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Structure of Bacterial L Forms and Their Parent Bacteria

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ABSTRACT

WEIBULL, CLAES (Rocky Mountain Laboratory, Hamilton, Mont.). Structure of bacterial L forms and their parent bacteria. J. Bacteriol. **90**:1467–1480. 1965.—Light and electron microscopic studies were done on normal cells and L forms of *Proteus mirabilis*, *Staphylococcus aureus*, and *Corynebacterium* sp. grown in liquid media. Under the prevailing growth conditions, the L forms studied were morphologically indistinguishable from one another. They appeared as approximately spherical elements occurring singly or more often connected with each other by thinner portions of cell material. In sections of large L forms, the following structures were seen: a peripheral, triple-layered ("unit") membrane, a granular cytoplasm, nuclear regions, and vacuoles limited by membranes. Small bodies often were present inside the vacuoles. These bodies also contained a peripheral membrane and a granular cytoplasm but usually no nuclear regions. The normal bacteria from which the L forms were derived differed markedly in structure from one another, especially in the surface layers of the cells.

Studies on the structure of bacteria have been greatly facilitated during the past 10 years thanks to increased resolution of the available electron microscopes and to advances in electron-microscopical techniques, e.g., the fixation and embedding methods developed by Kellenberger, Ryter, and Séchaud (1958). However, relatively few detailed reports on the structure of bacterial L forms have appeared so far (Hofschneider and Lorek, 1962; Tulasne, Minck, and Kirn, 1962; Ryter and Landman, 1964; Weibull, Mohri, and Afzelius, 1965). The aim of the present investigation was to compare the structure of L forms derived from different bacterial strains and species. Comparisons were also made between the L forms studied and their parent bacteria.

MATERIALS AND METHODS

Organisms and growth conditions. The organisms used, *Proteus mirabilis* strains VI, 9, and D52, the *Proteus* L forms L VI, L 9, and L D52, *Staphylococcus aureus* ATCC 6538P, an L form derived from it, and a *Corynebacterium* sp. (strain NM1) with its L form, have been described (Dienes and Sharp, 1956; Weibull and Gyllang, 1965). Stock cultures of the *Proteus* L forms were grown in Erlenmeyer flasks containing Albimi Brucella Broth (Albimi Laboratories, Inc., Flushing, N.Y.) with 1,000 units of penicillin G per ml. The *p*H of the medium was adjusted to 7.5. The same medium, further supplemented with 1.5% NaCl and 0.2% $Na_2HPO_4 \cdot 12H_2O$, was used for growing the *Staphylococcus* and *Corynebacterium* L forms. The cultures were incubated at 37 C for 24 hr and were stored at 4 C. Subcultures were made monthly.

Cultures of L forms and the normal bacteria for experimental work were obtained by growing the organisms in 250-ml Erlenmeyer flasks, each containing 100 ml of medium. L forms and normal cells of *P. mirabilis* were grown in the Brucella Broth or in the medium described by Abrams (1955). The former medium, supplemented with salts as described above, was used for growing the remaining organisms. The cultures were incubated on a rotary shaker (100 cycles per min) at 37 C, and were harvested during the phase of active growth (4- to 8-hr cultures).

Light microscopy. A Spencer 97× phase-contrast objective and a 10× eyepiece were used. Photomicrographs of unfixed L forms mounted in the growth medium between slide and cover slip were taken with a microcamera equipped with 35-mm Kodak High Contrast Copy film, with an Ascorlight 300-w electronic flash (American Speedlight Corp., New York, N.Y.) as the light source. The magnification given by the negative was 290 times.

Electron microscopy. Preparations of unsectioned L forms were made by fixing the organisms directly in the growth medium with formaldehyde (final concentration, 4%) for 1 hr and dialyzing

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the fixed suspension against distilled water for 2 days. The dialyzed suspension was suitably diluted with distilled water, and drops of it were allowed to dry on Formvar-coated grids.

For sectioning, cultures of L forms and normal cells were prefixed with formaldehyde as described above, centrifuged, and suspended in OsO_4 fixative (Kellenberger et al., 1958). Agar-block preparations of the bacterial material were made, treated with uranyl acetate, and embedded in Vestopal W as described by Kellenberger et al. Sections of embedded material were cut on a Porter-Blum model MT-1 microtome and mounted on uncoated 300- or 400-mesh grids. As a rule, no staining was done after sectioning. In a few cases, however, lead staining according to the procedure of Reynolds (1963) was applied. Sections were examined in a Siemens Elmiskop I electron microscope at 80 kv and at an instrumental magnification of 20,000 times; 20- or 30-μ objective apertures were used.

