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Folic Acid and L-5-Methyltetrahydrofolate

Comparison of Clinical Pharmacokinetics and Pharmacodynamics

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Abstract

There is a large body of evidence to suggest that improving periconceptional folate status reduces the risk of neonatal neural tube defects. Thus increased folate intake is now recommended before and during the early stages of pregnancy, through folic acid supplements or fortified foods. Furthermore, there is growing evidence that folic acid may have a role in the prevention of other diseases, including dementia and certain types of cancer.

Folic acid is a synthetic form of the vitamin, which is only found in fortified foods, supplements and pharmaceuticals. It lacks coenzyme activity and must be reduced to the metabolically active tetrahydrofolate form within the cell. L-5-methyl-tetrahydrofolate (L-5-methyl-THF) is the predominant form of dietary folate and the only species normally found in the circulation, and hence it is the folate that is normally transported into peripheral tissues to be used for cellular metabolism. L-5-methyl-THF is also available commercially as a crystalline form of the calcium salt (Metafolin®), which has the stability required for use as a supplement.

Studies comparing L-5-methyl-THF and folic acid have found that the two compounds have comparable physiological activity, bioavailability and absorption at equimolar doses. Bioavailability studies have provided strong evidence that L-5-methyl-THF is at least as effective as folic acid in improving folate status, as measured by blood concentrations of folate and by functional indicators of folate status, such as plasma homocysteine.

Intake of L-5-methyl-THF may have advantages over intake of folic acid. First, the potential for masking the haematological symptoms of vitamin B₁₂ deficiency may be reduced with L-5-methyl-THF. Second, L-5-methyl-THF may be associated with a reduced interaction with drugs that inhibit dihydrofolate reductase.

The importance of periconceptual folic acid supplementation in reducing the risk of neural tube defects and other congenital malformations is generally accepted. Furthermore, evidence is accumulating to support a possible role of folic acid in the reduction in risk of other diseases, including dementia and certain types of cancer, making folic acid the subject of intense discussion by the medical community and by the public.

Despite the important functions of the vitamin folic acid, its chemical nature has seldom been the focus of scientific discussion. Folic acid is an oxidized synthetic form of the vitamin, which does not exist in nature except in fortified foods, supplements and pharmaceuticals. Folic acid itself is not active as a coenzyme, and it undergoes several metabolic steps to be activated. The advantage of folic acid over natural folates has been its greater stability and lower cost, which make it particularly suitable for use in vitamin supplements, pharmaceuticals and fortified foods with a sufficient shelf-life. In addition, until recently, the natural forms of folate (reduced derivatives) have only been available as mixed diastereoisomers, of which only half possess biological activity.

The predominant active form of folate in the body is L-5-methyltetrahydrofolate (L-5-methyl-THF), which accounts for approximately 98% of folates in human plasma. L-5-methyl-THF is also the predominant active metabolite after intake of folic acid. Because of its limited stability, pharmaceutical use of L-5-methyl-THF was not feasible until its stable calcium salt was developed (Metafolin®; Merck Eprova AG, Schaffhausen, Switzerland). Since then, folic acid has been replaced by the calcium salt of L-5-methyl-THF in several vitamin supplements

and pharmaceuticals to enable direct use of the predominant natural form of the vitamin.

The objective of this review is to provide evidence that L-5-methyl-THF is as effective as folic acid in improving folate status. Differences in the metabolic pathways and the mode of action are discussed, with particular emphasis on genetic polymorphisms and the potential to mask vitamin B₁₂ deficiency symptoms. In this review, L-5-methyl-THF refers to the biologically active diastereoisomer, also known as 6S-5-methyl-THF.

1. Clinical Pharmacokinetics

1.1 Absorption

Folic acid (pteroylmonoglutamate) is absorbed as such, whereas food folates (polyglutamate derivatives) are hydrolysed to monoglutamates in the gut by a brush border hydrolase prior to absorption. Both forms are absorbed in the proximal small intestine via the proton coupled folate transporter (PCFT), a saturable transporter that transports oxidized and reduced folates with similar efficiency.^[1] In addition to sharing a common transport mechanism, most dietary folates and folic acid that is added to the diet share a common metabolic fate, as they are metabolized to L-5-methyl-THF during their passage across the intestinal mucosa (figure 1). Folic acid is reduced to dihydrofolate (DHF) and then to tetrahydrofolate (THF) by DHF reductase (DHFR) in the mucosal cell, and it is

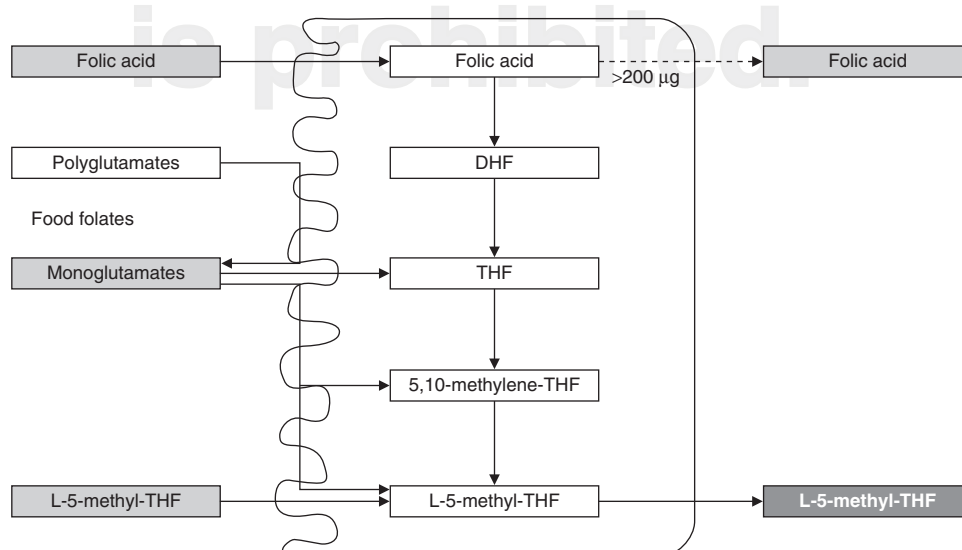


Fig. 1. Intestinal folate absorption (from the brush border membrane through the mucosal cell via the portal vein into the peripheral circulation). DHF = dihydrofolate; THF = tetrahydrofolate.

then metabolized via serine hydroxymethyltransferase and 5,10-methylenetetrahydrofolate reductase (MTHFR) to L-5-methyl-THF. Some of this metabolism may occur in the liver prior to the release of L-5-methyl-THF into the peripheral circulation. At high intakes, unmetabolized folic acid and reduced folates appear unchanged in the circulation.^[2]

The average dose of folic acid expected from food fortification in the US and Canada is approximately 100 µg/day, which would be almost completely converted to L-5-methyl-THF. A study by Lucock et al.^[3] showed a direct relationship between the quantity of L-5-methyl-THF that appeared in the blood and the amount of folic acid that was ingested. However, above 200 µg/day, small amounts of folic acid appear unchanged in the circulation.^[4] The capacity of the intestine and liver to convert absorbed folate to L-5-methyl-THF is quite high, particularly if the folates are reduced. In a study by Stern et al.,^[5] 12 subjects received an oral dose of 5 mg of L-5-formyl-THF (leucovorin, folinic acid), and plasma folate was monitored at various times. In all subjects, a large increase in plasma folate, consisting almost entirely of L-5-methyl-THF, was detected.

Perry and Chanarin^[6] showed that the increase in the plasma folate concentration was the same for folic acid as for the other reduced folates in subjects who received oral doses (10 µg/kg of bodyweight) of folic acid and different reduced folate derivatives. Folate concentrations were determined microbiologically with *Lactobacillus casei*, which detects all forms of folate, and with *Streptococcus faecalis*, which only measures non-methylated folates. For folic acid, the increase in *S. faecalis* activity, while noticeable, was nevertheless short lived and quite small when compared with the increase in *L. casei* activity, indicating that during absorption, a major part of the reduced folates and folic acid was converted to L-5-methyl-THF.

Results obtained after a single oral administration of folic acid and L-5-methyl-THF in humans have indicated that the bioavailability of L-5-methyl-THF is at least as high as that of folic acid.

Pentieva et al.^[7] conducted a double-blind, placebo-controlled study with a crossover design in 21 healthy male subjects, comparing single oral doses of folic acid (500 µg, equivalent to 1132 nmol) and L-5-methyl-THF (500 µg, equivalent to 1089 nmol). To minimize interindividual differences in baseline plasma folate concentrations, subjects were given folic acid to saturate their body stores. Before the test and during the intervals between the crossover tests, subjects received a daily oral dose of folic acid (5 mg for 1 week), followed by a 2-day period without folic acid. Plasma folate concentrations determined by a microbiological assay with *L. casei* showed no

differences in the area under the plasma concentration-time curve (AUC) [$p=0.9$] and maximum plasma concentration (C_{\max}) values between folic acid and L-5-methyl-THF after time-matched baseline correction.

