Nitrous Oxide Has Multiple Deleterious Effects on Cobalamin Metabolism and Causes Decreases in Activities of Both Mammalian Cobalamin-dependent Enzymes in Rats

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ABSTRACT In man, use of the general anesthetic nitrous oxide, N_2O, is associated with hematologic and neurologic abnormalities that mimic those seen in cobalamin (Cbl, vitamin B_{12}) deficiency. We have measured a number of aspects of Cbl metabolism in rats exposed to various concentrations of N_2O for various periods of time.

As little as 2% N_2O given for 15 h resulted in 30% inhibition of methionine synthetase (MS) in rat liver. With 50% N_2O, inhibition of 70% occurred within 1 h and did not change during the next 48 h. Under these conditions, no inhibition of methylmalonyl-CoA mutase (MMCoAM) was observed. The recovery of MS activity was slow and was only 80% of control values 72 h after N_2O was stopped. Studies employing rats previously injected with [^{57}Co]Cbl showed that N_2O displaced [^{57}Co]Cbl from MS in a manner that temporally and quantitatively paralleled the loss of MS activity. Recovery of MS activity paralleled the reappearance of [^{57}Co]Cbl on MS. N_2O also caused the hepatic content of CH_3-[^{57}Co]Cbl to decrease by 20–60%. When [^{57}Co]-Cbl was extracted from liver and analyzed by paper chromatography, [^{57}Co]Cbl analogues were present (10–40% of total [^{57}Co]Cbl) in rats exposed to N_2O, but not in control animals. When rats were exposed to 50% N_2O for 33 d, the total of endogenous Cbl and Cbl analogues in liver decreased to 35% of control values and endogenous Cbl decreased to 10% of control values. At this time, MS activity was 15% of control values and MMCoAM was only 26% of control values.

We conclude that N_2O causes multiple defects in Cbl metabolism that include the following: (*a*) rapid inhibition of MS activity with a slow recovery when N_2O is stopped; (*b*) displacement of Cbl from MS; (*c*) decreased CH_3-Cbl; (*d*) conversion of Cbl to Cbl analogues; (*e*) the gradual development of Cbl deficiency and (*f*) an eventual decrease in MMCoAM activity with a further decrease in MS activity.

INTRODUCTION

Cobalamin (Cbl, vitamin B_{12})[1] is synthesized only by microorganisms (2) but is required by man and other mammals that use it as an obligatory coenzyme for methionine synthetase (MS) (3) and methylmalonyl-CoA mutase (MMCoAM) (4). MS binds (5, 6) and uses methyl-cobalamin (CH_3-Cbl) and catalyzes the conversion of N^5-methyltetrahydrofolate and homocysteine to tetrahydrofolate and methionine, respectively. MMCoAM binds (5, 6) and uses adenosyl-cobalamin (Ado-Cbl) and catalyzes the conversion of L-methylmalonyl-CoA to succinyl-CoA. Cbl deficiency in man causes a megaloblastic anemia or neurologic abnormalities (7). The biochemical bases for these abnormalities are unknown although it is likely that a decrease in MS activity is responsible for the hematologic abnor-

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[1] *Abbreviations used in this paper*: Cbl, cobalamin; MS, methionine synthetase; MMCoAM, methylmalonyl-CoA mutase; CH_3-Cbl, methyl-cobalamin; Ado-Cbl, adenosyl-cobalamin; IF, intrinsic factor; EU, enzyme unit.

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malities in that indistinguishable absormalities occur in folate deficiency (7). A decrease in MMCoAM activity may be responsible for the neurologic abnormalities (8) because these are not observed in folate deficiency. Obtaining unequivocal answers to these questions has been hindered, however, by the fact that Cbl deficiency does not cause a megaloblastic anemia in animals and only causes neurologic abnormalities in certain primates (9, 10).

Amess et al. (11) have shown that 50% nitrous oxide (N_2O) administered to surgery patients causes megaloblastic changes in the bone marrow within 6 h. N_2O administered for several weeks to patients with tetanus appears to have been responsible for the sometimes fatal megaloblastic anemia that they developed (12). N_2O has been shown to cause neutropenia in rats (13–16) and to inhibit the growth of malignant mouse (17), and normal (18) and malignant (19–21) human cells. Chronic exposure to N_2O can cause a peripheral neuropathy in humans (22–24), and an increased incidence of birth defects in rats (25) and in humans (26–28).

The hematologic and neurologic abnormalities associated with N_2O in man are similar to those seen in Cbl deficiency (7). N_2O does interact with the reduced forms of Cbl (29) and Deacon et al. (30) have shown that N_2O causes inhibition of MS activity in rat liver. McCaan et al. (31) have shown that giving 50% N_2O to monkeys for 6 wk induces a peripheral neuropathy and demyelinization of the spinal cord. Methylmalonicaciduria was not observed in these animals (30, 31) and this has suggested that MMCoAM activity was not affected. In the current study, we administered N_2O to rats and measured a number of aspects of Cbl metabolism including direct measurements of MMCoAm.

METHODS

CN-[^{57}Co]Cbl (200 μCi/nmol), CN-[^{58}Co]Cbl (4 μCi/nmol), and N^5-[*methyl*-^{14}C]tetrahydrofolic acid, (barium salt, 56 mCi/mmol) were obtained from Amersham Corp. (Arlington Heights, Ill.). The [^{58}Co]Cbl was obtained in the form of capsules that are used in the Dicopac Schilling Kit (Amersham Corp., Des Plaines, Ill.). Each capsule was opened and the contents were dissolved in 5 ml of H_2O. After centrifugation at 10,000 *g* for 15 min at 4°C, the supernate containing the [^{58}Co]Cbl was stored at −20°C. DL-2-[*methyl*-^{14}C]-methylmalonyl coenzyme A (54 mCi/mmol) was obtained from New England Nuclear, Boston, Mass.). Methylmalonyl coenzyme A, glutathione (GSH), L-homocysteine thiolactone, L-methionine, *S*-adenosylmethionine (iodide form), N^5-methyltetrahydrofolic acid (barium salt), flavin adenine nucleotide (FAD), SP-Sephadex C-25, CN-Cbl, OH-Cbl, CH_3-Cbl, and Ado-Cbl were obtained from Sigma Chemical Co. (St. Louis, Mo.). DEAE-cellulose (DE52) was obtained from Whatman, Inc. (Clifton, N. J.) and converted to the phosphate form as described in the package insert. Amberlite XAD-2 was obtained from Mallinckrodt Inc. (St. Louis, Mo.). Sprague-Dawley rats, 150–200 g males, were obtained from Charles River Breeding Laboratories (Wilmington, Mass.). Purified hog intrinsic factor (IF) (32), hog R protein (32), and hog R protein-Sepharose (33) were prepared as described previously.

Holo-MMCoAM was assayed at 30°C by a modification (5) of the method of Cannata et al. (34), which measures the conversion of 2-[*methyl*-^{14}C]methylmalonyl coenzyme A to [^{14}C]-succinyl coenzyme A. Total MMCoAM was assayed in an identical manner except that assays were performed in the dark in the presence of 30 nmol of Ado-Cbl. Apo-MMCoAM was calculated by subtracting values obtained for holo-MMCoAM from those obtained for total MMCoAM. 1 enzyme unit (EU) catalyzes the formation of 1 μmol of product/min.