RESULTS

Under the prevailing growth conditions, the *Proteus*, *Staphylococcus*, and *Corynebacterium* L forms could not be distinguished from one another when studied with the phase-contrast or the electron microscope. Figure 1 shows light-microscopic (phase-contrast) pictures of *Proteus* L forms grown in Brucella Broth. As can be seen, the L forms consisted of approximately spherical units connected with each other by thinner portions of cell material. Single spherical units were also observed, but not so often as in cultures of *Proteus* L forms grown in the medium described by Abrams (1955) and in cultures of *Staphylococcus* and *Corynebacterium* L forms grown in broth prepared from fresh meat (Weibull, 1963; Weibull and Lundin, 1963).

The appearance of electron micrographs of unsectioned L forms was in agreement with the light-microscopic observations (Fig. 2). This indicates that the L bodies essentially retained their shape when fixed and dried.

From the shape of unsectioned L forms, it could be expected that sections of these forms would have the appearance of approximately circular or ellipsoidal figures often merging into one another. This proved to be the case (Fig. 5, 6, and 8). Hourglass-shaped sections were rare (Fig. 3).

As reported earlier (Weibull and Mohri, 1965), the nature of the growth medium may be important for obtaining good fixation of L forms. This was borne out by the present investigation. Only L forms grown in Brucella Broth could be fixed with formaldehyde or OsO_4 without undergoing lysis. Figure 11 shows a sectioned *Proteus* L form grown in Abrams' medium and fixed as described in Materials and Methods. In comparison with pictures shown in most electron micrographs of L forms grown in the Brucella Broth (Fig. 4 to 8), this L form contains much less intracellular material, indicating extensive lysis. Indications of disruption of the membrane were also seen (Fig. 11).

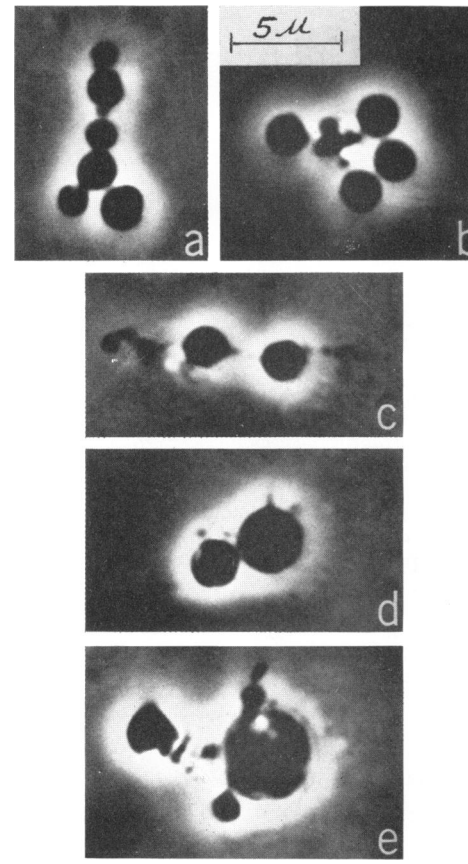

FIG. 1. *Proteus L forms grown in Albimi Brucella Broth. A drop of the culture was placed between slide and cover slip, and the organisms were photographed under a phase-contrast microscope with an electric flash as the light source.* × *2,900. (a, b, and c) Proteus mirabilis L VI. (d and e) P. mirabilis L 9.*

The periphery of many L forms seemed to be covered with some electron-dense material, probably precipitated during the fixation or embedding processes (Fig. 3, 5, 6, and 7). This material to some extent obscured the surface structure of the L forms. Similar precipitates also occurred around many normal *Proteus* cells (Fig. 13). However, L forms and normal cells without this material were also seen (Fig. 8, 9, and 12). Organisms with and without dense material at the periphery often were located close to each other in the Vestopal sections. In the absence of the peripheral electron-dense material, the surface

