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# Folic Acid

Jacob Selhub and Irwin H. Rosenberg

Folic acid is the term most commonly used to refer to a family of vitamers with related biological activity. Other terms, folate, folates, and folacin, are generally interchangeable. Folic acid was isolated in 1943 by a team at Lederle Laboratories that included E. L. Robert Stokstad, and this isolation was followed by the identification and synthesis of pteroylglutamic acid in 1945.<sup>1</sup> Fifteen years earlier Lucy Wills of the Royal Free Hospital in London had described a “new haemopoietic factor” in yeast that cured tropical macrocytic anemia in India. This unknown substance was referred to as “Wills factor” by Watson and Castle when they confirmed that the new hemopoietic principle was found in a different fraction of liver extract from that which was curative for pernicious anemia. Concurrently, factors identified by other methods were assigned candidate vitamin names during that period of the 1930s when it was still possible to aspire to the identification of a new vitamin. Some of those names included “vitamin M” and “vitamin Bc” relating to assay animals, monkey and chick. The relationship of these factors to other factors such as the growth factor for *Lactobacillus casei* was not appreciated.<sup>2</sup> The term “folic acid” was used by Mitchell et al. in 1941<sup>3</sup> to refer to a growth factor in spinach leaves, thus the term folic acid or folate. Other designations for this yet to be determined growth principle were vitamin B-10 and vitamin B-11, and when folic acid was identified as pteroylglutamic acid, those numerical designations dropped out and thus the next and last vitamin to be identified became vitamin B-12.

The metabolic interactions of vitamin B-12 and folic acid and their common association with megaloblastic anemia were an important part of the history of both vitamins. Only in retrospect was it possible to note that “vitamin Bc” was folic acid, “vitamin Bc conjugate” was folylpolyglutamate, and there was yet an additional “extrinsic factor” or anti-pernicious-anemia factor that would eventually become vitamin B-12. If the first half of this century was the era of the identification of all of the known vitamins, including folic acid, and the elaboration of the use of synthetic forms of the vitamin for the treatment of deficiency disease and anemia, then the second half of the century might be appreciated as the

period when new research on the absorption, metabolism, and assay characteristics of this vitamin has led to an appreciation of its importance in the diet and its potential for adding to the nutritional defenses against cancer, heart disease, stroke, and birth defects.

## Folate Metabolism

**Structure and function of folate coenzymes in one-carbon transfer reactions.** Folates are a family of compounds that have pteroylglutamic (PteGlu) as a common structure (Figure 1). They differ from each other in the pyrazine ring, which can assume different forms of substitutions, and by the p-aminobenzoylglutamate (PABAGlu) moiety, which can contain additional glutamate residues in gamma peptide linkage. The pyrazine ring may be partially reduced at the 7,8 position ( $H_2PteGlu_n$ ) or fully reduced ( $H_4PteGlu_n$ ). Reduction is catalyzed by dihydrofolate reductase (reaction 2, Figure 2), a target enzyme for cancer chemotherapy. Fully reduced folates ( $H_4PteGlu_n$ ) may be present as such or substituted with a carbon unit of various oxidation levels. At the formate oxidation level the substitution may occupy the 5 position (5-formyl- $H_4PteGlu_n$ ), the 10 position (10-formyl- $H_4PteGlu_n$ ), or both positions (5,10-methenyl- $H_4PteGlu_n$ ). Both the 10-formyl and 5,10-methenyl derivatives serve as respective substrates for the synthesis of C-2 and C-8 of purines (reactions 16 and 17, Figure 2).

At the oxidation level of formaldehyde, the substitution occupies both the 5 and 10 positions (5,10-methylene- $H_4PteGlu_n$ ). Unlike other substitutions, 5,10-methylene- $H_4PteGlu_n$  is unstable and readily dissociates to formaldehyde and  $H_4PteGlu_n$ . In spite of this instability, 5,10-methylene- $H_4PteGlu_n$  participates in a number of key reactions. In the de novo synthesis of thymidylate (reaction 14, Figure 2) the methylene group together with two electrons from the pyrazine ring are transferred to form a methyl group that substitutes a hydrogen in the 5 position of the deoxyuridine monophosphate (dUMP). This transfer results in  $H_2PteGlu_n$  as a product. Reduction to  $H_4PteGlu_n$  is a necessary step to start a new cycle of thymidylate or other folate-dependent syntheses. This

