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Disruption of Folate and Vitamin B_{12} Metabolism in Aged Rats Following Exposure to Nitrous Oxide

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The ability of nitrous oxide (N_2O) to disrupt folate and vitamin B_{12} metabolism was examined in young (2-month), middle-aged (12-month), and elderly (24-month) Fischer 344 rats. Abnormalities in folate metabolism were assessed in a noninvasive manner by measuring the urinary excretion of formic acid and formiminoglutamic acid (FIGLU), compounds that are elevated in the urine of mammals with a deficiency in folate. After a 6-h exposure to 60% N_2O/40% O_2, urinary formic acid excretion increased 3- to 25-fold the first day following N_2O exposure and returned to background levels by the second day after exposure in all age groups. Urinary FIGLU excretion increased 100- to 300-fold in the first day following N_2O exposure, with the highest FIGLU excretion rates found in the elderly rats and the lowest in the young rats. By the second day after N_2O exposure, FIGLU excretion rates returned to baseline levels in all age groups. Plasma folate progressively decreased with increasing age, whereas no age-dependent changes were observed in red cell folate, liver folate, or plasma vitamin B_{12} levels. The elderly rats demonstrated the highest vitamin B_{12} content in the liver and the lowest vitamin B_{12} content in the kidney compared to the other age groups. Hepatic methionine synthase activities (measured 16–21 days after N_2O exposure) were elevated in the elderly compared to the middle-aged or young rats, but methionine synthase activities in kidney and brain were not different among the three different age groups. It was concluded that in rats, aging *per se* only slightly influences the disruption of folate metabolism produced by exposure to N_2O. (Key words: Age factors. Anesthetics, gases: nitrous oxide. Enzymes: methionine synthase. Vitamins: folate, B_{12}.)

NITROUS OXIDE (N_2O) inactivates methionine synthase (EC 2.1.1.13), a vitamin B_{12}-dependent enzyme that plays a pivotal role in folate metabolism, in both animals[1–3] and humans.[4,5] Although signs of vitamin B_{12} and folate deficiency develop in humans given N_2O for prolonged periods (days), the clinical significance of N_2O use for a limited duration remains less certain.[6,7]

The toxic effects produced by N_2O appear to be greatest in critically ill patients and in those with a pre-existing vitamin B_{12} and/or folate deficiency. Critically ill patients given N_2O for 0.5–6 h develop megaloblastic bone marrows and have an increased mortality rate.[8,9] In patients with subclinical deficiencies of vitamin B_{12}, neurologic dysfunction and megaloblastic changes have occurred after routine surgical procedures of approximately 2 h duration in which N_2O was a component of the anesthetic.[10,11] Furthermore, in a rat animal model, vitamin B_{12} deficiency and N_2O act in a synergistic fashion to retard growth[12] and to impair the use of deoxyuridine in bone marrow.[13] Such observations are consistent with the thought that "sick" individuals and those with deficient or marginal stores of vitamin B_{12} might be harmed by N_2O exposure.

The elderly may constitute another subpopulation of patients susceptible to the inactivation of methionine synthase by N_2O. Elderly patients are often debilitated and tend to have deficiencies in vitamin B_{12} or folate, with the incidence of subnormal values of these vitamins being as high as 25% in patients over 60 yr of age.[14–17] Such findings would appear to invite an examination of the role of aging *per se* on the disruption of folate metabolism after exposure to N_2O. However, variables such as diet, vitamin and mineral intake, disease processes, medications, duration of anesthesia, and type and extent of surgery are difficult to control in the perioperative period, and moreover, tissue biopsies required for certain measurements of folate status are difficult to obtain in patients because of ethical and practical reasons. Therefore, in the current study, we used the Fischer 344 rat, a rodent model extensively used in research on aging,[18] to examine the combined influence of age and N_2O on folate metabolism.

The disruption of folate metabolism in rats was noninvasively assessed by measuring the excretion of formic acid and formiminoglutamic acid (FIGLU) in urine. Increased urinary excretion of these compounds has been found in young rats exposed to N_2O,[19,20] presumably due to a N_2O-induced depletion of free tetrahydrofolate that is required for the further metabolism of formic acid and FIGLU (fig. 1). In addition, blood and tissues were analyzed for folate and vitamin B_{12} levels and methionine synthase activities in rats of different ages. Our hypothesis was that the older animals would exhibit a relative deficiency in folate and vitamin B_{12}, have lower methionine synthase activities, and demonstrate an enhanced and prolonged excretion of urinary formic acid and FIGLU after exposure to N_2O when compared to the younger animals.

