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# Degradation of Group A Streptococcal Cell Walls by Egg-White Lysozyme and Human Lysosomal Enzymes

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Group A streptococci and their isolated cell walls, normally resistant to egg-white lysozyme and the lysosomal enzymes of human phagocytes, were converted to lysozyme-sensitive forms by partial removal of cell wall carbohydrate, substitution of free amino groups, and by saponification of O-acyl groups. The resultant modified streptococcal cell walls showed rapid degradation when treated with leukocyte granule extract derived from human peripheral blood polymorphonuclear leukocytes and monocytes and when subjected to phagocytosis by living human leukocytes. These results indicate that the factors responsible for lysozyme resistance of the group A cell wall also influence its resistance to human leukocyte granule enzymes and suggest that the chemical composition of the cell wall, in addition to the presence of cell wall carbohydrate, determines this resistance.

Group A streptococcal cell walls or their components persist in animal tissues (1, 12, 28, 29, 34, 39, 40) and, theoretically, may play a role in post-streptococcal sequelae in humans. However, their intracellular fate has not been extensively studied at the ultrastructural level and the reasons for their persistence are poorly defined. According to one report (6), some but not all of the streptococci phagocytized by human polymorphonuclear leukocytes (PMN) in vitro were completely digested (including cell wall) within 3 hr. In contrast, streptococci ingested by human monocytes in a comparable in vitro system, although undergoing loss of intracytoplasmic contents, retained undegraded walls over the 6- to 8-hr period of observation (13). Although radiolabeling studies (5) showed a slow and incomplete phagocytic digestion of cell wall polysaccharide, no studies have focused on the fate of the mucopeptide portion of wall nor on the influence of its nature or accessibility to enzymes upon its dissolution or persistence. The present study was designed to test effects of polysaccharide removal and subsequent chemical modification on the susceptibility of the residual cell wall to intraphagocytic degradation in human leukocytes as compared to its sensitivity to egg-white lysozyme and extracts of leukocyte lysosomal granules. The results indicate that treatments which render the cell wall sensitive to lysozyme also make it susceptible to extracted granule enzymes and phagocytic degradation, and suggest that the chemical nature of the cell wall, in addition to the presence of cell wall polysaccharide, determines its resistance to degradation.

## MATERIALS AND METHODS

**Organisms.** Group A *Streptococcus pyogenes* (strain T 1 Av), M antigen-negative by precipitin tests, was derived from strain T 1/155, originally obtained from R. Lancefield of Rockefeller University. Stationary-phase cultures (18 to 48 hr), grown in Brain Heart Infusion broth (Difco) at 37 C, were used in all preparations. Ultraviolet light-killed *Micrococcus lysodeikticus* (Difco) was used to test lysozyme activity.

**Preparation of cell walls.** Cell walls were prepared as described by Huff et al. (19). Streptococci were suspended in water and shaken with 66-$\mu$m diameter glass beads in a Braun disintegrator with cooling until there was a 10-fold reduction in optical density (OD; 5 to 7 min). After centrifugation for 15 min at 8,700 $\times$ *g*, the sediment was suspended in 1 M KCl and layered over sucrose gradients (18). Cell walls were stored in sucrose–1 M KCl at $-40$ C and washed three times with water before use.

**Preparation of modified cell walls.** Formamide extraction was carried out as described by Heymann et al. (14). Washed, lyophilized, streptococcal cells or cell walls were suspended in formamide (10 mg/ml of formamide) and heated at 165 to 170 C for 1 hr. The

suspension was then centrifuged, and the pellet was washed with 1% HCl in 95% ethanol, followed by three washes with water.

Acid-extracted streptococci were prepared by suspending lyophilized organisms in 0.2 N HCl and heating at 100 C for 10 min (23). Acylation and de-O-acylation were achieved by the use of acetic anhydride and sodium hydroxide, respectively, by the methods of Heymann et al. (15). Aggregates of extracted cells or cell walls were disrupted by brief sonic treatment (Branson sonifier, model LS 75).

**Preparation of leukocyte granule extract.** A modification of the method of Janoff and Scherer (20) was used to obtain human leukocyte granules. Venous blood from normal human donors was anticoagulated with heparin (10 units/ml) and sedimented at room temperature for 45 min in a 5:1 ratio with 6% low-molecular-weight dextran in isotonic saline. The supernatant fluid, containing 80 to 90% PMN, was centrifuged, and the leukocytes were washed in saline. Erythrocytes were lysed by hypotonic shock in 0.2% NaCl. The leukocytes were suspended in 0.34 M sucrose ($10^8$ cells/ml) and disrupted by sonic treatment. Cell breakage was confirmed by phase contrast microscopy. The suspension was then centrifuged at 500 × *g* to deposit unbroken cells and nuclei, and the supernatant fluid was centrifuged at 17,000 × *g* for 20 min to sediment lysosomal granules.

The granule pellet was suspended in 0.1 M phosphate buffer, *p*H 8, and disrupted by repeated freezing and thawing (10 times). The suspension was then centrifuged at 80,000 × *g* to sediment debris, and the supernatant fluid was dialyzed against cold phosphate-buffered saline, *p*H 7.4, for 5 hr. The resultant extract, containing 420 to 460 μg of protein/ml, was stored at −40 C.

