LASER LIGHT SCATTERING MEASUREMENT OF DEXTRAN-INDUCED *STREPTOCOCCUS MUTANS* AGGREGATION

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ABSTRACT Intensity fluctuation spectroscopy was used to study dextran-induced aggregation of *Streptococcus mutans* bacteria. Smoluchowski's theory of colloidal flocculation provided a consistent model of the agglutination process. Our experiments indicated that aggregation was inhibited by the negatively charged surfaces of the cells, while dextran polymers effectively bound organisms together. Our experimental data were consistent with the quantitative predictions of a polymer bridge model of agglutination.

INTRODUCTION

We have used quasi-elastic laser light scattering to study dextran-induced aggregation of the bacterial species *Streptococcus mutans*. The organism is found in the human oral cavity and is strongly implicated as a major agent of tooth decay. In the presence of sucrose, these bacteria synthesize both soluble and insoluble branched dextrans which enhance cell aggregation and cell adherence to the enamel substrate (1).

The organisms have negatively charged surfaces (2). They must make contact to aggregate and hence require sufficient thermal energy to overcome the repulsive interaction of the overlapping electrical double layers of the cells in solution. Van der Waals interactions and polymer bridging have been proposed as mechanisms for achieving stable adherent configurations of cells in contact (3). However, when *S. mutans* is examined after growth in sugarless media, the cells coagulate very slowly in the absence of both cell-bound and extracellular polymer agglutinins, an indication that under our particular experimental conditions the nonspecific van der Waals force is weak. We assume that the van der Waals force changes insignificantly upon addition of polymers in our experiments because for the most part the dextran concentrations used were low and we expect to coat only a small fraction of the bacterial surface. On the other hand, the nonionic dextran molecules are capable of agglutinating *S. mutans* rapidly by polymer bridging through attachment to receptor sites on adjoining bacterial surfaces. To be an efficient agglutinin in this model, the average diameter of the polymer must exceed twice some effective barrier length extending from each bacterium beyond which the dextran bonds can survive the electrical repulsive force and the thermal movements of the bound bacteria relative to each other.

From the work of other investigators with both erythrocytes (4) and bacteria (5), we anticipate that the magnitude and the sign of the cell charge and the dimensions of the double layer might be altered by changing the qualities of the cell surface and the nature of the

suspension medium. Thus the rate of aggregation will be affected by such factors as the ionic strength and pH of the medium, the concentrations of multivalent ions, and the numbers and sizes of nonionic polymers attached to the cell surface.

Since in our experiments the bacteria are nonmotile, the fluid medium is quiescent, and the intercellular forces are short range, Brownian motion must be the primary mechanism for promoting cell-dextran and cell-cell encounters. These interactions lead respectively to the binding of molecular dextran to receptor sites and to enhanced cell aggregation. We note light scattering experiments on our dextran samples showed that generally dextran molecules did not bind to one another, thus excluding the third possible form of interaction. Some aggregation was detected in the higher molecular weight fraction work, but the rate was so slow that it was insignificant in comparison to the rate of bacterial aggregation.

In such circumstances it is reasonable to propose Smoluchowski's theory of colloidal flocculation as an appropriate description of these processes. The theory predicts that agglutinins whose dimensions exceed those of a barrier length should enhance the rate of aggregation. In addition, if polymer bridging is the major active cause of flocculation with the polymers anchored to cell-bound receptor sites, then the aggregation rate should show a maximum value at a particular concentration of dextrans. For a specific reaction model for the macromolecules and their binding sites, the concentration of polymer at the maximum rate of aggregation determines the average number of receptors on the bacterial surface. Furthermore, in an approximate version of the theory, the rate should be directly proportional to the initial concentration of bacteria in suspension. Our experiments agree with these predictions of the Smoluchowski theory. Some of the above dependencies have been found previously for other bacterial species (5), while experiments on erythrocytes showed a disaggregation phase at high dextran concentrations (4) which has not been observed for bacteria.

In addition, we have found as expected (6) that the rate of bacterial aggregation as a function of pH has a single maximum, peaking at the isoelectric point of pH 3.7 and then decreasing for both lower and higher pH values as the bacterial surface acquires net positive and negative charges, respectively.

