Distribution of Enzymes Forming Polysaccharide from Sucrose and the Composition of Extracellular Polysaccharide Synthesized by *Streptococcus mutans*

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The distribution of polysaccharide-forming activity from sucrose was investigated in cultures of three strains of *Streptococcus mutans* by using an assay which conveniently determines total polysaccharide. The enzymatic activity for polysaccharide formation from sucrose is almost exclusively extracellular. The ratio of the fructan to glucan in the polysaccharide differs among the three strains investigated. The enzymatic activity for the formation of polysaccharide from sucrose has been shown to be bound to the cell-free polymer itself.

Extracellular polysaccharides are synthesized by a variety of bacteria and occur either as a discrete capsule or as diffuse slime (22). The most common polysaccharides synthesized from sucrose are the dextrans and levans, the composition and distribution of which were recently reviewed by Jeanes (16) and Hestrin (14). Recently, the formation of extracellular polysaccharide from sucrose by *Streptococcus mutans* has been shown to be causally related to the formation of smooth surface dental caries in animals and to be implicated in this process in humans (6, 7, 10, 24). Polymers identified as dextran and levan have been found in dental plaque and in cultures of *S. mutans*, whereas the constitutive enzymes responsible for polysaccharide formation have been demonstrated both in a cell-associated form and in the cell-free culture medium (9). Recently, several extracellular enzymes forming glucans from sucrose have been purified, and the products of the reactions have been shown to differ in physical appearance with suspensions described as flocculent, gel-like, or uniformly turbid (12). Levanusucrase has also been purified from the culture medium of strains of this organism (4). Although both dextranusucrase and levansucrase have been shown in cell-free and cell-bound forms, the relative distribution of these activities in *S. mutans* cultures has not been unequivocally established.

In the past, dextranusucrase and levanusucrase have been assayed by following the release of free glucose or fructose in the reaction mixture. The indirect assay has certain inherent disadvantages, e.g., when polymer formation is determined in whole cultures, other enzyme systems may be present which convert sucrose, the substrate of the polysaccharide-forming system, to its constituent monosaccharides. We have developed a simple and convenient assay which allows us to estimate the total amount of polysaccharide formed from sucrose in the culture and to distinguish the relative fractions of glucan and fructan. This assay has been applied to the study of the relative distribution of the total polysaccharide-forming activity in *S. mutans* cultures. In addition, we have determined some of the properties of the polysaccharide and the enzymes which form it.

**MATERIALS AND METHODS**

*Organisms.* The strains of *S. mutans* (5) used in this study were SL1, IB1600, and 21TYP. Organisms were grown on 500 to 1,000 ml of Brain Heart Infusion medium (Difco) in either a Chemapce vibromixing fermentor or a fermentation design 2-liter fermentor (New Brunswick Scientific Co.). Unless specified, sterile glucose (2% final concentration) was added aseptically after the medium had been sterilized in the autoclave. Cultures were incubated at 37 C, in an atmosphere of 95% N₂ + 5% CO₂, and at

1 Deceased 1 July 1971.
constant pH by using accessory equipment described in detail elsewhere (20). Small batches of organisms were grown under similar constant conditions of temperature, pH, and atmosphere in tissue culture spinner flasks by methods described in detail elsewhere (19).

Cell breakage by sonic, ballistic, and extrusion devices. Cells were broken in a sealed chamber with a Branson sonifier model W185D by exposing them to the full power output of the instrument for periods of time ranging from 15 sec to 10 min. Cooling was achieved by continuous pumping of ice water through the jacket of the chamber. Before treatment, the air was replaced by flushing the chamber with nitrogen, or a nitrogen-\(\text{CO}_2\) (95\(:\)5\%) mixture. Treatments were made with and without glass beads in the chamber. Cells were broken in a Braun model MSK mechanical cell homogenizer at 4,000 cycles/min with 0.18-mm glass beads. The samples were kept cool by running liquid CO\(_2\) through the cooling system. Whole culture, culture medium, or washed cells in 10-ml volumes, were treated by extrusion in a French pressure cell.