**Chemical determinations.** Protein content of granule extracts was measured by the Lowry method (24), using bovine serum albumin as the standard. Rhamnose was determined by the method of Dische and Shettles (21).

**Turbidometric studies.** In general, 0.1 M phosphate buffer, *p*H 6.1, 0.1 ml of the suspension to be tested, and either egg-white lysozyme (twice crystallized, 10,015 units/mg, Worthington Biochemical Corp., New Jersey) or leukocyte granule extract were added to make a final volume of 1 ml. The OD was then read against a buffer blank at 750 nm with a Beckman Model B spectrophotometer. The initial optical densities ranged from 0.053 to 0.081. The tubes were then incubated in a 37 C water bath, and readings were taken at various time periods. All data are expressed as a percentage decrease in OD, based on the zero time reading as the 100% value.

**Phagocytic and ultrastructural studies.** A sample (0.05 ml/ml of blood) of an aqueous suspension (OD of 0.5 to 0.6) of untreated or chemically treated streptococci was added to heparinized human blood and incubated at 37 C with constant shaking. Specimens were fixed for electron microscopy at intervals between 0.5 and 6 hr by centrifuging at 1,000 × *g* for 5 min and by replacing the supernatant plasma with 2.5% glutaraldehyde in sodium cacodylate, *p*H 7.2 (37). After fixation, buffy coats were removed with applicator sticks and sectioned into small fragments. The tissue was post-fixed in acetate-Veronal-buffered 1% osmium tetroxide and stained with uranyl acetate (36). Dehydration was performed by passage through graded ethanol concentrations to propylene oxide, from which embedment was made in Epon 812. Sections were cut on an LKB Ultratome III and placed on Formvar-coated grids. All sections were stained with alkaline lead citrate (33) and examined with an Hitachi electron microscope, model HU-11c, operated at 75 kv.

Measurements of cell wall thickness were made with a Bausch and Lomb measuring magnifier with a scale marked in 0.1-mm gradations. The dimensions given represent an average of at least 20 measurements on electron micrographs printed at magnifications of 69,000 and 120,000.

## RESULTS

**Unmodified cell walls.** Cell walls of untreated streptococci varied between 15 and 17 nm in thickness (Fig. 1). During incubation of suspensions of walls or intact organisms in buffer alone, or in the presence of high concentrations (up to 140 μg of protein/ml) of lysozyme or leukocyte granule extract in buffers ranging from *p*H 6 to *p*H 8, the electron microscopic appearance of wall was essentially unchanged and no decreases in OD were found over an 18-hr period (Table 1, line 1). Lower concentrations of either enzyme preparation (20 μg of protein per ml) at *p*H 6.1 caused immediate lysis of comparable suspensions of *Micrococcus lysodeikticus*, and electron microscopy showed that these bacteria and their walls were completely degraded within PMN as soon as material could be fixed. After phagocytosis of the group A streptococci, as shown previously, (13), little change occurred. The maximal decrease in cell wall thickness was less than 5 nm. Following treatment of the streptococci with acetic anhydride (acylation) or saponification with sodium hydroxide (de-O-acylation), or both in that order, there were no changes in the appearance of the walls and, moreover, there was no decrease in OD of enzyme-treated suspensions (Table 1, lines 2–4).

**Formamide-extracted cell walls.** A single extraction of walls or whole streptococci with hot formamide removed 75% of the rhamnose originally present. In most experiments, cell wall "shells" were prepared in this manner from intact streptococci, rather than from mechanically prepared wall fragments, in order to facilitate ultrastructural observations in phagocytosis experiments. These structures (Fig. 2) retained the approximate shape of the cell, but the wall thickness was reduced 50% to an average of 8 nm and the cytoplasmic contents were usually lost. Suspensions of such shells, incubated with

