CONVERSION OF C\textsuperscript{14}-LABELED ACETATE TO CITRIC AND MALIC ACIDS IN THE TOMATO FRUIT\textsuperscript{1}

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The possible intermediary position of the plant acids in respiration and carbohydrate metabolism as indicated for some plant tissues, suggests that a similar situation may exist in fruit. Many fruits contain comparatively large amounts of various acids (8) and apparently have a very active acid metabolism, as shown by the changes in concentration which occur during growth, ripening and storage. Turner (10), for example, found that most of the citric acid in apple tissue was actively engaged in the metabolic flux during the post-harvest cold storage period. He concluded that the data of his experiments are compatible with the participation of a Krebs cycle in carbohydrate oxidation in the apple, but do not prove that such a cycle exists.

While C\textsuperscript{14}-labeled substrates have been used extensively for studying intermediary metabolism in some plant tissues, their use in connection with fruit has not been reported, so far as known. In preliminary experiments with tomato fruit, it was found that following injection with C\textsuperscript{14}-labeled sodium acetate, radioactivity appeared in the respiratory CO\textsubscript{2}, the acids, and in certain other fractions isolated from the juice and tissue. Data from degradation studies on citric and malic acid which suggest the participation of a Krebs cycle in tomato fruit metabolism are presented.

Experimental

The fruits used in this experiment were greenhouse grown Michigan State Forcing tomatoes picked at the mature green state. Samples were collected in the evening and kept in a refrigerator at 3° C until 7:30 A.M. when treatments were started. Carboxyl C\textsuperscript{14}-labeled sodium acetate with a specific activity of $3.00 \times 10^8$ counts per minute per mM (c.p.m.) was prepared by the conventional Grignard reaction and dissolved in water to provide a concentration of 1 mg./ml. By means of a hypodermic syringe, 6 ml. of this solution were injected into the locules of two tomatoes weighing 284 and 199 gm., respectively. Preliminary experiments with dye solutions showed that solutions injected by this method were absorbed mainly by the locular tissue and to a lesser extent by the pericarp. The treated samples

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were transferred immediately to a desiccator at 28°C and kept at this temperature during the course of the experiment.

The evolved CO₂ was aspirated from the chamber with CO₂-free air and collected in sodium hydroxide solution contained in a gas washing bottle fitted with a sintered glass disperser. The solution was replaced every four hours and the absorbed CO₂ was precipitated as barium carbonate for radioactivity assay. The specific activity of the respiratory CO₂ reached a maximum in approximately 14 hours and declined thereafter. Meanwhile the rate of increase in the cumulative radioactivity began to level off in approximately 28 hours, indicating that the maximum rate of incorporation had been reached and that further incubation would probably reduce the net incorporation of the labeled substrate in the fruits.

The fruits, therefore, were transferred to a blender and finely ground. The juice was separated by centrifuging and adjusted to pH 3 with sulfuric acid. After standing overnight the precipitated protein was removed by centrifuging. The clear juice thus obtained was further acidified with sulfuric acid and extracted with ether for 72 hours in a liquid-liquid extractor. The ether extract was evaporated to dryness under vacuum, the residue was taken up in 10 ml. distilled water, and after being chilled in a refrigerator the insoluble fatty material was separated by filtration. By titration it was found that the filtrate contained 11.7 mE total acids. The titrated solution was counted by direct plating and was found to have $2.18 \times 10^6$ c.p.m., which
represented 10% of the total input and 18% of the radioactivity incorporated. By chemical analyses for citric (7) and malic (6) acids, the organic acid fraction was found to contain 398 mg. (6.2 mE) of citric acid and 354 mg. (5.3 mE) of malic acid. The close agreement of the total milliequivalents of acids found by titration (11.7) and by analysis for citric and malic acids (11.5) indicate that only traces of other acids could have been present.

To determine the incorporation and distribution of C\(^{14}\) in citric and malic acids, one-half of the acid solution was neutralized with sodium hydroxide, evaporated to dryness under vacuum, and the hydroxy acids separated by means of partition chromatography according to the method of BULEN, VARNER, and BURRELL (1). Only citric and malic acids could be identified positively on the chromatogram. The citric acid fraction was concentrated to 25 ml. and was found by analysis to contain 2.51 mg./ml. Similarly, the malic acid fraction concentrated to 10 ml. was found to contain 5.85 mg./ml.

Each of the pure acids thus obtained was diluted with non-radioactive carrier, acidified with sulfuric acid and extracted with ether in a liquid-liquid extractor. The ether extracts were dried over sodium sulfate and evaporated. Citric acid was degraded according to WEINHOUSE, MODES, and FLOYD (11). Malic acid was degraded according to WOOD, WERKMAN, HEMMINGWAY, and NIER (12).

All samples were counted as barium carbonate to a standard error of two per cent. and corrected for background and self-absorption.

