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Effect of cobalamin inactivation on folate-dependent transformylases involved in purine synthesis in rats

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N_2O oxidizes and inactivates cob[I]alamin, and animals exposed in this way serve as models for cobalamin 'deficiency'. Such animals show a fall in activity of glycinamide ribotide transformylase and a rise in that of 5-amino-4-imidazolecarboxamide ribotide transformylase. The fall in glycinamide ribotide transformylase activity was prevented by parenteral 5'-methylthioadenosine derived from methionine. Methylthioadenosine in turn is converted into formate. Activity of glycinamide ribotide transformylase recovers after 7 days despite continued N_2O inhalation, and this is probably related to restoration of methionine synthesis by induction of betaine:homocysteine transmethylase.

10-Formyltetrahydrofolate is the formyl donor for C-2 and C-8 in the synthesis of the purine nucleus. The enzymes are GAR transformylase (EC 2.1.2.2), which links formate to GAR, giving C-8, and AICAR transformylase (EC 2.1.2.3), which completes the closure of the nucleus by providing C-2.

There have been few studies of purine biochemistry in cobalamin or folate deficiency. Utilization of [^{14}C]formate for purine synthesis is depressed by folate antagonists (Skipper *et al.*, 1950). There was no difference in the amount of [^{14}C]uric acid excreted in urine within 48 h of a dose of [^{14}C]formate in controls and in patients with megaloblastic anaemia due to folate deficiency. However, the folate-deficient group showed a late peak, not present in controls, of urinary excretion of the label 7–8 days after the oral dose.

Aminoimidazolecarboxamide, which is normally converted into inosinic acid by AICAR transformylase, may be excreted in the urine of patients with both cobalamin and folate deficiency (Luhby & Cooperman, 1962; Herbert *et al.*, 1964; Middleton *et al.*, 1964). Increased aminoimidazolecarboxamide excretion has also been found in cobalamin-depleted rats (Oace *et al.*, 1968).

Animals exposed to the anaesthetic gas N_2O provide a good model for cobalamin deficiency. N_2O is cleaved on exposure to transition-metal complexes such as cobalamin. At the same time the cobalamin is oxidized to an inactive form (Banks *et al.*, 1968). The activity of methionine synthetase, of which cobalamin is a coenzyme, falls within 30 min of N_2O exposure in the rat and is very low after 3 h (Deacon *et al.*, 1978). A similar result has been reported in man in liver biopsy material taken during abdominal surgery (Koblin *et al.*, 1982). The enzyme activity remains depressed as long as N_2O exposure is continued.

Inactivation of cobalamin by N_2O in turn has profound effects on the folate coenzymes. The failure to transfer the methyl group of 5-CH_3-H_4PteGlu to homocysteine to form methionine in the methionine synthetase reaction leads to at least transient increases in tissue concentrations of methyltetrahydrofolate polyglutamates (Lumb *et al.*, 1980). There is impaired hepatic uptake of methyltetrahydrofolate, so that plasma concentrations rise (Lumb *et al.*, 1981). There is marked urinary excretion of methyltetrahydrofolate (Lumb *et al.*, 1982) and a marked fall in tissue folate concentrations.

There is impaired conversion of folate monoglutamate into folate polyglutamate in the N_2O-

Abbreviations used: GAR, glycinamide ribotide (phosphoribosylglycine amide); AICAR, 5-amino-4-imidazolecarboximide ribotide (phosphoribosylaminoimidazolecarboxamide); H_4PteGlu, tetrahydropteroylglutamic acid; 5-CH_3-H_4PteGlu, 5-methyltetrahydropteroylglutamic acid; 5-CHO-H_4PteGlu, 5-formyltetrahydropteroylglutamic acid; 10-CHO-H_4PteGlu, 10-formyltetrahydropteroylglutamic acid; 5,10-CH=H_4PteGlu, 5,10-methenyltetrahydropteroylglutamic acid.

treated rat when H_4PteGlu and CH_3-H_4PteGlu are used as substrates. However, the conversion is normal when formyl-substituted folates, such as 5- or 10-CHOH_4PteGlu (Perry *et al.*, 1979), are given. The suggestion was made that cobalamins were required for the supply of a formyl group to form formyltetrahydrofolates. The source of the formyl group appeared to be methionine via the pathway concerned with polyamine synthesis and the formation of methylthioribose (Perry *et al.*, 1983), which is converted into formate (Trackman & Abeles, 1981).

