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Concentration-Dependent Multiple Binding Sites on Saliva-Treated Hydroxyapatite for *Streptococcus sanguis*

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The influence of bacterial cell concentration on estimates of the number of binding sites and the affinity for the adsorption of a strain of *Streptococcus sanguis* to saliva-treated hydroxyapatite was determined, and the possible presence of multiple binding sites for this organism was tested. The range of concentrations of available bacteria varied from 4.7×10^6 to $5,960 \times 10^6$ cells per ml. The numbers of adsorbed bacteria increased over the entire range tested, but a suggestion of a break in an otherwise smooth adsorption isotherm was evident. Values for the number of binding sites and the affinity varied considerably depending upon the range of available bacterial concentrations used to estimate them; high correlation coefficients were obtained in all cases. The use of low bacterial cell concentrations yielded lower values for the number of sites and much higher values for the affinity constant than did the use of high bacterial cell concentrations. When data covering the entire range of bacterial concentrations were employed, values for the number of sites and the affinity were similar to those obtained by using only high bacterial cell concentrations. The simplest explanation for these results is that there are multiple binding sites for *S. sanguis* on saliva-treated hydroxyapatite surfaces. When present in low concentration, the streptococci evidently attach to more specific high-affinity sites which become saturated when higher bacterial concentrations are employed. The possibility of multiple binding sites was substantiated by comparing estimates of the adsorption parameters from a computer-simulated isotherm with those derived from the experimentally generated isotherm. A mathematical model describing bacterial adsorption to binary binding sites was further evidence for the existence of at least two classes of binding sites for *S. sanguis*. Far fewer streptococci adsorbed to experimental pellicles prepared from saliva depleted of bacterial aggregating activity when low numbers of streptococci were used, but the magnitude of this difference was considerably less when high streptococcal concentrations were employed. This suggests an association between salivary components which possess bacterial-aggregating activity and bacterial adsorption to high-affinity specific binding sites on saliva-treated hydroxyapatite surfaces.

Teeth are covered by a membranous film, the acquired pellicle, which is formed by the selective adsorption of salivary components onto the apatitic mineral of enamel (8, 12, 17). Experimental pellicles can be formed by exposing teeth or preparations of hydroxyapatite (HA) to saliva. Such experimental pellicles are comparable to natural pellicles in the salivary macromolecules they contain and in their overall amino acid and carbohydrate composition (5, 6, 14, 23, 24). Therefore, the adsorption of bacteria to saliva-treated HA (S-HA) surfaces has frequently been studied as a model of bacterial attachment to teeth (1, 3, 4, 18, 21, 26, 28, 30, 34).

It has been shown that the adsorption of several common oral bacterial species to S-HA surfaces is adequately described by a Langmuir adsorption isotherm, and the adsorption parameters (maximum number of binding sites and the affinity constants) for several prominent human plaque bacteria have been reported (1, 3, 10). However, although qualitatively similar results have been obtained by different laboratories, significant quantitative differences have been noted. For example, Appelbaum and co-workers (1), using the same adsorbent, reported higher numbers of binding sites and lower affinities for strains of *Streptococcus sanguis*, *S. mitis*, and *S. mutans* on S-HA than those initially reported by Clark et al. (3). The former investigators used cell concentrations from 10^9 to 6×10^9 cells per ml, whereas Clark et al. used much lower concentrations (10^7 to 6.5×10^7 *S. sanguis* cells per ml).

There have been other discrepancies between laboratories concerning the interactions of bacteria with salivary components. It has been suggested that components of human saliva which are able to bind to bacterial cells and cause their aggregation also promote bacterial adsorption to S-HA surfaces if they are present in experimental pellicles (3, 18–20, 28, 30). However, it was recently reported that saliva-induced aggregation of *S. sanguis* occurred by a separate and distinct mechanism from that involved in adsorption to S-HA (32). High bacterial cell concentrations (up to 4×10^9 streptococci per ml) were used in this last study; the studies mentioned earlier involved lower bacterial cell concentrations.

These apparent concentration-dependent differences could reflect multiple types of binding sites on S-HA for various oral bacteria. This prompted us to evaluate the influence of bacterial cell concentration on the parameters for the adsorption of *S. sanguis* to S-HA and to test the hypothesis that there are multiple types of binding sites for this organism.

MATERIALS AND METHODS

Cultures and cultural conditions. *S. sanguis* C5 and FC-1, *S. mitis* 26, and *S. mutans* JBP were originally isolated from human dental plaque. The strains were maintained by weekly transfer in Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, Md.) and on blood agar plates. All cultures were incubated at 35°C in Brewer Anaerobic Jars (Becton, Dickinson & Co., Rutherford, N.J.) filled with 80% N_2–10% H_2–10% CO_2.