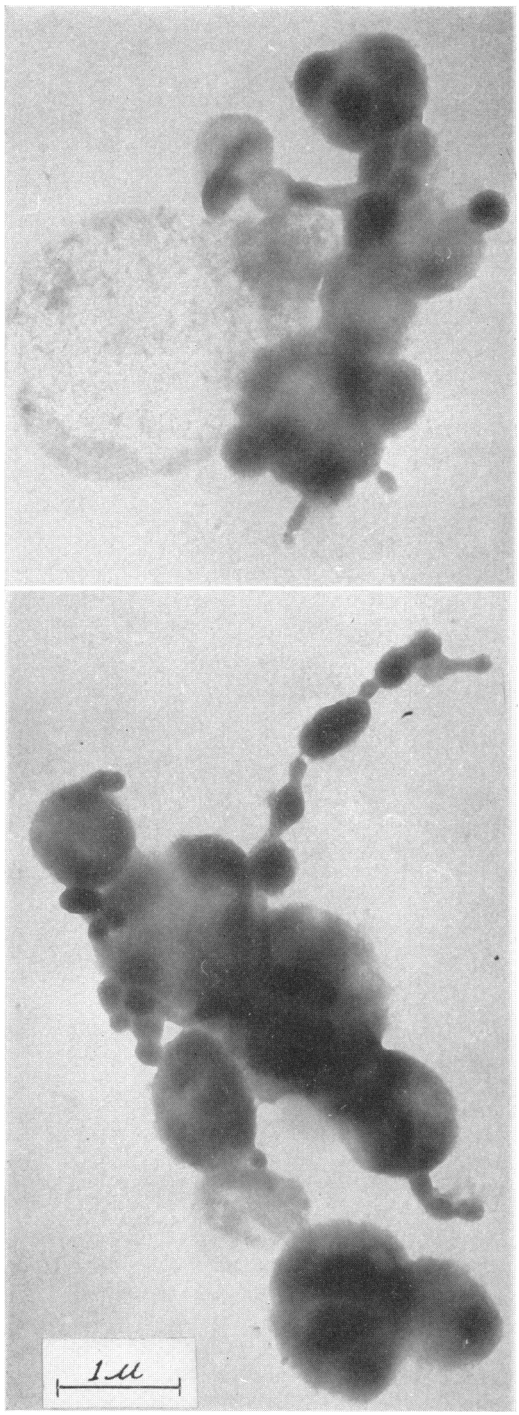

FIG. 2. *Electron micrographs of drop preparations of unsectioned Corynebacterium L forms. These L forms and those shown in all of the following figures were grown in Albimi Brucella Broth except that shown in Fig. 12, which was grown in Abrams' semisynthetic medium.* × *16,000.*

layer of the L forms appeared as a triple-layered structure (Fig. 9). This structure, a "unit" membrane according to Robertson (1959), had a thickness of about 75 A, and was indistinguishable from the membranes bounding the internal vacuoles of the L forms and the small bodies inside these vacuoles (see below). The two innermost layers of the unit membrane could be discerned in L forms, the surface of which was covered with dense material (Fig. 7).

Typical mesosomes were not seen in any of the L forms studied.

A granular cytoplasm, nuclear regions containing filamentous material, and vacuoles were the main intracellular structures seen in medium-sized and large L forms (Fig. 3 to 10). The cytoplasmic granules measured 100 to 200 A (Fig. 9), suggesting that they represented ribosomes. Multiple membranes, which consisted of dense and light strata exhibiting a periodicity of 40 to 50 A, were seen in some cytoplasmic regions (Fig. 4 and 5). The vacuoles were bounded by unit membranes (Fig. 9). Inside the vacuoles, small bodies were often present (Fig. 4 and 8). These bodies were bounded by unit membranes (Fig. 9), and contained a granular cytoplasm but usually no nuclear regions. Connections between these small bodies and the main cytoplasm could be seen in some sections (Fig. 10). Some bodies had a shape suggesting an incipient division process (Fig. 4, 8, and 9).

The normal *Proteus* cells from which the L forms were derived also contained a peripheral unit membrane, but this membrane was surrounded by a thicker structure in part of which three dark layers could be discerned (Fig. 12 and 13). The appearance of lysed or plasmolyzed cells (Kellenberger and Ryter, 1958; Cota-Robles, 1963) indicated that the latter structure represented the bacterial cell wall, and that the unit membrane was the cytoplasmic membrane. Actually, the cell shown in Fig. 12a may have undergone a slight plasmolysis, since indications of a connection between cell wall and cytoplasmic membrane can be seen at some points of the cell periphery. However, plasmolysis does not necessarily represent an artifact, since the viability of plasmolyzed bacteria has been demonstrated (Taubeneck, 1955; Weibull, 1965).

