Mechanism of Adherence of *Streptococcus mutans* to Smooth Surfaces

II. Nature of the Binding Site and the Adsorption of Dextran-Levan Synthetase Enzymes on the Cell-Wall Surface of the Streptococcus

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The mechanism of adsorption of the *Streptococcus mutans* enzymes responsible for the synthesis of insoluble dextran-levan to the *S. mutans* cell-wall binding sites has been studied. Certain characteristics of these binding sites are presented. The adsorption of these enzymes to the cell surface occurred rapidly without the addition of a source of energy and over a pH range of 3 to 11. The adsorption was inhibited by soluble dextran, probably due to the strong affinity of the polymer to the enzyme. All other polymers and sugars studied showed little or no inhibition. The adsorption was also inhibited by antibody globulin to the α-d immunologically specific group antigen surface polysaccharide of *S. mutans* and by anti-dextran globulin. The inhibition by anti-α-d globulin is considered to be due to a restriction of access of enzyme to the binding site of the enzyme which may be located in close proximity to the group antigen. On the other hand, anti-dextran globulin appeared to directly inhibit the adsorption by covering the binding site. Dextranase destroyed the binding site and released glucose from the *S. mutans* cells. These data indicate that *S. mutans* grown in media containing glucose possesses a small amount of dextran on the cell surface, and that this dextran is, or is a part of, the binding site for enzymes which synthesize the insoluble dextran-levan polymer. Trypsin inhibited the synthesis of insoluble polysaccharide and the adherence of cells. It is not clear in this case that destruction of the binding sites occurred. These data present a partial explanation of the processes which may be concerned in the formation of dental plaque on the smooth surfaces of teeth.

In our previous study (12), the adherence of *Streptococcus mutans* cells to a smooth glass surface was shown to require (i) the presence of dextran-sucrase and levan-sucrase enzymes which had adsorbed to the surface of the streptococcal cells, and (ii) the synthesis of insoluble dextran-levan polysaccharide by the cell-bound enzymes. It is considered essential to know the nature of the site to which the polysaccharide synthetases are bound to determine the mechanism of adherence of the streptococcal cell to a smooth surface. The nature of the site may then indicate a possible approach to a means of inhibiting the binding of the synthetases to the *S. mutans* cell. Such information may be of value for the control of plaque formation on the smooth surfaces of teeth and subsequent dental caries.

The data presented here deals with (i) the energy requirement and pH dependence of the binding of the enzymes on the *S. mutans* cell, (ii) the inhibition of the binding by sugars, polymers, and antisera, and (iii) the nature of the binding site.

**MATERIALS AND METHODS**

*S. mutans* cells. *S. mutans* strains were received from sources previously indicated (11). The cells were grown in THB broth (Todd-Hewitt broth, Difco) fortified with 1.8% glucose and salts (19). For the adherence test, viable cells and cells killed by exposure to 100°C for 20 min were used (12). The heat-treated cells were devoid of enzymes necessary for the synthesis of insoluble polysaccharide; however, the binding sites of the enzymes remained intact (12). Therefore, heat-killed cells (strain HS6, group a) were used throughout the study.

**Crude enzyme preparation.** Crude enzyme preparation (CEP) for the synthesis of insoluble dextran-levan polysaccharide was prepared from the culture supernatant of HS6 cells by ammonium sulfate pre-
cipitation as described previously (12). The CEP (1 ml) converted 0.416 μmol of glucose and 0.153 μmol of fructose per min into the insoluble polymers (12).

**Antisera.** Anti-group a whole cell serum and anti-a-d globulin were prepared as described (10). Anti-a serum and anti-a-d globulin reacted with the a and a-d serological sites of the *S. mutans* group a polysaccharide antigen, and anti-a-d globulin also reacted with a polysaccharide antigen in several group d strains (10). Anti-CEP was obtained by injecting CEP with incomplete adjuvant into rabbits (12). This antisemur completely inhibited the synthesis of insoluble dextran-levan polymer; however, it did not contain a and a-d antibodies (12).

Anti-10449 (S. *mutans*, group C) serum, obtained by the injection of whole cells into rabbits (19), was used as the source of anti-dextran globulins. The globulin solution was prepared as follows: 10 ml of anti-10449 (S. *mutans* group C) whole serum was placed on a column (1.5 by 12 cm) containing G-200 Sephadex in 0.025 M phosphate (pH 6.8) in 0.07 M NaCl. The column was washed with 40 ml of the buffer before use. The upper white, turbid part of the column was transferred to a smaller column (0.8 by 5 cm) after suspending it in a small amount of buffer. After washing the small column with 5 ml of buffer, anti-dextran globulin was eluted with 0.025 M glycine-hydrochloride buffer (pH 2.4) in 0.07 M NaCl. Fractions (2 ml) were collected and neutralized with 1 N NaOH to pH 7 to 7.5. The anti-dextran activity was measured by the capillitary precipitin test (18) by using soluble dextran (molecular weight 2 × 10⁶; Sigma Chemical Co., from *Leuconostoc mesenteroides*). The globulin preparation gave a strong precipitin reaction with a dextran solution containing 10 μg/ml (Tables 1 and 2). Whole-cell sera against HS56 (group A), PA1 (group B) and B13 (group D) did not react with the dextran. In addition, anti-CEP gave a similar negative result. The anti-dextran globulins also agglutinated 10449 whole cells to a 1:16 dilution, whereas group a and b cells were weakly positive at a 1:2 dilution. These results showed that the anti-dextran globulin preparation was satisfactory for further use. The total protein in the preparation, by the Folin procedure (18), was 14.6 mg/ml.

