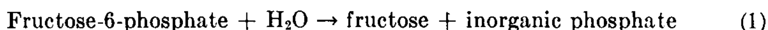
THE BIOSYNTHESIS OF SUCROSE PHOSPHATE*

BY LUIS F. LELOIR AND C. E. CARDINI

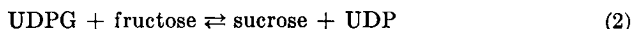
(From the Instituto de Investigaciones Bioquímicas, Fundación Campomar,† Buenos Aires, Argentina)

(Received for publication, October 18, 1954)

The enzymic formation of sucrose from UDPG[1] and fructose has been described in the preceding paper (1). While some preparations of the enzyme were found to be almost devoid of action of fructose-6-phosphate, other extracts catalyzed the formation of free sucrose either from fructose or from its phosphate. Since these extracts contain phosphatase, it seemed likely that the action on fructose phosphate was the sum of Reaction 1 plus Reaction 2.

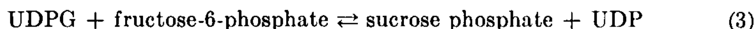


Fructose-6-phosphate + H_2O → fructose + inorganic phosphate (1)



UDPG + fructose ⇄ sucrose + UDP (2)

However, further work has shown that it is possible to obtain enzyme preparations which catalyze Reaction 3.



UDPG + fructose-6-phosphate ⇄ sucrose phosphate + UDP (3)

Phosphatase acts on sucrose phosphate according to Reaction 4.

Sucrose phosphate → sucrose + phosphate (4)

Crude extracts of wheat germ catalyze all four reactions, and it has not been possible to obtain a complete separation of the enzymes. Nevertheless, with some of the preparations the rate of Reaction 3 was 2 or 3 times faster than that of Reaction 2, and sucrose phosphate has been obtained in amounts which have allowed the determination of its structure with reasonable certainty.

Methods

Preparation of Enzyme—The method of separation of the enzymes acting on fructose and on fructose phosphate was based on the observation that the fructose phosphate enzyme has a greater tendency to become insoluble

* This investigation was supported in part by a research grant (No. G-3442) from the National Institutes of Health, United States Public Health Service, and by the Rockefeller Foundation.

† J. Alvarez 1719.

[1] The following abbreviations are used: UDP for uridine diphosphate, UDPG for uridine diphosphate glucose, Tris for tris(hydroxymethyl)aminomethane, and TPN for triphosphopyridine nucleotide.

This is an Open Access article under the CC BY license.

when dialyzed against water. The procedure described in the preceding paper was used, but with some changes. The mixing with a blender was omitted, thus making centrifugation at high speed unnecessary, as well as the first dialysis. The wheat germ, 100 gm., was suspended in 300 ml. of 0.05 M phosphate buffer of pH 7.1 and left standing at 5° during 1 hour. The paste was centrifuged 15 minutes at 3000 r.p.m. Without dialyzing, the supernatant fluid was treated with ammonium sulfate, manganous chloride, and ammonium sulfate again, as previously described. The precipitate obtained in the second ammonium sulfate treatment (Fraction IV) was dissolved in the smallest possible amount of water and dialyzed overnight at 5° against several changes of distilled water. The precipitate was

TABLE I

Purification of Enzyme

The activity is expressed in micromoles of total sucrose (free plus esterified) formed in 30 minutes per mg. of protein under the conditions described in the text.

Preparation	Protein	Activity on		$\frac{A}{B}$
		Fructose (*A*)	Fructose-6-phosphate (*B*)	
	mg. per ml.			
Fraction IV	23	1.16	0.20	5.8
1. 0.05 M $(NH_4)_2SO_4$ extract	40	0.70	0.65*	1.06
2. 0.1 " " "	22	0.50	0.90†	0.55
3. 0.1 " " "	10	0.40	1.20	0.33
4. 0.1 " " "	4.4	0.90	1.10	0.82

* and † 65 and 82 per cent, respectively, of the total sucrose formed was esterified.

separated by centrifugation and washed three to four times by suspending in 1 ml. of distilled water and centrifuging. The precipitate was then extracted successively with 2 ml. of 0.05 M and 0.1 M ammonium sulfate. The pooled extracts were dialyzed overnight. The precipitate was washed and extracted successively with 2 ml. of ammonium sulfate at the concentrations shown in Table I (Preparations 1 to 4). In some cases, the precipitation by dialysis and extraction had to be repeated in order to obtain a preparation with a low content in phosphatase and in UDPG fructose transglycosylase.

