The Mechanism of Polysaccharide Production from Sucrose

By S. HESTRIN and S. AVINERI-SHAPIRO, *Chemistry Department, Cancer Laboratories, and Department of Hygiene and Bacteriology, The Hebrew University, Jerusalem*

(*Received* 8 *October* 1943)

The recent preparation of levansucrase, like that of dextransucrase and phosphorylase, brings a new and powerful tool to bear on the study of macromolecular biosynthesis (Hestrin, Avineri-Shapiro & Aschner, 1943; Hestrin & Avineri-Shapiro, 1943; Hehre & Sugg, 1942; Stacey, 1942, 1943; cf. also Cori, 1939; Hanes, 1940; Behrens & Bergmann, 1939).

Recent work on polysaccharide production *in vitro* has largely been dominated by the discoveries of the Cori school concerning the central role of phosphorylase and phosphate in the biological breakdown and formation of glycogen and starch. The experiments reported below describe the reaction balance-sheet, the substrate requirement, and the inhibitors and activators of a levan-forming enzyme system. Inorganic phosphate does not appear to be necessary for levan production. This strengthens the view that phosphorylation is but one of several processes used by living cells for the synthesis of macromolecules of the polysaccharide class.

The reactions leading to levan and dextran production may have implications in the field of oligase specificity (Harrison, Tarr & Hibbert, 1930; Norman, 1937; Hehre, 1941; Hestrin & Avineri-Shapiro, 1943), and in the development of cell properties such as virulence, resistance, and antigenicity, known to be conditioned by the dextran- and levan-containing bacterial capsule (Beijerinck, 1912; Heidelberger, 1927; Evans, Hawkins & Hibbert, 1941; Hehre & Sugg, 1942). Moreover, levan and dextran occur not only in many bacteria but also in several fungi (Kopeloff, Welcome & Kopeloff, 1920; Anderson & Raistrick, 1936) and in a variety of green plants (Hibbert, Tipson & Brauns, 1931; Challinor, Haworth & Hirst, 1934; Hassid, 1939); and it is thus possible that the mechanism of polysaccharide production from sucrose, exemplified by the *Aerobacter levanicum* levansucrase used in the present investigation, is of widespread occurrence throughout microbe and plant life.

METHODS

Enzyme preparations

Levansucrase was prepared in sterile solution from cells of *Aerobacter levanicum* (Hestrin *et al.* 1943). The autolyzed, particle-free enzyme solutions were stored in a refrigerator in the presence of thymol and chloroform and were used within a week of preparation. The enzyme preparation was non-reducing to Fehling's solution and, being free from levan and furanosides, failed to liberate reducing sugar after hydrolysis for 1 hr. with boiling 0·5% oxalic acid. Sterility at 37° was maintained by addition to reaction mixtures of a drop of a solution of thymol in chloroform.

Yeast sucrase. A commercial preparation supplied by Fraenkel and Landau, Berlin.

Inulase was in the form of an ethanol- and acetone-dried preparation obtained by us from an inulin-fermenting *Torula* yeast.

Analytical methods

Levan in the presence of hexoses and oligosaccharides was determined as described by Hestrin *et al.* (1943), and is expressed as mg. glucose/100 ml. Levan in the presence of ethanol-precipitable substrates (inulin, phosphoric esters) was assessed qualitatively by observation of the change in turbidity, with the aid of a photoelectric nephelometer during the course of the incubation. The minimum amount thus detectable was 30 mg./100 ml. Further tests were made for levan, in the presence of inulin, as follows: samples of the reaction mixture were added to two parts of 96% ethanol and a drop of dilute $CaCl_2$ solution. Levan was precipitated immediately and could be separated by centrifuging within 5 min., i.e. before inulin precipitation set in. By repetition of the procedure complete separation of levan from inulin was effected. The amount of the levan could then be determined in the ethanol precipitate in the usual manner.

Reducing sugar was determined according to Somogyi (1926).

Sucrose was determined in the presence of levan, (*a*) in the heat-inactivated but otherwise untreated reaction mixture, on the basis of the final increase in reducing power produced therein by incubation with invertase; and (*b*) in reaction mixtures which had been freed from levan by addition of ethanol to 72% strength, from the increase of reducing power produced, in a suitably diluted sample, by treatment for 1 hr. with 0·5% oxalic acid in a boiling water-bath.

Total fructose (free and combined) was determined colorimetrically (Hestrin *et al.* 1943).

Free fructose was estimated in the presence of aldose, sucrose and levan by deducting from the total fructose the sum of the amount of fructose equivalent to the determined sucrose and that present as levan. The results were confirmed by applying the same procedure to reaction mixtures which had previously been freed from levan by ethanol precipitation.

Free glucose was determined as the difference between the total reducing sugar and free fructose. Hypoiodite methods were unsuitable.

Substrates

The sugars used were mostly purchased from British Drug Houses, Ltd. Phosphoric esters were prepared in this laboratory, fructose-1-6-diphosphate and fructose-6-phosphate as recommended by Kobel & Neuberg (1932), and glucose-1-phosphate as described by Hanes (1940).

