The Mechanism of Polysaccharide Production from Sucrose

3. DONOR–ACCEPTOR SPECIFICITY OF LEVANSUCRASE FROM *AEROBACTER LEVANICUM**

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

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Some years have passed since the hypothesis was advanced that chains of levan and dextran, like starch, grow stepwise by the repeated transfer of a hexosyl group from a donor to a growing acceptor molecule (cf. Hestrin, 1949; Hehre, 1951). The validity of this hypothesis has been firmly established for dextrans by recent investigations (Koepsell *et al.* 1953; Hehre, 1953; Nadel, Randles & Stahly, 1953; Tsuchiya *et al.* 1955; Hellman *et al.* 1955). Knowledge concerning the mode of growth of levans, on the other hand, is still relatively meagre (cf. review by Barker & Bourne, 1953; Dedonder & Noblesse, 1953; Hestrin, 1953).

It has been suggested (Hestrin, Feingold & Avigad, 1955) that the reactions catalysed by levansucrase can be written in the form of a readily reversible primary step and a subsequent 'irreversible' step (eqns. 1 and 2). (The term levansucrase denotes the total enzyme system concerned in fructosyl transfer rather than any single enzyme component. Reactions whose reversal could not be detected by the methods used have been designated as 'irreversible'.)

$$\mathrm{fr}\sim\mathrm{R}+\mathrm{enz}\rightleftharpoons\mathrm{fr}\sim\mathrm{enz}+\mathrm{R}, \quad (1)$$

$$\mathrm{fr}\sim\mathrm{enz}+\mathrm{acceptor}\rightarrow\mathrm{fr}<\mathrm{acceptor}+\mathrm{enz}, \quad (2)$$

where $\sim$ (Lipmann's symbol for the high-energy bond) designates linkage of fructofuranose (fr) to carbonyl of aldose (R) as in the donor ($\mathrm{fr}\sim\mathrm{R}$) or to enzyme (enz), and where $<$ represents the linkage of fructofuranose to acceptor, which may be water or the carbinol site in a saccharide.

Reversible shuttling of a fructosyl residue from carbonyl to carbonyl by the action of levansucrase has been described (Hestrin *et al.* 1955):

$$\mathrm{fr}\sim\mathrm{R}_1+\mathrm{R}_2\rightleftharpoons\mathrm{fr}\sim\mathrm{R}_2+\mathrm{R}_1. \quad (3)$$

For substrate serving at once as donor and acceptor, however, the net observed reaction assumes an 'irreversible' form (eqn. 4):

part reactions:

$$(m)\ \mathrm{fr}\sim\mathrm{R}+(m)\ \mathrm{water}\rightarrow(m)\ \mathrm{fructose}+(m)\ \mathrm{R},$$

$$v(q-1)\,\mathrm{fr}\sim\mathrm{R}+(v)\,\mathrm{fr}\sim\mathrm{R}\rightarrow(v)\,\mathrm{fr}_q\sim\mathrm{R}+v(q-1)\,\mathrm{R},$$

$$(n)\ \mathrm{fr}\sim\mathrm{R}+\mathrm{P}\rightarrow\mathrm{fr}_n<\mathrm{P}+(n)\ \mathrm{R},$$

sum:

$$(s)\ \mathrm{fr}\sim\mathrm{R}+(m)\ \mathrm{H_2O}+\mathrm{P}\rightarrow(m)\ \mathrm{fructose}+(v)\ \mathrm{fr}_q\sim\mathrm{R}+\mathrm{fr}_n<\mathrm{P}+(s-v)\ \mathrm{R}, \quad (4)$$

where symbols in parentheses are molar amounts, s being the total substrate ($\mathrm{fr}\sim\mathrm{R}$) consumed, m the fructose released, v the substrate serving as initial acceptor affording oligosaccharides $\mathrm{fr}_q\sim\mathrm{R}$ (i.e. $\mathrm{fr}_{q-1}<\mathrm{fr}\sim\mathrm{R}$), P is an unspecified 'primer' serving as initial acceptor affording branched or linear levan ($\mathrm{fr}_n<\mathrm{P}$), and $(s-v)$ is the total aldose released. The 'irreversible' transfer of fructosyl from carbonyl to carbinol and the nature of levan formed by the action of *A. levanicum* levansucrase are the subject of the present communication.

METHODS AND MATERIALS

Production of A. levanicum *cells.* The bacteria were cultured at 30° in Roux flasks. Each flask contained 120 ml. of nutrient agar overlayered thinly with 10 ml. of 5% (w/v) sucrose solution (Hestrin, Avineri-Shapiro & Aschner, 1943). As starter, a 24 hr. culture in 5% sucrose–nutrient broth was used (2·5 ml./flask). The crop was harvested after 36 hr., washed three or four times with cold water to free from medium constituents and packed by centrifuging; yield, approx. 50 g. of packed cell paste/m.^{2} of culture surface.

Extraction of A. levanicum *levansucrase from cells.* All operations were carried out at 4°. Washed cells were suspended in 5 parts of water and disrupted during 35–45 min. in a Mickle disintegrator. Insoluble matter was removed by centrifuging at 18 000 *g* for 15 min. More than 90% of the total levansucrase activity was found in the solution. The faint yellow and slightly turbid fluid ('enzyme') contained 1–2% of solutes.

Assay of levansucrase activity. This activity was determined in a standard mixture containing 6% (w/v) of sucrose at pH 5·4 [McIlvaine phosphate–citrate buffer, diluted 1:5 (by vol.)] at 37° under toluene. Deviations from these standard conditions are indicated. Levansucrase in solution had lost less than 30% of activity after 3 months at 4°; a lyophilized preparation retained full activity after one year. Enzyme was not inactivated by maintenance during 2 hr. at pH values ranging from 4 to 10 at 6°, but was rapidly destroyed at pH 3·5 and at pH above 10·5. Glucose was not metabolized by the whole extract of *A. levanicum* under the conditions used for levansucrase reaction. When sucrose was incubated with the enzyme under toluene overnight, the

* Part 2: Avineri-Shapiro & Hestrin (1945).

reducing power of the sucrose–enzyme solution on hydrolysis in 0·01 N-HCl remained constant.

Paper test of levansucrase activity. Concentrated aqueous solution of the substrate (about 0·1 mg.) was applied from a micropipette to Whatman no. 2 filter paper to a spot about 5 mm. in diameter. The spot was dried (room temp.) and covered with 10 μl. of buffered levansucrase solution. The reaction on the paper was continued at 35° in an enclosed humid space for 4 hr. Control spots contained enzyme without substrate and substrate without enzyme. To terminate the enzyme reaction, the paper was dried in an oven at 50–80°. Products of reaction were resolved by paper chromatography (see below).

Capillary-tube test of levansucrase activity. Controlled reaction conditions and the high sensitivity of the paper-spot test were combined by the use of a glass capillary tube as reaction vessel in the manner described by Porter & Hoban (1954).

The components were arranged in measured drops on a sheet of Parafilm (La Pine and Co., Chicago, U.S.A.), mixed and lifted quantitatively by contact from the sheet to a capillary reaction vessel. Finally a drop of toluene was introduced. Control mixtures were prepared similarly. The assay system contained 1 vol. of enzyme, 0·5 vol. of phosphate–citrate solution (McIlvaine buffer, final dilution 1:20), pH 5·4, and 1 vol. of 2·5% substrate solution in a final total volume of about 25 μl. The capillaries, sealed at about 10 cm. distance from the meniscus, were immersed in a water bath at 37°. After 3 hr. their contents were transferred to filter paper and the nature and number of reaction products ascertained by paper chromatography.