Bacterial adsorption to S-HA. For adherence assays, the organisms were grown in Todd-Hewitt broth containing 10 μCi of [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.) per ml as previously described (3). Organisms from overnight cultures were harvested by centrifugation, washed twice, and suspended in 0.05 M KCl containing 1 mM phosphate (pH 6.0), 1 mM $CaCl_2$, and 0.1 mM $MgCl_2$ (buffered KCl) at concentrations ranging from 6×10^6 to $8{,}000 \times 10^6$ cells per ml. The suspensions contained 800 cpm per 10^6 streptococcal cells.

Whole unstimulated saliva was collected from a single donor (blood type O) with containers chilled in ice. The saliva was clarified by centrifugation at 12,000 × *g* for 10 min and was frozen before use. It was not heat inactivated because we wished to follow closely the method of Rosan et al. (32).

Adsorption to S-HA was performed essentially as described previously (11), except that the assay was scaled down to use 5 mg of HA beads (Gallard-Schlessinger Chemical Corp., Carle Place, N.Y.) and a reaction volume of 125 μl in wells in microtitration plates fitted with polyethylene caps. After incubation of the S-HA–bacteria mixtures, duplicate 25-μl samples of the supernatant liquors were removed and placed in scintillation vials to determine the concentration of free bacteria at equilibrium (C). The beads were then washed three times with buffered KCl and were transferred to scintillation vials to determine the numbers of bacteria which had attached (Q). These values were corrected for quench owing to the S-HA. The initial concentration of bacteria available for adsorption was calculated from the concentration of free streptococci at equilibrium plus the number of organisms which had adsorbed to the S-HA beads adjusted to a 1-ml basis. These values were 20 to 40% lower than the concentrations of bacteria added, presumably because of adsorption to the walls of the reaction chambers. All assays were performed at least in duplicate.

Bacterial adsorption to pellicles formed from saliva depleted of bacterium-aggregating activity. To determine whether the depletion of saliva components that aggregate bacteria affected bacterial attachment, samples of whole clarified saliva were absorbed three times with washed cells of the different organisms by the method of Rosan et al. (32); a portion of the saliva was left untreated for comparison. Before absorption, the clarified saliva agglutinated the streptococci when diluted 1:16 and 1:32 for *S. sanguis* C5 and FC-1, respectively, and 1:8 for both *S. mutans* JBP and *S. mitis* 26. After absorption, the saliva no longer aggregated the organisms. Samples of the untreated and depleted saliva were then used to form pellicles on the HA beads, and the numbers of [^{3}H]thymidine-labeled streptococci which attached to the pellicles were determined.

Adsorption model. The Langmuirian model used has been described in previous publications (3, 10, 20) and is expressed by equation 1, $C/Q = 1/KN + C/N$, in which C is the concentration of free bacterial cells at equilibrium, Q is the number of cells adsorbed to the S-HA, K is the affinity constant (reflecting the strength of the attachment bond to the S-HA surface), and N is the maximum number of adsorption sites on the S-HA adsorbent. In this paper, the units of these quantities are: for C, the number of cells per milliliter; for Q, the number of cells adsorbed per 5 mg of S-HA; for K, milliliters per number of cells (i.e., concentration^{-1}); and for N, the maximum number of adsorption or binding sites per 5 mg of S-HA.

Computer simulations. A set of values for K and N was used to generate a theoretical isotherm according to the Langmuirian relationship of $Q = KNC/(1 + KC)$; increasing values for C produced corresponding values of Q, thus describing the theoretical isotherm for the set of parameters chosen. A series of points was then selected on this isotherm to match closely the concentration values obtained in our experiments. Then, errors of ±5% were randomly assigned to the values of Q calculated for each of the selected concentrations. In this way, the simulation corresponded to an experimental isotherm for adsorption onto homogeneous binding sites. The dependence of the values of K and N on the adsorption coverage used in their derivation could then be found by selecting concentration ranges, calculating K and N (equation 1) with points on each range, and comparing these values with those obtained from points which describe the isotherm to approximately its saturation plateau.

Also explored was the possibility that multiple sites are involved in the adsorption of bacteria onto S-HA. If it is assumed that there are n types of adsorption sites that function independently, and each type has adsorption parameters N_i and K_i, the total number of

bacteria adsorbed, Q, will be given by equation 2, $Q = \Sigma_n[N_iK_iC)/(1 + K_iC)]$, which implies that the adsorption onto each kind of site is of a Langmuirian nature. The present results suggested that there were mainly two discernible adsorption sites. Under these conditions, the number of bacteria adsorbed is given by equation 3, $Q = [(N_1K_1C)/(1 + K_1C)] + [(N_2K_2C)/(1 + K_2C)] = Q_1 + Q_2$, in which Q_1 and Q_2 represent the adsorption onto sites 1 and 2, respectively. The four adsorption parameters N_1, K_1, N_2, and K_2 were evaluated by a nonlinear least-squares procedure (13).

