matographed on DEAE-Sephadex (*7*), the antigen became bound to the DEAE-Sephadex and was eluted from the resin in the monosialoganglioside fraction by 0.02*M* ammonium acetate in methanol. No antigen was detected either in the disialoganglioside fraction eluted by 0.12*M* ammonium acetate in methanol or in the tri- and tetrasialoganglioside fraction eluted by 0.5*M* ammonium acetate in methanol. On the basis of this property and its chromatographic mobility (Fig. 1), we conclude that the antigen is probably a large monosialoganglioside.

The antigen is not detected by the autoradiographic method described here in ganglioside mixtures from human tissues other than colorectal carcinoma (*8*). However, the antigen is present in human meconium, which is a rich source of fetal glycolipids (*9*). The glycolipids of tumor cells differ from their normal counterparts (*10*) and some may reflect their embryonic origin.

JOHN L. MAGNANI
MANFRED BROCKHAUS
DAVID F. SMITH
VICTOR GINSBURG
National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205
MAGDALENA BLASZCZYK
KENNETH F. MITCHELL
ZENON STEPLEWSKI
HILARY KOPROWSKI
Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

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Nitrate Synthesis in the Germfree and Conventional Rat

Abstract. *Metabolic balance studies show that germfree and conventional Sprague-Dawley rats synthesize nitrate. Equivalent results for germfree and conventional rats eliminate the microflora as obligatory components of nitrate production. Nitrate synthesis appears to be a mammalian process.*

Nitrate (NO_3^-) and nitrite (NO_2^-) (*1*) react in the body to form carcinogenic nitrosamines (*2*). The diet is not the only source of NO_3^- for such reactions, as humans and laboratory animals on low-NO_3^- diets can excrete more NO_3^- than they ingest (*3*, *4*). We report here isotopic studies with rats that confirm that NO_3^- is synthesized in the body and show by the use of germfree rats that the flora is not obligatory for its formation.

Germfree or conventional (cesarean-derived, specific pathogen–free) male Sprague-Dawley rats (initially weighing 150 g; Charles River Breeding Laboratories, Wilmington, Massachusetts) were housed individually in metal metabolism cages and were given unlimited amounts of distilled water and a casein- and cornstarch-based, low-NO_3^-, low-NO_2^- diet (*5*). Germfree rats were maintained in flexible film isolators (*6*). The basal diet was supplemented as indicated with either 0.001 or 0.005 percent $Na^{15}NO_3$ (KOR Isotopes, Cambridge, Massachusetts). Food consumption was determined at 24-hour intervals for the conventional animals and estimated to be comparable for germfree animals (*7*). Urine and feces were collected during each 24-hour period. Urine for the conventional animals was preserved with isopropyl alcohol (final concentration, ≥ 8 percent). All samples were maintained at −15°C until analyzed.

We determined the NO_3^- content of food, urine, or feces by reducing it to NO_2^- on a cadmium column and assaying the NO_2^- by diazotization and coupling with a Griess reagent (*8*). The relative abundance of $^{15}NO_3^-$ and $^{14}NO_3^-$ was determined by conversion of NO_3^- to nitrobenzene (*9*), which was then analyzed by gas chromatography–mass spectrometry (GC-MS) (*10*). This method was also used to confirm the reliabil-

