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Effects of Lysozyme and Inorganic Anions on the Morphology of *Streptococcus mutans* BHT: Electron Microscopic Examination

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The effects of hen egg white lysozyme and the inorganic salt sodium thiocyanate on the integrity of *Streptococcus mutans* BHT were studied by transmission electron microscopy. Both control cells and cells exposed to NaSCN possessed thick outer cell walls and densely staining inner cell walls juxtaposed to the plasma membranes. In the presence of NaSCN, however, the *S. mutans* BHT nucleoid was coagulated into thick electron-dense filaments. Exposure of *S. mutans* BHT to 150 μg of hen egg white lysozyme per ml resulted in the progressive destruction of both the cell walls and the plasma membranes. The enzyme appeared to affect the region of the cell wall septum, and exposure to 150 μg of hen egg white lysozyme per ml for as short a time as 10 min resulted in visible morphological cell wall alterations. At 30 min, ultrastructural observations revealed that the majority of the cells were in the process of expelling a portion of their cytoplasmic contents from the septal and other regions of the cells at the time of fixation. After 3 h of incubation in the presence of this high lysozyme concentration, gelled protoplasmic masses, which were free from the cells, were evident. In addition, extensive damage to the outer and inner cell walls and to the plasma membranes was apparent, although the cells maintained their shape. On some areas of the cell surface, the outer cell wall and plasma membrane were completely absent, whereas at other locations the outer cell wall was either split away from the inner cell wall and plasma membrane or distended from an area free of inner cell wall and plasma membrane. Upon addition of NaSCN to the hen egg white lysozyme-treated cells, both the gelled protoplasmic masses and the damaged cells exhibited an exploded appearance and existed as membrane ghosts, cell wall fragments, or dense aggregates of cytoplasmic components. The effects of a low lysozyme concentration (22.5 μg/ml) on *S. mutans* morphology were less pronounced at short incubation times (i.e., 10 and 30 min) than those that were observed with a high enzyme concentration; however, breaks in the cell walls and dissolution of the plasma membranes with resulting cell lysis were visible after a prolonged (3-h) incubation and after subsequent addition of NaSCN.

Salton (23) and Grula and Hartsell (11), in their early investigations of the mechanism of lysozyme action, used electron microscopy to demonstrate that the enzyme caused destruction of bacterial cell walls. More recent studies have stressed the need for electron microscopy in investigations of lysozyme bacteriolysis because of possible problems of interpretation sometimes associated with the results of biochemical assays (2, 27). A variety of microorganisms have been previously examined for their interaction with hen egg white lysozyme (HEWL, EC 3.2.1.17) by this technique (3, 6, 13, 26, 28). In this communication, the results of ultrastructural studies of the oral microorganism *Streptococcus mutans* BHT are presented.

We are currently using *S. mutans* BHT as a model bacterium to explore the bacteriostatic, bactericidal, and bacteriolytic properties of hen and human lysozymes. *S. mutans* BHT is very sensitive to the growth inhibitory and killing effects of these enzymes (15). However, radiochemical and biochemical assays suggest that the presence of lysozyme alone does not cause a release of DNA from cells with resultant cell lysis (10). Bacteriolysis does take place when appropriate concentrations of inorganic salts are added to lysozyme reaction mixtures (21). Bio-

chemical analyses also indicate that the majority of the cell wall peptidoglycan of *S. mutans* BHT is hydrolyzed by lysozyme treatment alone and that additional strategically important peptidoglycan is lost from these cells when inorganic salts are added to reaction mixtures (9). Electron microscopic observations noted in this investigation confirm that cell lysis occurs rapidly with salt addition and surprisingly illustrate that a significant percentage of the cells are in fact lysed with lysozyme alone even though released DNA is not detectable in cell lysates (10). Moreover, our ultrastructural studies provide visual clues to a determination of how lysozyme exerts its antibacterial effects.

MATERIALS AND METHODS

Bacterial cultures and growth. *S. mutans* BHT, a serotype *b* strain (18), was kindly provided by H. Jordan, Forsyth Dental Center, Boston, Mass. Primary cultures were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) to the late log phase (optical density of 0.80 at 675 nm, 1-cm light path) and then inoculated as a 1:400 dilution into FMC synthetic medium (25). Incubations were carried out at 37°C, and cultures were harvested at the late log phase of growth (optical density of 0.90 at 675 nm). Cell pellets were washed three times in ice-cold distilled water and suspended in 0.025 M ammonium acetate, pH 6.8, to an optical density at 700 nm of 0.40 (10^9 cells per ml, Petroff-Hausser bacterial chamber) immediately before lytic assays were performed.

Assays. Cell suspensions were preincubated with stirring at 37°C. After 1 h, HEWL was added to a final concentration of either 22.5 (low concentration) or 150 (high concentration) μg/ml, and incubation was continued for an additional 3 h. Subsequently, either NaSCN or NaF was added at a 0.1 M final concentration for an additional 1-h incubation. Control suspensions contained neither HEWL nor inorganic salts. Reaction mixtures in which inorganic salts were added to the cells in the absence of HEWL were also examined. In preparation for electron microscopy, samples (9 ml each) were removed during the time course of the reaction and mixed with glutaraldehyde (1 ml, 50% [vol/vol]; Polysciences Inc., Warrington, Pa.) in thick-walled glass centrifuge tubes (12 ml; Ivan Sorvall, Inc., Norwalk, Conn.).

Electron microscopy..After prefixation in 5% glutaraldehyde (vol/vol) for 2 h at 4°C, the cells were centrifuged at 27,500 × *g* for 1 h. The pellets were sliced into approximately 2-mm^2 sections, and the sections were washed for 15 min (three times, 5 min each) in 0.1 M sodium cacodylate buffer (pH 7.4) and postfixed in 1% osmium tetroxide in cacodylate buffer (pH 7.4), for 1 h at room temperature. The sections were then washed (three times, 5 min each) with 0.1 M maleate buffer, pH 5.15, and stained en bloc for 1 h at room temperature with 1% (wt/vol) uranyl acetate in 0.1 M maleate buffer, pH 6.0. The stained sections were washed in maleate buffer, pH 5.15, and dehydrated in a graduated cold ethanol series (50 to 100%). Propylene oxide acted as a transitional solvent for embedding in Epon (17).

