

Dissociation of methionine synthetase (*EC* 2.1.1.13) activity and impairment of DNA synthesis in fruit bats (*Rousettus aegyptiacus*) with nitrous oxide-induced vitamin B₁₂ deficiency

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1. The effect of methylcobalamin inactivation by the anaesthetic gas nitrous oxide on the activity of the cobalamin-dependent methionine synthetase (5-methyltetrahydrofolate homocysteine methyltransferase; *EC* 2.1.1.13) reaction, and on DNA synthesis, in the fruit bat *Rousettus aegyptiacus*, was examined.
2. Methionine synthetase activity in the liver of bats exposed to N₂O–oxygen (50:50, v/v) for 90 min/d averaged 32% of that of controls after 4 d of exposure and only 5% after 12–14 weeks of exposure.
3. DNA synthesis in the bone marrow, as reflected by the deoxyuridine suppression test, was unaffected by 4 d of exposure to N₂O and only minimally affected after 5–10 weeks of exposure.
4. These results suggest that DNA synthesis in the fruit bat is unusually resistant to inhibition of methionine synthetase and imply the existence of a non-methylated circulating folate pool in this species.

Nitrous oxide oxidizes active reduced cob(I)alamin to inactive cob(III)alamin (Banks *et al.* 1968). Exposure to the gas results in inhibition of one of the mammalian vitamin B₁₂-dependent enzymes, methionine synthetase (5-methyltetrahydrofolate homocysteine methyltransferase; *EC* 2.1.1.13) (Deacon *et al.* 1978), which requires methylcobalamin as cofactor. In humans (Amess *et al.* 1978), monkeys (Dinn *et al.* 1978) and rats (Deacon *et al.* 1980 *a, b*) the inhibition of methionine synthetase induced by short-term exposure to N₂O is accompanied by abnormal *de novo* synthesis of DNA, as demonstrated by the deoxyuridine (dU) suppression test (Killman, 1964; Metz *et al.* 1968). It is probable that inhibition of methionine synthetase is related directly to abnormal DNA synthesis (Herbert & Zalusky, 1962; Noronha & Silverman, 1962; reviewed by Das & Herbert, 1976). Prolonged exposure to N₂O also depletes the animal's vitamin B₁₂ stores (Kondo *et al.* 1981; van der Westhuyzen *et al.* 1982).

In the fruit bat (*Rousettus aegyptiacus*) deprived of dietary vitamin B₁₂, severe deficiency of the vitamin ensues (Green *et al.* 1975) but, in contrast to man and other animals, DNA synthesis remains unaffected as shown by a normal dU suppression test (Miller *et al.* 1980). This unexpected observation led to the present study of the dU suppression test in fruit bats exposed to N₂O, for the degree of inhibition by N₂O of the methylcobalamin-dependent methionine synthetase reaction may be greater than that associated with dietary vitamin B₁₂ deprivation since, in the latter situation, some metabolically-active coenzyme is likely always to be present.

The results indicate that short-term exposure to N₂O caused severe inhibition of methionine synthetase activity without affecting DNA synthesis. With long-term exposure, methionine synthetase activity fell to almost undetectable levels, yet the effect on DNA synthesis was minimal.

* For reprints.

MATERIALS AND METHODS

Experimental animals

Fruit bats (*Rousettus aegyptiacus*) were captured in the wild and maintained on a pest-free, all-fruit diet (Green *et al.* 1975; van Tonder *et al.* 1975), supplemented with an oral vitamin B₁₂-free multivitamin preparation ('Abidec'; Parke Davis) to prevent the development of deficiency of other vitamins (van der Westhuyzen *et al.* 1982).

