CARBON DIOXIDE AND AMMONIA FIXATION IN THE BIO-
SYNTHESIS OF CITRULLINE*

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In the preceding paper (1) an enzyme preparation was described which catalyzed the synthesis of citrulline from ornithine in the presence of adenosinetriphosphate (ATP), ammonia, magnesium and bicarbonate ions, and carbamylglutamate. Glutamic acid could not replace the latter compound.

Balance studies have failed to provide satisfactory evidence for the role of carbamylglutamate in this system. Therefore we have attempted to obtain more certain information as to the source of the carbamyl group of citrulline by the use of isotopically labeled compounds. Experiments were carried out with C\textsubscript{14}-labeled carbon dioxide, carbamylglutamic acid labeled with C\textsubscript{14} in the carbamyl group, and with N\textsuperscript{15}-labeled ammonia.

The experiments reported in this paper show clearly that carbamylglutamate does not participate in the synthesis of citrulline by the transfer of its carbamyl group to ornithine as previously postulated (2). It will be shown in the present paper that the carbamyl group of the citrulline synthesized can be accounted for in terms of the free ammonia and bicarbonate ions added to the system.

Procedures

Analytical methods as well as enzymatic preparations have been described previously (1, 2). In all the experiments, the enzymatic Fraction B obtained by ethanol precipitation of extracts of acetone powder from rat liver residues was used. The enzymatic synthesis of urea from citrulline was carried out with the enzymatic preparation described by Ratner and Pappas (3). The \textit{L} forms of carbamylglutamic acid, ornithine, and citrulline were used in the experiments as indicated. N\textsuperscript{15} analysis was performed with a consolidated Nier mass spectrometer. Radioactivity was estimated by standard techniques. Carbamylglutamic

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acid labeled with C\textsuperscript{14} in the carbamyl group was synthesized by means of Reactions 1 to 3.\textsuperscript{1,2}

\[
\text{KC}^{14}\text{N} + S \rightarrow \text{KC}^{14}\text{NS} \quad (1)
\]

\[
\text{KC}^{14}\text{NS} + \text{ZnO} \rightarrow \text{ZnS} + \text{KC}^{14}\text{NO} \quad (2)
\]

\[
\begin{array}{c}
\text{COOH} \\
\text{CH}_2 \\
\text{CH}_2 \\
\text{H-C-NH}_2 \\
\text{OOOH}
\end{array} + \text{KC}^{14}\text{NO} \rightarrow
\begin{array}{c}
\text{COOH} \\
\text{CH}_2 \\
\text{CH}_2 \\
\text{H-C-NH}-\text{C}^{14}-\text{NH}_2 \\
\text{COOH}
\end{array} \quad (3)
\]

Ammonium chloride-N\textsuperscript{15} was obtained by conversion of commercial ammonium nitrate containing 32 atom per cent N\textsuperscript{15} excess.

In all experiments dealing with the fate of ammonia, large incubation volumes were used in order to facilitate chemical isolation. Smaller volumes were used when radioactive carbon was employed since it was possible in these experiments to use larger dilutions with non-isotopic material without affecting the analytical accuracy.

In all cases colorimetric analyses were performed on small aliquots of the incubation mixtures after deproteinization with perchloric acid. The main part of the incubation mixture was deproteinized with trichloroacetic acid, since deproteinization with perchloric acid results in precipitation of insoluble perchlorates upon concentration, which are particularly disturbing when carbamylglutamate is to be isolated. Aliquots of the clear supernatant fluid after centrifugation were conveniently diluted with non-isotopic citrulline or carbamylglutamate. In experiments in which isotopic ammonia was used the samples were freed of ammonia by steam distillation at an alkaline pH. When isotopic carbon dioxide was used, the experimental samples were freed of isotopic carbon dioxide by gassing the medium with non-isotopic carbon dioxide.

Isolation of Citrulline—Citrulline was separated as the insoluble copper

\textsuperscript{1} Carbamylglutamate was recrystallized to constant radioactivity. The specific activity was unchanged after conversion of the carbamylglutamate to 5-propionic hydantoin, which was recrystallized four times from water. Further, carbamylglutamate was degraded with acid potassium permanganate according to an unpublished method of Cohen and Koritz. The urea formed was decomposed with an excess of urease; the liberated carbon dioxide was then collected as BaCO\textsubscript{3} and its radioactivity determined. The specific activity of the BaCO\textsubscript{3} sample was of the same order as that of the carbamylglutamate.

