PLANT PHYSIOLOGY

Vol. 40 No. 4

July 1965

Biosynthesis of Sucrose Phosphate with Sugar Cane Leaf Chloroplasts[1, 2]

S. Haq and W. Z. Hassid

Department of Biochemistry, University of California, Berkeley, California

Introduction

Leloir and his collaborators (7, 11, 12) and others (1, 3, 6, 9, 13, 15, 16) have shown that synthesis of sucrose in extracts of a number of plants can occur by 2 different enzymic reactions: A) UDP[3]-D-glucose + D-fructose ⇌ sucrose + UDP; B) UDP-D-glucose + D-fructose 6-P ⇌ sucrose-P + UDP.

In reaction (A) D-fructose serves as the glucosyl acceptor and the reaction is freely reversible. In reaction (B) in which D-fructose 6-P is the acceptor, the sucrose-P formed is hydrolyzed by a phosphatase in a reaction which is practically irreversible, producing free sucrose. It is of interest to note that small amounts of sucrose-P have been detected among labeled photosynthetic products in plants (2, 5).

In a previous communication Frydman and Hassid (8) showed that extracts of sugarcane leaves contained an enzyme which produced sucrose from UDP-D-glucose and D-fructose, but neither sucrose nor sucrose-P was formed when D-fructose was replaced by D-fructose 6-P in the reaction mixture. This indicated that only the enzyme which catalyzed reaction (A) was present in the extract. However, when chloroplasts isolated from the sugarcane leaves were used as the enzyme source, sucrose was formed from UDP-D-glucose and D-fructose 6-P as well as from UDP-D-glucose and D-fructose. Since sucrose-P was not detected in the incubation mixture containing UDP-D-glucose and D-fructose 6-P, it could not be decided whether the sucrose was formed by enzymic removal of P from sucrose-P or by glucosylation of D-fructose produced by the action of a phosphatase on D-fructose 6-P. Bird and Stocking (4) have demonstrated the synthesis of sucrose from UDP-D-glucose and D-fructose or D-fructose 6-P by chloroplasts isolated in a nonaqueous medium; but they were also unable to detect the formation of sucrose-P.

Evidence is presented in this paper that the chloroplasts of sugarcane leaves and spinach leaves contain, in addition to the enzyme that forms sucrose from UDP-D-glucose and D-fructose, the enzyme that catalyzes the formation of sucrose-P from UDP-D-glucose and D-fructose 6-P.

Materials and Methods

Sugarcane leaves were obtained from the Southwestern Irrigation Field Station, United States Department of Agriculture, Brawley, California. Spinach leaves were purchased locally.

UDP-glucose-U-C^{14} and sucrose-P uniformly labeled with C^{14} in the D-fructosyl moiety were prepared enzymatically by Mr. Tsau-Yen Lin of this laboratory. C^{14}-labeled D-fructose 6-P was prepared by the reaction of D-fructose-U-C^{14} with yeast hexokinase and ATP in the presence of Mg^{++}.

Invertase free of melibiose was obtained from the Nutritional Biochemical Corporation. The source of phosphatase was human seminal sperm which was centrifuged and the fluid dialyzed against water or 0.05 M acetate buffer solution.

The enzymatic reactions involving labeled compounds were carried out in capillary tubes, 1.2 to 1.5 mm diameter according to Porter and Hoban (10), with the modification that the incubation mixture was mixed on parafilm by gentle blowing and sucking with the capillary tubes having fine tapered tips, before finally sucking up the mixture into the tube and sealing both ends. The mixtures were then incubated at 37°.

Paper electrophoresis was carried out on oxalic acid-washed paper (Whatman No. 1) in 0.2 M ammonium formate buffer, pH 3.6 at 30 v/cm, and the material was chromatographed on Whatman No. 1 paper with the organic phase of ethyl acetate: acetic acid: water (3:1:3, v/v).

Isolation of Chloroplasts. Unless otherwise stated,

[1] Received November 27, 1964.

[2] This investigation was supported in part by a research grant A-1418 from the National Institutes of Health, United States Public Health Service and by a research grant G-23763 from the National Science Foundation. Support of this work by the Agricultural Experiment Station is also acknowledged.

[3] UDP = Uridine diphosphate.

all operations were performed between 0° and −5°. Chloroplasts were isolated essentially by the method of Whatley, Allen and Arnon (17). Approximately 50 g of immature sugarcane leaves were cut into small pieces and homogenized 3 times in a mortar with sea sand and 100 ml portions of 0.35 M sodium chloride solution containing 0.02 M Tris, pH 8.3, and 0.01 M sodium ascorbate. The homogenates were combined and passed through a double layer of cheesecloth. The solution of approximately 250 ml was centrifuged at 40 × *g* for 5 minutes to remove the sand and suspended cell-wall materials. The supernatant solution was centrifuged at 1500 × *g* for 10 minutes. The residue was washed twice with the sodium chloride solution and centrifuged. The chloroplasts were then suspended in 5 ml of cold, distilled water.

