Short-term Metabolic Fate of [13N]Ammonia in Rat Liver in Vivo*

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The short-term metabolic fate of [13N]ammonia in the livers of adult male, anesthetized rats was determined. Following a bolus injection of tracer quantities of [13N]ammonia into the portal vein, the single pass extraction was ~93%, in good agreement with the portal-hepatic vein difference of ~90%. High performance liquid chromatographic analysis of deproteinized liver samples indicated that labeled nitrogen is exchanged rapidly among components of: (a) mitochondrial aspartate aminotransferase and glutamate dehydrogenase reactions and (b) cytoplasmic aspartate aminotransferase and alanine aminotransferase reactions (tα, for the exchange of label toward equilibrium is on the order of seconds). Comparison of specific activities of glutamate and ammonia suggests that at 5 s most labeled glutamate was mitochondrial, whereas at 60 s ~93% was cytosolic; this change is presumably brought about by the combined action of the mitochondrial and cytosolic aspartate aminotransferases and the aspartate carrier of the malate-aspartate shuttle. Specific activity measurements of glutamate, alanine, and aspartate are in accord with the proposal by Williamson et al. (Williamson, D. H., Lopes-Vieira, O., and Walker, B. (1967) Biochem. J. 104, 497-502) that the components of the aspartate aminotransferase reaction are in thermodynamic equilibrium, whereas the components of the alanine aminotransferase reaction are in equilibrium but compartmented in the rat liver. Despite considerable label in citrulline at early time points, no radioactivity (~0.25% of the total) was detected in carbamyl phosphate, suggesting very efficient conversion to citrulline with little free carbamyl phosphate accumulating in the mitochondria. Our data also show that some portal vein-derived ammonia is metabolized to glutamine in the rat liver, but the amount is small (~7% of that metabolized to urea) in part because liver glutamine synthetase is located in a small population of perivenous cells "downstream" from the urea cycle-containing perportal cells. Finally, no tracer evidence could be found for the participation of the purine nucleotide cycle in ammonia production from aspartate. The present work continues to emphasize the usefulness of [13N]ammonia for short-term metabolic studies under truly tracer conditions, particularly when turnover times are on the order of seconds.

Nitrogen-15 has been the major tracer for studying nitrogen metabolism since the pioneering work of Schoenheimer and colleagues in the late 1930's (see Ref. 1 for a review). However, the use of 15N as a tracer may present some problems (1). It has a natural abundance of ~0.4%, so that unphysiologically high 15N precursors are often used in biological experiments in order to obtain a measurable enrichment in product. Furthermore, 15N is nonradioactive and requires elaborate mass spectral analysis (e.g., Ref. 2). In the present work, we show that 13N is an ideal isotope for the study of short-term nitrogen metabolism. Because 15N does not occur naturally and the specific activity of the 15N-labeled precursor can be very high, truly tracer studies are possible under conditions in which such studies with 15N are virtually impossible. In addition, analysis by HPLC is relatively simple and accurate.

We showed previously that in rat brain, the tα for conversion of blood-borne [15N]ammonia to L-[amide-15N]glutamine is rapid (tα < 3 s) (3). We have now extended these studies to show that the metabolism of portal vein-derived [13N]ammonia in rat liver in vivo to largely irreversibly labeled products (i.e., urea plus glutamine) is also rapid (tα < 10-11 s). (Irreversible in the present context implies little redistribution of this label over the time periods employed (~60 s) in the current experiments.) The present work with [13N]ammonia shows that in rat liver: (a) label is exchanged rapidly among components of the glutamate dehydrogenase, aspartate aminotransferase, and alanine aminotransferase reactions; (b) only a small fraction of portal blood-derived ammonia is metabolized to glutamine; and (c) little carbamyl phosphate is present in the mitochondria. It is important to emphasize point a. It is well-known that the components of the glutamate dehydrogenase and aspartate aminotransferase reactions are at thermodynamic equilibrium in rat liver (4, 5). However, it has not been appreciated just how rapidly nitrogen is exchanged among the components of these reactions (within seconds) in vivo. Conclusions drawn from long-term studies with 15N-labeled ammonia or amino acids must take the present findings into account.

MATERIALS AND METHODS

Biochemicals—L-Amino acids, carbamyl phosphate (dilithium salt), L-amino-acid oxidase purified from Corynebacterium adamansae venom (aqueous solution, 8 units/mg), bovine liver catalase (aqueous suspension, 36,000 units/mg), Bacillus subtilis alanine dehydrogenase (suspension in 2.4 M ammonium sulfate, 30 units/mg), bovine liver argininosuccinate lyase (0.5 unit/mg), and Escherichia coli glutaminase.

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1The abbreviation used is: HPLC, high performance liquid chromatography.

