Recycling in the Pentose Phosphate Pathway: Comparison of \( C_6/C_1 \) Ratios Measured with Glucose-\( C^{14} \) and Fructose-\( C^{14} \)

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The pentose phosphate pathway can be regarded as catalyzing: 3 glucose-6-P \( \rightarrow \) 3CO\(_2\) + 1 glyceraldehyde-3-P + 2 fructose-6-P. We use the term recycling to describe the situation in which fructose-6-P formed in the above reactions is converted to glucose-6-P which re-enters the pentose phosphate pathway. The net reactions of the pathway would then be:

\[
glucose-6-P \rightarrow 3CO_2 + 1 \text{glyceraldehyde-3-P}.
\]

This formulation does not mean that carbons 1, 2 and 3 of glucose-6-P are completely oxidized but merely that 3 molecules of CO\(_2\) and 1 molecule of glyceraldehyde are the net yield when 3 molecules of glucose-6-P pass through the pathway. We stress that the above definition does not imply that the pentose phosphate pathway is a formal sequence of reactions which act as a closed system.

The extent to which recycling is a general feature of the pentose phosphate pathway is not known. Katz and Wood (7) have assumed that recycling is unrestricted. Cheddelin and his colleagues (4) have questioned this assumption. Recent studies with animal cells have indicated that under certain conditions recycling is restricted (8). There is little experimental evidence that bears directly on the question of recycling in plants. The distribution of \( C^{14} \) from specifically labeled glucose fed to carrot tissue suggests that in this tissue recycling may be restricted (1). A similar conclusion has been drawn from data obtained with tomatoes (15).

In the work reported in this paper recycling has been investigated by comparing the rates at which \( C^{14}O_2 \) was released from carbons 1 and 6 of fructose-\( C^{14} \) and glucose-\( C^{14} \) supplied to a variety of plant tissues. The relationship between fructose-6-P and glucose-6-P could determine the degree of recycling. Complete interconversion of the 2 sugar phosphates would be expected to result in unrestricted recycling. If the fructose-6-P formed in the pentose phosphate pathway was not readily converted to glucose-6-P then recycling would probably be restricted. Consider a tissue in which an active pentose phosphate pathway resulted in a very low \( C_6/C_1 \) ratio. If in such a tissue fructose-6-P and glucose-6-P were completely interconvertible, then \( C_6/C_1 \) ratios measured with fructose-\( C^{14} \) would be the same as those measured with glucose-\( C^{14} \). However, if fructose-6-P was not readily converted to glucose-6-P, ratios measured with fructose-\( C^{14} \) would be expected to be higher than those measured with glucose-\( C^{14} \).

The above argument assumes that the fructose-\( C^{14} \) added to the tissues is converted to \( C^{14}O_2 \) via fructose-6-P. For higher plants there appears to be no data contrary to this assumption. However, there is evidence that in liver, fructose is metabolized via fructose-1-P (6). The extensive operation of a pathway similar to that proposed for liver would invalidate the use of \( C_6/C_1 \) ratios with fructose and glucose as a means of studying recycling. The fructose pathway in liver is dependent upon an aldolase that cleaves fructose-1-P. Aldolase activity with fructose-1-P has not been separated from that with fructose-1,6-diP. Recent evidence strongly suggests that in liver a single enzyme cleaves both fructose-1,6-diP, fructose-1-P (5). The presence in plants of a pathway of fructose metabolism similar to that proposed for liver would be marked by an ability of aldolase preparations to cleave both fructose-1,6-diP and fructose-1-P. Accordingly, for 2 of the tissues studied, a comparison has been made of the relative activities of aldolase preparations with fructose-1,6-diP and fructose-1-P.

Materials and Methods

Reagents. These were obtained as follows: glucose-1-\( C^{14} \) and -6-\( C^{14} \) from the Radiochemical Centre, Amersham, United Kingdom; NAD, NADH\(_2\), ATP, fructose-1-P, hexokinase, alkaline phosphatase, and aldolase from Sigma Chemical Company; fructose-1,6-diP, triose phosphate dehydrogenase, and \( \alpha \)-glycerophosphate dehydrogenase from Boehringer and Sons; triose phosphate isomerase from California Corporation for Biochemical Research.

