Mechanism of the Adherence of *Streptococcus mutans* to Smooth Surfaces

III. Purification and Properties of the Enzyme Complex Responsible for Adherence

HIDEHIKO MUKASA AND HUTTON D. SLADE

Department of Microbiology, Northwestern University Medical and Dental Schools, Chicago, Illinois 60611

Enzymes which possess the ability to cause the adherence of *Streptococcus mutans* cells to a smooth glass surface were purified 1,100 times by chromatography on agarose gel followed by hydroxylapatite gel. During the purification procedures, the enzymes from strain HS6 (group *a*) were examined for the synthesis of water-soluble and water-insoluble polysaccharide and the ability to produce adherence. The enzyme preparations producing adherence of the *S. mutans* cells in the presence of sucrose possessed a molecular size of about 400,000 to 2,000,000 and were composed of approximately equivalent amounts of dextran and levan sucrases and 5 to 30% polysaccharide. The most highly purified preparation contained a negligible amount of contaminating protein as judged by polyacrylamide gel electrophoresis, immunoelectrophoresis, and gel diffusion. In these three tests, the location of the enzyme responsible for the synthesis of insoluble polymer was detected by embedding or covering the enzyme-containing gel with a layer of sucrose-containing agarose gel and observing the formation of insoluble polymer. During purification the ability of all fractions to produce adherence was parallel with the enzyme activity responsible for the synthesis of insoluble polysaccharide from sucrose. About two-thirds of the sucrase enzyme complex in the *S. mutans* culture fluid synthesized water-soluble polymer. This complex, obtained by filtration through agarose gel, was smaller in molecular size, lower in sugar content, and did not produce adherence, in contrast to the enzyme complex which possessed adherence activity. The inhibition of the enzyme complex synthesizing soluble polymer required more anti-synthetase serum than that required to inhibit the synthesis of water-insoluble polymer. It is not known whether the lack of adherence activity in this enzyme was due to its smaller size and lower sugar content or the absence of unknown factors which are essential for its activity. The carbohydrate in these enzyme preparations, composed of glucose, may represent a primer molecule and/or a remnant of the polymer synthesized by the enzyme. The enzyme activity was not inhibited by anti-dextran globulin.

In our previous studies (20, 21) it was shown that the sucrase enzymes in a crude cell-free enzyme preparation from the culture supernatant of *Streptococcus mutans* (Strain HS6, group *a*) were able to bind to the surface of the *S. mutans* cell and subsequently synthesize water-insoluble dextran and levan from sucrose. These conditions resulted in the adherence of the *S. mutans* cell to a smooth glass surface. The *a–d* antigen polysaccharide and dextran on the *S. mutans* cell surface functioned in the binding of the enzyme(s) (20, 21). The same enzymes synthesized insoluble dextran and levan from sucrose in the absence of cells.

It was not clear, however, whether the dextran sucrase responsible in part for adherence existed as a single enzyme or in multiple forms, and whether it was complexed with a carbohydrate polymer. Also, it was not known if additional components present in the crude enzyme preparation were necessary for adherence of *S. mutans* cells to a glass surface. In depth studies on the purification of sucrases from *S. mutans* have been reported (1, 2, 9), however a precise determination of the purification and properties of the enzymes based on their ability to promote adherence of *S. mutans* cells has not been carried out. Such a study would contribute to

our knowledge of the mechanism of adherence of these streptococci to a smooth surface and would be relevant to the formation of an *S. mutans* plaque in vivo.

MATERIALS AND METHODS

Streptococcal mutans cells. *S. mutans* (group *a*, strain HS6) was used throughout the study (21). The cells were grown in THB broth (Todd-Hewitt Broth, Difco) fortified with 1.8% glucose and salts (24).

Adherence activity. The adherence activity of the enzyme preparations was measured, using heat-treated cells as described previously (20), with the following modifications. In a glass tube (10 by 75 mm) instead of a tube (13 by 105 mm), 2 μliters of 10% merthiolate, a varying quantity of 0.05 M phosphate buffer (pH 6.8) and enzyme fraction, 0.2 ml of 0.5% cell suspension, and 0.2 ml of 5% sucrose in buffer were added in the order named. The final volume was 1.2 ml instead of 6 ml. About 60% of the cells adhered firmly on the glass surface under optimal conditions (20). The optical density increase due to the synthesis of insoluble polysaccharide during the assays was less than 5%. The relative adherence activity as given in the tables is the corrected value. One unit of adherence activity is defined as the amount of enzyme complex which produces an adherence of 30% of the cells to the glass surface. Relative adherence activity is the adherence unit value per milligram of protein of the enzyme fractions compared with the adherence unit value per milligram of protein of the culture fluid.

Synthesis of polysaccharide from sucrose by cell-free enzyme complex. A semiquantitative analysis of the synthesis of water-insoluble polysaccharide was measured as described by reading the turbidity increase at 550 nm of the reaction mixture (20). Using 0.1 ml of enzyme fraction, the turbidity increases of 0.003 to 0.005, 0.006 to 0.01, and above 0.011 per 3-h incubation were expressed as +, +2, and +3.

Quantitative analyses of the water-insoluble and water-soluble polysaccharide synthesized were measured as follows. The reaction mixture was composed of 0.2 ml of 5% sucrose, 2 μliters of 10% merthiolate, enzyme, and 0.05 M phosphate buffer (pH 6.8) to 1.2 ml (20). After incubation, the water-insoluble polymer was obtained by centrifugation at $5,000 \times g$ for 10 min, washed three times with water, and the dextran and levan sucrase activities were measured by counting [^{14}C]glucose and [^{3}H]fructose incorporated into the polymer (20).

The water-soluble polysaccharide was precipitated with 67% ethanol from the supernatant. The precipitate was collected after 2 h by centrifugation and washed with 67% ethanol three times. Dextran and levan sucrase activities which synthesized the water-soluble, ethanol-insoluble polymer were measured as described above. Dextran sucrase activity is expressed as dextran sucrase units which convert 1 mg of sucrose to dextran in 1 h (11). Levan sucrase activity is also expressed as levan sucrase units which convert 1 mg of sucrose to levan in 1 h. Relative dextran and levan sucrase activities are the activities of each per milligram of protein of the enzyme fractions compared

with those of the culture fluid.