Prinz-Langenohl et al.^[8] performed a randomized, double-blind study with a four-period crossover design. Twenty-one healthy female subjects were given single oral doses of folic acid (400 µg) and equimolar amounts (906 nmol) of L-5-methyl-THF (416 µg), either without or with a folic acid preload (1 mg/day for 10 days). Plasma folate concentrations were determined using a competitive immunoassay, which measured all folate forms. The AUC from time zero to the time of the last measurable concentration (AUC_{last}) and C_{\max} values (table I), as well as the mean concentration-time profiles, did not differ significantly between folic acid and L-5-methyl-THF, regardless of whether the subjects were preloaded with folic acid or not. Folic acid and L-5-methyl-THF exposures were within the limits generally accepted as bioequivalent (table I).

Single-dose studies have several drawbacks. For example, most of these studies use unphysiologically high doses to obtain measurable responses, and the data may not be comparable to those from low-dose studies, as high doses may be absorbed passively as well as actively, unlike lower doses, which are only absorbed actively. Melse-Boonstra et al.^[9] concluded that a multiple-oral-dose design using isotope-labelled folate vitamers is the best approach for measuring folate bioavailability. Since such studies are rare, data from long-term studies do provide a better understanding of the relative absorption of different forms of folate.

Fohr et al.^[10] performed a randomized, placebo-controlled, double-blind study of 160 young female subjects receiving 400 µg of folic acid with equimolar amounts of [6R,S]-5-methyl-THF. After 8 weeks of treatment, serum folate concentrations were significantly higher (~3-fold) in the [6R,S]-5-methyl-THF group than in the folic acid group, whereas the increase in red blood cell (RBC) folate in both treatment groups was comparable. This higher increase in serum folate after administration of the racemic mixture depended on the fact that the unphysiological 6R-isomer had better binding to the protein in the immunoassay that was used, indicating false positive results. Obviously, the 6R-isomer would not be metabolized to L-5-methyl-THF, which is a prerequisite for incorporation into RBCs. The data indicated that the mean increase in RBC folate concentrations was comparable in the folic acid and [6R,S]-5-methyl-THF groups (69% and 66%, respectively).

Additionally, Fohr et al.^[10] measured folate metabolites in plasma in a subgroup of 20 women who were heterozygous (CT genotype) for the 677C→T polymorphism in the *MTHFR* gene

Table 1. Bioavailability of equimolar doses of L-5-methyl-THF and folic acid (baseline uncorrected data from Prinz-Langenohl et al.,^[8] re-evaluated)

| Compound | Parameter | | | |
|-------------------------|----------------------------------|--|---|---|
| | C_{\max} (nmol/L) ^a | C_{\max} ratio L-5-MTHF : folic acid ^b | AUC_{last} (nmol • h/L) ^a | AUC_{last} ratio L-5-MTHF : folic acid ^b |
| Without preload | | | | |
| L-5-methyl-THF (416 µg) | 39.4 [14.6] | | 210 [17.7] | |
| Folic acid (400 µg) | 36.5 [31.8] | | 191 [28.8] | |
| | | 108.0 [96.9, 120.3] | | 109.9 [99.6, 121.3] |
| With preload | | | | |
| L-5-methyl-THF (416 µg) | 50.0 [16.8] | | 278 [14.4] | |
| Folic acid (400 µg) | 55.9 [21.6] | | 277 [18.3] | |
| | | 89.9 [80.2, 100.8] | | 100.2 [90.3, 111.2] |

a Geometric mean [%CV].

b Estimate (%) [90% CI].

AUC_{last} = area under the plasma concentration-time curve from time zero to the time of the last measurable concentration; **C_{max}** = maximum plasma concentration; **CV** = coefficient of variation; **THF** = tetrahydrofolate.

(ten in the folic acid group and ten in the L-5-methyl-THF group), using a high-performance liquid chromatography (HPLC) method coupled with a microbiological assay. Un-metabolized folic acid was detected in 8 of 10 women in the folic acid group, whereas unmetabolized folic acid was not detected in the L-5-methyl-THF group. Other metabolites such as THF and 5-formyl-THF were detected in both the folic acid and L-5-methyl-THF groups but could not be quantified because their concentrations were below the limit of accurate detection.

Lamers et al.^[11] conducted a similar follow-up study using the [6S] isomer of 5-methyl-THF instead of the racemic mixture. After 24 weeks of treatment, the RBC and plasma folate concentration increases were significantly greater in the group receiving [6S]-5-methyl-THF than in the folic acid group.

Houghton et al.^[12] conducted a 16-week, randomized, placebo-controlled, intervention trial to evaluate the relative effectiveness of folic acid (400 µg) versus equimolar amounts of [6S]-5-methyl-THF in maintaining blood folate concentrations during lactation (n=72). At the end of the study, the RBC folate concentration in the [6S]-5-methyl-THF group was higher (2178 nmol/L; 95% CI 1854, 2559) than that in the folic acid group (1967 nmol/L; 95% CI 1628, 2307) [$p < 0.05$]. The distribution of folate forms in RBCs did not differ significantly between the [6S]-5-methyl-THF and placebo groups when evaluated by stable isotope dilution HPLC mass spectrometry. However, the folic acid group had higher amounts of 5-formyl-THF ($p < 0.03$).

De Meer et al.^[13] investigated the absorption of equimolar amounts of [6S]-5-methyl-THF and folic acid (400 µg) in two different age groups (<30 years [n = 12] and >50 years [n = 12]).

Although folate absorption was about 20% lower in the older group than in the younger group, the investigators found no differences in absorption in each age group after supplementation with either form of the vitamin.

Isotopes have been used to evaluate the relative bioavailability of folic acid and a series of THF derivatives, including L-5-methyl-THF, in both animals^[14] and humans.^[15,16] When physiological doses were used in the animal study, the results indicated that the bioavailability of orally administered folic acid and L-5-methyl-THF was equivalent.^[14] Gregory et al.^[15] reported differences in the relative bioavailability of various monoglutamyl forms of folates in humans. However, the relatively large doses required in the human protocol made the two act differently, since the metabolic capacity of the intestine may have been exceeded. This was confirmed and extended by Wright et al.^[16] in a study that included a food (spinach) source of L-5-methyl-THF.

1.2 Distribution

L-5-methyl-THF circulates in its free form or loosely bound to plasma proteins. L-5-methyl-THF (and any other reduced folate) in plasma is transported into most peripheral tissues via reduced folate carrier-1, which has a very poor affinity for folic acid and is specific for reduced folates.^[17] Transport into some tissues such as the liver can be by PCFT, the transmembrane carrier responsible for intestinal transport of both oxidized and reduced folate.^[1,18]

A second distinct folate transporter, known as folate binding protein or the folate receptor, is expressed in a more limited

range of tissues. High levels of folate binding protein are expressed in the choroid plexus, the kidney proximal tubes, the placenta and a number of human tumours, while lower levels have been found in a variety of other tissues.^[1] This transporter is responsible for reabsorption of folate in the kidney by a receptor-mediated endocytotic process and is believed to play a similar role in folate transport in other tissues. This transporter has high affinity for L-5-methyl-THF and folic acid.

Folate monoglutamate transported into cells is metabolized to polyglutamate forms by the enzyme folylpolyglutamate synthetase (FPGS). Most of the entering folate is L-5-methyl-THF, which is a poor substrate for FPGS.^[19] This has to be metabolized to THF via the methionine synthase reaction before effective polyglutamylation and tissue retention is achieved (figure 2). Folic acid is also a poor substrate for FPGS.^[19] Any unmetabolized folic acid that entered tissues would have to be metabolized to a reduced folate before it could be effectively polyglutamated and retained.

When tissue levels of folate are high, competition between endogenous L-5-methyl-THF polyglutamates and transported L-5-methyl-THF monoglutamate for methionine synthase reduces the demethylation of L-5-methyl-THF and reduces its conversion to retainable polyglutamates.^[20,21] This homeostatic mechanism prevents the accumulation of excessive levels of folate in tissues, even when plasma folate concentrations are very high.

1.3 Metabolism

Folate is used as a generic term for a family of chemically and functionally related compounds based on the folic acid structure (figure 3). Folic acid lacks any coenzyme activity until it is reduced, within the cell, to the metabolically active THF form. The enzymatic reduction of folic acid to THF occurs in a two-step process via DHF, which is catalysed by DHFR. The initial reduction to DHF is rate limiting.^[23] The second reduction results in an active L (or 6S) stereoisomer due to asymmetry at carbon-6 of THF. Folates in tissues act as donors and acceptors of one-carbon units in metabolic reactions known as one-carbon metabolism. These one-carbon units can be at the oxidation level of methanol (L-5-methyl-THF), formaldehyde (5,10-methylene-THF) or formate (5- or 10-formyl-THF or 5,10-methenyl-THF).

