METABOLISM OF L-ASPARTIC ACID

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Aspartic acid, like glutamic acid, occupies a central position in amino acid metabolism. In most tissues it takes part in the transamination reaction (1). In the liver and the kidney, where all amino acids undergo deamination, the rate of this process for aspartic acid is second only to glutamic acid (2). For these reasons, one would expect the exchange of amino nitrogen of aspartic acid with the nitrogen of the metabolic pool to be more rapid than in the case of other amino acids. This is indeed true, as the present experiment with isotopic L-aspartic acid shows.

EXPERIMENTAL

Preparation of L-Aspartic Acid Containing N$^{15}$—L-Aspartic acid containing N$^{15}$ was prepared by the action of Escherichia coli on fumaric acid and ammonia containing N$^{16}$. Quastel and Woolf (3) have shown that E. coli catalyzes the reversible reaction, fumarate + NH$_3$ $\rightarrow$ L-aspartic acid.

E. coli was grown on 2 per cent agar in ten Roux bottles. The agar medium contained 2 per cent of Bacto-tryptone and 0.5 per cent each of glucose, sodium chloride, and concentrated yeast extract. The 2 day culture of E. coli was separated from the agar by gentle rocking with normal saline, filtered through gauze, centrifuged, washed, and made up to 200 ml. with normal saline.

15 gm. of fumaric acid were suspended in water, neutralized with sodium hydroxide, and the solution made up to 130 ml. 4 gm. of ammonium chloride, containing 31.8 atom per cent excess of N$^{15}$, were added, followed by 100 ml. of Clark and Lubs 0.05 M phosphate buffer at pH 7.4, 25 ml. of E. coli suspension, and 20 ml. of toluene. The mixture was placed in a suction flask, which was evacuated and filled with nitrogen. The flask was placed in an incubator at 37°. The ammonia content of the mixture fell in 24 hours to 19.2 per cent and in 48 hours to 18 per cent of the original. Apparently, an equilibrium between fumaric acid and ammonia on the one hand and aspartic acid on the other was reached in 2 days under the conditions of the experiment.

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After 2 days the mixture was boiled, centrifuged, and the precipitate washed with hot water. 60 ml. of saturated copper sulfate solution were added and the mixture was placed in an ice box overnight. The copper aspartate crystals were washed with a little cold water, suspended in 600 ml. of hot water, and decomposed with hydrogen sulfide. The filtrate was evaporated to about 150 ml. and 2 volumes of 95 per cent alcohol were added. After standing overnight, the aspartic acid crystals were filtered off, washed with alcohol, and finally with ether. Yield, 6 gm.

The nitrogen of the aspartic acid thus prepared contained 31.3 atom per cent excess N\textsuperscript{15} corresponding to an atomic weight of 14.32. The theoretical nitrogen content of this aspartic acid (mol. wt. 133.4) is 10.72 per cent; found, 10.86 per cent.

Rat Feeding—Three male rats weighing between 240 and 290 gm. each were placed on a stock diet containing starch 71, casein 15, yeast 5, Osborne-Mendel salt mixture 4 (4), Wesson oil 3, and cod liver oil 2 per cent. After 3 days, 0.3 gm. of isotopic aspartic acid and 0.2 gm. of sodium bicarbonate were given to each rat daily for 3 days. The aspartic acid and bicarbonate were mixed with 8 to 12 gm. of the stock diet, which was made into a dough with a little water.

The food was all eaten by the rats. Feces and urine were collected daily. The combined weights of the rats were 786 gm. before, and 739 gm. after the isotopic feeding.

Preparation of Material for Analysis—24 hours after the last isotopic feeding, the rats were killed by exsanguination under ether anesthesia. Blood was removed from the heart, some saline being used for washing, and mixed with some oxalate. Stomach and intestines were removed and washed free from their contents. Other internal organs, liver, spleen, kidney, testes, lungs, etc., were removed and treated together. The bodies were skinned, leaving the muscles and bones and brain as carcass. The four fractions, namely stomach-intestine, other internal organs, skin, and carcass, were worked up separately.

Blood—The blood with saline washing was centrifuged. Part of the cells was used for the preparation of hemin. The cells were laked with water and mixed in a Waring blender, centrifuged, and dropped slowly into 3 volumes of glacial acetic acid at 97° containing some sodium chloride. The mixture was heated on a water bath for an hour. The hemin crystals were separated by centrifuging, washed twice with 50 per cent acetic acid, twice with water, twice with 95 per cent alcohol, and once with ether. The N\textsuperscript{18} concentration of hemin was determined.

