Electron Microscopy During Release and Purification of Mesosomal Vesicles and Protoplast Membranes from *Staphylococcus aureus*

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The mesosomes of log-phase *Staphylococcus aureus* ATCC 6538P and *Staphylococcus aureus* phage-type 80/81, as seen in situ in ultrathin sections, were of the vesicular type. The constituent vesicles ranged from 35 to 50 nm in diameter when the glutaraldehyde-osmium-uranium-lead sequence of fixation and staining was used. During protoplasting in hypertonic buffer containing a muralytic enzyme, vesicles of the same size were extruded and required magnesium ion to maintain structural integrity. The vesicles, purified from the protoplasting supernatant medium by density gradient centrifugation, maintained size and configuration in a homogeneous preparation. Cytoplasmic membranes, produced by osmotic shock and nuclease treatment of protoplasts, were similarly concentrated in gradients. However, they were not free of membrane-associated ribosomes nor of mesosomal vesicles except when prepared in the absence of magnesium.

Mesosomes (10, 17), the intracytoplasmic membranous organelles which are most prominent in (though not restricted to) gram-positive bacteria, may occur in several configurations in the fixed, intact cell (3, 16). Their possible functions have been often discussed, but few studies have been made of their isolation, purification, composition, and activities in vitro (5, 8, 9, 12, 13, 14, 21, 22, 29, 36). Few of these reports show the sequence of ultrastructural changes during protoplasting and mesosome vesicle release, and most utilized rod-shaped bacteria.

We present here the ultrastructure of some staphylococcal mesosomes in situ, during release of their components by protoplasting with a muralytic enzyme, and after purification of the resultant vesicles by differential and density gradient centrifugation. Cytoplasmic membrane preparations from disrupted and enzymatically treated protoplasts were similarly concentrated and examined. A preliminary report of the findings has been made (T. J. Popkin, T. S. Theodore, and R. M. Cole, Bacteriol. Proc., 1970, p. 24). Details of preparative methods and preliminary chemical analyses are given elsewhere (33).

MATERIALS AND METHODS

Cultures. *Staphylococcus aureus* ATCC 6538P (obtained from the American Type Culture Collection), *S.*

aureus phage type 80/81 (isolated from a clinical source at the National Institutes of Health) and *S. aureus* strain LS (obtained from Archibald Scott, Department of Bacteriology, University of Dundee, Scotland) were used throughout. Stock cultures were maintained on Trypticase Soy Agar slants (BBL) at 4 C and subcultured every 3 months.

Growth conditions and lysis. Cells were grown aerobically to late log phase by shaking in 500-ml lots at 250 rev/min and 37 C for 12 to 14 hr in either Trypticase Soy Broth (TSB) or AOAC Broth (Difco), washed once, and suspended in 0.01 M phosphate buffer (*p*H 6.5) which contained 5% (for TSB) or 20% (for AOAC) sodium chloride—with or without 0.02 M magnesium sulfate. They were then incubated at 37 C in the presence of a cell-wall-lytic enzyme (LS enzyme) obtained from a 22 to 44% ammonium sulfate fraction of the supernatant fluid of a culture of *S. aureus* strain LS (33). Samples were removed at intervals and processed for electron microscopy.

Electron microscopy. After incubation, samples were mixed with an equal volume of 5% glutaraldehyde buffered to *p*H 7.2 in 0.2 M sodium cacodyalate (28) and kept at room temperature for 2 hr. The fixed whole cells or protoplasts were collected by centrifugation at 10,000 × *g* for 1 hr. The supernatant fluids, containing the mesosomal vesicle fraction, were centrifuged at 100,000 × *g* for 2 hr. The pellets were placed in 0.02 M sucrose buffered at *p*H 7.2 in 0.1 M phosphate (28) and held overnight at 4 C.

Secondary fixation was in acetate-Veronal-buffered 1% osmium tetroxide overnight at 25 C. This and subsequent agar embedment and uranyl acetate washings

were done by the methods of Ryter and Kellenberger (26).

Pellets or band materials from gradients were similarly fixed.

Dehydration in graded solutions of ethanol was followed by propylene oxide and final embedment in Epon 812 (19). Thin sections (silver-gray) were cut on an LKB Ultratome I with a diamond knife, placed on Formvar-coated copper grids, and stained for 3 min with alkaline lead citrate (23). Negatively stained preparations were made with either 1% aqueous uranyl acetate or 2% sodium phosphotungstate, pH 7.0. Micrographs were recorded on Kodak Electron Image plates with a Hitachi HU-11-C electron microscope operating at 75 kv.

Measurements were made on photographic prints by use of a Bausch & Lomb magnifier with an ocular micrometer scale calibrated in 0.1-mm gradations. Results were consistent when made on micrographs of different magnifications.

Purification of mesosomal vesicles and membranes. Details of treatments and separation procedures are reported elsewhere (33). In brief, after optimal time of exposure to LS enzyme in hypertonic buffer, protoplasts and any residual cell walls or whole cells were removed by centrifugation at 10,000 × g for 1 hr. After washing in the buffer, followed by osmotic shock in hypotonic buffer and treatment with deoxyribonuclease and ribonuclease, these preparations were again washed and sedimented on cesium chloride or sucrose density gradients to give bands of the resultant membranes. The original protoplasting supernatant was centrifuged at 100,000 × g for 2 hr and similarly washed and sedimented on cesium chloride or sucrose density gradients to give bands of mesosomal vesicles.

