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Studies on the Role of Folic Acid and Folate-Dependent Enzymes in Human Methanol Poisoning

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SUMMARY

Methanol toxicity is observed in monkeys and humans but is not seen in rats or mice. The expression of methanol poisoning is related to the ability of an animal to metabolize formate to carbon dioxide. Since the rate of formate oxidation is related to hepatic tetrahydrofolate (H_4folate) content and the activities of folate-dependent enzymes, studies were designed to determine hepatic concentrations of H_4folate and activities of folate-dependent enzymes of human liver and livers of species considered insensitive to methanol poisoning. An excellent correlation between hepatic H_4folate and maximal rates of formate oxidation has been observed. In human liver, H_4folate levels were only 50% of those observed for rat liver and similar to those found in monkey liver. Total folate was also lower (60% decreased) in human liver than that found in rat or monkey liver. Interestingly, mouse liver contains much higher hepatic H_4folate and total folate than rat or monkey liver. This is consistent with higher formate oxidation rates in this species. A second important observation has been made. 10-Formyltetrahydrofolate dehydrogenase activity, the enzyme catalyzing the final step of formate oxidation to carbon dioxide, was markedly reduced in both monkey and human liver. Thus, two mechanisms may be operative in explaining low formate oxidation in species susceptible to methanol toxicity, low hepatic H_4folate levels and reduced hepatic 10-formyltetrahydrofolate dehydrogenase activity.

Methanol poisoning has been recognized in humans since the early part of the century (1). Methanol is a mild central nervous system depressant, which produces no obvious signs or symptoms of toxicity until about 12–24 hr after ingestion (2–4). Following this period, metabolic acidosis develops along with visual disturbances which are often accompanied by nausea, vomiting, tachypnea, abdominal pain, and headaches. The ocular toxicity is characteristically described as optic disc edema leading to blindness if treatment is not instituted (2–4). Interestingly, this toxic syndrome, which has been clearly described in humans, is not seen in lower species, and common laboratory animals do not adequately serve as models for the study of this disease (5–7).

A number of laboratories (5–7) have shown that several species of monkeys are susceptible to methanol poisoning and are affected in a manner similar to that observed in humans. Monkeys develop central nervous system depression followed by an asymptomatic latent period of 12–24 hr, after which time metabolic acidosis and blindness develop (5, 8–10). McMartin *et al.* (7) showed that, in monkeys, the latent period observed in methanol poisoning is a period of compensated metabolic acidosis and, once the respiratory and metabolic compensating mechanisms are exhausted, the pH of the blood drops rapidly and the characteristic signs of methanol poisoning are observed. Furthermore, the metabolic acidosis was caused by formic acid production and accumulation in the body (6, 7, 11).

The accumulation of formic acid in a species sensitive to methanol poisoning is characteristic of this syndrome. In rats and most other rodents methanol administration does not lead to accumulation of formic acid in the body and no metabolic acidosis is observed (7). The sensitivity of a species to methanol poisoning appears to be related to its ability to metabolize formate. Clay *et al.* (6) demonstrated that the rate of disappearance of methanol from the blood in both rats and monkeys is approximately the same; however, rats metabolize formic acid at about twice the rate of that seen in monkeys (6, 7, 12). This appears to be a major factor leading to methanol poisoning in monkeys.

The pathway of formate oxidation to carbon dioxide is dependent upon folic acid in both rats and monkeys (12–16). If rats are made folate deficient (14), they metabolize formate at a reduced rate and develop metabolic acidosis following the administration of methanol. Folate deficiency in monkeys leads to the production of high levels of formate at relatively low

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ABBREVIATIONS: H_4folate, tetrahydrofolate; H_2folate, dihydrofolate; 10-HCO-H_4folate, 10-formyltetrahydrofolate; 5,10-CH=H_4folate, 5,10-methenyltetrahydrofolate; 5,10-CH_2-H_4folate, 5,10-methylenetetrahydrofolate; 5-CH_3-H_4folate, 5-methyltetrahydrofolate; 5-HCO-H_4folate, 5-formyltetrahydrofolate; HPLC, high performance liquid chromatography; EDTA, ethylenediaminetetraacetate.

