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Effect of Lysozyme on Enterococcal Viability in Low Ionic Environments

ROBERT H. METCALF[1] AND R. H. DEIBEL

Department of Bacteriology and The Food Research Institute, College of Agricultural and Life Sciences, The University of Wisconsin, Madison, Wisconsin 53706

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The action of lysozyme on the enterococcal cell differed markedly as a function of the ionic strength of the environment. In high ionic environments ($I \simeq 0.3$), the traditionally slow lytic response and decrease in viability were noted. In a low ionic environment the majority of the cell wall was hydrolyzed, but cellular integrity was preserved and almost all cellular protein, deoxyribonucleic acid and ribonucleic acid remained with the lysozyme-cell complex. However, under these conditions, lysozyme inactivated energy-yielding metabolism, and a rapid extensive loss of viability was observed. Some other basic compounds without lytic activity on the cell wall also effected a substantial reduction in viability. The data suggest that lysozyme acts on the cell membrane to effect disruption of cellular metabolism.

We observed marked differences in the lytic activity of lysozyme (EC 3.2.1.17) on enterococcal suspensions in low and high ionic environments (8; Metcalf, Ph.D. thesis, University of Wisconsin, Madison, 1970). In a high ionic environment (e.g., I = 0.3), variation in the lytic rate was observed as a function of the strain used, the growth medium, and the phase of growth from which the cells were harvested. Viability decrease paralleled the lytic reaction. Addition of lysozyme to an enterococcal suspension in a low ionic environment (i.e., distilled water) resulted in an increase in the optical density of the mixture and a change in cell morphology from optically dense to refractile-like cells as observed under phase microscopy. A rapid loss of viability occurred. The optical density increase and morphological change were associated with the adherence of lysozyme to the cells, forming a complex which sedimented at low centrifugal forces. These changes were stable for several days. However, addition of salts to this lysozyme-cell mixture after a brief incubation period caused a complete and rapid clearing of the suspension, which was associated with disruption of the cellular integrity. The time required for the incubation of the cells with lysozyme so that the addition of salt would result in at least a 90% decrease in the optical density is referred to as the time required to obtain "sensitized" cells.

In this investigation we endeavored to substantiate and study the rapid loss of viability with lysozyme under conditions of low ionic strength. Thus, the purpose of this communication is to describe the loss of cell viability and to correlate this loss of viability with the action of lysozyme on some cellular components.

MATERIALS AND METHODS

Cultures. The enterococcal strains used in the majority of experiments were *Streptococcus faecium* F24 (ATCC 6057) and *Streptococcus faecalis* K2A. *Micrococcus lysodeikticus* was obtained from D. J. Tipper, University of Wisconsin, and *S. faecium* var. *durans* and its lytic phage 1051 came from Paul Hartman, Iowa State University. Except for the experiments with radioactive materials, cultures were grown in a medium (TYE) consisting of tryptone (1%), yeast extract (0.5%), glucose (0.5%), NaCl (0.5%), K_2HPO_4 (0.5%), *p*H 7.0 to 7.2. Cultures were grown statically at 37 C, except *M. lysodeikticus*, which was grown at 30 C on a shaker, and *S. faecium* var. *durans*, which was grown at 30 C under static conditions.

Treatment of cultures. After incubation, the test cultures were chilled and centrifuged at 4,000 × *g* in a Sorvall RC2-B refrigerated centrifuge at 4 C. The cells were washed with water or with the respective suspending solution and resuspended in this solution to an optical density of approximately 0.6 (dry weight about 0.2 mg/ml). After addition of the variables and volume adjustment, the mixtures were incubated at 37 C. In most experiments a final lysozyme concen-

[1] Present address: Department of Biological Sciences, Sacramento State College, Sacramento, Calif. 95819.

tration of 200 μg/ml was used. All water used was distilled, deionized water. The optical density determinations were made in matched 18-mm Pyrex tubes and read at 600 nm against a water blank by using a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.).

Semisynthetic medium. Several experiments were performed with *S. faecium* and radioisotopes. For these experiments it was necessary to use a semisynthetic medium in order to obtain good incorporation of the radioisotope. The composition of this medium was similar to that of Deibel and Niven (5), but differed in the following respects: glucose concentration was halved; sodium citrate, xanthine, thiamine, folic acid, and pyridoxal hydrochloride were omitted; and asparagine (20 mg/100 ml), L-methionine (1 mg/100 ml), and D-alanine (10 mg/100 ml) were added.

To obtain good growth of *S. faecium*, it was necessary to autoclave glucose and K_2HPO_4 together (autoclaved glucose effect). When these compounds were autoclaved separately and added to the semisynthetic medium, *S. faecium* grew at a relatively poor rate, about 5% of that obtained when components were autoclaved together. The rate of autolysis of cells grown in the semisynthetic medium and suspended in water was slow, with approximately a 10% decrease in turbidity after 20 hr.

Assays. *N*-Acetylhexosamines were determined by the procedure of Reissig et al. (10), with *N*-acetyl-D-glucosamine Mann Research Laboratories, New York, N.Y.) as the standard.

