INHIBITION OF RAT FETAL METHIONINE SYNTHASE BY NITROUS OXIDE
AN IN VITRO STUDY

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Nitrous oxide administered to pregnant rats at the end of gestation inhibits both maternal and fetal hepatic methionine synthase in a concentration- and time-dependent manner (Baden, Serra and Mazze, 1984). Inhibition in the fetus, however, lags behind that in the mother and it is uncertain whether this difference is attributable to the time taken for the fetal concentration of nitrous oxide to reach the maternal concentration or whether there is an intrinsic difference between the fetal and maternal enzyme. To help answer this question, we have examined the inhibition of fetal and maternal methionine synthase by nitrous oxide in vitro. We also have made observations about the in vitro stability of the enzyme and the effect of oxygen on its activity.

MATERIALS AND METHODS

Nine timed-pregnant Sprague-Dawley rats weighing 200–300 g were obtained from the breeder (Hilltop Lab Animals, Chatsworth Ca. 91311) on day 15 of gestation. They were bedded on ground corn cob (Bed O'-Cobs, Anderson's Cob Division, Maumee, Oh. 43527) and housed in polypropylene cages with stainless steel lids. Food (Wayne Lab-Blox, Allied Mills, Inc., Chicago, Il 60951) and water were allowed ad libitum.

On day 19 of gestation, the rats were killed by exposure to 100% carbon dioxide. They were bedded on ground corn cob (Bed O'-Cobs, Anderson's Cob Division, Maumee, Oh. 43527) and housed in polypropylene cages with stainless steel lids. Food (Wayne Lab-Blox, Allied Mills, Inc., Chicago, Il 60951) and water were allowed ad libitum.

On day 19 of gestation, the rats were killed by exposure to 100% carbon dioxide. Maternal livers were removed, perfused with cold 1.15% potassium chloride, and immediately frozen in liquid nitrogen. Fetuses were delivered by Caesarean section and placed on ice for about 10 min to ensure complete insensibility. Livers of fetuses from each litter were then removed, pooled and frozen in liquid nitrogen.

At the time of the study, approximately 1 g of liver was thawed and homogenized in 5 ml of potassium phosphate buffer 0.05 mol litre⁻¹ (pH 7.4) with a Brinkman Polytron homogenizer for 20–30 s at three-quarters speed. The resultant homogenate was centrifuged in a Sorvall RC2-B centrifuge at 20000 g for 80 min at 4°C. The protein content of the supernatant was measured by a modification of the method of Lowry and co-workers (1951), and adjusted to 10 mg ml⁻¹ by addition of potassium phosphate buffer.

In vitro exposures were carried out in capped, 13 x 100-mm silicon-coated test tubes containing
0.5-ml aliquots of supernatant (protein 5 mg). The head space of each tube was filled with a 50% nitrous oxide–50% oxygen mixture. Concentrations of nitrous oxide and oxygen were measured at the beginning and end of exposure with a Miran 1A infra-red gas analyser and an Instrumentation Laboratory 401 oxygen analyser, respectively. Samples were incubated with gentle agitation for 15, 30, 45, 60, 90 or 120 min in a water bath at 37 °C. In addition, five maternal and fetal samples were incubated for 24 h. At the end of exposure, tubes were uncapped, flushed with air and the samples frozen immediately at —4 °C. Control samples were treated in exactly the same way except that they were exposed to 50% oxygen and 50% nitrogen. In a further study, maternal and fetal samples were incubated for 24 h at 37 °C with air alone, to determine the stability of the enzyme over this period.

Methionine synthase was assayed according to the method described by Koblin and colleagues (1982). A 200-μl total volume of the methionine synthase reaction mixture contained: sodium phosphate buffer 20 μmol (pH 7.4), mercaptoethanol 25 μmol, adenosine methionine 50 mmol, vitamin B<sub>12</sub> 10 nmol, homocysteine 50 nmol (freshly prepared from the lactone form), methyltetrahydrofolate 120 nmol (1 μCi of [5-<sup>14</sup>C]-MTHF), and 40 μl of either supernatant or phosphate buffer. Following incubation for 1 h at 37 °C, the reaction was stopped by the addition of 800 μl of cold distilled water. The resultant 1.0-ml mixture was layered on a Dowex 1-X8 anion exchange resin (200-400 mesh; chloride form) column, previously equilibrated with sodium phosphate buffer 0.1 mol litre<sup>−1</sup> (pH 7.4). The eluate was collected after washing with 3 ml of buffer. An aliquot of eluate was mixed with Aquasol 2, 10 ml and counted in a Beckman LS330 scintillation counter. Methionine synthase activity was expressed as nmol of methionine produced in 1 h per mg of protein.

Enzyme concentrations in maternal and fetal liver at the same time point were compared using the Student t test. Values at different time points within the same group were compared using analysis of variance and Newman-Keuls post hoc test. The difference between the inhibition of maternal and fetal enzymes was analysed by calculating areas under the activity-time curves using the trapezoid rule and comparing them using the Student t test. P < 0.05 was considered significant.

**RESULTS**

Control methionine synthase activity in fetal liver was 65% of that in maternal liver (table I).
Activity in both fetal and maternal liver decreased progressively in a time-dependent manner following exposure to 50% nitrous oxide (fig. 1). The patterns of inhibition during the first 2 h of exposure were very similar. In particular, areas under the percentage activity-time curves during this period were not statistically different from each other. After 24 h exposure, activity was about 14 and 17% of fetal and maternal control activity, respectively (table I).