**Inhibition of in vitro synthesis of insoluble polysaccharide.** Inhibitor (5 or 50 mg) was added to the reaction mixture, which consisted of 1 ml of 5% sucrose, 5 μlitors of 10% merthiolate, and 50 μlitors of CEP in 0.05 M phosphate buffer, pH 6.8. The volume was adjusted to 6 ml with buffer (12). After 3 h of incubation at 37 C, the turbidity increase at 550 nm was recorded, and the percentage of inhibition was calculated by comparison with that of the control.

**Adherence of cells on glass surface.** In a glass test tube, 5 μlitors of 10% merthiolate, 4 ml of 0.05 M phosphate buffer (pH 6.8), 50 μlitors of CEP, 1 ml of 0.5% cells, and 1 ml of 5% sucrose in buffer were added. After incubation at 37 C for 16 h at a 30° angle, the concentration of the cells which adhered to the glass was determined by measuring the optical density at 550 nm, as described previously (12). In the case of viable cells or heat-killed cells which had bound CEP, CEP was not added to the suspension.

Inhibition of the adherence of cells was measured by adding 5 or 50 mg of inhibitor to the above mixture.

**Adsorption of enzymes on cells.** Heat-killed cells (5 mg) were incubated with 50 μlitors of CEP in 1 ml of phosphate buffer at 37 C for 30 min. The suspension was centrifuged. The quantity of enzyme which remained unadsorbed in the supernatant was measured by the adherence test with 1 ml of heat-killed cells, 1 ml of 5% sucrose, and 4 ml of phosphate buffer (12), and also by the incorporation of [14C]glucose and [14C]fructose from 50 μg labeled sucrose (2.69 × 10⁴ counts/min of [14C]glucose and 3.36 × 10⁴ counts/min of [14C]fructose) into insoluble polymers.

After washing the cells with phosphate buffer, the amount of enzyme adsorbed on the cells was measured by the adherence test with 5 μlitors of 10% merthiolate, 1 ml of 5% sucrose, and 5 ml of buffer (12), and also by the incorporation of [14C]glucose or [14C]fructose from sucrose. The cells or insoluble polymer, or both, were washed with water three times by centrifugation, added to mix L scintillation fluid (21) after lyophilization, and counted in a Beckman LS-100 scintillation counter.

The enzyme adsorbed on the cells was also determined by observing a decrease in the number of bands in agar-gel diffusion between anti-CEP and CEP which had been adsorbed with the heat-killed cells. To 50 μlitors of CEP, 1.25 to 10 mg of heat-killed cells were added and incubated at 37 C for 30 min. After centrifugation, the supernatant was reduced to 50 μlitors by lyophilization and analyzed by agar-gel diffusion (15).

**Speed of adsorption of enzyme to cells.** Heat-killed cells (5 mg), 5 μlitors of 10% merthiolate, and 25 μlitors of CEP in 5 ml of 0.05 M phosphate buffer, pH 6.8, were incubated for 1 to 30 min at 5 C, 30 C, or room temperature. After incubation, cells were immediately collected by centrifugation at 4 C, washed once with buffer, and measured for ability to adhere.

**pH effect on synthesis of insoluble polysaccharide, adsorption of enzyme, and adherence of cells.** pH effects on insoluble polymer synthesis and adherence of cells were measured in various buffers (pH 1 to

**Table 1. Anti-dextran antibody precipitin reaction with dextran**

<table>
<thead>
<tr>
<th>Antiserum or antibody globulin</th>
<th>Precipitin reaction with dextran (mol wt 2 × 10⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:12</td>
</tr>
<tr>
<td>Antidextran globulin</td>
<td>–</td>
</tr>
<tr>
<td>Anti-10449 serum</td>
<td>–</td>
</tr>
</tbody>
</table>

* Dilutions of dextran solution with initial dextran content of 2.5 mg/ml.
heat-killed cells
various by
tion of
above,
adsorption
added
and incubated
heat-killed
antisera
by
centrifugation,
supernatant
30
was
also measured
Those cells which had
the cells
of
enzymes
inhibition
by
described above.

Dilutions
of anti-dextran globulin fraction.

Heat-killed cells (0.5%) in phosphate buffer.