The emphasis on the role of folate deficiency in producing megaloblastic anaemia has been on the impairment of thymidylate synthesis, and this reaction is depressed in marrow from N_2O-treated rats (McKenna *et al.*, 1980; Deacon *et al.*, 1980). There are no data on effects on purine synthesis. Since both carbon atoms donated by folate in purine synthesis are at the formate level of oxidation and the supply of formate appears to be impaired in N_2O-treated rats, it was of particular interest to study the effect of N_2O on these pathways and whether methionine and its derivatives could reverse any defects that might be present.

We have reported briefly that 24h exposure to N_2O depressed the activity of GAR transformylase while increasing the activity of AICAR transformylase (Deacon *et al.*, 1983).

Materials and methods

Animals

Male 80–100g Sprague–Dawley rats were used. Animals were killed by exsanguination from cardiac puncture after an injection of sodium pentabarbitone. During exposure to the N_2O/O_2 (1:1) gas mixture, the animals were housed in a special Perspex [poly(methyl methacrylate)] environmental chamber in which humidity and CO_2 were controlled. Control animals were kept in air.

In some studies methionine (Sigma Chemical Co.) and 5′-deoxy-5′-methylthioadenosine (Sigma Chemical Co.) were injected intraperitoneally at a dose of 16μmol in 0.5ml of 0.9% NaCl at 0h, 48h and 76h during the 4-day exposure to the N_2O/O_2 gas mixture. Animals were killed at either 24h after the first injection or 24h after the 76h injection.

Reagents

AICAR, ATP, azaserine, folinic acid, ribose 5-phosphate, 3-phosphoglycerate, ammonium sulphamate (0.5%, w/v) and naphthylethylene dihydrochloride were obtained from Sigma Chemicals Co. Dowex 50W (H^+ form; 8% cross-linked; 200–400 dry mesh) (Sigma Chemical Co.) was converted into the NH_4^+ form with 1M-NH_3. AG 1-X8 (acetate form; 100–200 mesh) was obtained from Bio-Rad Laboratories. [1-^{14}C]-Glycine (51.2mCi/mmol) was obtained from Amersham International.

Preparations of liver extracts

Livers were quickly removed and homogenized in cold 0.03M-potassium phosphate buffer, pH7.0 (approx. 50mg/ml), in a hand-operated glass homogenizer. The homogenates were centrifuged at 3000*g* for 45min at 4°C and the supernatants used for assay.

5,10-CH=H_4PteGlu and 10-CHO-H_4PteGlu

These compounds were prepared from folinic acid (5-CHO-H_4PteGlu) (Rabinowitz, 1963). Conversion on acidification was monitored spectrophotometrically by the appearance of an absorption peak at 348nm. On neutralization, the formation of 10-CHO-H_4PteGlu was indicated by the disappearance of the peak at 348nm and the presence of a peak at 258nm.

GAR

This was prepared from acetone-dried chicken liver powder (Hartman *et al.*, 1956; Lukens & Flaks, 1963). Formyl-GAR and GAR were identified by the incorporation of [1-^{14}C]glycine, by a positive orcinol test for pentose (Mejbaum, 1939) and by a specific assay for each of the ribotides with the ammonium sulphate fraction (0–60% saturation) of acetone-dried chicken liver as enzyme source (Lukens & Flaks, 1963; Flaks & Lukens, 1963).

Determination of protein

This was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Assay of AICAR transformylase activity

The method was that described by Flaks *et al.* (1957) and Flaks & Lukens (1963). The reaction was terminated by the addition of 0.4ml of 10% (w/v) trichloroacetic acid, and 0.1ml of acetic anhydride was added to 0.5ml portions of the supernatant. After 20min the remaining non-acetylated diazotizable amine was measured by the Bratton–Marshall reaction (Bratton & Marshall, 1939). AICAR transformylase activity was calculated by measuring the disappearance of AICAR at 540nm by using a molar absorption coefficient of 26400$M^{-1}\cdot cm^{-1}$.

