The Nonenzymatic Decarboxylation of Diketosuccinate and Oxaloglycolate (Dihydroxyfumarate)*

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Both plant and animal tissues have been shown to contain enzyme systems capable of oxidizing meso- and D(-)tartaric acid (1–4). The first oxidation product has been assumed to be oxaloglycolate (or an enol form thereof), as shown in Equation 1.

\[ \text{COO}^- \quad \text{COO}^- \]
\[ \text{HCOH} \quad \text{C=O} \quad + \quad 2\text{H} \]

Equation 1

This substance, available in solid form as dihydroxy-fumaric acid (5–7), has been shown to be a substrate for peroxidases (8–10). Unless it is very carefully purified, oxaloglycolate also readily undergoes nonenzymatic autoxidation (10, 11), presumably to diketosuccinate (Equation 2).

\[ \text{COO}^- \quad \text{COO}^- \]
\[ \text{C=O} \quad \quad \quad \text{C=O} \]
\[ \text{HCOH} \quad \text{HCOH} \]

Equation 2

Malic dehydrogenase has been shown to catalyze the reduction of diketosuccinate by DPNH (4). Furthermore, both oxaloglycolate and diketosuccinate undergo spontaneous decarboxylation in aqueous solution. These nonenzymatic reactions complicate the analysis of the metabolic path of degradation of tartrate. As a prerequisite for the use of labeled compounds to study the biochemical synthesis and degradation of tartrate, it seemed essential to know the manner of the nonenzymatic decarboxylation of both oxaloglycolate and diketosuccinate. The present paper describes such a study.

EXPERIMENTAL

Methods and Materials

Preparation of Labeled Compounds—Fumaric acid-1-C\(^{14}\), purchased from Nuclear-Chicago Corporation, was oxidized to \(\text{dl-tartaric acid-1-C}^{14}\) by \(\text{KClO}_3\) with \(\text{OsO}_4\) as catalyst, according to Miles and Terry (12). \(\text{dl-tartaric acid-1-C}^{14}\) was further oxidized to oxaloglycolic acid-1-C\(^{14}\) by \(\text{H}_2\text{O}_2\) according to Hartree (7). The oxidation of oxaloglycolic acid-1-C\(^{14}\) to diketosuccinic acid-1-C\(^{14}\) by \(\text{Br}_2\) was carried out as described by Fenton (13). \(\text{dl-tartaric acid-2-C}^{14}\), oxaloglycolic acid-2-C\(^{14}\), and diketosuccinic acid-2-C\(^{14}\) were prepared similarly from fumaric acid-2-C\(^{14}\) obtained from Volk Radiochemical Co. Because of symmetry, the acids labeled at C-1 contain an equal amount of isotope at C-4, and the acids labeled at C-2 contain an equal amount of isotope at C-3.

Preparation of Diphenacyl Tartronate—This substance was prepared according to the method of Rather and Reid (14), as modified by Loewus et al. (15). The melting point was 148° with no change on further recrystallization. The analysis\(^2\) is as follows:

\[
\begin{align*}
\text{C}_{12}\text{H}_{10}\text{O}_2 \\
\text{Calculated: C 64.1, H 4.5} \\
\text{Found: C 64.23, H 4.73} \\
\text{C 64.24, H 4.83}
\end{align*}
\]

Other reagents and materials were the same as those previously described (1, 16, 17).

