Oxidation of 5-methyltetrahydrofolate in cobalamin-inactivated rats

Michael LUMB, Rosemary DEACON, Janet PERRY and Israel CHANARIN*

Haematology Section, M.R.C. Clinical Research Centre, Northwick Park Hospital, Harrow, Middx. HA1 3UJ, U.K.

Rats were exposed to nitrous oxide, which inactivates cob(I)alamin (Cbl). As in air-breathing rats methionine administration led to the conversion of hepatic 5-methyltetrahydrofolate (MeH₄folate) in to formyltetrahydrofolate. The recovery of MeH₄folate levels in liver after its oxidation initiated by methionine was noted and the rate compared with that for air-breathing rats. Oxidation of MeH₄folate was less complete and occurred more slowly in Cbl-inactivated rats as compared with controls. However, recovery of MeH₄folate levels was more rapid in Cbl inactivation. S-Adenosylmethionine did not produce a significant change in MeH₄folate levels in Cbl-inactivated rats, whereas it did so in air-breathing animals.

INTRODUCTION

5-Methyltetrahydrofolate (MeH₄folate) is formed by the reduction of a transferable one-carbon unit on tetrahydrofolate (H₄folate) to the methyl form. The methyl group is thereafter taken up by homocysteine to form methionine. Both folate and cobalamin (Cbl) are required for this step.

The final step in MeH₄folate synthesis is reduction of 5,10-methylenetetrahydrofolate (CH₂H₄folate) to MeH₄folate by the enzyme 5,10-methylenetetrahydrofolate reductase. The thermodynamics of the reaction (CH₂H₄PteGlu → MeH₄folate) *in vitro* is strongly to the right; so much so that it is suggested that the reverse reaction, which is oxidation of MeH₄folate, is unlikely to occur [1]. On this basis the methyl group of MeH₄folate can only be disposed of by transfer to homocysteine. In Cbl deficiency or Cbl inactivation there is severe impairment, or even cessation, of methyl transfer to homocysteine. On this basis, no further metabolism of MeH₄folate is possible in Cbl deficiency, and folate is said to be trapped as MeH₄folate [2,3].

This interpretation of data has been questioned. By preparing MeH₄folate labelled with ¹⁴C in the methyl group and ³H in the pteridine moiety, the half-life of the methyl group on MeH₄folate in rat liver has been studied [4]. In the liver of a control group, the half-life of the methyl group on MeH₄folate was 2 h. In rats in which Cbl had been inactivated by exposure to N₂O for 24 h there was no methyl-group turnover. In rats breathing N₂O for 72 h there was significant metabolism of the methyl group. Instead of methyl transfer to homocysteine, in Cbl inactivation MeH₄folate was used as substrate for synthesis of the folate coenzyme, that is, additional glutamic acid residues were added to form MeH₄folate polyglutamates. The methyl group was then oxidized from the hexaglutamate. The oxidation of MeH₄folate in Cbl-inactivated rats was confirmed by giving [Me-¹⁴C]MeH₄folate, when increased amounts of ¹⁴CO₂ were exhaled [5].

A further way to explore methyl-group oxidation of

MeH₄folate is by administration of methionine. Noronha & Silverman [2] showed that, when methionine was added to rat diet, there was a fall in MeH₄folate levels in liver, accompanied by a rise in formylH₄folate and H₄folate. A similar effect is obtained with a single parenteral dose of methionine [5,6]. In control rats there was a loss of over 90% of MeH₄folate within 30 min of an injection of methionine. S-Adenosylmethionine (AdoMet) was also effective, though less so than methionine. After a dose of 100 μmol of methionine, MeH₄folate levels started to return to control values after 2.5 h, when the liver methionine levels had already returned to baseline. At this time AdoMet and S-adenosylhomocysteine (AdoHcy) levels (both derived from the dose of methionine) were still raised. However, the ratio had returned to normal [7]. Thus it is uncertain, in the situation *in vivo*, whether the MeH₄folate level is responsive to methionine concentrations, as seems likely, or to AdoMet, as suggested by Kutzbach & Stokstad [8].

The enzyme responsible for the oxidation of MeH₄folate to CH₂H₄folate in the first instance is CH₂H₄folate reductase [9,10]. This enzyme *in vitro* is inhibited by AdoMet, but not by methionine [1,8].

Our previous studies on MeH₄folate oxidation have been on air-breathing rats. Here we report studies in rats in which Cbl has been inactivated by N₂O and the enzyme methionine synthetase thereby inactivated [11].

MATERIALS AND METHODS

Approx. 100 g male Sprague–Dawley rats were used in groups of four at each time period or at each dose of methionine or AdoMet. Control animals were left in air. N₂O-exposed groups were placed in wire cages, which in turn were placed in a Perspex (Lucite) cabinet through which a mixture of N₂O and oxygen (1:1, v/v) (Entonox) was passed. Water vapour and CO₂ were removed as described elsewhere [11].

