Folate polyglutamate synthetase activity in the cobalamin-inactivated rat

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Exposure to N_2O inactivates cob[I]alamin and interferes with the activity of methionine synthetase, of which cob[I]alamin is a coenzyme. Less directly, it stops the formation of folate polyglutamate from tetrahydrofolates. Studies on the activity of folate polyglutamate synthetase in rat liver *in vivo* were carried out. The synthetase activity increased after exposure to N_2O for up to 48 h, but longer exposure was accompanied by a return of activity to baseline values. The rise in synthetase activity was prevented by supplying methionine, 5'-methylthioadenosine or 5-formyltetrahydrofolate. The fall in folate polyglutamate synthetase activity after 48 h was accompanied by a restoration of hepatic synthesis of folate polyglutamate despite continuation of N_2O exposure.

N_2O , in the presence of transition-metal complexes such as cobalamin, is cleaved into N_2 and O_2 (Banks *et al.*, 1968). At the same time cob[I]alamin is oxidized into the inactive cob[III]-alamin form. Exposure of animals to N_2O similarly inactivates cobalamin, and this provides a very satisfactory model for cobalamin 'deficiency'. In man long-term N_2O exposure produces a megaloblastic anaemia (Lassen *et al.*, 1956; Amess *et al.*, 1978; Skacel *et al.*, 1983) as well as a neuropathy indistinguishable from that due to cobalamin deficiency (Layzer, 1978). Amess *et al.* (1978) showed that the effects of N_2O in man were due to interference with cobalamin function.

Exposure to N_2O leads to inactivation of methionine synthetase in the rat (Deacon *et al.*, 1978) followed by induction of betaine:homocysteine transmethylase, an alternative pathway for methionine synthesis (Lumb *et al.*, 1983). After 24 h of N_2O exposure there is virtually no synthesis of folate polyglutamate in liver with $H_4PteGlu$ as substrate. However, folate polyglutamate synthesis was normal when $CHO-H_4PteGlu$ was used as substrate (Perry *et al.*, 1979), and hence it was suggested that, firstly, cobalamin was concerned in the provision of the formyl group required for the formylation of $H_4PteGlu$, and, secondly, that $CHO-H_4PteGlu$ was the normal substrate for poly-

glutamate synthesis. Further, the effect of N_2O on folate polyglutamate synthesis was overcome by an injection of either methionine or products of methionine metabolism such as S-adenosylmethionine or 5'-methylthioadenosine (Perry *et al.*, 1983). The latter compound yields formate (Trackman & Abeles, 1981).

As prolonged exposure to N_2O induces an alternative pathway for the synthesis of methionine, the present study was planned to determine whether this was accompanied by a restoration of folate polyglutamate synthesis, whether there were corresponding changes in the activity of polyglutamate synthetase and, if so, whether these were affected by provision of methionine, 5'-methylthioadenosine and $CHO-H_4PteGlu$.

Materials and methods

Materials

L-Methionine, 5'-methylthioadenosine, DL-5- $CHO-H_4PteGlu$, L-glutamic acid and ATP were obtained from Sigma Chemical Co. Other reagents were AnalaR grade (BDH Chemicals). L-[G- 3H]-Glutamic acid (specific radioactivity approx. 22 Ci/mmol) and [2- ^{14}C]pteroylglutamic acid (specific radioactivity approx. 50 μ Ci/mmol) were obtained from Amersham International. [2- ^{14}C]- $H_4PteGlu$ was prepared from [2- ^{14}C]pteroylglutamic acid by sodium dithionite reduction (Silverman & Noronha, 1961). [G- 3H]5- $CHO-H_4PteGlu$ was obtained by tritium-exchange labelling in aqueous medium of the unlabelled com-

Abbreviations used: $H_4PteGlu$, tetrahydropteroylglutamic acid; 5- $CHO-H_4PteGlu$, 5-formyltetrahydropteroylglutamic acid; 5- $CH_3-H_4PteGlu$, 5-methyltetrahydropteroylglutamic acid.

pound, carried out for us by Amersham International, and purified as previously described (Nixon & Bertino, 1971).

