Tissue folates in fruit bats (*Rousettus aegyptiacus*) with nitrous oxide-induced vitamin B₁₂ deficiency and neurological impairment

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1. Long-term exposure of the fruit bat *Rousettus aegyptiacus* to nitrous oxide, which inactivates methylcobalamin, leads to neurological impairment and ataxia.
2. In N₂O-exposed animals, liver concentrations of total folates and methyl folates decreased to less than one-fifth that of control animals. *Pediococcus cerevisiae*-active folates were also reduced.
3. In brain, there were no changes in total or methyl folates, but *P. cerevisiae*-active folates were lower in N₂O-exposed animals.
4. Supplementation with methionine retarded the development of neurological impairment and the fall in liver total and methyl folates, but not that in *P. cerevisiae*-active folates.
5. Supplementation with serine failed to retard the development of neurological impairment or fall in hepatic folates.
6. The present results suggest that the N₂O-induced neurological impairment in the bat is not related to depletion of cerebral folates, but do not exclude changes in the subcellular distribution of folates.

Study of the mechanism of the neurological impairment associated with vitamin B₁₂ deficiency has been difficult owing to the lack of a suitable small-animal model in which vitamin B₁₂ deficiency with neurological changes can be induced rapidly. This has been overcome by the observation that exposure of the fruit bat (*Rousettus aegyptiacus*) to the anaesthetic gas nitrous oxide leads to severe neurological impairment progressing to paralysis and death after 8–12 weeks' exposure (van der Westhuyzen et al. 1982a). N₂O inactivates methylcobalamin by oxidizing cob(I)alamin to cob(III)alamin (Banks et al. 1968), thereby inactivating the enzyme methionine synthetase (5-methyltetrahydrofolate homocysteine methyltransferase; *EC* 2.1.1.13; MS) which requires cob(I)alamin as a cofactor (Deacon et al. 1980a; Chanarin et al. 1985). Depletion of tissue vitamin B₁₂ stores follows long-term exposure to N₂O (van der Westhuyzen et al. 1982a).

The basic lesion whereby vitamin B₁₂ deficiency or inactivation leads to neurological impairment is uncertain. Ever since the clinical observation that folic acid apparently aggravated the neurological changes in humans suffering from vitamin B₁₂ deficiency (in the form of pernicious anaemia), these changes have been thought to be related somehow to folic acid metabolism.

Inactivation of cobalamin by N₂O has profound effects on folic acid metabolism in the rat, including changes in plasma levels (Lumb et al. 1981a), loss of tissue folates (Lumb et al. 1981b), impairment of folate polyglutamate synthesis from unsubstituted folates (Perry et al. 1979a) and impaired uptake of folate analogues by the liver (McGing et al. 1978; Chanarin et al. 1985). However, the rat does not develop neurological lesions on exposure to N₂O and is thus not a suitable animal model. Furthermore, in the reported studies in the rat, exposure to N₂O has been acute (up to 10 d), and thus not entirely relevant to the development of vitamin B₁₂-related neurological changes, which require longer periods of N₂O exposure.
The neurological complications of vitamin B₁₂ deficiency may be related to changes in folates in the brain. In the present report, cerebral and hepatic folates in control and neurologically-impaired fruit bats following long-term exposure to N₂O have been examined. As methionine has been shown to protect partially against the neurological changes in the N₂O-exposed bat (van der Westhuyzen et al. 1982a; van der Westhuyzen & Metz, 1983), a group of animals who received dietary supplementation with methionine was included. In a further group, serine was given as a dietary supplement instead of methionine to detect a possible effect of an augmented supply of methylene groups on tissue folates.

MATERIALS AND METHODS
The study was approved by the Animal Ethics Committee of the University of the Witwatersrand Medical School.

