The extremely slow and variable activity of dihydrofolate reductase in human liver and its implications for high folic acid intake

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Numerous clinical trials using folic acid for prevention of cardiovascular disease, stroke, cognitive decline, and neural tube defects have been completed or are underway. Yet, all functions of folate are performed by tetrahydrofolate and its one-carbon derivatives. Folic acid is a synthetic oxidized form not significantly found in fresh natural foods; to be used it must be converted to tetrahydrofolate by dihydrofolate reductase (DHFR). Increasing evidence suggests that this process may be slow in humans. Here we show, using a sensitive assay we developed, that the reduction of folic acid by DHFR per gram of human liver ($n = 6$) obtained from organ donors or directly from surgery is, on average, less than 2% of that in rat liver at physiological pH. Moreover, in contrast to rats, there was almost a 5-fold variation of DHFR activity among the human samples. This limited ability to activate the synthetic vitamer raises issues about clinical trials using high levels of folic acid. The extremely low rate of conversion of folic acid suggests that the benefit of its use in high doses will be limited by saturation of DHFR, especially in individuals possessing lower than average activity. These results are also consistent with the reports of unmetabolized folic acid in plasma and urine.

folate | provitamin utilization | unmetabolized folic acid | vitamin supplements | nutrition

The physiological function of dihydrofolate reductase (DHFR) is the reduction of 7,8-dihydrofolate (7,8-DHF) (1) produced in the reaction of thymidylate synthase and possibly by autooxidation of tetrahydrofolate. It also may aid in the regeneration of tetrahydrobiopterin in endothelial cells needed for coupled nitric oxide synthase activity (2). However, since the synthesis of folic acid (FA, pteroylglutamic acid) as a provitamin in 1945, DHFR has taken on another role: reduction of FA to 7,8-DHF (Fig. 1). Folic acid, in itself, has no known cofactor activity and is not found to a significant extent in nature. By the time one-carbon metabolism was recognized as actually being carried out by derivatives of tetrahydrofolic acid (THF), folic acid already had been found to alleviate the anemia of folate deficiency and was in wide use in vitamin supplements. Folic acid has only a single chiral center in its glutamate residue and is therefore easier to synthesize than diastereomeric 6*S*-THF. Thus, there appeared to be little immediate reason to look beyond folic acid.

The U.S. Reference Daily Intake (Daily Value) for FA is 0.4 mg for adults to aid in the prevention of birth defects and anemia, or double this amount for pregnant or lactating women. Consumers of fortified ready-to-eat cereals can have an intake, due to nutrient overages, of up to 0.8 mg of FA per standard serving size (U.S. Department of Agriculture National Nutrient Database for Standard Reference, Release 18, www.ars.usda.gov). Higher doses, up to 5 mg/d, have been used in clinical trials for secondary prevention of heart disease and stroke. All of these potential exposures are on top of that from the Food and Drug Administration mandated grain fortification program in the U.S. which has been estimated to provide up to 0.2 mg/d of FA to the average person (3, 4). One factor that has not been fully considered is whether DHFR has sufficient capacity in humans to efficiently metabolize these potentially high loads of FA, particularly in light of a frequent misconception that FA is reduced as it crosses the intestinal wall [see review (5)].

Concern has been expressed that the presence of unmetabolized FA (pteroylglutamic acid in plasma or tissues not yet converted to active forms of folate) may be detrimental (6–9). Although folate deficiency has been linked to cancer susceptibility via increased uracil misincorporation (10), recent evidence suggests that high intake (≥1 mg/d) of FA may exacerbate some preexisting cancers or progression of precancerous lesions, such as in the colon (11) or prostate (12). The activity of natural killer cells has been reported to be decreased in older women having higher plasma levels of unmetabolized FA (13). Unmetabolized FA has also been hypothesized to cause masking of B12 deficiency and/or aggravate the neurological damage associated with prolonged B12 deficiency (14–16). Therefore, it is important to develop a better understanding of the mechanism of accumulation of unmetabolized FA in humans.