As expected, the normal *Staphylococcus* and *Corynebacterium* cells had a structure quite different from normal *Proteus* cells (Fig. 14 and 15). Well-developed mesosomes were seen in most of the staphylococci and the corynebacteria, sometimes connected with the cell periphery (Fig. 14). A very thick but more homogeneous surface structure was seen in the staphylococci (Fig. 14). The cell walls and cytoplasmic membranes of the

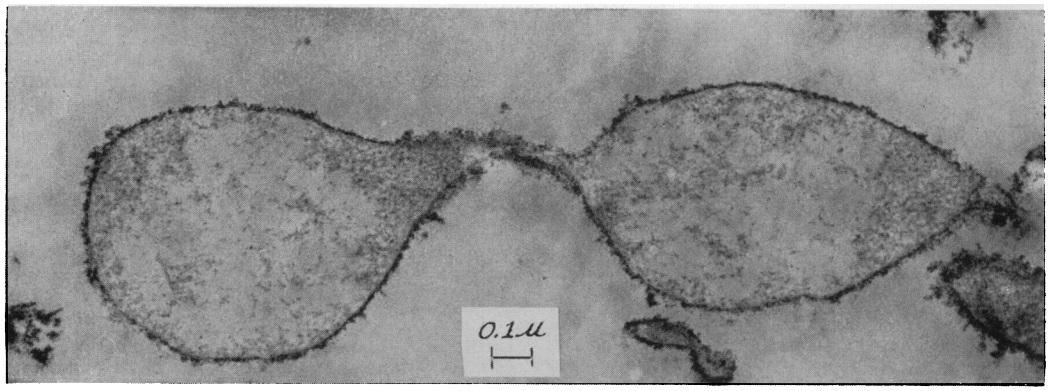