MS was assayed by a modification (5) of the method of Brot and Weissbach (35). Reaction volumes were 1.0 ml and contained the following: KPO_4, pH 7.5, 50 μmol; NaCl, 200 μmol; 2-mercaptoethanol, 200 μmol; L-homocysteine, 2.5 μmol; *S*-adenosylmethionine, 100 nmol; N^5-methyltetrahydrofolic acid, 75 nmol containing 20 nCi of N^5-[*methyl*-^{14}C]tetrahydrofolic acid; CN-Cbl, 30 nmol; and $FADH_2$, reduced with platinum and H_2 (35), 100 nmol. After incubating for 40 min at 37°C, assay tubes were boiled for 3 min and cooled to 4°C. Methionine-coated charcoal, 1 ml, was added and after standing for 10 min at 4°C, the assay tubes were centrifuged at 40,000 *g* for 30 min. The supernate, 1 ml, was then assayed for ^{14}C as described (5). 1 EU catalyzes the formation of 1 μmol of product/min. Methionine-coated charcoal binds N^5-[*methyl*-^{14}C]tetrahydrofolic acid but does not bind [^{14}C]methionine and was prepared by adding 50 g of L-methionine to 30 g of Norit charcoal in 500 ml of H_2O. The mixture was stirred for 2 h at 22°C, stored at 4°C, and warmed to 22°C just before being used in the MS assay. When studied with 0–300 μl of rat liver, kidney, and brain supernate, and with boiled supernates (>97% loss of activity), direct comparisons showed that methionine-coated charcoal was equivalent to ion exchange resins (5, 35) in terms of its ability to separate N^5-[*methyl*-^{14}C]tetrahydrofolic acid from [^{14}C]methionine when used in assays for MS. Both techniques gave linear values for incubations from 5 min to 3 h provided that <20% of the substrate was converted to product.

Tissues were frozen at −70°C for at least 30 min and homogenates were prepared by adding 2.5 ml of 14 mM $NaPO_4$, pH 7.0, containing 2.8 mM GSH for each gram of tissue, followed by homogenization in a Waring blender (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.) at 4°C for 1 min. The homogenates were centrifuged at 30,000 *g* for 45 min at 4°C and the supernate was used for enzyme assays and for DEAE-cellulose chromatography. Chromatography was performed at 4°C on 2.5 × 20-cm columns of DEAE-cellulose (phosphate form) equilibrated with 20 mM $NaPO_4$, pH 7.0, containing 2 mM GSH, and run at a flow rate of 100 ml/h. Homogenate supernate, 50 ml, was applied to each column followed by washing with 150–550 ml of equilibrating solution. A linear gradient was then developed in which the mixing flask contained 600 ml of the equilibrating solution and the reservoir contained 600 ml of the equilibrating solution with 0.5 M NaCl. Fractions of 10 ml were collected.

For Cbl assays, Cbl was extracted from tissues, homogenate supernates, and DEAE-cellulose column fractions, by a modification (36) of the method of Frenkel et al. (37). To each 1 g or 1 ml of material, 1 ml of 0.5 M sodium acetate-HCl at pH 4.5 and 2 ml of 0.15 M NaCl containing 50 μg of KCN/ml were added followed by, in the case of tissues, homogenization in a Waring blender at 4°C for 3 min. The samples were heated for 45 min at 100°C and centrifuged at 30,000 *g* for 30 min at 4°C. The supernates were assayed for Cbl using a described (36) radioisotope dilution assay employing human IF as the Cbl-binding protein, and for the total of Cbl and Cbl analogues using a described (36) radioisotope dilution assay employing human R protein as the Cbl-binding protein. [^{57}Co]Cbl and [^{57}Co]Cbl analogues were purified from supernates prepared as just described for Cbl assays, by subjecting them to reverse-

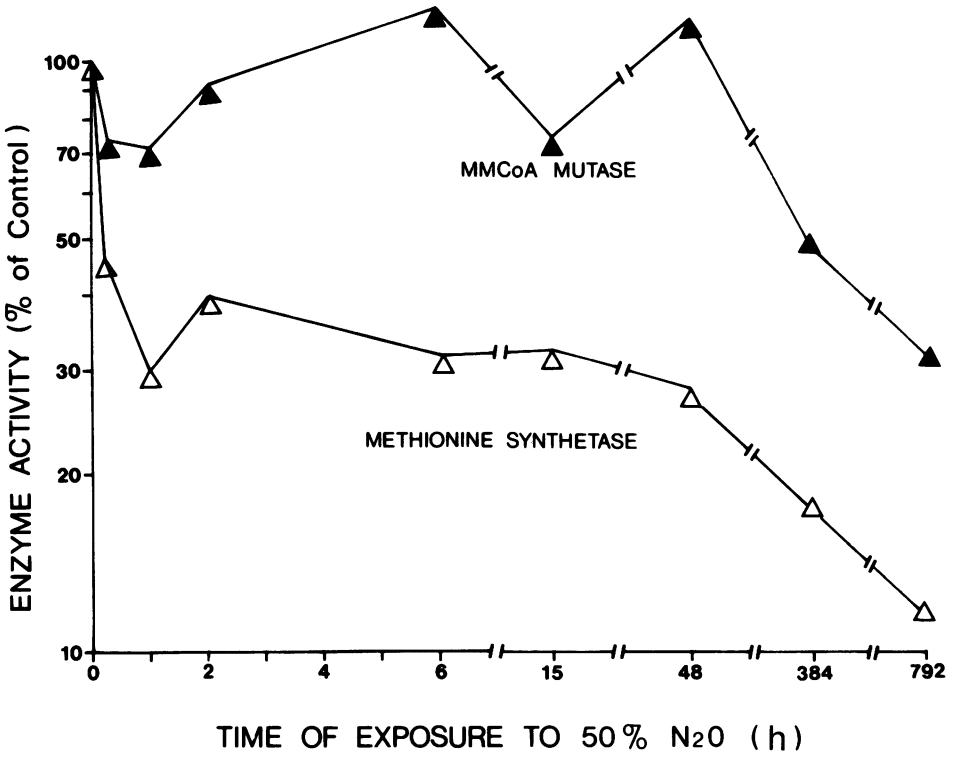

FIGURE 1 Activity of MS and holo-MMCoAM in liver from rats that were exposed to 50% N_2O/ 50% O_2 for various periods of time. Each point represents the mean value obtained for livers from three to six rats that were assayed simultaneously with three to six control rats. For MS, all of the experimental values were significantly different ($P < 0.01$) from the control values. For holo-MMCoAM, only the values at 384 and 792 h were significantly different ($P < 0.01$) from the control values.

affinity chromatography on hog R protein-Sepharose as described (33) except that Cbl and Cbl analogues were eluted with 60% pyridine. The eluates were dried with a Speed-Vac concentrator (Savant Instruments, Inc., Hicksville, N. Y.) and subjected to descending paper chromatography on Whatman No. 3MM paper at room temperature for 30 h. The developing solvent was 880 ml of sec-butanol, 8.2 ml of glacial acetic acid, 6.2 μmol HCN, and a saturating amount (~425 ml) of H_2O. The paper was dried in a fume hood, cut into 38 equal fractions, and assayed for ^{57}Co and ^{58}Co using a Beckman Gamma 8000 system (Beckman Instruments, Inc., Fullerton, Calif.).

For [^{57}Co]Cbl and [^{58}Co]Cbl coenzyme determinations, tissues were extracted (38), and partially purified using Amberlite XAD-2 (Rohm & Haas Co., Philadelphia, Pa.) (38). Nonradioactive CH_3-Cbl, Ado-Cbl, and OH-Cbl, 100 nmol of each, were added as carriers, and the samples were fractionated by chromatography on 0.9 × 20 cm columns of SP-Sephadex (39). Individual fractions were assayed for ^{57}Co and ^{58}Co as described above.

CN-[^{57}Co]Cbl and CN-[^{58}Co]Cbl were converted to OH-[^{57}Co]Cbl and OH-[^{58}Co]Cbl, respectively, by performing incubations in 5 mM acetic acid-NaOH, pH 4.5, at 4°C at a distance of 20 cm from a 60 watt tungsten light bulb for 16 h. The samples were applied to 0.7 × 2-cm columns of SP-Sephadex that were equilibrated with H_2O. The columns were washed with 5 ml of H_2O to remove residual radioactive CN-Cbl and radioactive OH-Cbl was then eluted with 1.5 ml of 0.2 M NaCl.