Ultraviolet absorption measurements. The supernatant fluid from the sedimentation of an enterococcus-lysozyme mixture in water was scanned for absorption maxima in the ultraviolet region with a Cary 15 spectrophotometer (Applied Physics Corp., Monrovia, Calif.).

Radioisotope experiments. L-Tryptophan-*3*-^{14}C (specific activity = 23 mCi/mmole) and uracil-*2*-^{14}C (31 mCi/mmole) were obtained from New England Nuclear Corp., Boston. Thymine-*2*-^{14}C (28.1 mCi/mmole) was purchased from Schwarz BioResearch, Inc., Orangeburg, N.Y.), and D-alanine-*1*-^{14}C (8.7 mCi/mmole) was obtained from International Chemical and Nuclear Corp., City of Industry, Calif. All radioisotopes were checked for purity by thin-layer chromatography.

Total radioactive estimations of *S. faecium* cells grown in the presence of L-tryptophan-^{14}C and suspended in either water or water containing lysozyme (200 μg/ml) were determined by drying duplicate 0.1-ml samples in scintillation vials overnight at 70 C, adding 15 ml of toluene-base scintillation fluid (2,5-diphenyloxazole [PPO], 4 g; dimethyl-1,4-bis-2-[5-phenyloxazolyl] benzene [dimethyl POPOP], 0.5 g; toluene to 1 liter), and assaying for radioactivity with a scintillation counter. When 5 ml of the cell suspension was sedimented at 12,000 × *g* for 10 min, duplicate 0.1-ml samples of the supernatant fluid were assayed for radioactivity in a similar manner.

The same techniques were used in experiments dealing with radioactive thymine, uracil, or D-alanine. The total radioactive count of *S. faecium* cells grown in the presence of the radioisotope and suspended in either water or water containing lysozyme (200 μg/ml) was estimated by adding 0.5 ml of these suspensions to 10 ml of Bray solution (60 g of naphthalene, 20 ml of ethylene glycol, 100 ml of methanol, 4.0 g of PPO, 0.2 g of dimethyl POPOP, dioxane to 1 liter) in a scintillation vial. When 5 ml of the cell suspension was sedimented at 12,000 × *g* for 10 min, 0.5 ml of the supernatant fluid was assayed for radioactivity as mentioned above. All radioactive counts were made for 10 min with a Packard Tri-Carb scintillation spectrometer, model 3375.

Streptococcus durans-phage procedure. The low ionic strength medium used to suspend *S. faecium* var. *durans* phage-infected cells consisted of 0.4% enzymatic hydrolyzed casein, 0.08% K_2HPO_4, 0.03% glucose, *p*H 6.8 to 7.0.

The number of plaque-forming units of *S. faecium* var. *durans* phage 1051 was estimated by overlaying a petri dish containing TYE agar with 2.5 ml of the TYE medium containing 0.75% agar to which one drop of a *S. faecium* var. *durans* culture and 0.1 ml of the phage dilution was added. Plates were incubated overnight at 30 C.

Estimation of oxygen reduction. *S. faecalis* was grown in TYE (50 ml in a 500-ml flask) on a shaker for 5 hr at 37 C. The cells were then chilled, harvested, washed, and suspended in Sorenson phosphate buffer, *p*H 7.0, 0.002 M. The cell suspension (2.9 ml) was placed in the well of the Biological Oxygen Monitor (model 53, Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio; with a Sargent SRG Recorder attached) and equilibrated at 31 C for 30 sec. Then either buffer (0.1 ml) or 2% glucose in buffer was added, the oxygen probe was inserted, and oxygen consumption was followed. Lysozyme was added by injecting 0.1 ml of a water solution (containing 620 μg of lysozyme) into the well through small Tygon tubing.

Preparation of osmotically fragile bodies. A culture of *S. faecium* taken from the exponential phase of growth was washed and resuspended in 0.01 M phosphate buffer, *p*H 7.0, in 0.5 M sucrose, so that the optical density was approximately 0.5 when diluted 1:10 in water. Lysozyme in 0.5 M sucrose was added to a concentration of 200 μg/ml, and the cells were incubated for 1 hr at 37 C. The cells were osmotically fragile after 15 min of incubation, and dilution of the cells in water resulted in about 10% of the absorbance of cells similarly diluted in 0.5 M sucrose. Phase microscopy examination of cells after 60 min of incubation showed almost all to be dense, spherical bodies, quite distinct from oval whole cells.

Chemicals. The following compounds were obtained from Sigma Chemical Co., St. Louis, Mo.: cytochrome *c*, type III from horse heart; ribonuclease A, type XII-A from bovine pancreas; ribonuclease B, type XII-B from bovine pancreas; cadaverine, free base; spermine, free base; poly-D-lysine HBr, molecular weight 40,000 to 100,000; poly-L-arginine-hydrochloride, molecular weight approximately 50,000. Poly-DL-lysine HBr, molecular weight 15,000 to 20,000, and poly-L-lysine HBr, molecular weight 3,000 to 5,000, were obtained from Nutritional Biochemi-

cals Corp. Lysostaphin was the gift of E. F. Harrison and P. A. Tavormina, Mead, Johnson and Co., Evansville, Ind. Salt-free, twice crystallized lysozyme was purchased from Worthington Biochemical Corp., Freehold, N.J..

