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THE ROLE OF THE TRICARBOXYLIC ACID CYCLE IN AMINO ACID SYNTHESIS IN *ESCHERICHIA COLI*

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For the past three years we have been using a variety of C¹⁴ compounds in studies of amino acid synthesis in *Escherichia coli*. Some of the results obtained with C¹⁴O₂ have been published¹ and a report of other experiments using C¹⁴-acetate is in manuscript.² In addition to these tracers C¹⁴-labeled glucose and C¹⁴-labeled amino acids have been used. As a result we have accumulated a wide variety of data concerning the utilization of various carbon compounds in amino acid synthesis. A large part of the results bear on the synthesis of aspartic acid and glutamic acid and can be interpreted in terms of the Krebs cycle. This paper describes the operation of the cycle and the following paper⁹ shows the utilization of the aspartic and glutamic acids as precursors for the synthesis of two families of amino acids. The interpretation of the data shows that the Krebs cycle is of importance in amino acid synthesis, accounting for more than 50 per cent of the protein carbon, but relatively unimportant as a mechanism for oxidizing glucose.

Methods.—Cultures of *E. coli*, strain B, were grown with aeration at 37°C. in mineral media containing appropriate carbon sources, one source being labeled with C¹⁴. After one hour's exponential growth, during which the bacterial mass doubled, the cells were harvested and their protein was

purified by successive extraction with cold trichloroacetic acid, alcohol, alcohol-ether, hot trichloroacetic acid, and ether. The protein was then hydrolyzed (6 *N* HCl, 106°, 18 hrs.) and two-dimensional paper chromatograms made of the hydrolysates. *Sec.* butanol/formic acid/water (70:-20:10) and phenol/water/NH₃ (80:20:0.3) were used as solvents. The radioactive regions were located by making radioautograms, thus allowing the relative radioactivity of the various spots to be counted directly on the paper. Using C¹⁴-glucose† as a sole carbon source, all the bacterial carbon contained C¹⁴ and measurements of the radioactivity of the spots gave the relative abundance of the amino acids of the hydrolysate. These relative abundance values were then used to calculate the specific radioactivity of the amino acids in other experiments. This method gave values in good agreement with those obtained by eluting the amino acids from the paper and measuring the radioactivity and amino acid content of the eluate.

The C¹⁴-glucose was prepared from *Canna* leaves³ exposed to C¹⁴O₂ and the C¹⁴-amino acids were isolated from *Chlorella*⁴ grown in the presence of C¹⁴O₂.

Location of C¹⁴.—Cells were grown in the presence of C¹²-glucose and either C¹⁴O₂ or CH₃ C¹⁴OOH. Aspartic acid and glutamic acid were isolated from the purified protein and degraded⁵⁻⁷ to determine the position of the C¹⁴. The observed values are listed in table 1 and compared with the values predicted from the Krebs cycle model.

In figure 1 the schematic diagram of the Krebs cycle shows that when C¹⁴O₂ condenses with C¹²-pyruvic acid and is then converted to aspartic acid without passing through a symmetrical intermediate all of the C¹⁴ should be located in the C-4 of aspartic acid. On the other hand, if the pathway involves a symmetrical intermediate, or a rapid exchange with a symmetrical intermediate, then the C¹⁴ should be equally distributed between the C-1 and C-4 of aspartic acid. The observed values indicate that roughly two-thirds of the aspartic acid is formed by way of a symmetrical intermediate. The spread in observed values is probably due to variations in the state of the cells rather than to an experimental error.

Fumaric acid appears to be the symmetrical compound involved. When C¹²-fumaric acid was added to the medium, C¹⁴-fumaric acid was formed by the cells and released into the medium. This C¹⁴-fumaric acid was formed by the cells from both C¹⁴O₂ and from C¹⁴-glucose. In contrast to this, C¹²-succinic acid caused the release of C¹⁴-succinic acid into the medium when the cells were supplied with C¹⁴-glucose and C¹²O₂ but not when they were using C¹²-glucose and C¹⁴O₂. The carbon of C¹⁴O₂ evidently does not enter succinic acid.

