Turnover of the Methyl Moiety of 5-Methyltetrahydropteroylglutamic Acid in the Cobalamin-Inactivated Rat

By M. Lumb, I. Chanarin, Janet Perry, and Rosemary Deacon

The metabolism of the methyl group of 5-methyltetrahydrofolate was studied in rats in which cobalamin had been inactivated by exposure to nitrous oxide and in air-breathing control animals. Methylfolate labeled with $^{[3]H}$ in the methyl group and with $[^{14}C]$ in the pteridine-PABA portion of the folate molecule was injected and the disappearance of $[^{14}C]$H$_2$ relative to $[^{3}H]$folate was measured in liver. The half-time of the methyl group was two hours. There was no turnover of the methyl group in the livers of control rats during absorption from the small gut, and 5-methyltetrahydrofolate (5-CH$_3$H$_4$PteGlu [methylfolate]) and transported in this form to tissues.$^1$ Within tissue cells, the methyl group is detached by linking it to homocysteine to form methionine. The enzyme transferring the methyl group of methylfolate, methionine synthetase, is present in most tissues including liver, marrow, small gut, testis, and brain, and the reaction requires both folate and cobalamin coenzymes.

It is widely agreed that the activity of methionine synthetase is depressed in cobalamin deficiency states such as pernicious anemia.$^2,3$ In the experimental situation, such as the inactivation by oxidation of reduced cobalamin by the anesthetic gas nitrous oxide (N$_2$O),$^4,6$ there is similarly a rapid loss of methionine synthetase activity.$^7$ The fate of the methylfolate moiety in cobalamin deficiency or in cobalamin inactivation is not known.

In untreated pernicious anemia, there is an increase in the concentration of serum folate$^8$ (largely methylfolate) but a decrease in the concentration of red cell methylfolate polyglutamates.$^9,10$ In the N$_2$O-exposed rat, there is a rise in the liver concentration of methylfolate polyglutamate within the first 24 hours of N$_2$O inhalation, followed by a marked loss, so that 80% of liver folates, including methylfolates, have disappeared after ten days.$^11$ This process is accompanied by a rise in plasma folate levels$^9$ and a marked loss of methylfolate into urine.$^12$ The rise in the plasma methylfolate levels appears to be due to impaired tissue uptake of methylfolate.$^13$ While the marked loss of folates into urine accounts for the development of folate deficiency, it does not tell us whether any metabolism of the methylfolate moiety occurs. Is it trapped in that form, as suggested in the methylfolate trap hypothesis?$^14,15$ Can the methyl group be oxidized back to methylene and formate?

The purpose of this study was to explore problems by, firstly, determining the relative amounts of folate carrying a formate substituent, a methyl substituent, or no substituent and, secondly, determining the turnover of the methyl moiety of methyltetrahydrofolate using a $[^{14}C]$ label in the methyl group and a $[^{3}H]$ label in the pteroylglutamate portion of the folate molecule. These observations were made in both control and cobalamin-inactivated rats.

NATURAL FOLATES, during absorption from the small gut, are converted into 5-methyltetrahydrofolate (5-CH$_3$H$_4$PteGlu [methylfolate]) and transported in this form to tissues.$^1$ Within tissue cells, the methyl group is detached by linking it to homocysteine to form methionine. The enzyme transferring the methyl group of methylfolate, methionine synthetase, is present in most tissues including liver, marrow, small gut, testis, and brain, and the reaction requires both folate and cobalamin coenzymes.

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MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats 4 to 5 weeks old and weighing between 80 and 120 g were used. Those exposed to N$_2$O were placed in cages in a specifically constructed perspex (ICI, Welwyn Garden City, Herts, UK) chamber, through which a mixture of N$_2$O/O$_2$ (1:1, vol/vol) was passed. Water vapor and CO$_2$ were removed. Control animals were left in air.