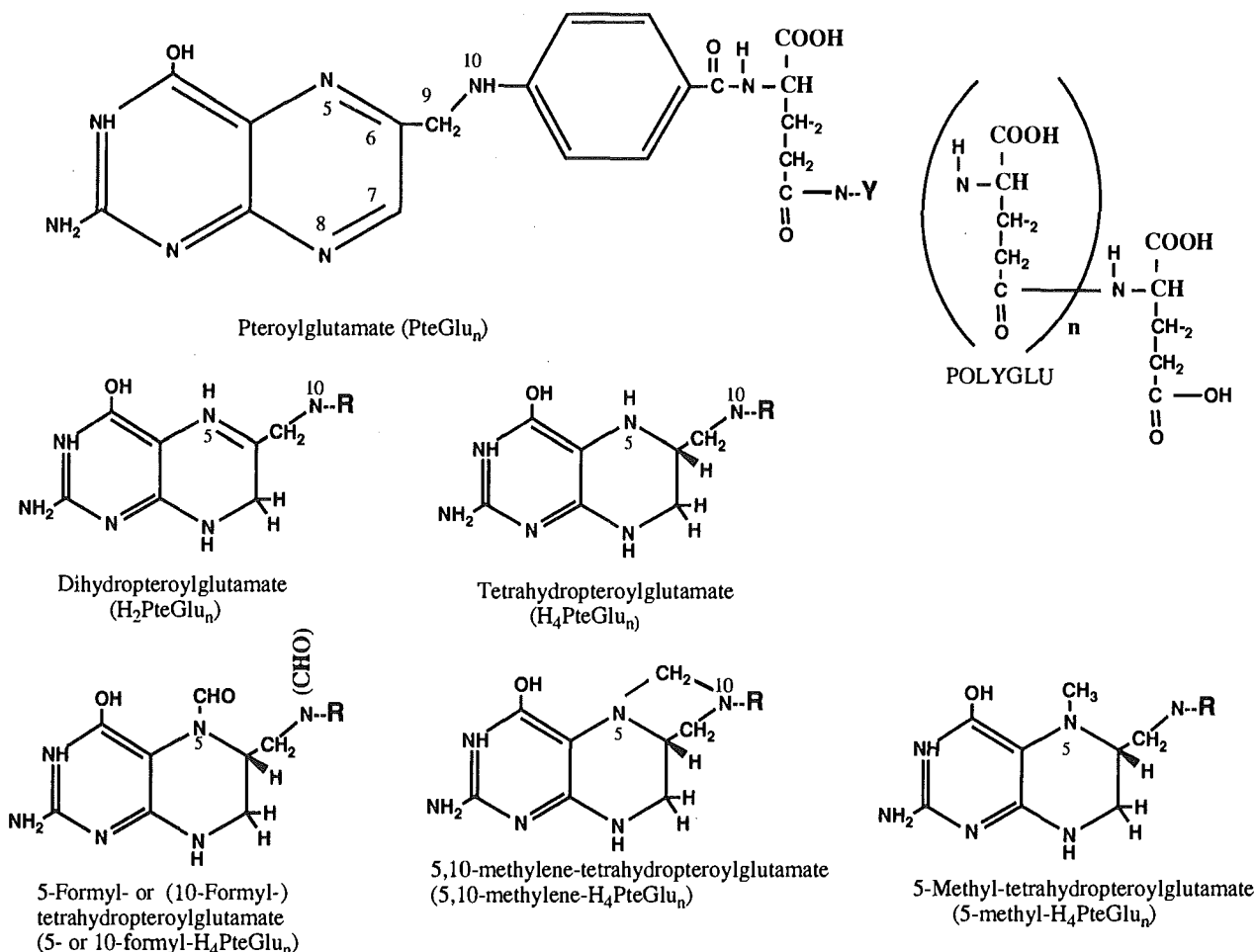


Figure 1. Chemical formulas for pteroylglutamic acid and its derivatives.

necessity provided the basis for choosing dihydrofolate as a target enzyme for cancer chemotherapy. Compounds in the family of antifolate agents, in which the hydroxyl group in the 4 position is replaced by an amino group (aminopterin, methotrexate), are potent inhibitors of dihydrofolate reductase with affinities for the enzyme that are three orders of magnitude higher than the affinity for H<sub>2</sub>PteGlu<sub>n</sub>.

Another reaction for which 5,10-methylene-H<sub>4</sub>PteGlu<sub>n</sub> serves as substrate is the reversible synthesis of serine from glycine (reaction 4, Figure 2)). The importance of this reaction lies primarily in the opposite direction, where a new carbon unit from serine is acquired by folate coenzymes for use in numerous syntheses. Because serine is formed from glucose, such folate-dependent syntheses are de novo syntheses, as opposed to salvage pathways where proteins and nucleic acids, either from the diet or from within the cell, are degraded to release the respective compounds (purines, pyrimidines, methionine etc.).

At the oxidation level of methanol, substitution occurs at the 5 position (5-methyl-H<sub>4</sub>PteGlu<sub>n</sub>). This methyl-tetrahydrofolate serves as the methyl donor for homocysteine methylation to methionine (reaction 7, Figure 2) in a reaction that is catalyzed by a methyltransferase which, in animals, contains methyl-B-12 as the prosthetic group and requires catalytic amounts of S-adenosyl-methionine (SAM).

Circulating folates are monoglutamyl derivatives mostly in the form of 5-methyl-H<sub>4</sub>PteGlu. Within the cell, acquisition of additional glutamates (polyglutamylation, reaction 3, Figure 2) renders these folates incapable of cellular exit unless they are converted back to monoglutamyl derivatives.<sup>4-11</sup> H<sub>4</sub>PteGlu is the preferred substrate for polyglutamylation while 5-methyl-H<sub>4</sub>PteGlu exhibits poor substrate activity.<sup>5-9</sup> The assimilation of circulating folate, i.e., 5-methyl-H<sub>4</sub>PteGlu, by the cell necessitates demethylation. Such a demethylation can occur only through homocysteine methylation catalyzed by the B-12-dependent methyltrans-

