The Metabolism of the Organic Acids of Tobacco Leaves *

XVIII. EFFECT OF CULTURE OF EXCISED LEAVES IN SOLUTIONS OF POTASSIUM L-ISO CITRAT E

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Among the unsolved problems of the biochemistry of organic acids in green leaf tissue is the position of isocitric acid in the metabolic system. This acid accumulates in substantial amounts in the leaves of plants of the botanical family Crassulaceae, and has been found in moderate amounts in a number of other species, notably the fruit of the blackberry (Rubus fruticosus), where it was first discovered in nature (1), and the leaves of the foxglove (Digitalis purpurea) (2). Nevertheless, inasmuch as isocitric acid is a member of the tricarboxylic acid cycle, there is little doubt that its distribution, at least in trace amounts, is far wider than these and a few other observations would suggest. Indirect evidence for this is the identification of isocitric dehydrogenase in such plant tissues as cucumber and pea seeds (3), Avena coleoptile (4), and parsley root (Petroselinum hortense) (5), and in the leaves of chickweed (Stellaria mati a), elder (Sambucus nigra) (6), and tomato (Lycopersicon esculentum) (7).

The accumulation of isocitric acid in high concentrations in leaves of crassulaceous plants, and to a moderate extent also in a few species in other botanical families, suggests, however, that this substance fulfills some function in them which is quite different from what may be regarded as its usual function as a member of the tricarboxylic acid cycle. Leaves of Bryophyllum calycinum normally contain from 12 to 16 g of isocitric acid per kg of fresh weight. Extensive study of the acids of these leaves during exposure to diurnal alternation of light and dark, or to extended periods of light or of darkness, has shown that isocitric acid remains essentially unaltered in amount throughout the treatment. Notwithstanding this, the malic acid present may decrease from 14 to 2 g per kg during a 10-hour period of illumination and return almost to the initial high level during the following night (8).

Citric acid undergoes diurnal variation in amount in Bryophyllum leaves in a manner essentially parallel with that of malic acid, although to a much smaller extent with respect to the quantity involved (10).

It would appear that the isocitric acid stored in such remarkable amounts in the leaves of crassulaceous plants is used up in the course of the organic acid metabolism only under conditions of stress, and even then to only a small extent. Long exposure of Bryophyllum leaves to light constitutes one of the conditions that have been observed to bring this about, and another is the situation that arises when leaves that are unusually low in starch content are maintained in darkness for many hours in addition to the normal period (11). This is not to say that isocitric acid is a wholly inactive component of the tissue, but merely that its metabolic activity in comparison with that of other organic acid components is low. For example, Stutz and Burris (12) have found that Bryophyllum calycinum leaves exposed to air containing C¹⁴O₂ acquire radioactivity readily in the organic acids, but about 90% was found in malic acid and only about 5% in isocitric acid. Similar results have been published by Thomas and Ranson (13).

In view of the apparent biochemical indifference of isocitric acid in leaves of crassulaceous plants, it has seemed essential to examine the behavior of the acid when introduced in substantial amounts into the leaves of another species. Leaves of the tobacco plant have accordingly been cultured in solutions of potassium L-isocitrate in darkness, and the effect upon the organic acid composition has been studied. The outcome has been a demonstration that isocitric acid is extensively metabolized in tobacco leaves, the major identified product being citric acid. This result confirms an earlier test (14) in which, because of the scarcity of optically active isocitric acid at the time, only a single sample of leaves could be treated.

EXPERIMENTAL PROCEDURE

For the large scale quantitative experiment, 10 samples of 20 leaves each were collected on July 20, 1958 by the statistical method (15) from 20 plants of Nicotiana tabacum, var. Connecticut shade-grown, raised in the greenhouse. The leaves were cut from 10 successive nodes on each plant counting from the lowest fully expanded leaf. The coefficient of variation of the fresh weight was 2.1% and that of the total nitrogen content 1.7%. Of these samples, 8 were used for the present experiment. The control culture solutions were water, 0.2 M potassium sulfate, and 0.2 M potassium succinate adjusted to pH 5.0. The mono-potassium L-isocitrate used was isolated from Bryophyllum leaves by the procedure of Vickery and Wilson (16), and a 0.15 M solution was prepared at pH 4.0 and 0.2 M solution at pH 5.0 by adjustment with potassium hydroxide; 2 samples were cultured in each of these, the culture periods being, respectively,
24 and 48 hours. All control samples were cultured for 48 hours. During the experiment, the leaves were supported in V-shaped troughs which contained about 1200 ml of culture solution. These were placed in a completely dark room maintained at 24° and 50% relative humidity. The leaves were then weighed and dried at 80°, and the tissue was equilibrated in the air-conditioned room until it attained constant weight; it was then ground to powder for analysis. The isocitric acid in the used culture solutions was recovered as monopotassium salt in a yield of 88%. 