Dextran and levan sucrase activities were also measured by liquid-gas chromatography by determination of the fructose and glucose released from sucrose. The culture fluid of *S. mutans* does not contain significant quantities of invertase (6, 15). After the incubation of the reaction mixture (20) for 16 h, 0.24 ml of the supernatant obtained by centrifugation was lyophilized and directly silated with 100 μliters of silation reagent (pyridine-hexamethyldisilazane-trimethylchlorosilane, 3:2:1) at room temperature for 5 min. One microliter of the mixture with 1 μg of mannose as internal standard was injected into the column and chromatographed as described previously (18).

Chemical analysis. The protein content of the enzyme preparations was estimated by the Folin phenol reagent (14) using bovine serum albumin as a standard. Total sugars were measured by the phenol-sulfuric acid method (3). The glucose content of the enzyme preparations was analyzed as follows. The sample (50 to 200 μg) was hydrolyzed with 150 μliters of 4 N HCl at 100 C for 4 h. The material was dried and methylated with 100 μliters of 0.5 N hydrochloride-methanol at 78 C for 20 h. After drying in a vacuum at room temperature, it was silated and analyzed by liquid-gas chromatography as described above.

Antisera. Anti-dextran sucrase serum (anti-CEP [crude enzyme preparation]) was obtained by injecting crude dextran-levan sucrase (CEP) with incomplete adjuvant intravenously into rabbits (20). This antiserum completely inhibited the synthesis of insoluble dextran-levan polymer. However, it did not inhibit the adsorption of sucrase enzymes to the surface of *S. mutans* and was free of antigroup polysaccharide and anti-dextran globulins (21).

Anti-dextran globulin was purified by Sephadex G-200 chromatography from anti-*S. mutans* (strain 10449, group *c*) whole cell serum (22).

Serological procedures. Agar gel diffusion and immunoelectrophoresis were carried out as previously described (16, 17). In both cases, the gel was made of 0.6% pure agarose (Schwarz/Mann, Orangeburg, N.Y.), 0.02% NaN₃, and 0.8% NaCl in 0.05 M phosphate buffer (pH 6.8). After the antigen-antibody bands of precipitate developed, the position of the enzyme was determined by a modification of an earlier method (9). A gel composed of 0.3% agarose, 0.024% NaN₃, and 5% sucrose, in the same buffer, was layered to a thickness of 2 mm in each case to determine which precipitin band possessed the ability to synthesize insoluble polysaccharide. In this procedure, the upper gel layer was poured at 40 to 45 C so as not to denature the enzyme. After the incubation at 37 C for 24 h, the presence of the sucrase enzyme was shown by the appearance of a cloudy white precipitate of glucan.

The inhibition of dextran sucrase activity in the enzyme preparations by anti-CEP and anti-dextran was carried out as follows. The reaction mixture described above for the quantitative analysis of polysaccharide synthesized was used minus the sucrose solution. To this mixture anti-CEP or anti-dextran (21 to 184 μliters per dextran sucrase unit) was

added, and the reaction was incubated for 2 h at 37 C. The mixture was centrifuged at 6,000 $\times$ g for 10 min. The supernatant was added to 0.2 ml of sucrose solution (containing labeled sucrose), and the water-soluble and water-insoluble polysaccharides were measured by incorporation of [^{14}C]glucose and [^{3}H]fructose, as described above.

Polyacrylamide gel electrophoresis. Polyacrylamide gel was composed of 5% acrylamide, 0.25% methylene-bisacrylamide, 0.05% ammonium persulfate, and 0.05% N,N,N',N'-tetramethylethylenediamine in 0.05 M phosphate buffer (pH 6.8). A softer gel containing 3% acrylamide and 0.15% methylenebisacrylamide was also used. Duplicate columns of each sample were electrophoresed in the same buffer at 75 V, 6 mA/tube for 2 h. One column was stained for protein with 1% Amido Black 10B in methanol-water-acetic acid (5:5:1) for 10 min. The negative print of the stained gel was taken directly by an enlarger (Charles Beseler Co., East Orange, N.J.). The other column was embedded in agarose gel which contained 5% sucrose, as described above, to detect the synthesis of insoluble polymer. After incubation for 1 day at 37 C, the gel was photographed by an immunodiffusion camera (Cordis Corp., Miami, Fla.).

Purification of enzyme. Strain HS6 was grown in 4 liters of broth as described above. After overnight incubation, the culture supernatant was obtained by centrifugation: The CEP was obtained from the supernatant by precipitation with (NH$_4$)$_2$SO$_4$ (20). The following methods were used for further purification of CEP.

(i) Purification by filtration through agarose gel. CEP (15 ml) was applied to a column (2.5 by 54 cm) of BioGel A-50m in 0.05 M phosphate buffer (pH 6.8). The flow rate was adjusted to 0.7 ml/min and each fraction (5.6 ml) was collected and analyzed (see Fig. 1). The fractions which showed water-insoluble polymer synthetase activity were combined to give fraction A (tube no. 38 to 44) and fraction B (no. 45 to 56). Fractions A and B were each concentrated to 3 ml by lyophilization and further fractionated on a column (1 by 54 cm) of BioGel A-0.5m in the same buffer. The flow rate was adjusted to 0.41 ml/min and each fraction (1.66 ml) was collected and analyzed (see Fig. 2). Blue Dextran 2000, serum albumin, and phenylalanine were used as standards for the estimation of molecular size in both these gel-filtrations.

CEP (20 ml) was applied to a column (2.5 by 53 cm) of BioGel A-0.5m in the same buffer. The flow rate was adjusted to 3.3 ml/min and each 13.3-ml portion was collected. Fractions no. 11 and 12, which possessed a strong water-insoluble polymer synthetase activity, were combined. This fraction was designated AF.

