

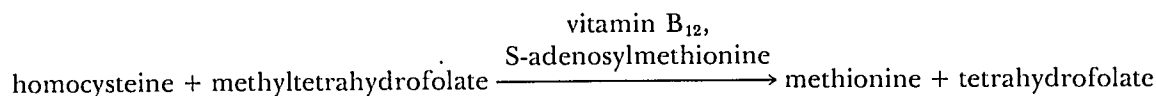
Inactivation of Methionine Synthetase by Nitrous Oxide in Mice

Donald D. Koblin, Ph.D.,* Joe E. Watson,† Joan E. Deady, M.S.,‡
E. L. R. Stokstad, Ph.D.,§ Edmond I. Eger II, M.D.¶

To characterize the nitrous oxide-induced inhibition of the enzyme, methionine synthetase, we measured enzyme inactivation as a function of nitrous oxide concentration and exposure time. Mice exposed to 0.8 atm nitrous oxide exhibited more than a 50 per cent decrease in liver methionine synthetase activity within 30 min, and activity dropped to 5-25 per cent of the original value after a 4-hour exposure. Although 4-hour exposures to low nitrous oxide partial pressures (less than 0.05 atm) did not significantly alter methionine synthetase activity, higher concentrations of nitrous oxide caused a progressive inhibition over this time period. Continuous exposure to trace levels of nitrous oxide (approximately 1100 ppm) for eight to 22 days produced a small but significant reduction in liver and brain methionine syn-

thetase activity. Methionine synthetase activity returned to control levels two to four days following inactivation. Other anesthetics (xenon, halothane, isoflurane, enflurane) did not produce inactivation. (Key words: Anesthetics, gases: nitrous oxide; xenon. Anesthetics, volatile: halothane; enflurane; isoflurane. Brain. Liver: enzymes, methionine synthetase. Metabolism.)

IN RATS, exposure to nitrous oxide decreases methionine synthetase activity in the liver¹⁻³ and brain.³ Methionine synthetase is a vitamin B₁₂-dependent enzyme that synthesizes the amino acid methionine from homocysteine, according to the following scheme:



Nitrous oxide inhibits this enzyme by oxidizing the cobalt atom of vitamin B₁₂ from an active Co(I) state, which arises as an intermediate in the transfer of the methyl group of methylcobalamin to produce methionine,⁴ to an inactive Co(II) or Co(III) state.^{5,6} In the process, nitrous oxide becomes reduced to nitrogen and oxygen.^{5,6}

Inactivation of methionine synthetase by nitrous oxide prevents the conversion of methyltetrahydrofolate to tetrahydrofolate through the enzymatic pathway given above. This inactivation may decrease

the intracellular concentration of tetrahydrofolate and inhibit the production of 5:10 methylene tetrahydrofolate, a requirement for DNA synthesis.⁷ A transient increase in the amount of methyltetrahydrofolate is found in rat liver 24 hours after exposure to nitrous oxide, but declines thereafter.⁸ The inactivation of methionine synthetase induced by nitrous oxide and the possible depletion of tetrahydrofolate may account for the abnormalities found in bone marrow and the signs of vitamin B₁₂ deficiency that occur in patients chronically treated with nitrous oxide.^{7,9,10} The fact that DNA synthesis is altered in bone marrow taken from humans⁷ and rats¹¹ treated with nitrous oxide, also supports this theory. Inactivation of methionine synthetase by nitrous oxide may also play a role in the development of polyneuropathy¹²⁻¹⁵ and spinal cord lesions¹⁶ seen after prolonged exposure to subanesthetic and even trace levels of nitrous oxide.¹⁵

Previous studies of inhibition of methionine synthetase by nitrous oxide have examined only the influence of relatively high concentrations (50-80 per cent) of nitrous oxide.¹⁻³ Because the effects of this process may be important to patients and operating room personnel, we studied the inactivation of methionine synthetase produced by different concentrations of nitrous oxide, and whether prolonged exposure to trace levels would also produce inactivation. In addition, we examined the time course of inactivation.

* Assistant Research Chemist, University of California, San Francisco.

† Staff Research Associate, University of California, Berkeley.

‡ Staff Research Associate, University of California, San Francisco.
§ Professor of Nutritional Sciences, University of California, Berkeley.

¶ Professor of Anesthesiology, University of California, San Francisco.

