CARBON DIOXIDE UTILIZATION BY PIGEON LIVER

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Direct experimental evidence that carbon dioxide is used in the formation of α-ketoglutaric acid from pyruvic acid by a suspension of minced pigeon liver has been previously outlined (1, 2). The present report comprises a detailed description of this reaction and of the process of carbon dioxide assimilation by pigeon liver. The principal facts found are: (1) α-Ketoglutaric acid synthesized from pyruvic acid in a radioactive bicarbonate medium contains radioactive carbon. (2) On the average about 5 to 10 per cent of the total radioactivity of the system can be accounted for by the isolated α-ketoglutaric acid. (3) All of the radioactive carbon in the α-ketoglutaric acid is present in the carboxyl group α to the ketonic oxygen. (4) The addition of citric acid to the system during the synthesis of α-ketoglutarate from pyruvate causes no change in the ratio of activity per mg. of carbon of the α-ketoglutarate to that of the medium. (5) Only about 25 per cent of the assimilated carbon dioxide can be accounted for as α-ketoglutaric acid. (6) A part of this residual (non-α-ketoglutarate) radioactivity can be released as carbon dioxide by treatment with ninhydrin and with chloramine-T. (7) An assimilation of CO₂ similar to that in liver does not occur in the formation of α-ketoglutarate by minced pigeon muscle.

Preparation of C¹⁴O₂—Radioactive C¹⁴ was prepared by the bombardment of amorphous boron with 8.5 m.e.v. deuterons in the Chicago cyclotron, the reaction being

\[ {B^{10}} + \text{D}^2 \rightarrow {C^{14}} + \text{He} \]

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CO₂ Utilization by Pigeon Liver

After bombardment, the boron was mixed with twice its weight of powdered CuO and heated at 900° in a stream of oxygen in a quartz combustion tube. The C₁₂O₂ was frozen out over 0.1 ml. of NaOH contained in a glass trap immersed in liquid nitrogen. The small quantity of alkaline solution containing the radioactive carbon was transferred quantitatively to the experimental vessel which contained from 50 to 100 ml. of Krebs' saline-bicarbonate medium, previously equilibrated with 95 per cent oxygen, 5 per cent CO₂. The introduction of the small quantity of carbonate containing the radioactive carbon caused no appreciable change in the pH or ionic concentration of the medium. The time required for these operations, from the removal of the target from the cyclotron to the introduction of the radioactive carbonate into the experimental solution, was about 15 minutes. More than 95 per cent of the radioactivity of the target material could be recovered as C₁₂O₂ and the addition of C₁₈ as a carrier was not necessary. The activity was sufficient to permit a working period of 3.5 to 4 hours, despite the short half life of the radioactive carbon and dilution of the carbon dioxide in the course of the experiment.

Measurement of Radioactivity—Activities (expressed as divisions per second) were measured with a Lauritsen electroscope. To make the readings comparable and to correct for self-absorption all measurements were made in solution and under identical geometrical conditions. In every case the unknown was dissolved in 0.5 ml. of either water or alkali. Duplicate samples could be read with an error of less than 2 per cent. In Tables I to IV, values for radioactivity have been corrected for decay and are comparable within a given experiment.

Distribution of Radioactive Carbon Dioxide in Bicarbonate Medium—The method of experimentation consisted in shaking the minced tissue in Krebs' Ca-free saline medium, pH 7.4, in an atmosphere of 95 per cent oxygen, 5 per cent CO₂. It was desirable to know, therefore, how rapidly the radioactivity is distributed between the gas and liquid phases of the experimental system after its addition as radioactive carbonate. Experiments to answer this question were devised as follows: 50 ml. of the bicarbonate buffer, pH 7.4, to which has been added 0.3 ml. of radioactive carbonate were shaken in an atmosphere of 95 per cent oxygen, 5 per cent CO₂ in an experimental vessel having a volume of 203 ml. At
intervals during the shaking 0.5 cc. portions of the liquid phase were withdrawn by means of a calibrated syringe through the rubber stopper which closed the shaking vessel, immediately mixed with a known volume of strong alkali by way of a 2-way stop-cock inserted in the syringe barrel, and an aliquot of this solution was used for radioactivity measurements. The same procedure was adopted in similar experiments in which 7.6 gm. of freshly minced pigeon breast muscle were added. Fig. 1 depicts two such ex-

![Graph](https://via.placeholder.com/150)

**Fig. 1.** Exchange rate of radioactive CO₂ between liquid and gas phases, in bicarbonate medium.

periments carried out at 25° and 40° respectively. At 25° complete equilibrium is attained 23 minutes after the addition of the radioactive carbonate, while at 40° equilibrium is reached in 14 minutes. It will be noted that at neither temperature does the presence of tissue markedly influence the rate of attainment of equilibrium.

