KINETICS OF AMMONIA METABOLISM IN VIVO*

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The studies of Schoenheimer and his colleagues (1) amply demonstrated that N\textsuperscript{15}, introduced into the animal as ammonia or an \(\alpha\)-amino acid, may later be found not only in urea but in the amino acids of tissue protein. Particularly rich were glutamic and aspartic acids and the amide nitrogen of glutamine and asparagine. These findings, coupled with the great activity of hepatic transaminases and glutamic dehydrogenase and the relatively weak activity of hepatic \(\alpha\)-amino acid oxidases when assayed in vitro (2), led to the concept that deamination of \(\alpha\)-amino acids is accomplished by the consecutive action of specific transaminases and glutamic dehydrogenase. Incorporation of administered NH\textsubscript{3}-N\textsuperscript{15} into amino acids has been considered to occur by reversal of the process. The collective actions of glutamic dehydrogenase, glutamine synthetase, and carbamyl phosphate synthesis have been suggested (3) as the explanation of the extremely low concentration of ammonia in animal tissue (4).

These concepts have not been subjected to a direct experimental test in vivo. Although several reactions have been observed in which the amide nitrogen of glutamine is transferred to various acceptors (5–9), the metabolic role of the large amounts of glutamine in animal tissues is but poorly understood. There have been repeated suggestions that glutamine, rather than ammonia, may be specifically employed for urea synthesis; the status of this problem has recently been reviewed (3). In the present studies NH\textsubscript{3}, glutamine, \(D\)- and \(L\)-leucine, labeled with N\textsuperscript{15}, have been given intravenously to rats, and the N\textsuperscript{15} content of the non-protein nitrogenous components of liver and other tissues has been determined after various time intervals in the hope that the data so obtained might elucidate the problems cited above.

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EXPERIMENTAL

Materials—Glutamine amide-N\textsubscript{15}, 32.2 atoms per cent excess, was a product of the Mann Biochemical Company, New York. Separate samples of N\textsubscript{15}H\textsubscript{4}NO\textsubscript{3}, containing 34 and 36.2 atoms per cent excess were obtained from the Eastman Organic Chemicals, Rochester, New York, and converted to the lactate salt. DL-Leucine-N\textsubscript{15}, 10 atoms per cent excess, was prepared by the procedure of Schoenheimer and Ratner (10) from phthalimide-N\textsubscript{15} obtained from the Eastman Organic Chemicals and resolved into its optical isomers with the use of hog kidney deacylase as described by Birnbaum et al. (11).

Procedure—The N\textsubscript{15} compounds, in no more than 1 ml. of 0.85 per cent NaCl, were administered by injection into the tail vein of fasted male rats, weighing about 300 to 400 gm., while under light ether anesthesia. The rats were sacrificed by decapitation, and tungstic acid filtrates of appropriate tissues were prepared as previously described (4). When desired, whole carcass was first passed through a meat grinder several times, and a filtrate was prepared from the mixture so obtained. Data obtained on such filtrates were corrected for the loss suffered during the grinding procedure. In all cases, to remove any ammonia present, the filtrate was passed through a 1 cm. column containing 1 gm. of Permutit per 7 ml. of filtrate. To facilitate handling, the filtrate was concentrated in vacuo at room temperature, a procedure which does not cause hydrolysis of glutamine. The concentrate was diluted to 21 ml., and two 10 ml. aliquots were withdrawn.

Isolation of Urea N—To an aliquot of the filtrate 1 ml. of a 1 per cent solution of Arlington jack bean urease in 0.5 M phosphate buffer, pH 6.5, was added, and the solution was incubated at 37° for 30 minutes. The ammonia was removed by vacuum distillation by using a modification of Speck's (12) procedure and test tubes calibrated at 10 ml., 2 ml. of saturated borate buffer, pH 10.6, a silicone antifoam agent,\textsuperscript{1} and, as indicator, 1 part 0.1 per cent phenolphthalein plus 2 parts 0.2 per cent Nile blue A in 95 per cent ethyl alcohol. The pH of the solution was maintained above 10.0 to insure complete distillation.

Isolation of Glutamine Amide N—After neutralizing the residue from the urea distillation, glutamine was hydrolyzed by addition of 1 ml. of 10 N H\textsubscript{2}SO\textsubscript{4} and heating in a boiling water bath for 11 minutes. The hydrolysate was neutralized with 10 N NaOH, with chlorophenol red as indicator, and the ammonia was distilled in the usual fashion after addition of 3 ml. of 5 N NaOH rather than borate.