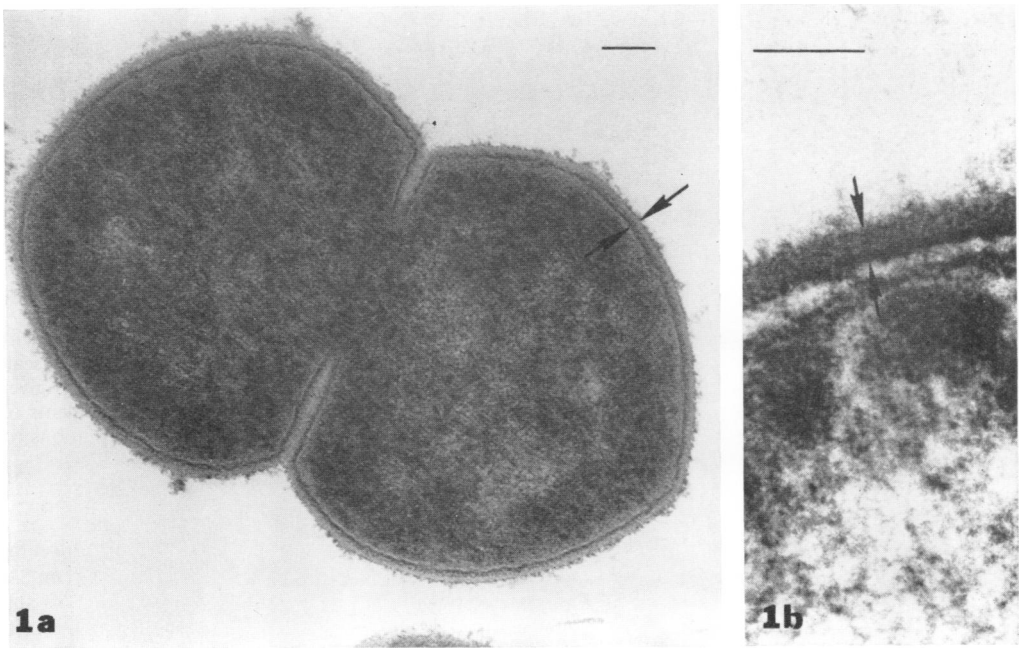


FIG. 1. *a, Untreated group A streptococcus. Cell wall is between arrows. ×69,000. Bar, 0.1 μm. b, Plasmolysing cell showing cell wall thickness between arrows. ×300,000. Bar, 0.05 μm.*

TABLE 1. *Effects of various chemical treatments on the group A streptococcal cell wall*

| Treatment | Electron microscope observation | | Response to: | | Electron microscope observations after phagocytosis |
|---|---|---|---|---|---|
| | Wall thickness | Other | Lysozyme | Granule extract | |
| 1. None | 15 to 17 nm | | None | None | Loss of cytoplasm, no dissolution of wall |
| 2. Acetic anhydride (AA) | 15 to 17 nm | | None | None | |
| 3. NaOH | 15 to 17 nm | | None | None | |
| 4. AA + NaOH | 15 to 17 nm | | None | None | |
| 5. Formamide (F) | 8 nm | Loss of cytoplasm | 50% OD[a] Decrease | 25% OD Decrease | Usually no change in wall, occasional fragmentation |
| 6. F + AA | 8 nm | Loss of cytoplasm | 50% OD Decrease | 25% OD Decrease | Usually no change in wall, occasional fragmentation |
| 7. F + NaOH | 8 nm | Loss of cytoplasm | 85% OD Decrease | 70% OD Decrease | Complete wall degradation |
| 8. F + AA + NaOH | 8 nm | Loss of cytoplasm | 85% OD Decrease | 70% OD Decrease | Complete wall degradation |
| 9. HCl | 7.7 nm | Coagulated cytoplasm | No change[b] | | Usually no change in wall |
| 10. HCl + AA | 7.7 nm | Coagulated cytoplasm | No change[b] | | Usually no change in wall |
| 11. HCl + NaOH | 7.7 nm | Coagulated cytoplasm | No change[b] | | Considerable wall degradation |
| 12. HCl + AA + NaOH | 7.7 nm | Coagulated cytoplasm | Complete loss of wall[b] | | Complete wall degradation |

[a] OD, optical density.
[b] Electron microscope observation.

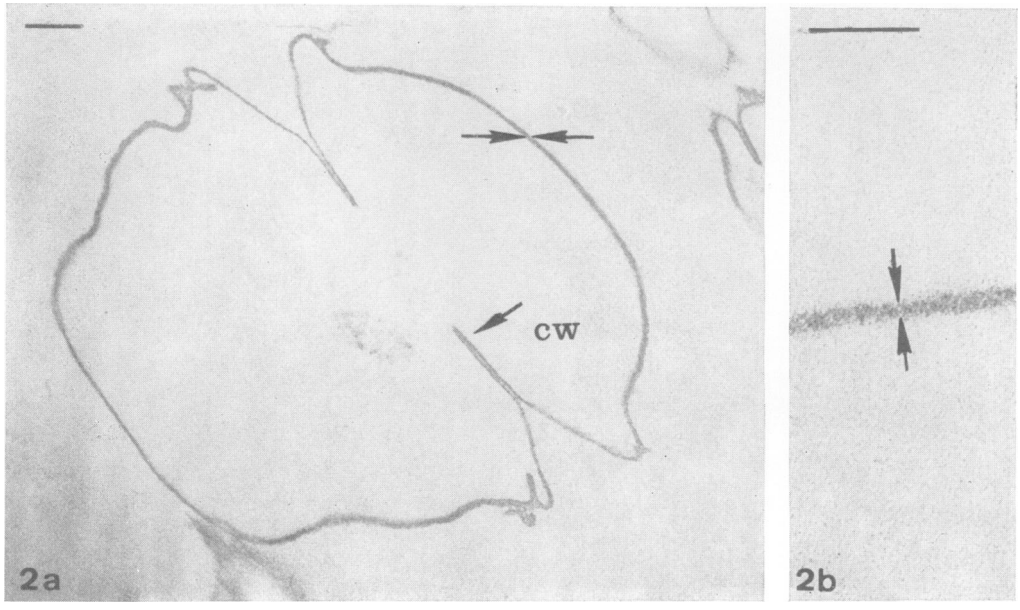


FIG. 2. *a, Cell wall shell prepared from intact streptococci by formamide extraction. Cell wall, between arrows; cw, cross wall. ×69,000. Bar, 0.1 μm. b, High magnification of cell wall, between arrows. ×300,000. Bar, 0.05 μm.*

