The addition of alkali to the oxidized conjugated acids produced a marked increase in absorption in the longer wave lengths with the development of a maximum near 3750 Å. This maximum was not apparent in alkaline oxidized ethyl linolenate, linolenic acid, or elaidolinolenic acid. However, it was observed in the spectra of alkaline oxidized linoleates.[4] The cause of this absorption or its significance is not known.

Summary

1. The changes in ultraviolet absorption spectra were followed during the oxidation of linolenic acid, ethyl linolenate, elaidolinolenic acid, pseudoeleostearic acid, α-eleostearic acid, and β-licanic acid.

2. Oxidation of the non-conjugated trienes is accompanied by an increased absorption with the production of maxima at 2350 and 2750 Å. Oxidation of conjugated trienes is accompanied by decreased absorption in the region of 2600–2800 Å. and increased absorption in the region of 2300 and above 3200 Å.

3. Since the absorption spectra of purified fatty acids, conjugated or unconjugated, are not affected by cold alkali, it is concluded that the absorption bands which appear with autoxidation are due to oxygen-containing chromophores.

MINNEAPOLIS, MINNESOTA RECEIVED APRIL 3, 1945

[CONTRIBUTION FROM THE DIVISION OF PLANT NUTRITION, COLLEGE OF AGRICULTURE, AND THE DEPARTMENT OF BACTERIOLOGY, UNIVERSITY OF CALIFORNIA]

Isolation and Structure of an Enzymatically Synthesized Crystalline Disaccharide, D-Glucosido-L-sorboside

BY W. Z. HASSID, M. DOUDOROFF, H. A. BARKER AND W. H. DORE

It has been shown that the bacterium *Pseudomonas saccharophila* contains a phosphorylase which catalyzes the reversible reaction: sucrose + inorganic phosphate ⇄ glucose-1-phosphate + fructose.[1,2] Sucrose prepared from glucose-1-phosphate and fructose was isolated in crystalline form and shown to be identical with natural sucrose.[3] In a preliminary report[4] evidence was presented indicating that the bacterial enzyme preparation which synthesizes sucrose from glucose-1-phosphate and fructose, can also combine glucose-1-phosphate with L-sorbose or D-ketoxylose to form the corresponding disaccharides. The present investigation is concerned with the preparation and the chemical constitution of the crystalline disaccharide formed from α-D-glucose-1-phosphate and L-sorbose under the influence of the partially purified phosphorylase from *Pseudomonas saccharophila*.

The procedure used for the isolation and crystallization of the disaccharide is a modification of that employed for the isolation and crystallization of synthetic sucrose. The empirical formula of the disaccharide obtained by elementary analysis is $C_{12}H_{22}O_{11}$. The compound does not reduce Fehling solution or alkaline ferricyanide. It has a sweet taste and gives a positive Seliwanoff reaction. It appears to be very slightly affected by invertase, but it is easily hydrolyzed with acid. The reducing value obtained after acid hydrolysis corresponds to a disaccharide consisting of glucose and sorbose. A mixed glucosazone and sorbosazone was obtained from the products of hydrolysis. After fermenting out the glucose from the hydrolyzate, a pure sorbosazone could be prepared. The melting point of the carbohydrate is 178–180°. The specific rotation is $[\alpha]_D$ + 33°. Hydrolysis with acid changes the rotation to +7.5°; this value agrees well with the expected rotation for an equimolar mixture of D-glucose and L-sorbose. The rate of acid hydrolysis is approximately twice that of sucrose.

The acetylated disaccharide has a rotation in chloroform, $[\alpha]_D$ +38°. The molecular weight of this derivative determined by the Rast method is 578. This is 85% of the theoretical value for a completely acetylated disaccharide consisting of two hexose units. The low value is probably due to partial decomposition of the compound during the determination which involves heating to about 180°.

