Metabolic Fate of an Oral Dose of $^{15}$N-labeled Nitrate in Humans: Effect of Diet Supplementation with Ascorbic Acid

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ABSTRACT

The metabolic fate of a p.o. dose of 3.5 mmol $^{15}$N-labeled nitrate has been investigated in 12 healthy young adults. Samples of urine, saliva, plasma, and feces were collected over a period of 48 hr following administration of the dose. Subjects received either 60 mg of ascorbic acid, 2 g of ascorbic acid, or 2 g of sodium ascorbate per day. An average of 60% of the $^{15}$NO$_3^-$ dose appeared in the urine as nitrate within 48 hr. Less than 0.1% appeared in the feces. The $^{15}$N label of nitrate was also found in the urine (3%) and feces (0.2%) in the form of ammonia or urea. The fate of the remaining 35% of the $^{15}$NO$_3^-$ dose administered is unknown. No effect of ascorbic acid or sodium ascorbate on the nitrate and nitrite levels of plasma, saliva, urine, or feces was observed. A one-compartment pharmacokinetic model was used to describe the relationships between intake, plasma concentration, and urinary excretion of nitrate. The half-life of nitrate in the body was found to be approximately 5 hr, and its volume of distribution was about 30% of body weight. Daily endogenous biosynthesis of nitrate was estimated to be about 1 mmol/day.

INTRODUCTION

There is widespread concern over exposure to nitrate from dietary and environmental origins and its potential risk to human health. Several epidemiological studies have suggested an association between exposure to high levels of nitrate and increased incidence of stomach cancer (2, 11, 35). While nitrate is a stable chemical species, it can be converted to nitrite by bacterial reduction in the oral cavity (20) or other areas of the body containing high concentrations of bacteria (20, 23, 27, 37). Salivary nitrite concentration has been reported to be directly proportional to the amount of nitrate ingested (25). The swallowing of saliva exposes the stomach to the nitrite formed in the oral cavity. Considerable evidence suggests that nitrite in the stomach has the potential to nitrosate nitrogenous constituents of food products, drugs, and agricultural chemicals to form N-nitroso compounds (4, 6, 16). Endogenous production of N-nitroso compounds has been demonstrated in humans by a number of investigators (14, 21, 30). The specific involvement of N-nitroso compounds in human disease has not been shown, but it is well established that most N-nitroso compounds are potent carcinogens in laboratory animals (17).

Recent studies (7, 8, 15, 26, 34) have confirmed earlier reports (19) that the diet is not the only source of nitrate but that nitrate is synthesized endogenously from reduced nitrogen compounds. Hence, not all of the nitrate excreted in the urine is of dietary origin, although during periods of large nitrate intake, the contribution of endogenous nitrate biosynthesis to urinary nitrate is small. Urinary excretion of nitrate in humans after high nitrate intakes relative to typical dietary levels (1.2 mmol/day for the United States population (33) range from 50 to 90% (5, 12, 22). The recovery of large p.o. doses of nitrate in laboratory animals has been found to be between 35 and 92% (10, 13, 32). Since urinary recovery is incomplete and fecal excretion of nitrate has been found to be negligible (23), it is clear that nitrate is metabolized to a significant extent in the body.

The administration of $^{15}$N-labeled nitrate allows one to study the metabolic fate of ingested nitrate more directly. Wang et al. (31) administered $^{15}$N-labeled nitrate to rats and recovered approximately 60 to 70% of the $^{15}$N label in the urine, about one-half of which was in the form of nitrate. We reported previously that about one-half of the $^{15}$N-labeled nitrate administered to humans appeared in the urine as nitrate (7); however, the urine or feces was not analyzed for the presence of $^{15}$N label in other nitrogen-containing compounds.