L-5-methyl-THF is the predominant folate form entering the human metabolism and the transport form of folate in plasma. Other important forms of the coenzyme that are synthesized by the described metabolic pathways are shown in figure 3. Each of the folate forms plays a specific role in intracellular metabolism.

Practically all tissue folates are polyglutamate forms. Metabolism of folates to polyglutamates is required for their biological activity because the polyglutamate forms are much more effective substrates for folate-dependent enzymes than are the monoglutamate derivatives, which are the transport forms of

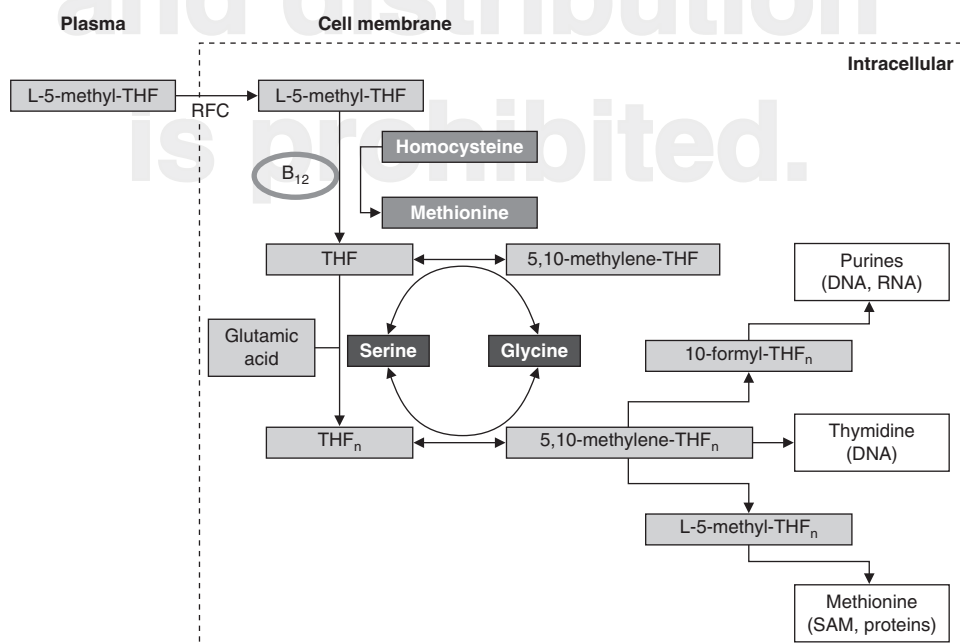


Fig. 2. Transport and metabolism of L-5-methyl-THF by peripheral tissues. **B₁₂**=vitamin B₁₂ (cobalamin); **RFC**=reduced folate carrier; **SAM**=S-adenosyl-methionine; **THF**=tetrahydrofolate; **THF_n**=THF polyglutamate, where n is the total number of glutamate residues.

the vitamin.^[21,24] Conversion of folates to polyglutamates of a chain length greater than three or more is also required for the accumulation and retention of folate by tissues.^[21]

Folate coenzymes are involved in three major interrelated metabolic cycles in the cytosol of cells. These cycles are required for the synthesis of thymidylate and purines, which are precursors for DNA and RNA synthesis, and for the synthesis of methionine from homocysteine and the interconversion of serine and glycine (figure 4).

5,10-methylene-THF plays a central role in these cycles, as it can be used directly for thymidylate synthesis, or reduced to L-5-methyl-THF in the methionine synthesis cycle, or oxidized to 10-formyl-THF to be used in purine synthesis. Although these synthesis cycles are located in the cytosol, mammalian cells also contain a large mitochondrial folate pool, which is also involved in the provision of one-carbon precursors for cytosolic one-carbon metabolism.

Serine is the major provider of one-carbon units for folate-dependent one-carbon metabolism.^[21,25] It donates its β -carbon to THF to generate glycine and 5,10-methylene-THF.

Folate is required for the synthesis of thymidylate, a nucleotide required specifically for the synthesis of DNA (figure 4). Thymidylate synthase catalyses the transfer of the one-carbon group from 5,10-methylene-THF to generate deoxythymidine

monophosphate. The folate molecule also provides the reducing component in this reaction, and the THF is oxidized to DHF. The DHF that is generated has to be reduced back to THF before it can be re-utilized in one-carbon metabolism in a reaction catalysed by DHFR. Some DHFR enzymes, including the mammalian one, can also reduce folic acid to DHF, although folic acid is a much poorer substrate than DHF for this enzyme.^[23] The enzymes involved in the synthesis of thymidylate are believed to translocate to the nucleus of the cell during the S phase of the cell cycle when DNA is synthesized.^[26]

The methylation of homocysteine to produce methionine uses L-5-methyl-THF as the methyl donor in a reaction catalysed by methionine synthase, one of only two vitamin B₁₂-dependent enzymes in mammals (figure 4). L-5-methyl-THF is generated from 5,10-methylene-THF in a reaction catalysed by the flavoprotein MTHFR. Methionine can be metabolized to S-adenosylmethionine, which acts as the methyl donor in many reactions, including the methylation of DNA, histones and other proteins, neurotransmitters and phospholipids, and the synthesis of creatine. These methylation reactions play important roles in development, gene expression and genomic stability.^[27]

The folate-dependent methionine cycle is sensitive to inadequate folate status. When folate status is poor, the decreased

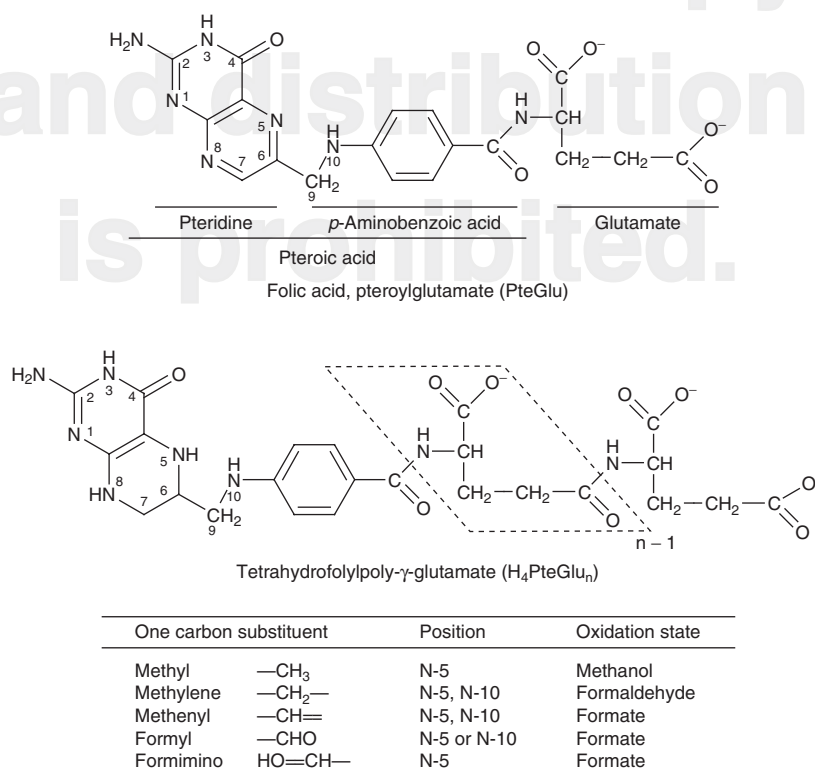


Fig. 3. Structure of folic acid and reduced folate polyglutamates. [Reproduced from Shane,^[22] with permission. Copyright Elsevier (2000).]