Received from the Department of Anesthesia, University of California, San Francisco, California 94143, and the Department of Nutritional Sciences, University of California, Berkeley, California 94720. Accepted for publication October 9, 1980. Supported in part by NIH Anesthesia Research Center Grant GM-15571, NIH Research Grant AM08171, the Anesthesia Research Foundation, and the Cryogenic Rare Gas Laboratories. Presented at the annual meeting of the American Society of Anesthesiologists, St. Louis, Missouri, October 1980.

Address reprint requests to Dr. Koblin: Department of Anesthesia, University of California, Room HSE 1386, Third Avenue and Parnassus Street, San Francisco, California 94143.

tion, the time course of recovery of activity, and the possibility that other anesthetics may also produce inactivation.

Methods

Adult, male CD-1 mice (Charles River) initially weighing 23–27 g were provided Purina® Chow and water *ad libitum*. The methionine content of this diet is 0.43 per cent (w/w). Animals were exposed to a light:dark cycle of 12 hours:12 hours.

To study short-term exposures (0–4 hours) to nitrous oxide, groups of eight mice were placed in plastic cages with wire-mesh tops and loaded into a 20-l pressure chamber.¹⁷ The chamber was flushed with 100 per cent oxygen for 10 min, and the desired dose of N₂O was added to produce an ambient pressure between 1.0 and 1.8 atm. The use of slightly increased atmospheric pressures allowed all short-term experiments to be conducted at the same background partial pressure of oxygen (1 atm). Chamber gases were blown through a soda-lime container with an induction motor-driven fan to remove carbon dioxide. Chamber temperature was approximately, 27–30° C during the run. For a 4-hour exposure, the eight mice would typically consume enough oxygen to reduce the chamber pressure approximately 0.20 atm.

At the end of the exposure period, a sample of gas was removed from the chamber through a bleed valve with a glass syringe and was analyzed by gas chromatography with thermal conductivity detection. The exact partial pressure of nitrous oxide at the end of the exposure was calculated. Following removal, the mice were killed by cervical dislocation, and their livers were rapidly removed and frozen at –20° C. All livers were removed within 25 min of the end of the run.

Experiments with xenon or nitrogen were carried out in a similar manner. However, partial pressures for these two gases were calculated by measuring the partial pressure of oxygen in the pressure chamber with a Beckman® E2 oxygen analyzer at the end of the run, and by subtracting this pressure from the total pressure.

Exposures to the volatile agents halothane, isoflurane, and enflurane were carried out in the same chamber but occurred at atmospheric pressure. Oxygen (4 l/min) and controlled amounts of anesthetic (delivered from a temperature-compensated vaporizer) continuously passed through the chamber for the 4-hour exposure period. Concentrations of volatile anesthetics were measured by gas chromatography with a stainless steel column, 120 cm in length and 1/8 in in diameter, and packed with 10 per cent

SF-96 on Chromasorb® WHP (60/80 mesh). Oven temperature was 50° C and nitrogen flow was 20 ml/min. Anesthetic concentrations were determined by peak height measurements from calibration curves prepared from standard tanks of each anesthetic.

For each series of experiments, the methionine-synthetase activity of eight shelf control mice was also measured. These mice were obtained in the same shipment from the supplier but were not exposed to anesthetic or placed in the pressure chamber.

To study chronic exposure (8, 15, or 22 days) to trace levels of nitrous oxide, experimental and control mice were housed in two neighboring 225-l environmental chambers. Air inflow rates into each chamber were approximately 15 l/min. In addition, the experimental chamber received N₂O at a rate of approximately 20 ml/min, as regulated by a Matheson® 610 flowmeter. Each environmental chamber was equipped with circulating blowers that withdrew the gas mixture from the chamber, passed it through a soda-lime container to remove CO₂ and through a thermoelectric cooling device to remove water vapor, and then returned it to the chamber. Environmental chamber temperatures were kept from 23.0–25.5° C. Mice were housed four to a cage in these chambers and had Absorbri® bedding. Each cage had a filter-top cover. Cages were cleaned twice weekly, at which time the mice were removed from the environmental chambers for less than 30 min. Concentrations of nitrous oxide in the experimental chamber were monitored daily. These concentrations varied from 700 to 1600 ppm over the 22-day period, the average being 1100 ppm. No nitrous oxide could be detected in the control chamber. Experimental and control mice were killed after spending 8, 15, or 22 days in the environmental chambers; their livers and brains were removed and frozen at –20° C.

