STUDIES IN PROTEIN METABOLISM

V. THE UTILIZATION OF AMMONIA FOR AMINO ACID AND CREATINE FORMATION IN ANIMALS*

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There is no doubt that animals can synthesize certain amino acids, the ultimate source of the nitrogen being that of the food protein. This process probably occurs under normal conditions, as the average composition of food proteins is never the same as that of the body proteins into which they have to be converted. According to the generally accepted theory, amino acid synthesis in the animal follows the scheme by which ammonia reacts with α-keto acids to form imino acids which are reduced to amino acids. Knoop and Oesterlin (1) have followed this reaction in

\[
\text{NH}_3 + \text{RCH}_2\text{CCOOH} = \text{RCH}_2\text{COOH} + 2\text{H}_2\text{O} \quad \text{Hz} \rightarrow \text{RCH}_2\text{CHCOOH}
\]

According to this scheme amino acids could be formed either from ammonia administered directly to the animal or from that liberated by deamination of other amino acids.

A large amount of work has been carried out on the utilization of ammonia for protein synthesis in animals, but the results have not been conclusive. Partial replacement of protein nitrogen in the diet of laboratory animals or ruminants by ammonia or urea gave no proof of the utilization of these substances by tissue cells (2). Embden and Schmitz (3) by using a more direct method per-

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fused organs with α-keto acids and ammonia and obtained the corresponding amino acids. Neber (4) treated mixtures of pyruvic acid and ammonia with large quantities of tissue slices and observed a small increase of amino nitrogen above the control values. Although these experiments demonstrate amino acid synthesis by animal tissue, they offer no definite proof that the amino nitrogen formed was derived from the added ammonia. In all these experiments the perfused organs or the tissue slices contained amounts of protein nitrogen (or free amino nitrogen) far greater than those recovered in the newly formed amino acids. The new amino acid could have been formed by a direct shift of the amino nitrogen from an amino acid to the keto acid.

A reaction of this kind has recently been observed in in vitro experiments by Herbst (5). An amino acid treated with an α-keto acid gave rise to a new amino acid corresponding to the keto acid, a Schiff base being formulated as the intermediate step. Braunstein and Kritsman (6) have recently advanced strong evidence that the reaction studied by Herbst actually occurs in organs: treatment of pyruvic acid with glutamic or aspartic acid and minced muscle gives rise to the formation of alanine and loss of glutamic acid. The authors suggest that these two aminodicarboxylic acids, or their corresponding keto acids, are intermediates in the formation of other amino acids. In contrast to the mechanism for amino acid formation, discussed above, the reactions of Herbst and of Braunstein do not involve ammonia.

The isotope of nitrogen can be applied to investigations of the role of ammonia in amino acid synthesis in animals. If isotopic ammonia is given, it will mix with the ammonia present in the animal organism, including that which is formed as an intermediate product. As the animal organ does not discriminate between the isotopes of the same element, both the ammonia administered and the ammonia formed in the cell must be treated alike. If ammonia is used in the synthesis, the newly formed amino acid should contain an increased amount of isotope. As administered ammonia is not directly excreted in the urine, but first converted into urea, it is involved in metabolic reactions. As will be shown later, it is available also for other syntheses.

We have carried out two exploratory experiments by feeding isotopic ammonia to animals under conditions in which amino acid
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synthesis was to be expected. An adult rat was kept on a stock diet, the protein of which was casein. This does not contain appreciable amounts of glycine. About 300 mg. of benzoic acid were added to the diet for 10 days; this resulted in a continuous excretion of glycine (830 mg.) in the form of hippuric acid, for which an equivalent amount of glycine must have been synthesized. The diet also contained 1.2 per cent nitrogen (with 1.21 atom per cent N$_{15}$ excess) in the form of ammonium citrate. The animal kept its weight and consumed the same amount of food as the controls on the same diet without benzoic acid and ammonium citrate.

