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Mammalian nitrate biosynthesis: Incorporation of $^{15}NH_3$ into nitrate is enhanced by endotoxin treatment

(metabolism/stable isotope/carcinogenesis)

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ABSTRACT **Incorporation of an oral dose of [^{15}N]ammonium acetate into urinary [^{15}N]nitrate has been demonstrated in the rat. Investigation of the regulation of nitrate synthesis has shown that *Escherichia coli* lipopolysaccharide potently stimulates urinary nitrate excretion (9-fold increase). It was further shown that the enhanced rate of nitrate excretion by lipopolysaccharide was due not to a reduction in nitrate metabolic loss but rather to an increased rate of synthesis. This conclusion was based on finding a proportionally increased incorporation of [^{15}N]ammonium into nitrate nitrogen with lipopolysaccharide treatment. Nitrate biosynthesis was also increased by intraperitoneal injection of carrageenan and subcutaneous injection of turpentine. It is proposed that the pathway of nitrate biosynthesis may be the result of oxidation of reduced nitrogen compounds by oxygen radicals generated by an activated reticuloendothelial system.**

Several epidemiological studies have suggested an association between exposure to high levels of nitrate (NO_3^-) and increased incidence of stomach cancer (1–4). Nitrate can be converted to nitrite (NO_2^-) by bacterial reduction in areas of the body containing high concentrations of bacteria (5–7). The primary concern of nitrate and nitrite exposure in humans is the possibility of reaction with nitrogen compounds in the body, such as amines or amides, to produce *N*-nitroso compounds. The carcinogenicity of *N*-nitroso compounds has been observed in many animal species (8), suggesting that these compounds may represent cancer risks when formed in tissues and body fluids in humans.

Previously, total body burden of nitrate and nitrite was thought to be essentially a function of dietary intake and environmental exposure (9). Recent studies (10, 11) have confirmed earlier reports (12) that an additional and significant source presented to the body is via endogenous nitrate biosynthesis. This observation is based on excess excretion of nitrate in urine by human subjects or animals ingesting a low-nitrate diet. The origin of this nitrate was subsequently shown to occur in mammalian tissues as assessed by nitrate balance studies in germ-free and conventional rats (13–15). It is well known that the oxidation of ammonia to nitrate is a continuous process of the nitrogen cycle (16, 17). Therefore, we have investigated whether isotopically labeled ammonia, when administered to rats, appears as labeled nitrate in urine.

We observed an unexpected stimulation of nitrate biosynthesis during the course of metabolic balance studies in healthy adults (18). One human subject consuming a low and constant intake of dietary nitrate developed a fever and nonspecific intestinal diarrhea. Urinary nitrate excretion increased 9-fold during the illness compared with that before symptoms appeared. A similar elevation of urinary nitrate excretion has been noted by Hegesh and Shiloach (19) in infants suffering from diarrhea. Therefore, we explored the possibility that rats exposed to *Escherichia coli* lipopolysaccharide, which results in an endotoxin-induced fever, have an enhanced nitrate biosynthesis.

MATERIALS AND METHODS

Ammonia Incorporation into Nitrate. Six male Sprague–Dawley rats (mean weight, 150 g; Charles River Breeding Laboratories) were housed individually in stainless steel metabolic cages. Rats were given unlimited access to a diet low in protein and also in NO_3^- and NO_2^-. The diet is similar to the AIN-76 diet (20), except that the protein source is lactalbumin [5% (wt/wt)] and dextrin is added [15% (wt/wt)] to equalize calories. Dietary intake of nitrate was less than 1 μmol/day. A low content of dietary protein was used to favor the incorporation of ammonia into nitrogen compounds and to reduce its loss via urea synthesis (21). After 7 days of urine collection, 2 mmol of [^{15}N]ammonium acetate (99% ^{15}N, from Stohler Isotopes, Waltham, MA) was given intragastrically (i.g.), at 0900 hours, after an overnight feeding period. Urine was collected for the following 24 hr and examined for [^{15}N]nitrate. The abundance of $^{15}NO_3^-$ was determined by conversion of NO_3^- to nitrobenzene, which was then analyzed by gas chromatography/mass spectrometry (22) on a HP5992 (Hewlett Packard, Palo Alto, CA). The gas chromatographic separation was carried out on a Durabond DB-5 fused silica capillary column (Quadrex, New Haven, CT) with a helium carrier flow rate of 1 ml/min, initial column temperature of 110°C, and temperature programming of 4°C/min. Total urinary nitrate was measured by an automated modified Griess procedure (22).