The fact that the disaccharide is non-reducing shows that the glucose and sorbose are linked through the carbonyl groups. Evidence that the L-sorbose exists in the disaccharide as sorbofuranose was obtained by oxidizing the compound with sodium periodate. In a disaccharide consisting of glucopyranose and sorbofuranose glycosidically united through positions 1 and 2 of the ketose and aldose monosaccharides, the glucose residue would possess three adjacent free hydroxyls, on carbon atoms 2, 3 and 4, and the sorbose residue would possess two free hydroxyls, on carbon atoms 2 and 3. On oxidation of such a disaccharide with periodate, the glucose residue should consume two moles of periodate and form one mole of formic acid, while the sorbose residue should consume one mole of periodate. A total

(1) M. Doudoroff, N. Kaplan and W. Z. Hassid, *J. Biol. Chem.*, **148**, 67 (1943).

(2) M. Doudoroff, *ibid.*, **151**, 351 (1943).

(3) W. Z. Hassid, M. Doudoroff and H. A. Barker, THIS JOURNAL, **66**, 1416 (1944).

(4) M. Doudoroff, W. Z. Hassid and H. A. Barker, *Science*, **100**, 155 (1944).

of 3 moles of periodate would thus be consumed and one mole of formic acid would be formed per mole of disaccharide. If the sorbose residue were to exist in the disaccharide in the pyranose form, it would also contain three free hydroxyl groups on carbon atoms 3, 4 and 5-and, as in the case of the glucose, it should consume two moles of periodate and give rise to one mole of formic acid. In this case a total of 4 moles of periodate would be consumed and two moles of formic acid formed per mole of disaccharide. Actually, on oxidation of the carbohydrate with sodium periodate, 2.97 moles of periodate are consumed and 0.96 mole of formic acid are formed. These data agree with the assumption that the disaccharide under discussion contains a furanose and pyranose ring. The possibility that the disaccharide is made up of glucofuranose and sorbopyranose can be eliminated on the basis of the periodate oxidation data. Glucofuranose would contain two pairs of adjacent hydroxyls, on carbon atoms 2 and 3 and 5 and 6, and the sorbopyranose would have three adjacent hydroxyls, on carbon atoms 3, 4 and 5. In oxidizing such a disaccharide, a total of four moles of periodate would thus be used giving rise to one mole of formic acid. This is inconsistent with the experimental data.

Since glucose whether in a free or combined state (oligosaccharides, polysaccharides and hemicelluloses) is always found in nature as glucopyranose, its existence in this form in the glucosido-sorboside is very probable. Further evidence to support this view is derived from the fact that this disaccharide is formed by the same enzyme through an identical phosphorolysis mechanism as synthetic sucrose[3] in which the glucose component is known to exist as glucopyranose. All these lines of evidence strongly indicate that the glucose residue in the new disaccharide has the pyranose structure; L-sorbose, therefore, probably has the furanose configuration. In this connection, it is noteworthy that fructose, which has a pyranose structure when existing in the free state, assumes a furanose configuration whenever it combines with another sugar to form an oligosaccharide or polysaccharide. Apparently, the ketohexose, L-sorbose, shows the same behavior.

In the known "de-phosphorolytic" condensations of α-glucose-1-phosphate, to form starch, glycogen and sucrose, the α-linkage of the phosphoric acid ester is retained in the glycosidic linkage with another monosaccharide unit. It is, therefore, reasonable to assume that the α-linkage is similarly retained in the condensation of α-glucose-1-phosphate and L-sorbose. The resulting disaccharide would then be α-glucosido-L-sorboside.

The new disaccharide gives a blue-green color with diazouracil, a reaction shown by Raybin[5] to be specific for sucrose or any other compound sugar containing the same type of glycosidic glucose–fructose linkages that exist in sucrose, such as raffinose, gentianose, and stachyose.[6]

The fact that the phosphorylase from *Pseudomonas saccharophila*, which can effect the synthesis of sucrose from glucose-1-phosphate and D-fructose, can also effect the synthesis of a disaccharide from glucose-1-phosphate and L-sorbose, strongly suggests *a priori* that the local structure existing where the linkage occurs between the two monosaccharide units in the glucosido-sorboside disaccharide is the same as it is in sucrose. The observation that the glucosido-sorboside disaccharide gives the Raybin color reaction, which has so far been found to be positive only for carbohydrates containing the sucrose linkage, adds force to the suggestion that the new disaccharide has the same linkage that exists in sucrose.

