In vivo oxidation of the methyl group of hepatic 5-methyltetrahydrofolate

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SUMMARY Methionine given parenterally to rats caused rapid disappearance of methyltetrahydrofolate from the liver and a corresponding rise in tetrahydrofolate and formyl-tetrahydrofolate concentrations. When [^{14}C]H₃—H₄folate was given, methionine caused an increased [^{14}C]O₂ excretion, indicating that oxidation of the methyl group had occurred. Methionine was more effective than S-adenosylmethionine at causing oxidation, but serine was ineffective.

The lowest dose of methionine to produce an effect was 0.5 μmol , which is less than the daily dietary intake in a rat. The data suggest that the concentration of methylfolate in rat livers is controlled by the concentrations of methionine.

Folic acid functions in the transfer of single carbon units. These are formyl (CHO—) required for purine synthesis, methylene (—CH₂—) required for thymidine synthesis, and methyl (—CH₃—) required for methionine synthesis. 5-methyltetrahydrofolate (5—CH₃—H₄PteGlu, methylfolate) is also the form in which natural folates are absorbed from the gut into portal blood. It is also the dominant analogue in plasma and the only form of folate in the cerebrospinal fluid. The manner in which methylfolate is formed and metabolised is central to understanding the interaction between cobalamin and folate, and these aspects have been reviewed.¹

5-Methyl-tetrahydrofolate is formed by reduction of 5,10-methylenetetrahydrofolate. The enzyme is 5,10-methylenetetrahydrofolate reductase, and in vitro the reaction is noticeably shifted in the direction of methylfolate synthesis. On the basis of in vitro studies the reverse reaction, involving oxidation of methylfolate, is regarded as unlikely.

The methyl group of 5—CH₃—H₄PteGlu is transferred to homocysteine by methionine synthetase in the de novo synthesis of methionine. This is said to be the only means for the disposal of the methyl group of methylfolate. Failure of the methionine synthetase reaction, which occurs in cobalamin deficiency or in cobalamin inactivation by nitrous oxide, may lead to accumulation of 5—CH₃—H₄PteGlu and subsequent "trapping" of folate. This has been termed the methylfolate trap hypothesis.^{2,3}

When these ideas were put forward,³ it was also noted that methionine had considerable effects on the distribution of folate analogues in rat livers. The authors found that 1% methionine added to the rat diet and fed for 24 hours before sacrifice considerably increased the amount of tetrahydrofolate and formyl-tetrahydrofolate at the expense of methyltetrahydrofolate. This occurred in both control and experimental animals. The mechanism of this redistribution was not established, although it was conjectured that methionine might provide a methyl group acceptor via S-adenosylhomocysteine derived from demethylation of S-adenosylmethionine.

The effect of methionine in lowering CH₃—H₄PteGlu and increasing H₄PteGlu and CHO—H₄PteGlu concentrations in liver has been confirmed most recently by Brody *et al*, who showed that 300 μmol of methionine in the rat virtually removed all hepatic methylfolate within 30 minutes. They too did not comment on how this occurred. They used cobalamin-inactivated rats so that the methyl group could not have been passed on to homocysteine.⁴

S-adenosylmethionine in vitro inhibits the activity of the enzyme methylene tetrahydrofolate reductase and so inhibits further synthesis of methylfolate. On this basis the concentration of S-adenosylmethionine has been assumed to be the main regulatory factor in the rate of methylfolate synthesis.

The validity of these views are of importance in understanding folate and one-carbon unit metabolism and how cobalamin deficiency produces its effects. The purpose of this study was to explore how methionine and related compounds changed the concentrations of

methylfolate in rat livers, and in particular to obtain evidence for the in vivo oxidation of the methyl group of methylfolate to formate and carbon dioxide.

Material and methods

Methionine, 5-deoxy-5'-methylthioadenosine, glycine, serine and betaine were obtained from Sigma. Sodium formate was obtained from Koch Light. S-adenosylmethionine was generously donated by Dr G Stramentinoli of Bioresearch, Milan.

Male Sprague-Dawley rats weighing between 80 and 120 g were used. Animals were given the compounds in a standard volume of 0.5 ml in water by intraperitoneal injection. Thirty minutes later, animals were killed, livers removed and heated for five minutes in 20 volumes of 1% ascorbic acid (pH7) at 95°C. Samples were then cooled, homogenised, and stored at -20°C.

IDENTIFICATION OF FOLATES

Liver homogenates were treated with γ -glutamyl carboxypeptidase prepared from pig kidney. Homogenate (0.1 g) was adjusted to pH4.5 and incubated with 1.5 ml of enzyme preparation for five hours at 37°C, then diluted to 10 ml in 0.1M sodium phosphate buffer (pH 5.7).