***E. coli* Lipopolysaccharide Endotoxin-Induced Fever.** Six male Sprague–Dawley rats (200–250 g) were housed in individual metabolic cages and given a diet low in nitrate but nutritionally complete (20). After 1 wk of urine collection, rats were injected intraperitoneally (i.p.) with *E. coli* lipopolysaccharide (LPS) at 1 mg/kg of body weight (Serotype 0127:B8 obtained from Sigma). Core rectal temperatures were measured 6 hr later (Yellow Springs Instrument Digital Thermometer) to confirm that a fever was induced. Six control rats fed *ad lib* with the same diet were injected with 0.9% sterile saline solution. Additional studies were carried out similarly on six male Wistar and six male Fischer 344 rats (Charles River Breeding Laboratories).

Administration of LPS and Ammonia. Six male Sprague–Dawley rats were given the low-protein low-NO_3^- diet for 5 days and then received 2 mmol of [^{15}N]ammonium acetate i.g. [^{15}N]-Nitrate was measured in the urine collected for 24 hr after administration of the isotope. After a 1-wk break, these rats were injected i.p. with LPS (1 mg/kg) and simultaneously given 2 mmol of [^{15}N]ammonium acetate i.g. The enrichment of

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Abbreviations: i.g., intragastrically; i.p., intraperitoneally; LPS, lipopolysaccharide.

$^{15}NO_3^-$ in urine over the next 24 hr was determined.

Metabolism of Nitrate with LPS. The effect of LPS on nitrate metabolism was investigated by administering $Na^{15}NO_3$ (99% ^{15}N; Stohler Isotopes) (10 μmol per rat) i.g. and monitoring urinary excretion of [^{15}N]nitrate. After a 1-wk break, these same rats were injected i.p. with LPS (1 mg/kg) and simultaneously given 10 μmol of $Na^{15}NO_3$ i.g. $^{15}NO_3^-$ was measured in the urine over the next 24 hr.

Effect of Inflammation on Nitrate Biosynthesis. Two additional procedures resulting in an experimentally induced inflammation were examined for their effect on nitrate biosynthesis. Twelve adult male rats (250 g) were placed on the low-nitrate diet for 5–7 days and urine was collected to determine baseline nitrate excretion. Six rats were subsequently injected i.p. with carrageenan (0.15 g per rat) and the remaining rats were treated subcutaneously with turpentine (0.5 ml per rat). Urine was collected for 3 days following carrageenan or turpentine treatment and analyzed for nitrate.

RESULTS

Orally administered [^{15}N]ammonia was found to be incorporated into nitrate nitrogen. The ^{15}N enrichment of NO_3^- (atom % excess ^{15}N) appearing in urine of six rats after an oral dose of [^{15}N]ammonium acetate was 2.2 ± 0.57% (mean ± SD). The amount of $^{15}NO_3^-$ in urine was 73 ± 32 nmol, corresponding to 0.004% of the administered dose of labeled ammonia. This finding shows that a pathway exists for the formation of nitrate from ammonia.

E. coli LPS endotoxin-induced fever had a striking effect on urinary nitrate excretion. Core rectal temperatures measured 6 hr after administration of LPS revealed an increased temperature of 0.8 ± 0.1°C. Food intake during the course of the fever was significantly reduced. Urinary nitrate excretion increased 9-fold (45 ± 6.3 μmol/day) during the first day of the fever compared with an average nitrate excretion of 5.2 ± 0.13 μmol/day during the week preceeding LPS administration (Fig. 1). As the fever subsided, nitrate excretion decreased, and nitrate levels returned to initial values 5 days after injection. Control rats injected with 0.9% saline solution showed no increase in nitrate excretion (data not shown). Further experiments on 12 additional rats have confirmed these findings; the average excretion of nitrate was enhanced 9-fold by LPS treatment. However, there was a large variation in the degree of enhancement among rats. This correlated with the degree of fever induced ($r = 0.80$, $P < 0.05$).