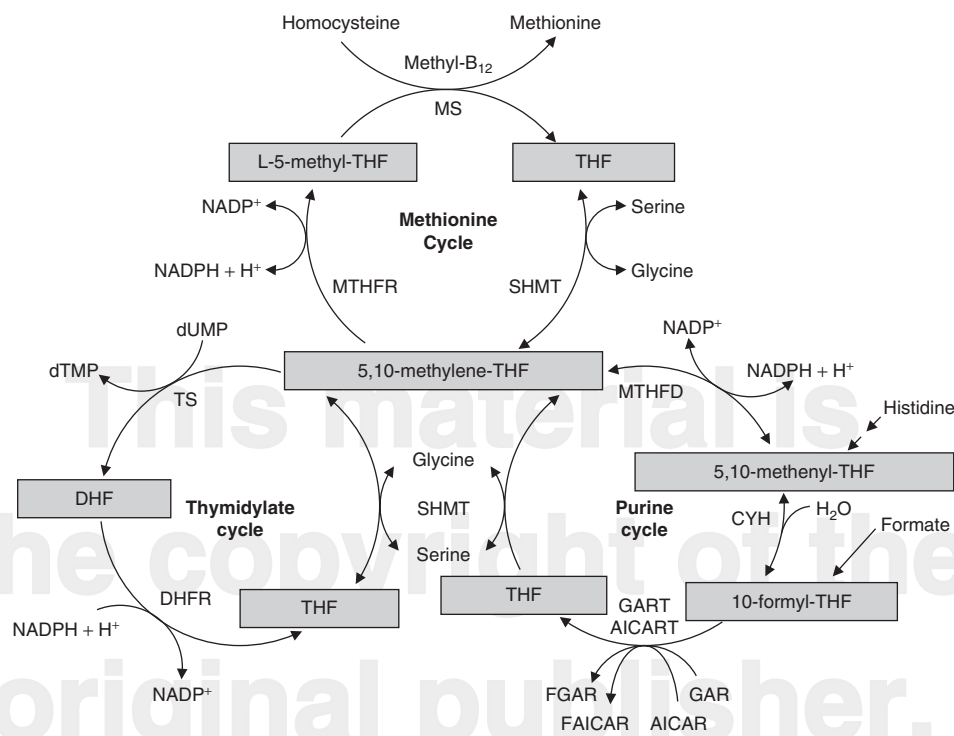


Fig. 4. Folate-mediated metabolism. **AICAR**=aminomidazole carboxamide ribonucleotide; **AICART**=AICAR formyltransferase; **B₁₂**=vitamin B₁₂ (cobalamin); **CYH**=methenyl-THF-cyclohydrolase; **DHF**=dihydrofolate; **DHFR**=DHF reductase; **dTMP**=deoxythymidine monophosphate; **dUMP**=deoxyuridine monophosphate; **FAICAR**=formyl-AICAR; **FGAR**=formyl-GAR; **GAR**=glycinamide ribonucleotide; **GART**=GAR formyltransferase; **MS**=methionine synthase; **MTHFD**=methylene-THF-dehydrogenase; **MTHFR**=5,10-methylene-THF-reductase; **NADP**=nicotinamide adenine dinucleotide phosphate; **NADPH**=reduced NADP; **SHMT**=serine hydroxymethyltransferase; **THF**=tetrahydrofolate; **TS**=thymidylate synthase. [Reproduced from Shane,^[22] with permission. Copyright Elsevier (2000).]

ability to remethylate cellular homocysteine due to inadequate L-5-methyl-THF results in an increased plasma homocysteine level, and the plasma homocysteine level is an indirect indicator of folate insufficiency.

1.4 Elimination

Under normal conditions of dietary intake and status, whole-body folate turns over quite slowly, with a half-life in excess of 100 days.^[28] Urinary excretion of intact folate accounts for only a very small proportion of this turnover. Over 99% of tissue folate is in the polyglutamate form. The actual mechanism of catabolism is poorly understood but primarily involves cleavage at the C9-N10 bond to generate *p*-aminobenzoylpolyglutamates and a pterin moiety.^[29] The *p*-aminobenzoylpolyglutamates are hydrolysed to the monoglutamate by a lysosomal glutamylhydrolase, acetylated and then excreted in urine as *N*-acetyl-aminobenzoylmonoglutamate. The pterin moiety is excreted in bile and appears in the faeces.

Some L-5-methyl-THF is secreted in the bile, but at intakes of 400 µg/day or less, a large proportion of this is reabsorbed in

the intestine. Some intact folate is also excreted in the faeces and urine; the proportion depends on the dose and resulting plasma concentrations. The proportion of L-5-methyl-THF excreted in the urine increases as the plasma concentration increases above 45 nmol/L.^[13]

2. Clinical Pharmacodynamics

2.1 Physiological Actions

Plasma and RBC folate concentrations and plasma homocysteine concentrations are commonly used biochemical parameters for assessing folate status in humans. Plasma folate concentrations change immediately after intake of folate and are therefore often used as a parameter for bioavailability studies.^[30] Because RBCs circulate for about 120 days but only accumulate folate during erythropoiesis, RBC folate concentrations change slowly and are an indicator of long-term folate status. Plasma homocysteine concentrations increase when inadequate quantities of L-5-methyl-THF are available

Table II. Effects of dietary folate and folic acid on plasma folate, RBC folate and plasma homocysteine concentrations^[17]

| Compound | RBC folate (nmol/L) | | Plasma folate (nmol/L) | | Plasma homocysteine (μmol/L) | |
|---|---------------------|---------------------------------------|------------------------|---------------------------------------|------------------------------|---------------------------------------|
| | baseline | net change at 4 wk [% of baseline] | baseline | net change at 4 wk [% of baseline] | baseline | net change at 4 wk [% of baseline] |
| Dietary folate (additional folate 350 μg/d) | 338 | 59.3 [17.5] | 13.8 | 6.5 [47.1] | 11.0 | -1.5 [-13.6] |
| Folic acid (additional folic acid 500 μg/2 d) | 339 | 42.9 [12.6] | 14.6 | 5.8 [39.7] | 10.8 | -1.8 [-16.7] |
| Placebo (no additional folate or folic acid) | 347 | -1.2 | 13.2 | -0.6 | 10.2 | 0.6 |

RBC = red blood cell.

to donate the methyl group that is required to convert homocysteine to methionine. The inverse association between blood folate concentrations and plasma homocysteine concentration is well established.^[31] Thus changes in fasting plasma homocysteine concentrations are considered to be a sensitive functional indicator of changes in folate status – although in population studies, homocysteine concentrations can also be affected by other conditions such as vitamin B₁₂ deficiency.

Examination of dietary intake patterns of an elderly population, originating from the Framingham Heart Study cohort prior to food fortification with folic acid, identified a clear dose-response relationship for plasma folate concentration and a clear inverse dose-response relationship for homocysteine concentration with increased intake of fruits and vegetables (major sources of L-5-methyl-THF).^[32] These relationships were similar to those for breakfast cereals and vitamin supplements (sources of folic acid).

A controlled human dietary study^[33] compared folate status and plasma homocysteine lowering after 4 weeks of intake of either dietary folate (mainly 5-methyl-THF) from fruits and vegetables, or folic acid, or no additional folate or folic acid. The results (table II) yielded the same conclusion, i.e. that folate intake lowers plasma homocysteine concentrations and improves folate status (measured as plasma and RBC folate) independently of the source of folate.

Equimolar doses of L-5-methyl-THF and folic acid given over 24 weeks are equally effective in increasing plasma and RBC folate concentrations and in reducing plasma total homocysteine concentration. Two studies by Venn et al.^[34,35] and a study by Lamers et al.^[11,36] demonstrated that RBC folate concentration increases in a similar, time-dependent manner, plasma folate and RBC folate concentrations increase in a dose-dependent manner, and plasma homocysteine concentrations decrease by a comparable fraction.

Venn et al.^[34,35] investigated the long-term effects of folic acid and L-5-methyl-THF in 167 healthy subjects and 104 women of childbearing age, respectively. Both studies were conducted in a randomized, double-blind, placebo-controlled manner. For 24 weeks, the subjects received either folic acid (100 μg), equimolar amounts (227 nmol) of L-5-methyl-THF as a calcium salt (113 μg), or placebo. Folate status was assessed by a microbiological assay using *L. casei*. In both studies, the increase in plasma folate and RBC folate concentrations did not differ significantly ($p > 0.05$) between folic acid and L-5-methyl-THF after 24 weeks of intake. Also, no differences in the concentration-time-profiles of plasma folate ($p = 0.48$) and RBC folate ($p = 0.70$) could be detected between the folic acid group and the L-5-methyl-THF group. L-5-methyl-THF was at least as effective as folic acid in lowering homocysteine concentrations (table III).

Table III. Equivalence of effects of L-5-methyl-THF and folic acid on RBC folate, plasma folate and plasma homocysteine concentrations

| Study and compound | RBC folate (nmol/L) | | Plasma folate (nmol/L) | | Plasma homocysteine (μmol/L) | |
|--|---------------------|--|------------------------|--|------------------------------|--|
| | baseline | net change at 24 wk [% of baseline] | baseline | net change at 24 wk [% of baseline] | baseline | net change at 24 wk [% of baseline] |
| Venn et al. (2002)^[34] | | | | | | |
| L-5-methyl-THF (113 μg/d) | 837 | 253 [30.2] | 20.0 | 6.9 [34.5] | | |
| Folic acid (100 μg/d) | 932 | 275 [29.5] | 25.2 | 9.2 [36.5] | | |
| Venn et al. (2003)^[35] | | | | | | |
| L-5-methyl-THF (113 μg/d) | 814 | 170 [20.9] | 17.5 | 8.1 [46.3] | 8.8 | -1.4 [-15.9] |
| Folic acid (100 μg/d) | 915 | 222 [24.3] | 23.3 | 11.2 [48.1] | 8.4 | -0.8 [-9.5] |

RBC = red blood cell; **THF** = tetrahydrofolate.