The feasibility of explaining the experimental results in terms of equation 3 can be assessed by comparing the experimental value of Q with the corresponding calculated value for the same concentration. Also the statistical criteria for goodness of fit can be compared in using equation 2 for one adsorption site and equation 3 for the binary model. It is pertinent to point out that the selection of only two adsorption sites in no way precludes the possible existence of other sites that may contribute to a greater or lesser degree to total bacterial adsorption.

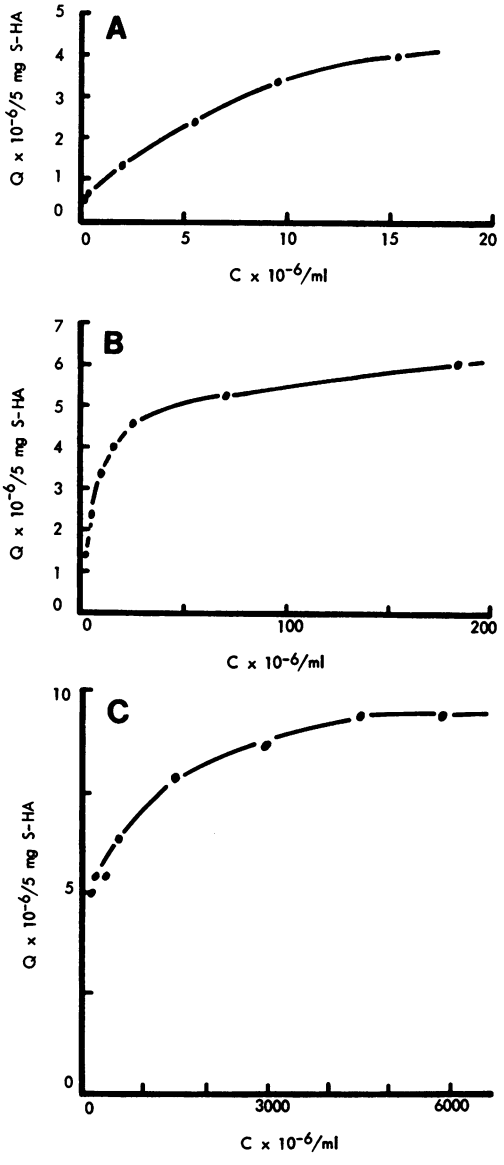

FIG. 1. Adsorption isotherms for the attachment of *S. sanguis* C5 to S-HA.

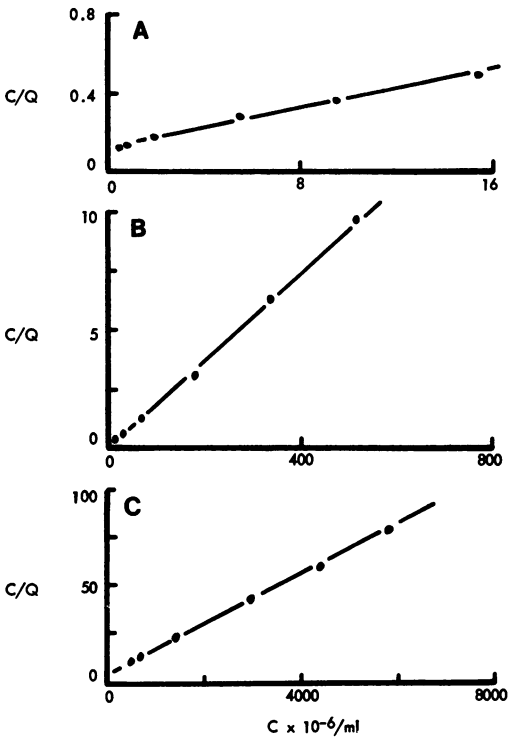

FIG. 2. Plots of C/Q versus C for the adsorption of *S. sanguis* C5 to S-HA.

