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Mechanism of Coaggregation Between *Actinomyces viscosus* T14V and *Streptococcus sanguis* 34

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Actinomyces viscosus T14V and *Streptococcus sanguis* 34 coaggregate by a mechanism which is not inhibited by 1 M NaCl, is dextran independent, requires calcium, is pH dependent with an optimum at pH 8.0 to 8.5, and appears to require the interaction of a protein or glycoprotein on *A. viscosus* with a carbohydrate on *S. sanguis*. The coaggregation is inhibited more than 80% by 0.01 M lactose, 0.02 M β-methyl-D-galactoside, or 0.05 M D-galactose; inhibition of coaggregation was less than 10% in 0.1 M α-methyl-D-galactoside, melibiose, maltose, cellobiose, sucrose, and a number of monosaccharides. At very high concentrations of enzyme, protease from *S. griseus* destroyed the reactive site on *A. viscosus* but not on *S. sanguis*. Both were totally resistant to dextranase. Periodate (0.01 M; pH 4) inactivated both bacteria. The ability of *S. sanguis* to coaggregate with *A. viscosus* was not destroyed by phenol-water extraction at 65°C for 15 min. When the bacteria were cultured under specified conditions, the coaggregation was highly reproducible. Under the same conditions, T14AV, the avirulent mutant of *A. viscosus* T14V, did not coaggregate with *S. sanguis* 34. Electron microscopic studies of coaggregates, labeled immunochemically with antibody to *A. viscosus*, indicated that fibrils on *A. viscosus* may be involved in the coaggregation.

An important goal of oral biology is to understand the forces by which the bacteria in dental plaque are held together and the whole mass adheres to teeth. In the development of plaque, the potential importance of specific surface interactions between and among bacterial species was proposed by Gibbons and colleagues (15, 16), who reported many examples of specific coaggregation between paired oral bacteria of different species. Among these pairs of bacteria were certain strains of *Streptococcus sanguis* coaggregating with specific strains of *Actinomyces viscosus* and *A. naeslundii*, which appeared attractive to us and to other laboratories (2, 11, 26) as models for studying the molecular mechanisms involved in these phenomena. Particular reasons for our interest in these models are the prominence of *S. sanguis* and probably *A. viscosus* in the earliest stages of plaque formation (6, 42, 44, 45), and the possible importance of the actinomyces in root caries and periodontal disease (21–23, 41; A. C. R. Crawford, S. S. Socransky, E. Smith, and R. Phillips, J. Dent. Res. **56**:B120, 1977).

As a starting point for studying the coaggregation between strains of *S. sanguis* and actinomyces, we were especially interested in the virulent *A. viscosus* T14 (T14V) and in the avirulent mutant that appeared spontaneously (T14AV). T14V was isolated from a human patient with periodontal disease and caused periodontal disease in gnotobiotic rats (S. S. Socransky, personal communication). Preliminary experiments indicated that T14V would coaggregate with *S. sanguis* 34, and we have studied this system (i) to establish conditions for growth of the bacteria and for their coaggregation that would afford highly reproducible results, (ii) to probe the general nature of the interaction and of the surface molecules involved, and (iii) to compare T14AV with T14V. In view of the recent evidence that the surface of T14V carries many fibrils that are laden with the "virulence-associated antigen" (7), we attempted to determine whether these structures have a role in the coaggregation.

Early in our investigation, T14V and *S. sanguis* 34 appeared to coaggregate by two different mechanisms, one of which was observed only in very low electrolyte concentrations and was inhibited by a buffer solution containing 0.025 M potassium phosphate and 0.025 M NaCl. Because the coaggregation that is not inhibited by electrolytes is probably the more relevant to

events in the mouth (in the presence of saliva), we chose to concentrate on that mechanism.

MATERIALS AND METHODS

Bacteria. Cultures of *A. viscosus* T14V and T14AV were kindly provided by B. F. Hammond of the University of Pennsylvania, Philadelphia (19). Cultures of *S. sanguis* 34, *S. salivarius* 9GS2, and *S. mutans* 6715 were kindly provided by R. J. Gibbons, Forsyth Dental Center, Boston, Mass. Stock cultures were grown on Trypticase soy agar (Baltimore Biological Laboratory [BBL]) in screw-cap tubes, and were stored at −20°C. To minimize genetic variation through subculturing, working cultures were used for no more than 6 weeks after starting from frozen stocks. Small working cultures were carried in tubes of Trypticase soy broth at 36 to 37°C that were transferred twice weekly. For coaggregation studies, the bacteria were cultured in a medium which contained, per liter, 5 g of NaCl, 2.5 g of K_2HPO_4, and the dialyzable portion of 17 g of Trypticase (BBL) and 4 g of yeast extract (BBL), incubated at 36 to 37°C while rotating at 150 cycles per min. For the streptococci, 0.5% glucose was added to the medium after autoclaving. Cultures were flushed with nitrogen and sealed tightly either with a screw cap or with several layers of Parafilm. When the absorbance at 650 nm (A_{650}) reached approximately 1.0 to 1.5, cells were collected by centrifugation, washed with buffer, tested for coaggregation and, if satisfactory, were stored in 50% glycerol at −20°C.

We have estimated relative cell population densities only by A_{650} measurement without attempting to relate these values to cell numbers.

Chemicals. The following were obtained from Sigma Chemical Co., St. Louis, Mo.: Dextran 2000 (average molecular weight, 2×10^6), α- and β-methyl D-galactosides, purified protease from *Streptomyces griseus* (type V; no. P-5005), and Dextranase no. D1508.

N-acetyl succinimide (NASI) was obtained from ICN Pharmaceuticals Inc., Cleveland, Ohio; trypsin, crystallized three times, was from Miles Laboratories, Inc., Kankakee, Ill. (code A0, 36-555-1).

Potassium lactobionate was prepared by mixing equal volumes of 2 M calcium hemilactobionate and 1.5 M tripotassium phosphate, freezing to break the gel, and thawing and centrifuging to remove the $Ca_3(PO_4)_2$. The supernatant was adjusted to pH 8.0 and was assumed to be 1 M with respect to potassium lactobionate.

Rabbit antiserum specific for the fibrils ("V-antigen") of T14V was generously supplied by John Cisar (7). Peroxidase-labeled goat immunoglobulin G from antisera against rabbit IgG was obtained from the Research Division, Miles Laboratories.

