Bacterial Levans of Intermediate Molecular Weight[1]

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The demonstration by Grönwall and Ingleman (1944, 1945) that a satisfactory plasma extender could be obtained from the dextran produced from sucrose by cultures of *Leuconostoc mesenteroides* greatly stimulated research on bacterial polysaccharides. The work on dextran from *Leuconostoc mesenteroides* eventually led to the commercial production of a good quality plasma substitute (clinical dextran) which is now used extensively by the military. The extender is prepared by partial hydrolysis of the high-molecular-weight polysaccharide and subsequent methanol fractionation of the hydrolyzed product to obtain a material ranging in molecular weight from 25,000 to 200,000 (Hines *et al.*, 1953). This clinical dextran may be used to maintain the blood volume of a patient suffering severe loss of blood by virtue of its high colloid osmotic pressure. The dextran is gradually excreted in the urine and is replaced by newly-synthesized plasma proteins.

In the present study, a large number of polysaccharide-producing organisms were investigated in the hope of finding an organism which produced directly a material of molecular size suitable for use as a plasma extender. The necessity of hydrolysis, which is one of the primary disadvantages in clinical dextran preparation, would thereby be eliminated. Those cultures which passed a preliminary screening were subjected to further examination. Solvent fractionations and molecular weight determinations were carried out, and polysaccharide synthesis by one strain was studied in detail in an attempt to find conditions favorable for the production of high yields of polysaccharide of the required size. Both growing cultures and cell-free systems were examined. The effect of addition of low-molecular-weight polysaccharide as "acceptor molecules" or "primer" (Tsuchiya *et al.*, 1953) was of particular interest.

MATERIALS AND METHODS

Screening

Preliminary selection. Eighty-eight aerobes producing at least 5 mg of polysaccharide per ml of broth were selected from 850 soil isolates by workers in the Department of Bacteriology. A semisynthetic medium (medium 1-A) and salts medium (medium 2-A) were employed. The composition of these media is given in table 1.

Polysaccharide production and large-pore dialysis. After 2 to 3 transfers of a culture on agar slants, duplicate 25 x 200 mm test tubes containing 10 ml of one of the two media were loop-inoculated and incubated on a rotary shaker. An incubation temperature of 30 C was used for slants and tubes. After an incubation time of 9 or 24 hours, the broth was centrifuged to remove cells and any insoluble polysaccharide which might be present. The supernatant was then dialyzed for 96 hours against running water at 10 C or below through a large-pore membrane prepared in the following way: One layer of cheesecloth (36 x 44-mesh gauze) was fastened with a rubber band over the end of a stainless steel cylinder, 54 mm in diameter. The covered end was immersed in a solution containing 2 per cent agar and 10 per cent gelatin at 70 to 80 C. After dipping, the tube was allowed to drain at an angle of about 45 degrees until the dripping rate was one drop every two seconds. The tube was placed in the cold for 5 minutes to allow the gelatin to set, and was then ready for use. The agar and gelatin concentrations were such that molecules slightly smaller than clinical dextran would not pass the membrane. Therefore, the undialyzed fraction should contain all molecules in the size range from just below clinical dextran size to very large molecules. The pore size of such membranes may be increased somewhat by decreasing the relative gelatin concentration.

Viscosity and osmotic pressure determinations. Relative viscosity measurements were performed on samples which contained at least 2 mg of polysaccharide per ml after dialysis. Determinations were made with a 2-ml Ostwald viscometer at 37.5 C. Those samples which had a viscosity quotient[2] of 0.85 or less were concentrated under reduced pressure to a concentration of at least 1.5 per cent for osmotic pressure determinations.

The osmotic pressures of the samples were determined with the apparatus shown in figure 1. After the cellophane membrane had been soaked in water for 1

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[2] Viscosity quotient =

$$\frac{\text{ln relative viscosity}}{\text{concentration in g solute per 100 g solution}}$$

TABLE 1

Substance	Amount/100 ml medium
Medium 1-A	
Sucrose	5.0 g
Difco Casamino acids	1.0 g
K_2HPO_4	50. mg
KH_2PO_4	50. mg
DL-tryptophane	40. mg
L-cystine	20. mg
$MgSO_4 \cdot 7H_2O$	20. mg
$FeSO_4 \cdot 7H_2O$	1.0 mg
$MnSO_4 \cdot H_2O$	1.0 mg
$ZnSO_4$	1.0 mg
Adenine-SO_4	1.7 mg
Guanine-HCl	1.2 mg
Uracil	1.0 mg
Thiamine-HCl	20. μg
Riboflavin	20. μg
Nicotinic acid	20. μg
Calcium pantothenate	20. μg
Pyridoxal-HCl	40. μg
p-Aminobenzoic acid	4.0 μg
Folic acid	2.0 μg
Biotin	0.02 μg
Vitamin B_{12}	0.02 μg
Medium 2-A	
Sucrose	5.0 g
Difco Casamino Acids	50. mg
Na_2HPO_4	0.75 g
KH_2PO_4	0.25 g
$K_2SO_4 \cdot 7H_2O$	100. mg
$MgSO_4 \cdot H_2O$	10. mg
$CaCl_2$	1. mg
$FeSO_4 \cdot 7H_2O$	0.1 mg
Medium 2-B	
Sucrose	10.0 g
$(NH_4)_2SO_4$	0.5 g
K_2HPO_4	2.0 g
$MgSO_4 \cdot 7H_2O$	20.0 mg
$K_2SO_4 \cdot 7H_2O$	1.0 mg
$MnSO_4 \cdot H_2O$	1.0 mg
$ZnSO_4 \cdot H_2O$	1.0 mg

The pH was adjusted to 6.7 before autoclaving.

to 2 hours it was placed over the osmometer cup and held in place by the aluminum washer which is fastened to the cup by 3 screws. One ml of a polysaccharide solution was then placed in the cup through the hole in the top, followed by enough Skellysolve C (a high-boiling petroleum ether) to fill the remaining space. Next, the bushing holding the capillary was screwed into place and any excess Skellysolve C removed from the capillary. The osmometer was submerged in water containing 1 per cent phenol so that only the glass capillary was above the surface. The osmometer was kept in this bath in a 25 C room until the height of the Skellysolve C in the capillary remained constant (about 4 to 5 days). A water blank was included with

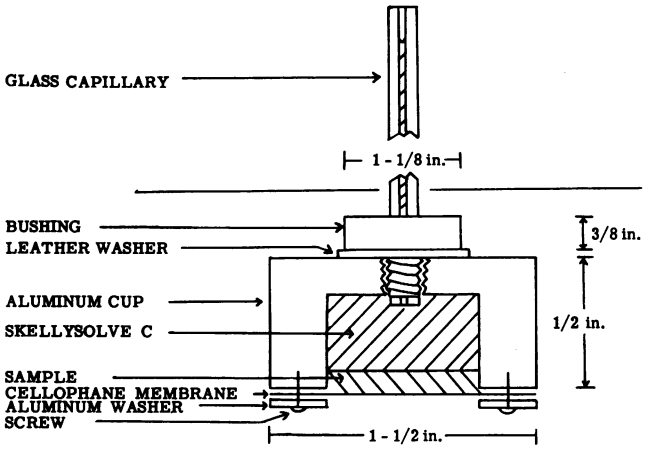

FIG. 1. Osmometer

each determination, and the difference in height between the sample and the blank was taken as the osmotic pressure in mm of Skellysolve C. The phenol prevented bacterial growth in the osmometers.

Fermentation and Enzymatic Procedures

Growth of organisms. Fermentations were carried out in 500-ml Erlenmeyer flasks containing 100 ml of medium and incubated on a rotary shaker at 25 or 30 C. Inoculum for these flasks was taken from liquid cultures grown under the same conditions but on a smaller scale.

