SEROLOGICALLY REACTIVE POLYSACCHARIDES PRODUCED THROUGH THE ACTION OF BACTERIAL ENZYMES*

I. Dextran of Leuconostoc mesenteroides from Sucrose

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A preliminary paper (1) reported that a serologically reactive polysaccharide of dextran nature is produced from sucrose through the action of an enzyme or some similar heat-labile agent contained in sterile filtered extracts of *Leuconostoc mesenteroides*. The object of the present paper is to describe the preparation of the sterile extracts and to compare the chemical and serological properties of the dextran formed by the bacteria-free extracts with those of the dextran formed in cultures of the living bacteria.

Previous workers have reported the production of gum-like material from sucrose by sterile filtrates derived from various species of spore-forming bacilli. In 1910 Beijerinck (2) described the formation of "slime" on sucrose agar by filtered preparations of *Bacillus mesentericus*; he considered the active principle to be a "synthetically active enzyme" which he termed *viscosaccharase*. Harrison, Tarr, and Hibbert (3) reported that a sterile filtrate of similar bacilli formed from sucrose an alcohol-precipitable material which gave opalescent solutions. Also, it is possible that the production of bacteria-free "colonies" on sucrose agar plates by filtrates of sucrose broth cultures of *Bacillus fluorescens* which has been reported by Dienes (4) and others represents another manifestation of the same phenomenon. However, in none of the earlier studies were the products identified chemically or tested serologically, whereas in the present study the product is proved to be an immunologically reactive polysaccharide similar in both chemical and serological properties to the product formed in cultures of the living bacteria.

EXPERIMENTAL

Materials and Methods

Preparation of the Extracts.—The extracts utilized in the present study were made from cultures of a strain of *Leuconostoc mesenteroides* isolated in this laboratory; it gave mucoid growth on sucrose mediums and produced acid from both xylose and

arabinose. With this strain of leuconostoc the fundamental principle that serologically reactive material could be formed in the absence of the bacterial cells was demonstrable with Berkefeld filtrates of sucrose broth cultures. That is, these filtrates which were apparently sterile as far as could be told by microscopic or cultural tests showed a gradual increase in opalescence and in serological reactivity when stored in the ice box. However, for a study of the formation of the polysaccharide the filtrates had the disadvantage of containing only a low concentration of the active agent in comparison to the large amounts of the preformed product. That disadvantage was met by applying the principle introduced by Sevag (5) for the separation of proteins from polysaccharides: when the fluids of sucrose broth cultures were shaken with chloroform most of the preformed polysaccharide remained in the supernatant, whereas the active principle which would subsequently produce the specific polysaccharide (when added to sucrose) was contained in the chloroform emulsion layer. With this strain of leuconostoc (which has a highly soluble polysaccharide) the active agent could also be separated by salting-out procedures but the chloroform treatment was more convenient and was utilized to prepare all of the extracts used in the present experiments.

Some extracts were made from the supernatant fluids of centrifuged cultures but for convenience most of them were prepared from the whole cultures without preliminary removal of the bacterial cells. The usual starting material was a total of 5 one-liter lots of culture which had been grown in separate flasks for about 20 hours at 23°C.; the cultures had received a large inoculum and by the time they were used had already become gummy or viscous; microscopic observation showed no evidence of autolysis or disintegration of the bacterial cells. The medium in which the cultures were grown consisted of 1 per cent bacto-peptone (without meat extract or infusion), 0.5 per cent salt, and 5 per cent sucrose. This medium afforded adequate growth and for the preparation of the extracts had the advantages of giving little foam when shaken and of containing only a minimum amount of material adsorbable upon chloroform.

Each flask of culture was chilled, shaken by hand for 5 minutes with 25 cc. of cold chloroform, and then centrifuged in the cold at 1500 R.P.M. for 5 to 10 minutes. The supernatant fluids which contained most of the serologically reactive polysaccharide and most of the bacteria were decanted from the chloroform emulsion precipitates which contained the active principle. In order to get further separation from the preformed polysaccharide the emulsion material from all of the flasks was collected and washed two or three times with cold distilled water; each time the emulsion was suspended in 500 cc. of water and then sedimented by low speed centrifugation and carefully separated from the wash fluid. In order to free the active principle from its combination with the chloroform the washed emulsion material was treated with 500 cc. of cold 95 per cent ethyl alcohol, in which the active agent was insoluble. The particles were brought into a finely dispersed state and this alcohol suspension was centrifuged in the cold at high speed for 40 minutes. The alcohol was decanted and, after the tubes had drained for an hour in the refrigerator, the precipitate was extracted with 50 cc. of distilled water and adjusted to about pH 7.5 with 0.1 N NaOH. After removal of insoluble particles by centrifugation at high speed the extracts were filtered through Berkefeld W filters which had been

tested by the "bubbling pressure" method and found to emit air only at or above a pressure of 680 mm. of mercury. The filtered extracts were adjusted to pH 6.0 and then stored in a brine-cooled chamber at a temperature slightly below 0°C.