Comparison of Sprague–Dawley, Wistar, and Fischer 344 rats showed that all three strains had enhanced urinary nitrate excretion after LPS administration. Urinary nitrate excretion increased 9- to 10-fold for Sprague–Dawley rats (53.0 ± 32 μmol/day), Wistar rats (50.6 ± 33 μmol/day), and Fischer 344 rats (35.6 ± 15 μmol/day) 24 hr after LPS administration compared with an average nitrate excretion of 5 μmol/day during the week preceeding LPS treatment. Similarly, as the fever subsided, nitrate excretion decreased.

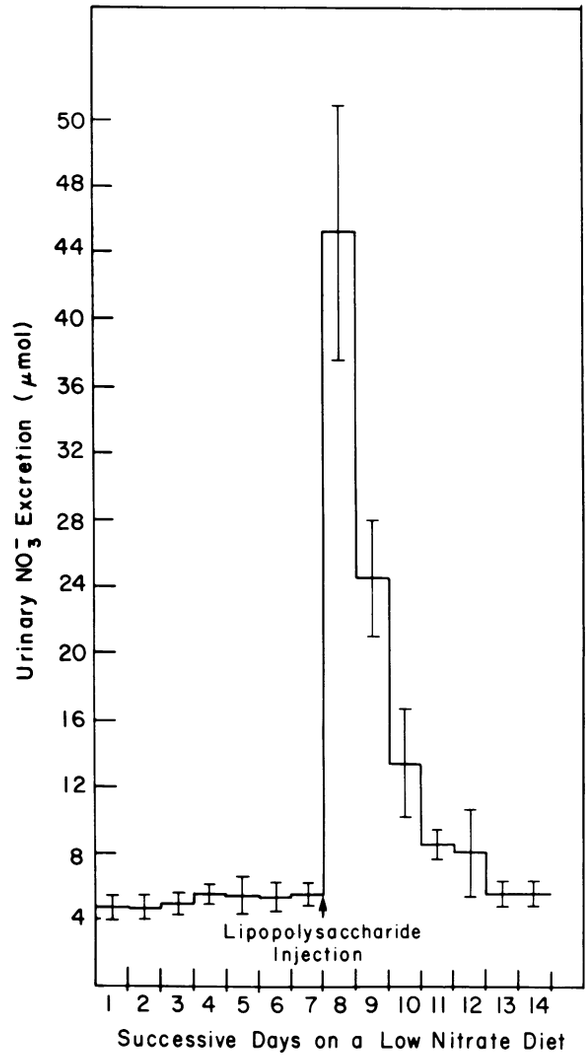

FIG. 1. Induction of nitrate excretion in the rat during endotoxin-induced fever. On day 8, rats were injected i.p. with *E. coli* LPS. Results represent mean ± 95% confidence interval for a group of six rats.

The enhanced urinary excretion of nitrate during LPS administration was shown to be the result of an increased rate of ni-

Table 1. Effect of LPS administration on incorporation of [^{15}N]ammonium acetate into [^{15}N]nitrate

	Control			LPS administered		
Rat	Total urinary NO_3^-, μmol/24 hr	^{15}N in NO_3^-, nmol	% dose incorporated	Total urinary NO_3^-, μmol/24 hr	^{15}N in NO_3^-, nmol	% dose incorporated
1	2.68	40.5	0.002	17.0	602	0.030
2	3.41	87.0	0.004	71.6	3,000	0.150
3	2.60	39.0	0.002	21.7	710	0.036
4	2.56	56.6	0.003	25.3	660	0.033
5	3.62	80.4	0.004	7.52	266	0.013
6	1.58	18.6	0.001	27.0	1,345	0.067

