

The folate cycle and disease in humans

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The folate cycle and disease in humans. The prevalence of hyperhomocysteinemia in renal disease patients, its treatment by folate administration, and its aggravation by the 677 C→T mutation of methylene-tetrahydrofolate (methylene-THF) reductase has established the folate cycle as an important factor in the pathogenesis and management of renal disease. Proper function of the folate cycle depends on normal function of involved enzymes adequate of the vitamin and its correct disposition within the body. Vital processes in folate disposition include conversion of dietary folylpolyglutamates to monoglutamates, intestinal absorption, receptor and carrier-mediated transport across cell membranes, and cellular export. Folate coenzymes are responsible for the one-carbon unit transfer in intermediary metabolism and are required for several reactions in key metabolic processes, for example of purine, pyrimidine and methionine synthesis, and glycine and serine metabolism. Methionine synthase and its recently discovered reducing protein as well as methylene tetrahydrofolate reductase are key folate enzymes in homocysteine metabolism. Deficiencies of these enzymes are important causes of severe disease in the rare remethylation defects causing homocystinuria. Knowledge of their catalytic and molecular properties is important in understanding possible causes of moderate hyperhomocysteinemia, as for example, the well-known 677 C→T transition of methylene tetrahydrofolate reductase.

Folate is vital in humans for several metabolic reactions involved in the formation and transfer of C1 units. As with other vitamins such as B₁₂ [1], a large number of different physiological and metabolic processes are necessary to convert folic acid in the diet into intracellular, metabolically active forms. These processes include interconversion of polyglutamate and monoglutamate forms of folate, intestinal uptake, transport across cell membranes, and export processes and metabolic reactions catalyzed by specific enzymes.

Deficiency of folate caused by renal loss or poor nutrition may be important in relationship to renal disease. For example, low dietary intake [2] and low blood levels [3] of folate in dialysis patients are well known, and anemia in renal failure patients, whether or not on dialysis, is a common finding [4]. Monitoring of nutritional

status [5] and adjuvant therapy, including folate, irrespective of hyperhomocysteinemia [6], plays an important role in the management of renal disease.

In recent years, the link between folate homeostasis and homocysteine metabolism has been established to be important in a number of disease states, including various types of vascular disease [7] as well as in renal disease. The prevalence of hyperhomocysteinemia in renal disease patients [8, 9], its treatment by folate administration [10] and its aggravation by the 677 C→T mutation of methylene-tetrahydrofolate (methylene-THF) reductase [11] have established the folate cycle as an important factor in the pathogenesis and management of renal disease.

This article reviews current knowledge of processes involved in folate homeostasis and metabolism. Particular emphasis is placed on folate enzymes directly related to homocysteine metabolism.

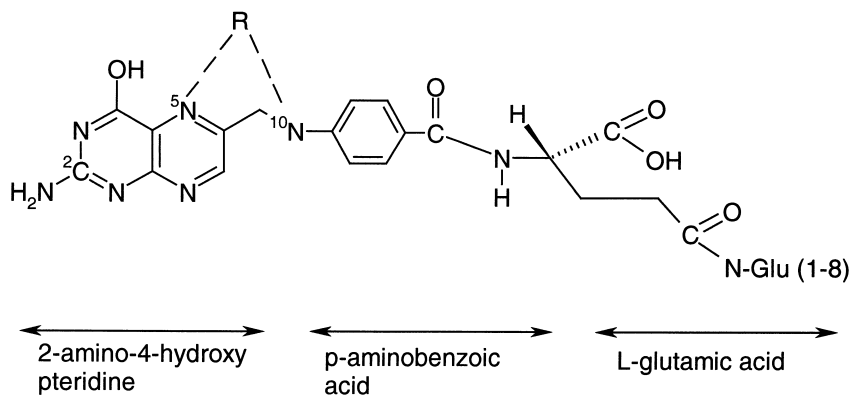
PROCESSING OF INGESTED FOLATE AND INTESTINAL TRANSPORT

The folic acid molecule is complex and consists of 2-amino-4-hydroxy-pteridine linked to *p*-aminobenzoic acid (that is, pteric acid) combined with a variable number (one to nine) of glutamic acid residues (Fig. 1). These glutamate moieties are bound to pteric acid and to each other by amide linkages involving the gamma carboxyl group of glutamate [12]. Dietary folates exist mainly as 5-methyl- and 10-formyl-THF in polyglutamate forms that cannot cross cell membranes [13] and must be enzymatically hydrolyzed by folylpolyglutamate conjugase (E.C. 3.4.19.9) to the monoglutamyl form in the intestine to be absorbed. The conjugases are widely distributed. The human conjugase isolated from jejunal brush borders is a lysosomal exopeptidase cleaving terminal γ glutamate residues [14–16]. A second distinct enzyme from jejunal mucosa is located intracellularly and is reported to have endopeptidase activity [17].