Ultrathin sections were cut on a Porter-Blum MT2 ultramicrotome and placed on 200-mesh grids coated with 0.3% Formvar film for low-magnification observations and on 300-mesh uncoated grids for high-magnification observations. The thin sections were sequentially stained for 25 min with freshly saturated uranyl acetate in 50% ethanol and for 7 min with lead citrate (22). All thin sections were examined and photographed in a JEOL 100B or JEOL 100S transmission electron microscope operating at 60 and 80 kV, respectively.

Biochemicals. Sodium thiocyanate and ammonium acetate were purchased from Fisher Scientific Co., Pittsburgh, Pa., and sodium fluoride was purchased from J. T. Baker Chemical Co., Phillipsburg, N.J. HEWL (3× crystallized) was obtained from Sigma Chemical Co., St. Louis, Mo., and the enzyme concentration was determined from the extinction coefficient of $E^{1\%}_{1\,cm}$ = 26.9 at 280 nm (16).

RESULTS

Control cells (Fig. 1) of *S. mutans* BHT incubated for a period of 4 h in 0.025 M ammonium acetate, pH 6.8, were morphologically similar to *Lactobacillus casei* and *Lactobacillus plantarum* cells (26). The cells possessed a cell wall approximately 28 to 33 nm in diameter, consisting of an outer cell wall (17 to 22 nm thick) and an inner cell wall (approximately 11 nm thick). The inner cell wall was juxtaposed to the lightly staining plasma membrane. *S. mutans* BHT exposed to NaSCN (Fig. 2) was identical in morphology to untreated control cells (Fig. 1), with the exception of a markedly coagulated nucleoid. The coagulated nature of the nucleoid in the presence of the inorganic salt (i.e., NaSCN) is in agreement with the observations of Gilpin and co-workers (7) in their studies of salt activation of autolytic enzymes in a mutant of *Staphylococcus aureus*.

Exposure of *S. mutans* BHT to high concentrations (150 μg/ml) of HEWL resulted in significant alterations in gross morphology. As early as 10 min of incubation and characteristic of cells incubated for 30 min was the ejection of swollen cytoplasmic material (Fig. 3 and 4), with little apparent morphological alteration of the cell wall and plasma membrane. In a majority of instances, the electron-opaque cytoplasmic material appeared gelled or coagulated at the septal regions of dividing cells. However, gelled masses were also noted attached along the periphery of many cells. After a 3-h incubation in the presence of 150 μg of HEWL per ml, there was extensive damage to the cell wall and plasma membrane of *S. mutans* BHT (Fig. 5). Not only had the cell wall detached from the plasma membrane, but it was also broken and markedly degraded (Fig. 5). Figure 5 (inset) illustrates where portions of the cell wall had been stripped away, leaving thin and amorphous fragments. In

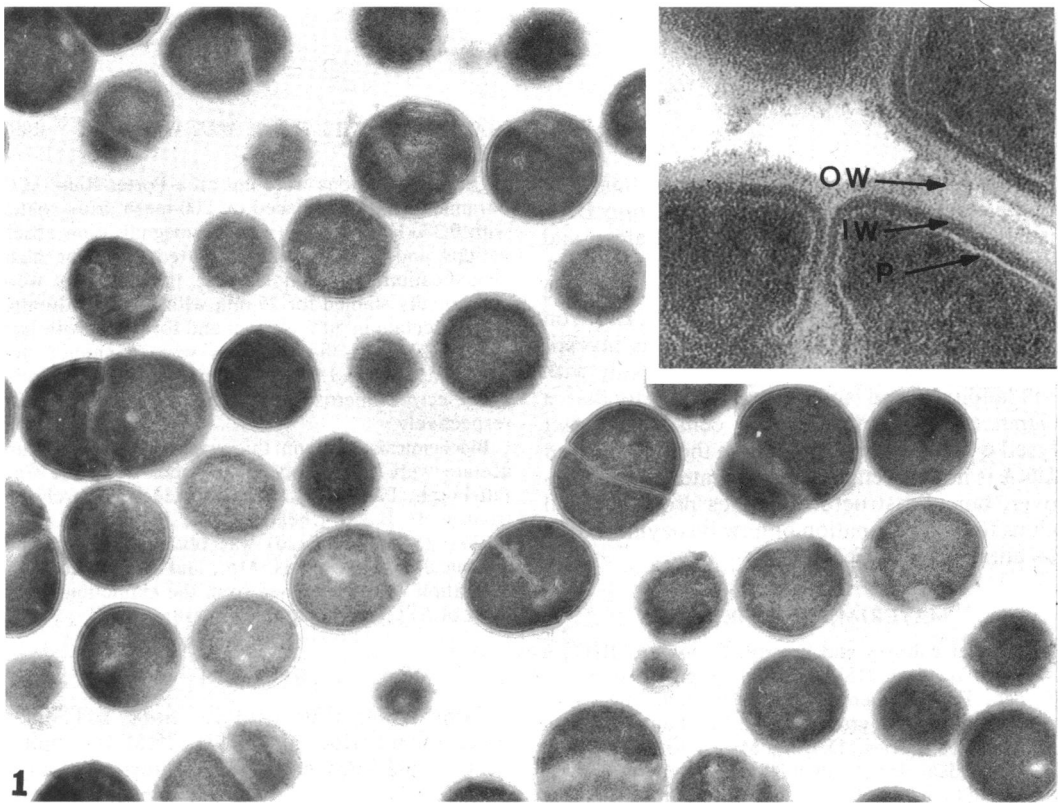

FIG. 1. Electron micrographs of *S. mutans* BHT control cells. Cells were incubated for a period of 4 h. Low magnification (×27,000) demonstrates a homogeneous population of normal cells. (Inset) High-magnification electron micrograph showing well-defined outer cell wall (OW), dense inner cell wall (IW), and plasma cell membrane (P) (×207,000).

