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Adsorption of Lysozyme from Human Whole Saliva by *Streptococcus sanguis* 903 and Other Oral Microorganisms

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Several strains of *Streptococcus sanguis*, *Streptococcus mutans*, *Streptococcus mitis*, *Actinomyces viscosus*, and *Actinomyces naeslundii* plus fresh isolates of *Streptococcus salivarius* were surveyed for their abilities to deplete lysozyme from human-whole-saliva supernatant. Bacteria were incubated in saliva for 60 min at 37°C and then removed by centrifugation, and the recovered supernatant solutions were assayed for lysozyme activity by using whole cells of *Micrococcus lysodeikticus* as the substrate. Mean lysozyme depletions by bacterial strains varied over a wide (eightfold) range. The greatest mean depletion of lysozyme (60 to 70%) was observed with *S. sanguis* (biotype I), serotype *b* of *S. mutans*, and the fresh *S. salivarius* isolates. The lowest mean depletion was noted with *S. mitis* (15%) and biotype II *S. sanguis* (ca. 30%). The remaining species and strains exhibited an intermediate degree of depletion. In studies with *S. sanguis* 903, lysozyme was depleted by normal or heated (90°C, 30 min) bacteria and could be recovered from the organism. Furthermore, under appropriate conditions, lysozyme depletion by cells at 0 and 37°C was very similar. On the basis of these observations, we concluded that depletion was due to the adsorption of lysozyme by the organism. With *S. sanguis* 903, lysozyme adsorption depended on the concentration of bacteria, time of incubation, and the ionic strength of the medium. The extent of adsorption, however, was independent of pH's of 3.9 to 8.3. When a low concentration of *S. sanguis* 903 was used, lysozyme adsorption reached saturation (4 μg of adsorbed lysozyme per 10^7 cells) at 20 μg of lysozyme added per ml. Salivary lysozyme adsorption by several other oral microorganisms (*A. viscosus* WVU 626 and WVU 627, *S. sanguis* 73x11, *S. mutans* BHT, and *S. salivarius* NG) was similar to that of *S. sanguis* 903 in sensitivity to ionic strength. Lysozyme adsorption by *S. sanguis* 903 from either a buffer solution or a saliva supernatant was more sensitive to ionic strength at 0 than at 37°C. On the basis of results from experiments in saliva versus buffer, we concluded that saliva had no major effect on the extent of lysozyme adsorption by *S. sanguis* 903 other than providing a source of ionic strength. A comparison of pH and ionic strength effects on lysozyme adsorption by *S. sanguis* 903 with literature reports of lysozyme lysis of whole cells and hydrolysis of cell walls, peptidoglycan, and $(GlcNAc)_4$ suggested that adsorption by *S. sanguis* 903 was more dependent on electrostatic interactions than was lysozyme catalysis. The possibility is discussed that anionic bacterial surface components mediate lysozyme adsorption and temper the potential effects of lysozyme on the microorganisms.

Saliva plays a major role in the maintenance of oral health by providing lubrication for oral tissues, mineralizing constituents for enamel, and providing buffering capacity. Another important function of saliva is the maintenance of a stable microbial flora. The means by which saliva exerts an influence on the oral flora are not fully understood, but it is evident that several saliva substances have the potential to directly influence the composition of the oral flora. For example, lysozyme, lactoferrin, and lactoperoxidase are thought to be inhibitory saliva components (21). Immunoglobulin A and salivary mucins also influence oral microbial composition via their potential to inhibit (35, 36) as well as promote (33) bacterial adherence.

Salivary lysozyme is presumed to contribute to the antibacterial defense mechanisms in saliva as a result of its bacteriolytic activity. It is of interest that oral bacteria appear to be resistant to the lytic activity of hen egg white lysozyme (HEWL) (11). HEWL has been reported to aggregate oral streptococci and to sensitize such aggregated bacteria to subsequent salt- or deter-

gent-induced lysis (28). In fact, when the HEWL active site is blocked, it still promotes aggregation of *Streptococcus mutans*, and the aggregated organisms are still sensitized to either salt or detergent lysis (28). The above observations suggest that lysozyme could exert effects independent of its muramidase function. Recently, human lysozyme has been reported to inhibit the growth of *S. mutans* BHT without lysis (16). Although lysis of *S. mutans* by HEWL alone was not observed, it has recently been shown that significant peptidoglycan hydrolysis occurs (12) and that lysis of the organism upon the addition of salt is not accompanied by a corresponding significant increase in additional peptidoglycan hydrolysis (12). These observations are suggested to indicate several possible mechanisms of lysozyme interaction with *S. mutans*, including association with only unrestricted (noncritical) areas of peptidoglycan, activation of autolytic enzymes, and lysozyme-membrane interactions (12, 13).

In addition to its enzymatic property, lysozyme is an extremely cationic protein (pI, ca. 10.5) and is thus positively charged at all physiological pH's. The cationic properties of lysozyme may allow it to also interact with nonsubstrate bacterial components. Nonlysozyme cationic proteins found in granulocytes damage bacterial membranes (24). Lysozyme (HEWL) has also been observed to cause membrane leakage in *S. mutans* (27). These reports thus suggest that lysozyme possesses, together with muramidase activity, nonenzymatic properties which could be involved in modulating the microbial population in the oral cavity.

In light of the possible modes of interaction between oral microbes and lysozyme, it is necessary to investigate these putative interactions to eventually evaluate the role of salivary lysozyme in the oral ecosystem. Most of the above investigations of oral microbe-lysozyme interactions were performed in the absence of saliva and with nonhuman lysozyme. Since lysozyme is suspected of interacting with other saliva constituents (9, 34), it is possible that the degree and mechanism of lysozyme interaction with the oral flora in situ may not be accurately reflected by studies conducted in the absence of human saliva. Furthermore, oral bacteria are known to adsorb saliva substances (33). These substances may also affect lysozyme-microbe interactions. We chose, therefore, to initiate studies of the interaction of salivary lysozyme with prominent oral microbes in saliva. In this initial phase of our investigations, we observed a range of interactions between salivary lysozyme and oral bacteria. Furthermore, using a strain of *Streptococcus sanguis* as a model, we report here that the adsorption of lysozyme from saliva is saturable, time and temperature dependent, sensitive to ionic strength, and relatively independent of pH.