Once folate has entered the enterocyte by specific membrane transport, intracellular polyglutamate synthesis occurs to meet the cellular needs for folate metabolism and also to maintain a concentration gradient in favor of entry of folate monoglutamate into the cell. Reconversion

Key words: folic acid, hyperhomocysteinemia, methylene tetrahydrofolate reductase, vitamin B₁₂, anemia, renal failure.

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Folic acid species	Substituent at N-5	Substituent at N-10
Tetrahydrofolic acid	- H	- H
5-methyltetrahydrofolic acid	- CH ₃	- H
5,10- methylenetetrahydrofolic acid	- CH ₂ -	- CH ₂ -
5,10- methenyltetrahydrofolic acid	- CH=N ⁺ -	- CH=N ⁺ -
5-formyltetrahydrofolic acid	- CHO	- H
10-formyltetrahydrofolic acid	- H	- CHO
10-formiminotetrahydrofolic acid	- H	- HCNH

Fig. 1. Structure of folic acid and related compounds.

sion of polyglutamates to monoglutamates allows transport across the basolateral membrane into the portal circulation, mainly as 5-methyl-THF, most of which is nonspecifically bound to albumin [18].

CELLULAR TRANSPORT OF FOLATE

Transport of folates across cell membranes is facilitated by both receptor-mediated and carrier-mediated mechanisms, which are variably expressed in different tissues. Membrane folate receptors, which are attached through glycosyl-phosphatidyl-inositol anchors, have a high affinity for folic acid as well as for reduced folates [19]. Their distribution in cell membranes is diffuse, and internalization of folate probably involves classic endocytosis [20]. Folate receptors have been described in three isoforms, two of which are widely distributed, while one seems to be specifically associated with placenta and is likely to be important in the maternal-fetal transfer of folate [21, 22].

Carrier-mediated systems (RFC) exhibit a much higher affinity for reduced folates (1 to 5 $\mu\text{mol/L}$) than for folic acid (about 200 to 400 $\mu\text{mol/L}$) and facilitate active transport involving exchange with organic anions [23].

One example of a folate uptake mechanism is that existing in the brush border membrane of rat small intestine luminal epithelium with a pH optimum of 5.5 to 6 and high saturability ($K_m < 5 \mu\text{mol/L}$), which can internalize all folate compounds, including folic acid. Another system in mouse luminal epithelium does not appear to

be related to folate absorption, and appears to be located in the plasma membrane at the basolateral surface [24]. The functional expression and mRNA distribution of a RFC-1 cDNA involved in folate transport have been described [25]. Subsequent studies in the mouse revealed a higher capacity for 5-methyl-THF uptake in mature absorptive cells than in proliferative crypt cells, and this was associated with RCF-1 gene expression, which codes for a 58 kD brush border membrane protein [24].

Folate exit pumps, which are directly linked to adenosine 5'-triphosphate (ATP) hydrolysis, will act in opposition to folate uptake so that maintenance of the transmembrane folate gradient will depend on the net effect of these two processes [26, 27].

Finally, passive diffusion has also been shown to play a role in folate receptor-mediated transport across the human placenta [28].

Once transported into cells, monoglutamyl folates must be converted to their polyglutamyl forms by folylpoly γ -glutamate synthetase (EC 6.3.2.17), the latter being preferentially retained in cells [29]. Cytosolic and mitochondrial isoforms of folylpoly γ -glutamate synthetase exist, probably coded for by the same gene whose variable transcription has been proposed to regulate enzyme levels in different tissues [30]. The degree of polyglutamylation may be important in the effectiveness of antifolate drugs such as methotrexate [31].