Sterility Controls.—Each extract was examined carefully by microscopic and cultural methods and in no instance were any microorganisms detected. No disinfectants were added to the extract-substrate mixtures but all of the constituents had been sterilized either by filtration or by autoclaving and aseptic technique was employed in the preparation and subsequent handling. The sterility of the extract-substrate mixtures was well controlled by microscopic and cultural tests made at appropriate intervals during the incubation periods as well as at the beginning and end of the experiments. The cultural tests consisted of inoculations in sucrose peptone broth and agar slants and also in broth and agar slants enriched with sterile (filtered) unheated cane juice; all these mediums were known to be adequate for growth of leuconostoc bacteria and those enriched with the unheated cane juice can be regarded as furnishing an especially rigorous test. From the results of the numerous sets of controls we feel certain that all of the observed reactions occurred in the complete absence of bacteria.[1]

Potency of the Extracts.—The present paper does not include any systematic data on the minimal time and the minimal concentration of extract which are required for the production of demonstrable amounts of the polysaccharide, but some information on both those points was obtained. In respect to time, if the extract was used in dilutions of 1:2 or 1:4 in mixtures containing 5 per cent sucrose significant amounts of reactive material were regularly produced within 1 to 2 hours at either 23 or 37°C. In respect to minimal amount of extract, dilutions of 1:1000 of all and of 1:10,000 of most of them would form demonstrable amounts of the polysaccharide in test mixtures containing 5 per cent sucrose if incubated for 20 days at 23°C.

Serological Tests.—All of the antiserums were from rabbits. The dextran of the present strain of leuconostoc reacts with Types 2 and 20 antipneumococcus as well as with antileuconostoc serum (6, 7); in the many comparative titrations which we have made the range of dilutions of the dextrans which gave precipitation was usually essentially the same for those three kinds of antiserum. Hence, in most experiments of this paper the dextran produced by action of the extracts was measured on the basis of tests with only one of the antiserums, usually Type 2 antipneumococcus. The dextran also reacts to some extent with Type 12 antipneu-

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[1] Tubes of sucrose broth if inoculated with as much as 0.5 cc. of the extracts became opalescent but the opalescence can be accepted simply as an intrinsic property of the polysaccharide product formed from sucrose by the active principle of the extracts; no bacteria were demonstrable in the opalescent solutions either by direct examination or by subculture, and no change in pH or titratable acidity was evident whereas with leuconostoc bacteria an increase in acidity would have occurred. Further evidence that the opalescence did not represent microbial growth was that its development was neither more rapid nor finally greater in amount when the extracts were inoculated in sucrose mediums (broth or unheated sugar cane juice) which could support luxuriant growth of the bacteria than when the same extracts were added to aqueous sucrose solutions in which leuconostoc bacteria could grow poorly if at all.

mococcus serum but since this cross reaction occurs only with relatively high concentrations of the antigen the Type 12 antiserum by itself was unsuitable for routine measurement of the product of the action of the extract.

The leuconostoc antiserums were produced by immunization with bacteria grown in sucrose peptone broth; none of the antiserums we have obtained with leuconostoc grown on glucose mediums have been reactive with the dextran. The pneumococcus antiserums had been produced by immunization with bacteria grown in infusion broth containing no added carbohydrate; with the pneumococci growth in a sucrose medium apparently is not essential in order to get an antiserum reactive with the leuconostoc dextran. It should be noted that an occasional lot of either Type 2 or 20 antiserum although of high homologous titre may lack the capacity to give the usual leuconostoc reaction (6).

Action on Sucrose

A number of lots of sterile extract prepared at different times by the described procedure have been tested and all have had the capacity of forming serologically reactive material from sucrose. The extracts obviously do not represent purified solutions of the active agent but were adequate to establish the general principle of the reaction with sucrose and to permit the isolation of the purified polysaccharide product in amounts large enough for chemical as well as serological study. None of the extracts was entirely free of preformed polysaccharide. However, the amount contained was extremely small in comparison to the yield obtained after incubation with sucrose, so that there could be no doubt that by far the principal part of the polysaccharide found in the incubated test mixtures represented material synthesized through the action of the sterile extract.

The reaction with sucrose was recognizable not only by serological test but also by simple observation of the development of opalescence and by chemical tests for alcohol-precipitable material and for reducing sugars. The set of phenomena which always characterized the action of the sterile extract is illustrated by the data of the following experiment.

