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

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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 [3H] in the methyl group and with [14C] in the pteridine-PABA portion was injected and the disappearance of [14C]H3 relative to [3H]folate was measured in liver. The half-time of the methyl group in the livers of control rats was two hours. There was no turnover of the methyl group for the first 72 hours after cobalamin inactivation. After 72 hours, there was a slow turnover of the methyl group, with a half-time of 43 hours. In control rats, it is assumed that the methyl group was metabolized by transfer to homocysteine to form methionine. In cobalamin-inactivated rats, it was shown that methylfolate was used as the substrate for forming folate polyglutamate, and analogues with 3, 4, and 5 glutamic acid residues were present. It is likely that oxidation of the methyl group by methylene tetrahydrofolate reductase occurs from folate polyglutamate containing six and seven glutamic acid residues. (Brody et al, Biochemistry 21:276, 1982), since we were unable to demonstrate labeled methyl in longer chain analogues.

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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 NO were placed in cages in a specifically constructed perspex (ICI, Welwyn Garden City, Herts, UK) chamber, through which a mixture of NO/O2 (1:1, vol/vol) was passed. Water vapor and CO2 were removed. Control animals were left in air.

Tissue preparation for determination of folate analogues. Animals were anesthetized by an injection of sodium pentobarbital 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. 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.

Samples, other than those required for folate polyglutamate analysis, were incubated with folate conjugase (γ-glutamylhydro-lase) prepared from pig kidney 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 CH2-H,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). Folate polyglutamates were separated on a 1.5 × 50-cm diethylaminoethyl (DEAE) cellulose column (Whatman DE52, Waidstone, Kent, England). From the Section of Haematology, Medical Research Council’s Clinical Research Centre, Harrow, Middlesex, 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. H$_4$PteGlu, 10-CHO-H$_4$PteGlu, 5-CHO-H$_4$PteGlu, and 5-CH$_3$-H$_4$PteGlu all eluted before fraction 50. Peaks were identified initially by microbiological assay with Lactobacillus casei (American Type Culture Collection [ATCC] [Torey Research Station, Aberdeen, Scotland] 7469) and Pedicoccus cerevisiae (ATCC 8081) and by co-chromatography with labeled marker compounds.

As 5-CHO-H$_4$PteGlu and 5-CH$_3$-H$_4$PteGlu 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-H$_4$PteGlu supporting the growth of _P cerevisiae—or by chromatography on QAE-A25 Sephadex (Pharmacia, Milton Keynes, Bucks, 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. In our hands, 5-CHO-H$_4$PteGlu elutes from the column in the ten to 12 mL fractions before 5-CH$_3$-H$_4$PteGlu. Assay with _L casei_ was carried out at pH 6.1 since, at this pH, all folate analogues produced a similar growth response.

Isoptes. D,L,5-[^14]C]H$_3$-H$_4$PteGlu (58 mCi/mmol) was purchased from Amersham International (Aylesbury, Bucks, UK). D,L,5-CH$_3$-[G-[^14]C]H$_4$PteGlu (1,250 mCi/mmol) was prepared from [^1H]-H$_4$PteGlu 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-[^14]C]H$_3$-H$_4$PteGlu and 1 μCi of 5-CH$_3$-[G-[^14]C]H$_4$PteGlu in 0.5 mL of normal saline. The ratio of [^14]C] to [^1H] was 1. Because of the lower specific activity of[^14]C]-labeled folate, about 20% of this compound was required to 1 part of the [^1H]-labeled folate.

Labeling in the methyl group of 5-CH$_3$-H$_4$PteGlu 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-[^14]C]H$_3$-H$_4$PteGlu showed the expected high level of[^14]C] activity. Methionine formed in the presence of 5-CH$_3$-[G-[^14]C]H$_4$PteGlu 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.

**RESULTS**

The effect of N$_2$O exposure on distribution of folate analogues. Following exposure to N$_2$O 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 < 0.0001) between the rates of decrease of CH$_3$-H$_4$PteGlu and the other two analogues (H$_4$PteGlu and CHO-H$_4$PteGlu). 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. H$_4$PteGlu and CHO-H$_4$PteGlu did not disappear. To determine whether this folate in the CH$_3$-H$_4$PteGlu form is trapped or not, the turnover of the labeled methyl group was studied.

Turnover of the methyl group in ^14C]H$_3$-H$_4$PteGlu and CH$_3$-[G-[^14]C]H$_4$PteGlu in air-breathing rats. The ratio of [^14]C] to [^1H] in injected methyltetrahydrofolate was 1.0. Any transfer of the [^14]C]-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 [^1H]-labeled H$_4$PteGlu 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]H$_3$-H$_4$PteGlu and CH$_3$-[G-[^14]C]H$_4$PteGlu in N$_2$O-exposed rats. Rats exposed to N$_2$O for up to 72 hours did not show any significant change in the [^14]C]/[^1H] ratio during this period (Fig 2); that is, there appeared to be no significant metabolism of the methyl group on 5-CH$_3$-H$_4$PteGlu. 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.
Evidence that the methyl group is metabolized in the 

N\textsubscript{2}O-treated rat. We explored whether the N\textsubscript{2}O-treated rat exposed to N\textsubscript{2}O for >24 hours could make folate polyglutamate using the [\(^{14}\text{C}\)]H\textsubscript{3}H\textsubscript{4}PteGlu. Labeled CH\textsubscript{3}-H\textsubscript{4}PteGlu was given to rats exposed to N\textsubscript{2}O for four days to and controls, and the liver was processed as described. Incubation with folate conjugase enzyme was omitted.

Thirty-nine percent of the [\(^{14}\text{C}\)] originally present as [\(^{14}\text{C}\)]H\textsubscript{3}H\textsubscript{4}PteGlu was present as polyglutamate in the N\textsubscript{2}O-treated animals six hours after injection of the [\(^{14}\text{C}\)]-labeled folate. The methylfolate peak eluting between 28 and 34 mL (Fig 4) accounted for 42% of the radioactivity. Twenty percent of the [\(^{14}\text{C}\)] 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\textsubscript{2}O-exposed rat, 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 \textit{L casei} but not for \textit{P cerevisiae}. It was concluded that in the N\textsubscript{2}O-exposed rat, folate polyglutamate was formed with CH\textsubscript{3}-H\textsubscript{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\textsubscript{3}H\textsubscript{4}PteGlu. There is an accumulation of 5-methyltetrahydrofolatepolyglutamate in the liver of N\textsubscript{2}O-treated rats within the first 24 hours of exposure.\textsuperscript{15} Thereafter, the methylfolate level falls due to excretion of
Methyl group turnover. Methyl folate 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.$^{32}$ It has been known since 1962$^{39}$ 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.

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