Extraction of Chloroplasts with Acetone. Chloroplasts from 50 g lots of sugarcane leaves were isolated by the method previously described, but instead of suspending them in water they were extracted with acetone to remove chlorophyll and fatty materials. The chloroplasts were stirred for 5 minutes in 10 ml of cold (0°) acetone in a centrifuge tube, and then centrifuged at 12,000 × *g* for 10 minutes. This operation was repeated (3 or 4 times) until the acetone extract was colorless. The acetone extract which was devoid of enzymatic activity was discarded. The extracted chloroplasts were suspended in 5 ml of cold (5°) distilled water prior to further use.

Assays. The reaction mixture for the synthesis of sucrose and sucrose-P contained 0.5 μmole of D-fructose or D-fructose 6-P, 0.5 μmole of NaF, 2 μmoles of Tris-HCl, pH 7.5, 0.3 μmole of $MgCl_2$, 4.3×10^{-3} μmole (0.16 μc; 1 μc = 60,000 cpm) UDP-glucose-U-C^{14} and 50 μl of enzyme suspension (either chloroplasts or acetone extracted chloroplasts) in a total volume of 65 μl. The reaction mixture was incubated for 60 minutes at 37° and then subjected to paper electrophoresis. The radioactive materials were located on the electrophoretogram by autoradiography.

The nonmobile radioactive compounds were eluted from the paper with water and the eluates were passed through a mixed Amberlite MB-3 resin (H^+, OH^- form) column to eliminate charged impurities. The eluates were chromatographed along with radioactive samples of sucrose (R_g, 0.47), D-glucose (R_g, 1.0) and D-fructose (R_g, 1.3) which served as reference compounds. Radioactive sugars were again located on the chromatogram by exposure of the paper to x-ray film, and the radioactivity in individual compounds was estimated with a Geiger-Müller tube and ratemeter.

Sucrose-P and glucose 1-P (degradation product of uridine diP D-glucose) were eluted from the electrophoretogram and again subjected to electrophoresis, thereby effecting a clear separation of the 2 sugar phosphates.

Sucrose-P was eluted from the paper and dephosphorylated by incubation at 37° with 30 μl of crude seminal phosphatase for 60 minutes in the presence of 2.5×10^{-3} M Mg^{++}. After passing the incubation mixture through a mixed resin, the eluate was chromatographed as before, using radioactive sucrose as a reference compound. The radioactive sucrose was located by radioautography and its radioactivity was determined as before. The synthetic sugar was eluted from the paper and hydrolyzed with invertase in 0.1 M Tris-maleate buffer, pH 4.5 at 37° for 60 minutes. After passing the solution through mixed resin, the eluate was chromatographed and the C^{14} labeled sugars were detected by exposing the paper to x-ray film.

Hydrolysis of Sucrose-P and D-Fructose 6-P by Chloroplast Preparations. The phosphatase activity of the different chloroplast preparations containing sucrose synthetase was determined by observing the extent to which radioactive sucrose-P and D-fructose 6-P were hydrolyzed within a certain period of time.

The incubation mixture contained 2.2×10^{-4} μmole (8.3×10^{-3} μc) sucrose-P labeled uniformly with C^{14} in the D-fructosyl moiety or 3.5×10^{-3} μmole (0.13 μc) D-fructose-U-C^{14} 6-P, 2 μmoles of Tris-HCl, pH 7.5, 0.3 μmole of $MgCl_2$, and 40 μl of enzyme suspension in a total volume of 49 μl. The mixture was incubated for 60 minutes at 37° and subjected to paper electrophoresis. The nonmobile sugars were eluted from the paper and passed through Amberlite MB-3 mixed resin and then chromatographed, using radioactive sucrose and D-fructose as reference compounds. The eluates were chromatographed and the radioactive sugars were located by radioautography as before.

Results

The data presented in table I show that in the incubation mixture containing UDP-D-glucose C^{14} and D-fructose and chloroplasts about 17 % of the radioactivity originally present in the labeled UDP-D-glucose appeared in sucrose. The sucrose was identified by comparing its migration with that of authentic sucrose on paper chromatograms and by its complete hydrolysis to radioactive glucose with yeast invertase. A small amount (1 %) of radioactive sucrose was also produced when D-fructose 6-P was substituted for D-fructose in the reaction mixture. Formation of sucrose-P could not be detected.