Even in the absence of exogenous agglutinins, the bacterial strain we tested, *S. mutans* 10449, will aggregate if grown in tryptic soy broth (TSB) supplemented with 2% sucrose. Presumably, the active agglutinating agent is cell-bound dextran synthesized by the bacteria from the sucrose in the growth medium (1). The dextran remains firmly attached to the cells despite several washings. In contrast, glucose grown cells do not produce cell-bound dextran and thus aggregate very slowly in the absence of extracellular agglutinins. This fact further suggests a secondary role for the van der Waals interaction in bacterial binding, at least for pH values in the physiological range.

In our experiments, the *S. mutans* cells were cultured in a commercial, sucrose-enriched medium. With identical washed and resuspended samples, the same time was required for the bacteria to reach a predetermined state of aggregation. The control suspension could then serve as a standard of comparison for bacteria under other conditions. This reference sample is free of extra-cellular polymers whose presence in solution might modify the characteristic rate of cell aggregation.

THEORY

Aggregation Kinetics

In 1916, Smoluchowski (7) developed a theory for the kinetics of the aggregation of colloids. He assumed that a sphere of influence surrounds each particle and that there is a fixed probability for sticking in the course of a collision, viz., the intersection of one sphere of influence with another. Initially the suspension consists only of single spherical particles with spheres of influence of radius R. Particles execute Brownian motion until they collide. If the result is doublet formation, the new unit undergoes Brownian movement with a reduced velocity until entering the sphere of influence of some other single or multiple particle. As aggregation proceeds, the average diffusion constant of the ensemble of aggregates decreases. The change can be observed continuously in time by monitoring the intensity spectrum of scattered laser light which has been Doppler shifted in frequency because of the Brownian movements of the aggregates.

The Smoluchowski theory is reasonably successful in describing colloidal flocculation (8). The model also appears to be valid as a description of bacterial aggregation. A prediction which follows from the theory is that the rate of heteroflocculation (for different sized particles) is greater than homoflocculation (for like sized particles) by a factor R_2/R_1 if $R_2 \gg R_1$ (7, 9). Here R_2 is the radius of the larger aggregate and R_1 that of the smaller. If R_2 is the bacterial radius and R_1 the effective hydrodynamic radius of a dextran molecule, then in our experiments R_2/R_1 ranges from ~10 to 55. We therefore have assumed that the adsorption of dextran on the bacterial surface is essentially complete before bacterial aggregation actually commences (9). The experimental data appear to justify this assumption.

Smoluchowski's equations for the rate of aggregation can be solved analytically if one makes certain simplifying assumptions. The basic assumptions are that the radii of all aggregates are equal and that, once formed, aggregates do not dissociate. The resulting solutions provide an adequate description of the bacterial processes we have observed. Given these assumptions, Smoluchowski showed that the density of k-fold aggregates as a function of time is:

$$\nu_k/\nu_0 = \rho^{k-1}/(1 + \rho)^{k+1}. \tag{1}$$

In Eq. 1, ν_k is the number density of aggregates consisting of k single bacteria. ν_0 is the initial density of bacteria in the monodisperse suspension before aggregation begins. The dimensionless variable ρ is given as

$$\rho = 4\pi DRP\nu_0 t. \tag{2}$$

with D an average diffusion constant for the ensemble of aggregates and R the radius of an average sphere of influence. The time is measured by t, with $t = 0$ the starting point of the experiment. P, the probability that a collision will result in sticking, depends on such factors as the concentration of dextran molecules added to the suspension, the number of dextran receptor sites on the surface of each cell, the pH of the medium, and other factors determining the outcome of a collision between aggregates.

Modifications of the suspension medium which alter the rate of cell aggregation affect

either the value of P or ν_0 in Eq. 1. However, in this equation P and ν_0 appear on the right only in the factor ρ. If the theory is valid, we then would expect that measurements of aggregation rates under different experimental conditions would all be equivalent to one another modulo a scale transformation in time, $t = \alpha t'$. Thus, for bacteria in a given medium, we can measure some function of the ν_k/ν_0 and graph it as it changes in time. If we vary the medium's aggregating properties so that $P' = \alpha P$, or $\nu_0' = \alpha\nu_0$, with α a constant, the graph of the function in general will also change. However, if we simultaneously modify the time scale of aggregation by an appropriate uniform stretch or contraction, $t = \alpha t'$, the two curves should transform into one another. In each of the different experiments described in this paper, we have consistently found the above to be the case for dextran-induced bacterial aggregation. This fact strongly supports the applicability of the Smoluchowski theory to the aggregation of *S. mutans* and the validity of the assumptions and approximations which have led to the solutions of Eq. 1.

