DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration

Memorandum

Date: MAY 23 2001

From: Director, Division of Standards and Labeling Regulations, Office of Nutritional Products, Labeling and Dietary Supplements, HFS-820

Subject: 75-Day Premarket Notification for New Dietary Ingredients

To: Dockets Management Branch, HFA-305

New Dietary Ingredient:	L-5-Methyl-THF
Firm:	Merck KGaA
Date Received by FDA:	March 13, 2001
90-Day Date:	June 11, 2001

In accordance with the requirements of section 413(a) of the Federal Food, Drug, and Cosmetic Act, the attached 75-day premarket notification for the aforementioned new dietary ingredient should be placed on pubic display in docket number 95S-0316 **after** June 11, 2001.

Felicia B. Satchell

Felicia B. Satchell

95S-0316

RPT95

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington, DC

APR – 2 2001

Dr. Najib Sehat
Merck KGaA
CHN-BS
Regulatory Affairs, C11/243
Frankfurter Str. 250
64293 Darmstadt, Germany

Dear Dr. Sehat:

This is to inform you that the notification dated March 9, 2001, you submitted pursuant to 21 U.S.C. 350b(a)(2) was received and filed by the Food and Drug Administration (FDA) on March 13, 2001. Your notification concerns the substance described as "the calcium salt of L-5-methyltetra-hydrofolate (L-5-methyl-THF)" that you assert is a new dietary ingredient. This notification is a resubmission of a notification dated September 22, 2000 for the same ingredient that FDA responded to in a letter dated December 26, 2000.

In accordance with 21 C.F.R. § 190.6(c), FDA must acknowledge its receipt of a notification for a new dietary ingredient. For 75 days after the filing date (i.e., after May 27, 2001), you must not introduce or deliver for introduction into interstate commerce any dietary supplement that contains "L-5-methyl-THF." Please note that the acceptance of this notification for filing is a procedural matter and thus, does not constitute a finding by FDA that the new dietary ingredient or the dietary supplement that contains the new dietary ingredient is safe or is not adulterated under 21 U.S.C. 342. As another procedural matter, your notification will be kept confidential for 90 days after the filing date. After June 11, 2001, your notification will be placed on public display at FDA's Docket Management Branch in docket number 95S-0316. However, any trade secret or otherwise commercial confidential information in the notification will not be disclosed to the public.

Please contact us at (202) 205-4168, if you have any questions concerning this matter.

Sincerely yours,

Rhonda R. Kane, M.S., R.D.
Consumer Safety Officer
Dietary Supplements Team
Division of Standards
and Labeling Regulations
Office of Nutritional Products, Labeling
and Dietary Supplements
Center for Food Safety
and Applied Nutrition

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Food and Drug Administration
Center for Food Safety and Applied Nutrition, CFSAN
Office of Nutritional Products, Labeling, and Dietary Supplements
HFS-820
200 C Street, S.W.
Washington, DC 20204
USA

Attention: Ms. Felicia B. Stachell

Dear Ms. Stachell:

Pursuant to Section 8 of the Dietary Supplement Health and Education Act of 1994, Merck KGaA ("Merck"), located at Darmstadt, Frankfurter Str. 250, 64293 Darmstadt, Germany, submits this new dietary ingredient notification to the Food and Drug Administration (FDA) for the calcium salt of L-5-methyltetra-hydrofolate ("L-5-methyl-THF"), a derivative of folic acid to be manufactured by Merck for use in dietary supplements.

Merck's L-5-methyl-THF is intended for use in dietary supplements as a source of the naturally occurring, predominant form of folate.

Attached is a discussion of the scientific data and information demonstrating that Merck's L-5-methyl-THF, when used under the conditions suggested in the labeling of the dietary supplements, is reasonably expected to be safe. Included in the attachments are the following:

(1) a description of the chemistry, manufacturing, stability and intended use, "Chemistry and Properties of L-5-Methyltetrahydrofolic acid, Calcium Salt Intended for the Use in Dietary Supplements as a Source of the Naturally Occurring Predominant Form of Folate," (Attachment I);

(2) a discussion of the safety and vital role of L-5-methyl-THF in folate physiology, "Nutritional Functions and Safety of Consumption of L-5-Methyltetrahydrofolate, the Naturally Occurring Predominant Form of Dietary Folate and its Vital Role in Folate Physiology," by Jacob Selhub, Ph.D., Jesse F. Gregory, III, Ph.D., Walter H. Glinsmann, M.D. (Attachment II);

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MERCK

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(3) and a list and three copies of the cited references, including English translations of the two cited German references.

Sincerely,

Merck KGaA

ppa.

Dr. Arnulf Heubner

i. V.

Dr. Najib Sehat

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New Dietary Ingredient Notification

Attachment I

Chemistry and Properties of L-5-Methyltetrahydrofolic acid, Calcium Salt Intended for the Use in Dietary Supplements as a Source of the Naturally Occurring Predominant Form of Folate

Kommanditgesellschaft auf Aktien
Handelsregister AG Darmstadt HRB 6164
Sitz der Gesellschaft: Darmstadt

Geschäftsleitung und pers. haftende Gesellschafter:
Bernhard Scheuble (Vorsitzender),
Michael Römer (stellvertretender Vorsitzender),
Michael Becker, Thomas Schreckenbach.

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1. CHEMISTRY CONSIDERATIONS CONCERNING L-5-METHYL-THF

1.1 CHEMICAL NAME

(6S)-N-[4-[[(2-amino-1,4,5,6,7,8-hexahydro-5-methyl-4-oxo-6-pteridinyl)methyl]amino]benzoyl]-L-glutamic acid, calcium salt

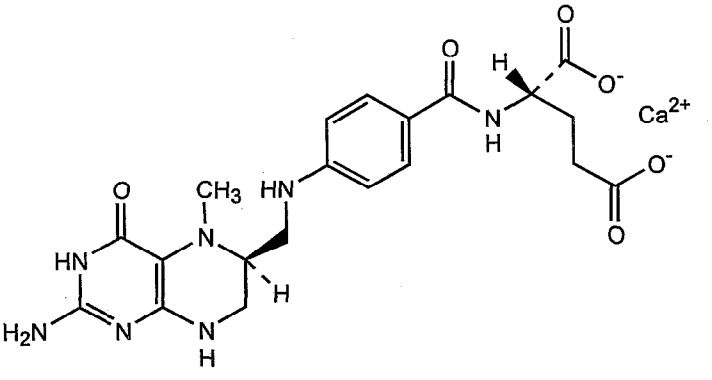
1.2 CHEMICAL ABSTRACT SERVICE (CAS) REGISTRY NUMBER

129025-21-4 (151533-22-1, L-5-methyl-THF : Calcium = 1 : 1 complex)

1.3 CHEMICAL SYNONYMS

Calcium L-Mefolinate
L-5-methyl-THF, calcium salt
(6S)-5-methyltetrahydrofolic acid, calcium salt
(6S)-5-methyl-5,6,7,8-tetrahydropteroyl-L-glutamic acid, calcium salt

1.4 CHEMICAL STRUCTURE



1.5 MOLECULAR FORMULA

$C_{20}H_{23}CaN_7O_6$

1.6 MOLECULAR WEIGHT

497.5

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1.7 PHYSICAL AND CHEMICAL PROPERTIES

Physical state, color:	Yellowish crystalline powder
Odor:	Almost odorless
pH (2.5%)	Between 7 and 8
Melting point:	Degradation > 300 °C
Flash point:	Degradation > 300 °C
Explosion properties:	No special properties
Density:	About 0.25 g/cm^3
Solubility:	Soluble in water, insoluble in organic solvents
Degradation:	Subject to oxidative and nonoxidative modes of degradation (Gregory 1989, 1996)

1.8 MANUFACTURING METHOD

1.9 SPECIFICATIONS

The calcium salt of L-5-methyl-THF is a beige to yellow crystalline powder that has a characteristic infrared absorption spectrum. Merck's specifications for L-5-

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methyl-THF, calcium salt are shown below in Table 1. These specifications indicate a high degree of purity generally consistent with the use of this substance as a nutritional dietary supplement.