Experimental procedure

Bats were exposed in a chamber to an atmosphere of N₂O-oxygen (50:50, v/v) for 90 min daily. During exposure, levels of carbon dioxide and water vapour were controlled. Both short-term (4 d) and long-term (5-14 weeks) exposures were studied. At the end of the experiment, bats were killed by exsanguination via cardiac puncture and the liver removed immediately after death and stored on ice. Bone marrow was removed from the long bones and placed in ice-cold Hanks balanced salt solution (without pH indicator). Paired control and N₂O-exposed bats were killed at the same time. The dU suppression test on marrow, and methionine synthetase assay on liver, were carried out immediately after the death of the animals.

Methionine synthetase assay

Methionine synthetase activity in the liver was assayed by the method of Taylor & Wiessbach (1971) in which the rate of formation of [¹⁴C]methionine from homocysteine in the presence of [¹⁴C]methyltetrahydrofolate and S-adenosylmethionine is measured. All assays were performed in triplicate. The [¹⁴C]methyltetrahydrofolate was obtained from Amersham International (Amersham, Bucks, UK), L-homocysteine from Sigma Chemical Co. (Poole, Dorset, UK) and S-adenosylmethionine from Boehringer Mannheim.

dU suppression test

The dU suppression test was performed on bone marrow cells as described previously (Metz *et al.* 1968; Miller *et al.* 1980). dU, which is incubated with the cell suspension in the first part of the test, is normally converted into thymidine in a folate-requiring step and incorporated into DNA. Vitamin B₁₂ coenzyme activity is assayed indirectly, since in the methionine synthetase reaction it is required for the conversion of the circulating form of folate (methyltetrahydrofolate) into the form involved in thymidine synthesis (5,10-methylenetetrahydrofolate). In the second part of the test, preformed [³H]thymidine is added to the cell suspension so that any requirements for DNA synthesis not met by synthesis from dU is met by incorporation of [³H]thymidine. The result is expressed as:

$$\frac{[3\text{H}]\text{thymidine uptake after dU}}{[3\text{H}]\text{thymidine uptake alone}} \times 100.$$

Normal human marrow meets more than 90% of its thymidine requirements from dU so that less than 10% [³H]thymidine is utilized. Abnormal values (> 10%) are obtained in cobalamin deficiency (Metz *et al.* 1968).

Statistical analyses

Statistical evaluation was carried out using Student's *t*-test (two-tailed). The level of significance was chosen as $P < 0.05$. Results are expressed as means with their standard errors.

Table 1. *Methionine synthetase (5-methyltetrahydrofolate homocysteine methyltransferase; EC 2.1.1.13) activity in the liver of control and nitrous oxide-exposed fruit bats (Rousettus aegyptiacus)*

(Mean values with their standard errors)

Group	n	Enzyme activity		
		nmol/h per mg protein		Percentage of control
		Mean	SE	
Control	6	3.9	0.8	100
N ₂ O				
4 d	7	1.3*	0.5	32
12-14 weeks	3	0.4*	0.1	5

Mean value was significantly different from control value: * $P < 0.02$.Table 2. *Results of the deoxyuridine (dU) suppression test with marrow cells of normal and nitrous oxide-exposed fruit bats (Rousettus aegyptiacus)*

(Mean values with their standard errors)

Group	n	$\frac{\text{Counts with dU} + [^3\text{H}]\text{thymidine}}{\text{counts with } [^3\text{H}]\text{thymidine alone}} \times 100$								
		dU alone			dU + PGA			dU + cyanocobalamin		
		Mean	SE	Range	Mean	SE	Range	Mean	SE	Range
Control	6	1.5	0.3	0.9-3.1	1.3	0.3	0.8-2.9	1.3	0.2	0.7-2.5
N ₂ O										
4 d	5	1.4	0.3	0.7-2.0	1.2	0.4	0.6-2.0	1.4	0.4	0.5-2.4
5-10 weeks	10	4.4*	1.0	0.9-10.5	2.7	0.6	0.8-7.0	3.4	0.7	0.8-8.4

PGA, pteroylglutamic acid.

Mean value was significantly different from control value: * $P < 0.05$.