Methionine synthetase was assayed after the method of Sauer and Jaenicke.¹⁸ One part of tissue was added to nine parts of 0.01 M potassium phosphate buffer (pH 7.3) and homogenized with a Brinkmann Polytron homogenizer. The suspension was centrifuged at 4° C for 80 min at 20,000 × *g*. Aliquots (100 μl) of the supernatant and 100 μl of a substrate mixture (containing 20 μM cyanocobalamin, 58 mM dithiothreitol, 0.5 mM S-adenosylmethionine, 15 mM homocysteine, 14 mM β-mercaptoethanol, 1 mM methyltetrahydrofolate with 0.25 μCi of [5-¹⁴C]-methyltetrahydrofolate, and 175 mM phosphate buffer, pH 7.5) were placed in 1.5 ml conical centrifuge tubes, which were covered with nitrogen and capped. The samples were incubated at 37° C for one hour, placed in a bath of boiling water for 2 min to stop the reaction, mixed and centrifuged. The supernatant

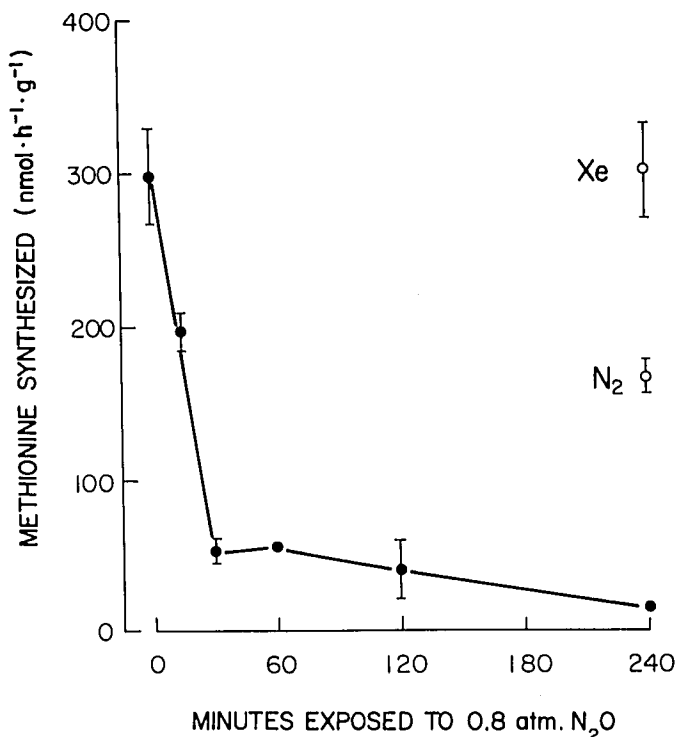


FIG. 1. Time course of inactivation of methionine synthetase in the liver by nitrous oxide. Animals were exposed to approximately 0.8 atm N₂O (range 0.78 to 0.82 atm N₂O) for varying periods of time in the presence of 1.0 atm oxygen. Also shown is methionine-synthetase activity following exposure to 0.80 atm nitrogen or 0.77 atm xenon, both in the presence of 1.0 atm oxygen for four hours. Each point represents the mean \pm SE for eight mice. For the points for which the error bars are not exhibited, the errors are within the size of the symbols. The eight shelf control mice were not exposed to anesthetic or placed in the pressure chamber.

(100 μ l) was added to the top of a Biorad[®] AG1-X8 (200–400 mesh) column, and the column was washed three times with 0.5 ml water. The total effluents were collected in a scintillation vial, 8 ml of a Triton[®] X-100 scintillation cocktail was added, and the samples were counted for radioactivity. A standard sample from rat liver was also included in each series of assays to ensure that the absolute activity was the same from experiment to experiment. Methionine-synthetase activity is expressed as the nanomoles of methionine produced per hr/g of original tissue. All assays were performed in duplicate.

Statistical significance was calculated using an unpaired *t* test for the chronic exposure studies, in which the control group was compared with the group exposed to trace levels of nitrous oxide for a given period. For the other series of experiments, in which mice were exposed to a range of nitrous oxide concentrations, to a single nitrous oxide concentration for

different time periods, or to a spectrum of different anesthetics, statistical significance was calculated using an analysis of variance employing the Newman-Keuls test for multiple comparisons.

Results

We first examined the time course of methionine-synthetase inactivation after the introduction of 0.8 atm N₂O in the presence of 1.0 atm oxygen (fig. 1). Methionine-synthetase activity in the liver progressively decreased as exposure times increased, and exposure periods as short as 15–30 min produced significant inactivation. After four hours of exposure, methionine-synthetase activity decreased to five per cent of the control value. Exposure to 0.77 atm xenon, and 1.0 atm oxygen did not alter methionine-synthetase activity. However, in this series of experiments, exposure of mice to 0.8 atm nitrogen, and 1.0 atm oxygen resulted in a 44 per cent decrease in activity from control value.