(ii) Purification by filtration through hydroxylapatite. Four-times concentrated CEP (25 ml) was applied to a column (2.5 by 20 cm) of BioGel HTP in 0.01 M phosphate buffer (pH 6.8) and eluted with a linear concentration gradient of 500 ml of 0.01 M phosphate and 500 ml of 0.8 M phosphate buffers (pH 6.8). The flow rate was adjusted to 4.5 ml/min and each 9-ml portion was collected. The active fractions (no. 41 to 47) were combined, precipitated with ammonium sulfate at 75% saturation, and dialyzed

against 0.02 M phosphate buffer (pH 6.8). The material was applied again to a HTP column (1.5 by 24 cm) and eluted with a linear concentration gradient of 300 ml of 0.02 M phosphate buffer (pH 6.8) and 300 ml of 0.2 M phosphate buffer (pH 6.8). The flow rate was 0.5 ml/min and each 9-ml portion was collected. The active fractions (no. 3 and 4) were combined, lyophilized, and dialyzed against 0.05 M phosphate buffer (pH 6.8). The volume was adjusted to 4 ml. This preparation was designated as fraction HF.

(iii) Purification by agarose gel and hydroxylapatite. CEP (50 ml) was applied to a column (2.5 by 51 cm) of BioGel A-0.5m in 0.05 M phosphate buffer (pH 6.8). The flow rate was adjusted to 4.9 ml/min and each 9.8-ml portion was collected. Fractions (no. 10 to 13) which possessed strong enzyme activity were combined and directly applied to the second column (1 by 23 cm) of BioGel HTP in 0.05 M phosphate buffer (pH 6.8) (see Fig. 4). After washing with 30 ml of the above buffer and 10 ml of 0.1 M phosphate buffer, linear gradient chromatography with 130 ml of 0.01 M phosphate buffer (pH 6.8) and 130 ml of 0.35 M phosphate buffer (pH 6.8) was performed at a flow rate of 0.12 ml/min. Each 3.3-ml portion was collected. Fractions of positive activity (no. 29 to 36 and no. 38 to 44) were combined to make fractions 1 and 2, respectively, each was dialyzed, brought to 12 and 8 ml, and designated as AH1 and AH2.

All column chromatography was done at room temperature.

RESULTS

The adherence assay. The assay as used in these studies measures the *S. mutans* cells which are (i) attached to the smooth glass surface (cell absorption) and (ii) those which in turn attach to the former cells (cell-to-cell binding). In the latter case, the binding may occur on the glass surface or in the medium as a preliminary to attachment to the surface. In addition, some strains of *S. mutans* will adhere to an in vitro cell-free polysaccharide layer (13). The term adherence as used in our studies (20, 21) refers to the total cells which have absorbed in this manner. No attempt has been made to differentiate the enzyme activity which promotes adherence of *S. mutans* to the glass surface from that enzyme activity, if different from the former, which promotes cell-to-cell binding.

Fractionation of CEP on agarose gel columns. CEP from culture fluid of *S. mutans* strain HS6 (group *a*) was fractionated by passage through BioGel A-50m column (Fig. 1). The enzyme responsible for the synthesis of water-insoluble polysaccharide was found over a wide range. To fractionate the active preparation on the basis of molecular size, the fraction of higher molecular size (Fig. 1A, tubes 37 to 45) and lower size (Fig. 1B, tubes 46 to 57) were each applied

to a BioGel A-0.5m column. Almost all of the protein and enzyme activity of fraction A appeared near the void volume of the column as expected (Fig. 2A). The active material of fraction B appeared to contain several molecular sizes (Fig. 2B). Judging from K_d values of the first (Fig. 1A) and of the last (Fig. 2B) active fractions, the approximate molecular size of the water-insoluble polysaccharide synthetase ranged from 400,000 to 2,000,000.

Relations between molecular size, adherence activity, sucrase activities, and sugar content. The fractions which were positive for polysaccharide synthesis were tested for their ability to produce adherence of *S. mutans* cells to a glass surface in the presence of sucrose.

FIG. 1. *BioGel A-50m gel filtration of CEP obtained from culture fluid. Column length, 2.5 by 54 cm; buffer, 0.05 M phosphate (pH 6.8); flow rate, 0.7 ml/min; each tube, 5.6 ml. Symbols:* ●, *absorbance at 254 nm; shaded area, insoluble polysaccharide synthesis. A control experiment showed that the first and second peaks of Blue Dextran 2000 (B1 and B2) and that of serum albumin (SA) appeared at tubes marked in the figure.*

FIG. 2. *BioGel A-0.5m gel filtration of fractions A and B from Fig. 1. A and B were each reduced to 3 ml and applied on a column (1 by 54 cm) of BioGel A-0.5m in the same buffer as used in Fig. 1. Flow rate, 0.41 ml/min; each tube, 1.66 ml. Symbols are the same as in Fig. 1. Peaks of Blue Dextran 2000 (BD) and phenylalanine (PH) appeared as indicated in the figure.*

Table 1 shows that CEP activity per milligram of protein was 39 times more active than the original culture supernatant. Fractions 9, 10, and 11 in Fig. 2A and 11 in Fig. 2B, however, were 400 to 800 times more active. The sharp drop in relative activity to a negligible value (fractions 12 to 21, Fig. 2B) corresponded to decrease in molecular size. The total sugar and glucose content also decreased (Table 2) as the relative adherence activity decreased (Table 1). It is also evident (Table 1) that the fractions possessing a high adherence activity were responsible for the synthesis of significant quantities of water-insoluble dextran and minor quantities of water-soluble polymer.

Previous studies have shown that the polymer synthesized by CEP contained about two-thirds dextran and one-third levan (7, 20). Table 1 also shows that levan sucrase activity was present in all fractions, in those containing the water-soluble as well as water-insoluble polymers.

The indication that the particle which synthesized water-insoluble polymer possessed a molecular size near 2,000,000 was supported by polyacrylamide gel electrophoresis. When 5% polyacrylamide gel was used, all the activity of the insoluble polymer synthetase remained within 2 mm of the top of the gel columns; however when 3% gel was used, the activity moved to a depth of 9 mm in cases of supernatant, CEP, fraction 10 of Fig. 2A, and 11 of Fig. 2B (Fig. 3). Fractions 15 and 21 (Fig. 2B) showed no active band, as expected. In addition to the main broad band, a very weak narrow band was seen in fractions 10 and 11 at a depth of 15 mm. Staining of the CEP gel for protein showed that the proteins spread to a depth of 35 mm with two distinct bands at about 20 mm (Fig. 3Cᵃ). Protein-staining patterns of 10, 11, 15, and 21 showed they all contained nonactive proteins at a depth of 20 mm (not shown).