Rats were administered 2 mmol of [^{15}N]ammonium acetate i.g. during the control period. Urinary nitrate was measured 24 hr after administration of the dose. After a break of 1 wk, rats were given LPS (1 mg/kg) i.p. and 2 mmol of [^{15}N]ammonium acetate.

trate synthesis using [^{15}N]ammonium acetate as a N donor. Table 1 shows that the increased excretion of nitrate after LPS administration was accompanied by increased incorporation of [^{15}N]ammonium acetate into nitrate nitrogen. Although the mean total urinary nitrate excretion increased 10-fold with LPS administration, the mean increase in $^{15}NO_3^-$ production from ammonia was 25-fold.

Furthermore, LPS administration did not decrease the metabolic loss of nitrate in the rat. When a 10-μmol dose of [^{15}N]-nitrate was administered orally to rats, the average recovery of nitrate in the urine was 72.8 ± 7.4% for the control period vs. 58.1 ± 8.8% after simultaneous administration of [^{15}N]nitrate and LPS [$P \leq 0.05$ (not significant) by Student's t test].

Two other types of inflammatory states produced changes in nitrate biosynthesis. A carrageenan-induced inflammation produced a greater than 2-fold increase in nitrate synthesis (Fig. 2). Nitrate levels returned to baseline values after three days. On the other hand, a turpentine-induced inflammation produced a delayed pattern of enhancement of nitrate biosynthesis (Fig. 3). Nitrate levels were not significantly increased for the first 24 hr after turpentine administration, but thereafter were increased approximately 3-fold.

DISCUSSION

Orally administered ammonia is incorporated into urinary nitrate in the rat, suggesting that ammonia is either a precursor or is in rapid equilibrium with a precursor to endogenously form nitrate. The conversion of ammonia to nitrate was small, only 0.004% of the administered dose of labeled ammonia. This low conversion of ammonia to nitrate is not surprising because ammonia is rapidly cleared from plasma (23) and incorporated into the body urea and glutamine pools (24). Nitrate biosynthesis thus appears to be a minor component of ammonia metabolism.

LPS stimulated nitrate excretion in three strains of rats. When rats are consuming a low-nitrate diet, urinary nitrate is an approximate measure of endogenous nitrate biosynthesis (11) because nitrate excretion in the feces is negligible (5, 13, 14). However, urinary nitrate excretion accounts for only 60–70%

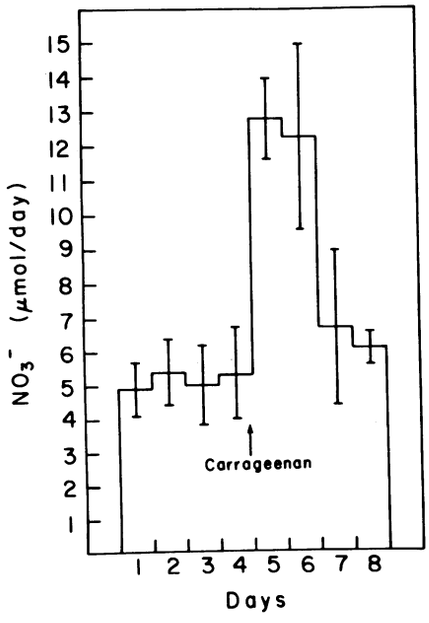

FIG. 2. Enhanced nitrate excretion after carrageenan administration. On day 5, rats were injected i.p. with 0.15 g of carrageenan. Results represent mean ± SD for six rats.

