SUGAR TRANSFORMATION IN LEAVES OF CANNA INDICA

I. SYNTHESIS AND INVERSION OF SUCROSE*

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It was previously demonstrated that, when solutions of hexose sugars such as glucose, fructose, or mannose are introduced into green leaves under aerobic conditions, an increase in sucrose is observed (1-3). The sucrose appears to be synthesized at the expense of the monosaccharides. Excised barley leaves infiltrated with 5 per cent glucose or fructose accumulated sucrose to a level of about 6 per cent of dry weight (2).

The obvious questions that arise from these infiltration experiments are these: are the infiltrated sugars used per se in the synthesis of sucrose or is the disaccharide formed as a result of the infiltration of unphysiological concentrations of reducing sugars which have altered the metabolism of the leaf in such a way as to cause the synthesis of sucrose from starch or some other storage product? We have undertaken to answer these questions by the use of C¹⁴-labeled sugars.

Methods

Selection and Preparation of Plant Leaf Material—The selection of the leaf of Canna indica for this study was based on the fact that in photosynthesis approximately 80 per cent of the CO₂ fixed is incorporated in the sugar fraction (4), suggesting that this tissue has a high capacity for sugar synthesis. Repeated analyses of Canna leaf, carried out in this laboratory in conjunction with the preparation of radioactive sugars (4, 5), resulted in identification of its major chemical constituents. The leaf tissue does not contain a detectable amount of starch, and infiltration experiments have shown that Canna leaf is as effective as other plants in synthesizing sucrose from glucose or fructose. Furthermore, intact leaves are not required but small disks of tissue can be used.

Canna leaves were selected which had not yet attained full maturity. In an attempt to obtain more uniform experimental material and to conserve the radioactive sugars, leaf disks were employed in these studies. The blades of the leaves used were about 18 cm. wide and 30 cm. long, with

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a weight of 14 to 16 gm. They were washed under cold running tap water, placed on a sheet of cardboard, and the leaf disks punched out with an 18 mm. cork borer. The disks were then washed three times with water by decantation and blotted between paper towels. A single leaf provided from 70 to 80 disks, having an average weight of 90 mg. per disk. From this total a random sample of forty-nine disks was selected. A group of seven of these disks was placed in boiling 80 per cent alcohol to serve as a control.

_Infiltration_—Forty-two _Canna_ leaf disks were placed in a 50 ml. beaker and a dilute solution of radioactive sugar\(^1\) was pipetted onto them. In an attempt to label the carbohydrate pools without disturbing the normal metabolism of the leaf tissue, radioactive sugars were used in concentrations below 0.5 per cent, which is lower than that existing in normal leaves. In previous infiltration experiments (1, 2), sugar concentrations of 5 to 10 per cent were used. The disks were kept submerged by placing a rubber stopper on them. The beaker containing the disks was placed in a desiccator, and the system was slowly evacuated to about 120 mm. pressure. As soon as the evolution of gas bubbles from the periphery of the disks had ceased, the vacuum was released by a slow admission of air. This process was repeated twice in order to insure infiltration of the solution. The excess infiltrating solution was removed by decantation and the disks were washed twice with 3 ml. portions of water to recover the adhering radioactive sugar. The disks were then transferred to a 400 ml. beaker filled with distilled water and washed five times by decantation. Seven of these washed disks were put at once in boiling 80 per cent alcohol in order to observe the early transformations of the infiltrated sugar. The time required to infiltrate and wash the disks was about 12 minutes.

_Respiration Conditions_—The remaining thirty-five disks were placed on edge around the inside of a 50 ml. beaker containing a 2.85 cm. circle of moistened filter paper at the bottom. The outside walls of the beaker had previously been completely covered with black friction tape. The beaker in turn was placed in a respirometer consisting of a tape-covered micro bell glass equipped with a side tubulation and a bottom plate (Fig. 1). Moist air (not freed of CO\(_2\)) was forced over the surface of the leaf disks, sweeping the respired CO\(_2\) into the absorber containing 5.0 ml. of 0.2 N sodium hydroxide. The rate of air flow through the system was 10

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\(^1\) The C\(^14\)-labeled glucose, fructose, and sucrose used in these experiments were isolated from _Canna indica_ leaves exposed to photosynthesis in the presence of C\(^14\)O\(_2\). The distribution of C\(^14\) activity was equal among the individual atoms of the carbon chains of these sugars. The glucose was contaminated with 0.13 per cent fructose, the fructose with 0.17 per cent glucose, and the sucrose with 0.15 per cent fructose and 0.37 per cent glucose (5). These small amounts of contaminants did not interfere with the interpretation of the results.
to 15 ml. per minute. At various successive time periods, samples of seven disks each were removed from the respirometer and placed in boiling 95 per cent ethanol.

