latterly was occupied in administrative problems and in the early development of the Oxford Nutrition Survey, which owes much to his wise guidance. Appraising the work as a whole, we realize the careful chemical interest in completion, combined with a wide knowledge and appreciation of biological applications; he had learnt fully the necessary physiology and bacteriology required. It indeed seems that his studies on micro-organisms were only at an initial stage.

The Secretary has told me that as a member of the Committee of the Society, Walker displayed a quiet ability combined with a sense of humour that proved to be invaluable in finding a way through the complicated results of a discussion on procedure or the like. His complete familiarity with the rules of the Society and of syntax alike could on occasions be a little disconcerting but extremely helpful.

Quiet and modest in manner, unfailingly honest in his judgements and dealings with men, he proved a wise, independent-minded, courageous and respected counsellor in the laboratory. He had the capacity for carrying through administrative detail without appearing flurried or perturbed; in part this was due to an unfailing sense of humour which never deserted him, and which so often proved disconcerting. One of his many hobbies was chess playing.

In 1932 he married Dr Vera Reader, whose work upon nutritional problems is well known to biochemists, and is survived by his wife and two children. R. A. PETERS

The Enzymic Production of Levan

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This paper describes the preparation and study of cell-free levan-synthesizing enzyme systems (levansucrase) from two bacterial species [cf. Aschner, Avineri-Shapiro & Hestrin, 1942]. A particularly convenient source of synthesizing enzyme has been found in a newly isolated non-sporulating bacterium to be referred to as *Aerobacter levanicum*. Recently Hehre has achieved the cell-free synthesis of dextran by the action of an enzyme isolated from *Leuconostoc mesenteroides* [Hehre, 1941; Hehre & Sugg, 1942; Stacey, 1942]. Levan- and dextran-synthesizing enzymes are thus now available in sterile form. The two principal polysaccharides which bacteria form specifically from sucrose may henceforth be included, together with glycogen and starch, in the category of macromolecular materials whose production can be effected *in vitro* at will.

Nomenclature

Beijerinck [1910, 1912] coined the word viscosaccharase in generic designation of enzyme systems which mediate polysaccharide-gum production from sucrose. We propose to designate individual enzymes within this group in each case in accordance with the polysaccharide whose production is catalysed. Thus for levan-synthesizing enzyme: levansucrase; for dextran-synthesizing enzyme: dextransucrase. Since the viscosucrases are very different in specificity from phosphorylase, the enzyme whose activity mediates the production of starch or glycogen, it seems correct to retain for the dextran- and levan-synthesizing systems the nomenclature and classification proposed by Beijerinck. It should not be overlooked, however, that the over-all processes of levan and dextran production fit ill in any recognized class of carbohydrate-mediated reactions. The assignation of the dextran- and levan-synthesizing enzyme group to the sucrases can therefore at best be only tentative.

The enzymes of levan *hydrolysis* are, as will be shown elsewhere, distinct from levansucrase. They belong by definition, together with inulase, to the class of polyfructosidases. They may conveniently be referred to, therefore, as levanases.

EXPERIMENTAL

Bacterial strains used. Three bacterial strains were selected for investigation after preliminary trials with a sucrose-agar medium had indicated their excellence as levan formers. The first was *Bacillus subtilis*, Marburg strain, the levan of which has been the subject of extensive chemical study [Hibbert & Brauns, 1931]. This species is typical of a large group of sporulating bacteria which induce levan deposition in sucrose-agar medium at a distance from the actual growth site of the colony. The second organism selected was an aerobic gas-producing spore former received from the National Collection of Type Cultures under the name *B. polymyxa* (Prazmowski) Migula [*B. asterosporus* (Meyer)] N.C.T.C. no. 4744. It differs morphologically in the mode of its levan production from organisms of the *B. subtilis* type, depositing levan in sucrose medium at the cell-medium interface in the form of a giant capsule. The third test organism was a lactose-fermenting non-sporulating bacterium isolated from a fruit-tree root in Jerusalem. Like *B. polymyxa* it produced levan at the cell-medium interface only. The organism is actively motile, possessing peritrichous flagella. It ferments glucose, fructose, lactose, sucrose, raffinose and mannitol with the production of acid and gas, but ferments glycerol, inulin and levan with pro-