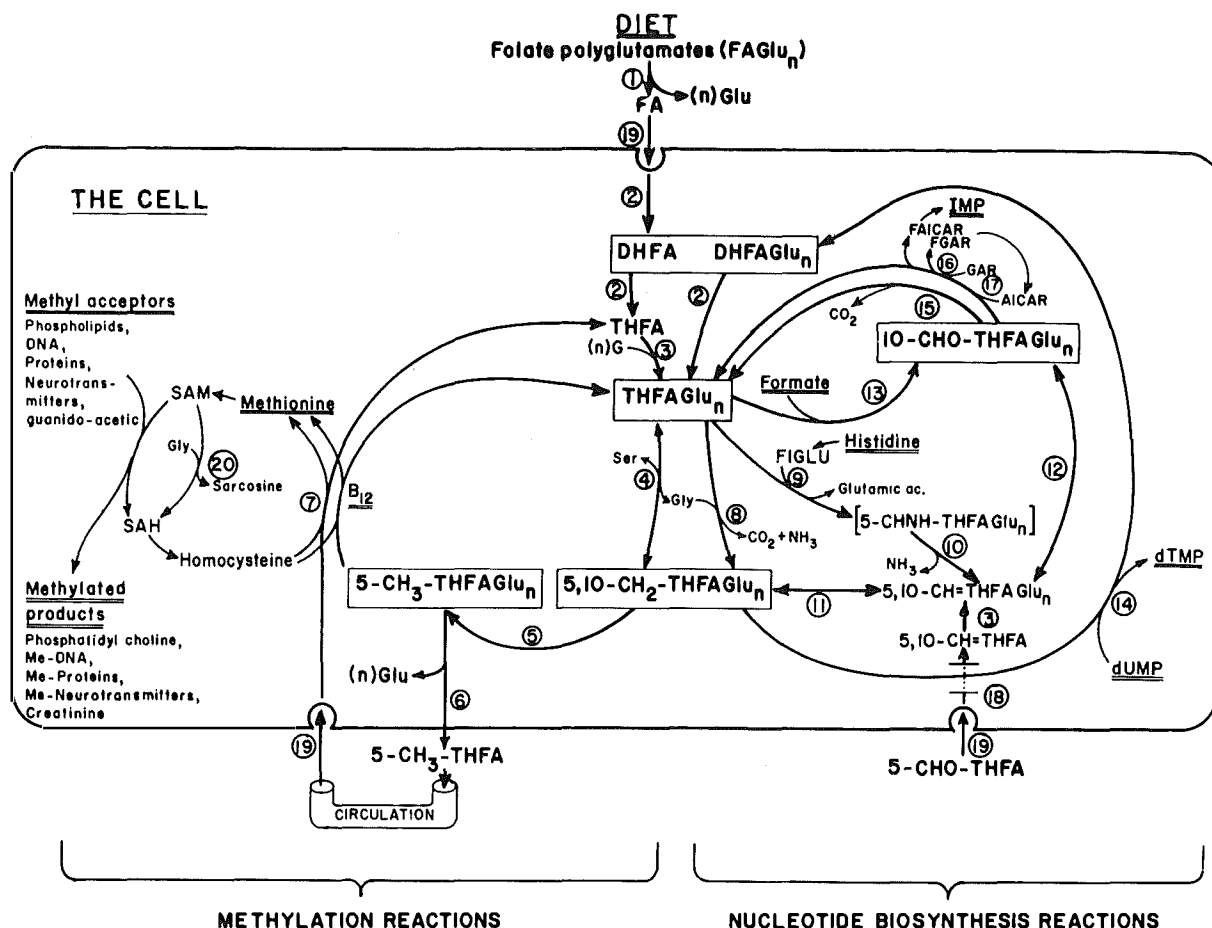


Figure 2. Enzymes and reactions of folate metabolism: 1)  $\gamma$ -glutamyl hydrolase (brush border?) (EC 3.4.22.12), 2) dihydrofolate reductase (EC 1.5.1.3), 3) folylpoly-glutamate synthase (EC 6.3.2.17), 4) serine hydroxymethyl transferase (EC 2.1.2.1), 5) methylenetetrahydrofolate reductase (EC 1.7.99.5), 6)  $\gamma$ -glutamyl hydrolase (lysosomal?) (EC 3.4.22.12), 7) cobalamin-dependent methionine synthase (EC 2.1.1.13), 8) glycine cleavage enzyme system (EC 1.4.4.2;2.1.2.10), 9) glutamate formiminotransferase (EC 2.1.2.5), 10) formiminotetrahydrofolate cyclodeaminase (EC 4.3.1.4), 11) methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5), 12) methylenetetrahydrofolate cyclohydrolase (EC 3.5.4.9), 13) formyltetrahydrofolate synthetase (EC 6.3.4.3), 14) thymidylate synthase (EC 2.1.1.45), 15) formyltetrahydrofolate dehydrogenase (EC 1.5.1.6), 16) phosphoribosyl glycinamide (GAR) formyl transferase (EC 2.1.2.2), 17) phosphoribosyl aminoimidazole carboxamide (AICAR) formyl transferase (EC 2.1.2.3), 18) 5-formyltetrahydrofolate cycloligase (EC 6.3.3.2), 19) folate/MTX transport mechanism, and 20) glycine methyl transferase (EC 2.1.1.20).

ferase. Cellular folate depletion is one consequence of vitamin B-12 deficiency.

Polyglutamylation is also associated with greater affinity of enzymes toward their respective substrates, and if a particular substrate acts as an inhibitor of a particular reaction, inhibition potency is increased with increase in the number of glutamate residues.<sup>5-12</sup>

The mechanism of polyglutamylation is consistent with findings showing that the majority of cellular folates contain a total of five or six glutamate residues. However, conditions (folate deficiency, alcoholism, methotrexate therapy, and others) have been described that were found to be associated with elongation of the glutamate chains. The mechanistic basis for these extensions of the glutamate chain remains uncertain.

The involvement of folate in the synthesis of purines, thymidylate, methionine, and serine-glycine interconversion lies in the propensity of  $H_4PteGlu_n$  to serve both as a one-carbon acceptor and a one-carbon donor, and also in the capacity of the cell of folate coenzyme interconversions.

The acquisition of a carbon unit by  $H_4PteGlu_n$  occurs primarily through the transfer of serine 3-carbon and formation of 5,10-methylene- $H_4PteGlu_n$  (reaction 4, Figure 2). Aside from serving as the source of the methyl group for thymidylate synthesis, the methylene group of 5,10-methylene- $H_4PteGlu_n$  may undergo oxidation to formate or reduction to methanol levels of oxidation.

Oxidation is a reversible reaction that results in the formation of 5,10-methenyl- $H_4PteGlu_n$  (reaction 11,

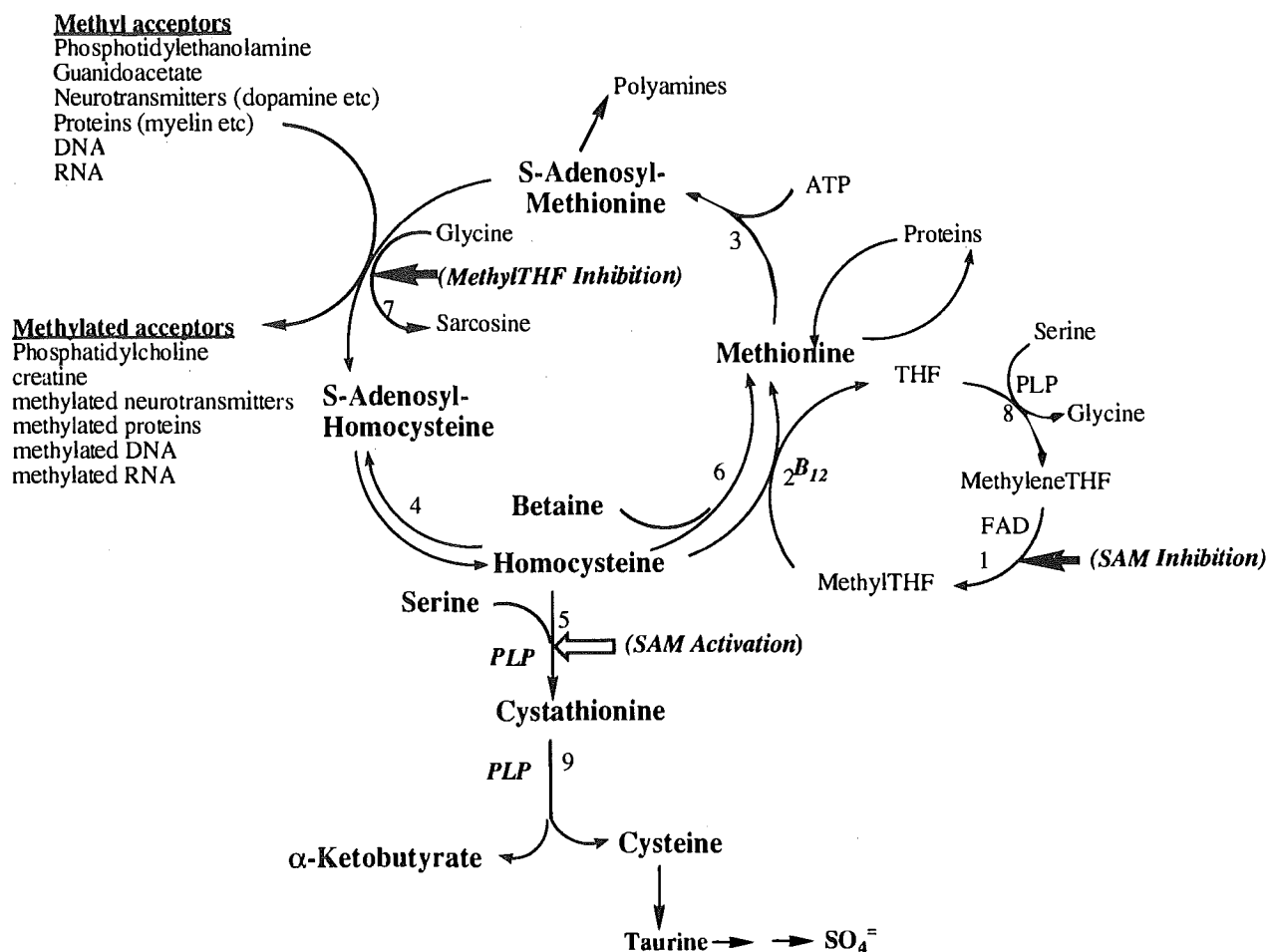


Figure 3. Homocysteine metabolism in animals. Enzyme reactions that are regulated by SAM and 5-methyltetrahydrofolate (Methyl-THF) are indicated by large arrows. Open arrows indicate activation, closed arrows indicate inhibition. Enzymes: 1) 5,10-methylenetetrahydrofolate reductase; 2) N-5-methyltetrahydrofolate:homocysteine methyltransferase; 3) S-adenosylmethionine synthase; 4) S-adenosylhomocysteine hydrolase; 5) cystathionine  $\beta$ -synthase; 6) betaine:homocysteine methyltransferase; 7) glycine N-methyltransferase; 8) serine hydroxymethylase; 9)  $\gamma$ -cystathionase.