Assay for total polysaccharide formation. Total polysaccharide formation was assayed as follows unless specified otherwise. A 0.2-ml amount of the material to be assayed was dispensed into duplicate tubes, one pair for each desired time interval. The tubes were kept in an ice bath while 0.3 ml of buffered sucrose (0.05 M potassium phosphate, pH 6.8) was added to each tube. Thus, each tube contained 3 \(\mu\)moles of sucrose with 0.3 \(\mu\)Ci of sucrose-\(\text{U}^{14}\)C (Schwarz BioResearch; final specific activity, 0.1 \(\mu\)Ci/\(\mu\) mole of sucrose). The assay period was started by transferring the tubes from the ice bath to a 37 C water bath. At the appropriate time intervals, 4.5 ml of absolute methanol was added to a pair of duplicate tubes. The contents of each tube were filtered by vacuum on 2.5-cm glass filter discs (no. 984H; Arthur H. Thomas Co.) held in a microanalysis glass filter holder (Millipore Corp.). The sample tube was washed with 5-ml amounts of absolute methanol three times, and each washing was filtered over the sample. The residual methanol was removed by vacuum and the dry disc with the labeled polysaccharide was transferred to a scintillation vial. A 10-ml amount of a mixture containing 4 g of 2,5-di-\(\beta\)-phenylloxazole (PPO) per liter of toluene was added to each vial and the radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

Quench correction was not necessary since the channel ratios were essentially constant within each experiment. The count rate counts per minute for each tube was plotted against the time at which the reaction was stopped. With appropriate dilution of the enzyme, this reaction was found to be linear and proportional to the enzyme concentration. The linear relationship was observed up to rates of incorporation of 0.5\(\%\) (0.015 \(\mu\)mole) of the substrate per hour. Activity is expressed as the number of counts per minute per hour precipitated after correction for the zero-time blank.

Polysaccharide was synthesized by the enzymes from strain SL1 between pH 5 and 9. Polysaccharide formation by spent cell-free culture medium was assayed at six different pH values with the following buffer systems: Borax-NaOH, pH 10; tri(hydroxy-methyl)aminomethane (Tri)-chloride, pH 9; Tri-chloride, pH 8.2; phosphate, pH 6.8; phosphate, pH 6.0; Tris-acetate, pH 5. The only sample which had significantly lower activity was that incubated at pH 10. The standard for assay chosen was pH 6.8.

The filtrates from samples precipitated at zero time, 30 min, 1 hr, and 2 hr were collected, and methanol was removed by gentle heating while a stream of nitrogen was run over the surface. A sample of the remaining syrups was applied to a 250 \(\times\) 18 mm Biotin (Calbiochem) P-30 column previously equilibrated with 0.1 M NaCl. The column was eluted with 0.1 M NaCl, and fifty 2.75-ml fractions were collected. Regardless of the time at which the reaction was stopped, the radioactive material was eluted from the column at a position corresponding to sucrose. No material larger than sucrose could be detected in any of the filtrates.

Specifically labeled substrates. Glucosyl- and fructosyl-\(\text{U}^{14}\)C specifically labeled sucroses were prepared by Bruce Chassy of this laboratory. The intact sugars and acid hydrolysis products of the sugars were chromatographed on Whatman 3MM paper strips with unlabelled carrier glucose, sucrose, and fructose using acetic acid-isooamyl acetate-water (3: 3:1, v/v/v) as the developing solvent. The specifically labeled sucroses were found to have no detectable impurities or cross contamination of the hexose moieties.

Formation of water-insoluble polysaccharide. A 100-ml amount of spent culture medium, after removal of the SL1 cells by centrifugation and subsequent membrane filtration, was incubated overnight with 5 g of sucrose at 37 C. Bacterial growth was inhibited by adding three drops of chloroform to the mixture before incubation. The resulting white polysaccharide was separated from the incubation mixture by centrifugation. The polysaccharide was suspended in water with a homogenizer, sedimented in the centrifuge, and washed three times with water. A sample of this preparation was reserved for infrared analysis by drying in vacuo at 50 C. The remainder of the polysaccharide was washed once more in buffer (0.05 M potassium phosphate, pH 6.8), resuspended in buffer, and stored at 4 C.

Preparation of samples for infrared spectroscopy. Small batches of whole culture or washed cells in 0.05 M potassium phosphate buffer were treated with either sucrose or glucose (1 g of substrate) as described above. After 2 hr of incubation, the sample was sedimented in a centrifuge, washed once with water, and washed twice with absolute methanol. The sedimented samples were further extracted with chloroform-methanol (2:1, v/v) at 55 C for 4 hr to remove the lipid from the cells. Methanol-precipitable material from cell-free culture medium was dissolved in water and reprecipitated in 95\% methanol. The samples were washed three times with methanol and dried in a vacuum oven at 55 C. All dried samples to be examined by infrared spectroscopy were mixed with potassium bromide and pressed into a pellet. The pellets were examined in a Perkin-Elmer model 421 double-beam infrared spectrophotometer.
RESULTS

Effect of heat inactivation on polysaccharide formation. Portions (10 ml) of a stationary-phase culture of SL1 were dispensed into screw-cap tubes, the tubes were put into a boiling water bath, and were removed at the following time intervals: 0, 5, 10, 20, and 30 min. These samples were assayed for polysaccharide-forming activity in the usual manner, and the slopes of the lines (i.e., enzyme activity) were plotted against the boiling time (Fig. 1). There was a very rapid loss of activity during the first 5 min of boiling followed by a slower loss for the balance of the 30-min boiling time. Significant activity was found through 20 min of boiling as shown by the "F" ratio of the regression analysis, whereas, with 30 min of boiling, the activity was no longer significant.

