THE BIOSYNTHESIS OF CARBON-14-LABELED COMPOUNDS.
II. THE CHROMATOGRAPHIC SEPARATION OF THE MONOSACCHARIDES, DISACCHARIDES, AND TRISACCHARIDES FROM PLANT EXTRACTS

G. R. NOGGLE and M. ELEANOR SCHUMACHER

(with one figure)

Received November 16, 1951

During the development of a method for the separation of biosynthetically prepared C\(^{14}\)-labeled glucose and fructose (4), it was found necessary to remove sucrose from the plant extracts before the monosaccharide separation could be effected. MONTGOMERY et al. (3) used the Tiselius displacement technique for isolating 6-(\(\alpha\)-D-glucopyranosyl)-D-glucose from an enzymatic starch hydrolyzate, and indicated in a footnote that the method could be adapted to a separation of monosaccharides and disaccharides. WHISTLER and DURSO (7) demonstrated that a mixture of charcoal (Darco G-60) and Celite (Celite 535) could be used for the column separation of monosaccharides, disaccharides, and trisaccharides. Using the former technique, we found that glucose and sucrose could be separated but that the method involved the use of three charcoal columns in series, and rather large volumes of solution were required to carry out the separation. The latter method does not need elaborate equipment and enables one to handle rather large amounts of sugar without resorting to excessively large volumes of desorbing solutions. It was found possible, by using this method to separate sucrose from the monosaccharide fraction of plant sugar extracts and, in addition, to isolate a trisaccharide fraction.

The general procedure followed is that of WHISTLER and DURSO (7) and is here briefly summarized. A mixture of Darco G-60 and Celite 535 was selected as the adsorbent. Other charcoals were tried but the Darco G-60 gave the most consistent results. Equal amounts by weight of the charcoal and Celite were mixed, washed with water, filtered, and dried. The adsorbent (35 gm.) was packed dry in a glass chromatographic tube 25 mm. in diameter and 300 mm. long, the bottom of which was attached to a receiving flask, which in turn was connected to a vacuum line. The adsorbent was slowly added to the tube (vacuum on) until a column 250 mm. long was attained. During the filling process the sides of the tube were vigorously tapped to aid in packing the adsorbent. Before the addition of the sugar solution 150 ml. of water were passed through the column, the flow rate then being maintained at approximately 200 ml. per hour. Whistler

\(^{1}\) Work performed under contract no. W-7405-eng-26 for the Atomic Energy Commission.
and Durso state that a column 34 mm. x 230 mm. has a loading capacity of 2 gm. of sugar. Our columns had roughly the same capacity but the amount of sugar placed on the column was dependent upon the relative proportions of monosaccharides and disaccharides present in the solution. If the disaccharide fraction predominated, then not more than 1 gm. of sugar was loaded on the column. If the disaccharide fraction was small then up to 2 gm. of sugar could be put on the column.

The sugars were removed from the column by the successive additions of water and increasing concentrations of ethanol. The monosaccharide fraction was desorbed with 800 ml. of water, the disaccharide fraction with one liter of 5% ethanol, and the trisaccharide fraction with 500 ml. of 15% ethanol. From the time that the adsorbent was wetted with water, the sugar solution and desorbing solutions were so added that the column was not permitted to run dry. The columns could not be reused and a new one was prepared for each separation.

The desorbed sugar solutions were concentrated and aliquots analyzed for reducing sugar and total sugar after acid hydrolysis. Samples of the aliquots were also run on paper chromatograms (6) to check on the completeness of separation. Radioactive sugars were detected on the paper chromatograms by exposing them to Eastman No-Screen X-ray film.

We found, as did Whistler and Durso, that the monosaccharides were desorbed with 800 ml. of water. A smaller volume of water decreased the recoveries of the monosaccharides, while a larger volume increased the chance for the disaccharide fraction to appear as a contaminant. Occasionally, however, small amounts of disaccharides were found in the monosaccharide fraction when 800 ml. of water was used. Most of the difficulty was found to be due to inadequately mixed charcoal and Celite. As the mixture had a tendency to layer out when stored, a column containing an excessively high proportion of Celite was occasionally prepared. Under these conditions the disaccharide fraction appeared in the water eluate along with the monosaccharides. If, on the other hand, the column was prepared with the adsorbent containing a high proportion of charcoal, a very slow flow rate was obtained and the complete separation was difficult to carry out. Poor separation was also found with one batch of Darco G-60, but a number of subsequent batches have given no difficulty.

The desorption of a number of monosaccharides that might be expected in plant extracts was carried out and the results are shown in table I. Two different charcoal-Celite mixtures were prepared and tested for improved sugar recovery. The results of the desorption of glucose, fructose, and galactose from adsorbent columns prepared from the various charcoals are shown in table II. Bone charcoal gave much better recoveries than the other charcoals but, because of the difficulty in obtaining uniform batches for routine work, the Darco G-60 has been used in all subsequent work.

