K_A K_B K_C}{ABC}\right) \tag{12.23}$$

Ordered A, Random B–C

$$\frac{V_1}{v_o} = \left(1 + \frac{\alpha K_B}{B} + \frac{\alpha K_C}{C} + \frac{\alpha K_B K_C}{BC} + \frac{\alpha K_A K_B K_C}{ABC}\right) \tag{12.24}$$

Random A–C, Ordered B

$$\frac{V_1}{v_o} = \left(1 + \frac{\beta K_A}{\alpha A} + \frac{\beta K_C}{C} + \frac{\beta K_B K_C}{BC} + \frac{\beta K_A K_B}{AB} + \frac{\beta K_A K_B K_C}{ABC}\right) \tag{12.25}$$

Again, the usual procedure for the graphical analysis of initial rate data would be to treat each substrate as the varied substrate at different fixed concentrations of another substrate, maintaining a fixed concentration of the third substrate. In this way, all kinetic constants can be determined and, what is equally important, each mechanism can be identified unequivocally.

12.2.3 Steady-State Sequential Mechanisms

The general rate equations for the steady-state sequential mechanisms, in the presence of products, are developed in the following sections. The rate equations in the absence of products can be written down directly from the general rate equations, simply by omitting the terms in the denominator and the numerator that contain the concentration terms for products P, Q, and R, and eliminating the K_{eq} with the aid of Haldane relationships. In all trisubstrate systems, the inhibition constants will always represent true dissociation constants.

Ter Bi mechanisms

(a) *Ordered Ter Bi mechanism.* In the presence of all three substrates and the absence of products, the rate equation is

$$v_o = \frac{V_1 ABC}{K_{iA}K_{iB}K_C + K_{iB}K_C A + K_{iA}K_B C + K_C AB + K_B AC + K_A BC + ABC} \tag{12.26}$$

or, in the reciprocal form

$$\frac{v_o}{V_1} = 1 + \frac{K_A}{A} + \frac{K_B}{B} + \frac{K_C}{C} + \frac{K_{iA}K_B}{AB} + \frac{K_{iB}K_C}{BC} + \frac{K_{iA}K_{iB}K_C}{ABC} \tag{12.27}$$

If the variable substrate is separated from the constant substrates, we obtain

$$\frac{1}{v_o} = \frac{1}{V_1}\left(1 + \frac{K_B}{B} + \frac{K_C}{C} + \frac{K_{iB}K_C}{BC}\right) + \frac{K_A}{V_1}\left(1 + \frac{K_{iA}K_B}{K_A B} + \frac{K_{iA}K_{iB}K_C}{K_A BC}\right)\frac{1}{A} \tag{12.28}$$

$$\frac{1}{v_o} = \frac{1}{V_1}\left(1 + \frac{K_A}{A} + \frac{K_C}{C}\right) + \frac{K_B}{V_1}\left(1 + \frac{K_{iA}}{A}\right)\left(1 + \frac{K_{iB}K_C}{K_B C}\right)\frac{1}{B} \tag{12.29}$$

$$\frac{1}{v_o} = \frac{1}{V_1}\left(1 + \frac{K_A}{A} + \frac{K_B}{B} + \frac{K_{iA}K_B}{AB}\right) + \frac{K_C}{V_1}\left(1 + \frac{K_{iB}}{B} + \frac{K_{iA}K_{iB}}{AB}\right)\frac{1}{C} \tag{12.30}$$

Equations (12.28) and (12.30) are symmetrical, but not Eq. (12.29). Thus, from the initial velocity studies one can identify the substrate B, but cannot tell apart the substrate A from the substrate C. Binding studies, however, will identify A as the only substrate which binds to the free enzyme.

The same initial velocity equations fit the mechanism where the addition of A and B is ordered, but C adds randomly. For such a mechanism (and for ordered ones as well), the best distinction is made by dead-end inhibitors or isotopic exchange at equilibrium.

In the reverse direction, in the presence of products and the absence of substrates, the rate equation is identical with that for the Ordered Bi Bi mechanism (Section 9.2).

(b) *Bi Uni Uni Uni Ping Pong Ter Bi mechanism.* In the presence of substrates and the absence of products of reaction, the rate equation is

$$v_0 = \frac{V_1 ABC}{K_{iA}K_B C + K_C AB + K_B AC + K_A BC + ABC} \tag{12.31}$$

or, in the reciprocal form:

$$\frac{V_1}{v_0} = 1 + \frac{K_A}{A} + \frac{K_B}{B} + \frac{K_C}{C} + \frac{K_{iA}K_B}{AB} \tag{12.32}$$

If the variable substrate is separated from the constant substrates, we obtain

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B} + \frac{K_C}{C}\right) + \frac{K_A}{V_1}\left(1 + \frac{K_{iA}K_B}{K_A B}\right)\frac{1}{A} \tag{12.33}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_A}{A} + \frac{K_C}{C}\right) + \frac{K_B}{V_1}\left(1 + \frac{K_{iA}}{A}\right)\frac{1}{B} \tag{12.34}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_A}{A} + \frac{K_B}{B} + \frac{K_{iA}K_B}{AB}\right) + \left(\frac{K_C}{V_1}\right)\frac{1}{C} \tag{12.35}$$

Equations (12.33) and (12.34) are symmetrical, but not Eq. (12.35). Thus, from the initial velocity studies, one can identify the substrate C, but cannot tell apart the substrate A from the substrate B. Binding or product inhibition studies are necessary.

In the reverse direction, in the presence of products and the absence of substrates, the rate equation is identical with that for the usual Ping Pong Bi Bi mechanism (Section 9.5).

Ter Ter mechanisms

(c) *Ordered Ter Ter mechanism.* In the forward direction, the rate equation is identical with that for the Ordered Ter Bi mechanism (preceding section). In the forward and the reverse direction, the rate equations are completely symmetrical.

(d) *Bi Uni Uni Bi Ping Pong Ter Ter Mechanism.* In the forward direction, the rate equation is identical with that for the Bi Uni Uni Uni Ping Pong Ter Bi mechanism.

(e) *Bi Bi Uni Uni Ping Pong Ter Ter Mechanism.* In the forward direction, the rate equation is identical with that for the Bi Uni Uni Bi Ping Pong Ter Ter mechanism.

(f) *Hexa Uni Ping Pong Ter Ter mechanism.* In the forward direction, in the presence of substrates and the absence of products, the rate equation is

$$v_0 = \frac{V_1 ABC}{K_C AB + K_B AC + K_A BC + ABC} \tag{12.36}$$

or, in the reciprocal form:

$$\frac{V_1}{v_0} = 1 + \frac{K_A}{A} + \frac{K_B}{B} + \frac{K_C}{C} \tag{12.37}$$

If the variable substrate is separated from the constant substrates, we obtain

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B} + \frac{K_C}{C}\right) + \left(\frac{K_A}{V_1}\right)\frac{1}{A} \tag{12.38}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_A}{A} + \frac{K_C}{C}\right) + \left(\frac{K_B}{V_1}\right)\frac{1}{B} \tag{12.39}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_A}{A} + \frac{K_B}{B}\right) + \left(\frac{K_C}{V_1}\right)\frac{1}{C} \tag{12.40}$$

Equations (12.38)–(12.40) are completely symmetrical. Thus, from the initial velocity studies one can not identify the substrates A, B, and C. Product inhibition studies, however, will identify all the substrates unequivocally.

12.3 FULL RATE EQUATIONS FOR TER BI SYSTEMS

Let us proceed with the much more demanding derivation of full rate equations, in the presence of all substrates and products of reaction, for the Steady-State Ordered Ter Bi systems (Cleland, 1963; Hsu *et al.,* 1967; Plowman, 1972).

12.3.1 General Rate Equations

There are two basic nonequilibrium Ter Bi systems:

(a) compulsory-order ternary-complex Ter Bi and Bi Ter mechanism, and a
(b) random-order ternary-complex Ping Pong Ter Bi mechanism.

The Ordered Ter Bi and Bi Ter systems can be represented as

$$\mathrm{E} \underset{k_2}{\overset{k_1\mathrm{A}}{\rightleftharpoons}} \mathrm{EA} \underset{k_4}{\overset{k_3\mathrm{B}}{\rightleftharpoons}} \mathrm{EAB} \underset{k_6}{\overset{k_5\mathrm{C}}{\rightleftharpoons}} (\mathrm{EABC} \leftrightarrow \mathrm{EPQ}) \underset{k_8\mathrm{P}}{\overset{k_7}{\rightleftharpoons}} \mathrm{EQ} \underset{k_{10}\mathrm{Q}}{\overset{k_9}{\rightleftharpoons}} \mathrm{E} \tag{12.41}$$

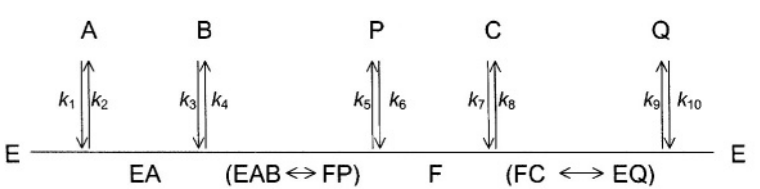
An Ordered Ter Bi reaction will take place in the forward direction:

$$\mathrm{A} + \mathrm{B} + \mathrm{C} \rightarrow \mathrm{P} + \mathrm{Q}$$

and an Ordered Bi Ter reaction will take place in the reverse direction:

$$\mathrm{P} + \mathrm{Q} \rightarrow \mathrm{A} + \mathrm{B} + \mathrm{C}.$$

The Ter Bi Ping Pong system, in the forward direction, is called Bi Uni Uni Uni Ping Pong Ter Bi system (Cleland, 1967). The reaction sequence for this mechanism can be presented as



(12.42)

A complete rate equation for the Ordered Ter Bi system, in the presence of all substrates, A, B, and C, and both products, P and Q, has the general form

$$v_0 = \frac{(k_1k_3k_5k_7k_9ABC - k_2k_4k_6k_8k_{10}PQ)E_0}{\text{denominator of rate equation}} \tag{12.43}$$

Equation (12.43) can be expanded into the general rate equation in the form of rate constants, taking appropriate denominator terms for the Ordered Ter Bi system from Table 3.

Table 3 Denominator terms in Ter Bi mechanisms (Cleland, 1963; Plowman, 1972; Segel, 1975)

Denominator	Kinetic constants	Ordered Ter Bi	Ter Bi Ping Pong
Constant	$V_2K_{iA}K_{iB}K_C$	$k_2k_4k_9(k_6+k_7)$	—
A	$V_2K_{iB}K_C$	$k_1k_4k_9(k_6+k_7)$	—
C	$V_2K_{iA}K_B$	$k_2k_5k_7k_9$	$k_2k_7k_9(k_4+k_5)$
AB	V_2K_C	$k_1k_3k_9(k_6+k_7)$	$k_1k_3k_5(k_8+k_9)$
AC	V_2K_B	$k_1k_5k_7k_9$	$k_1k_7k_9(k_4+k_5)$
BC	V_2K_A	$k_3k_5k_7k_9$	$k_3k_5k_7k_9$
ABC	V_2	$k_1k_3k_5(k_7+k_9)$	$k_1k_3k_7(k_5+k_9)$
P	$\frac{V_1K_Q}{K_{eq}}$	$k_2k_4k_6k_8$	$k_2k_4k_6(k_8+k_9)$
Q	$\frac{V_1K_P}{K_{eq}}$	$k_2k_4k_{10}(k_6+k_7)$	$k_2k_8k_{10}(k_4+k_5)$
PQ	$\frac{V_1}{K_{eq}}$	$k_8k_{10}(k_2k_4+k_4k_6+k_2k_6)$	$k_6k_{10}(k_2k_4+k_2k_8+k_4k_8)$
AP	$\frac{V_1K_Q}{K_{iA}K_{eq}}$	$k_1k_4k_6k_8$	$k_1k_4k_6(k_8+k_9)$
BQ	$\frac{V_2K_AK_{iC}}{K_{iQ}}$	—	$k_3k_5k_8k_{10}$
CQ	$\frac{V_2K_{iA}K_B}{K_{iQ}}$	$k_2k_5k_7k_{10}$	$k_2k_7k_{10}(k_4+k_5)$
ABP	$\frac{V_1K_Q}{K_{iA}K_{iB}K_{eq}}$	$k_1k_3k_6k_8$	$k_1k_3k_6(k_8+k_9)$
BCQ	$\frac{V_2K_A}{K_{iQ}}$	$k_3k_5k_7k_{10}$	$k_3k_5k_7k_{10}$
BPQ	$\frac{V_2K_AK_{iC}}{K_{iP}K_{iQ}}$	$k_3k_6k_8k_{10}$	$k_3k_6k_8k_{10}$
CPQ	$\frac{V_2K_{iA}K_B}{K_{iQ}K_{iP}}$	$k_2k_5k_8k_{10}$	—
$ABCP$	$\frac{V_1K_Q}{K_{iA}K_{iB}K_{iC}K_{eq}}$	$k_1k_3k_5k_8$	—
$BCPQ$	$\frac{V_2K_A}{K_{iQ}K_{iP}}$	$k_3k_5k_8k_{10}$	—

Expanded form is

$$v_0 = \frac{(k_1k_3k_5k_7k_9ABC - k_2k_4k_6k_8k_{10}PQ)E_0}{\begin{array}{lll} k_2k_4k_9(k_6+k_7) & +k_1k_3k_5(k_7+k_9)ABC & +k_1k_3k_6k_8ABP \\ +k_1k_4k_9(k_6+k_7)A & +k_2k_4k_6k_8P & +k_3k_5k_7k_{10}BCQ \\ +k_2k_5k_7k_9C & +k_2k_4k_{10}(k_6+k_7)Q & +k_3k_6k_8k_{10}BPQ \\ +k_1k_3k_9(k_6+k_7)AB & +k_8k_{10}(k_2k_4+k_4k_6+k_2k_6)PQ & +k_2k_5k_8k_{10}CPQ \\ +k_1k_5k_7k_9AC & +k_1k_4k_6k_8AP & +k_1k_3k_5k_8ABCP \\ +k_3k_5k_7k_9BC & +k_2k_5k_7k_{10}CQ & +k_3k_5k_8k_{10}BCPQ \end{array}} \tag{12.44}$$

The general rate equation for the Ter Bi Ping Pong system in reaction (12.42) has the same form as Eq. (12.43) and can be expanded in the same way by writing the denominator terms for this mechanism from Table 3.

The general rate Eq. (12.43) for both Ter Bi systems, written in terms of rate constants, can be transformed into the rate equation written in terms of kinetic constants, in the general form:

$$v_0 = \frac{V_1V_2\left(ABC - \dfrac{PQ}{K_{eq}}\right)}{\text{denominator of rate equation}} \tag{12.45}$$

The rate equation of the Ordered Ter Bi mechanism can be written by expanding the denominator with appropriate kinetic constant terms multiplied by substrate concentration terms given in Table 3.

$$v_0 = \frac{V_1V_2\left(ABC - \dfrac{PQ}{K_{eq}}\right)}{\begin{array}{l} V_2K_{iA}K_{iB}K_C + V_2K_{iB}K_CA + V_2K_{iA}K_BC + V_2K_CAB + V_2K_BAC + V_2K_ABC \\ + V_2ABC + \dfrac{V_1K_Q}{K_{eq}}P + \dfrac{V_1K_P}{K_{eq}}Q + \dfrac{V_1}{K_{eq}}PQ + \dfrac{V_1K_Q}{K_{iA}K_{eq}}AP + \dfrac{V_2K_{iA}K_B}{K_{iQ}}CQ \\ + \dfrac{V_1K_Q}{K_{iA}K_{iB}K_{eq}}ABP + \dfrac{V_2K_A}{K_{iQ}}BCQ + \dfrac{V_2K_AK_{iC}}{K_{iP}K_{iQ}}BPQ + \dfrac{V_2K_{iA}K_B}{K_{iQ}K_{iP}}CPQ \\ + \dfrac{V_1K_Q}{K_{iA}K_{iB}K_{iC}K_{eq}}ABCP + \dfrac{V_2K_A}{K_{iQ}K_{iP}}BCPQ \end{array}} \tag{12.46}$$

The rate equation for the Ter Bi Ping Pong mechanism can be expanded in the same way by taking denominator terms from Table 3.

12.3.2 Definition of Kinetic Constants in Terms of Rate Constants

Ordered Ter Bi mechanism

$$K_A = \frac{\text{coefBC}}{\text{coefABC}} \qquad K_{iA} = \frac{\text{const}}{\text{coefA}} = \frac{\text{coefP}}{\text{coefAP}} = \frac{\text{coefC}}{\text{coefAC}} = \frac{k_2}{k_1}$$

$$K_B = \frac{\text{coefAC}}{\text{coefABC}} \qquad K_{iB} = \frac{\text{coefA}}{\text{coefAB}} = \frac{\text{coefAP}}{\text{coefABP}} = \frac{k_4}{k_3}$$

$$K_C = \frac{\text{coefAB}}{\text{coefABC}} \qquad K_{iC} = \frac{\text{coefBPQ}}{\text{coefBCPQ}} = \frac{\text{coefABP}}{\text{coefABCP}} = \frac{k_6}{k_5}$$

$$K_P = \frac{\text{coefQ}}{\text{coefPQ}} \qquad K_{iP} = \frac{\text{coefCQ}}{\text{coefCPQ}} = \frac{\text{coefBCQ}}{\text{coefBCPQ}} = \frac{k_7}{k_8} \tag{12.47}$$

$$K_Q = \frac{\text{coefP}}{\text{coefPQ}} \qquad K_{iQ} = \frac{\text{const}}{\text{coefQ}} = \frac{\text{coefC}}{\text{coefCQ}} = \frac{\text{coefBC}}{\text{coefBCQ}} = \frac{k_9}{k_{10}}$$

$$\frac{V_1}{E_0} = \frac{\text{numer}_1}{\text{coefAB}} \qquad \frac{V_2}{E_0} = \frac{\text{numer}_2}{\text{coefPQ}} \qquad K_{eq} = \frac{\text{numer}_1}{\text{numer}_2}$$

Ter Bi Ping Pong mechanism

Definition of K_A, K_B, K_C, K_P, and K_Q constants is the same as in the Ordered Ter Bi mechanism; in addition,

$$K_{iA} = \frac{\text{coefP}}{\text{coefAP}} = \frac{\text{coefC}}{\text{coefAC}} = \frac{k_2}{k_1} \qquad K_{iP} = \frac{\text{coefBQ}}{\text{coefBPQ}} = \frac{\text{coefAB}}{\text{coefABP}} = \frac{k_5}{k_6}$$

$$K_{iB} = \frac{\text{coefAP}}{\text{coefABP}} = \frac{k_4}{k_3} \qquad K_{iQ} = \frac{\text{coefC}}{\text{coefCQ}} = \frac{\text{coefBC}}{\text{coefBCQ}} = \frac{k_9}{k_{10}} \tag{12.48}$$

$$K_{iC} = \frac{\text{coefQ}}{\text{coefCQ}} = \frac{\text{coefBQ}}{\text{coefBCQ}} = \frac{k_8}{k_7}$$

12.3.3 Definition of Rate Constants in Terms of Kinetic Constants

Ordered Ter Bi mechanism

$$k_1 = \frac{V_1}{E_0 K_A} \quad k_2 = \frac{V_1 K_{iA}}{E_0 K_A} \quad k_3 = \frac{V_1}{E_0 K_B}$$

$$k_4 = \frac{V_1 K_{iB}}{E_0 K_B} \quad k_5 = \frac{V_1}{E_0 K_C}\left(1 + \frac{k_6}{k_7}\right) \quad \frac{1}{k_6} = \frac{E_0}{V_2} - \frac{1}{k_2} - \frac{1}{k_4} \tag{12.49}$$

$$\frac{1}{k_7} = \frac{E_0}{V_1} - \frac{1}{k_9} \quad k_8 = \frac{V_2}{E_0 K_P}\left(1 + \frac{k_7}{k_6}\right) \quad k_9 = \frac{V_2 K_{iQ}}{E_0 K_Q}$$

$$k_{10} = \frac{V_2}{E_0 K_Q}$$

Ter Bi Ping Pong mechanism

$$k_1 = \frac{V_1}{K_A E_0} \qquad k_2 = \frac{V_1 K_{iA}}{K_A E_0} \tag{12.50}$$

The other rate constants cannot be determined.

12.3.4 Effects of Isomerization

Ordered Ter Bi mechanism

EABC + EPQ isomerize: Calculation of k_5, k_6, k_7 or k_8 is invalid.

EA isomerizes: E/E_o, EAB/E_o, EQ/E_o, k_4, k_9, and k_{10} may still be determined.

EAB isomerizes: E/E_o, EA/E_o, EQ/E_o, k_1, k_2, k_9, and k_{10} may still be determined. (12.51)

EQ isomerizes: E/E_o, EA/E_o, EAB/E_o, k_1, k_2, k_3, and k_4 may still be determined.

Ter Bi Ping Pong mechanism

EAB + FP and/or FC + EQ isomerize: The rate constants and distributions shown below can still be calculated. (12.52)

EA isomerizes: Only E/E_o and F/E_o can be calculated.

12.3.5 Distribution Equations

Ordered Ter Bi mechanism

$$\frac{E}{E_o} = \frac{\frac{V_1K_Q}{K_{eq}}P + V_2K_ABC + V_2K_{iA}K_BC + V_2K_{iA}K_{iB}K_C}{\text{denominator of rate equation}}$$

$$\frac{EA}{E_o} = \frac{\frac{V_1K_Q}{K_{iA}K_{eq}}AP + \frac{V_2K_A}{K_{iA}K_{eq}}PQ + V_2K_BAC + V_2K_{iB}K_CA}{\text{denominator of rate equation}} \qquad (12.53)$$

$$\frac{EAB}{E_o} = \frac{\frac{V_1K_Q}{K_{iA}K_{iB}K_{eq}}ABP + \frac{V_2K_AK_{iC}}{K_{iP}K_{iQ}}BPQ + \frac{V_2K_B}{K_{iB}K_{eq}}PQ + V_2K_CAB}{\text{denominator of rate equation}}$$

$$\frac{(EABC + EPQ)}{E_o} = \frac{\left\{\begin{array}{l}\frac{V_1K_Q}{K_{iA}K_{iB}K_{iC}K_{eq}}ABCP + \frac{V_2K_A}{K_{iP}K_{iQ}}BCPQ + \frac{V_2K_{iA}K_B}{K_{iP}K_{iQ}}CPQ \\ + \left[\frac{V_1}{K_{eq}} - \left(\frac{K_A}{K_{iA}} + \frac{K_B}{K_{iB}}\right)\frac{V_2}{K_{eq}}\right]PQ + \left(V_2 - \frac{V_1K_Q}{K_{iQ}}\right)ABC\end{array}\right\}}{\text{denominator of rate equation}}$$

$$\frac{EQ}{E_o} = \frac{\frac{V_1K_Q}{K_{iQ}}ABC + \frac{V_2K_A}{K_{iQ}}BCQ + \frac{V_2K_{iA}K_B}{K_{iQ}}CQ + \frac{V_1K_P}{K_{eq}}Q}{\text{denominator of rate equation}}$$

Ter Bi Ping Pong mechanism

$\frac{E}{E_0}$: the same as for Ordered Ter Bi, without the numerator constant term.

$\frac{EA}{E_0}$: the same as for Ordered Ter Bi, without the numerator A term. (12.54)

$$\frac{F}{E_0} = \frac{V_2K_CAB + \frac{V_1K_P}{K_{eq}}Q + \frac{V_2K_AK_{iC}}{K_{iQ}}BQ}{\text{denominator of rate equation}}$$

The distribution between the central complexes, EAB + FP and FC + EQ, cannot be determined.

12.3.6 Haldane Relationships

Ordered Ter Bi mechanism

$$K_{eq} = \frac{V_1K_{iQ}K_P}{V_2K_{iA}K_{iB}K_C} = \frac{K_{iQ}K_{iP}}{K_{iA}K_{iB}K_{iC}} \tag{12.55}$$

Ter Bi Ping Pong mechanism

$$K_{eq} = \frac{K_{iQ}K_{iP}}{K_{iA}K_{iB}K_{iC}} = \frac{V_1K_{iQ}K_P}{V_2K_{iA}K_BK_{iC}} = \frac{V_1K_{iP}K_Q}{V_2K_{iA}K_{iB}K_C} = \left(\frac{V_1}{V_2}\right)^2\frac{K_QK_P}{K_{iA}K_BK_C} \tag{12.56}$$

12.4 FULL RATE EQUATIONS FOR TER TER SYSTEMS

12.4.1 General Rate Equations

There are four basic steady-state Ter Ter systems: an Ordered Ter Ter mechanism, and three Ping Pong Ter Ter mechanisms. In trisubstrate reactions with three substrates and three products of reaction, it is possible to envisage three different Ping Pong Ter Ter systems:

(a) Bi Uni Uni Bi Ping Pong Ter Ter,
(b) Bi Bi Uni Uni Ping Pong Ter Ter, and
(c) Hexa Uni Ping Pong Ter Ter.

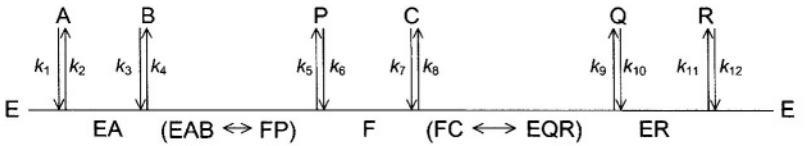
Further variations of the Ter Ter systems, the Random A, B, ordered C; Ordered A, B, random C; and Ordered A, random B, C, were discussed in Section 12.1.

The Ordered Ter Ter sequence is

$$\begin{aligned} &E \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3B}{\rightleftharpoons}} EAB \underset{k_6}{\overset{k_5C}{\rightleftharpoons}} (EABC \rightleftharpoons EPQR) \\ &(EABC \rightleftharpoons EPQR) \underset{k_8P}{\overset{k_7}{\rightleftharpoons}} EQR \underset{k_{10}Q}{\overset{k_9}{\rightleftharpoons}} ER \underset{k_{12}R}{\overset{k_{11}}{\rightleftharpoons}} E \end{aligned} \tag{12.57}$$

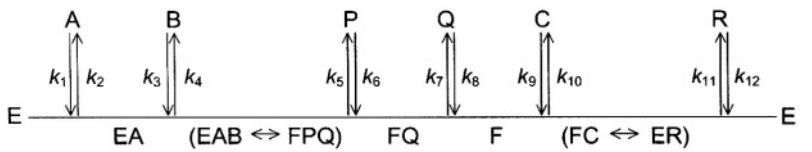
The reaction sequences for the three Ping Pong Ter Ter mechanisms are

Bi Uni Uni Bi Ping Pong Ter Ter system



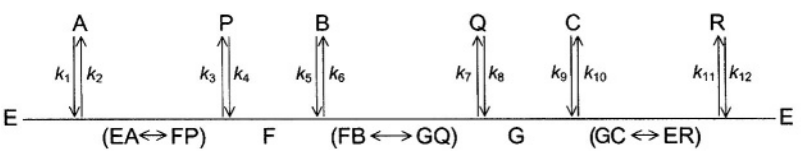
(12.58)

Bi Bi Uni Uni Ping Pong Ter Ter system



(12.59)

Hexa Uni Ping Pong Ter Ter (Goss & Wood, 1982)



(12.60)

The general rate equation, in terms of rate constants, for all Ter Ter mechanisms has always the same form:

$$v_0 = \frac{(k_1k_3k_5k_7k_9k_{11}ABC - k_2k_4k_6k_8k_{10}k_{12}PQR)E_0}{\text{denominator of rate equation}} \tag{12.61}$$

The various Ter Ter mechanisms differ only by the composition of their denominators. Thus, Eq. (12.61) can be expanded into the general rate equation in rate constant form for each Ter Ter mechanism in turn, taking the appropriate denominator terms from Table 4 (Cleland, 1963).

The general rate equation, in terms of kinetic constants, for all Ter Ter mechanisms has always the same form

$$v_0 = \frac{V_1V_2\left(ABC - \dfrac{PQR}{K_{eq}}\right)}{\text{denominator of rate equation}} \tag{12.62}$$

The various Ter Ter mechanisms differ only by the composition of their denominators. Thus, Eq. (12.62) can be expanded into the general rate equation in kinetic constant form for each Ter Ter mechanism in turn, taking the appropriate denominator terms from Table 4.

Note again that the kinetic constant composition next to a given substrate concentration term is always identical for all Ter Ter mechanisms. For example, the kinetic constant composition next to a PQR concentration term is always V_1/K_{eq}, although the rate constant composition may be very different. The same is true for all other denominator terms in all Ter Ter reactions (Table 4).

Table 4 Denominator terms in Ter Ter mechanisms (Cleland, 1963; Plowman, 1972; Fromm, 1975; Segel, 1975)

Denominator	Kinetic constants	Ordered Ter Ter	Bi Uni Uni Bi Ping Pong	Bi Bi Uni Uni Ping Pong	Hexa Uni Ping Pong
Constant	$V_2K_{iA}K_{iB}K_C$	$k_2k_4k_9k_{11}(k_6+k_7)$	—	—	—
A	$V_2K_{iB}K_C$	$k_1k_4k_9k_{11}(k_6+k_7)$	—	—	—
C	$V_2K_{iA}K_B$	$k_2k_5k_7k_9k_{11}$	$k_2k_7k_9k_{11}(k_4+k_5)$	$k_2k_7k_9k_{11}(k_4+k_5)$	—
AB	V_2K_C	$k_1k_3k_9k_{11}(k_6+k_7)$	$k_1k_3k_5k_{11}(k_8+k_9)$	$k_1k_3k_5k_7(k_{10}+k_{11})$	$k_1k_3k_5k_7(k_{10}+k_{11})$
AC	V_2K_B	$k_1k_5k_7k_9k_{11}$	$k_1k_7k_9k_{11}(k_4+k_5)$	$k_1k_7k_9k_{11}(k_4+k_5)$	$k_1k_3k_9k_{11}(k_6+k_7)$
BC	V_2K_A	$k_3k_5k_7k_9k_{11}$	$k_3k_5k_7k_9k_{11}$	$k_3k_5k_7k_9k_{11}$	$k_5k_7k_9k_{11}(k_2+k_3)$
ABC	V_2	$k_1k_3k_5(k_7k_9+k_7k_{11}+k_9k_{11})$	$k_1k_3k_7(k_5k_9+k_5k_{11}+k_9k_{11})$	$k_1k_3k_9(k_5k_7+k_5k_{11}+k_7k_{11})$	$k_1k_5k_9(k_3k_7+k_3k_{11}+k_7k_{11})$
P	$\frac{V_1K_{iR}K_Q}{K_{eq}}$	$k_2k_4k_6k_8k_{11}$	$k_2k_4k_6k_{11}(k_8+k_9)$	—	—
R	$\frac{V_1K_{iQ}K_P}{K_{eq}}$	$k_2k_4k_9k_{12}(k_6+k_7)$	—	$k_2k_7k_{10}k_{12}(k_4+k_5)$	—
PQ	$\frac{V_1K_R}{K_{eq}}$	$k_2k_4k_6k_8k_{10}$	$k_2k_4k_6k_8k_{10}$	$k_2k_4k_6k_8(k_{10}+k_{11})$	$k_2k_4k_6k_8(k_{10}+k_{11})$
PR	$\frac{V_1K_Q}{K_{eq}}$	$k_2k_4k_6k_8k_{12}$	$k_2k_4k_6k_{12}(k_8+k_9)$	$k_2k_4k_6k_{10}k_{12}$	$k_2k_4k_{10}k_{12}(k_6+k_7)$
QR	$\frac{V_1K_P}{K_{eq}}$	$k_2k_4k_{10}k_{12}(k_6+k_7)$	$k_2k_8k_{10}k_{12}(k_4+k_5)$	$k_2k_8k_{10}k_{12}(k_4+k_5)$	$k_6k_8k_{10}k_{12}(k_2+k_3)$
PQR	$\frac{V_1}{K_{eq}}$	$k_8k_{10}k_{12}(k_2k_4+k_2k_6+k_4k_6)$	$k_6k_{10}k_{12}(k_2k_4+k_2k_8+k_4k_8)$	$k_6k_8k_{12}(k_2k_4+k_2k_{10}+k_4k_{10})$	$k_4k_8k_{12}(k_2k_6+k_2k_{10}+k_6k_{10})$

(*continued*)

Denominator	Kinetic constants	Ordered Ter Ter	Bi Uni Uni Bi Ping Pong	Bi Bi Uni Uni Ping Pong	Hexa Uni Ping Pong
AP	$\frac{V_1 K_{iR} K_Q}{K_{iA} K_{eq}}$	$k_1k_4k_6k_8k_{11}$	$k_1k_4k_6k_{11}(k_8 + k_9)$	—	—
AQ	$\frac{V_1 K_{iP} K_R}{K_{iA} K_{eq}}$	—	—	—	$k_1k_3k_6k_8(k_{10} + k_{11})$
BR	$\frac{V_2 K_A K_{iC}}{K_{iR}}$	—	—	$k_3k_5k_7k_{10}k_{12}$	$k_5k_7k_{10}k_{12}(k_2 + k_3)$
CP	$\frac{V_1 K_{iR} K_Q}{K_{iC} K_{eq}}$	—	—	$k_2k_4k_6k_9k_{11}$	$k_2k_4k_9k_{11}(k_6 + k_7)$
CR	$\frac{V_2 K_{iA} K_B}{K_{iR}}$	$k_2k_5k_7k_9k_{12}$	$k_2k_7k_9k_{12}(k_4 + k_5)$	$k_2k_7k_9k_{12}(k_4 + k_5)$	—
ABP	$\frac{V_1 K_{iR} K_Q}{K_{iA} K_{iB} K_{eq}}$	$k_1k_3k_6k_8k_{11}$	$k_1k_3k_6k_{11}(k_8 + k_9)$	—	—
ABQ	$\frac{V_1 K_{iP} K_R}{K_{iA} K_{iB} K_{eq}}$	—	$k_1k_3k_5k_8k_{10}$	$k_1k_3k_5k_8(k_{10} + k_{11})$	$k_1k_3k_5k_8(k_{10} + k_{11})$
ACP	$\frac{V_1 K_Q K_{iR}}{K_{iA} K_{iC} K_{eq}}$	—	—	$k_1k_4k_6k_9k_{11}$	$k_1k_4k_9k_{11}(k_6 + k_7)$
APQ	$\frac{V_1 K_R}{K_{iA} K_{eq}}$	$k_1k_4k_6k_8k_{10}$	$k_1k_4k_6k_8k_{10}$	$k_1k_4k_6k_8(k_{10} + k_{11})$	$k_1k_4k_6k_8(k_{10} + k_{11})$
BCR	$\frac{V_2 K_A}{K_{iR}}$	$k_3k_5k_7k_9k_{12}$	$k_3k_5k_7k_9k_{12}$	$k_3k_5k_7k_9k_{12}$	$k_5k_7k_9k_{12}(k_2 + k_3)$

(*continued*)

Denominator	Kinetic constants	Ordered Ter Ter	Bi Uni Uni Bi Ping Pong	Bi Bi Uni Uni Ping Pong	Hexa Uni Ping Pong
BQR	$\frac{V_2 K_A K_{iC}}{K_{iQ} K_{iR}}$	—	$k_3k_5k_8k_{10}k_{12}$	$k_3k_5k_8k_{10}k_{12}$	$k_5k_8k_{10}k_{12}(k_2 + k_3)$
CPR	$\frac{V_1 K_Q}{K_{iC} K_{eq}}$	—	—	$k_2k_4k_6k_9k_{12}$	$k_2k_4k_9k_{12}(k_6 + k_7)$
CQR	$\frac{V_2 K_{iA} K_B}{K_{iQ} K_{iR}}$	$k_2k_5k_7k_{10}k_{12}$	$k_2k_7k_{10}k_{12}(k_4 + k_5)$	—	—
ABCP	$\frac{V_1 K_Q K_{iR}}{K_{iA} K_{iB} K_{iC} K_{eq}}$	$k_1k_3k_5k_8k_{11}$	—	$k_1k_3k_6k_9k_{11}$	—
ABCQ	$\frac{V_1 K_{iP} K_R}{K_{iA} K_{iB} K_{iC} K_{eq}}$	$k_1k_3k_5k_7k_{10}$	$k_1k_3k_5k_7k_{10}$	—	—
ABPQ	$\frac{V_1 K_R}{K_{iA} K_{iB} K_{eq}}$	$k_1k_3k_6k_8k_{10}$	$k_1k_3k_6k_8k_{10}$	$k_1k_3k_6k_8(k_{10} + k_{11})$	—
BCQR	$\frac{V_2 K_A}{K_{iQ} K_{iR}}$	$k_3k_5k_7k_{10}k_{12}$	$k_3k_5k_7k_{10}k_{12}$	—	—
BPQR	$\frac{V_2 K_A K_{iC}}{K_{iP} K_{iQ} K_{iR}}$	$k_3k_6k_8k_{10}k_{12}$	$k_3k_6k_8k_{10}k_{12}$	$k_3k_6k_8k_{10}k_{12}$	—
CPQR	$\frac{V_2 K_{iA} K_B}{K_{iP} K_{iQ} K_{iR}}$	$k_2k_5k_8k_{10}k_{12}$	—	—	—
ABCPQ	$\frac{V_1 K_R}{K_{iA} K_{iB} K_{iC} K_{eq}}$	$k_1k_3k_5k_8k_{10}$	—	—	—
BCPQR	$\frac{V_2 K_A}{K_{iP} K_{iQ} K_{iR}}$	$k_3k_5k_8k_{10}k_{12}$	—	—	—

12.4.2 Definition of Kinetic Constants in Terms of Rate Constants

Ordered Ter Ter System

Kinetic coefficients for the constants K_A, K_B, K_C, K_P, K_Q, V_1 and V_2 are the same as in the Ordered Ter Bi system. In addition, inhibition constants are

$$
\begin{aligned}
K_A &= \frac{\text{coefBC}}{\text{coefABC}} & K_{iA} &= \frac{\text{const}}{\text{coefA}} = \frac{\text{coefC}}{\text{coefAC}} = \frac{\text{coefP}}{\text{coefAP}} = \frac{\text{coefPQ}}{\text{coefAPQ}} = \frac{k_2}{k_1} \\
K_B &= \frac{\text{coefAC}}{\text{coefABC}} & K_{iB} &= \frac{\text{coefA}}{\text{coefAB}} = \frac{\text{coefAP}}{\text{coefABP}} = \frac{\text{coefAPQ}}{\text{coefABPQ}} = \frac{k_4}{k_3} \\
K_C &= \frac{\text{coefAB}}{\text{coefABC}} & K_{iC} &= \frac{\text{coefABP}}{\text{coefABCP}} = \frac{\text{coefABPQ}}{\text{coefABCPQ}} = \frac{\text{coefBPQR}}{\text{coefBCPQR}} = \frac{k_6}{k_5} \\
K_P &= \frac{\text{coefQR}}{\text{coefPQR}} & K_{iP} &= \frac{\text{coefCQR}}{\text{coefCPQR}} = \frac{\text{coefABCQ}}{\text{coefABCPQ}} = \frac{\text{coefBCQR}}{\text{coefBCPQR}} = \frac{k_7}{k_8} \\
K_Q &= \frac{\text{coefPR}}{\text{coefPQR}} & K_{iQ} &= \frac{\text{coefR}}{\text{coefQR}} = \frac{\text{coefCR}}{\text{coefCQR}} = \frac{\text{coefBCR}}{\text{coefBCQR}} = \frac{k_9}{k_{10}} \\
K_R &= \frac{\text{coefPQ}}{\text{coefPQR}} & K_{iR} &= \frac{\text{const}}{\text{coefR}} = \frac{\text{coefP}}{\text{coefPR}} = \frac{\text{coefC}}{\text{coefCR}} = \frac{\text{coefBC}}{\text{coefBCR}} = \frac{k_{11}}{k_{12}}
\end{aligned}
\tag{12.63}
$$

Bi Uni Uni Bi Ping Pong Ter Ter system

The definition of Michaelis constants, V_1, and V_2 is the same as above. In addition,

$$
\begin{aligned}
K_{iA} &= \frac{\text{coefC}}{\text{coefAC}} = \frac{\text{coefP}}{\text{coefAP}} & K_{iP} &= \frac{\text{coefAB}}{\text{coefABP}} = \frac{\text{coefABQ}}{\text{coefABPQ}} \\
&= \frac{\text{coefPQ}}{\text{coefAPQ}} = \frac{k_2}{k_1} & &= \frac{\text{coefBQR}}{\text{coefBPQR}} = \frac{k_5}{k_6} \\
K_{iB} &= \frac{\text{coefAP}}{\text{coefABP}} = \frac{\text{coefAPQ}}{\text{coefABPQ}} = \frac{k_4}{k_3} & K_{iQ} &= \frac{\text{coefCR}}{\text{coefCQR}} = \frac{\text{coefBCR}}{\text{coefBCQR}} = \frac{k_9}{k_{10}} \\
K_{iC} &= \frac{\text{coefQR}}{\text{coefCQR}} = \frac{\text{coefABQ}}{\text{coefABCQ}} & K_{iR} &= \frac{\text{coefP}}{\text{coefPR}} = \frac{\text{coefC}}{\text{coefCR}} \\
&= \frac{\text{coefBQR}}{\text{coefBCQR}} = \frac{k_8}{k_7} & &= \frac{\text{coefBC}}{\text{coefBCR}} = \frac{k_{11}}{k_{12}}
\end{aligned}
\tag{12.64}
$$

Bi Bi Uni Uni Ping Pong Ter Ter system

The definition of kinetic constants K_A, K_B, K_C, K_P, K_Q, K_R, V_1, and V_2, in a coefficient form, is the same as in the Ordered Ter Ter system. In addition,

$$
\begin{aligned}
K_{iA} &= \frac{\text{coefC}}{\text{coefAC}} = \frac{\text{coefPQ}}{\text{coefAPQ}} & K_{iP} &= \frac{\text{coefABQ}}{\text{coefABPQ}} \\
&= \frac{\text{coefCP}}{\text{coefACP}} = \frac{k_2}{k_1} & &= \frac{\text{coefBQR}}{\text{coefBPQR}} = \frac{k_5}{k_6}
\end{aligned}
$$

$$K_{iB} = \frac{\text{coefAPQ}}{\text{coefABPQ}} = \frac{\text{coefACP}}{\text{coefABCP}} = \frac{k_4}{k_3} \qquad K_{iQ} = \frac{\text{coefR}}{\text{coefQR}} = \frac{\text{coefBR}}{\text{coefBQR}} = \frac{\text{coefAB}}{\text{coefABQ}} = \frac{k_7}{k_8}$$
$$K_{iC} = \frac{\text{coefBR}}{\text{coefBCR}} = \frac{\text{coefR}}{\text{coefCR}} = \frac{\text{coefPR}}{\text{coefCPR}} = \frac{k_{10}}{k_9} \qquad K_{iR} = \frac{\text{coefC}}{\text{coefCR}} = \frac{\text{coefBC}}{\text{coefBCR}} = \frac{\text{coefCP}}{\text{coefCPR}} = \frac{k_{11}}{k_{12}} \tag{12.65}$$

Hexa Uni Ping Pong Ter Ter system

The definition of kinetic constants K_A, K_B, K_C, K_P, K_Q, K_R, V_1, and V_2, in a coefficient form, is the same as in the Ordered Ter Ter system. In addition,

$$K_{iA} = \frac{\text{coefPQ}}{\text{coefAPQ}} = \frac{\text{coefCP}}{\text{coefACP}} = \frac{k_2}{k_1} \qquad K_{iP} = \frac{\text{coefAC}}{\text{coefACP}} = \frac{\text{coefAQ}}{\text{coefAPQ}} = \frac{k_3}{k_4}$$
$$K_{iB} = \frac{\text{coefAQ}}{\text{coefABQ}} = \frac{\text{coefQR}}{\text{coefBQR}} = \frac{k_6}{k_5} \qquad K_{iQ} = \frac{\text{coefBR}}{\text{coefBQR}} = \frac{\text{coefAB}}{\text{coefABQ}} = \frac{k_7}{k_8} \tag{12.66}$$
$$K_{iC} = \frac{\text{coefBR}}{\text{coefBCR}} = \frac{\text{coefPR}}{\text{coefCPR}} = \frac{k_{10}}{k_9}$$

12.4.3 Definition of Rate Constants in Terms of Kinetic Constants

Ordered Ter Ter mechanism

Equations for rate constants k_1, k_2, k_3, k_4, k_5, k_6, k_8, k_9, and k_{10}, are the same as for the Ordered Ter Bi system, but k_7 is different:

$$k_1 = \frac{V_1}{E_0 K_A} \qquad k_2 = \frac{V_1 K_{iA}}{E_0 K_A}$$
$$k_3 = \frac{V_1}{E_0 K_B} \qquad k_4 = \frac{V_1 K_{iB}}{E_0 K_B}$$
$$k_5 = \frac{V_1}{E_0 K_C}\left(1 + \frac{k_6}{k_7}\right) \qquad \frac{1}{k_6} = \frac{E_0}{V_2} - \frac{1}{k_2} - \frac{1}{k_4}$$
$$\frac{1}{k_7} = \frac{E_0}{V_1} - \frac{1}{k_9} - \frac{1}{k_{11}} \qquad k_8 = \frac{V_2}{E_0 K_P}\left(1 + \frac{k_7}{k_6}\right) \tag{12.67}$$
$$k_9 = \frac{V_2 K_{iQ}}{E_0 K_Q} \qquad k_{10} = \frac{V_2}{E_0 K_Q}$$
$$k_{11} = \frac{V_2 K_{iR}}{E_0 K_R} \qquad k_{12} = \frac{V_2}{E_0 K_R}$$

Bi Uni Uni Bi Ping Pong Ter Ter system

Rate constants k_1, k_2, k_{11}, and k_{12}, have the same meaning as in the Ordered Ter Ter system. The other rate constants cannot be determined. (12.68)

Bi Bi Uni Uni Ping Pong Ter Ter system

$$k_1 = \frac{V_1}{E_0 K_A} \quad k_2 = \frac{V_1 K_{iA}}{E_0 K_A}$$
$$k_7 = \frac{V_2 K_{iQ}}{E_0 K_Q} \quad k_8 = \frac{V_2}{E_0 K_Q} \tag{12.69}$$

The remaining constants cannot be calculated from the kinetic constants.

Hexa Uni Ping Pong Ter Ter system

None of the rate constants can be calculated. (12.70)

12.4.4 Distribution Equations

Ordered Ter Ter mechanism

$$\frac{E}{E_0} = \frac{V_2 K_{iA} K_{iB} K_C + V_2 K_A BC + V_2 K_{iA} K_B C + \frac{V_1 K_Q K_{iR}}{K_{eq}} P + \frac{V_1 K_R}{K_{eq}} PQ}{\text{denominator of rate equation}}$$

$$\frac{EA}{E_0} = \frac{V_2 K_{iB} K_C A + V_2 K_B AC + \frac{V_1 K_Q K_{iR}}{K_{iA} K_{eq}} AP + \frac{V_1 K_R}{K_{iA} K_{eq}} APQ + \frac{V_2 K_A}{K_{iA} K_{eq}} PQR}{\text{denominator of rate equation}}$$

$$\frac{EAB}{E_0} = \frac{V_2 K_C AB + \frac{V_1 K_Q K_{iR}}{K_{iA} K_{iB} K_{eq}} ABP + \frac{V_1 K_R}{K_{iA} K_{iB} K_{eq}} ABPQ + \frac{V_2 K_B}{K_{iB} K_{eq}} PQR + \frac{V_2 K_A K_{iC}}{K_{iP} K_{iQ} K_{iR}} BPQR}{\text{denominator of rate equation}}$$

$$\frac{(EABC + EPQR)}{E_0} = \frac{\left\{ \begin{array}{l} \frac{V_1 K_Q K_{iR}}{K_{iA} K_{iB} K_{iC} K_{eq}} ABCP + \frac{V_2 K_A}{K_{iP} K_{iQ} K_{iR}} BCPQR + \frac{V_2 K_{iA} K_B}{K_{iP} K_{iQ} K_{iR}} CPQR \\ + \frac{V_1 K_R}{K_{iA} K_{iB} K_{iC} K_{eq}} ABCPQ + \left[\frac{V_1}{K_{eq}} - \left(\frac{K_A}{K_{iA}} + \frac{K_B}{K_{iB}} \right) \frac{V_2}{K_{eq}} \right] PQR \\ + \left[V_2 - V_1 \left(\frac{K_Q}{K_{iQ}} + \frac{K_R}{K_{iR}} \right) \right] ABC \end{array} \right\}}{\text{denominator of rate equation}}$$

$$\frac{EQR}{E_0} = \frac{\frac{V_1 K_Q}{K_{iQ}} ABC + \frac{V_1 K_{iP} K_R}{K_{iA} K_{iB} K_{iC} K_{eq}} ABCQ + \frac{V_2 K_A}{K_{iQ} K_{iR}} BCQR + \frac{V_2 K_{iA} K_B}{K_{iQ} K_{iR}} CQR + \frac{V_1 K_P}{K_{eq}} QR}{\text{denominator of rate equation}}$$

$$\frac{ER}{E_0} = \frac{\frac{V_1 K_R}{K_{iR}} ABC + \frac{V_2 K_A}{K_{iR}} BCR + \frac{V_2 K_{iA} K_B}{K_{iR}} CR + \frac{V_1 K_P K_{iQ}}{K_{eq}} R + \frac{V_1 K_Q}{K_{eq}} PR}{\text{denominator of rate equation}} \tag{12.71}$$

Bi Uni Uni Bi Ping Pong Ter Ter system

$\frac{E}{E_0}$: the same as Ordered Ter Ter, without the numerator constant term.

$$\frac{EA}{E_o}\text{: the same as Ordered Ter Ter, without the numerator } A \text{ term,}$$
$$\frac{ER}{E_o}\text{: the same as Ordered Ter Ter, without the numerator } R \text{ term.} \qquad (12.72)$$

$$\frac{F}{E_o} = \frac{V_2K_CAB + \frac{V_1K_{iP}K_R}{K_{iA}K_{iB}K_{eq}}ABQ + \frac{V_1K_P}{K_{eq}}QR + \frac{V_2K_AK_{iC}}{K_{iQ}K_{iR}}BQR}{\text{denominator of rate equation}}$$

The distribution between the central complexes, EAB+FP and FC+EQR, cannot be determined.

Bi Bi Uni Uni Ping Pong Ter Ter system

$$\frac{E}{E_o} = \frac{V_2K_ABC + V_2K_{iA}K_BC + \frac{V_1K_R}{K_{eq}}PQ + \frac{V_1K_QK_{iR}}{K_{iC}K_{eq}}CP}{\text{denominator of rate equation}}$$

$$\frac{EA}{E_o} = \frac{V_2K_BAC + \frac{V_1K_QK_{iR}}{K_{iA}K_{iC}K_{eq}}ACP + \frac{V_1K_R}{K_{iA}K_{eq}}APQ + \frac{V_2K_A}{K_{iA}K_{eq}}PQR}{\text{denominator of rate equation}}$$

$$\frac{FQ}{E_o} = \frac{\frac{V_1K_Q}{K_{iQ}}ABC + \frac{V_1K_{iP}K_R}{K_{iA}K_{iB}K_{eq}}ABQ + \frac{V_1K_P}{K_{eq}}QR + \frac{V_2K_AK_{iC}}{K_{iQ}K_{iR}}BQR}{\text{denominator of rate equation}} \qquad (12.73)$$

$$\frac{F}{E_o} = \frac{V_2K_CAB + \frac{V_1K_{iQ}K_P}{K_{eq}}R + \frac{V_1K_Q}{K_{eq}}PR + \frac{V_2K_AK_{iC}}{K_{iR}}BR}{\text{denominator of rate equation}}$$

The distribution between central complexes can not be determined.

Hexa Uni Ping Pong Ter Ter system

$$\frac{E}{E_o} = \frac{V_2K_ABC + \frac{V_1K_QK_{iR}}{K_{iC}K_{eq}}CP + \frac{V_1K_R}{K_{eq}}PQ}{\text{denominator of rate equation}}$$

$$\frac{F}{E_o} = \frac{V_2K_BAC + \frac{V_1K_{iP}K_R}{K_{iA}K_{eq}}AQ + \frac{V_1K_P}{K_{eq}}QR}{\text{denominator of rate equation}} \qquad (12.74)$$

$$\frac{G}{E_o} = \frac{V_2K_CAB + \frac{V_2K_AK_{ic}}{K_{iR}}BR + \frac{V_1K_Q}{K_{eq}}PR}{\text{denominator of rate equation}}$$

The distribution between central complexes can not be determined.

12.4.5 Effects of Isomerization

Ordered Ter Ter mechanism

(EABC + EPQR) isomerize: Distributions valid. The calculation of $k_5, k_6, k_7,$ and k_8 constants is invalid.
EA isomerizes: The calculation of $k_1, k_2, k_3, k_5, k_6, k_7,$ and k_8 constants is invalid. The calculation of EA/E_0 and $(EABC + EPQR)/E_0$ is invalid.
EAB isomerizes: The calculation of k_3, k_4, k_5, k_6, k_7, and k_8 constants is invalid. The calculation of EAB/E_0 and $(EABC + EPQR)/E_0$ is invalid. (12.75)

Bi Uni Uni Bi Ping Pong Ter Ter system

(EAB + FP) and/or (FC + EQR) isomerize: All the distribution and rate constants shown above may be calculated.
EA isomerizes: The calculations of k_1, k_2, and EA/E_0 are invalid.
ER isomerizes: The calculations of k_{11}, k_{12}, and ER/E_0 are invalid. (12.76)

Bi Bi Uni Uni Ping Pong Ter Ter system

EAB + FPQ and/or FC + ER isomerize: All the rate constants and distributions shown can be calculated.
EA isomerizes: The calculation of k_1 and k_2, and EA/E_0 is invalid.
FQ isomerizes: The calculation of k_7 and k_8, and FQ/E_0 is invalid. (12.77)

Hexa Uni Ping Pong Ter Ter System

Isomerization of the central complexes has no effect on the distribution equations. (12.78)

12.4.6 Haldane Relationships

Ordered Ter Ter mechanism

$$K_{eq} = \frac{K_{iP}K_{iQ}K_{iR}}{K_{iA}K_{iB}K_{iC}} = \frac{V_1K_PK_{iQ}K_{iR}}{V_2K_{iA}K_{iB}K_C} \tag{12.79}$$

Bi Uni Uni Bi Ping Pong Ter Ter system

$$K_{eq} = \frac{K_{iP}K_{iQ}K_{iR}}{K_{iA}K_{iB}K_{iC}} = \frac{V_1K_{iQ}K_PK_{iR}}{V_2K_{iA}K_BK_{iC}} = \frac{V_1K_{iP}K_QK_{iR}}{V_2K_{iA}K_{iB}K_C} = \left(\frac{V_1}{V_2}\right)^2 \frac{K_QK_PK_{iR}}{K_{iA}K_BK_C} \tag{12.80}$$

Bi Bi Uni Uni Ping Pong Ter Ter system

$$K_{eq} = \frac{K_{iP}K_{iQ}K_{iR}}{K_{iA}K_{iB}K_{iC}} = \frac{V_1K_{iQ}K_PK_{iR}}{V_2K_{iA}K_BK_{iC}} = \frac{V_1K_{iP}K_{iQ}K_R}{V_2K_{iA}K_{iB}K_C} = \left(\frac{V_1}{V_2}\right)^2 \frac{K_PK_{iQ}K_R}{K_{iA}K_BK_C} \qquad (12.81)$$

Hexa Uni Ping Pong Ter Ter system

$$\begin{aligned} K_{eq} &= \frac{K_{iP}K_{iQ}K_{iR}}{K_{iA}K_{iB}K_{iC}} = \frac{V_1K_{iP}K_QK_{iR}}{V_2K_{iA}K_BK_{iC}} = \frac{V_1K_{iP}K_{iQ}K_R}{V_2K_{iA}K_{iB}K_C} = \frac{V_1K_PK_{iQ}K_{iR}}{V_2K_AK_{iB}K_{iC}} \\ &= \left(\frac{V_1}{V_2}\right)^2 \frac{K_PK_QK_{iR}}{K_AK_BK_{iC}} = \left(\frac{V_1}{V_2}\right)^2 \frac{K_PK_{iQ}K_R}{K_AK_{iB}K_C} = \left(\frac{V_1}{V_2}\right)^2 \frac{K_{iP}K_QK_R}{K_{iA}K_BK_C} \\ &= \left(\frac{V_1}{V_2}\right)^3 \frac{K_PK_QK_R}{K_AK_BK_C} \end{aligned} \qquad (12.82)$$

12.5 PRODUCT INHIBITION IN TRISUBSTRATE MECHANISMS

The general rate equation for all Ter Ter mechanisms has always the same form

$$v_0 = \frac{V_1V_2\left(ABC - \dfrac{PQR}{K_{eq}}\right)}{\text{denominator of rate equation}}$$

Thus, the various Ter Ter mechanisms differ only by the composition of their denominators, as is shown in Table 4.

Similarly, the general rate equation for all Ter Bi mechanisms has always the same form

$$v_0 = \frac{V_1V_2\left(ABC - \dfrac{PQ}{K_{eq}}\right)}{\text{denominator of rate equation}}$$

and the various mechanisms differ only by the composition of their denominators (Table 3).

Although each trisubstrate mechanism has a unique general rate equation, the rate equations in the absence of the products of reaction sometimes have identical forms for several Ter Bi and Ter Ter mechanisms. Therefore, in order to identify unequivocally the mechanism, we must revert to the product inhibition analysis and the use of dead-end inhibitors.

The rate equations in the presence of one of the products of reaction can be written down directly from the general rate equations, simply by omitting the terms in the denominator and in the numerator that contain the concentration terms for other two products of reaction. The initial rate equations, in the presence of the products of reaction, are usually very complex for trisubstrate reactions.

Let us illustrate the product inhibition patterns with an example of the Ordered Ter Ter mechanism (Section 12.4). In the presence of all the substrates of reaction, A, B, C, and the product Q, the rate equation is

$$v_0 = \frac{V_1 ABC}{K_{iA}K_{iB}K_C + K_{iB}K_C A + K_{iA}K_B C + K_C AB + K_B AC + K_A BC + ABC + \dfrac{K_C K_{iP} K_R}{K_{iC} K_P K_{iQ} K_{iR}} ABCQ} \tag{12.83}$$

The rate Eq. (12.83) can be written down directly from the general rate equation for the Ordered Ter Ter mechanism (Table 4), simply by omitting all the concentration terms that contain P and R and eliminating the K_{eq} with the aid of Haldane relationships. Equation (12.83) is identical with the rate Eq. (12.26) for the Ordered Ter Ter mechanism, in the absence of products, except that a new term, $\Phi ABCQ$, is added in the denominator:

$$\Phi = \frac{K_C K_{iP} K_R}{K_{iC} K_P K_{iQ} K_{iR}} \tag{12.84}$$

Equation (12.83) can be rearranged to show any of the three substrates as the varied ligand at fixed concentrations of the other two ligands:

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_B}{B} + \frac{K_C}{C} + \frac{K_{iB}K_C}{BC} + \Phi Q\right) + \frac{K_A}{V_1}\left(1 + \frac{K_{iA}K_B}{K_A B} + \frac{K_{iA}K_{iB}K_C}{K_A BC}\right)\frac{1}{A} \tag{12.85}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_A}{A} + \frac{K_C}{C} + \Phi Q\right) + \frac{K_B}{V_1}\left(1 + \frac{K_{iA}}{A}\right)\left(1 + \frac{K_{iB}K_C}{K_B C}\right)\frac{1}{B} \tag{12.86}$$

$$\frac{1}{v_0} = \frac{1}{V_1}\left(1 + \frac{K_A}{A} + \frac{K_B}{B} + \frac{K_{iA}K_B}{AB} + \Phi Q\right) + \frac{K_C}{V_1}\left(1 + \frac{K_{iB}}{B} + \frac{K_{iA}K_{iB}}{AB}\right)\frac{1}{C} \tag{12.87}$$

Equations (12.85)–(12.87) illustrate a rather complex nature of rate equations for trisubstrate reactions if they are written in the presence of one of the products of reaction.

Therefore, in order to simplify the product analysis of trisubstrate reactions, and for a proper interpretation of product inhibition patterns, we shall need a suitable comparative overview of trisubstrate mechanisms. Table 5 lists the product inhibition patterns for the major Ter Bi and Ter Ter mechanisms. Table 5 shows that, ultimately, each mechanism can be identified unequivocally on the basis of its unique product inhibition patterns. Product inhibition analysis is also able to identify unequivocally each substrate in a given mechanism (Plowman, 1972; Fromm, 1975, 1995).

Product inhibition analyses are laborious procedures in enzyme kinetics, but their power is well illustrated by examples given in Table 5.

Table 5 Product inhibition patterns for trisubstrate reactions (Plowman, 1972; Fromm, 1975; Segel, 1975)

		Varied substrate								
		A			B			C		
Mechanism	Product inhibitor	Unsaturated	Saturated with B	Saturated with C	Unsaturated	Saturated with A	Saturated with C	Unsaturated	Saturated with A	Saturated with B
Ordered Ter Bi	P	N	UC	UC	N	N	UC	N	N	N
	Q	C	C	C	N	—	N	N	—	UC
Ordered Bi Ter	P	N	UC		N	N				
	Q	UC	UC		UC	UC				
	R	C	C		N	—				
Bi Uni Uni Uni	P	N	UC	—	N	N	—	C	C	C
Ping Pong Ter Bi	Q	C	C	C	N	—	N	N	—	N
Ordered Ter Ter	P	N	UC	UC	N	N	UC	N	N	N
	Q	UC	UC	UC	UC	UC	UC	UC	UC	UC
	R	C	C	C	N	—	N	N	—	UC
Bi Uni Uni Bi	P	N	UC	—	N	N	—	C	C	C
Ping Pong Ter Ter	Q	UC	UC	UC	UC	UC	UC	N	N	N
	R	C	C	C	N	—	N	UC	—	UC
Bi Bi Uni Uni	P	N	UC	N	N	N	N	UC	UC	UC
Ping Pong Ter Ter	Q	UC	UC	—	UC	UC	—	C	C	C
	R	C	C	C	N	—	N	N	—	N
Hexa Uni	P	N	—	N	C	C	C	UC	UC	—
Ping Pong Ter Ter	Q	UC	UC	—	N	N	—	C	C	C
	R	C	C	C	UC	—	UC	N	—	UC

Type of inhibition: C = competitive; N = noncompetitive; UC = uncompetitive.

12.6 EXAMPLES OF ENZYME TRISUBSTRATE MECHANISMS

Mechanism	Enzyme		References
Random AB, Random QR	Guanylate cyclase	EC 4.6.4.15	Garbers *et al.*, 1974; Jonhson & Corbin, 1991
Random BC, Random PQ	Citrate cleavage enzyme	EC 4.1.3.8	Plowman & Cleland, 1967
	γ-Glutamyl cysteine synthetase	EC 6.3.2.2	Yip & Rudolph, 1976; Fahey & Sundquist, 1991
Ordered Bi Ter	Malic enzymes	EC 1.1.1.38	Hsu *et al.*, 1967
Ordered Ter Bi	Glyceraldehyde-3-P dehydrogenase	EC 1.2.1.13	Orsi & Cleland, 1972; Harris & Waters, 1973
Ordered Ter Ter	Glutamate dehydrogenase (pH 8.8)	EC 1.4.1.4	Silverstein, 1974; Colman, 1990
Random Ter Ter	Adenylosuccinate synthase	EC 6.3.4.4	Rudolph & Fromm, 1969; Markham & Reed, 1977; Cooper & Rudolph, 1995
	Glutamine synthetase	EC 6.3.1.2	Wedler & Boyer, 1972; Villafranca & Nowak, 1992; Purich, 1998
	Glutathione synthetase	EC 6.3.2.3	Wendel & Heinle, 1975; Meister, 1985
	Formate-tetrahydrofolate ligase	EC 6.3.4.3	Joyce & Hines, 1966; Rader & Huennekens, 1973
Bi Uni Uni Uni Ping Pong Ter Ter	NAD^+-specific glyceraldehyde-3-P dehydrogenase	EC 1.2.1.12	Duggleby & Denis, 1974; Harris & Waters, 1973
Bi Uni Uni Bi Ping Pong Ter Ter	Meliolate hydroxylase	EC 1.14.13.4	Strickland & Massey, 1973; Massey & Hemmerich, 1975
	Threonyl-tRNA synthetase	EC 6.1.1.3	Allende *et al.*, 1970
Bi Uni Uni Bi (random) Ping Pong Ter Ter	Leucyl-tRNA synthetase	EC 6.1.1.4	Hampel & Tritz, 1988
Hexa Uni Ping Pong Ter Ter	Pyruvate, phosphate dikinase	EC 2.7.9.1	Milner *et al.*, 1978; Frey, 1992; Raushel & Villafranca, 1988

REFERENCES

Books

Plowman, K.M. (1972) *Enzyme kinetics,* McGraw-Hill, New York.
Fromm, H.J. (1975) *Initial rate enzyme kinetics,* Springer-Verlag, Berlin.
Segel, I.H. (1975) *Enzyme kinetics,* Wiley, New York.

Review articles

Cleland, W.W. (1967) *Ann. Rev. Biochem. 36,* 77–112.
Colman, R.F. (1990) *The Enzymes,* 3rd ed., Vol. 19, 283–300.
Cooper, B.F. & Rudolph, F.B. (1995) *Methods Enzymol. 249,* 188–211.
Fahey, R.C. & Sundquist, A.R. (1991) *Adv. Enzymol. Relat. Areas Mol. Biol. 64,* 1–5.
Fromm, H.J. (1979) *Methods Enzymol. 63,* 42–53.
Fromm, H.J. (1995) *Methods Enzymol. 249,* 123–144.
Frey, P.A. (1992) *The Enzymes,* 3rd ed., Vol. 20, 141–170.
Goss, H. & Wood, H.G. (1982) *Methods Enzymol. 87,* 51–66.
Hampel, A. & Tritz, R. (1988) *Methods Enzymol. 166,* 260–265.
Harris, J.I. & Waters, M. (1973) *The Enzymes,* 3rd ed., Vol. 13, 1–50.
Johnson, R.A. & Corbin, J.D. (eds.) (1991) *Methods Enzymol. 195,* 100–120.
Massey, V. & Hemmerich, P. (1975) *The Enzymes,* 3rd ed., Vol. 12, 191–230.
Meister, A. (1985) *Methods Enzymol. 113,* 185–199.
Meister, A. (1985) *Methods Enzymol. 113,* 393–400.
Purich, D.L. (1998) *Adv. Enzymol. 72,* 9–42.
Rader, J.I. & Huennekens, F.M. (1973) *The Enzymes,* 3rd ed., Vol. IX, 197–240.
Raushel, F.M. & Villafranca, J.J. (1988) *CRC Crit. Rev. Biochem. 23,* 1–26.
Rudolph, F.B. & Fromm, H.J. (1979) *Methods Enzymol. 63,* 138–159.
Villafranca, J.J. & Nowak, T. (1992) *The Enzymes,* 3rd ed., Vol. 20, 63–100.
Viola, R.E. & Cleland, W.W. (1982) *Methods Enzymol. 87,* 353–366.