Procedure for Decarboxylation of Labeled Compounds—The decarboxylation of diketosuccinic acid was carried out in aqueous solution in a reaction flask connected by a wide tube to a receiver flask containing a solution of \(\text{Ba(OH)}_2\) for trapping the \(\text{CO}_2\). Between 0.2 and 1 mmole of the acid were dissolved in 5 to 10 ml. of \(\text{H}_2\text{O}\). The system was evacuated through a stopcock attached to the connecting tube. The receiving flask was cooled in an ice bath, and the reaction flask was submerged in a water bath kept at 70°. Incubation was continued with occasional gentle agitation for 2 to 3 hours. In this time, the decarboxylation of diketosuccinic acid is complete, and much of the \(\text{CO}_2\) is converted to a precipitate of \(\text{BaCO}_3\) in the receiving flask. The \(\text{BaCO}_3\) was collected by centrifugation, washed three times with hot water and twice with methanol, and then plated out and dried for radioactivity assays in standard size planchets. Results are given as “thick sample” counts per minute corrected for background. In some experiments the acid residue remaining from the decarboxylation was dried by lyophilization, and oxidized to \(\text{CO}_2\) over hot platinum in a combustion tube. The \(\text{CO}_2\) was collected as \(\text{BaCO}_3\), washed, and then oxidized to oxaloglycolic acid-1-C\(^{14}\) by \(\text{H}_2\text{O}_2\) according to Hartree (7).

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† Deceased March 24, 1958.

\(^1\) The terms hydroxymalonic acid and tartronic acid are used interchangeably, as are the terms diketosuccinic acid and dihydroxytartaric acid.

\(^2\) Analysis performed at Micro-Tech Laboratories, Skokie, Illinois.
The decarboxylation of labeled oxaloglycolic acid was carried out by a procedure similar to that employed for the decarboxylation of diketosuccinic acid. In one experiment the evacuated reaction vessel was flushed with N₂ and provided with an inert of yellow phosphorus, to remove all traces of O₂. The results thus obtained were not appreciably different from those obtained when the reaction vessel was evacuated. Samples of BaCO₃ were prepared from the CO₂ released on decarboxylation, and from the CO₂ obtained by combustion of the residue from the decarboxylation, which should consist mainly of glycolaldehyde.

**Procedure and Results**

### Decarboxylation of Labeled Acids—Oxaloglycolate is decarboxylated to give 1 mole of glycolaldehyde and 2 moles of CO₂ (18, 19) as shown in Equation 3, and the decarboxylation of diketosuccinate gives tartronate and CO₂ (20-22), as shown in Equation 4. To determine the origin of the CO₂ formed in these decarboxylations, the compounds were labeled with C¹⁴ in the external carboxyl groups on the one hand, and in the 2 internal carbon atoms on the other hand. These labeled compounds were decarboxylated in aqueous solution, and the radioactivity of the products was ascertained. Each type of experiment was repeated at least twice. Representative results have been assembled in Table I. The figures in the first column are expressed as specific activities of the BaCO₃ samples which were actually counted. In the second column are listed the calculated values for the specific activities of those carbon atoms which contain the label. The results show that the CO₂ is derived primarily from the carboxyl groups of the acids. The specific activity of the collected CO₂ obtained on decarboxylation of the C₁ labeled acids was generally lower than theory, probably because complete collection of the total CO₂ was not achieved. The main objective was to determine whether diketosuccinate is decarboxylated to tartronate by a benzoic acid type rearrangement, which had formerly been suggested (22), and the results proved that this is not the case.

In separate experiments, with appropriate modifications in technique, diketosuccinate-2-C¹⁴ was decarboxylated in phosphate-buffered medium at pH ranging from 5.4 to 8.5. The results were essentially the same as those obtained on decarboxylation of the free acid in unbuffered medium, with the CO₂ formed containing about twice as much label at the higher pH values as at the lower.

### Exchange of CO₂ into Tartronic Acid—When the decarboxylation of unlabeled diketosuccinic acid was carried out in the presence of C¹⁴O₂, a small amount of C¹⁴ was incorporated into the tartronic acid. Details of two representative experiments are given in Table II. In the first experiment, the tartronic acid residue was oxidized directly to CO₂. In the second experiment, the tartronic acid was converted to diphenacyl tartronate and recrystallized several times with no change in specific activity. The third experiment of Table II shows that a small amount of exchange occurred when tartronic acid itself was incubated with C¹⁴O₂. As controls, oxaloglycolic acid was also decarboxylated in the presence of C¹⁴O₂, and experiments were performed to measure C¹⁴O₂ exchange into dl-tartaric acid and into mesoxalic acid. In all cases, the amount of exchange

