The Mechanism of Polysaccharide Production from Sucrose

5. TRANSFER OF FRUCTOSE TO C-1 OF ALDOSE BY LEVANSUCRASE*

BY S. HESTRIN AND G. AVIGAD
Laboratory of Microbiological Chemistry, Department of Biochemistry, Hebrew University—Hadassah Medical School, Jerusalem, Israel

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Levansucrase catalyses the reversible transfer of fructose from an aldosyl β-fructofuranoside (R~fr) to C-1 of free aldose (R—H) (eqn. 1):

$$R_1 \sim fr + R—H \rightleftharpoons R \sim fr + R_1—H. \quad (1)$$

This enzymic ability has enabled a series of new sucrose analogues to be synthesized (Hestrin, Feingold & Avigad, 1955; Avigad, Feingold & Hestrin, 1956; Feingold, Avigad & Hestrin, 1957; Avigad, 1957; Péaud-Lenoël & Dedonder, 1957; Péaud-Lenoël, 1957*a*).

This paper defines the range of eqn. 1. A preliminary account of some of these experiments has been presented (Avigad & Hestrin, 1957). An example (eqn. 2) of the aldosyl exchange reaction is now described quantitatively:

$$\text{Sucrose} + \text{melibiose} \rightleftharpoons \text{raffinose} + \text{glucose} \quad (2)$$

Competitive inhibition of levansucrase by methyl α-D-glucopyranoside and methyl β-D-fructofuranoside is also demonstrated. To account for the specificity of levansucrase towards substrates and inhibitors, the idea is advanced that the active surface of this enzyme comprises an aldophilic alongside a fructophilic centre.

Nomenclature. The relationship to sucrose of compounds (R~fr) in which the glucose moiety has been replaced by another aldose is indicated by attaching a prefix to sucrose. Thus α-galactosyl β-fructofuranoside is named 'galsucrose'; α-lactosyl β-fructofuranoside (4^G-β-galactosylsucrose) is named 'lactsucrose', etc.

MATERIALS AND METHODS

Enzyme. Levansucrase was prepared from cells of *Aerobacter levanicum* grown on sucrose medium as described previously (Hestrin, Feingold & Avigad, 1956). The enzyme solution formed 20 mg. of levan/hr./mg. of protein N (0·15 M-sucrose, 37°, pH 5·4).

Aldoses. Many aldoses and several sucrose analogues were provided by the generosity of colleagues (see acknowledgements). Uniformly labelled [^{14}C]sucrose and [^{14}C]-glucose were obtained from the Radiochemical Centre, Amersham, Bucks. Fructosylglucose isomers (Feingold *et al.* 1956) and R~fr compounds which differed from sucrose at C-4, C-5 or C-6 of the aldose moiety were prepared enzymically by the action of levansucrase. D-Xylulose and D-ribulose were tested as a mixture (hydrolysate of the equilibrium mixture of products formed from ribose 5-phosphate by pentose phosphate isomerase). Methyl β-D-fructofuranoside was prepared as described by Breuer & Bacon (1957).

Dextran (Commercial Solvents Corp., Terre Haute, Ind., U.S.A.) and glycogen (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) were hydrolysed partially in acid as described by Barker, Bourne, James, Neely & Stacey (1955), and xylan (Pfanstiehl Chemical Co., Waukegan, Ill., U.S.A.) as described by Whistler & Tu (1952). The partial hydrolysates were partially desalted, concentrated and finally freed of monoses and residual salt by washing with water on a carbon–Celite column. Solutions of the homologous polymer series were obtained by extracting the extruded contents of the columns in 50% (v/v) ethanol and concentrating the extracts under reduced pressure.

The purity of each of the substrates used was checked by means of paper chromatography. Commercial preparations of L-xylose and D-arabinose were shown to be substantially pure substances by measurement of the optical rotation.

Standard conditions. Digests contained 0·1 M-donor (sucrose, raffinose) and approx. 10% (w/v) acceptor in phosphate–citrate buffer [McIlvaine solution, pH 5·4, diluted 1:5; Gomori, 1955) at 37° under toluene. The enzyme concentration was 0·5 unit/ml. (unit as defined by Dedonder & Péaud-Lenoël, 1957), representing 0·02% protein N.

Technique of survey of carbohydrates. Depending on chromatographic convenience, either a disaccharide (sucrose) or a trisaccharide (raffinose) was employed as the donor system. Raffinose was used in tests of hexoses, sucrose in tests of oligoaldoses and either sucrose or raffinose in tests of pentoses, heptoses and deoxy sugars. Acceptor activities of the carbohydrates were surveyed in sets of parallel experiments, each with a control test with either glucose (raffinose as donor) or melibiose (sucrose as donor). Under the conditions selected as standard (see above), the enzyme was at saturation in relation to glucose and at about half saturation in relation to melibiose. To conserve rare carbohydrates, microtechniques were employed. The reaction vessel consisted of glass capillary tubes (1·5 mm. diam.) and contained a total volume of reaction mixture of 30–60 μl. (Hestrin *et al.* 1956). Portions (4–10 μl., containing 0·5–1·0 mg. of total carbohydrate) were removed with the aid of calibrated capillary pipettes (0·5 mm. diam.), applied to Whatman no. 1 filter paper along a start line in spots of approx. 4 mm. diam. and immediately dried in a stream of warm air. Mercuric

* Part 4: Feingold, Avigad & Hestrin (1956).

chloride incorporated previously into the paper (0·05 μmole of mercuric chloride/spot) ensured the prompt arrest of the reaction.

Solvent systems and spray reagents are listed in Table 1. Chromatograms were resolved at room temperature (approx. 25°). The minimum amount of fructose which could be detected on paper by means of the urea–phosphoric acid reagent was found to be 3 μg. (free or combined).

A product is described in this paper as an aldosylfructoside only if it exhibited the following behaviour: ketose test, +; aldose test, +; test for reducing-group activity, −; mobility quotient (mobility of free aldose/mobility of formed aldosylfructoside), 1·57±0·05 (in solvent *b* (Table 1)); the same quotient measured in solvent *a* was 1·42±0·05. Aldosylfructoses and fructosylaldose could be differentiated readily from aldosylfructosides on the basis of the test for reducing-group activity.