MATERIALS AND METHODS

Bacteria. The bacteria used included *S. sanguis* strains 73x11, 903, 66x49, B-4, ATCC 10558, and M-5 and *Streptococcus mitis* strains 72x41, ATCC 10557, and 9811, obtained from B. Rosan, The University of Pennsylvania, Philadelphia. *Actinomyces* strains, obtained from G. Bowden, University of Manitoba, Winnipeg, included *Actinomyces viscosus* WVU 626, WVU 627, H103, and WA2 and *Actinomyces naeslundii* ATCC 12104, X600, and W1048. *S. mutans* strains AHT, BHT, FA-1, Ingbritt, 6715, LM-7, K1-R, and SL-1, obtained from laboratory stocks, were also studied. *Streptococcus salivarius* strains were isolated from swabs of the tongues of healthy volunteers. The swabs were streaked onto mitis salivarius agar (Difco Laboratories, Detroit, Mich.), and the plates were incubated anaerobically at 37°C for 48 h. The plates were then held in air at room temperature (48 h), and several colonies typical of *S. salivarius* were selected, purified, and used for study. All culture stocks were maintained at −70°C in 10% skim milk broth. Strains to be tested were transferred to blood agar not more than 1 week before use. For adsorption experiments, several colonies were transferred from blood agar to 3% tryptic soy–0.5% yeast extract broth and cultured for 18 h at 37°C. The streptococcal and *A. naeslundii* strains were grown anaerobically (GasPak; BBL Microbiology Systems, Cockeysville, Md.); *A. viscosus* strains were incubated in a CO_2-enriched atmosphere (GasPak). Bacteria in late log phase were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C and washed twice with an equal volume of 10 mM potassium phosphate-buffered saline, pH 7 (PBS).

Saliva. Unstimulated whole saliva was collected from at least four healthy adult donors, pooled, and clarified by centrifugation as described (9). All saliva used in this study was freshly collected, except for certain experiments in which saliva supernatant was dialyzed. Dialysis of saliva was done by using Spectrapor 3 membrane tubing against 200 volumes of either water or PBS at 4°C for 18 h, except as noted otherwise. For pH adjustments of saliva, samples were titrated with either 1 N HCl or 1 N NaOH. In some cases, salivary lysozyme was depleted by adsorption with *Micrococcus lysodeikticus* as described previously (9). Additional treatments and supplements of the saliva supernatant are described in the figure legends. All saliva supernatants were kept in an ice bath until used and were then prewarmed to 37°C 5 min before the adsorption experiments.

Lysozyme depletion by bacteria. Washed bacteria were suspended in PBS to an absorbance of 1.0 (540 nm), unless noted otherwise. For each experimental sample, one volume of bacterial suspension was centrifuged as described above, and the bacterial pellet was resuspended uniformly in an equal volume of prewarmed (37°C) adsorption medium (saliva supernatant or buffers supplemented with human urinary lysozyme [HUL]; Worthington Diagnostics, Freehold, N.J.). Incubation was then continued at 37°C for 60 min, unless stated otherwise. Controls consisted of adsorption media without added bacteria. Control and test samples were treated identically. After incuba-

tion, samples were chilled in ice, transferred to microfuge tubes, and centrifuged for 5 min in the cold in a Microfuge B (Beckman, Instruments, Inc., Fullerton, Calif.). Supernatant solutions were assayed for lysozyme activity by a spectrophotometric method in PBS (32). Several volumes of supernatant solution were assayed to ensure that enzyme activity was proportional to the volume of sample assayed. Lysozyme depletion was determined by comparing the lytic units obtained in the control sample (no bacteria) with the lytic units present in the sample exposed to bacteria. The percent lysozyme depletion was calculated from the following formula: {[(lytic units in control sample) − (lytic units in test sample)]/(lytic units in control sample)} × 100.

RESULTS

Depletion of salivary lysozyme by oral bacteria. The initial phase of this investigation included a survey of several prominent oral microorganisms to determine their abilities to deplete lysozyme in human-whole-saliva supernatant. Twenty-two strains of oral streptococci (four species) and seven strains of actinomyces (two species) were examined (Table 1). During the experiments shown in Table 1, lysis of the organisms was not evident by visual inspection. An additional experiment was performed to verify this impression. Two strains of each species shown in Table 1 were incubated for 60 min at 37°C in whole-saliva supernatant. The absorbance (540 nm) of each suspension was measured at zero time and at 60 min. No (i.e., <6%) saliva-dependent decrease in absorbance was found. Within each species, the following ranges of lysozyme depletion were observed: *S. sanguis*, 9 to 80%; *S. mitis*, 0 to 29%; *S. mutans*, 9 to 75%; *S. salivarius*, 27 to 86%; *A. viscosus*, 28 to 81%; *A. naeslundii*, 35 to 60%. In general, repeat determinations of lysozyme depletion, using independent saliva pools, yielded consistent results for a given strain. Notable exceptions to this general consistency were single determinations with *S. sanguis* 66x49 (9%), *S. mitis* 10557 (0%), and *S. mutans* Ingbritt (9%). Although we have no explanation for these inconsistent values, they were not a result of lysis of the test organisms. In any case, they represented only 4% of the total number of determinations reported in Table 1. In general, type I strains of *S. sanguis* were more active than were type II strains (mean depletion, 61 versus 33%). Serotype *b* strains of *S. mutans* were observed to be the most active *S. mutans* strains in our studies, a finding that agreed with those of earlier reports (16). *S. mutans* strains representing serotypes *a*, *c*, *d*, *e*, and *g* were only ca. 50% as active as the serotype *b* strains studied. The *S. mitis* strains exhibited a uniformly low ability to deplete lysozyme from saliva (mean depletion, 15%) relative to the other streptococcal species. A range of activity was also noted among the *S. salivarius* isolates (mean depletion, 36 to 85%), although as a group they were relatively active (67%) and similar to the serotype *b* strains of *S. mutans* (70%) and type I *S. sanguis* (61%). The *A. viscosus* and *A. naeslundii* strains were similar in their abilities to deplete lysozyme from saliva (49 and 44%, respectively). Because of its consistently high activity (mean depletion, 74%), *S. sanguis* 903 was chosen for further study.

TABLE 1. Depletion of salivary lysozyme by oral bacteria

Microorganism	Strain (serotype)	% Lysozyme depletion	Saliva pool[a]
S. sanguis	73x11 (I)	56, 52, 66	a, b, d
	903 (I)	75, 80, 66	a, c, d
	66x49 (I)	9, 78	a, c
	B-4 (II)	20, 36	a, c
	10558 (II)	38, 35	a, b
	M-5 (II)	24, 42	a, b
S. mitis	72x41	3, 7, 15	a, b, d
	10557	0, 17, 29	a, c, d
	9811[b]	16, 20, 28, 14	a, c, e, e
S. mutans	AHT (*a*)	34, 36	f, g
	FA-1 (*b*)	71, 64, 75	f, g, d
	BHT (*b*)	73, 71, 66	f, g, d
	Ingbritt (*c*)	9, 35, 36	f, g, d
	SL-1 (*d*)	32, 41	f, g
	LM-7 (*e*)	38, 30	f, g
	6715 (*g*)	32, 44	f, g
	K1-R (*g*)	18, 13, 15	f, g, d
S. salivarius	CT	74, 82	h, i
	NL	52, 77	h, i
	MW	27, 44	h, i
	LT	57, 80	h, i
	NG	83, 86	h, i
A. viscosus	WVU 626	42, 75, 32	j, k, l
	WVU 627	28, 65, 81	j, k, m
	H103	44, 49	j, k
	WA2	32, 49	j, k
A. naeslundii	12104	35, 35	j, k
	X600	44, 45	j, k
	W1048	60, 45	j, k

[a] Lysozyme activities (U/ml) for saliva pools were: a, 81; b, 71; c, 59; d, 110; e, 218; f, 186; g, 138; h, 226; i, 256; j, 188; k, 130; l, 206; m, 190.