Specific references

Allende, C.C., Chaimovich, H., Gatica, M., & Allende, J.E. (1970) *J. Biol. Chem. 245,* 93–100.
Cleland, W.W. (1963) *Biochim. Biophys. Acta* 67, 104–137.
Cleland, W.W. (1963) *Biochim. Biophys. Acta* 67, 173–187.
Cleland, W.W. (1963) *Biochim. Biophys. Acta* 67, 188–196.
Duggleby, R.G. & Dennis, D.T. (1974) *J. Biol. Chem. 249,* 167–174.
Frieden, C. (1959) *J. Biol. Chem. 234,* 2891–2900.
Fromm, H.J. (1967) *Biochim. Biophys. Acta 139,* 221–231.
Garbers, D.L., Hardman, J.G., & Rudolph, F.B. (1974) *Biochemistry 13,* 4166–4176.
Heyde, E., Nagabhushanam, A., Vonarx, M., & Morrison, J.F. (1976) *Bioch. Biophys. Acta 429,* 645–660.
Hsu, R.Y., Lardy, H.A., & Cleland, W.W. (1967) *J. Biol. Chem. 242,* 5315–5322.
Joyce, B.K. & Hines, R.H. (1966) *J. Biol. Chem. 241,* 5716–5724.
Markham, G.D. & Reed, G.H. (1977) *Arch. Biochem. Biophys. 184,* 24–34.
Milner, Y., Michaels, G., & Wood, H.G. (1978) *J. Biol. Chem. 253,* 878–883.
Morrison, J.F. & Ebner, K.E. (1971) *J. Biol. Chem. 246,* 3977–3984.
Orsi, B.A. & Cleland, W.W. (1972) *Biochemistry 11,* 122–132.
Plowman, K.M. & Cleland, W.W. (1967) *J. Biol. Chem. 242,* 4239–4247.
Rife, J.E. & Cleland, W.W. (1980) *Biochemistry 19,* 2321–2328.
Rose, A., O'Connell, E.L., Litwin, S.L., & Bar-Tana, J. (1974) *J. Biol. Chem. 249,* 5163–5168.
Rudolph, F.B. & Fromm, H.J. (1969) *J. Biol. Chem. 244,* 3832–3839.
Silverstein, E. (1974) *Biochemistry 13,* 3750–3754.
Strickland, S. & Massey, V. (1973) *J. Biol. Chem. 248,* 2953–3963.
Wedler, F.C. & Boyer, P.D. (1972) *J. Biol. Chem. 247,* 984–992.
Wendel, A. & Heinle, H. (1975) *Hoppe-Seyler's Z. Physiol. Chemie 356,* 33–43.
Yip, B.P. & Rudolph, F.B. (1976) *J. Biol. Chem. 251,* 3563–3568.

This page intentionally left blank

Chapter 13

Cooperative and Allosteric Effects

All biological events, at some point, require the precise and selective binding of one molecule to another, usually a ligand by an enzyme or a protein, or a substrate by an enzyme. The specific nature of such specific recognitions and their consequences is the basis of all chemical processes in nature, diverse as the immune response, nervous activity, mobility, selective gene expression, signaling within and between cells, and an ordered and regulated metabolism (Chock *et al.,* 1988; Engel, 1996; Klotz, 1997).

13.1 COOPERATIVE VERSUS ALLOSTERIC EFFECTS

It is obvious that all living organisms have a need for a high degree of control over metabolic processes so as to permit orderly change without precipitating unwanted progress towards thermodynamic equilibrium.

The activity of majority of enzymes is regulated by the concentration of their own substrates in a Michaelis–Menten fashion. However, such a control may be insufficient for some metabolic purposes. For example, in order to increase the velocity of a simple Michaelian enzyme from $0.1V$ to $0.9V$, it is necessary to increase the substrate concentration from $K_M/9$ to $9K_M$, that is, an 81-fold increase. Similarly, an 81-fold increase in inhibitor concentration is required to reduce the velocity from 90% to 10% of the uninhibited value. On one hand, the concentration of metabolites *in vivo* vary within relatively narrow limits while, on the other hand, the activity of specific enzymes must be increased or decreased within very large limits. Consequently, in addition to a simple Michaelian kinetics, nature has a need for additional control mechanisms for the regulation of enzyme activity *in vivo.*

Thus, many of the enzymes at control points in metabolism display the property of responding with exceptional sensitivity to changes in metabolite concentrations, a response that does not obey the usual Michaelis–Menten kinetics. This property is reserved for *regulatory enzymes,* which are usually found at the beginning, at the end or at a branchpoint of a given metabolic pathway.

Two types of phenomena are responsible for such regulatory properties of enzymes. First are the *cooperative* phenomena in proteins and enzymes, and second are the *allosteric* properties of proteins and enzymes (Kurganov, 1982). Cooperativity is the apparent change in affinity or activity–that deviates from Michaelis–Menten kinetics–of an enzyme or protein with its substrate or other ligand as the concentration of the ligand changes. Cooperativity often requires that the enzyme or protein is built up of interacting subunits, but may also arise

for steady-state random mechanisms or alternative reaction partways (Charlier & Plapp, 2000).

Allosteric effects arise when binding of substrate or ligand at a site other than active site affects activity or binding. Allosteric effects are possible in a monomeric enzyme, for instance by a simple noncompetitive inhibitor, or by binding of the effector to a distant site, perhaps on another subunit of a multisubstrate protein. The term allosteric enzyme was originally coined by Monod *et al.* (1963) for enzymes that display altered kinetic properties in the presence of effectors that have no structural resemblance to substrates; therefore, the term allosteric is somewhat ambiguous. The ambiguity in the use of the "allostery" is that binding of a substrate to another subunit can affect activity in an apparent "cooperative" manner. An extensive discussion of differences between the phenomena and the mechanism is presented by Neet (1995).

Lactate dehydrogenase, an enzyme isolated from the beef heart, is an example of a typical Michaelian enzyme. This enzyme is a tetramertic oligomer, composed of four identical subunits, but each subunit binds its substrates independently of other subunits, in a typical noncooperative fashion (Holbrook *et al.,* 1975).

A well-documented example of an allosteric enzyme is provided by the studies of a dimeric enzyme yeast pyruvate kinase (Hess *et al.,* 1966; Hess & Wurster, 1970). The binding parameters were obtained from measurements of the enzyme catalyzed reaction:

$$\text{Phosphoenolpyruvate} + \text{ADP} + \text{H}^+ \rightarrow \text{Pyruvate} + \text{ATP} \qquad (13.1)$$

Figure 1 shows the response of enzyme activity to phosphoenolpyruvate and fructose-diphosphate concentration. It is obvious from Fig. 1 that the response of enzyme activity to increasing concentrations of substrate is not hyperbolic (Michaelian), but sigmoid. Such a sigmoid response increases the sensitivity of enzyme to small changes in the concentration of substrate and thus increases

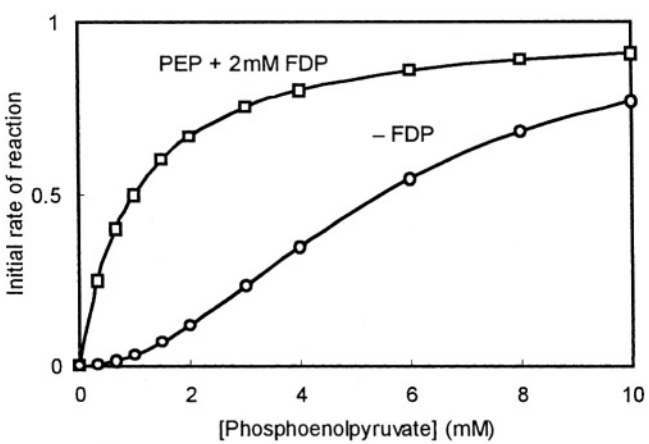


Figure 1. Response of yeast pyruvate kinase activity to increasing concentrations of phosphoenolpyruvate (PEP) and fructose-diphosphate (FDP).

the metabolic control over the enzyme activity. In the presence of fructose-diphosphate (a metabolite structurally totally unrelated to phosphoenolpyruvate), a response of enzyme to increasing substrate concentrations becomes hyperbolic. Thus, pyruvate kinase displays *sigmoid kinetics,* that is abolished in the presence of fructose-diphosphate, which is its *allosteric activator.*

What is the structural explanation for the occurrence of a simgoid, non-Michaelian kinetics?

Pyruvate kinase is a dimeric enzyme, composed of two identical subunits, with a single active site on each subunit. Each active site binds a pair of substrates, phosphoenolpyruvate and ADP and thus the entire molecule of enzyme binds two pairs of substrates (Fig. 2).

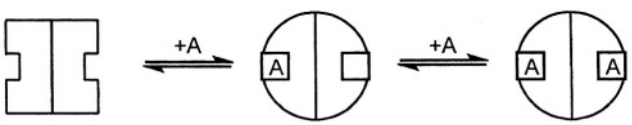


Figure 2. Conformational changes in pyruvate kinase, induced by the binding of phosphoenolpyruvate (A).

The kinetic response of pyruvate kinase to its substrate may be explained in the following way. If the enzyme is saturated with ADP, it is found in an "open" (square) conformation in Fig. 2. After the binding of the first molecule of the other substrate, phosphoenolpyruvate, the conformation of the entire molecule is changed into a "closed" (round) form. A closed conformation has a higher affinity for the substrate than the open conformation. Thus, the binding of the first substrate molecule may induce a conformational change in the entire dimeric molecule of enzyme and thus increase its affinity for the second substrate molecule. Or, in other words, a conformational change, induced by the binding of a substrate molecule at the active site, is propagated through the protein molecule to another binding site, which may be far away from the first binding site. This induction of the conformational change in enzyme may explain the sigmoid kinetics shown in Fig. 1. One should realize, however, that the graphical presentation in Fig. 2 may be exaggerated, because even a small conformational change in enzyme can cause an allosteric or a cooperative effect.

Homotropic effect refers to allosteric effects produced by enzyme's own substrate and heterotropic effects are due to metabolites that are not structurally related to enzyme's substrates. Positive effects are related to enzyme activation and negative effects to enzyme inhibition. Thus, fructose-diphosphate is a positive allosteric effector of pyruvate kinase.

The first observation of a sigmoid binding of a ligand to a protein was reported by Bohr (1903), for the cooperative binding of oxygen to hemoglobin. In 1910, Hill proposed the empirical equation which gave a reasonably good fit to the data. In 1925, Adair obtained a closer fit using an equation containing four different binding constants for the four different heme groups. Thus, the first mathematical models for cooperative binding were developed to describe quantitatively the binding of oxygen to hemoglobin.

More recently, two general types of models for allosteric enzymes have been proposed. These are

(a) the sequential interaction models (KNF models), and
(b) the concerted-symmetry model (MWC model).

As the name suggests, the KNF sequential interaction models assume sequential or progressive changes in the affinities of vacant sites as sites are occupied. The MWC concerted-symmetry model assumes that the enzyme preexists as an equilibrium mixture of high affinity oligomer and a low affinity oligomer. Ligands, including the substrate itself, act by displacing the equilibrium in favor of one state or the other. During the transition, the conformation of all subunits change at the same time and the oligomer retains its symmetry.

Allosteric and cooperative effects may be of different kind and Table 1 shows the main types encountered in enzymology.

Table 1. Different types of cooperative and allosteric phenomena

Types of binding			Examples	Source
Non-cooperative binding			Lactate dehydrogenase + NADH	Holbrook *et al.* (1975)
Cooperative binding	Homotropic	Positive	Pyruvate kinase + PEP Hemoglobin + O_2	Hess *et al.* (1966) Perutz *et al.* (1998)
		Negative	Alcohol dehydrogenase + NADH	Charlier & Plapp (2000)
	Heterotropic	Positive	Pyruvate kinase + FDP	Hess *et al.* (1966)
		Negative	Threonine deaminase + isoleucine	Davies & Metzler (1972)

It is important to note, and should always be remembered, that all the rate and binding equations for cooperative and allosteric enzymes were derived under rapid equilibrium assumption. Therefore, all kinetic models for the cooperative phenomena can be equally well applied to enzyme reactions that are in the rapid equilibrium and to the binding of ligands to enzymes and proteins.

Thus, we can conclude that a ligand or a substrate can bind to an enzyme or a protein (a) in a *noncooperative* fashion and (b) in a *cooperative fashion.* A cooperative binding can take place with *positive cooperativity* or with a *negative cooperativity,* but the difference is purely comparative. A positive cooperativity takes place when the ligand tightens the binding of other ligands compared to a protein which is devoid of cooperativity, and the negative cooperativity is exactly the opposite.

13.2 NONCOOPERATIVE BINDING

The binding of a ligand (L) to a protein molecule (E) can be described by the following equilibrium:

$$E + L \rightleftharpoons EL \tag{13.2}$$

The stability of the EL complex is an intrinsic property of the atomic contacts made between the partners and so has a constant value, irrespective of their concentration. This value is defined by a *dissociation constant* (K_d) or an *association constant* (K_a), where

$$K_d = \frac{[E]_F \cdot [L]_F}{[EL]} \quad K_a = \frac{[EL]}{[E]_F \cdot [L]_F} \tag{13.3}$$

It cannot be sufficiently stressed that $[E]_F$ is the concentration of *free binding sites* on the protein, and $[L]_F$ is the concentration of *free ligand.* When a system is in equilibrium, the *net* concentrations of each of the three components do not change; they are in a dynamic equilibrium (Winzor & Sawyer, 1995; Engel, 1996).

The crucial values to be gained from binding experiments are the affinity of the binding sites and the concentration of such sites in the sample. The way in which the binding data are used entirely depends on the method used to collect them.

13.2.1 Scatchard Analysis

This method depends on the ability to measure the concentration of free ligand $[L]_F$ or bound ligand $[L]_B$ (Scatchard, 1949; Scatchard *et al.,* 1957). Methods of this type usually exploit differences between the molecular properties of the free ligand and the protein–ligand complex.

In the usual application of the method, to a solution of a protein a ligand is added, that is, a protein solution is titrated with a ligand. The total concentration of the added ligand $[L]_T$ is known, and can be represented as

$$[L]_T = [L]_B + [L]_F \tag{13.4}$$

The concentration of free ligand $[L]_F$, or the concentration of bound ligand $[L]_B$ is measured, as the $[L]_T$ is added. It is important to remember that the number of binding sites on a protein molecule is not known initially, so even if its molar concentration is known, the concentration of the sites is not. The protein may be even a crude and impure preparation of a cell extract; this is not problematic because only a knowledge of the total ligand concentration and the free or bound ligand concentrations can be used to perform a titration.

Now, Eq. (13.3) can be rearranged as

$$K_d = \frac{[E]_F \cdot [L]_F}{[L]_B} \tag{13.5}$$

where $[E]_F$ is the concentration of unoccupied or free binding sites on the target protein molecule, and $[L]_B$ represents the concentration of occupied binding sites or, equally the concentration of bound ligand molecules. It is known that

$$[L]_B = [L]_T - [L]_F \quad \text{and} \quad [E]_F = [E]_T - [L]_B \tag{13.6}$$

so that

$$K_d = \frac{([E]_T - [L]_B)[L]_F}{[L]_B} \tag{13.7}$$

and, rearranging

$$[L]_B = [E]_T - K_d \frac{[L]_B}{[L]_F} \tag{13.8}$$

The graphical presentation of Eq. (13.8), the *Scatchard plot,* can be used to determine the relevant quantities, $[E]_T$ and K_d (Fig. 3).

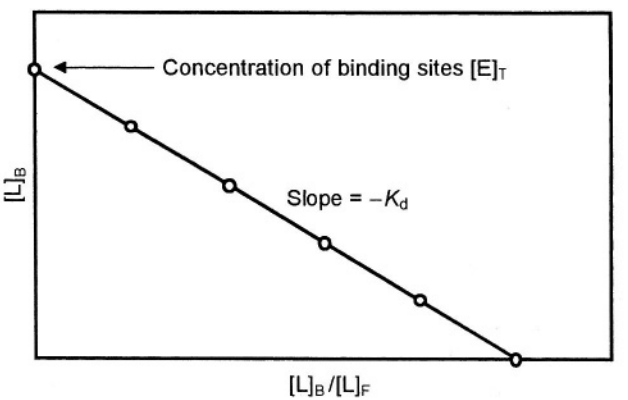


Figure 3. The Scatchard plot.

13.2.2 Measurement of the Saturation of Binding Sites

This method usually exploits a spectroscopic difference between the free protein and the protein–ligand complex or, less frequently, a difference between the properties of the free ligand and the bound ligand. For example, if the binding of a ligand induces a fluorescence change in a protein, then in this case the complex is more fluorescent than the free protein; ligand is added to the solution of a protein and the fluorescence is recorded. The total amount of ligand added, $[L]_T$, and the degree to which the protein sites are saturated with the ligand are known. The degree of saturation, or fractional saturation, is defined by

$$\alpha = \frac{[EL]}{[E]_F + [EL]} = \frac{[EL]}{[E]_T} = \frac{\Delta F}{\Delta F_{max}} \tag{13.9}$$

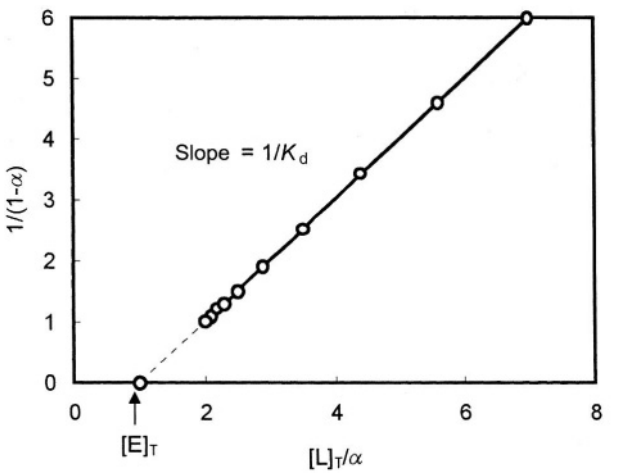
where ΔF is the fluorescence of a protein in the presence of a subsaturating amount of a ligand and ΔF_{max} the fluorescence of a protein in the presence of a saturating concentration of a ligand. Since

$$K_d = \frac{([E]_T - [EL])([L]_T - [EL])}{[EL]} \quad \text{and} \quad [EL] = \alpha[E]_T \tag{13.10}$$

The following expression can be derived:

$$\frac{1}{1-\alpha} = \frac{[L]_T}{\alpha K_d} - \frac{[E]_T}{K_d} \tag{13.11}$$

The graphical presentation of Eq. (13.11) is shown in Fig. 4.



$$\frac{1}{1-\alpha} = \frac{[L]_T}{\alpha K_d} - \frac{[E]_T}{K_d}$$

Figure 4. Graphical presentation of Eq. (13.11). The data points are drawn assuming that $[E]_T = K_d = 1$.

Note that the data points in Fig. 4 are converging to 1 (on ordinate) and to $K_d + [E]_T$ (on abscissa), as α tends to zero. This type of analysis is very popular in ligand-binding studies with enzymes and proteins (Leskovac & Pavkov-Peričin, 1975).

While this is a valid form of linear analysis, it has become more usual, and is statistically more accurate, to use nonlinear fitting methods in which the data are fitted directly to a nonlinear equation. $[L]_T$, an independent variable, is represented as a function of α, the dependent variable; thus Eq. (13.9) is defined by

$$\alpha = \frac{([L]_T + [E]_T + K_d) - \sqrt{([L]_T + [E]_T + K_d)^2 - 4[L]_T[E]_T}}{2[E]_T} \tag{13.12}$$

This is the most important general relationship used in binding studies with proteins that bind ligands noncooperatively. It can be applied in any conditions

and allows direct fitting to a set of data and can extract values of K_d and $[E]_T$ in any standard nonlinear fitting computer program (Engel, 1996).

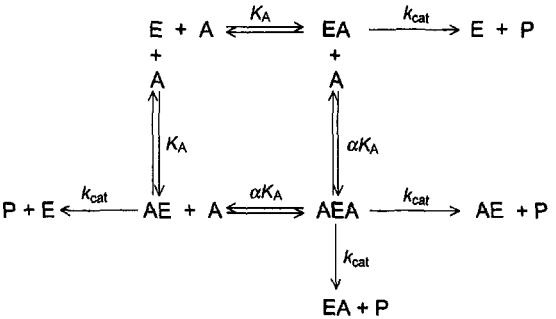
A linear method described above requires the knowledge of the measure of the saturation of binding sites (α); sometimes, α cannot be determined with precision. In such cases, a differential procedure developed by Leskovac *et al.* (1993) provides a useful extension of the above method. The differential procedure provides a precise estimation of K_d and $[E]_T$ even without the prior knowledge of α.

13.3 POSITIVE COOPERATIVITY

13.3.1 The Adair Equation

If the binding of one substrate molecule to an enzyme induces structural changes that result in altered affinities for the vacant sites, the velocity curve will no longer obey the Michaelis–Menten kinetics. Let us consider an enzyme with two identical substrate binding sites (Adair, 1925).

If the binding of one substrate molecule changes the dissociation constant, K_A, by a factor α, and the k_{cat} is the same at each site regardless whether the other site is occupied, the reaction sequence is



(13.13)

If $V_{max} = 2k_{cat}E_0$, and the derivation of the velocity equation is based on a rapid equilibrium assumption, we shall obtain

$$\frac{v_0}{V_{max}} = \frac{\dfrac{[A]}{K_A} + \dfrac{[A]^2}{\alpha K_A^2}}{1 + \dfrac{2[A]}{K_A} + \dfrac{[A]^2}{\alpha K_A^2}} \tag{13.14}$$

An enzyme with four identical sites may be treated similarly. If the binding of the first substrate molecule changes the dissociation constants of the vacant sites by a factor, a, the binding of the second substrate molecule changes the dissociation constant for the remaining vacant sites by a factor, b, and the third substrate molecule introduces an interaction factor, c, than the effective dissociation constants for the binding of first, second, and the third substrate molecule are

$$K_{A1} = \frac{K_A}{4} \quad K_{A2} = \frac{a2K_A}{3} \quad K_{A3} = \frac{ab3K_A}{2} \quad K_{A4} = abc4K_A \tag{13.15}$$

Note that the change induced by the first molecule of substrate is retained, so is the change induced by the second and the third molecule of substrate; that is, the interactions are cumulative (DeLand, 1994; Imai, 1994; Johnson, 1994).

From Eqs. (13.15), one can calculate the distribution of the total enzyme represented by each of the four enzyme complexes plus the free enzyme. From the distribution equations, the rate equation for a tetrametic enzyme can be derived:

$$\frac{v_0}{V_{max}} = \frac{\dfrac{[A]}{K_A} + \dfrac{3[A]^2}{aK_A^2} + \dfrac{3[A]^3}{a^2bK_A^3} + \dfrac{[A]^4}{a^3b^2cK_A^4}}{1 + \dfrac{4[A]}{K_A} + \dfrac{6[A]^2}{aK_A^2} + \dfrac{4[A]^3}{a^2bK_A^3} + \dfrac{[A]^4}{a^3b^2cK_A^4}} \tag{13.16}$$

where $V_1 = 4k_{cat}E_0$.

The above treatment can be applied to enzymes with 6, 8, or more subunits.

In the rate Eq. (13.16), *a, b,* and *c* are the factors by which the intrinsic binding constant, K_A, has been altered. In the positive cooperativity, these *interaction factors* have the values less than 1, and in the negative cooperativity they have the values larger than 1. If the interaction factors are equal to one, we have no cooperativity, and expression (13.16) reduces to the Michaelis–Menten equation. The shape of the velocity or the binding curve and the distribution of enzyme species strongly depends on the numerical values of interaction factors (Fig. 5).

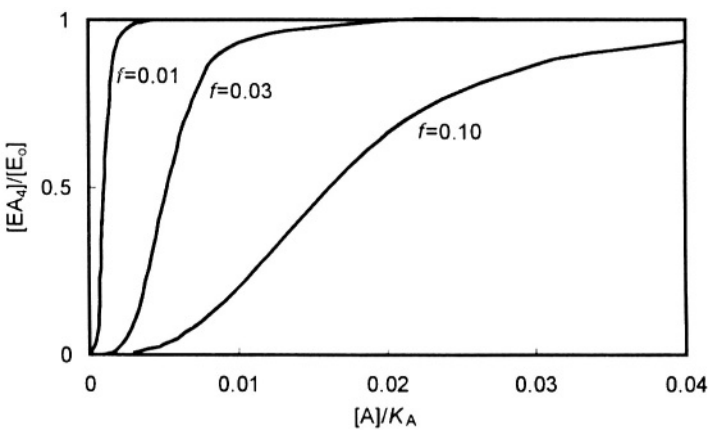


Figure 5. Relative concentration of the completely liganded tetrameric enzyme, $[EA_4]$. The curves were drawn with the aid of Adair equation (13.16), assuming that $K_A = 1$, and that the interaction factors are equal ($f = a = b = c$) and increasing gradually.

Figure 5 shows that, even if the cooperativity is modest ($a = b = c = 0.03$), the enzyme exists predominantly as a completely liganded species EA_4, already at a very low concentration of substrate ($A > 0.01K_A$). The concentration of a free enzyme is substantial only at very low concentrations of A and the concentration of EA_2 and EA_3 species is negligible at all concentrations of the substrate.

13.3.2 The Hill Equation

Consider an enzyme with n equivalent substrate binding sites. If the positive cooperativity in substrate binding is very strong, that is, if the factors *a, b, c,* and so on, are very small numbers, then the concentrations of all enzyme–substrate complexes containing less than n molecules of substrate will be negligible at any concentration of A that is appreciable compared to K_A.

Under this condition, we have

$$E + nA \rightleftharpoons EA_n \tag{13.17}$$

and

$$K_X = \frac{[E][A]^n}{[EA_n]} \tag{13.18}$$

In this case, the velocity equation will be dominated by the $[A]^n$ terms (Hill, 1910).

For example, the rate Eq. (13.16) for the tetrameric enzyme in such cases reduces to

$$\frac{v_0}{V_{max}} = \frac{[A]^4}{K_X + [A]^4} \quad \text{where} \quad K_X = a^3b^2cK_A^4 \tag{13.19}$$

In general,

$$\frac{v_0}{V_{max}} = \frac{[A]^n}{K_X + [A]^n} \tag{13.20}$$

where v_0/V_{max} is the fractional saturation of enzyme with the substrate, n, the number of substrate binding sites per molecule of enzyme, and K_X, the constant comprising the interaction factors *a, b, c,* and so on, and the intrinsic dissociation constant K_A. Thus,

$$K_X = K_A^n(a^{n-1}b^{n-2}c^{n-3}\ldots z^1) \tag{13.21}$$

Equation (13.20) is known as the *Hill equation.*

As an example, Fig. 6 shows the graphical presentation of Eq. (13.20) for a tetrameric enzyme with four different values for K_X.

If the cooperativity of substrate binding is not very high, then the rate equation will not be reduced to the Hill equation. Nevertheless, velocity curves can be expressed by the Hill equation, although n will no longer equal the actual number of substrate binding sites.

We can write the Hill equation in the Lineweaver–Burk fashion:

$$\frac{1}{v_0} = \frac{1}{V_{max}} + \frac{K_X}{V_{max}}\left(\frac{1}{[A]^n}\right) \tag{13.22}$$

A plot of $1/v_0$ versus $1/[A]$ will afford a curve that is concave and bent upward, providing a limiting value of $1/V_{max}$ on the ordinate. However, a plot of $1/v_0$ versus $1/[A]^n$ will provide a straight line with an intercept on abscissa equal to $-1/K_X$ and an intercept on ordinate equal to $1/V_{max}$.

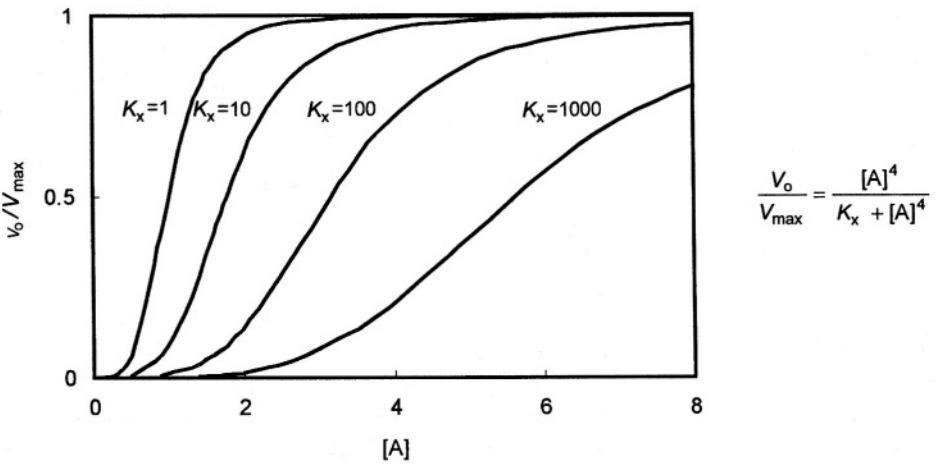


Figure 6. Sigmoid activity for a tetrameric enzyme drawn according to the Hill equation (13.20), assuming that $n = 4$ in all cases, and K_X gradually increases.

The simplified Hill Eq. (13.20) can be converted into a useful linear form:

$$\log\left(\frac{v_o}{V_{max} - v_o}\right) = n \log[A] - \log K_X \qquad (13.23)$$

The linear form of the Hill equation permits a direct estimation of the apparent number of substrate binding sites per molecule; a plot of $\log[v_o/(V_{max} - v_o)]$ versus log[A] is a straight line with a slope of n. In practice, however, such plots are usually not linear, and asymptotes at low and high [A] have slopes of 1; the slope one uses is the maximum one taken in the middle of the curve.

The Hill equation is equally well applicable to ligand-binding studies with proteins and to enzyme kinetics, although only under specific conditions. If equilibrium ligand binding is measured instead of initial velocities, then v_o/V_{max} is substituted by Y, where Y is now a fractional saturation of a protein with the ligand. The various forms of the Hill equation will now have different shapes in ligand-binding studies compared to initial rate studies (Table 2).

Table 2. The forms of Hill equation in ligand binding as opposed to initial rate studies

Types of equation	Initial rate studies	Ligand binding studies
Hill equation	$\frac{v_o}{V_{max}} = \frac{[A]^n}{K_X + [A]^n}$	$Y = \frac{[A]^n}{K_X + [A]^n}$
Lineweaver–Burk form	$\frac{1}{v_o} = \frac{1}{V_{max}} + \frac{K_X}{V_{max}}\left(\frac{1}{[A]^n}\right)$	$\frac{1}{Y} = 1 + K_X\left(\frac{1}{[A]^n}\right)$
Logarithmic form	$\log\left(\frac{v_o}{V_{max} - v_o}\right) = n \log[A] - \log K_X$	$\log\left(\frac{Y}{1 - Y}\right) = n \log[A] - \log K_X$

Should one use the Hill plot in practice to examine the initial velocity behavior of enzymes? Because infinite cooperativity is assumed to be the basis of the Hill treatment, only rapidly equilibrating systems are suitable for the Hill analysis. However, enzyme systems displaying steady-state kinetic behavior will not satisfy this requirement; for this reason, one must avoid the use of kinetic data in any application of the Hill equation to steady-state enzyme systems.

13.3.3 Binding of Oxygen by Hemoglobin and Myoglobin

Let us examine the application of the Hill equation to a well-known example of equilibrium binding of molecular oxygen by the cell protein myoglobin and by the blood protein hemoglobin. Myoglobin and hemoglobin are structurally closely related transport proteins, but myoglobin is a monomeric protein that binds one molecule of oxygen while hemoglobin is a tetrameric protein that binds four molecules of oxygen at saturation (Henry *et al.,* 1997; Perutz *et al.,* 1998). Since oxygen is a gas, its concentration in aqueous solutions may be expressed by its partial pressure in solution (Fig. 7).

Figure 7A shows the equilibrium binding curve for oxygen with hemoglobin and myoglobin in solution, with a vertical axis representing the fraction of myoglobin or hemoglobin molecules saturated with oxygen, or the fractional saturation of the protein molecule with oxygen (Y value). Both binding curves fit the simple Hill Eq. (13.20), described above. However, the number of binding sites calculated for myoglobin is $n = 1$, and for hemoglobin $n = 2.8$. It is immediately obvious that, with myoglobin, the Hill equation reduces to the usual Michaelis–Menten case, while the binding of oxygen to the molecule of hemoglobin is cooperative (Neet, 1995).

The number of apparent binding sites for oxygen on a molecule of myoglobin or a molecule of hemoglobin may be determined with the aid of the linear form of the Hill Eq. (13.23). According to this equation, if $\log[Y/(1-Y)]$ is plotted versus $\log(pO_2)$ a straight line is obtained with a slope equal to n, the number of apparent binding sites per molecule of protein (Fig. 7B).

From the linear form of the Hill equation, one can see that when log [A] becomes zero (Table 2), then

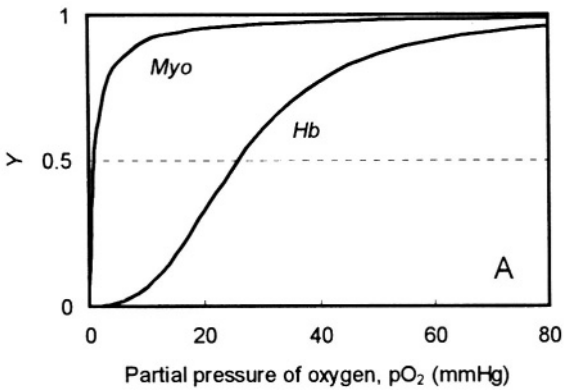
$$\log K_X = -\log\left(\frac{Y}{1-Y}\right) \tag{13.24}$$

In initial rate studies, if the initial rate of reaction is equal to half the maximal velocity, that is, $v_0 = V_{max}/2$, then $n \log[A]_{0.5}$ is equal K_X. Thus, K_X may be calculated from yet another relationship:

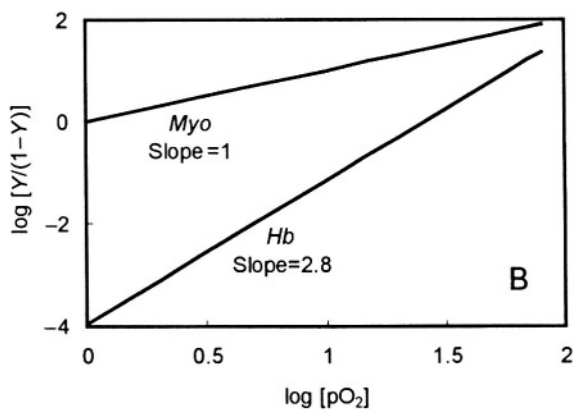
$$[A]_{0.5} = \sqrt[n]{K_X} \tag{13.25}$$

where $[A]_{0.5}$ is the concentration of a substrate at which the enzyme operates with a half maximal velocity.

The numerical values of K_X and n for hemoglobin and myoglobin can be obtained from Fig. 7. The n-values are obtained directly from the slopes of lines



$$Y = \frac{[A]^n}{K_X + [A]^n}$$



$$\log\left(\frac{Y}{1-Y}\right) = n\log[A] - \log K_X$$

Figure 7. (A) The equilibrium binding curve for molecular oxygen with hemoglobin and myoglobin in solution, at pH 7.0; the ordinate represents the fractional saturation of myoglobin or hemoglobin molecules with oxygen, abscissa is the partial pressure of oxygen ([A] = pO_2). (B) A replot of binding data from above figure according to the linear form of the Hill equation.

in Fig. 7B. From the data in Fig. 7A, one can calculate that the half-saturation with oxygen for myoglobin is at 1 mm Hg, and for hemoglobin at 26 mm Hg pressure; these values correspond to the value of $\sqrt[n]{K_X}$ in Eq. (13.25).

The constants K_X are determined with much more precision from the linear plot in Fig. 7B; here, the value of K_X for myoglobin is obvious, while the value for hemoglobin is obtained from $\log K_X = 3.95$, or $K_X = 9160$.

With hemoglobin, one can see that if the experimental binding data are analyzed in terms of the Hill equation, the calculated value of n is less than the actual number of binding sites; the value of n cannot be higher than the number of binding sites, and it is almost always lower. Thus, the value of n does not determine the number of binding sites, but it is just a model-independent fitting parameter.

13.4 NEGATIVE COOPERATIVITY

In addition to positive cooperativity, negative cooperativity is also possible. In this case, the binding of each substrate molecule decreases the intrinsic affinities of the vacant sites. A common reason for apparent negative cooperativity is the presence of two forms of the enzyme with different K_M values; such a situation always gives reciprocal plots that are convex upward.

Consider the influence of the substrate concentration on an enzyme with two identical active sites and an interaction factor α. The Adair Eq. (13.14) for this case is

$$\frac{v_0}{V_{max}} = \frac{\dfrac{[A]}{K_A} + \dfrac{[A]^2}{\alpha K_A^2}}{1 + \dfrac{2[A]}{K_A} + \dfrac{[A]^2}{\alpha K_A^2}}$$

When both sites are identical and there is no cooperativity between them, then $\alpha = 1$. In this case, the above equation reduces to the Michaelis–Menten equation.

Consider three different values for an interaction factor α that will produce the: (a) positive cooperativity, $\alpha = 0.3$; (b) no cooperativity, $\alpha = 1$; and (c) negative cooperativity, $\alpha = 5$ (Fig. 8).

The double reciprocal plot is linear for the enzyme with no cooperativity (the middle line in Fig. 8); in the case of positive cooperativity, the plots are concave up, whereas negative cooperativity gives the plots that are convex upwards. Thus, the shape of the double reciprocal plot may already detect the

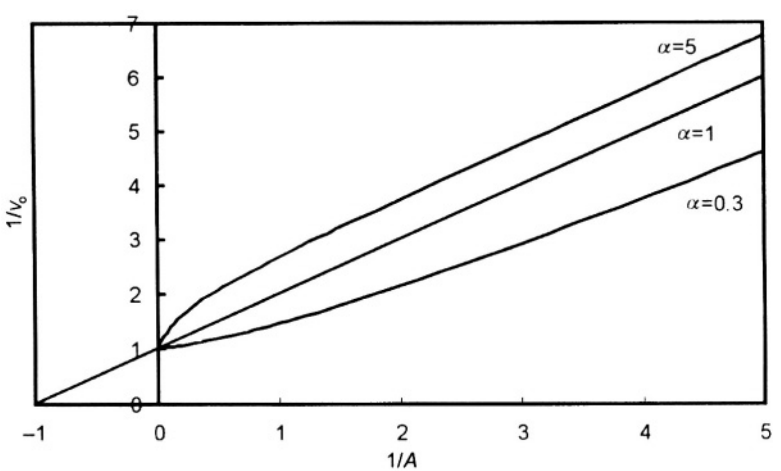


Figure 8. Double-reciprocal plots showing the positive and negative cooperativity in a dimeric enzyme. The lines were drawn with the aid of an Adair Eq. (13.14), assuming that $V_{max} = K_A = 1$.

presence of cooperativity. Kugranov (1982) proposed a simple graphical method for the estimation of the shape of the cooperative curve; the Kurganov method is a procedure for estimating the cooperativity of an enzyme by using the initial rates at only three different concentrations of substrate to estimate the shape of the curve.

13.5 MONOD, WYMAN, AND CHANGEUX MODEL (MWC MODEL)

In 1965, Monod, Wyman, and Changeux proposed an important unified model for allosteric proteins (Monod *et al.,* 1963; Monod *et al.,* 1965). They have studied many examples of cooperative and allosteric effects, and concluded that they were closely related and that conformational flexibility probably accounted for both phenomena. They have proposed a structural model for allosteric enzymes and proteins, which comprises the following postulates:

(1) Allosteric proteins are oligomers composed of protomers which are associated in such a way that they all occupy equivalent positions. This implies that the molecule possesses at least one axis of symmetry.
(2) To each ligand able to form a stereospecific complex with the protein, there corresponds one, and only one, site on each protomer. In other words, the symmetry of each set of stereospecific receptors is the same as the symmetry of the molecule.
(3) Two (at least two) states are reversibly accessible to allosteric oligomers. These states differ by the distribution and/or energy of inter-protomer bonds, and therefore also by the conformational constraints imposed upon the protomers.
(4) The conformation of each protomer is constrained by its association with the other protomers.
(5) As a result, the affinity of one (or several) of the stereospecific sites towards the corresponding ligand is altered when a transition occurs from one to the other state.
(6) When the protein goes from one state to another state, its molecular symmetry is conserved.

Let us first analyze the interactions of such a model in a *tetrameric protein* with a single ligand (F) endowed with differential affinity towards the two accessible states, T state (tight, tense) and R state (relaxed)(Fig. 9).

The T state represents the conformation with the lower affinity for the ligand, and the R state represents the conformation with the higher affinity for the ligand (Fig. 9). In the absence of a ligand, the two states, symbolized as R and T, are assumed to be in equilibrium; thus L is an equilibrium constant for the $R_o \leftrightarrow T_o$ transition. In order to distinguish this constant from the dissociation constants of the ligand, we shall call it the *allosteric constant.* Further, let K_R and K_T be the microscopic dissociation constants of a ligand F bound to a stereospecific site, in the R and T states, respectively.

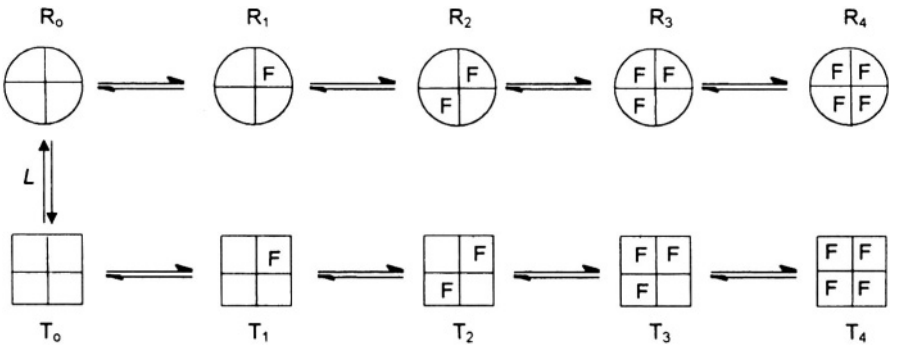


Figure 9. The concerted-symmetry model of Monod, Wyman, and Changeux. T represents a low affinity form of enzyme which is in equilibrium with R, which is a high affinity form of the enzyme.

13.5.1 Derivation of Rate Equations

The distribution of complexes for the tetrameric enzyme (or protein) can be written in the usual manner from the rapid equilibrium assumptions, taking into account the number of different ways of arranging the ligand F in any given complex. Thus, we may write the successive equilibria as follows:

Dissociation constants are K_R	Dissociation constants are K_T	
$R_0 + F \rightleftharpoons R_1$	$T_0 + F \rightleftharpoons T_1$	
$R_1 + F \rightleftharpoons R_2$	$T_1 + F \rightleftharpoons T_2$	(13.26)
$R_2 + F \rightleftharpoons R_3$	$T_2 + F \rightleftharpoons T_3$	
$R_3 + F \rightleftharpoons R_4$	$T_3 + F \rightleftharpoons T_4$	

All equilibria in Eq. (13.26) are endowed with a single *intrinsic dissociation constant* K_R for the R-states, and with a single *intrinsic dissociation constant* K_T for the T states. The transition

$$R_0 \overset{L}{\rightleftharpoons} T_0 \tag{13.27}$$

is endowed with the *allosteric equilibrium constant L.*

Taking into account the probability factors for the dissociation of the R_1, R_2, R_3, R_4, and T_1, T_2, T_3, T_4 complexes, the concentrations of the 10 forms of protein in Fig. 9 are related by the following expressions:

$$[R_1] = \frac{4[F]}{K_R}[R_0] = 4\alpha[R_0] \qquad [T_1] = \frac{4c[F]}{K_R}L[R_0] = 4\alpha cL[R_0]$$

$$[R_2] = \frac{6[F]^2}{K_R^2}[R_0] = 6\alpha^2[R_0] \qquad [T_2] = \frac{6c^2[F]^2}{K_R^2}L[R_0] = 6\alpha^2c^2L[R_0]$$

$$[R_3] = \frac{4[F]^3}{K_R^3}[R_0] = 4\alpha^3[R_0] \qquad [T_3] = \frac{4c^3[F]^3}{K_R^3}L[R_0] = 4\alpha^3c^3L[R_0]$$

$$[R_4] = \frac{[F]^4}{K_R^4}[R_0] = \alpha^4[R_0] \qquad [T_4] = \frac{c^4[F]^4}{K_R^4}L[R_0] = \alpha^4c^4L[R_0] \tag{13.28}$$

$$[T_0] = L[R_0]$$

Equations (13.28) are obtained with the aid of the rapid equilibrium assumptions, from the distribution of complexes; the dissociation constants K_R and K_T are disguised as c, the nonexclusive binding coefficient:

$$c = \frac{K_R}{K_T} \tag{13.29}$$

In addition, α represents the reduced, or normalized concentration of the ligand F:

$$\alpha = \frac{[F]}{K_R} \tag{13.30}$$

Let us now depart from the tetrameric case and take the general case, in order to define two functions corresponding, respectively, to

(a) the fraction of protein in the R state ($\bar{R}$ = *function of state* R):

$$\bar{R} = \frac{[R_0] + [R_1] + [R_2] + [R_3] \cdots + [R_n]}{([R_0] + [R_1] + [R_2] + [R_3] \cdots + [R_n]) + ([T_0] + [T_1] + [T_2] + [T_3] \cdots + [T_n])} \tag{13.31}$$

(b) the fraction of sites actually bound by the ligand (Y_F = *saturation function*):

$$Y_F = \frac{([R_1] + 2[R_2] + 3[R_3] \cdots + n[R_n]) + ([T_1] + 2[T_2] + 3[T_3] \cdots + n[T_n])}{n([R_0] + [R_1] + [R_2] + [R_3] \cdots + [R_n]) + n([T_0] + [T_1] + [T_2] + [T_3] \cdots + [T_n])} \tag{13.32}$$

Using the equilibrium equations, and Eqs. (13.29) and (13.30), we shall obtain two novel useful functions. The *function of state* R is

$$\bar{R} = \frac{(1+\alpha)^n}{L(1+c\alpha)^n + (1+\alpha)^n} \tag{13.33}$$

and, similarly, the *function of state* T is

$$\bar{T} = \frac{L(1+c\alpha)^n}{L(1+c\alpha)^n + (1+\alpha)^n} \tag{13.34}$$

The ratio of the fraction of protein in the R state to the fraction of protein in the T state (the "quotient function") is

$$\bar{Q} = \frac{\bar{R}}{\bar{T}} = \frac{\bar{R}}{1-\bar{R}} = \frac{(1+\alpha)^n}{L(1+c\alpha)^n} \tag{13.35}$$

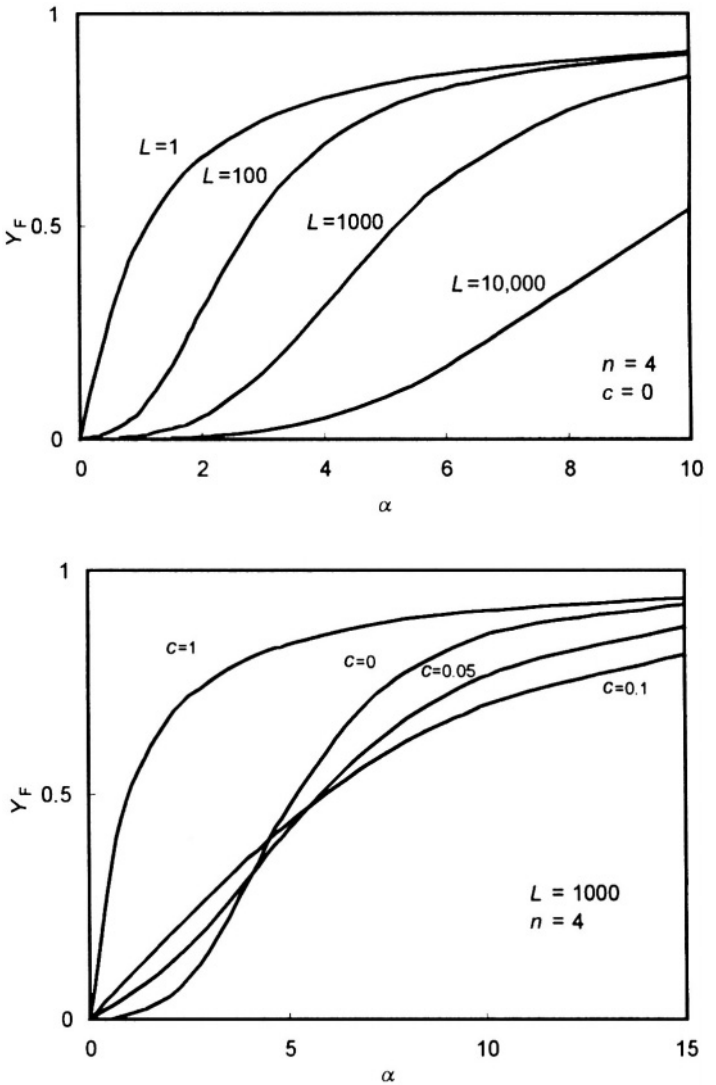


Figure 10. Theoretical curves of the saturation function Y_F. The curves are drawn assuming the various values of constants L and c, with $n = 4$.

For an enzyme under rapid equilibrium conditions, the fraction of total sites occupied by the ligand F is equal to v_o/V_{max}. Thus, for the *saturation function* Y_F, we have

$$Y_F = \frac{v_o}{V_{max}} = \frac{Lc\alpha(1+c\alpha)^{n-1} + \alpha(1+\alpha)^{n-1}}{L(1+c\alpha)^n + (1+\alpha)^n} \tag{13.36}$$

Equation (13.36) may be written in a simplified form

$$Y_F = \frac{v_0}{V_{max}} = \frac{c\alpha}{(1+c\alpha)}(1-\bar{R}) + \left(\frac{\alpha}{1+\alpha}\right)\bar{R} \qquad (13.37)$$

where $\bar{R}$ has a meaning given by Eq. (13.33). Equation (13.37) is a general rate or binding expression, that includes several special cases which are described further.

13.5.2 Effects of *L* and *c* on Cooperativity

Figure 10 illustrates the effects of *L* and c on the shape and sigmoidicity of saturation curves. In this figure, theoretical curves for the Y_F function have been drawn, corresponding to different values of the constants *L* and *c*. In such graphs, the cooperative homotropic effect of the ligand, predicted by the symmetry properties of the model, is expressed by the curvature of the lower part of the curves. The graphs illustrate the fact that the "cooperativity" of the ligand depends on the values of *L* and *c*. The cooperativity is more marked when the allosteric constant *L* is large, that is, when the $R_0 \leftrightarrow T_0$ equilibrium is strongly in favor of T_0. The cooperativity is also favored when the ratio of microscopic dissociation constants, *c,* is small.

Thus, one can conclude that the cooperativity of ligand binding depends heavily on magnitudes of *L* and *c*. The saturation curve becomes more sigmoidal as *L* increases, that is, as the T state is favored. Also, the saturation curve becomes more sigmoidal as *c* decreases, that is, as the affinity of the T state for F decreases relative to the affinity of the R state for F.

13.6 APPLICATIONS OF THE MWC MODEL TO ENZYMES

The concerted symmetry model is much more versatile than simple sequential models described by Adair or Hill. This model is endowed by the variable values of K_R, *L,* and *c*, and therefore may provide explanations for many properties of allosteric enzymes and proteins, including

(a) partial allosteric inhibition,
(b) increased, decreased, or constant substrate cooperativity in the presence of effectors, and
(c) activation by low concentrations of competitive inhibitors.

In the following discussion, we shall review several applications of the MWC model, concerning the activation and/or inhibition of allosteric enzymes with substrates or ligands.

It should be borne in mind that in almost all enzyme systems, the saturation functions with respect to substrates or effectors cannot be determined directly, and are only inferred from kinetic measurements. This is not the case with ligand binding to proteins, such as the binding of oxygen to hemoglobin, where the saturation of protein with the ligand can be measured directly. We shall, therefore, discuss only the most characteristic qualitative predictions of the model in its application to enzymes.

In any enzyme system, activating or inhibitory effects are measured in terms of variations of the two kinetic constants, K_M and V_{max}, as a function of the concentrations of substrate, A, or effectors, F. Two classes of effects may then be expected in allosteric enzymes.

(a) *"K systems"* (= binding). Both F and A have differential affinities towards the T and R states, that is, both A and F are allosteric ligands. Then, evidently the presence of F will modify the apparent affinity of the enzyme for A, and conversely.

(b) *"V systems"* (= rate). A has the same affinity for the two states. Then, there is no effect of F on the binding of A, nor of A on the binding of F. F can exert an effect on the reaction only if the two conformational states of the enzyme differ in their catalytic activity. Depending on whether F has maximum affinity for the active or for the inactive state, it will behave as an activator, X (positive *V* system), or as an inhibitor, I, (negative *V* system).

Furthermore, the MWC model predicts that

(a) In allosteric enzyme systems, an allosteric effector, that is, a specific ligand endowed with different affinities towards the two states, should always exhibit cooperative homotropic interactions.

(b) In those systems in which an allostreric effector modifies the apparent affinity of the substrate, the substrate should also exhibit cooperative homotropic interactions.

(c) In those systems in which the effector does not modify the affinity of the substrate, the latter should not exhibit homotropic cooperative interactions.

13.6.1 Substrate Binding in the Absence of Effectors

Exclusive ligand binding (Substrate has the affinity only for the R state)

The simplest MWC model assumes that *c* is very small, that is, the T state has essentially no affinity for the substrate A. Under this condition,

$$\bar{R} = \frac{(1+\alpha)^n}{L + (1+\alpha)^n} \tag{13.38}$$

and the velocity equation reduces to

$$Y_F = \frac{v_o}{V_{max}} = \left(\frac{\alpha}{1+\alpha}\right)\bar{R} = \frac{\alpha(1+\alpha)^{n-1}}{L + (1+\alpha)^n} \tag{13.39}$$

Equation (13.39) may be expanded, in order to illustrate the binding of substrate to a four-site enzyme:

$$\frac{v_o}{V_{max}} = \frac{\dfrac{[A]}{K_A} + \dfrac{3[A]^2}{K_A^2} + \dfrac{3[A]^3}{K_A^3} + \dfrac{[A]^4}{K_A^4}}{L + 1 + \dfrac{4[A]}{K_A} + \dfrac{6[A]^2}{K_A^2} + \dfrac{4[A]^3}{K_A^3} + \dfrac{[A]^4}{K_A^4}} \tag{13.40}$$

where $K_A = K_R$.

V Systems (Substrate has the same affinity for the R and the T state)

In *V* systems, T and R states may have the same affinity for A, but different intrinsic catalytic activities, that is, $k_{cat}^{R} > k_{cat}^{T}$. The rate equation for such *V* systems is derived from the rapid equilibrium assumption:

$$Y_F = \frac{v_o}{V_{max}} = \frac{\alpha(1+gL)}{(1+\alpha)(1+L)} \tag{13.41}$$

where $V_{max} = nk_{cat}E_o$, and (g) is the ratio of catalytic activities in the T and the R state, respectively.

In writing Eq. (13.41), it is assumed that $g < 1$, because the R state has the higher catalytic activity ($k_{cat}^{R} > k_{cat}^{T}$). The Michaelis plot for such a *V* system is hyperbolic, just as would be observed for a mixture of two enzymes with the same K_M but different V_{max} values (Schuller *et al.,* 1995; Grant *et al.,* 1996).

Mixed K and V systems (Substrate has different affinities for the R and the T State)

If the R and T states have different affinities for the substrate, A, that is, if $c \neq 1$, as well as different catalytic activities, that is, if $k_{cat}^{R} \neq k_{cat}^{T}$ or $g \neq 1$, the rate equation becomes

$$Y_F = \frac{v_o}{V_{max}} = \frac{Lc\alpha g(1+c\alpha)^{n-1} + (1+\alpha)^{n-1}}{L(1+c\alpha)^n + (1+\alpha)^n} \tag{13.42}$$

where $V_{max} = nk_{cat}E_o$.

If $g < 1$ and $c < 1$, the R state has both the higher affinity for A as well as the higher catalytic activity. Alternatively, it is possible for the higher affinity state to have the lower catalytic activity ($g > 1$ and $c < 1$), a situation leading to substrate inhibition.

13.6.2 Exclusive Binding of Effectors

Inhibition in exclusive binding K systems (Substrate binds exclusively to the R state and inhibitor binds exclusively to the T state)

Let us assume that, in the *K* system, an allosteric inhibitor has a much higher affinity for the T state than for the R state. As a result, an allosteric inhibitor will displace the $T_o \leftrightarrow R_o$ equilibrium in favor of T_o. If we assume that the substrate, A, binds exclusively to the R state, that is, c=0, and inhibitor, I, binds exclusively to the T state, that is, $K_i^R/K_i^T \gg 1$, the velocity equation becomes

$$Y_F = \frac{v_o}{V_{max}} = \left(\frac{\alpha}{(1+\alpha)}\right)\bar{R} = \frac{\alpha(1+\alpha)^{n-1}}{L(1+\beta)^n + (1+\alpha)^n} \tag{13.43}$$

where

$$\bar{R} = \frac{(1+\alpha)^n}{L(1+\beta)^n + (1+\alpha)^n} \tag{13.44}$$

$$\alpha = \frac{[A]}{K_A} = \frac{[A]}{K_A^R} \tag{13.45}$$

$$\beta = \frac{[I]}{K_i} = \frac{[I]}{K_i^T} \tag{13.46}$$

In an expanded form, and for the tetrameric enzyme, Eq. (13.43) becomes

$$Y_F = \frac{v_o}{V_{max}} = \frac{\dfrac{[A]}{K_A} + \dfrac{3[A]^2}{K_A^2} + \dfrac{3[A]^3}{K_A^3} + \dfrac{[A]^4}{K_A^4}}{L\left(1 + \dfrac{4[I]}{K_i} + \dfrac{6[I]^2}{K_i^2} + \dfrac{4[I]^3}{K_i^3} + \dfrac{[I]^4}{K_i^4}\right) + \left(1 + \dfrac{4[A]}{K_A} + \dfrac{6[A]^2}{K_A^2} + \dfrac{4[A]^3}{K_A^3} + \dfrac{[A]^4}{K_A^4}\right)} \tag{13.47}$$

Equation (13.47) has no terms containing both [I] and [A], because of the assumption of exclusive binding of each ligand to only one of the two states. Equation (13.43) predicts that, as the concentration of substrate A increases, the plot of v_o/V_{max} versus [I] becomes more sigmoidal (Fig. 11).

The graphical determination of constants K_i and L in this system is possible with an appropriate transformation of Eq. (13.43). Thus, Eq. (13.43) can be transformed into a nonlinear form:

$$\frac{v_o}{V_{max}^{\alpha} - v_o} = \frac{1}{L_\alpha (1+\beta)^n} \tag{13.48}$$

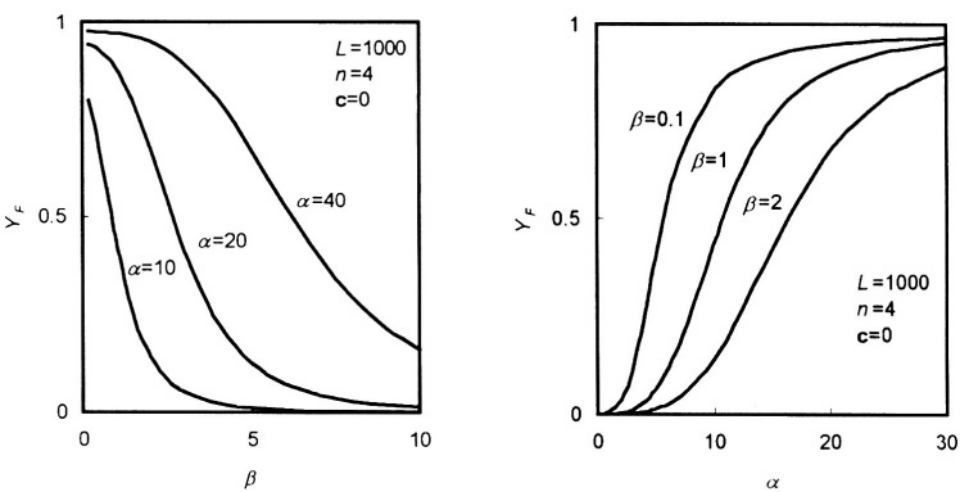


Figure 11. Inhibition of the tetrameric enzyme in the *K* system. The curves in figures were drawn according to Eq. (13.43). As the concentration of substrate A increases ($\alpha = [A]/K_A$), the curves are becoming more sigmoidal.

where

$$V_{max}^{\alpha} = V_{max}\left(\frac{\alpha}{1+\alpha}\right) \quad \text{and} \quad L_{\alpha} = \frac{L}{(1+\alpha)^n} \tag{13.49}$$

Equation (13.48) may be further converted into a linear form:

$$\sqrt[n]{\frac{V_{max}^{\alpha} - v_0}{v_0}} = \left(\frac{\sqrt[n]{L_{\alpha}}}{K_i}\right)[I] + \sqrt[n]{L_{\alpha}} \tag{13.50}$$

Thus, if the concentration of substrate is held constant ($\alpha = [A]/K_A$), a double reciprocal plot of $\sqrt[n]{(V_{max}^{\alpha} - v_0)/v_0}$ versus [I], will provide a straight line with intercepts on abscissa and ordinate equal to K_i and $\sqrt[n]{L_{\alpha}}$, respectively.

Activation in exclusive binding K systems (Substrate binds to both states but an activator binds preferentially to the R state)

Let us assume that, in the *K* system, an allosteric activator, X, binds preferentially to the R state at sites distinct from the substrate binding sites. In this particular case, $K_X^R/K_X^T \ll 1$. An activator mimics the substrate by displacing the $T_0 \leftrightarrow R_0$ equilibrium in favor of R_0. The general form of rate equation for this case is

$$Y_F = \frac{v_0}{V_{max}} = \frac{\alpha(1+\alpha)^{n-1}}{\frac{L}{(1+\gamma)^n} + (1+\alpha)^n} \tag{13.51}$$

where

$$\alpha = \frac{[A]}{K_A} = \frac{[A]}{K_A^R} \quad \text{and} \quad \gamma = \frac{[X]}{K_X} = \frac{[X]}{K_X^R} \tag{13.52}$$

Because both A and X can occupy an R subunit independently, there are a large number of possible complexes of R state with ligands that can be formed. The velocity equations are now much more complex than in the preceding case. For a dimeric enzyme, the rate Eq. (13.51) in an expanded form is

$$Y_F = \frac{v_0}{V_{max}} = \frac{\frac{[A]}{K_A} + \frac{[A]^2}{K_A^2} + \frac{2[A][X]}{K_A K_X} + \frac{2[A]^2[X]}{K_A^2 K_X} + \frac{[A][X]^2}{K_A K_X^2} + \frac{[A]^2[X]^2}{K_A^2 K_X^2}}{L + 1 + \frac{2[A]}{K_A} + \frac{[A]^2}{K_A^2} + \frac{2[X]}{K_X} + \frac{[X]^2}{K_X^2} + \frac{4[A][X]}{K_A K_X} + \frac{2[A]^2[X]}{K_A^2 K_X} + \frac{2[A][X]^2}{K_A K_X^2} + \frac{[A]^2[X]^2}{K_A^2 K_X^2}} \tag{13.53}$$

where $V_{max} = 2k_{cat}E_0$.

Equation (13.53) can be simplified to

$$Y_F = \frac{v_0}{V_{max}} = \frac{\frac{[A]}{K_A}\left(1 + \frac{[A]}{K_A}\right)\left(1 + \frac{[X]}{K_X}\right)^2}{L + \left(1 + \frac{[A]}{K_A}\right)^2\left(1 + \frac{[X]}{K_X}\right)^2} \tag{13.54}$$

Equation (13.54) can be further simplified to

$$Y_F = \frac{v_o}{V_{max}} = \frac{\alpha(1+\alpha)(1+\gamma)^2}{L+(1+\alpha)^2(1+\gamma)^2} \tag{13.55}$$

where α and γ have the meaning given by equation (13.52). Figure 12 shows the graphical presentation of the activation in the *K* system (Eq. (13.51)).

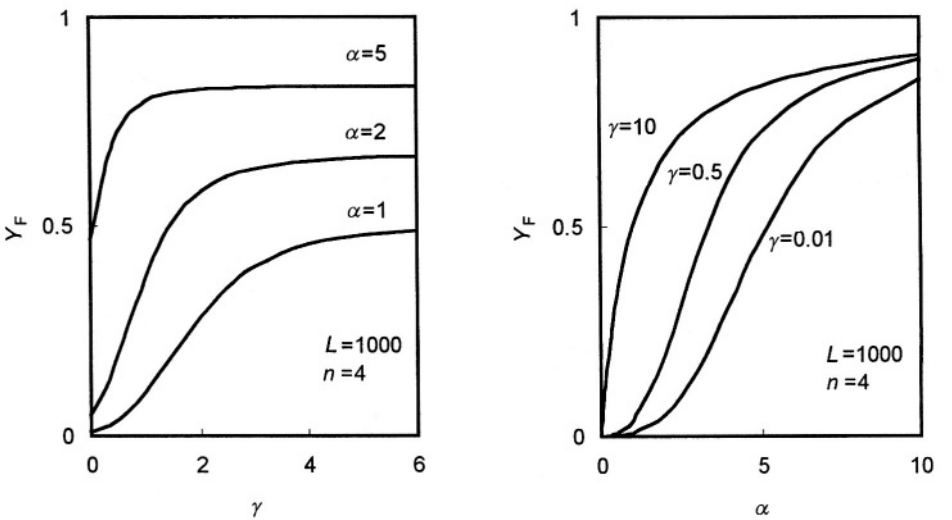


Figure 12. Activation of the tetrameric enzyme in the *K* system. The curves in figures were drawn according to Eq. (13.51). The concentration of an activator, γ, increases gradually in the presence of different concentrations of a substrate, α, and vice versa.

Determination of *L* and K_X in this system is similar to the former system. For this purpose, the general rate equation in the presence of the activator (13.51) can be transformed into a linear form:

$$\sqrt[n]{\frac{v_o}{V^{\alpha}_{max} - v_o}} = \left(\frac{1}{K_X \cdot \sqrt[n]{L_\alpha}}\right)[X] + \frac{1}{\sqrt[n]{L_\alpha}} \tag{13.56}$$

where V^{α}_{max} and L_α have the meaning given by Eq. (13.49).

Heterotropic interactions with multiple allosteric ligands

Let us now analyze the properties of the model with respect to heterotropic interactions between different allosteric ligands. For this purpose, consider a system involving three stereospecific ligands, each binding at a different site. Assume that one of these ligands is the substrate (A) and, for the sake of simplicity, that it has significant affinity only for the sites in one of the two states

(for example, R). Assume similarly that, of the two other ligands, one (the inhibitor I) has affinity exclusively for the T state, and the other (the activator X) for the R state. Let Y_F be the fractional saturation of the enzyme with A.