duction of acid only. The gelatine liquefaction test, and the methyl-red and citrate tests were all negative, and the Voges-Proskauer reaction positive. The organism therefore appeared to belong to the *Aerobacter* group. We propose for it the name *A. levanicum*.

Estimation of levan. Levan is defined for the purposes of this investigation as material precipitable by 72% ethanol and hydrolysable to fructose by hot dilute acid of *p*H 2·0. A method was required which would permit a fairly precise assay of levan, as thus defined, to be made in small amounts of fluid when large amounts of sucrose or reducing sugar are also present. A satisfactory method was developed from data given for *Bacillus subtilis* levan by Hibbert, Tipson & Brauns [1931].

The following procedure was adopted: 1·0 ml. test solution is added to 3·0 ml. ethanol in a centrifuge tube of 15 ml. capacity. As a rule all levan present separates immediately as a flocculent sediment. If flocculation is slow, it can be hastened and rendered quantitative by the addition of a drop of 1% $CaCl_2$. The suspension is centrifuged, and the sedimented levan is freed from reducing sugar and sucrose residues by twice-repeated solution in H_2O over a water-bath and precipitation each time with ethanol. Addition of a glass bead to the mixture facilitates these operations. The final sediment is taken up in 3·0 ml. 0·5% oxalic acid and complete hydrolysis effected by heating in a boiling water-bath for 1 hr. Evaporation is restricted during this treatment by placing a glass bulb at the tube aperture. The hydrolysate obtained is neutralized, cleared with $Zn(OH)_2$, and diluted to a suitable volume. The reducing power of the filtrate is estimated by the method of Somogyi. The amount of levan is calculated from the 'glucose value' by multiplying by a factor which allows both for the small difference in reducing power between glucose and fructose, and for the entry of H_2O during hydrolysis. The efficacy of the method was controlled on solutions of levan from *B. subtilis* of known concentration. Assays in the range 40–400 mg./100 ml. yielded results reproducible within about 5 mg./100 ml. Since the method measures ethanol-precipitated labile furanosidic polysaccharide as distinct from less easily hydrolysed pyranosidic polysaccharide, it is of high specificity.

A rapid qualitative spot-test for polysaccharide in the agar gel medium was found useful. It is based on observation of the turbidity change undergone by pieces of agar gel when they are immersed in methanol. If they contain levan they rapidly become milky white in the area of levan deposition.

Estimation of fructose. Fructose was determined by means of the specific reducing action of the keto-hexose on phosphomolybdotungstic acid. Reaction conditions indicated for method (1) of Davidson, Kermack, Mowat & Stewart [1936] were adopted, and the concentration of fructose was estimated colorimetrically against solutions of known fructose concentration similarly treated. Measurements were made in a Pulfrich photometer using filter S 61. Over a wide range of concentration the extinction values were found to be a linear function of the fructose concentration.

Sterility of enzyme reaction mixtures. Numerous sterility controls were carried out. In the experiments with *B. subtilis* enzyme, the danger of contamination was very great and strict asepsis had to be practised at all steps of the test. In the experiments with *Aerobacter* enzyme sterility was successfully maintained by the addition of a drop of thymol in chloroform to the mixtures. Enzyme preparations and reaction mixtures were considered sterile only if direct microscopic examination and growth tests using both heavy and diluted inocula at the beginning and at the end of the experiments gave a uniformly negative result. Experiments in which contamination occurred were rejected.