Some of the aldoses examined were of types (pentose, deoxyhexose, methylpentose and heptose) which gave differentiating colour reactions with listed spray reagents. With these the results of the spray tests provided a direct confirmation of the assumption that the aldose added to a test system had been incorporated into the formed aldosylfructoside.

Analyses of radioactive substrates. Components of ^{14}C systems were resolved chromatographically as described above. Portions were adjusted to contain 83 μmc±10%, unless otherwise is indicated. The distribution of radioactivity on the paper chromatogram was recorded (Roberts & Carleton, 1956) with a model C100 Actigraph with a D34 thin-window counter (Nuclear Chicago Co., Chicago, Ill., U.S.A.). A slit width of ⅛ in. was used on the 250 scale and one of ¼ in. on the 1K scale range of the rate meter. The recordings were analysed planimetrically. To estimate an area beneath a peak, a base line was drawn at a level corresponding to the background activity. The total area enclosed by the base line and the traced curve was taken as 100, the increments contributed by the single peaks being expressed as percentages. Overlapping areas under successive peaks were estimated by extrapolation. In the systems in which ^{14}C had been supplied in the form of uniformly labelled [^{14}C]sucrose, relative molar concentrations were calculated from radioactivities on the basis of the following relationship (A = counts/min.; M = moles): M (fructose): M (sucrose): M (1^F-β-fructosylsucrose) = A (fructose): ½A (sucrose): ½A (1^F-β-fructosylsucrose). The experimental results were in good accord with this theoretically expected relationship (see Hestrin *et al.* 1956). Planimetric measurements of components representing ⩾5% of total ^{14}C were found to be reproducible within ±5%. Components were identified by both their mobility and reactions with spray reagents.

In several of the experiments, paper regions corresponding to radioactivity peaks were eluted with hot water and assayed for fructose colorimetrically (Roe, Epstein & Goldstein, 1949). The colorimetric and planimetric assay were found to agree within ±6%. Internal checks on the validity of planimetric assays were obtained by demonstrating that in systems in which [^{14}C]sucrose was the sole source of added ^{14}C the results of radioactivity measurements conformed within <5% to predicted relationships, namely the following (A = counts/min.): A (Δ sucrose) = A (products formed from sucrose); A (glucose) = ½[A (Δ sucrose) − ⅔A (1^F-β-fructosylsucrose)]; A (glucose) = A (fructose) + A (levan) + ⅓A (1^F-β-fructosylsucrose).

RESULTS

Survey of aldoses

On the basis of the net amounts of aldosylfructoside found to be formed from aldoses in standard reaction conditions, three classes of aldose were distinguished (numbers in parenthesis give the range of yield of aldosylfructoside in μmoles/ml., as observed at 30–60% decomposition of donor): (*a*) apparently inert compounds (<1·5); (*b*) poor acceptors (1·5–5·0); (*c*) good acceptors (⩾20).

Table 1. *Methods used in paper chromatography*

Designation	Composition of solvent system (by vol.)	Solvent systems: Reference	Flow time (hr.)	Type of carbohydrate resolved
a	Propanol–ethyl acetate–water (7:1:2)	Albon & Gross (1952)	24–36	Neutral
b	Butanol–ethanol–water (5:2:2)	—	60–70	Neutral
c	Pyridine–ethyl acetate–acetic acid–water (5:5:1:3)	Fischer & Dörfel (1955)	12	Uronic acid, lactone
d	Propanol–aq. NH_3 soln.–water (6:3:1)	Hanes & Isherwood (1949)	10	Phosphate ester*

* Papers were pretreated with ethylenediaminetetra-acetic acid (Eggleston & Hems, 1952).

Component located	Spray tests: Spray reagent	Reference
Neutral sugar		
Reducing group	Triphenyltetrazolium chloride (alkaline)	Wallenfels (1950)
	Aniline oxalate	Horrocks & Manning (1949)
Ketose	Urea–phosphoric acid	Wise, Dimler, Davis & Rist (1955)
Aldose	Aniline oxalate	Horrocks & Manning (1949)
Uronic acid	*p*-Anisidine	Hough, Jones & Wadman (1950)
Phosphate ester	Ammonium molybdate	Hanes & Isherwood (1949)

Table 2. *Compounds tested for acceptor activity at* C-1 *in respect of fructose transferred from sucrose or raffinose*

Definitions of classes (*a*), (*b*) and (*c*) are given in the text. The figure in parenthesis after the name of an aldose is the $R_{sucrose}$ (solvent *b*) of the aldosylfructoside formed.

Compound*	Class *a*	Class *b*	Class *c*
Aldopentose	D-Ribose	D-Arabinose (1·12)	D-Xylose (1·20)
	D-Lyxose	L-Xylose (1·18)	L-Arabinose (1·14)†
Aldohexose	D-Mannose	L-Glucose (1·05)	D-Glucose (1·00)
	D-Gulose	L-Galactose (0·90)	D-Galactose (0·92)
	D-Talose	D-Altrose (1·26)	—
	—	D-Idose (1·32)	—
Aldoheptose	D-Glycero-L-mannose	D-Glycero-L-glucose (0·66)	D-Glycero-D-galactose (0·60)
	D-Glycero-D-gulose	D-Glycero-L-galactose (0·57)	—
Aldo-octose	—	D-Threo-L-galactose (0·58)	—
Ketopentose	D-Ribulose	—	—
	D-Xylulose	—	—
Ketohexose	D-Fructose	—	—
	L-Sorbose‡	—	—
	D-Tagatose	—	—
Ketoheptose	D-Mannoheptulose	—	—
	D-Glucoheptulose	—	—
Deoxyaldose	2-Deoxy-D-ribose	—	—
	2-Deoxy-D-glucose	—	—
	2-Deoxy-D-allose	—	—
	6-Deoxy-L-mannose (L-rhamnose)	6-Deoxy-L-galactose (L-fucose) (1·52)	—
	2:6-Dideoxy-D-allose (D-digitoxose)	—	—
Uronic acid	D-Glucuronic	—	—
	D-Glucurono-δ-lactone	—	—
	D-Galacturono-δ-lactone	—	—
Aldosamine	D-Glucosamine	—	—
	N-Acetyl-D-glucosamine	—	—
Aldose ester	D-Glucose 6-phosphate	—	—
Aldose ether	3-*O*-Methyl-D-glucose	—	—
	2:6-Dideoxy-3-*o*-methyl-D-*lyxo-hexose* (D-diginose)	—	—
	2:6-Dideoxy-3-*o*-methyl-D-*ribo-hexose* (D-cymarose)	—	—
	6-Deoxy-3-*o*-methyl-D-galactose (D-digitalose)	—	—
	6-Deoxy-3-*o*-methyl-D-glucose (D-thevetose)	—	—
Disaccharide	Trehalose	—	Maltose (0·38)
	2-β-Fructosylglucose	—	Cellobiose (0·37)
	3-β-Fructosylglucose	—	Lactose (0·35)
	Laminaribiose	—	Melibiose (0·32)
	Turanose	—	6-β-Fructosylglucose (0·56)
	Leucrose	—	Gentiobiose (0·35)
	Inulobiose	—	Isomaltose (0·35)
	Levanbiose	—	—
Trisaccharide	—	—	Maltotriose (0·19)
	—	—	Panose (0·17)
	—	—	Isomaltotriose (0·15)
	—	—	Manninotriose (0·13)
Homologous polymer series	—	—	Oligodextrans of DP§ 2–7
	—	—	Oligoamyloses of DP 2–7
	—	—	Oligoxylans of DP 2–6

