The Effect of Nitrous Oxide Inactivation of Vitamin B₁₂ on Rat Hepatic Folate

IMPLICATIONS FOR THE METHYLFOLATE-TRAP HYPOTHESIS

Michael LUMB,* Rosemary DEACON,* Janet PERRY,* Israel CHANARIN,* Barbara MINTY,†
Michael J. HALSEY† and John F. NUNN†

*Section of Haematology and †Division of Anaesthesia, Clinical Research Centre,
Northwick Park Hospital, Harrow, Middlesex, U.K.

(Received 12 September 1979)

Rats exposed to N₂O show a decrease in liver folate to about 25% of the initial value after 10 days. There is a transient increase in the amount of 5-methyltetrahydropteroylpolyglutamate in the first 24 h, but thereafter the content decreases. The level of 5-methyltetrahydropteroylmonoglutamate declines without any transitory increase. The transient accumulation of 5-methyltetrahydropteroylpolyglutamate is due to failure of methionine synthetase. Thereafter the decrease in the amount of methylfolate makes it improbable that trapping of methylfolate is the explanation for failure of folate metabolism in vitamin B₁₂ deficiency.

Megaloblastic marrow change in man after inhalation of the anaesthetic gas N₂O was reported by Lassen *et al.* (1956) and Amess *et al.* (1978). The latter group in addition showed that the defect in thymidine synthesis was partly corrected by the addition of vitamin B₁₂ to marrow-cell suspensions. N₂O is cleaved by contact with vitamin B₁₂ *in vitro*, with oxidation of vitamin B₁₂ [cob(I)alamin] to a form [cob(III)alamin] inactive in the methionine synthetase reaction (Banks *et al.*, 1968; Blackburn *et al.*, 1977).

Further evidence for a toxic effect of N₂O was provided by reports of neuropathy after self-medication with N₂O over relatively long periods (Layzer *et al.*, 1978; Sahenk *et al.*, 1978; Layzer, 1978) with numbness and tingling of the extremities, loss of dexterity, poor balance, impotence and interference with sphincter control.

More basic observations have been made in laboratory animals and these include neurological changes in monkeys (Dinn *et al.*, 1978), impaired folate metabolism (McGing *et al.*, 1978; Deacon *et al.*, 1978), but normal metabolism of methylmalonic acid, which is also a vitamin B₁₂-dependent pathway (Deacon *et al.*, 1978).

The importance of achieving an understanding of the action of N₂O is, first, that it may prove possible to use it more extensively for analgesia after surgery or injury and, second, that, as the biochemical changes appear to be identical with those found in pernicious anaemia in relapse, it may lead to the

elucidation of the manner in which vitamin B₁₂ controls and regulates folate metabolism.

In the present paper we report data on the effect of N₂O on folate stores in rat liver and the implication of the data on current theories of vitamin B₁₂ action.

Materials and Methods

Sprague-Dawley rats (4 weeks old) were maintained in an atmosphere of O₂/N₂O (1:1) for up to 14 days in a specially constructed cabinet in which CO₂ and water vapour were controlled. At the start three to five animals were killed by exsanguination by cardiac puncture and livers (as well as brains and marrows) were removed. Thereafter two to four animals were killed at various intervals throughout the period of N₂O exposure.

Livers were weighed, ascorbate was added (5 ml of a 1% solution at pH 7.0/g of liver), and the livers were boiled for 5 min to destroy endogenous folate conjugase activity and then homogenized. Homogenates were cooled and stored at –20°C. For assay the homogenate was extracted by heating 10 µl added to 20 ml of 0.2 M-phosphate buffer with 0.15% ascorbate, pH 5.7, at 69 kPa for 10 min. A further portion was incubated with chick pancreas overnight (0.1 ml of liver homogenate to 1 ml of 1% ascorbate, pH 7.0, and 0.1 ml of chick pancreatic conjugase at 37°C). Folate content of liver extracts was determined by microbiological assay with

Lactobacillus casei and *Pediococcus cerevisiae* as test organisms.