Each time that a group of seven disks was taken for analysis, the alkali in the CO₂ absorber was transferred quantitatively to a stoppered 125 ml. Erlenmeyer flask and replaced with 5.0 ml. of fresh alkali. 10 ml. of 0.05 M barium chloride were added to the alkali containing the CO₂ respired by the disks, and the total amount of the barium carbonate formed was estimated by titrating the excess of sodium hydroxide with 0.050 N hydrochloric acid with a phenolphthalein indicator. The precipitated barium carbonate was collected by centrifugation, washed with water, dispersed in alcohol, and pipetted onto tared copper disks for subsequent determination of radioactivity.

Sample Preparation—The leaf disk samples were extracted three times by boiling for approximately 15 minutes with 10 ml. portions of 80 per cent ethanol. The extracts were reduced in volume to about 0.5 ml. by evaporation on a steam bath, toluene was added as a preservative, and the extracts were dried in a vacuum oven at 40°. The dried extracts were then taken up in 1.00 ml. of water, and 0.50 ml. of each sample was taken for a quantitative analysis of the sugars, 0.25 ml. for isolation of sucrose, and 0.04 ml. for paper chromatographic analysis.

Determination of Sugars—The 0.50 ml. aliquots of the extracts were clarified by treatment with 0.5 ml. of a saturated solution of neutral lead acetate, followed by 1.5 ml. of a saturated solution of disodium phosphate and a pinch of charcoal. The mixtures were filtered through Celite on
small Büchner funnels, the filter cakes washed five times with 5 ml. portions of water, and the clear filtrates diluted to 50 ml.

The reducing values of these solutions were determined before and after inversion with invertase by the colorimetric method of Folin and Malmros (6), and the difference was taken as a measure of the sucrose content. Total fructose was determined by Roe's method (7). This fructose value, minus half of the reducing value of sucrose after hydrolysis, gave the amount of free fructose. The amount of free glucose was estimated by subtracting the free fructose value from the reducing value of the original solution.

**Chromatographic Analysis**—The chromatographic procedure used was similar to that described by Benson et al. and Calvin (8, 9). The extracts were applied at a point 3 inches in from each of two adjacent edges of 18 × 24 inch Whatman No. 1 filter paper sheets in four 0.01 ml. aliquots, allowing time for drying between applications. The amount of activity applied to each paper was then determined with a Tracerlab SU-3A rate meter, and the chromatograms were developed with water-saturated phenol in the short dimension. When the solvent had reached the lower edges (24 hours), the papers were removed from the cabinet and dried overnight in a current of air at room temperature. The chromatograms were then developed in the long dimension with a mixture of 52 per cent n-butanol, 13 per cent acetic acid, and 35 per cent water. After 18 hours, when the solvent reached the lower edges of the papers, they were removed from the cabinet and dried in air for 3 hours.

Radioautograms were made by placing the papers in contact with 14 × 17 inch Eastman medical "no-screen" x-ray films. The exposure times used in the preparation of these radioautograms were inversely proportional to the amount of C\(^14\) activity initially applied at the origin of the chromatogram, so that visual comparisons of the relative distributions of radioactivity could easily be made between the members of a series. The standard exposure was arbitrarily chosen as 4 days for a paper with 4000 c.p.m.

Preliminary identification of the radioactive compounds was based upon their \(R_F\) values in the two solvents employed. To facilitate identification, a reference chromatogram of the major constituents normally occurring in Canna leaf extracts (sugars, amino acids, and organic acids) was prepared. The identification of these constituents was made by preparing two-dimensional chromatograms of non-radioactive Canna extracts of varying concentrations to which an authentic sample of a suspected constituent had been added. When an added component was found to exhibit the same chromatographic properties as a component in the extract, it was assumed that the two were the same. For making a tentative identifica-
tion of the major organic compounds by paper chromatography, the spray reagents used were a 0.2 per cent solution of m-phenylenediamine dihydrochloride (10) for sugars, a 0.1 per cent alcoholic solution of ninhydrin for amino acids, and a 0.04 per cent alcoholic solution of neutral bromocresol green for organic acids (11).

Confirmation of the identified compounds was made by cochromatography of the eluted radioactive spots with authentic specimens in a third solvent. A butanol-ethanol-water solvent (5) was used to differentiate between sugars and amino acids; butanol-formic acid-water (11) and ethanol containing 5 per cent ammonia were used for identification of organic acids (12).

The hexose monophosphates were identified by their $R_F$ values, and their authenticity was confirmed by identification of the sugars obtained after enzymatic hydrolysis. Treatment with phosphatase (General Biochemicals, Inc.) yielded products that occupied the same spots as authentic glucose, fructose, and mannose when chromatographed together.