RESULTS

A. Bacillus subtilis

Production of levan by living cells

Cultures of *B. subtilis*, grown in fluid medium containing sucrose, caused the appearance of a marked opalescence and an increasing viscosity associated with the appearance of a polysaccharide whose behaviour when isolated was that of typical levan. The concentration level of levan in the medium depended on the conditions of the experiment [cf. Cooper & Preston, 1935]. It was particularly high in a medium which ensured a uniform supply of sucrose and a continuous removal, by dilution, of reaction products other than levan. To this end *B. subtilis* was inoculated into buffered 0·25% peptone in phosphate solution contained within a cellophane sac suspended in a large volume of 10% sucrose in 0·25% peptone. After 4 days at 30° the concentration of levan in the internal phase had reached a record level of 12·5%; outside the sac, the levan content remained nil.

In sucrose-2·5% agar medium, growth of *B. subtilis* and similar species is remarkable because of the appearance of a peculiar non-continuous coacervate pattern at a considerable distance from the actual growth site of the colony [Beijerinck, 1912]. Levan powder sprinkled on the sucrose agar diffused through the medium only very slowly and failed altogether to form within it characteristic coacervate patterns of the type observed near *B. subtilis* colonies. There seemed to be strong evidence, therefore, that levan coacervation at a distance from the bacterial colony growing on sucrose agar is due to the activity of a diffusible enzyme within the region involved. Resting cells of *B. subtilis* grown on sucrose medium and suspended in pure sucrose solution synthesized levan actively. In peptone cultures containing levan as sole source of carbohydrate, *B. subtilis* grew satisfactorily with formation of much acid.

Specificity. Corresponding substrate specificity in levan production between the strain of *B. subtilis* here employed and that studied by Harrison, Tarr & Hibbert [1930] was noted. In the experiments with our strain, a resting-cell technique was employed. Mixtures were composed of 0·4 ml. physiological saline containing the thoroughly washed cell crop of 10·0 ml. sucrose-peptone cultures of *B. subtilis* (harvested after 10 days of growth at 30°) and 1·0 ml. of sterile 1·0 *M* solution of sugar in Sörensen phosphate buffer *p*H 6·8. Levan assays were carried out both at the beginning of the experiment and

after 2 days' incubation at 30°. Glucose, fructose, invert sugar, trehalose, maltose, melezitose, and methyl-β-fructofuranoside (syrup preparation after Menzies [1922]) failed to support levan production. Sucrose and its galactoside raffinose, on the other hand, were readily built up into levan. Inulin was fermented by the cells with formation of some reducing sugar. Levan coacervation was not evident, however, in inulin agar on which *B. subtilis* was grown (cf. also *Streptococcus salivarius* [Niven, Smiley & Sherman, 1941]).

The negative findings with methyl-β-fructofuranoside and inulin are in apparent disagreement with a generalization on levan production formulated by Harrison *et al.* [1930]. This states that substances with a fructofuranoside terminal unit, hydrolysable by fructosidases, are suitable indirect substrates of levansucrase activity. Experiments with cell-free enzyme preparations may elucidate this question.

Experiments on cell-free levansucrase from B. subtilis

Sterile Seitz filtrates of *B. subtilis* cultures in fluid sucrose medium failed to produce levan in a detectable amount. It was concluded either that levansucrase in the fluid culture is predominantly endocellular or that it is exocellular but absent from the filtrate because it is adsorbed during filtration on the Seitz filter pad. The situation in sucrose-agar medium is very different. Here levansucrase is actively secreted into the medium and can be effectively separated free from living cells by means of a technique of selective diffusion through agar gel originally invented by Beijerinck [1912].

Pieces of sterile agar medium composed of 0·5% peptone, 0·1% K_2HPO_4 (*p*H 7·2), 2·5% agar and 2·5% sucrose were laid out on a Petri dish and covered by corresponding pieces of gel medium similarly compounded but without sucrose. The pieces were about 1 cm. square and 3 mm. in thickness. *B. subtilis* was sown in heavy inoculation in the central area of each top piece. After incubation for 24 or 48 hr. at 30°, the top layers were aseptically removed. The sterile bottom layers contained levansucrase.