The isolated chloroplasts after extraction with acetone were found to synthesize 14 % labeled sucrose from UDP-D-glucose-U-C^{14} and D-fructose. Substitution of D-fructose 6-P for D-fructose in the incubation mixture produced 1 % labeled sucrose-P and 1 % labeled sucrose. After hydrolysis of the sucrose-P with seminal phosphatase, the product had the same R_g value as sucrose (R_g, 0.47), and its complete hydrolysis with yeast invertase yielded radioactive glucose.

Table I. *Sucrose and Sucrose-P Synthesis from UDP-D-Glucose-U-C^{14} and D-Fructose or D-Fructose 6-P by Sugarcane Chloroplasts*

For quantities of substrates and conditions of incubation see the text under Assays.

Source of enzyme	Acceptor	Percentage of total radioactive counts recovered as:			
		Sucrose		Sucrose-P	
		cpm	%	cpm	%
Chloroplasts	D-Fructose	1743	17	...	None
Chloroplasts	D-Fructose 6-P	107	1	...	None
Acetone extracted chloroplasts	D-Fructose	1409	14	...	None
Acetone extracted chloroplasts	D-Fructose 6-P	103	1	97	1

When the whole leaves (50 g) were homogenized with acetone, a precipitate was obtained after centrifuging the homogenate at 10,000 × *g* for 10 minutes. This precipitate contained an enzyme which produced 1 % sucrose-P when incubated with UDP-D-glucose-U-C^{14} and D-fructose 6-P. The precipitates from spinach leaves also produced sucrose-P under these conditions.

Phosphatase activity of the chloroplasts and that of the acetone extracted chloroplasts was determined using C^{14} labeled sucrose-P and D-fructose-C^{14} 6-P as substrates. As shown in table II, the extent of hydrolysis of sucrose-P in the presence of the acetone extracted chloroplasts was considerably greater than that of D-fructose 6-P. An attempt to obtain soluble preparations of phosphatase or sucrose-P synthetase by extracting the chloroplasts of sugarcane leaves with various buffer solutions, sodium lauryl sulphate solution, and by sonic rupture proved unsuccessful.

Discussion

The results of previous experiments showed that sugarcane (8) and tobacco leaf chloroplasts (4) contain an enzyme capable of transferring D-glucose from UDP-D-glucose-C^{14} to D-fructose 6-P, forming sucrose but not sucrose-P. This indicates that a sucrose-P might have been formed, but was subsequently hydrolyzed by a phosphatase to free sucrose. However, the possibility remained that the formation of sucrose did not result from a direct hydrolysis of sucrose-P by phosphatase, but was produced by a D-glucose transfer from the glucosyl nucleotide to D-fructose which was derived from the hydrolysis of the D-fructose 6-P by a phosphatase present in the chloroplasts.

Table II. *Hydrolysis of C^{14}-labeled Sucrose-P and D-Fructose-U-C^{14} 6-P with Sugarcane Leaf Chloroplasts as a Phosphatase Source*

Incubation period 60 minutes at 37°.

Plant constituent	Percentage of radioactive sugars obtained after hydrolysis:			
	Sucrose		Fructose	
	cpm	%	cpm	%
Chloroplasts	133	26	239	3
Acetone extracted chloroplasts	372	74	245	3

Attempts to show that sucrose-P is formed by the ordinary chloroplast preparations proved to be unsuccessful. However, by using chloroplasts which had been extracted with acetone as a source of enzyme, sucrose-P was produced. Although the amounts of this phosphorylated disaccharide were small (approximately 1 % of the radioactivity in the substrate) its presence nevertheless indicates synthesis of sucrose-P in the chloroplasts. The amount of sucrose-P as indicated in table I is reported on the basis of the radioactive sucrose formed from the hydrolysis of the sucrose-P with phosphatase. However, the quantity of the sucrose-P synthesized was probably considerably greater than 1 %, because some loss of this disaccharide undoubtedly occurred during electrophoresis, hydrolysis with phosphatase and adsorption by passing through the Amberlite column.

The results in table II show that sucrose-P was hydrolyzed to a greater extent than D-fructose 6-P. Since no sufficient amount of nonradioactive sucrose-P was available to determine the rate of hydrolysis reaction to compare with that of D-fructose 6-P, it cannot be concluded from these data that a specific phosphatase is present in the chloroplasts which preferentially hydrolyzes sucrose-P. However, since Mendicino (13) showed that a purified sucrose synthesase from wheat contains an enzyme that hydrolyzes sucrose-P at a greater rate than D-fructose 6-P, the possibility of the presence of such a specific phosphatase in sugarcane leaves cannot be excluded. This problem requires further investigation.

