Tissue S-adenosylmethionine levels in fruit bats (*Rousettus aegyptiacus*) with nitrous oxide-induced neuropathy

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1. The effect of cobalamin inactivation by the anaesthetic gas nitrous oxide on the concentration of S-adenosylmethionine (Ado Met) in brain and liver of fruit bats (*Rousettus aegyptiacus*) was examined.

2. Test animals exposed to N_2O–oxygen (50:50, v/v) developed ataxia and paralysis leading to death after an average of 9·8 weeks (*n* 6). Animals receiving pteroylmonoglutamic acid supplements in the diet became ataxic earlier (mean 8·8 weeks) while those receiving methionine supplements survived for significantly longer periods (12·5 weeks, $P < 0.01$).

3. Plasma cobalamin levels indicated severe depletion of cobalamin stores in N_2O-exposed animals.

4. The mean concentration of Ado Met in the brain of N_2O-treated bats was nearly 50% higher than that of untreated controls. Ado Met levels in treated bats receiving pteroylmonoglutamic acid or methionine supplements were respectively 18 and 25% higher than in controls. In contrast, the concentration of Ado Met in the liver of all the N_2O-treated groups was slightly lower than in controls.

5. These results suggest that the N_2O-induced neuropathy in the fruit bat is not related to a depletion of Ado Met in the nervous system.

S-adenosylmethionine (Ado Met) is required for a variety of essential methylation reactions, including those in nervous tissue. Ado Met is derived from methionine, which is obtained partly from the diet and partly from two homocysteine methylase reactions (Fig. 1), namely the betaine–homocysteine methyltransferase (*EC* 2.1.1.5) reaction and the 5-methyltetrahydrofolate homocysteine methyltransferase (*EC* 2.1.1.13) reaction. The latter reaction requires methylcobalamin as an essential cofactor and methyltetrahydrofolate as a methyl donor.

The mechanism whereby cobalamin deficiency may lead to neurological changes in man is unknown. Recently, experimental animals exposed to nitrous oxide have been used as animal models for the study of the pathogenesis of the neurological changes. Nitrous oxide oxidizes cob(I)alamin to cob(III)alamin (Banks *et al.* 1968) and administration of the gas to the monkey or the fruit bat (*Rousettus aegyptiacus*) leads to severe neurological dysfunction (Scott *et al.* 1981; van der Westhuyzen *et al.* 1982). The oxidation of cobalamin leads to severe inhibition of the cobalamin-dependent enzyme 5-methyltetrahydrofolate homocysteine methyltransferase (Deacon *et al.* 1980*b*) and Scott *et al.* (1981) have postulated that the cobalamin-related neuropathy is the result of deficient methionine synthesis leading to deficient Ado Met and defective methylation reactions in the nervous system.

To test this hypothesis, Ado Met was measured in fruit bats in which neuromuscular impairment had been induced by exposure to N_2O, and the results were compared with control animals. In addition, the effect of other specific nutrients, observed to prevent or aggravate the neuropathy, on Ado Met concentrations in the brain and liver of N_2O-treated bats, was examined.
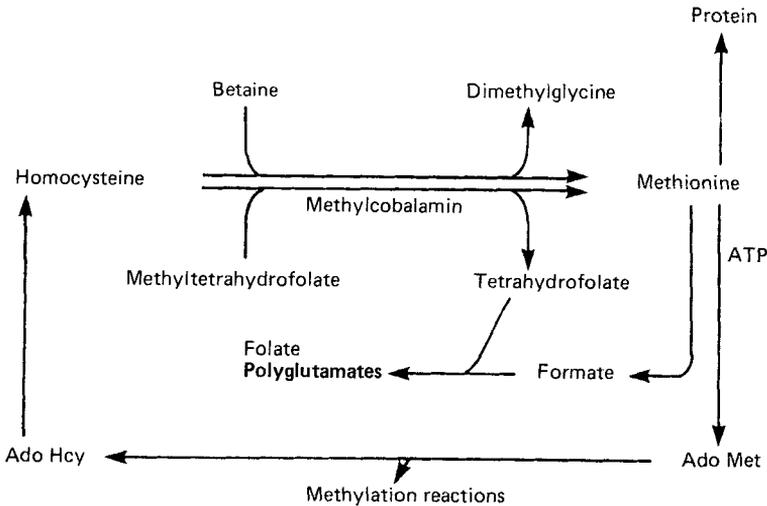
Fig. 1. Synthesis and fate of methionine. Ado Hyc, adenosylhomocysteine; Ado Met, adenosylmethionine.

