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THE EFFECT OF VITAMIN B_{12} INHIBITION IN VIVO: IMPAIRED FOLATE POLYGLUTAMATE BIOSYNTHESIS INDICATING THAT 5-METHYLTETRAHYDROPTEROYL-GLUTAMATE IS NOT ITS USUAL SUBSTRATE

P. McGing, B. Reed, D.G. Weir and J.M. Scott
Departments of Biochemistry and Medicine, Trinity College, Dublin 2, Ireland

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SUMMARY

Using 5-[^{14}C] methyltetrahydropteroylglutamate and [^{3}H] pteroylglutamate it was found that vitamin B_{12} dependent methyltransferase can be inhibited in vivo in mice by nitrous oxide. Examination of the liver [^{3}H] folates showed as expected that both controls and nitrous oxide treated mice had synthesised the reduced 5-methyl form. However, the controls had converted [^{3}H] almost exclusively into a polyglutamate with virtually no monoglutamate present, while nitrous oxide treated mice had failed to add glutamates to over half of the incorporated folate. Thus in vivo demethylation may be necessary prior to polyglutamate biosynthesis and act as a method of controlling folate accumulation, or be used to create a concentration gradient with the circulating folate.

Vitamin B_{12} deficiency is known to cause decreased folate polyglutamate levels in red blood cells (1,2) lymphocytes (3) and liver (4). A possible explanation for this would be that 5-CH_3-H_4PteGlu[1] cannot act as a substrate for the ligase that adds glutamates to folate within the cell. Thus inability to metabolise 5-CH_3-H_4PteGlu, as would be expected in vitamin B_{12} deficiency, would result in decreased polyglutamate biosynthesis. Conflicting evidence exists as to whether this is in fact the case. A study with Neurospora crassa indicated that 5-CH_3-H_4PteGlu does not act as a substrate (5) while a contrary finding was reported for sheep liver (6). The fact that methotrexate is converted into a polyglutamate, albeit poorly (7), would indicate that the specificity of the ligase is not absolute.

Nitrous oxide (N_2O) has been used to inhibit vitamin B_{12} dependent ethanolamine ammonia ligase (8) by inactivating Co(I) formed during catalysis probably by converting it to Co(II)(9). The Co(I) state also arises as an

1. Abbreviations: 5-methyltetrahydropteroylglutamate, 5-CH_3-H_4PteGlu; 5-methyldihydropteroylglutamate, 5-CH_3-H_2PteGlu; pteroylglutamate, PteGlu; nitrous oxide, N_2O.

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intermediate in the vitamin B_{12} dependent demethylation of 5-CH_3-H_4PteGlu. The finding that transient megaloblastic changes have been found in patients receiving N_2O (10) suggested to us that N_2O might represent a specific model for the inactivation of vitamin B_{12} dependent reactions in mammals *in vivo*. We have now demonstrated the inhibition *in vivo* of vitamin B_{12} dependent demethylation of 5-CH_3-H_4PteGlu by N_2O in mice and examined the effect of this inhibition of folate polyglutamate biosynthesis.

MATERIALS AND METHODS

Radiochemicals: (3'5'9 n [^{3}H]) PteGlu (58 Ci/mmole) and 5-[^{14}C]-CH_3-H_4PteGlu (56 mCi/mmole) were supplied by The Radiochemical Centre Amersham, U.K. The latter was used to make 5-[^{14}C]-CH_3-H_2PteGlu. Labelled [^{14}C] folate polyglutamate standards for use as column markers were prepared as previously described (11,12).

Animals: Adult Laca random bred white mice were maintained in metabolism cages with food and water supplied. Urine was collected into 10% sodium ascorbate giving a final concentration after collection of 1%. Mice for N_2O treatment were placed in chambers, through which was flowing 50% N_2O; 50% O_2 (polyglutamate distribution studies) or 20% N_2O; 80% O_2 (all other studies) for a period of 30 minutes prior to injection of the folate tracer, and returned to the same chamber immediately after injection for further periods of up to 48 hours. Control mice were kept in similar cages in the atmosphere. Mice were injected intraperitoneally with either 5 µCi of [^{3}H] PteGlu or of 5-[^{14}C]-CH_3-H_4PteGlu in 0.2 ml of saline. All animals were killed by cranial fracture.

