CARBON DIOXIDE AND ACETATE UTILIZATION BY CLOSTRIDIUM KLUYVERI

II. SYNTHESIS OF AMINO ACIDS*

BY N. TOMLINSON†

(From the Department of Plant Biochemistry, University of California, Berkeley, California)

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The isolation and identification of labeled amino acids from the acid hydrolysates of cells of Clostridium kluyveri grown in synthetic medium containing ethanol, acetate, and CO₂ as carbon sources, and with C¹⁴O₂ or acetate-1-C¹⁴ as labeled substrate, have been reported previously (28). Alanine, serine, glycine, threonine, and aspartic acid so obtained have been degraded to determine the position of the C¹⁴ in each compound, and the results are reported in the present paper.

Methods and Materials

Degradation of Amino Acids—Before the degradation procedures described below were carried out, suitable amounts of unlabeled carrier were added to the labeled compounds. The carrier was recrystallized twice, once from water with ethanol, then from water with acetone, and was examined for purity by paper chromatography before being used. After the carrier was added to a labeled compound, it was recrystallized twice more from water by the addition of acetone, then dissolved in water and dried on the steam bath. Samples from each crystallization were converted to CO₂, which was trapped as barium carbonate. The purity of the labeled amino acid was considered to be satisfactory if the second crystallization did not cause any significant decrease in specific activity.

The specific activity of the separate carbon atoms was determined as barium carbonate, except when the method of degradation yielded a carbon atom as formaldehyde. When this occurred, the dimedon derivative of formaldehyde was prepared and counted as such, the self-absorption correction being the same as that used for barium carbonate (34). The barium carbonate first obtained in the degradation procedures was always decomposed with freshly boiled lactic acid and the CO₂ released was again converted to barium carbonate prior to plating.

Glycine was degraded with ninhydrin by the method of Van Slyke et al.

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† Present address, Laboratory of Plant Pathology, Summerland, British Columbia.
The carbon dioxide liberated from the carboxyl group was trapped in barium hydroxide solution, and the residual formaldehyde was distilled into 0.4 per cent dinedon solution (34).

Ninhydrin degradation was used to obtain the carboxyl carbon of alanine as barium carbonate (33). The \( \alpha \) and \( \beta \)-carbon atoms were converted to acetate (14), which was degraded by pyrolysis (3).

Serine was degraded by the periodate method of Sakami (23) except that the formaldehyde was plated as the dinedon derivative. The specific activity of the carboxyl carbon was checked by ninhydrin degradation.

Ninhydrin degradation (33) was used to obtain the combined carbon of the two carboxyl groups of aspartic acid, and the chloramine-T method of Ehrensvärd et al. (10) was used for the determination of the 2 carbon atoms separately. The hypochlorite method of Langheld (18) was used to obtain the \( \alpha \)- and \( \beta \)-carbon atoms as acetaldehyde. The validity of this method has been checked by Ehrensvärd et al. (10). The acetaldehyde was oxidized to acetic acid with acid permanganate and degraded by pyrolysis (3).

Threonine was degraded by the periodate method (23). The acetaldehyde formed from C-3 and C-4 was distilled from the neutral reaction mixture into an ice-cooled receiver and oxidized to acetic acid with acid permanganate. The acetic acid was steam-distilled, purified by passage through a silica gel column (12), checked for purity and identity by paper chromatography (17), and degraded by the method of Phares (21).

Determination of Specific Activity of Labeled Amino Acids—The colorimetric method of Troll and Cannan (29) was used to determine the quantity of the amino acids in solutions of known \( ^{14} \text{C} \) content and a correction was made by the method of Thompson et al. (27) for interfering ninhydrin-positive material eluted from the chromatographic sheets with the amino acids. The accuracy of the determinations is \( \pm 10 \) per cent.

Assay of Radioactivity—This was conducted as described previously (28). The standard error of the count was less than \( \pm 3 \) per cent unless otherwise indicated in Tables I and II.

RESULTS AND DISCUSSION

The data obtained by degradation of the labeled amino acids, plus unlabeled carrier, are given in Table I. When \( ^{14} \text{C}^{4} \text{O}_{2} \) was the labeled substrate, the \( ^{14} \text{C} \) found in alanine, glycine, and aspartic acid was located almost exclusively in the carboxyl groups, the two carboxyl groups of aspartic acid being equally labeled. The larger portion of the \( ^{14} \text{C} \) in serine and threonine was found in their carboxyl groups, but about 8 per cent of the \( ^{14} \text{C} \) of serine was in the \( \beta \)-carbon atom, and about 42 per cent of the \( ^{14} \text{C} \) of threonine was in the \( \gamma \)-carbon atom. When acetate-1-\( ^{14} \text{C} \) was the labeled substrate, the \( ^{14} \text{C} \) was found nearly exclusively in the \( \alpha \)-carbon atom of each of the compounds degraded.
In Table II are recorded the actual specific activities of the labeled amino acids synthesized in the presence of $\text{C}^{14}\text{O}_2$. The data of this table, when considered with those of Table I, indicate that the carboxyl carbon atoms of each of these amino acids (specific activity 6.6 to 7.6 $\times 10^6$ c.p.m. per $\mu$M) must have originated mainly, if not exclusively, from carbon dioxide (initial specific activity 7.71 $\times 10^6$ c.p.m. per $\mu$M). It should be noted that the average specific activity of the carbon dioxide during the fermentation was about 5 per cent lower than the initial specific activity (28).