Tissue preparation for determination of folate analogues. Animals were anesthetized by an injection of sodium pentobarbitone and killed by exsanguination by heart puncture. Liver was removed and processed immediately. Ascorbic acid, 1% in water at pH 6, was heated to 95 °C, and 1 g of liver was added to 20 vol of hot ascorbate, left for five minutes, cooled, homogenized, and stored at −20 °C.$^16$ Preliminary studies showed that methods that were accompanied by any delay in heating the liver gave significantly higher formyltetrahydrofolate levels at the expense of tetrahydrofolate. Delay in processing liver may account for the published variation of formylfolate levels in normal rat liver from 10% to over 50% of total folate.$^18$−$20$

Samples, other than those required for folate polyglutamate analysis, were incubated with folate conjugase (γ-glutamylhydrolase) prepared from pig kidney$^{21}$ by adding 2 mL (0.1 g) of liver homogenate to 1.5 mL of conjugase preparation at pH 4.5. This was left at 37 °C for five hours and then the sample was diluted to 10 mL with 0.1 mol/L of sodium phosphate buffer, pH 5.7. This was centrifuged at 40,000 g for ten minutes and supernatant was retained for chromatography.

Tissue preparation for CH$_3$H$_4$PteGlu turnover studies. Livers were processed as described above. Forty milliliters of homogenate (2 g) were adjusted to pH 4.5 and incubated for five hours at 37 °C with 6 mL of conjugase enzyme preparation. The pH returned to 6.0 and the sample was centrifuged at 3,000 g for 30 minutes. The supernatant was removed and concentrated to 10 mL under reduced pressure at 40 °C.

Column chromatography and identification of folates. Folate monoglutamates were separated on an 0.9 × 50-cm diethylaminoethyl (DEAE) cellulose column (Whatman DE52, Waidstone, Kent, England).

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UK) using a nonlinear salt gradient, pH 6.0, for elution. The mixing chamber contained 150 mL of 0.1 mol/L potassium phosphate buffer, pH 6.0, and 0.2 mol/L 2-mercaptoethanol. The reservoir contained 1 mol/L potassium phosphate buffer, pH 6.0, and 0.2 mol/L 2-mercaptoethanol. Five-milliliter fractions were eluted. 

H₄PteGlu, 10-CHO-H₄PteGlu, 5-CHO-H₄PteGlu, and 5-CH₃-H₄PteGlu all eluted before fraction 50. Peaks were identified initially by microbiological assay with Lactobacillus casei (American Type Culture Collection [ATCC] [Torrey Research Station, Aberdeen, Scotland]) 7469 and Pediococcus cerevisiae (ATCC 8081) and by co-chromatography with labeled marker compounds. As 5-CHO-H₄PteGlu and 5-CH₃-H₄PteGlu elute very close together in this system, these fractions were further separated either by microbiological assay—both compounds supporting the growth of L. casei but only 5-CHO-H₄PteGlu supporting the growth of P. cerevisiae—or by chromatography on QAE-A25 Sephadex (Pharmacia, Milton Keynes, Bucks, UK) using 0.9 × 50-cm columns. The compounds were eluted using a linear salt gradient consisting of 300 mL of 50 mmol/L Tris-HCl, pH 7.5, 0.25% 2-mercaptoethanol in the mixing chamber, and 300 mL of the same buffer containing 0.6 mol/L NaCl in the reservoir. In our hands, 5-CHO-H₄PteGlu elutes from the column in the ten to 12 5-mL fractions before 5-CH₃-H₄PteGlu. Assay with L. casei was carried out at pH 6.1 since, at this pH, all folate analogues produced a similar growth response.

Isotopes. D,L-5-[14C]-H₃-H₄PteGlu (58 mCi/mmol) was purchased from Amersham International (Aylesbury, Bucks, UK). D,L-5-CHO-[G-3H]-H₄PteGlu (1.250 mCi/mmol) was prepared from [1H]-5-CHO-H₄PteGlu as described elsewhere. The compounds were 95% to 97% pure and were retested for purity before a new experiment. Animals were given an intraperitoneal injection containing 1 μCi of 5-[14C]-H₃-H₄PteGlu and 1 μCi of 5-CHO-[G-3H]-H₄PteGlu in 0.5 mL of normal saline. The ratio of [14C] to [3H] was 1. Because of the lower specific activity of [14C]-labeled folate, the ratio of [14C] to [3H] in injected methyltetrahydrofolate was 1.9. Any transfer of the [14C]-labeled methyl group would reduce the ratio and hence results were expressed in terms of this ratio. In the air-breathing rats, the ratio in liver fell markedly within three hours of administration of the dose and half the methyl group had left the [3H]-labeled H₄PteGlu portion at two hours (Fig 2). It is probable that this was due to transfer of the methyl group to form methionine in the methionine synthetase reaction. The mean ratios in three rats were 0.49 at two hours, 0.37 at four hours, and in the two rats, 0.34 at six hours.