The activity of DHFR in human liver and other tissues has not been well characterized. The few previous measurements mostly used autopsy tissue, which can lead to artifacts due to autolysis. Moreover, earlier DHFR activities were determined at a nonphysiological pH, and/or by consumption of NADPH cofactor, which necessitates a large correction for nonspecific oxidases (17–19).Valid comparison of rates among species using values measured in different laboratories is greatly hindered by the use of variable reaction conditions (e.g., salt concentration, impure substrate preparations, and temperature, in addition to pH).

In this study, we determined the activity of DHFR at physiological pH in *fresh* samples from human livers with FA as substrate and compared this to the rate with 7,8-DHF. To accurately measure the very low activity with FA as substrate at physiological pH, a new procedure has been developed for the determination of DHFR based on analysis of the THF product by HPLC. This work reveals that the activity of DHFR in fresh human liver is extremely low in comparison to that in rat liver and suggests that it becomes limiting when FA is consumed at levels higher than the Tolerable Upper Intake Level (1 mg/d for adults).

Results

HPLC Assay for Tetrahydrofolate. A linear response was observed down to the limit of detection of 3 fmol (S/N = 3) [supporting information (SI) Fig. S1]. A typical chromatogram of a reaction with 100,000 × *g* supernatant of human liver as the source of

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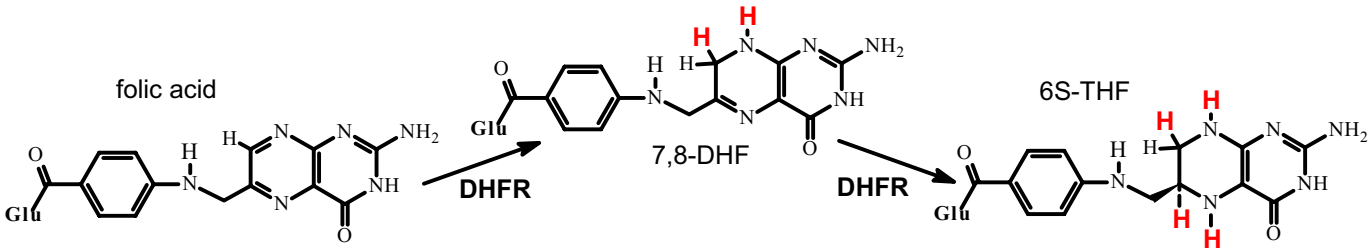

Fig. 1. The fully aromatic pteridine ring of folic acid (FA) presents a considerable barrier to the action of DHFR. Compared to 7,8-DHF, FA requires the loss of more resonance stabilization energy during the initial unsaturation of its pyrazine moiety. Thus, the V_{max} for DHFR with FA is typically several orders-of-magnitude slower than with 7,8-DHR, regardless of the source of the enzyme. Glu, glutamate.

DHFR shows a well-isolated peak for THF (Fig. S2). Due to the lability of THF, especially at low concentrations, it is essential that the HPLC mobile phase contain a reducing agent (e.g., DTT) to prevent on-column oxidation. Measurement of the THF product of DHFR circumvents interference by nonspecific oxidases that hinder assays which monitor disappearance of NADPH. The HPLC/fluorescence assay is optimized by use of highly purified 7,8-DHF substrate to minimize background THF and inhibitory folic acid.

Levels of DHFR Activity in Rat Liver with 7,8-DHF as Substrate. DHFR activity in all rat liver extracts with 7,8-DHF as substrate was measured both spectrophotometrically and by HPLC. Activity was determined as a function of time and of DHFR concentration to ascertain that rates were measured within a valid range. The same activities were found whether or not the livers were frozen before homogenization, indicating the absence of significant DHFR in subcellular organelles. The ratio of the 2 methods, HPLC/spectrophotometric, was 1.02 ± 0.09 for 100,000 × *g* supernatants from 7 different rats, or pooled rat preparations. The values ranged from 483 to 767 (average 577) nmol THF produced/min/gm wet weight liver (Table 1). All rats were adult and were from 2 different strains and from both sexes. Even so, there was less than a 2-fold variation in the activity of DHFR in their livers.