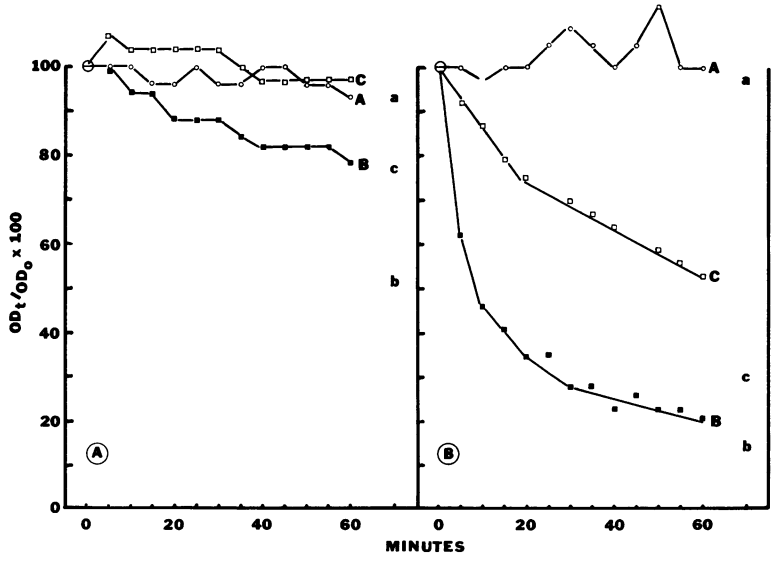


FIG. 3. *A, Response of formamide-extracted group A streptococci to lysozyme and leukocyte granule extract. Curve A, control cells in phosphate buffer; curve B, cells treated with lysozyme (100 μg/ml); curve C, cells treated with granule extract (80 μg of protein/ml). Points designated a, b, and c are overnight values. B, Response of formamide-extracted, de-O-acylated streptococci to lysozyme and granule extract. Curve A, control cells in phosphate buffer; curve B, cells treated with lysozyme (100 μg/ml); curve C, cells treated with granule extract (80 μg of protein/ml). Points a, b, and c represent overnight values.*

lysozyme or leukocyte granule extract, decreased slowly in OD so that 50 to 75% of the original OD remained (Fig. 3A, curves B and C). When phagocytized, most shells retained their shape and wall thickness during the 6-hr period of observation, even though numerous lysosomal granules were seen discharging into the phagocytic vacuoles (Fig. 4). Some

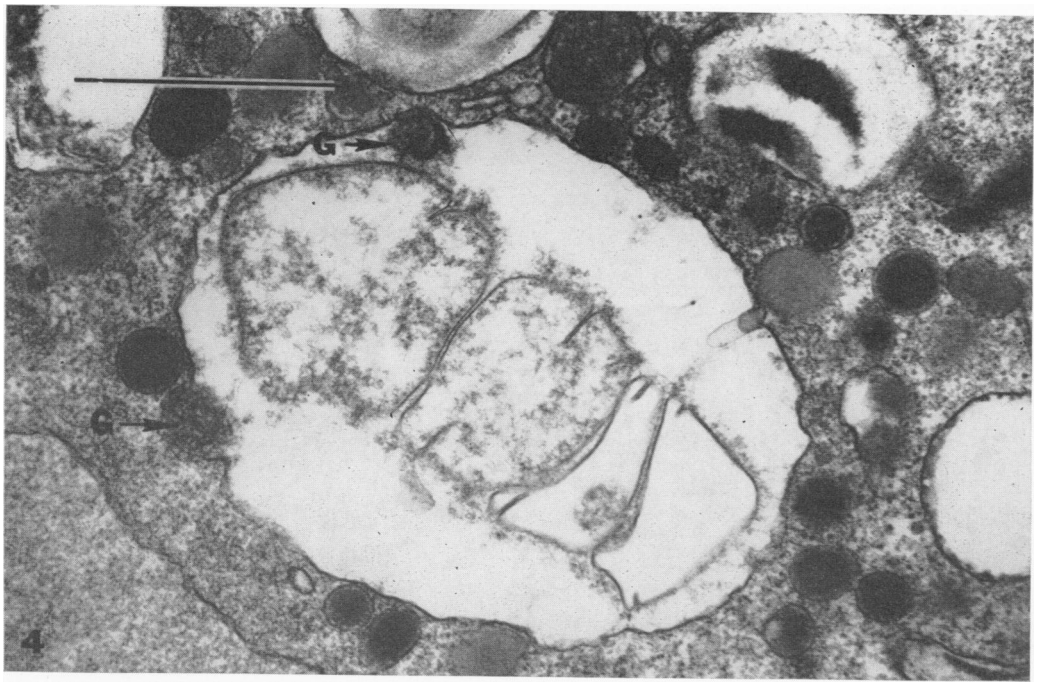


FIG. 4. *Phagocytic vacuole containing a chain of ingested cell wall shells. Lysosomal granules (G) are shown releasing their contents into the vacuolar space. ×34,000. Bar, 1.0 μm.*

walls became fragmented, but there was no complete dissolution. Treatment with acetic anhydride did not alter the results (Table 1, line 6). However, saponification alone or after acylation, although not altering the initial wall thickness, resulted in increased sensitivity to the enzymes and to intraphagocytic dissolution (Table 1, lines 7, 8). There were marked and rapid decreases in OD values of enzyme-treated suspensions (Fig. 3B, curves B and C). Within phagocytic vacuoles of PMN, the saponified shells showed rapid and random fragmentation (Fig. 5) which progressed (Fig. 6) to dissolution until no residual cell wall structure could be recognized (Fig. 7). All stages of this degradation could be seen in specimens fixed as early as 0.5 hr after adding shells to phagocytes.

