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Biotransformation of Nitric Oxide

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Previous investigations into the health effects of nitrogen oxides (NO_x) have mostly been conducted with special reference to nitrogen dioxide (NO_2) and its direct effects on the respiratory system, while the study of nitric oxide (NO) has been disregarded. We carried out a study on NO by exposing rats and mice to ^{15}NO or administering ^{15}N-nitrite and ^{15}N-nitrate to these animals by IP injection in order to elucidate the metabolic fate of NO.

The results of our study and previous findings led us to assume that the major metabolic path of inhaled NO is as follows: inhaled NO reacts with hemoglobin, forming nitrosyl-hemoglobin (NOHb), and from NOHb, nitrite (NO_2^-) and nitrate (NO_3^-) are generated. Major quantities of NO_3^- are discharged into the urine and a certain amount is discharged into the oral cavity through the salivary glands and transformed to NO_2^-. Part of this NO_2^- is converted to N_2 gas in the stomach. Nitrate in the intestine is partly reduced to ammonia (NH_3) through NO_2^-, reabsorbed into the body, and converted to urea. Most of the metabolites of inhaled NO are excreted rapidly from the body within 48 hr.

Introduction

In order to clarify the health effects of nitrogen oxides (NO_x), investigation of the direct pathological effects on the respiratory organs and basic studies on the absorption and biotransformation of NO_x are important. However, for investigation of the absorption or metabolic transformation of NO_x, the half-life of ^{13}N is too short for it to be used as a radioisotope, and in the case of ^{15}N, considerable amounts of the element are contained in the protein of the living body, so little work has been done with ^{15}N (*1–8*). In this report, the biotransformation of nitric oxide (NO) and its intermediate metabolites, nitrite (NO_2^-) and nitrate (NO_3^-), are reviewed on the basis of results obtained in our own investigations.

Absorption and Conversion of NO in Blood

In physicochemical comparison with SO_2, NO is less readily absorbed in the airway because of its low solubility in water [7.340 cm^3/100 mL cold water (*9*)]. Such observations concerning NO absorption have been made in studies with isolated and perfused lung by Yokoyama and Poslethwait (*10*) and Mustafa (*11*). We have also shown that less than 10% of NO is absorbed and oxidized in perfused rabbit lungs (*12*). However, the results in the case of perfused lung would be expected to differ from those in the case of a living system. With regard to NO absorption into the living body, Wagner (*13*) reported that more than 80% of NO was absorbed in normal breathing and more than 90% was absorbed in deep breathing. We obtained similar results with inhalation of 10 ppm NO (unpublished data). Despite its low solubility in water, the absorption of NO into the body is almost complete. Goldstein et al. (*14*) showed in experiments with monkeys that in the case of inhalation of 0.3 to 0.9 ppm ^{13}NO, 50 to 60% of the inhaled NO was found in the lung and spread into the other organs through the bloodstream. We found a high ^{15}N content in serum and urine after inhalation of 138 to 880 ppm ^{15}NO, and within 24 hr, about 40% of the inhaled ^{15}N was excreted into the urine (*5*).

NO is known to combine strongly with hemoglobin to form nitrosyl-hemoglobin (NOHb) *in vitro*. According to an investigation by Oda et al. (*15*), a very small amount of NOHb (0.13% of total hemoglobin) was found in the blood of mice after inhalation of 10 ppm NO. This suggested the possibility of rapid change of the absorbed NO in the blood. In addition, a number of studies have been made on the interaction between NO and blood or hemoglobin (*16–20*). Our experimental results with ^{15}NO showed that NO entered the blood, combined with Hb in the first stage, and was oxidized rapidly to $^{15}NO_2^-$ and $^{15}NO_3^-$ (*5*). Though the amount of NOHb in the blood is very small and in *in vivo* exposure, NOHb is of important significance as an intermediate in NO metabolism (*21*). In the reaction process of NO and Hb, degeneration of Hb and damage to erythrocyte membranes are observed (*22–24*).

From our results, it is thought that due to its low solubility in water, the major proportion of inhaled NO

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reaches the deeper portion of the lung, reacts with hemoglobin in erythrocytes to form NOHb, and is converted immediately to NO_2^- and NO_3^-. The major proportion of inhaled NO is excreted in urine in the form of NO_3^-.