L-Isocitric acid was determined by means of isocitric dehydrogenase as described by Stern (17). The other analytical methods used are described elsewhere (18, 19).

For the experiment with radioactive isocitrate, three successive leaves were cut from about the middle of the stalk of a tobacco plant of the variety Havana Seed grown in the greenhouse in the late winter season of 1960. The intermediate leaf was used for the experiment, and the others were immediately dried and prepared for analysis. It was assumed that the initial composition of the intermediate leaf could be calculated with sufficient accuracy as the mean of the composition of the two control leaves.

Monopotassium d,L-isocitrate 3, 4 C14 was synthesized by the method of Fitting and Miller (20) as recently modified (21) from 1 g of anhydrous sodium succinate to which 50 μC of neutralized succinic acid-2,3-C14 (0.4 mg) had been added. The yield was 0.8 g (42%) of a product which contained 49% of l-isocitrate by enzymatic analysis. The activity was 18 × 106 c.p.m. per mg. When tested by chromatography on paper, all of the radioactiv-

ity was found. Further examination showed that all of the activity was present in the α-ketoglutarate (isolated as 2,4-dinitrophenyl-

hydrazone) produced by isocitric dehydrogenase as described by Stern (17). The other analytical methods used are described elsewhere (18, 19).

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Table I

Effect of culture in solutions of potassium L-isocitrate upon organic acid composition of excised tobacco leaves

The data represent milliequivalents per kg of initial fresh weight of leaves, unless otherwise stated.

<table>
<thead>
<tr>
<th>Line</th>
<th>Component</th>
<th>Control before culture</th>
<th>Changes during culture in darkness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Water (0.2 M)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>48 hrs</td>
</tr>
<tr>
<td>1</td>
<td>Fresh weight, %</td>
<td>0</td>
<td>+9</td>
</tr>
<tr>
<td>2</td>
<td>Ash, g.</td>
<td>15.8</td>
<td>+0.2</td>
</tr>
<tr>
<td>3</td>
<td>Organic solids corrected for CO₂, g.</td>
<td>72.7</td>
<td>-2.8</td>
</tr>
<tr>
<td>4</td>
<td>Alkalinity of ash</td>
<td>304</td>
<td>+1.3</td>
</tr>
<tr>
<td>5</td>
<td>Potassium</td>
<td>157</td>
<td>-3.3</td>
</tr>
<tr>
<td>6</td>
<td>Extract of dry tissue, pH</td>
<td>5.38</td>
<td>+0.04</td>
</tr>
<tr>
<td>7</td>
<td>Uptake of acid</td>
<td>174</td>
<td>84</td>
</tr>
<tr>
<td>8</td>
<td>Total nonvolatile acids (except oxalic acid)</td>
<td>145</td>
<td>+1</td>
</tr>
<tr>
<td>9</td>
<td>Malic acid</td>
<td>106</td>
<td>-44.0</td>
</tr>
<tr>
<td>10</td>
<td>Citric acid</td>
<td>22.0</td>
<td>+42.2</td>
</tr>
<tr>
<td>11</td>
<td>Isocitric acid</td>
<td>0</td>
<td>+62.5</td>
</tr>
<tr>
<td>12</td>
<td>Succinic acid</td>
<td>1.5</td>
<td>+0.4</td>
</tr>
<tr>
<td>13</td>
<td>Minor acids</td>
<td>6</td>
<td>+2</td>
</tr>
<tr>
<td>14</td>
<td>Oxalic acid</td>
<td>25</td>
<td>+0</td>
</tr>
<tr>
<td>15</td>
<td>Loss of acid groups</td>
<td>68</td>
<td>24</td>
</tr>
<tr>
<td>16</td>
<td>Loss of acquired acid</td>
<td>152</td>
<td>21</td>
</tr>
<tr>
<td>17</td>
<td>Line 16 as % uptake</td>
<td>87</td>
<td>25</td>
</tr>
<tr>
<td>18</td>
<td>Citric acid possibly derived from malic acid</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>19</td>
<td>Citric acid derived from another source</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>Line 19 as % of Line 16</td>
<td>5</td>
<td>13</td>
</tr>
</tbody>
</table>

