Nitrate biosynthesis in the rat

Precursor–product relationships with respect to ammonia

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The endogenous biosynthesis of nitrate in rats was investigated by using $^{15}\text{NH}_3$ administered as a continuous intravenous infusion for as long as 96 h. A comparison of the enrichment of ^{15}N in urinary nitrate after a 24 h infusion revealed that it was 36% of the enrichment of plasma NH_3 and about 50% of the enrichment of plasma urea and urinary NH_3 . Continuous infusion of $^{15}\text{NH}_3$ for 96 h showed that a plateau for the incorporation of NH_3 into nitrate is reached by 24 h, whereas the enrichment of urinary NH_3 and urea increase during the 96 h. After the infusion of progressively larger doses of $^{15}\text{NH}_3$, the concentration of nitrate synthesized *de novo* increased. Although there was a significant correlation between plasma $^{15}\text{NH}_3$ concentration and $^{15}\text{NO}_3^-$ appearance, a given change in plasma NH_3 concentration does not produce a direct proportional change in nitrate synthesis. Our findings indicate that NH_3 is a quantitatively significant nitrogen precursor for nitrate, but that approx. 50% of nitrate nitrogen is derived from other, as yet unidentified, sources.

INTRODUCTION

Several epidemiological studies have demonstrated an association between exposure to nitrate and the incidence of stomach cancer (Cuello *et al.*, 1976; Zaldivar, 1977; Juhasz *et al.*, 1978; Hartman, 1983). The conversion of ingested nitrate into nitrite by the endogenous microflora in the body, and the subsequent reaction of nitrite in the acidic stomach with nitrogen compounds, can lead to the formation of carcinogenic *N*-nitroso compounds. Evidence for the formation of *N*-nitroso compounds has been shown in human volunteers after administration of an oral dose of nitrate and proline, resulting in the endogenous synthesis of *N*-nitrosoproline (Ohshima & Bartsch, 1981; Wagner *et al.*, 1982).

Previously the total body burden of nitrate was thought to be purely a function of dietary intake and environmental exposure (Hawthornthwaite *et al.*, 1975). However, recent studies (Green *et al.*, 1981a) have confirmed previous reports (Mitchell *et al.*, 1916) that an additional and significant source presented to the body is via endogenous nitrate biosynthesis. This appears to be a property of mammalian tissues rather than the intestinal microflora, since nitrate synthesis occurs in germ-free animals (Green *et al.*, 1981b; Witter *et al.*, 1981).

Our initial investigations of nitrate biosynthesis in rats revealed that an oral dose of [^{15}N]ammonium acetate could be incorporated into nitrate nitrogen (Wagner *et al.*, 1983a). This has been confirmed in similar experiments by Saul & Archer (1984). Furthermore, the oxidation of NH_3 to nitrate has been reported to occur in rat liver *in vitro* (Dull & Hotchkiss, 1984). These findings suggest that a pathway exists for the formation of nitrate from NH_3 and that NH_3 is either a direct

precursor or is in rapid equilibrium with a precursor, which ultimately is converted into nitrate. Furthermore, nitrate synthesis could be enhanced more than 10-fold by inducing inflammation in rats (Wagner *et al.*, 1983a). There was a proportionate increase in the incorporation of $^{15}\text{NH}_3$ into nitrate during inflammation.

It is important to determine the source of nitrogen for nitrate synthesis and the metabolic factors that may affect the rate of conversion of nitrogen precursors into nitrate. Therefore we have investigated the biosynthesis of nitrate in normal rats by using $^{15}\text{NH}_3$ administered as a continuous intravenous infusion for as long as 96 h. Our findings indicate that NH_3 serves as a quantitatively significant source of nitrogen for nitrate biosynthesis and also that approx. 50% of nitrate is derived from other, as yet unidentified, sources.