Methyl fructofuranoside was prepared in the form of a crude syrup according to the method of Menzies (1922). Of the total acid-labile fructoside in this preparation, 64% was invertase-labile. Levan was prepared enzymically from aqueous sucrose solution by the action of *Aerobacter* autolysate. The polysaccharide was precipitated several times from water by 72% ethanol and was free from sucrose or reducing sugars. Its properties have previously been described (Hestrin *et al.* 1943).

RESULTS

The substances formed when levansucrase acts upon different sugars

Sucrose

Typical results of an analysis of the products formed as the result of incubating sucrose with a solution of levansucrase are given in Part I of Table 1. Part II of this table gives the results of calculations pertaining to the amounts of various substances present after incubation of the mixture of sucrose and enzyme. Where they overlap, the results in Part II of Table 1 agree satisfactorily with those in Part I.

As the total reducing power released from the mixture by mild acid hydrolysis is apparently not significantly reduced by incubation (Exp. 2, Table 1), it is to be assumed that no significant amount of acid-resistant (non-furanosidic) oligo- or polysaccharide has been formed by the action of the enzyme. The diminution, as the result of incubation, in the amount of reducing power released from the mixture by the action of invertase (Exp. 3), is apparently wholly accounted for by the formation of levan (Exp. 4), for we have previously shown that this polysaccharide is completely resistant to the hydrolytic action of invertase (Hestrin *et al.* 1943). As the total (free plus bound) fructose content of the mixture is not significantly diminished

Table 1. *Analysis of the products of the action of levansucrase upon sucrose*

(2 ml. of levansucrase solution, 2 ml. of citrate buffer (Sorensen, pH 5·0) and 8 ml. of 3% sucrose solution were incubated together at 37°. The incubated mixture was then subjected to the procedure described in column A below.)

Part I. Experimental data

Exp.	Treatment of incubated mixture (A)	Reducing power (mg. glucose/100 ml.) of incubated mixture after the treatment described in column A: Incubation time 0 hr. (B)	Incubation time 24 hr. (C)	Difference Q (=C − B)	Reaction during incubation believed to be responsible for the observed difference Q (=C − B)
1	None	0	1160	+1160	—
2	Hydrolysis by heating with 0·5% oxalic acid for 1 hr.	1942	1910	− 32	Disappearance of furanosidic oligo- and polysaccharides
3	Hydrolysis by yeast invertase	1945	1531	− 414	?
4	Hydrolysis of ethanol-precipitable fraction (levan) by hot oxalic acid	0	400	− 400	Formation of levan
5	Hydrolysis of ethanol-soluble fraction (sucrose) by hot oxalic acid	1922	335	−1587	Disappearance of sucrose
6	Colorimetric estimation of total (free plus bound) fructose	974	924	− 50	Disappearance of total (bound and free) fructose

Part II. Interpretation of experimental data in Part I

Exp.	Calculation (references are to the results of the exps. in Part I above)	Calculated reducing value (mg. glucose/100 ml.) for: Incubation time 0 hr. (B)	Incubation time 24 hr. (C)	Difference Q (=C − B)	Reaction during incubation believed to be responsible for the difference Q (=C − B)
5	Difference between 3 and 1	1945	371	−1574	Disappearance of sucrose
6	50% of 2	971	955	− 16	Disappearance of total (bound plus free) fructose
7	50% of 5	966	176	− 790	Disappearance of fructose bound as sucrose
8	6 − (4 + 7)	6	358	+ 352	Appearance of free fructose
9	1–8	−6	802	+ 808	Appearance of free glucose

by incubation (Exp. 6), it is deduced that no significant interconversion of aldose and ketose takes place. Since the sum (1560) of the reducing powers released as the result of incubation (Exp. 1) and by hydrolysis of the levan formed during incubation (Exp. 4) is almost identical with the mean reducing equivalent (1580) of the sucrose which disappears (Exp. 5, Table 1, Parts I and II), it is to be concluded that all the free reducing components of the incubated mixture possess the reducing capacities of hexoses; the results of Exp. 8 (Table 1, Part II) show that fructose must be one contributor to the reducing activity of the incubated mixture.

The non-reducing component of the incubated mixture consists wholly of an ethanol-precipitable, acid-labile, invertase-resistant material which we describe as levan, together with an acid-labile sugar, hydrolyzable by invertase but not precipitable by ethanol, which must be sucrose or a mixture of this substance with a higher sugar similar to sucrose in all these respects. Since the latter alternative is extremely improbable and unsupported, it may be concluded that the reaction products of levansucrase action on sucrose are solely three: glucose, fructose and levan.

The total amount of sucrose ($Q_5 = 1580$; Exp. 5, Table 1) transformed by the enzyme in the course of the reaction is seen to be practically equivalent to the sum: levan formed + free glucose liberated + free fructose liberated;

$$Q_4 + Q_9 + Q_8 = 400 + 808 + 352 = 1560.$$

Hence the net reaction result may be represented by the following equation:

$$(n+m)\ \text{sucrose} + (n)\ H_2O \rightarrow (n+m)\ \text{glucose} + (n)\ \text{fructose} + (\text{levan}), \quad (1)$$

where m and n are weights of sucrose respectively converted into levan and glucose, and hydrolyzed to fructose and glucose, and where (levan) represents m fructosidic residues of sucrose converted into levan.

