UTILIZATION OF CARBON DIOXIDE IN THE SYNTHESIS OF PROTEINS BY ESCHERICHIA COLI. I

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Since carbon dioxide is always present in living systems where synthesis of proteins is occurring, it is desirable to study the fate of tracer isotopes of this rather simple metabolite before turning to more complicated substrates. Metabolic activity of most organisms leads to formation of CO₂ which in many cases is subsequently incorporated into amino acids, purines, pyrimidines, and other biologically important compounds. The studies leading to formulation of the Wood-Werkman reaction (1) demonstrated the importance of carbon dioxide as a participant in metabolism of heterotrophic organisms. Later investigations by Ruben and Kamen (2) and Evans and Slotin (3) indicated that carbon dioxide is utilized by mammalian tissues. The incorporation of isotopic carbon into proteins of mammalian liver was first demonstrated by Delluva and Wilson (4), who studied fixation of C¹³ derived from NaHCO₃ and found the isotope in aspartic and glutamic acids and arginine. These workers were hindered in their investigation by relatively low metabolism of the organisms used and the unavoidable dilution of the isotopic carbon with inactive carbon. Anfinsen et al. (5) have studied incorporation into liver slices of C¹⁴ derived from NaHCO₃. They also found isotopic carbon in aspartic and glutamic acids and arginine. Ehrensvärd (6) has studied incorporation of C¹³O₂ by the yeast Torulopsis utilis and has found isotopic carbon in the three amino acids already mentioned and lesser amounts in other amino acids.

We have investigated the metabolism of Escherichia coli in the presence of C¹⁴O₂ and have found that as much as 50 per cent of the isotopic tracer added to the medium was fixed by the organisms. Two-dimensional paper chromatograms of hydrolysates of these organisms followed by radioautographs revealed at least sixteen different radioactive components. Studies have been made to determine the components into which the C¹⁴ was synthesized. It has been possible to account for most of the radioactivity in proteins and nucleic acids. Isotopic carbon has been found in amino acids from protein hydrolysates including aspartic and glutamic acids, arginine, lysine, proline, and threonine. The location of isotopic tracers in these amino acids has been partially determined.
Carbox Dioxide in Protein Synthesis

Methods

Cells of *E. coli* strain B growing in the logarithmic phase were harvested and resuspended in a freshly boiled medium containing 6 gm. of Na₂HPO₄, 3 gm. of KH₂PO₄, 1 gm. of NH₄Cl, 5 gm. of NaCl, 0.2 gm. of MgSO₄·7H₂O per liter. Concentration of the organisms was adjusted to an optical density of 0.180 (about $5 \times 10^6$ cells per ml.) which amounted to 90 mg., wet weight, in the 150 ml. volume of medium employed. The cell suspension was placed in a glass-stoppered 1500 ml. flask, a solution containing 60 mg. of glucose and some NaHCO₃ was added, the flask was securely stoppered, and the mixture vigorously agitated on a shaking machine at 37° for 1 hour. In this time all the glucose was metabolized and the organisms about doubled in amount. Sufficient oxygen was present in the flask for aerobic growth and none of the CO₂ was lost from the system. The cells were harvested by centrifugation, washed, and chemically fractionated.

The high specific activities of the radioactive products of *E. coli* metabolism permitted rapid and simple radioactive assays. It was possible to limit samples to 2 mg. which, with the planchettes employed, amounted to no more than 0.3 mg. per sq. cm. For samples of this thickness no correction for self-absorption was necessary. It was also possible to employ an end, thin window Geiger counter, the stability and reproducibility of which are markedly superior to the flow type device. Use of paper and resin chromatography for chemical separations avoided introduction of extraneous chemicals into the assay samples.

Experimental

A first step in studying the metabolism of CO₂ by an organism is to determine the amount of exogenous CO₂ incorporated per mole of substrate consumed. The measurement is complicated by the dilution of the isotopic carbon which occurs during metabolism. It was therefore convenient first to measure the evolution of CO₂ per mole of glucose substrate consumed, and then to conduct a separate CO₂ uptake experiment in the presence of a large quantity of labeled CO₂. Another experiment showed that CO₂ incorporation was independent of its concentration in the medium over the experimental range involved. The measurement of CO₂ production was conducted in a 1500 ml. flask fitted with a stop-cock. An *E. coli* inoculum in the logarithmic phase was resuspended in 80 ml. of freshly boiled medium at pH 6.8. Glucose was added, the container securely stoppered, and the culture shaken at 37° until all the glucose (60 mg.) had been consumed (1 hour). The container was inverted and the liquid contents drawn out. The container was rinsed with 10 ml. of 0.01 N HCl and the CO₂ in the container absorbed by addition of 20 ml. of...
0.2 N NaOH and shaking for an hour. After collection of the hydroxide, the container was rinsed once again with 10 ml. of 0.2 N NaOH. The portions of hydroxide were combined and the carbonate precipitated as BaCO₃. The pH of the bacterial culture was measured and the quantity of CO₂ present in the medium determined by the Henderson-Hasselbalch equation. The experiment is summarized in Table I.