FOLATE METABOLISM

One carbon unit metabolism and coenzyme function involve modification of the carbon atom existing at the

Table 1. Summary of the main folate enzymes

Enzyme official name (other name)	Reaction	EC number	Comments (human enzyme unless specified)
Formate-tetrahydrofolate ligase (10 formyl-THF synthetase)	THF + formate + ATP \rightleftharpoons 10 formyl-THF + ADP + P _i	6.3.4.3	Trifunctional C ₁ -THF synthetase Cytosolic
Methenyl-tetrahydrofolate cyclohydrolase	10 formyl-THF \rightleftharpoons 5,10-methenyl-THF + H ₂ O	3.5.4.9	Prot. Cry-Str. [70], Enzyme kinetics [71],
Methylenetetrahydrofolate dehydrogenase	5,10-methenyl-THF + NADPH \rightleftharpoons 5,10-methylene-THF + NADP ⁺	1.5.1.5	DNA [34], Chr-loc. 14q24 [72]
Formyltetrahydrofolate dehydrogenase	10-formyltetrahydrofolate tetrahydrofolate + NADP ⁺ + H ₂ O \rightleftharpoons tetrahydrofolate + CO ₂ + NADPH	1.5.1.6	Cytosolic, Prot. [73] DNA [74]
Glycine hydroxymethyltransferase (serine hydroxymethyltransferase)	5,10 methylene-THF + H ₂ O glycine \rightleftharpoons serine + THF	2.1.2.1	Cofactor PLP, cyt./mit. Cyt. Cry-Str. [75] Cyt. DNA [40] Chr-loc. mit. 12q13, cyt. 17p11.2 [39]
Methylenetetrahydrofolate reductase	5,10-methylene-THF + NAPH \rightleftharpoons 5-methyl-THF + NADP ⁺	1.5.1.20	Cofactor FAD, cytosolic. Prot. [45], DNA, Chr-loc. 1p36.3 [49]
5-Methyltetra- hydrofolate:homocysteine S-methyltransferase (methionine synthase)	homocysteine + 5-methyl-THF \rightleftharpoons methionine + THF	2.1.1.13	Cofactor cobalamin, cytosolic Prot. [59] Cry-Str. E. Coli [56] DNA, Chr-loc. 1q42.3-q44 [62–64]
[methionine synthase]- cobalamin methyltransferase (cob(II)alamin reducing) (Methionine synthase reductase)	MS-cob(II)alamin + NADPH + AdoMet \rightleftharpoons MS- methylcob(I)alamin + AdoHcy + NADP ⁺	2.1.1.135	Cofactor S-adenosylmethionine, cytosolic, DNA, Chr-loc. 5p15.3-15.2 [68]
Glutamate formimino transferase	THF + formiminoglutamate \rightleftharpoons 5-formiminoTHF + glutamate	2.1.2.5	Bifunctional protein, cofactor PLP, Golgi associated. Cry-Str. [76]
Formimino-tetrahydrofolate cyclodeaminase	FormiminoTHF \rightleftharpoons 5,10-methenyl- THF + NH ₃	4.3.1.4	Prot./DNA Rat [36], canine kidney cells [37]
Dihydrofolate reductase	THF reduces to DHF	1.5.1.3	Cyt. Prot. Cry-Str. [77], Chr-loc. 5q11.1-q13.3 [78]

Abbreviations are: Chr-Loc, human chromosome location; Cry-Str, protein crystal structure; Cyt., cytosolic; DNA, cloning or gene structure details; FAD, flavine adenine dinucleotide; Mit., mitochondrial; PLP, pyridoxal 5'phosphate; Prot., details on protein purification and/or structure; THF, tetrahydrofolate.

oxidation levels of formyl ($-\text{HCO}$), methylene ($-\text{CH}_2^-$), or methyl ($-\text{CH}_3$; Fig. 1), and are covalently attached to the nitrogen atoms at position 5 and/or 10. These modifications involve several enzyme reactions related to different essential metabolic pathways such as the synthesis of purines, pyrimidines and methionine, glycine and serine metabolism, and the breakdown of histidine [12]. Table 1 summarizes the main reactions and lists special features and references to main data on enzymes of the folate cycle. The interactions of folate coenzymes and C1 unit metabolism are illustrated in Figure 2.

Folate compounds must be in the reduced and polyglutamate form to function as coenzymes. Reduction of folic acid or dihydrofolate to THF is catalyzed by dihydrofolate reductase. This enzyme plays an important role in folate homeostasis and has received much attention as the site of action of cancer treatment [32].