The values m and n are readily measurable as $2\,Q_4$ and $Q_1 - Q_4$ (Table 1), respectively. In the experiment reported in Table 1, they equalled respectively 800 and 760 mg. glucose/100 ml. of reaction mixture. The efficiency ratio of sucrose conversion to polysaccharide and to fructose respectively (m/n) was therefore 1·05; i.e. roughly one molecule of sucrose was hydrolyzed for each molecule converted into levan and glucose.

Raffinose

Raffinose is hydrolyzed by emulsin to give galactose and sucrose, and by invertase to give fructose and melibiose. An experiment accounting for the fate of raffinose which is incubated with levansucrase is summarized in Table 2.

Table 2. *Analysis of the products of the action of levansucrase upon raffinose*

(2 ml. of levansucrase solution, 2 ml. of citrate buffer (pH 5·0) and 2 ml. of a 6% solution of raffinose hydrate were incubated at 37°.)

Exp.	Treatment of incubated mixture (A)	Reducing power (mg. glucose/100 ml.) of incubated mixture after the treatment described in column A: Incubation time 0 hr. (B)	Incubation time 20 hr. (C)
1	None	0	342
2	Hydrolysis by heating with 0·5% oxalic acid for 1 hr.	1205	1213
3	Hydrolysis of ethanol-precipitable fraction (levan) by hot oxalic acid	0	126

It follows from the earlier analysis of the sucrose transformation (Table 1) that sugar is not desmolyzed by the levansucrase preparation. Hence, if galactose is liberated from raffinose during levan production, the reducing power of the mixture following mild acid hydrolysis (Exp. 2, Table 2) should rise with the incubation time. Actually, the value remained unchanged throughout incubation. It may be concluded, therefore, that sucrose is not liberated from raffinose through removal of galactose by the action of the enzyme preparation; and that the reducing sugar produced from raffinose consists either of melibiose or fructose, or of both sugars together. Since, however, the amount of levan formed (Exp. 3) is considerably less than the reducing component calculated in equivalents of mg. glucose/100 ml. (Exp. 1, Table 2), it is necessary to conclude that fructose is formed as well as melibiose. It is, therefore, possible to describe the transformations undergone by raffinose in the presence of the levansucrase preparation by the following equation:

$$(n+m)\ \text{raffinose} + (n)\ H_2O \rightarrow (n+m)\ \text{melibiose} + (n)\ \text{fructose} + (\text{levan}), \quad (2)$$

where (levan) represents m fructosidic residues of raffinose converted into levan, and where n and m are mols of substrate hydrolyzed to melibiose and fructose, and converted into melibiose and levan. The efficiency of raffinose conversion into levan in the experiments of Table 2 was similar to that for levan production from sucrose (Table 1):

$$m/n = \frac{2 \times 126}{342 - 126} = 1{\cdot}11.$$

The properties of levansucrase

Inhibition by glycolytic and respiratory poisons

None of the poisons tested possessed any significant inhibitory activity in the concentrations we used, with the possible exception of phlorrhizin (Table 3).

Table 3. *Effect of respiratory and glycolytic poisons on the activity of levansucrase*

(1 ml. of levansucrase solution, 1 ml. of citrate buffer (pH 5·0) and 1 ml. of 15% sucrose were incubated together at 37° for 6 hr. after the poison had been added to give the recorded concentration.)

Poison added	Concentration of poison (M)	Levan produced (mg./100 ml.)	Effect of the poison on levansucrase activity
None	—	163	—
Monoiodoacetate	1/1000	174	None
NaF	1/50	163	,,
KCN	1/500	187	,,
Phlorrhizin	1/150	119	? Slight inhibition
,,	1/1000	170	None

Possible activators of levansucrase

Substances known to participate in the enzyme system concerned with the production of starch or glycogen were tested for implication in the levansucrase system.

(*a*) *Adenylic acid.* By a procedure for the estimation of adenylic acid suggested by Shapiro (1943), it has been possible to show that levan-synthesizing autolysates of *Aerobacter levanicum* are without coenzyme effect on the synthesis of glycogen by a suitable phosphorylase.

(*b*) *Levan.* Since a 'levan-free' enzyme solution has been employed throughout this investigation, it is probable that the presence of levan is not, as is the case with glycogen in glycogen synthesis, a necessary condition of levan production. But the levansucrase autolysate was prepared from cells grown on sucrose, and it is possible that there were traces of undetected levan. Since, however, cells grown on glucose yield an active autolysate, this possibility must be considered highly unlikely (Hestrin *et al.* 1943).

(*c*) *Dialyzable enzyme constituents.* From Table 4 it is seen that dialysis has little effect on the activity of levansucrase.

(*d*) *Phosphate.* Active dialyzed enzyme solution is free from inorganic phosphate. Levan synthesized by the dialyzed enzyme from sucrose is likewise free from phosphate. Experiments reported below show that various phosphate esters of sugars fail to support levan production. The participation of phosphate ions in the process of levan production must therefore be regarded as highly unlikely.

Table 4. *Influence of dialysis on activity of levansucrase*

(1 ml. of levansucrase solution, 1 ml. of citrate buffer (pH 5·0) and 1 ml. of 15% sucrose were incubated at 37° for 4 or 24 hr. The dialyzed enzyme solution had been dialyzed in cellophan against running tap water for 10 hr. and then against distilled water for 10 hr.)