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Materials and Methods

These experiments were approved by the Animal Studies Subcommittee at the Veterans Administration Medical Center, San Francisco. Male Fischer 344 rats (National Institute of Aging colonies, Indianapolis, IN) of three different ages (2-month [young], 12-month [middle-aged], and 24-month [old or elderly]) were used in these studies. We obtained eight rats in each of the three age groups. The rats were housed in individual plastic metabolic cages (Nalge; 36 cm length × 28 cm width × 52 cm height). The animals were given Purina Rodent Laboratory Chow (Diet #5001, Purina Mills Inc.) and tap water *ad libitum* except during the 6-h period of exposure to N_2O.

Animals were allowed to adapt to their new surroundings for at least 1 week before the start of the experiments. To obtain background values for the urinary metabolites (formate, FIGLU) and to test for their day-to-day variability, six consecutive 24-h urine samples were collected from each of the animals before N_2O exposure. The urine collection vials contained 0.2 ml concentrated hydrochloric acid to minimize bacterial growth. After the background urine samples were obtained and frozen, rats were placed into individual, cylindrical, Plexiglass chambers (30 cm in length and 9–10 cm in diameter) with one-holed rubber stoppers at both ends. Plastic connectors inserted into the stoppers and attachment of tygon tubing allowed for the passage of gases. A sampling port attached to a three-way stopcock permitted sampling of chamber gases. Exposures to N_2O were performed between 0900 and 1500 hours. The chambers were initially flushed with

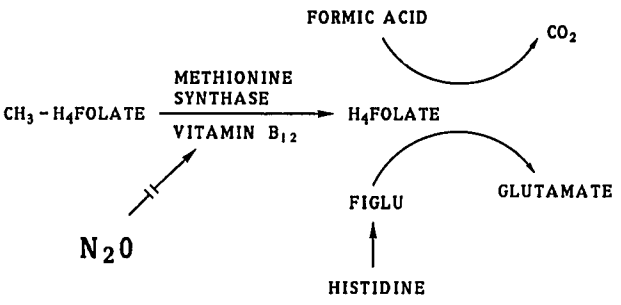

FIG. 1. A simplified scheme to illustrate the buildup of formic acid and formiminoglutamic acid (FIGLU) that occurs after exposure to nitrous oxide. Methionine synthase generates tetrahydrofolate (H_4FOLATE) from 5-methyltetrahydrofolate (CH_3-H_4FOLATE) and in the process converts homocysteine to methionine. Nitrous oxide-induced inactivation of methionine synthase *via* oxidation of the vitamin B_{12} cofactor results in relative depletion of tetrahydrofolate. Because tetrahydrofolate is required for the oxidation of formic acid to carbon dioxide and for the further breakdown of FIGLU to glutamate, formic acid and FIGLU levels tend to increase after exposure to nitrous oxide, resulting in an increased urinary excretion of these compounds. A more complete description of vitamin B_{12}-folate interrelationships and the metabolic processes involved is provided in reference 44.

100% oxygen for more than 2 min, and an inspired mixture of 60% N_2O/40% O_2 was administered for 6 h. Flow rates per rat were approximately 1 l/min. Gas samples were taken at approximately 90-min intervals from individual chambers during the 6-h exposure and analyzed with a SARA mass spectrometer to confirm the gas concentrations. Chamber CO_2 levels remained below 0.6%. Following the 6-h N_2O exposure, the chambers were flushed with 100% oxygen for 2 min. After the animals were removed from the N_2O environment, they were returned to their metabolic cages in room air and again provided with rodent chow and water.

A 17-h urine collection followed the N_2O exposure period. Twenty-four-hour urine samples were also collected on days 2, 3, 4, and 9 following N_2O exposure. Sixteen to 21 days following nitrous oxide exposure, the rats were killed with 100% carbon dioxide. Blood was collected from the inferior vena cava using heparin (20 units/ml blood) as the anticoagulant. Livers, kidneys, and brains were isolated from the animals, weighed, and stored at −20°−−30°C. Animal weights at the time of killing ranged from 206–330 g for the young rats, 384–436 g for the middle-aged rats, and 324–422 g for the elderly rats.