Utilization of CO₂ in α-Ketoglutarate Synthesis—The experimental procedure was as follows: 100 ml. of Ca-free Krebs' bicarbonate-saline were shaken at 40° for 10 minutes while a vigorous stream of 95 per cent oxygen, 5 per cent CO₂ was bubbled through
the solution. The radioactive carbonate (0.3 ml.) was added together with 1.7 ml. of 0.1 M malonate (3) and the experimental vessel closed and shaken for 15 minutes. 7.6 gm. of freshly minced pigeon liver were next added together with 10 ml. of 0.2 M pyruvate. The vessel was closed and shaken for a few minutes and 0.5 ml. of the suspension was withdrawn for a determination of the total original activity of the solution. The reaction was then let proceed while the closed vessel was shaken vigorously for 40 minutes. At the end of this time practically all of the pyruvate had been utilized. Three 0.5 cc. samples were withdrawn from the solution. One of these was mixed immediately with strong alkali and measured for total radioactivity. The second sample was pipetted immediately into 3 cc. of 2 N NaOH contained in Warburg vessels and, after acidification, the quantity of CO₂ determined manometrically. The third aliquot was pipetted into an alkaline solution, acidified, and the liberated CO₂ swept over by a stream of nitrogen into an absorbing tube containing 5 ml. of 20 per cent KOH. An aliquot of this was removed to determine the radioactivity of the liberated CO₂. These determinations permit a measure of the amount and radioactivity of the total CO₂ of the medium at the end of the experimental period.

After removal of these samples, the reaction mixture was deproteinized with 25 ml. of 10 per cent metaphosphoric acid. The volume of the filtrate was measured and two 5 cc. aliquots were removed for simultaneous determinations of α-ketoglutarate and succinate as described by Krebs and Johnson (4). 25 mg. of α-ketoglutaric acid were added to the remaining filtrate to act as a carrier and the solution mixed in centrifuge cups with an equal volume of a saturated solution of 2,4-dinitrophenylhydrazine in 2 per cent HCl. The hydrazone separated immediately and was collected by centrifuging. The precipitate was dissolved in boiling 60 per cent alcohol and the solution filtered into centrifuge tubes contained in an ice bath. The hydrazone crystallized quickly and was collected by centrifuging and dried in vacuo at 100°. The precipitate was weighed and dissolved in 0.5 ml. of 2 N NaOH for measurement of its radioactivity. The hydrazone melted at 223° and gave no depression of the melting point when mixed with a known sample.

On recrystallization the hydrazone gave material with a con-
stant rate of decay per mg. (Experiment 3, Table I), indicating the identity of the radioactivity with the α-ketoglutarate dinitrophenylhydrazone.

Control experiments in the absence of tissue were carried out in which amounts of pyruvic acid and α-ketoglutaric acid, comparable to those used in the tissue experiments, were shaken for 40 minutes at 40° in a radioactive bicarbonate buffer, pH 7.4. The dinitrophenylhydrazones of the keto acids were isolated and found to be completely devoid of radioactivity.

It is theoretically possible to ascertain the number of moles of CO₂ used per mole of α-ketoglutarate synthesized by a com-

### Table I

**Synthesis of α-Ketoglutarate in Radioactive Bicarbonate Medium**

The activities are expressed as divisions per second.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>α-Ketoglutarate synthesized mg.</th>
<th>Activity of α-ketoglutarate</th>
<th>Total activity of medium</th>
<th>Terminal activity of medium per mg. inorganic C</th>
<th>Ratio, α-ketoglutarate activity to original activity of medium</th>
<th>Ratio, medium activity per mg. C to α-ketoglutarate activity per mg. C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31.4</td>
<td>0.471</td>
<td>0.036</td>
<td>8.74</td>
<td>0.078</td>
<td>0.054</td>
</tr>
<tr>
<td>2</td>
<td>27.5</td>
<td>4.09</td>
<td>0.362</td>
<td>67.0</td>
<td>1.13</td>
<td>0.061</td>
</tr>
<tr>
<td>3</td>
<td>55.0</td>
<td>3.85</td>
<td>0.171</td>
<td>79.8</td>
<td>1.17</td>
<td>0.048</td>
</tr>
</tbody>
</table>

*Activities after successive recrystallizations of α-ketoglutarate dinitrophenylhydrazone.