Enzyme preparation	Activity of enzyme (levan produced (mg./100 ml.) in 4 hr.)	Activity of enzyme (levan produced (mg./100 ml.) in 24 hr.)
Undialyzed	128	398
Dialyzed	103	390

Reversibility of the action of levansucrase

Reversal of the reactions brought about by levansucrase was not demonstrated in mixtures composed as follows, which were incubated for 48 hr. at 37°: (1) Levan (300 mg./100 ml.); levansucrase (1 ml.); phosphate-citrate buffer (pH 5·0, 2 ml.). (2) As mixture (1), but with addition of glucose (300 mg./100 ml.). (3) As mixture (1), but with a levan concentration of 500 mg./100 ml. (4) As mixture (2), but with further addition of fructose (300 mg./100 ml.). In control experiments the enzyme solution used was shown to be active on sucrose.

No decrease of levan content nor liberation of reducing sugar could be detected during incubation in any of the mixtures. Mixtures 1, 2 and 3, after removal of levan by ethanol precipitation, gave a negative fructose reaction with phosphomolybdic acid. Moreover, it is also evident that, at least under the conditions of our tests, the *Aerobacter* autolysate does not act as a levan hydrolase.

It is to be noted, however, that living cells of the *Aerobacter* slowly ferment the levan formed by them, as also levan formed by *Bacillus subtilis* (Hestrin *et al.* 1943). This effect might conceivably be the outcome of a reversal *in vivo* of the direction of levansucrase action, but can be plausibly explained also by the assumption that breakdown of levan by the living cells is due to the action of a special polyfructosidase which is lost in the course of autolytic preparation of our levansucrase solution.

Substrate specificity

Sugars were tested by incubation, for 40 hr. at 37°, of mixtures composed of 1 ml. enzyme, 1 ml. citrate buffer (pH 5·0), and 1 ml. substrate (final conc. M/10). Inulin in similar mixtures was tested at 3% concentration. No levan production means formation of less than 20 mg. levan/100 ml., the limit of sensitivity of the tests employed.

(1) *Hexoses.* Glucose, fructose and invert sugar were not converted into levan.

(2) *Oligosaccharides.* Trehalose, maltose and lactose failed to produce levan. Raffinose produced 162 mg. levan/100 ml. in 40 hr. as against the production from sucrose of 320 mg. under the same conditions.

(3) *Glycosides.* Methylfructofuranoside was neither hydrolyzed nor converted into levan by the levansucrase preparation. Inclusion of yeast invertase in the reaction mixtures resulted in rapid hydrolysis, but did not induce production of levan. Sucrose, when added to the reaction mixture containing the methylfructoside, was converted into levan.

(4) *Polysaccharides.* Inulin was neither hydrolyzed nor converted into levan by the levansucrase preparation, though sucrose in the presence of inulin was so converted (cf. Niven, Smiley & Sherman, 1941). Inclusion of inulase, in the form of a dry powder, in reaction mixtures of inulin and levansucrase led to liberation of 1204 mg. reducing sugar/100 ml. in 20 hr., i.e. about as much as was produced from sucrose by levansucrase in a control mixture, but did not induce production of levan.

(5) *Phosphate esters.* K-glucose-1-phosphate, Na-fructose-1-6-diphosphate, and Ca-fructose-6-phosphate were dissolved in water, brought to pH 5·0 by addition of HCl and tested as to the availability for levan production after suitable dilution (2 parts substrate solution : 1 part enzyme). Levan was in no case formed, but sucrose, when added to the mixtures, was converted into levan. Glycogen was not formed from glucose-1-phosphate by the levansucrase preparation under the conditions of the test.

Sugar inhibitor specificity

The results of experiments to test the effect of different sugars and sugar alcohols on levan production from sucrose are summarized, and classified in groups of rising inhibitory power, in Table 5. It is evident from the data that certain carbohydrates exert a marked inhibitory effect on levansucrase activity, that the inhibitory effect under the conditions of these experiments may vary with the enzyme sample, and that the degree of inhibition produced by a carbohydrate depends on its configuration. It is possibly significant that, but for one exception (sorbose), all reducing sugars (*d*-glucose, *d*-galactose, maltose, *d*-xylose, *l*-arabinose, lactose) which produced appreciable inhibition (> 15 %) are similar in configuration at C_2, whereas all tested reducing sugars (*d*-mannose, *d*-fructose, *d*-glucosamine) which differ from glucose at C_2, are non-inhibitory. The non-reducing sugar derivatives tested were, except for α-methylglucoside, non-inhibitory (mannitol, sorbitol, trehalose). It should be noted that Koser & Saunders (1932) have shown that strains of *Aerobacter* ferment α-methylglucoside promptly.

Table 5. *Inhibition of levansucrase activity by sugars and sugar derivatives*

(1 ml. of levansucrase solution, 1 ml. citrate buffer (pH 5·0) together with substrate (sucrose) and inhibitor solutions to make 3 ml. totally were incubated together at 37° for 20 hr. The initial concentration of sucrose was M/34, and that of the inhibitor M/45. In control experiments without inhibitors present 180–245 mg./100 ml. of levan were produced.)