Animals

Male 80–120g Sprague–Dawley rats were used in all experiments. Test animals breathed N_2O/O_2 (1:1) in an environmental chamber in which CO_2 and humidity were controlled, with access to commercial rat diet and water *ad lib*. Control rats breathed air. For experiments on the effect of various periods of N_2O exposure on polyglutamate synthesis in the intact animal, rats were caged in the environmental chamber for up to 6 days. At 24h before the end of the experiment they were injected intraperitoneally with $[2-^{14}C]H_4PteGlu$ ($0.02\mu mol$, $1\mu Ci$) and then killed by exsanguination after intraperitoneal Nembutal. Animals whose livers were used for folate polyglutamate synthetase were treated in a similar manner, but were either injected with a non-radioactive compound 24h before death or killed immediately after the appropriate N_2O -exposure period. The number of animals used in each study is indicated in the Results section.

Folate polyglutamate synthesis *in vivo*

Livers from animals injected with $[2-^{14}C]H_4PteGlu$ were processed as described previously (Perry *et al.*, 1983).

Polyglutamate synthetase assay

Immediately on removal livers were weighed, placed in 2 vol. (w/v) of ice-cold 0.1M-potassium phosphate buffer, pH 7.4, and homogenized in a Sorvall homogenizer. The homogenates were centrifuged for 60 min at $74000g$ at $0^\circ C$, and 1 ml of each supernatant was dialysed overnight in 1 litre of 20mM-potassium phosphate buffer, pH 7.4, containing 20mM-2-mercaptoethanol. The dialysis residues were assayed without further purification. Protein was determined by using a colorimetric method (Bio-Rad Laboratories), with bovine serum albumin as standard.

Folate polyglutamate synthetase assay

Enzyme activity in freshly dialysed liver extracts was assayed by the method of Taylor & Hanna (1977), which measures the incorporation of radio-labelled glutamate into folate polyglutamates with unlabelled folate monoglutamate as substrate. Reaction mixtures (1 ml) contained: Tris/HCl buffer, pH 8.5, $200\mu mol$; KCl, $30\mu mol$; $MgCl_2$, $10\mu mol$; ATP, $5\mu mol$; $H_4PteGlu$, $0.1\mu mol$; 2-mercaptoethanol, $100\mu mol$; L-[G- 3H]glutamic

acid, $1\mu mol$ ($5\mu Ci$); liver extract protein, 1.5mg. Blank reaction mixtures omitted folate. After 2h incubation under N_2 at $37^\circ C$ reactions were stopped by adding 0.2ml of sodium ascorbate ($60mg/ml$) and heating for 10 min at $90^\circ C$. Protein was removed by centrifuging, the precipitate was washed with 1.4 ml of 5mM-potassium phosphate buffer, pH 7.0, and both supernatants were chromatographed on a $1cm \times 5cm$ column of Cellex D (Bio-Rad Laboratories) equilibrated with 5mM-potassium phosphate buffer, pH 7.0, containing 0.14M-2-mercaptoethanol. Assay products were eluted with a linear gradient of 60ml of 5–500mM-potassium phosphate buffer, pH 7.0, containing 0.14M-2-mercaptoethanol. Fractions (1 ml) were collected and their radioactivities counted, after the addition of liquid scintillant NE 260 (Nuclear Enterprises, Lighthill, Edinburgh, U.K.), in an LKB–Wallac liquid scintillation spectrometer with external standardization. This 1 ml assay system, followed by gradient elution of the columns, was used to establish the formation of folate polyglutamate, and also when 5-CHO- $H_4PteGlu$ was used as substrate. L-[G- 3H]Glutamate was eluted at fractions 5–10, the product from $H_4PteGlu$ at fractions 50–54, and that from 5-CHO- $H_4PteGlu$ at fractions 38–42. Comparisons of enzyme activity after N_2O inhalation were done routinely with 0.5 ml assay volumes and 'strip' elution of $0.5cm \times 3cm$ Cellex columns as described by Taylor & Hanna (1977). The formation of a radioactive folate diglutamate from L-[G- 3H]glutamic acid was confirmed by microbiological assay with *Lactobacillus casei* and *Pediococcus cerevisiae* and by co-chromatography with $[2-^{14}C]H_4PteGlu$, which is eluted from $1cm \times 5cm$ Cellex D columns at fractions 33–39.