RESULTS

Influence of the concentration of available bacteria on the parameters for the adsorption of *S. sanguis* C5 to S-HA. The numbers of *S. sanguis* C5 cells which adsorbed to the S-HA increased as the concentration of available bacteria increased, and saturation of the beads did not occur until the streptococcal concentrations at equilibrium exceeded 4,500 $\times$ 10^6 cells per ml (Fig. 1). The numbers of adsorbed bacteria (Q) increased in a curvilinear fashion, but a suggestion of a break in an otherwise smooth curve was evident at values of C between 50 $\times$ 10^6 and 500 $\times$ 10^6 cells per ml (Fig. 1B and C). Plots of C/Q versus C yielded straight lines at low, intermediate, and high bacterial concentration ranges (Fig. 2). This suggests that the Langmuir model adequately describes the adsorption of the streptococci over all of the concentration ranges tested. This was also reflected by the high

TABLE 1. Effect of bacterial concentration on parameters for adsorption of *S. sanguis* C5 to S-HA

Concn range of bacteria available (no. of cells × 10^6 per ml)	*n*	Correlation coefficient	No. of binding sites (*N*) per 5 mg of S-HA (×10^6)[a]	Affinity constant (*K*) (ml × 10^{-9} per cell)	*KN* (×10^{-2})
4.7–47.2	6	0.99	5.18 ± 0.16	191 ± 1	98.9
4.7–113	8	0.99	5.64 ± 0.03	162 ± 4	91.4
47.2–677	7	0.99	6.02 ± 0.11	93 ± 53	56.0
113–1,480	6	0.99	8.20 ± 0.3	7.8 ± 1.5	6.4
557–5,960	6	0.99	10.0 ± 0.05	2.5 ± 0.1	2.5
4.7–5,960	16	0.99	9.3 ± 0.04	9.4 ± 0.8	8.7

[a] Mean ± standard error of the mean.

correlation coefficients (0.99) obtained for each of the bacterial concentration ranges analyzed (Table 1).

The number of binding sites (*N*) and the affinity constant (*K*) were calculated for each of several ranges of concentrations of available bacteria (Table 1). The values for these adsorption parameters varied considerably depending upon the range of concentrations of available bacteria analyzed. Low bacterial cell concentrations yielded lower values for *N* and much higher values for the affinity constant (*K*) than those derived with high bacterial cell concentrations (Table 1). When data covering the entire range of concentrations of available bacteria were used, values for *N* and *K* were similar to those obtained with only high bacterial cell concentrations.

Appelbaum and co-workers (1) suggested using the product of the number of binding sites (*N*) and the affinity constant (*K*) to describe bacterial adherence to experimental pellicles. However, values for *KN* varied almost 40-fold when calculated from adsorption parameters derived from data with low versus high streptococcal concentrations (Table 1).

The experimental error inherent in estimating the number of bacteria which adsorbed to S-HA appeared to increase as the concentration of available bacteria increased (Table 2). Thus, the range of standard errors expressed as a percentage of the numbers of bacteria which adsorbed to the S-HA (*Q*) was higher when high numbers of bacteria were available for adsorption and lower when low numbers of bacteria were available (Table 2). These differences were statistically significant. The relatively high specific activity of the streptococcal cells used permitted reliable quantitation at the ranges of low concentration studied. The greater experimental error when high bacterial cell concentrations were used may be due to erratic removal of more weakly adhering organisms adsorbed to low-affinity binding sites during washing, or to steric interactions between high concentrations of bacteria competing for limited numbers of binding sites, or to cell-to-cell interactions resulting from multi-layer formation.

Comparisons of computer-generated and experimentally determined adsorption parameters. The values of *K* and *N* used in the computer simulation (theoretical) were 9.4×10^{-9} and 9.4×10^6; these parameters were derived from all 16 points obtained in the experimental isotherm. The variability in the values of *K* and *N* obtained through the use of the simulated points was about the same regardless of the concentration range used (Table 3), and it could be ascribed to the assigned random errors. The maximum deviations of these values from the assigned parameters (*K*, 9.4×10^{-9}; *N*, 9.4×10^6) were only 21 and 6.4% for *K* and *N*, respectively. In contrast, deviations of 2,500 and 40% were obtained for *K* and *N*, respectively, when they were derived from experimental points within comparable concentration ranges. Therefore, it is apparent that the experimentally obtained data in Table 3 are inconsistent with the adsorption of bacteria onto homogenous binding sites.

Analysis of model for binary adsorption sites. Table 4 shows the values obtained statistically for the four adsorption parameters associated with two adsorption sites (equation 3). Table 4 also shows the values obtained for *K* and *N* by assuming a single adsorption site operating over the whole concentration range. It is apparent that a much better goodness of fit is obtained

TABLE 2. Range of standard errors as percentage of numbers of streptococci which adsorbed to S-HA at various concentrations

Concn range of bacteria available (no. of cells × 10^6 per ml)	*n*	No. of streptococci adsorbed (*Q*) per 5 mg of S-HA (×10^6)	SE (% of *Q*)
4.7–47.1	7	0.53–4.52	0.2–2.9
62.1–557	5	4.52–5.6	1.6–3.3
557–5,960	6	5.5–9.3	2.5–7.8[a]

[a] $P > 0.005$, different from standard errors for the range of *Q* from 0.53×10^6 to 4.52×10^6.