\textsuperscript{2} We wish to thank Dr. Heidelberger of the McArthur Memorial Laboratory, University of Wisconsin, for his generosity in providing us with KC\textsuperscript{14}N and for helpful discussion.
salt. In the first experiments the separation was carried out essentially by the scheme previously described (4). It has been found simpler and equally satisfactory to precipitate the amino acid with copper carbonate at neutral pH, followed by conversion to the free amino acid by treatment with hydrogen sulfide and reconversion of the citrulline to the insoluble copper salt which has a characteristic melting point (5). The same amount of non-isotopic citrulline was added to both the control and experimental vessels and the citrulline was then isolated from both. By this procedure the possibility of coprecipitation of other isotope-containing metabolites could be determined and corrected for.

Isolation of Carbamylglutamate—This compound was separated from the incubation mixtures, after deproteinization and convenient dilution with non-isotopic carbamylglutamate, by conversion into the corresponding hydantoin. This was carried out by evaporation to near dryness on a hot-plate in the presence of 20 per cent hydrochloric acid (6). The 5-propionic acid hydantoin was recrystallized five times from water. Control solutions were diluted with non-isotopic carbamylglutamate and further treated in the same manner as the experimental solutions.

Isolation of Urea

Urea was isolated as the dixanthydrol urea, as previously described (4).

Results

As can be seen from Table I, Experiment 1, the enzymatic incubation of ornithine with N\textsuperscript{15}-labeled ammonia and magnesium and bicarbonate ions in the presence of ATP and carbamylglutamate results in the incorporation of the ammonia into the carbamyl group of citrulline. The magnitude of the incorporation is such that it must be concluded that ammonia is fixed without exchange with or replacement of the carbamyl group nitrogen of carbamylglutamate. If exchange had taken place under the experimental conditions used, the citrulline isolated would have contained a lower N\textsuperscript{16} concentration. Experiments 2 to 4, Table I, were conducted in order to ascertain whether or not the N\textsuperscript{15} fixation in citrulline could be accounted for in terms of either a direct exchange reaction between citrulline and free ammonia or indirectly by way of carbamylglutamate. From the results of Experiments 2 and 3 shown in Table I, it is seen that incubation of the enzymatic system in the absence of ATP with isotopic ammonia in the presence of either carbamylglutamate or citrulline does not result in appreciable incorporation of the N\textsuperscript{15}-nitrogen.

\*Since copper carbonate is insoluble, the precipitation of the copper citrullinate complex was carried out with slightly less than the theoretical amount required for total precipitation of the amino acid present.
in either of the compounds. Nevertheless, in the presence of ATP there is an extensive exchange between N\textsuperscript{15}-labeled ammonia and the carbamyl group nitrogen of citrulline, although carbamylglutamate itself picks up very little N\textsuperscript{15} (Experiment 4, Table I). It must thus be concluded that

Table I

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Compound isolated</th>
<th>Observed N\textsuperscript{15} concentration</th>
<th>N\textsuperscript{15} concentration corrected for dilution</th>
<th>NH\textsubscript{3} fixation</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Citrulline</td>
<td>0.500 (atom per cent excess)</td>
<td>25.50 (atom per cent excess)</td>
<td>79.6</td>
<td>Complete system</td>
</tr>
<tr>
<td>2</td>
<td>Citrulline</td>
<td>0.024 (atom per cent excess)</td>
<td>2.34 (atom per cent excess)</td>
<td>7.3</td>
<td>ATP and ornithine excluded; citrulline added</td>
</tr>
<tr>
<td>3</td>
<td>5-Propionic acid-hyoxanthine</td>
<td>0.020 (atom per cent excess)</td>
<td>0.80 (atom per cent excess)</td>
<td>2.5</td>
<td>ATP and ornithine excluded</td>
</tr>
<tr>
<td>4</td>
<td>Citrulline</td>
<td>0.210 (atom per cent excess)</td>
<td>14.16 (atom per cent excess)</td>
<td>43.8</td>
<td>Ornithine excluded; citrulline added</td>
</tr>
<tr>
<td>5</td>
<td>5-Propionic acid-hydantoin</td>
<td>0.007 (atom per cent excess)</td>
<td>0.73 (atom per cent excess)</td>
<td>2.3</td>
<td>Ornithine excluded; citrulline added</td>
</tr>
<tr>
<td>6</td>
<td>Citrulline</td>
<td>0.607 (atom per cent excess)</td>
<td>28.20 (atom per cent excess)</td>
<td>88.0</td>
<td>Complete system</td>
</tr>
<tr>
<td></td>
<td>Citrulline</td>
<td>0.187 (atom per cent excess)</td>
<td>3.94 (atom per cent excess)</td>
<td>12.3</td>
<td>Ornithine excluded; citrulline added</td>
</tr>
</tbody>
</table>

