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Effect of Nitrous Oxide and Methionine Treatments on Hepatic *S*-Adenosylmethionine and Methylation Reactions in the Rat

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SUMMARY

Nitrous oxide administration to experimental animals leads to significant alterations in the hepatic folate pathway. This pathway is closely linked to the metabolism of methionine and *S*-adenosylmethionine (AdoMet), two compounds that play a central role in biologically important methylation reactions. This study was carried out to assess whether nitrous oxide administration to animals can affect the metabolism of AdoMet and the AdoMet-dependent methylation reactions. Exposure of rats to a mixture of nitrous oxide and oxygen (50:50) for 2 hr reduced hepatic AdoMet levels. However, when methionine was administered to these rats, hepatic AdoMet rapidly increased to levels that were significantly higher than those observed in air-exposed animals. Concomitant with this increase, there was a significant and marked increase in the rate of methylation of phospholipids and carboxymethylation of proteins. Thus, nitrous oxide, in addition to its inhibitory effect on 5-methyltetrahydrofolate:homocysteine methyltransferase (methionine synthase, EC 2.1.1.13) activity, possesses another effect. It increases the rate of conversion of exogenously administered methionine into AdoMet with a subsequent increase in the rate of methylation of key cellular constituents.

INTRODUCTION

There is an increase in the number of reports linking nitrous oxide treatments and certain toxic manifestations in humans and experimental animals. These include megaloblastic bone marrow changes, neurological changes that appear to resemble a state of vitamin B_{12} deficiency, and alteration of one-carbon metabolism (1). The mechanism of nitrous oxide toxicity has been proposed to be due to the inhibition of the methyl cobalamin-dependent, 5-methyltetrahydrofolate:homocysteine methyltransferase (methionine synthase, EC 2.1.1.13) activity in tissues (2). This enzyme appears to have an important role in the regulation of H_4folate[1] regeneration in the folate biochemical pathway (3) (Fig. 1). Within 2 hr of exposure of rats to nitrous oxide, there is a decreased level of hepatic H_4folate and an elevation of 5-methyl-H_4folate levels (4). As a consequence of this alteration, nitrous oxide decreased the *in vivo* rate of formate oxidation to CO_2, a process that is dependent upon hepatic H_4folate through the action of 10-formyl-H_4folate synthetase (EC 6.3.4.3) activity and 10-formyl-H_4folate dehydrogenase (EC 1.5.1.6) activity. It has been postulated that this process is critical for the oxidative metabolism of formate to carbon dioxide and plays a role in determining whether a species is sensitive to methanol poisoning (5). Methanol treatment following a brief exposure of rats to nitrous oxide leads to the production of metabolic acidosis in this species, whereas this animal is otherwise insensitive to the effects of methanol (3, 5).

It is also known that methionine administration to nitrous oxide-treated rats reverses the elevation of the ratio of 5-methyl-H_4folate to H_4folate concentrations in liver produced by nitrous oxide. The ability of methionine or ethionine (4) to reverse the alteration of H_4folate concentration in liver also leads to a reversal in the inhibition of formate oxidation to carbon dioxide. Although reversal of the effects of nitrous oxide by methionine or ethionine is rapid and dramatic, the mechanism of this reversal remains somewhat in doubt. It is possible that methionine administration leads to increases in AdoMet which may act by inhibiting 5,10-methylene-H_4folate reductase (6, 7) and thereby reducing the flux of folate to 5-CH_3-H_4folate. Methionine administration to nitrous oxide-treated rats does not lead to reversal of nitrous oxide inhibition of methionine synthase activity.[2]

Since alteration of methionine synthase activity should produce changes in methionine synthesis and subsequent modulation of AdoMet levels, we examined the effect of nitrous oxide on steady-state levels of AdoMet; the effect of the combination of methionine and nitrous oxide on

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[1] The abbreviations used are: H_4folate, tetrahydrofolate; AdoMet, *S*-adenosylmethionine.

[2] K. A. Black and T. R. Tephly, unpublished observation.