Labeling in the methyl group of 5-CH₃-H₄PteGlu was checked by transferring the methyl group to homocysteine in the methionine synthetase reaction. Each labeled compound was incubated with homocysteine and normal rat liver extract as described elsewhere. Methionine was isolated from the final reaction mixture by high-performance liquid chromatography (HPLC) after volume reduction and counted for radioactivity. Methionine formed in the presence of 5-[14C]-H₃-H₄PteGlu showed the expected high level of [14C] activity. Methionine formed in the presence of 5-CHO-[G-3H]-H₄PteGlu did not show radioactivity above background levels.

Column fractions were counted in 5 mL of NE 260 (Nuclear Enterprises, Sighthill, Edinburgh, Scotland) in an LKB (Coseydon, Surrey, UK) Betarak liquid scintillation counter using a double isotope-counting program. Correction for quenching was made by the channels ratio method using an external standard.

Experimental procedures. Control animals were those breathing a mixture of equal parts of O₂ and N₂O. They received a normal animal diet. Rats were exposed to N₂O for a period that varied from one to 14 days. They were then given CH₃-H₄PteGlu labeled with [3H] and [14C], returned to an O₂/N₂O environment, and killed after a period that varied from two to 30 hours later. Livers were removed and processed as described. Three animals were used in each observation.

RESULTS

The effect of N₂O exposure on distribution of folate analogues. Following exposure to N₂O and inactivation of the cobalamin-requiring methionine synthetase pathway, there was a fall in the level of the three folate analogues measured (Fig 1). There were also significant differences (P < .0001) between the rates of decrease of CH₃-H₄PteGlu and the other two analogues (H₄PteGlu and CHO-H₄PteGlu). There was a lag of 24 hours before any fall in methylfolates appears. The ratio of methyl- to nonmethylfolates was 0.76 in control livers. This became 1.9 on day 3, indicating a relative increase in the proportion of methylfolate, and 3.6 and 3.5 on days 10 and 18. Thus, a greater proportion of folates were in the methyl form following inactivation of cobalamin, but this increase stabilized after the tenth day. H₄PteGlu and CHO-H₄PteGlu did not disappear. To determine whether this folate in the CH₃-H₄PteGlu form is trapped or not, the turnover of the labeled methyl group was studied.

Turnover of the methyl group in [14C]H₃-H₄PteGlu and CH₉-[G-3H]-H₄PteGlu in air-breathing rats. The ratio of [14C] to [3H] in injected methyltetrahydrofolate was 1.0. Any transfer of the [14C]-labeled methyl group would reduce the ratio and hence results were expressed in terms of this ratio. In the air-breathing rats, the ratio in liver fell markedly within three hours of administration of the dose and half the methyl group had left the [3H]-labeled H₄PteGlu portion at two hours (Fig 2). It is probable that this was due to transfer of the methyl group to form methionine in the methionine synthetase reaction. The mean ratios in three rats were 0.49 at two hours, 0.37 at four hours, and in the two rats, 0.34 at six hours.

Turnover of the methyl group in [14C]H₃-H₄PteGlu and CH₉-[G-3H]-H₄PteGlu in N₂O-exposed rats. Rats exposed to N₂O for up to 72 hours did not show any significant change in the [14C]/[3H] ratio during this period (Fig 2); that is, there appeared to be no significant metabolism of the methyl group on 5-CH₃-H₄PteGlu. Presumably, this is because of inactivation of cobalamin which is a cofactor in the methionine synthetase reaction and which is the major pathway for the methyl group transfer.