Pieces of agar containing levansucrase prepared as described were suspended in several volumes of 4% sucrose solution at 37°. Large amounts of reducing sugar appeared in the medium and there was a progressive increase in turbidity and viscosity with the formation of a non-reducing substance precipitable by 72% ethanol and hydrolysed in hot dilute oxalic acid with liberation of fructose. After 12 hr. incubation, 300 mg. levan/100 ml. were demonstrable in the fluid phase as against virtually none in the fluid phase of control suspensions consisting of similar pieces of agar in H_2O. The control and test mixtures remained sterile. It was therefore clear that production of levan had been accomplished in the absence of living cells.

Attempts to extract the *B. subtilis* levansucrase from the pieces of agar met with only limited success. Enzyme-containing pieces of agar, prepared as described above, were ground up with 4% sucrose solution. A particle-free fluid which possessed synthesizing activity was separated from the mixture by centrifugation. After incubation for 24 hr. at 30°, this sucrose eluate contained 61 mg. levan/100 ml.; a boiled sample similarly incubated was found to be almost devoid of levan. In both cases the solution remained sterile.

Diffusion of levansucrase and enzymically induced formation of levan as a coacervate in agar gel. It is evident from the method of preparation that levansucrase is secreted into sucrose agar by growing cells of *B. subtilis*, the secreted enzyme diffusing through the agar. To demonstrate this, a levansucrase-impregnated piece of agar was placed on a similarly sized piece containing sucrose but devoid of enzyme. After 15 min. at room temperature the top piece was removed and the bottom piece, which at this time contained no sign of levan, was incubated at 30° for 24 hr. Although the bottom piece remained sterile, a typical levan coacervate was found throughout its interior at the end of the incubation. Further trials involving several superposed layers of agar suggested that the rate of diffusion of measurable amounts of levansucrase through sucrose agar at room temperature was at least 0·1 mm./hr. (cf. Table 2). These results explain the ability of living *B. subtilis* to induce formation of levan coacervates in agar at a distance from its site of growth.

Effect of sugar on levansucrase production. Presence of sucrose within the growth medium is a prerequisite for production of levansucrase by *B. subtilis*. When this organism was grown on an agar medium devoid of sugar or containing maltose or glucose instead of sucrose, the levansucrase activity of the gel medium was found to be nil. The secretion and probably also the formation of levansucrase by *B. subtilis* in agar medium would appear from this result to be strictly adaptive. Cells harvested from broth cultures actively produced levan when suspended in sucrose solution if the latter had also been present in their growth medium, but produced little if any levan from sucrose when harvested from broth containing invert sugar as the sole carbohydrate.

Stability of the enzyme. The enzyme is thermolabile and is completely inactivated by brief exposure to 100°. Trituration under ethanol and acetone followed by rapid desiccation did not deprive levansucrase-impregnated agar of synthe-

sizing power [cf. Dienes, 1935]. By this treatment the enzyme could be obtained in a form suitable for prolonged storage.

B. Bacillus polymyxa

Production of levan by living cells

The growth of *B. polymyxa* in fluid culture media containing sugar is associated with increases in viscosity and opalescence which are particularly marked when the medium contains sucrose. The cells are found enclosed within a capsule which is large when sucrose, and small when another sugar, e.g. glucose or lactose, is the sole source of carbohydrate. The polysaccharide formed in the sucrose medium was separated by ethanol precipitation after partial removal of cells by centrifugation. After repeated solution in H_2O and precipitation by ethanol, the sticky precipitate was converted into a friable powder by trituration under methanol. In the ease with which it could be hydrolysed, liberating fructose, it resembled *B. subtilis* levan. The polysaccharide formed by *B. polymyxa* from lactose, on the other hand, was of a different type. This product, itself non-reducing, failed to form reducing sugar when heated in *N*/100 acid, and formed reducing sugar only when hydrolysed by hot 2*N* HCl (cf. *Chromobacter viscosum* [Carruthers & Cooper, 1936]).

