Sucrose Synthesis from Acetate in the Germinating Castor Bean: Kinetics and Pathway*

DAVID T. CANVIN† AND HARRY BEEVERS

From the Department of Biological Sciences, Purdue University, Lafayette, Indiana

(Received for publication, November 28, 1960)

During the germination of fatty seeds such as the castor bean, a rapid conversion of fat to sugar occurs (2). The fatty acids are probably broken down to acetyl units via $\beta$-oxidation (3); the discovery of the glyoxylate cycle enzymes (4) in the castor bean endosperm (5) provided a mechanism whereby these acetyl units could be quantitatively converted to malate. Since malate could be viewed as a sugar precursor, Kornberg and Beevers (6) suggested that the glyoxylate cycle, coupled with a reversal of glycolysis, afforded a plausible route for the conversion of acetate to sugar, which had been previously shown to occur with high efficiency in this tissue (7).

A considerable amount of evidence has now been collected on the existence of the enzymes required for such a conversion (7-11) in the tissues of fatty seedlings, on the rather strict confinement of malate synthetase and isocitritase to such tissues in higher plants, and on the striking changes in activity of these enzymes which occur in phase with the changing pace of fat breakdown in the developing seedling (8, 9). This evidence, all of it encouraging, must nevertheless be complemented by work with the intact tissue to establish the validity of the proposal in hand.

Bradbeer and Stumpf (12) have provided valuable data from experiments with peanut and sunflower tissues that show that the products and some of their labeling patterns after several hours in contact with specifically labeled acetate-C14 were those expected from the operation of the pathway of conversion were those predicted if the pathway of conversion were that proposed. In this report, more extensive short term experiments are described in which a variety of labeled substrates was provided to slices of castor bean endosperm (5) and slices 0.5 mm thick were cut by hand. The slices were washed twice in distilled water before use.

Castor bean endosperm which had been allowed to fix C402 for 10 minutes. The amount of acid was determined by titration with standard base and the total activity by persulfate oxidation (13). The radioactivity was confined to the carboxyl groups with 66% of the C4 in carbon 4 as determined by Lactobacillus arabinosus fermentation (14).

Feeding Experiments—One-gram (fresh weight) castor bean endosperm slices were incubated with 120 $\mu$moles of potassium phosphate, pH 5.0, and 5 $\mu$moles of acetate-1-C14 (330,000 c.p.m.) or 5 $\mu$moles of acetate-2-C14 (629,000 c.p.m.) in a total volume of 4.0 ml. The incubations were carried out in large Warburg flasks (125 ml) at 25°. The dicarboxylic acids were supplied under similar conditions as shown in Table XI.

Carbonate-free 20% NaOH was added to the center wells to absorb C402. The carbonate was converted to BaCO3 and assayed for radioactivity as described below.

After removal of the NaOH, the tissue was killed by the addition of 20 ml of boiling 80% ethanol; the tissue was then successively extracted for 10-minute periods on the steam bath with 20 ml of 80% ethanol, 20 ml of 20% ethanol, 20 ml of water, and, finally, 20 ml of 80% ethanol. The extracts were filtered and combined; the insoluble residue was retained by the filter. The combined extracts were taken to dryness in a vacuum on a water bath at 40°. The dry residue was successively extracted with ethyl ether and water, to yield, respectively, the lipid fraction and the water-soluble fraction.

Analytical Methods—The water-soluble fraction was separated into three fractions through the use of ion exchange resins. These fractions were acidic (mainly organic acids), basic (mainly amino acids), and neutral (mainly sugars). The aqueous extract was first passed through a 6- × 1-cm column of Dowex 50-X8 (II) and the effluent was then passed through a 6- × 1-cm column of Dowex 1-X10 (formate)—generated from the chloride form with 60 ml of 1 m sodium formate and 30 ml of 0.1 m formic acid. The effluent from this column constituted the sugar fraction. The amino acids were eluted from the Dowex 50 resin with 50
ml of 1 N NH₄OH and the organic acids were eluted from the Dowex 1 resin with 40 ml of 4 N formic acid.

After evaporation of these fractions to dryness in a vacuum on a water bath at 40°, the residues were redissolved in 25 ml of water and an aliquot taken to determine the radioactivity.

The sugar fraction was concentrated to a small volume and further separated chromatographically on Whatman No. 1 paper. Radioactive areas were ascertained by exposing strips with the reagent of Zimmerman (15), they were eluted from the paper. Radioactive areas were ascertained by exposing strips of the chromatogram to Kodak "no screen" x-ray film. Sucrose was hydrolyzed with crystalline invertase (Nutritional Biochemicals) and the products separated chromatographically. The amounts of the sugars were determined using the method of Dubois et al. (16).

