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Nitrate biosynthesis in man

(metabolism/stable isotopes/carcinogenesis)

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ABSTRACT **Nitrate metabolism was investigated in long-term metabolic balance studies on healthy young men. Under conditions of constant low ingestion of nitrate (<180 μmol/day per subject), the amount of nitrate excreted in urine was an average of 4-fold greater than the amount ingested. Balance studies with $^{15}NO_3^-$ showed that the source of the excess nitrate in urine was the endogenous biosynthesis of nitrate, rather than the emptying of a body pool. Nitrate biosynthesis occurred when nitrate ingestion was high as well as low, and the amounts synthesized appeared to be independent of intake and comparable to the amounts ingested from normal diets. Analysis of the $^{15}NO_3^-$ data also revealed that half of ingested nitrate was recovered as urinary nitrate. Because nitrate in urine is the net result of (*i*) intake, (*ii*) endogenous synthesis, and (*iii*) metabolic losses, the magnitude of the losses is such that, despite ongoing synthesis, the amount of nitrate in the urine of people consuming most diets will be less than the amount ingested.**

Nitrosamines are potent animal carcinogens (1). Small amounts of these compounds are formed in some foods and in the animal and human body from the reaction of amines and nitrite. A common precursor to nitrite is nitrate; higher than average levels of nitrate in some environments have been linked to elevated incidences of human cancer (2, 3). The environment is not the only source of nitrate: metabolic balance studies performed early in this century (4) and preliminary studies from our laboratory (5) have suggested that man biosynthesizes nitrate. We now have completed long-term metabolic balance studies that demonstrate the biosynthesis of nitrate in healthy adult men. These studies also show, by the use of $^{15}NO_3^-$, that a large amount of ingested nitrate is metabolized or otherwise not excreted in urine as nitrate.

MATERIALS AND METHODS

The study protocols were approved by the Massachusetts Institute of Technology Committee on the Use of Humans as Experimental Subjects and the Policy Committee of the Massachusetts Institute of Technology Clinical Research Center. The subjects—young adult male Massachusetts Institute of Technology students—were paid volunteers, judged to be healthy on the basis of medical histories, physical examinations, and standard blood and urine analyses. Participation in the studies was on an out-patient basis at the Clinical Research Center, and meals were consumed under supervision in the Massachusetts Institute of Technology Department of Nutrition and Food Science diet kitchen. Estimates of energy and fluid intakes were made prior to the studies to establish each subject's caloric and fluid requirement for the experimental periods. The subjects were asked to maintain usual levels of physical activity during the studies.

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In the first study, a soy protein-based diet was given to eight young men (mean age, 21; mean weight, 69 kg) for 84 days. The soy preparation consisted of a high-quality protein isolate (Supro-710, Ralston-Purina; ref. 6) and was incorporated to supply 0.8 g of protein per kg of body weight. The remainder of the calories was provided by carbonated and sucrose beverages, a corn starch dessert, and protein-free cookies (7). Mean energy intake was 46 kcal (1 cal = 4.184 J) per kg of body weight, with an average of total calories from protein of 7.0%, from carbohydrates of 53%, and from fat of 40%.

Another group of eight young men (mean age, 22; mean weight, 72 kg) participated in a second study lasting 56 days. During wk 1 of the study, the subjects consumed their free-choice diet. For the next 4 wk, they consumed the basal formula to which milk protein had been added. In addition, twice daily they consumed an omelette made of dried egg, water, and salt. Carbonated and sucrose beverages, a cornstarch dessert, and protein-free cookies provided the remainder of calories in the diet.

For the final 3 wk of the study, 25 g of standardized wheat bran preparation (American Association of Cereal Chemists certified food grade wheat bran) was added to the basal formula. The diet otherwise was identical to that of the previous 4 wk. Daily protein intake was higher in this second study—1.5 g of protein per kg of body weight. Mean daily calories consisted of 14% from protein, 45% from carbohydrate, and 41% from fat.

A third study involved another six young men (mean age, 24; mean weight, 79 kg) and lasted for 1 wk. The subjects consumed the high protein soy, milk, and egg diet utilized in the second study, with no addition of bran. On the penultimate morning of this study, after an overnight 10-hr fast, each subject was given a 300-mg (3500 μmol) dose of $Na^{15}NO_3$ (≥99% N = ^{15}N) in 10 ml of distilled water *per os*, followed by 200 ml of distilled water. Complete urine collections were made at 1, 3, 6, 12, 24, and 48 hr after dosing.

