FOLATE IN HEALTH AND DISEASE

edited by

Lynn B. Bailey

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To my mother, whose devotion to family and to the library named in her honor is an inspiration; to my husband Gary and son Gene for their love and delightful sense of adventure.

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Series Introduction

Nutrition has always been recognized as having a significant impact on health. Initially, the primary interest was the prevention of nutritional deficiencies. As concern with overt deficiencies diminished in developed countries, emphasis has shifted to the role of nutrition in maintenance of optimal health. For example, in the last 30 years, we have seen considerable interest in the effects of overnutrition (i.e., obesity), the role of saturated and polyunsaturated fats in cardiovascular disease and cancer, and the effects of sugar on dental caries.

We are now entering a new era of nutrition. In the case of macronutrients such as fat, our knowledge is becoming more precise, and significant progress is being made in defining which specific fatty acids in fats are responsible for the deleterious or beneficial health benefits. In the case of vitamins and minerals, information on their role in health and disease is increasing at an exponential rate.

We are starting to recognize that many micronutrients have a significant impact on the development—and therefore the risk—of many, if not the majority, of chronic diseases including cardiovascular disease, cancer, osteoporosis, diabetes, periodontal disease, and cataracts as well as various inflammatory and neurological diseases. Nutrition also impacts on the resistance to infectious diseases, sensory and cognitive function, and the risk of birth defects. The aim of this series is to bring to readers a detailed discussion of the more active and exciting aspects of this new era of nutrition.

This volume of the series, *Folate in Health and Disease*, is an excellent example of how our sophistication in understanding both the biochemical function and relevance to health has progressed for a particular nutrient. Dr. Bailey, a recognized authority on folate, has gathered other leaders in this field whose expertise covers all pertinent aspects of folate nutrition. This book is of particular interest

and timely in view of the recently acknowledged roles of folate in neural tube defects, cancer, and cardiovascular disease. It will be a valuable reference for everyone interested in this subject.

Lawrence J. Machlin

Preface

The importance of folate as the cornerstone of one-carbon metabolism has been recognized for decades. The clinical relevance of the metabolic role of this vitamin is a current area of intense investigation. The aim and scope of this book are to present scientists, including clinical practitioners, with an evaluation of recent data relevant to understanding: how folate is involved in the maintenance of normal metabolism; the changes in folate requirements associated with physiological stresses such as pregnancy: the metabolic and cellular alterations that result from inadequate folate intake; interactions of folate and other nutrients, drugs, and alcohol; the association between folate inadequacy and increased risk of chronic disease and neural tube defects; and the application of antifolate drugs for the treatment of neoplastic and nonneoplastic diseases. The basic scientist, as well as the practitioner, is presented with an up-to-date synopsis and interpretation of data that can be applied to either the research or clinical setting.

The first chapter reviews current knowledge related to mechanisms by which folate is handled in the body and how tissue folate levels and homeostasis are regulated. The following chapter provides an understanding of one-carbon metabolism as the biochemical basis for the effect of folate deficiency on cellular methylation reactions and manifestations of folate inadequacy such as defective cell division. A description of the sequence of events occurring in response to progressive folate depletion is presented in the third chapter and attention focused on appropriate diagnostic measures to characterize a folate deficiency in the clinical setting.

The potential for folate to modulate one of the risk factors for atherosclerosis, elevated homocysteine, is an intriguing area of research investigation addressed in the fourth chapter. The biochemistry of homocysteine metabolism and the var-

ious causes of hyperhomocysteinemia are reviewed. Data are presented relative to therapeutic interventions with vitamins, including folic acid.

Recommended Dietary Allowances (RDAs) are compared in Chapter 5 with other dietary folate recommendations available worldwide, while the experimental data on which they are based are critiqued. Physiological changes that may influence folate requirements are reviewed with a special emphasis on the increased folate utilization associated with pregnancy. Dietary sources of folate are contrasted and folate-dense foods identified to assist nutrition educators. In Chapter 6, the influence of maternal folate adequacy on human milk folate content and the status of the nursing infant and lactating woman are reviewed. Folate status of the U.S. population is then summarized in Chapter 7 based on data available from national surveys and studies of population subgroups.

The review of folate bioavailability presents a description of the efficiency of utilization, including an overview of the physiological and biochemical processes involved in folate absorption, transport, metabolism, and excretion, and the influence of food composition on these processes. Clarification of these issues is important to predict both the ability of diets to provide available folate to meet nutritional requirements and the impact of dietary modifications such as enrichment of foods with folic acid.

In light of the recommendations to increase folate intake in large segments of the population, the metabolic and clinical interrelationships of folate, vitamin B_{12}, and zinc are reexamined. The concern relative to the ability of folic acid to interfere with the diagnosis of a vitamin B_{12} deficiency is explored and an in-depth review of research data presented.

The remaining chapters focus on the potential association between folate status and alcoholism, neural tube birth defects, and cancer. Excess alcohol consumption increases the risk for folate deficiency which mediates several aspects of the disease of chronic alcoholism, including anemia, diarrhea with intestinal malabsorption, and increased prevalence of colonic neoplasia.

Data from large-scale clinical intervention trials provide a strong basis on which to base conclusions relative to the reduction in the occurrence of neural tube defects in women supplemented with folic acid during the periconceptional period. A detailed critique of data demonstrating a positive association between folate intake and risk reduction for neural tube defects is presented and a proposed metabolic basis for this birth defect discussed.

Cancer is a devastating disease that ranks second only to cardiovascular disease as a cause of death in the United States. A growing body of clinical studies suggests that folate status can modulate the process of carcinogenesis, with the strongest evidence associated with colorectal cancer. Diminished folate status is associated with a higher risk of carcinogenesis whereas supraphysiological status may convey some protective effect.

Folate antagonists occupy a central position in cancer treatment, and intervention in folate metabolism has been a long-standing target for cancer chemotherapeutic agents. The search for the best antifolate drug, or combination of drugs, to use with appropriate folate therapy has resulted in an enormous amount of data, which are condensed into a brief review in this book. The biochemical basis for the action of the antifolate drugs is addressed and current new areas of investigation highlighted.

Compounds that interfere with folic acid metabolism now also play important roles in the therapy of nonmalignant diseases, especially autoimmune diseases such as rheumatoid arthritis. The use of specific antifolates in the treatment of non-neoplastic diseases is reviewed and toxicities seen in clinical practice discussed. The final chapter reviews data suggesting that regardless of whether folate deficiency is primary or secondary to psychiatric disorders, folate replacement may improve mental function.

In summary, this book provides an in-depth review of new areas of clinical interest related to folate metabolism. Maintenance of folate metabolism in health and disease is approached by integrating a review of basic biochemical pathways and physiological functions with innovative approaches to the application of this knowledge in the clinical setting.

Lynn B. Bailey

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FOLATE IN HEALTH AND DISEASE

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1

Folate Chemistry and Metabolism

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I. INTRODUCTION

The vitamin folic acid was initially investigated as a dietary antianemia factor distinct from the pernicious anemia factor [1] and a recent historical review details early studies on the isolation of the vitamin and on the establishment of its role as a cofactor in one-carbon metabolism [2]. Because of the role of folate coenzymes in the synthesis of DNA precursors, folate antagonists have found widespread clinical use as antiproliferative and antimicrobial agents. It has been known for many years that the pernicious anemia that results from defects in vitamin B_{12} availability is due to induction of a secondary functional folate deficiency [3], but the metabolic interrelationship between vitamin B_{12} and folate remains controversial. More recently, the demonstration [4,5] that periconceptional supplementation with low doses of folic acid reduces the incidence of neural tube defects has generated considerable clinical and public health interest, with proposals that the food supply be supplemented with folic acid, although the metabolic basis for this effect is not understood.

Regimens for the treatment of folate-related disorders, the use of folate antagonists, and the advantages or potential disadvantages of folate supplementation require an understanding of normal folate metabolism and of the metabolic consequences of derangements in folate metabolism. This chapter presents an overview of current knowledge of the mechanisms by which folate is handled in the body and how tissue folate levels and homeostasis are regulated. The role of folates as coenzymes in one-carbon metabolism and the clinical consequences of derangements in folate metabolism are discussed in greater detail in accompanying chapters.

II. CHEMISTRY

Folic acid (pteroylmonoglutamate, PteGlu) consists of a 2-amino-4-hydroxypteridine (pterin) moiety linked via a methylene group at the C-6 position to a *p*-aminobenzoyl-glutamate moiety. Folate metabolism involves the reduction of the pyrazine ring of the pterin moiety to the coenzymatically active tetrahydro form (Fig. 1), the elongation of the glutamate chain by the addition of glutamate residues in γ-peptide linkage, and the acquisition and oxidation or reduction of one-carbon units at the N-5 and/or N-10 positions. Folate coenzymes function as acceptors and donors of one-carbon moieties in reactions involving nucleotide and amino acid metabolism. These reactions, known as one-carbon metabolism, are discussed in Chapter 2. The active coenzymatic species used in these reactions are folylpolyglutamates. The physiological roles of polyglutamate derivatives have been reviewed [6,7]. Folylpolyglutamates are more effective substrates and inhibitors than pteroylmonoglutamates of most folate-dependent enzymes and usually exhibit increased affinity and lower K_m values for these enzymes. Folylpolyglutamates are also preferentially retained by tissues.

Mammals lack the ability to synthesize folates de novo and require preformed folates in the diet. Naturally occurring folates are reduced derivatives, and consequently fully oxidized folic acid is only found in the diet when foodstuffs are supplemented with folic acid or when dietary folates are oxidized. Reduced folates are less stable than folic acid, and their stability varies depending on the one-carbon substitution. Large losses in food folate can occur during food preparation such as heating, particularly under oxidative conditions [8]. Oxidation of reduced folates usually results in cleavage products lacking vitamin activity, although a small proportion may be converted to biologically active oxidized forms [8]. Additional losses can also occur by leaching out folate during food preparation [8]. Frozen storage

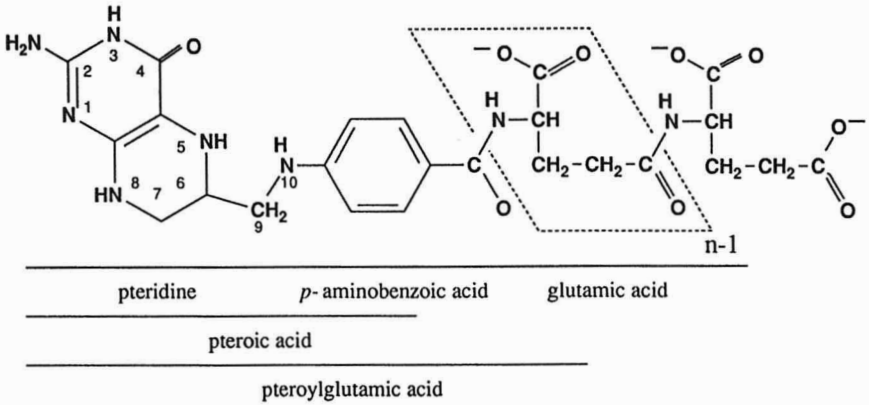

Figure 1 Structure of tetrahydropteroylpoly-γ-glutamate.

of foods leads to only slow loss of folate, although polyglutamate derivatives may be hydrolyzed to monoglutamates during storage or subsequent food preparation [8].

Folates are stabilized by storage in the absence of oxygen or in the presence of reducing agents such as ascorbate. Ascorbate-protected plasma, red blood cell, or urine samples can be stored frozen for long periods without appreciable loss of folate.

III. ABSORPTION AND TRANSPORT

Most dietary folates are polyglutamate derivatives and are hydrolyzed to monoglutamate forms in the gut prior to absorption across the intestinal mucosa. The hydrolysis of dietary folylpolyglutamates, which is catalyzed by γ-glutamylhydrolase, and the intestinal absorption of pteroylmonoglutamates are described in Chapter 8. Little is known concerning the stability of folate during its passage through the different microenvironments in the gut. The mechanism by which folate crosses the mucosal cell and is released across the basolateral membrane into the portal circulation is not understood. Some metabolism of folate, primarily to 5-methyl-H_4PteGlu, can occur during this process, but metabolism is not required for transport. The degree of metabolism in the intestinal mucosa is dependent on the folate dose given. When pharmacological doses of various folates are given, most of the transported vitamin appears unchanged in the portal circulation [9].

A. Plasma Transport

Pteroylmonoglutamates, primarily 5-methyl-H_4PteGlu, are the circulating forms of folate in plasma, and mammalian cells are not believed to transport polyglutamates of chain length three and above. After folate absorption into the portal circulation, much of this folate can be taken up by the liver, where it is metabolized to polyglutamate derivatives and retained or released into blood or bile. Enterohepatic circulation of folate has been described involving release of hepatic 5-methyl-H_4PteGlu into bile with reabsorption in the small intestine [10]. The predominance of 5-methyl-H_4PteGlu in plasma probably reflects that this is the major cytosolic folate in mammalian tissues and consequently is the major form that would be released (see Sec. IV.A). The extent, if any, of release of short-chain folylpolyglutamates from tissues is unknown. Plasma contains γ-glutamylhydrolase activity, and any polyglutamate released into plasma would be hydrolyzed to the monoglutamate.

A variable proportion of plasma folate is bound to low affinity protein binders, primarily albumin (K_d folate ~ 1 mM), which accounts for about 50% of bound folate, and the proportion is increased in folate deficiency [11]. Plasma also contains low levels of a high affinity folate binder (K_d folate ~ 1 nM). The levels of

the high affinity binder are increased in pregnancy and are very high is some leukemia patients [11]. The high affinity binder appears to be the same protein as the cellular high affinity folate binding protein (see Sec. III.B), which can be released from cells after hydrolysis of its glycosylphosphatidylinositol anchor. It has been suggested that protein binding may facilitate folate transport by tissues such as liver [12]. However, increased levels of the high affinity binder may reflect tissue damage or incidental release from tissues, and the effect of protein binding on folate availability for tissues remains an open question [11].

Red blood cells contain higher levels of folate than plasma, and practically all the red cell folates are 5-methyl-H_4PteGlu polyglutamates. Mature red cells and lymphocytes have a negligible capacity to transport and accumulate folate (see Sec. IV.D) and their folate stores accumulate during erythropoiesis and appear to be retained, probably due to protein binding, through the life span of the cell. Because of this, red cell folate levels are often used as a measure of long term folate status. Fasting plasma folate levels are also a good indicator of status, but plasma levels can also be influenced by recent dietary intake.

B. Cellular Transport

Folate transport by mammalian tissues and cells has been the subject of several recent reviews [11,13]. A number of different folate transport systems have been described, which fall into two classes: membrane carrier—and folate-binding protein—mediated systems.

1. *Folate Membrane Carriers*

A variety of membrane carrier—mediated folate transporters have been characterized kinetically in various mammalian tissues and in cultured mammalian cells. The folate carrier that is expressed in tissue culture cells, which has been the most extensively investigated, is also found in some tumor cells and fetal tissues but is distinct from the carriers in normal adult tissues. The transporter is saturable and has a fairly low affinity for reduced folates with K_t values in the low micromolar range and a similar or slightly lower affinity for the antifolate methotrexate [11,13]. The carrier has a greatly reduced affinity for folic acid ($K_t \sim 100\ \mu M$), which explains why most cultured cells require much higher levels of folic acid compared to reduced folates for growth. Several mechanisms have been identified for methotrexate mono- and diglutamate efflux by mammalian cells [14]. Efflux is anion dependent, and there is some disagreement as to whether the efflux systems are distinct from the folate influx systems.

Although this carrier may be expressed at low levels in some tissues, the major folate carriers in adult tissues are different. Normal mammalian tissues appear to possess a diverse range of folate transporters with differing affinities for various folate derivatives [11]. Folate transport in hepatocytes is energy dependent and

complex with saturable and nonsaturable components [15]. The nonsaturable component may represent a low affinity transporter for folate and methotrexate. Basolateral membranes from rat and human liver possess an electroneutral folate-H^+ cotransporter for reduced and oxidized folates and methotrexate [16,17]. The basolateral membrane of the small intestine mucosa possesses a folate transporter that is an anion exchanger [18]. Initial studies on folate transport across the mitochondrial membrane suggested a specificity for oxidized folates [19]. However, recent studies suggest that the mitochondrial transporter is specific for reduced folates, has little affinity for folic acid, and does not transport methotrexate [20,21].

As all these transporters have affinities for reduced folates in the micromolar range and higher, they would not be saturated by folate under physiological conditions, and folate influx into tissues should be responsive to any elevation in plasma folate levels found after folate supplementation.

2. *Folate-Binding Protein*

In recent years, studies of a high affinity plasma folate-binding protein and a plasma membrane—associated folate-binding protein have demonstrated extensive homology between the two proteins [22—25]. The protein is attached to the plasma membrane via a glycosylphosphatidylinositol anchor and can be released from the membrane by a specific phospholipase C [26]. The binding protein shows high affinity for a variety of folates with K_ds in the nanomolar range. Folic acid shows the highest affinity, while methotrexate has relatively poor affinity for the protein.

In normal tissues, the distribution of the binding protein is limited to the apical membrane of some epithelial cells [27]. High levels are found in the choroid plexus, and the protein is also found in kidney proximal tubules, erythropoietic cells, the vas deferens, the ovary, fallopian tubes, the uterine epididymus, trophoblastic cells of the placenta, lung aveolar, acinar cells of the breast, submandibular salivary and bronchial cells [27]. Low levels are found in the thyroid gland, and very low levels may be present in gut mucosal cells. Although most tissue culture cells express the low affinity folate carrier, some cells of epithelial origin also express the folate-binding protein, and the protein can be induced by culturing cells in medium containing very low levels of folic acid [28]. The protein is overexpressed in some human malignancies, and differences in affinities of some antifolates for the protein compared to their affinities for the folate carriers in normal tissues suggest an exploitable mechanism for selectivity in chemotherapeutic regimens [29]. The level of the binding protein may also be regulated by cell differentiation [30]. Antisera to the binding protein inhibit folate uptake and reduce intracellular folate accumulation in erythroid progenitor cells [31].

Folate transport via the folate-binding protein occurs by a receptor-mediated process, but the actual mechanism is not fully understood. In studies with some tissues and cells, the binding protein-folate complex has been shown to internalize

by a non—clathrin-mediated classical endocytotic pathway not involving lysosomes [32]. Other studies have localized the complex to plasmalemma vesicles (caveolae) and have suggested a process called potocytosis for recycling of the binding protein [33]. In either case, folate is released from the vesicle into the cytosol and the binding protein rapidly recycles back to the plasma membrane. The mechanism by which folate is transported out of these vesicles has not been established. It has been suggested that acidification of the vesicles effects release of folate from the binding protein and that the vesicles may also contain the lower affinity membrane folate carrier. As folate would be concentrated in the vesicles, this would allow efficient transport of folate via the lower affinity membrane carrier. This plausible mechanism would allow cells and tissues to accumulate folate at exogenous folate concentrations that would be inefficiently transported if the cell was dependent solely on the lower affinity carrier.

IV. TISSUE ACCUMULATION AND STORAGE

A. Folate Distribution

The types of folates that predominate in different tissues and species have been extensively documented in several reviews [7,34,35]. Endogenous folates in mammalian tissues are almost entirely folylpolyglutamate derivatives, while pteroylmonoglutamates are the only forms in plasma and urine. Although pentaglutamates are the predominant derivatives in rat liver, longer-chain-length derivatives are found in most other mammalian tissues. Intracellular metabolism to polyglutamates is required for the retention and concentration of transported folate. Hepatic folate concentrations in the rat are several hundred—fold higher than in plasma, while pteroylmonoglutamate concentrations are similar to plasma concentrations [36]. Although metabolism to polyglutamate derivatives might be considered a mechanism for folate storage, folylpolyglutamates are the active coenzyme species, and metabolism to polyglutamates is required for normal one-carbon metabolism. The ability of tissues to accumulate or store high levels of the vitamin, that is levels in excess of that required for normal metabolism, is quite limited [37] (see Sec. IV.D).

A significant proportion of cellular folate—up to 50% depending on the tissue or cell type—is associated with the mitochondria [38,39]. The mitochondrial folate pool is quite distinct from the cytosolic pool and displays a distinct one-carbon distribution. Practically all the cellular 5-methyl-$H_4PteGlu_n$ is located in the cytosol, while most of the cellular 10-formyl-$H_4PteGlu_n$ is in the mitochondria (38; R. F. Huang, S. Yoo, and B. Shane, unpublished data). Mitochondria contain folylpolyglutamates of similar or longer glutamate chain length than cytosolic folates [39]. Folate-dependent glycine synthesis and cleavage and choline degradation occur in the mitochondria, and it has been proposed that mitochondrial one-carbon metabolism also provides one-carbon moieties, via formate, for cytosolic one-carbon

metabolism [40]. The role of mitochondrial folate metabolism is discussed in Chapter 2.

B. Folylpoly-γ-glutamate Synthetase

The synthesis of folylpolyglutamates, and factors that regulate this synthesis, play a major role in the regulation of folate homeostasis and in the regulation of one-carbon metabolism. Mammalian cells possess two enzymes that can potentially directly regulate this process: folylpolyglutamate synthetase, which catalyzes the synthesis of folylpoly-γ-glutamates, and γ-glutamylhydrolase, a peptidase that can hydrolyze the folate polypeptide chain. The general properties of both of these activities have been the subject of recent reviews [6,7].

1. *Role and Distribution of Folylpolyglutamate Synthetase*

Chinese hamster ovary (CHO) cell mutants that lack folylpolyglutamate synthetase activity have greatly reduced folate pools, due to an inability to retain folates, and are auxotrophic for methionine, glycine, purines, and thymidine [41—43]. This auxotrophy is not relieved by elevating intracellular pteroylmonoglutamate to folate levels typically found in wild-type CHO cells [37], indicating that the auxotrophy is not caused solely by low folate levels but is also due to an inability to synthesize folylpolyglutamates. Wild-type CHO cells normally contain hexa- and heptaglutamates. However, metabolism of folate to the triglutamate is sufficient for normal cellular folate retention [43], and triglutamates function approximately as effectively as the longer-chain-length derivatives in the metabolic cycles of purine, thymidine, and glycine synthesis [37]. Folylpolyglutamate synthetase activity is highest in liver, and appreciable levels are found in most mammalian tissues, although it appears to be absent or present in negligible amounts in muscle tissue and mature blood cells [44]. Total body stores of folate in humans have been estimated at about 5—10 mg, with half of this in liver [9,45].

Folylpolyglutamate synthetase is located in the cytosol and mitochondria of mammalian tissues and cells, and the mitochondrial isozyme is required for the accumulation of normal mitochondrial folate pools [39]. Although folylpolyglutamates cannot be transported into the mitochondria, folylpolyglutamates synthesized in the mitochondria are slowly released into the cytosol (B. F. Lin and B. Shane, unpublished data). Model cells expressing folylpolyglutamate synthetase solely in the mitochondria have normal cytosolic and mitochondrial folate pools (B. F. Lin and B. Shane, unpublished data). It is not known whether the mitochondrial and cytosolic isozymes are encoded by separate genes, although genetic evidence supports a single gene [46].

2. *Properties*

Because of the marked lability and low abundance of the protein, mammalian folylpolyglutamate synthetases have only received detailed study in the last few

years. The enzyme has been purified to homogeneity from pig liver [47], a human folylpolyglutamate synthetase cDNA has recently been isolated from Epstein-Barr virus—transformed human lympohcytes [46], and the human enzyme has been overexpressed and purified (T. Garrow and B. Shane, unpublished data). The human gene has been localized to chromosome band 9q34.1 (T. Garrow, J. Korenberg, and B. Shane, unpublished data).

General characteristics of folylpolyglutamate synthetases from pig, mouse, rat, and beef liver have been described [7]. All mammalian folylpolyglutamate synthetases have native molecular sizes of 60,000—70,000 and appear to be monomeric proteins. The most effective pteroylmonoglutamate substrates for the pig liver and human enzymes are tetrahydrofolate and dihydrofolate. 10-Formyl-tetrahydrofolate has a reduced k_{cat}, while 5-substituted folates, such as 5-methyl- and 5-formyl-tetrahydrofolate, and folic acid are very poor substrates [48]. Increasing the glutamate chain length of the folate molecule causes a decrease in catalytic rate. k_{cat} values for polyglutamates of tetrahydrofolate, dihydrofolate, and folic acid decrease with increasing glutamate chain length, although long-chain folylpolyglutamates still retain affinity for the protein. The substrate specificity of the enzyme for pteroylmonoglutamate derivatives is not necessarily a good indicator of which compounds are the most effective polyglutamate substrates. For the pig liver enzyme, activity drops off faster with elongation of the polyglutamate chain of dihydrofolate and 10-formyl-tetrahydrofolate than with tetrahydrofolate. Tetrahydrofolate polyglutamates are the only effective long-chain substrates for the enzyme [49]. 5-Methyl-tetrahydrofolate is a relatively poor substrate, and its diglutamate derivative is essentially inactive.

4-Aminofolates, such as aminopterin and methotrexate, are more effective substrates of folylpolyglutamate synthetase than their parent 4-oxo-pteroylmonoglutamate derivatives [50—52]. However, the 4-amino substitution significantly impairs catalysis with polyglutamate derivatives, and the diglutamate derivatives of all 4-aminofolates tested have proven to be poor substrates for the enzyme [53].

C. Intracellular Folate-Binding Proteins

Binding of folylpolyglutamates to cellular proteins would be expected to reduce the availability of some of these compounds for enzymes of one-carbon metabolism and to assist in folate retention by tissues. Rat liver contains five major folate-binding proteins [25]. A large proportion of hepatic mitochondrial $H_4PteGlu_n$ are associated with sarcosine and dimethylglycine dehydrogenases [54]. The major cytoplasmic folate-binding protein, glycine *N*-methyltransferase, is present in high concentrations and contains bound 5-methyl-$H_4PteGlu_n$, which are inhibitors of the enzyme [55,56]. Rat liver contains additional cytosolic folate-binding proteins including 10-formyltetrahydrofolate dehydrogenase [57]. This enzyme binds $H_4PteGlu_n$, which are products, and consequently inhibitors, of the enzyme.

Serine hydroxymethyltransferase is also a high-abundance protein in liver cytosol and mitochondria [58] but has not been identified as a folate-binding protein despite the high affinity of folylpolyglutamates for the enzyme. Dilution of proteins and cellular metabolites occurs under the conditions used to isolate and identify binding proteins, and folates have high affinity for the hydroxymethyltransferase-glycine binary complex. Under normal cellular conditions, a significant proportion of cellular folates could potentially be associated with this enzyme.

Based on the affinities reported for folylpolyglutamates for enzymes involved in one-carbon metabolism, it is probable that a large proportion, if not most, of the intracellular folate is associated with proteins under physiological conditions and this proportion is likely to be increased when tissue folate levels are low. For instance, most of the hepatic 5-methyl-$H_4PteGlu_n$ is associated with glycine methyltransferase in folate-depleted rats [59].

D. Physiological and Pharmacological Factors Affecting Folate Accumulation

1. *Tissue Folylpolyglutamate Synthetase Activity*

The effect of folylpolyglutamate synthetase activity on folate accumulation has been investigated using model CHO transfectants expressing various levels of human folylpolyglutamate synthetase activity [37,43]. Under conditions where medium folate mimics physiological conditions, there is little effect of folylpolyglutamate synthetase activity on folate accumulation and accumulation is limited primarily by influx. Essentially all transported folate is metabolized to retained polyglutamate derivatives. Folate accumulation only becomes limited by folylpolyglutamate synthetase activity when activity levels are very low [37]. A different pattern is seen when cells are exposed to pharmacological levels of folate. Under these conditions, competition between entering folate and diglutamate in the cell limits the extent of metabolism to retainable polyglutamates (triglutamates and longer), and only a small proportion of the folate that is transported is retained by the cell [37]. This competition limits the ability of the cell to accumulate high levels of folate despite high folate levels in the medium. Folate accumulation under these conditions is directly proportional to the level of folylpolyglutamate synthetase activity.

Although these observations suggest that folate accumulation by tissues under physiological conditions may not be very responsive to differences in folylpolyglutamate synthetase levels, enzyme levels in tissue culture cells are usually considerably higher than those found in many mammalian tissues. At the levels of folylpolyglutamate synthetase activity found in many tissues, accumulation of folate would be expected to be limited by the level of folylpolyglutamate synthetase activity and, in some tissues, by the activity of the tissue's folate transporter(s). Although developing blood cells accumulate folate, mature human

blood cells possess negligible folylpolyglutamate synthetase activity and do not accumulate exogenous folate. Mitogen-stimulated human lymphocytes regain the ability to accumulate folate, and the time course of mitogen activation of folate accumulation correlates with an induction of folylpolyglutamate synthetase mRNA [60].

When physiological doses of folate are administered to experimental animals, tissue accumulation of the dose is similar regardless of the type of folate derivative administered (S. Yoo and B. Shane, unpublished data). When very large pharmacological doses are administered to tumor-bearing experimental animals, tissue and folate levels are increased to only a limited extent (1.5- to 8-fold), which may reflect differences in folylpolyglutamate synthetase activity in the different tissues and tumors [61]. Pharmacological doses of 5-formyl-H_4PteGlu are more effective than 5-methyl-H_4PteGlu or PteGlu at elevating tissue folate levels [61].

Polyglutamate formation is more rapid in dividing cell cultures [62] probably reflecting that folylpolyglutamate synthetase levels appear to be growth related and are higher in growing cells and fall when cells are induced to differentiate [63]. Increased net accumulation of a labeled folate dose occurs in the regenerating rat liver [64] while endogenous folate levels drop slightly and slightly longer endogenous polyglutamates are found [65].

Elevated expression of folylpolyglutamate synthetase should increase the accumulation of pharmacological levels of folate and should increase the accumulation and metabolism of poor substrates of the enzyme, such as 4-aminofolates. Methotrexate uptake and metabolism in tissue culture cells, and the cytotoxic efficacy of methotrexate, are directly correlated with enzyme activity levels [21]. The ability of blast cells from leukemia patients to synthesize methotrexate polyglutamates has been correlated with efficacy of drug treatment and has been proposed as a prognostic indicator for methotrexate treatment [66]. Some human leukemia blast cells express very high levels of folylpolyglutamate synthetase mRNA and activity [67], and these cells should accumulate very high levels of methotrexate and should be very sensitive to antifolate treatment.

Antifolate retention by tissues, and consequently cytotoxicity, should be very sensitive to relatively minor differences in folylpolyglutamate synthetase levels, in efflux rates for di- and triglutamate derivatives, and in differences in substrate specificity of synthetases from different sources, especially for di- and/or triglutamate derivatives [21,49]. Decreased polyglutamylation of methotrexate in cells from patients resistant to this drug has been described, and several tumor cell lines have been described in which resistance to methotrexate is due to decreased folylpolyglutamate synthetase levels [68–70].

2. *Folate Status*

Endogenous folate levels are reduced in the livers of folate-depleted animals [71], but the chain lengths of endogenous folylpolyglutamates are increased. This

phenomenon, which is also observed with cultured cells, reflects the slow rate of synthesis of long-chain folylpolyglutamates, which is limited by competition with entering pteroylmonoglutamate and shorter-chain-length folylpolyglutamates, the preferred substrates for folylpolyglutamate synthetase. Under folate-restricted conditions, this competition is decreased. Apparent folate turnover rates are also decreased (S. Yoo and B. Shane, unpublished data), which may reflect an increased proportion of tissue folate bound to folate-binding proteins. Plasma folate levels are elevated in rats receiving a diet containing high levels of folate. However, endogenous hepatic folate levels are increased only slightly, and the chain lengths of endogenous folylpolyglutamates are decreased and apparent tissue folate turnover rates are increased (S. Yoo and B. Shane, unpublished data). Again, similar effects are seen in tissue culture cells [49] and can be explained by increased competition between substrates for folylpolyglutamate synthetase. Brain folate levels are unresponsive to changes in dietary folate levels. Folate transport across the choroid plexus is mediated by a folate-binding protein. The high affinity of folate for this transporter suggests that transport into the brain would operate at V_{max} conditions except when plasma folate levels are extremely depressed.

While significant changes occur in the types of folylpolyglutamates that accumulate in some tissues with changes in folate status and intake, it is unlikely that this has regulatory significance for control of metabolic cycles of one-carbon metabolism. As described above, studies with model mammalian cells have suggested that the metabolic cycles involving purine, thymidine, and glycine synthesis show little discrimination between folylpolyglutamates of chain length three and above [37].

3. *Vitamin B_{12} Deficiency*

Tissue folate retention would be expected to be regulated by physiological and nutritional factors that affect the types of folate one-carbon derivatives that accumulate in tissues. In humans and experimental animals, vitamin B_{12} deficiency induces a secondary folate deficiency and tissue levels of folate are reduced up to 60%. The interaction between the vitamins is best explained by the "methyl trap" hypothesis [3]. Vitamin B_{12} deficiency reduces the activity of methionine synthase, one of two mammalian vitamin B_{12}-dependent enzymes, and a functional folate deficiency results due to accumulation of 5-methyl-H_4PteGlu$_n$, a substrate for methionine synthase, at the expense of other folate one-carbon forms, including H_4PteGlu$_n$ [3,72,73]. The megaloblastic anemia that occurs in humans can be explained by a lack of folate coenzymes for DNA precursor synthesis in blood cells. The reduction in tissue levels of folate is due to an impaired ability to retain folate rather than impaired tissue uptake of the vitamin [3]. The impaired retention of folate can be explained by the decreased level of H_4PteGlu$_n$ under these conditions and the poor substrate activity of 5-methyl-H_4PteGlu for folylpolyglutamate synthetase, as well as complete lack of substrate activity with polyglutamate forms of this compound.

Methionine ameliorates some of the effects of vitamin B_{12} deficiency in experimental animals and corrects abnormal hepatic folate metabolism in the rat but does not normalize folate metabolism in the bone marrow [3]. Methionine is believed to act via adenosylmethionine, which inhibits methylenetetrahydrofolate reductase, the enzyme responsible for the synthesis of 5-methyl-H_4PteGlu$_n$ [74]. The reaction catalyzed by the reductase is essentially irreversible under physiological conditions [75], and adenosylmethionine inhibition of this enzyme would slow the formation of 5-methyl-H_4PteGlu$_n$ and its consequent "trapping" when methionine synthase activity is inhibited.

Although the "methyl trap" hypothesis can explain most, if not all, of the metabolic derangements of folate metabolism in vitamin B_{12} deficiency, an alternate explanation for the interaction between the two vitamins has been proposed, namely, that vitamin B_{12} deficiency by some unknown mechanism causes a deficiency of "active" formate, oxidized one-carbon units utilized in one-carbon metabolism [76,77]. Several lines of evidence have been presented in favor of the "active" formate hypothesis. Many of the studies have used nitrous oxide treatment of experimental animals as a paradigm for vitamin B_{12} deficiency, as this agent causes inactivation of methionine synthase. In nitrous oxide-treated animals, 5-methyltetrahydrofolate and tetrahydrofolate injections failed to restore normal hepatic folate levels and polyglutamates, while formyl-tetrahydrofolate was effective in normalizing hepatic folate accumulation [76,77]. Similarly, formyl-tetrahydrofolate was more effective than tetrahydrofolate in restoring one-carbon metabolism, such as thymidylate synthesis, in tissues from these animals. The increased efficacy of formyl-tetrahydrofolate compared to tetrahydrofolate has been interpreted to indicate a lack of one-carbon units at the oxidation state of formate under vitamin B_{12} -deficiency conditions and to indicate that formyl-tetrahydrofolate is the preferred substrate for folylpolyglutamate synthetase and consequently tissue folate accumulation [77], although the latter is inconsistent with the known properties of purified folylpolyglutamate synthetase. Based on amelioration of defective folate metabolism by methylthioadenosine as well as by methionine, it has also been suggested that the methionine effect is due to its metabolism via decarboxylation of adenosylmethionine via the polyamine pathway with subsequent production of formate via the methylthioadenosine pathway [76,77].

Some recent studies, however, cast doubt on this interpretation and are consistent with the "methyl trap" hypothesis. In animals exposed to nitrous oxide, cytosolic folates are almost completely trapped as 5-methyl-H_4PteGlu$_n$ and the failure to detect complete trapping in previous studies was due to nonmethyl folates in the mitochondria [38]. Hepatic folate accumulation is reduced by up to 85% in the nitrous oxide—treated rat regardless of the type of folate administered, and all folates, including formyl-tetrahydrofolate, are trapped as 5-methyl-H_4PteGlu$_n$ (S. Yoo and B. Shane, unpublished data). Previous studies indicating "normal" accumu-

lation of formyl-tetrahydrofolate under these conditions used mixed isomers of the reduced folate, and the accumulation observed represented metabolites of the unnatural isomer of formyl-tetrahydrofolate (S. Yoo and B. Shane, unpublished data). While formyl-tetrahydrofolate is more effective than tetrahydrofolate in normalizing thymidine metabolism in marrow cells from vitamin B_{12}-deficient rats, it is also more effective at normalizing metabolism in marrow cells from folate-deficient animals [78]. Consequently, a specific effect of vitamin B_{12} deficiency on formyl-$H_4PteGlu_n$ production cannot be inferred from these data.

Because folate administration can induce a hematological response in vitamin B_{12}-deficient subjects, concerns have been raised that folate supplementation of the food supply may lead to a masking of the anemia that normally develops in these subjects and a higher incidence of the irreversible neurological manifestations of vitamin B_{12} deficiency. This issue is discussed in Chapter 9.

V. TURNOVER AND EXCRETION

A. Mechanism of Turnover

Folylpolyglutamates turnover in mammalian cells and tissues, but the mechanism of turnover has not been well characterized. Hepatic folate stores turn over rapidly in rats fed a high-folate diet, while turnover is very slow in folate-depleted animals (S. Yoo and B. Shane, unpublished data). Dietary folate that is not accumulated by liver is released primarily as 5-methyl-tetrahydrofolate, as this is the major cytosolic form of the vitamin. It is not known whether folylpolyglutamates are released as such by tissues or require hydrolysis to monoglutamates prior to release. Mammalian γ-glutamylhydrolases and transpeptidases that can hydrolyze folylpolyglutamates directly to the mono- or diglutamate derivatives, which would not be retained by tissues, have been described [6]. Any folylpolyglutamates released directly into the circulation would be hydrolyzed rapidly by plasma γ-glutamylhydrolase.

The half-life of labeled folates, primarily folylpolyglutamates, in cultured cells is similar to the generation time of the cells when cells accumulate levels of folate that support normal rates of growth, while the half-life is reduced, indicative of folate efflux, when cells contain high levels of folate [43]. Studies with a variety of cell lines have demonstrated a rapid efflux of mono- and diglutamate forms of aminopterin and methotrexate, while efflux of longer-chain derivatives is greatly reduced or negligible [43,79].

The major route of whole body folate turnover appears to be via catabolism to cleavage products [80,81]. After administration of a labeled folate dose to animals, a small amount of the dose is recovered as folate derivatives in urine in the first 24—48 hours. The bulk of the dose is recovered as pterin derivatives and *N*-acetamidobenzoylmonoglutamate. These cleavage products account for the bulk of

whole body folate turnover in humans and experimental animals. It has been proposed that the initial step in folate catabolism involves the cleavage of intracellular folylpolyglutamates at the C9-N10 bond, presumably due to oxidative damage, and the resulting *p*-aminobenzoylpolyglutamates ($pABAglu_n$) are hydrolyzed to the monoglutamate, which is *N*-acetylated in liver prior to excretion.

Any condition that increases oxidative cleavage of folate and consequently catabolism would be expected to increase folate requirements. Catabolism of folate is increased in pregnancy, particularly in the second trimester, and this increased catabolism is distinct from any increase in food intake [82].

B. Role of γ-Glutamylhydrolase

Mammalian tissues contain γ-glutamylhydrolases that can hydrolyze the polyglutamate chain of folates. The properties of these enzymes have been previously reviewed [6]. The hydrolases lack specificity for the pterin moiety and will hydrolyze $pABAglu_n$ with equal efficacy and probably also poly(γ-glutamate). Longer-chain folylpolyglutamates are usually better substrates than the shorter-chain-length glutamate derivatives. γ-Glutamylhydrolases in most tissues are located in the lysosome, have an acid pH optimum, and appear to be primarily endopeptidases [6]. A second distinct lysosomal endopeptidase has been isolated from beef liver [83], which appears to be identical to a previously described rat liver folylpolyglutamate:amino acid transpeptidase that catalyzes transfer of a variety of amino acids to folic acid di- to heptaglutamates with formation of folic acid—amino acid and a polyglutamate peptide [84]. The transpeptidase activity has a physiological pH optimum, although its physiological role is not understood.

The glutamylhydrolases from mouse liver [85] and rat intestinal mucosal cells [86] are lysosomal endopeptidases that specifically cleave folates to the monoglutamate derivative, while the human jejunal enzyme appears to be a random endopeptidase [87]. Human intestinal brush border membranes also contain a membrane-bound glutamylhydrolase with exopeptidase activity [88]. This enzyme, and other hydrolases found in the gut, are the only hydrolases that function well at neutral pH, and this appears to be related to their specialized role in the hydrolysis of dietary folates prior to folate absorption by the intestinal mucosa (see Chapter 8). Some plasma γ-glutamylhydrolases also show neutral pH optima. There is no compelling evidence for the existence of cytoplasmic hydrolases that function at physiological pH, although this possibility has not been rigorously addressed.

The role of lysomal glutamylhydrolases in folate homeostasis has not been well established but presumably is involved, at a minimum, in catabolism of folate cleavage products. Addition of putative γ-glutamylhydrolase inhibitors or lysosomal function inhibitors to CHO cells does not influence folate or antifolate accumulation or metabolism [21,37] and, as discussed previously, it is unlikely that the hydrolase plays a role in modulating the glutamate chain of folates to effect regu-

lation of metabolic cycles of one-carbon metabolism in this cell line. However, methotrexate metabolism in some tumor cell lines is influenced by γ-glutamylhydrolase inhibitors [89]. Differences between cell lines may reflect the fact that most tissue culture cells secrete γ-glutamylhydrolase into the medium and are depleted in the lysosomal enzyme [90]. A lysosomal uptake system for methotrexate polyglutamates has been characterized [91], which presumably is involved in methotrexate turnover in the cell. A methotrexate-resistant cell has recently been described in which resistance has been correlated with increased γ-glutamylhydrolase activity [92].

C. Excretion

Folate is freely filtered at the glomerulus and is reabsorbed in the proximal renal tubule [93,94]. The net effect is that most of the secreted folate is reabsorbed. The renal clearance of folate derivatives is inversely proportional to their affinities for the folate-binding protein in the kidney proximal tubes [95]. Increased excretion of folate has been reported in alcoholics, although it is unclear whether this reflects increased secretion or impaired reabsorption or if body stores of the vitamin are significantly depleted [96]. As described above, although urine does contain some folate derivatives, the bulk of the excretion products in humans are folate cleavage products [81].

Biliary excretion of folate has been estimated to be as high as 100 μg per day in humans [9,93]. Much of this would be reabsorbed in the small intestine, but loss of folate via this route could be significant in malabsorption syndromes. Fecal folate excretion is variable and is not a measure of folate availability due to folate biosynthesis by the intestinal flora. The extent of the contribution of this endogenously synthesized folate to folate stores in humans is not known. In experimental animals, sulfa drug treatment is required to produce a complete folate deficiency, and it has been demonstrated that bacterially synthesized folates contribute to hepatic folate stores, although the extent of this contribution has not been quantitated [97].

VI. SUMMARY

This chapter has summarized our current understanding of the mechanisms by which folate stores in tissues and in the body are regulated. Over the last few years, considerable progress has been made in our understanding of the properties of the folate transporters responsible for folate influx into tissues and the mechanisms by which folate is metabolized and retained in tissues. Our understanding of potential defects in these processes under pathological conditions is more limited. The mechanisms governing folate turnover and excretion are still poorly understood. Under normal conditions, folate accumulation by tissues can be governed in part by the level of the folate transporter and by folylpolyglutamate synthetase activity.

Metabolic disturbances that affect substrate availability for folylpolyglutamate synthetase, such as vitamin B_{12} deficiency, can influence tissue folate levels. Large changes in folate intake cause only relatively small changes in tissue folate stores. As small increases in dietary folate intake can influence the incidence of conditions such as neural tube defects, it appears that very modest changes in tissue folate levels can have significant metabolic effects.

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2

Biochemical Role of Folate in Cellular Metabolism

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I. INTRODUCTION

As described in the previous chapter, natural folate compounds exist in tissues as polyglutamates. These serve to keep the folates within the cell since only the monoglutamate forms are transported across membranes and only the monoglutamates are found in the plasma and urine. In most cases the polyglutamate forms of the folate coenzymes are better substrates for the enzymes than their monoglutamate counterparts. In this chapter all references to the folate coenzymes assume that it is the polyglutamates that are involved. An excellent review of the folate–vitamin B_{12} relationship has been written [1] , and a three-volume monograph [2,3] is available that contains articles on detailed aspects of folate metabolism. This chapter will emphasize some of the more recent studies with clinical significance.

The role of folate is, with a few rare exceptions, to carry one-carbon units. These one-carbon units exist at various levels of oxidation. The entire range of oxidation states for one-carbon compounds extends from methane, the most reduced, to carbon dioxide, the most oxidized. With the exception of carbon dioxide, one-carbon units are generated in the cell during metabolism and are carried from one reaction to another as folate derivatives. The oxidation levels differ from each other by the gain or loss of two electrons. When the one-carbon units are incorporated as folate derivatives, they may also be converted from one oxidation level to another by the gain (reduction) or loss (oxidation) of electrons. Table 1 lists the oxidation levels of the folate coenzymes.

One-carbon units are carried by folate only when the coenzyme is in the fully reduced tetrahydro form. It is important not to confuse the oxidation state of the folate coenzymes with the oxidation level of the one-carbon unit that is carried. As

Table 1 Oxidation Levels of the One-Carbon Substituents of the Folate Coenzymes

Oxidation level	Corresponding folate coenzyme
Methane, CH_4	None
Methanol, CH_3-OH	5-Methyl-THF
Formaldehyde, CH_2=O	5,10-Methylene-THF
Formic acid, HCOOH	5,10-Methenyl-THF
	5,10-Formimino-THF
	5-Formyl-THF
	10-Formyl-THF
Carbon dioxide, CO_2	None

noted in Chapter 1, folic acid is not a naturally occurring compound but can be converted to the dihydro-and tetrahydro- forms through the action of the enzyme dihydrofolate reductase. Dihydrofolate is generated during the synthesis of thymidylic acid and must be converted back to tetrahydrofolate in order to accept a one-carbon unit.

II. FOLATE AND THE ONE-CARBON POOL

The one-carbon pool is a functional concept. It describes the family of folate coenzymes that are in metabolic equilibrium [4]. Members of the pool are shown enclosed in large rectangles in Figure 1. Rectangles surrounded by a frame indicate that the folate carries a one-carbon unit. An unshaded frame indicates that the one-carbon unit is at the oxidation level of formate. The cross-hatched frame indicates that the one-carbon unit is at the oxidation level of formaldehyde, and the doubly cross-hatched frame indicates that the one-carbon unit is at the oxidation level of methanol.

Going from one oxidation level to another involves a redox reaction. The arrows indicate enzymatic reactions that convert one member of the pool to another or that move one-carbon units in and out of the pool. Before folate can carry a one-carbon unit, it must be reduced to the tetrahydro- reduction state. This is carried out by a single enzyme, dihydrofolate reductase, (1, Fig. 1), that reduces folic acid to dihydrofolate (DHF) and also reduces dihydrofolate to tetrahydrofolate (THF). Folic acid is not a natural compound, but it is much more stable than the majority of the reduced folates [5,6]. DHF is the natural substrate for the enzyme, and it is because folic acid is also reduced by the enzyme that it can serve as a vitamin.

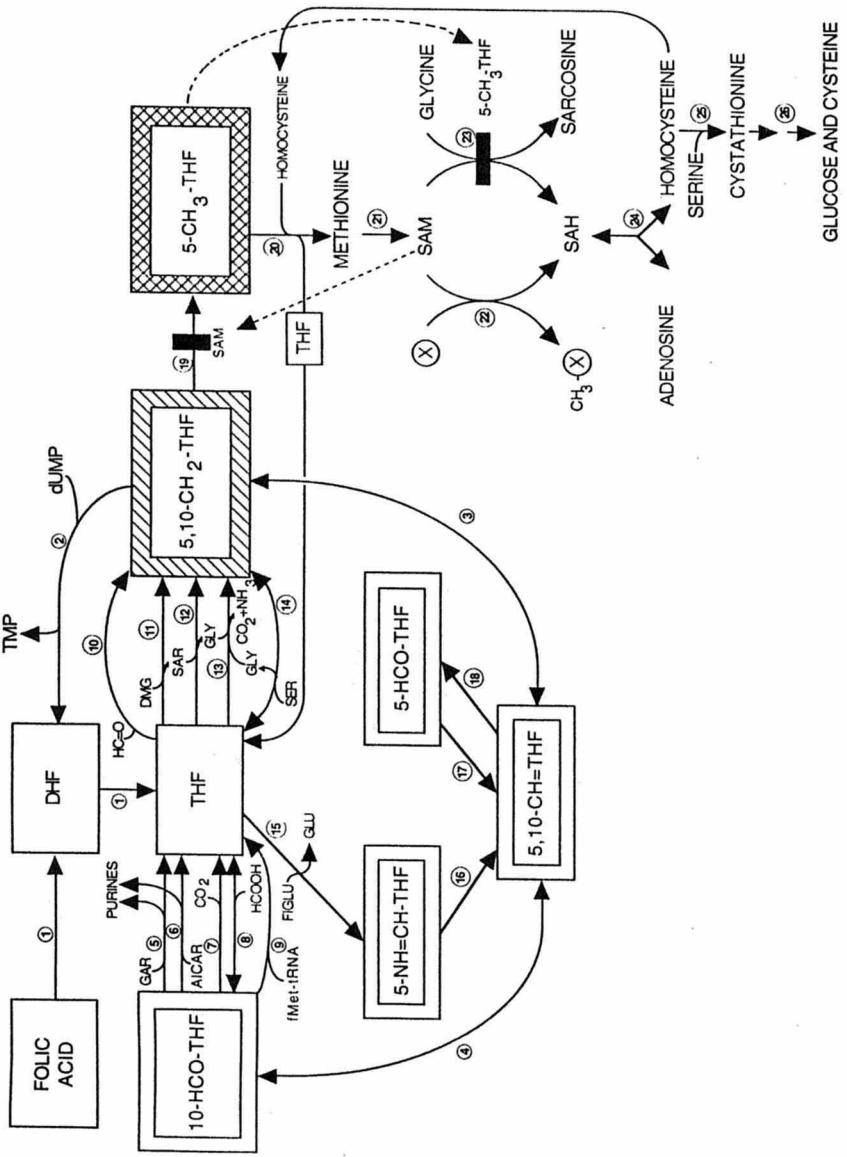
A. Reactions That Bring One-Carbon Units Into the Pool

One-carbon units at the oxidation level of formate can enter directly as formic acid (reaction 8, Fig. 1). This is catalyzed by 10-formyl-THF synthase. This reaction uses ATP for synthesis of the formyl-amide bond with the production of ADP and

inorganic phosphate. In mammalian tissue this activity is found as part of a trifunctional enzyme [7,8] together with reactions 4 and 3, which serve to "channel" one-carbon units back and forth between the formate and formaldehyde levels of oxidation [9,10]. "Channeling" is a method of directing a metabolic pathway since the product of one enzymatic reaction can become the substrate of another without equilibrating with the medium. Entry at the formate level of oxidation can also take place via reactions 15 and 16. Again, in mammalian tissue, both enzymatic reactions are carried out by a single peptide, and "channeling" between the two enzymatic sites is believed to occur [11]. Formiminoglutamic acid (FIGLU) arises from the catabolism of histidine [12]. Reaction 15 in Figure 1 is catalyzed by glutamate formiminotransferase with the production of 5-formimino-THF. This is then rapidly converted to 5,10-methenyl-THF by formimino-THF cyclodeaminase (reaction 16). The third mode of entry at the formate level of oxidation involves formation of 5-formyl-THF, commonly known as folinic acid or leucovorin. This compound is the most stable of all the reduced folate coenzymes and is widely used clinically in chemotherapeutic protocols. It is rapidly converted to all the other forms of folate after cyclizing to 5,10-methenyl-THF via 5,10-methenyl-THF synthetase (reaction 17).

The metabolic origin of 5-formyl-THF has been a mystery for some time. For a long while there was doubt as to whether it was a natural product since it makes up only a small part (10–15%) of the total folate pool [13,14] and can be formed nonenzymatically from 5-formimino-THF [6]. Recent studies have shown, however, that 5-formyl-THF is not an artifact of isolation. The presence of an enzyme that catalyzes the utilization of 5-formyl-THF (reaction 17) supported the suggestion that it is a natural compound. The question of the enzymatic reaction that is responsible for the formation of 5-formyl-THF has now been conclusively answered [15]. Schirch and Stover have shown that 5-formyl-THF can be formed enzymatically by serine hydroxymethyltransferase. This enzyme's primary function is the interconversion of serine and glycine (reaction 14). Serine hydroxymethyltransferase can also convert 5,10-methenyl-THF to 5-formyl-THF (reaction 18) [16,17]. The actual substrate of the reaction may be a hydrated form of 5,10-methenyl-THF, called anhydroleucovorin B, first described in 1952. Schirch and Stover have shown that 5-formyl-THF is a potent inhibitor of many folate-utilizing reactions and suggest that it may play a regulatory role in one-carbon metabolism [17].

Entry of one-carbon units at the formaldehyde level of oxidation occurs primarily through the action of serine hydroxymethyltransferase (reaction 14, discussed above). Although this reaction is freely reversible, studies have shown that the β-carbon of serine is the major source of metabolically available one-carbon units and, in vivo, formation of glycine is predominant [18]. Another source of entry at the formaldehyde level of oxidation is the glycine cleavage reaction (reaction 13). This is a complex reaction requiring the participation of four separate proteins and found


FOLIC ACID
DHF
THF
10-HCO-THF
5,10-CH2-THF
5-CH3-THF
5-HCO-THF
5-NH=CH-THF
5,10-CH=THF
TMP
dUMP
HC=O
DMG
SAR
GLY
CO2+NH3
SER
GAR
AICAR
CO2
HCOOH
PURINES
fMet-tRNA
FIGLU
GLU
THF
SAM
METHIONINE
HOMOCYSTEINE
GLYCINE
5-CH3-THF
SARCOSINE
SAH
ADENOSINE
HOMOCYSTEINE
SERINE
CYSTATHIONINE
GLUCOSE AND CYSTEINE

exclusively in the mitochondria. Carbon 2 of glycine is transferred to THF with the formation of 5,10-methylene-THF and the release of NH_3. Carbon 1 is oxidized to CO_2. Mutations that interfere with this reaction are responsible for a lethal metabolic disease, hyperglycinemia [19]. This is consistent with the observation that the physiological direction of serine hydroxymethyltransferase does not favor serine synthesis. Other sources of entry at the formaldehyde level of oxidation are reactions 11 and 12, catalyzed by dimethylglycine dehydrogenase and sarcosine dehydrogenase, respectively [20,21]. Both are mitochondrial enzymes. Dimethylglycine arises from the catabolism of choline, which takes place exclusively in liver mitochondria. One methyl group of dimethylglycine, which is at the methanol level of oxidation, is transferred to THF by dimethylglycine dehydrogenase and simultaneously oxidized to form 5,10-methylene-THF. The other product of the reaction is *N*-methylglycine, commonly known as sarcosine. Sarcosine dehydrogenase acts upon the product of this reaction in a similar manner to simultaneously oxidize and transfer the methyl group of sarcosine to THF with the production of methylene-THF and glycine [22]. Reaction 10 indicates that free formaldehyde can react with THF nonenzymatically to produce 5,10-methylene-THF. There is little if any free formaldehyde produced in vivo, however.

There are no reactions that permit entry of a one-carbon unit at the methanol level of oxidation. As mentioned above, reactions 11 and 12 catalyze the oxidation of the one-carbon units of dimethylglycine and sarcosine from the methanol to the formaldehyde oxidation level before incorporation into the folate pool.

Figure 1 Reactions involving enzymes and coenzymes of the one-carbon pool. The circled numbers refer to the following enzymes: **1**, dihydrofolate reductase; **2**, thymidylate synthase; **3**, 5,10-methylenetetrahydrofolate dehydrogenase; **4**, 5,10-methenyltetrahydrofolate cyclohydrolase; **5**, phosphoribosylglycinamide transformylase; **6**, aminocarboxamide ribotide transformylase; **7**, 10-formyltetrahydrolate dehydrogenase; **8**, 10-formyltetrahydrofolate synthase; **9**, methionyl *t*-RNA formyltransferase; **10**, nonenzymatic formation of 5,10-methylenetetrahydrofolate; **11**, dimethylglycine dehydrogenase; **12**, sarcosine dehydrogenase; **13**, glycine cleavage enzyme system; **14**, serine hydroxymethyltransferase; **15**, glutamate formiminotransferase; **16**, formiminotetrahydrofolate cyclodeaminase; **17**, 5,10-methenyltetrahydrofolate synthetase; **18**, minor reaction catalyzed by serine hydroxymethyltransferase; **19**, 5,10-methylenetetrahydrofolate reductase; **20**, methionine synthase; **21**, methionine adenosyltransferase; **22**, a variety of methyltransferase reactions; **23**, glycine *N*-methyltransferase; **24**, S-adenosylhomocystein hydrolase; **25**, cystathionine synthase; **26**, enzymes that convert cystathionine to cysteine and eventually glucose. DHF, dihydrofolic acid; THF, tetrahydrofolic acid; 10-HCO-THF, 10-formyltetrahydrofolic acid; 5-NH=CH-THF, 5-formiminotetrahydrofolic acid; 5,10-CH=THF, 5,10-methenyltetrahydrofolic acid; 5-HCO-THF, 5-formyl-tetrahydrofolic acid; 5-CH_3-THF, 5-methyltetrahydrofolic acid; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

B. Reactions That Move One-Carbon Units Out of the Pool

10-Formyl-THF synthase is reversible (reaction 8, Fig. 1), as discussed above. The energetically favorable direction of the reaction is the synthesis of 10-formyl-THF, which requires ATP and also produces ADP and inorganic phosphate [4]. This is in keeping with its role in removing formate that is generated metabolically and serves as the source of the one-carbon units needed for purine synthesis (reactions 5 and 6). In the presence of sufficient ADP and inorganic phosphate, however, ATP and formate are produced. Reaction 7 is catalyzed by 10-formyl-THF dehydrogenase [23,24]. During this reaction the formate is oxidized to CO_2. Although this enzyme can also hydrolyze 10-formyl-THF to THF and formate, it appears to be a minor activity, and it is uncertain whether simple hydrolysis to THF and formate occurs in the cell [25]. The purpose of the 10-formyl-THF dehydrogenase reaction is probably to return any folate not needed for purine synthesis to THF where it is available for accepting other one-carbon units. Reactions 5 and 6 utilize one-carbon units at the oxidation level of formate for the synthesis of purines [26]. Glycinamide ribotide (GAR) and aminoimidazole-carboxomide ribotide (AICAR) each accept a one-carbon unit from 10-formyl-THF during the biosynthesis of the purine ring. The two enzymes responsible are GAR transformylase and AICAR transformylase. Reaction 9, restricted to mitochondria, is responsible for the formylation of methionine in tRNA-fMet needed to initiate protein synthesis in mitochondria [27]. This is not necessary for cytosolic protein synthesis in eukaryotes.

One-carbon units at the formaldehyde level of oxidation, i.e., 5,10-methylene-THF, are transferred in two reactions. Reaction 14, catalyzed by serine hydroxymethyltransferase, has been discussed. The formation of serine via this reaction is probably not physiologically important since serine can also be synthesized via glycolysis from phosphoserine. Reaction 2, however, is extremely important. It is responsible for the synthesis of thymidylic acid (TMP) from deoxyuridylic acid (dUMP). Thymine, the pyrimidine base needed for DNA synthesis, is made in this reaction from uridine, while both are deoxynucleotides (i.e., deoxyuridylate and thymidylate). An important feature of this reaction is the fact that the one-carbon unit transferred to form thymidylic acid appears at the methanol level of oxidation, while the source of the one-carbon unit, 5,10-methylene THF, is at the formaldehyde level of oxidation. No external electron donors are needed for this reduction. Instead, the electrons needed for this reduction come from the tetrahydrofolate portion of the molecule itself, and the product is dihydrofolate (DHF). Dihydrofolate reductase (reaction 1) is necessary to regenerate THF so that it can accept one-carbon units.

The only one-carbon unit at the methanol level of oxidation is 5-methyl-THF, and the only reaction in which it participates is the transfer of the methyl group to homocysteine for the synthesis of methionine (reaction 20). This might seem strange

since methionine is an essential amino acid, but it is the homocysteine portion of the molecule that cannot be synthesized. As will be discussed below, the methyl group of methionine is metabolically labile. The enzyme that carries out this reaction is methionine synthase [28]. It requires a form of vitamin B_{12} (cyanocobalamin) as a cofactor. During the reaction the methyl group is transferred first from 5-methyl-THF to the cofactor to form methylcyanocobalamin, and the methyl group is then transferred to homocysteine [29,30].

C. Enzymes That Interconvert One-Carbon Units Within the Pool

The interconversion of 10-formyl-THF and 5,10-methenyl-THF (reaction 4) is catalyzed by methenyl-THF cyclohydrolase. Both this reaction and the reduction of 5,10-methenyl-THF to 5,10-methylene-THF (reaction 3) are catalyzed by enzyme activities that are part of the trifunctional enzyme discussed earlier [7,8]. Reaction 16 is catalyzed by the cyclodeaminase that is found as part of a bifunctional enzyme along with the glutamate formiminotransferase that catalyzes reaction 15 [12]. The conversion of 5-formyl-THF to 5,10-methenyl-THF (reaction 17) is catalyzed by 5,10-methenyl THF synthetase, as described above. The formation of 5-formyl-THF from 5,10-methenyl-THF is a minor activity associated with serine hydroxymethyltransferase (reaction 14). As discussed earlier, this is probably the physiological source of 5-formyl-THF [15]. Reaction 19 carries out an extremely important interconversion, the reduction of 5,10-methylene-THF to 5-methyl-THF. This is catalyzed by the enzyme 5,10-methylene-THF reductase [28]. The electron donor for this reduction is tightly bound $FADH_2$, and the reduction is essentially irreversible. It should also be noted that this reaction is inhibited by S-adenosylmethionine (SAM) [31,32]. SAM is the universal methyl donor for most reactions that occur in the cell. The methyl group of methionine can only be transferred after methionine has been converted to SAM in reaction 21. This has important regulatory implications.

D. Enzymes Regulated by Folate

In addition to those enzymes for which various forms of folate act as a substrate, there is one particular case in which folate acts as an allosteric regulator: the enzyme glycine *N*-methyltransferase (GNMT) catalyzes reaction 23 [33]. There are many examples of enzymes inhibited by a particular form of folate because of structural similarity with the substrate form of folate. In the case of GNMT, no form of folate is a substrate of the reaction. GNMT is a typical methyl transferase in which the methyl donor is SAM [34,35]. The methyl group is transferred to glycine to form *N*-methylglycine (sarcosine) and S-adenosylhomocysteine (SAH). The specific inhibitor of the reaction is 5-methyl-THF. There are more than 100 methyl transferase enzymes that act on a wide variety of substrates. Both small molecules and macromolecules are methylated, which results in the formation of compounds

that are metabolically and structurally important, e.g., epinephrine, phosphatidylcholine, and carnitine. The methylation of DNA is important in gene expression [36]. The different methyltransferases are collectively depicted in Figure 1 as reaction 22, where X denotes a compound that serves as a methyl acceptor. Each of these methyltransferases is present in small amounts. In contrast, GNMT is very abundant. In the livers of various species it makes up 1–3% of the soluble protein of the cytosol [35,37]. GNMT catalyzes a reaction that synthesizes sarcosine, a compound with no known metabolic role (it is rapidly oxidized back to glycine via reaction 12). Thus the methyl transferases that synthesize essential products are each present in small quantities, while the enzyme that synthesizes a nonessential product appears to be present in very large amounts. It has been suggested therefore that the function of GNMT is not to synthesize sarcosine, but to convert excess SAM to SAH through a reaction that does not form a product that might have physiological consequences. The ratio of SAM to SAH is important because most methyl transferase reactions are product-inhibited by SAH and their activities are governed by the SAM/SAH ratio [38]. GNMT provides an alternate route from SAM to SAH. Once SAH is formed it is cleaved to adenosine and homocysteine by SAH hydrolase (reaction 24). The homocysteine may be remethylated in reaction 20 or may combine with serine to form cystathionine (reaction 25) catalyzed by cystathionine synthase. Cystathionine can eventually be converted to cysteine and to glucose for gluconeogenesis. It is significant that this major pathway for methionine catabolism begins with activation to form SAM and requires methyl transfer to an acceptor in order for homocysteine to be produced [39].

Note that reactions 19 and 23 complement each other in that the synthesis of 5-methyl-THF is inhibited by SAM and the utilization of SAM is inhibited by 5-methyl-THF. This links the de novo synthesis of methyl groups (reaction 19) with regulation of the SAM/SAH ratio and availability of dietary methionine. When there is sufficient methionine in the diet, reaction 21 ensures that enough SAM will be made to inhibit reaction 19. The synthesis of 5-methyl-THF is unnecessary under these conditions since its only function is for remethylation of homocysteine. The decreased amount of 5-methyl-THF means that GNMT (reaction 23) is not inhibited and can proceed at a high rate to remove excess SAM and maintain the SAM/SAH ratio. When dietary methionine is low, however, there will be less SAM formed via reaction 21 and less inhibition of reaction 19. The increased levels of 5-methyl-THF contribute to the remethylation of homocysteine and inhibit GNMT (reaction 23). This acts to conserve the limited amount of methionine for the essential methylation reactions denoted by reaction 22.

III. COMPARTMENTATION OF FOLATE METABOLISM

Recent studies have shown that there is a compartmentation of folate metabolism between the cytosol and the mitochondria, at least in the liver and probably in other

tissues as well. This was first shown by Cook and Blair [40], who demonstrated that radioactive folate injected into rats was distributed differently between the cytosol and mitochondria. Horne et al. [41] showed that most of the folate in liver is distributed between the cytosol and mitochondria and that these two pools are not in equilibrium. This was done by exposing rats to nitrous oxide gas. This agent destroys the cyanocobalamin cofactor needed for reaction 20, methionine synthase. It mimics many of the effects of vitamin B_{12} deficiency. A functional folate deficiency develops because reaction 19 cannot be reversed and 5-methyl-THF is trapped and accumulates. It was found that although 5-methyl-THF accumulated in the cytosol fraction of the livers of nitrous oxide–treated rats, there was no change in the distribution of mitochondrial folates. Horne et al. [42] have now characterized the uptake of folate by intact mitochondria. They showed that the reduced folates, 5-methyl-THF and 5-formyl-THF, are taken up by a carrier-mediated process, while oxidized forms are not. This is consistent with the absence of dihydrofolate reductase in the mitochondria. Although folates are transported into the mitochondria by a carrier-mediated process, it is not energy dependent and is slower than the uptake of compounds such as serine and glycine.

In addition to the compartmentation of the folate coenzymes, there is a curious compartmentation of the enzymes. Some are found in the cytosol, some in the mitochondria, and some are found in both locations. A number of early studies that dealt with the isolation and characterization of these enzymes employed vigorous methods of tissue disruption which permitted mitochondrial contents to mix with the cytosol. Enzymes isolated in this way and found to be soluble were assumed, perhaps incorrectly, to have been derived from the cytosol.

Table 2 lists the probable location of each of the enzymes in Figure 1 that metabolize folate. Although many enzymes are present either in the cytosol or in the mitochondria, a number of activities (reactions 3, 4, 7, 8, and 14) are present in both compartments. In the cytosol, reaction 3, 4, and 8 are found as part of a trifunctional enzyme on a single peptide chain [7,8]. All three activities are present in rat liver mitochondria, but they have not been purified and it is not known whether they are on the same peptide [43]. Serine hydroxymethyltransferase (reaction 14) has been purified from both the cytosol and mitochondria. They are clearly different proteins with different primary structures but with identical active sites. Only the sequence of the cytosolic form of 10-formyl-THF dehydrogenase (responsible for reaction 7) is known.

Figure 2 shows the compartmentation of folate-mediated one-carbon metabolism [44]. The movement of reduced folates across the mitochondrial membrane is slow, but serine, glycine, and formate are rapidly equilibrated. Appling [45] has emphasized the interdependence of one-carbon metabolism between the two compartments. This was demonstrated in yeast, where it is much easier to obtain mutants lacking certain reactions but was also shown in higher animals. Certain mutants of Chinese hamster ovary (CHO) cells that are deficient in only the mitochondrial form

Table 2 Subcellular Localization of Folate Reactions

Reaction	Cytosol	Mitochondria
1	X	
2	X	
3	X	X
4	X	X
5	X*	
6	X*	
7	X	X
8	X	X
9		X
11		X
12		X
13		X
14	X	X
15	X	
16	X	
17	X	
18	X	
19	X	
20	X	
23	X	

Numbers refer to the reactions shown in Figure 1.
*Unpublished data, Glenn and Wagner.

of serine hydroxymethyltransferase (reaction 14) are unable to grow unless glycine is added to the medium [46]. Clearly the cytosolic form of the enzyme is unable to satisfy the glycine requirement of the cell. In other studies, it was shown that intact rat liver mitochondria could convert carbon 3 of serine or the *N*-methyl group of sarcosine to formate, which then was incorporated into purines by the addition of cytoplasmic 10-formyl-THF synthetase (reaction 8) and a cytoplasmic purine-synthesizing system [43]. It is believed that serine, the major source of one-carbon units, or dimethylglycine and sarcosine, which are products of choline catabolism in the liver only, enter the mitochondria and generate 5,10-methylene-THF. This can be converted to 10-formyl-THF via reactions 3 and 4 and used for mitochondrial protein synthesis. The glycine that is generated is degraded by the glycine cleavage reaction (reaction 13). Excess 10-formyl-THF can be converted to formate via reaction 8 in the mitochondria and transported back to the cytosol where it can reenter the one-carbon pool through reaction 8 in the cytosol.

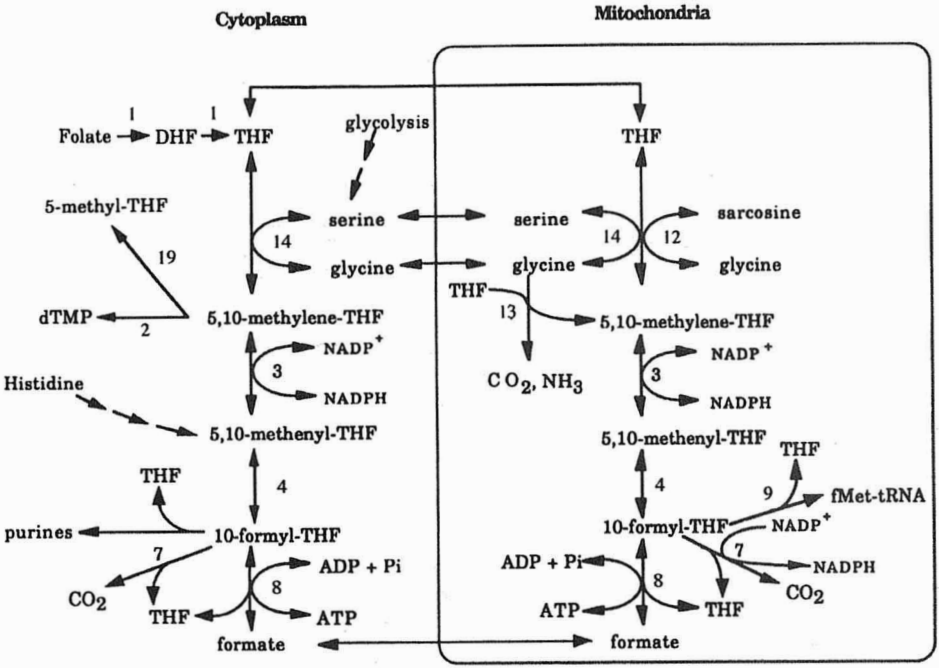


Figure 2 Compartmentation of folate-mediated one-carbon metabolism. (Adapted from Garcia-Martinez and Appling [44].)

IV. MANIFESTATIONS OF FOLATE DEFICIENCY

A. Defective Cell Division

Folate deficiency results in a specific type of anemia, a megaloblastic anemia, in humans [47]. Megaloblasts are large, abnormal, nucleated cells that are precursors of erythrocytes, and in folate deficiency they accumulate and are found in the bone marrow. These cells arise as a result of a failure of the red cell precursors to divide. The anemia, the deficiency of erythrocytes, is only one manifestation of diminished cell division. There are also decreased numbers of white cells and platelets. It is possible to conclude that there is general impairment of cell division, which is more apparent in tissues which turn over rapidly, such as the hematopoieitic system and the cells lining the digestive tract. These latter cells have "megaloblastic" changes in severe folate deficiency and appear abnormally large with enlarged nuclei. The impairment of cell division can be related to the role played by folate in nucleic acid synthesis. Two separate steps in the de novo pathway for purine biosynthesis utilize 10-formyl-THF, and the formation of thymidylic acid needed for DNA

synthesis utilizes 5,10-methylene-THF. A number of studies have indicated that it is the synthesis of thymidylate that is most susceptible to the deficiency of folate in the bone marrow rather than purine biosynthesis [48,49]. Studies on the pathogenesis of the anemia in folate deficiency have been hampered by the absence of a convenient experimental animal model. Until recently, only primates developed a typical megaloblastic anemia when fed a folate-deficient diet. Other species only demonstrated a decreased growth rate. Walzem and Clifford have developed a diet which, for the first time, rapidly produces anemia in both rats and mice [50,51]. This employs a mixture of amino acids to replace the casein previously used as the source of protein. Traces of folate present in the "vitamin-free" casein are eliminated, and the animals demonstrate decreased levels of red cells, white cells, and platelets in 3–5 weeks. Studies carried out on cultures of erythropoietic cells from the marrow of these folate-deficient mice showed that the block in erythropoiesis was corrected by thymidine but not by deoxycytidine or inosine (Koury and Horne, unpublished observations).

B. Accumulation of Metabolites

The overall metabolism of the folate coenzymes is shown in Figure 1. This interrelates reactions that generate and reactions that utilize one-carbon units and also shows how the different folate coenzymes are interconverted. In the case of folate deficiency, all of these reactions will be compromised to varying degrees depending on the relative affinities of the enzymes for their folate coenzymes and their subcellular location since, as described above, mitochondrial folates are not in rapid equilibrium with those in the cytosol. When reactions generating one-carbon units are affected by folate deficiency, substrates of those enzymes will accumulate and may be excreted. 5-Formiminoglutamic acid (FIGLU), a product of histidine breakdown, is such a compound and appears in the urine during folate deficiency [52]. It is easily measured and has been used as an indictor of folate deficiency. The accumulation of FIGLU has not been associated with any toxicity.

AICAR, the substrate for the incorporation of the second formate group in purine biosynthesis, likewise accumulates during folate deficiency. Both AICAR and its dephosphorylated product can be detected in the urine of folate-deficient humans and rats [53,54]. On the other hand, GAR, which is the substrate for the incorporation of the first formate, does not accumulate. When both of the enzymes from chicken liver were examined, as might be expected, the K_m for 10-formyl-THF, the formyl donor, was much lower for transfer of the formyl group to GAR than for transfer to AICAR, suggesting that the latter reaction is more easily affected by folate deficiency [55,56]. No toxic effects have been attributed to elevated levels of AICAR.

In contrast to the studies described above, accumulation of homocysteine, as a result of folate deficiency, may be a potential source of serious health problems.

As described above, the methyl group of methionine is lost during metabolism, and the homocysteine that eventually arises from this process is remethylated by the transfer of a methyl group from 5-methyl-THF. Failure to remethylate homocysteine results in elevation of both homocysteine and homocystine levels in the blood (homocystinemia) and in the urine (homocystinuria). A number of inherited metabolic defects are known to produce homocystinuria [39]: (1) methylene-THF reductase deficiency, which results in a failure to form 5-methyl-THF, (2) defects in processing of the cobalamin coenzyme needed for the transfer of 5-methyl-THF to homocysteine, and (3) absence of cystathionine synthase. During folate deficiency it might be expected that the remethylation of homocysteine would be compromised. This has been confirmed in a number of studies, and there is an inverse correlation between the levels of folate and homocysteine in blood in humans [57]. The elevation of homocysteine levels in the blood is not benign, however. Homocystinemia is associated with an increased risk of vascular disease [58]. Clinical studies have established that increased plasma homocysteine is associated with premature atherosclerosis in the coronary, cerebral, and peripheral vessels. There is thus some concern that folate deficiency itself might contribute to the development of atherosclerosis. This topic is dealt with in more detail in a later chapter.

C. Neural Tube Defects

There is now convincing evidence that folate supplementation reduces the incidence of neural tube defects in certain high-risk populations. There is no conclusive evidence, however, that individual mothers who have given birth to children with such defects were folate deficient. Although folate is necessary for cell division, the metabolic link between folate or one-carbon metabolism and development of the neural tube is unknown. This topic is dealt with in detail in a Chapter 12.

D. Defective Methylation

The importance of methylation reactions has been mentioned above. Not only are these reactions needed for the biosynthesis of many important products, but the methylation of DNA has been shown to regulate the expression of genes in eukaryotic cells. Methyl groups are transferred to the N5 position of cytosine in DNA by a specific DNA methylase. In most species about 3–5% of the cytosine in DNA appears as methylcytosine [59]. The extent of methylation of specific genes varies from tissue to tissue and changes during development. It has been shown that, in most cases, undermethylation favors gene expression and increased methylation is associated with gene "silencing" [59]. It is therefore of considerable interest that when rats were fed a diet containing no methyl groups, there was a very high incidence of spontaneous hepatic cancer [60]. This was a synthetic amino acid diet containing no choline in which methionine had been replaced with homocysteine. Animals fed the methyl-deficient diet were shown to have

undermethylated DNA when compared with animals fed a control diet [61]. The decrease in DNA methylation was accompanied by altered gene expression, which resembled the pattern of gene expression seen in animals exposed to carcinogens and in hepatomas. It is suggested that the undermethylation of the DNA causes increased expression of certain oncogenes.

In the diet described above all of the methyl groups needed for normal metabolism must be synthesized de novo from the one-carbon folate pool. Presumably de novo synthesis is not sufficient to provide enough SAM for methylation of DNA, and perhaps for other reactions as well. In addition, the high levels of homocysteine in the diet led to elevated amounts of SAH (due to reversibility of reaction 24) and thus decreased SAM/SAH ratios, which might be expected to inhibit methyltransferases generally [62]. Even when a normal diet containing choline is fed the amount of methionine it is not enough to satisfy the requirements for protein synthesis and for metabolic methylations. In humans, between 50 and 80% of the homocysteine generated is remethylated, depending on the dietary content of methionine and choline [63,64]. It might be expected, therefore, that folate deficiency itself could impair homocysteine metabolism by decreasing the amount of 5-methyl-THF available for reaction 20 and generally depressing the size of the one-carbon folate pool. This would also result in the elevation of homocysteine levels and decrease the SAM/SAH ratio. Both effects would be expected to impair cellular methylation reactions. This is indeed the case. When rats were fed the amino acid–defined folate-deficient diet developed by Clifford for 2 weeks, the SAM/SAH ratio in the liver decreased from 9.7 to 1.8 [65]. After 4 weeks the methylation of total liver DNA was significantly reduced [66].

The negative effect of folate deficiency on cellular methylation reactions may also be apparent in other ways. A number of recent studies have suggested that one-carbon compounds may play an important role in the function of the exocrine pancreas. The major function of this organ is the secretion of enzymes used in the digestion of a variety of nutrients. Ethionine, an analog of methionine that blocks cellular methylation reactions, has a specific toxic effect on the exocrine function of the pancreas [67]. The metabolic defect is an inability to release preformed zymogen granules from the pancreas [68]. A possible role for methylation in the secretion process is suggested by recent evidence showing that hormone-stimulated secretion in a number of tissues requires the interaction of G-proteins with components in the plasma membrane [69]. The carboxyl-terminal amino acid of these G-proteins is cysteine, which is first prenylated and then must be methyl esterified on the free carboxyl by reaction with SAM in order for interaction with the membrane to take place. Because secretion in the exocrine pancreas is such an active process, one might expect this to place a high demand on tissue levels of SAM. Alternatively, the extensive turnover of plasma membrane components during secretion may place a large burden on the synthesis of phosphatidylcholine from phosphatidylethanolamine. The enzyme, GNMT, is concerned with the regulation

of the SAM/SAH ratio and the de novo synthesis of methyl groups, as described above. Although GNMT is very abundant in the liver, it is almost as high in the exocrine pancreas [70], where it is also subject to allosteric inhibition by 5-methyl-THF [71], suggesting that it may have a function similar to that in the liver. The level of total folate in the pancreas is also very high, about 25% of the level in the liver, which is higher than any other extrahepatic organ [72]. Folate deficiency produces many of the same changes in the pancreas as it does in the liver, and the SAM/SAH ratio is decreased about 50% after 4 weeks [72]. It has recently been shown that after feeding the folate-deficient diet for 5 weeks the secretory response of the pancreas to cholecystokinin was significantly decreased (Balaghi and Wagner, unpublished observations). It is not clear whether this is a direct result of impaired methylation in the folate-deficient animals or whether it is due to a more general effect on amino acid and nucleic acid metabolism.

There is a well-known association between alcohol consumption and impaired folate absorption [73,74]. Alcoholism is the highest single cause of nonobstructure pancreatitis in humans [75,76]. If the pancreas is particularly sensitive to folate deficiency because of its high requirement for methyl groups, it might explain the high incidence of pancreatitis among alcoholic patients.

V. SUMMARY

The principal function of the folate coenzymes is to carry one-carbon units. Folic acid is converted to the active coenzyme by reduction to tetrahydrofolate. One-carbon units are carried at the formate, formaldehyde, or methanol levels of oxidation. Interconversion of these forms is accomplished by a series of enzymes. Many of these enzymes are multifunctional and are capable of "channeling" the one-carbon unit from one reaction to the next without equilibrating with the medium. 5-Formyltetrahydrofolate, previously believed to be an unnatural, but pharmacologically important, form of folate, has been shown to arise physiologically as a minor product of the enzyme serine hydroxymethyltransferase.

In addition to enzymes that metabolize folate, glycine N-methyltransferase is an enzyme whose activity is regulated by folate. This enzyme is important in controlling the methylating capacity of the cell.

Folate coenzymes and folate enzymes are compartmentalized between the cytosol and the mitochondria. Metabolic products are readily transported between compartments, but the folate coenzymes are not.

Folate deficiency leads to defective cell division and accumulation of metabolites, some of which, e.g., homocysteine, may be toxic. Folate deficiency can also lead to defective methylation since the de novo generation of methyl groups requires folate and the dietary supply of methyl groups coming from choline and methionine are not ordinarily sufficient to supply the needs of body.

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3

Clinical Spectrum and Diagnosis of Folate Deficiency

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I. INTRODUCTION

Folate deficiency is an important problem in areas of the world where there is poverty and malnutrition and is also seen less frequently due to a variety of other causes. It may be second only to iron deficiency (and in some situations the anemia of chronic disease) as a cause of anemia. Yet the recognition and diagnosis of folate deficiency often present difficult and confusing problems. In fact, although a very extensive literature has accumulated on the subject, much of it is difficult to interpret.

There are a number of reasons for this. It has not been easy to identify a "pure" folate deficiency state, either experimentally or clinically. Unlike pernicious anemia, for example, in which the entire clinical picture results from an isolated inability to absorb cobalamin, usually without associated deficiency of other nutrients, lack of folate usually develops in patients who have been ingesting a diet that is deficient in multiple nutrients or as part of a malabsorption syndrome in which the ability of the upper small bowel to absorb virtually everything may be compromised. Thus, a clinical finding that may be encountered in a patient who is clearly folate deficient (e.g., a neurological abnormality or a morphological picture such as a marrow crowded with ringed sideroblasts) may result from lack of folate or may be attributable to some other associated deficiency state or disorder. Furthermore, since malnutrition of multiple nutrients is frequently corrected at the same time folic acid therapy is given, an apparent response to the vitamin may be caused by the feeding of other substances or, in other situations, by the spontaneous remission of concomitant disease states. Even the few experimental studies in which human volunteers have been placed on a folate-deficient diet and depletion of the vitamin has been successfully achieved have not always been completely free of

these problems. For example, in the landmark study of Herbert [1] in which he induced the deficiency in a single subject (himself), unanticipated depletion of potassium and iron occurred during the first 10–15 weeks of the experiment. This needs to be taken into consideration in interpreting the "staging" of folate deficiency based on this study, since the bone marrow morphology and the number and size of the circulating erythrocytes may have been affected [1]. In other instances, folate deficiency has often been produced experimentally by combining a low-folate diet with alcohol intoxication [2–5], with its attendant complexities.

In this chapter we will begin by attempting to outline the sequence of events as progressive folate depletion develops. Attention will then be focused on the diagnostic measures available to identify deficiency of the vitamin.

II. SEQUENCE OF HEMATOLOGIC EVENTS IN DEVELOPING FOLATE DEFICIENCY

Since Herbert's classic experiment [1], a number of other investigators have used his diet or similar ones (requiring prolonged boiling of foodstuffs) to produce a state of folate deficiency in human volunteers [2–5]. However, only a handful of subjects have been studied in this fashion. Multiple serial assessments of marrow morphology or red cell folate concentrations were usually not performed, and only Herbert's original paper reported serial erythrocyte mean cell volumes (MCVs). Furthermore, for ethical or practical reasons, the experiments were terminated after only a moderate deficiency state was produced. In addition to these studies, however, many relevant clinical studies are available from which inferences may be drawn. Although such observations are commonly cross-sectional rather than longitudinal, they nonetheless provide useful information if interpreted with caution.

One is tempted to summarize the "stages" of developing folate deficiency in diagrammatic form in a single table or figure. Indeed, there have been brave attempts to do this [6]. However, this temptation will be resisted here. As will be noted, there are so many exceptions to the rules that any attempt at rigid classification of the stages of developing folate deficiency is doomed in our opinion to be so oversimplified as to be clinically misleading. Nonetheless, there is no doubt that as deficiency develops in a human being, usually over a period of several months to years, progressively severe depletion of the vitamin will be experienced and manifest *more or less* in the sequence summarized below.

A. Decrease in Serum Folate Concentration

In all the experimental studies of human volunteers subjected to folate deprivation, a fall in the serum folate level, usually occurring within 1–3 weeks, is the first event [1–5], although in some subjects this may require as long as 2 months to occur [2,5]. There ensues a period of weeks or months when the serum folate concentration is

low in which there is no other evidence of deficiency. Because of this, this stage has been referred to as one of "negative folate balance," rather than real deficiency [6]. Certainly the circulating folate level may be depressed even in situations in which no detectable alteration in total body folate has occurred, e.g., after acute alcohol administration [7]. The majority of low serum folate values encountered in clinical practice will not prove to be associated with any other evidence of deficiency. Therefore, a low serum concentration alone should not be considered, as has frequently occurred in the literature, to be a form of "folate deficiency." However, this does not indicate (as discussed below) that the serum level is a worthless diagnostic test if used and interpreted correctly in conjunction with the serum cobalamin level and the clinical and hematological findings.

B. Early Biochemical and Morphological Evidence of Tissue Folate Depletion

As folate stores begin to be depleted and progressive deficiency develops, the red cell folate concentration as measured by microbiological assay with *Lactobacillus casei* will fall to subnormal levels over a period of months [1]. Observations in patients at high risk for deficiency suggest that the serum homocysteine level usually becomes elevated at more or less the same time, as discussed later in this chapter.

In many patients at high risk, such as actively drinking chronic alcoholics who are also ingesting a folate-poor diet and unsupplemented pregnant women, one frequently encounters low serum and red cell folate levels. Elevated serum homocysteine levels are also commonly found in malnourished alcoholics in the absence of the hematological abnormalities characteristic of folate deficiency.

Although this was not clearly shown in the original subject studied by Herbert, perhaps due to concomitant iron deficiency, in a subsequent investigation megaloblastic changes in the bone marrow developed in both of two volunteers studied by Eichner and coworkers before abnormalities on the peripheral blood film were detectable [3]. The morphological abnormalities in the marrow also occurred in these subjects before erythrocyte folate levels became abnormal and in two of five volunteers studied by Cowan [4]. Usually the changes noted in the bone marrow at this early point are only mild to moderate in severity. By the time a florid megaloblastic marrow is seen, changes in the peripheral blood can most often be demonstrated. Giant bands and metamyelocytes are typically found during the stage of early marrow abnormalities (e.g., in pregnant women and in alcoholics) and may precede clear-cut alterations in the morphology of erythroid precursors.

C. Early Peripheral Blood Changes

Within weeks of the development of early morphological abnormalities in marrow, rather subtle changes appear in the peripheral blood [3]. This undoubtedly occurs in folate deficiency but has been more abundantly documented in states of cobalamin

depletion. It has been possible to observe the unfolding of mild deficiency in patients with pernicious anemia in whom maintenance cobalamin therapy was interrupted or in neglected first episodes of deficiency [8–14]. As the earliest peripheral blood changes appear, the MCV begins to rise above the baseline level for the patient but is not yet above the upper limit of normal for the test. Very early hypersegmentation or increments in neutrophil lobe average are also usually detectable at this time and often precede the increase in the MCV [14]. In some persons, macrocytes and macroovalocytes will also be seen on blood smears, but in others the red cells may show only minimal anisocytosis or no abnormalities. Thus, the peripheral blood picture prior to the development of a clearly increased MCV or anemia is variable. Some patients' smears show macroovalocytes and hypersegmented neutrophils that are apparent to the trained observer even though an elevation in the MCV has not yet developed [14,15].

D. Macrocytosis Without Anemia

As folate (or cobalamin) depletion progresses further, the MCV becomes unequivocally increased above normal. Neutrophil hypersegmentation (defined as more than 5% five-lobed or any six-lobed cells/100 granulocytes) is typically present in the peripheral blood at this stage and the neutrophil lobe average is elevated. In some patients, however, these changes may be very subtle and of questionable reproducibility (e.g., a single six-lobed neutrophil per 100 white cells or 6% five-lobed cells, hardly findings that will be identified by the average routine hospital laboratory) or may be undetectable. In the bone marrow, although in some instances the red cell changes are rather mild, increased numbers of giant bands and metamyelocytes are invariably seen at this stage of folate depletion in our experience.

E. Macrocytic Anemia

Eventually anemia develops. This will first be evident as a depression of the red blood cell count (RBC). As the defect in the production of erythrocytes appears, the hemoglobin and hematocrit remain normal for a period of time because of the increased red cell size, and the only abnormality in electronic counter measurements besides the high MCV (and MCH) is the low RBC. Eventually all three measures of anemia (hematocrit, hemoglobin concentration, and the red cell concentration) are depressed. At this point, macroovalocytes and macrocytes are usually detectable in the peripheral blood and hypersegmentation is more impressive. Anisocytosis and poikilocytosis of varying degrees will also be seen. The patient frequently remains asymptomatic.

F. Severe Anemia

As the anemia becomes progressively more severe, other findings appear or become more prominent. With hematocrits below 30%, the serum lactic dehydrogenase

(LDH) is usually elevated [16,17] (Table 1). Marked elevations (>600 μ/ml) are not seen in the majority of patients until the hematocrit falls below 20% and appear to be more common in cobalamin deficiency with lesser degrees of anemia (Table 1). The serum unconjugated bilirubin often rises and the plasma haptoglobin falls so that at this stage megaloblastic anemias may be confused with hemolytic anemias. The reticulocyte count, however, typically is not elevated, particularly if it has been corrected for the degree of anemia. We have found it most useful to calculate the absolute reticulocyte count by multiplying the RBC concentration by the percent reticulocytes rather than make more complicated adjustments.

The bone marrow becomes obviously megaloblastic with marked erythroid hyperplasia, although in occasional patients erythroid hypoplasia is seen [17]. With advancing anemia, the platelet count often becomes subnormal. Less commonly, the white blood cell count will also become depressed. Mild neutropenia may precede thrombocytopenia in some persons. If granulocytopenia is severe, neutrophil hypersegmentation may be difficult to detect [19]. Hypersegmentation, macroovalocytosis, anisocytosis, and poikilocytosis become more florid as anemia worsens. Howell-Jolly bodies, teardrop erythrocytes, and nucleated red blood cells may be seen on the blood film. In some patients the serum LDH rises to very high levels (1000–10000 μ/ml) (Table 1). With profound anemia, the degree of elevation of the MCV actually lessens [20,21], and in some instances the MCV may be normal even in the absence of a coexisting microcytic disorder.

Symptoms of anemia, such as weakness, fatigue, difficulty concentrating, irritability, headache, palpitations, and shortness of breath, typically appear at this stage. They may be seen at milder degrees of anemia in certain patients, particu-

Table 1 Serum Lactic Dehydrogenase (LDH) Levels in Patients with Cobalamin and Folate Deficiency with Varying Degrees of Anemia[a]

Hematocrit (%)	>35	30-34		20-29		<20	
Deficiency	CBL	CBL	Folate	CBL	Folate	CBL	Folate
Number of patients (%)	87	34	16	85	26	66	48
Serum LDH (μ/ml)							
<225	61 (70.1)	19 (55.9)	2 (12.5)	4 (4.7)	2 (7.7)	1 (1.5)	2 (4.2)
225–300	20 (22.9)	6 (17.6)	6 (37.5)	7 (8.2)	5 (19.2)	0	7 (14.6)
301–600	6 (7.0)	7 (20.6)	8 (50.0)	21 (24.7)	15 (57.7)	8 (12.1)	10 (20.8)
>600		2 (5.9)		53 (62.4)	4 (15.4)	57 (86.4)	29 (60.0)
>1000		1 (2.9)		31 (36.4)	1 (3.8)	32 (78.8)	24 (50.0)

[a] 272 and 90 consecutive patients with proven clinical deficiency (18) of cobalamin (CBL) and folate, respectively, studied at two New York City hospitals from whom serum LDH levels were available. All of the folate-deficient patients were anemic. The majority of the patients were studied in collaboration with Dr. David Savage.

larly the elderly. Angina pectoris may occur or be aggravated in persons with underlying coronary artery disease. Congestive heart failure may develop. Pulmonary edema may ensue, which may be fatal. In rare patients a severe, even lethal, lactic acidosis may occur. Tachycardia and postural hypotension are other frequent developments.

G. Warning

It must be emphasized that the above outline of events is that seen in the "average" person with evolving deficiency. Many patients will be encountered in clinical practice that do not appear to have read this chapter. For example, as will be discussed subsequently when diagnostic tests are considered, the *L. casei* red cell folate concentration is normal in a minority of patients with clear-cut megaloblastic anemia due to folate deficiency [22,23]. The serum folate level, as measured by microbiological or radiodilution assays, has also repeatedly been found to be normal in a substantial percentage of patients with frank megaloblastic anemia due to lack of the vitamin [18,23–26]. The serum homocysteine concentration was not increased in 9% of a recent consecutive series of patients with anemia due to folate deficiency [18]. Coexisting disease may mask some of these outlined changes as well. For example, the anemia of chronic disease, iron deficiency, or thalassemia minor may obviate the MCV elevation, and iron deficiency may obscure the erythroid marrow abnormalities [26].

III. INVOLVEMENT OF OTHER SYSTEMS

A. Glossitis

In current clinical practice, by the time folate depletion is severe enough to cause megaloblastic anemia, about a quarter of patients will have atrophic glossitis [18]. Some will complain of sore tongue or pain on swallowing. The tongue may be swollen, beefy red, or shiny in appearance and show atrophy of the papillae, occurring first at the edges and tip of the tongue. Whitish apthous ulcers with red borders may be seen [27]. The most common finding, however, is merely atrophy of the papillae, in the absence of tongue symptoms. Clinically detectable involvement of the tongue usually occurs at the stage in which anemia has developed. On occasion, however, it may be seen when the hematocrit is still normal.

B. Other Gastrointestinal Manifestations

Nausea, vomiting, and diarrhea occur in some patients with megaloblastic anemia due to lack of folate (or cobalamin). Abdominal pain and vomiting frequently appear after meals. It has been hard to estimate the exact frequency of these symptoms that are attributable to folate deficiency. In many patients, the complaints may be

caused by the underlying disorder that caused the deficiency rather than lack of folate itself. In tropical sprue, these gastrointestinal symptoms are frequent. However, since they often rapidly disappear within days of beginning folate therapy, they may actually be caused by the deficiency state rather than the underlying disease. The mechanism of the gastrointestinal symptoms caused by folate and cobalamin deficiency has not been demonstrated.

Anorexia is a very common complaint in folate-deficient patients, usually but not always related to anemia, often accompanied by substantial weight loss. Rather than a gastrointestinal symptom, however, anorexia may be an effect of anemia or of folate deficiency itself on the central nervous system.

Reversible malabsorption of test substances (e.g., xylose, fat, or vitamin B_{12} given with intrinsic factor) and jejunal morphological abnormalities have been demonstrated in patients with untreated folate deficiency [28–33]. However, no relationship has been demonstrated between the presence or severity of the defects in absorptive function and the occurrence of gastrointestinal symptoms.

The "megaloblastic gut" caused by lack of folate is thus often inapparent clinically. It may play a major role, however, in the development or clinical precipitation of symptoms in patients with tropical sprue. A vicious cycle appears to occur, in which the damage to the small bowel (probably induced by intestinal bacteria) results in folate malabsorption, which then causes folate deficiency of the small intestinal mucosa and aggravates the malabsorption state [34,35].

C. Hyperpigmentation

Areas of darkening of the skin and mucous membranes may be caused by severe folate deficiency [36–41]. Similar hyperpigmentation is also encountered in cobalamin-deficient patients [42,43]. The pigmentation is often patchy. It typically involves the dorsal surfaces of the fingers and toes, including the nails, and the creases of the palms and soles. Facial areas around the mouth and forehead and the tongue and buccal mucosa may also be affected. It may be more common in nonwhite populations [43]. In white patients a diffuse faint greyish-brown pigmentation, particularly affecting exposed parts, has been described [37]. The hyperpigmentation is gradually reversed over weeks to months by vitamin therapy. It was reported in 13 of 15 patients with nutritional megaloblastic anemia in Singapore [38] and in all of 7 women in Bristol with the disorder [37]. However, most observers have found abnormal pigmentation much less frequently [40]. The mechanism has not been established [42].

D. Fever

Even in the absence of infection, a low-grade fever occurs in many patients with megaloblastic anemia. Fever of unknown origin has been reported in approximately

40% of patients with either cobalamin or folate deficiency [44]. The temperature elevations are usually modest (< 102°F in most instances), begin to fall within 24–48 hours of vitamin therapy, and return to normal within a few days. These modest temperature elevations are more frequent in severely anemic patients [44]. The fever is almost certainly a reflection of the underlying deficiency state, but its pathogenesis is obscure. The role of cytokines would be worthy of study.

All of the above manifestations of lack of folate are not unique to deficiency of the vitamin, since they are also seen in cobalamin deficiency. Identical changes in the bone marrow and peripheral blood, glossitis, gastrointestinal symptoms, secondary malabsorption, hyperpigmentation, and fever occur in cobalamin-deficient patients.

E. Neuropsychiatric Abnormalities

This controversial and challenging area is the subject of Chapter 16 and will only be discussed briefly here. A sizable literature has accumulated, much of it involving anecdotal case reports, in which a variety of neurological disorders have been attributed to folate deficiency, including depression, dementia, peripheral neuropathy, and spinal cord damage resembling that seen in combined system disease due to cobalamin deficiency [45,46]. In some of the reported cases, evidence for depletion of folate stores was equivocal. In others, the patients were clearly folate deficient, but associated deficiencies or other disorders may have been responsible for the neurological manifestations. All of the problems in interpretation of folate-deficient patients due to the infrequency of "pure" deficiency states discussed above are relevant to the interpretation of these cases.