Concentrations of oxygen and nitrous oxide within the capped test tubes remained constant—even throughout the 24-h incubations. Also, there was no loss of activity of methionine synthase in frozen liver over the 3 months during which the studies were conducted. Even in diluted supernatant at 37 °C, the enzymatic activity decayed only slowly. After incubation for 24 h with gentle agitation at 37 °C in the presence of air, activity for both fetal and maternal enzyme was about 75% of initial activity (table I). A similar loss of activity was found after incubation with 50% oxygen under the same conditions, suggesting that this oxygen concentration had no inhibitory effect on methionine synthase.

**DISCUSSION**

When 50% nitrous oxide is administered to pregnant rats at the end of gestation, the time-dependent inhibition of methionine synthase in fetal liver lags considerably behind that in maternal liver (Baden, Serra and Mazze, 1984). For example, after 15-min exposure, activity of the enzyme decreases by 15% in fetal liver, but by 70% in maternal liver; a loss of 70% activity in fetal liver is achieved only after about 30 min. The most likely explanation for the difference is that equilibration to nitrous oxide is achieved more slowly in the fetus than in the mother and, therefore, the fetus is exposed to a lower average concentration. However, an alternative explanation might be that fetal and maternal enzymes are different or that the fetal enzyme is protected from the effects of nitrous oxide. Results from the present study support the equilibration hypothesis, since the difference between the pattern of inhibition of fetal and maternal methionine synthase was eliminated after *in vitro* exposure to nitrous oxide.

Methionine synthase proved to be fairly stable in dilute solution and was, therefore, easy to work with *in vitro*. After 24 h of incubation at 37 °C, only a 25% loss of activity was observed in untreated samples. Although the loss was small, it emphasizes the importance of running concurrent controls, especially if incubations are prolonged. Methionine synthase was not affected additionally by 24 h of incubation in the presence of 50% oxygen instead of air. This finding is consistent with that of other investigators (Sharer, Monk and Nunn, 1983) who found that hepatic methionine synthase activity in rats was unaffected by 4 h of exposure to two atmospheres of oxygen.

The significance of fetal exposure to nitrous oxide near the end of gestation has not been established. During routine Caesarean section, exposure probably is not long enough to decrease significantly the activity of methionine synthase. In a recent study, Landon and Toothill (1986) reported that methionine synthase activity in 11 placentae after Caesarean section during which nitrous oxide was administered, was not significantly different from that in 20 placentae after normal vaginal delivery. Exposure to nitrous oxide averaged less than 20 min. Our previous findings that exposure *in vivo* for 15 min did not significantly decrease enzyme activity in fetuses of rats (Baden, Serra and Mazze, 1984)—a species more sensitive to the biochemical effects of nitrous oxide than is man (Koblin et al., 1982)—is consistent with the data from the human placenta.

Longer exposure near the end of gestation would be expected to decrease fetal enzyme activity but, again, with uncertain consequences. Gross structural abnormalities would not be expected, and have not been reported, because the period of organogenesis is well passed. However, prolonged nitrous oxide administration is known to produce neurotoxicity in adult humans (Layzer,
1978; Brodsky et al., 1981). Animal studies have confirmed that the mechanism is a demyelination process resulting from an inadequate supply of methionine (Scott et al., 1981; van der Westhuyzen, Fernandes-Costa and Metz, 1982). Although neurotoxicity in adults occurs only after chronic exposure to nitrous oxide, the developing nervous system undergoing maturation, including myelination, may be sensitive to insult. Thus, a one-time exposure of fetuses to nitrous oxide could possibly induce discrete structural changes and result in neurobehavioural deficits. This was suggested in a review by Brackbill (1979), who noted that administration of inhaled anaesthetics, including nitrous oxide, to the mother at delivery was associated with poorer infant performance scores than was administration of local anaesthetics. However, most of the studies she reviewed were confounded by other factors which occurred at the time of delivery, such as the administration of additional drugs and the presence of underlying medical disease.

There is, however, experimental evidence from animals to suggest that nitrous oxide causes histological changes in the brain and neurobehavioural abnormalities. Cell proliferation in developing brains of mice exposed to 75% nitrous oxide for 6 h on day 14 of pregnancy showed some deviation from normal (Rodier et al., 1986). More significantly, exposure to nitrous oxide, but not to halothane, for 4 h on day 2 of the postnatal period produced a pattern of reduced cell proliferation followed by rebound that is characteristic of many antimitotic teratogens. Under the same conditions, long-lasting hypoactivity and behavioural deficits indicative of developmental neural damage occurred, although these effects were not unique to nitrous oxide, but were seen also with halothane (Koeter and Rodier, 1986; Rodier and Koeter, 1986). In another study, Rice and Millan (1985) exposed mice to 5, 15 or 35% nitrous oxide for 4 h per day on days 6–15 of pregnancy. Startle reflex hyporeactivity occurred in all exposed to nitrous oxide and was still present 3 months after delivery. Although these data are preliminary and not entirely unique to nitrous oxide, they point to the need for further clinical studies to determine whether nitrous oxide is a behavioural teratogen and, if so, whether inhibition of methionine synthase may play a role in this effect.

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REFERENCES