Elution of the sucrose area and subsequent hydrolysis of the solution with invertase produced products that gave single spots when with glucose and fructose. The sucrose identified by paper chromatography was not attacked by the yeast *Torula monosa*, which ferments glucose, fructose, and mannose; however, the inversion products were readily fermented. The authenticity of glucose and fructose obtained from the hydrolyzed sucrose was confirmed by the fact that, when the eluted glucose spot was oxidized with bromine, a compound resulted that gave a single spot when with a known sample of gluconic acid in a propanol-ammonia solvent (13), whereas the suspected fructose eluate remained unchanged. Treatment of this radioactive gluconic acid with hydrogen peroxide in the presence of ferric acetate (14) yielded a compound that occupied the same spot as authentic arabinose when chromatographed together. Treatment of the suspected fructose eluate with dilute alkali in an atmosphere of oxygen resulted in a compound that yielded the same spot as authentic arabinose when chromatographed together (15).

*Isolation and Hydrolysis of Sucrose*—The sucrose was isolated as follows. 0.25 ml aliquots of the aqueous solutions of the 80 per cent alcohol extracts were pipetted into 10 ml beakers containing 1 ml of water and 1 gm. of moist Duolite C-3 cation exchange resin. The mixtures were agitated for about 5 minutes, the supernatant solutions were filtered, and the resins washed twice with 1 ml portions of water. The filtrates were collected in 10 ml beakers containing 1 gm. of moist Duolite A-3 anion exchange resin, and after 5 minutes shaking the resins were filtered off and washed as before. A few drops of toluene were added to the partially deionized filtrates, and the solutions concentrated in a vacuum oven at 40°.
The concentrates were dissolved in 0.15 ml. of water, and 0.05 ml. of each solution was applied, 0.01 ml. at a time, on filter paper for one-dimensional chromatographic development in the butanol-ethanol-water solvent. An authentic mixture of sucrose, glucose, and fructose was added at the margins, the lower edge of the paper was serrated, and the chromatogram was run for 3 days, the solution dripping off the edge. A radioautogram was made in order to indicate the positions of the radioactive sugars. Identification of the sugars was made by comparison of their positions with those of the mixture of known sugars at the margins, after color development with m-phenylenediamine. The sucrose areas were eluted, hydrolyzed with invertase, and the products of hydrolysis rechromatographed in the identical manner. The radioautogram of this second chromatogram indicated the positions of the glucose and fructose moieties of the sucrose samples so that the relative distributions of C\textsuperscript{14} activity between them could be determined.

**Counting Methods and Calculations**—Radioactivity measurements were made with a Tracerlab rate meter (SU-3A) equipped with a detachable Geiger tube probe having a screened end window 1 inch in diameter. The instrument can be read with an accuracy of ±5 per cent of its full scale deflection in ranges of 200, 2000, and 20,000 c.p.m.

The relationship between the activity observed in counts per minute from samples counted on filter paper and the activity in microcuries is such that a solution containing 0.2 \textmu c. of C\textsuperscript{14}-glucose, absorbed on Whatman No. 1 filter paper in an area 14 mm. in diameter, gives a reading of 15,000 ± 1000 c.p.m.

The total activity of each extract was determined by counting the 0.04 ml. aliquots applied on filter paper sheets prior to chromatographic development. After two-dimensional chromatographic development and preparation of radioautograms, the radioactive areas corresponding to the positions of glucose, fructose, and sucrose were located on the radioautogram and were traced onto the chromatograms. By counting the active areas of the sugars and comparing the counts with those obtained from the originally applied extracts before they had been chromatographed, an estimation of the percentage distribution of activity among the glucose, fructose, and sucrose in the various alcohol extracts was made.

In those cases in which the radioactive areas on the chromatogram were larger than the window of the Geiger tube, the area was counted in sections by the use of metallic foil masks, and the total count in the area was estimated by summing the counts of the individual sections. When mixtures of radioactive sugars of known activity were subjected to this chromatographic method of separation and subsequent radioassay, the recoveries were within the range of error of the counting instrument.
Because of the variation of the individual disks with respect to sugar content, the amount of radioactive sugar infiltrated, and the amount of carbon dioxide respired, it was preferable to plot the results in terms of the percentage distribution of activity in the 80 per cent alcohol extracts rather than in terms of specific activity. Such a representation conveys the same picture as that obtained by a visual examination of the radio-autograms obtained from the radioactive extracts.

The residual activity of each set of the extracted disks which was not soluble in 80 per cent alcohol was determined by directly counting the activity of each individual disk. The dried extracted disks were of a thickness similar to that of Whatman No. 1 filter paper. Since the weight and composition of these two materials are similar, it was assumed that no appreciable error was introduced when the values obtained by direct counting of the flattened disks were compared with those obtained by counting aliquots of the alcoholic extracts absorbed on filter paper.