The plasma, containing some saline, was treated with 3 volumes of water and 2 volumes of 10 per cent trichloroacetic acid. The protein precipitate was filtered off and washed with 5 per cent trichloroacetic acid.
The total nitrogen and the N\(^{15}\) content of the cells, the plasma protein precipitate, and the plasma filtrate were determined in the usual manner. The results are shown in Table I.

**Tissues**—Stomach-intestine, internal organs, and carcass were separately ground in a meat grinder and homogenized in a Waring blender. Skin was cut into small pieces with scissors. Each group of tissues was treated with 6 volumes of 95 per cent alcohol. After 3 days, the mixture was filtered on a Büchner funnel and the tissue suspended again in a fresh portion of alcohol. After three changes of alcohol, the tissues were treated similarly twice with ether. The defatted tissues were dried in air and used for isolation and analysis, as described below.

The combined alcohol and ether extracts were concentrated in vacuo until most of the alcohol had been removed. The residue was taken up in chloroform plus water and the mixture was shaken and then allowed to separate. The total nitrogen and N\(^{16}\) concentration of the two layers were determined separately. The nitrogen in the aqueous layer was taken as non-protein nitrogen, while that in the chloroform layer was taken as lipoid nitrogen.

From the aqueous layer, some colorless crystals deposited on standing. These were identified as creatine hydrate by crystalline form and nitrogen content (28.0 per cent). The substance was recrystallized from water with the addition of alcohol and used for N\(^{16}\) determination.

**Stomach-Intestine**—The entire stomach-intestine preparation was used for the extraction of nucleic acid by the method of Hammarsten (5). The yield was about 90 mg. from 5 gm. of dried tissue. It was analyzed for N\(^{16}\). Part of the nucleic acid was hydrolyzed and the purine precipitated by the method of Graff and Maculla (6). The precipitate and the filtrate, which should contain the pyrimidines, were analyzed for total nitrogen and N\(^{16}\). From 21.2 mg. of nucleic acid, 1.79 mg. of nitrogen in purine precipitate and 1.17 mg. of nitrogen in purine filtrate (total 2.96 mg. of nitrogen) were obtained. The ratio of purine nitrogen to pyrimidine nitrogen is about 3:2, but the total nitrogen (14 per cent)
is lower than that (18.5 per cent) calculated for deoxyribonucleic acid (C₃₉H₄₁N₁₄P₂O₂₉).

The protein remaining after the extraction of nucleic acid was washed with hot 5 per cent trichloroacetic acid until free from sulfate and ammonia. The washed residue was dried and used for nitrogen and N¹⁵ determinations.

*Internal Organs, Carcass, and Skin*—The dried tissues were hydrolyzed with hydrochloric acid in the usual manner and the nitrogen and N¹⁵ contents determined. The hydrolysates were treated with cuprous oxide according to the suggestion of Bailey *et al.* (7).

Tyrosine crystals separated from all the hydrolysates and were filtered off and recrystallized. From the filtrates of internal organs and carcass, aspartic acid and glutamic acid were precipitated as barium salts. Aspartic acid was isolated as copper salt and the glutamic acid as hydrochloride in the usual manner. Lysine was isolated from the carcass hydrolysate as benzoyllysine by the method of Kurtz (8). Arginine was isolated from the internal organs and skin as monooclavinate. This was decomposed with concentrated hydrochloric acid, the liberated flavianic acid was filtered off, and the filtrate was diluted with water, boiled with norit to remove the last trace of flavianic acid. The filtrate was evaporated to dryness *in vacuo*. The residue was taken up in alcohol and an excess of pyridine added. Arginine monochloride crystallized on standing. This was recrystallized by dissolving in a little water and adding alcohol to a concentration of 80 per cent.