[b] Three independently carried stocks of this strain were examined. Lysozyme depletions were: stock 1, 16 and 20%; stock 2, 28%; stock 3, 14%.

Basis of lysozyme depletion. The depletion of lysozyme from saliva supernatant by the test bacteria was estimated by loss of enzyme activity from the saliva supernatant. Lysozyme depletion might then result from (i) inactivation of the

lysozyme by some extracellular or cell-associated bacterial products, (ii) inactivation by salivary enzymes promoted by the presence of bacteria, or (iii) adsorption of lysozyme onto the microorganisms. To examine the possibility that extracellular products might account for lysozyme depletion, the following experiment was performed. Whole-saliva supernatant was preadsorbed with *M. lysodeikticus* (9) at 0°C to remove lysozyme. The preadsorbed specimen was then divided; one portion was incubated with *S. sanguis* 903 (37°C, 60 min), and the other portion was incubated as described above, with no added bacteria. After incubation, the saliva supernatants were recovered, and each was supplemented with HUL (21 U/μg) to give 248 U/ml. The HUL-supplemented samples were incubated for a further 120 min at 37°C to allow time for possible inactivation of HUL. In the control (no *S. sanguis* exposure), at 60 and 120 min of incubation, 93 and 88%, respectively, of the added HUL activity remained. In the sample exposed to *S. sanguis*, 98 and 86% of the added HUL activity was recovered after 60 and 120 min, respectively, of incubation. Thus, it is unlikely that depletion of salivary lysozyme by *S. sanguis* is owing to inactivation of lysozyme by extracellular substances.

To examine the possibility of cell-associated degradation of lysozyme, recovery of depleted enzyme from saliva-exposed bacteria was attempted. *S. sanguis* 903 cells were incubated with saliva supernatant (60 min, 37°C), collected by centrifugation, and suspended in 10 mM phosphate buffer, pH 7. After incubation (40 min, 37°C), the buffer suspension of bacteria was centrifuged, and the supernatant solution was assayed for eluted lysozyme. Of the originally depleted lysozyme, 83% was recovered. No significant degradation of cell-associated lysozyme under the standard depletion conditions was observed. Thus, depletion of salivary lysozyme was not due to degradation of lysozyme but appeared to be consistent with adsorption of the enzyme onto the organism. Accordingly, depletion of lysozyme by *S. sanguis* 903 will hereafter be referred to as adsorption of lysozyme.

In the case of the streptococcal species listed in Table 1, it also seemed possible that production of H_2O_2 over the 60-min incubation period might lead to some loss of lysozyme activity through the salivary lactoperoxidase-SCN-H_2O_2 system (10). To test this possibility, a sample of whole-saliva supernatant was supplemented with 100 μM H_2O_2 and incubated for 60 min at 37°C. A control saliva specimen (no H_2O_2 added) was also included. The H_2O_2-supplemented sample did not exhibit a loss of lysozyme activity as compared with the control (226 versus 190 U/ml). Therefore, we concluded that any production of H_2O_2 by bacteria during the saliva incubation period would be inconsequential to lysozyme activity.

Conditions for lysozyme adsorption to *S. sanguis*. In the following studies, we examined the effects of bacterial and lysozyme concentration, saliva pH, time and temperature of incubation, and effects of salts on the adsorption of salivary lysozyme by *S. sanguis* 903. The proportion of total salivary lysozyme adsorbed was dependent on the cell density present and reached maximal adsorption (81 to 85%) at an absorbancy of 1 to 1.5 (Fig. 1). Cells were not saturated with lysozyme under this maximal condition, since recovery of cells by centrifugation, followed by resuspension in another sample of a saliva pool, resulted in a further adsorption of lysozyme similar to that seen in the first cycle. Lysozyme adsorption was observed to decrease markedly on a third and fourth cycle of exposure to new saliva supernatant. Thus, cells at an absorbancy of 1.0 were ca. 30 to 50% saturated with respect to lysozyme binding capacity after a single exposure to saliva supernatant.

The adsorption of salivary lysozyme by *S. sanguis* 903 was also dependent on lysozyme concentration (Fig. 2). In this experiment, whole-saliva supernatant was first depleted of lysozyme by preadsorption with *M. lysodeikticus* (9). Next, the depleted saliva was supplemented with HUL at the indicated levels. HUL-supplemented specimens were then incubated in the presence and absence of *S. sanguis* 903. In this case, the microorganism was used at a concentration of about 1% of that required to achieve maximal lysozyme adsorption in an ex-

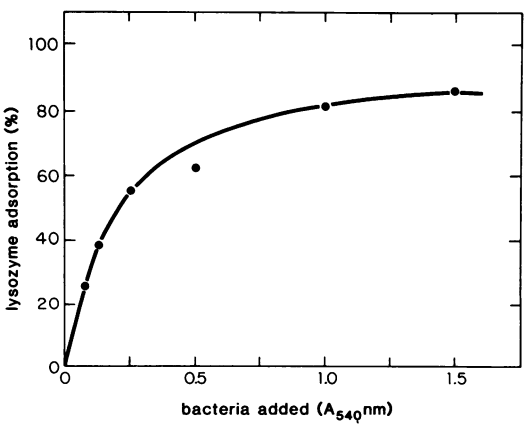

FIG. 1. Effect of bacterial concentration on salivary lysozyme adsorption. *S. sanguis* 903 at the indicated absorbances (540 nm) was exposed to saliva supernatant at 37°C for 60 min. Control saliva supernatant (without bacteria) contained 110 U of lysozyme activity per ml.