2Except where specified, ammonia refers to the sum of ammonium ions (NH4+ and ammonia free base (NH3). At physiological temperatures and pH, approximately 1% of ammonia will be in the form of the free base (NH3).
nase A (25 units/mg) were obtained from Sigma. Beef liver glutamate dehydrogenase (solution in glycérol, 25 units/mg) was purchased from Boehringer Mannheim. Cyanogen bromide-activated Sepharose 4B was obtained from Pharmacia P-L Biochemicals. E. coli carbamylphosphate synthetase (~200 units/mg) (5) was kindly donated by Dr. D. B. Kappus (Department of Biochemistry, Cornell University Medical College).

[15N]Ammonia was produced by reducing [15NO]/[15NO] (produced in a CS-15 cyclotron by the [15O(p, 15)N] reaction) with Devarda’s alloy and sodium hydroxide, bubbled into 3 ml of 50 mM potassium phosphate buffer, pH 7.2–7.4 (7), and made isotonic with sodium chloride. Yields of radioactivity at the end of collection were typically 200–400 mCi. The final concentration of ammonia in this buffer was 50–100 μM.

Experimental Animals—Adult male Wistar rats weighing 250–450 g were fed ad libitum up to the time of the experiment. The rats were anesthetized with ketamine-HCl-ether, and an incision was made through the ventral body wall (laparotomy). The portal vein was exposed and catherized, and a bolus (0.2 ml) of [15N]ammonia was quickly (~2 s) injected. From 5 to 60 s after beginning the injection, a small piece of liver was freeze-clamped and deproteinized while still frozen in a glass-to-glass homogenizer containing a ~3-fold excess of ice-cold 1% (w/v) picric acid. The mixture was centrifuged for 30 s in a microcentrifuge, and the supernatant was filtered by means of a syringe attached to a Millipore GS (0.22 μm) membrane. An aliquot (20 μl) of the clear supernatant was analyzed by HPLC (see below).

In preliminary studies utilizing identified small amounts of blood (100–200 μl) from the portal and hepatic veins by means of a 30-gauge catheter, and specimens of liver (~100 mg) were freeze-clamped for later metabolic analysis; the blood was quickly transferred to snap-top plastic microcentrifuge tubes and frozen in liquid nitrogen, also for later metabolism analysis (see below). In one experiment, the hepatic vein was clamped and severed and then quickly reopened at the moment of the [15N]ammonia bolus injection; all the blood emanating from the severed and then quickly reopened hepatic veins by means of the catheter was collected and treated with 50 pl of 1 M potassium phosphate buffer (pH 8.0), 900 units of celulase suspension, and 0.5 mg of tL-amino-acid oxidase. After incubation at 37 °C for 15 min, the solution was added to a Dowex 50-Tris+ column (50 cm) and eluted with 3 volumes of water. α-Keto-γ-aminobutyric acid was eluted in the wash and represents label originally present in the carbamido group of citrulline. [15N]Ammonia (generated from L-amino-γ-[15N]amino acids) and unreacted L-[15N]-aminomino acids are retained on the column. In this experiment, 80% of the radioactivity was eluted in the water wash verifying that radioactivity in peak 6 is due to citrulline. In another experiment, peak 5 was analyzed for L-[15N]alanine. Alanine dehydrogenase (i.e. pellet obtained by centrifuging 100 μl of an ammonium sulfate suspension, ~750 μg, 1.54 units), 50 μl of 1 M ammiodol, 50 μl of 1 M semicarbazide HCl (pH 8.5), and 0.5 mg of semi-carbazide lyase were added to this homogenate. After incubation at 37 °C for 10 min, the mixture was added to a Dowex 50–Trais+ column (2.5 × 0.5 cm) and eluted with 3 column volumes of water. The radioactivity remaining on the column was due to ammonia nitrogen originally present in alanine. In this experiment, 80% of the radioactivity in peak 5 was converted to [15N]ammonia, which we considered as definitive proof that peak 5 is [15N]alanine since there is some overlap between peaks 5 and 6 (Fig. 1).

Because argininosuccinate is relatively unstable (13), we analyzed peak 8 to be certain that it represented N-labeled argininosuccinate.

In this fraction was added 100 μl of 1 M potassium phosphate buffer (pH 8.0) and solid arginine (5 mg). After incubation at 37 °C for 10 min, the mixture was added to a Dowex 50–Trais+ column (2.5 × 0.5 cm) and eluted with 3 column volumes of water. Under these conditions, only the basic amino acids are retained on the column. Therefore, the radioactivity remaining on the column must have been due to L-[15N]arginine and represented label originally present in argininosuccinate. The conversion was 73%.

Analysis of Nonradioactive Metabolites—Samples of frozen liver and blood were deproteinized with ice-cold 3 and 1.2 M perchloric acid, respectively, and neutralized essentially as described previously (3). Amino acids were measured by HPLC of the o-phthaldialdehyde derivatives (14) using α-aminoadipate as an internal standard. For the reference, we assume that label in these peptides is negligible (<1%).

α-Glutamylcysteine and glutathione coelute with aspartate and glutamate, respectively. One minute after infusion of L-[15N]-glutamate into the portal vein, ≤2% of the recovered radioactivity in the rat liver was in these two peptides (data not shown). Therefore, in the present work, we assume that label in these peptides is negligible (<1%).

