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Saliva-Induced Aggregation of Oral Streptococci

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Cells of several species of oral microorganisms have been shown, in earlier studies, to be aggregated by saliva. In the present study some of the basic properties of the aggregation system are examined. The observation is made that the saliva-induced aggregates of *Streptococcus sanguis* and *S. mitis* can be dissociated to stable particles which consist of about 100 cells and have a median diameter of about 4.5 μm. It is proposed that these are subunits, or core aggregates, of the large primary aggregates. Counts of the core aggregates can be taken as a precise and accurate measure of aggregation. Experiments based on this procedure show that the aggregation of *S. sanguis* is maximal at 10 C and at 1 meq of Ca^{2+} ions per liter and is not affected by a change in *p*H between 3.9 and 8.7 or by a change in the phase of growth of the microorganisms. Core aggregates diminish in number with prolonged incubation, suggesting that the aggregating factors break down with time. Formalinized cells yield stable aggregates. However, with Formalinized cell aggregation is maximal between 20 and 30 C and proceeds in the absence of calcium ions. Evidence is presented that whole saliva contains separate aggregating factors for *S. sanguis* and *S. mitis*. The factors differ in their affinity for intact cells and for hydroxyapatite and differ in their stability to dialysis. These findings suggest that many different aggregating factors exist in saliva, each of which may be capable of interacting with cells of one or several bacterial species.

Human dental plaque consists of a complex mixture of bacteria and intercellular matrix. However, certain organisms predominate, and the selection of some of these appears to be determined by the ability of the organisms to adhere to the tooth surface (6). Hillman, van Houte, and Gibbons (3) were able to show that saliva enhanced the attachment of certain plaque-forming bacteria to enamel powder, while Gibbons and Spinell (1) and Hay, Gibbons and Spinell (2) showed that these plaque-forming organisms are aggregated by saliva. However, measurement of saliva-induced aggregation, like the measurement of aggregate formation in general, is difficult (5, 7). In the present paper a method is developed which makes it possible to measure aggregation electronically. With this procedure it has been possible to study various parameters of the aggregating system and to examine some of the basic mechanisms of aggregation.

MATERIALS AND METHODS

S. sanguis strain H7PR and *S. mitis* strain 26 were grown overnight at 37 C in Trypticase soy broth (Baltimore Biological Laboratory). The cells were harvested, washed with saline (0.154 M NaCl), and resuspended to one-fifth of the original volume with saline. Formalinized cells were prepared from 1 liter of overnight cultures to provide a stable source of cells. Washed cells were suspended in one-fiftieth of the original culture volume with 1% Formalin (Mg^{2+}-free) in phosphate-buffered saline, *p*H 7.5. The mixture was kept at room temperature for 16 hr, after which it was centrifuged, and the cells were resuspended in the same volume of 0.2% Formalin in phosphate-buffered saline. The treated cells were stored at 4 C and diluted 6- to 12-fold with saline for use. Whole saliva was collected over ice, without stimulation, and clarified by two centrifugations at 25,000 $\times$ *g* for 15 min at 4 C. Saliva was deionized by treatment with ethylenediaminetetraacetate (EDTA). One-tenth volumes of 0.1 M EDTA, *p*H 8.6, were added to the saliva, and the mixture was kept at 4 C for 15 min and then was dialyzed overnight in the cold against two changes of distilled water. The calcium content of different samples of saliva was reduced from about 1 meq/liter (range 0.5 to 1.5 meq/liter) to 0.05 meq/liter or less. Calcium and magnesium were measured by a Perkin-Elmer atomic absorption spectrophotometer, model 403.

For the assay, a mixture was prepared containing 0.05 M sodium succinate plus 0.001 M $CaCl_2$, *p*H 6.0, 0.1 ml; 0.154 M NaCl, 0.05 ml; and saliva, 0.10 ml, and mixed well. Cells (0.05 ml containing about 5×10^5 particles/μm^3) were added to this mixure by rapid injection with an automatic pipetting device (Schwarz/Mann, Orangeburg, N.Y., or Baltimore Biological Laboratory, Baltimore, Md.), bringing the volume to 0.30 ml. The whole sample was mixed well and incubated for 15 min at room temperature. Control samples lacking either saliva or cells also were prepared.