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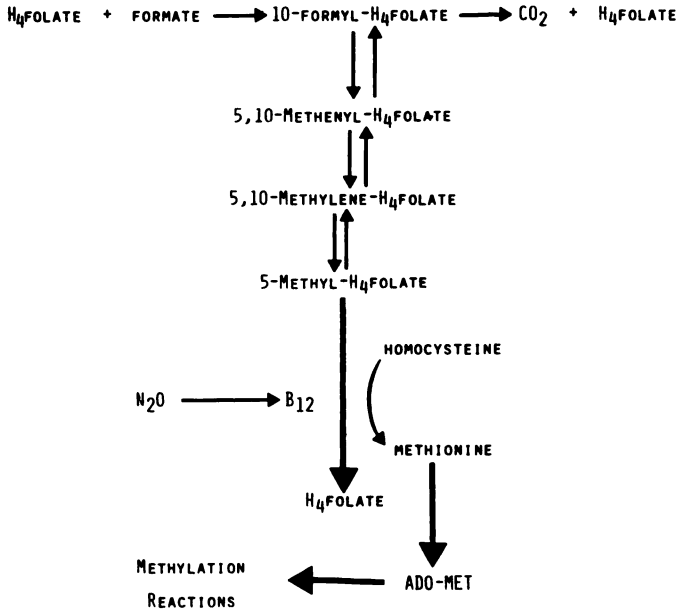

FIG. 1. *H_4folate regeneration in the folate biochemical pathway*

AdoMet-dependent methylation reactions was also examined.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (200–250 g) were obtained from Bio-Labs (Madison, Wisc.) and were housed in wire-bottomed cages. Temperature, humidity, and lighting were controlled. Purina laboratory chow and water were provided ad libitum.

Chemicals. *S*-Adenosyl-L-[*methyl*-^{14}C]methionine (59 mCi/mmole), [^{14}C]sodium formate (58.6 mCi/mmole), L-[1-^{14}C]methionine (51 mCi/mmole), and L-[*methyl*-^{14}C]methionine (56.7 mCi/mmole) were purchased from the Amersham-Searle Corporation (Arlington Heights, Ill.). Preblend 3a70B scintillation cocktail was purchased from Research Products International Corporation (Elk Grove, Ill.). The cation exchange resin, Cellex P, was purchased from Bio-Rad Laboratories (Richmond, Calif.). All other chemicals were purchased from Sigma Chemical Company (St. Louis, Mo.) and were of the highest available purity.

Metabolic experiments. Exposure of rats to nitrous oxide was carried out in glass metabolic chambers to measure the rate of $^{14}CO_2$ generation. A mixture of nitrous oxide-oxygen in the ratio of 50:50 was introduced into the chamber at a rate of 4 liters/min. One rat was placed in each chamber and was exposed to N_2O/O_2 for at least 2 hr before any injections were made. Exposure was continued throughout the course of the experiment. In control studies, rats were exposed to room air. L-[1-^{14}C]Methionine or L-[*methyl*-^{14}C]methionine (500 dpm/nmole) was administered i.p.

Hepatic AdoMet assay. A modification of the method of Eloranta *et al.* (8) was employed for the determination of AdoMet in hepatic tissue. Rats were killed by decapitation. Livers were rapidly removed and homogenized in 2 volumes of 0.25 M sucrose containing 1 mM mercaptoethanol, 0.1 mM EDTA, and 1 mM dithiothreitol. Homogenates were mixed with 50% (w/v) trichloroacetic acid to give a final concentration of 8% trichloroacetic acid. The homogenates were centrifuged at 20,000 × *g* for 15 min at 4°. The supernatant fraction was separated and an aliquot was extracted three times with an equal volume of ether, filtered through a filter paper, and applied to a Cellex-P column (1 cm × 7 cm) (H^+ form). The column was washed with 100 ml of 1 mM HCl followed by 70 ml of 10 mM HCl and 80 ml of 50 mM HCl. AdoMet was then eluted with 30 ml of 500 mM HCl. The amount of AdoMet was determined by measuring A_{257} and using a molar extinction coefficient of 1500 liters mole^{-1} cm^{-1}.