For testing the killing effect of these basic compounds, 2 mg/ml was prepared in sterile, distilled, deionized water. In an attempt to minimize contamination, these solutions were sedimented for 10 min at approximately 4,000 × *g*, and part of the supernatant fluid was aseptically removed. This supernatant fluid was the source of the compound used in the killing experiments.

Growth in tubes employing the three-tube most probable numbers (MPN) technique (6) was checked for purity by the catalase test and by a microscopic examination of a wet mount.

Cell wall isolation. A culture of *S. faecium* was grown in TYE (18 liters) for 14 hr (into the stationary phase of growth), and the cells were harvested by centrifugation. After the cells were washed twice with cold water, 45 g of cells (wet weight) was mixed with 45 ml of water. In each trial, the cartridge for the Nossal Disintegrator was filled with 14 ml of this cell suspension and 9 ml of glass beads (SuperBrite Glass Beads, type 120-5005, 3M Co., St. Paul, Minn.). The beads had been washed in 20% sulfuric acid overnight and then washed repeatedly with water before oven drying. The cartridge underwent nine 15-sec bursts from the Nossel Disintegrator, interspersed by cooling in ice.

The pooled broken cell suspension was sedimented at 1,000 × *g* for 10 min. The pellet was discarded, and the supernatant fluid was decanted, and its contents were sedimented at 12,000 × *g* for 10 min. The supernatant fluid was discarded and the pellet was resuspended in water, and these steps were repeated six times. The resulting crude cell wall preparation was suspended in 10 ml of 0.1 M phosphate buffer, pH 7.7, to which 3 mg of trypsin (Sigma) and a crystal of ribonuclease XII A (EC 2.7.7.16) were added, and incubation was allowed for 4 hr at 37 C. The differential centrifugation was then repeated six times, and the pellet which resulted from the last centrifugation at 12,000 × *g* was used for cell wall experiments.

RESULTS

Initial viability studies. When lysozyme is added to a growth medium (i.e., high ionic strength), *S. faecium* is unaffected by its presence and the organism grows as it does in the absence of the enzyme. Even small inocula are unaffected by the enzyme in the growth medium (9). This observation afforded the exposure of *S. faecium* to lysozyme in a low ionic environment and the subsequent introduction of a diluted suspension to a growth medium for the estimation of cell viability (the growth medium was also used as the diluent).

In a typical experiment, a *S. faecium* culture was harvested from the exponential phase of growth. The cells were washed, suspended in water, and divided equally, and either lysozyme (test series, 200 μg/ml) or water (control series) was added. At various times samples were withdrawn and the three-tube MPN technique, with TYE broth as diluent, was used to estimate the number of viable cells present (Fig. 1). In the control series, there was hardly any change in viable counts during the test period. With lysozyme added, there was a rapid decrease in viability; a decrease of more than 5 logs within 3 min and over a 7-log decrease by 12 min.

After 2 min of incubation of cells with lysozyme in this experiment (as opposed to the normal 5- to 15-min incubation period for complete sensitization for the lytic response), over 99.99% of the cells were not viable, but there was only a 40% decrease in turbidity when salts were added to a portion of this mixture (to a concentration of 0.22 M). Thus, sensitization of the cells, so that addition of salts decreases turbidity by at least 90%, must come after the killing of enterococci. This suggests that the killing action of lysozyme under low ionic conditions might involve something in addition to its enzymatic activity on the cell wall.

In a related experiment, a comparison was made between the lysozyme-killing rate by the high ionic procedure (suspending cells in 0.064 M phosphate buffer, pH 7.0, containing 0.15 M NaCl) and the low ionic procedure (suspending cells in water). Cells of *S. faecium* taken from both the exponential and stationary phases of growth were tested using the MPN procedure. In both instances, after 20 min at 37 C in the presence of 200 μg of lysozyme/ml, the killing of cells by lysozyme in water far exceeded the killing of cells in buffer-saline. With an expo-

