Chronic Cobalamin Inactivation Impairs Folate Polyglutamate Synthesis in the Rat

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ABSTRACT Nitrous oxide, by inactivating cobalamin in vivo, produces a suitable animal model for cobalamin 'deficiency.' The synthesis of folate polyglutamate with tetrahydrofolate as substrate is severely impaired in the N_2O-treated rat, but is normal with formyltetrahydrofolate as substrate. Methionine restores the capacity of the N_2O-treated rat to utilize tetrahydrofolate the minimum effective dose being 16 μmol. *S*-Adenosylmethionine was somewhat less effective than methionine but 5'methylthioadenosine, a product of *S*-adenosylmethionine metabolism, was significantly more effective than methionine in correcting the defect in folate polyglutamate synthesis. 5'Methylthioadenosine is metabolised to yield formate. It is suggested that these compounds have their effect in correcting folate polyglutamate synthesis by supplying formate for the formylation of tetrahydrofolate. Formyltetrahydrofolate, at least in the cobalamin-inactivated animal, is the required substrate for folate polyglutamate synthesis. Cobalamin is concerned with the maintenance of normal levels of methionine and this in turn is a major source of formate through *S*-adenosylmethionine and 5'methylthioadenosine.

INTRODUCTION

The anesthetic gas, nitrous oxide (N_2O), inactivates cobalamin (vitamin B_{12}) and has proved an extremely useful tool in investigating the biochemical effects of loss of cobalamin activity. On exposure to transition-metal complexes, of which cobalamin is one, N_2O, which is otherwise inert, is cleaved into nitrogen and oxygen (1). It thus acts as a two-electron oxidizing agent and oxidizes cob[I]alamin to the inactive cob[III]alamin. In man too, exposure to N_2O inactivates cobalamin and, if exposure exceeds 6 h, megaloblastic hemopoiesis and marrow failure (2–4), which can be fatal (2, 5), develops. Prolonged intermittent exposure leads to a neuropathy essentially similar to that found in cobalamin deficiency (6).

Exposure of animals to N_2O leads to inactivation of the enzyme methionine synthetase (7), which has a methylcobalamin cofactor, but there is no increase in urinary methylmalonic acid excretion, which too requires cobalamin. However, long-term exposure of rats to N_2O is accompanied by a fall in hepatic methylmalonylCoA mutase activity (8).

The inactivation of cobalamin by N_2O also leads to major effects on folate metabolism. There is a rise in plasma folate on exposure to N_2O in both man (4) and rat (9) and a marked loss of folate from the tissues in rats (10, 11) due to excretion of 5-CH_3-H_4-pteroyl-glutamate (5-CH_3-H_4PteGlu) into the urine (12). Recovery of tissue folate on returning N_2O-exposed rats to air requires a dietary source of folate (13). There is an increased urinary formiminoglutamic acid excretion in the N_2O-treated rat (unpublished observations) but the activity of the enzyme, formiminotetrahydrofolate formiminotransferase, is unaffected (14). Exposure to N_2O also leads to impaired synthesis of thymidine, as assessed in the deoxyuridine suppression test, in both rats (15, 16) and man (3), and this impairment is corrected in part by the addition of cobalamin to the test system.

Exposure to N_2O also affects other enzymes concerned with folate metabolism. There is a rise in activity of 10-formyltetrahydrofolate synthetase (linking formate to H_4PteGlu), a fall in activity of 5,10-methylenetetrahydrofolate cyclohydrolase (10-CHO-H_4PteGlu → 5,10-CH=H_4PteGlu) (14) and an increase in activity of thymidylate synthetase (17). Other enzymes, including serine transhydroxymethylase, 5,10-methylenetetrahydrofolate dehydrogenase, and 5,-

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10-methylenetetrahydrofolate reductase, are unaffected (14).