In vivo ^{14}C-methionine incorporation into phospholipids. Rats were exposed to N_2O/O_2 (50:50) for 3 hr prior to decapitation. L-[*methyl*-^{14}C]Methionine or L-[1-^{14}C]methionine (500 dpm/nmole) was administered i.p. 1 hr prior to sacrifice. Livers were rapidly removed. Initial lipid extraction was carried out essentially as described by Folch *et al.* (9). Liver tissue was extracted with 20 volumes of a mixture of chloroform and methanol (2:1, v/v). After centrifugation and filtration through Whatman paper No. 1, the filtrate was mixed with 15% of its volume of 0.1 M KCl and allowed to separate. The lower phase was taken, dried under nitrogen, redissolved in chloroform, and applied to a silicic acid column (1 × 30 cm). Neutral lipids, glycosphingolipids, and phospholipids were separated by passing through the column, in sequence, 100 ml of chloroform, 100 ml of acetone/methanol (9:1, v/v), and 200 ml of methanol. Part of the methanol fraction (containing the phospholipids) was evaporated under nitrogen (to dryness), mixed with 15 ml of the scintillation cocktail 3a70B, and counted. Another part of the methanol fraction was evaporated to dryness under nitrogen and its phospholipid phosphorus content was determined by the method of Bartlett (10).

A third part of the methanol fraction was evaporated to dryness under nitrogen, dissolved in a minimal volume of chloroform, and applied to a silica gel plate. After development in chloroform/methanol/ammonium hydroxide (60::35:5, v/v), the plate was dried and the various lipids were detected by iodine. Authentic standards were used to locate the specific phospholipids. Each lipid fraction was separately scraped off from the plate into a vial, mixed with 10 ml of the scintillation counting cocktail 3a70B, and counted.

In vivo [^{14}C]methionine incorporation into proteins. Total, stable, and labile incorporation of radioactivity into liver proteins was determined by the method described by Schatz *et al.* (11). In this method two types of incorporation were examined: stable incorporation (nonhydrolyzable by heating at 100° for 5 min in a pH 7.4 medium), representing incorporation into the backbone of the protein, and labile incorporation (hydrolyzable under the described conditions), representing the carboxymethylation of the protein. Proteins were determined by the method of Lowry *et al.* (12).

In vivo [^{14}C]formate incorporation into hepatic 5-methyl-H_4folate. Rats were exposed to an N_2O/O_2 (50:50) mixture or to atmospheric air for two hr prior to the i.p. administration of [^{14}C]formate (10,000 dpm/mmole; 10 mg/kg) at zero time. The animals were killed 30 min or 60 min after the [^{14}C]formate injection. Livers were rapidly removed and placed in liquid nitrogen. Various folate derivatives were determined in liver samples as described by McMartin *et al.* (13). The high-pressure liquid chromatography fractions containing the 5-methyl-H_4folate were pooled, and a sample was mixed with the scintillation cocktail 3a70B and examined for radioactivity.

Statistical analysis. Student's *t*-test for unpaired data was used to compare group means. The minimal level of significance was taken at $p < 0.05$.

RESULTS

Effect of nitrous oxide and methionine on hepatic AdoMet levels in vivo. Figure 2 presents kinetic studies performed *in vivo* at three doses of methionine in rats which had been exposed for 2 hr to nitrous oxide/oxygen (50:50). At zero time, about a 30% decrease in AdoMet levels was observed in nitrous oxide-treated animals. Following methionine administration, there was a dose-dependent increase in AdoMet levels in both air-exposed rats and in nitrous oxide-treated animals. However, the magnitude of increase in the nitrous oxide-treated rats was consistently and significantly above that seen in air-exposed animals. Peak levels of AdoMet were reached within 15–30 min following methionine administration; and at the higher doses of methionine, AdoMet levels remained significantly increased over the course of the

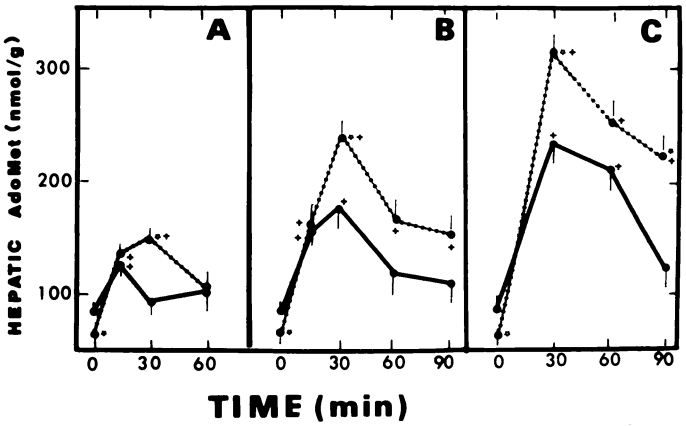

FIG. 2. *Effect of nitrous oxide and methionine on hepatic AdoMet levels*

Rats were exposed to an N_2O/O_2 mixture (●– – –●) or atmospheric air (●——●) for 2 hr prior to the i.p. administration of methionine at zero time. Doses of methionine injected (in milligram per kilogram of body weight) were 22, 50, and 100 in A, B, and C, respectively. Hepatic AdoMet was assayed as described under Materials and Methods. Each point represents the mean of at least three animals ± standard error of the mean. * Statistically significantly different from the corresponding control ($p < 0.05$). † Statistically significantly different from corresponding zero time value ($p < 0.05$).