RESULTS

In thin section, cells of *S. aureus* 80/81 and ATCC 6538P appeared similar, but not identical, to other staphylococci (1, 2, 5, 18, 30-32, 35, 36; also see Fig. 1a-1c). There were no apparent differences in morphology between cells grown on TSB or AOAC media. The cell wall appeared trilaminar as in some other gram-positive bacteria (2, 4, 16, 34), and a well-defined submural periplasm or "gap substance" (20) separated wall from cytoplasmic membrane (Fig. 1d). The cytoplasm and nucleoid resembled those of other bacteria fixed by glutaraldehyde plus osmium, but ribosomes (except in lysing cells) were indistinct. The mesosomes in situ were conspicuous and usually arose from the membranous septum preceding the cross-wall, although peripheral mesosomes also occurred (Fig. 1a and 1c). Unlike those reported in other staphylococci (1, 2, 5, 18, 30, 31, 35, 36), these mesosomes were entirely vesicular. The component vesicles measured 35 to 50 nm in diameter. Occasionally, areas of apparent contact between nucleoid and mesosome could be seen (Fig. 1a).

Because strain ATCC 6538P has more meso-

somes per cell as compared with strain 80/81 under various growth conditions, most studies were performed with the former strain. However, the procedures employed were found equally suitable for either strain.

When exposed to the LS enzyme, the cells incurred rapid lysis of cell wall with concurrent initiation of protoplasting in the hypertonic buffer. In a manner previously described (11, 12, 25, 27), a pocketing of vesicles appeared between membrane and wall, and vesicles were released within 2 min as breaks appeared in the cell wall (Fig. 2a, 2b). As protoplasting progressed, chains of vesicles could occasionally be seen attached to the protoplast membrane (Fig. 3a, 3b, 3c). At 60 min of incubation, some whole cells, intact separated protoplasts, fragments of cell wall, and extracellular mesosomal vesicles were seen in the mixture (Fig. 4). The diameters of the released vesicles were the same as those seen in situ. The protoplasting process and the appearance of the resulting mixture were the same whether 5 or 20% NaCl was used in the buffer.

Examination of the crude vesicle fraction (100,000 × g) from samples taken after 2 min of protoplasting, or later, showed primarily vesicular and probably spheroidal elements in the sections (Fig. 5). The predominant small vesicles were bounded by a membrane of approximately 8.5 to 9.5 nm in total width, the inner layer of which was usually obscured by its approximation to the homogeneous granular vesicular content. The outer dense layer of membrane was approximately 3 nm in width. Vesicle diameters ranged from 35 to 50 nm, as in vesicles in intact cells and those released during protoplasting. Usually these crude preparations also contained some larger, membrane-bounded vesicles of a different internal appearance, apparently due to the presence of ribosomes (Fig. 5, arrows). The membranes of these bodies were of the same dimensions as those of the smaller vesicles but were seen more clearly. These large contaminating vesicles could often be seen to originate from cells during protoplasting (Fig. 6). In addition, the crude fraction contained some tubular bodies and masses of free ribosomes and other cytoplasmic debris (Fig. 5).

