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Electron Microscopy of the Lysis of *Staphylococcus aureus* Cell Walls by *Aeromonas* Lytic Factor

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Isolated and purified cell walls of *Staphylococcus aureus* were treated with a purified fraction of the culture supernatant fluid of a species of *Aeromonas*. The course of lysis of the cell walls was followed over a period of time by examination of samples under an electron microscope. The undifferentiated cell wall was rapidly digested, but the equatorial rings were more resistant. The undifferentiated cell wall became a very thin sheet before completely dissolving, leaving a series of equatorial rings of various widths. As digestion proceeded, solubilization of the entire cell wall occurred. Analogous findings were obtained with purified *S. aureus* mucopeptide. It is concluded that the *Aeromonas* lytic principle is an enzyme, and that susceptible bonds are more concentrated in the undifferentiated cell wall mucopeptide.

A morphological study of the lysis of *Staphylococcus aureus* cell walls has been reported by Virgilio et al. (10). Agents used in this work were lysozyme, trypsin, and trichloroacetic acid. Cell walls with residual autolysin activity, when treated with lysozyme, slowly dissolved in a completely nonspecific fashion. A similar result was obtained with lysozyme treatment of isolated mucopeptide. This result contrasts with the morphological changes observed when *S. aureus* cells are allowed to undergo autolysis (5). Mitchell and Moyle (5) were able to isolate 75% of the original cell walls from an autolysate as hemispherical shells, and indicated that the lytic action of the autolysin was confined to a narrow ribbon of cell wall at right angles to the equatorial ring of the cell. We have recently reported that culture supernatant fluids of an *Aeromonas* species cause lysis of *S. aureus* cells (2). Isolated cell walls, devoid of autolysin activity, have also been found to be lysed by these fluids. An electron microscope study of the digestion of cell walls and mucopeptide was therefore undertaken with purified *Aeromonas* lytic agent to complement the work cited above.

MATERIALS AND METHODS

Preparation of S. aureus cell walls. S. aureus, strain 8325 ($\alpha i^- p^+$) (7) was grown overnight in the CY medium of Novick (6). The cells were harvested by centrifugation and washed twice with distilled water. To a 10% (w/w) suspension of cells in distilled water, an equal volume of glass beads (Superbrite type 120-5005, Minnesota Mining and Manufacturing Co., St. Paul, Minn.) was added, and the mixture was stirred in a Servall Omni-mixer at full speed for 10 min at 0 to 5 C. The beads were removed by filtration through a coarse sintered-glass filter and were washed with water; the washings were added to the broken cell suspension. The suspension was further fractionated by alternate cycles of slow and fast centrifugation at 0 C. The cell walls were not sedimented at 2,500 × *g* for 5 min, but were deposited at 12,000 × *g* for 10 min. Ten to twelve such cycles were required for removal of unbroken cells. The cell walls were then suspended in distilled water and heated at 56 C for 2 hr to destroy any autolytic activity. They were collected by centrifugation and resuspended in 0.05 M sodium phosphate (*p*H 7.6), containing 2 mg of trypsin per ml (Worthington Biochemical Corp., Freehold, N.J.) and 125 μg of ribonuclease per ml (Sigma Chemical Co., St. Louis, Mo.). Incubation at 37 C was continued until there was no further fall in turbidity at 660 mμ. The cell walls were then washed several times with distilled water and freeze-dried.

Preparation of mucopeptide. Cell walls were extracted with 50 ml/g of 5% (w/w) aqueous trichloroacetic acid (with stirring) at 4 C for 64 hr; the concentration was then increased to 10% (w/w) by the addition of more trichloroacetic acid. Extraction was continued at 4 C for an additional 36 hr to remove the teichoic acid component (1) of the cell wall. The mucopeptide was isolated by centrifugation, washed several times with distilled water, and freeze-dried.