Certain things are clear, however. There is excellent evidence that the developing nervous system in utero and in infancy and childhood is dependent on adequate folate nutrition for normal function. The occurrence of neural tube defects in utero will be discussed in another chapter. In addition, infants and children with congenital folate malabsorption represent a rare example of "pure" folate deficiency. Of nine cases of congenital folate malabsorption reported in the literature, seven were mentally retarded and four had seizures [47–53]. Cerebral calcifications were detected either in the basal ganglia and/or the parieto-occipital cortex in three of four patients studied. In addition, cerebral calcifications in these areas have been described in children with acquired folate deficiency [54–56]. In two adolescent children with congenital folate malabsorption, peripheral neuropathy appears to have been well documented [49,53]. In one adult placed on a folate-deficient diet, mental symptoms, including irritability and forgetfulness as well as sleeplessness, developed and were promptly reversed after folate therapy [1]. Neuropsychiatric manifestations have not been reported by other investigators who studied human subjects on a folate-deficient diet [2–5].

Whether peripheral neuropathy, dementia, depression, or spinal cord damage occur in adults with folate deficiency remains to be established. Although tempt-

ing, it is problematic to extrapolate to the adult from documented syndromes in infants or children. For example, cobalamin deficiency in infants has neurological manifestations (including choreoathetotic movements), which are not encountered in adults with lack of cobalamin [57,58].

F. Infertility

Although cobalamin deficiency is a well-established cause of female and male infertility, it has been more difficult to establish a similar role for lack of folate. Patients with celiac sprue have been reported in whom inability to conceive, or recurrent abortion, was noted with a subsequent response to a gluten-free diet, sometimes given along with vitamins. Some of these patients were deficient in folate during the period of infertility [59–62]. One such patient conceived and had a normal pregnancy while taking folic acid, although she refused to follow a gluten-free diet [62].

IV. THE SYNDROME OF "ACUTE FOLATE DEFICIENCY"

A number of case reports and series of cases have been presented in whom the usual sequence of events in evolving folate deficiency as summarized earlier in this chapter has not been followed [63–78]. Although the documentation has varied from case to case and the picture has not always been uniform, certain findings have been common. Typically the patient has been very ill, often in an intensive care setting. Recent major trauma or surgery and/or hepatic and renal failure were often present. A majority of the reported patients have received parenteral nutrition containing amino acid solutions. Many have been given agents that interfere with folate metabolism such as ethanol, nitrous oxide, trimethroprim, or sulfasalazine. Many have had an underlying condition that tends to deplete folate stores such as malabsorption, pregnancy, or chronic alcoholism. *A common theme recurring in most of the case reports is a septic febrile illness*. The patient typically had a normal or relatively normal complete blood count and MCV before the various disastrous events occurred. Over a period of several weeks or 1–2 months, a shorter interval than has usually been required to cause frank folate deficiency by dietary means in normal volunteers, severe thrombocytopenia has developed, in some cases with neutropenia. Although the peripheral blood film has either been normal or has shown hypersegmentation and varying numbers of macroovalocytes, the MCV has not been elevated (in those cases in which its diagnostic value was not obscured by blood transfusions). Bone marrow changes have varied from very subtle megaloblastic abnormalities to unusually florid ones. In some instances, serum and red cell folate levels have been low, but in others these tests have been normal. In one patient, hepatic folate stores were also normal [73]. In some cases, bone marrow deoxyuridine suppression test values were consistent with folate deficiency [73,78]. This syndrome has usually been interpreted as due to acute megaloblastic arrest of

the bone marrow that is manifest primarily by severe thrombocytopenia and sometimes neutropenia as well. Folate deficiency has supposedly evolved so rapidly that the erythrocyte abnormalities have not had time to develop and only the cells with a very short life span have been affected.

An alternative interpretation is that in most instances the acute thrombocytopenia is primarily due to other causes, most frequently invasion of the blood stream by bacteria. The megaloblastic changes in the bone marrow may well be due to folate deficiency and may limit the response of the marrow to the shortened platelet life span characteristic of the thrombocytopenia of sepsis [79], but they are not the primary cause of the cytopenias. In some patients, clearly, agents that interfere with folate metabolism, including ethanol, nitrous oxide, antifols and methionine [80], may have caused or contributed to the acute megaloblastic arrest of the bone marrow. Loss of folate-rich bile through gastrointestinal suction may be a contributory factor in some patients [65]. Frequently, multivitamin therapy has been given without folate and it is possible that supplying all the other vitamins may increase the relative demands for folate. Folate requirements may well be increased in these desperately ill patients, but the exact contribution of infection or renal and hepatic failure to increasing requirements or the relevant mechanisms are obscure. In summary, rather than an acute folate deficiency limited to only one or two hematological cell lines, in our view most of these patients are best explained as cytopenias of complex origin in which folate deficiency plays a contributory role but is often not the main actor.

It is interesting that serum LDH levels go virtually unmentioned or have been normal in "acute folate deficiency" suggesting that severe ineffective erythropoiesis is not present despite the finding of a megaloblastic marrow. Since megaloblastic change precedes the development of any abnormalities in the peripheral blood in experimental folate deficiency, the finding of a megaloblastic marrow in a given patient does not necessarily imply that decreased levels of one or another cell line in the peripheral blood are the result of the megaloblastic change in the marrow.

V. "LOCALIZED" FOLATE DEFICIENCY

Macrocytic changes in a variety of epithelial tissues have been observed in patients with megaloblastic anemias [16]. Could such changes occur in a single organ in the absence of a generalized deficiency state? It is likely that increased demands for folate occur locally in a given tissue that is subject to stimuli causing increased cellular division. Thus, for example, in a group of patients treated with oral contraceptive hormones there was no evidence of systemic folate deficiency but megaloblastic changes were present on Papanicolaou smears of cells obtained from the uterine cervix, which reverted to normal after folic acid therapy [81,82]. Others have reported analogous syndromes involving white cells [83], colonic epithelial cells in patients with inflammatory bowel disease [84], and bronchial epithelial cells

in smokers [85]. Further studies are needed to define the exact nature and pathogenesis of these findings. Responsiveness to large doses of folic acid does not prove that the observed morphological abnormalities are caused by folate deficiency since the effects of the vitamin in large doses may be pharmacological rather than "physiological." Some of these localized cytological abnormalities may be precursor lesions to the development of cancers of various organs [86] (see Chapter 13).

VI. DIAGNOSIS OF FOLATE DEFICIENCY

Consideration will now be given to the use of various tests in the diagnosis of folate deficiency in patients. The focus will be on diagnostic considerations in the clinical setting in the individual patient in contrast to the use of the various tests in nutritional surveys of populations. As will be seen from the following discussion, no test is perfect in the diagnosis of folate deficiency, either in its specificity or sensitivity. Standard measurements, like the serum or red cell folate concentration, often give results that may mislead. It is still necessary for the clinician to put together all the clinical, morphological and biochemical information and come up with a diagnosis of deficiency or its absence. In most instances this is straightforward, but in a substantial minority of cases, diagnostic uncertainty is encountered and one may only be able to achieve a tentative or probable diagnosis.

A. Serum Folate Concentration

Since a low serum folate level is the first detectable abnormality in subjects placed on a folate-deficient diet, it might appear to be an ideal screening test for deficiency, at least from the point of view of sensitivity. Surprisingly, however, in a sizable minority of patients with clear-cut megaloblastic anemia due to folate deficiency, the serum folate concentration is only slightly decreased or is within the normal range [18,23,24,26,87]. Many investigators have noted a significant overlap in serum folate values between normal subjects and patients with clear-cut deficiency. This had led to the frequent use of "borderline," "indeterminate," "suggestive," or "highly suggestive" zones or ranges of serum folate, in which most patients will not turn out to be clinically deficient but a few will. Typical grey zones are in the range of 3–6 ng/ml using the *Lactobacillus casei* microbiological assay and 2–4 ng/ml using radioisotope dilution methods. Perhaps in 25% (in some series in as many as 50%) of patients with megaloblastic anemia due to folate deficiency, the serum level will fall in such a zone or may even be normal [23,24]. The use of such indeterminate, nondiagnostic ranges for a laboratory test markedly compromises its specificity.

In addition, an unequivocally *low* serum folate is frequently seen in patients who do not have tissue depletion of folate. In part this is to be expected from the way in which the normal range is defined for most diagnostic tests, excluding 2.5%

of normal subjects who will have low values by definition [88]. A low serum folate level also occurs in very early phases of folate deficiency (so-called negative folate balance) [6], as well as in a substantial fraction of pregnant women, of patients taking anticonvulsants, and of persons who recently have ingested large amounts of alcohol [2,16]. Acute alcohol administration to normal volunteers with presumed normal folate stores causes an acute fall in the serum folate concentration to low levels within hours [7]. The specificity and the sensitivity of the serum folate level are therefore both poor in the diagnosis of the clinically significant deficiency state. This has led some to abandon the serum folate completely and to use the red cell folate instead. In a study of alcoholics with anemia it was concluded that neither the serum nor red cell folate provided useful information and the tests were therefore not recommended in the routine workup of alcoholics with anemia [23].

The explanation for patients with folate deficiency and normal serum levels of the vitamin has not been clear. Although in some instances serum may not have been obtained in the fasting state, the recent feeding of folate does not appear to account for many of these anomalous results [23].

The serum folate level is still clinically useful, however, when employed in conjunction with the serum cobalamin concentration and the clinical and hematological data, as will be discussed below. Since most radioassay systems measure the serum cobalamin and folate levels simultaneously, the additional folate measurement adds little in the way of expense. In contrast, the red cell folate is a separate test and is therefore more costly. It also has its limitations.

B. Erythrocyte Folate Concentration

The erythrocyte folate concentration, in contrast to the serum level, appears to be a direct measurement of tissue coenzyme concentrations in cells produced by the bone marrow. In a comprehensive initial study of the performance of this test as measured by microbiological assay using *L. casei*, it correlated much more strongly with the presence of megaloblastic hematopoiesis than the serum folate measurement [89]. For these reasons it has often been argued that the erythrocyte concentration as measured by microbiological assay is a better test of tissue folate "stores" than the serum level and is a more reliable diagnostic test. Unfortunately, the red cell folate level has serious limitations in both sensitivity and specificity. Patients with clear-cut megaloblastic anemia due to folate deficiency may have normal red cell folate values [22,23,90,91]. The *L. casei* erythrocyte folate level was normal in 11 and 31% of two series of folate-deficient alcoholic patients with megaloblastic anemia [22,23] and in 26 and 61% of patients with megaloblastic anemia of pregnancy [90,91], respectively. Normal red cell folates are most commonly seen in patients with little or no anemia due to deficiency [22,23,92,93]. In a study of the hospital use of the red cell folate concentration, Jaffe and Schilling concluded that it added little useful information to the serum folate value [94]. Nonetheless,

the erythrocyte folate level is low in the majority of patients with megaloblastic anemia due to folate deficiency and the degree of depression of the level correlates with the severity of the anemia [89]. There are major problems with specificity, however [23,93]. The test is often subnormal in patients at risk for folate deficiency who have not yet developed it, e.g., chronic alcoholics or pregnant women [16,23]. A more serious problem in specificity is the depression of the erythrocyte folate concentration seen in 60% of patients with megaloblastic anemia due to cobalamin deficiency [16], since the differentiation between the two deficiency states is usually the most important issue in a patient with a proven or suspected megaloblastic anemia. In contrast, the serum folate level is low in only 2–10% of patients with pernicious anemia.

The above comments, however, depict the virtues and limitations of the erythrocyte folate measurement as assessed by *microbiological assays*. Such assays are virtually unavailable to clinicians as diagnostic tests at this time. A very important problem that has hardly been addressed is the current increasingly widespread use of *radioassays* for erythrocyte folate concentrations employing commercial radiodilution assay kit systems primarily designed for measurement of serum folate levels. The performance of many commercial radioassays of serum folate concentrations appears to be adequate and generally comparable to *L. casei* methods, with the advantages of technical simplicity and lack of interference by antibiotics [92,95–97], although there remains considerable variability in performance between commercial methods and between laboratories [98–100]. A host of technical problems in the measurement of erythrocyte folate by radioassay, however, has been repeatedly documented in the literature [96,98–102], raising serious questions about the precision, accuracy, and general reliability of the assays. Furthermore, with a single exception involving a single kit [93], there has been no meaningful clinical validation of these commercial assays comparing values from patients with well-defined deficiency with those of control subjects. The problem is further compounded by the tendency of manufacturers to modify kit methods without reevaluation. (It should be remembered that radiodilution assays for serum cobalamin became widely used in the 1970s in the absence of clinical validation leading to major errors in diagnosis before technical problems were resolved [88].) In current clinical practice, then, we cannot recommend radioassay measurements of the erythrocyte folate concentration as a diagnostic test in the evaluation of patients. This constitutes an additional persuasive argument in favor of the serum folate level.

C. Serum Homocysteine Concentration

The serum total homocysteine level is a functional test of folate coenzyme activity, which reflects tissue deficiency of methyltetrahydrofolate as a substrate in the methionine synthase reaction [88,103]. The sensitivity of the serum homocysteine concentration in the diagnosis of a large series of patients with proven megaloblastic

anemia due to folate deficiency was of the order of 90% [18]. However, the serum homocysteine level may be elevated in alcoholic patients who have not yet developed anemia or morphological evidence of folate deficiency [104,105]. In addition, the level of this metabolite may be increased in the serum of subjects with congenital disturbances in homocysteine metabolism as well as in renal insufficiency or intravascular volume depletion [18,88,106,107]. In two recent series, the serum homocysteine concentration was elevated in 40–46% of patients with serum creatinine levels greater than 2.0 mg/dl (Pogue, Lindenbaum, and Allen, unpublished observations).

The serum homocysteine value is elevated in 96% of patients with proven cobalamin deficiency [18]. Therefore, to be properly interpreted the serum homocysteine level needs to be used in conjunction with the serum cobalamin and methylmalonic acid (MMA) levels and tests of renal function [18,88]. The serum MMA is a very useful test for cobalamin deficiency in the evaluation of patients with possible deficiency of these vitamins [18,88,108–110].*

The relative merits of the serum folate, red cell folate, and serum total homocysteine concentrations in the diagnosis of folate deficiency have not been assessed in the literature in a series of patients whose hematological status was well defined. We therefore compared the performance of these three tests in a series of consecutive alcoholic patients with anemia studied in collaboration with Dr. David Savage (Tables 2 and 3). Clinical and hematological features of these patients have been published in detail [23]. In Table 2 the findings in 24 patients with megaloblastic bone marrow morphology in aspirates obtained within 24–48 hours of admission are compared with those of 71 who lacked megaloblastic changes. In half of the patients with megaloblastic changes the serum folate was above 2.1 ng/ml, the lower limit of normal for the radiodilution assay used [23]. If the cutoff point for detection of folate deficiency was raised to 4.0 ng/ml, excellent sensitivity was achieved at the expense of extremely low specificity. The sensitivity of the erythrocyte folate level as measured by *Lactobacillus casei* and the serum homocysteine concentration were both good, but about 30% of patients with normoblastic marrows had values for these tests consistent with folate deficiency. The majority of patients with abnormal values for each of the three measurements were in the normoblastic rather than megaloblastic group. In Table 3, patients with normoblastic bone marrow morphology and an elevated serum homocysteine level are compared to those without such an elevation. Depressions in serum and *L. casei* erythrocyte folate levels were more common in the patients with increased homocysteine values.

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*Aspects of the serum methylmalonic acid and total homocysteine assays are the subject of patents and patent applications filed on behalf of the University of Colorado, Columbia University, and the authors. A company has been formed by the University of Colorado to perform these assays.

Table 2 Diagnostic Tests of Folate Status in 95 Chronic Alcoholics with Anemia[a]

	No. patients/No. tested (%)	
	Megaloblastic bone marrow	Normoblastic bone marrow
Hypersegmented neutrophils present	20/24 (83.3)	4/71 (5.6)
Serum folate[b]		
<2.1 ng/ml	12/24 (50.0)	18/71 (25.4)
<4.0 ng/ml	23/24 (95.8)	46/71 (64.8)
Erythrocyte folate		
<150 ng/ml RBC[c]	7/8 (87.5)	10/33 (30.3)
Serum total homocysteine > 21.3 μM	21/24 (87.5)	22/71 (31.0)

[a] Of 121 patients studied consecutively, sera for measurement of folate and homocysteine were available in 95, and RBC folate was assayed in 41 of the 95. The serum cobalamin was greater than 300 pg/ml in every patient.
[b] Measured by radioassay.
[c] Measured by microbiological assay with *Lactobacillus casei*.
Source: Ref. 23.

Table 3 Diagnostic Tests of Folate Status in 71 Anemic Alcoholics with Normoblastic Bone Marrow Morphology and Elevated or Normal Serum Homocysteine Levels[a]

Serum total homocysteine	No. patients/No. tested (%)	
	Serum total homocysteine > 21.3 μM	⩽ 21.3 μM
Neutrophil hypersegmentation present	2/22 (9.1)	2/49 (4.1)
Serum folate		
<2.1 ng/ml	7/22 (31.8)	11/49 (22.4)
<4.0 ng/ml	16/22 (72.7)	30/49 (61.2)
Erythrocyte folate		
<150 ng/ml RBC	6/10 (60.0)	4/23 (17.4)
Serum creatinine increased	8/22 (36.4)	9/49 (18.4)

[a] The 71 patients are those listed in the right-hand column in Table 2. Serum homocysteine elevations defined as >3SD above the mean in normal controls. Increased serum creatinine ⩾1.4 mg/dl in men, >1.2 mg/dl in women.
Source: Ref. 18.

The data summarized in Tables 2 and 3 are consistent with the notion that folate deficiency that is relatively early or latent, i.e., not expressed hematologically as a megaloblastic anemia, is common in hospitalized alcoholic patients [105]. None of these tests can be used alone to establish the cause of anemia in a given patient. As discussed subsequently, whichever test or tests are obtained by the physician must be employed in combination with the hematological findings to make a secure diagnosis of clinically significant folate deficiency.

D. Urinary Excretion of Formiminoglutamic Acid

The measurement of the urinary excretion of formiminoglutamic acid (FIGLU) was widely used in the 1950s and 1960s as a diagnostic test for folate deficiency, but there were major problems with both specificity and sensitivity [16,87]. Therefore, the test is no longer used.

E. Deoxyuridine Suppression Test

The deoxyuridine suppression test, in which the ability of added deoxyuridine to suppress the incorporation of tritiated thymidine into DNA by bone marrow cells is measured (i.e., using the de novo rather than the salvage pathway of DNA synthesis), has been employed by a number of investigators as a research tool in the diagnosis of folate and cobalamin deficiency states [111]. It has often proved useful as a research procedure. However, the test is too cumbersome and time consuming (and therefore too costly) to justify its use as a routine diagnostic measure.

F. Tests for Cobalamin Deficiency

Before the introduction of the serum and red cell folate assays, the serum cobalamin concentration was widely used to differentiate folate from cobalamin deficiency. It is still an extremely useful test in the diagnosis of folate deficiency, although it has certain limitations.

Let us assume that the patient has morphological evidence of the presence of a megaloblastic anemia. In most instances, we are dealing with an either/or situation, i.e., the patient has either cobalamin or folate deficiency (let us put aside for the moment the minority of patients in which both deficiencies, or neither deficiency, may be present). If the serum cobalamin level is greater than 300 pg/ml in a patient with a megaloblastic anemia, it is highly likely that an underlying folate deficiency state is present, even if the serum and/or red cell folate levels are normal or equivocal. The serum cobalamin will be less than 300 pg/ml in at least 99% of patients with a hematological or neurological syndrome due to deficiency of cobalamin [14,109].

It was previously thought that a low serum cobalamin (i.e., less than the lower limit of normal, which is usually approximately 200 pg/ml using current radiodilution assay systems) was a highly sensitive test for cobalamin deficiency. A minority of patients, however, probably of the order of 5–10%, will have clear-cut hematological or neurological damage due to cobalamin deficiency that will respond to cyanocobalamin therapy, even though pretreatment serum cobalamin levels are in the 200–300 pg/ml range [14,15]. If a level of 300 pg/ml is therefore set in order to attain high sensitivity, the diagnosis of folate deficiency is facilitated when the serum cobalamin level is above this cut-off point.

There are problems with specificity, however, if the serum cobalamin level is low or in the low-normal (200–300 pg/ml) range [109,110]. Most patients with serum values between 200 and 300 pg/ml and many patients with *low* serum cobalamin levels (<200 pg/ml) do not appear to be deficient in the vitamin or have underlying disorders that put them at risk for deficiency. As with the serum folate level, by definition 2.5% of the normal reference population will have a low serum cobalamin concentration [88]. As the test is used in current clinical practice, probably about 25–50% of patients with low serum levels are not deficient in the vitamin [109,110]. Furthermore, in a minority of patients with folate deficiency (in one series as many as a third), a low serum cobalamin concentration occurs, which appears to be secondary to lack of folate [87,112,113]. It is seen less frequently in folate-deficient alcoholics, in whom liver damage tends to elevate the serum cobalamin concentration [114]. The serum cobalamin level often increases after folate therapy [87,112,113]. Since neither the serum cobalamin nor the serum folate is a specific test for deficiency of the respective vitamin, the patient in whom both levels are low or borderline may present a diagnostic conundrum.

In our experience and those of others [110] the serum methylmalonic acid (MMA) is a very useful confirmatory diagnostic test in the patient with a low or low normal serum cobalamin. Its specificity is high; most false positives occur in patients with intravascular volume depletion or renal insufficiency, in which a moderate increment in serum MMA may be seen [18]. The sensitivity of the test for tissue deficiency of cobalamin in a series of 434 patients with lack of cobalamin was 98% [18]. In virtually all of the patients who did not have an elevated serum MMA, the serum homocysteine value was increased, so that the combined use of the two serum metabolite levels identified 99.8% of patients with cobalamin deficiency [18].

Frequently, in the absence of the information provided by metabolite levels, a Schilling test has been done in the patient in whom both the serum cobalamin and folate level are depressed or borderline, in an attempt to rule out cobalamin deficiency, on the assumption that the test will be abnormal in patients with cobalamin deficiency and normal in those with lack of folate. However, a significant minority of persons with clinically important cobalamin deficiency (probably of the or-

der of 10–20%) will have normal absorption of crystalline vitamin B_{12} under the conditions of the Schilling test [109,115]. Some of these patients are unable to absorb dietary sources of cobalamin, often due to decreased acid-peptic hydrolysis of food cobalamins [115–118]. Conversely, in patients with primary folic acid deficiency there may be a transient impairment in vitamin B_{12} absorption as assessed by the Schilling test [28,33]. Therefore, the combined use of the serum metabolite levels is a better way of differentiating folate from cobalamin deficiency than the Schilling test [18]. The serum assay for antibodies to intrinsic factor may also be helpful because of its very high specificity for pernicious anemia of the adult type [16,119]. Its sensitivity is low, however, since it is positive in only approximately 50% of patients with pernicious anemia [16]. In the patient with both a low serum cobalamin and a low serum folate or with borderline levels of one or both vitamins and an elevated serum MMA in the absence of renal insufficiency or intravascular volume depletion, the diagnosis of cobalamin deficiency is established. It remains possible, however, that the patient has both deficiency states. Most commonly this is in the setting of a malabsorption syndrome. Such patients may be safely treated with both cobalamin and folic acid.

Therefore, in the patient with a megaloblastic anemia, low or borderline serum levels of both vitamins and normal renal function, an elevated serum MMA concentration will establish the diagnosis of cobalamin deficiency. If the serum MMA is normal in such a patient, and only the serum homocysteine is high, either cobalamin or folate deficiency may be present. Approximately 95% of such patients will prove to be deficient in folate rather than cobalamin [18]. A normal Schilling test, the absence of serum antibodies to intrinsic factor, and a history of a disorder (such as alcoholism) associated with lack of folate would provide further support to the diagnosis of folate deficiency in this situation.

In the patient in whom both the serum cobalamin is greater than 300 pg/ml and the serum folate is greater than 4.0 ng/ml, the possibility of folate deficiency, as discussed above, still exists.* This is particularly true if the serum folate is measured several days after admission to hospital in an alcoholic patient, since resumption of a normal diet and cessation of ethanol intake may normalize the serum folate level. In such patients, an elevation in the serum homocysteine may be a useful indicator of the presence of underlying folate deficiency. However, in patients with megaloblastic anemia in whom the serum levels of cobalamin, folate, and both metabolites are normal, it is likely that the anemia is due to some other cause, e.g., an underlying bone marrow malignancy (myelodysplasia or acute nonlymphocytic leukemia) or one of the rare congenital causes of megaloblastic anemia such as orotic aciduria.

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*Rarely, cobalamin deficiency may present with a serum cobalamin level above 300 pg/ml, associated with an elevation in serum MMA [14].

G. Increased MCV

An elevation in the MCV, although a typical finding in megaloblastic anemia due to folate deficiency, is neither specific nor sensitive. Most patients with increased MCVs in clinical practice will prove to have neither folate nor cobalamin deficiency [119]. In a recent series of 123 episodes of folate deficiency in 119 patients seen in New York City, the MCV was within normal limits in 25% of the patients, even though lack of folate was well proven based on morphological criteria and responses to folic acid therapy [18]. In most instances the normal MCV was attributable to a coexisting disorder, most commonly the anemia of chronic disease (less frequently, iron deficiency or thalassemia trait). Such examples of "masked megaloblastic anemia" [120] are most frequently encountered in sick hospitalized patients in contrast to office practice, where the average patient with a megaloblastic anemia due to folate or cobalamin deficiency will have an elevated MCV.

H. Megaloblastic Bone Marrow

As mentioned, the megaloblastic changes in the erythroid and myeloid series in the bone marrow are seen in both cobalamin and folate deficiency. Such changes in adults in the absence of lack of folate or cobalamin are observed occasionally in acute nonlymphocytic leukemia and myelodysplasias, such as refractory sideroblastic anemias, and after the administration of a variety of cancer chemotherapeutic agents.

In folate deficiency, the megaloblastic changes may be more prominent in the white cell series (i.e., giant bands and metamyelocytes) than in erythroid cells, particularly when a coexisting disorder is present, such as iron deficiency. Predominant granulocytic changes are also common in pregnant and alcoholic patients with folate deficiency. In addition, after treatment with folic acid or in patients responding to diet and the withdrawal of alcohol after admission to hospital, the giant bands and metamyelocytes revert to normal less quickly than the abnormalities in the red cell series [16,121].

It should be emphasized that the presence of a megaloblastic bone marrow, even in patients in whom other tests such as serum and erythrocyte folate levels are abnormal, although indicating the presence of folate deficiency, does not prove that the anemia is due to folate deficiency. Since megaloblastic changes develop early in folate depletion, they may be seen before erythropoiesis or leukopoiesis has been seriously compromised. Anemia in such a patient may be due to another cause. An example would be an alcoholic with a lung abcess who has developed the anemia of chronic disease but on bone marrow examination is found to have megaloblastic changes in the bone marrow associated with a low serum or red cell folate level. In such a patient, the anemia may not respond to folic acid therapy but will be reversed when the lung abscess is properly treated. Alternatively, the anemia may be caused by both folate deficiency and the anemia of chronic disease; in that case

one would expect to find abnormalities in peripheral blood films indicative of folate deficiency [23].

I. Neutrophil Hypersegmentation

Neutrophil hypersegmentation is a very early sign of dysfunctional granulopoiesis caused by folate deficiency, often present before anemia develops in patients with elevated MCVs due to depletion of the vitamin. The frequency of hypersegmentation was as high as 98% in a large series of patients with megaloblastic anemia, many of whom were seen in consultation [19]. However, the sensitivity of this finding was only 78% in 41 consecutive anemic alcoholic patients with megaloblastic change in the bone marrow attributed to folate deficiency [23]. The specificity of hypersegmentation for the presence of megaloblastic hematopoiesis in the latter study was 95%, however [23]. Thus, even though neutrophil hypersegmentation is an early development in experimental folate deficiency, it may be absent in frankly deficient patients. It may not be seen in infections and other stresses to granulopoiesis that cause a "shift to the left" in the differential count or in severely deficient patients who have a marked granulocytopenia [19].

Hypersegmentation is not completely specific for cobalamin and folate deficiency. It is very frequently found in patients with renal insufficiency, even with only modest serum creatinine elevations (>2 mg/dl) (Nath and Lindenbaum, unpublished). Hyperlobulated granulocytes also occur as a congenital anomaly, in some patients with myelodysplasia and chronic myelocytic leukemia and after therapy with corticosteroids and a variety of anticancer drugs. They are also frequently seen in apparently uncomplicated iron deficiency anemia [16,122]. Although in some instances this finding may indicate coexistent folate or cobalamin deficiency [16,123], a number of observers (including ourselves) have found that the neutrophil hypersegmentation in iron-deficient patients is often unaccompanied by any evidence of folate deficiency and completely responds to iron therapy alone [122]. We were unable to demonstrate decreased folate concentrations in neutrophils from such patients (Nath and Lindenbaum, unpublished).

J. Macroovalocytosis

Macroovalocytes are usually apparent on the peripheral blood films of patients with folate deficiency that is severe enough to cause anemia. They are also seen in nonanemic patients, although they may be few in number or undetectable. Oval macrocytes occur, however, in a variety of other conditions unassociated with cobalamin or folate deficiency, such as autoimmune hemolytic anemias, therapy with agents such as zidovudine, azathioprine, or anticancer drugs, and even severe uncomplicated iron deficiency anemia. In a series of anemic alcoholic patients, the specificity of macroovalocytosis for the presence of megaloblastic change in the

marrow was only 68%, although the sensitivity was 90% [23]. If the cut-off point was changed from the presence of any macroovalocytes to more than 3% of these cells, the specificity rose to 96%, but the sensitivity fell to 56% [23]. We have found similar limitations in specificity, even using the >3% cut-off point, in a recent series of 300 consecutive patients with high MCVs, most of whom were not alcoholics (Ogundipe et al., unpublished).

K. Combined Macroovalocytosis and Hypersegmentation

Neither neutrophil hypersegmentation alone nor macroovalocytosis alone is highly specific for the diagnosis of folate or cobalamin deficiency. However, the combination of the two findings makes it much more likely that a patient has one of these deficiency states. In a recent series of 300 patients with elevated MCVs, the specificity for either cobalamin or folate deficiency of the combination of neutrophil hypersegmentation and macroovalocytosis was >98% and the positive predictive value of these two findings together for either cobalamin or folate deficiency was >94% (Ogundipe et al., unpublished). In a series of 121 anemic alcoholic patients, the specificity for megaloblastic bone marrow changes of this combination of findings was 96% [23]. The combined finding is therefore very useful, although not completely specific. Some patients treated with chemotherapeutic drugs or with myelodysplasias or acute nonlymphocytic leukemia, may have both abnormalities in the absence of vitamin deficiency. However, if the combination of hypersegmentation and macroovalocytosis is accompanied by a low serum level of either folate or cobalamin, then the diagnosis of the deficiency state is virtually certain. A bone marrow examination will usually be unnecessary in this situation.

It should be emphasized that these morphological findings must be actively pursued by a trained observer. They are frequently missed by hospital laboratories under the stress of daily routine work [109].

L. Moderate to Severe Megaloblastic Anemia

In patients with moderate to advanced degrees of anemia caused by folate deficiency, a number of other "classic" findings are frequently seen. These include a markedly elevated serum LDH (Table 1), an increased serum concentration of unconjugated bilirubin, a low plasma haptoglobin level, thrombocytopenia and, less commonly, neutropenia. None of these findings is specific for a megaloblastic anemia; added to the finding of neutrophil hypersegmentation and macroovalocytosis, however, they constitute further strong evidence for deficiency of folate or cobalamin. It should be emphasized that the majority of patients with deficiency of either of these vitamins seen in current practice are not severely anemic, and these textbook findings are much more frequently absent than present [18,109].

M. Atrophic Glossitis

Atrophic glossitis is a useful nonhematological diagnostic clue. It was found in 22% of a recent series of 123 anemic folate-deficient patients [18]. Its sensitivity is therefore low. Other conditions, in addition to cobalamin deficiency, may cause atrophic glossitis, including lack of other B vitamins and iron. Nonetheless, the finding of atrophy of the tongue should lead to a search for other evidence of deficiency of folate or cobalamin. Like careful observation of the blood smear, this aspect of the physical examination is frequently omitted in current practice.

N. Therapeutic Trials

Before the serum folate level became widely available, various investigators undertook therapeutic trials with "physiological" doses of either cobalamin or folate in patients with proven megaloblastic anemias as a way to differentiate between the two deficiency states [16,87,124–126]. This was based on the concept that patients would only respond to the vitamin responsible for the deficiency state present if dosage was limited to so-called physiological doses, i.e., 100–200 μg daily of folic acid or 1 μg of vitamin B_{12}. Such trials, which were only used for research purposes in the past, are no longer done. They are cumbersome to perform, necessitating limitation of the diet and preparation of special doses of the vitamins, which are not currently marketed at the required dosage level. Furthermore, reticulocyte responses even using these "physiological" doses were sometimes misleading.

There is still a role in current practice for a therapeutic trial with a single vitamin, for example, using 1 mg of folic acid given by mouth on a daily basis for a period of 10 days or 1000 μg of vitamin B_{12} given intramuscularly for the same period. The main value of such therapeutic trials is if the patient fails to respond. This strongly suggests that deficiency of the other vitamin was present or that the underlying condition is not due to a deficiency of either vitamin. Alternatively, failure of a truly deficient patient to respond to a therapeutic trial may indicate a second disorder that also requires correction, e.g., iron deficiency or the anemia of chronic disease. Large doses of both vitamins are often given simultaneously in current practice either because the patient is very ill or one does not wish to wait for the results of laboratory tests to correct the underlying deficiency state. The results of such trials obviously provide little diagnostic information unless there is no response. Therapeutic trials should mainly play a confirmatory rather than a diagnostic role in current practice. This should be the case if there has been a careful examination of the peripheral blood and serum vitamin levels have been obtained. If pretreatment levels of both the serum MMA and total homocysteine are also available, in the great majority of cases one is able to put together all the information and come up with a correct diagnosis that does not depend on the results of a therapeutic trial.

An occasional challenge occurs if the patient has low or borderline levels of both cobalamin and folate, a normal serum MMA, and no antibodies to intrinsic factor. As mentioned, if such a patient has an elevated total homocysteine level, either cobalamin or folate deficiency may be present, although 95% of patients with hematological evidence of deficiency of one or the other vitamin in whom only the serum homocysteine is elevated will prove to have folate deficiency [18]. Since the serum homocysteine may be elevated in either deficiency state, it would be useful to have a laboratory test (other than the serum methylmalonic acid), which would help in differentiating these two conditions, a test that would be only abnormal in folate deficiency and not in patients with lack of cobalamin. Preliminary data indicate that the serum *N*-methylglycine level is elevated in more than 50% of patients with folate deficiency but is not increased in cobalamin deficiency [127].

In patients in whom both vitamin levels are decreased and only the serum homocysteine is elevated, sequential therapeutic trials with one vitamin at a time may be diagnostic, since in all patients studied to date who were treated in this manner the serum homocysteine has not responded to pharmacological therapy with the wrong vitamin [88].

O. Importance of the History in Diagnosis

Table 4 lists the underlying causes of folate deficiency seen at two New York City hospitals [128]. In contrast to the typical patient with cobalamin deficiency who usually presents with a history featuring only the symptoms of lack of cobalamin, in most patients with folate deficiency the history will reveal an underlying disorder that predisposes to lack of folate. In the United States, most commonly this is

Table 4 Causes of Megaloblastic Anemia Due to Folate Deficiency at Two New York City Hospitals[a]

Etiology	Number (%) of patients
Alcoholism and poor diet[b]	180 (89.6)
Sprue syndromes	13 (6.4)
Poor diet	4 (2.0)
Pregnancy (nonalcoholic)	2 (1.0)
Sulfasalazine	1 (0.5)
?Oral contraceptives	1 (0.5)

[a] 201 consecutive patients seen at Harlem Hospital Center and Columbia-Presbyterian Medical Center 1968–1978.
[b] Includes three pregnant patients, four patients taking phenytoin, and two patients with cancer.
Source: Ref. 128.

chronic alcoholism. The majority of patients with folate deficiency have a history of alcohol intake as well as a diet poor in sources of folate, although some patients may not be truthful about their intake of alcoholic beverages. Less commonly a patient proven to be folate deficient is found to be pregnant or lactating, taking an anticonvulsant drug, sulfasalazine or methotrexate or has a chronic hemolytic anemia [129,130]. Most of these causes of folate deficiency are becoming rare, at least in the industrialized countries.

Therefore, the history is very valuable in the diagnosis of folate deficiency, and in the average patient with proven deficiency no further workup is indicated if the cause is apparent from the history (e.g., the patient is a chronic alcoholic or is pregnant). However, when celiac or tropical sprue is suspected, a workup for a malabsorption syndrome may be undertaken. In the absence of gastrointestinal symptoms in a patient with proven folate deficiency who does not appear to be an alcoholic, pregnant, taking drugs that are associated with deficiency, or on a markedly deficient diet, a search for an underlying malabsorption syndrome is in order, since 10–15% of patients with either tropical or celiac sprue present with a megaloblastic anemia in the absence of digestive complaints.

The history taken alone may be misleading, however. For example, 19% of chronic alcoholic patients seen by a hematology consultation service at two New York City hospitals for megaloblastic anemia were found to have underlying cobalamin deficiency due to pernicious anemia [23]. In these patients the history of alcohol intake was irrelevant to the cause of the deficiency.

VII. CONCLUSIONS

The sequence with which the manifestations of folate deficiency unfold in human subjects placed on a deficient diet has been variable. There is also abundant clinical evidence for such variability. All diagnostic tests for folate deficiency have limitations in both specificity and sensitivity. Significant numbers of patients with unequivocal, severe folate deficiency will have either a normal or borderline serum or normal or borderline erythrocyte folate concentration or both. Conversely, many patients with low serum cobalamin and low serum folate levels will not prove to be deficient in these vitamins. Patients with serum cobalamin values that are normal or that have normal Schilling tests may nonetheless be deficient in cobalamin. The serum methylmalonic acid and total homocysteine concentrations appear to be important advances in our diagnostic armamentarium, if interpreted judiciously. All of the above diagnostic tests may be useful in diagnosis but when taken alone may mislead.

Despite these limitations in the sensitivity and specificity of tests used to diagnose folate deficiency, in most instances a correct diagnosis can be readily achieved by a synthesis of the morphological and biochemical findings. The starting point will usually be the demonstration of megaloblastic changes in the bone

marrow or the combination of hypersegmented neutrophils and macroovalocytes in the peripheral blood smear. We favor the measurement of serum concentrations of cobalamin and folate as the next diagnostic maneuver. Although some workers prefer the erythrocyte folate determination to that in serum, we do not. There are major problems with the specificity and sensitivity of the red cell vitamin level as assayed microbiologically. Furthermore, erythrocyte folate concentrations are now almost always measured in clinical laboratories by radioassay using commercially marketed kits that have not been validated as to reproducibility and effectiveness in differentiating deficient from nondeficient subjects.

Serum methylmalonic acid and homocysteine levels, interpreted together with the serum vitamin concentrations, often prove to be extremely useful ancillary tests in establishing the presence of cobalamin or folate deficiency and in differentiating the two disorders. The combination of the clinical and morphological findings with the serum vitamin and metabolite concentrations should allow the clinician to achieve an accurate diagnosis in the great majority of cases.

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Clinical Implications of Hyperhomocysteinemia

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I. INTRODUCTION

A. Historical Perspective

Thirty years elapsed between the discovery of homocysteine by DuVigneaud in 1932 [1] and the recognition of an inborn error of metabolism in which levels of this amino acid were increased in the blood and urine. Carson and Neill [2] and Gerritsen et al. [3] independently described children and infants with mental retardation who displayed homocysteinemia in association with other congenital abnormalities and precocious thromboembolism. Two years later, Mudd et al. [4] identified a lack of the enzyme cystathionine β-synthase in a patient with homocystinuria. Several additional reports followed and then in 1969, McCully [5] described an infant with homocystinuria who died with multiple vascular lesions but in whom the underlying biochemical defect was an abnormality in cobalamin (vitamin B_{12}) metabolism. Subsequently, similar lesions were described by Kanwar et al. [6] in a child who was found to have a deficiency of 5,10-methylene-tetrahydrofolate reductase.

This led McCully in 1975 to promulgate a new theory of arteriosclerosis in which homocysteine was implicated [7]. Since then, interest in homocysteine as a putative aggravating factor in atherosclerosis and thrombosis has waxed and waned. But in the last few years there has been a resurgence of interest in this theory, with a plethora of published clinical and experimental reports supporting a link between high levels of homocysteine and an increased predisposition to vascular disease.

B. Scope of the Chapter

A number of excellent reviews on the subject of hyperhomocysteinemia have been published in the last 5 years [8–18]. The present chapter will focus mainly on the

more recent literature in this area and, in keeping with the overall theme of this text, will pay particular attention to the role of folate in homocysteine metabolism.

Cellular and plasma homocysteine levels are controlled by two major metabolic pathways, the *transsulfuration* pathway and the *remethylation* pathway. In the transsulfuration pathway, homocysteine is irreversibly converted to cysteine. In the remethylation pathway, homocysteine is recycled back to methionine by two alternative mechanisms. The transsulfuration and remethylation pathways are regulated by both positive and negative allosteric effectors, the most important of which is S-adenosylmethionine. Vitamins, hormones, and metabolic factors also influence these pathways, as described below. Consequently, alterations in homocysteine levels occur in a number of pathological states, which may be either congenital or acquired (Table 1).

After a review of the biochemistry of homocysteine metabolism, a systematic examination of the various causes of hyperhomocysteinemia will be presented, beginning with classical homocystinuria, caused by a homozygous deficiency of cystathionine β-synthase. The most serious consequence of hyperhomocysteinemia is the occurrence of vascular complications including premature atherosclerosis and widespread vascular thrombosis. This association will be examined in detail, as well

Table 1 Causes of Hyperhomocysteinemia

A. Inherited Defects
   1. Enzyme deficiencies
      a. Cystathionine β-synthase
      b. Methylenetetrahydrofolate reductase
      c. Methionine synthase (Cbl E, Cbl G)
      d. Cobalamin coenzyme synthesis (Cbl C, Cbl D)
   2. Transport defects
      a. Transcobalamin II deficiency
      b. Cobalamin lysosomal transporter (Cbl F)

B. Acquired Defects
   1. Nutritional
      a. Cobalamin deficiency
      b. Folic acid deficiency
      c. Pyridoxine deficiency
   2. Metabolic
      a. Chronic renal disease
      b. Hypothyroidism
   3. Drug-induced
      a. Methotrexate and other folate antagonists
      b. Nitrous oxide and other cobalamin antagonists
      c. Azaribine and other pyridoxine antagonists
      d. Estrogen antagonists

as the several hypotheses that have been proposed to explain the link between hyperhomocysteinemia and vascular disease. Experimental hyperhomocysteinemia in animal models will be examined followed by a discussion of in vitro experiments that have investigated the effect of homocysteine on cultured vascular endothelial cells and blood coagulation factors. The spectrum of hyperhomocysteinemia ranging from mild to severe will be examined, including other inborn errors of metabolism affecting different steps in the remethylation pathway. The review will also cover other types of hyperhomocysteinemia that result either from heterozygous deficiency of the enzymes involved in the metabolism of homocysteine or acquired vitamin and cofactor deficiencies that give rise to varying degrees of hyperhomocysteinemia. The link between hyperhomocysteinemia and vascular disease that has been identified in several clinical and epidemiological studies will be reviewed. The chapter will conclude with several more recent reports on therapeutic interventions using vitamin replacement or supplementation.

II. METABOLISM OF HOMOCYSTEINE

Homocysteine is an intermediary metabolite of methionine, an essential amino acid obtained from the diet. Foods contain only trace amounts of homocysteine since tissue levels of this compound are generally low in most plants and animals. As shown in Figure 1, homocysteine is formed in the methionine cycle. Approximately 50% of the homocysteine formed in this pathway is remethylated to methionine. The other major route of homocysteine metabolism is through the transsulfuration pathway that leads to the formation of cysteine. Further catabolism of cysteine eventually results in the oxidation of its sulfur to inorganic sulfate. The methionine cycle and the transsulfuration pathway are quantitatively the two most important metabolic pathways for homocysteine metabolism in the body and appear to be under tight regulatory control (for recent reviews, see Refs. 19 and 20).

A. The Methionine Cycle

The first product of the methionine cycle is S-adenosylmethionine (AdoMet) (Fig. 1, reaction 1), which is synthesized from methionine and ATP by methionine adenosyltransferase (MAT; EC 2.5.1.6) (reviewed in Ref. 19). AdoMet is the major donor of methyl groups in biomethylation reactions and, after decarboxylation, is the source of aminopropyl groups for polyamine biosynthesis. The other products of the reaction are inorganic pyrophosphate (PPi) and phosphate (Pi), which are derived from a tripolyphosphatase activity inherent in the active site of MAT [21]. Three forms of the enzyme have been found in mammalian tissues [22]. The liver contains two isozymes designated MAT I (low K_m [for methionine] or α form) and MAT III (high K_m or β form). Fetal liver, kidney, and other extrahepatic tissues contain a low K_m form designated MAT II isozyme (γ form). An understanding

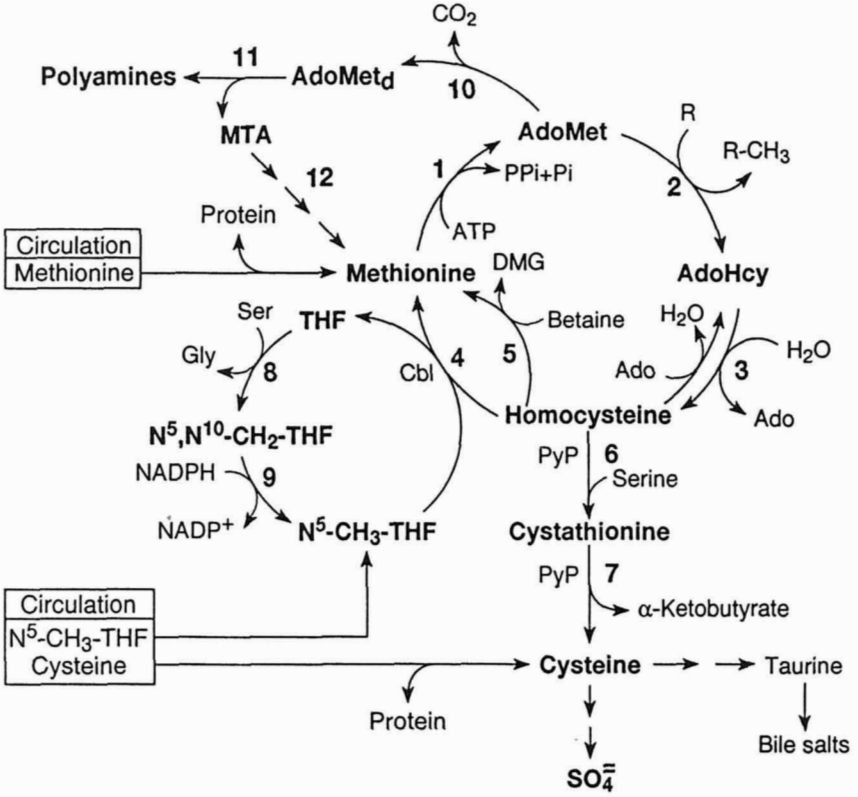


Figure 1 Metabolism of homocysteine. The enzymes of the methionine cycle are methionine adenosyltransferase (reaction 1), AdoMet-dependent methyltransferases (reaction 2), S-adenosylhomocysteine hydrolase (reaction 3), and cobalamin (Cbl)-dependent methionine synthase (reaction 4). An alternate remethylation pathway is catalyzed by betaine: homocysteine methyltransferase (reaction 5). The transsulfuration pathway is initiated by pyridoxal phosphate (PyP)–dependent cystathionine ß-synthase (reaction 6) and continues with the conversion of cystathionine to cysteine by PyP-dependent cystathionase (reaction 7). The methionine cycle is driven by two additional enzymes that are needed for the production of N^5-methyltetrahydrofolate (N^5-CH_3-THF): serine transhydroxymethylase (reaction 8) and N^5,N^{10}-methylenetetrahydrofolate reductase (N^5,N^{10}-CH_2-THF) (reaction 9). S-Adenosylmethionine (AdoMet) enters the pathway leading to polyamines after undergoing decarboxylation by AdoMet decarboxylase (reaction 10). Decarboxylated AdoMet serves as a donor of aminopropyl groups in the synthesis of spermidine and spermine from putrescine and spermidine, respectively (reaction 11). The methylthioadenosine (MTA) formed in the latter reaction is returned to the methionine cycle via a multistep pathway (pathway 12).

of tissue-specific regulation of the expression of the MAT isoforms may be close at hand since the human gene has been cloned recently [23,24].

S-Adenosylmethionine is utilized as the major methyl group donor (Fig. 1, reaction 2). Also, after undergoing decarboxylation, it is capable of donating an aminopropyl group in polyamine synthesis. The rate-limiting steps in the synthesis of polyamines are AdoMet decarboxylase (EC 4.1.1.50; Fig. 1, reaction 10) and ornithine decarboxylase (not shown) (reviewed in Ref. 25). In rats, approximately 33% of AdoMet administered intravenously was metabolized through the polyamine pathway [26]. Since the aminopropyl moiety, which does not recycle, is derived from methionine, the polyamine pathway can be considered as a catabolic pathway for homocysteine. However, methylthioadenosine (MTA), the other AdoMet product of spermidine and spermine synthases (Fig. 1, reaction 11), is salvaged by a multistep pathway (Fig. 1, pathway 12) and returned to the methionine cycle as methionine [27,28].

Biological methylation reactions utilizing AdoMet as the methyl group donor are numerous in all mammalian cells. Most cells contain several AdoMet-dependent methyltransferases (Fig. 1, reaction 2) that can transfer the methyl group to oxygen, nitrogen, or sulfur atoms of both small and large molecules. As examples, the synthesis of creatinine from guanidinoacetate, sarcosine from glycine, phosphatidylcholine from phosphatidylethanolamine, epinephrine from norepinephrine, and the methylation of tRNA and DNA all use AdoMet as the methyl donor. The other product of AdoMet-dependent methyltransferases is S-adenosylhomocysteine (AdoHcy), a potent inhibitor of most methyltransferase reactions. S-Adenosylhomocysteine is hydrolyzed by S-adenosylhomocysteine hydrolase (also called adenosylhomocysteinase) (EC 3.3.1.1; Fig. 1, reaction 3) to adenosine and homocysteine. Although reaction 3 is a reversible reaction, the equilibrium constant of the reaction strongly favors AdoHcy formation [29]. However, under normal metabolic conditions, the equilibrium is shifted in favor of hydrolysis by the removal of products. Adenosine is converted to inosine by adenosine deaminase and homocysteine is removed by the action of the three enzymes described below. AdoHcy hydrolase has received considerable attention as a target site for antiviral and antiparasitic chemotherapy [30] and has been cloned from *Leishmania donovani* [31].

The methionine cycle is completed by the remethylation of homocysteine to methionine. This reaction is catalyzed by N^5-methyltetrahydrofolate:homocysteine methyltransferase (methionine synthase; EC 2.1.1.13; Fig. 1, reaction 4) and betaine:homocysteine methyltransferase (EC 2.1.1.5; Fig. 1, reaction 5). Methionine synthase is found in most, if not all, mammalian tissues [19] and plays an essential role in both homocysteine and folate metabolism (reviewed in Ref. 32). The enzyme has an absolute requirement for cobalamin, more specifically the coenzyme form methylcobalamin. Conditions that limit the availability of coenzyme to the

enzyme (see below) lead to reduced or absent methionine synthase activity and hyperhomocysteinemia. Since the conversion of N^5-methyltetrahydrofolate, the major form of folate in circulation, to tetrahydrofolate (THF) is thought to occur exclusively via cobalamin-dependent methionine synthase, a diminution of its activity leads to a general impairment of folate metabolism. Most mammalian cells grown in culture contain both apo- and holo-methionine synthase. The ratio of apo- to holo-methionine synthase activity in cell extracts is influenced by cell culture conditions [33]. Mammalian tissues also contain both apo- and holo-forms of the enzyme. Apo-methionine synthase has been isolated and characterized from human placenta [34]. In addition to the substrates homocysteine and N^5-methyltetrahydrofolate, the enzyme is dependent upon reducing agents and AdoMet for maximal activity [35], and the reaction catalyzed by this enzyme is essentially irreversible. The enzyme has been cloned from *Escherichia coli* [36].

B. Fueling the Methionine Cycle

Because homocysteine is taken out of the methionine cycle when it is diverted to the transsulfuration pathway, additional methionine must be supplied to maintain homeostasis of the methionine cycle. Dietary methionine is the major refueling source while relatively small amounts are derived from the proteolysis of endogenous proteins. Because methyl groups are removed from the cycle by the action of AdoMet-dependent methyltransferases, methyl groups must also be provided. The folate substrate for methionine synthase, N^5-methyltetrahydrofolate, is generated intracellularly by pyridoxal phosphate (PyP)–dependent serine hydroxymethyl-transferase (SHMT; EC 2.1.2.1; Fig. 1, reaction 8) and N^5,N^{10}-methylenetetrahydrofolate reductase (MTHFR; EC 1.1.99.15; Fig. 1, reaction 9). In the first reaction SHMT converts tetrahydrofolate and serine to N^5,N^{10}-methylenetetrahydrofolate and glycine. In the second reaction MTHFR reduces N^5,N^{10}-methylenetetrahydrofolate with NADPH to N^5-methyltetrahydrofolate. Thus, a significant portion of the tetrahydrofolate generated by methionine synthase is recycled. Cells can also obtain N^5-methyltetrahydrofolate from the circulation since it is the major form of folate in serum, ranging in concentration from 10 to 35 nmol/liter. Intracellular folate concentrations range from 0.27 μmol/liter (rat muscle) to 15.6 μmol/liter (rat liver) [37]. Most cells express folate-binding proteins or receptors for internalizing circulating N^5-methyltetrahydrofolate (see Chapter 1).

An alternate pathway for the remethylation of homocysteine to methionine is mediated by betaine:homocysteine methyltransferase (BHMT; EC 2.1.1.5; Fig. 1, reaction 5). In contrast to methionine synthase, this enzyme is not widely distributed. Although trace levels of activity have been reported in some tissues, mammalian liver and kidney are the only tissues where BHMT has been reproducibly detected [38]. The enzyme catalyzes the conversion of homocysteine and betaine to methionine and dimethylglycine (DMG). The enzyme is inactivated by AdoMet

in vitro, but whether or not AdoMet regulates enzyme activity in vivo is not known [39]. Perhaps due to its limited tissue distribution, BHMT appears to be incapable of handling excessive homocysteine accumulation in pathological conditions leading to hyperhomocysteinemia. Thus, for example, in the congenital and acquired defects affecting the major cobalamin and folate-dependent remethylation pathway, the alternate BHMT route for conversion of homocysteine to methionine is unable to compensate sufficiently and hyperhomocysteinemia results. On the other hand, administration of betaine to patients with homocystinuria may improve their clinical condition as described below.

C. The Transsulfuration Pathway

Cells that contain active cystathionine β-synthase, (CBS; EC 4.2.1.22; Fig. 1, reaction 6) divert homocysteine from the methionine cycle into the transsulfuration pathway (reviewed in Refs. 19,40, 41). The pathway is a significant catabolic route for methionine metabolism, and its products include the nonessential amino acid cysteine, cysteamine, taurine (a constituent of bile salts), and sulfate (for synthesis of polysulfates such as heparin, heparan sulfate, dermatan sulfate, and chondroitin sulfate). Cystathionine β-synthase has been isolated and characterized from human liver [42,43]. This PyP-dependent enzyme exists as a tetramer with subunits of MW 63 kDa. The subunits undergo posttranslational modification by proteolysis and form dimers with MW of 48 kDa. The dimeric species has a 60-fold lower K_m for homocysteine and a specific activity that is 30 times higher than the tetrameric species [44,45]. Rat liver CBS has been cloned from immunopurified mRNAs of which there are four distinct types formed by alternate splicing patterns [46,47]. Each mRNA isoform is translated in transfected eukaryotic cells, and types I and III protein isoforms have enzymatic activity [48]. Human CBS has been cloned recently [49], and the gene is located on chromosome 21 [50]. Cystathionine is converted to cysteine and α-ketobutyrate by the PyP-dependent enzyme cystathionase (EC 4.4.1.1., Fig. 1, reaction 7), which completes the transsulfuration process.

D. Regulation of the Methionine Cycle and Transsulfuration Pathway

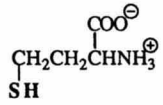
In mammalian liver approximately half of the methionine entering the methionine cycle is recycled, while the other half is irreversibly committed to cysteine synthesis through the transsulfuration pathway [51]. However, not all tissues (e.g., adrenal, lung, testis, and heart) have detectable levels of CBS and may, therefore, lack a trassulfuration pathway (reviewed in Ref. 19). In tissues that have an active methionine cycle and transsulfuration pathway, AdoMet appears to play a key role in regulating the flow of homcysteine towards remethylation or transsulfuration by interacting with three enzymes; CBS (reaction 6), MTHFR (reaction 9), and BHMT

(reaction 5). Finkelstein et al. [52] and Koracevic and Djordjevic [53] reported that AdoMet activates CBS. The mechanism of this activation has been elucidated recently by Roper and Kraus [48]. They found that AdoMet reduced the K_m for homocysteine approximately eightfold for isoforms type I and III without affecting the V_m. Thus, when intracellular concentrations of AdoMet are relatively high, as would occur after methionine loading, CBS is allosterically activated and a greater proportion of homocysteine is diverted into the transsulfuration pathway. On the other hand, AdoMet is a negative effector of both MTHFR [54–56] and BHMT [39]. Inhibition of MTHFR by AdoMet would decrease the concentration of N^5-methyltetrahydrofolate, thereby limiting the activity of methionine synthase, while inhibition of BHMT by AdoMet would limit the alternate remethylation pathway. However, as discussed by Finkelstein [19], regulation of the methionine cycle and transsulfuration pathway is achieved not only by AdoMet acting as an allosteric effector, but also by tissue levels of individual enzymes, induction of their synthesis by hormones and dietary methionine, and perhaps also by the action of other effector molecules such as S-adenosylhomocysteine acting on BHMT [39], MTHFR [55], and CBS [52,57]. Selhub and Miller [20] have proposed that hyperhomocysteinemia occurs when a single impaired homocysteine metabolic pathway affects other homocysteine metabolic pathways.

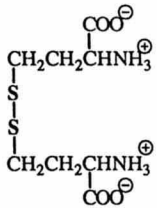
III. CLINICAL ASSESSMENT OF HYPERHOMOCYSTEINEMIA

Hyperhomocysteinemia is defined as a sustained elevation above normal of homocysteine and closely related analogs in plasma or serum. Plasma from patients with homocystinuria have levels of homocystine ("oxidized homocysteine") (Fig. 2) and the mixed disulfide of homocysteine-cysteine that are easily detected by amino acid ion-exchange analysis. Recent advances in methodology now make it possible to routinely determine homocysteine in normal subjects as well as patients with hyperhomocysteinemia. Protein-bound homocysteine accounts for 80% or more of the total homocysteine in normal and hyperhomocysteinemic plasma [58–61]. Proteins that carry homocysteine in circulation have not been identified with certainty, although there is some evidence suggesting that albumin may be a major carrier [60]. The concentration of "free reduced homocysteine" is very low and accounts for less than 5% of total plasma homocysteine in normal subjects [62]. The concentration of homocystine and the mixed disulfide of homocysteine-cysteine represent approximately 10–15% of total plasma homocysteine. It has been reported that homocysteine thiolactone is found in high concentrations in both normal individuals and patients with arteriosclerosis [63], but this observation has not been substantiated [64,65]. Moreover, nonspecific esterases, present in plasma as well as on the surface of endothelial cells, rapidly hydrolyze the thiolactone to homocysteine [66]. In this laboratory the mean concentration of total homocysteine

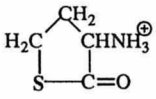
HOMOCYSTEINE AND RELATED COMPOUNDS



Homocysteine

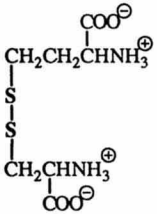


Homocystine

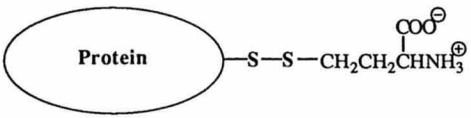


Homocysteine thiolactone

HOMOCYSTEINE MIXED DISULFIDES



Homocysteine-cysteine



Protein-bound homocysteine

Figure 2 Structures of homocysteine and related compounds including homocysteine bound to protein by a covalent disulfide bond.

in plasma from healthy female and male donors is 7.85 and 9.26 μmol/liter, respectively ($p = 0.002$). The mean concentration in serum is 10.34 and 12.30 μmol/liter, respectively ($p = 0.002$). The difference between plasma and serum for both sexes is statistically significant ($p < 0.001$) [67]. Similar concentrations have been found by other investigators (reviewed in Ref. 68).

A. Determination of Total Plasma Homocysteine

Total plasma homocysteine assays appear to be very reliable for assessing homocysteine status in patients (reviewed in Refs. 8,68). All methods designed to determine total homocysteine require a chemical reducing step to generate free homocysteine from the oxidized forms present in plasma. Refsum et al. [60] used dithioerythritol to release homocysteine from protein in an early radioenzymatic-HPLC assay for total plasma homocysteine. Stabler et al. [69] reduced the serum with 2-mercaptoethanol, derivatized homocysteine with *N*-methyl-*N*(*t*-butyldimethylsilyl) trifluoroacetamide, and utilized capillary gas chromatography-

mass spectrometry (GC-MS). Simultaneously other sulfur amino acids (e.g., cysteine, methionine, and cystathionine) were determined by this method. Araki and Sako [70] determined total plasma homocysteine using HPLC with fluorescence detection (HPLC-FD). Plasma was reduced with tri-*n*-butylphosphine and homocysteine was derivatized with the thiol-specific reagent ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F). Ubbink et al. [71] modified the HPLC chromatographic conditions of the latter method and were able to resolve SBD-conjugates of cysteine, cysteinylglycine, homocysteine, and glutathione in human serum. Vester and Rasmussen [72] have also used this method for total homocysteine but have included the internal standard mercaptopropionylglycine. Jacobsen et al. [61] developed a HPLC-FD assay for total serum homocysteine using monobromobimane (mBrB) as the fluorochromophore. Serum disulfide bonds were reduced with sodium borohydride and free thiols were derivatized with mBrB. Refinements of this assay have shortened sample processing and chromatography so that up to 85 unknowns are analyzed within a 24-hour period [67]. Refsum et al. [73] have described a fully automated assay based on the mBrB HPLC-FD for total plasma homocysteine using a combined sample processor-injector. Andersson et al. [74] used a conventional amino acid analyzer to determine total plasma homocysteine. Malinow et al. [75] developed an assay for total plasma homocysteine using borohydride reduction and HPLC with electrochemical detection (ED). Hyland and Bottiglieri [76] described an assay for total plasma homocysteine using 2-mercaptoethanol as reductant and homocysteic acid as an internal standard. After derivatization with *o*-phthaldialdehyde (OPA), the OPA-thiol adducts were resolved by HPLC-FD. Total plasma and serum homocysteine measurements by these methods are generally in good agreement with healthy subjects usually having plasma homocysteine levels between 5 and 15 μmol/liter.

B. Oral Methionine Loading

Oral methionine loading has been used to detect heterozygosity for CBS deficiency [77]. In this method oral methionine is administered (usually 100 mg/kg body weight) and blood samples are taken immediately before loading and then at 2, 4, 6, and 8 hours. Methionine and homocysteine metabolites such as homocystine and homocysteine-cysteine mixed disulfide are determined in plasma by amino acid ion-exchange chromatographic analysis. Plasma levels of methionine reach a peak within 2 hours, while homocysteine is highest only after 6–8 hours. The rate of fall from peak methionine levels is slower and the increase in homocysteine metabolite levels is severalfold greater in CBS heterozygotes than in normal controls. Based on results from methionine loading studies and determination of skin fibroblast CBS activity, Boers et al. [78] concluded that heterozygosity for CBS deficiency predisposes individuals to the development of premature occlusive arterial disease and ischemic cerebral vascular disease but not coronary artery disease. Similar results were

obtained by Clarke et al. [79] except that evidence for CBS heterozygosity was also found in 30% of the patients with coronary artery disease. More recent methionine loading studies have used total plasma homocysteine, which includes protein-bound homocysteine, as an indicator. Andersson et al. [80] found that neither methionine clearance nor postload total plasma homocysteine was affected by excess dietary methionine for 2 weeks in normal individuals. In a large study involving 169 individuals, postload total plasma homocysteine levels were similar in pre- and postmenopausal women and in men of similar age [81]. McGill et al. [82] have reviewed the results from a number of methionine loading studies and conclude that because of overlap between normal controls and obligate heterozygotes for CBS deficiency, it is difficult to accurately identify these individuals using this single metabolic loading test.

IV. PATHOGENESIS OF HYPERHOMOCYSTEINEMIA

A. Inherited and Congenital Defects

Homocystinuria is a heterogeneous group of diseases caused by inherited defects of homocysteine, cobalamin, or folate metabolism. The most common form of homocystinuria is caused by CBS deficiency with well over 600 cases reported up to 1989 [41]. Homocystinuria due to inherited defects of cobalamin transport and metabolism, although less frequent, may be the result of a wide variety of defects that involve cobalamin coenzyme synthesis, methionine synthase mutations, and abnormalities of cobalamin transport [83,84]. The only known inherited defect of folate metabolism that results in homocystinuria (apart from methionine synthase, which utilizes a folate substrate) is methylenetetrahydrofolate reductase deficiency [85].

1. *Cystathionine β-Synthase (CBS) Deficiency*

The enzymatic defect of the transsulfuration pathway responsible for homocystinuria was identified as CBS in 1964 by Mudd et al. [4]. The inactivation of CBS results in a blockage of the transsulfuration pathway and intracellular accumulation of homocysteine, which is then exported to the circulation (reaction 6, Fig. 3). The literature, both clinical and biochemical, on CBS deficiency is extensive and beyond the scope of this review. The reader is referred to the comprehensive review by Mudd et al. [41]. CBS deficiency is inherited as an autosomal recessive trait [86], and the gene is located on the subtelomeric region of chromosome 21 [50,87]. Based on enzyme activity levels in the presence and absence of pyridoxal phosphate, Fowler et al. [88] found evidence for three distinct classes of CBS mutants using cultured skin fibroblasts from pyridoxine-responsive and nonresponsive patients. One class has no detectable CBS activity in fibroblast extracts with or without pyridoxal phosphate. The second class has reduced activity and normal affinity for

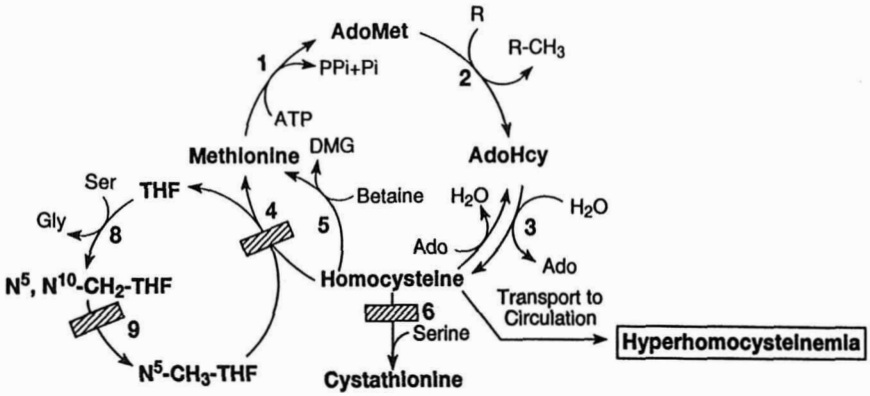


Figure 3 Metabolic dysfunctions leading to hyperhomocysteinemia. Impairment of Cbl-dependent methionine synthase (reaction 4), PyP-dependent cystathionine ß-synthase (reaction 6), or N^5,N^{10}-methylenetetrahydrofolate reductase (reaction 9) may lead to unregulated overproduction of homocysteine, transport to circulation, and hyperhomocysteinemia.

coenzyme, while the third class has reduced activity and reduced affinity for coenyzme. However, there was no consistent correlation with clinical response to pyridoxine. Later studies showed that by controlling the level of pyridoxal phosphate in the growth medium of the cultured skin fibroblasts from normal controls, pyridoxine-responsive, and pyridoxine nonresponsive patients, it was possible to calculate the apparent K_m values for coenzyme. In fibroblast extracts from responsive and nonresponsive patients, K_m values were 2–4 and 16–63 times higher, respectively, than those of controls (52–85 μM). The obligate heterozygous parents of homozygous children with CBS deficiency usually have less than 50% enzymatic activity in extracts of liver [86] and cultured skin fibroblasts [89–91] compared with controls.

2. *Methylenetetrahydrofolate Reductase (MTHFR) Deficiency*

Of the three documented inherited disorders of folate transport and metabolism only MTHFR deficiency causes hyperhomocysteinemia and homocystinuria (for reviews, see Refs. 85,92). This enzymatic block (reaction 9, Fig. 3) reduces or eliminates the supply of MeTHF for methionine synthase (reaction 4). Hyperhomocysteinemia also occurs in individuals who have an inherited deficiency of cobalamin-dependent methionine synthase. Congenital defects of this enzyme, which requires 5-methyltetrahydrofolate as substrate, will be discussed below.

Methylenetetrahydrofolate reductase deficiency was first described by Mudd et al. [93] in 1972 in a 16-year-old boy who suffered from muscle weakness, seizures, and abnormal electroencephalograms and in a 17-year-old girl who suffered

from schizophrenia and mental deterioration. Her 15-year-old sister also had homocystinuria and hyperhomocysteinemia. In contrast to patients with CBS deficiency, these patients had slightly reduced to normal levels of methionine in their plasma and normal levels of CBS activity in skin fibroblast extracts. More than 25 cases of MTHFR deficiency have been described, and the common clinical findings, which include developmental delay, motor and gait abnormalities, abnormal EEG, and psychiatric disorders, may be seen in infancy or not until adolescence [85]. Although the hyperhomocysteinemia associated with MTHFR deficiency can be moderate to severe with a mean plasma concentration of 57 μmol/liter (range = 12–233 μmol/liter), the prognosis for these patients is generally worse than in patients with CBS deficiency [94]. This difference in prognosis relates, at least in part, to the unresponsiveness to any form of treatment in MTHFR deficiency. About half of all CBS-deficient patients, on the other hand, respond to treatment with pyridoxine. Most patients with MTHFR deficiency have hypomethioninemia (mean = 12 μol/liter) [94], but in contrast to patients who have hypomethioninemia due to inborn errors of cobalamin metabolism (described below) and who develop severe megaloblastic anemia [84], patients with MTHFR deficiency do not become anemic. However, these patients, like those with CBS and methionine synthase deficiencies, develop thromboses of arteries and cerebral veins, which are often the cause of morbidity and mortality [95]. In fact the vascular lesions seen in patients with MTHFR, CBS, and methionine synthase deficiencies are remarkably similar, as pointed out by Kanwar et al. [6]. Recently Visy et al. [96] described a family of six siblings in which two brothers and a sister had MTHFR deficiency, but the clinical onset of severe and recurrent strokes, resulting in the death of one brother and the sister, did not occur until the patients were in their early twenties.

There appears to be variation in the expression of MTHFR deficiency as originally suggested by Mudd et al. [93] and later by Rosenblatt and Erbe [97]. This is based on differences between MTHFR activity measurements before and after heat treatment. The clinical hallmarks of severe MTHFR deficiency (<2% of normal detectable enzyme activity in skin fibroblasts, lymphocytes, or liver) are moderate to severe hyperhomocysteinemia, neurological complications, atherosclerosis, and thromboembolism. Kang et al. [98] described a thermolabile variant of MTHFR deficiency that resulted in mild homocysteinemia without apparent neurological complications, but associated with the development of coronary artery disease [99]. In a recent study of 212 patients with proven coronary artery disease and 202 controls without clinical evidence of cardiovascular disease, Kang et al. [100] found that 36 patients (17%) and only 10 controls (5%) had the thermolabile variant of MTHFR deficiency. The finding of a 5% incidence of thermolabile MTHFR in a control population, should this be confirmed by other investigators, suggests that the gene frequency for this trait is very high in the general population. The specific activities of MTHFR in peripheral lymphocytes (measured as nmol formaldehyde formed per mg protein per hour), from thermolabile variant

patients and controls were 5.58 ± 0.91 and 10.33 ± 2.89, respectively ($p < 0.01$). After heat inactivation residual MTHFR activity was 11.2 ± 1.43% in thermolabile variants and 36.3 ± 5.18% in the control group. Patients with thermolabile MTHFR deficiency had only slight hyperhomocysteinemia (13.19 ± 5.32 μmol/liter versus 8.50 ± 2.80 μmol/liter in controls; $p < 0.05$). Thermolabile MTHFR deficiency has also been found in individuals who are obligate heterozygotes for severe MTHFR deficiency and who have approximately 25% of normal MTHFR activity in lymphocyte extracts [101]. These individuals, who have slight to moderate hyperhomocysteinemia, may be compound heterozygotes with one allele for severe MTHFR deficiency and a separate distinct allele for the thermolabile MTHFR variant. Based on a recent study involving 339 individuals undergoing coronary angiography, Kang et al. [102] reported an incidence of 18.1% thermolabile MTHFR deficiency in patients with severe coronary artery stenosis (≥70% occlusion in one or more coronary arteries or ≥50% occlusion in the left main coronary artery; Group 1), an incidence of 13.4% in patients with mild to moderate disease (<70% occlusion in one or more arteries or <50% occlusion in the left main coronary artery; Group 2), and an incidence of 7.9% in subjects without coronary artery disease based on coronary angiography (Group 3). Interestingly, all three groups had hyperhomocysteinemia (14.86 ± 5.85, 15.36 ± 5.70, and 13.39 ± 3.80 μmol/liter in Groups 1, 2, and 3, respectively; not significantly different from one another) compared to normal (8.50 ± 2.80 μmol/liter). However, based on discriminant function analysis, thermolabile MTHFR was predictive of coronary artery disease, independent of other traditional risk factors. Although severe MTHFR deficiency has been difficult to treat [85], individuals with thermolabile MTHFR or with compound heterozygosity and mild to moderate hyperhomocysteinemia generally respond well to oral folic acid therapy. Whether the lowering of total plasma homocysteine by oral folate will prolong the development of cardiovascular disease in these subjects has not been established.

3. *Inborn Errors of Cobalamin Transport and Metabolism*

Vitamin B_{12}, an essential micronutrient, is converted to methylcobalamin, which functions as a cofactor for methionine synthase, and to 5'-deoxyadenosylcobalamim, the coenzyme for the mitochondrial enzyme methylmalonyl-CoA mutase (EC 5.4.99.2). In the small intestine cobalamins bind to intrinsic factor (IF), a glycoprotein secreted by parietal cells in the stomach, and are transported into ileal enterocytes by IF-receptor–mediated endocytosis. Newly absorbed cobalamin appears in circulation on the serum cobalamin-binding protein transcobalamin II (TCII), a serum protein of 45.5 kDa with high avidity for cobalamins ($K_d \approx 10^{-12}$ M) which serves as a cobalamin delivery protein to cells that express specific receptors for TCII [103]. The TCII-cobalamin complex is internalized by receptor-mediated absorptive endocytosis [104,105]. After proteolytic degradation of the TCII, cobalamin is transported from lysosomes into the cytosol. A portion of the

cytosolic cobalamin is transported into mitochondria and converted into 5'-deoxyadenosylcobalamin. The remaining cytosolic cobalamin is converted into methylcobalamin by a series of reactions that are poorly understood. Several distinct genetic defects of cobalamin transport and metabolism have been described that result in hyperhomocysteinemia. All of these defects result in a decrease of methionine synthase activity (reaction 4, Fig. 3). For comprehensive reviews on the biochemistry and clinical findings of the genetic defects of cobalamin transport and metabolism, see Refs. 83–85.

(*a*) *Transcobalamin II Deficiency* The first report of inherited TCII deficiency appeared in 1971 from Hakami et al. [106], who described two infant siblings presenting at 3 and 5 weeks of age with megaloblastic anemia despite normal serum cobalamin levels. They were able to demonstrate deficiency of TCII in the patients' sera by anion-exchange chromatography. Parenteral administration of 2000 μg of cyanocobalamin was able to effect a complete hematological remission. Approximately 42 cases have been reported to date [107]. The clinical presentation is usually within the first few months of life with failure to thrive, vomiting, weakness, and megaloblastic anemia. Clinical tests for hyperhomocysteinemia and/or homocystinuria were apparently not carried out in a number of cases or were not observed [108]. However, there are at least two documented cases in which homocystinuria has been reported [109,110]. Methylmalonic aciduria has also been detected in some but not all cases [83]. A consistent finding in patients with TCII deficiency is malabsorption of cobalamin [106] suggesting that TCII may also play a role in absorption. Some patients appear to have normal or elevated levels of immunologically reactive TCII, but the protein is incapable of supporting transmembrane delivery of cobalamin [108]. For a summary of the clinical findings in 17 cases, see Ref. 83.

(*b*) *Cbl C and D Mutations* The first inherited disorder of cobalamin intermediary metabolism was reported by Mudd et al. [111] in an infant with homocystinuria, cystathioninemia, and methylmalonic aciduria. The synthesis of methylcobalamin and 5'-deoxyadenosylcobalamin appeared to be impaired in cultured skin fibroblasts from the patient [112]. Several patients have now been described who appear to have a defect in the early processing of newly internalized cobalamin and lack the ability to retain cobalamin in cultured skin fibroblasts [83,84]. On the basis of complementation analysis [113,114], there appear to be two separate genetic loci (*cbl c* and *cbl d*) involved. Although the precise biochemical defect is unknown, it may be related to an inability to reductively cleave the upper-axial ligand of newly internalized cobalamin (e.g., removal of the cyanide group from cyanocobalamin) and form the proper intermediate for further processing [115].

(*c*) *Cbl E and G Mutations* The first description of this class of mutations appeared in 1984 [116,117]. Since then 11 patients have been documented with these

mutations, which present within the first 2 years of life, an exception being a 21-year-old initially diagnosed as having multiple sclerosis [118]. The heterogeneity in this case has been established by complementation analysis. Activity of methionine synthase seems to be impaired and skin fibroblasts from these patients have less methylcobalamin but normal 5'-deoxyadenosylcobalamin compared to controls. Clinical presentation includes megaloblastic anemia, neurological defects, and homocystinuria without methylmalonic aciduria. All of the patients have responded to therapy, and hydroxocobalamin appears to be more effective than cyanocobalamin.

(*d*) *Cbl F Mutation* The Cbl F mutation, first described by Rosenblatt et al. [119] in 1985, affects lysosomal transport of cobalamin. In skin fibroblasts from patients with the Cbl F mutation, cobalamin becomes trapped within the lysosome and can be visualized by electron microscope radioautography [120]. Although cobalamin is internalized, the cells become cobalamin deficient because of the lysosomal trapping. Normal lysosomal transport of cobalamin is a saturable, temperature-dependent process that appears to be specific for selected cobalamins [121]. These patients, being unable to synthesize sufficient quantities of methylcobalamin and 5'-deoxyadenosylcobalamin, have both homocystinuria and methylmalonic aciduria.

4. *Down's Syndrome*

Whereas the above inherited and congenital disorders are associated with hyperhomocysteinemia, Down's syndrome is a congenital chromosomal disorder that appears to be associated with lower than normal plasma homocysteine levels. The gene for CBS is located on chromosome 21 [122]. Individuals affected by Down's syndrome (trisomy 21) therefore have one additional copy of the gene, which results in approximately 150% of normal CBS activity [123]. Plasma homocysteine levels both before and after methionine loading in a group of eight children with Down's syndrome were significantly below normal [124]. However, in another study of nine adults with this chromosomal abnormality, the plasma homocysteine levels were not below normal [125]. What is of particular interest, however, is that Down's patients are remarkably free of atherosclerosis. In three of four reported studies, the apparent protective effect of CBS gene dosage has been reported [126–128]. In conflict with these findings, Moss et al. reported preatherosclerotic lesions in Down's syndrome [129]. These observations, which tie increased gene dosage for the major homocysteine-metabolizing enzyme to lowered homocysteine levels and an apparent protection against atherosclerosis, lend further indirect support to the homocysteine theory of atherosclerosis.

B. Acquired Disorders

1. *Nutrient Deficiencies*

Folate, cobalamin, and pyridoxine are required as cofactors or substrates in the metabolism of homocysteine. Deficiency of one (or more) of the three vitamins,

arising either from nutritional lack, malabsorption, increased utilization, or inactivation caused by various chemicals or drugs, interferes with homocysteine metabolism causing an increase in the serum level of homocysteine. The degree of rise in serum homocysteine caused by these nutrient deficiencies is variable but is sometimes extreme and may attain the levels encountered in patients with homozygous CBS deficiency [130]. Individuals with low-normal serum folate or cobalamin concentrations may also have elevated levels of plasma homocysteine [131].

(*a*) *Folate Deficiency* The causes and manifestations of folate deficiency are reviewed in Chapter 3. Since dietary folate deficiency accounts for a major proportion of all causes of folate deficiency, the prevalence of folate deficiency is highest among poor, malnourished populations and frequently affects the elderly and alcoholics. The various nutrient sources of folate are discussed in Chapters 5 and 8.

Folate, in the form of 5-methyltetrahydrofolate, is an obligatory cosubstrate in the conversion of homocysteine to methionine by methionine synthase. Consequently, folate deficiency, whatever the cause, decreases homocysteine conversion through this pathway, with a resulting increase in the serum homocysteine concentration. It has been estimated that, under normal circumstances, approximately half of all available homocysteine is metabolized through remethylation [132].

Elevated levels of serum homocysteine have been reported in several studies on patients with folate deficiency [133,134]. Kang et al. [133] reported moderate elevations of serum homocysteine in 53% of individuals with low-normal serum folate levels. Elevated levels of serum total homocysteine were described in 18 of 19 of patients with folate deficiency by Stabler et al. [134]. Administration of folic acid to one of these patients resulted in a fall of serum homocysteine to within normal limits. That not all patients with folate deficiency have elevated levels of homocysteine may be explained in part on the basis that the alternative pathway for transmethylation of homocysteine through betaine-homocysteine methyltransferase is not folate dependent. Moreover, homocysteine may be metabolized through the transsulfuration pathway, which is also not folate dependent.

Just as homocysteine levels are elevated in folate deficiency, it has also been demonstrated that the administration of folic acid to folate-sufficient individuals results in a diminution in their serum homocysteine concentrations [135–138]. Brattström et al. [136] reported that a daily 5 mg supplement of folic acid to a group of healthy subjects resulted in a significant lowering of serum homocysteine after 2 weeks. This effect was most marked in individuals whose original serum levels of homocysteine were high. Ubbink et al., studying a group of men with hyperhomocysteinemia, found low serum folate levels in 59.1% [139]. Administration of 1.0 mg folic acid together with 10 mg pyridoxal and 0.4 mg cyanocobalamin daily resulted in normalization of serum homocysteine levels after 6 weeks. In several studies, vascular occlusive disease has been associated with

elevated levels of plasma homocysteine (see below). However, in relatively few of these studies has the hyperhomocysteinemia been linked to nutrient deficiencies. Such an association has been noted by Mölgaard et al. [140], who found that among the 23% of their patients with claudication who had raised levels of plasma homocysteine, most had low serum folate levels (< 11.0 nmol/liter). Brattström et al. [141], studying patients with stroke, identified an inverse relationship between homocysteine levels and serum levels of folate as well as cobalamin and pyridoxine.

A variety of diseases of the small intestine that result in malabsorption may cause folate deficiency. Thus tropical and nontropical sprue as well as extensive inflammatory bowel disease cause folate deficiency [142]. Folate deficiency may also occur in conditions associated with increased rates of cellular proliferation as a result of greater utilization of folate. Chronic hemolysis, myeloproliferative disorders, rapidly growing hematological and other malignancies and skin disorders associated with widespread desquamation, such as psoriasis, may all be associated with folate deficiency [142]. Although these disorders have not been systematically studied with regard to circulating homocysteine levels, there are some reports of hyperhomocysteinemia in association with myeloproliferative disorders [143] and chronic psoriasis [144], and there is an increased incidence of vascular thrombosis in patients with myeloproliferative disorders [145] and psoriasis [146]. Several mechanisms have been implicated in the thrombotic complications associated with myeloproliferative diseases, most of which relate to platelet dysfunction. In this regard, however, numerous studies on the mechanisms responsible for vascular occlusion in hyperhomocysteinemia have focused on an interaction with platelets, as will be discussed below.

Antifolate drugs result in a state of functional folate deficiency. Methotrexate is the most widely used, both as an anticancer agent and also in low doses for the treatment of various nonmalignant disorders including rheumatoid arthritis and psoriasis. The patients with psoriasis described by Refsum et al. [144] were receiving chronic low doses of methotrexate. Thus, hyperhomocysteinemia may have been the result of the drug interfering with folate metabolism. In patients with psoriasis who received low-dose methotrexate (25 mg daily), plasma homocysteine levels rose after 2 days [144]. Patients on higher doses showed an increase in plasma homocysteine within hours, and this was reversed by folic acid [147,148]. It has been proposed that the increased incidence of thromboembolism seen in patients receiving methotrexate may be related to the observed elevation in plasma homocysteine level [11]. The effect on homocysteine levels of other drugs that interfere with folate metabolism has not been studied systematically. Thus, other inhibitors of dihydrofolate reductase such as trimethoprim, widely used in a combination antibiotic, as well as phenytoin and other anticonvulsant drugs, antituberculous drugs, phenothiazines, and trycyclic antidepressants are all known to be associated with folate deficiency [142]. It may therefore be anticipated that

such drugs could also result in elevations of plasma homocysteine. However, studies of these various agents have not yet been undertaken.

There is evidence that the long-term use of oral contraceptives is associated with folate deficiency [142]. The effect of these hormones on homocysteine levels may be more complex, since plasma homocysteine levels appear to be lower in women before the menopause and also during pregnancy [149–151]. Estrogen-containing contraceptives also affect pyridoxine metabolism and may exert an influence on homocysteine levels through this mechanism [152]. The effect of contraceptive steroids and the antiestrogen tamoxifen on plasma homocysteine levels is variable, and the mechanism of any effect of estrogen and its analogs on homocysteine metabolism has not been resolved [10]. There is, however, clear evidence of an increased risk of venous and arterial thromboembolism in women taking hormonal contraceptives [153].

(*b*) *Cobalamin Deficiency* Like folate, cobalamin is required for the major metabolic pathway of homocysteine remethylation. However, folate is required as a cosubstrate for the methionine synthase reaction, whereas cobalamin, in the form of methylcobalamin, functions as an essential cofactor for methionine synthase (see Fig. 1). Cobalamin deficiency, whatever the cause, is associated with an increase in plasma levels of homocysteine [134,154,155].There are two known cobalamin-dependent enzymes in humans: methylmalonyl CoA mutase and methionine synthase. In cobalamin deficiency, the substrates of these reactions accumulate so that plasma levels of both methylmalonic acid and total homocysteine rise. However, the rise in serum methylmalonic acid occurs earlier and more consistently than that of homocysteine in patients with cobalamin deficiency [155]. The reason for this difference is not clear, although a possible explanation is that metabolism of methylmalonate proceeds uniquely via its cobalamin-dependent enzymatic conversion. Homocysteine, on the other hand, has both an alternative remethylation pathway and the possibility of conversion to cysteine by transsulfuration. However, this explanation is probably an oversimplification since S-adenosylmethionine levels are known to tightly regulate conversion of homocysteine through its various metabolic pathways. The complex and reciprocal allosteric effects of S-adenosylmethionine on homocysteine metabolism have not been fully elucidated [20].

There is one situation in which the rise of homocysteine may precede that of methylmalonic acid. Cobalamin deficiency caused by the anesthetic gas nitrous oxide is due to the oxidation of cob(I)alamin in the active site of methionine synthase leading to enzyme inactivation [156].

Serum cobalamin and folate levels showed a statistically significant inverse correlation with serum homocysteine in groups of normal volunteers [67,81] and the administration of a cobalamin supplement resulted in a fall in plasma homocysteine levels in normal individuals [139]. The effect of cobalamin and folate deficiencies on homocysteine and other metabolites has been recently reviewed by Allen and coworkers [157].

(*c*) *Pyridoxine Deficiency* Both enzymes involved in the two reactions of the transsulfuration pathway for homocysteine metabolism require vitamin B_6 (pyridoxal phosphate). Nutritional deficiency of pyridoxine in humans is rare, but several studies have established a link between pyridoxine deficiency and artherosclerosis [158–160]. In one of these studies, an inverse correlation between plasma pyridoxal phosphate levels and plasma homocysteine was found in patients with stroke [141]. In an earlier study from the same group, such a correlation was not found in patients with cerebral vascular and peripheral vascular disease [158]. The most clear-cut evidence that pyridoxine deficiency is associated with hyperhomocysteinemia and vascular disease comes from observations and experiments using drugs that inhibit or interfere with pyridoxine metabolism. Thus, azauridine (azaribine), which was used in the treatment of psoriasis, inhibits the synthesis of uridine-5'-monophosphate. The compound was found to inhibit CBS activity [161], giving rise to hyperhomocysteinemia. The use of this drug was also associated with vascular occlusive complications. Other drugs that interfere with pyridoxine metabolism include the antituberculous agents isoniazid and cycloserine. In addition, hydralazine, penicillamine, phenelizine, and procarbazine are listed as interfering with vitamin B_6 metabolism [11].

(*d*) *Vascular Disease in Homocysteinemia Due to Nutrient Deficiencies* A vexing question is raised by the homocysteine theory of atherosclerosis: Why, if hyperhomocysteinemia predisposes to vascular disease and thrombosis, is there not a known strong association between nutrient deficiencies of folate, cobalamin, or pyridoxine and thromboembolic disease? Folate- and cobalamin-deficiency syndromes are certainly well-defined clinical entities, and both can give rise to elevated plasma homocysteine levels that approach those typically seen in patients with inherited homocystinuria [130,133,134,154,155,162,163]. No entirely satisfying explanation for this apparent lack of correlation has been put forward. Although cobalamin deficiency usually occurs in older individuals, it is certainly not restricted to the elderly. Hence, the argument that a predisposition to vascular disease among cobalamin-deficient patients is not appreciated because of the generally high prevalence of cardiovascular disease in this age group [13] does not fully address the question. Furthermore, folate deficiency does not have the same demographic age distribution as cobalamin deficiency. It has also been proposed that some of the other complications of folate and cobalamin deficiencies might mitigate against possible thromboembolic complications [13]. For example, megaloblastic anemia is often associated with diminished platelet numbers as well as defects in their function [142]. Also, in both folate deficiency and pernicious anemia, low blood cholesterol and lipid levels have been reported, which rise following correction of the underlying vitamin deficiency [164,165]. Although this suggestion may have some merit, the interactions between various discrete risk factors for cardiovascular disease are complex and it is not known whether and to what extent one favorable circumstance can offset the deleterious effect of another.

The most plausible explanation for an apparent lack of correlation between hyperhomocysteinemia-associated vitamin deficiencies and vascular disease is the issue of disease duration [41]. In general, by the time that plasma homocysteine levels rise into the range encountered in homocystinuria, the clinical features associated with the deficiency state result in the patient seeking medical attention, which leads to diagnosis, treatment, and correction of the underlying nutrient deficiency as well as the hyperhomocysteinemia. Thus, the symptoms of moderate to severe anemia with or without neurological complications usually declare the existence of vitamin B_{12} or folate deficiency. This focuses attention on the disorder and prompts vitamin replacement, curtailing the derangement in homocysteine metabolism. Still, cobalamin deficiency, in particular, may be insidious in onset, and may present clinically with predominantly or exclusively neurological complications [162], so that the time to diagnosis and therefore the hyperhomocysteinemic state may be protracted.

These issues notwithstanding, there have been a few reports in the literature that refer either to the presence or the absence of vascular complications in patients with pernicious anemia. It has been noted that life expectancy in men, but not women, with pernicious anemia is shorter than in the general population. This has been attributed to the higher incidence of gastric cancer in pernicious anemia [142,166]. However, in the study by Mosbech and Videbaek, of 115 patients studied, 24 (21%) died of cerebral vascular accident, and in a further 32 (28%), cardiac failure and coronary thrombosis was given as the cause of death [166]. Wilkinson, in a study of 301 patients in 1949, reported cerebral vascular accident in 38 (12.6%) and cardiac failure with coronary thrombosis in 31 (10.3%) [167]. On the other hand, in a matched control study, Mitchell and Mitchell [168], in an autopsy study, found no differences in the degree or severity of vascular pathology, including thromboembolism in a small number of patients with pernicious anemia.

Other observations that may have some bearing on the possible association of vascular disease with pernicious anemia were reported by Lawson et al. [169]. These investigators recorded sudden death, which was attributed to cardiac arrest in patients with pernicious anemia following treatment. They attributed death to arrhythmia caused by hypokalemia resulting from a rapid shift of potassium into a suddenly expanding red cell mass, producing hypokalemia. Not studied was the degree of underlying coronary artery disease. It is possible that these individuals might have been susceptible to sudden cardiac death caused by arrhythmia because of underlying coronary vessel disease. In this regard, it is interesting to note that cardiac deaths have not been reported in other groups of patients in whom there is a rapid production of red cells such as treated patients with severe iron deficiency anemia or patients receiving recombinant human erythropoietin for the anemia associated with chronic renal disease.

2. Metabolic Abnormalities

(*a*) *Renal Failure* There are several reports in the literature of mild to moderate levels of hyperhomocysteinemia occurring in patients with chronic renal disease [170–175]. Since renal excretion of homocysteine accounts for 1% of the total homocysteine produced in the body [132], it is unlikely that raised plasma levels of homocysteine in chronic renal disease may be attributed solely to reduced renal excretion. Increases in total plasma homocysteine [134] have been found in patients with chronic renal disease as well as protein-bound homocysteine [13], homocystine, and homocysteine-cysteine mixed disulfide [176,177]. It is however, also unlikely that the hyperhomocysteinemia observed in patients with chronic renal failure is due to metabolic factors. On the contrary, there is indirect evidence to suggest that homocysteine metabolism through the transsulfuration and remethylation pathways may actually be increased in chronic renal disease. Serine is also consumed in both pathways (see Fig. 1), and it has been reported that plasma serine levels are reduced in chronic renal failure [170,171]. However, it seems more likely that the reduced plasma serine levels result from increased metabolism of homocysteine through the transsulfuration pathway since supplementation of folic acid to patients with chronic renal failure reduces the plasma homocysteine level [170,178]. This implies that the remethylation pathway is curtailed through lack of folate and is in accord with other evidence, suggesting that there may be intracellular lack of folate in chronic renal disease, particularly in patients on chronic dialysis [179].

There is a high prevalence of vascular disease in patients with chronic renal insufficiency and cardiovascular disease is a major cause of morbidity and mortality in patients undergoing chronic hemodialysis [180,181]. Several mechanisms have been proposed to explain the occurrence of vascular disease in association with chronic renal failure, and it certainly seems probable that hyperhomocysteinemia may be a contributory factor.

(*b*) *Thyroid Disease* Both hypothyroidism and cobalamin deficiency are frequently the result of an underlying autoimmune disease. Autoimmune thyroiditis causes hypothyroidism and pernicious anemia results in cobalamin deficiency. Furthermore, autoimmune thyroid disease and pernicious anemia frequently coexist [182–185]. It has recently been reported that serum homocysteine levels are raised in patients with hypothyroidism [186–188]. In a proportion of these patients with raised homocysteine, there was evidence of an underlying cobalamin deficiency, usually caused by pernicious anemia. However, in others, the hyperhomocysteinemia was an isolated finding, not associated with any coexistent deficiency of cobalamin or folate. In a group of 40 hypothyroid patients, serum homocysteine was significantly higher than the normal range [188]. It is not clear whether the hyperhomocysteinemia observed in patients with hypothyroidism who are not cobalamin or folate deficient is caused by an effect on the transsulfuration or the remethylation pathway.

V. CLINICAL IMPLICATIONS OF HYPERHOMOCYSTEINEMIA

A. Classical Homocystinuria

Several inborn errors of metabolism may give rise to the clinical syndrome of homocystinuria with its associated multiple organ system complications [41]. All share in common a metabolic defect involving either the transsulfuration or remethylation pathways for homocysteine metabolism. Affected individuals are homozygous for one of these mutations and phenotypically display features similar to those first described in individuals with severe hyperhomocysteinemia and homocystinuria [2,3]. Thus, deficiency of CBS [4], 5,10-methylenetetrahydrofolate reductase [6], and defects in cobalamin metabolism resulting in abnormalities of methionine synthase [5] all lead to a similar clinical syndrome characterized by hyperhomocysteinemia with multiple connective tissue defects and precocious cardiovascular disease. The underlying defects that arise from inherited abnormalities affecting each of the three enzymes identified as causes of homocystinuria may be heterogeneous. Thus, for example, several types of CBS deficiency may be recognized according to the responsiveness of affected individuals to large doses of pyridoxine [189]. The inherited defects affecting the remethylation pathway have been more precisely characterized, as described above. Thus, the several Cbl mutants, C, D, E, F, and G, which are characterized either by inadequate production of methylcobalamin, or by a defective methionine synthase enzyme, have all been well defined [83,84]. Methylenetetrahydrofolate reductase deficiency disrupts the pathway for generation of methyltetrahydrofolate, the cosubstrate with homocysteine for the methionine synthase reaction [94]. The relative frequency of inherited homozygous defects in the transsulfuration pathway is approximately 20 times greater than all of the defects in the remethylation pathway combined [190].

Severe hyperhomocysteinemia with homocystinuria caused by various inborn errors of metabolism result in similar clinical syndromes, regardless of the underlying mechanism. The underlying mechanisms that give rise to exaggerated and premature thrombosis and atherosclerosis in CBS deficiency as well as other genetic causes of hyperhomocysteinemia are not clear. It is, however, fairly certain that these complications are the direct consequence of the hyperhomocysteinemia, rather than to any other associated biochemical abnormalities that are known to occur as a result of the metabolic block in homocysteine metabolism. The most compelling evidence that incriminates homocysteine as the agent directly responsible for the vascular and other complications of hyperhomocysteinemia comes from a comparison of the metabolic derangements seen in the various disorders associated with hyperhomocysteinemia. McCully [5] and later others [6,191] have noted that vascular lesions occur in individuals affected by remethylation disorders, which are essentially the same as those seen in CBS deficiency. Thus, for example, methionine levels are diametrically different in individuals with defects of the transsulfuration

pathway (CBS deficiency) and those of the remethylation pathway (methionine synthase or methylenetetrahydrofolate reductase). As pointed out by Mudd et al. [41], the former disorder is characterized by hypermethioninemia, whereas in the latter group there is hypomethioninemia.