RESULTS

Methionine synthetase activity (Table 1)

Methionine synthetase activity was decreased in the liver of all bats exposed to N₂O compared with that of control animals. In short-term exposure, enzyme activity averaged 32% of that of control animals, and with prolonged exposure, the activity was almost undetectable ($\pm 5\%$). The difference in degree of reduction in both short-term and long-term exposures, as compared with control animals, was statistically significant ($P < 0.02$).

dU suppression test (Table 2)

The mean degree of suppression by dU in the bone marrow of bats after 4 d of exposure to N₂O (mean 1.4 (SE 0.3)%) did not differ significantly from that in control animals (mean 1.5 (SE 0.3)%). However, after long-term exposure to N₂O (mean 6.6 (SE 0.4)weeks) the degree of suppression by dU (mean 4.4 (SE 1.0)%) was significantly less than that of control

animals ($P < 0.05$) and of animals following short-term exposure to N_2O , although in the latter case the difference just failed to be statistically significant. The addition of either folate in the form of pteroylglutamic acid (PGA) or cyanocobalamin to the marrow cultures of animals after long-term exposure to N_2O produced a greater degree of dU suppression, so that the differences in the results of the dU suppression test between these and control animals was no longer statistically significant (Table 2). The corrective effect of PGA was greater than that of cyanocobalamin.

DISCUSSION

The effect of methionine synthetase activity in the liver or brain, or both, of rats exposed to N_2O has been reported by Deacon *et al.* (1978, 1979, 1980*a, b*) and Kondo *et al.* (1981). Deacon *et al.* (1978, 1979, 1980*a, b*) found the levels of enzyme activity in the liver of control rats to range from 1.4 to 3.4 nmol/h per mg protein; the synthetase activity after 30 min exposure to N_2O-O_2 (50:50, v/v) declined to 0.35–0.7 nmol/h per mg protein (30% of control) and, after 6 h of exposure, was virtually absent (0.03–0.04 nmol/h per mg protein; 6% of control). The dU suppression test became abnormal shortly after the inactivation of the enzyme. Brain methionine synthetase was depressed in a similar manner by exposure to N_2O . Kondo *et al.* (1981) also showed a fall in activity in the liver of rats exposed to N_2O-O_2 (50:50, v/v), but the degree of inhibition of the enzyme was less than that reported by Deacon *et al.* (1978, 1979, 1980*a, b*). Thus 15 min of exposure caused the level of methionine synthetase activity to decrease to 45% of control values, and activity fell to 30% after 1 h and remained at this level for exposure periods up to 2 d. With longer exposure, methionine synthetase activity decreased further to 18 and 12% of control values after 16 and 33 d respectively.

The results of the present study show that in the fruit bat too, exposure to N_2O-O_2 (50:50, v/v) results in inhibition of the methionine synthetase enzyme. Deacon *et al.* (1978, 1979, 1980*a, b*) and Kondo *et al.* (1981) exposed rats continuously to N_2O . This is not possible in the bat, a free-flying animal, and in the present experiments exposure was intermittent (90 min/d). However, it is most unlikely that the effects of intermittent exposure would be different from that of continuous exposure, for the rate of recovery of methionine synthetase following exposure to N_2O is extremely slow (Deacon *et al.* 1979; Kondo *et al.* 1981). The degree of inhibition of the enzyme in relation to the length of exposure was less in the bat than that reported by Deacon *et al.* (1979) in the rat, and more like that reported by Kondo *et al.* (1981) in the rat. The values reported by Deacon *et al.* (1979) and Kondo *et al.* (1981) for the rat differ in the degree of methionine synthetase inhibition produced by N_2O-O_2 (50:50, v/v) making comparison with the values for the bat difficult. However, the important finding was that with adequate exposure (12–14 weeks), methionine synthetase activity virtually disappeared from bat liver.