Growth was assayed turbidimetrically. The per cent transmission at 660 mμ was determined in an Evelyn colorimeter for crude broth diluted 1 to 20 with distilled water, and growth was expressed as the optical density of the diluted broth. Another indication of the amount of growth was given by the pH change.

Enzyme preparations. Extracellular enzyme preparations were obtained from broths of strain 248 by centrifugation at 14,000 g for 20 minutes in an International centrifuge at 0 C. After the supernatant had been adjusted to a pH of 5.5 to 5.7, a drop of toluene was added, and the preparation was stored in a refrigerator. The material was used within 10 days. During this time no activity loss occurred.

Yeast invertase was prepared by adding 15 ml of water to the contents of one envelope (7.3 g) of Red Star active dry yeast and allowing the mixture to stand at room temperature for 30 minutes, after which time 2 ml of ethyl acetate were added. The mixture was stirred for one minute and then allowed to stand 20 minutes. Finally, 10 ml of water were added, the cells removed by centrifugation, and the supernatant retained as the source of invertase.

Analytical Procedures

Total carbohydrate analysis. Total carbohydrate was determined by a modification of the anthrone method of Koehler (1952). Two-ml samples of aqueous solution

containing from 5 to 50 μg carbohydrate per ml were pipetted into matched 18 x 150-mm test tubes. Standard tubes of glucose and fructose solutions (about 20 mg of glucose or fructose per ml) and distilled water blanks were included. Five ml of anthrone reagent, prepared by dissolving 1.5 g anthrone in 1 liter of concentrated sulfuric acid (C.P.), were then added rapidly to each tube from an automatic, syringe-type pipet. The mixture was immediately mixed by vigorous shaking and placed in a cool water bath to prevent the heat of the solution from developing the color unevenly. As soon as all the tubes were so treated, they were immersed in boiling water for 6 minutes to develop color, then placed immediately in cool water and allowed to come to room temperature. Per cent transmission at 620 mμ was determined in an Evelyn colorimeter. When only levan was present in the samples, the glucose control was omitted and the heating time was decreased to 2 minutes.

Measurement of polysaccharide and per cent "high-molecular-weight" material. Direct measurement of the polysaccharide present in fermentation broths was carried out in the following manner: A strip of Eaton-Dikeman No. 613 filter paper, one-half inch wide, was marked off in 3-cm sections. A 5- to 50-μl sample of centrifuged broth was pipetted on to the center of each section. For total polysaccharide determination, the dried filter paper was immersed in 2 liters of methanol (90 per cent by volume) for 15 minutes. During this period, residual sucrose, reducing sugars, and some low-molecular-weight oligosaccharides were eluted from the paper. The strip was rinsed for five minutes in a second bottle of 90 per cent methanol and finally dried. The sections were then cut apart, and each one was placed in a 16 x 150-mm test tube. Approximately 0.1 N HCl was added in 4- or 8-ml aliquots, depending upon the amount of polysaccharide on the paper. From the same strip, sections containing no broth were cut and treated with HCl for blanks and standards. For a standard, 2 ml of fructose solution containing 40.5 μg per ml and 2 ml of approximately 0.2 N HCl were used. When elution volumes were doubled for samples, blanks and standards were adjusted accordingly. After elution of polysaccharide had proceeded 10 minutes, 2 ml of eluate were removed for anthrone analysis.

When zero per cent methanol (that is, pure water) is used to elute polysaccharide, some polysaccharide still appears in the HCl eluate. This is termed "high-molecular-weight" polysaccharide. Determination of this material is identical with that just described except in the initial elution step.

Reducing sugar and sucrose determinations. Reducing sugars were determined by the method of Shaffer and Somogyi (1933). When this analysis was applied to fermentation broths, a correction was made for volatile reducing material (probably acetoin). This correction factor was determined by distilling an aliquot of broth and determining the reducing power of the distillate as "glucose". Sucrose was estimated by subtracting this corrected reducing sugar value and the polysaccharide value from the total carbohydrate value.

Physical Chemical Methods

Paper chromatography. The solvent system for paper chromatograms was water-butanol-pyridine in the volume ratio 3:6:4. Development was carried out at 37 C with downflow technique. Mono- and disaccharides were detected with a 5 per cent ammoniacal silver nitrate spray, and the anthrone spray of Johanson (1953) was used to detect levan. For quantitative estimation, levan was eluted from the appropriate paper sections with 0.1 N HCl, while water was used as eluant for the mono- and disaccharides. Approximately 90 per cent recoveries were obtained.

Polysaccharide purification and fractionation. Separation of polysaccharide from fermentation broths was accomplished by first heating the cell-free extract in a boiling water bath to destroy enzymes and then dialyzing the material in cellophane bags against distilled water at 4 C for 48 hours. Large volumes were concentrated *in vacuo* after dialysis. When dried product was desired, 9 volumes of methanol were added to the dialysate to precipitate the polysaccharide. The precipitate was then dried in a vacuum oven or desiccator at 50 C. When exact concentrations of material were not required, it was convenient to precipitate the polysaccharide before dialysis.

Methanol fractionations were carried out at 0 C by gradual addition from a burette of increasing increments of methanol to a series of test tubes containing equal volumes of 5 per cent polysaccharide solutions. After methanol addition, the tubes were centrifuged at 0 C in a refrigerated centrifuge to precipitate any suspended material. The supernatants were assayed for carbohydrate by the anthrone method. Plots of per cent polysaccharide in supernatant against volume per cent methanol were made. No correction was made for the volume shrinkage which occurs when methanol and water are mixed.

Results

Screening Procedure

The screening procedure described in the previous section was designed to select aerobic microorganisms which produce polysaccharides meeting the following requirements: 1) Polysaccharide yields must be at least 5 mg per ml of broth. 2) At least 2 mg of polysaccharide (of molecular size near, or somewhat greater than, that of clinical dextran) per ml must be present after large-pore dialysis. 3) The ratio of the osmotic pressure to the viscosity quotient ("R" value) of the

TABLE 2. *Characteristics of selected polysaccharides*

Sample	Viscosity Quotient*	R†
A5	0.53	204
248	0.51	168
1605	0.85	300
1624	0.66	155
6227	0.58	156
6472	0.35	160
6718	0.43	277
English Dextran (Tell No. 51002)	0.290	165
Swedish Dextran (Macrodex No. 6793A)	0.175	254
American Dextran (C. S. 84688A)	0.267	256
Plasma (approximate calculated values)	0.053	1300

* Viscosity quotient = $\frac{\text{ln (relative viscosity)}}{\text{concentration in g per 100 g soln.}}$

† R = $\frac{\text{Osmotic pressure}}{\text{Viscosity quotient}}$

polysaccharide should be greater than 150. Polysaccharides meeting these requirements should be large enough to be retained in the blood stream, and it should be possible to inject solutions sufficiently concentrated to maintain a colloid osmotic pressure similar to that obtained with clinical dextrans without increasing the blood viscosity excessively.

From the 850 isolated cultures, seven were selected which passed all these tests. A comparison of "R" values and viscosity quotients of the screened polysaccharides and clinical dextrans is given in table 2. Since the viscosity quotients are considerably higher than those of the dextrans, it might be supposed that a considerable number of relatively large molecules are present in the polysaccharide preparations. Such molecules would not only limit the concentration of extender which could be used, but would also have no appreciable colloid osmotic pressure. In order to prepare a suitable plasma extender from one of these materials it would be necessary to find some means for removing these large molecules. Some idea of the amount of such material present can be obtained by measuring the percentage "high-molecular-weight" polysaccharide with the elution technique described previously.

One of the cultures selected by the screen, number 284 (which produces a polysaccharide that is nonantigenic in the guinea pig), was chosen for detailed studies in which an attempt was made to find favorable conditions for the production of high yields of polysaccharide in the desired molecular-weight range. Polysaccharide synthesis in growing cultures and in cell-free systems was examined. Physical chemical studies of polysaccharide from several of the selected cultures were carried out.