Preparation of Fructose-\( C^{14} \). Fructose-1-\( C^{14} \) and -6-\( C^{14} \) were prepared from correspondingly labeled...
glucose-C\textsuperscript{14}. Glucose-1-C\textsuperscript{14} (0.35 mg, 0.05 mc) and glucose-6-C\textsuperscript{14} (0.4 mg, 0.05 mc) were incubated separately in reaction mixtures containing: Tris-HCl buffer, pH 7.4, 25 \(\mu\)moles; ATP 32 \(\mu\)moles; hexokinase (Type III) 10 mg; glucose-6-P isomerase 1.0 ml in a total volume of 2.3 ml. The incubations were carried out at 30\(^\circ\) for 105 minutes. The reactions were stopped with heat. The reaction mixtures were centrifuged and the pH of the supernatants was adjusted to pH 8.5. The supernatants were then incubated for 90 minutes at 30\(^\circ\) with alkaline phosphatase (10 mg). The phosphatase reaction was stopped with heat. Protein was removed by centrifuging after the reaction mixtures had been left at 0\(^\circ\) overnight. The labeled hexoses were then isolated by paper chromatography, using water-saturated phenol as solvent. Phenol was removed from the paper by extraction with ether.

The distribution of C\textsuperscript{14} in the glucose-1-C\textsuperscript{14} and -6-C\textsuperscript{14} isolated from the above reaction mixtures was shown to be similar to that of authentic glucose-1-C\textsuperscript{14} and -6-C\textsuperscript{14}. The degradation technique described by Busse et al. (3) was used. It is assumed that fructose-C\textsuperscript{14} and glucose-C\textsuperscript{14}, derived from the same incubation mixture, had identical labeling patterns.

All comparisons of C\textsubscript{6}/C\textsubscript{1} ratios were made with correspondingly labeled hexose obtained from the same incubation mixture. Glucose-6-P isomerase was prepared from pea seeds (2).

**Plant Material.** Fresh carrots (*Daucus carota* L.), white turnips (*Brassica rapa* L. ssp. *rapa*), and pumpkins (*Cucurbita pepo* L.) were bought locally. Discs 1 mm \(\times\) 9 mm were cut from the storage tissue of carrots and turnips and the mesocarp of pumpkin. Before use the discs were washed for 24 hours in the manner described previously (2). Replicate samples of 30 discs were used for comparison of C\textsubscript{6}/C\textsubscript{1} ratios.

Freshly picked carrot petioles were taken and the portion below the first branching point sliced into sections 1 mm thick. These sections were rinsed, blotted, and divided into replicate samples of 2.0 g fresh weight. Discs 7 mm in diameter were cut from the lamina of fresh, mature leaves of broad bean (*Vicia faba* L.). The discs were rinsed and divided into replicate samples of 0.9 g fresh weight.

Tobacco tissue cultures were of the single cell line H160 originally derived from the petiole of the hybrid *Nicotiana glutinosa* L. \(\delta\) \(\times\) *Nicotiana tabacum* L. var. Havana 38 \(\varphi\). The cells were grown on tobacco medium (12) supplemented with: 2.5 mg per liter calcium pantothenate, 0.1 mg per liter \(\alpha\)-naphthaleneacetic acid, 6.0 mg per liter 2,4-D, 1.0 g per liter Difco Bacto yeast extract, and coconut milk (15% by volume). An inoculum of 80 ml of cell suspension was added to 500 ml of medium in a 5-liter Erlenmeyer flask. The flask was then shaken continuously on a reciprocal shaker at 26\(^\circ\). After 3 weeks the cells were harvested, washed, resuspended in 0.01 M KH\textsubscript{2}PO\textsubscript{4} at pH 5.5, and returned to the shaker for a further 18 hours. At the end of this period of starvation the cells were harvested by filtration and replicate samples of 3.5 g fresh weight were prepared.

**Measurement of C\textsubscript{6}/C\textsubscript{1} Ratios.** The samples of tissue were suspended in 3.6 ml 0.03 M KH\textsubscript{2}PO\textsubscript{4}, pH 5.2. Hexose-C\textsuperscript{14}, 0.4 ml at 5 \(\times\) 10\textsuperscript{-6} m, was added to each sample. Incubations were carried out in 100-ml Erlenmeyer flasks at 30\(^\circ\). The methods used in the measurement of C\textsubscript{6}/C\textsubscript{1} ratios, hexose uptake, and radioactivity have been described (2). Light was excluded from flasks containing photosynthetic tissue.