Experimental animals
Fruit bats caught in the wild were kept in vivaria partially exposed to the natural day–night cycle. All animals were fed on a pest-free, all-fruit diet supplemented with 0·2 ml of a vitamin B₁₂-free oral vitamin preparation (Abidec; Parke-Davis, New York) fortnightly. The folic acid content of the diet was approximately 0·22 mg/kg edible portion (largely sliced banana). Since the bats consume food equivalent to three-quarters of their body-weight/d, the daily intake of folic acid was similar to that of rats consuming a diet mixture containing 1 mg folic acid/kg. Control animals received 5 μg cyanocobalamin/kg body-weight fortnightly, and maintained normal vitamin B₁₂ nutrition (van der Westhuyzen et al. 1982a). Test animals which were exposed to N₂O were randomly allocated to the following dietary groups: (1) N₂O group, standard all-fruit diet; (2) N₂O + met group, fruit supplemented with L-methionine (99%; Riedel-de Haën, Hanover, West Germany) at the rate of 600 mg/kg fruit. This supplement provided approximately 60 mg methionine/animal per d (0·3 g/kg body-weight), sufficient to delay significantly the onset of neurological impairment but low enough not to lead to sudden death (van der Westhuyzen & Metz, 1984). (3) N₂O + ser group, fruit supplemented with L-serine (Sigma, St Louis, Mo., USA) at the rate of 700 mg/kg fruit, which provided 70 mg serine/animal per d (0·53 g/kg body-weight).

Exposure to N₂O
Experimental bats were exposed to an atmosphere of oxygen–N₂O (50:50, v/v) daily for 90 min in a specially constructed cabinet in which water vapour and carbon dioxide were controlled. Exposure was continued until the animals showed unequivocal neurological impairment in the form of ataxia, at which stage they were killed (7·5–9·5 weeks) by exsanguination.

Extraction of tissue
Within 90 s of death, the brain and liver were removed, weighed, mashed and placed in 10 vol. ascorbate (10 g/l solution, pH 6·0) in a 95° water-bath. After boiling for 7 min to destroy endogenous folate conjugase activity, the tissue was cooled on ice, homogenized with a Potter–Elvehjem homogenizer and centrifuged at 15000 g for 20 min at 4°. The supernatant fractions were stored in the dark at −20° until assayed.

Folate assays
Folates were measured by microbiological assay after papain treatment. This method of folate release yields results comparable to those obtained with conjugase prepared from
Table 1. Physical changes in fruit bats (Rousettus aegyptiacus) exposed to nitrous oxide and the effect of dietary supplements

(Mean values with their standard errors)

<table>
<thead>
<tr>
<th>Group</th>
<th>$n$</th>
<th>Initial</th>
<th>Final</th>
<th>Duration of exposure (d)</th>
<th>Condition of group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td></td>
</tr>
<tr>
<td>$N_2O$</td>
<td>13</td>
<td>124</td>
<td>4</td>
<td>107**</td>
<td>Neurologically impaired, ataxic</td>
</tr>
<tr>
<td>$N_2O$+serine</td>
<td>7</td>
<td>132</td>
<td>4</td>
<td>111***</td>
<td>Neurologically impaired, ataxic</td>
</tr>
<tr>
<td>$N_2O$+methionine</td>
<td>6</td>
<td>119</td>
<td>3</td>
<td>138**</td>
<td>No neurological impairment</td>
</tr>
</tbody>
</table>

Mean values were significantly different, indicating a change in weight: ** $P < 0.01$, *** $P < 0.001$.

chicken pancreas (Kelly & Davis, 1965) and hog kidney (own unpublished observation). Papain (Difco, Detroit), 50 mg/ml in saline (9 g sodium chloride/l, 0.2 ml), was added to 4 ml homogenate, mixed and incubated in a water-bath at 50° for 1 h and then steamed for 20 min. After cooling, the samples were centrifuged at 2500 g for 10 min and stored at $-20^\circ$ (Kelly & Davis, 1965).