Results

Exposure to N_2O resulted in a marked decrease in the folate content of the liver. This was shown in four separate experiments with almost identical results. The greatest decrease in liver folate took place in the first 48 h (Fig. 1, Table 1) and was generally from approx. $5.6 \mu\text{g}$ of folate/g to $2.5 \mu\text{g}$ of folate/g. Thereafter there was a slower decrease over the next 8 days to about $1.5 \mu\text{g}$ of folate/g so that some three-quarters of the folate initially present in the liver had disappeared after 10 days exposure to N_2O/O_2 (1:1).

Table 1 also shows the results of assaying total folate after exposure to conjugase enzyme with *L. casei* and *P. cerevisiae*. With *L. casei* all the folate is

Fig. 1. Concentration of total folate (■), folate assayable only after treatment with a folate conjugase enzyme (▲) folate assayable before conjugase treatment (●) using *L. casei* in the livers of N_2O -treated rats

assayed, and with *P. cerevisiae* folates having a methyl substitution at position-5 are not assayed, but all other tetrahydrofolates in the monoglutamate form are assayed. The decrease in folate in the early stages is greater with *L. casei*-active forms and somewhat less with forms active only with *P. cerevisiae*. The ratio of total *L. casei*-active folate to *P. cerevisiae*-active folate on day 0 was 3.3 and on day 10 was 2.78.

Comparison of the decrease in folates as polyglutamates and those having relatively few glutamate residues (termed free folates, since they are available for microbiological assay without pretreatment with conjugase enzyme to remove glutamate residues in excess of one) shows that polyglutamates appear to be preserved for the first 24 h (Fig. 1). This is better shown in a 3-day study (Fig. 2a), where there is an increase in folate polyglutamate when assayed with *L. casei* at 24 h. This increase in the methylfolate polyglutamates indicates a 'trapping' of folate as postulated in the methylfolate-trap hypothesis (Herbert & Zalusky, 1962), owing to cessation of methionine synthetase activity. After 24 h there is either an escape from the trap by oxidation of the methyl group by methyl-enetetrahydrofolate reductase, catabolism of the methylfolate, or its redistribution from the liver. Loss of methylfolate in the free form occurs without any preliminary 'trapping'. This is of special interest as it has been postulated that the methylfolate trap operates at the folate monoglutamate level by blocking the methyltetrahydrofolate to tetrahydrofolate stage and hence making unavailable the correct substrate for folate polyglutamate synthesis, which has been postulated to be tetrahydrofolate. Our results show that this is incorrect. The inactivation of methionine synthetase leads to short-

Table 1. Hepatic folate concentrations in rats during inhalation of N_2O
Results are means $\pm$ S.E.M., except for those for two animals, which are averages $\pm$ range.

Duration of exposure (days)	Number of animals	Folate ($\mu\text{g/g}$)			
		<i>L. casei</i> assay		<i>P. cerevisiae</i> assay	
		'Free'	After conjugase	'Free'	After conjugase
0	5	2.56 ± 0.31	5.64 ± 0.33	0.35 ± 0.09	1.70 ± 0.24
1	3	1.17 ± 0.06	4.20 ± 0.58	0.06 ± 0.03	1.08 ± 0.11
2	3	1.07 ± 0.06	2.93 ± 0.42	0.11 ± 0.00	0.99 ± 0.09
4	3	0.67 ± 0.18	2.77 ± 0.16	0.07 ± 0.02	0.87 ± 0.08
6	3	0.07 ± 0.00	2.13 ± 0.20	0.04 ± 0.02	0.65 ± 0.05
8	3	0.63 ± 0.14	1.87 ± 0.23	0.04 ± 0.02	0.60 ± 0.05
10	3	0.53 ± 0.08	1.53 ± 0.25	0.02 ± 0.02	0.55 ± 0.00
0	3	4.33 ± 0.85	7.50 ± 0.61	0.97 ± 0.31	2.57 ± 0.44
1	2	2.30 ± 0.10	6.70 ± 0.30	0.22 ± 0.00	1.55 ± 0.15
2	4	1.88 ± 0.13	4.50 ± 0.41	0.32 ± 0.05	1.57 ± 0.22
3	4	1.98 ± 0.15	4.15 ± 0.30	0.35 ± 0.07	1.45 ± 0.07

Fig. 2. Three-day studies of the effect of (a) N_2O on liver folate, showing the rise in the content of polyglutamate forms active with *L. casei* in the first day and (b) N_2O on liver folates other than the methyl forms (*P. cerevisiae*-active)

Symbols: ■, total folate; ▲, folate present in polyglutamate form; ●, 'free' folate.

term accumulation of methylfolate polyglutamate, i.e. at a stage after the polyglutamate has been formed.