With regard to the determination of the respired radioactive carbon dioxide, each sample in the series, except for the last one, represented the respiratory CO₂ of multiple sets of disks, and the value for one set of seven was calculated by dividing the value of carbon dioxide obtained at a particular time interval by the appropriate number of sets. Inasmuch as the activity of the carbon dioxide respired in each set was calculated on the assumption that the activity respired by any one set in a given time interval is equal to the average amount of activity respired per set during that interval, the values are considered to be only approximate.

The specific activity of the barium carbonate derived from the respired CO₂ was determined by a radioassay of known amounts of the barium carbonate spread on copper plates. From the determination of total carbon dioxide collected and the specific activity of the barium carbonate obtained, the total amount of activity respired per set during a given time interval was calculated.

C¹⁴ determinations with the Tracerlab meter showed that sugar samples counted either as anhydrous syrups on copper plates or as barium carbonate after combustion gave the same calculated specific activity values after correction for mass absorption of the compound employed. However, the count obtained on an aliquot of radioactive sugar solution absorbed and dried on Whatman No. 1 filter paper gave only 33 per cent of the activity of a similar aliquot when dried and counted on a copper disk. This value appeared to be constant over a range of 20 to 160 γ of sugar. Therefore, in order to compare the activities of the fractions obtained by counting on paper with those counted as barium carbonate, the values obtained by the latter method were divided by 3.

The amounts of activity in the three fractions (80 per cent alcohol-
soluble, insoluble residue, and respired carbon dioxide) for each time period were added together and the percentage of this total contributed by each of the fractions was calculated.

Results

Glucose Infiltration—Examination of the data in Table I indicates that the six sets of disks varied considerably with respect to the amount of radioactive glucose initially infiltrated. The total activity accounted for was 366,000 c.p.m., indicating that approximately 0.11 ml. of the infiltrating solution was retained in the disks. This represents an average of approximately 18 γ of glucose per set of seven disks.

When the data in Table I are expressed as a percentage of the total C¹⁴ accounted for in each set of disks (Table II), it is evident that the loss in activity in the alcohol extracts with time is due to a rapid initial incorporation of activity in the leaf disk residue and a progressively increasing amount of activity respired as CO₂.

Fig. 2 represents the distribution of activity in the sugars during the 6 hour period in terms of percentage of the total activity in the alcohol extracts. It shows that the decrease in glucose activity is accompanied by an increase in sucrose activity and that both the glucose and fructose moieties of the sucrose possess C¹⁴ activity. Initially the sugar fraction

### Table I

Distribution of Activity in Fractions Derived from Respiring Canna Leaf Disks after Infiltration with 0.1 Per Cent Solution of C¹⁴-Glucose

<table>
<thead>
<tr>
<th>Alcohol extract</th>
<th>0 min.</th>
<th>15 min.</th>
<th>23 min.</th>
<th>83 min.</th>
<th>161 min.</th>
<th>363 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.p.m.</td>
<td>100,000</td>
<td>32,250</td>
<td>50,000</td>
<td>37,500</td>
<td>47,000</td>
<td>23,800</td>
</tr>
<tr>
<td>Leaf residues</td>
<td>1,450</td>
<td>4,900</td>
<td>8,200</td>
<td>10,500</td>
<td>13,900</td>
<td>12,000</td>
</tr>
<tr>
<td>0–15 min.</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>16–23</td>
<td>1,900</td>
<td>1,900</td>
<td>1,900</td>
<td>3,800</td>
<td>3,800</td>
<td>8,000</td>
</tr>
<tr>
<td>24–83</td>
<td>600</td>
<td>600</td>
<td>600</td>
<td>600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>84–161</td>
<td>600</td>
<td>600</td>
<td>600</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>162–363</td>
<td>1,900</td>
<td>1,900</td>
<td>1,900</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total CO₂</td>
<td>150</td>
<td>750</td>
<td>2,650</td>
<td>6,450</td>
<td>14,400</td>
<td>8,000</td>
</tr>
<tr>
<td>C¹⁴ accounted for</td>
<td>101,000</td>
<td>37,400</td>
<td>59,000</td>
<td>50,000</td>
<td>37,000</td>
<td>50,200</td>
</tr>
</tbody>
</table>

² Hydrolysis of the leaf disk residues and identification of the products by chromatographic analysis showed glucose, galactose, xylose, and arabinose to be the principal radioactive constituents.
Table II
Per Cent Distribution of C\textsuperscript{14} in Respiring Canna Leaf Disks after Infiltration with 0.1 Per Cent Solution of C\textsuperscript{14}-Glucose

Forty-two Canna leaf disks, having a fresh weight of 3.8 gm., were infiltrated with a solution containing 3 mg. of glucose of 44 \mu c. per mg. of specific activity.