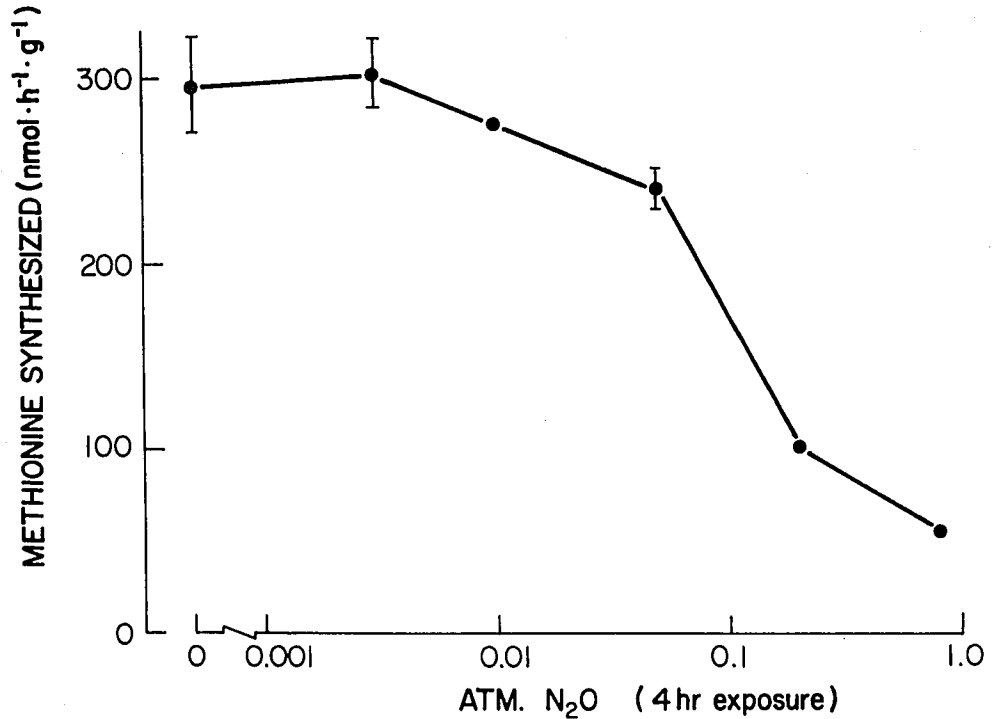
We next measured the inactivation of methionine synthetase produced by different concentrations of nitrous oxide (fig. 2). Exposures to less than 0.05 atm N₂O for four hours had no significant effect. However, if we interpolate from the curve in figure 2, enzyme activity was inhibited approximately 50 per cent after a four-hour exposure to 0.1 atm N₂O.

Chronic exposure of mice to trace levels (approximately 1100 ppm) of nitrous oxide marginally decreased methionine-synthetase activity (fig. 3). Although mice exposed for eight days had a significant ($P < 0.005$) reduction (27 per cent) in liver activity, a significant decrease in activity could not be detected in the liver after a 15- or 22-day exposure to 1100 ppm N₂O (fig. 3A). Methionine-synthetase activity in the brain was not detectably altered after an exposure of 8 or 15 days, but decreased significantly ($P < 0.005$, 13 per cent) after exposure to 1100 ppm N₂O for 22 days (fig. 3B).

Recovery of methionine-synthetase activity was nearly complete 2–4 days after a four-hour exposure to 0.8 atm N₂O (fig. 4). Activity returned to 70 per cent of its control value one day after exposure.

Volatile anesthetics did not alter methionine-synthetase activity. Four-hour exposures to halothane, isoflurane, or enflurane at concentrations that block the righting reflex had no significant effect on enzyme activity (fig. 5). It should also be noted in this series of experiments (fig. 5), that mice exposed to 0.75 atm nitrogen and 1.0 atm oxygen for four hours had the same amount of enzyme activity as did shelf controls, whereas a significant reduction was observed in a previous series of experiments (fig. 1).

FIG. 2. Concentration dependence of inactivation of methionine-synthetase activity in the liver by nitrous oxide. All exposures occurred in the presence of 1.0 atm oxygen and lasted four hours. Each point represents the mean \pm SE for eight mice. The eight shelf control mice were not exposed to anesthetic or placed in the pressure chamber. For the points for which the error bars are not exhibited, the errors are within the size of the symbols.