Assay of GAR transformylase activity

The method used was that of Warren & Buchanan (1957), with 0.2M-maleate buffer, pH6.8 (Hartman & Buchanan, 1959). The reaction was terminated by the addition of 0.1ml of 30%

(w/v) trichloroacetic acid, and 0.4ml portions were assayed for diazotizable amine by the Bratton–Marshall reaction (Bratton & Marshall, 1939). GAR transformylase activity was calculated from the amount of *p*-aminobenzoylglutamate (derived from H_4PteGlu produced) at 540nm by using a molar absorption coefficient of $40500\,M^{-1}\cdot cm^{-1}$.

Statistics

Data were analysed by using the Sheffé procedure for multiple comparison of means modified to take account of the non-homogeneous variance found (Gill, 1978).

Results

GAR transformylase activity

The activity of this enzyme (Table 1) declined after 21 h of exposure to N_2O and remained low for 5 days. However, although N_2O exposure was continued, the enzyme activity returned to normal values on day 7 and remained unaffected for the 18-day duration of the study (Fig. 1). The results showed a highly significant quadratic trend ($P<0.00001$) throughout the exposure time. There was a significant linear change during the first 24h and again from day 2 to 18.

AICAR transformylase activity

There was a rise in AICAR transformylase activity during the first 24h, and this was maintained. There was a significant quadratic trend during the first 24h ($P=0.00002$) and a significant cubic trend throughout the whole period of exposure ($P=0.0003$).

Effect of methionine and 5'-methylthioadenosine

This was studied only in relation to GAR transformylase. Methionine had no effect when given either 24h before or for several doses over 76h. Methylthioadenosine had no effect when given as a

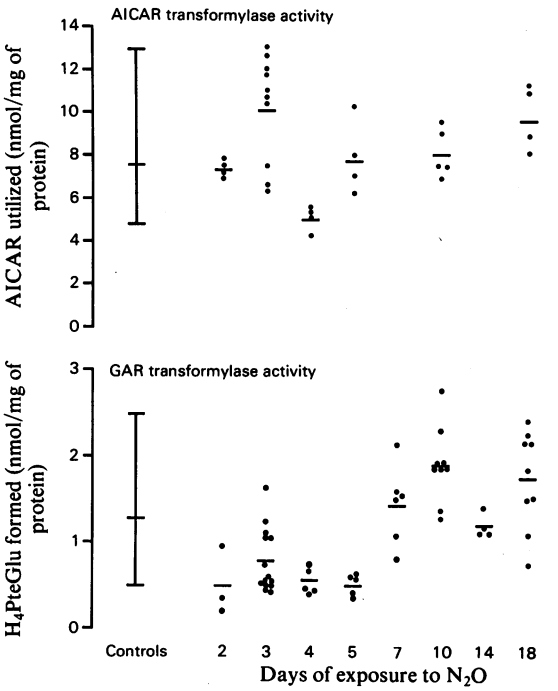

Fig. 1. *Activities of AICAR transformylase and GAR transformylase in the livers of control rats breathing air and O_2/N_2O (1:1) for up to 18 days*
For full experimental details see the text.

Table 1. *GAR transformylase and AICAR transformylase activities in rat liver*
For full experimental details see the text. Results are given as means ± S.D. for the numbers of determinations given in parentheses.

Duration of exposure to N_2O	GAR transformylase activity (nmol of H_4PteGlu formed/mg of protein)	AICAR transformylase activity (nmol of AICAR utilized/mg of protein)
0	1.27 ± 0.47 (60)	7.71 ± 1.87 (45)
6h	1.20 ± 0.19 (6)	7.24 ± 0.78 (6)
12h	1.36 ± 0.32 (6)	9.01 ± 0.32 (6)
18h	1.29 ± 0.26 (6)	11.68 ± 1.08 (6)
21h	0.83 ± 0.22 (6)	9.16 ± 1.61 (6)
24h	0.62 ± 0.30 (19)	8.57 ± 1.47 (19)
2days	0.49 ± 0.34 (4)	7.38 ± 0.38 (4)
3days	0.78 ± 0.37 (14)	10.12 ± 2.51 (10)
4days	0.54 ± 0.11 (8)	4.96 ± 0.92 (4)
5days	0.51 ± 0.10 (8)	7.83 ± 1.70 (4)
7days	1.42 ± 0.45 (7)	—
10days	1.90 ± 0.47 (9)	8.0 ± 1.01 (6)
14days	1.18 ± 0.15 (4)	—
18days	1.73 ± 0.57 (9)	9.7 ± 1.53 (4)

