THE BIOSYNTHESIS OF CARBON-14-LABELED COMPOUNDS. III. THE SEPARATION AND ISOLATION OF SUGARS BY ION-EXCHANGE CHROMATOGRAPHY[1]

G. R. NOGGLE[2] AND L. P. ZILL

BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY, OAK RIDGE, TENNESSEE

Received December 16, 1952

Introduction

One of the major difficulties in the preparation of labeled compounds by biosynthetic procedures is the isolation of chemically and radiochemically pure compounds. Often compounds which appear chemically pure are found to contain radioactive contaminants which may make such labeled compounds virtually useless in biological experiments. UDENFRIEND and GIBBS (**11**) and more recently PUTMAN and HASSID (**8**) used paper chromatography to effect the separation and isolation of several labeled sugars. The latter workers pointed out that such a procedure enabled them to prepare sugars of very high specific activity since it was unnecessary to add any carriers. Furthermore, they were able to obtain labeled sugars containing extremely small amounts of radioactive contaminants. NOGGLE and BOLOMEY (**5**) used column chromatography to effect the separation of glucose and fructose while GIBBS *et al.* (**2**) prepared labeled sucrose by fermenting out the monosaccharides. NOGGLE and SCHUMACHER (**6**) separated the sugars present in plant extracts into a monosaccharide, a disaccharide, and a trisaccharide fraction by column chromatography on charcoal. Certain disadvantages are found in all of these methods, such as limited capacity, inability to obtain radiochemically pure products, or the necessity of adding carrier, so that it seemed desirable to explore other methods that would minimize these difficulties. It has been known for a long time that sugars and certain other polyhydroxy compounds react readily with borate ions to form borate complexes which are negatively charged ions. KHYM and ZILL (**4**) demonstrated that a mixture of these sugar-borate complexes could be resolved by ion-exchange chromatography on strong-base anion exchange resins. Subsequently, NOGGLE and ZILL (**7**) showed that this procedure could be used for the quantitative analysis of the sugars found in plant extracts and when this method was tried as a preparative method for separating labeled sugars prepared biosynthetically, it was found to give results that overcame many of the difficulties encountered in other preparative procedures. The experimental results are presented in this paper.

[1] Work performed under contract no. W-7405-eng-26 for the Atomic Energy Commission.

[2] Present address: Southern Research Institute, 917 South 20th Street, Birmingham 5, Alabama.

Apparatus

PHOTOSYNTHESIS CHAMBER.—The chamber described by NOGGLE and BOLOMEY (5) was used.

ION-EXCHANGE COLUMNS.—The columns have been described elsewhere (9, 10). They were 0.85 sq. cm. in cross sections and 18.0 cm. long.

ION-EXCHANGE RESIN.—Dowex-1 strong-base anion exchange resin (Dow Chemical Company, Midland, Michigan) was used. The preparation of the resin has been described (4).

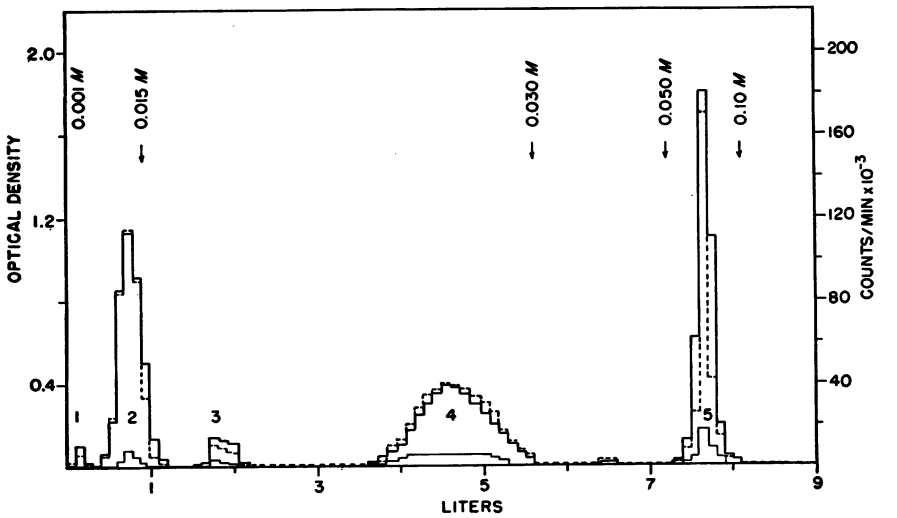

FIG. 1. Separation of the sugars in *Phytolacca decandra* by ion-exchange chromatography of their borate complexes. Resin bed, 0.85 sq. cm. × 12 cm.; Dowex-1 resin (200–400 mesh), borate form. Elution started with 0.001 M potassium tetraborate, changed to 0.015 M at 900 ml., to 0.030 M at 5600 ml., to 0.050 M at 7200 ml., and to 0.10 M at 8100 ml.