Folate Polyglutamate Extraction and Analysis: Mice livers were extracted by homogenising as previously described and analysed on columns of DEAE 52 cellulose (Whatman)(13) following permanganate oxidation to cleave the C9-N10 bond.

Folate Monoglutamate Extraction and Analysis: Urinary and liver folates were analysed by chromatography on QAE A25 Sephadex (14). The livers were extracted as previously described (13), but with 5% ascorbate being added to the extraction buffer, and 1% to the column buffers.

Thin Layer Chromatography (TLC): This was carried out on cellulose plates polygram Cel 400 UV_{254} as previously described (15) using three different solvent systems: 3% ammonium chloride; 0.05M potassium phosphate buffer, pH 6.0; 0.05M potassium phosphate buffer, pH 8.0.

RESULTS

When 5-[^{14}C]-CH_3-H_4PteGlu was administered to N_2O treated mice and controls urinary collection indicated that over two days the former excreted on average between 40-50% of the label while the latter excreted 70-80%. Examination of the radioactive material in each instance showed it to co-

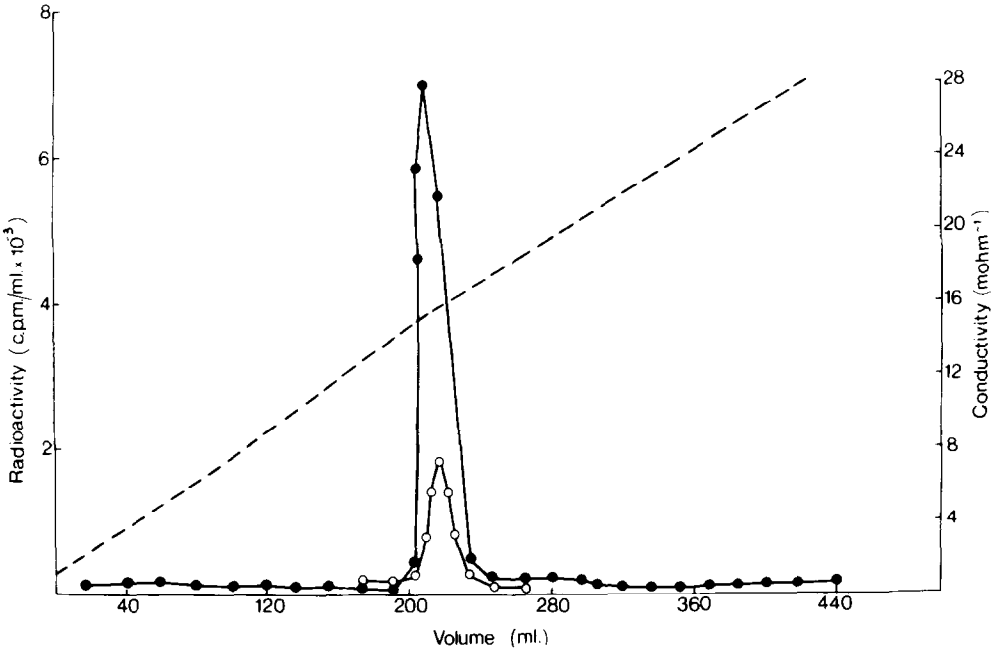

Figure 1: Chromatographic separation on QAE A25 Sephadex using a linear salt gradient (---) of a 48h sample of mouse urine collected from 5 animals maintained in an atmosphere of 20% N_2O; 80% O_2, following the administration of 5 µCi of 5-[^{14}C]-CH_3-H_4PteGlu to each animal (●—●). The elution position of authentic 5-CH_3-H_2PteGlu is also indicated (o——o).

chromatograph with authentic 5-CH_3-H_2PteGlu, the usual oxidation product of 5-CH_3-H_4PteGlu, on three TLC systems (not shown) and on a QAE Sephadex analytical column (Fig. 1). Similarly when [^{3}H] PteGlu was injected increased urinary excretion was seen in the N_2O treated mice. Analysis of the form of radioactive folate present in the livers of both the control and N_2O treated animals was carried out at various times (Fig. 2). As expected both groups contained the reduced 5-methyl form but there was a marked difference in their polyglutamate pattern. The controls as expected had formed polyglutamates (Fig. 2a) while substantial amounts of unaltered monoglutamate existed in the livers of N_2O treated animals (Fig. 2b). A comparison of the livers of N_2O treated mice and controls showed the retention of the tracer dose to be substantially diminished in the former (Table 1).