The contaminating particles were eliminated by subjecting the crude vesicle fraction to either cesium chloride or sucrose density gradient centrifugation. Preparations purified by cesium chloride (density 1.38 g cm⁻³) consisted of both vesicles (of uniform size range, 35 to 50 nm) and small tubular bodies (Fig. 7). Preparations purified by banding in a 60 to 85% stepwise sucrose gradient (33) were more uniform (Fig. 8a, 8b). These particles, which were found as a single
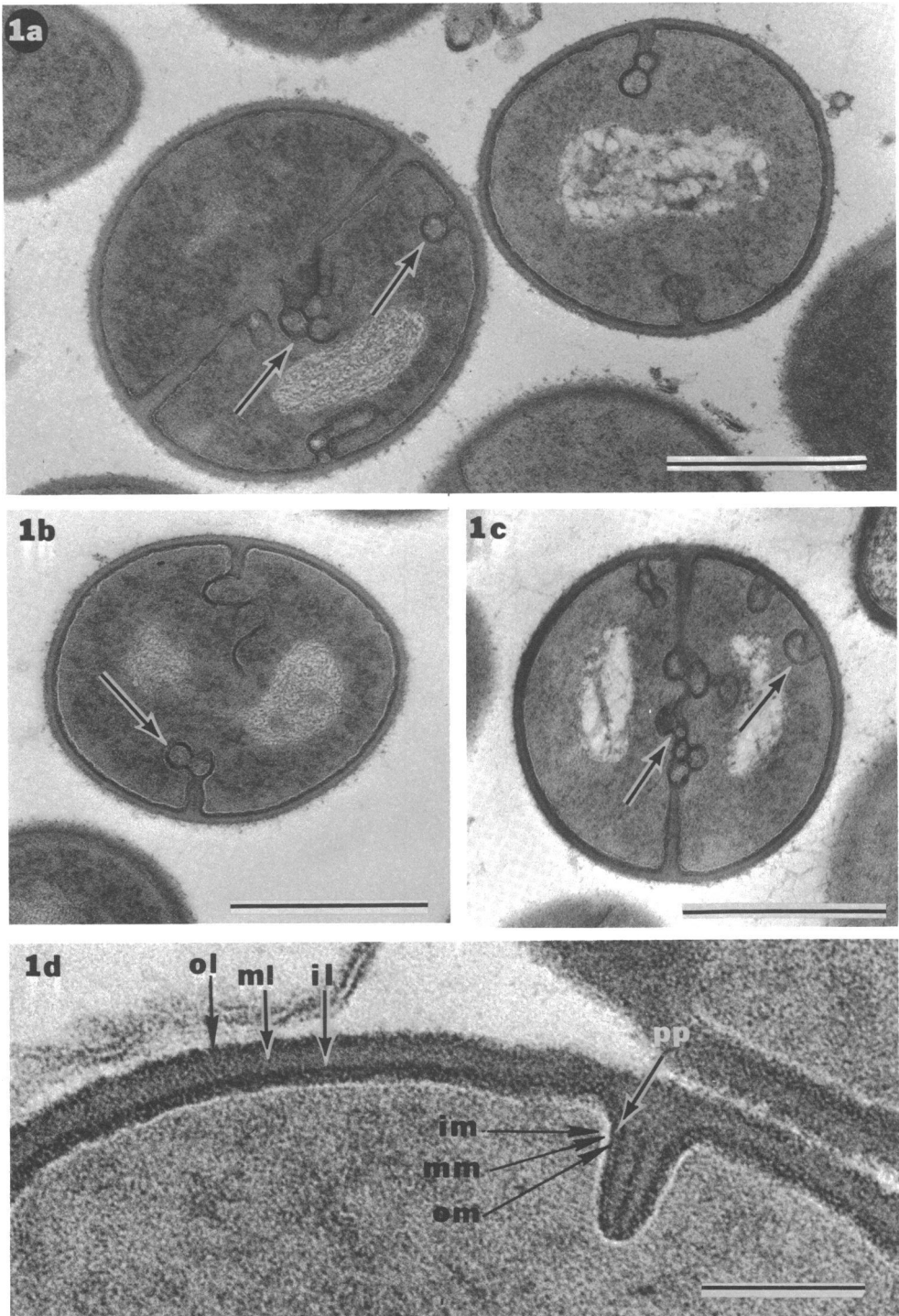
Fig. 1. *Ultrathin sections of log-phase cells of Staphylococcus aureus 80/81 and 6538P. Note the peripheral and septal vesicular mesosomes (arrows) in a, b, and c. ×57,500; bars, 0.5 μm. d, Wall detail; ol, outer dense layer of wall; ml, middle layer of wall; il, inner dense layer of wall; pp, periplasm; om, outer layer of cytoplasmic membrane; mm, middle nondense layer of membrane; im, inner dense layer of membrane. Bar, 0.1 μm.*
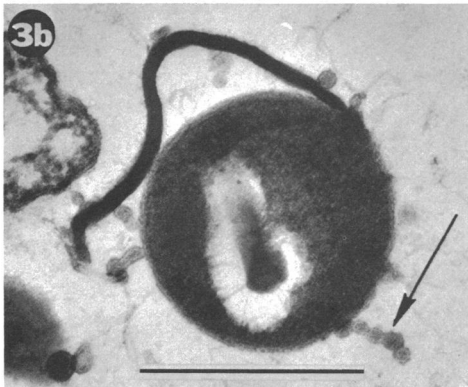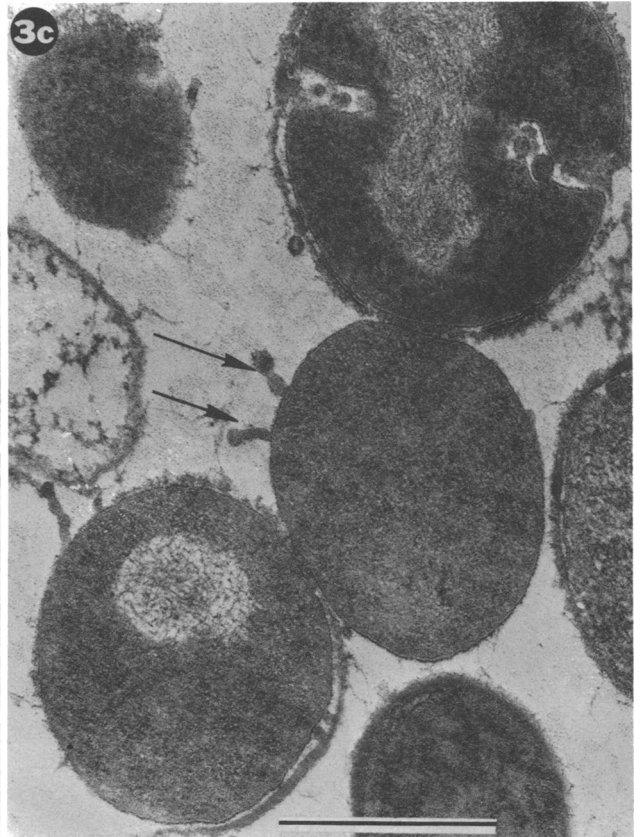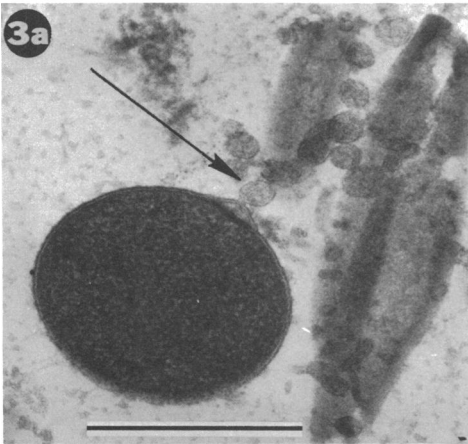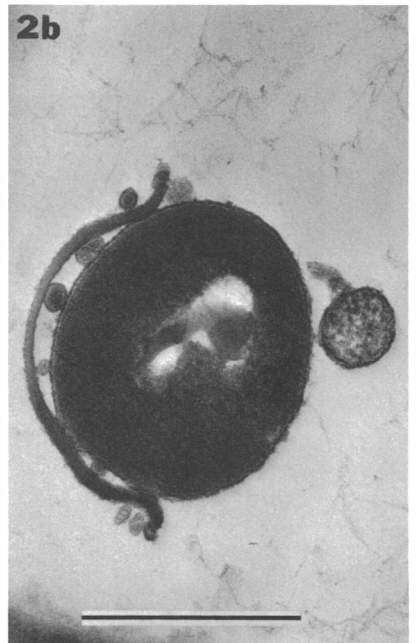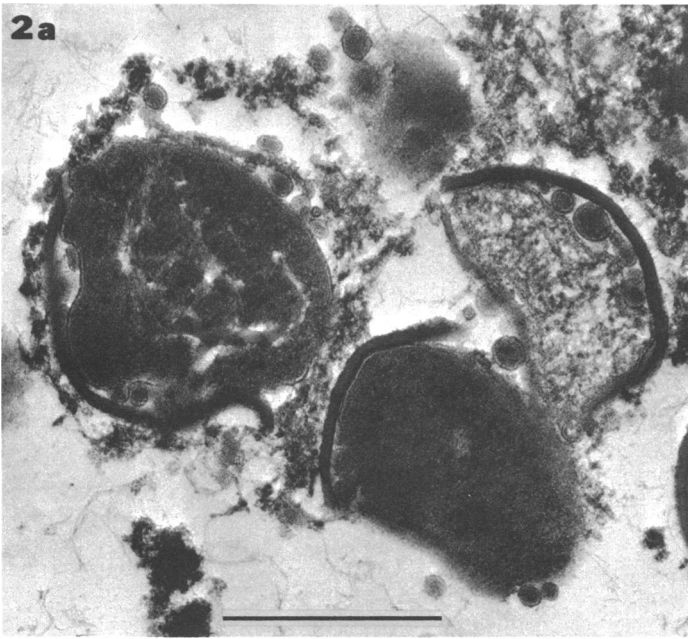
FIG. 2. *Ultrathin sections showing the enzymatic initiation of protoplasting in Staphylococcus aureus 80/81 and 6538P. a, Early stage; b, late stage. Note pocketing of vesicles between wall and cytoplasmic membrane and free extracellular mesosomal vesicles. Bars, 0.5 μm.*