Levels of DHFR Activity in Human Liver with 7,8-DHF as Substrate. DHFR activity in extracts of human liver was found to be very low compared to rat. Measurements of activity in crude extracts were not sufficiently accurate by the spectrophotometric method since baseline rates due to nonspecific NADPH oxidation were high compared to utilization by DHFR. Therefore, all quantitative analyses were obtained using the HPLC procedure. The coefficient of variation for replicate measurements of DHFR activity was 7%. Livers from 6 different donors were analyzed. As with the assay of enzyme from rat liver, activity was linear with reaction time for at least 10 min. (Fig. S3). The rate was also linear with the volume of extract added to the reaction (Fig. S4), demonstrating a lack of inhibition by endogenous compounds within the range examined. With 7,8-DHF as substrate, the levels of activity varied from 5.4 to 26 nmol/min/g wet weight liver (average 16.3) and did not appear to depend on age or gender (Fig. 2*A*). Formation of THF due to potential hydrolysis of endogenous THF polyglutamates was found to be insignificant in control reactions performed without added substrate. Even within these 6 liver samples, there is almost a 5-fold variation in the activity of DHFR in human liver. With the assumption that the preponderance of human liver DHFR activity is in the soluble fraction (as is thought to be the case with other mammalian species), the activity in human liver is 35 times lower on average (range: 22 to 106) than the average for rat liver with 7,8-DHF as substrate (Fig. 2*B*).

Folic Acid as Substrate for DHFR. Due to the slow rate of DHFR with FA, activity with this substrate was measured only by the HPLC method. The rate of THF formation by DHFR from rat liver with FA as substrate was 850 times slower than with 7,8-DHF (Table 2). The rate followed Michaelis-Menton kinetics; no substrate inhibition was observed, and K_m for FA was 1.8 μM (Table 3).

The level of DHFR activity in human liver with FA as substrate was also found to be extremely low. The liver with the highest activity with FA was only 0.02 nmol/min/g wet weight liver, i.e., 1300 times slower than with 7,8-DHF as substrate (Table 2). The K_m for FA was found to be 0.5 μM (Table 3). No substrate inhibition was observed, and the rate followed Michaelis-Menton kinetics. The activity in human liver is 56 times lower

Table 1. Rat liver DHFR activity with 7,8-DHF as substrate

Rat strain*	No. of rats†	Sex	No. of expts‡	Activity, nmol of THF produced per min per g wet weight of liver		
				HPLC assay	spec. assay§	HPLC/spec.§
CD (1)	1	M	1	767	840	0.91
CD (2)	1	M	5	737	769	0.96
CD (3)	1	M	1	526	516	1.02
CD (4)	1	M	2	508	491	1.03
CD (5)	1	M	1	483	508	0.95
Wistar	3	F	1	502	451	1.11
Wistar	16	M	3	513	437	1.17

*Rats were adults of 300 to 480 g body weight.
†Extracts from Wistar rats were pooled from the number of animals listed.
‡Activity was measured ≥4 times per experiment, each of which was a separate homogenization.
§spec. = spectrophotometrically at 340 nm for the consumption of NADPH and 7,8-DHF.