For some years it has been regarded as probable that sucrose is α-D-glucopyranosido-β-D-fructofuranoside[7] and this tentative conclusion has become virtually a certainty as a result of the more recent work of Purves and Hudson.[8] The accepted structure of sucrose is commonly written as shown in Fig. 1 which expresses the linkage as an α-D-glucoside and a β-D-fructoside in conformity with the nomenclature proposed by Hudson.[9]

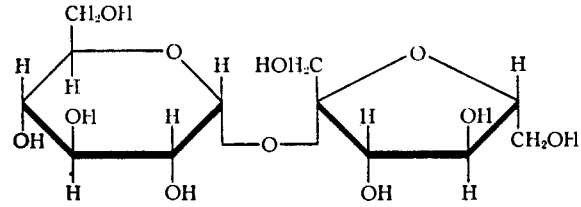

Fig. 1.—Structural formula for sucrose. (α-D-glucopyranosido-β-D-fructofuranoside.)

The probable structure of the new disaccharide is derived by analogy from that of sucrose. It is represented by the formula shown in Fig. 2 in

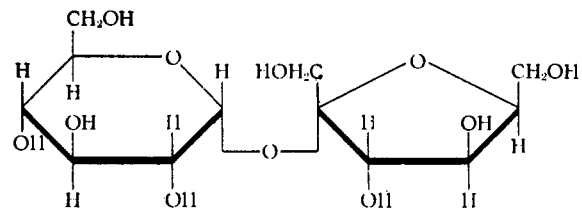

Fig. 2.—α-D-glucopyranosido-α-L-sorbofuranoside.

(5) H. W. Raybin, THIS JOURNAL, **55**, 2603 (1933); **59**, 1402 (1937).

(6) We have tested the diazouracil reagent on a number of carbohydrates not used by Raybin, namely: L-sorbose, trehalose, glucose-1-phosphate, fructose-1-6-diphosphate, fructose-6-phosphate and glucose-6-phosphate. All these compounds give a negative reaction with the reagent. The disaccharide, *iso*-sucrose, which consists of glucose and fructose but has different glycosidic linkages than sucrose, also fails to give this reaction (5). Synthetic sucrose (3), however, gives a positive reaction.

(7) E. F. Armstrong and K. F. Armstrong, "The Carbohydrates," Longmans, Green and Company, London, 1934, pp. 181–182.

(8) C. B. Purves and C. S. Hudson, THIS JOURNAL, **59**, 1170 (1937).

(9) C. S. Hudson, *ibid.*, **60**, 1537 (1938).

which the glycosidal linkages have the same configuration as in sucrose. The sorbose unit in the new disaccharide is a L-sugar in contrast to the D-fructose unit in sucrose. Since β-D-fructose and α-L-sorbose have the same configuration for their second carbon atoms[9] (see Fig. 3) it is necessary to designate the ketose portion of the new disaccharide as α-L-sorboside.

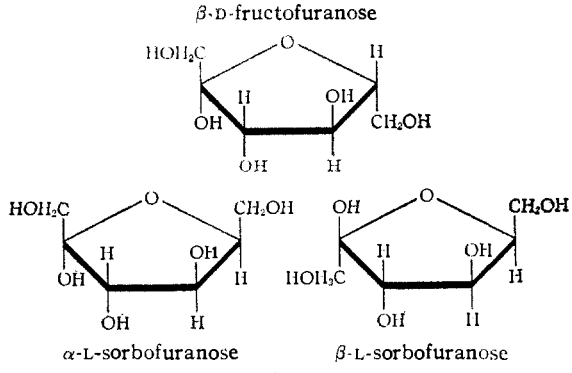

Fig. 3.

In comparing the X-ray diffraction data of the glucosido-sorboside with those of sucrose, considerable similarity is observed between the numerical values of the spacings of the patterns of the two sugars (Table I).