A series of mixtures consisting of one part of sterile extract plus one part of sterile (filtered) 10 per cent sucrose[2] solution in 0.2 molar acetate buffer pH 5.6 was prepared in a series of tubes; for controls similar mixtures with heat-inactivated (30

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[2] The sucrose used throughout the investigation was a sample of beet sugar known by previous test to be free from material reactive with the antiserums (leuconostoc and Types 2, 20, and 12 pneumococci) with which the leuconostoc dextrans are reactive. It was used instead of reagent sucrose because the latter usually contains significant amounts of material reactive with leuconostoc or the related pneumococcus antiserums (9, 10). The use for substrate of sucrose free of polysaccharide which is either identical or closely related to the polysaccharide product of the action of the extract seemed important for theoretical reasons, particularly because of the possible stimulatory influence of the presence of small amounts of the final product

minutes at 55°C.) extract were included; the incubation was at 23°C. At the time intervals listed in Table I the mixtures were observed for opalescence and one tube of each series was used for tests for alcohol-precipitable material, serological reactivity, and reducing sugar (8). The alcohol-precipitated material was sedimented by centrifugation and redissolved in salt solution equivalent to the original volume of the sample; this solution was observed for opalescence, tested for serological reactivity and for amount of reducing sugar before and after acid hydrolysis; for the hydrolysis the solution was heated with 1.0 N sulfuric acid for 6 hours in a sealed tube immersed in boiling water. The serological reactivity was determined by test against 1:15 dilutions of a Type 2 antipneumococcus rabbit serum; the pre-

TABLE I

Action of the Sterile Extract upon Sucrose

Test mixture	Incu-bation period	Properties of test mixtures				Properties of solutions of the alcohol precipitate		
		Opal-escence	Precipita-bility with 1.5 volumes of alcohol*	Sero-logical reactiv-ity‡	Reducing sugars (without hydroly-sis)	Opal-escence	Sero-logical reac-tivity‡	Reducing sugars after acid hydroly-sis§
	hrs.				*mg./cc.*			*mg./cc.*
	0	0	±	10	0.07	0	10	0.04
	6	+	++	200	0.4	+	200	0.3
Unheated extract plus sucrose	12	+±	+++	400	1.0	++	400	0.9
	24	++	++++	1000	2.4	++	1000	2.0
	48	+++	++++	1500	4.0	+++	1500	3.9
	96	++++	++++	3000	7.8	++++	4000	6.6
	192	++++	++++	7000	10.8	++++	7000	9.0
Heated extract plus sucrose	96	0	±	10	0.08	0	10	0.02
	192	0	±	10	0.09	0	10	0.02

* Test mixture diluted 1:5 in 10 per cent sodium acetate before addition of the alcohol.
‡ Dilution which gave precipitation with Type 2 antipneumococcus serum.
§ Only traces (0.02 to 0.03 mg. per 1 cc.) of reducing sugars before hydrolysis.

cipitation reaction was observed after 1½ hours incubation at 37°C. In the summary of the results presented in Table I the reducing sugars are calculated as glucose.

It is evident (Table I) that the development of the different phenomena (opalescence, serological reactivity, material precipitable with 1.5 volumes of

of the enzymatic action. From the practical standpoint of possible confusion in the final serological tests the presence of reactive material in the sucrose substrate would be important only in the serological test of relatively low dilutions of enzyme-substrate mixtures. Since all the experiments reported were done with sucrose derived from beet it should be noted that a sufficient number of well controlled experiments were made with cane sugar as substrate to prove that the sterile extracts can also form the reactive polysaccharide from sucrose of that origin.

alcohol, and free reducing sugar) proceeded at an orderly rate when the un-heated extract was incubated with sucrose, and that none of those phenomena occurred in the control mixture of sucrose plus heated extract.

The alcohol-precipitable material can be accepted as the product responsible for both the opalescence and the serological reactivity of the test mixture; the free reducing sugar represents another product of the action of the bacterial extract upon sucrose. It is important that throughout the course of the reaction the amount of free reducing sugar which had accumulated in the test mixtures was about the same as the amount of reducing sugar obtained by acid hydrolysis of the alcohol-precipitated material. This approximate agreement suggests that the action of the extract consists of the conversion of x molecules of sucrose into a polymer of x glucose anhydride units plus x molecules of fructose. Although that mechanism is not proved, data to be presented in Table II show that the polysaccharide is a dextran and other experiments which we have made indicate that the free reducing sugar is fructose.[3]

Chemical and Serological Properties of the Purified Polysaccharide Product

The facts (Table I) that the serological reactivity which developed in the sucrose-extract mixtures was resident in the alcohol-precipitable fraction and

[3] The supernatant fluids of extract-sucrose mixtures from which the dextran had been removed by alcohol precipitation, yielded large amounts of glucosazone (identified by crystalline appearance and by melting point) when heated with phenyl-hydrazine and sodium acetate. That the material from which the glucosazone was derived was a product of the action of the extract upon sucrose was proved by the lack of formation of glucosazone when the supernatant fluids of mixtures of heat-inactivated extract plus sucrose were subjected to similar treatment. These results indicated that the reducing substance was either glucose, mannose, or fructose.