The organic acid fraction was separated into its constituents by gradient elution on ion exchange resins (17). The gradient elution apparatus that was used yielded a linear gradient and was based on the theory of Lakshmanan and Lieberman (18). Formic acid (4 N) was used in the reservoir and the organic acids were eluted from an 11-×1 cm column of Dowex 1-X10 (formate). The eluate was collected in 3-ml fractions and the radioactive fractions were determined by counting a 0.3-ml aliquot on a metal planchet. The radioactive "peaks" were identified by their position of elution from the resin, by chromatography on paper with authentic samples in other formic acid-water (5:2:1 by volume), butanol-acetic acid-water (12:3:5 by volume), ethanol-ammonia-water (16:1:3 by volume), and by the fluorescence of the derivatives formed with resorcinol and H₂O₄ (19). Glucose 6-phosphate was identified by chromatography in methanol-ammonia-water (60:10:30 by volume) and by chromatography of the free sugar after treatment of the ester with Schwarz's Polidase. The paper chromatograms were exposed to Kodak "no screen" x-ray film; exact correspondence of authentic sample and radioactive area was used as proof of identity.

The amino acid fraction was separated by a modification of the procedure of Hirs, Moore, and Stein (20). The eluate was collected in 3-ml fractions and radioactive "peaks" were determined by counting a 0.3-ml aliquot on a metal planchet; amino acid "peaks" were determined with ninhydrin (21). The amino acids were identified by cochromatography in phenol-water (80:20 by volume) and butanol-acetic acid-water (12:3:5 by volume).

The glucose moiety of sucrose was degraded by the Leuconostoc mesenteroides procedure of Gunsalus and Gibbs (22). The ethanol was recovered by distillation and converted to acetic acid (23). The residue was acidified to pH 2.0 and the lactate extracted with ether. The lactate was degraded to CO₂ and acetic acid (24). Acetic acid was recovered by steam distillation and converted to its silver salt by the method of Jourdian (25). The silver acetate was degraded by a Schmidt reaction in a Stutz and Burris apparatus (26) in which the zinc trap was replaced with a permanganate scrubber (45% KMnO₄ in 1.0 N H₂SO₄). A weighed amount (10 to 15 mg) of silver acetate was placed in a round-bottomed combustion flask and the whole cooled to 0° in an ice bath. Two tenth milliliters of 100% H₂SO₄ (precooled in the refrigerator) was added and the flask again cooled to 0°. After this, the flask was removed from the ice bath, allowed to warm, and shaken until all the silver acetate had dissolved. After recoiling the solution to 0°, about 20 mg of solid NaN₃ was placed on the surface of the H₂SO₄ and the flask placed on the combustion apparatus. After flushing with pure N₂ for 2 to 3 minutes, the system was closed. The flask was immersed in a water bath at 70-75° and the reaction allowed to proceed for 60 minutes. The CO₂ was flushed from the system with N₂ and collected in baryta. The methylamine was recovered by distillation and combusted using perchlorate (13).

Malate was degraded with Lactobacillus arabinosus 17-5 (14) to CO₂ and lactate. The lactate was further degraded by the above procedures.

Assay of Radioactivity—All radioactive fractions were assayed as BaCO₃ on sintered porcelain disks with the use of a Mylar window continuous gas flow Geiger-Müller tube. The counts are corrected for background and self absorption.

The water-soluble compounds were converted to CO₂ by the perchlorate method of Katz et al. (13).

The insoluble material and lipids were combusted in a Stutz and Burris apparatus (26) using the wet combustion reagents of Van Slyke and Folch (27).

RESULTS

Experiments with Acetate-C¹⁴—Acetate daily and rapidly utilized by castor bean endosperm slices; at the end of 2 hours, over 60% of the added acetate had been metabolized. The total incorporation into water-soluble components and CO₂ was linear over this period and began without any apparent lag. Previous experiments had shown that the amounts of acetate that were used did not affect the respiratory gas exchanges of the tissue.

The fate of the metabolized acetate during the course of the experiment is shown in Fig. 1. The results with acetate-2-C¹⁴ are a composite of three separate experiments; because of the agreement between the proportional amounts of C¹⁴ incorporated at the different times, they show the consistency with which acetate is utilized by the castor bean endosperm at this stage of its germination. The organic acids contained the major part of the incorporated C¹⁴ during the early periods of incubation, regardless of the position of label in acetate. In a short time, however, the sugars became the major repository of the methyl

FIG. 1. The incorporation of C¹⁴ into various fractions obtained from castor bean endosperm slices incubated for various times with acetate-1-C¹⁴ or -2-C¹⁴.
carbon of acetate; the carboxyl carbon of acetate, in addition to being deposited in sugars, made a sizable contribution of C\textsuperscript{14} to CO\textsubscript{2}. It should be noted that, at any time, the carboxyl carbon of acetate was incorporated into sugars only one-half as efficiently to CO\textsubscript{2} as PO\textsubscript{4} at the end of 2 hours and largely accounted for as CO\textsubscript{2} at earlier times. The extremely low conversion of C-2 of acetate to CO\textsubscript{2} (less than 0.07 of that of C-1) should be especially noted.