As indicated earlier, the factor P is the probability that a collision of two aggregates will result in sticking. If dextran is used as a flocculating agent for bacteria, P depends on the ratio of occupied dextran sites to available sites on the cell surfaces (9). Let N be the number of dextran receptors per cell with ν_0 the density of single cells initially in suspension. If dextran in concentration c is added to the solution before aggregation begins, the molecules rapidly attach themselves to the cells' receptor sites. If one dextran molecule binds only two bacteria, the likelihood that a collision between bacterial aggregates will result in sticking depends on the product of the fraction of occupied sites F and the fraction of unoccupied sites $(1 - F)$,

$$P = KF(1 - F) \tag{3}$$

where

$$F = c/N\nu_0. \tag{4}$$

The coefficient K in Eq. 3, a function of the dimensions of both the dextrans and the double layer, may be varied by changing the dextran size, ionic concentrations in the medium, and the number of other neutral macromolecules attached to the cell surface (4). K also depends on the strength of the van der Waals interaction.

Intensity Fluctuation Spectroscopy

The Brownian motion of the suspended bacterial aggregates causes the intensity of the scattered light to fluctuate in time and its frequency spectrum to be Doppler shifted. These fluctuation and frequency shifts appear in the power output of the photomultiplier tube. We measure directly the homodyne time autocorrelation function of the current fluctuations about the average value defined as

$$C_i(\tau) = \lim_{T\to\infty} \frac{1}{T}\int_0^T i(t)\, i(t + \tau)\, \mathrm{d}t, \tag{5}$$

where $i(t)$ and $i(t + \tau)$ are the current outputs measured from the average value of the current at times t and $t + \tau$, respectively. For a dilute polydisperse suspension of noninteracting spherical scatters, $C_i(\tau)$ is given as (10, 11):

$$C_i(\tau) = [\int_0^\infty A(D)e^{-DK^2\tau}\, \mathrm{d}D]^2. \tag{6}$$

$K = (4\pi/\lambda) \sin(\theta/2)$ is the scattering wave vector with θ the scattering angle and λ the wavelength of light in the suspension medium. $A(D)\,dD$ is the intensity of light scattered by all of the suspended objects in the laser beam with diffusion constants between D and $D + dD$.

To apply Eq. 6 in actual experiments, Koppel (10) expanded the exponential in the integrand in powers of τ, took the square root of the natural logarithm of $C_i(\tau)$ and found

$$\ln[C_i(\tau)]^{1/2} = A - B\tau + C\tau^2 + \ldots, \tag{7}$$

where

$$A = \ln \int_0^\infty A(D)\,dD,\ B = \overline{D}K^2, \text{ and } C = \frac{1}{2} M_2 K^4,$$

while:

$$\overline{D} = \int_0^\infty k\, A(D) D\,dD / \int_0^\infty A(D)\,dD \tag{8}$$

and

$$M_2 = \int_0^\infty A(D)\,(D - \overline{D})^2\,dD / \int_0^\infty A(D)\,dD. \tag{9}$$

As aggregation proceeds, the values of $\overline{D}$ and M_2 change. For our experimental arrangement, we found $\overline{D}$ to be a monotonically decreasing function of the time. We assume that Eq. 1 accurately describes the course of aggregation and that the Mie-Rayleigh (12) theory of light scattering is applicable to our system of bacterial scatterers. With these assumptions, $\overline{D}$ may be calculated by computer as a function of time. We defer full details of our theoretical analysis to a future paper. The theoretical predictions (solid line) and the experimental results (circles) are presented in Fig. 1. Their reasonable agreement lends further credence to the appropriateness of the Smoluchowski theory as a model for bacterial aggregation.

If the agglutinating factors in the solution are modified, the graph of $\overline{D}$ as a function of time changes. For an increased aggregation rate, the $\overline{D}$ curve approaches the time axis more

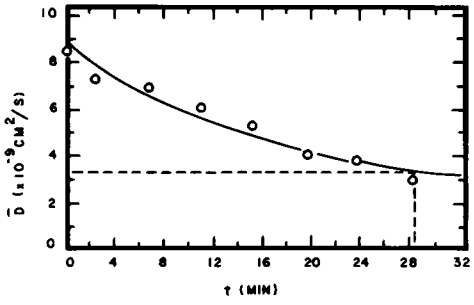

FIGURE 1 Mean diffusion constant $\overline{D}$ vs. time t for control sample. The origin of the time scale corresponds to the instant after dispersion of the sample into single cocci. The uncertainty in the value of D for each data point is indicated by the diameter of the open circles. The t coordinate for each data point corresponds to the midpoint of the time interval during which the photocurrent was sampled. The best fit curve predicted by Smoluchowski aggregation theory is also shown and gives an aggregation time t_{agg} of 28.5 min.