FIG. 3. *Section of a Corynebacterium L form.* × *51,000.*

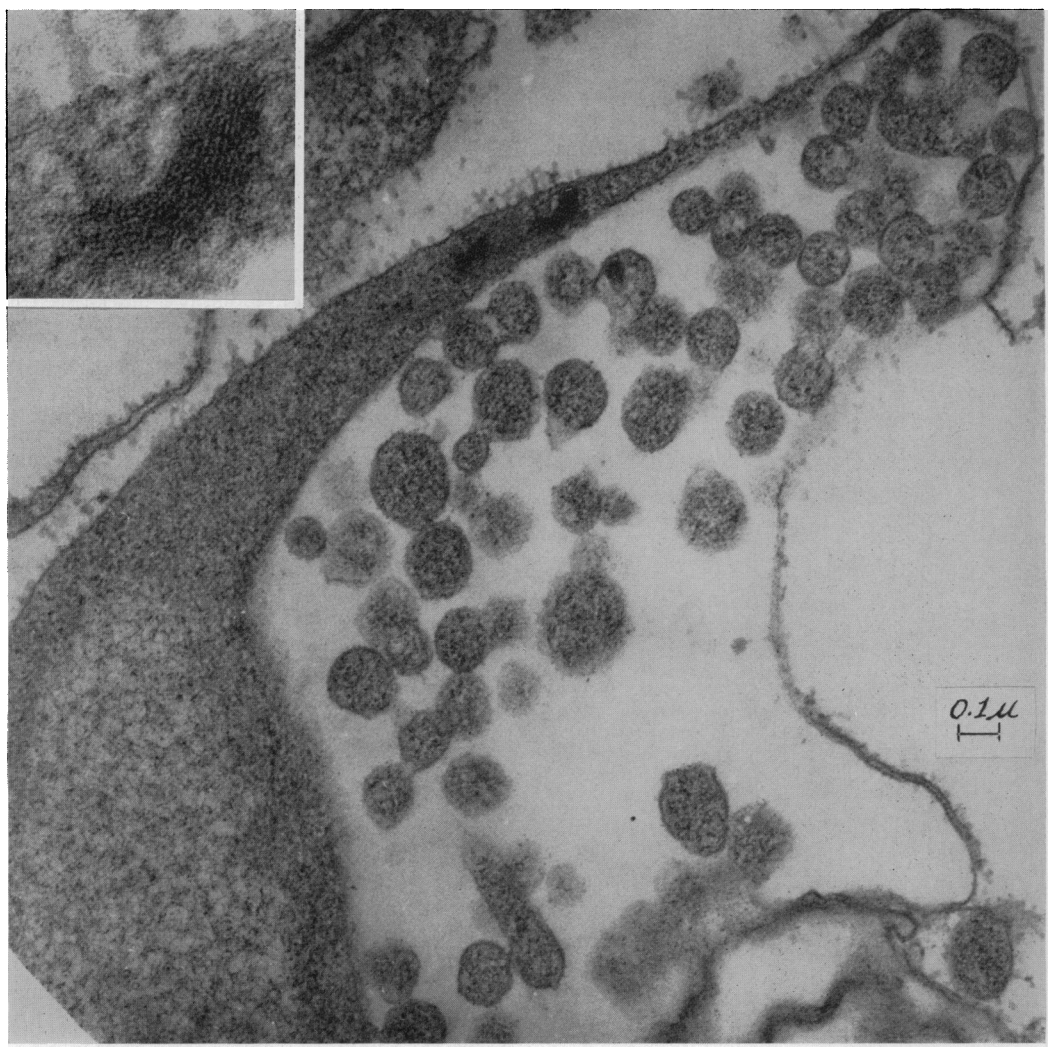

FIG. 4. *Section of a Staphylococcus L form. Inset shows cytoplasmic region consisting of alternating dense and light layers. Section stained with lead citrate.* × *50,000; inset,* × *220,000.*

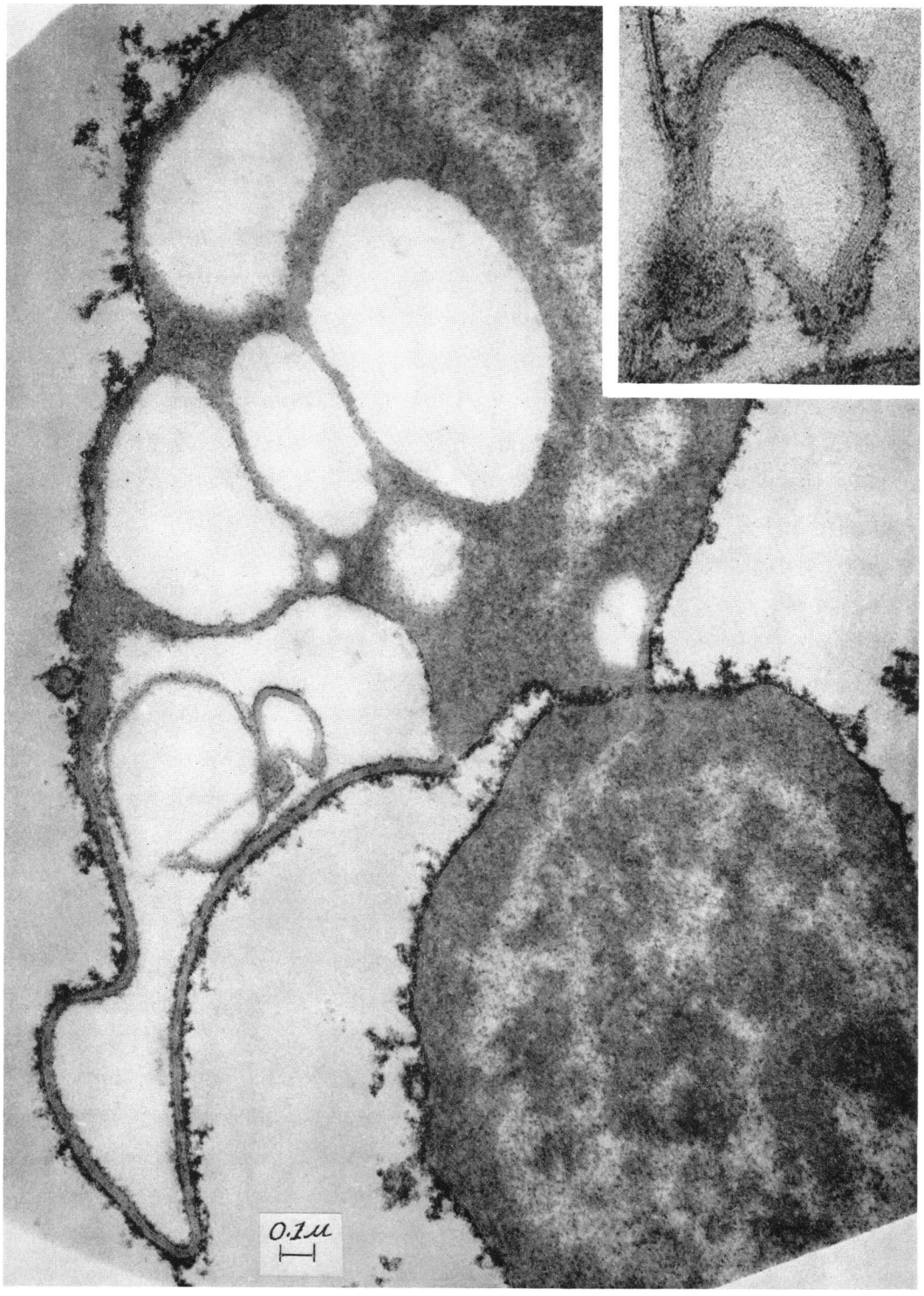

FIG. 5. *Section of Proteus L form, strain L 9. Inset shows cytoplasmic region consisting of alternating dense and light layers.* × *54,000; inset,* × *170,000.*