The nitrogen of the urinary hippuric acid contained a small but definite increase of the nitrogen isotope (0.025 to 0.045 atom per cent), which indicates that at least a small amount of the dietary ammonia was utilized for the glycine synthesis. As the dietary ammonia contained 1.21 atom per cent N$_{15}$ excess, about 3 to 4 per cent of the urinary glycine was formed by use of nitrogen of the administered ammonia. The creatine isolated from the carcass also contained a small but significant increase of the isotope.

A similar result was obtained in the second experimental series: Two immature rats of 60 gm. each were kept for 5 days on a low protein diet (3 per cent as yeast protein) which contained 2.3 per cent nitrogen as ammonium citrate (1.21 atom per cent N$_{15}$ excess). The animals lost weight as was expected. These unphysiological conditions were chosen in the expectation that the rats in the need of protein might synthesize at least a moderate amount of amino acids by utilizing the isotopic ammonia. This was the case. The protein nitrogen of the carcass contained a slight excess of N$_{15}$ above normal.

In order to locate the isotopic nitrogen in the body constituents, samples of the following seven different amino acids, in addition to creatine, were isolated in pure state: glycine, glutamic acid, aspartic acid, proline, histidine, arginine, and lysine. All these substances, with the exception of lysine, contained a small but definite excess of N$_{15}$. The isotope concentration in the lysine was normal (Table II).

The highest values were found in glutamic acid and in aspartic acid. It is interesting to note that the dicarboxylic acids, which according to the work of Braunstein and Kritzmann (6) seem to
play an especially active role in amino acid metabolism since they are intermediates in the formation of other amino acids, demonstrate this activity also by their high uptake of isotopic nitrogen. The findings on lysine and on the dicarboxylic acids are similar to those of the experiments in which animals were given heavy water (7). Glutamic and aspartic acids isolated from these animals had the highest deuterium content, while lysine was free of the isotope.

The arginine from the animals given isotopic ammonia also contained N\textsuperscript{15}. In order to locate the isotope in the molecule of this amino acid, the arginine isolated was hydrolyzed with strong alkali into ornithine and ammonia (8). The isotope was found in the ammonia, while the ornithine contained normal nitrogen. The isotope was thus present in the \text{HN=CNH}_2 part of the guanido group of the arginine. It is the same part which is liberated as urea by arginase,\textsuperscript{1} and which according to the theory of Krebs and Henseleit (9) is potential urea and is involved in normal urea formation. As ammonia given to animals is always converted into urea, our finding on arginine is additional support for this theory.

The high isotope content of the amide nitrogen of the protein is noteworthy. More than 10 per cent of this amide nitrogen was derived from the administered ammonia. According to the well accepted theory, the amide nitrogen in proteins is linked to the free carboxyl group of the combined dicarboxylic acids (as glutamine and asparagine). The result is of interest in connection with the finding of Leuthardt (10) that glutamine and asparagine may form urea independently of the ornithine cycle. The relation of urea formation to arginine and amide nitrogen will be discussed in a subsequent publication.

The experiments leave no doubt as to the ability of animals to utilize at least a small amount of ammonia for amino acid and creatine formation. However, the unphysiological conditions employed in both series make it uncertain that we are dealing with a process which takes place under normal conditions.

\textsuperscript{1} We have not employed arginase for the splitting as the normal nitrogen in the enzyme solution would have diluted the isotope in the amino acid.
EXPERIMENTAL

Feeding Benzoic Acid and Isotopic Ammonia to an Adult Rat—A male rat of 198 gm. was kept in a metabolism cage on the following diet: casein 16, corn-starch 47, lard 25, yeast 5, salt mixture (Osborne and Mendel (11)) 4, and sodium benzoate 3 per cent. After 2 days on this diet the animal received for another 6 days an addition of isotopic ammonia (1.21 atom per cent N¹⁵ excess) as the citrate. The diet thus contained 3.5 per cent total nitrogen, of which 0.6 per cent was that of the isotopic ammonia. As the average daily food intake was 12.1 gm., the animal consumed 72 mg. of ammonia nitrogen daily. Its weight remained constant (197 gm. at the end of the experiment). The urine was collected under toluene and pure hippuric acid was isolated daily in the usual manner. From the urines of the 8 day period a total of 1.89 gm. of hippuric acid, corresponding to 0.83 gm. of glycine, was isolated. For the isotope determination the samples obtained on the 5th, 6th, and 8th days were analyzed separately. The creatine of the carcass was isolated as described later. The results are given in Table I.