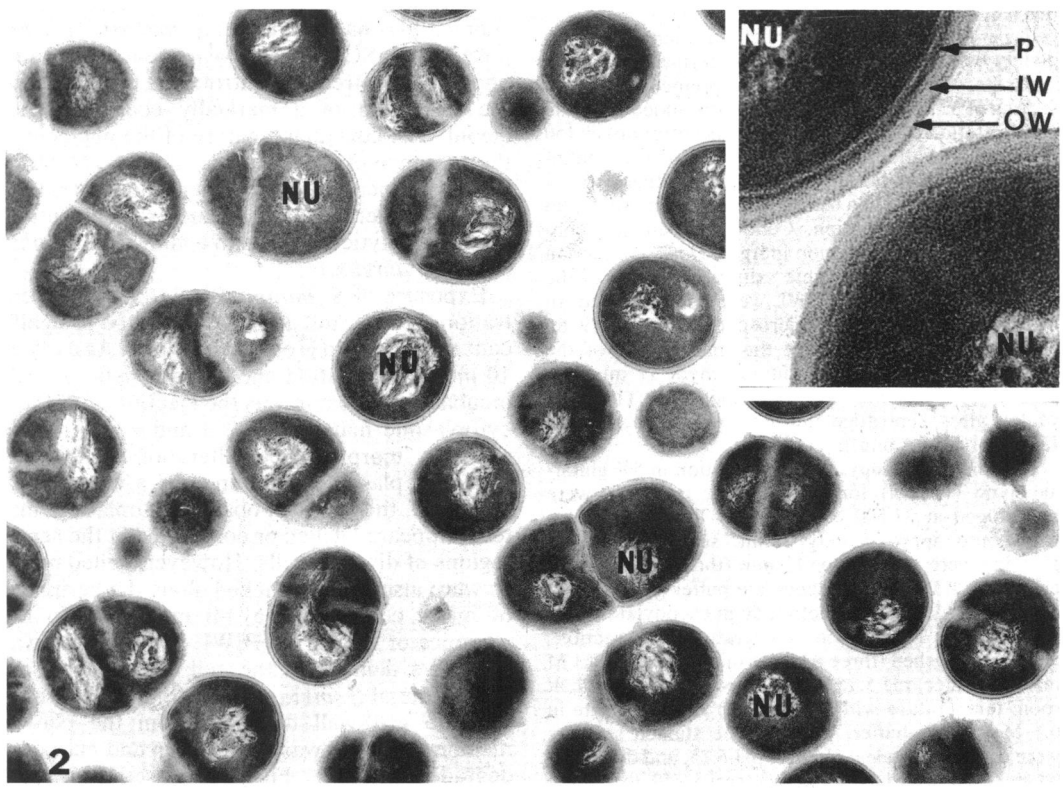

FIG. 2. Electron micrographs of *S. mutans* BHT cells treated with NaSCN. Cells were preincubated for 1 h and then treated with NaSCN for an additional 3 h (see text for details). Low magnification (×27,000) illustrates that the nucleoid (NU) material of the cells becomes pronounced in the presence of inorganic salts. (Inset) High-magnification electron micrograph, showing that the outer cell wall (OW), inner cell wall (IW), and plasma membrane (P) are apparently intact (×175,500).

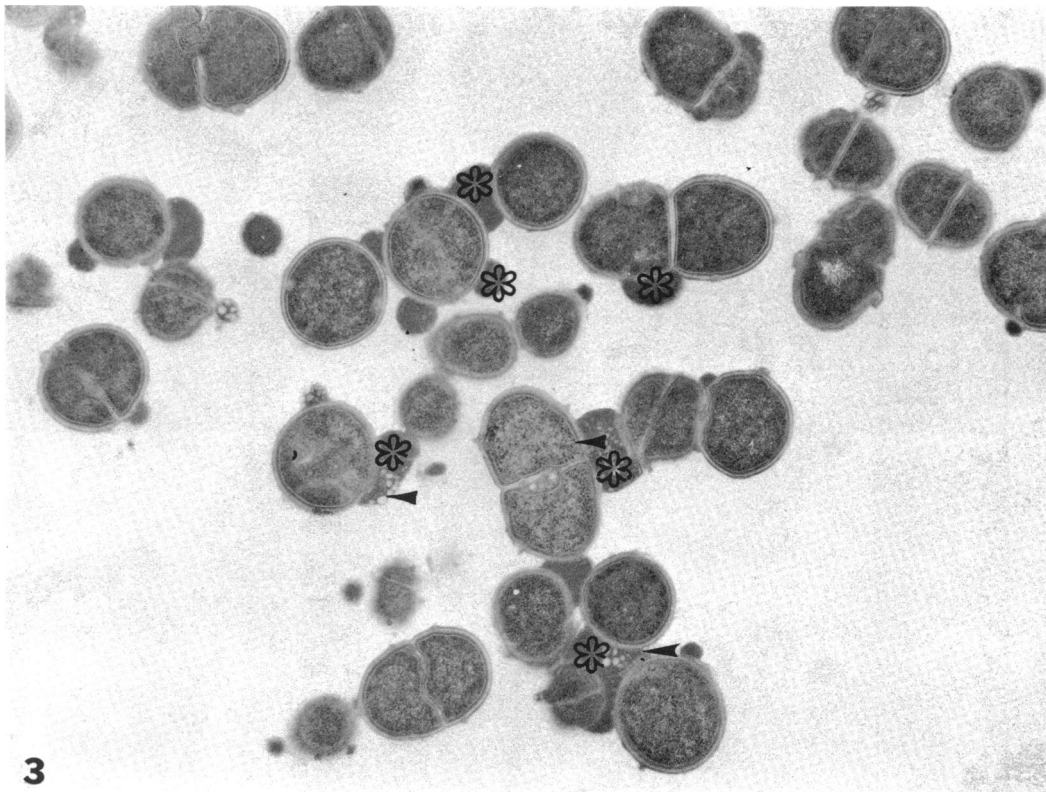

FIG. 3. Electron micrograph of *S. mutans* BHT cells treated for 10 min with a high concentration of HEWL. Cells were preincubated for 1 h and then treated with HEWL for 10 min (see text for details). Low magnification (×27,000) shows cells in the early stages of lysis. Gelled or coagulated cytoplasmic masses (❀) are in the process of expulsion from the cells, a process which occurs mainly at septal regions. Granules can also be clearly seen within the cytoplasm of the cells and within the coagulated masses (arrowhead).

addition, numerous holes or breaks in the plasma membrane occurred after a 3-h incubation in the presence of 150 μg of the enzyme per ml. Interestingly, the cells maintained an oblong-spherical shape even in the absence of significant portions of their cell wall. Note also that, except for the released coagulated protoplasmic masses, essentially all of the cytoplasmic ribonucleoprotein was still confined within the plasma membrane of the distorted cells.