Further purification and properties of the adherence enzyme. The adherence enzyme(s) and the water-insoluble polysaccharide synthetase enzyme(s) appear to be the same, as shown above. An increase in specific activity of glucosyltransferase after hydroxylapatite chromatography has been reported (9), therefore the enzyme complex, obtained by filtration through BioGel A-0.5m, was chromatographed on a BioGel HTP column. The chromatographic pattern showed two peaks of insoluble synthetase activity (Fig. 4). The fractions were designated as AH1 and AH2. To evaluate the effectiveness of each purification procedure, two other enzyme preparations were obtained as described above: fraction AF obtained by BioGel A-0.5m gel filtration of CEP; and fraction HF, obtained
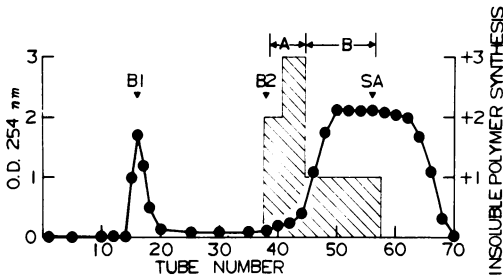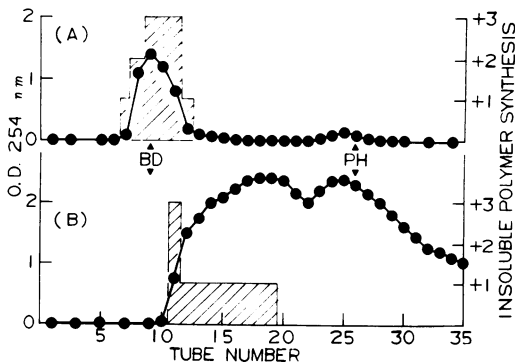
TABLE 1. *Relation between adherence activity and sucrase activities of fractions obtained by gel filtration*

Enzyme fraction	Relative adherence activity[a]	Relative dextran sucrase activity[b]		Insoluble dextran sucrase activity (%)[c]	Levan sucrase/dextran sucrase[d]	
		Insoluble	Soluble		Incorporation	Release
Supernatant	1.0	1.0	1.0	29.7	1.1	[e]
CEP	39.1	14.1	18.1	24.7	1.0	0.9
9A[f]	409.8	183.6	1.0	98.7	0.8	1.2
10A	543.4	328.8	1.1	99.2	0.9	1.6
11A	803.8	448.1	2.5	98.7	0.9	1.6
11B[f]	720.3	533.4	79.1	74.0	1.1	2.3
12B	8.9	20.0	212.8	3.8	1.1	1.3
13B	1.4	5.4	179.1	1.3	1.1	1.4
15B	1.4	3.8	102.2	1.5	1.2	1.6
17B	2.3	3.8	17.4	8.4	0.9	1.2
19B	2.1	2.5	22.6	4.8	1.2	1.2
21B	4.9	3.1	13.4	8.7	1.1	1.1

[a] Adherence units (2.0) per mg of protein.

[b] Insoluble measured at 0.016 dextran sucrase units per mg of protein; soluble measured at 0.036 dextran sucrase units per mg of protein.

[c] Total dextran sucrase activity.

[d] Incorporation of [¹⁴C]glucose and [³H]fructose from sucrose. Release of glucose and fructose during polymer synthesis.

[e] Could not be measured due to free sugars in the supernatant.

[f] Fractions as shown in Fig. 2A and 2B.

TABLE 2. *Sugar content of enzyme fractions*

Fraction	Total sugar[a] (%)	Glucose[b] (%)
Supernatant	[c]	[c]
CEP	1.3	1.0
9A	24.4	11.3
10A	15.4	11.3
11	9.8	7.2
11B	24.6	7.7
12B	9.9	2.9
13B	4.2	3.4
15B	2.5	2.0
17B	3.4	2.9
19B	2.8	2.5
21B	2.4	2.2

[a] Phenol-sulfuric acid method.

[b] The fractions were hydrolyzed in 4 N HCl at 100 C for 4 h and methylated in 0.5 N hydrochloride-methanol at 78 C for 16 h. The glucose derivative was analyzed by gas-liquid chromatography as described.

[c] Not analyzed due to free sugars in supernatant.

after two passages of CEP through a BioGel HTP column.

The enzymatic activity and sugar content of these preparations are shown in Table 3. The adherence and insoluble dextran synthetase activities of HF and AH2 increased 800 and 1,100 times, respectively. It can also be seen (Table 3) that these preparations synthesized mainly water-insoluble polysaccharide (column 5), and that comparable activity of levansucrase co-existed in the preparations.

Figure 5 illustrates the adherence of HS6 cells to glass in the presence of sucrose and several of the enzyme fractions. The crude culture supernatant, the $(NH_4)_2SO_4$-precipitated enzyme (CEP), and the highly purified fraction (AH2) each show maximal adherence. Fraction 15, which possesses a very little water-insoluble synthetase activity (Table 1), does not show adherence.

Glucose was a significant component of the AH2 preparation (33%), however, the glucose content of HF was only one-sixth that of AH2 (Table 3). AF, HF, and AH1 each showed one wide band of activity in gel electrophoresis (Fig. 6). The mobility indicates that the molecular size of AF is smaller and/or possesses a different charge than that of HF and AH1. These preparations still revealed bands of contaminating protein at a depth of 18 mm (not shown). AH2 seems to be composed of a large molecule because almost all the activity remained on top of the gel. The protein band (Fig. 6, AH2a) corresponded to the band with enzyme activity and no other protein bands appeared in the gel.