Because carbamyl phosphate is somewhat unstable, particularly at pH values ≤2.0 (10), we were concerned at first that the low counts in liver may have been due to destruction of carbamyl phosphate by the nonrecruited glutaminase and carbamyl phosphate transaminase. Therefore, in the present work, we assumed that label in these peptides is negligible (<1%).

The system was described in detail at a recent symposium (Nieves, E., Rosenapple, K. C., File, S. M., and Gelbard, A. S. (1966) Fifth International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Nov. 4–6, 1985, Toronto, Canada, p. 19) (see also Ref. 8).

With the SAX column used in the original work, we were unable to separate alanine and citrulline as well as ammonia and argininosuccinate. With newer columns, such separations are now possible.
monia was measured enzymatically with glutamate dehydrogenase (3).

Picric acid (1%) has been used for many years as a deproteinizing reagent, and we have found that brief exposure of standard mixtures of the amino acids of interest (i.e. glutamine, glutamate, aspartate, alanine, citrulline, arginine) and of ammonia (0 °C, <1 min) has no effect on their recovery.

Statistical Analysis—Data are expressed as mean ± S.E. Statistical significances were established using the one-tailed Mann-Whitney U test. The ratios of specific activities of glutamate to aspartate, glutamate to alanine, glutamate to ammonia, and citruline to ammonia were determined from the general term (*X*/Y)/(X/Y), where *X*/Y is the ratio of radioactivity and X/Y is the ratio of concentration. *X*/Y and X/Y were determined in two separate groups of rats. In order to determine the standard error of the mean (a) of the ratio of specific activity (A), we assume that the errors are propagated randomly. The standard equation for this type of error propagation is

\[
a^2/A^2 = b^2/B^2 + c^2/C^2,
\]

where B and C are the means of the ratio of radioactivity (*X*/Y) and ratio of concentration (X/Y), respectively; b and c are the respective standard errors of the mean.

RESULTS

Ammonia and Selected Amino Acid Concentrations in Liver of Adult Male Rats and Portal-Hepatic Vein Difference for Ammonia across Liver

The concentrations of aspartate, glutamate, glutamine, citrulline, alanine, and ammonia in freeze-clamped rat liver are given in Table I. From paired data of individual animals, the glutamate/aspartate, glutamate/alanine, glutamine/ammonia, and citruline/ammonia concentration ratios were 1.83 ± 0.36, 1.31 ± 0.42, 1.60 ± 0.48, and 0.988 ± 0.017, respectively. The concentrations of aspartate, glutamate, and glutamine given in Table I are somewhat lower than those reported previously (15) for freeze-clamped liver from adult male rats, possibly because of differences in the age of rats used in the two studies, on the other hand, levels of alanine and ammonia were comparable in the two studies. The portal venous and hepatic venous blood ammonia concentrations were 0.353 ± 0.040 and 0.036 ± 0.017 mM (n = 4), respectively, consistent with the known ability of liver to remove a large percentage of portal blood ammonia (e.g. Ref. 16).

Uptake and Metabolism of [15N]Ammonia by Rat Liver in Vivo

Single Pass Extraction of [15N]Ammonia—Pardridge (17) has calculated that the portal blood flow in the laparotomized rat is 0.93 ml/g/min. Assuming an average liver weight of 15 g for the rats employed in the present study, the portal blood flow is predicted to be ~14 ml/min. The bolus injection of [15N]ammonia (0.2 ml containing 50–100 µM ammonia) took ~2 s to inject into the portal vein and mixed almost instantly. Therefore, steady state levels of ammonia delivered to the liver were unlikely to be affected by this treatment. The single pass extraction of [15N]ammonia by the liver was 93 ± 1% (n = 5). This value is in good agreement with the portal-hepatic vein difference of ammonia (~90%), suggesting that the passage of ammonia is almost entirely one way. The recovery of label in liver at 30 s drops to 60% (average of two determinations of 59, 61%).

Changes of Relative Specific Activities of Glutamate, Aspartate, Alanine, and Ammonia with Time—At intervals from 5 to 60 s after the bolus injection of [15N]ammonia, a sample of liver was freeze-clamped from each rat, deproteinized, and analyzed for 15N-metabolites by HPLC (Fig. 2 and Table I). Within 5 s after beginning the bolus injection, 43% of the label was in a metabolized form. The ratio of label in glutamate to that in aspartate at 5 s is 4.36 ± 0.60 (range 3.2 to 6.6, n = 5), a value that is higher (p = 0.025) than the glutamate/aspartate concentration ratio of 1.83. The apparent 2.4-fold higher specific activity of gluta-