Complete 24-hr urine collections were made throughout each study. For the first 84-day study, urine was preserved by placing 15 ml of a 30% (vol/vol) HCl solution into each 2-liter collection jug; the collected urine was made up to 3 liters, if necessary, with distilled water, and samples were frozen until used for measurement of creatinine, total nitrogen, urea nitrogen [all by methods previously described (8–10)], and nitrate. For the second 56-day study, urine was preserved by placing 200 ml of 2-propanol into each 2-liter collection jug. For the third study, 100 ml of 2-propanol, 10 g of NaH_2PO_4, and 1.5 g of $NaHSO_3$ were placed in each jug. Urine was treated as above but analyzed only for creatinine and nitrate.

Subjects were weighed daily, and routine clinical analyses of blood and urine were performed weekly to monitor the health of each volunteer.

Diet components and urine were treated as follows for nitrate analysis. Diet components were diluted 1:5 (vol/vol) with 80%

Table 1. Nitrate* content of diet components

Component	Concentration†
Water	≤2.0
Supro 710	8.0
Supro 710 + milk	8.2
Omelette	12
Bran	8.0
Corn oil	1.1
Cornstarch	3.1
Sugar	1.4
Apple juice‡	2.0
Kool Aid	<0.1

* Samples contained no detectable nitrite (detection limit, 0.2 mg of $NaNO_2$ per kg of diet component).
† Values are mg of $NaNO_3$ per kg of diet component.
‡ Value taken from ref. 15.

reagent grade ethanol, incubated in a shaking water bath for 3 hr at 50°C and centrifuged for 15 min at 10,000 × *g* and 4°C; the supernatant was decanted and analyzed. Urine was simply diluted 1:20 (vol/vol) in distilled water.

An automated method was used to reduce the nitrate in samples to nitrite on a cadmium column, to couple the nitrite with a Griess reagent, and to detect the diazo chromophore at 546 nm. ^{15}N-Labeled nitrate was assayed by nitration of benzene to form [^{15}N]nitrobenzene (11), followed by gas chromatography/mass spectrometry (5992 system from Hewlett–Packard) with selected ion monitoring at a mass-to-charge ratio (*m/e*) of 123 (M^+) and *m/e* 124 ($M+1^+$).

The nitrogen dioxide content of midday air at the Massachusetts Institute of Technology was determined by the Saltzman–Griess procedure (12, 13), which involves drawing a measured volume of air through a bubbler containing Griess reagent and determining the absorbance at 550 nm of the solution.

Statistical analyses were performed by using the Biomedical Computer Programs (P-Series) prepared at the Health Sciences Computing Facility of the Department of Biomathematics and the University of California, Los Angeles School of Medicine (14).

RESULTS

The primary result of these studies is that considerably more nitrate is excreted than ingested, when the amount of nitrate ingested is small. Table 1 shows the low level of nitrate found in the components of the diet. During each of the three studies, nitrate ingestion was at most 180 μmol of NO_3^- (15 mg of $NaNO_3$) per subject per day. Inspired NO_2 also contributes to NO_3^- in urine (16–18). Midday air at Massachusetts Institute of Technology was found to contain 30 μg of NO_2 per m^3. If this were absorbed, metabolized, and excreted as NO_3^-, it would contribute only another 7 μmol to urinary nitrate daily. However, the amount of nitrate excreted averaged per subject per day 690 μmol for the first study, 880 μmol for the second study, and 890 μmol for the third study (Table 2).

Differences in daily urinary nitrate excretion were apparent, both among individuals and among days for a given individual (Fig. 1). The reasons for these variations are not known. No statistically significant correlations were found during long-term study between daily urinary nitrate excretion and body weight, urinary total nitrogen, urine volume, urinary creatinine, urinary urea nitrogen, day of study (time), or nitrate excretion during the previous 1 or 2 days. For the second study, daily urinary nitrate excretion appeared to be slightly higher with the addition of wheat bran (Table 3), but a repeated analysis of variance measure showed this difference to be insignificant ($P = 0.09$).