C₁-THF synthetase

Three enzymes, 10 formyl-THF synthetase, 5,10 methenyl-THF cyclohydrolase, and 5,10 methylene-THF dehydrogenase, which constitute the multi-enzyme complex C₁-THF synthetase, are responsible for the interconversion of (1) 10-formyl-THF with THF and formate, (2) 10-formyl-THF with 5,10-methenyl-THF, and (3) 5,10-

methenyl-THF with 5,10-methylene-THF [33]. The cDNA codes for a protein of 101 kD corresponding well with the properties of the enzyme purified from human liver [34]. 10-formyl-THF can also be converted to THF and carbon dioxide in the reaction catalyzed by the liver-specific 10-formyl-THF dehydrogenase, whereby excess C-1 units are released and the THF pool is maintained [35].

5,10-Methylene-THF

5,10-Methylene-THF is a central compound in the folate cycle since (1) it provides the entry point of the quantitatively important supply of C1 units from serine through the interconversion of glycine and serine catalyzed by serine hydroxymethyltransferase; (2) it links this supply of C1 units to the formation of thymidylate for the synthesis of pyrimidine nucleotides; and (3) it is reduced to 5-methyl-THF by methylene-THF reductase.

This provides methyl groups for the formation of methionine from homocysteine in the methionine synthase catalyzed reaction, which links folate metabolism intricately to homocysteine and finally allows THF to re-enter the pool of reduced folates.

Purine nucleotide synthesis

The folate cycle is also linked directly to purine nucleotide synthesis through the provision of carbon atoms