TABLE 3. Comparison of adsorption parameters derived from computer-simulated and experimentally determined isotherms

Concn range of bacteria at equilibrium (C) (free bacteria $\times 10^6$ per ml)	No. of values	Affinity (K) ($\times 10^{-9}$)	No. of sites (N) ($\times 10^6$)	% Coverage ($[Q/N] \times 100$)
Computer-simulated isotherm				
1–150	9	9.3	9.7	59.8
1–300	10	8.8	10.0	74.3
1–600	11	9.7	8.9	78.5
1–3,000	14	11.4	9.5	99.8
1–6,000	20	11.1	9.5	101.6
600–6,000	5	10.0	9.4	100.0
Experimentally determined isotherm				
0.5–184	9	115	6.4	65.5
0.5–339	10	173	5.8	65.5
0.5–513	11	248	5.6	65.5
0.5–3,000	14	13.8	8.6	92.6
0.5–5,888	16	9.4	9.4	99.5
626–5,888	5	2.7	10.0	99.5

with the two-site model. Thus, about 99% of the variance of Q (the adsorbed bacteria) can be explained by variance in the values of C (the equilibrium concentration); with the single-site model, only about 86% of the variance in Q can be so explained. Similarly, the standard deviation for the overall fitting for the binary model is almost one-third the value for the single-site model.

The values obtained for the adsorption parameters were then used to calculate the total numbers of adsorbed bacteria (Q) under equilibrium concentrations according to equation 3. A comparison of these quantities with the experimental adsorption results is shown in Fig. 3. Although there is some scattering, the overall agreement between the calculated and experimental adsorption values is good, and it substantiates the presence of at least two adsorption sites on the S-HA.

It is instructive to analyze the model used for binary sites (equation 3) in more detail. In Fig. 4 are plotted the total calculated bacterial adsorption and the contributions to it from sites 1 and 2 versus the concentration of free cells at equilibrium (C). It is apparent that adsorption onto the second adsorption site starts contributing significantly to the total adsorption when the concentration at equilibrium is in the order of 30×10^7 to 50×10^7 cells per ml. Interestingly enough, Tables 1 and 3 show that the values for N are quite similar when the first 9 to 11 points are used to calculate them; inclusion of additional points results in significantly higher values for N. Figure 4 indicates that at a concentration of 50×10^7 cells per ml, the contribution of site 2 to the total adsorption becomes conspicuous. At concentrations higher than about 3×10^8 cells per ml, site 1 is saturated for all practical purposes, and above this concentration, the increase in adsorption is essentially the contribution of site 2, which apparently approaches saturation at the highest experimental concentration used.

TABLE 4. Results of nonlinear regressions for binary and single-site adsorption models

Model	N ($\times 10^6$)[a]	K (ml $\times 10^{-9}$ per cell)[a]	Variance (R^2)	SD of Q ($\times 10^6$)
Binary site			0.987	0.37
Site 1	5.3 ± 0.3	170.8 ± 38		
Site 2	6.3 ± 1.1	0.36 ± 0.2		
Single site	7.5 ± 0.4	62.2 ± 21	0.859	1.1

[a] Mean ± standard deviation.

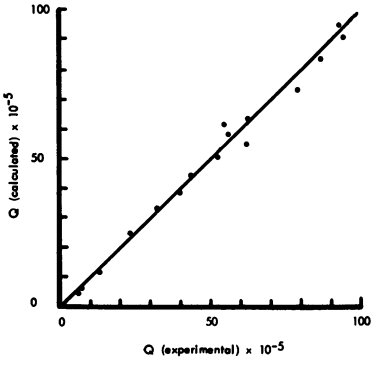

FIG. 3. Comparison of values of Q calculated from a model for adsorption to binary sites (equation 3) with values derived from experimental results. The solid line represents perfect agreement.

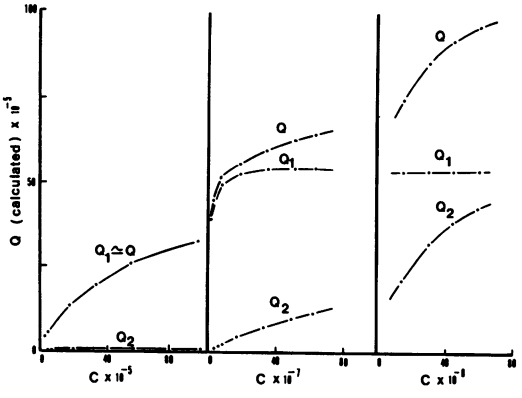

FIG. 4. Plots of the total calculated bacterial adsorption (Q) and contributions to it (Q_1 and Q_2) from adsorption to two presumed binding sites derived from the binary adsorption model (equation 3). The ordinate scale of Q is common for all plots. However, the abscissa scales cover three concentration ranges of C.