Final substrate concentrations, MgSO\textsubscript{4}, 5 \times 10^{-3} M; phosphate buffer, pH 7.2, 1 \times 10^{-2} M; NaHCO\textsubscript{3}, 6 \times 10^{-3} M; NH\textsubscript{4}Cl (containing 32 atom per cent N\textsuperscript{15} excess), 2.5 \times 10^{-3} M; ATP (no ATP was used in Experiments 2 and 3), 3 \times 10^{-3} M; carbamyl-L-glutamate, 1.8 \times 10^{-3} M; L-ornithine in Experiment 1 (which resulted in a synthesis of 100 \muM of citrulline), 1 \times 10^{-3} M; L-ornithine in Experiment 5 (which resulted in a synthesis of 98 \muM of citrulline), 2 \times 10^{-3} M. In Experiments 2, 4, and 6, L-citrulline, 1 \times 10^{-3} M, was used. Each incubation mixture contained in addition to the above components 150 mg. of purified enzyme Preparation B. Final volume, 100.0 ml., gas phase, air at 38°C. Incubation time, 9 hours for Experiments 1 to 4; 1 hour for Experiments 5 and 6.

neither the exchange reaction nor the over-all synthesis occurs through direct participation of the carbamyl group nitrogen of carbamylglutamate.

Since the above experiments were conducted under long incubation periods in order to determine the extent of exchange reaction, it seemed desirable to obtain additional information under conditions which would not permit an extensive exchange. This was done by shortening the incubation time and by using an excess of substrate (ornithine).

The results of the control experiments, shown in Experiment 6, Table I, indicate that only a small exchange has occurred between N\textsuperscript{15}H\textsubscript{3} and citrulline. On the other hand, it can be seen from the data given in
Experiment 5, Table I, that almost quantitative incorporation of the labeled N\textsuperscript{15}-ammonia into citrulline has taken place. That the incorporation of ammonia occurs exclusively in the carbamyl group nitrogen of citrulline is clearly seen from the per cent incorporation values shown in Experiments 1 and 5, Table I. Additional evidence for this was obtained by converting a sample of the citrulline, isolated in Experiment 1, Table I, to urea by the system described by Ratner and Pappas (3). Urea was isolated as the dixanthydrol derivative and found to contain 0.794 per cent N\textsuperscript{15} excess. This figure, corrected for dilution, as well as

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Compound isolated</th>
<th>Specific activity of isolated compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Controls (c.p.m. per μM)</td>
</tr>
<tr>
<td>1</td>
<td>Carbon dioxide</td>
<td>2750</td>
</tr>
<tr>
<td></td>
<td>Citrulline</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5-Propionic acid hydantoin</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>392</td>
</tr>
<tr>
<td></td>
<td>Citrulline</td>
<td>0</td>
</tr>
</tbody>
</table>

Final substrate concentrations, MgSO\textsubscript{4}, 5 \times 10^{-3} M; phosphate buffer, pH 7.2, 1 \times 10^{-5} M; NaHCO\textsubscript{3} (in Experiment 1 the bicarbonate ions contained C\textsuperscript{14} of specific activity, as indicated), 1 \times 10^{-2} M; NH\textsubscript{4}Cl, 5 \times 10^{-3} M; ATP, 5 \times 10^{-3} M; carbamyl-L-glutamate, 5 \times 10^{-3} M (in Experiment 2, C\textsuperscript{14}-labeled carbamyl-L-glutamate was used, the specific activity of which is indicated in Table II); L-ornithine (the control experiments were without ornithine), 5 \times 10^{-3} M; 27 mg. of purified enzyme Preparation B were used in each experiment. Final volume, 4.0 ml.; temperature 38°C; gas phase, air; time of incubation, 78 minutes. Under these conditions the system synthesized about 12 μM of citrulline. In Experiments 1 and 2, no citrulline synthesis was detectable in the control vessels. Nitrogen atoms present in urea, gave a corrected figure of 27.6 per cent (0.794 \times 17 \times 2 = 27.6), which is 84.5 per cent of the starting N\textsuperscript{15} concentration of the free ammonia and practically the same value as that calculated from the analysis of the isolated citrulline in the same experiment. If citrulline had contained significant amounts of the N\textsuperscript{15} incorporated during the synthetic reaction in any other nitrogen atom but that of the carbamyl group, a large dilution would have been observed in the isolated urea, as a result of the transimination reaction of citrulline with aspartic acid.