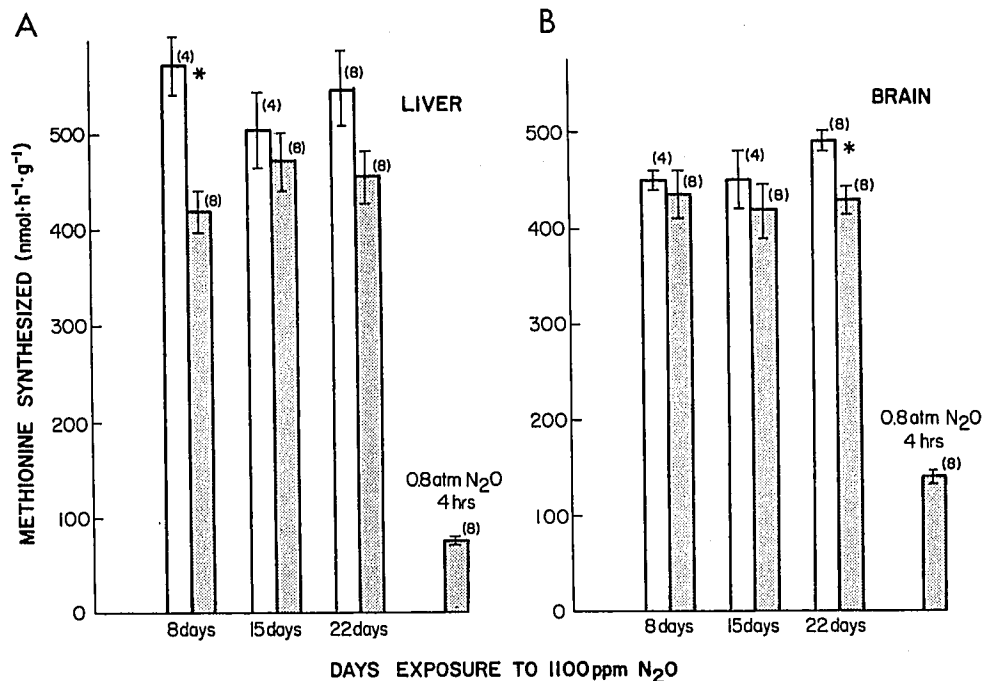


Discussion

Our goal was to quantitate the concentration and time-dependence of the inhibition of methionine-synthetase activity induced by nitrous oxide. We found, as have others,^{1,3} that methionine synthetase is inhibited by high partial pressures (0.8 atm) of

nitrous oxide for periods of exposure (15 min to 4 hours) typically employed in operating room procedures (figs. 1 and 2). However, over this same time span, nitrous oxide at partial pressures of 0.05 atm or less did not affect methionine-synthetase activity (fig. 2). Continuous exposure of mice to 1100 ppm nitrous oxide, a concentration commonly found in un-

FIG. 3. Methionine-synthetase activity in the liver (A) and whole brain (B) of mice continuously exposed to trace levels (approximately 1100 ppm) of nitrous oxide for 8, 15, and 22 days; and in control animals raised in an air environment. Each bar represents the mean \pm SE. The number of mice examined are given in parentheses. The methionine-synthetase activity in the liver of mice exposed to N₂O for 8 days and that occurring in the brain of mice exposed for 22 days differed significantly ($P < 0.005$) from control levels for mice raised in air. On the right of each graph are positive controls for this series of experiments that show the degree of enzyme inactivation after a four-hour exposure to 0.8 atm N₂O. The open bars represent the activity occurring in control mice, and shaded bars, the activity in animals exposed to N₂O.