<table>
<thead>
<tr>
<th>Respiration time, min.</th>
<th>0</th>
<th>15</th>
<th>23</th>
<th>83</th>
<th>161</th>
<th>363</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity in alcohol extract</td>
<td>98</td>
<td>86</td>
<td>85</td>
<td>74</td>
<td>70</td>
<td>47</td>
</tr>
<tr>
<td>&quot; &quot; leaf residue</td>
<td>1.5</td>
<td>13</td>
<td>14</td>
<td>21</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>&quot; &quot; respired CO\textsubscript{2}</td>
<td>0</td>
<td>0.4</td>
<td>1.3</td>
<td>5.2</td>
<td>9.6</td>
<td>29</td>
</tr>
</tbody>
</table>

![Graph of per cent extracted activity over time](http://www.jbc.org/)

Fig. 2. Distribution of activity in the sugar fraction of the alcohol extracts of C\textsuperscript{14}-labeled glucose-infiltrated Canna leaf disks.

Examination of the radioautogram (Fig. 3) shows that, immediately after infiltration of C\textsuperscript{14}-glucose, hexose phosphates and sucrose were formed, and, as would be expected, free glucose is the dominant spot on the film. After 15 minutes, the intensity of the sucrose spot approximated that of the glucose, and the hexose phosphates were still prominent as lesser constituents. The organic acids, citric, malic, glyceric, succinic, and lactic, and the amino acids, aspartic, glutamic, glutamine, and alanine, were beginning to appear. After 23 minutes, the sucrose contained more activity than the glucose, but the other compounds maintained an activity distribution similar to that after 15 minutes. By the end of 83 minutes (Fig.
4) the glucose activity was greatly diminished, the organic phosphates had disappeared, and there appeared to be a shift in activity from alanine to lactic acid. At the conclusion of the experiment, after 363 minutes, the

Fig. 3. Radioautogram showing the compounds formed from C^{14}-labeled glucose immediately after infiltration. The small amount of activity due to fructose, which is present as an impurity in the C^{14}-labeled glucose, has fallen to background intensity after 15 minutes.

Fig. 4. Radioautogram showing the compounds formed from infiltrated C^{14}-labeled glucose after 83 minutes of respiration in the dark.

bulk of the remaining activity resided in the sucrose. Some residual glucose activity was evident, but the glutamic acid possessed most of the activity among the lesser constituents.

Although no labeling was found in free fructose, considerable activity was present in the fructose moiety of the radioactive sucrose synthesized
during the experiment. The $^{14}C$ activity ratio of the glucose component of sucrose to that of the fructose component was 1.5:1. This ratio remained fairly constant during the experiment.

Whereas an interpretation of the data obtained from the radioautograms indicated that the radioactive glucose pool in the tissue was exhausted at the expense of sucrose synthesis, a quantitative analysis of the sugars present in the extracts, though showing considerable variation in the sugar content of the samples, indicated neither synthesis of sucrose nor an exhaustion of glucose (Table III).

<table>
<thead>
<tr>
<th>min.</th>
<th>Total sugar</th>
<th>Sucrose</th>
<th>Glucose</th>
<th>Fructose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg.</td>
<td>per cent</td>
<td>per cent</td>
<td>per cent</td>
</tr>
<tr>
<td>Control</td>
<td>12.7</td>
<td>61</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>0</td>
<td>9.9</td>
<td>51</td>
<td>18</td>
<td>30</td>
</tr>
<tr>
<td>15</td>
<td>13.1</td>
<td>61</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>23</td>
<td>11.4</td>
<td>54</td>
<td>7</td>
<td>36</td>
</tr>
<tr>
<td>83</td>
<td>9.7</td>
<td>46</td>
<td>19</td>
<td>32</td>
</tr>
<tr>
<td>161</td>
<td>9.8</td>
<td>43</td>
<td>20</td>
<td>34</td>
</tr>
<tr>
<td>363</td>
<td>5.7</td>
<td>49</td>
<td>16</td>
<td>34</td>
</tr>
</tbody>
</table>

**Table IV**

Per Cent Distribution of $^{14}C$ As Function of Time in Respiring Canna Leaf Disks, after Infiltration with 0.17 Per Cent Solution of $^{14}C$-Fructose

Forty-two Canna leaf disks, having a fresh weight of 3.75 gm., were infiltrated with 10 mg. of fructose of 29 μc. per mg. of specific activity dissolved in 6 ml. of water.

