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Metabolism of Nitrous Oxide by Human and Rat Intestinal Contents

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Nitrous oxide labeled with a stable heavy nitrogen isotope was used for *in-vitro* studies of nitrous oxide metabolism in man and rat. At 5 per cent oxygen tension, which is comparable to normal oxygen tension in the intestine *in vivo*, each gram of intestinal contents during a 16-hr *in-vitro* incubation produced 47 ± 13 nmol of molecular nitrogen for the rat and 103 ± 17 nmol for man. Active reductive metabolism of nitrous oxide by intestinal contents was significantly inhibited by antibiotics and by 20 per cent oxygen tension. It is suggested that the reduction of nitrous oxide to nitrogen may proceed through a single-electron transfer process with formation of free radicals. Under these circumstances, metabolism of nitrous oxide could produce toxic intermediates, even though the end-metabolite is inert. (Key words: Anesthetics, gases: nitrous oxide. Biotransformation: nitrous oxide. Metabolism: metabolites; free radicals.)

NITROUS OXIDE, the most commonly used inhalational anesthetic, has long been considered chemically inert in the body. Sawyer *et al.*[1] concluded that nitrous oxide was not metabolized by the liver after a study demonstrating that the fraction of nitrous oxide removed from the blood during a single pass through the liver was only 0.03 ± 0.05 per cent at 4.65 ± 0.21 per cent alveolar concentration. However, Matsubara and Mori[2] have shown that nitrous oxide is an intermediate in the reduction of nitrite to nitrogen by the soil bacteria, *Pseudomonas denitrificans*. Metabolic reduction of aromatic nitro compounds has also been shown to occur *in vitro* and *in vivo* in both mammalian tissue and gastrointestinal microflora. Compounds that are reduced in the intestine were shown to be reabsorbed into the blood, and thus may be toxic to the animal.[3,4] Recent evidence that nanomolar amounts of certain nitrogenous substances, such as N-nitroso compounds, may be carcinogenic[5] or teratogenic[6] has suggested that metabolism of nitrous oxide might be potentially toxic even if this were to occur in very small amounts.

It has been suggested that transition metal complexes such as the cobalt-ligand complex present in vitamin B_{12} may cleave nitrous oxide[7] and that vitamin B_{12} may be inactivated by nitrous oxide.[8,10] It has been proposed that the latter effect of vitamin B_{12} may relate to the action of nitrous oxide in producing megaloblastic anemia.[9,11] This demonstrated reactivity of nitrous oxide suggests the possibility of its interaction with other electron-transport systems in the body.

A major problem in investigating the metabolism of nitrous oxide is that living systems are already rich in nitrogen-containing compounds. A search for metabolites of nitrous oxide using chromatographic separation techniques would involve searching for nanogram quantities of metabolite among the grams of amino acids and related amines found in the body. There is no useful long-lived radioactive isotope of nitrogen that can be used in a tracer study. Fortunately, although stable isotopes are more difficult to detect and quantify than radioisotopes, we were able to use ^{15}N-labeled nitrous oxide, $^{15}N_2O$, for our studies of nitrous oxide metabolism.

The possibility that the intestinal contents of man and rat can reduce N_2O to N_2 was tested by exposing intestinal contents to $^{15}N_2O$. Then all the headspace gases above the incubation mixture were removed. Nitrogen gas was analyzed for any increased incorporation of ^{15}N, following the removal of the remaining nitrous oxide, water vapor and oxygen in the headspace gases.

Materials and Methods

The sensitivity of our analytic techniques was sufficiently high that the $^{15}N_2O$ used could contain no more than 10 ppm of contaminating ^{15}N compounds. The commercially prepared¶ $^{15}N_2O$ was purified by the manufacturer by successive condensation into phosphoric acid, basic potassium permanganate, dry sodium hydroxide, followed by three distillations at high vacuum. We subsequently redistilled the $^{15}N_2O$ on a vacuum line before use to ensure removal of any residual $^{15}N-$ impurities. Testing the end-product for nitrate or nitrite impurities or for volatile nitrogen compounds showed no detectable impurity.