rapidly. However, as stated previously, the curves for the different experiments can be transformed into one another by appropriate scaling of the measured time. Since different aggregation processes apparently pass through identical stages but at different times, we can use the graph of $\overline{D}$ vs. t to specify the state of aggregation uniquely. We do this by arbitrarily defining the aggregation time, t_{agg}, as the period required for $\overline{D}$ to be reduced to e^{-1} of its initial maximum value. At that moment, if the approximate Smoluchowski theory is a proper description of bacterial aggregation, the value of ρ in Eq. 1 is the same in all cases, since each process has reached a similar stage of aggregation. Thus we expect $\nu_0 DRPt_{agg}$ to be a constant. Our interpretation of the optical data is based on this assumption and we may regard the theoretical solid line of Fig. 1 as a universal curve form to be fit to all aggregation data by adjustment of the abscissa scale.

The Dimensions of Dextran Molecules in Different Molecular Weight Fractions

Sellen (13) has shown through light scattering measurements that the dextran synthesized by *Leuconostoc mesenteroides* strain B512 and separated into different molecular weight fractions consists of spherical polymers. Dextran from this bacterial strain is a homogeneous polymer of glucose with predominantly $\alpha - 1, 6$ links. Thus for these fractions the diffusion constant D is related to the molecular weight M by (13, 14):

$$D = AM^{-\alpha}, \tag{10}$$

and the intrinsic viscosity $[\eta]$ is given by a similar relation (14):

$$[\eta] = BM^{\beta}. \tag{11}$$

A homologous sequence of macromolecules can be fit by a single set of constants A, B, α, and β. We found this to be the case for the different molecular weight dextrans used in this experiment.

For spherical molecules, D is related to the effective hydrodynamic radius of translation, R_e, through the Einstein-Stokes relation:

$$D = kT/6\pi\eta_0 R_e. \tag{12}$$

In Eq. 12, T is the absolute temperature and η_0 is the viscosity of the solvent. Measurement of D at a known temperature yields the value of R_e which we assume approximates the dimension of the polymer effective in binding together two separate cells.

EXPERIMENTAL PROCEDURE

Sample Preparation

The phosphate buffer saline used for suspension (Beckman, Fullerton, Calif.) has a pH of 7.2. To reduce the amount of dust present in the sample, the buffer, after preparation, was continuously filtered for 2 days through a Diatom Filter (Vortex, Burton, Mich.). Just before use, the suspension was filtered twice more through 0.2-μm Nuclepore filters (Nuclepore Corporation, Pleasanton, Calif.).

Stock cultures of *S. mutans* 10449 were obtained from the School of Dental and Oral Surgery, Columbia University. To prepare a sample for aggregation study, several aliquots from a stationary phase culture grown in TSB broth (BBL) enriched with 2% sucrose were washed three times with saline, resuspended in saline to one half of their original volume, pooled together, and allowed to stand for 30 s so that the visible clumps could settle to the bottom of the tube. The supernate was filtered once through

a 5.0-μm Nuclepore filter to remove large intractable aggregates. Sufficient saline was then added to obtain an optical density of 0.3 at 700 nm in a Turner spectrophotometer. A ½-ml aliquot from the bacteria suspension was diluted with eleven parts of saline in a scattering cell. We vortex-mixed the sample for 15 s in the scattering cell to disperse aggregates and chose the instant after mixing as the zero of the time scale. The dispersion of the sample into single cocci was verified by microscopic examination of slides prepared from the sample. An *S. mutans* sample prepared by this procedure contains 8×10^6 single cocci/ml as determined by the pour plate technique using a TSB and agar medium. All experiments were conducted at 21°C.

When the pH was varied, the approximate buffer was substituted for saline throughout the sample preparation. Commercial buffers (Fisher, Springfield, N.J.) were used in the pH range >4.0. In the pH range <4.0, buffers were prepared from 50 ml of 0.1 M potassium biphthalate diluted to 100 ml using varying amounts of 0.1 M hydrochloric acid and distilled water.