Aggregates that formed after cells were incubated with saliva were large enough to be seen by the eye. Under a dissecting microscope the individual clusters were seen clearly and were readily distinguishable from the background turbidity due to the free cells remaining in the system. Scores of from 0 to 4 were assigned to designate samples ranging from no aggregation to complete aggregation (i.e., large clumps) with complete clearing of the supernatant fluid. However, it was observed that the large aggregates were fragile and broke apart even with gentle agitation. Attempts were made, therefore, to establish a condition whereby stable aggregates would be obtained and by which reproducible measurements of aggregation could be carried out. A Coulter Counter, model B (Coulter Electronics, Inc., Hialeah, Fla.) was used to measure the number and volume of bacteria (4) although, as commonly employed, it measures only single cells or small clusters. In the present system, the counter was able to distinguish the aggregates from the single cells. A 0.05-ml portion of the incubated sample was added to 50 ml of saline, and counts were taken of the particles at various settings of the counter. The thresholds, aperture current, and amplification were set as indicated in the legends to the figures. A 100-μm diameter orifice was used, and conditions were such that 0.50 ml of the diluted sample was drawn through the orifice in approximately 15 sec. All counts were corrected for background measured in saline. Counts were made in the linear response range of the instrument, and standard coincidence corrections were applied.

In Fig. 1, curve A, are shown the size and volume distributions of saliva-aggregated *S. sanguis*. Particles ranging in size from 3 to 9 μm in diameter were detected. However, it was apparent both from visual observation of the samples (where aggregates measuring several hundred micrometers across were seen) and from the shearing nature of the sample flow in the Coulter Counter system (i.e., through the 100-μm diameter orifice), that breakdown of the aggregates had occurred. It was decided, therefore, to deliberately dissociate the aggregates and to see whether it was possible to obtain stable, reproducible particle counts. The samples were rapidly agitated on a standard laboratory stirrer (Vortex Jr. mixer, model K-500-J, Scientific Industries, Inc. Springfield, Mass.), and the results shown in Fig. 1, curve B, were obtained. Aggregates with a relatively narrow range and symmetrical distribution of sizes were obtained, the median diameter being about 4.5 μm. No particles were detected in the range of 7 to 100 μm in diameter. Free, unaggregated cells (curve C) exhibited a narrow range of particle sizes with the median value being about 1.3 μm in diameter. This was consistent with the doublets and triplets that were found by micro-

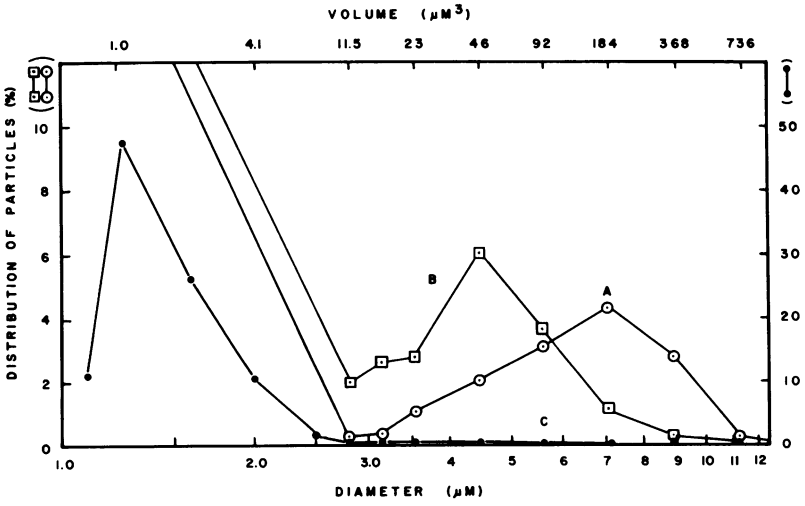

FIG. 1. *Distribution of particle sizes. Cells of S. sanguis alone (C) or cells plus saliva (A, B) were mixed and incubated as described in the text. The samples were either shaken gently (A) or agitated for 20 sec on a Vortex Jr. mixer (B, C), and 0.050 ml was removed and added to 50 ml of 0.154 M NaCl. Measurements were made of total particles at each of the following settings on a Coulter Counter: Lower threshold, 10.0; upper threshold, open; "1/aperture current," from 8 to ¼; "1/amplification," from 4 to ⅛. Coincidence factor was 2.50. A 0.50-ml portion of the diluted sample was drawn through the 100-μm diameter orifice in approximately 15 sec. Duplicate readings were taken at each setting, and background readings for saline alone were subtracted from each measurement. No particles larger than 9 μm in diameter were detected.*