Rats were injected with radioactive OH-Cbl bound to hog IF,[2] in the ventral tail vein and were killed by placing them in an ether jar for 1 min followed by cervical dislocation. Tissues were removed within 2 min of death, and were frozen at −70°C for at least 30 min before being homogenized in one of the various ways described above. Rats exposed to N_2O were placed in cages in an air-tight plexiglass box equipped with gas inlet and outlet ports, and a refrigeration system that maintained the temperature at 22°C. Rats were permitted unlimited access to food (Wayne Lab-blox, 22 pmol CN-Cbl/g, Allied Mills, Inc., Chicago, Ill.) and H_2O. Their cages were cleaned daily at which time the rats were exposed to air for 15 min. Various mixtures of N_2O and O_2 were administered to the plexiglass box (vol = 2 m^3) at a flow rate of 2.5 liters/min. Separate tanks of N_2O and O_2 were used. In the range of 2–80% N_2O, the gas mixture was monitored with an O_2 analyzer. At concentrations of N_2O below 2%, N_2O was measured directly with an N_2O analyzer.

[2] Previous studies (5, 40, 41) have shown that native IF-Cbl and R protein-Cbl are cleared exclusively from plasma by hepatocytes within minutes after their intravenous injection. The protein moieties are degraded intracellularly and the liberated Cbl is then either utilized by hepatocytes or released into plasma where it is bound to transcobalamin II and transported to other tissues.

TABLE I

Effect of Deleting CN-Cbl and 2-Mercaptoethanol from Assay Mixtures on the Amount of MS Activity Measured in Rat Liver after Rats Were Exposed to 50% N_2O/50% O_2 for Various Periods of Time

Assay conditions*	Enzyme activity after exposure to 50% N_2O					
	0 h		1.5 h		16 h	
	U/g	%	U/g	%	U/g	%
Experiment I						
Complete	0.0052	(100)	0.0015	29	0.0017	33
− CN-Cbl	0.0020	38	0.0022	42	0.0015	29
− 2-mercaptoethanol	0.0017	33	0.0035	67	0.0012	23
− CN-Cbl, − 2-mercaptoethanol	0.0017	33	0.0032	62	0.0012	23
Experiment II						
Complete	0.0062	(100)	0.0020	32	0.0017	27
− CN-Cbl	0.0027	44	0.0032	52	0.0017	27
− 2-mercaptoethanol	0.0032	52	0.0055	89	0.0025	40
− CN-Cbl, − 2-mercaptoethanol	0.0030	48	0.0052	84	0.0022	35

* MS was assayed as described under Methods.

‡ Experiment I used one rat at each time and experiment II, which was a repeat of experiment I, used two rats at each time period.

RESULTS

Effect of N_2O on MS and MMCoAM activity. The data in Fig. 1 indicate that 15 min of exposure to 50% N_2O caused the level of MS activity in rat liver to decrease to 45% of control values. MS activity fell to 30% of control values after 1 h and remained at this level for exposure periods as long as 48 h. Similar decreases in MS activity were observed in rat kidney and brain and in mouse liver and kidney (data not presented). As shown in Fig. 1, significant changes in the activity of holo-MMCoAM in rat liver were not observed during the first 48 h of exposure to 50% N_2O. These results demonstrate that N_2O causes rapid inhibition of MS activity but not of MMCoAM activity.

With longer exposures to 50% N_2O, MS activity in rat liver decreased further to 18 and 12% of control values after 384 and 792 h as shown in Fig. 1. Prolonged exposure to 50% N_2O also caused significant inhibition of holo-MMCoAM activity with levels of 48 and 32% of control values being observed at 384 and 792 h, respectively. These results indicate that prolonged exposure to N_2O causes a decrease in the activity of both mammalian Cbl-dependent enzymes.

MS and MMCoAM assays in liver, kidney, and brain from N_2O treated rats remained linear with respect to time and protein concentration, as was true with control rats (Methods). No evidence was observed for the presence of an inhibitor to MS in liver from N_2O-treated rats since assays for MS performed with combined control and N_2O-treated liver supernate gave values equal to the sum of the values obtained when the supernates were assayed separately. No change in MS or MMCoAM activity was observed in the livers of rats given 100% O_2 for 72 h. When four rats were exposed to 50% O_2/50% air for 384 h, values for MS and holo-MMCoAM actually increased by 29.5 and 39.5%, respectively when compared with four control rats that were exposed only to air ($P < 0.01$ in both cases). These results demonstrate that N_2O is responsible for both the early and the late decreases in Cbl-dependent enzyme activity in rats and that these decreases are not due to O_2.

No significant changes in apo-MMCoAM were observed at any of the times shown in Fig. 1. It is not clear if the MS assay described under Methods measures only holo-MS or if it also measures apo-MS. Data presented in Table I indicate that the MS activities in rat liver after 0, 1.5, and 16 h of exposure to N_2O each behave differently from one another when CN-Cbl, 2-mercaptoethanol or both are removed from the assay. This suggests that different forms of MS, including different amounts of apo-MS, may exist in liver before, shortly after, and after prolonged exposure to N_2O although additional experiments will be required to define the molecular bases for these differences.

The time-course of recovery of MS activity in rat liver was studied by exposing rats to 50% N_2O for 15 h and then returning them to air for various periods of time. As shown in Fig. 2, MS activity recovered slowly and was only 29 and 80% of control values after being returned to air for 6 and 72 h, respectively.

Levels of MS activity in rat liver after the exposure of rats to various concentrations of N_2O for 15 h are shown

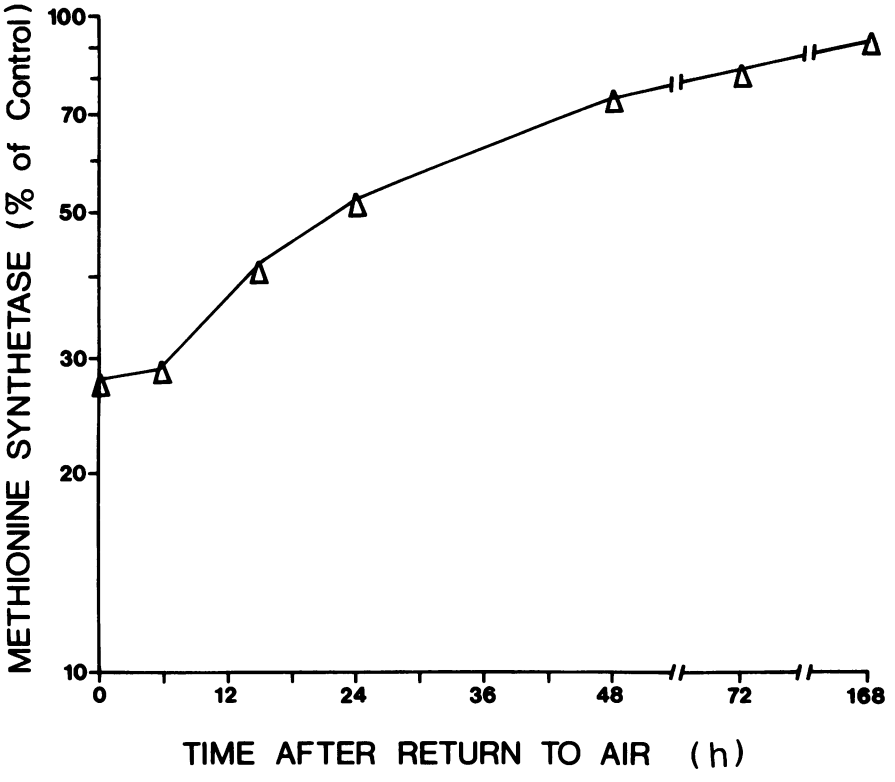

FIGURE 2 Recovery of MS activity in liver from rats that were exposed to 50% N_2O/50% O_2 for 15 h and then returned to air for various periods of time. Each point represents the mean of three to five rats that were assayed simultaneously with three to five control rats. All of the values except that at 168 h are significantly different ($P < 0.05$) from the control values.

in Fig. 3. As little as 2% N_2O caused significant inhibition of MS activity and gave values that were 68% of control values. Inhibition appeared maximal in the range of 6 to 80% N_2O where values of 32–24% of control values were observed. A similar concentration effect was observed for MS activity in rat brain and kidney (data not presented).

