BIOSYNTHESIS OF L-RHAMNOSE FROM D-GLUCOSE IN BUCKWHEAT

J. E. WATKIN and A. C. NEISH

The Prairie Regional Laboratory, National Research Council, Saskatoon, Saskatchewan, Canada

(Received 28 March 1961)

Abstract—D-Glucose-1-C\textsuperscript{14}, -2-C\textsuperscript{14}, and -6-C\textsuperscript{14} were fed to cuttings of buckwheat (Fagopyrum tataricum Gaertn.) and 24-48 hr later the rutin was isolated and hydrolysed. This gave D-glucose, L-rhamnose and quercetin all with the same specific activity and suggests that all components of rutin came from the same carbohydrate metabolic pool. Both rhamnose and glucose were degraded by methods which allowed estimation of C\textsuperscript{14} in each of the six carbon atoms. The rhamnose was apparently derived from glucose without rearrangement of the carbon skeleton. L-Rhamnose-1-C\textsuperscript{14} and L-fucose-1-C\textsuperscript{14}, fed to buckwheat, were not incorporated into rutin.

INTRODUCTION

Eight methyl pentoses (6-deoxyaldohexoses) are known to occur in plants.\textsuperscript{1} These are found in glycosides or polysaccharides rather than as free monosaccharides. The most common ones are L-rhamnose (6-deoxy-L-mannose) and L-fucose (6-deoxy-L-galactose). The other 6-deoxyhexoses are rare and found in the cardiac glycosides.

Three of the 6-deoxyhexoses, namely D-quinovose and D- and L-fucose, can be formally derived from the natural aldohexoses, D-glucose and D- and L-galactose respectively, by loss of the hydroxyl on carbon 6. The parent sugars of the other five 6-deoxyhexoses are not known to occur naturally. Thus on structural grounds it would seem unlikely that the biosynthetic route to these 6-deoxysugars is by formation of the appropriate hexose followed by reduction on carbon 6. We are unaware of any studies on the biosynthesis of rhamnose in higher plants.\textsuperscript{2} The present paper reports the biosynthesis of rhamnose found in rutin (quercetin-3-β-rhamnoglucoside) of buckwheat. An improved method of degrading rhamnose gives the C\textsuperscript{14} content of each carbon. Apparently rhamnose is formed from glucose in buckwheat without rearrangement of the carbon chain.

RESULTS

The results of feeding various labelled sugars to buckwheat cuttings (stem plus leaves) on the incorporation of C\textsuperscript{14} into rutin and its hydrolysis products, quercetin, rhamnose and glucose, are shown in Table 1. All the products were isolated and purified chromatographically. The plants used in each experiment were from the same batch. Large plants grown outdoors were used for the first experiment so large amounts of glucose and rhamnose (from rutin) could be obtained for degradation. The amount of labelled glucose fed was roughly equal to the natural pool in the plant.

Previous experience\textsuperscript{3} has shown that the per cent incorporation of glucose-C\textsuperscript{14} into rutin
is roughly equivalent to the percentage of rutin in the plant. This was found in experiment 2. The amount incorporated in experiment 1 was only about one quarter that anticipated, probably because the plants were more mature and the percentage of rutin was beginning to fall.\textsuperscript{4} The specific activities of all rutin components (quercetin, glucose and rhamnose) were about the same no matter which labelled glucose was fed (experiment 1).

Free rhamnose was not appreciably incorporated into rutin by buckwheat (experiments 2 and 3). The small amount of C\textsuperscript{14} incorporated from rhamnose in experiment 2 was

Table 1. Relative activities of rutin components from buckwheat plants fed C\textsuperscript{14}-labelled glucose, rhamnose or fucose

<table>
<thead>
<tr>
<th>Compound Fed*</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>Position of C\textsuperscript{14}</td>
<td>1</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Metabolic period (hr)</td>
<td>48</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>Wt. of compound fed (mg)</td>
<td>50</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Dry weight of plant (g)</td>
<td>18-1</td>
<td>21-3</td>
<td>19-1</td>
</tr>
<tr>
<td>Dose (\mu mole/g)</td>
<td>15</td>
<td>13</td>
<td>29</td>
</tr>
<tr>
<td>Total C\textsuperscript{14} fed (\mu c)</td>
<td>51-4</td>
<td>50</td>
<td>37</td>
</tr>
<tr>
<td>Rutin isolated (mg)</td>
<td>290</td>
<td>468</td>
<td>427</td>
</tr>
<tr>
<td>Specific activity of quercetin (\mu c/mole carbon)</td>
<td>17-2</td>
<td>9-7</td>
<td>7-5</td>
</tr>
<tr>
<td>Specific activity of glucose (\mu c/mole carbon)</td>
<td>150</td>
<td>0-3</td>
<td>1-5</td>
</tr>
<tr>
<td>Specific activity of rhamnose (\mu c/mole carbon)</td>
<td>13-9</td>
<td>10-8</td>
<td>7-7</td>
</tr>
<tr>
<td>Rutin as % of dry weight plant (%)</td>
<td>2-2</td>
<td>2-2</td>
<td>2-2</td>
</tr>
<tr>
<td>% of C\textsuperscript{14} fed recovered in rutin</td>
<td>0-50</td>
<td>0-41</td>
<td>0-39</td>
</tr>
</tbody>
</table>