According to the model, heterotropic effects would be due exclusively to displacement of the equilibrium between the R and T states of the protein. Thus, the saturation function for substrate in the presence of activator and inhibitor may be written as

$$Y_F = \frac{v_o}{V_{max}} = \frac{\alpha(1+\alpha)^{n-1}}{L' + (1+\alpha)^n} \tag{13.57}$$

where L' is an *apparent allosteric constant,* defined as

$$L' = \frac{\sum_o^n T_I}{\sum_o^n R_X} \tag{13.58}$$

where $\sum_o^n T_I$ and $\sum_o^n R_X$ stand, respectively, for the sum of the different complexes of the T state with I and of the R state with X. Following the same derivation as above, it will be seen that

$$L' = L\frac{(1+\beta)^n}{(1+\gamma)^n} \tag{13.59}$$

with $\beta = [I]/K_I$ and $\gamma = [X]/K_X$, where K_I and K_X stand for the microscopic dissociation constants of inhibitor and activator with the R and T states, respectively. Substituting the value of L' in Eq. (13.57), we have

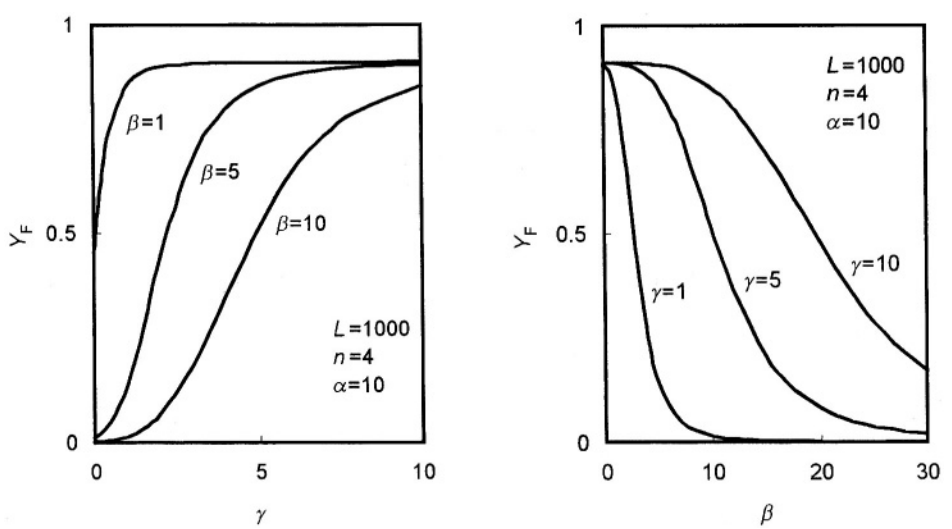


Figure 13. Theoretical curves showing the heterotropic effects of an allosteric activator (γ) or inhibitor (β) upon the shape of the saturation function, drawn according to Eq. (13.60).

$$Y_F = \frac{v_o}{V_{max}} = \frac{\alpha(1+\alpha)^{n-1}}{L\dfrac{(1+\beta)^n}{(1+\gamma)^n} + (1+\alpha)^n} \tag{13.60}$$

This equation expresses the second fundamental property of the MWC model, namely, that the heterotropic effect of an allosteric ligand upon the saturation function for another allosteric ligand should be to modify the homotropic interaction of the latter.

When the substrate itself is an allosteric ligand, the presence of the effector should, therefore, result in a change of the shape of the substrate saturation curve, as in Fig. 13.

The model, therefore, accounts for both homotropic and heterotropic interactions and for their interdependence. No particular assumptions has been, or need be, made about the structure of the specific sites or about the structure of the protein, except that it is a symmetrically bonded oligomer, the symmetry of which is conserved when it undergoes a transition from one to another state.

13.6.3 Nonexclusive Substrate and Effector Binding

The MWC model can also account for considerably more complex cases. For example, Eq. (13.60) becomes much more complicated, although much more realistic, if the ligands were assumed to have significant affinity for both of the two states (Monod *et al.,* 1963).

A substrate and an effector bind to both states

For example, if the substrate, A, and effector, F, bind to both the R and T states and both states are equally active, the complete velocity equation is

$$Y_F = \frac{v_o}{V_{max}} = \frac{Lc\alpha(1+c\alpha)^{n-1}(1+e\gamma)^n + \alpha(1+\alpha)^{n-1}(1+\gamma)^n}{L(1+c\alpha)^n(1+e\gamma)^n + (1+\alpha)^n(1+\gamma)^n} \tag{13.61}$$

where

$$\gamma = \frac{[F]}{K_F}, \text{ the specific effector concentration, and} \tag{13.62}$$

$$e = \frac{K_F^R}{K_F^T}, \text{ the ratio of effector dissociation constants} \tag{13.63}$$

The numerator of Eq. (13.61) now represents the contribution of all possible R and T complexes containing substrate. If $c < 1$ and $e < 1$, the effector is an activator, while if $c < 1$ and $e > 1$, the effector becomes an inhibitor.

A substrate and two effectors bind to both states

Nonexclusive substrate and effector binding may be even more complex; thus, the binding of substrate, A, can take place in the presence of an inhibitor, I, and an activator, X. In this case, the rate equation becomes even more complex:

$$Y_F = \frac{v_o}{V_{max}} = \frac{L'c\alpha(1+c\alpha)^{n-1} + \alpha(1+\alpha)^{n-1}}{L'(1+c\alpha)^n + (1+\alpha)^n} \tag{13.64}$$

This time, an apparent allosteric constant L' is a complex function:

$$L' = L\frac{(1+e\gamma)^n(1+f\beta)^n}{(1+\gamma)^n(1+\beta)^n} \tag{13.65}$$

where

$$e = \frac{K_X^R}{K_X^T} \quad \text{and} \quad f = \frac{K_i^R}{K_i^T} \tag{13.66}$$

Determination of c in nonexclusive systems

In the course of their study of an allosteric enzyme phosphofructokinase, Blangy *et al.* (1968) have developed the method for the estimation of binding constants K_A^R K_A^T and c, in nonexclusive K systems.

In order to illustrate their method, consider a system in which the substrate has an appreciable affinity for the T state, although c is still significantly less than unity ($c = 0.01$). Suppose now that an inhibitor, I, is added and binds almost exclusively to the T state; if the concentration of an inhibitor becomes high and

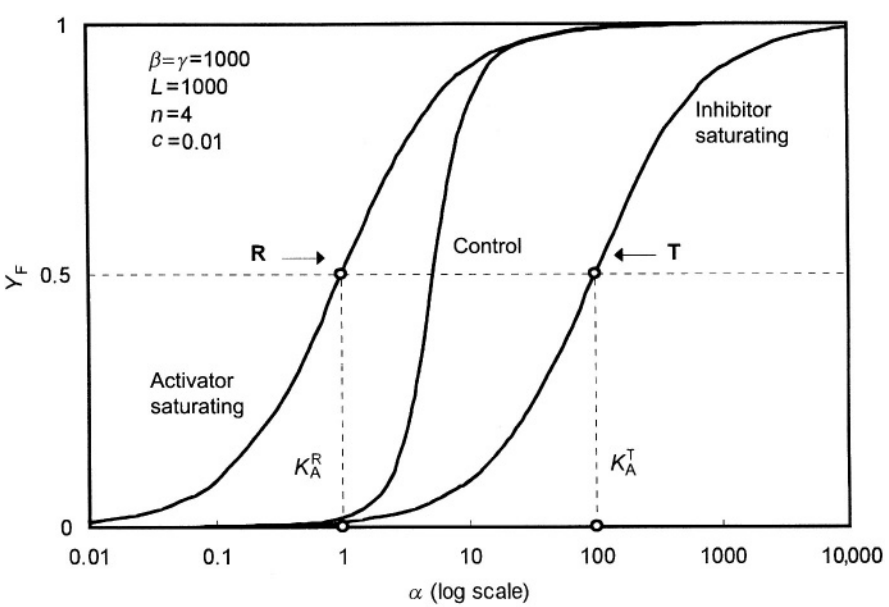


Figure 14. Nonexclusive binding of effectors. Velocity curves were drawn according to Eqs. (13.68)–(13.70), assuming that the substrate, A, binds preferentially to the T state ($c = 0.01$), the inhibitor, I, binds almost exclusively to the T state ($f = 1000$), and the activator, X, binds exclusively to the R state ($e = 0$).

Table 3. Examples of allosteric enzymes that obey the MWC model (Monod *et al.*, 1965; Neet, 1995)

Enzymes	Substrate	+ Inhibitor	+ Activator	Inhibitor + Activator	References
Pyruvate kinase (yeast) (EC 2.7.1.40)	Phospho-enolpyruvate ADP	—	Fructose-1,6-bisphosphate (Fig. 1)	—	Haekel *et al.*, 1968; Colman 1990
Isocitrate dehydrogenase (*Neurospora crassa*) (EC 1.1.1.41)	D-Isocitrate NAD^+	—	AMP (Eq. (13.51)) (Fig. 12)	—	Hathaway & Atkinson, 1963; Colman 1990.
Deoxythimidine kinase (*Escherichia coli*) (EC 2.7.1.21)	Deoxythymidine ATP	—	dCDP (Eq. (13.51)) (Fig. 12)	—	Okazaki & Kornberg, 1964
Threonine deaminase (*Escherichia coli*) (EC 4.2.1.16)	L-Threonine (Eq. (13.36)) (Fig. 10)	L-Isoleucine (Eq. (13.43)) (Fig. 11)	L-Norleucine (Eq. (13.51)) (Fig. 12)	L-Isoleucine + L-Norleucine (Eq. (13.60)) (Fig. 13)	Changeux, 1962, 1964
dCMP deaminase (donkey spleen) (EC 3.5.4.12)	dCMP	dTTP	dCTP	dTTP + dCTP (Eq. (13.60)) (Fig. 13)	Scarano *et al.*, 1963, 1964
Phosphofructokinase (*Bacillus stearothermophilus*) (EC 2.7.1.11)	Fructose-6-phosphate ATP	Phospho-enolpyruvate	ATP	Phosphoenol-pyruvate + ATP (Eq. (13.64)) (Fig. 14)	Blangy *et al.*, 1968

saturating, the conformation of the enzyme will be driven almost exclusively to the T state throughout the whole substrate concentration range used, and the resulting velocity curve will be hyperbolic, with $[A] = K_A^T$ at half the maximal rate of reaction.

On the other hand, in the presence of a saturating concentration of an activator, X, that binds exclusively to the R state, the enzyme will be driven entirely to the R state. This time [A] is equal K_A^R at half the maximal rate of reaction. From these two estimations, the nonexclusive binding coefficient, c, is calculated from

$$c = \frac{K_A^R}{K_A^T} \tag{13.67}$$

In order to draw the appropriate plots for the graphical estimation of K_A^R and K_A^T, the general rate Eq. (13.64) will be transformed into three partial forms:

(a) Control, in the absence of effectors:

$$Y_F = \frac{v_0}{V_{max}} = \frac{Lc\alpha(1+c\alpha)^{n-1} + \alpha(1+\alpha)^{n-1}}{L(1+c\alpha)^n + (1+\alpha)^n} \tag{13.68}$$

(b) In the presence of the substrate and the activator, X:

$$Y_F = \frac{v_0}{V_{max}} = \frac{Lc\alpha(1+c\alpha)^{n-1}(1+e\gamma)^n + \alpha(1+\alpha)^{n-1}(1+\gamma)^n}{L(1+c\alpha)^n(1+e\gamma)^n + (1+\alpha)^n(1+\gamma)^n} \tag{13.69}$$

(c) In the presence of the substrate and inhibitor, I:

$$Y_F = \frac{v_0}{V_{max}} = \frac{Lc\alpha(1+c\alpha)^{n-1}(1+f\beta)^n + \alpha(1+\alpha)^{n-1}(1+\beta)^n}{L(1+c\alpha)^n(1+f\beta)^n + (1+\alpha)^n(1+\beta)^n} \tag{13.70}$$

Figure 14 shows the graphical presentation of Eqs. (13.68)–(13.70). From this figure, one can read the values of binding constants K_A^R and K_A^T directly from the graph, and calculate their ratio ($c = K_A^R/K_A^T = 1/100 = 0.01$).

MWC model is very versatile, compatible with a kinetic behavior of a large number of allosteric enzymes (Neet, 1995). Table 3 lists examples of allosteric enzymes, kinetically compatible with most kinetic models described in this section.

13.7 KOSHLAND, NEMETHY, AND FILMER MODELS (KNF MODELS)

In 1966, Koshland, Nemethy, and Filmer described several models for oligomeric proteins or enzymes with different permissible site–site interactions (Koshland *et al.,* 1966). The KNF models avoid the assumption of symmetry of the MWC model, but use another simplifying features. They assume that the progress from T to the ligand-bound R state is a sequential process. The conformation of each subunit changes in turn as it binds the ligand, and there is no dramatic switch from

one state to another. While the MWC model uses a quaternary structural change, the KNF models use a series of tertiary structural changes (Koshland, 1968).

The KNF models were developed in a framework of a protein involving four subunits, one binding site per subunit, and one type of ligand. Variables that were investigated included

(a) geometrical relationship of subunits,
(b) the binding constants of ligand to protein,
(c) the strength of interaction between subunits, and
(d) the effect of nonidentical subunits.

In all KNF models, it was assumed that the individual subunits of the protein can exist in two conformations, A and B, and that only the conformation B binds the ligand, S, in significant amounts. Upon binding, the ligand induces a conformational change in the subunit to which it is bound. This change may be transmitted to neighboring vacant subunits via the subunit interfaces.

The *substrate (ligand) binding constant,* K_S, which represents the intrinsic affinity of a ligand or a substrate for an individual subunit, is given as

$$K_S = \frac{[BS]}{[B][S]} \tag{13.71}$$

The *transformation constant*, K_T, represents the equilibrium constant for the conformational change from the subunit in conformation A to the subunit in conformation B:

$$K_T = \frac{[B]}{[A]} \tag{13.72}$$

This constant does not include the effect of changing interactions of subunits.

Thus, the overall binding sequence $S + A \rightleftharpoons BS$ is, therefore, described by the product of two equilibrium constants:

$$K_S K_T = \frac{[BS]}{[A][S]} \tag{13.73}$$

To represent the interaction between subunits of different conformational structure, the *interaction constants,* K_{AA}, and K_{BB} will be employed; in this connection, it will be assumed that $K_{AA} = 1$. K_{AB} and K_{BB} are defined by Eqs. (13.74) and (13.75) in which (AB) refers to interacting subunits, whereas (A) and (B) refer to noninteracting subunits:

$$K_{AB} = \frac{[AB][A]}{[AA][B]} \tag{13.74}$$

$$K_{BB} = \frac{[BB][A][A]}{[AA][B][B]} \tag{13.75}$$

It is to be noted that Eqs. (13.74) and (13.75) contain equal stoichiometric amounts of A and B in numerator and denominator and, therefore, the equilibrium constants in these expressions relate the changes in the strengths of interaction

between subunits and do not contain the energy of the conformational change itself. Thus, these constants represent the increased ($K > 1$) or decreased ($K < 1$) stabilization of some conformations of the protein brought about by the interaction of subunits. For example, $K_{AB} > 1$ means that the interaction of AB is more favorable than the interaction of AA and tends to stabilize an AB neighbor with respect to an AA pair.

13.7.1 The Binding Sequence

Thus, upon binding, the ligand induces a conformational change in the subunit to which it is bound; the sequence of conformational transitions in KNF models can be visualized in three ways (Fig. 15):

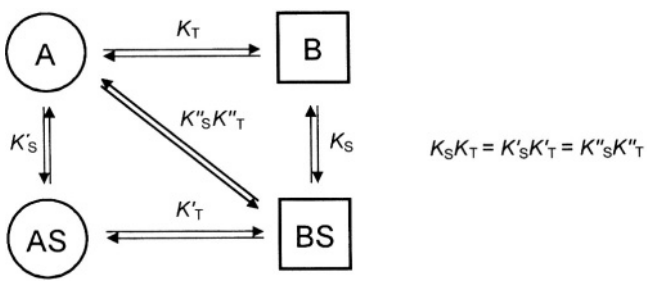


Figure 15. Overall conversion of a vacant A subunit to an occupied B subunit as induced by substrate binding.

(a) The substrate binds to the subunit in the A conformation with a binding constant K'_S and thereby induces a conformational change to the B form:

$$\begin{aligned} A + S &\rightleftharpoons AS \qquad K_{eq} = K'_S \\ AS &\rightleftharpoons BS \qquad K_{eq} = K'_T \\ \text{Sum: } A + S &\rightleftharpoons BS \qquad K_{eq} = K'_S K'_T \end{aligned} \tag{13.76}$$

(b) An equilibrium preexists between conformations A and B. The substrate binds to conformation B and pulls the coupled reactions to the right.

$$\begin{aligned} A &\rightleftharpoons B \qquad K_{eq} = K_T \\ B + S &\rightleftharpoons BS \qquad K_{eq} = K_S \\ \text{Sum: } A + S &\rightleftharpoons BS \qquad K_{eq} = K_S K_T \end{aligned} \tag{13.77}$$

(c) The binding and transformation occur simultaneously, that is,

$$S + A \rightleftharpoons BS \qquad K_{eq} = K''_S K''_T \tag{13.78}$$

Thus, the overall, combined transformation constant is always $K_S K_T$, regardless of the sequence.

13.7.2 Dimer Model

In order to illustrate the simplest KNF model, let us consider first a protein composed of only two subunits. The binding sequence can be represented by the Fig. 16.

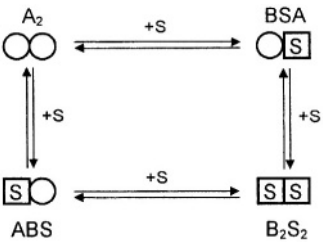
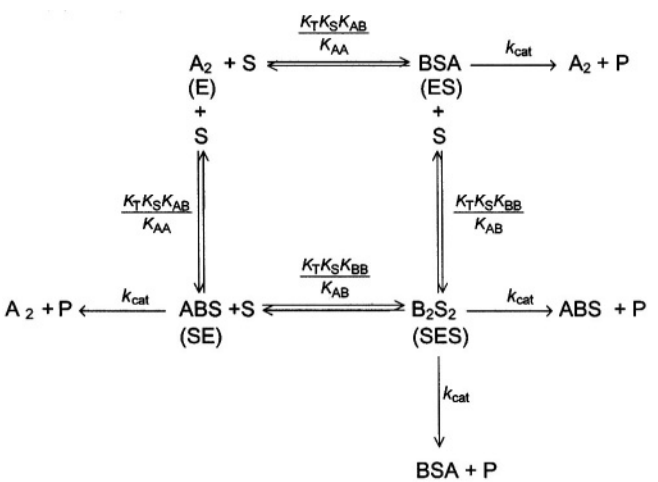


Figure 16. The dimer model.

Note that there are two different singly occupied complexes, BSA and ABS, and, therefore, there are two pathways to obtain a singly occupied complex.

Let us carry over the binding sequence in a dimeric protein in Fig. 16 to a dimeric enzyme operating under the rapid equilibrium conditions, in order to express the binding sequences in terms of all three types of constants: the substrate binding constant, K_S, the transformation constant, K_T, and the interaction constants, K_{AA}, and K_{BB} (Eq. (13.79)).



(13.79)

The velocity equation for the above enzymatic reaction is obtained in the usual manner from the rapid equilibrium assumptions:

$$\frac{v_o}{V_{max}} = \frac{K_T K_S K_{AB}[S] + K_T^2 K_S^2 K_{BB}[S]^2}{1 + 2K_T K_S K_{AB}[S] + K_T^2 K_S^2 K_{BB}[S]^2} \tag{13.80}$$

whereby $V_{max} = 2k_{cat}[E_o]$.

From the binding sequences in reaction (13.79), we can see that the general KNF model takes into account a variety of possible subunit interactions that are able to affect affinities of vacant sites. Therefore, the general KNF model is far more versatile than the simple sequential interaction model or, for that matter, than the MWC model.

13.7.3 Tetramer Model

In this model, several geometric models of subunit interactions will be evaluated. All interactions will be assumed to occur in a single protein containing four subunits. For convenience in visualizing the allosteric effects, a geometrical designation of the subunit interactions will be used, and the various models will be referred to by three descriptive phrases:

(1) *tetrahedral* – each subunit can interact with the other three,
(2) *linear* – two of the subunits can interact with two others, while two of the subunits can interact with only one other, and
(3) *square* – each subunit can interact with only two of the other three.

Figure 17 is a schematic illustration of the various modes of binding the ligand S to the tetrameric enzyme with a concerted mechanism included for comparison.

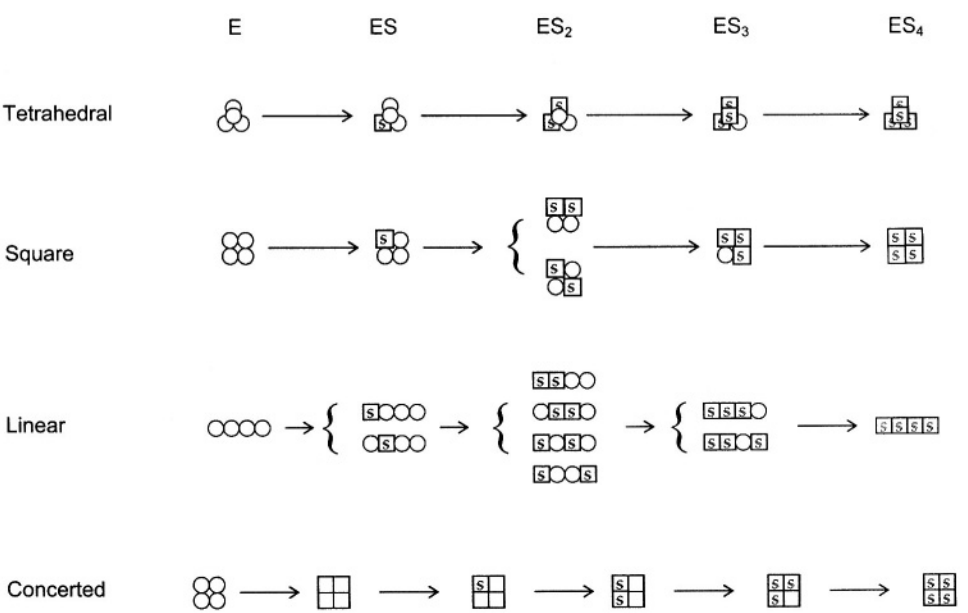


Figure 17. Interaction of subunits in KNF models. Conformation A ≡ ○ B ≡ □.

The terms “tetrahedral”, “square”, etc., are used to clarify the permissible subunit interactions and do not necessarily correspond to the actual arrangement of subunits in three-dimensional space.

13.7.4 Derivation of Binding Equations

In order to derive the saturation equations in the KNF models, the symbol Y is used to represent the fraction of binding sites occupied on the protein by the ligand; this value can vary from 0 to 1.0. The symbol N_S represents the average number of molecules of ligand bound per molecule of protein, as defined by

$$N_S = nY = \frac{[\text{ligand bound}]}{[\text{total protein}]} = \frac{\sum_{i=1}^{i=n} i[ES_i]}{\sum_{i=0}^{i=n} [ES_i]} \tag{13.81}$$

where i represents the number of molecules of S bound to an individual molecular species and n represents the number of subunits per molecular species. It is clear that both N_S and i can range from 0 to n but i can have only integral values, whereas N_S can have fractional values.

In dealing with binding equations, we shall generally refer to Y versus [S], or N_S versus [S] plot as "saturation curves". For convenience in visualizing the allosteric effects, a geometrical designation of the subunit interactions will be used, and the various models will be referred to by three descriptive phrases in Fig. 17.

A *tetrahedral* arrangement results in four equivalent ways to bind one molecule of (S), six ways to bind two, four ways to bind three, and one way to bind four. The number of interacting pairs will be three A–B and three A–A for the A_3BS species; one B–B, four A–B, and one A–A for $A_2B_2S_2$; three B–B and three A–B for AB_3S_3; and six B–B for B_4S_4. When $K_{AB} \neq 1$ and $K_{BB} \neq 1$, the average number of molecules of ligand bound per molecule of protein, N_S, is defined by

$$N_S = \frac{4K_{AB}^3X + 12K_{AB}^4K_{BB}X^2 + 12K_{AB}^3K_{BB}^3X^3 + 4K_{BB}^6X^4}{1 + 4K_{AB}^3X + 6K_{AB}^4K_{BB}X^2 + 4K_{AB}^3K_{BB}^3X^3 + K_{BB}^6X^4} \tag{13.82}$$

where $X = K_S K_T[S]$.

The *square case* assumes four subunits in a square pattern in which no interactions across the diagonal occur. The values of N_S are given by equation

$$N_S = \frac{4K_{AB}^2X + 4(K_{AB}^4 + 2K_{AB}^2K_{BB})X^2 + 12K_{AB}^2K_{BB}^2X^3 + 4K_{BB}^4X^4}{1 + 4K_{AB}^2X + (2K_{AB}^4 + 4K_{AB}^2K_{BB})X^2 + 4K_{AB}^2K_{BB}^2X^3 + K_{BB}^4X^4} \tag{13.83}$$

In the *linear model,* the two interior subunits react with each of the two neighbors, whereas the two terminal subunits react with only one neighbor. Therefore, the subunits can behave differently even though they may have identical tertiary structure. The saturation equation is given by

$$N_S = \frac{\left[\begin{array}{l} 2(K_{AB} + K_{AB}^2)X + 2(K_{AB}^2K_{BB} + 2K_{AB}^3 + K_{AB}^2 + 2K_{AB}K_{BB})X^2 \\ +6(K_{AB}^2K_{BB} + K_{AB}K_{BB}^2)X^3 + 4K_{BB}^3X^4 \end{array}\right]}{\left[\begin{array}{l} 1 + 2(K_{AB} + K_{AB}^2)X + (K_{AB}^2K_{BB} + 2K_{AB}^3 + K_{AB}^2 + 2K_{AB}K_{BB})X^2 \\ +2(K_{AB}^2K_{BB} + K_{AB}K_{BB}^2)X^3 + K_{BB}^3X^4 \end{array}\right]} \tag{13.84}$$

In the *concerted model,* the conformation of all subunits changes simultaneously. Since the subunit interactions all change simultaneously with the change of conformation, only one constant, K_{TC}, is needed to encompass the equilibria previously allocated to K_T, K_{AB}, K_{BB}, etc. The saturation equation is now reduced to

$$N_S = \frac{4K_S[S](1 + K_S[S])^3}{(K_{TC})^{-4} + (1 + K_S[S])^4} \tag{13.85}$$

Illustrative saturation curves for the square, tetrahedral, and concerted interaction geometries are shown in Fig. 18.

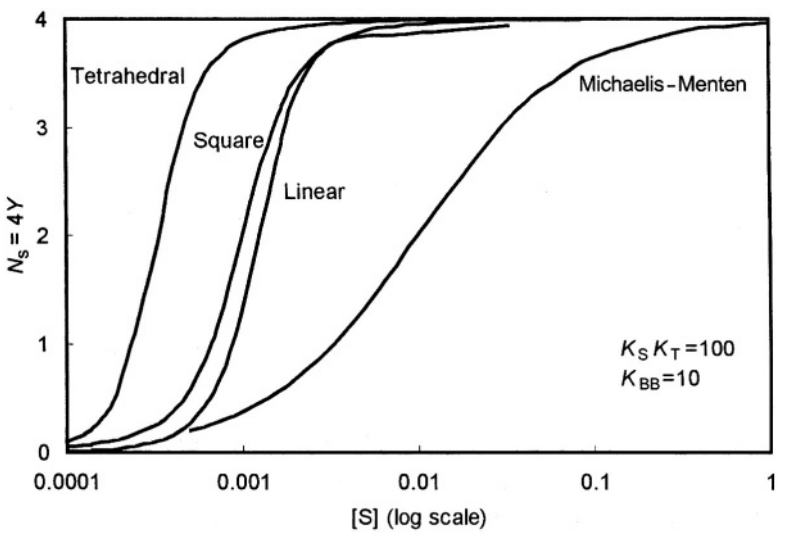


Figure 18. Examples of saturation curves for the various interaction geometries in KNF models and comparison with a Michaelis–Menten curve, having the same binding and transformation constants (K_S and K_T), but no subunit interactions ($K_{AB} = K_{BB} = 1$). $K_{AB} = 1$ for all curves.

On comparing the curves with similar values for constants K_S and K_T and moderate interactions between B conformations, that is, $K_{BB} = 10$, it can be seen that the steepness of the curve increases as the total number of subunit interactions increases, that is, linear < square < tetrahedral. Moreover, the midpoint of the N_S–log[S] curve shifts to lower S concentrations, that is, for the same binding and conformation constants, less ligand concentration is required for the half-saturation of enzyme or a protein. Although the different geometries give different saturation curves if the same intrinsic constants are chosen, the curves still can be made to coincide closely by the proper selection of the various equilibrium constants.

Similarly, we can examine the effect of changing the various equilibrium constants on the binding curves. Thus, we can examine the effect of changing

the $K_S K_T$, K_{AB} or K_{BB}, respectively, on the shape of the binding curves; for examples of this kind, the reader is directed to appropriate literature (Koshland *et al.,* 1966; Neet, 1980, 1995; Neet & Ainslie, 1980).

13.8 COMPARISON OF VARIOUS MODELS

What are the relative merits of the simple sequential model, the KNF model, and the MWC model? Let us compare the rate equations for the tetrameric allosteric enzyme, derived with the aid of each model, and establish the differences (Table 4).

Table 4. Rate equations for a tetrameric enzyme in different allosteric models

Simple interaction model (Eq. (13.16))	$Y_F = \dfrac{v_o}{V_{max}} = \dfrac{\dfrac{[A]}{K_A} + \dfrac{3[A]^2}{aK_A^2} + \dfrac{3[A]^3}{a^2bK_A^3} + \dfrac{[A]^4}{a^3b^2cK_A^4}}{1 + \dfrac{4[A]}{K_A} + \dfrac{6[A]^2}{aK_A^2} + \dfrac{4[A]^3}{a^2bK_A^3} + \dfrac{[A]^4}{a^3b^2cK_A^4}}$
MWC model (Eq. (13.36))	$Y_F = \dfrac{v_o}{V_{max}} = \dfrac{Lc\alpha(1+c\alpha)^3 + \alpha(1+\alpha)^3}{L(1+c\alpha)^4 + (1+\alpha)^4}$
KNF model (Eq. (13.82))	$N_S = \dfrac{4K_{AB}^3X + 12K_{AB}^4K_{BB}X^2 + 12K_{AB}^3K_{BB}^3X^3 + 4K_{BB}^6X^4}{1 + 4K_{AB}^3X + 6K_{AB}^4K_{BB}X^2 + 4K_{AB}^3K_{BB}^3X^3 + K_{BB}^6X^4}$

13.8.1 Comparison of the Simple Sequential and the MWC Model

Comparison of the simple interaction model (Eq. (13.16)) with the MWC model (Eq. (13.36)) shows that, for any finite values of K_R, L, or c, in the latter model, one can calculate the corresponding K_A, a, b, and c values in the former model. Thus, Eqs. (13.16) and (13.36) will give the same v_o/V_{max} values if the following conditions shown by Eqs. (13.86) are fulfilled:

$$K_A = K_R\left(\frac{1+L}{1+Lc}\right) \qquad \frac{1}{a} = 1 + \frac{\dfrac{(1-c)^2}{2c}}{1 + \dfrac{1}{2Lc} + \dfrac{Lc}{2}}$$

$$\frac{1}{b} = 1 + \frac{\dfrac{(1-c)^2}{2c}}{1 + \dfrac{1}{2Lc^2} + \dfrac{Lc^2}{2}} \qquad \frac{1}{c} = 1 + \frac{\dfrac{(1-c)^2}{2c}}{1 + \dfrac{1}{2Lc^3} + \dfrac{Lc^3}{2}} \tag{13.86}$$

In other words, a sigmoidal substrate saturation curve that fits the velocity equation of the former model can be shown to fit the velocity equation of the latter model as well.

The simple sequential interaction model is more general than the concerted-symmetry model in that there are many combinations of values for a, b, and c, for which there are no equivalent values of L and c. Furthermore, the

concerted-symmetry model explicitly excludes the negative cooperativity, a property displayed by many allosteric enzymes. Nevertheless, the MWC model provides a simple explanation for many properties of allosteric enzymes and, most importantly, provides a simple, easily visualized physical explanation for all cooperative effects.

13.8.2 Comparison of the MWC Model with the KNF Model

How does the MWC model compare with the KNF model? Comparing Eq. (13.82) for the KNF model with Eq. (13.36) derived for the MWC model, we find that in place of the simple dissociation constants L and c, we now have the more complex $K_S K_T$ (combined binding-transformation constants) plus two kinds of interaction factors, K_{AB} and K_{BB}.

The MWC model is basically a structural theory. The KNF model avoids the assumption of symmetry but uses another simplifying feature. It assumes that the progress from T to the ligand-bound R state is a sequential process. The conformation of each subunit changes in turn as it binds the ligand, and there is no dramatic transition from one state to another. Thus, the general KNF model is far more versatile than the MWC model. The finding of negative cooperativity excludes the simple MWC theory, but positive cooperativity is consistent with both models. In the latter case, the analysis of the shape of the binding curve is usually ambiguous, as the analysis of the shape of the binding curve with the KNF and MWC models often show similar shapes.

Research workers in areas involving structural properties of enzymes and proteins usually prefer the MWC model because it is essentially a structural theory and provides a simple framework for the prediction and interpretation of data. In theory, measurements of the rates of ligand binding can distinguish between the two models (Eigen, 1967; Austin & Rothberg, 1994; Mathews & Olson, 1994).

In practice, very few critical experiments were designed to decide between the MWC model and the KNF models in specific cases. Definitive ligand-binding studies were performed with a complex allosteric enzyme aspartate transcarbamylase from *Escherichia coli* by Parmentier *et al.* (1992). Aspartate transcarbamylase is an oligomer, responding to increased concentration of its substrates in a cooperative fashion. The holoenzyme is composed of six catalytic and six regulatory subunits; isolated catalytic subunits are catalytically active but devoid of allosteric properties (Stryer, 1988). Parmentier *et al.* (1992) have measured the ^{13}C kinetic isotope effects with physiological substrates, carbamyl phosphate and L-aspartate, for the holoenzyme and the isolated catalytic subunit in the presence of an allosteric effector, a substrate analog *N*-(phosphono-acetyl)-L-aspartate. Because an isotope effect reports back only from the active enzyme, it can distinguish between a loss of activity due to removal of enzyme from participation in catalytic turnover (the MWC model, which will not change the isotope effect on the remaining activity), and a loss due to modulation of rate constants contributing to catalytic turnover (the KNF model, which will change the intrinsic isotope effects).

Comparison of steady-state kinetic data and ^{13}C kinetic isotope effects of the holoenzyme with the isolated catalytic subunit, in this case, were consistent with a two-state MWC model for homotropic regulation of the enzyme in which catalysis occurs only via the R state of the enzyme, and the T state is catalytically inactive; the data effectively ruled out the KNF model.

13.8.3 The General Model

The MWC concerted-symmetry and KNF sequential interaction models may be considered as extreme cases of the more general model shown in Fig. 19. A general model for a four-site allosteric enzyme involves the hybrid oligomers. The first and the fourth column in Fig. 19 represent the concerted-symmetry model. The diagonal represents the sequential interaction model. As shown, there are 25 different types of enzyme forms. If the potential nonequivalent complexes are included (such as, e.g., two different T_3RS_2), the number raises to 44 possible enzyme forms (Hammes & Wu, 1971).

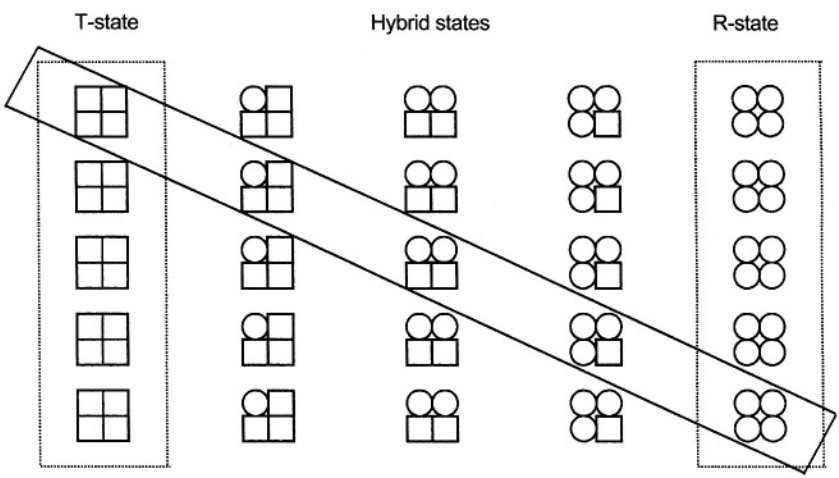


Figure 19. General model for a four-site allosteric enzyme involving hybrid oligomers.

All the models discussed in this chapter have been essentially equilibrium models that can be applied to kinetic experiments only by assuming that v_o/V_{max} is a true measure of Y_F. However, cooperativity can also arise for purely kinetic reasons in mechanisms that would show no cooperativity if binding could be measured at equilibrium (Ricard *et al.,* 1966, 1974; Ricard & Noat, 1984, 1986). Examples for such models are rarely described in the literature, and the kinetic model for hexokinase-D represents an example (Neet, 1995).

13.8.4 Hypotheses for Substrate Binding to Enzymes

There are two conceptual frameworks for interpreting the binding of substrates to enzymes. The first hypothesis was advanced by Fischer (1894), who proposed that the rigid substrate fits the rigid active site of enzyme just as the key fits the

lock (*key-and-lock hypothesis*). The second theory (*induced-fit hypothesis*) was proposed by Koshland (1958, 1960). Koshland's theory is a conceptual framework for interpreting the action of substrate binding in reorienting otherwise inactive groups on the enzyme into their catalytically active configuration; this implies an essential isomerization step. Yeast hexokinase provides a good example of induced fit, since the enzyme undergoes a dramatic conformational change upon binding of glucose (Jencks, 1969) (see Fig. 3 of Chapter 1).

It is probable that most enzymes undergo conformational changes upon binding of substrates. However, if such conformational changes are small, the substrate binding may be interpreted within the framework of Fischer's rigid stereochemical hypothesis.

REFERENCES

Books

Changeux, J.P. (1964) *These Doctorat des Sciences,* Paris.
Chock P.B., Huang, C.Y., Tsou, C.L., & Wang, J.H. (Eds.) (1988) *Enzyme dynamics and regulation,* Springer-Verlag, Berlin.
Engel, P.C. (Ed.) (1996) *Enzymology Labfax,* Bios Scientific Publishers, Oxford.
Jencks, W.P. (1969) *Catalysis in chemistry and enzymology,* McGraw-Hill, New York [Also available as *Catalysis in chemistry and enzymology* (1986) Dover Publications, New York].
Klotz, M. (1997) *Ligand-receptor energetics,* Wiley, New York.
Kurganov, B.I. (1982) *Allosteric enzymes: kinetic behaviour,* Wiley, New York.
Stryer, L. (1988) *Biochemistry,* 3rd ed., W.H. Freeman, New York.
Winzor, D.J. & Sawyer, W.H. (1995) *Quantitative characterization of ligand binding,* Wiley-Liss, New York.

Review articles

Austin, R.H. & Rothberg, L.J. (1994) *Methods Enzymol. 232,* 176–205.
Colman, R.F. (1990) *The Enzymes,* 3rd ed., Vol. 9, 283–300.
Davies, L. & Metzler, D.E. (1972) *The Enzymes,* 3rd ed., Vol. VII, 33–50.
DeLand, E.C. (1994) *Methods Enzymol. 232,* 632–655.
Eigen, M. (1967) *Nobel Symp. 5,* 333–350.
Holbrook, J.J., Liljas, A., Steindel, S.J., & Rossmann, M.G. (1975) *The Enzymes,* 3rd ed., Vol. XI, 191–240.
Imai, K. (1994) *Methods Enzymol. 232,* 559–576.
Johnson, M.L. (1994) *Methods Enzymol. 232,* 597–606.
Koshland, D.E. (1960) *Adv. Enzymol. 22,* 45–70.
Koshland, D.E. (1968) *Annu. Rev. Biochem. 37,* 359–380.
Mathews, A.J. & Olson, J.S. (1994) *Methods Enzymol. 232,* 363–387.
Neet, K.E. (1980) *Methods Enzymol. 64,* 139–192.
Neet, K.E. & Ainslie, R. (1980) *Methods Enzymol. 64,* 192–226.
Neet, K.E. (1995) *Methods Enzymol. 249,* 519–567.
Perutz, M.F., Wilkinson, A.J., Paoli, M., & Dodson, G.G. (1998) *Annu. Rev. Biophys. Biomol. Struct. 27,* 1–34.

Specific references

Adair, G.S. (1925) *J. Biol. Chem. 63,* 529–545.
Adair, G.S. (1925) *Proc. Royal Soc.* A *109*, 292–300.
Blangy, D., Buc, H., & Monod, J. (1968) *J. Mol. Biol. 31,* 13–35.

Bohr, C. (1903) *Zentralbl. Physiol. 17,* 682–688.
Changeux, J.P. (1962) *J. Mol. Biol 4,* 220–230.
Chariler, H.A. & Plapp, B.V. (2000) *J. Biol. Chem. 275,* 11569–11575.
Fischer, E. (1894) *Berichte Dtsch. Chem. Ges. 27,* 2985–2993.
Grant, G.A., Schuller, D.J., & Banaszak, L.J. (1996) *Protein Sci. 5,* 34–41.
Hammes, G.G. & Wu, C.-W. (1971) *Science 172,* 1205–1210.
Hathaway, J.A. & Atkinson, D.E. (1963) *J. Biol. Chem. 238,* 2875–2885.
Haeckel, R., Hess, B., Lauterhorn, W., & Wuster, K.-H. (1968) *Hoppe-Seyler's Z. Physiol. Chemie 349*, 699–714.
Henry, E.R., Jones, C.M., Hofrichter, J., & Eaton, W.R. (1997) *Biochemistry 36,* 6511–6528.
Hess, B., Haeckel, R., & Brand, K. (1966) *Biochem. Biophys. Res. Commun. 24,* 824–831.
Hess, B. & Wurster, B. (1970) *FEBS-Lett. 2,* 73–77.
Hill, A.V. (1910) *J. Physiol. 40,* 4–7.
Koshland, D.E. (1958) *Proc. Natl. Acad. Sci. USA 44,* 98–99.
Koshland, D.E., Nemethy, G., & Filmer, D. (1966) *Biochemistry 5,* 365–385.
Leskovac, V. & Pavkov-Perič in, D. (1975) *Biochem. J. 145,* 581–590.
Leskovac, V., Trivić, S., & Pantelić, M. (1993) *Anal. Biochem. 214,* 431–434.
Monod, J., Changeux, J.-P., & Jakob, F. (1963) *J. Mol. Biol. 6,* 306–329.
Monod, J., Wyman, J., & Changeux, J.-P. (1965) *J. Mol. Biol. 12,* 88–118.
Okazaki, R. & Kornberg, A. (1964) *J. Biol. Chem. 239*, 275–285.
Parmentier, L.E., O'Leary, M.H., Schachman, H.K., & Cleland, W.W. (1992) *Biochemistry 31,* 6598–6602.
Ricard, J., Mouttet, C., & Nari, J. (1966) Eur. *J. Biochem. 41,* 479–489.
Ricard, J., Meunier, J.-C., & Buc, J. (1974) *Eur. J. Biochem. 49,* 195–208.
Ricard, J. & Noat, G. (1984) *J. Theor. Biol. 111,* 735–753.
Ricard, J. & Noat, G. (1986) *J. Theor. Biol 123,* 431–451.
Scarano, E., Geraci, G., Polzella, A., & Campanille, E. (1963) *J. Biol. Chem. 238,* 1556–1566.
Scarano, E., Geraci, G., & Rossi, M. (1964) *Biochem. Biophys. Res. Comm. 16,* 239–243.
Scatchard, G. (1949) *Ann. N. Y. Acad. Sci. 51,* 660–672.
Scatchard, G., Coleman, J.S., & Shen, A.L. (1957) *J. Am. Chem. Soc.* 79, 34–40.
Schuller, D.J., Grant, G.A., & Banaszak, L.J. (1995) *Nature Struct. Biol. 2,* 69–76.

Chapter 14

The pH Dependence of Enzyme Catalysis

14.1 INTRODUCTION

Most enzymatic reactions *in vivo* and *in vitro* take place in aqueous solutions. Most enzymes are extremely sensitive to changes in pH of their environment. Whole-cell extracts, and crude enzyme preparations in general, are well buffered by enzyme and other polyelectrolyte impurities, but this natural buffering is lost when an enzyme is purified, and must be replaced by artificial buffers.

The simplest type of pH effect on enzyme, involving only a single acidic or basic group, is not different from the general case of inhibition or activation that was described in Chapters 5–7. Conceptually, the protonation of a basic group on an enzyme is simply the special case of the binding of a modifier at a specific site. However, there are several differences between protons and other modifiers that make it worth examining protons separately. First, virtually all enzymes are affected by protons, so that the proton is far more important than any other modifier. Second, the proton concentration can be measured and controlled over a range that is enormously greater than that available for any other modifier and therefore one can expect to observe very versatile effects on enzyme kinetics. Also, finally, protons normally bind to many sites on an enzyme, so that it is often insufficient to consider binding at one site only.

It is obvious that, in polymers like proteins, the acidic and basic properties are the consequence of protonation and dissociation of acidic and basic groups in side chains of polypeptides.

Out of many available definitions of acids and bases, perhaps the best and most accurate was given by Brönsted (1923): "An acid is a species having a tendency to lose a proton, and a base is a species having a tendency to add on a proton." Accordingly, Table 1 gives the classification of amino acid side chains in proteins according to their tendency to lose or gain the protons. The values given in Table 1 are average values for typical environments, however, and may differ substantially from the pK_a values of individual groups in special environments, such as the active sites of enzymes. A clear-cut example is provided by lysozyme, which has two catalytically active amino acids in the active site, Glu35 and Asp51; the pK_a value of the side chain carboxyl group in the former amino acid is perturbed to 3.5 and of the latter to 6.5, due to their microenvironment within the protein. Thus, a pair of amino acid side chains in the active site of lysozyme, Asp–Glu, serves as a general acid catalyst that helps to hydrolyze the *O*-glycosidic linkage of the polysaccharide substrate of lysozyme (Phillips, 1966).

Table 1. Ionizable groups in proteins

Amino acids	Types of group	Brönsted character at pH 7.0	pK_a	
			Small peptides[a]	Proteins[b]
C-Terminal	Carboxylate	Basic	3.6	3.0–3.4
Aspartate	Carboxylate	Basic	4.0	3.0–5.0
Glutamate	Carboxylate	Basic	4.5	3.0–5.0
Histidine	Imidazole	Mainly basic	6.4	5.5–7.0
N-terminal	Ammonium	Mainly acidic	7.8	7.5–8.5
Cysteine	Thiol	Acidic	9.1	8.0–8.5
Tyrosine	Phenol	Acidic	9.7	9.8–10.5
Lysine	Ammonium	Acidic	10.4	9.5–10.6
Arginine	Guanidinium	None	~12	11.6–12.6

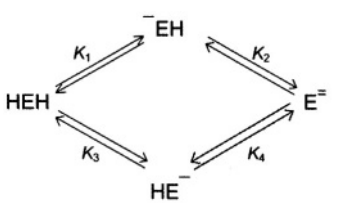
[a]Adopted from the data of Tanford (1962).
[b]pK_a values are based on average values at 25°C for groups in typical environments in proteins (Steinhardt & Reynolds, 1969).

14.2 DISSOCIATION OF DIBASIC ACIDS

14.2.1 Group Dissociation Constants

The first practical model for the pH dependence of enzyme catalysis was proposed by Michaelis (Michaelis & Davidsohn, 1911). The pH behavior of many enzymes can be interpreted as a first approximation in terms of this model, in which only two ionizable groups are considered.

The active site of an enzyme is represented as a dibasic acid, with two non-identical acidic groups (Adams, 1916; Michaelis, 1922; Brockelhurst, 1994).



(14.1)

With the dissociation constants defined as shown in this scheme, the concentrations of all forms of enzyme can be represented in terms of hydrogen-ion concentration:

$$[^{-}\mathrm{EH}] = \frac{[\mathrm{HEH}]K_1}{[\mathrm{H}^+]} \tag{14.2}$$

$$[\mathrm{HE}^{-}] = \frac{[\mathrm{HEH}]K_3}{[\mathrm{H}^+]} \tag{14.3}$$

$$[\mathrm{E}^{=}] = \frac{[\mathrm{HEH}]K_1K_2}{[\mathrm{H}^+]^2} = \frac{[\mathrm{HEH}]K_3K_4}{[\mathrm{H}^+]^2} \tag{14.4}$$

If the total enzyme concentration is $E_0 = [HEH] + [^-EH] + [HE^-] + [E^{=}]$, then:

$$[HEH] = \frac{E_0}{1 + \frac{K_1 + K_3}{[H^+]} + \frac{K_1K_2}{[H^+]^2}} \tag{14.5}$$

$$[^-EH] = \frac{\frac{E_0K_1}{[H^+]}}{1 + \frac{K_1 + K_3}{[H^+]} + \frac{K_1K_2}{[H^+]^2}} \tag{14.6}$$

$$[HE^-] = \frac{\frac{E_0K_3}{[H^+]}}{1 + \frac{K_1 + K_3}{[H^+]} + \frac{K_1K_2}{[H^+]^2}} \tag{14.7}$$

$$[E^{=}] = \frac{\frac{E_0K_1K_2}{[H^+]^2}}{1 + \frac{K_1 + K_3}{[H^+]} + \frac{K_1K_2}{[H^+]^2}} \tag{14.8}$$

Thermodynamic reasons dictate that

$$K_1K_2 = K_3K_4 \tag{14.9}$$

Dissociation constants K_1, K_2, K_3, and K_4, are termed *group dissociation constants*. Expressions (14.5)–(14.8) show how the concentrations of the four species vary with $[H^+]$, that is, with pH. Figure 1 shows the graphical presentation of these equations, with arbitrary values assumed for the dissociation constants.

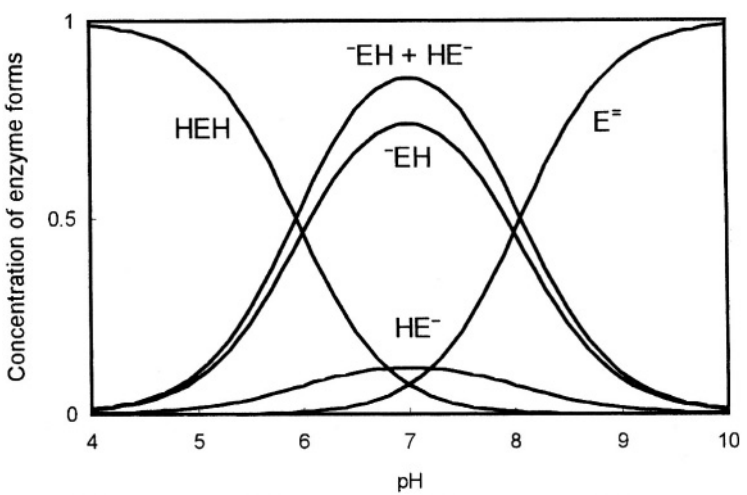


Figure 1. Relative concentrations of enzyme forms as a function of pH. Graphical presentation of Eqs. (14.5)–(14.8), with the following group dissociation constants: $pK_1 = 6.0$, $pK_2 = 8.0$, $pK_3 = 6.8$, and $pK_4 = 7.2$.

14.2.2 Molecular Dissociation Constants

Although K_1 and K_4 refer to dissociation of a proton from the same group, they are not in general identical. Usually, K_1 will be greater than K_4, because a negative charge on HE^- can help to hold a proton on group HE^-. The ratio of the concentrations of the two singly protonated species is given by

$$\frac{[HE^-]}{[^-EH]} = \frac{K_1}{K_3} \tag{14.10}$$

and this ratio is constant and independent of $[H^+]$.

For this reason, there is no way of telling how much of a given pH effect is due to one of these two species. Therefore, it is more convenient to treat them as a single species whose concentration is given by

$$[HE^-] + [^-EH] = \frac{\dfrac{E_0(K_1 + K_3)}{[H^+]}}{1 + \dfrac{K_1 + K_3}{[H^+]} + \dfrac{K_1K_2}{[H^+]^2}} \tag{14.11}$$

which may be written as follows:

$$[HE^-] + [^-EH] = \frac{E_0}{1 + \dfrac{[H^+]}{K_A} + \dfrac{K_B}{[H^+]}} \tag{14.12}$$

If the new constants are defined as follows:

$$K_A = K_1 + K_3 \text{ and } K_B = \frac{K_1K_2}{K_1 + K_3} \tag{14.13}$$

The constants K_A and K_B are termed *molecular dissociation constants* to distinguish them from the group dissociation constants (K_1–K_4). Only the molecular dissociation constants can be measured experimentally, although evidence can be obtained indirectly on the magnitude of group constants (Dixon, 1976).

14.3 EFFECTS OF pH ON KINETICS OF MONOSUBSTRATE REACTIONS

From the illustrations shown in Fig. 1, one can easily appreciate the significance of above theoretical considerations for enzyme kinetics. If the activity of an enzyme depends on the protonation of a single acidic group in the active site, and if the HEH species is the only active form, then the profile of enzyme activity versus pH will show the constant and maximal activity in the acid and gradually decrease to zero in the alkaline. Similarly, if the $E^=$ species is the only active form, the pH profile will show the constant and maximal activity in the alkaline and gradually decrease to zero in the acid. On the other hand, if the single

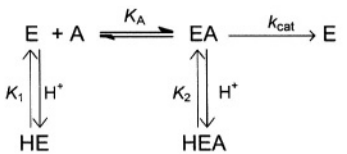
protonated species (^{-}EH and HE^{-}) afford the catalytically active enzyme, then the pH profile will appear as a bell-shaped curve, and the initial rate of enzyme-catalyzed reaction varies with $[H^+]$ according to equation

$$v_0 = \frac{k_{cat}E_0}{1 + \frac{[H^+]}{K_A} + \frac{K_B}{[H^+]}} \tag{14.14}$$

From the arbitrary group dissociation constants given in Fig. 1, one can calculate the values of molecular dissociation constants, pK_A (5.9) and pK_B (8.1), which are, in this case, 2.2 pH units apart. Since only the molecular dissociation constants can be measured experimentally, they have to be sufficiently apart to be distinguished from each other from the experimental data.

14.3.1 Free Enzyme and Enzyme–Substrate Complex are Protonated

Let us consider the influence of pH on a monosubstrate reaction of the Michaelis–Menten type, a case when both forms of enzyme are protonated (Mahler & Cordes, 1966).



(14.15)

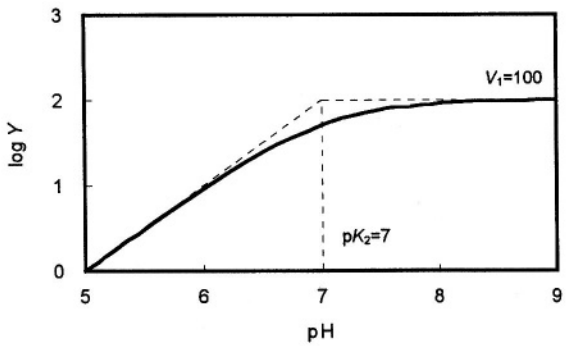
If $V_1 = k_{cat}E_0$, the steady-state treatment of this mechanism yields the initial velocity equation:

$$v_0 = \frac{\left(\frac{V_1}{1 + \frac{H}{K_2}}\right)A}{K_A\left(\frac{1 + \frac{H}{K_1}}{1 + \frac{H}{K_2}}\right) + A} \tag{14.16}$$

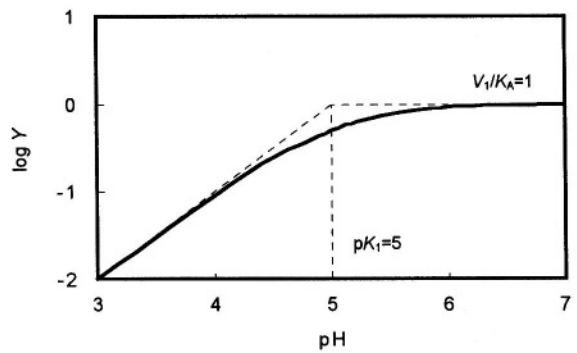
The three main kinetic functions are affected by pH in the following way:

$$V_1^{APP} = \frac{V_1}{1 + \frac{H}{K_2}} \tag{14.17}$$

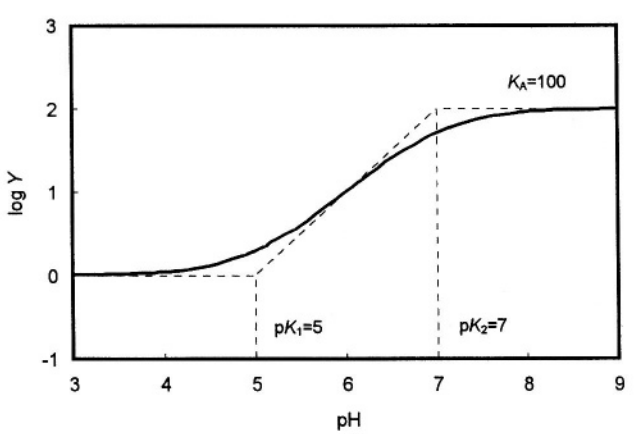
$$K_A^{APP} = K_A\left(\frac{1 + \frac{H}{K_1}}{1 + \frac{H}{K_2}}\right) \tag{14.18}$$



$$V_1^{APP} = \frac{V_1}{1 + \frac{H}{K_2}}$$



$$\left(\frac{V_1}{K_A}\right)^{APP} = \left(\frac{\frac{V_1}{K_A}}{1 + \frac{H}{K_1}}\right)$$



$$K_A^{APP} = K_A\left(\frac{1 + \frac{H}{K_1}}{1 + \frac{H}{K_2}}\right)$$

Figure 2. Free enzyme and enzyme–substrate complex are protonated. Dixon–Webb plots of Eqs. (14.17)–(14.19) are drawn assuming that $V_1 = K_A = 100$.

$$\left(\frac{V_1}{K_A}\right)^{APP} = \left(\frac{\dfrac{V_1}{K_A}}{1+\dfrac{H}{K_1}}\right) \tag{14.19}$$

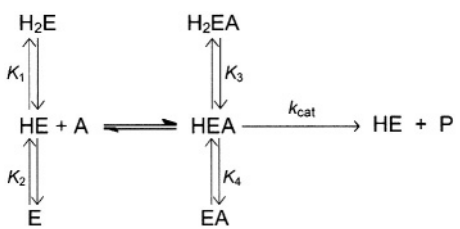
Figure 2 shows the Dixon–Webb plots of Eqs. (14.17)–(14.19).

Figure 2 shows graphically an important property of Eq. (14.16). The pH profile of log V_1/K_A will show the pK_a of the *free enzyme* only, while the pH profile of log V_1 will show the pK_a of the *enzyme–substrate complex* only. On the other hand, the pH profile of log K_A will show both pK_a values (Fersht, 1999).

This simple rule is also valid with multisubstrate reactions. If the enzyme is saturated with all substrates, except one (which is variable), the pH profile of *V/K* will always reflect dissociation in the free enzyme and the pH profile of *V* will always reflect dissociation in the enzyme–substrate complex.

14.3.2 Free Enzyme and Enzyme–Substrate Complex Are Protonated Twice

Let us consider a more complex case, when the free enzyme and the enzyme–substrate complex are each protonated twice (Mahler & Cordes, 1966):



(14.20)

where K_1 and K_2 represent the molecular dissociation constants for the free enzyme, and K_3 and K_4 those for the enzyme–substrate complex. If the k_{cat} is substituted with V_1/E_o, the steady-state treatment of this mechanism yields the following initial velocity equation (Euler *et al.*, 1924; Waley, 1953; Alberty & Massey, 1954; Brockelhurst, 1994):

$$v_o = \frac{\left(\dfrac{V_1}{1+\dfrac{H}{K_3}+\dfrac{K_4}{H}}\right)A}{K_A\left(\dfrac{1+\dfrac{H}{K_1}+\dfrac{K_2}{H}}{1+\dfrac{H}{K_3}+\dfrac{K_4}{H}}\right)+A} \tag{14.21}$$

where V_1 is the maximal velocity of reaction and K_A the Michaelis constant; thus, V_1 and K_A are the limits to which V_1^{APP} and K_A^{APP} tend at pH values between the two relevant pK_a values, if these pK_a values are sufficiently apart.

Inspection of Eq. (14.21) shows that the hydrogen ion concentration will affect both the apparent K_A and V_1 values; thus, hydrogen ion can be regarded as a noncompetitive effector of the enzyme. The apparent maximum velocity will be given by

$$V_1^{APP} = \frac{V_1}{1 + \frac{H}{K_3} + \frac{K_4}{H}} \tag{14.22}$$

It will, therefore, depend only on the ionizations of the enzyme–substrate complex. The apparent K_A value will be given by

$$K_A^{APP} = K_A \frac{\left(1 + \frac{H}{K_1} + \frac{K_2}{H}\right)}{\left(1 + \frac{H}{K_3} + \frac{K_4}{H}\right)} \tag{14.23}$$

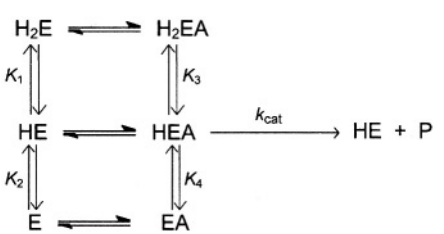
and will, therefore, be affected by ionizations of both the free enzyme and the enzyme–substrate complex. Combination of Eqs. (14.22) and (14.23) gives

$$\left(\frac{V_1}{K_A}\right)^{APP} = \frac{\left(\frac{V_1}{K_A}\right)}{\left(1 + \frac{H}{K_1} + \frac{K_2}{H}\right)} \tag{14.24}$$

Equation (14.24) shows that V_1/K_A varies with pH in a way that depends only on ionizations of the free enzyme. Figure 3 shows the Dixon–Webb plots of Eqs. (14.22)–(14.24).

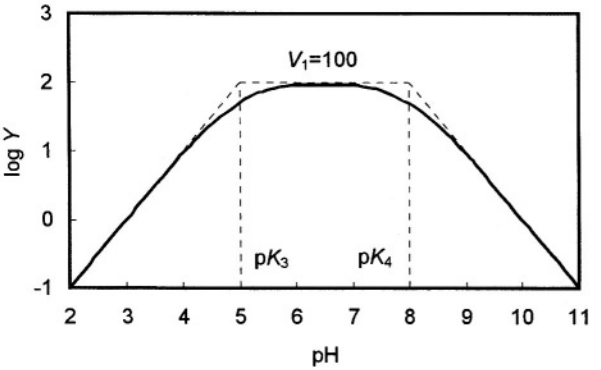
14.3.3 More than One Form of Enzyme Can Bind Substrate

Direct interconversion in only one ionization state (Reaction 14.20) is implausible in the general case, and the lack of recognition of the parallel pathways may lead to serious errors. It is more realistic to assume that differently protonated forms of the enzyme combine with substrate to afford differently protonated enzyme–substrate complexes with, however, only one form of enzyme–substrate complex being catalytically active (Reaction (14.25)).

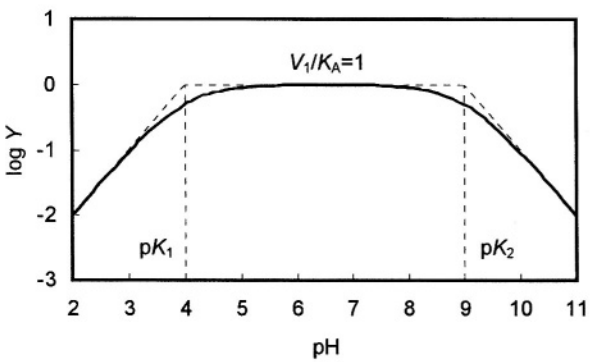


(14.25)

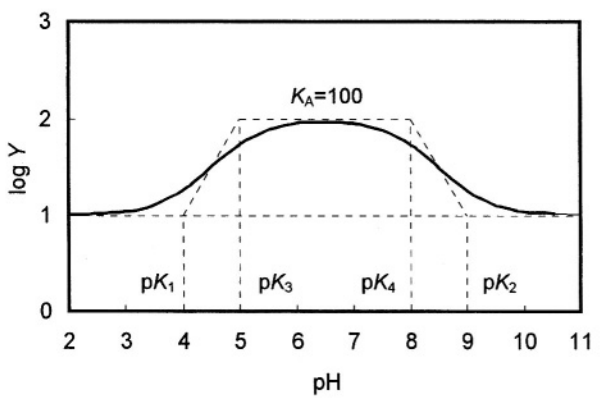
If it is assumed that the rate of breakdown of the HEA complex to yield products is slow relative to dissociation steps, so that the latter remain at



$$V_1^{APP} = \frac{V_1}{1 + \frac{H}{K_3} + \frac{K_4}{H}}$$



$$\left(\frac{V_1}{K_A}\right)^{APP} = \left(\frac{\frac{V_1}{K_A}}{1 + \frac{H}{K_1} + \frac{K_2}{H}}\right)$$



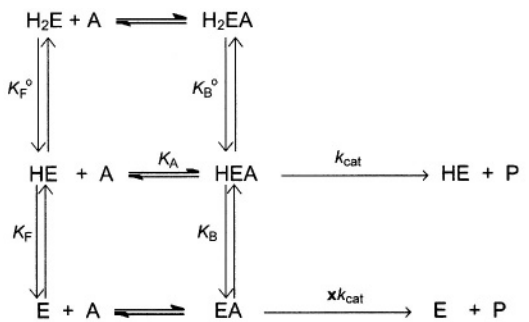
$$K_A^{APP} = K_A \frac{\left(1 + \frac{H}{K_1} + \frac{K_2}{H}\right)}{\left(1 + \frac{H}{K_3} + \frac{K_4}{H}\right)}$$

Figure 3. Free enzyme and enzyme–substrate complex are protonated twice. Dixon–Webb plots of Eqs. (14.22)–(14.24) are drawn assuming that $V_1 = K_A = 100$.

thermodynamic equilibrium, the situation does not differ from reaction (14.20), because the addition of a pathway cannot affect an equilibrium, and thus Eq. (14.21) will apply to the mechanism shown in reaction (14.25) (Tipton & Dixon, 1979; Brockelhurst, 1994).

14.3.4 More than One Form of Enzyme–Substrate Complex Can Yield Products

So far it was assumed that the ionizations that affect the breakdown of enzyme–substrate complex to products completely prevent this reaction. It is, however, possible that loss or gain of a proton near the active site may just change the rate of the reaction, without preventing it. So, reaction (14.25) must be modified to allow for another route to products. In this case, the enzyme has three states of protonation (E, EH, and EH_2), the substrate binds to all of them, while two enzyme–substrate complexes (EA and HEA) are transformed to products (Reaction (14.26)).



(14.26)

As in Section 14.3.3, we shall deal only with the rapid equilibrium of substrate and hydrogen-ion binding, because the full steady-state equations are very complex. Hence, the Michaelis constant represents the true dissociation constant of respective enzyme–substrate complexes, and K_F and K_B represent the acid dissociation constants of amino acids in the free and the bound enzyme, respectively.