Water soluble dextrans were obtained from Pharmacia (Piscataway, N.J.). Appropriate dextran concentrations in saline solvent were added to the final suspension of bacteria in the scattering cell to adjust the dilutions. The suspensions were then dispersed by vortexing in the sample cells.

Scattering Apparatus

A time-correlation laser homodyne system was constructed. The light source was a Hughes Model 3235H-PC helium-neon laser (wavelength = 6328 Å). The power output of the laser was 10 mW and stable. The laser beam was polarized horizontally with the electric field vector in the scattering plane. The Mie scattering form factors for both vertical and horizontal polarizations are found in Kerker (12) for scatterers in the appropriate size range. Based on these form factors, the horizontal polarization was preferred because it descriminated against Rayleigh scattering from small particles and also because the scattering angular pattern exhibited less extreme variations when the scatterer size was slowly increased from the monodispersed value.

The corona of the laser beam was largely eliminated by a 2.2-mm diam aperture mounted 30 cm in front of the scattering cell. The length of the beam constituting the scattering volume was defined by two apertures aligned between the sample and the photomultiplier. One vertical slit of 1.0-mm width was placed next to the scattering cell. The height of the scattering volume was approximately twice the radius of the laser beam, 1.36 mm. The second aperture, a 0.8-mm diam circle, was mounted on the front of the photomultiplier located 15 cm from the sample.

The collection optics illuminated the photomultiplier at a 90° scattering angle. An Amperex XP1002 Photomultiplier with an S-20 spectral response was employed. An amplifier with a grain of ~1,000 was constructed to increase the photocurrent.

The time correlation function which gives the mean diffusion constant was determined using a Northern Scientific Multichannel Analyzer Model 625C. The amplified photopulses were fed simultaneously into the input and the trigger. The dwell time per channel was 10^{-4} s, and total collection time was 30–45. Counts from the first 100 channels were recorded after every run. Fig. 2 presents a typical autocorrelation function obtained 30 s after dispersion of a control sample.

The optical cells (Hellma, Jamaica, N.Y.) which housed the samples for aggregation studies are made of fused quartz silica glass with Teflon plugs. They have a rectangular cross section in the scattering plane with dimensions 22 × 7 mm and a total volume of 7.25 ml.

Data Acquisition and Analysis

For each sample studied, the time correlation function was recorded every 5 min for a period of 30 min after the sample was prepared. The baseline of the time dependent function used for analysis was determined by measuring the correlation function at very large correlation times for each run.

The data were analyzed by Koppel's method of cumulants (10). The logarithm of the square root of the time correlation data for the first 15 channels was evaluated after the baseline had been subtracted. Then, the slope at each channel was calculated using three successive channels (i.e., 1-2-3, 2-3-4, 3-4-5, etc). The function slope vs. the correlation time was graphed and the intercept of the graph at $\tau = 0$ was used to determine the value of the second polynomial coefficient $\overline{D}K^2$.

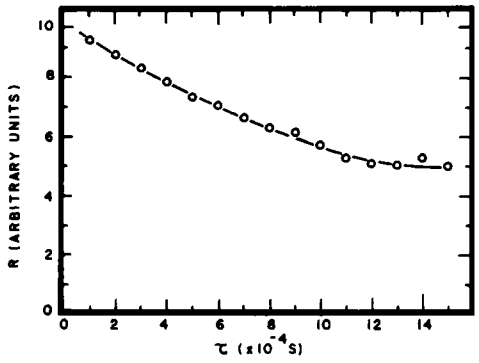

FIGURE 2

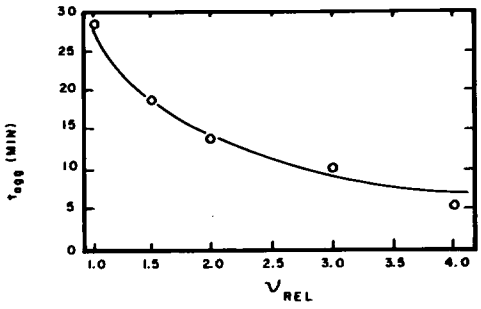

FIGURE 3

FIGURE 2 Typical autocorrelation function R vs. correlation time τ. This data was obtained for a control sample in early stages of aggregation. Only the first 15 channels are shown. R corresponds to $C_i(\tau)$ in the text plus a constant baseline term.