In untreated pernicious anemia there is a fall in the concentration of folate polyglutamate in erythrocytes without any change in the level of folate monoglutamate (18, 19). An impaired incorporation of PteGlu into hepatic folate polyglutamate has been demonstrated in sheep (20) and rats (21) with dietary-induced cobalamin deficiency. The N_2O-treated rat likewise showed an impairment in the synthesis of folate polyglutamate from parenteral PteGlu (15) as well as from H_4PteGlu (22). However, folate polyglutamate synthesis was normal when the N_2O-treated rats were given either 5-CHO-H_4PteGlu (folinic acid) or 10-CHO-H_4PteGlu as substrate (22). This suggested that formyltetrahydrofolates rather than tetrahydrofolate were the required substrates for folate polyglutamate synthesis in rats and that cobalamin was concerned with the provision of CHO-H_4PteGlu. This view was reinforced by the increased activity of formyltetrahydrofolate synthetase following exposure to N_2O (14).

The purpose of this study was to explore in greater detail the synthesis of folate polyglutamate in control and N_2O-treated rats and to determine how this could be related to the provision of 'formate' by testing the effect of compounds that might contribute a single carbon unit into the folate pool.

METHODS

Materials. L-Methionine, *S*-adenosyl-L-methionine (SAM)[1], 5'-methylthioadenosine (MTA), adenosine, L-serine, glycine, betaine, sarcosine, choline, and L-homocyteine thiolactone hydrochloride were obtained from Sigma, Poole, Dorset, U.K. [2-^{14}C]PteGlu (Radiochemical Centre, Amersham, U.K.), sp act ~52 μCi/μmol was reduced to [2-^{14}C]H_4PteGlu using sodium dithionite (23). Column chromatography of the product on DE52 cellulose showed the yield of H_4PteGlu to be between 82–85% of PteGlu. The remaining carbon-labeled fractions, which eluted in fractions 5–15, were not microbiologically active and appeared to be impurities in the [2-^{14}C]PteGlu eluting from the column before folate compounds. The ^{14}C-labeled fractions eluting in fractions 5–15 were concentrated, 0.5 μCi injected into rats, both air-breathing and N_2O-breathing, animals killed and livers removed at interval up to 24 h. No labeled activity was detectable in liver. We have shown elsewhere that these impurities are excreted into the urine (12). [2-^{14}C]H_4PteGlu, made the day before use, was stored overnight at −30°C under nitrogen and was used without further purification. There was no deterioration of [2-^{14}C]H_4PteGlu kept under these conditions for at least 9 d after preparation.

Animals. Male, 4–5-wk Sprague–Dawley rats, 80–120 g, were used in all experiments. For each compound to be tested three animals were placed for 18 h in a perspex chamber in which CO_2 and excess water vapour were removed, and through which was passed N_2O/O_2 (vol/vol 1:1). They were then injected intraperitoneally with [2-^{14}C]H_4PteGlu plus the test compound and returned immediately to the chamber. Control animals receiving [2-^{14}C]H_4PteGlu alone were treated identically. A further group of control rats were left in air but received the same compounds. All animals were allowed water and standard commercial rat diet ad lib. 24 h following injection the animals were killed by exsanguination following intraperitoneal nembutal and the livers removed rapidly. A 24-h time period was chosen because folate polyglutamate reaches maximal levels at this time (51).

Preparation of doses. Unless stated otherwise the administered injections comprised 16 μmol of the compound under study, together with 1 μCi (0.02 μmol) of [2-^{14}C]DL,H_4PteGlu in a total volume of 0.5 ml. Larger doses of L-methionine were administered in a 1.0 ml volume. 5'-MTA was dissolved by heating in a water bath at 75°C for 15 min and syringes containing the measured dose were left at 37°C immediately before injection. Adenosine was warmed gently before administration, as was L-methionine at concentrations of 125 and 250 μmol.