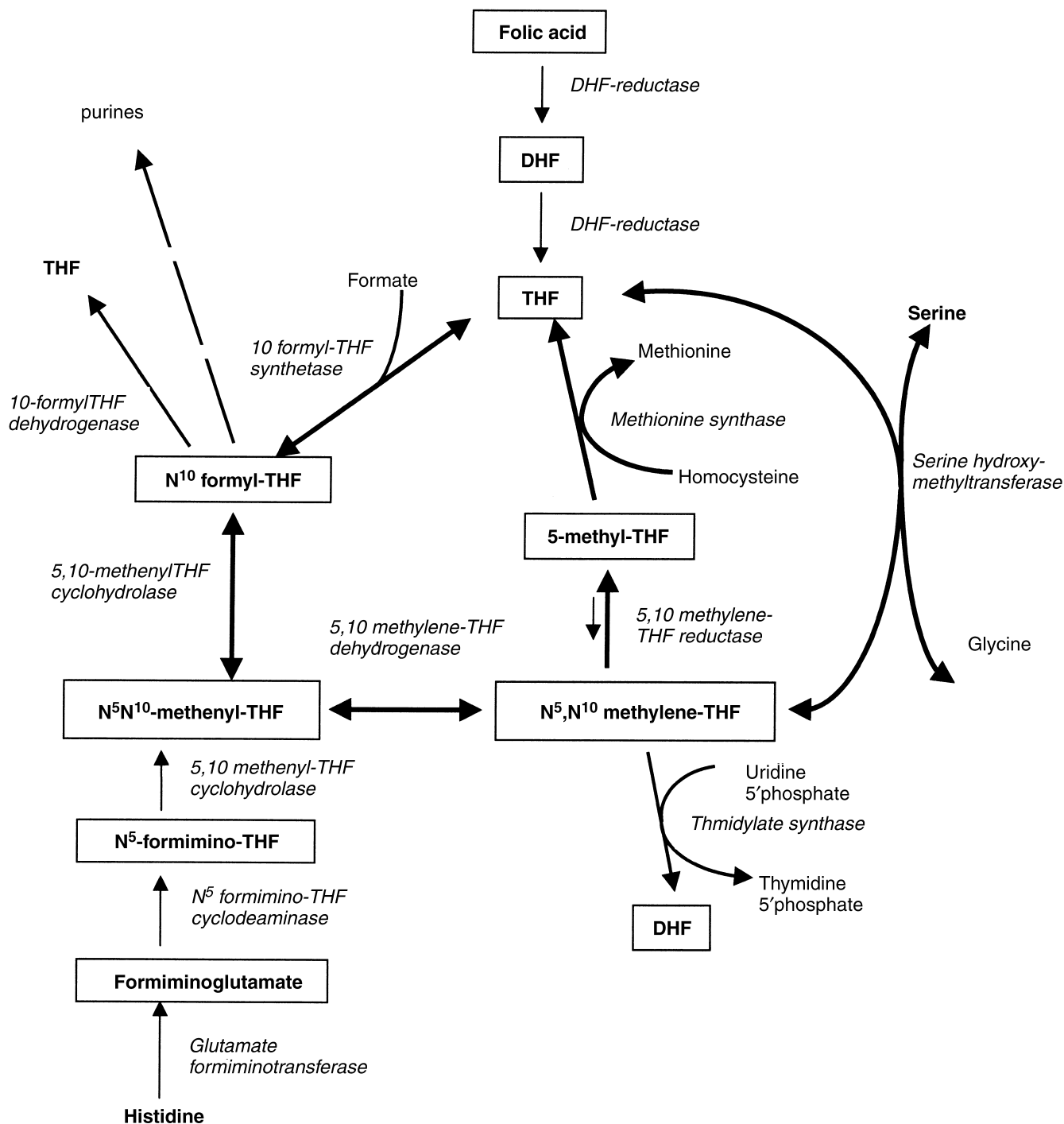


Fig. 2. Summary of the metabolic interactions of folic acid, related derivatives, pathways, and enzymes. Abbreviations are: THF, tetrahydrofolate; DHF, dihydrofolate.

numbers 2 and 8 of the purine ring by 10-formyl-THF, which is the folate coenzyme in the formation of α -N-formyl-glycinamide ribonucleotide and 5-phosphoribosyl-5-aminoimidazole-4-carboxamide, catalyzed by 10 formyl-THF:5 aminoimidazole 4 carboxamide ribonucleotide formyltransferase and 10 formyl-THF:glycinamide ribonucleotide formyltransferase, respectively [12].

Histidine metabolism

Folate coenzymes are also involved in the breakdown of histidine, which occurs in only a few tissues, such as liver and kidney, and has minor importance in the total turnover of C1 units. Histidine is converted to formiminoglutamate, whose formimino group is transferred to THF-forming N5-formimino-THF. This is further con-

verted to methenyl-THF, linking this amino acid to the folate cycle. The two reactions are catalyzed by a bifunctional octameric enzyme with glutamate formimino transferase and N⁵ formimino-THF cyclodeaminase activities. Recent studies report cloning and characterization of a rat bifunctional enzyme of 58 kD localized to the Golgi apparatus [36, 37].

Serine metabolism and folate

Serine hydroxymethyltransferase plays a key role in C1-unit metabolism [38].

Both cytosolic and mitochondrial forms exist, and cDNAs have been cloned for the human enzyme [39, 40]. The genes for the cytosolic form and mitochondrial form have been characterized [39], and a pseudo gene has been found on chromosome 1p32.3-33 [41]. The exact role of the different isoenzymes in C1 unit metabolism is not fully understood, but it has been proposed that their regulation might influence cell proliferation and be a target for antineoplastic drugs [42].

One important role of the enzyme is the channeling of C1 units of serine (carbon number 3), derived from intermediates of glycolysis, via 5,10-methylene-THF, into the folate cycle with the concomitant formation of glycine. Glycine cleavage occurs in only the mitochondria in a four reaction sequence involving the mitochondrial form of serine hydroxymethyltransferase [43]. The activity of the enzyme and degree of metabolism of glycine compared with serine varies considerably between different tissues [42]. In the reverse direction of this reaction, THF is regenerated from 5,10-methylene-THF, thereby converting glycine to serine and completing the sequence whereby the possibly toxic formate is converted to serine [38].

5,10-Methylene-THF reductase

This enzyme, which catalyzes the nicotinamide adenine dinucleotide phosphate (NADPH)-linked reduction of 5,10-methylene-THF to 5-methyl-THF, has been purified from pig liver [44] and human liver [45] with similar properties. The reaction is essentially irreversible in vivo, and its activity is a control point for entry of folate coenzymes into the remethylation of homocysteine pathway [46]. The purified enzyme is a dimer of equal subunits of 77 kD molecular mass, each of which contains one mole of noncovalently bound flavinadenine dinucleotide. Flavin acts as a conductor of reducing equivalents from NADPH to the natural substrate or in artificial systems to alternative electron acceptors such as menadione. The binding of S-adenosylmethionine results in allosteric inhibition, which can be reversed by S-adenosylhomocysteine [47]. This inhibition by S-adenosylmethionine acts synergistically with the activation of cystathionine β -synthase and is an important factor in the regulation of homocysteine metabolism [48].