Isolation of Amino Acids—It was found necessary to remove serine and

\textsuperscript{1} Dow-Corning Anti-Foam AF emulsion.
threonine, as they prevented adequate resolution of aspartic and glutamic acids by column chromatography. Accordingly, the residue from the glutamine distillation was adjusted to pH 8.5 and filtered through Celite. After adding 5 ml. of 1 N periodic acid, the pH was readjusted to 8.5, and the mixture was permitted to stand for several hours or overnight. A warm saturated solution of lead acetate was then added which precipitated iodate and excess periodate as well as sulfate. The precipitate was removed by centrifugation, and more lead acetate was added until it was in excess. The supernatant fluid was refrigerated overnight to complete precipitation, and excess lead was removed with H₂S. After clarification by passage through Celite, the solution was placed on a Dowex 1 hydroxide column, 20 mm. × 250 mm. The column was washed with water until the eluate, initially alkaline, was neutral, and amino acids were eluted with 1.2 N HCl. Elution was complete when the eluate no longer smelled of acetic acid, and this was confirmed by the ninhydrin test. The eluate was evaporated to dryness in vacuo to remove volatile acids, and the residue was dissolved in a small volume of 1.2 N HCl.

Aspartic acid, glutamic acid, glycine, and alanine were separated on a column of Dowex 50 (H⁺), 1.5 cm. × 95 cm., according to Stein and Moore (13), with 1.2 N HCl as the eluent. Ninhydrin determinations were performed directly on the eluates without prior neutralization. Aspartic and glutamic acids were judged to be free of one another by paper electrophoresis on Whatman No. 1 paper in 0.05 M acetate, pH 4.7, at 600 volts for 2 hours. Separation of glycine and alanine was established by chromatography on Whatman No. 1 paper in 77 per cent ethanol for 4 hours. Appropriate fractions were pooled, concentrated on a steam bath, and degraded by Kjeldahl digestion. After neutralization of the digest, distillation of the ammonia was carried out as previously described.

Ammonia was determined by potentiometric titration or colorimetrically with Nessler's reagent (14) or by the phenol-hypochlorite method (4).

Amino acids were determined by the ninhydrin method of Troll and Cannan (15) with L-leucine as standard.

The ammonia samples were evaporated to dryness following the addition of carrier ammonium sulfate as required. After transfer to Rittenberg tubes, ammonia was converted to nitrogen with 1 ml. of Van Slyke's hypobromite reagent (16), and the N¹⁵ was assayed in a Consolidated-Nier isotope ratio mass spectrometer, model 21-201.

RESULTS AND DISCUSSION

It will be seen in Fig. 1 that, 5 minutes after administration of NH₃-N¹⁵, the specific activity of glutamate was one-third that of the amide nitrogen of glutamine. Thereafter, ammonia was incorporated into the amide
position of glutamine considerably more rapidly than into any of the other nitrogenous components of liver. When the peak of activity was attained, the specific activity of glutamine was approximately 7 times that of either urea or glutamic acid. Aspartic acid and alanine equilibrated with glutamic acid after 15 minutes. However, in these and subsequent experiments, the amounts of aspartic acid and alanine isolated were rather small, necessitating addition of relatively large amounts of carrier before analysis; hence their specific activities have only qualitative significance. It is noteworthy, however, that in neither of the experiments of Fig. 1 was any isotope found in glycine which is sufficiently abundant in liver to assure confidence in the data.

After administration of amide-labeled glutamine, as shown in Fig. 2, isotope appeared slowly in the urea and glutamic acid fractions while aspartic acid, alanine, and glycine, not shown in the figure, exhibited significant quantities of isotope only when the specific activity of glutamine was at a maximum. Thus, the data offer no suggestion that the amide nitrogen of glutamine might be a direct precursor of any of the materials investigated, nor support to the concept that glutamine may contribute to urea.

Fig. 1. Appearance of N\textsuperscript{15} in various nitrogenous components of liver after administration of N\textsuperscript{15}H\textsubscript{3}. Male rats, weighing approximately 350 gm., each received 58.5 \( \mu \) moles of ammonium lactate, 34 atoms per cent excess N\textsuperscript{15}, in 0.5 ml. of saline. Three rats were sacrificed at each time, and their livers were pooled for analysis in duplicate. A, N\textsuperscript{15} incorporation in the first 15 minutes. B, N\textsuperscript{15} incorporation over a 6 hour period. The experiments in A and B were conducted independently.
synthesis by a pathway other than the ornithine-arginine cycle. When
the data from Fig. 2 relating to the specific activity of glutamine are re-
plotted as shown in Fig. 3, the limiting slope yields a turnover time of
2.7 hours. The significance of this value will be discussed later.