* Ketoses as well as aldoses are listed, since a ketose could be regarded for the purposes of the present classification as an aldose in which CH_2OH had replaced H at C-1.

† In addition to arabinosylfructoside, a β-fructofuranosylarabinose ($R_{sucrose}$ 1·24) was formed in this system. The yield of the fructosylarabinose exceeded that of the arabinosylfructoside.

‡ A fructosylsorbose ($R_{sucrose}$ = 0·96) was formed in good yield.

§ DP, Degree of polymerization.

Table 3. *Influence of specific alterations in atomic arrangement of aldose on the acceptor activity of* C-1

Carbon site of the alteration in aldose	Nature of alteration	Example (active parent compound→tested product)	Influence of alteration on acceptor activity of C-1*
1	CH_2OH for H on C	D-Glucose→D-glucoheptulose	−
	Glucosyl for H in OH	D-Glucose→trehalose	−
	CH_3 for H in OH	D-Glucose→methyl α-D-glucoside	−
2	Reduction	D-Glucose→2-deoxy-D-glucose	−
	Epimerization	D-Galactose→D-talose	−
	NH_2 for OH	D-Glucose→D-glucosamine	−
	Acetylamino for OH	D-Glucose→*N*-acetyl-D-glucosamine	−
	Hexosyl for H in OH	D-Glucose→2-β-fructosylglucose	−
3	Inversion	D-Xylose→D-ribose	−
	CH_3 for H in OH	D-Glucose→3-*o*-methyl-D-glucose	−
	Glucosyl for H in OH	D-Glucose→laminaribiose	−
4	Inversion	D-Glucose→D-galactose	+
	Glucosyl for H in OH	D-Glucose→lactose	+
5	Inversion	D-Altrose→L-galactose	+
	CH_2OH for H on C	D-Xylose→D-glucose	+
6	Reduction	L-Galactose→L-fucose	+
	Oxidation	D-Glucose→D-glucuronic acid	−
	Hydroxyalkyl for H on C	D-Galactose→D-glycero-D-galacto-heptose	+
	Phosphoryl for H in OH	D-Glucose→D-glucose-6-phosphate	−
	Glycosyl for H in OH	D-Glucose→isomaltose	+
2 and 3	Inversion at each C atom	D-Glucose→D-altrose	±
2 and 4		D-Glucose→D-talose	−
3 and 4		D-Glucose→D-gulose	−
2, 3 and 4		D-Xylose→L-xylose	±
2, 3, 4 and 5		D-Glucose→L-glucose	±
3 and 6	Oxidation with ring closure	D-Glucose→D-glucuronolactone	−

* +, Product has the same order of acceptor activity as the parent; ±, product exhibits much less acceptor activity than the parent; −, product, in contrast to parent, lacks acceptor activity.

A classified list of the aldoses that have been examined is in Table 2. Appropriate examples of the effect exerted by local alterations of atom arrangement in a parent aldose on the acceptor activity of C-1 are collected in Table 3. Additional examples can be derived from Table 2.

The findings underlying the above-given aldose classification were obtained by us in repeated experiments which involved three separate batches of the enzyme. However, in experiments carried out on another enzyme batch, Mr I. Abramovici, in this Laboratory, observed that the aldopentoses (L-xylose, D-arabinose) which had formerly been found within class (*b*) showed reactivities similar to those of members of class (*c*).

All the chromatographically resolved members of three homologous polymer series of oligoaldoses (partial hydrolysates of dextran, glycogen and xylan) accepted fructose at C-1 of the reducing-end unit. The reaction was effected readily, irrespective of the lengths of the polymer chains. Plots of log α' against degree of polymerization (French & Wild, 1953) are shown in Fig. 1.

Oligofructoses [degree of polymerization (DP), 2–8] prepared from partial acid hydrolysates of both inulin and levan all failed to accept fructose at C-2 of the reducing-end unit. On the other hand, carbinol sites in some oligolevans are known to be active acceptors (Mattoon, Holmlund, Schepartz, Varva & Johnson, 1955; Avigad, 1956; Dedonder & Péaud-Lenoël, 1957; Péaud-Lenoël, 1957*b*). This aspect will be the subject of a separate communication.

With one exception (L-sorbose), the compounds in classes (*a*) and (*b*) failed to inhibit either levan or fructose production to an extent evident chromatographically (>50%). On the other hand, the compounds in class (*c*) all did produce such an effect and also noticeably inhibited the formation of 1^F-β-fructosylsucrose. Accordingly, it can be inferred that the members of classes (*a*) and (*b*) possess a markedly lower affinity for the enzyme than any member of class (*c*).

Although L-sorbose did not accept fructose at C-2, it contained a carbinol site which was a good acceptor. This ketose thus exerted a marked inhibitory effect both on levan and fructose formation. The fructosylsorbose so formed reduced alkaline triphenyltetrazolium chloride. On this basis (Feingold *et al.* 1956), it has been inferred that the position of substitution in L-sorbose was not C-1.