When the amount of nitrate ingested was large, in contrast, less nitrate was excreted than ingested. During the penultimate day of the third study, subjects ingested a total of 3700 μmol of NO_3^-—3500 μmol of $^{15}NO_3^-$ from a single oral dose, 30 μmol of unlabeled nitrate from this dose, and the usual 180 μmol of unlabeled NO_3^- from the basal diet (Table 4). However, urinary excretion of nitrate for the 24-hr period averaged only 2700 ± 300 μmol of NO_3^-. The isotope data for the urinary nitrate (Table 4) show that a considerably larger amount of unlabeled (i.e., $^{14}NO_3^-$) nitrate is excreted than ingested, which is also the case in the absence of nitrate dosing. The urinary recovery of $^{15}NO_3^-$ within 24 hr, however, amounts to only 53 ± 6% of the ingested $^{15}NO_3^-$ dose (Table 4). Another 7 ± 2% of the dose was found as $^{15}NO_3^-$ in the urine collected between 24 and 48 hr. Urine was not collected at later times because preliminary studies had shown that urinary clearance of a nitrate dose was essentially completed within 2 days, Therefore, the overall recovery of ingested $^{15}NO_3^-$ as urinary $^{15}NO_3^-$ was 60 ± 8% within 48 hr. This incomplete recovery of ingested nitrate accounts for the overall deficit of excreted nitrate relative to ingested nitrate after nitrate dosing.

DISCUSSION

We conclude that nitrate is biosynthesized in man. This conclusion follows from the result that more nitrate is excreted in urine than ingested when ingestion of nitrate is low. The nitrate in urine in excess of intake could have had two sources: (*i*) a pool of stored nitrate in the body or (*ii*) the endogenous synthesis of nitrate.

Were a pool of nitrate the source, one would postulate that nitrate ingested by the subjects from their free-choice diets prior to the studies would accumulate in the body and would then turn over and empty during the studies. To be the source of excess nitrate observed in the present studies, the pool would have to be sufficiently large to release at least an average of 690 μmol of nitrate per day for at least 84 days and would have to empty with zero-order kinetics. The data from several laboratories on nitrate storage and excretion, however, suggest that such a body pool does not exist. Whelan (19) showed that dogs

Table 2. Summary of data from three metabolic balance studies

				Nitrate, μmol/day per subject			
					Excretion		
Study	Subjects	Duration, days	Daily protein intake, g/kg body wt	Ingestion	Mean	SEM within subjects	SEM between subjects
1	8*	84	0.8	≤180	690	62	190
2	8†	56	1.5	≤180	880	110	200
3	6	7	1.5	≤180	890	120	180

* Nitrate excretion not stable for one subject.
† Nitrate excretion not stable for two subjects.