Table 1. Specifications for L-5-methyl-THF, calcium salt

1.10 STABILITY

The stability of L-5-methyl-THF has been examined most extensively with regard to both the conditions of thermal processing and to changing pH. All folates are subject to chemical deterioration (reviewed by Gregory 1989,1996). Folic acid is very stable under physiological conditions and most conditions of food processing and storage. Tetrahydrofolate is highly susceptible to oxidative cleavage, while substituents at the N-5 position impart improved stability. L-5-methyl-THF is easily oxidized to 5-methyldihydrofolate, which retains vitamin activity by virtue of its ability to undergo facile reduction by thiols (e.g. cysteine or glutathione) or ascorbate. Irreversible deterioration of L-5-methyl-THF appears to occur mainly by chemical rearrangement of 5-methyldihydrofolate to form a pyrazino-s-triazine derivative. Exposure of 5-methyldihydrofolate to acidic conditions also yields cleavage of the C9-N10 bond. L-5-methyl-THF is often found to exhibit intermediate stability; it is often described as significantly less stable than folic acid. The degradation products of L-5-methyl-THF

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breakdown do not appear to be of toxicological significance as they are common constituents of most foods.

The manufacturer has developed a stability indicating HPLC-method, which is now validated. Tests on the stability of L-5-methyl-THF, calcium salt are performed according to the Guidelines of the International Conference of Harmonization (ICH). In stress-tests and as seen in many previous studies, the compound degrades completely over several days in aqueous solution in the absence of ascorbate or other reductant. The data regarding the stability of the powdered, crystalline L-5-methyl-THF calcium salt during extended storage at various temperatures and relative humidities indicate excellent stability (6 batches tested, data from a typical batch see table 2).
Initial tests in multivitamin preparations (where folic acid was replaced by L-5-methyl-THF, calcium salt) over 18 month show the compound's potential regarding stability for the intended use in supplements (Table 3).

Storage stability of the crystalline L-5-methyl-THF, calcium salt is comparable to or better than that of folic acid.

Table 2. Stability of L-5-methyl-THF, calcium salt during storage under varying conditions of temperature and relative humidity (r.h.), batch no. LMCA-7079

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Table 3. Stability of folic acid and L-5-methyl-THF, calcium salt in standard multivitamin tablets stored in blister packs

2. INTENDED USE OF L-5-METHYL-THF IN DIETARY SUPPLEMENTS

The intended use of L-5-methyl-THF, calcium salt is in dietary supplements as a source of folate. L-5-methyl-THF is the naturrally occurring predominant form of dietary folate.

3. NOMENCLATURE AND STRUCTURE OF FOLATES

Folate is the generic term for the family of pteroylglutamates that exhibit the qualitative vitamin activity of folic acid. As outlined below in Figure 1, folic acid (also pteroylglutamic acid, PteGlu) is the form of the vitamin having a fully aromatic ("oxidized") pteridine ring system, only traces appear in nature. This is the chemical form used most frequently for nutritional supplements because of its relative ease of synthesis and chemical stability.

Most naturally occurring dietary folates and folates in mammalian tissues are 5,6,7,8-tetrahydrofolates, primarily as polyglutamyl conjugates (Figure 1). The tetrahydrofolates function in metabolism as acceptors, carriers, and donors of one-carbon units. An additional important function of tetrahydrofolates is to mediate oxidation and reduction of folate-bound one-carbon units (*i.e.*, conversions among formyl (-CHO), methenyl ($-CH^{+}=$), methylene ($-CH_2-$). formimino (-CH=NH) and methyl ($-CH_3$) moieties). The tetrahydrofolates carry

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one-carbon units as substituents at the N-5 or N-10 positions or as methylene (-CH_2-) or methenyl (-CH^+=) carbons bridging from N-5 to N-10 (Figure 1). The tetrahydrofolates have asymmetric centers at the pteridin carbon no. 6 and at the alpha-carbon of the L-glutamate residue(s). Enzymatic reduction of folic acid yields only the L-diastereoisomer of tetrahydrofolate (THF) at the carbon no. 6, also termed L-tetrahydrofolate (L-THF), while chemical reduction yields an equimolar mixture of D- and L-diastereoisomers. The L-isomer has nutritional activity while the D-isomer appears to be metabolically inert in most instances. Of note is the fact that the R,S-nomenclature may create some confusion, because the natural form of folates substituted in the 10-position are in the 6R-form whereas all other natural forms are (as estimated) in the 6S-form.

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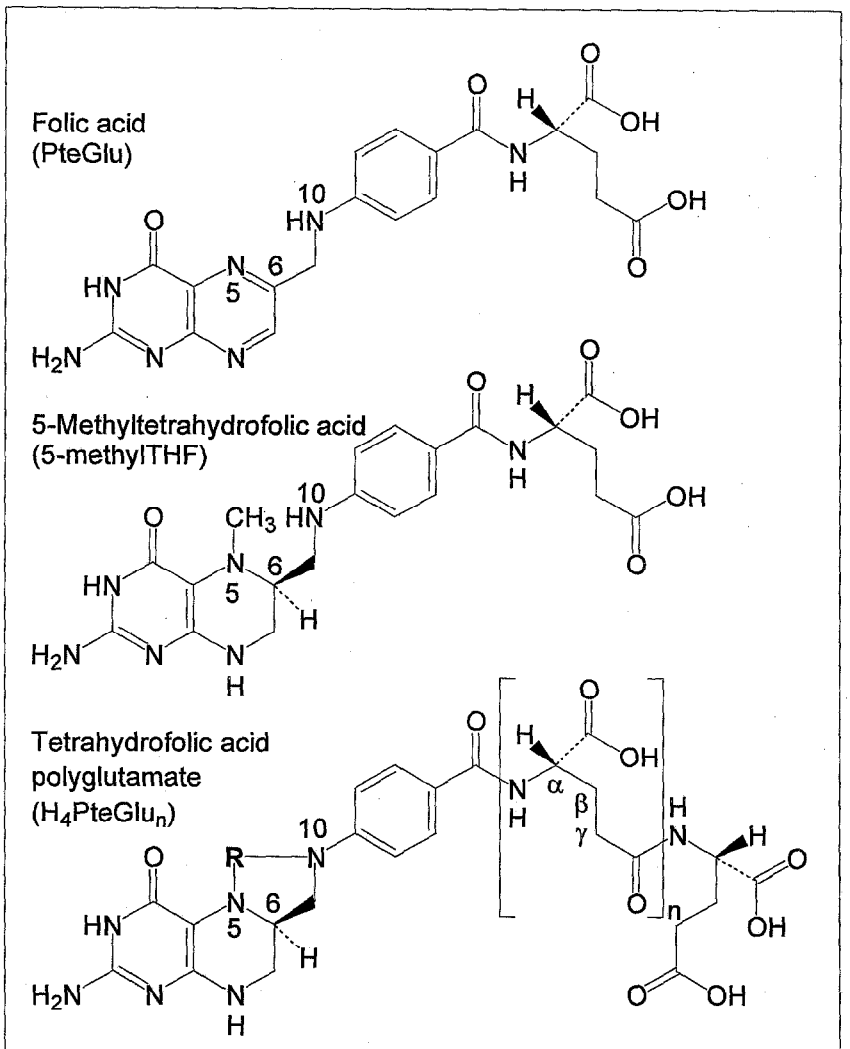
Figure 1. Chemical structures of folates



Substituent (R)	Position	Chemical name
-CH_3	5	5-Methyltetrahydrofolic acid
-CHO	5	5-Formyltetrahydrofolic acid
-CHO	10	10-Formyltetrahydrofolic acid
-CH=NH	5	5-Formiminotetrahydrofolic acid
-CH_2-	5 and 10	5,10-Methylenetetrahydrofolic acid
-CH^+=	5 and 10	5,10-Methenyltetrahydrofolic acid

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4. CONCLUSION

At the request of Merck, an independent panel of recognized experts, qualified by their scientific and/or medical training and relevant national and international experience to evaluate the safety of food and food ingredients, was convened in order to assess the generally recognized as safe (GRAS) status of L-5-methyl-THF, calcium salt for use as a source of folate in dietary supplements.
A comprehensive search of the scientific literature concerning nutritional, safety, and toxicity information was conducted and made available to the panel. The panel independently and critically evaluated the pertinent articles as well as the other information discussed above and concluded that, under the conditions of intended use, L-5-methyl-THF, meeting appropriate food grade specifications and produced in accordance with current good manufacturing practices (cGMP), is GRAS based on scientific procedures (Borzelleca et al. 1999). Additional data and informations for L-5-methyl-THF are summarized in the enclosed Attachment II.