doses of methanol (12). The first enzymatic step leading formate to carbon dioxide involves H_4folate and formate whereby 10-HCO-H_4folate formation is catalyzed by 10-HCO-H_4folate synthetase (EC 6.3.4.3), an ATP-dependent reaction (Fig. 1, reaction *1*). 10-HCO-H_4folate is then oxidized to carbon dioxide via 10-HCO-H_4folate dehydrogenase (EC 1.5.1.6) (Fig. 1, reaction *2*). Recent studies in our laboratory have shown an excellent correlation in rats between hepatic H_4folate levels and the maximal rate of formate oxidation *in vivo* (17, 18). The current study will demonstrate the relationship of the maximal formate oxidation rates to hepatic H_4folate levels in a number of species, and provide one explanation for the mechanism of methanol poisoning in humans. In addition, we show that the specific activity of 10-HCO-H_4folate dehydrogenase in human liver is low. This finding provides another possible explanation for the low formate oxidation rates in species susceptible to methanol poisoning.

Methods

Chemicals. The radiochemicals [^{14}C]formate and [5-^{14}C]CH_3-H_4folate were purchased from Amersham-Searle Corp. (Arlington Heights, IL). The chemicals H_4folate, 5-CH_3-H_4folate, 5-HCO-H_4folate, and 2-mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium ascorbate was purchased from Fisher Scientific Co. (Fair Lawn, NJ). Folic acid casei medium was purchased from Difco Laboratories (Detroit, MI). All other chemicals were of analytical or HPLC grade.

Animals. Male Sprague-Dawley rats (Biolabs, Madison, WI) which weighed 175–300 g, Swiss-Webster mice (Biolabs, Madison, WI) which weighed 25–30 g, and female cynomolgus (*Macaca fascicularis*) monkeys which weighed 2.5–5.5 kg, were used in these studies. The animals were maintained in wire bottom cages and the rodents were fed Purina Rodent Chow (Ralston Purina), while the monkeys were fed Purina Monkey Chow and fresh fruit daily. Water was available to the rats, mice, and monkeys *ad libitum* and chow was available to the rats and mice *ad libitum*.

Humans. Human liver tissue was obtained from the organ donor program. Livers were obtained from cadaver donors who were determined by established procedures to have cerebral death but in whom the cardiopulmonary function had been maintained. Donors were almost exclusively victims of traumatic head injuries. Livers used in this study did not have histologic or serologic evidence of liver disease and were not exposed to nitrous oxide. Liver tissue from individuals with chronic disease and those on chronic medication were excluded. Livers were harvested using standard retrieval techniques; livers used for the enzymatic studies were perfused with Collins solution while those used for the folate derivative studies were not perfused. The livers were immediately placed in ice for transport to the laboratory where, after sectioning into 50–100-g blocks, they were frozen in liquid nitrogen and stored at −70° until analyzed. Rat and monkey livers were frozen and stored in a similar manner to compare the effects of this handling protocol. No effect could be demonstrated.

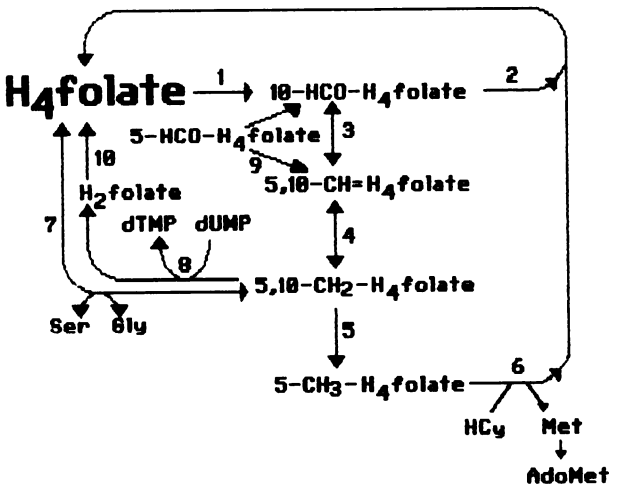

Fig. 1. Numbered reactions are represented by the following enzymes: *1*, 10-HCO-H_4-folate synthetase; *2*, 10-HCO-H_4folate dehydrogenase; *3*, 5,10-CH=H_4folate cyclohydrolase; *4*, 5,10-CH_2-H_4folate dehydrogenase; *5*, 5,10-CH_2-H_4folate reductase; *6*, methionine synthease; *7*, serine hydroxymethyltransferase; *8*, thymidylate synthetase; *9*, 5,10-CH=H_4folate synthetase; *10*, H_2folate reductase.