MATERIALS AND METHODS

Experimental animals

Egyptian fruit bats were caught in the wild and maintained in vivaria on a pest-free fruit diet as previously described (van der Westhuyzen *et al.* 1982). The animals received cobalamin supplementation by injection (5 µg cyanocobalamin/kg body-weight every 14 d) except in the test animals during the period of the experiment. Groups of test animals receiving the following diets were exposed to N_2O: group 1, basal fruit diet; group 2, fruit diet supplemented with methionine in the proportion of approximately 200 mg/kg fruit. This diet provided approximately 20 mg methionine/animal per d (140 mg/kg body-weight); group 3, fruit diet supplemented with 2 mg pteroylmonoglutamic acid (PGA)/kg fruit. The PGA was added to the food as a 0·6 mg/ml solution in 0·1 M-sodium hydroxide (van der Westhuyzen *et al.* 1982). This provided a supplement of 200 µg PGA/animal per d (1·4 mg/kg body-weight).

Plasma cobalamin levels

Blood was drawn by cardiac puncture at the end of the experiment, or from moribund animals before death. Cobalamin was measured by the radioisotope-dilution technique of Green *et al.* (1974).

S-adenosylmethionine assay

Brain or liver tissue (1–2 g) was removed immediately after death, washed in ice-cold 0·25 M-sucrose and homogenized in several volumes of ice-cold 0·25 M-sucrose containing 1 mM-mercaptoethanol, 0·1 mM-EDTA, and 1 mM-dithiothreitol. The homogenates were processed and the Ado Met and S-adenosylhomocysteine (Ado Hcy) separated on a Cellex P (H^+ form) column as described by Eloranta *et al.* (1976). Ado Met was measured by its absorption at 257 nm and the concentration of Ado Met in the eluate calculated by means of its known molar extinction coefficient (1500 l/mol per mm). Recoveries, which were determined by adding known amounts of S-adenosyl-L-[methyl-^{14}C]methionine to the assay, were similar to those previously reported (90–97%, Eloranta *et al.* 1976).

Exposure to N_2O

Test animals were exposed to an atmosphere of N_2O–oxygen (50:50, v/v) daily for 90 min in a specially constructed cabinet in which carbon dioxide and water vapour were controlled. Exposure was continued for up to 13 weeks or until the animals became ataxic and moribund. Control animals received the cobalamin-supplemented diet and were not exposed to N_2O.

RESULTS

Induction of neuromuscular impairment

All the animals exposed to N_2O developed neuromuscular impairment manifesting as weakness and inability to use the hind-limbs. With further exposure, paralysis supervened and flying was reduced to short hops. Abrasions developed on the chin as a result of repeated contact with the ground. Animals became moribund after 9·8 (0·7) weeks (mean with SEM).

Animals receiving PGA supplementation became ataxic and moribund at 8·8 (0·5) weeks (mean with SEM), somewhat earlier than those which did not receive PGA. By contrast, bats receiving methionine supplements survived for significantly longer, becoming ataxic after 12·5 (0·5) weeks. This is also significantly longer than the survival period of N_2O-treated bats receiving the basal diet ($P < 0.01$). Control animals not exposed to N_2O remained healthy throughout the period of study, with no evidence of neurological impairment.

Plasma cobalamin concentrations

All the animals were cobalamin-replete at the start of the experiment, with plasma cobalamin levels greater than 1500 pg/ml. Cobalamin deficiency developed during N_2O exposure (Table 1). The lowest cobalamin levels occurred in the animals receiving the basal fruit diet (mean with SEM 52 (13) pg/ml), followed by the PGA-supplemented group (110 (26) pg/ml) and then the methionine-supplemented group (170 (54) pg/ml). The differences in the mean values for the N_2O-exposed groups were not statistically significant. The plasma cobalamin levels were maintained in control animals.