——— — optical density (at 620 mμ) with anthrone reagent.
——— — optical density (at 620 mμ) with orcinol reagent.
- - - - - - - — radioactivity of 1-ml. aliquots.

Procedure

The leaves of pokeweed (*Phytolacca decandra*) were removed from field-grown plants and placed in the dark for 40 hours. The leaves (70 gm. fresh weight) were then exposed to 1.0 mc. of C^{14} as $C^{14}O_2$ in the photosynthesis chamber for 20 hours at a light intensity of approximately 700 f.c. At the conclusion of the illumination period, the leaves were cut up and dropped into boiling 80% ethanol for 30 minutes. The alcohol was decanted from the leaves, fresh boiling 80% ethanol was added, and the leaves were then macerated in a Waring Blendor for 10 minutes. The macerate was filtered and washed with hot ethanol. The alcohol filtrates were combined, and the alcohol was removed under reduced pressure after which the solution was deproteinized and deionized (5) and made up to a known volume.

Preliminary tests indicated that the solution contained approximately 200 mg. of sugar. Since the capacity of the ion-exchange resin bed (0.85 sq. cm. × 12.0 cm.) was approximately 60 mg. of sugar, a 20-ml. aliquot equivalent to roughly 40 mg. of total sugar was separated by the procedure of KHYM and ZILL (**4**). The results are shown in figure 1. Each collected fraction was analyzed for sugar with the anthrone reagent and with orcinol (**7**), and for radioactivity by plating aliquots on aluminum planchets and counting in a gas-flow proportional counter. The first peak through the column was not identified. It is suspected of being a saponin but additional tests must be made. Peaks 2, 3, 4, and 5 were found to be sucrose, raffinose, fructose, and glucose, respectively. The actual amounts, as measured by the optical densities of their anthrone derivatives, were 12.3 mg. of sucrose, 2.1 mg. of raffinose, 13.4 mg. of fructose, and 16.9 mg. of glucose, or a total of 44.7 mg. of sugar in the 20-ml. aliquot. The coincidence of the sugar concentration and radioactivity in each peak suggests that the individual sugars were radiochemically pure. When the isolated sugars were chromatographed and autoradiographs were prepared, no radioactive contaminants could be found.

The separated sugars were isolated by pooling the individual fractions containing a single sugar. Each pooled sample then contained one sugar in a solution of potassium tetraborate which was used to effect the separation. The solutions were treated with a strong-acid cation exchange resin (Dowex-50) in the hydrogen form to remove the potassium ions. The resin was then removed from the solution by filtering through a sintered-glass filter. It is imperative that the potassium ions be removed quickly from the sugar sample because the potassium tetraborate solution used to effect the separation is alkaline and prolonged standing of the sugars in alkaline solutions results in a considerable breakdown. Following removal of the potassium ions the sugar was in a solution of boric acid. The solution was concentrated under reduced pressure (water pump) at 30° C until the boric acid started to crystallize. Absolute methyl alcohol was added, and the volatile methyl borate formed was removed under reduced pressure at 30° C. This step was repeated three times after which practically all the boric acid had been removed. ZILL, KHYM, and CHENIAE (**12**) have shown that 5 to 7

TABLE I

AMOUNT, SPECIFIC ACTIVITY, AND TOTAL ACTIVITY OF SUGARS EXTRACTED FROM POKEWEED LEAVES.*

Sugar	Amount	Specific activity	Total activity
	mg.	*μc./mg.*	*μc.*
Sucrose	61.5	1.89	116.2
Raffinose	10.8	0.96	10.4
Fructose	67.1	1.78	119.4
Glucose	84.7	1.27	107.7

*Pokeweed leaves, 70 gm. fresh weight; had been administered 1.0 mc. of C^{14} as $C^{14}O_2$.

p.p.m. of boric acid remains in the sugar solutions after such treatments with methyl alcohol. The sugars may then be crystallized out by conventional techniques. Some sugar is lost during the manipulations necessary to free the solutions of potassium ions and boric acid, but recovery experiments indicated that approximately 90% of added sugar could be recovered.