Male and female Sprague-Dawley rats** weighing 200–300 g were maintained on a normal laboratory

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diet of unrestricted standard Purina Lab Chow® and water until the time of sacrifice. Tetracycline hydrochloride, neomycin sulfate, and bacitracin were administered to two rats by gavage twice daily for three days prior to the metabolism studies.[12]

Human large-intestinal contents were prepared from feces from one of the investigators. Three specimens obtained on different days provided material for the five homogenate studies. Rats were sacrificed and the small and large intestines were removed. The contents were removed and the walls were first washed thoroughly with 1.15 per cent KCl in 20 mM *p*H 7.4 Tris–HCl buffer to minimize contamination with fecal contents, then homogenized at 4 C in three parts (w/v) 0.1 M *p*H 7.4 potassium phosphate buffer containing 10 mM sodium lactate. The separated contents from both the small and large intestines were suspended and homogenized in three parts (w/v) of the phosphate–lactate buffer described above. The homogenates were diluted with the same buffers to the desired concentration before incubation with $^{15}N_2O$.

In a typical experiment 20 ml of a homogenate of 2.5 g of intestinal wall or intestinal contents were slowly degassed on a vacuum line at room temperature. Degassing by a freeze–thaw technique was avoided to prevent inactivation of the bacteria, which we observed to occur after freezing. The 80-ml reaction vessel was then evacuated and either pure $^{15}N_2O$ or a desired mixture of $^{15}N_2O$ and O_2 was introduced until atmospheric pressure was reached. The reaction vessel was sealed and incubated at 37 C for three hours in the case of intestinal-wall homogenates and 16 hours in the case of intestinal-content homogenates. Following the incubation period the reaction vessel was remounted on the vacuum line, and the headspace gas that was incondensable at liquid nitrogen temperature was removed by distillation into an activated charcoal trap held at liquid nitrogen temperature. The headspace gas was then released from the activated charcoal trap by heating. The remaining $^{15}N_2O$ in the headspace gas was removed by condensation, and any residual oxygen was removed by treatment of the gas in a vacuum line with copper at 500 C. Sample runs showed that this treatment effectively removed all nitrous oxide, oxygen, and water vapor from the headspace sample and provided pure nitrogen gas. A known amount of N_2 of normal isotopic abundance from $(NH_4)_2SO_4$ was added as an internal standard to this small amount of $^{15}N^{15}N$ gas produced by metabolic reduction of $^{15}N_2O$. The nitrogen gas produced was then introduced into a Micromass 602C® isotope ratio mass spectrometer to measure the amount of $^{15}N^{15}N$ produced by the homogenate in comparison with the N_2 internal standard.

TABLE 1. Production of $^{15}N^{15}N$ by Intestinal Contents and Intestinal Wall Following Exposure to $^{15}N_2O$*

Incubation Conditions	Human Large-intestinal Contents (16 Hr)	Rat Intestinal Contents (16 Hr)	Rat Intestinal Wall (3 Hr)
Oxygen			
None	561 ± 35 (5)†	118 ± 14 (8)	0.0 ± 1.0 (4)
5 per cent	103 ± 17 (4)	47 ± 13 (6)	—
10 per cent	—	6 ± 4 (3)	—
20 per cent	—	6 ± 5 (3)	—
Antibiotics	—	0.1 ± 0.4 (3)	—
Boiled	0.3 ± 0.2 (3)	0.0 ± 0.3 (3)	—

* nmol $^{15}N^{15}N$ produced per g wet sample. A background contribution corresponding to 5 nmol/g is found when $^{15}N_2O$ is exposed to buffer and the headspace gases are carried through the analysis. This amount appears to be from residual impurities in our very highly purified $^{15}N_2O$. This amount has been subtracted from each experimental value in the table.

† Number of samples in parentheses.

Two milliliters of gas were removed from the lumen of the large intestine of three rats with a hypodermic syringe. The partial pressure of oxygen was determined with a Severinghaus oxygen electrode.

Results

The amounts of $^{15}N^{15}N$ gas produced by 20-ml homogenates of rat intestinal contents, rat intestinal wall, human feces, and intestinal contents from rats pretreated with antibiotics indicated that both rat intestinal contents and human feces actively metabolize nitrous oxide (table 1). Metabolism was greatly inhibited by oxygen, 10 and 20 per cent, and partially inhibited by a lower oxygen concentration (5 per cent). This finding is consistent with a reductive route of nitrous oxide metabolism and with the previous studies of the soil bacteria, *Pseudomonas denitrificans*.[2] The very low rate of metabolism by washed intestinal wall demonstrates that the observed metabolism is not due to enzymatic activity of the intestinal wall. Essentially complete inhibition of metabolism following treatment of the intestinal contents with antibiotic agents indicates that metabolism is dependent upon an active metabolic role of intestinal bacteria. The absence of metabolism by boiled intestinal contents or by buffer alone further supports the role of active metabolism by intestinal microflora. The mean concentration of oxygen in the large intestines of these rats was approximately 4 per cent.