S-adenosylmethionine levels

The pattern of change in Ado Met levels was different in the liver compared with the brain (Table 1).

Liver. The concentration of Ado Met in the liver of N_2O-treated bats was not significantly different from that of controls (Table 1). In N_2O-treated bats receiving PGA supplementation, the mean (with SEM) concentration (63·0 (4·6) nmol/g) was lower still, but in animals receiving methionine, the mean (with SEM) value (71·0 (10·0) nmol/g) was closest to that of the control group. None of these differences reached statistical significance.

Brain. The mean (with SEM) concentration of Ado Met in the brain of the N_2O group receiving the basal diet (60·2 (6·7) nmol/g) was significantly higher than that of controls (40·6 (1·5) nmol/g) by approximately 50% ($P < 0.05$). The levels of Ado Met in the brain of the PGA- (47·2 (2·2) nmol/g) and methionine- (50·3 (2·2) nmol/g) supplemented groups were also significantly higher than in controls ($P < 0.05$, $P < 0.01$ respectively) but the mean values were lower than the N_2O-treated animals without dietary supplementation.

It is of interest that the mean values for the sum of the brain and liver Ado Met contents varied over fairly narrow limits in the various groups (377·9–420·1 nmol).

Table 1. *Plasma cobalamin and S-adenosylmethionine (Ado Met) levels in nitrous oxide-treated fruit bats* (Rousettus aegyptiacus) *with and without dietary supplementation*

(Mean values with their standard errors for six bats/group)

| | Plasma cobalamin (pg/ml) | | Tissue Ado Met nmol/g wet wt | | | | Total (nmol) in brain and liver† |
| | | | Brain | | Liver | | |
Group	Mean	SE	Mean	SE	Mean	SE	
Controls	1818	131	40·6	1·5	73·3	3·6	411·0
N₂O	52	13	60·2*	6·7	66·6	12·8	420·1
N₂O+PGA	110	26	47·2*	2·2	63·0	4·6	377·9
N₂O+ methionine	170	54	50·3**	2·2	71·0	10·0	420·1

Statistically significantly different from controls: * $P < 0.05$, ** $P < 0.01$.
† Average brain and liver weights (g) were 2·0 and 4·5 respectively.

DISCUSSION

In the present study, the administration of the cobalamin-inactivating gas N_2O to fruit bats led to neuromuscular impairment, ataxia and death, as reported previously (van der Westhuyzen *et al.* 1982). N_2O inhibits the activity of the cobalamin-dependent 5-methyltetrahydrofolate homocysteine methyltransferase enzyme by up to 90% in bat brain and 80% in the liver (A. Ruck, unpublished results). As the levels of L-methionine in animal tissues appear to exert an important controlling influence on the concentration of Ado Met (Schlenk, 1965; Baldessarini, 1966), a reduction in tissue Ado Met levels would be expected following severe inhibition of the cobalamin-dependent methyltransferase reaction. However, the results of the present study failed to reveal any reduction in Ado Met levels, and thus the postulate of Scott & Weir (1981) that the neurological impairment in the N_2O-treated animal is the result of impaired methylation reactions in the nervous system, owing to a reduction in Ado Met, does not appear to hold for the fruit bat.

In the same way, the effects of added PGA in aggravating, and methionine in delaying, the onset and progression of the neuromuscular impairment were not reflected by changes in the Ado Met concentration in the brain, which were very similar in these two groups of animals. These findings add further evidence to the suggestion that the N_2O-induced neuropathy in the bat is not related to changes in Ado Met concentration in the nervous system.

Tissue Ado Met levels have been reported in dietary cobalamin deficiency in the rat (Vidal & Stokstad, 1974) but not to our knowledge in N_2O-induced cobalamin inactivation. However, in a letter to the *Lancet*, Chanarin (1981) mentions that hepatic levels of Ado Met were not significantly different in control rats and rats in which cobalamin and methionine synthetase activities had been inactivated by N_2O. The actual data were not presented. Vidal & Stokstad (1974) measured Ado Met following column chromatography in the liver of rats given a diet low in cobalamin and with only 2 g methionine/kg in the protein supplement. Hepatic Ado Met was reduced in the deficient group, averaging 33 nmol/g wet tissue, and returned to normal levels of approximately 60 nmol/g wet weight upon the addition of cobalamin or methionine to the diet. By contrast, in the present study, the hepatic Ado Met levels were only slightly reduced in the N_2O-treated bat. This could

