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Electron Microscopy of *Staphylococcus aureus* Cell Wall Lysis

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ABSTRACT

VIRGILIO, RAFAEL (Escuela de Química y Farmacia, Universidad de Chile, Santiago, Chile), C. GONZÁLEZ, NUBIA MUÑOZ, AND SILVIA MENDOZA. Electron microscopy of *Staphylococcus aureus* cell wall lysis. J. Bacteriol. **91**:2018–2024. 1966.—A crude suspension of *Staphylococcus aureus* cell walls (strain Cowan III) in buffer solution was shown by electron microscopy to lyse slightly after 16 hr, probably owing to the action of autolysin. The lysis was considerably faster and more intense after the addition of lysozyme. A remarkable reduction in thickness and rigidity of the cell walls, together with the appearance of many irregular protrusions in their outlines, was observed after 2 hr; after 16 hr, there remained only a few recognizable cell wall fragments but many residual particulate remnants. When autolysin was previously inactivated by trypsin, there was a complete inhibition of the lytic action of lysozyme; on the other hand, when autolysin was inactivated by heat and lysozyme was added, a distinct decrease in the thickness of the cell walls was observed, but there was no destruction of the walls. The lytic action of lysozyme, after treatment with hot 5% trichloroacetic acid, gave rise to a marked dissolution of the structure of the cell walls, which became lost against the background, without, however, showing ostensible alteration of wall outlines. From a morphological point of view, the lytic action of autolysin plus lysozyme was quite different from that of trichloroacetic acid plus lysozyme, as shown by electron micrographs, but in both cases it was very intense. This would suggest different mechanisms of action for these agents.

Staphylococcus aureus cells have proved resistant to treatment with several lytic agents; even some enzymes, such as lysozyme, that dissolve completely certain gram-positive bacteria, have little or no action on cells of *S. aureus* (3, 13, 14).

Nevertheless, Mitchell and Moyle (5) were able to obtain protoplasts of *S. aureus* in a suitable medium, and they suggested that an autolytic enzyme ruptures the cell into two hemispheres. Aldrich et al. (1) reported that *S. aureus* cells are lysozyme-resistant, but that protoplasts have been obtained with this enzyme and the concurrent action of methicillin.

Isolated cell walls are resistant to lysozyme (6, 13, 15), but they become sensitive after treatment with trichloroacetic acid (4). A lytic effect on cell walls by autolysins, phagolysins, and virolysins was reported by Ralston and McIvor (11). The morphological changes caused by the action of these enzymes seem not to have been studied in detail.

This paper describes morphological changes in cell walls of *S. aureus* subjected to the action of lysozyme, autolysin, trypsin, and trichloroacetic acid, as shown by electron microscopy.

MATERIALS AND METHODS

Preparation of S. aureus cell walls. An 18-hr culture of *S. aureus* (strain Cowan III) was inoculated into four Roux bottles, containing nutrient agar prepared from fresh meat, and the bottles were incubated for 16 hr at 37 C. The culture from each bottle was collected in 10 ml of sterile distilled water, and was centrifuged for 20 min at 3,000 rev/min; the sediment was suspended in sterile distilled water. This suspension was mixed with 0.12-mm Ballotini glass beads (Jencons no. 15) and shaken for 60 min in a Mickle mechanical disintegrator. The beads were eliminated by filtration through a sintered-glass filter (Jena G3). The suspension was washed, and was then centrifuged at 10,000 × *g* for 30 min at 4 C (three times); the cell walls were separated and purified in a sucrose gradient (12). The cell walls were washed again (three times) with distilled water, and, as a control, they were studied under the electron microscope. The final enzymatic treatments were omitted,

to preserve the residual autolysin(s). The purified cell walls were suspended in sufficient distilled water to provide a suspension of 0.6 to 0.8 mg (dry weight) of bacteria per milliliter, according to a standardized opacity curve, in a Klett-Summerson photocolorimeter (2). The standard cell wall suspension was stored at −20 C.

Lysozyme treatment. A 1-ml sample of the standardized cell wall suspension was centrifuged, and the sediment was treated with 2 ml of a solution containing 1 mg of lysozyme (3× crystallized; Sigma Chemical Co., St. Louis, Mo.) per ml of 0.066 M phosphate buffer (*p*H 6.2). This suspension was incubated for 2, 4, 8, or 16 hr at 37 C.