FIG. 3. *Ultrathin sections of protoplasts with attached chains of mesosomal vesicles. These "chains" are associated with the cytoplasmic membrane as shown clearly in Fig. 3a. Bars, 0.5 μm.*
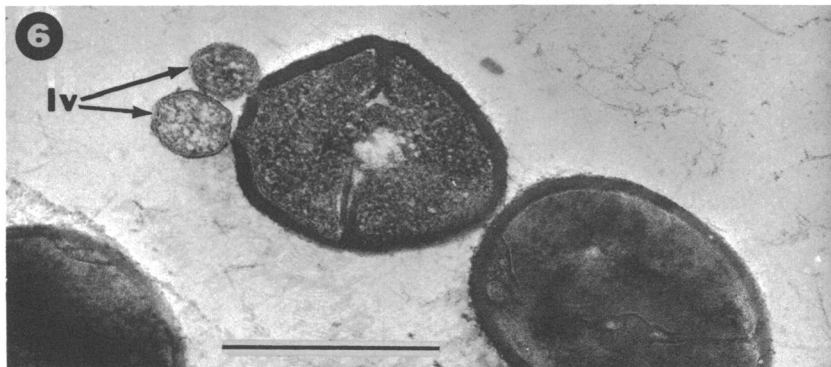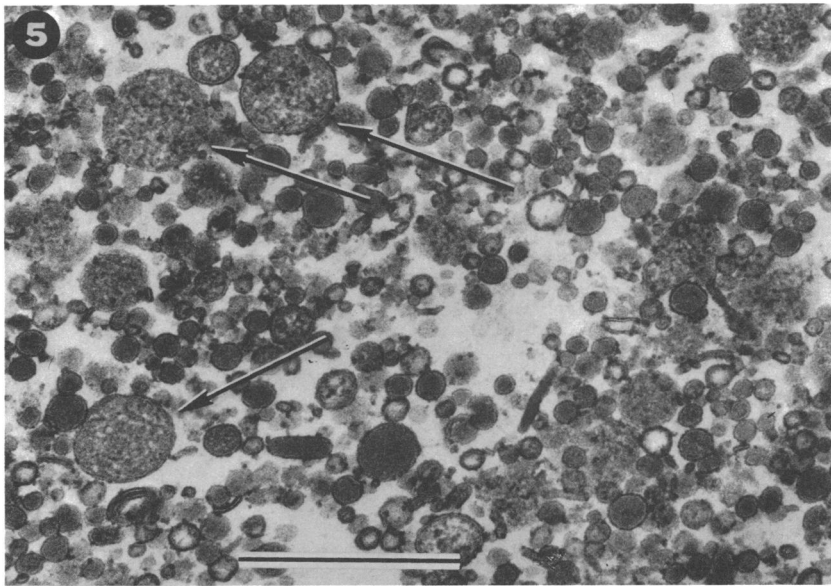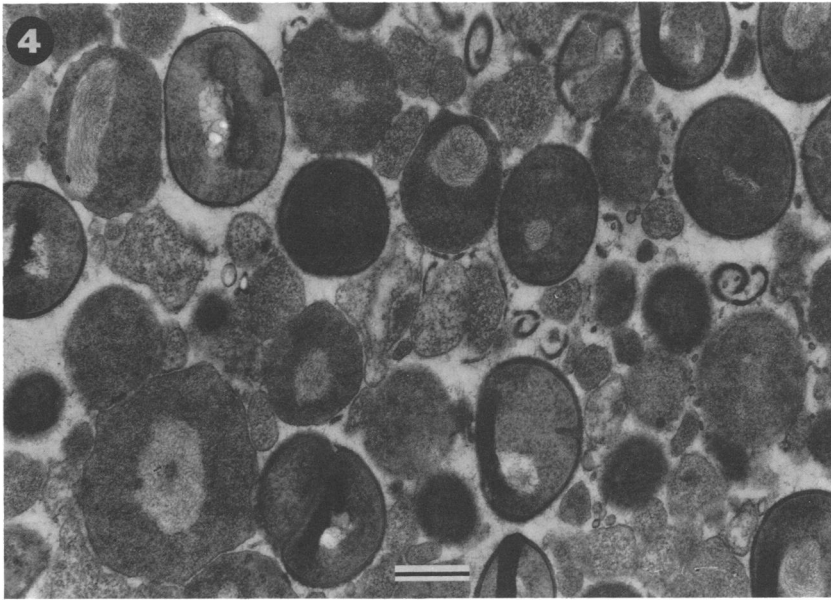
FIG. 4. *Crude protoplast pellet (10,000 × g). Note the presence of whole cells, intact protoplasts, cell wall fragments, and a few extracellular mesosomal vesicles. Bar, 0.5 μm.*