Discontinuous patterns of coacervated levan were not induced in sucrose agar on which *B. polymyxa* was grown, although the cells themselves were found embedded in a fluid mass from which levan has been isolated in good yield. Tests on pieces of gel from the vicinity of the cell colonies, carried out as described in the section on *B. subtilis* enzyme, failed to demonstrate the presence of levansucrase in the gel medium. Levansucrase is therefore obviously present in *B. polymyxa*, but is endocellular, unlike the enzyme in *B. subtilis* and other levan-producing spore-formers on sucrase-agar medium. Peptone medium containing levan (formed by *B. subtilis* or *B. polymyxa*) as the source of carbohydrate supported active growth of *B. polymyxa* with vigorous production of acid and evolution of gas.

C. Aerobacter levanicum

Levan synthesis with living cells

In a fluid medium with sucrose or raffinose as the source of carbohydrate, *A. levanicum* became heavily encapsulated, and the increase of viscosity and opalescence of the medium was found to be associated with the appearance of a levan. This was not bound to the cells, and was isolated by rapid centrifugation to remove cellular material, followed by precipitation in 75% ethanol and trituration of the gummy precipitate with absolute methanol. The white powder thus obtained gave viscous opalescent aqueous solutions and yielded fructose on heating in 0·5% oxalic acid. The cells were not encapsulated nor was the polysaccharide formed when disaccharides other than sucrose and raffinose constituted the source of carbohydrate in the medium. Negative results were also obtained with invert sugar and all common hexoses.

When *A. levanicum* was grown on sucrose agar, discontinuous patterns of coacervated levan were not seen within the gel. The cells themselves, however, were found entirely and deeply embedded in a viscous semi-fluid mass from which polysaccharide material having the properties of levan could be isolated in high yield.

A. levanicum on a medium containing levan as the sole source of carbohydrate grew slowly with some formation of acid but not of gas. Resting cells of *A. levanicum* were active levan producers (Table 3). Strains of *A. aerogenes* ferment sucrose and raffinose but, unlike *A. levanicum*, failed to induce levan production.

Levan synthesis with dried cells

After 2 days' growth at 30°, cells were harvested from 50 ml. nutrient broth containing 4% sucrose. Immersion of the thoroughly washed cells in ethanol and ether, followed by rapid desiccation *in vacuo*, converted them into a dry sterile powder. When suspended in 2·0 ml. sterile 5% sucrose at 37°, the cell powder induced formation of levan to a level of 324 mg./100 ml. within 20 hr. When an equal amount of powder was suspended in H_2O instead of sucrose, only negligible amounts of levan were demonstrable after the same period.

Experiments on cell-free levansucrase from A. levanicum

Attempts to isolate levansucrase from *A. levanicum* by methods dependent on ultrafiltration, e.g. the technique of selective diffusion in agar as applied in the case of *B. subtilis*, were negative. Levansucrase in *A. levanicum* appeared to be entirely endocellular. Methods of direct preparation from the cell itself were in this case feasible, since *A. levanicum* was readily killed by treatments which were not destructive of its activity towards sucrose. By ethanol-ether treatment or by autolysis in the presence of thymol and chloroform, sterile preparations of levansucrase could be obtained which possessed good synthetic power and were sufficiently stable for quantitative study.