At the appropriate time, rats were anaesthetized by intraperitoneal injection of Sagatal (0.4 ml) and killed by exsanguination. Livers were removed and rapidly washed

Abbreviations used: Cbl, cob(I)alamin; MeH₄folate, 5-methyltetrahydrofolate; formylH₄folate, formyltetrahydrofolate; AdoMet, S-adenosylmethionine; CH₂H₄folate, 5,10-methylenetetrahydrofolate; AdoHcy, S-adenosylhomocysteine.

* To whom correspondence and reprint requests should be sent.

in 0.15 M-NaCl, and excess liquid blotted with tissue paper. The livers were then weighed, cut into small pieces and placed in 20 vol. (w/v) of 0.06 M-ascorbic acid, pH 7, at 95 °C to inactivate the folate coenzymes. After 5 min, samples were cooled in ice, homogenized and stored at -20 °C.

Folate analogues were assayed by microbiological assay. Folate polyglutamate forms were converted into monoglutamates by incubation with γ -glutamyl hydrolase. γ -Glutamyl hydrolase (0.2 ml) was added to 0.4 ml of liver homogenate and 0.4 ml of 0.06 M-ascorbic acid and incubated for 5 h at 37 °C, diluted to 10 ml with 0.1 M-sodium phosphate, pH 5.7, containing ascorbic acid (1.5 g/l), and autoclaved for 10 min at 116 °C to precipitate protein. The samples were filtered through Whatman no. 1 filter paper and the clear filtrate was assayed.

In some studies, folate analogues were separated by DEAE-cellulose chromatography [12] and the eluate fractions assayed microbiologically with *Lactobacillus casei* and *Pediococcus cerevisiae*. In other studies liver extracts were assayed without prior separation of folate analogue by using *L. casei* and *Streptococcus faecalis*. *L. casei* responds to all hepatic folate analogues, but *S. faecalis* responds to folate analogues other than MeH₄folate, and therefore the difference between the two assays represents the amount of MeH₄folate.

RESULTS

All results are shown as mean values $\pm$ S.D. The data on air-breathing rats have been reported elsewhere [5], but are included in the Figures to contrast with data from N₂O-exposed rats. The levels of folate analogues in liver at the start of the studies are shown in Table 1. Exposure to N₂O causes a marked loss of folate from all tissues, owing to loss of folate into urine [13]. The total folate fell from 14.08 nmol/g of liver in controls to 12.2 nmol/g after 1 day of exposure to N₂O and to 4.7 nmol/g after 4 days of N₂O.

Effect of methionine

Methionine (16–300 μ mol) was given intraperitoneally and rats were killed 60 min later. Fig. 1 shows the effect on MeH₄folate and formylH₄folate. H₄folate levels changed in a similar way to those of formylH₄folate.

A larger dose of methionine was needed in the 'N₂O' group to produce a fall in MeH₄folate concentration equivalent to that found in air-breathing rats. In controls, MeH₄folate fell from 45% of total folates to 3%; in the '1-day N₂O' group the level fell to 12% and in the '4-day N₂O' group to 17%.

Fig. 1. Effect of intraperitoneally administered methionine on control and N₂O-exposed rats

Animals were killed 30 min after the dose of methionine, and liver was removed for assay of folate analogues. These are shown as a percentage of the folate in controls not receiving methionine. Data for H₄folate levels are not shown, but they follow the same pattern as those of formylH₄folate. Data on air-breathing rats are from Lumb *et al.* [5]. ○, Air; ●, N₂O for 1 day; ▲, N₂O for 4 days.

The fall in MeH₄folate was accompanied by corresponding increases in concentration of formylH₄folate (shown) and H₄folate (not shown). These rises lagged behind those noted in controls.

Recovery of MeH₄folate levels

When rats exposed to N₂O for 1 day were given single intraperitoneal injection of 100 μ mol of methionine and killed at 30 min intervals thereafter, both the fall and recovery of MeH₄folate levels in liver were seen (Fig. 2). The rate of recovery appears to be more rapid in Cbl-inactivated rats than in air-breathing rats. The lower MeH₄folate levels after 2 h in controls may be due to transfer of the methyl group to homocysteine, which does not occur in the N₂O-exposed group. Fig. 3, in addition, shows the rise and fall of H₄folate and formylH₄folate, contrasting with the changes in MeH₄folate levels. The changes were similar in rats exposed to N₂O for 1 day (Fig. 3) and for 4 days (results not shown).

Effect of AdoMet

This compound had no significant effect on the concentration of folate analogues in the livers of Cbl-

Table 1. Hepatic folates in air- and N₂O-exposed rats

Results are means $\pm$ S.D. Values significantly different from baseline values are indicated thus: $P < 0.01^*$; $P < 0.05^{**}$.