FIG. 5. *Crude vesicle pellet (100,000 × g). Note the predominance of small vesicular bodies of same size and homogeneous content as mesosomal vesicles in situ (Fig. 1) and during release (Fig. 2 and 3). Occasional large vesicles with cytoplasmic content were seen (arrows). Bar, 0.5 μm.*

FIG. 6. *Emergence of large "cytoplasmic" vesicles during protoplasting. Compare with Fig. 5. Bar, 0.5 μm.*
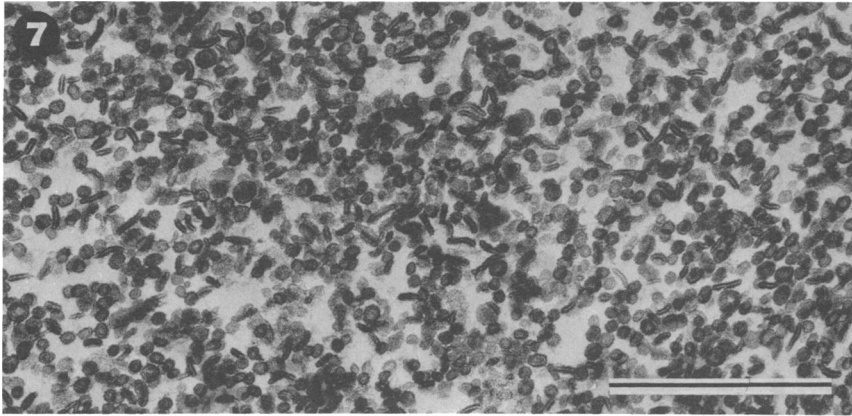
FIG. 7. *Mesosomal vesicles purified by cesium chloride density gradient centrifugation. Both uniform vesicles and small tubular structures are present. The latter are probably collapsed mesosomal vesicles. Bar, 0.5 μm.*

reddish-brown band in the upper region of the gradient (33), were entirely vesicular, ranged from 35 to 50 nm in diameter, and contained a homogeneous material (Fig. 9).

The upper band of mesosomal vesicles from the sucrose gradient were devoid of the larger membrane-bounded bodies present in the crude supernatant fraction. They were found in a lower, uncolored, diffuse band (33) of heterogeneous content (not shown here). Some membranous bodies of similar size, but predominantly protoplast membranes, were isolated from the osmotically shocked and nuclease-treated protoplast pellet (10,000 × g) after centrifugation in stepwise 60 to 75% sucrose gradients (33). These membranes appeared in a single, sharp upper band; a diffuse, lower band was contaminated with cell walls, cellular debris, and a few unlysed protoplasts. Most of these bodies, which were distorted in shape, appeared relatively empty, but all contained what appeared to be membrane-associated ribosomes (Fig. 10a, 10b, 10c). In addition, this band contained some free ribosomes and mesosomal vesicles.

When protoplasting was performed in the usual hypertonic buffer devoid of magnesium or other divalent cation, the supernatant fluid, after centrifugation at 100,000 × g, contained no mesosomal vesicles, and only a few ordered structures of uncertain nature were seen in sections from such a preparation (Fig. 11). On the other hand, the protoplast fraction (10,000 × g) following osmotic shock and nuclease treatment contained few visible or membrane-associated ribosomes or mesosomal vesicles, but intact membranes, some unlysed protoplasts, and fragments of cell wall were present (Fig. 12). These fractions were not subjected to density gradient centrifugation in this study.

DISCUSSION

This study shows that, although other features of intact cells were similar, the vesicular mesosome configurations seen in the strains of *S. aureus* examined were different from the tubules or lamellae reported in other staphylococci. It is uncertain whether these differences may be due to strain, media, conditions of growth, phase of growth, or preparative procedures for electron microscopy. Pretreatments (16) or conditions of fixation may influence mesosomal form, and Burdett and Rogers (3) recently emphasized the importance of ionic strength and divalent cation content of the fixing solution. They conclude that (in bacilli) constricted tubules and vesicles, and not lamellar membranes, represent the normal state in rapidly growing cells fixed successively by glutaraldehyde and the Ryter-Kellenberger (26) procedures, if initial ionic strength and divalent cation content are adequate. Our procedures for fixing log-phase cells, in which magnesium replaced calcium, appear to meet their requirements. Our holding period in sucrose-phosphate buffer (28) occurred after glutaraldehyde fixation, and the sucrose concentration (0.02 M) was below that said to influence mesosomal vesiculation (3). Therefore, we believe that the vesicular appearance of these staphylococcal mesosomes was not caused by fixation.