Based on the above-described data and information, Merck concludes that its L-5-methyl-THF, calcium salt, when used as a source of folate under the conditions recommended or suggested in the labeling of such dietary supplements, is reasonably expected to be safe.

5. REFERENCES

Borzelleca, J.F., Glinsmann, W.H., Gregory, J.F. (1999) Expert Panel Report. (Unpublished report prepared for Merck KGaA).

Gregory, J. F. (1989). Chemical and nutritional aspects of folate research: Analytical procedures, methods of folate synthesis, stability and bioavailability of dietary folates. Chapter in: Advances in Food and Nutrition Research 33:1-101, Academic Press, San Diego, CA.

Gregory, J.F. (1996). Vitamins. Chapter in: Food Chemistry, 3rd ed. (O.R. Fennema, ed.), M. Dekker, pp. 531-616.

Müller et al., (1994). Process for the Preparation of (6S)- and (6R)-Tetrahydrofolic acid. United States Patent No. 5,324,836.

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Müller et al., (2000). Stabile kristalline Salze von 5-Methyltetrahydrofolsäure (stable crystalline salts of 5-methyltetrahydrofolic acid). Europäische Patentanmeldung EP 1 044 975 A1 (translation enclosed).

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New Dietary Ingredient Notification

Attachment II

Nutritional Functions and Safety of Consumption of L-5-Methyl-tetrahydrofolate the Naturally Occurring Predominant Form of Dietary Folate and its Vital Role in Folate Physiology

Jacob Selhub, Ph.D., Jesse F. Gregory, III, Ph.D., Walter H. Glinsmann, M.D.

The objective of this review is to summarize the natural occurrence, nutritional properties, and biological function of L-5-methyltetrahydrofolate (L-5-methyl-THF), leading toward the conclusion that a supplement containing L-5-methyl-THF is nutritionally equivalent to folic acid in terms of bioavailability and its ability to be safe and effective for use as a source of folates.

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1. PREFACE

As recognized by the Food and Drug Administration (FDA),

- ..... the diet/disease relationship is more accurately described as being related to all of the biologically active vitamin forms of folate rather than just to the synthetic form of the vitamin (i.e., folic acid).

  61 Fed. Reg. 8752, 8758 (Mar. 5, 1996) NTD Health Claim Final Rule

As recently as October 10, 2000, FDA noted that

- Once absorbed, both food folate and folic acid are converted to the same metabolically active reduced derivatives..... Because folic acid and naturally occurring food folate are converted into active coenzyme forms for use in the body, the evidence for a protective effect from folic acid also supports the same protective effect for naturally occurring folates. FDA, Letter Regarding Dietary Supplement Health Claim for Folic Acid With Respect to Neural Tube Defects (Oct. 10, 2000)

Consistent with these principles, the scientific evidence described below support the biological equivalence of folic acid and L-5-methyl tetrhydrofolate (L-5-methyl-THF).

2. PROPERTIES AND BIOLOGICAL RELATIONSHIP OF FOLIC ACID AND L-5-METHYL-THF

Folic acid is the parent structure of all folates. Only traces of folic acid are found in nature. The synthetic preparation is currently used for food enrichment and supplementation. Folic acid is incapable of serving as a coenzyme for one carbon transfer, unless reduced to tetrahydrofolate (THF). The chemical reduction results in an asymmetric carbon in the 6 position. Only the L-isomer is biologically active. The enzymatic reduction occurs intracellularly in a two step process, which is catalyzed by dihydrofolate reductase (Figure 1). The resulting L-THF acquires a methylene group from serine or glycine to form L-5,10-methylene-THF (L-5,10-CH_2-THF) for subsequent utilization for the synthesis of thymidylate, oxidation to formyl-THF for the synthesis of purines or reduction to L-5-methyl-THF for the synthesis of methionine (via the B12-dependent reaction).

Alternatively the L-THF is converted to L-THF-polyglutamates after acquiring additional glutamate residues at the p-aminobenzoylglutamate moiety (L-THFGlu_n). This acquisition of additional glutamate residues prevent the folate from escaping the cell and at the same time increases their coenzyme activities.

L-5-methyl-THF is the principal form of circulating folate in the body, hence the form which is taken up by peripheral tissue (see below). Its entry into the cellular folate metabolism is in many respect identical to that of folic acid in that its incorporation into this metabolism is preceded by conversion to unsubstituted L-THF (Figure 2). This conversion is achieved through the propensity of practically all animal living cells, to promptly react the incoming L-5-methyl-THF with the B12 dependent methyltransferase and homocysteine to form methionine and L-THF.