The protein fraction isolated from the juice was combined with the protein obtained from the pulp by sodium borate extraction (2). The combined fraction weighed 1.65 grams, contained 7.0% nitrogen and had a radioactivity of 1.51 \(\times 10^6\) c.p.m.

**Discussion of results**

That acetate is readily metabolized by the tomato fruit is apparent from the data obtained. Approximately 45% of the radioactivity of the injected substrate was found in the respiratory CO\(_2\) within 28 hours. Radioactivity was incorporated also in the acid and protein fractions isolated from the juice and pulp (table I).

As shown by the C\(^{14}\)-labeling pattern in table II, only the carboxyl groups of citrus and malic acids were found to be labeled. In the case of

**TABLE I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Weight in gms.</th>
<th>Radioactivity c.p.m. (\times 10^6)</th>
<th>Per cent. of incorporated radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total acids</td>
<td>0.40</td>
<td>2.18</td>
<td>10</td>
</tr>
<tr>
<td>Protein</td>
<td>1.65</td>
<td>1.51</td>
<td>7</td>
</tr>
<tr>
<td>CO(_2)</td>
<td>0.57</td>
<td>9.89</td>
<td>45</td>
</tr>
</tbody>
</table>
malic acid, the labeling pattern could be explained on the basis of Krebs cycle operation, whereby the radioactive acetate carboxyl group is introduced into the cycle through citrate formation and eventually appears equally distributed in both carboxyl groups of malic acid. This mechanism, however, could not account for any net synthesis of plant acid, which apparently also is an important process in the fruit, since removal of any of the Krebs cycle acids would immediately discontinue the cyclic process. Consequently, it is necessary that some other means are available for synthesizing C₄ acids. Such synthesis could occur by the Wood-Werkman reaction (12), by the "malic fixation" scheme (4), or by a tail-to-tail condensation of the Thunberg type (9) which also results in an exclusive carboxyl labeling of the C-4 acids when starting from carboxyl labeled acetate.

### TABLE II

**DISTRIBUTION OF RADIOACTIVITY IN CITRIC AND MALIC ACIDS FROM TOMATO FRUITS UTILIZING CH₃C¹⁴OOH.**

<table>
<thead>
<tr>
<th>Citric acid</th>
<th>C.p.m. x 10⁵ per mM</th>
<th>Malic acid</th>
<th>C.p.m. x 10⁵ per mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole molecule</td>
<td>5.67</td>
<td>Whole molecule</td>
<td>3.03</td>
</tr>
<tr>
<td>C₁ + C₅</td>
<td>4.28*</td>
<td>C₁ + C₄</td>
<td>3.02*</td>
</tr>
<tr>
<td>C₆</td>
<td>1.39</td>
<td>C₂ + C₃</td>
<td>0.00</td>
</tr>
<tr>
<td>C₂ + C₃ + C₄</td>
<td>0.00</td>
<td>....</td>
<td>....</td>
</tr>
</tbody>
</table>

*Activity per 2 mM of carbon.

If citric acid in the tomato fruit is formed by the oxalacetate-acetate condensation via the Krebs cycle, assigning C₄ and C₅ of citric acid to the acetate residue, then C₆ and C₁ of citric acid should correspond to C₁ and C₄ of malic acid, respectively. When the specific activities of these carbon atoms are calculated to equal concentrations in the fruit to eliminate the dilution factor, the values obtained are in close agreement. Thus,

- Specific activity of C₁ or C₄ of malic acid = \( \frac{1}{2} (3.02 \times 10^5) \) = \( 1.51 \times 10^5 \) c.p.m./mM
- Specific activity of C₆ of citric acid = \( 1.39 \times 10^5 \) c.p.m./mM
- Malic acid content = 2.65 mM
- Citric acid content = 2.08 mM
- Specific activity of citric acid on the same concentration basis = \( 1.39 \times 10^5 \times 2.65/2.06 \) = \( 1.79 \times 10^5 \) c.p.m./mM

Similarly, if it is assumed that the specific activities of C₆ and C₁ of citric acid are equal as a result of the randomization process of oxalacetic acid equilibrating with symmetrical C₄ acids prior to the condensation reaction, then the specific activity of C₅ and the ratio C₅/C₁ can be calculated from the data. The observed ratio of 2.08 is in good agreement with the theoretical ratio of 2.0, derived on the basis of Ogston's (3) concept of the asymmetric nature of citric acid (4, 5).
Summary

Carboxyl C¹⁴-labeled sodium acetate was injected into the locules of mature green tomatoes. Radioactivity appeared in the respiratory CO₂, which accounted for 45% of the original amount injected. Labeling also occurred in the protein and acid fractions isolated from the juice and tissue. Degradation of malic and citric acids showed only the carboxyl groups to be labeled. The relation of the data to the possibility of Krebs cycle operation in the tomato fruit are discussed.

LITERATURE CITED