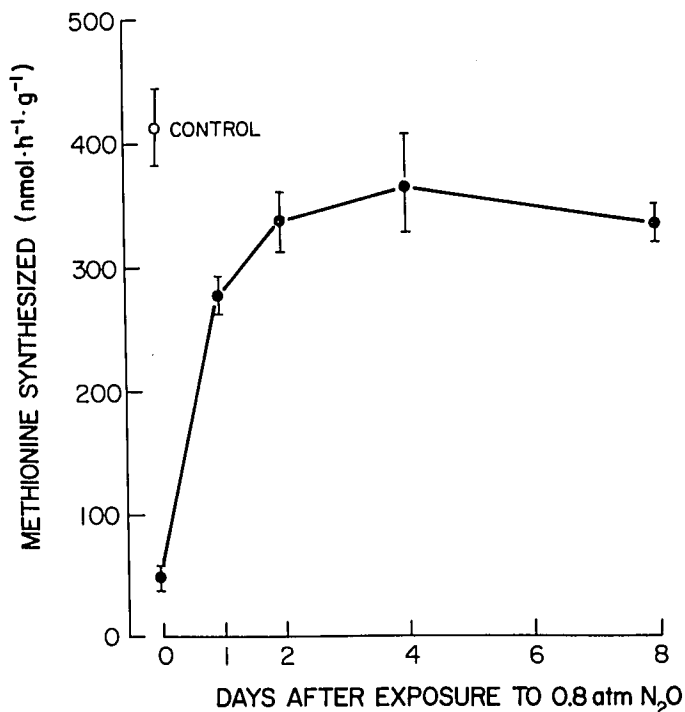


FIG. 4. Time course of recovery of liver methionine synthetase activity after a four-hour exposure to approximately 0.8 atm N_2O (range 0.76 to 0.81 atm) in the presence of 1.0 atm oxygen. Each point represents the mean \pm SE for eight mice killed either immediately after, or one, two, four, or eight days after exposure. The eight shelf control mice were not exposed to anesthetic or placed in the pressure chamber.

scavenged operating rooms, but 44 times the time-weighted average concentration recommended by NIOSH,**¹⁹ produced a small inactivation of methionine-synthetase activity in the liver and brain (fig. 3).

After methionine synthetase inactivation was induced in the liver by nitrous oxide, activity returned to control levels within 2–4 days (fig. 4). This finding qualitatively agrees with that of a previous report, which demonstrated that activity in rat liver slowly recovered over four days.³ This recovery may be due to *de novo* synthesis of the enzyme, since the addition of exogenous B_{12} to the assay medium has little influence on methionine-synthetase activity.²⁰ This lack of influence indicates that the endogenous B_{12} inactivated by nitrous oxide is tightly bound to the enzyme and cannot easily be displaced to reactivate the enzyme.

If the product of dose and exposure time is an important factor in determining the magnitude of the inactivation produced by nitrous oxide, then ex-

posure of mice to 1100 ppm N_2O for 22 days [*i.e.*, $(0.0011 \text{ atm}) \times (22 \text{ days}) \times (24 \text{ hr/day}) = 0.58 \text{ atm-hr}$] should produce essentially the same inactivation as occurs when mice are exposed to 0.15 atm N_2O for four hours [*i.e.*, $(0.15 \text{ atm}) \times (4 \text{ hr}) = 0.60 \text{ atm-hr}$]. However, a greater percentage of inactivation is found for the 4-hour period (fig. 2) compared with the 22-day period (fig. 3) for the equivalent dose-time product. The relatively higher activity found in mice chronically exposed to trace concentrations may arise from synthesis of new enzymes over this time span.

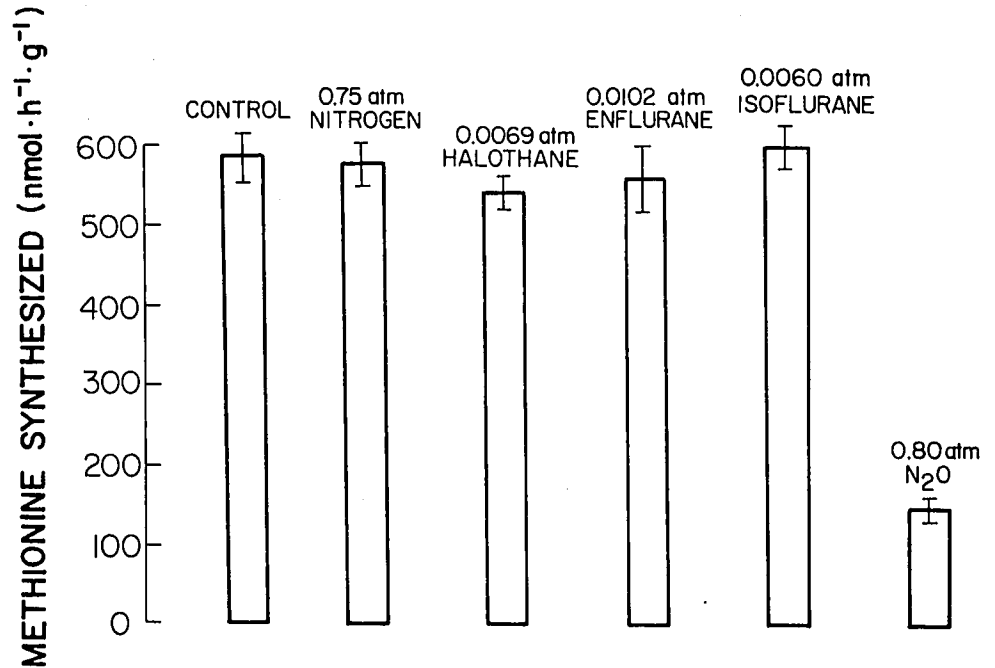
Other anesthetics (figs. 1 and 5) had no significant effect on methionine-synthetase activity, and thus anesthesia *per se* was not responsible for inactivation. A previous report³ showed that two rats exposed to 0.0035 atm halothane for six hours tended to have lower methionine synthetase-activity than did control animals. We found no significant differences between groups exposed to halothane and control groups (eight mice in each group), although there was a trend toward lower activity in the former (fig. 5).