Incubation of *S. mutans* BHT in 22.5 μg of HEWL per ml for 30 min to 3 h resulted in changes in cell morphology similar to those observed in cells incubated in 150 μg of HEWL per ml; however, in the presence of the lower enzyme concentration, the morphological changes were less pronounced during the early incubation times. Figure 6a is representative of *S. mutans* BHT incubated for 30 min in the presence of 22.5 μg of HEWL per ml. The cells were, for the most part, typical of control cells (Fig. 1 and 2), displaying an intact cell wall and uniformly electron-opaque cytoplasm. However, there was some evidence of the effects of the enzyme even at this low concentration and short incubation time. For example, there was a recognizable thinning and "loosening" of the outer cell wall in the septal region. Incubation for 3 h in the presence of 22.5 μg of HEWL per ml resulted in recognizable damage to the cells (Fig. 6b). Figures 6b and c indicate both the destruction of the cell wall and the loss of rigidity at the septal region. Note how, in one cell (Fig. 6c), the fragmented end of the outer cell wall peeled back over itself and was associated with significant damage to the plasma membrane. In addition, cytoplasmic material had leaked out through the destroyed plasma membrane.

Exposure of *S. mutans* BHT to the low concentration of lysozyme plus 0.1 M sodium thiocyanate resulted in complete cellular destruction (Fig. 6d and inset). Both cell wall and plasma membrane were fragmented and destroyed, exposing the cells to plasmolytic events which resulted in both the loss of the characteristic streptococcal shape and the loss of the cytoplasmic contents. Sequential treatment with high concentrations of HEWL followed by 0.1 M NaSCN resulted in the separation of two fractions after centrifugation at 27,500 × *g*. The

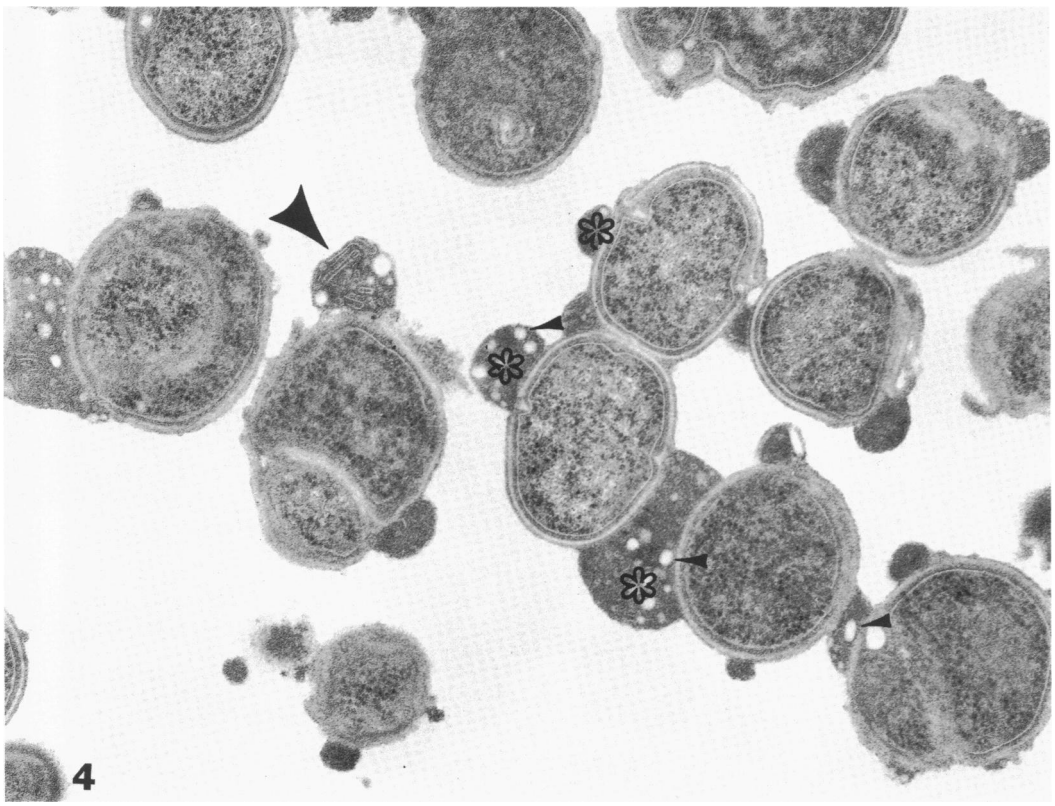

FIG. 4. Electron micrograph of *S. mutans* BHT cells treated for 30 min with a high concentration of HEWL. High magnification (×53,100) illustrates that after a 30-min treatment with HEWL, the majority of the outer cell walls, inner cell walls, and plasma membranes are essentially intact. The gelled protoplasmic masses (❀) contain both granules (small arrowhead) and vesicular material (large arrowhead).

bottom or rapidly sedimentable fraction contained what appeared to be small pieces of exploded cytoplasmic debris (Fig. 7a), whereas the top fraction was more representative of lysed cells. Cell walls with attached plasma membrane ghosts, cell wall fragments, and dense "aggregates" of cytoplasmic components (Fig. 7b) were also found in this fraction. The aggregates of cytoplasmic components could be seen as either separate masses or attached within the plasma membrane ghosts (Fig. 7c). Note in Fig. 7c that both outer and inner cell wall fragments were associated with the plasma membrane ghosts. The plasma membrane ghosts themselves showed breaks in their structures and were not of uniform shape or size (Fig. 7b and c). In contrast to NaSCN, the addition of NaF at the concentration tested (0.1 M) did not result in bursting of the HEWL-treated cells, although more debris was present in comparison with HEWL treatment alone (data not shown).