Analytical—Sucrose phosphate was estimated as described by Cardini *et al.* (1) with a sucrose standard. In order to distinguish sucrose from its phosphate, the samples were analyzed with and without precipitation with the zinc sulfate-barium hydroxide reagent (2). The Fiske and Subbarow (3) method was used for the estimation of phosphate.

Fructose-6-phosphate was estimated by using a preparation of glucose-phosphate dehydrogenase (4), which also contained phosphohexose isomerase.

Substrates—Fructose-6-phosphate was a commercial sample. A synthetic sucrose phosphate was prepared by direct phosphorylation of sucrose as described by Neuberg and Pollak (5).

Fructose-1-phosphate was synthesized according to Raymond and Levene (6).

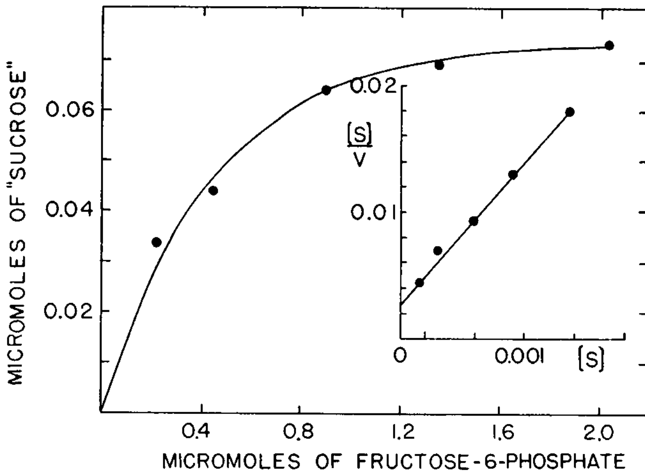


FIG. 1. Influence of fructose-6-phosphate concentration. System as described in the text. The amount of purified enzyme corresponds to 0.05 mg. of protein. Fructose-6-phosphate concentration as indicated. Incubated 30 minutes at 37°. Section at right, a Lineweaver-Burk (18) plot. $K_m = 2.2 \times 10^{-3}$.

Estimation of Enzyme—The procedure was as described in the previous paper, but the pH of the buffer was 6.4 and fructose-6-phosphate was used instead of fructose.

Results

Properties of Enzyme—A comparison of "sucrose" formation from free fructose and fructose-6-phosphate with different preparations of the enzyme has shown that the ratio of activities can vary from 5.8 to 0.33 (Table I), and this is considered proof that two different enzymes are involved. Even the best preparations still contained some phosphatase, and hence the reaction product was usually a mixture of sucrose phosphate and free sucrose. This contamination decreased considerably the yield of sucrose phosphate, thus rendering the isolation more difficult. The substitution of fructose-1-phosphate for fructose-6-phosphate as a substrate for the en-

zyme led to a great decrease in the yield of "sucrose," most of which was free. Nevertheless, there was a small amount which appeared to be esterified, but this point could not be clarified because the sample of fructose-1-phosphate contained a small amount of the 6 ester and because of the presence of phosphatase. This point will have to be investigated further.

The effect of changing the concentration of fructose-6-phosphate is seen in Fig. 1. Calculation of the apparent Michaelis constant gave a value of 2.2×10^{-3}. Fig. 2 shows the influence of changing the pH. The optimum was at pH 6.4.

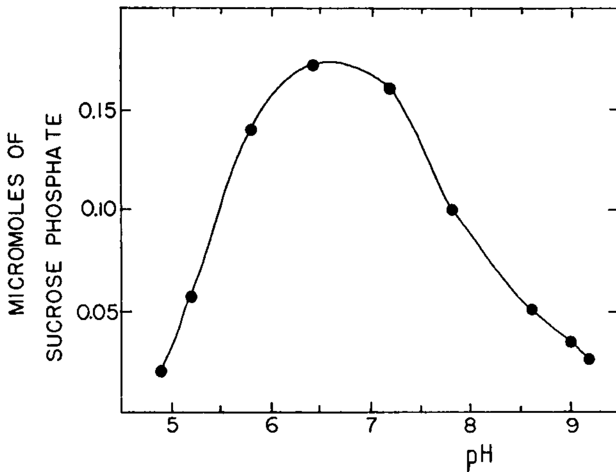


Fig. 2. pH optimum curve. System as described in the text with Tris or acetate buffer at 0.15 M final concentration. Incubated 60 minutes at 37°. The pH was determined on aliquots with a glass electrode.