Levan synthesis with cell autolysate. Preparation in this form was possible by autolysis of the cells in the presence of thymol and chloroform. The following routine was adopted: *A. levanicum* was cultivated in 1 l. conical flasks at 30° in a two-phase

medium consisting of a bottom solid layer of nutrient agar devoid of sucrose, and a thin top layer of 2% aqueous sucrose. After 20 hr. the cells were harvested, rinsed repeatedly in H_2O to free them from medium constituents and levan, and finally left to autolyse at 37° under H_2O containing a little thymol and chloroform. The volume ratio of culture medium to autolysate fluid was 100:1. After 24 hr. autolysis, the suspension was sterile. The dry-matter content of the faintly opalescent supernatant fluid from the autolysate was approx. 0·3%. If properly prepared it was entirely free from visible suspended particles. It contained no levan and did not reduce Fehling's solution. It had little or no protein precipitable by trichloroacetic acid. It contained constituents which reduced I_2 in alkaline medium; they were neither removed by dialysis nor by clearing with $Zn(OH)_2$. The fluid rapidly decolorized alkaline permanganate in the cold.

In sucrose solution buffered at *p*H 5·0, to which a little thymol in chloroform was added to maintain sterility, the autolysate induced progressive changes similar to those described above for *B. subtilis* levansucrase. The solution mixtures, which remained uniformly sterile, showed a progressive accumulation of levan polysaccharide (see Table 1). The rate of reaction was maximal at the outset of incubation, and then fell off rapidly. Within the first hour the fluid autolysate, in a mixture composed as indicated in Table 1, synthesized about 100 mg. polysaccharide/100 ml., i.e. an amount approximately equal to the dry weight of enzyme used. The activity of different lots of enzyme varied however, being dependent among other things on the number of bacterial cells harvested.

Table 1. *Formation of levan, reducing sugar, and associated changes in viscosity, induced by an autolysate of* Aerobacter levanicum *in sucrose solution*

Mixtures: 1·0 ml. supernatant enzyme fluid; 1·0 ml. 15% sucrose; and 1·0 ml. Sörensen Na-citrate buffer *p*H 5·0. Temp. 37°.

Viscosity was determined in an Ostwald viscosimeter.

Incubation time min.	Levan as mg. glucose/ 100 ml.	Relative viscosity	Reducing sugar as mg. glucose/ 100 ml.	Ratio of reducing sugar to levan
0	0	1·00	0	—
120	248	1·06	820	3·3
255	330	1·12	1110	3·4
345	341	1·17	1285	3·8

The changes noted failed to develop in the absence of sucrose, or with boiled enzyme. Dialysis against distilled H_2O left the activity of the autolysate towards sucrose unchanged. The catalytic properties of the cell extracts must be ascribed, therefore, to the action on sucrose of an enzyme system, levansucrase, which is typically thermolabile and non-dialysable.

The amount of reducing sugar liberated is considerably larger than the amount of fructoside material found as polysaccharide (Table 1). In no experiment was the ratio of reducing sugar to levan appreciably less than 3, and it was frequently greater. The ratio of reducing sugar to levan as shown by Table 1 is variable, and rises markedly with increasing incubation time. Both glucose and fructose can be identified in the reducing fraction. Moreover, *Aerobacter* strains other than that here described readily ferment sucrose and raffinose though they form no levan from these substrates. It may be assumed, therefore, that side by side with a levan-forming enzyme there is present in *A. levanicum* autolysate an ordinary invertase. It is still undecided whether the latter is indispensable to the process of levan formation [cf. Owen, 1923; Harrison *et al.*, 1930; Norman, 1937].

Diffusibility of levansucrase from A. levanicum *and deposition of formed levan in agar gel.* Levansucrase of *A. levanicum*, unlike that of *B. subtilis*, is non-diffusible in 2·5% agar gel. An experiment which shows the difference in diffusibility between the sterile levansucrase of the two bacteria strains is given in Table 2. This finding suggests that levansucrases of *B. subtilis* and of *A. levanicum* respectively are of different micellar size.