In one experiment (fig. 1), exposure of mice to 0.8 atm nitrogen plus 1.0 atm oxygen resulted in lower methionine-synthetase activity in the liver than occurred in control animals. Therefore, exposure of an animal to relatively high partial pressures of oxygen might also oxidize the cobalt atom in vitamin B_{12} from its Co(I) state, and thus produce inactivation. Indeed, a previous study showed that two rats exposed to 1.0 atm oxygen for six hours tended to have less activity than control animals.³ However, when the above experiment was repeated, no significant differences in enzyme activity could be detected between control mice and those exposed to 0.75 nitrogen and 1.0 atm oxygen for four hours (fig. 5). Furthermore, mice exposed to xenon, halothane, enflurane, and isoflurane in the presence of 1.0 atm oxygen (figs. 1 and 5), and those exposed to low concentrations of nitrous oxide in the presence of 1.0 atm oxygen (fig. 2), had the same level of enzyme activity in the liver as did control mice. Thus (except for the one instance), exposure to oxygen at a partial pressure of 1.0 atm for four hours did not decrease activity.

The importance of including a separate set of controls for each series of experiments can be seen from figures 1 and 5. Methionine-synthetase activity varied from 300 to 600 nmol of methionine produced per hr/g of liver in shelf control mice, depending upon the given series of experiments. These differences in activity were not due to variations in the assay procedure, since the standard rat liver sample showed essentially the same activity in each series of assays. We have no explanation for this

** National Institute of Occupational Safety and Health.

FIG. 5. Methionine synthetase activity in the liver of mice exposed to volatile anesthetics at approximately ED_{50} concentrations, which abolish the righting reflex in mice,^{††} and in mice exposed to 0.75 atm nitrogen for four hours. Also shown is a positive control for inactivation of methionine synthetase after exposure to 0.8 atm N_2O for four hours. All exposures (except for the shelf control mice) occurred in the presence of 1.0 atm oxygen. After exposure to halothane, enflurane, isoflurane, and nitrogen, methionine synthetase activity did not differ significantly from that of shelf control mice. Each bar represents the mean \pm SE for eight mice.



variation in control levels from experiment to experiment, except to note that other investigators have also found a variation in methionine-synthetase activity of over two-fold in the liver of control rats.³ The degree of inactivation induced by nitrous oxide also varied from experiment to experiment. A 4-hour exposure to 0.8 atm nitrous oxide decreased activity to between 5–25 per cent (figs. 1 and 5, respectively) of the control value. In addition, the relative control values for methionine-synthetase activity in the liver and brain may vary from experiment to experiment. We found approximately the same amount of enzyme activity in both brain and liver of control animals (figs. 3A, B). Although this finding agrees with that of a previous study in rats,²¹ another study reported that methionine-synthetase activity in rat brain was only one-half of that occurring in the liver.³ Nevertheless, brain and liver enzymes seem to be inactivated to approximately the same extent following exposure to 0.8 atm N_2O for 4 hours (fig. 3).

In conclusion, short exposures (4 hours) to nitrous oxide at high partial pressures (0.8 atm) markedly inhibit methionine-synthetase activity in mouse liver and brain. Short exposures to low partial pressures

(less than 0.05 atm) do not alter activity, and only a small decrease in activity is observed after 8 to 22 days of *continuous* exposure to concentrations of nitrous oxide that commonly occur in an unscavenged operating room. If these results can be extrapolated to the clinical situation, they suggest that inhibition of methionine-synthetase activity *per se* is more directly related to the apparent anemia and polyneuropathy seen in patients and abusers exposed to high concentrations of nitrous oxide than to the harmful effects occurring in those chronically exposed to trace levels.