* G = D-Glucose, Rh = L-Rhamnose, Fu = L-Fucose.
† Calculated from the dry weight of the alcohol extracted marc, the percent solubles being determined on a control plant.
§ Amount of compound fed expressed in \mu moles/g of dry weight of plant.
$ Samples lost, and specific activity assumed to be that of the quercetin on the evidence of experiment 1 and specific activity of the isolated rutin.

only 0-05 per cent of that fed (2\mu g) and this might be due to labelling of the general carbohydrate pool by metabolic products of rhamnose, as the glucose and quercetin were labelled to roughly the same extent. The free sugars of a buckwheat plant were isolated and partitioned on a cellulose column but no free rhamnose was found. It is unlikely that free rhamnose occurs naturally in buckwheat.

The isolated sugars were degraded; glucose by fermentation\textsuperscript{6,4} and further degradation of the products,\textsuperscript{7} rhamnose by an adaptation of the procedure of Boothroyd \textit{et al.}\textsuperscript{8} outlined in Fig. 1. The labelling patterns of glucose and rhamnose from experiment 1 are shown in Table 2. The percentages are based on the sum of the activities of the 6 carbons. This total varied, depending on the degradation, from 84 to 98 per cent of the activity of the original sample as measured by combustion of an aliquot before degradation. Generally speaking the rhamnose has the same labelling pattern as the glucose from the same sample, in spite of the fact that appreciable redistribution of the label has occurred in glucose.

The main findings of this work are (a) all three components of rutin have the same specific activity, (b) free rhamnose is not incorporated into rutin although the rhamnose in rutin is formed readily from glucose and (c) no appreciable redistribution of the labelling pattern occurs during conversion of glucose to rhamnose.

There have been seven other studies on the conversion of C\textsuperscript{14} labelled glucose to deoxyhexoses in intact organisms—six studies used micro-organisms\textsuperscript{9-14} and one the milk of lactating women\textsuperscript{16}. In five of these studies bound glucose was isolated as well as deoxyhexose. In each case the labelling pattern of the glucose fed had been redistributed to some extent in the bound glucose but the label in the deoxyhexose in all cases paralleled the bound glucose. An extreme example of redistribution of C\textsuperscript{14} is in the work of Taylor

\footnotesize
Table 2. \% Distribution of C\textsuperscript{14} in each carbon of glucose and rhamnose obtained from rutin by hydrolysis after feeding labelled glucose

<table>
<thead>
<tr>
<th>Fed</th>
<th>G-1-C\textsuperscript{14}</th>
<th>G-2-C\textsuperscript{14}</th>
<th>G-6-C\textsuperscript{14}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
<td>Rh</td>
<td>G</td>
</tr>
<tr>
<td>Isolated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>70.4</td>
<td>72.8</td>
<td>0.6</td>
</tr>
<tr>
<td>C2</td>
<td>2.3</td>
<td>4.5</td>
<td>82.5</td>
</tr>
<tr>
<td>C3</td>
<td>4.4</td>
<td>4.5</td>
<td>8.7</td>
</tr>
<tr>
<td>C4</td>
<td>3.9</td>
<td>4.1</td>
<td>5.2</td>
</tr>
<tr>
<td>C5</td>
<td>1.6</td>
<td>1.3</td>
<td>1.6</td>
</tr>
<tr>
<td>C6</td>
<td>17.6</td>
<td>12.8</td>
<td>1.6</td>
</tr>
</tbody>
</table>