Figure 2). This reaction is catalyzed by a trifunctional enzyme ( $C_1$ -THF synthase) that, in addition to the methylenetetrahydrofolate dehydrogenase that is responsible for oxidation, contains methenyltetrahydrofolate cyclohydrolase (reaction 12, Figure 2) and formyltetrahydrofolate synthase (reaction 13, Figure 2). Reactions 11 and 12 provide the substrates for purine synthesis.

The reduction of 5,10-methylene- $H_4$ PteGlu<sub>n</sub> to 5-methyl- $H_4$ PteGlu<sub>n</sub> (reaction 5, Figure 2) is a physiologically irreversible reaction. Irreversibility renders the regeneration of  $H_4$ PteGlu<sub>n</sub> dependent on the methylation of homocysteine to methionine by the B-12-dependent methyltransferase.

**Regulatory mechanisms.** The realization that folates are involved in a number of diverse reactions has prompted efforts to investigate regulatory mechanisms of folate metabolism. According to Krumdieck,<sup>13</sup> folate

metabolism can be regarded as two crucial groups of reactions that compete in the cell for available folates: reactions that lead to the de novo synthesis of methionine and those that lead to the synthesis of nucleic acids (purines and thymidylate). According to this hypothesis, the synthesis of methionine is the more crucial of the two. In addition to its function as a building block for proteins and as source of polyamines, methionine is the precursor of SAM, the universal methyl donor for >100 reactions. Many of these reactions, e.g., phospholipid synthesis and creatine-phosphate synthesis, are vital to the survival of the cell and the organism. This "tug of war" theory proposes that when folate is in short supply, folate coenzymes are directed toward methionine synthesis at the expense of nucleic acid synthesis. The above hypothesis assumes the existence of a coordination between nucleic acid synthesis and methionine synthesis. Such existence is yet to be proven.

The methyl folate trap hypothesis proposes that in vitamin B-12 deficiency, the combination of impaired B-12-dependent methyltransferase and irreversibility of the methylenetetrahydrofolate reductase-dependent reaction will lead to the trapping of folates as 5-methyl- $H_4PteGlu_n$ , resulting in the impairment of other folate-dependent syntheses, e.g., thymidylate and purines.<sup>14</sup> This hypothesis explains the common clinical symptoms (i.e., megaloblastic anemia) seen in folate and vitamin B-12 deficiencies. A modified version of the "methyl folate trap hypothesis" proposed by Scott and Weir<sup>15</sup> was constructed to account for the early reports which showed that large doses of folic acid could ameliorate the hematological symptoms seen in B-12-deficient patients but may mask or may even precipitate neurological manifestations. According to this hypothesis, the normal response of the cell to a low methionine level is an increase in methyltetrahydrofolate synthesis. This is because SAM, a potent inhibitor of this synthesis, is also at a low level. As a consequence, folate coenzymes are diverted away from nucleic acids synthesis and toward increased methionine synthesis.

The cell responds to vitamin B-12 deficiency in the same manner as when methionine is limited. However, because in B-12 deficiency the (B-12-dependent) methylation of homocysteine is impaired, the 5-methyl- $H_4PteGlu_n$  formed will accumulate (be trapped) because other folate coenzymes are needed for nucleic acid synthesis, and their absence will result in the cessation of cell proliferation (megaloblastic anemia). The limited methionine that is available is now directed for methylation reactions, which include brain myelin. Intake of large doses of folic acid will provide the necessary coenzymes for nucleic acid synthesis and cell proliferation. Since methionine synthesis is still limited, the increase in cell proliferation will divert the limited supply of methionine away from methylation reactions and toward protein building blocks for newly formed cells. Brain myelin may become increasingly unmethylated under these conditions.

In recent years interest in plasma homocysteine has been growing because epidemiologic data link moderate elevations of plasma homocysteine (hyperhomocysteinemia) to higher prevalence of occlusive vascular disease and stroke.<sup>16-20</sup> Homocysteine is a sulfur amino acid whose metabolism is at the intersection of two metabolic pathways, remethylation and transsulfuration (Figure 3). In remethylation, homocysteine acquires a methyl group from 5-methyl- $H_4PteGlu_n$  or from betaine to form methionine. The reaction with 5-methyl- $H_4PteGlu_n$  occurs in all tissues and is vitamin B-12-dependent, while the reaction with betaine is confined mainly to the liver and is independent of vitamin B-12. A considerable proportion of methionine is then activated by ATP to form SAM. SAM serves primarily

as a universal methyl donor to a variety of acceptors including guanidinoacetate, nucleic acids, neurotransmitters, phospholipids, and hormones. S-adenosylhomocysteine (SAH), the by-product of these methylation reactions, is subsequently hydrolyzed, thus regenerating homocysteine, which then becomes available to start a new cycle of methyl-group transfer.

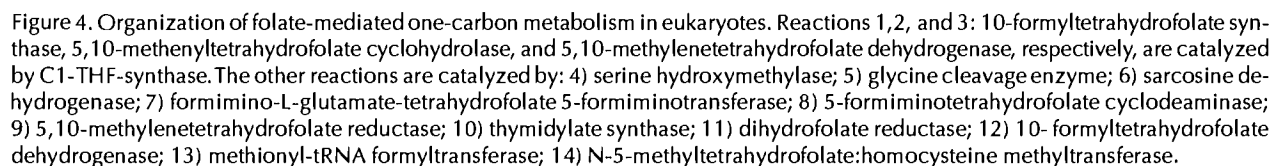
In the transsulfuration pathway, homocysteine condenses with serine to form cystathionine in an irreversible reaction catalyzed by the pyridoxal-5'-phosphate (PLP)-containing enzyme, cystathionine  $\beta$ -synthase (CBS). Cystathionine is hydrolyzed by a second PLP-containing enzyme,  $\gamma$ -cystathionase, to form cysteine and  $\alpha$ -ketobutyrate. Excess cysteine is oxidized to taurine or inorganic sulfates and excreted in the urine. Thus, in addition to the synthesis of cysteine, this transsulfuration pathway effectively catabolizes excess homocysteine that is not required for methyl transfer. Note that since homocysteine is not a normal dietary constituent, the dietary precursor of homocysteine is methionine.