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**Table I**

<table>
<thead>
<tr>
<th>Decarboxylated substances and their products</th>
<th>Specific activity (c.p.m. of BaCO₃)</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diketosuccinic acid-1-C¹⁴</td>
<td>976</td>
<td>2 x 1952</td>
</tr>
<tr>
<td>CO₂</td>
<td>1475</td>
<td></td>
</tr>
<tr>
<td>Tartronic acid</td>
<td>686</td>
<td>3 x 2088</td>
</tr>
<tr>
<td>Diketosuccinate acid-2-C¹⁴</td>
<td>1195</td>
<td>2 x 2990</td>
</tr>
<tr>
<td>CO₂</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Tartronic acid*</td>
<td>1552</td>
<td>2 x 2328</td>
</tr>
<tr>
<td>Oxaloglycolic acid-1-C¹⁴</td>
<td>671</td>
<td>2 x 1012</td>
</tr>
<tr>
<td>CO₂</td>
<td>1727</td>
<td></td>
</tr>
<tr>
<td>Glycolaldehyde</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>Oxaloglycolic acid-2-C¹⁴</td>
<td>1012</td>
<td>2 x 2024</td>
</tr>
<tr>
<td>CO₂</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Glycolaldehyde</td>
<td>1788</td>
<td></td>
</tr>
</tbody>
</table>

*This acid was converted to diphenacyl tartronate. The BaCO₃ obtained by combustion of the recrystallized derivative had a specific activity of 245. This figure was multiplied by 1/3 to obtain the average specific activity of the 3 carbon atoms of the tartronic acid.
observed was only about 0.1 of that observed with tartronic acid. All of these experiments were performed under comparable conditions and precautions were always taken to remove CO₂ itself by careful flushing with unlabeled CO₂. The low positive values observed with oxaloacetic acid, tartric, and meso-xalic acids require confirmation, and are here regarded primarily as controls for the validity of the relatively higher rate of CO₂ incorporation into tartronic acid. Though quantitatively small, this exchange has significance in relation to the mechanism of the decarboxylation. The determination of the exchange of CO₂ into tartronic acid was also carried out in buffered media at pH 5.4, 6.0, 7.0, and 7.5. The tartronate from these experiments was always purified in the form of the diphenacyl derivative before determination of its radioactivity. The exchange at the higher pH values was about one-third of the amount observed with the unbuffered free acid.

Effect of Cations on Decarboxylation of Diketosuccinate—
Pederson has studied the kinetics of the decarboxylation of dihydroxystearic acid (diketosuccinic acid) under various conditions (23). The present studies included similar measurements. The evolution of CO₂ was measured manometrically in Warburg vessels by conventional techniques (24). The diketosuccinic acid was weighed into the vessel side arm as a solid. The reactions were carried out at 30° in 3 ml. of 0.2 M acetate buffer of pH 5.0, and observations were continued until the decarboxylation was complete. The CO₂ evolution followed first order kinetics over its entire course, in agreement with Pederson’s findings. Table III shows the stoichiometry and rate constants observed over an 8-fold range of initial concentration. The first column shows the amount of diketosuccinic acid calculated from the weights, and is in good agreement with the total amount of CO₂ evolved as measured manometrically and shown in the second column. The effect of pH on the rate of decarboxylation is shown in Table IV. The data obtained are in approximate agreement with those of Pederson. Exact comparisons cannot be made because of the different conditions used. The present studies served as a check on the quality of the diketosuccinic acid employed, and provided a baseline for examination of the effects of some cations on the decarboxylation.