Figure 1. Intracellular metabolism of folic acid

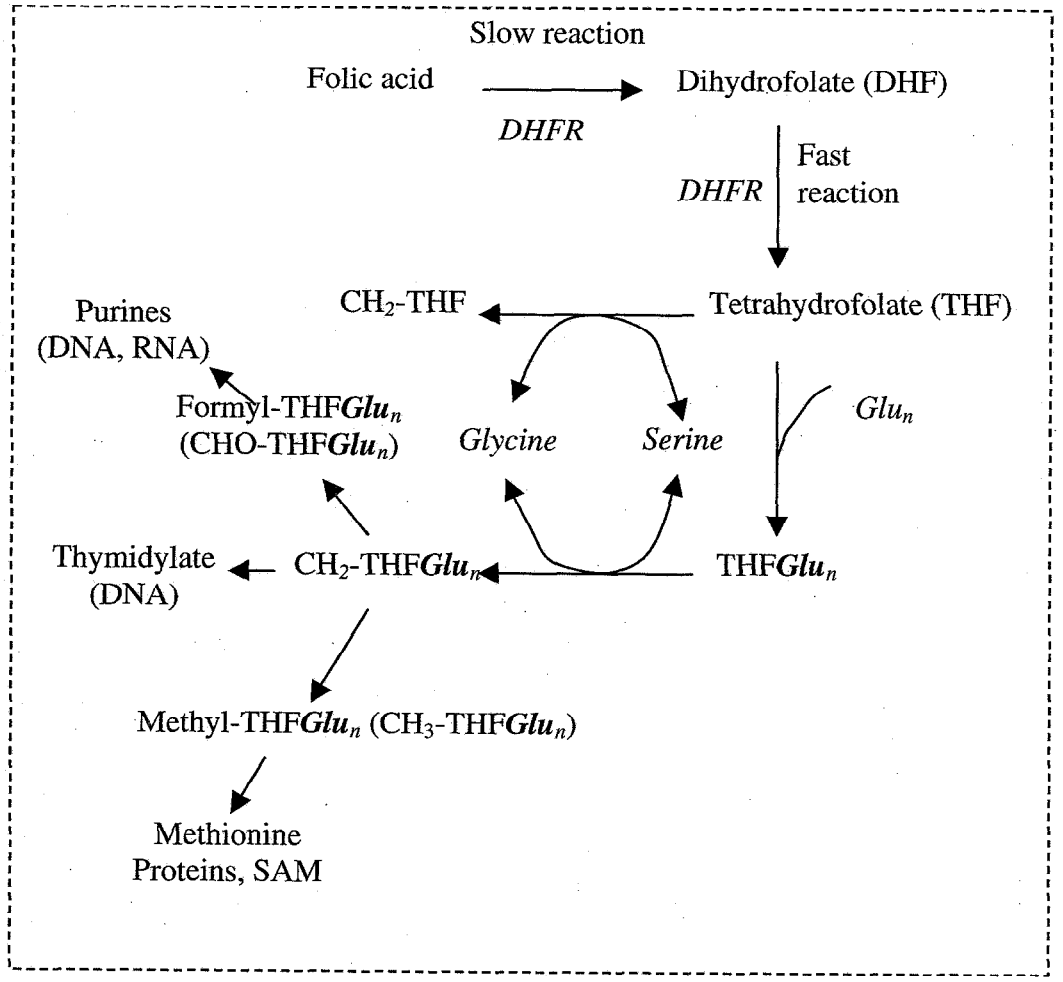
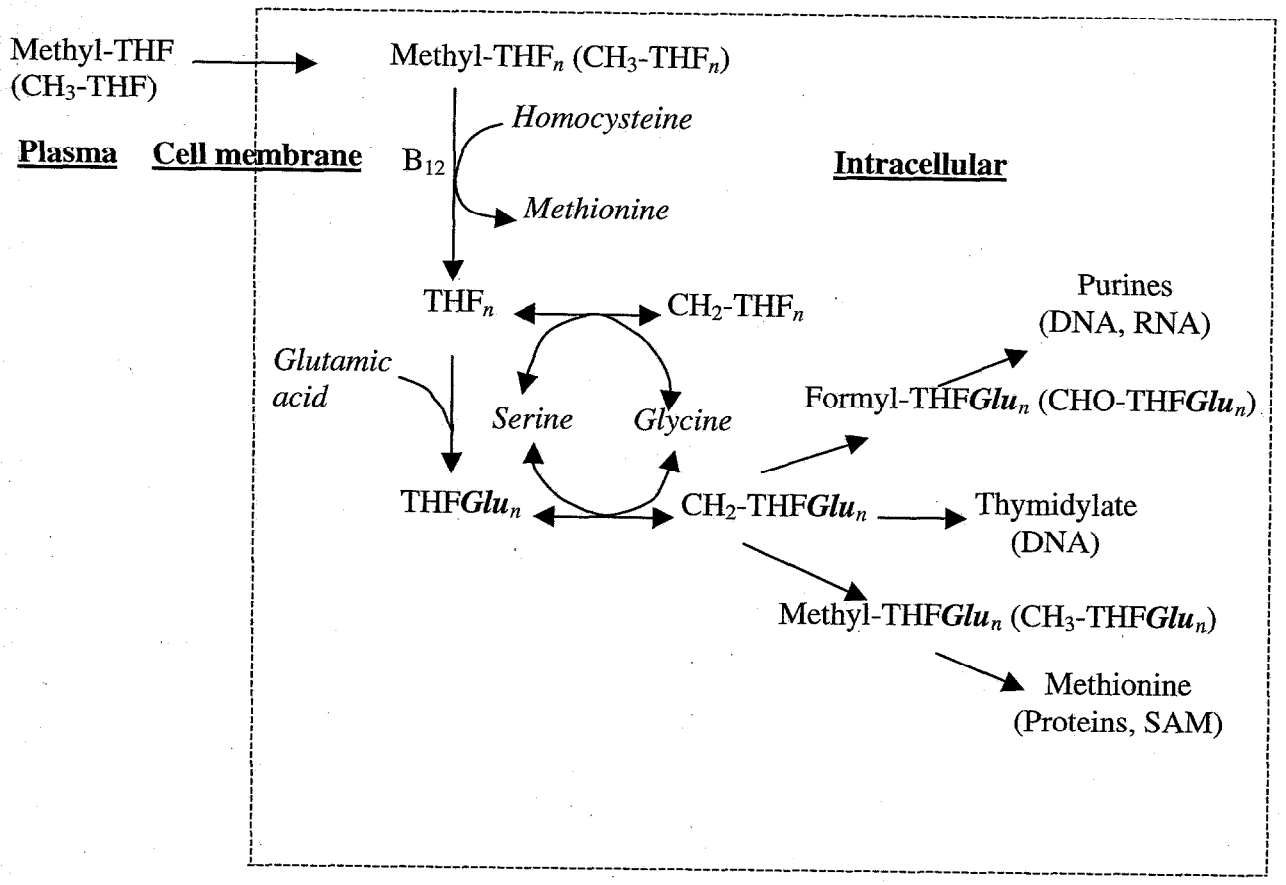


Figure 2. Transport and intracellular metabolism of L-5-methyl-THF by peripheral tissues



3. L-5-METHYL-THF AS THE MOST ABUNDANT AND, OFTEN THE ONLY FORM OF FOLATE IN FOOD

Unlike folic acid which is not found in nature, much of the naturally occurring folates are reduced polyglutamyl derivatives, 70% of which are forms of L-5-methyltetrahydrofolate (L-5-methyl-THF). Figure 3 is an example of folate distribution in 3 foods determined by Seyoum and Selhub (1993):

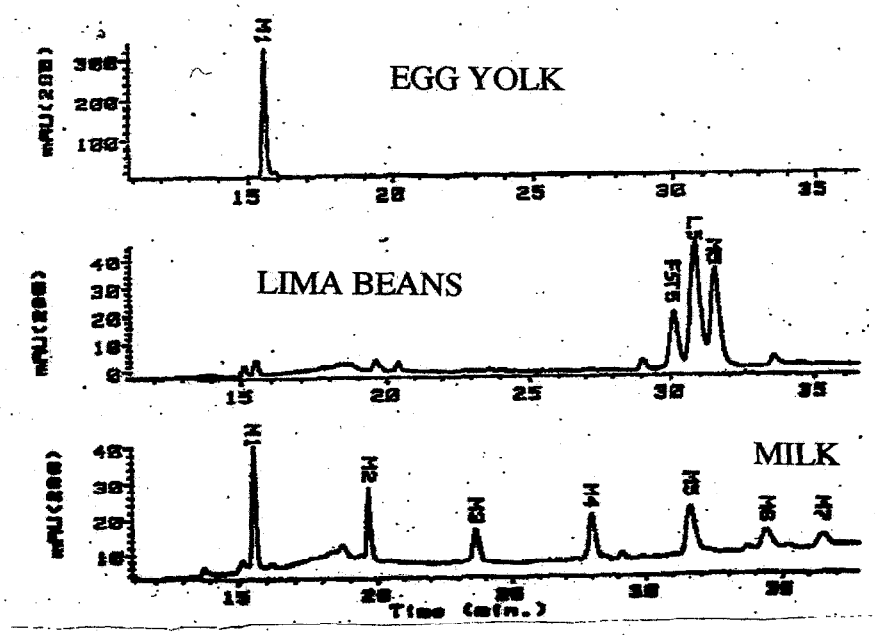
- egg yolk contains exclusively monoglutamyl L-5-methyl-THF;

- soybeans contain different forms of pentaglutamyl folates, about a third of which is methylated tetrahydrofolate, while
- milk comprises exclusively of a series of L-5-methyl-THF with different number of glutamate residues.

L-5-methyl-THF derivatives are also the folate forms found in orange juice (which is a major source of dietary folate), lettuce and cabbage (Table 1).

This is particularly evident from our understanding of the process of intestinal folate absorption (Figure 4).