Buffer-salt solution. With very few exceptions, all of which will be noted, the buffer system used for washing bacterial cells and for coaggregation studies was 0.025 M potassium phosphate (pH 8) containing 0.025 M NaCl. This system was chosen because it contains major inorganic constituents of saliva within the concentration ranges found there.

Methods for measurement of activity of enzymes. The activity of dextranase was determined by the conversion of dextran 2000 to isomaltose, in 0.1 M potassium phosphate buffer (pH 6) containing 1 mM $CaCl_2$. Isomaltose was measured by the 3,5-dinitro salicylic acid method of Miller et al. (28). The activity of proteases was determined by the caseinolytic assay of Kunitz (24) in 0.067 M potassium phosphate containing 0.1 mM $CaCl_2$ (pH 7.6); the casein cleavage products which were soluble in 5% trichloroacetic acid were measured by their A_{280}.

Enzyme treatment of T14V and *S. sanguis* 34. All enzyme treatments were at 36°C for 1 h. In the dextranase treatment, washed cells equal to approximately 5 mg (dry weight) were suspended in 5 ml of dextranase solution in 0.1 M potassium phosphate–1 mM $CaCl_2$ (pH 6); 0.1 ml of this dextranase solution, diluted to 1 ml with buffer containing 19 mg of dextran 2000, released 8.8 mg of isomaltose in 1 h. In the protease treatment, washed cells equal to approximately 5 mg (dry weight) were suspended in 5 ml of 0.067 M potassium phosphate–0.1 mM $CaCl_2$ (pH 7.6), containing (i) 5 mg of trypsin, crystallized three times, (ii) 35 mg of purified protease from *S. griseus*, or (iii) 3.5 mg of purified protease from *S. griseus*. Treated cells were collected by centrifugation, rinsed with pH 8 buffer, and then suspended in pH 8 buffer for coaggregation testing.

In protease activity determinations, 0.01 mg of trypsin digested 2.1 mg of casein, and 0.07 mg of bacterial protease digested 4 mg of casein per ml at 36°C in 1 h.

Amino group acetylation. NASI has been shown to react quite selectively with free amino groups in proteins (3). A 10-ml amount of T14V or *S. sanguis* cells suspended in pH 8 buffer, at a cell concentration to give an A_{650} reading of 2.0, was stirred gently with 15 to 17 mg of NASI for 1 h, and the pH was maintained between 7.5 and 8.5 by careful addition of KOH. Cells were collected by centrifugation, washed with buffer, and tested for coaggregation as indicated below.

Hot phenol-water extraction of *S. sanguis* 34. A dense suspension of washed *S. sanguis* 34, approximately 50 mg (dry weight) in 10 ml of 0.1 M NaCl, was heated with an equal volume of 88% phenol at 65 to 70°C for 15 min with frequent agitation (46). The cell residues were collected by centrifugation, resuspended in 0.1 M NaCl, and centrifuged. The hot phenol-water extraction was repeated, and the washed cell residues were suspended in buffer for coaggregation testing.

Formamide extraction of cell wall polysaccharide. Washed *S. sanguis* 34 cells, approximately 100 mg (dry wt), were suspended in 10 ml of formamide and heated at 150°C for 1 h; the formamide solution was fractionated by the method of Fuller (12). The acid alcohol precipitate was collected by centrifugation, washed twice with absolute alcohol, drained well, and suspended in pH 8 buffer.

Extraction of bacteria with high molarity salt solutions. T14V or *S. sanguis* cells were suspended in 8 M LiCl, 4 M KCl, or 5 M NaCl, adjusted to pH 7, and stirred for 1 h at room temperature. The cells were collected by centrifugation and washed with pH 8 buffer.

Electron microscopy. Bacterial cells for thin sections were washed three times by centrifugation in

phosphate-buffered saline (PBS, pH 7.2; 0.15 M NaCl, 0.01 M phosphate) and adjusted to an A_{650} of 2.0. Samples (0.25 ml each) were added to small tubes and were fixed for 1 h at room temperature either with formaldehyde (1.5%) and tannic acid (0.5%) (29, 39, 40) in PBS adjusted to pH 6.5, or with glutaraldehyde (2%) and tannic acid (0.5%) in 0.1 M sodium cacodylate at pH 6.5. (Formaldehyde solutions were prepared fresh by dissolving paraformaldehyde in boiling buffer.) The samples were then washed with PBS to remove fixative, fixed in 1% osmium tetroxide, buffered to pH 7.3 with 0.1 M sodium cacodylate, at room temperature for 30 min, rinsed, dehydrated in acetone, and embedded in Epon. Thin sections were examined without staining with a Phillips 300 electron microscope.

To distinguish T14V from *S. sanguis* 34 in the coaggregates, the immunochemical labeling method of Cisar et al. (7) was employed. Coaggregates were collected and washed without centrifugation. The washed bacteria were fixed for 10 min at room temperature with 1.5% formaldehyde in PBS (pH 7.2), washed with PBS three times to remove the fixative, and then incubated for 30 min at room temperature with either normal rabbit serum or rabbit antiserum specific for T14V fibril antigen. This step was done with 25 μl of serum diluted 1:20 in PBS. All preparations were washed three times with PBS to remove unreacted serum proteins and then incubated for 30 min at room temperature with 25 μl of the peroxidase-labeled goat anti-rabbit immunoglobulin G reagent diluted 1:20 in PBS. Samples were washed three times in PBS to remove unreacted labeled antibody and fixed for 5 min at room temperature in 2.5% glutaraldehyde buffered at pH 7.3 in 0.1 M sodium cacodylate. The peroxidase reaction was performed on samples treated with control serum and with antibody, followed by staining with osmium tetroxide by the method of Graham and Karnovsky (18). Samples were then dehydrated in acetone and embedded in Epon for the preparation of thin sections. The thin sections were cut on the Porter-Blume MT-2 ultramicrotome and examined unstained.

Procedure and conditions for coaggregation studies. Bacterial cells stored in 50% glycerol-distilled water were used for all coaggregation studies, except for the initial testing of each new lot of cells. For each experiment, bacteria were removed from the 50% glycerol by centrifugation at 4°C and washed twice by suspending in the pH 8 buffer and centrifuging. They were then very finely and uniformly suspended in buffer to give an A_{650} of 2.0 to 2.1.