In the previous study (21), CEP possessed more than four bands in agar gel against anti-CEP globulin. The antigen which showed the slowest rate of diffusion was selectively adsorbed by heat-treated *S. mutans* cells, and the cells recovered adherence activity. As seen in Fig. 7, a slowly diffusing band limited the diffusion of the insoluble polysaccharide synthetase, resulting in a distorted area of dextran
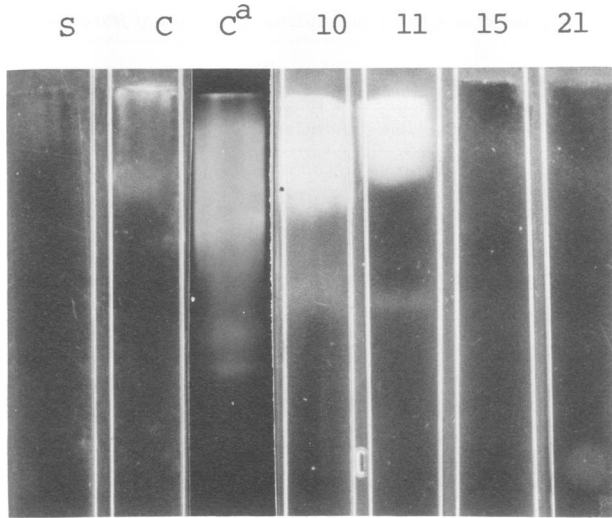
FIG. 3. *Polyacrylamide gel electrophoresis. Fractions from Fig. 2 were electrophoresed in 3% acrylamide gel in 0.05 M phosphate buffer (pH 6.8) at 75 V, 6 mA/tube for 2 h. C^a was stained for protein by Amido Black 10B. The remaining tubes were embedded in agarose gel containing 5% sucrose for the detection of insoluble polymer synthesis. White bands indicate the presence of the enzyme or protein. S, 200 μliters of supernatant; C, 25 μliters of CEP; 10, 50 μliters of no. 10 fraction of Fig. 2A; 11, 50 μliters of no. 11 fraction of Fig. 2B; 15, 100 μliters of no. 15 fraction of Fig. 2B; 21, 100 μliters of no. 21 fraction of Fig. 2B.*
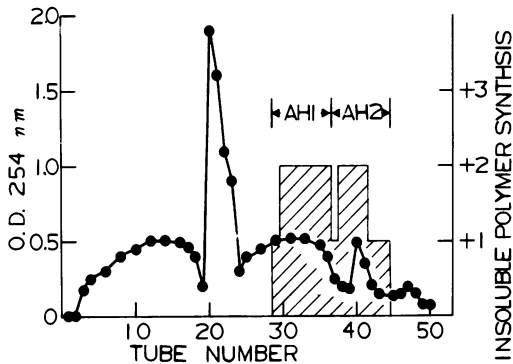
FIG. 4. *Purification by BioGel HTP of BioGel A-0.5m fraction. The fraction obtained by BioGel A-0.5m gel filtration of 50 ml of CEP was applied on BioGel HTP column (1 by 23 cm). After washing with 30 ml of 0.05 M phosphate buffer and 0.1 M phosphate buffer (pH 6.8), the column was eluted by linear gradient chromatography with 130 ml of 0.1 M phosphate buffer and 130 ml of 0.35 M phosphate buffers (pH 6.8) at flow rate of 0.12 ml/min. Each fraction: 3.3 ml. Symbols are the same as in Fig. 1.*

around the outer wells. Figure 8 shows that the presence of anti-CEP serum in the trough completely inhibited the formation of dextran between the well and the trough. The slow rate of diffusion of AH2 may be related to its large molecular size (Fig. 7, no. 6). As shown in gel diffusion and immunoelectrophoresis, CEP, AF, HF, and AH1 showed two or more precipitin bands with anti-CEP serum. It can be seen that very little contaminating antigen is present in the AH2 preparation (Fig. 7, no. 6, and Fig. 8), indicating a high degree of purity.

Inhibition of synthesis of water-insoluble and water-soluble dextran by anti-CEP. An inhibition study of soluble and insoluble dextran synthetase by anti-CEP was carried out to distinguish between the two. The supernatant fraction, AH2, and fraction 15 of Fig. 2B were used. The percentage of activity of insoluble synthetase per total dextran sucrase activity of each preparation was 29.7, 79.3, and 1.5, respectively (Table 1 and 3). Figure 9 shows that the insoluble synthetase complex of AH2 was inhibited 85% by 20 μliters of CEP antiserum, whereas the soluble synthetase activity was only inhibited about 10%. Similar results are evident for the supernatant and no. 15 fractions.

Anti-dextran serum, when tested under the conditions used in Fig. 9, did not inhibit the synthetase activity or adherence. This is possibly due to the presence of a polymer in the enzyme which does not possess the same chemical structure and antigenic specificity as that of the polymer present on the surface of the 10449 cells (21). Conversely, if the antibody was bound, the site was not associated with the enzymatically active part of the molecule.

DISCUSSION

The enzyme complex which enables *S. mutans* cells to adhere to a glass surface in the

TABLE 3. *Enzymatic activities and sugar content of purified enzyme fractions*

Enzyme preparation	Relative adherence activity[a]	Relative dextran sucrase activity[a]		Insoluble dextran sucrase activity[a] (%)	Levan sucrase/dextran sucrase[a]		Total sugar (%)	Glucose (%)
		Insoluble	Soluble		Incorporation	Release		
Supernatant	1.0	1.0	1.0	29.7	1.1	[b]	[b]	[b]
AF[c]	347.2	473.6	49.6	80.1	1.0	1.4	9.1	10.3
HF[d]	1193.3	870.0	114.2	76.3	0.8	1.5	6.1	4.6
AH1[e]	680.0	193.5	2.8	96.7	1.5	1.4	28.8	26.7
AH2[e]	1116.0	748.0	82.5	79.3	1.7	2.6	33.5	32.9

[a] See Table 1.
[b] Could not be measured due to free sugars in the supernatant.
[c] Obtained by BioGel A-0.5m filtration of CEP.
[d] Obtained by BioGel HTP filtration of CEP.
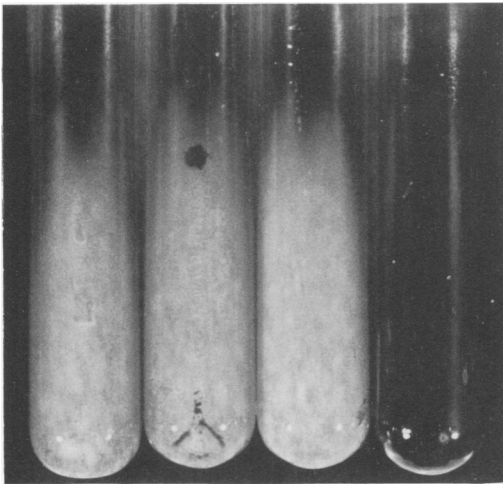[e] Fraction I and II of BioGel A-0.5m—BioGel HTP filtration (Fig. 3).