Figure 3. Total Folate and 5-methyl-THF Content in Nonfortified Selected Foods
(Peaks containing M are L-5-methyl-THF)



Food type	Total folate by HPLC assay	Percent L-5-methyl-THF of total folate	Reference
White Bread	21.3±0.69 µg/100g	22.5%	Pfeiffer et al. 1997
Wheat Bread	29.8±1.94 µg/100g	11%	Pfeiffer et al. 1997
White Rice	10.8±0.57 µg/100g	34.9%	Pfeiffer et al. 1997
Spagetti	22.3±1.77 µg/100g	12.7%	Pfeiffer et al. 1997
Orange juice	0.23-0.40 µg/ml	100%	Gregory et al. 1984, White et al. 1991, Seyoum & Selhub 1993
49 Vegetable & fruit products	10-187 µg/100g	mean 70%	Muller 1993a
15 Egg, meat & fish products	1-963 µg/100g	4.5-90.6%	Muller 1993b
10 Dairy products	0.3-398 µg/100g	5.1-36.2%	Muller 1993b
Egg yolk	1.93 nmol/g	100%	Seyoum & Selhub 1993
Cow liver	7.69 nmol/g	19.4%	Seyoum & Selhub 1993
Lima beans	2.27 nmol/g	35.2%	Seyoum & Selhub 1993
Baker's yeast	69.1 nmol/g	86.4%	Seyoum & Selhub 1993
Cabbage	0.8nmol/g	100%	Seyoum & Selhub 1993
Lettuce	3.0 nmol/g	100%	Seyoum & Selhub 1993

4. INTESTINAL TRANSPORT AND BIOAVAILABILITY OF FOOD AND SUPPLEMENTAL FOLATE

The intestinal transport of folate is a carrier-mediated process. This process is equally active for folic acid and all monoglutamyl reduced folate derivatives. The intestinal absorption of naturally-occurring food folates follows a somewhat different process. First polyglutamyl folates are hydrolyzed to monoglutamyl derivatives by a zinc-dependent pteroylglutamate hydrolase found at the brush border membrane of the small intestine (Reisenauer et al., 1977). Subsequently the monoglutamyl derivatives are transported across the intestine by the same carrier mediated process. Because some reduced folates are unstable and undergo oxidative cleavage, food folates are often less bioavailable than folic acid. The extent of decreased bioavailability depends on the presence of food antioxidants (e.g. vitamin C), cellular matrix and the effects of food processing (Scott et al., 2000).

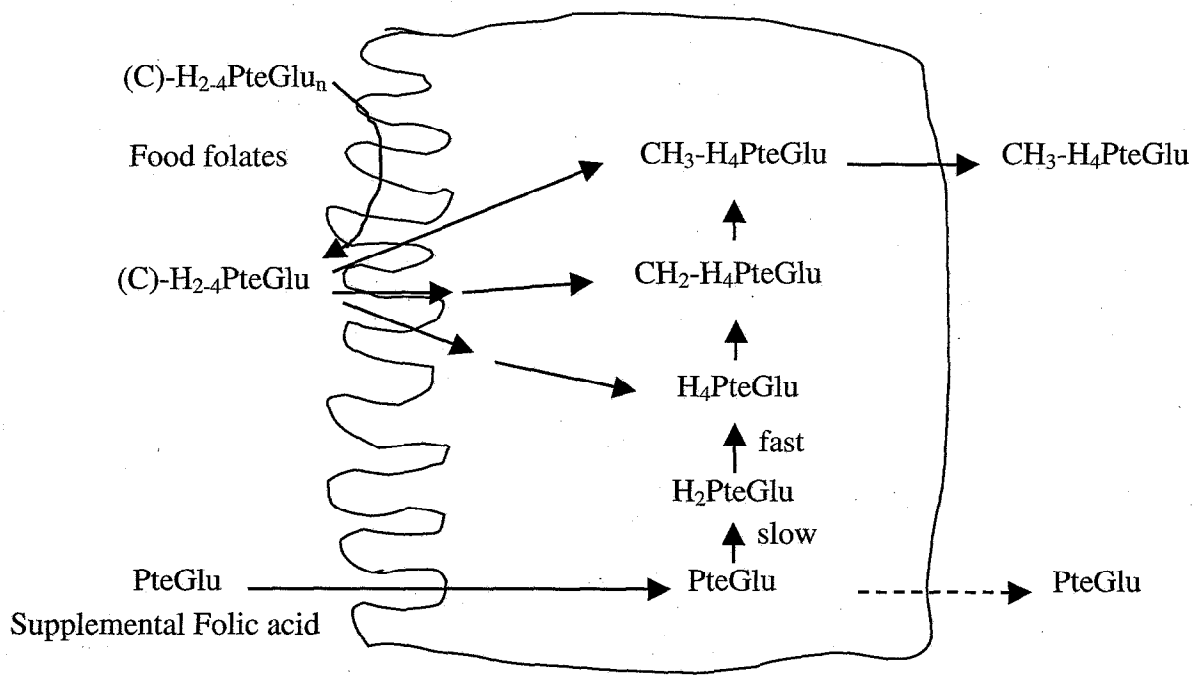
As shown in Figure 4, in addition to sharing the same intestinal transport mechanism, absorbed folic acid and food folate, including L-5-methyl-THF share a common metabolic outcome. During absorption by the intestine, folic acid is reduced to DHF and subsequently to L-THF followed by the acquisition of a carbon unit, which forms L-5,10-methylene-THF that is reduced to L-5-methyl-THF, the compound at issue.

Folic acid at an intake 100 µg/d, as would be obtained in food fortification, would be totally converted to L-5-methyl-THF. At the 400 µg/d intake of folic acid in typical supplements, most of the absorbed folic acid is converted to L-5-methyl-THF during the absorption process. Only small amounts of a 400 µg/d folic acid supplement are not reduced and methylated during absorption and are detectable in the plasma at this intake level. Other studies have shown a direct relationship between the quantity of L-5-methyl-THF appearing in the plasma and the amount of folic acid ingested (Lucock et al., 1989).

Non-methylated folate derivatives, that would ordinarily derive from food, also acquire a methyl group and are converted to L-5-methyl-THF.

Figure 4. Intestinal folate absorption

Schematic presentation of events occurring at the mucosal surface (brush border membrane) and following absorption into intestinal mucosal cells



These features are best illustrated in the data by Perry and Chanarin (1970) presented in Table 2. In this study, Perry and Chanarin determined the temporal increase in folate activity in plasma from volunteers which were given oral doses of folic acid and reduced folate derivatives (10 µg/kg body weight). Folate activity was determined microbiologically with *Lactobacillus casei* which is active for all forms of folate and with *Streptococcus faecalis*, which is active only for non-methylated folates. As shown in the Table, the temporal increase in plasma folate (*L. Casei*) was the same for folic acid as for the other reduced folates. More important however that with exception of folic acid, the increase in *S. faecalis* activity following the oral dose was negligible. For folic acid the increase in this activity, while noticeable, is nevertheless quite small when compared to the *L. casei* activity increase.