More specific evidence was obtained by experiments utilizing the procedure of Ekkert (11) (the low concentration of the reducing substance in our test mixtures prevented the use of the methylphenylhydrazine method and the presence of sucrose in the mixtures prevented the use of the usual color tests for fructose). The method consists simply of the addition of two or three drops of 4 N NaOH and a small piece of solid NaOH to two or three drops of the test solution on a porcelain plate: if fructose is present a pink to blood red color develops which is not given by other common carbohydrates. When this procedure was applied to mixtures of sucrose plus active extract which contained from 0.3 to 1.0 per cent of free reducing sugar, they always gave the pink to red color. Control tests with 0.3 to 1.0 per cent solutions of reagent fructose gave pink to red colors of intensities comparable to those shown by the sucrose-extract mixtures of corresponding reducing sugar content. Similar tests made upon mixtures of heat-inactivated extract plus sucrose, upon solutions containing 1 and 10 per cent sucrose, glucose, galactose, xylose, arabinose, maltose, or lactose, gave either a yellow color or no color; the purified leuconostoc dextran (1.0 per cent) gave no color. Thus, although the reducing sugar formed by the action of the extract upon sucrose was not actually isolated and identified there is considerable reason to believe that it is fructose.

that this fraction yielded abundant amounts of reducing sugar upon acid hydrolysis indicated that the product responsible for the serological reactivity was a polysaccharide. It seemed important to obtain purified preparations of this product in sufficient amount for an adequate comparison of its chemical and serological properties with those of the dextran polysaccharide which is formed in sucrose broth cultures of the leuconostoc bacteria from which the extracts had been derived. A preparation of dextran from sucrose broth cultures was available from another investigation (6) and for the present experiments two lots of polysaccharide were prepared utilizing sterile extracts made at times several months apart.

For lot 1 the test mixture was 20 cc. of bacterial extract plus 380 cc. of sterile 10 per cent sucrose in 0.1 M acetate buffer solution pH 5.6; for lot 2 the mixture was 300 cc. of the buffered sucrose solution plus 15 cc. of the bacterial extract; the first lot was incubated 9 days and the second lot 7 days at 23°C. Control mixtures (heat-inactivated extract plus sucrose and unheated extract plus buffer) were also prepared and incubated for the same periods. At the end of the incubation period the test mixtures were opalescent and apparently contained adequate amounts of the polysaccharide product; the control mixtures remained clear and furthermore gave no precipitate upon addition of 10 per cent sodium acetate and 1.5 volumes of alcohol.

The polysaccharide material from lots 1 and 2 was isolated and purified by the procedure described for preparation of the dextran from broth cultures (6). After addition of 10 per cent sodium acetate, 1.5 volumes of alcohol were added to the extract-substrate mixtures; the abundant gummy precipitate was dissolved in a volume of water equal to that of the original mixture and was then reprecipitated in the presence of acetate by the addition of 1.25 volumes of alcohol. This precipitate was redissolved in 300 cc. of water and after the addition of 10 gm. of sodium acetate and 5 cc. of glacial acetic acid was shaken mechanically with chloroform to remove any traces of protein (5, 12). The chloroform-protein emulsion layer was only small in amount after the first treatment and was entirely absent after the third treatment. The protein-free material was then twice precipitated by 1.25 volumes of alcohol in the presence of acetate; the final precipitate was ground under absolute alcohol and dried in vacuum over $CaCl_2$ at room temperature. The yield was 0.70 gm. of the purified polysaccharide from lot 1 and 0.45 gm. from lot 2, which represents, respectively, 175 and 143 mg. per 100 cc. of the extract-sucrose mixtures. It is noteworthy that the yields obtained through the action of these sterile extracts is greater than the yields of polysaccharide ordinarily obtained from whole cultures of pneumococci and many other bacteria.

Both of the purified preparations were analyzed chemically and tested serologically. The specific optical rotation (sodium D line at 24°C.) of the original products was determined with 0.4 per cent solutions. For determining the properties after hydrolysis 0.2 gm. of each product was heated for 6 hours with 1.0 N sulfuric acid in a sealed tube immersed in boiling water; in addition to the analyses for reducing sugars (8) and for optical rotation, the hydrolysate was treated with phenylhydrazine and oxidized with nitric acid (13) in order to get more specific information on the nature of the reducing sugar in the hydrolyzed material.