The formation of a 3-carbon unit from acetate, or a derivative thereof, and carbon dioxide has been investigated in cell-free bacterial systems, and

\begin{table}
\begin{center}
\begin{tabular}{|l|c|c|c|}
\hline
\text{Amino acid} & \text{Carbon atom} & \text{Labeled substrate, } \text{C}^{14}\text{O}_2 & \text{Labeled substrate, acetate-1-}\text{C}^{14} \\
\hline
\text{Alanine} & Total$\dagger$ & 590 & 6.0 \\
 & COOH & 1787 & 0.3 $\pm$ 0.1 \\
 & CH$_2$NH$_2$ & 0.5 & 17.4 \\
 & CH$_3$ & 2.3 & 0.5 $\pm$ 0.3 \\
\hline
\text{Serine} & Total & 245 & 4.3 $\pm$ 0.2 \\
 & COOH & 673 & 0.4 $\pm$ 0.1 \\
 & CH$_2$NH$_2$ & 1.3 $\pm$ 0.1 & 12.0 \\
 & CH$_2$OH & 61 & 0.5 $\pm$ 0.3 \\
\hline
\text{Glycine} & Total & 517 & 8.1 \\
 & COOH & 1038 & 0.4 $\pm$ 0.1 \\
 & CH$_2$NH$_2$ & $<0.4$ & 15.1 \\
\hline
\text{Aspartic acid} & Total & 533 & 3.2 \\
 & Combined COOH C & 1066 & 0.4 $\pm$ 0.1 \\
 & COOH & 1010 & \\
 & CH$_2$NH$_2$ & 0.6 $\pm$ 0.5 & 11.8 $\pm$ 0.4 \\
 & CH$_3$ & 2.2 $\pm$ 0.5 & 0.5 $\pm$ 0.3 \\
 & COOH & 1054 & \\
\hline
\text{Threonine} & Total & 278 & 3.5 \\
 & COOH & 618 & 0.4 $\pm$ 0.1 \\
 & CH$_2$NH$_2$ & 1.3 & 11.7 \\
 & CHOH & 1.1 & 0.5 $\pm$ 0.1 \\
 & CH$_3$ & 465 & 0.7 $\pm$ 0.2 \\
\hline
\end{tabular}
\end{center}
\end{table}

$*$ After dilution by addition of carrier amino acids.
$\dagger$ Determined on barium carbonate obtained by complete oxidation.
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more than one mechanism may be involved (22, 32). In Clostridium butylicum (35) carbon dioxide exchanges with the carboxyl group of pyruvate. Another condensation reaction, brought about by Escherichia coli extracts, requires the preliminary reduction of carbon dioxide to formic acid before it is incorporated in pyruvate (31). Although the mechanism of the reaction in C. kluyveri is unknown, the present data indicate the net production of a 3-carbon unit from carbon dioxide and acetate, or a 2-carbon unit derived from acetate.

Alanine and serine appear to arise from the same 3-carbon unit. The difference in labeling of the two compounds may result from exchange of

TABLE II
Specific Activity of Amino Acids Isolated from Acid Hydrolysate of Cells Grown in Presence of C¹⁴O₂

The isolation and identification of these amino acids have been described previously (28). The cells from which they were obtained were grown in 35 ml. of synthetic ethanol-acetate medium to which had been added 70 μM of C¹⁴-labeled carbonate of specific activity 7.71 × 10⁴ c.p.m. per μM and a 15 per cent inoculum grown in the same medium. The carbonate added with the inoculum diluted the labeled carbonate by 1 to 2 per cent.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Specific activity* c.p.m. per μM × 10⁻⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>6.6</td>
</tr>
<tr>
<td>Serine</td>
<td>7.4</td>
</tr>
<tr>
<td>Glycine</td>
<td>6.9</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>15.3</td>
</tr>
<tr>
<td>Threonine</td>
<td>12.5</td>
</tr>
</tbody>
</table>

* Before the addition of unlabeled carrier amino acids.

the β-carbon of serine with a 1-carbon unit. A number of investigators (1, 23, 25) have shown, in the rat, that serine can be converted reversibly to glycine plus formate. It also is possible that formaldehyde or a derivative thereof may be the 1-carbon intermediate (24). Although it has not been demonstrated that C. kluyveri carries out the conversion of carbon dioxide to formate, this is known to occur in other bacteria (36).