Three other standardized samples, with lysozyme concentrations of 125, 250, and 500 μg/ml, respectively, were incubated for 16 hr at 37 C.

Heat treatment. A 1-ml portion of the standardized cell wall suspension was heated for 1 hr in a boiling-water bath.

Trypsin treatment. The sediment from 1 ml of standardized cell wall suspension was suspended in 2 ml of a 0.1 mg/ml solution of crystallized trypsin (Calbiochem) in a (*p*H 8.4) $NaHCO_3$ buffer (8) and incubated for 90 min at 37 C.

Treatment with trichloroacetic acid. The sediment from 1 ml of standardized cell wall suspension was treated with 2.5 ml of 5% trichloroacetic acid at 90 C for 6 min (8).

The cell walls subjected to heat, trypsin, and trichloroacetic acid were immediately washed, centrifuged, and suspended in a 1 mg/ml lysozyme solution, and were incubated for 16 hr at 37 C.

In each case, a control was run, subjecting the cell walls to an identical treatment, but omitting the final addition of lysozyme.

The action of lysozyme on cell walls treated successively with trypsin and trichloroacetic acid was studied in another sample.

Electron micrographs. Samples of cell walls treated as described above were centrifuged and suspended in distilled water. Preparations on collodion films were dried, shadowed with chromium or palladium at an angle of 15°, and observed under a Siemens electron microscope (V. B. G.).

RESULTS

Action of lysozyme. Figure 1 shows the effect of lysozyme on *S. aureus* cell walls. When lysozyme was added to a cell wall suspension, some effects were observed within 2 hr. The edges of the cell walls began to dissolve, losing their smooth and homogeneous circular outline and becoming oval-shaped with many irregular prolongations. It was evident that there was some loss in thickness and rigidity, giving the whole structure the appearance of a semisolid flattened mass. This effect became more marked after several hours; after 16 hr, only a few cell walls could be recognized. There was an increasing number of particles of 250 to 700 A, apparently produced by the partial destruction of the cell walls.

Variation of lysozyme concentration. S. aureus cell wall preparations were treated with 125, 250, or 500 μg/ml of lysozyme for 16 hr at 37 C. An effect similar to that obtained with a lysozyme concentration of 1,000 μg/ml (Fig. 1) was observed with the 500 μg/ml concentration. With the other two concentrations, the effect was much less marked.

To clarify whether the "disintegrating lysis" was due only to the action of lysozyme or whether it was the result of the concurrent action of the residual autolysin bound to the cell wall despite the repeated washings, a control was prepared by macerating the cell walls for 16 hr in phosphate buffer (*p*H 6.2) at 37 C without lysozyme. On the other hand, autolysin was inactivated by heat, by trypsin, and by hot trichloroacetic acid, followed by lysozyme addition.

When the cell walls were suspended in phosphate buffer without lysozyme, it was observed that many of them retained their homogeneous and smooth edges, that they remained thicker than lysozyme-treated cell walls, and that they kept much intracellular material; some others showed the effect of a quite similar lytic action, but of obviously lesser degree than that caused by lysozyme (Fig. 2a).

Action of heat. There was no evidence of special alterations in *S. aureus* cell walls heated in a boiling-water bath for 60 min. There was no visible difference between them and untreated cell walls (Fig. 2b).

Action of trypsin. The thickness of cell walls from a crude preparation was decreased by trypsin, but some cytoplasmic fragments remained inside. There was a notable decrease in the visibility of the intracellular cross walls. The outlines and edges were circular, smooth, and well defined (Fig. 2c).

Action of trichloroacetic acid. The action of hot trichloroacetic acid was evident in a general decrease in the thickness of the cell walls; the cross walls persisted, and more cytoplasmic material was observed than in the trypsin-treated preparation (Fig. 2d).

Autolysin(s) was inactivated by all three agents. The effect obtained when lysozyme was added to these preparations could be ascribed only to the action of this enzyme; however, the possible alterations of the substrate resulting from these pretreatments must be considered.