The tests of the serological properties consisted of titrations of a series of dilutions of the two preparations against 1:15 dilutions of antileuconostoc (homologous to the strain from which the extracts had been prepared) and of Types 2, 20, and 12 antipneumococcus serums which were known to react with the previously studied (6) dextran produced by living cultures of the leuconostoc bacteria; as controls, tests were also included against 1:6 dilutions of samples of 29 other types of pneumococcus antiserum.

The data of the chemical properties are summarized in Table II and those on the serological properties in Table III. For purposes of comparison data on the dextran isolated (6) from a sucrose broth culture are included.

It is evident (Table II) that, with the exception of a difference in viscosity,[4] the chemical properties of the purified preparations of the alcohol-precipitable

TABLE II

Comparison of the Chemical Properties of Purified Preparations of the Dextrans Formed by Sterile Extracts with the Dextran Formed in Broth Culture

Source of the dextran	Color with iodine	Ash (as sodium)	Nitrogen* (by micro-Kjeldahl)	Relative‡ viscosity	Specific optical rotation*	Acid hydrolysate	
						Specific optical rotation*	Reducing sugars*,§ (as glucose)
		per cent	*per cent*		*degrees*	*degrees*	*per cent*
Extract plus sucrose (lot 1)	None	0.8	0.00	1.03	+179	+48.0	90
" " " (" 2)	"	0.8	0.04	1.05	+178	+49.9	89
Sucrose broth culture	"	0.7	0.02	1.22	+182	+48.1	94

* Calculated on ash-free anhydrous basis.

‡ Determined in an Ostwald viscosimeter at 20°C. with 0.2 per cent solution of the dextran in 0.9 per cent NaCl.

§ No reducing sugars before hydrolysis.

product formed from sucrose by the extracts are similar to those of the polysaccharides produced in sucrose broth cultures of the bacteria. The polysaccharides produced by the bacteria have been proved by others (14) to be dextrans and the polysaccharide formed by the extracts can be classified similarly on the basis of the high positive optical rotation, the high content of reducing sugars after acid hydrolysis, and the close approach of the optical

[4] Both lots of the dextran which had been formed by action of the sterile extract had a lower relative viscosity than did the dextran produced in the living bacterial culture. Since the same chemical procedure had been employed in their preparation the difference in viscosity indicates that the product of the sterile extract is of lower molecular (or particle) weight than the corresponding product of the broth culture. This difference if confirmed by examination of a large number of preparations from the two sources would present an interesting distinction between the polysaccharides formed in the usual culture and those formed in the absence of the bacterial cells.

rotation of the hydrolysate to the rotation of glucose. Additional evidence that glucose (which is the basic component of dextran) represents at least the principal portion of the reducing sugar in the hydrolysate is furnished by the fact that abundant amounts of glucose-phenylosazone were produced by treatment of the hydrolysate with phenylhydrazine, and by the fact that potassium acid saccharate (identified by its characteristic crystalline appearance) was obtained in large amount when the hydrolysate was oxidized with nitric acid (13); the absence of galactose was indicated by the fact that no mucic acid was formed during the nitric acid oxidation, and the absence of fructose, pentoses, or uronic acids was indicated by negative qualitative color tests. As shown in

TABLE III

Comparison of the Serological Properties of Purified Preparations of the Dextrans Formed by Sterile Extracts with the Dextrans Formed in Broth Culture

Source of the dextran	Reactivity versus different antiserums				
	Antileuco-nostoc	Antipneumococcus			
		2	20	12	Other types*
Extract plus sucrose (lot 1).........	+++	+++	+++	+	0
" " " (" 2).........	++++	++++	++++	±+	0
Sucrose broth culture.............	++++	++++	++++	+	0

0 = no precipitation with 1:5,000 or higher dilutions of the dextran.
+ = precipitation with 1:20,000   dilution of the dextran.
±+ =   "   " 1:50,000   "   "   "   "
+++ =   "   " 1:500,000   "   "   "   "
++++ =   "   " 1:1,000,000   "   "   "   "
* One of the two samples of Type 22 which were included gave positive reactions with 1:5,000 and 1:20,000 dilutions of all three dextrans.

Table II the leuconostoc dextrans of both extract and of culture source were distinguished from starch or glycogen by the fact that they gave no color with iodine.