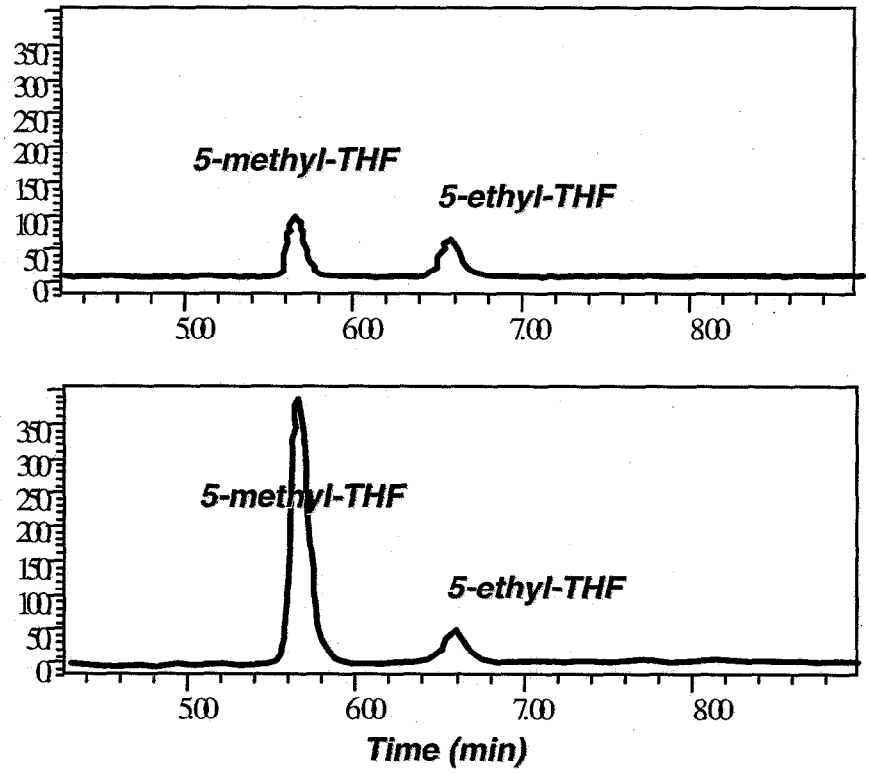
Since *S. faecalis* does not respond to methylated folates, these studies therefore, are confirmatory to the notion that during absorption much of the folates are converted to L-5-methyl-THF.

Table 2. Intestinal absorption and metabolism of folic acid and reduced folates (Perry and Chanarin, 1970)

Folate derivative	N	Plasma folate (ng/mL)				Urine
		0 hr	1 hr	2 hr	3 hr	(µg)
PteGlu						
L.casei	15	13.0	21.7	30.8	20.0	79.8
S. faecalis	15	0	4.8	1.1	0.3	18.0
H_2PteGlu						
L.casei	11	9.2	23.1	20.5	15.6	14.2
S. faecalis	11	0	0	0	0	4.6
H_4PteGlu						
L.casei	13	10.5	20.7	20.9	16.2	20.5
S. faecalis	13	0	0	0	0	3.4
5-CHO-H_4PteGlu						
L.casei	13	12.0	27.6	25.5	21.5	21.5
S. faecalis	13	0	1.5	1.6	1.6	1.0
5-CH_3-H_4PteGlu						
L.casei	16	11.9	30.9	24.4	21.2	62.0
S. faecalis	16	0	0	0	0	9.7

This capacity of the intestine to convert absorbed folate to L-5-methyl-THF is quite high particularly if these folates are already reduced or partially reduced. In the study by Stern et al., (2000) subjects were given an oral dose of D,L-5-formyl-THF (leucovorin, folinic acid) followed by HPLC analysis of plasma folates at various time intervals. Figure 5 is a typical chromatogram of plasma folate, 2 hours before (top) and after (bottom) the oral dose of folinic acid. As seen the oral dose resulted in a large increase in plasma folate. However only 5-methyl-THF could be detected. There were no traces of 5-formyl-THF, which is consistent with the notion that the intestine has a high capacity to convert reduced folates to the methyl derivative.

Figure 5. Affinity/HPLC of plasma 5-methyl-THF before (top) and after (bottom) oral dose of 5 mg D,L-5-formyl-THF (Stern et al., 2000)
(5-ethyl-THF is an internal standard)



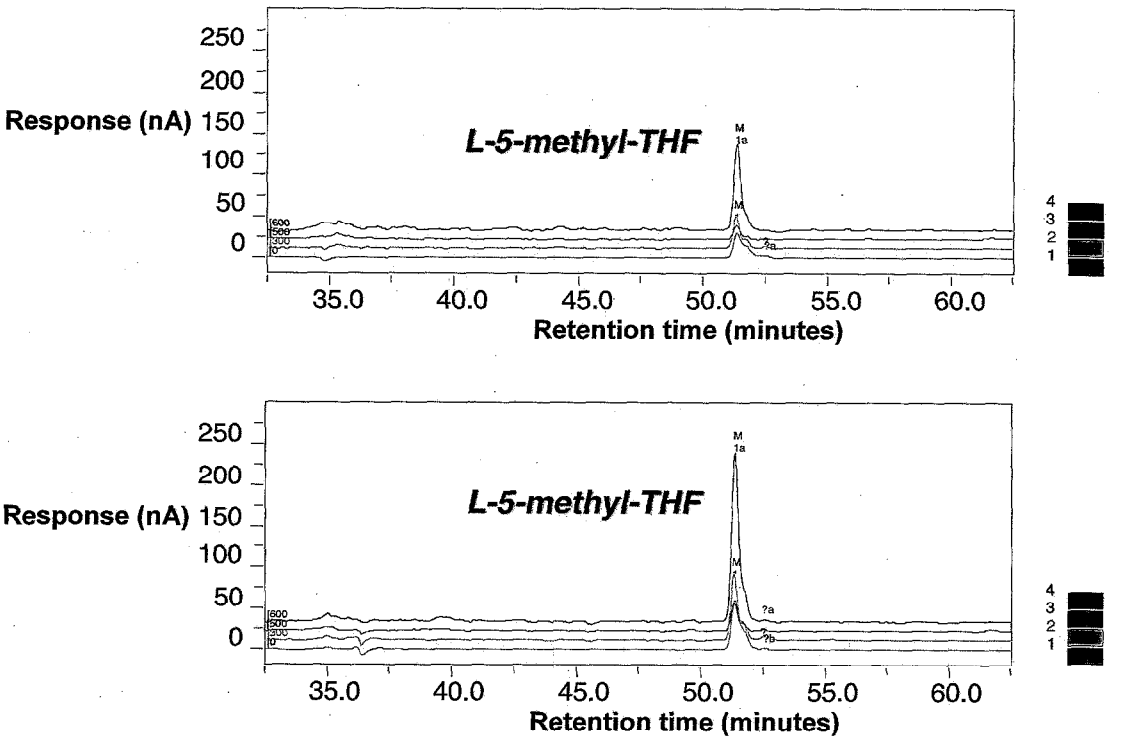
5. L-5-METHYL-THF IS THE SOLE CIRCULATING FORM OF FOLATE IN HUMAN

The reduction and methylation of absorbed folate by the intestine is likely to be a part of a physiological process which is aimed to maintain L-5-methyl-THF as the sole form of circulating folate. Figure 6 is a typical chromatography of post fortification plasma folate form distribution. Even though this fortification resulted in the doubling of plasma folate (Jacques et al., 1999), L-5-methyl-THF still remains the sole form of plasma folate.

Figure 6. Affinity/HPLC of folate in human plasma

The single peak in the two chromatograms corresponds to L-5-methyl-THF

(from Selhub and Bagley, unpublished)



6. THE PHYSIOLOGICAL ROLE OF PLASMA L-5-METHYL-THF

Plasma folate circulates as free or loosely bound to plasma proteins. Folate hemoestasis depends on a number of factors:

1. The amount ingested
2. The amount taken up by the peripheral tissues
3. The amount mobilized by tissue stores (liver) and
4. The amount excreted in the urine

It is important to point out that all these activities in plasma are mediated by L-5-methyl-THF.

Mobilization of folate from tissue stores for exit into the plasma compartment involves an intracellular hydrolysis of the L-5-methyl-THF-polyglutamates to the monoglutamyl derivatives and cellular exit of the resulting L-5-methyl-THF.

Because plasma folate is mainly not bound to proteins, glomerular filtration of this folate is massive, however very little of this folate ends up in the urine. This is because the brush border membrane of the proximal tubular cells of the kidney contains a high affinity folate binding protein (FBP) which has high affinities for folic acid and L-5-methyl-THF, but not for L-5-formyl-THF and the folate analogue, methotrexate. Other folates, i.e. DHF, L-THF and L-10-formyl-THF bind avidly to the FBP, but they are unstable and will not persist in the plasma environment.