Coaggregation experiments were performed in culture tubes (10 by 75 mm) as follows. Controls were 1 ml of T14V (A_{650} = 2.0 to 2.1) and 1 ml of *S. sanguis* 34 (A_{650} = 2.0 to 2.1). The coaggregation mixture consisted of 0.5 ml of T14V plus 0.5 ml of *S. sanguis* 34. The procedure for controls as well as coaggregation mixture was: (i) mix tubes well (10 s) with a Vortex mixer; (ii) let tubes stand 10 min at room temperature, then mix again; (iii) let tubes stand at room temperature for at least 30 min, preferably 1 to 2 h; (iv) centrifuge all tubes at approximately 7 × *g* for 2 min; and (v) carefully pipette off the top 0.6 ml and read the A_{650} (1-cm quartz cell, Beckman model 24 spectrophotometer).

Percent coaggregation =

$$\frac{\dfrac{A_{650}\,\text{T14V} + A_{650}\,\text{Ss}}{2} - A_{650}(\text{T14V} + \text{Ss})}{\dfrac{A_{650}\,\text{T14V} + A_{650}\,\text{Ss}}{2}} \times 100$$

When chemicals were added to test for inhibition of coaggregation, the protocol was modified as follows. (i) The cell suspensions were made slightly more concentrated (A_{650} = 2.25 to 2.3). (ii) Controls were 0.8 ml of T14V plus 0.2 ml of buffer, and 0.8 ml of *S. sanguis* 34 plus 0.2 ml of buffer. The coaggregation mixture consisted of 0.2 ml of buffer containing test material plus 0.4 ml of T14V plus 0.4 ml of *S. sanguis* 34. Calculation of inhibition of coaggregation was by the equation:

Percent inhibition =

$$\frac{\%\ \text{coaggregation without inhibitor} - \%\ \text{coaggregation with inhibitor}}{\%\ \text{coaggregation without inhibitor}} \times 100$$

RESULTS

Effect of culture conditions upon reproducibility of coaggregation. With bacteria cultured under favorable conditions, coaggregation at pH 8 was rapid and highly reproducible. The percent coaggregation was seldom less than 80 and was usually in the 85 to 90 range; replicates in the same experiment usually agreed to within 3%. This degree of reproducibility was dependent upon the composition of the culture medium; when T14V was cultured in a medium containing whole Trypticase and yeast extract in place of the dialyzable portion of these constituents, the cells did not coaggregate with *S. sanguis* 34. When 0.1% glucose was included in the medium with the dialyzable portion of Trypticase and yeast extract, the coaggregation was variable from one T14V harvest to another. When glucose was omitted from this medium, 100% of the cultures coaggregated with the high degree of reproducibility indicated. On the other hand, *S. sanguis* 34 coaggregated only when cultured in the presence of glucose; moreover, whole Trypticase and yeast extract in the culture medium did not interfere with the ability of *S. sanguis* 34 to coaggregate.

Although we have not made a careful study of the importance of the phase of growth at harvest, no apparent coaggregation difference was found between cultures harvested at an A_{650} of 0.4 and others harvested at an A_{650} greater than 1.0.

Stability of cells stored in 50% glycerol. We have stored *S. sanguis* for 2 years and T14V for 1 year without detecting any significant

change in their ability to coaggregate. Obvious advantages afforded are convenience and consistency in cell supply.

pH dependence. The degree of coaggregation was investigated over the pH range of 6 to 8.5, keeping PO_4^{3-} and NaCl constant at 0.025 M and adjusting the pH by varying the proportions of KH_2PO_4 and K_2HPO_4. At pH 6, the degree of coaggregation was variable and always less than at pH 6.5 and above. The rate and the degree of coaggregation increased between pH 6.5 and 8. In two experiments, the coaggregation was 50 and 60% in 3 h at pH 6.5, 69 and 73% in 2 h at pH 7, and 86 and 86% in 2 h at pH 8. A near maximum value was reached within 30 min at pH 8, and we observed little or no difference in rate or degree of coaggregation between pH 8.0 and 8.5.

Species specificity. Other than *S. sanguis* 34, only *S. mutans* 6715 and *S. salivarius* 9GS2 were tested with T14V, and coaggregation did not occur in either case.

Lack of coaggregation between T14AV and *S. sanguis* 34. When the avirulent mutant T14AV was cultured under the best conditions to favor coaggregation, it did not coaggregate with *S. sanguis* 34. An obvious difference between the two organisms is the production of an extracellular viscous material by T14AV which was reported by Hammond et al. (19) and which we have observed also. The production of this material might not be the reason for T14AV failing to coaggregate with *S. sanguis* 34, because addition of the viscous material (a concentrate of the nondialyzable portion of T14AV culture supernate) before mixing with *S. sanguis* 34 did not inhibit coaggregation.

Effects of PO_4^{3-}, F^-, 1 M NaCl, chelating agents, and dextran. The data which are summarized in Table 1 bear upon the importance of metal ions, electrostatic forces, and dextran in the coaggregation. The inhibition by 0.25 M PO_4^{3-} and by 0.025 mM ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid (EGTA), and Mg_2EGTA strongly indicate that calcium retained by the washed cells is essential for coaggregation. EDTA could inhibit by chelating magnesium or other metals in addition to calcium, but EGTA is 10^4 times more selective for calcium than magnesium (4, 10), and the importance of magnesium is excluded by the fact that Mg_2EGTA inhibits as effectively as does EDTA or EGTA.

Complete inhibition of coaggregation was obtained by treating T14V alone with 0.1 M sodium EDTA (pH 8), washing thoroughly, and mixing with untreated *S. sanguis* 34. The addition of

TABLE 1. *A. viscosus T14V-S. sanguis 34 coaggregation; effects of PO_4^{3-}, F^-, 1 M NaCl, chelating agents, and dextran*

Chemicals added	% Inhibition of coaggregation
PO_4^{3-}, 0.25 M[a]	100
NaCl, 1 M	0
EDTA, 0.025 mM	100
EDTA, 0.01 mM	0
EGTA, 0.025 mM	100
EGTA, 0.01 mM	3
Mg_2EGTA, 0.025 mM	100
Mg_2EGTA, 0.01 mM	3
Sodium diethyl-dithio-carbamate, 0.01 M	5
8-hydroxyquinoline	
2 mM	35
0.2 mM	2
NaF, 0.1 M	0
Dextran, 0.5 mg/ml	0

[a] To provide this level of PO_4^{3-}, the concentration of pH 8 buffer-salt was increased 10-fold.

Ca^{2+} (0.2 mM) to the EDTA-treated T14V just before mixing with *S. sanguis* 34 restored 80 to 90% of the ability of T14V to coaggregate. At 0.2 mM, Mg^{2+} did not restore coaggregation ability; at 2 mM, Mg^{2+} restored only 25%. There was no inhibition when *S. sanguis* 34 alone was treated with sodium EDTA, washed, and mixed with untreated T14V; therefore, the Ca^{2+}-requiring system is present on T14V alone.