Lytic activity. Lytic factor from the culture supernatant fluid of a species of *Aeromonas* (2), grown in 2% yeast extract (Difco), was purified and concentrated 20- to 25-fold to give a solution containing 45,000 units per ml, as previously defined (2). Details of the purification procedure and chemical changes taking place during digestion of cell walls will be

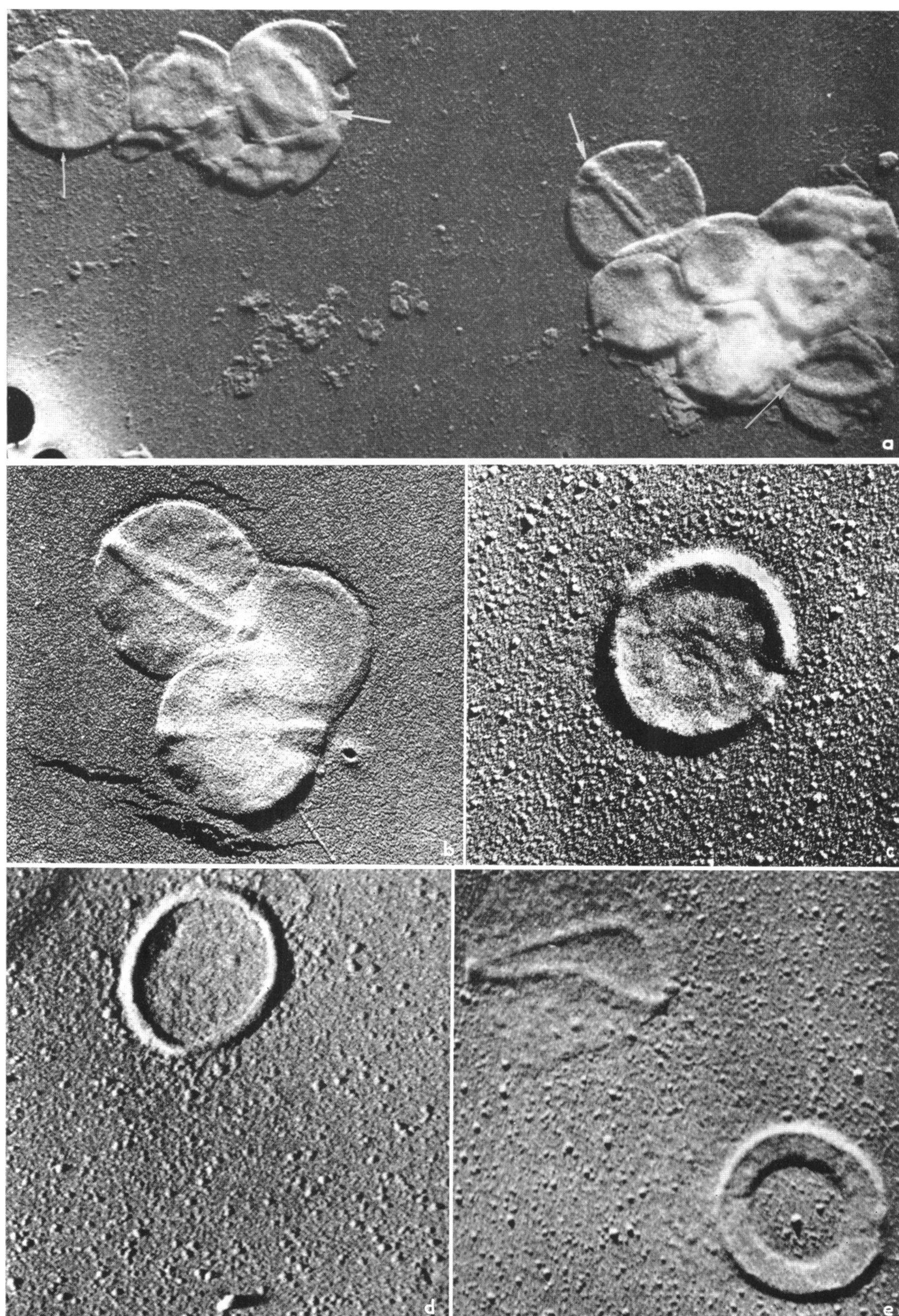

FIG. 1. *Stages in the digestion of Staphylococcus aureus cell walls by Aeromonas lytic factor. (a) Untreated cell walls.* × *21,000. (b–e) Treated cell walls which show successive stages.* × *28,000.*

published elsewhere. The rate of absorbancy change at 660 mμ with cell walls as substrate is approximately one-quarter of that with whole cells.