Fig 1. Rats were exposed to O₂/N₂O (1:1) for 18 days and groups of three were killed at intervals. There was a decrease in liver folate (■) up to ten days, which then stabilized. There was a delay in the fall in 5-CH₃-H₄PteGlu values (○) for 24 hours. The ratio of 5-CH₃-H₄PteGlu to H₄PteGlu (△) and CHO-H₄PteGlu (▲) stabilized at 3.5 after day 10.
Evidence that the methyl group is metabolized in the N$_2$O-treated rat. We explored whether the N$_2$O-treated rat exposed to N$_2$O for >24 hours could make folate polyglutamate using the [¹⁴C]H$_2$H$_4$PteGlu. Labeled CH$_3$-H$_4$PteGlu was given to rats exposed to N$_2$O for four days and to controls, and the liver was processed as described. Incubation with folate conjugase enzyme was omitted.

Thirty-nine percent of the [¹⁴C] originally present as [¹⁴C]H$_2$H$_4$PteGlu was present as polyglutamate in the N$_2$O-treated animals six hours after injection of the [¹⁴C]-labeled folate. The methylfolate peak eluting between 28 and 34 mL (Fig 4) accounted for 42% of the radioactivity. Twenty percent of the [¹⁴C] was not accounted for as folate and presumably represented utilization of the methyl group. The air-breathing controls did not form labeled polyglutamate.

To determine the nature of the labeled folate used for polyglutamate synthesis in the N$_2$O-exposed rats, the eluate fractions containing polyglutamate were pooled, exposed to conjugase enzyme, and chromatographed on QAE-A25 Sephadex. The folate in the eluate fractions eluted in the position of methyltetrahydrofolate (Fig 5). It was microbiologically active for _L casei_ but not for _P cerevisiae_. It was concluded that in the N$_2$O-exposed rat, folate polyglutamate was formed with CH$_3$-H$_4$PteGlu as substrate. The folate polyglutamate peaks corresponded to analogues with 3, 4, and 5 glutamic acid residues, as determined by their elution position (Fig 4).

**DISCUSSION**

Accumulation of CH$_3$-H$_4$PteGlu. There is an accumulation of 5-methyltetrahydrofolatepolyglutamate in the liver of N$_2$O-treated rats within the first 24 hours of exposure. Thereafter, the methylfolate level falls due to excretion of below) to methylene and formyl will still be counted as [¹⁴C]-methyl.

**Fig 2.** The ratio of [¹⁴C] to [³H] in the livers of rats given a 1:1 mixture of 5-[¹⁴C]H$_2$H$_4$PteGlu and 5-CH$_3$-[³H]H$_4$PteGlu intraperitoneally. Control animals breathed air and test animals were exposed to O$_2$/N$_2$O for one and three days. There were three animals at each point; the mean values are given.

**Fig 3.** Rats were exposed to N$_2$O for 72 hours or longer, however, metabolized the methyl group at a measurable rate. Methyl-tetrahydrofolate was lost into the urine at a considerable rate throughout the period of N$_2$O exposure, so that counting rates became very low after 20 hours. The mean [¹⁴C]/[³H] ratios in three rats were 0.87 after four hours, 0.76 at 15 hours, 0.64 at 20 hours, 0.68 at 24 hours, and 0.69 at 30 hours. Using a regression equation, we calculated a 50% level at 43.4 hours. The regression slope of the ratio of [¹⁴C]/[³H] in rats exposed to N$_2$O for three days was significantly different from 0 (t15, 5.14; P < .001).

Rats were also exposed to N$_2$O for up to 14 days. CH$_3$-H$_4$PteGlu labeled with [³H] and [¹⁴C] was given, and animals were killed six hours later. The results (Fig 3) show that after three days’ exposure to N$_2$O, there is no further significant change in the rate at which the methyl group is metabolized.

The disappearance of a [¹⁴C] label from tetrahydrofolate considerably underestimates the rate of “escape” from the methyl folate trap, since [¹⁴C], which has been oxidized (see below) to methylene and formyl will still be counted as [¹⁴C]-methyl.