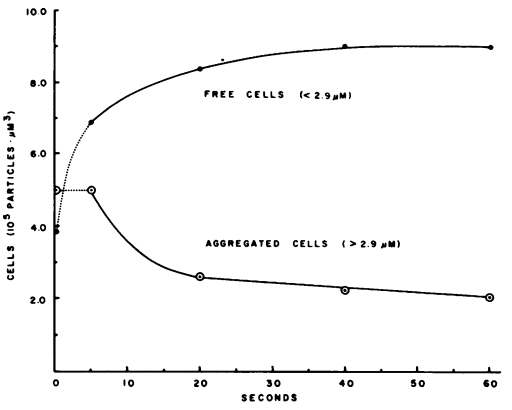

FIG. 2. *Mechanical disruption of aggregates. Aggregated samples of S. sanguis were agitated on a Vortex Jr. mixer for the indicated times. Measurements of particles were made with a Coulter Counter at 8, ½, and ¼ for the "1/aperture current" settings and at 2, 1, and ⅛ for the "1/amplification" settings. The ½ to 1 pair corresponds to 2.9 μm in diameter. The oridinate is the product of the number of particles detected between any two settings and their mean volume.*

scopic observation to predominate in the free cell preparations. Similar results were obtained with *S. mitis*. Because the maximum diameter of the free cells of both strains was about 2.9 μm, we adopted the view that all particles larger than 2.9 μm in diameter (determined by the "½" aperture current and "1" amplification settings on the Coulter Counter) were in the aggregated form. The course of release of the 4.5-μm diameter particles (or core aggregates) with increasing periods of agitation time is depicted in Fig. 2. A minimum in the number of core aggregates and a maximum in the number of free cells were reached after about 15 sec of agitation. Subsequently, all incubated samples were agitated for 15 sec and, with accurate control of agitation time, it was possible to obtain reproducible data from replicate samples and from different experiments. In a study of the reproducibility of the system four replicate samples gave a mean of 502×10^6 aggregated cells with a standard deviation of 26×10^6. However, occasional widely aberrant values were recorded so that routinely we prepared samples in triplicate. The presence of countable aggregates always was associated with visible evidence of aggregation. The higher the visible score of aggregation, the higher were the counts of the particles in the system. With no cells added, the counts were zero.

RESULTS

Cell and saliva concentrations. The aggregation of *S. sanguis* was dependent on the concentration of cells added to the system. Fig. 3A shows that the number of particles increased as the number of cells added to the system was increased. At the higher cell concentration, aggregation, as evaluated by visual means, appeared to be complete. However, the response to increasing cell concentration was limited by the aggregating activity of the saliva. A second experiment demonstrated the dependence of aggregate formation on saliva concentration (Fig. 3B). The number of counted particles increased to a maximum as the volume of deionized saliva added was increased. However, the linear portion of the curve could not be extrapolated to pass through the origin. A similar result was obtained with a partially purified preparation from saliva (*unpublished data*). Other experiments showed that the aggregating factor was removed from saliva and presumably was adsorbed to the cells during the aggregation period. Thus, the supernatant fluid from an aggregated cell system exhibited poor activity when incubated with fresh cells.

Phase of cell growth. Aggregating activity appeared to be independent of the phase of growth of the cells. Thus, the number of cells that was incorporated into aggregated form per unit number of cells added to the incubation system remained essentially the same whether the cells were harvested during logarithmic growth or in early stationary phase. It appears from this that the number of sites capable of reacting with salivary protein remained constant per cell throughout the growth curve. However, in late stationary phase the aggregating activity per unit cell increased. This was associated with an increase in the optical absorbance per cell and may have been related to an increase in cell wall thickness (8).