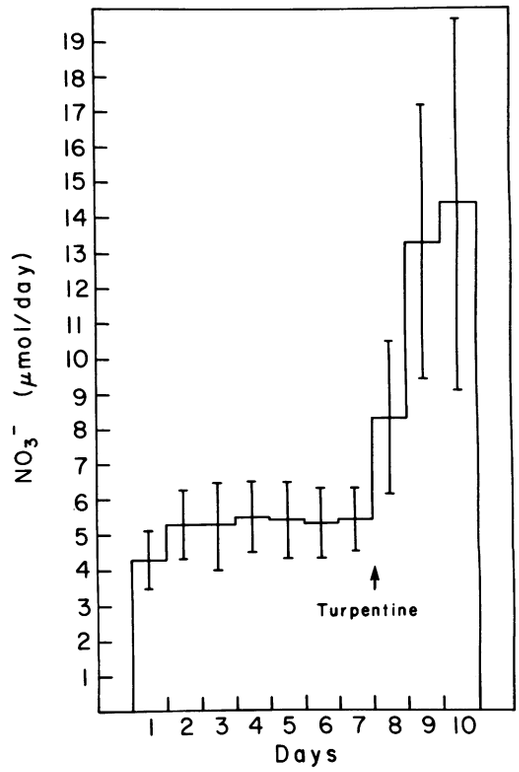

FIG. 3. Effect of turpentine administration on nitrate excretion. Turpentine (0.5 ml) was administered subcutaneously on day 8. Results represent mean ± SD for six rats.

of nitrate clearance from the body (25), so that the magnitude of endogenous nitrate biosynthesis is 1.4–1.7 times greater than urinary output. Since LPS treatment did not alter nitrate clearance, we conclude that the metabolism of nitrate is not significantly changed by LPS treatment. Therefore, the observed 9-fold increase in nitrate excretion after LPS administration was due not to a reduction in the metabolic loss of nitrate but to an increased rate of nitrate synthesis.

Moreover, LPS administration increased the incorporation of [^{15}N]ammonia into nitrate nitrogen. This shows that there was net nitrate synthesis (not recycling of label) after LPS administration. Since there was significantly more [^{15}N]nitrate produced from labeled ammonia with LPS treatment, this suggests that more ammonia nitrogen is shunted into nitrate biosynthesis during LPS treatment. In this context, however, a definitive conclusion would require knowledge of the ^{15}N enrichment of the immediate N precursor of NO_3^-.

Nitrate biosynthesis was also enhanced by carrageenan- and turpentine-induced inflammation. This is further evidence for induction of nitrate synthesis by an activated reticuloendothelial system. The enhancement of nitrate synthesis during these inflammations was less than observed with LPS treatment. Further studies will be required to assess whether activation of the immune system by antigenic stimuli or alterations in nitrogen metabolism during inflammation are responsible for the different degree of induction of nitrate synthesis.

These findings support the hypothesis that activation of the reticuloendothelial system significantly increases nitrate biosynthesis. One possible mechanism and one that is chemically logical is that the increased nitrate synthesis may have been due to an increased generation of reactive oxygen species, capable of oxidizing reduced nitrogen compounds to nitrate. Potential oxidizing species, derived from an activated immune system, that could oxidize reduced nitrogen compounds to nitrate in-

clude superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\cdot$), and singlet oxygen [$O_2(S = 1)$]. It has been shown that stimulation of phagocytic polymorphonuclear leukocytes produces substantial quantities of superoxide and hydrogen peroxide (26). The hydrogen peroxide results from the dismutation of superoxide. However, further reaction of these compounds, under certain conditions, yields more-reactive compounds such as hydroxyl radicals (26). Superoxide and hydrogen peroxide are produced by other systems in the body, including mitochondria during respiration (27), by a number of enzyme reactions such as those for xanthine oxidase and aldehyde oxidase (26), and during hyperbaric oxygen exposure (28). Further experiments designed to block the increased synthesis of reactive oxygen species after LPS administration will be necessary to test this hypothesis.

The existence of endogenous biosynthesis of nitrate has been reported for both humans (11) and rats (13, 15) and the present studies reveal that ammonia serves as a precursor for nitrate. However, our experiments do not permit an evaluation of the immediate nitrogen donor for nitrate formation, although they suggest that the conversion of ammonia N to nitrate might be modulated by activation of the reticuloendothelial system. We hypothesize that a pathway of nitrate biosynthesis involves oxidation of reduced nitrogen compounds by oxygen radicals generated within the body.

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