Displacement of Cbl from MS. Fig. 4 shows the chromatographic profiles of [^{57}Co]Cbl, endogenous Cbl, MS activity and MMCoAM activity when supernates from rat liver homogenates were fractionated on DEAE-cellulose. In Fig. 4A, rats were injected intravenously with 10 pmol of OH-[^{57}Co]Cbl and exposed to air for 96 h. Approximately 25% of the [^{57}Co]Cbl and endogenous Cbl was not adsorbed to DEAE-cellulose and appeared to be present in unbound form because >95% of both were adsorbed by albumin-coated charcoal. The remaining 75% of both forms of Cbl were adsorbed to DEAE-cellulose, and were eluted with a linear NaCl gradient in four peaks. Each of these appeared to represent protein-bound Cbl since neither form of Cbl was adsorbed by albumin-coated charcoal. The first two peaks cochromatographed with the two peaks of holo-MMCoAM activity that were observed, whereas the latter two peaks cochromatographed with the two observed peaks of MS activity. As shown in Table II, Ado-Cbl was the predominant coenzyme form of Cbl in the first two peaks, and CH_3-Cbl and OH-Cbl were the predominant forms in the last two peaks. These observations support the concept that most of the intracellular Cbl is tightly bound to MMCoAm and MS, as shown previously with both rabbits and rats (5, 6). The data in Fig. 4A also indicate that the injected OH-[^{57}Co]Cbl was nearly equilibrated with endogenous Cbl after 96 h of exposure to air.

In Fig. 4A, the ratio of MS activity to Cbl is lower in the first MS peak than in the second MS peak. The reason for this difference is unknown and could indicate that the two peaks of MS activity have different specific activities relative to bound Cbl, or that a previously unrecognized Cbl-binding protein is also present in the first MS peak.[3]

Fig. 4B shows the results of an experiment that was identical except that the rats were exposed to air for 48 h after the injection of OH-[^{57}Co]Cbl and then exposed to 50% N_2O for the second 48 h. Both peaks of MS activity

[3] Poston has presented evidence suggesting that leucine 2,3-aminomutase is present in mammalian tissues (42) and that this enzyme may be dependent on Ado-Cbl (43).

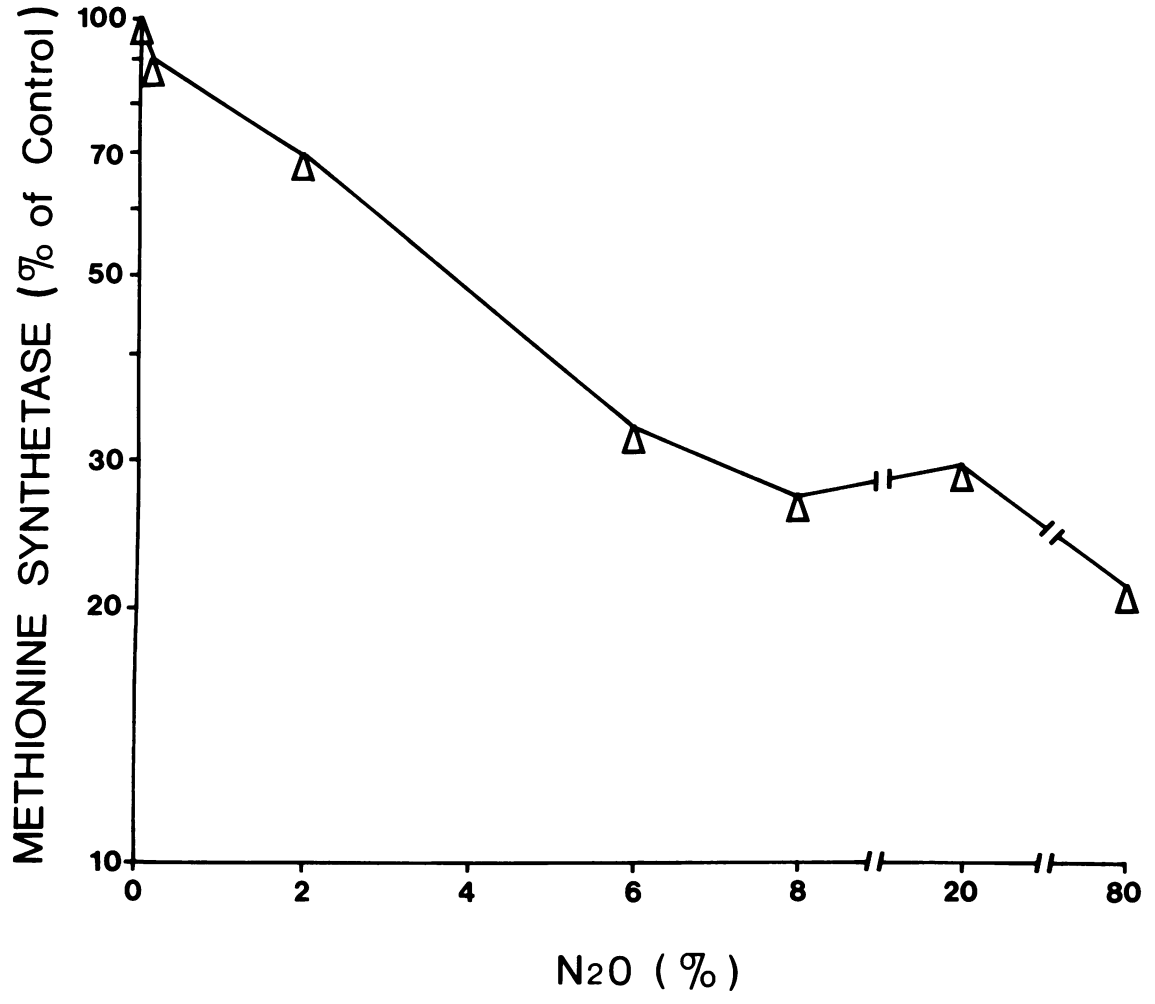

FIGURE 3 Activity of MS in liver from rats exposed to various concentrations of N_2O/O_2 for 15 h. Each value represents the mean of three to eight livers that were assayed simultaneously with three to eight control livers. The values obtained with 2–80% N_2O were significantly different ($P < 0.05$) from the control values.

were reduced. This was accompanied by a reduction in the amount of [^{57}Co]Cbl and endogenous Cbl present in the two MS peaks, with the decreases of both forms of Cbl being greater in the second MS peak. The fact that a marked reduction in Cbl was not observed in the first MS peak suggests either that another Cbl-binding protein is present in this region, or that Cbl is not rapidly displaced from the form of MS present in this region when it is inactivated by N_2O. The data in Fig. 4B also shows that [^{57}Co]Cbl remains nearly equilibrated with endogenous Cbl after 48 h exposure to air followed by 48 h exposure to N_2O.