Table IV. Effects of L-5-methyl-THF and folic acid on RBC folate, plasma folate and plasma homocysteine concentrations^[20,21]

| Compound | RBC folate (nmol/L) | | Plasma folate (nmol/L) | | Plasma homocysteine (μmol/L) | |
|---------------------------|---------------------|--|------------------------|--|------------------------------|--|
| | baseline | net change at 24 wk [% of baseline] | baseline | net change at 24 wk [% of baseline] | baseline | net change at 24 wk [% of baseline] |
| L-5-methyl-THF (208 μg/d) | 656 | 480 [73.2] | 14.6 | 13.3 [91.0] | 8.1 | -1.4 [-17.2] |
| L-5-methyl-THF (416 μg/d) | 603 | 829 [137.5] | 13.3 | 22.2 [166.9] | 8.3 | -1.5 [-18.1] |
| Folic acid (400 μg/d) | 668 | 631 [94.5] | 14.4 | 20.3 [140.9] | 8.2 | -1.1 [-13.4] |

RBC = red blood cell; **THF** = tetrahydrofolate.

In a randomized, double-blind, placebo-controlled study by Lamers et al.,^[11,36] 135 women of childbearing age were administered equimolar doses (906 nmol) of folic acid (400 μg) and L-5-methyl-THF (416 μg), as well as L-5-methyl-THF at a lower dose (208 μg), over 24 weeks.

Plasma and RBC folate concentrations increased significantly ($p < 0.001$) in all three supplemented groups, relative to placebo. The increase in plasma folate and RBC folate concentrations after 24 weeks of administration did not differ significantly between equimolar doses of folic acid and L-5-methyl-THF (table IV). The increase was dose proportional for the two L-5-methyl-THF doses that were tested. There was no difference in the concentration-time courses of folic acid and L-5-methyl-THF. Overall, the L-5-methyl-THF 208 μg and 416 μg doses were as effective as folic acid 400 μg in lowering plasma homocysteine concentrations. The maximum decrease in homocysteine concentrations was reached after 4 weeks.

With respect to folate derivatives, only small amounts of unmetabolized folic acid (<1.1 nmol/L) were detected in fasting plasma from subjects after 4, 12 and 24 weeks of administration of folic acid 400 μg, and no continuous increase or decrease was observed. No unmetabolized folic acid was found in RBCs. In all four groups (including the placebo group), the main folate derivative in RBCs was L-5-methyl-THF, accounting for 92–97% of total folate. In subjects who were homozygous for the 677C→T polymorphism in *MTHFR*, additional folate derivatives were found in RBCs, such as THF, 5,10-methenyl-THF and 5-formyl-THF, across the treatment groups.^[37]

Bostom et al.^[38] compared the effectiveness of daily oral administration of L-5-methyl-THF (17 mg) with that of equi-

molar amounts (34.0 μmol) of folic acid (15 mg) in reducing plasma homocysteine concentration in a randomized study in kidney dialysis patients (25 patients in each group). The data showed that L-5-methyl-THF intake for 12 weeks was as effective as folic acid (table V). Plasma folate was exclusively L-5-methyl-THF in those receiving L-5-methyl-THF. In those receiving folic acid, L-5-methyl-THF accounted for half of the increase in plasma folate, and free folic acid accounted for the remainder. Because high doses were used in this study, the results can be considered as general evidence of the safety of L-5-methyl-THF.

3. Potential Differences in Pathophysiological States

Supplementation with L-5-methyl-THF may provide additional benefits, compared with folic acid, in that the potential for masking vitamin B₁₂-deficiency symptoms may be reduced, potential drug interactions with drugs that inhibit DHFR may be reduced and potential adverse effects of some common polymorphisms in folate genes may be ameliorated. In addition, some concerns that have been raised about possible adverse effects of unmetabolized folic acid in the peripheral circulation would be alleviated.

3.1 Masking of Vitamin B₁₂-Deficiency Symptoms

Folate deficiency is usually due to a dietary insufficiency, although it can arise from other causes such as malabsorption syndromes. The classical symptom of folate deficiency is

Table V. Effects of L-5-methyl-THF and folic acid on plasma folate and plasma homocysteine concentrations^[24]

| Compound | Plasma folate (nmol/L) | | Plasma homocysteine (μmol/L) | |
|--------------------------|------------------------|--|------------------------------|--|
| | baseline | net change at 12 wk [% of baseline] | baseline | net change at 12 wk [% of baseline] |
| L-5-methyl-THF (17 mg/d) | 86.3 | 836.9 | 24.1 | -4.1 [-17.0] |
| Folic acid (15 mg/d) | 73.0 | 989.1 | 22.9 | -3.4 [-14.8] |

THF = tetrahydrofolate.

megaloblastic anaemia, a condition reflecting deranged DNA synthesis in erythropoietic cells. Folate deficiency megaloblastic anaemia is a condition also seen in pregnancy. The defect in DNA synthesis in folate deficiency has been ascribed to defective thymidylate synthesis under these conditions, with a resulting increase in uracil misincorporation into DNA.

Megaloblastic anaemia is also a classical symptom of a severe vitamin B₁₂ deficiency. This condition, which is quite prevalent in the elderly, is seldom due to a dietary deficiency but usually results from malabsorption of vitamin B₁₂. However, the anaemia that results is identical to that of folate deficiency. In severe vitamin B₁₂ deficiency, the B₁₂-dependent methionine synthase enzyme is inactive, and cytosolic folate is 'trapped' as L-5-methyl-THF, at the expense of other folate coenzyme forms required for one-carbon metabolism, such as thymidylate synthesis, leading to a functional folate deficiency in the cell.^[39] Because the defective DNA synthesis in megaloblastic anaemia is caused by an induced secondary folate deficiency (the 'methyl trap' hypothesis), high concentrations of folic acid can cause a haematological response in patients with megaloblastic anaemia caused by vitamin B₁₂ deficiency. However, folic acid is ineffective in preventing the severe neurological pathologies associated with vitamin B₁₂ deficiency involving degeneration of the spinal cord.^[40]

Vitamin B₁₂ deficiency is often undiagnosed and may affect a substantial percentage of the population, particularly the elderly. It may be associated with haematological symptoms (megaloblastic anaemia) and/or neurological symptoms (dementia, paresthesia and ataxia). One risk of folic acid supplementation is that high doses may delay the appearance of haematological manifestations of vitamin B₁₂ deficiency and consequently may delay diagnosis of the deficiency, allowing the associated neurological complications to progress. In 1998, this concern led the US Institute of Medicine^[41] to set an upper limit for folic acid intake at 1000 µg (1 mg) per day. This upper limit does not apply to food folate, only to folic acid obtained from fortified foods and from supplements.

The concern of high folic acid intake potentially 'masking' vitamin B₁₂-deficiency symptoms has been addressed in a number of studies comparing the incidence of undiagnosed vitamin B₁₂ deficiency, as measured by low plasma vitamin B₁₂ concentrations, before and after fortification of the food supply with folic acid in the US and Canada. Mills et al.^[42] found no increase in low vitamin B₁₂ concentrations without anaemia, as measured by the haematocrit and the mean corpuscular volume (MCV), after fortification in a Veteran Affairs population in the US. However, Ray et al.^[43] reported a significant increase in the prevalence of combined B₁₂ insufficiency and high folate

concentrations in elderly Canadians after fortification. Wyckoff and Ganji^[44] also found a significant increase in the proportion of subjects with low serum vitamin B₁₂ concentrations in the absence of macrocytosis after fortification, and suggested that MCV should not be used as a marker for vitamin B₁₂ deficiency in the post-fortification age.

In frank vitamin B₁₂ deficiency, where marrow methionine synthase is inactive, 5-methyl-THF would be metabolically inert and would not be able to supply the other folate coenzymes required for thymidylate synthesis. Consequently, L-5-methyl-THF would not be able to induce a haematological response in these patients. Several studies have looked into the response of bone marrow cells isolated from vitamin B₁₂-deficient patients, with respect to DNA synthesis, and reported that treatment with L-5-methyl-THF does not stimulate thymidylate synthesis, whereas folic acid does.^[45-49] There has also been an *in vivo* study of a single vitamin B₁₂-deficient individual, which found that administering L-5-methyl-THF did not mask the vitamin B₁₂ deficiency.^[50] Gutstein et al.^[50] administered L-5-methyl-THF orally and parenterally to a female patient with tropical sprue and combined folate and vitamin B₁₂ deficiencies with megaloblastic bone marrow. The 3-week treatment did not produce any haematological remission, which confirmed the 'methyl trap' hypothesis. Vitamin B₁₂ was then administered and, as expected, there was a rise in the reticulocyte count and haematocrit (since L-5-methyl-THF could be regenerated to THF again), and all folate-active compounds for DNA synthesis were formed. Finally, Smulders et al.^[51] reported that almost all of the features of the 'methyl trap' hypothesis were confirmed in a patient with vitamin B₁₂ deficiency. The authors compared various parameters before and after vitamin B₁₂ treatment with respect to folate metabolism in a patient who was vitamin B₁₂ deficient and homozygous for the *MTHFR* 677C → T polymorphism. In a comparison with post-vitamin B₁₂ supplementation values, analysis of RBC folate vitamers distribution revealed lower RBC folate concentrations, a much higher fraction of L-5-methyl-THF in the RBC folate and a slight shift towards shorter chain lengths in the polyglutamate chain length distribution of RBC L-5-methyl-THF. Furthermore, total DNA methylation was reduced by 22%, as shown by the L-5-methylcytosine/total cytosine ratio.