Donor activity among aldosylketosides

It was known from a previous investigation (Hestrin *et al.* 1956) that donor activity is common to several aldosyl β-fructofuranosides which contain an unsubstituted *trans*-disposed OH pair at C-2 and C-3 of the aldose moiety. It has now been shown that additional compounds in this class show the same behaviour, namely, β-L-arabinosyl β-fructofuranoside (L-arabsucrose), D-arabinosyl β-fructofuranoside (D-arabsucrose), 4^G-β-galactosylsucrose (lactsucrose) and 4^G-α-glucosylsucrose (maltsucrose). On the other hand, 2^G-α-galactosylsucrose (umbelliferose; Wickstrom & Baerheim-Svendsen, 1956) was not split by levansucrase. The rate of the enzymic decomposition of 0·2M-[^{14}C]-sucrose was not decreased by 0·2M-umbelliferose (inhibition < 5%). Thus it can be suggested that in sucrose, as in free glucose, ability to react with levansucrase was abolished by introduction of a hexosyl group at C-2 of the glucose moiety. The diazouracil reaction (Raybin, 1933) of umbelliferose was positive.

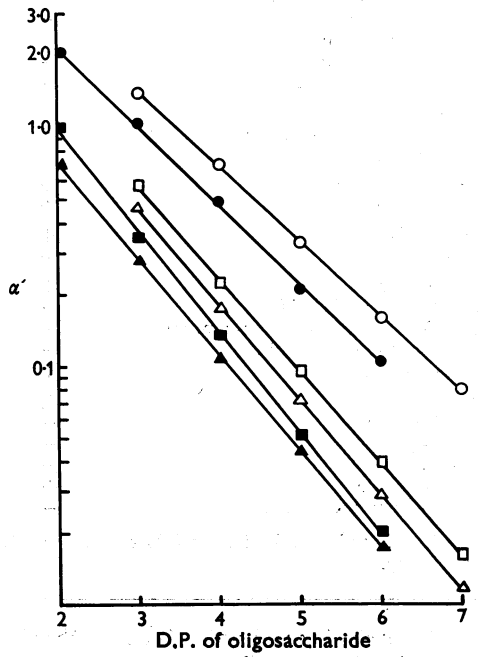

Fig. 1. Transfer of fructose from sucrose to C-1 of terminal reducing-end unit in homologous polymer series of oligoaldoses. Reaction mixtures containing 6% (w/v) of reducing oligosaccharide and 1·5% (w/v) of sucrose were incubated with levansucrase at pH 5·4 for 8 hr. at 37°. Chromatograms were developed with solvent *a* (Table 1). ▲, ■, ●, Homologous polymer series (reducing aldoses) prepared from dextran, glycogen and xylan respectively by partial acid hydrolysis. △, □, ○, Corresponding aldosylfructoside series formed from the polymer series. $\alpha' = R_{glucose}/(1 - R_{glucose})$ (French & Wild, 1953).

It was known from the previous study mentioned that introduction of a hexosyl group into the fructosylic moiety of sucrose at C-1, C-3 or C-6 abolishes donor activity. However, the question whether configurational changes in the ketose moiety of sucrose are able to alter the donor activity had still to be decided. Among disaccharides which differ from sucrose only in the ketose moiety at single carbons, two compounds (both synthesized by sucrose phosphorylase) have now been investigated: α-D-glucopyranosyl α-L-sorbofuranoside (an epimer of sucrose at C-5 of fructose) (Hassid, Doudoroff, Barker & Dore, 1945) and α-D-glucopyranosyl D-rhamnulofuranoside (6^F-deoxysucrose) (Palleroni & Doudoroff, 1956). Neither of these compounds was cloven by levansucrase.

Quantitative description of a typical example of the aldosyl-exchange reaction (eqn. 2)

As examples of the aldoses assigned to class (*c*), melibiose and glucose were chosen for quantitative study. Initial reaction rates in systems of donor (sucrose, raffinose) with and without exogenous added acceptor (melibiose in the system with sucrose, glucose in the system with raffinose) are compared in Table 4. Time courses are detailed in Figs. 2–4.

Table 4. *Initial relative rates of transfer of fructose to competing acceptors*

Initial reaction rates are calculated from measurements made within the first 2 hr. of reaction and are expressed on a molar basis in relative units. During the interval of observation, the measured rates remained substantially constant. Sucrose and glucose concn. was 0·2M; raffinose and melibiose concn. was 0·4M.

Component measured	[^{14}C]Sucrose only	[^{14}C]Sucrose + melibiose	Raffinose + [^{14}C]glucose	[^{14}C]Sucrose + raffinose*	Raffinose only
Sucrose	−100	−73	+13	−71	—
Raffinose	—	+46	−35	< +1	−50
Glucose	+97	+71	−14	+69	—
Fructose	+52	+14	+11	+37	+21
Anhydrofructose (levan)	+46	+16	+14	+30	+26
1^F-β-Fructosylsucrose	+2†	+1	—	+1	—

* Rates in this column refer only to the ^{14}C-labelled components formed from [^{14}C]sucrose in the presence of both sucrose and raffinose.

† Oligosaccharides other than those whose formation is reported were formed at rates <1.

The data confirm an earlier evaluation (Hestrin *et al.* 1956) of the relative order of the competitive powers of water and carbinol groups in sucrose. It is further noteworthy that the rates of transfer of fructose from sucrose or raffinose to C-1 of aldose (glucose, melibiose) were greater than was the rate of production of levan. Findings that will be detailed elsewhere have indicated that a many times slower transfer of fructose to C-1 of aldose can be effected by this enzyme from levan as donor. However, to demonstrate such activity on the part of levan, a degraded levan preparation had to be used as donor, and relatively high concentrations of both aldose and enzyme were maintained. The notion that the transfer of fructose from sucrose to C-1 of aldose occurs necessarily via levan (Péaud-Lenoël, 1957*a*) can be refuted on the basis of the above observations.

Decomposition of sucrose both in the system without any added aldose (Fig. 2) and in the system with added melibiose (Fig. 3) showed an abrupt intervening decrease in rate. This feature can be ascribed at least in part to an action of glucose formed during the reaction. Glucose competes with sucrose for the enzyme (Fig. 5) and exerts an inhibitory influence which appears to be even greater than that of melibiose (Table 4).