In the preceding paper of this series (28) it was shown that C¹⁴O₂ is converted to the methyl carbon atom of acetate to the extent of 200 c.p.m. per μM of C with no significant count in the carboxyl carbon atom. The total C¹⁴ recovered in the volatile fatty acids was 1 × 10⁶ c.p.m. in this culture. It has been shown recently that threonine is a good source of glycine in the rat (6) and is split to glycine and acetate (19). The labeling of threonine in the present experiments is such that it would require the splitting of only about 2 μM to account for the entire labeling of the
fatty acids. This explanation is attractive and is consistent with the data, but of course other possible reactions are not excluded.

Two possible explanations for the observed labeling of threonine are suggested. The first is the condensation of a 3-carbon unit, such as pyruvate, with a 1-carbon unit which originated for the most part in carbon dioxide, but which was diluted to the extent of 25 per cent from some other source, for example as a result of the production of a 1-carbon unit in the course of the formation of glycine from serine. The work of Hift and Mahler (15) has demonstrated the presence in beef liver of an enzyme which condenses formaldehyde and pyruvate to yield α-keto-γ-hydroxybutyrate. While no metabolic rôle has been ascribed to this compound, it might be converted to a precursor of threonine. In support of this idea is the finding of Teas et al. (26) that homoserine can replace threonine for growth of a Neurospora mutant.

Serine itself as a precursor of threonine appears to be ruled out by the lack of labeling of the β-carbon of threonine.

A second possible source of threonine has been suggested by Ehrensvärd et al. (10). They consider that threonine may originate from aspartic acid via homoserine, in view of the above mentioned finding of Teas et al. (26). However, in this case the γ-carbon and the carboxyl carbon should have equal specific activity, and it thus would be necessary to account for a reduction in the specific activity of the γ-carbon. This cannot occur by a reversal of the split of threonine to acetate and glycine (11), since C. kluyveri does not incorporate acetate carbon into the β- and γ-carbon atoms of threonine.

It is possible that the dilution may occur by formation of a portion of the threonine via the first mechanism.

The synthesis of aspartic acid with the carboxyls both formed from carbon dioxide and the α-carbon from the carboxyl of acetate may be accomplished by several known enzymatic reactions. The preliminary formation of a 3-carbon unit from acetate plus carbon dioxide has been discussed. The formation of a dicarboxylic acid from this 3-carbon unit or one derived from it, plus carbon dioxide, may occur by a number of possible mechanisms (2, 16, 20, 30, 32). The 4-carbon compound first arising in any of these reactions could be converted to oxalacetate and then to aspartic acid by transamination (5, 13). Any extensive equilibration of the aspartic acid precursor with a symmetrical compound such as fumaric acid is precluded by the virtual absence of acetate carboxyl carbon from the β position of aspartate.

The utilization of carbon dioxide and acetate by C. kluyveri and by the photosynthetic Rhodospirillum rubrum (7, 8) in the synthesis of the five amino acids considered in this paper appears to be very similar. The
observable differences probably arise from the ability of \( R. \) \textit{rubrum} to oxidize acetate to carbon dioxide, an ability which \( C. \) \textit{kluyveri} lacks (4, 28). On the other hand, \( C. \) \textit{kluyveri} differs markedly from \( E. \) \textit{coli} (9) and \( T. \) \textit{luppsi utilis} (10). When the two latter organisms are grown aerobically on acetate as sole carbon source, the \( \alpha \)- and \( \beta \)-carbon atoms of the five amino acids are formed from the methyl carbon atom of acetate and the carboxyl carbon atoms are derived from both the carboxyl and methyl carbons of acetate in about the same ratio as that found in the respiratory carbon dioxide.

**SUMMARY**

1. Alanine, serine, glycine, aspartic acid, and threonine obtained from the acid hydrolysate of cells of \( C. \) \textit{kluyveri} grown in a synthetic medium with ethanol, acetate, and carbon dioxide as carbon sources, and with either \( C^{14}O_2 \) or acetate-1-\( C^{14} \) as labeled substrates, have been degraded to determine the distribution of \( C^{14} \).

2. When \( C^{14}O_2 \) was the labeled substrate, it was shown that the carboxyl carbon atoms of each of the five amino acids was derived mainly, if not exclusively, from carbon dioxide. 8 per cent of the total \( C^{14} \) in serine was found in the \( \beta \)-carbon atom, and 42 per cent of the \( C^{14} \) in threonine was in the \( \gamma \)-carbon atom. The remainder of the carbon atoms of these and the other amino acids contained only negligible amounts of \( C^{14} \).

3. When acetate-1-\( C^{14} \) was the labeled substrate, the \( C^{14} \) found in the amino acids was almost all contained in their \( \alpha \)-carbon atoms.

4. The significance of these findings in relation to possible synthetic mechanisms is discussed.

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**BIBLIOGRAPHY**

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N. Tomlinson


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