FIGURE 3 Aggregation time t_{agg} vs. relative initial bacteria concentration $\nu_{rel} = \nu_0/\nu_0$ control. ν_0 is the bacterial concentration in the sample studied and ν_0 control is the bacterial concentration in the control sample (8×10^6 cocci/ml). The uncertainty in the value of t_{agg} for each data point is indicated by the diameter of the open circles. The relative concentrations are believed to be accurate within 0.1%. The best fit curve predicted by Smoluchowski aggregation theory, Eq. 13, is also shown (solid line).

Values of the average diffusion constant for all runs on a particular sample were plotted against the time. The resulting graph fit a curve of the form shown in Fig. 1. The time at which $\overline{D}$ reached a value equal to e^{-1} of its initial value was recorded as the aggregation time for that sample. Three identical samples were prepared and analyzed and the resulting aggregation times were averaged to determine the reported aggregation time for each set of conditions studied.

RESULTS AND DISCUSSION

Control Sample

S. mutans studied as control samples were prepared and analyzed by the standardized technique described under procedure. The changes in the average diffusion constant, $\overline{D}$, during aggregation for a typical control sample are shown in Fig. 1. Aggregation times, t_{agg}, for three identically prepared samples were 28.5, 28.5, and 28.7 min. The solid line in Fig. 1 represents the best fit curve to the data derived from the Smoluchowski aggregation theory.

$\overline{D}$ decreases monotonically in time because larger aggregates have smaller diffusion constants. The time of aggregation, t_{agg}, is a single parameter measure of the aggregation rate which may be used effectively to analyze a variety of factors which play a role in the flocculation process.

Aggregation Time as a Function of Concentration of Bacteria

To determine the effect on aggregation of different initial concentrations of single *S. mutans* cocci, several samples were analyzed over the range 8–36 $\times$ 10^6 cocci/ml. The concentration of bacteria was varied by changing the amount of saline used to dilute an aliquot of a washed bacterial suspension with an optical density of 0.3 at 700 mm.

If two different aggregating systems have arrived at the same stage of the agglutination process, the dimensionless variable ρ of Eq. 2 must have identical values for both. Consequently, if all of the experimental factors in Eq. 2 except ν_0 are held constant:

$$\nu_0 t_{agg} = \text{constant.} \tag{13}$$

Thus the initial concentration of single cocci should vary inversely with the time of aggregation. The experimental results and the theoretical prediction are plotted in Fig. 3. There is good agreement between theory and experiment.

Aggregation Time as a Function of Dextran Molecular Weight

We investigated the dependence of *S. mutans* aggregation time on extra-cellular dextran size. We used four commercial dextran functions whose molecular weights ranged from 40 to 2,000. The bacterial concentration in each sample tested was set at the value for the control sample, 8×10^6 cocci/ml. In every case 50 dextran molecules per coccus was added to the suspension.

The aggregation times for the dextran samples are shown in Fig. 4. The control aggregation time of 28.5 min is unchanged by the addition of dextran molecular weights of 40 and 70. However, the presence of 500 and 2,000 dextran in the suspension decreases the control aggregation time by 8 and 44%, respectively.

These experiments are consistent with the polymer bridge model of aggregation. Since we found the dextran fractions formed a homologous sequence, we assume that the strength of the possible bond between a dextran and bacterium is approximately equal for all fractions.

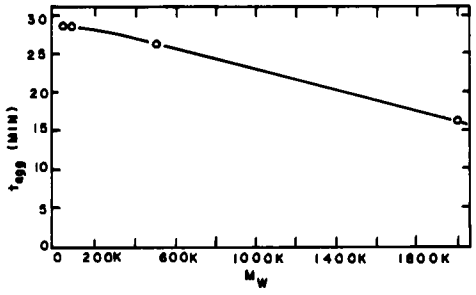

FIGURE 4

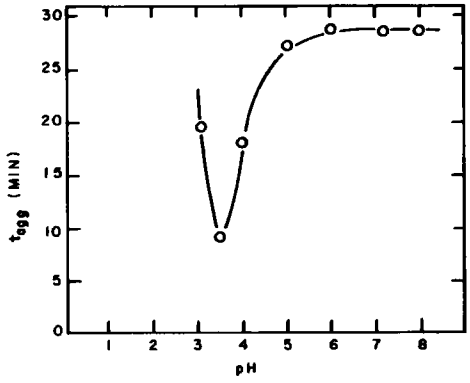

FIGURE 5

FIGURE 4 Aggregation time t_{agg} vs. dextran molecular weight M_w. In all samples, a concentration of 50 dextran molecules/coccus was added to control level bacterial suspensions just before monodispersion. The uncertainty in the value of t_{agg} for each data point is indicated by the diameter of the open circles. The average values of the dextran molecular weights in each fraction were supplied by the manufacturer. The solid line is simply a guide to the eye.