Enzyme assays. Rats were killed by decapitation. Livers were immediately removed and homogenized in the appropriate buffer or salt solution at 4°. Homogenates were 25% (w/v) and were prepared in a cold room (4°) with the use of a Potter-Elvehjem homogenizer. Homogenates were then centrifuged at 15,000 × *g* for 10 min. The supernatant was poured through cotton gauze into centrifuge tubes. Cytosol was prepared by centrifugation of supernatant at 100,000 × *g* for 60 min at 4°. The resultant cytosolic fraction was used as the source of enzyme for all enzyme assays except for the measurement of serine hydroxymethyltransferase (5,10-methylenetetrahydrofolate; glycine hydroxymethyltransferase) (EC 2.1.2.1); this activity was determined in the whole hepatic homogenate. All homogenates and cytosolic preparations were maintained on ice and assayed within 6 hr of homogenizing.

10-HCO-H_4folate synthetase (EC 6.3.4.3) was determined by a modification of the method of Rabinowitz and Pricer (19) in that the homogenizing buffer was 50 mM Tris-HCl, pH 7.4/0.1 mM EDTA. The 10-HCO-H_4folate dehydrogenase (EC 1.5.1.6) was determined by the method of Kutzbach and Stokstad (20). The cytosolic fraction was prepared with 50 mM potassium phosphate buffer, pH 7.4/0.1 mM EDTA.

Serine hydroxymethyltransferase activity was determined by the method of Taylor and Weissbach (21). Homogenates prepared in 75 mM potassium phosphate buffer, pH 7.4, were employed because activity was present in both the cytosolic and mitochondrial fractions. Liver cytosol prepared in 75 mM potassium phosphate buffer, pH 7.4, was used for the analysis of 5,10-CH_2-H_4folate reductase (EC 1.1.171) activity. This enzyme activity was measured as described by Black *et al.* (18) and Billings *et al.* (22). Methionine synthase (methionine synthetase; 5-methyltetrahydropteroyl-L-glutamate; L-homocysteine *S*-methyltransferase) (EC 2.1.1.13) activity was performed as described by Weissbach and co-workers (23, 24). This method uses a reducing system of platinum oxide and $FMNH_2$ in a reaction system of dithiothreitol, [5-^{14}C]methyl-H_4folate, and potassium phosphate buffer at pH 7.4. Incubations were conducted under reduced lighting in an H_2 atmosphere in Thunberg tubes as described by Billings *et al.* (22). H_2folate reductase (EC 1.5.1.3) activity was measured by the procedure of Hillcoat and Blakley (25) using a homogenizing buffer of 50 mM Tris-HCl, pH 7.0/0.1 mM EDTA. The H_2folate for this enzyme assay was prepared by the procedure of Blakley (26).

Clostridium cylindrosporum, ATCC #7955, was cultured and grown in broth under anaerobic conditions with the use of the technique of Rabinowitz and Pricer (19). The clostridia-laden broth was then used for the isolation and purification of clostridial 10-HCO-H_4folate synthetase (19). This enzyme was used to determine 10-HCO-H_4folate dehydrogenase enzyme activity as described by Kutzbach and Stockstad (20).

Folate determination. Hepatic folates were determined by the method of McMartin *et al.* (27). All folates were assayed as monoglutamates after enzymatic hydrolysis with hog kidney polyglutamate hydrolase. This technique combines HPLC separation with a microbiologic growth assay. Sample fractions were collected directly into folate-deficient *Lactobacillus casei* growth media and then they were inoculated and incubated as previously described by McMartin *et al.* (27).

Metabolic experiments. Metabolic experiments were carried out in metabolic chambers where $^{14}CO_2$ was collected by bubbling the expired air through a 2 N NaOH solution (12, 13). The dose of ^{14}C-formate was 500 mg/kg intraperitoneally for rats and monkeys, and 1.0 g/kg intraperitoneally for mice. These doses provided maximal and linear rates of oxidation over the course of measurement. All animals were given 3-amino-1,2,4-triazole (1 g/kg) 2 hr prior to formate injection to inhibit hepatic catalase activity.

Statistical analysis. Statistical evaluations were made with the use of the Student's *t* test for unpaired data. Probability values of <0.05 were considered statistically significant.

Results

The relationship of hepatic H_4folate and formate oxidation. Rat, mouse, and human liver were studied in order to determine the steady state levels of the intermediates of the folate biochemical pathway (Table 1). The concentrations of H_4folate, 5-CH_3-H_4folate, and total folate in human liver are significantly lower than those observed in mouse or rat liver. The mouse possesses extraordinarily high amounts of hepatic H_4folate and very high levels of total folate. The levels in rat liver have been used as a reference to those obtained in mouse and human liver because a great deal of information has been published for the metabolism of formate in this species. Black *et al.* (18) have recently analyzed hepatic folate derivatives in monkeys and these are included for comparative purposes in Table 1. Human and monkey levels of hepatic H_4folate are similar, although the total folate level is much lower in human liver.