Extraction of folates, column chromatography, and identification. Immediately on removal livers were weighed, diced with a sharp knife, and placed in 5 vol of 1% potassium ascorbate pH 7.0 in a boiling water bath for 5 min, to destroy conjugase enzyme activities. Thereafter the suspension was placed in an ice bath and homogenized. 0.2 ml of this homogenate was solubilized in NCS (Amersham Corp., Arlington Heights, IL) scintillation fluid (toluene 1 liter, PPO 6 g, POPOP 0.075 g) added and counted for radioactivity in an LKB-Wallace liquid scintillation counter (LKB Producter, Bromma, Sweden) with external standardization to determine total hepatic uptake of labeled folate. Homogenate equivalent to 2 g liver (10 ml) was diluted with an equal volume of 0.1 M potassium phosphate buffer pH 5.7 containing 0.1% ascorbic acid, autoclaved for 10 min at 10 psi and centrifuged. The clear supernatant was applied to a 40 × 0.9-cm column of DE52 cellulose (Whatman Ltd., Maidstone, Kent, U.K.). Columns were run at 4°C, and folates were eluted using a nonlinear gradient of potassium phosphate buffer pH 6.0 containing 0.2 M 2-mercaptoethanol. The gradient was made using 150 ml of 0.1 M phosphate in the mixing chamber and 500 ml 1.0 M phosphate in the reservoir. 5-ml fractions were collected and 1 ml of fractions 20–65 counted for radioactivity after addition of 5 ml of a micellar scintillation fluid (NE260, Nuclear Enterprises Lighthill Edinburgh, Scotland). The proportion of the radioactive dose converted into folate polyglutamate was expressed as a percentage of the total radioactive folate eluted from the column, using the formula dpm in polyglutamate fractions/dpm in fractions 20–65 × 100. Radioactivity in the folate fractions is shown in the chromatograms as a percentage of the intraperitoneal dose incorporated into a gram of liver i.e., (dpm/fraction ÷ 2)/(dpm/g liver) × 100.

Folates in the eluant fractions were identified by their position of elution from the column and by cochromatography with known folate compounds. The monoglutamate standards used were [G-^{3}H]5-CHO-H_4PteGlu, prepared by the Radiochemical Centre, Amersham, from 5-CHO-H_4PteGlu (Lederle Laboratories, Pearl River, NY) by exchange with tritiated water in the presence of a platinum catalyst and purified as described by Nixon and Bertino (24), and 5[^{14}C]CH_3-H_4PteGlu (Amersham Corp.). The elution position of polyglutamyl folates of chain lengths 4–6 were determined by comparison with those obtained from columns previously standardized with marker compounds of 5CH_3 [^{3}H]H_4PteGlu$_{4-6}$ made by incubation of *Lactobacillus casei* in a medium containing [^{3}H]PteGlu (25). Column eluates were also assayed microbiologically with *L. casei*

[1] *Abbreviations used in this paper:* MTA, methylthioadenosine; SAM, *S*-adenosylmethionine.

(ATCC 7469) and *Pediococcus cerevisiae* (ATCC 8081). Polyglutamyl folates were made available to the assay organisms by deconjugation to monoglutamyl forms following incubation of 0.1 ml of each fraction with 0.05 ml of human plasma (which contains conjugase enzyme activity) in 1 ml 0.1% ascorbate pH 4.5.

RESULTS

Hepatic content of [^{14}C]H_4PteGlu. The amount of labeled folate in the liver 24 h after 0.02 μmol parenteral H_4PteGlu averaged 9.6±0.4% (mean ± SEM) in 15 air-breathing rats and was 2.0±0.01% in 32 rats inhaling N_2O. This difference was highly significant (P = <0.001). There was an improvement in hepatic uptake of H_4PteGlu with increasing doses of intraperitoneal methionine (Fig. 1) but even with 250 μmol the uptake was 5.7% of the dose of H_4PteGlu, which was still well below that in air-breathing controls.

The effect of an injection of 16 μmol of various additives on the hepatic content of labeled folate of rats inhaling N_2O expressed as a percentage of the injected dose of [^{14}C]H_4PteGlu is as follows: serine, mean±SEM, 4.4±0.4; betaine 3.9±0.5; sarcosine 2.1±0.2; choline 1.4±0.1; homocysteine 1.7±0.3; glycine 3.7±1.5; adenosine 1.1±0.1; SAM 2.6±0.1 and MTA 3.0±0.6. There were three rats in each group other than MTA where eight animals were studied. There were no significant differences.