Fig. 5 shows the results of experiments in which rats were injected intravenously with 100 pmol of either OH-[^{57}Co]Cbl or OH-[^{58}Co]Cbl with both groups of rats being exposed to air subsequently for 48 h. The rats that received the OH-[^{57}Co]Cbl were then exposed to 50% N_2O for an additional 0–384 h, whereas the rats that received the OH-[^{58}Co]Cbl were continued in air for the same time periods. Animals from both sets were killed at the times indicated in Fig. 5, portions of the livers were homogenized together, and the supernates were chromatographed on DEAE-cellulose. As shown in Fig. 5A, when neither set of animals were exposed to N_2O the [^{57}Co]Cbl and [^{58}Co]Cbl gave essentially identical chromatographic profiles. Fig. 5B shows that exposure of the [^{57}Co]Cbl injected animals to 50% N_2O for 0.5 h caused a decrease in the amount of [^{57}Co]Cbl from the second MS peak, relative to the amount of [^{58}Co]Cbl from the animals exposed only to air. A further decrease in [^{57}Co]Cbl, relative to [^{58}Co]Cbl, in the second MS peak was observed in the animals exposed to N_2O for 48 h (Fig. 5C) and for 384 h (Fig. 5D). These results indicate that the displacement of Cbl from MS effected

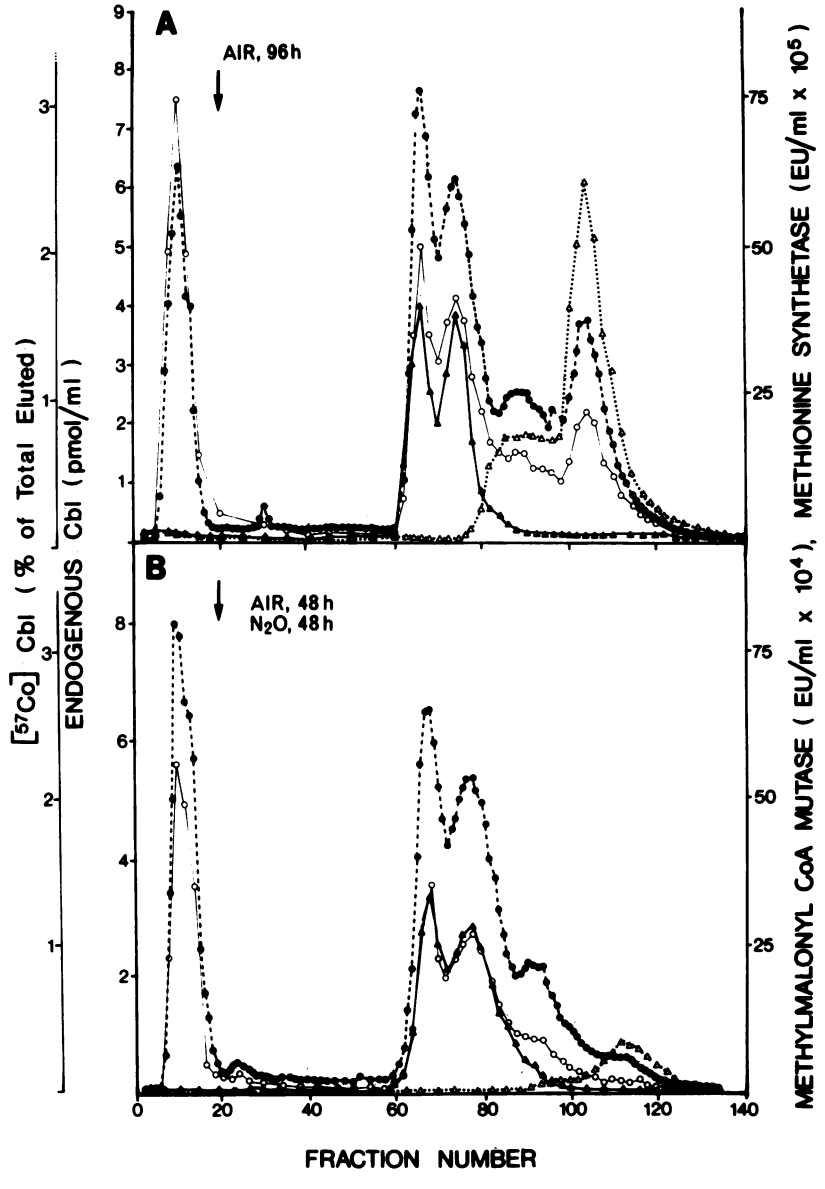

FIGURE 4 Effect of N_2O on the DEAE-cellulose chromatographic profile of MS, holo-MMCoAM, [^{57}Co]Cbl, and endogenous Cbl from rat liver. Six rats were each injected with 10 pmol i.v. of OH-[^{57}Co]Cbl bound to hog IF. Three of the rats were then exposed to air for 96 h (A) and the other three rats were exposed to air for 48 h followed by exposure to 50% N_2O/50% O_2 for 48 h (B). The animals were sacrificed, the livers from each set of animals were homogenized together, and 50 ml of supernate was chromatographed on DEAE-cellulose as described under Methods. The arrows indicate the start of the linear NaCl gradient. Fractions were assayed for [^{57}Co]Cbl (●), endogenous Cbl using the R protein radioisotope dilution assay (○), holo-MMCoAM (▲), and MS (△), as described under Methods. Assays for apo-MMCoAM (data not presented) gave values that paralleled holo-MMCoAM and were ~20 times higher.

by N_2O parallels the decrease in MS activity both temporarily and quantitatively. This correspondence also appeared to hold during the recovery phase because [^{57}Co]Cbl reappeared in the second MS peak (data not shown) in proportion to the recovery of MS activity shown previously in Fig. 2.

The data in Fig. 5B and C indicate that the [^{57}Co]Cbl displaced from the second MS peak was present as an excess of [^{57}Co]Cbl in the first and second MMCoAM peaks, i.e., the ratio of [^{57}Co]Cbl/[^{58}Co]Cbl was >1. This suggests that during the first few days of exposure to N_2O, Cbl displaced from MS becomes bound to

TABLE II

Coenzyme Forms of Cbl in Whole Rat Liver Supernate and in Various Fractions Obtained by DEAE-Cellulose Chromatography

Item	Fraction nos.*	CN-Cbl or SO_3-Cbl	CH_3-Cbl	Ado-Cbl	OH-Cbl
		%	%	%	%
Whole liver		7.5	15.4	71.4	5.7
1st and 2nd MMCoAM	62–80	4.5	3.5	83.7	8.3
1st MS	82–96	29.4	31.3	13.1	26.2
2nd MS	100–120	19.3	31.0	14.0	35.7

Whole liver supernate and DEAE-cellulose fractions from the experiment described in Fig. 4A were analyzed for their contents of the various coenzyme forms of [^{57}Co]Cbl as described under Methods.

* Refers to pooled fractions from Fig. 4A.

MMCoAM. This possibility may explain the slow recovery of MS activity and the slow replacement of Cbl bound to MS when the administration of N_2O is stopped since previous studies indicate that Cbl bound to MMCoAM turns over relatively slowly (5, 6). The data in Fig. 5B and C suggest that sufficient unbound Cbl may be available for replacing Cbl on MS but, it is not known if this apparent unbound Cbl is actually unbound in the cytosol in vivo since it could be artifactually released from other compartments, MMCoAM, MS, or other Cbl-binding proteins during the homogenization and chromatographic procedures.

Effect of N_2O on Cbl coenzyme levels. Fig. 6 shows the results obtained when portions of livers from the animals used in the double label experiments described in Fig. 5 (see above) were extracted and analyzed for their Cbl coenzyme content using chromatography on SP-Sephadex. Fig. 6A shows that the coenzyme profiles of [^{57}Co]Cbl and [^{58}Co]Cbl were essentially identical when neither group of animals were exposed to N_2O. After exposure to N_2O for 0.5, 48, and 384 h, the amount of [^{57}Co]Cbl present as CH_3-Cbl decreased by 20–60% relative to the [^{58}Co]Cbl from the unexposed animals, as shown in Fig. 6B, C, and D. These results indicate that N_2O causes a decrease in CH_3-Cbl levels although the magnitude of the decrease in relative terms is not as great as the decrease in MS activity or in the decrease of Cbl bound to MS.

The data in Fig. 6B, C, and D, indicate that the decreases in CH_3-[^{57}Co]Cbl are accompanied by proportionate increases in the amounts of Ado-[^{57}Co]Cbl. This observation supports the concept (see above) that the [^{57}Co]Cbl displaced from MS rapidly becomes bound to MMCoAM.