Bacterial adsorption to pellicles formed from saliva depleted of bacterial-aggregating activity. Far fewer streptococci adsorbed to experimental pellicles prepared from saliva depleted of bacterial-aggregating activity when low numbers of streptococci were available (Table 5). This indicates that their adsorption was dependent upon the presence of specific salivary components in the pellicles.

DISCUSSION

The data obtained in the present study indicate that the values for adsorption parameters derived from isotherms of *S. sanguis* adsorption to S-HA surfaces are markedly affected by the range of concentrations of available bacteria. Isotherms derived from initial streptococcal concentrations of 10^7 to 10^8 cells per ml gave lower estimates of binding sites and higher values for the affinity constants than did isotherms with bacterial concentrations in the range of 5×10^8 to 5×10^9 per ml. The simplest explanation for these results is that there are multiple binding or adsorption sites on S-HA surfaces to which *S. sanguis* cells attach with different affinities. Thus, when low concentrations of streptococci are available, the organisms adsorb to sites to which they have the highest affinity. At higher bacterial concentrations, these sites become saturated, and the organisms then adsorb to less specific sites to which they have a lower affinity. In the present investigation, the affinity constant of the low-concentration site is two orders of magnitude higher than the high-concentration affinity constant. Although the data were derived from studies of the adsorption of *S. sanguis*, the principles appear applicable to other organisms since low bacterial concentrations have yielded lower estimates of N and higher estimates of K than high bacterial concentrations for several other oral bacterial species (1, 3).

The proposed existence of multiple binding sites on S-HA surfaces for bacteria is reasonable because pellicles consist of complex mixtures of salivary macromolecules which include blood group-reactive mucins; proteins which are rich in tyrosine, proline and histidine; immunoglobulin A and lysozyme; and often amylase and immunoglobulin G as well (15, 16, 27, 29, 33). Furthermore, several of these components are known to bind to bacteria. For example, oral streptococci may be agglutinated by immunoglobulin A antibodies (35), lysozyme (31), and

TABLE 5. Streptococcal adsorption to HA treated with unabsorbed or bacteria-absorbed saliva

Organism	Initial bacterial concn (cells per ml)	No. of streptococci ($\times 10^5$) adsorbed ± SE per 5 mg of HA treated with:		% Adsorption[a]
		Unabsorbed saliva	Bacterium-absorbed saliva	
S. sanguis C5	1×10^7	7.4 ± 0.08	0.9 ± 0.03[b]	12.6
	5×10^9	117 ± 3.0	102 ± 3.0	87.2
S. sanguis FC-1	1×10^7	3.4 ± 0.26	0.35 ± 0.10[b]	10.3
	5×10^9	126 ± 9.1	48.8 ± 2.5[b]	38.7
S. mutans JBP	1×10^7	0.64 ± 0.07	0.32 ± 0.09[c]	50.0
	5×10^9	30.8 ± 1.1	26.1 ± 2.1	84.7
S. mitis 26	2×10^7	14.9 ± 0.05	0.46 ± 0.006[b]	3.1
	5×10^9	196 ± 3.2	167 ± 4.6[c]	85.2

[a] Number of streptococci adsorbed with bacterium-absorbed saliva as percentage of number adsorbed with unabsorbed saliva.
[b] $P < 0.001$ from unabsorbed saliva.
[c] $P < 0.05$ from unabsorbed saliva.

high-molecular-weight salivary glycoproteins (22, 36). They have also been shown to bind blood group-reactive salivary mucins on their surfaces (11). In the case of *S. sanguis*, there is evidence that it may bind to salivary receptors containing sialic acid residues (9, 25) and also to hydrophobic sites (26) in experimental pellicles. It is also clear that many bacteria possess several types of adhesins on their surfaces (8, 12). It therefore seems likely that bacterial attachment to salivary pellicles on teeth, and to other host tissues, involves multiple interactions between different types of bacterial adhesins and different classes of receptors present on macromolecules on the adsorbent surfaces. Such interactions would also be expected to occur with various affinities.

Computer simulations and a binary adsorption model suggest that there are at least two classes of binding sites for *S. sanguis* in experimental salivary pellicles. These sites could be comprised of sialic acid-containing receptors (9, 25) and hydrophobic residues (26), as mentioned previously, or combinations of these. Alternatively, there could be two distinct receptors whose bonds with streptococcal adhesins become stabilized by the occurence of secondary hydrophobic interactions. Because the data obtained suggest the existence of two types of binding sites, a binary model (equation 3) was adopted. However, we consider it probable that several classes of binding sites exist for various bacteria in view of the complex nature of bacteria surfaces and the array of salivary macromolecules present in pellicles.