Table 2. *Diffusibility of levansucrase of* Bacillus subtilis *and* Aerobacter levanicum *in agar gel*

Agent	Levan reaction at various depths of agar gel: 0–2 mm.	2–4 mm.	4–8 mm.	6–8 mm.
B. subtilis:				
Living cells	+ + +	+ + +	+ +	+ +
Levan powder	+ + +	−	−	−
Levansucrase (gel)	+ + +	+ +	+ +	+
A. levanicum:				
Living cells	+ + +	−	−	−
Levan powder	+ + +	−	−	−
Levansucrase (fluid)	+ +	−	−	−
Levansucrase (cell-residue)	+ +	−	−	−

2 mm. layers of 2·5% agar gel containing 4% sucrose were superposed and the source of levan allowed contact with the top layer. Tests for levan were carried out on the different agar layers after 24 hr. incubation at 30°. +, positive levan reaction; −, negative levan reaction.

When *A. levanicum* autolysate is left in contact with sucrose-agar gel under sterile conditions for several days, levan is formed, which is particularly concentrated in the fluid layer immediately above the gel, and is in greater dilution throughout the fluid phase. A very thin layer of levan is deposited

at the gel-enzyme interface, but no levan is found in the agar at depths greater than 1·0 mm. It seems therefore that the conditions of this experiment correspond with those which are associated with capsule formation in living cultures.

Effect of sugar on levansucrase production. In *B. subtilis* the production of levansucrase is adaptive, but in *A. levanicum* its production is constitutive (Table 3). The levansucrase activity of cells from media containing sucrose or invert sugar is seen to be very similar. From media without added sugar, enzyme preparations of lower activity were obtained, probably because cell growth in the absence of carbohydrate was poor.

Table 3. *Effect of sugar on production of levansucrase by* Aerobacter levanicum

Exp.	Synthesizing agent	Sugar in medium	Levan formation from sucrose mg./100 ml.
A	Resting cells	Sucrose	204
		Invert sugar	208
B	Autolysate	Sucrose	140
		Invert sugar	100
		None	34

50 ml. broth, containing 4% of the test sugar (if present), were incubated, after inoculation, for 2 days at 30°. The cell harvest (or the autolysate therefrom) was suspended in 3 ml. 0·9% NaCl and sucrose added to a final concentration of 5%; the mixture incubated for 20 hr. at 37°. In resting-cell mixtures antiseptic was not added. Levan was determined on mixtures cleared by centrifugation. In the absence of sucrose, levan content was nil.

Stability of enzyme. Levansucrase from *A. levanicum* possesses considerable stability and its solutions should prove well suited for chemical manipulation with a view to enzyme purification. Experiments showing the potency of the enzyme solution after storage at different temperatures are summarized in Table 4.

Table 4. *Stability of solutions of* Aerobacter levansucrase

Conditions of storing of enzyme		Activity of enzyme (mg. levan/100 ml. formed in 20 hr. at 37°)
Temp. (°C.)	Time (days)	
4	2	241
	21	243
22	2	248
	6	258
30	2	245
	6	228
37	2	215
	6	153
100	5 min.	0

Mixtures contained 1·0 ml. enzyme fluid; 1·0 ml. sucrose 15%; 1·0 ml. Na-citrate buffer (Sörensen) *p*H 5·0.

It has already been noted that cell-bound enzyme remains active after exposure to sterilizing treatment with ethanol and ether. The soluble enzyme is similarly stable. Its solution added to 3 parts of ethanol, with addition of a drop of dilute $CaCl_2$, deposited a light voluminous precipitate which, after separation by centrifugation and resolution in water, showed a synthesizing activity about one-half that of the original solution. Enzyme powder suitable for prolonged storage has also been obtained by desiccating the autolysate in the frozen state in high vacuum; there was some loss of activity.

Nature of polysaccharide produced by levansucrase from A. levanicum. More than 9 g. polysaccharide material have been prepared in the course of this work by the action of a solution of the enzyme on sucrose under conditions of strict sterility. This material has been converted into a powder by trituration under methanol. The material was non-reducing to Fehling solution, N-free (Lassaigne), not hydrolysed by yeast invertase, and gave no colour reaction with I_2. Its aqueous solutions, in contrast to those of inulin, were markedly turbid and viscous, and did not yield reducing sugar on boiling in neutral solution. It was rapidly converted to reducing sugar by boiling in *N*/100 HCl.