One of the 24-month old animals exhibited minimal food and water intake, a urine output of usually < 1 ml/day, and a body weight that was 38% less than its peers; thus, this aged rat was eliminated from the experiment and was not included in the pooled analyses. On autopsy, this rat had a large testicular tumor.

Methionine synthase activity was determined as described previously.[2] Activity was expressed as nanomoles of methionine produced $h^{-1} \cdot g^{-1}$ of original tissue or as nanomoles methionine produced $h^{-1} \cdot mg^{-1}$ of protein in the supernatant. Protein content was determined by the method of Lowry *et al.*[21]

Urinary FIGLU was analyzed by a spectrophotometric procedure as specified in Sigma Diagnostics Procedure No. 365-UV. Urinary formic acid was analyzed by the method used for formic acid in serum[22] with the exception that the acetonitrile precipitation step was not necessary for the analysis of urine specimens. Formic acid concentrations do not change when stored at −20° C for up to 6 months,[22] and all of the samples were assayed well within this time period. Excretion rates of FIGLU and formic acid were calculated from the product of the concentration and volume of urine collected over a given time period.

Vitamin B_{12} and folate concentrations in plasma, red cell folate concentrations, and vitamin B_{12} in liver, kidney, and brain were determined by a radioassay (MAGIC Vitamin B_{12} [^{57}Co]/Folate[^{125}I] No Boil Radioassay, CIBA Corning Diagnostics) that uses purified intrinsic factor for analysis of vitamin B_{12}. Vitamin B_{12} was extracted

from tissues after the method of Kondo *et al.*[23] Folate was extracted from liver after homogenization in 5% (w/v) ascorbate.[24] Supernatants of the tissue extracts were diluted (Immophase B12/Folate 0 Standard, CIBA Corning Diagnostics) to obtain values within the range of the assay standards. A linear response for vitamin B_{12} or folate was attained with various dilutions of the supernatants for each of the organs. A low folate/vitamin B_{12} control (BioRad) was included in each of the assays. Total tissue contents of vitamin B_{12} and folate were calculated from the product of the tissue concentrations of vitamins and the weight of the organs.

Statistical computations were performed by analysis of variance. Error bars associated with mean values indicate ± SD. Multiple comparisons were performed using the Newman-Keuls test or its analog when the nonparametric Kruskal-Wallis analysis of variance was used.[25] $P < 0.01$ was considered statistically significant.

Results

Background excretion rates of urinary formic acid in animals of the three different age groups and a measure of the day-to-day variability in formic acid excretion rates were obtained by six consecutive 24-h urine sample collections (fig. 2). Formic acid excretion rates (in μg/h) tended to be slightly lower in the middle-aged than in the young or old animals. For any given day, however, the formic acid excretion rates did not differ significantly among groups. No significant day-to-day variations in formic acid excretion rates were detected in any of the three different age groups.

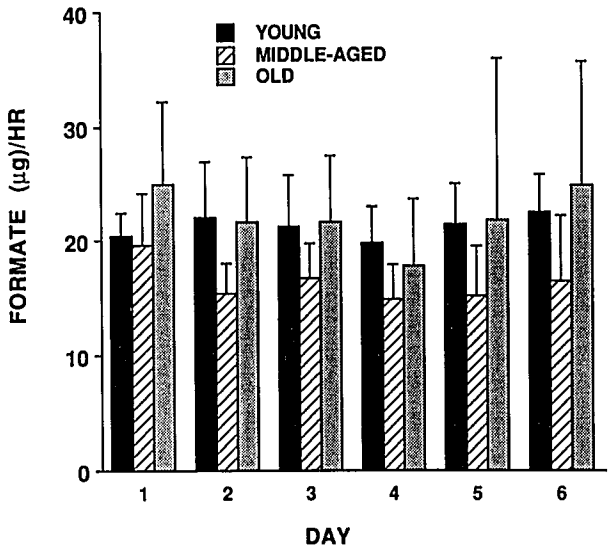

FIG. 2. Urinary formic acid excretion rates in eight young, eight middle-aged, and seven elderly rats in six consecutive 24-h urine samples obtained before exposure of the animals to nitrous oxide. Values are expressed as the total micrograms of formic acid excreted per h (mean ± SD). For any given day, formic acid excretion rates (A) were not significantly ($P < 0.01$) different amoung the young, middle-aged, or old animals. There were no significant day-to-day variations in formic acid levels among any of the three different age groups.