\textsuperscript{14} S. Segal and Y. J. Topper, Biochim. et Biophys. Acta, 25, 419 (1957).
\textsuperscript{15} S. Segal and Y. J. Topper, Biochim. et Biophys. Acta, 42, 147 (1960).
Biosynthesis of L-rhamnose from D-glucose in buckwheat

and Juni\textsuperscript{10} with an aerobic soil bacterium. When glucose-1-C\textsuperscript{14} or glucose-2-C\textsuperscript{14} were the substrates both glucose and rhamnose in the capsular polysaccharide were unlabelled. However when glucose-6-C\textsuperscript{14} was the substrate both glucose and rhamnose were labelled equally in positions one and six. Another example of the labelling pattern in the rhamnose varying greatly from that of the glucose administered was found in \textit{Pseudomonas aeruginosa}. Hauser and Karnovsky\textsuperscript{9} added glucose-6-C\textsuperscript{14} to the medium and isolated rhamnose labelled in positions one and six, from a rhamnolipid found in the medium. As there was no glucose in the rhamnolipid, bound glucose could not be isolated to check the labelling pattern. Using this same organism Kornfeld and Glaser\textsuperscript{16} have recently obtained a cell-free enzyme system that will convert thymidine diphosphate-D-glucose to thymidine diphosphate-L-rhamnose. A similar enzyme system that changes guanosine diphosphate-D-mannose to guanosine diphosphate-L-fucose has been isolated from \textit{Aerobacter aerogenes} by Ginsberg.\textsuperscript{17}

Glucose + Phosphate ester pool

\[ \text{Glucose} \rightleftharpoons \text{Phosphate ester pool} \rightleftharpoons \text{Glycoside pool} \]

\[ \text{Phenylalanine} \]

\[ \text{Acetate} \]

\[ \text{Glucose Nucleotide} \rightarrow \text{Rhamnose Nucleotide} \]

\[ \text{Quercetin} \]

\[ \text{Rutin} \]

\textbf{FIG. 2.}

In our view our investigation and those mentioned above show a common pattern for deoxyhexose biosynthesis. The first stage is the formation of a hexose nucleotide. During the formation of the nucleotide from glucose extensive redistribution of the C\textsuperscript{14} labelling pattern may occur in the phosphate ester pool depending on the peculiar carbohydrate metabolism of the organism. Buckwheat and some microorganisms\textsuperscript{15,14} show a C6-C1 interchange of C\textsuperscript{14} consistent with the EMP type of metabolism. Taylor and Juni's bacterium\textsuperscript{15} has a modified Entner–Doudoroff type of metabolism whereby the glucose carbon chain is first split and then the hexose molecule is rebuilt from carbons 4, 5 and 6 before a hexose nucleotide can be formed. In all organisms once the hexose nucleotide is formed it is converted directly without rearrangement of the carbon chain to a deoxyhexose nucleotide. These nucleotides are then used for polysaccharide or glycoside synthesis.

Application of the above facts and ideas leads to a scheme for the biosynthesis of rutin in buckwheat outlined in Fig. 2. When glucose enters the plant it probably enters the phosphate ester pool.\textsuperscript{18,19} This pool contains intermediates of glycolysis and of the pentose phosphate pathway. From this pool may be drawn erythro-4-phosphate and phosphoenol pyruvate required for synthesis of phenylalanine\textsuperscript{20} as well as pyruvate which is used as a source of acetate units. Quercetin is readily formed from phenylalanine and acetate.\textsuperscript{21}


The glycoside pool is postulated to contain glucose-1-phosphate and other glycosidic compounds such as uridine diphosphate glucose. Sugars can be interconverted as nucleotides e.g., uridine diphosphate glucose \( \rightarrow \) uridine diphosphate galactose, and a rhamnose nucleotide is probably formed here. Nucleotides act as donors for transglycosylation such as occurs in the synthesis of sucrose, oligosaccharides, glucuronides etc. Rutin could be formed by transglycosylation of quercetin from glucose and rhamnose nucleotides. Details of these reactions have not been investigated in rutin formation and it is not known whether a disaccharide is formed before reaction with the flavonol or whether monosaccharides are put on one at a time.

If indeed rhamnose is metabolized in plants as a nucleotide, the absence of a reversible enzyme which would make or break the nucleotide from the free sugar would explain both the absence of free rhamnose and its failure to be used for rutin synthesis. An analogous situation was found in wheat where D-xylose was not converted to xylan except after prior conversion to D-glucose\(^{28}\) whereas both D-glucose and L-arabinose could give rise to an xylan precursor in the glucoside pool.