The capillary-tube technique served as an equally useful means of conserving substrate in the examination of yeast-invertase action on fructose oligosaccharides.

Colorimetric and titrimetric methods. Reducing sugar was determined according to Somogyi (1945). Aldose was determined in presence of fructose by a micro-adaptation of the iodometric method (Macleod & Robison, 1929). The β-fructofuranoside linkage of oligosaccharides was completely hydrolysed by heating in a boiling-water bath at about pH 2 (0·5% oxalic acid, or 0·01 N-HCl) for 1 hr. Fructose (free plus combined) was determined specifically as by Roe, Epstein & Goldstein (1949). Levan, purified without loss by twice-repeated precipitation from aqueous solution with ethanol (Hestrin *et al.* 1943), was determined, without prior hydrolysis, by Roe's reagent. Protein N in the enzyme was evaluated colorimetrically (Lowry, Rosebrough, Farr & Randall, 1951).

Paper chromatography. Solvent systems and spray reagents are given in the next paper (Feingold, Avigad & Hestrin, 1956).

Macropolymeric levans. 'Culture levan' was separated from a 3-day-old culture of *A. levanicum* grown on 6% (w/v) sucrose in nutrient broth at 30°. The levan synthesized by cell-free extract ('enzyme-synthesized levan') was separated from a sucrose–levansucrase reaction mixture of standard composition (see assay of levansucrase activity) at 1 hr. reaction time. The crude preparations, separated by addition of 2 vol. of ethanol to the centrifugate of spent culture medium or enzyme digest, were purified by repeated cycles of ethanol precipitation from aqueous solution, and recovered finally as white non-hygroscopic powders containing 98% of fructosan (moisture-free basis) and less than 0·1% of N (Kjehldal).

'Levulans'. These degraded levans were separated as follows from a partial acid hydrolysate of culture levan. A concentrated solution of the native levan (10%, w/v) in phosphate–citrate buffer (McIlvaine) of pH 3·4 was held for 60 min. at 60° with stirring. The solution was cooled (30°) and brought to pH 7 with NaOH. Ethanol added up to a concentration of 57% (v/v) afforded a precipitate (I) and a supernatant fluid (II). Levulan A of degree of polymerization (DP) 150 (on basis of reducing power) or 190 (by ultracentrifugal analysis), representing 17% of the weight of original levan, was a subfraction precipitated from an aqueous solution of (I) by 57–65% (v/v) methanol. Levulan B of DP 20 (on basis of reducing power) or 40 (by ultracentrifugal analysis), representing 12% of the weight of original levan, was a subfraction precipitated from (II) by 57–75% (v/v) ethanol and further purified by reprecipitation with an excess of methanol from aqueous solution after dialysis.

Oligolevans and oligoinulins. Chromatographically homogeneous preparations of levanbiose, levantriose, levantetraose and a series of oligolevan mixtures with DP range 4–8, 6–9, 9–12 and 9–15 were obtained from partial hydrolysate of the culture levan. Inulobiose and inulotriose were prepared from a partial hydrolysate of inulin (Difco). For partial hydrolysis 2% (w/v) solutions of culture levan and inulin in 0·01 N-H_2SO_4 were held at 70° for 15 min., cooled and brought to pH 7. The partial hydrolysates were freed from monosaccharides by displacement chromatography from charcoal (cf. Whistler & Durso, 1950), and the fractions resolved by gradient elution from charcoal (Bacon, 1954*a*). Fractions were characterized by appropriate paper chromatographic tests and ratio of total ketose to reducing power.

'Kestosides.' Authentic specimens of 1^F-β-fructosylsucrose [*O*-α-D-glucopyranosyl-(1 → 2)-*O*-β-D-fructofuranosyl-(1 → 2) β-D-fructofuranoside; 1-kestose (Bacon & Bell, 1953)], 6^F-β-fructosylsucrose [*O*-α-D-glucopyranosyl-(1→2)-*O*-β-D-fructofuranosyl-(6→2) β-D-fructofuranoside; 6-kestose (Albon, Bell, Blanchard, Gross & Rundell, 1953)], and 6^G-β-fructosylsucrose [*O*-β-D-fructofuranosyl-(2→6)-*O*-α-D-glucopyranosyl-(1 → 2) β-D-fructofuranoside; neokestose (Gross, Blanchard & Bell, 1954)] were kindly supplied by Dr D. Gross, Tate and Lyle Research Laboratory.

The substances which we have shown to be mono-, di- and tri-fructosides of 1^F-β-fructosylsucrose, designated provisionally as oligosaccharides *a*, *h*, and *c*, were isolated from a levansucrase–sucrose reaction mixture (Feingold *et al.* 1956).

A second specimen of 6^F-β-fructosylsucrose was separated from a sucrose–yeast invertase reaction mixture (Bacon & Edelman, 1950) by fractionation on charcoal (Whistler & Durso, 1950). Its behaviour was found to be identical with that of the reference substance in all the experiments.

Disaccharides differing from sucrose in the aldosidic moiety. These substances are conveniently designated as sucroses with a prefix indicative of the nature of the aldosidic moiety. Thus α-D-xylopyranosyl β-D-fructofuranoside is designated as 'xylsucrose', and α-D-galactopyranosyl β-D-fructofuranoside as 'galsucrose'. Two disaccharides in this class—xylsucrose and galsucrose—were synthesized by the cell-free extract of *A. levanicum* acting on raffinose in the presence of the appropriate aldose as acceptor (Hestrin *et al.* 1955). The xylsucrose was isolated and characterized as described by Avigad, Feingold & Hestrin (1956). The galsucrose was a crystalline preparation whose isolation and properties will be described in a future communication.

Fructosylglucoses. 6-β-Fructosylglucose [6-*O*-β-D-fructofuranosyl-D-glucose (Bell & Edelman, 1954); spot I of sucrose–yeast-invertase oligosaccharide series (Bacon & Edelman, 1950)] was separated from the sucrose–yeast-invertase reaction mixture by elution from a carbon–Celite column and purified by band paper chromatography. The isomers 2-β-fructosylglucose and 3-β-fructosylglucose (2- and 3-β-D-fructofuranosyl-D-glucoses) were isolated from a levansucrase–sucrose reaction mixture (Feingold *et al.* 1956).

Other oligosaccharides. Stachyose and planteose were kindly given by Dr D. French. Melezitose and raffinose were commercial preparations.

Methyl ethers of fructose. Pure specimens of 3:4-di-, 1:3:4-tri- and 1:3:4:6-tetra-*O*-methyl-D-fructoses were kindly provided by Dr D. J. Bell.

EXPERIMENTAL AND RESULTS

Enzyme activity

Levansucrase of the soluble phase of an autolysate of *A. levanicum* was used as enzyme in an earlier investigation. The failure of this fraction to carry levan synthesis beyond a threshold concentration complicated the interpretation of the early experiments (Avineri-Shapiro & Hestrin, 1945). This effect was not observed in the present investigation in which the whole extract of the bacteria was used as enzyme. On 10% (w/v) sucrose, concentrations of levan as high as 2% were obtained. At 1% sucrose, complete disappearance of substrate was observed.