In the uptake experiment the 80 ml. of E. coli suspension were placed in a 1500 ml. glass container with 0.333 mole of glucose, and 4.0 moles of labeled CO₂ were added. The results of the experiment are shown in Table II.

In a given experiment the total uptake of isotopic tracer was governed by the inoculum, the amount of non-isotopic CO₂, the incubation time,

**TABLE I**

*Production of CO₂ by E. coli during Glucose Metabolism*

<table>
<thead>
<tr>
<th>Glucose consumed</th>
<th>Final pH</th>
<th>CO₂ in gas phase</th>
<th>CO₂ + HCO₃⁻ in medium</th>
<th>CO₂ formed per mole glucose consumed</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
</tr>
<tr>
<td>0.333</td>
<td>6.70</td>
<td>0.77</td>
<td>0.130</td>
<td>3.4</td>
</tr>
</tbody>
</table>

**TABLE II**

*C¹⁴O₂ Fixation by E. coli during Glucose Metabolism*

<table>
<thead>
<tr>
<th>Glucose consumed</th>
<th>Initial CO₂</th>
<th>Initial CO₂</th>
<th>Final CO₂</th>
<th>CO₂ fixed in cells</th>
<th>CO₂ fixed per mol glucose consumed</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>mM</td>
<td>c.p.s.</td>
<td>mM</td>
<td>c.p.s.</td>
<td>mM</td>
</tr>
<tr>
<td>0.333</td>
<td>4.0</td>
<td>16,000</td>
<td>4.8</td>
<td>252</td>
<td>0.21</td>
</tr>
</tbody>
</table>

and the nature and quantity of the substrate. For instance, in an experiment where the C¹⁴ was present in 0.001 mM of CO₂ and the calculated initial optical density of the culture was 0.0001, the organisms incorporated 50 per cent of the isotopic tracer while metabolizing 0.333 mM of glucose.

The over-all incorporation of C¹⁴O₂ into the growing organisms having been measured, a further step involved the study of the major chemical components of the cell into which the tracer was incorporated. The results of three growth experiments are shown in Table III.

The so called lipide fraction was obtained by extraction of the bacterial solids with hot 80 per cent alcohol followed by repeated ether-alcohol treatment. This fraction may contain free amino acids, Krebs cycle intermediates, phosphorylated carbohydrates, and a host of other compounds in addition to the lipides. It contained more than one radioactive component but was not examined in detail. The "nucleic acid" fraction
consisted principally of purines, pyrimidine nucleotides, and inorganic phosphorus. These were liberated by treating the bacterial solids with 5 per cent trichloroacetic acid at 90° for 15 minutes according to the procedure of Schneider (7). The residual solids which are termed "proteins" were hydrolyzed with 6 N HCl at 108° in a sealed tube for 15 hours. Aliquots of the three fractions were assayed for radioactivity.

### Table III

**Distribution of C<sup>14</sup> in E. coli**

<table>
<thead>
<tr>
<th>Radioactivity in counts per second</th>
<th>Experiment A</th>
<th>Experiment B</th>
<th>Experiment C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total radioactivity incorporated</td>
<td>2024</td>
<td>1940</td>
<td>2003</td>
</tr>
<tr>
<td>Lipide fraction</td>
<td>115</td>
<td>108</td>
<td>110</td>
</tr>
<tr>
<td>Nucleic acid</td>
<td>732</td>
<td>710</td>
<td>720</td>
</tr>
<tr>
<td>Protein hydrolysate</td>
<td>1261</td>
<td>1142</td>
<td>1173</td>
</tr>
<tr>
<td>Total recovered</td>
<td>2108</td>
<td>1960</td>
<td>2003</td>
</tr>
</tbody>
</table>

The initial optical density of all cultures was 0.180. The final optical densities of Experiments A, B, and C were 0.336, 0.333, and 0.335, respectively. The total radioactivity in the medium was 10,006 c.p.s.

### Table IV

**Fractionation of Radioactive Protein Hydrolysates by Using Dowex 2**

<table>
<thead>
<tr>
<th>Eluate fraction</th>
<th>Radioactivity in counts per second</th>
<th>Experiment A</th>
<th>Experiment B</th>
<th>Experiment C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>344</td>
<td>309</td>
<td>305</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>88</td>
<td>86</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Neutral amino acids</td>
<td>315</td>
<td>280</td>
<td>293</td>
<td></td>
</tr>
<tr>
<td>Aspartic and glutamic acids</td>
<td>455</td>
<td>416</td>
<td>423</td>
<td></td>
</tr>
</tbody>
</table>

The original radioactivities present before fractionation of the hydrolysates in Experiments A, B, and C were 1261, 1142, and 1173 c.p.s., respectively.