The results showing that the acetone-extracted chloroplasts from which it was possible to isolate sucrose-P dephosphorylates the product to a greater extent than with nonextracted chloroplasts containing active phosphatase cannot be explained.

Summary

The reactions leading to the formation of sucrose and sucrose-P were investigated with chloroplasts

from sugarcane leaves as a source of enzyme.

Chloroplasts isolated from these leaves synthesized sucrose from UDP-D-glucose and D-fructose; sucrose was also formed when D-fructose 6-P was substituted for D-fructose as the glucose acceptor; but sucrose 6-P was not formed.

When acetone extracted chloroplasts were used, sucrose-P and sucrose were formed from UDP-D-glucose and D-fructose 6-P; only sucrose was synthesized when D-fructose was used as an acceptor.

Sugarcane chloroplasts contain phosphatase capable of hydrolyzing D-fructose 6-P as well as sucrose-P.

Acknowledgments

The authors wish to thank Mr. Kenyon D. Beatty, research agronomist of the Southwestern Irrigation Field Station, United States Department of Agriculture, Brawley, California, and Dr. I. E. Stokes, leader of sugarcane and sweet sorgum investigations, United States Department of Agriculture, Beltsville, Maryland, for supplying the sugarcane leaves.

Literature Cited

1. Bean, R. C. 1960,. Carbohydrate metabolism of citrus fruits. I. Mechanism of sucrose synthesis in oranges and lemons. Plant Physiol. 35:429–36.
2. Bean, R. C., B. K. Barr, H. V. Welch, and G. G. Porter. 1962. Carbohydrate metabolism of the avocado. Relations between sugars in leaves during photosynthesis and subsequent dark periods. Arch. Biochem. Biophys. 96: 524–29.
3. Bean, R. C. and W. Z. Hassid. 1955. Synthesis of disaccharides with pea preparations. J. Am. Chem. Soc. 77: 5737–38.
4. Bird, I. F. and C. R. Stocking. 1964. Sucrose synthesis in chloroplast. Abstracts First Meeting of Federation of European Biochemical Societies. 56–57.
5. Buchanan, J. C. 1953. The path of carbon in photosynthesis. XIX. The identification of sucrose phosphate in sugar beet leaves. Arch. Biochem. Biophys. 44: 140–49.
6. Burma, D. P. and D. C. Mortimer. 1956. The biosynthesis of uridine diphosphate glucose and sucrose in sugar beet leaves. Arch. Biochem. Biophys. 62: 16–28.
7. Cardini, C. E., L. F. Leloir, and J. Chiriboga. 1955. The biosynthesis of sucrose. J. Biol. Chem. 214: 149–55.
8. Frydman, R. B. and W. Z. Hassid. 1963. Biosynthesis of sucrose with sugarcane leaf preparations. Nature 199: 382–83.
9. Hatch, M. D., J. A. Sacher, and K. T. Glasziou. 1963. Sugar accumulation cycle in sugarcane. I. Studies on enzymes of the cycle. Plant Physiol. 38: 338–43.
10. Porter, W. L. and N. Hoban. 1954. Ultramicrotechnique for enzymatic hydrolysis of sugars prior to chromatogram analysis. Anal. Chem. 26: 1846–47.
11. Leloir, L. F. and C. E. Cardini. 1953. The biosynthesis of sucrose. J. Am. Chem. Soc. 75: 6084.
12. Leloir, L. F. and C. E. Cardini. 1955. The biosynthesis of sucrose phosphate. J. Biol. Chem. 214: 157–65.
13. Mendicino, J. 1960. Sucrose phosphate synthesis in wheat germ and green leaves. J. Biol. Chem. 235: 3347–52.
14. Rorem, E. S., H. G. Walker, Jr., and R. M. McCready. 1960. Biosynthesis of sucrose and sucrose-phosphate by sugar beet extracts. Plant Physiol. 35: 269–72.
15. Schwimmer, S. and E. S. Rorem. 1960. Biosynthesis of sucrose by preparations from potatoes stored in the cold and at room temperature. Nature 187: 1113–16.
16. Turner, D. H. and J. F. Turner. 1958. Uridine diphosphate pyrophosphorylase of pea seeds. Biochem. J. 69: 448–52.
17. Whatley, F. R., M. B. Allen, and D. I. Arnon. 1959. Photosynthesis by isolated chloroplasts. Biochem. Biophys. Acta 32: 32–46.