Metabolism and Excretion of Inhaled NO

Biotransformation of NO after conversion to NO_2^-/NO_3^- is considered to be the same as the metabolism of NO_2^-/NO_3^- from foodstuffs. Many studies have been devoted to problems related to the fate of ingested nitrate and nitrite in the body (*25–35*). It is certain from the literature that increased ingested nitrate or nitrite results in increased urinary excretion of nitrate and nitrite, but the details of the major metabolic pathway are not sufficiently known.

In the case of ^{15}NO inhalation (145 ppm × 123 min) in rats (*5*), about 55% of the inhaled ^{15}N was excreted in urine, 75% of excreted ^{15}N being $^{15}NO_3$, and 24% ^{15}N-urea (Exp. A1 and A2 of Fig. 1). Compared with the case of IP injection of $Na^{15}NO_2$ or $K^{15}NO_3$, the main metabolites were $^{15}NO_3$ and ^{15}N-urea. (In the case of $^{15}NO_3$, a very small quantity of urea was found.) A pattern similar to the case of NO inhalation was found for $^{15}NO_2^-$ (Exp. B1 and B2 of Fig. 1), but the result for $^{15}NO_3^-$ differed. The reason for this is not well understood, but these observations are of some interest. The presence of ^{15}N-urea in urine was confirmed by the urease method and that of $^{15}NO_3^-$ was confirmed by gas-liquid chromatography/mass spectrometry method using the derivative of 3,4-xylenol (Fig. 2). The peak of M/E 168 in Figure 2*B* was apparently a result of ^{15}N-6-nitro-3,4-xylenol from inhaled ^{15}NO.

In order to elucidate exactly all the metabolites in the body, $Na^{15}NO_2$ (0.62 mg as ^{15}N per animal) was

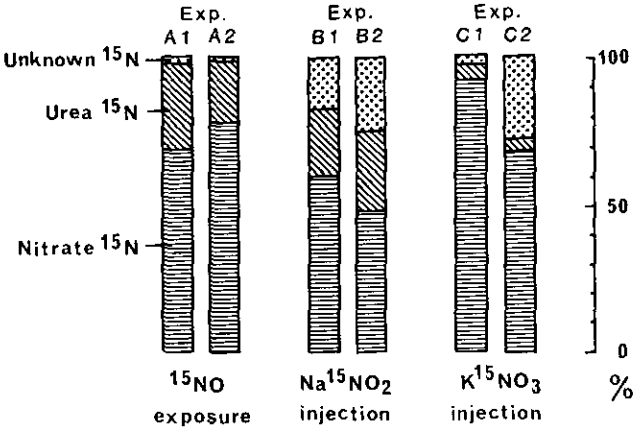

FIGURE 1. Distribution of ^{15}N (excess) in the urine from rats after the exposure to ^{15}NO or IP injection of $Na^{15}NO_2$ or $K^{15}NO_3$. Rats (2 animals per experiment) were exposed to 145 ppm ^{15}NO for 123 min, or were administered 9.33 mg $Na^{15}NO_2$ or 13.6 mg $K^{15}NO_3$ (2 mg as ^{15}N) per animal. The urine samples were taken for 48 hr after the exposure or injection.

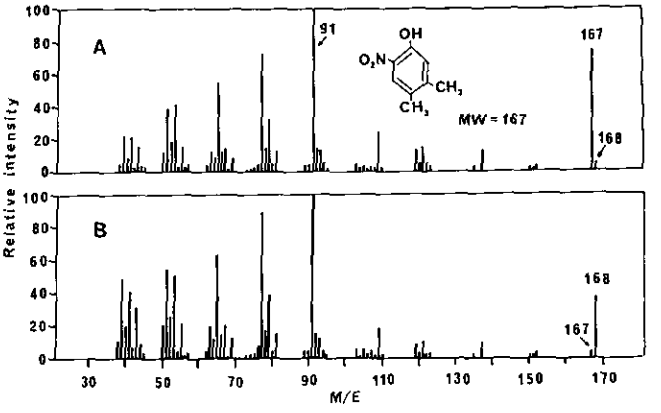

FIGURE 2. Mass spectra of 6-nitro-3,4-xylenol (*A*) and the derivative of 3,4-xylenol from the urine of rats exposed to ^{15}NO (*B*). The urine was from the samples described in Fig. 1.