For assay, samples were set up in duplicate by diluting 0·1 ml of prepared samples in 4·9 ml of the substrate previously inoculated with the test organism (Davis et al. 1970). All samples were assayed with antibiotic-resistant strains of Lactobacillus casei (ATCC 10463), Streptococcus faecalis (ATCC 9774) and Pediococcus cerevisiae (ATCC 7837). L. casei responds to all forms of folate; S. faecalis responds to non-methylated forms; thus the methylated forms are represented by the difference between the L. casei and S. faecalis values (Krumdieck et al. 1983). P. cerevisiae responds to tetrahydrofolate (THF), methylene- and formyl-THF (Lumb et al. 1981b; Krumdieck et al. 1983); the latter being quantitatively the most important form assayed by P. cerevisiae.

The statistical significance of differences between means was assessed by the Mann–Whitney test and all values are expressed as means with standard errors.

RESULTS
Clinical findings

The findings are summarized in Table 1. The effect of $N_2O$ exposure on the bats followed the pattern observed in previous studies (van der Westhuizen et al. 1982a; van der Westhuizen & Metz, 1983). Exposed animals lost weight and developed neurological changes manifest by impairment in climbing and flying, and in ataxia. Supplementation of the diet with methionine prevented the weight loss and retarded the development of neurological impairment, and none of these animals was ataxic after 9 weeks of exposure. Supplementation with serine did not prevent or retard the effects of exposure to $N_2O$.

Liver folates

The results are shown in Table 2. The mean concentration of total (L. casei) folates in the livers of control animals was 537 mg/g, of which 90% were methyl folates. In the $N_2O$-exposed animals, total folates had fallen severely to a mean value of 87 ng/g with 94% as
Table 2. The effect of nitrous oxide exposure on folates in the liver of the fruit bat (Rousettus aegyptiacus)  
(Mean values with their standard errors)

<table>
<thead>
<tr>
<th>Group</th>
<th>Group</th>
<th>Total folates (ng/g)</th>
<th>Methyl folates (ng/g)</th>
<th>Pediococcus cerevisiae-active folates (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
</tr>
<tr>
<td>Untreated controls</td>
<td></td>
<td>537</td>
<td>111</td>
<td>485</td>
</tr>
<tr>
<td>N₂O</td>
<td>12</td>
<td>87***</td>
<td>15</td>
<td>82**</td>
</tr>
<tr>
<td>N₂O + serine</td>
<td>7</td>
<td>70**</td>
<td>10</td>
<td>65**</td>
</tr>
<tr>
<td>N₂O + methionine</td>
<td>6</td>
<td>323</td>
<td>105</td>
<td>312</td>
</tr>
</tbody>
</table>

Mean values were significantly different from those of the control group: **P < 0.01, ***P < 0.001.  
† Percentage of total folates.

Table 3. The effect of nitrous oxide exposure on folates in the brain of the fruit bat (Rousettus aegyptiacus)  
(Mean values with their standard errors)

<table>
<thead>
<tr>
<th>Group</th>
<th>Total folates (ng/g)</th>
<th>Methyl folates (ng/g)</th>
<th>Pediococcus cerevisiae-active folates (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
</tr>
<tr>
<td>Untreated controls</td>
<td>6</td>
<td>44.8</td>
<td>2.1</td>
</tr>
<tr>
<td>N₂O</td>
<td>13</td>
<td>41.8</td>
<td>2.9</td>
</tr>
<tr>
<td>N₂O + serine</td>
<td>7</td>
<td>46.1</td>
<td>6.9</td>
</tr>
<tr>
<td>N₂O + methionine</td>
<td>6</td>
<td>44.3</td>
<td>7.6</td>
</tr>
</tbody>
</table>

Mean values were significantly different from those of the control group: *P < 0.05, **P < 0.01.  
† Percentage of total folates.

methyl folates. The ratio, methyl-THF: non-methylated THF therefore increased in the N₂O-exposed livers, but the increase was modest.

The fall in P. cerevisiae-active folates was even greater, from a mean concentration of 158 ng/g in control animals to one of 24 ng/g in the N₂O-exposed group.