Comparison of the data with the corresponding items in the right hand column of Table I suggests that the organic acid metabolism of this leaf was distinctly more vigorous than that of the 20-leaf sample; both malic acid and citric acid increased to a greater extent, and a significant amount of succinic acid accumulated. Nevertheless, the proportion of the isocitric acid taken up which underwent chemical change was about the same, as was also the increase of citric acid calculated as a percentage of the isocitric acid which disappeared.

The distribution of the radioactivity among the components of the leaf is shown in Table III, the data being calculated for convenience in terms of a single leaf. By far the greatest part of the radioactivity was present in substances extracted by water from the dried tissue, and only a negligibly small amount was found in the carbon dioxide evolved from the leaf during the culture period and in the residue from the extraction. The water extract, after being subjected to a partial lactonization process during which a small loss was encountered, was placed on a column of Dowex 1, and the water washings of the column were collected separately. They contained only a little radioactive material; accordingly, only a small part of the isocitric acid was converted into neutral and basic amino acids and sugars which would be the major known components of this fraction.

The organic acid components are listed in the order in which they are eluted from the column by increasing strengths of formic acid. This impurity was of the order of only 5 mg, or less than 2% of the total isocitrate acquired by the leaf, no consideration has been given to it save to subtract this quantity from the calculated uptake of D,L-isocitrate.

Disappeared from the system. The close agreement of three of the four results is of interest, and it is clear that a substantial part of the acquired isocitric acid may have been converted into citric acid. In support of this view is the fact that there is no organic acid component other than isocitric acid which diminished sufficiently in amount to account for the increase in citric acid.

Table II shows the effect of culture of a single tobacco leaf in 0.2 M radioactive isocitrate at pH 5.0 for 48 hours in darkness. The uptake was calculated from the loss of radioactivity from the culture solution, and the analytical data, expressed in milliequivalents per kg of initial fresh weight of the leaf, were calculated on the assumption that the initial composition of the treated leaf was the mean between that of the leaves above and below it on the stalk. The analytical errors are presumably similar to those of the data in Table I, but the sampling error is doubtless greater. In preparing the water extract of the dried leaf for organic acid analysis, the solution was repeatedly evaporated to dryness and heated in a boiling water bath with the object of converting a portion of the isocitric acid present to the lactone. Because the lactone is eluted from the Dowex 1 column at a point well separated from the citric acid fraction (which contains citric, both L- and D- isocitric, and phosphoric acids), a demonstration was provided of the presence of isocitric acid and a sample was obtained from which some information regarding the relative proportions of the two enantiomorphs present could be obtained.

1 The synthetic isocitrate used contained 8% of an impurity which was doubtless mainly alloisocitrate. Since the uptake of this impurity was of the order of only 5 mg, or less than 2% of the total isocitrate acquired by the leaf, no consideration has been given to it save to subtract this quantity from the calculated uptake of D,L-isocitrate.
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TABLE II
Effect of culture in 0.3 m solution of radioactive potassium isocitrate at pH 5.0 for 48 hours in darkness on organic acid composition of a tobacco leaf*

The data are expressed in milliequivalents per kg of initial fresh weight.

<table>
<thead>
<tr>
<th>Initial composition (calculated)</th>
<th>Change during culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uptake of isocitric acid.</td>
<td>+247</td>
</tr>
<tr>
<td>Total nonvolatile acids (except oxalic acid)</td>
<td>+185</td>
</tr>
<tr>
<td>Malic acid.</td>
<td>+25</td>
</tr>
<tr>
<td>Citric acid (pentabromoacetone method).†</td>
<td>+60</td>
</tr>
<tr>
<td>Isocitric acid (enzymatic method).</td>
<td>+58</td>
</tr>
<tr>
<td>Isocitric lactone (titration).</td>
<td>+14</td>
</tr>
<tr>
<td>Succinic acid.</td>
<td>+7</td>
</tr>
<tr>
<td>Minor acids.</td>
<td>+4</td>
</tr>
<tr>
<td>Loss of isocitric acid from system</td>
<td>168</td>
</tr>
<tr>
<td>Loss of isocitric acid as % of uptake</td>
<td>68</td>
</tr>
<tr>
<td>Increase of citric acid as % of loss of isocitric acid</td>
<td>36</td>
</tr>
</tbody>
</table>

* The initial fresh weight of the experimental leaf was 15.4 g. To convert the data to single leaf basis, divide by 64.9.