Feeding a Protein-Low Diet and Isotopic Ammonia to Immature Rats—Two male rats of 72 gm. each were kept in a metabolism cage on a diet of the following composition: corn-starch 63.75, lard 28.35, yeast 5.65, salt mixture (11) 2.25 per cent. To the diet was added 2.25 per cent of nitrogen (1.21 per cent N¹⁵ excess) in the form of ammonium citrate. A sample of the total nitrogen

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Feeding period</th>
<th>N¹⁵ concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>days</td>
<td>atom per cent excess</td>
</tr>
<tr>
<td>Hippuric acid</td>
<td>5</td>
<td>0.040</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.043</td>
</tr>
<tr>
<td>Body creatine</td>
<td>9</td>
<td>0.025</td>
</tr>
</tbody>
</table>
of the diet was analyzed for its isotopic composition. It contained 0.83 atom per cent N\(^{15}\) excess; i.e., about 70 per cent of the dietary nitrogen was present as ammonia (1.21 atom per cent N\(^{15}\) excess). The animals were kept on this diet for 5 days. They had consumed at the end of the experiment a total of 40 gm. of the diet, corresponding to an average of 180 mg. of ammonia nitrogen (1.4 gm. of N per kilo of body weight) per day.

The average weight of the animals had dropped from 72 gm. to 60.6 gm.; i.e., each animal lost about 2.2 gm. per day. The rats were killed; the intestinal tract was removed, washed with water, and added to the rest of the carcasses. The combined material (120 gm.) after being cut into small pieces was extracted three times with boiling alcohol for 8 hours each. The combined alcohol extracts were used for the isolation of creatine as described later.

**Isolation of Amino Acids**—The extracted carcasses were dried (weight 29 gm.) and boiled for 20 hours with 20 per cent hydrochloric acid. The solution was made neutral to phenolphthalein with barium hydroxide and the precipitate filtered off, whereby most of the calcium and phosphates of the skeleton were removed. From an aliquot of the solution free ammonia was distilled off in vacuo for the isotope analysis of the “amide nitrogen.” The remaining solution was freed of barium, and samples of the following amino acids were isolated by procedures previously described (7): glycine, glutamic acid, aspartic acid, proline, histidine, lysine, and arginine. The samples were recrystallized until pure (Kjeldahl analysis).

**Splitting of Isotopic Arginine**—24 mg. of the arginine monohydrochloride were boiled for 6 hours with 50 per cent KOH. A slow stream of nitrogen gas carried the liberated ammonia into a receiver containing dilute H\(_2\)SO\(_4\). Isotope analyses were carried out with the ammonia solution as well as with the alkaline solution containing the ornithine.

**Isolation of Creatine As Potassium Creatinine Picrate from the Carcasses**—The alcoholic extract of the carcasses was evaporated to a small volume in vacuo, and the aqueous residuum made acid (approximately 1 N) with hydrochloric acid and extracted with ether to remove fats. It was boiled for 4 hours to convert creatine into creatinine, brought to dryness in vacuo, and the residue dis-
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solved in 300 cc. of water. It was cleared by adding a solution of 1 gm. of lead acetate followed by NaOH until just alkaline to phenolphthalein; lead was removed from the filtrate by hydrogen sulfide. The solution was made faintly acid to Congo red with hydrochloric acid, and treated with 0.5 gm. of picric acid and 0.5 gm. of potassium picrate, which were dissolved by warming. The crystals formed after 2 days standing in the ice box were recrystallized from 300 cc. of a solution containing 0.12 per cent of picric acid and 0.12 per cent of potassium picrate. In this solution at ice temperature potassium creatinine picrate is practically insoluble