The maximal rates of formate oxidation are shown in Fig. 2. In three species, rat, mouse, and monkey, formate oxidation rates are clearly related to the level of hepatic H_4folate.

Hepatic folate-dependent enzymes. The activities of rat, monkey, and human folate pathway enzymes are shown in Table 2. Several human folate-dependent enzyme activities were significantly different from those observed in rat liver. 10-HCO-H_4folate dehydrogenase, the enzyme which catalyzes the final step in the conversion of formate to CO_2, was only 32% of the activity seen in rat liver. A reduced activity was also found for 5,10-CH_2-H_4folate reductase in human liver compared to that observed with rat liver. The activity of the H_2folate reductase in human liver was only 4% of that observed for rat liver. In contrast, the activity of the serine hydroxymethyltransferase was almost twice that observed for rat liver. Activities obtained for human liver 10-HCO-H_4folate synthetase and methionine synthase were similar to that of rat liver.

Activities of enzymes of the folate biochemical pathway in monkey liver were, in general, similar to those observed for human liver except for 10-HCO-H_4folate synthetase. The activity of this enzyme is extraordinarily high and probably accounts, in part, for the high level of 10-HCO-H_4folate found in monkey liver.

TABLE 1

Hepatic folate and folate coenzymes

Levels are expressed as nmol/g of liver ± SE. HCO-H_4folate represents the sum of 10-HCO-H_4folate and 5-HCO-H_4folate levels.

	Species			
	Mouse	Rat	Human	Monkey
HCO-H_4folate	6.4 ± 0.6	4.6 ± 1.3	3.3 ± 0.5	10.5 ± 0.8[a]
H_4folate	42.9 ± 1.2[a]	11.4 ± 0.8	6.5 ± 0.3[a]	7.4 ± 0.8[a]
5-CH_3-H_4folate	11.6 ± 0.4	9.3 ± 0.6	6.0 ± 0.7[a]	7.6 ± 1.1
Total folate	60.9 ± 2.1[a]	25.3 ± 0.9	15.8 ± 0.8[a]	25.5 ± 1.2
	$N = 4$	$N = 6$	$N = 5$	$N = 7$

[a] Value is significantly different from the rat liver ($p < 0.005$).

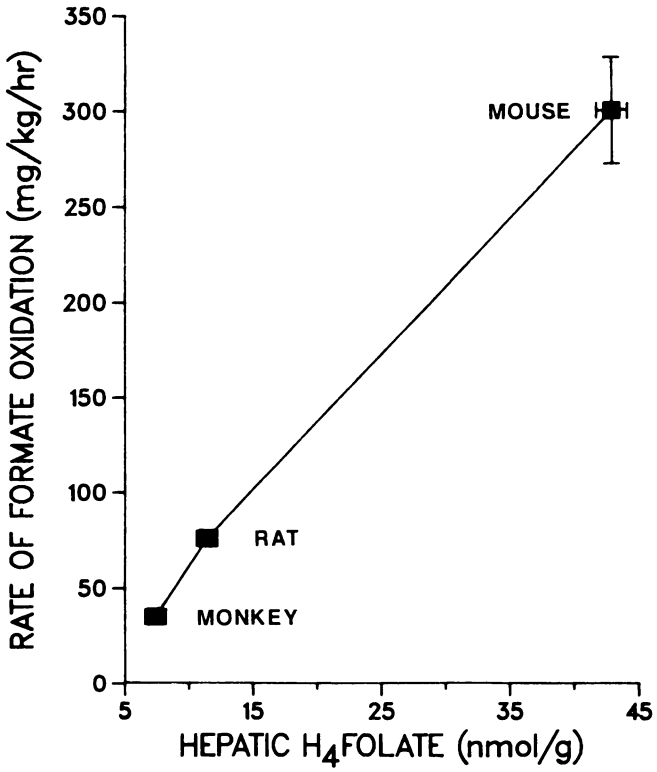

Fig. 2. The relationship of formate oxidation to hepatic H_4folate. Standard error bars are contained within the data points for the rat and monkey. Each point represents the mean value obtained for at least four animals.