The lack of inhibition by sodium diethyldithiocarbamate at 0.01 M is strong evidence against an important role for Cd, Co, Fe, Pb or Zn; the weak inhibition by 8-hydroxyquinoline may possibly suggest an essential role for Mn (27).

NaF was included because of the interest in F^- as an anti-dental caries agent. Apparently, the affinity of F^- for Ca^{2+} is not sufficient for 0.1 M F^- to inhibit the coaggregation.

Dextran was tested for inhibition because of its reported essential role in the coaggregation between other strains of *A. viscosus* and *S. sanguis* and its ability to cause aggregation of *A. viscosus* T6 (2, 26). At levels from 5 to 500 μg per ml, dextran 2000 neither inhibited coaggregation between T14V and *S. sanguis* 34 nor caused any aggregation of T14V alone.

The lack of inhibition by 1 M NaCl is an indication that electrostatic interaction between *S. sanguis* 34 and T14V is not a dominant factor and perhaps is unimportant in the coaggregation.

Effects of various treatments applied to T14V or *S. sanguis* separately. The data of Table 2 were obtained by treating either T14V or *S. sanguis* 34 alone as indicated and testing the treated species for coaggregation with the

TABLE 2. *A. viscosus T14V–S. sanguis 34 coaggregation inhibition by various treatments applied to either T14V or S. sanguis 34 alone before mixing with other (untreated) species*

Treatment[a]	% Inhibition by treating[b]: T14V	S. sanguis 34
95°C (6 min, pH 7)	100	0
HCl (0.01 N, 10 min, RT)	100	
HCl (0.01 N, 22 h, RT)		5
pH 4 (1 h, RT)	64	0
Amino group acetylation	100	0
IO_4^- (0.01 M, pH 4, 1 h, RT)	100	100
LiCl (8 M, pH 7, 1 h, RT)	100	0
KCl (4 M, pH 7, 1 h, RT)	79	NT
NaCl (5 M, pH 7, 1 h, RT)	0	NT
Bacterial protease (7 mg/ml)	98	10
Bacterial protease (0.7 mg/ml)	0	NT
Trypsin	14	5
Dextranase	0	0

[a] RT, Room temperature.
[b] NT, Not tested.

other species untreated. On the whole, these data indicate that the molecules essential for coaggregation (i) on *S. sanguis* 34 are primarily carbohydrate, (ii) on T14V are primarily protein or glycoprotein, and (iii) do not involve dextran in either case.

That the active molecules on *S. sanguis* 34 are primarily carbohydrate is indicated by their resistance to heat, their low pH, amino group acetylation, and proteolysis, and their rapid destruction by 0.01 M periodate at pH 4. These molecules are also resistant to hot phenol-water extraction (not shown in Table 2), a procedure to which carbohydrates are relatively resistant and proteins are very labile, and which has been used for the separation of proteins from polysaccharides (46). (*S. sanguis* 34 extracted with hot phenol-water gave 72% coaggregation with untreated T14V.) Moreover, the formamide procedure for preparing streptococcal cell wall polysaccharides (12) yielded a fraction from *S. sanguis* 34 which aggregated T14V slowly.

That the active molecules on T14V may be protein or glycoprotein is suggested by their complete inactivation by bacterial protease, aminoacetylation, heat, mild acid, 8 M LiCl, and 0.01 M periodate. Although the inactivation by periodate is complicated by the fact that pH 4 alone inactivated T14V very substantially, the results suggest that carbohydrate could be an essential part of molecules on T14V that are necessary for coaggregation. LiCl (8 M) inactivates levansucrase of T14V (M. Pabst, personal communication) and has been used to solubilize some proteins. Our experiments do not indicate whether the molecules essential for coaggregation were inactivated or were only removed from T14V by the LiCl.

The concentrations of enzymes used in these experiments were extremely high. Dextranase at 0.12 mg/ml failed to inactivate 1 mg of either bacterium per ml, but under the very same conditions 0.012 mg of dextranase per ml converted 8 mg of dextran 2000 per ml completely to isomaltose. At 1 mg/ml, trypsin only slightly inactivated 1 mg of T14V per ml, whereas 0.01 mg of trypsin per ml converted 2 mg of casein to trichloroacetic acid-soluble products. Similarly, bacterial protease at 0.7 mg/ml failed to inactivate 1 mg of either bacterium per ml per h, and yet 0.07 mg of bacterial protease per ml digested 4 mg of casein/ml per h to trichloroacetic acid-soluble products.

Inhibition of coaggregation by galactose and derivatives. The suggestion that the coaggregation of T14V with *S. sanguis* 34 depends upon a carbohydrate-protein interaction led to the testing of a number of monosaccharides, monosaccharide derivatives, and disaccharides for their ability to inhibit coaggregation. The compounds which inhibited significantly at 0.05 M or lower concentrations are shown in Table 3, with percent inhibition given for each concentration tested. All other sugars and derivatives tested gave less than 10% inhibition at 0.1 M and are listed in a footnote to Table 3. It is clear that the only significantly inhibitory compounds were β-galactosides, galactose, and *N*-acetyl-galactosamine. The most active compound, lactose, which is 4-O(β-D-galactopyranosyl)-D-glucopyranose, had roughly twice the activity of β-methyl-D-galactoside and fivefold the activity of D-galactose. Melibiose, which is an α-D-galactoside, and α-methyl-D-galactoside were essentially noninhibitory. The inhibition by galactose and *N*-acetyl-galactosamine may be attributed to the β forms in mutarotation.

Reversibility of the coaggregation. It should be noted that the coaggregation between T14V and *S. sanguis* 34 is reversible. The coaggregated cells have been completely disaggregated upon the addition of lactose, calcium chelating agents, or the amino group acetylating agent, *N*-acetyl-succinimide.

Electron microscopic observations. Transmission electron microscopy (Fig. 1) showed fibrils, approximately 4 nm in diameter, radiating outward from the periphery of T14V cells. These fibrils were not found on T14V cells which had been treated with 8 M LiCl, and which would not coaggregate with *S. sanguis* 34. Cells which had been boiled and did not coaggregate with *S. sanguis* 34 retained fibrils, although they appeared less abundant than on the untreated cells.