Isolation of Reaction Product—After many preliminary trials with paper chromatography and electrophoresis, the separation of the reaction product was carried out by anion exchange chromatography. The method of Khym and Cohn (7) with slight modifications was used. The effluents were analyzed by the resorcinol method (8), with and without previous alkaline treatment, and by ultraviolet absorption at 260 mμ. The procedure was as follows: The reaction mixture was prepared by mixing 160 μmoles of UDPG, 500 μmoles of fructose-6-phosphate, and 8 ml. of enzyme (total volume, 40 ml.) and incubating 1 hour at 37°. The proteins were coagulated by heating and filtered. The solution was passed through Dowex 50 and neutralized with ammonia. It contained 40 μmoles of "sucrose phosphate" in 155 ml. This was percolated into a column of Dowex 1 in the chloride form (4.15 sq. cm. × 12 cm.), followed by 300 ml. of 0.001 M ammonia. All the organic phosphate was retained in the

column. Gradient elution was carried out with an apparatus similar to that described by Alm, Williams, and Tiselius (9) by adding 0.03 M ammonium chloride to a 500 ml. mixing chamber filled with a solution containing 0.025 M ammonium chloride and 0.01 M sodium borate. Free sucrose and some ultraviolet-absorbing substances emerged first, and, after 450 ml. had passed through the column, "sucrose phosphate" began to emerge and was collected in 315 ml. Fructose-6-phosphate was retained even after several liters of this solvent had been passed through the column. The tubes containing "sucrose phosphate" were pooled, and the solution was passed through Dowex 50, neutralized with ammonia, and evaporated to dryness under vacuum. The borate was then removed by 3-fold addition of methanol and vacuum distillation. The product was dissolved in water, passed through Dowex 50, and neutralized with calcium carbonate. After filtering, the liquid was evaporated to dryness. Extraction with 95 per cent ethanol removed the calcium chloride, leaving the calcium salt of "sucrose phosphate." The latter was dissolved in 1 ml. of water, clarified by centrifugation, and precipitated with 6 volumes of ethanol, washed with ethanol, and dried. About 17 mg. of a white powder were obtained. The "organic phosphate" content was 1.4 μmoles per mg. (theoretical for the anhydrous Ca salt = 2.44). The fructose-phosphate ratio was 0.90.

Properties of Reaction Product—The product obtained was found to have no reducing power or ultraviolet absorption at 260 mμ. The estimation of fructose by Roe's (8) resorcinol method corresponded to 90 per cent of the organic phosphate. It is known that fructose esters give less color than free fructose; thus Lutwak and Sacks (10) obtained values as low as 58 per cent for fructose-6-phosphate and 87 per cent for fructose diphosphate, and there is always some uncertainty in respect to the purity of the samples.

The substance is stable to alkali. Thus, no phosphate was liberated by heating in 0.5 N alkali during 30 minutes, in contrast to fructose phosphates (Table II). The fructose content estimated by the resorcinol method did not change after heating 10 minutes in 0.2 N alkali during 10 minutes. The rate of removal of the phosphate group by 1 N acid at 100° is similar to that of fructose-6-phosphate and clearly different from that of fructose-1-phosphate and of synthetic sucrose phosphate, which is a mixture of isomers (Table II).

The appearance of reducing power during acid hydrolysis was measured (Fig. 3). Under those conditions, sucrose was hydrolyzed about 5 times faster than the "reaction product." The "reaction product" does not reduce TPN when incubated with glucose dehydrogenase plus isomerase (Table III). However, reduction of TPN takes place with the product of hydrolysis.

The results of experiments by paper chromatography (Table IV) may be summarized as follows: Mild acid hydrolysis gives a glucose spot and a

TABLE II

Acid and Alkaline Hydrolysis

The results represent the per cent of organic phosphate liberated. Alkaline hydrolysis was carried out with sodium hydroxide in silver test-tubes.