experiments. This apparent promotion of elevated AdoMet levels induced by methionine cannot be linked to effects of nitrous oxide on methionine synthase activity and suggests a second action of nitrous oxide beyond that previously described for its inhibitory effects on methionine synthase activity.

Studies on the rate of incorporation of L-[methyl-^{14}C] methionine and L-[1-^{14}C]methionine into hepatic AdoMet in vivo following nitrous oxide treatment. Since nitrous oxide treatment appeared to promote elevation of AdoMet levels above that produced by methionine administration alone, kinetic studies were carried out using [^{14}C]methionine in order to determine the rate of flux of methionine into hepatic AdoMet. This reaction is carried out in the liver through the mediation of ATP:L-methionine *S*-adenosyltransferase (methionine adenosyltransferase, EC 2.5.1.6). One experiment was conducted using methionine labeled with ^{14}C in the carboxyl group, and a second experiment was performed with [^{14}C]methylmethionine. A 30-min time period was chosen because it is at this time period where apparent maximal elevation of AdoMet level occurs *in vivo* (Fig. 2). Table 1 demonstrates that more radioactivity was incorporated into hepatic AdoMet in nitrous oxide-treated rats than in those which had been air-exposed. Also, the amount of radioactivity incorporated from methyl-labeled methionine was less than one-half that seen when carboxyl-labeled methionine was employed. This indicates that the methyl group of methionine and/or AdoMet is more active metabolically and thus has a faster metabolic turnover rate as compared with the carboxyl group of the molecule.

Effect of nitrous oxide, the incorporation of L-[methyl-^{14}C]methionine and L-[1-^{14}C]methionine on hepatic phospholipids, and on carboxymethylation of proteins.

TABLE 1

Effect of nitrous oxide on the incorporation of L-[methyl-^{14}C] methionine and L-[1-^{14}C]methionine into hepatic AdoMet

Exposure to nitrous oxide was carried out as described under Materials and Methods. L-[*methyl*-^{14}C]methionine and L-[1-^{14}C]methionine (500 dpm/nmole) were injected i.p. in a dose of 22 mg/kg. Thirty minutes after methionine injection, the rats were killed and hepatic AdoMet was determined as described under Materials and Methods. Values represent the mean ± standard error of the mean obtained from at least three rats.

Treatment	AdoMet	^{14}C Label in AdoMet
	nmoles/g liver	*nmoles/g liver*
L-[*methyl*-^{14}C]Methionine		
Control	87.2 ± 2.3	11.6 ± 1.6
Nitrous oxide	128.5 ± 4.8[a]	19.2 ± 1.3[a]
L-[1-^{14}C]Methionine		
Control	87.5 ± 3.5	28.5 ± 1.0[b]
Nitrous oxide	139.1 ± 5.2[a]	49.1 ± 1.6[a, b]

[a] Values are significantly different from air control values ($p < 0.01$).
[b] Values are significantly different from corresponding L-[*methyl*-^{14}C]methionine values ($p < 0.001$).

Table 2 demonstrates the extent of incorporation of [^{14}C] methylmethionine into hepatic phospholipids. Nitrous oxide treatment of rats markedly stimulated the methylation of phospholipids above that demonstrated with methionine alone. Further studies were performed in which phospholipids were separated by thin-layer chromatography into their various fractions; more than 85% of the label was associated with the phosphatidylcholine fraction. As expected, very little label was incorporated from the carboxyl-labeled methionine into phospholipids, and nitrous oxide had no effect on this very small amount of incorporation. These results are entirely consistent with the fact that hepatic AdoMet levels are higher in nitrous oxide-treated rats (Fig. 2).