DISCUSSION

It is commonly accepted that digestion of bacterial cell walls by lysozyme or other lytic enzymes leads to the bursting of the cell walls and the dispersal of the cell contents, yielding various endpoints of lysis. The degree of solubilization, however, has been suggested to depend upon the solubility of the released materials, the composition of the menstruum, and the presence and activity of the endogenous lytic enzymes of the cell (12). In terms of turbidimetric assays, which are used by most investigators to monitor enzymatic cell lysis, it has been suggested that clearing of the reaction mixture does not occur until the index of refraction of the bacterial suspension nears the index of refraction of water (14). In bacteria such as *Micrococcus luteus*, which are virtually completely sensitive to the bacteriolytic action of lysozyme, turbidimetric assays have been used successfully to quantitate the percentage of bacteriolysis (24). However, conclusions based on turbidimetric assays may for the following reasons yield problems in studies with bacteria not normally thought to be sensitive to the lytic action of lysozyme alone. (i) Lysozyme is known to aggregate a wide variety of bacteria determined to be lysis insensitive by biochemical assays. Thus, optical den-

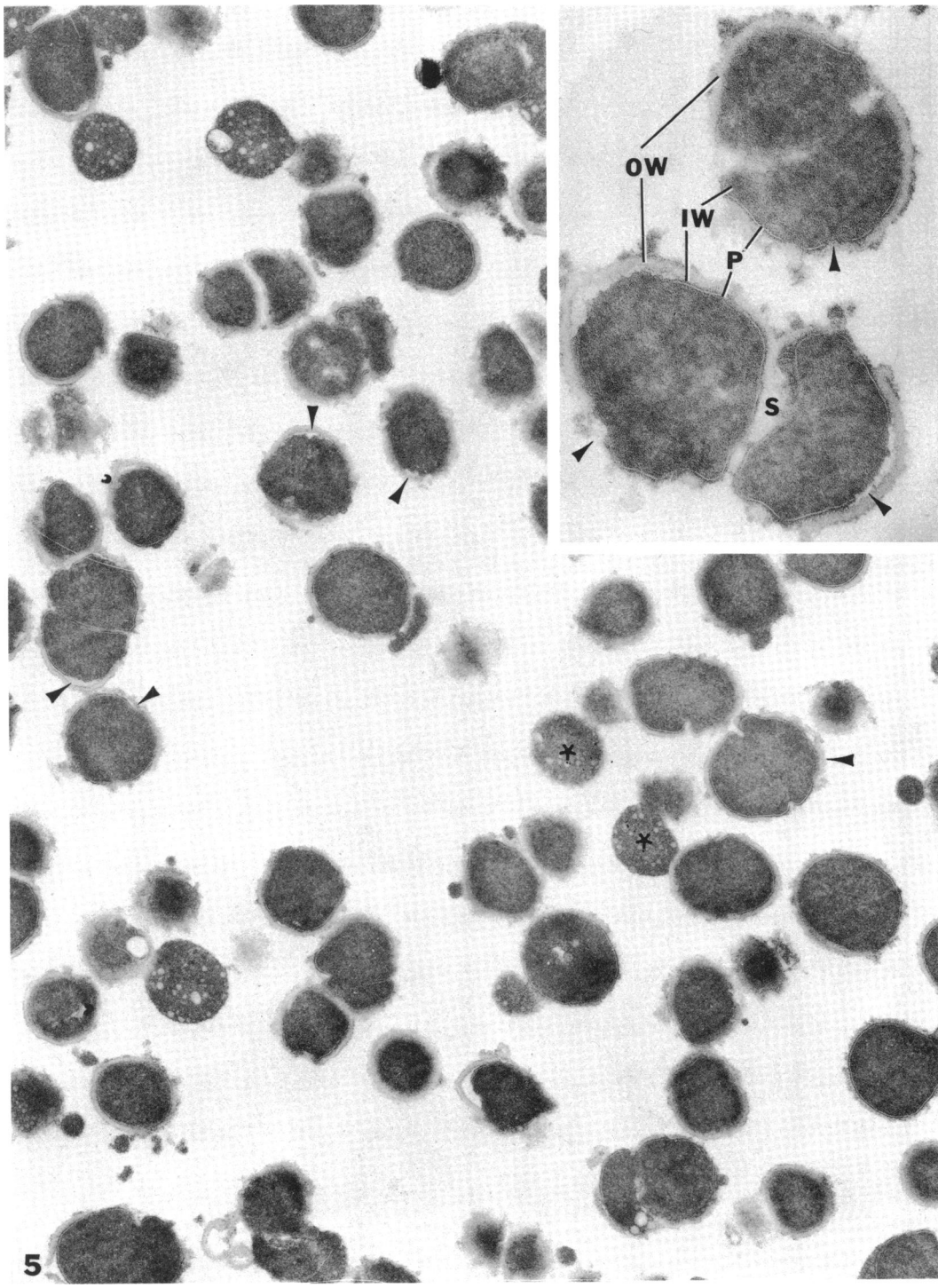

FIG. 5. Electron micrographs of *S. mutans* BHT cells treated with a high concentration of HEWL for longer incubation periods. Cells were preincubated for 1 h and then treated with HEWL for 3 h (see text for details). Low magnification (×40,320) illustrates extensive cell wall and cell membrane damage (arrowhead). Protoplasmic masses (*) have been expelled and are free of the cells. (Inset) High-magnification electron micrograph showing areas of the cell surface where the outer cell wall (OW), inner cell wall (IW), and plasma membrane (P) are no longer present (arrowhead), where the IW and P are still evident but most of the OW has disappeared, and where the OW is distended from an area where the IW and P are absent. The septal region (S) has been extensively damaged (×67,200).