The activity of whole extract (reaction time 1 hr.) rated 15–30 mg. of levan/hr./mg. of protein N.

Infrared absorption spectrum of enzyme-synthesized levan

Infrared absorption curves, kindly obtained for us by Professor D. Ginzburg, Chemistry Department, Israel Institute of Technology, are presented in Fig. 1. The absorption curves of culture levan, of cell-free enzyme-synthesized levan of *A. levanicum* and of the levan reported by Barker & Stephens (1954) were all practically interchangeable. The absorption curve of inulin, on the other hand, differed from that of the levans.

Methylation analysis of enzyme-synthesized levan

Levan was methylated exhaustively by the methods of Bell & Palmer (1952) as follows. Levan (1 g.) was treated three times with dimethyl sulphate in the presence of 30% (w/v) aqueous NaOH in presence of dioxan. The purified product contained 44·5% methoxyl. (Calc. for trimethyl levan: 45·6%.) The yield of dioxan-soluble material was 0·46 g. (38% of the theoretical), having $[\alpha]_D = -59°$ in $CHCl_3$ ($[\alpha]_D$ of trimethyl culture-levan $= -61°$).

Trimethyl levan was hydrolysed as by Arni & Percival (1951). The hydrolysate was applied to filter paper (Whatman no. 2) and resolved. The solvent system (Hirst & Jones, 1949) consisted of *n*-butanol–ethanol–water (5:1:4, by vol.). The paper-chromatogram components were treated by the urea–phosphoric acid spray (Wise, Dimler, Davis & Rist, 1955). Failure to find monomethylfructose confirmed that methylation had proceeded to completion. Three components were found: their mobilities corresponded respectively to those of authentic 1:3:4:6-tetra-, 1:3:4-tri- and 3:4-di-*O*-methyl-D-fructose (mobilities relative to 2:3:4:6-tetra-*O*-methyl-D-glucose: 1·01, 0·84 and 0·66). Mobilities in butanone–water azeotrope conformed to the same reference compounds. The trimethylfructose failed to reduce aklaline triphenyltetrazolium chloride and hence must have been largely free from 3:4:6-trimethylfructose (cf. Bell & Dedonder, 1954). Since the tetramethyl fructose gave a grey-blue rather than ochre colour reaction with the urea–hydrochloric acid spray, it could not be 1:3:4:5-tetra-*O*-methylfructose (cf. Bell & Northcote, 1954).

The relative amounts of di-, tri- and tetramethylfructose in the hydrolysate of the trimethyl levan were estimated by matching the intensity of chromatogram spots against spots formed from

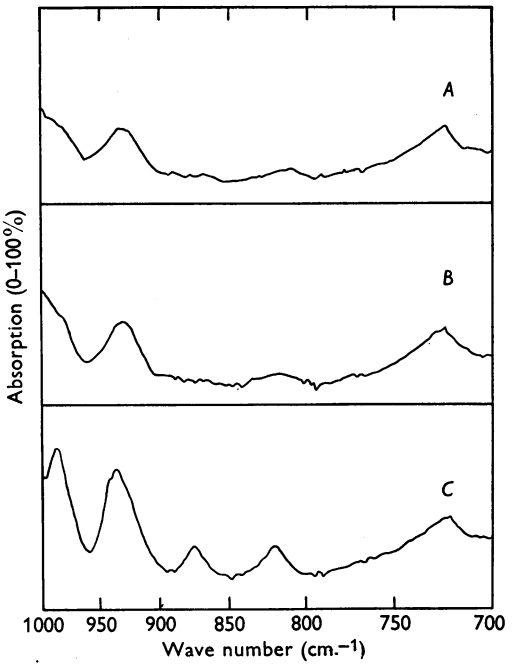

Fig. 1. Infrared absorption spectra of fructosans. Spectra were obtained with a Perkin–Elmer Model 21 infrared spectrophotometer by the Nujol-mull technique with a sodium chloride prism. Polysaccharides were dried by lyophilization. *A*, *A. levanicum* culture levan; *B*, enzyme-synthesized levan; *C*, inulin (Difco).

known amounts of the reference substances. The amounts of the di- and tetra-derivatives present seemed to be roughly equal, whereas the tri-derivative appeared to be 6 to 12 times as abundant as the di- or tetra-derivative.

The results of methylation analysis thus accord with the conclusion that levan synthesized *in vitro* has a branched structure which closely resembles *Bacillus subtilis* and *Pseudomonas prunicola* culture levans (Bell & Dedonder, 1954) and the culture levan of *A. levanicum* (Feingold, 1955). The main interfructosidic linkage must be 2:6-; the main or only branch linkage is 2:1-; the basal chain length is probably about 9, as in the culture levans. The failure to find 3:4:6-tri-*O*-methylfructose among the products of the hydrolysis of trimethyl levan suggests that the 2:1-linkage is limited to branch points.

Substrates serving as fructosyl donors with levansucrase

In an extensive substrate series (Table 1), donor activity (ability to form levan) was manifested only by compounds of type fr∼R, namely sucrose, xylsucrose, galsucrose, raffinose, 6^G-β-fructosylsucrose and stachyose (R equals respectively glucose, galactose, xylose, melibiose, 6-β-fructosylglucose and manninotriose). Formation of levan was accompanied in every instance by an appearance of free fructose and by release of a single aldose (Fig. 2).

Whereas sucrose afforded glucose as aldose product, xylsucrose afforded xylose, galsucrose afforded galactose, raffinose afforded melibiose, and stachyose afforded an aldose, presumably manninotriose (6-α-galactobiosylglucose); 6^G-β-fructosylsucrose afforded an aldobiose with the paper mobility and spray reaction of spot I of the sucrose–invertase series. The structure of (I) has been established by Bell & Edelman (1954) as 6-β-fructosylglucose (6-*O*-β-D-fructofuranosyl-D-glucose). Therefore *A. levanicum* extract must have acted directly on both raffinose and 6^G-β-fructosylsucrose, i.e. without prior hydrolysis of trisaccharide to an aldohexose and sucrose. Pazur (1952) has previously described the transfer of fructosyl from sucrose to trisaccharide (raffinose) by the action of a mould invertase. Finally it is also evident that stachyose was split by levansucrase specifically at the fructosidic linkage only.

No compound of type fr<R or R<fr∼gl was utilized as donor by the levansucrase. In particular, the replacement of fr∼ in sucrose by fr_n∼ (chain of *n* anhydrofructose units mutually linked 2:1- as in 1^F-β-fructosylsucrose and inulin, or 2:6- as in 6^F-β-fructosylsucrose) rendered sucrose inert as a donor. Compounds of the type fr_n∼gl transferred neither the whole of their fr_n∼ chain nor any part of it to a detectable extent. Their failure to afford levan was accompanied by failure to afford free fructose and by the absence of chain disproportionation (see also the next section on reactivity of fr∼R and fr<R with glucose). Fr< was equally inert as a donor in linkage to a secondary alcohol group (e.g. in 2- and 3-β-fructosylglucoses) as when in linkage to a primary alcohol group (e.g. in 1^F- and 6^F-β-fructosylsucrose and 6-β-fructosylglucose).