Conversion of Cbl to Cbl analogues by N_2O. Fig. 7 shows the results obtained when additional portions of livers from the dual label experiments described in Fig. 5 had their Cbl extracted, purified by reverse-affinity chromatography on hog R protein-Sepharose, and fractionated by paper chromatography. Fig. 7A shows that when both sets of animals were exposed only to air, the [^{57}Co]Cbl and [^{58}Co]Cbl chromatographed together in essentially a single peak that was in the same position as that of crystalline Cbl. After exposure to 50% N_2O for 0.5, 48, and 384 h, additional fast moving peaks of [^{57}Co]Cbl were observed. This indicates that N_2O causes the conversion of Cbl into Cbl analogues. The conversion of Cbl to Cbl analogues was not due to O_2 since [^{57}Co]Cbl analogues were not observed in the livers of four rats who were injected with 100 pmol of OH-[^{57}Co]Cbl and then exposed to air for 48 h followed by exposure to 50% O_2/50% air for an additional 384 h.

Somewhat larger amounts of Cbl analogues were also observed (data not shown) in rat brain, kidney, serum, small intestine, and spleen, although the patterns differed somewhat from that seen in liver, which lacked slower moving Cbl analogue fractions seen in these other tissues, particularly spleen. These differences could reflect differences in analogue production among tissues or could be due to differences in the cellular uptake or excretion of Cbl analogues among tissues. The latter possibility may be particularly pertinent with respect to liver in that this tissue does preferentially excrete certain Cbl analogues into bile (40).

Additional studies were performed (data not presented) in which various fractions from the DEAE-cellulose chromatogram shown in Fig. 5C were analyzed separately for the presence of [^{57}Co]Cbl analogues. The results indicated that similar total amounts of Cbl analogues were present in the free, MMCoAM, and MS regions, although the amounts of various Cbl analogues differed somewhat among these regions. Whether these Cbl analogues are formed from Cbl previously bound to MMCoAM and MS or whether they are formed elsewhere and become bound by these enzymes subsequently is unknown. The amount of Cbl activity that these Cbl analogues possess for MMCoAM and MS is also unknown.

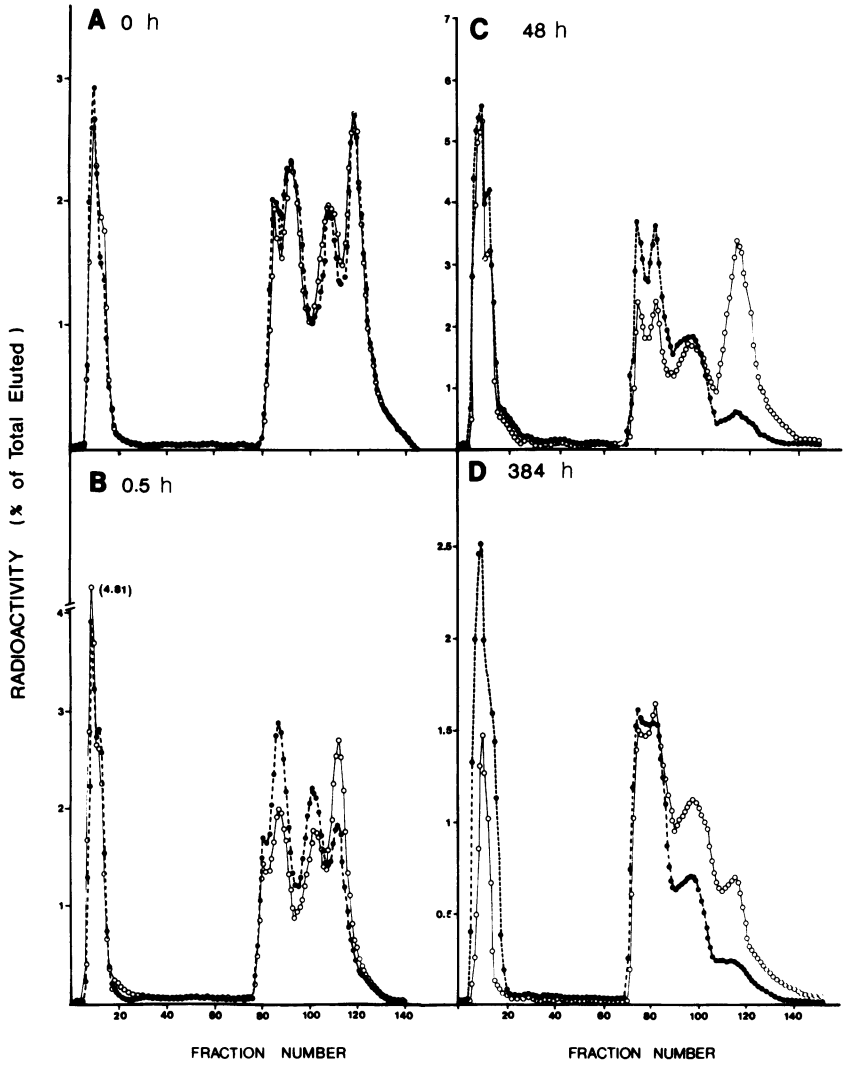

FIGURE 5 Time-course of the displacement of Cbl from MS when rats were exposed to N_2O. In each experiment, A–D, three rats were injected with 100 pmol i.v. of OH-[^{57}Co]Cbl bound to hog IF and three rats were injected with 100 pmol i.v. of OH-[^{58}Co]Cbl bound to hog IF. All of the animals in experiments A–D were then exposed to air for 48 h. The [^{57}Co]Cbl-injected animals were then exposed to 50% N_2O/50% O_2, and the [^{58}Co]Cbl-injected animals were continued in air, for (A) 0 h, (B) 0.5 h, (C) 48 h, and (D) 384 h. At the end of these times, the animals were killed, equal portions of the livers from the [^{57}Co]Cbl and the [^{58}Co]Cbl-injected animals were pooled and homogenized together, and the supernates, 50 ml, were chromatographed on DEAE-cellulose as described under Methods. Linear NaCl gradients were begun at fraction 30. Individual fractions were assayed for [^{57}Co]Cbl (●) and [^{58}Co]Cbl (○).

To determine the excretion rate of the Cbl analogues formed by N_2O and to determine if these Cbl analogues are converted back to Cbl, an experiment was performed in which 20 μCi of OH-[^{57}Co]Cbl (100 pmol) was injected intravenously into a rat that was exposed to 50% N_2O subsequently for 48 h. The [^{57}Co]Cbl was extracted from the liver, purified by reverse-affinity chromatography on hog R protein-Sepharose, and fractionated by paper chromatography. After elution from the paper with H_2O, the [^{57}Co]Cbl from the analogue fractions (3 pmol) was mixed with 100 pmol of CN-[^{58}Co]Cbl and 150 pmol of hog R protein, and injected intravenously into a rat. Urine and feces were collected daily for 5 d and showed that ~7% of the [^{57}Co]Cbl and ~1% of the [^{58}Co]Cbl were excreted each day with about half of each excreted label being present in the urine and in the feces. These studies thus indicate that Cbl analogues formed from Cbl in vivo by N_2O are excreted from the body at a faster rate than Cbl itself. We also determined the paper chromatography profile of the [^{57}Co]Cbl analogues and the [^{58}Co]Cbl that were injected into the rat, together with the profiles obtained