FIG. 5. *Effect of enzyme fractions on the adherence of S. mutans. Heat-killed cells (5 mg, HS6) were preincubated with enzyme fractions in 5 ml of 0.05 M phosphate buffer, pH 6.8, with 10 μliters of 5% merthiolate in a glass tube (13 by 150 mm) at 37 C for 30 min. After addition of 1 ml of 5% sucrose, the tube was incubated at a 30° angle at 37 C for 16 h and washed twice with the buffer. From left to right: supernatant, cell-free culture fluid (4.99 mg of protein); CEP, enzyme precipitated by ammonium sulfate (0.64 mg of protein); AH2, fraction shown in Fig. 4 (0.011 mg of protein); 15, fraction no. 15 in Fig. 2B (0.97 mg of protein).*
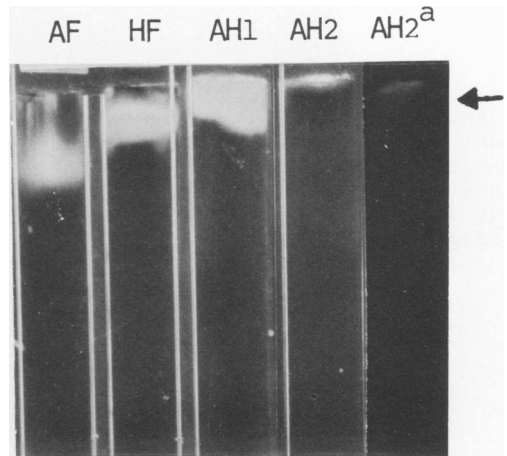

FIG. 6. *Electrophoretic patterns of purified enzyme preparations. Conditions for electrophoresis were the same as in Fig. 3. After electrophoresis, gels were incubated in agarose gel containing 5% sucrose for detection of enzyme activity, except gel a which was stained by Amido Black for protein. AF, 5 μliters containing 6.3 μg of protein; HF, 20 μliters containing 2.6 μg of protein; AH1, 50 μliters containing 24 μg of protein; AH2, 100 μliters containing 3.6 μg of protein.*

presence of sucrose was purified by chromatography. The complex demonstrated a molecular weight ranging from 400,000 to 2,000,000, contained both dextran and levan sucrases, and possessed a polysaccharide of glucose. The adherence activity of the material was parallel during purification with the enzyme activity that was responsible for the synthesis of insoluble polysaccharide.

The dextran sucrase enzymes from culture fluid of *S. mutans* strain OMZ176 and 6715 (group *d*) have been purified 900 to 1,800 times by hydroxylapatite chromatography followed by isoelectric focusing. The enzyme from OMZ176 was found in a number of fractions and most of the fractions showed several bands of enzymes or proteins on gel electrophoresis (9). The enzyme from 6715, however, was present in one band during chromatographic purification and had a molecular weight of 94,000. Several contaminating bands of protein appeared on gel electrophoresis (2). These dextran sucrase preparations were not tested for adherence activity, nor for the ratio of enzyme activities for the
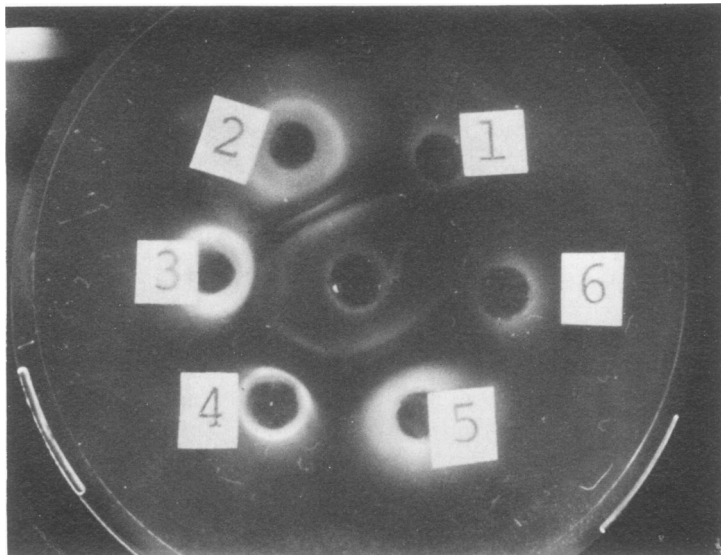
FIG. 7. Agar gel diffusion patterns of enzyme preparations. After development of precipitin bands with anti-CEP, the plate was covered with agarose gel which contained 5% sucrose and incubated to determine those precipitin bands which possessed water-insoluble polysaccharide synthetase activity. White clouds around outer wells indicate the presence of enzyme activity. Each outer well contained 10 μliters. The protein content is given in parenthesis. 1, culture supernatant (49.9 μg); 2, CEP (127.6 μg); 3, AF (12.6 μg); 4, HF (1.3 μg); 5, AH1 (4.8 μg); 6, AH2 (0.36 μg).
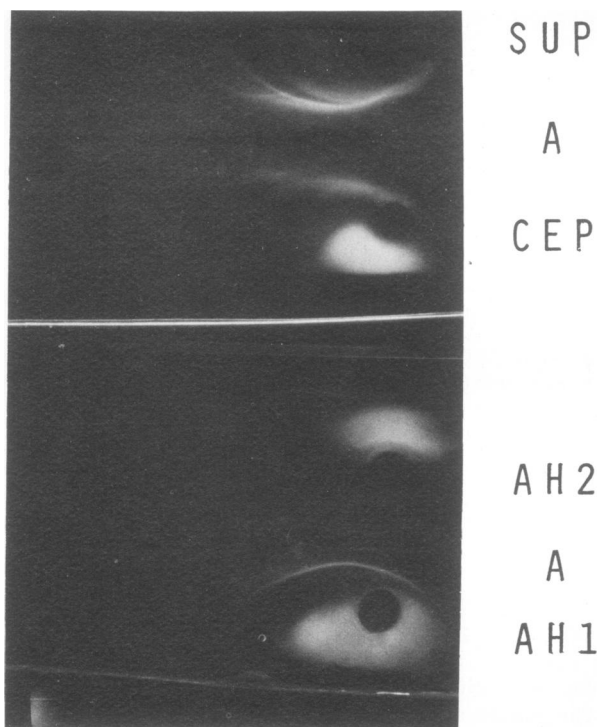