Table 1. *List of fructose compounds tested*

fr∼ designates the β-fructofuranosyl group (fr) linked to carbonyl of aldose, e.g. as in sucrose (fr∼gl), and *fr*< designates its linkage to a saccharide carbinol or water.

fr∼R

disaccharides: sucrose (fr∼gl); xylsucrose (fr∼xylose); galsucrose (fr∼galactose).
trisaccharides: 6^G-α-galactosylsucrose (raffinose); 6^G-β-fructosylsucrose (neokestose).
tetrasaccharides: 6^G-α-galactobiosylsucrose (stachyose).

R<fr∼gl

trisaccharides: 3^F-α-glucosylsucrose (melezitose); 6^F-α-galactosylsucrose (planteose).

fr<R (including fr_n∼gl)

monosaccharides: fructose; methyl β-fructofuranoside.
disaccharides: 6-β-fructosylglucose*; 2-β-fructosylglucose*; 3-β-fructosylglucose*; 6-β-fructosylfructose* (levanbiose); 1-β-fructosylfructose*† (inulobiose).
trisaccharides: 6-β-levanbiosylfructose (levantriose); 1-β-inulobiosylfructose† (inulotriose); 1^F-β-fructosylsucrose* (1-kestose); 6^F-β-fructosylsucrose* (6-kestose).
tetrasaccharides: 6-β-levantriosylfructose (levantetraose); fructosyl-1^F-β-fructosylsucrose* (fr_3∼gl = oligosaccharide *a*).
higher saccharides: reducing oligolevan series, DP 4–8, 6–9, 8–12, 9–15; difructosyl-1^F-β-fructosylsucrose* (fr_4∼gl = oligosaccharide *b*); trifructosyl-1^F-β-fructosylsucrose* (fr_5∼gl = oligosaccharide *c*); inulin (fr_n∼gl, *n* = about 35); levulans (preparations A and B); culture levan (DP∼300 000).

* This compound occurs in a levansucrase–sucrose system (Feingold *et al.* 1956).
† The crude preparation showed some donor activity which disappeared after purification and was traced to contamination of the sample by sucrose present in the partial hydrolysate of inulin.

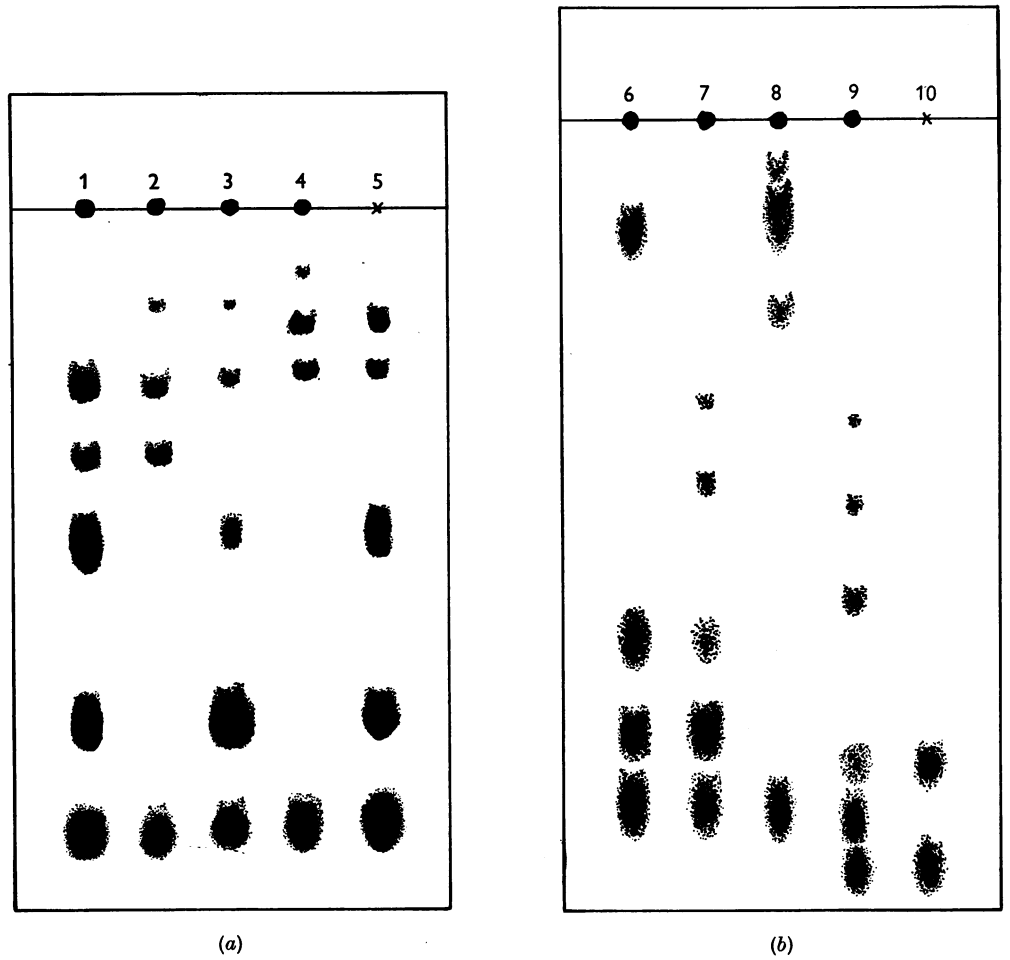

Fig. 2. Products formed by action of *A. levanicum* whole extract on donors. The substrate 1–2% at pH 5·4 (dilute McIlvaine buffer) with whole extract in dilution 1:3 under toluene at 37° for 4 hr.; 25 μl. of reaction mixture was spotted on filter papers (Whatman no. 1) which were developed with (*a*) *n*-butanol–acetic acid–water (4:1:5, by vol.) for 60 hr., and (*b*) *n*-propanol–ethyl acetate–water (7:1:2, by vol.) for 20 hr. Urea–phosphoric acid spray (specific ketose reagent) was applied in the region of the paper-chromatogram start lines. In the remaining areas of the chromatograms, benzidine–trichloroacetic acid was employed in (*a*); in (*b*) the spots are a composite of the patterns separately afforded by spraying with urea–phosphoric acid reagent and aniline oxalate reagent. Chromatogram components are listed in order of increasing mobility.

(*a*): 1. Reference sugars: levan, 6^G-β-fructosylsucrose, 6-β-fructosylglucose, sucrose, glucose, fructose. 2. 6^G-β-fructosylsucrose system: levan, tetrasaccharide (?1^F-β-fructosyl-6^G-β-fructosylsucrose), 6^G-β-fructosylsucrose, 6-β-fructosylglucose, fructose. 3. Sucrose system: levan, oligosaccharide *a* (fructosyl-1^F-β-fructosylsucrose), oligosaccharide *n* (1^F-β-fructosylsucrose), sucrose, glucose, fructose. 4. 6^G-α-Galactosylsucrose (raffinose) system: levan, tetrasaccharide (?1^F-β-fructosylraffinose), raffinose, melibiose, fructose. 5. Reference sugars: raffinose, melibiose, sucrose, glucose, fructose.

(*b*): 6. Reference sugars: levan, stachyose, sucrose, glucose, fructose. 7. Sucrose system: levan, oligosaccharides *a* and *n*, sucrose, glucose, fructose. 8. 6^G-α-Galactobiosylsucrose (stachyose) system: levan, pentasaccharide (?1^F-β-fructosylstachyose), stachyose, manninotriose (6-α-galactobiosylglucose), fructose. 9. Xylsucrose system: levan, pentasaccharide (?trifructosyl-xylsucrose), tetrasaccharide (?difructosyl-xylsucrose), trisaccharide (?1^F-β-fructosyl-xylsucrose), xylsucrose, fructose, xylose. 10. Reference sugars: xylsucrose, xylose.