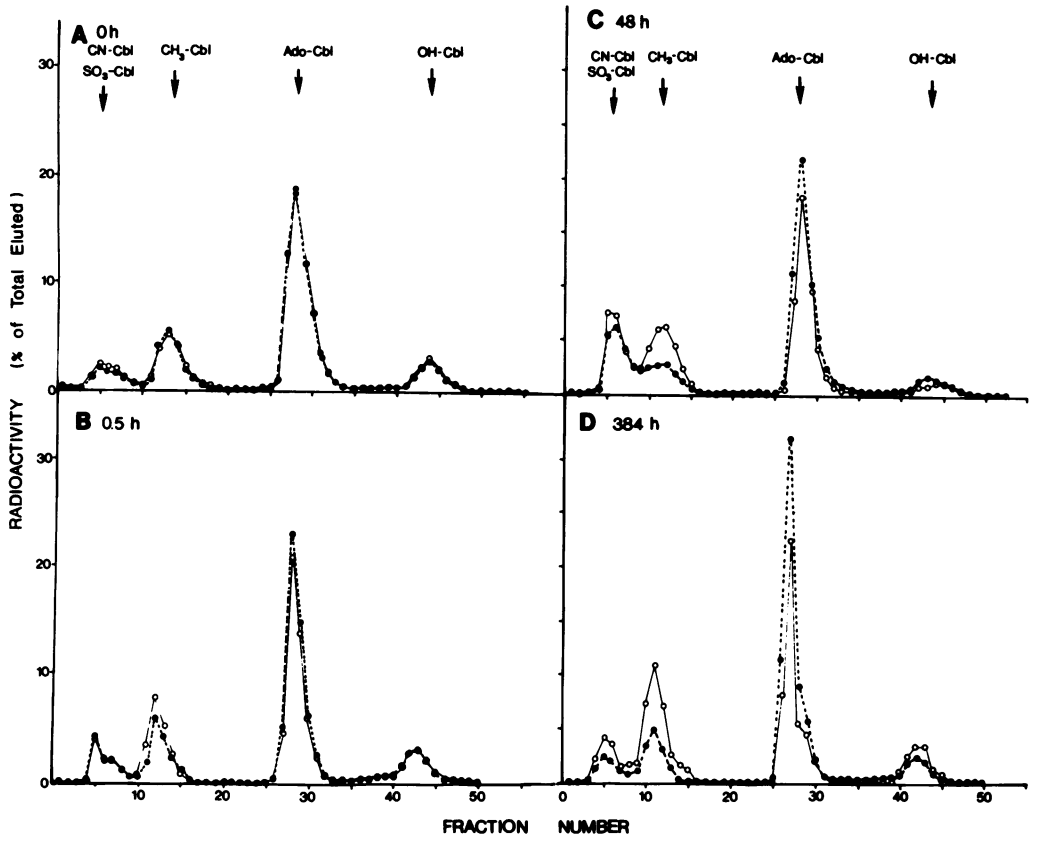

FIGURE 6 Time-course of the decrease of CH_3-Cbl and increase in Ado-Cbl in livers from rats exposed to N_2O. Portions, ~20%, of the livers from the double-label experiments described in the legend to Fig. 5 were extracted and partially purified as described under Methods. The coenzyme forms of [^{57}Co]Cbl and [^{58}Co]Cbl were separated by chromatography on 1.9 × 20 cm columns of SP-Sephadex equilibrated with H_2O at 22°C. After application of the samples in 2 ml of H_2O, the columns were washed sequentially with 30 ml of H_2O, 23 ml of 0.05 M Na acetate-acetic acid, and 23 ml of 0.2 M Na acetate-acetic acid, pH 5.0. Fractions of 1.5 ml were collected and assayed for ^{57}Co (●) and ^{58}Co (○) as described under Methods. Both sets of animals had been exposed to air for the first 48 h after injection. The [^{57}Co]Cbl-injected animals were then exposed to 50% N_2O/50% O_2, and the [^{58}Co]Cbl-injected animals were then continued in air, for (A) 0 h, (B) 0.5 h, (C) 48 h, and (D) 384 h.

after they were extracted, purified, and fractionated from the liver, kidney, and the carcass and other organs 5 d after injection (data not shown). The results indicated that most of the [^{57}Co]Cbl analogues were still present as Cbl analogues although 5, 9, and 20% of the material migrated in the Cbl region in liver, kidney, and the carcass and other organs, respectively. Whether this material represents the conversion of [^{57}Co]Cbl analogues back to [^{57}Co]Cbl or whether it only represents a percent increase in [^{57}Co]Cbl due to the preferential excretion of [^{57}Co]Cbl analogues is not known.

The effect of long-term administration of N_2O on the activities of MS and MMCoAM, and the levels of endogenous Cbl. The data in the preceding section suggested that the prolonged administration of N_2O might lead to a progressive decrease in tissue Cbl, due to its being converted to Cbl analogues, and that this might be responsible for the decrease in holo-MMCoAM activity and the further decrease in MS activity that were seen with prolonged N_2O administration. Data to support this concept are presented in Table III where 33 d of exposure to 50% N_2O caused significant decreases in holo-MMCoAM activity to 26, 40, and 62% of control values in liver, kidney, and brain, respectively. Levels of endogenous Cbl, as measured with the radioisotope dilution assay using IF, showed significant decreases to 10, 27, and 4% in liver, kidney, and brain, respectively. Levels of the total of Cbl and Cbl analogues, as measured with the radioisotope dilution assay using R protein,[4] showed smaller but significant decreases to 35, 46, and 24% of control values, in liver, kidney, and brain,

[4] Cbl analogues may not be measured to their full extent with the R protein assay since some Cbl analogues have slight to moderately reduced affinities for R protein, relative to Cbl (44).

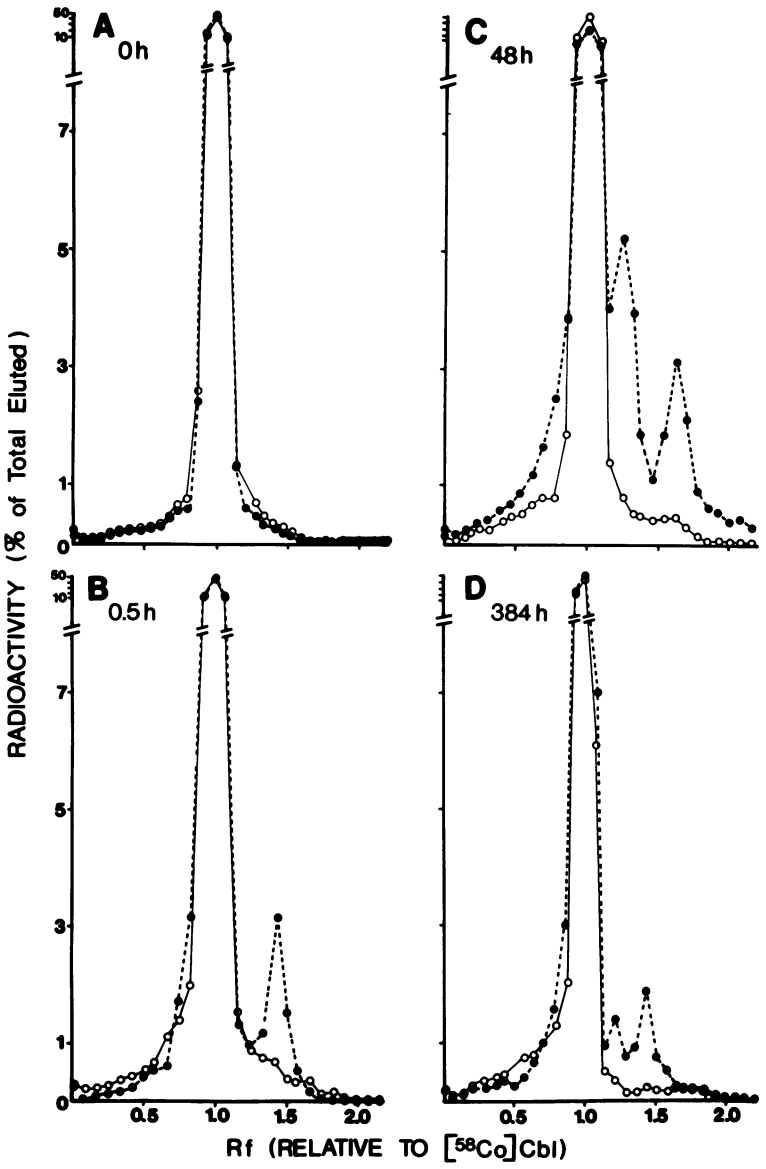

FIGURE 7 Time-course of the conversion of Cbl to Cbl analogues in livers from rats exposed to N_2O. Portions, ~15%, of the livers from the double-label experiments described in the legend to Fig. 5 were extracted, subjected to reverse-affinity chromatography on hog R protein-Sepharose and the [^{57}Co]Cbl (●) and [^{58}Co]Cbl (○) were then fractionated by paper chromatography, as described under Methods. Both sets of animals had been exposed to air for the first 48 h after injection. The [^{57}Co]Cbl-injected animals were then subjected to 50% N_2O/50% O_2, and the [^{58}Co]-Cbl-injected animals were continued in air for (A) 0 h, (B) 0.5 h, (C) 48 h, and (D) 384 h.

respectively. These results indicate that levels of Cbl were decreased to a greater extent than either levels of holo-MMCoAM activity or levels of Cbl analogues. This observation suggests that the Cbl analogues present after prolonged exposure to N_2O may possess at least some Cbl activity for MMCoAM.