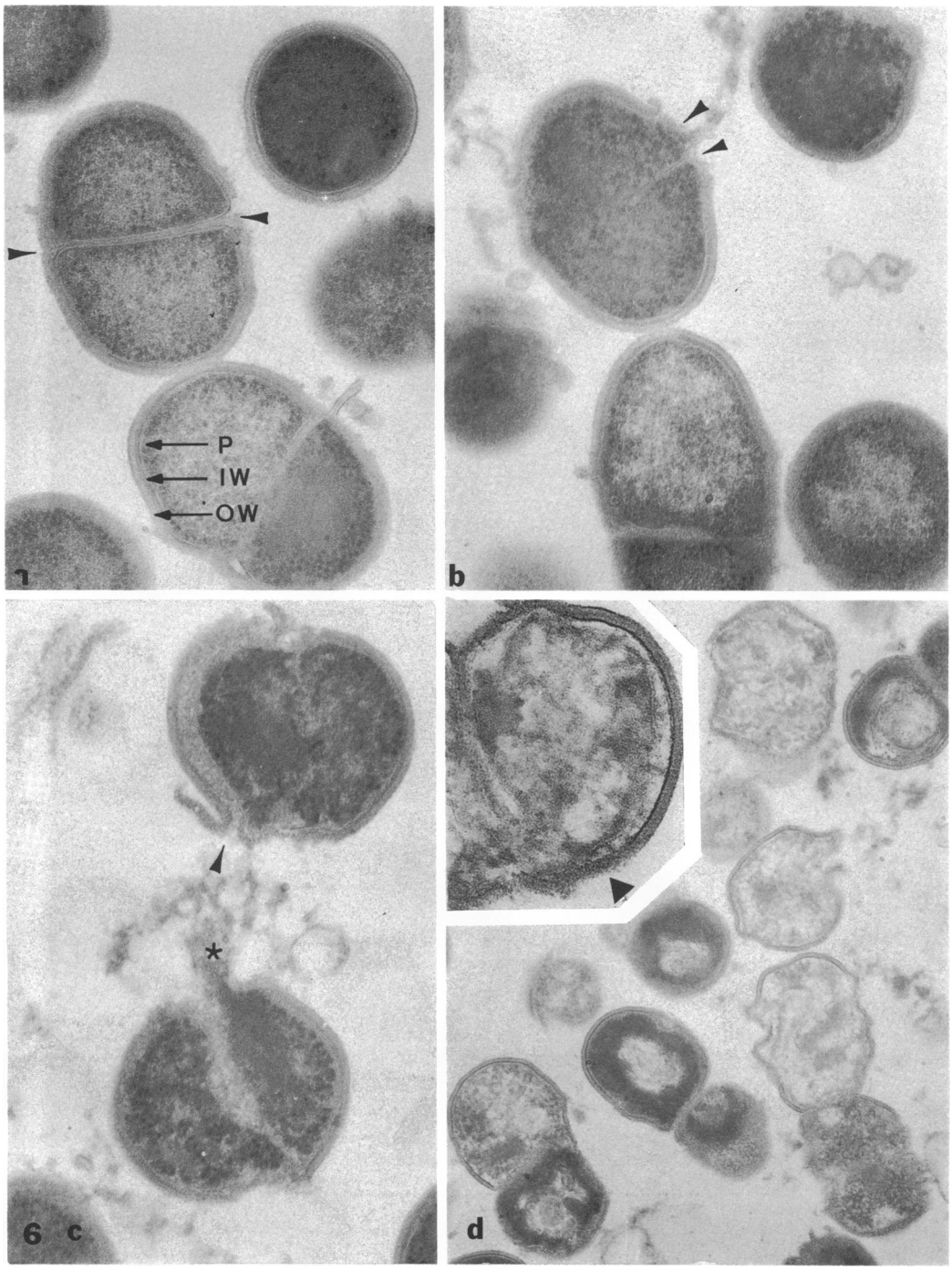

FIG. 6. Electron micrographs of *S. mutans* BHT cells treated with either a low concentration of HEWL alone or HEWL followed by NaSCN. (a) Cells preincubated for 1 h and then reacted with HEWL for 30 min (see text for details). At this early incubation time, the cells appeared intact with mild thinning of the cell wall in the septal region (arrowhead) (×54,600). P, Plasma membrane; IW, inner cell wall; OW, outer cell wall. (b) Cells incubated for 3 h with HEWL showing destruction of the cell wall (arrow-heads) initiating at the septal region (×54,600). (c) Cells showing more extensive destruction of the cell walls and cell membranes, with leakage of cytoplasmic ribonucleoprotein (*). The outer cell wall of one cell appears to be peeled back over itself (arrowhead) (×54,600). (d) Low magnification (×37,800) of HEWL-treated cells after incubation for 1 h with NaSCN showing partial destruction of cell wall and membrane areas on each cell. (Inset) High-magnification electron micrograph showing that many cells have lost the majority of their cytoplasmic contents through destruction of their cell surface (arrowhead), although a dense cytoplasmic structure remains (×75,600).