Several mechanisms have been proposed to explain the vascular damage and predisposition to thrombosis that are associated with hyperhomocysteinemia. These may be divided into three general categories, depending on whether they are concerned with endothelial cells, platelets, or soluble clotting factors and their inhibitors. The basic manifestations of homocystinuria consist of skeletal abnormalities, lens dislocation, mental retardation, and occlusive vascular disease including thrombosis and precocious atherosclerosis. The underlying biochemical mechanisms that give rise to these complications are not known, but theories abound. The vascular complications are the most serious, since they are the major cause of morbidity and mortality among affected individuals. Also, because of the intriguing association of hyperhomocysteinemia with these vascular complications, the potential role of homocysteine in the induction of both atherosclerosis and thrombosis has received increasing attention. The "experiment of nature" exemplified by homocystinuria has led to the investigation of milder degrees of hyperhomocysteinemia, both congenital and acquired, with respect to a potential role as an independent risk factor in atherosclerosis. No unifying concept to explain the chemical mechanism whereby hyperhomocysteinemia results in these various stigmata has been proposed, but it appears plausible that alteration of collagen metabolism or some other component of connective tissue may constitute the common underlying abnormality that is responsible for the skeletal, ocular, and vascular complications. The detailed features of CBS deficiency have been described by Mudd et al. [41].

1. *Skeletal Abnormalities*

Skeletal abnormalities in homocystinuria result in deformities of the axial skeleton and trunk such as scoliosis, kyphosis, pectus excavatum, and pectus carinatum. There is also lengthening of the appendicular skeleton, affecting both the upper and lower extremities and resulting in an overall increase in stature. There may also be limitation of joint movement and osteoporosis precipitating frequent bony fractures even following minor trauma.

2. *Ocular Features*

The most common eye abnormality is dislocation of the lens, usually in a downward direction, which develops at an early age. This frequently gives rise to glaucoma. Individuals with homocystinuria are also often myopic, probably due to laxity of the suspensory ligament of the lens. Retinal detachment occurs more frequently than in the general population.

3. *Mental Retardation*

The effect of homocystinuria on intellect is variable. There is a fairly high frequency of mental retardation, but in the series of 84 cases reported by McKusick [192] almost half of the patients were of average intelligence and some were college graduates. Carson et al. studied inmates of an institution for the mentally retarded in Northern Ireland and found homocystinuria among 0.3% [2]. In addition to the wide range of intellectual capabilities observed in patients with CBS deficiency, a bimodal distribution has been observed distinguishing pyridoxine-responsive patients, who have generally higher I.Q. scores, from pyridoxine nonresponsive patients [193]. In addition to mental retardation, some homocystinuric individuals suffer from seizure disorders. Additionally, a variety of neurological problems occur that are attributable to cerebral vascular occlusion, ranging from focal neurological signs to hemiplegia.

4. *Vascular Complications*

In an international survey of 629 patients with CBS deficiency reported in 1985, 42 of 59 deaths that appeared to be related to the genetic disorder were attributable to thromboembolism, and in a further five it was a contributory factor [193]. The fatal cardiovascular event occurred at a younger age among pyridoxine-nonresponsive patients than in the pyridoxine-responsive group. Although the vascular occlusion may affect any vessel, peripheral venous thrombosis with subsequent pulmonary embolism was relatively common. In all, 158 patients of the 629 surveyed had suffered a thromboembolic event after peripheral venous thrombosis (51%). The next most frequent occurrence was cerebral vascular accident (32%). Peripheral arterial thrombosis and myocardial infarction were reported in 11 and 4%, respectively. As for the vascular complications with fatal outcome, all thromboembolic complications occurred at a younger age among untreated pyridoxine-nonresponsive patients than in the untreated pyridoxine-responsive group.

Autopsy of patients with CBS deficiency often discloses multiple thrombi and emboli involving large and small arteries and veins. This has been reviewed by Mudd et al. [41]. Changes in the arterial wall have also been described, consisting of intimal thickening which may be concentric or patchy [194]. There is also a disruption of the media with increased deposition of collagen between split muscle fibers, and there may be damage to the internal elastic lamina [194]. Other less frequent changes are reviewed by Mudd et al. [41].

B. Mild to Moderate Hyperhomocysteinemia

Coronary artery disease, cerebral vascular disease, and peripheral vascular occlusive disease are presently the most common causes of mortality and morbidity in the

United States and many other economically advanced countries. Although there has been a declining mortality associated with cardiovascular diseases over the past 40 years, hundreds of thousands of Americans die of diseases affecting the heart and blood vessels each year. In 1988 there were nearly 1,000,000 deaths attributed to cardiovascular disease, and over 50% were due to coronary artery disease [195]. However, in recent years there has been a steadily declining mortality rate due to cardiovascular disease, related at least in part to our ability to identify and manage associated risk factors. The three major modifiable risk factors associated with coronary artery disease that have been identified to date are dyslipidemia, smoking, and hypertension [195,196]. Hyperhomocysteinemia is another risk factor in which interest has waxed and waned since McCully and Wilson [7] proposed the "homocysteine theory of arteriosclerosis" nearly two decades ago.

There has been a resurgence of interest in homocysteine because recent clinical evidence suggests that it is an independent risk factor for vascular diseases. This interest has been generated by a series of recent clinical studies (reviewed in Refs. 8–18) that indicate a high level of association between mild to moderate hyperhomocysteinemia and coronary artery disease [79,197–202], stroke [79,141,158,203–206], and peripheral vascular occlusive disease [75,79, 140,206–208] and by recent methodological advances that provide reliable data on total plasma and serum homocysteine levels [67,69,73,75,76]. The homocysteine theory states that elevated levels of homocysteine in the blood or hyperhomocysteinemia predispose an individual to the development of vascular disease. Total plasma (or serum) homocysteine is defined as the sum of all forms of homocysteine found in plasma and includes free homocysteine, free oxidized forms, and protein-bound homocysteine, as shown in Fig. 2. Hyperhomocysteinemia can vary in severity and for convenience may be defined as mild, moderate, or severe. Since there is no general consensus for defining these terms in absolute concentrations, we will define mild hyperhomocysteinemia as total plasma concentrations ranging from 15 to 25 μmol/liter, moderate hyperhomocysteinemia as 26 to 50 μmol/liter, and severe hyperhomocysteinemia as >50 μmol/liter. Severe hyperhomocysteinemia is usually, but not always, associated with the homocystinuria disease group. Detailed study of patients with homocystinuria could provide useful information on the mechanism of homocysteine-induced cardiovascular disease, but little work has been done in these patients other than clinical and postmortem observations. These individuals have plasma concentrations of total homocysteine ranging from 50 to 500 μmol/liter. The incidence of homocystinuria in the general population is approximately 1:200,000 although it may be higher in some populations such as in Ireland and New South Wales) [13].

A number of recent clinical studies have shown an association between mild hyperhomocysteinemia and premature coronary artery disease [79,197–202]. Genest et al. [197] in Boston determined total plasma homocysteine in 170 men who had

premature coronary artery disease diagnosed by angiography and found a significantly higher level (13.7 ± 6.4 μmol/liter) compared with 255 healthy controls (10.9 ± 4.9 μmol/liter). There was no significant correlation between homocysteine and either age, total cholesterol, LDL cholesterol, HDL cholesterol, or triglyceride levels. Based on elevated non–protein bound homocysteine in plasma after an oral methionine load, Clarke et al. [79] in Dublin detected hyperhomocysteinemia in 18/60 patients (30%) with premature coronary artery disease (<55 years), in 16/38 patients (42%) with cerebral vascular disease and in 7/25 patients (28%) with peripheral vascular disease and concluded that homocysteine was an independent risk factor for cardiovascular disease. Ubbink et al. [201] in Pretoria, South Africa, found that 42% of 163 male patients with coronary artery disease had hyperhomocysteinemia and that there was a positive correlation with the number of coronary arteries occluded. In contrast, the prevalence of hypercholesterolemia was 35%, and there was no correlation with the number of blocked arteries. Stampfer et al. [202] concluded that elevated levels of plasma homocysteine are associated with subsequent risk of myocardial infarction independent of other coronary risk factors after analyzing 271 physicians participating in the Physicians' Health Study who developed myocardial infarction and 271 paired controls from the same study. Finally, in a study involving 101 white males 50 years of age or younger with angiographically confirmed coronary artery disease and 108 white male controls, Pancharuniti et al. [209] found that increased total plasma homocysteine was an independent risk factor for coronary artery disease and that low plasma folate was associated with increased risk largely because of its effect on plasma homocysteine concentration.

Mild to moderate hyperhomocysteinemia is also thought to be a risk factor for cerebral vascular disease [79,141,158,203–206] and peripheral vascular occlusive disease [75,79,140,206–208]. In a study involving 99 patients with stroke and transient ischemic attack, Coull et al. [205] found that approximately one-third had elevated plasma homocysteine, a finding independent of other recognized risk factors for stroke. The combined mean homocysteine level for this group of patients was 15.5 ± 5.9 μmol/liter compared to 10.7 ± 3.2 μmol/liter ($p < 0.001$) for controls. There was no correlation between plasma homocysteine and other risk factors for stroke. However, impaired renal function was associated with elevated plasma homocysteine. Brattström et al. [141] also found a 40% incidence of mild hyperhomocysteinemia in 142 survivors of stroke. Although there was no correlation of hyperhomocysteinemia with other vascular disease risk factors, approximately 40% of the variation in plasma homocysteine was attributable to the serum concentrations of the vitamins (folic acid, pyridoxal phosphate, and cobalamin) or serum creatinine levels. Malinow et al. [207] found significantly higher levels of plasma homocysteine in 47 patients with peripheral vascular occlusive disease when compared with 103 controls. In a study of 214 patients with either cerebral vascular or peripheral vascular occlusive disease, Taylor et al. [206] found elevated plas-

ma homocysteine (above control mean + 2 SD) in 83 subjects (39%). The mean plasma homocysteine level in all patients was 14.4 ± 6.9 μmol/liter compared with 10.1 ± 2.2 μmol/liter controls ($n = 103$; $p < 0.05$). The rate of disease progression in patients with peripheral vascular occlusive disease but not cerebral vascular disease was more rapid in those with elevated plasma homocysteine. The high incidence of peripheral vascular occlusive disease and chronic renal failure has been recognized for many years [180]. Several groups have found moderate to high levels of plasma homocysteine in patients with chronic renal failure [170,173,177].

VI. MECHANISMS

A. Vascular Endothelium

Harker et al. carried out studies in baboons as well as patients with CBS deficiency [210,211]. In baboons given long-term infusions of homocysteine thiolactone, endothelial desquamation occurred, followed by shortened platelet survival. Based on these studies as well as observations in CBS-deficient patients who had shortened platelet survival compared with normal controls, they concluded that high levels of homocysteine were directly toxic to the endothelial cells, resulting in their exfoliation with secondary effects on platelets. However, the platelet survival studies were not reproduced by Uhlemann et al. [212], who studied six patients with CBS deficiency, including two of the four patients studied by Harker et al. [211]. Cytotoxic effects of homocysteine have also been demonstrated in experiments using cultured endothelial cells [213,214]. In cytotoxicity studies carried out by Wall et al. [213] using cultured human aortic endothelial cells, homocysteine caused release of radiochromium. Addition of catalase prevented this effect. However, in similar experiments using bovine aortic endothelial cells, Rodgers and Kane [215] found that high concentrations of homocysteine (10 mmol/liter) produced little if any endothelial cell damage. Mudd et al. [41] point out that an essential difference between these two experiments was the amount of copper present during incubation.

B. In Vivo Animal Models

McCully reported that weanling and yearling rabbits given subcutaneous injections of D,L-homocysteine thiolactone rapidly developed fibrous aortic plaques and arterial plaques in major organs [7,216]. However, Donahue et al. [217] were unable to reproduce the model. Desquamation of vascular endothelium, appearance of circulating endothelial cells, and decreased platelet survival were observed by Harker et al. [211] after continuously infusing baboons with 0.5 mg/min of L-homocystine for a 5-day period. A plasma level of about 200 μmol/liter homocystine was achieved over this time period. Similar results were obtained by this group when baboons were continuously infused with L-homocysteine (0.3 g/kg/day) over a 3-month period producing plasma levels of 100–200 μmol/liter homocystine (free

homocysteine and protein-bound homocysteine were not determined) [210]. These animals apparently received homocysteine thiolactone and not L-homocysteine (see Ref. 218). There was a marked reduction in intimal lesion formation associated with homocysteine-induced patchy endothelial cell injury when dipyridamole or sulfinpyrazone were administered suggesting that platelets played a role in lesion development [210,219]. The levels of *total* plasma homocysteine achieved in the Harker baboon studies were quite high and probably exceeded the levels usually found in homocystinuric patients [220]. However, these studies did focus attention on the vascular endothelium and endothelial-platelet interactions as the potential target site of homocysteine-induced injury.

C. In Vitro Endothelial Cell Studies

In the response-to-injury hypothesis of atherogenesis, Ross proposed that injury to the endothelium was the initiating event in the development of atherosclerosis [221]. Endothelial cells mediate numerous cytokine and growth factor responses that undoubtedly play important roles in atherogenesis [221–224]. During the 1980s in vitro studies were carried out on cultured endothelial cells to determine how homocysteine and homocysteine analogs affected viability and function. In these studies extremely high concentrations of D,L-homocysteine or D,L-homocysteine thiolactone (1–10 mmol/liter) were used, which exceeded the levels encountered even under the most severe pathological conditions. Wall et al. [213] found that 0.1–10 mmol/liter D,L-homocysteine thiolactone was cytotoxic to cultures of human umbilical vein endothelial cells (HUVEC) based on ^{51}Cr release and detachment assays. Catalase, but not superoxide dismutase, protected against cytotoxicity, suggesting that H_2O_2 production from thiol autooxidation might be involved. In a study using HUVEC from normal and CBS heterozygous donors, De Groot et al. [225] observed that the cells from heterozygous donors (assumed to contain 50% CBS activity) were more sensitive to 10 mmol/liter homocystine and methionine based on cytotoxicity assays. Not surprisingly, both cell types were equally sensitive to 10 mmol/liter homocysteine. Bovine aortic endothelial cells (BAEC) were lysed (^{51}Cr release) by a H_2O_2-generating system consisting of 0.5–1.0 mmol/liter homocysteine in the presence of cupric ions or ceruloplasmin as observed by Starkebaum and Harlan [226]. Recent in vitro studies by Dudman et al. [218] cast serious doubt on the significance of in vivo [210,211] and in vitro studies [213,219] that utilized desquamation and detachment, respectively, as an indicator of homocysteine-induced cell injury. They point out that the levels of homocysteine in these studies were excessive and that similar concentrations of cysteine, not known to be atherogenic, were equally effective in causing desquamation and detachment.

More recent studies have focused on impairment of endothelial cell resistance to thrombosis and the stimulation of procoagulant activities by homocysteine. Panganamala et al. [227] reported that prostacyclin synthesis was nonspecifically

stimulated by low concentrations of thiols (including homocysteine) but was inhibited at higher concentrations. Rodgers and Kane [215] found that 0.5–10 mmol/liter homocysteine (isomer not specified) promoted activation of Factor V to Va by proteolysis on BAEC and HUVEC cells. Also, surprisingly, no cytotoxicity was observed at 10 mmol/liter homocysteine. Methionine and cysteine also activated Factor V, indicating that the effect was not specific to homocysteine. Rodgers and Conn [228] found that homocysteine (isomer not specified) reduced protein C activation by up to 90% at homocysteine concentrations of >7.5 mmol/liter. Lentz and Sadler [229] found that 5 mmol/liter D,L-homocysteine increased thrombomodulin mRNA and protein synthesis in HUVEC and CV-1(18A) cells without affecting viability. However, thrombomodulin was not expressed on the surface of these cells, suggesting that homocysteine interfered with the secretory process by partially blocking glycosylation and sulfation of the protein. The authors also reported that homocysteine and other thiols blocked cell-free protein C activation by irreversibly inactivating protein C and thrombomodulin but not thrombin. Although these are perhaps the most interesting (and provocative) of all of the in vitro studies to date, their significance is open to question in view of the relatively high levels of homocysteine used in the study. Rodgers and Conn's interpretation of the relative levels of "free" and protein-bound homocysteine in homocystinuric patients is based on what seems to be erroneous information in the literature [230], which other investigators have failed to confirm [231]. Using HUVEC cells, Hayashi et al. [232] found that 10 mmol/liter homocysteine (isomer not specified) inactivated the cofactor activity of thrombomodulin, essentially confirming the results of Lentz and Sadler. Hajjar [233] recently reported that 1–5 mmol/liter D,L-homocysteine blocked tissue plasminogen activator (t-PA), but not plasminogen, binding to human endothelial cells (type not specified) in a time- and dose-dependent fashion. Interestingly, the blockade appeared to be specific. L-Cysteine did not block t-PA binding but did reverse homocysteine-associated reduction in t-PA binding. Lentz and Sadler [234] recently reported that 1.0 mmol/liter homocysteine significantly inhibits endoplasmic reticulum–dependent processing and secretion of von Willebrand factor by HUVEC cells.

Nitric oxide (NO), or endothelium-derived relaxing factor (EDRF), has potent vasodilatory and antiplatelet effects. Murphy et al. [235] reported that extracellular cysteine diminished NO production in cultured porcine aorta endothelial cells. Stamler et al. [236] found that exposure of BAEC, stimulated to secrete NO, to homocysteine resulted in its conversion to S-nitroso-homocysteine, which also has potent vasodilatory and antiplatelet effects. They speculated that nitrosothiol formation detoxifies homocysteine by blocking autooxidation and hydrogen peroxide generation and homocysteine thiolactone formation. However, they also pointed out that continuous exposure of the vascular endothelium to homocysteine impaired the NO-EDRF generating system.

D. Utilization of Homocysteine by Cultured Endothelial Cells

Cultured HUVEC transport homocysteine by sodium-dependent and sodium-independent transport systems [237]. Wang et al. [238] reported that cell-free extracts of cultured HUVEC had detectable levels of CBS and methionine synthase activity. The levels of CBS in the four isolates studied varied considerably. Interestingly, Wang et al. [238] were able to enhance the activity of methionine synthase by modifying the growth medium. This rendered the cells more resistant to homocysteine-induced injury.

E. Homocysteine-Modified Plasma Proteins

Although it has been known since 1979 that a substantial portion of homocysteine in the serum of homocystinuric patients and normal individuals is "protein-bound" [58], the protein(s) that carry homocysteine have not been identified with certainty. Individuals who may be mildly hyperhomocysteinemic (e.g., heterozygotes for CBS deficiency) are reported to have higher levels of protein-bound homocysteine [58,239,240]. Since homocysteine can participate in disulfide bond exchange reactions, it is possible that excessive homocysteine entering the circulation can alter plasma proteins by this process. Mansoor et al. [241] have recently shown that, after an oral methionine load, protein-bound homocysteine increased dramatically over a 6- to 8-hour period while protein-bound cysteine and cysteinylglycine decreased rapidly during the first hour. Although some evidence suggests that albumin carries homocysteine in circulation [60], other plasma proteins(s) that interact with homocysteine in vivo have not been identified. However, Harpel et al. [242] recently reported that concentrations of D,L-homocysteine as low as 8 μmol/liter dramatically increased the affinity of Lp(a) for plasmin-modified fibrin surfaces. These results suggest that homocysteine-modified Lp(a) could effectively compete with plasminogen for binding sites on fibrin-coated surfaces, thereby creating a more thrombogenic environment.

VII. TREATMENT AND PREVENTION OF HYPERHOMOCYSTEINEMIA

A. Severe Hyperhomocysteinemia

Many of the inborn errors of homocysteine metabolism described above that result in severe homocysteinemia are refractory to treatment, and yet, in some homozygous patients, response to therapy can be dramatic. The treatment of hyperhomocysteinemia is usually determined by its etiology and by the age of the patient at the time of diagnosis. Thus, newborns with CBS deficiency who are placed on a low-methionine cysteine-supplemented diet appear to avoid many of the early clinical complications and have higher mental capabilities than CBS-deficient children given normal diets [193]. Approximately half of the individuals with CBS

deficiency detected later in life can lower their homocysteine levels and prevent or delay clinical complications by taking oral doses of pyridoxine hydrochloride ranging from as little as 25 mg/day up to 1200 mg/day (reviewed in Ref. 41). The effectiveness of pyridoxine in preventing thromboembolism in pyridoxine-responsive patients with CBS deficiency has been shown statistically to be highly significant [193]. A combination of remethylation substrates and cofactors including betaine, folic acid, cobalamin, and pyridoxine may further lower homocysteine levels in plasma and urine of some patients [243,244]. Patients with hyperhomocysteinemia due to inherited disorders of cobalamin transport and metabolism respond to massive and frequent doses of cyanocobalamin or, in most cases, hydroxocobalamin as discussed above. In some of these patients betaine is also given to further lower homocysteine, but Allen et al. [245] have recently shown that betaine administration is ineffective as a homocysteine-lowering agent in these patients. Perhaps one of the more difficult conditions to treat is homozygous MTHFR deficiency. The degree of success in treatment and clinical severity appear to correlate with the severity of the deficiency [85,94]. Although numerous approaches to the treatment of this disease have been tried, including folic and folinic acid, methyltetrahydrofolate, betaine, cyanocobalamin, pyridoxine and methionine, in many cases these approaches have been unsuccessful, particularly if the diagnosis has been delayed and neurological complications are present.

Patients with severe hyperhomocysteinemia due to acquired cobalamin and folate deficiency generally respond very well to therapy with cyanocobalamin and folic acid, respectively. In pernicious anemia a single intramuscular dose of 1 mg cyanocobalamin can lower the plasma homocysteine level in severe hyperhomocysteinemia (>50 μmol/liter total plasma homocysteine) to within the normal range in 2–3 days [134]. Treatment of folate deficiency with daily oral doses of folic acid (1 mg) has a similar effect on lowering total plasma homocysteine [134].

B. Mild and Moderate Hyperhomocysteinemia

The etiology of mild to moderate hyperhomocysteinemia is generally complex and incompletely understood. Recent work suggests that heterozygosity for CBS deficiency [79], thermolabile MTHFR [98,100], compound heterozygosity for MTHFR deficiency [101], and suboptimal cobalamin, folate, and pyridoxine nutrition, particularly in the elderly [131,246,247], may, either singly or in combination, be contributory. As discussed above, patients with chronic renal disease generally have mild to moderate hyperhomocysteinemia [171,177] and a high risk of premature cardiovascular disease [180]. Efforts to lower moderately elevated levels of plasma homocysteine in hemodialysis patients by administering daily doses of 5 mg of folic acid have been successful [137,178], but whether this influences the long-term risk of developing vascular disease has not been reported. Although folic acid alone seems to be effective in lowering plasma homocysteine

concentration [135,136], there is a risk that this might mask an underlying cobalamin deficiency. Ubbink et al. [139], in a placebo-controlled study, used a daily regimen consisting of 10 mg pyridoxal, 1.0 mg folic acid, and 400 μg cyanocobalamin to normalize elevated plasma homocysteine levels within a 6-week period in apparently healthy men with moderate hyperhomocysteinemia. Cobalamin therapy was also effective in lowering elevated homocysteine levels in a group of elderly outpatients [131].

C. Prevention of Mild to Moderate Hyperhomocysteinemia

Until we gain a better understanding of the conditions that lead to the development of mild to moderate hyperhomocysteinemia, it is difficult to discuss what preventive measures can be taken. For example, how important is diet in determining basal levels of total plasma homocysteine? Does a high-protein diet necessarily equate with higher basal levels? Does basal plasma homocysteine concentration correlate with total daily methionine intake? And what are the effects of age and sex on plasma homocysteine levels? Andersson et al. [81] found that total homocysteine levels were similar in premenopausal women and comparably aged males. But in a recent study by Jacobsen et al. [67] there was a statistically significant difference in serum and plasma total homocysteine levels seen in males and premenopausal females of similar age. In both studies there was a significant inverse correlation between total serum homocysteine and serum cobalamin and folate levels in the groups of apparently healthy men and women [67,81]. Does this mean that what we now consider to be the normal range for total plasma homocysteine would be lowered by "optimal" nutrition with respect to cobalamin, folic acid, and pyridoxine? Clearly the effect of diet, age, sex, and nutritional status merit further study if we are to understand the factors responsible for homocysteinemia and hyperhomocysteinemia.

VIII. SUMMARY AND CONCLUSIONS

Homocysteine arises from dietary methionine. Disruption of the normal metabolic pathways that consist either of remethylation to methionine or transsulfuration to cysteine results in the accumulation of homocysteine in tissues and in the plasma giving rise to the clinical entity termed hyperhomocysteinemia. The degree of hyperhomocysteinemia may vary from mild to severe, depending on its cause. Several inherited and acquired causes of hyperhomocysteinemia are known. In the case of the inherited disorders, the severity of hyperhomocysteinemia depends on the gene dosage in the affected individual, the particular metabolic pathway involved, and the nature of the mutation. In homozygous affected individuals, the disorder usually but not always becomes apparent at a young age. Heterozygotes and compound heterozygotes, on the other hand, may only be discovered during adult life, or their condition may go undetected. The acquired disorders that give

rise to hyperhomocysteinemia are usually the result of deficiency of one or more of the three vitamin cofactors that are required for metabolism of homocysteine through the transsulfuration or remethylation pathways: pyridoxine, folic acid, and cobalamin. In addition, metabolic disorders such as chronic renal failure and hypothyroidism may also give rise to hyperhomocysteinemia.

Clinical interest in hyperhomocysteinemia first arose as a result of the recognition of precocious and severe vascular occlusive disease in individuals homozygous for inborn errors of metabolism that were associated with hyperhomocysteinemia. This compelling association led to the investigation of hyperhomocysteinemia as a possible risk factor for occlusive vascular disease including atherosclerosis and thrombosis. A large number of clinical and epidemiological studies have shown a link between mild to moderate hyperhomocysteinemia and an increased risk of vascular disease including cerebral vascular, peripheral vascular, and coronary artery disease. Noteworthy among these studies has been the observation that hyperhomocysteinemia is particularly evident as a risk factor for premature atherosclerosis and thrombosis affecting younger individuals.

Some of the risk factors for atherosclerosis can be modified by suitable intervention. In the case of hyperhomocysteinemia, it is clear that at least in those situations where it is associated with a cofactor deficiency, dietary supplementation may result in a substantial lowering of the plasma homocysteine concentration. It remains to be seen whether this is associated with a diminished risk of occurrence or recurrence of vascular occlusion. However, based on the available evidence to date, it appears promising that hyperhomocysteinemia may prove to be a modifiable risk factor for atherosclerosis.

Although considerable clinical and epidemiological data have been reported, there is relatively little information on the mechanisms whereby hyperhomocysteinemia results in vascular occlusion. Interaction of homocysteine with platelets or endothelial cells has been proposed as a possible mechanism. Most of the homocysteine in the plasma is covalently bound to protein. How this interaction of homocysteine with cellular or plasma proteins results in vascular damage and occlusion remains to be elucidated.

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5

Folate Requirements and Dietary Recommendations

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I. INTRODUCTION

This chapter presents the major dietary folate intake standards available worldwide and an overview of the types of experimental data on which these recommendations are based. A common database and similar experimental approaches were used to estimate the dietary folate intake standards regardless of the country of origin which explains their remarkable similarity. This chapter includes examples of key studies that illustrate the type of data on which the standards are based and different experimental approaches.

Each phase of the life cycle is associated with physiological changes that may influence folate requirements. In this chapter, emphasis is placed on the effect of pregnancy on folate requirements as well as the suggested inadequacy of current dietary recommendations for maintenance of folate status in nonpregnant women and adult men.

Estimated folate content of selected foods is presented in this chapter to illustrate the relative amount of folate different foods provide. Food composition data indicate the wide variation in folate content within each food group. Increased daily consumption of folate-dense foods including green leafy vegetables, orange juice, beans (pinto, navy), and fortified cereals may significantly enhance folate status. This chapter closes with a highlight of potential interactions between folate and drugs as well as selected nutrients.

II. DEFINITION OF FOLATE REQUIREMENTS, RECOMMENDED DIETARY ALLOWANCES, AND OTHER STANDARDS FOR DIETARY INTAKE

The folate requirement is the minimum quantity necessary to prevent a deficiency [1]. The translation of minimum requirement to a dietary recommendation for population groups necessitates that allowance be made for a number of variables, including (1) periods of low intake, (2) increased utilization, (3) individual variability, and (4) bioavailability. In the United States, the Recommended Dietary Allowances (RDAs) incorporate "margins of safety" intended to be sufficiently generous to encompass the variability in the minimum requirement among people and bioavailability of folate from different food sources [1]. The first step in establishing an RDA is to estimate the average requirement and then adjust by factors to compensate for incomplete utilization and variation in individual requirements [1]. In theory, population requirements follow a Gaussian distribution pattern, and the addition of two standard deviations to the mean requirement would cover the needs of most (i.e., 98%) individuals [1]. This value (minimum requirement plus "safety allowance") equates to the current definition of RDAs. The RDAs are derived from critical scientific evaluation of available data, and recommendations may undergo dramatic changes as illustrated by the 50% reduction in the folate RDA in the Tenth Edition (1989) compared to the Ninth Edition (1980) [1,2] (Table 1).

In addition to the RDA in the United States, there are a number of different recommendations from various governmental agencies and countries worldwide, including the Dietary Reference Value (DRV) in the United Kingdom [3], Recommended Nutrient Intake (RNI) in Canada [4], and the WHO/FAO Safe Level of Intake [5] (Table 1). In the United Kingdom, the decision was made to include three standards for folate intake, and these are collectively referred to as Dietary Reference Values (DRVs) [3]. DRVs include the Reference Nutrient Intake (RNI), which is similar to the RDA in the United States and is the value included in Table 1. The RNI is in theory two standard deviations above the average requirement referred to as the Estimated Average Requirement (EAR) [3]. The third value is the Lower Reference Nutrient Intake (LRNI), which represents the lowest intake that will meet the needs of some individuals in the group [3]. The future direction of RDAs in the United States is proposed to be similar to that adopted by the United Kingdom, with recommended intakes expressed as a set of different standards, one of which would be equivalent (by definition) to the RDA [6].

These reference values or recommended dietary intakes can be utilized in a variety of ways, including (1) planning food supplies for population subgroups, (2) interpreting food-consumption records of individuals and populations, (3) establishing standards for food assistance programs, (4) designing nutrition education programs, and (5) developing new food products [1]. In addition, an important application of the dietary recommendations is in food labeling. In the United States,

Table 1 Dietary Folate (μg/day) Recommendations for Population Groups

Age/Sex category	Recommended Dietary Allowance, 1980 [2]	Recommended Dietary Allowance, 1989 [1]	United Kingdom Dietary Reference Value [3]	Canadian Recommended Nutrient Intake [4]	WHO/FAO Safe Level of Intake [5]
Infants (months)					
0.0–0.5	30	25	50	50	16–24
0.5–1.0	45	35	50	50	32
Children (yr)					
1–3	100	50	70	65–80	50
4–6	200	75	100	90	50
7–10	300	100	150	125	102
Males (yr)					
11–14	400	150	200	170	170
15–18	400	200	200	150–185	200
19–24	400	200	200	210	200
25–50	400	200	200	220	200
51+	400	200	200	220–205	200
Women (yr)					
11–14	400	150	200	180	170
15–18	400	180	200	145–160	170
19–24	400	180	200	175	170
25–50	400	180	200	175	170
51+	400	180	200	190	170
Pregnancy	800	400	+100	300	370–470
Lactation					
1st 6 months	500	280	+60	+100	270
2nd 6 months	500	260	+60	+100	270

the seventh edition (1968) of the RDAs became the basis for establishing guidelines for the nutritional labeling of foods (U.S. Recommended Daily Allowances, or USRDAs) (7). The USRDAs are synonymous with Reference Daily Intakes (RDIs) used currently on food labels in the United States, and the term "Percent of Daily Value" refers to the percentage of the RDI that the food item provides per serving [7].

Comparable experimental and population-based data served as the basis for all of the dietary recommendations, which explains the remarkably similar estimates for the United States, Canada, the United Kingdom, and WHO/FAO (Table 1). The recommendations will inevitably change as new data become available and experimental approaches are standardized.

III. EXPERIMENTAL METHODOLOGY

Recommended dietary folate standards or allowances are based on various kinds of experimental approaches, including (1) "depletion-repletion" or studies of subjects maintained on folate-deficient diets followed by correction of the deficit with measured amounts of folate, (2) maintenance of folate status in controlled metabolic studies, (3) observations of folate status in populations in relation to intake, (4) quantitation of urinary folate excretory products, and (5) kinetic approach. In contrast to these methods, balance studies that involve a comparison of nutrient intake with the amount excreted in urine and feces are not reliable since variable quantities of folate are synthesized by intestinal microflora [8].

A. "Depletion-Repletion" Studies

An established approach to estimating nutrient requirements is to determine the quantity required to restore normal status following consumption of a diet low in the nutrient. The definition of "depletion" is variable; however, most studies are based on the progressive stages of folate deficiency as characterized by Herbert's classic depletion study in which he placed himself on a folate-deficient diet [9]. The reversal of folate deficiency in response to specified quantities of folic acid as measured by changes in status indices has been used by a number of investigators to estimate folate requirements [8,10]. For example, blood folate levels were normalized in folate-depleted women when an oral dose of 50 μg/day of supplemental folic acid was given, whereas 25 μg/day was insufficient [11]. Repletion of folate status has in general been accomplished by supplementing with oral or injected folic acid rather than dietary folate, thus not accounting for differences in bioavailability between synthetic folic acid and dietary folate [8,10,12].

A "depletion-repletion" protocol was used by Sauberlich et al. [13] to estimate folate requirements and dietary allowances for healthy nonpregnant women.

Subjects were fed a folate-deficient diet (9 μg/day for 28 days) followed by graded intakes of folate for a total of 92 days. A large number of assessment parameters were evaluated, some of which were more responsive than others to the "depletion-repletion" protocol. Unlike the study conducted by Herbert [9], the intent was not to severely deplete the subjects prior to repletion since the depletion phase was only 28 days compared to Herbert's depletion phase, which lasted 120 days [13]. The requirement estimate was primarily based on the quantity necessary to restore serum and red blood cell (RBC) folate concentrations to predepletion levels. Based on data from this study, Sauberlich et al. [13] estimated the minimum dietary folate requirement for nonpregnant women to be 200–250 μg/day and an allowance to be approximately 300 μg/day.

Similar types of studies involving repletion of folate-depleted subjects have been conducted with either diseased patients or those with multiple nutrient deficiencies [8]. For example, data obtained from a study in which folic acid was administered intramuscularly to a scorbutic patient with megaloblastic anemia was part of the basis for defining the estimated "absorbed" folate requirement [1,14]. There are two major problems with using this type of data to establish folate requirements for normal healthy individuals. First, the majority of injected folic acid enters the circulation while the absorption of dietary folate or orally supplemented folic acid is influenced by various conditions in the intestine. Second, malnourished subjects may not absorb folate to the same extent as normal healthy individuals [8].

B. Maintenance of Folate Status in Metabolic Studies

The "depletion-repletion" method of determining folate requirements is limited in scope due to the inappropriateness of utilizing the method during childhood, adolescence, pregnancy, or lactation. An alternative approach is to evaluate the level of folate intake in a controlled metabolic study that maintains normal folate status. Milne et al. [16] concluded that 200 μg/day of dietary folate was adequate to maintain normal folate status in adult males, and this metabolic study was cited as one basis for reducing the RDA from 400 to 200 μg/day in the Tenth Edition [1]. Our research group [15] used this method to evaluate the adequacy of folate intakes ranging from 200 to 400 μg/day in nonpregnant women and observed that 200 μg/day was insufficient to maintain status as discussed later in this chapter.

C. Observations of Folate Status in Population Groups in Relation to Intake

A third method used to determine dietary folate requirements and allowances is to estimate the amount of folate consumed by population groups who are maintaining "normal" folate status. The dietary folate intake estimated from food intake surveys in the United States, Canada, and the United Kingdom ranged from 185 to 305 μg/day for all groups (approximately 3 μg/kg of body weight) (Table 2) [17–22]. This

level of intake was associated with a relatively small prevalence (approximately 10%) of folate inadequacy based on blood folate levels from small subsamples of the populations surveyed in the United States and United Kingdom and liver folate levels in a Canadian study [3,23,24]. This approach is based on the following two assumptions: (1) dietary folate intake estimates are accurate and (2) estimates of folate status of populations are valid. The quantity of folate consumed by the U.S. population was estimated based on intake data from the U.S. Department of Agriculture (USDA) Food Consumption Surveys [18–20]. The USDA database [25] for food folate content was used for the analysis of the Food Consumption Surveys [18–20] and was also the primary database for the Second National Health and Nutrition Examination Survey (NHANES II) [23]. Considerable evidence suggests that dietary folate intake was underestimated in these surveys for reasons outlined below.

Food intake in general is underestimated using survey methodology, as illustrated by the fact that in the Nationwide Food Consumption Survey, the reported calorie intake was 1500 kcal for adult women 19–25 years old compared to the recommended average intake of 2200 kcal for this age group [1]. The discrepancy between reported intake and energy requirement has been confirmed in metabolic balance studies conducted by Mertz et al. [26] in which human subjects could not be maintained in energy balance when they were given energy intakes comparable to reported intakes.

Inadequacy of food composition data should be considered before assuming that food intake estimates for folate accurately reflect quantities consumed [27,28]. There are many questions concerning food sample preparation and the microbiological methods used for the generation of current folate databases. Accuracy in

Table 2 Dietary Folate Estimated Intake in United States, Canada, and United Kingdom

Origin	Category, age (yr)	Mean folate intake (μg/day)	Ref.
United States	Men, 19–74	281	17
	Women, 19–74	207	
	Women, 19–50	189	
	Children, 1–5	185	18
	Men, 19–50	305	19
	Women, 19–50	193	20
	Children, 1–5	197	
Canada	Men	205	21
	Women	149	
United Kingdom	Men	300	22
	Women	209	

determination of food folate is dependent, in part, on the completeness of extraction of folates from the sample matrix, their stability during extraction, and the extent of digestion of polyglutamates by folate conjugase [29,30]. Common procedures fail to completely extract folates [29] or enzymatically deconjugate the polyglutamate form [30]. Gregory et al. [29] reported that the selection of a buffer of pH 7.85 and two extraction steps is preferred to prevent underestimation of folate content. For example, frozen green peas were reported to contain either 1.2 or 2.2 nmol/g when extracted with buffers differing in pH (7.0 or 7.85) and composition [29]. A commonly cited folate data source indicated the folate content of frozen green peas to be 1.2 nmol/g. Therefore, the folate content of foods reported in commonly used databases appears to be low, which results in underestimations of dietary folate intakes of population groups (Table 2). It is imperative that research efforts be devoted to updating food folate composition data utilizing newer analytical techniques. This effort alone would greatly enhance our ability to relate dietary folate intake to status assessment data.

The potential loss of folate during cooking and thermal processing should also be considered when evaluating dietary folate intake [31]. In addition to chemical degradation, folate lost during food preparation by leaching into cooking water may be responsible for large differences between the quantity of folate contained in the food prior to cooking and in the food actually consumed [31].

The approach of relating folate status of population groups to intake data is also based on the assumption that the estimate of folate status in the population is accurate. The estimate in the United States that 10% of the population has blood folate levels below accepted norms was based on data from NHANES II in which folate analyses were limited to a small subgroup of individuals [23]. Another complicating factor was that two different assay methods (microbiological and radioassay) were used to measure serum and RBC folate concentrations, and all of the data could not be merged [23]. Conclusions relative to the folate status of populations in Canada and the United Kingdom are also based on a limited number of studies [3,24]. Therefore, it is inappropriate to conclude that data from national population surveys are currently sufficient to estimate the extent of folate inadequacy for populations as a whole. When available, data from NHANES III will provide more definitive information relative to folate status in the United States [32].

D. Quantitation of Urinary Folate Excretory Products

Studies conducted in both humans and rats have confirmed that the major route of whole-body folate turnover is by catabolism to cleavage products primarily excreted in the urine [33–35]. Metabolism of intracellular polyglutamyl folate is accompanied by catabolic breakdown of the vitamin at the C9-N10 bond, and the resulting *p*-aminobenzoylpolyglutamates are hydrolyzed to the monoglutamate (pABG), which is *N*-acetylated (apABG) in the liver prior to excretion [33–35]. The amount of folate

utilized for daily metabolic processes can be estimated by quantitating the urinary catabolic products excreted in the urine and expressing the sum as "folate equivalents" as demonstrated by McPartlin et al. [33]. This quantity can then be converted to a dietary recommendation by correcting for bioavailability and individual variability [33]. The interpretation of urinary data regarding excretion of folate catabolites and their relation to folate requirements in future studies should take into account the amount of catabolites derived from degradation of food folate since degradation occurs, to a variable extent, due to food processing, storage, and preparation [31].

E. Kinetic Approach

The requirement for a nutrient can be defined as the percentage of the total body pool utilized daily to maintain normal metabolic processes [1]. Folate requirements based on estimations of body pool size and rates of utilization have been derived from tissue biopsy analyses and assumptions related to the rate of utilization [1]. For example, Gailani et al. [36] documented the daily loss of hepatic folate stores determined by liver biopsy when patients with advanced cancer were fed a diet devoid of folate. Extrahepatic stores were estimated to be approximately half those in the liver and were assumed to be lost at the same rate [1,36]. The quantity of folic acid required to replace that catabolized daily was then estimated to be 60 μg [1,36]. Three problems with extrapolation of these data to healthy individuals are as follows:

1. The rate of folate utilization in patients with advanced neoplastic disease may not be the same as normal healthy subjects.
2. Subjects fed a diet devoid of folate may conserve folate.
3. The rate of extrahepatic folate utilization may not be the same as for utilization of hepatic folate.

The standard kinetic approach to estimating nutrient requirements involves chronic administration of a labeled nutrient and then fitting the data relative to appearance and disappearance of the isotope to mathematical equations. For example, Krumdieck et al. [37] estimated the biological half-life of folic acid to be 101 days based on excretion kinetics following several doses of ^{14}C-labeled folic acid in a subject with Hodgkin's disease.

The use of stable-isotopic methods permits calculation of in vivo folate pool sizes and kinetic parameters with no radiation exposure to subjects [38]. Assuming uniform distribution of labeled folate, the mass of the in vivo pool can be calculated from isotope dilution principles following administration of a given labeled dose of the vitamin [38]. Under conditions of repeated long-term dosing (analogous to continuous infusion) approaching steady-state conditions, pool size is calculated as the input rate divided by the fractional catabolic rate [38]. Our research group has conducted studies to determine folate kinetics in adult male and female

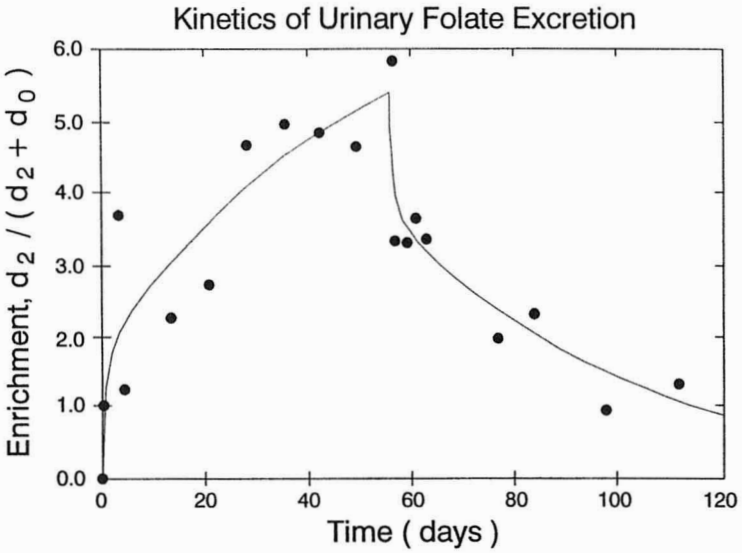

Figure 1 Stable isotopic enrichment of urinary folate during a metabolic study illustrating kinetic analysis of data consistent with a two-pool model [40].

subjects chronically supplemented with stable-isotopically labeled folates [38–40]. Preliminary kinetic analyses were consistent with a two-pool model: a minor rapid-turnover pool ($t_{1/2} < 1$ day) and a major slow-turnover pool ($t_{1/2} \cong 95$ days), as illustrated in Figure 1 [40]. Further refinement of this kinetic methodology, including measurement of additional pools, will provide the opportunity to estimate the fractional catabolic rate, characterize the size and half-lives of different body pools, and estimate rate constants for transfer between pools and excretion [38].

IV. REQUIREMENTS AND RECOMMENDED DIETARY INTAKES THROUGHOUT THE LIFE CYCLE

Physiological changes associated with the life cycle may increase folate requirements, as previously reviewed [27,42,43]. This is of particular concern during the periconceptional period and during pregnancy, when fetal growth and development may be compromised by folate inadequacy.

A. Pregnancy: Maternal Requirements for Optimal Fetal Growth

1. *Increased Folate Requirements During Pregnancy*

(*a*) *Prevalence of Inadequate Folate Status in Pregnant Population Groups.* Poor folate status is frequently associated with pregnancy [42,43] and documented in

economically deprived pregnant women in the United States [44–46]. The highest prevalence of low serum and RBC folate levels in NHANES II occurred among females (including pregnant women) aged 20–44 years, and impaired folate status was associated with increased parity in the small number of pregnant women surveyed [23].

(*b*) *Physiological Basis for Increased Folate Requirement.* Pregnancy is associated with a marked acceleration in cell multiplication due to uterine enlargement, expansion of blood volume, placental development, and fetal growth [47,48]. The quantity of folate required to adequately support these processes may be different at various gestational stages depending on many factors including large hormonal fluctuations and rates of cellular growth. Fetal folate blood concentrations appear to be maintained at maternal expense as evidenced by severalfold higher blood folate levels in newborns compared to maternal levels [49–51].

Several groups of investigators have reported increases in urinary folate excretion in pregnancy [8,52,53]. Glomerular filtration rate is increased during pregnancy accompanied by less efficient renal tubular reabsorption of nutrients [52]. It has been postulated that urinary folate loss contributes in part to the fall in serum folate, which begins before uterine, placental, or fetal growth can significantly increase the demand for folate [52].

The hypothesis that folate is absorbed less efficiently by pregnant women compared to nonpregnant women has been substantiated by data from some studies [8,54,55] but not others [8.56,57]. In our research group, Neuhaus et al. [58] found no differences in folate absorption in a study designed to evaluate the effect of pregnancy on folate absorption. Stable-isotopically labeled (deuterium) folate was used to compare the relative absorption of hexaglutamyl folate (d2-PteGlu$_6$) to folic acid (d4-PteGlu) determined at 16 weeks of gestation with the relative absorption at 32 weeks and in nonpregnant controls. In each absorption test, folate-saturated subjects were given an equimolar oral dose of d2-PteGlu$_6$ and d4-PteGlu, and isotopic labeling of urinary folates was determined by gas chromatography–mass spectrometry. The isotopic ratios of urinary folates excreted by the pregnant women were not different at 16 weeks compared to 32 weeks and did not differ from the ratios obtained in the nonpregnant controls [58].

(*c*) *Effect of Folate Deficiency During Pregnancy.* In malnourished impoverished human population groups, megaloblastic anemia during pregnancy is a common finding [5,8]. Folic acid supplementation and increased dietary folate intake during the periconceptional period have been shown to significantly reduce the risk of neural tube defects in numerous studies including observational as well as controlled-intervention protocols (see Chapter 12). Maintenance of prepregnancy folate status is of particular importance since approximately 50% of pregnancies in the United States are unplanned and the majority of women do not know they are pregnant during the first weeks of fetal development [59]. Folic acid supplementation has been associated with increased birth weight and reduced numbers of low-

birth-weight infants in folate-deficient women in Africa and India [43,60,61]. Goldenberg et al. [62] reported a positive association between folate intake, birth weight, and reduced risk of fetal growth retardation in the United States.

Adolescent pregnancy has been reported to be associated with a higher incidence of low-birth-weight infants than that observed in adult pregnancies [63]. Pregnant adolescents are especially at risk for folate deficiency due to the increased requirements for fetal and maternal tissue development coupled with normal adolescent growth [42,64].

2. *Estimates of Folate Requirements and Dietary Allowances During Pregnancy*

Controversy exists regarding the amount of folate required to meet the needs of the pregnant women and developing fetus [1,64,65]. The National Academy of Sciences Committee on Dietary Intake and Nutrient Supplements During Pregnancy did not have sufficient data on which to establish a specific supplementation recommendation for folic acid [64]. The Committee emphasized that the size of the folate body pool and equilibrium of the vitamin during pregnancy has not been assessed and that the lack of these data limits the ability to estimate folate requirements and make recommendations related to intake and/or supplementation during pregnancy [64]. Folic acid supplementation for pregnant women in general was not recommended by the Committee but was reserved for specific "high-risk" population groups, including pregnant adolescents [64].

The types of data used prior to 1993 as a basis for estimating folate requirements of pregnant women are illustrated by the following two studies. Chanarin et al. [66] reported that 100 μg/day of supplemental folic acid, in addition to dietary folate, met folate requirements based on maintenance of blood folate levels during pregnancy. In a second study, RBC folate response to supplementation in pregnant African women was cited as evidence that diets providing 300 μg/day were as effective in increasing blood folate levels as larger more bioavailable doses [67]. Based on these types of data, the RDA for pregnancy was reduced from 800 to 400 μg/day [1], which is similar to the RNI, DRV, and FAO/WHO recommendations for pregnancy (Table 1). Considering that the folate "allowance" for nonpregnant women was estimated to be approximately 300 μg/day by Sauberlich et al. [13,33], one may question whether an additional 100 μg/day of dietary folate is sufficient to cover the increased demands of pregnancy. Herbert [65] concluded that pregnant women should be advised to consume 500 μg/day of folate and that oral supplementation or food fortification appears desirable to maintain maternal stores and to keep pace with the increased folate demand in rapidly growing tissue.

More recently, McPartlin et al. [33] conducted a metabolic study to estimate folate requirements and dietary allowances for pregnant women. These workers quantitated folate breakdown products as a measure of folate requirements at three different stages of gestation and compared these data with those obtained during

the postpartum period as well as in nonpregnant control subjects. The mean total 24-hour excretion of pABG, apABG, and intact folate (expressed as folate equivalents) was significantly greater in the second and third trimesters than during the first trimester or postpartum period or in nonpregnant women (Fig. 2) [33].

The rationale for the method used to estimate folate requirements in the McPartlin et al. [33] study was that the 24-hour urinary concentration of folate catabolites reflects metabolic turnover of folate and is indicative of the requirement. The daily mean (±SD) folate requirement estimate in the nonpregnant group was 99 (±22) μg, and the corresponding value for the pregnant women was similar during the first trimester at 102 (±20) μg. Recommended allowances based on the addition of 2 SDs to the requirement estimates and correction for 50% bioavailability of dietary folate were 280, 660, and 470 μg/day for the first, second, and third trimesters, respectively [33]. McPartlin et al. [33] hypothesized that the increased requirement during the second trimester was due to increased DNA synthesis for rapid hyperplasia during this period. They speculated that the decrease in the rate of catabolism of folate from the second to the third trimester, despite increasing fetal-placental weight, may reflect a change in the nature of growth from hyperplasia to hypertrophy. The increase in folate equivalents excreted in the second and third

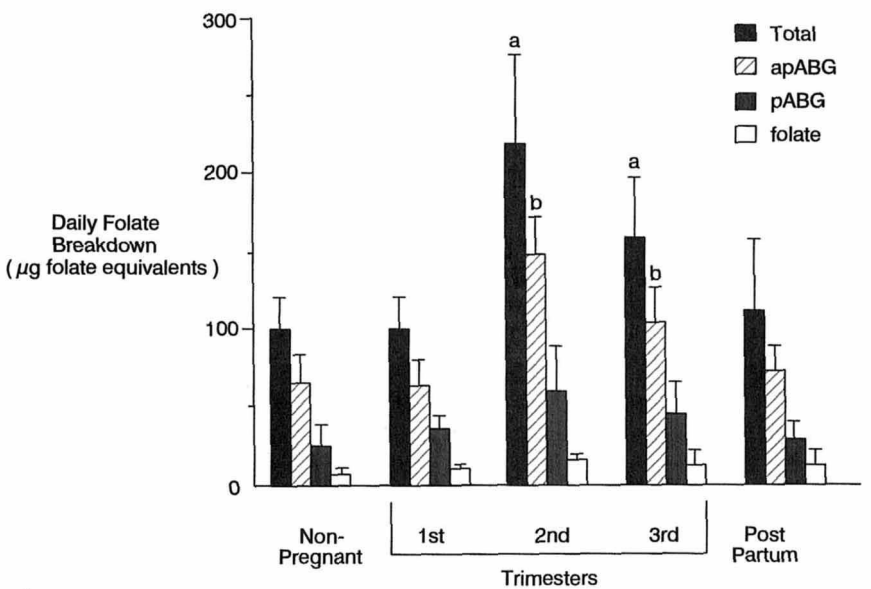

Figure 2 Urinary folate excretory products in pregnant women during the first, second, and third trimester and postpartum compared with that of nonpregnant controls. Bars with identical letters not significantly different. Bars without lettering are significantly different from those with letters [33].

trimester of about 200% and 150%, respectively, could not be attributed to an increased overall mass during pregnancy, since mean increases in mothers' weight in each of these periods were 5.8% and 12.4%, respectively. McPartlin et al. [33] concluded that the increased excretion of folate catabolic products was not due to changes in renal function. The basis for this conclusion was that glomerular filtration rate increases from about the eighth week of pregnancy and remains elevated until parturition [47], whereas folate catabolite excretion was greater in the second trimester and decreased significantly in the third before returning to normal after delivery [33]. The average estimated folate allowance based on these data [33] is higher than the current RDA [1], DRV [3], RNI [4], or WHO/FAO [5] dietary folate recommendations for pregnant women (Table 1).

B. Infancy, Childhood, and Adolescence

Blood folate levels of newborn infants are significantly higher than maternal blood levels, possibly reflecting an active transport process in utero [49–51]. These high blood folate values during early infancy decline to adult levels by 6 months of age, thus coinciding with the rapid rate of cell division and doubling of birth weight within the first 4–6 months of life [48].

A number of studies involving small numbers of malnourished infants supplemented with folic acid provided in formula or injections provided the basis of early estimates of infant folate requirements [8,68]. The amount of dietary folate recommended from birth to one year was reduced to 3.6 μg/kg (16–32 μg/day) from 5 μg/kg body weight in the Ninth Edition of the RDA [1] (Table 1). One study on which this change was based was a Lebanese estimation of the folate requirement in 2- to 6-month-old infants [69]. This study, upon which the FAO/WHO Safe Level of Intake [5] was also partially based, used growth, hematopoiesis, and clinical well-being as the criteria for adequacy of folic acid intake [69]. The blood folate levels of the infants fed 3.6 μg/kg maintained "borderline" serum and RBC folate blood levels that were normalized with intake levels of 5 μg/kg [68], suggesting that 3.6 μg/kg was inadequate to provide a "margin of safety."

The standards for dietary folate intake for the young infant (Table 1) are considerably lower than the amount of folate consumed by the exclusively breast-fed infant. This conclusion is based on current estimates of breast milk folate concentration, which approximates 100 μg/liter compared to previous estimates of 40–60 μg/liter [41] (see Chapter 6) and an average daily milk consumption of approximately 600 ml [1]. Even though the standards may underestimate the quantities consumed by the healthy breast-fed infant, intake is generally enough above the recommendations that a deficient intake is unlikely [70,71]. Breast-fed infants receive approximately 13–14 μg/kg body weight throughout the first 6 months, whereas formula-fed infants receive approximately 24 μg/kg in the first 6 months and 12–13 μg/kg in the second 6 months [70,71]. These differences in folate intake between

breast milk and formula significantly influence infant blood folate levels during the first 9 months of life (Fig. 3) [70]. The dramatic change in blood levels of formula-fed infants seen at 6–9 months of age reflects the displacement of the high-folate formula with solid food containing lower amounts of folate with possibly lower bioavailability [70,71]. The quantity of folate available to breast-fed infants appears to be sufficient to maintain normal blood folate levels throughout infancy (Fig. 3) [70,71].

Premature, low-birth-weight infants comprise a special group at risk of developing a folate deficiency partially explained by lower body stores at birth compared to full-term infants [8]. Shojania and Gross [72] showed that hematological signs of folate deficiency were evident within 2 weeks of age in low-birth-weight infants receiving 5 μg/kg body weight and that an additional 50 μg/day prevented development of deficiency signs. Dallman [73] has recommended folic acid supplements of 50 μg/day for well infants and 100 μg/day for sick infants weighing less than 2000 g at birth. A subcommittee of the American Society for Clinical Nutrition recommends daily parenteral folic acid intakes of 140 μg for full-term infants and 56 μg/kg for preterm infants [74].

Dietary folate recommendations for the adolescent have been set at levels comparable to those of mature adults (Table 1) since data from controlled metabolic studies are not available. It is recognized that adolescents undergo a rapid period of growth accompanied by a large increase in body mass, which may necessitate an increase in folate intake to support these changes [42,48,75–77]. A negative association between the stage of sexual maturation as an index of biological growth and folate status in adolescent males and females has been reported by Bailey et al. [78]. The RDA for children between 1 and 10 years of age is interpolated from the allowances for infants and adolescents since data for this age group are quite limited [1,8] (Table 1).

C. Adulthood

1. *Nonpregnant Women of Childbearing Age*

Three different research groups utilizing different methods have estimated folate requirements and allowances for nonpregnant women [13,15,33]. These methods include (1) "depletion-repletion" [13], (2) quantitation of urinary folate excretory products [33], and (3) maintenance of folate status in response to controlled intakes [15]. Sauberlich et al. [13] utilized the "depletion-repletion" protocol to estimate the minimum folate requirements to be 200–250 μg/day and 300 μg/day to be equivalent to an RDA level. McPartlin et al. [33] estimated the folate allowances for nonpregnant women based on urinary catabolite excretion to be 280 μg/day, which is similar to the estimate by Sauberlich et al. [13].

Our research group [15] conducted a comprehensive 70 day metabolic study with nonpregnant women to determine the response of folate status to different levels

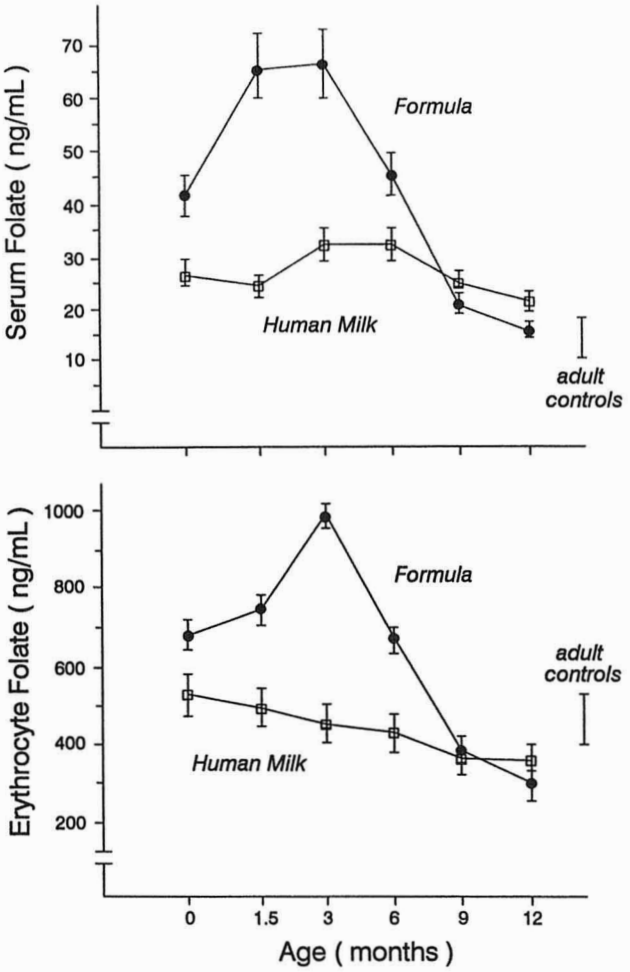

Figure 3 Mean serum and RBC folate levels of infants fed human milk (n = 14) or proprietary formula (n = 46) as the sole or primary source of nutrition during the first year of life. Values expressed as mean ±SEM [70].

of folate intake. Total intakes of 200, 300, or 400 μg folate/day were provided as conventional foods (30 μg/day) plus either 170, 270, or 370 μg/day of supplemental folic acid to permit precise control of folate intake. Consumption of either 200 or 300 μg/day resulted in significantly lower mean serum folate levels (2.9 ± 0.8 ng/ml; 3.3 ± 1.4 ng/ml, respectively) than 400 μg/day (7.0 ± 1.5 ng/ml) (Fig. 4). During this 10-week study period, serum and RBC folate decreased by 40% and

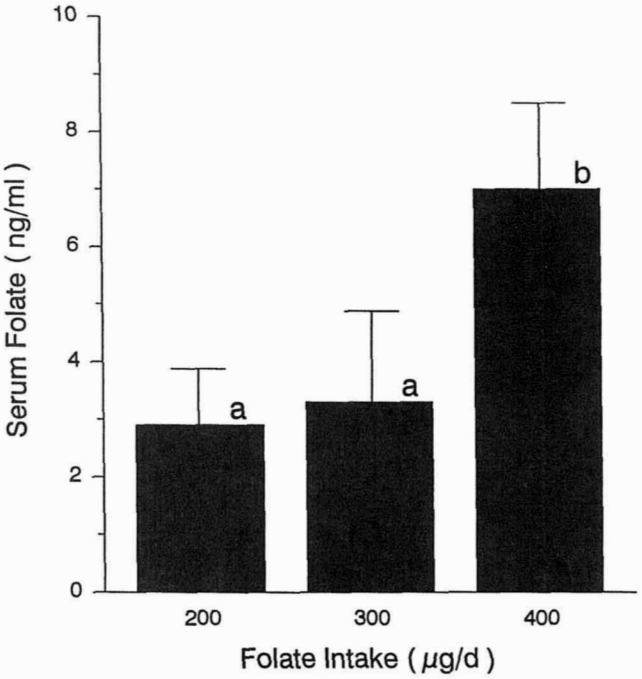

Figure 4 Mean ±SD serum folate (ng/ml) levels of three groups (n = 5) of nonpregnant women maintained on controlled dietary folate intakes of 200, 300, or 400 μg/day for 10 weeks.

20%, respectively, in the group consuming 200 μg/day compared to a 27% and 7% respective increase in the group consuming 400 μg/day (Fig. 5). Daily ingestion of 400 μg of dietary folate was adequate to maintain serum folate levels above accepted norms (>3 ng/ml) throughout the study in contrast to lower levels of intake. Plasma homocysteine levels were significantly higher in the group consuming 200 μg/day compared to that observed in either the 300 μg/day or 400 μg/day groups (Fig. 6). Plasma homocysteine levels were negatively correlated with serum, RBC, and urinary folate levels in the 200 μg/day group. The data suggest that the intake of 200 μg of dietary folate per day may be inadequate to maintain folate status in nonpregnant women and that 400 μg/day appears to more closely fulfill the criteria of a dietary allowance.

2. *Young Adult Men*

There are few data on which to base the folate requirements of adult men. Milne et al. [16] evaluated changes in folate blood levels in adult men fed an average of 200 μg folate/day. This study was not designed to estimate folate requirements, but the study is important in that folate intake was controlled for 6 months and changes

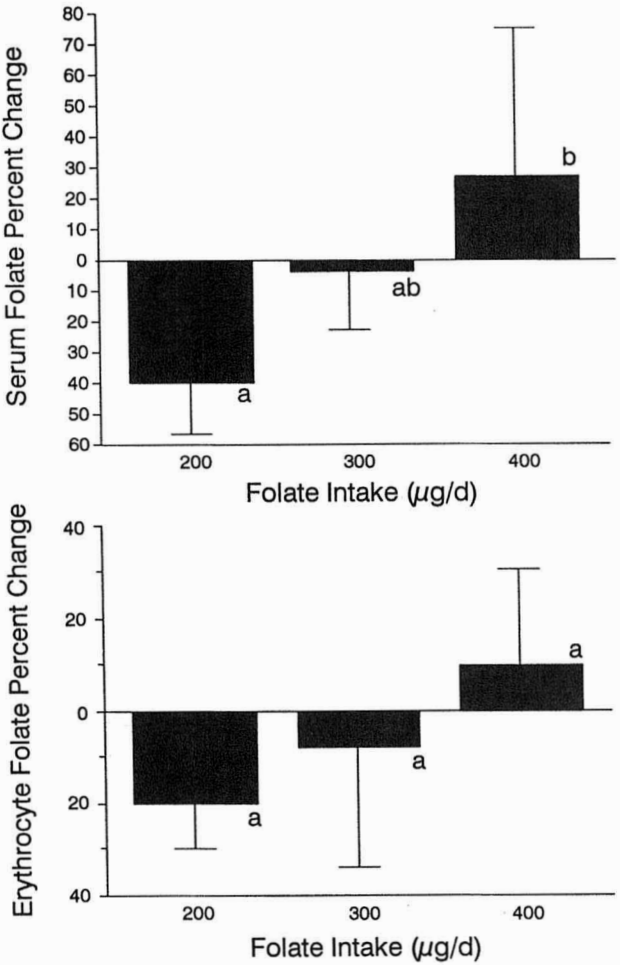

Figure 5 Mean ± SD percentage change in serum and RBC folate levels in response to either 200, 300, or 400 μg dietary folate/d for 70 days in three groups (n=5) of nonpregnant women. Identical letters indicate no significant difference [15].

in blood folate levels were monitored monthly. Reductions in both serum and RBC folate levels were significant and continuous throughout the 6-month period, suggesting that 200 μg/day of dietary folate was inadequate to allow for the "margin of safety" inherent in RDAs.

Jacob et al. (98) recently completed a controlled metabolic "depletion-repletion" study in adult men designed to assess the adequacy of the current RDA. One objective of the study was to evaluate plasma total homocysteine as an index of "functional" folate deficiency in response to graded intakes of dietary folate. Feeding

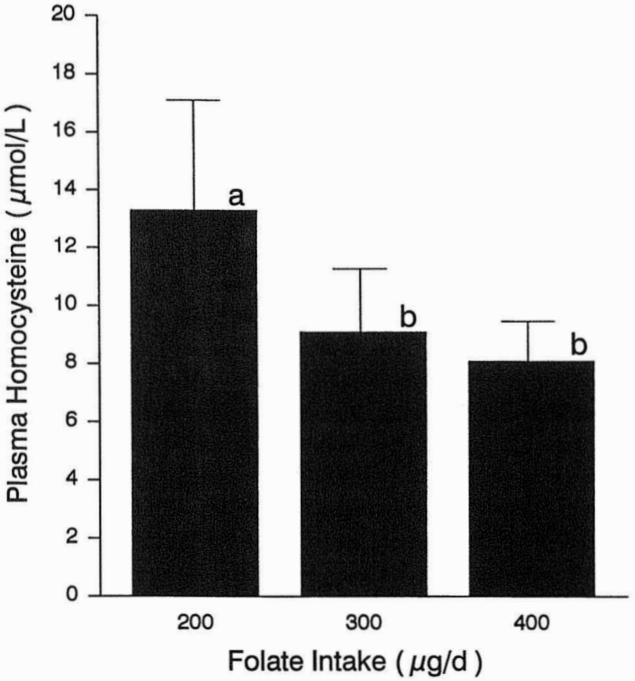

Figure 6 Mean ± SD plasma homocysteine (μmol/L) levels in three groups (n=5) of nonpregnant women in response to either 200, 300, or 400 μg dietary folate/d for 70 days. Identical letters indicate no significant difference [16].

of a low folate diet (~25 μg/day) for 30 days produced a moderate folate deficiency which increased plasma homocysteine levels significantly, but produced no hematologic symptoms. The lack of normalization of the high plasma homocysteine concentrations (>16 μM) with repletion at 84% of the current RDA led to the conclusion that the current RDA of 200 μg/day for adult men may not provide a large enough margin of safety to allow for periods of decreased folate intake, increased turnover, or individual variance in metabolism.

3. *Elderly Men and Women*

Data are insufficient to conclude that folate requirements and allowances for the elderly population are different from those for younger age groups due to the physiological effects of aging [42]. There are a number of potential age-related effects on folate utilization due to changes in organ systems, including those in the gastrointestinal tract [79,80,81]. Baker et al. [79] reported that the rise in serum folate following the oral ingestion of large pharmacological quantities of polyglutamyl folate supplied in yeast was significantly less in aged individuals than

that observed in young controls. The question of whether age affects folate absorption was investigated further using physiological quantities of radiolabeled folate [80]. In this study, Bailey et al. compared the absorption of polyglutamyl and monoglutamyl folates in aged and young adult male subjects. Luminal disappearance of both ^{3}HPteGlu and ^{14}CPteGlu$_{7}$ and the urinary recovery of each isotope as well as jejunal mucosal folate conjugase activities were compared. No age-related differences were found in luminal disappearance or urinary excretion of either isotope or in folate conjugase activity [80].

Environmental factors including chronic cigarette, drug, and alcohol use may have a negative impact on folate requirements of aged individuals [12,27,42,81] (see also Chapters 7 and 11). A large percentage of healthy aged individuals take numerous drugs daily including both over-the-counter and prescription medications [82,83]. The potential cumulative effect of these factors on folate utilization should be taken into account when estimating folate requirements of elderly individuals [42].

Dietary folate recommendations for elderly individuals are based on very few population-based studies since large-scale surveys generally have not included representative samples of the aged population [23,42]. Studies of elderly population subgroups may be limited in scope due to small sample size and inaccuracy of reported intake based on dietary recall. For example, one study cited in the 1989 RDA included only 21 elderly subjects with a reported intake of 135 μg/day based on dietary recall [84]. Even though this reported level of intake failed to maintain RBC folate levels of >150 ng/ml in approximately 50% of this group, these data were used to justify lowering the RDA from 400 μg/day to 180 and 200 μg/day for elderly females and males, respectively [1,84]. When available, data from NHANES III will provide folate status assessment data for a small but representative number of elderly individuals [32].

In future studies, the definition of "elderly" may need to be redefined and include several age categories over 65 years of age since life expectancy and the percentage of the population defined as "aged" has increased significantly [85]. When defining requirements of elderly individuals, it is important to recognize that aging varies between individuals so that at specific chronological ages, individuals exhibit differences in aging between tissues and organ systems [82].

Maintenance of adequate folate intake in aged individuals may also be one positive factor in reducing the risk of specific chronic diseases including coronary heart disease [86,87] (see Chapter 4) and cancer [88] (see Chapter 13).

V. DIETARY FOLATE INTAKE

Table 3 includes the folate content of a wide range of foods including those that are concentrated sources [89]. The specific quantities of folate in many of the food items included in Table 3 need to be validated using updated methodology, however, the relative quantities of folate provided by specific food items can be compared.

Table 3 Folate Content (μg) of Selected Foods

FOOD (average serving size)	μg/average serving	μg/100 g
FRUIT		
Orange juice, ready-to-drink (249g; 1C)	109	44
Orange (131g; 1 med)	40	30
Grapefruit juice, ready-to-drink (247g; 1C)	26	10
Grapefruit (146g; ½ med)	15	10
Apple juice (248g; 1C)	0	0
Apple, w/skin (138g; 1 med)	4	3
Banana (114g; 1 med)	22	19
Tomato juice (243g; 1C)	48	20
Tomato, raw (62g; ½ med)	9	15
Avocado (87g; ½ med)	54	62
Grapes (160g; 1C)	6	4
Cantaloupe (231g; ¼ med)	39	17
Strawberries, fresh (149g; 1C)	26	18
VEGETABLES		
Broccoli, raw (36g; ½C)	31	71
Broccoli, cooked (92g; ½C)	52	56
Spinach, raw (56g; 1C)	108	194
Spinach, cooked (95g; ½C)	102	107
Lettuce, romain (30g; 1C)	41	136
Lettuce, iceberg (55g; 1C)	31	56
Mustard greens, cooked (75g; ½C)	88	118
Turnip greens, cooked (72g; ½C)	85	118
Peas, green, cooked (80g; ½C)	47	59
Cauliflower, cooked (90g; ½C)	37	41
Squash, zucchini, cooked (90g; ½C)	15	17
Potato, Idaho, baked, skin (122g; 1 med)	11	9
Coleslaw (60g; ½C)	21	35
Corn, cooked (82g; ½C)	19	23
Asparagus, cooked (75g; 5 spears)	101	135
Carrots, cooked (78g; ½C)	11	14
Beans, green string, cooked (68g; ½C)	5	8
CEREAL, BREAD AND LEGUMES		
Ready-to-eat, superfortified, (28g; 1C)	395	1411
Ready-to-eat, moderately fortified, (23g; 1C)	80	350
Oatmeal, instant, cooked (234g; 1C)	161	69
Bread, white (25g; 1 sl)	9	35
Bread, whole wheat (29g; 1 sl)	16	55
Rice, white, cooked (205g; 1C)	6	3
Noodles, white, cooked (160g; 1C)	11	7
Grits, cooked (242g; 1C)	2	1
Beans, pinto, cooked (86g; ½C)	147	172
Beans, navy, cooked (91g; ½C)	127	140
Peanuts, raw, dried (73g; ½C)	92	126
Peanut butter (32g; ⅛C)	25	78

Table 3 Continued

FOOD (average serving size)	μg/average serving	μg/100 g
MEAT, EGGS, MILK, YOGURT		
Chicken breast, w/o skin (98g)	5	5
Chicken, leg or thigh, w/o skin (62g)	5	8
Chicken liver (70g)	539	770
Beef steak (85g)	10	12
Beef, ground, 25% fat (76g)	7	9
Beef liver (85g)	184	217
Pork chop (85g)	5	6
Ham (85g)	5	6
Bacon (113g; 2 sl)	0.6	5
Milk, whole (244g; 1C)	12	5
Yogurt, plain (245g; 1C)	17	7
Egg (50g; 1 Lg)	23	47

Source: Ref. 89

These food composition data illustrate that dietary intake advice needs to be quite specific due to the wide variation of folate within each food group. For example, 8 ounces (249 g) of orange juice contains approximately 100 μg of folate as compared to negligible quantities in the same volume of apple juice. Green leafy vegetables are excellent sources of folate, especially when cooking losses are minimized (Table 3). Whole wheat bread is a more concentrated source of folate than other types of bread and bread products. Other food items that are nutrient-dense sources of folate include asparagus, dried beans (i.e., pinto, navy), and peanuts. Meat is not a concentrated source of folate, with the exception of liver. In addition to these naturally occurring sources of folate, ready-to-eat breakfast cereals contribute significantly to folate intake in the United States since the majority of breakfast cereals in the U.S. marketplace contain approximately 100 μg/serving and a small number of products are "superfortified" with 400 μg/serving.

When assessing dietary folate intake and providing dietary advice, it is important to consider frequency of consumption of specific foods in addition to folate content. Some of the concentrated food sources of folate included in Table 3 are also major contributors of folate in the U.S. diet based on frequency of consumption (Table 4) [17]. For example, orange juice ranks number one as a contributor of folate to the U.S. diet because it is a popular component of the daily diet in contrast to green leafy vegetables such as spinach and turnip greens, which are less frequently consumed [17].

Table 4 Select Contributors of Folate in the U.S. Diet According to NHANES II Data, 1976–80

Ranking	Description	Percent of total folate
1	Orange juice	9.7
2	White bread, rolls, crackers	8.6
3	Pinto, navy, other dried beans	7.1
4	Green salad	6.8
5	Cold cereals (not superfortified or bran)	5.0
6	Eggs	4.6
9	Liver	3.1
10	Superfortified cereals	3.1
23	Hamburgers	1.2
25	Spinach	1.0
30	Green beans	0.8
34	Broccoli	0.7
36	Turnip greens	0.6

Source: Ref. 17.

VI. EXCESSIVE FOLATE INTAKES AND NUTRIENT/DRUG INTERACTIONS

Folic acid has been considered "nontoxic" not only in small supplemental doses but also quantities up to several hundredfold the requirement [90]. However, questions have been raised [91] related to potential negative effects of "excessive" supplemental folic acid intakes, including (1) the potential for folic acid to interfere with the efficacy of specific drugs, including anticonvulsants, and those that function as antifolates, (2) a negative influence on zinc utilization, and (3) interference with diagnosis of vitamin B_{12} deficiency.

Reports of an increase in seizure frequency in response to supplemental folic acid in the range of 5–30 mg/day have been noted in several isolated cases [92,93], however, no such effects were found in controlled studies including double-blind, crossover studies utilizing 15–20 mg folic acid per day in patients with drug-treated epilepsy (94,95). More recently, several studies of epileptic patients treated with diphenylhydantoin (Dilantin®) and given 3–5 mg folic acid daily for gingival hyperplasia for periods of 4 months to 1 year indicated no change in seizure frequency [96,97].

Chapter 15 includes an in-depth review of studies in which the potential interference of folate with the efficacy and/or increased toxicity of antifolate drugs used chronically in the treatment of nonneoplastic diseases was investigated. The primary conclusion from these data was that folic acid supplementation can safely

be combined with these medications with no significant reduction in drug efficacy or increase in negative side effects.

In the area of potential negative nutrient interactions, the potential for supplemental folic acid to interfere with zinc utilization has been the focus of a large number of research investigations (see Chapter 10). The majority of scientific evidence does not support the conclusion that supplemental folic acid will impair zinc status.

The primary area of concern related to "excessive" folic acid intake is the prevention of megaloblastic anemia associated with most but not all cases of vitamin B_{12} deficiency. This "masking" of anemia interferes with the diagnosis of a vitamin B_{12} deficiency and may result in severe and irreversible nerve damage if untreated. Experimental data related to this issue are extensively reviewed in Chapter 9. The Food and Drug Administration (FDA) considered this issue when estimating the safe upper limit for a proposal to fortify staple foods with folic acid [91]. The tentative conclusion of FDA with respect to individuals in the population with vitamin B_{12}-related problems was that safety issues could be resolved by setting a "safe upper limit" of intake of 1 mg of folate per day from all sources [91].

VII. SUMMARY AND CONCLUSIONS

Dietary folate intake recommendations are quite similar worldwide and are based on data obtained utilizing a diversity of experimental approaches, as reviewed in this chapter. Folate requirements and extrapolation to dietary intake recommendations can best be estimated by comparing and contrasting data obtained using diverse methodologies.

Dietary folate intake has been underestimated due to inaccuracies in nutrient composition in addition to underreporting, therefore, population groups are consuming more dietary folate than previously assumed. Reanalysis of food folate content with updated methodology will enhance the validity of relating folate intake data to estimates of folate status in population groups.

The observation that normal folate status cannot be maintained in nonpregnant women and adult men consuming recommended intakes in controlled studies suggest the need to reevaluate these recommendations. Folate requirements of the pregnant women appear to vary depending on the phase of gestation, and data suggest that requirements are higher during the second trimester than in other trimesters and in nonpregnant women. Major gaps in scientific knowledge related to folate requirements include estimation of folate requirements of children and adolescents as well as elderly individuals.

Dietary recommendations to increase consumption of folate-dense foods including green leafy vegetables and citrus fruit, if adhered to, will enhance folate intake as well as other nutrients with potential health benefits. Negative interactions with other nutrients or specific drugs are not issues of major concern in the gen-

eral population, with the exception of the potential for "excess" folic acid to interfere with the diagnosis of a vitamin B_{12} deficiency.

In conclusion, the availability of new data relative to folate requirements provides the opportunity to modify and improve dietary folate intake recommendations. The direction of the future appears to be a broader definition of dietary intake standards ranging from those consistent with minimum requirements to maximum intake levels.

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6

Folate Nutrition in Lactation

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I. INTRODUCTION

The benefits of human milk feeding have been emphasized by a number of U.S. governmental agencies [1–3] and health-professional organizations [4–6] over the past several years, recognizing that human milk not only provides nutrients in a highly bioavailable form but also enzymes, immunoglobulins, antiinfective and antiinflammatory substances, hormones, and growth factor [7–9]. These endorsements are reflected in *Healthy People 2000: National Health Promotion and Disease Prevention Objectives*, with a current health objective for the nation being to "increase to at least 75% the proportion of mothers who breast-feed their babies in the postpartum period and to at least 50% the proportion who continue breast-feeding until their babies are 5 to 6 months old" [10]. Human milk is the preferred food for the term infant, providing that the maternal diet is adequate and the quantity supplied is sufficient.

The nutritional cost for folate during lactation is directly related to the quantity of milk produced and its folate content. Thus, knowledge of the volume of milk produced or ingested by infants and its folate content is essential for an evaluation of maternal nutritional needs for this vitamin during lactation and/or an assessment of folate adequacy of human milk–fed infants. There is amazing consistency among reported values for the average volume of human milk ingested by apparently healthy breast-fed infants from both industrialized and developing countries of the world when appropriate methodology is applied to obtain data [11–12]. Typical milk volumes are 750–800 ml/day during established lactation, with values ranging from <400 to >1000 ml/day. This information, along with the average folate content of human milk, has traditionally been used to assess the maternal nutritional bur-

den for folate during lactation and has served as the basis for recommended levels of folate for the lactating woman.

The vitamin content of human milk is known to vary as a function of maternal dietary intake, but not always in a linear fashion. Maternal folate intake relates to human milk folate content most closely when maternal stores of this vitamin are low or absent. Milk folate secretion can and does diminish during maternal folate deficiency, and folate deficiency occurs in human milk–fed infants. However, maternal dietary folate adequacy cannot be assumed when folate secretion in milk is sufficient to maintain folate status of her nursing infant, because this is often accomplished by depletion or exhaustion of maternal folate. In this chapter, the influence of maternal folate adequacy on human milk folate content as well as the status of the nursing infant and the lactating woman will be reviewed.

II. HUMAN MILK FOLATE CONTENT

The wide range of mean values reported for the activity of folate in human milk from apparently well-nourished women undoubtedly reflects analytical difficulties rather than genetic differences or nutritional status of donors. Complete description of analytical methods, sampling and storage procedures, and folate status of donors are not always furnished in published reports, however, a few generalizations can be made. Folate in human milk is present in considerably greater amounts than reported by early investigators, a sizable fraction exists as pteroylpolyglutamates, and it is quantitatively bound to folate-binding proteins.

Early investigators significantly underestimated human milk folate content [13] because assays were made in the absence of ascorbate (to protect labile folates from oxidation), without pretreatments with a γ-glutamylhydrolase hydrolase (which cleaves long-chained folylpolyglutamates) and heat (for release from binding proteins), and/or with microorganisms that do not respond to all forms of folate in samples. A review of the current literature provides a greater than sixfold difference in reported mean values (22–141 μg folate/liter) [14–25]. Some investigators failed to detect a significant fraction of milk folate pteroylglutamates [17,21,23].

In a recent investigation, certain aspects of the microbiological protocol were evaluated for the detection of possible sources of error in the measurement of human milk folate activity [26]. Milk samples (50 ml) were collected from donors at 6 and 12 weeks of lactation. Samples were divided, immediately frozen in liquid nitrogen, and stored at −70°C until assay. Folate analyses were performed on aliquots of samples in two laboratories independently. The activity of human milk folate hydrolase was approximately 0.4 nmol ^{14}C-glutamic acid released/ml/min. This amount of endogenous hydrolase is roughly equivalent to one-twentieth of plasma activity, not sufficient to autolyze endogenous pteroylpolyglutamates in human milk, indicating that autolysis cannot account for the inability of some investigators to detect pteroylpolyglutamates in human milk samples. The mean folate content of

the samples analyzed in laboratory I was 100 ± 30 μg/liter (226 ± 68 nmol/liter) and in laboratory II was 110 ± 25 μg/liter (249 ± nmol/liter). The mean increase in milk folate content following treatment with exogenous hydrolase was 23 and 32% in respective laboratories (see Fig. 1), demonstrating that a significant fraction of human milk folates does exist as pteroylpolyglutamates with four or more glutamic acid residues [27]. Results from this study also demonstrated that heat treatment (121°C for 5 minutes) of milk prior to assay markedly increased (190%) the measurable folate content. Heat treatment of milk is known to destroy milk folate-binding proteins [28,29] and to release folate. Elimination of heat treatment or substitution of this step, for example, by filtration sterilization, can lead to serious underestimates of the folate content of human milk. The application of differential microbiological assays to the study of human milk folates indicates that methyl forms of folate are present (21–40%) but are not the sole variant in human milk [26,30]. Since circulating folate is principally 5-methyl-tetrahydropteroylmonoglutamate, the presence of a variety of folate forms in human milk notably both methylated and nonmethylated pteroylpolyglutamates implies that mammary epithelial cells are capable of extensive folate metabolism.

A positive relationship ($r = 0.71$) exists between the concentration of milk folate-binding proteins and folate activity [31]. The equation expressing this relationship is $y = 29.2 + 0.794x$, where y and x represent activity of folate-binding proteins and folates, respectively. The statistics of this relationship suggest that the

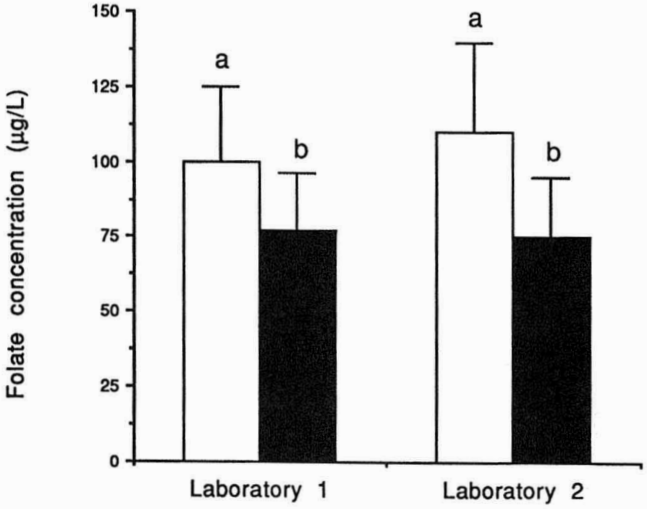

Figure 1 The folate content (μg/liter) of human milk samples with (open bars) and without (blackened bars) folate-hydrolase treatment, as measured by two independent laboratories. Different letters represent statistically significant differences ($p < 0.05$). (From Ref. 26.)

activity of folate-binding proteins is approximately 29 ng in excess of folate-binding capacity. This quantitative relationship (Fig. 2) is unique to milk. In other tissues, only a small fraction of total folate content exists in association with folate-binding proteins. The strong positive relationship between activity of human milk folate and folate-binding proteins indicates that their secretion is coordinated. Since the transport of folate from plasma to milk occurs against a steep concentration gradient (2- to 14-fold), it has been suggested that folate-binding proteins within the mammary epithelial cell serve the purpose of trapping folate for milk secretion [32]. However, the presence of many variants of folate in human milk, particularly the folylpolyglutamates, argues against this possibility. The complex of folate with milk folate-binding proteins is too stable ($K_d = 10^{-9}$ to 10^{-11} nmol/liter) to allow any significant enzymatic changes of the folate substrate [33]. When folate is complexed with its binding proteins, reduction by dihydrofolate reductase is inhibited. Additionally the presence of folate-binding proteins is not necessary to obtain higher folate concentrations in milk than in serum. The concentration of biliary folate is about 10 times that of serum folate, and evidence for folate-binding proteins in bile is lacking. The significance of folate-binding proteins in milk is unknown, but available evidence supports that they are required for folate secretion possibly via exocytosis as with other milk proteins. The presence of folate as protein-bound species in milk may enhance folate bioavailability to infants [34].

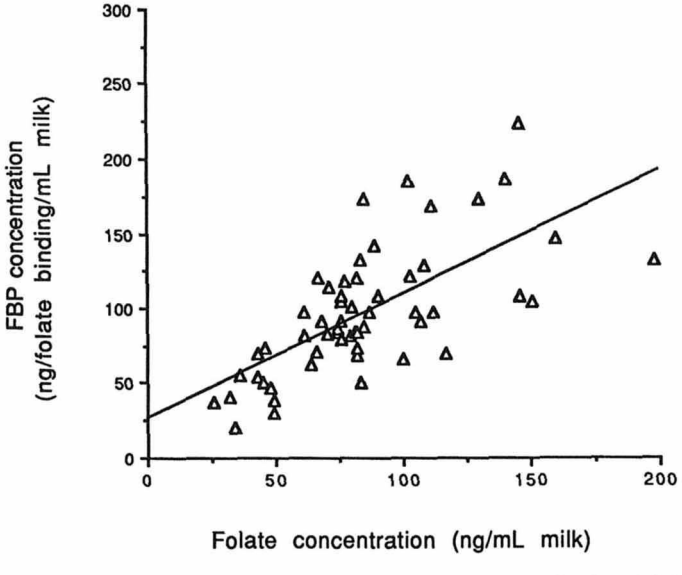

Figure 2 Relationship between folate concentration (ng/mL) and folate-binding proteins (ng folate binding/ml) in human milk samples. (Adapted from Ref. 31.)

Sampling techniques may influence the measurement of human milk folate. Results from longitudinal studies indicate that human milk folate concentrations increase as lactation advances [17,30,35,36]. Ek [17] noted that during weaning, the concentration of milk folate declines. At each stage of lactation, time of sample procurement at a feeding and during the day may also influence the measurement of human milk folate activity. Smith et al. [36] observed higher amounts of folate in hind-milk than in fore-milk and in samples collected in the evening compared to earlier in the day. These patterns of variation may account for a relatively minor amount of the variance in reported value for milk folate content.

III. MATERNAL FOLATE STATUS AND MILK FOLATE CONTENT

The quantity of folate needed to sustain lactation is not known with any degree of certainty. Most of the knowledge about the folate utilization by lactating women has been obtained indirectly from the approximate amount of folate secreted in milk plus the estimated maintenance requirement of the mother, which is assumed to be similar to that of nonreproducing females. Folate nutritional status of the lactating woman is usually presumed to be adequate if her infant is growing well and the folate content of her milk is within an acceptable range. Functional and/or health outcomes of lactating women have not been considered.

There is evidence that apparently healthy lactating women from affluent populations become folate depleted. In Sweden, Quist et al. [37] reported that 10% of mothers at term had low erythrocyte folate values (<66 μg/liter), and at 9 weeks postpartum the incidence was 33% among these same women during early lactation. In Finland, 3–5% of lactating women (n = 200) receiving 100 μg of supplemental folic acid per day exhibited low plasma folate values (<3 μg/liter), yet none of their exclusively breast-fed infants had inadequate concentrations from birth to 12 months of age [38]. In these Scandinavian studies, neither maternal folate intake nor human milk folate content was evaluated.

The preferential partitioning of folate to actively secreting mammary tissue was first described by Metz [39]. This investigator studied two lactating subjects with megaloblastic anemia and measured serum and human milk folate concentrations. Initial serum and milk folate contents were <5 μg/liter. One subject was given 100 μg of folic acid, while the other was given 200 μg per day. After 4 days, the milk folate concentration rose appreciably from 5 to 60 μg/liter. Serum folate levels remained low during the 18 days of the study in spite of persistently elevated human milk folate content. In the subject receiving 100 μg of folic acid per day no reticulocyte response was noted, and in the subject receiving 200 μg folic acid per day reticulocytosis was not evident until day 10 of the study. These findings indicate that supplemental folate is taken up by mammary epithelial cells preferentially over hemopoietic cells in lactating women with folate deficiency. Copperman et al. [21] noted a similar rise in milk folate content from approximately 6–9 to 45

μg/liter in a folate-deficient lactating woman following 3 days of supplementation with 5 mg of folic acid. However, when maternal blood folate values are within the acceptable range, oral folic acid supplements elevate plasma and erythrocyte folates but not milk folate [14,15]. Maternal folate status, even when judged to be adequate, deteriorates both in early and late lactation as evidenced by declines in plasma and erythrocyte folate, but milk folate concentration either stabilizes or increases [14,16,17]. Therefore, it appears that maternal reserves are depleted to maintain human milk folate content and some regulatory mechanism appears to control milk folate secretion since milk folate levels decline only in overt deficiency.

The high nutritional demand for folate during lactation is most dramatically illustrated in animal studies. The production of folate deficiency in the growing rat requires dietary supplementation with a sulfa drug such as succinylsulfathiazole to eliminate folate-producing gut flora in addition to a folate-free diet. A diet providing 200 μg folate /kg maintained pregnancy in the rat, but feeding of this diet during lactation resulted in 13% neonatal mortality [40]. Furthermore, a 200 μg folate/kg diet plus succinylsulfathiazole supported pregnancy. At parturition, newborns were indistinguishable from control animals as determined by birth weight. However, the continuation of this same diet through lactation resulted in 100% mortality by 5 days [41]. Nelson and Evans [41] also noted that maternal rats fed folate-deficient diets showed dramatic improvement in folate status following lactation. Thenen [42] produced three levels of folate deficiency in reproducing rats and likewise noted high neonatal mortality among pups born to the most severely deficient dams. This investigator extended her studies on experimental maternal folate deficiency to the new world primate, *Cebus albifrons* [43]. Pregnant monkeys fed low-folate diets exhibited progressively worsening features of folate deficiency throughout pregnancy and lactation including a reduction in milk folate. Notably, birth weight of infant monkeys from these mothers was indistinguishable from control infants, as were their plasma and erythrocyte folates. These findings in new world monkeys are remarkably similar to those from human studies. Since liver folates were dramatically reduced in infant monkeys from deficient mothers, these findings cast serious doubt on the common interpretation of human data that suckling infants are spared during maternal folate deficiency.

Studies with two animal species point to the possibility that dietary factors other than maternal folate intake may influence milk folate content [44]. Depressed milk folate secretion is an early manifestation of maternal iron deficiency [45], since mean milk folate content was depressed by 81% at day 18 of lactation in iron-deficient rat dams (Fig. 3). Iron-deficient rat dams not only secrete milk with less folate, they secrete milk with a substantially reduced percentage of long-chain folylpolyglutamates. Provision of additional dietary folate does not correct the defect in milk folate secretion during maternal iron deficiency. Although the mechanism is unknown, the defect is localized in the mammary tissue [46]. If and to what extent maternal iron deficiency affects milk folate content of lactating women has not been

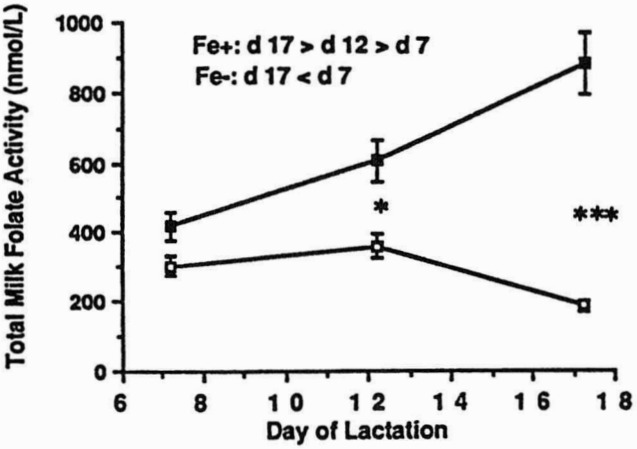

Figure 3 Mean total folate content from iron-sufficient (blackened squares) and iron-deficient (open squares) dams on days 7, 12, and 17 of lactation. Milk folate concentrations at each time point represents mean ± SEM of seven determinations. $^{*}p < 0.05$; $^{***}p < 0.001$. (Adapted from Ref. 45.)

investigated. However, iron and folate deficiencies are reported to coexist in reproducing women [44].

IV. DIETARY FOLATE INTAKE AND NUTRITIONAL STATUS OF LACTATING WOMEN

The Subcommittee on Nutrition During Lactation of the National Academy of Sciences recently reviewed available dietary data on lactating women in the United States published since 1976 [47]. The lack of available information is illustrated by the fact that only a total of 446 lactating women were studied. Only one study provided nationally representative data on lactating women ($n = 85$), while 16 studies provided data from nonrepresentative samples on only a total of 361 presumably well-nourished women, nearly all of these being well-educated Caucasians. Evaluation of the diets of the 85 lactating women from a nationally representative sample (USDA's 1977–78 Nationwide Food Consumption Survey) was performed by Krebs-Smith and Clark [48], who calculated a dietary score as well as nutrient adequacy ratios for selected nutrients. These estimates were then used to calculate two mean adequacy ratios: one for overall nutrient adequacy, the other representing "problem nutrients" (calcium, iron, magnesium, and vitamins A and C). In this analysis, only 19% of the lactating women studied had both high nutrient adequacy ratios and high mean adequacy ratios. Folate intake was not calculated in that study.

The subcommittee was able to locate only three studies in which dietary folate intakes of a total of 36 lactating women were tabulated: 6 "well-nourished" adult women from 4-day dietary records at month 6 of lactation [18]; 23 Navajo mothers from 24-hour recalls and 2-day records at unspecified times of lactation [49]; and 7 low-income women from 4-day records at days 5–7 and 43–45 of lactation [19]. Mean calculated folate intakes (μg/day) from these studies were 194 ± 139, 169 ± 139, 290 ± 100, and 340 ± 200, respectively. In two of these studies [18,49], mean folate intakes fell considerably below the current Recommended Dietary Allowance (RDA) of 280 μg/day [50], while mean intakes of low-income women exceeded the RDA. These scant data provide little insight into customary folate intakes of lactating women in the United States.

National data on dietary intakes of women in general are the best available for assessing whether or not folate intake is likely to be low in diets of women during lactation. This approach is based on the assumption that diets of women remain static during lactation. There are reasons to suspect that dietary practices may change due to advice from health care professionals, cultural beliefs, etc., however, when folate intakes are low for nonpregnant, nonlactating women, it also is likely that they will be low during lactation. The folate density of diets of U.S. women aged 19–50 was 137 μg/1000 kcal in the Continuing Survey of Food Intake by Individuals, Wave 1 [51]. At the recommended energy intake of 2700 kcal, folate intake of lactating women would be predicted to be 370 μg/day and at an energy intake of 2200 kcal, the value typically reported for lactating women, to be 301 μg/day [47].

These predicted intakes are remarkably close to the RDA for lactating women of 280 μg/day but the RDA for non-reproducing women of 180 μg/day represents a serious underestimation of folate requirements of women (see Chapter 5). Results of a study designed to determine the folate needs of women indicate that 300 μg/day is a more appropriate recommended intake [52]. At the predicted levels of intake, the increased requirement for the amount of folate secreted in milk is not likely to be met. Thus, it appears that U.S. lactating women are a population subgroup at risk for development of folate depletion. Nationally representative data on folate status of U.S. lactating women are not available for assessment of this possibility. However, support is provided from the analysis of laboratory folate data in a subsample of 10% of the adults from the Second National Health and Examination Survey (NHANES II) in which women aged 20–44 years were the group with the highest incidence of low folate values; 15% with plasma values <3.0 μg/liter and 13% with erythrocyte values <140 μg/liter [53]. Similarly, results from the Hispanic Health and Nutrition Examination Surveys (HHANES) show that the percentages of Mexican American, Cuban, and Puerto Rican women aged 18–44 years with low serum folates (<3 μg/liter) were 11.9, 10.1, and 8.1, and with low erythrocyte folates (<160 μg/liter) 7.8, 16.7, and 13.0, respectively [54]. According to this latter report, the functional consequences of these reduced stores are unknown. However, the ability of these women to maintain

normal hematopoiesis in times of increased demand, such as in pregnancy, may be compromised."

Folate nutritional status as measured by folate concentration of plasma or serum and erythrocytes of lactating women was evaluated in four studies, which also included measurement of human milk folate content [15–17,36]. In all four studies, no relationship was noted between laboratory indices of maternal folate status and human milk content as previously indicated. This lack of an association between milk and maternal blood folate has led to the conclusion that maternal folate status is adequate [17] even when mean laboratory values are below the acceptable range (<3 μg/liter for plasma folates and <140 μg/liter for erythrocyte folates). In that study, women received no supplemental folic acid during either pregnancy or lactation. Serum and erythrocyte folate levels in lactating women receiving prenatal supplements (1 mg/day) during pregnancy and throughout the first 3 months of lactation were 44 and 588 μg/liter at 6 weeks and 35 and 421 μg/liter at 3 months, respectively. Corresponding values for women receiving folic acid supplements during pregnancy but not during lactation were 6 and 285 μg/liter at 6 weeks and 21 and 233 μg/liter at 3 months for serum and erythrocyte folates, respectively [36]. In both of these groups of lactating women, erythrocyte folates decreased even though levels remained in the normal range (Fig. 4). Likewise, Butte and Calloway [16] reported that Navajo mothers who did not continue taking prenatal supplements containing folic acid during lactation showed a deterioration of maternal folate status from parturition to month 1 of lactation. Tamura and associates [15] observed lower but normal blood folate levels in nonsupplemented lactating women compared to those receiving 1 mg folic acid per day for 4 weeks. Only in the study with Navajo women was dietary folate intake assessed. Mean folate intake of lactating Navajo women from dietary sources was estimated to be 290 μg/day [49].