The pattern of isotope distribution obtained after the administration of
D-leucine-N\textsuperscript{15} (Fig. 4) was similar to that obtained with ammonia, although
all events were somewhat slower. Glutamine, urea, and glutamic acid all

showed linear rises in specific activity over the course of the 1st hour when
the specific activity of glutamine was almost 7 times that of urea. Only
trivial amounts of N\textsuperscript{15} were detected in the aspartic and alanine fractions
and none in glycine. These data are compatible with the concept that the
initial metabolic fate of D-leucine is oxidation by D-amino acid oxidase; the
subsequent fate of the ammonia so formed is identical with that observed
when ammonia is administered, \textit{viz.} predominantly synthesis into the amide
nitrogen of glutamine.

The relationships observed after administration of L-leucine, as seen in
Fig. 5, contrast markedly with those observed with ammonia, glutamine,
Fig. 3. Turnover of glutamine amide nitrogen. The broken line represents the best plot of the limiting slope of the experimental curve.

Fig. 4. Appearance of N\textsuperscript{15} in various nitrogenous components of liver after administration of d-leucine-N\textsuperscript{15}. Male rats, weighing approximately 350 gm., each received 119 mmoles of d leucine N\textsuperscript{15}, 10 atoms per cent excess, in 1.0 ml. of saline. Three rats were sacrificed at each interval, and their livers were pooled for analysis in duplicate.
or D-leucine. Glutamic acid rapidly became the most heavily labeled component, with isotope incorporated into urea and glutamine relatively slowly. The N\textsuperscript{15} contents of aspartic acid and alanine, not shown in Fig. 5, exceed that of glutamine throughout the experimental period. These data appear to offer direct evidence that oxidative deamination of L-leucine, and presumably of other amino acids, by either specific or non-specific L-amino acid oxidases is a relatively insignificant process and that the metabolism of L-leucine proceeds by consecutive transamination and oxidative deamination of the glutamic acid so formed. Noteworthy also is the fact that

![Graph showing the appearance of N\textsuperscript{15} in various nitrogenous components of liver after administration of L-leucine-N\textsuperscript{15}. Male rats, weighing approximately 350 gm., each received 126 \(\mu\)moles of L-leucine-N\textsuperscript{15}, 10 atoms per cent excess, in 1.0 ml. of saline. Three rats were sacrificed at each time, and their livers were pooled for analysis in duplicate.](image)

only after L-leucine administration was appreciable isotope observed in liver glycine, suggesting that the latter obtains its nitrogen by transamination from glutamate to glyoxylate or 3-phosphohydroxypyruvate (3). Neither alanine nor glycine appears to arise in significant quantity by reversal of the oxidative reactions catalyzed by L-amino acid or glycine oxidases, although the enzymatic feasibility of such reductive amination has been demonstrated (17).

Since both urea and glutamine were known to be relatively readily diffusible across cell membranes, further interpretation of the data presented required knowledge of the total distribution of isotope in the animal body. Table I shows the distribution of labeled urea and glutamine in various tissues of the rat 15 minutes after administration of NH\textsubscript{3}-N\textsuperscript{15}. It will be
seen that both urea and glutamine rapidly diffused from the tissues in which they are formed, although equilibrium had not been obtained 15 minutes after \( \text{NH}_3 \) administration. In the experiment shown, of the 52.2 \( \mu \text{moles of NH}_3\text{-N}^{16} \) given, 65 per cent was recovered as glutamine plus urea; most of the unrecovered isotope was unmetabolized ammonia. Data obtained from rats sacrificed 30 minutes after isotope administration indicated conversion of more than 90 per cent to glutamine plus urea. Particularly noteworthy is the fact that, of the ammonia fixed into organic linkage, somewhat more than 80 per cent was found as glutamine amide nitrogen.

Since the ammonia concentration of animal tissues is normally extremely low (4), even the minimal amounts of ammonia administered in the experiments described above must be considered as offering an ammonia “load” to the animal, although the doses were sufficiently small that it may be assumed that the data reflect the fate of ammonia normally arising in metabolism. However, these experiments gave no indication of the relative capacities of the urea- and glutamine-synthesizing systems. Accordingly, increasing amounts of ammonia were given to groups of rats which were sacrificed 20 minutes thereafter, and the amounts of isotope in the urea and glutamine of liver and carcass were determined. The data are shown in Fig. 6.