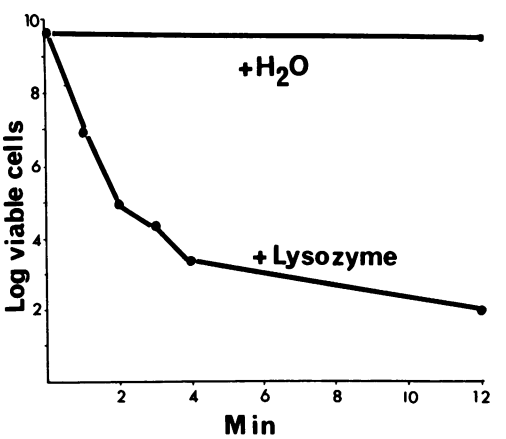

FIG. 1. *Viability of S. faecium suspended in water or in water containing lysozyme as a function of incubation time at 30 C.*

nential-phase culture, approximately a 1 log decrease in viable cells was observed in the high ionic environment, whereas greater than an 8 log decrease was observed with cells in water. No decrease in viable cells was observed with lysozyme treatment of the stationary-phase culture in the high ionic environment, in contrast to a viability decrease of over 6 logs when cells were suspended in water. The viability decrease for cells incubated in water with lysozyme was greater for *S. faecium* than it was for *S. faecalis*, but the viability decrease for *S. faecalis* was still extensive (3.5- to 6-log decrease). Thus, the rapid and extensive decrease in viability of enterococci caused by lysozyme in low ionic environments is not a characteristic of lysozyme activity in high ionic environments.

Viability of enterococci as a function of lysozyme concentration. An effort was made to ascertain the concentration of lysozyme which effected maximum death. A culture of *S. faecium* taken from the stationary phase of growth was washed and suspended in water. To 9 ml of the cell suspension, 1 ml of either water or a lysozyme solution was added, and the mixture was tempered to 37 C and incubated at this temperature for 5 min. The mixture was then diluted in TYE broth, and counts were made to estimate the remaining viable cells (Fig. 2). As the lysozyme concentration was increased to 100 μg/ml, a corresponding increase in the number of cells killed was observed. Further increases in lysozyme concentration (up to 200 μg/ml) did not greatly increase the apparent killing effect.

Metabolic aspects. The rapid loss in the viability of enterococci when cells were mixed with lysozyme in the absence of added ions corresponded with some structural changes as observed with phase microscopy. This offered little suggestion, however, as to the cause of death. But, it raised the question as to whether or not these dead cells still contained most of their cellular components or whether they were partial ghosts cells that had lost some of their internal components to the medium. Experiments were conducted to determine the fate of the cell wall and the cellular protein, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) when suspensions of enterococci in water were treated with lysozyme.

Previously, it had been observed that there was a release of *N*-acetyl-hexosamine material from the enterococci treated with lysozyme in water (9). However, this experiment did not provide information regarding the amount of cell wall material released in relation to the total amount of cell wall material present. To

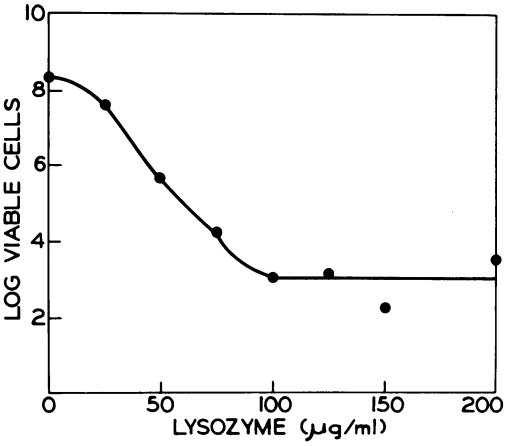

FIG. 2. *Viability of S. faecium suspended in water after incubation with various lysozyme concentrations for 5 min at 37 C.*

obtain this information an experiment was performed in which the cell wall of *S. faecium* was labeled specifically with D-alanine-^{14}C. The culture was grown for 16 hr in the presence of D-alanine-^{14}C (into the stationary phase of growth), chilled, harvested, washed, and resuspended in water or in water containing lysozyme. At various intervals, the total radioactivity and the radioactivity in the supernatant fluid after sedimentation of the cells were estimated. The radioactivity in the supernatant fluid represented the fraction of the cell wall that had been hydrolyzed and no longer sedimented with the cells. In the control series, where no lysozyme was added, the maximum percentage of radioactivity which did not sediment with the cells was 4%, as detected after 3 hr of incubation. When lysozyme was added to the cell suspension, radioactivity in the supernatant fluid increased rapidly at the start and eventually accounted for about 75% of the total radioactivity (Fig. 3). If the radioactive label had been uniformly taken up by the cell walls, this would mean that about 75% of the cell wall became solubilized when lysozyme reacted with enterococci in water. When the cells were sensitized to lysis, after approximately 12 min of incubation, only about 30% of the radioactive label failed to sediment with the cells. When cells incubated in the presence of lysozyme for 17 hr were added to a salt solution to lyse them, 84% of the total radioactivity did not sediment upon centrifugation. In comparison, 75% of the radioactivity failed to sediment with lysozyme-treated cells to which salt had not been added.