**Aldolase Assays.** Washed slices, 40 g fresh weight, were homogenized in a pestle and mortar with 25 ml 0.05 M Tris-HCl buffer, pH 8.1. The slurry was squeezed through 2 layers of muslin and centrifuged at 30,000 \(\times\) g for 10 minutes. The supernatant fraction was then centrifuged at 144,000 \(\times\) g for 40 minutes. The final supernatant fraction is termed crude extract. The crude extract was fractionated with \((\text{NH}_4)_2\text{SO}_4\). The fraction which precipitated between 40% and 60% saturation with \((\text{NH}_4)_2\text{SO}_4\) was resuspended in 2.5 ml 0.05 M Tris-HCl buffer, pH 7.5, and dialyzed overnight against 0.005 M Tris-HCl, pH 7.5. The dialysis residue was centrifuged at 20,000 \(\times\) g for 10 minutes and the supernatant assayed for aldolase. All the above preparations were carried out at 2\(^\circ\) to 4\(^\circ\). Rat liver aldolase was prepared according to Leuthardt and Wolf (9).

Aldolase activity was determined in 2 ways. In the first, the dihydroxyacetone-P formed in the aldolase reaction was assayed by measuring the rate of oxidation of NADH\textsubscript{2} in the presence of \(\alpha\)-glycerophosphate dehydrogenase. Reaction mixtures contained: NADH\textsubscript{2}, 0.25 \(\mu\)mole; fructose-1,6-diP or fructose-1-P, 2 \(\mu\)moles; Tris-HCl, pH 7.4, 100 \(\mu\)moles; \(\alpha\)-glycerophosphate dehydrogenase, 0.02 ml (concentrated enzyme diluted 1:10); plant extract, 0.2 ml, in a total volume of 1.0 ml. In the second method, aldolase activity was measured by the rate of NAD reduction in the presence of triose phosphate isomerase and triose phosphate dehydrogenase. Reaction mixtures contained: NAD, 0.25 \(\mu\)mole; fructose-1,6-diP or fructose-1-P, 2 \(\mu\)moles; Tris-HCl, pH 7.4, 100 \(\mu\)moles; sodium arsenate, 40 \(\mu\)moles; cysteine-HCl, 10 \(\mu\)moles; triose phosphate isomerase (concentrated enzyme diluted 1:20), 0.02 ml; triose phosphate dehydrogenase, 0.02 ml, and plant extract, 0.2 ml, in a total volume of 1.0 ml. The dehydrogenase was incubated with cysteine for 10 minutes before starting the reaction by adding extract and substrate. Spectrophotometric measurements were made with a Beckman DU spectrophotometer fitted with a Gilford automatic cuvette positioner and an absorbance indicator linked to a chart recorder.
Results and Discussion

Crude extracts and ammonium sulfate fractions of both pumpkin and turnip showed aldolase activity towards fructose-1,6-diP (table 1). No activity with fructose-1-P was detected when the triose phosphate dehydrogenase dependent assay was used. With the assay involving α-glycerophosphate dehydrogenase the addition of fructose-1-P gave a marked increase in the rate of oxidation of NADH₂. This increase was not affected by the addition of purified α-glycerophosphate dehydrogenase, either before or after the addition of fructose-1-P. Consequently the activity with fructose-1-P can not be ascribed to an aldolase reaction. Although the above extracts contain a system, possibly mannitol dehydrogenase in which metabolism of fructose-1-P can be linked to NADH₂ oxidation, it is concluded that there is no significant cleavage of fructose-1-P by pumpkin or turnip aldolase.

With the assay procedures used for the plant extracts an aldolase preparation from rat liver was shown to react with fructose-1,6-diP and fructose-1-P at rates of 0.34 and 0.035 μmoles/min per 10 g fresh weight, respectively. Thus it seems probable that the failure of plant aldolase preparations to cleave fructose-1-P is due to the absence of such activity rather than to unsatisfactory assays.

From the above results it is argued that it is very unlikely that pumpkin or turnip tissue metabolize fructose by the pathway proposed for liver in which fructose is phosphorylated to give fructose-1-P. Studies on the specificity of plant hexokinases support this view (11).