The two pathways are coordinated by SAM. This function of SAM stems from its ability to serve as an allosteric inhibitor of methylenetetrahydrofolate reductase and as an activator of CBS.<sup>19-24</sup> Thus, when intracellular SAM concentration is high, homocysteine is diverted through the transsulfuration pathway because synthesis of 5-methyl- $H_4PteGlu_n$  is inhibited and the CBS enzyme is activated. Conversely, when SAM level is low, homocysteine is diverted toward remethylation because synthesis of 5-methyl- $H_4PteGlu_n$  is uninhibited and the activity of CBS is diminished.

A second tier of coordination lies in the propensity of 5-methyl- $H_4PteGlu_n$  to serve as an inhibitor of glycine N-methyltransferase.<sup>25,26</sup> This enzyme serves to regulate the level of cellular SAM. A high intracellular level of SAM will result in an inhibited synthesis of 5-methyl- $H_4PteGlu_n$ , and that will release glycine N-methyltransferase from inhibition and allow it to rid the cell of excess SAM. When the concentration of 5-methyl- $H_4PteGlu_n$  is high because of low intracellular SAM level, glycine methylation is inhibited.

**Folate compartmentation.** Another important method of folate regulation is through compartmentation based on 1) asymmetric distribution of folate coenzymes between cytosol and mitochondria and 2) differential distribution of specific folate coenzymes because of binding proteins.

In liver cells the major pool of folate coenzymes are polyglutamyl derivatives, which are distributed in both the cytosol and mitochondria (Figure 3).<sup>27</sup> In cytosol 45% of total folates are methyl- $H_4PteGlu_n$ , 30% are formyl- $H_4PteGlu_n$ , and 25% are unsubstituted  $H_4PteGlu_n$ . In mitochondria only 7% of total folates are methyl- $H_4PteGlu_n$ ; the remainder are distributed between formyl- $H_4PteGlu_n$  (44%) and unsubstituted  $H_4PteGlu_n$  (48%).<sup>28</sup>



ation of initiator transfer RNA for organelle protein synthesis (Figure 4).<sup>35</sup>

Folate-binding proteins (FBP) are either membrane-bound or intracellular.<sup>36-38</sup> The membrane-bound proteins are thought to be involved in folate transport. The high-affinity FBP is located in the brush-border membranes of the proximal tubular cells of kidney and was shown to mediate transport of folate through an endocytic pathway.<sup>39-41</sup> This protein is also found in the choroid plexus and outer placental membranes, where it is thought to function in a similar manner to that in the kidney.<sup>42,43</sup> These high-affinity FBPs appear to transport 5-methyl tetrahydrofolate selectively; hence they are in a way responsible for folate compartmentation. Other folate transport proteins with lower affinity have been described. The recently isolated reduced folate transporter is responsible for the cellular sequestration of reduced folates and methotrexate but not oxidized folates.<sup>37,38</sup>

Intracellular FBPs are distributed in both mitochondria and cytosolic compartments.<sup>44</sup> Two of the three FBPs in the cytosol have been identified as the enzymes 10-formyltetrahydrofolate dehydrogenase and glycine N-methyltransferase.<sup>45,46</sup> The mitochondrial FBPs were shown to correspond to dimethylglycine dehydrogenase and sarcosine dehydrogenase.<sup>47</sup>

Glycine N-methyltransferase, which catalyzes the methylation of glycine by SAM, contains bound 5-methyl- $H_4PteGlu_5$ , which also acts as an inhibitor of this reaction.<sup>25,46,48</sup> It has been postulated that this inhibition of the glycine N-methyltransferase enzyme serves to regulate the level of SAM. Both of the mitochondrial FBPs catalyze the oxidative demethylation of their respective substrates to yield free formaldehyde. In the presence of their ligand ( $H_4PteGlu_5$ ) formaldehyde is trapped as 5,10-methylene- $H_4PteGlu_5$  for use in C1-dependent metabolism.

In liver 60% of cytosolic folates and 20% of mitochondrial folates were found to be bound to intracellular FBPs.<sup>49</sup> It is suggested, however, that much cellular folate is bound to the enzymes of folate metabolism, and this binding is important to the sequestration of folate co-enzymes for more efficient syntheses, particularly under conditions of limited folate supply.<sup>50-52</sup>

In substrate channeling, the intermediate products of a set of reactions are sequestered into the active site of the next enzyme without a dissociation step.<sup>27</sup> This phenomenon is caused by either a multifunctional enzyme or multienzyme complexes. Among the advantages predicted for such channeling is the high conversion rate that can be attained because the reactions are independent of bulk substrate concentrations and because the reaction intermediates are protected so they are not used for competing reactions. Channeling is susceptible to regulation through changes in the nature and extent of enzyme associations. C1-THF synthase is a trifunctional enzyme with two independent functional domains. One domain contains an overlapping active site shared by methylene-tetrahydrofolate dehydrogenase (reaction 3, Figure 4) and methenyl-tetrahydrofolate cyclohydrolase (reaction 2, Figure 4) on the  $NH_2$  terminal. The other domain contains 10-formyl-tetrahydrofolate synthase activity in the  $COOH$  terminal (reaction 1, Figure 4). Channeling occurs between reactions 2 and 3 in the first domain (but not between the two domains), so that when 5,10-methylene- $H_4PteGlu_n$  is used as substrate, no lag period is observed before the final product, 10-formyl- $H_4PteGlu_n$ , appears. Intermediates in these series of reactions (e.g., methenyl- $H_4PteGlu_n$ ) do not compete.

## Tissue and Food Folate Analysis

Naturally occurring folates are present in a variety of forms that differ by the state of oxidation and carbon

substitution of the pteridine ring and the number of glutamate residues in a  $\gamma$ -peptide linkage with the p-aminobenzoylglutamate moiety. Considerable effort has been directed to devising methods for the analysis of tissue and food folate distribution on the premise that information on folate distribution could provide the basis for understanding folate-dependent metabolism in tissues and folate bioavailability in food products. Initial information on the diversity of tissue and food folate distribution was derived from the use of the differential microbial assay.<sup>53</sup> The assay is based on the differing capacity of bacterial species to utilize different forms of folates. However, because bacteria cannot utilize polyglutamyl folates, extracts require prior treatment with pteroylpolyglutamate hydrolases (e.g., conjugase) before assay. The information provided by the differential microbial assay is thus limited.