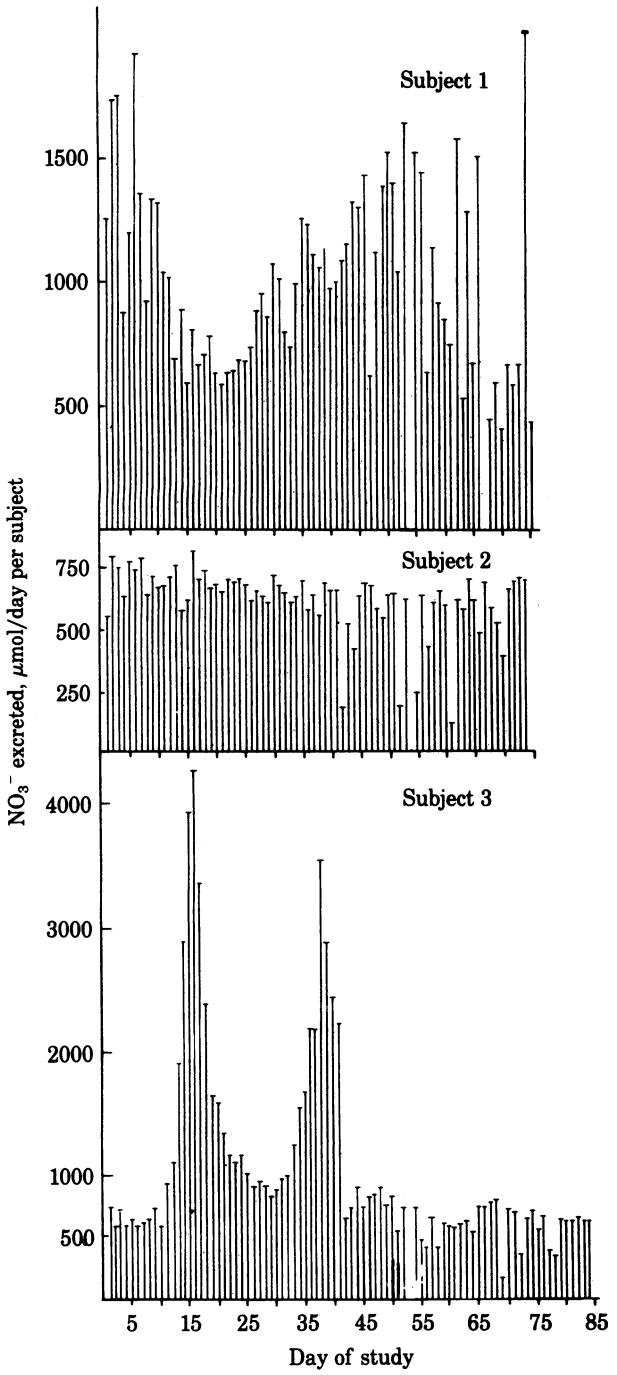

FIG. 1. Daily urinary nitrate excretion for three subjects during first metabolic balance study. Ingestion of nitrate was 180 μmol of NO_3^- per day. Missing values are due to lost urine samples.

chronically fed nitrate retained less than 2% of the total dose as nitrate in their tissues 24 hr after the final dose. Wang *et al.* (20) found that less than 0.003% of chronically administered $^{15}NO_3^-$ could be recovered as $^{15}NO_3^-$ from the blood, liver, or kidneys of rats 82 hr after the last $^{15}NO_3^-$ feeding. Urinary excretion of nitrate has been shown to be rapid and first order in the rat (20–22), in the dog (19, 23), and in man. In human subjects, Bartholomew *et al.* (24) found that urinary nitrate clearance was essentially completed within 1 day, and the results of Mitchell *et al.* (21) and the present results confirm that the bulk of urinary excretion of nitrate occurs within 24 hr of ingestion and is essentially completed within 48 hr.

In contrast, none of the experimental data is inconsistent with the hypothesis that nitrate is biosynthesized in the body.

Table 3. Daily urinary nitrate excretion during last week of each diet: second study*

Subject	NO_3^- excretion, μmol/day per subject: Without fiber, mean ± SEM	With fiber,† mean ± SEM
1	720 ± 33	1300 ± 96
2	980 ± 110	900 ± 49
3	800 ± 130	1200 ± 200
4	910 ± 73	910 ± 100
5	960 ± 120	990 ± 100
6	740 ± 67	1200 ± 300
7	700 ± 110	710 ± 80
8	730 ± 42	650 ± 64

* Daily nitrate ingestion was ≤180 μmol of NO_3^- per day per subject.
† Nitrate excretion during period with fiber was not significantly higher than excretion during period without fiber: $P = 0.09$.

Other investigators also have shown that urinary nitrate excretion exceeds nitrate ingestion when the latter is low (21, 25–27). When nitrate ingestion is high, however, it has repeatedly been shown that urinary nitrate is only a fraction of ingested nitrate (19–26). These contradictory findings might have been reconciled by either of two hypotheses: (*i*) endogenous nitrate synthesis occurs only when small amounts of nitrate are ingested and does not occur when large amounts of nitrate are ingested or (*ii*) endogenous nitrate synthesis occurs at all levels of nitrate intake but is somehow masked under conditions of high nitrate ingestion.

The first hypothesis has been disproved by the data from experiments using $^{15}NO_3^-$, both in a study in rats (22) and in this study in men. Table 4 shows that even after a dose of 3500 μmol of $Na^{15}NO_3^-$ [a nitrate dose twice as large as the average daily amount of nitrate ingested by individuals in the U.S. (15, 28)], unlabeled $^{14}NO_3^-$ excretion still exceeds the amount ingested by an average of 650 μmol. Therefore, nitrate biosynthesis is not abolished by high nitrate ingestion.

The data do in fact fit the second hypothesis. Thus, although during labeled nitrate dosing, the total amount of urinary nitrate (labeled and unlabeled) appears to be less than the total amount of ingested nitrate (Table 4), the reason is simply that much of the ingested nitrate is metabolized or otherwise does not appear as urinary nitrate. For all levels of nitrate ingestion, the relationship of nitrate excretion to nitrate intake is described by the generalized equation:

$$\text{Excretion} = \text{Intake} + \text{Endogenous synthesis} - \text{Metabolic losses},$$

with

$$\text{Metabolic losses} = f(\text{Intake}),$$

and

$$\text{Endogenous synthesis} = \text{Constant}.$$

In the present study, urinary $^{15}NO_3^-$ excretion accounts for only an average of 60% of the ingested dose. Incomplete recoveries of ingested nitrate in human urine have been reported by others: Radomski *et al.* (27) estimated that less than half of ingested nitrate was recovered as urinary nitrate; Maruyama *et al.* (29) reported incomplete recoveries of nitrate in subjects ingesting typical high-nitrate Japanese diets; Bartholomew *et al.* (24) found that 65–70% of a nitrate dose was recovered in urine; and Mitchell *et al.* (21) reported that 40–60% of ingested nitrate appeared in urine.