The serological properties (Table III) likewise were similar; that is, the dextrans produced by the sterile extracts like the dextran from the bacterial culture reacted not only with the antiserums of leuconostoc but also with the antiserums of Types 2, 20, and 12 pneumococci. The mutual cross reactions with the three different types of antipneumococcus serums, and especially the approximate correspondence in the "ratio" of the reactivities with Types 2 and 20 in comparison to Type 12, are evidence of a closer serological likeness between the dextrans from the two sources than could be furnished by a comparison based simply on their reactivities with the homologous antileuconostoc serums.

Especially convincing evidence was obtained by a series of absorption ex-

periments. It was known from a previous study (6) that absorption with leuconostoc bacteria would remove the capacity to react with the dextran of culture source from all of the antiserums listed in Table III provided the leuconostoc used for the absorption had been grown in sucrose broth, whereas absorption with pneumococci (2, 20, or 12) would fail to remove the dextran-reacting capacity from any of the antiserums other than the homologous anti-pneumococcus type. When these experiments were repeated with dextran of extract origin together with dextran of culture origin, the two behaved exactly alike; that is, the absorption treatment which removed the antibodies reactive with one always removed the antibodies reactive with the other, and the treatments which failed to remove the antibodies reactive with one always failed to remove the antibodies reactive with the other.[5]

In spite of the striking similarity in the serological properties from a qualitative standpoint, it should be noted that one lot (No. 1) of dextran of extract source did not react in as high dilution as did the dextran isolated from the culture. However, this difference seems relatively unimportant because both lots (Nos. 1 and 2) of extract origin reacted in sufficiently high dilution (1:500,-000 and 1:1,000,000) to prove the fundamental point that polysaccharides produced in the absence of living cells can approach if not equal the quantitative serological capacity of the polysaccharides produced in the usual sucrose broth cultures of the bacteria.

Influence of pH upon the Action of the Extract

The substrate-extract test mixtures in all the previously described experiments were maintained at about pH 5.6 by the use of acetate buffer. That routine was adopted at the beginning of the investigation because of an early observation that the extracts acted upon sucrose more rapidly at that pH than at pH 7.0. In order to get more systematic information on the influence of pH the following experiment was made.

A series of mixtures consisting of one part of extract, one part of 20 per cent sucrose, and two parts buffer solution were prepared, which gave test systems ranging from

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[5] In addition to furnishing evidence on the likeness of the dextrans from the two sources, these experiments illustrate an interesting difference between Type 2 pneumococci and leuconostoc bacteria in respect to the influence of growth in sucrose broth upon the antigenic properties of the two sorts of bacteria (6, 15). That is, the capacity to evoke antibodies reactive with the leuconostoc dextran or to remove the anti-dextran antibodies from a reactive serum, is possessed by leuconostoc bacteria which have been grown in sucrose broth but not by leuconostoc bacteria which have been grown in glucose broth, whereas Type 2 pneumococci which have been grown in plain or in glucose broth can not only evoke the dextran antibodies but also can absorb them from antiserums produced by immunization with Type 2 pneumococci which have been grown either in sucrose or non-sucrose broth.

pH 3.0–9.0; citrate buffer was used for the pH 3.0, acetate for the pH 4.0, 5.0, and 5.5, phosphate for the pH 6.0, 7.0, and 8.0, and borate for the pH 9.0 mixture. The pH which was tested at the beginning and again at the times of each serological determination, remained approximately constant in all the mixtures except the pH 9.0 borate which dropped to pH 8.5 early in the experiment. The test mixtures were incubated at 23°C. In order to measure the formation of serologically reactive material samples were removed from each mixture at the times shown in Fig. 1 and tested in a series of dilutions against a 1:15 dilution of Type 2 antipneumococcus serum; the samples were adjusted to about pH 7.0 by diluting them 1:10 in 0.1 M phosphate solution before using them in the serological tests.

It is evident (Fig. 1) that the extract formed significant amounts of the serologically reactive material over the relatively wide zone of pH 4.0–8.0 but that
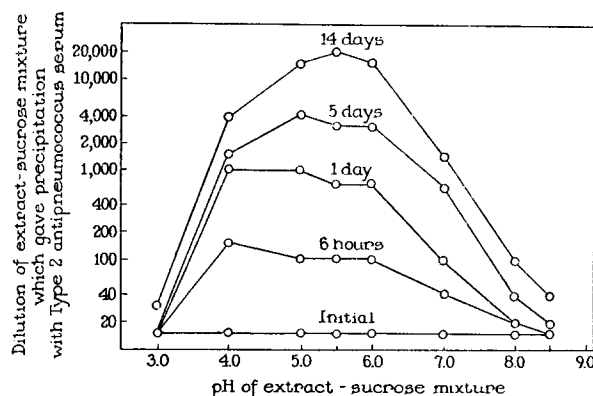

Fig. 1. The formation of serologically reactive material in extract-sucrose mixtures of different pH.

its action was more rapid between pH 4.0 and 6.0 than at pH 7.0 or above. The final yield of the reactive product was greater in the zone of pH 5.0–6.0 than in more acid or more alkaline systems. Four other lots of extract which had been prepared at different times over a period of 9 months were also compared in test systems of pH 5.5 and 8.2. All of these extracts produced at least 100 times more reactive material after 5 days incubation at 23°C. in the pH 5.5 mixture than in the pH 8.2 mixture, which indicates that the results in Fig. 1 are representative of extracts prepared from this strain of leuconostoc.