The labeling of T14V, alone and coaggregated with *S. sanguis* 34, by the indirect peroxidase-

TABLE 3. *A. viscosus 14V–S. sanguis 34 coaggregation inhibition by sugars and sugar derivatives*

Concn of inhibitor (M)	% Inhibition of coaggregation[a]				
	Lactose	Potassium lactobionate	β-Methyl-D-galactoside	D-Galactose	N-acetyl-D-galactosamine
0.05	97	91 (±4)	96	81 (±4)	70 (±9)
0.03	97	73 (±10)	94	52 (±8)	37 (±8)
0.02	98	45 (±12)	88 (±2)	29 (±3)	20 (±4)
0.01	88 (±5)	14 (±4)	39 (±36)	10	
0.005	60 (±4)				
0.0025	19 (±2)				

[a] These data are the means of two experiments, with duplicate samples in each; standard deviations (in parentheses) of 1 or less have been omitted. Certain sugars and derivatives were tested but did not inhibit coaggregation significantly; at 0.1 M, the mean percent inhibition was 9 for melibiose, 7 for sucrose, and less than 3 for D-glucose, D-mannose, xylitol, D-xylose, L-rhamnose, α-methyl-D-galactoside, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, maltose, and cellobiose.

labeled antibody method of Cisar et al. (7), is shown in Fig. 2. When normal rabbit serum was used in place of anti-T14V fibril serum, there was no labeling. It is clear that *S. sanguis* 34 alone is devoid of labeled fibrils, that the surface and fibrils of T14V are abundantly labeled, and that in the coaggregate the two bacteria are easily distinguished, with fibrils from T14V extending to and making contact with *S. sanguis* 34 cells. Without the immunochemical labeling, the distances between cells were like those shown in Fig. 2, but fibrils were difficult to see, and it was often impossible to distinguish between T14V and *S. sanguis* 34.

DISCUSSION

T14V and *S. sanguis* 34 cells, when cultured under the conditions reported here, will coaggregate rapidly and very reproducibly. In the buffer system we used, this phenomenon is pH dependent, Ca^{2+} dependent, dextran independent, levan independent (M. Pabst, personal communication), inhibited markedly by certain β-D-galactosides but not by α-D-galactosides or a number of other sugars, and probably not dependent upon electrostatic interaction between the two bacteria. We have presented strong evidence that the coaggregation results from a specific interaction between protein or glycoprotein molecules on T14V and carbohydrate molecules on *S. sanguis* 34.

Comparison of our study with other reports on coaggregation between pairs of oral bacteria reveals certain differences and similarities, as may be expected from experiments with different strains of bacteria cultured under different conditions. Contrary to our results, dextran caused the aggregation of several strains of *A. viscosus*, including T6 (26), and was essential in the coaggregation of *A. viscosus* 15987 with either *S. mutans* or *S. sanguis* (2); moreover, in these studies the aggregations and coaggregations were independent of pH and of Ca^{2+}. In another report (11) *S. sanguis* 34 coaggregated with three strains of *A. naeslundii* but not with *A. viscosus* T14. The failure of T14 to coaggregate with *S. sanguis* 34 possibly could be attributed to the growth of this organism in a medium which contained whole Trypticase and 1% glucose, because we found dependable coaggregation only when T14V was grown without added glucose and in the absence of the nondialyzable part of Trypticase and yeast extract. On the other hand, *A. viscosus* T14 and T14V may behave differently toward *S. sanguis* 34, even though they came from the same parent culture, since T14V was maintained in animals, whereas T14 was taken from a laboratory collection (R. P. Ellen, personal communication). The studies of Ellen and Balcerzak-Raczkowski (11) and our own concur in the evidence that the coaggregation-essential molecules on the actinomyces are protein and those on the streptococci are carbohydrate.

The dependence of coaggregation upon carbohydrate-protein interactions places these systems with the lectins in a general mechanism category of very broad biological importance, which includes the following phenomena: aggregation of erythrocytes, leukocytes, and tumor cells and mitogenic effects upon leukocytes (25, 43), sexual mating in yeast cells (9), the self-aggregation stage of slime molds (36), specific intraspecies aggregation in sponges (20), binding of various bacteria to animal tissue cells (13, 31, 37, 38), and the rhizobium-legume root nodule symbiosis (1). Perhaps this type of mechanism is essential in many examples of adherence of oral bacteria to each other and to the various tissues of the mouth. In this regard, it has been reported that strains of *S. mutans*, *S. sanguis*, *S. mitior*, and *A. viscosus* agglutinated red blood cells, and the suggestion was offered that oral bacteria may adhere to pellicle on teeth by re-