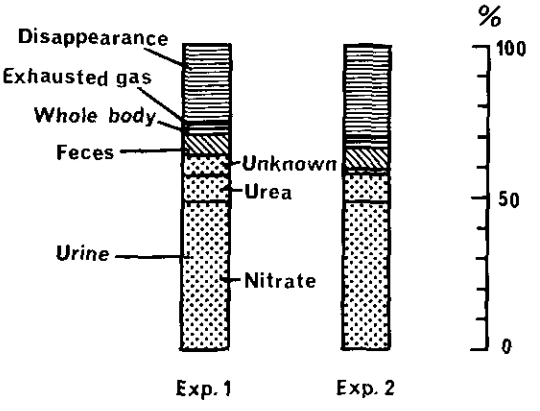

FIGURE 3. Distribution of ^{15}N (excess) in mice after IP injection of $Na^{15}NO_2$. Mice (five animals per experiment) were administered 2.88 mg $Na^{15}NO_2$ (0.62 mg as ^{15}N) per animal. The urine and feces were taken for 48 hr after the injection. The exhaled gas was collected for 48 hr to determine NH_3, NO, and NO_2. The mice were killed at 48 hr after injection, and carcasses were used to determine the residual ^{15}N ("whole body").

injected IP into mice, and the ^{15}N contents of the urine, feces, exhaled gas, and the body were estimated. As shown in Figure 3, 60.7% of the administered ^{15}N was found in the urine, 7.8% in feces, 0.3% in exhaled gas, and 1.6% in the body. The residual 30% was not found.

Wang et al. (*35*) administered ^{15}N-labeled nitrate and nitrite to rats and studied excretion and retention. They found that 60 to 70% of the dose was excreted in the urine, 10 to 20% was eliminated in the feces, and about 10% was retained in the body carcass (in the case of a single dose). The amount of unrecovered ^{15}N that we obtained was high compared to that of Wang et al., although this may have been due to differences in the animal species and administration methods. The unrecovered ^{15}N is assumed to have been in the form of N_2 gas, on the basis of both the recovery method used for exhaled gas and the *in vitro* experiments on the stomach contents of mice and on the presence of nitrite, as discussed in the next section.

Conversion of Nitrite and Nitrate in the Digestive Tract

The conversion of nitrite and nitrate in the digestive tract, especially in the stomach, should be evaluated in consideration of normal flora and injesta (*36*). A proportion of the NO_3^- in the blood is transferred to the oral cavity through saliva. Nitrate in the oral cavity is partly reduced to NO_2^- by oral bacteria. Thus, the produced NO_2^- reacts readily with amines from ingested foods or drugs under acidic conditions to form nitrosoamines. We observed that a large amount of N_2 gas and a small amount of NO are produced by anaerobic incubation with mouse stomach contents and NO_2^- in a Smith tube at 37°C, pH 3.5 (Fig. 4) (*7*). The production of N_2 gas is presumed to occur through the reaction shown below, which is used in the Van Slyke method of amine-N determination.

$$H_2N\text{-}\overset{\overset{R}{|}}{C}H\text{-}COOH + HNO_2 \rightarrow HO\text{-}\overset{\overset{R}{|}}{C}HCOOH + N_2 + H_2O$$

NO_2^- in the stomach was converted to N_2 gas by the proteins of the mouse diet as in the *in vitro* experiment. That is to say, NO_2^- entering the stomach is absorbed partly through the stomach wall, and some NO_2^- reacts with amines, ureides, or ascorbic acid (*37*), but a considerable amount of NO_2^- is changed to N_2 gas by reaction with proteins of the diet and disappears from the body. The unrecovered ^{15}N in our previous experiments (*5*) may have been the result of this reaction.