**The use of thymidylate synthase to determine 5,10-methylenetetrahydrofolates.** An electrophoretic assay method developed by Priest et al. is based on the incorporation of 5,10-methylene- $H_4PteGlu_n$  derivatives into a ternary complex with thymidylate synthase and 5-fluoro-2-deoxy[ $^3H$ ]uridylylate.<sup>54,55</sup> Folate with different pteridine ring structures (folic acid, dihydro- and tetrahydrofolate, formyl, and methyltetrahydrofolates) can be identified following their conversion to 5,10-methylene- $H_4PteGlu_n$  using selective combinations of chemical and enzymatic reactions.

An important extension of this procedure is the ability to analyze folate polyglutamates. At pH 8.2 the same ternary complex is negatively charged to an extent that is determined by the number of glutamate residues of the attached folate. The higher the number of residues the more negatively charged the complex, which allows separation by gel electrophoresis. Separated folates are quantified by densitometry after autoradiography.<sup>54,55</sup> This method is suitable for the analysis of folate in small tissue samples, as in cell culture, and it is the only method capable of determining 5,10-methylene-tetrahydrofolate derivatives. However the need to convert all folates quantitatively to 5,10-methylene-tetrahydrofolates renders this method quite tedious. Recently this method was used with some modifications for the analysis of rat liver folates.<sup>56</sup>

**Analysis of the glutamic acid chain length distribution.** Analysis of the glutamic acid chain length distribution requires cleavage of the folate molecule at the C9-N10 bond and detection of the resulting p-aminobenzoylglutamate moieties as naphthylethylenediamine azo dye derivatives. Krumdieck et al.<sup>57</sup> have taken advantage of the different susceptibility of reduced folates (with and without one-carbon substitution) for oxidative cleavage to develop a differential cleavage approach which divides folates into three pools: pool 1 includes 5,10-methylene-THF, THF, and DHF derivatives; pool

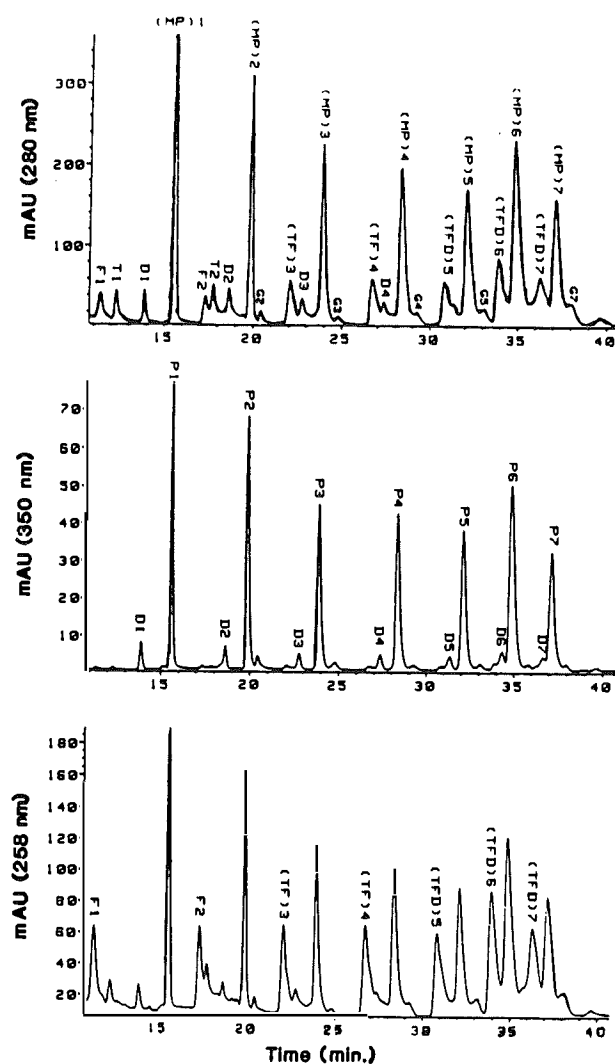


Figure 5. Chromatography of a mixture that contained PteGlu<sub>1-7</sub> and its various reduced forms, a total of 35 derivatives. Activities shown in the top panel represent absorption values at 282 nm, those in the middle panel 350 nm, and those in the bottom panel 258 nm. Symbols are as follows: F, 10-formyl-H<sub>4</sub>PteGlu<sub>1-7</sub>; T, H<sub>4</sub>PteGlu<sub>1-7</sub>; M, 5-methyl-H<sub>4</sub>PteGlu<sub>1-7</sub>; and P, PteGlu<sub>1-7</sub>. Numbers following symbols refer to numbers of glutamate residues. Reprinted with permission from reference 59.

2 consists of only 5-methyl-THF derivatives; and pool 3 consists of the various isomers of formyl-THF and 5-formimino-THF derivatives.

**Combined-affinity chromatography and reverse-phase liquid chromatography (affinity/HPLC) for the analysis of tissue folate distribution.** The affinity/HPLC method for folate analysis is based on the use of affinity chromatography for folate purification and a combination of HPLC separation and spectral analyses with a UV diode array detector.<sup>58,59</sup> Affinity chromatography uses a small column containing purified

milk folate-binding protein attached to a Sepharose-4B as the affinity matrix. The HPLC system employs a reverse-phase C<sub>18</sub> column and a mobile phase that is composed of an acetonitrile gradient and tetrabutyl amino phosphate as an ion-pairing reagent. Figure 5 is the chromatographic pattern of a mixture of 35 forms of folate (with and without a carbon substitution) with glutamate chains ranging from one to seven. The chromatographic pattern is composed of seven clusters representing groups of folates with increasing numbers of glutamate residues. In clusters containing mono- and diglutamyl folates there was a separation between the 10-formyl- (F), the tetrahydro- (T), and the dihydro- (D) derivatives. In clusters containing higher numbers of residues these three folates tended to elute in the same fraction. In each cluster PteGlu<sub>n</sub> (P) and corresponding 5-methyl-H<sub>4</sub>PteGlu<sub>n</sub> (M) eluted in the same peak, which was separate from the above three folates. The position of the eluting folate within the cluster provides a first screening for determining the pteridine ring structure. Spectral characteristics from the diode array detector provide the additional detail for final identification. Quantification of the activity peaks is based on individual peak area determined at 280, 258, and 350 nm (Figure 5), and corresponding "peak area extinction coefficients" are determined for each folate at these wavelengths.<sup>59</sup>

This new method of folate analysis has been applied to analysis of mammalian tissues and food products having diverse folate distribution.<sup>60-64</sup> The compositions determined by this method correspond closely with those published using other methods of analysis. Furthermore, the total folate contents estimated by summation of the concentration of the individual components closely correlate ( $r=0.954$ ) with those obtained from the microbial assay using *Lactobacillus casei* after conjugase treatment.<sup>63</sup> This method is technically simple and quite rapid. A total of 24-30 analyses can be accomplished within a week by one person.

## Functional Assessment of Folate Status

Folate deficiency can arise in a variety of settings, including alcoholism, low dietary intake, or malabsorption. It is seen under conditions of increased cellular turnover such as pregnancy, cancer, and hemolytic anemia. Several drugs, including sulfasalazine and diphenylhydantoin, interfere with folate absorption or use.