If the flow is clockwise in the Krebs cycle (figure 1) then only one of the two radioactive carbons found in aspartic acid will appear in glutamic acid and this carbon will appear as C-1 in glutamic acid. Table 1 shows

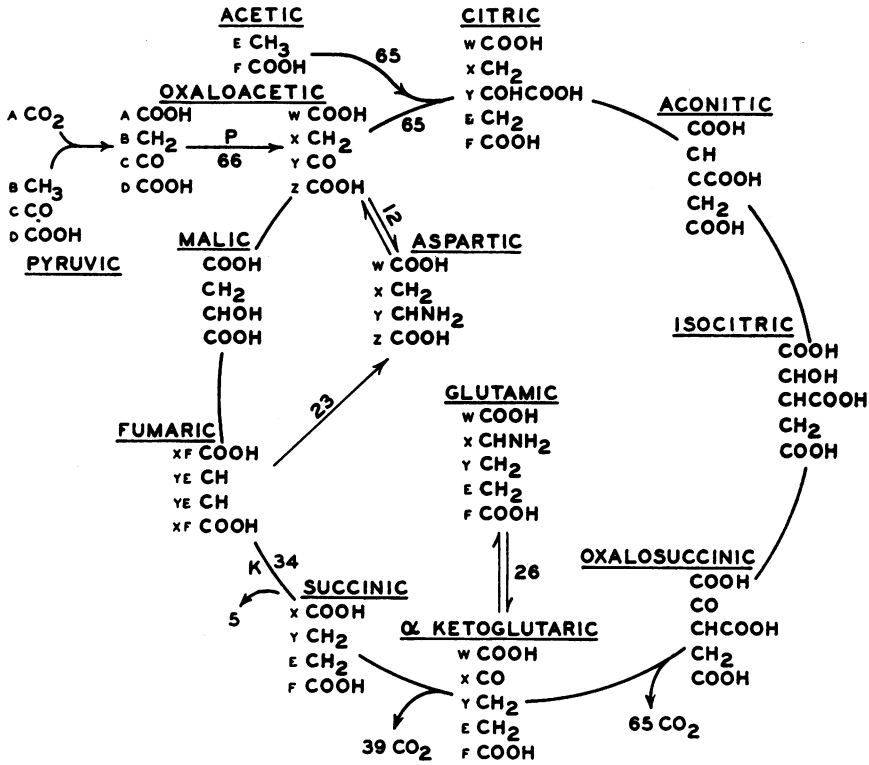


FIGURE 1

Schematic diagram of Krebs cycle. Various carbon atoms are lettered to facilitate identification. The numbers refer to the flow expressed in μ mol/g. dry cells/100 sec. These flow numbers apply only to cells growing exponentially with glucose, acetate, and CO₂ as carbon sources.

TABLE 1
DISTRIBUTION OF C¹⁴ IN ASPARTIC AND GLUTAMIC ACIDS

| CARBON ATOM | C ¹⁴ , % | | | | |
|---------------|---|--------------------------|--|--------------------------|----|
| | DERIVED FROM C ¹⁴ O ₂ | | DERIVED FROM CH ₃ C ¹⁴ OOH | | |
| | OBSERVED | PREDICTED BY KREBS CYCLE | OBSERVED | PREDICTED BY KREBS CYCLE | |
| Aspartic acid | 1 | 20-40 | 0-50 | ... | 50 |
| | 2 | 0 | 0 | ... | 0 |
| | 3 | 0 | 0 | ... | 0 |
| Glutamic acid | 4 | 80-60 | 100-50 | ... | 50 |
| | 1 | 97+ | 100 | 5+ | 12 |
| | 2 | 0 | 0 | ... | 0 |
| | 3 | 0 | 0 | ... | 0 |
| | 5 | 0 | 0 | 80+ | 88 |

that this prediction is fulfilled; the C-1 of aspartic acid is lost as CO₂ in passing around the cycle to glutamic acid.

The distribution in glutamic acid of C¹⁴ derived from CH₃C¹⁴OOH depends slightly on the flow in the various branches of the cycle. The predicted distribution is calculated for typical flow conditions (see below) and agrees with the observed distribution.