Hepatic folate polyglutamate synthesis from [^{14}C]H_4PteGlu. An average of 57.5±1.4% of H_4PteGlu in liver was converted into polyglutamate in 15 control animals (Table I). By contrast, there was no detectable folate polyglutamate formation from H_4PteGlu in 18 out of 32 rats given N_2O (Fig. 2) and the mean polyglutamate synthesis in the whole group was 5.3±1.3%. The difference was highly significant (P = <0.001).

Effect of methionine on folate polyglutamate synthesis. The impairment in the synthesis of folate polyglutamate from H_4PteGlu in N_2O-treated rats was corrected by the administration of methionine (Fig. 3). Folate polyglutamates elute in the fractions collected after tube 40. 'Titration' of the dose of methionine required to produce maximum folate polyglutamate synthesis (Fig. 4) indicated that this was of the order of 16 μmol. There was no further increase in polyglutamates formed even with amounts of 250 μmol methionine. By contrast, this large dose of methionine did not fully correct the impaired hepatic uptake of folate (Fig. 1). On the basis of the studies set out in Fig. 4, 16 μmol was selected as the dose level at which the effect of compounds in restoring folate polyglutamate synthesis in N_2O-treated animals was tested. At a dose of 16 μmol methionine 48.0±3.1% of labeled hepatic folate was in the polyglutamate form in the N_2O-treated rat (Table I). This was not significantly different from air breathing control rats but significantly different from N_2O-treated animals not given methionine (P = <0.001).

Effect of SAM. When H_4PteGlu was given to N_2O-treated rats with 16 μmol SAM the mean amount of folate polyglutamate formed in three animals was 31.0±0.7%, significantly <48.0±3.1% formed in the presence of an equimolar dose of methionine

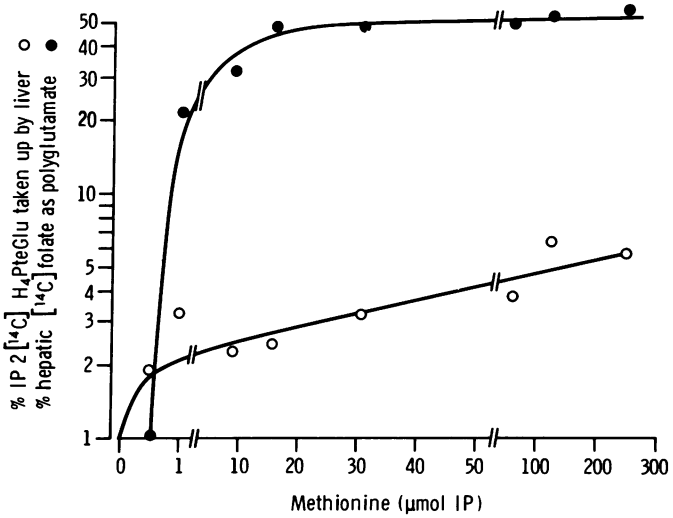

FIGURE 1 [^{14}C]H_4PteGlu (0.02 μmol) was injected intraperitoneally to groups of three rats together with increasing doses of methionine. The animals were maintained under N_2O/O_2 (1:1) and hepatic uptake of labeled folate (○) and the proportion converted into folate polyglutamate (●) determined.

TABLE I

Synthesis of Folate Polyglutamate from [^{14}C]H_4PteGlu (0.02 μmol). Effect of N_2O and Potential "Single Carbon" Donors (16 μmol)

Additive	Hepatic folate polyglutamate (percentage of labeled hepatic folate)	
	Air	N_2O
Nil	57.5±1.4 (15)	—
Nil	—	5.3±1.3 (32)
Methionine	70, 67	48.0±3.1 (3)
SAM	64, 62	31.0±0.7 (3)
Serine	ND	0.0 (3)
Betaine	66, 70	9.0±2.2 (3)
Sarcosine	57, 68	7.0±2.1 (3)
Choline	65, ND	15.0±1.4 (3)
Homocysteine	60	0.0 (3)
Glycine	61	0.0 (3)
Adenosine	65	0.0 (3)
5'MTA	68, 51	68.8±3.7 (8)

Data represent mean±SEM (number of rats). ND, not determined.