After centrifugation at $3000 \times g$ for 30 minutes the supernatant was applied to a column of DEAE cellulose (Whatman DE52). Folate monoglutamates were separated using a non-linear salt gradient. The mixing chamber contained 150 ml of 0.1M potassium phosphate (pH 6.0), to which was added 0.2M 2-mercaptoethanol. The reservoir contained 350 ml of 1.0M potassium phosphate (pH 6.0) with 0.2M 2-mercaptoethanol. Fractions (5 ml) were collected, all the folates eluting before fraction 50. Folates were identified by elution position and measured by microbiological assay with *Lactobacillus casei* (NCIMB 8081). 5-CHO-H₄PteGlu was measured using *Pediococcus cerevisiae* (NCIMB 7837)). As 5-formyl- and 5-methyl-H₄PteGlu elute very close together and 5-methyl-H₄PteGlu is inactive for *P. cerevisiae*, *P. cerevisiae* assay was used as a measure of 5-formylfolate.

Amounts of each folate were expressed as the percentage of the total folate.

[¹⁴C]₂ PRODUCTION

5-[¹⁴C]H₃-H₄PteGlu specific activity 60 mCi/mmol, was obtained from Amersham International. Ten Sprague Dawley rats were exposed to N₂O/O₂ (1/1) in a specially constructed perspex chamber. After four days half the rats were given an intraperitoneal injection containing 300 μ mol of methionine and 2 μ Ci 5-[¹⁴C]H₃-H₄PteGlu alone. The animals were then

placed in individual chambers through which nitrous oxide and oxygen continued to be passed. Expired carbon dioxide was collected by passing the outflow from the chambers through two vessels connected in series, each containing 50 ml of 2M potassium hydroxide. The efficiency of this system was checked by passing the outflow of the second vessel through a solution of barium hydroxide (50 g/l). No precipitate was formed. One ml samples of the potassium hydroxide were taken every hour into 10 ml NE260 (Nuclear Enterprises) and counted on a LKB Betarak liquid scintillation counter. Quench corrections were made by the channels ratio method using an external standard.

Results

PRODUCTION OF [¹⁴C]₂ FROM [¹⁴C]H₃-H₄PTEGLU Rats given [¹⁴C]H₃-H₄PteGlu exhaled [¹⁴C]₂, reaching a peak at two hours after the dose of methylfolate. When this was accompanied by an injection of 300 μ mol of methionine the total [¹⁴C]₂ excretion was greater (fig 1), reaching a peak at three hours, and this difference was significant at the 5% level.

EFFECT OF METHIONINE OF FOLATE ANALOGUES IN LIVER

The distribution of CH₃-H₄PteGlu, CHO-H₄PteGlu, and H₄PteGlu in rat livers is shown in table 1 and fig 2. Methionine (0.5 μ mol) produced a fall in the methylfolate concentrations from 45% to 36%, and the maximum decline to 2.6% was reached with a 50 μ mol dose. The fall in the CH₃-H₄PteGlu concentra-

Fig 1 Two groups of five rats breathing N₂O/O₂ (1/1) for three days were given an injection of [¹⁴C]H₃-H₄PteGlu. One group (O) was given 300 μ mol methionine intraperitoneally at the same time. Exhaled [¹⁴C]₂ was collected into potassium hydroxide.

Table 1 Effect of parenteral methionine on percentage of folate analogues present in rat liver

Dose of methionine (μmol)	CH ₃ -H ₄ PteGlu			CHO-H ₄ PteGlu			H ₄ PteGlu		
	(n =)	Mean* (SD)	Range	(n =)	Mean (SD)	Range	(n =)	Mean (SD)	Range
0	16	45.0 (7.0)	36.4-56.7	16	23.5 (3.6)	17.8-28.6	16	31.5 (5.7)	22.1-39.0
0.5	3	35.7 (4.2)	31.6-39.9	3	28.6 (2.6)	26.9-31.6	3	35.7 (5.1)	32.7-41.5
1.0	3	27.3 (5.7)	21.6-33.0	3	30.8 (3.2)	27.6-34.0	3	41.9 (5.0)	38.7-47.6
4.0	6	28.8 (10.1)	14.5-44.3	6	32.0 (5.8)	22.1-40.5	6	39.2 (10.6)	22.8-53.7
8.0	6	23.8 (5.5)	15.6-31.8	6	33.5 (3.8)	26.4-37.0	6	42.7 (4.1)	39.2-50.5
16.0	6	16.6 (7.9)	10.1-31.4	6	40.5 (5.2)	30.1-44.0	6	42.9 (4.3)	38.1-47.5
50.0	3	2.6 (1.5)	1.0-4.0	3	42.3 (4.3)	37.4-45.6	3	55.1 (3.0)	53.3-58.6
100.0	3	2.8 (0.5)	2.4-3.3	3	44.3 (2.7)	42.4-47.5	3	52.9 (2.5)	50.1-55.0
300.0	3	2.9 (0.3)	2.6-3.1	3	46.7 (3.1)	44.2-50.2	3	50.4 (2.9)	47.2-52.7

*Value is significantly less than in group not given methionine.

tion was accompanied by a parallel increase in CHO-H₄PteGlu and H₄PteGlu (table 1 and fig 3), implying oxidation of the methyl groups to formyl and carbon dioxide (fig 1).