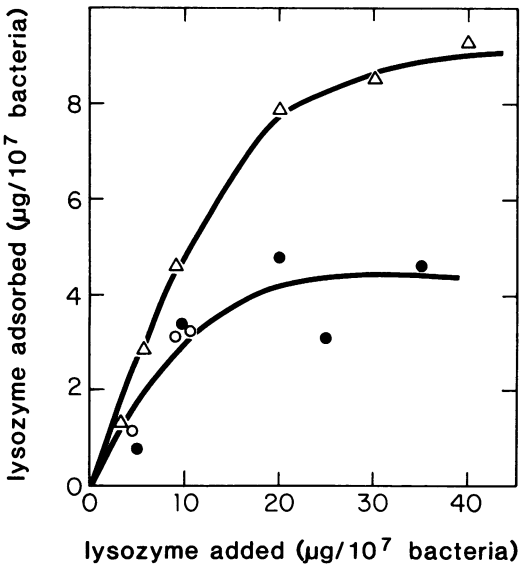

FIG. 2. Effect of enzyme concentration on lysozyme adsorption. *S. sanguis* 903 was suspended in PBS to give 10^7 colony-forming units per ml. Next, 1-ml samples were centrifuged, and the cell pellets were suspended in either lysozyme-depleted saliva (○, ●) or 25 mM ammonium acetate, pH 6.8 (△), supplemented with HUL at the indicated concentrations. Control samples at each HUL concentration lacked bacteria. Lysozyme-specific activities were: ○, 10.6 U/μg; ●, 13.6 U/μg; △, 10.2 U/μg.

periment of the type shown in Fig. 1 to create a situation in which the bacteria were limiting and lysozyme was in excess. Under these conditions, bacteria were saturated with ca. 4 μg of adsorbed lysozyme at an added HUL concentration of ca. 20 μg/ml. Adsorption was also examined in the absence of saliva. In this case, 25 mM ammonium acetate buffer (pH 6.8) was supplemented with HUL, followed by incubation in the presence and absence of limiting *S. sanguis* 903 (Fig. 2). Adsorption of lysozyme from buffer was also observed to be saturable, although the efficiency (i.e., slope) of adsorption was ca. twice that observed in the saliva experiment. Similarly, the lysozyme capacity of the cells in buffer (ca. 8 μg) was twice that observed for saliva-suspended cells.

The effect of pH on lysozyme adsorption by *S. sanguis* 903 was also examined (Fig. 3). In this case, lysozyme adsorption from pH-adjusted, dialyzed saliva and from HUL-supplemented (15 μg/ml), pH-adjusted, 10 mM sodium acetate solutions was studied. In general, pH had little influence on lysozyme adsorption in saliva or in buffer. Consistent with our earlier results (Fig. 2), lysozyme adsorption from buffer was more efficient than adsorption from saliva.

The effect of time and temperature of incubation on salivary lysozyme adsorption by *S. sanguis* 903 is shown in Fig. 4. Lysozyme adsorption was rapid at 37°C and essentially complete at 30 min. Lysozyme adsorption from saliva was also studied at an ice bath temperature. In this case, the time to reach maximal adsorption was ca. 60 min, and the extent of adsorption was only ca. 40% that obtained at 37°C (120 min). A comparison of the extent of adsorption of lysozyme at 0 and 37°C after 60 min of incubation in four other independent experiments yielded a mean value (ratio of adsorption at 0°C to that at 37°C) of 0.40.

Effect of ionic strength on lysozyme adsorption. In an effort to determine whether other saliva factors might be required to observe lysozyme adsorption to *S. sanguis* 903, a saliva specimen was dialyzed for either ca. 18 or 2 h against PBS. Upon examining the dialyzed specimens, we found no change in the lysozyme activities but a dramatic decrease in the extent of lysozyme adsorption to *S. sanguis*. For example, 2 h of dialysis against PBS reduced lysozyme adsorption ca. fivefold. Dialysis of saliva against water for 2 h had no effect on subsequent lysozyme adsorption by *S. sanguis*. At about the same time, we noted that dilution of saliva supernatant in either water or PBS had no effect on lysozyme activity but that subsequent adsorption of lysozyme from PBS-diluted saliva, but not water-diluted saliva, was markedly less than

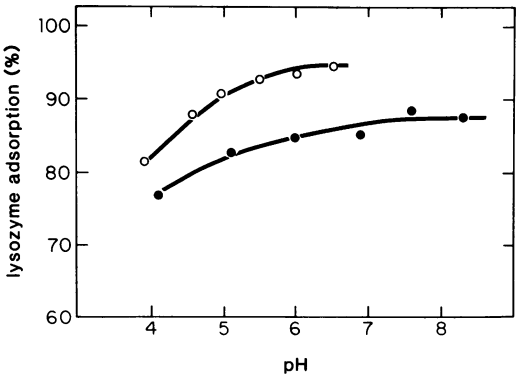

FIG. 3. Effect of pH on lysozyme adsorption. Sodium acetate (10 mM) with 15 μg of HUL per ml (○) and dialyzed saliva (●) were each incubated with and without *S. sanguis* 903. Saliva pH was titrated with either 1 N HCl or 1 N NaOH. Lysozyme activity in saliva supernatant was 244 U/ml; activity was unaffected at pH 8.3 (240 U/ml) to 5.1 (254 U/ml) but was reduced at pH 4.1 (166 U/ml). HUL activity in sodium acetate at pH 3.9 (186 U/ml) was reduced in a linear manner with an increasing pH, until it reached 154 U/ml at pH 6.

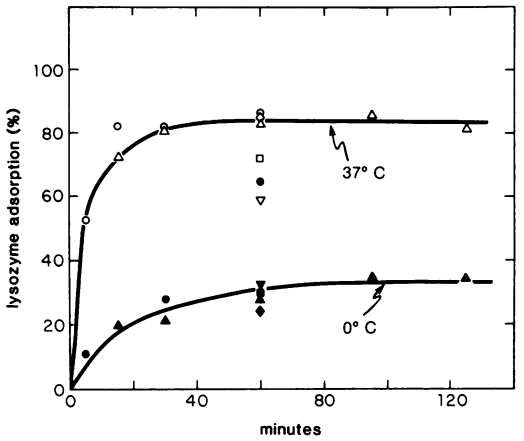

FIG. 4. Effect of time and temperature on salivary lysozyme adsorption. Saliva samples were incubated with and without *S. sanguis* 903 at 37°C (○, △, □, ▽, ◇) or in an ice bath (●, ▲, ■, ▼, ◆). At the indicated times, aliquots were removed from the incubated samples, chilled, and centrifuged, and the supernatants were assayed for lysozyme activity. Initial salivary lysozyme activities were: experiment 1 (○, ●), 212 U/ml; experiment 2 (△, ▲), 188 U/ml. Enzyme activities at the conclusion of the experiments were decreased in experiment 1 by 9% (0°C) and 16% (37°C) and in experiment 2 by 2% (0°C) and 0% (37°C). For remaining experiments, samples were taken at 60 min of incubation only. Lysozyme activities were: experiment 3 (□, ■), 205 U/ml; experiment 4 (▽, ▼), 193 U/ml; experiment 5 (◇, ◆), 182 U/ml.

that expected. These observations prompted a study of the effects of salts on lysozyme adsorption by *S. sanguis*.