Influence of Temperature of Incubation

The routine temperature of incubation of the substrate-extract test mixtures was 23°C. The enzyme, however, had the capacity of forming the serologically reactive material over a range of at least 3–37°C. In experiments made with 5 different lots of extract, evidence was obtained that considerable

inactivation of the enzyme occurs in mixtures incubated at 37°C. For example, the amount of serologically reactive material formed at 37°C. was about the same as that formed at 23°C. during the first 5 hours of incubation, but after periods of 3 days the mixtures incubated at 23°C. invariably contained at least 5 times as much of the reactive product as did the corresponding mixtures incubated at 37°C. This apparent slowing of the reaction at 37°C. became even more apparent after 10 and 20 days by which time the mixtures incubated at the low temperature of 3°C. contained about 5 times as much of the reactive product as that in the mixtures incubated at 37°C. The amount formed at 3°C. never equalled that produced by the same extract at 23°C. during the 20 day period of the experiment. However, the fact that 3°C. was sufficient for a reasonably high degree of activity on the part of the leuconostoc enzyme is of interest because at that temperature cultures of those bacteria will grow little if at all.

Lability of the Active Agent

The active agent of the extracts is heat-labile: control mixtures of heated extract (30 minutes at 55°C.) plus sucrose were included in each experiment throughout the investigation and in no instance was any reactive material formed. This routine heating treatment was evidently more than sufficient since 5 minutes exposure to either 50° or 55°C. usually resulted in practically complete inactivation whether the extracts were heated in systems of pH 5.5 or pH 7.0.

Observations on the question of lability at 37°C. were made with several lots of extract. The active agent in all of them was destroyed more rapidly when the exposure (in the absence of sucrose) was in systems of pH 7.0 than when in systems of pH 5.5. Individual extracts varied in the degree to which they showed this difference. With some the difference was great: for example, one extract lost at least 90 per cent of its original activity when it was exposed for 4 hours at pH 7.0 whereas only a slight loss in activity occurred when it was exposed for the same time at pH 5.5. Controls were included to show that the greater lability at 37°C. on the part of extracts held at pH 7.0 was not due to any action of the phosphate which was used as buffer. The difference in the degree of lability at 37°C. which occurred among the individual extracts suggests that some unrecognized impurity present in different concentrations in the various extracts may have participated in the destruction of the active principle of the extract. Experiments will be made to determine whether or not the inactivation at this comparatively low temperature represents an oxidative process.

Active Extract from a Glucose Broth Culture

Cultures of *Leuconostoc mesenteroides* produce abundant amounts of the reactive dextran when grown in sucrose medium but produce none of it when

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grown in mediums in which glucose or other common carbohydrates are sub-
stituted for sucrose. The failure of the bacteria to produce the dextran in the
latter mediums can reasonably be explained on the basis that these mediums
lack the substrate (sucrose) required for the synthesis of the dextran. Never-
theless, it seemed of interest to determine whether or not the bacteria after
repeated passage in a medium lacking sucrose would elaborate the enzyme in-
volved in the dextran synthesis. For the investigation of that question the
leuconostoc bacteria (which previously had always been grown in sucrose
mediums) were transferred 9 successive times in glucose broth, and an extract
was then prepared by exactly the same procedure as previously described.

This extract was found to possess the capacity to form the polysaccharide
when added to sucrose; but its potency was low: for example, the extract
derived from the glucose culture when used in a dilution of 1:2 produced about
the same amount of polysaccharide as did 1:50 or 1:100 dilutions of the extracts
derived from sucrose cultures. These results show that the leuconostoc bac-
teria can elaborate some amount of the enzyme involved in the dextran syn-
thesis when grown in the absence of sucrose. However, the lower degree of
reactivity of the extract derived from the glucose culture indicates that the
presence of sucrose during the growth of the bacteria has a stimulatory in-
fluence upon the elaboration of the enzyme.