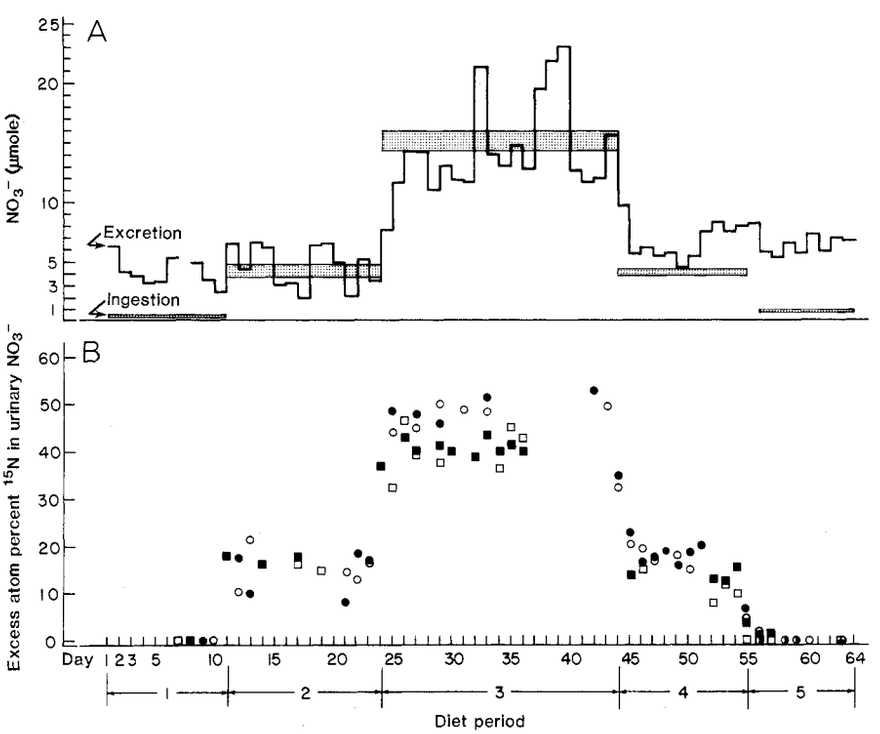

Fig. 1. (A) Ingestion of NO_3^- (mean ± the standard deviation) (the five shaded blocks) and urinary excretion of NO_3^- for one germfree rat. The minimal NO_3^- diet (Table 1) was consumed during periods 1 and 5; during periods 2 and 4, this diet was supplemented with 10 μg of $Na^{15}NO_3$ per gram and, for period 3, with 50 μg of $Na^{15}NO_3$ per gram. (B) Daily excess atom percent ^{15}N in urinary NO_3^- for two germfree (○, ●) and two conventional (□, ■) rats. Days 1, 11, 24, 44, and 55 correspond to the first 24-hour periods after diet change. The excess atom percent ^{15}N is the ratio of ^{15}N to (^{14}N + ^{15}N) in urinary NO_3^- from which this ratio for the natural isotope abundance has been subtracted.

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ity of the NO_3^- analyses based on the use of cadmium reduction and Griess reagent.

The results of a typical experiment comparing daily NO_3^- ingestion and urinary excretion for one germfree animal are shown in Fig. 1A. The data for all germfree and all conventional rats are summarized in Table 1. During diet period 1, both germfree and conventional animals excreted in the urine ten times more NO_3^- than they ingested. As both germfree and conventional rats excreted essentially the same excess of NO_3^-, an obligatory role for the flora in the origin of the NO_3^- is excluded.

During period 2, both germfree and conventional rats excreted slightly more NO_3^- than they ingested. During period 3, all animals excreted slightly less NO_3^- than they ingested. During period 4, when the diet was the same as during period 2, the rats resumed the slight excess excretion of NO_3^- observed during period 2. During period 5, when the diet was equivalent to that in period 1, the urinary excretion of NO_3^- again markedly exceeded the NO_3^- ingested.

These results raise two questions. What is the origin of the excess urinary NO_3^-? Why is the excess apparent only at low NO_3^- intakes?

The determination of the relative abundances of $^{14}NO_3^-$ and $^{15}NO_3^-$ in the diet and urine provides evidence relating to both of these questions. As shown in Fig. 2, $^{14}NO_3^-$ excretion consistently exceeded $^{14}NO_3^-$ ingestion, regardless of the NO_3^- content of the diet. Thus, during periods 2, 3, and 4, as well as during periods 1 and 5, the daily excretion of $^{14}NO_3^-$ was about 6 μmole per rat, despite the fact that the daily ingestion of $^{14}NO_3^-$ was 1 μmole or less.

One possible explanation for the excess excretion of $^{14}NO_3^-$ during all diet periods is that there is a body pool of NO_3^- that is turning over during the experiment. In such a case it would be expected that any NO_3^- pool would be labeled by ^{15}N during the 44 days (periods 2, 3, and 4) when $^{15}NO_3^-$ was ingested. When the $^{15}NO_3^-$ dietary supplement is removed during period 5, the $^{15}NO_3^-$ in this postulated pool should decrease and be detected as $^{15}NO_3^-$ in the urine. By day 3 of period 5, however, $^{15}NO_3^-$ was absent from the urine (Fig. 1B); the excess of NO_3^- in the urine over that ingested continued to be exclusively in the form of $^{14}NO_3^-$. In the other dietary periods as well, the labeling pattern of urinary NO_3^- promptly reflected the isotope content of that diet. Thus the labeling patterns in the urine during periods 4 and 5 are quite similar to those of periods 2 and 1, respectively, despite earlier exposure to $^{15}NO_3^-$. These data tend to exclude the possibility of a pool of NO_3^- in the body. Our results are consistent with those of Wang *et al.* (*11*), who also found NO_3^- to be rapidly excreted and not stored by the rat.

Table 1. Daily NO_3^- ingestion and urinary excretion for germfree and conventional rats. The values listed are the mean ± the standard error of the mean.