**Table I**

Distribution of 15N among various metabolites in liver from anesthetized adult male rats 5-60 s following a bolus (0.2 ml) injection of 15N/ammonia into the portal vein

| Metabolite          | 5 s (n = 5) | 10 s | 15 s | 60 s (n = 8) | amol/g, wet wt |
|---------------------|------------|------|------|-------------|---------------|
| Urea                | 2.29 ± 0.51| 8.61 | 18.3 | 62.3 ± 5.02| NM            |
| Aspartate           | 3.32 ± 0.36| 6.66 | 3.29 | 6.67 ± 1.26| 0.63 ± 0.03  |
| Glutamate           | 14.2 ± 1.54| 21.1 | 15.8 | 18.0 ± 1.9 | 1.12 ± 0.19  |
| Glutamine           | 1.41 ± 0.35| 1.49 | 1.85 | 3.56 ± 0.82| 2.63 ± 0.11  |
| Alanine             | 0.20 ± 0.16| 2.52 | 4.86 | 4.73 ± 1.39| 0.98 ± 0.12  |
| Citrulline          | 17.3 ± 1.84| 17.8 | 11.9 | 1.18 ± 0.45| 0.073 ± 0.006|
| Ammonia             | 57.4 ± 4.09| 43.4 | 33.9 | 4.64 ± 1.09| 0.83 ± 0.13  |
| Argininosuccinate   | 1.17 ± 0.53| 3.80 | 2.58 | ND          | NM            |
| Arginine            | 1.44 ± 0.57| ND   | 0.80 | ND          | NM            |

Ratios of radioactivity

- Glu/Asp
- Glu/Ala
- Glu/NH₃
- Cit/NH₃
- Cit/Arg-succ

| Metabolite          | 5 s (n = 5) | 10 s | 15 s | 60 s (n = 8) | amol/g, wet wt |
|---------------------|------------|------|------|-------------|---------------|
| Urea                | 2.29 ± 0.51| 8.61 | 18.3 | 62.3 ± 5.02| NM            |
| Aspartate           | 3.32 ± 0.36| 6.66 | 3.29 | 6.67 ± 1.26| 0.63 ± 0.03  |
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| Citrulline          | 17.3 ± 1.84| 17.8 | 11.9 | 1.18 ± 0.45| 0.073 ± 0.006|
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| Argininosuccinate   | 1.17 ± 0.53| 3.80 | 2.58 | ND          | NM            |
| Arginine            | 1.44 ± 0.57| ND   | 0.80 | ND          | NM            |

Ratios of specific activities

- Glu/Asp
- Glu/Ala
- Glu/NH₃
- Cit/NH₃
- Cit/Arg-succ

*NM, not measured; ND, not detected.

*p = 0.05 in comparison to the 5-s data.

*p = 0.001 in comparison to the 5-s data.
the hepatic vein (7%) in this was in glutamine position. Therefore, the ratio of 

\[
\frac{\text{ARG SUC}}{\text{NH}_3}
\]

ammonia to \([^{13}\text{N}]\text{urea}\) at 60

administered dose was retained by the liver; of the label arriving in

species leaving the liver by 30

portion of the labeled glutamine at 60 s will be labeled in the

vein, portions of rat liver were freeze-clamped and analyzed for

time. In fact, by 60 s, the [\(^{13}\text{N}\)]glutamate/[\(^{13}\text{N}\)]aspartate ratio declined to 2.92 ± 0.55 (range 1.2-6.1, n = 8, p = 0.05 compared

to the 5-s data), and the ratio of specific activities declined to

1.60 ± 0.44 (Table 1). (The final ratio of specific activities may differ slightly from 1.0 because of compartmentation of

\([^{13}\text{N}]\text{ammonia uptake; see below.})

Most (≥90%) of alanine aminotransferase activity in rat liver is cytosolic (18). Therefore, at early time points, when most of the labeled glutamate is in the mitochondria, little label should be present in alanine. Five seconds after [\(^{13}\text{N}\)]ammonia administration, the liver [\(^{13}\text{N}\)]glutamate/[\(^{13}\text{N}\)]alanine ratio was 61.1 ± 18.6, with a ratio of specific activities of 46.6 ± 20.6. By 1 min, the ratios had dropped to 4.5 ± 1.3 and 3.43 ± 1.46, respectively (p = 0.001 compared to the 5-s data). The latter value for ratio of specific activities is still far from unity. Again, the data stress the rapid labeling of alanine once the glutamate in the cytosol is labeled. The data are also in accord with the suggestion of Williamson et al. (5) that the components of the alanine aminotransferase reaction are in equilibrium, but that the \([\text{pyruvate]}[\text{glutamate}] / [\text{α-ketoglutarate}] [\text{alanine}]\) ratio is displaced from the thermodynamic equilibrium constant because of compartmentation of the reaction components.

The ratio of [\(^{13}\text{N}\)]glutamate/[\(^{13}\text{N}\)]ammonia changed considerably with time from 0.16 at 5 s to 4.6 at 60 s (p = 0.001). This change in ratio could have been due to the fact that, contrary to the generally accepted role of glutamate dehydrogenase, conditions in the liver favor a largely one-way incorporation of ammonia into glutamate. Much more likely is the possibility that, by 60 s, a large portion of the total [\(^{13}\text{N}\)]glutamate is cytosolic and thus is no longer in contact with mitochondrial glutamate dehydrogenase.