(P = <0.002). Increasing the dose of SAM to 32 μmol increased the amount of folate polyglutamate formed to the same level obtained with 16 μmol methionine. (Fig. 4).

Effect of other compounds. Serine, glycine, homocysteine, and adenosine had no effect on folate polyglutamate synthesis in the N_2O-treated rat. Choline and betaine produced 15 and 9% conversion of hepatic labeled folate into the polyglutamate form. Sarcosine, which yields formate directly by oxidation of its methyl group (26), yielded 7.0% folate polyglutamate formation.

Effect of 5'MTA. MTA was the most effective compound tested in overcoming the effect of N_2O in blocking folate polyglutamate synthesis (Fig. 5). The mean conversion in eight animals was 68.8±3.7% of the labeled folate in liver (Fig. 2, Table I). This value was significantly greater than the amount of folate polyglutamate formed in the presence of methionine or SAM (P = <0.001).

Effect of addition in control animals. Each addition was also given to 1–2 air breathing animals. There was no effect on folate polyglutamate formation, the result being the same as that obtained when H_4PteGlu was given alone.

DISCUSSION

Pteroylpolyglutamates have been the subject of two recent reviews (27, 28) but neither discuss the relation to cobalamin. In untreated pernicious anemia the fall in erythrocyte folate is due almost entirely to a fall in

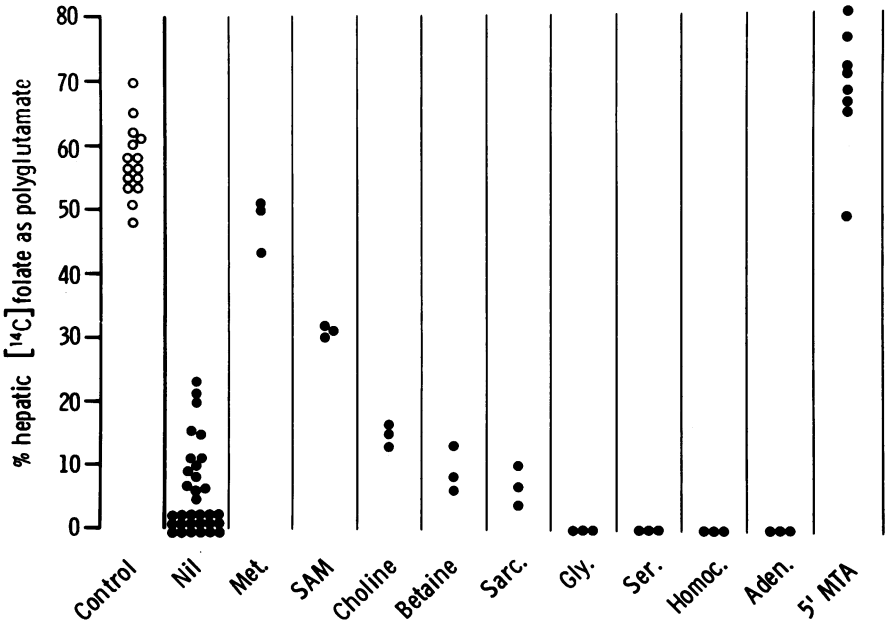

FIGURE 2 The percentage of [^{14}C]H_4PteGlu in liver converted into folate polyglutamate in N_2O-treated rats after an 0.02 μmol dose accompanied by 16 μmol of various compounds (●). Data in air-breathing rats (O) and in rats given only H_4PteGlu (second column) are also shown.