Effect of temperature. Aggregating activity was highest at about 10 C (Fig. 4). At lower temperatures the activity diminished, but aggregation was evident even at 0 C. Above 10 C the activity dropped progressively until, at 40 C, the activity was reduced to about 15% of the maximum. Additional experiments demonstrated that the progressive loss of activity was not the result of the inactivation of the salivary factor. Thus, preincubation of samples of saliva with buffer, but without cells, at the different temperatures did not bring about a differential loss of activity as measured by the subsequent addition of cells and incubation at room temperature.

Time course. Figure 5 shows that the number of core particles varied during extended incubation periods. Particle numbers reached a maximum at 10 to 20 min and then diminished. Interestingly, the rate of diminution was greater at 10 than at 37 C. The actual counts of the number of aggregated cells were considerably higher for the 10 C samples at 10 to 20 min

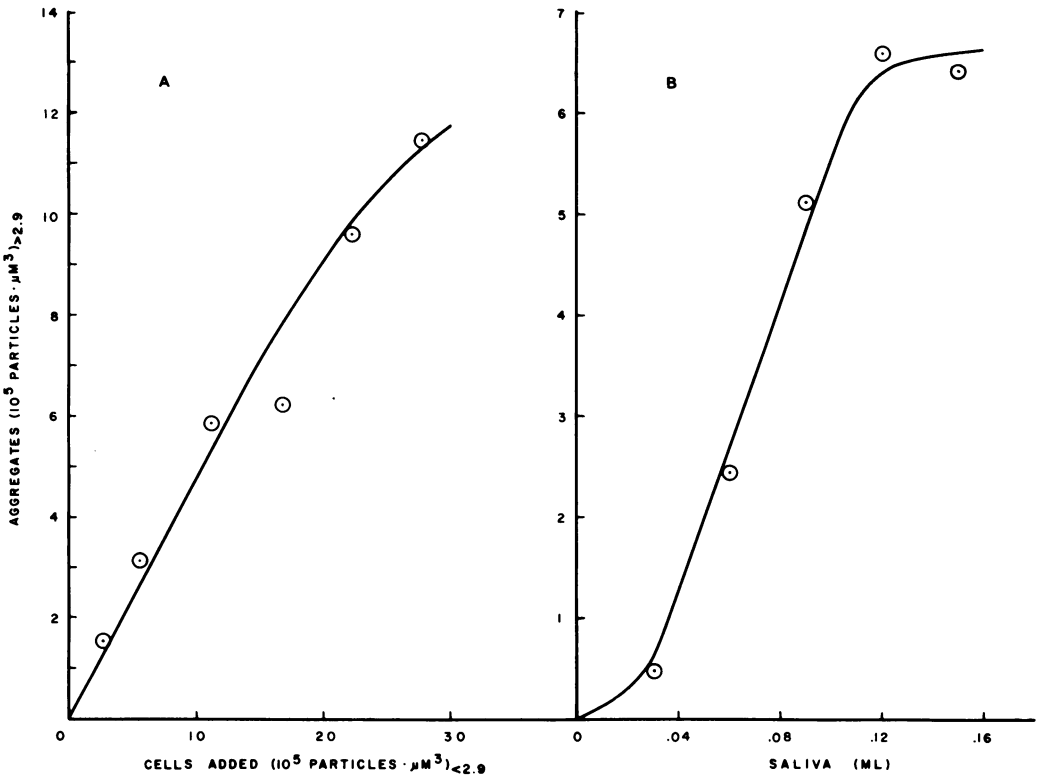

FIG. 3. *Cell and saliva concentrations. A, Cells of S. sanguis were added to the incubation mixture as indicated. B, Dialyzed saliva was added as indicated. The final concentration of added $CaCl_2$ in the assay system was 1 mM. The value for the aggregates formed with 0.10 ml of saliva in the absence of added $CaCl_2$ was 0.88 × 10^6. Samples were incubated for 15 min at room temperature.*

(cf. Fig. 4), but the counts for the two samples were essentially the same after 30 min. The course of visible aggregation was considerably different from the foregoing (Fig. 5). Very little aggregation was evident at 10 min, and there appeared to be a linear increase in the degree of visible clumping to at least 45 min.