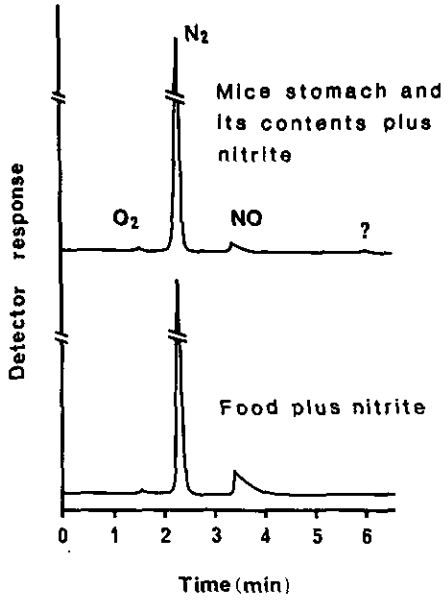

FIGURE 4. Gas appearance by *in vitro* incubation experiments with stomach and its contents or food plus nitrite in stomach pH. Mice stomach and its contents of 7.5 g or food of 5.0 g were homogenized with 25 mL 0.85% NaCl solution, and the homogenate was adjusted to pH 3.5 by addition of 2N HCl. After 25 mg $Na^{15}NO_2$ was added to the homogenate, which was incubated at 37°C for 5 hr in Smith tube, the gas produced was analyzed by gas-liquid chromatography on a Molecular Sieve 5A column.

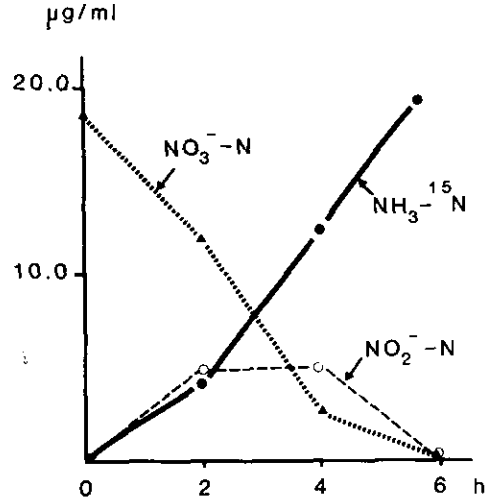

FIGURE 5. Transformation of $^{15}NO_3^-$ *in vitro* experiments with mice feces. Mice feces of 11.3 g were homogenized with pepton-NaCl solution of 200 mL. After 35 mg $Na^{15}NO_3$ was added to the homogenate, which was incubated at 37°C for 6 hr, part of the homogenate was centrifuged at 3000 rpm for 10 min, and the supernatant was used to determine $^{15}NH_3$, NO_3^-, and NO_2^-.

Table 1. NH_3 – ^{15}N concentration in intestine contents of mice after IP injection of $Na^{15}NO_2$.[a]

	Time	Total NH_3-N, mg	NH_3-^{15}N Atom % excess	NH_3-^{15}N μg Excess
Cecum and large intestine contents	After 1 hr	1.095	3.3008	36.1
	After 3 hr	1.270	1.5656	19.9
Control		1.984	0	0

[a]Mice (five animals per experiment) were injected IP with 3.73 mg $Na^{15}NO_2$ (0.8 mg as ^{15}N) per animal.

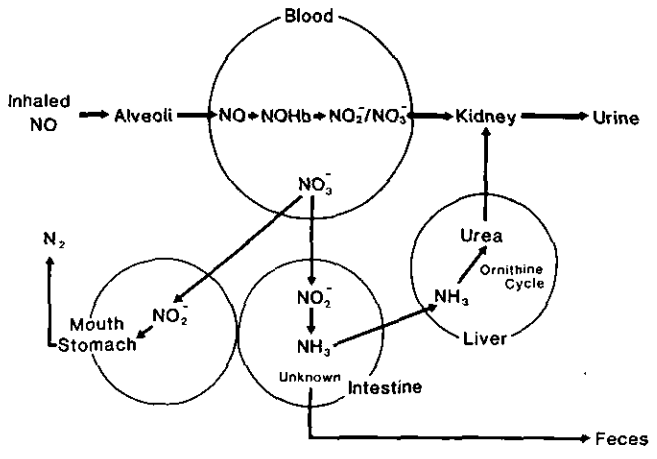

FIGURE 6. Hypothetical metabolic pathway of inhaled NO.

Nitrate in the stomach is transferred to the intestine without reduction and absorption in the healthy animal (*34*). However, in the stomach of ruminants such as cattle or sheep, NO_3^- is reduced to NO_2^- (*38*).

A considerable number of studies have been conducted regarding the fate of NO_2^- and NO_3^- in the intestine. Tannenbaum et al. (*33*) suggested that nitrate and nitrite are produced *de novo* in the intestine as a result of heterophic nitrification. According to the research of Witter et al. (*34*), who studied $^{13}NO_3^-$ in humans and rats, the ^{13}N compound administered was not rapidly absorbed through the stomach wall; the concentration was increased in the lower intestine, and a portion of the ^{13}N was retained in the body. The results of Wang et al. (*35*) suggested that NO_2^- and NO_3^- in the intestine are converted to the nitrogen compounds other than NO_2 and NO_3 by intestinal bacteria before reaching the large intestine. The investigations by Hill et al. (*39*) and Ishiwata et al. (*40*) showed that neither nitrite nor nitrate could be detected in the contents of the intestine nor in the feces.