**Acid-extracted cell walls.** A single acid extraction of whole streptococci resulted in shape-retaining shells morphologically similar to formamide-extracted shells and with a similar wall thickness (7.7 nm). However, the cell contents were coagulated (Fig. 8), in contradistinction to the loss of contents seen in formamide-extracted shells (Fig. 2 and 4). Residual rhamnose was not measured after acid treatment, but other studies (22) indicate that a single extraction does not remove all carbohydrate. After exposure to lysozyme, acid-extracted streptococci did not significantly differ from those shown in Fig. 8. Treatment of these shells with either acetic anhydride or sodium hydroxide resulted in no changes in ultrastructure, and such shells were not sensitive to lysozyme (Table 1, lines 9–11). However, treatment with acetic anhydride followed by saponification resulted in optimal degradability of the shells (Table 1, line 12). Although the sequential treatments did not change the appearance of the cell wall, even brief, subsequent exposure to lysozyme caused loss of recognizable wall from the coagulated protoplasts (Fig. 9), and no wall fragments were found in pelleted preparations. Phagocytosis of these acylated, saponified shells resulted in the same rapid wall dissolution as shown for formamide-extracted, saponified shells (Fig. 5–7).

Because of the coagulated protoplasts, no significant decrease in OD of lysozyme-treated, acid-extracted streptococci could be detected by turbidometry. Therefore, this method was not used for acid-extracted organisms, and the evidence for enzyme sensitivity is supplied by electron microscopy. Because of the qualitative comparability of results obtained with formamide-treated streptococci exposed to the enzyme preparations, only lysozyme was used to test acid-extracted shells in vitro.

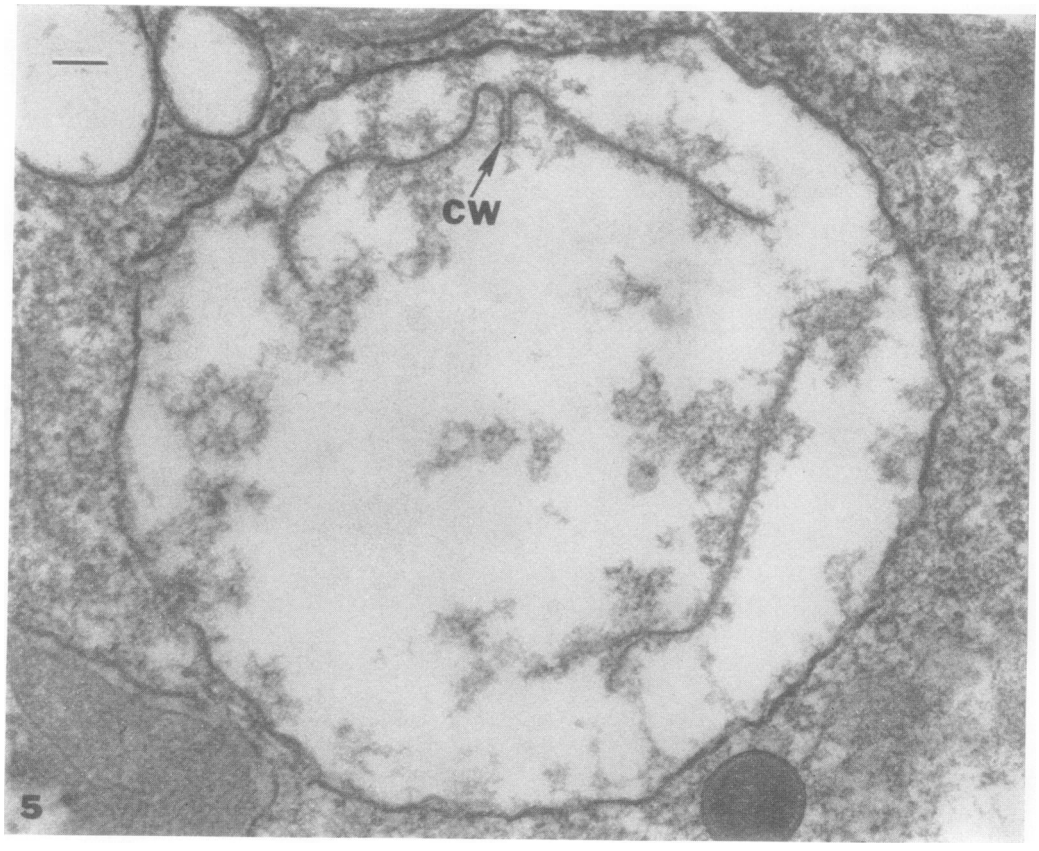


FIG. 5. *Fragmentation of de-O-acylated cell wall shell within phagocytic vacuole. CW, cross wall.* ×*69,000. Bar, 0.1 μm.*

## DISCUSSION

Our results confirm previous observations (10, 27, 30, 38, 46) that human PMN possess a lysozyme-like enzyme that can degrade susceptible bacterial cell walls, but also indicate that they do not contain enzymes, nor supply conditions, that can make the walls of group A streptococci susceptible to intraphagocytic degradation. Artificial conditions that render the wall sensitive to lysozyme also make it susceptible to leukocyte granule extract and phagocytic degradation. The observed events are best explained as follows.