DISCUSSION

The studies presented here demonstrate that N_2O causes many deleterious effects on Cbl metabolism. A number of these effects occur within 30 min and include inhibition of MS activity, displacement of Cbl from MS, a decrease in levels of CH_3-Cbl and the formation of Cbl analogues from Cbl. The displacement of Cbl from MS parallels the loss of MS activity both temporally and quantitatively and this suggests that this is an early and possibly the primary effect of N_2O that causes a decrease in MS activity.

The recovery of MS activity when N_2O administration is stopped is very slow with complete recovery requir-

TABLE III

Effect of Prolonged Exposure to 50% N_2O/50% O_2 on the Levels of MS, MMCoAM, Cbl, and Cbl plus Cbl Analogues in Rat Liver, Kidney, and Brain

Length of exposure to 50% N_2O	Number of animals	Tissue	MS*		MMCoAM‡		Cbl			
							IF assay§		R protein assay‖	
			Mean	Range	Mean	Range	Mean	Range	Mean	Range
d			*% control*		*% control*		*% control*			
0	7	Liver	(100)	91–115	(100)	67–140	(100)	47–125	(100)	77–124
2	4	Liver	27¶	23–35	112	102–128	87	75–96	104	88–126
16	5	Liver	16¶	12–21	58¶	44–74	35¶	27–45	74	56–92
33	5	Liver	15¶	12–19	26¶	22–32	10¶	2–15	35¶	28–40
0	3	Kidney	(100)	90–106	(100)	65–138	(100)	83–138	(100)	73–116
33	5	Kidney	4¶	4–5	40¶	37–47	27¶	17–42	46¶	36–63
0	3	Brain	(100)	98–102	(100)	86–121	(100)	95–111	(100)	93–108
33	5	Brain	5¶	3–7	62**	41–82	4¶	2–7	24¶	21–25

* Mean MS activity in liver, kidney, and brain from rats not exposed to N_2O was 0.0054, 0.0122, and 0.0018 EU/g of tissue, respectively.

‡ The values are for holo-MMCoAM. Mean holo-MMCoAM in liver, kidney, and brain from rats not exposed to N_2O was 0.0218, 0.0155, and 0.0027 EU/g of tissue, respectively. Corresponding values for apo-MMCoAM were 0.5047, 0.0631, and 0.0299 EU/g of tissue. Values for apo-MMCoAM did not change significantly (<10%) in any of the tissues after any of the exposures to N_2O.

§ IF is highly specific for Cbl and the IF assay does not measure Cbl analogues to a significant extent. Mean values for Cbl in liver, kidney, and brain from rats not exposed to N_2O were 22.9, 23.8, and 11.4 pmol/g of tissue, respectively.

‖ R protein binds Cbl and Cbl analogues and the R protein assay measures Cbl plus Cbl analogues. Mean values for Cbl plus Cbl analogues in liver, kidney, and brain from rats not exposed to N_2O were 38.4, 67.5, and 19.0 pmol/g of tissue, respectively.

¶ $P < 0.01$.

** $P < 0.05$.

ing ~1 wk. The recovery of MS activity appears to parallel the reappearance of Cbl bound to MS and supports the concept that the amount of Cbl bound to MS is of paramount importance concerning the activity of this enzyme. The slow return of Cbl bound to MS could be due to a necessity for synthesis of new MS molecules, which could occur slowly, but could also be due to the fact that Cbl displaced from MS appears to be sequestered rapidly by MMCoAM. The latter possibility is supported by the fact that tissues do contain large amounts of apo-MMCoAM (see footnote to Table III), that MMCoAM is present in mitochondria, whereas MS is present in the cytosol (6), and by the fact that Cbl bound to MMCoAM appears to turn over at a relatively slow rate (5, 6).

The studies presented here indicate that N_2O does not have a direct effect upon MMCoAM activity although the progressive conversion of Cbl to Cbl analogues and the preferential excretion of the latter does eventually lead to a decrease in holo-MMCoAM activity. Because of this, it is premature to conclude that a lack of MS activity is responsible for both the hematologic and neurologic abnormalities that are seen in humans in Cbl deficiency. The fact that methylmalonicaciduria was not observed in monkeys (31) who developed neurologic abnormalities after 6 wk of exposure to N_2O has suggested that MMCoAM was not affected, but the activity of MMCoAM was not measured directly. Thus, it is possible that levels of holo-MMCoAM were reduced sufficiently to cause neurologic abnormalities without being reduced sufficiently to cause methylmalonicaciduria. It is also possible that levels of holo-MMCoAM activity were reduced disproportionately in certain portions of the central nervous system and that more moderate decreases in other tissues prevented the development of methylmalonicaciduria.

It is interesting that as little as 6 h exposure to N_2O causes megaloblastic changes in the bone marrow in man (11), that <2 wk exposure is required for profound granulocytopenia and thrombocytopenia (12), and that neurologic abnormalities are not observed during these time periods. It is also interesting that intermittent exposure to N_2O over many months, as occurs with abuse by dentists and anesthesiologists, causes neurologic abnormalities in man but does not cause hematologic abnormalities (22–24). These observations, together with our observations in rats, suggest that the rapid inhibition of MS by N_2O may be responsible for the hematologic abnormalities seen in man. They also suggest that decreases in tissue Cbl levels, formation of Cbl

analogues, further decreases in MS, or decreases in MMCoAM are responsible for the neurologic abnormalities seen in man, although the mechanism by which these latter changes effect or are related to the neurologic abnormalities is unknown. The possibility that the formation of Cbl analogues might play a role in the neurologic abnormalities seen in chronic N_2O exposure is intriguing in view of the previous suggestion (36) that variations in levels of endogenous Cbl analogues in patients with Cbl deficiency might explain why some patients develop only hematologic or neurologic abnormalities while others develop both. Definitive answers to these questions as well as to the question of whether endogenous Cbl analogues present in mammalian tissues (44) are the same as those formed from Cbl by N_2O, will require further studies.

A number of studies (26–28) have suggested that spontaneous abortions and birth defects occur with increased frequency among anesthesiologists, dentists, and operating room and dental personnel, and that frequent exposure to low levels of N_2O is the most likely cause for these abnormalities. Our studies with rats are of interest in this regard because they show that levels of N_2O as low as 2% cause significant inhibition of MS and since the individuals just mentioned are sometimes exposed to levels of N_2O that approach this concentration. Future studies measuring levels of MS, MMCoAM, Cbl, and Cbl analogues in these individuals would be of interest.

The effects of N_2O on Cbl metabolism also raises questions concerning the use of N_2O as a general anesthetic. Because of the rapid and prolonged inhibition of MS, one wonders, for example, if exposing donors, recipients, or both to N_2O has deleterious effects on the results of bone marrow, kidney, or other transplants. Such inhibition might not be detrimental and might even be beneficial due to effects on the immune system. Studies concerning this question would be of interest as would studies concerning whether postoperative infections are more frequent or severe after the use of N_2O, and whether the incidence of birth defects is increased when N_2O is administered in various settings during the early stages of pregnancy.

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