In the N₂O-treated animals supplemented with methionine, the degree of fall in total folates was less and the mean was not significantly lower than the mean of the control animals. The percentage of methyl forms (96.6) was similar to that of animals treated with N₂O only. In contrast to its effect on total folates, methionine had no significant effect on the fall in P. cerevisiae activity following N₂O exposure.

Supplementation with serine had no significant effect on the fall in total folates, methyl forms or the P. cerevisiae values, compared with N₂O exposure without serine.

**Brain folates**

The results are shown in Table 3. In contrast to the fall in liver folates, exposure to N₂O failed to produce any significant change in the concentrations of methyl or total folates in
the brain. However, the ratio, methyl-THF: non-methylated forms decreased slightly in the 
N₂O-exposed groups, especially the serine-supplemented animals. The mean value for P. 
cerevisiae folate activity in the N₂O-exposed animals (9.8 ng/g) was significantly lower 
than that of the controls (16.0 ng/g, \( P < 0.01 \)). Supplementation with methionine or serine 
failed to prevent this decrease in P. cerevisiae-active folates.

**DISCUSSION**

The neurological impairment induced by N₂O in the fruit bat bears both resemblances and 
differences to that of man and non-human primates. The development of ataxia in the 
N₂O-exposed monkey (Scott *et al.* 1981) is similar (as far as comparisons allow) to that of 
the bat. Both exhibit shaking of the limbs at an early stage, followed later by difficulties 
with climbing leading to ataxia. In both species, the development of the neuropathy is 
considerably ameliorated by methionine supplementation. Pronounced histological changes 
resembling those of classical subacute combined degeneration in man, have been described 
in ataxic monkeys (Scott *et al.* 1981). However, although patchy spongiosie change 
suggestive of early demyelination has been observed in the spinal cord of fruit bats 
maintained on a vitamin B₁₂-deficient diet for several years (Green *et al.* 1975), clear 
histological changes have not been observed in bats with neurological impairment induced 
by N₂O (van der Westhuizen *et al.* 1982a). Time may be a factor here, as the monkey 
survives in the moribund state for 2 to 3 weeks, while the fruit bat dies within 1 to 2 d of 
becoming moribund. In contrast to the bat and monkey, mice and rats remain healthy 
when exposed to N₂O for protracted periods (Chanarin *et al.* 1985).

Changes in liver and brain folates have been reported in rats exposed for 5 or 10 d to 
N₂O (McGing *et al.* 1978; Lumb *et al.* 1980, 1981b). In rats there is a marked fall in L. casei 
folate activity in the liver to about 16% of that of control animals. P. cerevisiae activity 
shows a similar fall, of a somewhat lesser degree. There is little or no fall in L. casei folate 
activity in the brain but some fall in P. cerevisiae activity. The results of the present study 
of bats exposed long-term to N₂O are essentially similar.

The fall in total and methyl folates in the liver is related to the inhibition of the enzyme 
MS by N₂O. In the bat, MS activity in the liver after long-term N₂O exposure is only 5% 
that of control animals (van Tonder *et al.* 1986). Following inhibition of this enzyme, the 
methylation of homocysteine to methionine via donation of the methyl group of methyl-
THF is impaired. Hepatic uptake of folate analogues is impaired in the N₂O-exposed rat 
(McGing *et al.* 1978; Lumb *et al.* 1982). The non-metabolized methyl-THF is then 
excreted in the urine (Lumb *et al.* 1982) and the liver stores are depleted (Lumb *et al.* 
1980).