Polysaccharide Synthesis in Growing Cultures

Growth and polysaccharide production on various media. Because medium 1-A upon which strain 248 was first isolated is rather complex, a simpler medium was sought. The data of table 3 indicate that more polysaccharide was produced on medium 1-B (medium 1-A without any vitamins, purines or pyrimidines) than on medium 1-A. Likewise, medium 2-B (see table 1) was found to give results comparable to those obtained with 1-B.

TABLE 3. *Polysaccharide production on two media*

Medium*	Growth Time	Polysaccharide (as Fructose)	pH	Growth†
	hr	*mg/ml*		
1-A	24	6.7	5.6	0.24
	33	10.5	—	0.23
	48	9.9	5.6	0.28
1-B	24	12.0	5.6	0.24
	33	14.3	—	0.24
	48	13.9	4.1	0.26

* The composition of medium 1-A is shown in table 1. Medium 1-B is the same as 1-A except the vitamins, purines, and pyrimidines have been omitted. Incubation was at 25 C on a rotary shaker.

† Growth is expressed as optical density of a cell suspension diluted 1 to 20 with distilled water.

Composition of 248 polysaccharide. To identify the sugar which constituted 248 polysaccharide, two types of experiments were carried out. Koehler (1952) has reported that the anthrone reaction rate with a given carbohydrate is characteristic of its composition. In figure 2 the anthrone reaction rate curves of glucose, fructose, and purified 248 polysaccharide are compared. The reaction rate for 248 polysaccharide closely resembles that for fructose. Furthermore, a paper chromatogram of an acid hydrolysate of the purified polysaccharide revealed a single spot corresponding to fructose. Had glucose been present at a concentration of more than 8 per cent of the total, it would have been detected. It was concluded, therefore, that 248 polysaccharide is predominantly, if not completely, a fructose polymer, or levan. Subsequent identification of strain 248 as a variety of *Bacillus subtilis* supported these findings, since this organism characteristically produces a levan which consists of D-fructofuranose units linked predominantly in the 2 and 6 positions (Hibbert and Brauns, 1931). The energy for formation of these linkages appears to arise directly from the glucose-fructose bond of sucrose without the intermediation of hexose phosphates (Hestrin and Avineri-Shapiro, 1944, and Duodoroff and O'Neal, 1945). Evidence that *Bacillus subtilis* levan contains a very small amount of glucose in its structure (about 0.4 per cent) has been presented by Palmer (1951). Polysaccharides from the other screened cultures exhibited anthrone reaction rates similar to this levan.

Effect of inoculum on levan synthesis. Figure 3 demonstrates the importance of the amount of inoculum on

the ultimate production of levan. This experiment was carried out with medium 1-A at an incubation temperature of 30 C. It will be noted that the optimal inoculum concentration was at 2 per cent. The maximum levan yield was 56 per cent of the theoretical value.

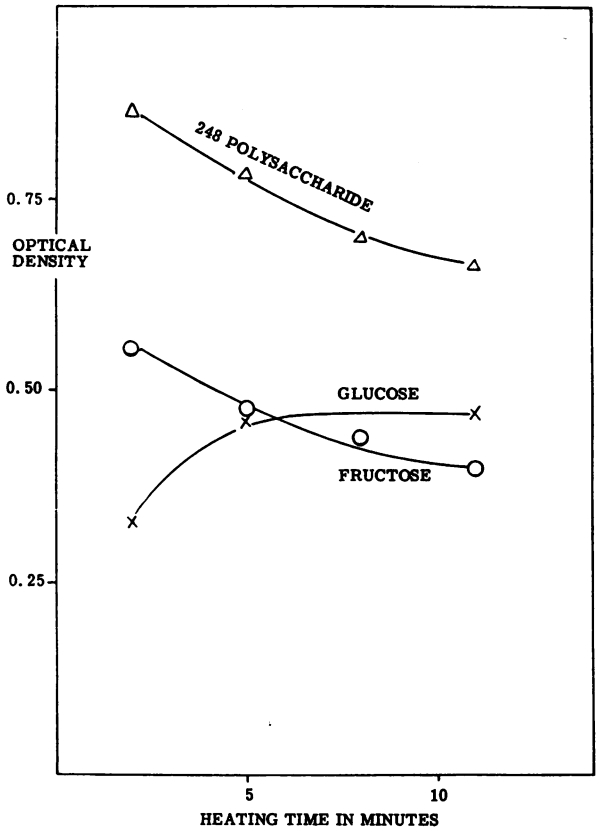

FIG. 2. Anthrone reaction rate curves. The concentration of the fructose and glucose solutions was 20.2 μg per ml. Optical density of the anthrone-carbohydrate reaction mixture was measured at 620 mμ with an Evelyn colorimeter.

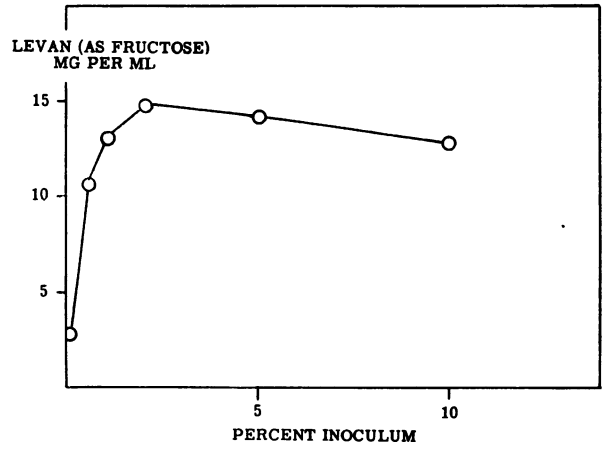

FIG. 3. The effect of inoculum on levan synthesis. Medium 2-B was used for this experiment, and incubation was at 30° C on a rotary shaker. After 24 hours the broths were assayed for levan by the elution method.

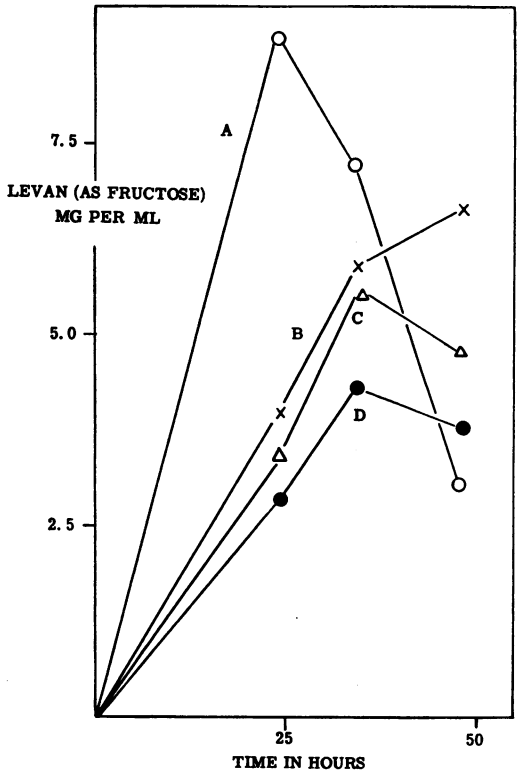

FIG. 4. Rate of levan synthesis with various inocula. Curve A, 2 per cent inoculum; curve B, 0.1 per cent inoculum plus 2 per cent centrifuged broth; curve C, 0.1 per cent inoculum plus 2 per cent centrifuged broth (boiled); curve D, 0.1 per cent inoculum. Medium 2-B was used, and incubation was at 30° C on a rotary shaker.