Under the described conditions of protoplasting, the mesosomal vesicles of the staphylococci were extruded as described in other bacteria (11–14, 25, 27). Infrequent examples were seen of their presumably temporary adherence to the protoplast membrane as chains of vesicles (11, 12, 21, 25), as might be expected from the low concentration of Mg^{2+} used. Other studies have shown that higher Mg^{2+} concentration enhances
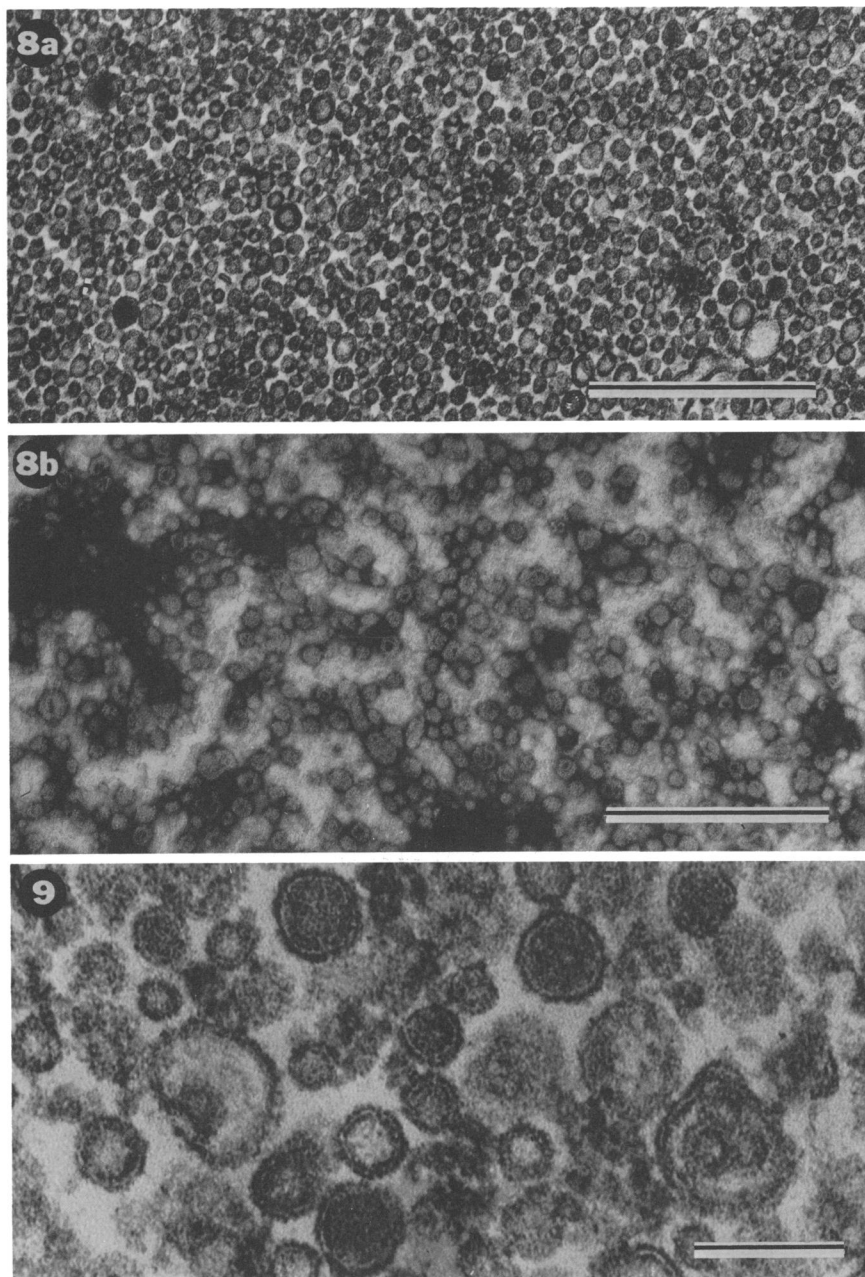
FIG. 8. *Mesosomal vesicles purified by sucrose density gradient centrifugation. These particles were entirely vesicular and uniform in size. a, Ultrathin section of mesosomal vesicles block-stained with uranyl acetate and post-stained with lead citrate; b, negatively stained with 1% aqueous uranyl acetate. Bars, 0.5 μm.*

FIG. 9. *Detail of mesosomal vesicles purified by sucrose density gradient centrifugation. The vesicles are homogeneous and membrane-bounded. Bar, 0.1 μm.*

adherence and that individual vesicles can be freed by lowering it (6, 22), as well as by applying osmotic shock (12).