In another series of experiments, the cell protein of *S. faecium* was labeled specifically by growing cells in a semisynthetic medium (see

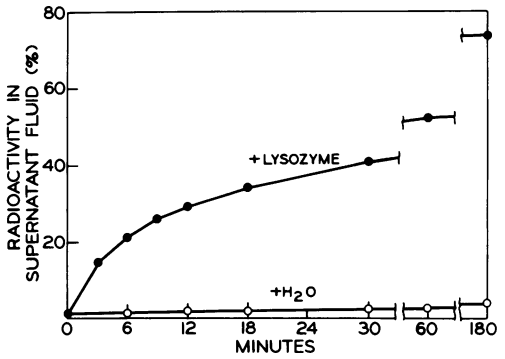

FIG. 3. *Percentage of D-alanine-^{14}C in the supernatant fluid after sedimentation of S. faecium cells in water at 12,000 × g for 10 min.*

Materials and Methods) containing L-tryptophan-^{14}C, one of the amino acids which *S. faecium* requires for growth. The culture was harvested, washed, and suspended in water or in water containing lysozyme. Total radioactivity and the radioactivity in the supernatant fluid after sedimentation of the cells were estimated. The radioactivity in the supernatant fluid represented the amount which was released from the cell-lysozyme complex. Only a small percentage (5.2%) of radioactivity was recovered in the supernatant fluid after sedimenting the cell-lysozyme complex (Table 1). The radioactivity in the amino acid pool was also estimated by boiling a cell suspension in water for 10 min, sedimenting the cells, and assaying the supernatant fluid for radioactivity. This amounted to 3% of the total radioactivity. After 60 min of incubation of the cells with lysozyme, a salt solution was added to lyse the cells. Upon centrifugation of this lysate, 65% of the radioactivity was found in the supernatant fluid.

The DNA of *S. faecium* was labeled by growing the cells in a semisynthetic medium containing ^{14}C-thymine into the stationary phase of growth. The culture was chilled, harvested, washed, and suspended in water or in water containing lysozyme. The total radioactivity and the radioactivity in the supernatant fluid after sedimentation of the cells were estimated. Even after 24 hr of incubation, only 2.6% of the total radioactivity failed to sediment with the cell-lysozyme complex (Table 1). After 15 min of incubation of the cells with lysozyme, a salt solution was added to lyse the cells. Upon centrifugation of this lysate only 30% of the radioactivity was found in the supernatant fluid.

The RNA of *S. faecium* was labeled by growing the cells in the semisynthetic medium containing ^{14}C-uracil into the stationary phase of growth. The culture was chilled, harvested, washed, and suspended in water or in water containing lysozyme. The total radioactivity and the radioactivity in the supernatant fluid after sedimentation of the cells were estimated. Treatment of *S. faecium* in water with lysozyme resulted in a rapid release (within 5 min) of about 5% of the total radioactivity to the supernatant fluid, but the rate of radioactivity released after this time slowed considerably (Table 1). After 1 hr of incubation of cells with lysozyme, a salt solution was added to lyse the cells. All of the radioactive label remained nonsedimentable after lysis of the cells. The radioactivity in the RNA pool was estimated by boiling a suspension in water for 10 min, sedimenting the cells, and assaying the supernatant fluid for radioactivity. This amounted to 9.5% of the total radioactivity. It is probable that the RNA which failed to sediment with the cell-lysozyme complex represented pool RNA, as it did not precipitate with cold trichloroacetic acid, indicating low-molecular-weight RNA.

To summarize, these experiments indicated that the rapid loss of cell viability when lysozyme was added to cell suspensions in a low ionic environment did not coincide with a mass leakage of intracellular macromolecules. Although the cell wall was solubilized under these conditions, the rate of solubilization was much slower than the rate of viability loss.

TABLE 1. *Radioactivity in supernatant fluid after lysozyme addition to a labeled S. faecium suspension in water and sedimented at 12,000 × g for 10 min*

Incubation (min)	Radioactivity in supernatant fluid[a] (cells grown with:)					
	^{14}C-uracil		L-Tryptophan-^{14}C		^{14}C-thymine	
	Control[b]	Test[c]	Control	Test	Control	Test
0 (Boiled)	9.5		3.0			
0	0.3		0		0.1	
5	0.8	5.2	0	1.1	0.2	0.7
30	1.7	6.3	0.4	1.2		
60	2.3	7.2	0.3	1.4	0.3	0.8
210					0.4	0.9
240			1.1	2.5		
330	5.4	8.6				
16 (hr)			0.8	5.2		
18 (hr)	10.1	10.7				
24 (hr)					0.9	2.6

[a] Percent of total counts per minute.
[b] No lysozyme added.
[c] Lysozyme added (200 μg/ml).

In an effort to ascertain whether or not total cellular metabolism was impaired, a series of experiments was performed to test the ability of phage to multiply in the lysozyme-treated cell. An exponential-phase culture of *S. faecium* var. *durans* was introduced to its lytic phage (1051) for 2 min in TYE to allow absorption and penetration. The multiplicity of infection was approximately three lytic phages per cell. The cells were then chilled by pouring over ice, sedimented, washed, and resuspended in distilled water. One volume of lysozyme (test series, 2 mg/ml) or water (control series) was added to the cell suspension (one volume), followed by eight volumes of the low-ionic-strength medium (described in Materials and Methods). This medium provided enough nutrients for the completion of the phage lytic cycle, but allowed for sensitization of the cells by lysozyme. The mixtures were incubated at 30 C, and at various intervals 5-ml samples were removed, chilled, and centrifuged at 12,000 × *g* for 10 min, and the resulting supernatant fluids were assayed for phage titer. In addition, phage-infected cells treated with lysozyme were resuspended in 4.5 ml of distilled water and incubated for 10 min at 30 C. One-half milliliter of a 2.0 M NaCl solution was added to lyse the cells. The resulting lysate was assayed for phage titer. A control experiment demonstrated that 0.2 M NaCl did not affect phage titer.