If it is assumed that $V_1 = k_{cat}E_0$ and $V_2 = xk_{cat}E_0$, the resultant rate equation can be easily derived from the rapid equilibrium assumptions (Tipton & Dixon, 1979):

$$v_0 = \frac{\left[\dfrac{V_1 + V_2\left(\dfrac{K_B}{H}\right)}{1 + \dfrac{H}{K_B^0} + \dfrac{K_B}{H}}\right]A}{K_A\left(\dfrac{1 + \dfrac{H}{K_F^0} + \dfrac{K_F}{H}}{1 + \dfrac{H}{K_B^0} + \dfrac{K_B}{H}}\right) + A} \qquad (14.27)$$

where V_1 and V_2 are the maximal velocities at low and high pH, respectively. The pH dependence of the apparent Michaelis constant, K_A, will give all four pK_a values, in the same way as the preceding model in reaction (14.20) (Eq. (14.23)),

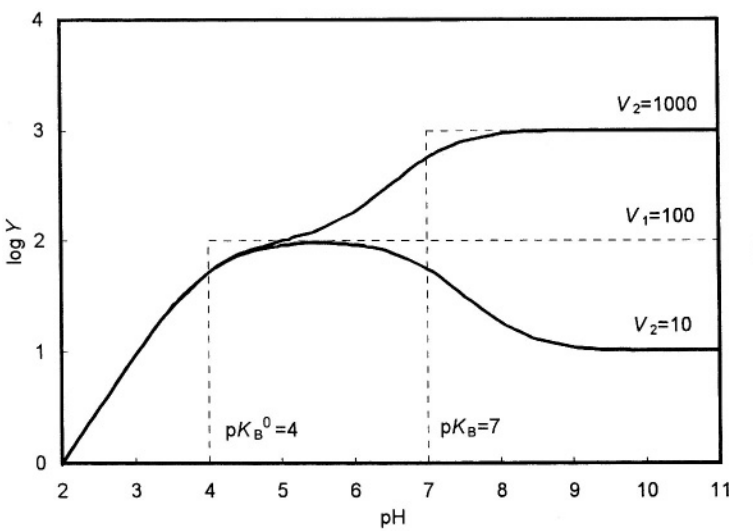
$$K_A^{APP} = K_A \left(\frac{1 + \frac{H}{K_F^o} + \frac{K_F}{H}}{1 + \frac{H}{K_B^o} + \frac{K_B}{H}} \right) \tag{14.28}$$

The pH dependence of the maximal rate, V^{APP}, and the specificity constant, $(V/K_A)^{APP}$, are now complex expressions:

$$V^{APP} = \frac{V_1 + V_2 \left(\frac{K_B}{H} \right)}{1 + \frac{H}{K_B^o} + \frac{K_B}{H}} \tag{14.29}$$

$$\left(\frac{V}{K_A} \right)^{APP} = \frac{\left[V_1 + V_2 \left(\frac{K_B}{H} \right) \right]}{K_A \left(1 + \frac{H}{K_F^o} + \frac{K_F}{H} \right)} \tag{14.30}$$

Figure 4 shows the pH profiles of the V^{APP} function, if $V_1 > V_2$, that is, if the maximal rate is higher in the acid than in alkaline, and vice versa, if $V_1 < V_2$. In each case, two plateaus are seen, each one for V_1 and V_2, because, in this particular case, the pK_a values are well separated by 3 pH units. Again, it is immediately apparent that the pH profile of the $\log V^{APP}$ function shows the pK_a on enzyme in the enzyme–substrate complex, that is, the values of pK_B and pK_B^o, respectively.



$$V^{APP} = \frac{V_1 + V_2 \left(\frac{K_B}{H} \right)}{1 + \frac{H}{K_B^0} + \frac{K_B}{H}}$$

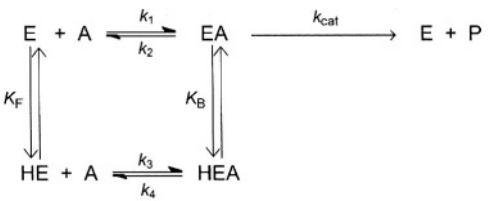
Figure 4. The Dixon–Webb plot of Eq. (14.29) is drawn assuming that $V_1 = 100$, and $V_2 = 10$, or 1000. Deprotonation increases ($V_1 < V_2$) or decreases ($V_1 > V_2$) the rate of the catalyzed reaction.

14.3.5 Multiple Reactive Sites

The preceding mechanism, shown in reaction (14.26), can be extended to activity for more than two protonic states in the enzyme–substrate complex. Provided the pK_a values are separated well enough so that plateaus exist, their values can be determined as above. The rate equations for kinetic mechanisms with multiple protonic states were developed by Brockelhurst and coworkers (Brockelhurst *et al.,* 1990; Topham *et al.,* 1991; Brockelhurst, 1996). For models in which there is multiplicity of reactive protonic species, each of which is considered to provide products in a single-step, reaction rate equations for reactions in any number of protonic states may be written down or generated within the computer without the need for extensive algebraic manipulation; this is achieved by using simple general expressions developed by Brockelhurst and coworkers.

14.3.6 Change in the Rate-Limiting Step with pH

Under normal circumstances, the pH dependence of k_{cat}/K_A follows the titration curve of the active site of the free enzyme. However, a special complication arises when the rate-limiting step changes considerably with pH (Reaction (14.31)).



(14.31)

In such cases, which are frequent in enzyme kinetics, an anomalous pH dependence of *V/K* may take place (Renard & Fersht, 1973). If the binding steps are fast compared to the chemical step (k_{cat}), and k_2 is much faster than k_{cat}, the specificity constant k_{cat}/K_A is then equal $k_{cat}/(k_1/k_2)$, and its pH dependence follows a titration curve of the ionization constant K_F, the value for the essential amino acid side chain in the free enzyme.

However, if $k_{cat} >> k_2$, then the pH dependence of k_{cat}/K_A may become more compex.

$$\frac{k_{cat}}{K_A} = \frac{k_{cat}[k_1 + k_3(H/K_F)]}{(1+H/K_F)[k_{cat} + k_2 + X(H/K_F)]} \qquad \text{where} \quad X = \frac{k_1 k_2}{k_3} \tag{14.32}$$

Figure 5 shows the Dixon-Webb plot of Eq. (14.32).

If $k_1 = k_3$ and $k_{cat} >> k_2$, then $k_{cat}/K_A - pH$ profile is a simple sigmoid curve with an apparent ionization constant given by $K^{APP} = K_F(1 + k_{cat}/k_2)$.

However, if $k_1 \neq k_3$ and $k_{cat} >> k_2$, then the $k_{cat}/K_M - pH$ profile is a complex double sigmoid curve defined by two ionization constants; the ionization constant at lower pH, K^{APP}, is equal:

$$K^{APP} = K_F\left[1 + \frac{k_1}{K_3}\left(1 + \frac{k_{cat}}{k_2}\right)\right] \tag{14.33}$$

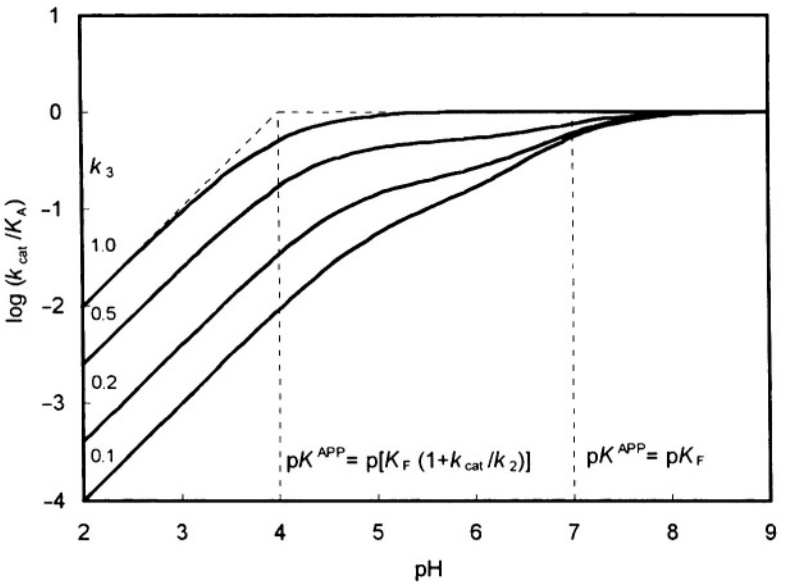


Figure 5. Change in the rate-limiting step with pH. The rate equation (14.32) is drawn assuming that: $K_A = k_1 = k_2 = 1$; $k_{cat} = 1000$; and $pK_F = 7$. The rate constant k_3 gradually decreases from the value of 1.0 to 0.1. The rate-limiting step at high pH is the diffusion-controlled encounter of the substrate and enzyme (defined by rate constant k_1).

The low pH pK_a is due to the change in rate limiting step from the chemical step (k_{cat}) to association (k_1); the pK_a at high pH is approximately equal to pK_F, the real pK_a of the free enzyme (Leskovac *et al.,* 1999).

The pH profiles of log *V/K* function may take the shapes similar to the pH profiles in Fig. 5, but the profile with a hollow is seen only if the substrate is sticky (i.e., dissociates more slowly than it reacts to give products) and certain ratios of rate constants occur; occasionally, the log *V/K* profile may also show a hump (Cleland, 1977). The profiles of log *V* function may also take the similar shapes, but the profile with a hollow is seen only when both the substrate that dissociates most rapidly from the central complex and the protons in the protonated central complex are sticky (Cleland, 1982).

14.4 EFFECTS OF pH ON KINETICS OF BISUBSTRATE REACTIONS

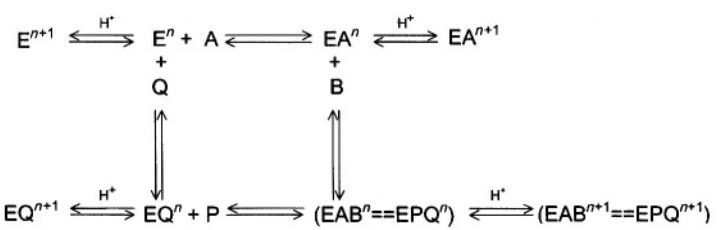
The theory developed in the preceding sections has, for the sake of simplicity, dealt with enzyme mechanisms that involve only one substrate. For simple kinetic models, pH dependent rate equations, such as (14.16), (14.21) and (14.27), may be derived by simple algebraic manipulation. If, however, steady-state conditions are

assumed to apply to the mechanism such as shown in reaction (14.26), the resulting kinetic equation is extremely complicated, usually too complicated to be of practical use (Alberty & Bloomfield, 1963; Peller & Alberty, 1963; Kaplan & Laidler, 1967).

For multistep intermediate complex models and those with multiple substrates, rate equations are most reliably written down by using the method of Cha (1968). Cha's method is a modification of the entire King–Altman schematic method of analysis in which simplification is achieved by treating some of the steps as quasi-equilibria (Chapter 4). It is particularly useful in the analysis of pH dependence, because rapid-equilibrium assumptions may be justified by the high rates of proton-transfer steps in aqueous media (Knowles, 1976).

Although usually high, the proton-transfer steps may become the rate-limiting steps in catalysis. If the proton movement into and out of the active site is restricted, the state of protonation of the enzyme–substrate complex is not equilibrated rapidly with respect to the rate of reaction to give products or the rate of substrate dissociation (Section 14.6.2); that is why the enzymes have acid–base catalysis. On the other hand, when the chemical step is exceptionally fast, the proton-transfer reactions may become the rate-limiting steps in catalysis; this case is verified in reaction catalyzed by carbonic anhydrase (Silverman & Tu, 1975).

If the rates of proton-transfer steps are high, in specific cases, the protons can be introduced into the rate equations for bisubstrate and even trisubstrate reactions in a straightforward manner, without a need for a complete derivation of rate equations (Schulz, 1994). If the protons are treated as dead-end inhibitors of enzymatic reactions, the concentration terms for protons can be introduced directly into the rate equations via the enzyme distribution equations, alleviating considerably the derivation procedure. In order to illustrate this type of analysis, consider the usual Ordered Bi Bi mechanism (Section 9.2).



(14.34)

In order to introduce the concentration terms for protons into the rate equation in a straightforward manner, certain hypothetical assumptions must be introduced into the kinetic model.

In this case, an assumption is made that the enzyme is distributed among four forms: (E), (EA), (EQ), and (EAB==EPQ). It is also assumed that each form can gain or lose a proton from the catalytically active site, but only the unprotonated dissociation form in each case, (E^n), (EA^n), (EQ^n) and $(EAB^n{=}{=}EPQ^n)$, is catalytically active.

The rate equation for the Ordered Bi Bi mechanism was derived in Chapter 4 (Section 4.3; Eq. (4.39)). The entire Eq. (4.39) can be divided by V_2AB to obtain an another useful form of the velocity equation:

$$v_o = \frac{V_1\left(1 - \frac{PQ}{AB}\frac{1}{K_{eq}}\right)}{1 + \frac{K_A}{A} + \frac{K_B}{B} + \frac{K_{iA}K_B}{AB} + \frac{P}{K_{iP}} + \frac{K_BK_Q}{K_pK_{iQ}}\frac{P}{B} + \frac{K_{iA}K_BK_Q}{K_PK_{iQ}}\frac{P}{AB} + \frac{K_A}{K_{iQ}}\frac{Q}{A} + \frac{K_{iA}K_B}{K_{iQ}}\frac{Q}{AB} + \frac{K_{iA}K_B}{K_{iB}K_PK_{iQ}}\frac{PQ}{A} + \frac{K_{iA}K_B}{K_PK_{iQ}}\frac{PQ}{AB}} \qquad (14.35)$$

Equation (14.35) is written for consideration of the forward reaction and all K_{eq} expressions are removed from the denominator with the aid of the Haldane relationship.

In the mechanism in reaction (14.34), all the rate constants are pH dependent and change with pH. For any rate constant in this mechanism, the observed pH dependent rate constant k is equal to

$$k = \overline{\overline{k}} \cdot \phi = \overline{\overline{k}}\left(1 + \frac{[H^+]}{K_1}\right) \qquad (14.36)$$

where $\overline{\overline{k}}$ is the pH independent constant and ϕ is the Michaelis pH function for a monobasic acid (Section 2.8). Thus, expression (14.35) is the velocity equation showing the pH dependent kinetic parameters.

It is possible, now, to transform Eq. (14.35) into a rate equation showing the pH independent kinetic parameters and their dependence on pH. The derivation of the velocity equation is simplified by treating the steps with protons as quasi-equilibria, because rapid equilibrium assumptions may be justified by the

Table 2. Numerator terms of enzyme distribution equations for the mechanism in reaction (14.34). Each numerator term is multiplied by a corresponding Michaelis pH function.

E/E_o	EA/E_o	EAB/E_o	EQ/E_o
$\phi_E\left(\overline{K_{iA}K_B}\right)$	$\phi_{EA}\left(\overline{\overline{K_B}}\right)A$	$\phi_{EAB}(1)AB$	$\phi_{EQ}(1)AB$
$\phi_E\left(\overline{K_A}\right)B$	$\phi_{EA}\left(\frac{\overline{K_BK_Q}}{\overline{K_{iQ}K_P}}\right)AP$	$\phi_{EAB}\left(\frac{\overline{K_{iA}K_B}}{\overline{K_PK_{iQ}}}\right)PQ$	$\phi_{EQ}\left(\frac{\overline{K_A}}{\overline{K_{iQ}}}\right)BQ$
$\phi_E\left(\frac{\overline{K_{iA}K_BK_Q}}{\overline{K_{iQ}K_P}}\right)P$	$\phi_{EA}\left(\frac{\overline{K_{iA}K_B}}{\overline{K_PK_{iQ}}}\right)PQ$	$\phi_{EAB}\left(\frac{\overline{K_{iA}K_B}}{\overline{K_PK_{iQ}K_{iB}}}\right)BPQ$	$\phi_{EQ}\left(\frac{\overline{K_{iA}K_B}}{\overline{K_{iQ}}}\right)Q$
		$\phi_{EAB}\left(\frac{1}{\overline{K_{iP}}}\right)ABP$	

high rates of proton-transfer steps. Thus, in this case, the protons may be treated simply as dead-end inhibitors, since each form of enzyme is catalytically active in only one state of ionization and becomes inactivated by the addition of a proton.

In order to introduce the terms for a dead-end inhibitor into the velocity equation, each enzyme distribution equation is multiplied by an appropriate Michaelis pH function. In order to do so, the corresponding distribution equations for the Ordered Bi Bi mechanism, found in Chapter 9 (Eq. (9.13)), were divided by V_2, followed by a partial elimination of K_{eq}. The entire procedure is shown in Table 2.

Thus, with the aid of enzyme distribution equations, the rate equation with the pH dependent kinetic parameters (Eq. (14.35)) is transformed into a rate equation with pH independent kinetic parameters.

$$v_0 = \frac{\left(\frac{\overline{\overline{V_1}}}{\phi_{EAB}+\phi_{EQ}}\right)\left(1-\frac{PQ}{AB}\cdot\frac{1}{\overline{\overline{K_{eq}}}}\right)}{1+\frac{\overline{\overline{K_A}}}{A}\left(\frac{\phi_E}{\phi_{EAB}+\phi_{EQ}}\right)+\frac{\overline{\overline{K_B}}}{B}\left(\frac{\phi_{EA}}{\phi_{EAB}+\phi_{EQ}}\right)+\frac{\overline{\overline{K_{iA}}}\,\overline{\overline{K_B}}}{AB}\left(\frac{\phi_E}{\phi_{EAB}+\phi_{EQ}}\right)}$$
$$+\frac{P}{\overline{\overline{K_{iP}}}}\left(\frac{\phi_{EAB}}{\phi_{EAB}+\phi_{EQ}}\right)+\frac{\overline{\overline{K_B}}\,\overline{\overline{K_Q}}}{\overline{\overline{K_P}}\,\overline{\overline{K_{iQ}}}}\left(\frac{\phi_{EA}}{\phi_{EAB}+\phi_{EQ}}\right)\frac{P}{B}+\frac{\overline{\overline{K_{iA}}}\,\overline{\overline{K_B}}\,\overline{\overline{K_Q}}}{\overline{\overline{K_P}}\,\overline{\overline{K_{iQ}}}}\left(\frac{\phi_E}{\phi_{EAB}+\phi_{EQ}}\right)\frac{P}{AB}$$
$$+\frac{\overline{\overline{K_A}}}{\overline{\overline{K_{iQ}}}}\left(\frac{\phi_{EQ}}{\phi_{EAB}+\phi_{EQ}}\right)\frac{Q}{A}+\frac{\overline{\overline{K_{iA}}}\,\overline{\overline{K_B}}}{\overline{\overline{K_{iQ}}}}\left(\frac{\phi_{EQ}}{\phi_{EAB}+\phi_{EQ}}\right)\frac{Q}{AB}$$
$$+\frac{\overline{\overline{K_{iA}}}\,\overline{\overline{K_B}}}{\overline{\overline{K_{iB}}}\,\overline{\overline{K_{iQ}}}\,\overline{\overline{K_P}}}\left(\frac{\phi_{EAB}}{\phi_{EAB}+\phi_{EQ}}\right)\frac{PQ}{A}+\frac{\overline{\overline{K_{iA}}}\,\overline{\overline{K_B}}}{\overline{\overline{K_{iQ}}}\,\overline{\overline{K_P}}}\left(\frac{\phi_{EA}+\phi_{EAB}}{\phi_{EAB}+\phi_{EQ}}\right)\frac{PQ}{AB} \qquad (14.37)$$

A comparison of Eq. (14.35), expressed in terms of pH dependent kinetic constants, with Eq. (14.37), expressed in terms of pH independent constants, shows that the following relationships exists between the pH dependent and pH independent kinetic constants:

$$V_1 = \frac{\overline{\overline{V_1}}}{\phi_{EAB}+\phi_{EQ}} \qquad (14.38)$$

$$K_A = \overline{\overline{K_A}}\left(\frac{\phi_E}{\phi_{EAB}+\phi_{EQ}}\right) \qquad (14.39)$$

$$K_B = \overline{\overline{K_B}}\left(\frac{\phi_{EA}}{\phi_{EAB}+\phi_{EQ}}\right) \qquad (14.40)$$

$$K_{iQ} = \overline{\overline{K_{iQ}}}\left(\frac{\phi_E}{\phi_{EQ}}\right) \qquad (14.41)$$

$$\frac{V_1}{K_A} = \frac{\overline{\overline{V_1}}}{\overline{\overline{K_A}}}\left(\frac{1}{\phi_E}\right) \qquad (14.42)$$

$$\frac{V_1}{K_B} = \frac{\overline{\overline{V_1}}}{\overline{\overline{K_B}}}\left(\frac{1}{\phi_{EA}}\right) \tag{14.43}$$

$$\frac{V_1 K_{iQ}}{K_A} = \frac{\overline{\overline{V_1}}\,\overline{\overline{K_{iQ}}}}{\overline{\overline{K_A}}}\left(\frac{1}{\phi_{EQ}}\right) \tag{14.44}$$

$$V_1 K_{iP} = \overline{\overline{V_1}}\,\overline{\overline{K_{iP}}}\left(\frac{1}{\phi_{EAB}}\right) \tag{14.45}$$

The Michaelis constants and inhibition constants are now rather complex relationships of Michaelis pH functions. However, the relationships expressed by Eqs. (14.42)–(14.45) are each a function of a single Michaelis pH function (Laidler, 1955; Schulz, 1994). Thus, in this particular mechanism, by a prudent choice of parameters, one can calculate the pK_a values of all forms of the enzyme in reaction (14.34). For example, Eq. (14.42) can be expanded to obtain

$$\log\left(\frac{V_1}{K_A}\right) = \log\left(\frac{\overline{\overline{V_1}}}{\overline{\overline{K_A}}}\right) - \log\left(1 + \frac{[H^+]}{K_E}\right) \tag{14.46}$$

Equation (14.46) has the same form as Eq. (14.19) (Fig. 2) and may be analyzed graphically in the same way.

The foregoing examples show the complexity of the pH dependence of full rate equations for bisubstrate reactions. Therefore, when dealing with the pH effect on the kinetics of reaction of two substrates, a *high concentration of one of the substrates is often used,* so that the reaction may be treated by equations that describe the single-substrate case (Tipton & Dixon, 1979).

14.5 pH PROFILES

14.5.1 Plotting of Experimental Data

There are three different ways of plotting the experimental data and finding the appropriate pK_a values by graphical methods:

(1) double reciprocal plots, H^+ as effector,
(2) plots of kinetic constants (or kinetic parameters) against pH, and
(3) plots of logarithms of kinetic constants against pH (Dixon–Webb plots).

The first graphical method is seldom in use. The first method of plots against $[H^+]$ or $1/[H^+]$ concentrates on a relatively narrow range of $[H^+]$ around the ionization constants and, in such plots, there is little useful information outside the range of 0.1–10 K_a (Ainsworth, 1977; Wang *et al.,* 1999). It has the advantage that it allows more complete analysis of hydrogen ions as activators and inhibitors using equations similar to those used in other aspects of kinetic studies (see Chapters 5–7).

The second method is often in use; it is illustrated in Fig. 1.

The third method is by far the most used, and the plots of logarithms of kinetic constants against pH are usually referred to as *Dixon–Webb plots* (Dixon & Webb, 1979); this method is illustrated in Figs. 2–9.

14.5.2 Monobasic Acids

In the practical work with enzymes, a relatively limited number of different types of titration curves is encountered for monobasic and dibasic acids. Equations (14.47) and (14.48) show the major types of equations for monobasic acids which are encountered in the technical and scientific literature (Cleland, 1977, 1982; Grimshaw *et al.,* 1981; Purich & Allison, 2000). Naturally, a rare occurrence of some rate equations and some pH profiles in the literature is in no correlation whatsoever with their importance in enzymology. A specific problem in enzyme kinetics will always raise the need for a specific kinetic model and the corresponding rate equations.

$$Y = \frac{C}{1 + \dfrac{H}{K_a}} \qquad Y = \frac{C}{1 + \dfrac{K_a}{H}} \tag{14.47}$$

$$Y = C\left(1 + \frac{H}{K_a}\right) \qquad Y = C\left(1 + \frac{K_a}{H}\right) \tag{14.48}$$

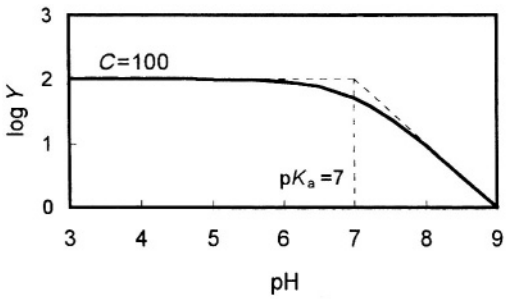
Figure 6 shows the Dixon–Webb plots of Eqs. (14.47) and (14.48).

The pH profiles in Fig. 6 may either show a change of a slope of ±1 at the pK_a, if the incorrectly protonated form can not bind a molecule of substrate or a molecule of inhibitor at all. Thus, pH profiles for titration curves of monobasic acids have some simple properties which should be remembered. Functions (14.47) are decreasing in alkaline or in the acid, with a slope of +1 or –1, respectively. Functions (14.48) are the mirror images of functions (14.47).

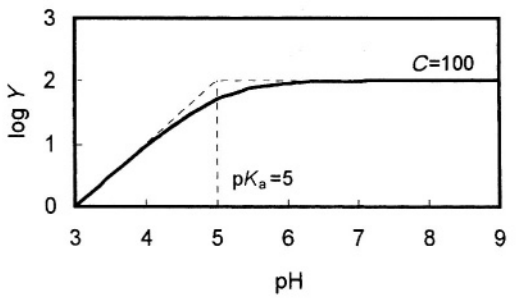
On the other hand, after initially decreasing with ±1 slope, the curve may plateau at a new level if the incorrectly protonated form can bind less strongly. In this case, the point at which the curve levels out is the pK_a in the bound complex. This leads to another type of equations frequently encountered in enzymology:

$$Y = \frac{YL + YH\left(\dfrac{K_a}{H}\right)}{1 + \dfrac{K_a}{H}} \tag{14.49}$$

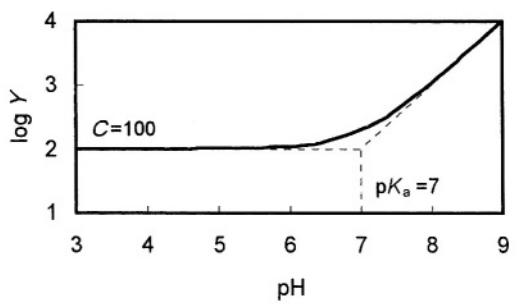
The Dixon–Webb plot of Eq. (14.49) displays two plateau values, one in the acid (*YL*) and the other in alkaline (*YH*). The value of log *Y* in Eq. (14.49) decreases with increasing pH with a slope of –1, or decreases with decreasing pH in the same manner, over a single pK_a value (Fig. 7).



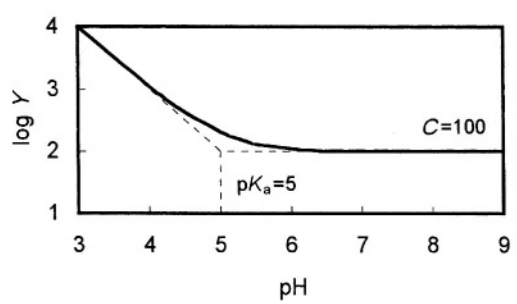
$$Y = \frac{C}{1 + \frac{K_a}{H}}$$



$$Y = \frac{C}{1 + \frac{H}{K_a}}$$

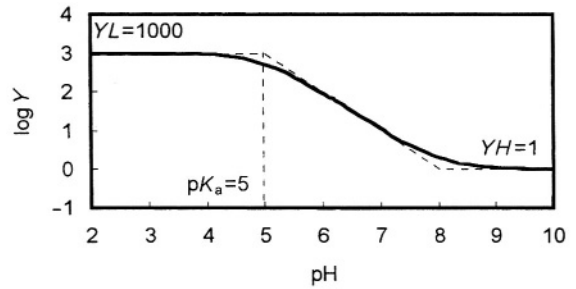


$$Y = C\left(1 + \frac{K_a}{H}\right)$$

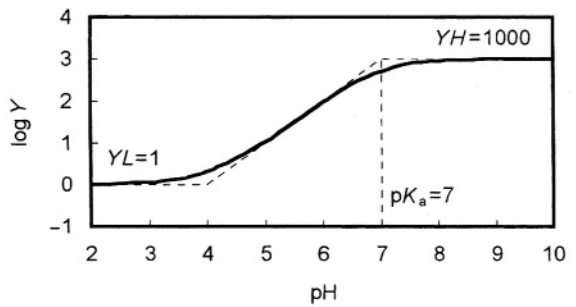


$$Y = C\left(1 + \frac{H}{K_a}\right)$$

Figure 6. pH Profiles for monobasic acids drawn according to Eqs. (14.47) and (14.48).



$$Y = \frac{YL + YH\left(\frac{K_a}{H}\right)}{1 + \frac{K_a}{H}}$$



$$Y = \frac{YL + YH\left(\frac{K_a}{H}\right)}{1 + \frac{K_a}{H}}$$

Figure 7. pH Profiles for monobasic acids drawn according to Eq. (14.49).

14.5.3 Dibasic Acids

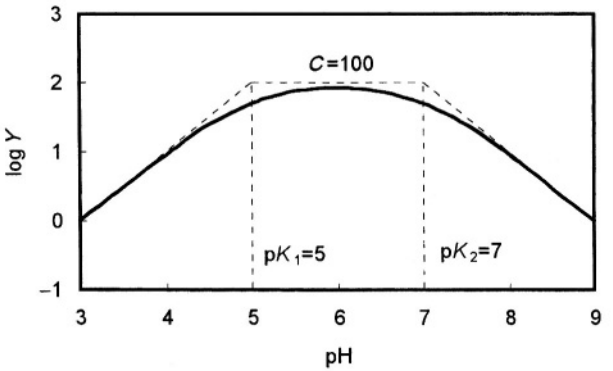
The kinetic models for dibasic acids, which are described in the literature, are rather numerous; Eqs. (14.50)–(14.54) are showing those which are encountered often in the practical work with enzymes (Cleland, 1977, 1982; Cook & Cleland, 1981; Grimshaw *et al.,* 1981).

$$Y = \frac{C}{1 + \frac{H}{K_1} + \frac{K_2}{H}} \tag{14.50}$$

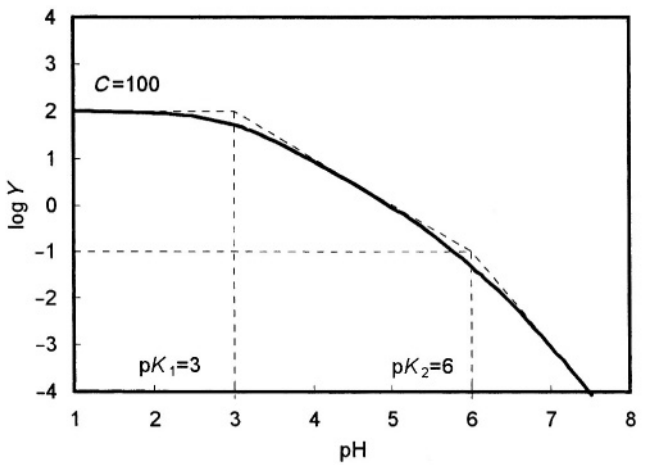
$$Y = \frac{C}{1 + \frac{K_1}{H} + \frac{K_2}{H} + \frac{K_1K_2}{H^2}} = \frac{C}{\left(1 + \frac{K_1}{H}\right)\left(1 + \frac{K_2}{H}\right)} \tag{14.51}$$

$$Y = \frac{C\left(1 + \frac{H}{K_2}\right)}{1 + \left(\frac{H}{K_3}\right)\left(1 + \frac{H}{K_1}\right)} \tag{14.52}$$

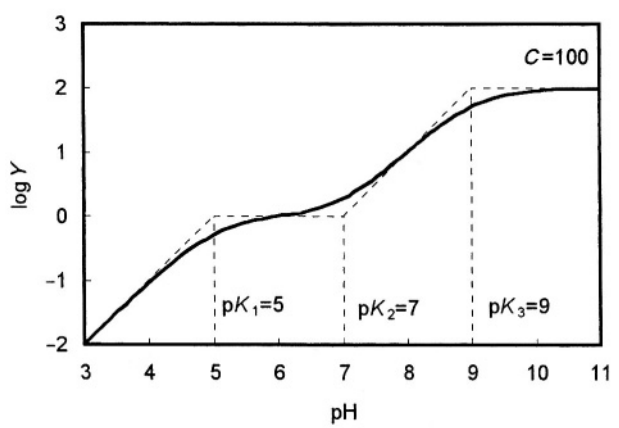
$$Y = \frac{C\left(1 + \frac{K_2}{H}\right)}{\left(1 + \frac{K_3}{H}\right)\left(1 + \frac{K_1}{H}\right)} \tag{14.53}$$



$$Y = \frac{C}{1 + \frac{H}{K_1} + \frac{K_2}{H}}$$



$$Y = \frac{C}{1 + \frac{K_1}{H} + \frac{K_2}{H} + \frac{K_1 K_2}{H^2}}$$



$$Y = \frac{C\left(1 + \frac{H}{K_2}\right)}{1 + \left(\frac{H}{K_3}\right)\left(1 + \frac{H}{K_1}\right)}$$

Figure 8. pH Profiles for dibasic acids drawn according to Eqs. (14.50)–(14.52).

$$Y = \frac{C}{1 + \frac{H}{K_2} + \frac{H^2}{K_1 K_2} + \frac{K_3}{H}} \tag{14.54}$$

Figure 8 shows the Dixon–Webb plots of Eqs. (14.50)–(14.52).

pH profiles for titration curves of dibasic acids are more complex. These titration curves have, usually, two different shapes: a pH profile has a plateau value in the acid and then decreases in alkaline, or vice versa, has a plateau value in alkaline and decreases in the acid. Note that the pH profile for Eq. (14.51) decreases in alkaline (Fig. 8), but, if we substitute pH with p(OH), the p(OH) profile will have the opposite shape, that is, a plateau value in alkaline with a decrease in the acid. On the other hand, the pH profile for Eq. (14.52) decreases in the acid, and in this case the same inversion is obtained if pH is substituted with p(OH). It is useful to keep these relationships in mind, especially when the need for the statistical analysis of data with computers arises (Cleland, 1979).

14.6 INTERPRETATION OF pH PROFILES

The use of pH studies to determine chemical mechanisms of action of enzyme-catalyzed reactions is probably their most important application in enzymology (Knowles, 1976; Cleland, 1982).

Which pH profiles do we look at, if we want to use the pH studies to determine the chemical mechanism? The pH profiles that will be of the most value will be (Cleland, 1970, 1982):

(a) log K_i or log $(1/K_i)$ for competitive inhibitors,
(b) log (V/K) for one or more slow, nonsticky substrates, and
(c) log V for these same substrates.

14.6.1 Log K_i Profiles for Competitive Inhibitors

The K_i values in these profiles represent the *equilibrium dissociation constants* from the enzyme form present under the reaction conditions. The pK_a values seen in these profiles are thus *the correct ones* in either the molecule which combines with the enzyme, or the enzyme form it combines with. Correct pK_a values are seen, and such profiles are good candidates for temperature variation or solvent perturbation studies to identify the groups involved.

Profiles of pK_i have the virtue of usually having simple shapes. However, they detect only groups whose protonation state affects *binding;* a group necessary for *catalysis,* but not directly participating in the binding process will not show up, or will show only small changes in affinity with change in protonation state.

These profiles usually have the following forms:

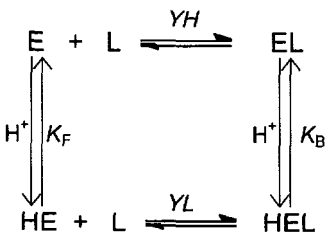
$$K_i^{APP} = \frac{K_i}{1 + \frac{H}{K_a}} \qquad K_i^{APP} = \frac{K_i}{1 + \frac{K_a}{H}} \tag{14.55}$$

$$K_i^{APP} = K_i\left(1 + \frac{H}{K_a}\right) \qquad K_i^{APP} = K_i\left(1 + \frac{K_a}{H}\right) \tag{14.56}$$

$$K_i^{APP} = \frac{C\left(1 + \frac{K_1}{H}\right)}{\left(1 + \frac{K_2}{H}\right)} \tag{14.57}$$

Note that Eqs. (14.55) and (14.56) are identical with Eqs. (14.47) and (14.48).

Let us consider the last case, Eq. (14.57) (Eftink & Bystrom, 1986; Leskovac *et al.,* 1998). A *non-ionizable ligand* L (inhibitor or a competitive dead-end inhibitor) binds to an enzyme. The enzyme has two states of protonation (E and HE) and the ligand can bind to both, although much stronger to the protonated form; that is, the dissociation constant *YH* (at high pH) is larger than *YL* (at low pH), and K_F is larger than K_B.



(14.58)

Thermodynamic equilibrium in reaction (14.58) is characterized by two *acid dissociation constants*:

$$K_F = \frac{[E][H^+]}{[HE]} \qquad K_B = \frac{[EL][H^+]}{[HEL]} \tag{14.59}$$

and two *ligand dissociation constants*:

$$YH = \frac{[E][L]}{[EL]} \qquad YL = \frac{[HE][L]}{[HEL]} \tag{14.60}$$

In addition,

$$K_F(YL) = K_B(YH) \tag{14.61}$$

Combination of Eqs. (14.60) and (14.61) gives the pH dependence of the apparent ligand dissociation constant (Y):

$$Y = \frac{YL\left(1 + \frac{K_F}{H}\right)}{\left(1 + \frac{K_B}{H}\right)} \tag{14.62}$$

After substituting K_F with $K_B(YH/YL)$ and rearranging, Eq. (14.62) becomes

$$Y = \frac{YL + YH\left(\frac{K_B}{H}\right)}{\left(1 + \frac{K_B}{H}\right)} \tag{14.63}$$

Note that Eqs. (14.62) and (14.63) are identical. Figure 9 shows the Dixon–Webb plots of Eq. (14.62). Thus, in Eq. (14.62) (Fig. 9A), the value of the ligand dissociation constant for the enzyme–ligand complex decreases from the plateau value in alkaline (*YH*), over a single pK_a (pK_B), to a new plateau value in the acid (*YL*); here, K_B is the dissociation constant of the acidic group on enzyme in the enzyme-ligand complex. A dissociation constant of an acidic group in the free enzyme can be calculated from Eq. (14.61) (shown in Fig. 9A as K_F). An opposite situation takes place if the enzyme has two states of ionization, the ligand binds to both, but this time much stronger to the unprotonated form; that is, the ligand dissociation constant in the acid (*YL*) is now higher than in alkaline (*YH*). The pH profile now has the opposite form, as shown in Fig. 9B, but the same equation applies.

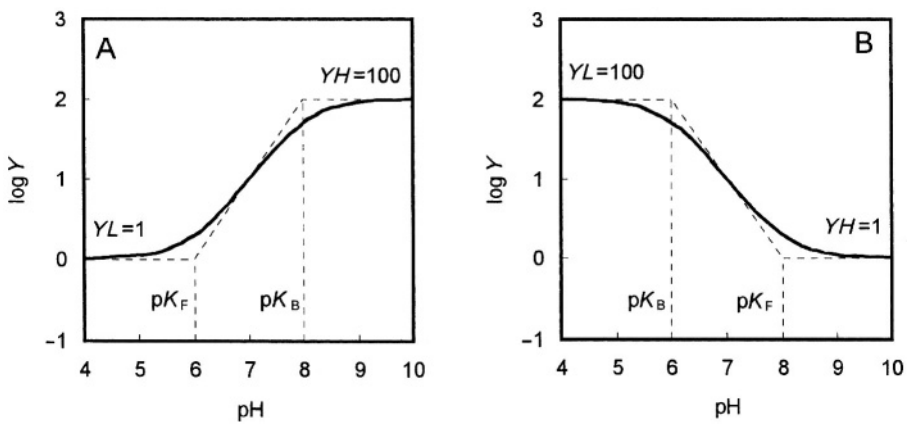


Figure 9. Binding of a nonionizable ligand: the Dixon–Webb plots of Eq. (14.62).

pH Profiles of K_i for competitive inhibitors have the virtue of usually having simple shapes; most often, they have the shapes shown in Fig. 9, because the affinity of enzyme for a ligand seldom drops to a zero value, even at extreme pH values. Sometimes, the pH profiles for K_i may have more complex shapes. Naturally, the situations shown by Eqs. (14.55)–(14.57) are valid only for nonionizable inhibitors. However, the p*K*'s of the inhibitor will not mask those of the enzyme, since one usually knows the p*K*s of the inhibitor and they can be factored out of the observed profile.

14.6.2 Log (*V/K*) Profiles

The *V/K* for a substrate is the apparent first-order rate constant for reaction of the variable substrate with the enzyme, when the concentration of the variable substrate is close to zero. *V/K is* the product of three factors (Cleland, 1982):

(1) The proportion of the enzyme in the correct form to react. This is pH dependent when only one protonation state of a group allows binding or catalysis.
(2) The bimolecular rate constant for the combination of enzyme and substrate. This will probably be limited by diffusion.
(3) The fraction of the collision complex that reacts to give products, as opposed to dissociating. This will be pH dependent whenever binding is not pH dependent, but the catalytic reaction requires one or more groups on enzyme to be in a given protonation state.

Thus, if a pK_a is seen in both the *V* and *V/K* profiles, the third case is probably responsible, while if it is seen only in the *V/K* profile, and not in the *V* profile, the first case is likely to be the cause.

Substrate is not sticky.

If the substrate is *not sticky* (i.e., dissociates from the collision complex faster than it reacts to give products), the *V/K* profile shows the *correct* pK_a values and the curve has usually a simple algebraic form. Such situations are amply illustrated in Section 14.3 with several models, and in corresponding Figs. 2–4.

In each case, the pK_a values seen in the *V/K* profiles will show correct pK_a values of dissociating groups on the free enzyme. While pH profiles of K_i for competitive inhibitors show only groups whose protonation state affects *binding,* the *V/K* profiles are showing groups that are responsible for *catalysis* as well as binding. The pH profiles for *V/K* functions may often have simple shapes. However, in Section 14.3, it was shown that more complicated profiles are often observed with two or more pK_a values on one limb.

Substrate is sticky.

If the substrate is *sticky* (i.e., dissociates more slowly than it reacts to give products), the pK_a values will not be seen in the correct position on the profile, but will be displaced *outward* (i.e., to lower pH when protonation decreases activity, and to higher pH when protonation increases *V/K*). The amount of displacement will be $\log(1 + k_3/k_2)$, where k_3 is the net rate constant for reaction of the collision complex to yield products, and k_2 is for dissociation. With a sticky substrate, the displacement can be a pH unit or more, although values of 0.5–1.0 pH unit are more common.

While the displacement of the pK_a is a sufficient problem, a further difficulty with sticky substrates is the alteration in shape of the pH profile in the vicinity of pK_a, which can occur with certain values of the rate constants. This alteration in shape can take the form of a hollow, and very infrequently a form of a hump. The hollow in the pH profile results when proton movement into and out of the active site is restricted, so that the state of protonation of the enzyme–substrate complex is not equilibrated rapidly with respect to the rate of reaction to give products or the rate of substrate dissociation (Section 14.4).

Substrates are normally sticky in the reaction direction with the higher maximum velocity, and not always then. In the direction with the lower maximum velocity, substrates cannot normally be sticky because their release rates from the enzyme must exceed the more rapid maximum velocity in the reverse direction. Thus, one needs to use the pH profiles of slow substrates only in the direction with the fastest maximum velocity; in the reverse direction, one can use the profiles of the normal substrates for comparison. Further, one should use the *V/K* profiles for nonsticky substrates to determine the pK_a values. Then, one should determine the nature of the catalytic groups by solvent perturbation and temperature variation of the pK_a values, and then, by comparison with the *V/K* profile of the normal sticky substrate determine the stickiness and, from the shape of the profile in the vicinity of the pK_a, how rapidly the protonation state of the group is equilibrated when the substrate is present (Rose *et al.,* 1974; Cleland, 1982).

14.6.3 Log *V* Profiles

Here again, the profile for the slow nonsticky substrate is likely to be more easily interpreted. In such cases, the *V* profile shows the *correct* pK_a values on the *enzyme–substrate complex.* Such situations are illustrated in Section 14.3, with several models (Figs. 2–4). In each case, the pK_a values seen in the *V* profiles will show correct pK_a values of dissociating groups on the enzyme–substrate complex, showing only the groups that are responsible for *catalysis.* Actually, what we see is the pH variation of whatever unimolecular step or steps are rate-limiting; this is usually catalysis, but may be a conformational change or product release. With a fast substrate (and especially in the direction with the faster maximum velocity), however, the product-release steps are often rate-limiting, or partly so. This displaces pK_a values outward on the pH profile (to higher pH's) until the portion of the reaction involving the chemical step does become rate-limiting.

While *V/K* profiles usually show total loss of activity when groups are incorrectly protonated, *V* profiles may show changes to a new plateau level when ionization of a group increases the rate of a step that is normally rate-limiting.

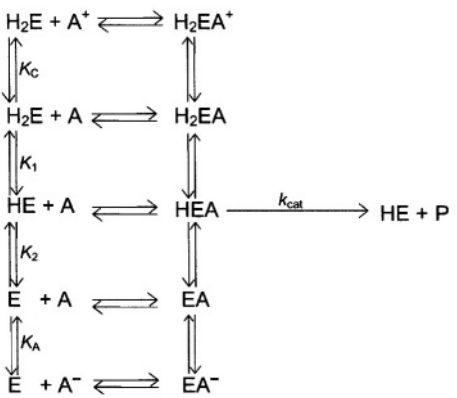
Thus, the main cause of perturbation of pK_a values in the *V* profiles is the fact that the chemical reaction is not rate-limiting. However, when the stickiness of a substrate does not displace the pK_a values in *V* profiles, it can lead to more complex shapes than the simple one corresponding in algebraic form to equation $(V/K)^{APP} = (V/K)/(1 + H^+/K_a)$, if proton access to the groups involved is restricted in the central productive complex of enzyme with substrates. When both the substrate that dissociates most rapidly from the central complex and the proton on the group in question are sticky, the curve will have a hollow in the vicinity of the pK_a.

14.6.4 Ionization of the Substrate

Many substrates ionize in the pH range used in kinetic experiments. If the substrate ionization is possible, one ought to consider whether observed pK_a

values refer to the enzyme or to the substrate. Enzymes often bind only one ionic form of the substrate or, more strictly, binding greatly favors one form. The theory in such cases is similar to this for enzyme ionizations and the results given above for enzymes require only slight modifications. The pH dependence of V and K still refer to the enzyme–substrate complex, but the pH dependence of V/K may refer either to the free enzyme or to the free substrate, or both.

Amino acids are typical ionizable substrates of enzymes; in aqueous solutions, they are present predominantly as zwitterions between pH 3 and 9 (Section 2.8.2). Consider an amino acid substrate with $pK_C = 2$ for a carboxylic group and $pK_A = 10$ for the α-amino group, a situation approximately corresponding to glycine (reaction (14.64)).



(14.64)

If the enzyme binds only the zwitterionic form of the substrate, the pH profile of log (V/K) will show the groups on enzyme responsible for catalysis (pK_1 and pK_2) in the broad pH region 3–9. Thus, if the enzymes were specific for the zwitterion, the variation of V/K with pH is given by

$$\log\left(\frac{V}{K}\right)^{APP} = \log\left(\frac{V}{K}\right) - \log\left(1 + \frac{H}{K_1} + \frac{K_2}{H}\right) - \log\left(1 + \frac{H}{K_c} + \frac{K_A}{H}\right) \qquad (14.65)$$

Equation (14.65) assumes that the enzyme binds only the zwitterionic form of substrate and that catalysis depends on two groups on enzyme with $pK_1 = 5$ and $pK_2 = 7$.

The Dixon–Webb plot of Eq. (14.65) will show all four pK_a values, two for the amino acid substrate and two for the free enzyme (Fig. 10).

As predicted by Eq. (14.65), the initial slope of the extrapolated line in Fig. 10 is 2, and the second linear segment has a slope of 1, before the pH profile levels out. Thus, in enzyme catalysis, the slope in excess of 1 usually indicates that the dissociation of a group on the substrate is involved (Schulz, 1994). However, the complications of several overlapping protonation ranges are increased by substrate ionizations, so it is always prudent, if possible, to use another substrates that do not ionize over the pH range under study (Tipton & Dixon, 1979; Cornish-Bowden, 1995).

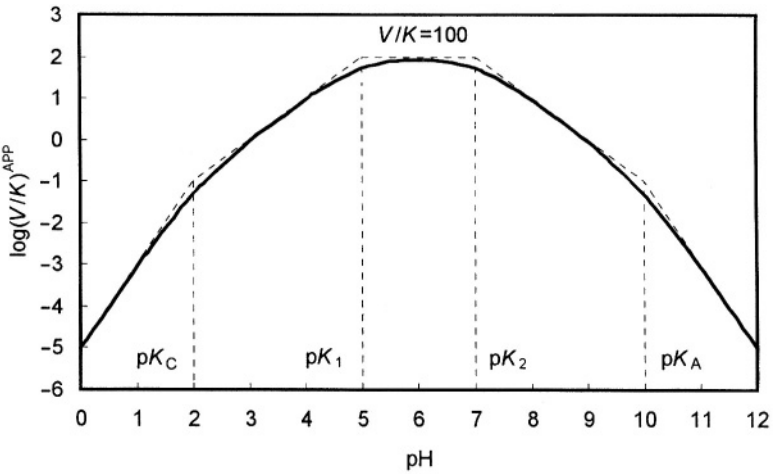


Figure 10. The Dixon–Webb plot of Eq. (14.65), showing the ionization of the enzyme and the amino acid substrate. The enzyme is maximally active ($V/K = 100$) between the pK's of two groups on the free enzyme responsible for catalysis ($pK_1 = 5$ and $pK_2 = 7$).

14.6.5 Reverse Protonation

The bell-shaped pH profiles arise when the free enzyme and enzyme–substrate complex are protonated twice, but only the middle protonation form is catalytically active (Sections 14.3.2 and 14.3.3). In interpreting such profiles, one problem remains that does not arise when only one pK_a is seen: which group on the enzyme must be protonated and which must be ionized for the proper catalytic activity? In this respect, a very frequent case is the reverse protonation, a situation when the protonation state of enzyme is different in the forward and reverse reactions.

As an example, consider a dehydration of L-malate catalyzed by fumarase, affording fumarate as the product of reaction; this reaction is fully reversible. Kinetic studies suggest a model in which a histidine residue and a carboxyl group in the active site of enzyme are necessary for catalysis. According to this model, from the malate side of reaction, the histidine must be protonated and the carboxyl ionized, while in the reverse direction, the states of ionization are reversed (Cleland, 1977) (Fig. 11). Note that, in Fig. 11, proton is not a part of the chemical reaction but, formally, the elements of water (proton and the hydroxyl ion) are removed from the substrate by the enzyme.

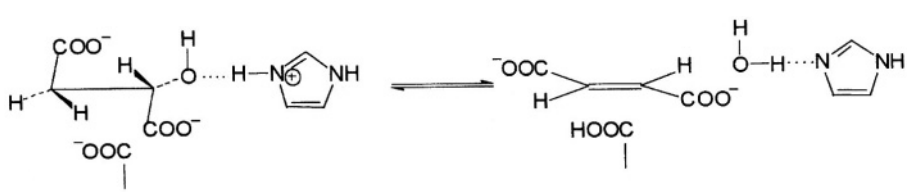


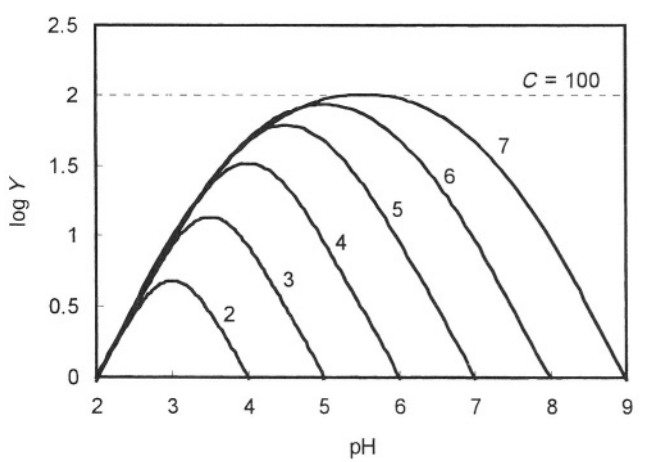
Figure 11. Reversible hydration of fumarate or dehydration of L-malate depends on a protonation state of a histidine residue and a carboxyl group in the active site of fumarase.

Which pH profiles do we look at, if we want to gain an experimental evidence for a mechanistic scheme in Fig. 11?

Obviously, the *V* profiles for the slow nonsticky substrate are the best choice, since these *V* profiles will show the correct pK_a values of amino acid side chains on enzyme in the enzyme–substrate complex that are responsible for catalysis. The apparent pK_a's of the catalytic groups of fumarase have been measured in both directions (Brandt *et al.,* 1963). When malate is used as a substrate, *V* profile is bell-shaped showing two pK_a's, 6.4 and 9.0, respectively; temperature variation of these groups indicates that pK_a 6.4 corresponds to dissociation of a carboxyl and pK_a 9.0 to dissociation of a histidine side chain in the active site of enzyme. Thus, from the malate side of reaction, the enzyme is active if histidine is protonated and the carboxyl ionized. With fumarate as a substrate, the *V* profile is again bell-shaped showing two pK_a's, 7.0 and 4.9, respectively; this time, however, the temperature variation indicates that pK_a 7.0 corresponds to dissociation of a carboxyl and pK_a 4.9 to dissociation of a histidine side chain. Thus, from the fumarate side of reaction, the enzyme is active if carboxyl is protonated and histidine is not.

Thus, it is in the *V* profiles that things cross over. This is possible only because the pK_a of imidazole shows a drastic swing, of more than 4 pH units, depending on which substrate is adsorbed.

The bell-shaped pH profiles, observed with fumarase, are very frequent in enzymology. However, the neighboring pK_a values in bell-shaped Dixon–Webb plots may be distinguished only if they are sufficiently apart, at least two pH units. Figure 12 shows an example of bell-shaped pH profiles with a gradually changing gap between the two pK_a values.



$$Y = \frac{C}{1 + \frac{H}{K_1} + \frac{K_2}{H}}$$

Figure 12. The Dixon–Webb plot of Eq. (14.50). pK_1 is kept constant at a value of 4, while pK_2 gradually increases from the value of 2–7.

14.6.6 Proton is a Part of the Chemical Reaction

In the preceding example, proton was not a part of the chemical reaction catalyzed by an enzyme. However, proton is often a part of a chemical reaction, especially with reactions catalyzed by dehydrogenases and kinases.

Consider an oxidation of ethanol by NAD^+, catalyzed by alcohol dehydrogenase:

$$CH_3CH_2OH + NAD^+ \rightleftharpoons CH_3CHO + NADH + H^+$$

Proton is a part of this fully reversible reaction, and the equilibrium constant is equal:

$$K_{eq}^{pH} = \frac{[CH_3CHO][NADH][H^+]}{[CH_3CH_2OH][NAD^+]}$$

From the Haldane relationship (Section 9.2), the equilibrium concentration of reactants is equal:

$$K_{eq} = \frac{[CH_3CHO][NADH]}{[CH_3CH_2OH][NAD^+]} = \frac{V_1 K_{iQ} K_P}{V_2 K_{iA} K_B}$$

Thus, we are dealing with two types of equilibrium constants, one which is pH dependent (K_{eq}), and the other which is pH independent (K_{eq}^{pH}) (Dalziel, 1975). These two equilibrium constants are related by

$$K_{eq} = \frac{K_{eq}^{pH}}{[H^+]} \quad \text{and} \quad \log K_{eq} = \log K_{eq}^{pH} + pH \tag{14.66}$$

Consequently, a plot of pH versus log K_{eq} will provide a straight line with a slope of one, and an intercept on ordinate will show the value of K_{eq}^{pH}.

Relationship (10.66) is typical for reactions catalyzed by NAD(P)-dependent dehydrogenases, such as the oxidation of alcohols catalyzed by the yeast (Leskovac *et al.,* 1996) or the horse liver alcohol dehydrogenase (Plapp *et al.,* 1986), and serves to calculate the K_{eq}^{pH} and to check the internal consistency of Haldane relationships.

14.7 SUMMARY OF PROCEDURES TO FOLLOW THE pH STUDIES

Let us summarize what we have said above in terms of the steps one takes to carry out a pH study of an enzyme mechanism (Cleland, 1970, 1982).

(1) Profiles of pK_i for competitive inhibitors will show the required state of protonation of the inhibitor or of groups on the enzyme for binding. Correct pK_a values are seen, and such profiles are good candidates for temperature variation or solvent perturbation studies to identify the nature of the groups involved.

(2) The log (V/K) versus pH profile for a nonsticky substrate shows the correct pK_a values of groups necessary for binding and catalysis. Those pK_a values not present in pK_i profiles are groups that act as acid–base catalysts during

the reaction, or whose protonation state is important for the chemical reaction, but not for binding. Profiles of *V/K* for nonsticky substrates can also be used in temperature variation and solvent perturbation studies to determine the nature of the groups involved. The *V/K* profiles for sticky substrates are not easy to use for the study of the chemical mechanism, but will indicate the degree of stickiness.

(3) The *V* profile will fail to show the pK_a values of groups that allow binding only when correctly protonated, while conversely, when the pK_a is seen in the *V* profile, one knows that binding is not totally pH independent, but that the group must be correctly protonated for catalysis. The displacement of the pK_a from its value in the *V/K* or pK_i profiles carries the information on: (a) the relative rates of steps in the mechanism and (b) on the environment in the enzyme–substrate complex.

(4) Profiles must be determined in both directions of the reaction. If the overall stoichiometry involves a proton, as do many kinases and dehydrogenases, the *V/K* profiles will show the pK_a of the acid-base catalytic group with different required protonation states in the two directions, and this pattern will tell which pK_a is that of the acid–base catalyst. When no proton is involved in the overall reaction, the *V/K* profiles should have similar shapes in forward and reverse reactions.

(5) The identity of catalytic groups when pK_a values are seen in pH profiles is established kinetically, by temperature variation and solvent perturbation studies.

Finally, it should be noted that one can use the NMR studies to determine directly the individual group pK_a values, a method especially practical with histidines. Or one can use UV studies for groups such as tyrosine, or rates of reaction with reagents for SH groups (Fersht, 1999).

14.7.1 Types of Acid Dissociation Constants

There are five types of pK_a's which we have encountered in steady-state kinetic analyses in this chapter: microscopic (or group), macroscopic (or molecular), titration, transition state, and "mirage" (Brockelhurst, 1996).

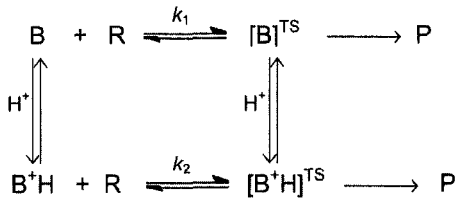
Microscopic or group. The value of pK_a of an individual group is of interest in connection with structural and mechanistic studies. Unfortunately, group pK_a values cannot be measured experimentally without making assumptions, although useful estimates can sometimes be obtained (Dixon, 1976).

Macroscopic. The macroscopic or molecular acid dissociation constants and their relationship to the group dissociation constants are defined in Section 14.2. The molecular pK_a are those which are obtained experimentally (Tipton & Dixon, 1979).

Titration. The fact that the titration curve of a multi-site acid is identical to that of a mixture of hypothetical one-site acids, each of the same concentration as the multiacid, permits the use of the dissociation constants of these hypothetical acids; thus, the pH dependence of any property of the multi-acid may be

represented by the sum of one-site titration curves characterized by the titration constants (Dixon, 1992).

Transition state. The transition state acid dissociation constant, K_a^{TS}, may be obtained from the observed rate constants k_1 and k_2 of two similar reactions, differing only in that a reactant in one case is the conjugate acid (B^+H) of the reactant (B) in another:



(14.67)

The virtual equilibrium constant, K_a^{TS}, defined in a usual way in terms of the concentrations of $[B]^{TS}$, $[B^+H]^{TS}$, and $[H^+]$, may be related to observable quantities via a thermodynamic cycle. The acidic hydrogen atom of the transition state serves as a reporter for factors affecting its acidity (Kurz, 1963, 1972; Thornton & Thornton, 1978).

"*Mirage*". The treatments described above assume that the same step is rate-limiting at all pH values; if this is not the case, a "mirage" pK_a value may appear in pH profiles. This case is treated in Section 14.3.6.

REFERENCES

Books

Ainsworth, S. (1977) *Steady-state enzyme kinetics,* MacMillan, New York.
Cornish-Bowden, A. (1995) *Fundamentals of enzyme kinetics,* revised edition, Portland Press, London.
Dixon, M. & Webb, E.C. (1979) *Enzymes,* 3rd ed., Academic Press, New York.
Engel, P.C. (ed.) (1996) *Enzymology Labfax,* Bios Scientific Publishers, Oxford.
Fersht, A. (1999) *Structure and mechanism in protein science,* W.H. Freeman, New York.
Gandour, R.D. & Schowen, R.L. (eds.) (1978) *Transition states of biochemical processes,* Plenum Press, London.
Mahler, H. & Codes, E. (1966) *Biological chemistry,* Harper & Row, New York.
Michaelis, L. (1922) *Die Wasserstoffionenkonzentration,* Springer-Verlag, Berlin.
Purich, D.L. & Allison, R.D. (2000) *Handbook of biochemical kinetics,* Academic Press, San Diego.
Schulz, A.R. (1994) *Enzyme kinetics,* Cambridge University Press, Cambridge.
Steinhardt, J. & Reynolds, J.A. (1969) *Multiple equilibria in proteins,* Academic Press, New York.

Review articles

Brockelhurst, K. (1996) in *Enzymology Labfax,* pp. 175–190.
Cleland, W.W. (1970) *The Enzymes,* 3rd ed., Vol. II, 1–65.
Cleland, W.W. (1977) *Adv. Enzymol. Rel. Areas Mol. Biol. 45,* 272–387.
Cleland, W.W. (1979) *Methods Enzymol. 63,* 103–137.
Cleland, W.W. (1982) *Methods Enzymol. 87,* 390–405.
Dalziel, K. (1975) *The Enzymes,* 3rd ed., Vol. XI, 1–60.
Dixon, H.B.F. (1992) *Essays Biochem. 27,* 161–181.

Knowles, J. (1976) *CRC Crit. Rev. Biochem.* 165–176.
Kurz, J.L. (1972) *Acc. Chem. Res. 5,* 1–9.
Tanford, C. (1962) *Adv. Protein Chem. 17,* 17–50.
Thornton, E.K. & Thornton, E.R. (1978) in *Transition states of biochemical processes,* pp. 3–100.
Tipton, K.F. & Dixon, H.B.F. (1979) *Methods Enzymol 63,* 183–234.

Specific references

Adams, E.Q. (1916) *J. Am. Chem. Soc. 38,* 1503–1510.
Alberty, R.A. & Massey, V. (1954) *Biochim. Biophys. Acta 14,* 347–359.
Alberty, R.A. & Bloomfield, V. (1963) *J. Biol. Chem. 238,* 2804–2816.
Brandt, D.A., Barnett, L.B., & Alberty, R.A. (1963) *J. Am. Chem. Soc. 85,* 2204–2216.
Brockelhurst, K. (1994) *Protein Engn. 7,* 291–299.
Brockelhurst, S.M., Topham, C.M., & Brockelhurst, K. (1990) *Biochem. Soc. Trans. 18,* 598–600.
Brönsted, A. (1923) *Rec. Trav.* Chim. *Pay-Bas 42,* 718–728.
Cha, S. (1968) *J. Biol. Chem. 243,* 820–825.
Cook, P.F. & Cleland, W.W. (1981) *Biochemistry 20,* 1796–1816.
Dixon, H.B.F. (1976) *Biochem. J. 153,* 627–629.
Efrink, M.R. & Bystrom, K. (1986) *Biochemistry 25,* 6624–6630.
v. Euler, H., Josephson, K., & Myrback, K. (1924) *Hoppe Seyler's Z. Physiol. Chemie 134,* 39–50.
Grimshaw, C.E., Cook, P.F., & Cleland, W.W. (1981) *Biochemistry 20,* 5655–5661.
Kaplan, H. & Laidler, K.J. (1967) Can. *J. Biochem. 45,* 539–549.
Kurz, J.L. (1963) *J. Am. Chem. Soc. 85,* 989–991.
Laidler, K.J. (1955) *Trans. Faraday* Soc. *51,* 528–561.
Leskovac, V., Trivić, S., & Anderson, M.B. (1996) *Indian J. Biochem. Biophys. 33,* 177–183.
Leskovac, V., Trivić, S., & Anderson, M.B. (1998) *Mol. Cell. Biochem. 178,* 219–227.
Leskovae, V., Trivić, S., & Anderson, M.B. (1999) *Eur. J. Biochem. 264,* 840–847.
Michaelis, L. & Davidsohn, H. (1911) *Biochem. Z. 35,* 386–396.
Peller, L. & Alberty, R.A. (1963) *J. Am. Chem. Soc. 81,* 5709–5711.
Plapp, B.V., Sogin, D.C., Dworschack, R.T., Bohlken, D.P., & Woenckhaus, C. (1986) *Biochemistry 25,* 5396–5402.
Phillips, D.C. (1966) *Sci. Amer. 215,* 7580–7590.
Renard, M. & Fersht, A.R. (1973) *Biochemistry 14,* 4714–4718.
Rose, I.A., O'Connel, E.L., Litwin, S., & Bar-Tana, J. (1974) *J. Biol. Chem. 249,* 5163–5168.
Silverman, D.N. & Tu, C.K. (1975) *J. A. Chem. Soc. 97,* 2263–2265.
Topham, C.M., Salih, E., Frazao, C., Kowlessur, D., Overinton, J.P., Thomas, M., Brockelhurst, S.M., Patel, M., Thomas, E.W., & Brockelhurst, K. (1991) *Biochem. J. 280,* 79–92.
Waley, S.G. (1953) *Biochim. Biophys. Acta 10,* 27–34.
Wang, J., Araki, T., Matsuoka, M., & Ogawa, T. (1999) *Biochim. Biophys. Acta 1435,* 177–183.

This page intentionally left blank

Chapter 15

Effects of Temperature on Enzyme Reactions

The thermodynamic treatment for the temperature dependence of simple chemical reactions, discussed in Chapter 2 (Section 2.6), applies equally well to enzyme-catalyzed reactions, but in practice several complications arise that must be properly understood if any useful information is to be obtained from temperature-dependence studies of enzyme reactions.

First, almost all enzymes become denatured if they are heated much above physiological temperatures and the conformation of the enzyme is altered, often irreversibly, with loss of catalytic activity. Exceptions are the thermophylic microorganisms, which are capable of working within a much broader range of temperature. The loss of catalytic activity if often due to denaturation; denaturation is chemically a very complex process, considering a large molecular size of proteins and the complexity of their three-dimensional structure.

The second influence of temperature is a usual influence of temperature on the rate of chemical reactions. The major effect of temperature is often not only on rates of chemical reaction, but also on the possible conformation changes before and after the chemical step, since these may have larger standard Gibbs free energies of activation than the chemical steps. Because of the overlapping influence on the structure of the protein molecule, straightforward analysis of the influence of temperature on the rates of enzyme-catalyzed reactions can be often obtained only within a fairly small range of temperature, usually between 0°C and 50°C.

A special branch of enzymology, cryoenzymology, deals with enzyme reactions in mixed solvents at subzero temperatures (Douzou, 1977; Fink & Greeves, 1979).