**Comments on Relative Specific Activities of Ammonia and Citrulline**—The specific activity of rat liver citrulline at 5 s following a bolus injection of [\(^{13}\text{N}\)]ammonia is 3.73 ± 0.574. Conceivably, our assay underestimated citrulline. Our value of 73 μmol/kg, wet weight, is considerably less than that of 210 pmol/kg reported by Matsuzawa and Ishiguro (19), but is in agreement with that reported by Saheki et al. of 72-85 and ~70 μmol/kg for the livers of rats fed a basal diet (20) and normal laboratory rat chow (21), respectively. Another possibility for the relatively high specific activity of citrulline is that the specific activity of whole liver citrulline is greater than that in the mitochondria. However, this possibility does not seem likely as citrulline is made in the mitochondrion. Owing to the high uptake of [\(^{13}\text{N}\)]ammonia by the liver, there is likely to be a gradient of [\(^{13}\text{N}\)]ammonia entering the cells along the sinusoid. Several authors (e.g. Ref. 22) have shown that the perportal hepatocytes are enriched in the urea cycle enzymes, whereas glutamine synthetase is located in a small group of perivenous hepatocytes. It is likely that the urea cycle-containing perportal hepatocytes are exposed to more [\(^{13}\text{N}\)]ammonia than are the more distant perivenous hepatocytes. Furthermore, the relatively high capacity urea cycle may act to maintain ammonia at a lower concentration in perportal hepatocytes than in perivenous hepatocytes. These two factors combined need not be very great. All that is required is that the specific activity of the [\(^{13}\text{N}\)]ammonia in the perportal hepatocytes be twice that of the liver as a whole.

**Calculation of in Vivo Urea Production Rates**—In order to calculate urea production rates, one must make allowances for ammonia delivery to the liver by the hepatic artery. Estimates of fractional blood flow to the liver supplied by the hepatic artery are 25–30% in dog, cat, and man (Ref. 23). In the following calculation, we assume that this value (25%) also holds for the rat and that the arterial blood ammonia
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concentration is 60 \mu M (cf. Ref. 34). Therefore, maximal urea production from blood-derived ammonia is (0.93 (hepatic blood flow in ml/g/min) \times 353 (nmol of ammonia/ml of portal blood) \times 0.93 (fractional extraction of labeled ammonia)) + (0.25 (arterial blood flow) \times 60 \times 0.93) = 319 nmol/g/min. Correcting for 7\% conversion of ammonia to glutamine (see below), the production rate becomes 297 nmol/g/min. This value does not take into account ammonia produced from endogenous sources. Theoretically, one could obtain the rate of production from blood-derived ammonia is (0.93 \times 353 \times 0.93 \times 0.25 = 141 nmol/g/min). However, as noted above, the specific activity of the urea cycle-containing cells is at least 1.82 \mu mol/g/min (where 0.83 is the concentration of ammonia in \mu mol/g, wet weight, and 2.22 is the (conservative) estimate of the labeled ammonia entering the liver available for urea synthesis). However, as noted above, the specific activity of the urea cycle-containing cells is at least 1.82 \mu mol/g/min too high by a factor of 2. Obviously, urea output from the liver depends strongly on nutritional factors. All that can be concluded from the present work is that the production of urea in the liver of fully fed, anesthetized male rats is on the order of 900 nmol/g/min, and a third of the precursor ammonia may be derived from portal blood ammonia.

It is instructive to compare our estimate for urea production obtained under in vivo conditions to some literature values obtained with isolated perfused liver. Hens et al. (25) report a value of 120 nmol/g/min (no ammonia added to perfusate) for urea released to medium. The output increased to 276 nmol/g/min with addition of ammonium chloride to the perfusate (25). Haussinger and Gerok (22) report a value for urea production in the isolated rat liver perfused with physiological NH4Cl (0.3 mM) of 648 nmol/g/min. With glutamine plus ammonium in the perfusate, the rate of synthesis increased to 972 nmol/g/min. Matsuzawa and Ishiguro (19) estimate a rate of urea production of 3-6 \mu mol/g/min in isolated rat liver.

Estimate of blood ammonia metabolism—Label in liver urea cycle components and glutamine synthesis—Label in liver urea cycle components and glutamine synthesis would appear to be 22.8 to 1.65 (i.e. 1.4 + 0.25) or approximately 14:1. The rate of glutamine synthesis from blood-derived ammonia is ~21 nmol/g/min (i.e. 297 \times 0.07).