Comparison of the radioactivity per mg. of carbon of the α-ketoglutarate at any given moment with the radioactivity per mg. of carbon of the inorganic carbon of the medium. Under the experimental conditions used, however, any such comparison is complicated by the continuous metabolic production of large amounts of non-radioactive CO₂ during the course of the experiments, the assimilation of CO₂ in reactions other than α-ketoglutarate synthesis, the length of time required for a uniform distribution of radioactive carbon between liquid and gas phases, and the possible preferential synthetic utilization of non-radioactive carbon dioxide which is produced in the tissue in the immediate vicinity of the synthesizing enzyme systems.
Quantitative data from a series of experiments are shown in Table I. In a typical experiment such as No. 3, the original radioactivity was 88 divisions per second for 60 mg. of CO₂ in the liquid and 14 mg. of CO₂ in the gas phase. During the 40 minutes of the experiment about 80 mg. of metabolic CO₂ were formed (3). Assuming no assimilation of CO₂, this would reduce the radioactivity of the medium to 2.09 divisions per second per mg. of carbon. Utilization of CO₂ occurs, however, as evidenced by the terminal activity of the medium being 1.17 divisions per second per mg. of carbon. Almost half of the activity originally present as carbonate has been converted into a bound form not liberated by acid. If the average activity of α-ketoglutaric acid is calculated on the basis of a constant rate of synthesis in a medium having an initial activity of 2.09 divisions per second per mg. of inorganic carbon and a final activity of 1.17 divisions, an average value for the acid of 1.63 divisions per second is obtained. The observed value for Experiment 3 is 0.171. On the basis of this very approximate calculation about 1 carbon atom in 10 of the synthesized α-ketoglutarate is derived from the medium. The experiments of the next section demonstrate that the number of carbon atoms of the α-ketoglutarate derived from the medium cannot exceed 1 in 5. Since calculation of the 1 in 10 value is approximate and can indicate only the order of magnitude of the synthesis, it is probable that 1 mole of CO₂ is derived from the medium for every mole of α-ketoglutarate synthesized.

Location of Radioactivity in Synthesized α-Ketoglutaric Acid

When the radioactive α-ketoglutarate dinitrophenylhydrazone obtained in the previous experiments is oxidized with potassium permanganate in acid medium at room temperature, a complete loss of radioactivity in the form of CO₂ occurs. In the experiments of Table II the hydrazone was dissolved in 5 cc. of dilute alkali, the solution acidified with 3 cc. of 50 per cent H₂SO₄, 10 ml. of saturated KMnO₄ added, and the resulting CO₂ swept over by N₂ into a receiver containing 3 ml. of 5 N NaOH.

A summary of the data in this section was reported (2) at the Symposium on Carbohydrate Metabolism held April 18, 1941, at the meeting of the American Society of Biological Chemists at Chicago. Wood, Werkman, Hemingway, and Nier (5) have independently obtained similar results using the carbon isotope C¹³.
If α-ketoglutaric acid was synthesized in pigeon liver by the reactions of the citric acid cycle (1), the intermediate formation of the symmetrical citric acid molecule would occur. On oxidation this would yield a mixture of α-ketoglutaric acid molecules in half of which the radioactive carbon was α and in the other half γ to the carbonyl group. On oxidation, such a mixture should give succinic acid with a loss of half the original radioactivity as carbon dioxide. The localization, however, of the entire radioactivity of the α-ketoglutarate in the carboxyl group adjacent to the carbonyl group as demonstrated in Table II is definite evidence against the suggested formation of this compound from citric acid.

If citrate was an intermediate in the formation of α-ketoglutaric acid from pyruvate and CO₂, the addition of citrate to the synthesizing tissue would give rise, presumably, to α-ketoglutaric acid of which the radioactivity had been considerably diluted. However, the experiments of Table III show that the addition of 25

### Table II

Oxidation of Radioactive α-Ketoglutarate Dinitrophenylhydrazone to Succinic Acid and CO₂

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>α-Ketoglutarate dinitrophenylhydrazone mg.</th>
<th>Activity</th>
<th>Activity of CO₂ liberated on oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>55.0</td>
<td>0.23</td>
<td>0.20</td>
</tr>
<tr>
<td>2</td>
<td>47.9</td>
<td>0.35</td>
<td>0.33</td>
</tr>
<tr>
<td>3</td>
<td>40.0</td>
<td>0.18</td>
<td>0.20</td>
</tr>
</tbody>
</table>