By contrast the unsubstituted or formyl-substituted folates (assayed with *P. cerevisiae*) show a steady decline in concentration more marked with free than with polyglutamate forms (Fig. 2b). In some of the animals virtually all *P. cerevisiae*-active folate appeared to be polyglutamate (Table 1, column 5).

Discussion

Observations on the South African fruit bat showed that there were differences in the way the animal used tetrahydrofolate and methyltetrahydrofolate for conversion into folate polyglutamate (Perry *et al.*, 1978). Both were taken up by liver to an equal extent. Tetrahydrofolate continued to be converted into the polyglutamate form over 4 days. Methyltetrahydrofolate was converted into polyglutamate in the first 24 h, but thereafter the amount of labelled methylfolate in the liver declined. The most likely explanation for the decline in liver methylfolate was that methylfolate that had not been converted into the polyglutamate form was either catabolized or recirculated via the enterohepatic route (Hillman *et al.*, 1978).

Inactivation of cob(I)alamin by oxidation with N_2O is accompanied by a loss of folate from the liver, being most marked in the first 48 h and thereafter continuing at a slower rate through the period of study. The current hypothesis is that methyltetrahydrofolate enters the cell, where it is converted into tetrahydrofolate. Further glutamate residues are added only to tetrahydrofolate to form folate polyglutamate, which is the active coenzyme. Vitamin B_{12} is required at the stage of conversion of

methyltetrahydrofolate into tetrahydrofolate in the methionine synthetase reaction. This reaction transfers the methyl group from methyltetrahydrofolate to homocysteine to form methionine and simultaneously gives rise to unsubstituted tetrahydrofolate. Several observations on the effect of N_2O on folate metabolism throw light on this hypothesis.

After exposure for 6 h to N_2O , methionine synthetase activity is almost undetectable (Deacon *et al.*, 1978) and presumably there is virtually no transfer of the methyl group from methyltetrahydrofolate to release tetrahydrofolate. This is the likely explanation for the increase in methyltetrahydrofolate at 24 h. However, this increase in methylfolate affects the polyglutamate forms and not the monoglutamates. This is not an unexpected result, since the polyglutamate coenzyme would be expected to be the active form. Under these circumstances it is more difficult to link this methyl transfer to the proposed requirement for tetrahydrofolate at a monoglutamate level as a substrate for polyglutamate synthesis. There is no accumulation of methyltetrahydropteroylmonoglutamate, the concentration of which declines (Fig. 1).

The decrease in liver methylfolate concentrations may be due to a combination of factors. There is no detectable synthesis of folate polyglutamate (McGing *et al.*, 1978) from either tetrahydrofolate or methyltetrahydrofolate (Perry *et al.*, 1979) in N_2O -treated rats. As there is a cessation of methyl transfer [methionine synthetase activity is virtually zero (Deacon *et al.*, 1978)], methylfolate must either be redistributed to other tissues via the enterohepatic route, or be catabolized presumably by cleavage at the 9–10-position, or the methyl group oxidized to give methylene and formyl analogues of tetrahydrofolate. We have some preliminary evidence that there is some redistribution of folate out of the liver, but there are no data on the other alternatives.

There is a danger of assuming that the acute events after oxidation of vitamin B_{12} by N_2O in the rat (and man) are similar to events in untreated pernicious anaemia, where adaptation to a slow onset of vitamin B_{12} deprivation may occur. All that can be said at present is that hitherto the similarity between the biochemical changes in pernicious anaemia and the N_2O -treated rat has been remarkable. On this basis our data do not support the methylfolate-trap hypothesis, i.e. there was no immobilization of folate in the methyl form, nor do the data support the view that the trap prevented the release of tetrahydrofolate. Indeed, there is no detectable formation of folate polyglutamate from tetrahydrofolate given as such, in the N_2O -treated rat, once again suggesting that a failure of folate polyglutamate synthesis is the major defect (Chanarin *et al.*, 1974).

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