The results of the experiments with fructose-C¹⁴ and glucose-C¹⁴ are presented in table II. All the tissues studied metabolized both hexoses readily. In general, the uptake of fructose-C¹⁴ lagged a little behind that of glucose-C¹⁴. This difference in rates

Table 1. Activity of Aldolase Preparations towards Fructose-1,6-diP and Fructose-1-P

<table>
<thead>
<tr>
<th>Assay</th>
<th>NADH₂ Oxidation with α-glycerophosphate dehydrogenase</th>
<th>without α-glycerophosphate dehydrogenase</th>
<th>NAD Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F-1,6-diP</td>
<td>F-1-P</td>
<td>F-1,6-diP</td>
</tr>
<tr>
<td>Pumpkin extract</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>crude</td>
<td>...</td>
<td>...</td>
<td>0.041</td>
</tr>
<tr>
<td>purified</td>
<td>0.362</td>
<td>0.021</td>
<td>...</td>
</tr>
<tr>
<td>Turnip extract</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>crude</td>
<td>0.710</td>
<td>0.0120</td>
<td>...</td>
</tr>
<tr>
<td>purified</td>
<td>0.0158</td>
<td>0.0048</td>
<td>0.00013</td>
</tr>
</tbody>
</table>

Table II. C¹⁴O₂ Production from Specifically Labeled Glucose-C¹⁴ and Fructose-C¹⁴ Supplied a Variety of Plant Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Hr from addition of hexose-C¹⁴</th>
<th>% Added hexose in medium at time of sampling C¹⁴O₂</th>
<th>% Added activity recovered as C¹⁴O₂</th>
<th>C-1</th>
<th>C-6</th>
<th>C₆/C₁</th>
<th>C-1</th>
<th>C-6</th>
<th>C₆/C₁</th>
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</thead>
<tbody>
<tr>
<td>Turnip storage</td>
<td>0-3</td>
<td>20</td>
<td>30</td>
<td>31.7</td>
<td>11.6</td>
<td>0.37</td>
<td>13.6</td>
<td>8.4</td>
<td>0.62</td>
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<tr>
<td>tissue</td>
<td>3-6</td>
<td>18</td>
<td>22</td>
<td>11.8</td>
<td>9.1</td>
<td>0.77</td>
<td>12.2</td>
<td>10.7</td>
<td>0.88</td>
</tr>
<tr>
<td>Carrot storage</td>
<td>0-2/5</td>
<td>21</td>
<td>29</td>
<td>31.4</td>
<td>8.9</td>
<td>0.28</td>
<td>15.7</td>
<td>8.1</td>
<td>0.52</td>
</tr>
<tr>
<td>tissue</td>
<td>2-5</td>
<td>18</td>
<td>22</td>
<td>8.7</td>
<td>7.9</td>
<td>0.91</td>
<td>9.3</td>
<td>7.6</td>
<td>0.82</td>
</tr>
<tr>
<td>Pumpkin mesocarp</td>
<td>0-3</td>
<td>8</td>
<td>19</td>
<td>30.2</td>
<td>7.5</td>
<td>0.25</td>
<td>21.0</td>
<td>9.9</td>
<td>0.47</td>
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<td>Carrot petiole</td>
<td>0-2</td>
<td>38</td>
<td>56</td>
<td>9.4</td>
<td>4.7</td>
<td>0.50</td>
<td>4.7</td>
<td>2.3</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>2-4</td>
<td>7</td>
<td>20</td>
<td>12.7</td>
<td>6.8</td>
<td>0.54</td>
<td>9.6</td>
<td>5.0</td>
<td>0.52</td>
</tr>
<tr>
<td>Broad bean leaf</td>
<td>0-5</td>
<td>43</td>
<td>68</td>
<td>7.2</td>
<td>5.9</td>
<td>0.82</td>
<td>4.4</td>
<td>3.8</td>
<td>0.86</td>
</tr>
<tr>
<td>Tobacco cells</td>
<td>0-4</td>
<td>...</td>
<td>...</td>
<td>14.1</td>
<td>11.1</td>
<td>0.79</td>
<td>6.0</td>
<td>5.2</td>
<td>0.87</td>
</tr>
</tbody>
</table>
of uptake is not sufficient to affect the C₆/C₁ ratios in the manner discussed previously (2).

The data obtained with storage tissue of carrot and turnip, and with pumpkin mesocarp are sufficiently similar to be discussed together. With glucose-C¹⁴ all these tissues showed the very low C₆/C₁ ratios indicative of an active pentose phosphate pathway. With fructose-C¹⁴ the initial ratios were strikingly higher than those obtained with glucose-C¹⁴. The difference between the ratios was largely due to the fact that carbon 1 of fructose made a much smaller contribution to CO₂ than did carbon 1 of glucose. Apart from this initial difference, the ultimate yields of C¹⁴O₂ from the 2 sugars were roughly similar.