MATERIALS AND METHODS

Male Sprague–Dawley rats, weighing 300–400 g, were housed throughout the experiment in stainless-steel metabolic cages in a light- and temperature-controlled room. The method of isotope administration was by continuous infusion via the external jugular vein as previously described (Moldawer *et al.*, 1980). A catheter was prepared with silastic tubing (0.6 mm int. diam.; Corning Glass Works, Corning, NY, U.S.A.), which is inserted through a 300 cm-long spring tether and connected to a flow-through swivel (Instech Laboratories, Baltimore, MD, U.S.A.). The swivel-tethering system permits normal ambulatory movement with the continuous infusion of solution. During the 3-day recovery period the catheter was kept open by administration of 0.9% NaCl twice a day. The animals were fed a low-nitrate

Abbreviation used: NAP, *N*-acetyl *n*-propyl ester.

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diet (Wagner *et al.*, 1983a) and tap water *ad libitum*. Only those animals that gained weight after this period and appeared healthy were used in the following experiments.

Animals received a constant infusion of [^{15}N]ammonium acetate (99 atom% ^{15}N ; Stohler Isotopes, Waltham, MA, U.S.A.) by using a Harvard infusion pump (Harvard Apparatus, Natick, MA, U.S.A.). A 60 ml syringe was used in the pump to deliver enough heavy-isotope solution for 24 h. Approx. 50 ml of solution was infused in 24 h, a rate of 2.1 ml/h. Three doses of NH_3 were infused into the rats for a 24 h period: 10–12 mmol, 20–25 mmol and 40–50 mmol/kg body wt.; four animals were evaluated at each dose level. One additional experiment involved infusion of the low dose of $^{15}\text{NH}_3$ into two rats for 96 h.

Urine was collected for 24 h before the infusion and during the last 6 h of the infusion period. Urine from the infusion period was analysed for [^{15}N]nitrate, $^{15}\text{NH}_3$ and [^{15}N]urea. Blood was collected after 24 h of infusion from the abdominal aorta under sodium pentobarbital anaesthesia while the NH_3 infusion was continued. Blood was analysed for $^{15}\text{NH}_3$ and [^{15}N]urea as well as for total NH_3 levels.

The blood samples were collected with a 12 ml plastic syringe and 19-gauge needle and placed in heparin-coated Vacutainer tubes, which were found to be free of NH_3 . Samples were immediately centrifuged at 4 °C for 25 min at 1000 g to separate the plasma. From this point on, the plasma samples were never placed in any glass container; only plastic test tubes and transfer pipettes were used in order to avoid NH_3 contamination. Two assays were performed simultaneously on the plasma samples: total plasma NH_3 and preparation and extraction of the $^{15}\text{NH}_3$. The NH_3 was extracted from the plasma for both assays in less than 1 h after removal from the animal. The speed of sample preparation was important in order to minimize glutamine breakdown to NH_3 , which would 'artefactually' change the NH_3 concentration.

The determination of the $^{15}\text{NH}_3$ enrichment in blood and urine is based on the procedure of Nissim *et al.* (1981). This procedure converts $^{15}\text{NH}_3$ into [^{15}N]glutamic acid, and the isotopic abundance of the glutamate NAP derivative is measured. A 1 ml portion of plasma or urine was transferred to an Eppendorf micro test tube containing 0.5 ml of Dowex 1 anion-exchange resin (X8; 100–200 mesh; OH^- form). The tube was vortex-mixed for 20 s and centrifuged immediately thereafter for 1 min. In this step glutamate and glutamine, but not NH_3 , bind to the resin. The supernatant was removed from the micro test tube and transferred to a 7 ml plastic test tube containing 1 ml of cold 0.2 M-Tris/HCl buffer, pH 8. Then, successively, were added 100 μl of 100 mM- α -oxoglutarate, 100 μl of freshly prepared 10 mM-NADH solution in 50 mM-phosphate buffer, at pH 8, and 10 μl of glutamate dehydrogenase [1450 units/ml in 50% (v/v) glycerol; Boehringer-Mannheim Biochemicals, Indianapolis, IN, U.S.A.]. The samples were incubated at 37 °C for 15 min and then 1 ml of Dowex 1 resin was added to stop the enzymic reaction and to separate the glutamate formed. The resin was washed once with 3 ml of water and then the glutamate was eluted by addition of 1 ml of 1 M-HCl. The sample was evaporated to dryness under N_2 at 100 °C and then the NAP derivative was prepared (Matthews *et al.*, 1979). The enrichment of the glutamate NAP derivative was analysed with the aid of a Hewlett-Packard 5995 g.l.c./m.s. system with an OV-1

Fig. 1. ^{15}N enrichment of nitrate, NH_3 and urea in blood and urine after 24 h of continuous infusion of $^{15}\text{NH}_3$.