The quantity of dietary folate needed to elicit a hemotological response in folate-deficient lactating women was evaluated in three studies [39,55,56]. Baumslag and Metz [55] reported that three heads of lettuce for 6 days was necessary to produce a reticulocyte response in a lactating woman with megaloblastic anemia. Assuming that a head of lettuce weighs 400–500 g and the folate content was 0.2 μg/g, the quantity of dietary folate fed to the lactating woman was approximately 240–300 μg. Coleman et al. [56] studied five folate-deficient South African lactating women fed a low-folate diet plus maize meal fortified with 100–500 μg folic acid and reported that 300–500 μg of folate per day were needed to produce an adequate hematological response. The minimum amount of folate needed to maintain serum folate levels of a lactating woman fed an experimental diet low in folate was 200–300 μg/day [39].

The studies reviewed above provide little insight into the amount of dietary folate needed to maintain both maternal folate status and milk folate in an acceptable range. It is clear, however, that milk folate content cannot be used as a proxy for maternal nutritional adequacy. Even though milk folate content is maintained

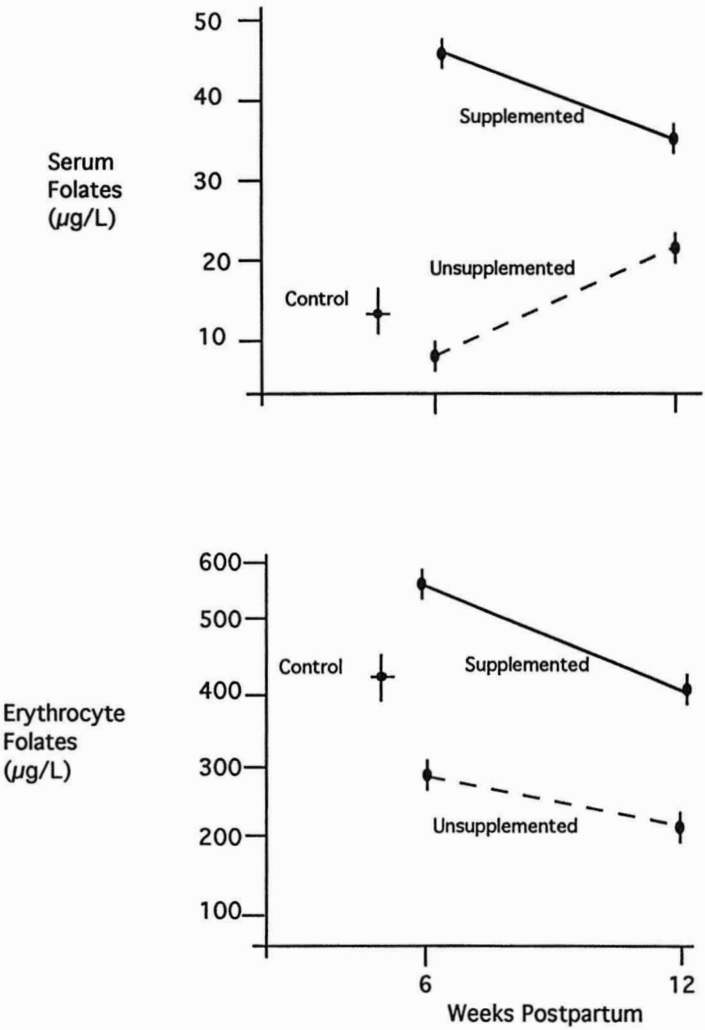

Figure 4 Erythrocyte and serum folates (mean ± SEM) of lactating women at 6 and 12 weeks postpartum who received supplemental folic acid (1 mg/day) during pregnancy. Women who continued taking folic acid supplements (supplemented group) and those who discontinued (unsupplemented group) are designated by solid and hatched lines, respectively. (Adapted from Ref. 36.)

over a wide spectrum of maternal folate status, an association between maternal and infant blood folate levels is often observed [15,17,36,38], suggesting that folate endowment at birth may influence subsequent folate status of nursing infants. Recognizing their limitations, results of studies with folate-deficient lactating women suggest that 300 μg/day is at or near the minimal folate requirement for lactating women and that intakes between 300 and 500 μg/day may be desirable to maintain maternal folate status.

V. FOLATE STATUS OF HUMAN MILK–FED INFANTS

There is general agreement that normal blood folate values of infants at birth are higher than maternal and "normal" adult values [14,15,38,57–59]. However, there is disagreement about whether the decline in plasma and erythrocyte folates reported by several investigators [57,58] represents normal physiology (i.e., analogous to the decline in hemoglobin concentration of early infancy) or folate status. Four reports support the concept that a decline in blood folate levels during early infancy is not normal [14,15,38,59]. Reported mean values for blood folate levels of Japanese [15], Norwegian [59], Finnish [38], and U.S. [14] human milk–fed infants are amazingly similar with serum or plasma values of 20–30 ng/ml and erythrocyte folate levels of approximately 400 ng/ml (396–429 ng/ml) during the first 6 months of life. In three of these studies from industrialized countries, serial blood samples were taken [14,38,59], and in none did blood folates diminish in early infancy; they either stabilized or increased slightly.

Several investigators [60,61] reported that breast-fed infants maintain blood folate levels greater than formula-fed infants. In these early reports formula feeding was either not defined [60] or referred to the use of home-made cow milk preparations [61]. In two studies, assessments were made of folate status of infants less than 6 months old fed proprietary formula preparations [14,62]. In both of those studies, quantity of folate declared on formula labels was substantially less (approximately 50%) than that contained in the product, and infants fed such products maintained serum and erythrocyte folates similar to or greater than cohort groups of breast-fed infants.

The amount of dietary folate ingested by infants is usually calculated from assumptions concerning the quantity of milk or formula typically ingested and the amount of folate contained in the type of milk consumed. The contribution of folate from foods other than milk is usually not considered, which may result in misleading conclusions. Dietary assessments of infants in the first year of life show that following the introduction of solid foods, the contribution of milk and/or formula to total kilocalories declines to 75, 50, and 33% at 6, 9, and 12 months, respectively [14]. For infants fed mixed diets at 9 and 12 months, the majority of dietary folate was provided by foods other than milk or formula. Folate intakes of contemporary

breast-fed and formula-fed infants from industrialized countries are well above those currently recommended (3.6 μg/kg) (see Chapter 5). Following the introduction of solid foods, blood folate levels declined in infants. During the period of mixed feeding, values for serum and erythrocyte folates were highly correlated with folate intake from milk (r = 0.5–0.7) and either not or weakly correlated with total folate intake. These findings in infants fed mixed diets indicate that milk folate (human milk, cow milk, and cow milk–based formula) is either highly bioavailable or that milk exerts a positive influence on folate bioavailability proportional to milk intake (see Chapter 8).

VI. RECOMMENDED FOLATE INTAKES DURING LACTATION

There have been no systematic studies of folate requirements of lactating women. The recommended intakes of folate for lactating women issued by The Food and Agricultural Organization/World Health Organization [63], British [64], Canadian [65], and American [50] scientific review committees are presented in Table 1. There is amazing consistency among these recommended folate intakes partially due to the fact that all of these organizations used a similar approach in deriving values. The amount of dietary folate needed to replace that secreted via milk is added to

Table 1 Recommended Folate Intakes for Nonreproducing and Lactating Women by Various National and International Organizations

	Recommended folate intakes	
Organization	Nonreproducing women	Lactating women
FAO/WHO, 1988	3.1 μg/kg or 180 μg for 58-kg woman	+100 μg/day or 280 μg for 58-kg woman
Committee on Medical Aspects of Food Policy, Panel on Dietary Reference Values (DRV), 1991	200 μg/day for 60-kg woman	+60 μg/day
Health and Welfare Canada, Report of the Scientific Review Committee: Nutrition Recommendations (RNI), 1990	3.1 μg/kg or 175 μg for 55-kg woman	+100 μg/day
Institution of Medicine, National Research Council, Food and Nutrition Board, Subcommittee on the 10th edition of the RDAs, 1989	180 μg/day for 58-kg woman	+100 μg/day (1st 6 months) +80 μg/day (2nd 6 months)

Source: Refs. 50, 63–65.

that recommended for the nonpregnant, nonlactating woman. The values used by these various agencies for the folate content of human milk was 40–60 μg/liter and for the absorption rate from dietary sources, 50–70%. Based on these values, a lactating woman secreting between 750 and 850 ml of milk per day is calculated to need between 60 and 100 μg of folate per day to cover that secreted in milk. As reviewed elsewhere in this book (see Chapter 5), there is good reason to believe that the recommended level of dietary folate intake for the nonreproducing woman should be approximately 300 μg/day or 5 μg/kg rather than 180 μg/day or 3 μg/kg. Sauberlich and associates [52] demonstrated that at an intake of 200 μg/day, women show a folate depletion state as evidenced by a decline in erythrocyte folate levels. This finding has recently been confirmed (see Chapter 5). Additionally, recent analyses show that human milk folate content in early lactation is approximately 100 μg/liter [26], indicating either that recommended increments do not cover the amount of folate secreted in milk even assuming a 100% absorption rate [64] or that an absorption rate of 75–85% [63,65,50] is needed to cover daily losses in milk. Available evidence indicates that folate absorption from a mixed diet is approximately 50% [52], suggesting that current recommended folate intakes for lactating women considerably underestimate actual need. The amount of folate needed to sustain lactation cannot be less than that secreted in milk. For a woman secreting 750 ml of milk per day, an additional 150 μg of folate is needed assuming that actively metabolizing mammary tissue is 100% efficient and does not require additional folate. Since milk secretion rates of lactating women can be as high as 1000 ml/day, an additional 200 μg/day of folate or a total of 500 μg/day is more likely to meet the folate requirement for most healthy lactating women than current recommended amounts.

VII. SUMMARY AND CONCLUSIONS

Knowledge of folate requirements during human lactation is scant. Recommended levels of folate intake have been made based on assumptions of the maintenance requirement of lactating women plus the quantity lost in milk. Recent evidence indicates that milk folate secretion is considerably greater than assumed and that recommended intakes underestimate actual need. Available information also indicates that maternal folate status can be compromised during lactation and that mammary tissue takes precendent over other maternal tissues. Adequacy of milk folate content and/or folate status of human milk–fed infants cannot be used as proxies for maternal folate adequacy, since both are maintained during folate depletion states of lactating women. Data from animal studies suggest that folate requirements during lactation are greater than during pregnancy. Research is sorely needed to determine if folate status of lactating women on self-selected diets is maintained during prolonged lactation.

ACKNOWLEDGMENT

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Folate Status of U.S. Population Groups

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I. INTRODUCTION

An increased interest in the folate status of population groups has occurred largely as the result of three aspects of the vitamin: (1) the association of low plasma folate levels with elevated plasma homocysteine concentrations and an increased risk of arteriosclerosis [1–6], (2) the benefits of folate in reducing the incidence of neural tube defects [7–12], and (3) the effects of folate on the occurrence of cancer [13–19].

Folate plays a crucial role in the biosynthesis of nucleotides that are essential for nucleic acid metabolism, cell division, and genetic expression. Folate-activated enzymes are required for the synthesis of certain amino acids and for various methylation reactions. Some of the brain deficits associated with fetal alcohol syndrome may be the result of reduced activities of these folate-containing enzymes [20,21]. Alcohol interferes with the activities of these enzymes as well as impairing the absorption of the vitamin [22–25]. In addition, plasma and erythrocyte folate levels are significantly lower in smokers than in nonsmokers [13,14]. The use of certain medications and oral contraceptives may also lower the folate concentrations in blood [18,20,26–33].

This chapter will consider briefly the procedures used to assess the folate status of populations. However, the major contribution of this chapter will be an evaluation of the folate nutritional status of the U.S. population by age groups, from infants to the elderly. Included will be information on factors that may influence folate status.

II. FOLATE STATUS ASSESSMENT PROCEDURES

Procedures used to diagnose a folate deficiency are considered in detail in Chapter 3. Nevertheless, since the assessment of the folate status of population groups is dependent upon suitable biochemical and dietary procedures, a few comments pertaining to these procedures appear appropriate here.

A. Biochemical Procedures

Folate nutritional status can be evaluated with reasonable reliability by biochemical procedures. Measurement of serum and erythrocyte folate concentrations are commonly performed and are the most practical procedure for application to population studies [27,28,34–37]. Erythrocyte folate levels are considered a better indicator of long-term folate status than serum or plasma folate levels [35,38–41]. Erythrocyte folate measurements do not distinguish between megaloblastic anemia due to vitamin B_{12} deficiency and that due to a folate deficiency. As a result of a primary vitamin B_{12} deficiency, folate concentrations in the serum may be elevated while low concentrations may be observed in the erythrocytes. If low folate levels are found in both the serum and the erythrocytes, the evidence is strong that a folate deficiency exists. When feasible, serum vitamin B_{12} measurements should be performed in conjunction with folate nutritional assessments [35].

Several guidelines or definitions have been used to evaluate folate status based on serum or erythrocyte folate concentrations. One approach is to classify populations into high-, moderate-, and low-risk groups, with cutoff points based on dietary intake or biochemical measures of folate status.

Guidelines should relate to folate levels in the plasma or erythrocytes at which the earliest biochemical, functional, morphological, physiological, or behavioral changes appear. Other biochemical measurements are available for the assessment of folate nutritional status but are generally not feasible for use in population studies [28,35,38,41–45].

B. Dietary Procedures

Dietary information on folate intake is often obtained, particularly when blood sampling is not possible. Dietary intake studies usually compare intake to the Recommended Dietary Allowances (RDA) [46,47]. Various procedures have been used to assess dietary intakes of folate including food frequency questionnaires, diet histories, diet diaries, diaries plus interviews, food purchases, and actual intakes [41,48–56]. Problems associated with these methods are recognized [41,57,58]. In addition, information on the folate content of foods is often missing or unreliable because of analytical methodology problems or uncertainties about folate bioavailability [43,59]. However, results of carefully conducted dietary intake studies have correlated with biochemical measurements of folate status in the same

population [48,49,60,61]. The estimated amount of folate provided by the U.S. food supply has fallen from 320 μg per capita per day in the year 1909 to the present level of 283 μg per capita per day largely due to changing eating habits [62].

III. NATIONAL HEALTH AND NUTRITION EVALUATION SURVEYS (NHANES)

A. Folate Analytical Procedures Used

Microbiological assays have served as the reference procedure for measuring folate concentrations in plasma and blood. With modern techniques, large numbers of samples can be analyzed for folate by microbiological methods [63]. Radioisotope procedures have also been utilized for the measurement of blood folate concentrations where microbiological assay facilities were not available. Radioisotope usage in itself has disadvantages, and difficulties have been encountered with the validation of commercial folate radioassay kits [64–66].

The NHANES II study measured serum and red blood cell folate levels with the use of the microbiological assay during the early phase and then changed to a radioassay procedure for the remaining samples. Merging the data obtained from the use of two differing assay methods proved difficult and unsatisfactory [27]. For the NHANES III, a radioassay folate procedure was used throughout the study. A recent report indicated that this radioassay procedure appeared to be significantly inaccurate analytically [65]. Folate data from the NHANES III study were not made available for inclusion in this report.

B. Survey Findings

The objectives, applications, and limitations of the NHANES have been reviewed by Yetley and Johnson [67] and by Senti and Pilch [27,68]. Serum and erythrocyte folate data were limited for the NHANES I [69] but were more extensive for the NHANES II [27,68]. For these surveys, serum folate concentrations of <6.8 nmol/liter (<3.0 ng/ml) and erythrocyte folate concentrations of <317 nmol/liter (<140 ng/ml) were considered low.

Although sampling and methodological problems were encountered in the NHANES, the results indicated that 10% of the U.S. population may have low folate stores [27,47,68]. Blood folate levels were obtained on 1466 male subjects and 1444 female subjects (Table 1–5). In the age range of 20–74 years, black males had a significantly higher percentage ($p = 0.01$) of low erythrocyte folate concentrations than did whites (Table 1). Dietary supplements improved the folate status of females in the 20–74 years group ($p = <0.05$) (Table 2). Women who smoked had a higher percentage of low folate values than nonsmokers ($p = <0.05$). Although not significant, females aged 20–44 years who used oral contraceptives had a higher percentage of low folate values (Table 3). Overall, females 20–44 years

Table 1 Proportion of Male Population Groups (20–74 years) with Low Serum or Red Blood Cell Folate Levels

	Serum folate (<6.8 nmol/liter; < 3.0 ng/ml)		RBC folate (<317 nmol/liter; <140 ng/ml)	
Group	n	Percent ± SE	n	Percent ± SE
White	748	14 ± 2.5	624	7 ± 1.5
Black	197	16 ± 5.7	167	17 ± 3.2
Smokers	381	18 ± 3.1	312	11 ± 2.9
Nonsmokers	587	13 ± 3.0	490	6 ± 2.4
Aspirin users	247	19 ± 4.7	214	13 ± 4.4
Aspirin nonusers	721	14 ± 2.3	588	7 ± 2.0
Supplement users	209	11 ± 4.7	178	5 ± 1.8
Supplement nonusers	759	15 ± 2.4	624	9 ± 1.7

Source: Refs. 27, 68.

of age appeared to be the group with the highest risk of developing a folate deficiency. In this group, 15% had low serum folate values, while 13% had low erythrocyte folate values [68]. In the children aged 6 months to 9 years, only 2% had low serum or erythrocyte folate values (Table 4). They also had higher median

Table 2 Proportion of Female Population Groups (20–74 years) with Low Serum or Red Blood Cell Folate Levels

	Serum folate (<6.8 nmol/liter; < 3.0 ng/ml)		RBC folate (<317 nmol/liter; <140 ng/ml)	
Group	n	Percent ± SE	n	Percent ± SE
White	804	13 ± 2.5	677	9 ± 1.6
Blacks	173	8 ± 3.5	142	12 ± 4.3
Smokers	308	19 ± 3.8	256	12 ± 2.1
Nonsmokers	686	9 ± 2.1	572	8 ± 1.8
Aspirin users	304	12 ± 3.0	569	7 ± 2.1
Aspirin nonusers	690	13 ± 2.7	569	10 ± 2.2
Supplement users	301	6 ± 2.4	253	4 ± 1.5
Supplement nonusers	693	15 ± 2.8	575	11 ± 1.8

Source: Refs. 27, 68.

Table 3 Proportion of Female Population Groups (20–44 years) with Low Serum or Red Blood Cell Folate Levels

Group	Serum folate (<6.8 nmol/liter; < 3.0 ng/ml)		RBC folate (<317 nmol/liter; <140 ng/ml)	
	n	Percent ± SE	n	Percent ± SE
OCA users	75	21 ± 6.9	63	20 ± 8.2
OCA nonusers	387	14 ± 2.9	326	11 ± 2.1
Pregnant	51	20 ± 8.1	42	21 ± 12.0
Nonpregnant	411	15 ± 3.2	347	12 ± 2.7

Source: Refs. 27, 68.

serum and erythrocyte concentrations than were found in the older groups (Table 5). Mean dietary intake of folate was estimated from dietary recall data to be 241 μg/day for all adults; mean intakes by sex were 197 μg/day for women and 263 μg/day for men [70,71].

The Hispanic Health and Nutrition Examination Survey (HHANES), conducted during 1982–1984, examined the nutritional status of Mexican-American,

Table 4 Proportion of Persons by Age Group with Low Serum or Red Blood Cell Folate Levels

Sex and age group	Serum folate (<6.8 nmol/liter; < 3.0 ng/ml)		RBC folate (<317 nmol/liter; <140 ng/ml)	
	n	Percent ± SE	n	Percent ± SE
Male				
6 months – 9 yr	294	2 ± 1.4	243	2 ± 1.6
10–19 yr	204	3 ± 1.3	178	5 ● 2.2
20–44 yr	362	18 ± 2.8	299	8 ± 2.5
45–74 yr	606	10 ± 2.5	503	8 ● 2.0
Female				
6 months – 9 yr	240	3 ± 2.1	201	2 ± 1.5
10–19 yr	210	12 ± 3.1	173	8 ± 2.8
20–44 yr	462	15 ± 3.1	289	13 ± 2.4
45–74 yr	532	9 ± 2.6	439	4 ± 0.8

Source: Refs. 27, 68.

Table 5 Mean Serum Folate Levels by Age Groups

Age group	Serum folate levels					
	Male			Female		
		Mean			Mean	
	n	nmol/liter	ng/ml	n	nmol/liter	ng/ml
6 months–10 yr	108	37.0	16.3	88	37.6	16.6
10–19 yr	73	20.6	9.1	79	18.4	8.1
20–44 yr	133	16.3	7.2	144	18.4	8.1
45–77 yr	208	17.0	7.5	197	20.6	9.1

Microbiological assay method used. *Source*: Refs. 27, 68.

Cuban, and Puerto Rican groups in selected areas of the United States [72]. Serum and erythrocyte folate concentrations were measured by a radioassay method on 1074 Mexican-American, 203 Cuban, and 426 Puerto Rican women aged 18–44 years. Low serum folate concentrations (<3.0 ng/ml; <6.8 nmol/liter) were found in 11.9% of the Mexican-American women, 8.1% of the Puerto Rican women, and 10.1% of the Cuban women. The incidence of low concentrations of folate in the erythrocytes (<160 ng/ml; 363 nmol/liter) was 7.8, 13.6, and 16.7% for the Mexican-American, Puerto Rican, and Cuban women, respectively. Of interest, the incidence of low plasma concentrations of folate was significantly reduced with the use of vitamin or mineral supplements [27,68,72] (Tables 1 and 2). Other studies have also indicated that vitamin supplements can provide a significant contribution to the daily intake of folate [73–75].

IV. FOLATE STATUS OF U.S. POPULATIONS

A. Infants and Children

Information on the folate status of infants is limited [26,34,37,76–82], and the studies reported usually involve small numbers of participants. Nevertheless, folate deficiency in infants is seldom observed. Hibbard and Kenna [79] found no evidence of folate deficiency in 57 low-birth-weight infants, however, low blood folate concentrations and megaloblastic anemia have been reported in premature low-birth-weight infants [82,83]. Folate deficiency was reflected in low serum and erythrocyte folate concentrations and in elevated urinary excretion of formiminoglutamic acid (FIGLU). Megaloblastic anemia occurred 2–4 months after birth.

Hibbard [84] reported normal infants to have a mean plasma folate concentration of 27.0 nmol/liter (11.9 ng/ml) and a mean erythrocyte folate concentra-

tion of 7.27 nmol/liter (321 ng/ml). Similar folate values for infants and children were found in the Ten State Nutrition Survey conducted between 1968 and 1970 [34]. White subjects had somewhat higher plasma and erythrocyte folate levels than those observed in black and Spanish-American subjects. Serum and erythrocyte folate concentrations have been reported to be higher in infants than in older children and adults [27] (Table 5). Smith et al. [76] also found that serum and erythrocyte folate concentrations of term infants were higher than or comparable to adult values.

As noted for infants, data on folate status of children in the United States are also limited. In an older study, Chase et al. [85] found that 10% of the migrant children examined in Colorado had serum concentrations of less than 13.6 nmol/liter (6 ng/ml) and 1% had erythrocyte folate concentrations of <363 nmol/liter (160 ng/ml). Low dietary intakes of folate have been reported for children 1–6 years of age [86,87]. Children from Kansas City, Missouri, 1–3 years in age (n = 66) consumed 20 μg/day, which represented only 40% of the RDA [86]. Children 4–6 years old (n = 58) had folate intakes of 40 μg/day, which was equivalent to 54% of the RDA [86]. Biochemical measurements were not conducted to assess the folate status of the children.

B. Adolescents

Nutrient deficiency may occur in adolescents because of their rapid growth and maturational changes. The results of a number of studies indicate that folate nutritional status may be compromised in adolescents [88–96] (Table 4). Daniel et al. [88] evaluated the folate status of 461 healthy adolescents. Adequate plasma folate concentrations were observed in 95% of the girls and 90% of the boys. The low dietary intake of folate of less than 0.1 mg/day reported was probably due in part to the incomplete food composition tables available.

More extensive studies of the folate status of adolescents were conducted by Bailey and associates [89–91]. In a study of 193 adolescents, mainly from black, urban, low-income households, 42% of the subjects had erythrocyte folate concentrations of <317 nmol/liter (140 ng/ml), and an additional 13% had levels of <363 nmol/liter (160 ng/ml) [89]. Serum folate concentrations of <6.8 nmol/liter (<3 ng/ml) were observed in 15% of the subjects. Serum folate concentrations decreased with increasing age and sexual maturity.

In another study, information was obtained on the dietary intakes of folate with the use of a food-frequency questionnaire [91]. The study involved 372 adolescents from low-income households. The urban black subjects selected folate-dense foods more frequently than did the rural black or urban Hispanic subjects. Rural whites consumed folate-dense food groups more frequently than blacks. Approximately 50% of the adolescents had erythrocyte and serum folate concentrations that were considered less than acceptable. The infrequent consumption of vegetables and fruit appeared to provide an explanation for the poor folate status of these adolescents.

McCoy and coworkers [92] used two 24-hour recalls to estimate the folate intakes of 1247 black and white adolescent girls from eight southeastern states. Mean intakes of folate were 185 μg/day for blacks and 217 μg/day for whites. Frequently the intakes were as low as one third of the RDA.

In 1985, Liebman [93] conducted a study in North Carolina of 91 girls aged 14–16 years. Of these girls, 32% had erythrocyte folate levels of less than 317 nmol/liter (140 ng/ml). The low levels were observed more frequently among black girls than white girls. Another study was conducted by Reiter et al. [96] with 39 black adolescent girls 12–14 years old from Petersburg, Virginia. Of these girls, 74% had marginal folate status and 3% had a deficient status.

Clark et al [94] investigated the folate status of 103 healthy adolescent girls 12, 14, and 16 years of age living in east central Alabama. Subjects were considered to be folate deficient if the folate concentrations in serum and erythrocytes were <6.8 nmol/liter (<3 ng/ml) and <317 nmol/liter (<140 ng/ml), respectively. Nearly 12% of the girls had serum folate concentrations of <6.8 nmol/liter (<3 ng/ml), and 47.6% had erythrocyte folate concentrations of <317 nmol/liter (<140 ng/ml).

In 1990, Tsui and Nordstrom [95] reported on the folate status of 164 adolescent boys and girls, age 12–16 years, from metropolitan Kansas City. Boys had higher folate concentrations in the serum and erythrocytes than did the girls. Forty percent of the girls and 13% of the boys were folate deficient as judged by erythrocyte folate concentrations of less than 317 nmol/liter (<140 ng/ml). Oddly, only 5% of the girls and no boys had serum folate concentrations of less than 6.8 nmol/liter (<3 ng/ml). The folate analyses were performed by a radioassay procedure. Hemoglobin values were significantly lower for the folate-deficient subjects than those of the normal subjects. Seventeen percent of the boys and 42% of the girls had folate intakes below the 1989 RDA.

C. Adults

In an early study, Hall et al. [59] studied the folate status in a group of 106 healthy male and female subjects. The subjects represented black migrant workers, clinic patients, and hospital personnel. Serum folate concentrations of less than 6.8 nmol/liter (<3 ng/ml) were observed in 17 of the 77 black subjects but in none of the 29 white subjects.

Butterworth et al. [14] studied the folate status in 235 women with a mean age of 25.0 years. The population studied was from a lower socioeconomic group in the Birmingham, Alabama, area [97]. The group was composed of 64% black and 36% white women. It was observed that 37% of the subjects had red blood cell folate concentrations of less than 410 nmol/liter (<181 ng/ml). Only 16% had red cell folate concentrations of 660 nmol/liter (290 ng/ml) or higher. Red cell folate concentrations above 450 nmol/liter (190 ng/ml) were considered acceptable.

In a related study, 464 similar women from the Birmingham area were investigated as to their folate status [16]. The women were attending county health department clinics for the purpose of family planning and routine health evaluation. Of this group, 36% had red blood cell concentrations of less than 410 nmol/liter (< 181 ng/ml). Twenty-nine percent had red blood cell concentrations above 660 nmol/liter (290 ng/ml).

Folate status has been evaluated in military personnel of the army, navy, marine, and air forces located at various military bases. Usually 95% of the male personnel fell within the age range of 17–34 years. The folate status of the military personnel was generally acceptable, indicating an adequate intake of the vitamin. For example, in a study of 681 men at the Twenty-Nine Palms Marine Corps Base in California, 5.7% had serum folate concentrations that were considered "at risk": < 13.6 nmol/liter (< 6.0 ng/ml) [60]. Red blood cell concentrations of folate were considered at risk in 8.4% of the men: < 363 nmol/liter (< 160 ng/ml) [60]. In a study conducted at the same military base on 294 female Marines, a comparable incidence of low folate values was observed.

The army meal ready-to-eat (MRE) operational rations were fed to 27 soldiers during a field exercise lasting 34 days [98]. Serum folate concentrations were acceptable but declined steadily with time (12.0 to 7.9 nmol/liter; 5.3 to 3.5 ng/ml). Dietary folate intakes were not described.

Drake [99] studied the nutritional status and dietary adequacy of single homeless women and their children. The subjects were residents in housing shelters in Kansas City, Missouri. The group of subjects was 11% Hispanic, 44% White, 41% black, and 4% of Asian descent. The adult subjects were 19–45 years of age. Dietary intake information indicated that the subjects in all age groups were consuming less than 50% of the 1989 RDA for folate, zinc, iron, and magnesium. Other studies have reported that women living under the poverty level have an average intake of folate of approximately 40 μg/day less than those with higher incomes [100,101].

In a study of 209 adult males, approximately 50% of the subjects had plasma homocysteine levels greater than 12 μmol/liter [102]. Levels of homocysteine greater than 12 μmol/liter have been considered elevated [103]. Elevated homocysteine concentrations in these men were associated with lower concentrations of plasma folate (Fig. 1) [102]. Men have been reported to have lower dietary intakes of folate than women [104,105]. Although the lower limit of normal plasma folate concentrations used in NHANES II was 6.8 nmol/liter (3.0 ng/ml) and by World Health Organization (WHO) was 13.6 nmol/liter (6.0 ng/ml), results of this study would suggest that the lower acceptable plasma concentration of folate was about 15 nmol/liter (6.6 ng/ml) [102].

Vitamin status was investigated in 1160 adult men and women survivors, aged 67–96 years, from the original Framingham Heart Study cohort [104]. Analysis of plasma concentrations of homocysteine revealed a strong inverse relationship with

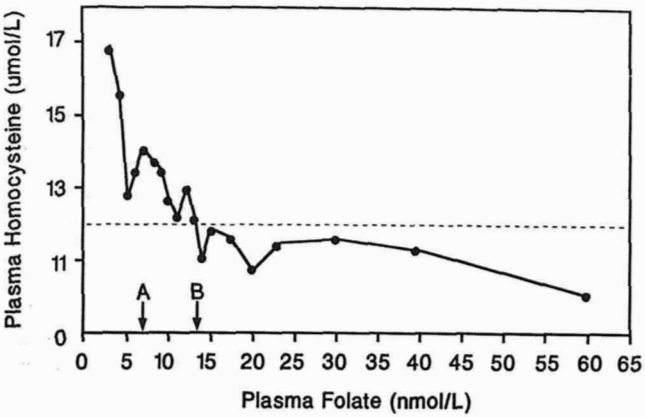

Figure 1 Relationship of plasma homocysteine levels to plasma folate levels (n = 209 adult males). Homocysteine levels above the dotted line are considered elevated. Point A indicates lower limit of normal plasma folate level as used by NHANES II (6.8 nmol/liter; 3.0 ng/ml). Point B indicates lower limit of normal plasma folate level as used by WHO (13.6 nmol/liter; 6.0 ng/ml). (From Ref. 102.)

plasma folate concentrations. Homocysteine concentrations were higher in men than in women and increased with age. Elevated homocysteine concentrations (>14 μmol/liter) were observed in 25% of the men 67–74 years of age, while 19% of the women in this age group had elevated homocysteine concentrations.

The interrelationship between homocysteine and folate was investigated in a case-control study that involved 101 white males aged 30–50 years with coronary artery disease, and 108 healthy white males of similar age as controls [106]. Plasma homocysteine concentrations were found to be higher in the coronary artery disease patients than in the controls. The plasma concentrations of folate, however, were lower in the patients than in the controls.

In the absence of a vitamin B_{12} deficiency, an elevated serum homocysteine level has been shown to be a strong predictor of folate status. Savage et al. [45] observed an increase in serum homocysteine in 91% of 123 patients considered folate deficient. In a study of 434 patients deficient in vitamin B_{12}, serum homocysteine was elevated in 95.9% and serum methylmalonic acid was elevated in 98.4% [45]. Thus, it would appear that normal levels of both homocysteine and methylmalonic acid would reliably rule out the presence of clinically significant vitamin B_{12} deficiency and, in most instances, also a folate deficiency.

Differences were also noted in the dietary intakes of folate [104]. Men 67–74 years of age had an average daily folate intake of 174 μg/4200 kJ, while women of the same age had an average folate intake of 214 μg/4200 kJ. Elevated homocysteine concentrations were observed among individuals with folate intakes

of up to 280 μg/day. Overall, the findings indicated a strong association between serum homocysteine concentrations and folate, vitamin B_{12}, and vitamin B_6 status [104].

In a further evaluation of the Framingham Heart Study, 548 surviving members were studied with regards to their serum folate and vitamin B_{12} concentrations [107]. Based on serum methylmalonic and total homocysteine concentrations, many elderly persons with serum vitamin B_{12} and folate concentrations that were considered normal were actually metabolically deficient in vitamin B_{12} or folate. Consequently, the incidence of inadequate folate status in population groups may be underestimated when based on folate concentrations of serum and red blood cells.

D. Elderly Persons

Earlier studies of folate status in the elderly population have been reviewed by Grinblat [39] and Rosenberg et al [40,108]. The incidence of low serum or erythrocyte levels of folate in this population varies widely, which may be attributable in part to inconsistent cutoff points used and differences in the analytical methods employed [22,40,69,109–118].

There appear to be no age-related changes in folate absorption or metabolism [39,110,113,119]. However, alcoholism and certain medications may increase the incidence of folate deficiency [20,23,24,30,119–124].

In the NHANES I, about 6% of the elderly (≥60 years) surveyed had low serum folate concentrations (<5.9 nmol/liter; < 2.6 ng/ml) [119,125]. The median serum folate concentrations ranged from 13.4 nmol/liter (5.9 ng/ml) in black men to 22.0 nmol/liter (9.7 ng/ml) in black women. Both white and black women had higher serum folate concentrations than men. NHANES II data were not reported separately on the folate status of person aged 65 years or older [27,68].

Based on the 1980 RDA of 400 μg/day of folate, Gary et al. [126] found in a survey of healthy elderly subjects that 37% of the men (n = 125) and 43% of the women (n = 145) had folate intakes less than 50% of the RDA. However, it was recognized that these estimates of intakes may be low as a result of inadequacies of the food composition tables used to estimate the content of folate in various foods.

In a subsequent report in 1984 by Garry et al. [127], folate concentrations in plasma and erythrocytes were determined for 270 healthy elderly persons in the Albuquerque area of New Mexico. Of these subjects, only 8% had low plasma levels of folate (<6.8 nmol/liter; <3.0 ng/ml) and only 3% had low erythrocyte levels of folate (<317 nmol/liter; <140 ng/ml). The investigators concluded that an inadequate folate status in the free-living healthy elderly population was not a major medical problem.

In the Boston survey of free-living or institutionalized elderly subjects, less than 2.5% had plasma folate concentrations under 7 nmol/liter (<3.0 ng/ml) [40]. In other reports, however, high incidences of folate deficiency (10–28%) have been

observed among institutionalized elderly subjects and among cognitively impaired elderly persons [20,39,121–123].

In 1978, Raper and Choudhury [109] observed that 39% of 400 patients admitted to an acute geriatric unit had erythrocyte folate concentrations of less than 385 nmol/liter (< 170 ng/ml). They observed that the low erythrocyte folate concentrations were associated with a megaloblastic bone marrow and a high mean corpuscular volume (MCV) greater than 95 μ^3.

Bailey and associates investigated the folate status of a group of predominantly low-income, urban, black elderly Florida persons [128]. Sixty percent were found to have erythrocyte folate concentrations of less than 317 nmol/liter (140 ng/ml). Although no evidence of an iron deficiency existed, 14% of the elderly persons were anemic. They also reported on the folate status of a group from Florida of low-income rural and urban elderly white and black men and women aged 60–97 years [124]. In this group, 39% of the blacks and 23% of the whites had erythrocyte folate concentrations of less than 317 nmol/liter (140 ng/ml). Overall, 28% of the group had serum folate concentrations of less than 13.6 nmol/liter (< 6 ng/ml). Anemia was observed in 12% of the persons, but only 3 of the 122 elderly persons had evidence of an iron deficiency. In this group, the folate deficiency may have contributed to the anemia observed.

Wagner et al. [69] also studied the folate status of elderly Florida women aged 55–87 years. The group consisted of 83 black women from a low-income urban area and 41 white women from middle and upper socioeconomic areas. Erythrocyte folate levels of less than 317 nmmol/liter (140 ng/ml) were found in 60% of the black women in contrast to only 6% of the white women.

In 1984, Smith et al. [129] studied the nutritional status of 146 elderly Nebraska residents in a long-term care facility. Of this group, 13% were found to have low serum folate concentrations (6.8 nmol/liter; < 3 ng/ml). The general nutritional health of this population appeared good.

Sahyoun et al. [130] provided details on the nutritional status of 260 elderly men and women, age 60–101 years, who resided in long-term care facilities in the Boston area. Dietary intakes of folate, based on three consecutive weekday dietary intake records, were less than the 1980 RDA. The mean intake was 271 μg/day of folate for the men and 239 μg/day for the women. Fifty-two percent of the men and 65% of the women had folate intakes of less than two thirds of the RDA. However, it was noted that the folate data in the nutrient composition data bank used were incomplete. Plasma folate levels were equal or higher than those observed in 124 noninstitutionalized males 60–94 years of age. The plasma folate levels of the institutionalized men, at the 10th percentile, was 8.8 nmol/liter (3.9 ng/ml). For the institutionalized women it was 10.4 nmol/liter (4.6 ng/ml). Although 49% of the institutionalized population received a nutrient supplement, the amount of folate contributed by the supplement was not stated. The investigators concluded that in

the Boston area, institutionalization in itself did not lead to an impairment of nutritional status.

In general, folate deficiency does not appear to be widespread or extensive among the free-living elderly population. The greatest risk of a folate deficiency appears to be among the low-income urban and rural elderly populations. However, if plasma concentrations of homocysteine are used as a functional measurement of folate status, then the prevalence of folate deficiency in elderly subjects has been observed to be considerably higher than the estimates based on plasma concentrations of folate [131]. Thus, in a European study, 19% of 286 elderly hospitalized subjects aged 65–88 years had a low folate status [131]. Nevertheless, information on the folate status of the old and very old in the United States is meager. Consequently, the NHANES III was designed to obtain nutritional information on the U.S. population 74 years of age and older.

E. Folate Status During Pregnancy and Lactation

The recognized occurrence of folate deficiency during pregnancy has resulted in the acceptance of routine prenatal folate supplementation [132,133]. Data from the NHANES project [27,68] indicated approximately 20% of the pregnant women had low serum and red blood cell folate levels (Table 3). An increased requirement for folate during pregnancy and lactation may place a stress on the maternal folate status [43,47,133–135]. For example, in a pregnancy and lactation study of 87 Navajo women, 9% of the women had low serum folate levels at term. After one month of lactation, 23% of the women studied had low serum folate levels [136]. The protective effects of folate on the risk of neural tube defects has given rise to a reconsideration of the folate requirements and pregnancy outcome [11,12,137–141]. These aspects are considered in detail in Chapters 5, 6, and 12.

F. Factors Influencing Folate Status

1. *Smoking and Folate Status*

In an early study in men by Nakazawa et al. [142], serum folate concentrations of smokers were significantly lower than those of nonsmokers (11.3 nmol/liter vs. 15.3 nmol/liter; 5.0 ng/ml vs. 6.7 ng/ml). Witter et al. [61] reported similar effects of smoking on serum and erythrocyte folate concentrations in a group of nonpregnant women.

Tables 1 and 2 present information obtained in the NHANES II study on the influence of smoking on the incidence of low serum and erythrocyte concentrations in adult men and women [27,68]. As noted previously, smoking had an adverse effect on blood folate concentrations that resulted in an increased incidence of subjects with low folate status.

Table 6 Effect of Smoking on Blood Folate Levels

Subjects	n	Folate Levels (ng/ml)[a]	
		Plasma	Red blood cells
Nonsmokers	25	8.0 ± 1.0	322 ± 27
All smokers	69	5.5 ± 0.4	261 ± 13
Smokers without metaplasia	27	6.3 ± 0.6	277 ± 18
Smokers with metaplasia	42	4.9 ± 0.5	251 ± 17

[a] Mean ± SD.
Source: Refs. 13, 15, 17.

These observations have led to the concept that smoking and environmental substances could inactivate folates in exposed tissues and produce localized folate deficiencies [13]. Evidence to support this concept has been reported by Heimburger et al. [13,15,17] (Table 6) and by Butterworth et al. [14,16,18,19].

Recently Piyathilake et al. [143] reported that folate concentrations in plasma, red blood cells, and buccal mucosa were significantly lower in smokers when compared to nonsmokers. At an intake level comparable to the RDA, smokers had 42% lower plasma folate concentrations when compared to nonsmokers. When the folate intake was threefold higher than the RDA, plasma folate concentrations were the same for both smokers and nonsmokers [143].

2. *Antifolate Drugs*

Numerous antifolate drugs are available, such as methotrexate, sulfasalazine, fluoropyrimidines, 10-deazaaminopterin, edatrexate, pyrimethamine, trimethoprim, and trimetrexate [144]. These folate antagonists are routinely used in the medical field in the treatment of forms of cancer, psoriasis, rheumatoid arthritis, inflammatory bowel disease, and bacterial and protozoal infections [144–146]. Their use may induce varying degrees of folate deficiency [146,147].

Prolonged use of some anticonvulsant drugs may interfere with folate metabolism to produce a megaloblastic anemia [20,26]. For example, phenytoin has been observed to induce a folate deficiency. Folate metabolism is also influenced by the anticonvulsants, phenobarbital, and primidone [148,149]. The use of aspirin appeared to increase the incidence of low serum and red blood cell folate levels in males [27,68] (Tables 1 and 2). Likewise, the use of oral contraceptive agents by women 20–44 years of age has been associated with an increased incidence of low serum and red cell folate levels [27,58] (Table 3). The effects of a number of therapeutic drugs on folate bioavailability are reviewed in Chapter 8.

3. *Alcoholism*

Poor folate nutritional status is frequently observed in chronic alcoholic patients due to impaired absorption, inadequate dietary intake, altered metabolism, and an increased excretion of folate [22–25,150,151]. From 50 to 87% of the chronic alcoholic patients may have low serum folate concentrations, and 43% may have low red blood cell folate concentrations. The interrelationship between alcohol and folate is considered in detail in Chapter 11.

4. *Nitrous Oxide*

Nitrous oxide anesthesia inactivates vitamin B_{12} rapidly. As a result, plasma concentrations of folate increase, reflecting the inactivation of the vitamin B_{12}-dependent methionine synthase enzyme. Consequently, a rapid rise in plasma concentrations of plasma homocysteine occurs [152]. The disturbance in vitamin B_{12} and folate metabolism returns to normal within a few days [152].

V. SUMMARY

The most common cause of a compromised folate status is a low dietary intake of the vitamin. Numerous factors, including drugs, alcohol, and smoking may impair folate utilization and contribute to the cause of a folate deficiency. Biochemical and dietary information indicate that a compromised folate status may exist in a significant proportion of adolescent subjects, women 20–44 years of age, and pregnant women. An increased use of plasma homocysteine measurements as a functional indicator of folate adequacy may reveal an increased incidence of a compromised folate status in some of the U.S. population.

The National Health and Nutrition Examination Surveys have provided extensive information on the folate status of various population groups. The information should provide guidance as to the need for folate supplements and for folate fortification of foods.

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8

The Bioavailability of Folate

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I. INTRODUCTION

The concept of nutrient bioavailability is often poorly defined, conceptually misused, or simply left to the reader to infer a working definition. In the context of folate, bioavailability is most appropriately used to describe the overall efficiency of utilization, including physiological and biochemical processes involved in intestinal absorption, transport, metabolism, and excretion. Thus, it is important to understand the basic chemistry of this vitamin, as well as the physiology and biochemistry of folate utilization and function, before folate bioavailability can be meaningfully assessed. Because of variation among individuals in folate digestion, absorption, and metabolism, considerable variability in the bioavailability of folate from any given food source is highly likely. Interactive effects of various foods on folate bioavailability would further complicate dietary assessments. Thus, attempts to predict the bioavailability of folate from specific foods for individual human beings may have little practical value.

Major interest in the bioavailability of folate arose in the 1970s following reports that (1) the bioavailability of endogenous folate in foods varied widely, with certain items exhibiting very low folate bioavailability, and (2) certain food items exerted a more pronounced inhibition of the utilization of added polyglutamyl folates than of the monoglutamyl form. The possible digestive and/or absorptive factors that influence the bioavailability of ingested folates could include (1) binding or entrapment in the food matrix, (2) altered pH at the jejunal mucosal surface, which could affect the extent of conjugase action and/or folate transport, (3) inhibition of jejunal brush border folate conjugase and/or transport by food components, and (4) effects on intestinal transit time, all of which could account for differences in folate

bioavailability among individual foods. Considerable evidence suggests that the bioavailability of *naturally occurring dietary folate*, mainly polyglutamyl, is governed largely by the extent of intestinal deconjugation by the jejunal brush border folate conjugase systems in humans and animals. However, the bioavailability of monoglutamyl dietary folates may also be incomplete. Reasons for incomplete bioavailability of monoglutamyl folates, whether present in the diet or formed through enzymatic action on dietary polyglutamyl folates, may include all of the above factors, although exact mechanisms are unknown at present.

Clarification of folate bioavailability is important for several reasons:

1. Probably most significant is the need to predict the ability of diets to provide sufficient available folate to meet nutritional requirements. It is currently impossible to estimate the quantity of folate available to a population group on the basis of food consumption data. Thus, the association of dietary folate intake, nutritional status, and diet/disease relationships cannot be fully interpreted.
2. The nutritional impact of dietary changes, such as enrichment of foods with folic acid, cannot be accurately predicted.
3. Attempts to define the folate requirement through kinetic modeling are limited by the absence of sound information regarding the bioavailability of dietary folate.
4. Improved methods for assessment of folate bioavailability in individuals would aid in the diagnosis and management of malabsorption conditions.

As discussed by Bronner [1], nutrient bioavailability is far more complex than mere assessment of intestinal absorption. In the context of folate bioavailability, key processes include the extent of intestinal deconjugation, binding, and absorption, in vivo distribution, the metabolic function of folate coenzymes, and the kinetics of catabolic and excretory processes. Complicating factors include the dose dependence of these processes, the influence of diet on the efficiency of the enterohepatic circulation of folate, and the potential role of intestinal microorganisms. Thus, folate bioavailability is a complicated, multifaceted phemomenon dictated by the relative efficiency of many physiological and biochemical processes. This chapter will review the current understanding of folate bioavailability from the standpoint of the physiology and biochemistry of folate utilization as well as the influence of food composition. Folate bioavailability has been the subject of several reviews [2–5]. This chapter is based on the review by Gregory [4], with an emphasis on summarizing and interpreting our present understanding of the utilization of dietary folate by human beings.

II. INTESTINAL ABSORPTION OF FOLATES

Intestinal absorption occurs in the jejunum by a complex process involving the transport of monoglutamyl forms of the vitamin. Jejunal folate transport occurs

mainly by a saturable, pH-dependent process, as previously reviewed [4,6–16]. At higher concentrations (>10 μM), a nonsaturable mechanism involving passive diffusion contributes to folate absorption [15,16]. The intestinal transport process exhibits K_m values of several micromolar for various monoglutamyl folates. Minor differences in K_m values for transport of various folates have been reported [15], These differences may not be of sufficient magnitude to yield significant differences in the extent of absorption, given the large absorptive capacity of the intestine. Competitive inhibition between folic acid, 5-methyl-H_4folate, 5-formyl-H_4folate, and several antifolates is evidence of a single transport protein for pteroylmonoglutamates [15–25]. The saturable transport is maximal at luminal folate concentrations of 10–20 μM, although absorption by the nonsaturable mechanism continues as a linear function of concentration at higher levels [19]. The nonsaturable process may be minimally significant in the absorption of endogenous dietary folate, but may be much more operative in the absorption of higher levels of supplemental folic acid.

Folate absorption exhibits pH dependence with optimal transport into brush border membrane vesicles at pH 4.9–5.5, while intact intestine exhibits maximal transport at pH 6.0–6.3 [19,25]. This difference between isolated vesicles and whole intestine may reflect a role of the unstirred water layer or mucosal coat in controlling the pH at the intestinal absorptive surface or its role as a diffusion barrier [26]. There may be several effects of pH on the absorptive process. Blair and associates [27,28] suggest that an acidic pH is necessary to minimize the charge on the folate molecule to facilitate its diffusion across the brush border membrane. The subsequent discovery of the transport process provided evidence for a pH effect on the intestinal folate binding and transport protein(s). In addition, a pH gradient between the acidic mucosal surface (e.g., pH 5.5) and the neutral cytosol drives folate transport through a folate/hydroxyl exchange process or folate/proton cotransport [22,23,29]. The mechanism of the nonsaturable transport process is unknown.

Two folate-binding proteins exist in the jejunal brush border membrane [30–32]. The role of these proteins in folate transport has not been determined. Covalent affinity labeling of the intestinal folate-binding protein(s) caused a loss of folate binding and transport [33], which suggests a role in the transport process. Although many types of cells have the ability to absorb folate by receptor-mediated endocytosis, including a colonic epithelial cell line (Caco-2 cells) [34], the significance of this process in the absorption of folate in the small intestine has not been determined.

Prior to entry into the portal blood, folic acid undergoes reduction to H_4folate and either methylation or formylation in mucosal cells [15,17,35–37]. This reduction process is readily saturated, such that significant amounts of folic acid are found in plasma and urine of humans ingesting 400–800 μg/day of supplemental folic acid (J. F. Gregory and L. B. Bailey, unpublished observations). In this regard, Lucock et al. [38,39] suggested that supplemental folic acid may be more efficiently re-

duced and methylated if divided into several smaller doses, thus improving its bioavailability by increasing the efficiency of metabolic utilization.

Approximately 10–20% of the absorbed folate (typically as monoglutamyl 5-methyl-H_4folate), is retained by the liver on the first pass, while the remainder circulates to other tissues [40]. The nature and function of various folate-binding proteins involved in the circulation and interorgan transport of folates has been reviewed [41–44]. Differences in binding to plasma proteins or differences in transport into peripheral tissues may account for differences in in vivo retention of folates observed in folate-saturated humans [45,46].

The liver has been shown to secrete folate mainly as monoglutamyl 5-methyl-H_4folate in the bile [40,47,48], which provides for an enterohepatic circulation of folate. The quantitative significance of this process and the efficiency of reabsorption have not been determined in humans. In this regard, Krumdieck et al. [49] observed in a study of the fate of [^{14}C]folate in a human subject that the rate of fecal excretion of ^{14}C was similar to that of urinary excretion. This suggests that a significant fraction of the biliary folates and/or catabolic products are not reabsorbed. Significant fecal isotopic excretion also has been reported in studies with monkeys and rats (10 and 30% of cumulative excretion, respectively) [50,51].

The hydrolytic deconjugation of polyglutamyl folates is an essential step in folate absorption [10,14]. Although pteroylpolyglutamate hydrolase (folate conjugase) enzymes have been detected in many tissues, their role in folate absorption in various animal species has not been fully determined. Studies of folate absorption in intestinal segments of dogs suggested that the deconjugation of polyglutamyl folates may occur at the surface of the absorptive cells [52,53]. However, the detection of unchanged polyglutamyl folates in the mucosal cells suggests at least partial transport of these forms prior to intracellular deconjugation in the dog. Jejunal perfusion studies later showed that the human intestinal mucosal surface is able to deconjugate polyglutamyl folate [12,13,54]. Perfusion of radiolabeled polyglutamyl folate yielded an array of shorter-chain pteroylpolyglutamates in the luminal contents, although little or no soluble conjugase activity was detected in the aspirated intestinal fluid assayed at pH 6.8. In such perfusion studies, the accumulation of polyglutamyl folates in the intestinal lumen also provided evidence that the rate of deconjugation is greater than the rate of the absorption mechanism. Chandler et al. [55] observed parallels between brush border folate conjugase activity, immunoreactive brush border conjugase protein, and in vivo hydrolysis of polyglutamyl folate throughout the length of the porcine small intestine. These results supported the proposed function of the brush border conjugase in the digestion of dietary polyglutamyl folates in species having the brush border enzyme.

Various animal species differ considerably with respect to the location and biochemical properties of conjugases associated with the digestive system. Intestinal conjugase activity was first identified and found to be associated with lysosomes

in the enterocytes [56], which was confirmed by Wang et al. [57]. An apparently cytosolic conjugase with endopeptidase activity was subsequently identified in chicken intestine [58]. This enzyme yields a pteroyldiglutamate product [58,59]. Intestinal mucosa from the rat, other rodents, and monkeys exhibits little or no conjugase activity associated with the brush border membrane [60,61]. Alternatively, most of the intestinal folate conjugase activity is in the soluble or lysosomal fraction with a pH optimum of 4–5 [57,60–62,65]. In vivo studies with dogs suggest the presence of significant brush border folate conjugase activity [52,53], although direct analysis of isolated canine brush border membrane has not been reported.

Contrary to initial reports of the absence of brush border conjugase activity in human mucosal tissue [63], a brush border conjugase activity distinctly different from the intracellular enyzme was reported by Reisenauer et al. [64]. The brush border conjugase differed from the intracellular conjugase with respect to its neutral pH optimum (pH $\sim$ 6.5), lack of inhibition by *p*–hydroxymercuribenzoate (a strong inhibitor of the intracellular enzyme), cation requirements, and chromatographic behavior. These findings have been extended in studies showing a high degree of similarity between the brush border conjugases of both swine and humans [60,61,64–67], with distinct differences between the properties of these enzymes and the intracellular conjugase purified from rat and human intestinal mucosa [57,62]. These differences in the properties of intestinal conjugases of various species are summarized in Table 1. On the basis of these findings, it appears that the pig would be the ideal animal model in terms of similarity to humans. Monkeys apparently do not exhibit significant intestinal brush border conjugase activity [61]. The nutritional significance of these differences among species has not been determined.

Reisenauer and Halsted [68] have calculated that the activity of human jejunal brush border conjugase exceeds that needed for hydrolysis of dietary polyglutamyl folates and, thus, is not rate limiting in the absorption process. Similar conclusions also have been reached concerning folate absorption in rats [69]. Because of the necessity for deconjugation prior to folate absorption, inhibition of the enzyme by food components or pharmaceuticals could reduce folate absorption, as discussed below.

The mechanism of hydrolysis of dietary polyglutamyl folates in animals lacking a brush border conjugase (i.e., rat and monkey) is unclear. Conjugase activity has been reported in the bile [70–72] and pancreatic juice [72–76] of rats, pigs, and humans. The biliary and pancreatic conjugases have not been fully characterized, and their activity has not yet been adequately quantified in humans. The results of Horne et al. [71] and Kesavan and Noronha [75,76] suggest that biliary and pancreatic conjugases may be responsible for the deconjugation of dietary polyglutamyl folates in the rat in view of the absence of a brush border conjugase.

Bhandari et al. [72] examined conjugase activity of porcine bile and pancreatic fluid and found insignificant activity in bile relative to that of pancreatic fluid.

Table 1 Comparison of Selected Enzymatic and Molecular Properties of Intestinal Folate Conjugases (Pteroylpolyglutamate Hydrolases) from Various Species

Fraction and property	Rat	Monkey	Pig	Human	Ref.
Brush Border Membrane					
pH optimum	—	—	6.5–7.0	6.5–7.0	64, 65, 66, 67
Specific activity (pmol/hr/mg protein)	~0	~0	178	341	57,60
Effect of 1 mM Zn^{2+}	—	—	stim	stim	60, 64, 65, 67
Effect of pHMB	—	—	none	none	60, 64
Mol. weight[a] (*1000)					55, 64, 65, 67
native	—	—	237–400	700	
subunits	—	—	120, 195	145, 115	
Mode of action	—	—	exo	exo	65, 67
K_m for $PteGlu_3$ (μM)	—	—	1.0–1.7	0.6–0.8	65, 66, 67
High-speed supernatant (cytosolic or lysomal conjugase)					
pH optimum	4.5	4.5–5.0	4.5–5.0	4.5–5.0	57, 60
Specific activity (pmol/hr/mg protein)	84	102	89	214	57
Efect of 1 mM Zn^{2+}	inhib	inhib	inhib	inhib	57, 60
Effect of pHMB	inhib	inhib	inhib	inhib	61, 62
Mol. weight (*1000), native	80	ND	ND	75	61, 62
Mode of action	mixed	ND	mixed	mixed	60, 61, 62, 65
K_m for PteGlu3 (μM)	0.21	ND	ND	1.2	61, 62

stim, Stimulation; inhib, Inhibition; pHMB, parahydroxymercuribenzoate; $PteGlu_3$, pteroyltriglutamate; ND, not determined.

[a] For molecular weights of subunits, the MW of the primary band from SDS-polyacrylamide gel is listed first. Molecular weights for native proteins were estimated by nondenaturing PAGE [67] or gel filtration chromatography [61, 62, 65, 66].

The pancreatic conjugase of pigs was present in sufficient quantity that, in spite of its pH optimum at pH 4.5, this enzyme could be involved in the initiation of folate deconjugation in concerted action with the brush border conjugase. The secretion of pancreatic conjugase in pigs was stimulated by food consumption, as occurs with trypsin and bicarbonate. Secretion of pancreatic conjugase in the rat has been shown to be stimulated by consumption of polyglutamyl folates [76]. Pancreatectomized rats exhibit impaired absorption of polyglutamyl folates, which indicates that pancreatic juice is the primary source of conjugase for hydrolysis of polyglutamyl folates in this species [75]. Conjugase in pancreatic juice from pigs exhibits endo/random specificity [72], in contrast to the exohydrolytic action of the brush border enzyme

[65,67]. This finding is consistent with a proposed function of the pancreatic enzyme in possibly initiating the deconjugation of dietary folates in the pig and potentially in humans prior to complete hydrolysis by the brush border conjugase [72].

Although the existence of conjugase activity in human intestinal fluid has been reported, its significance (if any) is unclear. Klipstein [77] detected conjugase activity in semi-quantitative assays of aspirated intestinal fluid, but Halsted et al. [54] detected little activity in conjugase assays at pH 6.8. Even in fasting subjects, the negative results of Halsted et al. [54] do not exclude the presence of pancreatic conjugase activity in view of its acidic pH optimum [71–73]. Chandler et al. [55] calculated that conjugase activity in porcine pancreatic juice (even when maximally stimulated) was minor relative to the activity of jejunal brush border. Further study is needed to clarify the physiological roles of the various conjugases in folate digestion and absorption.

III. METHODS FOR THE ASSESSMENT OF FOLATE BIOAVAILABILITY

Methods for the assessment of vitamin bioavailability include bioassays with animals (rat and chick), short-term single-dose protocols with humans and animals, intestinal perfusion studies in animals or humans, and in vivo isotopic methods in animals or humans [4,78]. Intestinal perfusion and cell culture studies have been useful in determining absorption rate in certain conditions; however, such methods provide little information that is applicable to predicting the bioavailability of vitamins in foods.

A. Bioassay Procedures with Animals

A fundamental issue in the study of folate bioavailability is the appropriateness of animal models. Many questions exist regarding the differences among species in specificity and location of folate conjugases involved in deconjugation of dietary polyglutamyl folates. In humans and pigs, deconjugation is primarily catalyzed by the conjugase of the jejunal brush border membrane, with minor secondary action apparently from a conjugase in pancreatic juice. In contrast, rats and most other species examined have little or no jejunal brush border conjugase; apparently the pancreatic conjugase is responsible for most folate deconjugation in rodent species [60,61,76]. Thus, studies of the bioavailability of polyglutamyl folate using rats may have little or no predictive value with respect to humans. Studies of the absorption of *monoglutamyl folate* in rats would be largely appropriate in estimating the bioavailability of monoglutamyl folate for humans since the absorption mechanism is the same in rats and humans [21]. These questions about the appropriateness of animal models provide a major impetus for the greater use of human subjects in many aspects of folate research. To date, the rat and chick have been employed

as primary bioassay animals [79,80]. As discussed previously, the pig is physiologically better suited in view of its similarity to the human, although its size and expense may preclude its routine use.

Bioassays with rodents are complicated by the ability of intestinal microflora to synthesize folate, which may be available to the animal by coprophagy [81,82]. In addition, microbial folate may be absorbed directly in the colon of the rat to a small extent [83]. Diet composition, especially the form and amount of dietary fiber, influences microbial populations and extent of folate synthesis [84]. This can yield differences in endogenous folate synthesis and utilization between control groups and those receiving test diets. Dietary antibiotics have been employed to suppress the microbial synthesis of the vitamin [85–87], but the use of antibiotics also complicates the interpretation [84] and introduces further questions as to the relevance of the rat model. Tail-cup devices that collect feces and prevent coprophagy reduce the contribution of microbial synthesis to the folate nutriture of the rat, although the interpretation of rat bioassays conducted using this technique also may be ambiguous [82].

Depletion-repletion bioassay procedures for available folate using the rat have been reported by Keagy and Oace [88] and Hoppner and Lampi [89,90]. These procedures, which do not employ oral antibiotics, were reported to yield a dose-response relationship between dietary folate and liver folate. Abad and Gregory [82] employed a slight modification of these procedures in studies of the bioassay response. In these studies, liver folate was found to be an inconsistent indicator of available dietary folate, while fasting plasma folate was a highly sensitive criterion. Abad and Gregory [82] also observed that the rat bioassay was sensitive to overestimation of available folate when conducted with or without the prevention of coprophagy.

Rat bioassay procedures have been extended by Clifford and associates through the use of amino acid–based diets [87]. The very low folate content of the basal diet and the use of a dietary antibiotic permit precise control of folate intake and sharp dose-response curves with respect to growth, blood folate, and tissue folate [91,92]. As with other rat bioassay procedures, the suitability of this animal model remains an unresolved question in view of the differences in digestive physiology.

The chick has been used for folate bioassays because of its sensitivity to and rapid onset of folate deficiency, ease of handling, high growth rate, and less incidence of coprophagy. Chick bioassays have been based mainly on methods developed by Scott et al. [93]. Although growth may not be a specific indicator of available dietary folate, plasma and liver folate are highly sensitive criteria [94]. This basic procedure has been used to assess the bioavailability of folate in foods and to determine folate activity of various forms of the vitamin [94–97]. A main concern in the use of the chick is its validity as an animal model in terms of the differences in mechanism of intestinal enzymatic deconjugation of polyglutamyl folates [58].

B. Short-Term Nonisotopic Studies with Human Subjects

The first use of human subjects in studies of vitamin bioavailability was reported by Melnick et al. [98] for studies involving ascorbic acid, niacin, riboflavin, and thiamin. These procedures were based on measurement of urinary excretion over a 24-hour period following ingestion of a reference dose or test substance. This general approach was first employed in studies by Tamura and Stokstad [99] and Perry and Chanarin [100,101] to assess folate bioavailability. Tamura and Stokstad [99] observed a weak and inconsistent relationship between the amount of ingested folate and urinary folate excretion in subjects receiving no chronic folate supplement. In contrast, linear relationships between ingested and excreted folate were found when the subjects were in a state of folate saturation achieved by the administration of 2 mg per day of folic acid orally. This technique, termed "folate saturation," has been used in additional studies involving the use of urinary excretion as the primary criterion of response and in many bioavailability studies with isotopically labeled folates.

The short-term (1–2 hr) increase in plasma folate concentration also has been used as an indicator of available folate (see Refs. 45,100–103). Advantageous because of their simplicity, such experiments must be interpreted with caution. The implicit assumption is that the rates of absorption are equivalent between reference and test doses and that their in vivo fates (i.e., distribution and metabolic utilization) are similar. If those conditions are met, then the plasma concentration at any sampling time is reflective of the quantity of folate absorbed [78]. Errors in interpretation can occur when folates differ in *rate of absorption* even though the *extent of absorption* may not differ significantly. Thus, methods based on a limited number of plasma folate measurements may be subject to underestimation of the bioavailability of folate in materials from which absorption is slower than that of the reference dose. Keagy et al. [103] employed such a procedure in a study of folate bioavailability and provided an excellent discussion of these limitations. Whenever possible, multiple blood samples should be taken up to 8 hours or more to permit an evaluation of the area under the plasma concentration-versus-time curve [104]. Such an approach provides greater validity in the experimental design and interpretation. A disadvantage of such protocols is the necessity for large doses to obtain a reliably quantifiable rise in plasma folate. Bailey et al. [104] reported that a single oral dose of 750 μg provided a more consistent response than 250- or 500-μg doses.

C. Isotopic Methods

The use of radioisotopes and stable isotopes provides several advantages over conventional methods of determining bioavailability [78]. Isotopic labeling of folate permits specific detection of the administered dose or its metabolites in blood, excreta, and tissues. Methods exist for the synthesis of a wide variety of folates

labeled with tritium or deuterium. While the use of radiolabeled folates provides a powerful tool for the study of folate bioavailability in animals and for diagnostic procedures in humans, stable isotopically labeled folates are better suited for experimental use in normal human subjects because of the greater safety of these compounds.

Experimental design is of critical importance, and questions regarding the most appropriate means of introducing the labeled folate(s) in isotopic studies of vitamin bioavailability have not been resolved [78]. Direct addition of exogenous folate to the test food or meal is termed extrinsic enrichment. Alternatively, incorporation into the tested food by administration to the parent plant or animal is termed intrinsic enrichment. Although the potential exists for intrinsic enrichment with labeled folates, practical limitations exist in achieving sufficient labeling for in vivo studies. In addition to isotopic protocols, many studies of folate bioavailability have been conducted using extrinsic enrichment with nonlabeled folates.

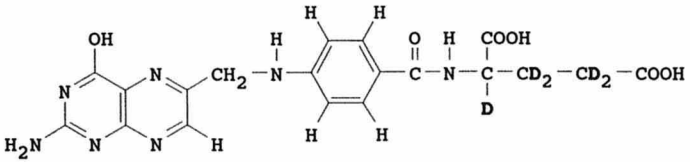
In isotopic studies, it is important to determine whether the labeled compound(s) are handled by the body in the same quantitative manner as the unlabeled nutrient. In this regard, the bioavailability of two deuterium-labeled forms of folic acid ([3′,5′-$^{2}H_2$] and [glutamate-$^{2}H_4$]) exhibit equivalent bioavailability in normal humans as judged by urinary excretion of labeled intact folates and the catabolite *p*-aminobenzoylglutamate [105,106]. This suggests that isotope effects, if any, are insignificant with these forms of the vitamin. The data of Connor et al. [107] indicate that differences in the rate of absorption between [^{3}H]- and [^{14}C]-labeled folates would be small.

Abad and Gregory [108] developed and used a single-dose protocol with rats to examine the inherent bioavailability of various [^{3}H] folates and the effect of various foods on the utilization of the labeled folates. A limitation of such studies using radiolabeled folates is the lack of labeled folates suitable for dual-isotope studies. Although [2-^{14}C] folic acid is commercially available, its specific radioactivity is too low to be useful in many studies of folate bioavailability at nutritionally relevant doses. Radioisotopic studies are generally limited to those in which a single tritiated form of the vitamin is administered. In addition, the suitability of the rat as a model to study the bioavailability of polyglutamyl folates is uncertain.

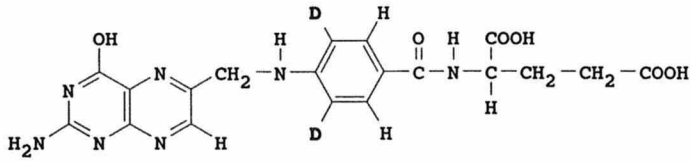
Stable isotopes are isotopes with stable nuclei, i.e., they do not undergo decay and emit no radiation. For organic nutrient compounds such as folate, stable isotopic labeling requires incorporation of one or more atoms of the desired isotope per folate molecule. This has the effect of increasing the molecular mass in proportion to the number of atoms incorporated. Isotopes suitable for labeling include deuterium (^{2}H), ^{13}C, ^{15}N, and ^{18}O. The use of stable isotopic methods requires mass spectrometry for the determination of extent of isotopic labeling (i.e., enrichment) of folates in biological samples (e.g., blood or urine). Folate molecules are poorly suited for many forms of mass spectrometry because of their lack of volatility and their thermal instability. These problems have been overcome through

the technique of cleavage of the C9-N10 bond and isolation of the *p*-aminobenzoylglutamate (pABG) fragment [109,110]. Derivatization of pABG using trifluoroacetic anhydride-trifluoroethanol forms an internal lactam that is well suited for gas chromatography–mass spectrometry (GCMS) in the negative-ion chemical-ionization electron-capture mode [110]. Affinity chromatography using immobilized folate-binding protein [111] provides suitable purification of folates in biological samples prior to preparation for GCMS analysis [109].

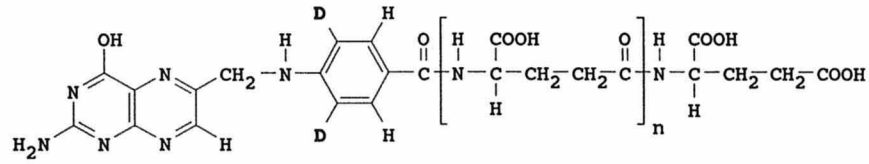
Labeling with deuterium was selected in methods developed by the author because of the ease of synthesis, relative to other potential methods, and the metabolic retention of the isotopes. Two approaches have been taken in deuterium labeling, as shown in Figure 1. [$^2H_2$]Folic acid monoglutamate can be prepared with deuterium at the 3′ and 5′ positions of the central benzene ring introduced by catalytic debromination [112,113] or acid-catalyzed exchange [114], and [$^2H_2$]pteroylpolyglutamates are synthesized using [3′,5′-$^2H_2$]pteroic acid [115,116]. Deuterated monoglutamyl folic acid also can be synthesized alternatively by cou-



Monoglutamyl Folate (glu-d5)



Monoglutamyl Folate (3',5'-d2)



Polyglutamyl Folate (3',5'-d2)

Figure 1 Structural formulas for deuterium-labeled folates suitable for in vivo studies in humans. Deuterium atoms (designated D) can be introduced in the 3′ and 5′ positions of the central benzenoid ring or on the glutamate chain.

pling pteroic acid to commercial [$^2H_4$] or [$^2H_5$]glutamic acid to yield [glu-$^2H_4$ or glu-$^2H_5$]folic acid [109]. Except for differences in molecular mass, these deuterated folates are chemically and metabolically identical to the nonlabeled species.

Many studies of folate bioavailability have involved predose saturation regimens to accentuate the excretion of the experimental dose. However, because of in vivo retention, catabolism, and fecal excretion, saturation regimens do not yield complete recovery of the isotopically labeled dose as intact urinary folate.

Using a 2 mg per day saturation dose for the preceding 7 days, the recovery of intact deuterium-labeled folates initially has been found to be 5–20% over 48 hours postdose following the oral administration of [$^2H_2$]- or [$^2H_4$]folate [105,109]. The metabolic equivalence of these two labeled forms of the vitamin permits direct comparisons in dual-label studies of folate bioavailability even with this comparatively low isotopic recovery. Repetitive use of this saturation protocol in six trials at 3-week intervals yielded a progressively increasing urinary recovery of injected [$^2H_4$] folate as intact urinary folate, from 25% in trial 1 to 51% in trial 6 [46]. These results, which showed a gradual increase in saturation, are consistent with the very slow turnover in in vivo kinetics observed for body folate [49] and indicate the difficulty in direct comparisons of bioavailability trials based on repeated saturation protocols. Alternate saturation protocols, as employed in studies with unlabeled folates, have involved the administration of 10–15 mg of folic acid per day for several days [100–102]. The relative merits of these various approaches have not been assessed fully.

Studies with radioactive folates have provided information that is potentially useful in establishing protocols. Rather than a saturation protocol with oral folate, Russell et al. [117,118] employed a "flushing" dose of 15 mg of folic acid injected intramuscularly 24 hours prior to absorption studies with [^3H]folic acid. The effectiveness of this procedure was shown by the recovery of typically 55–60% of the ingested tritium in 24-hour urine collected from normal subjects. The use of a postabsorptive "flushing" dose was originated by Klipstein [119], who employed a 15-mg injection of nonlabeled folic acid 3 hours after the oral dose of [^3H]folate. Other investigators have used a "flushing" dose with 10–15 mg of folic acid injected intramuscularly within 6 hours postdose, to enhance the urinary excretion of the administered isotope(s) [54,120–126]. It should be noted that the similarity of isotopic recoveries obtained using injections of 15 mg of folic acid 24 hours before or 3 hours after the radiolabeled dose suggest that the elevated level of tissue folate associated with predose saturation protocols does not inhibit folate absorption.

Several questions remain to be addressed in the development of optimal isotopic protocols for the study of folate bioavailability. (1) What is the appropriate saturation protocol? (2) What, if any, is the appropriate flushing dose? (3) If analytical sensitivity is sufficient, can stable isotopic studies of folate bioavailability be done without saturation or flushing? (4) Are single-dose or multidose protocols more useful in studies of nutrient bioavailability?

Table 2 Bioavailability of Various Monoglutamyl Folates as Determined in Human Subjects[a]

Study and response criterion[b]	THF	5-CH_3-THF	5-HCO-THF	10-HCO-THF	DHF	PteGlu
Perry and Chanarin [100]						
0- to 2-hr change in serum folate	117	218	179	ND	ND	100
6-hr urinary folate	25.7	77.7	59.0	ND	ND	100
Brown et al. [45]						
0- to 2-hr change in serum folate	40.5	135	101	159	156	100
Tamura and Stokstad [99]						
8-hr urinary folate	105	121	70	ND	ND	100
Gregory et al. [46]						
0- to 24-hr urinary excretion of [$^2H_2$] folates (derived from oral dose) relative to [$^2H_4$] folates (derived from injected control)	46.3	73.9	66.7	64.6	ND	100

All values are means. For tetrahydrofolates, the values quoted refer to the biologically active C-6 isomer. Abbreviations: THF, 5, 6, 7, 8-tetrahydrofolate; 5-CH_3, 5-methyl; 5-HCO, 5-formyl; 10-HCO, 10-formyl; DHF, 7, 8-dihydrolate; ND, not determined.

[a] Values are percentages relative to the response of folic acid. Note that all studies were conducted with some form of saturation protocol.

[b] The differences observed are indicative of large differences in in vivo retention and distribution under conditions of folate saturation. On the basis of animal experiments, little or no difference in the extent of intestinal absorption was expected in these studies involving fasting human subjects. Differences in renal reabsorption may also occur as a result of folate saturation.

IV. BIOAVAILABILITY OF FOLATE

The determination of the bioavailability of dietary folate is complicated by possible differences in inherent bioavailability among various folates, the effects of various dietary components and pharmaceuticals on the intestinal deconjugation and absorption processes, and the effects of various digestive abnormalities that may affect the extent of absorption. The following is a review of present knowledge concerning this topic.

A. Inherent Bioavailability of Various Folates

Superimposed upon the influence of diet composition on folate bioavailability are inherent differences in the apparent bioavailability of the various folates.

1. *Bioavailability of Polyglutamyl Folates*

The essentially complete absorption of folic acid, when administered under fasting conditions, has been well established, and the relative bioavailability of purified monoglutamyl and polyglutamyl folates has been extensively investigated in fasting subjects. The relative bioavailability of monoglutamyl folic acid versus the corresponding polyglutamyl species has been examined using isotopically labeled folates administered orally or by intestinal perfusion.

The extent of absorption of polyglutamyl folate in normal individuals, as evaluated by urinary excretion of the isotope after a flushing dose, ranges from approximately 60 to 80% relative to that obtained with monoglutamyl folic acid in fasting subjects [54,117,118,121,123–125]. In contrast, Rosenberg and Godwin [126] reported equivalent utilization of [^{3}H]folic acid and polyglutamyl [^{3}H]folate in a similar study. Gregory et al. [116] conducted a single-dose study with deuterated mono- and polyglutamyl folates in folate-saturated humans and found approximately 50% relative bioavailability of the polyglutamate as judged by urinary excretion.

Protocols with nonlabeled folates quantified by urinary folate excretion in folate saturated human subjects have indicated a mean bioavailability of 85–90% for triglutamyl and heptaglutamyl folates relative to folic acid [99]. Bailey et al. [104] compared the area under the curve of plasma folate concentration and found equivalent responses to 750 μg of folic acid and the molar equivalent of heptaglutamyl folate. Keagy et al. [103] reported that the bioavailability of heptaglutamyl folate repeatedly administered to humans in formula diets was 63% relative to folic acid. In marked contrast to these studies, Perry and Chanarin [101] reported approximately 25% apparent bioavailability, relative to folic acid, of polyglutamyl folates isolated from yeast. This low response may have been due to conjugase inhibitors (nucleic acids) possibly in the yeast folate preparation [127]. Overall, the results of these numerous studies indicate effective, but frequently incomplete, absorption of polyglutamyl folates, although the relative difference between monoglutamyl and

polyglutamyl folates appears to vary according to the protocol used. This incomplete bioavailability of polyglutamyl folates provides strong evidence for the potentially rate limiting nature of intestinal deconjugation processes. Any effects of inhibitory substances in food, or reduced conjugase activity in certain pathological conditions, could further lessen the bioavailability of polyglutamyl folates.

In spite of the lack of intestinal brush border folate conjugase in rats, effective utilization of polyglutamyl folates still occurs. Rat bioassays have indicated little or no difference in the bioavailability of folic acid and polyglutamyl folates when fed chronically in dehydrated diets [82]. Using single-dose administration in hydrated gelled diets, the bioavailability of radiolabeled polyglutamyl reduced folates was approximately 80% that of monoglutamyl reduced folates [108].

2. *Comparison of Monoglutamyl Folates*

Whether any nutritionally significant differences in bioavailability exist among the various monoglutamyl folates has not been fully resolved. Such differences, if any, must be assessed before the bioavailability of folate in complex diets can be accurately known. The various monoglutamyl folates have been reported to exhibit small differences in kinetic constants for intestinal transport [15,18,21], but transport studies simultaneously comparing all folates have not been reported.

In studies of tritiated folates administered in alginate gel orally to rats, 5-formyl-H_4folate exhibited similar absorption and urinary excretion but significantly greater hepatic retention of ^3H than rats given [^3H]folic acid [108]. Chick bioassays have shown that the bioavailability of 5-methyl-H_4folate was equivalent to that of folic acid when present in a purified diet [95].

Several studies have been conducted to determine the relative bioavailability of monoglutamyl folates in folate-saturated humans [45,46,99,100]. As summarized in Table 2, little agreement exists among these studies with respect to the relative bioavailability of monoglutamyl forms of various folates. Perry and Chanarin [100] conducted the only studies in which both serum folate and urinary folate measurements were evaluated in response to the single test dose. Relative to the response to folic acid, the tetrahydrofolates examined exhibited higher serum concentration and lower urinary excretion. These results suggest greater in vivo retention of orally administered tetrahydrofolates in folate-saturated subjects. In contrast, however, the results of Brown et al. [45] could not be attributed to specific structural characteristics of the folates examined. Similarly, the results of Tamura and Stokstad [99] showed little difference in urinary excretion among three tetrahydrofolates and folic acid.

Perhaps the most quantitatively sound study on this topic involved a dual-label stable-isotopic investigation in which the urinary excretion of [3′,5′-$^2H_2$]folates derived from the respective oral dose was evaluated relative to the simultaneous excretion of [glu-$^2H_4$]folates derived from an intravenous injection of [glu-$^2H_4$]folic acid [46]. Six trials were conducted to evaluate oral doses of folic acid,

tetrahydrofolate (H_4folate), 5-methyl-H_4folate, 5-formyl-H_4folate, and 10-formyl-H_4folate, each in comparison to its simultaneous injected control. The ratio of urinary $^2H_2$ to $^2H_4$folates was determined to provide a direct assessment of the relative bioavailability of each oral form of the vitamin. On this basis, all H_4folates examined showed lower urinary excretion relative to the injected control than was observed with folic acid. Because only urine could be analyzed, excretion data did not fully differentiate between differences due to variation in intestinal absorption or retention in tissues. These results strongly support the hypothesis of greater in vivo retention of oral H_4folates in folate-saturated subjects as reported by Perry and Chanarin [100]. Assuming that similar, nearly complete, absorption occurred in the fasting subjects (as seen in rats, discussed below), the results of Perry and Chanarin [100] and Gregory et al. [46] suggest that poorer in vivo retention occurred for oral folic acid than for 5-formyl, 10-formyl, 5-methyl, and unsubstituted forms of [$^2H_2$]H_4folate in folate-saturated subjects. Under conditions of folate saturation, differences among circulating folates with respect to tissue uptake and renal clearance and/or renal reabsorption could be accentuated beyond those normally occurring during typical folate intake. The reasons for the contrasting results of the other studies are unclear but may be related to differences in protocols, preparation of doses, variable purity of the tested compounds, etc.

A similar comparative study with rats fed normal levels of dietary folate showed no difference in absorption, tissue distribution and metabolism, and excretion between tritiated forms of folic acid, 5-formyl-H_4folate, and 5-methyl-H_4folate [50] (Fig. 2). More than 99% of each dose was absorbed in the fasting rats used

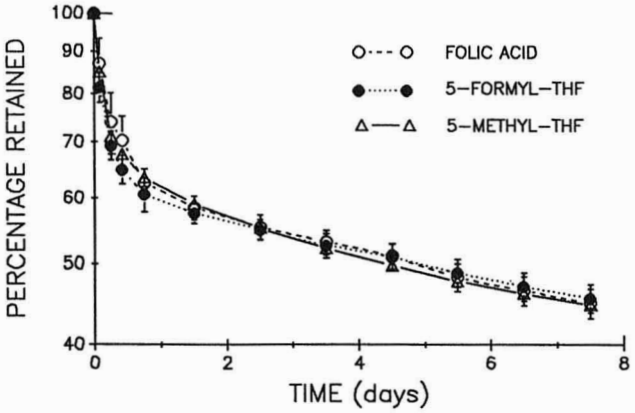


Figure 2 Whole body retention of radiolabeled folates in rats following a single oral dose of tritiated folic acid, 5-methyl-H_4folate, or 5-formyl-H_4folate. Retention was calculated as the administered dose minus urinary and fecal excretion. These results indicate essentially equivalent bioavailability and short-term in vivo kinetics of these folates.

in this study over the 8-hour observation period. Minor differences in tissue distribution of the labeled folates were observed at the 8-hour time point, but there were no significant differences in the overall kinetics of absorption and metabolism. Taken together, these results suggest little nutritionally significant difference in the in vivo absorption and utilization among the various monoglutamyl folates under normal dietary conditions.

B. Nondietary Factors Affecting Folate Bioavailability

1. *Altered Gastrointestinal Function and Folate Absorption*

Conditions in which the function of the digestive system is altered can markedly affect the bioavailability of ingested folate, as reviewed previously [7–10, 12,13,128–130]. The malabsorption of nutrients that occurs in tropical and celiac sprue and other intestinal disorders frequently involves impaired absorption of both mono-and polyglutamyl folates [7,13,124,125,131,132]. Patients with Crohn's disease do not exhibit folate malabsorption in normal regions of the jejunum [133]. Folate absorption and metabolism may be further impaired by anti-inflammatory drugs commonly used for treatment of these conditions (see Sec. B.3).

Because of the profound influence of intestinal pH on folate absorption, alteration of the pH of the intestinal contents can affect folate absorption. Reduced gastric acid secretion associated with atrophic gastritis in the elderly causes reduced absorption of [^{3}H]folic acid [117]. Folate absorption in atrophic gastritis could be enhanced by the simultaneous administration of acid, confirming a pH effect. The serum folate concentration was normal in the patients examined, apparently due to bacterial overgrowth in the small intestine associated with atrophic gastritis.

An opposite effect of intestinal pH may also occur. Patients with pancreatic insufficiency exhibited greater folate absorption than normal controls because of their slightly acidic intestinal pH (nearer the optimum for folate absorption) because of insufficient pancreatic bicarbonate secretion [118]. The oral administration of phosphate or bicarbonate buffering compounds normalized intestinal pH and reduced folate absorption to the normal range.

Surgical resection of portions of the small intestine may reduce folate absorptive capacity for nutrients. Compensatory increases in the absorption of certain nutrients has been observed, although the situation with folate is unclear. The specific activity of jejunal brush border folate conjugase has been reported to be increased in the remaining intestinal mucosa following partial surgical resection, which suggests that such an effect may occur with folate deconjugation [134,135].

Infection with human immunodeficiency virus (HIV) has been shown to cause a reduced serum folate response to a 5-mg oral dose of folic acid in fasting subjects [136]. This effect occurred regardless of the stage of the disease, gastrointestinal complications, or drug therapy. Whether this apparent malabsorption would also occur at doses consistent with dietary folate intake has not been determined.

2. *Effects of Age*

Little direct evidence supports the hypothesis that the elderly are especially prone to folate deficiency [137,138]; however, folate deficiency has been documented in certain segments of the elderly population [139]. The reasons for folate deficiency in the elderly could, at least in part, be related to marginally adequate diet coupled with possibly impaired folate bioavailability [138]. Aging does not alter the basic capacity for intestinal deconjugation and absorption of folate [123,140]. These issues are discussed further in Chapter 5.

3. *Antagonistic Effects of Ethanol and Other Drugs*

The existence of folate deficiency in chronic alcoholics has been well documented. The antifolate effects of ethanol could occur by impaired intestinal deconjugation, absorption, or transport processes, impaired metabolism, or enhanced catabolism or excretion of folates, as discussed in Chapter 11.

A number of therapeutic drugs act as antagonists of folate absorption, metabolism, or both. Cholestyramine impairs folate absorption in rats [141], presumably through binding folate as an anion exchanger [18]. The antifolates methotrexate, trimethoprim, and primethamine function as dihydrofolate reductase inhibitors and, thus, could interfere with the utilization of dietary folates. These folate analogs also exert antifolate activity by inhibiting intestinal absorption [15,24] and potentially other sites of folate transport [41].

Salicylazosulfapyridine (Azulfidine®, sulfasalazine) is a nonfolate drug used in inflammatory bowel disorder that exhibits antifolate effects. This compound has been shown to inhibit folic acid absorption in humans [142,143], and it behaves as a competitive inhibitor of folate absorption in vitro [17]. Salicylazosulfapyridine also has been shown to inhibit jejunal brush border conjugase [144] and several other enzymes involved in folate metabolism [145]. Thus, this compound would alter the bioavailability of dietary folate through effects on intestinal deconjugation and absorption as well as on folate metabolism.

Diphenylhydantoin (Dilantin®) is an anticonvulsant drug associated with folate deficiency. This compound retards the intestinal absorption of folic acid in humans [133,146] but, surprisingly, not in rats [147]. While the effects of diphenylhydantoin on the absorption of polyglutamyl folate have not been directly determined, Reisenauer and Halsted [144] found that diphenylhydantoin does not inhibit human jejunal brush border conjugase in vitro. Nelson et al. [148] reported that, as determined with jejunal perfusion, diphenylhydantoin did not inhibit the absorption of folate from orange juice (mainly as 5-methyl-H_4folate polyglutamates of various chain lengths). Krumdieck et al. [49] examined the influence of diphenylhydantoin administered to a single human subject. They observed no effect of the drug on the kinetics of folate excretion or on the pattern of excreted folate catabolites. An unrelated anticonvulsant, phenobarbitone, has been reported to impair folate status, possibly by enhancing folate catabolism through induction of

mixed-function oxygenases [149]. It should be noted, however, that the mechanism of folate catabolism has not been elucidated, and no role of mixed-function oxygenases has been reported.

Several nonprescription drugs have the potential of reducing folate absorption. Chronic consumption of aspirin and other salicylates may exert an antagonistic effect on folate status [150] by retarding absorption [151] or by enhancing excretion. However, incorporation of aspirin chronically in rat diets did not reduce the bioavailability of mono- or polyglutamyl folate in rat bioassays [152]. As discussed previously, compounds or conditions that alter intestinal pH could enhance or inhibit folate absorption. In this regard, antacids could reduce folate absorption by increasing the pH of the upper intestine. Russell et al. [118] reported that antacid consumption may inhibit folate absorption in normal human subjects, but dose-response effects were not fully determined. In contrast, chronic consumption of supplemental sodium bicarbonate in a rat bioassay did not reduce folate bioavailability [153]. This lack of effect could have been due to a buffering action of the diet components.

Many drugs used for their antiinflammatory properties in the treatment of arthritis have been associated with antifolate effects. Baggott et al. [154] reported that commonly used nonsteroidal antiinflammatory drugs inhibited several enzymes in folate-dependent one-carbon metabolism and suggested that this may be involved in their cytostatic and antiinflammatory properties. The extent to which these drugs may interfere with the metabolic utilization of dietary folate has not been determined.

C. Bioavailability of Folates from Foods

Quantitative aspects of the bioavailability of dietary folate for humans are poorly understood. Animal models provide, at best, qualitative information and may yield little useful information with respect to folate bioavailability in humans. Several extensive reviews have been published on this topic [2–5,155,156]. At the present time, the bioavailability of dietary folate cannot be predicted with acceptable accuracy and precision. This situation complicates the interpretation of nutrition surveys, assessment of diet-disease relationships, and evaluation of the merits of food-fortification programs.

1. *General Aspects of Folate Bioavailability*

Although the bioavailability of folate is undoubtedly incomplete in many foods, little reliable quantitative information exists at this time. Modern analytical methods have shown conclusively that dietary folate is comprised mainly of polyglutamyl species comprised of an array of chain lengths, oxidation states, and one-carbon substituents [157]. As discussed previously, many of the in vivo methods for determination of folate bioavailability have procedural or conceptual limitations. In addition, traditional methods for the measurement of total folate in foods are not well

standardized or verified. Many of the extraction methods used in traditional methods of folate assay are ineffective and yield an underestimation of the folate in foods [158]. The use of inefficient extraction procedures for assays in studies of folate bioavailability would cause an underestimation of the total folate concentration and, thus, render attempts to determine bioavailability essentially meaningless. Certain published studies probably suffer from this analytical problem.

Evidence of incomplete bioavailability of folate in foods was first obtained in short-term studies with human subjects. These studies indicated that foods differ widely with respect to the apparent bioavailability of endogenous folate and their impact on the utilization of added exogenous folate, as summarized in Table 3 [2,99,159,160]. Limitations of these studies include wide variation among subjects and the need for administration of large quantities of the food items tested to achieve measurable responses. Thus, the relevance of these results to normal human diets is unclear. For comparison, the results of folate bioavailability studies in animals are summarized in Table 4. On the basis of these studies, it is impossible to generalize with respect to relationships between source of the food or food composition and bioavailability of endogenous or exogenous folate.

In perhaps the most thorough assessment of folate requirements and the bioavailability of naturally occurring dietary folate, Sauberlich et al. [161] conducted a 92-day controlled metabolic study in women. The subjects received various amounts of folate from dietary sources, added folic acid, or a combination of dietary and added folate. These investigators concluded that a daily intake of 300 μg of total dietary folate (i.e., from food sources) was sufficient to meet the folate requirement, which was substantially higher than the intake of folic acid found to meet the requirement. In comparison to the synthetic folic acid, the response to dietary folate indicated no more than 50% bioavailability. At present this is the most conclusive estimate of the overall bioavailability of folate from food sources.

Probably the most definitive information regarding folate bioavailability was provided in studies by Colman and associates [102,162–164], who examined the efficacy of fortification of South African staple foods with folic acid. On the basis of 1–2 hours of plasma folate response, the apparent bioavailability of folic acid in fortified maize (as porridge) and rice was 50–60%, relative to the response observed with aqueous folic acid, and the apparent bioavailability of fortified bread was approximately 30–40% in the same study [102]. The long-term rate of change of erythrocyte folate during chronic administration of folic acid supplements, fortified maize meal or bread fortified with folic acid substantiated these findings and supported the validity of the short-term protocol in this context [162,163]. Bread fortified with folic acid to provide 900 μg per day yielded erythrocyte folate values similar to those obtained with 300 μg of folic acid in tablet form, which indicated approximately 33% bioavailability of the added folic acid in bread. Similarly, maize meal (prepared as a porridge) providing 500 μg added folic acid per day yielded erythrocyte folate values that were similar to those obtained with 300 μg

Table 3 Apparent Bioavailability of Endogenous Folate in Selected Foods Determined in Studies Using Human Subjects

Study and protocol	Amount of test food (g)	Percentage bioavailability	Range
Retief [159] (single subject, no folate saturation; 24-hr urinary folate, semiquantitative data)			
Tomato (raw)	1340	low	
Calf liver (fried)	505	high	
Cauliflower (cooked)	1400	moderate	
Peas (cooked)	1167	high	
Pumpkin (cooked)	780	low	
Spinach (cooked)	800	high	
Baker et al. [138] (no folate saturation; serum folate 2.5 and 5 hr postdose)			
Brewers' yeast (young adult subjects)	35	100	
Brewers' yeast (elderly subjects)	35	10	
Schertel et al. [167] (no folate saturation; 24-hr urinary folate) Brewers' yeast	45	30	
Tamura and Stokstad [99] (folate saturation; 8-hr urinary folate)			
Banana	890	82	0–148
Lima beans (dried, cooked)	560	70	0–138
Lima beans (frozen, cooked)	360	96	48–181
Liver (cooked)	63–94	50	22–103
Brewers' yeast	46–55	60	55–67
Cabbage (cooked)	500–700	47	0–127
Cabbage (raw)	500	47	0–93
Defatted soybean meal	200	46	0–83
Wheat germ	170	30	0–64
Orange juice concentrate	600	35	29–40
Egg yolk	250	82	61–100
Babu and Srikantia [160] (folate saturation; 24-hr urinary folate)			
Tomato	1000	37	24–71
Spinach	200	63	26–99
Banana	533–700	46	0–148
Egg, whole	300–500	72	35–137
Liver, goat	100	70	9–135
Rhode et al. [178] (long-term (7-wk) serum folate response to daily supplement as either folic acid (100 μg/day) or orange juice (100 μg folate/day), relative to unsupplemented control)			
Orange juice	~150 ml twice daily	~50	OJ and PteGlu groups not significantly different

Table 4 Bioavailability of Naturally Occurring Folate in Selected Foods as Determined by Animal Bioassay Procedures

Study and protocol	Percent bioavailability		
	Serum or plasma folate	Liver folate	Weight Gain
Abad and Gregory [82] (rat bioassay, multiple levels of test foods, slope ratio design)			
Orange juice	146		
Cabbage, blanched	68		
Ristow et al. [95] (chick bioassay, single level of each test food)			
Cabbage (raw)	100		
Cabbage (blanched)	45		
Calf liver (cooked)	134		
Hoppner and Lampi [147] (rat bioassay, multiple levels of test foods, slope ratio design)			
Brewers' yeast		104	
Babu and Lakshmaiah [86] (rat bioassay, single level of each test food)			
Brewers' yeast		56	
Spinach (preparation method not specified)		84	
Bengal gram (roasted)		83	
Clifford et al. [175] (rat bioassay, single level of most test foods)			
Orange juice	ND	120	62
Beans (refried)	ND	82	113
Broccoli (cooked)	82	58	103
Cabbage (cooked)	58	83	74
Cantaloupe	66	116	81
Clifford et al. [202] (rat bioassay, single level of most test foods)			
Beef liver	94	101	91
Orange juice	107	99	91
Mushrooms	123	89	73
Lima beans	75	85	111
Peas	68	75	97
Spinach	65	71	75
Collards	83	74	85
Wheat germ	63	68	77

Estimates are relative to the response predicted from folic acid standards. These values are calculated according to the response criterion specified at the column heading. Because of imprecision in assays (data not shown), the means presented should be considered to be approximations. The applicability of these data to human nutrition is unclear.

folic acid tablets, i.e., 60% bioavailability. The reasons for the incomplete absorption and/or utilization of the added folic acid from these foods are unclear, but entrapment in slowly digested residue may be involved. In spite of the incomplete response, however, folic acid fortification was effective in correcting folate deficiency in the subjects examined [164].

Currently, relatively few foods are fortified with folic acid (e.g., most breakfast cereals, meal replacement products, infant formulas, and medical formulas). At the time of this writing, the Food and Drug Administration is considering folic acid enrichment of cereal-grain products to reduce the incidence of folate-preventable neural tube defects [165]. This proposal would involve the addition of folic acid to the other nutrients already included in enrichment of a variety of products including enriched breads, flours, corn meal, grits, and various pastas. The proposed level of enrichment is substantially lower than that used in studies by Colman and associates [102,162–164] and would involve technologies of fortification that would, in all probability, differ from those employed in the South African studies. In addition, the traditional techniques of food preparation used in the South African studies differed considerably from those used in industrial baking and home preparation in the U.S. Thus, the data of Colman et al. may not be useful in predicting the bioavailability of folic acid used in the American fortification context. However, these data do indicate that added folic acid would, in all likelihood, exhibit only partial bioavailability.

The bioavailability of folic acid added to ready-to-eat breakfast cereal products has not been conclusively determined, although one would predict incomplete bioavailability. In studies of other types of foods experimentally fortified with folic acid, the results of chick bioassays indicated that certain dietary components reduced the bioavailability of the added folic acid [96].

The influence of food on the bioavailability of simultaneously ingested folic acid supplements has not been determined. This must be determined to assess the overall bioavailability of folate, since the use of vitamin-mineral supplements is common in the U.S. population.

Thermal destruction of folates occurs readily in food processing and home preparation [4]. To investigate the impact of cooking or thermal processing on the bioavailability of the *remaining* folate, Ristow et al. [95] prepared liquid model food systems (similar in composition to infant formulas), which were fortified with folic acid or 5-methyl-H_4folate, and subjected them to thermal processing (121°C for 20 min). After freeze drying, the solids from heated and unprocessed model food systems blended into a basal diet and were evaluated by chick bioassay. The concentration of available folate was equivalent to total folate determined by *L. casei* and HPLC assays, which indicated no adverse effect of heat on the bioavailability of the remaining folates. Several studies have examined the bioavailability of folate in raw and cooked foods; these will be discussed in later portions of this chapter.

The extent to which components of food could influence folate bioavailability by inhibiting the requisite intestinal deconjugation processes has not been determined. As discussed previously, the utilization of polyglutamyl folates is often less efficient than that of monoglutamyl folates, *even when polyglutamyl folates are given in the absence of food*. This suggests that deconjugation is a limiting factor and that any inhibition of conjugase activity would yield corresponding reductions of folate bioavailability. In vitro studies have shown that constituents of many foods can inhibit brush border folate conjugase from human and porcine intestine [165] (Table 5). The observed inhibition was specific for folate conjugase; brush border alkaline phosphatase and sucrase were not affected by the foods tested. The in vivo significance of the inhibition of folate conjugase by food components awaits experimental confirmation.

The following discussion examines specifically several topics concerning the bioavailability of dietary folates.