<table>
<thead>
<tr>
<th>Time, min.</th>
<th>0</th>
<th>17</th>
<th>40</th>
<th>92</th>
<th>215</th>
<th>380</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity, alcohol extract</td>
<td>100</td>
<td>99</td>
<td>97</td>
<td>90</td>
<td>80</td>
<td>73</td>
</tr>
<tr>
<td>&quot; leaf residues</td>
<td>0.3</td>
<td>1.3</td>
<td>2.4</td>
<td>3.3</td>
<td>3.3</td>
<td>5.0</td>
</tr>
<tr>
<td>&quot; respired CO₂</td>
<td>0</td>
<td>0.1</td>
<td>0.9</td>
<td>6.3</td>
<td>17</td>
<td>22</td>
</tr>
</tbody>
</table>

Fructose Infiltration—The results of infiltration with $^{14}C$-labeled fructose were similar to those obtained with $^{14}C$-labeled glucose. The relative distribution of activity is given in Table IV. The total activity accounted for in the fractions amounted to 813,000 c.p.m., indicating that about 0.23 ml. of the infiltrating solution had been retained in the tissue. In terms of fructose, this represents an average of 60 μ per set of seven disks. Here, as in the case of the glucose-infiltrated tissue, there can be noted a steady decrease in the percentage of activity remaining in the alcohol extracts,
although the decrease appears to be slower. Similarly, there is a steady increase in the percentage of CO activity respired as carbon dioxide. Only a relatively small percentage of the total activity was incorporated in the alcohol-insoluble fraction (Table IV).

![Graph](image)

**Fig. 5.** Distribution of activity in the sugar fraction of the alcohol extracts of C14-labeled fructose-infiltrated Canna leaf disks.

**Table V**

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Total sugar (mg.)</th>
<th>Sucrose (per cent)</th>
<th>Glucose (per cent)</th>
<th>Fructose (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.5</td>
<td>53</td>
<td>13</td>
<td>31</td>
</tr>
<tr>
<td>0</td>
<td>10.1</td>
<td>60</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>17</td>
<td>9.9</td>
<td>55</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>40</td>
<td>8.3</td>
<td>53</td>
<td>12</td>
<td>32</td>
</tr>
<tr>
<td>92</td>
<td>9.9</td>
<td>64</td>
<td>11</td>
<td>21</td>
</tr>
<tr>
<td>215</td>
<td>10.5</td>
<td>66</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>380</td>
<td>10.5</td>
<td>61</td>
<td>12</td>
<td>24</td>
</tr>
</tbody>
</table>

The shift in distribution of activity among the sugars was analogous to that observed when glucose was infiltrated. Fructose activity was lost at the expense of sucrose synthesis (Fig. 5) and catabolic transformations. The sugar fraction constituted 96 per cent of the activity in the alcohol extract at the beginning of the experiment. This value fell to 65 per cent at the end of the 6 hour respiration period.

The radioautograms obtained from the fructose-infiltrated disks were
similar to those obtained by infiltration of glucose, except for the reversal of the radioactive labeling.

Although free glucose never appeared on the radioautograms, resolution of the sucrose synthesized from active fructose into its constituents showed that the glucose contained approximately 40 per cent of the activity in the sucrose molecule (Fig. 5).

A quantitative analysis of the sugars present in the extracts showed that the total sugar concentrations as well as the per cent glucose, fructose, and sucrose did not appreciably change throughout the experiment (Table V).

**Sucrose Infiltration**—When radioactive sucrose containing equal amounts of C\(^{14}\) in the glucose and fructose moieties of the molecule was infiltrated into the leaf disks, the percentage distribution of C\(^{14}\) activity among the alcohol extract, residual-insoluble material, and respired carbon dioxide over a 6 hour period was similar to that obtained when either glucose or fructose was used. However, examination of the constituents in the alcohol extracts showed that, immediately after C\(^{14}\)-labeled sucrose infiltration (zero time), only 15 per cent of the C\(^{14}\) activity was found in sucrose, 40 per cent in free fructose, 30 per cent in glucose, and 15 per cent in the hexose monophosphates. As time progressed, the percentage of activity in the free hexose sugars and hexose phosphates decreased, whereas that in the sucrose, amino acids, and organic acids increased. After 6 hours respiration, sucrose appeared to be the major radioactive product, constituting 52 per cent of activity in the alcohol extract. These results indicate that, during infiltration, the sucrose must have been hydrolyzed to its monosaccharide constituents, which in the course of time were resynthesized to sucrose. As in the experiments with labeled glucose or fructose, chemical analysis of the samples revealed no major changes in the concentrations of the various sugars during the respiration period.

In order to verify these results and to ascertain whether or not any dif-

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**Table VI**

*Per Cent Distribution of C\(^{14}\) As Function of Time in Respiring Canna Leaf Disks after Infiltration with 0.4 Per Cent Solution of C\(^{14}\)-Fructose-Labeled Sucrose*

Forty-two Canna leaf disks, having a fresh weight of 2.9 gm., were infiltrated with 3 ml. of a solution containing 12 mg. of C\(^{14}\)-fructose-labeled sucrose with a specific activity of 14 \(\mu\)c. per mg.