C¹⁴-Glutamic and Aspartic Acids.—Experiments in which C¹⁴-glutamic and aspartic acids were added to the usual glucose medium showed that these amino acids were incorporated and interconverted by the cell. The Krebs cycle provides a mechanism for this interconversion and furthermore predicts that while glutamic acid could provide all four carbons for aspartic acid, aspartic acid could only contribute three of the five carbons of glutamic acid. When C¹⁴-glutamic acid was fed at high levels (1 mg./ml.) the specific radioactivity of the bacterial aspartic acid was found to be 78 per cent of the specific radioactivity of the bacterial glutamic acid indicating that 3.9 of the four carbons of aspartic acid were derived from glutamic acid. At high levels of aspartic acid, the specific radioactivity observed in glutamic acid was 72 per cent of the specific radioactivity of the bacterial aspartic acid showing that 2.9 of the five carbons of glutamic acid came from aspartic acid.

On the other hand, when 1-C¹⁴-glutamic acid (isolated from *E. coli* grown in the presence of C¹⁴O₂) was added to the medium, C¹⁴ was found in the glutamic acid (and in the proline and arginine derived from glutamic acid) but not in aspartic acid family. This observation confirms the prediction based on the Krebs cycle that the C-1 of glutamic acid should be lost in passing clockwise around the cycle to aspartic acid. This loss must occur before succinic acid is formed as succinic acid synthesized in the presence of C¹⁴O₂ does not contain C¹⁴.

Specific Radioactivities.—According to the cycle, oxaloacetic acid is derived partly from the carbon entering the cycle (*P* of figure 1) and partly from carbon which has passed around the cycle (*K* of figure 1). The previous paragraphs have shown that when C¹⁴O₂ is used as tracer the carbon passing around the cycle is entirely C¹². As a result the aspartic acid, assumed to have the same isotopic content as oxaloacetic acid, should have a specific radioactivity lower by a factor of $P/(P + K)$ than the specific radioactivity of the input CO₂. Values of 50 to 80 per cent have been observed indicating that the circulating flow *K* contributes from 20 to 50 per cent of the carbon.

The cycle also predicts that C-4 of oxaloacetic acid is the sole source of C-1 of glutamic acid. The same specific radioactivity is observed in C-1 of glutamic acid as in the C-4 of aspartic acid confirming the prediction and indicating that oxaloacetic acid has the same isotopic content as aspartic acid.

C¹⁴-Acetate.—The ratio of the specific radioactivities of glutamic acid and aspartic acid derived from *C¹⁴*-acetate also provides a sensitive measure of the relative flow in the branches *P* and *K*. Calculations based on the cycle show that when *C¹⁴H₃COOH* is used as tracer this ratio is $(7K^2 + 10KP + 4P^2)/(6K^2 + 4KP)$, and when *CH₃C¹⁴OOH* is used the ratio becomes $(2P + 3K)/2K$. The observed ratio varies from 2.5 to 5 indicating that the branch *K* contributes 22 to 50 per cent of the carbon.

The range of values obtained with acetate correspond to the range obtained with *CO₂*. Five parallel cultures were grown under identical conditions. In all cases the media contained glucose, acetate and *CO₂* as carbon sources. Five *C¹⁴*-labeled compounds were added, one to each

TABLE 2
PRODUCTS OF KREBS CYCLE

| | PROTEIN CARBON, % | |
|--|-------------------|-------|
| Glutamic acid | 12.7 | |
| Arginine | 6.4 | |
| Proline | 4.8 | |
| | — | |
| Glutamic acid family | | 23.9 |
| Aspartic acid | 9.9 | |
| Lysine | 7.4 | |
| Threonine | 4.3 | |
| Isoleucine | 6.9 | |
| Methionine | 2.0 | |
| | — | |
| Aspartic acid family | | 30.5 |
| | | — |
| Total protein from Krebs cycle | | 54.4 |
| Glutamic acid peptides | 6.3 | |
| Pyrimidines derived from aspartic acid | 5.7 | |
| | — | |
| Total non-protein | | 12.00 |
| | | — |
| TOTAL | | 66.4 |

tube. Under these conditions the flow calculated from one tracer should equal the flow calculated from the others. The ratio of *P/K* calculated from the culture incorporating *C¹⁴O₂* was 69/31; the cultures using *C¹⁴H₃COOH* and *CH₃C¹⁴OOH* gave ratios of 71/29. *C¹⁴*-glucose and 1-*C¹⁴*-glucose gave a ratio of 70/30.