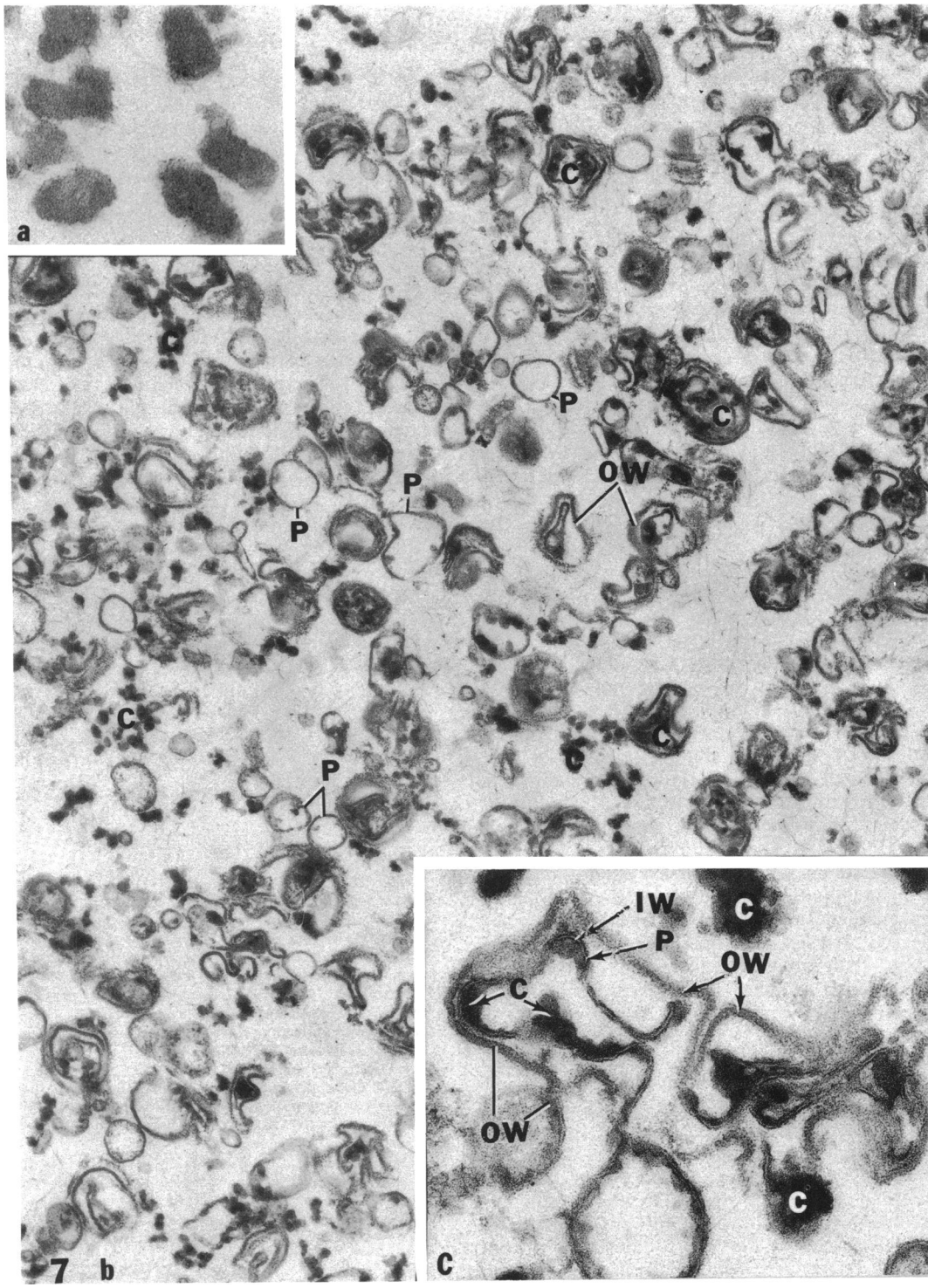

FIG. 7. Electron micrographs of *S. mutans* BHT cells treated with a high concentration of HEWL followed by NaSCN. Cells were preincubated for 1 h, reacted with HEWL for 3 h, and then incubated for a further 1 h with NaSCN (see text for details). (a) Lower portion of a high-speed pellet demonstrating cytoplasmic masses and aggregates which have been broken up into smaller pieces of debris with the addition of salt (×40,000). (b) Low magnification (×40,000) of the top portion of a high-speed pellet consisting of a crude membrane fraction (P), dense cytoplasmic aggregates (C), and outer cell wall fragments (OW). (c) High magnification (×90,000) of the top portion of a high-speed pellet showing the association of dense cytoplasmic aggregates (C) and outer (OW) and inner (IW) cell wall fragments with the damaged membrane fragments and vesicles. P, Plasma membrane.

sities may increase instead of decrease (20). (ii) Partial drops in turbidity may not represent true lysis, as the lysis cannot be correlated with degradation of cell wall peptidoglycan and DNA release (9, 10). (iii) In contrast to (ii), many gram-positive bacteria have been reported to be completely lysed, as measured by DNA quantitation, although they remain turbid (5).

An alternative biochemical assay based on DNA liberation has been used by just a few investigators in an attempt to achieve quantitation of the lytic process in systems in which lysozyme is coupled to inorganic salts or detergents to achieve the desired lytic effect (5, 9, 10, 21). However, as recently reported for the lysozyme-inorganic salt lysis of *Veillonella alcalescens*, absolute quantitation of the DNA may depend on the extent of liberation of the DNA from aggregated cytoplasmic components (27). Lysozyme may be entering the cell through its ability to alter the permeability of the plasma membrane (19) and may combine with and aggregate cytoplasmic components which are subsequently released as the coagulated or gelled protoplasmic masses observed in this study. Inorganic salts at appropriate concentrations seem to break up or explode not only these swollen cytoplasmic masses but also the damaged cells themselves, probably bringing about release of the DNA (Fig. 7c). However, the amounts of aggregated cytoplasmic debris remaining within or released from the cells (Fig. 6d and 7c) likely determine the final quantitation of the DNA (27). Thus, although DNA liberation can be corroborated by ultrastructural analyses (10), quantitation through this type of assay is questionable in terms of interpreting the exact degree of lysis. Moreover, the results demonstrate that DNA release is not of value for determining whether lysis takes place in the presence of lysozyme alone. Although we have reported that DNA is not detectable in supernatants when lysozyme is reacted with *S. mutans* BHT (10), clearly lysis in the form of released cytoplasmic masses was taking place, as observed by electron microscopy (Fig. 3 to 6).

Lysis of the *S. mutans* BHT cells with lysozyme alone might be expected on the basis of cell wall degradation studies, in which lysozyme treatment caused a loss of 70% of the insoluble peptidoglycan (9). Previous studies have shown that lysozyme can effect expulsion of "spherical bodies" in both *Bacillus megaterium* and *Bacillus anthracis* (8). In *B. megaterium*, the action of lysozyme appeared to be particularly located at the ends of the cells, whereas in *B. anthracis*, the entire cell wall appeared to be involved. The spherical bodies or gelled cytoplasms which lack an intact plasma membrane (4) have been observed gradually to become less opaque and finally disappear (8). For *S. mutans* BHT, cells lysed by lysozyme alone may represent cells harvested at a particular point in the cell division cycle, or lysis may initiate at the septal region where newly synthesized peptidoglycan would be expected to be sensitive to the enzyme. In addition, since lysis of *S. mutans* BHT by lysozyme is also observed at cell surface sites other than at the septa, some cells may have had an important fraction(s) of their cell wall peptidoglycan optimally positioned for lysozyme digestion, such that lysis of these cells occurred. Digestion of peptidoglycan located between the outer cell wall and the inner cell wall may affect a strategic site, leading first to a splitting away of the outer cell wall followed by cell lysis (Fig. 5). Barker and Thorne (1) have made similar electron microscopic observations for lysozyme effects on *L. casei*. Of interest, this splitting between the outer and inner cell walls could also be very important in terms of breakdown by lysozyme of cell-to-cell contact in chained microorganisms during bacterial growth (29; T. P. Byrnes, V. I. Iacono, J. J. Pollock, and M. L. Rochon, J. Dent. Res. [special issue A] **60**:571, abstr. 1047, 1981).