Table 5 Effect of Extracts of Selected Foods and Food Components on In Vitro Folate Conjugase Activity of Human and Porcine Jejunal Brush Border Membrane Vesicles

	Relative activity (% of control)	
Food item or constituent	Porcine	Human
Red kidney beans	64.5	84.1
Pinto beans	64.9	66.8
Green lima beans	64.4	64.8
Black-eyed peas	74.1	80.7
Yellow corn meal	64.7	71.3
Wheat bran	102	100.
Tomato	91.9	85.8
Banana	54.1	54.0
Cauliflower	74.8	84.3
Spinach	78.9	86.1
Orange juice	20.0	26.6
Whole egg	88.5	94.7
Evaporated milk	86.3	ND
Cabbage	87.9	ND
Lettuce	93.8	ND
Whole-wheat flour	99.7	ND
Medium rye flour	100.2	ND
Folate binding protein, bovine milk	97.8	ND

Food substances were compared for inhibitory activity on the basis of approximately equal concentration of solids in the in vitro conjugase assay. Values are means of three trials; values $\leqslant 85\%$ were significant at $p < 0.01$. ND, not determined.

Source: Bhandari and Gregory [166].

2. *Bioavailability of Folate in Yeast*

Yeast is significant in folate research mainly as a source of concentrated polyglutamyl folates used experimentally in crude or partially purified form and as used occasionally as a nutritional supplement. The bioavailability of polyglutamyl folates in yeast has been the subject of extensive and often conflicting research [99,152,160,167–170]. Studies by Grossowicz et al. [168] indicated that much of the apparent poor utilization of yeast folate in many studies with humans was the result of large doses that exceeded the capacity of intestinal deconjugation and absorption mechanisms. Yeast also contains conjugase inhibitors, apparently nucleic acids, that also contributed to the low bioavailability of yeast folate [127,169]. The potency of these inhibitors, when present at nutritionally relevant levels, and their effects on jejunal brush border conjugase *in humans* are unclear. Hoppner and Lampi [152] demonstrated by *rat bioassay* that the bioavailability of yeast folate was equivalent to that of folic acid. As indicated previously, low bioavailability of yeast folates has been reported in chronic alcoholics and the elderly [138,169]. The factors responsible are unclear, but these populations may be susceptible to poor absorption of dietary folate under certain conditions.

Crude preparations of polyglutamyl folates from yeast have been used to examine whether a defect in maternal absorption of dietary folate may contribute to the etiology of folate-related neural tube defects [171]. To a group of women who had given birth to an infant with a neural tube defect and appropriate controls, a formula meal was administered along with a yeast extract containing 4.5-mg equivalents of folate (primarily as polyglutamates). The bioavailability of the folate in this test meal was evaluated by comparing the rate of change of serum folate at 1, 2, and 3 hours after the test meal. On this basis, no evidence of impaired absorption of this large load of dietary polyglutamyl folate was detected. In spite of limitations of experimental design, this study provides strong evidence that any defect in folate transport or metabolism associated with the etiology of neural tube defects does not involve intestinal deconjugation or absorption of dietary folate.

3. *Bioavailability of Folate in Legumes and Vegetables*

The bioavailability of folate in legumes has been the subject of extensive investigation. A heat-activated component of legumes associated with the skin of the seed inhibits various folate conjugases [172,173], although its inhibition of human jejunal brush border folate conjugase has not been examined. As summarized in Table 3, Tamura and Stokstad [99] and Babu and Srikantia [160] reported generally high bioavailability of folate in legumes, although the precision of their data was low. Similar results were observed by Devadas et al. [174]. Keagy et al. [103] studied the effect of white beans fed in a formula meal on the utilization of added folic acid or heptaglutamyl folate bioavailability in humans. The beans had little effect on the apparent bioavailability of the added mono- or polyglutamyl folate. Similar studies were performed using [^{3}H]folates in rats [108] and indicated that

the presence of cooked red kidney beans caused a small (~20%) reduction in the bioavailability of both mono- and polyglutamyl folates. These results suggested that beans exert a weak inhibitory effect primarily on the absorption, rather than deconjugation, of folates in rats. Several other foods and food components (orange juice, pectic, blanched cabbage, and wheat bran) exerted a similar effect in these studies. Clifford et al. [175] observed ~82% bioavailability of endogenous folate in dried refried beans using a rat bioassay, on the basis of liver folate data (Table 4).

While the bioavailability of folate in many vegetables has been examined (Tables 3 and 4), cabbage is perhaps the most thoroughly studied. Tamura and Stokstad [99] reported a bioavailability of endogenous folate in cooked cabbage of 47% (range 0–97%) for human subjects. A similar mean value was observed in chick bioassays, which indicated approximately 40% bioavailability of the folate in cooked cabbage (comprised mainly of polyglutamyl folate) [95]. In the same chick bioassay, raw cabbage that had undergone deconjugation during diet preparation exhibited 100% bioavailability. Rat bioassays of available folate in blanched cabbage indicated 68% bioavailability of the endogenous, largely polyglutamyl folate [82], and similar values were obtained for cooked cabbage by Clifford et al. [175]. The incorporation of blanched cabbage in rat diets reduced the bioavailability of added tritiated mono- and polyglutamyl folates [109]. These studies suggest that cabbage may partially reduce the bioavailability of mono- and polyglutamyl folate in the rat. Similarly, on the basis of liver folate data, cooked and raw broccoli exhibited similar folate bioavailability (74 ± 9% and 83 ± 45%, respectively) [175].

The specific factors involved and the extent to which other vegetables act in a similar manner have not been determined. It should be noted that all values of bioassays with humans and animals are expressed as *a percentage relative to the folic acid standard*. Because folic acid in the reference diet may not exhibit complete bioavailability, most of the results summarized here do not reflect the *absolute bioavailability* of the vitamin.

4. *Bioavailability of Folate in Orange Juice*

Orange juice and other citrus products represent primary sources of dietary folate for Americans [176]. Folate in orange juice exists as 5-methyl-H_4folate of varying polyglutamyl chain lengths [177]. Questions concerning the bioavailability of naturally occurring folate in orange juice arose following the report by Tamura and Stokstad [99] that indicated a mean bioavailability of 31% in human subjects fed a 600-g dose of orange juice concentrate. Further studies indicated that the acidity of the large dose was mainly responsible for the low apparent bioavailability; neutralization increased the bioavailability of orange juice folate and added heptaglutamyl folate [177]. As mentioned previously, in vitro studies have indicated that components of many foods, including orange juice, have the ability to inhibit

brush border folate conjugase from human intestine [166]. This may also contribute to the observed low bioavailability of folate from large experimental doses.

The bioavailability of folate in more typical serving sizes of orange juice (200–400 ml) would probably be much greater. This was actually found by Rhode et al. [178] on the basis of a 30-day study in which human subjects were given either 100 μg of supplemental folic acid or orange juice to provide 100 μg of folate. Although the serum folate values of the group receiving orange juice were somewhat lower, the values did not differ significantly between these groups. This finding indicated that the folate from orange juice was absorbed and utilized reasonably effectively [178], in contrast to the acute studies reported previously [99].

The apparent bioavailability of endogenous folate in dried orange juice was found to be greater than that of folic acid in rat bioassays [82,175], and the orange juice did not inhibit the utilization of added polyglutamyl folate [82]. However, weak inhibitory effects were detected when [^{3}H]folic acid or polyglutamyl folates was fed to rats in the presence of orange juice solids in alginate gels [108].

The overall conclusion of these studies is that the bioavailability of orange juice folates, at normal levels of consumption, is high in humans and experimental animals. These results also suggest that orange juice components generally would not cause important reductions of the bioavailability of polyglutamyl folate in other foods in mixed diets.

5. *Bioavailability of Folate in Milk*

Folate in the milk of all species examined is associated primarily with both soluble and particulate folate-binding proteins (FBP) [41–44,179,180]. FBP in milk may serve multiple functions, including (1) concentration and/or transport of folate during secretion [181,182], (2) sequestering folate from intestinal bacteria, thus promoting folate absorption by the infant [183,184], (3) enhancement of absorption by interacting with mucosal cells [185], and (4) modulation of the rate of absorption of folate from milk [184,186,187]. Enhancement of folate absorption by FBP was suggested by the observation that FBP from bovine milk enhanced the uptake of folate by isolated rat intestinal mucosal cells [185], as confirmed in further studies using intestinal brush border membrane vesicles [188]. An enhancing role of FBP on folate absorption does not appear to be significant under in vivo situations in whole intestine, however. Several studies have indicated that FBP actually reduces the rate of folate absorption in vivo [184,186,187]. Mason and Selhub [189] showed that folate bound to FBP is absorbed by a different mechanism than the absorption of free folate in the small intestine.

Swiatlo et al. [190] examined, using rat bioassays, the influence of milk on the bioavailability of several concentrations of exogenous folic acid added to diets containing milk from either cows, goats, or humans (dried milk 20% by weight). As indicated by the slope of dose-response curves based on tissue and serum folate concentration, human milk enhanced the apparent bioavailability of folic acid relative to a milk-free control diet. Bovine milk also increased the apparent

bioavailability, while goat milk had little or no effect on folate bioavailability, relative to the milk-free basal diet. These results may have been related to the differences in folate-binding capacity, or they may have been associated with minor differences in diet composition and associated effects on intestinal microflora. It is unlikely that such an effect would be nutritionally significant in humans consuming a typical mixed diet.

The majority of folate in milk is protein bound, with ~28% as polyglutamyl forms [191]. Binding of polyglutamyl folate to folate-binding proteins in milk does not appear to impair its deconjugation by human and porcine jejunal conjugase, as indicated by in vitro studies with brush border membrane vesicles and purified bovine milk folate-binding protein [166].

With respect to infant nutrition, it has been reported that consumption of human milk or formula containing bovine milk protein was the major contributing factor affecting the folate status of infants even after the initiation of solid foods [192]. These important findings suggest that the bioavailability of folate in human milk and milk-based infant formulas is higher than that of folate from other infant food sources.

Typical pasteurization of fluid milk products has little effect on the folate-binding capacity [193]. More severe pasteurization conditions or thermal sterilization fully denature the protein and eliminate folate-binding activity [177]. In view of the dual ability of human infants to absorb both free and FBP-bound folate quite well, along with the documented nutritional adequacy of infant formulas that contain no active FBP, the role of FBP in infant nutrition is unclear. Further study is needed to assess the potential influence of FBP on intestinal function, microbial colonization, and disease resistance in infants.

6. *Effects of Dietary Fiber on Folate Bioavailability*

As reviewed previously [4], the influence of dietary fiber on folate bioavailability has been examined extensively. The primary objective in these studies was to explain the general tendency of certain plant-derived foods to exhibit incomplete bioavailability of folate. As will be seen, the hypothesis that dietary fiber, per se, reduces folate bioavailability is not supported by much of the experimental data.

Ristow et al. [94] reported that diets containing various forms of dietary fiber exhibited equivalent bioavailability of added folic acid in chick bioassays. Similar conclusions regarding other forms of dietary fiber were reached by Keagy and Oace [194] using a rat bioassay. Equilibrium dialysis experiments yielded no evidence of folic acid binding by several types of dietary fiber [94]. In apparent contrast, a report by Luther et al. [195] indicated that the insoluble residue of various diets, along with filter paper and cotton, could bind low concentrations of monoglutamyl folate. The chemical identity of the binding sites, and the analytical and nutritional significance of these observations, have not been determined. Russell et al. [196] examined the effects of fiber in humans and reported that bread with high or low

fiber content had little or no influence on the absorption of simultaneously ingested [^{3}H]folic acid, relative to fasting controls. Keagy et al. [103,197] observed that wheat bran delayed slightly the absorption of exogenous heptaglutamyl folate relative to folic acid. Wheat bran also reduced the plasma response to added heptaglutamyl folate in a rat bioassay [198]. In contrast, Bailey et al. [104] reported that wheat bran cereal retarded the absorption of polyglutamyl folate but did not influence that of monoglutamyl folic acid in humans. The presence of pectin and wheat bran in hydrated gelled diets weakly retarded the utilization of *both* mono- and polyglutamyl [^{3}H]folates in rats [109], which may reflect the difference in the deconjugation mechanism of these species.

Gregory et al. [65] examined in vitro the effect of various forms of dietary fiber on the activity of human and porcine intestinal conjugase in brush border membrane vesicles. Various ionic polysaccharides including neutralized forms of pectin, polygalacturonic acid, heparin, and sodium alginate did not inhibit the activity in a medium containing adequate Zn^{2+} (an essential cofactor). It was concluded that these polyanionic compounds probably would not directly retard the action of brush border conjugase in vivo. Although various foods contain constituents that may inhibit intestinal brush border folate conjugase [166], dietary fiber does not appear to be significantly involved. The human intestinal brush border conjugase is zinc dependent [65], and chelating compounds such as EDTA can inhibit conjugase activity in vitro [65,67]. It is unclear how dietary fiber exerts the inhibitory effects reported in humans, especially on polyglutamyl folates, in view of data indicating little or no binding of folate by dietary fiber and a lack of effect on brush border conjugase activity. The observation that certain forms of fiber inhibit the absorption of both mono- and polyglutamyl folates in the rat [108] suggests a possibly impaired diffusion of folates to the mucosal surface. Thus, the influence of food composition on the rate of diffusion of nutrients to the mucosal surface [26] may be an important area for future investigation.

7. *Potential Effects of Dietary Antifolates*

Many folate analogs inhibit folate absorption and metabolism. Such compounds, if found in foods, could impair the bioavailability of dietary folate by inhibiting the absorption, coenzymatic function, and/or interconversions of the vitamin. Little information is available concerning the existence of antifolates in foods, and this does not appear to be a significant factor influencing the bioavailability of folate in human diets [4].

8. *Effect of Nutrient Interactions on Folate Bioavailability*

The interactions of several other micronutrients may affect the bioavailability of folate. Severe folate deficiency causes impaired development of intestinal mucosal cells and can reduce folate absorption [199]. Vitamin B_{12} deficiency can indirectly reduce the metabolic function of folate. In vitamin B_{12} deficiency, the deficiency

of the vitamin B_{12}-dependent methionine synthetase leads to an unproductive accumulation of folate as 5-methyl-H_4folate [200]. Similarly, severe deficiency of riboflavin impairs the metabolic function of folate in rats (and presumably other mammalian species) because of the requirement of 5,10-methylene-H_4folate reductase for a flavin coenzyme [201]. Other metabolic interactions in the metabolic utilization and function of folate include functional roles of zinc and vitamin B_6. The sensitivity of folate utilization to normal variation in the intake of zinc and vitamin B_6 has not been determined.

The reported mutual interaction of zinc and folate, especially supplemental forms of these nutrients, has been a topic of recent interest, as discussed in Chapter 10. The preponderance of evidence suggests little nutritional significance to this potential interaction.

V. CONCLUSION

In summary, many uncertainties exist regarding the bioavailability of folate, whether present naturally or added to foods or taken as a supplement with a meal. Folate bioavailability appears to be frequently incomplete, although present knowledge does not permit quantitative estimates on the basis of the composition of the diet and the nutritional/physiological status of the person. Further information is needed regarding the biochemistry and physiology of folate absorption and in vivo behavior. Improved research techniques will aid in the development of a better understanding of factors affecting intestinal absorption and in vivo kinetics in human beings. In addition, a better understanding of the chemical and physical behavior of folate in foods is essential.

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Folate-Cobalamin Interactions

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I. INTRODUCTION

During the past 15 years several groups have reported that periconceptional folic acid supplementation reduces the risk of neural tube defects in pregnant women [1–10] (see also Chapter 12). A number of studies have also shown that plasma homocysteine (HCYS) concentrations are frequently elevated in occlusive vascular disease [11–14] and can be lowered by folic acid therapy [15,16]. In light of these developments, a variety of schemes to increase folate intake in the general population have been recommended [9,10,17] or are currently under consideration [9,10,15–18].

Although there is hope that these measures will reduce the incidence of neural tube defects and vascular disease, there is also concern that increasing folate intake will be deleterious. The primary focus of this concern is the risk of high folate consumption in persons with unrecognized cobalamin (Cbl, vitamin B_{12}) deficiency [19]. Numerous studies over the past 5 decades have shown that folic acid therapy causes hematological changes to improve or disappear in pernicious anemia,* but does not correct or prevent neurological complications. Thus, correction of megaloblastic anemia with folic acid may "mask" ongoing Cbl deficiency, allowing

*We will use the term *pernicious anemia* in this chapter to refer to the common adult-onset form of the disease, which is caused by atrophic gastritis of such severity as to lead to failure of intrinsic factor secretion.

neurological dysfunction to progress to irreversible Cbl neuropathy.* Moreover, some investigators have observed that folic acid appeared to precipitate or accelerate neurological complications [20–22].

In light of the recommendations to increase folate intake in large segments of the population, a reexamination of the metabolic and clinical interrelationships of folate and Cbl is appropriate. This chapter will review these issues, with special emphasis on the effects of folic acid therapy in pernicious anemia. Before addressing this principal topic, we will outline current hypotheses of the metabolic basis for the cardinal clinical features of Cbl- and folate-deficiency states and then briefly summarize recent developments in the diagnostic assessment of Cbl deficiency. A number of topics covered have been the subject of comprehensive and excellent reviews [19,23–28].

II. METABOLIC INTERACTIONS BETWEEN Cbl AND FOLATE

Cbl is known to be required for only two metabolic pathways in humans [19,26,29]. In the L-methylmalonyl–coenzyme A (CoA) mutase reaction (Fig. 1), Cbl serves as coenzyme in the form of adenosylCbl for the isomerization of L-methylmalonyl–CoA to succinyl-CoA, the final step in the conversion of propionic acid to succinyl-CoA. Folate has no role in this reaction. Both Cbl and folate are required in the methyl transfer reaction catalyzed by methionine synthase (Fig. 2). In this reaction Cbl serves as cofactor (in the form of methylCbl) for the recycling of homocysteine to methionine and the demethylation of 5-methyltetrahydrofolate (methylTHF) to tetrahydrofolate (THF) (Fig. 2). The methionine synthase reaction is the only metabolic pathway in which Cbl and folate are directly interrelated.

A. Megaloblastic Hematopoiesis Due to Disturbed Methionine Synthase Activity

THF serves as the source of active folate coenzyme forms for a host of biochemical reactions, including purine, pyrimidine, and amino acid synthesis [19,26]. Deficiency of either Cbl or folate leads to the same metabolic deficit: a lack of THF and folate coenzymes required for the synthesis of purines and pyrimidines, including thymidylate, which is incorporated into DNA. Failure of DNA synthesis causes megaloblastic hematopoiesis, with characteristic morphological abnormalities in bone marrow and peripheral blood and a progressive decline in erythrocyte, leukocyte, and platelet counts [19,29].

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*We will use the term *Cbl neuropathy* to refer to the neurological complications of Cbl deficiency, including peripheral neuropathy, spinal cord damage, and cerebral dysfunction. We have chosen Cbl neuropathy because it better encompasses the multiple manifestations of Cbl deficiency than other terms, such as *combined systems disease* and *subacute combined degeneration*. For a discussion of this topic, see Healton et al. [30].

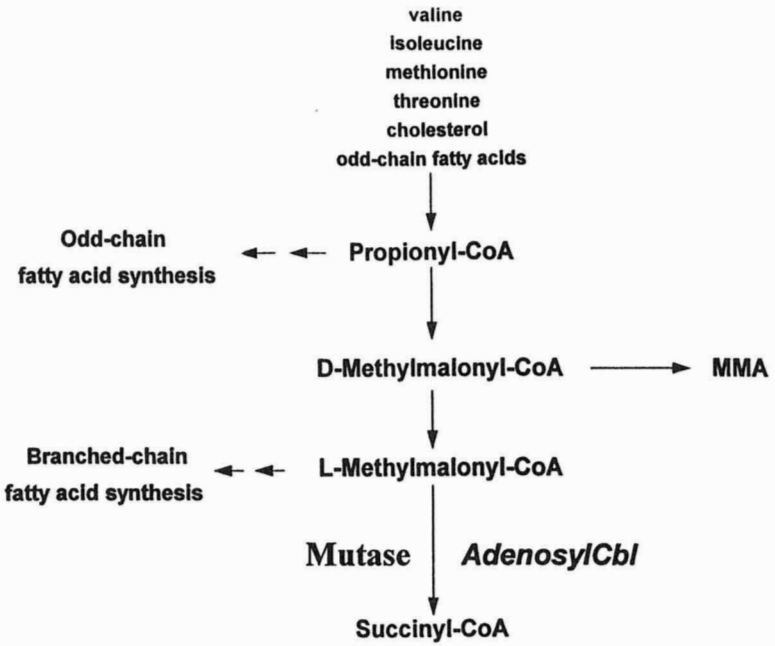


Figure 1 The L-methylmalonyl–coenzyme A mutase pathway. MMA = methylmalonic acid.

MethylTHF must be converted to THF in order for other folate coenzymes to be formed. In folate deficiency methylTHF is not available for conversion to THF. In Cbl deficiency, methylTHF is available but cannot be demethylated to THF (Fig. 2). According to the *methylfolate trap hypothesis* [31,32], the megaloblastic anemia of Cbl deficiency is due to impaired methionine synthase activity, which prevents conversion of methylTHF to THF. This metabolic block leads to "trapping" of methylTHF. Failure to convert methylTHF to THF deprives the cell of active folate coenzymes [31–33].

In Cbl-deficient cells, in addition to the failure to generate THF, methionine cannot be formed from HCYS (Fig. 2). The decrease in methionine synthesis leads to diminished S-adenosylmethionine (SAM) production. Low SAM levels promote the activity of 5,10-methyleneTHF reductase, which catalyzes the conversion of 5,10-methyleneTHF to methylTHF. This reduction step is irreversible [34] and leads to further "trapping" of methylTHF (Fig. 2).

Another consequence of the failure to generate THF in Cbl-deficient cells is a lack of polyglutamate folate. For folates to remain in the cell, a polyglutamate chain must be attached by the enzyme folate polyglutamate synthase (see Chapter 1). This enzyme has negligible activity towards methylTHF relative to other forms

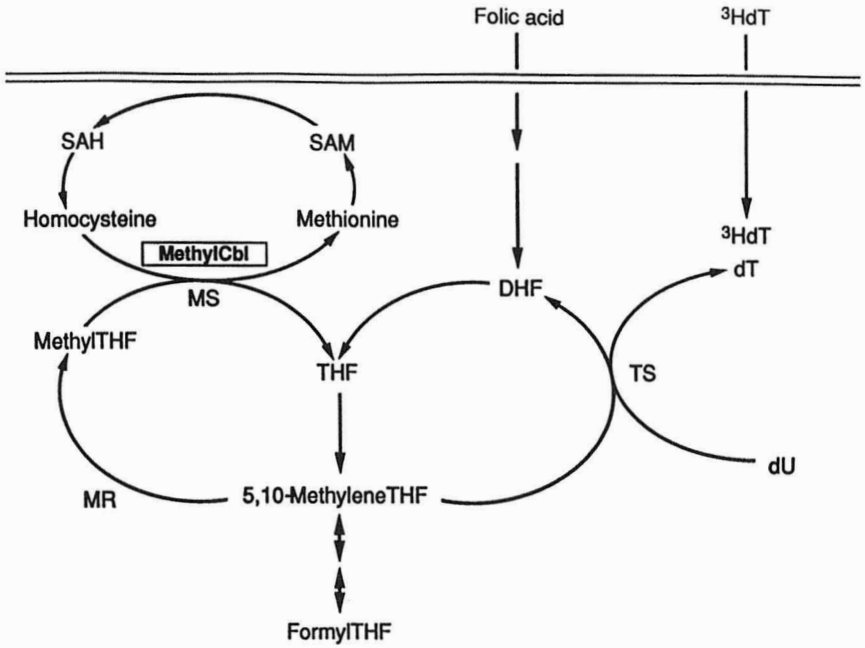


Figure 2 The cobalamin (Cbl)- and folate-dependent methionine synthase reaction. In the deoxyuridine suppression test (dUST), preincubation of normal bone marrow with dU suppresses the incorporation of H^3dT (tritiated deoxythymidine) into DNA; dU suppression is reduced in Cbl and folate deficiency. MS = methionine synthase; THF = tetrahydrofolate; DHF = dihydrofolate; SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine; MR = 5,10-methylene-THF reductase; TS = thymidylate synthase.

of folate, including THF, 5,10-methyleneTHF, and formylTHF (Fig. 2) [35–37]. In Cbl-deficient cells, polyglutamate folate is diminished [35,36,38–40] because most of the folate is methylTHF [35,41–43] and cannot be converted to THF. MethylTHF-monoglutamate leaks rapidly out of cells [36,44,45].

Hoffbrand and Jackson [43] have therefore suggested that the term "methylfolate trap" is misleading, since methylTHF does not accumulate in Cbl-deficient cells; rather, because it cannot be converted to THF and cannot itself be significantly polyglutamylated, it exits from cells, accumulates in plasma, and is then excreted in the urine [44,45]. These authors have suggested that a term such as "tetrahydrofolate starvation hypothesis" would be more appropriate for the mechanism of altered folate metabolism leading to megaloblastic hematopoiesis in Cbl deficiency.

Several clinical observations are consistent with the methylfolate trap (or tetrahydrofolate starvation) theory. That impairment of methionine synthase rath-

er than methylmalonyl-CoA mutase is responsible for the anemia of Cbl deficiency is strongly suggested by the finding that megaloblastic anemia is typically present in patients with inborn errors of metabolism that affect methionine synthase activity [46,47]. In contrast, inherited disorders that impair the mutase reaction alone do not cause megaloblastic anemia [25,27]. In addition, anemia is absent in patients with inherited deficiency of methyleneTHF reductase [46], in whom 5,10-methyleneTHF is not converted to methylTHF and thus remains available to serve as active coenzyme in the thymidylate synthase reaction (Fig. 2). The megaloblastic anemia of Cbl deficiency responds to synthetic folic acid (pteroylglutamic acid), which is reduced directly to THF and thus escapes the metabolic block (Fig. 2). Studies of the clearance and utilization of intravenously administered methylTHF in Cbl-deficient patients have been interpreted as indirect evidence in support of the methylfolate trap theory [48,49], as has the finding that the endogenous plasma folate concentration (almost all of which is methylTHF) is typically normal or elevated in Cbl deficiency [19,31,50–54] and falls with vitamin B_{12} therapy [19,31,50,51,54]. Certain in vitro observations are also consistent with the methylfolate trap hypothesis, namely, that THF but not methylTHF corrects the defective folate-dependent thymidylate synthase reaction in the bone marrows of Cbl-deficient patients in the deoxyuridine suppression test (dUST; Fig. 2) [43,55,56].

Chanarin and colleagues have challenged the methylfolate trap hypothesis [19,56–59]. Their view is that defective DNA synthesis in Cbl deficiency is due primarily to a deficit of "active formate" derived from methionine generated by the methionine synthase reaction. Deficient THF production is thus a related but less important complication of Cbl deficiency. The strongest evidence that Chanarin and colleagues have presented for the active formate (or formate starvation) hypothesis has been that formylTHF was superior to THF in correcting the abnormal dUST in the bone marrows of several of their patients with Cbl deficiency [19,56,57].

In investigations involving much larger numbers of patients than those included in the studies of Chanarin's group [19,56,57], Hoffbrand and colleagues [35,43,60–62] found that both THF and formylTHF corrected impaired thymidylate synthesis in Cbl-deficient marrow [43,60]. Although THF was not as effective as formylTHF in these experiments, this was equally so for the correction of abnormal thymidine synthesis in folate-deficient marrow; the superiority of formylTHF relative to THF in both deficiencies was attributed to its greater stability [35,43,60]. The data of Hoffbrand and associates are consistent with the methylfolate trap hypothesis, in which altered DNA synthesis in Cbl deficiency is due to a diminished supply of THF rather than active formate or formylTHF.

Although most of the experimental evidence favors the methylfolate trap (or THF starvation) hypothesis, this is not likely to be the sole mechanism for the hematological manifestations of Cbl deficiency. Based on the methylfolate trap hypothesis, the megaloblastic anemia of Cbl deficiency should respond fully to

treatment with folic acid. As will be discussed in Section V.B.2., however, responses to folic acid are often incomplete or transient, suggesting that the megaloblastic hematopoiesis of Cbl deficiency is not due solely to a deficit of THF.

B. Metabolic Basis for Neuropathy Due to Cbl Deficiency

There is thus good evidence that the megaloblastic anemia of Cbl deficiency is due to a block in the folate-dependent methionine synthase pathway. Whether impairment of methionine synthase is the cause of the neurological complications of Cbl deficiency is more controversial [27,28,63,64]. Anemia and neuropathy often occur independently of each other in Cbl deficiency; indeed, neuropathy tends to be most prominent in the least anemic patients [30,65,66]. The neuropathy of Cbl deficiency is not a typical feature of folate deficiency. Anecdotal reports that it may rarely occur in folate deficiency [67,68] are controversial. In addition, treatment with folic acid usually corrects the anemia of Cbl deficiency but may mask or exacerbate neurological complications (see Sec. V.B.2.c).

1. *Impairment of* L-*Methylmalonyl-CoA Mutase Pathway*

The dissociation between the hematological and neurological manifestations of Cbl deficiency as well as the differences in their responsiveness to folate therapy suggest that impairment of methionine synthase is not the primary or only disturbance in the neuropathy of Cbl deficiency and that a second, folate-independent pathway must be involved. The only other reaction in which Cbl is required in mammalian cells is the L-methylmalonyl-CoA mutase reaction, in which adenosylCbl serves as a cofactor for the conversion of L-methylmalonyl-CoA to succinyl-CoA (Fig. 1). Because folate has no role in this reaction, it has been suggested that the neuropathy of Cbl deficiency is due to impaired "mutase" activity. In this hypothesis, blockade of the mutase pathway causes accumulation of methylmalonyl-CoA and propionyl-CoA, which then substitute for malonyl-CoA and acetyl-CoA, respectively, in fatty acid synthesis. This leads to the incorporation of defective odd-chain [69] and branched-chain fatty acids in myelin [70]. Increased odd-chain fatty acids have been detected in affected nerve tissue of adult patients with neuropathy due to pernicious anemia [69] and both odd-chain and branched-chain fatty acids in the nervous systems of children with cellular deficiency of adenosylCbl [71,72]. Methylmalonic acid levels (Fig. 1; Sec. III) are markedly elevated in the serum [65,66,73,74], urine [19,75–77], and cerebrospinal fluid [78] of adult patients with neuropathy due to Cbl deficiency, demonstrating a block of the mutase pathway. However, serum homocysteine levels are also markedly increased in these patients.

The primary objection to this mechanism is that patients with inherited defects involving L-methylmalonylCoA-mutase do not develop the neurological complications of Cbl deficiency [25,27], despite the fact that serum MMA levels are higher than those of Cbl-deficient patients, which suggests that their mutase activity is

even more impaired [64]. In addition, in laboratory animals with neuropathy due to Cbl deficiency, abnormalities in the composition of fatty acids in myelin were only slight or were undetectable [79].

2. *Disturbed Methionine Synthase Pathway*

An alternative hypothesis for the neuropathy of Cbl deficiency is that impaired methionine synthase activity causes a deficiency of methionine and SAM, the primary source of methyl groups in the nervous system, which leads in turn to disturbed methylation reactions in myelin [28,80,81]. Several observations support this hypothesis. Abuse of the anesthetic gas nitrous oxide (N_2O), which inactivates methionine synthase, results in a clinical and pathological syndrome identical to that seen with Cbl deficiency [78,82]. Treatment of monkeys, fruit bats, and pigs with N_2O causes a similar neuropathy, which has now been shown in all three laboratory animals to be attenuated by the administration of methionine [80,83–85]. In addition, children with inborn errors of metabolism, such as 5,10 methyleneTHF reductase deficiency, associated with defective methionine and SAM production and with low cerebrospinal fluid SAM concentrations, exhibit demyelination [28,86–88].

In patients with inborn errors of Cbl metabolism affecting the methionine synthase pathway but not impairing the mutase reaction, nervous system disorders have been consistently observed [47,89]. Although the clinical neuropathy in these infants does not resemble Cbl neuropathy as seen in adults, it is not dissimilar to the neurologic dysfunction reported in children with acquired Cbl deficiency [89]. The predominant features of Cbl neuropathy in infants with disturbances in the methionine synthase pathway have consisted of hypotonia, developmental retardation, and seizures [47,89], which can hardly be considered to be highly specific for Cbl neuropathy. However, a single adult with the Cbl G mutation, which selectively impairs methionine synthase, was reported to develop a syndrome quite similar to adult-onset Cbl neuropathy (with the exception of some initially atypical features—minimal vibration sense defects and foot drop) [90]. This single case is perhaps the most convincing evidence in humans that interference with the methionine synthase reaction causes Cbl neuropathy.

Although the observations cited above support the hypothesis that methyl group deficiency is the cause of the neuropathy of Cbl deficiency, there are several caveats. First, although there is evidence of impaired methionine synthase activity in the form of elevated serum HCYS levels in Cbl-deficient patients with neuropathy, serum methionine levels are normal [91]. Second, in nerve tissue from animals with N_2O-induced neuropathy, methionine concentrations have been normal and SAM concentrations have been normal or increased [27,84,93]; the fall in the ratio of adenosylmethionine to adenosylhomocysteine reported by Weir and colleagues in the pig [84] could not be confirmed by Metz and coworkers in the fruit bat, suggesting that disturbed methylation reactions are not the cause of the neuropathy

[63,94]. Third, supplementation with amino acids other than methionine (valine and isoleucine), as discussed in the following section, also delays the onset of Cbl neuropathy in fruit bats [85,95]. Fourth, it is unclear whether data derived from children with inborn errors of methyl transfer metabolism are relevant to Cbl-deficient adults [27,63]. Fifth, as mentioned above, the neuropsychiatric abnormalities of Cbl deficiency are only rarely, if ever, seen in folate deficiency, despite the associated severe impairment in methionine synthase activity.

3. *Combined Impairment of Mutase and Methionine Synthase Pathways*

In our view, the two strongest arguments in favor of methionine synthase impairment as the cause of Cbl neuropathy appear to be the allevation of experimental damage to the nervous system by methionine and the clinical observations in congenital disorders, particularly the single case reported by Carmel and associates [90]. However, hypotheses suggesting that impairment of L-methylmalonyl-CoA mutase or methionine synthase activity causes neurological dysfunction in Cbl deficiency need not be mutually exclusive. In an attempt to resolve the conflicting data, Allen [29,64] has postulated a third possible mechanism: that the L-methylmalonyl-CoA mutase and methionine synthase pathways are in some way interrelated, and that both must be defective for the neuropsychiatric complications to develop. Two lines of evidence support this notion. First, a syndrome resembling Cbl neuropathy has been reported in patients with inherited defects that impair the synthesis of both adenosylCbl and methylCbl (i.e., Cbl C and D mutants) in which the mutase and methionine synthase pathways (Figs. 1 and 2) are both blocked [25]. One patient with the Cbl C defect developed neurological findings typical of Cbl deficiency as seen in adults [96]. In another with the Cbl D abnormality reported in two separate communications, there are conflicting accounts regarding the development of corticospinal tract signs [97,98], but the brain showed findings typical of Cbl deficiency at autopsy [98]; the spinal cord was not examined. Second, Cbl-deficient patients with neuropathy have far higher mean serum concentrations of both MMA and total HCYS than those without neurological dysfunction. Moreover, the concentrations of these metabolites differ between patients with and without neuropathy to a comparable degree [65]. Although serum concentrations may not reflect actual events in the nervous system, these observations could indicate that both L-methylmalonyl-CoA mutase and methionine synthase are more impaired in Cbl-deficient patients with neurological dysfunction than those without neurological involvement and that neither pathway is more impaired than the other [29,64,65].

It should also be remembered that although N_2O primarily inhibits methionine synthase, a secondary dysfunction of the mutase enzyme ensues during prolonged administration of the anesthetic [99]. In animals [84,85] and humans [78] with N_2O-induced neuropathy, serum [78,84,85] and cerebrospinal fluid [78] MMA levels are markedly elevated. In animals in whom methionine administration atten-

uates N_2O-induced neuropathy, plasma MMA levels are also lowered [85]. In a provocative recent experiment, valine and isoleucine [which are metabolized to propionylCoA and can therefore serve as precursors of methylmalonic acid (Fig. 1)] were found to be even more effective than methionine in protecting the fruit bat from neuropathy induced by N_2O combined with a Cbl-deficient diet [85,95]. Valine and isoleucine also caused a greater fall in plasma MMA than methionine and, in addition, led to an increase in hepatic methionine concentrations [85]. The mechanisms underlying these unexpected findings require clarification. They raise questions about the specificity of methionine supplementation in delaying the onset of Cbl neuropathy. They may also point to a currently obscure interrelationship between the methionine synthase and mutase reactions in the pathogenesis of neurological damage.

4. *Possible Metabolic Basis for Adverse Effects of Folate Therapy*

If folate therapy precipitates or accelerates neurological dysfunction while correcting hematological abnormalities in Cbl deficiency [20–22] (Sec. V.B.2.c), what could be the mechanism of this effect? There are several possibilities.

(*a*) *Toxicity of MethylTHF.* Because of the blocked methionine synthase pathway, large amounts of methylTHF might accumulate in the nervous system and cause damage [83,100]. However, there is no convincing evidence that folic acid or methylTHF is neurotoxic in Cbl deficiency.

(*b*) *Diversion of Cbl from the Nervous System.* Folate might divert depleted supplies of Cbl from the nervous system to the bone marrow, thus precipitating neuropathy [101–104]. A report that administration of folic acid to patients with untreated pernicious anemia caused the serum Cbl to fall concomitant with a reticulocytosis [103] was consistent with this hypothesis but was not confirmed in other studies [31,105,106]. Although Lear and Castle [107] reported that folate supplements coadministered with maintenance Cbl injections caused the serum Cbl level to fall in patients with pernicious anemia, Vilter and coworkers [108] were unable to confirm these findings. Moreover, the latter group reported that 0.7 μg/day of parenteral vitamin B_{12}, which is approximately equal to or slightly less than the estimated daily requirement [19], was as effective in preventing neurological relapse in patients receiving supplemental folic acid as in those not receiving supplements [108].

(*c*) *Diversion of Cbl from the Mutase Reaction.* In this postulate, folate therapy induces methionine synthase activity in the nervous system, thus increasing the methylCbl requirement and depriving L-methylmalonyl-CoA mutase of its adenosylCbl coenzyme. Although the coenzymes of Cbl have not been directly measured in neural tissue of Cbl-deficient patients treated with folic acid, administration of formylTHF to Cbl-deficient patients was not associated with significant alterations in adenosylCbl levels in plasma, red cells, or marrow [109]. The stron-

gest argument against this postulate is that it rests on the assumption that the mutase reaction is the critical pathway in the neuropathy of Cbl deficiency, for which there is no convincing evidence (see Sec. II.B.1).

(*d*) *Diversion of Methionine from the Nervous System.* Scott and coworkers have offered an interesting explanation for potential adverse neurological effects of folate in Cbl deficiency. In this hypothesis, administration of folic acid provides active folate coenzyme to dividing hematopoietic cells, promoting cell division and the synthesis of proteins such as hemoglobin, which require methionine. Methionine might thus be diverted to hematopoietic cells from the nervous system, leading to defective myelin synthesis and neuropathy. Consistent with this hypothesis are experiments in N_2O-treated fruit bats in which apparent folate-induced exacerbation of neuropathy was attenuated by co-administration of methionine [83].

In summary, a number of interesting hypotheses have been offered for potential neurotoxic effects of folic acid therapy in Cbl deficiency, but none of these postulates has been proven.

III. DIAGNOSIS OF Cbl DEFICIENCY

In Section V we will discuss the effects of folic acid administration in Cbl-deficient patients. Here we will review current approaches to the diagnosis of Cbl deficiency. The diagnostic approach to folate deficiency is discussed in Chapter 3.

The classic patient with Cbl deficiency presents with weakness, anorexia, weight loss, glossitis, neuropsychiatric disturbances (Sec. V.B.2.c), and macrocytic anemia associated with megaloblastic changes in blood and bone marrow [19,29,110]. In the modern era, many Cbl-deficient patients lack these textbook features, presenting with few or no symptoms and only subtle hematological abnormalities at diagnosis [111–113]. The trend towards earlier diagnosis is attributable at least in part to greater physician awareness and better diagnostic tools.

Nevertheless, early diagnosis of Cbl deficiency remains a challenging problem. It has become clear that several routine tests lack sensitivity and specificity for deficiency of Cbl. If one relies on the hematocrit and MCV as markers of Cbl deficiency, it is especially difficult to identify deficient patients with neuropsychiatric abnormalities, since approximately half have little or no anemia and almost one-quarter have a normal MCV [30,74]. In addition to its poor sensitivity for Cbl deficiency [74,111–116], an elevated MCV value lacks specificity for that diagnosis [117]. Neutrophil hypersegmentation [19,115,118,119] and macroovalocytosis [19,119] are present on the blood films of most patients with overt megaloblastic anemia, but these findings may be overlooked by routine hematology laboratories [112] or absent in patients with mild or no anemia [111,115, 119]; neither abnormality alone is specific for megaloblastic hematopoiesis, but this diagnosis is highly likely if both are present (see Chapter 3). Megaloblastic changes are invariably

present in the bone marrow of patients with anemia due to Cbl or folate deficiency, and the two conditions cannot be differentiated morphologically. In some patients without anemia, however, megaloblastic change in the marrow may be quite subtle or equivocal.

The use of laboratory tests in the diagnosis of Cbl deficiency and its differentiation from folate deficiency are discussed in detail in Chapter 3. A few points will be emphasized here. Cobalamin-folate interactions, some of which are poorly understood, may affect frequently used diagnostic tests. The decreased erythrocyte folate concentrations observed in 50% of Cbl-deficient patients [19] and the elevated serum folate levels seen in 10% or more [19,53] may be the result of the failure of cells with impaired methionine synthase to retain methylTHF-monoglutamate (Sec. II.A). On the other hand, the mechanism underlying the low serum Cbl concentration, which appears to be caused by folate deficiency, in a minority of patients with primary lack of folate is poorly understood [50,120–122].

The serum Cbl measurement continues to be a highly useful test for cobalamin deficiency. However, serum Cbl levels may be normal in patients with Cbl deficiency [123–128]. In a recent series of 173 deficient patients [124], serum Cbl levels were normal in 12 (5.2%) despite hematological evidence of deficiency. Five of the 12 patients also had unequivocal Cbl neuropathy. Serum MMA and total HCYS levels were elevated in all 12 and showed dramatic improvement after treatment with vitamin B_{12} in the 10 with follow-up measurements (Fig. 3). In addition to its suboptimal sensitivity, the serum Cbl measurement lacks specificity for Cbl deficiency [112,115,124,129–134] (see Chapter 3).

As discussed in Chapter 3, measurements of serum MMA and HCYS [29,65, 66,73,78,91,112,116,124–127,133,135–139] are highly useful ancillary tests in the diagnosis of Cbl deficiency. Serum MMA rises in Cbl but not in folate deficiency, whereas lack of either vitamin causes a rise in serum HCYS (Fig. 2). In a recent study of 434 episodes of Cbl deficiency, at least one metabolite was unequivocally elevated in 99.8% of cases and both were increased in 94.5% [116]. Hematocrit and MCV values were often normal and approximately one-third of patients had neurological complications of Cbl deficiency. Serum metabolite levels also helped to discriminate between Cbl and folate deficiency [116].

Therefore, normal levels of both MMA and HCYS can be used to rule out lack of Cbl with almost 100% certainty [116]. In addition, elevated serum levels of MMA (or of both MMA and HCYS) are more specific for Cbl deficiency than low serum Cbl levels. The most important limitation of the specificity of an increased serum MMA concentration is underlying renal dysfunction (or hypovolemia), which is not present in most Cbl-deficient patients [116]. Inborn errors of metabolism, such as deficiency of L-methylmalonyl-CoA mutase [25] or cystathionine-ß-synthase [140], may also cause metabolite elevations. The high cost of metabolite determinations relative to the serum Cbl measurement at this time

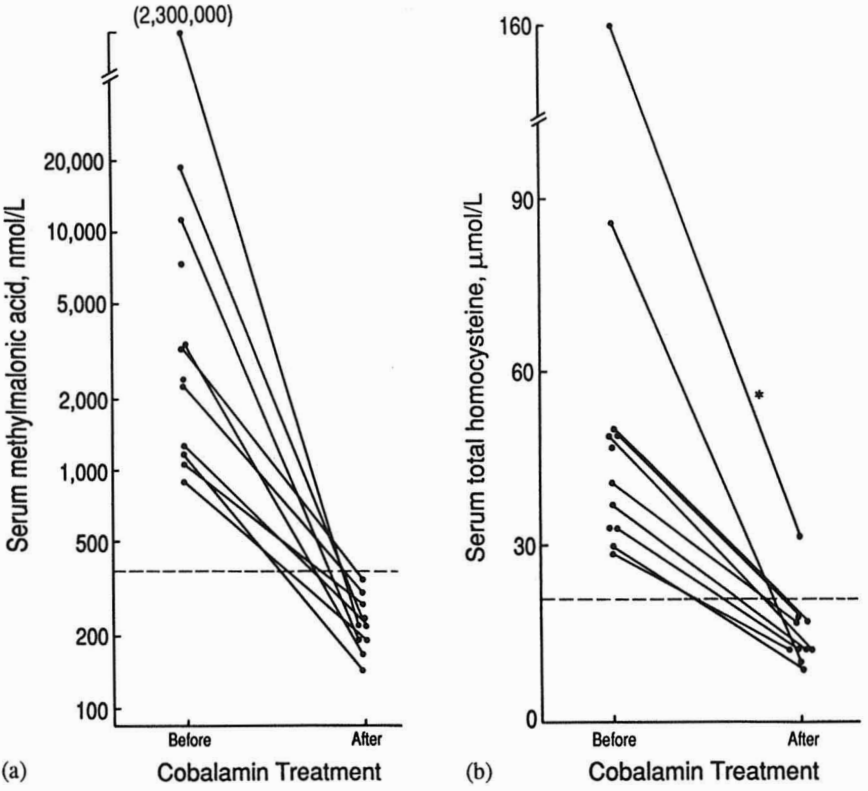


Figure 3 Serum methylmalonic acid (a) and total homocysteine (b) levels before and after treatment in 12 patients with serum Cbl levels between 215 and 475 pg/ml who responded hematologically to Cbl therapy. Dashed lines indicate 3 standard deviations above mean value in normal controls. Asterisk denotes patient studied 5 weeks after two Cbl injections. (Adapted from Ref. 124.)

argues in favor of their use as ancillary tests rather than a replacement for the serum Cbl level.

MMA and HCYS can also be measured in urine. Urinary MMA [19, 75–77,141,142] is more sensitive for Cbl deficiency than the serum Cbl [127]; however, its sensitivity relative to serum MMA and HCYS levels has not been established. The serum MMA and HCYS can be measured on the same sample as that taken for the serum Cbl determination. Either metabolite may be falsely elevated because of renal dysfunction [116]. The urinary MMA has the advantage of being noninvasive, but requires a simultaneous urine creatinine determination to correct for renal function. Thus far, screening studies using urinary MMA measurements

have not routinely included HCYS determinations. In the absence of a HCYS measurement, Cbl-deficient patients with isolated homocysteinemia may be missed [116]. Inclusion of the serum HCYS determination also allows identification of patients with folate deficiency [116] (see Chapter 3). Finally, serum MMA and HCYS measurements have been more extensively studied in patients with proven Cbl and folate deficiency [66,73,74,78,91,112,116,124–126,133].

A clear-cut response to Cbl therapy, if demonstrable, is the most reliable evidence that a hematological or neurological abnormality is due to Cbl deficiency. Three to six months of observation may be required to assess response, however, and amelioration of neurological abnormalities may be slight or equivocal. On the other hand, elevated MMA and HCYS levels due to Cbl deficiency invariably improve on Cbl therapy, with patients showing normal or near-normal values within days or weeks [138].

It should be emphasized that the Schilling test may be normal in 10–20% of patients with clinical evidence of Cbl deficiency; many of these patients have malabsorption of dietary cobalamin due to lack of acid and pepsin [143–150] (see Chapter 3).

IV. TREATMENT OF FOLATE DEFICIENCY WITH Cbl

Clinicians interested in Cbl and folate have long been intrigued by apparently anomalous responses of patients with deficiency of one vitamin to treatment with the other. We will discuss first the responses of folate-deficient patients to therapy with vitamin B_{12}. Although it has not been the subject of a systematic study in a large series of cases, most folate-deficient patients do not appear to respond to vitamin B_{12}. This was the experience of a number of observers in patients with megaloblastic anemia due to folate deficiency studied in the 1950s and early 1960s in whom little or no hematological effect was seen with Cbl therapy but full responses followed the administration of folic acid [151–155]. Similarly, in a recent report vitamin B_{12} in total doses of 1–4 mg produced no hematological response in three patients with megaloblastic anemia due to folate deficiency. Moreover, elevated serum levels of total HCYS were unaffected by Cbl therapy but fell into the normal range following treatment with folic acid [138].

Occasional folate-deficient patients, however, have been reported to have striking improvement in their blood counts on Cbl therapy [156–159]. Despite this, formiminoglutamic acid excretion remained elevated [157,158]. Megaloblastic change usually persisted in the bone marrows of folate-deficient patients responding to vitamin B_{12} [157–159], although normalization of the marrow was claimed in three partially documented cases [160]. Why responses to Cbl therapy occurred in these studies but not in other reports is unclear. In the dUST, the addition of Cbl to folate-deficient bone marrow produces little or no correction of dU suppression [43,56,161]. Although the folate-deficient patients responding to Cbl had been

maintained on relatively low folate diets [157–159], reticulocytosis was associated with a rise in the serum folate concentration in three of the patients [158], suggesting that the apparent hematological response to Cbl was in fact due to improving folate status. In Mollin's experience [154], all folate-deficient patients who showed a response to vitamin B_{12} eventually required treatment with folic acid.

Severe glossitis developed in a patient with combined Cbl and folate deficiency who was treated only with vitamin B_{12}. Although the glossitis was attributed to diversion of depleted folate stores from the tongue to hematopoietic cells [162], it may merely have been a manifestation of worsening folate deficiency unrelated to the Cbl administration.

V. TREATMENT OF Cbl DEFICIENCY WITH FOLIC ACID

A. Treatment with Wills Factor

Several years after it was shown that feeding of liver could induce remissions in pernicious anemia [163], Lucy Wills demonstrated that yeast extract corrected macrocytic anemia in malnourished Indian women [164] and laboratory animals [165]. On the basis of this discovery, therapeutic trials with a variety of yeast preparations were carried out in patients with pernicious anemia in Europe and America. Although treatment with yeast produced a reticulocytosis and rise in hemoglobin in some patients [166–170], most showed no response [170]. This experience contrasted strikingly with the excellent results of liver therapy in pernicious anemia, and suggested that the "Wills factor" of yeast and the "extrinsic factor" of liver were distinct. Subsequent workers proved that this hypothesis was correct by demonstrating that the yeast hematopoietin was folic acid [171,172] and that the active principle of liver therapy was Cbl [173].

B. Administration of Folic Acid in Pernicious Anemia

The synthesis of folic acid was described by Angier and colleagues [174] in August 1945. Three months later Spies and coworkers [175] reported its efficacy in the treatment of macrocytic anemia. Over the next 15 years numerous publications appeared on this topic, and much of the knowledge of the effects of folate therapy in Cbl deficiency derives from these reports. Interpretation of these studies is difficult for several reasons, including the absence of Schilling tests, serum folate and Cbl determination in most reports, the use of liver extracts of varying potency [176,177] in treatment, and frequent incomplete or duplicate reporting of patients. Nevertheless, certain themes emerge from these studies and an attempt will therefore be made to summarize the literature.

1. *Early Studies*

By the end of 1946, the results of folate therapy had been reported in approximately 60 patients with pernicious anemia [175,178–193]. In almost all cases pernicious

anemia was diagnosed by the presence of morphological abnormalities characteristic of megaloblastic anemia in blood and marrow and associated histamine-fast achlorhydria. Many of the patients had presented with relapsed pernicious anemia after stopping maintenance liver injections, or their liver therapy was discontinued to enter them into therapeutic trials with folic acid [184,187,191,193]. Several patients had neurological abnormalities, most commonly paresthesias or loss of vibration and position sense [179–183,187]. In most patients folic acid was given orally in a dose varying between 10 and 40 mg daily [179,183,186–188,192,193], but several received as much as 400 mg per day [192]. In many patients parenteral administration was used; most trials lasted 1–3 months.

The results were striking, with almost all patients found to show a reticulocytosis and improvement in hematopoiesis comparable to that seen following liver extract [180–194]. Associated with the hematological response was a return of appetite and weight gain and, in several cases, amelioration of glossitis [184,187]. Moreover, neurological complications did not occur or, if already present, did not progress; in fact, in several patients neurological findings were reported to improve [179,183,184,187].

2. *Limitations and Risks of Folic Acid Therapy in Pharmacological Doses in Cbl Deficiency*

Enthusiasm generated by these early reports [191,193,195,196] rapidly dissipated with larger trials and longer follow-up. In early 1947, several groups reported that initial remissions in pernicious anemia were often suboptimal on folate therapy alone, and that neuropathy in particular required treatment with injections of liver extract [20,197–200]. By the end of the year, editorials appeared in both the American [201] and British literature [202] stressing the limitations and dangers of folic acid therapy.

(*a*) *Hematological Effects of Large Doses of Folic Acid in Cbl Deficiency.* Although most groups reported that a brisk rise in reticulocyte count occurred following pharmacological doses of folic acid in patients with pernicious anemia in relapse [20,50,179–186,188,191–194,202–216], others found that the reticulocytosis was often blunted compared to that typically seen following liver therapy [20–22,175,197,217–229]. Correction of anemia was noted in most patients [20,180–192,204–209,212–215,219,222,230–236], but poor responses were also observed [20–22,102,197,210,219,221,223,224,237–239], even on doses of 15 mg/day or higher [102,197,239]. Although morphological abnormalities in the bone marrow resolved [183,185,187,193,219,220,237,240], some patients demonstrated persistent macrocytosis in the peripheral blood [219,228,239]. Similarly, leukopenia [20,180,184–188,205,209,212,219,222,231] and thrombocytopenia [184,187,188,206,211,212] were corrected in most, but not all, patients [180,219].

Other patients who were in remission and had been maintained on liver extract were switched to folic acid treatment. When compared to historical controls

with pernicious anemia whose liver extract therapy had been interrupted [241–245], the results in folate-treated patients strongly suggested that folic acid delayed the onset of hematological relapse. In the experience of Schwartz and coworkers [243,245], more than two-thirds of their historical controls experienced hematological relapse in the first year after discontinuation of liver therapy [243] versus only 19% of the prospectively studied patients receiving folate therapy (Fig. 4). Many patients remained in apparent hematological remission for periods of months or years on maintenance doses of 5–20 mg of folic acid daily, but eventually demonstrated macrocytic anemia [22,101,102,108,245–247] and megaloblastic change in the bone marrow [247]. The megaloblastic anemia often responded transiently to escalating doses of folic acid [108,246], but in virtually all instances therapy with liver extract (or vitamin B_{12}) was later required [22,108,245–247]. At the end of 10 years, over 90% of patients were in hematological relapse, neurological relapse, or both [108].

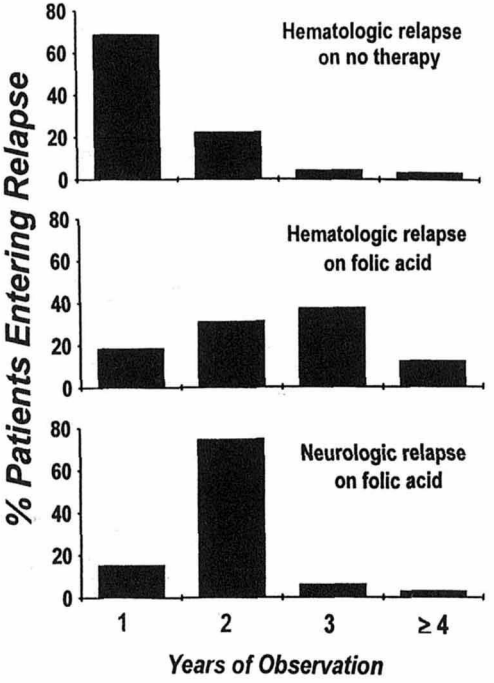


Figure 4 Summary of data provided in two reports describing the time course of relapse in patients with pernicious anemia. The first histogram depicts the percentage of patients with hematological relapse at 1, 2, 3, and 4 or more years on no treatment following discontinuation of liver extract therapy [243]. The next two histograms depict the percentage of patients with hematological and neurological relapse according to year while receiving folic acid 5 mg/day in place of liver treatment [245].

(*b*) *Effects of Large Doses of Folic Acid on Glossitis Due to Cbl Deficiency.* Glossitis due to Cbl deficiency also responded to folate therapy in variable fashion. In most patients tongue abnormalities resolved rapidly with folic acid [22,184,187,197,219,228]; in others glossitis was slow to respond, requiring 1–4 months for restoration of a normal tongue [219], or did not respond at all [22,185,214,219]. Glossitis frequently developed or recurred in the first year of treatment [219,248,249], but some episodes were reported after 3 or more years [101,245].

Tongue, hematological, and neurological complications were dissociated in Cbl-deficient patients receiving folic acid. Glossitis was the only manifestation of relapse in several patients [239,245], although it occurred in the presence of anemia or neuropathy in most instances [22,101,197,212,226,228,249–252]. Relapse of glossitis often developed (in association with neurological complications) in patients in hematological remission [101,226,239,250–254]. Glossitis sometimes responded to folate treatment despite ongoing hematological [22] or neurological relapse [214].

(*c*) *Effects of Large Doses of Folic Acid on the Neuropsychiatric Manifestations of Cbl Deficiency.*

1. Masking of Cbl Deficiency. Meyer [197] was the first to report that folic acid reversed the hematological complications of pernicious anemia but was unable to correct or prevent the neuropathy. These findings have been confirmed in approximately 40 subsequent publications [20–22,30,101,102,198,199,207,209, 212,214,218,219,223–226,228,233,235,237,238,245,247–262] (Fig. 5). In many of these reports neurological findings that were present before folate therapy persisted [20–22,214,218,219,224,228] or progressed on treatment [20–22,138, 209,214,218,219,228,237,238,247,251,252,254,257,259]. In others the neuropathy developed after folate therapy was begun [102,108,199,212,214,223,224,226, 228,233,235,236,248,249,251–253,260–262]. The neuropsychiatric abnormalities seen in Cbl-deficient patients treated with folic acid were identical to those of classic pernicious anemia [19,30]. Most presented with paresthesias and loss of vibration or position sense; in many there were other signs of Cbl neuropathy that was often severe [20–22,101,108,197,199,207,209,212,214,219,225,228,235,237, 238,248,249,251,252,254,257,259–262], including ataxia, abnormal reflexes, paraparesis [22,233,235,249,251,257,262], autonomic dysfunction [198,214,233, 249,251,257,259,260], and intellectual and psychiatric disturbances [21,22,197, 199,209,212,237,249,251,257,262,263]. In virtually all reports the neuropathy associated with folate therapy responded to treatment with liver extract or vitamin B_{12}, although often the responses were only partial [20,21,212,214,228,233, 235,248,257,262]. Neuropathy also occurred in patients receiving folic acid for other causes of Cbl deficiency, including gastrectomy [102,214], gastrojejunostomy and gastrocolic fistula [264], sprue [225,263], Crohn's disease [247,262,265], and fish tapeworm disease [266].

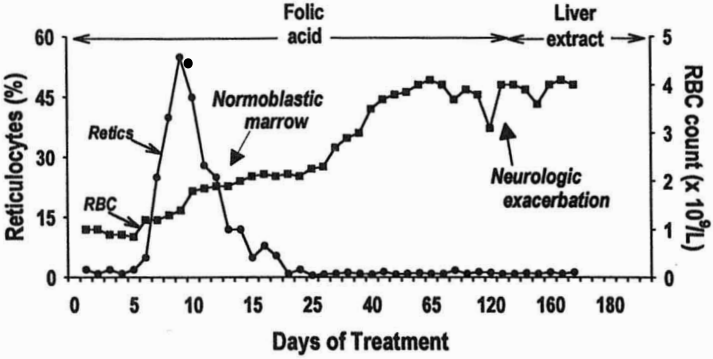


Figure 5 Clinical course of a 78-year-old woman with newly diagnosed pernicious anemia complicated by paresthesias, hyporeflexia, and loss of vibration sense. Folic acid was given by intramuscular injection at a dose of 25 mg/day for 9 days followed by oral therapy consisting of 65 mg/day for 30 days, 195 mg twice weekly for 24 days, and 10 mg/day for 70 days. Although both anemia and paresthesias initially disappeared on folate therapy, numbness and tingling of the fingers and toes returned on about day 135. The folic acid dose was increased to 25 mg/day, followed by 50 mg/day for several days, but the paresthesias worsened and were associated with increasing irritability and confusion. After 5 months of treatment, the folic acid was stopped and liver extract was given. (Adapted from Ref. 237.)

In prospective studies, the incidence of neurological relapse or progression has varied between 44.4 and 80% in patients with pernicious anemia on folate therapy for more than one year of observation (Table 1). In the study of Schwartz and coworkers [245], the majority of hematological relapses occurred in the second and third year on folic acid, whereas most neurological episodes developed in the first and second year (Fig. 4). In this report and others [20,108,209,212,214, 219,228,236,238,248,251,252,254,257,259,260], most patients with neurological relapse or progression were not anemic. Because of incomplete reporting, it is unclear how many may have had relatively subtle hematological abnormalities, such as macrocytosis [101,102,219] or neutrophil hypersegmentation [252]. In four nonanemic patients marrow morphology was described as normal [251,260,261]. Herbert stated, however, that he had never encountered a folic acid-treated patient who had normal hematological morphology [266].

2. Exacerbation or Precipitation of Cbl Neuropathy on Folic Acid. It is thus clear that neurological complications may arise in patients with pernicious anemia whose hematological and tongue abnormalities have been corrected or markedly improved by folic acid therapy. The claim that folate therapy may *precipitate* or *exacerbate* neurological dysfunction in Cbl deficiency [22,102,108,200,224] is more debatable. In studies of Cbl-deficient humans, there have been two lines of evidence

Table 1 Results of Studies of Patients with Pernicious Anemia Treated with Large Doses of Folic Acid

Folate dose (mg/day)	Observation time (months)	Patients (*n*)	Neurological relapse[a] or progression				
			With hematological relapse	Without hematological relapse	Total (*n*)	%	Ref.[b]
3–40	12	41	0	3	3	7.3	248
10	0.3–17	12	?	?	3	25.0	224
5–20	12	15	4	0	4	26.7	21
10–15	9	14	2	3	5	35.7	219
2.5–50	?	20	?	?	8	40.0	226
5	48	72	9	23	32	44.4	245
5–10	120	36	9	7	16	44.4	108
2.5–15	25	22	7	5	12	54.5	102
$\geqslant$10	24	38	?	?	28	73.7	212
5–600	12	10	0	8	8	80.0	214
5–40	35	20	5	11	16	80.0	238
Total		300			135	45.0	

[a] In many studies neurological and hematological relapse were not defined.

[b] The most pertinent references are listed for studies that were reported more than once.

that folate therapy is deleterious: (1) neurological events appeared to correlate with folate dose, and (2) neurological events occurred earlier and progressed more rapidly than seen in previous, untreated patients with pernicious anemia.

Ross and colleagues [22] reported that maintenance doses of 10 mg or more per day were associated with a higher incidence of neuropathy than doses of 5 mg/day or less; Vilter's group noted that neurological relapse consistently occurred when escalating doses of folic acid were required to maintain hemoglobin levels [108,199]. The possibility that the neuropathy was in fact due to progressive Cbl deficiency, however, and occurred coincidentally with the higher folic acid doses could not be excluded.

Several authors felt that neurological relapse was unusually rapid or "explosive" in patients receiving folic acid [20–22,197–199,209,214,218,219, 224,228,233,237,238,248,257]. If one examines the case reports of patients in whom disabling neuropathy occurred or progressed over 1–12 weeks, 25 are described in sufficient detail for review [20–22,197–199,209,214,228,233, 237,238,248,257]. All 25 received doses of 10 mg/day or higher. Eighteen of the 25 patients developed severe Cbl neuropathy (e.g., marked ataxia, Babinski signs, paraparesis, psychosis) in a span of 3 months; however, all 18 had objective neurological changes (e.g., loss of position and vibration sense, ataxia) prior to folate

treatment [21,22,197–199,209,228,233,237,238,248,257]. The progression or first appearance of neuropathy tended to be milder [248] and occur later [22,108,197,199,228] in patients who had previously received liver extract therapy than in those started on folic acid without prior liver treatment [20,21, 197–199,214,237,238], suggesting that the progressive decline in Cbl stores was at least as important as folate therapy in the development of neuropathy. Fulminant neuropathy has also been reported in patients with pernicious anemia *not* receiving folic acid therapy [340, 341].

In three prospective studies of folic acid treatment in pernicious anemia, 18.8–56.3% of neurological relapses occurred in the first year and 68.8–96.8% by the end of the second year [102,108,245] (Fig. 4). In contrast, of 15 patients with pernicious anemia who were observed on no treatment following discontinuation of liver therapy, none suffered neurological relapse over a period of 8–27 months [244]. In a later, retrospective study of Cbl-deficient patients relapsing after discontinuation of cyanoCbl therapy, only one of 40 hematological or neurological relapses occurred in the first 2 years [267]. Comparisons with historical controls may be misleading, however, in view of variation in definition of relapse and attention to clinical signs in the different reports, the unpredictable Cbl content of liver extract preparations [176,177], and the highly variable course of pernicious anemia in different patients [19,30,74,110,113,123,267–269]. For example, in a group of patients with pernicious anemia who were merely withdrawn from Cbl therapy and followed closely, neurological relapses occurred after a *shorter* time off treatment than hematological recurrences [123].

There is limited and inconclusive data on the effects of folic acid administration in Cbl-deficient laboratory animals [83,93,270,271]. In experiments with N_2O-treated fruit bats, ataxia and paralysis developed significantly more rapidly in animals receiving large supplements of folic acid (or of formylfolate) than in those on diet alone [83]. When these experiments were repeated in a second group of fruit bats, however, the onset of neuropathy was only slightly earlier in animals supplemented with folic acid [93]. Folic acid administration to a small number of monkeys on a Cbl-deficient diet [270,271] has produced inconclusive results. Spinal cord and optic nerve damage occurred earlier in monkeys receiving folic acid, but the visual defects were also milder in that group [271].

It remains an open question whether folic acid is deleterious to the nervous system of Cbl-deficient patients. The issue is not likely to be settled without a double-blind study in which subjects are randomized to receive either folic acid or placebo for a period of months or years in the absence of Cbl injections. It is unlikely that such a study will be performed.

3. Improvement in Neuropathy. In a number of Cbl-deficient patients, neurological findings improved on folate therapy [22,183,184,187,197,199, 204,207,215,222,237,239,250,252]. Paresthesias and sensory changes were the symptoms and signs that responded most commonly; occasional patients showed

improvement in more severe abnormalities, including vibration and position sense [204], ankle clonus [197,239], and Babinski [204] and Romberg signs [183]. In most patients the neurological findings improved during the first 2 months of folate treatment; relapse of neuropathy often occurred in those with long-term follow-up. The neurological improvement in Cbl-deficient patients on folate therapy is unexplained. It is possible that it was related to correction of anemia or a second underlying deficiency state (e.g., thiamine lack) or other neurological disorder.

(*d*) *Treatment with Other Forms of Folate in Cbl Deficiency*. Hematological responses have also been seen in several Cbl-deficient patients given folinic acid (formylTHF) [109,272–281], pteroyldiglutamic acid [282], pteroyltriglutamic acid [191,250,282, 342], and pteroylheptaglutamic acid [343]. Cbl neuropathy improved in two patients receiving folinic acid [274,275]; neurological deterioration occurred in one patient receiving pteroyltriglutamic acid [250]. Administration of pteroylheptaglutamate as yeast extract in doses equivalent to 2.3–5 mg/day of folic acid produced minimal or no reticulocyte responses in seven Cbl-deficient patients [283].

3. *Effects of Low Doses of Folic Acid in Cbl Deficiency*

(*a*) *Oral Folic Acid Doses < 5 mg Daily*. A folic acid dose of 5 mg/day produced excellent reticulocyte responses and ameliorated or prevented anemia in most of the Cbl-deficient patients cited above [21,22,204,207,214,215,218,225,228,245]. In the few patients who have been treated with lower folate doses, the hematological response has been variable. Kurnick reported reticulocyte and hemoglobin responses in two patients taking 2.5 mg/day and one receiving 1.25 mg/day [204]. Ross and colleagues maintained 11 patients on folic acid at a dose of 1.25 mg/day for periods of 3–9 months without recurrent anemia [22]. In other reports, two patients developed hematological relapse on 5 mg of folic acid twice weekly [228,247]. Of nine patients receiving an oral dose of 1 mg/day [52,218,284–287], eight showed a mild to moderate reticulocytosis [52,218,284–286] in association with normalization of marrow morphology in two [52] and improvement in hemoglobin levels in two others [218,286]. In a series of six patients treated with 1 mg daily for 23 days to 18 months, four showed an elevation in the hemoglobin, a fall in the MCV, or both, with two exhibiting progression of neuropathy [138].

Among Cbl-deficient patients receiving folic acid from multivitamin preparations [30,52,138,251–253,259–261,288,289], two had Cbl neuropathy in the absence of anemia on doses of 0.1–0.4 mg/day [138,260] (Table 2). One of the latter patients [138] had paresthesias and sensory deficits at the time that folic acid therapy was started; her anemia and macrocytosis resolved and neurological complaints worsened on a folate dose of 0.4 mg/day for 17 months. The second [260] had previously received vitamin B_{12} injections for pernicious anemia associated with ataxia, decreased vibratory sense, and hyporeflexia; the neurological abnormalities disappeared on Cbl treatment. Over a 5-year period she received only "occasion-

Table 2 Hematological and Neurological Findings in 17 Cobalamin-Deficient Patients with Neurological Abnormalities Taking Multivitamin Preparations Containing Folic Acid

Folate dose (mg/day)	Duration (mos)	Anemia	MCV (fl)	Marrow	Glossitis	Neuropathy	Ref.
?	12	yes	116	?MC	yes	vs,Rom,Bab	253
?	12	no	—	Nl	—	at,ps,vs,inc	260
0.3–1	3	no	—	—	—	par,Rom,vs	261
0.1–0.35	24	no	—	Nl	—	at,bed-ridden	260
0.4	17	no	79	—	no	par,vs,ps	138
0.5	31	yes	118	Nl[a]	yes	par,vs,ps,pain	252
1.5	8	yes	111	—	no	par,at,ps,vs,↓dtr, paraparesis	251
1.7–2.5	29	no	104	Nl	no	par,at,Rom,↓dtr,vs, ps,pain,touch	251
2–3	29	no	—	—	—	par,at,vs,ps,touch, ↓dtr	261
2.5	?	no	—	—	—	par,Rom,vs,pain,↓dtr	259
3.3–6.6	10	no	—	—	yes	par,at,Rom,Bab,vs,ps, pain,touch	251
4.5	29	yes	103	—	—	par,at,Bab,ps,vs,inc	251
4.5	48	yes	101	?MC	yes	par,at,Rom,vs,ps	253
5	3	no	86	—	no	par,Rom,at,pain,touch	253
5.1	5	yes	109	—	yes	dep,at,vs	253
6.3	60	no	—	Nl[a]	yes	par,vs,ps,Rom,- ↓dtr	252
8	30	no	92	—	yes	par,at,Rom,vs	253

par = paresthesias; vs, ps, touch, and pain = abnormalities of vibration, position, touch, and pain sense, respectively; at = ataxia; Rom = Romberg sign; Babinski = Babinski sign; dtr = deep tendon reflexes; inc = incontinence; dep = depression; ?MC = questionable megaloblastic change; Nl = normal marrow morphology.

[a] Normal marrow morphology, but neutrophil hypersegmentation present on blood film.

al" vitamin B_{12} injections and then none for 2 years, at which time she presented with recurrent ataxia. During these 2 years she had been taking two multivitamin preparations containing 0.1 mg and 0.25 mg of folic acid, respectively; the total daily dose of folic acid was not clearly stated [260]. It is uncertain whether the lack of anemia was related to folic acid therapy in this case, since many Cbl-deficient patients present with neuropathy in the absence of anemia whether or not they are receiving folate supplementation [74,123].

The reticulocyte count was approximately 3–4.2% on 0.35–0.40 mg/day of oral folic acid in two patients [266,284,291] and rose to 6% on the same dose of intramuscular folic acid in one [266]. Mild reticulocytosis was noted in 3 of 15 Cbl-deficient patients receiving 0.2 mg/day; none of these patients had a significant

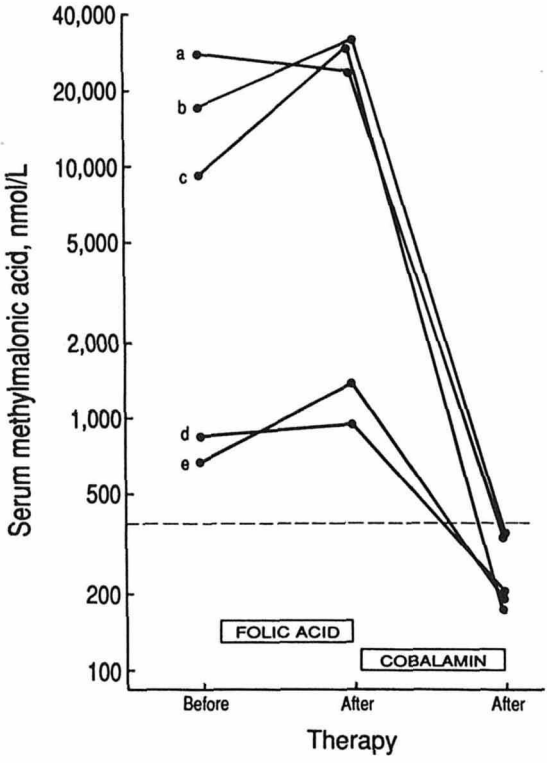
change in hemoglobin level [292]. In two patients with pernicious anemia, daily 0.1-mg doses of oral folic acid were said to normalize the bone marrow morphology, but no further documentation was provided [160]. No response was seen in five patients taking oral doses of 0.15–0.67 mg/day [290,293].

(*b*) *Parenteral Folic Acid Doses < 5 mg Daily.* In a patient with pernicious anemia treated with escalating intramuscular doses of folic acid, there was no response to daily doses of 0.05–0.1 mg, but the reticulocyte, white cell, and platelet counts rose and serum lactic dehydrogenase fell at a folate dose of 0.3 mg/day; bone marrow morphology was still abnormal at a dose of 0.7 mg/day and there was no clear-cut improvement in hemoglobin level until 1.0 mg was given [294]. Of nine Cbl-deficient patients given daily intramuscular injections of 0.1–0.4 mg by Hansen and colleagues [52], four showed a mild reticulocytosis on doses of 0.2, 0.3, and 0.4 mg/day, respectively; one of the patients receiving 0.4 mg daily had a rise in hemoglobin level. The other five patients, including one receiving 0.4 mg/day, had no response. Bone marrow morphology showed only slight improvement in six of the nine patients in this study, although it was unclear how many of those with a reticulocytosis underwent marrow examination. In other reports, a clear-cut reticulocyte response was noted in seven patients receiving 0.4–0.5 mg/day of folate given intramuscularly for 8–10 days [266,280,295].

Folate administration elicited no hematological response in six Cbl-deficient patients given intramuscular doses of 0.15–0.5 mg/day [138,286,296,297] and 12 receiving a single intravenous injection of 15 μg/kg [298,299].

(*c*) *Effect of Folic Acid Therapy on Serum MMA and HCYS Levels in Cbl Deficiency.* Serum MMA and total HCYS levels were reported in nine Cbl-deficient patients receiving 0.1–1.0 mg of folic acid per day [138]. Serum metabolite levels were available in five of them both before and after folate treatment (patients a–e, Fig. 6), two of whom showed hematological responses. In four additional patients who had received 0.4–1 mg daily by mouth for 3–18 months, metabolite measurements were available only after folate therapy. Three of these patients responded hematologically, two of whom showed progression of neuropathy, and a fourth patient developed neuropathy without anemia. In all nine patients in this report [138], serum MMA and HCYS levels were elevated during folic acid administration and fell into the normal range after treatment with Cbl. These observations demonstrate that oral administration of 0.4–1.0 mg doses of folic acid daily to Cbl-deficient patients is able to induce hematological improvement despite continuing biochemical evidence of Cbl deficiency and is some instances persistent or progressive neurological dysfunction.

(*d*) *Summary.* The effects of pharmacological doses of folic acid have been studied in several hundred Cbl-deficient patients (see Sec. V.B.2.c). When available for review, reticulocyte and hemoglobin responses were noted in more than 90% of these patients. In contrast, the hematological response to folic acid doses of 1 mg/day or less has been reported in only about 60 Cbl-deficient patients, many

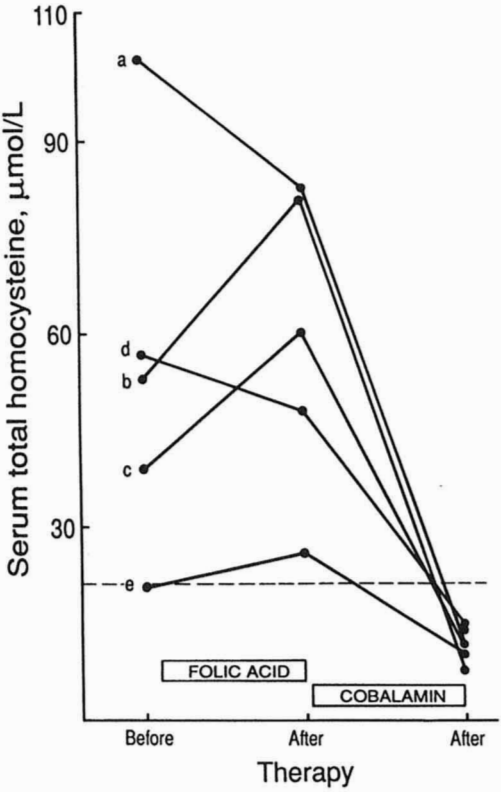


(a)

Figure 6 Serum methylmalonic acid (a) and total homocysteine (b) levels in five patients with cobalamin deficiency who were treated first with folic acid and later with cyanocobalamin. The duration of folic acid therapy between the first and second metabolite assays was 12, 13, 23, 65, and 67 days for patients a, b, c, d, and e, respectively. Patients a and b demonstrated no reticulocyte response to doses of 0.15–0.20 mg/day of parenteral folate. Patient c had no response to a course of 23 days of folate administered orally at a dose of 1 mg/day. Patients d and e received 1 mg/day orally for 2–2.5 months, with a fall in MCV in both cases and normalization of the blood film and correction of anemia in one each; neurological abnormalities did not progress. (Adapted from Ref. 138.)

of whom were observed only briefly and reported in sparse detail. Hematological responses have been commonly documented in patients on doses of 1 mg/day or less; occasional responses have been noted at doses as low as 0.1–0.2 mg/day [52,160,292].

On the other hand, many of those receiving low doses of folic acid had no apparent hematological response, in contrast to the Cbl-deficient patients taking pharmacological doses of folic acid. Seven of 12 patients receiving folic acid at doses



(b)

Figure 6 Continued.

of 0.5–1.0 mg/day failed to show a significant reticulocytosis or rise in hemoglobin [52,138,284,286,287,293,297]. Of 15 patients receiving 0.3–0.4 mg of folic acid per day [52,266,284,294–296], a reticulocytosis of 5% or higher was seen in only 8 [52,266,294,295]. Of three patients followed for more than 2 weeks on doses of 0.3–0.4 mg/day [138,266,294], only one showed an increase in the hemoglobin level [138]. Of the patients receiving 1 mg/day or less of folic acid, Cbl neuropathy was present in six [138,252,260,261], none of whom had a fulminant or "explosive" course. Thus the striking hematological and neurological changes observed in Cbl-deficient patients receiving pharmacological doses of folic acid have not been seen in those taking small amounts, but a larger number of patients need to be studied in prospective trials over longer periods of time to reach a definitive conclusion.

Several of the studies using small doses employed parenteral folic acid therapy [52,266,280,294,295,297]. It is unclear whether the hematological effects of

oral and parenteral folate administration would be equivalent, since malabsorptive defects are common in Cbl deficiency [300–303]. Moreover, there are no data on the clinical effects of synthetic folic acid added to foods in Cbl-deficient patients; this may be important for food-fortification schemes [9,10,17], since food folic acid may be destroyed by cooking [304] and less bioavailable than medicinal preparations [304,305]. The folate fortification of American breakfast cereals over the last two decades has not been associated with documented cases of masked Cbl deficiency, although there has been no systematic effort to detect such effects.

VI. CBL DEFICIENCY AND RISK OF INCREASED FOLATE INTAKE

For the prevention of neural tube defects, it has been recommended that women achieve a folate intake of 0.4 mg/day by ingesting folic acid supplements or increasing their dietary folate; in addition, statutory requirements are under consideration for additional folate fortification of cereal-grain products [9,10,17]. Recommendations may also be forthcoming to increase folate intake [15,16] in populations prone to vascular disease in an attempt to lower HCYS levels.

Because of inevitable variation in diet and self-medication practices, an intake of 0.4 mg/day is likely to be exceeded in some Cbl-deficient individuals. As mentioned, there is very limited experience with low doses of folic acid in Cbl deficiency (see Sec. V.B.3). The data suggest that a folate intake of approximately 0.3–0.8 mg/day may correct or prevent hematological abnormalities in a fraction (possibly as many as 50%) of Cbl-deficient patients; in some of these individuals, it is possible the neuropathy will develop or progress and be more difficult to diagnose because of the lack of overt hematological evidence of deficiency. There are no data to allow us to predict the number of such events that would occur in the general population at such levels of folate supplementation. Approximately 20% of Cbl-deficient patients who have *not* received folate supplementation present with neuropathy in the absence of macrocytosis or significant anemia [30,112]. Thus, in a patient with Cbl neuropathy discovered to have been taking low doses of folic acid, these findings may be unrelated. Masking of the neuropathy by folate treatment cannot be assumed unless it has been shown that there has been a prior hematological response to folic acid. With schemes to increase folate intake in the general population, it will be very difficult to establish whether the absence of obvious hematologic abnormalities in such patients reflects masking by folic acid supplementation or the natural history of their disease. These issues can only be untangled by carefully controlled prospective studies.

A. Prevalence of Cbl Deficiency

1. *Megaloblastic Anemia and Neuropathy Due to Cbl Deficiency*

Which population groups have the highest prevalence of cobalamin deficiency and are thus most at risk from intervention schemes to increase folate intake? In the

industrialized world, pernicious anemia accounts for most episodes of megaloblastic anemia and neuropathy due to Cbl deficiency [116]. Pernicious anemia occurs most frequently in the elderly [30,104,306] (Table 3) and in women [104,307]. Although traditionally considered a disease of northern Europeans, it occurs commonly in other ethnic groups, including blacks [66,116,307–311], Latinos [116,307,309,312], and people of the Indian sub-continent [19,313]. The overall prevalence of pernicious anemia in northern Europe is estimated to be 100–200 per 100,000 population [104]. Based on a comprehensive epidemiological study of the Rochester, Minnesota population, it was calculated that the lifetime risk of pernicious anemia was 10% [314], but a recent reexamination of the same data base indicated that 1.4% would appear to be a more accurate estimate of the lifetime risk at age 75 (L. J. Melton III, personal communication).

Clinically recognizable Cbl deficiency is uncommon in women of child-bearing age. Cbl deficiency was diagnosed in only 42 women under the age of 45 years over a 21-year period at two large New York City hospitals (D. Savage and J. Lindenbaum, unpublished data) and in only 10 women of that age over a period of 29 years in the Rochester, Minnesota area (Table 3) [306]. Although low serum Cbl levels are seen in about 20% of pregnant women [122], overt deficiency is extremely rare in pregnancy. In a survey of 17,000 pregnant women screened for megaloblastic anemia in Liverpool, England, only five (0.03%) had hematological or clinical evidence of Cbl deficiency [315]. Although it is unclear how vigorously Cbl deficiency was excluded in the neural tube defect prevention trials, no episodes of Cbl neuropathy were reported in more than 16,000 women receiving folic acid [1–8,316]. Because Cbl deficiency has an adverse effect on fertility [19], however, incidence data for pregnant females are not likely to reflect the true incidence of Cbl deficiency in young women. One concern is that Cbl

Table 3 Crude Incidence (per 100,000) of Pernicious Anemia in Rochester, Minnesota, 1950–1979

Age group (yr)	Men	Women	Total
25–34	0.0	3.6	1.9
35–44	2.7	5.1	4.0
45–54	8.5	8.7	8.6
55–64	26.7	19.9	22.8
65–74	56.0	75.9	68.3
75–84	119.2	85.8	97.7
⩾85	156.6	127.0	135.0
Total	17.3	23.9	20.9

Age and sex adjusted incidence rate for U.S. whites, 1990, is 23.7 per 100,000 (95% CI = 19.9–27.5).

Source: Based on data in Ref. 306, as modified by A. Zinsmeister and provided courtesy of L.T. Kurland.

deficiency due to pernicious anemia arises earlier in black and Latino women than whites; this trend has been observed in Washington, D.C. and Los Angeles [307–311] but not in New York City (D. Savage and J. Lindenbaum, unpublished observations).

2. *Subclinical Cbl Deficiency in the Apparently Healthy Elderly*

For several decades an increased prevalence of low serum Cbl levels have been reported in apparently healthy elderly people. Although a minority of subjects in these surveys have been found to have (usually subtle) morphological evidence of megaloblastic hematopoiesis, the majority have no clinical evidence of Cbl deficiency [125–128,317–329]. In addition, recent studies have shown that a sizable fraction (approximately 10–20%) of the elderly have elevated MMA levels [125,126,128,329] and that these MMA elevations respond to Cbl therapy [125,127]. Elevated serum metabolite levels are very commonly seen in elderly subjects with low normal serum Cbl concentrations (200–350 pg/ml) in addition to those with subnormal Cbl values (<200 pg/ml) [125,126,329]. The reason for the high prevalence of metabolic Cbl deficiency in the elderly is not established, but it does not appear to be due to classic pernicious anemia, since Schilling tests are usually normal in such subjects and serum antibodies to intrinsic factor are usually not detected [125,148,150,319,327,330]. These data suggest that estimates of the prevalence of Cbl deficiency based on the numbers of clinically diagnosed cases of pernicious anemia may be misleading. Other factors such as dietary Cbl deficiency [319,321], bacterial overgrowth [324,331,332], or food-Cbl malabsorption [144–150] are likely to be important in the elderly. In populations originating from tropical regions, such as Latinos from the Caribbean, tropical sprue is an important cause of Cbl deficiency [116,303].

The clinical significance of the low serum Cbl and high MMA measurements in apparently healthy elderly subjects without clinical evidence of deficiency has not been established. In asymptomatic subjects with low serum Cbl levels treated with vitamin B_{12} injections by Elwood and colleagues [318], there was no effect on hemoglobin level, neuropsychiatric status, or sense of well-being. In another study, only a slight fall in the erythrocyte MCV was demonstrable [125]. There was no relationship between serum Cbl concentration and mortality in a prospective, 10-year cross-sectional study of 673 subjects in Wales [317]. Long-term follow-up studies of such subjects suggest that most remain healthy despite a continued depression of the serum Cbl level [317,327,333], although some will develop clinical evidence of deficiency [327,333]. The published data are inadequate to estimate what percentage are at risk to develop clinically significant deficiency.

For people with low or low normal serum Cbl levels associated with high MMA and HCYS measurements, two vital issues need to be addressed: (1) What is the natural history of the individuals with these laboratory abnormalities? and (2) How will these people be affected by an increase in their consumption of folate?

These questions would best be answered by controlled interventional trials in which the effects of Cbl, folic acid, and placebo treatment are compared.

B. Assessing and Reducing the Risk of Increased Folate Intake in Cbl-Deficient Persons

1. *Educational Efforts and Supplementation with Cbl*

What should be done to minimize the potential risks of increased folate consumption in the potentially large fraction of the general public with unrecognized Cbl deficiency? First, physicians must be made aware of these interventions and reminded of the clinical effects of folate therapy in Cbl-deficient patients. Equally important, clinicians must be reminded that neuropathy occurs in approximately one-fifth of Cbl-deficient patients in the absence of anemia and macrocytosis, regardless of whether they have been taking folic acid supplements [112].

Second, the dangers of increased folate consumption are likely to be reduced by simultaneous supplementation with Cbl, even in patients with pernicious anemia. Despite their failure to secrete intrinsic factor, these patients absorb approximately 1% of cyanoCbl given by mouth and can be maintained indefinitely on oral doses of vitamin B_{12} of 0.3–1.0 mg/day without hematological or neurological relapse [334–338]. Hematological responses have been documented on oral doses as low as 5–16.8 μg/day [339]. In a study reported by Berlin and colleagues [336], 61 patients remained in hematological and neurological remission on doses of 0.5 or 1.0 mg/day for a period of 3 or more years; low serum Cbl levels were noted in occasional patients receiving the lower dose of vitamin B_{12}, but rose into the normal range when the dose was increased to 1.0 mg/day. Ingestion of Cbl-containing multivitamin tablets was associated with higher serum Cbl levels and lower serum MMA levels in elderly subjects in the Framingham Study [329]. The logic of including high doses of cobalamin in supplementation or food-fortification programs is even more compelling in view of recent evidence that suboptimal Cbl status may be an independent risk factor for neural tube defects [18] (see Chapter 12). We would recommend that multivitamin tablets containing folic acid should include 1 mg of Cbl as well. On the other hand, the fortification of food with such large concentrations of Cbl may pose insurmountable technical problems because of changes in the color of fortified foodstuffs.

2. *Detection of the Effects of Increased Folic Acid Intake*

Ideally, the effects of increased folate consumption in Cbl-deficient persons could be monitored by a reliable and inexpensive system of surveillance. Unfortunately, there is currently no diagnostic test for Cbl deficiency that combines high sensitivity and specificity with low cost (see Sec. III). Although a system of mass screening could be devised using tests such as serum Cbl, MMA, and HCYS measurements, it would be highly expensive and would only serve to identify persons at risk rather than those suffering the postulated adverse consequences of increased folate intake.

We would recommend a case-finding approach as an alternative to mass screening. Two or three defined regions where folate supplementation has been implemented would be selected for investigation. A laboratory with expertise in the diagnosis of Cbl and folate deficiency would be available in each region. A study group would be established, which would include clinicians and epidemiologists with a particular interest and expertise in nutritional anemias. Cases would be identified by the presence of typical hematological and neurological manifestations of Cbl deficiency (e.g., anemia, macrocytosis, paresthesias, ataxia); nondeficient age and sex-matched controls would be selected from the same patient population. Cases and controls would be fully investigated using established laboratory techniques and therapeutic trials with vitamin B_{12}, as appropriate, and an intensive evaluation of nutritional status (including folate and Cbl intake).

This case-finding approach should provide considerable useful information while minimizing cost. In our opinion, it would appear to be the best method of monitoring the effects of folate supplementation in large populations at this time.

VII. SUMMARY

Cbl and folate share a common metabolic pathway, the methionine synthase reaction. Deficiency of either vitamin causes a failure of THF and folate coenzyme production, leading to impaired DNA synthesis and megaloblastic hematopoiesis. At the present time, the methylfolate trap (or tetrahydrofolate starvation) hypothesis offers the best explanation for the hematological complications of Cbl deficiency. However, this theory fails to account for the incomplete or transient responses to folic acid in many Cbl-deficient patients.

The metabolic basis for the neuropathy of Cbl deficiency is not understood. Supplementation with methionine delays the onset of experimental Cbl neuropathy in animals. Although methionine deprivation may be the cause of Cbl neuropathy, this remains an unproven hypothesis. It is possible that the neuropathy is due to combined impairment of methionine synthase and the second Cbl-dependent enzyme, L-methylmalonyl-CoA mutase.

Serum Cbl measurements, although valuable in identifying Cbl deficiency, lack sensitivity and specificity for lack of the vitamin. Combined serum MMA and HCYS determinations are highly useful in identifying deficient patients and in distinguishing Cbl from folate deficiency. Serum metabolite determinations have the disadvantages of current high cost and limited availability and decreased specificity in patients with renal dysfunction. The combination of low-normal serum Cbl levels and high metabolite concentrations is extremely common in the apparently healthy elderly, but whether these laboratory abnormalities have important clinical relevance needs to be established through a well-designed interventional trial.

Administration of folic acid at doses of $\geq$5 mg/day ameliorates or corrects (at least temporarily) the megaloblastic hematopoiesis of Cbl deficiency. A normal

hematologic picture may mask the presence of neurological complications in that symptoms of anemia may be alleviated while nervous system damage may develop or progress. It has not been established whether such pharmacological doses of folic acid precipitate or exacerbate the neuropathy of Cbl deficiency in humans. Investigations of the effects of folate supplementation on Cbl neuropathy in laboratory animals have been inconclusive.

Data on the effects of low doses of folate in Cbl deficiency are very limited, but suggest that approximately half of Cbl-deficient patients will show a reticulocytosis after the administration of 0.4 mg of folic acid per day. There is virtually no data available on the hemoglobin response or risk of developing neuropathy on such doses. Whether supplementation with low doses of folic acid will be a significant clinical problem and the frequency with which this might occur cannot be predicted from the available information.

Approximately 20% of Cbl-deficient patients present with neurological complications with little or no anemia in the absence of macrocytosis regardless of whether they have received folate supplements. Therefore, the mere discovery of a Cbl-deficient patient with neurological abnormalities in the absence of anemia or macrocytosis who is receiving folic acid does not establish a cause-and-effect relationship. It is also unclear how increased folate intake will affect the large numbers of apparently healthy, mostly elderly people with low or low normal serum Cbl levels and high MMA and/or HCYS concentrations. The risks of increased folate consumption in persons with undiagnosed Cbl deficiency should be reduced by simultaneous supplementation with generous doses (1 mg/day) of vitamin B_{12}. All of these issues need to be addressed in well-designed prospective trials.

Because of the lack of a diagnostic test that combines low cost, high sensitivity and specificity, and a strong likelihood of being associated with Cbl neuropathy, mass screening strategies to detect Cbl deficiency in the general population would be extremely expensive at this time. To monitor the effects of vitamin supplementation and food-fortification schemes, we have recommended an alternative approach that emphasizes the detection and full evaluation of patients with clinically identifiable Cbl deficiency in selected centers with a special interest and expertise in megaloblastic anemia and Cbl neuropathy.

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Nutrient Interaction of Folate and Zinc

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I. INTRODUCTION

Zinc is essential for plants, microorganisms, and animals and is one of the 10 trace minerals that are essential for humans [1]. Since the discovery of a zinc metalloenzyme, carbonic anhydrase, in 1940 [2], a number of zinc-dependent enzymes have been identified. These enzymes play extremely critical roles in normal growth and development, biochemical functions including protein synthesis and nucleic acid metabolism, and cellular integrity [3]. Since the first discovery of human zinc deficiency in 1963, the importance of zinc has been well recognized in human nutrition [4]. Zinc is also known to be essential for the normal development of the fetus, and a deficiency of this nutrient has been shown to cause malformations in animals [5]. Excellent reviews by Mertz [1], Hambidge et al. [3], Prasad [6], and Vallee and Falchuk [7] on zinc in humans and animals are available.

The importance of maintaining adequate folate nutritional status during human pregnancy has been well recognized for decades, and the wide use of folic acid supplements for the prevention of megaloblastic anemias during pregnancy started in the late 1950s [8]. To date, no complications associated with folic acid supplementation on pregnancy outcome have been reported. However, in 1984 Milne et al. [9] and Mukherjee et al. [10] reported a possible adverse effect of folic acid supplementation on zinc nutriture and suggested its association with fetomaternal complications. Therefore, over the past decade, many researchers have investigated the relationship between folic acid supplementation and zinc nutriture. The results of these investigations have tended to generate some controversy rather than settling issues of interaction between the two nutrients. In light of recent reports demonstrating the beneficial effect of periconceptional folic acid supplementation

in reducing the risk of neural tube defects, it is important to clarify whether folic acid supplementation adversely affects zinc nutriture in humans [11,12]. The main objectives of this chapter are to discuss reports of interactions of folate and zinc published during the last 15 years and to identify gaps in our knowledge that may require further research.

II. HISTORY OF STUDIES ON FOLATE-ZINC INTERACTION

The first paper suggesting an interaction of folate and zinc was published in 1972 by Käferstein and Jaenicke [13] in which they reported that folate conjugase (pteroylgammaglutamylcarboxypeptidase, EC 2.4.22.12) purified from chicken pancreas to near homogeneity requires zinc for its activity. Williams and Mills [14], in 1973, reported that hepatic folate concentrations are reduced by approximately 35% in rats fed a low-zinc diet for a 6-week period as compared to rats fed a diet with adequate zinc. Silink et al. [15], in 1975, reported that zinc is essential for the stability and activity of bovine hepatic folate conjugase that was purified to homogeneity. The activity of the enzyme was reduced by 70% after a 24-hour dialysis against a chelating agent compared to a control dialysis against zinc acetate, however, when the chelator-treated enzyme was again dialyzed against zinc acetate, the activity was restored to 70% of the control enzyme activity. Furthermore, they reported that approximately 4.3 atoms of zinc exist per molecule of the enzyme. Studies by Käferstein and Jaenicke [13] and Silink et al. [15] established the essentiality of zinc for folate conjugase activity and suggested the possibility that folate conjugases from other sources are also zinc dependent.

III. ZINC REQUIREMENT FOR FOLATE CONJUGASE

A. Human Studies

In order to test the hypothesis that human intestinal folate conjugase is a zinc-dependent enzyme, Tamura et al. [16] investigated the intestinal absorption of synthetic folic acid (pteroylglutamic acid, PteGlu) and pteroylheptaglutamate ($PteGlu_7$) synthesized by the solid-phase technique [17] in experimentally induced zinc-depleted humans. Healthy adult male volunteers were fed a semipurified-formula diet containing 0.2 mg of elemental zinc per day for a period ranging from 4 to 9 weeks until the subjects became zinc depleted. Zinc depletion was defined by a decline in plasma zinc concentration to less than 0.7 μg/ml and by the appearance of certain clinical changes [18,19]. The diet contained 500 μg/day of PteGlu and oral administration of either PteGlu or $PteGlu_7$ (1.0 mg, 2.27 μmol) was given before the initiation of the low zinc diet and after the subjects became zinc depleted. The intestinal absorption of each folate was assessed by measuring the increase in serum folate concentrations after an oral dose, using *Lactobacillus*

casei microbiological assay. There were no differences between the absorption of PteGlu and $PteGlu_7$ before the subjects were given a low-zinc diet. However, after zinc depletion, the absorption of $PteGlu_7$ estimated by the area under the curve was reduced by an average of 52%, while the absorption of PteGlu remained unchanged (Table 1). Although direct determinations of intestinal mucosal folate conjugase activity were not performed, the data from this study suggest that zinc depletion causes decreased intestinal hydrolysis of $PteGlu_7$ and that human intestinal mucosal folate conjugase is a zinc-dependent enzyme. Because the experimental diet contained only synthetic PteGlu, Tamura et al. [16] were unable to evaluate whether the zinc-depleted subjects had reduced absorption of dietary folate, which exists mainly as pterolypolyglutamates ($PteGlu_n$) [20].