Comparison of curves A and D in figure 4 reveals that even with extended incubation times the levan synthesis in the cultures with 0.1 per cent inoculum never attained the value observed when 2 per cent inoculum was used. Addition to the 0.1 per cent inoculum of an amount of preformed levan (primer) equivalent to that present in the 2 per cent inoculum did not resolve this difference (curve B). Moreover, an amount of primer plus enzyme (centrifuged enzyme) equivalent to that in the 0.1 per cent inoculum added with 0.1 per cent inoculum was likewise insufficient (curve C). Therefore, while enzyme and primer are important, the presence of the larger number of cells is required to produce the complete effect. This suggests that a relatively high concentration of cells is required to utilize the glucose produced during levan synthesis:

$$n \text{ sucrose} \leftrightharpoons \text{levan} + n \text{ glucose}$$

If, as has been suggested by Doudoroff and O'Neal (1945), this reaction is reversible, the consequent rapid removal of glucose will drive the above reaction far to the right. Moreover, as will be shown later, glucose may act as an inhibitor of levan synthesis. Consequently, the removal of glucose by cells may con-

TABLE 4. *Influence of temperature on levan production by growing cultures of strain 248*

Time	25 C				30 C			
	Reducing sugar	Levan (as fructose)	High-mol-wt levan	Viscosity quotient*	Reducing sugar	Levan (as fructose)	High-mol-wt levan	Viscosity quotient*
hr	*mg/ml*	*mg/ml*	*% of total*		*mg/ml*	*mg/ml*	*% of total*	
24	27.0	9.3	24.6	0.32	5.8	6.8	25.6	0.33
48	15.8	9.0	34.6	0.42	12.4	3.2	48.7	0.52
72	10.6	6.2	46.9	0.64	8.0	0.4	—	—

* Viscosity quotient = $\frac{\text{ln (relative viscosity)}}{\text{concentration in g solute per 100 g solution}}$

ceivably affect levan synthesis favorably by decreasing this inhibition.

Effect of incubation temperature. Considerable difference in fermentation behavior was noted when incubation temperature was varied by 5 C. As shown in table 4, strain 248 incubated in medium 1-A produced more levan at 25 than at 30 C. A marked difference in sucrose utilization at the two temperatures is reflected by the appearance and disappearance of reducing sugars in the two fermentations. These data indicate that the lower temperature is much more favorable for levan production.

The "high-molecular-weight" fraction, as determined by the elution method described previously, increases as the fermentation continues beyond 24 hours. The effect was more apparent at 30 C. The increase in the percentage of this fraction reflects a more rapid decrease in the "lower-molecular-weight" levan, since it is accompanied by a decrease in total polysaccharide. Measurements of the viscosity quotient of the total levan in the broth indicated the same trend. Therefore, in order to get maximum yields of levan of lower molecular weight, the fermentation must be stopped before this degradation process begins. It is not known, of course, what fraction of this "lower-molecular-weight" levan is of desired molecular size.

General characteristics of a fermentation of strain 248. Figure 5 illustrates the important changes occurring in a fermentation by strain 248 on 2-B (6 per cent sucrose) medium. It will be noted that a decline in polysaccharide begins at about the same time that the sucrose is exhausted. It may be seen also that the appearance of large amounts of reducing sugar during levan synthesis is followed by a utilization of reducing sugar corresponding to a decreased rate of sucrose breakdown and a leveling-off in levan production.

Levan Production in Cell-Free Systems

Mechanisms for levan breakdown. The decrease in levan concentration noted in the later stages of a fermentation has also been noted in cell-free systems. Addition of purified levan to centrifuged broths which were free of sucrose resulted in the disappearance of levan and the appearance of reducing sugar. Three mechanisms may be suggested for this breakdown: 1) There may be a reversal of levan synthesis followed by sucrose inversion:

$$\text{n glucose} + (C_6H_{10}O_5)_n \rightleftharpoons \text{n sucrose} \rightarrow \text{n glucose} + \text{n fructose}$$

2) Alternatively, levan may be broken down directly to reducing sugar, or 3) small oligolevans may be formed and subsequently hydrolyzed to fructose. The term oligolevans, as applied here, refers to levan molecules so small that they do not appear as polysaccharide in the elution assay.

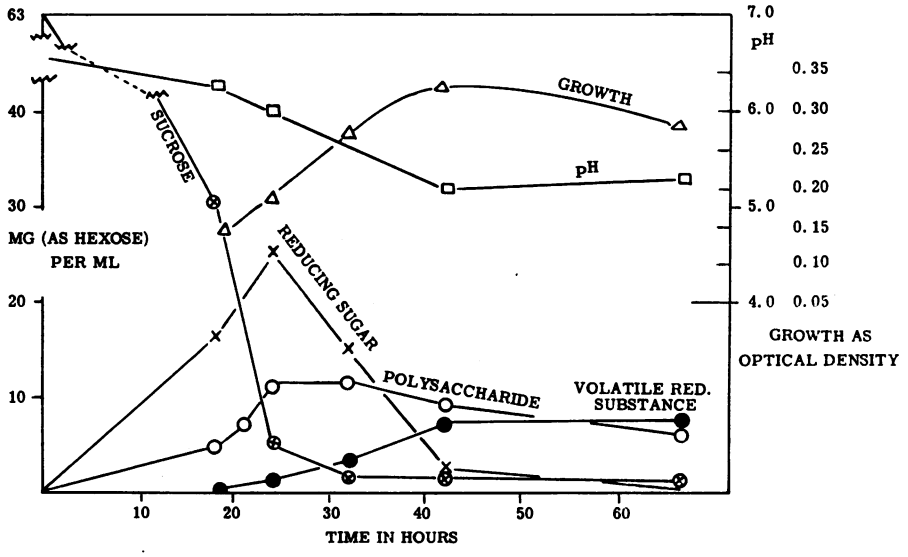

FIG. 5. Chemical changes during fermentation of 2-B (6 per cent sucrose) medium by strain 248. Incubation was on a rotary shaker at 25° C. Analyses were made as described under Experimental Methods.

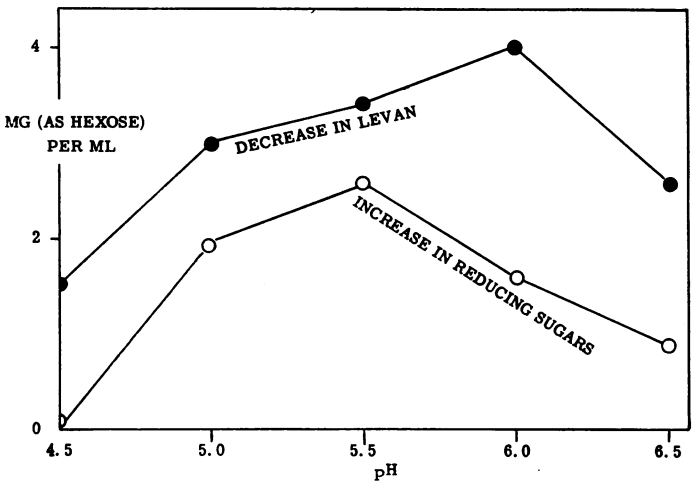

FIG. 6. pH optima for levan breakdown systems. Incubation of reaction mixtures was at 30° C. The enzyme preparation was made from a culture of strain 248 on 2-B (6 per cent sucrose) medium after 42 hours' incubation on a rotary shaker at 25° C. Levan hydrolysis was measured both by decrease in levan concentration and by increase in reducing sugar concentration. Initial levan concentration was 14.6 mg (as hexose) per ml.

In order to determine whether more than one enzyme was involved in this "levanhydrolase" reaction, the pH optima for levan disappearance and reducing sugar formation were determined simultaneously. The reaction mixture consisted of 4.0 ml of enzyme solution, 0.5 ml of a solution of purified levan containing 14.6 mg (as hexose) per ml, and 0.5 ml of distilled water. Adjustment of pH was made with concentrated H_3PO_4 or 5 N NaOH. The enzyme preparation was made from a culture of strain 248 grown on 2-B (6 per cent sucrose) medium for 42 hours. It can be seen from figure 5 that the sucrose concentration was negligible at this time so that further synthesis of levan should not interfere.