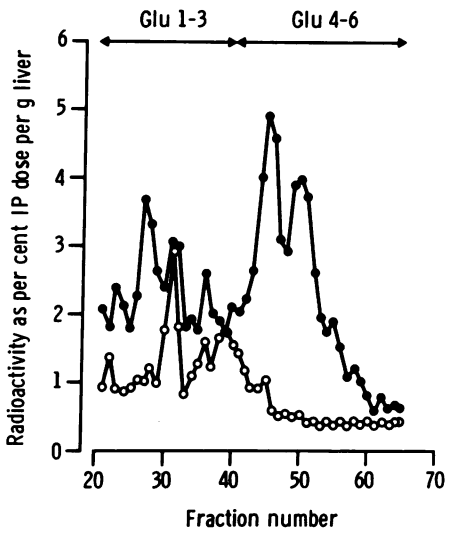

FIGURE 3 [^{14}C]H_4PteGlu (0.02 μmol) was given to two rats inhaling N_2O/O_2 (1:1) alone (O) and with 16 μmol methionine (●). The livers were removed and labeled folate analogues separated by chromatography on DE52 cellulose. The radioactivity in the eluate fractions are shown. The liver from the rat given H_4PteGlu alone failed to form any analogues containing longer glutamic acid chain folates (Glu 4–6) whereas the animal receiving methionine in addition formed folate polyglutamates in normal amount.

the predominant folate polyglutamate component (18, 19).

Impairment of folate polyglutamate synthesis was demonstrated in N_2O-treated mice (15) using PteGlu as substrate and in rats using H_4PteGlu and 5-CH_3-H_4PteGlu as substrates (22). As a normal proportion of hepatic folate is converted into polyglutamate in the N_2O-treated rat when CHO-H_4PteGlu is given, it appears that there is no impairment in the enzyme pteroylpolyglutamatic acid synthetase (22) but rather a failure to provide the appropriate folate substrate. The N_2O data suggest that formyltetrahydrofolates are the required substrates. Other studies of substrate requirements for folate polyglutamate synthesis in normal rat liver from air-breathing animals noted that H_4PteGlu was twice as active as 10-CHO-H_4PteGlu (29).

Further support for the suggestion that formylation of H_4PteGlu was impaired in the N_2O-treated rat arose from the increase in the activity of the enzyme formyltetrahydrofolate synthetase, which we interpreted as a response to lack of 'active formate'. Since the primary effect of N_2O is inactivation of methionine synthesis we looked for evidence for methionine being the source of active formate.

The observations reported in this paper suggest that the pathway from methionine to formate may be via SAM, decarboxylated SAM, 5'MTA, 5'methylthioribose to formate (Fig. 6). The last step in this cycle, namely metabolism of 1-phospho-5-methylthioribose to formate was demonstrated by Trackman and Abeles (30) in rat liver. 5'MTA was the most active of all the compounds we tested in overcoming the N_2O-induced impairment of folate polyglutamate synthesis. The lower activity of SAM as compared with methionine in restoring polyglutamate synthesis is probably related to the much lower hepatic uptake of SAM as opposed to methionine (31).

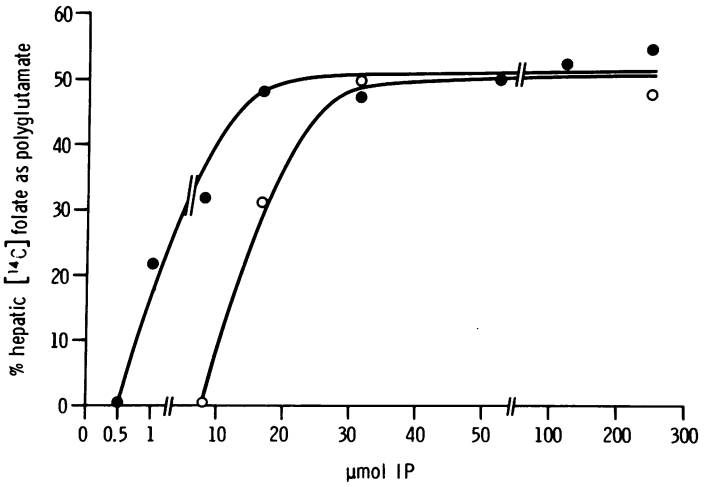

FIGURE 4 [^{14}C]H_4PteGlu (0.02 μmol) was given to rats inhaling N_2O/O_2 (1:1) with increasing doses of L-methionine (●) or SAM (O). The amount of folate polyglutamate formed from the labeled H_4PteGlu in liver was measured and expressed as a percentage of total uptake of substrate. Maximum values were reached with ~16 μmol methionine and 30 μmol SAM.