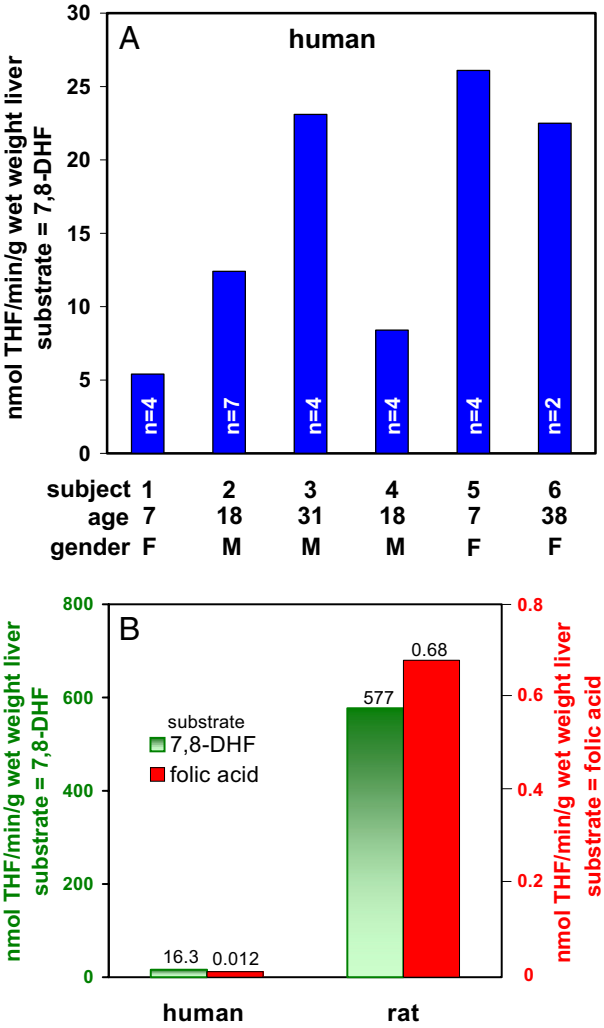

Fig. 2. The variation of DHFR activity in individual human livers and comparison of its average value to that in rat liver. (*A*) The activity of DHFR in the liver of 6 human subjects determined with 7,8-DHF as substrate. Subjects 1–5 were organ donors; 1 was a victim of smoke inhalation and 2–5 were from automobile accidents. Liver tissue samples from these subjects were frozen immediately and later extracted. Normal liver tissue was obtained from subject 6 during surgery for removal of a benign hepatoma and was extracted immediately without prior freezing. Homogenates of at least 2 separate samples from the liver of subjects 1–4 were examined. For both subjects 5 and 6 a single homogenate of at least 50 g of tissue was prepared. n = the total number of reactions with at least 4 time points in each. (*B*) Comparison of the average DHFR activity in human liver with the average value in rat liver.

on average (range: 34 to 164) than the average value for rat liver with FA as substrate.

Folic Acid as Inhibitor of DHFR. Inhibition by FA of the reduction of 7,8-DHF by DHFR was determined by measuring the rate of THF formation by the HPLC method. In the absence of FA both the rat and human enzymes were found to be slightly substrate inhibited above 0.5 and 1 μM 7,8-DHF for the rat and human enzymes, respectively, (Fig. S5) as has been previously reported (20, 21). The data were found to be well described by the equation for substrate inhibition detailed by Nakano et al. (22). Although this equation yields V_{max}/K_m, it does not provide values for these parameters separately. Thus, data at 7,8-DHF concentrations below 0.5 μM were used to estimate K_m, which was found to be 0.05 μM for human DHFR, about half of that reported for

Table 2. Folic acid vs 7,8-DHF as substrates for DHFR

Substrate	Activity, nmol of THF produced per min per g wet weight of liver	
	Human*	Rats†
Folic acid	0.02	0.6
7,8-DHF	26.1	513
7,8-DHF/folic acid	1300	850

*Subject No. 5.
†16 pooled male Wistar rats (see Table 1).

recombinant human enzyme measured by using progress curve analysis (21).

The presence of substrate inhibition hinders accurate assessment of K_i for FA. Inhibition of human DHFR by FA at concentrations of 7,8-DHF greater than 2 μM approached competitive. However, below 0.5 μM 7,8-DHF, inhibition by FA of human DHFR appeared to be noncompetitive. A nonlinear least squares fit to the equation for partial noncompetitive inhibition (23) yielded a K_i of 0.05 μM and a value of 0.07 for the parameter β. This K_i is about twice that reported previously for the human placental enzyme at pH 7.5, but the range of substrate used or the type of inhibition observed was not specified (17).