The effect of added $CaCl_2$, NaCl, and potassium phosphate on lysozyme adsorption by *S. sanguis* 903 was examined. Initial experiments were performed in which separate pools of saliva were used for each salt under study. Salt concentrations were expressed as ionic strength. The results clearly indicated that added salt was inhibitory (Fig. 5A). Fifty percent inhibition of adsorption occurred at added ionic strengths of 0.04 (NaCl and $CaCl_2$) and 0.01 (potassium phosphate). It also appeared that phosphate was particularly inhibitory, thus suggesting a specific ion effect. This turned out, however, not to be the case. Supplementation of a single saliva pool with either NaCl or potassium phosphate yielded essentially identical inhibition of lysozyme adsorption when the salt concentrations were expressed as ionic strengths (data not shown). Fifty percent inhibition of lysozyme adsorption occurred at a calculated ionic strength of ca. 0.05. A comparison of the effects of NaCl and potassium phosphate on lysozyme adsorption by *S. sanguis* 73x11, *S. mutans* BHT, *S. salivarius* isolate NG, and *A. viscosus* WVU 626, using single saliva pools, demonstrated that each salt was equally effective and was inhibitory for all of the organisms tested. For these organisms, 50% inhibition of lysozyme adsorption occurred at calculated added ionic strengths of 0.025 to 0.05 (data not shown). These initial salt studies suggested that the inherent variability in the salt concentrations (ionic strengths) of pooled saliva specimens would be troublesome and prompted treatment of saliva specimens to reduce the

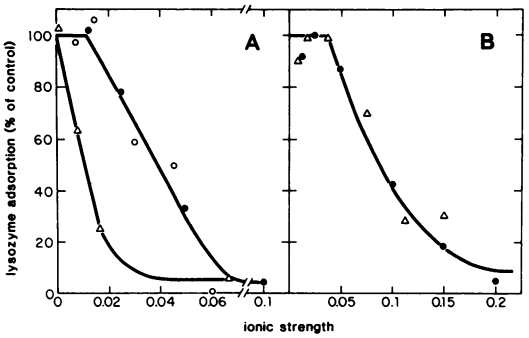

FIG. 5. Effect of ionic strength on salivary lysozyme adsorption. Salts are expressed as ionic strength added to saliva. Note ionic-strength scale difference in A and B. (A) Saliva was collected on each of 3 days. For experiment 1 (○), saliva samples (85 U of lysozyme activity per ml) were supplemented with $CaCl_2$ (range, 2.5 to 20 mM); activity was slightly reduced with 2.5 mM $CaCl_2$ (76 U/ml) and increased at 5 mM (98 U/ml) to 20 mM $CaCl_2$ (92 U/ml). For experiment 2 (△), saliva samples (146 U of lysozyme activity per ml) were supplemented with potassium phosphate (range, 1 to 20 mM); activity was slightly decreased at 1 mM (134 U/ml) and unchanged at 5 to 20 mM phosphate. For experiment 3 (●), saliva samples (146 U of lysozyme activity per ml) were supplemented with NaCl (range, 12.5 to 200 mM); activity was slightly increased at 12.5 mM (160 U/ml), 25 mM (154 U/ml), and 50 mM (156 U/ml) and slightly decreased at 100 mM (142 U/ml) and 200 mM NaCl (122 U/ml). Each supplemented saliva sample was incubated with and without *S. sanguis* 903. Control lysozyme adsorptions obtained without salt supplements were: experiment 1, 67%; experiment 2, 70%; experiment 3, 80%. (B) Samples of dialyzed (18 h, 4°C) saliva (152 U of lysozyme activity per ml) were supplemented with either NaCl (●) (range, 12.5 to 200 mM) or potassium phosphate (△) (pH 6.9; range, 6 to 100 mM). NaCl- and phosphate-supplemented samples were incubated with *S. sanguis* 903. Control samples incubated without bacteria consisted of saliva, saliva plus 200 mM NaCl, and saliva plus 100 mM phosphate. Salivary lysozyme activity was reduced with 200 mM NaCl (96 U/ml) and 100 mM phosphate (112 U/ml). Lysozyme activities (without bacteria present) at other NaCl and phosphate concentrations were estimated from a plot of activity versus salt concentration. Control lysozyme adsorption obtained without supplements was 86%.

ionic-strength variability so that further studies could be performed.

In an effort to minimize daily fluctuations in saliva ionic strength, pooled saliva samples were dialyzed against 200 volumes of water (18 h, 4°C). Such dialyzed specimens were then supplemented with the desired salts, and lysozyme adsorption was determined. A comparison of the effect of NaCl and potassium phosphate on lysozyme adsorption by *S. sanguis* 903 is shown in Fig. 5B. The two salts were equally effective, yielding 50% inhibition at an added ionic strength of 0.09. It should be recalled here that comparable inhibition of lysozyme adsorption in samples of nondialyzed saliva was noted at added ionic strengths of 0.01 to 0.05. A consideration of the differences in added ionic strength required to obtain 50% inhibition of adsorption suggests that undialyzed saliva pools contain salts within an ionic-strength range of 0.04 to 0.08. This estimate is certainly consistent with a reported calculated saliva ionic-strength range of 0.03 to 0.09 (22).

A comparison of the effect of ionic strength on lysozyme adsorption by *S. sanguis* 903 and *M. lysodeikticus* was performed, since abundant literature exists on lysozyme interactions with the latter organism (see Discussion). Dialyzed saliva was supplemented with up to 200 mM NaCl and incubated with each organism at 0°C for 10 min (to avoid lysis of *M. lysodeikticus*). The results (Fig. 6) revealed that *S. sanguis* 903 lysozyme adsorption was more sensitive to ionic strength (50% inhibition at an ionic strength of 0.025 versus 0.105 for *M. lysodeikticus*). Note also the increased sensitivity of the *S. sanguis* system to ionic strength at 0°C compared with that found in the earlier study conducted at 37°C (50% inhibition at an added ionic strength of 0.09).