15.1 FREE ENERGY PROFILES

The energetics of an enzyme-catalyzed reaction is usually discussed in terms of a free energy profile; this is a diagram showing the relative free energy levels of all enzyme–reactant complexes and the transition states for conversion between them, at some chosen set of standard conditions (Lumry, 1959,1995).

Figure 1 shows such a profile for an uncatalyzed and enzyme-catalyzed unimolecular chemical reaction. Free energy profiles are commonly shown as points representing the relative free energy changes, connected by a smooth curve. However, it should be pointed out that experimental observations typically can only provide information for the maxima and minima of these profiles and not on points in between (Thornton & Thornton, 1978; Purich & Allison, 2000).

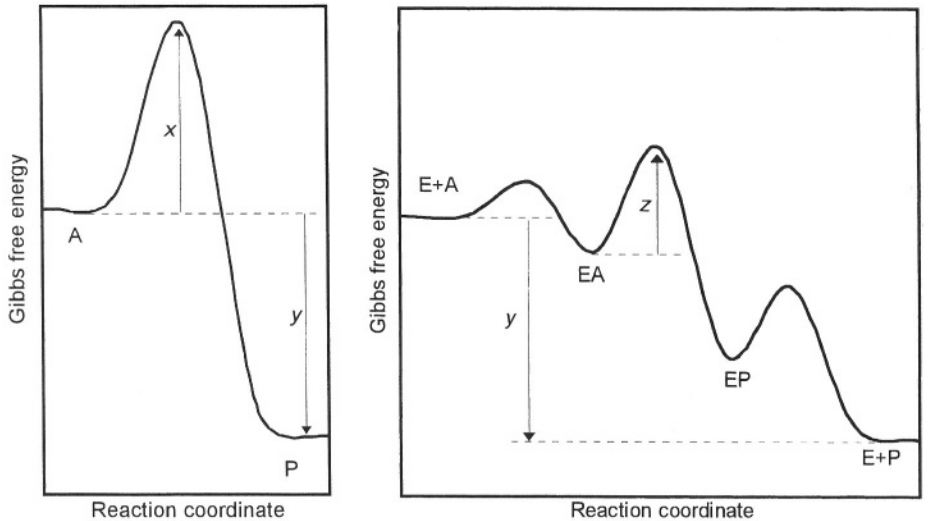


Figure 1. Free energy profile for an uncatalyzed (left) and enzyme-catalyzed (right) unimolecular reaction. In an uncatalyzed reaction, concentrations of A and P are at equilibrium; in the catalyzed reaction, their concentrations are also at equilibrium, and the off rate constants for A and P are the same.

For an uncatalyzed reaction, $A \rightleftharpoons P$ in Fig. 1 (left), x is the free energy of activation in the forward direction, and $(x + y)$ is the free energy of activation in the reverse direction; K_{eq} is the equilibrium constant for reaction. Since A and P are at equilibrium, the change in the free energy between substrate and product, y, is equal $-RT \ln K_{eq}$.

The free energy profile for an enzyme-catalyzed reaction is shown in Fig. 1 (right), when A and P are in equilibrium; the equilibrium constant, K_{eq}, is the same for both reactions. Here, the enzyme and substrate combine to form a Michaelis complex EA, which transforms into EP, followed by dissociation of P to give free enzyme again. The numerical value of y is equal $-RT \ln K_{eq}$, and is the same for both reactions. On the other hand, the value of z is now the Gibbs free energy of activation for conversion of EA to EP, which is less than x, a Gibbs free energy of activation for the uncatalyzed reaction. The value of y for an enzymatic reaction is equal $-RT \ln K_{eq}$ only if the reactants are at equilibrium.

According to the transition-state theory, the activation energies, $\Delta G^{\ddagger}$, are related to rate constants in forward and reverse directions by equation:

$$k = \left(\frac{k_B T}{h}\right) e^{-\Delta G^{\ddagger}/RT} \tag{15.1}$$

Equation (15.1) can be rearranged:

$$\ln\left(\frac{k}{T}\right) = \ln\left(\frac{k_B}{h}\right) - \left(\frac{\Delta G^{\ddagger}}{RT}\right) \tag{15.2}$$

Equation (15.2) is a nearly linear relationship over a moderate range of temperature; however, Eq. (15.2) can be used to obtain $\Delta G^{\ddagger}$ from the rate constant at any temperature (Chapter 2; Section 2.6).

The calculation of the rate enhancement resulting from enzymatic catalysis for a unimolecular reaction is simple.

$$\text{Rate enhancement} = 10^{(X-Z)/RT \ln 10} \tag{15.3}$$

Thus, if the difference in Gibbs free energy between x and z is 13.6 kcal/mol, the rate enhancement, k_{cat}/k_{uncat}, will be 10^{10}, because RT ln 10 is equal 1.36 kcal/mol at 25°C.

The rate enhancement in this case is not the same thing as the actual catalytic proficiency of an enzyme. Catalytic proficiency of the enzyme is a quantitative measure of enzyme's ability to lower the activation barrier for the reaction of a substrate, equal $(k_{cat}/K_M)/(k_{uncat})$ (Radzicka & Wolfenden, 1995; Northrop, 1998).

15.1.1 Standard States

The problems with construction of free energy profiles for enzyme-catalyzed reactions is the choice of *standard states* (Cleland & Northrop, 1999). The standard states are, in general, unit concentrations, using whatever unit is being used to evaluate rate constants. For this reason, the concentration terms are usually omitted from thermodynamic equations (Purich & Allison, 2000).

In enzyme reactions, however, the standard states must be accounted for. In a monomolecular enzyme reaction in Fig. 1, because the combination of E and A to give EA is a bimolecular step, the relative energy levels of (E + A) and EA depend on the concentration of A; the same applies to the dissociation of EP to give (E + P). The difference in energy levels of (E + A) and (E + P) must always equal $-RT \ln K_{eq}$, regardless of what standard state is chosen if one is representing an equilibrium free energy profile. If A and P are not at equilibrium, the energy levels of (E + A) and (E + P) will not differ by $-RT \ln K_{eq}$. Thus, the problem with profiles such as those in Fig. 1 is the choice of standard states.

Figure 2 shows the free energy profile when $K_{eq} = 0.1$ and the concentration of A equal to its dissociation constant from EA. If the concentration of A is equal P, the level of (E + P) lies 1.36 kcal/mol higher then the level of (E + A); however, if the concentration of P is equal 0.01 A, the level of (E + P) lies 1.36 kcal/mol below (E + A). Remember that RT ln 10 is equal 1.36 kcal/mol at 25°C.

This situation can cause problems with extrapolated limits. Enzymatic reactions are analyzed in terms of effects on V (substrate concentration extrapolated to infinity) and V/K (substrate concentration extrapolated to near zero). Therefore, one can envisage two simple limits for a unireactant enzyme reaction (Albery & Knowles, 1986):

$$\text{When } [A] = 0 \qquad v_o/[A] = k_{cat}/K_A E_o \qquad G^o_{(E+A)} = -\infty \tag{15.4}$$

$$\text{When } [A] = \infty \qquad v_o = k_{cat} E_o \qquad G^o_{(E+A)} = \infty \tag{15.5}$$

A further complication arises when the affinities of substrate and product for the enzyme are not the same, which is usually the case (Cleland & Northrop, 1999).

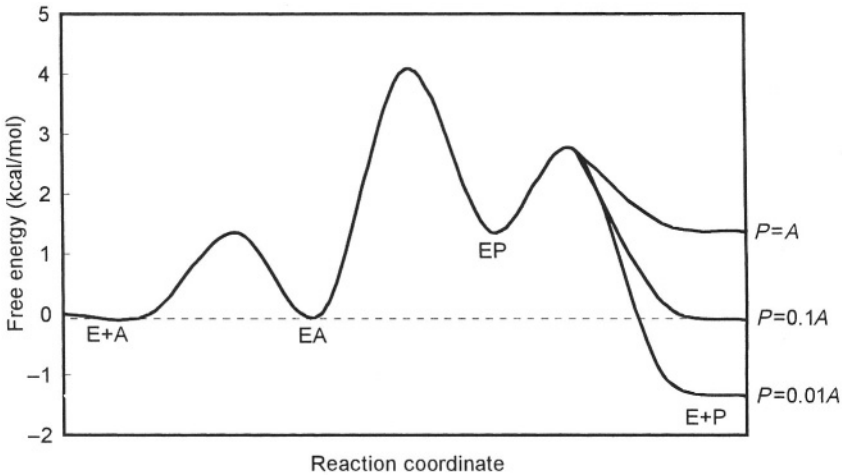


Figure 2. Free energy profile showing the influence of product concentration on the energy level of (E + P), assuming that [A] is equal to its dissociation constant from EA and that $K_{eq} = 0.1$.

15.1.2 Unimolecular and Bimolecular Steps in Free Energy Profiles

Consider again a monosubstrate enzyme-catalyzed reaction with two central complexes in more detail. Such a reaction will proceed via three transition states if it proceeds from A to P, or similarly in the reverse direction:

$$E + A \rightleftharpoons (EA)^* \rightleftharpoons EA \rightleftharpoons (EA \ldots EP)^* \rightleftharpoons EP \rightleftharpoons (EP)^* \rightleftharpoons E + P \tag{15.6}$$

Figure 3 shows the free energy diagram for this reaction.

As long as the levels of A and P chosen for the profile are kept at equilibrium, the energy change $\Delta 1$ is equal $-RT \ln K_{eq}$, where K_{eq} is the dimensionless equilibrium constant for the overall reaction: $E + A \rightleftharpoons P + E$. The overall reaction consists of a series of unimolecular and bimolecular steps. Bimolecular steps are characterized by free energy changes $\Delta 3$ and $(\Delta 7 + \Delta 6)$, and unimolecular steps by free energy changes $(\Delta 2 + \Delta 3)$, $\Delta 4$, $(\Delta 4 - \Delta 5)$, and $\Delta 6$.

As already pointed above, the bimolecular steps depend on the standard state chosen for substrate concentrations. Temperature analysis can be applied to unimolecular steps, provided the rates of these steps can be measured; this is often not easy, because many elementary steps in enzymatic reactions are very fast and rapid mixing techniques are usually needed to measure the rates of rapid reactions.

The equilibrium between intermediates EA and EP, $EA \rightleftharpoons EP$, is characterized by the free energy change $\Delta 5$ in Fig. 3, corresponding to $-RT \ln(K_{PT})$; K_{PT} is the dimensioneless internal equilibrium constant (i.e., $k_{forward}/k_{reverse}$) (Birnbaum *et al.*, 1989). The simplest way to determine the internal equilibrium constant would be to incubate a low level of substrate with increasing amounts

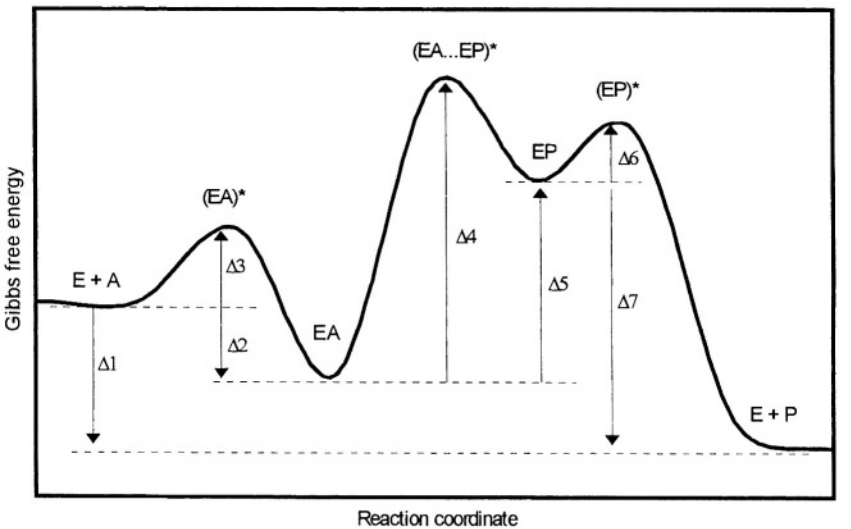


Figure 3. Energy profile for the overall reaction sequence:

$$E + A \rightleftharpoons EA \rightleftharpoons EP \rightleftharpoons P + E$$

of enzyme and denature each sample with cold acid. Then, the product/substrate ratio is measured and extrapolated to infinite concentration of enzyme. Sometimes, one can use the NMR methods and obtain the result with great simplification.

Thus, the main problems in constructing the free energy profiles is the knowledge of individual rate constants in the mechanism. With two substrates and two products, the problem of bimolecular steps is even greater. In this case, the energy levels of (E + A + B), (EA + B), and EAB will depend on the concentration of A and B, and the same is true for (E + P + Q), (EQ + P), and EPQ (Northrop, 1999).

15.2 SEPARATION AND ESTIMATION OF RATE CONSTANTS

As outlined above, temperature analysis may be successfully performed if the unimolecular and bimolecular rate constants are known; if the rate constants are known, and if we choose a standard state, a free energy profile can be drawn. Thus, the above thermodynamic analysis for a monosubstrate reaction shows that the meaningful application of $\ln(k/T)$ against $1/T$ plots requires that the individual uni- and bimolecular "microscopic" rate constants be separated.

A number of procedures have been used to separate rate constants in enzyme mechanisms. We shall mention only briefly: (a) rapid mixing techniques that can separate and measure almost all individual rate constants, (b) studies with alternative substrates that can provide information about the relative magnitude of some rate constants, and (c) work with alternative nucleophiles that can help to

separate some rate constants. Our main concern, however, is the steady-state kinetic methods as a means for the separation of individual rate constants.

15.2.1 Monosubstrate Reactions

Consider a simple Michaelis–Menten mechanism:

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_3} P + E$$

The limiting rate at low substrate concentrations is

$$v_0 = k_0[E_0][A] = \left(\frac{k_1 k_3}{k_2 + k_3}\right)[E_0][A] \tag{15.7}$$

The second-order rate constant k_0 is a composite value and the plot of $\ln(k_0/T)$ against $1/T$ will not necessarily apply to this constant directly. There are, however, two special cases under which this composite constant will obey the normal temperature behavior, as follows:

(a) When $k_3 >> k_2$. In this case, the k_2 may be neglected in comparison with k_3, and k_0 is equal to k_1. This means that the rate constant for the overall reaction at low substrate concentrations is simply that for the first step, the formation of the complex. If that is so, the plot of $\ln(k/T)$ against $1/T$ should apply to k_0, and the corresponding free energy of activation will be that for the initial complex formation.

(b) When $k_2 >> k_3$. In this case, the constant k_0 is now equal to $k_3 k_1/k_2$. The ratio of k_1/k_2 is simply the equilibrium constant for the complex formation:

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA$$

Its variation with temperature is given by

$$\frac{k_1}{k_2} = \frac{Xe^{-\Delta G_1^{\ddagger}/RT}}{Xe^{-\Delta G_2^{\ddagger}/RT}} = e^{-\Delta\Delta G^{\ddagger}/RT} \quad \text{where} \quad X = \frac{k_B T}{h} \tag{15.8}$$

and $\Delta\Delta G^{\ddagger}$ is the increase in Gibbs free energy per mole for the change from (E + A)to EA.

Since k_3 varies exponentially with temperature according to

$$k_3 = Xe^{-\Delta G_3^{\ddagger}/RT} \tag{15.9}$$

it follows that

$$\frac{k_1 k_3}{k_2} = Xe^{\frac{(\Delta\Delta G^{\dagger} + \Delta G_3^{\dagger})}{RT}} \tag{15.10}$$

In other words, the plot of $\ln(k/T)$ against $1/T$ should apply to this composite constant $k_1 k_3/k_2$, but the free energy of activation does not correspond to a

single elementary step; it is the sum of the free energy of activation for the second step ($EA \rightarrow E + P$) and the total energy for the first step ($E + A \leftrightarrow EA$).

When the situation corresponds to neither of these special cases the plots of ln (k/T) against $1/T$ will not necessarily be linear.

15.2.2 Bisubstrate Reactions

Free energy diagrams, such as the diagram shown in Fig. 3, are important tools for analyzing the structure and energy relationships that describe a reaction mechanism.

Very few complete thermodynamic analyses have been performed with enzymic reactions involving more than one substrate. This is understandable, considering the usual complexities of kinetic constants in bisubstrate and trisubstrate reactions. Kinetic constants in such reactions are usually composed of several individual rate constants, each of which may have its own dependence on temperature. In such cases, the separation of rate constants is usually a very difficult task. However, there are cases that allow a meaningful separation of rate constants.

In all rapid equilibrium systems, all Michaelis constants are always true dissociation constants of the complexes that dissociate. In all trisubstrate mechanisms, all inhibition constants are also always true dissociation constants of the corresponding complexes. The temperature dependence of the equilibrium constants can be treated as described in Section 15.2.1 for a monosubstrate reaction.

In some steady-state mechanisms, such as an Ordered Bi Bi mechanism, all or some of the rate constants can be calculated directly from the kinetic constants. Quantities like k_{cat} and K_M will not necessarily show the normal temperature behavior, since they are usually combinations of several rate constants, but if certain rate constants predominate, normal temperature behavior may be obeyed. This is often the case with bimolecular rate constants k_{cat}/K_A and k_{cat}/K_B in bisubstrate reactions.

A detailed thermodynamic analysis was performed with lactate dehydrogenase, in the lactate $\longrightarrow$ pyruvate direction, by means of steady-state kinetics and presteady-state kinetic methods, by Laidler and Peterman (1979). A particularly detailed kinetic studies of the energetics of two multistep enzymes, triosephosphate isomerase and proline racemase, has been described by the research team of Albery and Knowles (Albery & Knowles, 1976, 1986; Knowles, 1991). Apart from these examples, very few complete thermodynamic analyses have been performed with reactions involving more than one substrate or more than one intermediate in reaction.

15.3 HEAT OF IONIZATION OF AMINO ACID SIDE CHAINS IN ENZYMES

Most thermodynamic studies in enzyme kinetics have been performed by measuring the initial rates of reactions at a constant pH value and at several different temperatures, then proceeding with a construction of an ln(k/T) versus $1/T$

graph from the data. From such plots, one can calculate the thermodynamic parameters: enthalpy, entropy, and the Gibbs free energy of activation (Section 2.6).

Enzymatic reactions display wide variations in their thermodynamic properties, but less variations than the non-enzymatic reactions. Comparison of kinetic and thermodynamic properties of enzymatic reactions and, especially, the comparison between different substrates may provide valuable information about the properties and structure of various enzyme–substrate complexes. However, the amount of information, especially in structural terms, that is extracted from a thermodynamic study at a constant pH value, is usually limited to qualitative description of the various states of enzyme conformation, binding energies of substrates or the neutralization of enzyme with a substrate upon binding. Much more useful information can be obtained with thermodynamic studies at different pH values.

Amino acid side chains in enzymes that are taking part in catalysis can be identified kinetically from appropriate pH profiles of various kinetic constants, various groups of kinetic constants, or individual rate constants (Chapter 14). Usually, such amino acid side chains can be identified from their pK_a values, although the ranges of amino acid pK_a's overlap considerably (Table 1). A temperature change can have an effect on the degree of ionization of various groups on a protein as well as on substrates and effectors. Since proton dissociation constants are thermodynamic parameters, a change in temperature can result in a significant alteration of the pH–activity curve (Tipton & Dixon, 1979). Knowles (1976) has pointed out that factors that are sufficient to perturb a pK_a value in a protein can also affect changes in enthalpy and entropy. Ellis and Morrison (1982) have discussed the practical pK_a values under chosen experimental conditions and presented the programs for calculating pK_a values at different temperatures and ionic strengths.

Table 1 Heats of ionization of amino acid side chains in enzymes

		$\Delta H_{ionization}$	
Amino acid side chain	pK_a[a]	(kcal/mol)	(kJ/mol)
α-Carboxyl (end of polypeptide chain)	2–3	1.5	6.3
β- or γ-Carboxyl (aspartate or glutamate)	3–5	1.5	6.3
Imidazolium (histidine)	4–8	6.9–7.5	28.9–31.4
α-Amino (end of polypeptide chain)	7.5–8.5	10–15	41.9–54.5
Sulfhydryl (cysteine)	4–10[b]	6.5–7.0	27.2–29.3
ε-Amino (lysine)	6–10.5	10–15	41.9–54.5
Phenolic hydroxyl (tyrosine)	9–10.5	6	25.1
Guanidinium (arginine)	11.6–12.6	12–15	50.3–54.5

[a] The range of pK_a values observed in enzymes.
[b] Low values in ion pair with protonated histidine.

An exceptionally useful means for further identification of a given amino acid, participating in catalysis, is provided by a thermodynamic analysis, particularly the estimation of the heat of ionization.

The best way to understand the estimation of the heat of ionization of a particular amino acid side chain in the active site of enzyme is in relation to a specific example. Consider a reaction catalyzed by equine liver alcohol dehydrogenase (Maret & Makinen, 1991):

$$\text{Propan-2-ol} + \text{NAD}^+ \rightleftharpoons \text{Acetone} + \text{NADH} + \text{H}^+ \qquad (15.11)$$

In this enzymatic reaction, the catalytic constant, k_{cat}, is the rate-limiting step in the propan-2-ol $\longrightarrow$ acetone direction; a plot of log k_{cat} versus pH has a shape of a titration curve for a monobasic acid with pK_a value of 7.0, suggesting that a histidine residue is involved in catalysis in the ternary complex enzyme–NAD^+–alcohol (Maret & Makinen, 1991). However, this assignment is not unambiguous, since carboxyl groups are known to have pK_as as high as 8, and lysines to have pK_a's as low as 6.

Figure 4 shows the temperature dependence of the heat of ionization of the amino acid side chain in the active site of alcohol dehydrogenase, calculated from the catalytic constants for the above example.

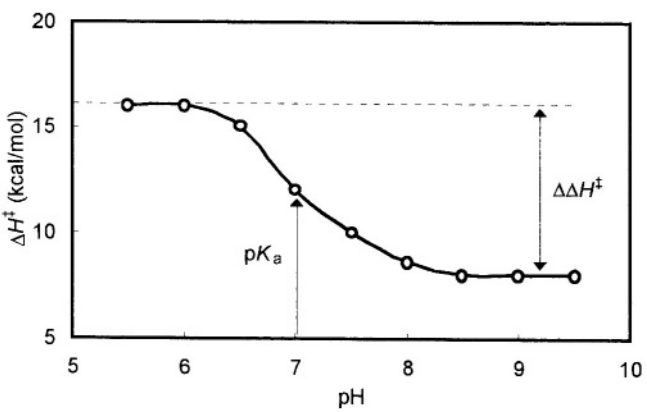


Figure 4. Thermodynamic profile for the heat of ionization of a histidine side chain in the active site of alcohol dehydrogenase, calculated from the catalytic constant (Maret & Makinen, 1991).

The pH profile in Fig. 4 shows a correct value of pK_a, the same value that is obtained from kinetic measurements. A difference in activation energies between the low and the high pH, $\Delta\Delta H^{\ddagger}$, shows the heat of ionization of the amino acid side chain in question, which is in this case 8.0 kcal/mol. This value further suggests that the histidine residue is responsible for catalysis (Table 1).

However, this heat of ionization is also consistent with a carboxyl if a conformational change accompanies the ionization, that is, if the pK_a is different in the two conformations; this is not a rare phenomenon. Therefore, a further

means are necessary to confirm the identity of the amino acid side chain in question. In this particular case, a solvent perturbation is the method of choice.

15.4 SOLVENT PERTURBATION

The solvent perturbation method depends on the different behavior of neutral and cationic acids when organic solvents are added to aqueous buffers, as indicated by the following examples:

$$R \cdot COOH \longrightarrow R \cdot COO^- + H^+$$

$$R_3 \cdot NH^+ \longrightarrow R_3 \cdot N + H^+$$

A neutral acid has no charge when protonated and generates a new negative and positive charge, while a cationic acid has a positive charge both before and after ionization. In the presence of an organic solvent, such as dimethylformamide, dioxane, formamide, etc., the charge separation involved in ionization of a neutral acid is suppressed and the pK_a is elevated, while there is little or no effect on ionization of a cationic acid. Addition of organic solvents, usually 15–25%, changes the pK_a of the buffer; in addition, the neutral acid buffers and the cationic acid buffers will have a different influence on the results of the solvent perturbation.

Therefore, the experimental protocol must include the comparison of the behavior of the pK_a in both neutral acid and cationic acid buffers in the presence and absence of solvent. One must run all four experiments, because there are some variations in the behavior of different neutral acid buffers (such as diethylamonate and phenolsulfonate) and cationic acid buffers (such as Tris and glycine) which must be corrected by control experiments. Table 2 shows the expected changes in apparent pK_a as a result of solvent perturbation (Cleland, 1977).

Table 2 Change in apparent pK_a as the result of solvent perturbation (Cleland, 1977)

	Nature of catalytic group	
Buffer	Neutral acid	Cationic acid
Neutral acid	No change	pK_a lowered
Cationic acid	pK_a elevated	No change

In the particular case of alcohol dehydrogenase, outlined in Section 15.3, the combination of temperature studies with solvent perturbation will separate the neutral amino acids from the cationic ones, provided the group is exposed to the solvent. Thus, by combining the knowledge of pK_a and the heat of ionization for a given amino acid side chain with a solvent perturbation method, a fairly accurate identification of an amino acid can be achieved.

The solvent perturbation method is a hazardous one, since one must always take care that the denaturation, or a time-dependent denaturation of enzyme,

does not take place in the presence of elevated concentrations of organic solvents at room temperature.

REFERENCES

Books

Douzou, P. (1977) *Cryobiochemistry,* Academic Press, New York.
Gandour, R.D. & Schowen, R.L. (eds.) (1978) *Transition states in biochemical processes,* Plenum Press, New York and London.
Purich, D.L. & Allison, D.R. (2000) *Handbook of biochemical kinetics,* Academic Press, San Diego.

Review articles

Cleland, W.W. (1977) *Adv. Enzymol. Rel. Areas Mol. Biol. 45,* 273–387.
Cleland, W.W. & Northrop, D.B. (1999) *Methods Enzymol. 308,* 3–27.
Ellis, K.J. & Morrison, J.F. (1982) *Methods Enzymol. 87,* 405–427.
Fink, A.L. & Geeves, M.A. (1979) *Methods Enzymol. 63,* 336–370.
Knowles, J.R. (1976) *CRC Crit. Rev. Biochem. 4,* 165–173.
Laidler, K.J. & Peterman, B.F. (1979) *Methods Enzymol. 63,* 234–257.
Lumry, R. (1959) *The Enzymes,* 2nd ed., Vol. 1, 157–160.
Lumry, R. (1995) *Methods Enzymol. 259,* 628–720.
Northrop, D.B. (1999) *Adv. Enzymol. Relat. Areas Mol. Biol. 73,* 25–55.
Thornton, E.K. & Thornton, E.R. (1978) in *Transition states in biochemical processes,* pp. 1–75.
Tipton, K.F. & Dixon, H.B.F. (1979) *Methods Enzymol.* 63, 183–234.

Specific references

Albery, W.J. & Knowles, J.P. (1976) *Biochemistry 15,* 5631–5640.
Albery, W.J. & Knowles, J.P. (1986) *Biochemistry 25,* 2529–2575.
Albery, W.J. & Knowles, J.P. (1986) *J. Theor. Biol. 124,* 173–183.
Birnbaum, J.J., Raines, R.T., Albery, W.J., & Knowles, J.R. (1989) *Biochemistry 28,* 9293–9305.
Knowles, J.P. (1991) *Nature 350,* 121–125.
Maret, W. & Makinen, M.W. (1991) *J. Biol. Chem. 266,* 20636–20644.
Northrop, D.B. (1998) *J. Chem. Educ.* 75, 1153–1157.
Radzicka, A. & Wolfenden, R. (1995) *Science 276,* 90–93.

This page intentionally left blank

Chapter 16

Isotope Exchange

16.1 PRINCIPLES OF ISOTOPE EXCHANGE

Study of the initial rates of bisubstrate and trisubstrate reactions in both directions, and in the presence and absence of products, will usually eliminate many possible reaction pathways and give a reasonably good idea of the main features of the mechanism, but it will not usually reveal the existence of minor alternative pathways. Even if a clear mechanism does emerge from the initial rate and product inhibition studies, it is valuable to confirm its validity independently. The important technique of *isotope exchange* can often satisfy these requirements.

This technique was initially developed by Boyer (1959) and subsequently considerably extended to involve rate measurement of loss, or exchange, of essentially all possible atoms or functional groups, the determination of kinetic isotope effects, the definition of stereochemical processes, and investigation of activators and inhibitors (Wedler & Shalango, 1982; Wedler, 1995; Purich & Allison, 2000).

All of the studies described in earlier chapters were concerned with the observed *net* velocity of a chemical reaction. In the steady state, the net velocity is a difference between the absolute forward and reverse velocity of any step. The unidirectional velocity of a step may be considerably faster than the observed net velocity. Isotope exchange is based on the simple fact that, in a chemical reaction, even if it is at equilibrium, when its net rate is zero by definition, the unidirectional rates through steps or groups of steps can be measured by means of isotopic tracers. Therefore, the isotope exchange studies provide a way of measuring the unidirectional rates of individual steps within a reaction sequence.

The introduction of a labeled substrate or product may be utilized to trace the course of the reaction quantitatively. Depending upon the position of the isotopic atom(s) in the labeled substrate or product, various exchanges may be possible. Various types of exchanges may be illustrated by considering a bisubstrate reaction in the following form:

$$\mathrm{A{-}X} + \mathrm{B{-}Y} \rightleftharpoons \mathrm{A{-}Y} + \mathrm{B{-}X} \qquad (16.1)$$

One might expect that there are a number of exchange reactions to be examined. However, not all hypothetical exchanges are possible in practice, instead only a limited number of exchanges is involved. Exchange can be measured between any substrate–product pair that shares a common part. For example, in the hexokinase reaction, it is possible to observe exchange reactions between glucose and glucose-6-phosphate, ADP and ATP, ADP and glucose-6-phosphate, but not

Table 1. Isotopes that may be used in kinetic exchange studies

Radioactive isotopes		Stable isotopes		
Isotope	Half-life	Isotope	Atomic weight	Natural abundance (%)
^{3}H	12.3 years	^{2}H	2.016	0.016
^{14}C	5730 years	^{13}C	13.008	1.1
^{22}Na	2.6 years	^{15}N	15.005	0.38
^{32}P	14.3 days	^{17}O	17.005	0.04
^{35}S	88 days	^{18}O	18.004	0.20
^{42}K	12.4 hours	^{33}S	32.982	0.74
^{45}Ca	163 days	^{34}S	33.980	4.2
^{59}Fe	46 days	^{35}Cl	34.981	75.4
^{125}I	60 days	^{37}Cl	36.978	24.6
^{203}Hg	47 days			

glucose and ADP. Likewise, NAD(P)-dependent dehydrogenases will never undergo exchange between the oxidized substrate and the oxidized coenzyme.

In the isotope exchange studies, virtually all stable and radioisotopically labeled atoms may be used (Table 1).

In principle, all isotopes listed in Table 1 may be used in isotope exchange studies. In practice, however, the only isotopes usually used are deuterium (monitored with NMR techniques), tritium, and ^{14}C, while a number of others are commonly used to measure kinetic isotope effects (^{13}C, ^{15}N, ^{18}O), but are not routinely used to measure isotope exchange.

16.2 ISOTOPIC EXCHANGE AT CHEMICAL EQUILIBRIUM IN SEQUENTIAL MECHANISMS

Most isotopic exchange experiments where the mechanism is sequential are conducted under equilibrium conditions, and most of the formal treatments have dealt with the equilibrium (Boyer, 1959; Morales *et al.,* 1962; Boyer & Silverstein, 1963, 1964; Allison *et al.,* 1977; Purich & Allison, 1980). However, some treatments have dealt with isotopic exchange in the steady state (Alberty *et al.,* 1962; Cleland, 1967; Boyer, 1978; Purich & Allison, 2000).

Let us consider an isotopic exchange under equilibrium conditions. The equilibrium constant of an enzymatic reaction is given by

$$K_{eq} = \frac{[P][Q]\cdots}{[A][B]\cdots} \tag{16.2}$$

In order to obtain equilibrium, attention must be given to the mass–action ratio, which is the product of each reaction product concentration divided by the product of each substrate concentration.

In isotope exchange studies, the usual approach is to treat the reaction system in terms of substrate–product pairs (such as $\alpha = [P]/[A]$ or $\beta = [Q]/[B]$). In a typical experiment, one substrate–product pair is held constant with respect to the absolute concentrations of each component; the other substrate–product pair may be adjusted to a variety of absolute levels. This pair is maintained at a constant ratio, so that the system remains at equilibrium. Then, the enzyme is added to fully equilibrate the system. Since the experimenter usually has a good estimate of K_{eq}, the composition of the system will not change much from the starting conditions. Then, a small aliquot of labeled reactant (substrate or product) is added, and the progress is followed by periodical sampling.

A better method is to make up the reaction mixture with the labeled reactant present and at equilibrium and add the enzyme to start the reaction; in this way, the time of exchange is shortened and denaturation of enzyme is prevented. In this case, the system must truly be at equilibrium.

Although the progress curve for the exchange is always *first order,* the magnitude of the exchange rate depends on the absolute concentrations of all reactants, level of enzyme, and the kinetic mechanism. The derivation of exchange-rate laws is based on the following assumptions (Boyer, 1959; Cleland, 1967; Fromm, 1975; Huang, 1979):

(1) The total enzyme concentration is much less than the concentration of any substrate or modifier.
(2) All unlabeled species are present at their equilibrium concentrations prior to the addition of the labeled species; this condition applies to the first method but not to the second method outlined above.
(3) Kinetic isotope effects, if any, are negligible; this condition is not required as lone as the label is a tracer.

16.2.1 Derivation of Rate Equations for an Ordered Bi Bi Mechanism

Isotope exchange is most easily understood in relation to a specific example. Consider the phosphorylation of fructose-6-P by ATP, a reaction catalyzed by *Lactobacillus plantarum* phosphofructokinase, which proceeds via an Ordered Bi Bi mechanism at neutral pH (Simon & Hoffer, 1978). If the coenzyme ATP, labeled in the γ-position with ^{32}P, is used for the equilibrium exchange studies, the terminal radioactive phosphate group will be transferred from ATP to fructose-6-P:

$$\underset{(A)}{\text{ADP}\mathbf{P}} + \underset{(B)}{\text{fructose-6-P}} \rightleftharpoons \underset{(Q)}{\text{ADP}} + \underset{(P)}{\text{fructose-1,6-}\mathbf{P}\text{P}} \qquad (16.3)$$

In this case, we shall have the transfer of a radioactive atom (represented by bold letters) from substrate **A** to product P. If the nucleotide in the adenine, or the ribose portion of ATP, is labeled with ^{14}C, we shall have the transfer of a radioactive atom from **A** to product Q. On the other hand, if the ^{14}C label is in the fructose part of fructose-6-P or ^{32}P label in the 6′-phosphate of fructose-6-P, we shall have the transfer of a radioactive atom from **B** to product P. Most isotope exchange studies are conducted between **A** and Q, or **B** and P.

There are several efficient methods for deriving steady-state rate equations for isotope exchange, all of which are described in Chapter 4. One has to be proficient at only one of them since, for a given reaction scheme, they all lead to the same equation.

Let us consider the Ordered Bi Bi mechanism in reaction (16.3) and derive the initial rate equation for the **A**–Q isotope exchange with the aid of the King–Altman method. The basic King–Altman figure for this case is shown in Fig. 1.

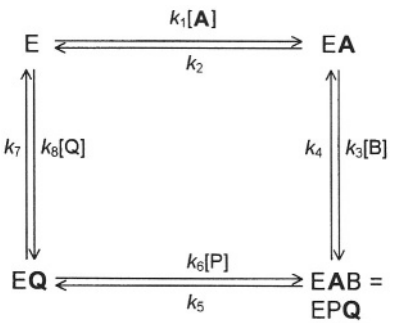
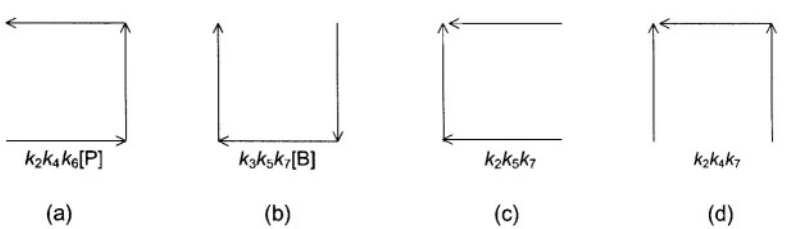


Figure 1. The basic King–Altman figure for an **A**–Q isotope exchange in an Ordered Bi Bi mechanism.

In the King–Altman figure, all enzyme forms are labeled and, therefore, there are four three-lined King–Altman patterns necessary to obtain the denominator term of the velocity equation.



The **A**–Q exchange in the Ordered Bi Bi system is given by

$$v_{A-Q} = k_7[\mathbf{EQ}] = \frac{k_7 \text{Numerator}_{EQ}}{\text{Denominator}} \tag{16.4}$$

The value of the numerator is given by

$$\text{Numerator}_{EQ} = k_1k_3k_5[\mathbf{A}][\mathrm{B}][\mathrm{E}] \tag{16.5}$$

and denominator is equal:

$$\text{Denominator} = k_2k_7(k_4 + k_5) + k_3k_5k_7[\text{B}] + k_2k_4k_6[\text{P}] \tag{16.6}$$

Therefore,

$$v_{\text{A-Q}} = \frac{k_1k_3k_5k_7[\text{A}][\text{B}][\text{E}]}{k_2k_7(k_4 + k_5) + k_3k_5k_7[\text{B}] + k_2k_4k_6[\text{P}]} \tag{16.7}$$

Before Eq. (16.7) can be used, we need an expression for [E]. If the experiment is done with the unlabeled reaction in the steady state, the expression for [E] becomes complicated, but, if the reaction rates are studied at equilibrium, the rate equations are less complicated. If the unlabeled reaction is at chemical equilibrium, the concentration of free enzyme in the Ordered Bi Bi mechanism is as follows:

$$[\text{E}] = \frac{[\text{E}_0]}{1 + \dfrac{k_1A}{k_2} + \dfrac{k_1k_3AB}{k_2k_4} + \dfrac{k_1k_3k_5AB}{k_2k_4k_6P}} \tag{16.8}$$

Equation (16.8) is derived in Section 16.2.2. Substituting Eq. (16.8) into Eq. (16.7), we obtain

$$v_{\text{A-Q}} = \frac{k_1k_3k_5k_7[\text{A}][\text{B}][\text{E}_0]}{\{k_2k_7(k_4 + k_5) + k_3k_5k_7[\text{B}] + k_2k_4k_6[\text{P}]\}\left(1 + \dfrac{k_1A}{k_2} + \dfrac{k_1k_3AB}{k_2k_4} + \dfrac{k_1k_3k_5AB}{k_2k_4k_6P}\right)} \tag{16.9}$$

In order to transform Eq. (16.9) from the rate constant form into a kinetic constant form, we shall divide the numerator and denominator with $k_1k_3(k_5+k_7)$, which is equal to CoefAB in Eq. (9.8) (Chapter 9); then, we shall transform the groups of rate constants into kinetic constants by using the appropriate definitions of kinetic constants from Eq. (9.9) (Chapter 9). The resulting rate equation for the **A**–Q exchange is

$$v_{\text{A-Q}} = \frac{V_1AB}{\left(K_{\text{iA}}K_{\text{B}} + K_{\text{A}}B + \dfrac{K_{\text{iA}}K_{\text{B}}K_{\text{Q}}}{K_{\text{P}}K_{\text{iQ}}}P\right)\left(1 + \dfrac{A}{K_{\text{iA}}} + \dfrac{AB}{K_{\text{iA}}K_{\text{eB}}} + \dfrac{Q}{K_{\text{iQ}}}\right)} \tag{16.10}$$

In Eq. (16.10), K_{eB} represents the dissociation constant of substrate B from the central complex EAB; it is an equilibrium constant equal k_4/k_3 in Fig. 1, and should not be confused with K_{iB}. The relationship between the two kinetic constants is

$$K_{\text{eB}} = K_{\text{iB}}\left(\frac{K_{\text{A}}}{K_{\text{iA}}}\right)\left(\frac{V_2}{V_1}\right) \tag{16.11}$$

K_{eB} will appear in all isotope exchange rate expressions for an Ordered Bi Bi mechanism (Table 2).

Table 2. Isotope exchange-rate expressions for sequential mechanisms (Purich & Allison, 1980, 2000).

1. *Uni Uni*

$$v_{A\leftrightarrow P} = \frac{V_1 A}{K_A\left(1+\frac{A}{K_{iA}}+\frac{P}{K_{iP}}\right)}$$

2. *Ordered Bi Uni*

$$v_{A\leftrightarrow P} = \frac{V_1 AB}{(K_{iA}K_B + K_A B)\left(1+\frac{A}{K_{iA}}+\frac{P}{K_{iP}}\right)} \qquad v_{B\leftrightarrow P} = \frac{V_1 B}{K_B\left(1+\frac{K_{iA}}{A}+\frac{K_{iA}P}{K_{iP}A}\right)}$$

3. *Rapid Equilibrium Random Bi Uni*

$$v_{all} = \frac{V_1}{1+\frac{K_A}{A}+\frac{K_B}{B}+\left(\frac{K_{iA}K_B}{AB}\right)\left(1+\frac{P}{K_{iP}}\right)}$$

4. *Rapid Equilibirum Random Bi Bi with dead-end EBQ*

$$v_{all} = \frac{V_1}{1+\frac{K_A}{A}+\frac{K_B}{B}+\frac{K_{iA}K_B}{AB}\left(1+\frac{P}{K_{iP}}+\frac{Q}{K_{iQ}}+\frac{PQ}{K_{iP}K_Q}\right)+\frac{K_A Q}{K_{eQ}A}}$$

5. *Ordered Bi Bi*

$$v_{A\leftrightarrow Q} = \frac{V_1 AB}{\left(K_{iA}K_B + K_A B + \frac{K_{iA}K_B K_Q}{K_{iQ}K_P}P\right)\left[1+\frac{A}{K_{iA}}\left(1+\frac{B}{K_{eB}}\right)+\frac{Q}{K_{iQ}}\right]}$$

$$v_{A\leftrightarrow P} = \frac{V_1 AB}{(K_{iA}K_B + K_A B)\left[1+\left(\frac{A}{K_{iA}}\right)\left(1+\frac{B}{K_{eB}}\right)+\frac{Q}{K_{iQ}}\right]}$$

$$v_{B\leftrightarrow P} = \frac{V_1 B}{K_B\left(1+\frac{K_{iA}}{A}+\frac{B}{K_{eB}}+\frac{K_{iA}Q}{K_{iQ}A}\right)}$$

6. *Theorell–Chance Bi Bi*

$$v_{A\leftrightarrow Q} = \frac{V_1 AB}{\left(K_{iA}K_B + K_A B + \frac{K_{iQ}}{K_{eq}}P\right)\left(1+\frac{A}{K_{iA}}+\frac{Q}{K_{iQ}}\right)}$$

$$v_{A\leftrightarrow P} = \frac{V_1 AB}{(K_{iA}K_B + K_A B)\left(1+\frac{A}{K_{iA}}+\frac{Q}{K_{iQ}}\right)}$$

$$v_{B\leftrightarrow P} = \frac{V_1 B}{K_B\left(1+\frac{K_{iA}}{A}+\frac{K_{iA}Q}{K_{iQ}A}\right)}$$

$v_{X\leftrightarrow Y}$ denotes the rate of exchange between the two reactants expressed in units of concentration/time.
In mechanism 4, K_{eQ} is equal the dissociation constant of Q from the EBQ complex.
In mechanism 5, K_{eB} is equal the dissociation constant of B from the EAB complex.

16.2.2 Distribution Equations at Chemical Equilibrium

Distribution equations for bisubstrate reactions in the steady state are often very complex expressions (Chapter 9). However, in the chemical equilibrium, the distribution equations for all enzyme forms are usually less complex. Consider an Ordered Bi Bi mechanism in reaction (16.12) with a single central complex:

$$\mathrm{E} \underset{k_2}{\overset{k_1\mathrm{A}}{\rightleftharpoons}} \mathrm{EA} \underset{k_4}{\overset{k_3\mathrm{B}}{\rightleftharpoons}} (\mathrm{EAB} = = \mathrm{EPQ}) \underset{k_6\mathrm{P}}{\overset{k_5}{\rightleftharpoons}} \mathrm{EQ} \underset{k_8\mathrm{Q}}{\overset{k_7}{\rightleftharpoons}} \mathrm{E} \tag{16.12}$$

At chemical equilibrium, there is no net reaction and the concentration of all enzyme forms is constant. Therefore,

$$\begin{aligned} \frac{[\mathrm{E}][\mathrm{A}]}{[\mathrm{EA}]} &= \frac{k_2}{k_1} \quad \frac{[\mathrm{EA}][\mathrm{B}]}{[\mathrm{EAB}]} = \frac{k_4}{k_3} \\ \frac{[\mathrm{EQ}][\mathrm{P}]}{[\mathrm{EPQ}]} &= \frac{k_5}{k_6} \quad \frac{[\mathrm{E}][\mathrm{Q}]}{[\mathrm{EQ}]} = \frac{k_7}{k_8} \end{aligned} \tag{16.13}$$

Now, one can eliminate stepwise each form of the enzyme simply by a stepwise substitution going from left to right and obtain directly the relationship

$$K_{\mathrm{eq}} = \frac{k_1 k_3 k_5 k_7}{k_2 k_4 k_6 k_8} = \frac{PQ}{AB} \tag{16.14}$$

Similarly, one can obtain the distribution equations in equilibrium,

$$\frac{E_0}{E} = 1 + A\frac{k_1}{k_2} + AB\frac{k_1 k_3}{k_2 k_4} + AB\frac{k_1 k_3 k_5}{k_2 k_4 k_6 P} \tag{16.15}$$

$$\frac{E_0}{EA} = 1 + B\frac{k_3}{k_4} + B\frac{k_3 k_5}{k_4 k_6 P} + \frac{1}{A}\frac{k_2}{k_1} \tag{16.16}$$

$$\frac{E_0}{EQ} = 1 + \frac{k_2 k_4 k_6 P}{k_1 k_3 k_5 AB} + \frac{k_4 k_6 P}{k_3 k_5 B} + \frac{k_6 P}{k_5} \tag{16.17}$$

$$\frac{E_0}{EAB} = 1 + \frac{1}{B}\frac{k_4}{k_3} + \frac{1}{AB}\frac{k_2 k_4}{k_1 k_3} + \frac{k_5}{k_6 P} \tag{16.18}$$

The four terms in Eqs. (16.15)–(16.18) refer to the four enzyme species E, EA, EQ, and (EAB + EPQ), and each is in the appropriate equilibrium ratio to the preceding one, that is $[\mathrm{EA}]/[\mathrm{E}] = k_1 A/k_2$, and so on. The equations do not contain Q, because, if equilibrium is to be maintained, only three of the four reactant concentrations can be chosen at will. Any one of A, B, and P can be replaced with Q by means of equality (16.14).

With the aid of the King–Altman method, one can derive the rate equations for the other two types of isotope exchange in the Ordered Bi Bi mechanism, in the same manner:

$$v_{B-P}=\frac{V_1 B}{K_B\left(1+\frac{K_{iA}}{A}+\frac{B}{K_{eB}}+\frac{K_{iA}Q}{K_{iQ}A}\right)} \tag{16.19}$$

$$v_{A-P}=\frac{V_1 AB}{(K_{iA}K_B+K_A B)\left[1+\left(\frac{A}{K_{iA}}\right)\left(1+\frac{B}{K_{eB}}\right)+\frac{Q}{K_{iQ}}\right]} \tag{16.20}$$

16.2.3 Survey of Rate Equations for Sequential Mechanisms

The equations for any other exchange reaction, and indeed for any other mechanism, can be derived similarly. However, in most cases, the rate equations for isotope exchange are derived by the method of King and Altman and various extensions of the same; also, the rate equations may be derived efficiently by the net rate constant method (Chapter 4). In most cases, the rate equations for isotope exchange are far too complicated to permit the determination of the usual kinetic constants. Nevertheless, there are a number of simplifying assumptions which will permit the derivation of manageable rate equations in specific cases (Boyer, 1959; Fromm *et al.,* 1964; Darvey, 1973).

Table 2 summarizes the isotope exchange rate expressions for selected common sequential mechanisms, in addition to the Ordered Bi Bi mechanism outlined above. Since there is no such mechanism as Rapid Equilibrium Random Bi Bi without a dead-end complex, at least a dead-end complex EBQ must form (mechanism 4). If both dead-end complexes, EBQ and EAP, are formed, an extra $K_B P/K_{eP}B$ term is added in the denominator of rate equation for mechanism 4; K_{eP} represents the dissociation constant of P from the EAP complex.

16.2.4 Estimation of the Rates of Isotope Exchange in Equilibrium

The first task is to estimate the rates of isotope exchange. The estimation of exchange rates may be accomplished by adding an isotopically labeled substrate (or product) to a fully equilibrated reaction system containing enzyme and by subsequently determining the extent of interconversion to labeled product (or substrate) by periodic sampling. The basic equation for relating the *rate of isotopic exchange* (v_{ex}) to the concentrations of two reactants, X and Y, undergoing exchange, is given by

$$v_{ex}=\frac{[X][Y]\ln(1-F)}{([X]+[Y])t} \tag{16.21}$$

where t is the time period elapsed between initiating and sampling the exchange process, and F is the fractional attainment of isotopic equilibrium (Frost & Pearson, 1961).

If the labeled species are designated as **X** and **Y**, the label will be distributed at isotopic equilibrium according to

$$\frac{[\mathbf{X}]}{[\mathbf{Y}]} = \frac{[\mathbf{X}]_E}{[\mathbf{Y}]_E} \tag{16.22}$$

Using the subscripts T and E to indicate [X] and [Y] at their time-dependent and equilibrium values, respectively, F may be expressed as the dimensionless number, as follows:

$$F = \frac{[\mathbf{X}]_T}{[\mathbf{X}]_E} = \frac{[\mathbf{Y}]_T}{[\mathbf{Y}]_E} \tag{16.23}$$

In practice, $[\mathbf{X}]_T$ and $[\mathbf{X}]_E$ may be expressed in any units that are proportional to their concentration or abundance. For radioactive determinations, either total activity or specific activity may be used; for stable isotopes, mole fraction may be employed. The expression given above for υ_{ex} indicates that the exchange process is always of first order, and it will remain so provided tracer quantities are employed. In practice, it means that the plot of $\log(1-F)$ versus time will be a straight line, with a slope proportional to the exchange velocity, υ_{ex} (Purich & Allison, 1980).

The above method is practical when one has to determine the rate of exchange after a considerable approach to equilibrium. Another approach is to measure the initial rate of exchange directly by sampling the first 10% of the reaction, which is then reasonably linear. Then, for analysis, one plots the amount of labeled product formed against time, and the slope of the line is equal to the initial velocity.

16.2.5 Determination of Equilibrium Constants

In order to establish accurate concentrations for estimating an isotope exchange rate, one must have an accurate value for the reaction *equilibrium constant.*

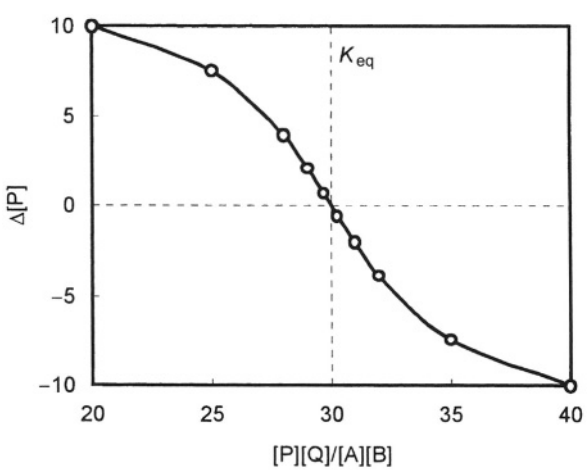


Figure 2. Determination of an equilibrium constant for a two-substrates, two-products reaction.

This is necessary not only for estimating $[\mathbf{X}]_E$ and $[\mathbf{Y}]_E$ in Eq. (16.23), but also for manipulating the experimental conditions. Various methods for precise estimation of equilibrium constants are described in the literature (Allison & Purich, 1979; Purich & Allison, 1980; Alberty, 1994).

A rapid method for the estimation of K_{eq} is to set up reaction mixtures containing known concentrations of all reactants at some ratio close to K_{eq}, and thus bracket the equilibrium position by varying the concentration of one reactant. After the addition of enzyme to each mixture, the change in concentration of one reactant is measured, and the change is plotted against the [product]/[reactant] ratio. The change in concentration of a chosen reactant can be positive or negative and where the line in the plot crosses zero, the [product]/[reactant] ratio equals K_{eq} (Fig. 2).

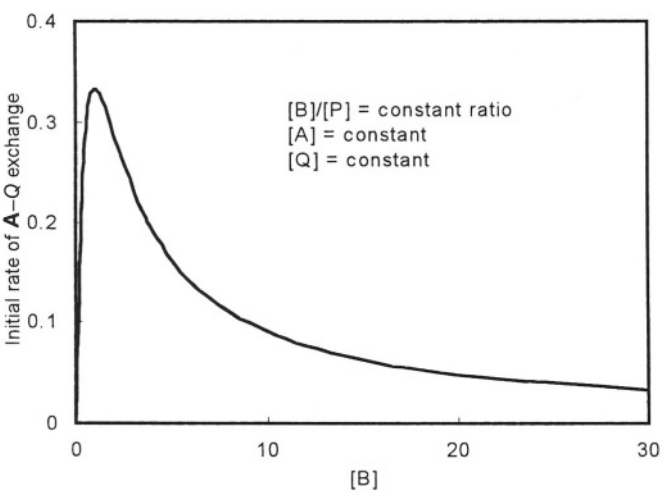
16.2.6 Graphical Presentation of Rate Equations

A–Q *exchange in an Ordered Bi Bi mechanism*

The rate equation for the **A**–Q exchange in an Ordered Bi Bi mechanism is given by Eq. (16.10). As the numerator of this equation is proportional to *B,* whereas the denominator is a quadratic in *B,* it is a rational polynomial of the order 1:2 in *B.* Provided *A* and *Q* are kept constant, Eq. (16.10) can be written in the following form:

$$v_{A-Q} = \frac{\text{constant}_1 B}{\text{constant}_2 + \text{constant}_3 B + \text{constant}_4 B^2} \qquad (16.24)$$

On the other hand, if *B* and *P* are kept constant, Eq. (16.10) is a rational polynomial of the order 1:1 in *A*, and can be written in the form:



$$v_{A-Q} = \frac{B}{1+B+B^2}$$

Figure 3. **A**–Q exchange in an Ordered Bi Bi system. Initial rate of **A**–Q exchange is drawn according to Eq. (16.24) assuming that all the constants are equal to unity.

$$v_{A-Q} = \frac{\text{constant}_5 A}{\text{constant}_6 + \text{constant}_7 A} \tag{16.25}$$

Let us first examine the effect of increasing B and P in constant ratio at some given values of A and Q. As B and P are increased from zero to saturation, the rate of exchange increases to a maximum and then decreases back to zero (Fig. 3).

This is an expected result, since Eq. (16,24) has the same form as the equation for the simple substrate inhibition (Chapter 11).

On the other hand, when the concentration of a substrate pair A/Q is increased in a constant ratio, keeping B and P constant, the rate of **A**–Q exchange increases in a hyperbolic fashion (Fig. 4). This is understandable, since Eq. (16.25) has a form of a usual hyperbola.

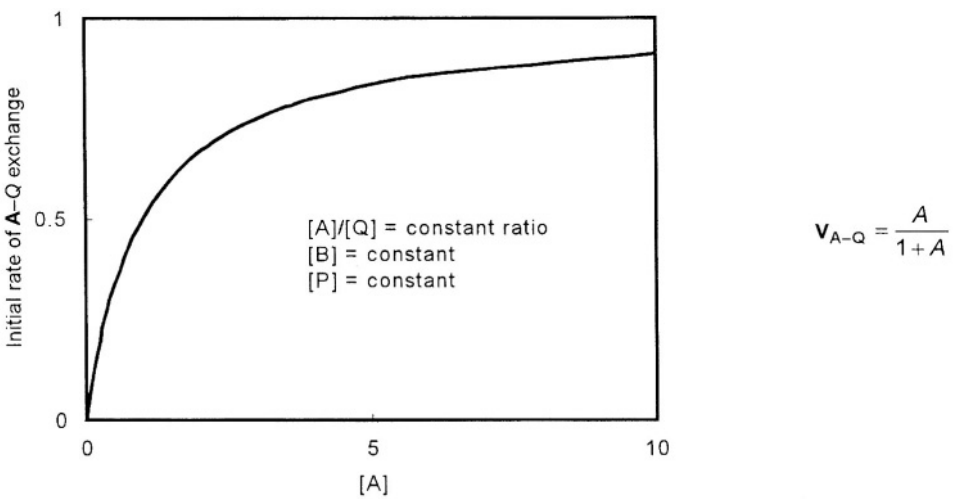


Figure 4. **A**–Q exchange in an Ordered Bi Bi system. Initial rate of **A**–Q exchange is drawn according to Eq. (16.25) assuming that all the constants are equal unity.

A–P *exchange in an Ordered Bi Bi mechanism*

From Table 2, the rate equation for the **A**–P exchange in an Ordered Bi Bi mechanism is

$$v_{A-P} = \frac{V_1 AB}{(K_{iA}K_B + K_A B)\left[1 + \left(\frac{A}{K_{iA}}\right)\left(1 + \frac{B}{K_{eB}}\right) + \frac{Q}{K_{iQ}}\right]} \tag{16.26}$$

This time, we shall represent the velocity equation in the form of double reciprocal plots when either the reactant pair A–Q or the reactant pair B–P is varied in constant ratio. If the concentration of the B–P pair is constant, and the concentration of the A–Q pair is varied, the double reciprocal plot is linear; on the other hand, if the concentration of the A–Q pair is constant, and B–P pair

is varied, the double reciprocal plot has a shape typical for substrate inhibition (Fig. 5).

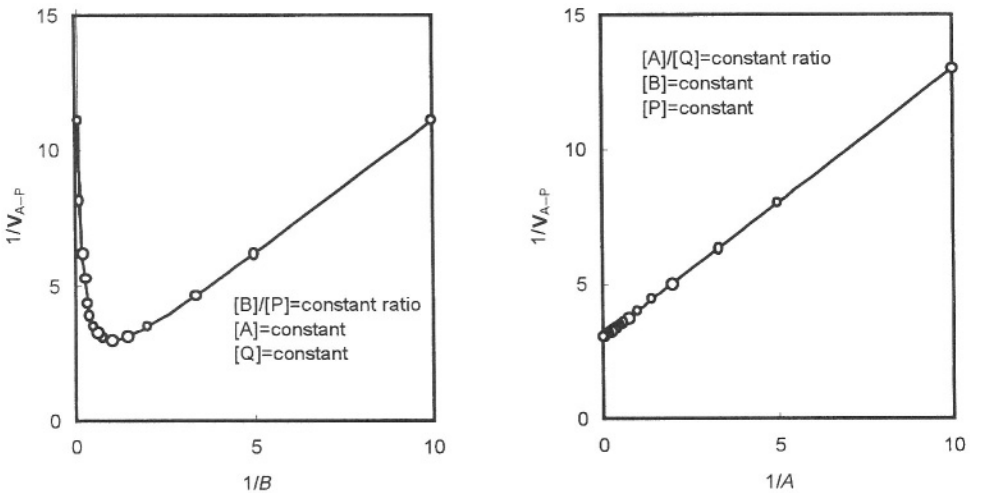


Figure 5. Double reciprocal plots for the A–P isotope exchange in an Ordered Bi Bi mechanism. The data points are drawn according to Eq. (16.26) assuming that all the constants are equal unity.

16.2.7 Types of Exchange-Rate Profiles

Equations given in Table 2 are relatively long and complex by comparison to initial-rate expressions. It is generally cumbersome, if not completely impractical, to obtain kinetic constants for each step from isotope exchange data; instead, one deals with the equations to obtain qualitative patterns of rate behavior.

The most frequently used method deals with the effect of raising the concentration of a particular substrate pair and then observing the effect on the rate of isotopic exchange. One can inspect the direct plots, as in Figs. 3 and 4, or can inspect the double reciprocal plots as in Fig. 5. In each case, we are looking for the shape of the exchange-rate profiles, and for the limiting values of functions at infinitely high concentration of variable reactants.

Usually, one can see by inspection which equations will have the various shapes. In complex cases, one may invoke the ancient l'Hopital's rule to determine the limits (de l'Hopital, 1696). Hospital's rule states that the limiting value of the quotient is equal to the limiting value of its first derivative; for example, for Eq. (16.24)

$$v_{\substack{\text{A-Q} \\ \text{limiting B-P}}} = \lim_{B\to\infty} \frac{f(B)}{g(B)} = \lim_{B\to\infty} \frac{f'(B)}{g'(B)} = \lim_{B\to\infty} \left(\frac{1}{B}\right) = 0 \qquad (16.27)$$

In isotope exchange studies, if we are plotting v_{ex} versus a substrate–product pair, we can encounter a number of types of exchange rate profiles, including hyperbolic (H), or sigmoidal (S) plots with no, partial or complete depression of the exchange rate (Fig. 6).

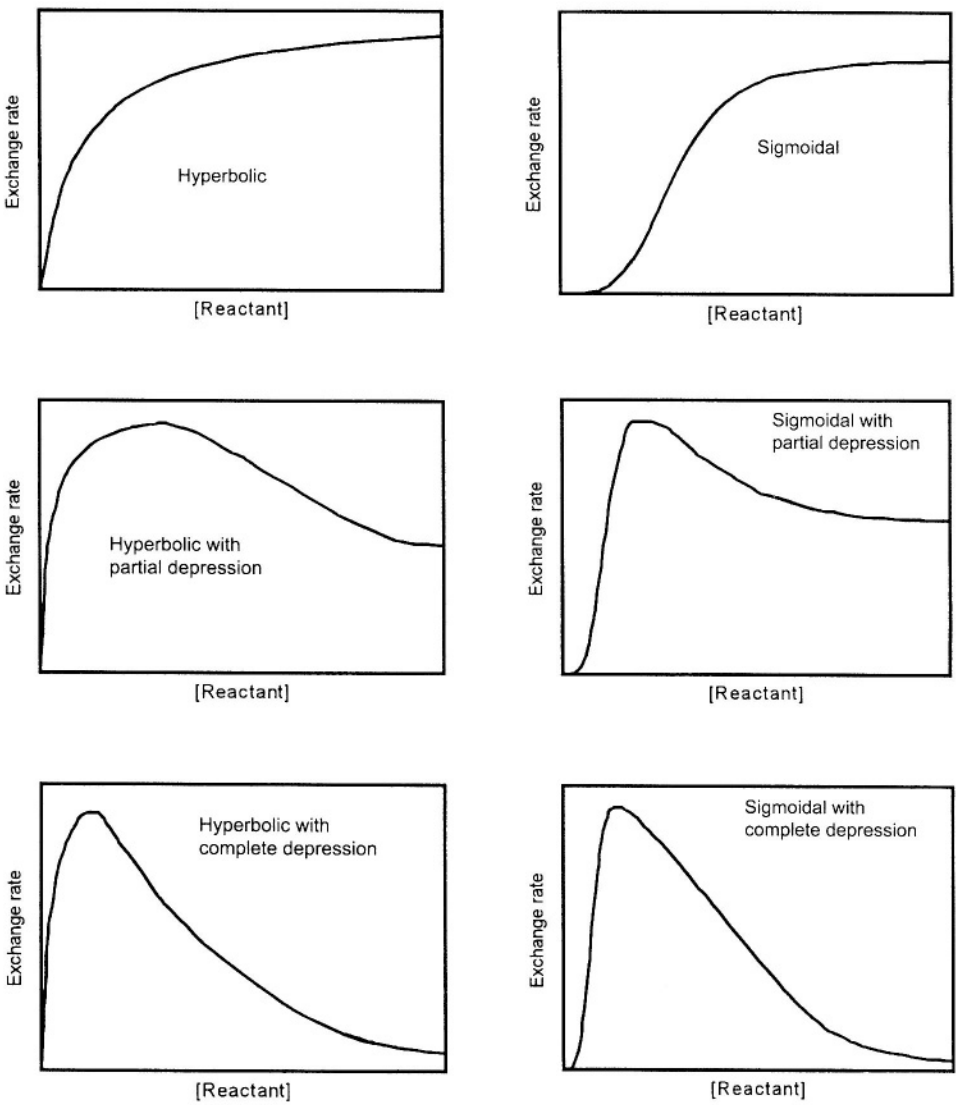


Figure 6. Types of exchange-rate profiles. Hyperbolic (H); hyperbolic with complete depression (HCD); hyperbolic with partial depression (HPD); sigmoidal (S); sigmoidal with complete depression (SCD); sigmoidal with partial depression (SPD).

Most isotopic exchange studies are done between A and Q, or B and P, and not with A and P; they usually afford exchange-rate profiles that are hyperbolic or

hyperbolic with complete depression. By definition, exchanges between B and Q are not possible.

Sigmoidal profiles are rarely encountered in practice. Such profiles are obtained only if the concentrations of all substrates and products are changed simultaneously in a constant ratio; such experimental protocols are rarely employed in practice (Wedler & Boyer, 1972).

The expected exchange-rate profiles for the sequential systems defined in Table 2 are explicitly described in Table 3. These do not take into account abortive complex effects, which are described later.

Table 3. Exchange profiles for sequential kinetic mechanisms

		Varied substrate–complex pair			
Mechanisms	Exchange	A–P	B–P	A–Q	B–Q
Uni Uni	A–P	H	—	—	—
Ordered Bi Bi	A–P	H	HCD	H	HCD
	B–P	H	H	H	H
	A–Q	HCD	HCD	H	HCD
Rapid Equilibrium Random Bi Bi with dead-end complex EBQ	Any	H	H	H	HCD
Rapid Equilibrium Random Bi Bi with a dead-end EBQ and EAP	Any	HCD	H	H	HCD
Random Bi Bi	Any	H	H	H	H
Random on — Ordered off Bi Bi	A–P	H	H	H	H
	B–P	H	H	H	H
	A–Q	HCD	HCD	H	H

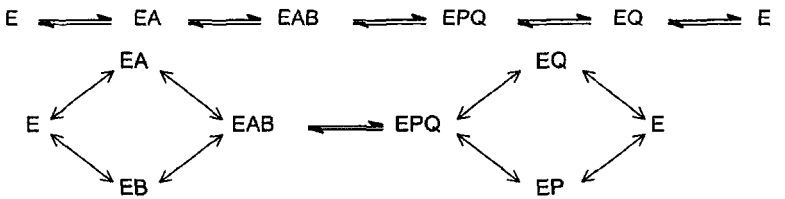
Types of exchange-rate profiles: H = hyperbolic; HCD = hyperbolic with complete depression.

16.3 SPECIFIC EXAMPLES OF SEQUENTIAL MECHANISMS

One problem with isotope exchange studies is that there are very few general rules about isotope exchange, so that one has the impression of an avalanche of arbitrarily chosen special cases. Therefore, it is useful to survey the most common cases in a qualitative fashion.

16.3.1 Qualitative Difference Between an Ordered Bi Bi and a Random Bi Bi Mechanism

The most common application of exchange experiments is the determination of the order of substrate binding. Let us examine the usual Bi Bi mechanism, which can proceed via an ordered or a random pathway.