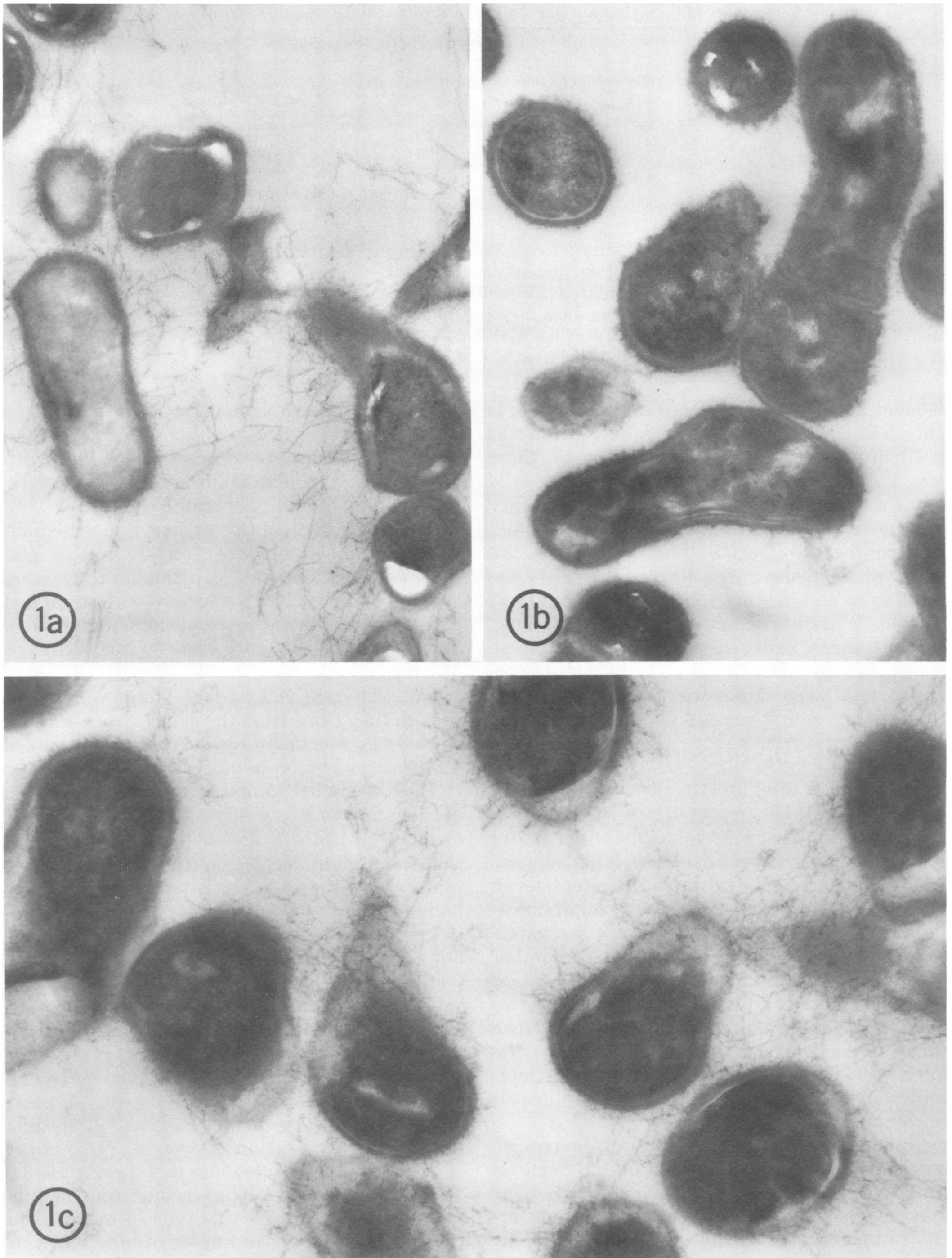

FIG. 1. *Thin-section electron micrographs of A. viscosus T14V fixed with formalin-tannic acid. (a) Controls untreated before fixation (note fibrils), ×26,000; (b) extracted with 8 M LiCl (fibrils absent) ×26,000; (c) boiled at pH 7 for 30 min (fibrils possibly altered) ×37,500.*

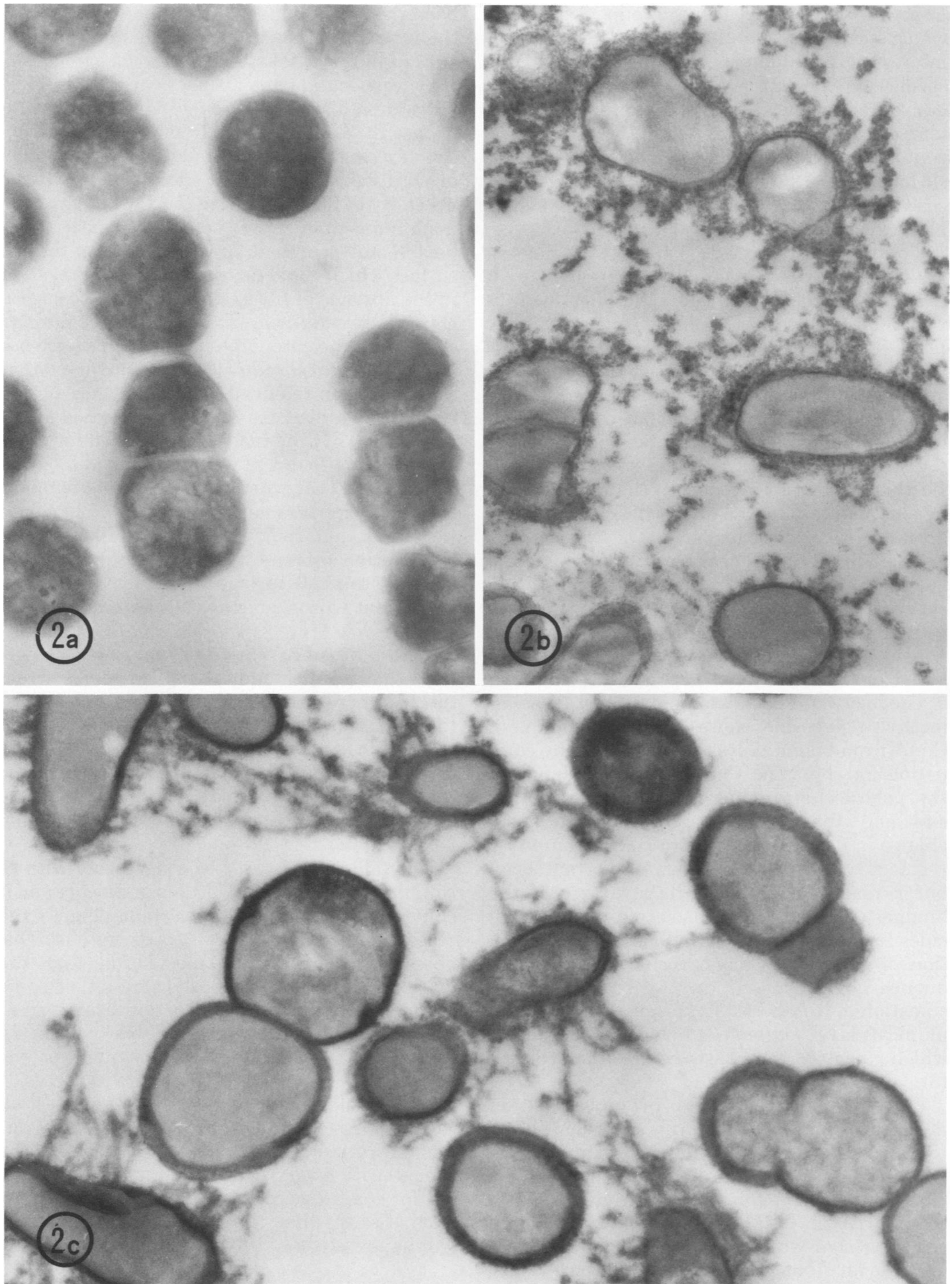

FIG. 2. *Thin-section electron micrographs of bacteria treated with rabbit antiserum to fibrils (V-antigen-specific antiserum), followed by peroxidase-labeled goat anti-rabbit IgG. (a) S. sanguis 34 alone, ×25,000. (b) A. viscosus T14V alone, ×25,000. (c) Coaggregate of S. sanguis 34 and T14V ×30,000.*

acting with specific blood group substances which have been found in the pellicle (33). An interesting possibility is that a single bacterial species may be competent to participate in several different specific carbohydrate-protein interactions, i.e., might carry carbohydrates which could interact with proteins on certain bacterial surfaces, and proteins which could interact with carbohydrates on certain other surfaces, such as blood group substances in pellicle.