Inhibitor present	Levansucrase activity (% of normal)	Inhibitor present	Levansucrase activity (% of normal)	
d-Mannose	98	*d*-Xylose	24	
d-Mannitol	97	*l*-Arabinose	24	
d-Fructose*	96	*d*-Glucose	24	
l-Sorbitol	90	,,	52	Mean = 38
d-Glucosamine	94	,,	29	
Trehalose	92	,,	48	
		l-Sorbose	38	
d-Galactose	58	Lactose	24	
,,	76			
α*d*-Methyl-glucoside	51			
Maltose	62			
,,	63			

* 16% of fructose failed to produce significant inhibition.

In view of the possible role of the glucose moiety of sucrose, and of the melibiose moiety of raffinose, in determining the combination of these substrates

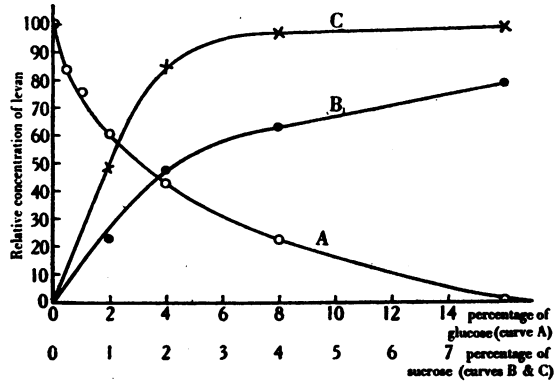


Fig. 1. Inhibition of levansucrase by glucose. *Curve A.* Formation of levan from 1% sucrose in the presence of varying concentrations of glucose. (Levan conc. 100 = 200 mg./100 ml.) *Curve B.* Formation of levan from different concentrations of sucrose in the presence of 4% glucose. (Levan conc. 100 = 368 mg./100 ml.) *Curve C.* Formation of levan from different concentrations of sucrose in absence of added glucose. (Levan conc. 100 = 368 mg./100 ml.)

with levansucrase, closer investigation of the nature of the inhibition of levansucrase by free glucose seemed desirable. The experiments are presented in Fig. 1.

It is evident from curve *A* that the effect of glucose is a function of its concentration and that the inhibitory effect increases more gradually as the concentration of glucose increases, complete inhibition of levan production from 1% sucrose being approached only when the glucose concentration is about 16%. From the curves *B* and *C* it is evident that the inhibitory effect of glucose diminishes as its concentration relative to that of sucrose falls. It may be concluded, therefore, that the inhibition of levan production from sucrose by free glucose corresponds in general form to an inhibition by a competition for the enzyme.

DISCUSSION

The experiments presented in this paper justify description of levansucrase as an enzyme system which forms levan from suitable aldose-fructofuranosides. It has been shown above for the case of a cell-free levansucrase preparation derived from *Aerobacter levanicum* that its net action on sucrose and raffinose can be represented in a general reaction:

$$(n+m)\ \text{aldose-fructofuranoside} + (n)\ H_2O \rightarrow (n+m)\ \text{aldose} + (n)\ \text{fructose} + [\text{levan}], \quad (3)$$

where aldose is glucose in sucrose, or melibiose in raffinose, where m and n are mol numbers experimentally measurable, and where [levan] represents m fructofuranose residues of substrate polymerized to molecules of levan. It follows that production of aldose ranks equally with levan formation as a product of levansucrase action. It remains to be ascertained, however, whether fructose production is a function of the same enzyme.

The absence of any measurable deficit in the balance-sheet summarized in formula (3) leads to the conclusion that the polymerized material is completely comprised in the fraction termed 'levan'. Since it is non-reducing to Fehling's solution, the polymer must either be a polysaccharide of chains which are sufficiently long to render the reducing power negligibly small or which are looped or otherwise terminated so as to eliminate the reducing groups. The non-dialyzable nature of the product, its viscosity in aqueous solutions, its lack of reducing power, its ready precipitation, even in high dilution, by ethanol, and its general resemblance to the levan from living cells, are all consistent with the view that it is indeed a levan.

The following facts establish a striking analogy between the production of polysaccharide from sucrose and from glucose-1-phosphate, respectively: (1) The absence of any energetic coupling of synthesis with sugar desmolysis (cf. Tables 1–3). (2) The colloidal character of the macromolecular end-product (cf. Hestrin *et al.* 1943). (3) The ease with which synthesis proceeds at moderate temperature with relatively low substrate concentration. (4) The substrate in both cases is not a simple hexose, but a glycosidic derivative higher in energy content than a simple hexose (cf. Hehre & Sugg, 1942). (5) The apparent virtual absence from the reaction mixtures of oligosaccharides. On the other hand, it needs to be pointed out that reversal of the direction of levansucrase activity has not so far been demonstrated though such reversal is, from analogy, to be expected. However, for reasons which have been given above, the negative result of the reversal experiments is not considered conclusive.

If the analogy is valid that polysaccharide production from sucrose is another example of synthesis-by-equilibrium, an interesting possibility emerges, namely, that the glucose in levan production from sucrose, the melibiose in levan formation from raffinose, and fructofuranose (though not the fructopyranose actually obtained) in dextran production from sucrose, all play a role formally analogous to that played by phosphate in the enzymic production of glycogen and starch. This possibility is supported by the present finding that glucose inhibits levan production as would a competitive inhibitor (Fig. 1).