Transport of folate into peripheral tissues also exhibits high specificity for L-5-methyl-THF. Transport of folate across membranes of these tissues is regulated by two different mechanisms:

- The reduced folate carrier which operates in many tissues, such as the hematoietic system. It is specific for reduced folates including L-5-methyl-THF, 5-formyl-THF and other reduced folates as well as the folate analogue, methotrexate. This carrier has 200 fold lower activity for folic acid than for reduced folate.
- The FBP in the kidney is found also in the choroid plexus where it acts as a folate transporter across the blood brain barrier. The FBP in mammary gland functions in folate transport into the milk compartment.

7. ADDITIONAL EVIDENCE OF EQUIVALENCE

As predicted from the above discussion of folate metabolism and physiology, folate forms are fully interconvertible (via L-5-methyl-THF). Indeed, a direct comparison of the metabolism of L-5-methyl-THF, L-5-formyl-THF, and folic acid in rats showed equivalent absorption, in vivo metabolism, and clearance patterns (Bhandari et al., 1992).

Examination of dietary intake patterns of elderly Framingham subjects prior to the initiation of food fortification with folic acid showed clear inverse dose-response relationships between fruit and vegetable intake (major sources of L-5-methyl-THF) and plasma homocysteine (Tucker et al., 1996). These relationships were similar to those for breakfast cereals and vitamin supplements (sources of folic acid). A recent controlled human study (Brouwer et al., 1999) yielded the same conclusion, i.e. folate (mainly L-5-methyl-THF) from fruits and vegetables lowers homocysteine and improves folate status.

The appropriateness of L-5-methyl-THF preparation for human consumption was recently addressed in two studies. Prinz-Langenohl et al. (2001) conducted a randomized, double blind, two period cross over study with 23 females of a child-bearing age, to compare the bioavailability of 400 µg folic acid vs an equimolar amount of L-5-methyl-THF. Blood samples were drawn from these volunteers before the oral dose and six times at time intervals up to 8 hours. Blood was separated and plasma was analyzed for folate activity by a commercially available immunoassay kit for IMx analyzer. The data obtained showed that the increase in the AUC (area under the curve) of plasma folate was 84.7 $\pm$ 21.9 nmol/L for L-5-methyl-THF and 59.3 $\pm$ 20,17 nmol/L for folic acid. The ratio of L-5-methyl-THF vs folic acid bioavailabilities was 1.54. The maximum increase in folate plasma level was 20.2 $\pm$ 7.1 nmol/L for folic acid and 23.5 $\pm$ 4.3 nmol/L for L-5-methyl-THF.

Dr. Selhub and colleagues (Bostom et al., 2000) have recently used L-5-methyl-THF at a level of 17 mg per day, in a study that compared the effectiveness of L-5-methyl-THF to reduce plasma homocysteine in kidney dialysis patients. The data showed that L-5-methyl-THF intake for 12 weeks was as effective as an equivalent molar dose of folic acid (Table 3). This study clearly shows equivalence and, as a high dose study, should be considered as general evidence of the safety L-5-methyl-THF. From the physiological standpoint, however, these large doses resulted in substantial increases in plasma folate (Table 4). In those receiving L-5-methyl-THF plasma folate was exclusively L-5-methyl-THF. In those who have received folic acid half of the increase in plasma folate is accounted for L-5-methyl-THF (Table 4 and Figure 7).

Table 3. Plasma total homocysteine (tHcy) in hemodialysis patients before and after 12 weeks supplementation with folic acid (15 mg/d) and an equimolar amount (17mg/d)of L-5-methyl-THF (Bostom et al. 2000)

	Folic acid Group (n = 25)	**L-5-methyl-THF** Group (n = 25)
Initial tHcy (µmol/L)	22.9 (21.0-24.8)	24.1 (22.1-26.1)
After treatment tHcy (µmol/L)	19.5 (18.3-20.7) (14.8% reduction)	20.0 (18.8-21.2) (17.0% reduction)

Figure 7. Affinity/HPLC of plasma folate after daily supplementation with 15 mg equivalent of L-5-methyl-THF and folic acid in hemodialysis patients (Selhub et al., unpublished)

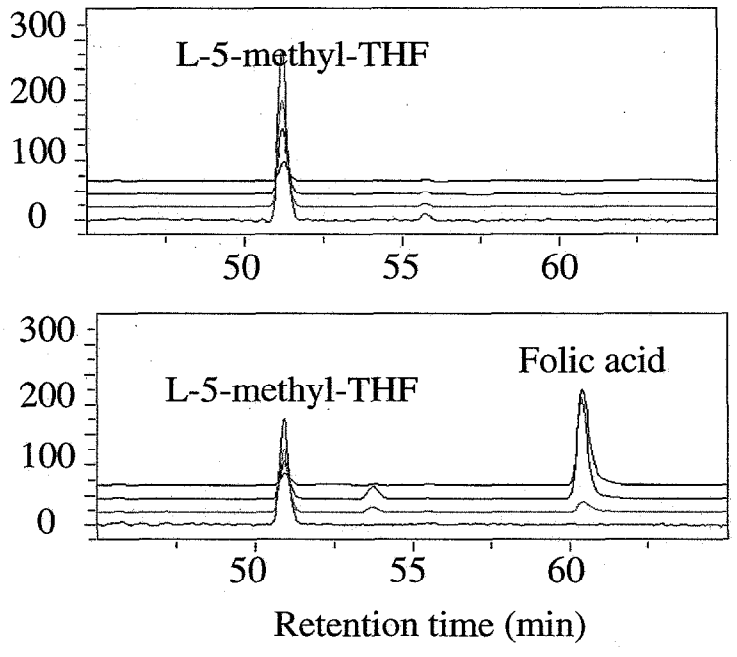


Table 4. Plasma folate distribution in hemodialysis patients before and after 12 weeks supplementation with folic acid (15mg/d) and L-5-methyl-THF (17 mg/d) (Bostom et al. 2000)

	Folic acid Group (n = 25)	L-5-methyl-THF Group (n = 25)
Plasma folate at baseline	32.2 ng/ml (76.2% L-5-methyl-THF)	38.1 ng/ml (86.3% L-5-methyl-THF)
Plasma folate after treatment	466 ng/ml (50% L-5-methyl-THF)	407 ng/ml (97% L-5-methyl-THF)

Microbial assay

The above studies by Prinz-Langenohl et al., (2001) and by Bostom et al., (2000) were conducted using Merck/Eprova compound which is the L-5-methyl-THF, calcium salt. The results of these studies demonstrate the bioavailability of this L-5-methyl-THF preparation and particularly its capacity to reduce plasma homocysteine levels as effectively as folic acid. This data is an indication that this preparation is biologically active.

In the study presented in Table 4 and Figure 7, the same L-5-methyl-THF preparation, as well as the D-5-methyl-THF and the racemic D,L-5-methyl-THF (equimolar mixture of the D- and L-isomers) were tested for their biological activity using *Lactobacillus casei* (ATCC 7469) as the test organism. This assay continues to be one of the most reliable method for the quantitative determination of biologically active folates. This organism will not respond to D-isomers of THF derivatives. Another advantage is that the growth response is quite similar for various monoglutamyl folates (Tamura 1990). Growth curve response of *L. casei* should be similar for folic acid as for L-5-methyl-THF, half for the racemic D,L-5-methyl-THF and none for D-5-methyl-THF. This prediction is confirmed in the data shown in Figures 8 and 9. In these studies 4 samples containing folate powders marked B1 to B4 were shipped from Merck/Eprova to Drs. Jacob Selhub and Jesse F. Gregory, III, laboratories. Weighed samples were dissolved in 0.025 M sodium hydroxide containing 10 mM dithioerytrytol. Folate concentrations were then determined spectrophotometrically and aliquots were used in

Dr. Selhub laboratory for an affinity/HPLC analysis with electrochemical detection (Bagley and Selhub, 2000).