The mucopeptide of group A cell walls contains a large number of free (unsubstituted) amino groups (25), no known substituted hydroxyl (O-) groups (16), and it is poorly cross-linked (25). The presence of either free amino groups (3, 31, 41) or of substituted O-groups (7, 8) can inhibit the activity of lysozyme on mucopeptide, the degree of inhibition depending probably on the balance between substituted and unsubstituted groups of each sort. This ratio may be affected by the large number of peptide chains bearing free amino groups (up to 40% on L-lysine; reference 25), and this could greatly hinder lysozyme action. In fact, native cell walls are not degraded by lysozyme nor within phagocytes (9, 13, 22, 39). This has been assumed usually to be due to a protective effect conferred by the presence of the cell wall polysaccharide (2, 22), but obviously cannot be shown without first removing this carbohydrate.

When removal is effected by the commonly used procedure of extraction with hot formamide (11, 31, 35), the remaining walls (or shells of whole bacteria) are partially sensitive to lysozyme (our results; references 15, 22). Possibly this is because formamide also acylates (i.e., formylates) both free amino and hydroxyl groups (16, 43). Substitutions of the former should improve lysozyme action, whereas substitution of the latter should decrease it. The partial sensitivity achieved suggests that more amino groups than

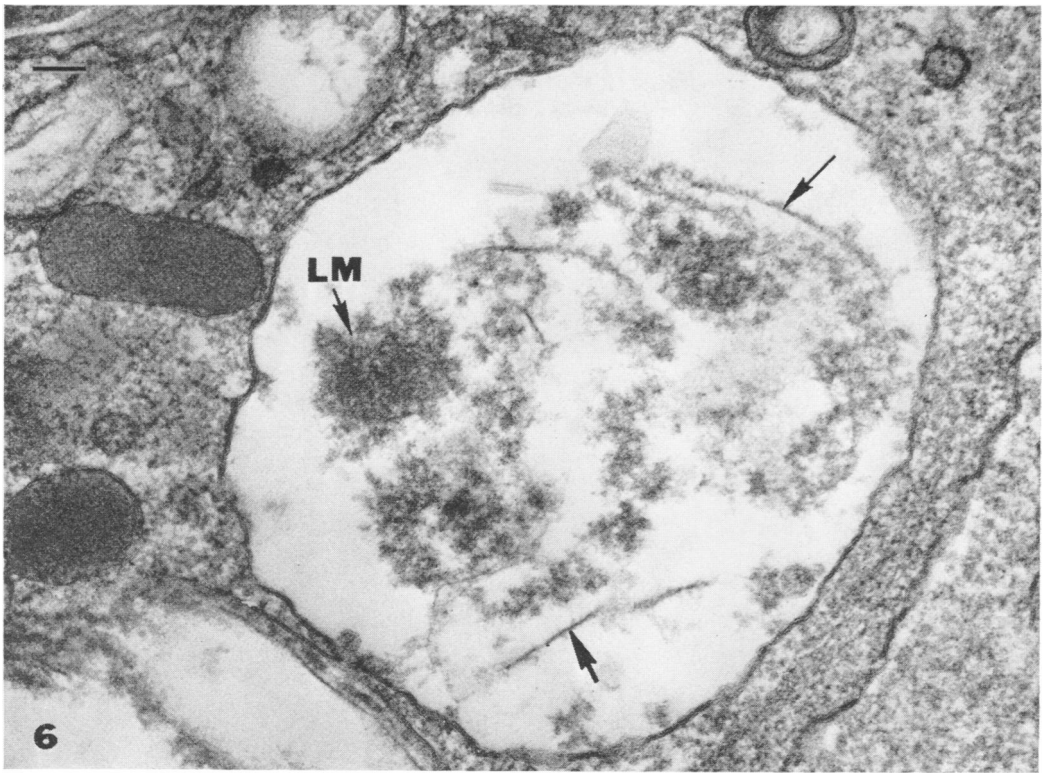


FIG. 6. *Later stage of cell wall dissolution showing phagocytic vacuole filled with lysosomal material (LM) and residual cell wall fragments (arrows). ×69,000. Bar, 0.1 μm.*