Lysis. Although the nature of the medium has a strong influence on the activity of the lytic agent, maximal lytic activity is obtained in any one of several amine-containing buffers at low ionic strength and at *p*H 9.0. Lysis of cell walls was carried out in 0.02 M tris(hydroxymethyl)aminomethane (Tris)-acetate buffer (*p*H 9.0) at an initial cell-wall concentration of 5 mg/ml, and enough lytic agent was added to give a final concentration of 4,500 units per ml. Mucopeptide at an initial concentration of 5 mg/ml in 0.01 M trimethylamine acetate buffer (*p*H 9.0) was digested with 1,600 units of lytic activity per ml. In both cases, incubation was at 28 C, and at suitable times samples were removed and frozen in a dry ice-acetone bath. After thawing, the suspensions were placed onto Formvar-coated grids and shadowed at $\tan^{-1}$ 0.2 (approximately 11°) with platinum-palladium (80:20, w/w), followed by examination and photography with a Siemens Elmiskop I electron microscope.

RESULTS

Action of lytic agent on cell walls. Figure 1 shows typical isolated cell walls and their progressive digestion by the *Aeromonas* lytic factor. Untreated cell walls are of uniform thickness except in the regions where equatorial rings, indicated by arrows, are visible (Fig. 1a). In undigested cell walls, the equatorial rings are seen to lie in random orientation. In the two cells on the left of Fig. 1b, the equatorial rings are diametrically oriented, and in the right one, circumferentially. Upon addition of lytic agent, a rapid digestion of cell wall takes place. Figure 1b shows cell walls which were frozen within 1 min after the addition of lytic agent. The undifferentiated cell wall has become thinner and some loss of definition can be seen around the edges, except in cells which are lying in such a manner that the equatorial ring forms the circumference.

In Fig. 1c, which shows a cell wall with its equatorial ring oriented circumferentially, the thinning of the undifferentiated cell wall, makes the equatorial ring appear more prominent. A further stage in this process is shown in Fig. 1d. In the lower specimen of Fig. 1e, the undifferentiated cell wall has been dissoved completely, leaving only a wide equatorial ring, whereas in the upper specimen a very thin sheet of undifferentiated cell wall is still visible surrounding the equatorial ring. Owing to the loss of rigidity produced by the removal of the undifferentiated cell wall, most residual equatorial rings are seen as complete circles on the grid surface. As digestion proceeds, complete solubilization of the equatorial ring gradually takes place.

It is not possible to give the exact times required to reach the stages shown in Fig. 1b to 1e. Very few undifferentiated cell walls could be found after 40 min of incubation, and almost no equatorial rings were seen after 3 hr. No visible changes in morphology occurred when cell walls were incubated at 28 C in buffer alone for up to 16 hr, confirming that autolysin activity had been destroyed by the heat treatment given in preparing the cell walls.