TABLE 2. Anti-dextran antibody agglutination activity

<table>
<thead>
<tr>
<th>Cells*</th>
<th>Agglutination of cells by anti-dextran globulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS6 (group a) . . . . .</td>
<td>+ + - - - - - -</td>
</tr>
<tr>
<td>FA1 (group b) . . . . .</td>
<td>+ ± + + + + + +</td>
</tr>
<tr>
<td>10449 (group c) . . . .</td>
<td>+ + + + + + + +</td>
</tr>
</tbody>
</table>

* Heat-killed cells (0.5%) in phosphate buffer.

Inhibition of enzyme adsorption on cells. Inhibition of the adsorption of enzymes on cells by monomers (50 mg), oligomers (50 mg), or polymers (5 mg) was measured. Heat-killed cells (5 mg) were incubated with the inhibitors and 50 μl of CEP at 30 C for 30 min. After centrifugation, the cells were washed twice with buffer. The amount of enzyme on the cells was measured by the adherence test and also by incorporation of labeled sugars, as described above, and the percentage of inhibition was calculated by comparison with the control.

Inhibition of the adsorption of enzyme on cells by antisera or antibodies was measured. To 1 ml of 0.5% heat-killed cells and 5 μl of 10% merthiolate, 5 to 10 μl of antisera or antibody globulin solution was added and incubated at 37 C for 30 min. After centrifugation, the effect of unabsorbed antibody in the supernatant on the inhibition of adherence was measured. The cells obtained by centrifugation were washed with buffer, incubated with 50 μl of CEP in 1 ml of buffer at 37 C for 30 min, and centrifuged. The amount of enzyme unadsorbed in the supernatant and enzyme adsorbed on cells after washing was also measured by the adherence test. In the case of the inhibition by anti-dextran globulin, the inhibition of adsorption of enzyme was measured by incorporation of labeled sugars from sucrose, as described above.

Destruction of enzyme-binding sites by hydrolytic enzymes. Heat-killed cells (5 mg) were incubated with 1 mg of dextranase (Worthington Biochemical Corp., Freehold, N.J.) or trypsin as described above, and the released sugars were analyzed by gas-liquid chromatography (8). The methylation and sialation procedures in preparation for gas chromatographic analysis release total glucose from whole cells, and consequently a separate hydrolysis is not necessary.

TABLE 3. Inhibition of cell-free synthesis of insoluble polysaccharide, adsorption of enzyme on cells, and adherence of cells

<table>
<thead>
<tr>
<th>Inhibitor*</th>
<th>Insoluble polysaccharide synthesized</th>
<th>Adhesion of enzyme on cells (measured by)</th>
<th>Synthesis of dextran levan polymer</th>
<th>Cell adherence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cell adherence</td>
<td>[14C] glucose*</td>
<td>[3H] fructose*</td>
</tr>
<tr>
<td>(i) None</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>(ii) Dextran (mol wt 2 × 10^9)</td>
<td>88.9</td>
<td>85.6</td>
<td>98.0</td>
<td>60.2</td>
</tr>
<tr>
<td>(iii) Dextran (mol wt 2 × 10^9)</td>
<td>44.5</td>
<td>83.2</td>
<td>99.0</td>
<td>60.7</td>
</tr>
<tr>
<td>(iv) Egg albumin</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>6.0</td>
</tr>
<tr>
<td>(v) Agarose</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>22.9</td>
</tr>
<tr>
<td>(vi) Fructose</td>
<td>61.5</td>
<td>0.1</td>
<td>0.1</td>
<td>40.2</td>
</tr>
<tr>
<td>(vii) Melibiose</td>
<td>77.0</td>
<td>0.1</td>
<td>0.1</td>
<td>34.0</td>
</tr>
</tbody>
</table>

* Concentration of inhibitors; numbers (ii) to (v): each 5 mg/6 ml; numbers (vi) and (vii): each 50 mg/6 ml.

Inulin, rhamnose, gentiobiose, N-acetylgalactosamine, raffinose, ribitol, glucose, galactose, glucosamine, lactose, glycerol, and dulcitol had no effect on any of the measurements.

* Incorporation of [14C]glucose from sucrose.

* Incorporation of [3H]fructose from sucrose.

* A 10 to 20% increase occurred rather than an inhibition.
required for this determination. The fungal dextranase was found to contain 12.8% galactose and 3.8% glucose. Therefore, the corrected values are shown in Table 6.

**Adsorption of enzyme during cell growth.** To 2 liters of the fortified THB broth, 200 ml of an 18-h culture in THB was added and incubated at 37°C. The cell growth was recorded at several intervals, and a volume of culture which contained cells equivalent to an optical density of 0.54 (in 10 ml) was withdrawn. Cells were heat-treated and washed as described above. The ability of the cells to adsorb the enzyme at different growth stages was measured by adherence of cells or by incorporation of labeled sugars from sucrose by using 200 µl of CEP, as described above.

**Agglutination of whole cells by antiserum.** Washed, lyophilized, heat-killed cells were tested for agglutination in capillary tubes as previously reported (11).