The requirement of Ca^{2+} by T14V for coaggregation with *S. sanguis* 34 is consistent with the essential role of Ca^{2+} and other metal ions in the binding of carbohydrates by Concanavalin A (ConA) and other lectins (14, 25). Physical-chemical studies have indicated that one Ca^{2+} and one Mn^{2+} per ConA monomer are necessary to maintain the proper molecular conformation for the binding of carbohydrates and hence for the various biological properties of ConA (32). Further studies are needed on the role of metal ions in maintaining the ability of T14V to coaggregate with *S. sanguis* 34.

The inhibition of coaggregation by lactose, lactobionic acid, and β-methyl-D-galactoside suggests that D-galactose in β-linkage is an important element of the site on *S. sanguis* 34 which binds to T14V. However, recent studies with ConA (32) justify caution in accepting such a conclusion. Those studies suggest that α-methyl-D-glucoside causes the dissociation of ConA from bound polysaccharides, not by competing for the same ConA site which binds to the polysaccharide, but by occupying another site and thereby causing a conformational change in ConA. Inhibition of coaggregation by β-galactosides may be a very important lead, but a real knowledge of the structure of the binding site may require isolation of the molecules from *S. sanguis* 34 and careful identification of component monosaccharides, their sequence, and linkage. In this regard, by the classification of Rosan (34, 35; Rosan, personal communication) *S. sanguis* 34 belongs to a group in which galactose is a constituent of the cell wall (8).

Fibril-like structures have been observed on *A. viscosus* and *A. naeslundii*, and their potential importance in the adherence of these bacteria in dental plaque has been suggested (11, 17, 33). Our electron microscopic studies suggest that fibrils on T14V may be important in the coaggregation with *S. sanguis* 34, but the evidence is far from conclusive. In the coaggregate (Fig. 2), labeling of the T14V fibrils by the indirect peroxidase-labeled antibody technique allowed a clear distinction between T14V and *S. sanguis* 34, and indicated that the two types of bacteria may be connected almost exclusively by the labeled fibrils. On the other hand, the presence of fibrils is not sufficient for coaggregation; they were found on heat-inactivated T14V (Fig. 1c) and on T14AV (7), which does not coaggregate with *S. sanguis* 34. Removal of fibrils by LiCl extraction (Fig. 1b) made T14V incapable of coaggregation, but this does not prove the necessity of fibrils, because the LiCl might have done more than simply remove them; it might have removed or denatured molecules at the cell surface which could have effected coaggregation in the absence of fibrils. In this regard, coaggregation between *A. naeslundii* 398 and *S. sanguis* strain S has been reported where fibrils were not found on *A. naeslundii* (11); it was assumed that they had been removed by the sonic oscillation employed to disperse the cells. There is a real possibility that the molecules required for coaggregation are found on the fibrils and also on the cell surface, that when the fibrils are abundant they provide the main contacts for coaggregation, and that removal of the fibrils without inactivation or removal of the essential molecules at the cell surface would leave the cells competent to coaggregate, but perhaps les efficiently.

With a broad definition of fimbriae (31), perhaps that term should be applied to the structures we have called fibrils; this seems well justified on a basis of morphology, function, and overall chemical composition (Cisar, personal communication). There is considerable evidence that fimbriae and pili may be important in the attachment of pathogens to host tissue cells (5, 30, 31, 37, 38).

The failure of T14AV to coaggregate with *S. sanguis* 34 might be of little significance, or it could be an extremely important clue to the reason for the avirulence of this mutant. Our observation encourages speculation that the avirulence may be primarily a result of some failure of adherence. Careful quantitative studies would be required to evaluate this hypothesis.

To assess the potential importance of the coaggregation between T14V and *S. sanguis* 34 in oral ecology, a knowledge of the effect of saliva is essential. Preliminary results in our laboratory indicate that saliva may inhibit the coaggregation very slightly and that the optimal pH for coaggregation may be lower in the presence of saliva than in its absence. These points will be investigated further.