An alternative to the view that levan is produced as the result of an equilibrium reaction would be the theory that it proceeds because it is coupled with some energy-yielding reaction. Sugar desmolysis has been excluded, but not so another energy-yielding process, that of sucrose hydrolysis to invert-sugar as represented in (3) by the n-term. For the *Aerobacter* levansucrase preparation used in this study m/n is about unity for the experiments on sucrose as well as those on raffinose 1. In the related case of dextran production from sucrose, however, Hehre (1941) has shown that the reaction proceeds in accordance with the equation:

$$(m)\ \text{sucrose} \rightarrow (m)\ \text{fructose} + [\text{dextran}], \quad (4)$$

where [dextran] represents m glucosidic residues of sucrose converted into dextran. It is clear from the equation that, in dextran production at least, accumulation of free invert sugar is completely dissociable from the conversion of sucrose into polysaccharide. For levan production a similar dissociability is therefore, by analogy, not impossible. In any case, energy coupling between the processes of levan formation and fructose production must, by the same analogy, be regarded as improbable.

It thus seems permissible provisionally to consider the reactions whereby levan and dextran are produced as energetically self-sufficient, and to describe the free energy transfer involved as an energy dismutation within the same or between similar substrate molecules, whereby part of the

substrate is built up into an energy-rich product (levan; dextran) while part is broken down into a relatively energy-poor product (aldose; ketose).

A different approach to the problem of levan and dextran production is opened up by specificity studies. Harrison *et al.* (1930) have shown—and we have earlier confirmed—that living cells of *Bacillus subtilis* do not form levan from melezitose. Since the latter sugar may be regarded as being derived from sucrose by glucose substitution in the fructose moiety, the unsuitability of melezitose for levan production suggested that an unsubstituted terminal fructofuranoside group, as found in sucrose or raffinose but not in melezitose, is the only group which is of qualitative value in determining the suitability of the substrate for levan production. According to Pascu, Wilson & Graf (1939), melezitose is 3-α-glucosido-β-fructosido $<2:1>$ α-glucoside. The furanose nature of the fructose moiety is not regarded as definitely established. The failure of several non-aldose-fructofuranosides cited above to yield levan in the presence of levansucrase shows, however, that the specificity of levansucrase is dependent not only on the fructosido- but also on the aldosido-residue of its substrate. The inhibition of levan production by free glucose provides additional evidence in support of this conclusion (see Fig. 1). It is clear that modification of the sucrose molecule, by galactose substitution at C_6 of the glucosidic residue to give raffinose, does not, as does glucose substitution in the fructosidic residue to give melezitose, deprive this substance of ability to act as a substrate for levansucrase. A similar conclusion with regard to the specificity of dextransucrase need not be drawn.

A question of fundamental importance in discussion of the reaction mechanisms leading from oligosaccharide to levan or dextran is the identity of the immediate precursor of the polysaccharide, i.e. the substrate directly involved in the polymerization. Is the metabolite in question a hydrolytic product *in statu nascendi*, i.e. glucopyranose, fructofuranose, or fructopyranose (Beijerinck, 1912; Kopeloff *et al.* 1920; Cooper & Preston, 1935), or, as will be suggested, a product other than a free hexose? The discussion of the above alternatives is limited to the case of levan synthesis.

It has been shown above that glucose-fructose interconversion does not occur in reaction mixtures of levansucrase and sucrose. Free glucose is therefore not a possible levan precursor. Free fructose is also excluded, since it has been shown not only that an equilibrium mixture of α- and β-fructopyranose is not a substrate of levan formation, but also that levansucrase does not produce levan from enzyme-substrate systems, e.g. methylfructofuranoside + invertase or inulin + inulase, wherein fructofuranose and fructopyranose are liberated *in statu nascendi* (see also Niven *et al.* 1941). It seems clear, therefore, that levansucrase is not an invertase admixed with a polymerase, which acts on free fructofuranose or fructopyranose.

That a hydrolyzing sucrase of the fructo-saccharase type is not causally concerned in levan production, i.e. in the reaction concerned with the *m*-terms of equation (3), is implicit in the above discussion. This corollary is consistent with the conclusion drawn below that the substrate specificity of the hydrolytic function of *Aerobacter* autolysate, i.e. the reaction concerned with the *n*-terms of equation (3), does not correspond to that of fructo-saccharase. Finally, it is further supported by the observation that the susceptibilities to inhibition by sugars of *Aerobacter* levansucrase, yeast fructo-saccharase and animal phosphorylase are all distinct (cf. Table 6).

Table 6. *Comparison of the sugar inhibitor susceptibilities of levansucrase, fructo-saccharase, and phosphorylase*

(+ = definite inhibitory effect; − = slight or no inhibitory effect; ... = no information.)

Sugar inhibitor	Levansucrase (*Aerobacter levanicum*)*	Fructosaccharase (yeast)†	Phosphorylase (animal)‡
Fructose	−	+	−
Glucose	+	+	+
Mannose	−	+	−
Galactose	+	+	−
Maltose	+	−	−
Lactose	+	−	...