Levansucrase is thus specific for fr~R compounds. For such compounds this enzyme is an even more sensitive reagent than is diazouracil (Raybin, 1933). Further, such sucrose analogues as sorbosylglucoside and ketoxylosylglucoside are Raybin-positive (Hassid, Doudoroff & Barker, 1947), yet might not be expected to give a positive reaction (polyketoside formation) with levansucrase. By the use of a capillary tube as reaction vessel and levansucrase as reagent, the positive identification of sucrose in a minute sample (15 μg.) of material was found feasible.

Acceptor function of fr~R compounds

Chromatographically non-mobile polymer fraction. This fraction, as formed in standard reaction mixture from sucrose, was removed from solution completely by centrifuging at 40 000 g for 30 min. This finding indicates that this material must be free from chains with DP below about 10^3. Even if sucrose had indeed served as initial acceptor in the series of the reactions that culminate in the formation of a macropolymeric product, the presence of a lone glucose residue in a product of high DP would not be expected to be demonstrable by any ordinary analytical technique. A search for aldose in chromatograms of hydrolysates of cell-free enzyme-synthesized levan has failed in fact to produce any sign that there is glucose or any other aldose in this material.

Chromatographically mobile polymer fraction formed by levansucrase from fr~R. This fraction in the case of the sucrose digest has been shown to be a mixture of ten substances whose DP values range from 2 to not more than 6, and which contain not more than one glucose residue in a chain (Feingold *et al.* 1956).

It was expected that when levan was formed from sucrose analogues (xylsucrose, galsucrose, raffinose, 6^G-β-fructosylsucrose, stachyose) analogous net formation of oligosaccharides would also occur. This expectation was confirmed (Fig. 2). It was noted, however, that the amount of oligosaccharide formed from raffinose and 6^G-β-fructosylsucrose was less than that in the corresponding sucrose and xylsucrose digests. The mobility on paper of the major oligosaccharide formed from trisaccharide, i.e. oligosaccharide formed from raffinose and oligosaccharide formed from 6^G-β-fructosylsucrose, was consistent with a tetrasaccharide structure. Xylsucrose and galsucrose, like sucrose, afforded visible amounts of a polymeric series of non-reducing fructosides. These contained xylose as well as fructose. Components up to DP 5 were discernible.

If the products formed had been of structural type fr~R>fr, they would have been degraded by levansucrase. Thus it can be inferred that oligosaccharides obtained from xylsucrose, galsucrose, raffinose, 6^G-β-fructosylsucrose and stachyose are the levansucrase-resistant form, i.e. the structural type represented by fr<fr~R (R is xylosyl when xylsucrose is the acceptor, galactosyl when galsucrose is the acceptor, melibiosyl when raffinose is the acceptor, 6-β-fructosylglucosyl when 6^G-β-fructosylsucrose is the acceptor, and manninotriosyl when stachyose is the acceptor).

The product fr_2~gl formed by levansucrase from sucrose has been shown to be predominantly the 1^F-β-fructosylsucrose (Feingold *et al.* 1956). By analogy it can be inferred that the interfructosidic linkage in fr_2~ aldosides formed respectively from xylsucrose, galsucrose, raffinose, 6^G-β-fructosylsucrose and stachyose is probably likewise predominantly 2:1.

Influence of growth in the presence of glucose on oligosaccharide production

Enzyme prepared from glucose-grown cells was only slightly less active in production of oligosaccharides, levan and fructose from sucrose than enzyme from sucrose-grown cells. It has therefore been concluded that the fructosyl transfers in *A. levanicum* are all mediated by a constitutive enzyme system.

Relative amounts of products formed from sucrose

It is apparent from equation (4) that the molar total of aldose released in levansucrase action is $m+n+v(q-1)$, where m represents moles of fructosyl transferred to water, n transfer to levan and $v(q-1)$ transfer to fr~R. Even if one enzyme is concerned with the several transfer kinds, the relative values of m, n and $v(q-1)$ can be expected to vary in different experiments according to the manner in which reaction conditions selectively influence the competitive power of the different acceptors.

To compute m, n and $v(q-1)$ for levansucrase acting on sucrose, three easily-obtained analytical data sufficed (in hexose equivalents): total reducing sugar, total free aldose and levan. Total free aldose as glucose $[m+n+v(q-1)]$ is obtained from the reducing power towards iodine. Since the participation of oligosaccharides in total reducing power is negligible, the free fructose (m) is obtained from the difference of reducing power towards copper and iodine. The part of glucose (n) released by levan formation is equal to the amount of fructose precipitated as fructosan (levan) by ethanol. The molar equivalent of fructose transferred to fr~R, $v(q-1)$, may then be calculated as the difference between total free glucose and the sum of glucose released by transfer to water and levan.

Proportions of n, m and $v(q-1)$ observed at two widely different concentrations of sucrose are

illustrated in Table 2. At 0·17 M sucrose and 54 M water, fructosyl transfer to water accounted for 40 % of the total transfer (Expt. *a*); on the other hand, at 1·5 M sucrose and 39 M water, transfer to water accounted for only 9 % of total transfer (Expt. *b*). In the first case, the 1^F-β-fructosylsucrose formed weighed about 30 % as much as levan, whereas in the second case the yield of 1^F-β-fructosylsucrose by weight was more than twice that of levan formed.

In Expt. *b* of Table 2 the absolute amounts of oligosaccharides accumulated were large, and the quantitative recovery of the oligosaccharides as pure fractions after resolution by band-paper chromatography was feasible. The sum of the weights of 1^F-β-fructosylsucrose and fructosyl-1^F-β-fructosylsucrose recovered was found to be in agreement with the value which could be deduced from calculated $v(q-1)$. The good agreement of recovery of oligosaccharides and the calculated amount served as a confirmation of the reliability of the analytical procedure outlined above. On a molar basis, 95 % of the total oligose formed in this experiment was 1^F-β-fructosylsucrose and 4 % was fructosyl-1^F-β-fructosylsucrose. All other oligosaccharide products therefore can have represented only 1 % of the total oligosaccharides formed.

Information about the relative molar acceptor activity of sucrose and products arising from it can be deduced from knowledge of the quantitative composition of the levansucrase–sucrose reaction mixture. A set of calculated values for relative acceptor activity (product formed/acceptor present) of major constituents of the system is presented in Table 3. The important features are the relatively low order of the activity of water and sucrose glucocarbinols as compared with C-1 of the sucrose fructocarbinols, the excellence of 1^F-β-fructosylsucrose over sucrose and the extremely high activity that has to be ascribed to an 'average' terminal group of levan. It is assumed here, but has not been proved, that all the reactions considered are mediated by the same enzyme entity.