The gum-like material precipitated by ethanol was completely hydrolysed by treatment for 1 hr. at 95° with 0·5% oxalic acid. The total reducing sugar thus liberated and calculated as 'glucose' was shown to be fructose by the specific colorimetric assay of keto-hexoses by means of phosphomolybdotungstic acid. The hydrolysate formed glucosazone (m.p. 210°) with phenylhydrazine. The alcohol-precipitable polysaccharide fraction therefore probably contains only fructose, and, because of the ease of hydrolysis of the polysaccharide, the sugar is probably in the furanose form.

The polysaccharide formed from sucrose by the action of *A. levanicum* autolysate is thus a levan. Whether the enzymically formed product is homogeneous and identical with the *Aerobacter* levan produced *in vivo*, and whether it is identical with other levans (e.g. that from cultures of spore-forming organisms [Hibbert & Brauns, 1931; Hibbert *et al.* 1931], from *Actinomyces* [Veibel, 1938], from certain bacterial plant pathogens [Lyne, Peat & Stacey, 1940], from a grass (*Pea trivialis*) and from barley [Challinor, Haworth & Hirst, 1934], is not known.

SUMMARY

1. A micro-method suitable for the specific estimation of levan in the presence of large amounts of reducing sugar and sucrose is described.

2. Levan production by *Bacillus subtilis* (I), *B. polymyxa* (II), and a newly isolated non-sporu-

lating species referred to as *Aerobacter levanicum* (III) has been studied. All three produced levan from sucrose and raffinose. In I, levansucrase is formed adaptively; in III it is constitutive. In I, growing on sucrose agar, levansucrase is exocellular and diffuses through the medium; in II and III on the same medium, it is endocellular.

3. Levansucrase has been prepared cell-free from III as an aqueous solution, as well as in soluble dry form. The cell-free enzyme systems act on sucrose with rapid formation of levan and of reducing sugar. Certain physical properties of cell-free levansucrase from I and III have been studied.

4. Levan has been prepared in substantial amount as a nitrogen-free powder by the action of sterile levansucrase from III on sucrose. Some properties of the enzymically formed polysaccharide are described.

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Complement Activity and Vitamin C

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In recent years evidence has accumulated that deficiency of ascorbic acid impairs the defensive reaction of the organism towards infection. There have been many attempts to correlate this anti-infective effect more specifically with factors participating in immunological reactions [cf. Perla & Marmorstan, 1941]. The greatest attention perhaps has been paid to the possible correlation between the complement activity of the serum and the vitamin C intake.

Although Zilva [1919, 1936] found no significant change of complement titre in scorbutic guinea-pigs, Simola & Brunius [1933] and Marsh [1936] claimed a lowering of the complement activity with a low intake of vitamin C. Chakraborty [1937] could not confirm the finding that vitamin C deficiency affected the complement titre in guinea-pigs.

More recently, Ecker, Pillemer, Martiensen, Wertheimer & Grandis [1938*a*], Ecker, Pillemer & Wertheimer [1938*b*], Ecker, Pillemer, Griffiths & Schwartz [1939] and Ecker & Pillemer [1940] claim to have established a direct correlation between the complement activity of the serum and its concentration of vitamin C. They based their conclusions on average results without any statistical evaluation of the significance of the means. On the other hand, Maccolini [1939] and Agnew, Spink & Mickelsen [1942] found no correlation between the complement titres of guinea-pigs and their intake of ascorbic acid. In view of these conflicting findings, we have reinvestigated the question.

The method of titrating the complement was based on the determination of 50% haemolysis, and the results were statistically evaluated. It may be said in advance that no significant changes were found in the complement titres of guinea-pigs at different levels of vitamin C intake.