The studies of Rozen and coworkers have consider-

ably advanced our knowledge of the molecular genetics of this important enzyme. They first cloned a cDNA that codes for the expected amino acid sequence based on data from the pig enzyme and maps to 1p36.3 [49]. This allowed elucidation of different mutations in patients with homocystinuria caused by severe methylene-THF reductase deficiency [50]. The cDNA of 2.2 kb codes for a 70 kD protein in an expression system, whereas a larger enzyme of 77 kD is found in tissues, suggesting the possibility of tissue-specific methylene-THF reductase protein isoforms and a complex regulation of the gene [51].

A thermolabile variant of this enzyme first described by Kang et al [52] is due to a 677 C→T transition [53] and is a common polymorphism with incidences as high as 23% in some populations [54].

5-Methyl-THF: Homocysteine S-methyltransferase (methionine synthase)

This complex enzyme catalyzes the transfer of a methyl group from 5-methyl-THF to homocysteine-producing methionine and THF, that is, remethylation. The enzyme plays a vital role in the provision of methionine for S-adenosylmethionine-dependent transmethylation reactions. Much of our understanding of the mechanism stems from elegant studies by Matthews, and coworkers on the *Escherichia coli* enzyme [55–57] as well as information on the human placenta [58] and the pig liver enzymes [59]. The porcine enzyme is a monomer of 151 to 155 kD molecular mass, contains one cobalt atom per mole of enzyme, and is dependent on S-adenosylmethionine for activity.

Cloning of the genes revealed that much homology exists between the human and *E. coli* forms, so that information gained from the extensive studies of the latter is indeed relevant for the human enzyme. Our present understanding of the catalytic cycle is deduced from kinetic studies that suggest an ordered sequential mechanism [60] as well as molecular modeling [55, 56]. In this cycle, the methyl form of the B₁₂ coenzyme donates its methyl group to homocysteine yielding the reduced form of the coenzyme cob(I)alamin and producing methionine. The cob(I)alamin moiety is remethylated by 5-methyl-THF-forming THF. Thus, reductive remethylation of the cobalamin molecule is a vital part of the reaction, particularly since the cob(I)alamin form occasionally becomes oxidized to the cob(II)alamin form, which is inactive. The methyl group is donated by S-adenosylmethionine in this process, and in *E. coli*, the electron is furnished by reduced flavodoxin, a protein lacking in mammals. Until its elucidation (discussed later in this article), there was much speculation on the nature of the human reducing component. Evidence was obtained that the mammalian enzyme utilizes two distinct proteins,

which are not related subunits, to transfer electrons from NADPH to the cobalamin moiety of the enzyme [61].

Three independent groups cloned the methionine synthase gene (designated MTR) at the same time, employing similar approaches and taking advantage of the highly conserved sequences in bacteria and *C. elegans* [62–64]. The cloned human gene encodes a protein of 1265 amino acids with a predicted molecular weight of 140 kD and is localized to chromosome 1q42.3-1q44. Mutations in the *MTR* gene have been identified in several patients with the cblG form of homocystinuria caused by methionine synthase deficiency [62, 65, 66]. Also, the 919 D→G polymorphism of *MTR* has been reported to be a determinant of plasma total homocysteine concentrations, albeit to a modest degree [67].

An important advance in understanding the methionine synthase function in humans was the discovery of the gene for methionine synthase reductase. Leclerc et al cloned a cDNA using consensus sequences for binding of potential reducing cofactors, FMN, FAD, and NADPH [68]. The isolated gene codes a mRNA of 3.6 kb and predicts a protein of molecular mass of 77,000 kD and 698 amino acids belonging to the family of ferredoxin-NADP + reductases. Different mutations in this gene, including large insertions or deletions caused by splicing defects and small deletions and point mutations, have been demonstrated in patients with the cblE form of homocystinuria [69]. This provides conclusive evidence that this gene and its protein are indeed necessary for proper function of human methionine synthase. Whether any additional reducing proteins are necessary for methionine synthase function remains to be established.

Metabolic importance of polyglutamate forms

The degree of polyglutamylation of coenzymes plays an important role in the regulation of folate enzymes as well as in their retention in cells (discussed previously in this article). For example, the affinity of formyl-THF synthetase for the substrate THF with four or five glutamate residues is 150 times higher than that for the monoglutamate form of THF. Furthermore, the conversion of glycine to serine by cytosolic serine hydroxymethyltransferase was three times higher with substrate containing five glutamate residues than with two [38].