Effect of pH. Changes in hydrogen ion concentration in the system affected aggregation very little. Activity was essentially constant between *p*H 3.9 and 8.7, although a small drop (about 20%) was observed at *p*H 4.9. Formate, acetate, succinate, 2-*N*-(morpholino) ethanesulfonate, or tris(hydroxymethyl)aminomethane buffers were used at different *p*H values. Because no discontinuities were observed in the *p*H response curve, it was concluded that the nature of the buffer anion was not a determinant for aggregation.

Calcium ions. The effect of calcium ions on aggregation was suggested first by the inhibitory effect of EDTA. In the presence of 6.7 mM EDTA, aggregation was inhibited by about 90%, whereas addition of EDTA after aggregation led to a dissociation of the particles to an equal extent. The particles that remained may have been aggregated by salivary antibody (immunogloublin A, IgA), because other experiments showed a 10 to 15% contribution by IgA to the aggregating system.

Direct demonstration of a calcium ion requirement was made with EDTA-treated, dialyzed saliva. Aggregation increased to a maximum at about 1 mM of added calcium salt (Fig. 6). However, there was no aggregation at the low concentrations of Ca^{2+} ions, perhaps because Ca^{2+} was bound by nonspecific proteins or by EDTA remaining in the system. At the higher concentrations of Ca^{2+} ions, aggregation was inhibited, perhaps because of competition for binding sites on the salivary factor or bacterial surface. The maximum for the aggregation of *S. mitis* was about 0.8 mM $CaCl_2$. It is of interest that untreated saliva contains about 1.5 meq of Ca^{2+} ions/liter. This concentration lies close to the observed optimum for aggregation of the two streptococci.

The aggregates, once formed, were stable in

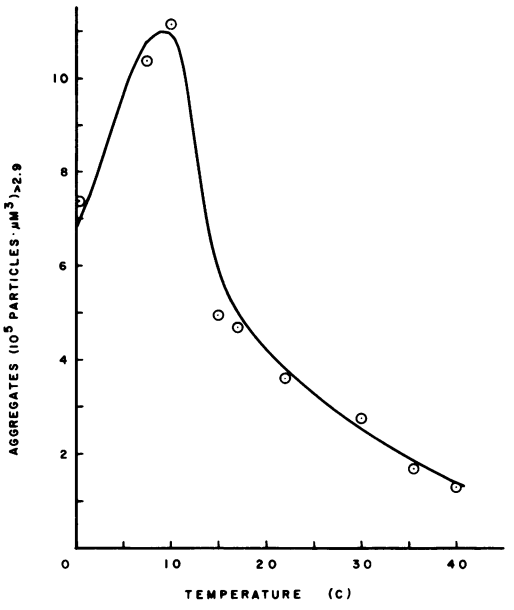

FIG. 4. *Effect of temperature. Replicate samples containing S. sanguis in the complete incubation system were incubated for 10 min at the indicated temperature, cooled in crushed ice, and sampled for counting. Similar results were obtained if the buffer-saline-saliva system was preincubated for 5 min at the appropriate temperature before the addition of cells. However, aggregation was reduced at all temperatures in the latter system.*

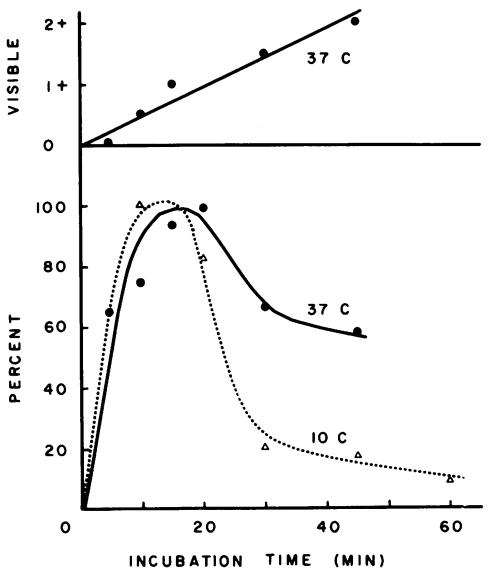

FIG. 5. *Time course of aggregate formation. Replicate samples containing S. sanguis in the complete incubation system were incubated at 10 or 37 C for the times indicated. The maximum number of aggregates at each temperature was taken as 100%, and other values were calculated from these. The degree of visible aggregation in the sample incubated at 37 C was evaluated with the aid of a dissecting microscope.*

media without Ca^{2+} ions. Thus, it was found that the counts of core particles were essentially the same whether samples of aggregated cells were diluted into saline or into succinate buffer containing 1 mM $CaCl_2$. Furthermore, the counts in saline were stable for at least 1 hr, and the subsequent decay was slow.