**RESULTS**

**Kinetics of adsorption of enzyme by heat-killed cells.** The adsorption of the synthetase enzymes by heat-killed cells was almost complete within 1 min at 5°C and at room temperature (Fig. 1). The same result was obtained at 37°C. These results also show that the adsorption of the enzyme on the binding sites did not require the addition of a source of energy. The adsorption of enzyme did not change significantly between pH 5 and 8 (Fig. 2). On the other hand, the synthesis of polymer and the adherence of cells were more sharply effected by pH (Fig. 3). The optimal pH for both systems was pH 5 to 7 and pH 6 to 8, respectively.

**Inhibition by sugars or polymers of enzyme adsorption by cells.** To obtain information on the mechanism of adsorption of the enzymes responsible for the synthesis of the insoluble dextran-levan polymer, a number of sugars, polysaccharides, and protein polymers were tested for possible inhibitory effects. The synthesis of insoluble dextran-levan and adherence of the cells was also measured in these experiments. Table 3 shows that insoluble polysaccharide synthesis was markedly inhibited by dextran, fructose, and melibiose. The action of dextran and melibiose may be due to a termination of the elongation of the polymer chain, and that of fructose may be due to a partial inactivation of dextranucrase (5, 20).
produced by polysaccharide and adherence on

different pH values (0) sucrose, and the

inhibit the serum was incubation. The

water, combined, and increase assay, buffer (pH

0 ~ ~ ~ ~ ~ ~ ~ 0 . 6

w0 1

0.5

0.4

0.3

0.2

0.1

0.12

0.10

0.08

0.06

0.04

0.02

5 7 9

POLYSACCHARIDE SYNTHESIS

ADHERENCE (O.D. 550 nm)

pH

0

0.5

1

1.5

2

2.5

3

3.5

4

4.5

5

5.5

6

6.5

7

7.5

8

8.5

9

9.5

10

10.5

11

Fig. 3. Effect of pH on synthesis of insoluble polysaccharide and adherence of cells. For the adherence assay, 5 mg of heat-killed cells in 1 ml of distilled water, 10 uliters of 10% merthiolate, 4 ml of 0.05 M buffer (pH 1 to 11), and 50 uliters of CEP were combined, and the mixture was incubated for 30 min at 37°C. After incubation, 1 ml of 5% sucrose was added, and the adherence (O) was measured as described in Materials and Methods after the usual incubation. The synthesis of insoluble polysaccharide at different pH values (●) was measured by the increase in turbidity. The reaction mixture was composed of 10 uliters of 10% merthiolate, 5 ml of 0.05 M buffer (pH 1 to 11), 250 uliters of CEP, and 1 ml of 5% sucrose, and was incubated at 37°C for 4 h.

Table 3 shows that dextran also produced a marked inhibition of enzyme adsorption, as illustrated in each of the three measurements. It is possible that dextran combines with the enzyme and that the complex is then unable to bind to the cell. Agarose produced a partial inhibition of enzyme adsorption. Fructose and melibiose had no effect.

Egg albumin showed a marked inhibition of cell adherence without an effect on polysaccharide synthesis and enzyme adsorption. This result indicates an inhibition of an unknown step in the adherence process. The same possibility may explain the result with agarose, although a partial inhibition of enzyme adsorption was obtained.

The partial inhibition of polysaccharide synthesis produced by fructose and melibiose resulted in inhibition of cell adherence.

The inhibition of cell adherence by dextran (Table 3) is due to an inhibition of polysaccharide synthesis and enzyme adsorption.

Inhibition by antibodies of enzyme adsorption on cells. In the previous investigation (12), anti-CEP serum was reported to completely inhibit the synthesis of insoluble polymer and, consequently, the adherence of cells. It was of interest to investigate the effect of various antibodies on the binding of these enzymes. Anti-CEP was not adsorbed by heat-killed cells (Fig. 4A). This is evident from the ability of the supernatant to completely prevent adherence. Anti-CEP-treated cells after washing were able to adsorb enzyme in a normal manner. Finally, the quantity of enzyme unadsorbed by the anti-CEP-treated cells did not change over the 0- to 50-uliter range of anti-CEP used.

Anti-a-d globulin was previously shown (12) to inhibit adherence of cells but not the synthesis of insoluble polymer. It was suggested that the a-d globulin inhibited the adsorption of enzyme. It is seen in Fig. 4B that the a-d globulin did indeed inhibit the adsorption of enzyme which resulted in a decrease of adherence to zero and an increase in the enzyme of the supernatant. It is also shown that the supernatant did not inhibit adherence and, therefore, did not contain any anti-a-d globulin.

Figure 4C shows that anti-a globulin was completely adsorbed by the heat-killed cells without inhibition of adherence by the adsorbed supernatant. It is also seen that a 20% inhibition of enzyme occurred within the range of antibody used. An increase in the quantity of enzyme in the supernatant has occurred; however, about four times more anti-a globulin was required to obtain complete inhibition.

In Fig. 4D, it is seen that antidextran globulin was completely adsorbed by the cells, with a simultaneous loss of ability to bind enzyme and a steady increase of unadsorbed enzyme in the supernatant.