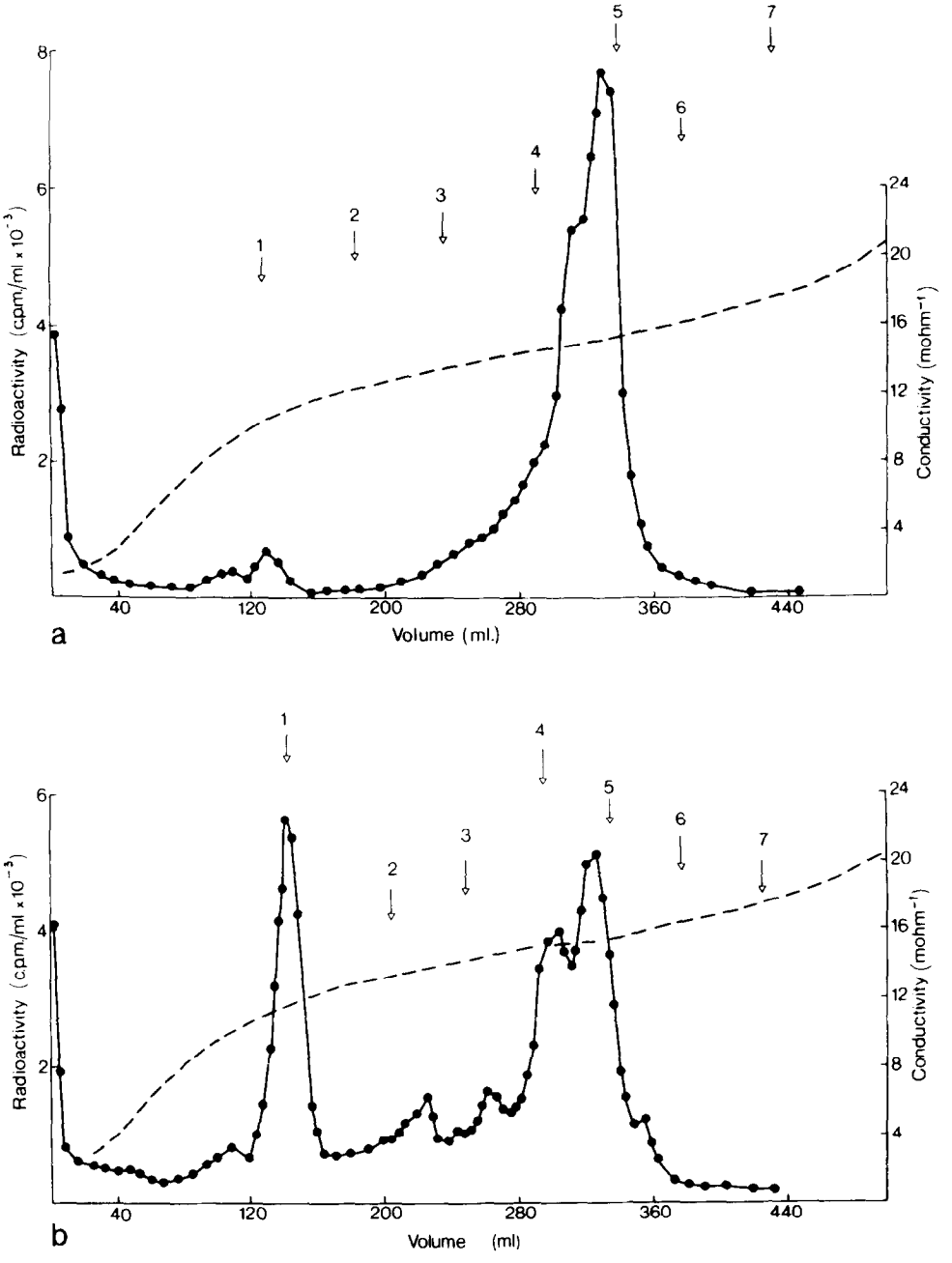

Figure 2: Chromatographic separation on DEAE 52 cellulose using a non-linear salt gradient (----) of the radioactive p-aminobenzoylglutamates formed on oxidative cleavage of the derivatives present in mouse liver 24h after the administration of 5 µCi of ^{3}H PteGlu (●——●) to each of 5 animals. (a) The mice were maintained under normal atmospheric conditions. (b) The mice were maintained in an atmosphere of 50% N_2O: 50% O_2. The numerals 1-7 indicate the elution positions of standard p-aminobenzoyl-glutamates $pABGlu_x$ (x = 1,2,3 ... 7).