Four rats per group were infused at doses of 10–12, 20–25 and 40–50 mmol/kg body wt. Data are presented as regression lines $\pm$ S.D., the ^{15}N enrichment being regressed to the size of the NH_3 dose.

capillary column (Hewlett-Packard, Palo Alto, CA, U.S.A.). The initial oven temperature was 120 °C and oven programming started to increase the temperature immediately after injection at a rate of 16 °C/min. Selected ion monitoring of ion masses $m/z = 274$ and 275 in the chemical-ionization mode using methane as the reagent gas was used to obtain the $^{15}\text{N}/^{14}\text{N}$ ratio for each sample.

The ^{15}N enrichment of plasma and urinary urea was quantified by the method of Matthews & Downey (1984). A 100 μl sample of protein-free [5% (w/v)-sulphosalicylic acid precipitated] plasma or urine was passed through a Dowex resin column [1 ml of Dowex 50W (X8; 100–200 mesh)]. The column was rinsed with 4 ml of 1 M-acetic acid, followed by 1 ml of water, and the eluate was discarded. Urea was eluted from the resin by the addition of 1.8 ml of 3 M- NH_3 . The NH_3 is evaporated under a stream of N_2 at 100 °C. The bis(trimethylsilyl) derivative of urea is prepared with the addition of 50 μl of NO-bis(trimethylsilyl)trifluoroacetamide (Supelco, Bellefonte, PA, U.S.A.) and 50 μl of acetonitrile. This derivative was chromatographed isothermally at 110 °C on a capillary OV-1 column. The methyl-loss fragment [$M - 15$, $m/z = 189$], as well as $m/z = 190$ and $m/z = 191$, were monitored by electron ionization to obtain the $^{15}\text{N}/^{14}\text{N}$ ratio and $^{15}\text{N}_2/^{14}\text{N}$ ratio.

The plasma NH_3 concentration was measured by a kit obtained from Hyland Diagnostics (Deerfield, IL, U.S.A.). The principle of this assay is the selective adsorption of NH_3 from plasma on to a strongly acidic cation-exchange resin. After elution of the NH_3 from the resin by 4 M-NaCl, the NH_3 is allowed to react with phenol and hypochlorite in the presence of sodium nitroferrocyanide. A stable dark-blue indophenol complex is formed, and when measured spectrophotometrically at 630 nm, is directly proportional to the concentration of NH_3 in plasma. The assay was very reproducible, with a 3.5% within-run, as well as day-to-day, coefficient of variance. Recovery of 'spiked' plasma samples was

Fig. 2. Relationship between plasma $^{15}\text{NH}_3$ concentration and urinary $[^{15}\text{N}]$ nitrate concentration after the three different doses of NH_3 infused

greater than 95%. Total urinary nitrate and $[^{15}\text{N}]$ nitrate were measured by methods previously described (Green *et al.*, 1982).