### Table III

Effect of Sodium Citrate on Radioactivity of Synthesized α-Ketoglutaric Acid

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Citrate added mg.</th>
<th>α-Ketoglutarate synthesized mg.</th>
<th>Activity of α-keto-glutarate per mg. C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>31.4</td>
<td>0.148</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>28.8</td>
<td>0.113</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>36.9</td>
<td>0.116</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>39.8</td>
<td>0.110</td>
</tr>
</tbody>
</table>
mg. of sodium citrate affects neither the yield of α-ketoglutaric acid nor the ratio of activity per mg. of carbon of the α-ketoglutarate to that of the medium. These experiments again support the view that citric acid is not an intermediate in the synthesis of α-ketoglutarate from pyruvic acid and CO₂ by liver under the conditions of these experiments.

*Non-α-Ketoglutaric Radioactivity and Its Nature*—In Table IV data are listed which would indicate that the total amount of CO₂ assimilated is much greater than can be accounted for by the amount of α-ketoglutaric acid formed. In these experiments the deproteinized reaction mixture was divided into two parts. To one 25 mg. of α-ketoglutaric acid were added and the dinitrophenylhydrazone isolated as previously described. A vigorous stream of CO₂ was bubbled through the other portion which was strongly acid. Samples were withdrawn from this solution at intervals until there was no further loss of activity. Under these circumstances it was found that the residual radioactivity was 3 to 4 times greater than that of the synthesized α-ketoglutaric acid.

Some indication as to the nature of this activity could be derived from the fact that on treatment with ninhydrin at boiling temperature (6) loss of radioactive CO₂ could be demonstrated. A similar reaction occurred with chloramine-T at 40° under the conditions described by Cohen (7). Here the radioactivity of the liberated CO₂ was about one-fourth of the total residual activity. Control experiments indicated that α-ketoglutaric acid was not decarboxylated by ninhydrin or chloramine-T under the

**Table IV**

*Effect of Ninhydrin and Chloramine-T on Non-α-Ketoglutarate Radioactivity*

The activities are expressed as divisions per second.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>α-Ketoglutarate synthesized mg.</th>
<th>Total activity of α-ketoglutarate</th>
<th>Total activity of medium after CO₂ removal</th>
<th>Activity of CO₂ released by ninhydrin</th>
<th>Activity of CO₂ released by chloramine-T</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35.0</td>
<td>5.18</td>
<td>31.8</td>
<td>7.8</td>
<td>10.39</td>
</tr>
<tr>
<td>2</td>
<td>39.0</td>
<td>6.2</td>
<td>31.2</td>
<td></td>
<td>0.78</td>
</tr>
<tr>
<td>3</td>
<td>53.0</td>
<td>1.2</td>
<td>4.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>31.6</td>
<td>0.22</td>
<td>0.929</td>
<td>0.209</td>
<td></td>
</tr>
</tbody>
</table>
conditions of these tests. It is probable, therefore, that the activity released by these agents is present as a carboxyl group of amino acids. In view of the existence of the transaminating enzymes in the liver such an amino acid as glutamic acid could be directly formed from the synthesized α-ketoglutaric acid and would liberate CO₂ under these conditions. Further examination of the compounds involved is being made.

Synthesis of α-Ketoglutaric Acid in Muscle—In the preceding sections it has been assumed that the appearance of radioactivity in α-ketoglutaric acid indicates a direct synthetic utilization of carbon dioxide in the formation of this substance. It is possible, however, that the radioactivity could result by a simple process of exchange between an intermediate in the reaction and carbon dioxide of the medium. Current theories of the mechanism of formation of α-ketoglutarate in pigeon liver postulate an initial condensation of pyruvic acid and CO₂ to form oxalacetic acid (1, 5, 8). Oxalacetic acid is known to break down in the presence of tissue to CO₂ and pyruvic acid and it might be argued that any reaction involving this compound would yield a radioactive end-product if the reaction medium contained radioactive carbon dioxide. Experiments to control this possibility are difficult to conceive but a comparison of the radioactivity of the α-ketoglutaric acid synthesized in muscle with that formed in liver offers some evidence on this point. In muscle α-ketoglutaric acid is formed by the oxidation of citric acid resulting from a condensation of oxalacetic acid and pyruvic acid (9). A formation of oxalacetic acid from pyruvate and CO₂ in this tissue does not occur, inasmuch as malonate will almost completely inhibit pyruvate utilization in the absence of added oxalacetic or other dicarboxylic acids (9). Under these circumstances the synthesis of muscle α-ketoglutarate in a radioactive bicarbonate medium would give rise to radioactive α-ketoglutarate only if a process of exchange between intermediates (such as oxalacetate) in the reaction and the carbonate of the medium took place. If the quantity of radioactivity found in muscle α-ketoglutarate were similar in magnitude to that found in the α-ketoglutarate synthesized by liver, it would eliminate the necessity for proposing a stoichiometric utilization of CO₂ in the latter process.