represent a species difference, for there is ample evidence that the rat and the bat do not react in the same way to dietary cobalamin deficiency or cobalamin inactivation by N_2O. Thus both dietary deficiency and cobalamin inactivation lead to neuromuscular changes in the bat (Green *et al.* 1975; van der Westhuyzen *et al.* 1982) but not in the rat (Dinn *et al.* 1978) and cobalamin inactivation is associated with a consistently abnormal deoxyuridine suppression test in the rat (Deacon *et al.* 1980*a*) but not in the bat (S. V. van Tonder, unpublished results).

The results of measurement of Ado Met concentrations in the brain suggest that either (1) the betaine-dependent synthesis of methionine can compensate (and possibly over-compensate) for the N_2O-impaired methyltransferase reaction, or (2) 80–90% inhibition of the cobalamin-dependent methyltransferase does not significantly impair the provision of methionine in vivo.

Finkelstein *et al.* (1982) have suggested that the betaine–methyltransferase plays an essential role in the synthesis of methionine (and hence of Ado Met) in rats given a limited-methionine diet (3 g/kg). Since fruit (such as banana) is low in protein (less than 15 g/kg wet weight), the diet of the bats is comparable with the low-methionine diet fed to the rats. Under these nutritional conditions, maintenance of methionine concentrations may be an essential function of the betaine-dependent reaction and is consistent with the increased level of this enzyme in the livers of rats given diets low in methionine (Finkelstein *et al.* 1982). The role of this synthetic pathway in the N_2O-treated bats remains to be evaluated.

The difference in the effect of N_2O exposure on the Ado Met concentrations in brain and liver may be of importance. N_2O exposure resulted in an increase of almost 50% in Ado Met levels in the brain compared with control animals. No such change was manifest in the liver, where a 10% reduction in Ado Met levels occurred although the difference did not reach statistically significant levels (Table 1). Thus it seems possible that in severe inhibition of cobalamin, as occurs in N_2O exposure, there is diversion of methionine or Ado Met from major sites in organs such as the liver to the nervous system. This may be a mechanism whereby the body ensures an adequate supply of Ado Met to critical organs such as the brain. Possible supportive evidence for this suggestion is the finding that the total Ado Met in brain and liver varied within relatively narrow limits in the control and test animals.

When additional methyltetrahydrofolate was given to N_2O-treated bats (PGA in the diet is converted to methyltetrahydrofolate during absorption from the gut and transport to the liver (Steinberg *et al.* 1981)) the mean level of Ado Met was lower in the brain and liver of these animals than in the brain and liver of N_2O-treated bats receiving the basal diet. This may be the result of an increased flux of methionine through the folate-dependent reaction with subsequent decreased flux through the betaine pathway. Methionine supplementation had a similar effect in reducing mean brain Ado Met concentrations in N_2O-treated bats, bringing them closer to those of the controls. This probably resulted from the decreased dependence on the synthetic pathway when more methionine was available in the diet.

Previous studies have indicated that with higher levels of methionine supplementation of the diet (1 g/kg), the N_2O-treated bats do not develop neuromuscular impairment for at least 14 weeks (van der Westhuyzen *et al.* 1982). Thus, although methionine can prevent the N_2O-induced neuromuscular impairment, the results of the present study indicate that this effect is not mediated through the correction of any deficiency of Ado Met. The action of methionine is thus through another pathway, possibly through the provision of additional formate groups (Chanarin *et al.* 1980). Formate is normally derived from the oxidation of methyl groups, with methionine being an important source (Case & Benevenga,

1977). Thus, failure of methionine synthesis may lead to a lack of formate and consequently to inadequate formylation of tetrahydrofolate (Fig. 1). Formyltetrahydrofolate is the preferred substrate for the synthesis of folate polyglutamate (folate enzymes) and, hence, impairment of folate polyglutamate synthesis compromises general folate metabolism (Chanarin *et al.* 1980). The relationship of these reactions to the N_2O-induced neuropathy remains to be evaluated.

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