Figure 6 demonstrates a pH optimum of 6.0 for levan disappearance and an optimum of 5.5 for reducing sugar formation. It may be concluded that at least two enzymes are involved in over-all levan hydrolysis, and that either the reverse-synthesis mechanism or the formation of oligolevans may be involved, while direct fructose formation from levan cannot be the sole means of breakdown.

Reactions competing with levan synthesis. The observation that during levan synthesis a quantity of reducing sugar was produced which considerably exceeded that which could be accounted for by the supposed synthesis mechanism led to a consideration of possible competing reactions. In this regard an examination of the relative activities of the various enzyme systems during different stages of a fermentation is of interest. Table 5 provides data for the comparison of the levan synthesis, reducing sugar production, and levan breakdown caused by enzyme preparations made from samples taken at three different times during a fermentation. The approximate levanhydrolase activity was measured as before, but 0.5 ml of yeast invertase preparation was first added to the 4.0 ml of enzyme and incubated for a period of time sufficient to invert all residual sucrose. The invertase preparation did not hydrolyze the levan. After sucrose inversion, 0.5 ml of levan solution was added and the assay continued as usual. Although the age of the broth had little or no effect upon the relative activities, some interesting relationships between the different activities may be seen from the data.

TABLE 5. *Relative enzyme activities of cell-free extracts**

Time†	Increase in Levan Concentration	Increase in Total Reducing Sugar	"Excess" Reducing Sugar‡	Approximate Levanhydrolase Activity	
				Levan decrease	Reducing sugar increase
hr	*mg/ml*	*mg/ml*	*mg/ml*	*mg/ml*	*mg/ml*
20	19.2	53.1	33.9	2.6	2.6
29	14.8	47.5	32.7	3.0	2.8
43	15.3	45.0	29.7	1.0	1.7

* Prepared from fermentations on 2-B (6 per cent sucrose) medium. Reaction mixtures were adjusted to pH 5.5 and incubated for 24 hours at 30 C. The changes in concentration of the various carbohydrate substances during this period were taken as measures of the various enzyme activities.

† Age of the fermentation broths from which the enzyme preparations were made.

‡ Difference between total reducing sugar and the change in levan concentration.

If the only mechanism for levan breakdown is that of reverse synthesis, the value for "levanhydrolase" activity has no meaning. In this case the reducing sugar produced in excess of that produced in synthesis arises solely from the action of sucrose. However, if levan is broken down by a hydrolytic mechanism, the amount of levan disappearing must be considered in evaluating total levan synthesis, while the reducing sugar produced by hydrolysis of levan and oligolevan must be considered as part of any "excess" reducing sugar.

It can readily be seen that even if the "levanhydrolase" action is considered, a large percentage of the sucrose has been broken down to reducing sugar by some other means. The following explanations may be considered for this behavior: 1) Sucrose action is responsible for the excess. 2) Large quantities of oligolevans which cannot be detected in elution analysis may have been synthesized, and the glucose arising from their synthesis accounts for the excess. 3) The formation of fructose by hydrolysis of oligolevans may provide some of the extra reducing sugar. 4) A combination of these processes is responsible.

No matter by what mechanism the "excess" reducing sugar is formed, the sucrose from which it came is no longer available for levan synthesis. Therefore, in order to obtain high levan yields, this sucrose loss must be held to a minimum. The pH of the reaction mixture

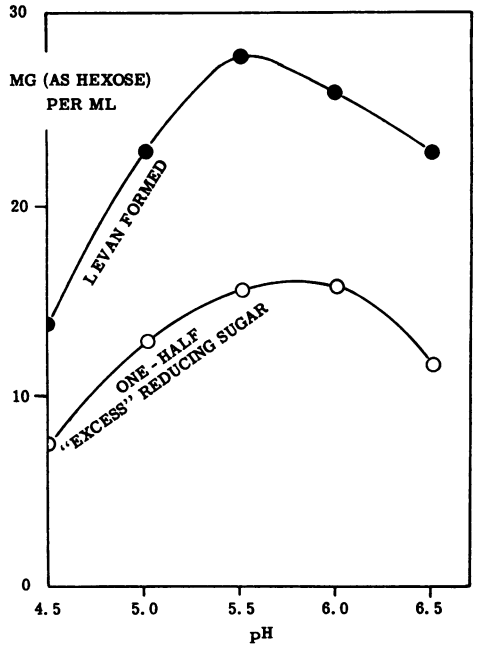

FIG. 7. Effect of pH on levan synthesis and competing reactions. The enzyme preparation was made from 2-B (6 per cent sucrose) broth after 20 hours' incubation for 24 hours at 30° C. One-half "excess" reducing sugar is used as a measure of the sucrose lost in reactions competing with levan synthesis.

and the addition of primer are two factors which influence the relative rates of sucrose utilization by the levan-synthesis and the competing sucrose-splitting reaction.

The relative rates of these two reactions at different pH values are compared in figure 7. Rates are expressed as the relative increase in levan and one-half the "excess" reducing sugar respectively. The pH optimum for the synthesis reaction appears to be somewhere between 5.4 and 5.7 while that for the competing reaction is in the vicinity of 5.7 to 6.0. The most favorable ratio of levan synthesis to sucrose splitting seems to be in the region of pH 5.3 to 5.5.

The effect of primer on levan synthesis and competing reactions. The effect of primer in enzymatic levan synthesis appears to hold considerable promise both for increasing yields and for controlling the molecular weight of the polysaccharide. The second aspect will be considered later. It was observed in preliminary experiments that enzyme preparations obtained from different fermentations containing increasing sucrose concentrations synthesized increasing amounts of levan. The possibility that this increased activity was due, at least in part, to the presence of increasing amounts of primer in the centrifuged extracts was tested in the following manner: Enzyme preparations were made from 3 fermentations on 2-B medium which contained respectively 2, 6, and 10 per cent sucrose and were incubated for 24 hours at 25 C. Primer was obtained by heating a 3.3-ml aliquot of the centrifuged broth from the 6 per cent sucrose fermentation for 5 minutes in a boiling water bath to destroy enzyme, followed by precipitation of levan with 9 volumes of methanol and subsequent drying of the precipitate. Enzymatic reaction mixtures were made which consisted of 3.5 ml enzyme preparation and 0.5 g sucrose. Primer was added to one of the mixtures containing enzyme from the 2 per cent sucrose medium. The reaction mixtures were all adjusted to pH 5.5 and incubated at 30 C. The changes in polysaccharide concentration are recorded in table 6. These data indicate that primer greatly stimulates levan production.

TABLE 6. *Effect of primer on levan synthesis*

Sucrose Content of Medium	Enzyme preparation*			
	Initial levan	Levan synthesized		
per cent		*4 hr*	*21 hr*	*45 hr*
2.0	3.0	2.0	8.5	18.1
2.0	12.9†	4.1	28.1	30.0
6.0	11.4	3.7	19.2	24.4
10.0	19.5	1.7	23.4	38.0

* Enzymatic preparations were made after 24 hours' incubation of the 2-B media at 25 C. Enzymatic reaction mixtures consisted of 3.5 ml of enzyme preparation and 0.5 g sucrose. The pH was adjusted to 5.5, and incubation was at 30 C. Levan values are expressed as mg of fructose per ml.

† Initial levan concentration increased by the addition of a primer polysaccharide preparation from the 6 per cent sucrose broth.

Further examination of this effect indicated that increasing amounts of added primer not only caused greater levan production, but diminished the amount of "excess" reducing sugar formed as well. Therefore, enzymatic reaction mixtures similar to that used in the previous experiment (2 per cent sucrose medium) were prepared, and increasing amounts of primer were added to them.