Discussion

Measurements of DHFR activity in fresh human liver samples obtained at surgery or from organ donors unambiguously reveal that the average value with 7,8-DHF as substrate is very low (35-fold) in comparison to rats. The majority of studies of DHFR with FA as substrate have been performed in acidic conditions since with most species the pH optimum of the reaction with this substrate is below pH 5 (1). There are many reports that at neutral pH, DHFR has no activity with FA as substrate, e.g., from human placenta (17) as well as chicken, sheep, mouse, and pig liver. The results of the current study show that the reaction with FA as substrate at physiological pH is measurable, but 1300 times slower than with the natural substrate 7,8-DHF for humans. A benefit of using an assay with high sensitivity, such as the current method, is that crude tissue homogenates can be analyzed at sufficient dilution to obviate effects of endogenous inhibitors. Of importance to the increasing population exposure to folic acid, the rate of DHFR with FA as substrate in human liver is 56 times slower per gram of tissue at physiological pH than in the rat. Thus, the disposition of FA in animal models may not meaningfully translate to human pharmacokinetics. The ratio of DHFR activity in human and rat liver reported here is comparable to protein abundances measured using a methotrexate binding assay (24).

This study permits estimation of the capacity of liver to convert FA to active THF. Considering the K_m of FA with DHFR (0.5 μM), a 1.5 L (approximately 1.8 kg) human liver containing more than 0.75 μmol (331 μg) FA would begin to saturate DHFR. The data in Fig. 2*A* and Table 2 show (see *SI Text*) that human liver can reduce FA to THF only in the range of 0.016 to 0.075 μmol FA/min, even at V_{max}. While a 0.4 mg (0.91 μmol) dose would be largely reduced in about an hour after peak absorption, a 5 mg dose of FA (11.3 μmol) could take up to 12 h in some individuals to be converted. This does not including the extra time needed once the level in the liver falls below its K_m. The kidneys, however, also excrete folate, especially after large doses. Based on the differential microbiological growth assays, it has been reported that humans administered intravenously with more than 20 μg/kg of FA excrete the majority still unmetabolized into the urine (25).

Several earlier studies have reported unmetabolized FA in human plasma following oral administration. For example, a

Table 3. Substrate and inhibitor properties of FA with human and rat liver DHFR

DHFR source	K_m for 7,8-DHF, μM	K_m for FA,* μM	K_i for FA,† μM	K_i for FA,‡ μM
Rat	0.06	1.8	0.15	0.02
Human	0.05	0.5	0.07	0.01

*Measured over the range of 0.3 to 300 μM FA.
†Apparent K_i for FA at 7,8-DHF concentrations below 0.5 μM, where inhibition appears noncompetitive.
‡Apparent K_i for FA at 7,8-DHF concentrations above 2 μM, where inhibition appears competitive.

substantial portion of FA appeared to remain unchanged in the plasma for at least 3 hours following oral administration of 10 μg/kg (26). Although there were early conjectures that FA might be converted to reduced folates during intestinal uptake, the liver was clearly established as a most important site of metabolism in humans by 2 studies of subjects fitted with portal (27) and also hepatic vein cannulas (28). A first pass effect in which a significant portion of an oral dose appears to accumulate initially in the liver has been reinforced by more recent isotope studies (29, 30). Unmetabolized FA has also been detected in plasma several hours after consuming more than 200 μg of FA (31). Recently, we have examined the plasma pharmacokinetics and urinary excretion of FA in healthy U.S. subjects given single oral doses over the range of 0.4 mg to 5.0 mg. Analysis of these data should reveal the extent of the variation among individuals. With the ever-increasing exposure to FA from fortification of prepackaged foods, use of supplements, and fortification of the basic grain supply, a total FA intake over 1 mg is now not uncommon in the United States (3, 4). Our results show that the low activity of DHFR in human liver is the fundamental cause of exposure to relatively high transients of plasma unmetabolized FA at doses greater than the Daily Value.