To check the completeness of the monosaccharide-disaccharide separation, two experiments were carried out with mixtures of C14-labeled glucose
TABLE I
RECOVERY OF GLUCOSE, FRUCTOSE, GALACTOSE, AND ARABINOSE FROM A DARCO G-60-CELITE 535 COLUMN AFTER DESORPTION WITH 800 ml. OF WATER.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Added</th>
<th>Recovered</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg.</td>
<td>mg.</td>
<td>%</td>
</tr>
<tr>
<td>Glucose</td>
<td>272</td>
<td>249</td>
<td>91</td>
</tr>
<tr>
<td>Fructose</td>
<td>259</td>
<td>259</td>
<td>100</td>
</tr>
<tr>
<td>Galactose</td>
<td>284</td>
<td>238</td>
<td>84</td>
</tr>
<tr>
<td>Arabinose</td>
<td>254</td>
<td>250</td>
<td>99</td>
</tr>
</tbody>
</table>

and sucrose. In the first case, 100 mg. of inactive glucose and 20 mg. of labeled sucrose (0.152 µc./mg.) were mixed, and then separated by the procedure described. The water and the 5% ethanol fractions were concentrated in vacuo to a small volume and placed on Whatman no. 1 filter paper for paper chromatography with a butanol-ethanol-water (40:10:50) solvent. Following the development of the chromatogram, the paper was dried and an autoradiograph was prepared. The developed autoradiograph revealed no sucrose spot in the monosaccharide fraction. Total and reducing sugar was determined chemically on the water and 5% ethanol fractions. The water fraction contained 92 mg. of reducing sugar and no non-reducing sugar. The 5% ethanol fraction contained 5 mg. of reducing sugar and 18 mg. of non-reducing sugar. A second mixture of 20 mg. of labeled glucose (0.64 µc./mg.) and 100 mg. of inactive sucrose was prepared and treated similarly. Autoradiographs of the two fractions indicated a small glucose contamination in the sucrose. The water fraction was found to contain 18 mg. of reducing sugar and no non-reducing sugar. The 5% ethanol fraction contained about 1 mg. of reducing and 94 mg. of non-reducing sugar.

Raffinose, the only trisaccharide commonly found in plant tissues, may be conveniently isolated from plant extracts by the method of Whistler and Durso. Using this technique we pooled a number of trisaccharide fractions from biosynthesis runs and isolated and crystallized the raffinose (1). At this writing a total of 600 mg. of C¹⁴-labeled raffinose (0.021 µc./mg.) have been prepared.

TABLE II
RECOVERY OF GLUCOSE, FRUCTOSE, AND GALACTOSE FROM DARCO G-60, NORIT A AND BONE CHARCOAL COLUMNS AFTER DESORPTION WITH 800 ml. OF WATER.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Darco G-60</th>
<th>Norit A</th>
<th>Bone charcoal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Glucose</td>
<td>91</td>
<td>87</td>
<td>97</td>
</tr>
<tr>
<td>Fructose</td>
<td>100</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>Galactose</td>
<td>84</td>
<td>87</td>
<td>100</td>
</tr>
</tbody>
</table>
The leaves of *Canna indica* were exposed to 5 mc. of C$^{14}$ as C$^{14}$O$_2$ in the photosynthesis apparatus described by Noggle and Bolomey (4), after which the sugars were extracted with 80% ethanol and then deproteinized and deionized (4). The extract, containing 300 mg. of sucrose and 230 mg. of reducing sugars was concentrated to 25 ml., and then separated on a Darco G-60-Celite 535 column. Paper chromatograms of the three separated fractions were prepared and then autoradiographed (fig. 1). The monosaccharide fraction was found to contain no sucrose. There was also a small spot indicating a compound with an $R_f$ value lower than sucrose, but in an amount too small to identify. This compound did not appear to be raffinose but it moved on the paper to a region that was slightly above raffinose. Phosphorylated sugars also are found in this region but the ion-exchange treatment should have removed them. The material did not interfere with the subsequent column separation of the glucose and fructose. The disaccharide fraction contained sucrose, a small amount of glucose, and some sugar that appeared to be raffinose. The trisaccharide fraction contained raffinose and a small amount of sucrose.

Another feature which enhances the value of the charcoal-Celite column when handling plant extracts is its clarifying power. Traces of color which frequently appear in the extract are partially removed in the deproteinization and deionization procedure. Such colored extracts, when passed through the charcoal-Celite columns, come out sparkling clear.

The problem of primary importance in the investigation was to find a method that would remove the disaccharides from plant extracts so that
the monosaccharides could be separated by column chromatography. The method of Whistler and Durso was found to be satisfactory for this purpose. In addition, the data of Whistler and Durso indicated that a disaccharide and a trisaccharide fraction might be separated from plant extracts. This was found to be true but there was some monosaccharide and trisaccharide contamination of the disaccharide fraction and a disaccharide contamination in the trisaccharide fraction. The amount of contamination was very small and under ordinary conditions would not interfere with the preparative separation of the sugar components from a plant extract. When handling labeled sugars, however, this contamination might give some trouble.

A second prerequisite of the method was that it be capable of handling at least one gram of sugar. The Whistler and Durso method also fulfilled this requirement.

The present method does not satisfactorily solve the problem of completely separating the sugar components from complex mixtures such as plant extracts. There are many important sugars such as the pentoses that can not be separated. The recent demonstration by Khym and Zill (2) that sugars could be separated by ion-exchange has opened up the possibility of making a complete separation of all the sugars present in plant extracts. The method has been adapted to the quantitative analysis of the sugars in plant extracts (5) and additional work has shown its application to preparative procedures.

LITERATURE CITED

5. Noggle, G. R. and Zill, L. P. The quantitative analysis of sugars in plants by ion-exchange chromatography. (To be published.)