Inspection of Table III reveals an excellent balance. Under the conditions of our experiments 20 per cent of the original C<sup>14</sup> was incorporated. The amount found in the lipide fraction was proportionally much higher for short growth periods. The radioactive nucleic acid fractions have been studied and the results will be presented elsewhere.

The protein hydrolysates were further fractionated by chromatography<sup>1</sup> on Dowex 2. This anion exchanger holds up aspartic and glutamic acids when in the chloride form. We have chosen to employ a bed 75 per cent

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<sup>1</sup> The results of a detailed study of the chromatography of amino acids on Dowex 2 will be published elsewhere.
chloride and 25 per cent hydroxide, which holds up all amino acids except arginine. A column consisting of 2 ml. of resin in a 10 ml. burette gave useful separations of amino acids. A hydrochloric acid-free hydrolysate was placed on the column. Washing with 10 ml. of water completely eluted arginine but no other amino acid. Lysine was eluted with 6 ml. of 0.02 M NaCl. Threonine and proline were eluted in that order with additional 0.02 M NaCl. Lysine was almost completely resolved while the other two amino acids were only partially separated. Aspartic and glutamic acids were ultimately eluted with 0.1 N HCl. These separations were made in the course of an hour. The results are shown in Table IV.

This partial separation of the amino acids gives a qualitative picture of the major components of the labeled amino acids. Aspartic and glutamic acids and arginine were the major labeled constituents. Examination of the crude fractions, however, showed that all were appreciably contaminated either with breakdown products of protein hydrolysis or with peptides. For instance, only 85 per cent of the radioactivity of the arginine fraction could be precipitated by repeated flavianic acid treatments by using additional arginine carrier with each precipitation. On the other hand paper chromatograms of the crude material showed no impurity of amino acids and no major radioactive component other than arginine.

**Isolation of Amino Acids**

The various eluates were further fractionated usually by the procedure of Stein and Moore (8) on Dowex 50 or split into their respective components by paper chromatography. Crude arginine from the Dowex 2 column has been purified in two ways: precipitation with flavianic acid and purification by paper chromatography. The two methods showed that 85 per cent of the radioactivity of the crude material was authentic arginine. In the experiment described herein arginine eluates from Dowex 2 were neutralized, evaporated to dryness, and purified by paper chromatography with phenol. The purity of the arginine subsequently eluted was confirmed by a paper chromatogram, with a butanol-acetic acid solvent. Aliquot portions of the material were assayed for total radioactivity.

The C\(^{14}\) content of carboxyl carbon was determined by a modification of the method of Van Slyke *et al.* (9) in which CO\(_2\) was liberated by ninhydrin at pH 2.5, collected in NaOH, and precipitated as BaCO\(_3\). The amidine carbon of another aliquot fraction was removed by arginase prepared according to the method of Hunter and Downs (10). The resultant urea was treated with urease and the product carbon dioxide removed by addition of HCl and boiling. The gas was trapped in alkali and assayed as BaCO\(_3\). It was found that the amidine carbon possessed 35 per cent and the carboxyl carbon 29 per cent of the C\(^{14}\) content of the radioactive
arginine. The residual C¹⁴ was apparently present in the remaining carbon chain.

The crude dicarboxylic acid fractions contained a brown material of unknown composition, methionine, phenylalanine, tyrosine, and probably cysteic acid. The material was subjected to paper chromatography by using phenol in the presence of ammonia. In this solvent aspartic and glutamic acids had $R_p$ values of 0.20 and 0.35, respectively, while the contaminating materials moved much farther on the paper. A radioautograph of the chromatogram permitted accurate determination of the location of the two amino acids, which were the major radioactive components. The appropriate regions were cut out of the chromatogram and eluted with 0.1 N HCl. These two eluates contained 70 per cent of the original crude radioactivity, which was equally divided between aspartic and glutamic acids. The procedure of Van Slyke et al. (9) was employed to liberate the α-carboxyl carbon of glutamic acid and both carboxyl carbons of aspartic acid. The δ-carbon of aspartic acid was liberated by an aspartic decarboxylase prepared by Meister, Sober, and Tice (11). Radioactivity was assayed as BaCO₃.

Action of the ninhydrin reagent liberated essentially all the C¹⁴ from aspartic and glutamic acids. In the latter case the results proved that the α-carboxyl group of glutamic acid possessed the tracer isotope. In the case of aspartic acid, use of the decarboxylase showed that about 80 per cent of the C¹⁴ was present in the δ-carbon.