Discussion

Our *in-vitro* studies demonstrated that both rat and human intestinal contents possess high nitrous oxide

reductase activity under anaerobic conditions and moderate activity under low oxygen tension normally present in the intestine. We have established that the 5 per cent oxygen tension used for our incubations is a good approximation of that normally present in the lumen of the intestine, although oxygen tension would probably be higher during clinical anesthesia. At the present time we are unable to determine whether the observed oxygen inhibition of metabolism is due to reoxidation of the reduced intermediate, inhibition of metabolic activity by an oxygen-sensitive control mechanism, scavenging of free radicals by oxygen, or decreased growth of bacterial nitroreductases. We also demonstrated that very little metabolism is carried out by enzymes of the intestinal wall, and that reduction within the intestine is active metabolism and is inhibited by boiling the intestinal content or by treatment with antibiotics. This study confirms the previously reported[2] reduction of nitrous oxide by *Pseudomonas denitrificans*. The inhibition of metabolism by antibiotics suggests that reduction is by bacteria and not through interaction with vitamin B_{12}.[7]

Although it is difficult to use our *in vitro* data to predict the amount of nitrous oxide metabolism occurring during clinical anesthesia in a human patient, it may be approximated at 0.004 per cent of the amount taken up. This calculation assumes that at 5 per cent oxygen tension the rates of metabolism *in vivo* and *in vitro* are comparable, and that on a relative weight basis, the uptakes of nitrous oxide in man and rat are similar, as are their average intestinal contents (3.3 per cent of total body weight††). Under these circumstances a 70-kg man breathing 75 per cent nitrous oxide for three hours at a total flow rate of 4 l/min would inspire 540 l and take up approximately 26.3 l of nitrous oxide.[13] Using our data obtained from direct *in-vitro* measurements of $^{15}N_2O$ metabolism by human intestinal contents, approximately 1.0 ml or 1.3 mg of metabolic nitrogen would be generated from the nitrous oxide absorbed during a three-hour period of anesthesia. The calculated metabolism of 0.004 per cent of nitrous oxide uptake (0.0002 per cent of the total inspired nitrous oxide) probably represents an underestimate, since it conservatively assumes steady production of N_2 by the intestinal incubate over a continuous 16-hour *in-vitro* exposure period.

There has been considerable interest in the toxicologic and pharmacologic implications of drug metabolism by gastrointestinal microflora.[14,15] Intestinal bacteria have been shown to be important in the metabolism of several classes of drugs. A study of 15 homothermic species, including man and common laboratory animals fed conventional diets, demonstrated that all tested species have similar gastrointestinal flora, although quantitative differences were found.[16]

Mason and Holtzman[17,18] have reported that enzymatic reduction of drugs containing nitro groups and other nitro aromatic compounds produces anion-free radicals. It is conceivable that the reduction of nitrous oxide may also proceed through such a single-electron transfer process.[19,20] If this were so, the pathway of nitrogen production might initiate free-radical reactions even though the end-metabolite is inert. Previous studies of intestinal bacterial metabolism indicate that such intermediates may be toxic within the intestine or be transferred from the intestine to the blood stream and produce generalized toxicity. In a study of *Escherichia coli* K-12 bacteria exposed to nitrous oxide and irradiated, long-lived radical species were observed in conjunction with a toxic effect on the *E. coli*.[21] These investigators suggested that an OH radical was responsible for the toxic effect. This radical may be produced during nitrous oxide reduction in the following reaction scheme:

$$N_2O \xrightarrow{e^-} [N_2O^-] \xrightarrow{H_2O} \cdot OH + OH^- + N_2$$

Electron transport system

Hydrogen donors, *e.g.*, lactic acid

This study indicates that N_2 is the major metabolite of nitrous oxide in intestinal microflora. Inasmuch as there is evidence that the metabolic route from nitrous oxide may include free-radical intermediates, in future studies it will be important to search for these possible radical intermediates. It is well established that free radicals are important in the etiology of carcinogenesis, teratogenesis,[22,23] and associated tissue damage.

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