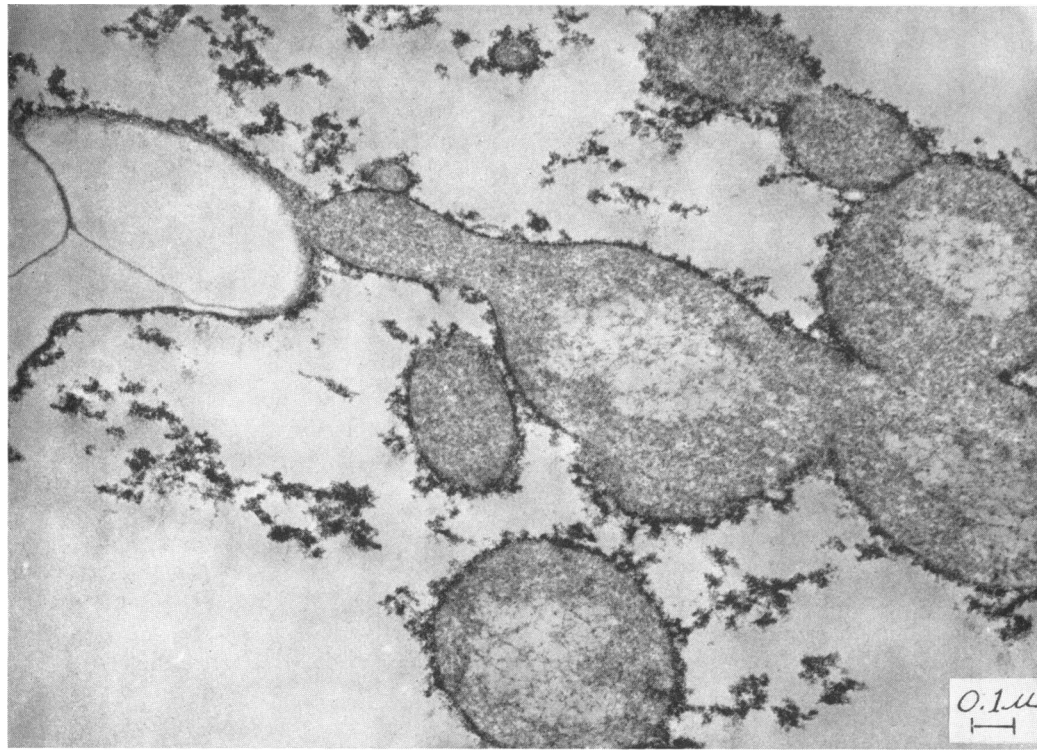

FIG. 6. *Section of Proteus L form, strain L VI.* × *51,000.*

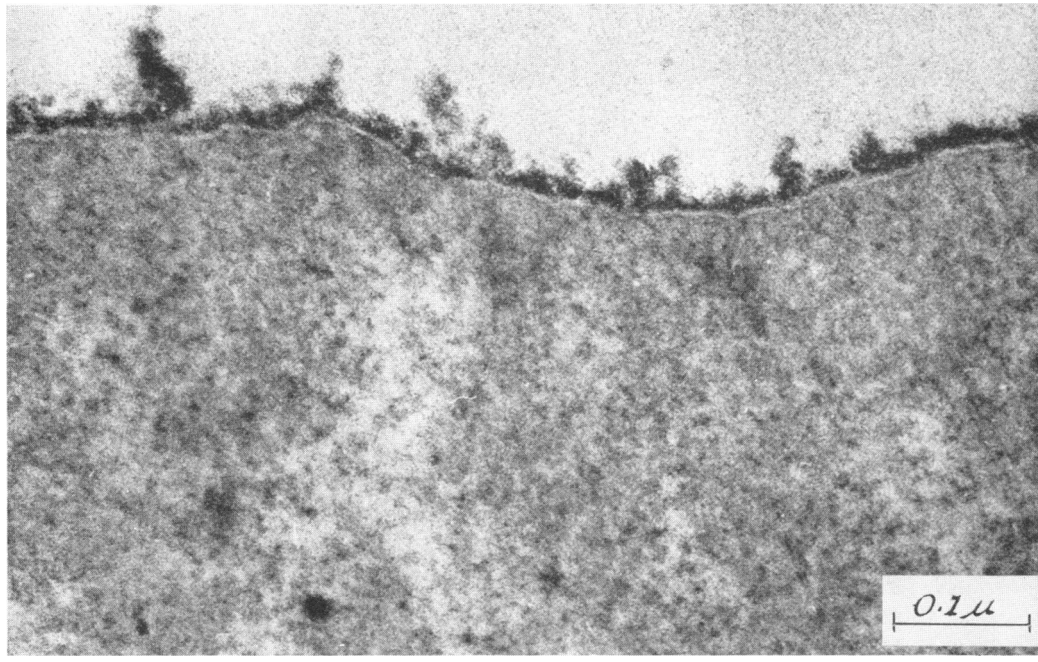

FIG. 7. *Section of a Corynebacterium L form showing surface structure consisting of outer dark material, probably precipitated during fixation, light intermediate layer, and inner dense layer.* × *180,000.*