FIGURE 5 Aggregation time t_{agg} vs. pH of the suspending medium. Samples were prepared as in control suspension with a range of buffers substituted for saline. The uncertainty in the value of t_{agg} for each data point is indicated by the diameter of the open circles. The value of the pH of the suspending medium was measured using a digital pH meter accurate to within 0.1%. The solid line is simply a guide to the eye.

The lower molecular weight dextran molecules are not large enough to withstand the electrical repulsive force between cells, but higher molecular weight dextran can bridge the barrier. The presence of these larger polymers on the cell surface decreases the aggregation time by increasing the probability of sticking when aggregates collide.

The dextran size capable of inducing aggregation can be estimated from Sellen's data. We determine the average radius of the molecules in each fraction by the Einstein-Stokes relation, Eq. 12. Table I presents the pertinent values.

Since the 40 and 70 mol wt fractions fail to enhance aggregation, we conclude that the critical length lies between 70 and 173 Å. A dextran molecule with dimensions greater than the critical length can simultaneously bind to two bacteria with the strength of the bonds exceeding the electrical repulsive force between the bacteria. We further recognize that the thickness of the electrical double layer surrounding each cell which shields the bacterial charge by a counter-ion cloud may be altered by the presence of macromolecules. Studies done on erythrocytes seem to indicate that the shielding distance increases remarkably upon addition of dextran (15, 16, 17).

It is also possible that 2,000=mol wt dextran is a more effective agglutinin than the smaller component 500 because the larger molecule may be able to bind several cells at once (18). Such a conclusion is consistent with the calculated radii of the dextran molecules from Sellen's data on diffusion constants (13).

On the other hand, the 2,000-mol wt dextran showed the greatest deviation from the homologous sequence tested by Eqs. 10 and 11 through separate experiments measuring the diffusion constants and intrinsic viscosities. Therefore, the high molecular weight dextran may also bind to bacteria more strongly than the low molecular weight dextrans because of a different molecular structure and hence aid the balance of foreces necessary to produce a stable aggregate (19).

Aggregation Time as a Function of pH

The aggregation time for an *S. mutans* suspension was investigated as a function of the pH of the suspending medium. Control level samples with a bacteria concentration of 8×10^6 cocci/ml were suspended in commercially prepared buffers for pH values >4.0 and in buffers prepared in this laboratory for pH values <4.0. Measurements performed with both types of buffers at pH 4.0 gave identical results. We therefore conclude that any chemical differences

TABLE I
MEASURED PHYSICAL PARAMETERS OF DEXTRAN FRACTIONS

Fraction	Molecular weight*	Diffusion constant (from Sellen)	Hydrodynamic radius
	$M_w \times 10^{-3}$	$D \times 10^7$	R_e
40	39.5 d	3.9 cm^2/s	55.4 Å
70	67.6	3.1	69.7
500	511	1.25	172.9
2,000	~2,000	0.71‡	303.9‡

*Supplied by Pharmacia.
‡Our estimate of the value.

in the buffers did not influence the aggregation process and, in fact, we were measuring the effect of pH on aggregation.

As in our previous experiments, the graph of the average diffusion constant as a function of time for each sample agreed module a time scale multiplicative factor with the control sample curve in Fig. 1. The results of this experiment are shown in Fig. 5. When compared with the control sample, little change in aggregation time for pH values between 5.0 and 8.0 was observed. However, a dramatic increase in the aggregation rate is evident in the pH range between 3.2 and 4.5, with maximum activity occurring at about pH 3.7.

The observed differences in the aggregation time due to changes in pH are related to electric charge effects. The cell walls of bacteria contain amphoteric and hydrophilic groups (6) which acquire charges through acid-base interactions and exhibit a net negative charge when suspended in a neutral medium.

