# AN ENZYMIC SYNTHESIS OF A SUCROSE ANALOG: α-D-XYLOPYRANOSYL-β-D-FRUCTOFURANOSIDE*

by

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Sucrose hydrolysis proceeds with $-\Delta F'$ of 6–10 kcal[2]. On the other hand, $-\Delta F'$ of hydrolysis of reducing disaccharides is thought to be 1–3 kcal[3]. On this basis, the observed apparent irreversibility of the growth of a levan chain might have been anticipated[4,5]. Furthermore, it has also been observed that the levansucrase system does not catalyze a disproportionation reaction between levan chains[5]. This result is understandable if indeed transfer of a fructose residue from the terminal end of a levan chain to the levansucrase system is a process costly in energy.

A scheme comprising a reversible primary step and an essentially irreversible subsequent step is proposed[1,5] to account for the above relationships (equations 1 and 2):

$$fr\sim R' + \text{enzyme} \rightleftarrows fr\sim \text{enzyme} + R', \qquad -\Delta F' = ca.\ 0 \text{ kcal} \tag{1}$$

$$fr\sim \text{enzyme} + R''\text{OH} \rightarrow fr < R'' + \text{enzyme}, \qquad -\Delta F' \geq 3 \text{ kcal} \tag{2}$$

where $R'$ is aldose liberated from a donor molecule ($fr\sim R'$), $fr\sim$ is the fructofuranosyl group linked either to the anomeric carbon of the aldosidic residue of the donor or to the enzyme in a postulated intermediary complex ($fr\sim$enzyme), $fr <$ is the fructofuranosyl group linked to a carbinol site in an acceptor ($R''$OH), $\sim$symbolizes the relatively high free-energy level of the lactol-lactol glycosidic bond as in sucrose, and $<$ symbolizes the relatively lower energy level of the ordinary lactol-carbinol type of glycosidic bond.

Since levansucrase forms levan from alternative donors[5] whose common feature is a terminal β-fructofuranosyl group in sucrose configuration, equation 1 suggests that levansucrase could catalyze reversible transfructosylation reactions of the following general form (equation 3):

$$fr\sim R' + R''' \rightleftarrows fr\sim R''' + R' \tag{3}$$

In particular, it has been shown[1] that the following equilibrium is catalyzed by this enzyme system (equation 4):

$$\text{sucrose} + \text{melibiose} \rightleftarrows \text{raffinose} + \text{glucose} \tag{4}$$

Glucose competitively inhibits levan production from sucrose[4]. Equation 1 accounts nicely for this inhibition, since it is evident that in an equilibrium:

$$K = [fr\sim \text{enzyme}]\,[R']/[fr\sim R']\,[\text{enzyme}]$$

* A preliminary note on the application of levansucrase to synthesis of sucrose analogs has been presented[1].

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added $R'$ (*i.e.* glucose) must decrease the concentration of $fr\sim$enzyme at equilibrium, and hence also the rate of synthesis of levan (equation 2). Apart from glucose, a diversity of aldoses are known to inhibit levan synthesis, and it is attractive to postulate that the mechanism of the inhibition is basically similar in all these cases.

These considerations imply that levansucrase catalyzes reversible shuttling of a β-fructofuranosyl group between the anomeric carbons of aldoses of different configurations and structures. The present communication reports an experimental demonstration of this property and its application to the synthesis of a sucrose analog, α-D-xylopyranosyl-β-D-fructofuranoside. "Xylsucrose" is perhaps an appropriate name for this compound. The method of preparation and proof of structure of xylsucrose are presented. The specificity of transfer reactions affected by levansucrase is being investigated by one of us (G.A.) as part of work leading to the Ph.D. degree.

## METHODS AND MATERIALS

*Enzymes*

*Levansucrase.* Cell-free crude extract of *Aerobacter levanicum* was used[5].

*Dextransucrase.* This preparation, kindly donated by Dr. H. M. TSUCHIYA, manifested weak levansucrase activity in addition to high dextransucrase activity.

*Amylosucrase.* A sonic extract of *Neisseria perflava* was used. The published method[6], which depends on spontaneous release of the enzyme from the bacterial cell during growth, proved unsuitable to our strain. The bacteria were grown at 37° C on liquid medium containing proteose peptone No. 3 (Difco) 1 %, yeast extract 0.2 %, NaCl 0.5 %, $Na_2HPO_4 \cdot 12\ H_2O$ 0.2 %, and glucose 5 % (added to the sterilized medium terminally); pH 7. The cells, harvested at the end of 48 hours, were washed twice with cold water, suspended in about 5 volumes of cold 0.025 *M* maleate buffer ($Na^+$; pH 6.5), and disrupted sonically (50 minutes with glass beads in a Mickle tissue disintegrator). The yellowish supernatant fluid afforded by centrifugation (15 minutes at 10,000 × *g*) polymerized 28 μ*M* sucrose in 24 hours at pH 6.4 at 10° C per ml enzyme fluid.