In 1986, Chandler et al. [21] demonstrated that the enzyme in human intestinal mucosal brush border requires zinc for its activity. These workers purified folate conjugase from the human jejunal mucosal brush border and showed that activity of the purified enzyme is reduced after dialysis against chelating agents and the activity restored by the in vitro addition of zinc. Chandler et al. [21] suggested that the activity of brush border folate conjugase (BBFC), hence the absorption of dietary folate, is influenced by the intestinal zinc levels. Wang et al. [22] reported that in contrast to the brush border enzyme, intracellular human intestinal mucosal folate conjugase is not zinc dependent. In 1987, Gregory et al. [23] reported that

Table 1 Response of Serum Folate Concentrations Following Oral Dose of PteGlu or $PteGlu_7$ (1 mg; 2.27 μmol) in Human Subjects[a]

	Concentration before zinc depletion (ng/ml)	Concentration after zinc depletion (ng/ml)	*p*
PteGlu			
0 hour	9.1 ± 4.8	10.7 ± 5.2	NS
1	51.2 ± 12.3	41.5 ± 6.3	NS
2	36.4 ± 8.4	39.6 ± 8.1	NS
Area under the curve above base line (ng/ml × hr)	55.9 ± 19.5	42.1 ± 8.2	NS
$PteGlu_7$			
0 hour	12.8 ± 3.2	13.9 ± 4.6	NS
1	45.8 ± 8.8	29.3 ± 8.7	< 0.01
2	36.0 ± 7.3	28.7 ± 6.1	< 0.05
Area under the curve above base line (ng/ml × hr)	47.6 ± 11.0	22.9 ± 9.0	< 0.005

[a] Mean ± standard deviation.
Source: Ref. 16.

the activity of partially purified human intestinal mucosal BBFC is reduced when the preparation was treated in vitro by EDTA, but activity is restored when an equimolar amount of zinc is added to EDTA. This additional evidence confirms that the human intestinal BBFC is zinc dependent.

The findings of Tamura et al. [16], Chandler et al. [21], and Gregory et al. [23] together firmly establish the zinc dependency of the activity of human intestinal folate conjugase, while it is not known whether zinc is required for the synthesis of the apoenzyme. These studies suggested that the availability of dietary $PteGlu_n$ is affected by zinc nutriture in humans. However, the clinical implication of these findings is unclear, since no well-controlled studies have indicated that poor zinc nutriture impairs the absorption of dietary folate in humans. Further studies are warranted to evaluate whether the absorption of dietary folate is affected among certain populations with inadequate zinc nutriture, such as infants and children whose growth responded to zinc therapy [24–26].

B. Animal Studies

Day and Gregory [27], in 1984, observed that zinc is required for the activities of human and porcine intestinal mucosal BBFC. Furthermore, in 1985 Wang et al. [22] evaluated the effect of in vitro zinc addition on the activities of folate conjugases in both brush border and intracellular fractions prepared from intestinal mucosal homogenates of monkey, pig, and rat. In vitro addition of zinc increased the activity of folate conjugase of the brush border preparations of pig, but it failed to stimulate the activities in the intracellular fractions of all three species tested. In contrast to human and pig, little or no folate conjugase activity was identified in the brush border fractions prepared from intestinal homogenates of monkey and rat [22]. Gregory et al. [23], reported that activity of partially purified intestinal BBFC from pig is inhibited by the in vitro addition of EDTA, but this inhibition was corrected by the addition of an equimolar amount of zinc. Thus, the mechanism of hydrolysis of $PteGlu_n$ in the pig resembles that in human. In addition, Bhandari et al. [28] reported that the activity of folate conjugase in the pancreatic juice of the pig is stimulated by the in vitro addition of zinc.

Canton et al. [29] reported that folate conjugase in the pancreas and washings of the intestinal lumen of the rat are zinc dependent. They measured the activities of folate conjugases and found that the activities are significantly lower in zinc-deficient rats than those in two groups of control rats that were either pair-fed with deficient rats or ad libitum fed a zinc-adequate diet. These results were confirmed by the decreased absorption of $PteGlu_n$ purified from yeast in zinc-deficient rats as compared to the other two control groups. However, the amount of folate conjugase in the intestinal lumen, which is apparently derived from pancreas, should have been sufficient to completely digest $PteGlu_n$ according to the calculation by this author based on the data reported by Canton et al. [29]. Total activity of folate conjugase in the intestinal luminal wash was estimated by plotting the changes of the enzyme

activity over a 3-hour period after loading yeast $PteGlu_n$ and by measuring the area under the curve for each group. These were approximately 900 and 1500 ng of $PteGlu_n$ hydrolyzed per mg of protein in zinc-deficient and pair-fed control groups, respectively. Assuming that the protein concentration in pancreatic juice was 10% and the amount of pancreatic juice secreted over the period was *only* 100 μl, the intestinal luminal folate conjugase should have been able to hydrolyze 9 and 15 μg of $PteGlu_n$ in zinc-deficient and pair-fed groups, respectively, although the amounts of yeast $PteGlu_n$ given to the rats ranged between 6.2 and 6.9 μg.

The findings of Canton et al. [29] are different from the data published by Tamura and Kaiser [30], who showed that the absorption of both chemically synthesized [^{3}H]-PteGlu and Pte-[^{14}C]Glu-Glu_6 in zinc-deficient rats was similar to that in controls. Tamura and Kaiser [30] showed that the intestinal folate conjugase activities were similar in zinc-deficient and control groups, indicating that rat intestinal folate conjugase is not zinc dependent. The findings by Tamura and Kaiser [30] are consistent with those of Wang et al. [22], who demonstrated that intestinal mucosal BBFC is absent in the rat and that intracellular folate conjugase is not zinc dependent. Canton et al. [31] demonstrated that the decline in folate conjugase activity in the pancreas occurs before depletion of zinc stores in many tissues of rats fed a low-zinc diet, indicating that the enzyme activity is sensitive to zinc nutriture.

Vickers et al. [32] reported that the activity of highly purified folate conjugase from bovine liver was inhibited by the in vitro addition of zinc chloride. The 50% inhibition was observed at a zinc chloride concentration of 2.5 mmol/liter using [^{3}H]-methotrexate diglutamate as the substrate. These findings are in contrast to those of Silink et al. [15], which indicate that folate conjugase in bovine liver is zinc dependent.

In summary, scientific data support the conclusion that zinc is required for the optimal function of folate conjugase from the following sources: human intestinal mucosal brush border, pig intestinal brush border and pancreas, bovine liver, chicken pancreas, and the pancreas and intestinal washings of the rat (Table 2). In contrast, intracellular folate conjugases in the intestinal mucosa from human, monkey,

Table 2 Zinc-Dependent Folate Conjugases

Species	Tissue	Ref.
Human	Intestinal mucosal brush border	16,21,23
Cow	Liver	15
Pig	Intestinal mucosal brush border	27
	Pancreas	28
Rat	Pancreas	29
	Intestinal washings	29
Chicken	Pancreas	13

pig, and rat are not zinc dependent. The absorption of $PteGlu_n$ is reduced in experimentally induced zinc-depleted humans. However, the clinical significance of this observation is not clear. Further studies are warranted to determine whether the decreased absorption of dietary $PteGlu_n$ occurs in humans with marginal zinc deficiency.

IV. FOLATE METABOLISM IN ZINC DEFICIENCY

A. Human Studies

As noted in the preceding section, only a few reports have been published on the effect of zinc nutriture on folate metabolism and absorption, particularly of $PteGlu_n$ in humans. Among the numerous reports of nutritional zinc deficiency in humans, it is rare to find any that include the assessment of folate nutriture. In the first series of investigations of human zinc deficiency, Prasad et al. [33] determined urinary excretion of formiminoglutamic acid (FIGLU) after histidine loading. The urinary FIGLU excretion in 10 zinc-deficient patients was similar to controls, indicating that these zinc-deficient patients were not folate deficient. Caggiano et al. [34] reported that serum folate concentration was within the normal range in a zinc-deficient patient with growth retardation and immune dysfunction. Furthermore, normal serum folate concentration has been reported in a patient who suffered from severe zinc deficiency secondary to alcohol abuse; manifestations of acrodermatitis in this subject were responsive to systemic zinc therapy [35]. In these reports, however, it was not clear whether the patients were given folic acid supplements before the tests were performed, since the interaction of folate and zinc had not been recognized at that point. In a case report describing a zinc-deficient patient with Crohn's disease, Heimburger et al. [36] reported normal concentrations of plasma and erythrocyte folate.

Jones and Peters [37] reported two patients with celiac syndrome who, after they were identified as having abnormally low plasma zinc concentrations, received a zinc supplementation trial (oral daily dose of approximately 150 mg for 8 weeks). Both patients had subnormal serum folate concentrations before the trial, and these concentrations remained low throughout the period of zinc therapy, indicating that the absorption of dietary folate was not improved by zinc therapy. It is known that the absorption of folate is reduced in patients with this disease by the mechanism of decreased absorption of hydrolyzed PteGlu derivatives, decreased digestion of $PteGlu_n$, or both. Although no determination of folate conjugase activity was performed in this study, it is possible to conclude that the zinc therapy neither improved nor impaired the folate status of the patients [37]. Jameson et al. [38,39] reported that signs of zinc deficiency almost always precede folate deficiency in patients with celiac disease. They suggested that the absorption of dietary folate is impaired due

to the reduced activity of intestinal folate conjugase which is zinc dependent. Furthermore, Jameson et al. [38] reported that PteGlu treatment increases the absorption of both PteGlu and zinc in these patients.

Milne et al. [40] reported that unexplainable disagreement exists between erythrocyte folate concentrations determined by microbiological assay using *L. casei* and radioassay methods when these erythrocytes were obtained from human subjects who were given a suboptimal amount of dietary zinc over a 4-month period. They suggested that a previously unrecognized role of zinc in folate metabolism occurs, namely, the conversion of a folate derivative(s) to a form(s) that did not respond to the radioassay. To date, however, such a phenomenon has not been reproduced, presumably due to the use of only one method (either microbiological or radiobinding assay) in the course of investigations.

Recently, Milne and Johnson [41] monitored the concentrations of serum and erythrocyte folate during a 208-day metabolic study in which 11 male subjects were given diets containing zinc (1, 2, 3, and 4 mg/day) for 36 days following a 28-day equilibration period (dietary zinc 10 mg/day). The subjects received a diet containing 10 mg of zinc at the end of the study for 36 days to replete zinc. Serum folate concentrations remained unchanged throughout the study, while erythrocyte folate levels during the periods of equilibration and of feeding 4.0 mg of dietary zinc were significantly lower than the repletion period. The authors indicated that the dietary intake of folate before admission was lower than the amount given during the study. The data indicate that short-term changes of low zinc intake do not affect blood folate concentrations.

Kauwell et al. [42] reported that there were no differences in serum and erythrocyte folate concentrations, urinary folate excretion, and the percentage urinary excretion of an orally administered deuterium-labeled PteGlu (800 μg/day) among a group of six adult males who received a restricted zinc intake (3.5 mg/day) for 24 days and another group of six men who received a zinc-adequate diet (14.5 mg/day) for the same period of time. This study was designed to determine erythrocyte metallothionein levels, which were previously shown to decrease within this time frame in response to a severely restricted zinc diet [43]. Folate utilization was found to be similar in both groups, however, the daily dose of 800 μg of deuterium-labeled PteGlu was potentially large enough to overcome subtle changes of folate metabolism secondary to zinc deficiency [42].

B. Animal Studies

Because of difficulties in conducting controlled metabolic studies in humans, several studies have been performed with laboratory animals in order to achieve better regulation of experimental conditions. The advantage of using the animal model includes the easy accessibility and the ability to develop well-defined severe zinc deficiency.

Hsu and his colleagues [44,45] performed several experiments on the metabolism of amino acids and formate in zinc-deficient rats in which folate metabolism may be directly or indirectly involved. An altered metabolism of sulfur amino acids in zinc-deficient rats was observed [44]. Findings included significantly increased production of $^{14}CO_2$ following administration of [$^{14}CH_3$]-methionine in zinc deficient rats, as compared to zinc-supplemented controls. However, increased production of $^{14}CO_2$ was not observed after the injection of 2-[^{14}C]-methionine and 1-[^{14}C]-methionine in zinc-deficient rats. These data indicated that transmethylation reactions, not transsulfuration reactions, are more active in zinc deficiency. Furthermore, they reported that the oxidation of [ring-2-^{14}C]-L-histidine and [^{14}C]formate is increased [44,45]. They further demonstrated that hepatic activities of both histidase and urocanase are increased in zinc-deficient rats. Therefore, they concluded that the increased oxidation of histidine is in part due to the increased activities of these enzymes caused by zinc deficiency.

These observations by Hsu et al. [44,45] appear to indicate that an increase in transmethylation, as well as the increases in oxidation of histidine and formate, are produced by altered folate metabolism secondary to zinc deficiency. The investigation of this topic was extended by Tamura et al. [46]. They produced zinc-deficient Sprague-Dawley male rats by feeding a semipurified egg-white diet containing less than 1 μg of zinc/g of diet for 6–7 weeks. Various parameters relating to folate metabolism were measured and compared with two other groups of rats that were either pair-fed with zinc-deficient rats or ad libitum fed a zinc-supplemented diet (100 μg of zinc/g of diet). They found that hepatic methyltetrahydrofolate: homocysteine methyltransferase (methionine synthase, EC 2.1.1.13) activity was increased approximately 60% in zinc-deficient rats, as compared to the other two controls. This increase resulted in a decreased proportion of 5-methyltetrahydrofolate (5-CH_3-H_4PteGlu$_n$) in the liver of zinc-deficient rats, although the total liver folate concentrations were similar in all groups. This change led to increased oxidation of [ring-2-^{14}C]-L-histidine and [^{14}C]-formate in zinc deficiency, presumably due to increased tetrahydrofolate. The mechanism of increased methionine synthase activity in zinc-deficient rats is unknown.

Decreased plasma and erythrocyte folate concentrations have also been observed in zinc-deficient rats, as compared to controls in published [46] and unpublished studies in the author's laboratory. In addition, a slight, but significant, decrease in the percentage of 5-CH_3-H_4PteGlu found in total plasma folate, estimated by the differential microbiological assay using *L. casei* and *Streptococcus faecium*, was observed in zinc-deficient rats. This finding may reflect the increased hepatic activity of methionine synthase due to zinc deficiency. The observation of reduced plasma folate concentrations in zinc-deficient rats is consistent with the findings of Canton et al. [29,31].

It is known that the activity of hepatic 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4PteGlu) reductase (EC 1.1.99.15) is controlled by thyroid function as well

as by the levels of S-adenosylmethionine (SAM) [47,48]. Stokstad et al. [47] reported that the activities of this enzyme decrease in rats with hypothyroidism. Tamura et al. [46] reported that 5,10-CH_2-H_4PteGlu reductase activities were not decreased in zinc-deficient rats, although the concentrations of plasma thyroid hormones (T_3 and T_4) were low. This finding is not surprising, as low levels of plasma T_3 and T_4 do not necessarily indicate hypothyroidism and circulating thyroid-stimulating hormone levels are not elevated in these rats [49]. Hepatic levels of SAM or methionine were not measured [46].

Tamura et al. [50] investigated the effect of zinc deficiency and testosterone administration on the activities of hepatic 10-formyltetrahydrofolate:NADP oxidoreductase (10-HCO-H_4PteGlu dehydrogenase, EC 1.5.1.6). They found that the activities of 10-HCO-H_4PteGlu dehydrogenase were significantly higher in the liver from zinc-deficient rats than in their pair-fed controls. They speculated that changes in enzyme activity are mediated by impaired testosterone function, which is known to occur in the zinc-deficient rat [51]. After injection of testosterone for 7 days, the increased activities of 10-HCO-H_4PteGlu in zinc-deficient rats became similar to the levels of those in pair-fed controls, substantiating the suggestion that administration of supplemental testosterone partially overcomes the effect of zinc deficiency [50].

It should be pointed out, however, that these experiments were performed using rats that were fed a low-zinc diet for several weeks: hence, the degree of zinc deficiency was extremely severe. It is unknown whether altered folate metabolism can be seen, for example, in rats with mild zinc deficiency. Certainly, it is difficult to extrapolate these data to human zinc deficiency, which is not generally as severe as in these experimental animals.

V. EFFECT OF FOLIC ACID ON THE ABSORPTION OF ZINC AND ZINC NUTRITURE

After the article by Milne et al. [9] appeared in 1984, interest was intensified to determine if PteGlu supplementation alters zinc nutriture in humans as well as in animals. Many studies were conducted to investigate the effect of PteGlu supplementation on zinc absorption and nutriture. As reviewed previously by Butterworth and Tamura [52], it has been difficult to judge the effect of PteGlu on zinc absorption and nutriture in humans because of the variation in the methods utilized and the molar ratios of folate to zinc used in these studies.

A. Human Studies

Milne et al. [9] studied the effect of PteGlu supplementation on zinc homeostasis using eight male volunteers who received diets containing three levels of zinc (approximately 8.0 mg/day for 31–42 days; 3.5 mg for 108–126 days; and 34 mg

for 18–24 days) in a total period of 6 months. Subjects were divided into two groups: one received 400 μg of PteGlu supplementation on alternate days in addition to the diet containing 150–180 μg per day of folate, and the other received no supplement. Zinc balance was measured throughout the study. Fecal zinc loss was significantly higher during periods of low-zinc diets in four subjects who received folic acid supplements than in those who did not (Table 3). However, these differences in fecal zinc loss were not observed during the repletion period when they received 35 mg of zinc per day. Zinc balance was not significantly different between the two groups. Milne et al. [9] suggested that the decreased zinc absorption was due to the formation of an insoluble compound of zinc that resulted from the binding to PteGlu in the intestine. However, it is difficult to accept this reason for the increased fecal zinc loss in PteGlu-supplemented subjects. The amount of zinc in the diet was approximately 3.5 mg (53.5 μmol) which is, on a molar basis, more than 60 times higher than the total folate ingested (0.86 μmol; dietary folate of 150–180 μg plus 200 μg of PteGlu). Even if all folate was available to form an insoluble compound by binding to zinc, more than 98% of dietary zinc should have been available for absorption. On the other hand, if it is assumed that one mole of PteGlu binds to one mole of zinc, the subjects should have had decreased folate absorption under the conditions of high zinc intake. Therefore, even the subjects in the PteGlu-supplemented group should have exhibited signs of folate depletion. However, no signs, such as decreased erythrocyte folate levels, were documented. In contrast, these values were increased when the subjects were given 35 mg of

Table 3 Effect of Folic Acid Supplementation on Fecal and Urinary Losses of Zinc in Men

	Folic acid supplementation in addition to dietary folate (150 μg/day)		
	0	400 μg (every other day)	*p*
Control period (dietary zinc 8.3 mg/day)			
Feces	5.83	7.30	<0.008
Urine	0.45	0.28	<0.001
Depletion period (dietary zinc 3.5 mg/day)			
Feces	2.37	2.87	<0.001
Urine	0.54	0.23	<0.001
Repletion period (dietary zinc 33.7 mg/day)			
Feces	28.94	27.88	NS
Urine	1.02	0.48	<0.001

Source: Ref. 9.

zinc during the third period, as compared to when the subjects were given only 3.5 mg of zinc. Therefore, it is reasonable to assume that some other mechanisms are responsible for the increased fecal zinc loss.

This report by Milne et al. [9] prompted many researchers to attempt to reproduce the observations. In a letter to the editor of the Journal of National Cancer Institute, Kakar and Henderson [53], without providing substantiating data, recommended that researchers monitor plasma zinc among women who were being given oral PteGlu supplementation in studies to evaluate the effect of this on cervical dysplasia [54]. In 1988, Butterworth et al. [55] responded to this recommendation by reporting that a daily oral dose of 10 mg of PteGlu for 4 months did not result in decreased plasma or erythrocyte zinc concentrations among women in the randomized-double blind clinical trial. This was confirmed by a further follow-up of plasma and erythrocyte zinc concentrations in many of these same subjects at 6 months after the initiation of PteGlu supplements (Table 4).

In 1987, Simmer et al. [56] reported evidence to support the findings of Milne et al. [9] that PteGlu supplementation interferes with zinc absorption in 10 healthy volunteers. They assessed zinc absorption by determining the increases in plasma zinc concentrations after 50 mg of oral zinc administration. These tests were performed before and after the subjects were given a daily oral supplementation of PteGlu (350 μg) for 2 weeks. The absorption of zinc was reduced after PteGlu supplementation (Table 5). Furthermore, in a study involving 10 pregnant women, zinc absorption was evaluated before and 24 hours after the termination of a

Table 4 Median Concentrations of Folate (ng/ml) and Zinc (μg/ml) in Plasma and Erythrocytes Obtained from Women with Cervical Dysplasia Supplemented with Oral PteGlu (10 mg/day) or Placebo[a]

	Months of supplementation			
	0	2	4	6
Folic acid–supplemented group (n = 49)				
Plasma folate	4.3	23.0	29.4	19.1
Erythrocyte folate	168	536	611	565
Plasma zinc	0.86	0.85	0.85	0.86
Erythrocyte zinc	11.3	11.4	11.2	11.6
Placebo group (n = 58)				
Plasma folate	5.5	4.9	4.4	4.7
Erythrocyte folate	181	183	172	178
Plasma zinc	0.81	0.83	0.85	0.85
Erythrocyte zinc	11.5	11.1	10.9	10.9

[a] Data of 0, 2, and 4 months have been published [55].

Table 5 Response of Plasma Zinc Concentrations after Oral Zinc Administration

Subjects	Treatment	Doses of zinc	Area under the curve (μg/ml × hr)	Peak concentration (μg/ml)	Ref.
10 healthy volunteers	before[a]	50 mg	4.30 ± 0.29[b]	1.67 ± 0.12	56
	after		3.39 ± 0.48	1.24 ± 0.16	
			$p < 0.05$	$p < 0.05$	
10 pregnant women	before[c]	25 mg	1.04 ± 0.12	0.53 ± 0.07	56
	after		0.51 ± 0.23	0.35 ± 0.09	
			$p < 0.02$	$p < 0.02$	
6 healthy young men	none	25 mg	3.70[d]	1.18	57
	PteGlu[e]		4.28	1.41	
			NS	NS	

[a] Zinc tolerance test was done before and after the subjects were supplemented with a daily dose of 350 μg PteGlu for 2 weeks.

[b] Mean ● standard deviation.

[c] Zinc tolerance test was done before and after the subjects were supplemented with a tablet containing 100 mg of ferrous iron and 350 μg of PteGlu daily for 2 weeks.

[d] Mean values; no standard deviations were reported. The areas under the curve were calculated by this author.

[e] PteGlu (10 mg) was administered together with 25 mg of zinc.

2-week prenatal vitamin supplementation containing 350 μg of PteGlu and 100 mg of ferrous iron. Zinc absorption, assessed by the same method as mentioned above, was reduced after supplementation with PteGlu and iron for 2 weeks. The degree of reduction in zinc absorption was larger in the study using pregnant women than in nonpregnant healthy volunteers given PteGlu alone, suggesting that PteGlu has a smaller effect on the absorption of zinc than the PteGlu-iron combination. In the latter study of pregnant women, oral administration of zinc was not combined with prenatal supplements of PteGlu and iron, therefore, Simmer et al. [56] suggested that the reduction of zinc absorption occurred at the site of intestinal mucosal cells rather than within the lumen. Based on these observations, they concluded that the hypothesis that an insoluble chelate of zinc and PteGlu forms within the lumen is unlikely to be correct.

Keating et al. [57], in 1987, performed studies to evaluate the effect of PteGlu on zinc absorption in humans and rats (Table 5). They assessed zinc absorption in six male volunteers using the response in serum zinc concentrations following an oral dose of zinc (382 μmol, 25 mg) with and without PteGlu (23 μmol, 10 mg; a molar ratio of PteGlu to zinc of 1 to 17). They observed no evidence that zinc bioavailability was altered by PteGlu. However, Milne [58] later raised a question as to the validity of zinc tolerance tests performed by Simmer et al. [56] and by

Keating et al. [57] for the following reasons: (1) the amount of zinc given for the absorption tests was much higher than normally consumed; (2) the initial rate of absorption, represented by the determination of zinc in circulation for only four hours after the oral dose, may not reflect net zinc absorption; (3) the tissue uptake of newly absorbed zinc is not considered; and (4) the rate of gastric emptying may influence the patterns of tolerance curves.

Krebs et al. [59] reported the effect of PteGlu on zinc nutriture as well as zinc absorption in two independent human populations. They evaluated zinc nutriture of eight patients with fragile X syndrome, who had been given a daily dose of 10–20 mg PteGlu for at least one year, by determining plasma zinc concentrations and the activities of plasma zinc metalloenzymes, including alkaline phosphatase and delta-amino-levulinic acid dehydrogenase. They found that the levels of these indices in patients were within the normal ranges, indicating no adverse effects of PteGlu supplementation on zinc nutriture in these patients. In addition, these researchers estimated the intestinal absorption of zinc using a stable isotope (^{70}Zn;0.81–1.83 mg) in three healthy adults with or without the administration of PteGlu (30 mg; molar ratio of PteGlu to zinc of 1 to 0.18–0.41). The absorption of ^{70}Zn was reduced by only 4.4% when PteGlu was given simultaneously. Based on the findings of these two experiments, they concluded that long-term administration of PteGlu does not interfere with zinc nutriture in humans and that the effect of a simultaneous PteGlu administration on the absorption of zinc is minor and not clinically significant.

Milne [58], in 1989, evaluated the effect of PteGlu (800 μg/day) on the intestinal absorption of a radioactive tracer dose of zinc (^{65}Zn, 0.1 μCi with 2.7 mg of zinc, molar ratio of PteGlu to zinc of 1 to 22.7) in a total of 13 human subjects who were allowed to consume their usual diet (average zinc intake of 11.4 mg/day by 3-day dietary diaries). The mean absorption of ^{65}Zn was not significantly different during the control and PteGlu-supplemented periods. Supplementation of PteGlu reduced the absorption of ^{65}Zn in only those subjects who demonstrated less than 30% of ^{65}Zn absorption when tested without the effect of PteGlu supplementation. In addition, the subjects whose ^{65}Zn absorption was affected by PteGlu tended to consume lower dietary zinc and to have lower plasma zinc and higher erythrocyte folate concentrations than those whose absorption was not affected by PteGlu. No difference was observed in the effect of PteGlu supplementation on the biological half-life of ^{65}Zn between periods when the subjects were supplemented with PteGlu and when they were not. Plasma zinc concentrations remained unchanged for a 10-week period during which the subjects received 800 μg of PteGlu daily.

Milne et al. [60], a year later, published another study of eight men fed a diet containing adequate zinc and folate (daily intake 12.6 mg and 300 μg, respectively) for a total of 168 days divided into four periods. During the first and third periods, the subjects received no PteGlu supplementation, and during the second and fourth, they received 400 and 800 μg/day, respectively, in random order (the

molar ratios of folate to zinc of 1 to 282, 120, or 75). Various indices of zinc nutriture were not affected by PteGlu supplementation, and the absorption of stable isotope zinc (^{67}Zn) was not significantly influenced by PteGlu supplementation. Zinc mobilization into the circulation was inversely related to the amount of PteGlu and was impaired by exercise. Furthermore, urinary and fecal zinc losses were not affected by PteGlu during each period. These findings are not consistent with the initial observation made in 1984 by the same group of researchers [9].

Fuller et al. [61] found a significant inverse relationship between serum folate and zinc concentrations among 60 preterm infants, 80% of whom received an oral dose (1.0 mg/day of PteGlu) during the early stages of life. They cautioned that such a large dose of PteGlu might adversely affect zinc nutriture of preterm infants and that a large dose of PteGlu should be given only when its benefit is warranted. However, the duration of oral PteGlu supplementation as well as the provision of parenteral zinc and PteGlu administration prior to oral PteGlu in some of the infants were not specified. Reid et al. [62] studied folate and zinc status in 21 patients who were receiving hemodialysis. They reported that neither folate (5 mg) nor zinc (25 mg) supplementation for 4–6 months affected zinc or folate nutritional status, respectively, as judged by the concentrations of serum folate and zinc and erythrocyte folate. They concluded that no apparent interaction between folate and zinc occurred among these patients.

Arnaud et al. [63] performed a zinc tolerance test to investigate the effect of PteGlu and 5-formyltetrahydrofolate (5-HCO-H_4PteGlu) on intestinal zinc absorption in 10 healthy subjects. They measured the changes in serum zinc concentrations and urinary zinc excretion following the administration of either zinc (30 mg), zinc with PteGlu (200 mg), or zinc with 5-HCO-H_4PteGlu (200 mg) at the folate to zinc molar ratio of 1 to 1. There were no significant differences in three loading tests as judged by the calculations of the area under the curves, peak zinc concentrations, half-life of zinc, and the urinary elimination of zinc.

Kauwell et al. [42,64] performed a study of 12 men who were assigned to consume diets containing either 3.5 or 14.5 mg of zinc/day for 25 days; 50% of the subjects in each group received 800 μg/day of supplemental PteGlu and the other 50% received no additional PteGlu. The study was repeated by reversing the PteGlu doses among subjects after an 80-day interval. No effect of PteGlu supplementation on any of the variables at either level of zinc intake was observed during this relatively short-term protocol.

In summary, the study by Milne et al. [9] stimulated interest in investigating the effect of PteGlu supplementation on zinc absorption and nutriture, and several articles have appeared on this topic during the last decade. Various researchers are not in agreement as to whether PteGlu adversely affects zinc nutritional status, and this disagreement may be due to the variations in the methods used in each study

[52]. These methods included the determinations of (1) zinc concentrations in serum, plasma, erythrocyte, or other blood cell components, (2) urinary excretion of zinc, (3) activities of zinc-dependent enzymes in serum or plasma, (4) absorption of zinc asesssed by increases in plasma zinc after oral dose and by the use of stable isotopes of zinc, (5) absorption and retention of radioactive zinc, and (6) zinc balance by measuring fecal and urinary losses in relation to intake. Another factor is the wide range in the ratios of folate to zinc used in the various studies, rendering it difficult to make direct comparison of results (Table 6).

Some of the techniques used in the investigations reviewed above are not always specific and sensitive indicators of zinc nutriture [65], therefore, some conclusions may be subject to reinterpretation or reexamination. It seems reasonable to conclude that PteGlu supplementation has either no effect on zinc nutriture or an extremely subtle effect. To modify beliefs based on early research, it will be necessary to conduct well-designed and well-controlled human studies that will lead to a definite conclusion.

B. Animal Studies

Shortly after the appearance of the paper by Milne et al [9] in 1984, McMaster et al. [66] evaluated the effect of two levels of dietary PteGlu (5 and 500 μg/day) on the intestinal absorption and tissue concentrations of zinc in rats fed a diet containing

Table 6 Effect of Folic Acid on Zinc Absorption and Zinc Nutriture in Humans

Methods	Molar ratios of folate to zinc[a]	Year of publication	Ref.
Zinc balance	1:135	1984	9
Plasma zinc response curve	1:750	1987	56
Plasma zinc response curve	1:17	1987	57
Plasma and red blood cell zinc	1:7	1988	55
Plasma zinc levels and zinc-dependent enzymes	1:7	1989	59
^{70}Zn absorption	1:0.18–0.41	1989	59
^{65}Zn absorption and blood zinc levels	1:23	1989	58
^{67}Zn absorption and zinc balance	1:75–282	1990	60
Serum zinc levels	1:2–30	1992	61
Serum and urinary zinc levels	1:1	1992	63
Serum zinc levels	1:20	1993	77
Zinc levels and zinc-dependent enzymes	1:22–336	1994	42

[a] Zinc intake of 12 mg/day was used to calculate the ratios in the studies where zinc intake data were not available.

50 μg of zinc/g of diet for 5–6 weeks. They found that the luminal uptake was significantly reduced by high intakes of PteGlu, while the amount of zinc released from the perfused gut was not affected. There was no obvious effect of high PteGlu intake on the net absorption of zinc as compared to that from low PteGlu intake. Furthermore, they showed that there was no difference in zinc concentrations in the serum, liver, and intestine among rats fed two levels of PteGlu. However, zinc concentrations in the kidney of rats given high PteGlu were significantly lower than those in rats fed low PteGlu. The significance of the latter finding is unclear. Ghishan et al. [67] observed a decreased absorption of zinc in rats when PteGlu was placed in the intestinal perfusate. The decreased zinc absorption was not found in the intestinal perfusion from rats given 500 μg/kg body weight of PteGlu for the preceding 14 days. They found that 250–500 μmol/liter of zinc inhibited mucosal-to-serosal transport of PteGlu in a study using everted sacs of rat jejunum and concluded that a mutual inhibition between folate and zinc exists at the intestinal absorption site. In vitro studies demonstrated that the interaction is pH dependent, since zinc and PteGlu formed insoluble complexes at pH 2.0, but these complexes dissolved at pH 6.0.

Keating et al. [57] reported no effect of PteGlu supplementation on the absorption of ^{65}Zn and the concentrations of zinc in the femur using a rat model. They tested the effect of PteGlu on ^{65}Zn absorption at molar ratios of folate to zinc of 2 to 1 and 1 to 20 by determining the retention of radioactive zinc in the liver and kidney four hours after intragastric administration. They also evaluated the zinc absorption by measuring zinc concentrations in the femur 3 weeks after feeding rats diets containing folate-to-zinc ratios of 4 to 1, 2 to 1, and 1 to 20.

Tremblay et al. [68] reported that a daily supplementation of PteGlu (5 mg/kg body weight) was associated with increased concentrations of both serum folate and zinc in pregnant sows at 30 days of gestation. The data indicated no apparent interference of PteGlu with zinc absorption, as judged by the determination of serum zinc. Extrapolation of these animal studies to humans must be done cautiously, since the process of the absorption of the nutrients in animals is not necessarily similar to that in humans.

To this author's knowledge, there are not many studies reporting the effect of folate deficiency on the metabolism of zinc. However, recently, Bills et al. [69] determined the concentrations of zinc in the liver of mice fed an amino acid-based diet with and without PteGlu for several weeks. They found that liver zinc concentrations of folate-deficient mice (47.1 μg/g) were significantly higher than those of mice given PteGlu (35.9 μg/g, $p = 0.001$). Although the exact mechanism of this finding is obscure, these researchers speculated that the greater zinc concentrations in the liver of folate-deficient mice are due to reduced use of zinc for synthesis of zinc-containing proteins.

VI. FOLATE-ZINC INTERACTION AND PREGNANCY OUTCOME

A. Human Studies

Mukherjee et al. [10], in a 1984 study of 450 pregnant women, reported that high maternal plasma folate concentrations during pregnancy are associated with an increased rate of pregnancy complications and fetal distress. They studied the relationship between 12 laboratory indices of maternal nutritional status and various pregnancy outcome measures including pregnancy and delivery complications, body weight, and condition of infants at birth. The blood samples were collected at various stages of pregnancy, and measurements included serum zinc, folate, and albumin concentrations. They concluded that (1) maternal plasma zinc levels were *inversely* correlated with birth weight of infants and (2) fetomaternal complications were significantly associated with plasma zinc and albumin levels in the lowest quartile among the samples obtained at a relatively early stage of pregnancy. A significant association was also identified between the lowest quartile of plasma zinc and albumin concentrations with the highest quartile of folate and fetomaternal complications. They suggested that, based on the findings of Milne et al. [9], PteGlu supplementation during pregnancy adversely affected the intestinal absorption of zinc (hence, impaired maternal zinc nutriture) and resulted in a high rate of fetomaternal complications.

Mukherjee et al. [70] later performed two other experiments in pregnant women. The first was to assess the absorption of zinc by determining the changes in plasma zinc for a 4-hour period following oral zinc administration in 12 pregnant women. They also monitored the changes of plasma folate levels, concluding that there is an inverse correlation between plasma zinc and folate levels. In the second study involving 13 pregnant women, they analyzed the effect of zinc administration on the absorption of PteGlu by comparing the increments in plasma folate concentration between giving PteGlu (10 mg) on day 1, and PteGlu and zinc (30 mg) together on day 2. The increment in plasma folate levels was reduced when zinc was given simultaneously (molar ratio of folate to zinc of 1 to 20) as compared to the increment by PteGlu alone. The area under the curve indicates that PteGlu absorption was reduced by approximately 21%, when PteGlu and zinc were orally administered together. Since the latter experiment was not repeated in reverse order using the same subjects, these data need to be confirmed.

Qvist et al. [71] monitored plasma folate and zinc concentrations along with other hematological parameters five times during pregnancy and 2 months after delivery in 45 women. They reported no relationship between plasma folate and zinc concentrations or between erythrocyte folate and zinc concentrations, however, no association between these values and pregnancy outcomes were discussed. They found that plasma zinc continued to decline as pregnancy progressed, while erythrocyte zinc levels showed a significant increase. They speculated that these

increases in erythrocyte zinc are due to the consequences of ineffective erythropoiesis due to folate deficiency during the later part of pregnancy. This may be analogous to the observation by Fredricks et al. [72] in 1964 that erythrocyte and leukocyte zinc concentrations were high in patients with megaloblastic anemia caused by folate deficiency. Furthermore, the observation is comparable to the findings of Bills et al. [69], who reported that liver zinc concentrations of folate-deficient mice were significantly higher than those of control mice. These findings may indicate that the utilization of zinc is altered in folate deficiency, however, it is important to distinguish whether these are due to the direct effect of folate deficiency or due to the redistribution of zinc among tissues under "stressed conditions" secondary to folate deficiency [73].

Sandstead et al. [74], in a study of 599 pregnant women under the age of 19, reported that PteGlu supplementation during pregnancy was associated with a decrease in plasma zinc levels, while zinc supplementation (30 mg) maintained constant levels of plasma zinc. Subjects with high plasma folate concentrations at the time of enrollment had a high incidence of infection; lower, but normal, maternal folate levels were associated with larger and heavier babies. Therefore, they suggested that a large amount of PteGlu during pregnancy may be undesirable. However, a detailed description of the study is yet to be published. Abir et al. [75] reported no correlation between folate and zinc concentrations in serum from women who had experienced miscarriages. They evaluated the in vitro effects of maternal serum samples on the development of mouse embryos, and determined folate and zinc concentrations in 18 serum samples because both nutrients are known to be important for normal fetal development.

Lehti [76] reported that, among eight towns tested in the Amazon basin of Brazil, the town with the highest stillbirth rate had the highest percentage of women with erythrocyte and serum folate concentrations that were below normal ranges during pregnancy. The same town was one of two in which the pregnant women had the lowest average serum zinc concentrations during the third trimester, indicating an association between poor nutriture in both folate and zinc and stillbirth rate. Further analyses of the data indicated significant positive correlations between serum folate and zinc ($r = 0.19, p < 0.001$) and between erythrocyte folate and serum zinc ($r = 0.17, p < 0.005$) in a total of over 300 pregnant women (K. Lehti, personal communication).

Many researchers became concerned about prenatal supplementation of PteGlu because of the reports by Muhkerjee et al. [10] and Milne et al. [9]. However, the findings of Mukherjee et al. [10] remained unchallenged until 1992, when Tamura et al. [77] reported the concentrations of folate and zinc in serum obtained from 285 pregnant women at around 18 and 30 weeks of gestation. They observed a weak, but statistically significant, positive relationship between serum folate and zinc concentrations at 30 weeks of gestation. Contrary to the report of Mukherjee et al. [10], Tamura et al. [77] found high serum folate levels to be associated with

favorable pregnancy outcome, which included (1) higher birth weight and Apgar scores of newborns, (2) reduced prevalence of fetal-growth retardation, and (3) lower incidence of maternal infection around delivery. Thus, they concluded that their data do not support the concept developed by Mukherjee et al. [10] and Sandstead et al. [74] that PteGlu supplementation adversely affects maternal zinc nutriture and pregnancy outcome. Furthermore, they recommended that earlier reports on vitamins or trace elements in relation to pregnancy outcome should be interpreted with extreme caution, because the relationships involved are complicated and the outcome of pregnancy is influenced by a large number of confounding factors.

Hambidge et al. [78] reported a prospective case-control trial evaluating the effect of PteGlu alone, PteGlu with multiple vitamins, or multiple vitamins alone on the concentrations of serum zinc in women who participated in a study concerning recurrence of neural tube defects conducted by the Medical Research Council (MRC) Vitamin Study Research Group [11]. These women consisted of two groups: 27 women with pregnancy associated with a neural tube defect and 108 matched controls with unaffected pregnancies. Blood samples were collected from women at entry to the trial, immediately before the women became pregnant, and at approximately 12 weeks of gestation. No association between serum zinc levels and neural tube defects was detected, and no change in serum zinc in response to PteGlu supplementation was observed. It was concluded that periconceptional PteGlu supplementation does not compromise zinc nutriture of the mother.

Few reports have attributed any undesired pregnancy outcome directly to prenatal PteGlu supplementation, even though such supplementation has been practiced for more than three decades. Furthermore, it is known that devastating neurological complications due to PteGlu supplementation among patients with pernicious anemia must be extremely rare, since the incidence of this disease is low among women of childbearing age and a deficiency of vitamin B_{12} is associated with infertility [8]. Considering all findings, it seems safe to conclude that adverse effects of standard PteGlu supplementation related to zinc status are minuscule, if any, among pregnant women.

B. Animal Studies

During the last several years, studies have been published concerning folate and zinc nutrient interaction using pregnant animals. Fuller et al. [79] evaluated the effect of PteGlu on the absorption of zinc in four groups of pregnant rats fed two levels of dietary PteGlu or zinc. They found that all groups of rats had a satisfactory reproductive outcome and that increased PteGlu in diets did not compromise tissue zinc concentrations. Therefore, they stated that their results, if they can be extrapolated to humans, provide some reassurance that prenatal supplementation of PteGlu may not cause zinc depletion in pregnant women. Southon et al. [80], in a

study of pregnant and nonpregnant rats, evaluated the effect of combined iron, calcium, and PteGlu on ^{65}Zn retention and tissue zinc levels. This combined supplementation during pregnancy reduced ^{65}Zn retention and plasma zinc concentrations as compared to nonsupplemented rats. Although zinc concentrations in maternal femur and fetal tissues were not affected, they concluded that such combined supplementation should be done with caution. Unfortunately, however, this study did not address the specific question of whether PteGlu alone affects retention of zinc and the tissue concentrations of zinc during pregnancy.

Record et al. [81] examined the interaction of zinc, iron, and folate in pregnant rats. They reported that concentrations of zinc were significantly lower in maternal serum and *higher* in fetal liver of rats fed a diet containing higher PteGlu (1.5 mg/kg of diet) than in those given lower PteGlu (0.3 mg/kg). Pregnancy outcome judged by fetal and placental weight and number of fetus per dam was not significantly affected by the amount of PteGlu in the diets. They concluded that, in rats, moderate supplementation of PteGlu is not detrimental to the development of the fetus.

Bremert et al. [82] examined the effect of dietary folate and/or zinc deficiency on teratogenesis in rats by identifying the malformations of embryos at midpregnancy. They found that either folate or zinc deficiency produced approximately 20% of malformations in embryos, but combined deficiency of folate and zinc demonstrated over 70% of teratogenesis. Serum folate concentrations were significantly decreased in both folate-sufficient and folate-deficient dams receiving low-zinc diets, as compared to dams fed zinc-sufficient diets. However, there was no indication that sufficient folate in diets reduced serum zinc levels in both zinc-sufficient and zinc-deficient dams. Similar studies were carried out by Quinn et al. [83] who examined the effect of supplemental PteGlu on teratogenesis in zinc-deficient rats. They reported that severe dietary zinc deficiency resulted in decreased maternal plasma folate concentrations and that these values were inversely correlated with litter size or weight of fetus among zinc-deficient rats. PteGlu supplementation did not reduce the high incidence of teratogenesis secondary to zinc deficiency, but it increased the incidence of club foot. This supplementation did not affect maternal and fetal zinc nutriture. The observations in these two studies that folate concentrations in serum or plasma were significantly reduced in zinc-deficient pregnant rats are consistent with the findings of nonpregnant rats reported by Canton et al. [29] and Tamura et al. [46]. Favier et al. [84] reported that the concentrations of liver and whole blood folate were significantly lower in zinc-deficient pregnant rats than those in pair-fed controls. A higher incidence of malformations of the fetus was observed in zinc-deficient dams than in controls, and this was not corrected by the supplementation of three forms of folates, including PteGlu, 5-HCO-H_4PteGlu, and polyglutamate prepared from yeast extract. The supplementation of PteGlu significantly reduced zinc concentrations in liver and femur of zinc-deficient pregnant rats, but the supplementation of 5-HCO-H_4PteGlu and

polyglutamate did not. The investigators speculated that the availability of the latter two folates are reduced in zinc deficiency.

VII. CONCLUSION

It is well known that certain folate conjugases require zinc for their activity and stability. In humans, the intestinal brush border folate conjugase is a zinc metalloenzyme, but the clinical significance of its zinc dependency is yet to be determined. Future research is likely to prove that folate conjugases in tissues other than intestinal mucosa are also zinc dependent in humans.

Zinc deficiency causes altered folate metabolism in rats. One of the consistent findings from various laboratories is that plasma folate concentrations are reduced in zinc-deficient rats as compared to zinc-sufficient controls; this might be explained by increased hepatic methionine synthase activity in zinc deficiency. Contrary to the findings in animals, overt changes in folate metabolism have not been identified in zinc-deficient humans.

There has been controversy as to whether folic acid supplementation adversely affects zinc absorption and nutriture in humans and animals. Considering the information available in the literature, it may be concluded that if there is any adverse effect of folic acid supplementation on zinc nutriture, it is extremely subtle. A similar conclusion can be drawn related to the question of an effect of prenatal folic acid supplementation on zinc nutriture and pregnancy outcome.

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11

Alcohol and Folate Intereactions: Clinical Implications

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I. INTRODUCTION

Ethanol is a common dietary component, with few ill effects on moderate drinkers. The estimated 5% of the population who consume excessive amounts—more than two drinks daily for women or more than four drinks daily for men—are at risk for the disease alcoholism. In addition to its mood-altering effects on the brain, ethanol diffuses through all tissues and has potentially deleterious effects on multiple organs, including the nervous system, bone marrow, stomach, pancreas, and liver. The clinical expression of alcoholism is often complicated by associated nutrient deficiencies. These include thiamine deficiency with progressive cerebellar dysfunction and peripheral neuropathy, protein-calorie malnutrition with alcoholic cirrhosis, and combined iron, folate, and pyridoxine deficiencies with the nutritional anemia of alcoholism. Binge drinking, or weeks of excessive ethanol intake to the exclusion of dietary nutrients, is often associated with progressive malnutrition and chronic diarrhea together with folate deficiency. Recent data also suggest a role for folate deficiency in alcohol-associated colon cancer. This chapter will describe the input of chronic alcoholism to folate deficiency and its potential clinical implications. In addition, the varied interactions of ethanol and folate metabolism will be reviewed in the context of different etiologies of folate deficiency.

II. FOLATE DEFICIENCY IN CHRONIC ALCOHOLISM

A. Incidence

The likelihood of developing folate deficiency depends upon the severity of the pattern of ethanol consumption. Data on the incidence of folate deficiency among

consumers of alcohol are difficult to interpret, owing to differences in population groups and lack of consensus on diagnostic criteria. For example, serum folate levels and general nutritional status were normal in a group of middle-class moderate drinkers [1], whereas serum folate levels were low in more than 80% of impoverished chronic alcoholics admitted to two different large municipal hospitals [2,3]. However, the serum folate level is a poor diagnostic tool in alcoholism, because it falls early in the course of folate deprivation [4] and decreases acutely in response to an ethanol challenge in well-nourished individuals [5]. Low red cell folate levels were seen in about 40% of derelict chronic alcoholic patients [3], but this measurement also lacks specificity in chronic alcoholism [6,7]. Megaloblastosis of the bone marrow, considered the ultimate measure of tissue folate depletion, occurred in 34–42% of alcoholic patients [6,8,9]. In these studies, the likelihood of megaloblastic anemia was greatest in generally malnourished patients with histories of poor dietary intake and did not correlate significantly with the incidence or severity of alcoholic liver disease [6,9]. In chronic alcoholism, megaloblastosis of the bone marrow usually implies folate deficiency, while vitamin B_{12} levels are usually normal or elevated. A recent report showed a strong association between elevated serum homocysteine and normal serum methylmalonic acid levels in 17 malnourished chronic alcoholics with low serum folate and normal serum vitamin B_{12} levels [10]. While the combination of elevated serum homocysteine and normal serum methylmalonic acid appears to be a sensitive measure of the metabolic abnormality of folate deficiency, the value of this measurement as a screening tool and predictor of tissue folate deficiency among alcoholics needs further evaluation.

B. Clinical Manifestations

1. *Anemia*

Anemia is the most clinically recognizable result of folate deficiency in chronic alcoholism (Table 1). Typically uncomplicated folate-deficiency anemia presents with macrocytosis of red blood cells and hypersegmented polymorphonuclear neutrophils, consistent with decreased DNA synthesis and delayed maturation of bone marrow. However, the anemia of chronic alcoholism is typically multifactorial and includes deficiencies of iron, pyridoxine, and folate as well as ethanol-induced bone marrow suppression (see Chapter 3). A substantial study of 121 chronic alcoholic patients with anemia identified the megaloblastic bone marrow of folate deficiency in 34%, overlapping with sideroblastic changes typical of pyridoxine deficiency in 23% and absence of iron stores in 13%. Megaloblastosis was attributed to ethanol-induced suppression of bone marrow in several patients with normal serum and red cell folate levels and incomplete therapeutic response to folic acid [7].

Table 1 Clinical Effects of Folate Deficiency in Alcoholism

Clinical effect	Comment	Ref.
Megaloblastic anemia	Often complicated by vitamin B_6 and iron deficiency	6–9
Diarrhea	Seen in binge drinkers, associated with intestinal malabsorption	14–19
Delayed liver regeneration	Still hypothetical	27
Colon cancer	Epidemiological association, may relate to hypomethylation of DNA	30–32

Comparative studies indicate that chronic alcoholic patients are at increased risk for developing megaloblastic anemia during periods of dietary inadequacy. A prospective study in a healthy adult male showed that 22 weeks of a folate-depleted diet were required for development of megaloblastic bone marrow changes [11], from which a minimal daily requirement of 50 μg folate was calculated using an estimated normal body storage of about 8 mg [12]. Subsequent prospective studies in recently drinking chronic alcoholics showed that only 5–10 weeks were required for diet-induced megaloblastic anemia [8,13], consistent with decreased body folate stores.

2. *Diarrhea*

Folate deficiency of the intestinal epithelium is functionally associated with altered transport and diarrhea. Because the intestinal mucosa undergoes continuous regeneration, with replacement of epithelial cells every 3 days, its folate requirement is greater than other tissues [14]. The high folate requirement of the intestinal mucosa may account for the finding of macrocytic epithelial cells in jejunal biopsies from severely folate-deficient and anemic binge drinkers [15,16]. Dietary induction of folate deficiency in rats was associated with macrocytosis of enterocytes and intestinal fluid malabsorption [17,18]. After normal human volunteers consumed low-folate diets for 2 weeks, water and electrolyte absorption from the perfused jejunum was lower than at baseline [19]. However, in another study of human volunteers, fluid and glucose malabsorption were observed only after combining low dietary folate with excessive ethanol consumption [13].

Two decades after these studies were performed, the mechanisms for the effect of folate deficiency—with or without ethanol exposure—on intestinal fluid movement remain unclear. Possible deleterious effects of folate deficiency on the intestinal mucosa include altered production or function of transporters and/or relative loss of effective villus-absorbing surface due to replacement of mature, densely packed cells with enlarged, immature cells. The synergistic contribution of etha-

nol to diarrhea in folate-deficient alcoholics may relate to its effects on small intestinal motility [20] and intestinal mucosal permeability [21].

3. *Alcoholic Liver Injury and Regeneration*

Circumstantial evidence suggests that folate deficiency plays a role in the normal defense against oxidative liver injury by limiting the availability of glutathione. Studies in animal models of alcoholic liver disease established the role of ethanol- and acetaldehyde-mediated lipid peroxidation in the pathogenesis of liver injury [22] as well as the importance of glutathione in defense against oxidant injury [23]. Methionine, a precursor of glutathione, may be diminished in alcohol-induced folate deficiency because endogenous methionine synthesis requires folate and ethanol inhibits methionine synthetase [24]. Others showed that S-adenosylmethionine administration prevents depletion of glutathione and attenuates liver injury in chronic ethanol-fed baboons [23].

Folate is central to DNA metabolism and cellular regeneration, but little is known about the potential effect of folate deficiency on these processes in alcoholic liver disease. Recent studies showed that chronic ethanol feeding blunts the normal cytokine-mediated stimulation of hepatic regeneration following partial resection, as measured by significantly decreased incorporation of ^{3}H-thymidine in regenerating liver tissue [25]. In the only study to address a potential role for folate in liver regeneration, addition of folic acid to liver biopsy slices taken from patients with alcoholic hepatitis increased ^{3}H-thymidine uptake for DNA synthesis [26]. Though separated by 30 years, these studies suggest a central role of folate deficiency in abnormal tissue repair in alcoholic liver disease.

4. *Cancer*

Folate deficiency may contribute to the increased incidence of cancer of the esophagus, lungs, and colon among alcoholics, possibly through the effect of reduced DNA methylation on proto-oncogene expression. Controlled clinical trials showing positive effects of folic acid supplements on bronchial dysplasia in smokers [27], on cervical dysplasia in women taking oral contraceptives [28], and on lowering the incidence of colonic neoplasia in patients with ulcerative colitis [29] suggest a role for folate deficiency in the pathogenesis of dysplasia and malignancies of these tissues. As described in Chapter 13, the association of folate deficiency with colon cancer was demonstrated prospectively in demethylhydrazine-treated rats; the incidence of colonic neoplasia was three times greater in folate-deficient than in folate-repleted animals [30]. Two recent studies showed an association among folate deficiency, chronic alcoholism, and increased risk of colon cancer [31,32]. The more recent study, which included more than 20,000 men and women, showed that the protective effect of high dietary folate on the risk of developing colorectal adenoma was obviated in those who drank more than two drinks (about 30 g ethanol) per day [32]. This study established that the risk of developing colorectal

adenoma was clearly lower in men and women consuming the greatest amount of dietary and supplemental folate than in those consuming the lowest amount of dietary folate. Among male drinkers, there was an inverse relationship between daily ethanol consumption and red cell folate levels. The risk of colorectal adenoma was significantly higher in both men and women who consumed more than two drinks of beer, wine, or liquor daily, and the relative risk of colorectal adenoma was three times greater in drinkers with low folate intake than in nondrinkers with high folate intake.

III. ETIOLOGIES OF FOLATE DEFICIENCY IN CHRONIC ALCOHOLISM

The pathogenesis of folate deficiency during chronic ingestion of ethanol involves many factors, including poor diet, intestinal malabsorption, altered hepatobiliary metabolism and renal excretion, and probable direct effects of ethanol metabolism on the folate molecule (Table 2). Consideration of etiologies must also distinguish effects of acute ethanol and its metabolites from effects secondary to complications of chronic ethanol exposure such as alcoholic liver disease. Etiologies must account for the rapid fall in serum folate level seen after acute administration of ethanol to

Table 2 Etiologies of Folate Deficiency in Alcoholism

Etiology	Comment	Ref.
Dietary inadequacy	Absence of folate in most alcoholic beverages	2,8,33
	Increased risk due to decreased body folate stores	4,8,12
Intestinal malabsorption	Inconsistent results among species; acute and	
Human	chronic effects of ethanol on folate hydrolysis	13,37,40,41
Monkey	and uptake	42
Miniature pig		38,39
Hepatobiliary metabolism		
Liver folate stores	Decreased in chronic alcoholic humans, monkeys	42,43
Hepatic PteGlu uptake	Acute ethanol increases, chronic ethanol inhibits	46,49
Synthesis of $PteGlu_n$	Unchanged in rat, monkey	48–50
Biliary folate excretion	Decreased in rat, increased in monkey	47,51
Renal excretion	Uncertain physiological significance; increased in rat, human; unchanged in monkeys	51,53–56
Oxidative folate destruction	May account for decreased response to therapy, rapid fall in serum folate; unconfirmed in vitro data	5,33,58,59
Membrane perturbations of hydrolase binding and transport proteins	Hypothetical	

human volunteers [5], the inhibition by acute ethanol of the availability of circulating folate to the recovering bone marrow [33], and the more rapid induction of dietary folate deficiency in alcoholic than nonalcoholic patient volunteers [4].

A. Dietary Inadequacy

Except for certain beers, little or no folate is present in alcoholic beverages [34]. Thus, binge-drinking alcoholics who substitute ethanol for other sources of calories typically deprive themselves of dietary folate. Two surveys of folate status in chronic alcoholics that included dietary estimates suggested that inadequate intake was a major cause of folate deficiency [2,8]. Decreased folate stores [4,8,12] place chronic alcoholic patients at increased risk for dietary folate deficiency.

B. Intestinal Malabsorption

Dietary folate, principally in the form of pteroylpolyglutamates ($PteGlu_n$), is absorbed in a two-step process. Hydrolysis by brush border jejunal folate hydrolase (often referred to as "folate conjugase") is followed by transport of the monoglutamyl derivative into the enterocyte by an anion carrier system [35,36]. Comparative human jejunal perfusion studies using doubly labeled folates showed that the availability of $PteGlu_n$ is about 50%, compared to the significantly greater availability of PteGlu (~75%) [37]. The kinetics of both folate hydrolysis and the monoglutamyl folate carrier require intraluminal folate in the physiological micromolar concentration range. Several studies from our laboratory provide evidence that both hydrolysis and transport are affected by chronic or acute alcoholism, although animal data are inconsistent among species.

1. *Hydrolysis of Pteroylpolyglutamates*

The intestinal perfusion system was used to study jejunal hydrolysis of $PteGlu_n$ by chromatographic quantitation of intraluminal products, using implanted tubes in miniature pigs fed controlled ethanol diets for a year [38]. Decreased in vivo folate hydrolysis [38] and decreased hydrolase activity were found in jejunal brush border vesicles from the same animals [39]. Folate hydrolysis was also inhibited acutely by preincubation of pig jejunal brush border vesicles in physiological concentrations of ethanol [39]. These data suggest that $PteGlu_n$ hydrolysis is inhibited by acute and chronic exposure to ethanol. Further confirmation in human consumers of alcohol is required.

2. *Uptake of Pteroylglutamate*

Intestinal absorption of orally administered ^{3}H-PteGlu was lower in binge drinkers than in normal volunteers [40]. When labeled ^{3}H-PteGlu was measured by the more precise jejunal perfusion method in recent binge drinkers, initial uptake was lower than uptake after 2 weeks of nutritional repletion and abstinence [41]. Because both studies included variables of poor nutrition and recent alcoholism, a third,

prospective study evaluated jejunal uptake of labeled PteGlu in hospitalized patients after induction of dietary folate deficiency, with and without added ethanol [13]. Compared to baseline measurements, jejunal uptake of labeled PteGlu, glucose, sodium, and water was decreased in two patients who developed megaloblastic bone marrow while consuming the folate-deficient diet supplemented with ethanol. Uptake was unchanged in one patient who received the folate-deficient diet without ethanol and in one other patient who received a normal diet with added ethanol. These clinical studies suggested that ethanol consumption and folate deficiency act synergistically to impair transport of water-soluble nutrients including PteGlu, whereas ethanol alone does not seem to affect PteGlu transport [13,41].

Results from animal models are conflicting between species. Monkeys fed ethanol at 50% of calories developed tissue evidence of folate deficiency and decreased ^{3}H-PteGlu absorption, as shown by decreased urinary excretion and increased fecal excretion of the label after oral administration of labeled PteGlu [42]. On the other hand, uptake of labeled PteGlu by perfused jejunal segments [38] and by isolated jejunal brush border vesicles was similar in miniature pigs fed ethanol at 50% of calories or control diets for one year [39]. Clinically, decreased intestinal absorption of PteGlu required the synergistic interaction of ethanol and folate deficiency [13,41]. Acute ethanol exposure did not affect labeled PteGlu absorption in human volunteers [41] nor uptake of labeled PteGlu by pig jejunal brush border vesicles [39].

C. Altered Hepatobiliary Metabolism

Because the liver is the major site of folate storage and metabolism and the major target of alcoholic injury, altered hepatobiliary function is a potential cause of folate deficiency. Indeed, liver biopsy studies showed half of normal levels of hepatic folate in chronic alcoholic patients with liver disease [43] and decreased hepatic folate in the monkey model of alcoholism [42]. Homeostasis in the normal liver is maintained by uptake of absorbed dietary PteGlu from the portal circulation by transport across the sinusoidal membrane [44]. Within the hepatocyte, $PteGlu_n$ is synthesized from PteGlu and used for storage and intracellular reactions. Following deconjugation to PteGlu, folate exits the hepatocyte by two routes, the bile and the systemic circulation. An estimated 10% of total folate is present in the enterohepatic circulation, most reabsorbed in the intestine and about 0.1% excreted daily in the feces [45]. Ethanol exposure could theoretically affect any of these steps, including membrane transport, storage (including binding to intracellular proteins), and excretion via biliary or systemic circulation.

Experimental evidence suggests different effects of acute and chronic ethanol on hepatobiliary folate metabolism. Acute incubation with ethanol increased uptake of methylPteGlu by isolated rat hepatocytes [46], while short-term ethanol feeding increased liver folate stores and decreased biliary folate excretion in rats [47]. On the other hand, chronic ethanol feeding had no effect on total liver folate in rats

[48] but significantly decreased liver folate in monkeys [42,49]. Other studies showed no effect of chronic ethanol feeding on the synthesis of labeled $PteGlu_n$ and its constituents after parenteral tracer folic acid in rats [48,50] and monkeys [49]. The combination of decreased exogenous hepatic folate and labeled folate following parenteral tracer ^{3}H-PteGlu in ethanol-fed monkeys suggested an inhibitory effect of chronic ethanol on the hepatic uptake of folic acid [49]. A 30-day folate turnover study in the same animals showed increased fecal excretion of radioactive folates in the chronic ethanol-fed group, consistent with an increase in biliary excretion and/or a decrease in intestinal reabsorption of the labeled folate [51]. Taken together, these studies in different species suggest that acute ethanol enhances hepatic folate uptake, whereas chronic ethanol appears to inhibit hepatic folate uptake and may promote its biliary excretion. Data from tracer studies in chronic ethanol-fed animals show no effect of ethanol on conversion of PteGlu to its $PteGlu_n$ storage form. Although little is known about the effect of ethanol on other pathways of intrahepatic folate metabolism, one group showed that chronic ethanol feeding inhibited methionine synthetase in rats [24].

D. Renal Excretion of Folate

The body folate pool is regulated in part by glomerular filtration of circulating 5-methylPteGlu, followed by efficient renal tubular reabsorption [52]. In a labeled folate turnover study, monkeys excreted less than 1% of the body folate pool in the urine daily [51]. The effect of ethanol administration on urinary folate excretion has been studied in humans, monkeys, and rats with somewhat conflicting results. Urinary folate excretion was moderately increased in human volunteers given folic acid supplements and alcohol for 2 weeks [53] or acutely [54]. Several studies in rats showed dose-related increases in urinary folate excretion following acute or chronic ethanol administration [54–56], independent of ethanol metabolism [55]. In monkeys that developed hepatic folate deficiency after 4 years of ethanol feeding, parenteral administration of labeled ^{3}H-PteGlu resulted in twice the normal urinary excretion of radioactivity in the first 3 days, followed by a normal slope of endogenous folate excretion [51]. These data are consistent with a block in renal tubular uptake of the exogenous tracer, although renal conservation of endogenous folate was not impaired. These studies suggest that acute or chronic ethanol exposure may affect renal folate conservation. However, the magnitude and overall significance of these findings is unclear.

IV. CELLULAR AND MOLECULAR BASES FOR FOLATE DEFICIENCY IN ALCOHOLISM

Current clinical and animal data suggest that multiple factors influence folate availability, storage, and homeostasis. Body stores are at risk of depletion if alcoholic

beverages with little or no folate are consumed [4,34]. Marginal folate deficiency with ethanol consumption appears to promote folate malabsorption [13]. Chronic exposure to ethanol appears to impair folate uptake by the liver [51] and its conservation by the kidney [55]. Acute ethanol appears to affect the availability of circulating folate, as shown by the abrupt fall in the serum folate level after intravenous administration of ethanol to a normal volunteer [5] and by the repeated inhibition by ethanol of bone marrow response to physiological administration of folic acid in three anemic, folate-deficient alcoholic patients [33]. Two separate hypotheses help to explain the acute and chronic effects of ethanol on folate metabolism and availability.

A. Oxidant Damage and Catabolism

In the presence of free iron, the reaction of the ethanol metabolite acetaldehyde with aldehyde oxidase produces reactive oxygen species that carry the potential for tissue damage [57]. Two in vitro studies suggest that free radical oxidant species may destroy the folate molecule, increasing folate catabolism [58,59]. In one experiment, incubation of 5-methylPteGlu with acetaldehyde, iron, and xanthine oxidase resulted in quantitative production of pteridine and *p*-aminobenzoic acid; this result was prevented by adding superoxide dismutase [58]. A somewhat similar in vitro experiment suggested that oxidation was targeted at the pteridine ring [59]. Although the oxidant destruction data have not been confirmed in vivo, another study identified urinary folate catabolites in ethanol-fed mice [60]. Folate catabolism also increases dramatically during pregnancy, thereby increasing the folate requirement and the risk of gestational folate deficiency [61].

B. Altered Membrane Composition and Function

Acute and chronic exposure to ethanol alters the lipid composition and physical properties of cellular membranes [62]. Folate homeostasis requires its interaction with many membrane-bound proteins. These include folate hydrolase and transport proteins for absorption in the jejunal brush border and binding and/or transport proteins for storage and metabolism in the liver plasma membrane and conservation in the renal tubular brush border [44]. Several folate-relevant proteins have been characterized: human and porcine jejunal folate hydrolase [63–65] and kidney brush border binding protein in the rat [66,67] and pig [68]. Folate-binding protein also appears to be present in several isolated cell systems: choroid plexus, placenta, serum [69], jejunal mucosa [70], and monkey kidney cells [71]. Because all these studies were done in isolation, the relevance of these proteins to folate homeostasis remains to be shown, and recent data question whether folate-binding protein is present in the jejunum or liver plasma membrane [72]. Nevertheless, the presence of these proteins in the lipid bilayer suggests the potential for significant perturbation by ethanol exposure.

Data from our pig model of chronic alcoholism suggest that changes in the physicochemical composition of several membranes may affect the activity of folate-relevant proteins required for folate homeostasis. As noted, jejunal folate hydrolase activity was reduced in jejunal brush border vesicles isolated from miniature pigs fed ethanol for 12 months as well as in vesicles from control animals after incubation with physiological concentrations of ethanol [39]. Using immunoblotting and activity measurements, we recently identified folate-binding protein in isolated micropig liver plasma membranes. The preliminary finding that the specified activity of folate-binding protein in liver plasma membranes correlates with altered membrane fatty acids in chronic ethanol-fed micropigs suggests that folate binding may be regulated by the phospholipid milieu of this membrane [73].

V. SUMMARY

Although threshold amounts have not been defined, the consumption of ethanol in the diet or in excess increases the risk for folate deficiency. Folate deficiency mediates several aspects of the disease of chronic alcoholism, including anemia, diarrhea with intestinal malabsorption, and increased prevalence of colonic neoplasia and may possibly mediate the development of alcoholic liver disease. There is no single etiology for folate deficiency among excessive consumers of alcohol. Although dietary deficiency is common among chronic alcoholics, evidence points to an inhibiting effect of ethanol on the processes of absorption and renal tubular conservation of folate. Other data indicate that chronic ethanol exposure alters the properties of cellular membranes with potential effects of folate binding and transport. Acute ethanol may accelerate folate catabolism by direct oxidative destruction of the folate molecule.

VI. FUTURE RESEARCH NEEDS

The large body of scientific data and literature accumulated over the past four decades have defined the incidence and potential significance of folate deficiency in chronic alcoholism and established a variety of possible etiologies. The advent of more precise biochemical, cellular, and molecular approaches permits much greater precision in these definitions. For instance, measurements of serum homocysteine and other methionine metabolites may detect populations at risk for folate deficiency according to levels of dietary alcohol consumption. The interactions of alcohol consumption and altered folate metabolism in neoplasia and the potential role of folate deficiency in the process of alcoholic liver injury and regeneration need greater definition. Even if no unifying hypothesis is found to describe the etiology of folate deficiency in alcoholism, focusing on molecular and cellular events

will greatly broaden our understanding of the biological interactions of ethanol and folate.

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12
Folate and Neural Tube Defects

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I. INTRODUCTION

Neural tube defects (NTDs) remain a major scourge of society. The epidemiology of NTDs demonstrates that an environmental factor causes an increase in the prevalence of NTDs during periods of extreme poverty and hunger. This suggested that deficiency of a dietary substance(s) is important in the pathogenesis of the lesion. This chapter reviews the evidence accumulated over the last 30 years that this factor is in fact dietary folate. The most recent evidence indicates that cobalamin is also an independent risk factor, which, as explained in the text, makes it very probable that, either directly or indirectly, the primary cause of NTDs is a defective enzyme, most probably in the affected fetus as well as in the mother, namely methionine synthase, since this is the only point in metabolism at which both folate and cobalamin interreact. These findings have significant implications for international and national nutrition authorities in formulating advice concerning the appropriate dietary requirements for pregnant mothers, women of childbearing age, and society in general. This chapter summarizes the recommendations of these bodies in light of the recent findings referred to above.

Throughout this chapter, folate is referred to using two terms: folic acid and folate. "Folic acid" refers to the oxidized form of folate, which as such is rarely seen in biological tissue. This is the compound used for pharmaceutical preparations since it is stable. When it is absorbed from the gastrointestinal tract, unless given in large doses, it is reduced and methylated to 5-methyltetrahydrofolate (5-CH_3THF) before passing into the blood circulation. On entering any biological cell, before it can be retained by the cell 5-CH_3THF must be demethylated by methionine synthase, and the resulting THF is polyglutamated to tetrahydrofolylpentagluta-

mate and hexaglutamates ($THFGlu_5$ and $THFGlu_6$), in which form it is biologically active and is involved in the intracellular synthesis of purines and pyrimidines, which are required for the production of DNA. Here this form is termed "folate" and is synonymous with these intracellular polyglutamyl forms, which also go to make up "dietary" or "food" folate.

The studies implicating folate in the pathogenesis of NTDs in humans can be classified, and are discussed, under the effect on the prevalence at birth of NTDs of four factors: (1) antifolates, (2) maternal folate status, (3) dietary folate, which includes food supplements and fortified food, and (4) intervention studies using folic acid. "Occurrence" refers to NTD births occurring to women for the first time and "recurrence" to an NTD birth to those women who have already delivered an NTD child.

In many situations where trials are being described, women who subsequently deliver an NTD baby are termed "cases," in contradistinction to those who deliver normal babies, who are termed "controls."

II. DEFINITION

In the developing human embryo, the neural plate changes to form the neural tube between days 24 and 28 postconception [1]. The final structures are the precursors of the spinal cord and its protrusion that eventually encases the brain. Incomplete closure of the former leads to spina bifida, which consists of an opening in the spinal cord, resulting in a child with physical disabilities ranging from very mild to severe. In the latter, there is paralysis of the bladder and lower limbs and hydrocephalus due to interruption of the flow of cerebrospinal fluid from the brain. Incomplete closure of the skull produces anencephaly, which is incompatible with life. These and similar conditions are collectively called neural tube defects (NTDs), with spina bifida and anencephaly accounting for 50% and 40%, respectively, and the others contributing 10%. There are marked geographical variations in the prevalence at birth of NTDs. During the 1980s, for example, the prevalence at birth (excluding terminations) was 0.8 per 1000 births in the United States, 3.6 per 1000 in the Republic of Ireland, and 10.6 per 1000 in a Chinese province [2].

III. GENETIC PREDISPOSITION TO NTDS

The strongest evidence that there is a genetic component in the etiology of NTDs is the fact that having had a previously affected pregnancy is the main predictive risk factor [2]. Recurrence rates are related to the prevalence at birth in any particular population, with the recurrence rate being usually 10–15 times higher than the prevalence at birth. After two or more such affected pregnancies, the recurrence risks are approximately 10% [2]. While risks to first-degree relatives are higher, risks to second-, third-, and higher degree relatives appear, in general, to be similar

to those for the general population, the number of births (or the number of studies) on which these conclusions are drawn are limited, and more research is required to confirm this [2]. Concordance rates for NTDs have been reported as being higher in monozygotic than in dizygotic twins [2], which also supports a genetic predisposition. There are pronounced racial differences in the prevalence of NTDs, with higher rates found in Caucasians than in Latin Americans, blacks, and Mongols [3]. Sikhs have an uncommonly high prevalence [4]. Traditionally very high prevalence rates have been found in populations of Celtic origin in Britain and Ireland [2], with similar high rates being found in migrants in Western Australia [5].

It is possible that these marked differences in prevalence could be due exclusively to environmental factors such as differences in the intake of a particular nutrient such as folate or any other environmental factors. However, it seems more likely that there is polygenic predisposition for a given genetic background. However, as discussed below, in any community, in addition to a genetic difference in prevalence, there appears to be a difference due to an environmental factor or factors. This latter may interact with the genetic predisposition or may be independent of it, but it is clearly susceptible to change as has recently been shown in prophylactic studies involving increased folate intake.

IV. NONGENETIC FACTORS AND NTDS

Epidemiological studies have provided strong evidence of the influence of nongenetic or environmental factors on the etiology of NTDs. The marked variations in prevalence over time and between geographical areas and, to a lesser extent, by season, social class, maternal age, and reproductive history suggest the effect of environmental factors. The associations between these and other environmental factors and NTDs are consistent with a dietary/nutritional etiology.

V. NUTRITION AND NEURAL TUBE DEFECTS

Historically there are well-established periods when a marked increase in prevalence of NTDs in a particular community followed a period of nutritional deprivation. The Great Depression in the late 1920s in the United States was followed by an increased prevalence of NTDs [6], as was the Dutch famine after the Second World War [7]. The prevalence in most western countries has been falling over recent decades [2]. Some but not all of this decline may be explained by termination of pregnancies, leaving improved nutrition as the most probable reason for the remainder. Seasonal variations have been noted in several studies, with most showing elevation in prevalence corresponding to conception during seasons when fresh vegetables—the most important source of folate—would be expected to be less available [8–10]. Some studies [11,12] but not others [13] have found increased

prevalence in families of lower social class, particularly in high-risk areas, supporting a nutritional role in the etiology.

VI. STUDIES IMPLICATING FOLATE

It has become clearer as this area develops that folate has a definite role in the etiology of NTDs. The magnitude of the protection effected by folic acid or folate may vary with the underlying prevalence in the area under study but the evidence is conclusive from several different sources, culminating in controlled intervention trials, that folate is involved.

A. Antifolates and NTD

Perhaps the earliest evidence implicating folate in the etiology of NTDs was a series of case reports linking the use of antifolates in pregnancy to NTD-affected pregnancies [14–16]. Antifolates have also been used to produce a variety of birth defects in mice [17] and golden hamsters [18]. However, as pointed out by Heid et al. [19], folate deficiency alone in the absence of antifolates does not produce NTDs in mice.

B. Maternal Folate Status and NTDs

Early studies by Hibbard suggested that folate deficiency, as well as leading to maternal anemia and abruptio placenta, might also cause congenital birth defects [20,21]. This view was supported by a study by Fraser and Watt [22], who found that of 17 mothers diagnosed as having megaloblastic anemia of pregnancy, 5 had congenitally abnormal babies, one of which was an NTD. However, a larger study by Giles [23] on 335 such cases found no support for a teratogenic effect for folate deficiency. A similar position was taken by Prichard et al. [24], who examined 86 infants born to women with severe folate deficiency and overt anemia. None of the infants had an NTD or any other birth defect, and the authors concluded that it seemed "quite unlikely that widespread public health measures focused on eradicating all suspicions of folate deficiency by providing folic acid supplements very early in pregnancy or even before conception would have a profound effect on reducing perinatal mortality or congenital malformations in the United States." This statement illustrates the hazards of drawing conclusions on large issues from small numbers.

Studies to address the question of inadequate numbers followed, as did studies using red cell folate (RCF) as an alternative to serum or plasma to assess folate status. In a large prospective study, Hall [25] measured serum folate in women attending their first antenatal clinic. The serum folate levels in 11 women who had children with CNS malformations were similar to those in women with unaffected pregnancies. A larger cohort of serum samples from some 18,000 pregnancies were

studied by Molloy et al. [26], and were also taken at the first booking clinic. Thirty-two NTD cases were compared to 395 randomly selected controls. No differences were found in median serum folate values or distribution of such values. These studies were on larger numbers and were based on the first antenatal booking clinic, which is approximately 10 weeks after the closure of the neural tube. While this was an improvement on previous studies, use of RCF rather than serum or plasma levels would be even better, since RCF reflects tissue folate much better than either plasma or serum [27]. In addition it represents the average folate status over the previous 120 days when the red cell population was being formed and is not subject to fluctuations caused by recent differences in dietary intake. However, unlike serum, RCF samples require special preparation and such collections are not routine. A small number of RBC samples were collected by Laurence et al. [28] during a folic acid intervention trial. Of the six RCFs from pregnancies affected by NTDs, two were at levels that were suggestive of folate deficiency and one had a value a little below the mean for 51 nonaffected pregnancies in the same group. However, the other three were well within the normal range for RCF, and none of the six had serum folates indicative of deficiency. Smithells et al. [29] prospectively collected serum and RCFs from women at 8 weeks gestation, six of whom subsequently went on to have an affected pregnancy. The mean $\pm$SD (ng/ml) for sera and RCF were, respectively, 4.9 ± 1.7 (if one case of acute folic acid ingestion is discarded) and 141 ± 25.5. They compared these to mean values (ng/ml) from 1000 pregnant controls of 6.3 and 288, respectively, and found them not to be statistically different using a two-tailed *t*-test. However, they argued that because of the large numbers in the control group, a one-tailed *t*-test was valid, which resulted in a significant difference for RCF ($p < 0.001$) but not for serum.

Recently Kirke and colleagues [30] have published a study on blood samples from 56,049 pregnant women obtained at their first antenatal clinic visit to the main Dublin maternity hospitals during the period 1986–1990. Subsequently, 81 of these women went on to deliver an NTD baby, and these were compared to 247 controls, which were a representative sample of nonaffected births in these hospitals during the same period. The median plasma B_{12} and folate and RCF (ng/ml) in NTD and control pregnancies were 243 and 296 ($p = 0.001$), 3.47 and 4.59 ($p = 0.002$), and 269 and 338 ($p < 0.001$), respectively. The plasma vitamin B_{12} and plasma folate influenced the maternal RCF in the cases only. Since plasma folate and plasma were B_{12}-independent risk factors for NTDs, this strongly suggests that the enzyme methionine synthase is implicated in the etiology of NTDs since this is the only place that vitamin B_{12} and folate interrelate in metabolism. See Section VIII.C for discussion of these findings.

The above studies determined folate status in women who went on to have an affected embryo in that pregnancy. An alternative approach to using the index pregnancy (where a birth defect actually occurred) is to study women with a history of an affected pregnancy in the nonpregnant state. These women, having an inherently

higher risk of a recurrence, might show intrinsic differences related to their folate status. In addition, Leck has shown that RCFs taken early in a normal pregnancy show a significant correlation with similar assays one year later [31]. This suggests that diet and/or folate metabolic processes may be fairly constant in any individual. Yates et al. [32] found a statistically significant difference ($p < 0.005$) in RCF in 20 women with a history of two or more NTDs compared to 20 controls matched for age, parity, and social class. Schorah et al. [33] found no differences in either RCF or serum folate in 68 nonpregnant women with a history of an NTD-affected birth compared to 100 controls matched for social class. More recently, the same group [34] compared 29 cases not in the index pregnancy with 29 controls and found no difference in dietary intake of folate, serum folates, RCF, and serum B_{12} levels. Multiple regression analyses showed that the relationship between RCF and serum folate differed between the two groups. Mooij et al. [35] recently evaluated RCF and serum folate levels in 62 women with a previous history of an NTD-affected pregnancy. While they do not compare the values obtained to a low-risk control group, the means for both parameters were well within the normal range.

C. Dietary Ingestion of Folate and Folic Acid (Including Supplements)

Six studies have attempted to relate differences in maternal ingestion of folate or folic acid with the prevalence of NTD-affected pregnancies. They vary significantly in design since supplements were not a significant factor in some studies [36] whereas in others they were [37–39]. Other studies reported effects for both food folate as well as supplements [40,41]. Of the six studies described briefly below, two demonstrated a positive protective effect of increased ingestion of supplements containing folic acid [37,39]. While two others agreed with the protective effect of supplements, they also demonstrated that adequate dietary folate might also be effective [40,41]. Ingestion of folic acid/folate was shown to be ineffective in two other studies [36,38].

Note in the section below that all studies except the first study (South Wales) compared occurrence of an NTD-affected pregnancy with women with normal pregnancies. The former we have called cases and the latter controls for convenience.

1. *South Wales Studies*

The first of these studies sought to establish a relationship between dietary intake as assessed by dietary recall and RCF and serum folate levels in 368 women all with a previously affected pregnancy [36]. Dividing their diets into good, fair, and poor with respect to folate intake, they found significant differences for both RCF and serum folate between the three groups. A relationship between diet and RCF and serum folate was established in 186 cases, of whom 103 were given dietary counseling and 71 were not. They reported that 72% of the counseled women improved their diet, compared to only 12% of the uncounseled women. There were

three recurrences in the former group and five in the latter, a nonsignificant difference. However, all eight recurrences occurred in the 45 pregnancies in women who had poor diets, while there was no recurrence in the 141 pregnancies on the "fair" or "good" folate-containing diets. It is not entirely clear if classification into the various diets was made in all instances before the outcome of the pregnancy was known.

2. *Centers for Disease Control (CDC) Study*

This study [37] compared the periconceptual use of multivitamin supplements, including folic acid, ascertained by telephone interview in 347 women who had had an NTD-affected birth in the Atlanta area between 1968 and 1980, with 2829 controls without birth defects. They found a significant protective effect for women who took multivitamin supplements. To allow for recall bias, a second control group of women who had children with birth defects other than NTD was also used. Using this group of controls, they found a protective effect of multivitamins for NTD births, but not for other birth defects. The major criticism of the study was that because it depended upon a woman's ability to recall multivitamin intake that had taken place 2.5–16 years earlier, recall bias was a possible confounding factor. Nevertheless, the authors concluded that either multivitamin use reduced the risk of an NTD-affected birth or that women who took multivitamins were intrinsically at lower risk for some other undetermined reason.

3. *National Institutes of Health Study*

This study [38] was designed to reexamine the question in such a way as to eliminate the recall bias produced by a long time delay as in the CDC study. Women were studied who had an NTD-affected fetus (n = 571), a fetus with a malformation other than an NTD (n = 546), or a normal birth (n = 573). The authors interviewed almost all of the women within 5 months of the diagnosis of a defect or the date of birth. Great care was taken with the methods of interviewing and analysis to eliminate bias. In this study multivitamin use including folic acid had no protective effect. Since this is the only study that failed to find a protective effect, and in the light of the positive effect of folic acid found in intervention trials (see later sections), much speculation has ensued as to why this study was negative. The most probable explanation was that the study populations were from California and Illinois, both areas of low prevalence of NTD. It is hypothesized that in such areas a higher proportion of the NTDs are intrinsically genetically determined or are due to factors other than folic acid, resulting in a situation where the protective effect of additional folic acid would be minimal, thus producing a negative result.

4. *Western Australia Study*

The study of Bower and Stanley [40] evaluated the diets and use of supplements in 77 cases of NTD, 77 controls with birth defects other than an NTD, and 154

controls with normal pregnancies. Cases and controls were matched for time of conception and interval from conception to interview. Women completed a questionnaire that enquired about food frequency and use of vitamin supplements for the 12-month period from 3 months before through the 9 months of the pregnancy. Daily dietary intakes of free and total folate were calculated from these questionnaires. Where supplements were taken, they were added to both the free and total folate. Crude and adjusted odds ratios controlled for a number of potentially confounding variables, e.g., social class, country of origin, etc., showed a significant protective effect of an increasing intake of free folic acid in the first 6 weeks of pregnancy, and folic acid supplements were also protective. A similar but weaker trend existed for total folate, and when folic acid supplements were discounted food folate alone was also effective, although conjugated folate on its own seemed to have no effect. The authors agree that this result is unexpected and suggest that it may point to a problem with deconjugation of food folate in cases. An alternative explanation is that the methodology used to determine the dietary level of conjugated and free folate is unreliable. A protective effect was also found for dietary fiber, calcium, vitamin C, and carotene. Dietary variables are closely interrelated, and the dietary methodology used by the authors make interpretation of the results difficult [42]. The study suggests that increasing the level of food folate reduces the risk of NTDs, but this could also be true of other dietary factors.

5. *The Boston Study*

Milunsky et al. [39] designed a prospective study with the methodological advantage that periconceptual vitamin supplementation usage was assessed for 93% of the study subjects at the time of prenatal screening and before the outcome of the pregnancy was known. This study thus overcame the problems of selective participation and of biased recall. Out of 22,776 women questioned about the use of multivitamins, 49 had an NTD-affected embryo. Women who took multivitamins containing folic acid before conception and in the first trimester were found to have a significantly reduced risk of an affected pregnancy compared to women who did not take supplements. Taking multivitamin supplements with folic acid had no more effect than folic acid alone. In the same study 11,944 women who did not receive folic acid in supplements had their diet analyzed by questionnaire. Using an arbitrary cutoff of intakes of less than 100 μg/day, the authors found that NTD prevalence was 7.3 per 1000 births for those with a daily folic acid intake of less than 100 μg compared with 3.1 per 1000 for those with daily intakes above this level. The findings suggest that a deficiency of total dietary folate may increase the risk of NTD.

6. *The Boston, Philadelphia, Ontario Study*

In a study based in Boston, Philadelphia, and Ontario, Werler et al. [41] compared multivitamin use including folic acid in the periconceptual period in 436 cases

compared to 2615 controls with infants with other birth defects. Interviews were within 6 months of delivery. The use of a multivitamin preparation containing folic acid was associated with a 60% reduction in risk. A semiquantitative food-frequency questionnaire was used to determine folate consumption in the 6-month period before the last menstrual period. Analysis of women who did not use multivitamin supplements was made dividing intake into quintiles according to the distribution of dietary folate intake among the control mothers. The risk of an NTD-affected pregnancy declined significantly as folate intake increased, supporting a role for dietary folate in the prevention of NTDs.

D. Intervention, Trials Involving Folic Acid

Most, but not all, of these trials have involved administration of folic acid to women with a previous history of an NTD-affected birth, the effect being measured by a reduction in the incidence of NTD recurrence. Two attempts were made to influence NTD recurrence by dietary advice to increase food folate ingestion. One trial examined women with no history of a previous NTD, i.e., it determined the capacity of folic acid to prevent occurrence.

1. *South Wales First Dietary Trial*

Laurence and colleagues [36] studied 905 women with a previous history of an NTD birth in two areas of South Wales, the women in one area being counseled to improve their diet and folate intake, the others acting as controls. A total of 109 pregnancies occurred in the counseled area, of which 71% were judged to have improved their diet, 27% had no change, and 2% had a worse diet. The corresponding figures for the 77 pregnancies in the noncounseled areas were 12% improved, 82% no change, and 5% worse. In the former group, there were 10 miscarriages and 3 NTDs, while in the latter there were 8 miscarriages and 5 NTDs. These results show no significant improvement due to counseling. However, if one disregarded the efforts at counseling and simply looked at the dietary intake, all eight recurrences occurred in women who were assessed as having a diet poor in folate as compared with those considered to have a fair or good dietary folate intake ($p < 0.001$). The above study was small and has many weaknesses, but it is important in two ways. First it was the first intervention protocol designed to reduce NTDs. Second and perhaps of greater relevance, it demonstrates that it may not be easy to prevent NTDs by dietary advice alone.

2. *South Wales Second Dietary Trial*

Laurence et al. [43] continued this type of work by examining the diet of 244 mothers with a previous NTD-affected birth and found with respect to folate that diets were good (14%), fair (45%), fair but unbalanced (14%), and poor (27%). All women were given dietary counseling and 176 subsequently became pregnant,

at which time the above figures had changed to good (40%), fair (44%), fair but unbalanced (9%), and poor (7%). There were five recurrences, three within the fair but unbalanced diet and two in the poor diet group. Thus, all of the recurrences affected subjects in the two inadequate dietary folate groups, giving a significant result ($p < 0.01$). However, as pointed out by Leck [44], in the initial study design, there was no group with a diet designated as "fair but unbalanced." After the outcome of the pregnancies were known, three women on what was initially classified as a "fair" diet had their diet judged retrospectively to be "fair but unbalanced." This introduces bias into the interpretation of the result. On the question of effectiveness of dietary advice, it must be remembered that all women were counseled and there were still five recurrences, which illustrates the ineffectiveness of dietary counseling alone as a measure of preventing NTDs.

However, the problems of dietary counseling observed in the above study might not be as great today, since there is now a greater certainty that there is a role for folate. Furthermore, in the 1970s when these studies were carried out, diet could not be improved with fortified foods such as breakfast cereals and bread, since none were then available. These studies underline the fact that while folate is widely available in food, even good sources of folate such as green leafy vegetables are not very high in folate. The exception is liver, which is really the only folate-rich food, but it is not recommended in early pregnancy because of concerns about its vitamin A content. Thus, changes in diet, without the inclusion of fortified foods, do not easily produce a marked change in folate intake, which reflects the lack of success in the two dietary intervention studies in preventing NTD recurrence.

3. *Multicenter Trial in England and Northern Ireland*

The first intervention trial using folic acid supplementation to attempt to reduce the recurrence of NTDs was conducted by Smithells and colleagues [45]. This group used a preparation, Pregnavite Forte F, that contained eight vitamins and trace elements. The preparation was taken three times per day, giving a daily ingestion of folic acid of 360 μg. Initially the authors planned to carry out a double-blind, randomized trial, but one of the three ethics committees to which this proposal was submitted insisted that all mothers receive treatment. This unfortunately resulted in the design being changed to an intervention trial, where all mothers recruited into the study received Pregnavite Forte F. Women who were pregnant when referred to the study or who declined to participate comprised a control or unsupplemented group. The women in the study were asked to take the supplement for at least 28 days before conception and to continue for the first 2 months of the pregnancy. Where supplementation occurred for less than 28 days or where it occurred after conception, such pregnancies were regarded as partially supplemented (see later). There was one NTD recurrence among 178 infants (0.6%) of fully supplemented women compared with 13 of 260 infants of unsupplemented women (5.0%). These studies were subsequently extended [46]. The results of all of the studies conducted

by this group were analyzed by Schorah and Smithells [47]. There were 14 recurrences in 1093 infants of fully supplemented women (1.3%), two in 211 infants of partially supplemented women (0.9%), and 24 in 486 infants of unsupplemented women (4.9%). Using the same methodology as Smithells et al. [45], Holmes-Siedle et al. [48] found one recurrence in 204 supplemented pregnancies (0.5%) and three in 28 unsupplemented pregnancies (10.7%).

4. *South Wales Folic Acid Intervention Trial*

After the results of the trial by Smithells et al. [45] had been published, Laurence and colleagues [28] published the results of a double-blind randomized control trial attempting to prevent recurrence in 60 women receiving 2.0 mg of folic acid twice per day (4.0 mg total) compared to 51 controls. There were two recurrences in the treated group and four in the untreated group, thus showing no significant effect of folic acid supplementation. However, one of the two recurrences in the supplemented group subsequently confessed to not having taken the folic acid, and she was then reclassified by Laurence et al. [28] as a noncomplier. All women had a blood sample taken for serum folate and RCF between the sixth and ninth weeks of estimated gestation. None of the placebo group had a serum folate level of greater than 12 ng/ml. The other mother with a recurrence in the treatment group had a serum folate level of 4.8 ng/ml at the time of sampling. The authors defined any woman with a serum folate of less than 10 ng/ml as a noncomplier and reclassified this recurrence along with 15 others in the treatment group with serum folate of <4.8 ng/ml to this new category in the control group. Based on this reclassification, there were six recurrences in the placebo or noncomplier group compared to none in the treated (complier) group, which was significant ($p = 0.04$).

The difficulties in interpreting the findings of the nonrandomized trial by the Smithells group [47] and the randomized trial by Laurence et al. [28] have been well documented [49]. In the studies by Smithells et al. [45,46], there were important differences between the supplemented and unsupplemented women, for example, in social class and history of a spontaneous abortion in the preceding pregnancy [49], factors known to influence recurrence risk [50,51]. The essential problem was that the two groups were not comparable and one could not determine whether the low recurrence rate of the supplemented group was due to the vitamin supplementation or explained by self-selection into the treatment group of women who were intrinsically at low risk of recurrence. Smithells and colleagues argued persuasively that the differences in characteristics between the supplemented and the unsupplemented groups could not explain the marked difference in the recurrence rate [52]. The main problems in the trial by Laurence et al. [28] were its small size and a methodological problem in data analysis [53]. Because of the problems with these studies, the role of folic acid in preventing NTDs was not accepted by many researchers. The continuing argument at that time effectively

precluded any serious public health measure to use folic acid supplementation (or food fortification) for the prevention of NTDs.

Three randomized trials commenced after this period to test the efficacy of folic acid and other vitamins in the prevention of NTDs.

5. *The UK Medical Research Council Trial*

The United Kingdom Medical Research Council Trial was a well-designed, randomized, double-blind trial that established conclusively the efficacy of folic acid in preventing the recurrence of NTDs [54]. In this large study, conducted in 33 centers in seven countries, 1817 women with a history of a previous pregnancy affected by an NTD were randomized into four groups: folic acid, folic acid plus multivitamins, multivitamins without folic acid, and placebo. Of 1195 completed pregnancies, there were six NTD recurrences in the 593 women (1.0%) who took folic acid compared with 21 in the 602 women (3.5%) who did not receive folic acid—a 72% protective effect (relative risk: 0.28; 95% confidence interval: 0.12–0.71). The multivitamin preparation without folic acid had no protective effect.

The only substantive criticism that can be made of this important study is the excessively high dosage of folic acid used—4.0 mg daily. This is similar to the amount used by Laurence et al. [28] and 10 times that used by Smithells et al. [45]. It is also 10–20 times the RDA for nonpregnant women, and one must remember that these women, when they started taking the supplements, were not pregnant. It is about 20 times the amount of dietary folate that most women ingest [55], and if one considers that folic acid is perhaps twice as available as food folate [56], this supplement may represent 40 times that in the diet. The MRC study group have subsequently defended the choice of this large dose in that it simultaneously tested both the hypothesis of Laurence et al. [28], which used 4.0 mg per day, and also tested the effectiveness of the smaller dose of 360 μg per day used by Smithells et al. [45]. They also anticipated that if the result was negative, which was quite possible, the high dose would show beyond any reasonable doubt that folic acid was not effective, whereas the use of a smaller dose would have left this question open. If one considers the use of this high dose to treat recurrence, any risks involved are clearly outweighed by the benefit, as such women have a recurrence risk of up to 4%. Weighed against such a benefit, are there any possible harmful effects of ingesting 4.0 mg of folic acid per day? The main risk would be that such levels would certainly give a sustained hematological response in subjects with vitamin B_{12} deficiency [57], thus preventing its timely diagnosis with allowing the neuropathy also associated with such deficiency to proceed undiagnosed. This, it can reasonably be argued, is not a problem for women who have a high risk of NTD recurrence. They are clearly identifiable in advance and can be checked for possible vitamin B_{12} deficiency. Furthermore, pernicious anemia, the only significant cause of clinical vitamin B_{12} deficiency, is rare in people under 40, so most women attempting to become pregnant would be at low risk for this condition. A

further consideration is that at such high levels, a large amount of folic acid survives unmetabolized for periods in the circulation. Thus, the developing embryo would be exposed to a nonnaturally occurring form of the vitamin [58]. This can be countered by saying that high levels of folic acid have been used in pregnancy for years with no obvious ill effects on the embryo. However, such prophylaxis is only in established pregnancy and not during the first trimester when the embryo is perhaps most vulnerable. While there were no obvious ill effects in the more than 500 births in the MRC trial, it must be remembered that birth defects of low frequency can be difficult to detect, for instance, the fetal alcohol syndrome, which emerged for the first time in 1973. However, the principal problem that arises is how the MRC trial result can be applied to the prevention of NTD occurrence. Since 95% of all affected pregnancies are first-time occurrences, this presents the real public health challenge. The prospect of giving all normal women who might become pregnant 4.0 mg of folic acid per day to prevent an event with a prevalence in such women of 1 to 2 per 1000 births would seem hard to justify.

6. *The Republic of Ireland Trial*

A randomized, double-blind trial was initiated in Ireland in 1981 to determine if periconceptional supplementation with either folic acid alone or a multivitamin preparation alone could reduce the recurrence risk of NTDs in women with a previously affected pregnancy from 5 to 1% or less [59]. This was similar to the reduction achieved by Smithells et al. [45,46]. There were three study treatments: folic acid, multivitamins without folic acid, and folic acid plus multivitamins, the daily dose of folic acid being 360 μg as used by the Smithells group [45,46]. There was no recurrence in the 172 infants in the folic acid groups and one recurrence in the 89 infants in the multivitamin without folic acid group, a nonsignificant difference. Women who were ineligible for the trial because they were pregnant when contacted constituted a nonrandomized control group; there were three recurrences among the 103 infants in this group. The difference in the recurrence rate between the folic acid groups and the nonrandomized controls was statistically significant, but the validity of this comparison is questionable. With only one NTD recurrence and its relatively small size (261 subjects), this trial did not provide clear evidence of a protective effect of folic acid. However, the findings are consistent with those of the MRC trial [54], and the absence of any NTD in the 172 infants whose mothers received folic acid suggests that folic acid may also have been protective at a daily dose which was one eleventh of that used in the MRC trial.

7. *The Hungarian Trial*

Czeizal and Dudas [60] carried out a randomized double-blind trial comparing a multivitamin preparation with a trace element preparation. While the trial had the disadvantage that it used two mixtures of nutrients, it made two significant advances. First the amount of folic acid used in the multivitamin preparations was the lower

level of 800 μg/day. More importantly, the trial tested the effect of prevention of occurrence, rather than the recurrence, of NTDs. There were no NTD-affected pregnancies in the 2104 women receiving the multivitamin preparation including folic acid compared to 6 NTD-affected births in the 2052 outcomes on the trace element preparation. While the numbers were smaller than the MRC trial, the result was significant at $p = 0.029$. This trial confirmed that folic acid prevents not only the recurrence of NTDs but also their occurrence, a suggestion that seemed likely from the MRC trial. It also revised downwards the required dosage of folic acid for supplementation.

VII. PUBLIC HEALTH RESPONSE TO THE EMERGENCE OF THE EFFECTIVENESS OF FOLIC ACID IN THE PREVENTION OF NTD

The suggested amounts of folate required for normal nutrition in various sectors of the population are reviewed periodically in different countries. They are expressed as the Recommended Dietary Allowances (RDAs) in the United States [61], but other terms have been introduced: Reference Nutrient Intake (RNI) [62] in the United Kingdom and, more recently, Population Reference Intake (PRI) by the European Community (EC) [63]. These values represent the amount of folate required daily to ensure adequate nutrition in 95–97.5% of the population. They are thus an overestimate of the amount needed for the vast majority of any given group. Most women who wish to protect themselves from having an affected pregnancy will not be aware at the time of closure of the fetal neural tube that they are pregnant. Thus the values recommended for pregnant women are of little consequence. The amount recommended for nonpregnant women have traditionally been of the order of 400 μg/day. However, recently these figures have been revised downwards to the following: United States, 180 μg/day; United Kingdom, 200 μg/day; EC, 200 μg/day. These values were set for the most part before the protective effect of folic acid in preventing NTDs had been clearly established. This is alluded to in the EC recommendations with the statement that "neural tube defects have been shown to be prevented in offspring by periconceptional ingestion of 400 μg folic acid in the form of supplements," although the value for non-pregnant women is still set at 200 μg per day. A future debate will arise as to the appropriateness or not of revising these recommendations upwards in the light of the present studies. In this context, we understand that the Food and Nutrition Board of the National Academy of Sciences is currently addressing the issue of revision of the RDA for folate.