Pederson noted that Cu⁺⁺ stimulates the decarboxylation of diketosuccinate, with complex effects on the kinetics (23). Kenten and Mann (9) reported that Mn⁺⁺ stimulates the decarboxylation rate of diketosuccinate, but gave little data. One might anticipate from the mechanism proposed by Steinberger and Westheimer (25) that divalent cations should definitely stimulate the decarboxylation rate. When the concentration of Mn⁺⁺ was increased, the initial stimulatory effect was also increased, but the rate of decarboxylation then declined more rapidly than expected from first order kinetics. The effect of Mg⁺⁺ was similar to that of Mn⁺⁺, except that higher concentrations of Mg⁺⁺ were required to give the same stimulatory effects as were observed with added Mn⁺⁺. With Ni⁺⁺, more striking deviations in the course of the decarboxylation were observed, as shown in Fig. 2. Even with low concentrations of Ni⁺⁺ (5 × 10⁻⁴ M), the expected stoichiometric amount of CO₂ (calculated from the weight of diketosuccinic acid) was not evolved, and with higher concentrations of Ni⁺⁺, the deviations became large.

Since the decarboxylations were generally carried out in air, controls were performed to show the absence of O₂ uptake.
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FIG. 1. Effect of Mn++ on decarboxylation of diketosuccinate.
O—O, no added metal ion; □—□, 10^{-2} M Mn++; and △—△,
5 \times 10^{-4} M Mn++.  

FIG. 2. Effect of Ni++ on decarboxylation of diketosuccinate.
O—O, no added metal ion; □—□, 10^{-2} M Ni++; and △—△,
5 \times 10^{-4} M Ni++.  

Thus, the sudden cessation of \( CO_2 \) evolution in the presence of \( 10^{-2} \) M Ni++ was shown not to be due to simultaneous \( O_2 \) consumption. In contrast, enzyme preparations, such as extracts of wheat germ, also caused an apparent decrease in the total amount of \( CO_2 \) evolved from diketosuccinate, but this effect was definitely associated with a simultaneous oxygen uptake catalyzed by the wheat germ extract. 

Oxidation of Labeled Tartrate by Mitochondria—Internally and externally labeled tartrate was oxidized separately by rat liver mitochondria supplemented with cytochrome c, DPN, and Mg^{2+} as described by Kun and Hernandez (2). The ratio of \( O_2 \) consumption to \( CO_2 \) evolution after correction for blank respiration was about 1. In addition, the \( CO_2 \) was collected for determination of its radioactivity. When tartrate labeled in the carboxyl groups was oxidized, the specific activity of the extra \( CO_2 \) evolved was the same as the specific activity of the carboxyl groups of the tartrate. When internally labeled tartrate was employed, only about 1 per cent of the extra \( CO_2 \) evolved was derived from the labeled carbon atoms of the tartrate. These results are in accord with a reaction path giving tartronic acid as a major end product of the oxidation of tartrate. Attempts to apply the molybdate method of Stafford (17) to determine tartronic acid quantitatively were not successful, primarily because of the high blank values given by the mitochondrial preparations without added substrate. There was, however, a definite increase in the color values obtained after tartrate oxidation, and this increase was approximately of the order of magnitude expected if tartronate were the major end product.

DISCUSSION

In 1879, Gruber first described diketosuccinic acid (20). Because of its ready conversion to \( CO_2 \) and tartronic acid, he assigned a branched chain structure

\[
\begin{array}{c}
\text{COOH} \\
\text{HOC-COOH} \\
\text{COOH}
\end{array}
\]

to the new compound and named it carboxytartronic acid. The fact that diketosuccinic acid was an oxidation product of pyrocatechol seemed to provide evidence for the presence of a branched carbon chain in benzene, and this led Kekulé to re-examine the question of the structure of diketosuccinic acid and to show that it was in fact a straight chain compound (21). Kekulé suggested that the first decarboxylation product of diketosuccinic acid was mesoxalyl semialdehyde, which then was converted to tartronic acid. Fenton (26, 27) later prepared mesoxalyl semialdehyde and showed that it could not be an intermediate in the conversion of diketosuccinic to tartronic acid, because mesoxalyl semialdehyde yields glyoxal and \( CO_2 \) in acid solution under conditions which favor the formation of tartronic acid from diketosuccinic acid.