† The citric acid fraction contained 50 meq of citric acid, 58 meq of L-isocitric acid, and 13 meq of phosphoric acid titrated to the phenolphthalein end point. The deficit from the titration of the entire fraction to the same end point, which was 168 meq, may be attributed to D-isocitric acid if no other acidic component was present as was the case in the large scale experiment. Accordingly, it is assumed that this fraction contained 17 meq per kg of D-isocitric acid.

acid. The fractions containing the minor acids (glycolic, D-glyceric, quinic, and aspartic acids are the chief components) acquired a little radioactivity, but succinic acid, although present as was the case in the large scale experiment. Accordingly, it is assumed that this fraction contained 17 meq per kg of D-isocitric acid.

The specific activity of the total isocitric acid in the lactone fraction was 1100 X 10³ c.p.m.; the specific activity of the L-isocitric acid in the lactone fraction at the end of the culture period can therefore be calculated to be 480 X 10³ c.p.m. It is doubtful if this is significantly different from the initial specific activity of 500 X 10³ c.p.m. of the L-isocitrate supplied.

The composition of the citric acid fraction (Table II, footnote) as established by determinations of total acidity and of citric, L-isocitric, and phosphoric acids, left a small deficit of acidity, presumably arising from the presence of D-isocitric acid. This amounted to 22% of the isocitric acid present, and if the D-enantiomorph is assumed to have remained unchanged during the culture period, the data in the last three lines of Table III can be calculated. These results lead to the conclusion that the citric acid present in the leaf had acquired a specific activity of approximately 300 X 10³ c.p.m. per mmole. In order to make certain that the citric acid was indeed radioactive, a citric acid fraction from a separate analysis was treated with potassium permanganate and potassium bromide under the conditions used for the quantitative determination of citric acid (24), and the pentabromoacetone was isolated by extraction with petroleum ether. This was in turn oxidized by the wet combustion method of Van Slyke et al. (25), the carbon dioxide was collected, and its radioactivity determined. The specific activity of the citric acid was found to be 170 X 10³ c.p.m. per mmole. It should be noted that this value refers only to the carbon atoms in positions 1, 3, and 4 of the acid.

TABLE III
Distribution of radioactivity among the components of a tobacco leaf after culture in potassium isocitrate-3,4-C¹⁴ at pH 5 for 48 hours in darkness

<table>
<thead>
<tr>
<th>C.p.m. per leaf X 10³</th>
<th>Distribution counts</th>
<th>Amount per leaf</th>
<th>C.p.m. per mmole X 10³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uptake of isocitric acid*</td>
<td>1100</td>
<td>100</td>
<td>1.27</td>
</tr>
<tr>
<td>Carbon dioxide liberated</td>
<td>16</td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td>Water extract of dry leaf</td>
<td>1110</td>
<td>96.5</td>
<td></td>
</tr>
<tr>
<td>Water extract after lactorization operation</td>
<td>1020</td>
<td>88.7</td>
<td></td>
</tr>
<tr>
<td>Insoluble residue</td>
<td>47.0</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>Total accounted for</td>
<td>1104</td>
<td>101.2</td>
<td></td>
</tr>
</tbody>
</table>

In water extract:
Nonacidic substances | 24.6 | 2.4 | | |
Minor acids (monovalent) | 14.8 | 1.5 | 0.42 | 35 |
Succinic acid | 11.1 | 1.1 | 0.076 | 149 |
Malic acid | 194 | 19.0 | 1.40 | 138 |
Acids in citric acid fraction (trivalent) | 671 | 66.5 | | |
Isocitric lactone (divalent) | 116 | 11.4 | 0.105 | 1100 |
Total in water extract | 1031 | 101.2 | | |