<table>
<thead>
<tr>
<th>Time, min.</th>
<th>0</th>
<th>15</th>
<th>40</th>
<th>100</th>
<th>175</th>
<th>355</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol extract</td>
<td>99</td>
<td>98</td>
<td>87</td>
<td>83</td>
<td>81</td>
<td>77</td>
</tr>
<tr>
<td>Leaf residues</td>
<td>0.8</td>
<td>1.6</td>
<td>9.9</td>
<td>11</td>
<td>10</td>
<td>6.2</td>
</tr>
<tr>
<td>Respired CO(_2)</td>
<td>0</td>
<td>0.1</td>
<td>2.6</td>
<td>6.1</td>
<td>8.9</td>
<td>17</td>
</tr>
</tbody>
</table>
ferences existed between the reactions involving the two moieties of the sucrose molecule, sucrose labeled with C\textsuperscript{14} only in the fructose half of the molecule\textsuperscript{3} was used as the infiltrating substrate. The results of this experiment (Table VI) showed a distribution of activity in the three fractions similar to that observed when C\textsuperscript{14}-labeled fructose was infiltrated. The total activity accounted for was 290,000 c.p.m., which is equivalent to an infiltration of 0.07 ml. of solution into the tissue. In terms of sucrose, this represents an average of approximately 47 \( \gamma \) per sample of seven disks.

Analysis of the alcohol-soluble fraction showed the same sequence of events as that noted when sucrose with equally labeled moieties was infiltrated; that is, an initial decrease of C\textsuperscript{14} activity in sucrose followed by a progressive increase in activity of this disaccharide (Fig. 6). Initially the sugar fraction constituted 100 per cent of the C\textsuperscript{14} activity in the alcohol extract; at the end of the experiment this value had fallen to 70 per cent, of which 60 per cent resided in the sucrose. There was no evidence for the occurrence of free glucose at any time during the 6 hour period, yet resolution of the synthesized sucrose into its constituents showed that both the glucose and fructose were radioactive.

When sucrose labeled only in the fructose moiety was used as a substrate, it was possible to differentiate between the infiltrated sucrose and that synthesized in the tissue from the inversion products of the sucrose, since

\textsuperscript{3} This sucrose was prepared by a modification of the enzymatic method of Wolo-chow et al. (16), but the procedure was modified so that the sugar could be isolated chromatographically with a high specific activity.
the resynthesized sucrose also contained activity in the glucose portion of the molecule. Fig. 6 shows that, at the time the first sample was taken (zero time), about 40 per cent of the infiltrating substrate had been hydrolyzed. Within the next 15 minutes approximately 85 per cent had been inverted before the hydrolysis was masked by resynthesis. When the zero time sample was taken, resynthesis of sucrose had already occurred to at least the extent of 3 per cent, as demonstrated by the amount of label introduced into the glucose moiety of the sucrose. As in the previous experiment, chemical analysis of the sugars in the tissue gave no indication of either an inversion or a synthesis of sucrose (Table VII).

### Table VII

<table>
<thead>
<tr>
<th>Time min.</th>
<th>Control</th>
<th>Total sugar mg.</th>
<th>Sucrose per cent</th>
<th>Glucose per cent</th>
<th>Fructose per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11.9</td>
<td>66</td>
<td>11</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>11.3</td>
<td>63</td>
<td>16</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>10.5</td>
<td>66</td>
<td>12</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>9.8</td>
<td>65</td>
<td>10</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>175</td>
<td>9.4</td>
<td>59</td>
<td>16</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>355</td>
<td>10.4</td>
<td>62</td>
<td>15</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

### DISCUSSION

Vacuum infiltration appears to be an effective method for introducing a solution into the vessels and intercellular spaces of plant tissue so as to bring compounds in intimate contact with the seat of metabolic activity. When radioactive sugars were infiltrated and the tissues allowed to respire in the dark, the metabolic transformations could be followed by an examination of a radioautogram series. Although the analytical data did not indicate any significant changes in the relative sugar concentrations within the tissues during the course of the experiments, the observed transformations of the infiltrated C14-labeled substrates disclosed the general metabolic changes in the tissue.

The significant fact brought out in this investigation is that, when either radioactive glucose or fructose is infiltrated into Canna leaf tissue, the metabolic products immediately formed are organic phosphates, chiefly hexose monophosphates and sucrose. The sucrose appears to be readily formed at the expense of either of the two monosaccharides. Thus, the uncertainty of the conclusions of the previous investigators (1–3), that
sucrose might have been formed from some reserve carbohydrate material because of a change in physiological conditions in the plant due to sugar infiltration, has been eliminated.