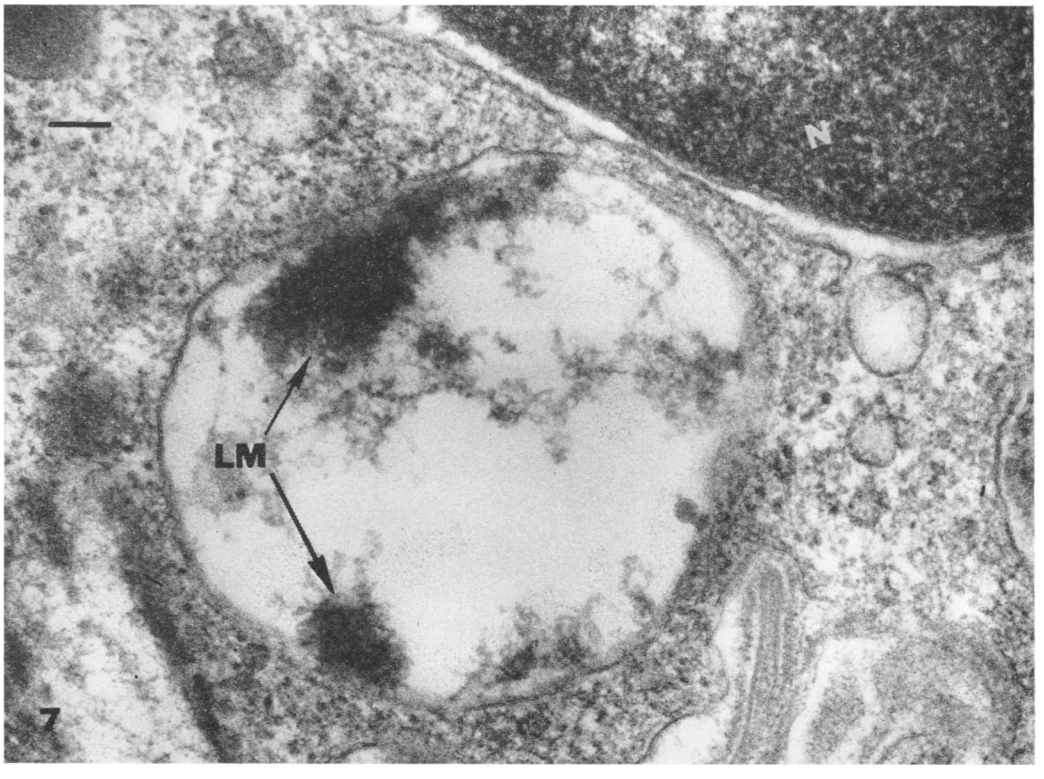


FIG. 7. *Phagocytic vacuole containing residue apparently derived from complete dissolution of cell wall. No recognizable cell wall remains. LM, lysosomal material; N, polymorphonuclear leukocyte nucleus. ×69,000. Bar, 0.1 μm.*

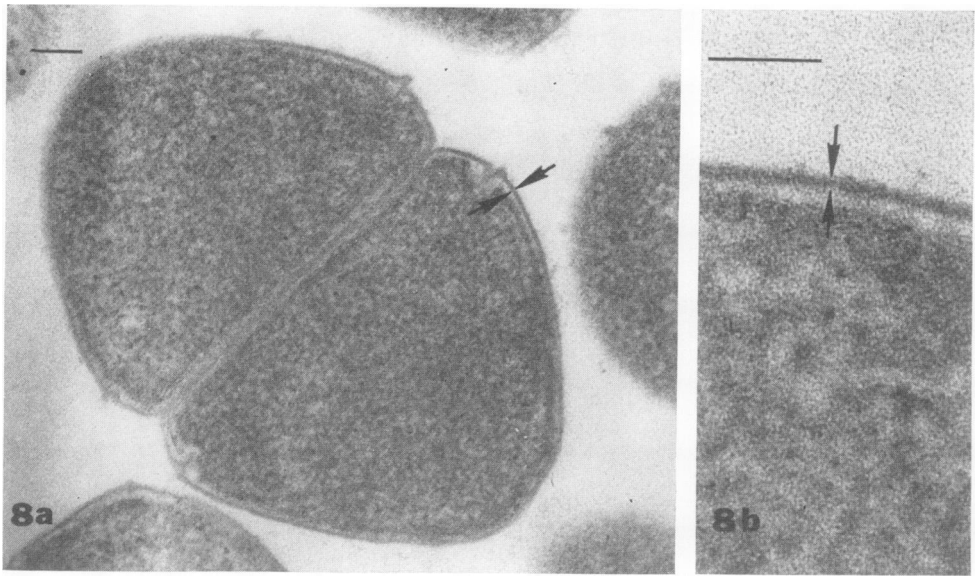


FIG. 8. *a, Acid-extracted group A streptococci. Note the coagulated cytoplasm. Cell wall, between arrows. ×69,000. Bar, 0.1 μm. b, High magnification of cell wall, between arrows. ×300,000. Bar, 0.05 μm.*