It will be seen that the data describing glutamine synthesis yield the typical hyperbolic curve expected of enzymatic reactions with an observed \( V_{\text{max}} \) of 37.2 \( \mu \text{moles per minute per kilo} \) and a \( K_m \) (dose level at which glutamine synthesis is \( V_{\text{max}}/2 \)) of 1.5 \( \mu \text{moles per kilo} \). The latter may be

### Table I

**Distribution of Labeled Urea and Glutamine after Administration of Ammonia-\( \text{N}^{15} \)**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Urea nitrogen</th>
<th>Glutamine amide nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu \text{moles per gm.} )</td>
<td>atoms per cent excess</td>
</tr>
<tr>
<td>Brain</td>
<td>6.7</td>
<td>0.043</td>
</tr>
<tr>
<td>Spleen</td>
<td>8.6</td>
<td>0.078</td>
</tr>
<tr>
<td>Heart</td>
<td>9.2</td>
<td>0.093</td>
</tr>
<tr>
<td>Kidney</td>
<td>36.8</td>
<td>0.059</td>
</tr>
<tr>
<td>Liver</td>
<td>10.6</td>
<td>0.150</td>
</tr>
<tr>
<td>Testes</td>
<td>9.3</td>
<td>0.029</td>
</tr>
<tr>
<td>Carcass</td>
<td>5.600</td>
<td>4.6</td>
</tr>
</tbody>
</table>

* Total in tissue.
compared with the $K_m$ (for NH$_3$) of 0.0025 M for rat liver glutamine synthetase which may be calculated from the data of Speck (12). At the highest dosage levels, glutamine synthesis was slightly inhibited, also in keeping with the observations in vitro of Speck (12). Thus, it may be inferred that there is some constant relationship between the amount of NH$_3$ administered and the intracellular NH$_3$ concentration. On this as-

![Graph](image)

**Fig. 6.** Urea and glutamine synthesis at various dosage levels of ammonia. Pairs of male rats, weighing approximately 350 gm., received ammonium lactate in the dosage shown, containing 10 μmoles of N$^{16}$H$_3$. After 20 minutes the rats were sacrificed, and the N$^{16}$ content of liver and carcass urea and glutamine was determined. A, total body urea and glutamine synthesized in 20 minutes (liver + carcass). B, newly formed glutamine and urea accumulated in liver and in carcass after 20 minutes.

assumption one may replot the data according to Lineweaver and Burk, *viz.* $1/v$ versus 1/dose. Such a plot yielded a $V_{\text{max}}$ of 50.1 μmoles per minute per kilo, presumably the maximal capacity of the glutamine-synthesizing system in the absence of substrate inhibition or failure of substrate or adenosine triphosphate supply, and 35 per cent greater than the $V_{\text{max}}$ which was physiologically realized.

The data describing urea synthesis in Fig. 6 are in marked contrast to those for glutamine synthesis. Over a wide range of substrate concentration, 50 to 1680 μmoles of ammonia administered, urea synthesis proceeded at the rate of 8.6 ± 0.9 per cent of the administered dose per 20 minutes.
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Only at the highest dose level, at which ammonia toxicity was manifest, did the rate fall to 6.0 per cent of the dose per 20 minutes. This is not the behavior usually expected of a single step enzymatic process, since it appears to follow first order kinetics; i.e., the rate of urea synthesis over the entire range experimentally feasible was dependent solely on the available substrate, and neither the necessary enzymes nor coenzymes seemed to limit the synthetic rate. The most obvious explanation for this surprising observation would be that, even at the highest doses, the intracellular ammonia concentration is well below $K_m$ and that only the rate of entry of ammonia into cells was limiting. However, were this so, an increased rate of urea synthesis would be expected at the higher levels of ammonia dosage at which glutamine synthetase was already saturated and glutamine synthesis, proceeding at a constant rate, was no longer competing for the available ammonia. At a dosage level of 50 μmoles of NH$_3$ per rat, 54 per cent of the isotope was recovered in the total body glutamine, whereas, at the dosage necessary to obtain maximal glutamine synthesis, only 15 per cent of the administered isotope was recovered in this form. Thus, it appears that the $K_m$ for the substrate of the rate-limiting step in urea synthesis from NH$_3$ is relatively high and that in these experiments the substrate concentration remains well below this $K_m$, thereby permitting a linear relationship between urea synthesis and NH$_3$ dosage. These observations are difficult to reconcile with the fact that it is the supply of ornithine and citrulline which limits the rate of urea synthesis from NH$_3$ (18), and present studies are addressed to this problem. Of interest is the fact that only at the highest level of ammonia administration was there indication that urea synthesis was proceeding in the liver at a rate in excess of diffusion from that organ. The maximal rate of urea synthesis in the rat may be estimated by extrapolating the linear portion of the curve to that expected when the administered dose is 3780 μmoles, viz. the LD$_{50}$ for ammonia (18). The value so obtained is 44 μmoles per minute per kilo. Thus, the total capacities of the rat urea- and glutamine-synthesizing systems are of the same order.