In the present study, the sugars were not crystallized since they were to be used in solution for another experiment. Aliquots of the individual sugars were burned, and the CO_2 converted to $BaCO_3$ for counting. The specific activities of the sugars are shown in table I.

Discussion

It can readily be seen that the ion-exchange chromatography method is capable of separating the borate complexes of a great many different sugars. Not only sugars but any compound capable of forming a borate complex may be separated. Sugar alcohols (**12**) and glycosides (**1**) as well as phosphorylated carbohydrates (**3**) have been separated and additional work is under way on other compounds. It is not always possible to predict the conditions necessary to effect a complete separation of the sugars present in a sample of biological origin, but preliminary tests by paper chromatography are usually sufficient to indicate the necessary steps.

The resin beds used in the present experiments were capable of handling 50 to 60 mg. of sugar without having the peaks run together. Larger columns can be used to handle larger amounts of sugar. The sugars can be obtained carrier-free by this method. Since sugars of high specific activity are not generally needed for biological experiments, carrier sugar can be added following removal of the potassium ions and before the removal of the boric acid.

Summary

Carbon-14-labeled sucrose, raffinose, fructose, and glucose were separated and isolated from plant extracts by ion-exchange chromatography of their borate complexes. The sugars were prepared carrier-free and free of chemical and radiochemical impurities as indicated by paper chromatography and autoradiography.

LITERATURE CITED

1. Chambers, M. A., Zill, L. P., and Noggle, G. R. The use of ion-exchange resins with glycosides. Jour. Amer. Pharm. Assoc., Sci. Ed. **41**: 461–464. 1952.
2. Gibbs, M., Dumrose, R., and Acher, F. Biochemical Preparations. Vol. 2. John Wiley & Sons, Inc., New York. 1952.
3. Khym, J. X. and Cohn, W. E. The separation of sugar phosphates by ion exchange with the use of the borate complex. Jour. Amer. Chem. Soc. **75**: 1153–1156. 1953.
4. Khym, J. X. and Zill, L. P. The separation of sugars by ion-exchange. Jour. Amer. Chem. Soc. **74**: 2090–2094. 1952.

5. NOGGLE, G. R. and BOLOMEY, R. A. The biosynthesis of carbon-14-labeled compounds. I. The chromatographic separation of glucose and fructose. Plant Physiol. **26**: 174–181. 1951.
6. NOGGLE, G. R. and SCHUMACHER, M. E. The biosyntheses of carbon-14-labeled compounds. II. The chromatographic separation of the monosaccharides, disaccharides, and trisaccharides from plant extracts. Plant Physiol. **27**: 422–426. 1952.
7. NOGGLE, G. R. and ZILL, L. P. The quantitative analysis of sugars in plants by ion-exchange chromatography. Arch. Biochem. Biophys. **41**: 21–28. 1952.
8. PUTMAN, E. W. and HASSID, W. Z. Isolation and purification of radioactive sugars by means of paper chromatography. Jour. Biol. Chem. **196**: 749–752. 1952.
9. TOMPKINS, E. R. Laboratory application of ion-exchange techniques. Jour. Chem. Educ. **26**: 32, 92. 1949.
10. TOMPKINS, E. R., KHYM, J. X., and COHN, W. E. Ion-exchange as a separations method. I. The separation of fission-produced radioisotopes, including individual rare earths, by complexing elution from Amberlite resin. Jour. Amer. Chem. Soc. **69**: 2769–2777. 1947.
11. UDENFRIEND, S. and GIBBS, M. Preparation of C^{14} uniformly labeled fructose by means of photosynthesis and paper chromatography. Science **110**: 708. 1949.
12. ZILL, L. P., KHYM, J. X., and CHENIAE, G. M. Further studies on the separation of the borate complexes of sugars and related compounds by ion-exchange chromatography. Jour. Amer. Chem. Soc. **75**: 1339–1342. 1953.