*β-Glucosidase.* This was prepared from extract of sweet almonds by precipitation with tannin ("Rohferment")[7].

*Yeast invertase.* A commercial concentrate obtained from B.D.H. was used.

*Sucrose-specific sucrase*[8]. This was prepared from a hybrid yeast (haploid strain 12836) kindly supplied by Prof. C. C. LINDEGREN. The use of this enzyme as a specific reagent in sugar analysis will be the subject of a later communication.

*Analytical methods*

*Ketose* (fructose) was determined by the resorcinol method[9], and *pentose* (xylose) by MEIJBAUM's orcinol method[10] with a heating time of 40 minutes.

*Reducing aldose* in presence of ketose was measured by a microadaptation of an iodimetric method[11]. Total *reducing power* of hydrolysate of xylsucrose towards copper was estimated colorimetrically[12].

Raybin's *diazouracil* test was used in a modified form[13].

Enzyme activity was examined qualitatively by the method of paper chromatogram analysis. Quantitative methods were employed in those cases in which the paper chromatograms gave evidence of detectable activity by the enzyme under study.

*Analysis of sugar acetates.* Crystalline sucrose octaacetate (m.p. 89° C) was prepared from sucrose[14]. A solution of the substance in ethanol was analyzed for acetyl colorimetrically with hydroxylamine[15], and for fructose by ROE's method[9] applied directly. The values conformed within ± 3 % to theoretical expectation. It was therefore assumed that the above noted analytical procedures are applicable to the acetates of both sucrose and its analogs.

*Paper chromatography.* Mixture consisting of *n*-propanol, ethyl acetate, and water (7:1:2, by volume) was mostly used as solvent system. Spray reagents used were: (1) urea-phosphoric acid[16] as a selective reagent for ketose and pentose (colour reactions: intense blue with ketose, faint or no colour with aldohexose, yellow with pentose, and brown with glyceraldehyde), (2) aniline-oxalate[17] as a selective reagent for aldoses, and (3) alkaline triphenyltetrazolium chloride[18] as a test for free reducing function.

*Column chromatography.* The columns contained activated charcoals filled 1:1 (w/w) with Celite #535 (Johns Manville Co.). Darco G60 (Atlas Powder Co.) was used in the composition of columns #1 and 2, and active carbon No. 130 (Sutcliffe, Speakman & Co., Leighs, Lancs., England) in column #3.

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## EXPERIMENTAL

### *Synthesis of xylsucrose from raffinose and* D-*xylose*

A reaction mixture consisting of raffinose (5%) and levansucrase solution (final dilution 1:2) at pH 5.4 in McIlvaine citrate buffer (final dilution 1:4) under toluene at 35° C was examined chromatographically at successive times (0–10 hours). The paper chromatograms showed the formation of a mixture of products (levan, a tetrasaccharide[5], melibiose and fructose) all of which increased in amount during the time of observation. When D-xylose (10%) was included in the system, the distribution of products of reaction was modified: the production of fructose and levan were both greatly depressed and a new non-reducing component appeared as a prominent product of reaction. The paper chromatogram mobility of the new component differed little from that of glucose. However, the spray reactions were typical for ketoside and for pentoside. It could be anticipated accordingly that the substance is a disaccharide,—xylosido-fructoside. Its amount increased until about 6 hours, then remained roughly constant during the next four hours while production of levan continued steadily though slowly.

### *Preparation and isolation of xylsucrose*

100 ml of solution of 20 g of D-xylose and 10 g of raffinose in McIlvaine buffer pH 5.4 (diluted 1:2) were added to 100 ml of levansucrase solution and incubated under toluene at 35° C for 7 hours.

At the end of the incubation period, the reaction was terminated by addition of 4 volumes of methanol to the mixture. The suspension was left in the cold overnight and filtered. The filtrate at neutral pH ($BaCO_3$) was reduced in volume *in vacuo* at 50° C to a thick syrup. The concentrate was applied to column #1 where it was freed from monosaccharides by washing with 5 liters of water. Melibiose and several minor reaction products were removed with 6 liters of 2% (v/v) ethanol. The remaining components—largely raffinose and xylsucrose—were recovered in 4 liters of 50% (v/v) ethanol. The solution was evaporated down to a thick syrup, and then transferred to column #2 where it was freed from remaining traces of melibiose by washing with 10 liters of 2.5% (v/v) ethanol. Partial separation of xylsucrose from raffinose was accomplished by gradient elution (20% v/v ethanol in the upper reservoir and water 1 liter in the lower). Eluate fractions containing both xylsucrose and raffinose were concentrated and applied to column #3. The separation of the sugars was accomplished from this column by gradient elution (15% v/v ethanol in the upper reservoir and water 1 liter in the lower). All the fractions containing xylsucrose in chromatographically pure form were pooled and evaporated. The residues were extracted into water, treated with charcoal, freed from salts (Amberlite IRC-4B and IRC-50 resins), and lyophilized. 0.4 g of hygroscopic white powder was recovered.