In 1921, Lachman (22) reopened the question of the mechanism of the decarboxylation reaction, and suggested that it occurs as “an analog of the rearrangement of benzil to benzilic acid.” This would make the branched chain structure first proposed by Gruber an intermediate in the decarboxylation. The data presented in Table I show that the rearrangement proposed by Lachman does not occur. If it did, the \( CO_2 \) formed from diketosuccinic acid must acquire one-third of its carbon from one of the carboxyl groups.
of the proposed intermediate are equivalent, and one of them is formed from a carbonyl group. The data obtained for the decarboxylation of diketosuccinic acid-2-C\textsuperscript{14} show that only about 1 per cent of the CO\textsubscript{2} acquires label from the carbonyl carbon atoms. As shown later, this small conversion of carbonyl carbon to CO\textsubscript{2} can be explained by a different mechanism than a benaeric acid rearrangement.

In accordance with modern concepts (25), the decarboxylation of diketosuccinate may be shown as in Scheme 1. (In

\[
\text{CHO-CO-COO}^- \quad \text{mesoxalic semialdehyde}
\]

\[
\begin{align*}
\text{COO}^- & \quad \text{COO}^- \\
\text{C}=\text{O} & \quad \text{C}=\text{O} \\
\text{HOC-OH} & \quad \text{HOC-OH} \\
\text{COO}^- & \quad \text{CO}_2 \\
\end{align*}
\]

Scheme 1 diketosuccinate is pictured as a monohydrate, in accordance with analytical data on its salts (13, 21) but the water is considered to be readily added to and removed from both of the carbonyl groups in aqueous solution.) Thus the intermediate is not the mesoxalic semialdehyde suggested by Kekulé, but an enol thereof, and this enol might ketogenic in two ways, giving tartronate or mesoxalic semialdehyde. The facts show that at acid and neutral pH, the reaction goes primarily to tartronate.

If the steps in the decarboxylation of diketosuccinate are slightly reversible, the incorporation of CO\textsubscript{4}O\textsubscript{2} into tartronic acid can readily be explained. Thus, when the enolic intermediate reacquires a carbonyl group from CO\textsubscript{4}O\textsubscript{2} to form labeled diketosuccinic acid, the symmetry of the latter compound would result in the retention of about half of the label in the tartronic acid formed when the labeled diketosuccinate was again decarboxylated. This explains the incorporation of CO\textsubscript{4}O\textsubscript{2} into tartronic acid during the decarboxylation. The exchange of CO\textsubscript{4}O\textsubscript{2} into tartronic acid itself shows that the second step in the decarboxylation is also reversible.

The postulated mechanism for the decarboxylation can account not only for the exchange of CO\textsubscript{4}O\textsubscript{2} into tartronic acid, but it can also account for the fact that a small but definite amount of the carbonyl carbon of diketosuccinate appears in the CO\textsubscript{2} during decarboxylation. This is because tartronate is a symmetrical compound. One of the carbonyl groups of tartronate is derived from a carbonyl carbon atom of diketosuccinate. When CO\textsubscript{2} is added back to tartronate there is a 50 per cent chance that it may add to either end of the molecule. Under these circumstances, half of the backcarboxylation will occur in such a way that a carbon atom originally present as carbonyl carbon in diketosuccinate, will now become a carbonyl group of diketosuccinate, and so contribute to the CO\textsubscript{2} formed when the diketosuccinate is again decarboxylated. The reaction sequence would not have to proceed through tartronic acid itself, since there are two equivalent resonating forms of the enolate (Scheme 2).

The data obtained in the present experiments are not sufficiently accurate to warrant a rigorous quantitative comparison of the rate of the exchange of CO\textsubscript{4}O\textsubscript{2} into tartronate with the amount of label which appeared in CO\textsubscript{2} formed from diketosuccinic acid-2-C\textsuperscript{14}.