The heated cell walls treated with lysozyme appeared to be lighter and more transparent to the electron beam; within the walls, some small granular and irregularly scattered cytoplasmic remains could be clearly observed (Fig. 3a).

The action of lysozyme on trypsin-treated *S.*

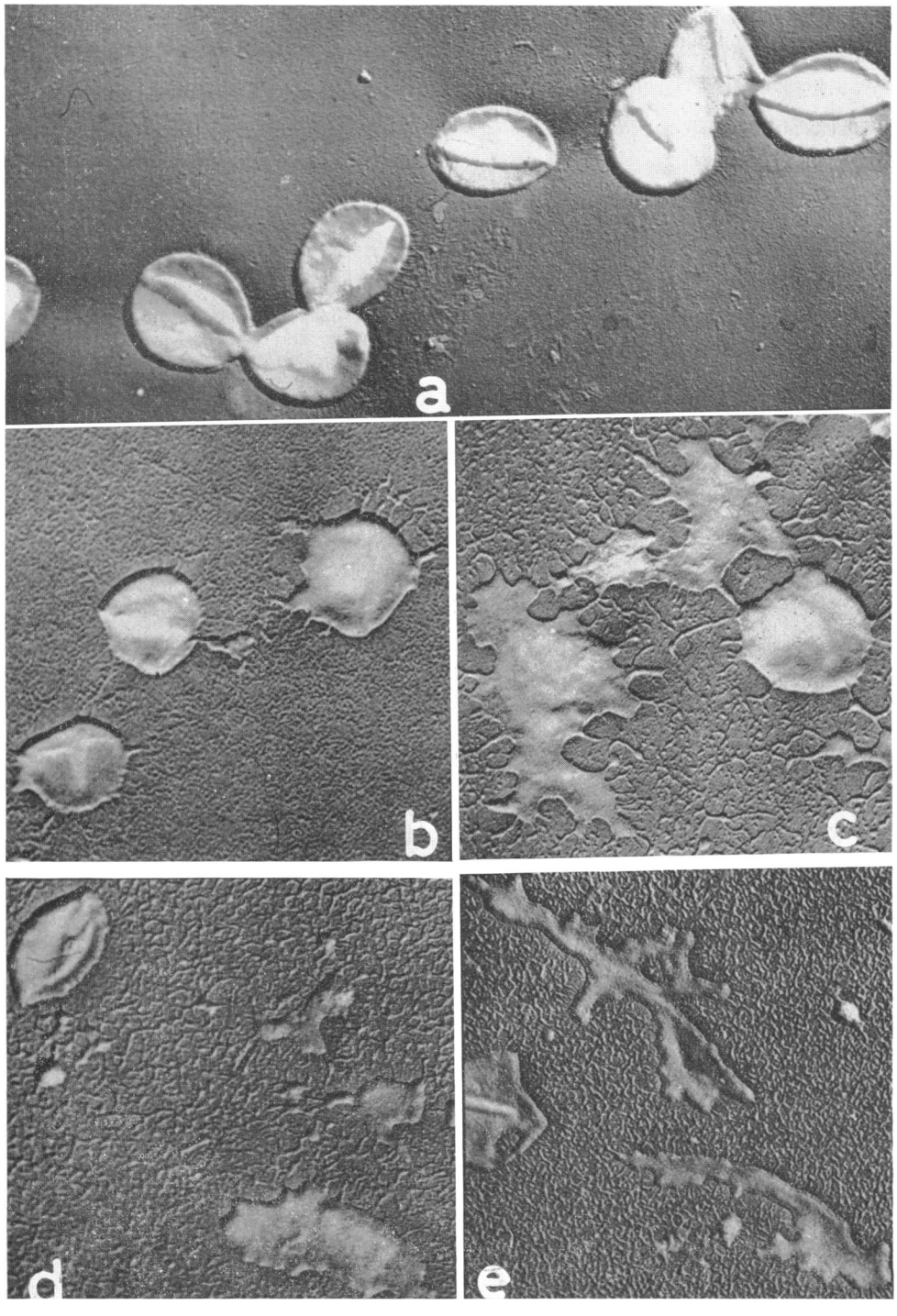

FIG. 1. *Crude preparation of Staphylococcus aureus cell walls treated with lysozyme. (a) Control cell wall preparation. (b, c, d, and e) After 2, 4, 6, and 16 hr of treatment with lysozyme (1 mg/ml) at 37 C. × 17,000.*