Digestion of mucopeptide. Structural changes analogous to those seen with cell walls were observed when isolated mucopeptide was treated with lytic agent (Fig. 2). Untreated mucopeptide with its thick equatorial ring is shown in Fig. 2a. Figure 2b illustrates the thinning and spreading of the undifferentiated mucopeptide over and

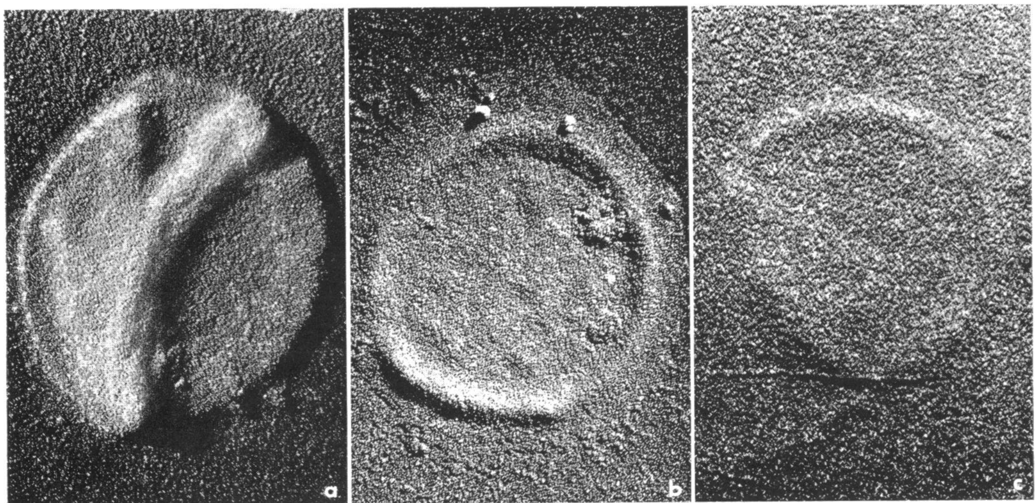

FIG. 2. *Stages in the digestion of Staphylococcus aureus mucopeptide.* × *56,000.*

around the equatorial ring. It would appear that, with some bonds split, the mucopeptide network is now much more loose and is able to cover a greater area. A mucopeptide sample in which dissolution is almost complete is depicted in Fig. 2c, where only a slight suggestion of the equatorial ring remains.

DISCUSSION

The cell walls of gram-positive bacteria contain two major types of structural polymer, the teichoic acid (1) and the mucopeptide (4). However, it is the mucopeptide that is responsible for the rigidity and mechanical strength of the cell wall (11), and the possibility that lysis of live *S. aureus* cells was caused by weakening of this component was therefore considered. The present studies demonstrate a direct action of the *Aeromonas* lytic agent, under physiological conditions, on isolated cell walls, and specifically on the mucopeptide structure. From the known chemical resistance of mucopeptide (8) this demonstration is *prima facie* evidence that the lytic agent itself is an enzyme.

It is evident from the results presented that different regions of the cell wall and mucopeptide are completely digested at different times by the lytic enzyme, suggesting that chemical differences may exist between the undifferentiated cell wall and the equatorial ring. At the time of complete digestion of the undifferentiated cell wall, equatorial rings of greatly varying widths are visible. As it is believed that the equatorial ring represents the plane of division in *S. aureus* (3), the varying width of the equatorial rings would be a reflection of the degree of septum formation that has occurred in the cell at the time of mechanical rupture. Thus, it is possible that these structures remain longer than the bulk of the wall in the lytic medium, simply because they are composed of a greater amount of the same substrate. Although this probably contributes to the differences observed, it is unlikely to be of great significance in view of the results of Virgilio et al. (10), whose studies showed no comparable structural changes when *S. aureus* cell walls and mucopeptide were digested with lysozyme. Moreover, when *S. aureus* cell wall mucopeptide was digested with lysostaphin, the whole structure appeared to be attacked at similar rates, and no structural differentiation was observed (9).

The results of Mitchell and Moyle (5) clearly demonstrated different regions of the cell wall of *S. aureus* sensitive and resistant to the action of autolysin. However, it was not established whether this differential action of autolysin was caused by chemical differences between different regions of the cell wall, or occurred because the autolytic enzyme itself was localized within the band of wall actually digested. Our own studies, in which an external supply of lytic enzyme was used, support the conclusion that chemical differences may exist between different regions of the mucopeptide of *S. aureus* cell walls.

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