The fact that no radioactive free fructose could be detected when C14-glucose was infiltrated, nor free radioactive glucose found when C14-labeled fructose was introduced, but that in each case both of the monosaccharide moieties in the sucrose were strongly radioactive, indicates that neither free glucose nor free fructose is involved in sucrose synthesis. A mechanism for sucrose synthesis in this plant, such as exists in the case of the enzyme from *Pseudomonas saccharophila* (17, 18), can therefore be eliminated. If this system were operating, labeled glucose-1-phosphate, presumably formed from the infiltrated C14-labeled glucose, would combine with free fructose through a dephosphorolytic condensation, forming sucrose. Inasmuch as no free radioactive fructose could be detected in the tissue, the fructose would inevitably have been derived from the unlabeled natural supply in the plant. The sucrose would therefore be expected to be labeled predominantly in the glucose moiety, which is not the case.

Since the sucrose contained activity in both of its monosaccharide constituents, and since there was no activity in the free fructose when C14-labeled glucose was infiltrated nor any activity in the free glucose when C14-fructose was infiltrated, the indication is that there was no active invertase system present in the intact leaf tissue that would hydrolyze the disaccharide during the course of the experiment. However, inasmuch as C14-labeled sucrose was rapidly inverted when an attempt was made to infiltrate it into the Canna leaf disks, it may be concluded that in the tissue the sites of invertase activity and sucrose synthesis are spatially separated from each other. In all probability, the infiltrated sucrose was hydrolyzed extracellularly, and the resultant glucose and fructose were resynthesized into sucrose after entry into the intact cells. It has been observed that the excess infiltrating solutions contained no sucrose when the C14-labeled monosaccharides were infiltrated; however, when C14-labeled sucrose was infiltrated, the external solutions contained both C14-labeled glucose and fructose. Additional experimental evidence is also available at present, which will be reported at a later date, that the hydrolysis of sucrose is the result of contact with the disrupted cells at the periphery of the leaf disks.

The C14 activity of the hexose monophosphates (phosphates of glucose, fructose, and mannose) appears to be highest at the first stages of sucrose synthesis when the rate of increase in activity of the disaccharide is at a maximum. When sucrose becomes the dominantly labeled compound, the activity in the hexose monophosphates disappears. These observations suggest that phosphorylated glucose and phosphorylated fructose serve as precursors of sucrose.
Whereas the $^{14}C$ activity data indicate about 95 per cent utilization of the infiltrated substrates, the analytical data show little change in the size or composition of the sugar pools in the tissue. This observation suggests that the infiltration of small amounts of $^{14}C$-labeled sugars inside the cells does not result in the labeling of the corresponding sugar pools. The fact that the carbohydrate pools are not labeled can be explained on the basis that the infiltrated labeled sugars are rapidly transformed into other compounds prior to equilibration with the relatively large amount of the non-radioactive sugars present in the plant. It is possible that the glucose and fructose are phosphorylated at the cell wall and do not become free sugars within the cell.

**SUMMARY**

The transformations of $^{14}C$-labeled glucose, fructose, and sucrose were studied in disks of *Canna indica* leaves during the course of 6 hour respiration periods in the dark. Infiltration of either radioactive monosaccharide caused a rapid appearance of radioactive sucrose and radioactive hexose monophosphates.

When $^{14}C$-labeled glucose was infiltrated into the leaf disks, the percentage of the total $^{14}C$ activity in this sugar rapidly decreased, while that of the sucrose showed a parallel increase. No activity could be detected in the free fructose. However, when the sucrose was hydrolyzed to its monosaccharide constituents, the activity of the fructose was comparable to that of glucose.

Likewise, infiltration of $^{14}C$-labeled fructose resulted in a decrease of $^{14}C$-fructose activity and in a simultaneous steady increase in the amount of $^{14}C$ activity in the sucrose. There was no activity in the glucose but, as in the case of $^{14}C$-labeled glucose infiltration, the glucose and fructose produced by hydrolysis of this sucrose did not vary greatly in their activities.

The sucrose does not seem to be formed by a mechanism involving either free fructose or free glucose. The synthesis of the disaccharide probably involves a dephosphorylolytic condensation of phosphorylated derivatives of glucose and fructose.

Although there was no indication of the presence of invertase or phosphatase activity in the intact cells, infiltration of $^{14}C$-labeled sucrose resulted in an immediate hydrolysis, presumably extracellular, into its monosaccharide constituents. The resultant glucose and fructose were rapidly resynthesized into sucrose after entry into the intact cells.

In addition to hexose phosphates and sucrose, the infiltrated radioactive sugars also produced labeled aspartic and $\gamma$-aminobutyric acids, alanine, glutamine, citric, malic, glyceric, succinic, and lactic acids.
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