The fate of the remainder of the nitrate is mostly unknown. Feces were not analyzed for nitrate in these studies, but the results of Saul *et al.* (30) and our results from rat feces (22) sug-

Table 4. Third study: 24-hr NO_3^- ingestion and excretion for each subject after dosing with $^{15}NO_3^-$

Subject	Nitrate, μmol/day per subject: Total NO_3^- excreted of 3700 ingested*	$^{14}NO_3^-$ excreted of 210 ingested†	$^{15}NO_3^-$ excreted of 3500 ingested*	$^{15}NO_3^-$ excreted ÷ ingested, %
1	2600	860	1770	51
2	3100	1300	1740	50
3	2400	780	1600	46
4	3000	870	2150	61
5	2500	660	1800	51
6	2800	700	2130	61

* Nitrate excreted is significantly less than amount ingested: $P < 0.005$.
† Nitrate excreted is significantly greater than amount ingested: $P > 0.005$.

gest that fecal excretion of nitrate is negligible. Some of the ingested nitrate is likely to be metabolized by the intestinal flora (31, 32). In our subjects, some 2–4% of ^{15}N ingested as $^{15}NO_3^-$ is metabolized to the oxidation state of ammonia, and excreted as [^{15}N]urea and $^{15}NH_4^+$ (unpublished data). Reduction of nitrate to nitrite might be followed by deamination or nitrosation reactions, complexation, formation of gaseous products, or further reduction (33)—all of which would lead to a net loss of nitrate in addition to the reoxidation of nitrite to nitrate (34).

Whatever the mechanisms involved, the metabolic losses of nitrate appear to be proportional to nitrate ingestion. In contrast, endogenous synthesis appears to be relatively constant and independent of ingestion. It follows that with high levels of nitrate intake, the subsequently large amounts lost will exceed the amounts biosynthesized, and the net amount of nitrate excreted will be less than that ingested.

The average amounts of nitrate biosynthesized daily in man can be estimated as follows. Table 2 shows that an average of 690 μmol of NO_3^- per 24 hr per person is excreted in urine during the first study, and averages of 880 and 890 μmol of NO_3^- per 24 hr per person are excreted in urine during the second and third studies. The slightly higher levels of urinary nitrate in the second and third studies may have been related to the higher level of protein in the diet, although we presently have no proof of this. In each study, subjects ingested a maximum of 180 μmol of NO_3^- from the daily diet and inhaled 7 μmol of NO_2 from air. By assuming a steady-state recovery of 60% of these two contributions as urinary nitrate, the remaining 580 μmol of NO_3^- from the first study and 770 and 780 μmol of NO_3^- from the second and third studies represent the average daily excretion of endogenously synthesized nitrate. If endogenously synthesized nitrate were metabolized as ingested nitrate is, then this excreted level of synthetic nitrate would represent about half of the amount of nitrate that is endogenously synthesized daily in man. By way of comparison, the U.S. per capita consumption of nitrate from the daily diet is 1600 μmol of NO_3^- (15, 28).

The amounts of endogenously synthesized nitrate excreted by the rat and by man are comparable on a body weight basis. A 400-g male rat excretes some 6 μmol of endogenously synthesized nitrate daily, which equals 15 μmol/kg per day (22). An average excretion for men of 700 μmol of endogenously synthesized nitrate is equivalent to 10 μmol/kg per day. Because the mechanism of biosynthesis is currently unknown, we cannot judge whether the interspecies similarity in the rate of biosynthesis is significant or is simply fortuitous.

After our preliminary findings of excess nitrate in human urine, we had hypothesized that microorganisms in the intestinal tract were generating the nitrate by means of nitrification (5). This was the reason that the effects of dietary fiber on excess nitrate excretion were explored in the second study reported here. We have since rejected this hypothesis, primarily because we have shown that both germ-free rats and conventional rats generate nitrate (22). This finding has been corroborated by others (35). The toxicological significance of extensive nitrate metabolism and of nitrate biosynthesis is unknown at this time, although the probable involvement of nitrite in these processes makes them of some toxicological interest.

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