Table 7 compares the values obtained, either by direct assay or estimation, for carbohydrate substances in the reaction mixtures with the corresponding values obtained directly after paper-chromatographic separation (see experimental methods). The spots for sucrose, glucose, and fructose were eluted and analyzed by the anthrone method. Although glucose and fructose were poorly resolved in this chromatogram, the general tendency for fructose concentration to decrease and glucose concentration to increase with increase in primer concentration can be noted. This behavior of glucose and fructose of course reflects respectively the increase in levan synthesis and the decrease in sucrose disappearance by a competing reaction previously mentioned.

It was observed in experiments similar to that just described that the primer effect was not as marked when the pH of the broth was somewhat lower at the end of fermentation step than the usual value of 6.0.

TABLE 7. *Analyses for carbohydrates in reaction mixtures and after chromatographic separation*

All values expressed as hexose in mg per ml

Primer	Analyses	Total Carbohydrate	Total Reducing Sugar	Polysaccaride	"Excess" Reducing Sugar*	Resid. Sucrose†	Fructose	Glucose
4.2	On mixture	98.0	55.0	18.8	36.2	24.2	—	—
	After chr. sep.	83.1	44.1	17.4	26.7	21.6	22.7	21.4
12.1	On mixture	105.0	58.0	33.4	24.6	13.6	—	—
	After chr. sep.	97.8	52.5	30.5	22.0	14.8	26.3	26.2
19.8	On mixture	112.0	58.0	46.0	12.0	8.0	—	—
	After chr. sep.	101.0	39.2	49.0	—	12.4	13.6	25.6
25.5	On mixture	120.0	58.0	58.4	0	3.6	—	—
	After chr. sep.	105.0	43.2	56.0	—	6.2	8.0	35.2

* "Excess" reducing sugar is the difference between the total reducing sugar value and the value for levan.

† Residual sucrose values were estimated in the mixtures by subtracting the values for levan and total reducing sugar from total carbohydrate values.

The enzymatic preparation was obtained from 2-B (2 per cent sucrose) medium after 20 hours' incubation at 25 C. Primer was obtained from 2-B (6 per cent sucrose) medium after 24 hours' incubation at 25 C. Enzymatic reaction mixtures consisted of 4.0 ml enzyme solution, 0.5 g sucrose, and primer solution in varying amounts. Total volume was 5.0 ml. The pH was 5.5. Assays were made after 24 hours' incubation at 30 C.

Since it was considered possible that the pH optimum for synthesis of levansucrose is greater than 6.0, the effect of primer was tested again, but a medium which maintained a pH value greater than 6.0 was substituted for medium 2-B. This new medium (3-A) was the same as 2-B except that (1) ammonium ion from ammonium nitrate replaces the ammonium ion of an equivalent amount of ammonium sulfate, and (2) the pH was adjusted to 7.0 instead of 6.8 before autoclaving. The pH was 6.6 after 23 hours' incubation. It was found that appreciably smaller amounts of primer were required to achieve an effect equivalent to that observed previously.

Effects of added reducing sugars on enzymatic activity. Attempts to inhibit selectively the enzyme system which produces "excess" reducing sugar by addition of glucose or fructose proved unsuccessful. Varying amounts of glucose or fructose were added to reaction mixtures consisting of 0.5 g of sucrose and 4.0 ml enzyme prepared from a 42-hour-old culture of strain 248 grown on 2-B (6 per cent sucrose). The final volume of each of the mixtures was 5.0 ml and the pH was 5.5. The data of table 8 indicate that glucose inhibited both levan synthesis and the competing reaction, but fructose inhibited slightly only the synthesis. It is obvious that addition of neither sugar selectively retards the competing reaction.

TABLE 8. *Effect of glucose and fructose on enzyme activity*

Added Hexose	Per Cent Inhibition	
	One-half "excess" reducing sugar	Levan synthesis
mg/ml		
Glucose		
0	0	0
10.2	9.4	3.9
40.8	32.5	41.3
76.5	50.0	67.0
Fructose		
9.8	0	16.1
39.2	0	7.7
73.5	7.7	4.5

Initial sucrose concentration (as hexose) in the reaction mixture was 105 mg per ml. Assays for enzymatic activity were made after 24 hours' incubation at 30 C. The reaction mixtures consisted of 4.0 ml enzyme preparation, 0.5 g sucrose, and solutions of glucose or fructose to a total volume of 5.0 ml. The pH was 5.5.

Effect of pH on stability to heat. Figure 8 is an illustration of an attempt to denature selectively the enzyme system which produces "excess" reducing sugar by heating the enzyme extract at different pH values. The enzymatic reaction mixture consisted of 4.0 ml of enzyme solution, 0.2 ml of an 0.8 per cent primer solution, and 1.0 ml of a 50-per cent sucrose solution. Desired pH values were obtained by addition of phosphoric acid or sodium hydroxide. The tubes were heated for 30 seconds without mixing and immediately placed in cold water to prevent further denaturation. The pH of each solution was then adjusted to 5.5, and the tubes were incubated for 24 hours at 30 C. It is

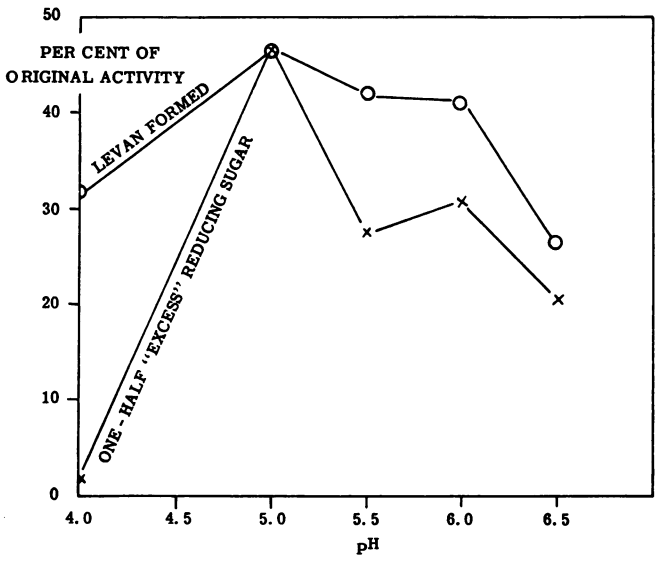

FIG. 8. Effect of pH on stability to heat. 5.2 ml of reaction mixture were heated in 18 x 150-mm Pyrex test tubes for 30 seconds in a boiling water bath without mixing. The tubes were then placed in a cold water bath. Enzymatic assays were performed after 24 hours' incubation at 30° C. All samples were adjusted to pH 5.5 prior to incubation.

apparent that both enzyme systems were most stable at pH 5.0. The enzyme system producing "excess" reducing sugar was almost completely inactivated by heating at pH 4.0, while 30 per cent of the levan-synthesizing activity still remained.

Physical Chemical Studies

Results of light-scattering experiments. The determination of molecular weights by the light-scattering method of Debye (1947) was found to be unreliable for the levan preparations obtained in this study because the complete removal of very high molecular-weight material from analytical fractions was not accomplished. In order to apply this method, there must be no particles larger than $\frac{1}{20}$ the wavelength of the light used. When such particles are present, the method of Zimm (1948a, 1948b) must be used. The latter technique was applied to levan fractions whose precipitation and dialysis behavior strongly indicated an average molecular weight of about 10,000 for the bulk of the material, but the weight-average molecular weight observed was about 50,000. It was concluded from the results of this and other similar experiments that either the fractions were contaminated by a small amount of very high-molecular-weight material, possibly protein, which was not removed by high speed centrifugation, or else some of the smaller particles in the solution formed large agglomerates. It follows, therefore, that neither method gives a reliable indication of the size of the *bulk* of the material. Since no good alternative method was available at the time of this work, only a qualitative indication of molecular weights could be obtained by the use of solvent fractionation and large-pore dialysis.