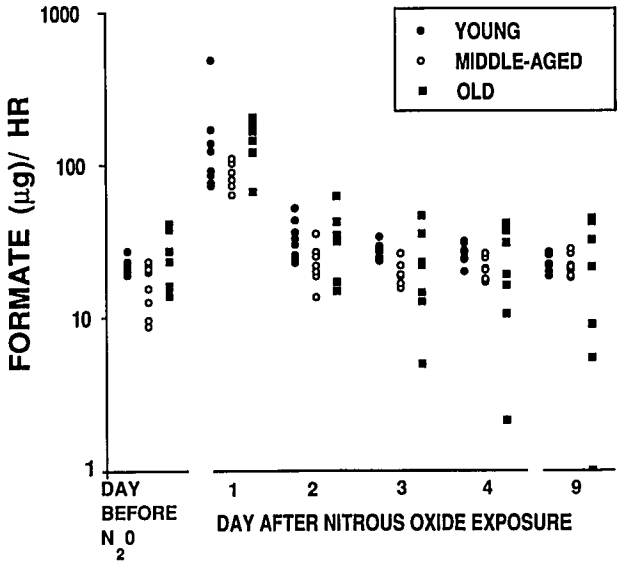

FIG. 3. Urinary formic acid excretion rates in young, middle-aged, and elderly rats on the day immediately before, and on days after exposure to 60% N_2O for 6 h. Values are expressed as the total micrograms of formic acid excreted per h. Note the logarithmic scale of the ordinates. For each of the time points measured there are eight values for the young and middle-aged rats and seven values for the old rats, although a lesser number of symbols are visualized because some of the points overlap. Data comparison for any given age group at all time points using the Kruskal-Wallis test for nonparametric analysis of variance revealed significantly ($P < 0.01$) elevated formic acid excretion rates for the young and middle-aged rats 1 day after N_2O exposure compared to the other time points; the formic acid elevations in the elderly rats 1 day after N_2O exposure compared to background levels of formic acid just failed to reach statistical significance at the $P < 0.01$ level. Formic acid excretion rates 1 day after N_2O exposure were not different among the three different age groups.

During the 24-h collection of urine samples at room temperature, the possibility existed that formic acid might be destroyed or produced by bacteria contaminating the urine samples or that formic acid might evaporate from the urine collection chambers that were exposed to room air. To test for these possible artifactual changes, 11 different urine samples were either frozen immediately or allowed to remain at room temperature for 24 h and then frozen. Formic acid concentrations in the samples allowed to remain at room temperature for 24 h were 100% ± 9% (± SD) of the fresh urine samples.

Urinary formic acid excretion rates increased in all three age groups by 3- to 25-fold for the first day following exposure to N_2O (fig. 3). Age did not influence formic acid excretion rates 1 day after N_2O exposure. Formic acid excretion rates returned to background levels by the

second day after exposure and remained at the background levels thereafter (fig. 3).

Prior to N_2O exposure, FIGLU excretion rates in urine were relatively small and did not differ with age. Background FIGLU excretion rates (mean ± SD) were 1.9 ± 1.9, 1.6 ± 2.0, and 4.5 ± 4.5 nmol/h in the young, middle-aged, and elderly animals, respectively. Urine collections obtained the day after N_2O exposure showed marked increases (more than 100-fold) in FIGLU excretion rates, and the elevations in FIGLU excretion rates were highest in the elderly and least in the young rats (fig. 4). The FIGLU excretion rates returned to those near background in all three age groups by the second day after N_2O exposure (fig. 4).

Methionine synthase activities in liver, kidney, and brain were measured 16–21 days after exposure of rats to N_2O. Hepatic methionine synthase activities, measured either on the basis of per gram of original tissue (fig. 5A) or per milligram of protein (fig. 5B), were significantly ($P < 0.01$) higher in the elderly as compared to middle-aged or young rats. Methionine synthase activities in kidney and brain did not vary significantly with age (fig. 5).