Table 1: Effect of nitrous oxide on mouse liver uptake of injected [^{3}H] PteGlu

Exposure Time Post Injection	Controls[a]			Nitrous oxide Treated[a]		
	Liver weight[b]	Incorporation of [^{3}H] PteGlu[c]		Liver weight[b]	Incorporation of [^{3}H] PteGlu[c]	
(hours)	(g)	µCi/g	(%)[d]	(g)	µCi/g	(%)[d]
6	5.39	0.26	(1.05)	6.39	0.14	(0.55)
17	5.00	0.56	(2.25)	5.83	0.24	(0.95)
23	4.94	0.53	(2.13)	4.58	0.24	(0.95)
28	4.54	0.53	(2.12)	5.66	0.23	(0.92)
42	4.46	0.36	(1.43)	4.86	0.16	(0.65)

a At each time five control and five nitrous oxide treated mice were used.
b Total weight of the livers of the five mice
c Each mouse received a total of 5 µCi [^{3}H] PteGlu by intraperitoneal injection
d % of the 25 µCi administered incorporated/g liver

DISCUSSION

Haematological changes have been reported in both rats (16) and man (10) as a result of N_2O exposure. A possible explanation could be that the Co(I) formed during vitamin B_{12} dependent demethylation of 5-CH_3-H_4PteGlu is inactivated by N_2O, as has been shown to occur with the bacterial enzyme ethanolamine ammonia ligase (8). This suggested to us that N_2O treatment might represent a useful model for inducing vitamin B_{12} inactivation in mammalian liver and marrow. It was found that N_2O did in fact inhibit 5-CH_3-H_4PteGlu metabolism both when this folate was given directly as 5-[^{14}C]-CH_3-H_4PteGlu or when it was formed from [^{3}H] PteGlu *in vivo*. There was a marked increase in urinary excretion of the label in N_2O treated mice compared to controls in both instances. All of the [^{14}C] present in the

urine was found to be the dihydro oxidation product of 5-[^{14}C]-CH_3-H_4PteGlu indicating that demethylation of the [^{14}C] methyl group had been impaired by N_2O treatment (Fig. 1).

Of greater interest was the effect of inhibiting the demethylation of 5-CH_3-H_4PteGlu on folate retention by the liver and on polyglutamate biosynthesis. The amount of label retained by the N_2O treated livers was always less than half that of the controls at the various times examined (Table 1). Analysis of the liver folates showed that in the control mice as expected virtually all of the incorporated label had been converted into a polyglutamate with virtually no monoglutamate pool present (Fig. 2a). In the N_2O treated mice almost half of the incorporated radioactive folate had had no glutamate residues added by the cell (Fig. 2b). The most probable explanation for this would be that either 5-CH_3-H_4PteGlu cannot act as a substrate for polyglutamate biosynthesis with some residual vitamin B_{12} activity accounting for the polyglutamate biosynthesis that had occurred, or that the ligase has a decreased activity for the 5-methyl form.

As a result of these findings the question which arises is to why mammalian cells cannot convert the form of the vitamin with which they are usually presented (i.e. 5-CH_3-H_4PteGlu$_1$), into the forms usually present in the cell (i.e. 5-CH_3-H_4PteGlu$_{5,6}$) without demethylation of the former. We feel that this may represent a method by which the cell controls the amount of folate that it accumulates. By regulating the rate of demethylation of the 5-CH_3-H_4PteGlu pool it could control the amount converted to a polyglutamate and thus the amount to be retained. In addition metabolism of the transported form may represent a type of metabolic trapping whereby a concentration gradient between 5-CH_3-H_4PteGlu in the circulation and an intracellular pool of the same derivative can be established.

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