In the control series, where no lysozyme was present, a 4-log increase in phage titer was observed during the incubation period (Fig. 4). In the test series, where lysozyme was added, no increase in phage titer was observed, even when the cells were lysed by the addition of salt and the resulting lysates were assayed for phage. Thus, normal phage development could not occur in enterococci treated with lysozyme in a low ionic environment.

The above experiments suggested a defective metabolism. Several additional experiments were undertaken to gain information on the possible nature of the metabolic defect(s). When a culture of *S. faecium* was washed and suspended in a phosphate buffer (*p*H 7.0, 0.001 M) containing glucose (0.5%) and bromocresol purple (0.004%), the indicator color did not change to show acid production from glucose during the 4-hr incubation period with any concentration of lysozyme which produced sensitized cells (40 μg/ml and higher). In the control without lysozyme, acid production was detected within 15 min.

In other experiments, the ability of cells to consume oxygen in the presence of lysozyme in a low ionic environment was estimated. *S. faecalis* consumed oxygen readily when grown aerobically in TYE medium and then suspended in a weak phosphate buffer (0.002 M, *p*H 7.0) containing glucose (Fig. 5). When lysozyme was added to a suspension of *S. faecalis* treated in an identical manner, oxygen uptake continued but at a decreased rate for about 1 min. In the second minute after lysozyme addition, oxygen consumption ceased completely. A similar cessation of oxygen uptake was observed immediately after lysozyme was added to a suspension of *M. lysodeikticus* in 0.002 M phosphate buffer solution containing yeast extract as the substrate instead of glucose. Thus, lysozyme appears to disrupt the energy-generating mechanisms of susceptible cells in low ionic environments.

Effect of lysozyme on osmotically fragile bodies. The defective carbohydrate catabolism focused our attention on the possible alteration of the cell membrane by lysozyme. Many previous investigators (see Discussion) have associated membrane disruption and metabolic impairment with various basic compounds. Experiments were performed in an effort to reveal an alteration of the cell membrane in the presence of lysozyme. Osmotically fragile bodies (OFB) were prepared (see Materials and Methods), washed carefully in 0.5 M sucrose (sedimented at 500 × *g* for 10 min), and

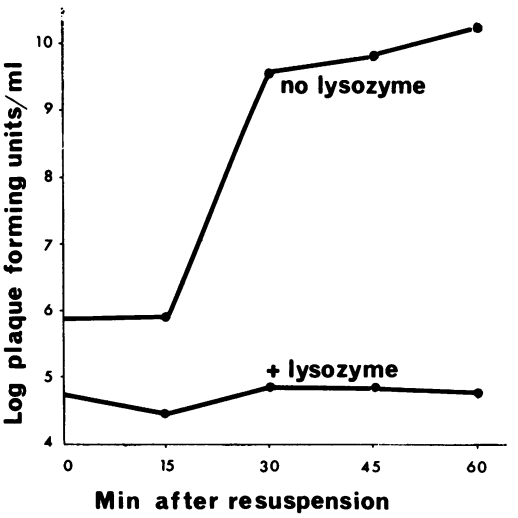

FIG. 4. *Effect of lysozyme on the reproduction of S. faecium var. durans phage 1051 in cells infected in TYE medium, washed, and resuspended in a low-ionic-strength medium. Phage titers for lysozyme-treated S. faecium var. durans cells were estimated by combining the phage count from the supernatant fluid with the count from the cells lysed by salt addition.*

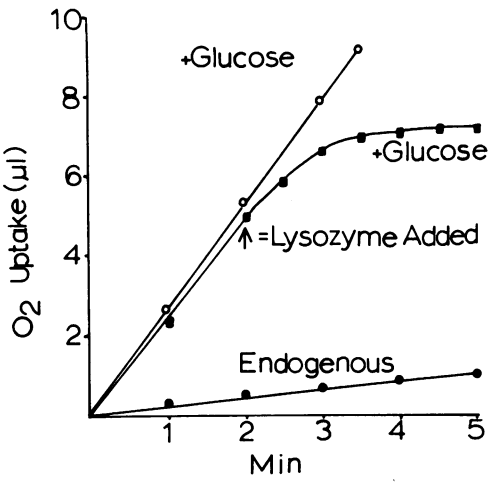

FIG. 5. *Effect of lysozyme on oxygen uptake of aerobically grown S. faecalis cells in 0.002 M phosphate buffer, pH 7.0. Where indicated, 0.1 ml of a 2% glucose solution in buffer was added to 2.9 ml of cell suspension (optical density approximately 0.95). A final lysozyme concentration of 200 µg/ml was added in one experiment (■) after 2 min as indicated by arrow.*