Advanced folate deficiency characteristically causes macrocytic or megaloblastic anemia, with abnormalities on peripheral blood smear and bone marrow examinations. Clinical manifestations of folate deficiency often resemble the hematologic features of vitamin B-12 deficiency. Status of both folate and B-12 should be evaluated in suspected cases.

Table 1. Laboratory assessment of folate nutriture

Measurement	Deficient levels	Low levels	Acceptable levels
Serum folate (nmol/L)	<6.7	6.7–13.2	>13.5
Red cell folate (nmol/L)	<315	315–358	>360

**Laboratory tests.** Measurement of serum folate is the most widely used method to assess folate status. For most clinical situations it is adequate because low body stores are reflected in low folate levels. A low serum folate level is not always a reflection of depleted body stores, however, because serum folate is sensitive to changes in intakes or temporary changes in folate metabolism.

Since red blood cell folate content is at least ten times the concentration of serum folate, another method of assessing folate status is the measurement of whole-blood folate. This method obviates the need to separate the two blood constituents. The serum acts as a source of conjugase to hydrolyze red cell folylpolyglutamates before microbial assay.<sup>65</sup> A standard method to determine folate levels is by microbial assay with *Lactobacillus casei*. Serum folate levels are determined directly while red cell levels are determined after treatment with conjugase. The method is sufficiently simple for routine use, but it is inadequate for blood samples containing antibiotics or methotrexate, compounds that interfere with bacterial growth.

In recent years, a widely applied technique is to use the high-affinity binding protein derived from milk to determine folate by competitive binding assay.<sup>66</sup> This method is not sensitive to antibiotics or methotrexate. It requires the use of a high pH in the incubation mixture because at physiologic pH the affinity for 5-methyltetrahydrofolate, the main constituent of serum folate, is much lower than for folic acid, which is used as the radioactive standard.<sup>67</sup> Before analysis serum samples must be boiled to destroy endogenous folate-binding protein.

**Functional and synthetic product assays.** Folate deficiency is associated with alteration of a number of biochemical reactions. The metabolism of histidine to glutamic acid is impaired in folate deficiency, which causes an increase in urinary excretion of formimino-glutamic acid (FIGLU) and urocanic acid. This occurs particularly after a loading dose (2–15 mg) of L-histidine.<sup>68</sup>

Folate deficiency also impairs the de novo synthesis of adenine compounds, and in consequence, there is an increased urinary excretion of adenine precursors such as aminoimidazolecarboxamide.<sup>68</sup>

The deoxyuridine suppression test is based on the capacity of a cell system to synthesize thymidylate de novo from added deoxyuridine (dUMP), a folate-dependent pathway, thereby diminishing (or competing for) incorporation of radioactive thymidine into cellu-

lar DNA. In folate deficiency, suppression by deoxyuridine of [<sup>3</sup>H]thymidine incorporation is diminished. The method uses aspirates of bone marrow or peripheral lymphocytes.<sup>69,70</sup>

Another functional test of folate status uses lymphocytes from patients to determine incorporation of [<sup>14</sup>C]formate into serine or methionine in the presence of excess glycine or homocysteine.<sup>71</sup> The method depends upon the presence of tetrahydrofolate coenzymes; hence in deficiency states a decrease in the incorporation of the radioactive formate is observed. The laboratory tests of folate status are summarized in Table 1.

The recognition of plasma homocysteine levels as a functional indicator of folate status stemmed principally from the recognition that moderate elevations of plasma homocysteine levels are associated with increased risk of occlusive vascular disease and stroke.<sup>16–19</sup> Vitamin status and plasma homocysteine concentrations are inversely correlated in nonlinear fashion.<sup>72,73</sup> There is a plasma folate level ( $\approx 6$  ng/mL) beyond which homocysteine concentrations remain unchanged and below which concentrations increase. Similar though weaker correlations have been observed between (nonfasting) plasma homocysteine and vitamin B-12 and PLP plasma concentrations.<sup>72,74</sup>

Plasma homocysteine also may serve to assess the adequacy of folate intake. Data from the Framingham Study demonstrated a strong inverse correlation between folate intake (estimated by food frequency questionnaire) and plasma homocysteine levels. The pattern is similar to that of plasma folate.<sup>72</sup> An estimated intake of  $\approx 400$   $\mu$ g/day appears to be adequate for maintaining low homocysteine plasma levels.

## Folate Absorption and Food Folate Bioavailability

Much of our present knowledge of intestinal absorption of folates is derived from studies that used crystalline folate compounds.<sup>75–78</sup> These studies can be summarized as follows: 1) Intestinal folate absorption occurs at the monoglutamate level; hence hydrolysis of folylpolyglutamates to monoglutamyl derivatives is a necessary step for this process. 2) In man and pig the brush-border membrane of the small intestine contains a pteroylpolyglutamate hydrolase (PPH) that is distinct from that found within the enterocyte, by its pH requirement, size, and sensitivity to inhibitors.<sup>79</sup> It

is suggested that in man and pig this brush-border enzyme is, at least in part, responsible for the hydrolysis of dietary folate polyglutamates.<sup>79,80</sup> 3) The intestinal transport of monoglutamyl folates occurs by a carrier-mediated process that is equally shared by reduced and oxidized monoglutamyl derivatives as well as by the antifolate methotrexate. This transport is highly pH dependent, with an optimum pH of 5.0–6.0, and declines sharply with increase in pH. A second transport mechanism is simple diffusion. This transport predominates at higher luminal pH or at pharmacological folate concentrations.

In spite of this information our knowledge of the bioavailability of food folates is fragmentary. The paucity of information stems principally from the complexity of dietary folates, their low level in various foods, the presence of dietary factors such as inhibitors of PPH that could affect folate bioavailability, and the inability to distinguish between newly absorbed folate and endogenous folates. The use of deuterated folate for studying intestinal folate absorption in man is a step in the right direction.<sup>81</sup> The use of these folates in their crystalline form has provided valuable new information about the characteristics of intestinal folate absorption in man.<sup>82,83</sup> Deuterated folates were used for a long-term study to determine body pool and body turnover of folates.<sup>84</sup> The possibility of applying this approach to the study of a variety of parameters, including assessment of folate status and folate requirement, is quite exciting. However, exactly how these deuterated folates can be used to study the absorption of food folates remains to be determined.

## Folic Acid and Disease

Beginning with the association of folic acid with macrocytic anemia and even its mistaken role as the anti-pernicious anemia principle in the 1930s, the primary clinical relationship of folic acid has been its hemopoietic effect in the prevention and treatment of macrocytic or megaloblastic anemia. Given the additional nutritional stress of pregnancy, folic acid deficiency and megaloblastic anemia of pregnancy continue to be of worldwide importance. The effects of excessive alcohol and alcoholism on the intake, absorption, metabolism, and utilization of folate have made that pathogenic relationship important. These relationships of folic acid to macrocytic anemia have been well described in previous issues of *Present Knowledge in Nutrition* and in numerous reviews. In the spirit of presenting present and newly emerging knowledge about folic acid nutrition in the clinical arena, we will address some emerging information on inherited abnormalities of folate metabolism and the connection of folic acid with neural tube defects, cancer, and heart disease.