What pressures might have led to the low DHFR activity in the liver of *Homo sapiens*? The turnover rate during steady state catalysis of pure recombinant human enzyme (32) is about 2- to 3-fold higher (taking into account the effect of salt) than that of pure rat DHFR (33). Thus, the differences in the activity in liver between these 2 species is not primarily related to the intrinsic properties of the enzymes. While amplification of the DHFR gene in some cancers (especially those that have become resistant to antifolates) has been found, there are several mechanisms whereby DHFR activity in normal tissues might be regulated. DHFR protein binds to the coding region of its own mRNA causing translational repression. The amino acids so far identified in this interaction (34) are all conserved between rat and human. However, of the 27 bases reported to be the DHFR binding site in human mRNA (35), A423 and C426 in a putative stem-loop are both present as G in the rat and might be responsible for differences in basal translation rate. In addition, DHFR and thymidylate synthase (TS) are up-regulated during the G_1 to S-phase transition and have several transcription factors in common, such as SP1, E2F-1, and a member of the ETS family (Elk3 for DHFR and NRF-2/GABP for TS) (36, 37). The turnover of hepatocytes varies markedly between rat and human. Mitotic figures present in nonregenerating adult rat liver range from about 1 to 3 per 1,000 cells (38). On the other hand, only 1 mitotic figure in 10,000 to 100,000 cells is found in normal human liver (39). Thus, the ratio of DHFR activity in rat and human liver roughly parallels their relative hepatic cellular replacement rates. E2F-1 has been shown to be overexpressed in hepatocellular carcinoma and several other cancers. Therefore, the low level of DHFR, and the other proteins under the control of E2F-1, in humans may have evolved to hinder the development of cancer. If this is the case, other animals with slow tissue turnover rates, possibly related to long life span, might also have low DHFR activity.

Several factors might be related to the high variability of DHFR activity among individuals. The oncogene product MDM2 is capable of binding to and monoubiquitinating DHFR. This has the effect not of decreasing cellular protein stability, but rather directly decreasing DHFR activity. Considerable diminution of DHFR activity was found in cell lines either overexpressing or transiently transfected with MDM2. Conversely, siRNA knockdown of MDM2 produced a modest, but significant increase in DHFR activity (40). On the other hand, MDM2 also inactivates retinoblastoma (Rb) tumor suppressor protein by ubiquitination, which in turn promotes E2F-1/DP1 transcriptional activity (41). Thus, individual variations in the levels of MDM2 may have counterbalancing effects on DHFR activity, e.g., increased transcription, but also inhibition by monoubiquination.

So far, no polymorphisms in the coding region of human DHFR have been reported (42). Several variations of DHFR have been found in noncoding regions, which may relate to the variability of DHFR activity found in the current study. A 19 bp deletion polymorphism within intron 1 (43) has been found to enhance gene expression (44, 45). This increased expression was thought to be due to loss of another Sp1 transcription factor binding site (43), but in fact contains a consensus binding site for the repressor, Sp2.* However, since this 19 bp deletion has recently been reported to be associated with increased unmetabolized folic acid in plasma taken from the fasting subjects in the Framingham Offspring Cohort (46), the nature of its functional significance remains to be resolved. Other polymorphisms in the DHFR promoter region, some of which occur with fairly high frequency, have been associated with decreased event free survival of children with acute lymphoblastic leukemia. Gene reporter assays in 3 human cancer cell lines showed as much as a 2-fold variation of transcription rate within some of these haplotypes. Additionally, carriers of the CC-1610 promoter genotype were found to have significantly higher (approximately 3-fold) q-PCR expression of DHFR mRNA than noncarriers (47). Furthermore, several microRNAs (e.g., miR-192 and miR-24) have been found to be important in the translational regulation and/or degradation of DHFR mRNA. Although these microRNAs are conserved, a relatively frequent SNP (829C→T) in the miR-24 binding site in the 3′UTR of DHFR message has been found to increase its stability (48). Combinations of these noncoding variants, and perhaps also post translational modification by ubiquitination may be sufficient to explain the variations of DHFR activity among human individuals found in the current study.