resuspended in this osmotically protected environment to an optical density of about 0.4. Lysozyme (in 0.5 M sucrose) was added to the test series and sucrose alone to the control series. There was no significant change in the optical density of the test series. Microscopically, however, there was a rapid change in OFB morphology in the test series as compared with the control series. The OFB looked damaged in that they lost much of their density and clumped together. In addition, cell debris was observed in the suspending medium, and the lysozyme-OFB mixture eventually became floccular. It thus appears as if lysozyme does cause some adverse effect on the cell membrane, an effect which may be related to the killing of the cell.

Effect of other basic compounds. The previous experiments led to a consideration of the effect of other basic compounds when added to an enterococcal suspension in a low ionic environment. We desired to compare not only the killing effect but also the peculiar and significant increase in optical density that is associated with lysozyme in the low ionic environment. The cause of the increased optical density is not known, and its relationship to the death of the cell has not been established.

A *S. faecium* culture, harvested in the stationary phase of growth, was washed and suspended in water. Optical densities were estimated at 20 min, and the number of viable cells was estimated by using the MPN technique. A salt solution was then added to the mixtures (final NaCl concentration was 0.3 M), and the optical densities were again determined.

In the first experiment (Table 2) some of the basic substances (especially cytochrome *c*) effected an increase in optical density that paralleled that of lysozyme. None, however, caused a clearing reaction similar to lysozyme upon NaCl addition. A slight decrease in the viable count was observed, but none was comparable to lysozyme. In the second trial, lysostaphin also effected a large increase in optical density, but neither lysis upon NaCl addition nor a viability decrease was noted. These results tend to negate any direct association of the increase in optical density with loss of viability. The high-molecular-weight polymers of lysine and arginine evidenced a moderate increase in optical density and practically no lysis upon salt addition. A significant killing effect was noted, however, as the 4-log decrease in viable cells represents a 99.99% reduction in the population. In terms of the number of cells killed, these substances approach the bactericidal potential of lysozyme.

Response of isolated cell walls. Although

TABLE 2. *Effect of basic substances on optical density, lysis, and viable count of Streptococcus faecium suspensions in water*

Substance added (200 µg/ml)	Optical density after		Log viable cells/ml after 20 min[b]
	20 min	20 min + NaCl[a]	
Trial 1			
Control	0.49	0.47	9.18
Cytochrome *c*	1.00	0.66	8.36
Ribonuclease A	0.85	0.70	8.36
Poly-DL-lysine[c]	0.67	0.55	8.79
Lysozyme	0.95	0.01	—[d]
Trial 2			
Control[e]	0.54	0.54	8.63
Poly-D-lysine[c]	0.66	0.59	4.63
Poly-L-arginine[c]	0.82	0.80	4.63
Lysostaphin	0.92	0.74	8.63
Lysozyme	1.07	0.01	—[d]

[a] Final NaCl = 0.3 M.

[b] Sample for viable counts taken before NaCl addition.

[c] Poly-DL-lysine, molecular weight = 15,000 to 20,000; poly-D-lysine, 40,000 to 100,000; poly-L-arginine, 50,000.

[d] Less than 1 cell/ml.

[e] Similar results obtained with poly-L-ornithine, poly-L-lysine (molecular weight 3,000 to 5,000), and spermine.

the initial increase in optical density associated with lysozyme activity in the low ionic environment could not be definitively related to viability, we nevertheless deemed it desirable to determine the cellular structure related with the reaction. The most probable structure in this reaction was the cell wall, and, in an experiment to test this supposition, cell walls of *S. faecium* were isolated (see Materials and Methods) and suspended in water. Water was added to the control series and lysozyme to the test series (200 μg/ml), and the mixtures were incubated at 37 C (Fig. 6). No change in the optical density of the control series was observed during the incubation period. When lysozyme was added to the cell wall preparation, there was an immediate increase in the optical density, from the value of the control (about 0.6) to approximately 0.9. As the incubation proceeded, there was a gradual decrease in the optical density, indicating dissolution of the cell wall. This was confirmed by assaying the supernatant fluid for *N*-acetylhexosamines after sedimentation of the wall-lysozyme mixture. An increasing amount of this cell wall constituent was found in the soluble fraction. In the control series, no *N*-acetylhexosamines were detected after centrifugation of the wall suspension.

DISCUSSION

Among the gram-positive cocci, the enterococci are intermediate in their sensitivity to lysozyme when tested in cell suspension by traditional, high-ionic-strength procedures. Because lysis of enterococcal cell suspensions proceeds at a much slower rate than it does with *M. lysodeikticus* suspensions, we believe that the enterococci serve as better models for investigation of lysozyme activity under varying ionic conditions.