In addition to collections of urine, feces, and saliva, the studies reported in this paper included blood sampling up to 48 hr after ingestion of Na$^{15}$NO$_3$. This permits a comparison of urinary nitrate clearance with total nitrate clearance from the body with the use of a one-compartment pharmacokinetic model and also is helpful in interpreting salivary nitrate and nitrite data. Since supplementing the diet with ascorbic acid has been suggested as a possible means of reducing endogenous N-nitrosation (18), the effect of ascorbic acid on the clearance of nitrate from the body and on the nitrate and nitrite levels of saliva was investigated.

MATERIALS AND METHODS

The protocols for work described were screened by the Committee on the Use of Humans as Experimental Subjects of M. I. T. Subjects were selected for experiments on the basis of a medical history, thorough physical examination, and normal routine blood and urine clinical chemistry analysis. Volunteers on these studies were selected from the M. I. T. student population and participated on an outpatient basis in the Department Diet Kitchen.

The first study was conducted on 6 young adult men, ages 20 to 28 years, with a mean body weight of 79 kg. Subjects received a low-nitrate diet for 7 days with complete mineral and vitamin supplements to meet all National Academy of Sciences/National Research Council recommendations, including trace elements. The diet, which has been described previously (7), consisted of a soy protein diet, supplying 0.1 g of protein (W x 6.25) per kg per day, to which egg and milk protein was added to increase protein intake to 1.5 g of protein per kg per day. The remaining calories were derived from protein-free cookies, cornstarch dessert, and carbonated and sucrose beverages. Energy intake to provide energy...
equilibrium was calculated from a dietary history interview and maintained without change during the study. During the final 48 hr of this period, a \(^{15}\)NO\(_3\) nonpharmacokinetic study was conducted as described below. A break period of 1 week followed in which subjects could choose a free-choice diet, but each subject took a 500-mg ascorbic acid supplement with each meal and evening snack (4 times per day = a 2-g total daily dose). After the break period, a final 1-week period followed, consisting of the low-nitrate diet plus a 2-g supplement of ascorbic acid. The \(^{15}\)NO\(_3\) nonpharmacokinetic study was repeated at the end of this period. The ascorbic acid supplements were taken at each meal during the pharmacokinetic studies.

A second study investigated nitrate pharmacokinetics in young adults using 3 male subjects and 3 female subjects (the group of 6 were between the ages of 19 and 24 years, with a mean weight of 64 kg). The protocol was similar to that of the first study, except that 500 mg of L-proline were administered to subjects 1 hr after the nitrate dose. The formation of nitrosoproline in these subjects will be reported elsewhere. An additional third diet period of 1 week was investigated in these subjects, consisting of a 2-g (500 mg 4 times a day) supplementation of sodium ascorbate (Hoffmann-La Roche Inc., Nutley, N. J.). A \(^{15}\)NO\(_3\) kinetic study was done at the end of this period.

A study of \(^{15}\)NO\(_3\) kinetics involved giving a p.o. dose of 3.5 mmol of Na\(_{15}\)NO\(_3\) (99% atom excess \(^{15}\)N; KOR Isotopes, Cambridge, Mass.) in 10 ml of distilled water, followed by 200 ml of distilled water. Complete urine collections were made for the periods 0 to 1, 1 to 3, 3 to 6, 6 to 12, 12 to 24, and 24 to 48 hr after dosing. Samples of whole saliva were collected by expectoration without stimulation just before the administration of nitrate and 0.5, 1, 2, 3, 6, 12, 24, and 48 hr afterwards. Blood samples were taken just prior to the nitrate dose and at 1, 3, 6, 12, 24, and 48 hr after dosing. Fecal samples were collected following the dose for 3 days and pooled.

Complete daily 24-hr urine samples were collected in prewashed 2-liter polypropylene bottles containing the following preservatives: 100 ml of pure ethyl alcohol, 10 g of Na\(_2\)HPO\(_4\), and 1.5 g of Na\(_2\)HSO\(_3\). Lower amounts but similar ratios of the preservatives were added to bottles collecting urine for shorter collection periods. Saliva was collected in sterile polypropylene centrifuge tubes containing 100 \(\mu\)l of 1.0 m NaOH as a preservative. Saliva was immediately centrifuged at 10,000 \(\times\) for 15 min after collection in order to move any microbiological sediment or large debris. The supernatant was removed and stored frozen (\(-20^\circ\)) until analyzed. Whole blood was collected in heparinized tubes and centrifuged for 30 min at 1000 \(\times\) g. Plasma was removed and stored at \(-20^\circ\) until analyzed.