In addition to being an exceedingly poor substrate, FA is also an inhibitor of DHFR in the reduction of its physiological substrate, 7,8-DHF. The apparent K_i values for FA for human and rat liver DHFR are 1 or 2 orders of magnitude lower than their respective K_m values. This is consistent with the kinetic mechanism proposed for the human (21) and bacterial enzymes (49). Inhibition of DHFR from various species by FA has been previously observed, although the type of inhibition was fre-

* Magee A, O'Brien K, Parle-McDermott A (2009) Investigation of the putative functional effect of the 19bp deletion polymorphism within intron 1 of the dihydrofolate reductase (DHFR) gene. 11th Meeting of the Irish Society of Human Genetics, September 12, 2008, Dublin, P7. *Ulster Med J* 78:68.

quently not reported (17, 50). Inhibition of DHFR from human KB cells was found to be competitive at high concentrations of 7,8-DHF (51), as in this report. However, we also observed that FA behaves as a noncompetitive inhibitor of human DHFR at low concentrations of 7,8-DHF. Mammalian DHFR is a monomer with a single binding site for folate. We, therefore, suggest that at low 7,8-DHF the apparent noncompetitive inhibition by FA may be due to the binding of these 2 folates to separate species (e.g., FA to a DHFR/NADP complex and 7,8-DHF to DHFR/NADPH). At high 7,8-DHF concentrations both folates would compete for the same enzyme species. The physiological product of thymidylate synthase would probably be a polyglutamate of 7,8-DHF rather than the monoglutamate studied here. But, unlike most other folate dependent enzymes, DHFR does not have a substantially greater preference for the polyglutamylated forms (50–52). Whether unmetabolized FA accumulates within cells to the extent of meaningfully decreasing the activity of DHFR is currently unknown, and in any event is unlikely to occur as a result of doses at or below the U.S. Daily Value.

The activity measurements reported here indicate that humans may have a very limited ability to efficiently metabolize FA, especially in high doses. Numerous clinical trials examining the prevention of heart disease, stroke, and Alzheimer's disease using FA have used doses up to 5 mg/day (or even higher). Although supplements containing FA have in some trials reduced the risk of stroke, no effect on risk for coronary heart disease has yet been reported. The low activity of DHFR in human liver suggests that increasing the dose of FA may at some point, with individual variation, no longer produce further increases in the active folate pool. It is clear, however, that the Daily Value of folic acid can improve folate status (53) and helps to reduce the risk of neural tube birth defects.

Our data in no way imply that FA taken in this amount is inappropriate. Indeed, they suggest that most of the FA given in the amount of the U.S. Daily Value (0.4 mg) is converted to active folate in most individuals. Our results, however, predict that intakes higher than the Tolerable Upper Intake Level (1.0 mg), whether from a combination of supplements and fortified foods, or from high dose folic acid administered for therapeutic purposes will considerably escalate exposure to circulating unmetabolized FA. This is consistent with a recent report showing increased levels of plasma unmetabolized FA as a result of the U.S. fortification program and in consumers of supplements compared to nonconsumers (54).

Materials and Methods

Reagents. FA, phenylmethylsulfonyl fluoride, NADPH, Tris base, and sodium ascorbate were from Sigma, 2-mercaptoethanol from Fluka, tetrahydrofolate from Schircks, trichloroacetic acid (TCA) from EM Science (TX1045), phosphoric acid from Baker (0260), DTT from Gold Biotechnology and HPLC grade methanol from Fisher. THF stock solutions were stored under argon at −80 °C. NADPH solutions were made in 25 mM Tris·HCl, pH 8.0, and stored frozen. Folic acid solutions were kept dark and cold and stored frozen.

Commercial sources of 7,8-DHF were found to be contaminated with FA, which inhibits the reaction, and/or with THF, which decreases the sensitivity of the HPLC assay. To avoid these problems, 7,8-DHF was synthesized in our laboratory by adaptation of the method of Blakley (see *SI Text*). Analysis by HPLC showed that after calcium precipitation the product contained less than 0.1% THF or FA. Solutions of 7,8-DHF were prepared daily in argon sparged 0.1 M sodium ascorbate, and the final concentration of ascorbate in all DHFR reactions was 4 mM.