From Experiment 1, Table II, it is clear that incubation of C\textsuperscript{14}-labeled sodium bicarbonate with ammonia and magnesium ions in the presence of ornithine, ATP, carbamylglutamate, and the enzyme system, results in a total incorporation of the carbon dioxide into citrulline, as indicated
from the specific activity of the isolated citrulline. The control incubation mixture, carried out under the same conditions as were used with the experimental vessels but in the absence of ornithine (under which condition no citrulline synthesis occurs), was treated with the same amount of non-isotopic citrulline as was used for dilution purposes in the experimental vessel, followed by isolation under similar conditions. No activity could be detected in the isolated amino acid, indicating that no contamination with other radioactive material occurred during the isolation procedure.

Carbamylglutamate was isolated as 5-propionic acid hydantoin from both experimental and control incubation mixtures, as described above. No radioactivity was detectable in the control experiments and such low activity was found in the samples from the experimental vessels that it must be concluded that carbamylglutamate does not exchange the carbon of its carbamyl group with the bicarbonate present in the medium.

In Experiment 2, Table II, similar experiments are recorded with the exception that non-isotopic carbon dioxide was substituted for the isotopic bicarbonate and carbamylglutamate labeled with C\textsuperscript{14} in the carbamyl group was used. As shown in Table II, no radioactivity could be detected in the isolated citrulline from both experimental and control incubation mixtures. A further indication of the lack of exchange of the carbamyl group carbon in carbamylglutamate has been obtained by incubating C\textsuperscript{14}-labeled carbamylglutamate with rat liver washed residue preparation by using the experimental conditions previously described (2). The carbon dioxide present was collected in KOH, converted to BaCO\textsubscript{3}, and assayed for radioactivity. No radioactivity could be detected.

**DISCUSSION**

On the basis of the isotopic studies reported in this paper, the over-all reaction leading to the synthesis of citrulline from ornithine can be formulated as in the accompanying structure.
While the over-all formulation is essentially that proposed by Krebs and Henseleit (7), it fails to provide information as to the nature of the intermediates. Since there is theoretical as well as experimental objection to assuming a direct carboxylation of the δ-amino group of ornithine (2), one must assume that the primary fixation of carbon dioxide takes place with another constituent of the system. The evidence that glutamic acid acts as the primary CO₂ acceptor has been presented previously from studies with washed liver residue preparations (2). However, still to be accounted for is the almost specific requirement for carbamyl-glutamate with the soluble enzyme used in the present studies. Glutamic acid cannot replace carbamylglutamic acid in this system. One is thus forced to conclude that carbamylglutamate or a closely related compound is more directly concerned with the fixation of carbon dioxide and NH₃ leading to citrulline synthesis. The possible rôle of a phosphorylated derivative of carbamylglutamate has been discussed in the preceding paper (1).

In accordance with the present findings, citrulline seems to be synthesized by a chain of reactions in which a carbamyl type of compound is formed. The following series of reactions has been postulated to account for the findings to date.

1. \[ \text{CO}_2 + \text{NH}_3 + \text{glutamate} \xrightarrow{\text{ATP}} \text{carbamylglutamate} \]
2. \[ \text{Carbamylglutamate} + \text{ATP} \xrightarrow{} \text{carbamylglutamate} \sim \text{P} + \text{ADP} \]
3. \[ \text{Carbamylglutamate} \sim \text{P} + \text{NH}_3 + \text{CO}_2 \rightarrow \text{intermediate} \]
4. \[ \text{Intermediate} + \text{ornithine} \rightarrow \text{citrulline} + \text{carbamylglutamate} \]
5. \[ 2\text{CO}_2 + 2\text{NH}_3 + \text{glutamate} + \text{ornithine} \xrightarrow{\text{ATP}} \text{citrulline} + \text{carbamylglutamate} \]

**SUMMARY**

Studies with N¹⁵H₃, C¹⁴O₂, and C¹⁴-labeled carbamylglutamic acid revealed that the carbon and nitrogen of the carbamyl group of the citrulline synthesized by a soluble enzyme system were derived from the NH₃ and CO₂ added to the medium. Neither the carbon nor nitrogen of the carbamyl group of carbamylglutamic acid appeared in the carbamyl group of citrulline.

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