STUDY OF CARBON DIOXIDE FIXATION IN THE SYNTHESIS OF CITRULLINE*

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(Received for publication, July 29, 1948)

The ornithine → citrulline → arginine cycle of urea synthesis originally proposed by Krebs and Henseleit (1) has had increasing experimental support (2-10). The position of citrulline in the cycle, however, has been questioned (11). With the advent of the successful separation of the two over-all enzymatic steps, ornithine → citrulline and citrulline → arginine (10), from the intact cellular system the position of citrulline appeared to be well established. However, it seemed desirable to determine the intermediary rôle of citrulline by the use of carbon dioxide containing C14. In the present paper it is demonstrated that the incorporation of C14 into the carbonyl group of citrulline and urea is of such a magnitude that citrulline must be considered as an obligatory intermediate in the urea synthesis cycle.

Procedures

Tissue Preparations—The enzyme preparations used in this study were the KCl-washed rat liver residue for the step ornithine → citrulline (10) and whole liver homogenate for the step citrulline → urea, both previously described by Cohen and Hayano (9).

Substrates—L-Ornithine and L-citrulline were prepared from L-arginine according to the method of Hunter (12) and Gornall and Hunter (13). Adenosine triphosphate (ATP) was prepared from rabbit muscle (14).

Analytical—Citrulline was estimated by the colorimetric method of Archibald (15). Urea was determined either by the manometric method of Krebs and Henseleit (1) or the colorimetric method of Archibald with isonitrosopropiophenone (16). The measurement of radioactivity was carried out according to Reid (17) with a thin mica window counter. Preparations of C14-containing samples for counting were collected and dried on thin aluminum cups of known area. For orientation purposes some preparations were counted directly as dry films after adsorption of small samples on a layer of lens paper filling the bottom of aluminum cups. With care, reproducible results are obtained with this technique.

* Aided in part by a grant from the Wisconsin Alumni Research Foundation.
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Results

Preliminary small scale experiments were carried out in which total C\textsuperscript{14}O\textsubscript{2} fixation was estimated for the step ornithine $\rightarrow$ citrulline (Table I). It will be noted that in all three experiments the elimination of either glutamic acid or ornithine results in a marked decrease in CO\textsubscript{2} fixation of the order of one-tenth to one-twelfth that of the complete system.

A large scale experiment was carried out under the conditions described in Table II. The reaction was stopped by the addition of 5.0 ml of 1 N HCl and deproteinized by heating. The CO\textsubscript{2} liberated on the addition of

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Incubation mixture</th>
<th>C\textsuperscript{14}O\textsubscript{2} fixed in protein-free medium*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Complete system</td>
<td>100</td>
</tr>
<tr>
<td>&quot;</td>
<td>Without ornithine</td>
<td>10</td>
</tr>
<tr>
<td>&quot;</td>
<td>Glutamic acid</td>
<td>3</td>
</tr>
<tr>
<td>&quot;</td>
<td>Glutamic acid and NH\textsubscript{3}</td>
<td>9</td>
</tr>
<tr>
<td>B</td>
<td>Complete system</td>
<td>100</td>
</tr>
<tr>
<td>&quot;</td>
<td>Without ornithine</td>
<td>8</td>
</tr>
<tr>
<td>&quot;</td>
<td>Fumaric acid replacing glutamic acid</td>
<td>10</td>
</tr>
<tr>
<td>C</td>
<td>Complete system</td>
<td>100</td>
</tr>
<tr>
<td>&quot;</td>
<td>Without ornithine</td>
<td>12</td>
</tr>
<tr>
<td>&quot;</td>
<td>Glutamic acid</td>
<td>10</td>
</tr>
</tbody>
</table>

Table I
Preliminary Experiments for Estimation of C\textsuperscript{14}O\textsubscript{2} Fixation