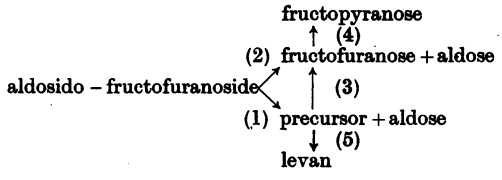
* Cf. Table 5. † Haldane (1930). ‡ Cori & Cori (1940).

There remain thus the following general possibilities: (1) That the levan precursor is a unit of which the macromolecular product is a multiple, i.e. is a monomeric fructose anhydride such as could be obtained from the original oligosaccharidic substrate by non-hydrolytic cleavage. (2) That the levan precursor is not such a unit but a substituted derivative thereof, as is the precursor (glucose-1-phosphate) of glycogen, and starch, i.e. is either sucrose or raffinose as such (Owen, 1923), or a replacement derivative of these substrates in which the original aldosidic moiety (glucose or melibiose) is replaced by an enzyme-linked group as yet undefined.

The enzyme mechanism of fructose production from sucrose and raffinose by the *Aerobacter* preparation is not easy to fit into any of the present systems of carbohydrase classification (cf. Hestrin, 1940). The view might seem tenable that the *n*-term of equation (3) exists simply because levansucrase is admixed in the enzyme preparation with ordinary hydrolyzing fructo-saccharase, but this is incompatible with the inability of the autolysate to

hydrolyze methylfructofuranoside, which by definition must be hydrolyzed by fructo-saccharase. The possibility that the production of free fructose in the reaction is due to the hydrolysis of some of the levan formed is excluded by the fact that our levansucrase solution does not contain levan-hydrolase. There remains therefore the interesting possibility that our levansucrase produces fructose from sucrose and raffinose by an oligase mechanism other than ordinary fructo-saccharase. Since the production of fructose by the action of levansucrase on raffinose does not proceed via sucrose, the enzyme in question cannot be gluco-saccharase or α-glucosidase. It must therefore be a sucrase of a new specificity type, and one which may or may not be an intrinsic component of the levan-producing enzyme system.

Summarizing then we may consider the following possibilities for the mechanism of action of *Aerobacter* levansucrase on sucrose and raffinose:



where the bracketed numerals represent steps in the reaction, (1) being catalyzed by a levansucrase component, and (2), if it is existent, by a hydrolyzing invertase. If fructose is liberated as well as levan by the action, on sucrose or raffinose, of the tested enzyme preparation, then reaction (3) must occur if reaction (2) does not, and conversely.

The bearing of the above scheme on the theory of 'direct' disaccharide fermentation deserves comment. As originally formulated two decades ago this theory was essentially negative in content, being the denial of prevailing belief that disaccharides are always hydrolyzed to hexoses by the action of known oligases before fermentation (Willstätter, 1928). In regard to the case of maltose, 'direct' fermentation acquired a positive meaning when it was shown that primary condensation of maltose units to glycogen by a path whch need not involve glucose as an intermediary was a probable initial step in maltose fermentation by certain yeasts (Willstätter & Rohdewald, 1937; Leibowitz & Hestrin, 1942). It was obvious, however, that the fermentation of disaccharides containing two different hexoses, such as lactose and sucrose, had not thus been covered. The results in the present paper are relevant to the theory of the existence of a direct sucrose fermentation in the sense that they reveal for the first time a cell-free enzyme system which transforms sucrose, at least in part, into readily fermentable material, yet apparently does not do so by hydrolysis.

SUMMARY

1. Levansucrase has been defined as an enzyme which catalyzes the following transformations:

$(n+m)$ sucrose + (n) H_2O
$\rightarrow (n+m)$ glucose + (n) fructose + [levan];

$(n+m)$ raffinose + (n) H_2O
$\rightarrow (n+m)$ melibiose + (n) fructose + [levan];

where n and m are mols of substrate converted into levan and aldose, and into fructose and aldose respectively, where [levan] represents the levan formed from m fructosidic residues of substrate and where the above reaction products represent the total amount of substrate acted upon by the enzyme.

2. Respiratory and glycolytic poisons do not significantly inhibit the action of levansucrase.

3. Phosphate, adenylic acid, levan itself, and the dialyzable components of the enzyme preparation are not essential components of the levan-forming enzyme system.

4. Reversal of the enzyme reaction which leads to the synthesis of levan has not been demonstrated but its possible existence has not been excluded. Although living cells of *Aerobacter levanicum* ferment levan, the levansucrase preparation obtained from them contained no levan-hydrolase.

5. The substrate specificity of levansucrase is high.

6. The susceptibility of levansucrase to inhibition by different sugars is distinct from that of yeast fructo-saccharase and also from that of phosphorylase.

7. The inhibition by glucose of levansucrase action on sucrose is competitive in type.

8. Our levansucrase preparation did not hydrolyze methylfructofuranoside or inulin. The hydrolyzing sucrase in the levansucrase preparation is considered to be different from either typical gluco-saccharase or fructo-saccharase.

9. Possible reaction paths leading from sucrose and raffinose to levan are discussed, and the bearing of the findings on the theory of direct sucrose fermentation is noted.

The authors wish to acknowledge with thanks a debt of gratitude to Dr M. Aschner and to Dr J. Leibowitz for their interest in this investigation and for many suggestions.