These results establish that in carrot, turnip, and pumpkin, carbon 1 of glucose is more readily converted to CO₂ than is carbon 1 of fructose. If we assume that the fructose-C¹⁴ is metabolized via fructose-6-P then the data show that in these tissues fructose-6-P is not in complete isotopic equilibrium with glucose-6-P. Thus it appears that in these tissues recycling is restricted because fructose-6-P is not completely interconvertible with glucose-6-P. The fact that the ratios with fructose-C¹⁴ are appreciably less than unity indicates that some of the fructose-6-P enters the pentose phosphate pathway and that the restriction on recycling is not total. The data in this paper and from previous work (1) are consistent with the view that in carrot slices much of the fructose-6-P formed in the pentose phosphate pathway is metabolized via the Embden-Meyerhof-Parnas pathway.

Results similar to those described above have been obtained with rat epididymal adipose tissue (8). However, lack of isotopic equilibration between fructose-6-P and glucose-6-P does not appear to be universal. In mouse brain (10) and human erythrocytes (13) there is strong evidence that the 2 hexose phosphates are completely interconvertible. The scant data available at present suggest that lack of isotopic equilibration between hexose-6-phosphates may be a characteristic of tissues with an active pentose phosphate pathway.

There is no obvious explanation why fructose-6-P is not readily interconvertible with glucose-6-P in carrot, turnip, and pumpkin. There is no lack of glucose-6-P isomerase, as high activities of this enzyme have been demonstrated in turnip (2) and pumpkin (Smillie and ap Rees, unpublished observations). It is known that glucose-6-P isomerase is inhibited by phosphogluconate (14). It is conceivable that in tissues with an active pentose phosphate pathway the level of phosphogluconate is high enough to cause partial inhibition of glucose-6-P isomerase. Such an inhibition would restrict recycling and favour the entry of glucose-6-P into the pentose phosphate rather than the Embden-Meyerhof-Parnas pathway. A second possibility is that the fructose-6-P formed in the pentose phosphate pathway does not have free access to the glucose-6-P isomerase and consequently is metabolized mainly via phosphofructokinase.

The data obtained with tobacco cells, broad bean leaves, and carrot peels serve to demonstrate that ratios measured with fructose-C¹⁴ are not always significantly higher than those measured with glucose-C¹⁴. With the high C₆/C₁ ratios of tobacco cells and broad bean leaves, restricted recycling would not necessarily lead to ratios with fructose that were significantly higher than the ratios with glucose. The effect of restricted recycling on the yields of CO₂ from carbon 1 of glucose and fructose could be small in relation to the yields from carbon 6. The results obtained with carrot peels are of interest in that they show that similar ratios with fructose-C¹⁴ and glucose-C¹⁴ can be obtained from tissues which have a ratio significantly less than unity. The carrot peels data indicate that in this tissue the hexose-6-phosphates are completely interconvertible and recycling is unrestricted.

Summary

Fructose-C¹⁴ and glucose-C¹⁴ were used to measure C₆/C₁ ratios of a variety of plant tissues. Initial C₆/C₁ ratios with glucose were about 0.3 in storage tissue of carrot (DAucus carota L.) and turnip (Brassica rapa L., ssp. rapa) and in mesocarp of pumpkin (Cucurbita pepo L.). In the above tissues initial C₆/C₁ ratios with fructose-C¹⁴ were about 0.55. The difference in the ratios was due mainly to the fact that carbon 1 of glucose was converted to CO₂ more readily than was carbon 1 of fructose.

Fructose-1,6-diphosphate aldolase preparations from pumpkin mesocarp and turnip storage tissue were shown to have no significant activity with fructose-1-phosphate. It was concluded that it was unlikely that these tissues metabolized fructose to C¹⁴O₂ via fructose-1-phosphate. It was argued that in carrot, turnip and pumpkin tissues fructose-6-phosphate is not readily interconvertible with glucose-6-phosphate and that consequently recycling in the pentose phosphate pathway is restricted in these tissues.

Comparison of C₆/C₁ ratios with glucose and fructose provided no evidence of restricted recycling in slices of carrot peele. suspensions of tobacco (Nicotiana glutinosa L. × Nicotiana tabacum L.) cells, and discs of leaves of Vicia faba L.

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Literature Cited