**Folate interaction with a common mutation.** Like many other congenital disorders, those that are caused by abnormalities in folate metabolism are rare and are in the domain of specialists in genetic and metabolic disorders.<sup>85</sup> In 1988 Kang et al.<sup>86</sup> reported a variant of methylenetetrahydrofolate reductase (MTHFR) that was distinguishable from the normal enzyme by its lower specific activity and its heat sensitivity (thermolability). In subsequent studies the authors suggested that this thermolabile variant was an inherited autosomal recessive trait present in ≈5% of the general population and in 17% of patients with coronary artery disease.<sup>87</sup> A group headed by Rozen<sup>88</sup> isolated the cDNA for human MTHFR and demonstrated that the thermolability is caused by a missense mutation of alanine (ala) to valine (val). Using PCR techniques, a number of laboratories have shown that the frequency in the normal population of homozygous mutant genotype is higher than that predicted by the enzymatic studies of Kang et al.<sup>86</sup> and stands at 12%.

It is too early to predict the importance of this mutation in the prevalence of disease. MTHFR thermolability may be associated with moderate hyperhomocysteinemia.<sup>89</sup> Early evidence implies a higher prevalence of homozygotes for alanine to valine mutations among mothers who have given birth to a child with neural tube defect and also among people with cardiovascular disease.

**Folate and cancer.** Early clinical studies of folic acid suggested a relationship between megaloblastic morphologic changes in tissues of individuals with folate deficiency and the appearance of precancerous cells. In 1954 Massey and Rubin<sup>90</sup> postulated that cytologically abnormal cells in the stomach might represent a transitional cell type between those cells that characterize the atrophic gastric epithelium in folate deficiency or pernicious anemia, and gastric cancer cells. Two decades later, prospective controlled clinical intervention trials by investigators at the University of Alabama found regression of dysplasia of epithelial cells in the uterine cervix and reversion of metaplasia in tracheobronchus epithelial cells.<sup>91,92</sup> Studies such as these are potentially confounded by the cytological similarities of megaloblastic and dysplastic cells. Since the Alabama studies several more epidemiologic studies have related folate nutrition to precancerous dysplastic changes in cervical epithelium and the colorectal epithelium.<sup>93–105</sup> Animal studies have demonstrated that carcinogen-induced colon cancer is induced more quickly and with greater severity in folate-deficient animals.<sup>104</sup> The relationship of folate to DNA methylation, DNA repair, and oncogene expression have provided the basis for hypotheses relating folate nutriture to cancer risk and prevention. Several trials now underway are exploring the possibility that increased folate intake or supplementation may lessen the risk of devel-

oping cancer in stomach and colon or the risk of precancerous colonic polyps progressing to colon cancer.

The most convincing epidemiologic evidence of an association between folate status and colorectal neoplasia was published after a study of >25,000 people showed that increased folate intake was inversely associated with the incidence of adenomatous polyps in the distal half of the colorectum.<sup>105</sup> Thus, a growing body of clinical studies suggest that folate status can modulate the process of carcinogenesis. The evidence is strongest with respect to colorectal cancer, where the effects occur with relatively modest alterations in folate status and may be present when vitamin status is altered within the range of what is presently considered to be normal.

**Folate and neural tube defects.** One of the most compelling studies relating vitamins and disease was the randomized controlled clinical trial which showed that a nutritional supplement containing folic acid successfully and markedly reduced the occurrence of neural tube defects (spina bifida or anencephaly) in babies when the intervention was administered before conception.<sup>106</sup> This was the culmination of a quarter century of research dominated by observational studies showing that folate supplements protected against some neural tube defects. Another randomized controlled clinical trial had shown that large doses of folic acid (4 mg) administered before pregnancy could successfully prevent the recurrence of a neural tube defect in children born to mothers who had previously had a child with such a defect.<sup>107</sup> Such studies have given rise to recommendations in several countries, including the United States, that women capable of becoming pregnant should consume 400 µg of folate daily. In several countries fortification of flour is under consideration as one approach, along with improved diet, to ensure adequate folate intake. The mechanism by which increased folate intake might affect neural tube defect continues to be uncertain. It is almost certain that neural tube defect occurs as a result of complex genetic and nutritional interactions. Some genetic alterations that have been proposed as participants in the pathogenesis are abnormalities of the methionine synthase gene and polymorphisms of methylene tetrahydrofolate reductase (MTHFR) gene. Preliminary studies suggest that the heat-labile variant of MTHFR is associated with risk of neural tube defect (Blom et al., personal communication). The possibility that elevated homocysteine levels secondary to nutritional or genetic abnormalities might have toxic effects on the fetus at the critical time of neural tube closure is currently under investigation.

**Folate, homocysteine, and cardiovascular disease and stroke.** The association between plasma homocysteine concentration and thrombo-occlusive heart disease has recently become the subject of many clinical studies that link moderate elevations of plasma homocysteine to symptomatic peripheral vascular, cerebrovascular, and

coronary heart disease.<sup>16-18</sup> Pooled results from retrospective studies indicate that fasting plasma homocysteine concentrations in patients with vascular disease are on average 31% higher than in normal subjects. Abnormal plasma homocysteine concentration after an oral methionine challenge is 12 times more prevalent in patients with cardiovascular disease than in normal subjects. Prospective studies of middle-aged male physicians in the United States indicated that plasma homocysteine concentrations of only 17 µmol/L, or 12% above the upper limit of normal, were associated with a 3.4-fold increase in the risk of acute myocardial infarction.<sup>18</sup> In the Framingham Heart Study, the longest observed cohort study on vascular disease, it has been demonstrated that folic acid, vitamin B-12, and vitamin B-6 are determinants of plasma homocysteine levels, with folic acid showing the strongest association (in this and several other studies).<sup>108,109</sup> In that same population, ultrasound demonstrated that homocysteine plasma levels were strong predictors of carotid artery narrowing. Such narrowing is predictive of the risk of both stroke and coronary disease. The pathogenetic mechanisms by which plasma homocysteine may be associated with increased thrombo-occlusive vascular disease have not been clearly established. Possible mechanisms include direct toxic effects of the sulfur amino acid on vascular endothelium, prothrombotic or antithrombotic effects on platelets or clotting control mechanisms, and the induction of changes in vascular reactivity.

Numerous studies have demonstrated that homocysteine levels are responsive to intervention, the most effective vitamin intervention being folic acid, with or without vitamins B-12 and vitamin B-6. Intervention trials to examine the impact of reduced homocysteine levels on the morbidity and mortality from heart disease and stroke are under development.

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