REFERENCES

Anderson, C. G. & Raistrick, H. (1936). *Biochem. J.* **30**, 16.
Behrens, O. & Bergmann, M. (1939). *J. biol. Chem.* **129**, 587.
Beijerinck, M. (1912). *Folia Microbiol.* **1**, 377.
Challinor, S. W., Haworth, W. N. & Hirst, E. L. (1934). *J. chem. Soc.* p. 676.
Cooper, E. A. & Preston, J. (1935). *Biochem. J.* **29**, 2267.
Cori, C. F. (1939). *Cold Spr. Harb. Symp. quant. Biol.* **7**, 260.
Cori, G. T. & Cori, C. F. (1940). *J. biol. Chem.* **135**, 733.
Evans, T., Hawkins, W. & Hibbert, H. (1941). *J. exp. Med.* **74**, 511.
Haldane, J. B. S. (1930). *Enzymes*, p. 48. London: Longmans, Green and Co.
Hanes, C. S. (1940). *Proc. Roy. Soc.* B, **129**, 174.
Harrison, F., Tarr, A. H. & Hibbert, H. (1930). *Canad. J. Res.* **3**, 449.
Hassid, W. Z. (1939). *J. Amer. chem. Soc.* **61**, 1223.
Hehre, E. (1941). *Science*, **93**, 237.
—— & Sugg, G. (1942). *J. exp. Med.* **75**, 339.
Heidelberger, M. (1927). *Chem. Rev.* **3**, 403.
Hestrin, S. (1940). *Enzymologia*, **8**, 193.
—— & Avineri-Shapiro, S. (1943). *Nature, Lond.*, **152**, 49.
—— —— & Aschner, M. (1943). *Biochem. J.* **37**, 450.
Hibbert, H., Tipson, R. S. & Brauns, F. (1931). *Canad. J. Res.* **4**, 221.
Kobel, M. & Neuberg, C. (1932). *Handb. d. Pflanzenanalyse*, **1**, 546. Berlin: Springer.
Kopeloff, N., Welcome, C. & Kopeloff, L. (1920). *J. biol. Chem.* **43**, 171.
Koser, S. & Saunders, F. (1932). *J. Bact.* **24**, 267.
Leibowitz, J. & Hestrin, S. (1942). *Biochem. J.* **36**, 772.
Menzies, R. (1922). *J. chem. Soc.* **121**, 2238.
Niven, C., Smiley, K. & Sherman, J. (1941). *J. biol. Chem.* **140**, 105.
Norman, A. G. (1937). *The Biochemistry of Cellulose, Polyuronides, Lignin, etc.* Oxford: University Press.
Owen, W. (1923). *J. Bact.* **8**, 420.
Pascu, E., Wilson, E. & Graf, L. (1939). *J. Amer. chem. Soc.* **61**, 2675.
Shapiro, B. (1944). *J. biol. Chem.* (in the Press).
Somogyi, M. (1926). *J. biol. Chem.* **70**, 599.
Stacey, M. (1942). *Nature, Lond.*, **149**, 639.
—— (1943). *Chem. Ind.* **62**, 110.
Willstätter, R. (1928). *Untersuchungen über Enzyme*, **2**. Berlin: Springer.
—— & Rohdewald, M. (1937). *Hoppe-Seyl. Z.* **247**, 269.

Addendum

(*Received* 15 *October* 1943)

Since the submission of this manuscript it has come to our notice that Kagan, Latker & Zfasman (1942) have demonstrated phosphorylysis of sucrose with intact cells of *Leuconostoc mesenteroides*. Doudoroff, Kaplan & Hassid (1943) have shown the occurrence of a *Pseudomonas saccharophila* and further made the important discovery that the phosphorylytic breakdown of sucrose to glucose-1-phosphate and fructose is reversible. Thus a mechanism for direct sucrose fermentation is disclosed, the suggested analogy as regards store of energy for polysaccharide synthesis between glucose-1-phosphate and sucrose is corroborated, and an enzyme system is revealed whereby levan and dextran can be formed from their constituent hexose units via sucrose.

REFERENCES

Doudoroff, M., Kaplan, N. & Hassid, W. Z. (1943). *J. biol. Chem.* **148**, 67.
Kagan, B., Latker, S. & Zfasman, E. (1942). *Biokhimiya*, **7**, 92.

The Reduction of Dehydroascorbic Acid in Plant Extracts

By E. M. CROOK and E. J. MORGAN, *Biochemical Laboratory, Cambridge*

(*Received* 23 *October* 1943)

Pfankuch (1934) observed that when 0·3 % cysteine was added to potato press juice in which the ascorbic acid (abbreviated to AA) had become oxidized, 10 mg./100 ml. of AA reappeared in 2–3 hr. at room temperature, after which time no more AA appeared. Further, he showed that this was probably an enzymic reaction, for it did not occur in heated juice or in the filtrate from a protein precipitate. Hopkins & Morgan (1936) independently discovered a similar enzyme in cabbage and cauliflower juices, which accounted for the 'protection' of AA by reduced glutathione ('GSH'). Crook & Hopkins [1938] showed the dependence of this protective system upon pH and indicated that the activities of