### *Properties of xylsucrose*

The product was chromatographically pure. It gave a positive Raybin test. It was non-reducing both to copper reagents and to iodine. $[\alpha]_D^{20} = +62$ ($H_2O$, *ca.* 1.15).

The paper chromatogram mobility in *n*-butanol-acetic acid-water (4:1:5, by volume) resembled glucose ($R_F = 0.19$) closely; in phenol with 1% $NH_3$ saturated by HCN, $R_F$ was 0.47.

The ketose content equalled 98% of theory for xylosido-fructoside. The molar ratio of pentose to ketose was 1.05.

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*Xylsucrose hepta-acetate.* Acetylation[14] of 25 mg of xylsucrose afforded 40 mg of colourless syrup. Acetyl content was found to be 7.10 equivalents per anhydro-fructose unit.

*Periodate oxidation.* The periodate oxidation of xylsucrose and sucrose was followed in the Warburg respirometer by the method of PERLIN[19]. Reactions conducted on 2.40 mg xylsucrose and 1.50 mg sucrose at 16.5° C proceeded smoothly and to a clearly defined end-point within 7 hours. Xylsucrose and sucrose formed respectively 0.97 and 1.10 $M$ of formic acid per mole of disaccharide.

*Yeast invertase* hydrolyzed the oligosaccharide totally to xylose and fructose. Aldose and ketose contents of the total hydrolysate were respectively 98 and 97% of the values expected on a weight basis. Copper reducing power corresponded within 1% to that of an equal weight of equimolar mixture of D-xylose and fructose.

*β-glucosidase* ("Rohferment") failed to affect xylsucrose under conditions which permitted near total hydrolysis of either cellobiose or salicin.

*Amylosucrase* failed to affect xylsucrose under conditions in which it converted sucrose to an extent of about 50% to fructose and amylopectin besides a small amount of an unidentified oligosaccharide containing fructose and glucose and presumably a trisaccharide.

*Sucrose-specific sucrase,* in striking contrast to the classical type of yeast invertase, failed to affect xylsucrose under conditions which permitted total hydrolysis of sucrose by the enzyme solution.

With *levansucrase,* xylsucrose behaved as a typical donor substrate manifesting both donor and acceptor function. With this enzyme xylsucrose afforded both levan, fructose, and xylose besides a small amount of three oligosaccharides of D.P. (degree of polymerization) presumably 3, 4 and 5 respectively and which contained xylose in addition to fructose[5].

The paper chromatogram mobilities ($R_{\text{fructose}}$) of the three xylo-fructo-oligosaccharides formed from xylsucrose by levansucrase were respectively 0.68, 0.55, and 0.43. The log $\alpha'$ values[20] (*i.e.* log $[R_F/1 - R_F]$) when plotted against D.P. afforded a straight line whose slope differed only slightly from that of the corresponding function of an oligosaccharide series (1-kestose series) synthesized by levansucrase from sucrose[13]. The findings suggest that the three xylo-fructo-oligosaccharides are successive members of a homologous polymer series. Presumably these products arise by a sequence of transfructosylations in which xylsucrose acts as initial acceptor and as the donor.

On incubation with *dextransucrase,* xylsucrose was converted slowly to levan and xylose with an attendant liberation of little fructose. The observed rate of degradation of xylsucrose was minute compared to the rate of the polymerization of sucrose to dextran by this enzyme system. The slight liberation of fructose from xylsucrose by the action of this dextransucrase preparation may be ascribed to the activity of the trace of levansucrase present. The possibility that xylsucrose may be polymerized by dextransucrase to xylan and fructose has not been ruled out, but it is clear that if this reaction occurs at all it must be very slow.