To further examine the possibility that lysozyme adsorption at 0°C was more sensitive to ionic strength than adsorption at 37°C, the following experiment was performed. Dialyzed saliva was supplemented with 25 to 100 mM NaCl, and each supplemented sample was incubated in the presence and absence of *S. sanguis* 903. Duplicate samples were incubated in an ice bath and at 37°C. The results (Fig. 7A) revealed that lysozyme adsorption at an ice bath temperature was indeed more sensitive to ionic strength (50% inhibition at an ionic strength of 0.045 versus 0.090 at 37°C). It was also noted that, in dialyzed saliva (no NaCl added), lysozyme adsorption was still temperature dependent (64% at 0°C versus 81% at 37°C). When the same experiment was repeated in 25 mM ammonium acetate with 10 μg of HUL added per ml (Fig. 7B), adsorption was again more sensitive to ionic strength at 0°C (50% inhibition at an ionic strength of 0.1 versus >0.2 at 37°C). In this case, however, it should be noted that, in ammonium acetate (0.025 ionic strength), lysozyme adsorption was identical at 0 and 37°C. In a second experiment, we attempted to further reduce the saliva ionic strength by dialysis of a saliva pool with 200 volumes of 10 mM EDTA (20 h, 4°C), followed by dialysis with 1,000 volumes of water (20 h, 4°C). The dialyzed saliva was incubated for 60 min in the presence and absence of *S. sanguis* 903 at 37°C and in an ice bath. The temperature effect on lysozyme adsorption was still observed (64% at 0°C versus 86% at 37°C). Because the temperature dependence of adsorption was negated in low-ionic-strength buffer but was persistent (albeit reduced relative to nondialyzed saliva) in exhaustively dialyzed saliva, some saliva component(s) seems to be required to observe the temperature effect.

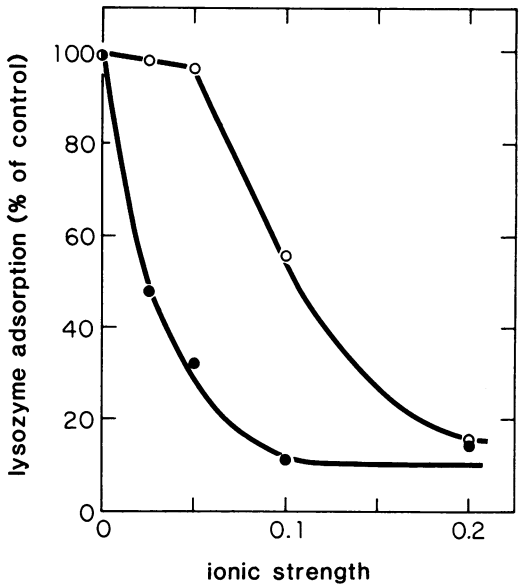

FIG. 6. Effect of ionic strength on salivary lysozyme adsorption to *S. sanguis* 903 and *M. lysodeikticus* at 0°C. Samples of dialyzed (18 h, 4°C) saliva (204 U of lysozyme activity per ml) were supplemented with NaCl (range, 25 to 200 mM). Lysozyme activity was reduced with an increasing NaCl concentration, until it reached 132 U/ml (200 mM NaCl). Each supplemented saliva sample was incubated with *S. sanguis* 903 (●) or *M. lysodeikticus* (○) or without bacteria for 10 min in ice. NaCl is expressed as ionic strength added to saliva. Control lysozyme adsorption obtained without NaCl supplements was 42% (*S. sanguis* 903) and 84% (*M. lysodeikticus*).

DISCUSSION

Basis of lysozyme depletion. The depletion of lysozyme from saliva by the oral microbes test-

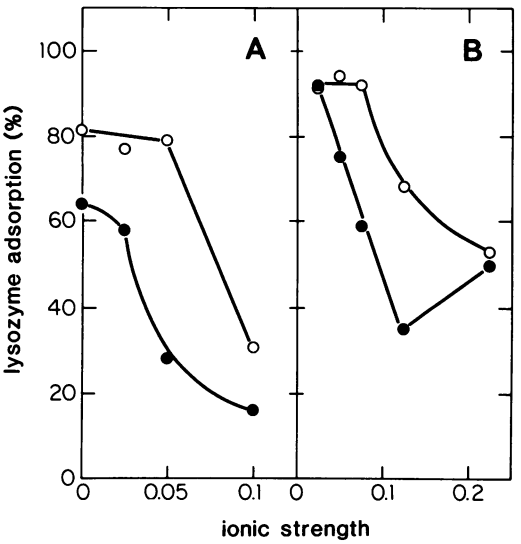

FIG. 7. Effects of temperature, medium, and ionic strength on lysozyme adsorption to *S. sanguis* 903. Samples at each ionic strength were incubated with and without *S. sanguis* 903 at 37°C (○) or in an ice bath (●). (A) Samples of dialyzed (18 h, 4°C) saliva (147 U of lysozyme activity per ml) were supplemented with NaCl (range, 25 to 100 mM). Increasing the concentration of NaCl reduced lysozyme activity to 124 U/ml (100 mM NaCl, 0°C, 37°C). NaCl is expressed as ionic strength added to saliva. (B) Ammonium acetate (pH 6.8, 25 mM) with 10 μg of HUL per ml (143 U of lysozyme activity per ml) was supplemented with NaCl (range, 25 to 200 mM). Activity was reduced after incubation with NaCl to 104 U/ml (200 mM NaCl, 0°C) and 80 U/ml (200 mM NaCl, 37°C). Ionic strength represents the total in buffer plus NaCl supplements. Note the ionic-strength scale difference in A and B.

ed could be due to adsorption of the enzyme to the microorganisms or inactivation of the enzyme by cell-free or cell-associated substances without adsorption of lysozyme. Further studies of lysozyme depletion by *S. sanguis* 903 yielded results consistent with adsorption of the enzyme to the organism and inconsistent with enzyme inactivation as the principal basis of lysozyme-microbe interaction. Consistent with the adsorption model were the following observations: (i) depletion occurred at 0°C, (ii) heat-killed cells (90°C, 60 min) depleted lysozyme (data not shown), and (iii) lysozyme could be recovered from the organism. Failure to observe lysozyme inactivation by saliva supernatants recovered from *S. sanguis* incubation mixtures clearly ruled out the possibility of extracellular inactivating substances. *S. sanguis* strains (including strain 903) have been reported to produce extracellular proteases active on certain salivary proteins (2, 18) and casein (18). EDTA (10 mM) has been shown to inhibit 60 to 90% of the proteolytic activity of these organisms. Supplementation of EDTA-inhibited proteases with Ca^{2+} resulted in virtually complete recovery of activity (18). In our experiments, however, EDTA (10 mM) reduced lysozyme depletion by only 25%. The observation that lysozyme depletion was enhanced by dialysis of saliva (even dialysis against EDTA) and was progressively inhibited by addition of $CaCl_2$ to dialyzed saliva is, thus, just the opposite of the results expected if lysozyme depletion was due to proteolytic inactivation of lysozyme. In addition, the pH optimum of two nonspecific (i.e., non-immunoglobulin A-specific) *S. sanguis* proteases was ca. 6.5 (18). At a pH of 5.0, 60 to 75% of the activity was lost. Similarly, at a pH of 8, a 40 to 50% decrease in proteolytic activity was reported. In our studies, less than a 10% change in lysozyme depletion from either saliva or acetate buffer was noted over a pH range of 3.9 to 8.3 (Fig. 3). Thus, the pH independence of lysozyme depletion is in marked contrast to the pH dependence of the nonspecific *S. sanguis* proteases. The extensive lysozyme depletion observed at 0°C (low ionic strength) is also inconsistent with enzymatic inactivation of lysozyme. Thus, in the case of *S. sanguis* 903, the depletion of lysozyme appears to be based upon adsorption of the enzyme to the organism. Whether this is the principal mode of interaction of lysozyme with all of the organisms included in the survey shown in Table 1 is unknown. On the basis of other considerations (see below), however, it also seems likely that lysozyme adsorption is the principal mode of interaction with two strains of *A. viscosus* (WVU 627, WVU 626), *S. sanguis* 73x11, *S. mutans* BHT, and *S. salivarius* NG.