Estimate of Radioactivity in Carbamyl Phosphate—No discrete radioactive peak was obtained in the region of the HPLC effluent assigned to carbamyl phosphate (i.e. between [15N] urea and [15N] aspartate). For an isolated peak (e.g. labeled arginine), the system is capable of detecting 0.1\% of the total radioactivity applied to the column, provided the counts injected on to the column are adequate. However, since carbamyl phosphate elutes relatively closely behind urea, we estimate the limit of detection of [15N] carbamyl phosphate in the presence of moderate amounts of labeled urea and aspartate as 0.25\% of the total radioactivity applied to the HPLC column. In one experiment, in which the liver was freeze-clamped at 15 s following the bolus injection of [15N] ammonia (Fig. 1), 0.64\% of the label in peak 1 plus the elute immediately before aspartate in the HPLC effluent was retained on a Dowex 1 acetate column (0.5 \times 5 cm) eluted with water. Since carbamyl phosphate and AMP are retained under these conditions, the maximum label present in carbamyl phosphate at 15 s is 0.0064 \times 18.5 (radioactivity in peak 1) = 0.12\% of the total. By 15 s, the relative radioactivity in citrulline was declining. If any free carbamyl phosphate is formed in the mitochondrion, its relative specific activity should peak at 5-10 s. Since no discrete peak of [15N] carbamyl phosphate was obtained at 5 s, we conclude that the maximum possible amount of label in the mitochondrial carbamyl phosphate pool is 0.25\% of the total label in the liver cell.

DISCUSSION

Much of our knowledge of liver metabolism has been obtained from studies of hepatocytes and isolated perfused livers, where it is possible to manipulate the environment and to carry out balance studies in a defined system. Because of the rapid exchange of many metabolites between blood and liver, it is often difficult to assess biological pathways by tracer techniques in vivo. Nevertheless, we have shown that within 5 s of administration of [15N] ammonia, less than 0.4\% of the total label taken up in the liver is lost to the blood as labeled metabolites. In addition, since the circulation time of an anesthetized adult male rat is about 6 s (26), the liver during this 5 s could only have been exposed to minimal amounts of recirculating label. Therefore, at least over this period, the liver may be regarded as a closed system to a good approximation. Since even in this short time, 43\% of the label was present in metabolites, this "closed system" has allowed us to estimate truly in vivo turnovers for several biological reactions that were heretofore only estimated from in vitro studies.

This type of analysis of rapid nitrogen flux exemplifies the usefulness of 15N as a tracer for short-term studies. Consider the following calculations. In the present studies, the bolus (0.2 ml) of [15N] ammonia injected into the portal vein contained a maximum of 100 nmol/ml ammonia, of which 93\% entered the liver. Approximately 1.4\% of the label in liver at 5 s was in glutamine (mostly amide). Thus, if the bolus had contained 100 \mu M [15N] ammonia, the amount of newly formed L-[amido-15N] glutamine would have been 0.26 nmol. Since the liver contains ~2.8 \mu mol of L-glutamine/g, wet weight, or ~39 \mu mol/whole liver and the natural abundance of 15N is ~0.4\%, then the typical rat liver contains ~160 nmol of L-[amido-15N]glutamine. The enrichment over background of this pool

4 This conclusion assumes that labeled urea is being synthesized only from [15N] ammonia. Theoretically, some label from asparagine may also be incorporated. However, at 5 s, the ratio of specific activities of total ammonia to asparagine is 16:1. Moreover, at 5 s, it is probable that most of this labeled asparagaine is still in the mitochondria. Therefore, the ratio of specific activities in the cytosol may be even greater. Consequently, we ignored the contribution from labeled asparagine in the calculation.
had $^1$H/ammonia been employed instead of $^1$H/ammonia, would have been only 0.16%.

As indicated above, the components of the glutamate dehydrogenase, aspartate aminotransferase, and alanine aminotransferase reactions are thought to be in equilibrium in rat liver (Ref. 5; see also Ref. 15). An equilibrium situation, however, gives no clue as to the rates of input and output of the components of the reactions. By 5 s, following a bolus administration of $[^{13}N]$ammonia, ~18% of the label had been transferred to glutamate (i.e. sum of label in glutamate, aspartate, and alanine) in the rat liver. These findings indicate that the rate of exchange between mitochondrial glutamate and ammonia nitrogen must be extremely rapid. It took ~2 s to inject the bolus, and the liver was frozen at 5 s after the start of the bolus injection; yet, label in glutamate was, within experimental error, at a maximum. Let us assume that the process of glutamate labeling is at least 90% complete after 4 s of exposure of the liver cells to $[^{13}N]$ammonia. This yields a $t_0$ for exchange of labeled nitrogen between ammonia and mitochondrial glutamate toward equilibrium of ~2 s. Since aspartate is labeled almost instantly, the rate of exchange of labeled nitrogen toward equilibrium among ammonia, glutamate, and aspartate pools in the mitochondria must be described by a $t_0$ also of ~2 s. It is more difficult to estimate rates of exchange of nitrogen between glutamate and aspartate and between glutamate and alanine in the cytosol because of the relatively slow exchange of $[^{13}N]$aspartate between mitochondria and cytosol (see below). However, because the inherent activities of soluble aspartate and alanine aminotransferrases are similar to that of mitochondrial aspartate aminotransferase in rat liver, it seems reasonable to assume that rates of exchange of nitrogen between components of these aminotransferase reactions are also defined by a $t_0$ value of the order of seconds.