Experiments with *C¹⁴*-glucose as a sole carbon source served to measure the relative abundance of the various products of the Krebs cycle (table 2). The quantity of Krebs cycle intermediates leaking out of the cell into the medium was estimated by examining the culture fluid after cells had grown in the presence of *C¹⁴O₂* and *C¹⁴*-acetate. From these data and the observed ratio *P/K*, the flow in the remaining branches of the cycle can be

calculated. The numbers in figure 1 show the flow (in micro-moles/gram dry cells/100 sec.) for a typical case of cells growing exponentially with glucose as the principal energy source.

The over-all validity of this flow diagram was checked independently by comparing the predicted and observed production of $C^{14}O_2$ from $CH_3C^{14}OOH$. (Predicted 18 per cent; observed 14 per cent.) However, it should be emphasized that the addition of other carbon sources may alter the flow drastically. The addition of glutamic acid for example reduces markedly the incorporation of CO_2 and acetate. Furthermore, the circulating flow K depends on the temperature and on the phase of growth;

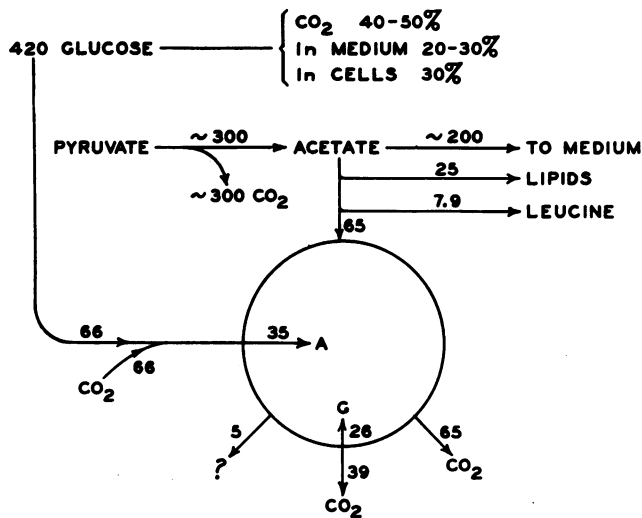


FIGURE 2

Utilization of glucose by *E. coli*. The numbers express the flows in μ mol./g. dry cells/100 sec. A symbolizes aspartic acid plus products; G symbolizes glutamic acid plus products.

as cells came out of a lag phase into exponential growth the fraction circulating increased from 18 to 29 per cent.

The flows in the Krebs cycle can also be related to the total flow of glucose. Four grams of glucose are utilized to produce 1 g. of dry cells. Roughly 50 per cent of glucose is converted to CO_2 . Since the distribution of carbon in the cell is known from experiments with C^{14} -glucose, the initial flow of glucose can be compared to the flow in the Krebs cycle. Figure 2 shows that less than one-sixth of the glucose carbon enters the Krebs cycle. Furthermore, the total CO_2 produced from the glucose is about 1260μ mol./g. cells/100 sec. whereas the net CO_2 production of the Krebs cycle is only 38μ mol./g. cells/100 sec.

Accordingly, the Krebs cycle accounts for only a small fraction of the total oxidation carried out by the cells and its importance as a possible source of energy is correspondingly small.

Experiments with other micro-organisms show patterns of C^{14} incorporation similar to those observed with *E. coli*. While the complete flow patterns have not been worked out, it appears that the Krebs cycle plays an important role in amino acid synthesis of *Torulopsis utilis* and *Neurospora crassa*. *Chlorella pyrenoidosa*, growing by photosynthesis, showed a pattern of acetate incorporation similar to *E. coli*.

Summary.—By observing the incorporation of C^{14} from various carbon sources into the amino acids of *E. coli* it is possible to demonstrate the occurrence of most of the features predicted by the Krebs cycle. It is then possible to determine the flow in the various branches of the cycle using one tracer and to check the flows so established by use of other tracers. This method does not prove the chemical constitution of the intermediates involved but it does establish so many of their properties that it furnishes strong support for the types of compounds postulated in the Krebs cycle.

The flows established show that the cycle provides more than 50 per cent of the carbon required for protein synthesis but it is relatively unimportant as a mechanism for oxidation. These experiments provide a quantitative basis for the suggestion of Krebs, Gurin, and Eggleston⁸ that “the component reactions of the cycle serve to supply intermediates for organic synthesis.”

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† When no position is assigned to C^{14} the compound under discussion is randomly labeled.

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