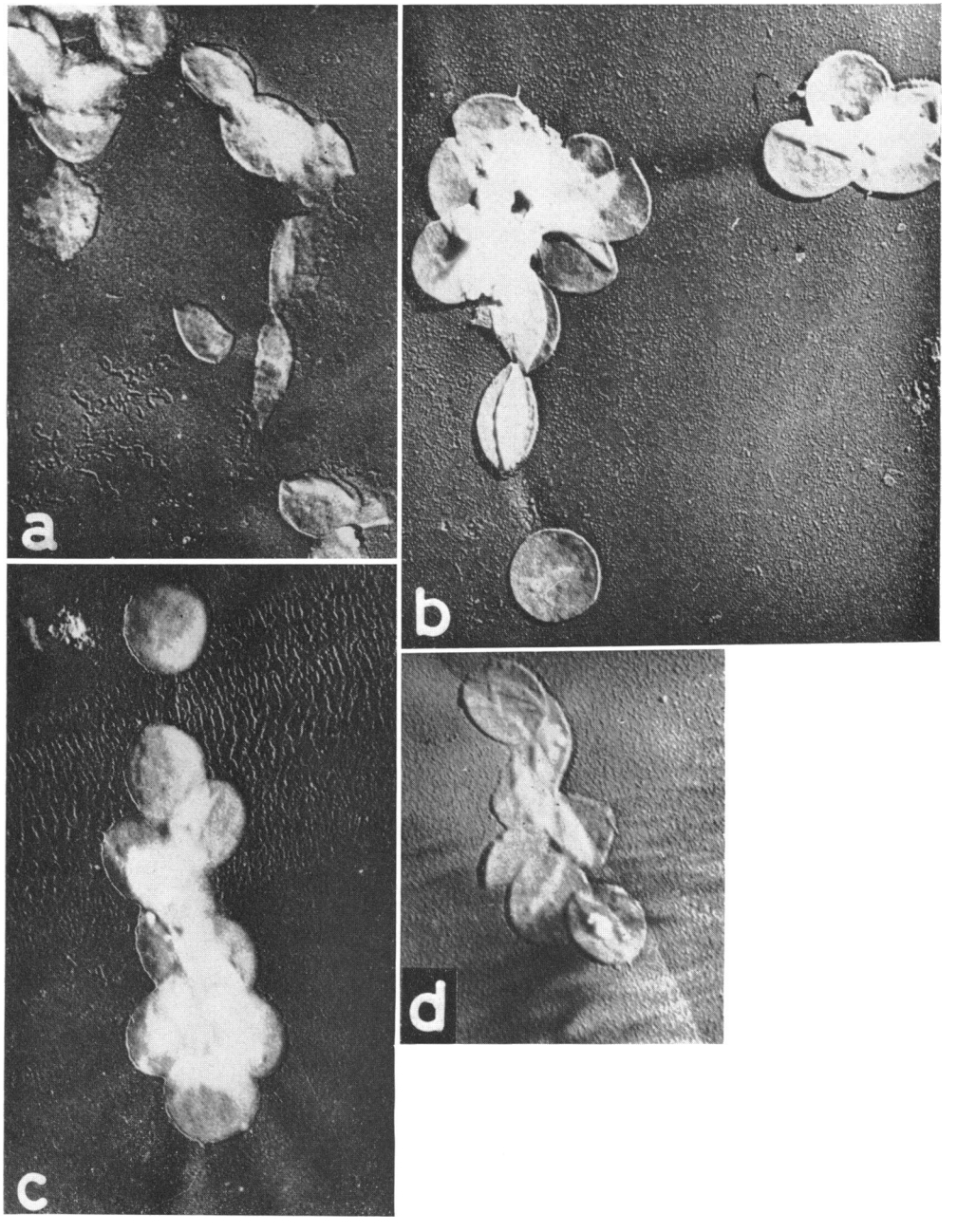

FIG. 2. *Autolysin, heat, trypsin, and trichloroacetic acid action on Staphylococcus aureus cell walls.* × *13,000.* (*a*) *Cell walls without lysozyme, maintained in a pH 6.2 phosphate buffer for 16 hr at 37 C.* (*b*) *Cell walls heated in a boiling-water bath for 60 min.* (*c*) *Cell walls treated with 0.05 mg/ml of trypsin for 90 min at 37 C.* (*d*) *Cell walls treated with a 5% trichloroacetic action at 90 C for 6 min.*

aureus cell walls did not usually modify either their outlines or their thickness, as compared with those treated only with trypsin. The surface, however, was altered, and presented some small depressions which gave it a gridlike appearance (Fig. 3b).