The finding that 24% of the label was in urea cycle components plus glutamine by 5 s suggests (assuming the bolus of $[^{13}N]$ammonia took ~2 s to inject) that the conversion of $[^{13}N]$ammonia to the irreversibly labeled products, urea and glutamine, in liver is described by a process with a $t_0$ of ~10–11 s ($k$ = 0.06 s$^{-1}$).

At this point, a few comments on the Häussinger/Gerok (22) interorgan glutamine cycle are appropriate. Our data certainly show that, in agreement with their suggestion, portal vein ammonia is converted, in part, to glutamine in the liver in vivo. However, our estimate of the partitioning of portal vein ammonia metabolism between glutamine and urea of 1:14 is less than that proposed by Häussinger and Gerok of 1:2 (22). Low incorporation of portal vein-derived ammonia into glutamine may be due to the fact that: 1) by the time the blood reaches the perivenous hepatocytes, it has already been considerably depleted of ammonia, and 2) the actual volume of cells containing glutamine synthetase activity is low. Our calculated value of glutamine synthesis from portal blood-derived ammonia (~21 nmol/g/min) is considerably less than that calculated from the data of Tate et al. (27) of 3.5 nmol/g/min for rat liver glutamine synthetase when measured under optimal conditions. Of course, the true rate of glutamine synthesis in the perivenous hepatocytes is likely to be much higher than 21 nmol/g/min because this calculated value does not take into account endogenously produced ammonia. Tate et al. report $K_m$ values of 5 and 2.3 mM, respectively, for glutamate and ATP; a value for ammonia was not reported, but it was stated that the rat liver enzyme is similar to the brain enzyme ($K_m$ has been reported for ammonia of 180 $\mu$M). We determined a glutamate concentration of ~1.2 mmol/g, whereas literature values for ATP level in whole rat liver are in the range 2–3 mM (Ref. 12). If these values also apply to the perivenous hepatocytes, then the glutamine synthetase is almost saturated with ammonia but not with glutamate and ATP. Assuming no in vivo stimulation or inhibition with effectors, the rat liver glutamine synthetase may be turning over at ~10% of its maximal rate (or ~350 nmol/g/min). Our data suggest that, at least under the conditions of our experiment, rat liver glutamine synthetase may be more important for the metabolism of endogenously produced ammonia than of portal vein-derived ammonia.

According to Häussinger and Gerok (22), glutamine is exported from the liver; this glutamine and extrahepatic glutamine on return to the liver act as a source of ammonia for urea synthesis. Concerning the former point, grss of labeled glutamine from the liver was detectable within seconds after injection of $[^{13}N]$ammonia into the portal vein. Thus far, our data are in accord with at least the first two parts of the Häussinger/Gerok cycle (glutamine synthesis in the liver from portal ammonia and export of this glutamine), but the magnitude is less than that suggested by Häussinger and Gerok, at least under the conditions of our experiment.

The purine nucleotide cycle has been proposed as a major source of ammonia for urea synthesis (28). However, Rognstad (29) showed that hadacidin (an inhibitor of adenylosuccinate synthetase) had no effect on urea production in rat hepatocytes. Krebs et al. (30) showed that the rate of incorporation of $^{14}$N derived from L-[$^{14}$N]alanine into urea was 18 times the rate of incorporation into the 6-amino group of the adenine nucleotides in rat hepatocytes. In the present experiments, we were unable to detect any (~0.25% of the total radioactivity) label in AMP at any time in the rat liver from 5 to 60 s after the bolus injection of $[^{13}N]$ammonia despite ~3–7% incorporation of label into aspartate. If aspartate nitrogen were readily incorporated into AMP, then one might expect at least some labeling of the AMP pool. Assuming that: 1) the upper limit of AMP labeling is 0.25% of the total label and 2) the concentrations of aspartate and AMP are 0.63 (present work) and 0.2 (Ref. 30) mmol/g, respectively, then the ratio of specific activities of aspartate to AMP is >80 (i.e. (6.67/0.63)/(0.25/2.0)) at 50 s. Thus, our data are also in accord with the notion that transfer of aspartate nitrogen to AMP is not an important route for ammonia production in liver. However, our comparison may not be altogether appropriate since we are considering a futile cycle (i.e. ammonia → aspartate → AMP → ammonia), and such cycles may be minimized by compartmentation of enzymes.

The present work provides some interesting information concerning individual steps of the urea cycle. First, we were able to detect little (~0.25% of the total) radioactivity in carbamyl phosphate despite appreciable labeling of citrulline. Values for carbamyl phosphate concentration in liver vary greatly in the literature. For example, Tatibans and Shigesada (21) report a value of 1.25 nmol/g for freeze-clamped mouse liver in the post-absorptive state, whereas Rajman (32) gives a value of 0.15 mmol/g for rat liver. Part of this difference may be due to species variation, but more recent values for carbamyl phosphate in freeze-clamped rat liver are of the order of 11 μmol/kg (33) and ~6 μmol/kg (34).