Reactivity of fr ~ R and fr < R with glucose

Glucose, like many other aldoses, is a powerful inhibitor of levan synthesis (Hestrin & Avineri-Shapiro, 1944). It seemed of interest to learn whether fructosyl transfer to glucose-carbinol sites could account for the inhibitory activity of glucose, as in the glucose inhibition of sucrose hydrolysis by yeast and mould fructosidases (Bealing, 1953; Edelman, 1954). The amount of fructosyl transferred to carbinol sites of glucose was found to be far too small to account for the marked inhibitory effect on levan formation. On the other hand, a rapid uptake of the fructosyl group of fr ~ enz by the carbonyl site of glucose probably accounts largely for the inhibitory effect of glucose on levan production (Hestrin *et al.* 1955).

Doudoroff & O'Neal (1945) suggested that terminal fr < groups in levan chains can be transferred to glucose carbonyl by levansucrase at a measurable rate provided that the sucrose thus formed is removed continuously by hydrolysis.

To examine the reversibility of fructosyl transfer by *A. levanicum* levansucrase, the di-, tri-, tetra- and higher saccharides of structure class fr < R as listed in Table 1 were incubated in 1 % (w/v) concentration with 2 % (w/v) glucose in the presence of levansucrase at 37°, pH 5·4, for 4 hr. Under the conditions of this experiment, the enzyme concentration used was sufficient to cause apparently complete cleavage of a 2 % sucrose solution. In control runs, similar reaction mixtures were incubated in the absence of glucose. The occurrence of reaction was examined by spotting on paper oligosaccharides containing 0·3 mg. of carbohydrate. The chromatograms were developed with solvent systems (Feingold *et al.* 1956) that were appropriate

Table 2. *Relative proportions of fructose, oligosaccharide and macropolymeric levan in levansucrase–sucrose system*

The procedure and method of computation are presented in the text. (*a*) Sucrose 6 % (w/v), reaction time 1 hr., standard enzyme preparation; (*b*) sucrose 50 % (w/v), reaction time 15 hr., enzyme solution prepared from cells grown on 12 % (w/v) sucrose in nutrient broth.

Reaction		Glucose formation (μmoles/ml.)	
Type	Designation*	Expt. *a*	Expt. *b*
Total transfer	$n+m+v(q-1)$	29·5	26·7
Levan synthesis	n	15·4	13·9
Sucrose hydrolysis	m	12·5	2·3
Oligosaccharide synthesis	$v(q-1)$	1·6	10·5†

* For explanation of symbols, see text.

† Contributions to the total glucose from the transfers of fructosyl leading to accumulations of 1^F-β-fructosylsucrose and fructosyl-1^F-β-fructosylsucrose were found to be 9·9 and 0·5 μmol. respectively. The amounts of 1^F-β-fructosylsucrose and fructosyl-1^F-β-fructosylsucrose were determined by weight following the chromatographic resolution and total recovery of the purified components.

to the detection of unit changes in DP in the original fr < R compound. No case was found in which incubation of the fr < R compound, with or without glucose, in the presence of enzyme resulted in the appearance chromatographically of macropolymeric levan, sucrose, and/or any oligosaccharide other than the fr < R introduced, or in the liberation of more than a trace of fructose. Had more than 5 % (5 μg. of fructose on the paper chromatogram) of the tested fr < R compound entered into reaction, the chromatogram would have revealed such reaction.

Like the oligosaccharides, levan macropolymer at 1 and 3 % concentrations failed to react with glucose to afford detectable amounts of sucrose or fructose.

In view of these results, it is concluded that fr < R compounds were not utilized by levansucrase as donors to a measurable extent. For the case of all fr_n < R compounds which have been shown (Feingold *et al.* 1956) to be synthesized by levansucrase, the findings accord with the assumptions that the position of equilibrium in the reaction:

$$fr_n < R + (n)\ \text{glucose} \rightleftharpoons (n)\ fr \sim \text{glucose} + R \quad (4a)$$

is far to the left, or that the velocity of reaction in the direction to the right was negligibly slow in the conditions used, or both.

For fructosylglucose isomers, in particular, interconversion of some of these isomers and formation of one of these isomers from others, specifically the formation of 6-β-fructosylglucose from 2- and 3-β-fructosylglucoses (cf. Feingold *et al.* 1956), should have been found if the establishment of equilibrium in glucose exchanges of the general type:

$$fr < \text{glucose}^* + \text{glucose} \rightleftharpoons \text{glucose}^* + fr < \text{glucose} \quad (4b)$$

had been significantly accelerated by this levansucrase system.

Table 3. *Acceptor activities (molar basis) of components of sucrose–levansucrase reaction system*

Expts. *a* and *b* (Table 2). All values are in μmoles/ml.

Computations of average [acceptor]:

[C-1 fructocarbinol in sucrose] = initial [sucrose], it being assumed that the latter approximates to the average [sucrose]; [water] is calculated from [sucrose] with the aid of standard tables;
[reactive carbinols in fructose] = [sum of C-1 and C-6 carbinols in final free fructose], this value being then multiplied by 0·5 to obtain average for time of the experiment = [final fructose] × 2 × 0·5;
[reactive glucocarbinols] = [sum of C-2, C-3 and C-6 glucocarbinols] = initial [sucrose] × 3;
[1^F-β-fructosylsucrose] = final [1^F-β-fructosylsucrose] averaged over the time of the experiment by multiplying by 0·5;
[levan terminal groups] = [anhydrofructose residues in levan] × degree of branching, the value thus obtained then being averaged over the time of the experiment by multiplying by 0·5 = [anhydrofructose residues in levan] × 0·1 × 0·5. Note that this computation does not take account of the possible occurrence of a chain-terminating reaction step.

Computations of total of fructosyl accepted:

Net formation of a product is taken as the measure of the total transfer of fructosyl to the designated acceptor, since the product accumulated is not hydrolysed or disproportionated and the maximum molar rate of incorporation of product into levan cannot exceed the very low molar rate of formation of levan ($\sim 5 \times 10^{-5}$ μmole/ml.).*

Concentrations of 1^F-β-fructosylsucrose, fructosyl-1^F-β-fructosylsucrose, free fructose and levan formed are from data in Table 2.

Amount of total transfer to glucocarbinols in sucrose is taken as being given by the amount of fructosylglucoses (2-, 3- and 6-β-fructosylglucoses) accumulated (cf. Feingold *et al.* 1956). The values for fructosylglucoses and that for difructoses (levanbiose and inulobiose) are both rough total values estimated by inspection of a chromatogram of a concentrate of the reaction mixture after it had been freed from levan and monosaccharides.

Acceptor	Product	Expt.	Transfer (y)	Average [acceptor] (x)	Acceptor activity manifested/mole (y/x, relative to sucrose = 1) Expt. *a*	Expt. *b*
C-1 fructocarbinol in sucrose	1^F-β-Fructosylsucrose	*b*	10	15×10^2	—	1
		a	1·6	$1{\cdot}7 \times 10^2$	1	—
Water	Fructose	*b*	2	4×10^4	—	0·01
		a	12	$5{\cdot}5 \times 10^4$	0·02	—
C-1 and C-6 carbinol in fructose	Difructoses	*a*	~0·01	12	~0·1	—
C-2, C-3 and C-6 glucocarbinol	β-Fructosylglucoses	*a*	~0·05	5×10^2	~0·01	—
1^F-β-Fructosylsucrose	Fructosyl-1^F-β-fructosylsucrose	*b*	0·5	5	—	14
Levan terminal	Levan	*b*	14	0·7	—	3×10^3
		a	15	0·8	2×10^3	—

* The molar rate of formation of levan is calculated on the basis of DP $\sim 3 \times 10^5$. This value has been arrived at by measurements of diffusion and sedimentation velocities which will be presented in detail elsewhere.