The most drastic action on the thickness of the cell walls was observed when they were subjected

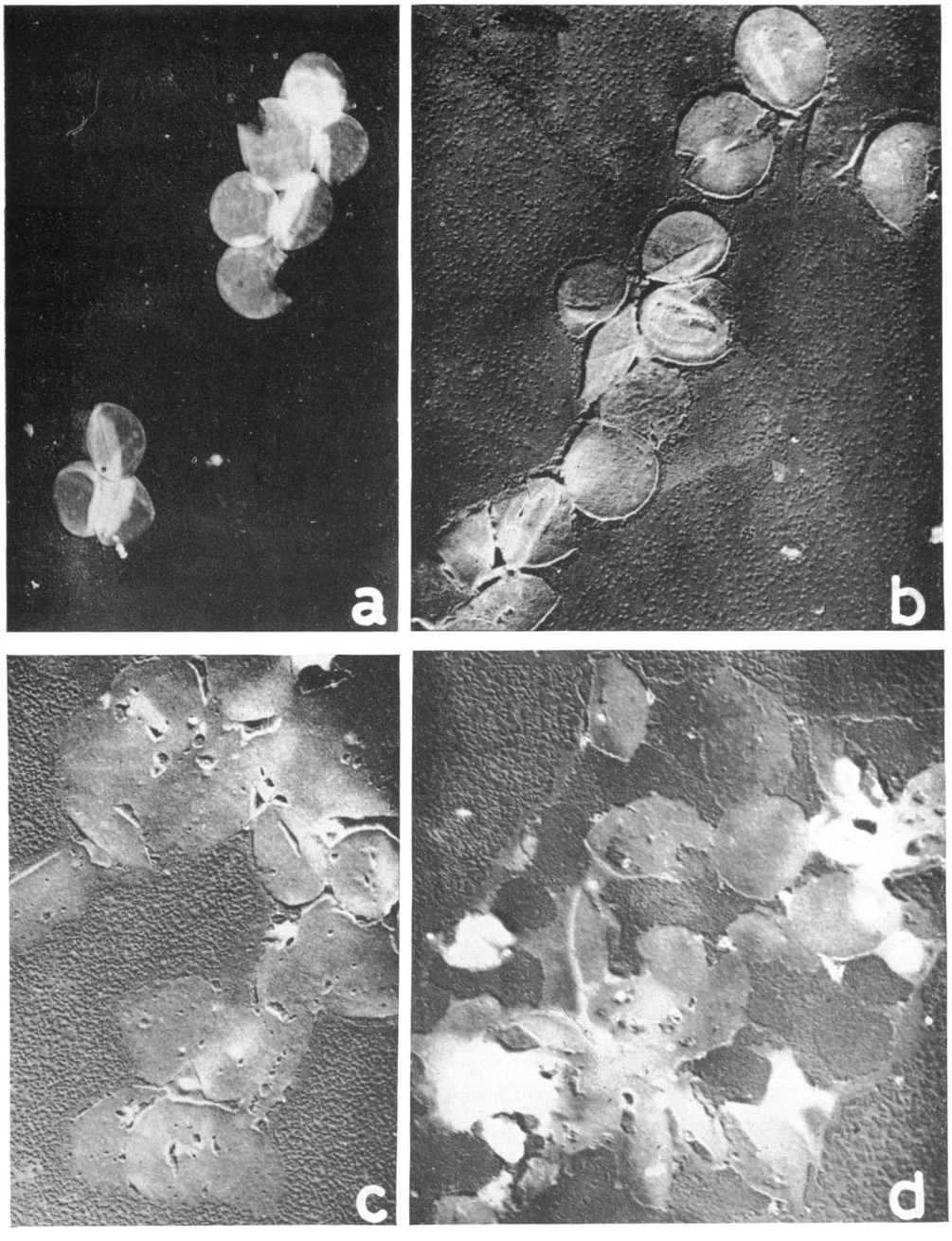

FIG. 3. *Action of lysozyme on Staphylococcus aureus cell walls previously treated with heat, trypsin, and trichloroacetic acid.* × *14,000.* (*a*) *Cell walls heated and treated with lysozyme.* (*b*) *Cell walls treated first with trypsin and then with lysozyme.* (*c*) *Cell walls treated first with trichloroacetic acid and then with lysozyme.* (*d*) *Cell walls treated with trypsin, trichloroacetic acid, and lysozyme.*

to the action of hot trichloroacetic acid and lysozyme (Fig. 3c). With this treatment, the cell walls, even though they almost disappeared against the background, did not lose their shape, and their outlines were clearly seen until their extreme thinness prevented them from being observed.

The effect of successive treatments with trypsin, trichloroacetic acid, and lysozyme on *S. aureus*

cell walls was similar to that just described (Fig. 3d).

DISCUSSION

The chemical constitution of bacterial cell walls makes them extremely resistant to the lytic action of solvents and strong chemicals (9). They are affected only by specific enzymes which can digest them. In *S. aureus*, one of its own enzymes, autolysin, has been shown to have this action. Virolysin, which is induced by a phage infection of the cells, has also been studied. Lysozyme does not act on the whole cell nor on isolated cell walls (13, 15), but Ralston (10) described a slight effect on whole cells which is reinforced by the simultaneous action of virolysin or autolysin ("synergism"). The methods mainly employed in study of these actions are the chemical determination of the components liberated by the enzymatic digestion of the cell wall, and a turbidimetric method.

Moderate lysis due to the action of the residual autolysin bound to our crude preparation was observed under the electron microscope. When lysozyme was added, both the rate and the intensity of the lytic action were greatly increased; the effect became perceptible within 2 hr, and, after 16 hr, the disintegration was almost complete. If the residiual autolysin(s) is eliminated by the action of trypsin, the cell walls are modified by the protease action of this enzyme, and later treatment with lysozyme produces no marked effects.