DISCUSSION

The experiments dealt with the production from sucrose of a serologically
reactive polysaccharide of dextran nature by the action of sterile filtered
extracts obtained from cultures of *Leuconostoc mesenteroides*. That other com-
mon carbohydrates do not yield the polysaccharide which is formed from su-
crose was shown previously (1) and further information on that point will be
given later. The active principle in the extracts was heat-labile and presum-
ably was an enzyme or combination of enzymes. From the method of prep-
aration it can be assumed that the active principle was "exocellular" or free
in the fluid of the cultures from which the extracts were obtained. However,
although it was a less convenient method, active extracts were also obtained
by grinding the bacterial cells which indicates that some of the active agent
also occurs within or upon the surface of the cells.

In regard to chemical properties the polysaccharide formed by the sterile
extract was similar to the polysaccharide produced in sucrose broth cultures;
both could be classified as dextrans on the basis of their high positive optical
rotation, high content of reducing sugars after hydrolysis and by evidence
that glucose comprised at least the principal portion of the hydrolyzed products.
In regard to serological properties the dextrans of extract and of culture source
were strikingly similar not only in tests against leuconostoc antiserum but also
in tests against Types 2, 20, and 12 antipneumococcus serums and in tests
against a series of variously absorbed antiserums. This agreement in the

antipneumococcus cross reactions and in the reactions against the absorbed serums represents evidence of a closer serological likeness between the dextrans of the two sources than would be furnished by tests against leuconostoc antiserum alone.

No intensive study of the mechanism of the action of the sterile extracts was made other than to show that the products are formed at a reasonably orderly rate and that the reaction, which occurs to some extent over the relatively wide zone of pH 4.0–8.0, is more rapid and gives a higher final yield in systems between pH 5.0 and 6.0 than at pH 7.0 or above. It is of interest to point out that the elaboration of the dextran by the active agent of leuconostoc origin has at least two points in common with the syntheses of glycogen and starch by enzymes of yeast, animal, and plant origins. That is, in regard to the final product of the reaction, the leuconostoc dextran like glycogen and starch is a complex polysaccharide composed apparently entirely of glucose units; and in regard to the substrate, the sucrose from which the dextran is derived, like the glucose-1-phosphate from which glycogen and starch are formed, is a glucoside. But the mechanism of formation of the dextran has not been studied enough to allow any closer comparison; for example the questions of whether or not phosphorus compounds participate and of whether or not the reaction is reversible have not been determined.

Although the experiments of this paper included extracts prepared from only one strain of *Leuconostoc mesenteroides*, the possession of a demonstrable polysaccharide-synthesizing enzyme is not peculiar to this strain. Sterile extracts which form serologically reactive polysaccharides similar to those produced in the corresponding bacterial cultures, have been prepared not only from 5 other strains of leuconostoc but also from group H streptococci, *Streptococcus salivarius*, and several non-spore-forming bacilli (presumably lactobacilli) isolated from plants. However, for the demonstration of the "enzymatic synthesis" of serologically reactive polysaccharides, all of these kinds of bacteria seem to have at least two advantages over the majority of microorganisms. First, the extraordinarily large amounts of the polysaccharide found in the whole cultures would indicate them to be a likely source from which to isolate a sufficient amount of enzyme or active agent to produce demonstrable amounts of the polysaccharide in the absence of the cells. Secondly, the formation of the polysaccharide by these bacteria can be referred to a definite constituent (sucrose or raffinose) of the medium. Of these two advantages the knowledge of the substrate is probably the more important, and indeed the other apparent advantage may depend entirely upon it. For example, in the case of leuconostoc and of the other kinds of bacteria from which we have prepared active extracts, the extraordinarily large amounts of polysaccharide are produced only when the medium contains relatively large amounts of the particular substance (sucrose and sometimes raffinose) from which the polysaccharide is elaborated; if none of that substance is available none of the polysaccharide

is produced. In the case of pneumococci and most other bacteria the actual substrates from which the specific polysaccharides are made are entirely unknown not only in regard to their chemical nature but also in regard to whether they represent original constituents of the medium or some intermediate products of bacterial metabolism. But in either event if it were possible to supply the appropriate substances in adequate amount the yield of specific polysaccharide might perhaps become as abundant as the yield of dextran in sucrose broth cultures of leuconostoc.

SUMMARY

A serologically reactive polysaccharide of dextran nature was produced from sucrose by the action of some enzyme or similar heat-labile agent contained in sterile filtered extracts derived from sucrose broth cultures of *Leuconostoc mesenteroides*. Rigorous controls were included to prove that this reaction occurred in the absence of microorganisms.

Purified preparations of the dextran formed by the sterile extracts were similar to the dextran elaborated in sucrose broth cultures of the bacteria in respect to both chemical and serological properties. The serological likeness was established not only by tests against leuconostoc antiserums but also by cross reactions with antiserums of Types 2, 20, and 12 pneumococci and by tests against a series of variously absorbed antiserums.

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