Features of lysozyme-microbe interactions. Lysozyme lysis of *M. lysodeikticus* is both pH and ionic strength dependent (7, 30). Furthermore, pH and ionic strength are interdependent parameters of lytic activity. In general, lytic activity at a low pH is favored by high ionic strengths and at a high pH by lower ionic strengths. Optimal lytic activity at pH 6.2 extended over an ionic-strength range of ca. 0.03 to 0.10 (7). At an ionic strength of 0.05, maximal lytic activity was observed over a pH range of 6 to 9.5. In contrast to whole-cell lysis, degradation of cell walls (7) or hydrolysis of peptidoglycan (17) and of chitin-like substrates such as $(GlcNAc)_4$ (7) by lysozyme is less sensitive to ionic strength at pH's of 5 to 6.5. Furthermore, catalysis exhibits narrow pH activity profiles at constant ionic strengths over a range of 0.05 to 0.10. In fact, the pH dependencies of hydrolysis of spin-labeled peptidoglycan (17) and of the hydrolysis of the neutral oligomer $(GlcNAc)_4$ (7) were very similar (pH optimum of about 5; 50% inhibition at pH 3.5 to 4 and ca. 7). On the basis of these

observations, it appeared that whole-cell lysis was more dependent upon electrostatic interactions between lysozyme and cell surfaces than was hydrolysis of peptidoglycan. This notion was supported by studies in which the charges associated with the lysine and arginine residues of lysozyme were neutralized by derivatization (6). Acetylation of all lysines was without effect on hydrolysis of $(GlcNAc)_4$ but resulted in a dramatic decrease in the optimal ionic strength for activity on whole cells (at pH 6.5, optimal ionic strength was reduced to ca. 0.004). However, under optimal pH and ionic-strength conditions, acetylated lysozyme was ca. 70% as lytically active as native enzyme. Derivatization of arginine residues of acetylated lysozyme further increased the ionic-strength sensitivity of bacteriolysis but had no effect on $(GlcNAc)_4$ hydrolysis. Therefore, lysozyme catalysis, but not interaction (and possibly binding) with whole cells, was only feebly dependent upon bulk electrostatic interactions, suggesting that initial lysozyme-cell interactions are principally between the positively charged lysozyme (pI, ca. 10.5) and negatively charged cell surface (pI, <4) and not between lysozyme and peptidoglycan.

The data we have obtained are consistent with a model of a positively charged lysozyme molecule interacting with a negatively charged bacterial cell. The features of salivary lysozyme-oral microbe interactions are not typical of the reported characteristics for HEWL interactions with cell walls, spin-labeled peptidoglycan, or $(GlcNAc)_4$, all of which are generally less sensitive to ionic strength and more pH dependent. The electrostatic forces of cationic lysozyme interaction with the whole cell would be reduced by an increase in the ionic strength of the medium. Several experiments provided evidence that salivary lysozyme-oral bacteria interactions were ionic strength dependent. Adsorption sensitivity to salts was demonstrated by dilution and dialysis of saliva in PBS. Comparable inhibition of adsorption occurred with phosphate, NaCl, and $CaCl_2$ when these saliva supplements were expressed as ionic strength, but not if expressed on a molar basis (Fig. 5B). Thus, inhibition resulted from increases in saliva ionic strength rather than from ion-specific effects. Ionic-strength inhibition of lysozyme (HUL) adsorption was also observed in buffer (Fig. 7B) and therefore was not dependent on a saliva medium. A variety of organisms were very similar in the ionic-strength sensitivities of their interactions with salivary lysozyme. These strains included *M. lysodeikticus*, *A. viscosus* WVU 626 and WVU 627, *S. sanguis* 73x11, *S. mutans* BHT, *S. salivarius* NG, and *S. sanguis* 903. Ionic-strength sensitivity was, therefore, a characteristic feature of salivary lysozyme interactions with a diverse collection of organisms.

The pH independence of lysozyme adsorption also supports the electrostatic model of lysozyme-oral microbe interaction. Owing to the isoelectric points of lysozyme (ca. 10.5) and of bacteria (<4) (25), one would expect lysozyme to remain positively charged and the bacteria to maintain their negative charges throughout the pH range we examined (pH 3.9 to 8.3). Saliva pH had minimal effects on lysozyme adsorption (Fig. 3). Similarly, HUL adsorption in sodium acetate (pH 3.9 to 6.5) was essentially unaffected by pH (Fig. 3). Since pH influences on HEWL activity are reported to be a function of medium ionic strength (7), buffer ionic strength at all pH's was maintained at a constant level (0.01), and adjustments of saliva pH added minimal ionic strength (<0.003). Thus, the flat pH adsorption profile we observed was a true reflection of the pH influence on the lysozyme interaction and not a result of combined pH–ionic strength effects.

Several bacterial molecules are likely candidates to provide the appropriate negative surface charge for lysozyme interaction. These components should contain ionizing groups with pK's of <4 (i.e., carboxyls, phosphates) (19, 25) and may include teichoic acids, lipoteichoic acids, teichuronic acids, and phospholipids of the cell membrane. Additional components might also contribute to salivary lysozyme adsorption, e.g., peptidoglycan, surface proteins, and capsular substances. Many examples of lysozyme binding to nonsubstrate cellular constituents have been reported. For example, HEWL binds to *Escherichia coli* pilin (23), tubulin (1), and phospholipid vesicles (31). HEWL interactions with bacterial membranes have also been observed (27). In addition, a teichoic acid-containing extract of *S. mutans* BHT was capable of binding HEWL (V. J. Iacono, B. L. Grossbard, F. J. MacKay, and J. J. Pollock, Program Abstr. Annu. Meet. Int. Assoc. Dent. Res. 59th, Chicago, Ill, abstr. no. 795, 1981). Thus, the cationic properties of lysozyme may promote several modes of interaction with bacteria that do not involve its muramidase function. Furthermore, the particular mode(s) of interaction may vary from organism to organism.