Plasma concentrations of folate consistently decreased with increasing age of the animals (table 1); however, there were no differences in red cell folate or liver folate (in terms of μg folate/g liver) among animals of the three different age groups. (The lower amount of total liver folate [in μg] in the young animals was related to the lower liver weights in young compared to middle-aged and elderly rats.) Plasma concentrations of vitamin B_{12} did not exhibit a consistent trend with age. Similarly, there were no consistent changes in tissue concentrations of vitamin B_{12} with age. Hepatic concentrations of vitamin B_{12} were highest in the elderly rats, whereas the elderly rats exhibited the lowest concentrations of vitamin B_{12} in kidney (table 1). Brain concentrations of vitamin B_{12} did not differ among young, middle-aged, and elderly animals.

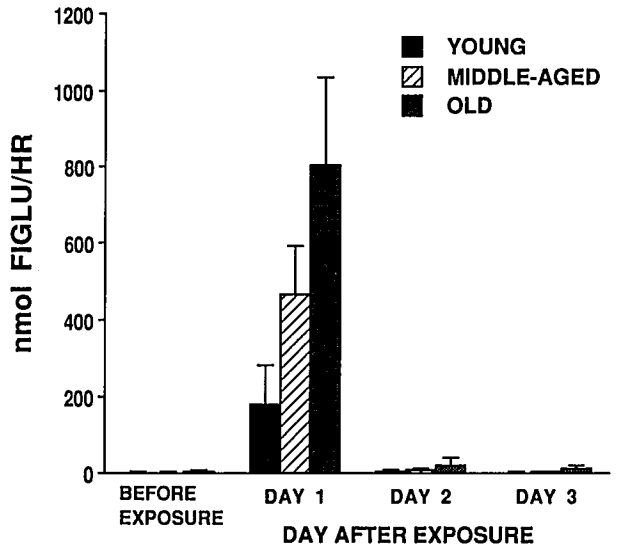

FIG. 4. Urinary FIGLU excretion rates (mean values ± SD) in young, middle-aged, and elderly rats on the day immediately before and on days after exposure to 60% N_2O for 6 h. Values are expressed as the total nanomoles of FIGLU excreted per hour. FIGLU excretion rates differed significantly ($P < 0.01$) in the three different age groups (old > middle-aged > young) in the urine collections obtained 1 day after N_2O exposure.

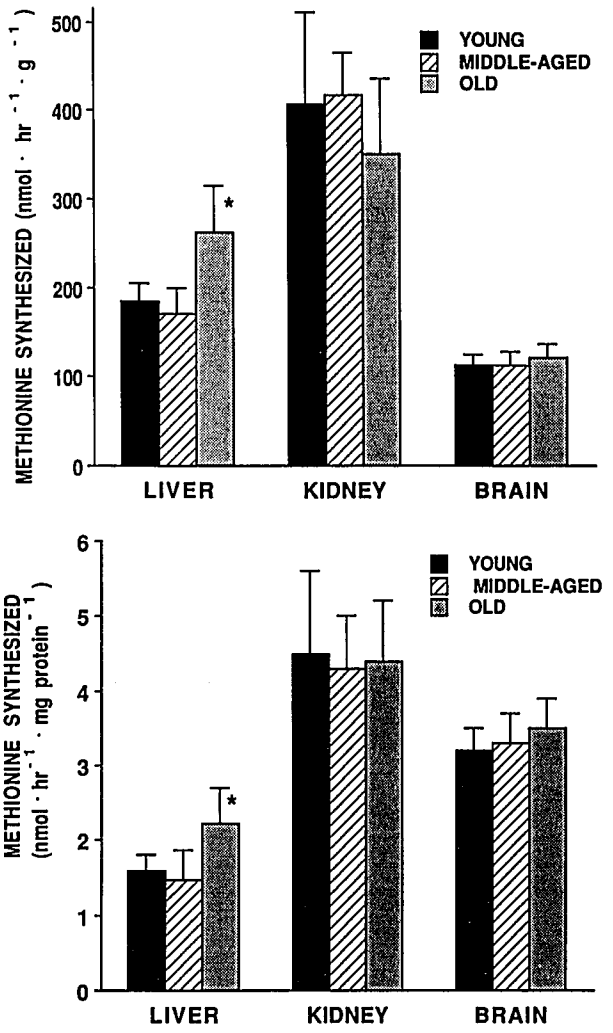

FIG. 5. Methionine synthase activities in livers, kidneys, and brains of young, middle-aged, and elderly rats measured 16–21 days after N_2O exposure. Activities are expressed as nanomoles of methionine produced · h^{-1} · g^{-1} of original tissue (*A*) or as nanomoles methionine produced · h^{-1} · mg^{-1} of protein in the supernatant (*B*) (mean ± SD). Liver methionine synthase activities in the elderly animals were significantly higher (*$P < 0.01$) than in the young or middle-aged rats.