Nitrate in urine and plasma and nitrate and nitrite in saliva were analyzed by an automated Griess procedure (9). Diet samples were analyzed for nitrate according to the method of Sen and Lee (24). Each diet component (10 g) was extracted for 30 min into an alkaline solution at 50\(^\circ\). After protein precipitation by 10 ml of 1.4 \(\mu\)l ZnSO\(_4\) and then centrifugation for 15 min (2500 \(\times\) g), the resulting supernatant was placed on a short (10 cm) anion-exchange column (Dowex 1, 50 to 100 mesh). Nitrate was eluted with 20 ml of 4 \(\mu\)l NaCl. Fecal samples were analyzed for nitrite similarly to food samples but with the addition of a final reverse-phase Sep-Pak (Waters Associates, Inc., Milford, Mass.) cleanup step (23).

\(^{15}\)N-Labeled nitrate in urine was measured by nitration of benzene to form \(^{15}\)N-nitrobenzene, followed by gas chromatography-mass spectrometry (HP5992 system from Hewlett-Packard) with selected ion monitoring at a mass:charge ratio of 123 (M) and 124 (M + 1) (9). The amount of \(^{15}\)N-nitrate in feces was analyzed in an identical manner. The total \(^{15}\)N enrichment of both ammonia and urea in urine was assayed by treating 5 ml of urine with urease in the outer well of a Conway dish (1). After a saturated K\(_2\)CO\(_3\) solution is added to liberate gaseous ammonia, the ammonia is trapped in 1% H\(_2\)SO\(_4\) in the center well of the Conway dish. The resulting ammonium sulfate solution was analyzed by isotope ratio mass spectrometry [Nuclide 3-60 RMS (8)]. The \(^{15}\)N enrichment of all nitrogen-containing compounds in feces was determined by Kjeldahl digestion of 5 g of feces (20) followed by steam distillation of ammonia into 1% H\(_2\)SO\(_4\). Isotope ratio mass spectrometry was used to quantitate \(^{15}\)NH\(_4\)\(^+\).

**RESULTS**

The daily urinary nitrate excretion for 12 subjects on a 5-day low-ascorbic acid diet (60 mg/day) was 0.78 ± 0.22 (S.D.) mmol/day. No significant effect of ascorbic acid or sodium ascorbate (both given at 2 g/day) on daily urinary nitrate excretion was found; the average daily nitrate excretion for each 5-day diet period was 0.70 ± 0.26 and 0.68 ± 0.36 mmol/day, respectively. However, on all 3 diets, more nitrate was excreted in the urine than was ingested. Since the average daily intake of nitrate from the diet for all subjects was 0.15 mmol/day, this represents excretion of about 0.6 mmol nitrate/day in excess of dietary intake.

The urinary excretion of a 3.5-mmol p.o. dose of \(^{15}\)NO\(_3\) is shown in Table 1. About 90% of all \(^{15}\)NO\(_3\) appearing in urine was excreted within 24 hr. Even during elevated nitrate intake, excess urinary nitrate excretion above dietary intake still occurs, as shown by the excretion of \(^{15}\)NO\(_3\). A high supplementation of ascorbic acid (2 g/day) or sodium ascorbate (2 g/day) had no significant effect on urinary excretion of the \(^{15}\)NO\(_3\) dose (data not shown).

The average fasting nitrate level in plasma was 0.03 mm for the low-ascorbic acid diet period (Table 2). After the administration of nitrate, the plasma levels rose sharply during the first hr, a 6-fold increase above fasting levels. Nitrate levels in plasma did not fully return to initial base-line levels until 24 to 48 hr later. The average fasting level or rate of decay of nitrate in plasma was not altered by supplementation of ascorbic acid or sodium ascorbate (data not shown).