TABLE II
Compounds from Immature Rats Given Low Protein Diet and Isotopic Ammonia (1.81 Atom Per Cent N15 Excess)

| Compound                         | N15 excess  
|----------------------------------|-------------
| Amide N                          | 0.114       
| Glycine                          | 0.050       
| Glutamic acid                    | 0.085       
| Aspartic "                       | 0.067       
| Proline                          | 0.037       
| Histidine                        | 0.012       
| Lysine                           | 0.003       
| Arginine                         | 0.033       
| "Urea" from arginine             | 0.069       
| Ornithine from arginine          | 0.004       
| Creatine                         | 0.036       

and recrystallization can be repeated many times without appreciable loss. The final product, 0.703 gm. (from the second series), was assayed colorimetrically for its creatinine content; found 18.5 per cent, calculated 18.6 per cent. N (Kjeldahl after reduction with Sn + HCl) found 20.8, 20.6; calculated 20.7. For the isotope analysis of the creatinine a sample of the potassium creatinine picrate was freed of picric acid by suspending in dilute H2SO4 and extracting with ether. The analysis was carried out with the aqueous solution.

All values obtained in the isotope analyses of the compounds from the animals are given in Table II.
DISCUSSION

All the isotope values listed in Tables I and II, with the exception of that of lysine and ornithine in Table II, are above normal. The increase of N\textsuperscript{15} is far above the limit of error discussed in Papers I and II of this series.

The isotope was given to the animals in the form of ammonia and could not have entered the organic compounds in the animals by mere physical exchange, as the nitrogen in the compounds is stably bound. This is further shown by the negative findings on lysine (and on ornithine). If the presence of isotope in the amino acids were due to physical exchange, it should be found in all amino acids, including lysine, as the carbon-nitrogen linkage at the $\alpha$-carbon atoms is the same. The isotopic nitrogen must have entered by a chemical reaction, either by a new formation of amino acids from substances with different carbon chains or by successive deamination and amination of the same carbon skeleton (see discussion in (7)).

The results of both experiments offer definite proof for the ability of rats to utilize at least a small amount of dietary ammonia. They suggest that ammonia liberated from amino acids may be utilized for the formation of other amino acids. They do not, however, exclude other mechanisms, such as the direct transfer of nitrogen from an amino acid to a keto acid as discussed before.

Both experiments were unphysiological. Hippuric acid formation is a normal process in herbivorous animals, but the amount of benzoic acid detoxified in our first experiment is much higher than is ever observed normally. This fact, together with the peculiar conditions under which the rats were kept in the second experiment, leaves it uncertain whether the observed slight utilization of ammonia plays an appreciable rôle under normal conditions.

SUMMARY

1. Two experiments were carried out on the utilization of ammonia for amino acid synthesis in rats by feeding ammonia N\textsuperscript{15}.
2. An adult rat was given a diet containing 16 per cent casein, together with benzoic acid and isotopic ammonia in the form of ammonium citrate. While the diet was practically devoid of glycine the animal excreted in the 8 day period more than 830 mg. of glycine in hippuric acid. At least part of this glycine must have
been synthesized. The nitrogen of the hippuric acid showed a small but definite increase of its isotope content, demonstrating the utilization of a small amount of dietary ammonia for glycine formation.

3. Two immature rats were fed a low protein diet to which a large amount of isotopic ammonia was added as the citrate. Creatine, glycine, glutamic acid, aspartic acid, proline, histidine, lysine, and arginine were isolated from the bodies of the animals. All, with the exception of lysine, contained an excess of isotope, the highest concentration being found in glutamic and aspartic acids. The animals had thus utilized a small amount of the dietary ammonia.

4. The ammonia liberated during protein hydrolysis ("amide nitrogen") had an isotope concentration much higher than that of any amino acid.

5. The arginine from the animals was hydrolyzed into ammonia and ornithine. The isotope was found in the ammonia, while the ornithine moiety had normal nitrogen. The isotope was thus present in the guanido group of the arginine.

6. The isotope content in the creatine of the tissues from both experiments was also above normal.

7. The experiments offer proof for the ability of rats to utilize at least a small amount of ammonia for amino acid and creatine formation.

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