Two further official responses to the new position have emerged, one in the United Kingdom under the auspices of a special committee set up by the Department of Health, and the other in the United States by the Public Health Service championed largely by the Centers for Disease Control (CDC).

Within weeks of the publication of the results of the MRC trial in August 1991, the U.K. Department of Health had written to all health care professionals in the United Kingdom advising them of the protective effect of folic acid [64]. It was suggested that all women who had a history of a previously affected pregnancy should be advised to take 5.0 mg of folic acid per day if they planned to become pregnant (4.0 mg used in the trial was not commercially available). In addition, a special committee was established to produce further recommendations, which it did in December 1992 [65].

From the folate standpoint these recommendations were (1) to prevent recurrence (previously affected pregnancy), women should take 4 mg of folic acid per day if they still intend to become pregnant after suitable genetic counseling; (2) to prevent occurrence (no previously affected pregnancy), women should ensure that they take an additional 400 μg of folic acid/folate per day prior to conception and for at least 12 weeks postconception. It is suggested that there are three possible ways of achieving an extra intake of folate/folic acid: eating more folate-rich foods, eating foods fortified with folic acid, or taking folic acid as a medicinal/food supplement. If a woman not taking this additional amount of folic acid realizes she is pregnant before 24 days postconception, when the neural tube begins to close, she should begin taking extra folic acid at once. The Committee recommended that appropriate amounts of folic acid should be added to fortify bread or breakfast cereals only to help accomplish these objectives. Initially, the objective of extending fortification appears to be very nondirective, leaving it to industry to make such additions at the prompting of the Departments of Health and Agriculture.

In August 1991, the CDC as part of the Public Health Service of the U.S. government issued its recommendations on the prevention of recurrence of NTDs, suggesting that such women should take 4.0 mg/day if they planned on becoming pregnant [66]. In September 1992 recommendations on the prevention of occurrence in the US were developed through the efforts of the CDC, the Food and Drug Administration (FDA), the Health Resources and Services Administration, and the National Institutes of Health. The development of these recommendations was coordinated through the Office of the Secretary for Health [67]. The following recommendations were issued on behalf of the Public Health Service:

> All women of child-bearing age in the United States who are capable of becoming pregnant should consume 0.4 mg of folic acid per day for the purpose of reducing their risk of having a pregnancy affected with spina bifida or other NTDs. Because the effects of higher intakes are not well known but include complicating the diagnosis of vitamin B_{12} deficiency, care should be taken to keep total folate consumption at $<$ 1 mg per day, except under the supervision of a physician. Women who have had a prior NTD-affected pregnancy are at high risk of having a subsequent affected pregnancy. When these women are planning to become pregnant, they should consult their physicians for advice.

Unlike the U.K. committee, the CDC and FDA have taken a much more active role in trying to ensure that increased folic acid is taken. They have presented a policy suggesting the addition of sufficient folic acid to a staple such as flour to ensure that the vast majority of women (>95%) possibly receive 400 μg of folic acid per day in their diet.

The task of making such recommendations in the United States, however, lies with the FDA. The following is an outline of their past and present positions on folic acid.

In 1971 FDA published conditions for the use of folic acid, defining it as a drug (36FR6843) and setting its level for perscriptions at 1.0 mg/day. They subsequently (1980) added the condition on labeling (45FR69043) that even at a low level it may obscure pernicious anemia while neurological disease associated with this condition progresses. They approved it as a food additive (21CFR172.345) provided that the maximum intake of the food consumed during a period of one day, or as directed for use in the case of a dietary supplement, will not result in the daily ingestion of the vitamin in excess of 0.4 mg (400 μg) for foods labeled without reference to age or physiological state. More recently (November 1990) FDA was asked to issue regulations authorizing nutrient content and health claims on food labels specifically relating to the claim that folic acid could prevent NTDs. In November 1991, FDA concluded that scientific agreement and data were not sufficient to support the use of health claims related to folic acid and the prevention of NTDs (55 FR 60610).

In November 1992, the FDA Folic Acid Subcommittee recommended that a plan of food fortification be developed by FDA to facilitate the implementation of the Public Health Service recommendation. In April 1993, FDA provided the Subcommittee with food fortification proposals and in October 1993 published a proposed regulation to "enrich" staple grain products at the level of 140 μg/100 g. This enrichment level was estimated to provide an additional 100 μg/day of folic acid to average diets. Two proposed regulations were also published including a food additive regulation, which was proposed to control and standardize the addition of folic acid to foods other than the "enriched" products. The third regulation proposed to permit a health claim related to folic acid and neural tube defect risk reduction that met specified criteria.

The view of countries other than the United States and United Kingdom on the need for a public health response has been more conservative. In Canada, the Health Promotion Branch, Health and Welfare Canada [68] recommended that women with a previous history of an NTD-affected pregnancy should consult their physician about folic acid supplements. Women with no such history ought to be encouraged to choose foods high in folate and if they planned to take a supplement of folic acid should consult their physician. The Netherlands has been even more conservative, concluding that at the moment research results do not justify recom-

mendations to encourage use of folic acid supplements for the primary prevention of NTDs [69]. The National Health and Medical Research Council of Australia has recommended that women with a history of an NTD birth should be referred for genetic counseling and should be advised to take periconceptional folic acid at 0.5 mg/day [70]. They go on to recommend that staple foods such as bread and cereals be fortified with folic acid. Their recommendations are under consideration by the Australian Food Authority. Indications are that they may allow fortification of food with folic acid (which is currently prohibited), but only to levels that would restore what was present prior to processing.

VIII. COMMENTS ON RECOMMENDATIONS CONCERNING THE USE OF FOLIC ACID/FOLATE TO PREVENT NTDS

Based on the effectiveness of the MRC trial and considering the risk:benefit ratio, both U.K and U.S. authorities recommended that women with a previous history of NTD should be counseled as to the risks and, if they intend to become pregnant, should take 4.0 mg of folic acid per day. The CDC added that if it was possible that they might become pregnant, they should take 400 μg daily as a prophylaxis. The recommendations from both groups to prevent occurrence include *all* women who might become pregnant. It was recommended that they should take an extra 400 μg of folic acid per day prior to conception and during early pregnancy. The lower dose is now supported by many observational [37,39,41] and intervention [45–60] studies.

The CDC recommendations use the term folic acid, while the U.K. report often refers to folate/folic acid. Both CDC/FDA and the U.K. Department of Health suggest three ways of achieving 400 μg of folic acid/folate per day: (1) eating more folate-containing foods, (2) eating more foods fortified with synthetic folic acid, and (3) taking supplements containing synthetic folic acid.

Clearly the objective of getting additional folic acid can be most obviously achieved in any one individual by taking supplements. However, as a public health measure, both groups saw it as being ineffective. Such supplements were unlikely to be taken by a majority of women who would become pregnant, no matter what public health measures were taken. In addition, over half of all pregnancies are thought to be unplanned. The suggestion that an additional or even a total of 400 μg per day of folic acid/folate can be achieved through changes in diet warrants some comment. Since there is no folic acid in the diet (if one uses that term to describe the synthetic form used in the vitamins trials), it is clear that one cannot hope to emulate the protection of the trials or of taking vitamin supplements through a nonfortified or nonsupplemented diet. The studies of Bower and Stanley [40] and Werler et al. [41] suggest that increased dietary folate may have a protective effect, but the evidence is by no means as strong or as unambiguous as the proven

effect of folic acid. In addition, while there may be an effect, the relative effectiveness of dietary folate versus folic acid is unclear: Were the changes seen optimum or just partial? In practical terms there are two further considerations. The CDC/FDA recommendations refer to a total of 400 μg of folic acid. per day and seem to include food folate. The U.K. recommendations refer to extra folate or to supplements or fortification with an extra 400 μg of folic acid. To achieve an *increase* of 400 μg of dietary folate per day without using fortified foods would be difficult, if not impossible, for the average women. Even to achieve a *total* of 400 μg per day would not be easy. Furthermore, since dietary folate is only half as bioavailable as folic acid, one would need to achieve an additional 800 μg of dietary folate per day, which, on any ordinary diet (excluding liver, because it is not recommended in pregnancy because of its high vitamin A content), is impossible. It may, and probably will, transpire that this arbitrary level of 400 μg used in the trials and in women taking supplements is unnecessarily high and that lower levels of folic acid or diets high in natural folates are just as effective. However, at the moment the positive result of most of the folic acid supplement studies and of the intervention trials refer to folic acid at levels of ≥400 μg per day. The remaining way of achieving an increase of 400 μg of folic acid per day is through the ingestion of suitable fortified foods. There are two subapproaches to the use of fortified foods. One could fortify a very limited range of food specifically directed to women who might become pregnant. Such an approach has the same problems of delivery to the right people. The alternative is to fortify the diet sufficiently widely so that almost all women of child-bearing age would receive an additional or total of 400 μg of folic acid per day. This is the approach being advanced by the CDC and FDA. While this approach would be very effective in preventing NTDs, it has one very real drawback. If one tries to ensure that say 95% of all women get a total of 400 μg of folic acid per day through fortification of dietary stable foods, such as bread or cereals, then, depending upon the diet and differences in eating habits, over half of the population would get approximately 700 μg and some 5% would get more than 1 mg per day. While this might not be a significant hazard to women of child-bearing age, the risk of masking pernicious anemia in the elderly at this latter level must be considered. A compromise may be to choose a lower target figure for universal fortifications as an interim measure. It could be argued that by using a lower fortification target of, say, 200 μg per day, one would probably prevent most NTDs and not put the elderly at risk. Perhaps allowing a wider range of foods fortified with relatively high levels of folic acid would allow women to elect to reach the target figure of 400 μg per day without the need to take daily supplements (tablets). Such more heavily fortified foods would have to contain a warning to the elderly of the possible ill effects of large intakes of folic acid for them.

IX. OTHER FACTORS THAT MAY INFLUENCE THE EFFECT OF FOLATE ON NTDS

The literature contains references to other factors that have been suggested as having some involvement in NTDs and that are also implicated directly or indirectly in folate metabolism.

A. Anticonvulsant Drugs

It is well established that anticonvulsant drugs impair folate status [57]. Some such drugs, most notably valproic acid, have also been reported as causing NTDs [71]. Valproic acid has been reported to cause NTDs in experimental animals [72], and it has been reported that this can be prevented by the reduced forms of folate/folinic acid.

B. Vitamin C

Smithells et al. [29] reported that the white cell vitamin C levels were significantly lower in blood samples taken from seven women during the first trimester of a pregnancy that subsequently led to an NTD when compared to normal pregnancies taken at the same time. Bower and Stanley [40] also found different vitamin C intakes, as estimated by dietary recall, in 77 cases with an affected pregnancy compared to 77 controls that had a pregnancy with a different birth defect. Vitamin C is a potent antioxidant and may protect the labile and easily oxidized forms of folate during food storage, intestinal absorption, and even during plasma circulation.

C. Vitamin B_{12}

Because of the close metabolic relationship known to exist between vitamin B_{12} and folate [73], any mention of this nutrient in connection with NTDs is of interest. The first such mention was the finding that of the vitamin B_{12} levels of serum taken during the first trimester of pregnancy in six women who subsequently went on to have an NTD-affected pregnancy, two had extremely low and another had a low normal level compared to controls [74]. Two subsequent larger studies using the index pregnancy have been reported. One compared first-trimester blood in 32 cases with 363 controls [26] but failed to detect any differences. A similar study by Mills et al. [75] also failed to find differences between cases and controls.

The levels of vitamin B_{12}–binding proteins (transcobalamins) have been reported to be elevated in the amniotic fluid of cases compared to controls [76–78], and the vitamin B_{12} level itself in amniotic fluid were found to be lower [77,79]. As discussed in Section V.B, we have recently studied blood samples from more than 56,000 pregnant women obtained at their first hospital maternity visit, 81 of whom subsequently delivered an NTD baby [30]. There were significantly lower levels

of plasma folate and RCF in cases compared to controls. However, the values found in the cases were mostly in the normal range, suggesting that a metabolic block is involved rather than simple folate deficiency. In addition, there was also a significantly lower plasma vitamin B_{12} level. The plasma folate and the plasma vitamin B_{12} levels were both independent risk factors for NTD. The risk of NTD in cases was examined in relation to both plasma folate and vitamin B_{12} simultaneously. Three ranges were defined for each of these vitamins using the 25th and 75th percentiles in controls as cutoff points. The NTD prevalence in the nine groups so formed is shown in Figure 1. It is apparent that the risk increased with decreasing levels of folate at each level of vitamin B_{12} and vice versa. This strongly suggests that the enzyme methionine synthase is involved either directly or indirectly, as this is the only enzyme in metabolism that requires both folate and vitamin B_{12} to function. Further evidence that methionine synthase is defective in mothers who have NTD-affected pregnancies can be found in Figure 2 from this same study [30]. In cases and controls there is a direct correlation between the plasma folate and RCF, as would be expected. However, no correlation would be expected (except at very low levels) between vitamin B_{12} and RCF as shown in the controls. The finding that a significant positive correlation *is* present in the cases strongly suggests that the methionine synthase in the cases is responsive to plasma vitamin B_{12} over a range where normally the enzyme would be fully functional except at very low levels of

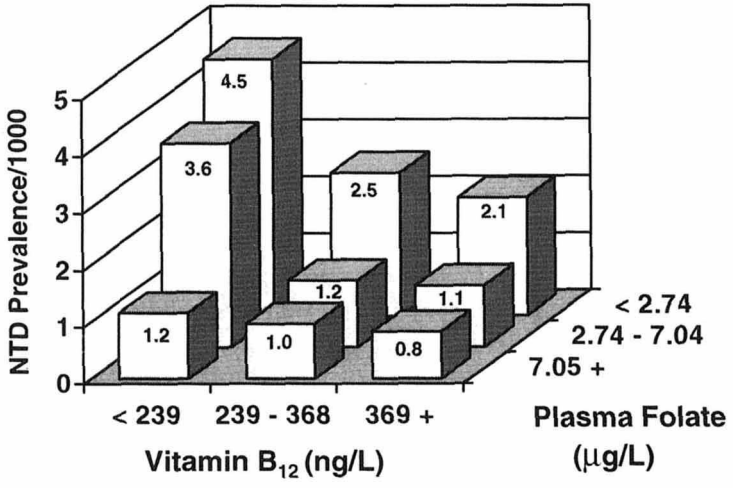

Figure 1 The effect of plasma vitamin B_{12} and plasma folate on the prevalence at birth of NTDs. Each plasma level was divided into three groups and the prevalence at birth of NTDs was calculated for the resultant nine categories. (Reproduced by kind permission of the *Quarterly Journal of Medicine*, 1993; 86:703–708. Oxford University Press.)

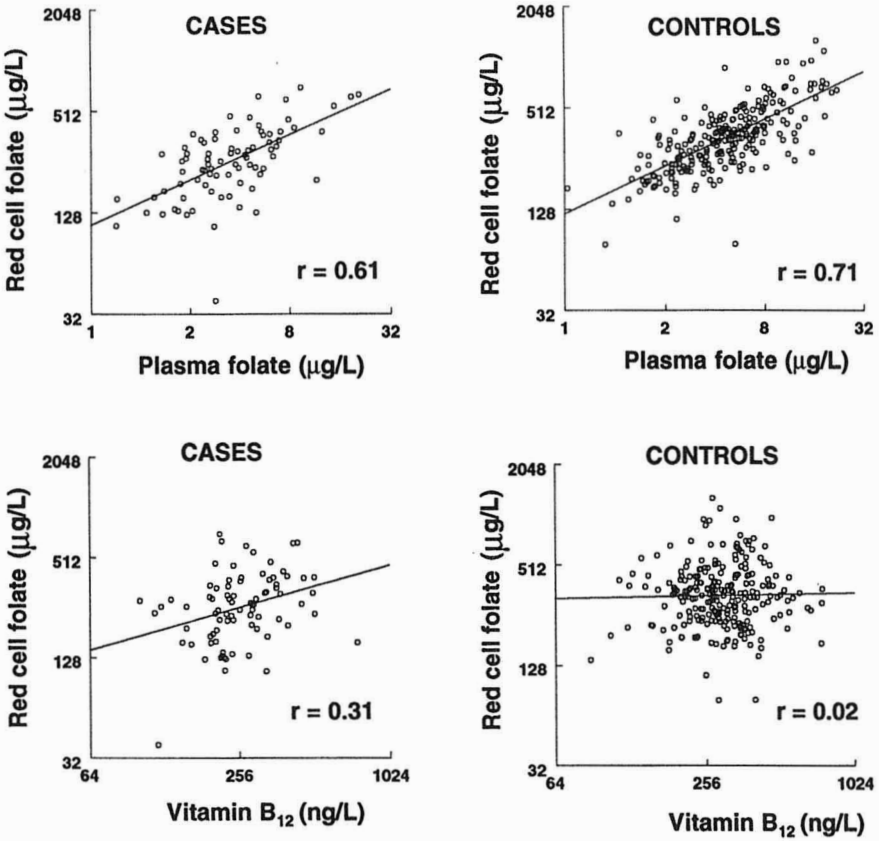

Figure 2 Correlations between RCF and plasma folate and plasma vitamin B_{12} in cases and controls. A log scale was used and the regression lines and correlations (r) are given. (Reproduced by kind permission of the *Quarterly Journal of Medicine*, 1993; 86:703–708. Oxford University Press.)

vitamin B_{12}. As a consequence, the enzyme is rate limiting in regard to access of folate into the cell.

Methionine synthase fulfills three functions [73]:

1. It is required to retain folate in the cell, since 5-CH_3THF entering the cell from the plasma has to be demethylated and then conjugated to produce THF Glu_5 and THF Glu_6.
2. In producing these latter forms it allows DNA biosynthesis to take place.
3. The methyl group taken from 5-CH_3THF is used to remethylate homocysteine to produce methionine and S-adenosylmethionine (SAM), the latter being required for methylation reactions.

It is postulated that at a time of rapid cell division in the fetal neural crest, if the cells have defective methionine synthase, they will fail to demethylate newly taken-up 5-CH_3THF, which in turn would lead to defective incorporation of folate into the dividing cells. This incorporation of new folate would be particularly important in dividing cells, since at each division the new cells will by definition only have 50% of the folate in the original cell. The lower levels of folate in these cells could impair synthesis of DNA and SAM. Alternatively, impaired activity of methionine synthase might in itself decrease DNA or SAM biosynthesis. Any of the above events could lead to an NTD.

All the evidence suggests that the defective methionine synthase while present in the mother must also be present in the fetus, since it is difficult to see how a primary defect in the mother could realistically cause an NTD in the fetus. The only methods whereby defective maternal methionine synthase might produce such a result would be, first, if there was a cellular barrier of maternal origin between the maternal uterine circulation and the fetal trophoblast and if this maternal barrier in some way impaired folate transport from the maternal circulation into the trophoblast by a method that was methionine synthase dependent. However, the interface between the mother and fetus insofar as it involves cells is of fetal origin [80]. A second way in which an impaired maternal methionine synthase might influence the fetus would be if some substance whose supply to the neural crest cells was critical was synthesized by maternal methionine synthase. We are unaware of any such substance. The mother, of course, may also have the defective enzyme, which would explain why the RCF is generally lower in cases than controls. Only a fetus who had inherited the defective enzyme would have a high susceptibility to a lesion. The degree of severity of the defective enzyme would determine the concentration of folate in the extracellular fluid necessary to overcome the block. The lesion might or might not occur, depending upon the relative supply of maternal folate and vitamin B_{12} to the fetus in comparison to the severity of the inherited enzyme defect. This could explain why only some of the progeny from the mother are affected.

A further consequence may be that subjects with NTDs will demonstrate defective methionine synthase in their cells and as such are carriers of the lesion. If this is confirmed it should be possible eventually to both locate the gene responsible and produce a test that would identify the carriers. This is the subject of ongoing work in our department. Such carriers of the defective enzyme might also be at risk of developing clinical diseases other than NTD at other times during their life. For instance, the expression of this enzyme defect would produce an impaired removal of its substrate homocysteine. It is now well established that homocysteinemia is a risk factor for coronary heart disease [81]. Lifelong hyperhomocysteinemia produced in this way could well induce atheromatous disease. It would also explain why patients with hyperhomocysteinemia have been shown to have low levels of folate and vitamin B_{12} [81], which, as in the maternal

NTD cases, would exacerbate the degree of malfunction of the defective methionine synthase and enhance the level of hyperhomocysteinemia. It would be expected that such patients would have low RCF levels in the same way as the maternal NTD cases, and this is part of an ongoing study.

D. Homocysteine

It has been suggested that women with a previously affected pregnancy have abnormal levels of the enzyme cystathionine synthase and thus have an impaired capacity to catabolize homocysteine via this enzyme [82]. This suggestion has been made as a result of impaired clearance of homocysteine after a methionine load in 6 out of 12 such women.

X. OTHER STUDIES ON THE ETIOLOGY OF FOLIC ACID–RESPONSIVE NEURAL TUBE DEFECTS

A. Animal Models

As mentioned earlier, folate deficiency in animals, in the absence of an antifolate, does not produce NTDs [19]. While animal models exist for NTDs, for example, the curly tailed mouse, in such models NTDs are not responsive to folic acid, folinic acid, methionine, homocysteine, or vitamin B_{12} [83]. They thus throw no light on the etiology of NTDs related to folate deficiency. Rat embryos have been induced to produce NTDs in response to serum from women carrying an affected embryo [84], and rat embryos also showed a high level of NTDs when cultured in the absence of methionine [85]. However, folate had no remedial effect on this model, making its value unclear.

B. Human Studies

Recently Habibzadeh et al. [86] reported that trophoblasts taken from controls and NTD-associated pregnancies showed differences in the way they metabolized the folate cofactor 5-methyltetrahydrofolate in its monoglutamate form. The methyl group was labeled with [^{14}C] and its incorporation into total protein and total nucleic acids was followed in trophoblasts from six cases and compared to six controls. Incorporation of the label into the nucleic acid pool in four of the cases was slower than in two other cases and six controls, although the final amount incorporated was equal. The only initial fate of the methyl group of 5-methyltetrahydrofolate is its conversion into methionine and its metabolism via S-adenosylmethionine. The authors suggest that the methyl group proceeds via sarcosine, formate to 5,10-methylenetetrahydrofolate, which is used for de novo nucleic acid biosynthesis.

However, 5-methyltetrahydrofolate is not an important or major source of carbon one units for the biosynthesis of purines or pyrimidines via this route. The more direct route for the methyl group to end up in nucleic acids would be via DNA and RNA methyltransferases that use S-adenosylmethionine as a cofactor.

The same group compared 29 women with a history of an NTD-affected pregnancy with 29 controls [34]. The former had been taking folic acid supplements a year previously but not at the same time of the study. The RCF folate levels were higher in cases than controls in contradiction of the findings of Yates et al. [32] and of their own group [33]. Multiple regression analyses suggested that the relationship between RCF and serum folates was different in the cases. Cases seemed to be better at making red cell folates than controls for a given plasma level. Bower et al. [87] have reported differences in response to food folate between cases and controls as measured by the subsequent elevation of the serum folate and have suggested that the intestinal hydrolysis of food folate polyglutamates may be impaired in cases. This model would attempt to explain the etiology of NTD by impaired utilization and, consequently, status of the mother. While differences in status do exist between cases and controls [30] and do contribute to risk of an NTD, they do not appear from our results to be directly involved in the etiology of NTDs.

XI. SUMMARY

The studies enumerated and discussed in this chapter are summarized in Tables 1 and 2 and demonstrate beyond a doubt that folate is an important determinant of NTD risk. It has been shown that folic acid given at 400 μg/day prevents the recurrence of NTD and that 800 μg prevents both the occurrence and recurrence of NTD in the majority of cases. It seems probable from other studies that 400 μg of folic acid is equally effective. The most effective way of delivering this extra 400 μg to women would be through general dietary fortification with folic acid rather than the use of supplements (tablets) because the latter would be difficult to implement. A major concern is that this extra folic acid would be eaten by all of society. This gives rise to the possibility that the autoimmune disease pernicious anemia, which is caused by vitamin B_{12} malabsorption, would be undiagnosed since folic acid at high levels prevents the emergence of the anemia and thus its diagnosis. Nerve damage in such circumstances continues and is irreversible.

The mechanism by which folic acid or folate prevents NTDs is still unknown, as a recent book and review indicate [88,89]. A recent study by our own group has found significantly lower levels of both plasma folate and RCF in cases compared to controls, which might have been anticipated from the success of folic acid in preventing NTDs. However, both plasma folate and RCF in the cases were distributed throughout the entire normal range, suggesting that a metabolic block rather than folate deficiency was involved. The finding that there were significant-

Table 1 Effect of Folic Acid/Folate on Prevelance of NTDs in All Studies

Name of study	Subject Description	Outcome	Comment	Ref.
South Wales Studies	Dietary intake 141 fair or good diet 45 poor diet	 Zero NTDs (0%) Eight NTDs (16%)	Not clear if dietary classification was made after outcome was known	47
Centers for Disease Control	347 with NTD 2829 controls without	Significant protective effect of multivitamin intake	Recall of vitamin intake was 2.5–16 years after pregnancy	41
National Institutes of Health	571 with NTD birth 546 other birth defect 573 normal births	No protective effect of multivitamin intake	Interviews within 5 months of diagnosis or at birth; only study not to show a protective effect	42
Western Australia Study	77 with NTD birth 77 normal births	Intake of supplements and food folate protective; evidence that free folate in food was protective	"Free" folate in food found protective while total folate in food was less effective; suggest either malabsorption or methological problem	44
Boston Study	Out of 22,776 who answered the questionnaire, 49 had NTDs	Significantly reduced NTDs; dividing dietary intake into greater or less than 100 μg/day gave 3.1 and 7.3 NTDs per 1000 births, respectively	Prospective study at prenatal screening before outcome known; dietary recall not very reliable; protective effect of diet just significant	43
Boston, Philadelphia, Ontario Study	436 with NTDs 2615 with other birth defects	Multivitamin supplementation gave 60% protective effect; also evidence of dietary protection without supplements	Interview 6 months post-delivery; dietary data depended upon recall and was just significant	45

Table 2 Effect of Folic Acid/Folate on Neural Tube Defect Risk

Name of study	Type of intervention[a]	Numbers subject (number relative risk NTDs)			Comments	Ref.
South Wales First Dietary Trial	Improvement in food intake	109 counseled 77 noncounseled	(3) (5)	0.43	First attempt at intervention	40
South Wales Second Dietary Trial	Improvement in food intake	176 counseled Good or fail	(0) (zero)		3 NTDs on fair diet reclassified to unbalanced	47
Multicenter Trial England and N. Ireland	Multivitamineral, 360 μg folic acid	1093 fully (14) 211 partial (2)	(13%) (0.9%)	0.14	Not double-blind; 486 non-supp. did not enter trial, used as controls	49–51
Holmes-Siedle Trial	Multivitamineral, 360 μg folic acid	204 (full 10) 28 none (3)	(0.5%) (10.7)		Not double-blind; 486 non-supp. did not enter trial, used as controls	52
South Wales Folic Acid Trial	Double-blind, 4.0 mg folic acid	60 full (2) 51 none (4)	3.3% 7.8%	0.40	Authors moved the 2 NTD on supplements to become "non–complier"; result then significant	33
U.K. Medical Research Council Trial	Double-blind folic acid (4.0 mg) folic acid/multivit multivit placebo	593 (6) 602 (21)	1.0% 3.3%		Well-designed conclusive trial showed protection by folic acid in 72% of mothers; very high levels of folic acid used	58
Republic of Ireland	Double-blind folic acid (400 μg) folic acid + multivitamin multivit/mineral	172 (0) 89 (1)	0% 1.1%	0.00	Numbers too small to be significant	63
Hungarian Trial	Multivitamin Multimineral	2104 (0) 2052 (6)	0% 0.3%	0.00	The only trial to prevent occurrence; small number of cases	60

[a] All interventions except the Hungarian Trial involved women with a previous history of an NTD-affected pregnancy and were thus attempts to prevent recurrence.

ly lower levels of plasma vitamin B_{12} in cases compared to controls and that plasma folate and plasma vitamin B_{12} are independent risk factors for NTDs indicates that this metabolic block, either directly or indirectly, involves the enzyme methionine synthase, the only enzyme whose activity is influenced independently by these two nutrients. The involvement of a defective methionine synthase is further suggested by the fact that a correlation exists between plasma vitamin B_{12} levels and RCFs in the cases, which would not be expected and does not occur in the controls. RCF biosynthesis is dependent upon methionine synthase, and the observation that it is vitamin B_{12} responsive at apparently normal levels of vitamin B_{12} indicates that this enzyme is deranged in most mothers with NTD-affected fetuses. It seems probable, however, that such events in maternal cells could not in themselves influence the closure of the neural tube in the fetus and that an affected fetus must also have an impaired methionine synthase. Whether or not the condition occurs would depend upon how much folate or vitamin B_{12} the mother was supplying to the affected fetus to overcome this metabolic block, thus making the circulating maternal level of these two nutrients independent risk factors for an NTD.

Carriers of the defective enzyme methionine synthase may also be at risk of other diseases in later life. Homocysteine, the substrate of the enzyme may be inadequately metabolized, leading to hyperhomocysteinemia, a condition that is known to predispose to atheroma. Such a defect would be exacerbated by low plasma folate and/or plasma vitamin B_{12} levels.

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13

Folate Status: Effects on Carcinogenesis

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I. INTRODUCTION

A provocative array of observations from both laboratory and clinical investigations indicate that alterations in folate status may modulate the process of neoplastic transformation in selected epithelial tissues. Diminished folate status appears to promote carcinogenesis. Considerably more speculative is the concept that supraphysiological folate status might afford some protective effect. This discussion will review these observations and outline some of the possible mechanisms by which these effects may be exerted.

The terminology utilized by the science of cancer biology is a complex one; in part because there remains some disagreement as to the precise definition of several basic concepts. It is therefore useful to begin this discussion by reviewing the definitions of some of these important concepts. *Neoplasm* refers to an abnormal mass of cells, "the growth of which exceeds and is uncoordinated with that of the normal tissues and [which] persists in the same excessive manner after cessation of the stimuli which evoked the change" [1]. *Neoplasia* refers to the abnormal growth process associated with a neoplasm. A *malignant neoplasm*, or *cancer*, is a neoplasm whose growth is sufficiently abnormal and excessive that it tends to encroach upon, and invade, adjoining or distant tissues, thereby interfering with normal physiological processes. The transition from normality to malignancy in a population of cells is usually accompanied by cytologic features such as enlarged, irregular-appearing nuclei, increased variability in cell size, and abnormal maturation; a group of features collectively referred to as *dysplasia*. *Metaplasia* is an abnormal process whereby cells acquire the appearance of a different cell type. An example of metaplasia is the transformation of bronchial epithelial cells, which are

normally columnar, to a squamous morphology—a process often seen in heavy cigarette smokers. Metaplasia is frequently indicative of an increased predilection towards malignancy.

II. HISTORICAL PERSPECTIVE

An interest in possible relationships between megaloblastic cells and cancerous ones dates back at least four decades. In 1954, Massey and Rubin described the persistence of abnormal gastric columnar cells in the stomachs of individuals with pernicious anemia, even after the anemia had been successfully treated with vitamin B_{12} [2]. Since the cytologic appearance of these cells exhibited some characteristics of both megaloblastic cells and cancer cells, and since several prior reports had observed an increased incidence of gastric cancer in individuals with pernicious anemia [3–5], these investigators postulated that these abnormal cells might represent a transitional cell type between those cells that characterize the atrophic gastric epithelium in pernicious anemia and gastric cancer cells.

Studies examining a relationship between megaloblastosis related specifically to folate deficiency and cancer date back to the 1960s when Van Niekerk first noted several cytologic similarities between epithelial cells of the uterine cervix from folate-deficient women and cervical cells that were dysplastic [6]. In both cases, the cells are larger than normal and have nuclei that are disproportionately large compared to the size of the cytoplasm, and in both the chromatin is hyperchromatic. Nevertheless, for many years there was little evidence to suggest that the cytologic similarity between megaloblastic and neoplastic cells was anything more than a coincidence.

It was only in the 1970s and 1980s that investigators began to effectively question whether there might be a functional association between megaloblastic and dysplastic cells. Two prospective, controlled clinical intervention trials, conducted in the 1980s by investigators at the University of Alabama, studied individuals with dysplastic changes in the epithelia of the uterine cervix [7] and metaplastic changes in the bronchi [8]. Each of these studies observed a significant degree of attenuation or regression in dysplasia or metaplasia after several months of supplementation with pharmacological doses of folic acid alone [7] (Fig. 1) or in conjunction with pharmacological doses of vitamin B_{12} [8]. This lent further support to the concept that folate and dysplasia might be functionally related, although it remains unclear whether the alterations in cervical cytology associated with supplementation were truly an attenuation of dysplasia or merely an attenuation of megaloblastosis that masqueraded as dysplasia. A subsequent clinical intervention trial [9], again conducted by the University of Alabama group, tested a larger population of women and followed the subjects for a longer period of time. This trial was unable to reproduce the attenuation of dysplasia noted in the earlier study. However, more than two-thirds of the subjects enrolled in the latter trial had the

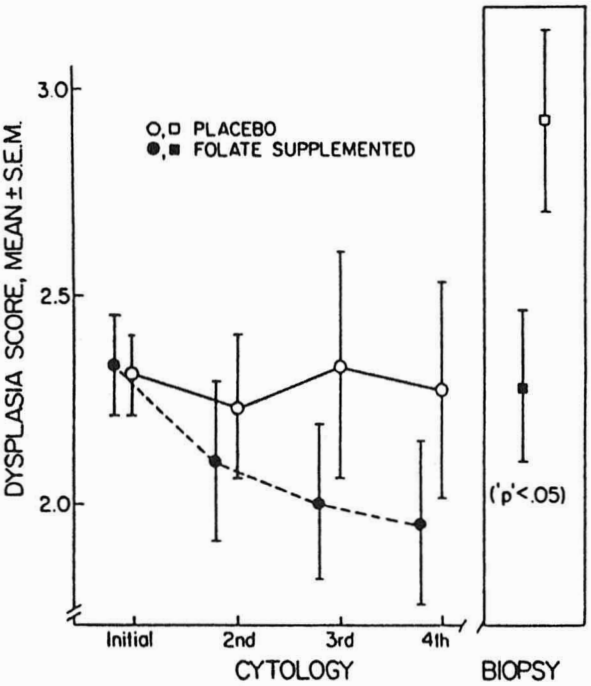

Figure 1 The course of cervical dysplasia in women supplemented with 10 mg of folic acid daily (closed circles) or placebo (open circles). Dysplasia score refers to the severity of dysplasia. (From Ref. 7.)

lowest grade of dysplasia upon entry, a stage of disease known to have a spontaneous rate of reversion to normal of more than 60% [10]. The investigators felt that this may have interfered with their ability to observe an effect of supplementation. A large, recently published case-control study designed to examine folate status and multiple other risk factors for cervical dysplasia did not find an overall association between red blood cells (RBC) folate and dysplasia [11]. However, subjects who had human papilloma virus-16 infection of the cervix, an important risk factor for cervical carcinogenesis [12], had a severalfold greater risk of having dysplasia if they also had diminished RBC folate levels. This suggests that whatever enhancement of cervical carcinogenesis is effected by diminished folate status requires concurrent factors predisposing toward carcinogenesis. The fact that diminished folate status was defined as a RBC folate level of <660 nmol/liter (291 ng/ml) in this study was of particular interest since that value is well within the range of folate levels conventionally accepted as normal, suggesting that the effect of diminished folate status does not require a frank folate deficiency as it is usually defined.

III. TISSUES IN WHICH FOLATE STATUS HAS BEEN IMPLICATED IN CARCINOGENESIS: CLINICAL OBSERVATIONS

A. Uterine Cervix

As indicated in the previous section, much of the groundwork in this field has been established in the uterine cervix. Several clinical trials utilizing case-control methodology have examined the association between dietary folate intake and cervical neoplasia [11,13–17] (Table 1). Although several of these studies demonstrate that the relative risk of cervical neoplasia is somewhat diminished in individuals consuming larger quantities of folate, the effect uniformly falls short of statistical significance when adjusted for confounding variables. Nevertheless, this negative consensus among case-control studies in cervical neoplasia should not be construed as proof that no effect exists: these studies suffer from several limitations. In most instances, folate status was assessed by estimating average dietary folate intake by means of food frequency questionnaires that have not been validated for folate intake. None of the trials, excepting the Butterworth study, stratified for human papillomavirus (HPV) infections. These studies were therefore unable to explore possible synergism between diminished folate intake and HPV, and such stratification may be necessary to observe a folate effect. Furthermore, all of the studies, excepting the Butterworth trial, examined women with either carcinoma in situ or invasive cancer. These represent the latter stages of neoplasia, by which time the progression to cancer may be far enough advanced that there is little opportunity for modulation by factors such as folate status. In summary, at the present time the data are too conflicting to make any definitive statement about whether alterations in folate status truly modulate the process of carcinogenesis in the cervix.

Table 1 Case-Control Studies of Folate and Cervical Neoplasia

Case diagnosis	Folate measure	*N*	Relative risk high vs low folate		95% Confidence interval	Ref.
			crude	adjusted		
In situ	Intake	313	0.6	1.3	0.3–5.8	13
Invasive	Intake	409	0.4	0.8	0.3–1.7	14
Invasive	Intake	773	0.9	0.8	0.4–1.4	15
In situ	Intake	731	0.4	0.7	0.4–1.5	16
Invasive	Intake	895	1.0	1.1	0.7–1.6	17
Dysplasia	RBC folate	464	0.6	0.7	0.4–1.3	11

Source: Adapted from Ref. 75.

B. Colorectum

Clinical observations from several different groups point to an association between diminished folate status, assessed either by dietary intake or by measurement of blood levels, and an enhanced risk of colorectal adenomas or cancer (Table 2). Lashner and colleagues first made this observation in a case-control study of individuals afflicted with chronic ulcerative colitis [18], a disease that carries with it not only a 10- to 40-fold increased risk of colonic cancer but a significant risk of folate deficiency [19,20] as well. The authors determined that individuals who had not been taking folate supplements over the long term had a rate of colonic neoplasia that was approximately 2.5-fold greater than those who had been taking supplements. Furthermore, chronic administration of sulfasalazine, a drug commonly used for the treatment of ulcerative colitis and one known to specifically inhibit both folate absorption [20,21] and folate metabolism [22], was associated

Table 2 Epidemiological Studies of Folate and Colorectal Neoplasia

Case diagnosis	N	Relative[a] risk	95% Confidence limits	Ref.[b]
Dysplasia	99	0.38[c]	0.12–1.20	18[d]
Cancer				24
colon, men	410	1.03	0.56–1.89	
rectum, men	454	0.31	0.16–0.59	
colon, women	446	0.69	0.36–1.30	
rectum, women	290	0.50	0.24–1.03	
Colorectal cancer	784	0.53–0.56	NA[e]	25
Colon cancer, men	462	1.24	NA	26
Colon cancer, women	376	0.54	NA	26
Distal colorectal adenomas, men	9490	0.63	0.41–0.98	27
Distal colorectal adenomas, women	15984	0.63	0.46–0.95	27
Dysplasia and cancer	67	0.82	0.68–0.99	23

[a] Relative risk for high folate intake, or level, compared to low intake or level. Adjusted for various colorectal cancer risk factors.

[b] All studies were case-control except for Ref. 27, which was a prospective cohort study. All studies measured habitual folate intake except Ref. 23, which measured RBC folate levels.

[c] Crude relative risk, but authors note that there was "no appreciable change when adjusted for known confounders of neoplasia or folate exposure."

[d] Folate intake from supplements only.

[e] Not available.

with a 50% increase in the risk of dysplasia. Both of these observations fell slightly short of conventionally accepted levels of statistical significance, but the study nevertheless established the importance of examining this issue in the colon. More recently, Lashner has confirmed these observations by prospectively assessing erythrocyte (RBC) folate levels in ulcerative colitis patients who harbor neoplastic changes in their colorectum with age-, sex-, and disease-matched controls who do not harbor any neoplastic lesions [23]: this analysis demonstrated that the RBC folate level was 66.2 ng/ml lower in the individuals with dysplasia or cancer than in the controls, a statistically significant difference. The group harboring neoplastic lesions consisted of four subjects with dysplasia and two with cancer. No evidence of general malnutrition was observed among these individuals, and therefore the lower mean folate level in this group was not felt to be a result of the underlying neoplasia. An 18% incremental increase in the relative risk of colorectal neoplasia was observed for every drop of 10 ng/ml in the RBC folate. It is noteworthy that although the group that harbored neoplasia had diminished RBC folate levels compared to the control group, the absolute level in the neoplasia group (454 ng/ml) was well within the range of values conventionally accepted as normal.

Epidemiological studies of the general population have generated mixed results: one based in western New York [24] observed that increased habitual intake of folate was associated with a decreased incidence of rectal, but not colonic, cancer, whereas a study that examined the population on the island of Majorca [25] observed similar reductions in relative risk for colonic and rectal cancer combined. A dissenting study, conducted in a population from western Washington State [26], only examined rates of colon cancer and did not observe a significant reduction in risk with increased dietary intake of folate. However, a strong trend towards such an effect was present in women.

The most convincing epidemiological evidence to date to establish an association between folate status and colorectal neoplasia was recently published by Giovannucci et al. [27]. The prospective design of this study, the large population included in the analysis (n = 25,474), and the control of several confounding dietary variables enabled these investigators to convincingly support the hypothesis that increased folate intake is inversely associated with the incidence of adenomatous polyps in the distal half of the colorectum. These studies in the general population are particularly important since they demonstrate an apparent effect in populations that do not clearly have other, predisposing, conditions to colorectal cancer. Although the results from the epidemiological studies pertaining to folate and colorectal cancer are not uniformly positive, the trend towards such an effect seems much stronger than has been observed in the cervix.

Results from a small, prospective, controlled intervention trial have been reported [28]. Eleven subjects with colonic polyps and eleven subjects with colorectal adenocarcinoma were randomized to receive either 10 or 0 mg of folic acid daily for 6 months following removal of their neoplasm. The major endpoint examined was DNA methylation of normal-appearing rectal mucosa, which has been suggested

as an intermediary marker of colorectal cancer. Alterations in DNA methylation are commonly observed as an early biochemical phenomenon in colonic carcinogenesis [29–33]. Global hypomethylation of DNA has been observed frequently in early, premalignant, colonic neoplasias [29,30] and hypomethylation of certain proto-oncogenes, such as k-*ras*, has been documented to occur [31]. Therefore, in addition to possibly serving as a mechanism by which folate deficiency enhances carcinogenesis (see Sec. IV), DNA hypomethylation may prove to be a satisfactory intermediary marker of colonic carcinogenesis. Interestingly, DNA hypomethylation precedes most of the other known molecular events that occur in colorectal tumorigenesis [34], suggesting that alterations in DNA methylation may play an active role in the evolution of carcinogenesis. Therefore, it is of interest to determine whether the DNA hypomethylation associated with early dysplasia can be modified by intervention. Although the DNA hypomethylation observed in early adenomatous polyps in humans is not likely a consequence of folate deficiency, it is of considerable interest that this preliminary study demonstrated that pharmacological doses of folic acid reversed the global DNA hypomethylation present in the normal-appearing rectal epithelium of individuals harboring either colorectal adenomas or cancers [28]. This has important implications for determining the mechanism by which folate status modulates colorectal carcinogenesis and has a potential role in the prevention of colorectal cancer.

C. Lung and Esophagus

A single clinical study, previously mentioned, has examined this issue in the lung [8]. Heimburger et al. performed a prospective, placebo-controlled, intervention trial in 80 male smokers with squamous metaplasia. Both folic acid and hydroxycobalamin were concurrently administered to the experimental group. After 4 months a significant improvement in the severity of metaplasia was observed in the experimental, but not control, group. One study has also examined this issue in the esophagus [35]. Individuals in areas with either a low or high prevalence of esophageal cancer underwent esophageal brush cytology. Those with either dysplasia or cancer and those from high-risk regions had lower serum folate levels than individuals with normal cytology. Lower levels of vitamins A, E, and B_{12} were also associated with dysplasia or cancer. In addition there was an association between lower levels of serum and RBC folate and cellular atypia.

There are insufficient data at this point to make any firm conclusions regarding folate and cancer in the lung and esophagus.

IV. ESTABLISHING A CAUSATIVE RELATIONSHIP BETWEEN FOLATE STATUS AND CARCINOGENESIS

However provocative these clinical studies might be, they can only establish an associative, and not causative, relationship. Two of the three intervention studies

demonstrate a positive effect [7,8], implying a causative relationship between folate status and cancer. However, the reversal of dysplasia with supraphysiological doses of folate is a maneuver that does not necessarily imply that diminished or deficient folate status promotes the process of carcinogenesis.

In order to more clearly establish whether a true cause-and-effect relationship exists between diminished folate status and colorectal carcinogenesis, an animal study was recently conducted [36]. The dimethylhydrazine rat model of colorectal carcinogenesis, which has been extensively studied [37], was chosen to examine this issue. Weekly subcutaneous injections of 1,2-dimethylhydrazine dihydrochloride, a procarcinogen whose metabolites are thought to exert their carcinogenic effect by alkylation of DNA, produces a sequence of hyperplasia, followed by increasing grades of dysplasia and finally cancer in the colorectal mucosa over a period of several months. Histologically, these events are similar to those thought to occur in human colonic carcinogenesis, although the time course is greatly accelerated. Many of the genomic events commonly observed in human colonic carcinogenesis, such as c-*myc* proto-oncogene overexpression and k-*ras* activation, are also observed in this model [38,39]. Folate-replete rats were fed an amino acid–defined diet containing 8 mg folic acid/kg of chow; the deplete group received an identical diet except that it contained 0 mg folic acid/kg [40]. Neither group received succinylsulfathiazole, a nonabsorbable antibiotic typically added to produce a more marked degree of folate deficiency. Severe deficiency was therefore avoided because of the endogenous synthesis of folate by intestinal bacteria and because of coprophagy [41]. The degree of folate deficiency that was created in the deficient groups was therefore moderate enough to maintain good health in the animals and to prevent any difference in the growth curves between the deficient and replete animals. After 5 weeks of carcinogen exposure and at 5-week intervals thereafter, rats from each of the groups were killed, tissue samples of blood, liver, and colonic mucosa were harvested for determination of folate concentrations, and the colorectum from each animal was fixed and stained for conventional histological examination. Two pathologists, blinded to the groupings of the rats, independently examined identical representative longitudinal sections of colorectum from each animal and the total number of dysplastic and cancerous foci were tallied. Figure 2 demonstrates that the folate-deficient rats with the underlying predisposition to colonic neoplasia started developing colonic neoplasia at a greater rate by 15 weeks and by 20 weeks had a significantly greater incidence of neoplasia than folate-replete controls [36]. No cancer was observed in the two control groups that received saline injections in conjunction with either a folate-replete or depleted diet.

In this rat model of colonic carcinogenesis, moderate folate deficiency does enhance the development of colonic dysplasia and cancer, thereby providing considerable evidence of a cause-and-effect relationship between diminished folate status and colorectal cancer.

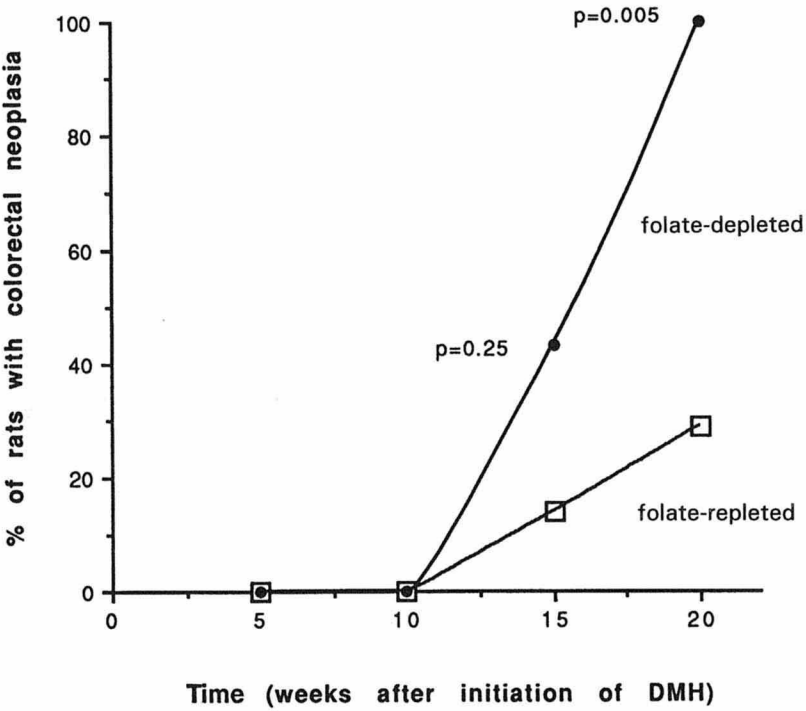

Figure 2 Incidence of colonic dysplasia and/or carcinoma in folate-depleted (closed circles) and folate-replete (open circles) rats treated with the procarcinogen dimethylhydrazine (DMH). At 20 weeks there was a significant difference between the two groups. (Adapted from Ref. 36.)

V. DIMINISHED FOLATE STATUS AND ENHANCED CARCINOGENESIS: POTENTIAL MECHANISMS

The mechanism by which diminished folate status acts to enhance carcinogenesis is not known. By necessity, therefore, any discussion pertaining to the mechanism must be speculative. There are many potential mechanisms by which this effect may occur, and there is no a priori reason to believe that multiple mechanisms might not play a role (Table 3). Since all the known biochemical functions of folate pertain to its ability to mediate the transfer of one-carbon fragments [42], it is possible that one or more of the mechanisms that have been hypothesized to mediate other examples of lipotrope deficiency–associated carcinogenesis [43] may also be responsible for enhanced carcinogenesis associated with the isolated depletion of folate.

Diminished folate status may have some influence on the patterns of DNA methylation and thereby might influence gene expression. Mammalian DNA is

Table 3 Candidate Mechanisms for Folate-Related Enhancement of Carcinogenesis

Induction of DNA hypomethylation
Secondary deficiency of choline
Diminution in natural killer cell surveillance
Increased chromosomal fragility
Misincorporation of uridylate for thymidylate in DNA synthesis
Facilitation of tumorigenic virus metabolism

methylated at deoxycytidine residues; methylation of the other nucleotides probably does not occur but has not been rigorously excluded [44]. Virtually all of these methylated residues reside in cytosine-guanine (5′-CpG-3′) sequences, a dinucleotide sequence disproportionately clustered in "CpG-rich islands" located at the 5′ end of genes [45]. Methyltetrahydrofolate provides the methyl group for methionine, and therefore S-adenosylmethionine (SAM), synthesis [46,47]. Since SAM is the proximal methyl donor for DNA methylation (Fig. 3) [48], it is not surprising

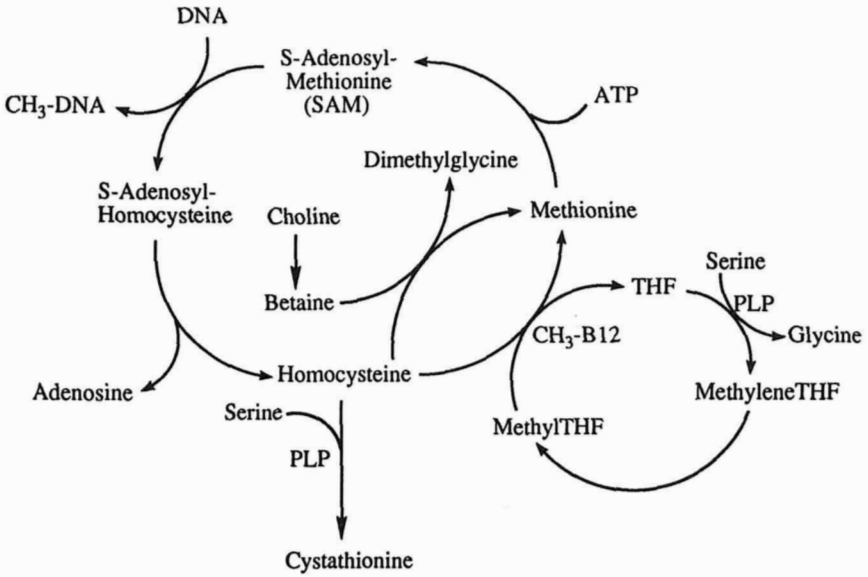

Figure 3 The sulfur-containing amino acid pathway. 5-Methyltetrahydrofolate, located in the lower righthand corner of this diagram, is a substrate that supplies the methyl group for methionine synthesis. Methionine is then adenylated to produce S-adenosylmethionine. This compound is the proximal methyl donor for DNA methylation. Alternatively, methionine can be synthesized from betaine, which is an oxidation product of choline. PLP, pyridoxal 5-phosphate; THF, tetrahydrofolate. (Adapted from Ref. 76.)

that rats who are fed diets devoid of methionine, choline, vitamin B_{12}, and folate have been observed to have low hepatic SAM concentrations and diminished hepatic DNA methylation [49]. Rats that are fed diets lacking choline, or lacking choline and other lipotropes, have marked enhancement of chemically induced hepatocarcinogenesis [43]; when such diets are prolonged, the animals appear to spontaneously develop these cancers [50]. Preceding the development of the cancers is the appearance of global DNA hypomethylation [49], specific hypomethylation of the c-*myc*, c-*fos*, and c-Ha-*ras* proto-oncogenes and elevated steady-state levels of the corresponding mRNAs [51]. All three of these proto-oncogenes have been associated with hepatocarcinogenesis in the rodent. These observations are consistent with a considerable body of literature, which indicates that altering the methylation of certain genes is an important means of regulating gene expression [52], probably by modulating the interaction between the promoter sites of genes and the transcription machinery [53]. In most instances, hypomethylation of the 5′ end of genes regulated by this phenomenon is associated with enhanced transcription. Further evidence supporting a mechanistic role for DNA hypomethylation in colonic carcinogenesis is provided by observations that indicate that global DNA hypomethylation is almost invariably observed in early neoplastic lesions of the colorectum in humans, often before other genomic events associated with colon carcinogenesis appear [29,30]. In some cases, hypomethylation of certain colon cancer-associated proto-oncogenes such as k-*ras*, has also been observed [31]. Aberrant DNA hypomethylation has also been proposed as the mechanism by which genomic imprinting, the heritable suppression of either the maternal or paternal allele, is lost in human cancer [54,55]. This provides another avenue by which folate deficiency might predispose to inappropriate gene expression. Nevertheless, this is an increasingly complex field, since site-specific DNA *hyper*methylation has also been observed in colonic carcinogenesis, including sites that contain important tumor suppressor genes such as p53 [32,33].

Whether diminished folate status alone—a nutritional manipulation known to decrease SAM levels [46,47]—may induce global DNA hypomethylation is still a matter of debate. One recent report suggests that severe folate deficiency in rats produces global DNA hypomethylation [56], but this observation needs to be confirmed. Whether gene-specific hypomethylation is present in folate deficiency is under active investigation.

Another mechanism that might play a role in the procarcinogenic effects of diminished folate status is by inducing a secondary depletion of choline. Choline deficiency in animals is known to serve as a promoter of hepatocarcinogenesis, and prolonged choline deficiency is, by itself, a sufficient condition to produce hepatocellular carcinomas [57,58]. Extrahepatic neoplasms sometimes arise with chronic choline deficiency, although, in the case of many tissues, an initiator or other underlying predisposition may need to be present in order for the choline deficiency

to result in cancer. The mechanism by which choline deficiency mediates these effects is not known, but it has been suggested that the increased intracellular 2,3-diacylglycerol levels observed in choline deficiency are of sufficient magnitude to activate protein kinase C (PKC) and induce its shift from a cytosolic to cell membrane compartment [59]. Activation of protein kinase C is associated with mitogenesis and enhanced expression of the c-*myc* proto-oncogene [60,61]; furthermore, fibroblasts transfected with a mutant form of PKC which is continuously activated acquire a neoplastic phenotype [62]. Activation of PKC is apparent in several malignancies. For instance, in the colon it has been shown to occur early in the development of chemically induced colonic neoplasia [63] as well as in human colorectal cancers [64,65]. In order to understand how folate deficiency might promote carcinogenesis by the same pathway, it is necessary to recall some in vitro observations of Finkelstein et al., which indicate that impairing methionine synthesis in rat liver homogenates by removal of methylfolate leads to enhanced activity of the alternative pathway for methionine synthesis, the transmethylation of homocysteine by betaine [66]. Betaine, in turn, is obtained from the oxidation of choline (Fig. 3). A long-term deficiency of folate might, therefore, lead to increased utilization of betaine and choline in the liver and to a significant enough depletion of choline in the liver and peripheral tissues to increase 2,3-diacylglycerol levels and to activate protein kinase C. This is a hypothesis that has yet to be proven.

Folate deficiency is known to have adverse effects on the integrity of lymphocyte function, as assessed by the blastogenic response to mitogens [67]. Natural killer cells, a subset of lymphocytes, are felt to be responsible for the surveillance and destruction of arising clones of neoplastic cells [68]. A rodent study has demonstrated that severe folate deficiency will suppress the ability of lymphocytes to kill heterologous cells to which the rodents had previously been sensitized [69]. One could hypothesize, therefore, that diminished folate status might impair the ability of natural killer cells to destroy dysplastic or cancerous cells.

Cancers in many tissues have been linked to disruptions in chromosomal integrity, often leading to the loss of tumor suppressor gene activity [33]. The induction of folate deficiency in cultured lymphocytes is known to induce a variety of such chromosomal abnormalities at foci called folate-sensitive "fragile sites," some of which are closely linked to known proto-oncogenes and antioncogenes [70,71]. There are also observations that suggest that folate deficiency may produce such an effect in vivo [72].

Another mechanism mentioned in Table 3 pertains to the creation of imperfect DNA copies during cell division due to the misincorporation of deoxyuridylate for thymidylate in the setting of folate deficiency. When human lymphoid cells are treated with potent inhibitors of folate metabolism, such as methotrexate, de novo thymidine synthesis, which is folate dependent, drops to low enough levels that uridylate is incorrectly substituted in newly synthesized DNA where thymidylate

should be [73]. The implications of this phenomenon as it pertains to cellular metabolism are not yet defined, but it suggests a means by which impaired folate status could play a role in the creation of missense or nonsense mutations.

Tumorigenic viruses are felt to play an important role in the process of carcinogenesis in tissues such as the uterine cervix. Diminished folate status may promote the development of such cancers by interacting with the biology of these viruses. The human papilloma virus-18 (HPV-18), which is highly associated with human cervical neoplasia, specifically incorporates into the human genome adjacent to four loci, three of which are in or near a constitutive fragile site [74]. Diminished folate status in vitro [70,71] and, perhaps, in vivo [72] is known to distort chromosomal architecture at such sites and might thereby facilitate incorporation of the viruses.

A great deal of work remains to be done in order to understand the means by which folate status might modulate the process of carcinogenesis.

VI. CONCLUSIONS

A growing body of clinical studies suggest that folate status can modulate the process of carcinogenesis. The evidence for this seems to be strongest in the colorectum; evidence in the uterine cervix is less consistent and only sparse data exists for the lung and esophagus. Diminished folate status is associated with a higher risk of carcinogenesis, whereas supraphysiological status may convey some protective effect. A controlled laboratory study in an animal model of colorectal cancer appears to have confirmed a true cause-and-effect relationship between folate deficiency and enhanced carcinogenesis.

Interestingly, these effects seem to occur with relatively modest alterations in folate status and may even be present when vitamin status is altered within the range of what is presently considered to be a normal. Folate, as a compound whose only known functions relate to its transfer of one-carbon units, therefore appears to have joined some of the other lipotropes as a compound that might modulate carcinogenesis. The mechanisms by which this may occur are diverse and are under intense investigation.

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This review is dedicated to the memory of Dr. Thomas Parsons, M.D., who was held in highest esteem by his colleagues for his insight, intelligence, and common sense, but most of all for his absolute dedication to a humanitarian approach to medicine.

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14

Folates and Folate Antagonists in Cancer Chemotherapy

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I. INTRODUCTION

Folate antagonists have occupied a central position in cancer treatment for nearly half a century. The folic acid analog, methotrexate (MTX), was discovered prior to 1950 and remains today perhaps the most widely used of any single cancer chemotherapeutic agent [1,2]. This success has played a key role in sustained efforts to better understand the enzymes related to folate metabolism and to develop new strategies for the disruption of this metabolism to improve cancer therapy. Because of the length of time and the magnitude of the disease, these studies have created a profoundly large literature, all of which cannot be considered within the scope of this chapter. Hence, an effort will be made to focus on those folate antagonists that are currently in clinical use or are undergoing clinical evaluation and on folates themselves that are currently in use as modulators of other antineoplastic drugs.

Cancer is a devastating disease that ranks second only to cardiovascular disease as a cause of death in the United States [3]. Solid tumors are typically adult diseases, and nonsolid tumors or leukemias most often affect children. Historically, treatment of solid tumors has relied on two approaches: physical removal by surgery or irradiation and chemical intervention with drugs or biological response modifiers [3,4]. Leukemias are usually treated chemotherapeutically, but bone marrow transplant therapy is becoming more widespread.

There has recently been a proliferation of experimental approaches to cancer treatment involving alteration of genetic expression, which collectively has been termed "gene therapy" [5]. While such approaches hold promise for the future, it is probable that over the next few years classical drug intervention, incorporating

biological response modifiers, will remain the backbone of systemic cancer therapy.

Cancer drugs fall into several classes, including agents that directly interact with and modify DNA, such as alkylating agents and cisplatin [6], interfere with enzymes that act on DNA, such as topoisomerase inhibitors [7], interact with tubulin and alter microtubule assembly and disassembly, such as the vinca alkaloids and taxol [6,8], and inhibit enzymes that catalyze critical metabolic steps, the so-called antimetabolites [6,9]. Folate antagonists are members of this latter category, and MTX is by far the most extensively used and studied folate antagonist.

II. METHOTREXATE AND OTHER FOLATE ANTAGONISTS

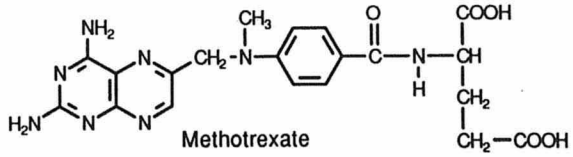
A. Structure and Enzyme Targets

A single modification of folic acid, substitution of an amino group for a hydroxyl group at position four of the pteridine ring, gives rise to compounds that are very tight binding inhibitors of the enzyme dihydrofolate reductase (DHFR). Methotrexate (MTX), the classic analog with this substitution, is also methylated at the N^{10} position. While a vast number of other folate derivatives with substitutions at various positions have been tested as antineoplastic agents, none have thus far shown sufficient improvement over MTX to replace it in the clinic. This is partially because of familiarity with activity and toxicity patterns, but there is also a lack of evidence that newer compounds are truly more effective therapeutically. Figure 1 shows the structure of MTX and several other folate antagonists currently being considered experimentally in the clinic. Numerous additional derivatives are under consideration in preclinical studies.

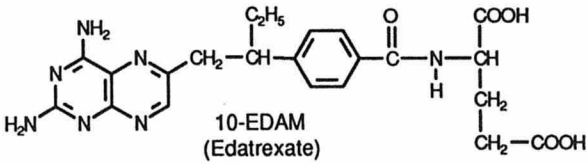
Folate antagonists are typically categorized as either "classical" or "nonclassical." This classification arose in relation to MTX and is based upon the presence or absence of a glutamyl functional group (Fig. 1). The presence of this charged acidic amino acid generally dictates whether uptake into cells occurs via a protein carrier system. Alternatively, nonclassical folate antagonists typically are taken up by diffusion. Trimetrexate (Fig. 1) is an example of a nonclassical folate antagonist currently undergoing clinical trials for both solid tumors and leukemias that are refractory to MTX [10].

An additional system by which folate antagonists have been categorized is based on their intracellular enzyme target (Fig. 2). Historically, the enzyme target that has received the most attention is DHFR. No doubt this is because MTX is a tight binding inhibitor of DHFR, and MTX has been the premier folate antagonist [2,11,12]. However, other DHFR inhibitors continue to be synthesized in an effort to obtain agents with superior antitumor properties. An example of one such derivative is 10-ethyl-10-deazaaminopterin (10-EDAM or edatrexate) (Fig. 1), which is currently undergoing clinical trials against head and neck cancer [13].

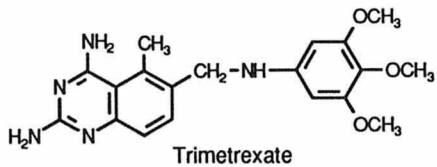
4-amino-10-methyl-pteroyl glutamate



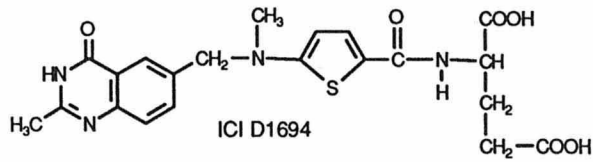
10-ethyl-10-deaza-aminopterin



2,4-diamino-5-methyl [(3,4,5-trimethoxyanilino) methyl]-quinalozine



N-(5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin -6-ylmethyl)-N-methyl amino]-2-thenoyl)-L-glutamic acid



5,10-dideaza 5,6,7,8-tetrahydrofolate

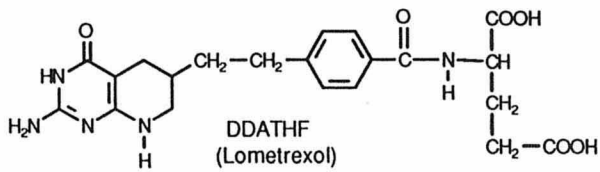


Figure 1 Methotrexate and other folate antagonists currently undergoing clinical investigation.

With a view toward both improvement of cancer therapy and overcoming resistance resulting from overexpression of DHFR, folate antagonists targeted against other folate-metabolizing enzymes are under active investigation (Figs. 1 and 2). The non-DHFR enzyme target that has received most attention is thymidylate synthase (TS). Several quinazoline-based inhibitors were found to be potent inhibitors of TS but were discontinued clinically because of various side effects [14,15]. A less toxic compound of this class currently undergoing clinical investigation, *N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl)-L-glutamic acid (ICI D1694) [9], is shown in Figure 1.

Another set of enzymes that have been targeted as an alternative to DHFR are glycinamide ribonucleotide transformylase (GARTF) and aminoimidazole carboxamide ribonucleotide transformylase (AICARTF), enzymes that participate in the de novo purine biosynthetic pathway and utilize 10-formyltetrahydrofolate (10-HCO-H_4folate) as a substrate (see Fig. 2C). Figure 1 shows the structure of 5,10-dideazatetrahydrofolate (DDATHF or Lometrexol) [9], a potent inhibitor of GARTF that is being investigated in clinical trials.

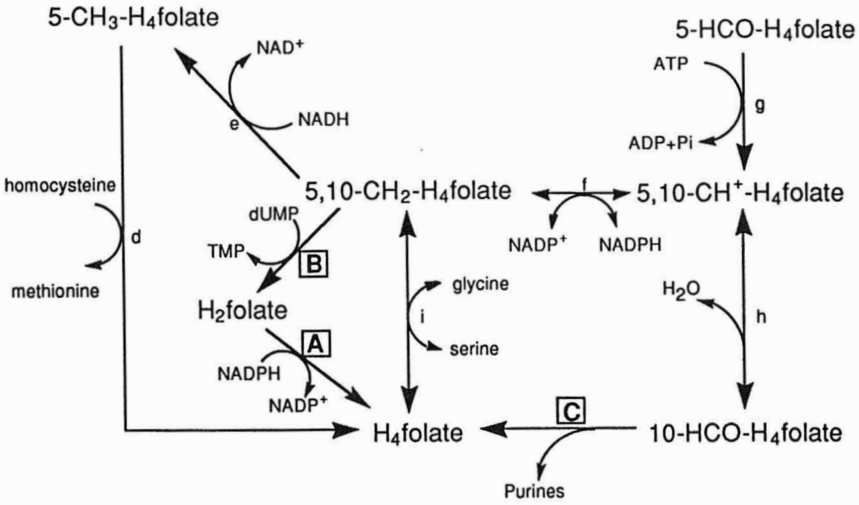


Figure 2 Folate metabolic interconversions affected by methotrexate and other folate antagonists. Target enzymes include (A) dihydrofolate reductase (methotrexate, trimetrexate, 10-EDAM), (B) thymidylate synthase (ICI D1694), (C) glycinamide ribonucleotide transformylase (DDATHF) and aminoimidazole carboxamide ribonucleotide transformylase. Other related folate metabolizing enzymes include (d) methionine synthetase, (e) methylenetetrahydrofolate reductase, (f) methylenetetrahydrofolate dehydrogenase, (g) methenyltetrahydrofolate synthetase, (h) methenyltetrahydrofolate cyclohydrolase, and (i) serine hydroxymethyl transferase.

B. Cellular Sensitivity and Resistance

Critical features of sensitivity and resistance to antineoplastic drugs include uptake or transport, intracellular transformation and impact on the regulation of biochemical events within the cell. Evaluation of each of these factors in cultured cell systems has made important contributions to the development of more effective drug therapies.

1. *Transport*

Natural folates and folate antagonists enter the cell through an energy-dependent process requiring one or more specific carrier proteins [2,11,12]. Since folates and folate antagonists utilize the same transport system, there is competition between them for uptake. Quantitatively, the K_m or half-maximal saturation concentration for MTX uptake has been estimated from kinetic studies with various cell lines to be in the range of 1–5 μM. Efforts to isolate an active transport protein system that can be reconstituted have thus far been unsuccessful, but investigators have obtained purified preparations of proteins from tumor cell membranes that have a high affinity for MTX, folic acid, and reduced folates [16]. Interestingly, many tumor cells express a so-called membrane "folate-binding protein" with a molecular weight different from the isolated carrier proteins. This protein can be upregulated significantly when cells are placed in folate-depleted media [16]. It has been postulated that regulation of this protein plays a physiological role in tissue adaptation to changing folate availability and to sensitivity to folate antagonists. With the recent cloning of the cDNA coding for this protein, it should be possible to rapidly gain a better understanding of its precise role in the transport process and in resistance mechanisms [17].

The proliferative state of tumor cells in culture is an important factor in the uptake of folates and folate antagonists. Generally, more rapidly dividing cells acquire MTX and folates more avidly [18]. While other factors no doubt also play a role, this natural attempt by tumor cells to increase their folate uptake for rapid proliferation is thought to be a contributing factor to selective toxicity of folate antagonists.

A number of agents used clinically in combination with MTX can affect intracellular accumulation of folate antagonists [12]. Vincristine, which is used extensively in cancer therapy, is a naturally occurring vinca alkaloid that disrupts microtubule structure. It reportedly enhances MTX accumulation in cell cultures by inhibiting MTX efflux. However, because the concentration required for this effect is near 10 μM and peak plasma levels achieved clinically are typically only about 0.1 μM, it is questionable whether this drug-retention effect has pharmacological significance. Nevertheless, synergism has been observed in tumor-bearing animals when vincristine is administered several hours subsequent to MTX. In another case, inhibition of MTX efflux by probenicid has also been reported [12].

This observation has resulted in inclusion of probenicid in some therapeutic regimens with MTX. MTX influx is inhibited in culture by several drugs, including ouabain, glucocorticoids, and cephalothin [19]. Again the clinical significance is unclear because very high doses are required for this inhibition in culture systems. Finally, 7-hydroxymethotrexate (7-OH-MTX), the major circulating metabolite of MTX in humans, also competes with MTX for transport in culture systems [11,12]. The clinical significance of this effect has not been explored.

Resistance is the primary reason for failure of MTX therapy, and a number of MTX-resistant mutants with transport defects have been obtained by exposure of tumor cells to the drug in culture [2]. However, since the same carrier system is used by folates and MTX, suppression of MTX uptake should also result in less uptake of required natural folates. Hence, there is somewhat of a conceptual enigma regarding these resistance models. Possibly the abnormally high folic acid levels (2–3 μM), typically found in culture media used to generate the resistant mutants, meet the demand for the natural folates even in the face of low-uptake characteristics. Much lower levels (10 nM) of another folate, 5-methyltetrahydrofolate (5-CH_3-H_4folate) are present in human plasma [20]. Factors such as these have led to uncertainty regarding the relevance of these cultured cell resistance models to clinical resistance.

Nevertheless, strategies have been developed with the intention of overcoming drug transport-related resistance. One such strategy is to develop drugs that can penetrate the tumor cell by passive diffusion and, thus, be taken up without involvement of the defective carrier system. To achieve a diffusable folate antagonist, the highly charged glutamyl functional group is usually deleted, yielding compounds such as trimetrexate (Fig. 1).

2. *Polyglutamylation*

Folates and classical folate antagonists acquire several additional glutamyl residues after entry into the cell. Generally, this polyglutamylation results in both improved retention within the cell and enhanced binding to target enzymes. The MTX target, DHFR, is exceptional in that it is relatively insensitive to the polyglutamylation state of substrates or inhibitors [11,21]. However, there have been reports, based on isolated enzyme studies, that MTX dissociates somewhat more slowly from DHFR when it is polyglutamylated. Regardless, it is clear that polyglutamylation of MTX significantly enhances intracellular retention and binding to secondary enzyme targets such as TS [21].

There is a single enzyme in human tissue responsible for polyglutamylation of folates and folate antagonists. Folylpolyglutamate synthetase (FPGS; EC 6.3.2.17) catalyzes the addition of up to eight glutamyl residues, in γ-carboxyl peptide linkage, to all of the natural reduced folates. This enzyme also catalyzes the polyglutamylation of classical folate antagonists, but they tend to be less active substrates and typically acquire fewer glutamyl residues. FPGS is a magnesium-

ATP-dependent enzyme that is stimulated by monovalant cations. It has been purified to homogeneity from bacterial sources as well as mammalian liver [21], and the human liver enzyme has recently been cloned by Shane and coworkers [22].

Inhibitors of FPGS have been investigated because of their potential as antitumor agents. While a variety of structural analogs have been prepared that are effective inhibitors, they have not proven active in cellular toxicity tests. An analog of aminopterin in which the terminal glutamyl group is replaced by ornithine is the most potent inhibitor ($K_i = 0.15\ \mu M$) prepared to date, but it has shown little cellular toxicity because of poor transport properties [23].

Intracellular polyglutamylation of MTX plays an important role in retention and, thus, antitumor activity. MTX polyglutamates with two or more residues are poorly transported into cells [11,21]. However, once polyglutamylation occurs within the cell there is preferential retention and extracellular levels of MTX can be lowered extensively with little loss of intracellular MTX polyglutamates. The rate of cell growth and the level of natural folate polyglutamates within the cell are both important determinants of intracellular polyglutamylation and retention [11,18,21].

Presumably, the intracellular content of MTX polyglutamates is maintained by a balance between synthesis and hydrolysis [24]. Folylpolyglutamate hydrolase, which is widely distributed in tissues, is highly specific for the γ-glutamyl peptide bond of polyglutamates but is relatively insensitive to substrate structure otherwise. This enzyme is more active at acid pH and apparently resides primarily in lysosomes within the cell. While intracellular activity is thought to be the primary function of this hydrolase, Galivan and coworkers have recently reported that it is secreted into media from cell cultures [25]. Thus far, the precise role of this enzyme in intracellular polyglutamate retention, or maintenance of a given chain length distribution, has been difficult to demonstrate directly. Attempts have been made to synthesize specific inhibitors of the enzyme to address this question but those prepared thus far have required relatively harsh measures, such as detergent treatment, for cellular uptake. Nevertheless, this approach, or some other means to modulate the activity of the enzyme in the cell, will be very helpful in assessment of its intracellular role in both folate and folate antagonist retention.

It has been proposed that MTX could be selectively toxic for tumor versus normal tissue as a result of differences in polyglutamylation efficiency. This proposal is based on reports that polyglutamylation of MTX is more avid in murine leukemia and Ehrlich ascities tumor cells than in intestinal epithelial cells [11,21]. Also, MTX was polyglutamylated better in a human leukemic cell line than in normal myeloid progenitor cells when exposed to [^{3}H] MTX in vitro [21].

Not only has MTX polyglutamylation been shown to enhance cytotoxicity in cultured cell lines, decreased polyglutamylation has been reported to be a mechanism of resistance to MTX. FPGS-deficient variants that are MTX resistant have been obtained by exposure to the drug in culture [26]. Likewise, cultures have been

derived directly from human tumors resistant to MTX that exhibited a low capacity to form polyglutamates.

3. *Target Enzyme Interaction*

DHFR is the primary target for most of the classical folate antagonists that have been investigated. There is striking similarity among different species in the amino acid sequence of the active site region of this single polypeptide protein. A large number of investigators using various techniques have contributed to elucidation of the DHFR active site and the binding of folate antagonists to this site [27]. Two of the most seminal are: (1) x-ray crystallography studies showing that MTX, which is a structural analog of the DHFR substrate dihydrofolate (H_2folate), binds in a different orientation in the active site than the folate, and (2) the 4-amino functional group of MTX, which appears to confer particularly avid binding, interacting directly with the carboxyl oxygen of an isoleucine in the active site. For a more complete discussion of this topic, the reader is referred to several excellent reviews [27–29].

MTX binds tightly to DHFR in a binary complex but binds even more tightly when the cofactor NADPH is present to form a ternary complex [27–29]. Estimates of dissociation constants in the picomolar range for the ternary complex have been reported. The dependence of binding on the secondary cofactor, and potential inhibition by the oxidized form, $NADP^+$, have lead to proposals that the ratio of NADPH/$NADP^+$ could play an important role in tumor sensitivity to MTX. However, it should be pointed out that the reactivity of the normal substrate H_2folate would also depend upon this ratio. Therefore, it is the balance between inhibitor and substrate sensitivity to the ratio that would govern activity.

It is generally thought that it is necessary to achieve an intracellular MTX concentration in excess of DHFR active sites to exert therapeutically relevant effects [2,11,27]. This concept has led investigators, both clinical and preclinical, to explore the use of relatively high-dose MTX regimens. In turn, this approach has led to therapies involving rescue by leucovorin following high, even potentially fatal, doses of MTX. This rescue therapy will be described in more detail later.

MTX resistance resulting from mutant DHFR with altered MTX-binding properties has been reported in model systems [2]. These mutant enzymes also catalyze the natural reaction poorly, consistent with impaired binding of the natural substrate, H_2folate. The clinical significance of this type of resistance has not yet been established, however, tumor samples from patients resistant to MTX have yielded enzymes with diminished ability to interact with MTX in vitro.

Elevated expression of DHFR is the most common MTX resistance mechanism [2,12]. This type of resistance can be stable (persistent after drug withdrawal) or unstable in cultured cell systems. In cancer patients, resistance due to elevated levels of the enzyme is typically stable. Elevated DHFR is associated with an increased number of gene copies. This "gene amplification" can occur in the

natural chromosome or in nonintegrated pieces of DNA called "double minute" chromosomes. Amplification in double minute chromosomes is less stable than amplification within the natural chromosome. It is probable that the double minute form typically precedes the more stable chromosomal amplification. A common approach to overcoming resistance to MTX resulting from overexpression of DHFR has been the design of drugs that target other enzymes. Two that are currently under investigation clinically are the TS inhibitor, ICI D1694, and the GARTF inhibitor, DDATHF (Figs. 1 and 2). Both are polyglutamylated efficiently within the cell and, in contrast to DHFR inhibitors, polyglutamylation leads to much improved binding to their target enzymes.

4. *Intracellular Consequences*

Direct assessment of intracellular folate pool changes in response to MTX and other folate antagonists is essential to understanding how they exert their cytotoxic effects [30–32]. Quantitative evaluation of individual folate pools originally relied on differential microbiological growth support assays [33]. This approach remains one of the most sensitive methods available but it is somewhat limited in the folate species that can be detected. A further limitation is that information regarding polyglutamate status of reduced folates is lost because their removal is necessary to permit uptake by the microbes. Additional methods have been developed more recently that are based on high-performance liquid chromatography (HPLC) separation of tissue folates [32,34]. These methods also generally require prior removal of polyglutamates to lower an otherwise unmanageable number of species. Folates separated by HPLC have been quantitated by spectroscopic, electrochemical, microbiological, and radioisotopic detection methods. In the latter case, labeling of intracellular folate pools with a radioactive folate source is necessary. While this detection method is very sensitive, difficulties can arise from failure to achieve uniform folate pool labeling. Further, animal and human investigation is limited when detection relies on radioactivity measurements because of the high quantity of isotope required and safety considerations.

Another method for reduced folate estimation has been developed based on entrapment of folates into an enzyme complex which is radioactively labeled by a secondary ligand. This method involves entrapment of 5,10-methylene-tetrahydrofolate (5,10-CH_2-H_4folate), and other folates after cycling them enzymatically to this form, into a very stable ternary complex with excess microbial TS and tritiated 5-fluoro-2′-deoxyuridine 5′-monophosphate ([^{3}H]FdUMP) [20,30,35–37]. Bound tritium from the labeled nucleotide derivative is proportional to original tissue folate. Hence, this approach does not require prior labeling of the folate pools. Further, since folates are trapped in their natural polyglutamylated state, the number of glutamate residues associated with each of the different natural folates can be evaluated. This is accomplished by electrophoretic or isoelectric focusing separation based on the charge associated with the free α-carboxyl groups of

glutamyl residues from the original folate polyglutamates [35,38]. Labeled complexes are typically evaluated fluorographically. While this method is highly sensitive and requires mild preparative conditions, it has the limitation that some enzymes are required for which there is currently not a commercial source.

As a result of DHFR inhibition, MTX can exert both antipyrimidine (thymidylate) and antipurine effects (Fig. 2). The antipyrimidine effect has typically been ascribed to depletion of 5,10-CH_2-H_4folate, a cosubstrate for TS (see Fig. 2B). Depletion of 5,10-CH_2-H_4folate is a consequence of depletion of the DHFR reaction product tetrahydrofolate (H_4folate) (see Fig. 2A). This mechanism was originally based on rational predictions of metabolic behavior, however, direct estimation of folate pools in culture systems exposed to MTX has made possible a better understanding of the quantitative aspects of the depletion mechanism. While Galivan and coworkers have observed nearly complete depletion of 5,10-CH_2-H_4folate in human hepatoma cells [39], other investigators have reported that significant levels remain under conditions where MTX was cytotoxic [40]. In a murine leukemia cell culture system in which all folate pools were measured, partial depletion of 5,10-CH_2-H_4folate occurred concomitantly with partial depletion of other folates except H_2folate [30]. This was thought to result from the fact that all folate pools are directly or indirectly linked enzymatically and, thus, respond in concert. However, all pools were not equally depleted. Certain pools, such as H_4folate and 5,10-CH_2-H_4folate, exhibited less depletion than others, suggesting that even though all pools are linked, each is individually regulated to provide maximal opportunity for cell survival. In effect, more critical reduced folate pools are protected at the expense of others.

The general depletion of reduced folate pools in favor of H_2folate following MTX exposure could also explain antipurine effects. 10-HCO-H_4folate is a substrate in two reactions involved in de novo purine biosynthesis (see Fig. 2C), and in cell culture systems depletion of this folate has been observed in response to MTX [30]. However, as with 5,10-CH_2-H_4folate, depletion is not necessarily complete [32]. Interestingly, an unusual formyl folate, 10-formyldihydrofolate (10-HCO-H_2folate), has been observed following MTX treatment in some culture systems [32]. This species is believed to result from formylation of elevated H_2folate. While 10-HCO-H_2folate has been demonstrated to inhibit GARTF as well as TS in isolated enzyme systems, the general role of this unusual folate in MTX cytoxicity remains somewhat unclear.

In addition to inhibition of DHFR, MTX as well as H_2folate that becomes elevated in response to MTX can inhibit TS and enzymes involved in de novo purine biosynthesis (see Fig. 2B,C). In contrast to DHFR, inhibition of these secondary targets is profoundly affected by the polyglutamylation status of both the folate and folate antagonist [21,32].

MTX and other folate antagonists are thought to be cytotoxic because they suppress DNA synthesis and/or repair as a result of both pyrimidine and purine

depletion [2,12,41]. The relative contribution varies from one cell line to another and with culture conditions, but generally thymidylate depletion appears to be the most important effect because it occurs at lower MTX concentrations than purine depletion. MTX concentrations in the 1- to 10-nM region can suppress thymidylate synthesis in culture systems, while 100 nM or greater levels are typically necessary to suppress purine synthesis.

Cell cycle kinetics is important to both the antipurine and antipyrimidine effects of MTX. The contribution of each is more prominent during S-phase [18,41]. DNA synthesis suppression resulting from metabolite depletion of precursors is thought to be the primary reason for S-phase specificity. However, a number of enzymes, including TS, also respond to cell-cycle kinetics, and these changes would also be expected to play a role in sensitivity to MTX. A secondary mechanism of MTX cytotoxicity can arise from elevation of deoxyuridine monophosphate (dUMP) in response to TS inhibition (see Fig. 2B). Elevation of this nucleotide can lead to unusually high levels of the trinucleotide with concomitant misincorporation of deoxyuridine into DNA. This abnormal base must be excised by uracil-DNA-glycosylase followed by a series of DNA repair steps involving hydrolysis of several nucleotides, resynthesis of the hydrolyzed region incorporating thymidine in place of uridine, and finally ligation to reconnect DNA strands [42]. An inadequate supply of thymidylate would be anticipated to inhibit this repair process, leading to further DNA damage.

Induction of a process known as "programmed cell death" or "apoptosis" has now been demonstrated to occur following treatment of cultured tumor cells with a number of cancer chemotherapeutic agents including folate antagonists [43]. This mode of cell death is thought to play an important role in the antitumor activity of these drugs. Apoptosis is distinct from necrosis with regard to several morphological and biochemical features. It is an energy-dependent process that requires protein synthesis and is characterized by intranucleosomal cleavage of DNA by an endonuclease. While there appears to be a general requirement for expression of the BCL2 gene to prevent induction of apoptosis, mechanistic elucidation of specific pathways is incomplete.

C. Preclinical Cytotoxicity Determinants

A number of factors affect the cytotoxic activity of folate antagonists, but perhaps the two most important are concentration and time of exposure [12]. Relatively low concentrations of MTX can be cytotoxic. In many culture systems, MTX exhibits an IC_{50} in the 1- to 10-nM range. But, depending on the origin of the cell line, higher concentrations can be required. Typically, the target is to obtain intracellular levels well in excess of DHFR sites to assure complete suppression of activity. With regard to exposure time, MTX is cell-cycle specific and at least one doubling of the cell population is generally required so that all cells are exposed to drug during

a period of sensitivity. More rapidly dividing tumor cells require shorter exposure times, and this can be exploited therapeutically to gain selective cytotoxicity versus slower growing normal tissue.

Because MTX exerts both antipyrimidine and antipurine effects, thymidine, adenosine, inosine, and hypoxanthine have an impact on cytotoxicity. Thymidylate synthesis is suppressed at lower MTX levels than is de novo purine synthesis, so thymidine tends to be more active in reversal of toxicity at low doses of MTX. However, thymidine alone cannot completely reverse toxicity at higher doses and a purine source is also needed [12]. This has led to the concept that in order to be therapeutically effective, MTX doses must be used that can overcome the relatively high levels of both thymidine and purines found in human plasma [44,45].

In animal models, use of thymidine and/or purines in combination with MTX has been investigated in an effort to reduce toxicity selectively in normal tissue and improve therapeutic effects. In tumor-bearing animals, the combination of thymidine with MTX yielded better therapeutic results than MTX alone [12]. However, direct extrapolation of these results to humans is difficult because of the different types of tumors, different metabolic rates, and particularly different levels of circulating thymidine in the animal models [46]. The basal level of circulating thymidine in mice is some 10 times greater than found in the human circulatory system. Nevertheless, clinical trials have shown that combinations of nucleosides with MTX can lessen human toxicity, although therapeutic improvement has not been demonstrated as yet.

Of particular importance to the cytotoxic action of folate antagonists is the presence of natural reduced folates. 5-Formyltetrahydrofolate (5-HCO-H_4folate or leucovorin) and other folates can reverse the cytotoxic effects of MTX in a competitive manner [2,11,12,47]. There are several possible reasons for the competitive nature of this cytotoxicity reversal. Since leucovorin and MTX utilize the same membrane transport system, competition can occur at the level of uptake [11]. Competition can also occur at the level of the intracellular enzyme FPGS, which in turn affects MTX retention as well as interaction with secondary target enzymes [48]. It should be pointed out that 5-HCO-H_4folate itself is a poor substrate for FPGS. However, it is metabolized intracellularly to other reduced folates that are much more active and, hence, are potentially more effective competitors of MTX polyglutamylation [47]. An additional and perhaps the most likely reason for the competitive behavior is elevation of intracellular reduced folate pools by leucovorin. Higher intracellular folate levels can cause displacement of MTX from DHFR and other enzymes, which in turn would facilitate escape [48].

D. Pharmacokinetics and Disposition

Accurate determination of plasma MTX levels is vital to evaluation of pharmacokinetic behavior and, hence, to the effective clinical use of the drug.

Several assays have been developed for this purpose. The most sensitive and accurate take advantage of the high specificity of MTX for the DHFR active site. Both inhibition of catalytic turnover and competitive protein binding of [^{3}H]MTX to DHFR have been used successfully [12,49]. HPLC separation prior to quantitation has usually been used when MTX metabolites were investigated [50]. Radioimmunoassays or fluorescence polarization immunoassays are typically used for routine clinical evaluation. These methods are based on use of antibodies prepared from MTX conjugated to bovine serum albumin [51]. Some cross-reactivity with metabolites of MTX, particularly 2,4-diamino-N^{10}-methylpteroic acid (DAMPA), have been reported with these antibody-based assays [12]. While not as accurate, these more rapid assays are necessary during therapy when plasma MTX must be monitored to assure that drug concentrations have fallen below toxic levels.

MTX has been administered both intravenously and orally. Oral doses are absorbed well from the gastrointestinal tract by a saturable active transport system, but bioavailability is somewhat unpredictable at high doses [52]. Metabolic breakdown of MTX to DAMPA occurs presystemically, probably catalyzed by enzymes from bacterial sources in the intestine. Once absorbed, the drug enters the portal circulation and passes through the liver where metabolism to 7-hydroxymethotrexate (7-OH-MTX) and polyglutamylation takes place. The latter metabolic fate tends to make the liver a storage site for MTX. While the metabolites, DAMPA and 7-OH-MTX, are inactive pharmacologically, they do cross-react in radioimmunoassays. The relatively high variability of plasma MTX estimates following oral administration has been ascribed to assay inconsistencies as well as differences in drug metabolism and disposition. Regardless of the reasons, the relative unpredictability of plasma levels following oral doses of MTX has led to more extensive use of intravenous administration.

In the circulatory system, MTX is bound substantially to serum albumin [12]. The impact of this binding on drug availability has not been thoroughly characterized. Generally, MTX exhibits a volume of distribution similar to that of total body water. MTX is absorbed extensively but relatively slowly into ascitic fluids, typically reaching steady-state levels after several hours. Retention in these fluids plays an important role in drug pharmacokinetics because efflux back into the circulatory system is very slow, contributing to the relatively long and variable half-lives that have been reported. Attempts have been made to take therapeutic advantage of this slow release from ascitic fluids by introducing MTX directly into the intraperitoneal cavity.

Plasma concentrations can reach peak levels of 1–10 μM when MTX is administered intravenously at conventional doses of 25–100 mg/m^2. However, high-dose regimens, involving infusion of gram quantities, can lead to millimolar levels [12]. Following intravenous administration, MTX is eliminated from plasma in

multiple phases. The initial phase (typically 12–24 hours) has a half-life of 2–3 hours, primarily due to renal excretion. The half-life during this phase has little dependence upon drug dose even in the "high-dose" range. Renal dysfunction can extend the initial phase half-life, as well as the terminal half-life, in a manner which is approximately proportional to the half-life of serum creatinine [53]. There is an age-dependent lengthening of MTX elimination half-life, which is poorly understood but leads to increased toxicity.

When MTX is administered at conventional doses it is primarily removed from plasma through urinary excretion during the first 12 hours. Clearance through the kidney is extremely efficient, in some cases reportedly exceeding creatinine clearance. At very high MTX doses, drug concentrations as high as 10 mM have been observed in urine. Because under such conditions renal failure can occur from drug precipitation, hydration and alkalization are typically used during these high-dose regimens [2].

The specific mechanism by which MTX is eliminated by the kidney has not been fully examined in humans. However, it is probably similar to the dog and the monkey, which have been studied extensively. MTX is actively secreted into the proximal renal tubule with reabsorption in the distal tubule. While a number of drugs inhibit MTX renal excretion, others, including antibiotics, enhance excretion, probably through competitive tubular reabsorption mechanisms. Interestingly, folate can inhibit MTX reabsorption, leading to the suggestion that a possible mode of rescue by the folic acid derivative leucovorin may be acceleration of MTX excretion.

E. Metabolism

Two metabolites of MTX have been observed in humans [12]: DAMPA, which arises presystemically, and 7-OH-MTX, which results from action of the enzyme aldehyde oxidase in the liver. 7-OH-MTX can account for nearly half of total drug present in urine during the latter part of the first day following administration and can represent an even greater proportion during the second day. DAMPA likewise becomes more prominent during later stages following MTX administration and reportedly accounts for up to one fourth of urinary material during the second day.

7-OH-MTX can compete with the parent drug for cellular transport, polyglutamylation, and enzyme binding. Galivan et al. have demonstrated that hepatoma cells have limited ability to hydroxylate MTX but can polyglutamylate the 7-hydroxy derivative fairly efficiently [54]. In contrast, normal rat hepatocytes can readily hydroxylate MTX but only polyglutamylate the metabolite to a limited extent. These observations have led to the suggestion that the relative ability to polyglutamylate and hydroxylate MTX could play a role in selectivity for normal versus tumor tissue.

F. Toxicity

The duration and intensity of MTX toxicity is dependent upon dose, schedule, route of administration, and individual pharmacokinetic variability [2,12]. Gastrointestinal mucositis and myeloid suppression are typically the main toxicities observed. The somewhat greater sensitivity of gastrointestinal epithelial tissue, compared to granulocyte and platelet precursors, is believed to be the result of greater accumulation and persistence of MTX in these tissues, possibly due to preferential polyglutamylation [55]. Both gastrointestinal mucositis and myeloid suppression are reversed within 2 weeks of discontinuation of the drug, provided renal function is normal.

Fatal toxicity caused by MTX is relatively rare and in almost all cases results from delayed MTX clearance from plasma [12,56]. Thus, routine monitoring of plasma MTX levels during therapy is critical. Because of differences in assay methods and differences in the dose and schedule of various regimens, the time and frequency of monitoring can vary. The target is to achieve plasma drug levels below 50 nM, which is considered to be nontoxic.

Leucovorin is commonly used to rescue from toxicity associated with MTX. Other rescue techniques including platelet transfusion, hydration, hemodialysis, and peritoneal dialysis have also been used on an experimental basis. Carboxylpeptidase G1, an enzyme that cleaves the glutamyl residue from MTX, has also been used to reverse toxicity experimentally. However, inactivation of the naturally occurring folates is a disadvantage of this approach. Activated charcoal and cholestyramine [57] have been used to reduce MTX toxicity by physical removal through binding to intestinal drug.

Use of thymidine to rescue from MTX toxicity has been reasonably successful, however, few antitumor responses have been obtained [12]. Because of the limited investigation of this rescue agent, the therapeutic value remains uncertain. Other agents that can interfere with cell cytokinetics and, hence, antagonize S-phase–specific MTX have also been used as rescue agents. An example is L-asparaginase, which antagonizes MTX toxicity by inhibiting protein synthesis and preventing cell cycle entry into S-phase. Following high doses of MTX, rescue from bone marrow toxicity and mucositis has been reasonably successful with this agent [58].

The nutritional status of cancer patients can have a profound effect on MTX-related toxicity [59]. Up to a twofold decrease in MTX clearance that results in a significant increase in toxicity can be attributed to nutritional status alone. In animals, increased toxicity of MTX has been demonstrated to result directly from a folate-deficient diet [60].

Chronic liver toxicity has been observed most often in patients receiving MTX therapy over a period of several years. Although not extensively studied, hepatotoxicity was reduced when weekly pulsed MTX therapy was used in place

of continuous daily treatment. Increased lipid deposition, which is possibly the result of impaired choline synthesis, could provide a biochemical rationale for liver toxicity [2].

Intrathecal injection of MTX for treatment of meningeal malignancy can lead to neurotoxicity, which is manifested by headache, rigidity, vomiting, and fever [2,12]. A second neurotoxic syndrome that can occur following prolonged MTX treatment of adult patients with meningeal leukemia is characterized by seizures and coma. The biochemical basis for MTX toxicity in the central nervous system is poorly understood.

III. MODULATION OF FLUOROURACIL BY LEUCOVORIN

Modulation can be described as the alteration of antitumor drug activity by a secondary therapeutic agent, typically a normal cellular metabolite. In a sense, rescue from high-dose MTX toxicity by leucovorin can be considered as a modulation therapy. However, modulation is usually considered in terms of potentiation of the cytotoxic effects of a primary agent. A classic example of this type of potentiation is enhancement of the antitumor activity of fluorouracil (FU) by leucovorin. Clinical trials with FU/leucovorin, such as those shown in Table 1, have led to use of this therapeutic approach not only in advanced colorectal cancer, but in the treatment of advanced breast and head and neck cancer as well [61].

A. Biochemical Rationale

There are several different ways in which FU can exert its cytotoxic action. Generally, it must first be activated by a series of metabolic steps giving rise to FUMP and FdUMP. These metabolites can be further metabolized to the triphosphate level and misincorporated into nucleic acids with concomitant destabilization [62]. Another mechanism of cytotoxic action of FdUMP, and one that is more important to leucovorin modulation, is potent inhibition of TS [63]. FdUMP binds to TS in an ordered process followed by binding of the normal substrate 5,10-CH_2-H_4folate. The resultant, very stable inhibitory ternary complex depletes intracellular thymidylate and ultimately suppresses DNA synthesis and/or repair [64]. The modulating effect of leucovorin is derived from its ability to elevate intracellular 5,10-CH_2-H_4folate, which in turn increases the stability of the inhibitory TS ternary complex [64,65].

B. Experimental Models

Early reports by Ullman et al. demonstrated that leucovorin in combination with fluorodeoxyuridine (FdUrd), an intermediate in the metabolism of FU to FdUMP,

Table 1 Selected Randomized Trials of Fluorouracil/Leucovorin in Colorectal Cancer

Fluorouracil	Leucovorin	No. of patients	Response (%)	Median survival (months)	Ref.
370 mg/m^2/day × 5 (IV bolus)	—	61	7	9.6	74
370 mg/m^2/day × 5 (IV bolus)	200 mg/m^2/day × 5 (IV bolus)	64	33	12.6	74
370 mg/m^2/day × 5 (IV bolus)	—	34	15	13.2	76
370 mg/m^2/day × 5 (IV bolus)	500 mg/m^2/day (IV 144 hr infusion)	29	45	14.6	76
600 mg/m^2/wk (IV bolus)	—	39	5	NR	73
600 mg/m^2/wk (IV bolus)	500 mg/m^2/wk (IV 2 hr infusion)	43	16	NR	73
375 mg/m^2/day × 3 (IV bolus)	100 mg hourly × 4 and then every 4 hr for 3 days (oral)	18	35	14.0	75
600 mg/m^2/wk × 6 (IV bolus)	125 mg/m^2 hourly × 4 for 6 weeks (oral)	31	45	9.5	72

NR, Not reported.

significantly enhanced murine leukemia cell cytotoxicity in culture [64]. Later, Berger and Hakala, as well as Houghton et al., reported that increased stabilization of the inhibitory ternary complex could be achieved by elevation of 5,10-CH_2-H_4folate [66,67]. Since that time, a number of tumor cell lines, including those of human origin, have been shown to have increased sensitivity to FU when leucovorin is present in growth media [61].