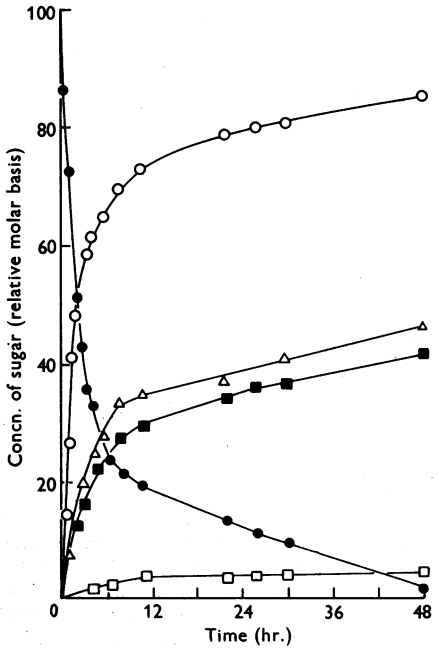

Fig. 2. Time course of reactions initiated by levansucrase acting on 0·2 M-[^{14}C]sucrose. ●, Sucrose; ○, glucose; ■, levan; △, fructose; □, 1^{F}-β-fructosylsucrose. Concentrations are expressed on a relative molar basis for sugars, and in moles of anhydrofructose for levan, the initial sucrose concentration being taken as 100.

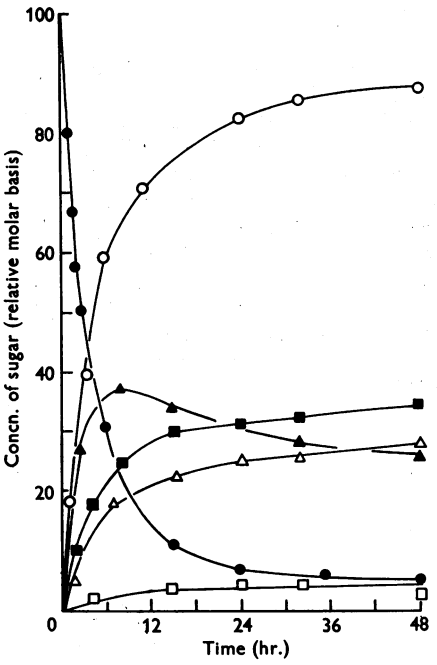

Fig. 3. Time course of reactions initiated by levansucrase acting on 0·2 M-sucrose and 0·4 M-melibiose. ▲, Raffinose; for explanation of other symbols see Fig. 2. Concentration is expressed as in Fig. 2.

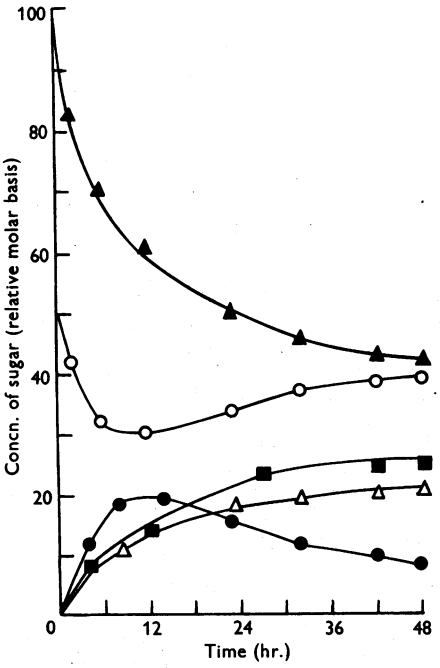

Fig. 4. Time course of reactions initiated by levansucrase acting on 0·4 M-raffinose and 0·2 M-[^{14}C]glucose. For explanation of symbols and ordinate see Figs. 2 and 3.

In good agreement with the assumption that different donor systems are activated by an identical enzyme, the decomposition of one donor (sucrose) was inhibited by an alternate donor (raffinose) (Table 4). Melibiosyl fructoside (i.e. raffinose) and free melibiose inhibited decomposition of sucrose about equally.

The rate of the incorporation of ^{14}C of sucrose into raffinose was found to be negligibly low at zero time in a substrate system consisting initially of only sucrose and raffinose (Table 4), and to increase slowly with progressing decomposition of donor. This behaviour has suggested that aldosyl radicals in an aldosyl fructoside pair cannot be exchanged directly by the levansucrase system.

Effects of concentration of aldoses and donor on the reaction velocity conformed to the Michaelis–Menten relation (Fig. 6). It was noted that the formations of fructose and levan both responded equally to an alteration in substrate concentration (sucrose, glucose) throughout the investigated ranges. The values of the Michaelis constants (K_m) of glucose (as acceptor at C-1) and sucrose (as donor) were very close to one another, being found to be 0·074 M and 0·062 M respectively. K_I of glucose, too, was a similar value, namely 0·072 M (Fig. 5), the value found being the same irrespective of whether decomposition of sucrose or formation of levan served as the basis of the calculation. On the other hand, K_m of melibiose (as acceptor at C-1) was a relatively high value, 0·42 M. Since K_m of sucrose and glucose are similar, one might anticipate K_m of raffinose to be like that of melibiose. However, K_m of raffinose has not as yet been measured. K_m of sucrose in *Bacillus subtilis* levansucrase has been found to be 0·09 M (Péaud-Lenoël, 1957*b*).