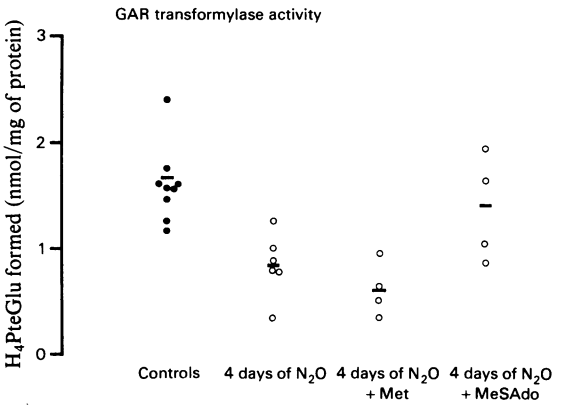

Fig. 2. *Activity of GAR transformylase in the livers of rats breathing air (●) and O_2/N_2O (1:1) for 4 days with or without intraperitoneal doses of methionine (Met) or methylthioadenosine (MeSAdo)*
For full experimental details see the text.

single dose, but it prevented the fall in GAR transformylase activity when given as three doses over 76h. The values in the methylthioadenosine-treated animals were not significantly different from air-breathing controls (Fig. 2), whereas those given methionine still showed a significant fall in GAR transformylase activity.

Discussion

The fall in activity in GAR transformylase on exposure to N_2O is not surprising, since there is evidence that inactivation of cobalamin leads to a failure in the supply of C_1 units at the formate level of oxidation (Perry *et al.*, 1979), which is the form necessary for purine synthesis. Unlike the effects on methionine synthetase, which are evident within 30min of exposure to N_2O, and on the impaired deoxyuridine utilization for thymidine synthesis, evident within 60min, the effect on GAR transformylase was detectable only after 21h.

The effect on thymidylate synthetase is similar to that noted in the present work with AICAR transformylase, and indicates an effect on both C_1 units. We have interpreted the rise in thymidylate synthetase with decreased methylation of deoxyuridine as indicating a failure of supply of C_1 units, and we interpret the results with the folate-dependent transformylases in the same way. It may be noted that the enzyme formyltetrahydrofolate synthetase, which links formate to H_4PteGlu, is also induced by exposure to N_2O, whereas the next enzyme in the sequence converting 10-CHO-H_4PteGlu into 5,10-CH=H_4PteGlu (5,10-methenyltetrahydrofolate cyclohydrolase) is diminished in activity (Perry *et al.*, 1980). This has been interpreted as indicating a need to conserve formyltetrahydrofolate, which is the required form for folate polyglutamate synthesis and purine synthesis.

We were unable to show any effect on GAR transformylase with parenteral methionine. Nor was a single dose of methylthioadenosine effective, but several doses over 4 days was effective in preventing a fall in GAR transformylase activity. This contrasts with the effectiveness of a 16μmol dose of methionine in restoring folate polyglutamate to normal in the N_2O-treated rat. It seems that the rat gives priority to the formation of folate polyglutamate rather than to restoring purine synthesis. We did not test larger doses of methionine, which might well have been effective.

After 1 week purine synthesis became normal despite continued exposure to N_2O. This follows the induction of an alternative pathway of methionine supply in the rat, namely that wherein betaine serves as the methyl donor to homocysteine instead of methyltetrahydrofolate (Lumb *et al.*, 1983). We have shown that methionine, through methylthioadenosine, is a source of formyl groups (Perry *et al.*, 1983).

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