Fractionation studies. An examination of the precipitation behavior of a levan gives an indication of its homogeneity. When a precipitant such as methanol is used, it may be expected that, in general, high-molecular-weight material is precipitated by relatively low methanol concentrations, while the more soluble material requires the addition of greater amounts of methanol. Hence, a methanol fractionation curve in which the concentration of unprecipitated levan is plotted against methanol concentration (per cent by volume) is useful in estimating the range of the relative molecular weights of the levan molecules and the percentages of these molecules within a given molecular weight range.

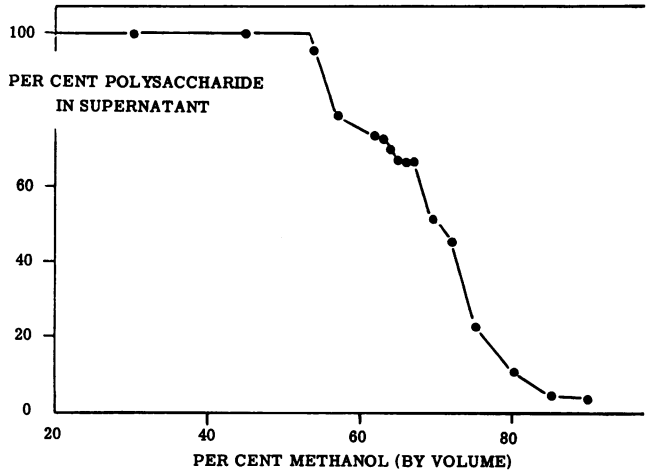

FIG. 9. Methanol precipitation of polysaccharide from strain 6718. Precipitation was carried out at 0° C. A 5 per cent solution was used. Residual polysaccharide was determined by the anthrone method.

TABLE 9. *Large-scale polysaccharide production*

Culture Number	Growth Time	Growth*	pH	Yield of Dry Product†
	hr			*g*
A5	32.5	0.16	5.9	37.4
1624	41.0	0.27	5.4	17.8
6718	18.0	0.086	6.0	39.4

* Growth is expressed as optical density of a cell suspension diluted 1 to 20 with distilled water.

† The theoretical yield of dry product was 99.5 g.

Figure 9 illustrates a typical methanol fractionation of a crude levan. The fractionation was carried out at 0 C on levan prepared from a culture of strain 6718 grown on medium 2-B (10 per cent sucrose). The material appears to have a fairly wide molecular-weight range, but it may be divided up into two rather heterogeneous fractions. The first of these precipitates at about 60 per cent methanol and contains relatively high-molecular-weight material. The percentage of this first fraction corresponds roughly to the percentage of levan which cannot be eluted from filter paper by water in 15 minutes. The weight-average molecular weight for this fraction, as obtained by the method of Zimm (1948a, 1948b) was about 1.8×10^6. The second fraction appears to contain molecules with a wide range of molecular weights.

The cultures which were selected by the screening procedure were tested for "high-molecular-weight" fraction by the elution method. Those cultures which produced the highest yields of levan and the lowest percentage of "high-molecular-weight" levan on medium 2-B (10 per cent sucrose) were selected for large-scale production and fractionation studies. For each culture, 21 individual fermentation flasks were prepared in the usual manner and inoculated simultaneously. After a suitable fermentation period, the broths were pooled and the levan purified as described previously. The results of this experiment are recorded in table 9. The fractionation curves of these polysaccharides were found to be very similar to each other (figure 9). It was concluded that none of these cultures produced predominantly a single homogeneous fraction under the given conditions.

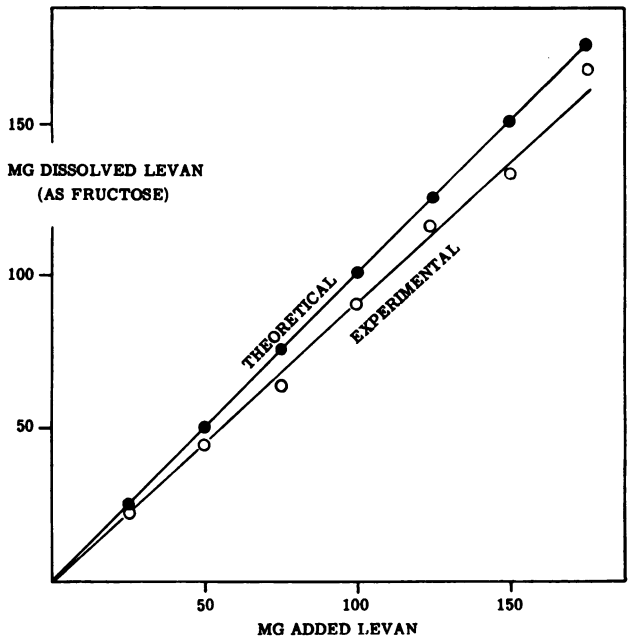

FIG. 10. Solubility of methanol-precipitated levan from strain 248. Levan was prepared from a fermentation by strain 248 on 1-A (6 per cent sucrose) medium.

In order to reproduce a fractionation it is essential to carefully control temperature, levan concentration, rate of methanol addition, and the rate of mixing of the solution. Particular care should be taken to insure temperature control. If methanol is added rapidly, or if mixing is inadequate, localized high concentrations of methanol may form sticky masses of levan of molecular weight lower than that desired in the given fraction being prepared. Since such masses are redissolved with difficulty, the precipitated fraction may be contaminated by this material. Other solvents such as dioxane, acetone, and isopropyl alcohol do not appear to be as satisfactory for fractionation as methanol, in as much as the latter causes a much more gradual precipitation of levan than do these other solvents.

The use of methanol as a levan precipitant appears to cause some retrogradation or agglomeration of levan, for it has been observed that polysaccharide precipitated by 90 per cent methanol will not redissolve completely in water. In figure 10, the solubility of previously precipitated levan from strain 248 is shown. A water-insoluble fraction amounting to 8.75 per cent of the total levan remains undissolved. Subsequent methanol precipitation and redissolving of this new levan solution revealed the formation of a second insoluble portion. This behavior has been noted with levans from a number of the different bacterial strains. If similar retrogradation takes place to a limited extent in aqueous solutions, the abnormally high molecular weights obtained by the Zimm method might be explained.

Effect of primer and high sucrose concentrations on molecular weight. Since it appears from fractionation experiments that none of the bacterial strains which passed the screen produce large homogeneous levan fractions in normal fermentation, attention was turned to cell-free preparations because they have fewer variables and can be more rigidly controlled. Of primary interest are the effects of added primer which not only increases the rate of formation and final yield of levan, but also alters the molecular weight distribution.

An example of the effect of primer on molecular weight is illustrated in figure 11. The polysaccharide for these experiments was produced by an enzyme preparation from a culture of strain 248 incubated on 3-A (2 per cent sucrose) medium for 22 hours at 25 C. Both reaction mixtures had a sucrose concentration of 10 per cent. To one of them primer was added to the amount of 2.41 per cent. The pH was adjusted to 5.5 and the tubes were incubated at 30 C for 36 hours. During this period, 96 per cent of the sucrose was utilized in each reaction mixture. A levan yield of 59.6 per cent was obtained when primer was present, compared to a 44 per cent yield obtained when no primer was added.