Deacon *et al.* (1980*b*) reported that in five marrows from control rats the dU suppression test mean was 6.6%. After 60 min of exposure to N_2O it became abnormal, and increasingly abnormal till 6 h. There was usually a small improvement in the test of the order of 2–3% when 10 μ g hydroxycobalamin was added, and a somewhat greater fall when folate in the form of folic acid (30 μ g) was added in vitro. In another paper, Deacon *et al.* (1980*a*) reported on studies of rats exposed to N_2O-O_2 (50:50, v/v) for 3 h. In forty control rats, the mean dU suppression was 7.3%. After 3 h of exposure to N_2O the dU suppression test was abnormal, the mean being 15.7%. There was no significant improvement in dU utilization on addition of 10 μ g PGA; an improved dU utilization occurred with the addition of formylfolate.

The results in man differ from those reported in rats by Deacon *et al.* (1980*a, b*). In

patients receiving N₂O–O₂ (50:50, v/v) for 24 h, the mean dU suppression was 19.9% (controls mean 4.0%) (Amess *et al.* 1978). The addition of 4 µg cyanocobalamin or 40 µg PGA produced significant correction in the dU suppression test, to 11.2 and 9.0% respectively. These results were confirmed in a further study of forty-two patients receiving N₂O–O₂ (50:50, v/v) (Amos *et al.* 1982). The dU suppression was abnormal in almost all patients, and significant partial correction occurred on addition of cyanocobalamin or PGA.

The effect of N₂O exposure on the dU suppression test in the bat, as shown in the present study, differed from those reported for man and the rat. Thus exposure of the bat to N₂O for 4 d failed to affect the dU suppression test. After 5–10 weeks of exposure to the gas, the dU suppression test in the bat was only mildly affected, in spite of the fact that methionine synthetase activity in the liver was virtually absent.

Methionine synthetase is the enzyme responsible for demethylating methyltetrahydrofolate (Poston & Stadtman, 1975), the major transport form of folate in man (Herbert *et al.* 1962) and probably in most other mammals (Scott & Weir, 1976). Without the initial demethylation step, folate is not available for intracellular metabolism (Scott & Weir, 1976) and normal dU utilization will not occur (reviewed by Das & Herbert, 1976) since its conversion to 5,10-methylenetetrahydrofolate is impaired.

Thus, depression of methionine synthetase appears to be the basis of the abnormal dU suppression test in vitamin B₁₂ deficiency in humans (Killman, 1964; Metz *et al.* 1968), and following exposure to N₂O in humans (Amess *et al.* 1978), rats (Deacon *et al.* 1980a) and monkeys (Dinn *et al.* 1978).

DNA synthesis in the fruit bat appears to be unusually resistant to inhibition of methionine synthetase since neither profound dietary vitamin B₁₂ deficiency (Miller *et al.* 1980) nor short-term N₂O exposure affects the dU suppression test. Only prolonged N₂O exposure results in abnormal dU suppression and even then the effect is slight. This is contrary to the findings in other mammalian species in which short-term exposure to N₂O has an immediate effect on the dU suppression test. As in other animals, dU suppression in the fruit bat is folate-dependent, for the addition of methotrexate, a potent inhibitor of the key enzyme dihydrofolate reductase (*EC* 1.5.1.3), results in a markedly abnormal dU suppression test (Miller *et al.* 1980). Thus the N₂O-treated bat has adequate metabolically active folate despite inhibition of the enzyme which in other species plays a key role in the provision of this form of folate.

The possible reason for the resistance of the fruit bat to the effects of dietary vitamin B₁₂ deficiency or N₂O exposure on DNA synthesis may be related to the finding that the fruit bat has a non-methylated pool of circulating folate which is separate from the methyltetrahydrofolate pool (Perry *et al.* 1979). Folate from the non-methylated pool would be able to enter the metabolically active pool directly, by-passing the methionine synthetase-dependent step. Such a mechanism may have evolved in the fruit bat since, in the wild, it subsists on an exclusive fruit diet (fruit contains no vitamin B₁₂), and thus its vitamin B₁₂ nutrition might be expected to be marginal. A protective pathway to serve as an escape from the effects of vitamin B₁₂ deficiency would obviously be advantageous.

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