We observed in $Na^{15}NO_2$ injection experiments that ^{15}N in the intestine was retained for a relatively long time in comparison with retention in the liver and kidney (*7*). From more detailed investigations on ^{15}N in the intestine, we showed that the greater part of ^{15}N in the intestine is composed of trichloroacetic acid (TCA)-soluble and TCA-insoluble ^{15}N, and that the amounts of NO_2^- and NO_3^- are very small except for those existing 1 hr after the injection.

The ratio of TCA-soluble ^{15}N to total ^{15}N increased with time. This suggests that low molecular weight ^{15}N compounds in the intestine are eliminated relatively rapidly, while high molecular weight ^{15}N compounds are retained for a considerable length of time. *In vitro* incubation experiments (*7*) with mice intestinal contents and NO_2^-/NO_3^- suggested that NO_3^- in the intestine is reduced to NO_2^- and converted to unknown nitrogen compounds by intestinal bacteria.

To investigate the end products from NO_3^-/NO_2^- in the intestine, a mixed solution of mouse feces and a peptone-NaCl solution of $Na^{15}NO_3$ was incubated at 37°C for 6 hr (Fig. 5). The concentration of NO_3^- in the mixture decreased with time and disappeared after 6 hr. As for NO_2^-, a temporary increase was found, but it disappeared after 6 hr. On the other hand, the concentration of ^{15}N-NH_3 ($^{15}NH_3$) in the incubation solution increased rectilinearly with time. The *in vitro* results suggested that NO_3^- in the intestine was converted to NH_3 through NO_2^- by intestinal bacteria.

To confirm the conversion of NO_2^- to NH_3 *in vivo*, mice were given an IP injection of ^{15}N-nitrite (0.8 mg ^{15}N per animal), after which the concentration of $^{15}NH_3$ in the intestine contents was estimated at 1 hr and 3 hr following the injection. As shown in Table 1, the atom percent excess of $^{15}NH_3$ in the intestinal contents after the injection showed high values compared with the control, indicating the conversion of $^{15}NO_2^-$ to $^{15}NH_3$. From these results, it is considered that NO_3^- in the intestine is reduced to NH_3 through NO_2^- by the intestinal bacteria, and NH_3 thus produced is absorbed through the intestinal walls and metabolized to urea.

Discussion

On the basis of the results mentioned above, the possible metabolic pathway of inhaled NO is illustrated in Figure 6. A small amount of inhaled NO reacts with tissue components in the lung, but most of it enters the blood through the alveoli and reacts with hemoglobin in erythrocytes. NO_2^- and NO_3^- are produced through NOHb (nitrosyl-hemoglobin) and these are transferred to the serum. The greater part of the NO_3^- is excreted into the urine through the kidney.

Part of the NO_3^- in the blood is secreted into the oral cavity through saliva, and is converted to NO_2^- by oral bacteria. Part of the NO_2^- that reaches the stomach is converted to N_2 gas with the proteins of the diet by the Van Slyke reaction and disappears. The intestinal NO_3^- transferred from the blood and stomach is converted to NH_3 or unknown compounds through NO_2^- by the intestinal bacteria. Ammonia thus produced is absorbed through the intestinal wall into the body. This reabsorbed ammonia is metabolized to urea through the urea cycle and is excreted into the urine.

The metabolism of NO/NO_2 in the living body greatly assists in its detoxification and disposal. Most of the NO/NO_2 is rapidly converted to low toxicity NO_3^- in the blood by oxidation with O_2, etc., and is eliminated from the body. However, a portion of NO/NO_2 and produced NO_2^- react with the living components and tissues, resulting in various injuries. NO_2^- is thought to be involved not only in pathological effects on the respiratory system (*41*), but also in injury of the cell membrane (*24,42*), disturbance of the information-mediating system (*43,44*), alteration of immunological functions (*45,46*), peroxidation of cell membrane lipids (*47,48*), carcinogenesis, and aging (*49–51*). A number of important questions regarding these problems cannot as yet be convincingly answered and still await further study.

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