	1 N sulfuric acid				0.5 N alkali	
	Heating at 100°					
	10 min.	20 min.	30 min.	60 min.	15 min.	30 min.
Fructose-1-phosphate	77	87	100		92	100
Fructose-6-phosphate			13	22	76	78
Reaction product			10	25	0	0
Synthetic sucrose phosphate			42	52	0	0

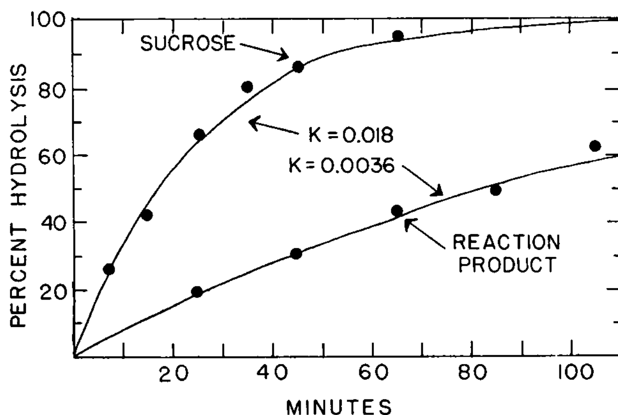


FIG. 3. Acid hydrolysis of reaction product. The curves represent the theoretical values calculated for a first order reaction ($\log_{10}$ and minutes). The points correspond to the results obtained by incubating 0.15 μmole of substance at 37° in 0.9 N hydrochloric acid. After the specified time, the samples were neutralized and the reducing power was measured by the ferricyanide method (19). Samples which were heated 4 minutes at 100° in 0.9 N acid were considered to give the value for 100 per cent hydrolysis.

phosphoric ester spot, but no free fructose. Treatment with kidney phosphatase or with a crude extract of wheat germ in the presence of Mg^{++} results in a substance migrating like sucrose. Hydrolysis of this "sucrose" with acid or with yeast invertase gives glucose and fructose. Other experiments showed that the "reaction product" can be separated from fructose-

6-phosphate by paper chromatography with several solvents. Thus, with methanol-formic acid (11) or with ethanol ammonium acetate of pH 3.8

TABLE III

Reduction of TPN by Reaction Product after Hydrolysis

The substances were mixed with 0.15 μmole of TPN, 0.4 ml. of 1 per cent sodium bicarbonate, 3 mg. of *Zwischenferment*, and water to 2.5 ml. The TPN reduced was calculated from the increase in absorbancy at 340 mμ. Results in micromoles.

Substance	TPN reduced (*A*)	Substance added* (*B*)	$\frac{A}{B}$
Fructose-6-phosphate	0.08	0.07	1.14
Fructose-1-phosphate	0.004	0.13	0.03
Reaction product	0.006	0.14	0.04
" " hydrolyzed†	0.068	0.074	0.92

* Calculated from the organic phosphate content.
† Heated 5 minutes at 100° in 0.1 N acid.

TABLE IV

Paper Chromatography of Reaction Product

Solvent, *n*-butanol-pyridine-water, 6:4:1.5. Benzidine-trichloroacetic acid reagent (20) as developer.

	R_{xylose}		
	Experiment I	Experiment II	Experiment III
Reaction product		0.04	
" " + acid hydrolysis*		0.1, 0.75	
" " + kidney phosphatase	0.56		0.59
" " + crude wheat germ	0.55		
" " + kidney phosphatase + invertase			0.76, 0.84
Sucrose	0.56	0.52	0.59
" + invertase			0.76, 0.85
Fructose	0.82	0.83	0.84
Glucose		0.75	0.75
Fructose-6-phosphate		0.04	

* Heated 5 minutes in 0.1 N acid at 100°.

(12), it migrates at about 75 per cent the rate of fructose-6-phosphate. About the same separation could be obtained by paper electrophoresis with borate buffer (13). These procedures were not used for the isolation of the substance because there was overlapping with ultraviolet-absorbing substances.

The reaction product was found to remain unaffected after treatment with yeast or honey invertase. The latter finding was unexpected, since honey invertase (14) is believed to be specific for the glucose moiety of the substrate.