The crude lysine fraction was relatively free of other amino acids. The principal contaminant was a small amount of threonine. Purification was carried out by paper chromatography by using a mixture containing butanol, acetic acid, and water. In this mixture threonine and other contaminants moved considerably farther than did lysine. Radioautographs of the chromatogram permitted isolation of the appropriate area of the paper. The purified lysine was eluted with 0.1 N HCl and 80 per cent of the original radioactivity was recovered. The carboxyl group was liberated with ninhydrin and assayed as BaCO₃. The carboxyl carbon was found to contain only 15 per cent of the total C¹⁴ in the lysine.

The eluate of neutral amino acids from Dowex 2 contained radioactive threonine and proline in addition to many unlabeled neutral amino acids. The inorganic solid components were NaCl and NaOH. After adjustment to pH 6 with HCl, the eluate was dried in vacuo and amino acids extracted with butyl alcohol, leaving behind much of the solid NaCl. The alcohol was in turn evaporated in vacuo and the solids subjected to paper chromatography by using a mixture of butanol, acetic acid, and water as the solvent. This procedure gave complete resolution of the radioactive components. A radioautograph of the paper chromatogram facilitated
accurate localization of the radioactive spots. These were cut out and individually eluted with 0.1 N HCl. Under the combined conditions of resin and paper chromatography, the only possible contaminant of proline was alanine. The proline was freed of this substance by paper chromatography with phenol. One-fourth of the radioactivity of the neutral fraction was found in the resultant purified proline. The carboxyl group was removed with ninhydrin and assayed as BaCO₃. More than half of the tracer isotope was thus shown to be present in this carbon.

The crude threonine fraction eluted from the paper chromatogram contained hydroxyproline and a small amount of glycine.

The eluate was subjected to chromatography on Dowex 50 with 1.5 N HCl according to the procedure of Stein and Moore (8). Threonine, hydroxyproline, and glycine were eluted in that order and completely resolved. The purified threonine possessed 30 per cent of the radioactivity of the neutral fraction. The carboxyl group was found to contain 20 per cent of the C¹⁴ of the threonine molecule.

In the paper chromatogram additional radioactivity occupied a region in which valine, leucine, and isoleucine and other relatively non-polar amino acids were also found. An eluate from the paper contained one-fourth of the radioactivity of the original neutral fraction. It has not been further identified.

DISCUSSION

E. coli grown in the presence of glucose as an energy source oxidizes about half of the sugar to CO₂, directly utilizing the remainder as a carbon source. Our tracer experiments show that exogenous CO₂ is used in numerous ways as a building block in synthesis. Much more CO₂ is released in the oxidation of glucose than is used in synthesis.

The observations of C¹⁴O₂ incorporation into aspartic and glutamic acids and into arginine have close parallels in mammalian studies. In work with liver, Delluva and Wilson (4) showed incorporation of isotopic tracer derived from NaH¹³CO₃ into these three amino acids. They observed that labeled carbon could be released from the two dicarboxylic acids by ninhydrin. The amidine carbon of arginine was proved to be isotopically labeled. Anfinsen et al. (5) have studied incorporation into liver slices of C¹⁴ derived from NaHCO₃. They have accounted for most of the fixed C¹⁴ in aspartic and glutamic acids and arginine.

Thus our findings with respect to these three amino acids are parallel to the work of others on liver in the following respects: The major portion of C¹⁴ fixed in protein is found in aspartic and glutamic acids and arginine. In so far as the positions of C¹⁴ in the respective compounds may be compared, they are identical.
Additional evidence for the validity of the identifications of the amino acids has been obtained through experiments in which the basal media were supplemented in turn by non-radioactive proline, threonine, lysine, and arginine. The presence of these supplements suppressed synthesis of the corresponding labeled amino acids. These experiments are described in detail elsewhere and possible synthetic pathways of these six C\textsuperscript{14}-labeled amino acids are discussed.

**SUMMARY**

*Escherichia coli* incorporates exogenous CO\textsubscript{2} during synthesis.

For each mole of glucose metabolized, 0.21 mole of labeled CO\textsubscript{2} is fixed by the organisms, principally in nucleic acids and proteins.

Examination of protein hydrolysates showed that aspartic and glutamic acids, arginine, lysine, proline, and threonine contained labeled carbon\textsuperscript{2} and that these accounted for virtually all of the C\textsuperscript{14} incorporation into proteins.

**BIBLIOGRAPHY**


\textsuperscript{2} The C\textsuperscript{14} used in these experiments was obtained from the Atomic Energy Commission, Oak Ridge National Laboratory, Oak Ridge, Tennessee.
UTILIZATION OF CARBON DIOXIDE IN THE SYNTHESIS OF PROTEINS BY ESCHERICHIA COLI. I
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