The apparent presence of multiple binding sites is consistent with the recent suggestion of Nesbitt et al. (26) that *S. sanguis* adsorption to S-HA is not Langmuirian but rather involves positive cooperativity. This suggestion was based on sigmoidal isotherms for *S. sanguis* adsorption to S-HA. However, in view of the apparent presence of two or more binding sites for *S. sanguis* on S-HA and in view of the biphasic isotherm observed in the present study, it is possible that the isotherms obtained by these investigators originated in the break in the biphasic curve, producing the apparent sigmoidal shape.

It is not clear what constitutes a receptor or binding site for a bacterial cell on a host tissue. Although a binding site for a single molecule may be a specific region of a molecule on the adsorbent, it is unlikely that a single bacterial adhesin molecule interacting with a receptor would be adequate to hold a bacterial cell on a surface. Therefore, a bacterial binding site may represent a region on the adsorbent surface with a high density of receptor molecules to interact with the adhesin molecules of the organism. Attachment would occur when enough adhesin and receptor molecules interact to overcome Brownian movement and the negative electrostatic energies which tend to repel bacterial cells from most natural surfaces. The receptors in pellicles are probably immobile because the salivary macromolecules are tightly adsorbed to the HA, and intermolecular bonding is also thought to occur (8). However, the glycoproteins and glycolipids which serve as bacterial receptors on epithelial cells are mobile in the lipid bilayer of the cell membrane (7), and consequently, receptor recruitment can occur after the bacterial cell first associates with the surface. This may increase the overall strength of the bacterial adsorption bond as time goes on.

Several conceptual models may be advanced to account for two or more categories of bacterial binding sites (Fig. 5), but the data obtained do not discriminate among them. Each site could represent a cluster of different randomly distributed receptor molecules with which different adhesins of the organism interact with a high or low affinity (Fig. 5A). It is also possible that high- and low-affinity binding sites are derived from differences in the density of a single type of receptor molecule on the surface which is capable of interacting with one type of bacterial adhesin (Fig. 5B). Still another possibility is that there may be two or more types of receptors for two or more adhesins with different spatial arrangements (Fig. 5C). These mechanisms are not mutually exclusive, and several could be

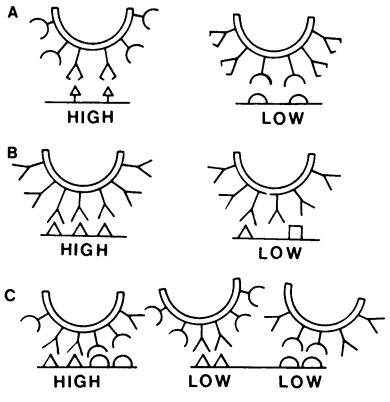

FIG. 5. Diagrammatic representation of three conceptual models for high- and low-affinity binding sites for bacteria, represented by: (A) Clusters of different receptor molecules which interact with different affinities with distinctive bacterial adhesins; (B) differences in the density of a single type of receptor molecule which interacts with one type of bacterial adhesin; and (C) differences in the spatial arrangement of clusters of two types of receptors which interact with two types of bacterial adhesins.

involved in a given bacterium-surface interaction. The complexity of these interactions would tend to mask the existence of clear-cut phases or steps in curves of plots of Q versus C. This may explain why there was only a suggestion of a break in the experimental adsorption isotherm generated in the present investigation, and why Appelbaum and coworkers (1) did not detect a step in the isotherms they produced.

The purpose of the computer simulation of a theoretical isotherm was to generate points to assess how the estimates of the adsorption parameters depended on the percentage of the adsorbent coverage used to derive them. The data (Table 3) clearly demonstrate that in the case of a single simulated adsorption site having properties similar to those reflected by the experimental results, the values obtained for the adsorption parameters are not significantly affected by the extent of site coverage or saturation over the range studied. However, the situation is quite different with the experimentally generated data. In this case, there was a marked dependence on the values of K and N on the bacterial concentrations used to estimate them. This dependence cannot be ascribed to the degree of adsorption coverage because the simulated data in Table 3 show that this is not the case when dealing with a single adsorption site. Thus, these observations further support the existence of multiple adsorption sites for *S. sanguis* attachment to S-HA.

The lack of a pronounced dependence of values of K and N on coverage as revealed by the computer simulations is explained by the relatively low values of K for *S. sanguis* adsorption to S-HA. With values for K in the order of 10^{-7} to 10^{-9}, the isotherm contains information about both K and N from the very beginning, i.e., starting with low percentages of coverage and low concentrations at equilibrium. This is because one cannot discern a sharp knee or bend close to the origin of the isotherm. The increase in coverage occurs gradually with increasing bacterial concentrations, reflecting the magnitude of both adsorption parameters. In contrast, in adsorption processes where the affinity constant is very high (in the order of 10^3 to 10^4), as in the adsorption of salivary proteins onto HA (16), we have verified that it is necessary to obtain coverages of about 75% of saturation to derive parameters within 20% of the values obtained with experimental points that define the saturation plateau (data not shown).