Protein carboxymethylation was also studied. Table 3 shows that nitrous oxide treatment of rats leads to an

TABLE 2

Effect of nitrous oxide on the incorporation of L-[methyl-^{14}C] methionine and L-[1-^{14}C]methionine into hepatic phospholipids

Exposure to nitrous oxide and [^{14}C]methionine injection were carried out as described in Table 1. Rats were killed 1 hr after methionine administration. Phospholipids were isolated and assayed as described under Materials and Methods. Values represent the mean ± standard error of the mean obtained from at least three rats.

Treatment	^{14}C Label in phospholipids	
	nmoles	*^{14}C/μmole phospholipid phosphorus*
L-[*methyl*-^{14}C]Methionine		
Control		36.01 ± 1.63
Nitrous oxide		55.16 ± 2.77[a]
L-[1-^{14}C]Methionine		
Control		0.64 ± 0.02[b]
Nitrous oxide		0.63 ± 0.02[b]

[a] Values are significantly different from the corresponding control values ($p < 0.001$).
[b] Values are significantly different from the corresponding L-[*methyl*-^{14}C]methionine values ($p < 0.001$).

TABLE 3

Effect of nitrous oxide on the incorporation of L-[methyl-^{14}C] methionine and L-[1-^{14}C]methionine into hepatic proteins

Exposure to nitrous oxide and [^{14}C]methionine injection were carried out as mentioned in Table 1. Rats were killed 1 hr after methionine administration. Proteins were fractionated and assayed as described under Materials and Methods. Values represent the mean ± standard error of the mean obtained from at least three rats.

Treatment	^{14}C Label in hepatic proteins		
	Total	Stable fraction	Labile fraction
	nmoles ^{14}C/mg protein		
L-[*methyl*-^{14}C]Methionine			
Control	1.94 ± 0.11	0.99 ± 0.04	0.95 ± 0.13
Nitrous oxide	2.94 ± 0.17[a]	1.02 ± 0.04	1.48 ± 0.14[a]
L-[1-^{14}C]Methionine			
Control	1.16 ± 0.01[b]	1.00 ± 0.03	0.16 ± 0.03[b]
Nitrous oxide	1.21 ± 0.01[b]	1.06 ± 0.04	0.15 ± 0.02[b]

[a] Values are significantly different from the corresponding control values ($p < 0.05$).

[b] Values are significantly different from the corresponding L-[*methyl*-^{14}C]methionine values ($p < 0.01$).

increased incorporation of methyl label from methionine into liver protein. Nitrous oxide treatment of rats led to an increase in ^{14}C labeling of hepatic proteins when methyl-labeled methionine was employed but not when carboxyl-labeled methionine was used. Furthermore, the incorporation increase was attributed to the labile fraction, which is generally ascribed to carboxymethylation. This is dependent upon AdoMet and is consistent with the elevated AdoMet levels in the livers of rats treated with nitrous oxide.

Effects of nitrous oxide on incorporation of [^{14}C]formate into hepatic 5-methyl-H$_4$folate. Studies were carried out to determine incorporation of [^{14}C]formate into 5-methyl-H$_4$folate. The amount of radioactivity appearing in 5-CH$_3$-H$_4$folate was increased significantly at both 30 and 60 min after [^{14}C]formate injection (Table 4). Results in Fig. 3 show the specific activity of ^{14}C in 5-CH$_3$-H$_4$folate. The results for specific activity (Fig. 3) are not significantly different at 30 min, probably because 5-CH$_3$-H$_4$folate levels are quite elevated in the N_2O-treated rats prior to [^{14}C]formate admainistration. Thus, 60 min are needed to reveal the difference in specific activity. These experiments represent the first direct demonstration of the trapping of ^{14}C label from formate in 5-methyl-H$_4$folate in nitrous oxide-treated animals.

TABLE 4

Effect of N_2O on [^{14}C]formate incorporation into 5-methyl-H$_4$folate

	Radioactivity	
	30 Min	60 Min
	dpm × 10^{-3}/g liver	
Control	5.5 ± 0.5	1.0 ± 0.4
N_2O-Treated	10.3 ± 1.0[a]	6.0 ± 0.7[a]

[a] Significantly different from control values ($p < 0.05$). The dose of formate employed was 10 mg/kg administered i.p. 2 hr after N_2O/O_2 (50:50) treatment. The 5-methyl-H$_4$folate level in control animals was 8.0 nmoles/g of liver and, in N_2O-treated animals, the value was 13 nmoles/g of liver.