It would not be surprising if these two sugars having molecular constitutions and steric configurations so closely related should find similar packing arrangements and so produce related crystal structures, although few examples of this kind have been noted among the sugars the crystal structures of which are understood. The data now available for the new sugar are too meagre to justify further speculation as to its crystal structure. The films of the two sugars do not resemble one another so much as the tabulation would suggest, since the intensities of corresponding reflections are, in general, quite different. The pattern for the glucosido-sorboside cannot be interpreted to suggest an admixture with sucrose since the three strongest reflections that are used for the identification of sucrose,[10] the 3.59, 4.71 and 7.6 Å. spacings are absent from the list for the new disaccharide.

Experimental

Enzymatic Synthesis of Glucosido-sorboside.—The phosphorylase was prepared and partially purified from 5 g. of dry bacteria (*Pseudomonas saccharophila*) by the method previously described.[2] The preparation was added to a solution containing 15 g. of the potassium salt of glucose-1-phosphate and 11 g. of L-sorbose, adjusted to *p*H 7.4 and barium acetate was then added to make the final concentration 0.1 *M*. The mixture was then diluted to 300 ml. and the *p*H adjusted and maintained at 7.4 during incubation. The reaction mixture was kept at 37° with frequent shaking for fifteen hours.

(10) J. D. Hanawalt, H. W. Rinne and L. K. Frevel, *Ind. Eng. Chem., Anal. Ed.*, **10**, 457 (1938); see also card index file of X-ray spacings issued by American Society of Testing Materials, 260 S. Broad St., Philadelphia, Pa.

Isolation of the Disaccharide.—The mixture was heated at 80° for five minutes, cooled and adjusted to *p*H 7.8. Two and a half volumes of 95% ethanol was added and the solution was allowed to remain for several days at 4°. The precipitate, containing most of the inorganic and esterified phosphate, was removed by filtration and the alcohol distilled off *in vacuo* at 40°. The solution was then made up to 500 ml. and the electrolytes, including the remaining traces of glucose-1-phosphate, removed by passage through ion exchange columns of Amberlite 1R-100 and Amberlite 1R-4.[11] The columns were washed with water and the combined solution and washings were concentrated to 100 ml.

The L-sorbose left in the reaction mixture was removed by conversion into sorbosazone and extraction of the osazone with ethyl acetate as follows: The solution was treated with a mixture of 20 ml. of glacial acetic acid and 20 ml. of phenylhydrazine, heated on a steam-bath for ten minutes and cooled. The sorbosazone which separated out was filtered, washed with a little ice water and the filtrate extracted four times with 200-ml. portions of ethyl acetate. The extracted solution was treated two more times with the same quantities of phenylhydrazine and acetic acid and the phenylosazone extracted with ethyl acetate as before.[12] Analysis showed that the solution contained approximately 0.5 g. of disaccharide, which was still contaminated with a small amount of reducing sugar. The solution was again passed through the Amberlite columns, concentrated to 10 ml., treated with a mixture of 2 ml. of phenylhydrazine and 2 ml. of acetic acid. The mixture was heated for ten minutes on the steam-bath, cooled and extracted four times with three volumes of ethyl acetate. After a similar treatment of the extracted solution with these reagents, the solution was purified by passing through the Amberlite columns and then extracting with ether. The solution was now devoid of reducing sugar. It was then treated with a small amount of vegetable charcoal, allowed to remain at room temperature for fifteen minutes and filtered. The solution was evaporated *in vacuo* at 40° to about 10 ml., diluted with a little alcohol and filtered again. The filtrate was transferred to a small beaker and evaporated to a thick sirup in the vacuum oven at 40°. The sirup was treated with hot absolute alcohol and stirred with a glass rod. A small quantity of petroleum ether was then added and the mixture was stirred again. After standing *in vacuo* for several hours, the sirup was converted into a crystalline mass. The crystals were washed with a few ml. of absolute alcohol and dried *in vacuo* at 60°. The yield was 0.38 g.

Properties of the Disaccharide.—The carbohydrate is very soluble in water, has a sweet taste and does not reduce Fehling or alkaline ferricyanide solution. It gives a positive Seliwanoff reaction. It is very slowly attacked by invertase, but is rapidly hydrolyzed with acid. The carbohydrate gives the blue-green color reaction with diazouracil,[5] a test known to be specific for sucrose or sugar containing the same type of glycosidic glucose-fructose linkages existing in sucrose.