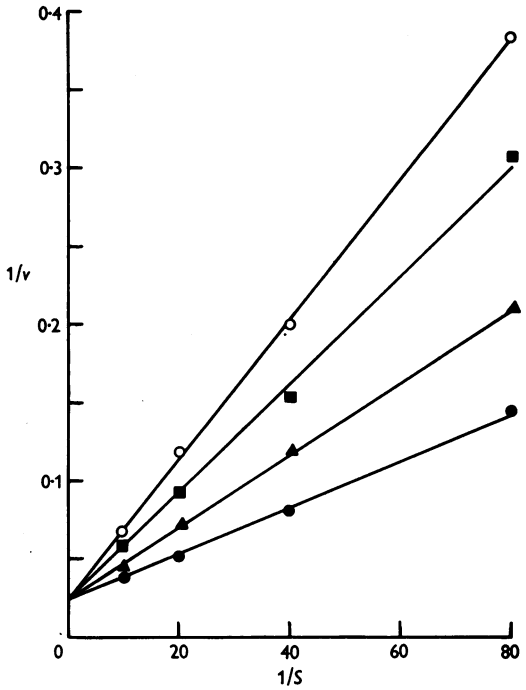

Fig. 5. Inhibition of levansucrase by methyl glycosides and glucose. Reaction mixtures contained varying concentrations of [^{14}C]sucrose in the presence of 0·1 M-methyl glycoside or -glucose. Samples (10 μl.) whose activity was 166 μmc were taken at 120 min. Within this time, reaction velocities remained practically constant in every case. In the experiment with added glucose, the net increase of glucose (Δglucose) was calculated as the sum (moles): levan anhydrofructose + fructose + 1^F-β-fructosylsucrose. In this system the amount of [^{14}C]-glucose which had been liberated from sucrose by exchange of the latter with free glucose was calculated from the difference (A = counts/min.): A (glucose) − A (Δglucose). The amount of this exchange was equivalent to approx. 15% of the sucrose found at 120 min. in the mixture. S = Molarity of sucrose; v = velocity of decomposition of sucrose (μmoles/ml./2 hr.). ●, Sucrose without added inhibitor; ▲, with methyl α-D-glucopyranoside; ■, with methyl β-D-fructofuranoside; ○, with D-glucose.

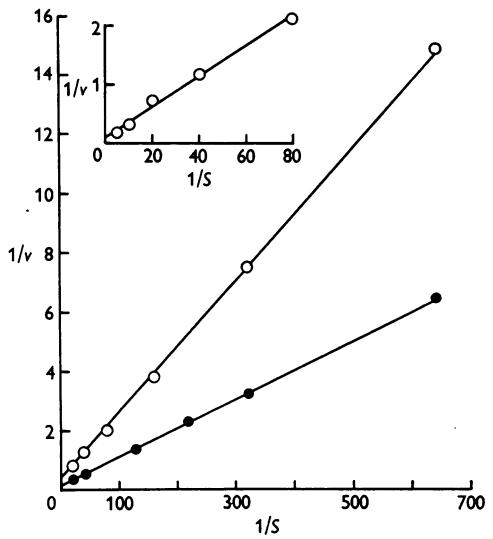

Fig. 6. Michaelis constants of levansucrase in respect of glucose, melibiose and sucrose. S = Concn. (molarity) of a varied component (glucose, melibiose or sucrose, as specified below) of substrate system. v = Reaction velocity in μmole/ml./t, where t is reaction time (min.) as specified. Within such times, all the reaction rates remained steady. Main figure: ●, sucrose formation at t = 90 in a reaction system of 0·2 M-raffinose with [^{14}C]-glucose, the concn. of the latter being varied; ○, decomposition of sucrose at t = 10 in a reaction system of sucrose without any added aldose, the concn. of sucrose being varied. Inset: ○, formation of raffinose at t = 120 in a reaction system of 0·1 M-[^{14}C]sucrose and melibiose, the concn. of the latter being varied.

Sucrose–raffinose systems attained the same 'steady state' whether sucrose or raffinose was the primary donor. The value of the molar quotient ([raffinose] [glucose]/[sucrose] [melibiose]) in these systems approached 2·2 (Fig. 7). If this value is taken as a true Nernst equilibrium constant (K), the loss of free energy ($-\Delta F'$) attending transfer of fructose from sucrose to the anomeric carbon position of α-melibiose can be estimated from the relationship $\Delta F' = -RT \cdot \ln K$ to be 0·48 kcal. (37°; pH 5·4), i.e. a small value consistent with the placement of both sucrose and raffinose on nearly the same energy level, somewhat below that of adenosine triphosphate (Robbins & Boyer, 1957; Vladimirov, Vlassova, Kolotilova, Lyzlova & Panteleyeva, 1957; cf. review by Mahler, 1957).

Time courses and equilibrium values of levansucrase-catalysed fructose transfer to aldose C-1 in several other donor–acceptor systems (sucrose–cellobiose, sucrose–maltose, raffinose–D-xylose, raffinose–L-xylose and raffinose–D-arabinose) have been determined by analogous methods in experiments carried out jointly with Mr I. Abramovici and to be detailed elsewhere. In all these systems, the reaction followed a more or less similar time course and values of K were in the range 1–2.

Inhibition of levansucrase by methyl β-D-fructofuranoside (Meβ-fr)

Fructosides in which C-2 of fructose is linked to a carbinol carbon (R < fr compounds) are not split at the glycosidic linkage by levansucrase (Hestrin & Avineri-Shapiro, 1944; Hestrin *et al.* 1956). In the course of the present work, the behaviour of a pure preparation of Me β-fr, a simple example of this class, was examined in some detail.

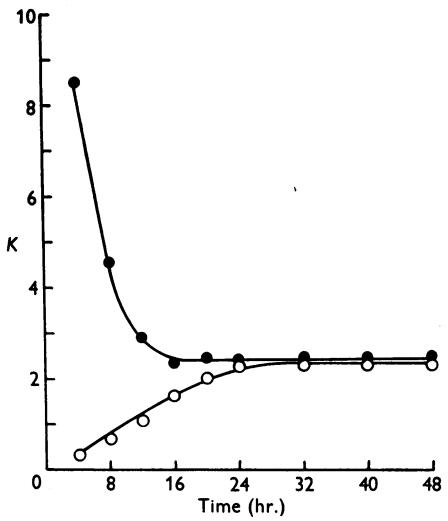

Fig. 7. Approach of a levansucrose–sucrose–raffinose system to apparent equilibrium. Curves are plotted from the data given in Figs. 3 and 4. K = [raffinose] [glucose]/[sucrose] [melibiose], the concentrations being expressed in moles. ○, Interaction of sucrose with melibiose; ●, interaction of raffinose with glucose.

Me β-fr did not serve as a donor with levansucrase though it was formed by the same enzyme in a reaction system consisting of methanol as acceptor and sucrose as donor. Mould fructosylases, on the other hand, both form and cleave Me β-fr (Schlubach & Rauchalles, 1925; Bealing, 1953; Edelman, 1954, 1956; Breuer & Bacon, 1957).