The amidine and ornithine nitrogen in arginine were determined separately by the method of Barnes and Schoenheimer (9). Arginine monochloride (17.3 mg.) from the internal organs was dissolved in a baryta solution (containing 5 gm. of Ba(OH)₂·8H₂O in 30 m. of water), boiled gently under a reflux, and the liberated ammonia blown by a stream of nitrogen into a known amount of standard acid. After 21 hours, the acid solution was titrated and the N¹⁵ determined. The baryta solution was treated with an excess of sulfuric acid, filtered, washed, and the filtrate analyzed for total nitrogen and N¹⁵. The result of nitrogen determinations was as follows: amidine nitrogen 2.20 mg. and ornithine nitrogen 2.36 mg. (total nitrogen 4.56), which amounts to 26.3 per cent; theoretical, 26.8 per cent. The experiment with skin arginine gave similar results.

A nucleic acid extract of the internal organs was prepared by Schneider's method (10) and analyzed for N¹⁵. The hydrolysate of the carcass was analyzed for amide nitrogen.

The results of N¹⁵ analysis of the tissue components are shown in Table II.

*Urine and Feces*—The combined feces were ground, digested with concentrated sulfuric acid, and made up to 200 ml. Aliquot portions were
taken for total nitrogen and $N^{15}$ determinations. About nine-tenths of the combined urine plus washing was used for the isolation of allantoin by the method of Shaffer and Greenbaum (11). The allantoin was recrystallized from hot water and analyzed for $N^{15}$.

**TABLE II**

*Distribution of Isotopes in Organ Components; Concentration in Atom Per Cent Excess*

<table>
<thead>
<tr>
<th>Component</th>
<th>Whole body</th>
<th>Stomach-intestine</th>
<th>Internal organs</th>
<th>Carcass</th>
<th>Skin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-protein N.</td>
<td>0.359</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipoid N.</td>
<td>0.134</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein N.</td>
<td></td>
<td>0.541*</td>
<td>0.379</td>
<td>0.121</td>
<td>0.109</td>
</tr>
<tr>
<td>Amide N.</td>
<td></td>
<td>0.062</td>
<td></td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>Creatine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td></td>
<td></td>
<td>0.155</td>
<td>0.044</td>
<td>0.034</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td></td>
<td></td>
<td>0.618</td>
<td>0.175</td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td></td>
<td></td>
<td>0.753</td>
<td>0.258</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td></td>
<td></td>
<td>0.534</td>
<td></td>
<td>0.008</td>
</tr>
<tr>
<td>Arginine</td>
<td></td>
<td></td>
<td></td>
<td>0.078</td>
<td></td>
</tr>
<tr>
<td>Amidine</td>
<td></td>
<td></td>
<td>0.595</td>
<td>0.072</td>
<td></td>
</tr>
<tr>
<td>Ornithine</td>
<td></td>
<td></td>
<td>0.480</td>
<td></td>
<td>0.085</td>
</tr>
<tr>
<td>Nucleic acid (Schneider)</td>
<td></td>
<td></td>
<td></td>
<td>0.226</td>
<td></td>
</tr>
<tr>
<td>&quot;     (Hammarsten)</td>
<td></td>
<td></td>
<td></td>
<td>0.785</td>
<td></td>
</tr>
<tr>
<td>Purine ppt.</td>
<td></td>
<td></td>
<td></td>
<td>0.706</td>
<td></td>
</tr>
<tr>
<td>&quot;     filtrate</td>
<td></td>
<td></td>
<td></td>
<td>0.628</td>
<td></td>
</tr>
</tbody>
</table>

* Exclusively of nucleic acid.

**TABLE III**

*Distribution of Isotopes in Excreta*

<table>
<thead>
<tr>
<th>Component</th>
<th>$N^{15}$ concentration</th>
<th>Total N</th>
<th>Total $N^{15}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>atom per cent excess</td>
<td>m.eq.</td>
<td>m.eq.</td>
</tr>
<tr>
<td>Feces</td>
<td>0.440</td>
<td>22.4</td>
<td>0.10</td>
</tr>
<tr>
<td>Urine</td>
<td>2.19</td>
<td>125.5</td>
<td>2.76</td>
</tr>
<tr>
<td>Urea</td>
<td>2.13</td>
<td>111.4</td>
<td></td>
</tr>
<tr>
<td>Ammonia</td>
<td>1.25</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Allantoin</td>
<td>0.275</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

With the remainder of the urine, total nitrogen, urea nitrogen, ammonia nitrogen, and the corresponding $N^{15}$ contents were determined. The results are shown in Table III.