Diet period	Daily total $NaNO_3$ intake (μmole)	Daily urinary $NaNO_3$ excretion*	
		Germfree rats (N = 6)	Conventional rats† (N = 6)
1	0.50 ± 0.03	4.9 ± 1.3	5.1 ± 0.8‡
2	4.3 ± 0.5	5.4 ± 1.3	5.4 ± 1.1§
3	15 ± 1	14 ± 2	13 ± 1‖
4	4.1 ± 0.2	6.3 ± 1.0	7.4 ± 0.8¶
5	0.75 ± 0.07	6.0 ± 0.7	6.9 ± 0.6‡

*Excretion data for the first 2 days of diet periods 2 through 5 are excluded to allow for the elimination of NO_3^- ingested during the previous diet period. †Analysis of variance shows no significant difference ($P > .1$) between the urinary excretion of $NaNO_3$ for germfree and conventional rats over all diet periods. ‡Significantly greater than intake, $P < .005$. §Significantly greater than intake, $P < .05$. ‖Significantly less than intake, $P < .01$. ¶Significantly greater than intake, $P < .01$.

Nitrogen oxides in inspired air are another possible source of urinary NO_3^-, but the concentrations measured are insufficient to account for the observed excess of urinary NO_3^- (*12*). Thus, mammalian synthesis is the most likely source of the excess excreted NO_3^-.

During the periods when the diet was supplemented with $^{15}NO_3^-$, the excretion of $^{15}NO_3^-$ in the urine accounted for from one-third to one-half of that ingested (Fig. 2). An average of 16 (±6) percent of the ingested label appeared as $^{15}NH_4^+$ and [^{15}N]urea in the urine of conventional animals (*13*); these labeled metabolites were not detected in the urine of germfree animals. A small amount of NO_3^- appears in the feces of germfree animals (about 0.5 μmole per day) whereas NO_3^- is not detectable in the feces of conventional animals. These results are consistent with the reduction by the gastrointestinal flora of a portion of the ingested NO_3^- (*14*).

The quantitative aspects of NO_3^- recovery in the urine help to provide an answer to the question of why urinary NO_3^- excretion appears to be in excess of NO_3^- ingestion only during periods of low NO_3^- intake. The data on $^{15}NO_3^-$ during period 4, for example, show that approximately one-third of the ingested $^{15}NO_3^-$ is excreted in the urine of the conventional rat (Fig. 2). As dietary $^{15}NO_3^-$ and $^{14}NO_3^-$ can be assumed to be metabolized identically, then of the 4.1 μmole of total NO_3^- ingested daily during this period, only about 1.4 μmole are recovered in the urine. The urinary $^{14}NO_3^-$ recovery during this period indicates that endogenous synthesis contributes about 6 μmole of urinary NO_3^-. Since this amount of endogenously derived NO_3^- is greater than the 2.7 μmole of dietary NO_3^- that does not appear in the urine, the net excretion of NO_3^- is greater than the ingestion. In contrast, during diet period 3, the rats ingested 15 μmole of NO_3^- daily, of which again about 40 percent, or 6 μmole, was recovered in the urine. The remaining 9 μmole were not recovered as urinary NO_3^-. The

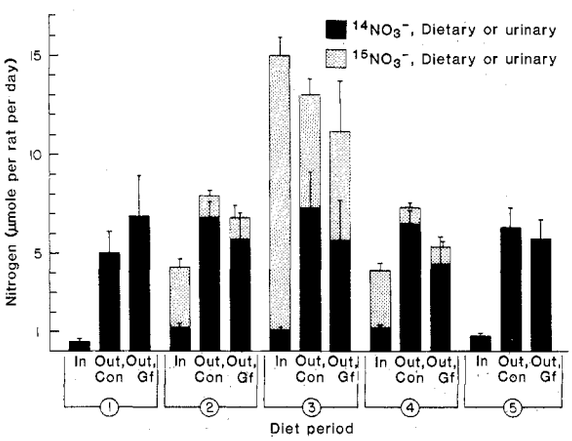

Fig. 2. Mean (± the standard deviation) daily $^{14}NO_3^-$ and $^{15}NO_3^-$ ingestion and excretion for one conventional (*Con*) and one germfree (*Gf*) rat for each diet period. Excretion data from the first 2 days of each diet period are excluded to allow for the elimination of NO_3^- ingested in the previous period. Diets ingested during each period are described in the Fig. 1 legend.

$^{14}NO_3^-$ data for this period indicates that endogenous synthesis contributes 7 μmole of urinary NO_3^-. Since this is less than the 9 μmole of dietary NO_3^- that do not appear in the urine, the net excretion of NO_3^- is now less than the amount ingested. Thus, although NO_3^- synthesis occurs at all concentrations of NO_3^- intake, urinary excretion in excess of intake will be apparent only when intake is low—that is, when the amount of dietary NO_3^- not appearing in the urine is less than the amount of endogenously synthesized NO_3^- that is excreted.