RESULTS

The ^{15}N enrichment of nitrate, NH_3 and urea in blood and urine after continuous infusion of ^{15}N -labelled NH_3 for 24 h is shown in Fig. 1. The data are presented as regression lines, the ^{15}N enrichment being regressed to the size of the NH_3 dose. It can be seen that the ^{15}N enrichment of urinary nitrate was significantly less than that of plasma NH_3 . The slope of the regression line for urinary $[^{15}\text{N}]$ nitrate was 0.19, whereas for plasma $^{15}\text{NH}_3$ the slope was 0.53. Thus the enrichment of nitrate was only 36% of the enrichment found in plasma NH_3 . The ^{15}N enrichment of urinary NH_3 , plasma urea and urinary urea were also significantly higher than the enrichment in nitrate; the slopes of the regression lines were 0.46, 0.43 and 0.34 respectively. In comparison, the ^{15}N enrichment of nitrate was approx. 50% of the enrichment found in these other nitrogen products whose precursor is blood NH_3 or nitrogen compounds in equilibrium with blood NH_3 . The regression line of plasma NH_3 was significantly different ($P < 0.05$) from the regression lines of all the other nitrogen products measured. There was no significant ($P < 0.1$) difference between the regression lines of urinary NH_3 , plasma urea and urinary urea.

The relationship between plasma $^{15}\text{NH}_3$ concentration and urinary $[^{15}\text{N}]$ nitrate concentration, after the three different doses of NH_3 infused, is presented in Fig. 2. The plasma $^{15}\text{NH}_3$ concentration is derived from the total plasma NH_3 concentration multiplied by the ^{15}N atom% excess. In a similar manner the urinary $[^{15}\text{N}]$ nitrate concentration can be derived. As the concentration of $^{15}\text{NH}_3$ in blood increased, due to larger amounts of NH_3 infused, the concentration of nitrate synthesized *de novo* was increased. Linear-regression analysis showed a significant ($P < 0.05$) correlation between the plasma $^{15}\text{NH}_3$ concentration and the appearance of $[^{15}\text{N}]$ nitrate in urine; the correlation coefficient was 0.89. However, the slope of the regression line was 0.10, which suggests

Fig. 3. ^{15}N enrichment $\pm$ S.D. of urinary nitrate, urinary NH_3 and urinary urea for two rats infused with $^{15}\text{NH}_3$ for 96 h

that a change in plasma NH_3 concentration does not produce a direct proportional change in nitrate synthesis.

Results for the ^{15}N enrichment of urinary nitrate, urinary NH_3 and urinary urea for two rats infused with $^{15}\text{NH}_3$ for 96 h are shown in Fig. 3. The enrichment of urinary nitrate had reached a plateau by 18–24 h after the start of the $^{15}\text{NH}_3$ infusion. Urine sampled at 6–8 h had a lower ^{15}N enrichment than had the 18–24 h sample. No urine was sampled between 8 and 18 h of the infusion, but it can be inferred that a plateau is reached between these time points, since there is no significant increase in the ^{15}N enrichment of nitrate after 24 h. Thus there is a fairly rapid equilibrium of NH_3 and the immediate nitrogen precursor of nitrate. The data presented in Fig. 3 show that the ^{15}N labelling of the precursors of urinary NH_3 and urea had not reached a plateau, since the ^{15}N enrichment of both N products continue to increase with time. Furthermore, throughout the 96 h infusion period the enrichment of urinary NH_3 and urea is significantly greater than that of urinary nitrate.

Since urea may have both nitrogen moieties labelled, the enrichment of di-labelled urea was measured. The enrichment of both single- and di-labelled urinary urea continued to increase with time. Therefore, although an 'apparent' steady-state level of labelling of nitrate is reached 24 h after $^{15}\text{NH}_3$ infusion, this was not true of other nitrogen compounds, specifically urinary NH_3 and urea, whose precursors equilibrate with plasma NH_3 at slower rates.

DISCUSSION

The ^{15}N isotope of nitrogen was used as a tool for the study of the biological conversion of NH_3 into nitrate. The results show conclusively that a pathway exists for the transfer of NH_3 nitrogen to nitrate. Infusing $218 \mu\text{mol}$ of $^{15}\text{NH}_3/\text{h}$ intravenously into a rat resulted in the appearance of 9.4 nmol of $[^{15}\text{N}]$ nitrate/h in urine. Assuming that urinary nitrate represents only 60% of total nitrate produced (because of metabolism; Wagner *et al.*, 1983b), the percentage conversion of NH_3 into nitrate was 0.007%. This is identical with results obtained with $^{15}\text{NH}_3$ given as an oral bolus (Wagner *et al.*, 1983a; Saul & Archer, 1984).