A reaction sequence for the decarboxylation of oxaloglycolate may be shown as in Scheme 3. In Scheme 3 it is assumed, in analogy with other \(\beta\)-keto acid decarboxylations (25) that the keto form of the acid goes to the enol of the product. As in the case of diketosuccinate, the enol formed in the first reaction step may ketogenic in two ways. If the preferred ketonization occurs in the same "direction" as in the decarboxylation of diketosuccinate, then hydrogen should add to the carbon atom which is closest to the carbonyl group, giving hydroxymalonlic semialdehyde rather than hydroxypyruvate. The fact that a second molecule of CO\textsubscript{2} is readily lost suggests that malonic semialdehyde is formed in preference to hydroxypyruvate. The data of Dickens and Williamson (28) on the decarboxylation of hydroxypyruvrate in hot aqueous solution suggest, however, that hydroxypyruvate would be decarboxylated to some extent under the conditions of the present experiments. Holzer and Holldorf (29) have identified hydroxypyruvate as a product of the decomposition of oxaloglycolate although their data do not indicate what proportion of the product accumulates in this form. The C\textsuperscript{14} label found in the residue of "glycolaldehyde" formed from oxaloglycolate-1-C\textsuperscript{14} (Table I) suggests that no more than about 10 per cent of this residue could have consisted of hydroxypyruvate, since glycolaldehyde itself should contain no label.

The present studies were the outcome of an interest both in the mechanism of decarboxylation reactions and in the metabolism of a group of organic acids of yet uncertain importance in intermediary metabolism. This group of compounds includes, among others, tartrate, hydroxypruvrate, oxaloglycolate, diketosuccinate, hydroxymalonate, and ketomalonate. Some of the possible interrelationships of this group of compounds have been summarized by Stafford (1). A path of conversion of hydroxymalonic acid to glycine has been suggested by the demonstration of a transaminase which forms aminomalonate from ketomalonate (30), and a decarboxylase which converts amino malonic acid to glycine (31). Furthermore, Davies and
Kun (4) have shown that malic dehydrogenase can apparently effect the oxidation of tartrate, oxaloglycolate, and tartronate approximately as well as it can effect the oxidation of malate.

Kun has identified glyoxylate and hydroxypyruvate as well as diketosuccinate as products of tartrate oxidation (2, 3), but the quantities of these products reported accounted for only a small fraction of the tartrate oxidized in his experiments. Although the data obtained in the present experiments with labeled tartrate suggest that hydroxymalonic acid may be a major product, they would also be consistent with a wide variety of possible mixtures of products. The complexities of the problem are great. It is hoped that the use of labeled tartrate may facilitate the quantitative determination of the products of tartrate oxidation. In the meantime, it is well to keep in mind that the conditions employed here to study the nonenzymatic decarboxylation mechanism are rather different from the conditions employed in the enzyme experiments.

SUMMARY

The decarboxylation of C4-labeled diketosuccinic acid in aqueous solution has been shown to yield CO2 derived primarily from a carboxyl group of the acid. Such a small contribution of the carboxyl carbon of diketosuccinic acid to the CO2 formed can be attributed mainly to the reversibility of a reaction sequence in which an enolate of tartronate (hydroxymalonic) acid is the first decarboxylation product. In keeping with such a mechanism, it has been shown that when diketosuccinic acid is decarboxylated in the presence of C14O2, C14 is fixed in the tartronic acid formed, and that CO2 also exchanges its carbon very slowly with tartronic acid.

The decarboxylation of labeled oxaloglycolic acid has been shown to yield CO2 derived from the carboxyl groups. The C14 content of the residue after the decarboxylation shows that the products probably consist of a mixture of about 9 parts of glycolic aldehyde and 1 part of hydroxypyruvic acid.

REFERENCES

The Nonenzymatic Decarboxylation of Diketosuccinate and Oxaloglycolate
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C. T. Chow and Birgit Vennesland