Anal. Calcd. for $C_{12}H_{22}O_{11}$: C, 42.08; H, 6.43. Found: C, 42.12; H, 6.54. Specific rotation: $[\alpha]_D$ + 33° (in water, *c*, 2); melting point, 178–180°.

Hydrolysis of the Disaccharide and Identification of Products.—When the carbohydrate was hydrolyzed with 0.1 *N* hydrochloric acid and analyzed for reducing sugars[13] a theoretical reducing value corresponding to a disaccharide, consisting of glucose and sorbose, was obtained. The hydrolyzed solution gave the theoretical yield of sorbose, determined with Roe's method.[14] The neutral hydrolyzate, when treated with phenylhydrazine hydrochloride and sodium acetate produced mixed crystals of glucosazone

(11) R. M. McCready and W. Z. Hassid, THIS JOURNAL, **66**, 560 (1944).

(12) Since no visible osazone precipitate separated out after the third treatment with these reagents, the solution was extracted with ethyl acetate without previous filtration.

(13) W. Z. Hassid, *Ind. Eng. Chem., Anal. Ed.*, **9**, 228 (1937).

(14) J. H. Roe, *J. Biol. Chem.*, **107**, 15 (1934).

and sorbosazone.[15] No mannose phenylhydrazone could be obtained from this solution. After fermenting out the glucose from the hydrolyzate with *Torula monosa*, an osazone was obtained which was identified as sorbosazone.[16] A 1% solution of the disaccharide in 1 *N* hydrochloric acid hydrolyzed at room temperature gave a final specific rotation of $[\alpha]_D + 7.5°$. The rotation of an equimolar mixture of D-glucose and L-sorbose, under the same conditions, was $[\alpha]_D + 8.0$, corrected for the water of hydrolysis. These rotation values agree well with the assumption that the disaccharide consists of D-glucose and L-sorbose.

Invertase acted very slowly on this disaccharide. Samples of the carbohydrate kept for twenty-four hours with 0.1% of Wallerstein invertase scales at 37° were hydrolyzed to the extent of 5%. A similar sample of sucrose was 99% hydrolyzed under the same conditions in ten minutes. Calculated on this basis, the rate of hydrolysis of the glucosido-sorboside with invertase is at least 13,000 times less than that of sucrose. Since this commercial invertase preparation contains melibiase, and probably other enzymes, it is possible that the slight hydrolysis of the disaccharide is due to some enzyme other than invertase.

Rate of Hydrolysis of the Disaccharide.—The change of rotation of a 2% solution of the disaccharide in 1 *N* hydrochloric acid was observed at 21.5° until a rotation of $[\alpha]_D + 8.0°$ was reached. The course of hydrolysis of this sugar was represented by a logarithmic curve, indicating a first order reaction. The velocity constant *K* of the reaction is 0.01835. The velocity constant *K* of a 2% sucrose solution, determined under the same conditions, is 0.0095, showing that the rate of hydrolysis of the new disaccharide with acid is approximately twice as great as that of sucrose.

Acetylation.—A 0.1-g. sample of the disaccharide was acetylated with 0.46 ml. of acetic anhydride in the presence of 0.7 ml. of pyridine at 0° as previously described.[3] The yield of the acetylated disaccharide was 0.11 g. The substance was insoluble in water, but soluble in alcohol and chloroform.

Anal. Calcd. for $C_{12}H_{14}O_{11}(CH_3CO)_8$: CH_3CO, 50.7. Found: CH_3CO, 49.8. Specific rotation: $[\alpha]_D$ +38° (in chloroform, *c*, 2).

The molecular weight of the acetylated derivative, determined by Smith and Young's modification of Rast's method,[17] was 578, a value which is 85% of the theoretical molecular weight of 679 for a disaccharide. The low value may be attributed to partial decomposition of the sample, which probably occurred when it was heated to approximately 180°.