Although the transfer of NH_3 nitrogen to nitrate has been demonstrated, it was not known how much nitrate nitrogen was derived directly (or indirectly from NH_3). Thus a continuous intravenous infusion of $^{15}\text{NH}_3$ was used to produce a constant ^{15}N enrichment in the nitrate precursor pool. Our initial hypothesis was that the nitrogen precursor for nitrate was derived from free NH_3 in blood. By sampling the plasma compartment, after achieving a constant plateau of $^{15}\text{NH}_3$ in blood, it was observed that the ^{15}N enrichment in nitrate was much lower than the enrichment in blood NH_3 . Possible reasons for the difference in the ^{15}N enrichment between nitrate and blood NH_3 are as follows: (1) a 24 h infusion was not long enough to allow full equilibration of NH_3 and the immediate nitrate precursor; (2) nitrate synthesis is an intracellular process and the intracellular enrichment of $^{15}\text{NH}_3$ is lower than that in plasma; (3) another nitrogen compound can provide nitrogen for nitrate synthesis, and this nitrogen compound does not equilibrate with the NH_3 pool, so that it dilutes the ^{15}N label of the nitrate pool.

For these reasons, the continuous infusion of $^{15}\text{NH}_3$ was extended for 96 h. It was clear that the ^{15}N enrichment in nitrate had reached a plateau by 24 h, since the enrichment remained constant from 24 to 96 h of infusion of NH_3 . Furthermore, nitrate reached isotopic equilibrium fairly rapidly, between 8 and 18 h, as inferred from the data. Thus it can be concluded that our protocol does achieve a maximum enrichment in nitrate within 24 h. Although the possibility exists of slow-equilibrating pools of NH_3 in the body, it would seem unlikely that they could contribute nitrogen to nitrate. If certain pools of NH_3 had not reached isotopic equilibrium by 24 h, it would be expected that the enrichment would have increased through the next 72 h of infusion. Such was the case with urinary NH_3 and urea. Waterlow *et al.* (1978) suggest that, owing to recycling of label, there is in fact a slow return of tracer to the sampled pool, so that the enrichment in the sampled pool will gradually rise until it is the same in all pools in the system. Thus the ^{15}N from NH_3 that is initially incorporated into protein via amino acid synthesis does not represent an irreversible disposal of label, but eventually appears in end products of nitrogen metabolism. The fact that the enrichment of nitrate did not change after 24 h of NH_3 infusion suggests that slower-equilibrating pools of NH_3 nitrogen do not contribute significantly to nitrate synthesis.

It is well known that, when a labelled amino acid is given by constant infusion, the plateau specific radioactivity in plasma is not the same as that in the free amino acid pools within tissues. For example, when $[^{14}\text{C}]$ lysine was infused into rats, the relative values of the plateau specific radioactivities in plasma, liver and muscle of free lysine were about 10:5:8 respectively (Waterlow & Stephen, 1968). Hence, we attempted to obtain an index of the intracellular enrichment of NH_3 in selected tissues by measuring the enrichment of urea and urinary NH_3 . The enrichment of urea is a good measure of the intracellular enrichment of NH_3 in the liver (Duda & Handler, 1958). However, the enrichment of nitrate was approx. 50% of the enrichment of urea. This result would suggest that the intracellular $^{15}\text{NH}_3$ enrichment in the liver was greater than the enrichment in nitrate. Therefore, even if nitrate synthesis is an intracellular process, our results reveal that not all of the nitrogen for nitrate synthesis is derived from NH_3 .