Decomposition of sucrose by levansucrase was found to be inhibited competitively by Me β-fr. In contrast, several other compounds of the type of R < fr, namely levanbiose and inulobiose (in which R is fructose) and 1^F-β-fructosylsucrose (in which R is sucrose), failed to inhibit decomposition of sucrose by levansucrase, under the standard conditions of test, to a chromatographically evident extent (> 50 %). Evidently the nature of the afructon group was exerting a marked effect on the ability of the β-fructofuranosyl radical to enter into combination with the active surface of the enzyme. Free fructose, even at a concentration as high as M, failed to inhibit decomposition of sucrose appreciably. The value of K_I of Me β-fr, as estimated on the basis of measurements of sucrose decomposition, was 0·085 M (Fig. 5), a value only slightly higher than K_m of sucrose.

Me β-fr retarded formation of levan more markedly than it did the formations of fructose and 1^F-β-fructosylsucrose (Table 5). To account for this disparity the possibility can be considered that, in addition to an ability to combine with the enzyme at the sucrose-binding site, Me β-fr is able to combine with the enzyme at a levan-binding site (cf. Péaud-Lenoël, 1957*b*). It can be seen in Table 5 that transfer of fructose to Me β-fr resulted in the formation of a methyl β-fructobioside. At equal acceptor concentration (0·2 M), the amount of the transfer of fructose to Me β-fr was almost twice that to sucrose. The following relationship applied (moles): sucrose decomposed with Me β-fr/sucrose decomposed without Me β-fr = fructose formed with Me β-fr/fructose formed without Me β-fr = (levan anhydrofructose formed with Me β-fr + methyl β-fructobioside)/levan anhydrofructose formed without Me β-fr. In other words, the excess of inhibitory effect of Me β-fr on production of levan, i.e. the difference between the extent of the inhibition of production of levan and that of the inhibition of production of fructose, was balanced by the amount of the transfer of fructose to Me β-fr.

The values of $R_{sucrose}$ of 1^F- and 6^F-β-fructosylsucrose have been found to be respectively 0·55 and 0·44 (in solvent *b*) (Feingold *et al.* 1956). The ratio

Table 5. *Influence of methyl β-D-fructofuranoside* (Me β-fr) *on transfer reactions catalysed by levansucrase*

Systems contained 0·2 M-[^{14}C]sucrose in the presence of stated concentrations of Me β-fr. The activity of portions (10 μl.) taken for analysis was 166 μmc.

Component	Rate of formation of component* (μmoles/ml./hr.)		Inhibition of formation of component (%)	
	Without Me β-fr	0·18 M-Me β-fr	0·18 M-Me β-fr	0·36 M-Me β-fr
Levan	7·20	2·50	65	76
Difructosylsucrose	0·13	0·07	46	—
1^F-β-Fructosylsucrose	1·28	0·78	39	64
Glucose†	12·60	7·60	40	56
Methyl β-fructobioside†	—	1·90	—	—
Fructose	3·56	2·22	38	54

* Values are calculated on the yields observed at 4 hr. From yields observed at 6 hr. values about 15% lower were found.

† $R_{sucrose}$ of methyl β-fructobioside was 1·37 (solvent *b*), i.e. a value close to that of glucose. To estimate the rate of formation of methyl β-fructobioside, the following theoretically established relationship was used (A = counts/min.): A (methyl β-fructobioside) = $\frac{1}{2}(A_x - A_s)$, where A_x is the radioactivity of the glucose area (i.e. the area which contains glucose and methyl β-fructobioside), and A_s is the sum: A (levan) + A (fructose) + $\frac{1}{3}A$ (1^F-β-fructosylsucrose) + $\frac{1}{2}A$ (difructosylsucrose). The amount of glucose was calculated from the difference: $A_x - A$ (methyl β-fructobioside).

of the chromatographic mobility of methyl β-fructobioside to that of Me β-fr in the same solvent system was 0·54. This suggests that fructo-C-1 in Me β-fr was the active acceptor site, i.e. that the preferred reaction sites both in Me β-fr and sucrose correspond to each other.

Inhibition of levansucrase by methyl α-D-glucopyranoside (Me α-gl)

The ability of an α-glucoside to combine with the active surface of levansucrase was found to be markedly influenced by the nature of the aglycon group. Thus Me α-gl (aglycon = CH_3) was an inhibitor of levan formation (Hestrin & Avineri-Shapiro 1944), yet trehalose (aglycon = α-glucosyl) and 1^F-β-fructosylsucrose (aglycone = β-difructofuranosyl) failed to inhibit formation of either levan or fructose noticeably in the standard conditions of reaction. In good agreement with the assumption that glucosyl in Me α-gl combines with the enzyme at a sucrose-binding site, it was found that the action of Me α-gl is competitive in respect of sucrose (Fig. 5). K_I of Me α-gl is 0·18 M.

Although Me α-gl combined with the enzyme the glucosidic linkage in Me α-gl was not split. Carbinol sites in Me α-gl, as those in free glucose, were all poor acceptors of fructose.

DISCUSSION

Several families of polymers consisting of chains of monosyl residues terminated by fructose in the sucrose configuration are known: 'raffinoses' in seeds (Herrisey, Fleury, Wickstrom, Courtois & LeDizet, 1954), 'maltsucroses' among the products formed by Schardinger enzyme (French & Wild, 1953) and in honeydew (Wolf & Ewart, 1955) and 'dextransucroses' in a sucrose culture of a *Streptococcus* (Hehre, 1956). It is the commonly held view and one which has been supported experimentally that such polymers can be generated by processes of repetitive transglycosylation initiated to an appropriate carbinol site in sucrose. However, results here described suggest that the catalysed transfer of fructose to C-1 of aldoses (eqn. 1) may represent still another means by which monosyl chains bearing a fructose terminal are formed in nature.

Synthesis of sucrose by a non-enzymic and unequivocal method has been accomplished recently (Lemieux & Huber, 1956). The same method could no doubt be applied to syntheses of sucrose analogues (R ~ fr). Presumably, however, such syntheses could be accomplished more conveniently in respect of a wide, now-defined range of R with the help of levansucrase. This enzyme is specific as between anomers of an aldose. Moreover, the transfer of a fructose group from donor to an acceptor is uniformly effected by this enzyme with net retention of fructose configuration. Thus only a single aldosyl fructoside (a product conforming in structure and configuration to sucrose) can be expected to be formed from any given aldose on its reaction with a fructose donor in the presence of levan sucrase.