DISCUSSION

Since the levan polymer is highly branched, its observed failure to react appreciably with glucose in the presence of levansucrase cannot be ascribed to insufficiency in the molar concentration of levan terminal groups. The available evidence suggests, further, that the absence of reaction with glucose need not be a consequence of the possible presence of a structural anomaly at the chain ends of native levan. Thus oligolevans (derived from native levan by partial acid hydrolysis) were readily hydrolysed by yeast invertase, yet like native levan they failed to react with glucose at an appreciable rate in the presence of levansucrase.

The free-energy change ($-\Delta F'$) during the hydrolysis of sucrose has been estimated to be at least 6 kcal. (cf. Burton & Krebs, 1953). The $-\Delta F'$ of hydrolysis of the maltose and isomaltose linkages of starch are thought to be 1–3 kcal. (cf. Kalckar, 1954; Hehre, 1951). If $-\Delta F'$ for the hydrolysis of polyfructoses and polyglucoses are taken to be similar, there is obtained for $-\Delta F'$ of transfer of fructosyl from glucocarbonyl in sucrose to fructocarbinol in a saccharide a value not less than 3 kcal. Reversal of the usual direction of transfer by adding glucose to a levansucrase–levan system might thus indeed be too small to have been detected.

Throughout an extensive series of representative fructo-oligosaccharides tested, those forming levan on incubation with levansucrase solution also formed free fructose and oligosaccharide of low DP, whereas compounds that did not form levan likewise formed little or no free fructose or oligosaccharide. Further, acceptor competition for fructosyl occurred between water and fr~R. It therefore appears probable that an identical intermediate, presumably fr~enz, is involved in the transfers of fructosyl to levan, water and donor.

A scheme of the transfer reactions that can be ascribed to the *A. levanicum* levansucrase system is presented in Fig. 3.

The nature of the primer P (eqn. 4) which initiates the growth of levan chains is still uncertain. If levan synthesis occurs by transfer of fructosyl to sucrose as initial acceptor, an F-β-fructosylsucrose compound must be the primary intermediate. On the other hand, it is also conceivable that the primary step in chain formation is the reversible transfer of the fructosyl group from sucrose to enzyme, and that subsequent growth of the levan chain occurs by further transfers of fructosyl to the formed fr~enz compound. In order to evaluate the possible roles of 1^F- and 6^F-β-fructosylsucroses and of the hypothetical fr~enz compound as intermediates in levan synthesis, further knowledge of their relative acceptor activities is required. Evidence has also been obtained by us (unpublished work) that partly hydrolysed levans can serve in levan synthesis as fructosyl acceptors. If fr~enz is a primary acceptor, an effect of enzyme concentration on the DP of the polymer formed could be expected. This question, and the effects of chain length and linkage type on the acceptor activity of fructosans, are being investigated and will be reported separately in a later communication.

Since the enzyme effected no evident transfer of either single fructosyl or of chains of fructosyl within levans, inulins and F-β-fructosylsucroses from one carbinol site to another, the levansucrase solution is probably free from any chain-transferring mechanism which can operate at carbinol

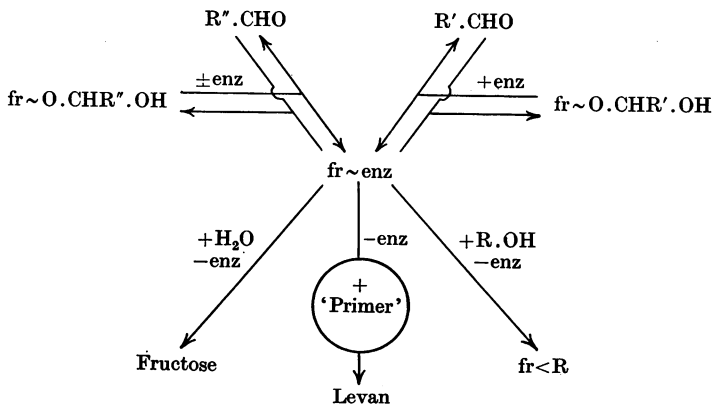

Fig. 3. Scheme of transfer reactions catalysed by *A. levanicum* levansucrase solution. ~ Designates linkage of fructofuranosyl (fr) to carbonyl of aldose (R.CHO) and to enzyme (enz); < designates linkage of fructofuranosyl to carbinol of alcohol (R.OH); R.CHO can be aldohexose (e.g. glucose), aldobiose (e.g. melibiose), aldopentose (e.g. xylose); R.OH can be sugar (e.g. sucrose) or alkyl alcohol (e.g. ethanol). Interactions of fr~enz with non-sugar alcohols will be described separately.

level, i.e. from mechanism with an action pattern analogous to that of plant *Q*-enzyme (Barker & Bourne, 1953) or animal branching factor (Larner, 1953). Instead, the branching step in levan growth may consist in the transfer of fructosyl from fr∼enz to a C-1 carbinol site of a growing levan chain. The isolation of 1^F-β-fructosylsucrose from the reaction system has shown directly that transfer of the single fructosyl group to the C-1 site of fructosyl in an acceptor is one of the activities manifested by a levansucrase preparation.

Since the primary carbinol of terminal fructosyl both in a range of tested donors (sucrose, raffinose, 6^G-β-fructosylsucrose) and in a number of non-donor compounds (Feingold *et al.* 1956; unpublished experiments) served with levansucrase as acceptor of fructosyl, it seems reasonable to suppose that fr∼enz itself could also function as acceptor and donor. A reaction series generating a protein-headed levan (fr_n∼enz) could then occur (eqns. 5 and 6), concomitantly with the reaction series generating R-headed polymer (eqns. 7 and 8):

$$fr\sim enz + fr\sim enz \rightarrow fr_2\sim enz + enz, \quad (5)$$

$$fr\sim enz + fr_{n-1}\sim enz \rightarrow fr_n\sim enz + enz, \quad (6)$$

$$fr\sim enz + fr\sim R \rightarrow fr_2\sim R + enz, \quad (7)$$

$$fr\sim enz + fr_{n-1}\sim R \rightarrow fr_n\sim R + enz. \quad (8)$$

The question arises whether the elongation and branching of levan chains represent an unrestricted type of polycondensation reaction, i.e. involve transfer of more than one fructosyl residue at a time. Levansucrase failed to transfer the $fr_n\sim$ group of $fr_n\sim R$ compounds ($n>1$) to acceptors; this suggests that transfer in the manner:

$$fr_{n1}\sim enz + fr_{n2}\sim enz \rightarrow fr_{n1+n2}\sim enz + enz \quad (9)$$

likewise probably does not occur. This is in accord with the view formulated by Hehre (1951) that the enzymic synthesis of polysaccharides from sucrose, as from glucose 1-phosphate, is a restricted polycondensation process.