Expression of results and statistics

Enzyme activity was expressed as pmol of L-[G- 3H]glutamate found in the folate polyglutamate fraction/h per mg of protein. The values were corrected for the activity in the blank sample. Statistical comparison between groups was done by using Student's *t* test.

Quantification of liver folate polyglutamate synthesis *in vivo*

Livers were prepared for column chromatography on DEAE-cellulose (DE-52; Whatman, Maidstone, Kent, U.K.) and for determination of radioactivity by methods previously described (Perry *et al.*, 1983). The proportion of the radioactive dose converted into polyglutamates was expressed as a percentage of the total radioactive folate eluted from the column.

Results

Folate polyglutamate synthesis in vivo on prolonged exposure to N₂O

The effect of 24 h of exposure to N₂O on synthesis of liver polyglutamates from injected [2-¹⁴C]H₄PteGlu in the intact animal has been reported (Perry *et al.*, 1979, 1983), and in 18 of 32 rats no radioactive polyglutamate was detected, with only small amounts in the remainder. In the present study, three or four rats were exposed to 2, 3 or 5 days of N₂O before being given [2-¹⁴C]H₄PteGlu intraperitoneally, and returned to N₂O for a further 24 h. Column chromatography of liver extract showed a pronounced radioactive peak of folate polyglutamate followed by a smaller one. Judged on elution position and differential microbiological assay with *L. casei* and *P. cerevisiae*, the major peak was identified as 5-CH₃-H₄PteGlu₅, the smaller one as H₄PteGlu₅. There was no evidence of an accumulation of short-chain folate. Thus impairment of folate polyglutamate synthesis due to N₂O exposure was restored after 24 h (Fig. 1).

Fig. 1. Hepatic folate polyglutamate synthesis from injected [2-¹⁴C]H₄PteGlu in the intact rat after timed exposure to N₂O/O₂.

For experimental details see the text.

Folate polyglutamate synthetase activity

The formation of labelled folate diglutamate (eluted between 45 and 57 ml) from unlabelled H₄PteGlu and [G-³H]glutamate in the presence of dialysed liver extract is shown in Fig. 2(a). Fig. 2(b) shows a similar experiment with 5-CHO-H₄PteGlu as substrate. There was no difference in assay results with liver dialysis residues from both control and N₂O-treated rats when either H₄PteGlu or 5-CHO-H₄PteGlu was used as substrate.

Folate polyglutamate synthetase activity in 12 air-breathing rats was 175.5 ± 23.1 pmol/h per mg of protein (mean $\pm$ S.E.M.) (Fig. 3). After 24 h of N₂O/O₂ administration to ten rats the value had risen to 258.8 ± 40.9 pmol/h per mg, and the value after 48 h in six rats was 275 ± 71.0 pmol/h per mg. Both these values were significantly higher ($0.01 < P < 0.025$) than in control animals. Further exposure resulted in a decrease in activity, the values being at 3 days 197.8 ± 58.9 , at 4 days 190.3 ± 78.9 , at 7 days 131.9 ± 9.2 , at 14 days 103 ± 6.5 and 18 days 156.8 ± 6.7 pmol/h per mg. None of these activities were significantly different from the control value.