The observation that all components of rutin have the same specific activity is now understandable since they all come from the same intermediates in the phosphate ester pool. Direct formation of L-rhamnose from D-glucose requires three epimerizations and a reduction of \(-\text{CH}_2\text{OH}\) at carbon 6 to \(-\text{CH}_3\). This reduction has been unknown until now, in the biochemistry of hexoses but an analogy in the triose sugars would be the formation of pyruvate from 2-phosphoglyceric acid. In the hexose series the intermediate would be a 5-6 hexose but as the order of the epimerization and dehydration is unknown it is not possible to suggest which hexose. However, stereospecific reduction of the hexose in the stable pyranose form (carbon 1 being linked to the nucleotide) would enable the formation of the 6 methyl group and the epimerization on carbon 5 to form the L-sugar to take place simultaneously. The epimerization of carbons 3 and 4 may take other enzymes and 3- and 4- epimerases are known, such as galactowaldenase, and L-ribulose-5 phosphate 3-epimerase which occurs in \textit{Aerobacter aerogenes}.\(^{28}\)

**EXPERIMENTAL**

\textbf{General}

Measurements of C\(^{14}\)O\(_2\) were carried out in a gas phase proportional counter using the technique of Buchanan and Nakao.\(^{34}\) The radioactive sugars were purchased from Atomic Energy of Canada Ltd. Unless stated otherwise, all solutions were evaporated by a stream of dry filtered air at room temperature. This was usually done in a tared beaker.

\textbf{Administration of Labelled Sugars}

Buckwheat (\textit{Fagopyrum tataricum} Gaertn. V. CD 4251) was grown outdoors for the first experiment (Table 1) and in a growth chamber for the second and third experiments. The administration of labelled compounds by absorption of a solution through the cut end of the stem has been described.\(^{22}\) The cuttings were kept in an incubator at 22°C with 400 ft c. (4000 lx) of fluorescent light for the time indicated in Table 1.


Isolation of Products

When the metabolic period was over, rutin was isolated,8 and hydrolysed by refluxing in 3 per cent sulfuric acid for one hour. The hydrolysate was cooled, filtered to remove quercetin, and the filtrate clarified by passage through a small column (2.5 x 4 cm) of a mixture of equal volumes of Darco-G-60 and Celite 535. The sulfuric acid was neutralized by barium carbonate, the insolubles filtered, and the solution evaporated to a hard syrup.

This syrup contained both rhamnose and glucose. These sugars were separated by chromatography on a cellulose column (2.6 x 20 cm). n-Butanol one-quarter saturated with water eluted the rhamnose first. The glucose was then eluted by n-butanol saturated with water. The sugars were detected by spotting on paper and spraying with p-anisidine. The appropriate fractions were pooled, evaporated, taken up in water, extracted twice by ether and evaporated again. The ether-soluble impurities were discarded.

Measurement of C\(^{14}\)—distribution in Glucose and Rhamnose

The samples of glucose and rhamnose obtained above were degraded on a one-mmole scale. This required addition of 2–3 parts of carrier. Glucose was degraded by fermentation with resting cells of \textit{L,euconostoc mesenteroides}\(^{6,6}\) and the products (carbon dioxide, ethanol, lactic acid) isolated and degraded as described by Neish and Blackwood.7

Rhamnose was degraded by an adaptation of the procedure of Boothroyd \textit{et al.}8 for the chemical degradation of glucose. The modified procedure (Fig. 1) incorporates several short cuts; i.e. the \(\alpha\)- and \(\beta\)-methyl glycosides were not separated prior to periodic acid oxidation and no attempt was made to isolate a crystalline strontium salt after oxidation. The solution obtained after bromine oxidation was aerated to remove bromine, acidified to pH 1 by hydrochloric acid, concentrated to 2 ml, and then more hydrochloric acid added to make the solution 2N. Hydrolysis and isolation of glyoxylic acid as the 2,4-dinitrophenyl-hydrazone was carried out as before. The filtrate from the hydrazone was passed through a small column of carbon (Darco-G-60), mixed with Celite 535, then concentrated to 30 ml and the lactic acid isolated by continuous extraction with ether for 3 hr. The ether was evaporated in the presence of a few ml of water and the lactic acid titrated to the phenol red end point with 0.05 N sodium hydroxide. The lactic acid was then purified on a silicic acid column8 and degraded by oxidation to carbon dioxide and acetic acid as described previously.7 The acetic acid was degraded further by the Schmidt reaction as described by Phares.88 Thus the concentrations of C\(^{14}\) in carbons 2 to 6 are determined separately whereas the C\(^{14}\) of carbon 1 is determined by difference.

Acknowledgements—The authors are indebted to Mr. J. Dyck for radioactivity measurements and to Mr. G. H. Kreutz for skilful technical assistance.

---