**DISCUSSION**

_**Recovery of Isotope**_—During the feeding period the rats consumed 2700 mg. of aspartic acid containing 6.34 m.eq. of $N^{15}$. About 90 per cent of
the isotope was accounted for in the fractions analyzed (Table IV). The remaining 10 per cent must be lost in the gastrointestinal content which was washed away.

It will be noted that 43.4 per cent of the isotope was excreted and 45.5 per cent retained in the animal body. Of this 45.5 per cent, 39.4 per cent was in the proteins, while only 6.1 per cent was in non-protein and lipoid constituents.

**Urinary Ammonia and Urea**—The relative concentrations of N\(^{15}\) in urinary ammonia and urea after feeding an isotopic nitrogenous substance depend not only upon the nature of the substance but also upon the rate of feeding and the interval of time during which the urine is collected.

### Table IV

| Distribution of Isotopes in Excreta and Animal Body |
|---------------------------------------------|-----------------|
|                                              | Total N\(^{15}\) in fraction | Per cent recovery |
|                                              | m.eq. | m.eq. |
| Feces                                        | 0.10  | 1.6   |
| Urine                                        | 2.76  | 43.4  |
| Non-protein N                                | 0.35  | 5.5   |
| Lipoid N                                     | 0.04  | 0.6   |
| Total protein N                              | 2.50  | 39.4  |
| Red cells                                    | 0.040 |       |
| Plasma                                       | 0.047 |       |
| Stomach-intestine                            | 0.292 |       |
| Internal organs                              | 0.378 |       |
| Carcass                                      | 1.200 |       |
| Skin                                         | 0.542 |       |
| Total recovery                               | 5.75  | 90.5  |
| Unaccounted for                              | 0.60  | 9.5   |

After a single feeding of an isotopic amino acid with N\(^{15}\) in the \(\alpha\)-amino group, there is a period when its concentration in blood is greater than that in the tissues because it must enter the blood before it can reach the tissues. Since urinary ammonia can be formed in the kidney directly from amino acid brought to it by the blood, the rate of this ammonia formation must depend upon the concentration of the amino acid in the blood. Urea formation, on the contrary, will depend upon the concentration of the amino acid in the liver. If this view is correct, the isotope concentration of the ammonia formed shortly after feeding should be greater than that of the urea; the ratio, N\(^{15}\) concentration in NH\(_3\) to N\(^{15}\) concentration in urea, should be high. This is shown by human experiments\(^1\) in which

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\(^1\) To be reported in a later paper.
urine samples were collected at short intervals after a single feeding of aspartic acid.

As more and more of the isotopic amino acid enters the liver and other tissues, two chief reactions can occur: (1) transamination and (2) oxidative deamination and subsequent urea formation. The isotopic amino acid formed by transamination and the original amino acid fed pass back and forth between the body proteins and the metabolic pool as equilibrium conditions may require. In the liver, both original and transaminated isotopic amino acids can yield their nitrogen for urea formation. In the kidneys, both can form ammonia. The ratio, N\(^{15}\) concentration in NH\(_3\) to N\(^{15}\) concentration in urea, in the urine collected 3 to 12 hours after a single feeding will depend primarily upon the relative rates of the redistribution of the amino group of the fed amino acid among the other amino acids and of urea formation. Under uniform conditions, this ratio should be characteristic of the amino acid fed.

With continuous feeding, as in the present experiment with rats, the total isotopic substance enters the blood in smaller amounts over a longer period of time than in the case of single feeding. Consequently, ammonia formation in the kidneys from the isotopic amino acid fed will be sustained, and the ratio, N\(^{15}\) concentration in NH\(_3\) to N\(^{15}\) concentration in urea, for the total urine over the entire feeding period will be higher than in the case of single feeding.

When the isotopic substance is mixed with food to which the rats have constant access, difference in habit of feeding may conceivably cause some variations in the ratio, N\(^{15}\) concentration in NH\(_3\) to N\(^{15}\) concentration in urea, in different experiments. Nevertheless, the results obtained under uniform conditions should be comparable.

The ratio, N\(^{15}\) concentration in NH\(_3\) to N\(^{15}\) concentration in urea, for aspartic acid in the present experiment is 0.59. Previous experiments with other natural amino acids with N\(^{15}\) in the \(\alpha\)-amino group have given values of 1.60 for L-leucine (12), 1.08 for L-lysine (13), and 0.96 to 1.2 for glycine (14, 15). For racemic amino acids, the values are much higher (16, 17). For ammonium citrate, the ratio of 0.38 has been reported (18).