**Fig 4.** 5-[¹⁴C]H$_2$H$_4$PteGlu was given to rats breathing air (O) or an N$_2$O/O$_2$ (1:1) (©) mixture for four days; the rats were killed after six hours. Liver was extracted and folate analogues were separated on DEAE cellulose as described. 5-CH$_3$-H$_4$PteGlu elutes between fractions 28 to 34 in both groups. The N$_2$O-treated animals have additional labeled peaks which are products of [¹⁴C]H$_2$H$_4$PteGlu eluting after fraction 34, which are folatepolyglutamates of varying chain length. These are absent in the air-breathing controls, in which the [¹⁴C]H$_3$CH$_2$ is passed on intact to homocysteine to form methionine.
CH$_3$H$_4$PteGlu into the urine.$^{15}$ The present study shows that, despite the urinary loss, the proportion of folate held in the CH$_3$ form increases in the N$_2$O-treated rat for the first ten days, but thereafter there is a stable relationship of methyl- and nonmethyl folate. Makar and Tephy$^{27}$ found an increased incorporation of [¹⁴C] formate into 5-CH$_3$H$_4$PteGlu in N$_2$O-breathing rats one hour after the formate dose. At no time did we observe a disappearance of nonmethyl folates and the same was true of the studies in cell culture by Fujii et al.$^{16}$ in which there was an accumulation of methylfolate, but a persistent peak of 10-CHO-H$_4$PteGlu was noted.

**Methyl group turnover.** Methylfolate turnover studies using murine lymphoma cells incubated with CH$_3$H$_4$PteGlu labeled with [³H] and [¹⁴C] were reported by Nixon et al.$^{17}$ They reported that between 81% and 85% of the methyl group was transferred within five minutes of cell uptake.

This work does not appear to have been repeated. These values differ considerably from our findings in the intact animal. We were able to use isotopes of much higher specific activity than were available to Nixon et al, although this is only a partial explanation of the differences. Our data show a slower turnover of the methyl group of 5-CH$_3$H$_4$PteGlu, with a half-time of two hours in control animals.

There was no measurable turnover of the methyl group of CH$_3$H$_4$PteGlu within the first 72 hours of cobalamin inactivation, but thereafter there was a slower turnover, with a calculated half-time of 43 hours. The first step in methylfolate metabolism in the N$_2$O-treated animal is the formation of folate polyglutamate using unaltered CH$_3$H$_4$PteGlu. Methylfolatepolyglutamates containing 3, 4, and 5 glutamic residues are demonstrable. Brody et al$^{11}$ showed that the methyl group is metabolized from six and seven chain folate polyglutamates and we suggest that, when Glu 6 is added, the methyl group is oxidized via methylenehydrofolate reductase to methylene and formyl. However, there is no evidence of induction of this enzyme, there being no change in hepatic methylenetetrahydrofolate reductase activity, even after 20 days of N$_2$O exposure.$^{29}$ Oxidation of the methyl moiety of CH$_3$H$_4$PteGlu has been demonstrated when this compound participates in the methylation of biogenic amines$^{29,30}$ and the enzyme identified as methylene tetrahydrofolate reductase.$^{29}$ Thorndike and Beck$^{31}$ have demonstrated oxidation of the methyl group by normal leukocytes as well as by the leukocytes from a patient with pernicious anemia.

The three-day delay before the N$_2$O-treated rats are able to use methylfolate appears to be due to the time necessary for recovery of methionine levels due to induction of an alternate pathway for methionine synthesis, namely, betaine homocysteine methyltransferase.$^{27}$ It has been known since 1962$^{19}$ that methionine restored formylfolate levels in the livers of cobalamin-deficient rats. In rats exposed to N$_2$O and given methionine, there was an almost total shift of methylfolate into formylfolates and to tetrahydrofolate with 30 minutes.$^{16}$ Thus, the level of methionine appears to be the major factor in determining the rate of methylfolate oxidation in cobalamin inactivation.

**REFERENCES**

METHYLFOLATE TURNOVER

on rat hepatic folate. Implications for the methylfolate-trap hypoth-


25. Makar AB, Tephly TR: Effect of nitrous oxide and methio-


28. Perry J, Deacon R, Lumb M, Chanarin I: The effect of nitrous oxide-induced inactivation of vitamin B\textsubscript{12} on the activity of formyl-methenyl-methylenetetrahydrofolate synthetase, methylene tetrahydrofolate reductase and formiminotetrahydrofolate trans-

29. Pearson AGM, Turner AJ: Folate dependent 1-carbon trans-


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