As shown in Table 2, the fasting salivary nitrate and nitrite concentrations for 12 individuals on the low ascorbic acid diet, measured before the nitrate dose, averaged 0.20 and 0.09 mm, respectively. The mean nitrate and nitrite levels in mixed saliva following the dose are also given in Table 2. Wide variations in the concentration of nitrate and nitrite in saliva were observed among individuals. The peak concentration of salivary nitrate and nitrite occurred in the vicinity of 1 hr. The peak level for salivary nitrate was roughly 10 times the fasting level, whereas the nitrite peak was about 5 times that of the fasting salivary nitrite level. The S:P ratio varied between 12 and 20, the higher values occurring shortly after the dose (Table 2). The S:P ratio averaged 17 following the dose, but 2 individuals showed S:P ratios of up to 50. The average ratio of salivary nitrite to salivary nitrate varied from 0.19 to 0.42 (Table 2). This ratio was highest at fasting but dropped significantly following the nitrate dose. Ascobic acid or sodium ascorbate did not alter nitrate and nitrite levels in saliva.

The recovery of \(^{15}\)N after the \(^{15}\)NO\(_3\) dose revealed that approximately 60% (2.0 mmol) of the \(^{15}\)NO\(_3\) appears as unmetabolized nitrate in urine within 48 hr after the dose. Less than 0.1% (0.001 mmol) of the nitrate dose appears in the feces as \(^{15}\)NO\(_3\). Three % (0.11 mmol) of the administered \(^{15}\)NO\(_3\) ap-

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was not recovered as either excreted nitrate, ammonia, or urea. Therefore, approximately 35% of the 15N of Kjeldahl-digested feces was considerably less (only 0.2% of the administered dose). Therefore, approximately 35% of the 15N was not recovered as either excreted nitrate, ammonia, or urea.

**DISCUSSION**

Nitrate balance studies as presented here and those conducted previously (7, 8, 15, 19, 26, 34) show net urinary nitrate excretion which cannot be attributed to dietary intake. During periods of low dietary nitrate intake (0.15 mmol/day), urinary nitrate excretion in excess of dietary intake is approximately 0.6 mmol/day. We have suggested that endogenous nitrate biosynthesis in man must account for this excess urinary nitrate excretion (7). When a high intake (3.5 mmol) of nitrate, as 15NO3-, was presented to subjects, this endogenous production of nitrate still occurred; excretion of 14N(V) NO3- averaged 1 mmol/day. When the diet was supplemented with 2 g of ascorbic acid or sodium ascorbate, there was no significant effect on endogenous production of nitrate. Furthermore, while ascorbic acid effectively competes with nitrogen-containing compounds for nitrosating species derived from nitrate metabolism (18), it appears to have no influence on nitrate excretion. Following the administration of 3.5 mmol of 15NO3-, the amount of nitrate appearing in the urine, plasma, or saliva was not different in supplemented and nonsupplemented ascorbic acid diet periods.

Fasting salivary nitrite levels averaged 0.09 mw, as has been reported previously for individuals consuming a low-nitrate diet (25, 29). A wide range of salivary nitrate and nitrite concentrations among the individuals was found following the nitrate dose. The variability found in this study may be associated with individual differences in salivary flow rates, oral cavity flora (25), and the capacity of the active transport mechanism for enriching saliva with nitrate (3). The ratio of nitrite to nitrate in saliva tended to average about 0.33, which is slightly higher than the value of 0.25 reported by Spiegelhalder et al. (25). This ratio appears lower at earlier times following the dose (high salivary nitrate levels) and is high at low salivary nitrate concentrations. A possible explanation is that the nitrate reductases of the oral bacteria are approaching saturation at the peak salivary nitrate concentrations achieved following the dose (2.0 to 3.0 mw).