At this time, the temperature dependence of lysozyme adsorption in saliva cannot be explained (Fig. 4). One would not expect simple electrostatic interactions between oppositely charged species to exhibit the magnitude of temperature dependence observed. Indeed, binding of an anionic dye and subsequent precipitation of HEWL were reported to be identical at 3 and 32°C (5). Lysozyme adsorption to *S. sanguis* 903, however, may be considerably

more complex than HEWL-dye binding. Since temperature is known to affect membrane fluidity (20), the temperature dependence of lysozyme adsorption could indicate an interaction with the bacterial membrane or membrane-bound structure. A lysozyme interaction with the membrane cannot fully explain the temperature dependence of adsorption, however, because adsorption in ammonium acetate was virtually identical in ice and at 37°C at a low ionic strength (Fig. 7B).

Lysozyme adsorption to *S. sanguis* 903 was more sensitive to ionic strength at 0 than at 37°C (Fig. 7). Thus, the influences of ionic strength and temperature on lysozyme adsorption to oral bacteria appear to be interdependent. Note also that adsorption of lysozyme from dialyzed saliva (Fig. 7) was more sensitive to ionic strength (especially at a low temperature) than in the corresponding ammonium acetate buffer system. Even dialysis against EDTA, followed by a second exhaustive dialysis, failed to remove this differential effect. Thus, saliva constituents also appear to promote a temperature–ionic-strength influence on lysozyme adsorption.

The adsorption of lysozyme to *S. sanguis* 903 was saturable in both buffer and saliva (Fig. 2), indicating possible site or spatial limitations to the lysozyme-microbe interaction. In this experiment, ca. 4 μg of lysozyme was adsorbed from saliva supernatant by ca. 10^7 organisms. Therefore, a typical salivary bacteria load (10^8 to 10^9 cells per ml) might have the potential to remove ca. 40 μg of salivary lysozyme. Since salivary lysozyme levels are estimated at 10 to 45 μg/ml (15, 34), a significant proportion of the total lysozyme present may be removed from saliva in appropriate intraoral areas via bacterial adsorption. A recent study of 10 adults indicated that lysozyme concentrations are 15-fold higher in dental plaque fluid than in whole saliva (3). It was suggested that bacteria were the vehicle of lysozyme entry into plaque.

Consistent differences in lysozyme adsorption were observed between buffer and saliva supernatants as adsorption media. Lysozyme adsorption was greater in ammonium acetate than in saliva (Fig. 2, 3, and 7). In the experiment shown in Fig. 2, the pooled saliva sample was not dialyzed before incubation with *S. sanguis* 903. Lysozyme adsorption in this case from saliva was ca. 50% that obtained in buffer. However, dialyzed saliva was used in the experiments shown in Fig. 3 and 7. In these two experiments, the presence of saliva decreased adsorption by only ca. 10% as compared with the buffer medium. Thus, the reduced efficiency of lysozyme adsorption noted in Fig. 2 may be primarily attributed to the increased ionic strength of undialyzed saliva.

The adsorption of salivary lysozyme to oral bacteria varied, depending on the species and strain tested (Table 1). Certain species and serotypes (*S. salivarius*, *S. sanguis* type I, and *S. mutans* serotype *b*) exhibited the greatest adsorption of salivary lysozyme. *S. mitis* and *S. sanguis* type II strains were notably weak in lysozyme interaction. The remaining *S. mutans* strains, representing several serotypes, and the *Actinomyces* were moderately efficient in salivary lysozyme adsorption. The two groups of organisms weakest in this interaction (*S. mitis*, *S. sanguis* type II) do have some common traits. The strains in these groups appear to be physiologically related (8), and classification distinctions are not clear-cut (4, 8). These organisms are also unique among oral streptococci, since both groups reportedly lack lipoteichoic acids (14, 29). Therefore, the presence or absence of certain structural components may determine bacterial capacity for lysozyme interaction.

The wide range (eightfold) of lysozyme adsorption shown in Table 1 could reflect variations in the electronegativity of the organisms. Electrophoretic estimates of surface potentials (Zeta potentials) have been reported for 28 strains (three species) of oral streptococci (26). Zeta potentials did vary over a 2.5-fold range, and some similarities were noted within species and serogroups of species. For example, *S. mutans* serotype *b* strains BHT and FA-1 were the most electronegative (Zeta potential, −40 mV) (26) and, similarly, were very efficient in lysozyme adsorption (Table 1). Further correlations between our lysozyme adsorption data and reported Zeta potentials were not obvious, however. Very few strains were common to both studies, and in the case of several *S. mitis* strains (designated *S. sanguis* IA by Olsson), Zeta potentials were very negative (−30 mV); however, lysozyme adsorption with strains of this species in our study was very low. It is possible that saliva constituents coat and thus alter surface potentials of oral microbes. If this is true, the relationship between the Zeta potential of cells in buffer and in saliva should vary among microorganisms. Thus, Zeta potentials may influence the interaction with salivary lysozyme. However, other parameters, such as specific bacteria structures (25), saliva environment, and even growth-related changes in Zeta potentials (19), may also affect lysozyme adsorption.

The biological outcome of lysozyme adsorption to oral microorganisms will depend on the nature of the interaction. For example, a primary association with non-peptidoglycan surface components may prevent subsequent hydrolysis of peptidoglycan and eventual cell lysis. Others (for review, see reference 12) have suggested that access of lysozyme to cell peptido-

glycan is modified by surface components, as may be reflected in differences in the extent of lysozyme adsorption and in the ionic-strength sensitivity of the adsorption process. For example, *M. lysodeikticus* exhibited maximal lysozyme adsorption from saliva at an added ionic strength (0.05) that caused ca. 70% reduction in adsorption to *S. sanguis* (Fig. 6). We also observed that lysis of *M. lysodeikticus* in dialyzed saliva was stimulated about 50% up to an added ionic strength of 0.10, whereas lysozyme adsorption by *S. sanguis* was reduced by 60% (unpublished data). The ready sensitivity of *M. lysodeikticus* and resistance of *S. sanguis* and other oral microbes to lysis by lysozyme, together with differences in response to ionic strength reported here, may reflect fundamental differences in the modes of initial interaction of salivary lysozyme with *M. lysodeikticus* and oral bacteria. Furthermore, it is possible that, in some cases, interactions of bacteria with other saliva constituents may reduce or promote lysozyme adsorption. Primary interaction of lysozyme with other saliva constituents already adsorbed to cells could prevent any cellular damage from resulting. Future studies of the biochemical and biological effects of lysozyme on bacteria in saliva-containing systems should aid in the eventual assessment of the role of lysozyme in control of the flora in the mouth.

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