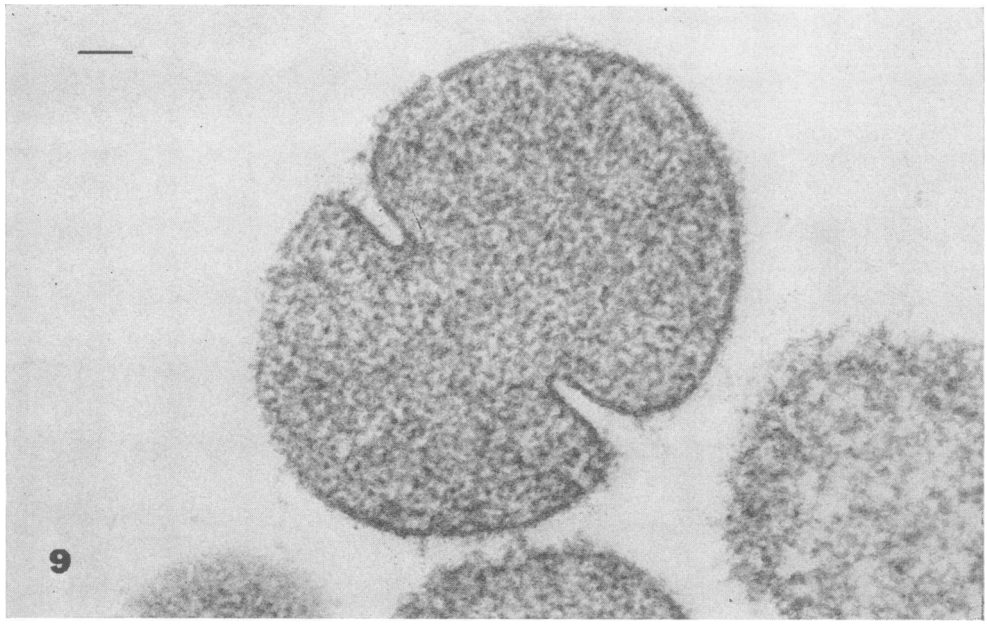


FIG. 9. *Acid-extracted, acylated, saponified group A cell after lysozyme treatment. The cell wall has disappeared from the coagulated protoplast. ×69,000. Bar, 0.1 μm.*

hydroxyl groups are substituted, which could depend on their differential sensitivities to acylation. However, the inhibitory action of the substituted O groups is made apparent by de-O-acylation (16) with weak alkali. After such treatment, formamide-extracted walls are highly sensitive to lysozyme, which suggests that the amino groups remain adequately substituted by formyl derivatives and that the inhibition was due only to the previously substituted O groups.

There is, therefore, no need for additional acylating procedures, such as acetic anhydride treatment (15), to cover free amino groups, and our results show that such treatment preceding saponification does not change the findings.

When removal of polysaccharide is brought about by hot dilute hydrochloric acid (23), the remaining wall is still insensitive to lysozyme or phagocytic degradation, possibly because free amino groups remain unsubstituted. After subsequent treatment with acetic anhydride, which should acylate both amino and hydroxyl groups, the wall remains insensitive. This suggests that this acylating procedure is somehow different from acylation by formamide, which produces partial lysozyme sensitivity. Whether this is because the introduced groups are acetyl in one instance and formyl in the other or because they acylate different proportions of N and O groups is unknown. Nevertheless, subsequent de-O-acylation by alkali results in complete sensitivity to enzymatic degradation after either procedure. Treatment with alkali is ineffective on non-acid-treated walls, either alone or after acetic anhydride. If used alone on acid-extracted walls it produced no sensitivity to lysozyme, but, oddly, resulted in considerable susceptibility to phagocytic degradation (Table 1, line 11). This difference between lysozyme sensitivity and phagocytic degradation did not occur under any other conditions of treatment. This phenomenon is at present unexplained, except on the basis of differences between egg-white lysozyme and human lysozyme or of the conditions present within phagocytic vacuoles.

All muramidases cannot be equated. Although human and egg-white lysozymes are of a similar nature, they differ in amino acid composition, antigenic structure, and activity (30). The *Streptomyces* $F_1$ enzyme (a $\beta$-1,4-endo-acetylmuramidase) is reported to completely dissolve group A cell walls, as well as those of other gram-positive bacteria that are not directly sensitive to lysozyme (26). However, these streptococcal walls were first treated with trypsin and were washed several times in sodium dodecyl sulfate (H. Heymann, *personal communication*), procedures which do not render them lysozyme-sensitive.

In considering our results, certain facts cannot be ignored. (i) There is no assurance that the mucopeptides of all group A streptococci are identical. Few strains have been examined quantitatively (15, 25). (ii) The amounts and chemical structures of all cell wall polysaccharides of different strains of group A streptococci may differ, and, therefore, their possible protective effects or susceptibility to removal by various chemical procedures or enzymes also may differ. (iii) The residual polysaccharide remaining on walls after relatively mild acid extraction may still be inhibitory to lysozyme. That this is not completely valid, however, is suggested by the partial sensitivity to lysozyme seen after formamide treatment which also leaves residual carbohydrate. There is also the possibility that formamide alters the residual carbohydrate to change its protective effect and acid does not. The presence of complete or unaltered carbohydrate, as on native cell walls, may also prevent the effects of acetic anhydride and alkali because these treatments do not affect lysozyme sensitivity until most of the carbohydrate is removed. There is no known enzyme capable of completely removing the cell wall carbohydrate; only determinants of group specificity are removed by the exo-$\beta$-*N*-acetylglucosaminidase found in some leukocytes (4, 44). Other methods of removing polysaccharide, such as autoclaving (32), or extraction with dilute nitrous acid (45), sodium deoxycholate (17), or trichloroacetic acid (42), have not been tested for their effects on the lysozyme sensitivity of the residual mucopeptide. (iv) The use of an M-negative strain in our experiments does not alleviate the possibility that other proteins, and therefore other sites susceptible to acylation or deacylation, may be present in the native cell wall. Although no surface fimbriae indicative of R protein (45) are present, the wall may contain other proteins, such as T antigen. Because these cell walls were not treated with proteolytic enzymes, any residual cell wall protein remaining after formamide or acid treatment could affect the action of lysozyme or lysosomal enzymes.

The best possible conclusion at the present time is that group A streptococcal cell walls are resistant to egg-white lysozyme and to phagocytic digestion, not only because of the presence of the polysaccharide and protein, but because their cell wall structure contains a sufficient number of free amino groups to hinder lysozyme activity and the activity of similar muramidases found in human leukocyte granules. If this is so, then the persistence of group A cell walls in tissues may be explainable. However, it must be added that persistence in vivo has been shown only in animals, and that our experiments have utilized human leukocytes.

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