The mean recovery of 15NO3- in urine was 60% of the administered dose. Previously reported recovery of nitrate in urine in human studies is between 50 and 90% (5, 7, 12, 22). The excretion of nitrate in the feces was negligible (less than 0.1%), as was similarly reported by Saul et al. (23). Excretion of nitrate in the feces has also been reported to be low in the rat following administration of nitrate (8, 31). It was found that nitrate undergoes reduction to reduced nitrogen, with 3% of administered nitrate appearing as either ammonia or urea in urine and 0.2% in feces. This is much less than the value of 16% obtained from studies with rats (8). About 35% of the 15NO3- dose cannot be recovered as excreted nitrogen-containing compounds in the urine or feces. This nitrate may undergo metabolism to gaseous products which are exhaled in the breath or appear in flatus.

A one-compartment pharmacokinetic model is a useful tool in analyzing the plasma and urinary data obtained in this study. Nitrate entry into the body can occur by 2 routes, namely, dietary intake and endogenous synthesis. Nitrate is removed by urinary excretion and reaction to reduced forms of nitrogen. When the nitrate inputs to the body are taken to be constant and the removal processes are assumed to be first order in nitrate concentration, a one-compartment pharmacokinetic model leads to the following equation to describe the plasma nitrate concentrations:

\[ V_o \frac{dC}{dt} = R - k_T V_o C \]

where \( V_o \) is the volume of distribution of the body, \( C \) is the plasma nitrate concentration, \( R \) is the net rate of input (primarily endogenous synthesis), and \( k_T \) is the total elimination constant (units of inverse time). The solution to this equation is:
From the data in Table 2, it appears that the steady-state concentration of nitrate in plasma has a mean value of about 0.03 mm. This value was subtracted from the mean plasma concentration following ingestion of the nitrate dose and plotted versus time on semilog coordinates. It was found that the removal of nitrate from the body was, indeed, primarily first order in plasma nitrate concentration (data not shown). $k_T$ was found to be 0.14 per hr, corresponding to a half-life for nitrate in the body of 5 hr. $C_0$ was determined by extrapolating the semilog plot to time 0 and was found to be 0.135 mm, indicating a volume of distribution for nitrate of 21.1 liters ($V_D = dose/C_0$). Since the mean weight of all 12 subjects was 71.4 kg, the nitrate space in humans is thus about 30% of body weight. The data of Ellen et al. (5), who administered a dose of up to 130 mmol of nitrate, also shows an exponential decay in plasma nitrate concentration following ingestion and suggests a similar volume of distribution of about 30% of body weight.

The total clearance of nitrate from the body can be estimated by multiplying $k_T$ and $V_D$, which yields 2.9 liters/hr for the subjects of this study. Urinary clearance was calculated by dividing the average rate of urinary excretion by the log mean plasma nitrate concentration for each urine collection period, which yielded a mean value of 1.6 liters/hr. This ratio of renal to total clearance (1.6/2.9), determined from the data for total nitrate ($^{14+15}$N), provides an independent prediction of the fraction of the nitrate presented to the body which will appear unmetabolized in urine. The urinary clearance was calculated to be 55% of total clearance, which is in good agreement with the recovery of 60% of the administered $^{15}$NO$_3^-$ in urine as nitrate.

The daily nitrate excretion in the urine was found to be in the range of 0.50 to 0.90 mmol/day. However, this only represents 60% of the total daily exposure to nitrate. Since the mean consumption of dietary nitrate was 0.15 mmol/day in this study, the amount of endogenous nitrate biosynthesis in humans can be estimated to be 0.60 to 1.4 mmol/day.

A one-compartment model is inadequate for addressing questions concerning such matters as the generation of nitrite in the oral cavity and the fate of nitrite presented to the stomach. However, since the nitrate concentration of the blood is a major factor in determining the nitrate and nitrite levels of the rest of the body, the simple analysis presented here is an important step toward developing models which will yield insight into these issues.

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