Note on the classification of fructosylases

The donor–acceptor specificity of levansucrase is of interest in the context of a current discussion on the classification of fructosylases (Bacon, 1954*b*). Some pertinent findings are summarized in Table 4. The relations shown can be accommodated in a sequence varying with the enzyme source. The reactions are:

for yeast invertase:

(*a*) fr∼R + enz → fr<enz + R,
(*b*) fr<enz + carbinol ⇌ fr<carbinol + enz,
(*c*) fr<enz + water → fructose + enz,

for levansucrase:

(*a*) fr∼R + enz ⇌ fr∼enz + R,
(*b*) fr∼enz + carbinol → fr<carbinol + enz,
(*c*) fr∼enz + water → fructose + enz,

for mould invertase:

(*a*) fr∼R + enz ⇌ fr∼enz + R,
(*b*) fr∼enz + carbinol → fr<carbinol + enz,
(*c*) fr<carbinol + enz ⇌ fr<enz + carbinol,
(*d*) fr<enz + water → fructose + enz,

and possibly:

(*e*) fr∼enz + water → fructose + enz.

The scheme suggests that whereas the transfer of fructosyl from sucrose to yeast invertase proceeds practically irreversibly, i.e. with relatively large $-\Delta F'$ (probably about 3 kcal.), the corresponding transfer either to mould invertase or levansucrase proceeds reversibly with little free-energy change ($-\Delta F'$ approx. zero). Mould invertase is shown as resembling levansucrase in the capacity to react reversibly with fr∼R, and as resembling yeast invertase in the ability to hydrolyse products of structure fr<R. This poses the question whether mould invertase is a single fructosidase or a mixture. The assumption that the mould preparation may contain distinct sucrases—one predominantly a hydrolase, the other predominantly a transfructo-

Table 4. *Types of transfructosylations catalysed by different fructosylases*

+, Designates demonstrated transfer; −, designates that reaction was investigated and found not to be detectable.

	Transfructosylation* types					
	From fr∼R to oxygen in			From fr<R to oxygen in		
Fructosylase	Water	R.OH	C-1 of aldose	Water	R.OH	C-1 of aldose
Yeast invertase	+	+	−†	+	+§	−†
Mould invertase	+	+	+†	+‡	+‖ −†	−†
A. levanicum levansucrase¶	+	+	+	−	−	−

* Bacon (1954*b*).
† Edelman (1954).
‡ Bealing (1953).
§ Whelan & Jones (1953).
‖ Pazur (1953).
¶ Data from present paper.

sylase—has been supported by Kurasawa, Saito, Honma & Yamamoto (1955).

Bealing & Bacon (1953) have suggested that the singular ability of levansucrase to form a macropolymeric product may depend on a high affinity of levansucrase for large acceptor molecules. A contributing factor in the synthesis of macropolymer by levansucrase may be that levansucrase, in contrast to mould and yeast invertase, lacks the ability to hydrolyse the anhydrofructose chains which it forms.

SUMMARY

1. An improved procedure for the extraction of *Aerobacter levanicum* levansucrase and a micro test for levansucrase activity are described.

2. Levan produced from sucrose by cell-free levansucrase solution was methylated. Examination by paper chromatography of the products of hydrolysis of the methylated polysaccharide indicated that it has a branched structure similar to that of levan from cultures of *A. levanicum* and other bacterial species. This conclusion has been further supported by infrared analysis.

3. The effect of levansucrase on representatives of several classes of oligofructosides has been examined. All compounds examined with a terminal β-fructofuranosidic group linked to the anomeric carbon of an aldose formed levan, whereas compounds with the same group linked to a carbinol carbon were not observed to be attacked by levansucrase. It is suggested that the fructosidic linkage in the former group, typified by sucrose, has the higher free-energy content. Members of this class are therefore described by a high-energy symbol fr~R, whereas compounds of the second class are designated fr<R.

4. Formation of levan from compounds of the type fr~R has been observed with R as follows: glucose, galactose, xylose, 6-α-galactosylglucose (melibiose), 6-β-fructosylglucose and 6-α-galactosylmelibiose.

5. Substitution of the fructosidic moiety of fr~R at position C-1, C-3 or C-6 by a glycosyl group led to loss of the capacity to form levan.

6. Formation of levan from fr~R was accompanied in all cases by concomitant formation of oligosaccharides (largely fr_n<fr~R), free fructose and free aldose (R).

7. Constituents of a sucrose–levansucrase reaction mixture differed markedly from each other in activity as fructosyl acceptors. The acceptor activities (molar basis) were: water≪sucrose≪1-β-fructosylsucrose≪average terminal group of the growing levan molecule.

8. The findings conform to the suggestion that the transformations catalysed by levansucrase comprise a readily reversible, followed by a not readily reversible, step:

$$fr\sim R + \text{enzyme} \rightleftharpoons fr\sim \text{enzyme} + R, \quad (1)$$

$$fr\sim \text{enzyme} + \text{acceptor} \rightarrow fr<\text{acceptor} + \text{enzyme}, \quad (2)$$

where the acceptor can be either water or carbinol.

9. Modes of chain elongation and branching in levan synthesis and a classification of fructosylases are discussed.

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Note added in proof. The recent work of Péaud-Lenoël & Dedonder (1955) and Péaud-Lenoël (1955) indicated that levansucrase of *Bacillus subtilis* forms compounds of type fr~R from a preparation of degraded levan in the presence of suitable aldohexoses (R). Further experiments testing the reversibility of transfructosylation catalysed by *Aerobacter levanicum* levansucrase were therefore undertaken.

Reaction mixture containing 2% (w/v) of glucose and 1% (w/v) of donor [levulan DP 38 and oligolevans of DP in the range 3–12 prepared from *A. levanicum* levan] were incubated at pH 5·4 with levansucrase [0·28 unit as defined by Péaud-Lenoël & Dedonder (1955)/ml. of reaction mixture] in the presence of excess of dextransucrase. Portions of the reaction mixtures containing 0·2 mg. of donor were transferred at spaced time intervals up to a total reaction time of 54 hr. to filter paper and examined chromatographically for fructose (sensitivity of analytical method: 3 μg. of fructose). Under these conditions, sucrose formed could be expected to be acted on by dextransucrase, and a continuing reaction leading finally to total net conversion of the donor into fructose should have occurred. In fact, the amount of fructose formed was found to be very small: 3 ± 1 mg. of fructose from 100 mg. of donor.

In the light of the results, formulation of equilibrium constants of reactions catalysed by levansucrase is deferred until more complete information on the structure of the reactants becomes available.

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The Mechanism of Polysaccharide Production from Sucrose

4. ISOLATION AND PROBABLE STRUCTURES OF OLIGOSACCHARIDES FORMED FROM SUCROSE BY A LEVANSUCRASE SYSTEM

By D. S. FEINGOLD, G. AVIGAD & S. HESTRIN
Laboratory of Microbiological Chemistry, Department of Biochemistry, Hebrew University-Hadassah Medical School, Jerusalem, Israel

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Levan-forming systems (levansucrase) transfer the β-fructofuranosyl group of sucrose in an essentially irreversible manner to carbinol sites in suitable acceptors (Hestrin, Feingold & Avigad, 1956). The levansucrase-mediated transfructosylation can be either 'polyrepetitive', leading to macropolymer (levan), or 'oligorepetitive', resulting in formation of oligosaccharides. Oligorepetitive transfructosylation is a property of ordinary mould and yeast fructosidases as well as of levansucrase. On the other hand, the capacity for polyrepetitive transfer appears to be specific for fructosylase of the levansucrase type (Bealing & Bacon, 1953; for review see Bacon, 1954*a*). The factors which determine the polymerization ceiling of the oligorepetitive process and the relation of concurrent oligorepetition and polyrepetition in a levansucrase system acting on sucrose are uncertain.