The continuous infusion of NH_3 for 96 h revealed that the urinary urea enrichment never achieved an isotopic steady state. The two nitrogen atoms of urea are derived from different sources. One of the nitrogen atoms comes from NH_3 , whereas the other is derived from aspartate. The probability of finding di-labelled urea is equal to the probability (i.e. enrichment) of labelled NH_3 entering the urea cycle times the probability (enrichment) of labelled aspartate entering the cycle. Since the enrichment of nitrate was less than the enrichment of di-labelled urea, we can conclude that not only is the enrichment of liver intracellular NH_3 higher, but also the enrichment of aspartate must be significantly greater than that found in nitrate. If these pools contributed nitrogen to nitrate, the enrichment of nitrate should have increased with time, as was found with urinary NH_3 and urea. This was not observed, suggesting that these nitrogen sources are not major precursors of nitrate.

Furthermore, the enrichment of nitrate was also less than that of urinary NH_3 ; the enrichment in nitrate was 44% of that in urinary NH_3 . The precursor of urinary NH_3 is glutamine (Lund, 1980). In effect, urinary NH_3 enrichment is a reflection of the enrichment of body glutamine (Golden, 1981). Glutamine appears to be a major transporter of body nitrogen, and glutamine synthesis appears to be the most significant means of maintaining the normally low intracellular concentration of NH_3 (Lund, 1980). On the basis of these observations the enrichment of urinary NH_3 that was achieved in these experiments should provide some index of the intracellular NH_3 enrichment, particularly in muscle. Muscle converts approx. 50% of arterial NH_3 into glutamine (Freed & Gelbard, 1982). Therefore, since the enrichment of nitrate was lower than the enrichment of urinary NH_3 , this further supports the view that another source of nitrogen, which is not in equilibrium with NH_3 , is diluting the nitrate precursor pool or nitrate pool.

By contrast, all of the experimental data are consistent with the hypothesis that there is a source of nitrogen for nitrate synthesis other than NH_3 . This nitrogen compound does not have to come from the diet, since nitrate synthesis occurs when rats receive only a 5% glucose solution (Green, 1981). In theory, nitrogen for nitrate synthesis could be provided by amino acids, purines and pyrimidines, creatine and other nitrogenous constituents of muscle, and other body nitrogen compounds, but the extent to which these potential precursors are important remains to be determined.

The flux of NH_3 can be calculated from the equation:

$$\text{Flux} = \frac{\text{rate of } ^{15}\text{NH}_3 \text{ infusion}}{\text{plateau } ^{15}\text{N enrichment of plasma } \text{NH}_3}$$

(Waterlow *et al.*, 1978). The mean flux in the present experiments was 6.0 mmol/h per kg body wt., and this was independent of the amount of NH_3 infused. Since flux is the sum of all processes that remove the metabolite from a pool, the nitrate flux is the sum of nitrate excretion and metabolism. Because the daily nitrate excretion is known, and the amount of metabolism is known from previous data, the nitrate flux was calculated to be 0.51 $\mu\text{mol/h}$ per kg body wt. Thus the nitrate flux is 0.008% of the NH_3 flux. This is in close agreement with the measured conversion of administered $^{15}\text{NH}_3$ into nitrate, namely 0.007%.

The ratio of the ^{15}N enrichment of urinary nitrate to

that of plasma NH_3 suggests that 36% of nitrate is derived from blood NH_3 , or about $0.18 \mu\text{mol/h}$ per kg body wt. The remaining $0.33 \mu\text{mol}$ of nitrogen/h per kg body wt. into the nitrate pool must be coming from an unknown nitrogen precursor. If nitrate synthesis is an intracellular process and the ^{15}N enrichment of intracellular NH_3 is 70% of that in plasma, then 51% of nitrate nitrogen may be derived from NH_3 , or $0.27 \mu\text{mol/h}$ per kg body wt.

Finally, nitrate synthesis was found to increase with the infusion of progressively larger amounts of NH_3 . However, the rate of increase of nitrate synthesis was not directly proportional to the size of the NH_3 dose. Thus, under normal physiological conditions, it appears that the regulation of nitrate synthesis does not occur through changes in plasma NH_3 concentration. On this basis, the regulation of nitrate biosynthesis may be controlled by the availability of the oxidizing species, which we have suggested elsewhere might be generated largely via the reticuloendothelial system (Wagner *et al.*, 1983a).

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