Distribution of polysaccharide-forming activity in whole cultures. A stationary-phase culture of SL1 was separated into samples of (i) whole culture, (ii) washed cells, and (iii) culture supernatant as follows. An amount of 290 ml of the culture was centrifuged, and the supernatant culture medium was decanted and saved for further use. The cell pellet was washed with 100 ml of 0.05 M phosphate buffer (pH 6.8). The cells were sedimented a second time, the washings were discarded, and the washed cells were made up to 50 ml, thus giving a cell concentration of 5.8 times that of the original culture. The fractions were assayed for polymer formation (Fig. 2). When the polysaccharide-forming activity of the whole culture is compared to the activity recovered in the culture medium and the washed cells, most of the activity is extracellular, while the washed cells have a small residual activity. Table 1 compares the distribution of polysaccharide-forming activities in three strains of S. mutans. The distribution of activities is nearly identical for SL1, IB1600, and 21TYP, and almost all of the activities of the culture are recovered in the spent culture medium.

![Figure 1: Effect of boiling on polysaccharide synthesis in whole culture of S. mutans. Portions of the boiled culture samples were incubated at 37°C with 14C uniformly labeled sucrose (0.2 µCi of sucrose-14C per µmole of unlabeled carrier sucrose) and samples were precipitated at 0, 1.5, 3, and 5 hr. The count rate of these samples was plotted against the time of incubation, and the activity units in counts per minute per hour were determined from the slope of the resultant linear plots.](image1)

![Figure 2: Distribution of activity of polysaccharide formation in a whole culture of strain SL1 grown on Brain Heart Infusion medium. A 0.5-ml amount of an assay mixture containing sucrose; 250 µmoles of sucrose (specific activity 1.6 x 10^4 µCi per µmole) was precipitated in 4.5 ml of absolute methanol at each point. Whole culture (O), culture medium (A), and washed cells (•). A zero time control of 600 counts per min was subtracted from each of the values of the washed cell series because of initial binding due to the higher cell concentration.](image2)

| Table 1: Distribution of polysaccharide-forming activity in cultures of three strains of Streptococcus mutans* |
|---------------------------------|-----------------|-----------------|-----------------|
| Organism | Whole culture | Culture medium | Washed cells |
| SL1 | 2,207 (100) | 1,972 (90) | 86 (4) |
| 21TYP | 3,240 (100) | 2,880 (89) | 324 (10) |
| IB1600 | 3,420 (100) | 3,840 (110) | 58 (2) |

* Cells from whole cultures of the organisms were sedimented by centrifugation, washed in buffer, and made to a new volume in buffer. All three fractions were assayed (specific activity is 2.22 x 10^4 counts per min per µmole of sucrose), and the data were fitted by using a linear regression analysis program. The numbers are the slopes of the activity curves in counts per minute per hour. The numbers in parentheses are percentages. The activity units of the washed cell fraction were normalized to that of the whole culture by correcting for the appropriate concentration factor.
addition, these polysaccharide-synthesizing enzymes found in the spent culture medium can be concentrated on an Amicon PM30 membrane. All of the active material is retained above the filter and none can be recovered in the filtrate.

In addition to the usual control (pH 6.8), we also grew cultures of SL1 to the end of the exponential phase with pH values maintained at 6.2 and 5.5. An additional culture, the final value of which was pH 4.4, was grown without pH control. There was no substantial difference in the distribution of polysaccharide-forming activities for any of these cultures grown at more acid conditions than pH 6.8. Essentially all of the polysaccharide-forming activity of the whole cultures was recovered in the culture medium after the cells had been removed by centrifugation.

**Broken cell experiments.** In order to determine if there was any intracellular polysaccharide-forming activity with sucrose as a substrate, the cells were disrupted and the broken cell mass was compared to the intact cells for activity. Attempts to use the sonic oscillator or agitation with glass beads in the Braun shaker substantially reduced the polysaccharide-synthesizing activity of the cell-free culture medium. Attempts to protect the activity with a nitrogen or carbon dioxide atmosphere during treatment proved unsuccessful.