Synthesis of NO_3^- in humans has been reported (*4*) and was attributed to intestinal nitrifying bacteria. Our studies indicate that NO_3^- synthesis occurs in the germfree rat as well as in the conventional rat and thus is independent of the flora. It appears that NO_3^- synthesis is a mammalian process.

LAURA C. GREEN
STEVEN R. TANNENBAUM
Department of Nutrition and *Food Science, Massachusetts Institute of Technology, Cambridge 02139*
PETER GOLDMAN
Department of Pharmacology, Harvard Medical School, and *Beth Israel Hospital, Boston, Massachusetts 02215*

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13. Urine samples were treated with urease (Sigma) to generate ammonia (NH_3), which is trapped as $(NH_4)_2SO_4$ [E. J. Conway and A. Byrne, *Biochem. J.* **27**, 419 (1933)] and determined on the basis of its reaction with hypochlorite and phenol to form the blue indophenol [H. N. Munro, Ed., *Mammalian Protein Metabolism* (Academic Press, New York, 1969), vol. 3, chap. 30]. The relative abundances of the $^{15}NH_4^+$ and $^{14}NH_4^+$ were determined by mass spectrometry after oxidation to molecular nitrogen with NaOBr (Nuclide 3-60 RMS, State College, Pa.).
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Nitrogen-13-Labeled Nitrite and Nitrate: Distribution and Metabolism After Intratracheal Administration

Abstract. *Radioactive nitrogen-13 from nitrite (NO_2^-) or nitrate (NO_3^-) administered intratracheally or intravenously without added carrier to mice or rabbits was distributed evenly throughout most organs and tissues regardless of the entry route or the anion administered. Nitrogen-13 from both anions was distributed uniformly between plasma and blood cells. We found rapid in vivo oxidation of NO_2^- to NO_3^- at concentrations of 2 to 3 nanomoles per liter in blood. Over 50 percent oxidation within 10 minutes accounted for the similar nitrogen-13 distributions from both parent ions. The oxidation rates were animal species–dependent. No reduction of $^{13}NO_3^-$ to $^{13}NO_2^-$ was observed. A mechanistic hypothesis invoking oxidation of $^{13}NO_2^-$ by a catalase–hydrogen peroxide complex accounts for the results. These results imply a concentration dependence for the in vivo fate of NO_2^- or nitrogen dioxide.*

The in vivo fate of nitrite (NO_2^-) and nitrate (NO_3^-) is being studied with radioactive ^{13}N (half-life = 10 minutes) tracer technology that offers insight into an enigma of marked contemporary importance. More than 100 years ago, Gamgee (*1*) first observed what is now known to be the conversion of oxyhemoglobin (HbO_2) to methemoglobin (MetHb) when NO_2^- was added to blood. The biochemical pathways of this pharmacologically and toxicologically important transformation are not fully understood (*2*). Newberne's (*3*) recent report that long-term NO_2^- ingestion by rats caused an increased incidence of cancer in the lymphoreticular system elicited immediate congressional attention (*4*) because removal of NO_2^- as a food additive in accordance with the Delaney Clause may increase the risk of food poisoning from botulism. The resolution (*5*) of this public health dilemma is complicated by earlier findings (*6*) that ingested NO_3^-, a natural component of many foods, especially vegetables, is reduced to NO_2^- by oral microflora to produce salivary NO_2^- concentrations of several hundred milligrams per liter. The formation and reactions of NO_3^- and NO_2^- in the intestinal tract are considered potentially important in understanding the etiology of human gastric cancer, but their significance is controversial (*7*).

The long-term health effects of NO_3^- and NO_2^- entering the body through the respiratory tract are largely unknown. These anions may form in the atmosphere (*8*) or in vivo from airborne nitrogenous compounds. Inhalation of nitrogen dioxide (NO_2), the most abundant nitrogenous air pollutant, can result in the formation of NO_2^- and NO_3^- (*9*). In a pioneering tracer experiment, Goldstein *et al.* (*10*) found that ^{13}N inhaled by rhesus monkeys as NO_2 gas [0.6 part per million (ppm)] was rapidly taken up by the blood and that a substantial portion was uniformly disseminated to extrapulmonary sites. They postulated from chemical arguments that a portion of the NO_2 reacted with aqueous vapor and mucus by disproportionation to form nitrous acid (HNO_2) and nitric acid (HNO_3), precursors to NO_2^- and NO_3^-. Pryor *et al.* (*11*) found in vitro evidence that NO_2 concentrations below 50 ppm probably react with lung components to form only NO_2^-. Svorcova and Kaut (*9*) observed both NO_3^- and NO_2^- in the blood of rabbits that had been exposed to 22.5 ppm of NO_2 for 4 hours. Recently, Igbal and his co-workers (*12*) observed the in vivo formation of a nitrosamine

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