References

1. Deacon R, Lumb M, Perry J, et al: Selective inactivation of vitamin B₁₂ in rats by nitrous oxide. *Lancet* 2:1023–1024, 1978
2. Horne DW, Briggs WT: Effect of dietary and nitrous oxide-induced vitamin B-12 deficiency on uptake of 5-methyltetrahydrofolate by isolated rat hepatocytes. *J Nutr* 110: 223–230, 1980
3. Deacon R, Lumb M, Perry J, et al: Inactivation of methionine synthetase by nitrous oxide. *Eur J Biochem* 104:419–422, 1980
4. Taylor RT, Weissbach H: N⁵-methyltetrahydrofolate-homocysteine methyltransferases, *The Enzymes IX, Part B*. Edited by Boyer PD. New York, Academic Press, 1973, pp 121–165
5. Banks RGS, Henderson RJ, Pratt JM: Reactions of gases in solution. Part III. Some reactions of nitrous oxide with transition-metal complexes. *J Chem Soc (A)* 2886–2889, 1968
6. Blackburn R, Kyaw M, Swallow AJ: Reaction of Cob(I)alamin with nitrous oxide and Cob(III)alamin. *J Chem Soc Faraday Trans* 73:250–255, 1977

†† Smith RA, Koblin DD, Smith M, et al: The anesthetic potency of volatile agents in mice. Abstracts of Scientific Papers, Annual Meeting of the American Society of Anesthesiologists, pp 741–742, 1977.

7. Amess JAL, Rees GM, Burman JF, et al: Megaloblastic haemopoiesis in patients receiving nitrous oxide. *Lancet* 2:339-342, 1978
8. Lumb M, Deacon R, Perry J, et al: The effect of nitrous oxide inactivation of vitamin B₁₂ on rat hepatic folate. *Biochem J* 186:933-936, 1980
9. Lassen HCA, Henrickson E, Neukirch F, et al: Treatment of tetanus. Severe bone-marrow depression after prolonged nitrous-oxide anaesthesia. *Lancet* 1:527-530, 1956
10. Cullen MH, Rees GM, Nancekivill DG, et al: The effect of nitrous oxide on the cell cycle in human bone marrow. *Br J Haematol* 42:527-534, 1979
11. McKenna B, Weir DG, Scott JM: The induction of functional vitamin B-12 deficiency in rats by exposure to nitrous oxide. *Biochim Biophys Acta* 628:314-321, 1980
12. Sahenk Z, Mendell JR, Couri D, et al: Polyneuropathy from inhalation of N₂O cartridges through a whipped-cream dispenser. *Neurology* 28:485-487, 1978
13. Layzer RB, Fishman RA, Schafer JA: Neuropathy following abuse of nitrous oxide. *Neurology* 28:504-506, 1978
14. Layzer RB: Myeloneuropathy after prolonged exposure to nitrous oxide. *Lancet* 2:1227-1230, 1978
15. Cohen EN, Brown BW, Wu M, et al: Anesthetic health hazards in the dental operator. *ANESTHESIOLOGY* 51(3S): 254, 1979 (abstract)
16. Dinn JJ, Weir DG, McCann S, et al: Methyl group deficiency in nerve tissue: A hypothesis to explain the lesion of subacute combined degeneration. *Ir J Med Sci* 149:1-4, 1980
17. Halsey MJ, Eger EI II, Kent DW, et al: High-pressure studies of anesthesia, *Progress in Anesthesiology, Volume 1*. Edited by Fink BR. New York, Raven Press, 1975, pp 353-361
18. Sauer HJ, Jaenicke L: Einfacher Test zur Messung der Methionin-Synthetase-(MS-)Activitat und seine Anwendungsmöglichkeiten in der Klinik. *Klin Wochenschr* 50:986-990, 1972
19. Mazze RI: Waste anesthetic gases and the regulatory agencies. *ANESTHESIOLOGY* 52:248-256, 1980
20. Coward JK, Chello PL, Cashmore AR, et al: 5-methyl-5,6,7,8-tetrahydropteroyl oligo- γ -L-glutamates: Synthesis and kinetic studies with methionine synthetase from bovine brain. *Biochemistry* 14:1548-1552, 1975
21. Ordonez LA, Wurtman RJ: Enzymes catalyzing the *de novo* synthesis of methyl groups in the brain and other tissues of the rat. *J Neurochem* 21:1447-1455, 1973