The inhibition of adherence by anti-dextran (Fig. 4D) did not indicate whether one or both enzymes were inhibited. Both dextran- and levansucrase enzymes were inhibited (Fig. 5). The inhibition is accompanied by an increase in dextranucrase and levansucrase in the supernatant fluid.

It can be concluded that anti-CEP did not prevent the adsorption of enzyme, whereas either anti-dextran or anti-a-d globulin produced a complete inhibition. Although antidextran was the most effective inhibitor and anti-a-d was second most effective, these data do not determine which was the specific inhibitor.

It is seen in Fig. 4B and C (shaded area) that antibodies to a and a-d antigens produced a strong agglutination of heat-killed cells. Antidextran globulin produced a very weak reaction (Fig. 4D). The experimental conditions responsible for the agglutination also resulted in inhibition of enzyme adsorption (Fig. 4B and C).
Fig. 4. Inhibition by antisera or antibody globulin of enzyme adsorption on cells. Heat-killed cells (5 mg) in 1 ml of buffer were pretreated with 5 μl of 10% merthiolate and 5 to 100 μl of antisera or antibody globulin solution at 37 C for 30 min. After incubation the suspension was centrifuged. The amount of antibody remaining in the supernatant (Δ) was measured by the inhibition of adherence by the adsorbed supernatant. The enzyme adsorbed on the cells obtained by centrifugation was washed with buffer and incubated with 50 μl of CEP in 1 ml of buffer at 37 C for 30 min. The amount of enzyme adsorbed on the cells (O) was measured by the adherence assay after washing the cells with buffer. The following antibodies were used: A, anti-CEP serum; B, anti-a-d globulin; C, anti-a serum; D, anti-dextran globulin. In B, C, and D, the shaded area represents agglutination of whole cells.

Destruction of enzyme-binding sites by hydrolytic enzymes. Dextranase- or trypsin-treated heat-killed cells were markedly reduced in their ability to bind the enzymes dextranase and levansucrase (Table 4). The synthesis of insoluble polysaccharide likewise was inhibited by dextranase and trypsin. However, other hydrolytic enzymes used did not inhibit either process (Table 4). To avoid the possibility that an incomplete removal of hydrolytic enzyme from cells would cause an apparent destruction of the enzyme-binding sites by inhibiting insoluble polymer synthesis due to inactivation of the enzyme, the cells were washed repeatedly or heat-treated again after the enzyme treatment. The inhibition by dextranase was about 70%, and the amount of unadsorbed enzyme in the supernatant increased to somewhat more than 100% (Table 5).

Additional heat treatment of the trypsin-treated cells markedly reduced the inhibition of enzyme adsorption; however, cell adherence
Fig. 5. Inhibition of adsorption of enzyme on heat-killed cells by antidextran globulin. Cells were pre-treated with globulin and incubated with CEP as described in Fig. 4. The amount of unadsorbed enzyme was measured by incorporation of \(^{14}C\)glucose (O) and \(^{3}H\)fructose (A) from labeled sucrose into insoluble polysaccharide. The amount of enzyme adsorbed on cells was measured by incorporation of \(^{14}C\)glucose (□) and \(^{3}H\)fructose (▲).

Table 5. Destruction of enzyme-binding sites by hydrolytic enzymes

<table>
<thead>
<tr>
<th>Hydrolytic enzyme</th>
<th>Inhibition (%) of enzyme adsorption on cell</th>
<th>Unabsorbed enzyme increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Synthesis of dextran-levan polymer</td>
<td>Synthesis of dextran-levan polymer</td>
</tr>
<tr>
<td></td>
<td>[^{14}C]glucose [^{3}H]fructose</td>
<td>[^{14}C]glucose [^{3}H]fructose</td>
</tr>
<tr>
<td>Control</td>
<td>0.0 0.0 0.0 0.0 0.0 0.0</td>
<td>0.0 0.0 0.0 0.0 0.0 0.0</td>
</tr>
<tr>
<td>Dextranase</td>
<td>69.6 70.2 72.7 106.5 119.3 0.0</td>
<td>69.6 70.2 72.7 106.5 119.3 0.0</td>
</tr>
<tr>
<td>Lysozyme(^a)</td>
<td>0.0 0.0 0.0 6.7 10.8 10.5</td>
<td>0.0 0.0 0.0 6.7 10.8 10.5</td>
</tr>
<tr>
<td>Trypsin(^a)</td>
<td>89.6 10.9 30.7 -46.9 -36.6 8.9</td>
<td>89.6 10.9 30.7 -46.9 -36.6 8.9</td>
</tr>
<tr>
<td>Pepsin(^a)</td>
<td>89.0 86.0 80.3 61.4 68.8</td>
<td>89.0 86.0 80.3 61.4 68.8</td>
</tr>
</tbody>
</table>

\(^a\) As measured by incorporation of \(^{14}C\)glucose from sucrose.