In vitamin B₁₂ deficiency, folates are 'trapped' as L-5-methyl-THF, which leads to a functional deficiency of other folate coenzymes. As L-5-methyl-THF is a very poor substrate for folylpolyglutamate synthase, it is not converted into the polyglutamate forms required for folate retention, and it is released by the tissue, leading to a true cellular folate deficiency. Initially, plasma folate concentrations increase but, in the long term, the

folate is catabolized and excreted, leading to lowered plasma folate concentrations. When folic acid is given to vitamin B₁₂-deficient subjects, some of the dose can be converted to polyglutamates in tissues, retained and converted to other folate coenzymes prior to its eventual trapping as L-5-methyl-THF. This would have the potential to delay the appearance of megaloblastic changes (masking). The haematological changes associated with vitamin B₁₂ deficiency generally occur before the neurological symptoms and are easily treatable before the onset of irreversible long-term neurological damage (funicular myelosis).

In asymptomatic vitamin B₁₂-deficient subjects (i.e. subjects at earlier stages in the progression to clinical disease), methionine synthase activity would be lowered but not completely absent. The rate of metabolism of L-5-methyl-THF to other folate forms involved in thymidylate synthesis would be reduced, but some metabolism would still occur. Consequently, L-5-methyl-THF could slow the appearance of megaloblastic blood changes in these subjects and potentially 'mask' the underlying vitamin B₁₂ deficiency. However, its potential for 'masking' would be lower than that of folic acid or other folate forms that can provide cofactors for thymidylate synthetase without first having to be metabolized through the vitamin B₁₂-dependent methionine synthase reaction.

The advantages of long-term therapy with L-5-methyl-THF, compared with folic acid, are several. First, as outlined above, L-5-methyl-THF has a lower potential for masking vitamin B₁₂-deficiency symptoms. Second, the cellular uptake of circulating L-5-methyl-THF is subject to tight cellular control, whereas folic acid, which is not subject to this cellular control, is

retained even in folate-replete subjects.^[3,52] For these reasons, it can be suggested that L-5-methyl-THF should be considered for use in long-term folate therapies.

3.2 Effect of Polymorphisms in the 5,10-Methylenetetrahydrofolate Reductase Gene

A variant (677C→T) in the *MTHFR* gene is the most established genetic risk factor for neural tube defects. MTHFR is a key regulatory enzyme in folate and homocysteine metabolism because it irreversibly catalyses the formation of L-5-methyl-THF from 5,10-methylene-THF (figure 5). The 677C→T substitution results in an amino acid change of alanine to valine at a site that is critical for flavin adenine dinucleotide (FAD) binding and enzyme stability.^[53]

Approximately 10% of the Caucasian and Asian populations are homozygous carriers of the 677T variant.^[54,55] The frequencies are lower in the African American population (about 1%) and higher in the Hispanic population (about 25%). Homozygous carriers of the 677T allele have reduced MTHFR activity, leading to impaired synthesis of L-5-methyl-THF and elevated homocysteine concentrations.

MTHFR regulates the remethylation of homocysteine to methionine by catalysing the rate-limiting reduction of 5,10-methylene-THF to the methyl group donor L-5-methyl-THF. Thus the metabolic effects of this polymorphism reflect defects in methyl group metabolism due to reduced L-5-methyl-THF synthesis via the MTHFR reaction. L-5-methyl-THF and its subsequent metabolism through the methionine synthase reaction are not affected by the 677T variant in its first-pass

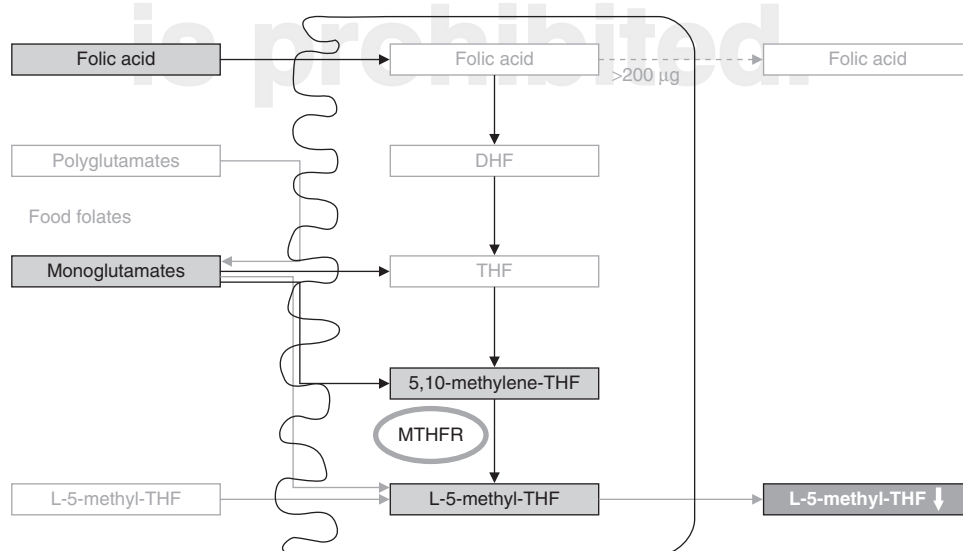


Fig. 5. Impairment of intestinal absorption of folic acid and some reduced dietary folates through inhibition of MTHFR. DHF = dihydrofolate; MTHFR = 5,10-methylene-THF reductase; THF = tetrahydrofolate; ↓ indicates a decreased concentration.

metabolism. Once it is converted to THF and other folate derivatives, however, further utilization for methyl group metabolism would require its metabolism through the MTHFR reaction, and any metabolic advantage over folic acid would be lost.

Several studies have shown that [6S]-5-methyl-THF is at least as effective as folic acid in increasing plasma folate and RBC folate concentrations in men. However, only a few of these studies took into consideration the genotype of the 677C→T polymorphism of *MTHFR*. Some of these studies had several limitations, including the use of high doses and racemic mixtures. In a single-dose study (5 mg), Willems et al.^[56] found higher bioavailability of L-5-methyl-THF (administered as a racemic mixture) compared with folic acid, irrespective of the (cardiovascular) patient's genotype. Pentieva et al.^[7] performed a bioavailability study in which the natural folate derivative [6S]-5-methyl-THF and folic acid were compared (a 500 µg dose of both forms). They excluded the TT genotype and found in the remaining genotypes that the plasma folate response curve was significantly higher after both folate treatments than after placebo, and the response did not differ between the treatments. Several long-term studies have been performed, with prior genotyping of the study participants and equal distribution of *MTHFR* genotypes between groups (Fohr et al.^[10] [racemic mixture], Venn et al.^[35] Litynski et al.^[57] and Lamers et al.^[11]). Because of the limited numbers of individuals with the TT genotype, each study was constrained by the small number of homozygous persons in the different treatment groups. Therefore, data on the effect of [6S]-5-methyl-THF or folic acid on plasma or RBC folate concentrations in TT individuals are rare and require further clarification.

3.3 Potential Drug Interactions

Several drugs, such as methotrexate, aminopterin, pyrimethamine, trimethoprim and triamterene, affect folate metabolism by inhibition of DHFR.^[23] Folate is required for the synthesis of thymidylate, a nucleotide required specifically for the synthesis of DNA. The normal function of DHFR is to reduce DHF produced in the thymidylate cycle back to THF. Thymidylate synthetase activity is only expressed in replicating cells and is highest in fast growing cells. Consequently, drugs targeted to DHFR, such as methotrexate, have proven to be effective chemotherapeutic agents, as they are selective inhibitors of rapidly growing cells.