By using the French pressure cell, we could break the cells and preserve the activity. Five successive treatments of a whole culture of strain IB1600 yielded a preparation which had the same activity as the original culture. When this preparation was examined in the phase-contrast microscope, only a small fraction of the intact cells was seen in the presence of a large number of ghosts of disrupted organisms. Spent culture medium treated in the same manner in the French pressure cell lost none of its activity when assayed for polysaccharide synthesis.

Washed cells of IB1600, adjusted to 12 times the cell density of the original culture, were disrupted by five passes through the French pressure cell. This broken cell preparation showed no substantial difference from the unbroken cell mass when assayed. These experiments establish that all of the polysaccharide-synthesizing activity of a culture could be accounted for in the cells and the culture medium, and no additional intracellular activity could be demonstrated.

**Ratio of glucosyl- and fructosyl-labeled polymers.** The relative amounts of glucose- and fructose-containing polysaccharide formed from the extracellular enzymes was easily determined using the specifically labeled sugars. Table 2 shows data obtained from three strains of *S. mutans* and from SL1 grown at three different pH control values. The SL1 strain of *S. mutans* synthesized polysaccharide of predominantly glucosyl moieties whereas very little fructose was incorporated into the polymer. When the SL1 strain was grown without pH control, there was no detectable amount of fructosyl-labeled polymer found. In contrast to the SL1 strain, the IB1600 and 21 TYP strains of *S. mutans* formed a substantial amount of fructosyl-labeled polymer.

**Infrared spectra.** Figure 3 shows a comparison of the infrared spectra of the water-insoluble polysaccharide, glucose-treated whole culture, and the sucrose-treated whole culture. The characteristic bands seen in the sucrose-treated whole culture comparable to those found in the water-insoluble polymer are 1,145, 923, 1,005, 845, and 785 cm⁻¹. The same bands were found in the difference spectrum of the glucose-treated vs. the sucrose-treated whole culture. These bands also occur in the methanol-precipitated medium fraction of the sucrose-grown culture and are absent in the methanol-precipitated medium of the glucose-treated culture. The spectra of sucrose-grown whole culture and water-insoluble polysaccharide are similar to those shown by Guggenheim and Schroeder (13). These bands are absent in the glucose-treated culture and the other glucose-treated fractions. In addition, no detectable bands corresponding to polysaccharide could be found in the sucrose-treated washed cultures.

**Table 2. Ratios of glucan and fructan in extracellular polymer from strains of Streptococcus mutans.**

| Organism | Final pH | Glucosyl (%) | Fructosyl (%) |
|----------|----------|--------------|---------------|
| IB1600   | 6.8      | 70           | 30            |
| 21 TYP   | 6.8      | 80           | 20            |
| SL1      | 6.8      | 96           | 4             |
| SL1      | 6.6      | 95           | 5             |
| SL1      | 5.5      | 96           | 4             |
| SL1      | 4.4      | 100          |               |

* Culture medium after growth and removal of organisms was incubated under normal assay conditions with either glucosyl-specific or fructosyl-specific ¹⁴C-sucrose solutions having equal specific activities. Pairs of tubes assaying each sample were removed at zero time and, at the end of 3 hr, were precipitated with methanol and filtered. Percentages are expressed as the fraction of the sums of the independent counts.
cells when the samples were examined by infrared spectroscopy with the spectra of the glucose- and sucrose-treated washed cells being identical. An infrared spectrum of the water-insoluble polysaccharide from strain IB1600 proved very similar to that made from SL1. The single difference was the appearance of a minor band at 760 cm⁻¹ although the preparation has a much higher fructosyl content than polysaccharide from the SL1 strain as determined by the specifically labeled sugars.

**Binding of enzymes to polysaccharide.** The enzyme or enzymes responsible for the formation of the heteropolymer can be bound in a very stable form to the polysaccharide itself. Table 3 shows the results of an assay in which water-insoluble polysaccharide was added back to a fresh sample of the cell-free culture medium from which it had been formed. The polysaccharide, made as described, has a substantial residual polysaccharide-forming activity. When the polysaccharide is added back to the spent culture medium from which it has been made, the individual activities seem to be additive, since 93% of the expected sum of the two components is recovered. The removal of the added polysaccharide by centrifugation coincided with a removal of more than 80% of more of the total activity.