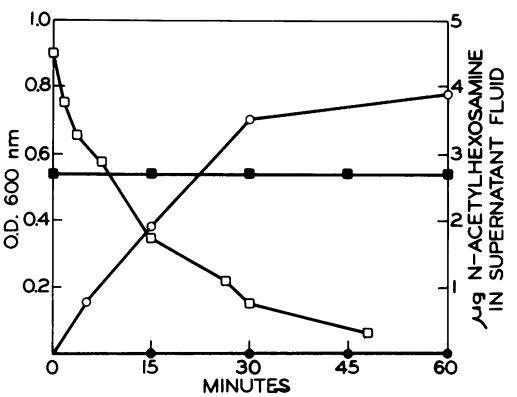

FIG. 6. *Optical density and the presence of nonsedimenting N-acetylhexosamines of a S. faecium cell wall suspension in water treated with lysozyme. Symbols:* ■, *optical density of control series, no lysozyme added;* ●, *nonsedimenting N-acetylhexosamine from control series;* □, *optical density of test series, lysozyme added;* ○, *nonsedimenting N-acetylhexosamine from test series.*

It was reported previously that the lytic rate of enterococci by lysozyme was increased when salts were initially absent from the suspending solution but added after a brief incubation period (9). Now, it is evident that there are additional differences aside from the lytic rate between lysozyme action on enterococci in high and low ionic environments.

When lysozyme is added to an enterococcal suspension in water at 30 or 37 C, it rapidly adheres to the cells, resulting in a turbidity increase of the suspension. Because a similar rapid increase in the turbidity of a suspension of isolated *S. faecium* cell walls upon lysozyme addition was observed, it appears as if the initial turbidity increase of whole-cell suspensions is the result of the adherence of lysozyme to the cell wall. It should be noted that this reaction only takes place in low ionic environments.

Within the first few minutes after lysozyme addition to an enterococcal suspension in water, the cells become metabolically inactive and they are killed. A 3- to 5-log decrease in viability is observed within 3 min, with 200 μg of lysozyme per ml at 30 C. Death to the cells comes before the cells are sensitized, so that they will display at least a 90% decrease in turbidity when a salt solution is added. Sensitization of the cells to lysis comes after approximately 30% of the cell wall is made soluble and no longer sediments with the cell. Further incubation of the cells with lysozyme results in dissolution of approximately 75% of the cell wall; but peculiarly enough, a very stable, discrete cell structure appears microscopically, which contains the large majority of the cell protein, DNA, and RNA. The forces holding this cell-lysozyme complex together remain to be identified. Colobert and Dirheimer (4) reported that a digestion product of *M. lysodeikticus* cell walls formed an insoluble complex with lysozyme in the absence of salt. Perhaps something similar is occurring with lysozyme being able to complex with any cell wall fragment in a manner which would maintain cellular integrity. This is especially difficult to envision when lysis of isolated *S. faecium* cell walls in water proceeded without any apparent end-product inhibition. Apparently, lysozyme is able to replace or greatly

fortify the remaining or residual cell wall of the enterococci.

In low ionic environments, lysozyme effected a rapid and extensive decrease in the viability of the enterococci, completely unlike lysozyme action in high ionic environments. Several lines of evidence suggest that the killing effect is associated with lysozyme action on the cell membrane. First, sensitization of enterococci and the subsequent lysis of cells upon salt addition proceeded in 0.5 M sucrose as it did in water (Metcalf, Ph.D. thesis, University of Wisconsin, Madison, 1970). As protoplasts were not produced in the osmotically protected medium, lysozyme appears to be doing something more than just removing the cell wall. Second, when lysozyme was added to a protoplast preparation of *S. faecium* in 0.5 M sucrose, damage to the protoplasts was observed microscopically. Third, two nonenzymatic, basic compounds (i.e., poly-D-lysine and poly-L-arginine) also effected a large killing of enterococci suspended in water.

The adverse effect of many basic compounds on different genera of microorganisms has been reported by various workers (1–3, 7, 12, 13). Yphantis et al. (13) reported that ribonuclease, protamine, lysozyme, cytochrome *c*, myoglobin, and serum albumin caused the release of 260 nm-absorbing material when added to suspensions of *Candida utilis* in water. They also reported that these compounds released the cellular amino acid pool into the medium and effected approximately a 2-log drop in viability by acting on the cell membrane. They suggested that the positively charged groups on the proteins combined with the negative charges on the membrane surface to cause a distortion of the membrane with ensuing lesions. The presence of cations strongly inhibited this interaction, except in the cases of protamine and bovine serum albumin. Svihla et al. (11) reported that, in a water suspension, cytochrome *c* penetrates into the yeast cell.

With enterococcal suspensions in water, there is no major leakage of proteins, pool amino acids, or nucleic acids in response to lysozyme, and only a few of the basic compounds tested possessed a significant bactericidal activity. The possible reaction of lysozyme with cell membrane components to cause loss of cell viability is in accord with the results obtained in this study. However, the mechanism by which lysozyme forms a stable complex with enterococcal cells in water, while still solubilizing the majority of cell wall, remains to be elucidated.

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