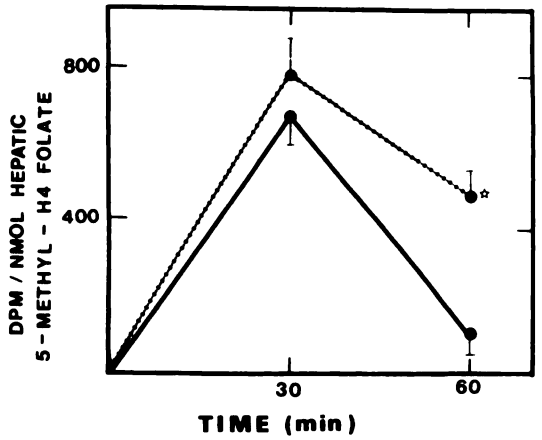

FIG. 3. *Effect of nitrous oxide on the incorporation of [^{14}C]formate into hepatic 5-methyl-H$_4$folate*

Rats were exposed to an N_2O/O_2 mixture (●- - -●) or atmospheric air (●——●) for 2 hr prior to the i.p. administration of [^{14}C]formate (10,000 dpm/nmole; 10 mg/kg) at zero time. Each point represents the mean of at least three rats ± standard error of the mean. * Statistically significant from the corresponding control value ($p < 0.01$).

DISCUSSION

Previous reports from this laboratory (4) as well as other labortories (14) showed that methionine is very rapidly and efficiently converted into AdoMet *in vivo*. The present study, using various doses of methionine, shows the time course of methionine conversion into hepatic AdoMet. In agreement with the previous reports, hepatic AdoMet sharply increased after the methionine injection. However, there was a somewhat unexpected observation; that is, in the nitrous oxide-exposed rats, AdoMet not only showed a sharp rise in its hepatic concentrations but reached levels that were significantly higher than those observed in the corresponding air-breathing rats. The mechanism by which nitrous oxide causes this increase in hepatic AdoMet concentration is not clearly understood. Two possibilities which might be considered are that it increases the rate of AdoMet synthesis from methionine by stimulating the methionine adenosyltransferase activity (directly or indirectly) or that it inhibits the further utilization of AdoMet mainly by inhibiting the AdoMet-dependent methylation reactions. Results obtained in this study clearly favor the former effect, since nitrous oxide does not inhibit the utilization of AdoMet. The rate of methylation of phospholipids and proteins is significantly increased in nitrous oxide-treated rats as compared with air-exposed rats. A probable mechanism would be a stimulatory effect of nitrous oxide on the rate of synthesis of AdoMet, resulting in high levels of AdoMet which lead to the increased rate of AdoMet-dependent methylation reactions. Further experiments are in progress to determine the site of action of nitrous oxide on the AdoMet metabolism.

Interest in this study can be more appreciated when the role of the AdoMet-dependent reaction in intermediary metabolism is considered (15). AdoMet is the methyl donor for numerous methylation reactions.

AdoMet-dependent methylation reactions are now considered to be a universal mechanism for regulating the activity of macromolecules; e.g., protein carboxymethylation has been shown to play a role in leukocyte chemotaxis (16, 17). Hirata and Axelrod (18) showed that the degree of methylation of the erythrocyte membrane phospholipids markedly affects the fluidity of these membranes. Furthermore, several studies showed that mRNA methylation is an important factor in the efficiency of translation of mRNA (19).

AdoMet is also the precursor of decarboxylated AdoMet, which is involved in the biosynthesis of the polyamines spermidine and spermine—compounds that play an essential role in cellular metabolism and regulation (20). The possibility that nitrous oxide can affect the metabolism of these polyamines should be seriously considered.

Another consequence of the effect of nitrous oxide is on the regulation of folate biochemistry. Previous reports have shown that nitrous oxide administration results in significant changes in the steady-state levels of various folate coenzymes. There is a significant decrease in hepatic H_4folate concentrations and a corresponding increase in the 5-methyl-H_4folate levels (4). This has been ascribed to a trapping of methyl groups in the form of 5-methyl-H_4folate form according to what is known as the methyl-trap hypothesis (21, 22).

Our results directly demonstrate this trapping. Thus [^{14}C]formate adminstration to air-breathing and nitrous oxide-breathing rats led to significantly higher radioactivity in 5-methyl-H_4folate in the nitrous oxide-breathing rats as compared with air-breathing controls. In other studies a marked reduction of H_4folate has been observed. These data highlight the perturbation that can be produced by an agent once thought to be relatively inert.

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