The HPLC analysis of these samples is described in Figure 8. Based on these analysis the concentration of these solutions is 4.5 pmole/ml (about 2.0 ng/ml) with the exception of B3 which was slightly higher 5 pmol/ml (2.2 ng/ml). The data in Figure 9 is the result of the microbial assay, using *L.casei* as the organism, of these four solutions as determined in the laboratory of Dr. Selhub. The same results were obtained in the laboratories of Jesse F. Gregory, III and Tsunenobu Tamura. The data in Figure 9 shows that samples B1 and B2 have equivalent superimposing activities. B1 is folic acid as indicated from the chromatogram in Figure 8. On the basis of equal activity with B1, sample B2 most correspond to the L-isomer of 5-methyl-THF. Sample B3 on the other hand, is exactly half as active for *L.casei* as B1 (folic acid) or B2 (L-5-methyl-THF) and therefore B3 corresponds to the D,L-isomer of 5-methyl-THF. B4 is totally inactive for *L.casei* and therefore it corresponds to the D-isomer of 5-methyl-THF. This microbial data corresponds with the analytical data of the manufacturer of the folate compounds.

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Germany

Figure 8. Affinity chromatography of approximately 10 pmol equivalent of compounds 1 through 4 supplied by Merck/Eprova

Compound 1 (top left) is folic acid (based on retention time and electrochemical characteristics). The rest correspond to 5-methyl-THF. Note all preparations have single peaks which is an evidence of high purity.

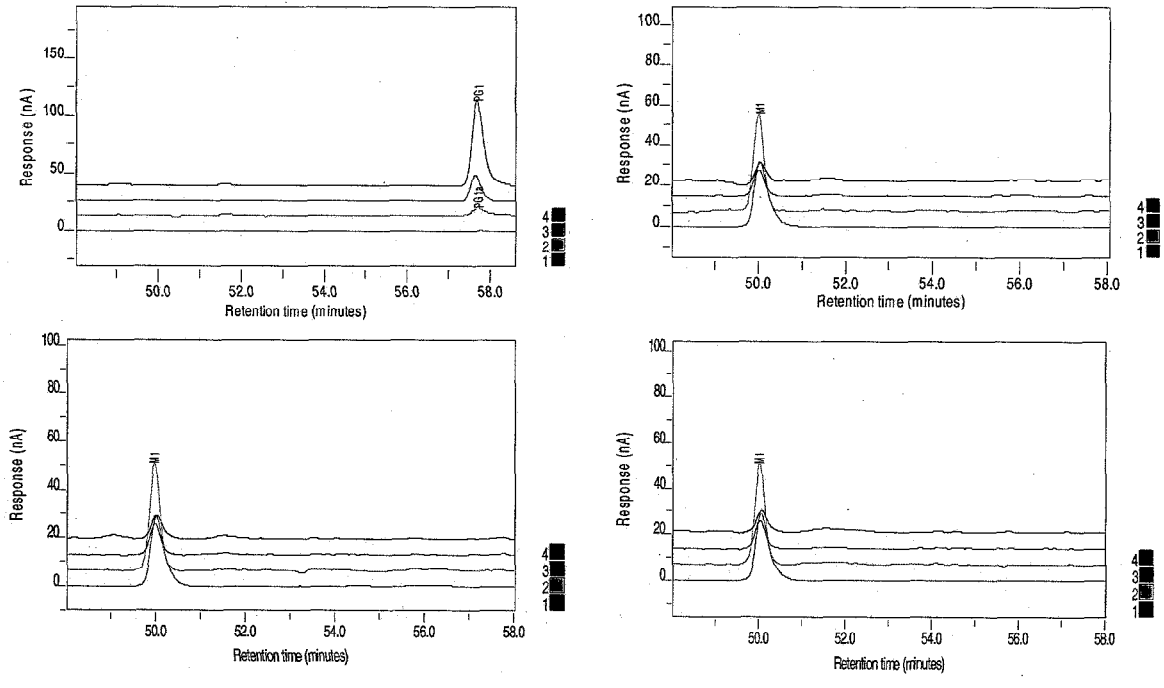
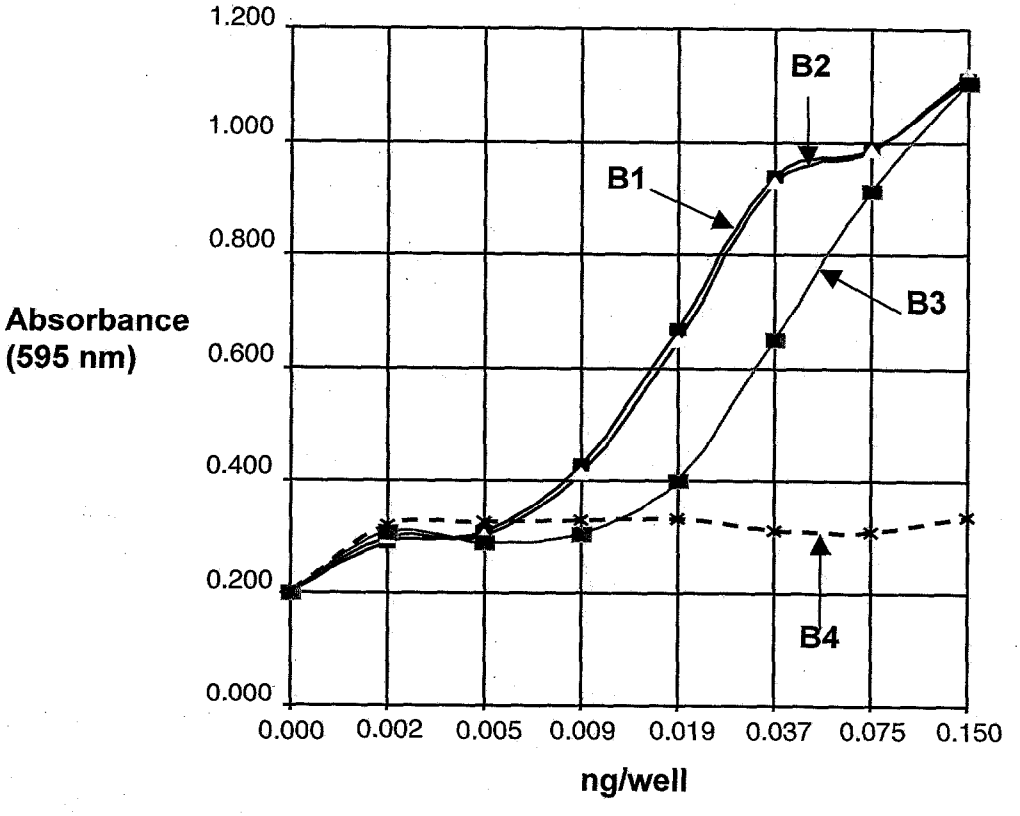


Figure 9. Microbial analysis of the 4 folate preparations from Merck/Eprova

The same solutions used for the analysis described in Figure 8, were diluted to 2 ng milliequivalent folic acid /ml (4.53 pmol/ml). Aliquots containing 0 to 0.15 ng were then added to 96 well plates and folate activity was determined, microbiologically with *Lactobacillus casei* (ATCC 7460). Plot depicts turbidity as determined by optical density vs folate equivalent in the respective plates.



Conclusion

The predominant form of dietary folate is L-5-methyl-THF. Folic acid itself is incapable of serving as a coenzyme for one carbon transfer, unless reduced to L-THF and further converted to L-5-methyl-THF.

The naturally occurring predominant form of folate is now available as the stable, crystalline L-5-methyl-THF, calcium salt. It is effective in the promotion of growth in microbiological assays, has a consistent bioavailability in human beings. Intake of high dose (17 mg/day) over 12 weeks was effective in lowering the homocysteine levels in hemodialysis patients without any side effects.
Based on the above-described data and information, we conclude that L-5-methyl-THF, calcium salt, when used as a source of folate under the conditions recommended or suggested in the labeling of such dietary supplements, is reasonably expected to be safe.

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