INHERITED DISORDERS OF FOLATE METABOLISM

Several rare congenital disorders of folate metabolism are known [1].

Hereditary folate malabsorption reported in less than 20 cases exhibits megaloblastic changes, diarrhea, failure to thrive, and neurological abnormalities in the first few months of life. Serum and cerebrospinal fluid (CSF) folate levels are low and respond poorly to oral folate

treatment. Little is known on the molecular defect, but involvement of a folate transporter seems plausible.

Other disorders of folate transport have been reported such as a postulated defect of 5-methyl-THF uptake of into bone marrow cells. One patient with dyserythropoiesis was shown to have reduced 5-methyl-THF uptake into erythrocytes.

An adult with progressive sensorineural hearing loss, dysarthria, dysgraphia, and gait ataxia was shown to have low folate levels in CSF that was postulated to be due to a defect of a folate binding protein of the choroid plexus.

Deficiency of dihydro-THF reductase has been reported but not substantiated as an inborn error of metabolism [12].

Also glutamate formiminotransferase/formimino-THF-cyclodeaminase deficiency has been observed in both asymptomatic subjects and in patients with neurological abnormalities, including three with associated malignancy, but not established as a cause of clinical abnormalities.

Several distinct, rare inherited disorders resulting in deficient methionine synthase activity are known. Functional deficiency of this enzyme, either isolated (due to the cblE or cblG cobalamin defects [79]) or combined with methylmalonyl CoA mutase deficiency (cblC/D [80] or cblF defects [81]), leads to severe disease and early death, sometimes even in treated patients. Neurological abnormalities, feeding difficulties, and delayed development, together with megaloblastic anemia, usually manifesting in the first weeks or months of life, increased homocysteine with low methionine levels, together with methylmalonic aciduria in the combined defects are all hallmarks of this enzyme deficiency.

In both isolated and combined deficiencies, treatment with high doses of intramuscular hydroxocobalmin usually leads to a marked improvement of the biochemical abnormalities, but neurological damage may be irreversible. As in other remethylation defects, betaine and methionine can be given to increase further the ratio of methionine to homocysteine as well as folic acid or folinic acid.

Acquired causes of methionine synthase deficiency are also known, such as exposure to nitrous oxide [82] and also B₁₂ deficiency [83].

Methylene-THF reductase deficiency is well known as a cause of severe hyperhomocysteinemia. Most patients have presented between the neonatal period and one year of age with severe neurological disease, including seizures, upper motor neuron signs, microcephaly, electroencephalogram (EEG) abnormalities, developmental delay, and psychomotor retardation. Later presenting patients with milder features, gait abnormalities, and psychiatric disorder are known. Vascular abnormalities have been identified at post-mortem in some patients [1].

In addition to increased plasma levels of homocysteine

with low methionine, folates can be low in serum, red cells, or CSF reflecting low 5-methyl-THF. Although a few exceptional patients have responded to pharmacological doses of folate, most are unresponsive to specific vitamin administration, and treatment is aimed at lowering homocysteine and correcting low methionine levels [1]. Most patients have responded poorly to treatment, and long-term outcome appears to be poor.

Finally, although not described in humans, deficiency of 10 formyl-THF dehydrogenase has been discovered in a mouse model [84].

CONCLUSION

An important question is whether renal disease per se can disturb folate metabolism. Clearly, if folate status is affected by poor nutrition or renal loss of water soluble vitamins, low folate levels may lead to reduced activity of folate enzymes, including those related to homocysteine metabolism leading to pathology, for example, anemia. Conversely, if homocysteine elevation occurs because of a disturbance of metabolism at the level of methylenetetrahydrofolate reductase or methionine synthase, independent of folate status, this may lead to disturbed availability of reduced folates essential for cellular integrity. There is little evidence that disturbed folate metabolism in the inherited disorders of folate metabolism causes renal disease except for the occurrence of hemolytic uremic syndrome in patients with cblC/D disease. However, this condition has a complex etiology with increased methylmalonic acid levels as well as deficiency of methionine synthase function.

Clearly, much work is still needed to elucidate both the mechanism for the cause of hyperhomocysteinemia in renal disease and also the interactions between metabolic changes in renal disease and folate enzymes, considering possible inhibition of folate enzymes by uremic compounds.

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