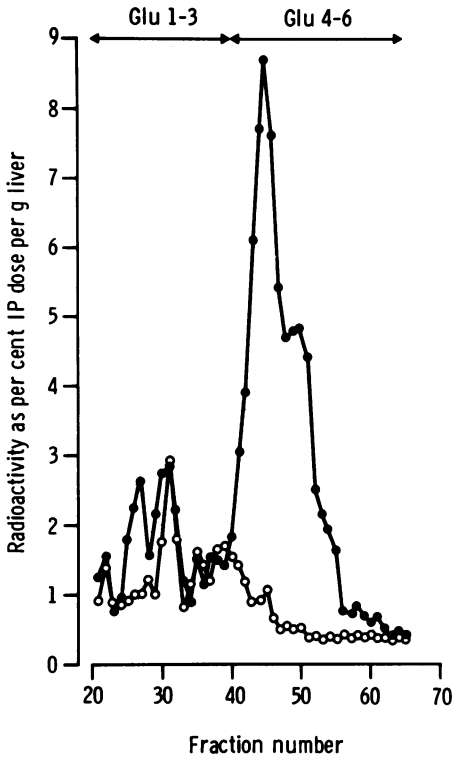

FIGURE 5 [^{14}C]H_4PteGlu (0.02 μmol) was given to two rats inhaling N_2O/O_2 (1:1) alone (O) and with 16 μmol 5'MTA (●). The livers were removed and labeled folate analogues separated by chromatography on DE52 cellulose. The radioactivity in the eluate fractions is shown. The liver from the rat given H_4PteGlu alone failed to form any analogues containing longer glutamic acid chain folates (Glu 4–6) whereas the rat given MTA converted 70% of substrate into folate polyglutamate.

Failure to form MTA results in a failure of cell division (32). The pathways shown in Fig. 6 leading to MTA and polyamine production are greatly accelerated by increased cell division (33). Limiting the amount of methionine or homocysteine causes a twofold increase in SAM decarboxylase activity (34). Thus, these pathways seem to be essential to cell division and the reason may be the requirement for formate, which is an important single carbon unit donor.

Further evidence for a role of cobalamin in metabolism of MTA was reported by Sugimoto and Fukui (35). In studying the cobalamin requirements of *Ochromonas malhamensis* they found that methionine partially replaced the requirement for vitamin B_{12}, and the latter was replaced completely by a factor present in yeast, identified as 5'MTA.

The second aspect of N_2O toxicity brought out in these studies is impaired hepatic uptake (or retention) of folate analogues. This too is well documented in untreated pernicious anemia (36, 37). It is only poorly corrected by methionine in vivo (21, 38). It has been suggested that the reduced amount of labeled folate in liver of N_2O-treated animals is due to increased loss rather than impaired uptake (39). The amount of labeled folate in the liver of N_2O-treated animals is 20% of that in control animals at 24 h, whereas the fall in total folate was from a mean of 5.6 to 4.2 μg/g over the same period (10). An alternative hypothesis is that cobalamin is required for normal folate uptake by an action on the cell membrane and that this is impaired in the N_2O-treated rat.

The failure of the cobalamin-deficient or the N_2O-treated animal to utilize either PteGlu or H_4PteGlu normally (15, 20, 22, 40, 41) and the impaired utili-