Over the 2-hour period, only about 2\% of the added C\textsubscript{14} from either acetate-1-C\textsubscript{14} or -2-C\textsubscript{14} was incorporated into the lipid fraction and about 2.5\% into the insoluble residues; these amounts are insignificant compared to the C\textsubscript{14} content of the water-soluble compounds and CO\textsubscript{2}.

The entire acetate molecule is initially incorporated into the organic acids (Fig. 2). As time progresses, a diminishing percentage of the C\textsubscript{14} was found in the organic acids and an increasing proportion appeared in the sugars and CO\textsubscript{2}. This inverse behavior, with the curves for organic acids extrapolating clearly to 100\% at zero time, is typical of a precursor-product relationship and means that the C\textsubscript{14}-labeled organic acids (no residual C\textsubscript{14}-labeled acetate is present in these fractions) were rapidly converted to sugars and CO\textsubscript{2}. The radioactivity even more rapidly spreads from the organic acids to the amino acids (note acetate-1-C\textsubscript{14}), as would be expected if they were in equilibrium with the corresponding keto acid. Only relatively small amounts of C\textsubscript{14} accumulate in the amino acids, however, and it appears likely that little net synthesis occurs.

Although almost all of the incorporated C\textsubscript{14} was present in the organic acid fraction at the end of 2 minutes, it was already in several compounds (Tables I and II) and it was not possible to indict any one compound as the prime recipient of the acetate molecule. At all times, most of the radioactivity was present in malate from both acetate-1-C\textsubscript{14} and acetate-2-C\textsubscript{14}; at the end of 2 minutes it contained 34\% to 37\% of the total C\textsubscript{14} present in the organic acids whereas after 2 hours it contained 70 to 75\% of the C\textsubscript{14}. With acetate-2-C\textsubscript{14}, citrate contained more or an equal amount of radioactivity than succinate. With acetate-1-C\textsubscript{14} at the 2- and 5-minute interval, succinate contained more C\textsubscript{14} than citrate. In these two samples, it should be noted, the total recovery of radioactivity was abnormally low and it is possible that much of this loss occurred in the citrate fraction.

One-gram castor bean endosperm (fresh weight) contained 8.7 ± 0.5 \(\mu\) equivalents of malic acid and 1.4 ± 0.2 \(\mu\) equivalents of other acids. The C\textsubscript{14} distribution among malate and the other acids may then only be a reflection of the pool sizes of the various acids. Certainly, the specific activity of succinate and

![Fig. 2. The time course distribution of C\textsubscript{14} among the various fractions obtained from castor bean endosperm slices incubated with acetate-1-C\textsubscript{14} or -2-C\textsubscript{14}.](image)

### TABLE I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time of incubation (min)</th>
<th>c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate</td>
<td>2 5 15 30 60 120</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>750 830 1,120 1,170 1,590 2,070</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>202 316 660 1,690 2,150 2,350</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>0 19 434 1,010 825 583</td>
<td></td>
</tr>
<tr>
<td>Glycolate</td>
<td>0 88 219 370 2,090 1,450</td>
<td></td>
</tr>
<tr>
<td>Fumarate and</td>
<td>unknowns</td>
<td></td>
</tr>
<tr>
<td>% Recovery</td>
<td>75.0 91.0 92.2 94.0 96.6</td>
<td></td>
</tr>
</tbody>
</table>

Total Malate: 2,907, 4,831, 13,363, 20,580, 31,515, 42,303; Total added: 3,070, 5,930, 14,500, 23,300, 33,500, 43,800; % Recovery: 75.6, 88.0, 94.2, 91.3, 95.5, 94.0

### TABLE II

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time of incubation (min)</th>
<th>c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate</td>
<td>2 5 15 30 60 120</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>1,073 5,680 10,759 16,300 34,000 42,600</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>472 1,090 1,252 1,900 3,230 2,710</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>652 1,206 1,478 1,935 3,150 2,360</td>
<td></td>
</tr>
<tr>
<td>Glycolate</td>
<td>296 1,509 3,120 4,650 5,450 6,080</td>
<td></td>
</tr>
<tr>
<td>Fumarate</td>
<td>203 195 943 805 1,393 912</td>
<td></td>
</tr>
<tr>
<td>Unknowns</td>
<td>0 134 327 882 2,192 3,470</td>
<td></td>
</tr>
</tbody>
</table>

Total Malate: 3,590, 10,704, 18,409, 27,309, 52,277, 69,069; Total added: 3,688, 12,187, 19,562, 29,999, 54,781, 63,900; % Recovery: 95.6, 88.0, 94.2, 91.3, 95.5, 94.0
citrate is equal to or greater than malate even though malate contained far more total radioactivity.