Oxidation of the Disaccharide with Sodium Periodate.—A 0.0342-g. sample of the disaccharide was dissolved in 5 ml. of water, 1 ml. of 0.5 *M* sodium periodate was then added and diluted to 10 ml. After allowing the mixture to remain at room temperature for forty hours, the solution came to a constant rotation of $[\alpha]_D +20.3°$. The amount of periodate consumed in the reaction[18] was estimated by treating 5-ml. samples with an excess of 0.1 *N* sodium arsenite in the presence of potassium iodide and sodium bicarbonate buffer and back titrating with 0.1 *N* iodine. The formic acid was determined by titrating a 5-ml. sample with 0.1 *N* of sodium hydroxide. The results showed that 2.97 moles of periodate were consumed, giving rise to 0.96 mole of formic acid in the oxidation of one mole of disaccharide. When a similar sample of sucrose was oxidized with the same oxidant, 2.99 moles of periodate were consumed and 0.96 mole of formic acid was liberated per mole of this sugar. These data show that like sucrose, the new disaccharide contains both a pyranose and a furanose monosaccharide.

X-Ray Examination of α-D-Glucosido-L-sorboside.—An X-ray diffraction pattern was obtained from the sugar by the powder method, using molybdenum radiation filtered through a zirconium oxide screen. Eighteen reflections were observed of which the three strongest were the 4.30, 6.9, and 5.8 Å. spacings. All spacings are given in Table I in comparison with the recorded spacings for sucrose.

TABLE I

INTERPLANAR SPACINGS FOR GLUCOSIDO-SORBOSIDE AND SUCROSE

Glucosido-sorboside		Sucrose[a]	
Spacing in ångström units	Intensity[b]	Spacing in ångström units	Intensity
		7.6	s.
6.9	s.	6.9	s.
		6.0	v.v.w.
5.8	m.s.	5.7	m.
5.4	v.w.	5.4	v.w.
4.62	m.	4.71	v.s.
		4.50	m.s.
4.30	v.s.	4.30	m.
4.15			
3.98	v.w.	4.00	s.
3.72	v.w.	3.79	w.
3.53	w.	3.59	v.v.s.
		3.37	v.v.w.
3.33	w.	3.32	v.w.
3.25	v.w.	3.10	v.w.
2.92	w.	2.88	m.s.
2.77	v.v.w.	2.80	v.w.
2.51	v.w., broad	2.49	v.v.w.
2.36	v.w.	2.34	m.
2.25	v.w.	2.24	m.
2.16	w.	2.18	v.w.
2.00	w.	2.06	w.m.
		1.95	v.v.w.
1.85	w.m.	1.90	v.w.
		1.80	v.w.

[a] The spacings for sucrose are those given by Hanawalt, *et al.*,[10] but several weak lines are omitted. The intensity designations have been changed to approximate qualitative estimates. [b] Intensity indicators: v.v.s. = very very strong, v.s. = very strong, s. = strong, m.s. = medium strong, m. = medium, w. = weak, v.w. = very weak, v.v.w. = very very weak, w.m. = weak to medium. Bracketed figures indicate the outer edges of a broad reflection.

Summary

A crystalline non-reducing disaccharide consisting of D-glucose and L-sorbose has been synthesized from glucose-1-phosphate and L-sorbose through the action of a phosphorylase from *Pseudomonas saccharophila*.

Periodate oxidation data show that the glucose residue in the disaccharide possesses the pyranose and the sorbose residue possesses the furanose configuration.

Evidence is presented indicating that both D-glucose and L-sorbose are joined through α-linkages.

The disaccharide is probably α-D-glucopyranosido-α-L-sorbofuranoside.

BERKELEY, CALIFORNIA RECEIVED FEBRUARY 10, 1945

(15) W. Z. Hassid and R. M. McCready, *Ind. Eng. Chem., Anal. Ed.*, **14**, 683 (1942).

(16) C. A. Browne and F. W. Zerban, "Physical and Chemical Methods of Sugar Analysis," John Wiley and Sons, Inc., New York, N. Y., 1941, p. 687.

(17) J. H. C. Smith and W. G. Young, *J. Biol. Chem.*, **75**, 289 (1927).

(18) E. L. Jackson, "Organic Reactions," Vol. II, edited by R. Adams, John Wiley and Sons, Inc., New York, N. Y., 1944, pp. 341–375.