Low-dose methotrexate is used extensively for the treatment of rheumatoid arthritis and is also used for the treatment of other systemic rheumatic disorders and psoriasis.^[58-60] Its therapeutic effectiveness has not been shown to be influenced by oral administration of folate derivatives.^[61] A meta-analysis of nine studies indicates that folic acid supplementation reduces the toxicity of methotrexate without reducing its efficacy.^[62]

While folic acid supplements do not appear to influence the efficacy of low-dose methotrexate treatment, the use of methotrexate and other drugs that target DHFR would be expected to interfere with the metabolic utilization of folic acid, which requires its reduction to THF by DHFR. Because folic acid is a very poor substrate for human DHFR,^[23,63] the initial reduction to DHF occurs slowly and would be greatly impaired by DHFR inhibitors. In addition, the human liver varies greatly in its ability to reduce folic acid.^[63] DHFR is not involved in the metabolic utilization of L-5-methyl-THF and other naturally

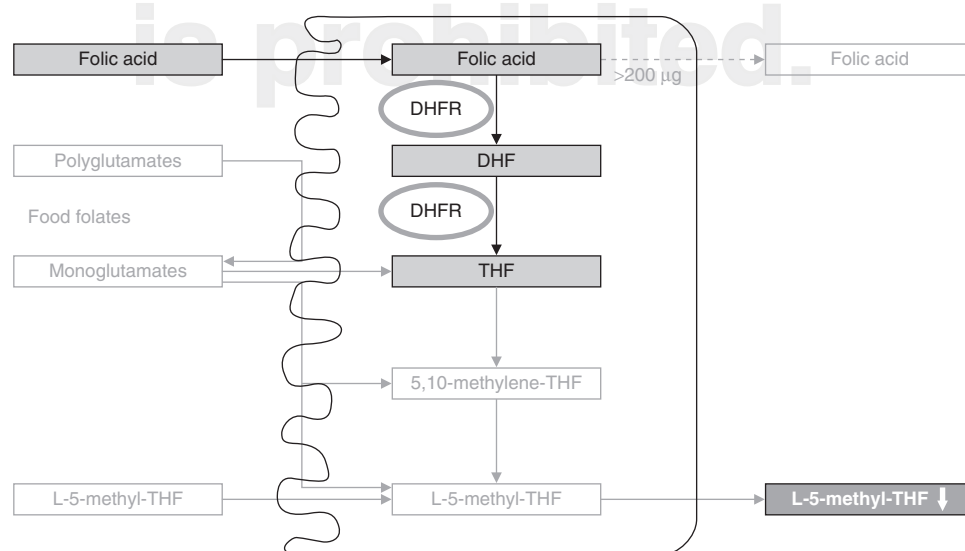


Fig. 6. Impairment of intestinal absorption of folic acid through inhibition of DHFR. **DHF** = dihydrofolate; **DHFR** = DHF reductase; **THF** = tetrahydrofolate; ↓ indicates a decreased concentration.

occurring reduced folates. Thus a considerably reduced interaction potential with DHFR inhibitors is expected during absorption after oral administration of L-5-methyl-THF as compared with folic acid (figure 6).

4. Conclusions

Folic acid is not naturally occurring, and it requires activation by reduction before it can serve as a coenzyme for one-carbon transfer reactions. L-5-methyl-THF is the predominant form of dietary folate, the principal form of circulating folate in the body and the preferred substrate for transport into peripheral tissues.

Bioavailability studies have provided strong evidence that L-5-methyl-THF is at least as effective as folic acid in improving folate status, as measured by blood concentrations of folate and by functional indicators of folate status, such as plasma homocysteine concentration.

Supplementation with L-5-methyl-THF may be preferable to folic acid, as L-5-methyl-THF may be less likely to mask haematological symptoms of severe vitamin B₁₂ deficiency and it exhibits a lower interaction potential with drugs that inhibit DHFR.

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References

- Zhao R, Matherly LH, Goldman ID. Membrane transporters and folate homeostasis: intestinal absorption and transport into systemic compartments and tissues. *Expert Rev Mol Med* 2009; 11: e4
- Schmitz JC, Stuart RK, Priest DG. Disposition of folic acid and its metabolites: a comparison with leucovorin. *Clin Pharmacol Ther* 1994; 55: 501-8
- Lucock M, Wild J, Smithells R, et al. Biotransformation of pteroyl-monglutamic acid during absorption: implications of Michaelis-Menten kinetics. *Eur J Clin Nutr* 1989; 43 (9): 631-5
- Kelly P, McPartlin J, Goggins M, et al. Unmetabolized folic acid in serum: acute studies in subjects consuming fortified food and supplements. *Am J Clin Nutr* 1997; 65 (6): 1790-5
- Stern LL, Bagley PJ, Rosenberg IH, et al. Conversion of 5-formyl-tetrahydrofolic acid to 5-methyltetrahydrofolic acid is unimpaired in folate-adequate persons homozygous for the C677T mutation in the methylenetetrahydrofolate reductase gene. *J Nutr* 2000; 130 (9): 2238-42
- Perry J, Chanarin I. Intestinal absorption of reduced folate compounds in man. *Br J Haematol* 1970; 18 (3): 329-39
- Pentieva K, McNulty H, Reichert R, et al. The short-term bioavailabilities of [6S]-5-methyltetrahydrofolate and folic acid are equivalent in men. *J Nutr* 2004; 134 (3): 580-5
- Prinz-Langenohl R, Lamers Y, Moser R, et al. Effect of folic acid preload on the bioequivalence of [6S]-5-methyltetrahydrofolate and folic acid in healthy volunteers [abstract no. 169-P]. *J Inher Metab Dis* 2003; 26 Suppl. 1: 124
- Melse-Boonstra A, Verhoef P, West C. Quantifying folate bioavailability: a critical appraisal of methods. *Curr Opin Clin Nutr Metab Care* 2004; 7 (5): 539-45
- Fohr IP, Prinz-Langenohl R, Brönstrup A, et al. 5,10-Methylenetetrahydrofolate reductase genotype determines the plasma homocysteine-lowering effect of supplementation with 5-methyltetrahydrofolate or folic acid in healthy young women. *Am J Clin Nutr* 2002; 75 (2): 275-82
- Lamers Y, Prinz-Langenohl R, Moser R, et al. Supplementation with [6S]-5-methyltetrahydrofolate or folic acid equally reduces plasma total homocysteine concentrations in healthy women. *Am J Clin Nutr* 2004; 79 (3): 473-8
- Houghton LA, Sherwood KL, Pawlosky R, et al. [6S]-5-Methyltetrahydrofolate is at least as effective as folic acid in preventing a decline in blood folate concentrations during lactation. *Am J Clin Nutr* 2006; 83 (4): 842-50
- De Meer K, Smulders YM, Dainty JR, et al. [6S]-5-methyltetrahydrofolate or folic acid supplementation and absorption and initial elimination of folate in young and middle-aged adults. *Eur J Clin Nutr* 2005; 59 (12): 1409-16
- Bhandari SD, Gregory JF. Folic acid, 5-methyl-tetrahydrofolate and 5-formyl-tetrahydrofolate exhibit equivalent intestinal absorption, metabolism and in vivo kinetics in rats. *J Nutr* 1992; 122: 1847-54
- Gregory JF, Bhandari SD, Bailey LB, et al. Relative bioavailability of deuterium-labeled monoglutamyl tetrahydrofolates and folic acid in human subjects. *Am J Clin Nutr* 1992; 55: 1147-53
- Wright JA, Finglas PM, Dainty JR, et al. Differential kinetic behavior and distribution for pteroylglutamic acid and reduced folates: a revised hypothesis of the primary site of PteGlu metabolism in humans. *J Nutr* 2005; 135: 619-23
- Sirotnak FM, Tolner B. Carrier mediated membrane transport of folates in mammalian cells. *Ann Rev Nutr* 1999; 19: 91-122
- Chiao JH, Roy K, Tolner B, et al. RFC-1 gene expression regulates folate absorption in mouse small intestine. *J Biol Chem* 1997; 272 (17): 11165-70
- Chen L, Qi H, Korenberg J, et al. Purification and properties of human cytosolic folylpolyglutamate synthetase and organization, localization and differential splicing of its gene. *J Biol Chem* 1996; 271: 13077-87
- Green JM, Ballou DP, Matthews RG. Examination of the role of methylenetetrahydrofolate reductase in incorporation of methyltetrahydrofolate into cellular metabolism. *FASEB J* 1988; 2: 42-7
- Shane B. Folylpolyglutamate synthesis and role in the regulation of one carbon metabolism. *Vit Horm* 1989; 45: 263-335
- Shane B. Folate, vitamin B12 and vitamin B6. In: Stipanuk MH, editor. *Biochemical and physiological aspects of human nutrition*. New York: WB Saunders Company, 2000: 483-518
- Blakley RL. Dihydrofolate reductase. In: Blakley RL, Benkovic SJ, editors. *Folates and pterins: chemistry and biochemistry of folates*. Vol. 1. New York: Wiley, 1984: 191-244
- Matthews RG, Ghose C, Green JM, et al. Folylpolyglutamates as substrates and inhibitors of folate-dependent enzymes. In: Weber G, editor. *Advances in enzyme regulation*. Vol. 26. Oxford: Pergamon Press, 1987; 157-71
- Gregory III JF, Cuskelly GJ, Shane B, et al. Primed, constant infusion with [2H3]serine allows in vivo kinetic measurement of serine turnover, homocysteine remethylation, and transsulfuration processes in human one-carbon metabolism. *Am J Clin Nutr* 2000; 72 (6): 1535-41