The unknowns in Tables I and II are the combined activities from three separate fractions from the ion exchange column. At least five distinct compounds were present (as shown by paper chromatography and autoradiography) but the low C14 content did not permit identification.

Aspartate, glutamate, and alanine contained most of the C14 in the amino acid fraction from acetate-2-C14 (Table III). In the longer time intervals, glycine and serine also became labeled. Glutamic and aspartic acids were also labeled from acetate-1-C14 (Table IV).

Malate was isolated from the castor bean endosperm slices and degraded stepwise to determine the internal distribution of C14. When acetate-1-C14 was the substrate, the C14 was equally distributed between the carboxyl carbons of malate at all time periods (Table V). When acetate-2-C14 was used as substrate, the radioactivity was initially preponderantly present in carbon 3 of malate (Table VI). After 5 minutes, the 2 center carbons of malate were equally labeled, and after 15 minutes there was a small amount of spreading to the carboxyl groups.

The results of the degradations of the glucose moiety of sucrose are shown in Tables VII and VIII. With carboxyl-labeled acetate as substrate, 70% of the C14 was roughly equally distributed in carbons 3 and 4. With methyl-labeled acetate, 90% of the C14 was equally distributed between carbons 1, 2, 5, and 6. Sucrose, glucose, and fructose were the only labeled compounds of the sugar fraction (Table IX). It can readily be seen that sucrose was the first free sugar to become labeled and that it contained the largest portion of the radioactivity at all times; from the specific activity it can also be seen that it was the major sugar present. There were at all times about equal amounts of free glucose and fructose present in the tissue and when they

<table>
<thead>
<tr>
<th>Table III</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Radioactivity of amino acids isolated from castor bean endosperm which was incubated with acetate-2-C14</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time of incubation (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Glutamate</td>
<td>128</td>
</tr>
<tr>
<td>Aspartate</td>
<td>369</td>
</tr>
<tr>
<td>Alanine</td>
<td>134</td>
</tr>
<tr>
<td>Glycine</td>
<td>0</td>
</tr>
<tr>
<td>Serine</td>
<td>0</td>
</tr>
<tr>
<td>Unknowns</td>
<td>130</td>
</tr>
<tr>
<td>Total</td>
<td>634</td>
</tr>
<tr>
<td>Total added</td>
<td>847</td>
</tr>
<tr>
<td>% Recovery</td>
<td>71.4</td>
</tr>
</tbody>
</table>

*Calculated by difference from the total radioactivity in the amino acid fraction.

<table>
<thead>
<tr>
<th>Table IV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Radioactivity of amino acids isolated from castor bean endosperm which was incubated with acetate-1-C14</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time of incubation (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Glutamate</td>
<td>49</td>
</tr>
<tr>
<td>Aspartate</td>
<td>197</td>
</tr>
<tr>
<td>Residual*</td>
<td>44</td>
</tr>
</tbody>
</table>

*Calculated by difference from the total radioactivity in the amino acid fraction.

<table>
<thead>
<tr>
<th>Table V</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Percentage of distribution (based on specific activity) of C14 in malate isolated from castor bean endosperm which was incubated with acetate-1-C14</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Time of incubation (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>49.7</td>
</tr>
<tr>
<td>C2 and 3</td>
<td>0</td>
</tr>
<tr>
<td>C4</td>
<td>50.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table VI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Percentage of distribution (based on specific activity) of C14 in malate isolated from castor bean endosperm which was incubated with acetate-2-C14</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Time of incubation (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COOH (1)</td>
<td>0</td>
</tr>
<tr>
<td>CHOH (2)</td>
<td>10</td>
</tr>
<tr>
<td>CH2 (3)</td>
<td>90</td>
</tr>
<tr>
<td>COOH (4)</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table VII</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Percentage of distribution (based on specific activity) of C14 in glucose moiety of sucrose isolated from castor bean endosperm material which was incubated with acetate-1-C14</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Time of incubation (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.2</td>
</tr>
<tr>
<td>2</td>
<td>6.0</td>
</tr>
<tr>
<td>3</td>
<td>32.9</td>
</tr>
<tr>
<td>4</td>
<td>43.5</td>
</tr>
<tr>
<td>5</td>
<td>7.4</td>
</tr>
<tr>
<td>6</td>
<td>7.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table VIII</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Percentage of distribution (based on specific activity) of C14 in glucose moiety of sucrose isolated from castor bean endosperm which had been incubated with acetate-2-C14</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Time of incubation (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.5</td>
</tr>
<tr>
<td>2</td>
<td>23.8</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
</tr>
<tr>
<td>4</td>
<td>3.7</td>
</tr>
<tr>
<td>5</td>
<td>23.0</td>
</tr>
<tr>
<td>6</td>
<td>24.5</td>
</tr>
</tbody>
</table>
did become labeled, they contained equal amounts of radioactivity.  