Final substrate concentrations were as follows: $3.8 \times 10^{-2}$ M L-glutamate, $2.5 \times 10^{-3}$ M L-ornithine, $5 \times 10^{-3}$ M NH\textsubscript{4}Cl, $1.25 \times 10^{-2}$ M phosphate buffer, pH 7.15, $3.3 \times 10^{-2}$ M Mg\textsubscript{2}SO\textsubscript{4}, $1.5 \times 10^{-3}$ M ATP, $6 \times 10^{-3}$ M NaH\textsuperscript{14}CO\textsubscript{3}, and potassium ions to bring the medium to isotonicity and a total volume of 4.0 ml. Each cup contained 3.5 mg of washed residue N. Incubation time 40 minutes at 38°.

* The radioactivity of the protein-free medium is expressed as relative values. 100 is equivalent to a total fixation of about 25 to 30 per cent of the C\textsuperscript{14} added.

acid was collected in alkali. Aliquots of the alkali samples containing the C\textsuperscript{14}O\textsubscript{2} were analyzed for total carbonate content by the standard Warburg manometric technique. The residual C\textsuperscript{14}O\textsubscript{2} was then precipitated as BaCO\textsubscript{3} for determination of radioactivity. Any residual C\textsuperscript{14}O\textsubscript{2} present in the reaction mixture was washed out by flushing with non-isotopic CO\textsubscript{2}, which was then removed by prolonged flushing with CO\textsubscript{2}-free air. The precipitated protein was centrifuged off, the supernatant filtered through retentive paper, and aliquots of the filtrate analyzed for citrulline and urea. 48 micromoles of citrulline were found and no urea was detected. Evapo-
ration of an aliquot to a dry film and assay for radioactivity indicated that a high CO₂ fixation had occurred.¹

The reaction mixture after incubation and deproteinization was divided into three fractions which were diluted with non-isotopic L-citrulline. The dilutions were 5, 50, and 150 times the original citrulline present. These samples were now treated with Ba(OH)₂ and ethyl alcohol, according to Jones and Moeller (18), in order to remove dibasic amino acids. The precipitates were collected and aliquots assayed for radioactivity. All showed some activity due in part to the presence of some adsorbed citrulline on the crude barium salts. The precipitates were then resuspended in H₂SO₄. The BaSO₄ which was separated by centrifugation contained no appreciable radioactivity. Treatment of the supernatant with non-isotopic L-citrulline

**Table II**

Radioactivity of Carbon Dioxide, Citrulline, and Urea

<table>
<thead>
<tr>
<th>Compound</th>
<th>Specific activity counts per min. per micromole compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon dioxide</td>
<td>420</td>
</tr>
<tr>
<td>Citrulline</td>
<td>413</td>
</tr>
<tr>
<td>Urea</td>
<td>410</td>
</tr>
</tbody>
</table>

The reaction mixture for citrulline synthesis contained the following components: 3.8 × 10⁻² M L-glutamate, 3.3 × 10⁻³ M L-ornithine, 6.6 × 10⁻³ M NH₄Cl, 2 × 10⁻³ M ATP, 1 × 10⁻³ M phosphate buffer, pH 7.15, 3.3 × 10⁻³ M MgSO₄, 9 × 10⁻³ M NaHCO₃, and KCl ions to bring the medium to isotonicity (activity of C¹⁴ measured in a Geiger-Müller counter, 36,000 counts per minute per mg. of carbon). Washed residue 28 mg. of N. The total volume of the reaction mixture was 30 ml. The mixture was incubated at 35° for 40 minutes in a closed vessel and in the presence of air.

and further precipitation with Ba(OH)₂ and alcohol resulted in a drop in radioactivity of the precipitate. As the activity of these precipitates was very low, isolation and identification of this fraction were not undertaken in the present studies. For precipitation of the citrulline the method of Vickery and Gordon (19) employed for other amino acids was used. The supernatant from the first Ba(OH)₂ treatment was freed of alcohol by evaporation and brought to pH 7.0 by the addition of H₂SO₄. After removal of BaSO₄, the solution was treated with HgCl₂ to twice the molarity of the citrulline present, and then Ba(OH)₂ was added to raise the pH to 9.3. The citrulline-Hg-Ba complex was separated by centrifugation, washed several times with water, alcohol, and ether, and then assayed for