DISCUSSION

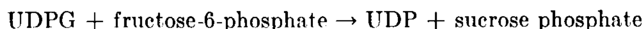
It can be concluded that the reaction product is sucrose with a phosphate group at position 6 of the fructose moiety, because dephosphorylation with phosphatase produces a substance behaving like sucrose, and mild acid hydrolysis gives free glucose and a fructose ester which behaves like fructose-6-phosphate when treated with acid or with isomerase plus glucose dehydrogenase. However, it should be pointed out that the samples obtained were only about 60 per cent pure, as judged by the phosphate content of the calcium salt. Further purification was not attempted because only small amounts were available. A point of interest is that sucrose phosphate is more stable to acid than is free sucrose. Thus, the ratio of the hydrolysis constants for the glycosidic links, (K for sucrose)/(K for sucrose phosphate), is 5 in 0.9 N acid at 100°. It was found previously (15) that the ratio of the hydrolysis constants of the phosphate in position 1, (K for glucose-1-phosphate)/(K for glucose-1,6-diphosphate), is 4 in 0.25 N acid at 37°. In both cases, the phosphate group at position 6 stabilizes the substituents at the other end of the molecule.

The sucrose phosphate detected by Buchanan (16) was believed to yield fructose-1-phosphate by hydrolysis, and hence it would be different from the product described in this paper.

A study of the distribution of the two enzymes which lead to the synthesis of sucrose would be of interest for plant physiology, but is difficult to carry out, owing to the presence of interfering enzymes (phosphatase and sucrase). Studies on green leaves with labeled substrates (17) have shown that the labels are introduced into both halves of sucrose before appearing in the free monosaccharides. This would prove that free fructose is not involved directly in the synthesis, were it not for the fact that the same experiments demonstrated that most of the sugars in leaves are stored in a metabolically inert compartment. Thus the introduction of the label in free fructose at the site of synthesis might have been faster than it appeared to be in these experiments.

SUMMARY

A wheat germ enzyme which catalyzes the following reaction has been studied:



$$\text{UDPG} + \text{fructose-6-phosphate} \rightarrow \text{UDP} + \text{sucrose phosphate}$$

It is concluded that the reaction product is sucrose with a phosphate group at position 6 of the fructose moiety, because dephosphorylation with phosphatase yields a substance behaving like sucrose and mild acid hydrolysis gives free glucose and a fructose ester which behaves like fructose-6-phosphate when treated with acid or with isomerase, glucose dehydrogenase, and TPN.

The glycosidic linkage of sucrose phosphate was found to be more stable to acid than was that of sucrose.

BIBLIOGRAPHY

1. Cardini, C. E., Leloir, L. F., and Chiriboga, J., *J. Biol. Chem.*, **214**, 149 (1955).
2. Somogyi, M., *J. Biol. Chem.*, **160**, 61 (1945).
3. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
4. LePage, G. A., and Mueller, G. C., *J. Biol. Chem.*, **180**, 975 (1949).
5. Neuberg, C., and Pollak, H., *Biochem. Z.*, **23**, 515 (1910).
6. Raymond, A. L., and Levene, P. A., *J. Biol. Chem.*, **83**, 619 (1929).
7. Khym, J. X., and Cohn, W. E., *J. Am. Chem. Soc.*, **75**, 1153 (1953).
8. Roe, J. H., *J. Biol. Chem.*, **107**, 15 (1934).
9. Alm, R. S., Williams, R. J. P., and Tiselius, A., *Acta chem. Scand.*, **6**, 826 (1952).
10. Lutwak, L., and Sacks, J., *Arch. Biochem. and Biophys.*, **39**, 240 (1952).
11. Bandurski, R. S., and Axelrod, B., *J. Biol. Chem.*, **193**, 405 (1951).
12. Paladini, A. C., and Leloir, L. F., *Biochem. J.*, **51**, 426 (1952).
13. Consden, R., and Stanier, W. M., *Nature*, **169**, 783 (1952).
14. White, J. W., and Maher, J., *Arch. Biochem. and Biophys.*, **42**, 360 (1953).
15. Cardini, C. E., Paladini, A. C., Caputto, R., Leloir, L. F., and Trucco, R. E., *Arch. Biochem.*, **22**, 87 (1949).
16. Buchanan, J. G., *Arch. Biochem. and Biophys.*, **44**, 140 (1953).
17. Putman, E. W., and Hassid, W. Z., *J. Biol. Chem.*, **207**, 885 (1954).
18. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).
19. Schales, O., and Schales, S. S., *Arch. Biochem.*, **8**, 285 (1945).
20. Bacon, J. S. D., and Edelman, J., *Biochem. J.*, **48**, 114 (1951).