However, the data of Figs. 1 and 4 indicate that, at low NH$_3$ concentration, glutamine synthesis is considerably more efficient than urea synthesis and glutamine synthesis is the major mechanism for detoxification of NH$_3$, whether the latter be administered or arises metabolically as from d-leucine. This is in accord with the observation of Du Ruisseau et al. (19) that administration to rats of a lethal dose of ammonia resulted in an increase in total body glutamine, and glutamine synthesis appears to be the most significant means of maintaining the normally extremely low cellular concentration of ammonia (4). Moreover, it can be calculated that glutamine turnover can suffice to provide the nitrogen necessary for normal
urea synthesis. A 300 gm. rat fed a commercial stock ration excretes of the order of 1 mmole of urea nitrogen per hour, of which half is derived from ammonia and half from aspartic acid (20). The apparent turnover time of the total body glutamine of 1.25 mmoles, calculated from the rate of loss of isotope in Fig. 3, is 2.7 hours. However, if the NH$_3$ so liberated is metabolized in a manner similar to administered ammonia, 80 per cent is resynthesized into glutamine; thus the real turnover time is about one-fifth the apparent rate or about 0.54 hour, or 2.5 mmoles per hour, of which 0.5 mmole leaves the glutamine pool. Thus, the rate of loss of ammonia from glutamine is of the same order as the synthesis of ammonia into urea under normal conditions.

Assuming that the linear relationship between ammonia concentration and urea synthesis observed here also obtains at the still lower ammonia concentration of the normal steady state, urea synthesis and, hence, nitrogen balance must be governed by those factors which determine ammonia concentration. Of these, the major factors would appear to be the concentration of $\alpha$-amino acids which by transamination could increase the glutamate available to glutamic dehydrogenase, $\alpha$-keto acids for glutamine transaminase-deaminase, $\alpha$-ketoglutarate and reduced pyridine nucleotides for reversal of glutamic dehydrogenase, and adenosine triphosphate for glutamine resynthesis. Of these, the first is most likely to be under endocrine control and the latter three to be limiting in the fasting or diabetic animal.

It is difficult to assess the role of glutamic dehydrogenase in maintaining the intracellular NH$_3$ concentration. As shown in Fig. 1, 5 minutes after N$^{15}$H$_3$ administration, the N$^{15}$ concentration of the $\alpha$-nitrogen of glutamate was only one-third that of the amide nitrogen of glutamine. This may reflect only a lack of available $\alpha$-ketoglutarate or reduced pyridine nucleotide and the high dilution suffered by N$^{15}$ incorporated into glutamate since, by virtue of the rapid turnover of glutamine, the effective pool of glutamate is actually the total $\alpha$-nitrogen of glutamate plus glutamine. Conceivably, in the absence of a "load" of administered NH$_3$ or a precursor thereof, in the steady state glutamate synthesis by reversal of the glutamic dehydrogenase reaction may be a more significant process than under the conditions of these experiments.

**SUMMARY**

Ammonia, glutamine, L- and D-leucine, labeled with N$^{15}$, were administered to rats, and the N$^{15}$ of liver urea, glutamine, glutamate, aspartate, alanine, and glycine, as well as total body glutamine and urea, was determined at various time intervals. Glutamine synthesis was found to be the major fate of ammonia. Urea synthesis, per unit time, was found to
represent a fixed percentage of available ammonia over a large concentra-
tion range. The data indicate that D-leucine undergoes initial oxidative
deamination, whereas the nitrogen of L-leucine is transferred to glutamate
before it becomes available for glutamine or urea synthesis. The signif-
icance of these findings is discussed.

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