Human Liver DHFR. Fresh human liver was obtained from organ donors for whom no recipient was available or in one case at surgery. All procedures were approved by the University of South Alabama Institutional Review Board. Liver was frozen in liquid nitrogen within 20 min of removal of life support and stored at −80 °C until processed. The sample of liver obtained at surgery was homogenized immediately after removal and extracted without prior freezing. To accurately determine DHFR levels in tissues, liver was extracted with minimal manipulation. Liver was homogenized on ice in a Potter-Elvejhem homogenizer with 1 g liver:3 mL homogenizing buffer. The buffer was 0.2 M Tris·HCl, pH 7.6 at 4 °C, a pH and ionic strength at which DHFR was found to be maximally stable, and contained 10 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 100,000 × *g* for 60 min at 4 °C, and the supernatant removed and stored in aliquots at −80 °C until assayed for activity. For measurement of K_m and K_i values, and ratio of activity with FA vs. 7,8-DHF, a 50% to 90% saturating ammonium sulfate fraction was prepared. This contained 97% of the activity and was then subjected to ultrafiltration with 0.1 M Tris·HCl, pH 7.4, to remove ammonium sulfate and endogenous THF from the retentate.

Rat Liver DHFR. Livers from CD or Wistar adult male or female rats were removed immediately after euthanasia. Livers were either immediately homogenized after removal or frozen in liquid nitrogen before homogenization. DHFR activity was measured in 100,000 × *g* supernatants of rat liver prepared exactly as for human liver.

DHFR Activity Assay. Two methods were used to monitor the activity of DHFR.

(*i*) The standard spectrophotometric assay was used when activities were sufficiently high to make this feasible. Reactions were run in water-jacketed cuvettes with the temperature maintained at 27 °C, and NADPH plus 7,8-DHF consumption monitored at 340 nm with a Cary 300 spectrophotometer. Formation of THF product was calculated using a millimolar extinction coefficient of 12.3 $mM^{-1}cm^{-1}$ with 7,8-DHF as substrate (55).

(*ii*) To measure very low activities in crude extracts a column switching HPLC method was developed in which the product, THF, was detected by its native fluorescence. A Perisorb C18 guard column was attached to a Kromasil C18, 5 μm, 50 × 4.6 mm column in series with a Kromasil C18, 5 μm, 250 × 4.6 mm column. The columns (in water jackets maintained at 30 °C) were eluted at 1.1 ml/min with 15 mM sodium phosphate, pH 2.3/methanol (83:17), containing 5 mM DTT, made fresh daily. Samples (20 μL) were injected onto the first column with a Varian 420 autosampler set at 4 °C. Just after all of the THF had entered the second column, a Rheodyne LabPro column switching valve reversed the flow on the first column which was flushed with the same eluant at 0.7 mL/min by a second pump while continuing the forward elution of material on the longer column. THF was detected with a Jasco FP-1520 or Waters 2475 fluorometer at 290 nm ex, 360 nm em. Data were collected and analyzed with Waters Empower software.

DHFR Reaction Conditions. Reactions were run in 0.1 M Tris·HCl, pH 7.4 at 27 °C, 0.1 mM NADPH, 50 mM 2-mercaptoethanol, and 60 μM FA or 7,8-DHF (except where noted), and varying amounts of human or rat liver extract as the source of DHFR. All components of the reaction mixture except substrate were temperature equilibrated for 5 min before initiation of reaction with 7,8-DHF or FA. For the spectrophotometric assay the reaction was monitored during this time to observe any baseline rate due to nonspecific oxidation of NADPH. To measure the reaction rate by HPLC, aliquots were removed every 2 min for a total of 10 min and added to a solution of ascorbic acid and TCA, final concentrations 0.1 M and 0.55 M, respectively. The precipitated protein was removed by centrifugation at 4 °C for 10 min at 30,000 × *g*, and the supernatant immediately adjusted to pH 4.0 to 4.5 to optimize the effectiveness of ascorbate in maintaining THF. Samples were stored at −80 °C until analyzed by HPLC. THF dilutions for HPLC standards were made fresh daily in the same solution composition and pH as in the DHFR reaction samples, and treated similarly to the samples. For K_m curves samples were taken every 30 s for 2 min. Further details of the assay are available in *SI Text*.

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