The polyglutamate status of 5,10-CH_2-H_4folate is an important determinant of the stability of the inhibitory TS ternary complex. Using TS obtained from human colon adenocarcinoma xenografts grown in nude mice, Radparvar et al. have demonstrated that longer-chain-length polyglutamates of 5,10-CH_2-H_4folate can stabilize the ternary complex as much as 200-fold better than the monoglutamate form [68]. Further, Romanini et al. have demonstrated in a cultured human T-lymphocyte cell line deficient in FPGS activity that shorter-chain-length polyglutamates are associated with diminished FU modulation [37].

Other folates, including 5-CH_3-H_4folate and folic acid, have also been used to modulate fluoropyrimidine activity in vitro [61]. In the case of folic acid, higher concentrations were required compared to leucovorin [64]. It should be pointed out, however, that this necessity for higher concentrations of folic acid may not be as important in vivo where systemic metabolism and activation can occur. Wright et al. have shown in mice that 5,10-CH_2-H_4folate levels can be elevated in tumors by folic acid as extensively as by leucovorin [69]. In addition, arrest of P388 leukemia cell growth in mice treated with FU and folic acid has been reported [70]. This latter response was only obtained if folic acid was administered 12–24 hours prior to FU. Simultaneous administration was ineffective. This schedule dependency suggests that the animal participates prominently in the metabolism of folic acid, making active metabolites available that cannot be achieved by tumor cells alone in culture.

The mouse has been the primary animal model system used to investigate modulation of FU activity by leucovorin. Tumors of both murine and human origin have been studied with various regimens of FU, FdUrd, and leucovorin [46,61]. In some cases tumor growth suppression was reported, however, in general it has been difficult to demonstrate the same efficacy in the mouse system as has been obtained in clinical trials with humans. This may be the result of much higher circulating levels of thymidine in mouse versus human plasma [46] and could also be influenced by dietary folic acid intake in enriched mouse diets [70].

C. Pharmacokinetics of Leucovorin

Only the [6S] isomer of [6R,S]-5-HCO-H_4folate is biologically active [61]. Because of concerns about potential interference at the level of uptake or metabolic activity by the inactive [6R] form, pharmacokinetic studies have addressed each of these

isomers in plasma following administration of the mixture. While the [6S] form is cleared rapidly ($t_{1/2}$, 30–45 min), primarily through metabolic conversion to 5-CH_3-H_4folate and other metabolites, the [6R] form is metabolically inert. An elimination half-life of 8 hours is almost entirely due to renal clearance. Oral administration of the mixture results in plasma accumulation of the [6R] isomer, but little elevation of the active [6S] isomer because it is metabolized very efficiently (>90%) presystemically [71]. The metabolic products that accumulate after oral doses of leucovorin are the same metabolites as observed after IV administration [20]. These metabolites include 5-CH_3-H_4folate, 5,10-CH_2-H_4folate, H_4folate, and 10-HCO-H_4folate. The predominant metabolite, 5-CH_3-H_4folate, is cleared somewhat more rapidly from plasma than renal clearance, suggesting some tissue absorption or metabolism. Other folate metabolites, 5,10-CH_2-H_4folate, H_4folate, and 10-HCO-H_4folate, are cleared from plasma with a half-life that is similar to that of the parent compound, [6S]-5-HCO-H_4folate. Because of their instability, these metabolites have not been studied in urine, however, it is likely that they are primarily eliminated by metabolism to 5-CH_3-H_4folate.

Because in culture systems relatively high levels of leucovorin (10 μM) are required to modulate FU activity [61], attempts have been made to achieve these same high levels in plasma during clinical trials. However, it remains questionable whether such levels of leucovorin itself are necessary. Culture system studies do not take into account systemic metabolic activation, and metabolites appear to be as active as the parent compound itself based on oral leucovorin clinical trial results [72]. It can be seen in Table 1 that when leucovorin was administered orally in two trials, responses were as great as following high IV doses. Yet it has been shown in several pharmacokinetic studies that oral administration of [6R,S]-5-HCO-H_4folate results in accumulation of metabolites but very little parent compound [20,33]. Hence, these metabolites, not [6S]-5-HCO-H_4folate, are apparently the agents giving rise to the very good responses obtained.

D. Clinical Applications

A number of different FU/leucovorin protocols have been investigated in clinical trials for colorectal, breast, and head and neck cancer [61]. Results from several of the colorectal clinical trials are shown in Table 1. Responses have varied but can be as high as 45%. Unfortunately, increases in median survival have been minimal. Both short-term and long-term infusion as well as bolus IV and oral administration using various doses and intervals between leucovorin and FU have been actively investigated [72–76]. These studies have led to acceptance of two basic treatment regimens. In the first, IV bolus leucovorin and short-term infusion of FU (15 min) are administered daily for 5 days. Leucovorin is typically administered at 200 mg/m^2 and FU at 370 mg/m^2 with adjustment of dosage, depending on

tolerance, during subsequent courses. Courses are repeated every 28 days. The interval between leucovorin and FU has varied from 0 to 2 hours. The leucovorin dose has varied between 60 and 500 mg/m^2 while FU doses have been as high as 770 mg/m^2 [77]. A second treatment regimen currently in use involves administration of high-dose leucovorin (500 mg/m^2) as a 2-hour infusion with escalating bolus FU doses given midway through infusion. This schedule is administered every week for 6 weeks followed by a 2-week rest. FU is typically administered initially at 600 mg/m^2, although higher doses have been used. Even higher doses of leucovorin have often been administered under this protocol, but improvement in response due to these extremely high doses remains unproven [78].

IV. SUMMARY

Intervention in folate metabolism has been a longstanding target for cancer chemotherapeutic agents. The DHFR inhibitor MTX was discovered more than 40 years ago and remains today one of the most widely used antitumor drugs. Other folate antagonists, particularly those with enzyme targets other than DHFR, continue to be considered in attempts to overcome resistance to MTX and improve therapeutic efficacy. In addition, folates themselves have been incorporated into therapies with other antitumor agents such as FU for treatment of solid tumors. Overall, investigation of the role of folates and folate antagonists in cancer chemotherapy has contributed not only to treatment of this important disease, but also to understanding the underlying biochemical basis for regulation of an important family of metabolites, the reduced folates.

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Folate Antagonists in Nonneoplastic Disease: Proposed Mechanisms of Efficacy and Toxicity

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I. THE USE OF ANTIFOLATES IN THE TREATMENT OF NONNEOPLASTIC DISEASES

A. History

Compounds that interfere with folic acid (FA) metabolism now play important roles in the therapy of nonneoplastic diseases (Fig. 1). One of the earliest uses of an antifolate in nonmalignant disease was by Gubner in 1951, who administered aminopterin (4-aminopteroylglutamic acid) to patients with rheumatoid arthritis (RA), rheumatic fever, and psoriatic arthritis [1,2]. In these studies there was remission of articular and periarticular inflammation in seven of the eight patients treated. Toxicities such as alopecia, mouth ulcers, sore throat, abdominal cramps, diarrhea, and pancytopenia were noted. One patient with extensive psoriasis had a remission of skin lesions lasting for several weeks. Following these observations, aminopterin became an established treatment for psoriasis [3]. However, methotrexate (MTX), which was introduced soon after aminopterin, became recognized as a preferable and less toxic therapeutic agent [4–6]. MTX was approved in the early 1970s by the U.S. Food and Drug Administration (FDA) for use in the treatment of psoriasis. Sulfasalazine (SASP), also an antifolate, was synthesized in the late 1930s in order to provide an agent with combined sulfonamide and salicylate effects for possible use in the treatment of RA. It is of interest that the use of SASP for arthritis antedates clinical trials of aminopterin for this purpose [7]. Although SASP and MTX are now known to interfere with several enzymes involved in folic acid metabolism, SASP was synthesized well before the chemical structure of folic acid was known and before purposeful synthesis of folic

acid analogs became possible. Thus, the antifolate effects of SASP were not only fully appreciated after aminopterin and MTX had been evaluated clinically.

This chapter will focus on the use of antifolates in RA and, to a lesser extent, in psoriasis and inflammatory bowel disease. RA is the most common of the above diseases, with a prevalence of 1–2% in the United States population [8]. RA affects individuals of all ages and races, with females affected approximately 2.5 times as often as males [8].

The use of specific antifolates in the treatment of nonneoplastic disease will be reviewed and toxicities seen in clinical practice discussed. A mechanism involving compromised folate nutriture is proposed to explain the toxicity of antifolates. The administration of leucovorin or FA will be discussed as possible methods to lessen antifolate toxicity. Finally, several mechanisms that may account for the efficacy of antifolates in autoimmune diseases will be reviewed, again focusing on RA and animal models of this disease.

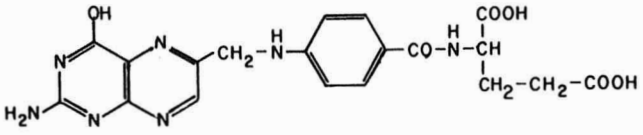
B. Specific Antifolates in the Treatment of Rheumatoid Arthritis and Other Nonneoplastic Diseases

1. *Methotrexate*

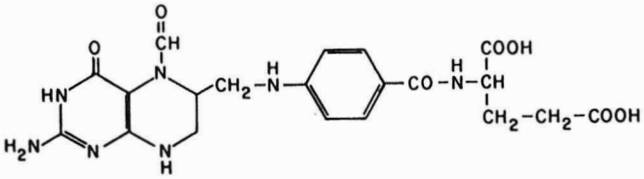
Since the 1970s antifolates have found increasing usefulness in the treatment of nonneoplastic diseases. MTX, a chemotherapeutic agent and antimetabolite of FA, is currently used in low doses (2.5–20 mg/week) for the treatment of a wide variety of nonneoplastic diseases such as RA, psoriasis, psoriatic arthritis, polymyositis, dermatomyositis, systemic lupus erythematosus, Reiter's syndrome, Wegener's granulomatosis, Behcet's syndrome, sarcoidosis, asthma, primary biliary cirrhosis, and inflammatory bowel disease [9–22].

MTX has been especially effective in the treatment of RA. A survey at the University of Alabama at Birmingham shows that 57% of patients with RA are currently being treated with MTX. This figure is likely typical of the use of MTX at other tertiary care institutions. Numerous trials since 1984 have established the short (23–26) and long-term (27–33) effectiveness of this agent as a disease-modifying antirheumatic drug (DMARD) for therapy of RA. Six of these studies are profiled below.

The Cooperative Systematic Studies of Rheumatic Diseases Programs [23] studied 189 patients with RA who were entered into a prospective, controlled, double-blind, multicenter trial. One hundred and ten patients remained on the trial for 18 weeks, receiving 7.5–15 mg of MTX orally per week. Patients receiving MTX had greater clinical improvement than patients receiving placebo as judged by the following criteria: duration of morning stiffness, grip strength in both hands, 50-foot walking time, pain analog scale, patient assessment of disease, and physician assessment of disease by standard indices. Approximately one third of the patients receiving MTX were withdrawn from the study for adverse drug reactions.

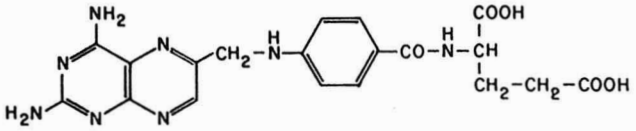


FOLIC ACID

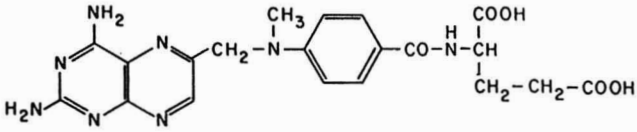


5-FORMYLTETRAHYDROFOLIC ACID

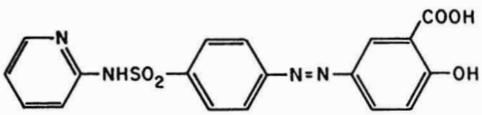
(Citrovorum Factor, leucovorin, folinic acid)



AMINOPTERIN



METHOTREXATE



SULFASALAZINE

Figure 1 Structures of folates and drugs with antifolate activity.

Weinblatt et al. [24] evaluated 28 patients with RA in a 24-week, double-blind crossover trial, which compared MTX in pulsed doses of 7.5–15 mg weekly with placebo. Significantly greater improvement was noted with MTX treatment compared to placebo, as judged by the number of swollen joints, number of tender/painful joints, joint-swelling index, joint pain/tenderness index, 50-foot walking time, duration of morning stiffness, and patient and physician assessment of disease activity. Most adverse effects of therapy occurred with equal frequency in the 7.5- and 15-mg MTX dose groups; however, nausea was more frequent during high-dose therapy. During MTX administration, 52% of the patients reported adverse manifestations while 15% of the patients had adverse manifestations during placebo administration. Immunological studies showed that there were no differences between MTX and placebo in mean rheumatoid factor titers, the levels of circulating immune complexes, and percentage of blood mononuclear cells expressing phenotypic and activation markers. Unexpectedly, the proliferative response of lymphocytes to mitogens was the same in the placebo and MTX-treated groups.

Andersen et al. [25] studied 12 patients treated with weekly pulsed doses of intramuscular MTX (5–15 mg/week), in a double-blind, placebo-controlled, crossover study. After 13 weeks of therapy, the patients receiving MTX had significant improvement in joint counts for swelling and pain/tenderness, joint scores for swelling and pain/tenderness, duration of morning stiffness, and patient and physician assessment of disease when compared to those receiving placebo. Adverse reactions during MTX treatment included pancytopenia, stomatitis, pruritic skin rash, and proteinuria (one patient each). Rheumatoid factor titers were not statistically different between treatment groups; however, there was a statistically significant decrease in IgG, IgM, and IgA during MTX treatment. There were no differences in absolute lymphocyte count, mononuclear cell subsets, or proliferative responses to phytohemagglutinin and pokeweed mitogen between treatment groups.

Weinblatt et al. published an 84-month update of 26 patients enrolled in an open prospective trial of MTX therapy [26]. A 50% improvement in joint pain/tenderness and swelling indices was noted in 12 of 26 of the patients still enrolled in the trial. At 84 months, 11.5% discontinued MTX because of toxicity. They concluded that MTX is still an effective therapy after 84 months of treatment.

Kremer et al. published a 90-month update of a prospective, open observational study [27]. Sixty-two percent of patients had remained on MTX therapy. The major reason for drug discontinuation was MTX toxicity rather than lack of efficacy. Significant improvements from baseline occurred for all clinical parameters except the number of tender joints.

Pincus et al. recently surveyed the probability of continuation of second-line drugs, including MTX, in 532 patients seen in seven private rheumatology practices [28]. MTX and prednisone were continued by greater than half the patients

for more than 60 months. For all MTX courses, 4% of patients discontinued the drug for lack of efficacy and 19% discontinued MTX because of toxicity. The probability of continuation of MTX was 75% at 2 years and 55% at 5 years.

It is now evident that low-dose MTX treatment is a valuable therapy for RA. A metaanalysis by Felson et al. of trials published through 1990 concluded that antimatarial drugs and MTX are the preferred drugs for RA therapy [34]. Compared to other disease-modifying antirheumatic drugs, MTX remains the drug with the best drug survival at 10 years [35].

2. *Sulfasalazine*

SASP is used extensively in the treatment of inflammatory bowel disease and to a lesser extent in the therapy of RA. Svartz et al. in 1942 reported that SASP was useful in both rheumatic polyarthritis and ulcerative colitis [36]. Several controlled trials have documented the efficacy of SASP compared to placebo and its ability to maintain remissions of ulcerative colitis [37–39]. A dose-response effect has been noted for a dose of 1 versus 2 g versus 4 g daily, however, side effects were more prevalent with higher doses [40].

Sulfasalazine proved effective in clinical trials for arthritis during the early 1940s, but interest waned until McConkey et al. [41] conducted an uncontrolled trial in 74 patients with RA treated with SASP. Thirty-eight patients (51%) showed improvement and were able to remain on the drug for at least one year. The most common adverse effect noted was dyspepsia; megaloblastic anemia and neutropenia were also reported. In a similar noncontrolled trial, Bird et al. [42] followed patients on a dose of up to 3.0 g of SASP per day for 6 months. The levels of acute-phase reactants (i.e., C-reactive protein and haptoglobin) in serum decreased in these patients and there was improvement in clinical variables such as grip strength and pain score.

More recently, controlled trials of SASP have been conducted. Pullar et al. [43] compared SASP to placebo and intramuscular gold sodium thiomalate in patients with RA. After 6 months, patients on SASP and gold had similar improvements in laboratory and clinical measures of RA. The placebo group had no significant change in rheumatoid factor levels, pain score, grip strength, and index of disease activity. The major toxic manifestations were nausea and vomiting. An additional study by Pullar et al. [44] showed that there was a positive dose-response relationship of SASP therapy and clinical improvement, with a dose of >40 mg/kg/day showing greatest benefit.

Pinals et al. [45] used 3 g of SASP per day, in a 15-week, randomized, parallel, double-blind trial. Compared to placebo, the SASP-treated group has significant improvement in duration of morning stiffness, grip strength in both hands, pain on an analog scale, joint pain/tenderness count and score, and joint swelling count and score. Twenty-eight percent of the SASP patients were withdrawn from

the study because of adverse drug reactions including gastrointestinal intolerance, rash or pruritus, elevated liver enzymes, and anemia. Only 2% of the patients in the placebo group were withdrawn for adverse drug reactions. The authors concluded that SASP is an effective drug for the treatment of RA.

The Cooperative Systematic Studies of Rheumatic Diseases Programs [46] compared SASP (2 g/day), gold sodium thiomalate, (50 mg/week), and placebo in 186 patients with RA in a double-blind randomized study for 37 weeks. The only significant differences between the SASP, injectable gold, and placebo groups were a decreased sedimentation rate and an improvement in grip strength in the right hand in the SASP and gold-treated groups. The excellent response to placebo was unexpected in this trial since injectable gold has previously been found to be superior to placebo therapy. Sixteen percent of patients were withdrawn from SASP therapy because of skin rash and gastrointestinal intolerance.

SASP has recently been studied in early nonerosive RA by the Australian Multicentre Clinical trial group [47]. One hundred and five patients were randomized to receive placebo or SASP. The patients receiving SASP were significantly better than the placebo group, with fewer tender and swollen joints after 6 months of therapy. There was a nonstatistically significant trend to fewer erosions on radiographs in the SASP treatment group. The group concluded that SASP is a useful treatment modality in early RA. Fourteen SASP-treated patients versus only four placebo-treated patients were withdrawn due to side effects.

3. *Other Antifolates*

Many other drugs with antifolate activity are useful in the treatment of nonneoplastic disease. The following diseases have been treated with drugs having antifolate activity: malaria with pyrimethamine [48,49], bacterial infections with trimethoprim [48,50], hypertension with triamterene [48,51], epilepsy with phenytoin [48,52], and *Pneumocystis carinii* infections with trimetrexate [48,53]. A number of nonsteroidal antiinflammatory agents (e.g., aspirin, salicylic acid, ibuprofen, naproxen, mefanamic acid, sulindac, and indomethacin) have been shown to inhibit folate-dependent enzymes [54]. An intriguing finding is that the analgesic and antipyretic drugs, acetaminophen (e.g., Tylenol) and antipyrene, possess little or no antifolate activity, suggesting that antiinflammatory activity may be mediated by interference of folate metabolism and that analgesic and antipyretic activities result from interference of the metabolism of other nutrients (e.g., ecosanoids) [54].

II. THE TOXICITY OF ANTIFOLATES IN THE TREATMENT OF NONNEOPLASTIC DISEASES

A. Methotrexate

Many rheumatologists have concluded that toxicity is the major factor limiting prolonged MTX therapy [34,55]. Toxic manifestations such as nausea, stomatitis,

elevated liver function tests, cytopenias, and pulmonary toxicity have generally been reported in 30–60% of patients using the drug [31,34,35,55]. A 2-year retrospective study documented that 93% of patients experienced an adverse drug reaction during treatment [32]. A trend toward more toxicity was seen comparing doses of 10 to 5 mg/m^2 in a recent double-blind trial, while 33% of patients receiving 20 mg/m^2 in this trial required dose reduction or MTX discontinuation [56]. These results indicate that there is a dose-response relationship for toxicity [56].

Hepatotoxicity and pulmonary toxicity are two of the most clinically important toxic manifestations. The determinants of serious liver disease in patients with RA taking MTX have recently been evaluated [57]. A 5-year cumulative incidence of cirrhosis is estimated to be 1 in 1000 treated patients. Late age at the initiation of MTX therapy and duration of therapy are independent risk factors for serious liver disease [57]. The authors indicated that liver disease is an uncommon complication of MTX therapy for RA. Thus, given the low incidence of hepatotoxicity, obtaining routine liver biopsies during MTX therapy for RA remains controversial practice [58]. Acute pneumonitis, from MTX therapy, manifests as an interstitial and alveolar infiltrate with hypoxia [59]. The etiology of the pneumonitis is not entirely clear, however, discontinuation of MTX and high-dose corticosteroids generally cause resolution of symptoms. Further studies are warranted to determine risk factors for MTX-induced pneumonitis.

B. Sulfasalazine

Das et al. estimated that the overall incidence of adverse side effects of SASP therapy to be quite variable [60]. The major adverse effects of SASP include nausea and vomiting, anemia, leukopenia, agranulocytosis, skin rash, hepatotoxicity, male infertility, and lung disease [61]. Khan et al. found that 38% of patients on 4 g/day of SASP experienced toxic manifestations including nausea, malaise, headache, myalgias, diarrhea, constipation, anal soreness, anal mucous discharge, flatulence, dysuria, anorexia, indigestion, insomnia, and dizziness [39]. A dose relationship is suggested since the incidence of toxicity on 2 g/day of SASP was reported to be low.

C. Antifolate Combinations

The toxicity of antifolates used in combination has generally been found to be at least additive [62,67]. There have been several reports of toxicity in patients receiving both MTX and trimethoprim-sulfamethoxazole. Pancytopenias, thrombocytopenia, rash, and stomatitis have been documented in patients receiving this combination for both psoriasis and RA [62–66]. A recent case report also documented nausea, vomiting, anemia, and lowered plasma and RBC folate status in a patient with RA taking combination MTX and SASP therapy [67].

III. THE MECHANISM OF TOXICITY OF ANTIFOLATES IN NONNEOPLASTIC DISEASE AND CONTROL OF TOXICITY

A. Inhibition of Folate-Dependent Enzymes as a Mechanism for Toxicity

1. *Methotrexate as a Prototype*

MTX (unmodified) is a powerful inhibitor of dihydrofolate reductase (DHFR) (Fig. 2, step I) [63]. This enzyme is necessary to reduce dietary FA and dihydrofolic acid (DHF) to tetrahydrofolic acid (THF), thus completing biochemical transformations required to convert the vitamin into the coenzyme form. The coenzyme form can now participate in one-carbon transfer reactions. DHFR also functions in the reduction of dihydro-folyl-polyglutamates ($DHFG_n$), produced during the biosynthesis of thymidylate in actively dividing cells (step I). It is clear, therefore, that inhibition of DHFR will interfere with both utilization of dietary folates and the recycling of intracellular $DHFG_n$s [68,69]. The accumulation of $DHFG_n$s in MTX-treated cells has other metabolic consequences. $DHFG_n$s are also inhibitors of 5,10-methylene tetrahydrofolate dehydrogenase (step V), TS (step IV), and AICAR T'ase (step VI) [70–72]. Thus, the decrease in the pool size of $THFG_n$ in concert with increases in the pool of $DHFG_n$s depletes coenzymes while producing inhibitors. Protracted MTX administration also depletes the total folate pool, an effect believed to be due to irreversible destruction of $DHFG_n$ [73]. Like folates, MTX itself is a substrate for folyl-polyglutamate synthetase (step II) and undergoes conversion to polyglutamyl derivates ($MTXG_n$). $MTXG_n$ were first identified at our institution by Baugh, Krumdieck, and Nair [74]. The additional polyglutamyl moieties markedly modify the properties of MTX. $MTXG_n$ are more effectively retained inside cells and acquire inhibitory activity over enzymes in addition to DHFR such as enzymes of purine biosynthesis (i.e., AICAR and GAR T'ase, steps VI and VII) and TS [70]. $MTXG_n$ may also inhibit 5,10-methylene tetrahydrofolate dehydrogenase (step V) [72]. In conclusion, marked and very complex changes in intracellular folate composition and pool size and in the activities of enzymes of one carbon metabolism are the hallmark of MTX administration.

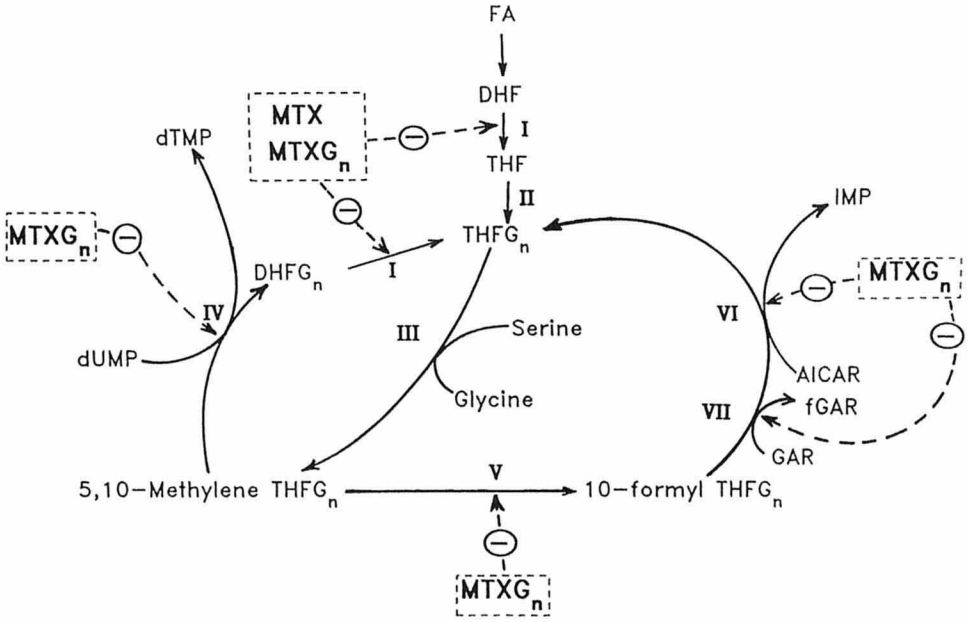
2. *Sulfasalazine*

Sulfasalazine also interferes with folate metabolism at several different anatomical and biochemical sites. SASP inhibits intestinal absorption of folate in human subjects and laboratory animals and inhibits the cleavage of folylpolyglutamates by conjugase [75–78]. Sulfasalazine has also been shown to inhibit a number of folate-dependent enzymes in cell-free preparations and in cultured lymphocytes [54,76,79,80].

B. Compromised Folate Nutriture and Depletion During Antifolate Therapy

1. *Inflammatory Bowel Disease*

In many studies, the folate status of patients taking antifolates for nonneoplastic diseases has been shown to be depressed. A depressed folate status has been seen



FA = Folic acid
DHF, $DHFG_n$ = Dihydrofolic acid and its polyglutamates, respectively
THF, $THFG_n$ = Tetrahydrofolic acid and its polyglutamates, respectively
MTX, $MTXG_n$ = Methotrexate and its polyglutamates, respectively
dUMP = deoxyuridylate
dTMP = deoxythymidylate
GAR = Glycinamide ribotide
fGAR = N-formyl-Glycinamide ribotide
AICAR = Aminoimidazolecarboxamide ribotide
IMP = inosine monophosphate

ENZYMES

I. Dihydrofolate reductase (EC. 1.5.1.3)
II. Folylpolyglutamate synthetase
III. Serine hydroxymethyltransferase (EC. 2.1.2.1)
IV. Thymidylate synthetase (EC. 2.1.1.6.)
V. 10-formyl tetrahydrofolate synthetase; 5,10 methenyltetrahydrofolate cyclohydrolase; 5,10 methylene tetrahydrofolate dehydrogenase (E.C. 6.3.4.3, EC. 3.5.4.9, E.C. 1.5.1.5, respectively)
VI. Aminoimidazole carboxamide ribotide transformylase (E.C. 2.1.2.3)- AICAR T'ase
VII. Glycinamide ribotide transformylase (EC 2.1.2.2)- GAR T'ase

------O----------> Indicates sites of inhibition

Figure 2 Folate-dependent pathways and methotrexate inhibited steps.

in patients taking SASP for inflammatory bowel disease. Pounder et al. [81] evaluated red blood cell morphology in 50 patients with ulcerative colitis receiving SASP, 50 normal controls, and 10 patients with UC not receiving SASP. Twenty-two percent of SASP-treated patients had elevated mean corpuscular volume (MCV), a classic indicator of folate deficiency, and 4% of SASP-treated patients had frank folate deficiency (as indicated by serum folate < 2.5 ng/ml and an elevated MCV). Impaired folate nutriture was not observed in controls. Swinson et al. [82] evaluated folate status and folate absorption in patients with inflammatory bowel disease taking SASP. Twenty-two percent of the patients taking SASP had macrocytic red cells, while macrocytosis was not observed in the control group not taking SASP. Forty-two percent of the patients in the SASP-treated group had an increased reticulocyte count compared to 7% in the untreated group. Macrocytosis, however, may be a poor indicator of impaired folate status in SASP-treated patients since hemolysis and reticulocytosis frequently occur. In addition, folate absorption was generally impaired in patients with ulcerative colitis and was further impaired by SASP administration. The authors concluded that folate deficiency can be a significant problem in patients taking SASP, especially if dietary folate intake is inadequate.

Longstreth and Green [83] studied patients with chronic colitis taking SASP and compared these to patients with colitis not given SASP. In both groups, disease symptomatology was mild and diets were not restricted. The groups did not differ from each other in hemoglobin, MCV, or final folate levels. However, only in the SASP-treated groups was there a correlation between drug dose and MCV. The authors concluded that folate depletion may not be inevitable in patients taking SASP; however, subclinical depletion of folates can occur with doses of SASP greater than 2 g/day.

Halsted et al. [77] examined patients with ulcerative colitis by performing jejunal perfusion studies and determining folate status. Seven patients were taking SASP, and 10 patients were not treated with SASP. Folic acid absorption was significantly lower in the SASP-treated patients. Also, the serum folate levels were found to be low in four of seven patients taking SASP. The authors concluded that there was a clear relationship between SASP use and low serum folate levels. In addition, the jejunal hydrolysis of polyglutamyl folates by conjugase and disappearance of polyglutamyl and monoglutamyl folates from the gut lumen was inhibited more than 50% by concentrations of SASP of more than 1 mM. The authors conclude that folate malabsorption and depletion of folate stores are therefore possible consequences of SASP administration.

2. *Psoriasis*

The folate status of 20 patients on long-term MTX therapy for psoriasis and of 17 patients not receiving MTX was studied [84]. Demographics, including disease duration, were similar in both groups. Red blood cell (RBC) folate levels were

significantly lower in the MTX-treated patients. The plasma levels of folate, as determined by an isotope-binding assay, approximately doubled 24 hours after a MTX dose in both groups. This finding is suggestive of leakage of folate from an intracellular site (e.g., liver) or displacement by MTX. In support of this conclusion, Johns and Plenderleith observed that MTX could increase urinary excretion of a dose of tritiated folic acid administered 24 hours previously [85]. Therefore, there may be a potential for intracellular folate depletion during long-term MTX therapy for psoriasis.

3. *Rheumatoid Arthritis*

The anemia found in RA patients receiving SASP has been investigated by Grindulis and McConkey [86]. They studied RA patients receiving SASP (2 g/day or more) or penicillamine (500 mg/day). The MCV increased significantly only in patients taking SASP, while serum and RBC folate levels did not change significantly in either group. The authors concluded that the elevated MCV is related to hemolysis, but a dose of SASP higher than 2 g/day may precipitate folate deficiency.

The folate status of RA patients taking low-dose MTX has been examined in our laboratories [72]. The activity of a folate-dependent metabolic pathway in peripheral blood mononuclear cells was determined as a measure of folate status in control subjects with no serious medical illness, patients with RA not taking MTX, and patients taking low-dose MTX for RA. This assessment of folate status, termed the C_1 index, is a measurement of the rate of radiolabeled serine formation from [^{14}C] formate and excess glycine. In control subjects, the C_1 index was found to be positively correlated with folate levels in PBMCs as measured by a *Lactobacillus casei* assay. The mean activity of the C_1 index in the MTX-treated RA group was approximately 50% of that of the non MTX-treated RA group and a group of normal controls. There was no difference in dietary intake of folate between the three groups, suggesting that MTX was responsible for the decreased activity. This is one of the first reports demonstrating that low-dose MTX compromised a functional assay of folate nutriture.

A retrospective analysis of 23 patients with RA taking MTX by Weinblatt and Fraser demonstrated that there is an association between increasing mean corpuscular volume (MCV), folate deficiency, and hematological toxicity [87]. In this study, six patients who experience hematologic toxicity all had low serum or RBC folates, and most had elevated MCVs. This report also indicates that folate status worsens during MTX therapy.

A trial in our clinics using FA supplementation (1 mg/day) during low-dose MTX therapy for RA showed that the C_1 index decreased during 6 months of MTX therapy [88]. There was a larger drop in the C_1 index in the placebo-supplemented group than the FA-supplemented group, suggesting that FA supplements delay or prevent folate deficiency in these patients.

Hine et al. also documented an impaired folate status during MTX therapy [89]. Serum samples of patients from the Cooperating Clinics trial [23] were assayed by MTX-resistant *L. casei*. A greater decrease in serum folate levels was noted in patients receiving 48 weeks of MTX therapy versus 18 weeks of therapy. In contrast, there was no difference in serum FA level before and after treatment with either oral or parenteral gold, D-penicillamine, or SASP.

C. Administration of Leucovorin During Low-Dose MTX Therapy: Attempts to Prevent Toxicity

The relationship between folate depletion and MTX toxicity was first demonstrated by Hellman et al. [90], who found that lower initial RBC folate levels were correlated with higher toxicity in patients being treated for tumors of the head and neck. Therefore it seemed reasonable to find measures to prevent decreases in folate status during therapy, and the high-dose MTX-leucovorin rescue protocol offers a rationale for the use of leucovorin to prevent or lessen MTX toxicity. Leucovorin, which bypasses the inhibition of dihydrofolate reductase (DHFR) (step I, Fig. 2), is the usual antidote of MTX when used in cancer chemotherapeutic doses. There is little doubt that high-dose MTX with leucovorin rescue reverses much of the toxicity of potentially lethal doses of MTX.

Attempts to dissociate toxicity from efficacy during low-dose MTX therapy by the simultaneous administration of leucovorin (5-formyl tetrahydrofolic acid, folinic acid, citrovorum factor) have produced varied results. Ive and DeSaram administered 3 mg of leucovorin for every 5 mg of MTX given for psoriasis and observed a reduction of the frequency of mouth ulcers, gastrointestinal intolerance (nausea, vomiting), and macrocytosis [91]. Roengik [92] used leucovorin, 4–8 mg intramuscularly (IM), 2 hours after IM MTX in psoriasis patients with mouth ulcers, and observed complete resolution of stomatitis and no diminution of therapeutic effect.

On the other hand, the effects of leucovorin administration to MTX-treated RA patients have not been as encouraging. Tisher et al. [93] used up to 45 mg/week of leucovorin with a median MTX dose of 15 mg/week. Nausea caused by MTX disappeared, but disease activity judged by subjective clinical assessment, Ritchie articular index, grip strength, and erythrocytic sedimentation rate showed deterioration. This ratio of leucovorin to MTX clearly reduced the efficacy of the drug. This effect may have an analogy in neoplastic disease where expansion of folate pools with leucovorin decreased tumor sensitivity to MTX because leucovorin interfered with the ability of cells to metabolize MTX to polyglutamates [94]. In a placebo-controlled study, Hanrahan and Russell [95] used 20 mg of leucovorin weekly in patients receiving weekly IM MTX for RA who were about to discontinue the drug because of side effects. They reported no significant differences in

efficacy as measured by tenderness and swelling indices and pain score. However, scrutiny of their data shows that there is a trend towards reduction of efficacy in the leucovorin-supplemented group. Buckley et al. [96] in a placebo-controlled trial gave 1 mg of leucovorin per mg of MTX, with no loss of efficacy and a trend toward less stomatitis and gastrointestinal upset. Shiroky et al. and the Canadian Leucovorin Study Group have recently presented the results of a trial using 2.5–5.0 mg of leucovorin weekly, administered 24 hours after MTX dose [97]. During 52 weeks of therapy, there was no difference in efficacy between leucovorin- or placebo-treated groups, and there was a significant decrease in side effects in the leucovorin-treated group. One patient treated with leucovorin failed to improve by at least 50% in one of the assessed variables despite a dose of 30 mg of MTX weekly and was withdrawn for lack of efficacy. Weinblatt et al. have recently shown that 1 mg of leucovorin administered concurrently with MTX (maximum MTX dose: 20 mg/week) did not interfere with the efficacy of MTX in an 8-week trial [98].

D. Concurrent Administration of Folic Acid During Antifolate Therapy

There are now several studies that indicate that oral FA (i.e., pteroylglutamic acid), when given with MTX, can reduce the toxicity without altering the efficacy in RA therapy. Wilke and colleagues at the Cleveland Clinic gave 6–9 mg FA orally, 5 days after the dose of MTX and reported limited toxicity from MTX [99,100]. A placebo-controlled, double-blind trial indicated that FA (1 mg/day) lessened toxic side effects without reducing efficacy during 6 months of low-dose MTX therapy for RA [88]. A recent long-term analysis of patients taking MTX for RA indicates that there is a subset of patients able to continue MTX despite developing significant toxicity if FA is added to the treatment regimen to ameliorate side effects of the drug [101]. However, the use of FA supplementation remains controversial. Stenger et al. concluded that a reduction in the dose of MTX may be more appropriate than oral folate supplementation [102].

E. A Possible Mechanism for Diminution of Antifolate Toxicity by the Simultaneous Administration of Folates

The mechanism by which FA reduces MTX toxicity is uncertain. However,it seems reasonable to postulate that supplements of FA may relieve the toxicity of MTX by replenishing or preventing the depletion of the cellular folate pools in tissues at risk. Since it is very likely that DHFR is only partially inhibited at low doses of MTX, enough of the administered FA supplement should become reduced to the tetrahydrofolate coenzyme to prevent the reduction of cellular folates to dangerously low levels. This would prevent the synergistic effects of MTX therapy during a superimposed state of folate deficiency.

It should be emphasized that the amount of FA incorporated into the biologically active pool of tetrahydrofolates will always be less than the amount administered because of the presence of a partially inhibited DHFR. This limitation is removed when leucovorin, a tetrahydrofolate that bypasses the inhibition of DHFR, is administered. All of the administered leucovorin supplement would then replete the cellular tetrahydrofolate pools. It can be anticipated, therefore, that low doses of leucovorin would protect against toxicity as effectively as FA. This has been shown by the work of Buckley et al. [96] and Weinblatt et al. [98]. At higher doses of leucovorin, the therapeutic effectiveness of MTX would also be eliminated, explaining why the joint symptoms actually worsened in the trial of Tishler et al. [93]. Leucovorin and FA are not metabolically equivalent substances in the presence of MTX. In the presence of MTX, relatively large doses of FA would not be completely reduced to tetrahydrofolates and would not be expected to behave like leucovorin and alter the therapeutic response. It is our contention, therefore, that the mechanism by which FA supplementation lessens toxic manifestations is by repleting somatic tissues (such as the hepatocyte and the enterocyte), whose folate nutriture is compromised by the antimetabolite activities of MTX. The immunologically active tissues, such as the WBC, are more resistant to folate repletion by FA in the presence of MTX.

Long-term, low-dose MTX administration not only inhibits a number of folate-requiring enzymes, but also produces a well-documented depletion of cellular folates [103,104]. We postulated that these two mechanisms could have an additive (or synergistic) adverse effect on folate status, leading to the often observed cytopenias, stomatitis, and gastrointestinal intolerance in MTX-treated RA patients. We further postulated that the depletion of cellular folates could be corrected by the administration of FA, without substantially affecting the MTX inhibition of folate-requiring enzymes necessary for therapeutic efficacy. Based on this rationale, a randomized, double-blind, placebo-controlled trial was set up to evaluate whether FA administration would significantly lessen toxic manifestations during MTX administration.

1. *A Clinical Trial Evaluating Placebo and Folic Acid Supplementation (1 mg/day)*

Thirty-two patients with rheumatoid arthritis completed a 24-week placebo-controlled, double-blind trial of FA (1 mg/day) supplementation during low-dose MTX therapy [88]. Patients were seen initially and after 12 and 24 weeks of MTX therapy and were evaluated at those times for joint symptomatology (joint counts and indices of pain/tenderness and swelling) and drug toxicity. Changes from baseline of the complete blood count with differential, liver function tests, and the C_1 index were noted. Folate status was assessed initially in the patients by plasma, RBC folate levels, and the C_1 index.

Cumulative MTX doses and dietary intakes of folate and vitamin B_{12} were the same in the FA and placebo groups. There was no difference between patient improvement in joint scores or joint indices between the two groups, indicating that the 1 mg/day FA supplement did *not* interfere with the effectiveness of the drug. A toxicity score was calculated as the summation of the duration of all toxic events for each patient multiplied by an intensity factor at the time of appearance of the toxic event (1 = mild, 2 = moderate, 3 = severe), multiplied by a clinical severity factor (1 = alopecia, pruritus, GI intolerance; 2 = diarrhea, vomiting, stomatitis, rash; 3 = elevated liver enzymes, gastrointestinal bleeding, elevated serum creatinine; 4 = cytopenias, documented infections, pulmonary toxicity), divided by the number of weeks on the protocol. The toxicity score was internally validated by the fact that high toxicity scores were observed in all patients who were removed from the protocol because, in the judgment of both the patient and rheumatologist, MTX toxicity was intolerable. The toxicity score was significantly lower in the FA-supplemented group than in the placebo-treated group. In addition, there were four dropouts in the placebo group due to MTX toxicity and no dropouts in the FA-supplemented group. Initial folate status, as measured by plasma and RBC levels, was predictive of future toxicity in the placebo-treated group. In addition, there was a relationship between folate status, as measured by the C_1 index, toxicity, and efficacy. When the C_1 index is viewed from the standpoint of efficacy, smaller decreases (or even increases) in the C_1 index occurred in patients who did not improve. When measures of efficacy and toxicity are combined, in patients who improve, $>50\%$ decreases in the C_1 index are associated with toxicity, while $>25\%$ decreases are associated with minor or no toxicity. Both of these findings indicate that moderate antagonism of folate-dependent metabolism is correlated with efficacy, while excessive antagonism is correlated with toxicity, suggesting the importance of careful titration of the activity of folate-dependent enzymes. Obviously, the optimal ratio of FA to MTX has yet to be determined and FA supplementation is still controversial [105]. There is a need for convenient and simple methods to monitor the intracellular ratio of folate to MTX, especially in immunologically active tissue (e.g., lymphocytes).

2. *A Clinical Trial Evaluating Placebo and Folic Acid Supplementation (5 vs. 25 mg/day): Preliminary Results*

A larger trial of FA supplementation during MTX therapy for RA is underway [105]. This trial is a prospective, placebo-controlled, double-blind trial comparing placebo, 5 mg, or 27.5 mg of FA supplementation per week during MTX therapy in an attempt to lessen drug toxicity. Because the supplement code has not yet been broken, the identity of the study groups is not known. At baseline, a joint count and score for pain/tenderness and swelling and a 24-hour dietary intake using food models is performed. A venous blood sample is drawn for analysis of plasma homocysteine (Hcy) levels and plasma, RBC, and peripheral blood mononuclear

cells (PBMC) folate levels using a MTX-resistant *L. casei*. In these patients, MTX is started in a dose ranging from 5.0 to 7.5 mg/week by the attending rheumatologist. Patients are instructed to take the designated vitamin/placebo capsules on 5 days of the week when MTX is not taken. Patients are evaluated every 3 months by the principal investigator (SLM). At each follow-up visit, a joint examination, dietary history, and blood work are repeated. A toxicity score is also calculated as described previously [88].

After 9 months of MTX therapy, there is no difference in efficacy as measured by joint counts and scores between the placebo, 5 mg/week, and 27.5 mg/week groups. Thus, a weekly 5- or 27.5-mg FA supplement does not seem to diminish the efficacy of MTX therapy [105].

IV. MECHANISM OF EFFICACY OF ANTIFOLATES IN NONNEOPLASTIC DISEASE

A. Inhibition of Inflammatory and Immunological Responses

The mechanism of action of low-dose MTX in autoimmune diseases, such as RA, remains obscure. However, several lines of evidence suggest that MTX has both an antiinflammatory and immunosuppressive effects. Substances often assayed to gauge immunosuppressive and antiinflammatory effects include complement proteins (C_3, C_4, CH_{50}), erythrocyte sedimentation rate, immunoglobulin levels (IgG, IgA, IgM, etc.), leukotriene and prostaglandin levels, and cytokine levels (IL-1,etc.). Other tests include chemotaxis and lectin stimulation (e.g., phytohemagglutinin, concanavalin A, and pokeweed mitogen). Antiinflammatory effects of MTX have been demonstrated and include inhibition of acute and chronic inflammatory responses in the rat and inhibition of polymorphonuclear chemotaxis [106,107]. Leroux et al. found that leukotriene B_4 (LTB_4) formation by neutrophils was depressed 32% after one dose of MTX [108]. Sperling et al. have demonstrated acute and chronic (6–8 weeks) suppression of the 5-lipoxygenase pathway with significant decreases in the ω-oxidation products of LTB_4 [109,110]. Changes in immunological parameters are more varied. No consistent changes in interleukin-1 (IL-1) have been demonstrated during MTX therapy, however MTX appears to affect both IL-1 production and mediated events in some patients [111]. Segal, et al. however, found that inhibition of IL-1 activity by MTX in vitro is dependent upon folate pathways, since the addition of leucovorin abolished the inhibitory effects of MTX [112]. Substantial decreases in IgM and IgA rheumatoid factor in MTX-treated patients have been demonstrated [113]. However, Anderson et al. reported smaller decreases in the circulating levels of IgG, IgM, IgA, and Westergren erythrocyte sedimentation rate and no changes in C_3, C_4, and CH_{50} during MTX therapy [25].

Recent work has demonstrated significant inhibition of the proliferative response of peripheral blood mononuclear cells (PBMCs) of MTX-treated patients

to phytohemagglutinin stimulation when the PBMCs are exposed to medium containing physiological levels of FA and deoxyuridine is used instead of thymidine as the radioactive tracer [114]. The antiproliferative effect of MTX is also supported by experiments demonstrating a subnormal proliferative response to lectin stimulation of PBMCs treated in vitro with MTX [115]. These recent reports disprove prior reports of normal or even supernormal responses to mitogens (phytohemagglutinin, concanavalin A, and pokeweed mitogen) by PBMCs of MTX-treated patients [24,25].

It has been somewhat controversial whether low-dose MTX therapy is immunosuppressive. In 1989, Shiroky et al. concluded that no reliable immunosuppressive effects of MTX had been demonstrated [116]. On the other hand, there have been several reports of opportunistic *Pneumocystis carinii* and herpes zoster infections in patients treated with low-dose MTX [117–119]. Later reports have indicated that complications related to immunosuppression are now seen commonly [119]. Rosenthal et al. demonstrated that both MTX and trimetrexate (nonpolyglutamylatable antifolate) can suppress immune responsiveness in humans [120]. Both drugs were inhibitors of T-dependent antibody formation, and inhibition of phytohemagglutinin-induced ^{3}H-uridine and ^{3}H-deoxyuridine incorporation was shown following in vitro addition of the drugs.

B. Antifolate Effects as the Mechanism of Action in Rheumatoid Arthritis

Some interference with folate metabolism appears to be involved in both the mechanism of efficacy and toxicity of MTX therapy of RA. Evidence regarding efficacy is demonstrated by the fact that several antifolates—MTX, 10-deazaaminopterin (10-DAM), and SASP—are currently used in RA therapy [23,35,41–47,121,122]. Even some nonsteroidal antiinflammatory drugs have been shown to inhibit some folate-dependent enzymes [54]. Supplementation with excessive doses of folinic acid (a reduced and one-carbon–substituted folate) abolishes toxicity but negates the efficacy of MTX therapy in RA [93]. These results clearly indicate that interference with folate metabolism is responsible for both efficacy and toxicity of MTX. However, the central question remains regarding which folate-dependent enzyme(s) to block to produce efficacy.

1. *Inhibition of Dihydrofolate Reductase as a Site of Efficacy*

Inhibition of DHFR does *not* seem to be necessary for the action of antifolates in the treatment of RA. Administration of 1 mg of supplemental FA per day to patients with RA taking MTX significantly lessened toxicity without reducing efficacy [88]. The ability of FA to lessen toxic manifestations indicates that DHFR is not completely inhibited. Recent work indicates that folate levels in the plasma, RBCs, and even to some extent PBMCs levels increase with FA supplementation without a loss of MTX efficacy [105]. This suggests that tetrahydrofolate coenzymes are

formed from the FA supplement, therefore, inhibition of DHFR is not complete and is not necessary for efficacy. In addition, the reported lack of efficacy of trimetrexate, a nonpolyglutamylatable inhibitor of DHFR, in the treatment of rat adjuvant arthritis also suggests that inhibition of DHFR alone is not sufficient for efficacy in this animal model [123].

2. *Inhibition of Thymidylate Synthase as a Site of Efficacy*

Inhibition of thymidylate synthase (TS) is another site that may be involved in the mechanism of efficacy (Fig. 2). Hine et al. demonstrated inhibition of TS in lymphocytes from patients with RA treated with MTX [114]. However, several lines of evidence seem to argue against inhibition of TS as the metabolic event responsible for the efficacy of MTX in RA. CB3717, an inhibitor of TS, is *not* effective in rat adjuvant arthritis [123]. In addition, 5-fluorouracil (5-FU), a specific inhibitor of TS, is a much less potent immunosuppressant than MTX in animal models of organ transplantation [124]. Orotic aciduria, an inborn error of metabolism where uridylate and thus thymidylate biosynthesis are blocked, is associated with only mild immunosuppression, compared to the severe immunosuppressive effects when some of the enzymes of purine metabolism are deficient [125].

3. *Inhibition of Purine Biosynthesis as the Site of Efficacy: A Hypothesis*

The evidence pointing to a blockade in purine nucleotide biosynthesis as the site of efficacy is intriguing and has been written as a hypothesis by the Antifolate Study Group at the University of Alabama at Birmingham [126]. This hypothesis states that inhibition of purine nucleotide biosynthesis at the site of aminoimidazole carboxamide ribotide transformylase (AICAR T'ase) is necessary to produce efficacy (Fig. 2). Several lines of evidence suggest that this enzyme is especially sensitive to low concentrations of MTX. The concentration of AICAR increases markedly when cultured lymphoid cells are exposed to only a 20 nM concentration of MTX [127]. A three to fivefold increase in urinary excretion of AICAR has been shown in MTX-treated patients receiving only 2.5 mg/day of drug [128]. A similar response has been observed in laboratory animals [129]. MTX also increases the intracellular concentrations of dihydrofolate polyglutamates ($DHFG_n$s) [70,71]. $DHFG_n$s are also an inhibitors of the transformylase [130].

AICAR and AICA riboside have been shown in our laboratory to inhibit adenosine deaminase (ADA) and S-adenosyl homocysteine hydrolase (SAH hydrolase) [131]. The integrity of ADA and SAH hydrolase are crucial to normal immune function and have been shown to be compromised in severe combined immunodeficiency syndrome (SCID) [132]. Over 85% of children with ADA deficiency have SCID, and children with SCID have recurrent bacterial, viral, and fungal infections which are consequences of their immunosuppression [133,134].

Immunosuppression in ADA deficiency is generally thought to be mediated by accumulation of adenosine and deoxyadenosine by the following mechanisms [132]:

1. Increased adenosine levels increase AMP, ADP, and ATP and lead to inhibition of phosphoribosyl pyrophosphate (PRPP) synthesis and ultimate pyrimidine starvation.
2. Increased adenosine levels lead to increased S-adenosyl homocysteine (SAH) levels, which ultimately inhibit S-adenosylmethionine–requiring transmethylation reactions. Deoxyadenosine also irreversibly inactivates SAH hydrolase.
3. Increased dATP levels resulting from the accumulation of deoxyadenosine inhibits ribonucleotide reductase.

All of the above mechanisms are reported to be toxic to the immune system and they are not mutually exclusive.

It is, therefore, our hypothesis that a mechanism of effectiveness of MTX in inflammatory arthritis is through AICAR accumulation with its inhibition of ADA and SAH hydrolase. The trapping of both adenosine and SAH is cytotoxic to immunologically active cells. This interference with normal adenosine metabolism leads to immunosuppression, though lesser in degree, by a mechanism similar to that found in the SCID patient.

Several lines of evidence support the link between MTX, AICAR, adenosine metabolism, and compromised immune function. Cronstein et al. have demonstrated that MTX and AICA riboside cause adenosine release and inhibit neutrophil adherence to fibroblasts and endothelium [135]. Adherence is normalized by the addition of exogenous ADA. Wolf et al. have shown that both adenosine and MTX attenuate leukocyte-endothelial cell adhesion in post capillary venules via adenosine receptors [136]. Adenosine then acts as an immunomodulator by occupying specific receptors on neutrophils, monocytes, and lymphocytes [137,138]. Firestein et al. have demonstrated that adenosine kinase inhibitors inhibit neutrophil adhesion to endothelial monolayers and significantly decrease paw swelling in Lewis rats with adjuvant arthritis [139]. An additional hypothesis of Nesher and Moore suggests that some immunosuppressive effects of MTX are due to an interference with S-adenosyl methionine (SAM)–dependent methylation and polyamine synthesis [140]. As discussed previously, the inhibition of transmethylation by the accumulation of SAH is one of the proposed mechanisms of immunosuppression in SCID.

C. Animal Studies of Antifolates in the Treatment of Arthritis

The MRL/lpr mouse has been previously show to have an inflammatory arthritis mimicking human RA and also has skin vasculitis, lymphadenopathy, and a

immune-mediated glomerulonephritis [141]. An initial trial evaluated the effects of 10-deaza aminopterin (10-DAM) (1,5 and 25 mg/kg/week) and MTX at the same doses plus an additional dose of 100 mg/kg/week [142]. 10-DAM has an enzyme inhibitory spectrum similar to that of MTX. MTX at 5, 25, and 100 mg/kg body weight per week and 10-DAMa 25 mg/kg/week significantly lessened skin-lesion-proteinuria scores. Animals receiving MTX at 25 mg/kg/week had a significantly longer median life span than animals receiving MTX at 100 mg/kg/week. Evaluation of hind limb histopathology indicated that MTX at 25 mg/kg/week and 10-DAM at 25 mg/kg/week reduced cartilage and bone erosion as well as synovitis and vasculitis in hind limbs. Thus, these histological features were lower in animals receiving MTX at 25 mg/kg/week when compared to animals receiving MTX at 1, 5, or 100 mg/kg/week. Unexpectedly the highest MTX dose (100 mg/kg/week) was not as efficacious as the intermediate dose of 25 mg/kg/week. One interpretation of these results, which is consistent with our hypothesis (Sec. IV.B), is that the highest dose of MTX blocked PBMC purine biosynthesis at the GAR T'ase step and prevented the accumulation of AICAR. In contrast, the lower dose of MTX primarily blocked AICAR T'ase and allowed accumulation of AICAR in these cells. The accumulated AICAR then interfered with normal adenosine metabolism and exerted a cytotoxic effect.

Additional animal model and cell culture studies also are consistent with an AICAR-mediated immunosuppression. Ward et al. [143] and Galivan et al. [123] found that intermediate doses (2–15 mg/kg/week) of MTX were much more effective in the treatment of rat adjuvant arthritis than were higher doses. Welles et al. [144] and Ridge et al. [145] successfully treated rat adjuvant arthritis with low doses (150–900 μg/kg/week) of MTX. Kahn et al. [146] found that an intermediate dose of MTX was more effective in extending cardiac allograft survival when compared to a higher dose. In cell culture experiments, Bokkerink et al. [127] observed that AICAR accumulated when MOLT-4 cells were exposed to 20 nM MTX but did not accumulate in a medium containing 200 nM MTX. Presumably the higher MTX concentration completely blocked the GAR T'ase step, preventing the accumulation of AICAR. The above experiments are consistent with the hypothesis that low or moderate amounts of MTX result in the accumulation of AICAR, which then exerts an antiadenosine and immunosuppressive effect.

V. SUMMARY

Antifolates play an increasing role in the therapy of nonmalignant diseases, especially autoimmune diseases. It appears that toxicity can be abated while efficacy is maintained by the administration of FA during MTX therapy. Further work is necessary to determine the optimal dosing schedule and amount of supplemental FA. Trials have primarily centered on folate supplementation during MTX treatment of RA. Other studies may center on combinations of folate and other antifolates in

treatment of other disease states. A critical question remains about which folate-dependent enzyme must be inhibited to allow for MTX efficacy in the treatment of autoimmune diseases. Our research group hypothesizes that a block in AICAR T'ase is most important.

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Folate and Neuropsychiatry

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I. INTRODUCTION

The metabolism of folate and vitamin B_{12} are intimately related, and this is reflected in the fact that deficiency of either vitamin may lead eventually in humans to a morphologically indistinguishable megaloblastic anemia. Indeed there is some evidence that the megaloblastic anemia of vitamin B_{12} deficiency, for example in pernicious anemia, is due, at least in part, to a block in folate metabolism [1]. This intimate relationship of the two vitamins is further emphasized by the fact that the administration of folic acid to patients with vitamin B_{12} deficiency may precipitate or aggravate the neurological complications of the latter deficiency [2], perhaps by aggravating the existing block in folate metabolism [3].

The neuropsychiatric complications of folic acid and vitamin B_{12} deficiency are much less clearly understood than the hematological manifestations. It has been recognized throughout this century that neurological and psychiatric disorders may sometimes precede, accompany, or follow megaloblastic anemia and that the correlation between the nervous system and hemopoetic symptoms and signs is usually a poor one [4]. However, it is only since the synthesis of folic acid in 1945 and the isolation of vitamin B_{12} in 1948, followed by the development of assay procedures to distinguish the two vitamin-deficiency states, that our understanding of the similarities and differences between their nervous system complications has gradually evolved. This has proved easier for the relatively purer vitamin B_{12}-deficiency states (for example, pernicious anemia) than for folate deficiency, which is so often accompanied by other deficiency states for dietary or gastrointestinal reasons. However, the clinical evidence of the harmful effects of folate deficiency

on the nervous system has been reinforced by studies of (1) the neurological manifestations of inborn errors of folate metabolism [5,6], (2) the neuropsychiatric complications of antifolate drugs [7,8], and (3) the prevention of neural tube defects by folic acid [9]. Furthermore, the importance of folate in nervous system metabolism is also emphasized by experimental and neurochemical studies, which will be discussed below.

Although folate deficiency, like vitamin B_{12} deficiency, can lead to disorders of peripheral nerve and, less commonly, spinal cord [3,10,11] this chapter is concerned with the mental complications of the deficiency, especially as there is evidence in some countries of a high incidence of folate deficiency among psychiatric and psychogeriatric patients. Previous reviews of the neuropsychiatric aspects of folate deficiency include those of Reynolds [3,12], Botez and Reynolds [10], Young and Ghadirian [13], and Botez and Botez [14].

II. NEUROCHEMICAL MECHANISMS

There is an active transport process for methyltetrahydrofolate across the blood-brain barrier [15], where it is present in the cerebrospinal fluid in humans in concentrations approximately three times greater than in serum [16], an unusual observation implying an important functional role. There are many possible metabolic mechanisms through which folates may influence nervous system function, including (1) its intimate relationship with vitamin B_{12} metabolism, (2) its role in the synthesis of methionine and S-adenosylmethionine (SAM), and therefore in many methylation reactions, and (3) its influence on monoamine metabolism, especially in the case of psychiatric disorders. These relationships are all illustrated in Figure 1.

Folates and vitamin B_{12} are required for the synthesis of purines and pyrimidines and therefore are important for nucleic acid and nucleoprotein synthesis. Impairment of these functions is thought to underlie megaloblast formation in the bone marrow leading to the hematological complications of both deficiency states [1]. Similar considerations could be relevant in the nervous system, but the remarkably high cellular turnover in blood compared to the nervous system may imply that such pathways are relatively less important in the latter and could contribute to the lack of correlation between hematological and neurological manifestations of each deficiency [3].

Particular interest is now focused on the role of folates in methylation in the nervous system. Methyltetrahydrofolate donates its methyl group to homocysteine to form methionine (a reaction catalyzed by vitamin B_{12}), which in turn transfers it to SAM (Fig. 1). SAM is the sole methyl donor in the brain in numerous methylation reactions involving nucleoproteins, proteins, membrane phospholipids, neurotransmitters, and monoamines [17,18]. It is remarkable that in independent studies

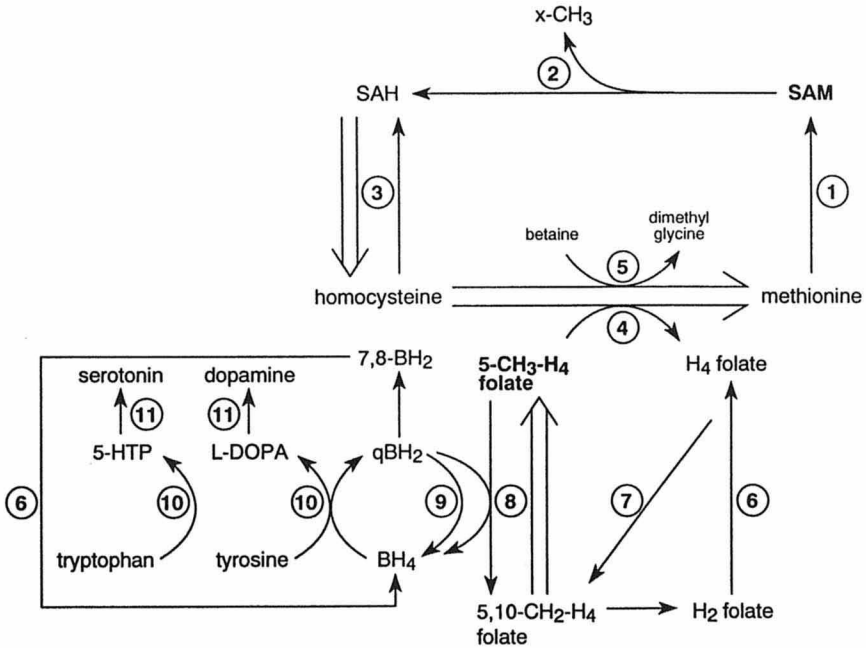

Figure 1 Interrelationship of folate, methylation, and monoamine metabolism. SAMe, S-adenosylmethionine; SAH, S-adenosylhomocysteine; 5-CH_3-H_4folate, 5-methyltetrahydrofolate; H_4folate, tetrahydrofolate; H_2folate, dihydrofolate; 5,10-CH_2-H_4folate, 5,10-methylenetetrahydrofolate; CH_4, tetrahydrobiopterin; qBH2, quininoid dihydrobiopterin; 7,8-BH_2, 7,8-dihydrobiopterin; L-DOPA, L-dihydroxyphenylalanine; 5-HTP, 5-hydroxytryptophan. 1, methionine adenosyltransferase; 2, x-methyltransferase; 3, S-adenosylhomocysteine hydrolase; 4, methionine synthetase; 5, betaine:homocysteine methyltransferase; 6, dihydrofolate reductase; 7, serine hydrox-methyltransferase; 8, 5,10-methylenetetrahydrofolate reductase; 9, dihydropterin reductase; 10, tyrosine or tryptophan nydroxylase; 11, amino acid decarboxylase.

in Italy [19,20], confirmed in the United Kingdom [21,22] and the United States [23], SAM has been shown to have antidepressant properties. These studies have all been reviewed [24]. The similarity in the clinical effects of folic acid (in folate-deficient patients) and SAM on mental function has led to the suggestion that methylation processes are important in the expression of mood, the failure of which may underlie some affective disorders [17,20].

Folic acid and SAM have both been shown to influence monoamine metabolism in humans and experimental animals, which links the methylation hypotheses with the longer-established and more well-known monoamine hypotheses of affective

disorder [17,20,25]. For example, experimental folate deficiency can lead to a fall in brain serotonin [26]. It is also of interest that in the rat brain the highest regional concentration of methyltetrahydrofolate is in areas of dense serotonergic innervation [27]. In children with inborn errors of folate metabolism and neurological disease, low levels of cerebrospinal fluid (CSF) 5-hydroocyindole acetic acid (5HIAA) and homovanillic acid (HVA) have been reported [28,29]. Low levels of CSF 5HIAA have also been related to folate deficiency in patients with folate-responsive neuropsychiatric disorders [30] and depression [31]. The administration of SAM in depression is associated with a rise in both CSF 5HIAA and HVA [32,33] and a fall in serum prolactin levels [34]. The mechanism of the influence of folate on monoamine metabolism is uncertain, but one possibility is through an influence of on tetrahydrobiopterin (BH_4) metabolism [35], since a pteridine cofactor is required for tryptophan hydroxylase, the rate-limiting enzyme in the synthesis of 5HT, and also for tyrosine hydroxylase in the synthesis of dopamine [36,37]. In depressed patients CSF BH_4, 5HIAA, and HVA are all highly correlated, and each in turn is significantly correlated with red cell folate [31]. Impaired BH_4 metabolism has been reported in temporal lobe tissue from depressed epileptic patients undergoing temporal lobectomy [38]. Coppen and coworkers [39] found a significant correlation between plasma folate and urinary total biopterin in depressed patients. The in vitro stimulation of rat brain BH_4 synthesis by methyltetrahydrofolate [40] is in keeping with these clinical observations of a link between biopterin, monoamine, and folate metabolism.

III. EVIDENCE IMPLICATING FOLATE DEFICIENCY IN NEUROPSYCHIATRIC DISORDERS

A. Folate-Deficient Megalobalstic Anemia

Reynolds and coworkers [41] compared the neurological findings in 24 patients admitted to a general medical ward with severe folate deficiency (23 of whom had a macrocytic anemia) and in an age- and sex-matched control group of admissions from the same ward without folate deficiency. There was a significant increase in "organic brain syndrome" (OBS), which was present in 71% of the folate-deficient group but only 31% of the control group. For the purpose of this study OBS included patients with "disabling apathy and depression."

Shorvon and coworkers [11] compared the neuropsychiatric associations of megaloblastic anemia due to folate deficiency with those of megaloblastic anemia due to vitamin B_{12} deficiency in patients presenting to physicians or hematologists. Two thirds of both series of patients exhibited nervous system disorders, which again illustrates the important point that as many as one third of patients with deficiency of either vitamin severe enough to produce a megaloblastic anemia may have no clinical evidence of neurological or psychiatric complications, at least at the point

in the evolution of the deficiency at which they present. Although there was a considerable overlap between the nervous system complications of the two deficiencies, it was found that spinal cord and peripheral nerve disorders were more common with vitamin B_{12} deficiency, whereas psychiatric disorders, mainly depression, were more common with folate deficiency. The folate-deficient nervous system disorders responded rather better to vitamin replacement therapy than the vitamin B_{12}–deficient group, perhaps because the latter, most of whom had pernicious anemia, were an older group. The most common causes of the folate deficiency were gastrointestinal and dietary, but in some patients the deficiency was unexplained.

B. Folate Deficiency in Psychiatric Patients

Table 1 summarizes studies of folate deficiency in psychiatric inpatient populations over the last 25 years. All the studies employed serum folate assays, but very few included assays of the more stable red cell folate, an important consideration to be discussed later. As Young and Ghadirian [13] have discussed, the assay procedures, control ranges, and "cutoff" points for deficiency have varied considerably between the studies. However, utilizing the serum folate assay, there has been fairly consistent agreement of a high incidence of deficiency varying between 8 and 50%, the great majority between 15 and 33%. In the few red cell folate studies the incidence of deficiency has varied between 22 and 33%. In those studies that included controls, the incidence of deficiency in the latter has varied between 0 and 10%. The studies have been undertaken predominantly in the United Kingdom and Scandinavia, but also in Canada, the United States, and Australia. It must not be assumed that the incidence of deficiency will be similar in other countries where dietary habits may be very different.

In most of the surveys summarized in Table 1, the patients have not been well categorized. The main exception is Carney [42], who in his original study of 423 admissions emphasized the association of folate deficiency with endogenous depression and organic psychosis. In a later study Carney and Sheffield [50] confirmed an association with both endogenous and neurotic depression and with organic mental change. However, folate deficiency can occasionally occur with any psychiatric diagnostic category. In this most recent study employing red cell folate assays, Carney and coworkers [54] divided their 285 patients into five diagnostic groups. As is illustrated in Table 2, borderline (<200 ng/ml) or severe (<150 ng/ml) folate deficiency was significantly more common in depressed and alcoholic patients than in euthymic, manic, or schizophrenic patients. Among depressed patients severe folate deficiency was twice as common in the endogenous than in the neurotic subgroup. In 100 consecutive admissions with depression, Reynolds and coworkers [35] found that 24% had low serum folate levels. The deficient subgroup were more depressed on admission and discharge and had responded less

Table 1 Studies of Folate Levels in Psychiatric Populations

Ref.	Year	Country	Nature population	No. of patients/ controls	% with low folate levels				Comments[a]
					Serum folate		Red cell folate		
					Patients	Controls	Patients	Controls	
42	1967	UK	Unselected acute psychiatric admissions	423	22	9.6			Low serum folate defined as <4.5 nmol/liter; patients with organic psychosis and endogenous depression had serum folate values significantly lower than controls
			Healthy controls	62					
43	1967	UK	Unselected psychiatric admissions	75	49				Low serum folate defined as <6.8 nmol/liter; deficiencies largely associated with alcohol abuse, and therapy with barbiturates or antiepileptic drugs
44	1967	Australia	Psychiatric hospital residents	48	15	0			Low serum folate defined as <4.5 nmol/liter
			Healthy controls	61					
45	1969	Denmark	Randomly selected female psychiatric inpatients	29	25		0		Low serum folate defined as <4.5 nmol/liter; low whole blood folate defined as <45 nmol/liter
46	1969	Sweden	Psychiatric admissions selected on basis of risk deficiency	115	21				Low serum folate defined as <6.8 nmol/liter
47	1969	Sweden	Unselected consecutive acute psychiatric admissions	84	51		22		Low serum folate defined as <5.7 nmol/liter; low whole blood folate defined as <102 nmol/liter. Low serum folate unrelated to age of patients but more common in women
48	1970	Australia	Unselected consecutive acute psychiatria admissions and readmissions	411	33				Low serum folate defined as <7.9 nmol/liter; folate deficient patients mostly epileptic or schizophrenic and using barbiturates, phenytoin, or phenothiazines

35	1970	UK	Consecutive admissions with depression to research unit	100	24				Low serum folate defined as <5.7 nmol/liter; higher depression scores, lower Marke-Nyman Validity scores and poor response to antidepressant therapy in those patients with low serum folate
49	1971	UK	Mixed inpatients psychiatric population	30	33	6	23	6	Low serum folate defined as <5.7 nmol/liter; low red cell folate defined as <227 nmol/liter
			Healthy controls	33					
50	1978	UK	Unselected acute psychiatric admissions	272	21				Low serum folate defined as <4.5 nmol/liter; low folate linked with depression and physical illness
51	1978	US	Unselected admissions to psychiatric service of general hospital	269	30	0			Low serum folate defined as <13.6 nmol/liter; diet frequently inadequate but not related to serum values
			Healthy controls	40					
52	1982	UK	Outpatients on long-term lithium therapy in remission	107	17				Low serum folate defined as <9.1 nmol/liter; patients on lithium had lower mean folate compared with controls; patients with lower serum folate had higher affective morbidity index
			Healthy controls	60					
53	1986	US	Unselected psychiatric admissions	60	1.7				Low serum folate defined as 5.5 nmol/liter
54	1990	UK	Consecutive psychiatric admissions	285			31		Borderline deficiency defined as red cell folate <453 nmol/liter (31%), definte deficiency as <340 nmol/liter (12%); higher rate of deficiency in affective and alcoholic patients, especially those with endogenous depression
55	1990	UK	Consecutive psychiatric admissions with depresssino and schizophrenia	123			33		Borderline and definite deficiency

[a] To convert nmol/liter to ng/ml, divide by 2.266.

Table 2 Relationship Between Clinical Diagnosis and Moderately Low [456 nmol/liter (<200 ng/ml)] or Severeley Low [340 nmol/liter (150 ng/ml)] Red Blood Cell Folate Levels

Clinical diagnosis	Total no. of patients	No. (%) of patients with low red cell folate levels	
		<423 nmol/liter[a]	<340 nmol/liter[a]
Depression			
endogenous	95	37 (39)	19 (20)
neurotic	57	21 (37)	6 (11)
subtotal	152	58 (38)	25 (16)
Euthymia	42	7 (17)	2 (5)
Mania	32	7 (22)	3 (9)
Schizophrenia	29	5 (17)	1 (3)
Alcoholism	30	12 (40)	4 (13)
Total	285	89 (31)	31 (12)

[a] To convert nmol/liter to ng/ml divide by 2.266.
Source: Ref. 54.

well to standard antidepressant drug treatment than the nondeficient group. The former subgroup also had lower Marke-Nyman Validity scores, a measure of personality related to psychic energy and drive.

In the only outpatient study we are aware of, Coppen and Abou-Saleh [52] reported significantly lower serum folate levels in 107 patients on long-term prophylaxis with lithium for recurrent affective disorder compared with 60 normal controls. Using an arbitrary cutoff point of 8 ng/ml, they also found that patients with serum folate values below this level had significantly greater affective morbidity over the previous 2 years on a global rating scale.

Not included in Table 1 are several studies of serum, red cell, and CSF folate in epileptic patients, all of which confirm a significant association between anticonvulsant-induced folate deficiency and psychiatric complications of epilepsy, including depression and organic mental deterioration. These studies were reviewed in detail by Reynolds [3], and have since been reinforced by further studies [56–59] and discussed by Reynolds [60].

Excluding epileptic patients, the causes of folate deficiency in psychiatric patients have not been very clearly established. Barbiturates and alcohol have contributed in some studies. It would not be surprising if dietary deficiency was a major cause, especially as some forms of psychiatric illness, including depression, may lead to anorexia or change in dietary habits. Certainly most authors assume that diet is the most important factor, although the few attempts to confirm this have

not been successful. Reynolds and coworkers [35] found no difference in serum folate levels between those patients whose diet was assessed as poor, moderate, or good. Thornton and Thornton [51] also found that poor diet was as common in patients with as in those without folate deficiency. Interestingly, red cell folate deficiency was as common in depressed patients as in alcoholic patients in the study of Carney and coworkers [54], although the latter were more obviously malnourished. Similarly, depressed patients were more folate deficient than schizophrenic or medical patients on the same hospital diet [61]. Carney and Sheffield [62] found that the main associations of folate deficiency were chronic psychiatric illness, physical illness, psychotropic drug therapy, and malnutrition. However, we are unaware of any evidence that phenothiazines or standard antidepressants depress folate levels [63]. It is apparent that in a large minority of psychiatric patients folate deficiency is unexplained. There have been no studies, to our knowledge, of folate absorption in psychiatric patients. This is an important omission because gastrointestinal disease is a common cause of severe folate deficiency in medical practice, and Botez and coworkers [64] have described a syndrome of folate deficiency, fatigue, mild or moderate depression, minor neurological signs, and functional gastrointestinal disorders or malabsorption.

C. Folate Deficiency in Geriatric and Psychogeriatric Patients

Several studies (Table 3) suggest a high incidence of folate deficiency in geriatric admissions, with an even higher incidence in patients with psychogeriatric disorders. These surveys have again relied mainly on serum folate assays with the same variations in technique and definition of deficiency already noted for psychiatric studies (Table 1). All authors have recorded at least an 18% incidence of low serum folate levels, rising to figures as high as 80–90% in some psychogeriatric studies. For example, in elderly admissions to a general hospital Hurdle and Williams [66] found low serum folate values in 22% of patients with physical disability without psychiatric morbidity but 67% in patients with additional mental disorder. As might be expected in both geriatric and psychogeriatric patients, dementia is the most common association with folate deficiency [67,69,78]. Again it is assumed, perhaps correctly, but without systematic study, that secondary dietary deficiency is the overwhelming etiological factor. However, as in the psychiatric studies reviewed above, the psychological disorders in these elderly population surveys have, in general, not been very carefully categorized or rated, and it is interesting that several of the studies refer to symptoms of apathy, withdrawal, lack of motivation, and depression as evidence in favor of the dietary theory without also considering the possibility that these symptoms might be, at least in part, the result of the deficiency either for primary or perhaps more commonly for secondary reasons [3].

Only three surveys have included red cell folate assays [69,71,76]. In the former study of 115 consecutive admissions to a geriatric unit at a district general

Table 3 Studies of Folate Levels in Elderly Populations With and Without Psychiatric Illness

Ref.	Year	Country	Nature population	No. of patients/ controls	% with low folate levels				Comments[a]
					Serum folate		Red cell folate		
					Patients	Controls	Patients	Controls	
65	1965	UK	Admissions of elderly patients to old people's home	51	80	29			Low serum folate defined as < 13.6 nmol/ liter
			Healthy elderly controls	51					
66	1966	UK	Elderly patients admitted to hospital	67	42				Low serum folate defined as < 11.33 nmol/ liter; incidence of folate deficiency highest in patients with mental symptoms (67%) compared with severe physical disability (38%) or mild physical disability (22%)
67	1967	UK	Mixed population of acute psychogeriatric admissions and established inpatients	59	78				Low serum folate defined as < 13.6 nmol/ liter
68	1967	UK	Elderly patients in hospital for > 3 months	39	18	7.5			Low serum folate defined as < 6.8 nmol/ liter; no difference between elderly control group and young control group; hospital inpatients had lower folate values
			Elderly patients living at home	72					
			Young healthy controls	62					
69	1973	UK	Consecutive admissions to acute geriatric assessment unit	115	22		16		Low serum folate defined as < 4.5 nmol/ liter; low red cell folate defined as < 453 nmol/liter patients with dementia had lowest red cell folate; dementia score correlated with red cell folate ($r = 0.41$)

70	1977	US	Cosecutive elderly admissions to psychiatric service of general hospital	26	35	0		Low serum folate defined as < 1.1 nmol/liter; low folate values noted particularly in confused, disoriented, and depressed patients
			Healthy age-matched controls	22				
71	1977	France	Elderly admissions to acute and long-stay units	165	61		37	Low serum folate defined as < 9.1 nmol/liter; low red cell folate defined as < 453 nmol/liter
72	1982	France	Elderly admissions	480	44			Low serum folate defined as < 6.8 nmol/liter
73	1984	France	Female patients admitted to psychogeriatric unit	27	92.6			Low serum folate defined as < 6.8 nmol/liter
74	1984	UK	Patients with senile dementia	29	23		3	Normal range not defined
			Age-matched controls	35				
74	1984	UK	Consecutive referrals to psychogeriatric unit	234	20			Normal range not defined; highest incidence of folate deficiency in functionally ill patients (23%), especially depressed subgroup
76	1984	France	Elderly patients in long-term geriatric unit	124	58		75	Low serum folate defined as < 4.5 nmol/liter; low red cell folate defined as < 453 nmol/liter; mostly dementia or cerebrovascular disease
77	1986	France	Hospitalized geriatric patients	1000	75			Low serum folate defined as < 11.3 nmol/liter
78	1986	UK	Inpatients with dementia in acute assessment unit	45	36			Low serum folate defined as < 5.7 nmol/liter

[a] To convert nmol/liter to ng/ml, divide by 2.266.

hospital, 16% had low red cell folate values and the 14 patients with dementia had significantly lower levels than any other diagnostic group. Furthermore, in this subgroup there was a significant correlation (0.41) between the folate levels and the severity of the dementia on the mental assessment score. In a small study of 13 outpatients presenting to a Memory Clinic, 10 of whom had "probable Alzheimer's disease," Sommer and Wolkowitz [79] noted a significant positive correlation (0.67) between red cell folate levels and mini Mental State Examination scores, even though all the folate values were within the normal range. In 63 elderly demented subjects Kral and coworkers [80] found no correlation between low serum folate and memory impairment, but the latter was correlated with the serum vitamin B_{12}-to-folate ratio.

In a survey of nutritional status and cognitive functioning in 260 healthy elderly subjects aged 60–94 years living in the community, Goodwin and coworkers [81] reported a significant relationship between abstract thinking ability and memory and low folate levels or folate intake. The details of the folate data were not presented, but it must be assumed that in such a population the great majority were in the normal range.

D. Folate Deficiency in Epileptic Patients

This subject has been reviewed by Reynolds [3,60]. Megaloblastic anemia was first noted in patients on anticonvulsant drugs in 1952 [82]. Although anemia occurs in less than 1% of patients, the incidence of hematological abnormalities, especially macrocytosis, and of folate deficiency as measured by both serum and red cell values is much higher [3]. The frequency of abnormally low serum folate values varies according to the nature of the epileptic population studied but ranges from 27 to 91% [3]. Subnormal serum folate levels are significantly correlated with low red cell and low cerebrospinal fluid folate levels [83–85]. Phenytoin, phenobarbital, and primidone are particularly implicated, and it has been suggested that the antifolate activity of these three drugs and their anticonvulsant properties may be linked [86,87]. Lowered red cell folate levels have, however, been reported in patients on monotherapy with carbamazepine and, to a lesser extent, with sodium valproate [88]. It is also apparent that the effects on serum, red cell, and CSF folate is most marked in patients taking more than one anticonvulsant. The mechanisms of the influence of these drugs on folate metabolism are still unclear, but interference with folate transport and/or liver enzyme induction have been implicated.

The complex relationship between interictal psychopathology and folate deficiency in patients with epilepsy has stimulated considerable interest and study. Epilepsy is in itself a condition with a range of organic and psychosocial aspects. The complexities of psychiatric morbidity in epileptic patients have been studied extensively and reviewed elsewhere [58,60,89]. Isolating a casual role for folic acid in such a complex condition appears daunting, but studies have consistently sup-

ported an association between impaired folate metabolism and psychopathology in patients with epilepsy [3,60]. Furthermore, correlations have also been made in epileptic patients between folate deficiency and the monoamine neurotransmitter systems that have been implicated in primary psychiatric disorders.

Following early case reports implicating folate deficiency in various psychiatric disorders in epileptic patients, including psychoses, depression, psychomotor retardation, and dementia, a number of systematic studies followed (Table 4). The majority of investigators have used serum folate levels, but some have included red cell and/or CSF folate levels [49,56,59,94]. The populations studied have varied greatly. For example, Reynolds and coworkers studied epileptic inpatients and demonstrated that in this population mean CSF folates were significantly lower in all groups with mental symptoms compared to the mentally normal group. Snaith and coworkers [92] found a higher incidence of serum folate deficiency in outpatients with psychiatric morbidity, and Reynolds and coworkers [49] found a similar association between low red cell folate and mental illness in outpatients. Of particular interest is the community-based study of Edeh and Toone [59], who found low serum folate to correlate with depression but not anxiety. Trimble and coworkers [56] found an association in children between organic mental deterioration and folate deficiency, but Deb and coworkers [95] failed to find any association between folate deficiency and psychiatric disturbance in mentally handicapped epileptic patients.

The studies summarized in Table 4 are methodologically sound. All make comparison between mentally normal epileptic patients and those with psychiatric symptoms, and several also use healthy controls. The majority, however, fail to define or operationalize "psychiatric disorders" or "behavioral disturbance," although some use standardized measures. The findings are striking. All studies, with the exception of the mentally handicapped group, show an association between mental symptoms and low folate levels. This association is strong enough to emerge despite the large number of potentially confounding organic, pharmacological, and psychosocial variables that may exist in patients with seizures.

E. Folate Metabolism and Infection with the Human Immunodeficiency Virus

Severe malnutrition and wasting frequently accompany the later stages of the acquired immunodeficiency syndrome (AIDS). It is not surprising therefore that most nutritional studies in human immunodeficiency virus (HIV)–infected patients have been performed at the end stage of the disease. The literature contains conflicting data on resting blood folate levels in HIV disease. Low serum and red cell folate levels have been reported in a group of 74 patients at all stages of HIV infection [96]. In contrast, high plasma and red cell folates levels were reported in a group of 24 asymptomatic HIV-infected patients [97], whereas no significant

Table 4 Studies of Folate Levels in Epileptic Populations With and Without Psychiatric Illness

Ref.	Year	Country	Nature population	No. of patients/ controls	% with low folate levels				Comments[a]
					Serum folate		Red cell folate		
					Patients	Controls	Patients	Controls	
90	1969	UK	Inpatients with:						
			dementia	15					Mean CSF folates were significantly lower in all groups with mental symptoms than in the mentally normal group of patients
			schizophrenia	16					
			depression	12					
			behavioral disturbance	8					
			normal mental state	10					
91	1969	Ireland	Inpatients with:						Low serum folate defined as <4.5 nmol/liter
			psychotic	40	38				
			nonpsychotic	40	15				
			normal controls			0			
92	1970	UK	Outpatients with:						Low serum folate defined as <6.8 nmol/liter
			schizophrenia	10	50				
			depressive psychosis	5	80				
			reactive depression	11	36				
			other neurosis	3	0				
			aggression	8	50				
			mentally normal	27	19				
			normal controls	31		0			

49	1971	UK	Inpatients with psychiatric illness	33	88		70		Low serum folate defined as <5.7 nmol/liter Low red cell folate defined as <227 nmol/liter Significant correlations found between serum and red cell folate levels in all groups. In the epileptic patients there is a significant association between low serum and red cell folate and the presence of psychiatric illness. "Psychiatric illness' heterogeneous group of patients with diverse psychiatric symptoms
			Outpatients with psychiatric illness	19	37		47		
			Outpatients without psychiatric illness	15	0		0		
			Nonepileptic patients	30	33		23		
			Normal controls	33		6		6	
56	1980	UK	Resident children at hospital school	312	48		69		Low serum folate defined as <6.8 nmol/liter; low red cell folate defined as 113 nmol/liter. Children with neuropsychiatric abnormalities, including neurotic disorders, depression, and a fall in IQ had significantly lower serum and red cell folates than children without these problems
			Controls attending general medical outpatients	96					
57	1983	US	Adult population of chronic residents with severe seizure disorders and recurrent admissions	140	25				Low serum folate associated with depressed mood. Weaker correlation of low folate and anxiety symptoms. 78% of low-folate group had mental symptoms, 15% of normal folate group had mental symptoms. Dividing the population into folate <4.5 nmol/liter and >4.5 nmol/liter showed a very strong correlation with depressed mood. Hematological parameters did not differ
			Above patients with serum folate <4.5 nmol/liter	32					
			Above patients with serum folate >4.5 nmol/liter	33					
93	1983	Spain	Chronic outpatients	95					Three factors associated with psychological disturbance: serum folate was significantly lower ($p = 0.005$), MCV was significantly higher ($p = 0.05$), polytherapy (3 or more drugs) significantly higher

Table 4 Continued

Ref.	Year	Country	Nature population	No. of patients/ controls	% with low folate levels				Comments[a]
					Serum folate		Red cell folate		
					Patients	Controls	Patients	Controls	
94	1984	Australia	Epileptic outpatients on monotherapy with:						Memory dysfunction correlated with red cell folate levels and serum drug levels
			sodium valporate	25					
			phenytoin	57					
			carbamazepine	34					
			Total	116					
59	1985	UK	Adult patients drawn from general practice population	82	11		3.6		Low serum folate defined as <6.8 nmol/liter. Low red cell folate defined as <330 nmol/liter. Used standardized measures of psychopathology. Close correlation between serum and red cell folate. Folate deficiency correlated with multiple therapy. Patients classified as having psychiatric morbidity had significantly lower red cell folate and serum folate. Serum folate levels in depressed group differed significantly from patients with no morbidity but not in anxiety group
95	1987	UK	Mentally handicapped	32					Significantly lower mean serum folate level in epileptic group. Serum folate lower in epileptic patients on phenytoin
			Mentally handicapped nonepileptic subjects						No association of low serum folate with psychiatric disturbance using standardized measures

differences were found in a larger series of 100 asymptomatic HIV-infected patients [98]. In another study erythrocyte folate levels were normal in 11 asymptomatic HIV patients and 9 AIDS patients, however, there was an upward trend for serum folates in both groups [99]. In these patients folate absorption was tested over a 3-hour period by giving 5 mg of folic acid orally after overnight fasting. There was a significant impairment in folate absorption in patients with HIV and AIDS compared to controls. Malabsorption may be due to an enteropathy that is commonly found in HIV-infected patients. There is some debate over whether the HIV virus itself may cause an enteropathy or if it is due to opportunistic infection of the gut [100–102]. The reduced ability to absorb folate in asymptomatic HIV patients without any opportunistic infections supports the hypothesis that the HIV virus itself can cause an enteropathy. Adequate folate absorption in patients infected with the HIV virus is vital as folinic acid is a necessary adjunct to therapy with trimetrexate, used in the treatment of *Pneumocystis carinii* (PCP) [103]. It is interesting to note that in a study of another dihydrofolate reductase inhibitor, using high-dose co-trimoxazole for treatment of PCP infections in AIDS, folate rescue with oral folic and folinic acid both failed to reduce the pancytopenia [104]. Folate rescue is also required after high-dose methotrexate, which is commonly employed as part of cytotoxic chemotherapy regimens in the treatment of high-grade lymphomas that are increasingly a problem in AIDS.

Resting serum folate levels may not be a reliable guide to folate status for several reasons. Numerous abnormal proteins are present in HIV infection which may affect the binding of folates. For instance, binding of folate is known to increase in malignancy and inflammation where increases in abnormal proteins are known to occur [105]. Furthermore, an increased incidence of vitamin B_{12} deficiency in HIV infection has been reported by several investigators [98, 106, 107] and may complicate a diagnosis of folate deficiency. In a study of asymptomatic HIV-infected patients (CDC stages II and III), those with low serum vitamin B_{12} levels performed more poorly than those with normal vitamin B_{12} status on specific measures of information processing, speed, and visuospatial problem-solving skills [108]. These findings suggest that vitamin B_{12} deficiency may be a factor in cognitive changes in the early stages of HIV infection. No similar studies have been reported that have examined the associations between folate deficiency and neuropsychiatric complications. However, a secondary folate deficiency may develop in vitamin B_{12}–deficient patients, due to the intimate metabolic relationship of these vitamins, that may be an underlying cause of depressive symptoms commonly found in HIV patients.

Folate deficiency in the central nervous system has been studied in six HIV-infected children by one group of researchers, who reported low CSF folate levels in five patients and low CSF methionine and SAM in all six patients [109]. Four of the six patients had calcification of the basal ganglia or focal white matter lesions as revealed by computed tomography. CSF levels of neopterin, which are

released by macrophage stimulation, were suggested to interfere with the metabolism of folates and lead to a CNS folate deficiency. The reductions in CSF folate and SAM in the HIV-infected children were similar in magnitude to those seen in children with inborn errors of folate metabolism with evidence of central demyelination. Reduced CSF SAM levels have also been reported in a group of 20 adult HIV-infected patients with and without neurological complications [110]. However, in this study there was no evidence of overt folate or vitamin B_{12} deficiency. These findings may have interesting implications because of the link between both folate and vitamin B_{12} deficiency and defective methyl group metabolism leading to subacute combined degeneration of the cord [29, 111, 112] and the fact that in some patients with AIDS a myelopathy pathologically resembling subacute combined degeneration of the cord has been described [113, 114].

IV. EFFECTS OF FOLATE TREATMENT

There are many reports in the literature of neurological disorders due to folate deficiency and responsive to folic acid therapy [3, 4, 10, 111]. Indeed, as mentioned before, in the study of the neuropsychiatric complications of megablastic anemia by Shorvon and coworkers [11], patients with folate deficiency responded better to treatment than those with vitamin B_{12} deficiency.

In 1967 Reynolds [115] reported an improvement in the mental state of varying degree in 22 out of 26 folate-deficient epileptic patients on vitamin therapy followed for 1–3 years. A consistent pattern of improvement in drive, initiative, alertness, concentration, and mood was described, which often took several months to be fully apparent. This observation proved controversial as, although other open studies were supportive, several placebo-controlled trials proved negative [3]. Possible reasons for conflicting reports included the short duration of the studies (usually 3 months), especially bearing in mind the efficient blood-brain barrier mechanism limiting the entry of folic acid into the nervous system [116]; the reliance on standard and insensitive psychometric batteries, which focused on cognitive function, but not mood and arousal; the inclusion of deteriorated epileptic subjects; and the reliance on serum as opposed to red cell folate levels as a measure of folate status [60].

Studies of folate treatment on the mental state of nonepileptic subjects have been more consistent. Botez and coworkers [64, 117, 118] have described the effect of folate supplements in the syndrome of long-standing functional gastrointestinal disorder, abnormal diet, or occult malabsorption and folate deficiency associated with depression, lassitude, and minor neurological signs. In a placebo-controlled trial of folic acid (15 mg daily) for 4 months in 24 patients, significant improvements in the Wechsler IQ memory scale and Kohs block design occurred in the vitamin-treated group. In a larger open label study, improvement was also seen in the verbal performance and total scores of the Ottawa Wechsler IQ and in the

Halstead Category and Kohs block design tests [119]. In 50 patients the improvement in mood was reported as very good or good in 86%.

In a double-blind controlled trial Coppen and coworkers [120] randomized 102 subjects attending the Lithium Clinic either to an additional small dose of 200 μg folic acid daily or to matching placebo. Seventy-five patients completed one year in the trial. In all of the patients on folic acid supplements there was a significant association between lower affective morbidity index (AMI) and end of trial plasma folate. In unipolar patients those with plasma folate greater than 13 ng/ml had significantly lower Beck mood and AMI scores. In a more recent double-blind controlled trial, Godfrey and coworkers [55] compared 15 mg of methyltetrahydrofolate against placebo in addition to standard psychotropic treatment in 41 acute psychiatric outpatients with borderline or definite folate deficiency, as indicated by a red cell folate level below 200 ng/ml. In both depressed and schizophrenic subgroups, methyl folate significantly improved clinical and social recovery and the differences with placebo increased with time over the 6 months of the trial.

In view of the benefits of folate supplements in addition to standard therapy, Crellin and coworkers [121] undertook a pilot double-blind comparison of methyl folate (50 mg daily) with a standard antidepressant (amitriptyline 150 mg daily) as monotherapy for 6 weeks in 31 outpatients with moderate depression, most of whom had normal red cell folate levels. Any patients showing >25% fall in Montgomery-Asberg Depression Scores (MADS) due a 2-week placebo run-in period were not randomized. In the trial the MADS response rate was very similar with amitriptyline and with methyltetrahydrofolate. Interestingly, of three amitriptyline nonresponders who were subsequently crossed over to methyltetrahydrofolate, two improved. A further remarkable observation was that methyl folate responders showed striking increases in red cell folate levels in comparison to methyl folate nonresponders.

There is a significant association between folate deficiency and organic mental impairment or dementia [41, 68] and there are case reports of some patients responding to treatment with folic acid [3, 122–124]. In 17 institutionalized elderly patients with dementia and low red cell folate levels, Shaw and coworkers [125] reported no significant improvement on a combination of oral folic acid and intramuscular vitamin B_{12} in a double-blind crossover placebo-controlled trial for 12 weeks. However, the authors comment on the severity of the dementia, the small numbers, and the relatively short duration of the trial. More recently Brocker and coworkers [77] studied openly 50 folate-deficient elderly subjects for 21 days prior to and 21 days after three weekly injections of 50 mg of folinic acid. There was a significant improvement in mood and organic mental function on vitamin therapy, including four patients with depression and three with dementia who recovered complete "self-sufficiency." In a recent double-blind study conducted for 8 weeks in 96 elderly depressed patients with mild to moderate dementia, Passari and co-

workers [126] found that methyltetrahydrofolate, 50 mg daily, was as effective as the standard antidepressant trazodone, 100 mg daily, in improving depressive symptoms, rated on the Hamilton scale, irrespective of folate status.

CONCLUSIONS AND CLINICAL CONSIDERATIONS

This review confirms that in many countries there is a high incidence of folate deficiency in psychiatric and psychogeriatric patients, especially inpatients. The deficiency can be associated with any diagnostic category but is more common in depression and dementia. Although dietary deficiency may be an important cause, the etiology of the deficiency is far from clearly established. In epileptic patients anticonvulsant drugs are an important factor. There is growing evidence that whether the deficiency is primary or secondary to the psychiatric disorder, folate replacement may improve mental function, whether or not it is added to standard psychotropic medication.

It is clear that because of the poor correlation between hematological and nervous system complications of both folate and vitamin B_{12} deficiencies, many cases of deficiency, especially folate deficiency, will be overlooked by relying solely on screening for macrocytosis with blood counts. As the red cell folate assay is a much more reliable guide to folate status than a serum folate level, in our opinion all psychiatric admissions as well as outpatients at highest risk, i.e., those with depression and organic mental states, including alcoholics, should ideally be screened with this assay.

There remains some uncertainty as to what level of red cell folate constitutes a deficiency state, and this will vary from laboratory to laboratory and depends also in part on the type of assay procedure employed. This is important as there is some evidence that even a borderline deficiency state can be harmful to the mental state [55]. Granted, however, that it is possible for a minority of patients to have a megablastic anemia due to folate or vitamin B_{12} deficiency without neuropsychiatric complications at a given point in time [11], there is no clear way of knowing whether a low or borderline red cell folate with a normal blood count is harmful to the mental state of the patient. In our opinion it is likely to be so, if not currently, then at least in the future. Therefore, the patient should be given the benefit of the doubt about treatment. Future studies utilizing plasma homocysteine levels will probably help to clarify the degree of functional deficiency present in the context of a low red cell folate level [25, 31].

Whether the patient should be treated with folic acid, folinic acid, or methyltetrahydrofolate is also uncertain, as is the question of the dose and the duration of treatment. Most studies have been with standard folic acid therapy, but folic acid is transported into the nervous system as methyl folate, and recent trials with this formulation have been encouraging [55, 121] but there are no compara-

tive studies. Furthermore, the amount of methyl folate entering the nervous system is limited by a very efficient blood-brain barrier mechanism [15, 16]. This may, in turn, perhaps reflect a necessary mechanism to prevent the excitatory properties of folates leading to undesirable consequences, such as seizures [126]. Whichever formulation is used, most of a pharmacological dose is unlikely to enter the nervous system. Coppen and coworkers [120] reported significant effects on mood of a physiological dose of folic acid given for one year, whereas Crellin and coworkers [121] noted antidepressant effects of methyltetrahydrofolate given in a large dose for 6 weeks. In his earliest report of the effect of folic acid in epileptic patients, Reynolds [115] emphasized that the beneficial effects on the mental state could take several months to be apparent, and this seemed to be confirmed in the controlled trial in nonepileptic subjects by Godfrey and coworkers [55] in which improvement increased over time for the 6 months of the study. Clearly further studies are required to clarify the issues of formulation, dose, and duration of treatment.

Whichever formulation is used, the vitamin is remarkably, but not totally, free of side effects. The risk of precipitating or exacerbating seizures occasionally has been mentioned and is probably greater with large doses given over long periods of time, especially in epileptic patients [60, 115, 127]. The occasional occurrence of sleeplessness, overactivity, increased anxiety, or poor concentration, as reported by Hunter and coworkers [128] in normal subjects, is not in our opinion a "toxic" effect as suggested by Young and Ghadarian [13] but reflects a tendency to overarousal and even hypomania, as can occur with any antidepressant and as has also been noted with a closely related metabolite, SAM [129].

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Index

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