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LITERATURE CITED

1. **Bohlool, B. B., and E. L. Schmidt.** 1974. Lectins: a possible basis for specificity in the rhizobium-legume root nodule symbiosis. Science **185**:269-271.
2. **Bourgeau, G., and B. C. McBride.** 1976. Dextran-mediated interbacterial aggregation between dextran-synthesizing streptococci and *Actinomyces viscosus*. Infect. Immun. **13**:1228-1234.
3. **Boyd, H., S. J. Leach, and B. Milligan.** 1972. N-acylsuccinimides as acylating agents for proteins: the selective acylation of lysine residues. Int. J. Peptide Protein Res. **4**:117-122.
4. **Bryant, R. E., and D. E. Jenkins.** 1968. Calcium requirements for complement dependent hemolytic reactions. J. Immunol. **101**:664-668.
5. **Buchanan, T. M., and W. A. Pearce.** 1976. Pili as a mediator of the attachment of gonococci to human erythrocytes. Infect. Immun. **13**:1483-1489.
6. **Carlsson, J., H. Grahnen, and G. Jonsson.** 1975. Lactobacilli and streptococci in the mouth of children. Caries Res. **9**:333-339.
7. **Cisar, J. O., A. E. Vatter, and F. C. McIntire.** 1978. Identification of the virulence-associated antigen on the surface fibrils of *Actinomyces viscosus* T14. Infect. Immun. **19**:312-319.
8. **Cole, R. M., G. B. Calandra, E. Huff, and K. M. Nugent.** 1976. Attributes of potential utility in differentiating among "group H" streptococci or *Streptococcus sanguis*. J. Dent. Res. **55**:A142-A153.
9. **Crandall, M. A., and T. D. Brock.** 1968. Molecular basis of mating in the yeast *Hansenula wingei*. Bacteriol. Rev. **32**:139-163.
10. **Des Prez, R. M., C. S. Bryan, J. Hawiger, and D. G. Colley.** 1975. Function of the classical and alternate pathways of human complement in serum treated with ethylene glycol tetraacetic acid and $MgCl_2$-ethylene glycol tetraacetic acid. Infect. Immun. **11**:1235-1243.
11. **Ellen, R. P., and I. B. Balcerzak-Raczkowski.** 1977. Interbacterial aggregation of Actinomyces naeslundii and dental plaque streptococci. J. Periodontal Res. **12**:11-20.
12. **Fuller, A. T.** 1938. The formamide method for the extraction of polysaccharides from hemolytic streptococci. Brit. J. Exp. Pathol. **19**:130-139.
13. **Fuller, R.** 1975. Nature of the determinant responsible for the adhesion of lactobacilli to chicken crop epithelial cells. J. Gen. Microbiol. **87**:245-250.
14. **Galbraith, W., and I. J. Goldstein.** 1970. Phytohemagglutinins: a new class of metalloproteins. Isolation, purification, and some properties of the lectin from *Phaseolus lunatus*. FEBS Lett. **9**:197-201.
15. **Gibbons, R. J., and M. Nygaard.** 1970. Interbacterial aggregation of plaque bacteria. Arch. Oral Biol. **15**:1397-1400.
16. **Gibbons, R. J., and J. van Houte.** 1973. On the formation of dental plaques. J. Periodontol. **44**:347-360.
17. **Girard, A. E., and B. H. Jacius.** 1974. Ultrastructure of *Actinomyces viscosus* and *Actinomyces naeslundii*. Arch. Oral Biol. **19**:71-79.
18. **Graham, R. C., and M. J. Karnovsky.** 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. **14**:291-302.
19. **Hammond, B. F., C. F. Steel, and K. S. Peindl.** 1976. Antigens and surface components associated with virulence of *Actinomyces viscosus*. J. Dent. Res. **55**:A19-A25.
20. **Henkart, P., S. Humphreys, and T. Humphreys.** 1973. Characterization of sponge aggregation factor. A unique proteoglycan complex. Biochemistry. **12**:3045-3050.
21. **Jordan, H. V., R. J. Fitzgerald, and H. R. Stanley.** 1965. Plaque formation and periodontal pathology in gnotobiotic rats infected with an oral actinomycete. Am. J. Pathol. **47**:1157-1167.
22. **Jordan, H. V., and B. F. Hammond.** 1972. Filamentous bacteria isolated from human root surface caries. Arch. Oral Biol. **17**:1333-1342.
23. **Jordan, H. V., P. H. Keyes, and S. Bellack.** 1972. Periodontal lesions in hamsters and gnotobiotic rats infected with *Actinomyces* of human origin. *J. Periodontal Res.* **7**:21-28.
24. **Kunitz, M.** 1947. Crystalline soybean trypsin inhibitor. II. General properties. J. Gen. Physiol. **30**:291-310.
25. **Lis, H., and N. Sharon.** 1973. The biochemistry of plant lectins. Annu. Rev. Biochem. **42**:541-574.
26. **McBride, B. C., and G. Bourgeau.** 1975. Dextran-induced aggregation of *Actinomyces viscosus*. Arch. Oral Biol. **20**:837-841.
27. **Martell, A. E., and M. Calvin.** 1952. Chemistry of the metal chelate compounds. Prentice-Hall, Inc., New York.
28. **Miller, G. L., R. Blum, W. E. Glennon, and A. L. Burton.** 1960. Measurement of carboxymethyl cellulase activity. Anal. Biochem. **1**:127-132.
29. **Mizuhira, V., and Y. Futaesaku.** 1971. On the new approach of tannic acid and digitonine to the biological fixatives. Proc. Electron Microsc. Soc. Am. **29**:494.
30. **Nagy, B., H. W. Moon, and R. E. Isaacson.** 1977. Colonization of porcine intestine by enterotoxigenic *Escherichia coli*: selection of piliated forms in vivo, adhesion of piliated forms to epithelial cells in vitro, and incidence of pilus antigen among porcine enteropathogenic *E. coli*. Infect. Immun. **16**:344-352.
31. **Ottow, J. C. G.** 1975. Ecology, physiology, and genetics of fimbriae and pili. Annu. Rev. Microbiol. **29**:79-108.
32. **Richardson, C. E., and W. D. Behnke.** 1976. Physical-chemical studies on the role of metal ions in concanavalin A. J. Mol. Biol. **102**:441-451.
33. **Rölla, G., and M. Kilian.** 1977. Hemagglutination activity of plaque-forming bacteria. Caries Res. **11**:85-89.
34. **Rosan, B.** 1973. Antigens of *Streptococcus sanguis*. Infect. Immun. **7**:205-211.
35. **Rosan, B.** 1976. Relationship of the cell wall composition of Group H Streptococci and *Streptococcus sanguis* to their serological properties. Infect. Immun. **13**:1144-1153.
36. **Rosen, S. D., R. W. Reitherman, and S. H. Barondes.** 1975. Distinct lectin activities from six species of cellular slime molds. Exp. Cell Res. **95**:159-166.
37. **Salit, I. E., and E. C. Gotschlich.** 1977. Hemagglutination by purified type I *Escherichia coli* pili. J. Exp. Med. **146**:1169-1181.
38. **Salit, I. E., and E. C. Gotschlich.** 1977. Type I *Escherichia coli* pili: characterization of binding to monkey kidney cells. J. Exp. Med. **146**:1182-1194.
39. **Semionescu, N., and M. Semionescu.** 1976. Galloylglucoses of low molecular weight as a mordant in electron microscopy. I. Procedure and evidence for mordanting effect. J. Cell Biol. **70**:608-621.
40. **Semionescu, N., and M. Semionescu.** 1976. Galloylglucoses of low molecular weight as mordant in electron microscopy. II. The moiety and functional groups possibly involved in the mordanting effect. J. Cell Biol. **70**:622-633.
41. **Socransky, S. S., C. Hubersak, and D. Propas.** 1970. Induction of periodontal destruction in gnotobiotic rats by human oral strain of *Actinomyces naeslundii*. Arch. Oral Biol. **15**:993-995.
42. **Socransky, S. S., A. D. Manganiello, D. Propas, V. Oram, and J. van Houte.** 1977. Bacteriological studies of developing supragingival dental plaque. J. Periodon-

tal Res. **12**:90–106.

43. **Springer, G. F., P. R. Desai, and J. C. Adye.** 1974. Lectin and agglutinin receptors of red cell components. Ann. N.Y. Acad. Sci. **234**:312–331.
44. **Theilade, E., O. Fejerskov, W. Prachyabraed, and M. Kilian.** 1974. Microbiologic study on developing plaque in human fissures. Scand. J. Dent. Res. **82**:420–427.
45. **Tinanoff, N., A. Gross, and J. M. Brady.** 1976. Development of plaque on enamel. Parallel investigations. J. Periodontal Res. **11**:197–209.
46. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. **5**:83–91.