On the basis of the present survey of many aldoses, the following generalizations concerning the effect of atomic arrangements on the acceptor activity of C-1 of aldose are presented:

(1) Among neutral unsubstituted aldopyranoses, those with configuration conforming at C-2 and C-3 to threose (D- or L-) exhibit activity, whereas those with configuration conforming to erythrose (D- or L-) lack activity.

(2) Activity is consonant with wide variation in atomic arrangement in aldose at C-4 and other atoms remote from C-1 to C-3. However, a carboxyl group on C-5 or a phosphoryl group at C-6 in aldose suppresses the activity. This behaviour is consistent with the assumption that a negative charge occurs in the enzyme surface and that the approach of an anionic aldose is thereby hindered.

(3) Activity is lacking in aldopyranoses in which the C-2 hydroxyl group has been replaced by H or

NH_2 or in which the C-2 or C-3 hydroxyl group is substituted (glycosyl, CH_3) or in which the C-1 hydrogen atom has been replaced by $-CH_2OH$.

Thus the suggestion can be made that the C-2 and C-3 hydroxyl groups play an active part in the mechanism of the interaction of levansucrase with an aldose.

In any hypothesis concerning the structure of the active surface of levansucrase, account must be taken of some general conclusions which may be derived from the present study. (*a*) Donor activity of sucrose is abolished if alterations of atomic arrangement or composition are made at 'sensitive' carbon sites either in the glucose or fructose moiety of the disaccharide, e.g. at gluco-C-2 or at fructo-C-5 and C-6. (*b*) Acceptor activity of C-1 is confined to aldopyranoses which possess an unsubstituted *trans*-disposed hydroxyl pair at C-2 and C-3 (cf. also, Péaud-Lenoël, 1957*a*). (*c*) The ability of glycosides containing either α-glucopyranosyl or β-fructofuranosyl as glycon to inhibit decomposition of sucrose is confined to those glycosides whose aglycon group satisfies a rigid specificity requirement. Inhibition was shown to be produced, for example, by appropriate methyl glycosides but not by several corresponding glycosyl glycosides. To account for the above properties of levansucrase, it is suggested that for an aldosyl fructoside to be attacked by the enzyme the active surface of the latter must establish close contact with several hydroxyl groups in substrate regions on both sides of the glycosidic oxygen. This hypothesis implies that the glucose moiety of sucrose is bound by an aldophilic region and that the fructose moiety is bound by a neighbouring fructophilic region of the surface of the enzyme.

Since a levansucrase system catalyses several kinds of fructose-transfer reactions, the question arises whether these reactions are all effected by the same catalyst. It has been shown above that both the transfer of fructose from donor to water (fructose formation) and that to anhydrofructocarbinol (levan formation) exhibit an equal response to alteration of the concentration of sucrose. Similarly, they have also been found to be retarded equally by an added aldose (glucose, melibiose). Furthermore, it has been shown that the following are able to enter into competition within a levansucrase system (Avigad, 1955; Hestrin *et al.* 1956; Péaud-Lenoël, 1957*b*; present results): (1) water, fructo-C-1 carbinol of sucrose, carbinols of levan, C-1-carbonyl of glucose and C-1-carbonyl of some other aldopyranoses (as acceptors); (2) sucrose and raffinose (as donors). Resolution of levansucrase into separate donor- and acceptor-activating systems has not proved possible so far. The results in hand are thus all in agreement with the assumption that donor and acceptors are activated in a levansucrase system by a single protein (Hestrin *et al.* 1956; Péaud-Lenoël, 1957*b*).

SUMMARY

1. Neutral unsubstituted aldopyranoses conforming configuratively to L-threose and D-threose at C-2 and C-3 formed aldosyl fructoside when incubated with a donor of fructose in the presence of *Aerobacter levanicum* levansucrase. Acceptor activity of C-1 in a free aldose was suppressed by any one of the following: epimerization at C-2, inversion at C-3, introduction of a substituent (glycosyl, CH_3) at C-2 or C-3; deoxygenation at C-2; replacement of hydrogen at C-1 by $-CH_2OH$. An anionic group in an aldose suppressed the activity of C-1. Other wide variations in atomic arrangement at C-4 and other atoms remote from C-1 to C-3 in free aldose did not suppress acceptor activity of C-1.

2. Reversible transfer of fructose from sucrose to C-1 of α-melibiose or some other aldoses proceeded with small loss of free energy ($-\Delta F' =$ approx. 0·5 kcal.).

3. Values of K_m for levansucrase in respect of glucose and melibiose (as acceptors at C-1) and in respect of sucrose (as donor) were 0·074, 0·42 and 0·062 M respectively.

4. Methyl β-D-fructofuranoside and methyl α-D-glucopyranoside competitively inhibited the decomposition of sucrose by levansucrase. Their K_I values were 0·085 and 0·18 M respectively.

5. Donor activity in sucrose was abolished by any one of the following: introduction of a glycosyl group at gluco-C-2 or at fructo-C-1, -C-3 or -C-6; inversion of configuration at fructo-C-5; deoxygenation at fructo-C-6.

6. The observed substrate and inhibitor specificities of levansucrase suggest the hypothesis that the active surface of this enzyme contains a fructophilic region alongside an aldophilic region and that the catalytic mechanism depends on an interaction of each of these regions with several hydroxyl groups of the substrate.

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The Sedimentation Characteristics of Deoxyribonucleic Acid from Normal and Diseased Human Tissues

By E. E. POLLI

Istituto di Clinica Medica Generale e di Terapia Medica, Universita de Milano, Via Francesco Sforza, 35, Milano

AND K. V. SHOOTER

Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London

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Clinical investigations of certain anti-metabolites which interfere with metabolism of nucleic acid have shown that they act in different ways upon the leucocytes present in the bloodstream in myeloid and lymphatic leukaemia. This suggests that the nucleic acids of these leucocytes may differ. The physical properties and chemical composition of the deoxyribonucleic acid from normal and leukaemic human leucocytes have been investigated by Polli (1957) and Polli & Semenza (1955, 1956). It was found that the effect of urea and of alkali on the viscosity of solutions of deoxyribonucleic acid prepared from myeloid leukaemia leucocytes was different from that of solutions of deoxyribonucleic acid from normal or lymphatic leukaemia leucocytes. Differences be-