In an experiment with 7.6 gm. of minced pigeon muscle in 50
ml. of radioactive bicarbonate medium, the addition of 4.0 ml. of 0.5 M pyruvate and 6.0 ml. of 0.1 M fumarate led to the synthesis of 55.5 mg. of \(\alpha\)-ketoglutaric acid. The \(\alpha\)-ketoglutarate isolated as the hydrazone as described above contained no appreciable amount of radioactivity. This strongly suggests again that the process of \(\text{CO}_2\) assimilation in pigeon liver is a metabolic reaction representing a stoichiometric utilization of inorganic carbonate.

**DISCUSSION**

Carbon dioxide participates in a variety of synthetic reactions in the tissues of higher animals: in the formation of urea (10–12), of carbaminohemoglobin (13), in the synthesis of \(\alpha\)-ketoglutarate from pyruvate in pigeon liver (1), and the formation of glycogen from lactic acid in the rat (14, 15). In the formation of urea, carbon dioxide is unquestionably used for synthetic purposes (i.e., the reaction involves an increase in free energy) and apparently the physiological objective of the reaction, to eliminate metabolic end-products, has distracted attention from its similarity to the assimilation of \(\text{CO}_2\) by the lower organisms (16). The more recent demonstrations of the metabolic utilization of carbon dioxide involve reactions suggesting that carbon dioxide, once formed, is not necessarily eliminated as such but can reenter the metabolism of the organism by several reaction paths to form a variety of tissue constituents. In such circumstances the exposure of the organism or cell to an environment containing radioactive carbon as \(\text{CO}_2\) would lead to the presence of the tagged atoms in many of the compounds present in the tissue. If the appearance of radioactivity represented merely an exchange between the \(\text{CO}_2\) of the medium and carboxyl groups of various organic acids, the use of radioactive \(\text{CO}_2\) would not give any demonstration of a direct metabolic utilization of \(\text{CO}_2\). For the reasons outlined in the preceding sections of this paper such an explanation is regarded as unlikely for the appearance of radioactive carbon in \(\alpha\)-ketoglutaric acid synthesized in pigeon liver and it is believed that \(\text{CO}_2\) participates directly as a reactant in this synthesis.

The mechanism by which carbon dioxide is utilized in \(\alpha\)-ketoglutarate synthesis in pigeon liver is unknown. The mechanisms already suggested (1, 5, 8) postulate the preliminary formation of oxalacetic acid from carbon dioxide and pyruvic acid, a reaction
for which there is as yet no direct experimental evidence. The condensation of oxalacetic acid with pyruvate to yield citrate as an intermediate can be excluded on the basis of the data presented in the present paper. The intermediate formation of isocitrate (5) rather than citrate by condensation of oxalacetate and pyruvate likewise seems improbable in view of the demonstrated equilibrium between citrate, isocitrate, and aconitic acid in most tissues (17).

SUMMARY

1. The synthesis of α-ketoglutarate from pyruvate by minced pigeon liver involves the direct participation of CO₂. It is probable that 1 mole of CO₂ is utilized for each mole of α-ketoglutarate synthesized.

2. The radioactivity of the α-ketoglutarate is confined entirely to the carboxyl group α to the carbonyl group. This precludes the intermediate formation of citric acid, a conclusion further supported by experiments in which non-radioactive citrate was added to the tissue during the synthetic reaction.

3. A maximum of 1 in 5 carbon atoms of the α-ketoglutarate is derived from the carbon dioxide of the medium.

4. The synthesized α-ketoglutarate accounts for about one-fourth the assimilated CO₂. Release of part of this residual activity as CO₂ by ninhydrin and chloramine-T suggests that it is present in part as amino acids or similar compounds.

5. The formation of α-ketoglutarate in muscle does not involve the participation of carbon dioxide.

6. Evidence is presented that the process of CO₂ assimilation in pigeon liver, as demonstrated by the use of radioactive C¹⁴, is a metabolic reaction representing a stoichiometric utilization of inorganic carbonate.

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CORRECTION

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