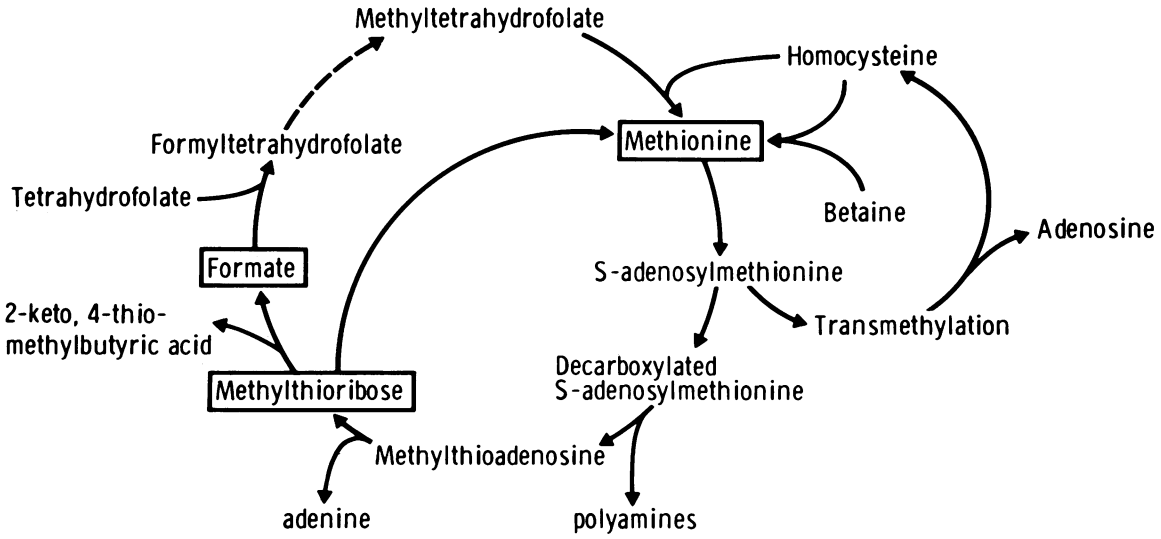

FIGURE 6 Proposed pathways for formate formation from SAM.

zation of H_4PteGlu in untreated pernicious anemia (42, 43, 44) are not readily explained on the basis of the methylfolate trap hypothesis (45, 46), which requires that forms of folate other than 5-CH_3-H_4PteGlu be used in a normal manner in cobalamin deficiency. We have not been able to demonstrate significant accumulation of folates in the methyl form in the N_2O-treated animal (10, 11), that is, we have not been able to demonstrate methyl folate trapping.

A third possibility is that MTA is influencing folate function by its resynthesis into methionine, a pathway demonstrated in rat liver by Backlund and Smith (42). However, if MTA acts merely through its resynthesis to methionine it would be difficult to explain why it is significantly more effective than methionine in overcoming impaired folate polyglutamate synthesis, particularly as both MTA and methionine are taken up by liver at similar rates (31).

Methionine may act, too, either through SAM via its role in transmethylation, or as a source of formate through oxidation of the methyl group. Impaired transmethylation via SAM has been proposed as an explanation for the N_2O effects (48) and interference with transmethylation by cycloleucine produces changes in the central nervous system not dissimilar to those occurring in cobalamin neuropathy. The biochemical lesion has been postulated as impaired methylation of arginine in myelin basic protein (49). This has not been demonstrated, however, in the neuropathy occurring in some species on prolonged exposure to N_2O.

Choline, betaine, and sarcosine all show some activity in overcoming the defect studied in the N_2O-treated rat. No doubt this is due to their potential as single-carbon unit donors and their role in methyl group transfer. It is notable that serine and glycine, which are purported to be the principal source of single carbon units, are totally ineffective in this system, although the pathways by which they donate a carbon unit to the folate pool remains intact in the N_2O-treated rat. The carbon unit from serine is a methylene group whereas, we suggest, the more oxidized formate is required. The fall in the methenyltetrahydrofolate cyclohydrolase activity in response to N_2O may hamper oxidation of methylene units to formate.

A criticism that has been levelled at studies dependent on the use of H_4PteGlu is that this compound is unstable and hence degraded. H_4PteGlu is stable if stringent reducing conditions are maintained and this has been the practice in our studies. Its integrity has been confirmed by the absorption spectrum and by its availability for *P. cerevisiae* on microbiological assay.

A further difficulty with our hypothesis is that sodium formate given as a single 250-μmol injection did not correct the impaired folate polyglutamate synthesis in the N_2O-treated rat (unpublished observation). We continue to explore this. It may be that, like formaldehyde (50), "active-formate" is required under the particular circumstances present in the N_2O-treated rat.

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