26. Woeller CF, Anderson DD, Szebenyi DME, et al. Evidence for SUMO-dependent nuclear location of the thymidylate biosynthesis pathway. *J Biol Chem* 2007; 282 (24): 17623-31
27. Wolffe AP, Jones PL, Wade PA. DNA methylation. *Proc Natl Acad Sci U S A* 1999; 96: 5894-6
28. Gregory III JF, Williamson J, Liao JF, et al. Kinetic model of folate metabolism in nonpregnant women consuming [2H₂]folic acid: isotopic labeling of urinary folate and the catabolite para-acetamidobenzoylglutamate indicates slow, intake-dependent, turnover of folate pools. *J Nutr* 1998; 128 (11): 1896-906
29. Suh JR, Herbig AK, Stover PJ. New perspectives on folate catabolism. *Annu Rev Nutr* 2001; 21: 255-82
30. Gregory III JF. Case study: folate bioavailability. *J Nutr* 2001; 131 (4 Suppl.): 1376-82S
31. Selhub J, Jacques PF, Wilson PW, et al. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. *JAMA* 1993; 270 (22): 2693-8
32. Tucker KL, Selhub J, Wilson PW, et al. Dietary intake pattern relates to plasma folate and homocysteine concentrations in the Framingham Heart Study. *J Nutr* 1996; 126 (12): 3025-31
33. Brouwer IA, van Dusseldorp M, West CE, et al. Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary controlled trial. *J Nutr* 1999; 129 (6): 1135-9
34. Venn BJ, Green TJ, Moser R, et al. Increases in blood folate indices are similar in women of childbearing age supplemented with [6S]-5-methyltetrahydrofolate and folic acid. *J Nutr* 2002; 132 (11): 3353-5
35. Venn BJ, Green TJ, Moser R, et al. Comparison of the effect of low-dose supplementation with L-5-methyltetrahydrofolate or folic acid on plasma homocysteine: a randomized placebo-controlled study. *Am J Clin Nutr* 2003; 77 (3): 658-62
36. Lamers Y. (6S)-5-methyltetrahydrofolate compared to folic acid supplementation: effect on risk markers of neural tube defects [dissertation; in German]. Bonn: University of Bonn, 2005
37. Lamers Y, Kehlenbach U, Prinz-Langenohl R, et al. [6S]-5-methyltetrahydrofolate as adequate alternative to folic acid supplementation [abstract]. 1st International Conference on Folate – Analysis, Bioavailability and Health (EuroFoodFolate 2004); 2004 Feb 11-14; Warsaw
38. Bostom AG, Shemin D, Bagley P, et al. Controlled comparison of L-5-methyltetrahydrofolate versus folic acid for the treatment of hyperhomocysteinemia in hemodialysis patients. *Circulation* 2000; 101 (24): 2829-32
39. Shane B, Stokstad EL. Vitamin B12-folate interrelationships. *Annu Rev Nutr* 1985; 5: 115-41
40. Pietrzik K, Golly I, Loew D. Handbuch Vitamine – für Prophylaxe, Therapie und Beratung. Munich: Urban und Fischer Verlag, 2008
41. Institute of Medicine. Dietary reference intakes (DRI) for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. Washington DC: National Academy Press, 1998 [online]. Available from URL: http://books.nap.edu/openbook.php?record_id=6015 [Accessed 2010 May 18]
42. Mills JL, Von Kohorn I, Conley MR, et al. Low vitamin B-12 concentrations in patients without anemia: the effect of folic acid fortification of grain. *Am J Clin Nutr* 2003 Jun; 77 (6): 1474-7
43. Ray JG, Vermeulen MJ, Langman LJ, et al. Persistence of vitamin B12 insufficiency among elderly women after folic acid food fortification. *Clin Biochem* 2003 Jul; 36 (5): 387-91
44. Wyckoff KF, Ganji V. Proportion of individuals with low serum vitamin B-12 concentrations without macrocytosis is higher in the post folic acid fortification period than in the pre folic acid fortification period. *Am J Clin Nutr* 2007 Oct; 86 (4): 1187-92
45. Metz J, Kelly A, Swett VC, et al. Deranged DNA synthesis by bone marrow from vitamin B12-deficient humans. *Br J Haematol* 1968; 14 (6): 575-92
46. Ganeshaguru K, Hoffbrand AV. The effect of deoxyuridine, vitamin B12, folate and alcohol on the uptake of thymidine and on the deoxynucleoside triphosphate concentrations in normal and megaloblastic cells. *Br J Haematol* 1978; 40 (1): 29-41
47. Zittoun J, Marquet J, Zittoun R. Effect of folate and cobalamin compounds on the deoxyuridine suppression test in vitamin B12 and folate deficiency. *Blood* 1978; 51 (1): 119-28
48. Das KC, Herbert V. In vitro DNA synthesis by megaloblastic bone marrow: effect of folates and cobalamins on thymidine incorporation and de novo thymidylate synthesis. *Am J Hematol* 1989; 31 (1): 11-20
49. Hoffbrand AV, Jackson BF. Correction of the DNA synthesis defect in vitamin B12 deficiency by tetrahydrofolate: evidence in favour of the methyl-folate trap hypothesis as the cause of megaloblastic anaemia in vitamin B12 deficiency. *Br J Haematol* 1993; 83 (4): 643-7
50. Gutstein S, Bernstein LH, Levy L, et al. Failure of response to N5-methyltetrahydrofolate in combined folate and B12 deficiency: evidence in support of the 'folate trap' hypothesis. *Dig Dis* 1973; 18 (2): 142-6
51. Smulders YM, Smith DE, Kok RM, et al. Cellular folate vitamers distribution during and after correction of vitamin B12 deficiency: a case for the methyl-folate trap. *Br J Haematol* 2006; 132 (5): 623-9
52. Scott J. Methyltetrahydrofolate: the superior alternative to folic acid. In: Kramer K, Hoppel PP, editors. *Nutraceuticals in health and disease prevention*. New York: Marcel Dekker, 2001: 75-90
53. Frosst P, Blom HJ, Milos R, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 1995; 10 (1): 111-3
54. Bailey LB, Moyers S, Gregory III JF. Folate. In: Bowman BA, Russell RM, editors. *Present knowledge in nutrition*. 8th ed. Washington, DC: ILSI Press, 2001: 214-29
55. Ueland PM, Rozen R. MTHFR polymorphisms and disease. Austin (TX): Landes Bioscience, 2005
56. Willems FF, Boers GH, Blom HJ, et al. Pharmacokinetic study on the utilization of 5-methyltetrahydrofolate and folic acid in patients with coronary artery disease. *Br J Pharmacol* 2004; 141 (5): 825-30
57. Litynski P, Loehrer F, Linder L, et al. Effect of low dose of 5-methyltetrahydrofolate and folic acid on plasma homocysteine in healthy subjects with or without the 677C → T polymorphism of methylenetetrahydrofolate reductase. *Eur J Clin Invest* 2002; 32: 662-8
58. Pincus T, Yazici Y, Sokka T, et al. Methotrexate as the 'anchor drug' for the treatment of early rheumatoid arthritis. *Clin Exp Rheumatol* 2003; 21: S179-85
59. Wong JM, Esdaile JM. Methotrexate in systemic lupus erythematosus. *Lupus* 2005; 14: 101-5
60. Warren RB, Griffiths CE. Systemic therapies for psoriasis: methotrexate, retinoids, and cyclosporine. *Clin Dermatol* 2008; 26: 438-47
61. Visser K, Katchamart W, Loza E, et al. Multinational evidence-based recommendations for the use of methotrexate in rheumatic disorders with a focus on rheumatoid arthritis: integrating systematic literature research and expert opinion of a broad international panel of rheumatologists in the 3E Initiative. *Ann Rheum Dis* 2009; 68: 1086-93
62. Katchamart W, Ortiz A, Shea B, et al. Folic acid and folinic acid for reducing side effects in patients receiving methotrexate for rheumatoid arthritis (an update systematic review and metaanalysis). *J Rheumatol* 2008; 58 (6): 1197-8
63. Bailey SW, Ayling JE. The extremely slow and variable activity of dihydrofolate reductase in human liver and its implications for high folic acid intake. *Proc Natl Acad Sci U S A* 2009; 106: 15424-9

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