It has long been supposed that carbamyl phosphate gener-
ated in the mitochondria is utilized for urea synthesis, whereas that generated in the cytosol is used for pyrimidine biosynthesis. The tacit assumption seems to be that the mitochondrial pool is the larger (e.g. Refs. 31-34). How does this notion fit in with the present results? At 5 s following the bolus injection of [15N]ammonia, considerable label was present in the liver as ammonia and citrulline with a smaller amount in arginosuccinate and arginine. The specific activity of citrulline appears to peak at ~5-10 s whereas that in arginosuccinate peaked at ~10 s. Using Ratner's (13) value for liver arginosuccinate of 11.2 μM, the expected product-precursor relationship between citrulline and arginosuccinate was obtained between 5 and 15 s (Table I). A classical product-precursor relationship requires that as the product specific activity reaches a maximum, the precursor specific activity should have declined to approximately the same value (e.g. Ref. 35). Thus, at 5-10 s, the specific activity of the mitochondrial pool of carbamyl phosphate should have been of the same magnitude as that of citrulline. Since at 5-10 s <0.25% of the label was in carbamyl phosphate (and the concentration of citrulline is 73 μmol/kg, wet weight, and the amount of label in citrulline was 17.9%), the maximum size of the mitochondrial carbamyl phosphate pool of rat liver is (0.25 × 73)/17.9 = 1.0 μmol/kg, wet weight, or (using the conversion factor of Meijer et al. (34)) ≤18 μM within the mitochondria. Interestingly, Meijer et al. (34) calculate that the intramitochondrial pool of rat liver carbamyl phosphate is <100 μM. In order to explain our 15N data, one can assume that the major pool of rat liver carbamyl phosphate is mitochondrial, but this requires the total concentration in liver to be ≤2.0 μmol/kg, wet weight. Such a low value is certainly possible in view of the findings of Meijer et al. (34) and Tatibana and Shigesada (31). Alternatively, contrary to the accepted idea that the major pool of carbamyl phosphate in liver is mitochondrial, the major pool is, in fact, cytosolic. It is possible that once formed in the mitochondria, carbamyl phosphate is rapidly "channeled" into citrulline with little or no free carbamyl phosphate occurring normally in the mitochondrion. Although not stated as such by the authors, some of the findings of Pausch et al. (33) are in agreement with the idea of channeling; thus, inhibition of carbamyl-phosphate synthetase II by acivicin blocked de novo pyrimidine biosynthesis in hepatocytes even in the presence of excess ammonia. Only when ornithine transcarbamylase activity was inhibited by t-norvaline in the presence of excess ammonia was the anti-inhibitor inducible block of pyrimidine biosynthesis overcome. A resolution of the alternatives discussed above hinges on a knowledge of the actual subcellular in vivo concentration of liver carbamyl phosphate. In any case, our findings suggest that the true in vivo concentration of carbamyl phosphate in rat liver mitochondria cannot be greater than 18 μM.

Finally, the change with time of the ratio of labeled glutamate to [15N]glutamate to [15N]ammonia deserves some comments. This change is explained by assuming an initial formation of labeled glutamate and aspartate in the liver mitochondria in which nitrogen flow between ammonia, glutamate, and aspartate is rapid. This rapid labeling of mitochondrial components is followed by a relatively slow process in which labeled glutamate and aspartate appear in the cytoplasm where they are no longer in contact with glutamate dehydrogenase. Rat liver mitochondria contain a carrier for the transfer of glutamate and aspartate as part of the malate-aspartate shuttle. Under normal conditions, it is thought that aspartate is transported in an energy-requiring process from the mitochondria to the cytoplasm (36). Labeled aspartate, once in the cytoplasm, will quickly exchange nitrogen with glutamate. Given that the present data are consistent with the in vivo operation of the glutamate/aspartate carrier of the malate-aspartate shuttle, can the data be used to obtain an estimate of flux for this carrier in vivo? By measuring the rate at which the relative specific activity of glutamate/aspartate approaches 1.0, the 4t, is calculated to be ~50 s. However, there is a large uncertainty in the actual calculation (Table I).

Despite this uncertainty, the data are in reasonable agreement with estimates from previous in vitro studies. Thus, the rate of loss of labeled aspartate in isolated rat liver mitochondria to the medium has been reported to occur with 4t values in the range of 0.5 to several minutes under a variety of conditions (36). Murphy et al. (37) report a rate of egress of aspartate from mitochondria of ~10 pmol/g, dry weight/min at an aspartate concentration of 14 nmol/mg of mitochondrial protein, dry weight. Using their conversion factor for mitochondrial protein, the 4t for turnover of mitochondrial aspartate to the exterior is ~22 s. Thus, it seems reasonable both from our present 15N data and from kinetic experiments with isolated rat liver mitochondria (36, 37) that mitochondrial aspartate is transported to the cytosol with a 4t value in the order of 20 s to several minutes in vivo.

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