Discussion

The current work supports the hypothesis that hepatic H_4folate is a major factor in determining whether a species will or will not be susceptible to methanol poisoning. Eells *et al.* (17) first showed the excellent correlation between hepatic H_4folate and formate oxidation in rats *in vivo*. This correlation has been examined in other species. Mice, which have a high capacity for formate oxidation, possess high hepatic H_4folate levels and monkeys, which have a low rate of formate oxidation *in vivo* (12), possess low hepatic H_4folate (18) (Fig. 2). Based on this information, one would predict that the maximal rate of formate oxidation to CO_2 in humans should be similar to the maximal rate of formate oxidation observed for monkeys. This would explain why both monkeys and humans accumulate formic acid and develop methanol poisoning, whereas other species such as rats and mice do not. In fact, the half-life of elimination of formate in humans has been reported to be 4–5 times longer than in the rat (28), but maximal rates of formate oxidation are not available for humans.

The human total hepatic folate levels are relatively low and are in excellent agreement with the data presented by Hoppner and Lampi (29) in a large post-mortem series and with the results of Chanarin *et al.* (30) in a smaller series where surgical specimens were analyzed. Folate derivatives have not been analyzed in human liver previously.

It is of no small interest that monkeys which have low rates of formate oxidation, 2–3 times slower than the rat, can be

TABLE 2

Specific activities of hepatic folate-dependent enzymes in various species

Enzyme activities are expressed as nmol of product formed/min/mg of protein. Values are means ± SE.

	Rat	Human	Monkey
10-HCO-H_4folate synthetase	65.9 ± 5.0 (4)[a]	75.0 ± 8.7 (4)	142 ± 16[b] (4)
10-HCO-H_4folate dehydrogenase	88.3 ± 11.7 (4)	23.0 ± 2.2[b] (4)	33.0 ± 4.0[b] (4)
Serine hydroxymethyltransferase	10.8 ± 0.6 (4)	18.5 ± 0.7[b] (4)	17.1 ± 9.7[b] (9)[c]
H_2folate reductase	19.8 ± 1.3 (3)	0.74 ± 0.17[b] (3)	4.1 ± 0.7[b] (6)[c]
5,10-CH_2-H_4folate reductase	1.21 ± 0.07 (5)	0.42 ± 0.07[b] (6)	0.22 ± 0.02[b] (7)[c]
Methionine synthase	0.09 ± 0.007 (4)	0.10 ± 0.008 (4)	0.09 ± 0.012 (7)[c]

[a] Numbers in parentheses, number of individual specimens used.
[b] Value is significantly different from that of the rat liver ($p < 0.005$).
[c] Black *et al.* (18).

treated with folinic acid (5-HCO-H_4folate) and protected against the metabolic acidosis arising from methanol administration (12, 16). In monkeys pretreated with 5-HCO-H_4folate, an increased rate of formate oxidation was observed and no metabolic acidosis resulted (12, 16). Furthermore, Noker *et al.* (16) showed, in monkeys, that once metabolic acidosis had been produced following methanol administration, folinic acid administration could reverse the metabolic acidosis (16). The observation that folinic acid given concurrently with methanol reduced or abated the formic acidosis in monkeys (16) takes on a new significance with the data presented here. Humans have reduced levels of both total folate and H_4folate, and the use of a folate analogue in methanol poisoning might be advocated in combination with the present regimen of hemodialysis, ethanol, and sodium bicarbonate administration (31). 5-HCO-H_4folate, thus, could function as a "rescue treatment" in methanol poisoning. A recent report dealing with a series of methanol-poisoned patients has been published where 5-HCO-H_4folate acid was used in humans (32) in an attempt to increase the rate of formate metabolism.

A second hypothesis has evolved from the current work which could also explain regulation of the rate of formate oxidation. A relative enzymatic deficit in the pathway of conversion of formate to carbon dioxide has been demonstrated. The first folate-dependent reaction involved in formate oxidation is catalyzed by the enzyme, 10-CHO-H_4folate synthetase (Fig. 1, reaction *1*), a protein which catalyzes the ATP-dependent reaction of formate and H_4folate leading to 10-HCO-H_4folate (33). The hepatic 10-HCO-H_4folate synthetase activity in rats and humans is essentially the same. However, the second catalyst in the conversion of formate to CO_2, 10-HCO-H_4folate dehydrogenase, is much lower in human liver. Thus, a second explanation for a low formate oxidation rate in humans may be offered, i.e., a reduced catalytic capacity for formate oxidation. This, coupled with low H_4folate, may be an important feature of the formic acidemia observed in humans after methanol ingestion. The data presented here represent specific activities which cannot be directly equated with kinetic data from purified enzymes, yet the implication is obvious. In addition, our data clearly refute the hypothesis presented in a recent review which concluded that humans are deficient in the enzyme 10-HCO-H_4folate synthetase and therefore have an impaired ability to handle formate (34). It is shown that 10-HCO-H_4folate synthetase activity is present in human liver and at a level similar to that found in rat liver.