The response of brain folates to N₂O is different to that of the liver in that there is no 
fall in total or methyl folates. Similar observations were made in the rat exposed to N₂O 
for 5 d (Lumb *et al.* 1981b) and in the vitamin B₁₂-deficient fruit bat (Perry *et al.* 1979b). 
The enzyme MS occurs in the brain of both the rat and the fruit bat, and is inhibited by 
N₂O in both animals (Deacon *et al.* 1980a; Lumb *et al.* 1983; van der Westhuizen & Metz, 
1983). This inactivation is comprehensive. Therefore, there must be some mechanism which 
protects brain folates following inhibition of MS. There is possibly some redistribution of 
folates from liver to other tissues, as in the N₂O-exposed rat (Lumb *et al.* 1981b). The 
extremely slow turnover of total folates in brain (Carl *et al.* 1980) is also important, since 
this leads to a limited demand for folates from the blood. Moreover, selective concentration 
of folates occurs in the cerebrospinal fluid (CSF) (Herbert & Zalusky, 1961) with rapid 
transport of 5-methyl-THF into CSF from serum (Spector & Lorenzo, 1975). In the fruit 
bat, only 5-methyl-THF is taken up by brain tissue, and uptake is similar in control and
vitamin B₁₂-deficient animals (Perry et al. 1979b). It is possible then that the uptake of circulating folates by brain tissue leads to preservation of total and methyl folates in the brain despite inhibition of MS.

The action of methionine in partially protecting the N₂O-exposed bat against the development of neurological impairment was confirmed in the present study. Methionine prevented, but not completely, the fall in liver folates, a finding compatible with the report by Eells et al. (1982) that methionine prevents the decrease in liver THF in rats exposed for 4 h to N₂O, and by Perry et al. (1983) who has demonstrated that methionine restores the capacity of the N₂O-treated rat to utilize THF.

The mechanism whereby methionine retards the depletion of total and methyl folates in the liver of the N₂O-exposed animal is uncertain. It has been suggested that methionine impairs the recycling of THF into 5-methyl-THF through its conversion to S-adenosylmethionine which inhibits the enzyme 5,10-methylene-THF reductase (FADH₃) (EC 1.7.99.5), responsible for the production of 5-methyl-THF (Kutzbach & Stokstad, 1967). THF is thus released for other metabolic functions. Perry et al. (1983) have suggested that the corrective effect of methionine is by supply of formate for the formylation of THF.

In the present study, methionine failed to preserve the concentration of P. cerevisiae-active folates in both liver and brain, and the lowest levels of unsubstituted reduced or formyl-substituted folates occurred in the brain of animals supplemented with methionine. The fall in these folates in the brain of N₂O-exposed bats is thus unlikely to be causally related to the neuropathy, for the effect of methionine in protecting against neurological impairment was not accompanied by preservation of the concentration of these folates (which include 5- and 10-formyl-THF). Furthermore, supplementation with serine retarded the fall of P. cerevisiae-active folates in the brain of half the animals but failed to protect against the neurological impairment.

The preservation of the concentration of total and methyl folates in the brain of bats with neurological impairment following vitamin B₁₂ inactivation, does not lend support to the theory that the neuropathy of vitamin B₁₂ deficiency is related to depletion of cerebral folates, mediated by inactivation of MS with its methylcobalamin cofactor. It has been suggested rather that the neurological changes may result from other vitamin B₁₂-dependent functions, such as impairment of the adenosylcobalamin-dependent methylmalonyl-CoA mutase (EC 5.4.99.2) reaction, which leads to limited changes in odd-chain fatty acid metabolism (Frenkel, 1973; Fehling et al. 1978; Peifer & Lewis, 1979; van der Westhuyzen et al. 1983), or some other undescribed metabolic functions of vitamin B₁₂. In the fruit bat, neurological changes associated with vitamin B₁₂ deficiency appear not to be related to the accumulation of cobalamin analogues (van der Westhuyzen et al. 1982).

In conclusion, it is possible that the overall level of folates in the brain is less important than the regional and subcellular distribution of these compounds. For example, the
activity of MS in the normal mouse is high in synaptosomes, suggesting that MS may have some synaptosomal-specific function (Carl et al. 1980). The results of the present study do not rule out the possibility of changes in the subcellular or regional distribution of folates.

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REFERENCES