FIG. 8. Immunoelectrophoretic patterns of enzyme preparations. After development of precipitin bands, plates were covered with sucrose-containing gel, as in Fig. 6. Trough A, anti-CEP; SUP, 10 μliters of culture supernatant (49.9 μg); CEP, 5 μliters (63.8 μg); AH2, 50 μliters (1.6 μg); AH1, 10 μliters (4.8 μg).
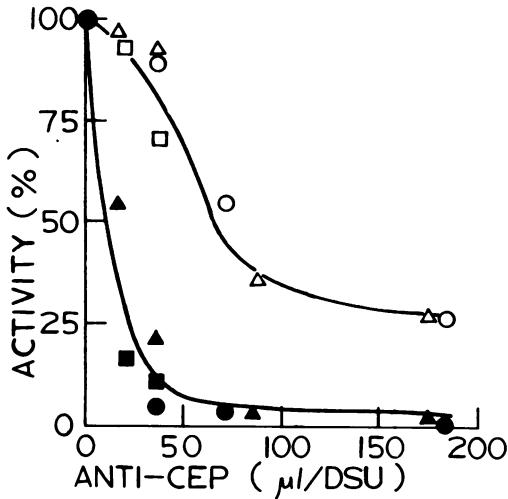
FIG. 9. *Inhibition of water-insoluble and water-soluble dextran sucrase activities by anti-CEP. Enzyme preparations and anti-CEP were incubated at 37 C for 4 h and centrifuged. The activities of the supernatant were measured by incorporation of [¹⁴C]glucose into water-insoluble and water-soluble polymers. Symbols: ● and ○, insoluble and soluble dextran sucrase activities of culture fluid; ▲ and △, those of AH2; ■ and □, those of fraction no. 15 of Fig. 2B.*

synthesis of water-insoluble and water-soluble polysaccharide. In the present study, the enzyme responsible for adherence and insoluble polysaccharide synthesis was different in molecular size from the dextran sucrase of OMZ176. The latter moved more rapidly in 3.5% acrylamide gel. The soluble polysaccharide synthetase was irreversibly adsorbed by hydroxylapatite (Table 4). Results with strain 6715 have shown that a low-molecular-weight enzyme fraction was easily eluted from hydroxylapatite (2). The reason for this difference is not at present apparent.

The recovery of enzymatic activity from crude culture supernatants in the various fractions (Table 4) showed that BioGel A-0.5m was effective in separating the activity responsible for the synthesis of soluble polymer from that responsible for the synthesis of insoluble polymer, with a recovery of 104% of the latter enzyme (Table 4, AF). A minor contaminant was present (Fig. 7, no. 3). By passage through a hydroxylapatite column, the recovery decreased significantly (HF and AH1) and the contaminating proteins were still present (Fig. 7 and 8). These proteins possessed no insoluble polymer synthetase activity and no serological identity with the adherence enzyme complex. AH2, which did not contain inactive contaminating protein, and HF showed the highest

relative adherence activity (Table 3). A mixture of AH2 and AH1, or either one when mixed with other fractions in Fig. 4, never showed an increase or decrease in adherence and sucrase activities. These results indicate that the contaminating proteins in AF, HF, and AH1 would not be a hinderance in further enzymatic and serological studies of the molecular basis of adherence. Isoelectric focusing was not attempted because a 90% loss was expected (9), and because the present fractions (especially AH2) were sufficiently pure.

In the present study, only a high-molecular-weight material which was composed of dextran and levan sucrases and polysaccharide possessed adherence activity. It is not clear whether the difference between the enzymes responsible for the synthesis of insoluble and soluble polymers is due only to molecular size, or whether additional factors are required. The enzyme unit responsible for the synthesis of the soluble polymer may be a subunit of the larger enzyme.

The previous study (20) indicated that the enzyme complex responsible for adherence diffused slowly in agar gel as judged by its selective adsorption on heat-killed cells. This property has been established by the present results (Fig. 3, 6, 7, 8).

In the present study, enzyme fractions of high adherence activity always revealed high activities of water-insoluble levan sucrase as well as water-insoluble dextran sucrase. A purified dextran sucrase fraction (1) from *S. sanguis* which synthesizes water-insoluble dextran is free of levan sucrase activity, whereas *S. salivarius* produces primarily fructose polymer (5, 22). On the basis that *S. mutans* is primarily responsible for the development of caries on the smooth surfaces of teeth rather than other streptococci (4, 8, 13), this ability of *S. mutans* might be due to the synthesis of both water-insoluble dextran and levan as suggested previously (7, 20, 21). A dextran sucrase preparation from OMZ176 was

TABLE 4. *Recovery of enzymatic activities*

Preparation	Adherence	Dextran sucrase recovery (%)		Levan sucrase recovery (%)	
		Insoluble	Soluble	Insoluble	Soluble
Supernatant .	100.0	100.0	100.0	100.0	100.0
CEP	50.0	90.2	115.7	105.3	106.3
AF	43.8	103.9	10.8	104.3	9.9
HF	25.0	18.2	2.4	13.8	1.9
AH1	19.8	5.6	0.1	8.7	0.1
AH2	2.0	1.4	0.1	2.4	0.2

shown to be free of levan sucrase activity as judged by hydrolysis of the product with 4 N H_2SO_4 at 97 C (9). However, we have found (results not shown) that hydrolysis with the same concentration of HCl at 100 C destroyed the fructose released from sucrose or inulin and also the free fructose in the control. A quantitative yield of fructose from *Aerobacter* levan was obtained by heating for 1 h at 100 C in 0.01 N HCl (10). However, such mild conditions did not release either glucose or fructose from fraction AH2 or the insoluble polymer synthesized by AH1. It is probable that the HS6 levan may possess a different structure or is complexed with dextran so that hydrolysis does not occur in weak acid solution. In the case of strain 6715, levan sucrase was separated from dextran sucrase by ammonium sulfate precipitation (2). The lack of levan sucrase in this case might explain the small size of the enzyme. However, in the present study, preparations of lower molecular size (Table 1, 13B to 21B) did not synthesize water-insoluble polysaccharide nor produce adherence.