An important point in arguing the reality of these vesicles is the size conformity among vesicles in situ: those in various stages of extrusion and liberation and those comprising the final gradient-purified bands. Their diameters (35 to
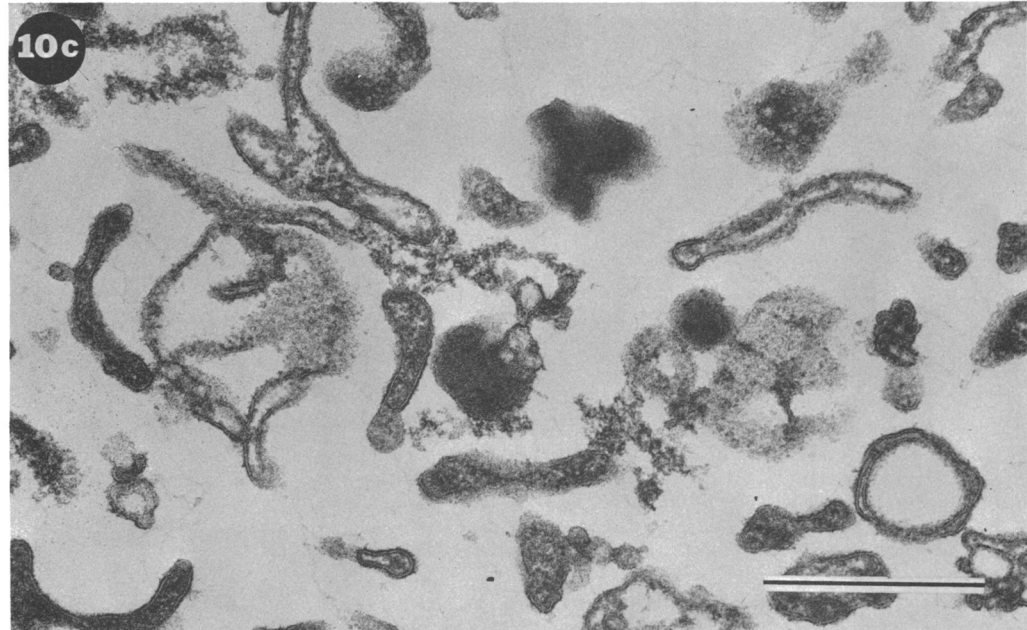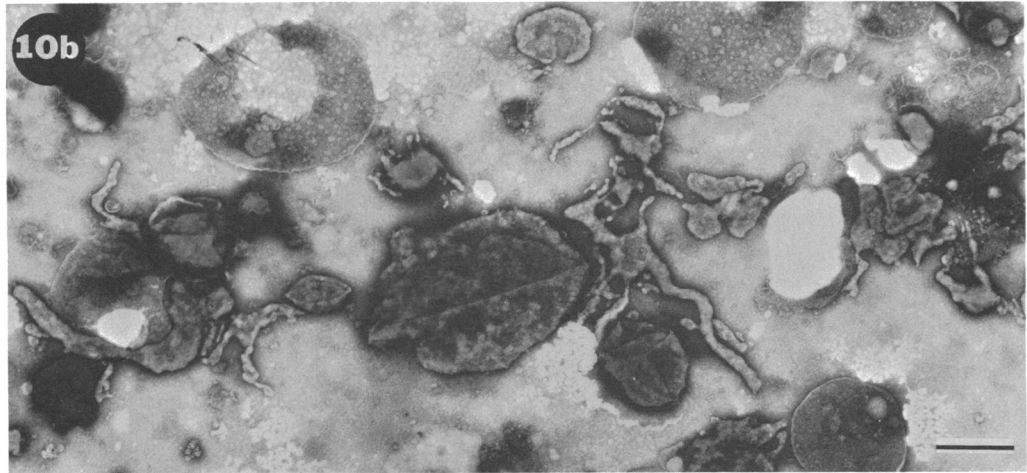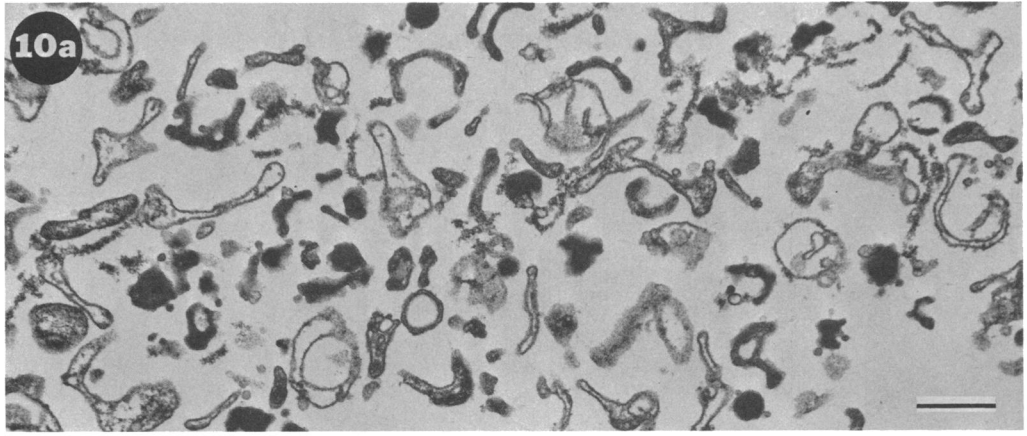
FIG. 10. *Cytoplasmic membrane fraction isolated by sucrose density gradient centrifugation. These preparations were contaminated with ribosomes, either free or associated with the membranes. Occasionally, a few mesosomal vesicles were found. a, Ultrathin section which was block-stained with uranyl acetate and poststained with lead citrate; bar, 0.5 µm. b, Negatively stained with 2% sodium phosphotungstate; bar, 0.5 µm. c, Detail of cytoplasmic membrane fraction in ultrathin section; bar, 0.5 µm.*
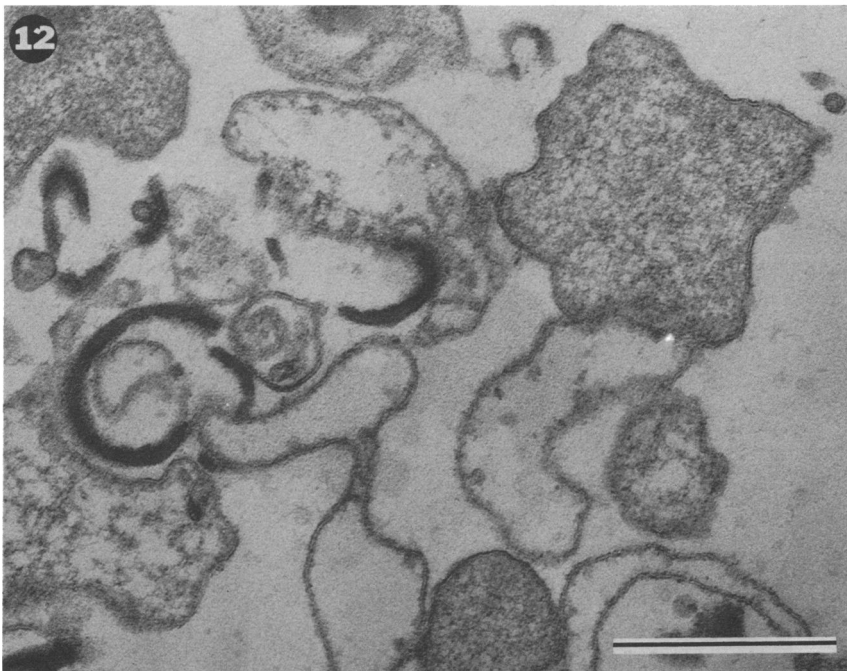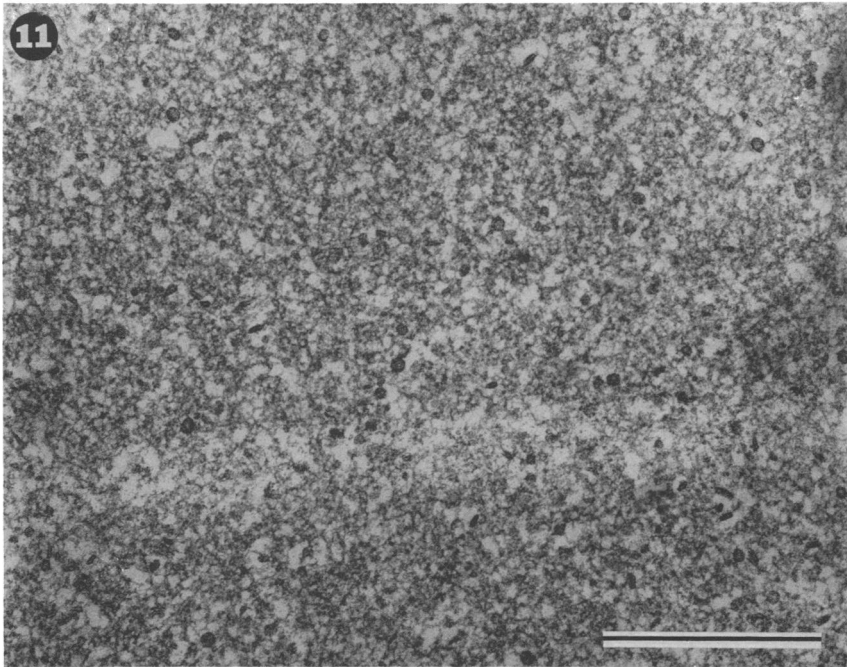
FIG. 11. *Ultrathin section of mesosomal vesicle fraction (100,000 × g) obtained in the absence of magnesium ion. Note absence of intact, small vesicles as seen in Fig. 5 and 8. Bar, 0.5 μm.*