The sucrose molecule was at all times equally labeled in the glucose and fructose moieties (Table X).

**Experiments with C4-Labeled Dicarboxylic Acids**—In view of the postulated role that succinate and malate play in the glyoxylate cycle, it was hoped that they would be rapidly utilized by castor bean endosperm tissue. The C4-labeled substrates were then supplied with the aim in mind of recovering most of the C4 as C4O2 and other end products. However, the experiments were hampered by poor absorption into the tissue and, for that reason, the results are more reasonably viewed in relation to the acid actually absorbed rather than in relation to the amount of acid added.

In a 4-hour period only about 15% of the added succinate was absorbed by the tissue and an additional 4-hour incubation period resulted in only an additional 5% absorption. In an 8-hour incubation period, only 18% of the malate was absorbed. Attempts to increase the absorption of these weak acids by decreasing the pH and by intermittent adjustments of the pH during the course of the experiment were unsuccessful.

Although many experiments were performed, only single representative results are shown in Table XI. With succinate-1-C4 the tissue and incubation medium were not separated before extraction; the major portion of the C4 was recovered in the organic acids and was shown to be almost entirely succinic acid. In an attempt to determine whether this was caused by poor absorption or by nonutilization of the acid, the tissue and incubation medium were separated before extraction in the subsequent experiments. Thus, with succinate-2-C4, it was readily demonstrated that the largest portion of the acid was not absorbed and could be recovered as such in the incubation medium. However, C4-labeled sugars and amino acids were also detected in the incubation medium but, since only the tissue was analyzed in the experiment with succinate-2-C4, in Table XI the amounts of radioactivity shown to be in these fractions are minimal ones. With malate C4 the tissue and incubation medium were separated before extraction, but both portions were analyzed for all constituents and the total radioactivity of each fraction is shown; it is seen that here also the major portion of the added C4 can be recovered as unabsorbed dicarboxylic acid.

In viewing the results, particular attention should be paid to the amounts of radioactivity deposited in the two end products of acid metabolism—the CO2 and sugars. A large portion of the acids which was absorbed was metabolized into these two fractions. Further, when the dicarboxylic acid was carboxyl-labeled (i.e., succinate-1-C4, malate-C4), equal amounts of C4 were released as CO2 and incorporated into sugar; when the acid was center-labeled (i.e., succinate-2-C4), about seven times as much C4 was incorporated into sugar as was released as CO2.

The glucose moiety of sucrose, obtained from castor bean endosperm incubated with succinate-2-C4 for 8 hours was degraded and 96% of the C4 was found to be equally distributed between carbons 1, 2, 5, and 6.

**DISCUSSION**

The problem of the mechanism of the fat to sugar conversion (2) in germinating fatty seeds has been one of long standing in plant physiology. Although glycerol can be readily converted to sugar by castor bean endosperm (28), the respiratory quotient (29) and the over-all efficiency of the conversion (2) demand that the fatty acids must also be converted to sugar. When the conversion is complete in the castor bean endosperm, 50 to 60% of the fat can be accounted for as sugar; this value is only a minimal value since losses of sugar caused by respiration and synthetic events in the embryo proper are not allowed for. The fatty acids are probably broken down to acetyl-CoA via β-oxidation (3) and thus any proposed mechanism for the fat to sugar conversion must be capable of converting acetyl units to hexose with at least 60% efficiency.

---

**Table IX**

Relative radioactivity and specific activity* (in parentheses) of free sugars isolated from castor bean endosperm which was incubated with acetate-2-C4

<table>
<thead>
<tr>
<th>Time of incubation (min)</th>
<th>Sugar</th>
<th>c.p.m.</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1,480 (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>10,580 (22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>270 (11)</td>
<td>221 (14)</td>
<td>31,360 (69)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>720 (42)</td>
<td>680 (47)</td>
<td>87,300 (132)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>2,010 (103)</td>
<td>1,836 (114)</td>
<td>1,480,600 (163)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>4,600 (182)</td>
<td>4,270 (170)</td>
<td>148,600 (163)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Expressed as c.p.m. per µmole of carbon.