It is shown in Table 2 and 3 that all the enzymatic fractions contained carbohydrate. The carbohydrate in the highly purified preparations (AH1 and AH2) was composed of significant quantities of glucose. Enzyme preparations from strain 6715 also contained carbohydrate (C. F. Schachtele, personal communication). The carbohydrate in these enzyme fractions may represent a primer molecule and/or a remnant of the polymer synthesized by the enzyme.

It is known that the addition of dextran stimulated the sucrose activity (2, 21, 22) of culture fluids, and that after hydroxylapatite chromatography the enzyme activity was dependent upon the addition of dextran (2). The absence of an inhibitory effect of our anti-dextran globulin on enzyme activity may indicate, if the globulin is bound, that the dextran in the enzyme does not directly function in polymer synthesis. The absence of antigenic specificity may also indicate an incomplete polymer or a remnant of a complete polymer originally present on the surface of the *S. mutans* cell.

Recently, two forms of cell-associated dextran sucrase were extracted from viable cells of *S. mutans* (GS5, group *c*) with 1.0 M NaCl (13a). These forms did not show any major difference either enzymatically or in molecular size with the extracellular enzyme from the same strain. The study also showed that the extracted cells still retained more than 50% of their original adherence activity. As shown previously (21), almost all of the adherence enzyme complex was tightly bound to a specific site on the cell surface of viable cells and was not extracted with 1.0 M NaCl, whereas nonspecifically bound enzyme complex was extracted without effecting the adherence ability of the cells. Therefore, the cell-associated enzymes which are extractable are probably not bound to the cell in the same manner as are those which are not extractable. This indicates that the two binding sites differ in their composition and/or location.

The enzyme purified in the present study is being used to obtain information on the nature of the enzyme-binding site complex on the surface of the *S. mutans* cell.

LITERATURE CITED

1. Carlsson, J., E. Newbrun, and B. Krasse. 1969. Purification and properties of dextran sucrase from *Streptococcus sanguis*. Arch. Oral Biol. **14**:469–478.
2. Chludzinski, A. M., G. R. Germaine, and C. F. Schachtele. 1974. Purification and properties of dextransucrase from *Streptococcus mutans*. J. Bacteriol. **118**:1–7.
3. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Kebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
4. Fitzgerald, R. J., and P. H. Keyes. 1960. Demonstrations of the etiologic role of streptococci in experimental caries in the hamster. J. Amer. Dent. Ass. **61**:9–19.
5. Garszczynski, S. M., and J. K. Edwards. 1973. Synthesis of a broth levan by a cell-bound levansucrase from *Streptococcus salivarius* (SS2). Arch. Oral Biol. **18**:239–251.
6. Gibbons, R. J. 1972. Presence of an invertase-like enzyme and a sucrose permeation system in strains of *Streptococcus mutans*. Caries Res. **6**:122–131.
7. Gibbons, R. J., and M. Nygard. 1968. Synthesis of insoluble dextran and its significance in the formation of gelatinous deposits by plaque-forming streptococci. Arch. Oral Biol. **13**:1249–1262.
8. Guggenheim, B. 1968. Streptococci of dental plaques. Caries Res. **2**:147–163.
9. Guggenheim, B., and E. Newbrun. 1969. Extracellular glucosyltransferase activity of an HS strain of *Streptococcus mutans*. Helv. Odontol. Acta **13**:84–97.
10. Hestrin, S., D. S. Feingold, and G. Avigad. 1956. The mechanism of polysaccharide production from sucrose. 3. Donor-acceptor specificity of levansucrase from *Aerobacter levanicum*. Biochem. J. **64**:340–351.
11. Koepsell, H. J., and H. M. Tsuchiya. 1952. Enzymatic

synthesis of dextran. J. Bacteriol. **63:**293-295.

12. Krasse, B., and J. Carlsson. 1970. Various types of streptococci and experimental caries in hamsters. Arch. Oral Biol. **15:**25-32.

13. Kuramitsu, H. 1974. Adherence of *Streptococcus mutans* to dextran synthesized in the presence of extracellular dextransucrase. Infect. Immunity **9:**764-765.

13a. Kuramitsu, H. 1974. Characterization of cell-associated dextransucrase activity from glucose-grown cells of *Streptococcus mutans*. Infect. Immunity **10:**227-235.

14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193:**265-275.

15. McCabe, M. M., and E. E. Smith. 1973. Invertase activity in *Streptococcus mutans* and *Streptococcus sanguis*. Arch. Oral Biol. **18:**525-531.

16. Matsuno, T., and H. D. Slade. 1970. Composition and properties of a group A streptococcal teichoic acid. J. Bacteriol. **102:**747-752.

17. Matsuno, T., and H. D. Slade. 1971. Group A streptococcal polysaccharide antigens. Infect. Immunity **3:**385-389.

18. Mukasa, H., and H. D. Slade. 1972. Chemical composition and immunological specificity of the streptococcal group O cell wall polysaccharide antigen. Infect. Immunity **5:**707-714.

19. Mukasa, H., and H. D. Slade. 1973. Structure and immunological specificity of *Streptococcus mutans* group *b* cell wall antigen. Infect. Immunity **7:**578-585.

20. Mukasa, H., and H. D. Slade. 1973. The mechanism of adherence of *Streptococcus mutans* to smooth surfaces. I. The roles of insoluble dextran-levan synthetase enzymes and cell wall polysaccharide antigen in plaque formation. Infect. Immunity **8:**555-562.

21. Mukasa, H., and H. D. Slade. 1974. The mechanism of adherence of *Streptococcus mutans* to smooth surfaces. II. The nature of the binding site and the adsorption of dextran-levan synthetase enzymes on the cell wall surface of the streptococcus. Infect. Immunity **9:**419-429.

22. Newbrun, E., and J. Carlsson. 1969. Reaction rate of dextran-sucrase from *Streptococcus sanguis* in the presence of various compounds. Arch. Oral Biol. **14:**461-468.

23. Schachtele, C. F., A. E. Loken, and M. K. Schmitt. 1972. Use of specifically labeled sucrose for comparison of extracellular glucan and fructan metabolism by oral streptococci. Infect. Immunity **5:**263-266.

24. Soprey, P., and H. D. Slade. 1972. Immunochemistry of the streptococcal group R cell wall polysaccharide antigen. Infect. Immunity **5:**91-97.