## DISCUSSION

As a non-reducing oligosaccharide which is totally hydrolyzed to equimolar amounts of xylose and fructose by yeast invertase, xylsucrose must be a disaccharide. The

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lability towards yeast invertase shows that the fructosyl moiety is β-fructofuranosyl. The ability of xylsucrose to serve with levansucrase as a donor confirms this conclusion. The specific optical rotations $[\alpha]_D^{20}$ of the methyl-α- and methyl-β-D-xylopyranosides are respectively +153.9 and −65.5 as compared to +158.9 for methyl-α-D-glucopyranoside. On this basis, the rotation of α-xylosyl-β-fructofuranoside would be expected to be a positive value only slightly lower than that of sucrose, whereas β-xylosyl-β-fructofuranoside would be expected to have a negative rotation. Hence the observed positive value $[\alpha]_D^{20} = +62$ for xylsucrose indicates that the xylosyl moiety is in the alpha configuration. This conclusion is further corroborated by the finding that β-glucosidase does not hydrolyze this disaccharide. Since periodate oxidation of xylsucrose afforded 1 mole of formic acid per mole of disaccharide, the ring structure of the aldosidic moiety of this β-fructofuranoside could not be xylofuranose but must be xylopyranose. The conversion of xylsucrose to a heptaacetate confirms that seven free hydroxyl groups are present in the molecule. The structure of xylsucrose is established by these findings as α-D-xylopyranosyl-β-D-fructofuranoside (Fig. 1). In experiments in which D-glucose has served as the acceptor of the fructose residue from raffinose, one non-reducing disaccharide was found to be formed and this has proved to be sucrose[1]. The structure assigned above to xylsucrose is therefore in full accord with that which can be anticipated on the basis of analogy.

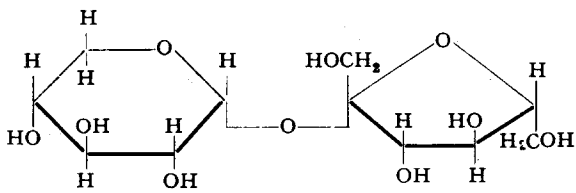

Fig. 1. Xylsucrose: α-D-xylopyranosyl-β-D-fructofuranoside.

Xylsucrose may serve as a tool in the elucidation of the role of the C-6 glucocarbinol group of sucrose in the substrate specificity of sucrases. The observation that xylsucrose is hydrolyzed by ordinary yeast invertase accords with the established concept of the specificity of this enzyme as a β-fructofuranosidase[21]. It has been shown above, on the other hand, that even though the C-6 glucocarbinol group of sucrose is unnecessary to the action of ordinary yeast invertase this group is nevertheless requisite for sucrose hydrolysis by a special type of yeast sucrase. Furthermore, the intact C-6 glucocarbinol is also requisite for the action of amylosucrase on sucrose. Finally, the C-6 glucocarbinol is also required by dextransucrase. The failure of this last enzyme to act on xylsucrose could conceivably be due to incapacity of the substrate to serve either as a donor molecule or as an acceptor. Since α-xylose-1-phosphate is not attacked by sucrose phosphorylase[22,23], it can be deduced that this sucrase, too, requires an intact C-6 glucocarbinol group for activity.

The circumstance merits mention that when levansucrase transfers β-fructofuranosyl from raffinose to the anomeric carbon of xylose, the non-reducing disaccharide formed is predominantly or exclusively the α-D-xylopyranoside rather than the β-anomer. This suggests that the substrate specificity of levansucrase is not directed solely to the β-fructofuranosidic moiety of the substrate. The specificity of the enzyme to the configuration obtaining at the anomeric carbon of the aldosidic moiety of the substrate contrasts strikingly with the lack of discrimination which

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characterizes the relation of the enzyme to configurational arrangements obtaining at carbon positions 4, 5, and 6 of the aldosidic moiety[4,24].

The present work provides a basis for the belief that directed use of levansucrase as a tool of oligosaccharide synthesis will make available for study a rich crop of new sucrose analogs which differ from sucrose only in the aldosidic moiety. A complementary series of sucrose analogs has previously been synthesized with the aid of sucrose phosphorylase[23]. The versatile activity of sucrose as a substrate of polymer synthesis suggests the hypothesis that the structural analogs of this sugar function in metabolism analogously to sucrose, *i.e.* that they act as substrates of self-motored processes of polymerization and give rise to a diversity of polyaldoses. Experiments to explore this possibility are planned.

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SUMMARY

Levansucrase transferred the β-fructofuranosyl group of raffinose to the anomeric carbon of xylose. The disaccharide "xylsucrose" formed by this reaction has been isolated and is shown to be α-D-xylopyranosyl-β-D-fructofuranoside.

Xylsucrose was hydrolyzed by ordinary yeast invertase and was utilized both as donor and acceptor by levansucrase. Some other sucrases (dextransucrase, amylosucrase, and a special yeast sucrase) were inert towards this sucrose analog.

The substrate specificity of levansucrase and the possible role of sucrose analogs in biological polymer synthesis are discussed.

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