H_2folate reductase (Fig. 1, reaction *10*) catalyzes the reduction of folate to H_2folate which, in turn, is further reduced to H_4folate. In actively dividing cells, thymidylate synthesis is dependent on the regeneration of H_4folate by H_2folate reductase. In nonproliferating tissue such as the liver, which has virtually undetectable levels of thymidylate synthetase (5,10-methylenetetrahydrofolate; dUMP C-methyltransferase) (EC 2.1.1.45) (Fig. 1, reaction *8*), there would be less H_2folate reductase needed due to the relative lack of purine biosynthesis. It is of interest that chronic low dose methotrexate exposure in humans can result in hepatic injury, fibrosis, and even cirrhosis (35–37). Methotrexate is a potent inhibitor of H_2folate reductase. The low levels of hepatic H_2folate reductase in humans may account for the increased susceptibility to the hepatic toxicity seen with this agent.

Serine hydroxymethyltransferase (Fig. 1, reaction *7*), a pyridoxal-dependent enzyme, catalyzes a carbon transfer from serine to H_4folate and results in the formation of 5,10-CH_2-H_4folate and glycine (38). The serine hydroxymethyltransferase reaction has been considered one of the most important physiologic means for entry of one-carbon units into the folate pathway. Arnstein and Neuberger (39) demonstrated that carbon-3 of serine is catabolized to form glycine which thereby provides 70% of the methyl groups ultimately transferred to methionine. Rowe *et al.* (40) demonstrated that, in human lymphocytes, serine is essentially the sole source of one-carbon units in *de novo* synthesis of purines and in the methyl group donated to thymidylate. The data presented here demonstrate an elevated activity of serine hydroxymethyltransferase in human liver compared to that of rat liver. The importance of this increased activity is not known at this time and is the subject of another study.

The enzyme 5,10-CH_2-H_4folate reductase (Fig. 1, reaction *5*) catalyzes the NADPH-dependent reduction of 5,10-CH_2-H_4folate to 5-CH_3-H_4folate and has been shown here to be low in human liver. A central regulatory role for 5,10-CH_2-H_4folate reductase in one-carbon metabolism has been advocated (41). Because one of the key sources of one-carbon units in the folate pathway is through serine hydroxymethyltransferase, the active flux via carbon-3 of serine into this pathway results in continuous production of 5,10-CH_2-H_4folate as a co-substrate for thymidylate synthesis or for 5-CH_3-H_4folate synthesis. In a cell that is not actively forming DNA, there is little requirement for 5,10-CH_2-H_4folate for DNA synthesis and the irreversible reduction of 5,10-CH_2-H_4folate to 5-CH_3-H_4folate diverts one-carbon units ultimately to the synthesis of methionine. The 5,10-CH_2-H_4folate reductase may therefore serve a regulatory role in maintaining 5-CH_3-H_4folate. It should be noted that this enzyme and 5-CH_3-H_4folate are low in human liver. Black *et al.* (18) hypothesized that, in monkey liver, the low 5,10-CH_2-H_4folate reductase coupled with a high activity of 10-HCO-H_4folate synthetase might account for the high level of 10-HCO-H_4folate observed in livers of this species. Thus, 5,10-

CH_2-H_4 folate reductase may be an important factor in determining the steady state levels of one-carbon folate intermediates in an animal. It has also been suggested that inhibitors of this enzyme such as *S*-adenosylmethionine, can affect the ratio of hepatic levels of H_4folate and 5-CH_3-H_4folate (42).

Methionine synthase (Fig. 1, reaction *6*) catalyzes the transfer of a methyl group from 5-CH_3-H_4folate to homocysteine leading to the formation of methionine and H_4folate (24). The data presented here suggest that methionine synthase may not normally provide a regulatory role in the relative flux of the folate derivatives, as the relative activities of this enzyme in different species are essentially identical. However, studies where nitrous oxide is used to inhibit methionine synthetase activity have shown that this enzyme is very important in the regeneration of H_4folate *in vivo* (17, 22).

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