On the other hand, *S. mutans* bacteria grown in sucrose synthesize cell-bound dextran polymers which probably have varying lengths. We know that if dextran is to be effective as a bridge between bacteria, its radius must exceed some critical length determined in part by the electrostatic barrier surrounding each cell. As the pH of the medium is lowered, the magnitude of the cell surface charge is reduced, as is the size of the associated barrier. Thus smaller dextran molecules bound to the cell surface now qualify as bridging polymers since their dimensions exceed the critical barrier distance. In addition, because the cells can now approach one another more closely than previously, the attractive van der Waals force becomes more effective. At the isoelectric point, the rate of aggregation is a maximum. When the pH is lowered below this value, the bacteria become positively charged and a charge barrier appears again. Similar conclusions have been reported for dextran-induced aggregation of human erythrocytes (16, 17).

Aggregation Time as a Function of Concentration of High Molecular Weight Dextran

The aggregation time of *S. mutans* was measured upon addition of various concentrations of a high molecular weight dextran (mol wt 2,000). Dextran concentrations of up to 10^7 molecules (mol wt 2000) per coccus were added to the control sample which had an *S. mutans* concentration of 8×10^6 cocci/ml, in pH 7.2 buffer. The dextran molecules were observed to aggregate among themselves at high concentrations, but at a much slower rate than the bacteria. For example, a 1% decrease of the average diffusion constant of a suspension of dextran of mol wt 2,000 was observed during the 48 hr time period after suspension preparation. Hence the effects of aggregation of even high concentrations of dextran in the sample solution did not warrant modification of the diffusion model of bacterial agglutination.

The results of this experiment are shown in Fig. 6. At very low dextran concentrations, the aggregation time equals the value for the control sample, 28.5 min. As the extra-cellular dextran concentration increases to ~1 molecule/coccus the aggregation time begins to decrease, then sharply falls with increasing dextran concentration, and finally achieves a minimum value of 9.4 min at ~450 molecules/coccus. Increasing the dextran concentration beyond the level of maximum aggregation activity results in a sharply increasing aggregation

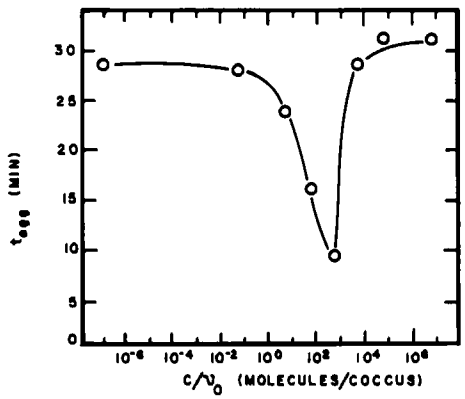

FIGURE 6 Aggregation time t_{agg} vs. high molecular weight dextran concentration c. Bacteria suspensions were prepared as in the control sample ($\nu_0 = 8 \times 10^6$ cocci/ml). Dextran having mol wt 2,000 R was added just before monodispersion. The aggregation time is plotted against the dextran concentration expressed in units of molecules per cocci on a logarithmic scale. The uncertainty in the value of the aggregation time for each data point is indicated by the diameter of the open circles. The relative concentrations are believed to be accurate to within 0.1%. The solid line is simply a guide to the eye.

time which plateaus to a maximum value of 31 min at dextran concentrations above $\sim 10^4$ molecules/coccus.

The bridging model of aggregation is qualitatively consistent with these experimental results. From Eq. 3, we find that the likelihood, P, that two aggregates will stick upon collision is a parabolic function of dextran concentration, provided that there is no change in any of the other factors appearing in ρ of Eq. 2. According to the theory, the shortest aggregation time occurs when

$$c/\nu_0 = N/2. \tag{14}$$

On the basis of the data and the assumption of the validity of the polymer bridge model of aggregation, we estimate a minimum number of 10^3 dextran receptor sites per cell.

This model is not fully valid for experiments with erythrocytes which include a nonspecific dextran binding mechanism (4). However, in our case it would appear that the rate of bacterial aggregation on dextran concentration is in general agreement with the theory of specific binding sites.

The slow aggregation plateau observed at high dextran concentrations may result from an intermediary binding mechanism. After saturation of receptor sites, bacteria may aggregate if parts of dextrans attach to each other while other segments on the molecules are in turn bound to bacteria.

CONCLUSIONS

We have shown that aggregation of *S. mutans* is consistent with Smoluchowski's theory of colloidal flocculation. We have also confirmed that polymer bridging is the predominant attractive mechanism available to overcome repulsive charge interactions between cells. We varied cell concentration, dextran concentration, dextran size, and pH of the medium to test

both the quantitative and the qualitative predictions of the model. There is good agreement between theory and experiment.

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