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LITERATURE CITED

1. **Barker, D. C., and K. J. I. Thorne.** 1970. Spheroplasts of *Lactobacillus casei* and the cellular distribution of bactoprenol. J. Cell Sci. **1**:755–785.
2. **Bladen, G., G. Hageague, R. Harr, and F. Pollock.** 1972. Lysis of certain organisms by the synergistic action of complement and lysozyme. J. Dent. Res. **52**:371–376.
3. **Bladen, H. A., and S. E. Mergenhagen.** 1964. Ultrastructure of *Veillonella* and morphological correlation of an outer membrane with particles associated with endotoxic activity. J. Bacteriol. **88**:1482–1492.
4. **Brown, R. J., and T. R. Corner.** 1977. Influence of ambient pH on the osmotic properties of protoplasts from *Bacillus megaterium*, p. 93–99. *In* D. Schlessinger (ed.), Microbiology—1977. American Society for Microbiology, Washington, D.C.
5. **Chassy, B. M., and A. Giuffrida.** 1980. Method for the lysis of gram-positive, asporogenous bacteria with lysozyme. Appl. Environ. Microbiol. **39**:153–158.
6. **Coleman, S. E., I. van de Rijn, and A. S. Bleiweis.** 1970. Lysis of grouped and ungrouped streptococci by lysozyme. Infect. Immun. **2**:563–569.
7. **Gilpin, R. W., A. N. Chatterjee, and F. E. Young.** 1972. Autolysis of microbial cells: salt activation of autolytic enzymes in a mutant of *Staphylococcus aureus*. J. Bacteriol. **111**:272–283.
8. **Gladstone, G. P., and H. H. Johnston.** 1955. The effect of cultural conditions on the susceptibility of *Bacillus anthracis* to lysozyme. Br. J. Exp. Pathol. **36**:363–372.
9. **Goodman, H., J. J. Pollock, V. J. Iacono, W. Wong, and G. D. Schockman.** 1981. Peptidoglycan loss during hen egg white lysozyme-inorganic salt lysis of *Streptococcus mutans*. J. Bacteriol. **146**:755–763.

10. **Goodman, H., J. J. Pollock, L. I. Katona, V. J. Iacono, M.-I. Cho, and E. Thomas.** 1981. Lysis of *Streptococcus mutans* by hen egg white lysozyme and inorganic sodium salts. J. Bacteriol. **146**:764–774.
11. **Grula, E. A., and S. E. Hartsell.** 1954. Lysozyme and morphological alterations induced in Micrococcus lysodeitious. J. Bacteriol. **68**:171–177.
12. **Grula, E. A., and S. E. Hartsell.** 1954. Lysozyme action and its relation to the Nakamura effect. J. Bacteriol. **68**:302–306.
13. **Grula, E. A., and S. E. Hartsell.** 1957. Lysozyme in the bacteriolysis of Gram-negative bacteria. I. Morphological changes during the use of Nakamura's technique. Can. J. Microbiol. **3**:13–21.
14. **Grula, E. A., and S. E. Hartsell.** 1957. Lysozyme in the bacteriolysis of Gram-negative bacteria. II. Factors influencing clearing during the Nakamura technique. Can. J. Microbiol. **3**:23–24.
15. **Iacono, V. J., B. J. MacKay, S. DiRienzo, and J. J. Pollock.** 1980. Selective antibacterial properties of lysozyme for oral microorganisms. Infect. Immun. **29**:623–632.
16. **Kuramitsu, S., K. Ikeda, K. Hamaguchi, S. Miwa, and K. Nakashima.** 1975. Binding of N-acetyl-chitotriose to human lysozyme. J. Biochem. (Tokyo) **78**:327–333.
17. **Luft, J. H.** 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. **9**:409-414.
18. **Perch, B., E. Kjems, and T. Ravn.** 1974. Biochemical and serological properties of *Streptococcus mutans* from previous human and animal sources. Acta Pathol. Microbiol. Scand. Sect. B **82**:357–370.
19. **Pollock, J. J., H. Goodman-Bicker, L. I. Katona, M.-I. Cho, and V. J. Iacono.** 1979. Lysozyme bacteriolysis, p. 429–447. *In* I. Kleinberg, S. Ellison, and I. D. Mandel (ed.), Proceedings: Saliva and Dental Caries (a special supplement to Microbiology Abstracts). Information Retrieval, Inc., Washington, D.C.
20. **Pollock, J. J., V. J. Iacono, H. Goodman-Bicker, B. J. MacKay, L. I. Katona, L. B. Taichman, and E. Thomas.** 1976. The binding, aggregation and lytic properties of lysozyme, p. 325–352. *In* H. M. Stiles, W. J. Loesche, and T. C. O'Brien (ed.), Proceedings: Microbial Aspects of Dental Caries (a special supplement to Microbiology Abstracts), vol. 3. Information Retrieval, Inc., Washington, D.C.
21. **Pollock, J. J., L. I. Katona, H. Goodman, M.-I. Cho, and V. J. Iacono.** 1981. Bacteriolysis of *Streptococcus mutans* BHT by lysozyme and inorganic anions normally present in human saliva. Arch. Oral Biol. **26**:711–716.
22. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. **17**:208–213.
23. **Salton, M. R. J.** 1953. Cell structure and the enzymic lysis of bacteria. J. Gen. Microbiol. **9**:512–523.
24. **Salton, M. R. J.** 1956. Studies of the bacterial cell wall. The action of lysozyme on cell walls of some lysozyme sensitive bacteria. Biochim. Biophys. Acta **22**:495–506.
25. **Terleckyj, B., N. P. Willett, and G. D. Shockman.** 1975. Growth of several cariogenic strains of oral streptococci in a chemically defined medium. Infect. Immun. **11**:649–655.
26. **Thorne, K. J. I., and D. C. Barker.** 1972. The occurrence of bactoprenol in the mesosome and plasma membranes of *Lactobacillus casei* and *Lactobacillus plantarum*. J. Gen. Microbiol. **70**:87–98.
27. **Tortosa, M., M.-I. Cho, T. J. Wilkens, V. J. Iacono, and J. J. Pollock.** 1981. Bacteriolysis of *Veillonella alcalescens* by lysozyme and inorganic anions present in saliva. Infect. Immun. **32**:1261–1273.
28. **Virgilio, R., C. González, N. Muñoz, and S. Mendoza.** 1966. Electron microscopy of *Staphylococcus aureus* cell wall lysis. J. Bacteriol. **91**:2018–2024.
29. **Westmacott, D., and H. R. Perkins.** 1979. Effects of lysozyme on *Bacillus cereus* 569: rupture of chains of bacteria and enhancement of sensitivity to autolysins. J. Gen. Microbiol. **115**:1–11.