(16.28)

The $\mathbf{A} \leftrightarrow \mathbf{Q}$ exchange velocity, as a function of the absolute levels of the [B]/[P] pair, is the diagnostic for an ordered mechanism, where one substrate, A, must precede the other's binding and one product, P, must precede the other product's release.

As the [B]/[P] pair is raised, the central complexes become the favored enzyme species, and the uncomplexed enzyme form becomes scarce. Since the uncomplexed enzyme form is required for **A** (a labeled substrate) to be converted to **Q** (a labeled product), and vice versa, the $\mathbf{A} \leftrightarrow \mathbf{Q}$ exchange will increase as we go from low to moderate levels of the [B]/[P] pair, but the $\mathbf{A} \leftrightarrow \mathbf{Q}$ exchange will be depressed at high [B]/[P] absolute levels. The $\mathbf{A} \leftrightarrow \mathbf{Q}$ exchange will not be depressed at high [A]/[Q] absolute levels since increasing [A] and [Q] concentrations favor the binding of **A** or **Q**. Thus, raising the [A]/[Q] or [B]/[P] pair will result in an increased $\mathbf{B} \leftrightarrow \mathbf{P}$ exchange in a hyperbolic fashion, consistent with the rate saturation kinetics. In summary, the depression of the $\mathbf{A} \leftrightarrow \mathbf{Q}$ exchange at high [B]/[P] serves to identify the ordered mechanism.

On the other hand, in the random mechanism, we have alternative routes for exchange processes, and no exchange will be inhibited by high levels of any substrate–product pair. This is true, since **A** may bind to E and EB, and raising the [B]/[P] pair will not inhibit the combination of **A** to the EB species. Because the mechanism is fully symmetrical, a similar statement is true for all exchanges.

16.3.2 Ordered Bi Bi Mechanism

In an Ordered Bi Bi mechanism, we can examine the $\mathbf{A} \rightarrow \mathbf{Q}$, $\mathbf{B} \rightarrow \mathbf{P}$, or the $\mathbf{A} \rightarrow \mathbf{P}$ exchange.

In an $\mathbf{A} \rightarrow \mathbf{Q}$ exchange, as the concentrations of B and P are raised, the exchange velocity increases at first simply because B is needed for the reaction in which part of A becomes part of Q. The E**A** that is formed must react with B in order to pass thorough the central complexes and form E**Q**, which liberates the labeled product. However, after a certain point, a further increase in [B] and [P] causes the exchange velocity to decrease. The reason for this substrate inhibition is as follows. The rate at which labeled Q appears depends on the level of E**Q**, that is, $\mathbf{v_o} = k_X[\mathbf{EQ}]$; consequently, and since the system is at equilibrium, as [P] increases, the level of E**Q** decreases. At saturating [P], the level of E**Q** is zero and total substrate inhibition is observed. Similarly, saturating [B] drives all the E and EA into the central complexes. If no E is present, there is nothing for **A** to react with, and we observe again a total substrate inhibition.

Although varying [A] and [Q] together and [B] and [P] together gives all the information required to determine the reaction sequence, varying [A] and [P]

together and [B] and [Q] together can be used to confirm the presence or absence of possible dead-end complexes.

Cleland (1970) has formulated a simple rule by which one can predict whether substrate inhibition will be obtained in reactions with no alternate sequences: "Total substrate inhibition is observed if either of the varied reactants (of a pair) adds between the points of combination of the exchange reactants, and both varied reactants do not add to the same stable enzyme form".

16.3.3 Random Bi Bi

One of the major uses of isotope exchange studies is in discriminating between Ordered Bi Bi and Random Bi Bi mechanisms. In a Random Bi Bi sequence, with no preferred pathway, the $\mathbf{A} \rightarrow \mathrm{Q}$ or $\mathbf{Q} \rightarrow \mathrm{A}$ exchange increases in a hyperbolic fashion as [B] and [P] are increased. Similarly, $\mathbf{B} \rightarrow \mathrm{P}$ and $\mathbf{P} \rightarrow \mathrm{B}$ exchange shows normal hyperbolic kinetics. Saturating [B] and [P], in reaction (16.28), simply forces all the A–Q flux through the lower pathway, and saturating [A] and [Q] forces all the flux through the upper pathway. Thus, the exchange pair become the inner pair of an Ordered Bi Bi system when the concentrations of the varied pair are infinitely high.

Isotope exchange is the only way to determine whether a random system is a rapid equilibrium one. If the conversion of the central complexes is the sole rate-limiting step, then all exchanges in a given direction must proceed at the same rate at any given set of reactant concentrations. Therefore, $\boldsymbol{v}_{\mathrm{A-Q}} = \boldsymbol{v}_{\mathrm{A-P}} = \boldsymbol{v}_{\mathrm{B-P}}$, and $\boldsymbol{v}_{\mathrm{Q-A}} = \boldsymbol{v}_{\mathrm{P-A}} = \boldsymbol{v}_{\mathrm{P-B}}$. The equality of exchange rates holds even if dead-end complexes form.

Isotope exchange can also be useful in detecting partially random segments in what otherwise appears to be an ordered sequence; this situation appears frequently in enzyme kinetics. For example, if increasing [B] and [P] in an Ordered Bi Bi mechanism yields significant substrate inhibition, but the exchange velocity cannot be driven to zero, that is, the plot intersects the $1/\mathbf{v}_0$-axis at a finite value, then we may suspect that some degree of randomness is present.

16.4 SEQUENTIAL MECHANISMS AWAY FROM EQUILIBRIUM

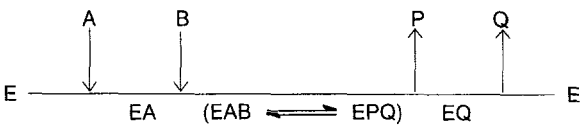
Although most exchange studies have been designed for processes at equilibrium, the back exchange of labeled product while the reaction is proceeding in the forward direction can provide valuable information about catalysis. Under favorable conditions, investigators may utilize such isotope exchange data to learn about the order of product release and presence of covalent enzyme–substrate compounds. However, the derivation of rate equations for isotope exchange in such cases are rather complex, and usually not of practical utility (Hass & Byrne, 1960; Purich & Allison, 1980, 2000; Nordlie, 1982).

The derivation of rate equations for isotope exchange away from equilibrium may be understood in terms of Eq. (16.7) for the Ordered Bi Bi mechanism

(Section 16.2.1). Equation (16.7) contains rate constants, the concentration of labeled reactant, the concentration of unlabeled substrates, and the enzyme form the labeled reactant reacts with. The concentration of this enzyme form must be expressed in terms of rate constants and the concentration of reactants. In chemical equilibrium, this expression is relatively simple (Eq. (16.8)). Under the steady-state conditions, when the concentration of reactants is away from equilibrium, this enzyme form must be replaced from the steady-state distribution equation, which is usually more complex (Eq. (9.13)). Therefore, the resulting velocity equations for isotope exchange away from equilibrium are usually more complex and, consequently, their practical application becomes cumbersome.

Nevertheless, isotopic exchange under nonequilibrium conditions were employed to establish the order of product release or the order of substrate addition in sequential mechanisms.

For example, consider an Ordered Bi Bi system:



Label from P can appear in the corresponding substrate even in the absence of Q. All that is needed is the presence of a sufficient level of EQ in the steady state. The exchange of label from Q into a substrate will not occur unless a significant concentration of P is present. Thus, one can test both products separately at levels at or above their inhibition constants, and establish the order of product release. Thus, the order of product release can be established: the first product released is that which exchanges with a substrate in the absence of the another product, and the second product released is that which does not. If both products show exchange, than their release must be random.

If the reaction is freely reversible, one can test in the same way the order of addition of substrates on the substrate side of reaction. One of the first systems to be characterized in this way was glucose-6-phosphatase (Hass & Byrne, 1960).

A more complex case was described by Nandi (1978). δ-Aminolevulinic acid synthase from *Rhodopseudomonas spheroides* is a pyridoxal phosphate-dependent enzyme that catalyzes the formation of δ-aminolevulinic acid from glycine and succinyl coenzyme A; in this case, the release of products is random and more than one product can exchange back into substrate.

16.5 ISOTOPE EXCHANGE IN PING PONG MECHANISMS

16.5.1 Rate Equations for Ping Pong Mechanisms

Equations for the initial rate of isotope exchange in Ping Pong mechanisms may be derived by one of the methods described in Chapter 4; the usual procedure will be to apply the King–Altman method or the net rate constant method (Cleland, 1975) (Table 4).

Table 4. Isotope exchange-rate expressions in Ping Pong mechanisms

Ping Pong Bi Bi mechanism

$$v_{A\leftrightarrow Q} = \frac{V_1 AB}{\left(K_A B + \frac{K_{iA}K_B P}{K_{iP}}\right)\left(1 + \frac{A}{K_{iA}} + \frac{Q}{K_{iQ}} + \frac{K_{iP}A}{K_{iA}P}\right)}$$

$$v_{A\leftrightarrow P} = \frac{V_1 A}{K_A\left(1 + \frac{A}{K_{iA}} + \frac{K_{iP}A}{K_{iA}P} + \frac{Q}{K_{iQ}}\right)}$$

$$v_{B\leftrightarrow Q} = \frac{V_1 B}{K_B\left(1 + \frac{B}{K_{iB}} + \frac{P}{K_{iP}} + \frac{K_{iQ}B}{K_{iB}Q}\right)}$$

Bi Bi Uni Uni Ping Pong Ter Ter mechanism
(Rate equation in the absence of C and R)

$$v_{A\leftrightarrow Q} = \frac{V_1 AB}{\left(K_{iA}K_B + K_A B + \frac{K_{iA}K_B K_Q P}{K_P K_{iQ}}\right)\left(1 + \frac{A}{K_{iA}} + \frac{AB}{K_{iA}K_{iB}} + \frac{ABK_{iP}}{K_{iA}K_{iB}P} + \frac{ABK_{iP}K_{iQ}}{K_{iA}K_{iB}PQ}\right)}$$

The last equation in Table 4 for the Bi Bi Uni Uni Ping Pong mechanism (in the absence of C and R) is relatively long and complex. The right-hand portion of the denominator in this equation represents the reciprocal concentration of free enzyme in chemical equilibrium multiplied by $[E_o]$. If the products C and R are present, the distribution equation for $[E]/[E_o]$ would be more complex (Eq. (12.73)); consequently, the resulting full rate equation for the **A** → Q isotope exchange becomes rather cumbersome.

Table 5. Exchange profiles for Ping Pong kinetic mechanisms

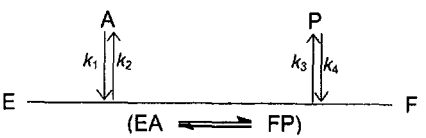
		Varied substrate–complex pair			
Mechanisms	Exchange	A–P	B–P	A–Q	B–Q
Ping Pong Bi Bi	A–P	H	H	H	HCD
	B–Q	HCD	H	H	H
	A–Q	HCD	H	H	HCD
Bi Bi Uni Uni Ping Pong Ter Ter (in the absence of C and R)	A–P	H	HCD	H	HCD
	B–P	H	H	H	H
	A–Q	HCD	HCD	H	HCD

Types of exchange profiles: H = hyperbolic; HCD = hyperbolic with complete depression.

Thus, due to the complexity of rate equations, the most frequently used method for measuring exchange rates deals with the effect of raising the concentration of a particular substrate pair and then observing the effect on rate exchange. In each case, we are looking for the shape of the exchange-rate profiles, and for the limiting values of functions at infinitely high concentration of variable reactants. Usually, one can see by inspection which equations will have the various shapes (Table 5).

16.5.2 Partial Ping Pong Mechanism

Isotope exchange is a powerful method for probing Ping Pong systems, because isolated portions or the partial reactions of the overall reaction sequence can be studied. For example, in a Ping Pong Bi Bi system, the $\mathbf{A} \rightarrow \mathbf{P}$ and $\mathbf{P} \rightarrow \mathbf{A}$ exchange can be measured in the absence of B and Q:



(16.29)

Almost immediately after mixing E, A, and P, the system will come to equilibrium at essentially the established [P]/[A] ratio. If no exchange takes place between the suspected A and P pair unless all reactants are present, then the system is sequential.

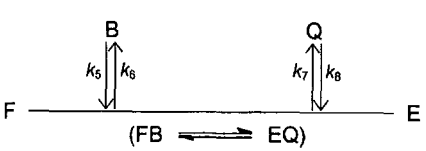
The exchange velocity equations for an isolated portion of a Ping Pong Bi Bi system are relatively simple. Thus, the initial rate of isotope exchange, v_0, for an $\mathbf{A} \rightarrow \mathbf{P}$ exchange is given by

$$v_0 = \frac{V_{\max A-P} \cdot AP}{K_{iA}P + K_{iP}A + AP} \tag{16.30}$$

where

$$V_{\max A-P} = \frac{V_1 K_{iA}}{K_A} = \frac{V_2 K_{iP}}{K_P} = \frac{k_2 k_3}{k_2 + k_3} \tag{16.31}$$

Similarly, when E, B, and Q are mixed, in the absence of A and P, the other partial reaction is automatically established at chemical equilibrium:



(16.32)

Again, if no exchange takes place between the A–P and B–Q pairs unless all reactants are present, then the system is probably sequential.

The initial rate of isotope exchange, v_o, for an $\mathbf{B}\rightarrow\mathbf{Q}$ exchange is given by

$$v_o = \frac{V_{\max\ B-Q} \cdot BQ}{K_{iB}Q + K_{iQ}B + BQ} \tag{16.33}$$

where

$$V_{\max\ B-Q} = \frac{V_1 K_{iB}}{K_B} = \frac{V_2 K_{iQ}}{K_Q} = \frac{k_6 k_7}{k_6 + k_7} \tag{16.34}$$

The critical test for the Ping Pong mechanism is the relationship between the maximum chemical velocities and the maximum exchange velocities:

$$\frac{1}{V_{\max\ A-P}} + \frac{1}{V_{\max\ B-Q}} = \frac{1}{V_1} + \frac{1}{V_2} \tag{16.35}$$

Equation (16.35) is derived from expressions for maximal chemical velocities (Eq. (9.9)) and from Eqs. (16.31) and (16.34); thus, Eq. (16.35) can be written in the rate constant form:

$$\frac{k_2 + k_3}{k_2 k_3} + \frac{k_6 + k_7}{k_6 k_7} = \frac{k_3 + k_7}{k_3 k_7} + \frac{k_2 + k_6}{k_2 k_6} \tag{16.36}$$

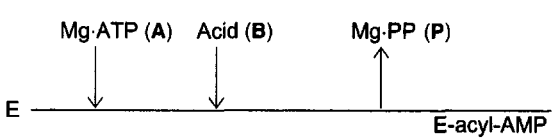
Equation (16.35) is the critical test for this mechanism and it *must* hold for the mechanism to be Ping Pong.

16.5.3 Determination of the Order of Addition of Reactants in Ter Ter Ping Pong Mechanisms

One of the most useful applications of isotope exchange studies comes in determining the order of addition of substrates in trisubstrate Ping Pong mechanisms. The best way to understand this problem is in relation to a specific example. Consider, for example, the reaction catalyzed by a large number of synthetases (Walsh, 1998):

where R · COOH is a carboxylic or an amino acid, and a true substrate is Mg · ATP.

The reaction sequence can be depicted as follows:



(16.38)

This reaction sequence is a Bi Uni segment of the trisubstrate Ping Pong mechanism, the Bi Uni Uni Bi Ping Pong Ter Ter mechanism. The kinetic

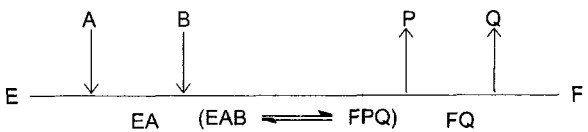
Table 6. ATP–PP_i exchange in reactions catalyzed by synthetases. The Bi Uni segment of the Bi Uni Uni Bi Ping Pong Ter Ter mechanism

Patterns	Order of addition of substrates	Primary reciprocal plot of $1/v_0$ versus 1/[ATP] at constant [PP_i]	Secondary plots	Conclusion
First	ATP adds before acid in steady-state ordered fashion	Intersecting	Uncompetitive substrate inhibition by the acid	ATP is identified as substrate A and acid as substrate B
Second	Addition of ATP and acid is ordered but in rapid equilibrium	Intersecting	No substrate inhibition by acid is observed; the replot of slopes versus 1/[acid] goes through the origin	The same
Third	Acid adds before ATP	Equilibrium ordered crossing on the vertical axis	—	Acid is identified as substrate A and ATP as substrate B
Fourth	Addition of reactants is random	Intersecting	No substrate inhibition observed.	Random addition of substrates

question is whether Mg · ATP and acid add in a random or in a sequential order, and in the latter case whether Mg · ATP adds first or the second (Cleland, 1977).

In this case, one can easily measure the PP_i–ATP exchange; if one varies the concentration of Mg · ATP at different fixed levels of acid, and with PP_i held constant, there are four patterns that can be observed (Table 6). Thus by measuring the exchange of product P with its corresponding substrate, one can tell whether the pattern is random or ordered, and, if ordered, whether P picks up label from substrate A or B. This method was applied initially in asparagine synthetase reaction (Cedar & Schwartz, 1969) and the corresponding equations were written in detail (Santi *et al.,* 1974).

The above analysis can also be applied to the Bi Bi segment of a trisubstrate Ping Pong mechanism, the Bi Bi Uni Uni Ping Pong Ter Ter mechanism:



(16.39)

In the same way, as in the preceding case, one can identify the order of addition of substrates and the identity of substrates A and B, respectively (Segel, 1975).

16.6 ABORTIVE COMPLEX FORMATION

A number of enzymes may form abortive complexes that are nonproductive forms of the enzyme. Such complexes appear as a result of the adsorption of ligands under conditions where the enzyme may not carry out its usual chemistry. For example, the binding of NAD^+ and acetaldehyde to alcohol dehydrogenase leads to the formation of an enzyme–NAD^+–acetaldehyde complex, which cannot allow for hydrogen transfer because both ligands are already in their oxidized states.

To detect the presence of abortive ternary complexes, the kineticist may raise the concentration of dissimilar substrate–product pairs (such as glucose–ADP and ATP–glucose-6-P in the hexokinase reaction, or acetaldehyde–$NAD^+$and ethanol–NADH in alcohol dehydrogenase reaction). This will lead to the inhibition of all exchanges irrespective of the kinetic mechanism, and provide useful information about the abortive complex formation (Wong & Hanes, 1964; Wedler & Boyer, 1972; Purich & Allison, 1980, 2000). Nevertheless, product inhibition is still unrivaled as the means for detecting abortive complex formation.

REFERENCES

Books

Fromm, H.J. (1975) *Initial rate enzyme kinetics,* Springer-Verlag, Berlin.
Frost, A.A. & Pearson, R.G. (1961) *Kinetics and mechanism,* 2nd ed., Wiley, New York.
de L'Hopital, G.F. (1696) *Analyse des Infiniment Petits,* Paris.

Purich, D.L. & Allison, R.D. (2000) *Handbook of biochemical kinetics,* Academic Press, New York.
Segel, I.H. (1975) *Enzyme kinetics,* Wiley, New York.
Walsh, C. (1998) *Enzymatic reactions and mechanisms,* W.H. Freeman, San Francisco.

Review articles

Allison, R.D. & Purich, D.L. (1979) *Methods Enzymol. 63,* 3–22.
Boyer, P.D. (1978) *Acc. Chem. Res. 11,* 218–230.
Cleland, W.W. (1967) *Annu. Rev. Biochem. 36,* 77–100.
Cleland, W.W. (1970) *The Enzymes,* 3rd ed., Vol. II, pp. 1–50.
Cleland, W.W. (1979) *Adv. Enzymol. Rel. Areas Mol. Biol. 45,* 273–387.
Huang, C.H. (1979) *Methods Enzymol. 63,* 54–75; 76–84.
Nordlie, R.C. (1982) *Methods Enzymol. 87,* 319–340.
Purich, D.L. & Allison, R.D. (1980) *Methods Enzymol. 64,* 3–46.
Santi, D.V., Webster, R.W., & Cleland, W.W. (1974) *Methods Enzymol. 29,* 620–627.
Wedler, F.C. & Shalango, W.H. (1982) *Methods Enzymol. 87,* 647–711.
Wedler, F.C. (1995) *Methods Enzymol. 249,* 443–478.

Specific references

Alberty, R.A. (1994) *Biochim. Biophys. Acta 1207,* 1–11.
Alberty, R.A., Bloomfield, V., Peller, A., & L. King, E.L. (1962) *J. Am. Chem. Soc. 84,* 4381–4390.
Allison, R.D., Todhunter, J.A., & Purich, D.L. (1977) *J. Biol. Chem. 252,* 6046–6056.
Boyer, P.D. (1959) *Arch. Biochem. Biophys. 82,* 387–397.
Boyer, P.D. & Silverstein, E. (1963) *Acta Chem. Scand. 17,* 195–205.
Boyer, P.D. & Silverstein, E. (1964) *J. Biol Chem. 239,* 3901–3910.
Cedar, H. & Schwartz, J.H. (1969) *J. Biol. Chem. 244,* 4122–4127.
Cleland, W.W. (1975) *Biochemistry 14,* 3220–3230.
Darvey, I.G. (1973) *J. Theor. Biol. 42,* 55–62.
Fromm, H.J., Silverstein, E., & Boyer, P.D. (1964) *J. Biol. Chem. 239,* 3645–3655
Hass, L.F. & Byrne, W.L. (1960) *J. Am. Chem. Soc. 82,* 947–957.
Morales, M.F., Horrovitz, M., & Botts, J. (1962) *Arch. Biochem. Biophys. 99,* 6046–6056.
Nandi, D.L. (1978) *J. Biol Chem. 253,* 8872–8887.
Simon, W.A. & Hoffer, H.W. (1978) *Eur. J. Biochem. 88,* 175–181.
Wedler, F.C. & Boyer, P.D. (1972) *J. Biol. Chem. 247,* 984–994.
Wong, T.F. & Hanes, C.S. (1964) *Nature 203,* 492–496.

This page intentionally left blank

Chapter 17

Kinetic Isotope Effects

Kinetic studies of reactions in which isotopic substitutions have been made in the reacting molecules can provide insight into the molecular details of the reaction mechanism. The most important property of a chemical bond that is being broken in a chemical reaction for kinetic isotope effects is the mass of the two atoms joined together covalently and the force constant that defines it. Therefore, substitution of deuterium (^{2}H) or tritium (^{3}H) for protium (^{1}H) produces much larger rate changes than isotopic substitution of heavier elements, which is why hydrogen isotopes have been primarily used in kinetic studies. The heavy isotopes (^{13}C, ^{15}N, and ^{18}O) can be very useful, however, for analysis of kinetic and chemical mechanisms, since the precision of mass-spectrometric isotope ratios is very high.

Isotope effects can be divided into three categories: primary, secondary, and solvent. Primary isotope effects are ones where bonds to the isotopic atom are made or broken during reaction. Secondary ones involve isotopic atoms where bonding changes during reaction, but no bonds are made or broken to them. Isotope effects can be normal or inverse; normal isotope effects are ones where the rate is slower with the heavy isotope and inverse ones show faster rates with the heavier atom. Solvent isotope effects result from running reactions in heavy water; solvent isotope effects may also be primary and secondary.

17.1 ORIGIN OF ISOTOPE EFFECTS

17.1.1 Kinetic Isotope Effects of Deuterium

Primary isotope rate effects are due to the fact that bonds involving deuterium have smaller zero-point energy than the analogous bonds involving hydrogen. A rigorous theoretical explanation of kinetic isotope effects is a complex and difficult task, as discussed by Melander & Saunders (1980); a less rigorous treatment is given by other authors (Jencks, 1969; O'Leary & Kleutz, 1972; Cleland, 1977; Klinman, 1978; Moore & Pearson, 1981; Atkins & de Paula, 2002). Here, we shall limit ourselves to an oversimplification that seems to have practical and productive utility; this simplification is the argument that (qualitatively and quantitatively) the most important contribution to kinetic isotope effects is the difference in vibrational zero-point energies of bonds to hydrogen versus bonds to deuterium.

Consider, for example, the vibrational energy levels for a C–H bond. They are, of course, quantized, and the lowest level (zero-point) has a nonzero energy $E = (1/2)h\nu$, where ν is the frequency of the C–H vibration. If we further assume

this bond to be represented by a simple harmonic oscillator, then we can envision Hooke's law relationship for ν, such that $\nu = (1/2)(K/M)^{1/2}$, where K is a simple force constant, and M is the reduced mass of the system (here, the isolated C–H bond). The infrared stretching frequencies of C–H and C–D bonds in many compounds cluster at 2,900 and 2,100 cm^{-1}. These values convert to zero-point energies of 4.15 kcal/mol for a C–H and 3.0 kcal/mol for a C–D bond. Thus, the zero-point energy for the C–D bond is 1.15 kcal/mol lower, just estimating for one vibrational stretch.

Consider a chemical reaction in which the C–H or C–D bond is broken in the transition state, that is, where this stretching vibrational frequency becomes the reaction coordinate motion and has an imaginary frequency in the transition state. In this case, the same transition state is reached for the C–H or for the C–D molecule. However, because C–D bond is at lower zero-point energy, deeper in the potential well than the C–H bond, more energy will be required to bring C–D to the transition state than for C–H, a difference of 1.15 kcal/mol due just to the change in zero-point energy alone (Fig. 1).

At room temperature, we can calculate that this energy difference will show up as a 7-fold rate difference in favor of C–H (Westeheimer, 1961; Jencks, 1969). In other words, for this effect

$$\frac{k_H}{k_D} \simeq 7 \tag{17.1}$$

Strictly speaking, one must worry about the smaller mass of C–H than of C–D (or N–H, O–H, or S–H than N–D, O–D, or S–D) combinations. However, the masses of C, N, O, and S are so much greater than the masses of either H or D, that the heavier atom remain essentially fixed in the vibration, the force constants are about the same, and so the mass difference between H and D is paramount in

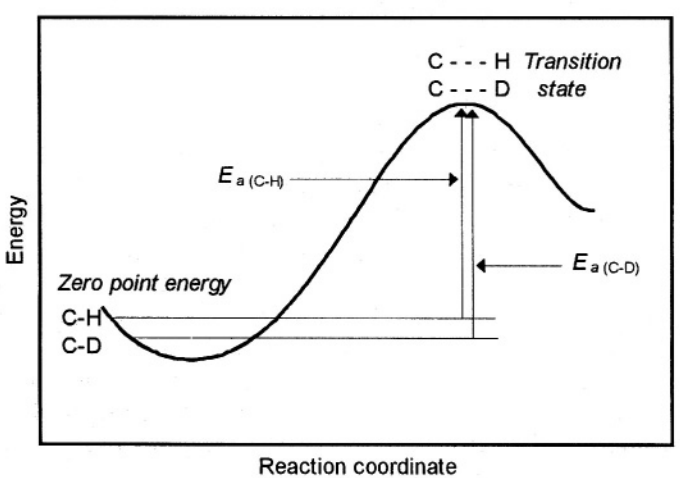


Figure 1. Schematic representation of a primary isotope effect for C–H and C–D bonds, where E_a is the activation energy.

determining the frequency of such vibrations. In an analogous calculation, one can show that O–H versus O–D differences in zero-point energies should correspond to a 10-fold rate enhancement for O–H versus O–D reacting at room temperature (Jencks, 1969).

Isotopic substitution in reacting positions in molecules may affect the rates of reactions, but they can also affect the equilibrium of reactions.

17.1.2 The Theory of Equilibrium Isotope Effect

Isotope effects on equilibrium constants can be understood on the basis of statistical thermodynamics (Sühnel & Schowen, 1991). If the hydrogen is substituted by deuterium, the overall equilibria are

$$C + AH \underset{}{\overset{K_H}{\rightleftharpoons}} BH \qquad C + AD \underset{}{\overset{K_D}{\rightleftharpoons}} BD \tag{17.2}$$

The isotope effect expressed as the ratio of equilibrium constants, K_H/K_D, is a ratio of molecular partition functions for reactants and products:

$$\frac{K_H}{K_D} = \frac{\left(Q^H_{product}/Q^H_{reactant}\right)}{\left(Q^D_{product}/Q^D_{reactant}\right)} \tag{17.3}$$

The molecular partition functions, Q, can be related to molecular properties of reactants and products. The partition function expresses the probability of encountering a molecule, so that the ratio of partition functions for the products versus the reactants of a chemical reaction expresses the relative probability of encountering products versus reactants and, therefore, the equilibrium constant. The partition function can be written as a product of independent factors at the level of various approximations, each of which is related to the molecular mass, the principal moments of inertia, the normal vibration frequency, and the electronic energy levels, respectively. When the ratio of isotopic partition function is calculated, the electronic part of the partition function cancels, at the level of the Born–Oppenheimer approximation, an approximation stating that the motion of nuclei in ordinary molecular vibrations is slow relative to the motions of electrons.

What remains, can be expressed in terms of equilibrium isotope effect:

$$\frac{K_H}{K_D} = \frac{(Q_{BH}/Q_{AH})}{(Q_{BD}/Q_{AD})} = (\mathrm{MMI})(\mathrm{ZPE})(\mathrm{EXC}) \tag{17.4}$$

Without engaging in detailed derivation and development of Eq. (17.4), we can state that the symbols on the right-hand side of equation represent the isotopic ratios of the following functions: MMI is the "mass-moments of inertia", which express the contribution of the isotope effect on the translational and rotational energies; ZPE is the "zero-point energy", which expresses the contribution of the isotope effect on the zero-point energies; and EXC is the "excited vibrational states", which expresses the contribution of the isotope effect on the population of excited vibrational states. Thus, the quantities MMI, ZPE, and EXC, are determined

by the masses of atoms, the position of the atoms in space, and the force field of the molecule (Melander, 1960; Melander & Saunders, 1980; Sühnel & Schowen, 1991).

It is the vibrational effect of isotopic substitution, and for hydrogen isotopes the effect on vibrational zero-point energy that is the predominant source of equilibrium isotope effects. Equilibrium isotope effects can, therefore, be considered to be vibrational effects to give structural information that is closely related to that obtainable by vibrational spectroscopy (Sühnel & Schowen, 1991). Thus, an equilibrium isotope effect, whether primary or secondary, measures the difference in stiffness of bonding of an atom in the reactant and product. That is why the heavier atom enriches in the more stiffly bonded position.

Equilibrium isotope effects are readily measured and can also be calculated if suitable force field is available from vibrational spectroscopy, such as infrared or Raman frequencies.

Like other vibrational properties, isotope effects are local properties reaching out only one or two bond distances. Thus, introduction of deuterium into a C–H bond of an sp^3 carbon center in a steroid molecule, for example, will give the same molecular partition–function ratio as for substitution of deuterium at a similar carbon in nearly all other organic molecules. Therefore, tabulations of partition–function ratios have been constructed in the literature, as values relative to a chosen standard; the standards are usually: water for hydrogen and oxygen, CO_2 for carbon; and ammonia in aqueous solution for nitrogen. These values are generally known as *isotopic fractionation factors,* ϕ, usually defined as the ratio heavy/light, and they can be used to calculate equilibrium isotope effects for reactions (Cleland, 1980; Schowen & Schowen, 1982).

The utility of fractionation factors is that the standard-molecule contribution cancels from a ratio of fractionation factors and the calculation is independent of the arbitrary standard molecule chosen for comparison.

For example, for reaction in which a molecule bearing an isotope label in a site of type X (say, an sp^3 C–H bond) is transferred into a molecule with the label now located in a site Y (say, an sp^2 C–H bond), the equilibrium isotope effect is

$$\frac{K_H}{K_D} = \frac{(Q_{H_x}/Q_{D_x})}{(Q_{H_y}/Q_{D_y})} = \frac{\phi_x}{\phi_y} \tag{17.5}$$

An equilibrium isotope effect can be either normal or inverse. Equilibrium isotope effects in the forward and reverse reactions are inversely related; thus, the isotope effect for the forward reaction is the reciprocal of the isotope effect in the reverse reaction. Equilibrium isotope effects are temperature dependent and go to unity at infinite temperature.

17.1.3 The Transition-State Theory of Kinetic Isotope Effects

Isotope effects on rate constants of reaction can also be understood on the basis of statistical thermodynamics (Sühnel & Schowen, 1991). The basic concept is that a molecular configuration corresponding to the point of maximum energy separating the reactant from the product state can be identified; this configuration can be thought of as a molecular species.

$$\underset{\text{reactant state}}{A} + B \longrightarrow \underset{\text{transition state}}{[T]} \longrightarrow \underset{\text{product state}}{D} + E \tag{17.6}$$

The concentration of T is expressed in terms of a thermodynamic equilibrium constant, $K^{\ddagger}$, for conversion of the reactant-state molecule to the transition-state molecule. According to the transition-state theory (Eyring, 1935), for most cases the rate of chemical reaction is equal:

$$k_L = \left(\frac{k_B T}{h}\right) K_L^{\ddagger} \tag{17.7}$$

where the subscript L stands for H or D. The kinetic isotope effect, in this case, can be again related to molecular properties of reactants and the transition state, as the transition state can be thought of as a molecular species.

$$\frac{k_H}{k_D} = \frac{K_H^{\ddagger}}{K_D^{\ddagger}} = (\text{MMI})(\text{ZPE})(\text{EXC}) \tag{17.8}$$

The symbol MMI has exactly the same meaning as in expression for the equilibrium isotope effect (Eq. (17.4)), but the symbols ZPE and EXC are different. Without engaging in detailed derivation and development of Eq. (17.8), we can state that the contribution of ZPE and EXC are different in the transition state compared to product state, because the transition-state quantity contains contributions from only 3N-7 stable vibrations of the transition-state and not from the single unstable (barrier-crossing) quasi-vibration (Sühnel & Schowen, 1991).

In the transition state, taking into account the thermodynamic formalism, one can again express the isotope effects in terms of fractionation factors. Since, most commonly, a single site is isotopically substituted in both reactant and transition states, the isotope effect is simply the ratio of reactant state to the transition-state fractionation factor.

$$\frac{k_H}{k_D} = \frac{\phi_{\text{reactant}}}{\phi_{\text{transition}}} \tag{17.9}$$

Thus, the kinetic isotope effects reflect the difference in fractionation factor between reactant and transition states.

Part of the isotope effect is temperature dependent, but for the primary isotope effect the reaction coordinate motion effect, which is always normal, is temperature independent. The primary isotope effects are almost always normal. Secondary isotope effects can be either normal or inverse.

Kinetic isotope effects, k_H/k_D, ranging from 2 to 15 are observed in reactions where other evidence suggests that hydrogen transfer occurs in the transition state (Leskovac & Trivić, 1988). Low isotope effect ratios around 2 or less (above 1.5) may mean that the proton-transfer step is only partially rate-limiting (Cleland, 1977). Larger values are seen in some chemical reactions, although an increasing number of enzymatic reactions give large isotope effects; for example, lypoxigenase from soybean gives an isotope effect of 80 (Glickman *et al.,* 1994). If

the ratio $k_H/k_D = 1$ is observed, this observation is diagnostic, indicating that bond breakage is one of the slow steps in the overall reaction.

17.1.4 Hydrogen Tunneling

Larger values ($k_H/k_D = 25–30$) are seen in some chemical (Jencks, 1969; Sühnel & Schowen, 1991) and some enzymatic reactions (Glickman *et al.,* 1994). These large ratios may reflect quantum-mechanical tunneling through potential barriers, occurring selectively for the light isotope of hydrogen.

Semi-classical mechanical treatments of primary kinetic isotope effects require the reactants to surmount an energy barrier in order for them to form products. By contrast, quantum mechanics allows for the phenomenon of tunneling, whereby reactants of insufficient energy to pass over the top of the energy barrier can still be converted to product, provided that the width of the barrier is small. Only particles having de Broglie wavelengths ($\lambda = h/mv$) that are on the same scale as the barrier thickness can "penetrate" the barrier by quantum-mechanical tunneling. Proton and hydride ions have 1–2 Å de Broglie wavelength, and several enzymatic reactions have been demonstrated to exhibit tunneling. In fact, hydrogen probably always tunnels, especially in enzymatic reactions (Klinman, 1991; Bahnson & Klinman, 1995). Tunneling is favored by high, thin potential energy barriers, by low temperature, and by steric hindrance which appears to influence solvent exclusion.

17.2 TRITIUM VERSUS DEUTERIUM KINETIC ISOTOPE EFFECTS

If substitution of deuterium for protium in a chemical reaction produces a detectable kinetic isotope effect, substitution of tritium will produce an even larger kinetic isotope effect (k_H/k_T), because of the larger mass of tritium and the resultant lower zero-point vibrational energy of a C–T relative to C–D and C–H bond. In a number of chemical and enzymatic reactions, a simple logarithmic proportionality is observed, known as a *Swain–Schaad relationship* (Swain *et al.,* 1958):

$$\log\left(\frac{k_H}{k_T}\right) = 1.44 \log\left(\frac{k_H}{k_D}\right) \quad \text{or} \quad \log\left(\frac{k_H}{k_D}\right) = 0.69 \log\left(\frac{k_H}{k_T}\right) \tag{17.10}$$

There is an important experimental distinction between ^2H and ^3H kinetic isotope experiments. Deuterium isotope effects are usually measured by comparing reciprocal plots from separate experiments with deuterium-labeled reactants and with unlabeled reactants, which allows determination of the *V* isotope effect from the ratio of intercepts and the *V/K* one from the ratio of slopes. In contrast to that, tritium isotope effects are measured by the internal competition method, that is, by changes in specific activity in product or residual substrate, and thus provide only the isotope effect on *V/K*. The kinetic isotope effects on *V* with tritium can be measured only with the aid of carrier-free tritiated molecules.

Breakdown of the Swain–Schaad relationship leads to different exponentials in Eq. (17.10) and represents a sign that the rate of the chemical reaction is partially influenced by the quantum mechanical tunneling (Bell, 1980; Devault, 1984; Klinman, 1991; Bahnson & Klinman, 1995; Bruice & Benkovic, 2000; Knapp & Klinman, 2002).

17.3 KINETIC ISOTOPE EFFECTS ON V_{max} AND V_{max}/K_M IN ENZYMATIC REACTIONS

The two independent kinetic parameters of the Michaelis–Menten equation are V_{max} and V_{max}/K_M. Many enzymologists who have analyzed for isotopic effects have looked for rate reductions in V_{max}, because V_{max}, for most enzymes, contains a rate term for the chemical step involving the covalent change being analyzed. In probing with a deuterated substrate, for instance, if $V_{max(H)}/V_{max(D)} > 1$, then V_{max} rates may be controlled totally or in part by a slow catalytic step involving C–H (C–D) fission. The magnitude of V_{max} difference observed for substrate-deuterium kinetic isotope effects varies widely in enzymatic reactions.

For example, the oxidation of L-[2-^{2}H]-lactate by a hydroxyacid oxidase from rat kidney shows a V_{max} isotope effect of 8, suggesting that C–D cleavage is probably fully rate-determining (Cromartie & Walsh, 1975). Such a large isotope effect on V_{max} is relatively rare. In many cases, observedV_{max} isotopeeffects may be in the range 2.0–3.0, and interpretation can be complex (Leskovac *et al.*, 2002). One possible interpretation of the small isotope effects is that the V_{max} of the enzymatic reaction is rarely determined by a single step involving chemical change. Rather, the enzyme as catalyst may permit a reaction path where a single high energy barrier is replaced by several smaller barriers of about equivalent heights. That is, multiple steps may occur at similar rates, with each step being partly rate-limiting. A low value for an isotope effect may reflect such a behavior. Cleland (1975) argues that conformational changes in the proteins leading to release of products often place limits on V_{max}; such slow steps could completely suppress expression in V_{max} of the isotope effect from the chemical step.

17.3.1 Monosubstrate Reaction

Let us start the derivation of rate equations for kinetic isotope effects with analysis of a simple monosubstrate enzyme reaction:

$$\mathrm{E} \underset{k_2}{\overset{k_1\mathrm{A}}{\rightleftharpoons}} \mathrm{EA} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{EP} \underset{k_6\mathrm{P}}{\overset{k_5}{\rightleftharpoons}} \mathrm{E} \qquad (17.11)$$

The chemical step in the forward direction is k_3, and this step will experience the isotope effect, such as in the C–H cleavage; we may safely assume that other steps are unaffected by the isotope exchange, because they only reflect the binding of substrate (Walsh, 1998).

Under initial velocity conditions, when k_6P is insignificant, and under the steady-state assumption, we obtain the following form of the velocity equation, the simple Michaelis–Menten equation:

$$v_0 = \frac{k_1k_3k_5AE_0}{(k_2k_4 + k_2k_5 + k_3k_5) + k_1(k_3 + k_4 + k_5)A} \qquad (17.12)$$

Suppose that the exchange of deuterium for protium in the chemically reactive position in the substrate reduces the magnitude of the rate constant k_3 fivefold, without affecting the other rate constants (Fig. 2).

How will a kinetic isotope effect on k_3 be expressed in v_0? The kinetic expression for observed isotope effect is the ratio of both entire rate equations describing the disappearance of hydrogen and deuterium substrates.

One can see from Fig. 2 that the ratio of intercepts, *D* versus *H,* in the double reciprocal plot is equal to $V_{max(H)}/V_{max(D)}$. On the other hand, the ratio of slopes is equal to $(V_{max(H)}/K_{M(H)})/(V_{max(D)}/K_{M(D)})$. Although an isotope effect on v_0 may not be easily interpretable, one can see from Fig. 2, that, as [A] becomes saturating, the isotope effect tends to the value of $V_{max(H)}/V_{max(D)}$; contrary to that, as [A] tends to zero, the isotope effect tends to the value of $(V_{max(H)}/K_{M(H)})/(V_{max(D)}/K_{M(D)})$.

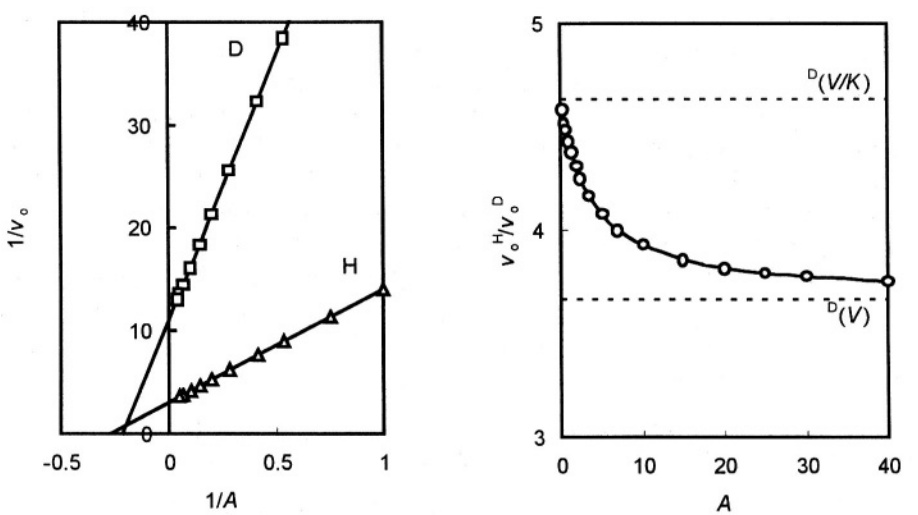


Figure 2. Kinetic isotope effect for a monosubstrate reaction depicted in reaction 17.11. The substrate concentration-dependence of initial rates of reaction were drawn for a hydrogen substrate (v_0^H) and a deuterated substrate (v_0^D) according to Eq. (17.12), assuming that $k_1=k_4=k_5=E_0=1$, $k_2=5$, and that $k_{3(H)}=1$ is reduced by isotope exchange fivefold to $k_{3(D)}=0.2$.

How will a kinetic isotope effect on k_3 be expressed in V_{max} and in V_{max}/K_M? The expressions for the basic kinetic constants, derived from Eq. (17.12), have the following forms:

$$V_{max} = \frac{k_3 k_5 E_0}{(k_3 + k_4 + k_5)} \tag{17.13}$$

$$\frac{V_{max}}{K_A} = \frac{k_1 k_3 k_5 E_0}{(k_2 k_4 + k_2 k_5 + k_3 k_5)} \tag{17.14}$$

The extent to which the intrinsic isotope effect is expressed in V_{max} depends on the ratio of k_3 to k_5, the ratio of the rate constant for the chemical step to the rate constant for product release. When k_3/k_5 is small, that is less than 1, then the observed isotope effect on V_{max} approaches the true isotope effect; when k_3/k_5 is large (>1), product release is rate-limiting and the isotope effect tends to be suppressed when V_{max} is measured.

$$\left(\frac{V_{max(H)}}{V_{max(D)}}\right) = \left[\frac{\frac{k_{3(H)}}{k_{3(D)}} + \frac{k_3}{k_5} + \left(\frac{K_{eq(H)}}{K_{eq(D)}}\right)\frac{k_4}{k_5}}{1 + \frac{k_3}{k_4} + \frac{k_4}{k_5}}\right] \tag{17.15}$$

Thus, it is clear that the value of $V_{max(H)}/V_{max(D)}$ will tend to 1, when $k_3 \gg k_5$, and will tend to $k_{3(H)}/k_{3(D)}$, when $k_5 \gg k_3$.

On the other hand, the expression for the intrinsic isotope effect in V_{max}/K_A depends on a different ratio: the ratio of k_3 to k_2. Thus, we obtain

$$\frac{(V/K)_H}{(V/K)_D} = \left[\frac{\frac{k_{3(H)}}{k_{3(D)}} + \frac{k_3}{k_2} + \left(\frac{K_{eq(H)}}{K_{eq(D)}}\right)\frac{k_4}{k_5}}{1 + \frac{k_3}{k_2} + \frac{k_4}{k_5}}\right] \tag{17.16}$$

It can be seen that the apparent isotope effect on *V/K* varies inversely with the ratio k_3/k_2, between values of 1 and the true intrinsic isotope effect. When $k_3 \gg k_2$, the effect is suppressed; when $k_3 \ll k_2$, the effect is fully expressed:

$$\frac{(V/K)_H}{(V/K)_D} \quad \begin{cases} \text{tends to 1, when } k_3 \gg k_2 \\ \text{tends to } k_{3(H)}/k_{3(D)}, \text{ when } k_3 \ll k_2. \end{cases} \tag{17.17}$$

In other words, an isotope effect does not show up specifically in *V/K* when the enzyme–substrate complex breaks down to enzyme-product complex many times for each time it reverts to E + A. This situation is often called a *"high commitment to catalysis"*. Conversely, when dissociation of A from EA occurs much more readily than covalent change to form EP, then the *"commitment to catalysis is low"*, and the isotope effect show up in *V/K*, but not in V_{max}. Note that the isotope effects on *V* and *V/K* are also influenced by the ratio k_4/k_5; if this

ratio is high, both isotope effects will tend to the value of $(K_{eq(H)}/K_{eq(D)})$. The commitment factors, and their calculation, is described in the following sections.

17.3.2 Nomenclature of Isotope Effects in Bisubstrate Reactions

In bisubstrate enzyme reactions, an effort must be made to reduce the complexity of kinetic expression. Therefore, and according to the nomenclature of Northrop (1977), we shall write the conventional expression for an isotope effect, k_H/k_D, as ^Dk; similarly, the deuterium isotope effect on V_{max}/K_M [i.e., $(V_H/K_H)/(V_D/K_D)$] will be written as $^D(V/K)$, and so forth.

Let us take the Ordered Bi Bi mechanism depicted in Fig. 3; this time, we refer to an enzymatic reaction in which the isomerization of both central complexes, EAB and EPQ, takes place. The family of deuterium isotope effects on a chemical reaction following this mechanism is illustrated in Fig. 3.

The diagram shown in this figure illustrates that one may link an observed kinetic isotope effect to a certain segment of an enzyme-catalyzed reaction. This description should be regarded as an idealized situation, because the magnitude of the isotope effect can be greatly influenced by the relative magnitude of the rate constants in certain steps.

For the sake of clarity, the terminology of Northrop (1977, 1982) will be introduced, in which a distinction is made among following types of isotope effects:

- observed
- apparent
- limiting
- internal
- intrinsic
- diminished isotope effects
- isotope effect on equilibrium constant.

(a) What one measures directly is an *"observed"* isotope effect, ^Dv_o, depicting a single data point for one set of reaction conditions, and as such it depends upon the entire steady-state distribution of enzyme forms. The kinetic expression for an observed isotope effect is the ratio of both entire rate equations describing the disappearance of the hydrogen and deuterium substrate.

(b) $^D(V/K)_{APP}$ and $^DV_{APP}$ are *"apparent"* isotope effects extrapolated from observed effects to very low and very high substrate concentrations, respectively, and termed apparent because the cosubstrate is fixed at an arbitrary level.

(c) $^D(V/K)$ and ^DV are *"limiting"* isotope effects at low and high substrate concentrations, respectively, and represent the true kinetic constants describing the steady-state expression of the isotope effect.

(d) The limiting isotope effects share a portion of the reaction sequence in common. The *"internal"* isotope effect, $^Dk'$, is the effect on this common portion, representing all steps of the catalytic sequence following substrate binding and leading up to and including the first irreversible step.

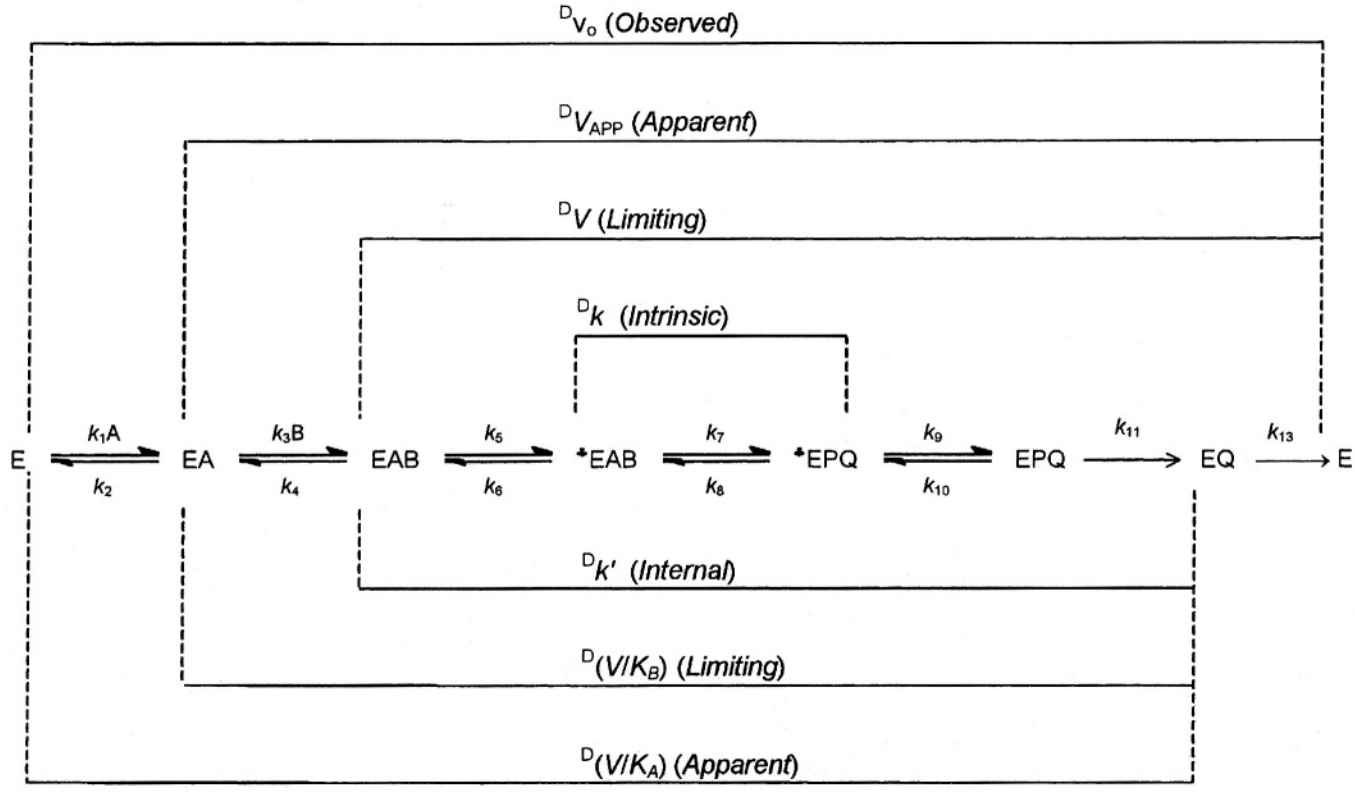


Figure 3. Kinetic isotope effects in an Ordered Bi Bi system.

(e) The *"intrinsic"* isotope effect, ${}^{D}k$, expresses the full effect imposed on the chemical step, which is analogous to isotope effects in uncatalyzed chemical reactions.

(f) Comparisons between isotope effects in this family requires a zero point of reference. By the convention of expressing isotope effects as a ratio, the absence of an effect has a value of 1. Correction to a zero point of reference is, therefore, accomplished by subtracting 1 from the value of isotope effect; an isotope effect diminished by one is, therefore, referred to as a *"diminished"* isotope effect. Thus, the diminished isotope effect is equal (isotope effect –1).

(g) Kinetic isotope effect on equilibrium constant, ${}^{D}K_{eq}$, is expressed by Eq. (17.30) (Wolfsberg, 1972; IUPAC, 1979).

17.3.3 Ordered Bi Bi Mechanism

Bisubstrate enzyme reactions are among the most common in nature, and we must leave the monosubstrate and turn to bisubstrate reactions if we are to understand real enzymes (Northrop, 1975, 1981, 1982, 1995; Cleland *et al.,* 1977; Blanchard & Wong, 1991).

Consider a familiar Ordered Bi Bi mechanism shown in Fig. 3.

$$\mathrm{E} \underset{k_2}{\overset{k_1\mathrm{A}}{\rightleftharpoons}} \mathrm{EA} \underset{k_4}{\overset{k_3\mathrm{B}}{\rightleftharpoons}} \mathrm{EAB} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} {}^{*}\mathrm{EAB} \underset{k_8}{\overset{k_7^{*}}{\rightleftharpoons}} {}^{*}\mathrm{EPQ} \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} \mathrm{EPQ} \xrightarrow{k_{11}} \mathrm{EQ} \xrightarrow{k_{13}} \mathrm{E} \quad (17.18)$$

This time, we refer to an enzymatic reaction in which the isomerization of both central complexes, EAB and EPQ, takes place.

As already pointed out in Chapter 9, the steady-state expressions for the catalytic constant, V_1, and for the specificity constant, V_1/K_B, for bisubstrate mechanisms are rather complex. For the ordered mechanism in reaction (17.18), as we have already pointed out in Section 9.2.2, even if we leave out the isomerizations, that is, the complexes *EAB and *EPQ, expressions for V_1/K_B and V_1 are rather complex. If we include the isomerization complexes, *EAB and *EPQ, the rate equations for the catalytic constant, V_1, and for the specificity constant, V_1/K_B, appear quite formidable compared to equations for the monosubstrate reaction (Eqs. (17.13) and (17.14)). Further, if we remember that the kinetic expression for isotope effects is the ratio of both entire rate equations describing the disappearance of hydrogen and deuterium substrates (or other isotopes), than the rate equations for isotope effects may appear awesome.

Surprisingly, however, the isotopic expressions for complex steady-state bisubstrate mechanisms have a common and highly specific form that, once understood, enables complex mechanisms to be treated practically.

Isotope Effect on (*V/K*)

In an Ordered Bi Bi mechanism shown in reaction (17.18), it is possible to rearrange the rate equation for the specificity constant, *V/K,* into the following simple form (Northrop, 1975, 1977, 1981, 1991):

$$^{D}\left(\frac{V}{K}\right) = \frac{(^{D}k + C_F + C_R\,{}^{D}K_{eq})}{(1 + C_F + C_R)} \tag{17.19}$$

where C_F is the "*forward commitment to catalysis*", C_R the "*reverse commitment to catalysis*", and ^{D}k is the "*intrinsic isotope effect*".

This equation is written here for deuterium isotope effects, but applies for any other isotope equally well. In Eq. (17.19), C_F represents the tendency of the enzyme complex poised for chemical reaction to continue forward through catalysis, as opposed to its tendency to partition back to free enzyme and an unbound substrate. Similarly, C_R is the tendency for the first enzyme complex following the chemical step to undergo reverse reaction, as opposed to partitioning forward through the first irreversible step leading to unbound product.

For the ordered mechanism shown in reaction (17.18) expressions for the "commitment to catalysis" have the form of a series of ratios of rate constants from adjacent steps. For example, at low concentrations of substrate A and fixed levels of substrate B, the commitment factor C_{FA} is:

$$C_{FA} = \frac{k_7^*}{k_6} + \frac{k_5 k_7^*}{k_4 k_6} + \frac{k_3 k_5 k_7^* B}{k_2 k_4 k_6} = \frac{k_7^*}{k_6}\left[1 + \frac{k_5}{k_4}\left(1 + \frac{k_3}{k_2}B\right)\right] \tag{17.20}$$

where asterisk shows the isotope sensitive step.

A forward commitment for a given reactant is defined as the ratio of the rate constant for the isotope-sensitive step to the net rate constant for release of that reactant from the enzyme, where a net rate constant corresponds to the net flux through a series of steps.

In this expression, those partition ratios that involve intermediates other than the initial collision complex make up what are called the *internal commitment,* while the term containing the partition ratio for the collision complex is the *external portion of the commitment*; the total of the external and internal commitment is the total commitment. Thus, the internal commitment for A, and the external commitment for A, in mechanism (17.18) are, respectively,

$$\left(\frac{k_7^*}{k_6} + \frac{k_5 k_7^*}{k_4 k_6}\right) \quad \text{and} \quad \left(\frac{k_3 k_5 k_7^* B}{k_2 k_4 k_6}\right) \tag{17.21}$$

For $^{D}(V/K)$, the reverse commitment, C_R, is for the first irreversible step following the isotope sensitive step:

$$C_R = \frac{k_8}{k_9}\left(1 + \frac{k_{10}}{k_{11}}\right) \tag{17.22}$$

Thus, equations for commitments are easily derived from the mechanism, and consist of combinations of partition ratios for intermediates.

Commitment factors vary in different experiments; this is an important distinction between deuterated substrates where the noncompetive method is

used, and the tritiated substrates where the competitive method is used. Thus for both competitive and noncompetitive experiments, the reverse commitment includes steps through the first irreversible step and is the same. The forward commitment for a competitive experiment, however, is for the labeled substrate. Thus with lactate dehydrogenase, which obeys the Ordered Bi Bi mechanism, if we are using tritiated NADH (substrate A), it is necessary to keep pyruvate (substrate B) as low as possible in order to minimize the external commitment and to determine isotope effects on (V/K_{pyruvate}) rather than ones on (V/K_{NADH}). In a noncompetitive experiment, the commitment is for the varied substrate. Thus, we can vary pyruvate with either unlabeled or deuterated NADH and we shall be always looking at (V/K_{pyruvate}), for which the commitment is minimized (Cleland, 1982). This is an important distinction when comparing *V/K* values in Northrop's method.

Isotope Effect on V

For the ordered mechanism in reaction (17.18), at high concentration of both substrates, isotope effects on maximal velocities can be expressed as

$$^{D}V = \frac{^{D}k + R_F/E_F + C_R\,{}^{D}K_{eq}}{1 + R_F/E_F + C_R} = \frac{^{D}k + R_{VF} + C_R\,{}^{D}K_{eq}}{1 + R_{VF} + C_R} \tag{17.23}$$

where C_R is the reverse commitment to catalysis as defined by Eq. (17.22). The new terms R_F and E_F are the "*ratio of catalysis*" and the "*equilibration preceding catalysis*", respectively (Northrop, 1982).

R_F consists of the arithmetic sum of ratios of the rate constant for the chemical step to net rate constants, one for each of the other forward steps contributing to the maximal velocity, leading towards and away from the isotopically sensitive step.

For reaction (17.18), at *saturating* [A], it is

$$R_F = \frac{k_7}{k'_3} + \frac{k_7}{k'_5} + \frac{k_7}{k'_9} + \frac{k_7}{k'_{11}} + \frac{k_7}{k'_{13}} \tag{17.24}$$

with net rate constants equal:

$$k'_3 = \frac{Bk_3k'_5}{k_4 + k'_5} = \frac{k_3k_5B}{k_4 + k_5} \tag{17.25}$$

$$k'_9 = \frac{k_9k_{11}}{k_{10} + k_{11}} \tag{17.26}$$

$$k'_5 = k_5 \qquad k'_{11} = k_{11} \qquad k'_{13} = k_{13} \tag{17.27}$$

For calculations involving *V*, [B] is saturating and, therefore, the first term in Eq. (17.24) is zero.

E_F is the "equilibrium preceding catalysis" and consists of the sum of equilibrium constants; for reaction (17.18), it is

$$E_F = 1 + \frac{k_6}{k_5} + \frac{k_4k_6}{k_3k_5B} = 1 + \frac{k_6}{k_5}\left(1 + \frac{k_4}{k_3B}\right) \tag{17.28}$$

The quotient of the ratio of catalysis to the equilibration preceding catalysis, at *saturating* [A], is, now, a rather complex expression:

$$R_{VF} = \frac{R_F}{E_F} = \frac{\dfrac{k_7}{k_3 B}\left(1 + \dfrac{k_4}{k_5}\right) + \dfrac{k_7}{k_5} + \dfrac{k_7}{k_9}\left(1 + \dfrac{k_{10}}{k_{11}}\right) + \dfrac{k_7}{k_{11}} + \dfrac{k_7}{k_{13}}}{1 + \dfrac{k_6}{k_5}\left(1 + \dfrac{k_4}{k_3 B}\right)} \tag{17.29}$$

Again, for calculations involving *V,* [B] is saturating and, therefore, the terms containing B in denominators of Eqs. (17.28) and (17.29) will drop out.

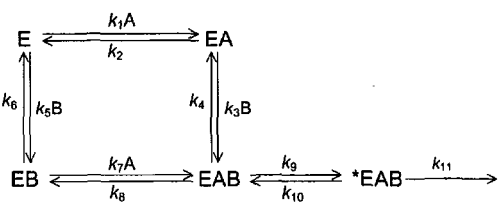
Intrinsic isotope effects are usually different in forward and reverse chemical reactions, since their ratio is the equilibrium isotope effect, given by

$${}^{D}K_{eq} = \frac{{}^{D}k_{forward}}{{}^{D}k_{reverse}} \tag{17.30}$$

which is often easier to measure than to calculate. A frequent case is also that ${}^{D}K_{eq}$ is not equal to unity (Cleland, 1982).

17.3.4 Random Bi Bi Mechanism

For the Random Bi Bi mechanism, expressions for commitment factors are more complex due to the branch points: the expression forward through the common sequence is divided by the net rate constant away through the separate branches.



(17.31)

For example, in the random segment in reaction (17.31), the net rate constant for the release of A is

$$k_{off} = k_8 + \frac{k_2 k_4}{k_2 + k_3 B} \tag{17.32}$$

The forward commitment to catalysis at low [A] is, therefore,

$$C_{FA} = \frac{k_{11}}{k_{10}}\left[1 + \frac{k_9}{k_8 + (k_2 k_4)/(k_2 + k_3 B)}\right] \tag{17.33}$$

Similarly, in the random segment in reaction (17.31), the net rate constant for the release of B is

$$k_{off} = k_4 + \frac{k_6 k_8}{k_6 + k_7 A} \tag{17.34}$$

Therefore, the forward commitment of catalysis at low [B] is

$$C_{FB} = \frac{k_{11}}{k_{10}}\left[1 + \frac{k_9}{k_4 + (k_6k_8)/(k_6 + k_7A)}\right] \tag{17.35}$$

Examination of Eqs. (17.33) and (17.35) reveals that the isotope effects at low concentrations of one substrate should be determined at low concentrations of the other, in which case both equations reduce to

$$C_{FAB} = \frac{k_{11}}{k_{10}}\left(1 + \frac{k_9}{k_4 + k_8}\right) \tag{17.36}$$

If either substrate is rapidly released from the ternary complex, that is, either k_4 or k_8 is large, the forward commitment factor simplifies to

$$C_{IF} = \frac{k_{11}}{k_{10}} \tag{17.37}$$

Equality (17.37) is usually referred to as an "internal" commitment to catalysis for the mechanism in reaction (17.31) (Northrop, 1982).

17.3.5 Ping Pong Mechanism

Consider the following reaction scheme for the Ping Pong mechanism:

$$E \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} EA \xrightarrow{k_3} F \underset{k_6}{\overset{k_5B}{\rightleftharpoons}} FB \xrightarrow{k_7} E \tag{17.38}$$

In a Ping Pong mechanism, usually only one of the two half-reactions is sensitive to isotopic substitution. Suppose that only the rate constant k_3 is sensitive to isotopic substitution. In mechanism (17.38), rate constants k_3 and k_7 are not simple microscopic rate constants, but are the net rate constants for conversion of EA to F and FB to E, respectively, and contain contributions from conformational changes, the bond-breaking steps, and release of products.

Reaction (17.38) is sufficient for consideration of such Ping Pong mechanisms since k_3 will not depend on the concentration of substrates (Cook, 1991). The expressions for V, V/K_A, and V/K_B are given by equations

$$\frac{V}{E_0} = \frac{k_3k_7}{(k_3 + k_7)} \quad \frac{V}{K_AE_0} = \frac{k_1k_3}{k_2 + k_3} \quad \frac{V}{K_BE_0} = \frac{k_5k_7}{k_6 + k_7} \tag{17.39}$$

The expressions for the isotope effects are given by equations

$${}^DV = \frac{\left({}^Dk_3 + \dfrac{k_3}{k_7}\right)}{\left(1 + \dfrac{k_3}{k_7}\right)} \quad {}^D\left(\frac{V}{K_A}\right) = \frac{\left({}^Dk_3 + \dfrac{k_3}{k_2}\right)}{\left(1 + \dfrac{k_3}{k_2}\right)} \quad {}^D\left(\frac{V}{K_B}\right) = 1 \tag{17.40}$$

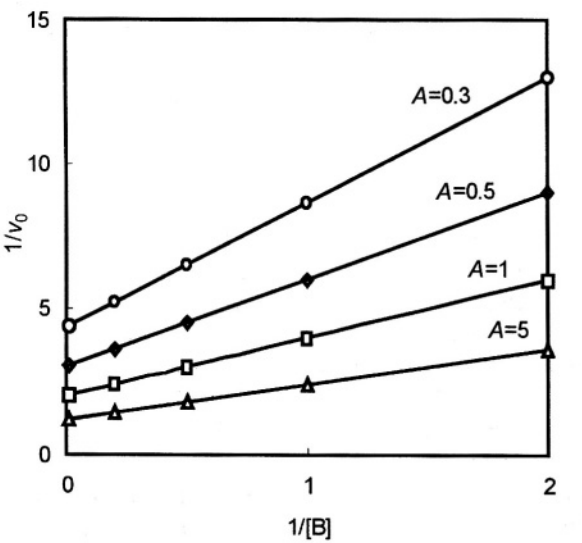
The isotope effect on V/K_B, ${}^D(V/K_B)$, is equal to 1 due to lack of an isotope-sensitive step in the second half-reaction. If it is not known whether the kinetic mechanism is sequential or Ping Pong, the isotope effects on the V/K_A and V/K_B values will not distinguish between a Steady-State Ordered and Ping Pong mechanisms.