**Table X**

Specific activity of component hexoses of sucrose isolated from castor bean endosperm which was incubated with acetate-2-C4

<table>
<thead>
<tr>
<th>Hexose</th>
<th>Time of incubation (min)</th>
<th>Sugar</th>
<th>c.p.m.</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5</td>
<td>11.2*</td>
<td>132</td>
<td>385</td>
<td>736</td>
<td>1,138</td>
</tr>
<tr>
<td>Fructose</td>
<td>14.3</td>
<td>111</td>
<td>377</td>
<td>782</td>
<td>1,025</td>
<td></td>
</tr>
</tbody>
</table>

* Specific activity expressed as c.p.m. per µmole of hexose.

**Table XI**

Radioactivity of various fractions obtained from castor bean endosperm which was incubated with some C4-labeled dicarboxylic acids

The incubation mixtures were as follows. Succinate-1-C4, 2 µmoles (32,900 c.p.m.); potassium phosphate, pH 5.0, 100 µmoles; endosperm slices, 500 mg; total volume 2.0 ml. Succinate-2-C4, 4 µmoles (65,100 c.p.m.); potassium phosphate, pH 4.0, 144 µmoles; endosperm slices, 20 g; total volume 4.0 ml. Malate-C4, 4.05 µmoles (30,100 c.p.m.); potassium phosphate, pH 5.0, 134 µmoles; endosperm slices, 1.0 g; total volume 4.0 ml.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Succinate-1-C4* (4 hours*)</th>
<th>Succinate-2-C4* (4 hours*)</th>
<th>Malate-C4* (8 hours*)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c.p.m.</td>
<td>c.p.m.</td>
<td>c.p.m.</td>
</tr>
<tr>
<td>CO2</td>
<td>617</td>
<td>662</td>
<td>1,189</td>
</tr>
<tr>
<td>Sugars</td>
<td>610</td>
<td>4,559</td>
<td>886</td>
</tr>
<tr>
<td>Amino acids</td>
<td>1,090</td>
<td>1,474</td>
<td>1,715</td>
</tr>
<tr>
<td>Organic acids</td>
<td>31,500</td>
<td>8,857</td>
<td>1,110</td>
</tr>
<tr>
<td>Lipids</td>
<td>208</td>
<td>150</td>
<td>22,700</td>
</tr>
</tbody>
</table>

* Time of incubation.
† Tissue separated from incubation medium before extraction.
The glyoxylate cycle, described by Kornberg and Krebs (4), provides a mechanism for the utilization of C2 units in synthetic events. The reactions of the cycle can be summarized as follows:

\[
\text{acetyl-CoA} + \text{oxaloacetate} \rightarrow \text{citrate} + \text{CoA} \\
\text{citrate} \rightarrow \text{isocitrate} \\
\text{isocitrate} \rightarrow \text{malate} + \text{glyoxylate} \\
\text{malate} \rightarrow \text{CO}_2 + \text{phosphoenolpyruvate} \\
\text{glycolysis} \rightarrow \text{carbohydrate}
\]

The net effect of one turn of the cycle is the conversion of 2 C2 units to a C3 dicarboxylic acid. Kornberg and Beevers (6) have provided evidence that the glyoxylate cycle is present in castor bean endosperm and the complete pathway from fat to sugar was envisaged as:

\[
fatty \text{acyl-CoA} \rightarrow \text{acetyl-CoA} \rightarrow \text{glyoxylate cycle} \rightarrow \text{malate} \rightarrow \text{CO}_2 + \text{phosphoenolpyruvate} \\
\text{glycolysis} \rightarrow \text{carbohydrate}
\]

This proposal would allow for a 75% conversion of acetate to hexose.

The results of the present experiments clearly establish that the entire acetate molecule is initially incorporated into the organic acids (Fig. 2). Malate, citrate, and succinate contain the major portion of the C4 from either acetate-1-C14 or -2-C14 in the shortest time periods (Tables I and II) and the curves in Fig. 2 thus show that these are the earliest stable compounds formed from acetate. If the glyoxylate cycle indeed accounts for the utilization of acetate, both malate and citrate would be the initial repositories of C4 in the experiments with acetate-C14. From acetate-2-C14 the C4 in the malate first produced would be expected to be in carbon 3. However, two "turns" of the cycle would be sufficient to bring about equal labeling in positions 2 and 3, and, in all but the very earliest time period, the malate was in fact symmetrically labeled in these carbons (Table VI). In the experiments with acetate-1-C14, apparently none of the exposures were short enough to demonstrate a preponderance of C4 in C-4 of malate. We conclude that the observed concomitant entry of acetate-C14 into citrate and malate and the distribution of the C4 in the malate are consistent with the operation of the glyoxylate cycle.