Other ions. Sodium chloride at 50 mM inhibited the aggregation of *S. sanguis* by about 20%. This was the concentration of the salt used routinely in the assay system. With 77 mM sodium chloride, the inhibition was about 35%.

We made the interesting observation that cations of a valency higher than calcium brought about the aggregation of *S. sanguis* in the absence of saliva. Thus, divalent iron, copper, zinc, mercury, tin, magnesium, and calcium were without effect, whereas trivalent iron and aluminum and tetravalent tin ions were potent aggregators. Ferric ions at 25 μeq/liter gave particle counts that were as high as those obtained in the complete Ca^{2+} plus saliva system. The large number of bonding options available for ferric, aluminum, and stannic ions may have

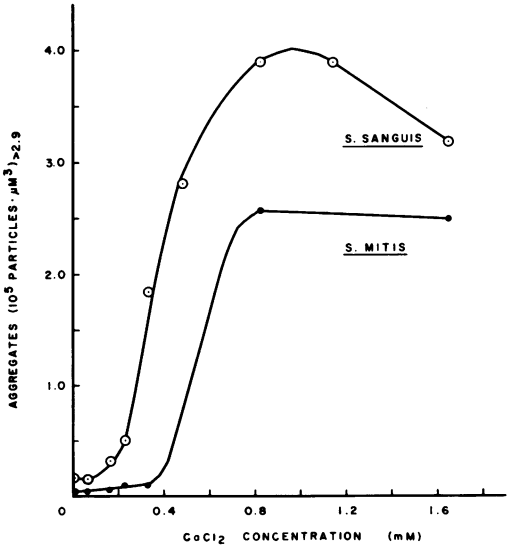

FIG. 6. *Effect of calcium ions. Dialyzed saliva was incubated in the complete incubation system containing the indicated final concentrations of $CaCl_2$. Samples contained cells of either S. sanguis or S. mitis and were incubated for 15 min at room temperature.*

accounted for the ability of these cations to effect cellular aggregation.

Formalinized cells. The responses of Formalinized cells of *S. sanguis* to changes in several of the above parameters were different from those of untreated cells. Thus, Formalinized cells exhibited a broad region of optimal aggregating activity between 20 and 30 C, with activity decreasing rapidly both below and above these temperatures. Also, aggregates of Formalinized cells were stable with time so that the particle counts increased up to about 15 min and remained unchanged until at least 45 min. Most interestingly, the aggregation of Formalinized cells of *S. sanguis* or *S. mitis* appeared to be independent of calcium ions (Table 1). An inhibition of aggregation was observed, however, at concentrations above 2 mM $CaCl_2$. Calcium was not found in the Formalinized cell preparations, but about 0.2 meq of Mg^{2+} ions/liter was detected. Mg^{2+} ions at this concentration did not affect the aggregation of untreated cells.

Differences in aggregating factors. The question was considered whether *S. sanguis* and *S. mitis* were aggregated by a common salivary factor. Accordingly, untreated cells of both strains were incubated with saliva under standard conditions, after which free and aggregated cells were removed by centrifugation. The aggregating activity of each of the resulting supernatant fractions was assayed by incubation with fresh cells. It was found (Table 2) that *S. sanguis* removed all of the factor needed for its aggregation, but only 36% of the factor needed to aggregate *S. mitis*. *S. mitis* removed the factor for its own aggregation but very little of the factor for *S. sanguis*.

The factors also exhibited different affinities for hydroxyapatite. Hay, Gibbons, and Spinell (2) and Hillman, van Houte, and Gibbons (3) demonstrated that the aggregating factor for *S. mitis* was absorbed to hydroxyapatite. Their experiment was repeated and samples of saliva were incubated with increasing weights of hydroxyapatite. Assays were carried out for aggregating activity remaining in the saliva with cells of both strains. It was found that 50 mg of hydroxyapatite added to 1 ml of saliva reduced the aggregating activity for *S. sanguis* by 100%, whereas the activity for *S. mitis* was reduced by 30%. Finally, the aggregating factors for the two strains exhibited different degrees of stability to treatment with EDTA and dialysis. It was found during the preparation of Ca^{2+}-free saliva that recoveries of aggregating activity for *S. sanguis*, even after the addition of optimal concentrations of $CaCl_2$, were low and frequently were zero. The recovery of activity for *S. mitis*, on the other hand, was complete.