¹ It has been observed that the metabolic CO₂ production under these conditions will account for a 5 per cent maximum dilution of the C¹⁴O₂ added.
radioactivity. The analytical values obtained with all three dilutions were consistent within 5 per cent. The samples were then taken up in dilute H$_2$SO$_4$, decomposed with H$_2$S, centrifuged, washed, and the supernatant and washings filtered through retentive paper. The filtrate was aerated and analyzed for citrulline. The samples were concentrated in vacuo to a small volume, and aliquots were converted enzymatically to urea according to Cohen and Hayano (10) except that aspartic acid was used instead of glutamic acid. Urea was separated either as xanthydrol urea according to the method of Allen and Luck (20) or decomposed with urease and the CO$_2$ precipitated as BaCO$_3$ in the usual way.

As can be seen from Table II the specific activity per micromole of urea and citrulline is practically the same as that of the bicarbonate of the medium.

Another procedure$^2$ employed in a large scale experiment for estimating the incorporation of C$^{14}$O$_2$ into citrulline involved the quantitative adsorption of citrulline from the deproteinized reaction mixture by Zeo-Karb according to the method reported by Archibald (15). Measurements of citrulline and radioactivity indicated quantitative adsorption of both by Zeo-Karb. Approximately 80 per cent of the citrulline was eluted from the Zeo-Karb by treatment with 30 per cent H$_2$SO$_4$. Analysis of the eluate after removal of sulfate ions with Ba(OH)$_2$ revealed the same ratio of radioactivity to citrulline content as before adsorption. The eluted citrulline solution was then heated at 105° for 1 hour with concentrated sodium hydroxide to decompose the terminal ureide group to carbon dioxide which was collected as BaCO$_3$. Measurement of radioactivity of this carbon dioxide showed a specific activity per mg. of carbon which was 98 per cent that of the original bicarbonate in the medium. Of interest was the finding that washing Zeo-Karb with 30 per cent H$_2$SO$_4$ gives rise to a substance in the washings which when heated with alkali reacts with diacetyl monoxime, thus interfering slightly with the determination of citrulline.

**DISCUSSION**

The synthesis of citrulline and urea with practically the same specific activity as that of the C$^{14}$O$_2$ present originally in the medium strongly supports the position of citrulline as an obligatory intermediate in the urea cycle. The relatively low rate of C$^{14}$O$_2$ fixation in the absence of ornithine or glutamic acid indicates that in this system citrulline synthesis is by far the most active CO$_2$ fixation reaction.

Attempts to demonstrate the formation of an intermediate carbamyl-glutamic acid derivative (21) in this study were unsuccessful owing chiefly

$^2$ This part of the study was carried out with the collaboration of Dr. M. Hayano.
to the instability and rapid conversion of this compound. Experiments in this direction are now in progress.

The authors are indebted to Dr. Robert H. Burris, Department of Biochemistry, for samples of C\textsuperscript{14}O\textsubscript{2} and for facilities for estimation of radioactivity.

**SUMMARY**

1. The synthesis of citrulline from ornithine in the presence of C\textsuperscript{14}O\textsubscript{2} has been studied in washed rat liver residue. The specific activity of the isolated citrulline is of the same order of magnitude as that of the C\textsuperscript{14}O\textsubscript{2} added.

2. The conversion of citrulline containing C\textsuperscript{14} in the carbonyl position to urea by liver homogenates results in the formation of urea with the same specific activity as that of the added citrulline.

3. The significance of these findings on the intermediary rôle of citrulline in the Krebs-Henseleit cycle is discussed.

**BIBLIOGRAPHY**

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