Succinate-C4 is also present at the earliest times during acetate utilization, as might have been anticipated from its role as an intermediate in the glyoxylate cycle. At no time was succinate-C4 present without citrate-C4 and malate-C4 and, in the experiments with acetate-2-C14, where fairly complete recovery of the acids was achieved (Table II), the radioactivity in succinate was clearly less than that in either malate or citrate. As a whole, then, the results speak against the participation of the persisting but heavily criticized (30) theoretical condensation of two acetate units to form succinate (31) for which, it should be emphasized, we have no evidence from enzymatic work with this material.

Although radioactive glyoxylic was not isolated, the occurrence of C4-labeled glycine and serine (Table III) and glycolate (Tables I and II) is strong presumptive evidence for its production in this tissue. Glycine and serine can easily arise from glyoxylic acid (32) and glycolate could be formed via the mediation of glyoxylic reductase (6). Oxaloacetate may be presumed to become labeled since radioactive aspartate was isolated (Tables III and IV). Thus, the only intermediates of the glyoxylate cycle that were not shown to become labeled from acetate-C14 were oxaloacetate and isocitrate. Isocitrate must be rapidly metabolized since it could not be demonstrated in spite of several attempts; the absence of this acid from castor bean endosperm has been reported previously (33).

The glyoxylate cycle is a variant of the tricarboxylic acid cycle (4). Since isocitric dehydrogenase is present in the castor bean endosperm (6), and since castor bean mitochondria are capable of oxidizing all the tricarboxylic cycle acids (34), it would appear that the complete machinery for both cycles is present. \(\alpha\)-Ketoglutarate is the only member of the tricarboxylic acid cycle that is not a member of the glyoxylate cycle, and a measure of its radioactivity would indicate whether the tricarboxylic acid cycle is operative. Glutamate may be considered to be in equilibrium with \(\alpha\)-ketoglutarate through direct amimation (35). The presence of C4-labeled glutamate (Tables III and IV) shows that some isocitrate is converted to \(\alpha\)-ketoglutarate. However, it should be recalled that at no time does the C4 in the amino acids produced from acetate represent more than a small percentage of that utilized and that aspartate, rather than glutamate, is the most heavily labeled of the amino acids in the early periods (Table IV). In previous experiments with pyruvate-C14 the glutamate pool contained very low amounts of C4 (36). Other evidence which indicates that the \(\alpha\)-ketoglutarate is largely by-passed is as follows.

1. With acetate-1-Cl4 the C4 released as CO2 is never above that incorporated into sugars and with acetate-2-C14 only a very small amount is released as CO2; it is almost all incorporated into sugars. If the tricarboxylic acid cycle were operative, a much larger proportion of the C4 would be released as C4O2.

2. The recycling of acetate-2-C14 in the tricarboxylic acid cycle would yield malate with the internal distribution of C4 as

\[
\text{COOH} \rightarrow \text{CHOH} \rightarrow \text{CH}_2 \rightarrow \text{COOH}
\]

whereas recycling in the glyoxylate cycle would yield malate with the distribution of

\[
\text{COOH} \rightarrow \text{CHOH} \rightarrow \text{CH}_2 \rightarrow \text{COOH}
\]

If both are operative, an intermediate distribution will be obtained and this is what is found in bacteria (37) in which aspartate was obtained with 20% of the label equally distributed in the carboxyl groups and 74% of the C4 in the center carbons. In the castor bean, however, 90% of the C4 was retained in the center carbons even after 2 hours; this suggests that the malate must arise almost exclusively by the glyoxylate cycle.

3. Acetate metabolized via the tricarboxylic acid cycle is converted to CO2 and lost from the tissue. If the losses of sugar by respiration and synthetic events in the embryo proper are added to the observed 60% conversion of fat to sugar, the efficiency comes even closer to the maximal figure of 75% predicted from the mechanism proposed, and certainly does not allow for the loss of acetate as CO2 which would occur if isocitrate were metabolized by way of \(\alpha\)-ketoglutarate.

We conclude that, in the castor bean endosperm, acetate is utilized almost exclusively in the glyoxylate cycle, in spite of the fact that all of the enzymes of the tricarboxylic acid cycle are present. Although the intracellular distribution of the enzymes may be of importance here (11), the precise mechanism of the
complete diversion of isocitrate through isocitritase remains in doubt.