Injection of methionine (16 μ mol) into eight rats followed by N₂O/O₂ inhalation for 24 h gave synthetase assay values of 211 ± 36.9 pmol/h per mg, and 5'-methylthioadenosine (16 μ mol) into five animals 147.2 ± 19.4 pmol/h per mg. Although methionine and methylthioadenosine prevented the rise in polyglutamate synthetase activity due to exposure to N₂O, the values did not reach statistical significance.

The effect of 5-CHO-H₄PteGlu (8 μ mol) intraperitoneally on synthetase activity was also studied in four air-breathing controls and five N₂O/O₂-breathing animals. Livers were assayed 24 h after the dosage (for synthetase activity) with 0.5-ml assay systems with H₄PteGlu as folate substrate. Mean control values were 133 ± 25.8 pmol/h per mg, and after N₂O/O₂ for 24 h 152.2 ± 20.1 pmol/h per mg. Although the rise in ligase activity was not detectable after formyl-H₄PteGlu, the values were not different statistically from those without intraperitoneal 5-CHO-H₄PteGlu. There was no difference between air-breathing and N₂O/O₂-breathing rats.

Discussion

Our previous studies on the effect of N₂O on folate polyglutamate synthesis had been concerned with events in the first 24 h after exposure to N₂O and the mechanism whereby these changes occurred. It was concluded that methionine, via S-adenosylmethionine, 5'-methylthioadenosine and

Fig. 2. Cellex D chromatography of the products of 1 ml folate polyglutamate synthetase assays in air-breathing (○) and N_2O/O_2 -breathing (●) rats (a) with $H_4PteGlu$ as substrate and (b) with 5-CHO- $H_4PteGlu$ as substrate. For experimental details see the text.

Fig. 3. Folate polyglutamate synthetase activity after various periods of N_2O/O_2 exposure.

The effect of injected methionine, 5'-methylthioadenosine and 5-CHO- $H_4PteGlu$ on enzyme activity after 24 h of N_2O/O_2 exposure is also shown.

5'-methylthioribose, provided a C_1 unit at the formate level of oxidation, which was required to form formyl- $H_4PteGlu$ (Perry *et al.*, 1983). Since the latter compound appears to be the substrate to which further glutamic acid residues are added to form the active coenzyme (folate polyglutamate) *in vivo*, failure to formylate $H_4PteGlu$ leads to impaired folate polyglutamate formation.

This study extends these observations by showing that the events of the first 24 h after cobalamin inactivation are transient, being followed by recovery of folate polyglutamate synthesis. The initial impairment of folate polyglutamate synthesis is accompanied by a rise in activity of the enzyme folate polyglutamate synthetase, and this activity returns to baseline after 48 h despite continued exposure to N_2O and despite continued depression of the enzyme methionine synthetase (Chanarin, 1980). The likely explanation is a restoration of methionine synthesis, with betaine serving as the methyl donor for methylation of homocysteine (Lumb *et al.*, 1983). The rise in polyglutamate synthetase activity is prevented by methionine and 5'-methylthioadenosine.

Though these data apply to the rat, it remains to be shown that they are equally applicable to man. Man alone develops megaloblastic anaemia in response to cobalamin deficiency. Further, patients with megaloblastic anaemia consistently show diminished erythrocyte folate polyglutamate activities (Chanarin, 1979). Thus it is possible that man may not be able to compensate for the loss of methionine synthetase activity by induction of betaine transmethylase, as does the rat. If this were so, it would explain man's greater susceptibility to the effects of cobalamin deficiency. Unfortunately, betaine transmethylase is present only in liver, and attempts to demonstrate its presence in human fibroblast culture with or without exposure to N_2O have been uniformly negative (R. Deacon, unpublished work).

The N_2O -treated rat develops folate deficiency (Lumb *et al.*, 1980) as a result of massive loss of 5-Me- H_4 PteGlu into the urine secondary to an impairment of tissue uptake of this folate analogue (Lumb *et al.*, 1982). The low erythrocyte folate concentration in man with cobalamin deficiency may also be secondary to increased urinary folate loss over a prolonged period and not necessarily to a failure to induce a betaine transmethylase.

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