The presence of multiple binding sites for *S. sanguis* in experimental pellicles formed on HA surfaces appears to resolve discrepancies in the literature about different estimates of binding sites and affinities made for oral bacteria with either low or high bacterial concentrations (1, 3, 35). In this regard, it is important to note that values for adsorption parameters calculated from data over the entire range of bacterial concentrations tested yielded values for N and K which approximated those obtained with only the high bacterial concentrations (Tables 1 and 3). Thus, the inclusion of data derived from high bacterial concentrations masks the higher affinity and the more specific binding sites on the S-HA surface.

Appelbaum and co-workers (1) suggested using the product of the number of binding sites (N) and the affinity constant (K) to describe the adherence properties of bacteria to experimental pellicles. However, estimates of KN were found to vary almost 40-fold when they were calculated from adsorption parameters derived from data based on high or low streptococcal concentrations (Table 1). Appelbaum et al. (1) also noted such variation. However, they did not consider it likely that there was more than one type of binding site on the S-HA surface for each of the organisms studied, and therefore, they assumed that estimates obtained with low bacterial concentrations were inaccurate. However, since values of KN can vary so widely and do not discriminate between binding sites of differing affinities and specificities, they would appear to be of limited usefulness. Furthermore, since K and N are independent parameters, equal values of KN could refer to very dissimilar systems. For example, high K and low N for one organism and low K and high N for another could yield similar values for KN, yet the adsorption processes of these bacteria would be quite different. Consequently, equating the behavior of adsorption systems on the basis of the product of their adsorption parameters may not only be misleading, but it may also be erroneous. Clark and co-workers (4) recently expressed a similar view.

It has been suggested that components of saliva which bind to bacterial cells and cause their aggregation also promote bacterial attachment if present in the pellicle (3, 18, 19, 22, 28, 30). However, Rosan et al. (32), using high concentrations of bacteria, recently reported that similar numbers of *S. sanguis* cells adsorb to experimental pellicles formed from saliva depleted of bacterial-aggregating components and to pellicles formed from untreated saliva. Therefore, we repeated their experiments with both high and low bacterial concentrations (5×10^9 and 10^7 cells per ml). Much lower numbers of *S. sanguis*, *S. mitis*, and *S. mutans* adsorbed to pellicles prepared from saliva depleted of bacterial-aggregating activity than to pellicles formed from unabsorbed saliva when low numbers of streptococci were used (Table 5). This indicates that adsorption of bacteria from low-

concentration suspensions to S-HA is dependent upon specific salivary components. However, as noted by Rosan et al. (32), the numbers of streptococci which attached to the two types of S-HA surfaces showed less difference when high streptococcal concentrations were used. It would appear from these data that salivary components which can aggregate bacteria are associated with bacterial adsorption to high-affinity binding sites on S-HA. Use of high bacterial concentrations apparently saturates these high-affinity sites, and less specific interactions between bacteria and salivary pellicle components occur.

Since the bacterial concentrations employed in assays of adherence to S-HA surfaces appear to determine the nature of the receptor-bacterial interactions which occur, the question therefore arises as to what reaction conditions most closely mimic those occurring in the human mouth which relate to bacterial colonization of teeth. It is thought that individuals become colonized by oral bacteria after exposure to saliva from other humans in their environment. Because the volume of saliva transferred is small, the low number of bacteria introduced in the mouth results in only low concentrations available for attachment to teeth or oral mucosal surfaces. Thus, the organisms would be expected to attach to those binding sites for which they have the highest affinity. These interactions would also be the most specific. After colonization has taken place, the numbers of a species present in the saliva would increase considerably. In individuals who are colonized by *S. sanguis*, approximately 10×10^6 cells of the organism can be found per ml of saliva as a result of dislodgement or desorption from colonized tooth surfaces (2). A tooth having a surface area of 2 to 3 cm^2 could therefore be exposed to 3×10^6 to 5×10^6 *S. sanguis* cells per cm^2 of pellicle surface. The spheroidal HA beads most often used in adherence assays have been estimated to have an average surface area of 0.63 cm^2 per mg (1). The reaction mixtures used by Clark et al. (3) contained up to 2.6×10^6 *S. sanguis* cells per cm^2 of S-HA surface and therefore approximate these conditions. Those used by Appelbaum et al. (1) and Rosan et al. (32) are an order of magnitude higher. Therefore, the class of receptors studied with the bacterial concentrations of Clark and co-workers (3) would appear to be those most likely involved in the formation of dental plaques after the cleaning of teeth.

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