Table 4. Inhibition of the cell-free synthesis of insoluble polysaccharide and destruction of enzyme-binding sites by hydrolytic enzymes

<table>
<thead>
<tr>
<th>Hydrolytic enzyme</th>
<th>Inhibition (%) of enzyme adsorption by cells as measured by syntheses of dextran-levan polymer</th>
<th>Unabsorbed enzyme increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell adherence</td>
<td>Synthesis of dextran-levan polymer</td>
</tr>
<tr>
<td></td>
<td>[^{14}C]glucose [^{3}H]fructose</td>
<td>[^{14}C]glucose [^{3}H]fructose</td>
</tr>
<tr>
<td>Dextranase</td>
<td>74.3 64.2 52.9 49.4</td>
<td>74.3 64.2 52.9 49.4</td>
</tr>
<tr>
<td>Trypsin</td>
<td>59.0 94.2 75.3 82.9</td>
<td>59.0 94.2 75.3 82.9</td>
</tr>
</tbody>
</table>

\(^a\) As measured by incorporation of \(^{14}C\)glucose from sucrose.

These results indicate that the binding site contains dextran and that sucrose-glucose grown cells might possess sufficient dextran to serve as the binding site. Therefore, an analysis was made to determine the release of dextran from dextranase. The total glucose content in heat-killed and washed HS6 cells was found to be 2.60% and in 10449 cells 2.97% of the dry weight. Table 6 shows the quantity of this glucose released by dextranase. The trypsin control was negative. Gas chromatographic analysis showed that the glucose used in the culture medium contained less than 0.05% sucrose. From this sucrose, 1.5 \(\mu\)g of dextran/100 mg of dry cells could have been synthesized. These results show that only a limited quantity of dextran on the cell surface can satisfy the requirement for adherence.

Dissociation of bound enzyme from the cells. An attempt was made to separate the
insoluble polymer synthetase from viable cells or heat-killed cells which had bound the enzyme. Previous results had shown (Fig. 2) that the enzyme remained on the cells between pH 3 and 9. The enzymes were inactivated at pH 1 and 2 and at 10 and 11 (Fig. 2). The treatment of the cells with 0.5 to 8.0 M LiCl or 0.5 to 6.0 M NaCl did not release the enzymes. The various sugars and polymers listed in Table 2 were each tested and found to be inactive. However, soluble dextran, with a molecular weight of either $2 \times 10^4$ or $2 \times 10^6$, was very effective in reducing the ability of the cells to adhere to a glass surface (Table 7). Although dextran markedly reduced adherence, these results do not determine whether the effect of adherence was due to a release of enzyme from the cells or an inactivation of enzyme. Anti-a-d globulin was ineffective in releasing the bound enzymes (Table 7), although it was effective in preventing the binding of the enzymes (Fig. 4B).

**Further proof on the enzyme-binding site association.** In the previous investigation (12, Fig. 5), all of the synthetase activity in 25 $\mu$liters of CEP was shown to be adsorbed by 5 mg of heat-killed cells. The binding sites had become saturated, and the further addition of CEP resulted in an increase in the enzyme in the supernatant (12). Anti-CEP serum (Fig. 6, center well) formed at least four bands with CEP (no. 1). The two slower-diffusing bands were completely adsorbed from 25 $\mu$liters of CEP by 5 mg of cells (no. 2). As the ratio of CEP to cells increased (no. 3-5) (50, 100, and 200 $\mu$liters of CEP per 5 mg of cells), the quantity of the slower component increased. Therefore, these data indicate that the slower-diffusing antigen on the plate may be the insoluble polysaccharide synthetase enzyme.

The immunoelectrophoretic pattern of CEP against anti-CEP showed two major slow-moving bands (Fig. 7, no. 1). When CEP was

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**Table 6. Glucose released from heat-killed cells by hydrolytic enzymes**

<table>
<thead>
<tr>
<th>Hydrolytic enzyme</th>
<th>Heat-killed cells</th>
<th>Glucose released (mg/1 mg of cells)</th>
<th>Glucose released per total glucose in cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextranase</td>
<td>HS6</td>
<td>1.48</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>10449</td>
<td>1.01</td>
<td>3.4</td>
</tr>
<tr>
<td>Trypsin</td>
<td>HS6</td>
<td>&lt;0.1</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td></td>
<td>10449</td>
<td>&lt;0.1</td>
<td>&lt;0.3</td>
</tr>
</tbody>
</table>

* Heat-killed cells (5 mg) were incubated with 1 mg of enzyme, as described in Table 3, and the released sugars were analyzed by gas-liquid chromatography.

**Table 7. Dissociation of enzymes from viable cells and CEP-treated heat-killed cells**

<table>
<thead>
<tr>
<th>Material</th>
<th>Loss of adherence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viable cells</td>
</tr>
<tr>
<td>Control</td>
<td>0.0</td>
</tr>
<tr>
<td>Dextran (mol wt $2 \times 10^4$)</td>
<td>79.5</td>
</tr>
<tr>
<td>Dextran (mol wt $2 \times 10^6$)</td>
<td>96.2</td>
</tr>
<tr>
<td>Anti-a-d globulin</td>
<td>13.6</td>
</tr>
</tbody>
</table>

* Enzyme-treated cells (5 mg) were incubated with various materials in 2 ml of buffer at 37 C for 30 min. The cells were centrifuged, washed with buffer, and measured for adherence ability.