EFFECT OF S-ADENOSYLMETHIONINE ON FOLATE ANALOGUES IN LIVER

S-adenosylmethionine was far less effective on a molar basis than methionine in producing a fall in methylfolate analogues (table 2 and fig 4). The rate of decline of methylfolate with increasing doses of S-adenosylmethionine paralleled that achieved with methionine, and suggested that the effect of S-adenosylmethionine

was caused by methionine which had been regenerated after further metabolism of S-adenosylmethionine.

EFFECT OF 5'-METHYLTHIOADENOSINE ON FOLATE ANALOGUES IN LIVER

This compound, derived from decarboxylated S-adenosylmethionine via synthesis of polyamines, is metabolised back to methionine with release of a formate unit. At a dose of 16 μmol 5'-methylthioadenosine produced a fall of 30% in methylfolate concen-

Fig 2 Fall in the concentration of 5-CH₃-H₄PteGlu in rat liver in animals killed 30 minutes after an intraperitoneal dose of methionine. Dose of methionine varied from 0.5 to 300 μmol.

Fig 3 Mean fall in the concentration of 5-CH₃-H₄PteGlu in rat liver after methionine and concomitant rise in the concentration of H₄PteGlu and CHO-H₄PteGlu.

Table 2 Effect of parenteral S-adenosylmethionine on percentage of folate analogues present in rat liver

Dose of S-adenosylmethionine (μmol)	CH ₃ -H ₄ PteGlu			CHO-H ₄ PteGlu			H ₄ PteGlu		
	(n =)	Mean (SD)	Range	(n =)	Mean (SD)	Range	(n =)	Mean (SD)	Range
0	16	45.0 (7.0)	36.4–56.7	16	23.5 (3.6)	17.8–28.6	16	31.5 (5.7)	22.1–39.0
31	6	32.3* (6.5)	25.1–42.9	6	32.2 (6.2)	23.4–38.0	6	35.5 (4.9)	30.1–43.1
97	3	28.0* (4.7)	21.7–35.0	6	33.1 (2.7)	30.2–37.6	6	38.9 (2.7)	33.7–40.7
194	3	23.7* (5.6)	18.9–29.9	3	28.4 (1.3)	26.9–29.3	3	47.9 (6.5)	41.2–54.2

*Value is significantly less than in group not given S-adenosylmethionine.

Fig 4 Mean fall in the concentration of 5-CH₃-H₄PteGlu in rat liver after methionine, S-adenosylmethionine, 5'-methylthioadenosine and serine.

trations (table 3 and fig 4). Due to toxicity of 5'-methylthioadenosine, doses greater than 16 μmol were not tested.

EFFECT OF OTHER COMPOUNDS ON FOLATE ANALOGUES IN LIVER

Neither serine nor formate (table 4 and fig 4) had any

effect on methylfolate concentrations in liver but both glycine and betaine had an effect that was comparable with an equimolar dose of S-adenosylmethionine.

Discussion

Kutzbach and Stokstad suggested that the concentration of methylfolate in liver is regulated by control of the rate of its synthesis.⁵ S-adenosylmethionine inhibited the activity of methylenetetrahydrofolate reductase which reduces methylene-H₄PteGlu to methyl-H₄PteGlu. Our data suggest that the concentration of methylfolate in liver is more finely attuned to that of methionine. Excess methionine is toxic and its increase leads to rapid conversion of the methyl group of methylfolate to more oxidised forms, and presumably to the end of methionine synthesis. This oxidation occurs in both B₁₂-deficient² and B₁₂-inactivated animals (unpublished observations) as well as in healthy animals. The amount of methionine producing this effect can be as little as 0.5 μmol , which is well below the normal daily intake in a rat. S-adenosylmethionine was less effective than methionine and suggests that S-adenosylmethionine concentrations are less important than methionine in regulating methylfolate metabolism. Indeed, the activity of S-adenosylmethionine was related to the amount of methionine regenerated.