17.4 DETERMINATION OF KINETIC ISOTOPE EFFECTS

17.4.1 Measurement of Kinetic Isotope Effects in Bisubstrate Reactions

The kinetic expression for observed isotope effects is the ratio of both entire rate equations describing the disappearance of hydrogen and deuterium substrates. The isotopically sensitive step appears in multiple terms and cannot be factored out. In order to achieve factoring and subsequent simplification to useful kinetic equations, it is necessary to examine the limits of rate equations at low and high substrate concentrations, where enzyme reactions approach first-order and zero-order kinetics, respectively. To understand this, we must consider how isotope effects in bisubstrate reactions are measured.

Consider an Ordered Bi Bi reaction depicted in Fig. 3. For deuterium effects, and if the exchangeable position is completely or nearly completely substituted by deuterium, one simply runs reciprocal plots with deuterated and hydrogen-containing molecules and takes the ratio of slopes as the *V/K* effect, and the ratio of the intercepts as the *V* effect. The computation of limiting isotope effects from apparent values requires extrapolation as a function of the cosubstrate concentration (Fig. 4).



$$\frac{1}{v_0} = \frac{1}{V_1}\left(1+\frac{K_A}{A}\right)+\frac{K_B}{V_1}\left(1+\frac{K_{iA}}{A}\right)\frac{1}{B}$$

Figure 4. Measurement of kinetic constants in an Ordered Bi Bi mechanism. Data points are drawn according Eq. (9.15), assuming that $V_1=K_A=K_{iA}=K_B=1$.

Extrapolations must be made prior to computing the isotope effects, using several point patterns at low and high concentrations of the substrate B; then, the plots obtained with a hydrogen-containing substrate are repeated with the deuterium-containing substrate.

The ratios of $(V/K_B)_H$ versus $(V/K_B)_D$, at different constant values of [A], are calculated from the slopes in Fig. 4, and the ratios of $(V)_H$ versus $(V)_D$, at different constant values of [A], are calculated from the intercepts in Fig. 4. Thus, in an Ordered Bi Bi mechanism, the simplest way to determine V/K_B is to vary [B]. In this way, we obtain V/K_B directly, regardless of the [A] level, and this minimizes the commitments; this works regardless of which substrate carries the label.

There are two principal methods for obtaining the isotope effects: noncompetitive and competitive. The noncompetitive method with full substitution for obtaining the rate data is commonly used for deuterium isotope effects. A competitive method is usually associated with tritium or ^{13}C, because it is difficult to obtain chemically a full isotopic substitution with these isotopes. In a competitive method, with less then total substitution of reactants with isotopes, the fraction of label is unimportant; tritium or ^{13}C are always used as trace labels. What we do have to correct for in the competitive method is the level of the non-labeled substrate if it adds after the labeled one (Northrop, 1982). From competitive experiments only the $^{D}(V/K)$ values are obtained.

17.4.2 Determination of the Limiting Isotope Effects on *V/K*

The theory of kinetic isotope effect outlined in Section 17.3.3 is essential because it provides an insight into how a change in one step of kinetic mechanism is expressed in kinetic parameters that can be measured. Consider an ordered bisubstrate mechanism depicted in Fig. 3:

$$E \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3B}{\rightleftharpoons}} EAB \underset{k_6}{\overset{k_5}{\rightleftharpoons}} {}^{*}EAB \underset{k_8}{\overset{k_7}{\rightleftharpoons}} {}^{*}EPQ \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} EPQ \xrightarrow{k_{11}} EQ \xrightarrow{k_{13}} E$$

It is important to note that commitment factors may depend on the level of other reactants present, and this variation can be used to determine the kinetic mechanism. For an ordered bisubstrate mechanism, the commitment of B (the second substrate to add) is independent of A (the first substrate to add), and depends only on how fast B is released from the enzyme relative to the forward rate constant for the bond-breaking step. Conversely, the commitment for A varies from infinity at saturating B to the value for B at near zero B, and thus the actual rate constant for release of A from the EA complex does not affect the commitment of B, even if it is quite small.

At low substrate concentrations, the observed isotope effect approaches $^{D}(V/K)$, as the limiting isotope effect at zero substrate concentration. However, one can see from Eq. (17.19) that, if the reverse commitment to catalysis becomes large, the reaction step containing the bond-breaking reaction nears a chemical equilibrium, and the isotope effect expressed on *V/K* approaches the value of $^{D}K_{eq}$, the equilibrium isotope effect. Obviously, the lower the commitments to catalysis, the more fully the kinetic isotope effect will be expressed on *V/K*.

For the Ordered Bi Bi mechanism in Fig. 3, substitution of C_F (Eq. (17.20)) and C_R (Eq. (17.22)) into Eq. (17.19) reveals that the apparent isotope effect on *V/K* is dependent on the concentration of B.

$$^{D}\left(\frac{V}{K_A}\right)_{APP} = \frac{^{D}k_7 + \frac{k_7}{k_6}\left[1+\frac{k_5}{k_4}\left(1+\frac{k_3}{k_2}B\right)\right] + \frac{k_8}{k_9}\left(1+\frac{k_{10}}{k_{11}}\right)^{D}K_{eq}}{1+\frac{k_7}{k_6}\left[1+\frac{k_5}{k_4}\left(1+\frac{k_3}{k_2}B\right)\right] + \frac{k_8}{k_9}\left(1+\frac{k_{10}}{k_{11}}\right)} = \frac{\text{constant}_1 + B}{\text{constant}_2 + B} \tag{17.41}$$

At very high [B], the commitment of A becomes large and the isotope effect is abolished (Fig. 5).

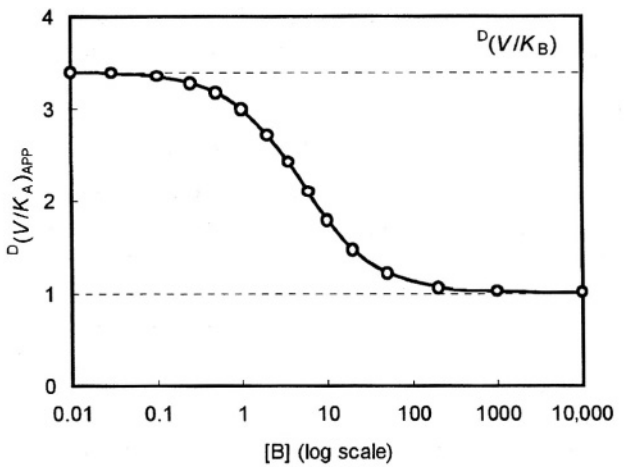


Figure 5. Dependence of the isotope effect, $^{D}(V/K_A)_{APP}$, on the concentration of B. The data points are calculated according to Eq. (17.41), assuming that all the rate constants in commitment factors C_{FA} and C_R are equal to unity, except that $^{D}k_7$=5, and $^{D}K_{eq}$=$^{D}k_7/^{D}k_8$=5.

At very low [B], the expression approaches the limiting isotope effect on *V/K*, that is, tends to $^{D}(V/K_B)$, where the forward commitment factor reduces to

$$C_{FB} = \frac{k_7}{k_6}\left(1+\frac{k_5}{k_4}\right) \tag{17.42}$$

Hence, Eq. (17.41), describing the limiting isotope effect on *V/K*, is independent of the concentration of A. In practice, what we do is to vary [B] at different levels of labeled or unlabeled [A] and determine $^{D}(V/K_B)$ from the slope ratio; this minimizes any external commitment factors.

A special case occurs if the system in Fig. 3 is a rapid equilibrium ordered one. In this case, k_2 is very large and, therefore, Eq. (17.20) approximates the expression:

$$C_{FB} = \frac{k_7^*}{k_6}\left(1+\frac{k_5}{k_4}\right) \tag{17.43}$$

In this case, the limiting *V/K* isotope effect is obtained at either low [A] or low [B].

17.4.3 Determination of the Limiting Isotope Effects on V

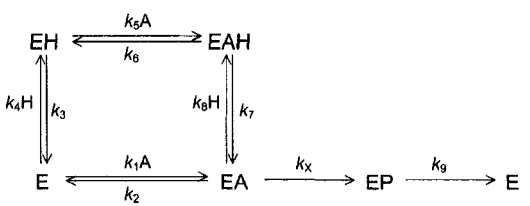
At high substrate concentrations, measured isotope effects approach ^{D}V, governed by Eq. (17.23). For the Ordered Bi Bi mechanism in Fig. 3, a quotient of R_F (Eq. (17.24)) and E_F (Eq. (17.28)) becomes a complex expression (Eq. (17.29)) which can be substituted into Eq. (17.23); the resulting equation then becomes far too complex for a practical use (Northrop, 1982). For practical purposes, the extrapolation of apparent values of ^{D}V obtained at saturating concentrations of A, to saturating concentrations of B, will afford the limiting isotope effect on V.

Isotope effects on V cannot be determined when tritium or a heavy atom is used as a trace label; in such cases, only the V/K isotope effects are obtained.

17.4.4 Determination of Internal Isotope Effects

Internal isotope effects cannot be obtained by raising or lowering of substrate concentrations, but may be approached by studying limiting isotope effects as a function of some other variable, such as pH dependence, temperature, or substrate structure. For example, to enhance observed isotope effects and obtain more useful information on the degree of rate limitation of the bond-breaking step, and the location of other rate-limiting steps along the reaction path, the pH can be raised or lowered so that the chemical reaction or the pH dependent pathway including the chemical reaction becomes rate limiting (Northrop, 1982).

Consider the simple model shown in reaction 17.44, where k_X includes the bond-breaking step and is thus isotope dependent.



(17.44)

Lowering the pH draws the enzyme away from EA and the catalytic sequence, thus, lowering the external commitment to catalysis:

$$C_{EF} = \frac{k_x}{k_2 + \dfrac{k_6 k_8 [H]}{k_6 + k_7}} \tag{17.45}$$

Thus, at low pH (high [H]), $^{D}(V/K)$ approaches the limit:

$$^{D}\left(\frac{V}{K}\right) = \frac{^{D}k_x + C_{EF}}{1 + C_{EF}} \rightarrow |^{D}k_x| \quad \left(\text{when} \quad pH < pK_a = \frac{(k_2 + k_x)(k_6 + k_7)}{k_6 k_8}\right) \tag{17.46}$$

Similarly, for the limiting isotope effect on V, at low pH, ^{D}V approaches the limit:

$$^{D}(V) = \frac{^{D}k_x + \left[\frac{(k_x/k_7) + (k_x/k_9)}{1 + (k_8[H]/k_7)}\right]}{1 + \left[\frac{(k_x/k_7) + (k_x/k_9)}{1 + (k_8[H]/k_7)}\right]} = \frac{^{D}k_x + \frac{R_{EF}}{E_F}}{1 + \frac{R_{EF}}{E_F}} \rightarrow |^{D}k_x| \quad (\text{when pH} < \text{p}K_a) \tag{17.47}$$

The pK_a value in this equation is a very complex function (Cleland, 2001).

17.4.5 Determination of Intrinsic Isotope Effect

The term ^{D}k in Eqs. (17.19) and (17.23) is the intrinsic isotope effect in the forward direction, the value we need to interpret the structure of the transition state in enzyme-catalyzed reactions and the effectiveness of enzyme catalysis (Cleland, 1982, 1995; Northrop, 1982, 1991; Northrop & Duggleby, 1990).

The determination of the intrinsic isotope effect is based on the Swain-Schaad relationship (Eq. (17.2)). For diminished isotope effects, a fixed relationship between deuterium and tritium is maintained,

$$^{T}k - 1 = ^{D}k^{1.44} - 1 \tag{17.48}$$

In the absence of an equilibrium isotope effect, that is, if $^{D}K_{eq} = 1$, the general expression for diminished *V/K* isotope effect becomes a simpler function of the diminished intrinsic effect:

$$^{D}\left(\frac{V}{K}\right) - 1 = \frac{^{D}k - 1}{1 + C_F + C_R} \tag{17.49}$$

The commitment factors do not contain isotopic steps; therefore, the parallel tritium expression for Eq. (17.49) has the same denominator, leading to a cancellation of commitment factors in the ratio

$$\frac{^{D}(V/K) - 1}{^{T}(V/K) - 1} = \frac{^{D}k - 1}{^{T}k - 1} \tag{17.50}$$

Substitution in the Swain–Schaad relationship gives

$$\frac{^{D}(V/K) - 1}{^{T}(V/K) - 1} = \frac{^{D}k - 1}{^{D}k^{1.44} - 1} \tag{17.51}$$

Consequently, one can calculate ^{D}k from the values of $^{D}(V/K)$ and $^{T}(V/K)$.

If the equilibrium isotope effect, $^{D}K_{eq} = ^{D}k_F/^{D}k_R$, is present, the expression for diminished isotope effects is not cleared of commitment factors, but takes the form (Cleland, 1977)

$$^{D}\left(\frac{V}{K}\right) - 1 = \frac{^{D}k_F - 1 + C_R(^{D}K_{eq} - 1)}{1 + C_F + C_R} \tag{17.52}$$

Similar treatment of equation for the tritium effect gives

$$^{T}\left(\frac{V}{K}\right) - 1 = \frac{(^{D}k)^{1.44} - 1 + C_R(^{T}K_{eq} - 1)}{1 + C_F + C_R} \tag{17.53}$$

By taking the ratio of both equations, we obtain

$$\frac{^{D}(V/K) - 1}{^{T}(V/K) - 1} = \frac{^{D}k - 1 + C_R(^{D}K_{eq} - 1)}{(^{D}k)^{1.44} - 1 + C_R(^{D}K_{eq}^{1.44} - 1)} \tag{17.54}$$

If $^{D}K_{eq}=1$, or $C_R=0$, this equation reduces to Eq. (17.51).

When C_R is finite and K_{eq} is different from unity, solutions of Eq. (17.49) give only an apparent value of ^{D}k. With two unknowns (^{D}k and C_R), an exact solution is not possible, but at $^{D}K_{eq} > 1$, limits can be set for the intrinsic isotope effect because

$$^{D}K_{eq} \geq \left(\frac{^{D}k_F}{^{D}k_R}\right)_{CALC} \tag{17.55}$$

$$\left(^{D}k_R\right)_{CALC}$$

Therefore, it follows that the intrinsic isotope effect lies between the calculated limits:

$$^{D}K_{eq}(^{D}k_R)_{CALC} \geq ^{D}k_F \geq (^{D}k_F)_{CALC} \tag{17.56}$$

To obtain the upper limit, $(^{D}k_R)_{CALC}$ can be calculated from data in the forward direction by first multiplying the limiting isotope effect by the equilibrium isotope effect, because

$$^{D}K_{eq} = \frac{^{D}(V/K)_F}{^{D}(V/K)_R} \tag{17.57}$$

Therefore,

$$\frac{\left(\frac{^{D}(V/K)_F}{^{D}K_{eq}} - 1\right)}{\left(\frac{^{T}(V/K)_F}{^{D}K_{eq}^{1.44}} - 1\right)} = \frac{^{D}\left(\frac{V}{K}\right)_R - 1}{^{T}\left(\frac{V}{K}\right)_R - 1} = \frac{^{D}(k_R)_{CALC} - 1}{^{D}(k_R)_{CALC}^{1.44} - 1} \tag{17.58}$$

17.4.6 Stickiness of Substrates

The stickiness of substrate is determined by the ratio of the net rate constant for reaction of the first collision complex through the first irreversible step, to the rate constant for dissociation of the collision complex. This ratio can be expressed as

$$\frac{C_{F-external}}{(1 + C_{F-internal} + C_R)} \tag{17.59}$$

For the mechanism in reaction (17.18), the stickiness of substrate is expressed by

$$\frac{\left(\frac{k_3k_5k_7B}{k_2k_4k_6}\right)}{\left[1+\left(\frac{k_7}{k_6}+\frac{k_5k_7}{k_4k_6}\right)+\left(\frac{k_8}{k_9}+\frac{k_8k_{10}}{k_9k_{11}}\right)\right]} \tag{17.60}$$

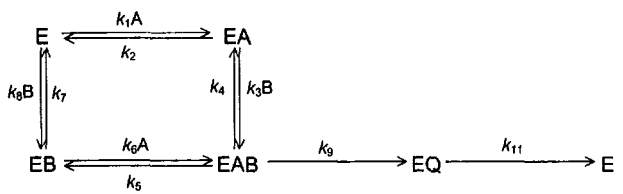
Thus, it is possible for a substrate to have a finite external commitment and still not be sticky, if C_R is larger than the external forward commitment (Cleland, 1977).

17.5 KINETIC MECHANISM FROM THE VARIATION OF SUBSTRATES AND PRODUCTS

17.5.1 Substrate Dependence of Isotope Effects

Kinetic isotope effects are sensitive to changing concentrations of substrates, products of reactions, and allosteric effectors (Cook, 1991). This sensitivity of isotope effects may be profitably applied to analyze kinetic mechanisms.

Consider the following reaction scheme for a bisubstrate reaction:



(17.61)

The kinetic isotope effects in bisubstrate reactions are measured by placing an isotope label in one of the substrates, followed by a complete kinetic analysis with a pair of unlabeled substrates, and then by a complete analysis with a pair of labeled and unlabeled substrate. In this way, the kinetic isotope effect on the maximal velocity, ^{D}V, and the isotope effects on V/K for both substrates, $^{D}(V/K_A)$ and $^{D}(V/K_B)$, are obtained. Table 1 gives typical examples from the literature.

Generally, the following can be stated qualitatively concerning the dependence of isotope effects on V and V/K.

One of the $^{D}(V/K)$ values is equal to unity

In a sequential mechanism, an isotope effect equal to or close to one on one of the two substrate V/K values suggests a Steady-State Ordered mechanism. The V/K for the first substrate bound will have the isotope effect of unity. The isotope effect of one may also apply to the second substrate in a Ping Pong mechanism, but a distinctive, initial velocity pattern with parallel lines is obtained in this case.

In Table 1 such examples are alcohol dehydrogenase, dihydrofolate reductase and dopamine β-hydroxylase; in the former cases, the nucleotide coenzyme adds first and in the latter case it is dopamine that adds first. In the case of alcohol dehydrogenase, the intrinsic isotope effect is 6 (Cook & Cleland, 1981), while in the case of dopamine β-hydroxylase it is 10 (Ahn & Klinman, 1983), clearly showing that the isotope-sensitive step is not completely rate-limiting.

Table 1. Substrate dependence of deuterium isotope effects

Enzyme	Substrate pair	Parameter	Isotope effect	pH	Reference
One of the $^{D}(V/K)$ values is unity					
Liver alcohol dehydrogenase	Cyclohexanol-1-(H,D) NAD^+	V V/K_{NAD} $V/K_{alcohol}$	1.10 0.85 3.14	8.0	Cook & Cleland, 1981
Dihydrofolate reductase (*E. coli*)	NADP(H,D) Dihydrofolate	V V/K_{NADPH} V/K_{DHF}	1.90 1.10 2.80	7.8	Morrison & Stone, 1988
Bovine dopamine β-hydroxylase	Dopamine-2,2,-$(H,D)_2$ Oxygen	V $V/K_{dopamine}$ V/K_{oxygen}	2.3 1.0 4.8	4.5	Ahn & Klinman, 1983
$^{D}(V/K)$ *Values are finite but not equal*					
Yeast alcohol dehydrogenase	Ethanol-$(H,D)_5$ NAD^+	V V/K_{NAD} $V/K_{alcohol}$	1.8 1.8 3.2	7.3	Ganzorn & Plapp, 1988
	NAD^+ 2-Propanol-d_8	V V/K_{NAD} $V/K_{alcohol}$	2.20 3.23 2.54	7.0	Trivić & Leskovac, 1994
Liver glutamate dehydrogenase	L-Norvaline-2-(H,D) $NADP^+$	V V/K_{NADP} $V/K_{norvaline}$	2.46 4.68 1.49	8.0	Rife & Cleland, 1980
$^{D}(V/K)$ *Values are finite and equal*					
Malic enzyme (*Ascaris suum*)	L-malate-(H,D) NAD^+	V V/K_{NAD} V/K_{malate}	1.45 1.49 1.45	7.5	Kiick *et al.*, 1986
Ping pong mechanism					
Glucose oxidase (*Aspergillus niger*)	D-glucose-(H,D) Oxygen	V $V/K_{glucose}$ V/K_{oxygen}	7.8 3.8 1.0	5.0	Bright & Gibson, 1967

For alcohol dehydrogenase, ^{D}V is close to unity, indicating that the overall rate is probably completely limited by the off-rate of NADH, the rate constant k_{11} in reaction (17.61); in other words, this enzyme has a Theorell-Chance mechanism.

$^{D}(V/K)$ *Values are finite but not equal*

Finite but unequal isotope effects on the two substrate *V/K* values suggests a Steady-State Random kinetic mechanism. The smaller of the *V/K* isotope effects reflects the stickier substrate. In reaction (17.61), it means that the rate constant k_5 is substantial, and that the central complex is able to dissociate back to EA as well as to EB.

From the data in Table 1, one can say the following qualitatively: (a) a finite isotope effect on all three kinetic parameters indicates that the isotopic-sensitive step is partially rate-limiting under all conditions, (b) the lower value of ^{D}V relative to one of the $^{D}(V/K)$ values suggests that a step after the first irreversible step limits the reaction, and (c) the substrate released fastest from the Michaelis complex has the larger isotope effect. Thus, with yeast alcohol dehydrogenase operating with 2-propanol and with glutamate dehydrogenase operating with L-norvaline, coenzyme is released faster than the other substrate.

$^{D}(V/K)$ *Values are finite and equal*

Equal isotope effect on the two *V/K* values suggests one of several possibilities, including an Equilibrium Ordered mechanism with or without a dead-end EB complex, a Rapid Equilibrium Random mechanism, or a Steady-State Random mechanism in which the rates of release of A and B from the central complex are equal.

If the value of k_2 is very fast with respect to k_3B at any concentration of B used, the mechanism approximates rapid equilibrium order addition of A. Under these conditions, the isotope effect will be constant, that is, $^{D}(V/K_A)=^{D}(V/K_B)$, whatever substrate is varied at any concentration of the fixed substrate.

Based on isotope effects only, it is not possible to distinguish the Rapid Equilibrium Ordered from the Rapid Equilibrium Random mechanism. However, the first mechanism gives a distinctive initial velocity pattern that intersects on the ordinate with B as the varied substrate. To tell the difference between the Rapid Equilibrium Random and the Steady-State Random mechanism will require other methods, such as the isotope trapping method (Rose *et al.*, 1974), or isotopic exchange.

Ping Pong mechanism

The reaction scheme for the Ping Pong mechanism of glucose oxidase is given by reaction (17.38):

$$\mathrm{E} \underset{k_2}{\overset{k_1\mathrm{A}}{\rightleftharpoons}} \mathrm{EA} \xrightarrow{k_3} \mathrm{F} \underset{k_6}{\overset{k_5\mathrm{B}}{\rightleftharpoons}} \mathrm{FB} \xrightarrow{k_7}$$

where A is D-glucose and B is oxygen.

$^{D}(V/K_{oxygen})$ is equal to 1, due to the lack of isotope-sensitive step in the oxidative half-reaction. A value of ^{D}V larger than $^{D}(V/K_{glucose})$ suggests that glucose is sticky, that is, k_2 is slower or is not much greater than k_3 (Blanchard & Wong, 1991). An inverse situation, when ^{D}V is smaller than $^{D}(V/K_{glucose})$ would suggest a partial limitation by the oxidative half-reaction, whereas complete limitation will give a value of unity for ^{D}V.

17.5.2 Product Dependence of Isotope Effects

Isotope effects will also depend on the concentration of products present in the reaction mixture and this effect will be mechanism dependent. A theoretical basis for this type of isotope effects was developed (Cook, 1991), but very few experiments were carried out to date (Cook *et al.,* 1993).

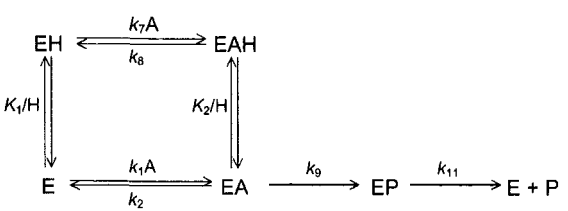
17.5.3 Dependence of Isotope Effects on Allosteric Effectors

These effects have been characterized theoretically (Cleland, 1991), but practically they have found only a limited application (Parmentier *et al.,* 1992).

17.6 KINETIC MECHANISM FROM THE VARIATION OF KINETIC ISOTOPE EFFECTS WITH pH

Isotope effects on enzyme-catalyzed reactions are only rarely fully expressed, since bond-breaking steps are often faster than other steps such as release of reactants. In order to enhance observed isotope effects and obtain information on the degree of rate limitation of the bond-breaking step, and the location of other rate-limiting steps along the reaction path, the pH can be raised or lowered so that the chemical reaction becomes rate-limiting (Cook & Cleland, 1981; Cook, 1991). Further, the variations of kinetic isotope effects with pH may be used in a profitable way to distinguish between different reaction mechanisms.

Consider a simple model shown in reaction (17.62).



(17.62)

From this model, one can derive four different and independent kinetic models, simply by omitting some reaction pathways (Fig. 6).

Table 2 shows the variation of the rate constant composition of V and V/K with pH, for the four kinetic models in Fig. 6.

Fig. 7 shows the variants of the mechanisms shown in Fig. 6, together with the form of expected plots of isotope effects as a function of pH. In reaction (17.62), only the chemical step, depicted by the rate constant k_9, is sensitive to deuterium for protium isotope exchange in the substrate.

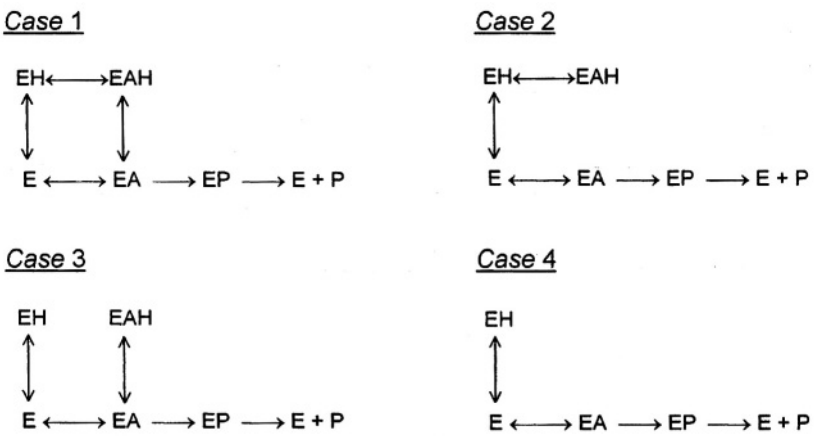


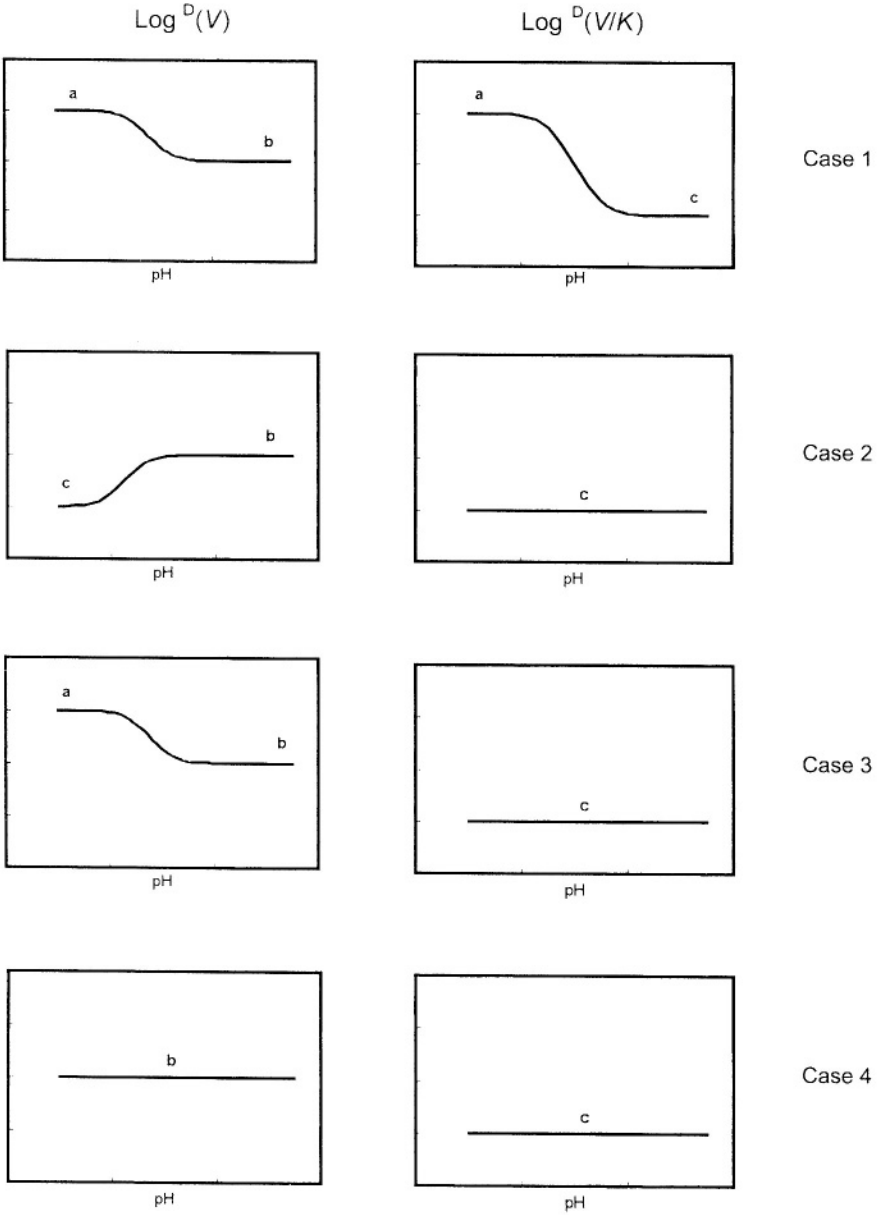
Figure 6. Four kinetic models derived from reaction 17.62.

Table 2. Limiting expressions for isotope effects at low and high pH for kinetic models shown in Fig. 6

Parameter		Case 1	Case 2	Case 3	Case 4
^{D}V	Low pH[a]	$^{D}k_9$	$\frac{^{D}k_9 + k_9/k_2}{1 + k_9/k_2}$	$^{D}k_9$	$\frac{^{D}k_9 + k_9/k_{11}}{1 + k_9/k_{11}}$
	High pH[a]	$\frac{^{D}k_9 + k_9/k_{11}}{1 + k_9/k_{11}}$	$\frac{^{D}k_9 + k_9/k_{11}}{1 + k_9/k_{11}}$	$\frac{^{D}k_9 + k_9/k_{11}}{1 + k_9/k_{11}}$	$\frac{^{D}k_9 + k_9/k_{11}}{1 + k_9/k_{11}}$
$^{D}(V/K)$	Low pH[a]	$^{D}k_9$	$\frac{^{D}k_9 + k_9/k_2}{1 + k_9/k_2}$	$\frac{^{D}k_9 + k_9/k_2}{1 + k_9/k_2}$	$\frac{^{D}k_9 + k_9/k_2}{1 + k_9/k_2}$
	High pH[a]	$\frac{^{D}k_9 + k_9/k_2}{1 + k_9/k_2}$	$\frac{^{D}k_9 + k_9/k_2}{1 + k_9/k_2}$	$\frac{^{D}k_9 + k_9/k_2}{1 + k_9/k_2}$	$\frac{^{D}k_9 + k_9/k_2}{1 + k_9/k_2}$

[a]High pH means at least a pH unit below the apparent pK_a which represents the pH where the isotope effect is the average of those at low and high pH.

For Case 1, when the entire mechanism is used, the full isotope effect ($^{D}k_9$) on *V* and *V/K* will be observed below the p*K*s seen in the *V* or *V/K* profiles, but not above the pKs in the profiles unless $k_2 \gg k_9$ (for *V/K*) or $k_{11} \gg k_9$ (for *V*). In any event, the *V* and *V/K* effects become equal as the pH is lowered.



$$a = \log\left({}^{D}k_9\right) \quad > \quad b = \log\left(\frac{{}^{D}k_9 + k_9 / k_{11}}{1 + k_9 / k_{11}}\right) \quad > \quad c = \log\left(\frac{{}^{D}k_9 + k_9 / k_2}{1 + k_9 / k_2}\right)$$

Figure 7. Variation of kinetic isotope effects with pH for the mechanisms shown in Fig. 6.

For Case 2, the *V/K* isotope effect will be pH independent and will not be the full effect on k_9. The *V/K* isotope effect is also pH independent for Case 3, but again is not the full effect on k_9. For Case 4, both *V* and *V/K* effects are pH independent, and are not the full effect on k_9 (Cook & Cleland, 1991).

While only the last variant may be readily distinguished from the other three by the usual pH kinetics, the additional use of the isotope effect may distinguish among all four. This example clearly illustrates the power of the pH variation of kinetic isotope effects, a method that can considerably extend the usefulness of pH studies to detect the subtle variations in kinetic mechanisms (Northrop, 1982).

17.6.1 Principal Use of Substrate Isotope Effects

The principal uses of substrate isotope effects for the enzymologist are listed below (Cleland, 1977,1995; Northrop & Rebholz, 1997; Northrop & Simson, 1998; Northrop, 2001):

(a) The absolute values for bond-breaking steps give clues about the chemical mechanism and the nature of the transition step.
(b) The degree to which the absolute value expresses in *V* or *V/K* tells where the rate-limiting steps are in the mechanism.
(c) One of the major application of isotope effects in random mechanisms is to detect a degree of randomization, such as it is in a random or partially random bisubstrate mechanisms.

Examination of the general equation for the kinetic isotope effect on *V/K* (Eq. (17.19)) shows that one must lower the commitment factors to obtain a reliable estimate of the intrinsic isotope effect, ^Dk. The value of commitment factors can be lowered in two ways.

The external commitment can usually be eliminated by changing the pH, because the internal commitment is usually independent of pH. Internal commitments may be lowered by a proper choice of slow substrates. Thus, for a serious isotope effect studies, one should try all possible substrates for an enzyme and use those with the lowest commitments; then, one should try to change the pH in order to lower the commitments further.

In an ordered bisubstrate mechanism one must vary the second substrate, B, and determine V/K_B, regardless of whether the label is in A or B, since V/K_A will not show an isotope effect. For a random mechanism one must vary both A and B, since one may see different isotope effects on V/K_B and V/K_A, a distinction that may help to characterize the mechanism. The effects on V/K_B and V/K_A should be different when one or both substrates are sticky, that is, dissociate more slowly from the enzyme than they react to give products. The substrate with the lower *V/K is* the sticky one. Larger effects on *V* than on either *V/K* show that both substrates are sticky; smaller ones show that a slow step follows release of the first product. A rapid equilibrium random mechanism will show equal isotope effects on *V*, V/K_B, and V/K_A, all larger than unity.

Comparison between the diminished form of isotope effects can be employed to determine the relative steady-state contribution of different components to the limiting values of kinetic parameters. For example, comparing diminished

intrinsic to diminished *V/K* isotope effect provides a measure of the sum of commitment factors, as shown by the following rearrangement of Eq. (17.49):

$$C_F + C_R = \left(\frac{^Dk - 1}{^D(V/K) - 1}\right) - 1 \qquad (17.63)$$

Similarly, comparing diminished internal to diminished limiting isotope effects provides a measure of the "stickiness" of substrates (Eqs. (17.36) and (17.37)).

17.7 ISOTOPE TRAPPING

The method of isotope trapping allows one to determine the stickiness of all substrates but the last to combine in an ordered mechanism and of all substrates in a random mechanism (Rose *et al.,* 1974; Rose; 1995). This method was developed initially by Rose for the yeast hexokinase reaction, and it is essentially a single turnover experiment in which one determines analytically the proportion of an enzyme–substrate complex that reacts to give products, as opposed to dissociating.

An experimental protocol is depicted in Fig. 8.

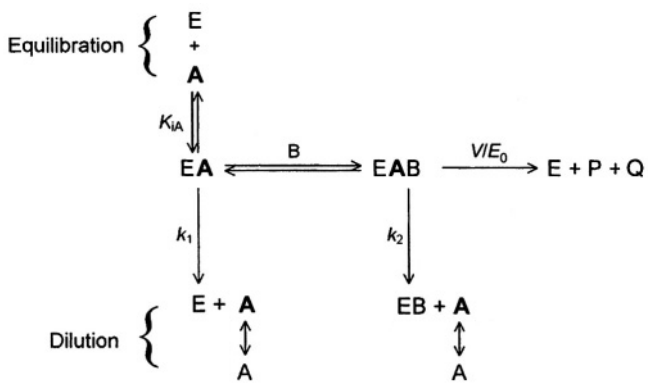


Figure 8. Isotope trapping experiment.

An experimental protocol in Fig. 8 is shown for an ordered bisubstrate mechanism. A small volume of enzyme is incubated with sufficient labeled substrate, **A**, to convert most or all of the enzyme into a binary complex, E**A.** This solution is then diluted into a large volume containing the unlabeled substrate, A, plus variable amounts of cosubstrate, B. After several seconds, acid is added to stop the enzymatic reaction, and labeled product, **Q**, is determined analytically. A blank is then run with the labeled reactant already diluted in the large solution, plus only the enzyme present in the small volume. The experiment is then repeated at different levels of the second substrate B, and a reciprocal plot is made of the amount of labeled product, **Q**, as a function of the reciprocal of the substrate B concentration; thus, by extrapolation, the maximum amount of labeled product formed, $\mathbf{Q}_{max}$, is obtained.

The initial E**A** complex can dissociate into E and **A,** which is immediately diluted by the excess of unlabeled A. Also the E**A** complex can add B and either dissociate into EB and **A,** which again results in dilution, or reaction to give products.

Because of the large dilution factor and a short incubation time, the only reversible step in Fig. 8 is addition of B, and thus one can easily determine the partitioning of the EA complex between products and dissociation.

After introducing appropriate corrections for blank reactions, the rate equations involved are

$$k_2 = \frac{V}{E_0}\left(\frac{\dfrac{E_0}{\mathbf{Q}_{max}}}{1+\dfrac{K_{iA}}{\mathbf{A}_{free}}} - 1\right) \tag{17.64}$$

$$\frac{V}{E_0}\left(\frac{K^{APP}}{K_B}\right) \leqslant k_1 \leqslant \frac{V}{E_0}\left(\frac{\dfrac{E_0}{\mathbf{Q}_{max}}}{1+\dfrac{K_{iA}}{\mathbf{A}_{free}}} - 1\right)\frac{K^{APP}}{K_B} \tag{17.65}$$

where $\mathbf{A}_{free}$ is the concentration of free labeled substrate, **A**, present in the original solution, K^{APP} is the apparent trapping constant from the reciprocal plot, K_B is the Michaelis constant of B in the chemical reaction. In Eq. (17.64), if the second product release limits *V,* the ratio V/E_0 increases and becomes larger than the true value of V/E_0. Equations (17.64) and (17.65) allow calculation of the values of k_1 and k_2 relative to V/E_0.

The isotope trapping method clearly distinguishes the Ping Pong from the sequential mechanisms, in cases when the initial velocity patterns do not provide an unambiguous answer; in a Ping Pong mechanism, the amount of trapped label will not depend on the concentration of B (Cleland, 1977).

17.8 HEAVY-ATOM KINETIC ISOTOPE EFFECTS

To this point, our discussion has concentrated only on substitution with isotopes of hydrogen. We can also look at rate effects that occur on substitution with isotopes of heavier atoms, including the stable isotopes ^{13}C for ^{12}C, ^{15}N for ^{14}N, and ^{18}O for ^{16}O. Studies have been performed on heavy-atom isotope effects, both in chemical reactions (Jencks, 1969) and in enzyme-catalyzed reactions (Cleland, 1977; O'Leary & Kleutz, 1972; O'Leary, 1980; Huskey, 1991). One immediate problem is that the expected rate differences in heavy-atom isotope effects are much smaller because of the much smaller percentage change in atomic mass on isotopic substitution.

For example, ^{2}H is twice as heavy as ^{1}H, but ^{13}C is only 8% heavier than ^{12}C, and the zero-point vibrational energy levels for the carbon isotopes are much closer. Maximal observed isotope effects for $^{13}C/^{12}C$ are about 1.06, those for $^{15}N/^{14}N$ about 1.04, and those for $^{18}O/^{16}O$ about 1.06. Enzymologists who study secondary or heavy-atom isotope effects often refer to them in percentage terms. For example, an effect of 1.06 is frequently referred to as an "isotope effect of six percent".

To obtain credible distinction, great experimental precision is required in measurements of these small differences in rates. One technique for detection of heavy-atom isotope effects has been to measure isotopic composition of products early in the reaction and again at 100% completion with high-precision ratio mass spectrometers (O'Leary & Kleutz, 1972). Another approach is a sensitive equilibrium-perturbation method for enzymatic experiments, where freely reversible reactions are studied (Schimerlik *et al,* 1975; Cleland, 1980).

17.9 SECONDARY ISOTOPE EFFECTS

The primary isotope effects discussed so far take place when the isotopic substitution occurs at the bond broken in the reaction in question, and we have pointed out that, for $k_H > k_D$, the C–H or C–D bond breakage is significantly advanced in the rate-limiting transition state. Numerous studies have also been made on the effect of isotopic substitution of deuterium or tritium at a bond adjacent to the reacting bond, such that the C–D or C–T bond is not itself broken during the reaction. Isotopic substitution at such positions can lead to either slower or faster rates, leading to *secondary kinetic isotope effects* (Dahlquist *et al*., 1968; Jencks, 1969; Smith *et al.,* 1973; Melander & Saunders, 1980; Cook, 1991).

Consider the hydride-transfer reaction catalyzed by alcohol dehydrogenase, shown in Fig. 9.

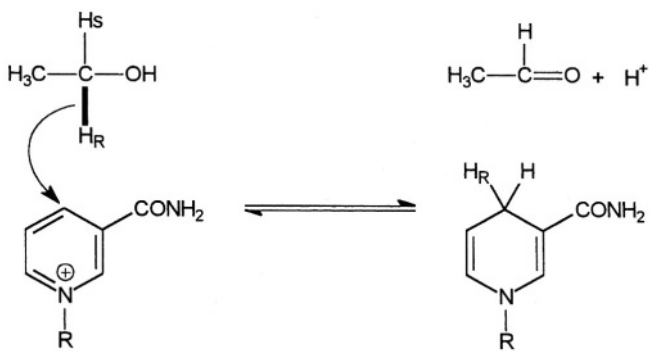


Figure 9. Alcohol dehydrogenase-catalyzed oxidation of ethanol with NAD^+.

In this reaction, we can distinguish the following isotope effects:

Primary	Arises from the deuterium substitution of the H_R proton at C1 of ethanol, ^{13}C substitution at C1 of ethanol, ^{18}O substitution in ethanol, or ^{13}C substitution at C4 of the nicotinamide ring of NAD^+
α-Secondary	Arises from the deuterium substitution of the H_S proton at C1 of ethanol, or at 4-position of the nicotinamide ring of NAD^+
β-Secondary	Arises from the substitution in the methyl group of ethanol
Remote	Arises from the ^{15}N substitution at N1 of the nicotinamide ring of NAD^+

Remote substitution usually gives no appreciable isotope effect, but ^{15}N substitution at N1 of the nicotinamide ring of NAD^+ gives small secondary isotope effects because of the bonding change at the nitrogen during reaction (Rothberg & Cleland, 1991). Primary and secondary isotope effects are often treated as separate phenomena, since with deuterium the primary ones tend to be much larger than the secondary ones. With heavier atoms such as ^{13}C, ^{15}N, and ^{18}O, the primary and secondary isotope effects are of the similar size. However, both effects have the same cause, namely a difference in the fractionation factor of the reactant and transition state, and with both effects the same equations apply.

17.10 SOLVENT ISOTOPE EFFECTS

The introduction of deuterium in place of proton in water, and its consequent exchange into some positions of enzymes and substrates, produces *solvent isotope effect* on the kinetic and equilibrium constants associated with the enzymatic reactions. These effects, usually expressed as ratios of the appropriate constants in two isotopic solvents HOH and DOD, are useful in the study of reaction mechanisms (Gandour & Schowen, 1978; Cook, 1991; Quinn & Sutton, 1991).

At this point, we may note that the most immediate characteristic of an overall solvent isotope effect on steady-state kinetic parameters, k_{HOH}/k_{DOD}, V_{HOH}/V_{DOD}, or $(V/K)_{HOH}/(V/K)_{DOD}$, is its direction. The customary nomenclature attributes a *normal solvent isotope effect* when the kinetic parameters are larger in HOH, and an *inverse solvent isotope effect* when the kinetic parameters are larger in DOD. Solvent isotope effects can also be primary and secondary.

17.10.1 Nomenclature

The nomenclature of solvent isotope effects is the same as the Northrop's nomenclature for kinetic isotope effects described in preceding sections (Cook, 1991). Consider a simple monosubstrate reaction,

$$E \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EP \xrightarrow{k_5} E$$

Under initial velocity conditions and the steady-state assumption, we obtain the following form of kinetic constants for V and V/K, respectively:

$$V_{max} = \frac{E_0 k_3 k_5}{(k_3 + k_4 + k_5)} \tag{17.66}$$

$$\frac{V_{max}}{K_A} = \frac{E_0 k_1 k_3 k_5}{k_2(k_4 + k_5) + k_3 k_5} \tag{17.67}$$

The corresponding solvent isotope effects on V and V/K are given by equations

$$^{D_2O}V_{max} = \frac{V_{max}^{H_2O}}{V_{max}^{D_2O}} = \frac{^{D_2O}k_3 + C_{VF} + C_R^{D_2O}K_{eq}}{1 + C_{VF} + C_R} \tag{17.68}$$

$$^{D_2O}\left(\frac{V_{max}}{K_A}\right) = \frac{\left(\frac{V_{max}}{K_A}\right)^{H_2O}}{\left(\frac{V_{max}}{K_A}\right)^{D_2O}} = \frac{^{D_2O}k_3 + C_F + C_R^{D_2O}K_{eq}}{1 + C_F + C_R} \qquad (17.69)$$

In Eqs. (17.68) and (17.69), the forward commitment factors are $C_F = k_3/k_2$ and $C_{VF} = k_3/k_5$, respectively, and the reverse commitment factor is $C_R = k_4/k_5$. The equilibrium isotope effect is for the chemical step in the forward direction,

$$^{D_2O}K_{eq} = \frac{^{D_2O}k_3}{^{D_2O}k_4} \qquad (17.70)$$

$^{D_2O}k_3$ is the intrinsic solvent isotope effect for the chemical step in the forward direction and equals the ratio of k_3 values in HOH and DOD; similarly, $^{D_2O}k_4$ is the intrinsic solvent isotope effect for the chemical step in the reverse direction and equals the ratio of k_4 values in HOH and DOD.

Equations (17.67)–(17.69) are based on the assumption that only the rate constants of the chemical step are subject to a kinetic solvent isotope effect. This assumption simplifies the derivation of rate equations, but one must always keep in mind that, although the substrate binding and product release are usually solvent isotope-insensitive, the entire mechanism may contain more than a single isotope sensitive step.

The rate equation for solvent isotope effects with more complex bisubstrate mechanisms may be derived in the same way as was described in Section 17.3.3 for the kinetic isotope effects, using the corresponding nomenclature.

17.10.2 Proton Inventory

Solvent isotope effects, since they refer to rate or equilibrium constants, have to do with the effect of the isotopic solvent on both initial (reactant) states and final (transition or product) states. We shall make an attempt to dissect these effects and to deal with the effects on the individual states (Quinn & Sutton, 1991).

Simply by measuring the rate of reaction in HOH and DOD reveals almost nothing about the mechanism, but measuring the rate as a function of the mole fraction of DOD in isotopic mixtures of various composition can be much more informative. Thus, kinetic studies of solvent isotope effects in a series of HOH and DOD can, in some cases, allow the dissection of the isotope effect into its component contributions from different sites in the reactant and transition states. In this way, one can construct a list of the contributing hydrogenic or protonic sites and the magnitude of the isotope effect for each; this is called a *proton inventory.*

The conduct of proton inventory consists of determining the kinetic parameters of interest in a number of isotopic water mixtures of deuterium atom fraction n, so that the data set comprises values of k_n (n). The data are then fit, by an appropriate statistical procedure, to a corresponding theoretical equation, and contributing effects are calculated from this.

The simplest model is when a single site determines the entire solvent isotope effect. Consider a mixture of HOH and DOD in which the mole fraction of DOD is *n,* so that DOD/HOH ratio is $n/(1-n)$. For an exchangeable hydrogen in a reactant molecule AH, the deuterium/protium ratio will also be [AD]/[AH] = $n/(1-n)$ if the equilibrium constants for protons and deuterons are the same at that position. There will be normally some selection, so that the actual ratio is $\phi_R n/(1-n)$, where ϕ_R is the *fractionation factor* for that exchangeable reactant position. Such exchanges of hydrogen can take place not only in the reactant molecule itself but also in the transition state of reaction in which it participates. In this transition state, there is a similar deuterium/protium ratio $\phi_T n/(1-n)$, where ϕ_T is the fractionation factor for the same exchangeable position in the transition state. The total rate of reaction is now the sum of the rates for the protonated and deuterated species, and so the rate of reaction is proportional to

$$\left[\frac{1+\dfrac{\phi_T n}{(1-n)}}{1+\dfrac{\phi_R n}{(1-n)}}\right] = \frac{(1-n+\phi_T n)}{(1-n+\phi_{RT} n)} \tag{17.71}$$

From Equations (17.71), it follows that the observed rate constant k_n at mole fraction n of DOD may be expressed as a function of n and the normal rate constant k_o in pure HOH as follows:

$$k_n = k_o\left(\frac{1-n+\phi_T n}{1-n+\phi_R n}\right) \tag{17.72}$$

In the simplest cases, the reactant and the transition state will have only one and the same exchangeable position for hydrogen or deuterium. In such cases, the shape of the curve obtained from Eq. (17.72) provides a way of calculating the fractionation factors in the reactant and the transition state of reaction, directly from the experimental data (Gandour & Schowen, 1978).

Consider an example from the literature, a proton inventory for the equine liver alcohol dehydrogenase-catalyzed oxidation of ethanol with NAD^+ (Chandra Sekhar & Plapp, 1990) (Fig. 10).

In this case, mechanistic information suggests that the reactant and the transition state have one and the same exchangeable position for hydrogen or deuterium, which is a hydroxyl group in ethanol (Fig. 9). The rate of reaction was faster in DOD, and an inverse solvent effect on k_{cat} of 2.0 was observed; the data in Fig. 10 were fitted with Eq. (17.72), assuming one protonic site having different fractionation factors in reactant and transition states.

The low fractionation factor of 0.37 suggests that the reactant state is an enzyme-bound alkoxide with a low barrier hydrogen bond between it and Ser-48 in the primary structure of enzyme. This proton transfers to Ser-48 during hydride transfer, so that its fractionation factor approaches unity. The value of 0.73 for the transition state fractionation factor shows that the hydrogen bond is weakening as the proton is transferred during the reaction.

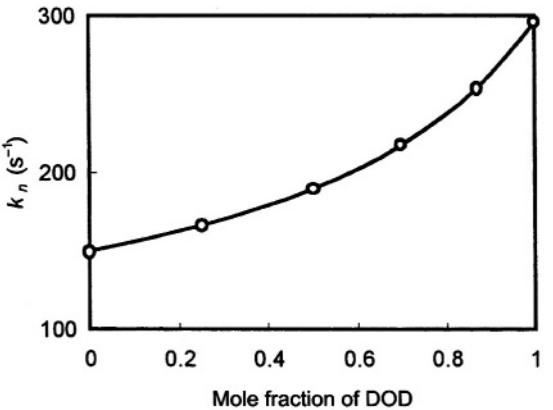


Figure 10. Proton inventory for alcohol dehydrogenase-catalyzed oxidation of ethanol with NAD^+. The data points are the measured k_{cat}-values (k_n) and the curve was drawn according to Eq. (17.72), assuming that k_0=150 s^{-1}, ϕ_R=0.37, and ϕ_T=0.73 (Chandra Sekhar & Plapp, 1990).

17.10.3 Kresge (Gross–Butler) Equation

To this point, we have considered just one hydrogen, but a typical enzyme molecule contains a large number of exchangeable hydrogens, each of which may or may not contribute to the solvent isotope effect. If all hydrogens exchange independently, the effects of all the different hydrogens are multiplicative and Eq. (17.72) can be generalized into the equation:

$$k_n = k_0\left[\frac{\prod_i^x (1-n+n\phi_i^T)}{\prod_i^x (1-n+n\phi_i^R)}\right] \tag{17.73}$$

Equation (17.73) is the Kresge equation that describes the functional dependence of k_n on the isotopic composition, n, of the solvent (Kresge, 1964; Kresge *et al.*, 1987). A conduct of a full proton inventory from the Kresge equation is never practical. However, if some simplifying assumptions are introduced into the Kresge equation, useful information may be obtained under special circumstances. For example, if we assume that ϕ_R is equal to unity, Eq. (17.73) reduces to a polynomial function of n:

$${}^n k = {}^{D_2O}k\left[\prod_i^x (1-n+n\phi_i^T)\right] \tag{17.74}$$

Therefore, if a single transition-state proton contributes to the solvent isotope effect, ${}^n k$ will be linear function of n; if two protons contribute, the dependence will be quadratic; if three protons contribute, cubic, etc. In practice, even the most carefully collected data in the proton inventory experiments can distinguish

only between one and two protons in the transition state. When x is greater than 3, proton inventories cannot be interpreted. Moreover, if the solvent isotope effect is 2.0 or less, the distinction between one and two exchangeable sites becomes difficult, and the distinction between two or more sites almost impossible (Schowen & Schowen, 1982; Quinn & Sutton, 1991).

The proton inventory analysis is further complicated by the fact that, in enzymatic reactions, the reactant state fractionation factor is often not unity, as described for the alcohol dehydrogenase case above.

REFERENCES

Books

Atkins, P. & de Paula, J. (2002) *Physical chemistry,* 7th ed., Oxford University Press, Oxford.

Bell, R.P. (1980) *The tunnel effect in chemistry,* Chapman & Hall, New York.

Buncel, E. & Lee, C.C. (eds.) (1987) *Isotopes in organic chemistry,* Vol. 7, Elsevier, Amsterdam.

Cook, P.F. (ed.) (1991) *Enzyme mechanism from isotope effects,* CRC Press, Boca Raton, Florida.

Cleland, W.W., O'Leary, M.H., & Northrop, D.B. (eds.) (1977) *Isotope effects on enzyme-catalyzed reactions,* University Park Press, Baltimore.

Devault, D. (1984) *Quantum mechanical tunneling in biological processes,* 2nd ed., Cambridge University Press, Cambridge.

Gandour, D.R. & Schowen. R.L. (eds.) (1978) *Transition states in biochemical processes,* Plenum Press, New York.

Jencks, W. P. (1969) *Catalysis in chemistry and enzymology,* McGraw-Hill, New York [Also available as *Catalysis in chemistry and enzymology* (1986) Dover Publications, New York].

Melander, L. (1960) *Isotope effects on reaction rates,* Raven Press, New York.

Melander, L. & Saunders, W.H. (1980) *Reaction rates of isotopic molecules,* Wiley, New York.

Moore, J.W. & Pearson, R.G. (1981) *Kinetics and mechanism,* Wiley, New York.

Walsh, C. (1998) *Enzyme reaction mechanisms,* W.H. Freeman, New York.

Review articles

Bahnson, B.J. & Klinman, J.P. (1995) *Methods Enzymol. 249,* 373–397.

Blanchard, J.S. & Wong, K.K. (1991) in *Enzyme mechanism from isotope effects,* pp. 341–366.

Cleland, W.W. (1975) *Acc. Chem. Res. 8,* 145–160.

Cleland, W.W. (1977) *Adv. Enzymol. Rel. Areas Mol. Biol. 45,* 273-387.

Cleland, W.W. (1980) *Methods Enzymol. 64,* 104–125.

Cleland, W.W. (1982) *Methods Enzymol. 87,* 625–641.

Cleland, W.W. (1982) *Methods Enzymol. 87,* 641–646.

Cleland, W.W. (1991) in *Enzyme mechanism from isotope effects,* pp. 247–268.

Cleland, W.W. (1995) *Methods Enzymol. 249,* 341-373.

Cook, P.F. (1991) in *Enzyme mechanism from isotope effects,* pp. 203–230.

Cook, P.F. (1991) in *Enzyme mechanism from isotope effects,* pp. 231–246.

Huskey, W.P. (1991) in *Enzyme mechanism from isotope effects,* pp. 37–72.

IUPAC (1979) International Union of Pure and Applied Chemistry, *Pure and Appl. Chem. 51,* 1725-1740.

Klinman, J.P. (1978) in *Transition states in biochemical processes,* pp. 150–170.

Klinman, J.P. (1991) in *Enzyme mechanism from isotope effects,* pp. 127–150.

Kresge, A.J., O'Ferrall, M., & Powell, M.F. (1987) in *Isotopes in organic chemistry,* pp. 122-150.

Leskovac, V., Trivić, S., & Peričin, D. (2002) *FEMS Yeast Research 2,* 481–496.

Northrop, D.B. (1977) in *Isotope effects on enzyme-catalyzed reactions,* pp. 100–130.

Northrop, D.B. (1981) *Annu. Rev. Biochem. 50,* 103–131.

Northrop, D.B. (1982) *Methods Enzymol. 87,* 607–625.
Northrop, D.B. (1991) in *Enzyme mechanism from isotope effects,* pp. 181–202.
Northrop, D.B. (1995) *Methods Enzymol. 249,* 211–240.
Northrop, D.B. (2001) *Methods: A Companion to Methods Enzymol. 24,* 114–124.
O'Leary, M.H. (1980) *Methods Enzymol. 64,* 83–104.
Quinn, D.M. & Sutton, L.D. (1991) in *Enzyme mechanism from isotope effects,* pp. 73–126.
Rose, I.A. (1995) *Methods Enzymol. 249,* 315–340.
Schowen, R.L. & Schowen, L.A. (1982) *Methods Enzymol. 87,* 551–606.
Sühnel, J. & Schowen, R.L. (1991) in *Enzyme mechanism from isotope effects,* pp. 3–36.
Westeheimer, F.H. (1961) *Chem. Rev. 61,* 265–280.
Wolfsberg, M. (1972) *Acc. Chem. Res. 5,* 225–240.

Specific references

Ahn, N. & Klinman, J.P. (1983) *Biochemistry 22,* 3096–3100.
Bright, H.J. & Gibson, H.Q. (1967) *J. Biol. Chem. 242,* 994–1003.
Bruice, T.C. & Benkovic, S.J. (2000) *Biochemistry 39,* 6267–6274.
Chandra Sekhar, V. & Plapp, B.V. (1990) *Biochemistry 29,* 4289–4295.
Cleland, W.W. (2001) Personal communication.
Cook, P.F. & Cleland, W.W. (1981) *Biochemistry 20,* 1796–1816.
Cook, P.F., Yoon, M.-Y., Hara, S., & McClure, G.D. (1993) *Biochemistry 32,* 1795–1802.
Cromartie, T. & Walsh, C. (1975) *Biochemistry 14,* 3482–3490.
Dahlquist, F.W., Rand-Meir, T., & Raftery, M.A. (1968) *Proc. Natl. Acad. Sci. USA 61,* 1194–1204.
Eyring, H. (1935) *J. Chem. Phys. 3,* 107–115.
Ganzhorn, A.J. & Plapp, B.V. (1988) *J. Biol. Chem. 263,* 5446–5455.
Glickman, M.H., Wieseman, J.S., & Klinman, J.P. (1994) *J. Am. Chem. Soc. 116,* 793–794.
Kiick, D.M., Harris, B.G., & Cook, P.F. (1986) *Biochemistry 25,* 227–236.
Knapp, M.J. & Klinman, J.P. (2002) *Europ. J. Biochem. 269,* 3113–3121.
Kresge, A.J. (1964) *Pure Applied Chem. 8,* 243–258.
Leskovac, V. & Trivić, S. (1988) *J. Org. Chem. 53,* 6123–6125.
Morrison, J.F. & Stone, S.R. (1988) *Biochemistry 27,* 5499–5506.
Northrop, D.B. (1975) *Biochemistry 14,* 2644–2651.
Northrop, D.B. & Duggleby, R.G. (1990) *Bioorg. Chem. 18,* 435–439.
Northrop, D.B. & Rebholz, K.L. (1997) *Arch. Biochem. Biophys. 342,* 317–321.
Northrop, D.B. & Simpson, F.B. (1998) *Arch. Biochem. Biophys. 352,* 288–292.
O'Leary, M.H. & Kleutz, M.D. (1972) *J. Amer. Chem. Soc. 94,* 3585–3589.
Parmentier, L.E., O'Leary, M.H., Schachman, H.K., & Cleland, W.W. (1992) *Biochemistry 31,* 6598–6602.
Rife, J.E. & Cleland, W.W. (1980) *Biochemistry 19,* 2321–2333.
Rose, A., O'Connell, E.L., Litwin, S.L., & Bar-Tana, J. (1974) *J. Biol. Chem. 249,* 5163–5173.
Rothberg, N.S. & Cleland, W.W. (1991) *Biochemistry 30,* 4068–4071.
Schimerlik, A., Rife, J.E., & Cleland, W.W. (1975) *Biochemistry 14,* 5347–5354.
Smith, L.E.H., Mohr, L.H., & Raferty, M.A. (1973) *J. Amer. Chem. Soc. 95,* 7497–7500.
Swain, C.G., Stivers, E.C., Reuwer, D.F., & Schaod, L.J. (1958) *J. Amer. Chem. Soc. 80,* 5885–5890.
Trivić, S. & Leskovac, V. (1994) *Indian J. Biochem. 31,* 387–391.

Chapter 18

Statistical Analysis of Initial Rate and Binding Data

The first task in enzyme kinetics is a proper measurement and collection of initial rate data, binding data, or other kinetic data. Thus, the most important part of enzyme kinetics is a correct choice of kinetic data to be measured and the technically correct estimation of the same. The second important part of enzyme kinetics is an appropriate statistical analysis of experimental data.

18.1 STATISTICS

18.1.1 Statistical Parameters

At this point, it is appropriate to introduce the definitions of basic statistical parameters which are extensively covered in textbooks on biochemical statistics (Mandelhall *et al.,* 1981; Ratkowsky, 1983; Wiener *et al.,* 1991; Cornish-Bowden, 1995; Daniel, 1995; Rosner, 1996; Zar, 1996; Punch & Allison, 2000) and applied here to enzyme kinetics.

The first statistic is the *arithmetic mean* or average. One may measure the initial rate of a reaction, repeat the same measurement several times, and observe several slightly different values. If the sum of the several values were designated ΣY_i and n were the number of values, the average initial rate would be

$$\overline{Y} = \frac{\sum Y_i}{n} \tag{18.1}$$

The arithmetic mean does not give any indication of the amount of scatter in the observations. The *variance* is defined as the sum of the squares of the deviations from the mean divided by the number of degrees of freedom. If only one parameter is measured, the degrees of freedom are given by $n-1$. Therefore, the expression for the *sample* variance is

$$\text{sample variance} = s^2 = \frac{\sum (Y-\overline{Y})^2}{n-1} \tag{18.2}$$

Thus, the *standard deviation* is defined as the square root of the variance:

$$\text{standard deviation} = \sqrt{\frac{\sum Y^2 - \frac{(\sum Y)^2}{n}}{n-1}} \tag{18.3}$$

The standard deviation is an error of a given value expressed in the units of this value. The standard deviation is a statistic which measures the variability or dispersion of the set of data.

Standard error is the standard deviation of the sampling distribution of a statistic. As such, it measures the precision of the statistic as an estimate of a population parameter. A commonly computed standard error is the standard error of the sample.

The coefficient of variation, or the relative standard deviation of the mean, is defined as follows:

$$\text{coefficient of variation} = \frac{\sqrt{\text{variance}}}{\bar{Y}} \times 100 = \frac{\text{standard deviation}}{\bar{Y}} \times 100 \quad (18.4)$$

Coefficient of variation is the error of a given value expressed as a percentage.

18.1.2 Linear Regression by a Least Squares Method

Before the advent of computer technology and the computerized statistical methods for data analysis, a procedure that was employed extensively in the analysis of enzyme kinetic data was linear regression. It is important to point out that *linear regression performed by the least squares method should not be used unless the values are weighted.* If it is used without proper weighting one can get bad results.

In general terms, the assumption of a linear relationship implies that a dependent variable Y, is a linear function of an independent variable X. In the case of the linear Lineweaver–Burk plot, $1/v_0$ is the dependent variable while $1/A$ is the independent variable. In the case of the linear Hanes plot, A/v_0 is the dependent and A is the independent variable. The linear regression, as presented here, is based on four assumptions:

(1) It is assumed that the independent variable, X, is measured without error.
(2) The expected value of the dependent variable for a given value of the independent variable is

$$\hat{Y} = A + BX \quad (18.5)$$

(3) For any given value of X, the observed Y values are distributed independently and normally. This is represented by

$$Y_i = A + BX_i + \varepsilon_i \quad (18.6)$$

where ε_i is the error in the estimate, or the measurement error.
(4) It is assumed that the variance around the regression line is constant and, therefore, independent of the magnitude of X or Y.

The aim of the linear regression is to calculate the values A and B in Eq. (18.5) such that the dependent variable Y can be estimated for any given value of the independent variable X. Thus, the difference between the observed values of Y_i and the values estimated from the regression line would be

$$r_i = \hat{Y}_i - Y_i \tag{18.7}$$

where r_i is the residual and $\hat{Y}_i$ is the estimated value of Y_i.

The solutions for A and B are (Johansen & Lumry, 1961):

$$A = \frac{\sum X^2 \sum Y - \sum X \sum XY}{n\sum X^2 - (\sum X)^2} = \frac{\dfrac{(\sum X^2 \sum Y - \sum X \sum XY)}{n}}{\sum X^2 - \dfrac{(\sum X)^2}{n}} \tag{18.8}$$

$$B = \frac{n\sum XY - \sum X \sum Y}{n\sum X^2 - (\sum X)^2} = \frac{\sum XY - \sum X\left(\dfrac{\sum Y}{n}\right)}{\sum X^2 - \dfrac{(\sum X)^2}{n}} \tag{18.9}$$

The variance about the regression line is estimated as

$$s^2 = \frac{\sum (Y - A - BX)^2}{n-2} = \frac{\sum Y^2 - A\sum Y - B\sum XY}{n-2} \tag{18.10}$$

The degrees of freedom in this equation are equal to $(n-2)$ because both A and B are estimated from the observed data. The standard error on the estimate of A is

$$\text{standard error}_A = \sqrt{s^2\left(\frac{\sum X^2}{n\sum X^2 - (\sum X)^2}\right)} \tag{18.11}$$

In the same manner, the standard error for the estimation of B, the regression coefficient, is

$$\text{standard error}_B = \sqrt{s^2\left(\frac{n}{n\sum X^2 - (\sum X)^2}\right)} \tag{18.12}$$

The quantities that are required for the estimation of A and B and their standard errors are n, ΣY, ΣY^2, ΣX, ΣX^2, and ΣXY.

18.1.3 Weighting

Statistical methods commonly give little attention to the possibility that the experimental values analyzed may not all be equally reliable. Although it may sometimes be true that the primary observations, a set of v_o values, for example, have uniform variance, we must transform them before analysis into secondary values, such as $1/v_o$ or A/v_o, that are certainly not of uniform variance (Cleland, 1979; DiCera, 1992; Cornish-Bowden, 1995).