It should also be pointed out that, although limited tricularboxylic acid cycle operation will account for the release of $\text{C}^{14}O_2$ from acetate 2 $C^{14}$ and the observed spreading of $C^{14}$ from the center carbons to the carboxyl carbons of malate (Table VI), there is an additional mechanism apparently operative that will accomplish the same end result. The production of radioactive alanine (Table III) indicates that $C^{14}$-labeled pyruvate is produced. Thus, if center-labeled malate underwent the reactions:

\[
\begin{align*}
\text{COOH} & \quad \text{COOH} \quad \text{COOH} \\
*\text{CHOH} & \quad *\text{CO} \quad *\text{CO} \quad *\text{COCoA} \\
*\text{CH} & \quad *\text{CH} \quad *\text{CH} \quad *\text{CH}_2 \\
\text{COOH} & \quad \text{COOH}
\end{align*}
\]

uniformly labeled acetate would be produced. The entry of this acetate into the glyoxylate cycle would produce carboxyl-labeled malate and, upon decarboxylation of this malate, $C^{14}$o$_2$.

Although the organic acids were the initial acceptors of acetate, the time studies make it clear that they were only the precursors of sugar (Fig. 2), and more specifically sucrose (Table IX), which was the main product of acetate utilization.

The labeling patterns that are found throughout the experiments in glucose (Tables VII and VIII) are similar to those reported by Bradbeer and Stumpf (12). The distribution is that expected from acetate-$C^{14}$ incorporation if the oxaloacetate derived from malate is converted to sugar with the loss of carbon 4 and a condensation of the remaining three carbons. The labeling pattern of the glucose produced from succinate-$2-C^{14}$ would indicate that this $C_4$ acid is directly converted to glucose by the above mechanism. In addition to the labeling patterns, the relatively close 1:1 stoichiometry between the amount of $C^{14}$ in the CO$_2$ and the sugars from carboxyl-labeled dicarboxylic acids clearly shows that one carboxyl group is lost as CO$_2$ for every carboxyl group incorporated into sugars (Fig. 1, Table XI). If the dicarboxylic acids are center-labeled, however, both carbons are converted to sugar with little loss as CO$_2$ (Fig. 1, Table XI).

Pyruvate is not directly converted to sugar in this tissue (36) so it is highly probable that the three-carbon fragment from oxaloacetate which enters the glycolytic sequence is phosphorypyruvate. Phosphoenolpyruvate carboxykinase is present in the castor bean endosperm (10), and is the most likely enzyme in this tissue. The evidence suggests that the tricarboxylic acid cycle is not operative in the material used and shows that the major fate of the conversion to sucrose is by far the most important fate of acetate in the endosperm of the castor bean.

**SUMMARY**

Acetate-1-$C^{14}$ and -2-$C^{14}$, succinate-1-$C^{14}$ and -2-$C^{14}$, and malate-$C^{14}$ were supplied to slices of endosperm from five-day-old castor bean seedlings.

Acetate was rapidly and linearly incorporated into water-soluble constituents and CO$_2$. The methyl carbon of acetate accumulated solely in sucrose whereas the carboxyl carbon contributed label equally to sucrose and CO$_2$. The time course study proved that the organic acids were the initial recipients of the acetate molecule. The distribution of $C^{14}$ in the various acids coupled with the internal distribution of label in malate showed that acetate was incorporated via the glyoxylate cycle.

Glucose produced from acetate-1-$C^{14}$ contained 70% of the $C^{14}$ equally distributed in carbons 3 and 4 whereas glucose produced from acetate-2-$C^{14}$ contained 90% of the label equally distributed in carbons 1, 2, 5, and 6. The glucose produced from succinate-2-$C^{14}$ had an internal distribution of label similar to that produced from acetate-2-$C^{14}$. Succinate-2-$C^{14}$ contributed about seven times as much $C^{14}$ to sugars as to CO$_2$ whereas succinate-1-$C^{14}$ and malate-$C^{14}$ contributed about equal amounts of radioactivity to sugars and CO$_2$. The distribution of label in the sugars and the stoichiometry between the $C^{14}$ in the sugars and CO$_2$ indicated that the hexose is formed from $C_4$ acids by a decarboxylation and reversal of glycolysis.

The evidence suggests that the tricarboxylic acid cycle is not operative in the material used and shows that the major fate of acetate is conversion to sucrose by way of dicarboxylic acids produced in the glyoxylate cycle.

**REFERENCES**

29. Murlin, J. K., J. t. i e m. Physiol., 17, 283 (1934).
Sucrose Synthesis from Acetate in the Germinating Castor Bean: Kinetics and Pathway
David T. Canvin and Harry Beevers


Access the most updated version of this article at http://www.jbc.org/content/236/4/988.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/236/4/988.citation.full.html#ref-list-1