Time under N ₂ O (days)	No. of animals	[Folate] (nmol/g)			
		Total	MeH ₄ folate	FormylH ₄ folate	H ₄ folate
0	16	14.08 $\pm$ 1.97	6.31 $\pm$ 1.06	3.31 $\pm$ 0.73	4.46 $\pm$ 1.06
1	10	12.2 $\pm$ 2.33**	8.56 $\pm$ 1.76*	1.48 $\pm$ 0.44*	2.16 $\pm$ 0.42*
4	8	4.71 $\pm$ 0.75*	3.11 $\pm$ 0.51*	0.71 $\pm$ 0.22*	0.89 $\pm$ 0.22*

Fig. 2. Effect of intraperitoneal administration of methionine to rats, exposed to N₂O for 24 h and killed between 0.5 and 4 h later

The MeH₄folate concentration in the liver (as a percentage of the control values from rats not given methionine) is shown and compared with those from a similar study in air-breathing rats. Data on air-breathing rats are from Lumb *et al.* [5]. —, N₂O; ----, air.

inactivated rats in a dose varying from 16 to 200 μmol (Fig. 4). The latter was the maximum dose we were able to give.

DISCUSSION

The data extend observations, obtained by the different approaches set out in the Introduction, that MeH₄folate is not trapped in Cbl deficiency but can be oxidized to formate and presumably to CO₂. The daily intake of methionine by rats on a normal laboratory diet in the present study was 400 μmol. Thus the doses of methionine used in the study are within physiological limits.

There appears to be some MeH₄folate in liver (3% in controls and about 12–17% in Cbl-inactivated rats) that is not shifted by methionine. Perhaps this might represent 'trapped' MeH₄folate, but the difference between the controls and the N₂O-exposed group is one of degree; that is, 'trapping', if present, is not a phenomenon unique to Cbl deficiency or Cbl inactivation.

Recovery of MeH₄folate in controls and N₂O-exposed groups, are similar, and differences may relate to the greater ease with which the methyl group can be metabolized in control animals.

The most interesting data relate to the failure of AdoMet to affect MeH₄folate levels. In control rats, AdoMet is effective, though less so than methionine. In the situation *in vitro*, AdoMet inhibits CH₂H₄folate reductase without oxidizing the methyl group. Methionine, however, was found to be ineffective *in vitro*.

This contrasts with the situation *in vivo*, where methionine remains effective in both control [5] and Cbl-inactivated groups; AdoMet is partially effective in controls [5] and ineffective in Cbl inactivation. The reason for this is uncertain. The evidence favours meth-

Fig. 3. Effect of intraperitoneal administration of methionine to rats, exposed to N₂O for 24 h and killed between 0.5 and 4 h later, on folate-analogue concentrations

The concentrations of folate analogues in the liver are expressed as a percentage of the level in animals not given methionine. —, MeH₄folate; ----, formylH₄folate; ····, H₄folate.

Fig. 4. Effect of AdoMet administration to rats exposed to N₂O for 4 days on liver folate-analogue concentrations

There was no significant change from the levels in a group of rats not given AdoMet. —, MeH₄folate; ----, H₄folate; ····, formylH₄folate.

ionine as the principal regulating agent of MeH₄folate levels, and AdoMet is active only by virtue of its methionine moiety.

REFERENCES

- Green, J. M., Ballou, D. P. & Matthews, R. G. (1988) FASEB J. 2, 42–47

2. Noronha, J. M. & Silverman, M. (1962) Vitamin B₁₂ und Intrinsic Factor, p. 728, Enke, Stuttgart
3. Herbert, V. & Zalusky, R. (1962) *J. Clin. Invest.* **41**, 1263–1276
4. Lumb, M., Chanarin, I., Perry, J. & Deacon, R. (1985) *Blood* **66**, 1171–1175
5. Lumb, M., Chanarin, I., Deacon, R. & Perry, J. (1988) *J. Clin. Pathol.* **41**, 1158–1162
6. Brody, T., Watson, J. E. & Stokstad, E. L. R. (1982) *Biochemistry* **21**, 276–282
7. Lumb, M., Bottiglier, T., Deacon, R., Perry, J. & Chanarin, I. (1989) *Biochem. J.*, in the press
8. Kutzbach, C. & Stokstad, E. L. R. (1971) *Biochim. Biophys. Acta* **250**, 459–477
9. Pearson, A. G. M. & Turner, A. J. (1975) *Nature (London)* **258**, 173–174
10. Taylor, R. T. & Hanna, M. L. (1975) *Life Sci.* **17**, 111–120
11. Deacon, R., Lumb, M., Perry, J., Chanarin, I., Minty, B., Halsey, M. J. & Nunn, J. F. (1978) *Lancet* **ii**, 1023–1024
12. Perry, J., Chanarin, I., Deacon, R. & Lumb, M. (1983) *J. Clin. Invest.* **71**, 1183–1190
13. Lumb, M., Perry, J., Deacon, R. & Chanarin, I. (1982) *Br. J. Haematol.* **51**, 235–242

Received 29 November 1988/9 January 1989; accepted 16 January 1989