* Polymers (5 mg) or 50 $\mu$liters of anti-globulin solution in 2 ml of buffer were used. Other sugars (50 mg each) and polymers (5 mg each) listed in Table 2 did not cause a loss of adherence by the enzyme-treated cells.

**Fig. 6. Adsorption of enzymes on heat-killed cells.** To 50 $\mu$liters of CEP, 1 ml of cells (containing 1.25, 2.5, 5, or 10 mg) and 10 $\mu$liters of 10% merthiolate were added, and the mixture was incubated at 37 C for 30 min. After centrifugation, the supernatant in each case was reduced to 50 $\mu$liters by lyophilization, and 10 $\mu$liters of each was added to the outer wells. The center well contained 10 $\mu$liters of anti-CEP. The remaining wells contained the following: 1. CEP; 2. CEP adsorbed with 10 mg of cells; 3. CEP adsorbed with 5 mg of cells; 4. CEP adsorbed with 2.5 mg of cells; 5. CEP adsorbed with 1.25 mg of cells.
ADHERENCE OF S. MUTANS

The binding sites for the insoluble polysaccharide synthetases were studied by using heat-killed cells of S. mutans group a strain HS6. The binding sites were specifically blocked by anti-dextran globulin (Fig. 4B) and hydrolyzed by dextranase (Table 4-6). The insoluble polymer synthetases from S. mutans made a stable complex with soluble dextran (Fig. 7), and the adsorption of the enzyme to the binding sites was markedly inhibited by the same dextran (Table 3). These data strongly indicate that the binding sites contained dextran. The presence of a significant quantity (2.6%) of glucose polymer on cells grown in glucose media supports this conclusion. This conclusion also explains the binding of enzyme by the cells in the absence of an added source of energy (Fig. 1), and indicates that a dissociation of enzyme and binding sites did not occur under various conditions (Fig. 2, Table 7).

DISCUSSION

The binding sites for the insoluble polysaccharide synthetases were studied by using heat-killed cells of S. mutans group a strain HS6. The binding sites were specifically blocked by anti-dextran globulin (Fig. 4B) and hydrolyzed by dextranase (Table 4-6). The insoluble polymer synthetases from S. mutans made a stable complex with soluble dextran (Fig. 7), and the adsorption of the enzyme to the binding sites was markedly inhibited by the same dextran (Table 3). These data strongly indicate that the binding sites contained dextran. The presence of a significant quantity (2.6%) of glucose polymer on cells grown in glucose media supports this conclusion. This conclusion also explains the binding of enzyme by the cells in the absence of an added source of energy (Fig. 1), and indicates that a dissociation of enzyme and binding sites did not occur under various conditions (Fig. 2, Table 7).

incubated with S. mutans group a antigen before electrophoresis, no change in the movement occurred (no. 2). Similar results were obtained with inulin (no. 4) and the antisera or antiglobulins given in Fig. 4 (except anti-CEP), and the sugars and polymers given in Table 3. However, when CEP was incubated in the presence of soluble dextran (molecular weight 2 x 10^5) (no. 3) or low-molecular-weight dextran (molecular weight 2 x 10^4), the slower-diffusing band of the two major bands did not move during electrophoresis. The soluble dextran did not show any bands with anti-CEP. These results show that the antigen whose mobility was drastically changed by dextran is probably the insoluble polymer synthetase. The slower-diffusing components of CEP which complexed with dextran (no. 3) correspond to the slower-diffusing components of CEP (Fig. 6) which had adsorbed to the heat-killed cells.

Synthesis of enzyme-binding sites during cell growth. Cells were harvested at different stages (Fig. 8), heat-killed, and washed. The adherence increased from a value of 40% at 1 h to the maximum, which was reached at the resting stage (O). However, the amount of dextranase and levansucrase adsorbed on the cells did not undergo a significant change during the growth of the culture. These results indicate that the cells contain the same quantity of binding site material per cell during growth. The increase in adherence indicates that the cell-bound enzyme is more effective in developing adherence as the culture reaches the stationary phase of growth.

Fig. 7. Complex formation of CEP with polymers. A 50-μliter amount of polymer (2.5 mg) or antisem was added to 50 μliters of CEP, and the mixture was incubated at 37 C for 10 min. A 10-μliter amount was added to each of the four wells, and was electrophoresed at 100 V for 2 h. After electrophoresis, 50 μliters of anti-CEP was added to the A trough. In each well, 7.5 μliters of each of the following was added. 1, CEP + saline; 2, CEP + a antigen; 3, CEP + dextran (mol wt 2 x 10^5); 4, CEP + inulin.