In citric acid fraction:
L-isocitric acid (enzymatic method) | 164 | 24 | 0.208 | 550 |
D-isocitric acid, calculated | 360 | 54 | 0.087 | 4140 |
Citic acid (pentabromoacetone method) | 147 | 22 | 0.413 | 300† |

Total in citric acid fraction | 671 | 100 | | |

* The mixture of L-isocitrate and D-L-isocitrate administered had a specific activity of 900 X 10³ c.p.m. per mmole. The uptake of L-isocitrate was 0.908 mmole and that of D-L-isocitrate-3,4-C¹⁴ of specific activity 4140 X 10³ c.p.m. per mmole was 0.277 mmole. Accordingly, the specific activity of the L-isocitrate available to the leaf tissue was 550 X 10³ c.p.m. per mmole when a correction for the presence of 8% of impurity, presumably mainly alloisocitrate, in the synthetic material is applied. It is assumed that the D-isocitrate does not become involved in the metabolism. It is known to be stable to isocitric dehydrogenase and aconitase.

† See the text for discussion of this presumably maximal figure.
3 and 4 in citric acid. Any radioactive carbon present in carboxyl groups as a result of randomization reactions or derived from fixation of radioactive carbon dioxide would have been eliminated during the formation of the pentabromoacetone. Accordingly, this figure would be expected to be a minimal one.

\[
\begin{align*}
\text{CH(OH)} & \rightarrow \text{COOH} \\
\text{CH} & \rightarrow \text{COOH} \\
\text{C} & \rightarrow \text{HBr}_2 \\
\text{C} & \rightarrow \text{O} \\
\text{C} & \rightarrow \text{Br}_3 \\
\text{Isocitric acid-} & \text{3,4-Cl}^4 \\
\text{Citric acid-} & \text{3,4-Cl}^4 \\
\text{Pentabromoacetone-} & \text{Cl}^4
\end{align*}
\]

**DISCUSSION**

The extensive metabolic transformations undergone by isocitrate when introduced into the tobacco leaf contrast sharply with the behavior of this substance in the leaves of crassulacean plants, in some species of which it is present in concentrations that approach 0.1 M. Furthermore, it appears to share in the metabolic reactions of the leaf system in these plants only under conditions of stress. In the tobacco leaf, on the other hand, it promptly undergoes reactions of which two kinds can be clearly distinguished. Titratable carboxyl groups equivalent to from one-quarter to one-third of the added acid disappear from the system, and citric acid is generated in an amount equivalent to about 40% of the isocitric acid which undergoes chemical change. When radioactive isocitrate-3,4-Cl^4 is supplied, the appearance of radioactivity in both succinic and malic acids accounts for approximately 14 to 1 (27). In Bryophyllum leaves there may be from 6 to 15 times as much isocitric acid as citric acid present, depending on the time of day when the leaves are collected for examination. Observations such as these may, however, mean that in tobacco leaves isocitric acid, as is apparently also the case with citric acid (28), is present in two different situations with respect to its availability as a substrate of the enzyme systems concerned. If, in crassulacean plants, isocitric acid is likewise so segregated, its stability during the normal processes of organic acid metabolism may be to some extent accounted for.

**SUMMARY**

Although L-isocitric acid is normally a stable organic acid component of the leaves of plants in the family Crassulaceae, it is readily and extensively metabolized when introduced into the leaves of the tobacco plant. Decarboxylation reactions occur and moderately extensive conversion to succinic, malic, and citric acids has been detected by the use of synthetic isocitric acid labeled in positions 3 and 4. Citric acid accumulates in an amount of the order of 40% of the isocitric acid that undergoes chemical change. A part of the newly formed citric acid may have arisen through the operation of the reactions of the tri-carboxylic acid cycle, but the evidence favors the view that a part is also formed through the agency of aconitase.

**Acknowledgments**—The skillful technical assistance of Lauren S. Nolan, Katherine A. Clark, and Edna Baker Breedis is gratefully acknowledged, as well as the help from many discussions with Dr. Israel Zelitch.

**REFERENCES**


\(^2\) Bacon, Palmer, and de Kock (26) found aconitase in low concentration in Bryophyllum crenatum leaves although they detected none in three other crassulacean species. Evidence for its presence in B. calycinum leaves has been obtained by Dr. I. Zelitch in this laboratory (unpublished observation).
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