TABLE 1. *Effect of calcium ions on the aggregation of fresh and Formalinized cells of streptococcus sanguis*

Ca^{2+} concn (meq/liter)	Aggregation (10^5 particles μm^3)$_{>2.9\ \mu m}$	
	Fresh cells	Formalinized cells
0	0.17	2.56
1.65	3.19	3.0

TABLE 2. *Specificity of adsorption of salivary factors*

Saliva adsorbed with:	Aggregation (10^5 particles μm^3)$_{>2.9\ \mu m}$	
	S. sanguis	*S. mitis*
No cells	2.01	5.09
S. sanguis	0 (0)[a]	3.25 (63.9)
S. mitis	1.93 (96.0)	0.55 (10.7)

[a] Values in parentheses indicate the percent of aggregating activity remaining in the salivas compared with those of untreated saliva.

DISCUSSION

The fragility of the large, or primary, aggregates is a remarkable feature of the aggregative phenomenon. When the aggregates break down, it is found that they dissociate to particles that are always less than 9 μm in diameter. This finding can best be interpreted by assuming that the primary aggregates are comprised of small "core aggregates" whose median diameter is about 4.5 μm. If the diameter of the individual cells is taken to be 1 μm (i.e., ca. 0.5 μm^3), then the core aggregates (ca. 50 μm^3) contain about 100 cells each. During the formation of aggregates saliva-coated free cells probably combine to form core particles, and core particles then combine to form large aggregates. During mechanical disaggregation the reverse transitions probably take place between primary aggregates, core aggregates, and free cells. It is significant that agitated samples, if left undisturbed, reassociate to form large aggregates. An interesting consequence of this proposal is that the bonds holding cells together within the core aggregates may be different from those that join the core aggregates within the large aggregates. It is possible that two aggregating factors exist in saliva, one which is specific for core aggregate formation and the other which participates in the adhesion between core aggregates.

Perhaps a related feature of the present observations is the demonstration of the instability of the core aggregates with time. It was found that the number of core aggregates in the cell-plus-saliva system reaches a maximum after about 15 min and decreases with additional time of incubation. Thus, core aggregate formation is at a maximum when the large, primary aggregates are barely discernible. Although primary aggregates continue to grow to visible size, the core aggregates continue to diminish in number. The finding can best be explained on the basis that intercellular bonds within the core aggregates break down with time. The internally unstable core aggregates evidently remain associated within the primary aggregates and it is only when the latter are mechanically disturbed that the core aggregates disintegrate. It seems not unreasonable to suggest that cellular enzymes bring about the breakdown of the entrapped salivary factors, especially since aggregates of Formalinized cells are stable, once formed under the same conditions.

Demonstration of the existence of separate salivary factors for the aggregation of two different oral microorganisms is of importance for general understanding of the processes whereby bacteria are deposited on teeth and tissue surfaces. Saliva was shown in the past to be involved in the adsorption of a number of oral microorganisms to teeth (6) and also to be responsible for the aggregation of many of these organisms (1, 2). The present finding suggests the possibility that many different factors exist in saliva and that each one is responsible for the interaction with one or several of the different bacterial species found in the mouth. It is interesting that cells of either *S. sanguis* or *S. mitis* adsorb small amounts of the aggregating factor for the other strain, because this suggests that the factors are not completely specific but that they cross-react with the different cells. Preliminary data show that the *S. sanguis* factor becomes attached to the peptidoglycan of the cell wall, whereas the *S. mitis* factor appears to react with some other constituent of the cell surface. The accessibility of the peptidoglycan in the intact cells of *S. mitis* may determine whether the *S. sanguis* factor will be adsorbed and participate in the aggregation of these cells. The observed cross-reactivity may be related, then, to the nature of the bacterial surface rather than to the specificities of the salivary factors.

ACKNOWLEDGMENT

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