As a source of urinary ammonia and urea, L-aspartic acid behaves like ammonia and unlike other natural amino acids so far studied in this laboratory. This finding suggests that the amino group of L-aspartic acid is more rapidly removed to form urea than its deamination to form ammonia in the kidneys, either directly or through glutamine (19).

Whether the amino group is actually first deaminated to give ammonia which then forms urea, or whether it gives rise directly to urea, it is impossible to decide at present. It is interesting to note in this connection that a mechanism exists for the direct formation of urea from aspartic acid.
Ratner (20) has prepared a soluble enzyme system from beef liver which catalyzes the formation of arginine from citrulline, aspartic acid, and L-phosphoglyceric acid. If this is an important mechanism for the formation of urea in the intact rat, a high isotope concentration in the ammonia relative to the urea might be expected.

Distribution of Isotope in Proteins—The general pattern of distribution of N\(^{15}\) in various organ proteins after feeding isotopic aspartic acid is about the same as after feeding with other amino acids (see Table IV). These concentrations reflect the rate of turnover of the proteins of the various organs.

Distribution of Isotope in Protein Components—As in previous experiments in this laboratory with labeled amino acids, the N\(^{15}\) concentration in any component follows the same order as the N\(^{15}\) concentration in the total protein of various organs. Thus, the N\(^{15}\) concentrations of tyrosine isolated from internal organs, carcass, and skin are 0.155, 0.044, and 0.034 atom per cent excess respectively, while the corresponding concentrations for the total proteins are 0.379, 0.121, and 0.109 respectively.

In previous experiments in this laboratory, the protein component that had the highest N\(^{15}\) concentration was the amino acid fed. Glutamic acid usually came next, followed by aspartic acid. In the present experiment glutamic acid had the highest concentration of N\(^{15}\), although aspartic acid was the amino acid fed. This is similar to the finding after feeding isotopic ammonia (21, 22) and is in harmony with the hypothesis that the amino group of aspartic acid behaves like ammonia.

The high N\(^{15}\) content of amidine nitrogen of arginine from internal organs is to be expected in view of the part arginine plays in the urea formation in the liver. Whereas in the internal organs, which include the liver, the amidine nitrogen had a higher N\(^{15}\) concentration than the total protein, this is not true for the skin, which is not a site of urea formation.

The negligible N\(^{15}\) content of lysine is to be expected, as this amino acid, considered as an essential amino acid in nutrition, cannot be synthesized in the body.

The high concentration of N\(^{15}\) in the nucleic acid of stomach-intestine suggests that it has a high rate of turnover. This is noteworthy, especially as the N\(^{15}\) concentration of nucleic acid of stomach-intestine is higher than that of the total protein, while in the other internal organs the reverse is the case. This point, however, requires further investigation.

The nucleic acid of internal organs has about the same N\(^{15}\) concentration as the allantoin in the urine. This is to be expected, as the allantoin is derived from the purines.

The low N\(^{15}\) contents of creatine and hemin found in the present experiment are in general agreement with previous findings (23, 24) after feeding ammonia or other amino acids.
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1. L-Aspartic acid was synthesized from fumaric acid and ammonia containing $^{15}N$ in the presence of *Escherichia coli.*

2. The isotopic aspartic acid was added to the stock diet of three rats for 3 days. Only 1.6 per cent of the $^{15}N$ was excreted in the feces and 43.4 per cent in the urine, and 45.5 per cent was retained in the body.

3. The concentration of $^{15}N$ was the highest in the plasma proteins, and least in the red cell protein, with stomach-intestine, internal organs, carcass, and skin occupying the intermediate positions.

4. The $^{15}N$ content of urinary ammonia was lower than that of urea.

5. The $^{15}N$ concentration of aspartic acid was lower than that of glutamic acid isolated from the same organs.

6. These last two findings together suggest that aspartic acid is so rapidly deaminated that its amino group behaves metabolically like ammonia.

We are indebted to Mr. I. Sucher for the $^{15}N$ determinations, to Miss Laura Ponticorvo and Miss Martha Masako Yamasaki for technical assistance, and to Dr. D. Shemin and Dr. G. L. Foster for their kindly interest and helpful suggestions.

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