The data have implications for the methylfolate trap hypothesis, which assumes that the methyl group of methylfolate is not oxidised. Even in the absence of a methionine supplement the B₁₂-inactivated animal is able to dispose of the methyl group of methylfolate by

Table 3 Effect of parenteral 5'-methylthioadenosine on percentage of folate analogues present in rat liver

Dose of MTA (μmol)	CH ₃ -H ₄ PteGlu			CHO-H ₄ PteGlu			H ₄ PteGlu		
	(n =)	Mean (SD)	Range	(n =)	Mean (SD)	Range	(n =)	Mean (SD)	Range
0	16	45.0 (7.0)	36.4–56.7	16	23.5 (3.6)	17.8–28.6	16	31.5 (5.7)	22.1–39.0
4	3	40.0 (4.9)	36.3–45.6	3	22.6 (4.2)	18.9–27.1	3	37.4 (2.4)	35.5–40.1
8	3	28.4* (3.3)	25.1–31.6	3	26.6 (1.3)	25.2–27.7	3	45.0 (3.8)	40.7–47.9
16	3	15.6* (6.2)	8.5–19.3	3	34.1 (2.4)	31.7–36.5	3	50.3 (4.2)	46.8–55.0

*Value is significantly less than in group not given methylthioadenosine.

Table 4 Effect of parenteral glycine, serine, betaine and formate given at a dose of 16 μ mol on percentage of folate analogues present in rat liver

Compound	(n =)	CH ₃ -H ₄ PteGlu		(n =)	CHO-H ₄ PteGlu		(n =)	H ₄ PteGlu	
		Mean (SD)	Range		Mean (SD)	Range		Mean (SD)	Range
0	16	45.0 (7.0)	36.4–56.7	16	23.5 (3.6)	17.8–28.6	16	31.5 (5.7)	22.1–39.0
Glycine	6	31.2* (7.2)	24.5–40.7	6	28.6 (5.5)	19.8–34.8	6	40.2 (4.7)	33.8–45.3
Serine	3	44.2 (3.1)	41.7–47.7	3	17.4 (0.9)	16.8–18.4	3	38.4 (2.5)	35.5–39.9
Betaine	6	34.0* (9.6)	24.1–47.2	3	28.5 (7.4)	17.1–36.0	3	37.5 (6.1)	30.1–48.4
Formate	3	39.4 (8.22)	31.6–48.0	3	27.7 (5.2)	22.5–32.8	3	32.9 (3.1)	29.5–35.6

*Value is significantly less than in group not given either glycine nor betaine.

oxidation.⁶ This is enhanced considerably by raising the methionine intake.

Accumulation of methylfolate occurs in B₁₂-inactivation, primarily because of the failure of the methionine synthetase reaction. The hepatic concentration of methylfolate reaches its peak within hours of exposure to nitrous oxide but thereafter falls and remains at a plateau after seven days. This methylfolate is metabolically active in so far as it serves as a substrate for the synthesis of polyglutamate with up to five glutamic acid residues. The absence of methylhexaglutamate has suggested oxidation of the methyl group from the hexaglutamate in accordance with the observations of Brody, Watson, and Stokstad.⁴ Addition of a methionine supplement is accompanied by oxidation of methylfolate within minutes, so that it seems unlikely that this occurs from methylhexaglutamate alone but probably from methylfolates of variable chain length, including monoglutamates.

The confirmation of the in vivo oxidation of the methyl group of methylfolate in both healthy and B₁₂-inactivated animals removes one of the central pivots of the methylfolate trap hypothesis. There are other equally important observations, however, that are not accounted for by methylfolate trapping.¹ Firstly, the most important is the failure of B₁₂-deficient or B₁₂-inactivated cells to use H₄PteGlu in a normal manner. Thus H₄PteGlu failed to correct the defect in the dU suppression test in marrow cells from patients with untreated pernicious anaemia. This was confirmed with rat marrow from animals exposed to nitrous oxide. Further H₄PteGlu was not used as substrate for folate polyglutamate synthesis in liver in rats exposed overnight to nitrous oxide.

Secondly, the impairment of both thymidine synthesis in the dU suppression test and of folate polyglutamate synthesis in the B₁₂-inactivated rat was reversed completely if folate carrying a carbon unit at the formate level of oxidation was provided. The most convenient was 5-formyl-tetrahydrofolate, but 10-formyl-tetrahydrofolate and 5,10-methenyl-tetrahydrofolate were equally effective.

Thirdly, the defects due to B₁₂-inactivation were reversed by supplying a compound that yielded formate. The most interesting was 5'-methylthioadenosine, which is derived from S-adenosylmethionine and is reconverted to methionine, yielding a mole of formate for each mole methionine regenerated. This compound was much more effective than methionine itself in reversing the effects of B₁₂ deficiency.

We therefore suggest that the effect of B₁₂ deficiency is a lack of active formate which is normally derived directly from methionine and indirectly through S-adenosylmethionine and 5'-methylthioadenosine.

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