**Table 3. Binding of polysaccharide-forming system to water-insoluble polysaccharide**

| Component                                | Activity (counts per min per hr) |
|------------------------------------------|----------------------------------|
| Water-insoluble polysaccharide           | 431                              |
| Culture medium                           | 1,022                            |
| Polysaccharide and medium                | 1,347                            |
| Activity remaining                       | 256                              |

* A 0.2-ml amount of a suspension of water-insoluble polysaccharide (2 mg) was added to culture medium after growth of strain SL1 and subsequent removal of cells. The components were assayed individually and in combination. The polysaccharide added was removed by centrifugation and the residual activity was assayed in the supernatant liquid. All components were incubated for 1 hr at 37°C before assay.

**DISCUSSION**

The direct assay of polysaccharide-synthesizing activity by the incorporation of labeled substrate has many advantages over the indirect assay which measures the release of the constituent monosaccharides of sucrose. While dextransucrase has been shown to be highly specific, Carlson (4) has isolated a levan-sucrase from *S. mutans* which has substantial invertase activity after extensive purification. Growing cultures and washed cells of *S. mutans* are capable of using sucrose as an energy source and divert only a small fraction of the sugar to polysaccharide (19, 21). Although the details of the catabolism of sucrose have not been clarified, it is likely that an invertase is present. Using the release of fructose or glucose as a measure of dextransucrase or levan-sucrase, or both, assumes no contribution of monosaccharide from other sources.

Composition and structural linkages of the extracellular polymer of *S. mutans* were determined from the analysis of hydrolysis products of the polymer and by infrared spectroscopy. Guggenheim and Shroeder (13) and Guggenheim (11) have investigated the composition and linkages of the polysaccharide formed by a strain of *S. mutans* grown on sucrose. They reported an infrared spectrum for the polysaccharide which is almost identical to the one we have shown of the water-insoluble polysaccharide. They reported very little fructose, whereas our results show almost identical infrared spectra for two polymer preparations which have substantially different amounts of the fructosyl component. More detailed work is needed before infrared spectroscopy can be
used in the analysis of these polysaccharides.

The bulk of the polysaccharide-synthesizing activity, with sucrose as a substrate, from cultures of S. mutans is extracellular. Furthermore, the activity recovered in the whole, washed cells is an upper limit associated with this fraction, since no additional intracellular activity can be demonstrated when sucrose is the substrate. Polysaccharide-forming capacity associated with the cells is of two types: that formed at the surface, and an intracellular storage polysaccharide which has been shown to accumulate in substantial amounts under certain circumstances (15). This intracellular storage compound should be accounted for in any further analysis of the cell-associated polysaccharide synthesis. Gibbons and Fitzgerald (8) emphasized the importance of extracellular dextran for the adherence of the organism to surfaces. The precise locus of the enzymes responsible for polysaccharide formation on the surface of the cell is unknown.

We found that there are substantial losses in polysaccharide-synthesizing ability when the cell-free enzymes are exposed to two common cell breakage methods. The reason for the inactivation by exposure to sonic treatment or agitation with glass beads is not clear. It would be wise to avoid the use of these methods if it is desirable to preserve the activity of the cell-free enzyme. This sensitivity to sonic and ballistic treatment is in contrast with the stability of the enzymes when exposed to heat or washing with distilled water.

The binding of the polysaccharide-forming enzymes to the polymer is significantly related to the analysis of the locus of the enzyme on the cell and to the accumulation of these polysaccharides in nature. Bailey (3) reported the binding of a dextranase from Streptococcus bovis to the extracellular dextran formed by the organism. The transglycosidase activity from S. bovis responsible for extracellular slime formation has been investigated (1-3, 18, 23) and is similar to that of S. mutans in that it is constitutive. The polysaccharide-forming activity of S. bovis differs from S. mutans in that the polymer from the former is exclusively composed of glucose units and the activity of the S. bovis enzyme is much more sensitive to extremes in temperature and pH. We were able to determine that the polysaccharide-forming activity from S. mutans is extracellular. This interpretation is made possible because the enzyme or enzymes are constitutive. If the enzymes were inducible, sucrose would necessarily be present during growth, and the enzymes would be bound to the resultant polysaccharide which would be cell-associated. Under these conditions, the polysaccharide-forming activity would appear to be cell-associated. Preliminary evidence indicates that no polysaccharide-forming enzymes appear in the culture medium when strain SL1 is grown on sucrose. The binding of the enzymes to polymer and the stability of the enzymatic activity on the polymer may be of particular significance in the accumulation of these polymers in dental plaque. The bound enzyme would allow the continued accumulation of polymer in plaque which would be independent of the presence of live organisms.

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