FIG. 12. *Crude membrane fraction (10,000 × g) obtained in the absence of magnesium ion after deoxyribonuclease and ribonuclease treatment. Note intact membranes and relative absence of ribosomes. Some intact protoplasts and cell wall fragments are present. Bar, 0.5 μm.*

50 nm) are smaller and more uniform than the 30- to 300-nm spheres (22) or mixed tubules and vesicles (13, 29) isolated from *Bacillus licheniformis*, or than vesicles obtained from *B. subtilis* (8) or *Listeria monocytogenes* (14). Although membrane-bounded and with homogeneous content of unknown nature, they are clearly distinct from the fraction containing cytoplasmic membranes (Fig. 10a, 10b, 10c). The small "tubules" (Fig. 7) in cesium chloride gradient preparations are not usually seen during protoplasting nor in sucrose gradients (Fig. 8a, 8b), and we consider them to be collapsed mesosomal vesicles resulting from dehydration or other unknown effects of cesium chloride. Clearly, these vesicles lose structural integrity in the absence of magnesium ion (Fig. 11). On the other hand, under the same conditions, protoplast membranes remain intact whereas ribosomes decrease (Fig. 12). A similar effect of magnesium deficiency on the membrane-associated ribosomes of *E. coli* envelope preparations has been recently described by Haywood (15).

We wish to emphasize that in this or any similar studies it is the internal membranous bodies (be they vesicular or other in shape) of the mesosome that are extruded and purified. There is no evidence that the portion of membrane which forms the enclosing mesosomal sac is included in the concentrated preparations often referred to as purified *mesosomes*. An interesting question is whether the evaginated sac portion of the mesosome—if it could be distinguished from the rest of protoplast membrane—may have functions different from those of its contained vesicles or of cytoplasmic membrane. However, our present efforts are directed to the chemical composition and in vitro functions of mesosomal vesicles of *S. aureus* purified as described, and to comparison with protoplast membranes and with results obtained in other bacteria (6, 8, 9, 12, 14, 21, 22).

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