Fig. 8. Synthesis of binding site during growth of cells. Cells (5 mg) were removed from the culture, washed with water, held at 100 C for 20 min in water, and incubated with 100 μliters of CEP in buffer at 37 C for 30 min. After washing with buffer, the amount of enzyme adsorbed by the cells was measured by incorporation of [14C]glucose (△) and [3H]-fructose (△) from labeled sucrose. The adherence ability of the cells (O) and the optical density (•) were measured on each volume of culture fluid removed at the times indicated.

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Kinetic studies of dextran synthesis have shown that dextranase has a high affinity for dextran. The soluble dextrans are also a strong acceptor and also an initiator of dextran synthesis (2, 6, 13). In the present study, the addition of soluble dextran also inhibited synthesis of the insoluble polymer (Table 3). It also inhibited the enzyme adsorption by possibly competing with the enzymes for the binding sites and preventing the adherence of cells. The other polymers used were not effective inhibitors. Therefore, it is likely they could not inhibit the synthesis of insoluble polymer and could not compete with enzymes for the binding sites. However, they could possibly be incorporated into growing polymer during incubation and could terminate the elongation of polymer resulting in the prevention of adherence. More direct proof of specific affinity of enzyme for dextran was observed by immunoelectrophoresis (Fig. 7), indicating the sucrase enzymes could form a stable complex with dextran.

Levansucrase has been reported to be extracted from S. salivarius cells by LiCl (3). In our study, extracts from viable cells showed several bands with anti-CEP, indicating that some dextran-levan sucrases were extracted by this method. However, dextran-levan sucrases bound to heat-killed cells could not be released from the binding sites by any methods tested, including various pH's and exposure to salts, sugars, polymers, or antibodies (Table 7). After such treatment, the heat-killed or viable cells did not lose their ability to adhere or to synthesize polymers. Dextran-treatment of enzyme-treated cells decreased the adherence ability of the cells (Table 7). However, this might have been due to the inactivation of enzymes on cells by the added dextrans. These results could mean that the cell-bound sucrases were not released, even though the unbound enzymes or those inside the cells were extractable.

Fungal dextranase released about 6% of the insoluble glucose polymer from heat-killed cells (Table 6) and inactivated 70% of the binding ability of the dextran sites (Tables 4 and 5). The incomplete destruction of the binding sites by the enzyme corresponds to the reports that insoluble glucan from S. mutans 6715 (group d) was partially (27%) degraded (16) and that the polymer from HS6 was more resistant to the enzyme (14). Therefore, it appears that the dextran in the binding site may possess a similar sugar conformation with that of insoluble dextran.

As shown in the previous report (12) and in Tables 3, 4, and 5, the dextran and levan sucrase enzymes behave the same in regard to their binding to the S. mutans cell. This suggests that the two enzymes exist as a complex which binds to the dextran site. It is not known whether the enzymes bind to the cell as they are synthesized or whether they are bound after their release from the cell. It has been shown (16) that glucose-grown S. mutans cells contained 2 to 10% of the total enzyme activity and the culture fluid contained the remainder.

In the previous study (12), anti-a-d globulin inhibited adherence, whereas anti-a was ineffective. It was suggested that the a-d site served as the binding site for the sucrase enzymes or was located in close proximity to the binding sites (12). However, the anti-a serum has been found to inhibit adherence when a fourfold quantity was used (Fig. 4C). Antibodies to a and a-d prevented the adsorption of sucrases (Fig. 4B and C), contrary to the action of anti-CEP serum (Fig. 4A). Also, purified a antigen, which carries both a and a-d serological sites in a single molecule (12), did not inhibit the binding of enzyme by the cells. Furthermore, the a antigen molecule did not complex with enzyme (Fig. 7), contrary to the action of dextran. These results indicate there was no direct interaction between the sucrase enzymes and the a antigen molecule. Therefore, the only possible explanation of the results presented previously (12) and those on cell agglutination and enzyme inhibition in Fig. 4B and C is that sufficient amounts of anti-a-d or a globulin reacted with a-d or a sites on the cell surface and at the same time interfered with the binding activity of those sites which are located in close vicinity to the antigen. The failure of glucose-grown HS6 cells (Tables 1 and 2) to stimulate anti-dextran formation might also indicate that the binding sites are partially covered with the a antigen on the cell surface. Glucose-grown 10449 (group c) cells, however, stimulated the formation of anti-dextran (Tables 1 and 2) and most likely possess a somewhat different surface configuration.

In summary, these results and those presented previously (12) show that the S. mutans cell possesses a binding site for the dextran-levan sucrase enzymes which contains dextran and is hydrolyzed by dextranase. The a-d cell wall polysaccharide antigen is adjacent to, or a part of, this site. Adsorption to this site by the synthetases is essential for the synthesis of the insoluble polysaccharide polymer and the resulting adherence of the cell. Antibodies against the S. mutans polysaccharide cell wall antigen, synthetase enzymes, and dextran effectively
block the sequence of events responsible for adherence. The application of these results remains for future investigation.

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LITERATURE CITED