Discussion

Using formic acid and FIGLU in urine as indirect markers to characterize alterations in folate metabolism, we found that aging *per se* minimally influences the N_2O-

TABLE 1. Folate and Vitamin B_{12} Concentrations (Mean ± SD)

	Young	Middle-Aged	Elderly
Folate			
Plasma (ng/ml)	81.2 ± 8.5	67.8 ± 6.4‡	50.5 ± 7.4*†
Red cell (ng/ml)	278 ± 69	268 ± 64	297 ± 48
Liver (μg/g)	7.7 ± 0.89	8.56 ± 2.04	8.73 ± 2.08
Total liver (μg)	92.7 ± 11.6	141 ± 34.1‡	152 ± 46.0*
Vitamin B_{12}			
Plasma (pg/ml)	1220 ± 135	954 ± 80‡	1090 ± 181
Tissue Vitamin B_{12} (ng/g)			
Liver	51.5 ± 10.8	42.1 ± 8.2	64.2 ± 15.7†
Kidney	1020 ± 252	971 ± 188	432 ± 217*†
Brain	32.9 ± 2.6	31.7 ± 2.7	33.8 ± 3.5
Total Tissue Vitamin B_{12} (ng)			
Liver	618 ± 123	697 ± 146	1110 ± 310*†
Kidney	2480 ± 739	3080 ± 395	1750 ± 714†
Brain	59.0 ± 7.0	62.2 ± 5.2	64.3 ± 6.3

* Elderly different from young rats at $P < 0.01$.
† Elderly different from middle-aged rats at $P < 0.01$.
‡ Middle-aged different from young rats at $P < 0.01$.

induced perturbation of vitamin B_{12} and folic acid metabolic pathways in rats. For all age groups, the marked increases in urinary excretion of formic acid (fig. 3) and FIGLU (fig. 4) that occurred the first day after exposure to N_2O returned to background excretion rates by the second day after exposure. If a sustained functional deficiency in folate occurred in elderly rats after N_2O exposure, the markedly increased excretion rates of formic acid and FIGLU would be expected to persist for days after N_2O exposure. Although the magnitude of the increase in FIGLU excretion rates increased with increasing age the first day after N_2O exposure (fig. 4), this same trend was not observed with the excretion of formic acid (fig. 3). It remains possible that measurements over shorter time periods (hours) would have revealed a prolonged recovery of urinary excretion rates of FIGLU and formate in the elderly compared to the younger animals.

The elevations of urinary formic acid and FIGLU that occur following exposure to N_2O and inactivation of methionine synthase are associated with a depletion in H_4folate (fig. 1). This form of the coenzyme is required for the enzymatic pathways involved in the oxidation of formic acid to carbon dioxide[20] and in the catabolism of FIGLU.[19,26] Exposure of young rats to 50–80% N_2O for 2 h to 4 days results in a 40–87% depletion in hepatic levels of H_4folate.[27–31] After young rats are exposed to 50% N_2O for 4 h and returned to a room air environment, hepatic levels of H_4folate return to control values within 1–2 days of the recovery period.[27] Although individual tissue folate derivatives were not directly measured in the current experiments, measurements of the urinary FIGLU and formate excretion rates suggest that the time course of alterations in tissue H_4folate levels after N_2O exposure is similar among rats of various ages. Nevertheless, it must be emphasized that these urinary metabolites are only indirect markers of folate status and that other factors can contribute to the excretion of these compounds. For example, excretion of FIGLU in urine is enhanced with increased intake of histidine, and the administration of methionine reduces FIGLU excretion after N_2O exposure.[19] Thus, it is possible that the differences observed in FIGLU excretion rates between animals of different ages for the first day after N_2O exposure (fig. 4) were related to age-dependent differences in the dietary intake or absorption of these amino acids.