The primer was synthesized by an enzyme preparation made from 2-B (2 per cent sucrose) broth after 22 hours' incubation at 25 C. An initial sucrose concentra-

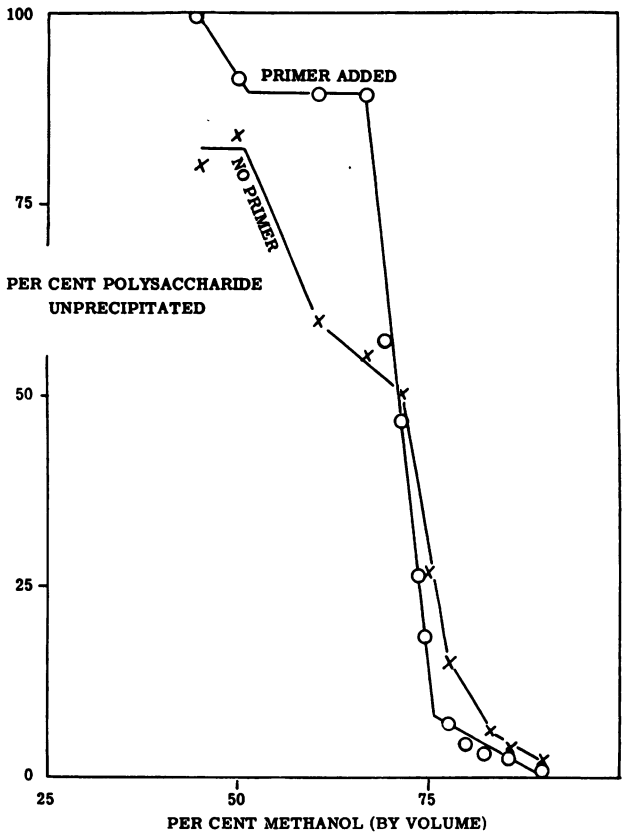

FIG. 11. Fractionation of 248 levan synthesized with and without primer. Fractionations were performed at 0° C. Levan in the supernatant was determined by anthrone assay.

tion of 44 per cent was used in order to decrease the size of the levan. During purification 30.2 per cent of this material was lost through dialysis, and was therefore of low molecular weight. From the remaining levan, the fraction precipitating between 77 and 90 per cent methanol, which was assumed to be of lowest molecular weight, was retained for use as primer. Since the rate of dialysis of this material through a large-pore membrane was somewhat greater than the rate for a dextran fraction known to possess a weight-average molecular weight of 11,000, it was assumed that the primer possessed an average molecular weight somewhat lower than this value.

It is apparent that the levan produced in the presence of primer consists predominantly (to the extent of 80 per cent) of one fraction, precipitated between 67.5 and 76 per cent methanol. On the other hand, when primer is not included, it can be seen that the synthesized levan possesses several fractions and contains more material of high molecular weight, as indicated by the precipitation at lower methanol concentrations.

It seems probable that the molecular weight of polysaccharide could be controlled fairly well by proper regulation of sucrose concentration, primer concentration, and primer molecular weight. Furthermore, if the enzyme system which produced polysaccharide synthesis can be separated from the competing system, much higher yields of levan should be obtainable.

SUMMARY

A screening procedure is described which was designed to select bacterial strains capable of producing polysaccharides displaying the properties required of suitable plasma extenders. From 850 random soil isolates, 7 strains were selected which produced relatively large amounts of polysaccharide of molecular size as large as, or greater than, clinical dextrans, and which displayed ratios of osmotic pressure to viscosity quotient, that is, ln relative viscosity/concentration, near those of clinical dextrans.

One of the selected culture, number 248, was identified as a strain of *Bacillus subtilis*, which produces levan by an extracellular mechanism. An inoculum concentration optimum for the production of levan in growing cultures of this strain was observed. This effect requires the presence of an optimal concentration of intact cells.

Two primary types of enzymatic activity were noted in cell-free extracts from cultures of strain 248—levan synthesis and a competing reaction which forms a large amount of reducing sugar in excess of the glucose expected as a result of synthesis by the mechanism:

$$\text{n sucrose} \leftrightharpoons (C_6H_{10}O_5)_n + \text{n glucose}$$

A minor percentage of the "excess" reducing sugar may be accounted for by a third type of reaction in which levan is slowly degraded. This "levanhydrolase" activity appears to involve at least two enzymes. Possible mechanisms of these reactions are proposed and their interrelationships discussed.

The most favorable ratio of the rate of levan synthesis to the rate of the primary competing reaction appears to occur between pH 5.4 and pH 5.7. Added glucose was found to inhibit both the levan-synthesis reaction and the formation of "excess" reducing sugar to the same degree. Fructose showed no appreciable inhibition of either reaction even at very high concentrations.

It is possible at pH 4.0 to destroy almost completely the activity of the enzyme system producing "excess" reducing sugar, while still retaining 30 per cent of the synthesis activity, by heating 5-ml volumes in a boiling-water bath for 30 seconds. Both enzyme systems were most stable to heat at pH 5.0.

It was not possible in this study to obtain levan samples which were entirely free of very large particles. These particles caused weight-average molecular weight, as determined by light scattering, to be so high that no reliable indication of the average molecular weight for the *bulk* of the sample could be obtained.

Methanol fractionation of polysaccharides produced by several selected cultures always revealed the presence of significant amounts of polysaccharide of very high molecular weight. The remainder of the polysaccharide consisted of particles apparently varying widely in molecular weight. Methanol precipitation of levans appears to cause about 9 per cent of the material to retrograde.

The addition of preformed levan acceptor molecules (primer) to a levan-synthesizing system accelerates synthesis, increases final levan yield, and effects the production of a high percentage of relatively homogeneous product, with respect to molecular weight.

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Virucidal Activity of Representative Antiinfective Agents Against Influenza A and Vaccinia Viruses[1]

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The literature on the virucidal activity of various chemical agents has been recently reviewed by Dunham (1954). Many of the reports are fragmentary and widely scattered, and the data available were obtained under a wide variety of conditions of test.

This study was undertaken to compare the virucidal activities of representative, commonly employed, antiinfective agents against two dissimilar viruses under the same experimental conditions. Because viruses themselves lack demonstrable metabolic activity, the methods employed were based primarily upon the phenol coefficient method (Reddish, 1954). Vaccinia virus and influenza A virus were chosen as the two test viruses because of their biological dissimilarity and the quantity of information available about them.

MATERIALS AND METHODS

Preparation of viral inocula. Influenza A virus. The PR-8 strain of influenza A virus was maintained by serial passage of 0.2 ml amounts of dilute inocula (10^{-4} to 10^{-7}) in the allantoic cavity of chicken embryos in the tenth or eleventh day of embryonic development. Eggs were incubated at 36 C for 40 hours after infection, and all viable eggs were chilled in the refrigerator (8 C) overnight before collection of allantoic fluid. Dead embryos were discarded. Infected allantoic fluids were pooled and enough inactivated (56 C for 30 minutes) normal horse serum was added to make a final concentration of 20 per cent. The inoculum was then distributed in 2.0-ml. amounts into ampuls which were stored at −70 C (in a dry ice chest). One frozen ampul from each pooled inoculum was thawed and titrated for infectivity in eggs before that inoculum was used in virucidal tests. *Vaccinia virus.* The New York City Board of Health strain of vaccinia virus was maintained by serial passage of 0.2-ml amounts of diluted virus (10^{-3} to 10^{-6}) on the chorioallantoic membrane of 10- to 11-day chicken embryos. The creation of the false air sac in the eggs was greatly facilitated by the use of the Vir-Tis egg punch.[2] The infected eggs were incubated for 4 days at 36 C. Chorioallantoic membranes showing well-developed pocks were removed, pooled, and homogenized in saline to make a 10 per cent suspension by weight. The suspension was clarified by centrifugation, and sufficient inactivated normal horse serum was added to make 20 per cent by volume. The pooled inoculum was then distributed into ampuls and stored at −70 C as described above.

Infectivity titrations in embryonated eggs. Serial decimal dilutions of inocula or virus-agent mixtures were made in physiological saline and 0.2-ml amounts of each dilution were inoculated into groups of 6 to 10 eggs each. *Influenza A virus.* Eggs inoculated with influenza A virus into the allantoic cavity were incubated for 48 hours at 36 C. All viable eggs were chilled overnight at 8 C before collection of allantoic fluid. Dead embryos were discarded. The presence or absence of viral hemagglutinin in allantoic fluids from individual eggs was used to indicate that infection and viral

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[2] Virtis Co., Yonkers, New York.