Suppose that in a set of n values of b_i each has a known *population* variance, σ_i^2. The sample variance, s^2, (defined by Eq. (18.2)) is actually an unbiased estimate of the population variance, σ_i^2, which is defined as

$$\text{population variance} = \sigma^2(b_i) = \lim_{n\to\infty} \frac{1}{n} \sum (b_i - \beta)^2 \tag{18.13}$$

where b_i is a measured quantity and β is an ideal, true value of b. We should expect that in estimating β one should allow for this variation of variances by calculating a weighted mean:

$$\bar{b} = \frac{\sum w_i b_i}{\sum w_i} \tag{18.14}$$

in which the values of the weights w_i are larger for the more reliable observations. Now, we must calculate how much larger they should be. If the individual b_i values are independent, the variance of $\bar{b}$ follows from the definition of variance in Eq. (18.14):

$$\sigma^2(\bar{b}) = \frac{\sum w_i^2 \sigma_i^2}{(\sum w_i)^2} \tag{18.15}$$

and, after the partial differentiation with respect to any individual weight w_j, we can show that the partial derivative is zero if

$$w_j = \frac{\sum w_i^2 \sigma_i^2}{\sigma_j^2 (\sum w_i)} \tag{18.16}$$

It is only possible for all such partial derivatives to be simultaneously zero if every weight is inversely proportional to the corresponding variance, that is, if

$$w_i = \frac{1}{\sigma_i^2} \tag{18.17}$$

In enzyme kinetics, one usually measures the initial rate v_o at substrate concentration A, but transforms it into $1/v_o$ or A/v_o before plotting. It can be shown that the variance of $1/v_o$ and A/v_o must be as follows:

$$\sigma^2\left(\frac{1}{v_o}\right) = \left(\frac{1}{v_o^4}\right)\sigma^2(v_o) \tag{18.18}$$

$$\sigma^2\left(\frac{A}{v_o}\right) = \left(\frac{A^2}{v_o^4}\right)\sigma^2(v_o) \tag{18.19}$$

where $\sigma^2(v_o)$ is the variance of v_o and the concentration of A is assumed to be known without error. Since the weights for v_o are usually not known, in practice, two hypotheses for the appropriate weights for v_o are commonly applied:

(a) Hypothesis that the v_o values are of uniform variance, so that

$$w(1/v_o) = v_o^4 \tag{18.20}$$

$$w(1/v_o) = v_o^4/A^2 \tag{18.21}$$

(b) Hypothesis that the v_o values have uniform coefficient of variation, so that their variances are proportional to the squares of the true v_o values, in which case

$$w(1/v_o) = v_o^2 \tag{18.22}$$

$$w(A/v_o) = v_o^2/A^2 \tag{18.23}$$

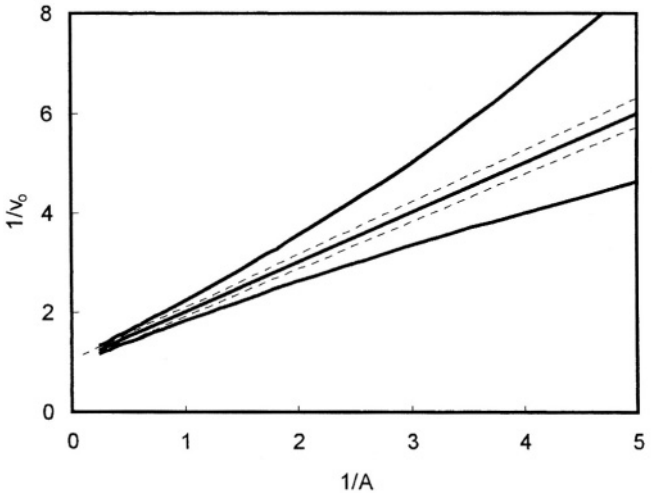


Figure 1. Envelopes of expected error for fitting the reciprocal plot, with K and V equal to unity. (——), standard error of v_0 assumed to be constant at $\pm 0.05V$; (---), standard error of v_0 assumed to be proportional to v_0 at $\pm 5\%$.

All of the expressions in Eqs. (18.20)–(18.23) are approximate, because they are derived from approximate expressions for the variances.

For practical purposes, one can choose the first weighting assumption, that is, a constant variance, if the measured values span less than a factor of 5. If the measured values span more than a factor of 10, the coefficient of variation is constant, and one chooses the second assumption (Cleland, 1979).

The necessity of using weighted fits can be seen visually from Fig. 1, which shows envelopes of probable error for data obeying the linear Lineweaver–Burk equation, for the cases where the initial rates have either equal standard errors or the standard errors proportional to initial rates of reaction. When the standard error is proportional to v_0, the variance is proportional to v_0^2 and the estimation of kinetic parameters by linear regression becomes more reliable (Fig. 1).

In fitting pH profiles for V or V/K, the parameters vary by a factor 10 per pH unit above or below the pK_a which causes loss of activity. In such cases, it is better to assume that the variance of the initial rates is proportional to the square of the initial velocity. This corresponds to a constant percent error (i.e, 1 ± 0.1 and 10 ± 1), and requires v^2 weights in reciprocal plots.

18.1.4 Polynomial Regression

Polynomial regression analysis proceeds in a manner similar to linear regression (Carnahan *et al.,* 1969). As an example, we shall analyze the second-order polynomial regression; the regression for the second-order polynomial is

$$Y = b_0 + b_1X + b_2X^2 + \varepsilon \qquad (18.24)$$

The aim of regression is to obtain estimates of parameters b_0, b_1, and b_2 which describe a regression line which fits the experimental data best. The assumptions listed in Section 18.1.3 for the linear regression will apply in this treatment of regression analysis of a second-order polynomial. For any value of the independent variable, the observed value of the dependent variable will deviate from the regression line by a residual equal to $Y - \hat{Y}$, where $\hat{Y}$ is the expected value from the regression equation. The procedure of regression analysis is to obtain estimates of the parameters which will minimize the sum of the squared residuals:

$$\sum r^2 = \sum (Y - b_0 - b_1X - b_2X^2)^2 \tag{18.25}$$

The expansion of the right-hand side of this equation, followed by differentiation with respect to each of the parameters gives

$$\frac{d\sum r^2}{db_0} = -2\sum Y + 2nb_0 + 2b_1\sum X + 2b_2\sum X^2 \tag{18.26}$$

$$\frac{d\sum r^2}{db_1} = -2\sum XY + 2b_0\sum X + 2b_1\sum X^2 + 2b_2\sum X^3 \tag{18.27}$$

$$\frac{d\sum r^2}{db_2} = -2\sum X^2Y + 2b_0\sum X^2 + 2b_1\sum X^3 + 2b_2\sum X^4 \tag{18.28}$$

Minimization of the sum of the squared residuals with respect to the parameters is achieved by setting each of the foregoing three equations equal to zero and solving the system of equations for the parameters. Thus, the following equations are obtained:

$$\sum Y = nb_0 + b_1\sum X + b_2\sum X^2 \tag{18.29}$$

$$\sum XY = b_0\sum X + b_1\sum X^2 + b_2\sum X^3 \tag{18.30}$$

$$\sum X^2Y = b_0\sum X^2 + b_1\sum X^3 + b_2\sum X^4 \tag{18.31}$$

The terms that must be obtained from the experimental data are n, ΣX, ΣX^2, ΣX^3, ΣX^4, ΣY, ΣY^2, ΣXY, and ΣX^2Y.

The variance for a second-order polynomial is given by

$$s^2 = \frac{\sum Y^2 - b_0\sum Y - b_1\sum XY - b_2\sum X^2Y}{n-3} \tag{18.32}$$

The standard error of the estimates of the parameters is given by equation:

$$[\text{standard error}(b_{i-1}) - \sqrt{s^2 \cdot c_{ii}}]_i \qquad \text{where} \quad i = 1, 2, 3, \ldots, m \tag{18.33}$$

where the correction factor, "c_{ii}", may be estimated independently (Schultz, 1994).

Polynomial regression is especially useful in substrate inhibition systems which give rise to rate equations which are a 1: 2 function of substrate concentration (Chapter 11). It is important to emphasize that the present type of

analysis is not applicable to rational polynomials of the order 2:2 or higher. Further application of the polynomial regression is in the analysis of reaction progress curves (Section 18.4.3).

18.1.5 Nonlinear Regression

When good preliminary estimates of kinetic constants are available, either from properly weighted fits to the equation in reciprocal form, or from graphical analysis, the kinetic data are best analyzed by fitting a curve using nonlinear regression (Ratkowsky, 1983). Nonlinear regression is a procedure for fitting data to any selected equation. As with linear regression, nonlinear regression procedures determine values of the parameters that minimize the sum of the squares of the distances of the data points from the curve. Unlike linear or polynomial regression, a nonlinear regression problem cannot be solved in one step. The computer programs that perform these calculations are readily available (Cleland, 1979; Watts, 1994; Cornish-Bowden, 1995; Duggleby, 1995) (Section 18.2.4).

Several methods for the nonlinear least squares fitting are known, but we shall describe only the Gauss–Newton method (Cornish-Bowden, 1995). The nonlinear regression method, based on an iterative fitting by the Gauss–Newton method, was initially proposed by Wilkinson for the estimation of the steady-state enzyme kinetic parameters (Wilkinson, 1961). The Gauss–Newton method of iteration converges rapidly and allows simple calculation of standard errors of the fitted constants. Nonlinear regression requires a preliminary estimate of the parameters, and these are obtained by linear regression similar to that described in Section 18.1.4. It should be noted that, in his original work, Wilkinson employed a weighted linear regression of A/v_0 versus A. The following expressions provide for the preliminary estimates of the parameters:

$$|D| = \sum v_0^3 \sum \frac{v_0^4}{A^2} - \sum \frac{v_0^3}{A} \sum \frac{v_0^4}{A} \tag{18.34}$$

$$K_A = \frac{\left(\sum v_0^4 \sum \frac{v_0^3}{A} - \sum v_0^3 \sum \frac{v_0^4}{A} \right)}{|D|} \tag{18.35}$$

$$V_{max} = \frac{\left[\sum v_0^4 \sum \frac{v_0^4}{A^2} - \left(\sum \frac{v_0^4}{A} \right)^2 \right]}{|D|} \tag{18.36}$$

The nonlinear aspect of Wilkinson method is based on the assumption that if a function is nonlinear in a parameter, c, the following linear approximation may be used:

$$f_{V,C} \cong f_{V,C^0} + (c - c^0) f'_{V,C^0} \tag{18.37}$$

where c^0 is a provisional estimation of c and f' is the first derivative of f with respect to c. In terms of enzyme kinetics, Eq. (18.37) becomes

$$V_0 \cong \frac{V_{max}}{V^0_{max}}\left[\frac{V^0_{max}\cdot A}{K^0_A + A} - (K_A - K^0_A)\frac{V^0_{max}\cdot A}{(K^0_A + A)^2}\right] \tag{18.38}$$

To initiate the calculations, the preliminary estimates of K_A and V_{max} from linear regression are used as the provisional estimates of the parameters. The following calculations lead to updated estimates of the parameters.

$$f = \frac{V^0_{max}A}{K^0_A + A} \tag{18.39}$$

$$f' = \frac{V^0_{max}A}{(K^0_A + A)^2} \tag{18.40}$$

$$|D| = \sum f^2 \sum f'^2 - \left(\sum ff'\right)^2 \tag{18.41}$$

$$b_1 = \frac{(\sum f'^2 2 \sum v_0 f - \sum ff'^2 \sum v_0 f')}{|D|} \tag{18.42}$$

$$b_2 = \frac{(\sum f^2 2 \sum v_0 f' - \sum ff' \sum v_0 f)}{|D|} \tag{18.43}$$

$$K_A = K^0_A + \frac{b_2}{b_1} \tag{18.44}$$

$$V_{max} = V^0_{max} b_1 \tag{18.45}$$

The updated estimate of K_A is tested against the provisional estimate. If the two are sufficiently close, for example, if $\text{abs}(K_A - K^0_A)$ is ≤ 0.005, the updated parameters are accepted as the best estimate.

If, on the other hand, the updated K_A does not pass the test, the provisional estimates of the parameters are replaced by the updated estimates and the calculations embodied in Eqs. (18.39)–(18.45) are repeated. It should be understood that, as K^0_A approaches the best estimate, the value of b_1 approaches unity and b_2 approaches zero. After the best estimates have been obtained, the variance can be calculated as

$$s^2 = \frac{\sum v_0^2 - b_1 \sum v_0 f - b_2 \sum v_0 f'}{n - 2} \tag{18.46}$$

The standard error for K_A is

$$\text{standard error}_{K_A} = \frac{1}{b_1}\sqrt{s^2\left(\frac{\sum f^2}{|D|}\right)} \tag{18.47}$$

while the standard error for V_{max} is

$$\text{standard error}_{V_{max}} = V_{max}\sqrt{s^2\left(\frac{\sum f'^2}{|D|}\right)} \tag{18.48}$$

The Wilkinson procedure converges rapidly on the best estimate of K_A if Eq. (18.38) is appropriate. If a computer program is written to execute the

Wilkinson procedure, it is advisable to count the number of iterations required for convergence. If more than 4 or 5 iterations are required, one would be well advised to question whether Eq. (18.38) is appropriate for the enzyme involved.

In fitting pH profiles for V or V/K, the parameters vary by a factor 10 per pH unit above or below the pK_a which causes loss of activity. In such cases, it is better to assume that the variance of the initial rates is proportional to the square of the initial velocity. This corresponds to a constant percent error (that is 1 ± 0.1 and 10 ± 1), and calls for v_0^2 weights in reciprocal plots. The final iterative fit may then be unweighted if the equation is expressed in the logarithmic form, since the variance of $\log v_0$ is the variance of v_0 divided by v_0^2, and the variance of v_0 in this case is proportional to v_0^2.

Iterative fitting by the Gauss–Newton method may be applied to equations that are nonlinear in two or more constants, simply by expanding Eq. (18.37).

$$v_0 = f_0 + (a - a_0)\left(\frac{\partial F}{\partial a}\right)_0 + (b - b_0)\left(\frac{\partial F}{\partial b}\right)_0 + \cdots \qquad (18.49)$$

where there are as many terms containing partial derivatives as there are non-linear constants in the rate equation. The constants $a_0, b_0, \ldots$ are the preliminary estimates of the constants $a, b, \ldots$, and F_0 is the function calculated using these preliminary values $a_0, b_0, \ldots$ More detailed information about the statistical complexities involved in fitting complex kinetic data are found in the review by Garfinkel *et al.* (1977) and the textbook of Ratkowsky (1983).

18.2 ANALYSIS OF INITIAL RATE DATA

In the analysis of initial rate data it is imperative to keep the time elapsed between data acquisition and data analysis as short as possible.

The first task is a proper choice of the substrate concentration range for kinetic measurements. For one-substrate systems, choice of the substrate concentration range is not particularly a problem. In preliminary trials, one chooses the widest range about the Michaelis constant with due care to avoid substrate inhibition. A rough value of K_M may thus be obtained, and the range can be refined. Since the greatest velocity change occurs in the region around K_M, it is frequently satisfactory to vary the concentration from about 0.2 to 5.0 times this constant. This changes the fractional attainment of maximal velocity from 0.14 to 0.84, and reasonable estimates of K_M can be made (Fromm, 1975; Endrenyi, 1981; Davis, 1992).

Once the range of substrate concentrations to be used is chosen, the intermediate levels must be decided upon. If substrate concentrations are spaced in an arithmetic series, too many of the points will be in the high concentration range. A geometric series is better, but the best procedure is probably to space the points evenly on a reciprocal plot.

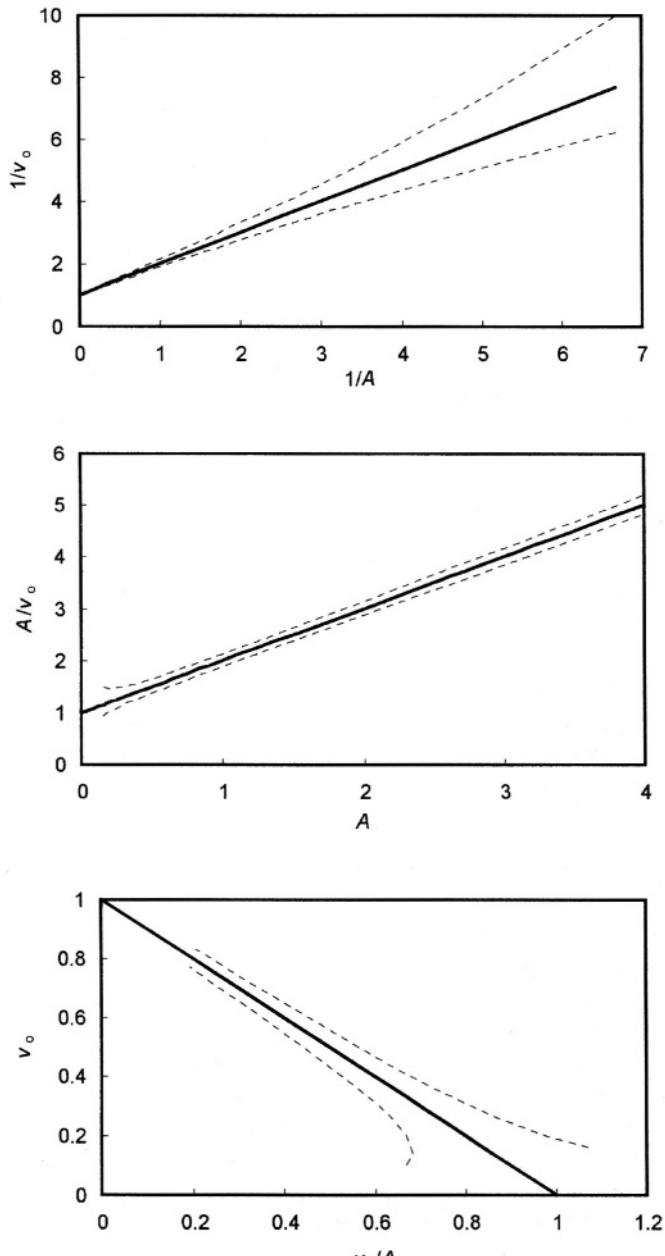


Figure 2. Envelopes of expected errors for data fitting to the linear forms of the Michaelis–Menten equation, with K and V equal to unity, and a constant standard error in v_o of $\pm 0.03V$ (dotted lines).

18.2.1 Linear Plots

If one fits the data in the form of one of the reciprocal graphs, a linear plot is always obtained. Under no circumstances should one attempt to fit data in reciprocal form for statistical evaluations of kinetic constants, since this requires weighted fits using v_0^4 weights (when equal variance in velocities is assumed) or v_0^2 weights (if the error is proportional).

Dowd & Riggs (1965) performed a statistical analysis of the three linear forms of the Michaelis–Menten equation, using the least-squares analysis. Their conclusion was that the Lineweaver–Burk is the least reliable and that, on balance, the Hanes plot is the most reliable for the graphical visualization of data. Figure 2 shows the distribution of expected errors in three linear plots, indicating that the errors are best distributed in the Hanes plot over a widest range of substrate concentrations. In the Lineweaver–Burk plot, the errors are highest at the lowest substrate concentrations and lowest at high substrate concentrations. The advantage of the Lineweaver–Burk plot is that it separates the variables.

The three linear plots in Fig. 2 show that the choice of substrate concentrations above or well above the K_M will afford a relatively precise estimation of the maximal velocity, V_{max}, but a very poor estimation of the specificity constant, V_{max}/K_M. On the other hand, a choice of substrate concentrations below or well below the K_M will afford a good estimate of the specificity constant, V_{max}/K_M, but a very poor estimate of the maximal velocity, V_{max}.

18.2.2 Analysis of Bisubstrate Reactions

For two-substrate and three-substrate enzymatic systems, the problems of data gathering become more cumbersome (Allison & Purich, 1979); a consultation of the more rigorous statistical treatment of the rate data may be necessary in these cases (Cleland, 1967, 1979; Fromm, 1975; Mannervik, 1982; Wiener *et al.,* 1991; Duggleby, 1995).

The problems of the statistical analysis of kinetic data are most easily understood in relation to a specific example. Therefore, let us consider a bisubstrate reaction that proceeds in an ordered fashion: the leading substrate A is added first, followed by substrate B, and the initial velocity equation is

$$v_0 = \frac{V_1 AB}{K_{iA}K_B + K_B A + K_A B + AB} \tag{18.50}$$

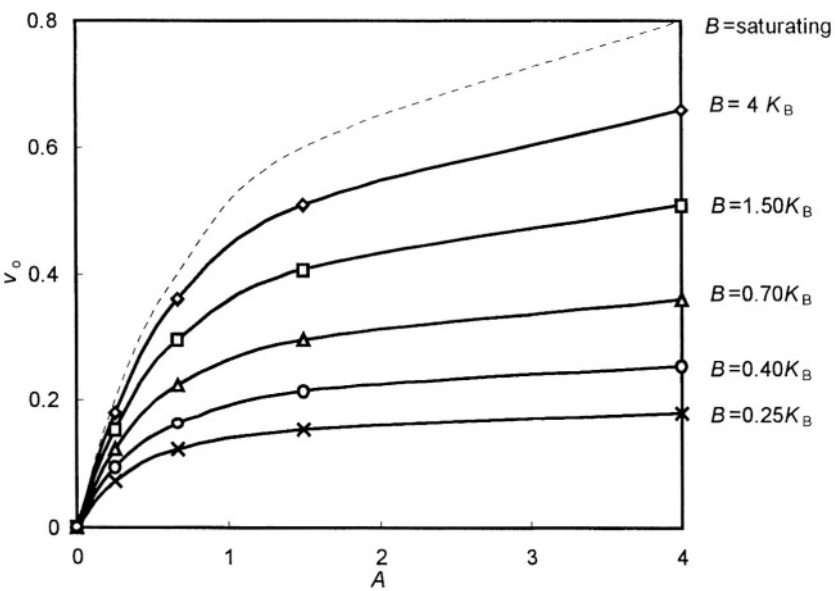
Let us calculate the initial rates of reaction with several increasing concentrations of a variable substrate A, in the presence of a fixed concentration of substrate B, and then repeat this procedure with several different concentrations of a constant substrate B (Table 1).

The first task is a proper choice of substrate concentrations. One could use the data points where no two concentrations are the same. However, it is much better to run a grid of points, because, in this way the pattern can be plotted with either substrate as the variable one. The best procedure is to space the points evenly on reciprocal plot; the spacing of lines should be picked so that the optimal replots

Table 1. Initial rates of reaction calculated according to Eq. (18.50), assuming that $V_1 = K_A = K_B = 1$, and $K_{iA} = 0.3$

A	*B*				
	0.25	0.40	0.70	1.50	4.00
0.25	0.0725	0.0952	0.1228	0.1546	0.1802
0.7	0.1228	0.1667	0.2237	0.2958	0.3590
1.5	0.1546	0.2143	0.2958	0.4054	0.5085
4.0	0.1802	0.2540	0.3590	0.5085	0.6584

are obtained. Figure 3 shows an approximately optimal choice of substrate concentrations for a bisubstrate reaction, both in A as well as in B.



Figuer 3. Ordered bisubstrate reaction. Data points are drawn according to numerical values in Table 1.

Sometimes, a need may arise to measure the initial velocities at very low or at very high substrate concentrations. For example, the latter case arises when substrate inhibition takes place at high concentrations of one of the substrates.

After a correct choice of substrate concentrations, the initial reaction rate is measured with each chosen pair of substrates. It is necessary to repeat each measurement at least once. Running duplicates or even triplicates is very useful, since it gives a rough measure of the precision of measurement and provides an insurance against exceptionally bad measurements. The number of data points is

arbitrary; it is obvious that 30 data points are better than 20, and 20 are better than 10, but the choice of the number of data points depends on many factors and must be decided upon for each specific case.

The analysis of initial rate data is performed *graphically* and *statistically.*

18.2.3 Graphical Analysis

Although graphical analysis is a quick and useful way to visualize enzyme kinetic data, for any definitive work, the data must be subjected to statistical analysis so that the precision of the kinetic constants can be evaluated. However, there are good reasons why plotting methods are essential. The human eye is much less easily deceived than any computer program and is capable of detecting unexpected behavior even if nothing currently available is found in the literature.

Figure 4 shows a plot of initial rate data in the linear form, in the Lineweaver–Burk and the Hanes fashion, replotted from the data in Table 1.

In the graphical analysis of initial rate data, it is prudent to use all three plots shown in Figs. 3 and 4. The direct plot of v_o versus [A] will show directly the influence of substrate concentration on initial rate of reaction. The two linear plots should be used together, because the Lineweaver–Burk plot serves to visualize the influence of low concentrations whereas the Hanes plot serves to visualize the influence of high concentrations of substrates. The third plot, the Eadie–Hofstee plot, is useful in detecting exceptionally bad measurements (Section3.11).

Figure 5 shows the secondary graphs, the replots of intercept and slope function from the Lineweaver–Burk plot in Fig. 4. The spacing of lines in the primary plot is important for obtaining good replots.

18.2.4 Statistical Analysis

After a kinetic experiment has been carried out, the initial velocities should be plotted for preliminary examination, so that one can tell which rate equation or equations to use in fitting the data. With the rapid advent of computer technology and personal computers, computer programs for the statistical analysis of kinetic data are now the main instrument for statistical analysis.

The problems of parameter estimation and model discrimination by statistical analysis are most easily understood in relation to a specific example. Let us consider a bisubstrate reaction that obeys the rate Eq. (18.50). The initial rate data from Table 1 are presented graphically in Figs. 3 and 4. The reciprocal plot of $1/v_o$ versus $1/A$ is a family of straight lines which, in this case, may look either parallel or intersecting due to the fact that the crossover point is distant from the vertical axis, because $K_{iA} < K_A$. If K_{iA} is much smaller than K_A, it would be impossible to distinguish between the parallel and the intersecting pattern in the double reciprocal plot. Thus, especially with a normal scatter of experimental measurements, both linear plots shown in Fig. 4 may indicate either a parallel or an intersecting pattern, that is, either a Ping Pong or a sequential bisubstrate mechanism (Chapter 9). Our task is to distinguish these two mechanisms by computer analysis.

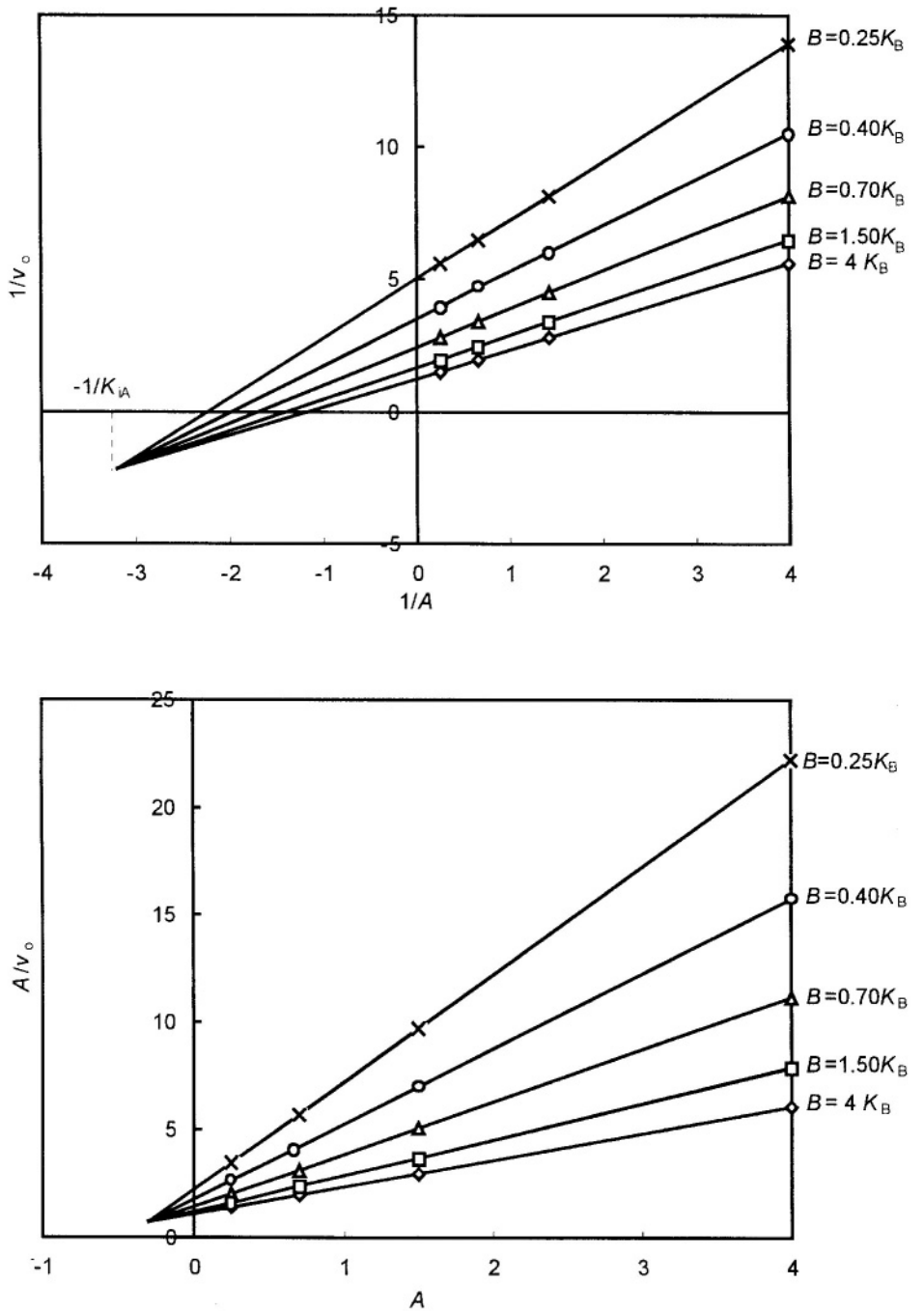


Figure 4. Ordered bisubstrate reaction. The initial rate data from Table 1 are presented in the linear form, according to Lineweaver–Burk (top) or Hanes (bottom).

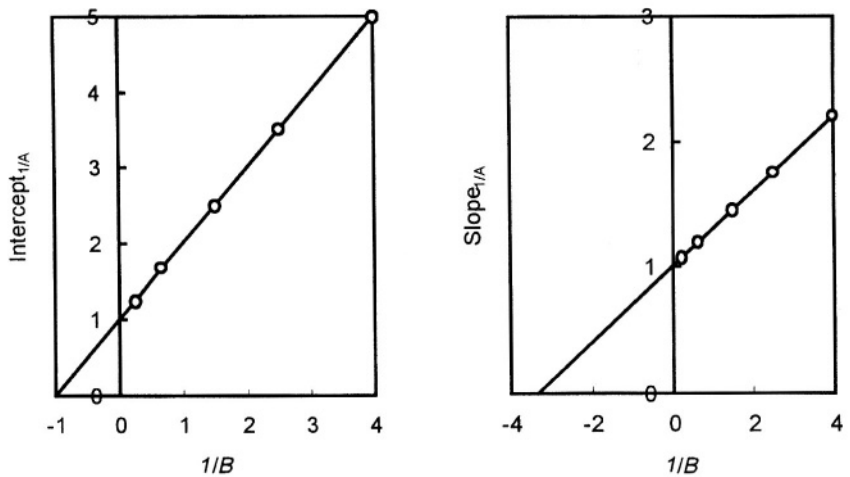


Figure 5. Replot of the intercept and the slope function from the Lineweavar–Burk plot in Fig. 4.

How to distinguish a sequential from a Ping Pong bisubstrate mechanism

In order to analyze a set of experimental data for this reaction, 20 data points are collected in Table 1, calculated with the aid of Eq. (18.50); the numerical values of data points are listed again in Table 2, showing the concentrations of each substrate pair (entries 2 and 3) and the corresponding initial velocities (entry 4). In order to perform a meaningful statistical analysis, let us produce from the data in Table 2 a set of data that are slightly biased in favor of the sequential mechanism. For this purpose, let us scramble the theoretical rate data (entry 4 in Table 2) by using the random-number generator to give the data a proper noise (entry 5, shown as "*Input* v_0"); programs for random-number generators are available in every modern statistical computer program. Thus, an average error of 5% was introduced into the calculated or "ideal" data; this is a typical random error one may expect in initial rate measurements in enzyme kinetics.

Then, let us fit the scrambled data from Table 2, shown in column 5 as "*Input* v_0", to a rate equation for two suspected mechanisms: an ordered bisubstrate mechanism (18.50) and a Ping Pong bisubstrate mechanism:

$$v_0 = \frac{V_1 AB}{K_B A + K_A B + AB} \tag{18.51}$$

The analysis of data is performed by a computer program, and thus the choice of an appropriate program must be made.

The choice of computer programs

There are numerous academic and commercial computer programs that have been developed for the purpose of statistical analysis of initial rate and binding

Table 2. Statistical analysis of initial rate data for the bisubstrate reaction

	Theoretical data from Table 1				Calculated by a computer program			
					Ordered		Ping Pong	
No.	A	B	v_0	Input v_0	Calculated	Difference	Calculated	Difference
1	2	3	4	5	6	5−6	7	5−7
1	0.25	0.25	0.0725	0.062	0.07494	−0.01294	0.09732	−0.03532
2	0.70	0.25	0.1228	0.139	0.12443	0.01457	0.13710	0.01900
3	1.50	0.25	0.1546	0.144	0.15470	−0.01070	0.15599	−0.01199
4	4.00	0.25	0.1802	0.193	0.17844	0.01456	0.16870	0.02430
5	0.25	0.40	0.0952	0.100	0.09810	0.00190	0.11751	−0.01751
6	0.70	0.40	0.1667	0.162	0.16860	−0.00660	0.18090	−0.01890
7	1.50	0.40	0.2143	0.212	0.21421	−0.00221	0.21530	−0.00330
8	4.00	0.40	0.254	0.239	0.25140	−0.01240	0.24030	−0.00130
9	0.25	0.70	0.1228	0.141	0.12588	0.01512	0.13797	0.00303
10	0.70	0.70	0.2237	0.228	0.22585	0.00215	0.23438	−0.00638
11	1.50	0.70	0.2958	0.286	0.29535	−0.00935	0.29558	−0.00958
12	4.00	0.70	0.3590	0.371	0.35511	0.01589	0.34482	0.02618
13	0.25	1.50	0.1546	0.164	0.15762	0.00638	0.15745	0.00655
14	0.70	1.50	0.2958	0.298	0.29776	0.00024	0.29677	0.00123
15	1.50	1.50	0.4054	0.397	0.40425	−0.00725	0.40223	−0.00523
16	4.00	1.50	0.5085	0.499	0.50254	−0.00354	0.49925	−0.00025
17	0.25	4.00	0.1802	0.189	0.18283	0.00617	0.17063	0.01837
18	0.70	4.00	0.3590	0.358	0.36053	−0.00253	0.34734	0.01066
19	1.50	4.00	0.5085	0.498	0.50635	−0.00835	0.50111	−0.00311
20	4.00	4.00	0.6584	0.658	0.65015	0.00785	0.66119	−0.00319
	K_A		1.0		0.96±0.08	(149)	1.25±0.11	(87)
	K_{iA}		0.3		0.27±0.08	(176)	—	
	K_B		1.0		0.99±0.08	(170)	1.27±0.10	(104)
	V_1		1.0		0.98±0.03	(877)	1.07±0.05	(422)
	SIGMA				0.0105		0.0154	
	VARIANCE				0.000110		0.000237	

Data in parentheses are the weighting factors.

data (Purich & Allison, 2000). We shall list some of the common and most used programs.

Academic programs

FORTRAN (Cleland, 1979)
LEONORA (Cornish-Bowden, 1995)

Commercial software packages

CURVE FIT (GraphPad Software Inc.)
ENZFITTER (Sigma-Aldrich)
FIT-REGRESSION ANALYSIS (Sigma-Aldrich)
GRAFIT (Sigma-Aldrich)
INSTAT (Sigma-Aldrich)
KELL (Sigma-Aldrich)
MATHEMATICA (Wolfram Research)
MATLAB (The Math Works Inc.)
MATHCAD (Mathsoft Engineering & Educatian Inc., Cambridge, Massachusetts)
ORIGIN (OriginLab, Massachusetts)
PRISM (GraphPad Software, San Diego, California)
SAS (SAS Institute Inc., SAS Campus Drive, Cary, North Carolina)
SIGMA PLOT (SPSS Science, Chicago, Illinois)
SIGMA STAT (SPSS Inc., Chicago, Illinois)
SPSS (SPSS Inc., Chicago, Illinois)
STATISTICA (StatSoft Inc., Tulsa, Oklahoma).
TABLECURVE 2D and 3D (Sigma-Aldrich)
ULTRAFIT (Sigma-Aldrich)
XPLORE (MD. Tech. Method and Data Technologies)

Computer analysis of data

Out of many available computer programs for statistical evaluation of initial rate data, we shall apply the first program from the above list, which is based on the iterative fitting by the Gauss–Newton method, due to its simplicity and reliability.

This computer program will be applied for analysis of rate data in Table 2, in order to decide between the two mechanisms. Remember that the computer program will fit the equation to the data, and not the data to an equation. From the given set of initial rate data, the computer program will calculate the kinetic parameters with a standard error, the SIGMA value and the VARIANCE, for both mechanisms and the weighted mean for each kinetic constant. The data calculated by computer are given in Table 2 in columns (6) and (7).

Once one has fitted the given rate equation to the data or, as in this case two rate equations, one has to evaluate the results and draw proper conclusions. The following criteria are used in picking the best equation (Mannervik, 1982).

Table 3. *F*-test for the data in Table 2 (Ordered Bi Bi is the true model)

	Sum of squares	Degrees of freedom	Mean square	
Ordered Bi Bi	0.080554	16		
Ping Pong	0.214497	17	0.0126174	$F = 10.6$
Difference	0.133943	1	0.133943	$P = 0.001$

(1) A good model is expected to give a rapid convergence in the regression analysis.

(2) VARIANCE, the residual sum of squares, or SIGMA, its square root. Adding extra terms to a rate equation will lower SIGMA only when the fit is really improved; thus, the fit with the lowest SIGMA is usually the best.

(3) *F-Test. F*-Test is the analysis of variance for comparing models, which is performed by comparison of the experimental data for both models with the aid of *F*-test. For the application of this test, it is necessary to calculate the sum of squares, SS, for each model,

$$SS = \sum \left(\frac{v}{\hat{v}} - 1 \right)^2 \qquad (18.52)$$

where v and $\hat{v}$ are the observed and calculated value for each data point. For a specific example in Table 2, we shall obtain the following (Table 3).

Degrees of freedom are equal to the number of data points minus the number of estimated parameters; mean square is the quotient of the sum of squares and degrees of freedom. When the mean square value for the difference is divided by the mean square value for the tested Ping Pong model, a *F*-value of 10.6 is obtained. The answers about the significance of *F*-values can be found in the tables of *F*-statistics (Winer *et al.,* 1991). In the above case, one can find in such tables that the level of significance, for this particular case, is $P = 0.001$, which indicates a highly significant difference between the two models; conventionally, we take 5% as the dividing line between "significant" and "not significant", and so we conclude that the improvement due to introduction of parameter K_{iA} is statistically significant.

(4) *Standard errors of the constants.* When the standard errors are less than 20% of the values, one can consider the values to be well determined, and thus the term containing this constant definitely present. On the other hand, values such as 2 ± 2 or -1 ± 1 show complete lack of significance, and suggest that the term may be absent. The meaning of this is that the data do not detect the presence of the term.

(5) *Randomness of the residuals* (Straume & Johnson, 1992; Cornish-Bowden, 1995). If the observed rates truly have uniform coefficient of variation, the difference should tend to increase in absolute magnitude as the calculated rate increases. On the other hand, if the observed rates really have constant standard deviations, the simple differences should be scattered in a parallel band about zero, as in Fig. 6.

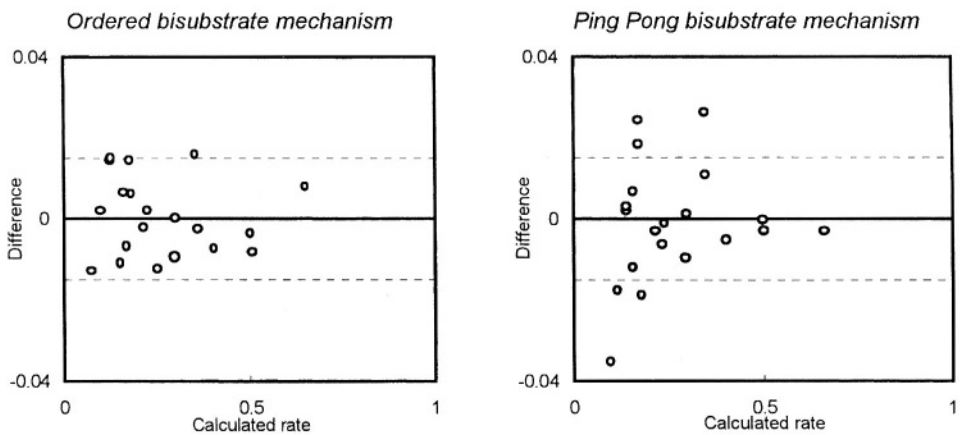


Figure 6. Residual plots for assessing the correctness of a weighting scheme.

In Table 2, the calculated values of the weighted mean *(W)* are shown for each kinetic constant, respectively; the W_i-values are larger for more reliable observations (Section 18.1.4). In this case, the *W*-values were computed from the simplified relationship, $W_i = 1/(\text{standard deviation})^2 = 1/\text{variance}$.

If the statistical parameters in Table 2 are compared, the equation for an ordered mechanism fits the data better than equation for a Ping Pong mechanism by all criteria. The standard error of all kinetic constants are lower, the SIGMA value is substantially lower, and the residual plots are better with the ordered compared to the Ping Pong mechanism, since the spread of data in Fig. 6 is larger for the latter mechanism. *F*-test also shows that the sequential mechanism is the correct one.

18.2.5 Least Squares Surfaces

The common academic and commercial computer programs using the iterative fitting by the Gauss–Newton method will handle most well-measured data with little difficulty, but in some cases where the data are bad, where preliminary estimates cannot be obtained by fitting in reciprocal form, or where the equation is simply a difficult one to fit, the program may fail to converge to a position of minimum residual sum of squares. In such cases, it is useful to examine the actual shape of the least squares surface.

In order to construct the least squares surfaces, consider the Michaelis–Menten equation with only two constants:

$$v_0 = \frac{VA}{K + A} \tag{18.53}$$

In this case, the surface is simply a contour map of residual least square as a function of *V* and *K*; the residual least square is the sum of differences between experimental value of v_o and the value of v_o calculated with the assumed values of *V* and *K*.

The construction of such a least square surface is very simple; one simply progresses through a grid of V and K values and calculates

$$\sum \left[v_i - \frac{VA_i}{K + A_i} \right]^2 \tag{18.54}$$

If the results are printed out in a grid form, one can then draw contours directly on the printed sheet; this can be done with the aid of simple computer programs.

Contour of equal residual ΣS^2 for a set of an arbitrary chosen set of data is shown in Fig. 7. The elongated diagonal shape of contours result from V being in the numerator and K in denominator, and shows that V and K can be raised or lowered together with much smaller effect on residual sum of squares than if one is raised and the other is lowered.

Examination of the least squares surfaces is useful in telling whether any minimum really exists. When no minimum is found, the contours of residual least square are of an irregular shape, forming a long shallow valley with one end closed and the another end opened. This shows that one of the constants cannot be properly estimated. The commercial statistical programs will plot the least square surfaces automatically and thus simplify the analysis.

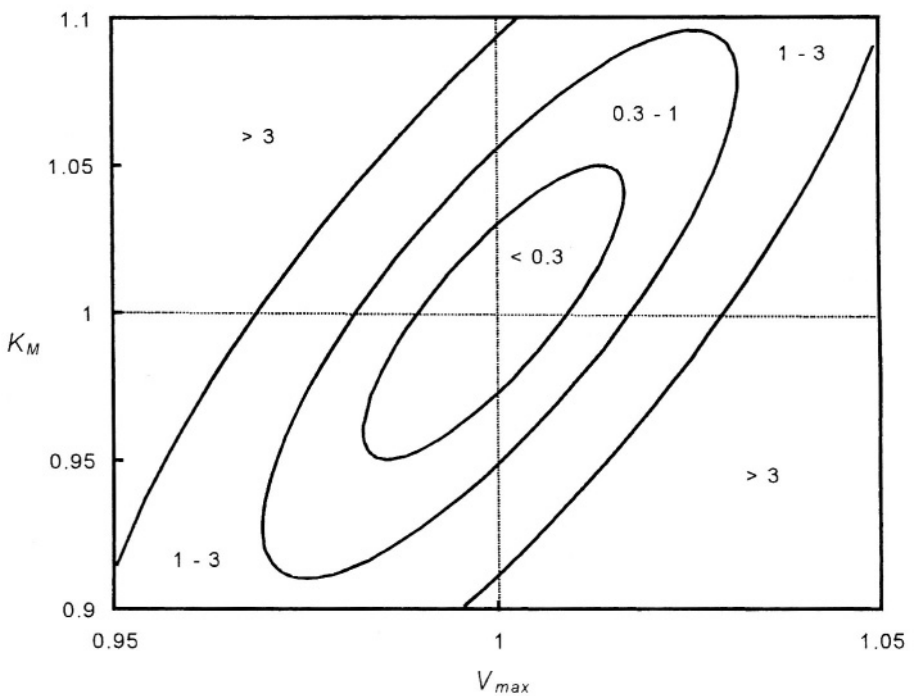


Figure 7. Contours of equal residual least square for a set of arbitrary set of data (nine data points ranging from 0.25 to 10 K_M) obeying the Michaelis–Menten equation. Contours are calculated with the aid of Eq. (18.54) and the limits are given by numerical values (multiplied by 1000).

18.3 MODEL DISCRIMINATION

The main objective in data analysis in not only the parameter estimation but also model discrimination.

Graphical analysis must always precede the statistical analysis. It is imperative to keep short the time elapsed between data acquisition and data analysis, and in most cases, it is advisable to perform the graphical analysis even while the experiment is still in progress. When the data clearly define the nature of the rate or binding equation, statistical analysis is not needed to do this. Nevertheless, for a definitive work, statistical methods are necessary for parameter estimation as well as for model discrimination (Senear & Bolen, 1992). Computer programs are now available for even the most sophisticated problems in enzyme kinetics (see Section 18.2.4).

The proper combination of graphical and statistical analysis will usually provide definitive answers for most problems in model discrimination. Several specific examples are given below.

Linear Inhibition (Chapter 5). In linear inhibition, it is particularly important to distinguish between competitive and noncompetitive types of inhibition, that is, to decide between Eqs. (18.55) and (18.56):

$$v_0 = \frac{V_1 A}{K_A\left(1 + \frac{I}{K_{iS}}\right) + A} \tag{18.55}$$

$$v_0 = \frac{V_1 A}{K_A\left(1 + \frac{I}{K_{iS}}\right) + A\left(1 + \frac{I}{K_{ii}}\right)} \tag{18.56}$$

In competitive inhibition, the $(1+I/K_{ii})$ term is missing in denominator, and consequently there is no intercept effect on the vertical axis in the $1/v_0$ versus $1/A$ plot, at different constant concentrations of I. The presence of the missing term may be detected graphically, but if K_{ii} is very large, the pattern may appear competitive; in such cases, statistical analysis may be able to detect the presence of the missing term.

Nonlinear inhibition (Chapter 6). In nonlinear inhibition, the replots of slope or intercept function from the primary plot of $1/v_0$ versus $1/A$ plot, at different constant concentrations of I, are not linear. The replots may be curved parabolically or hyperbolically, according to equations:

$$y = a + bX + cX^2 \tag{18.57}$$

$$y = a\left(\frac{1 + \frac{X}{K_{int}}}{1 + \frac{X}{K_{slope}}}\right) \tag{18.58}$$

In this case, the statistical problem is to detect the curvature of replots, that is, to distinguish by statistical methods between the nonlinear equations given above and the equation of a straight line, given by $y = a + bX$.

Bisubstrate reactions (Chapters 8 and 9). In bisubstrate reactions, a frequent case is a need to distinguish between the Steady-State Ordered, Ping Pong, and Equilibrium Ordered mechanism; the rate equations involved are

$$v_0 = \frac{V_1 AB}{K_{iA}K_B + K_A B + K_B A + AB} \tag{18.59}$$

$$v_0 = \frac{V_1 AB}{K_A B + K_B A + AB} \tag{18.60}$$

$$v_0 = \frac{V_1 AB}{K_{iA}K_B + K_B A + AB} \tag{18.61}$$

In the Ping Pong mechanism, the constant term is missing and in an Equilibrium Ordered, the $K_A B$ term is missing relative to the Steady-State Ordered mechanism. Steady-State Ordered may not be easily distinguished graphically from the Ping Pong mechanism if $K_{iA} \ll K_A$; also it cannot be easily distinguished from the Equilibrium Ordered mechanism if $K_{iA} \gg K_A$. A statistical analysis by computer must detect the presence or the absence of the given term (as described in Section 18.2).

Substrate Inhibition (Chapter 11). A general rate equation for substrate inhibition in monosubstrate and bisubstrate reactions (when the concentration of a noninhibitory substrate is held constant), is

$$v_0 = \frac{V_1 A}{K_A + A + A^2/K_i} \tag{18.62}$$

In such cases, it is important that the data cover both high and low concentrations of substrate so that the initial velocities fall within several orders of magnitude. In substrate inhibition, graphical analysis precedes statistical analysis, and data are usually plotted in the form of v_0 versus log [A] (Fig. 7); from such plots, the initial estimate of K_i can be obtained by graphical methods (Section 11.2.7).

Trisubstrate reactions (Chapter 12). In trisubstrate reactions, initially, one is trying to detect the absence of particular term(s) from the general rate equation for trisubstrate mechanisms by graphical methods, simply by trying the proper combinations of all three substrates in double reciprocal plots (Section 12.1). When the kinetic mechanism is established in this way, the rate data are fitted to an appropriate rate equation by computer.

pH studies (Chapter 14). In pH studies, one is usually dealing with an enormous span of hydrogen ion concentrations and, therefore, all plots are always presented in a logarithmic form, both in graphical and in statistical analysis. In pH studies, one of the typical problems is to distinguish between the pH plot for a monobasic from the pH plot for a dibasic acid, depicted by equations:

$$Y = \frac{C}{\left(1 + \frac{H}{K_a}\right)} \tag{18.63}$$

$$Y = \frac{C\left(1 + \frac{H}{K_2}\right)}{1 + \frac{H}{K_3}\left(1 + \frac{H}{K_1}\right)} \qquad \text{where} \qquad K_1 > K_2 > K_3 \tag{18.64}$$

Equation (18.63) has a form of a titration curve of a monobasic acid with a plateau in alkaline which is decreasing in the acid over a single pK_a value (Fig. 6). Equation (18.64) has a form of a titration curve of a dibasic which is also decreasing in the acid, but this time over two pK_a values, pK_3 and pK_1 (Fig. 8). In this case, if pK_3 and pK_1 are not sufficiently apart, separated by at least two pH units, the titration curve may resemble that for the monobasic acid, with a single pK_a value, and Eq. (18.64) is reduced to Eq. (18.63); in such cases, even the statistical analysis can fail to detect the presence of two dissociating groups in the enzyme.

18.4 INTEGRATED MICHAELIS–MENTEN EQUATION

A kinetic equation can be written in two distinct forms, either as an expression that shows how the concentration of reactants change with time, or as one that shows how the rate of reaction varies with the concentrations of the reactants. The enzymologist normally considers the second of these to be the usual form, whereas to the chemist the first is the usual form. Chemists have continued to prefer integrated rate equations, which have the merit to express what is actually measured. Michaelis and Menten (1913) showed that the behavior of enzymes could be studied much more simply by measuring initial rates, when the complicating effects of product accumulation and substrate depletion did not interfere.

Nevertheless, in spite of various problems which arise when the progress curves of enzyme reactions are studied, there is no doubt that the analysis of progress curves forms a very valuable part of enzyme kinetics, provided that it is carried out cautiously (Strayton & Fromm, 1979; Duggleby, 1995).

18.4.1 Irreversible Reaction

Consider an irreversible monosubstrate reaction, according to Michaelis and Menten:

$$E + A \rightleftharpoons EA \rightarrow E + P$$

Before the Michaelis–Menten equation can be integrated, it is necessary to introduce into it some relationship that exists between the reactant concentrations. Therefore, let us write v_o as $-dA/dt$, and substitute it into the Michaelis–Menten equation to form:

$$v_0 = -\frac{dA}{dt} = \frac{V_{max}A}{K_A + A} \qquad V_{max}dt = -\left(\frac{K_A + A}{A}\right)dA \tag{18.65}$$

Integrating between any two times (i.e., zero-time, t_0, and any other time, t) and the corresponding substrate concentrations A_0 and A:

$$V_{max}\int_{t_0}^{t} dt = -\int_{A_0}^{A}\left(\frac{K_A + A}{A}\right)dA \tag{18.66}$$

Separating the terms in the right-hand expression,

$$V_{max}\int_{t_0}^{t} dt = -K_A\int_{A_0}^{A}\frac{dA}{A} - \int_{A_0}^{A} dA \tag{18.67}$$

$$V_{max}t = -K_A \ln\frac{A}{A_0} - (A - A_0) \tag{18.68}$$

Equation (18.68) is the integrated form of the Michaelis–Menten equation. Rearranging, we obtain

$$\frac{A_0 - A}{t} = V_{max} - K_A\left[\frac{\ln(A_0/A)}{t}\right] \tag{18.69}$$

where $(A_0 - A)$ is the concentration of substrate utilized by time t.

Equation (18.69) resembles the Hanes plot in the initial rate case. Similarly, Eq. (18.69) can be transformed in various ways in order to permit plotting as a straight line:

$$\frac{t}{\ln(A_0/A)} = \frac{1}{V_{max}}\left[\frac{A_0 - A}{\ln(A_0/A)}\right] + \frac{K_A}{V_{max}} \tag{18.70}$$

$$\frac{t}{A_0 - A} = \frac{K_A}{V_{max}}\left[\frac{1}{(A_0 - A)}\right]\ln\left(\frac{A_0}{A}\right) + \frac{1}{V_{max}} \tag{18.71}$$

Thus, a reaction progress curve may be analyzed along the entire course of reaction.

18.4.2 Product Inhibition in an Irreversible Reaction

Equations (18.69)–(18.71) were derived with an assumption that the overall reaction is irreversible, and that the product inhibition is insignificant. However, it is safer to assume that it does occur and to calculate the product inhibition constant as a part of the analytical procedure. Thus, in an irreversible reaction, with a competitive product inhibition, we obtain

$$v_0 = -\frac{dA}{dt} = \frac{dP}{dt} = \frac{V_{max}(A_0 - P)}{K_A\left(1 + \frac{P}{K_P}\right) + (A_0 - P)} \tag{18.72}$$

Equation (18.72) is obtained from Eq. (18.65) by substituting A with (A_0-P) and multiplying the Michaelis constant K_A with a factor $(1+P/K_P)$, because a competitive inhibition with P is assumed.

Equation (18.72) can be integrated in the same way as Eq. (18.65) to obtain

$$V_{max}t = \left(1-\frac{K_A}{K_P}\right)(A_0-A) + K_A\left(1+\frac{A_0}{K_P}\right)\ln\frac{A}{A_0} \qquad (18.73)$$

This equation can also be written in three linear forms, as was shown for Eq. (18.68).

Equation (18.73) has found only a limited use, because it is impossible to distinguish in a single run between the slowing down that results from depletion of substrate and slowing down that results from competitive inhibition by the accumulating product; thus, Eq. (18.73) is not providing the means of calculating the inhibition constant K_P, and the kinetic parameters V_{max} and V_{max}/K_A.

18.4.3 Estimation of Initial Rates of Reaction

A correct estimation of initial rates of reaction is an important part of analytical procedures in enzyme kinetics. The methods for the estimation of initial rates of reaction are most easily understood in relation to a specific example. Thus, for example, consider an oxidation of NADH by acetaldehyde, catalyzed by yeast alcohol dehydrogenase (Leskovac, 2000):

$$\underset{(B)}{\text{Acetaldehyde}} + \underset{(A)}{\text{NADH}} + \text{H}^+ \rightleftharpoons \underset{(P)}{\text{Ethanol}} + \underset{(Q)}{\text{NAD}^+} \qquad (18.74)$$

Figure 8A shows the entire course of this reversible reaction.

Reaction was started with a saturating concentration of acetaldehyde (20.3 mM), nonsaturating concentration of NADH (30.5 μM), and enzyme (0.2 nM), at pH 7.0, 25°C; the reaction progress curve shows the appearance of product, the NAD^+. It is obvious from Fig. 8A that the rate of reaction decreases with time with depletion of substrate and that the initial rate is a tangent to the reaction progress curve going through the origin.

In order to estimate the initial rate, the entire course of this reversible reaction was integrated according to Eq. (18.73), and redrawn in a linear form in Fig. 8B. Since the reaction was started with a saturating concentration of one of the substrates and, in this case, $K_P >> K_A$ and $[B_0] >> [A_0]$, it is admissible to treat reaction (18.74) as an irreversible monosubstrate reaction and fit the entire reaction progress curve to Eq. (18.73). From the linear plot, one can calculate the maximal velocity V (equal to a reciprocal intercept on ordinate) and V/K (equal to a reciprocal slope).

Thus, from Fig. 8B, one can calculate the numerical values of kinetic constants:

$$k_{cat} = V_1/E_0 = 0.42\ \mu M\ s^{-1}/0.0002\ \mu M = 2100\ s^{-1}$$

$$V/K = V_1/K_A E_0 = 0.0052\ \mu M^2\ s^{-1}/0.0002\ \mu M = 2600\ \mu M\ s^{-1}$$

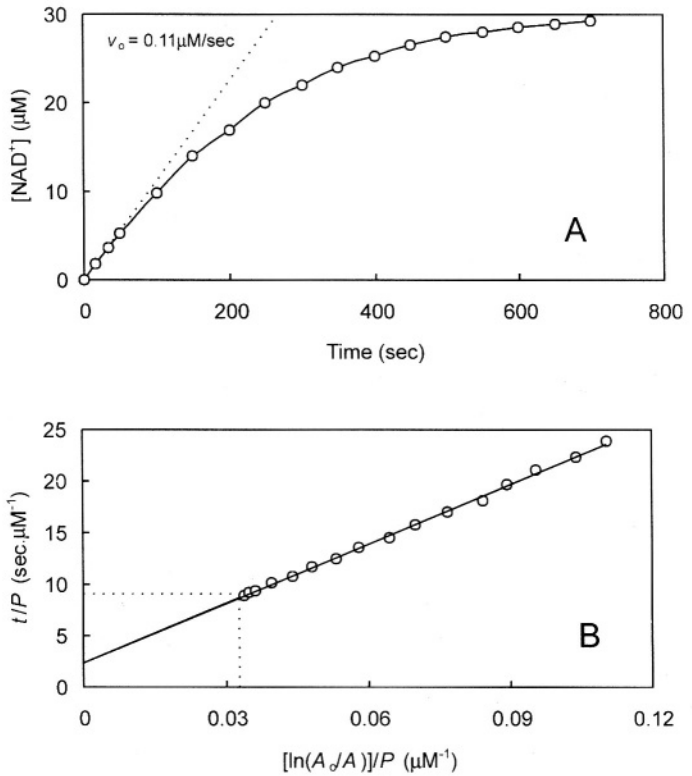


Figure 8. (A) Reaction progress curve for the yeast alcohol dehydrogenase-catalyzed oxidation of NADH with acetaldehyde and (B) the replot of the data from the progress curve according to the linear transformation of the rate equation (18.73) (Leskovac, 2000).

Note that the data points in Fig. 8B are converging to $1/v_0$ (9 s μM^{-1} on ordinate) and to $1/A_0$ (0.033 μM^{-1} on abscissa), as the time tends to zero. Thus, the main advantage of the integrated forms of the Michaelis–Menten equation is the possibility to obtain an accurate estimate of the initial rate of reaction, v_0.

In cases when it is not convenient to use the integrated rate equation, it is still possible to use progress curve data advantageously: instead of fitting the data to the true equation, they can be fitted to an equation of the form

$$[P] = \beta_0 + \beta_1 t + \beta_2 t^2 + \beta_3 t^3 + \cdots \tag{18.75}$$

which defines any single-values continuous function. The coefficients β_0, β_1, β_2, β_3, and so on, can be estimated by the method of least squares, preferably by a computer program, and then the initial velocity can be estimated from

$$\frac{d[P]}{dt} = \beta_1 + 2\beta_2 t + 3\beta_3 t^2 + \cdots \tag{18.76}$$

as v_o tends to β_1 when t tends to zero. In principle, the more terms are included in an equation, the better the fit becomes. However, in practice, with enzymatic progress curves, almost all of the useful information is contained in the first three terms of Eq. (18.75) and the values of β_4 and higher terms are determined largely by random error.

The initial rate of reaction, in the above example with alcohol dehydrogenase, was calculated from the reaction progress curve with the aid of the polynomial (18.75) (Table 4). The value of the initial rate of reaction, v_o, is found in the first t-term in Table 4. Consequently, it is not advisable to go beyond the term t^3 and for many purposes the term in t^2 will define the curvature accurately enough (Knowles, 1965).

Table 4. Fit of Eq. (18.75) to experimental data points from the Fig. 6A

Order of the polynomial	t^5	t^4	t^3	t^2	t	R
2				−0.0001	0.0979	0.9954
3			8×10^{-8}	−0.0002	0.1138	0.9999
4		-7×10^{-12}	9×10^{-8}	−0.0002	0.1143	0.9999
5	-3×10^{-14}	4×10^{-11}	8×10^{-8}	−0.0002	0.1139	0.9999

The estimation of v_o by fitting the progress curve to a power series in t has the advantage when compared with the use of integrated equations. It can be done regardless of whether the correct rate equation is known or not, and is not affected by complications such as progressive denaturation of enzyme during assay.

18.4.4 Computer Analysis of Reaction Progress Curves

The determination of kinetic parameters is normally made by using initial velocities. However, initial velocities are not useful, or at least become more difficult to deal with, under a variety of conditions, including when the enzyme concentration is equal to or higher than the substrate concentration, when there is a strong product inhibition or a strongly reversible reaction, when dealing with single turnover reactions, and so on. Under such conditions, it is better to simply analyze the complete time-course of the reaction. Unfortunately, expressions describing the substrate depletion as a function of time, that is, an integrated rate equation, are complex and difficult to obtain (Foster & Niemann, 1953; Orsi & Tipton, 1979; Rebholz & Northrop, 1995).

Advances in computer technology have allowed investigators to develop computer programs that will numerically integrate differential equations, even for complex equations. Several of such programs have been described in the literature and are currently in use; one of these is a KINSIM/FITSIM computer software package for numerical integration of rate equations developed by Frieden (Barshop *et al.,* 1983; Zimmerle & Frieden, 1989; Frieden, 1994). The application of such computer programs to complex kinetic mechanisms that contain several enzyme forms and a number of rate constants, usually requires a preliminary

estimate of most rate constants. Once the numerical values of the majority of rate constants are already known, an appropriate computer program may be able to estimate the values of the remaining rate constants and refine the values of all rate constants in the given mechanism by a variety of fitting procedures. The most powerful kinetic analysis of an enzyme mechanism assigns rate constants to each step, and with transient kinetics and simulations, such mechanisms can be described in full (Chandra Sekhar & Plapp, 1990; Plapp, 1995).

REFERENCES

Books

Carnahan, B., Luther, H.A., & Wilkes, J.O. (1969) *Applied numerical methods,* Wiley, New York.
Cornish-Bowden, A. (1995) *Analysis of enzyme kinetic data,* Oxford Science Publications, Oxford.
Daniel, W.W. (1995) *Biostatistics,* 6th ed., Wiley, New York.
Endrenyi, L. (ed.) (1981) *Kinetic data analysis: design and analysis of enzyme and pharmacokinetic experiments,* Plenum Press, New York & London.
Fromm, H.J. (1975) *Initial rate enzyme kinetics,* Springer-Verag, Berlin.
Mandelhall, W., Schaffer, R.L., & Wackerly, D.D. (1981) *Mathematical statistics with applications,* 2nd ed., Duxbury Press, Boston.
Purich, D.L., & Allison, D.R. (2000) *Handbook of biochemical kinetics,* Academic Press, San Diego.
Ratkowsky, D.A. (1983) *Nonliner regression modeling,* Marcel Dekker, New York.
Rosner, B. (1996) *Fundamentals of biostatistics,* 4th ed., Duxbury Press, Belmont, California.
Schultz, A.R. (1994) *Enzyme kinetics,* Cambridge University Press, Cambridge.
Winer, B.J., Brown, D.R., & Michels, K.M. (1991) *Statistical principles in experimental design,* 3rd ed., McGraw-Hill, New York.
Zar, J.H. (1996) *Biostatistical analysis,* 3rd ed., Prentice-Hall, New York.

Review articles

Allison, R.D. & Purich, D.L. (1979) *Methods Enzymol. 63,* 3–21.
Cleland, W.W. (1967) *Adv. Enzymol. 29,* 1–32.
Cleland, W.W. (1979) *Methods Enzymol. 63,* 103–138.
Davies, J.S. (1992) *Methods Enzymol. 210,* 374–390.
Duggleby, R.G. (1995) *Methods Enzymol. 249,* 61–90.
DiCera, E. (1992) *Methods Enzymol. 210,* 54–67.
Frieden, C. (1994) *Methods Enzymol. 240,* 311–322.
Garfinkel, L., Kohn, M.C., & Garfinkel, D. (1977) *Crit. Rev. Bioenerg. 2,* 329–361.
Mannervik, D. (1982) *Methods Enzymol. 87,* 370–389.
Orsi, B.A. & Tipton, K.F. (1979) *Methods Enzymol. 63,* 159–183.
Plapp, B.V. (1995) *Methods Enzymol. 249,* 91–122.
Rebholz, K.L. & Northrop, D.B. (1995) *Methods Enzymol. 249,* 211–240.
Senear, D.F. & Bolen, D.W. (1992) *Methods Enzymol. 210,* 463–480.
Strayton, M.M. & Fromm, H.J. (1979) *Methods Enzymol. 78,* 309–320.
Straume, M. & Johnson, M.L. (1992) *Methods Enzymol. 210,* 87–105.
Watts, D.G. (1994) *Methods Enzymol. 240,* 23–36.

Specific references

Barshop, B.A., Wrenn, R.F., & Frieden, C. (1983) *Anal. Biochem. 130,* 134–145.
Chandra Sekhar, A. & Plapp, B.V. (1990) *Biochemistry 290,* 4289–4295.
Dowd, J.E. & Riggs, D.S. (1965) *J. Biol. Chem. 240,* 863–869.
Foster, R.J. & Niemann, C. (1953) *Proc. Natl. Acad. Sci. USA 39,* 999–1010.

Johansen, G. & Lumry, R. (1961) *C. R. Trav. Lab. Carlsberg 32,* 185–214.
Knowles, J.R. (1965) *Biochem. J. 95,* 180–188.
Leskovac, V. (2000) Unpublished results.
Michaelis, L. & Menten, M.L. (1913) *Biochem. Z. 49,* 333–369.
Wilkinson, G. (1961) *Biochem. J. 80,* 324–330.
Zimmerle, C.T. & Frieden, C. (1989) Biochem. *J. 258,* 381–386.

This page intentionally left blank

Subject Index