An unexpected finding in this study was the increase in hepatic methionine synthase activity (but not kidney or brain activities) in elderly compared to middle-aged or young rats (fig. 5). This increase in enzyme activity was apparent when expressed either in terms of original liver weight or in terms of milligrams of protein in the supernatant. Thus, it is unlikely that a simple alteration in the chemical composition of the liver with age could explain the increase in methionine synthase activity in liver in the 24-month-old rats. In previous experiments (using Sprague-Dawley rats), lower hepatic methionine synthase activities were reported in young adult rats compared to weanling rats[32] and in 6-month-old compared to 2-month-old rats.[33] In this latter study,[33] however, rats were switched from a solid diet to a liquid diet at 2 months of age, and the decrease in hepatic methionine synthase activity may have been related to a change in dietary constituents (*e.g.*, methionine[32] or zinc[34]).

Methionine synthase activities were determined 16–21 days after exposure to N_2O. Since previous studies showed a return of methionine synthase activities to near background levels 1–4 days after acute exposure of young adult rodents to N_2O,[2,3,23,28,35,36] it is assumed that the 2–3-week time period allowed for complete recovery of enzyme activity. Because of the limited supply and considerable cost of the elderly rats, complete recovery curves (requiring large numbers of animals) of methionine synthase activity after acute exposure to N_2O were not performed in the current experiments. Although it remains uncertain why a prolonged time (days) is needed for recovery of methionine synthase activity after N_2O exposure, it is likely that reaction with N_2O causes irreversible damage to the enzyme and that synthesis of new apoenzyme is needed for recovery.[37,38] The present results, showing a return of urinary FIGLU and formic acid excretion rates to background levels in all age groups within 2 days after N_2O exposure (figs. 3 and 4), suggest that the recovery of methionine synthase activity in the elderly animals occurs over a time course that is similar to that of the younger rats and that synthesis of new enzyme occurs at similar rates in young and old rats.

Plasma concentrations of folate in the rat (table 1) are

approximately 10 times the values in humans, a finding that is in agreement with the work of other investigators.[39] A progressive decrease in plasma folate concentrations was observed with increasing age of the rats (table 1). However, plasma folate concentrations are considered to be poorly representative of tissue folate stores.[40] Red cell folate and liver folate did not differ among the three age groups (table 1).

The patterns observed in vitamin B_{12} status with age depended on the tissue examined. No consistent age-related changes were seen in plasma vitamin B_{12} concentrations (table 1). Hepatic content of vitamin B_{12} was highest in the elderly animals, whereas kidney concentrations of vitamin B_{12} in the elderly rats were approximately one-half of the values in the young and middle-aged rats (table 1). Vitamin B_{12} concentrations in the kidney were 7- to 23-fold higher than those found in liver, which is in agreement with previous studies.[41,42] Liver is partially depleted of vitamin B_{12} after prolonged exposure of rats to 50% N_2O[12,23]; however, tissue vitamin B_{12} concentrations in the present studies are within the range of control values reported by others,[41,42] indicating that a single 6-h exposure to 60% N_2O did not markedly reduce the tissue content of vitamin B_{12}.

If these results can be extrapolated to the clinical situation, they suggest that aging *per se* only plays a minor role in the toxic effects produced by brief exposures to N_2O in critically ill patients and those with a pre-existing vitamin B_{12} deficiency. Nevertheless, such an extrapolation needs to be interpreted in light of known species differences. A slower rate of methionine synthase inactivation in humans compared to rodents[2,4,5] might imply a lesser susceptibility of humans to N_2O, but the lower content of hepatic folate in humans compared to rats[43] may enhance the relative toxicity of N_2O in humans. Furthermore, the limitations of the human studies need to be recognized. Although a high incidence of megaloblastic anemia and mortality was found by Amos *et al.*[8] in critically ill patients who received N_2O, these studies were not controlled and the same high mortality rate might be found in critically ill patients who undergo anesthesia and surgery and do not receive N_2O. In addition, only two reports are available[10,11] that associate an exacerbation of a pre-existing vitamin B_{12} deficiency with a brief exposure to N_2O. It is not known how many patients with vitamin B_{12} deficiencies receive N_2O without the production of untoward effects.

In summary, urinary excretion rates of formic acid and FIGLU were measured in 2-month-, 12-month-, and 24-month-old rats before and after a 6-h exposure to 60% N_2O. Excretion rates of these compounds were markedly elevated during the first day following N_2O exposure and returned to near background rates of excretion by the second day after exposure in animals of all ages. Thus, in this animal model, it appears that aging *per se* exerts little influence on the magnitude and time course of the disruption of vitamin B_{12} and folate metabolism that occurs after acute exposure to nitrous oxide.

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