Trypsin has no action on the mucopeptide (the specific substrate of lysozyme), which remains susceptible to its enzymatic effect after trypsinization of the walls in sensitive bacteria; *S. aureus* cell walls do contain the basal mucopeptide, but are insensitive to lysozyme (9). The marked lytic effect of lysozyme on crude cell walls, and its subsequent inactivation by trypsin, may be explained by means of a necessarily concurrent action of autolysin and lysozyme, as suggested by Ralston (10) from work with whole cells. As it is not our purpose to discuss fully the chemical implications of this work, no attempt has been made to rule out the possibility that, in the special case of *S. aureus*, trypsin may produce an alteration on the substrate, presumably bound in a complex molecule.

It can be assumed that heat completely destroys the autolysin, and at the same time causes a change in the structure of the cell walls which makes them subject to the action of lysozyme; this would explain the distinct decrease in thickness of the cell walls shown by our electron micrographs. Ralston explains the "synergism" between autolysin and lysozyme as the uncovering of sites sensitive to the action of lysozyme. It is possible that the action of hot water has a similar explanation, since it has been shown that it removes some antigenic material (7). The difference between the effect of lysozyme on heated cell wall (Fig. 3a) and that of lysozyme together with autolysin (Fig. 1e) may be attributed to their action on different sites or substrates, which leads to complete lysis in the latter case.

The combined action of trichloroacetic acid and lysozyme differs from those mentioned above. A uniform decrease in cell wall thickness is observed, which is probably due to a homogeneous and gradual dissolution of its components (Fig. 3c). The action of lysozyme on *S. aureus* cell walls previously treated with trichloroacetic acid has been described by Maldemstam and Strominger (4), who explained it as the extraction of teichoic acids by trichloroacetic acid; this would leave the specific substrate available for the attack by lysozyme. Apparently, dissolution observed under the electron microscope is greater than that described by these authors, and this could be due to the different composition of the cell walls of the strains used.

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