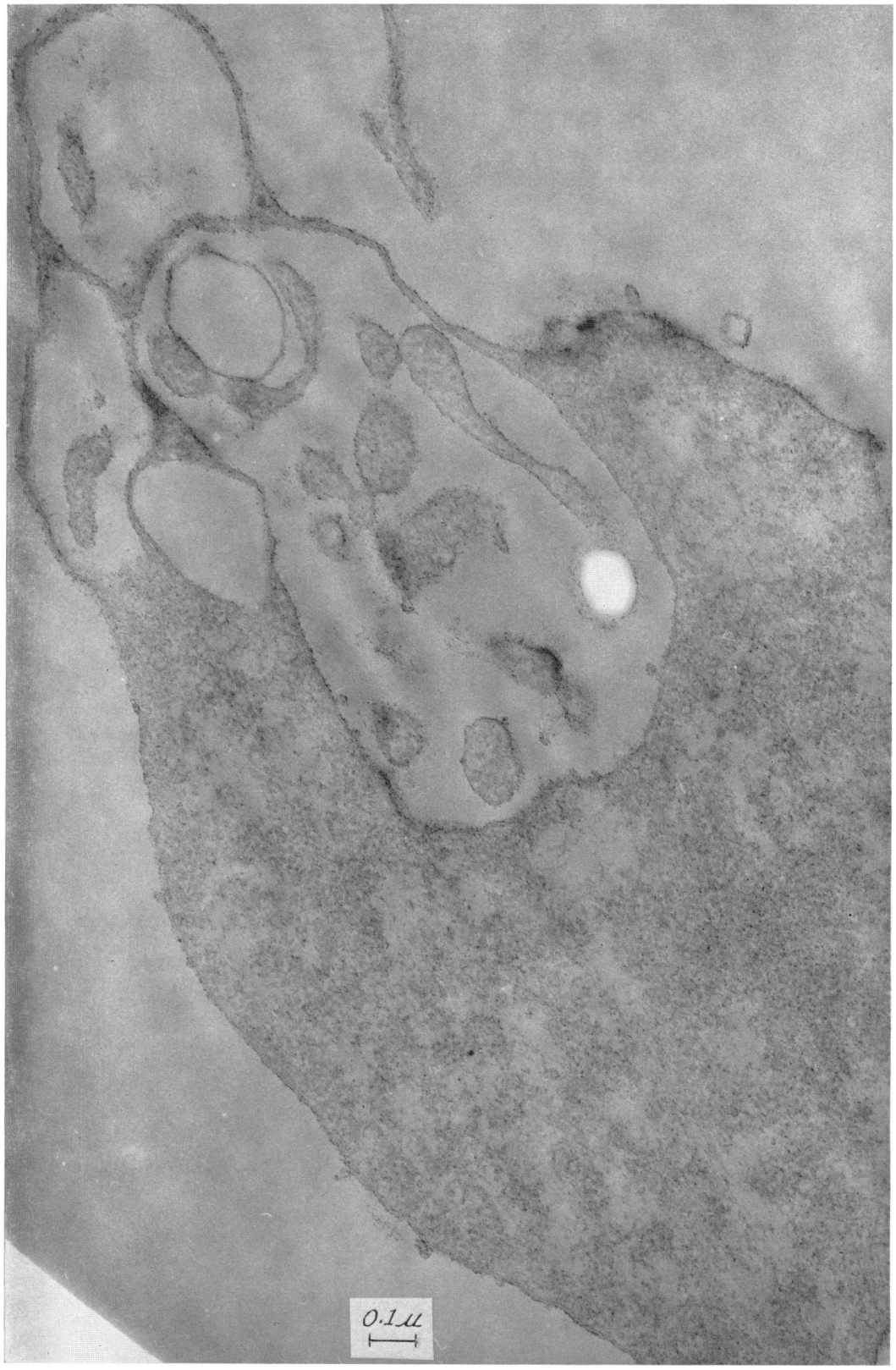

FIG. 8. *Section of Proteus L form, strain D52.* × *68,000.*

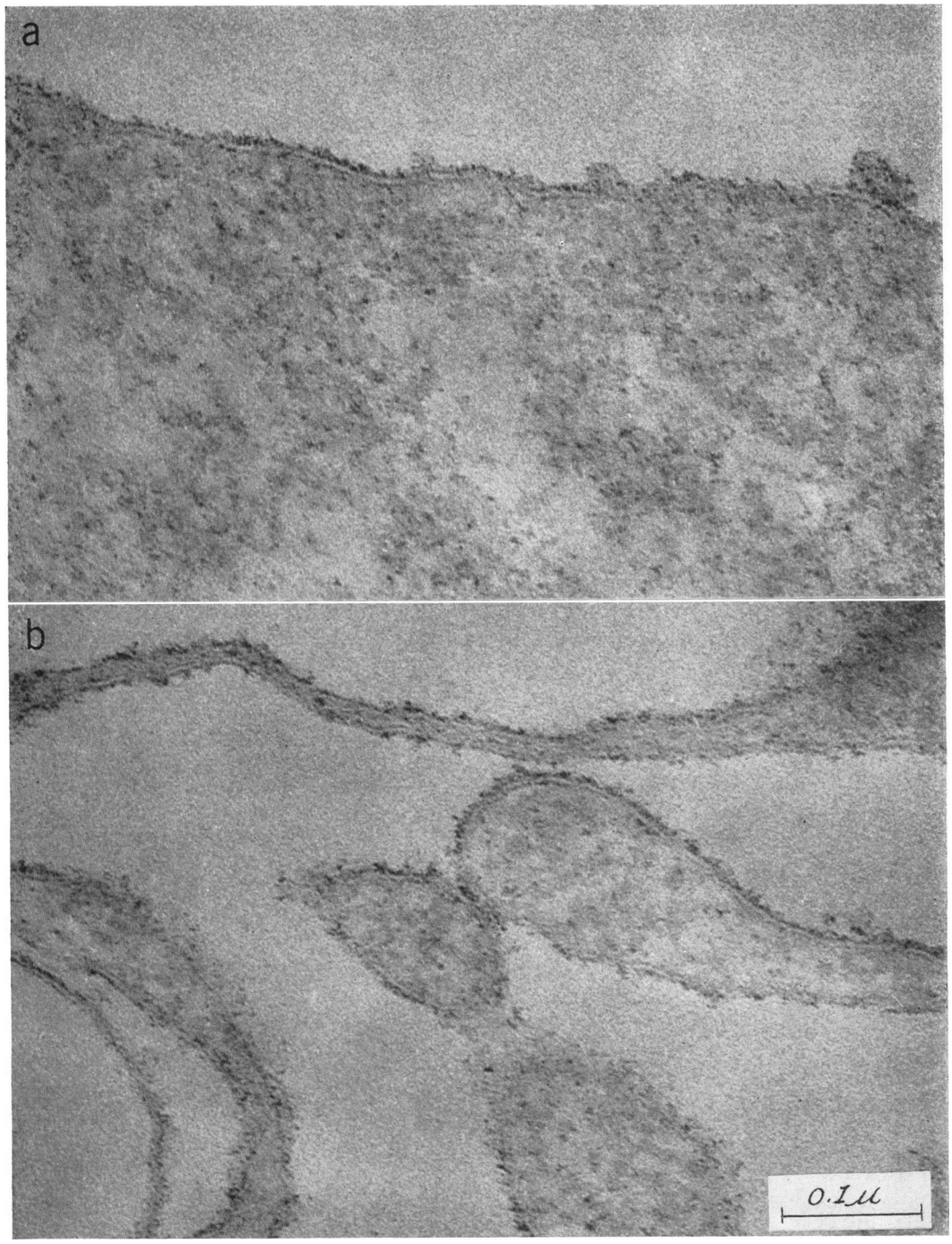

FIG. 9. *Details of Fig. 8 at higher magnification,* × *210,000. (a) Peripheral unit membrane consisting of two dense layers and an intermediate light one. (b) Part of vacuole containing small bodies. Bodies and vacuole are bounded by unit membranes.*

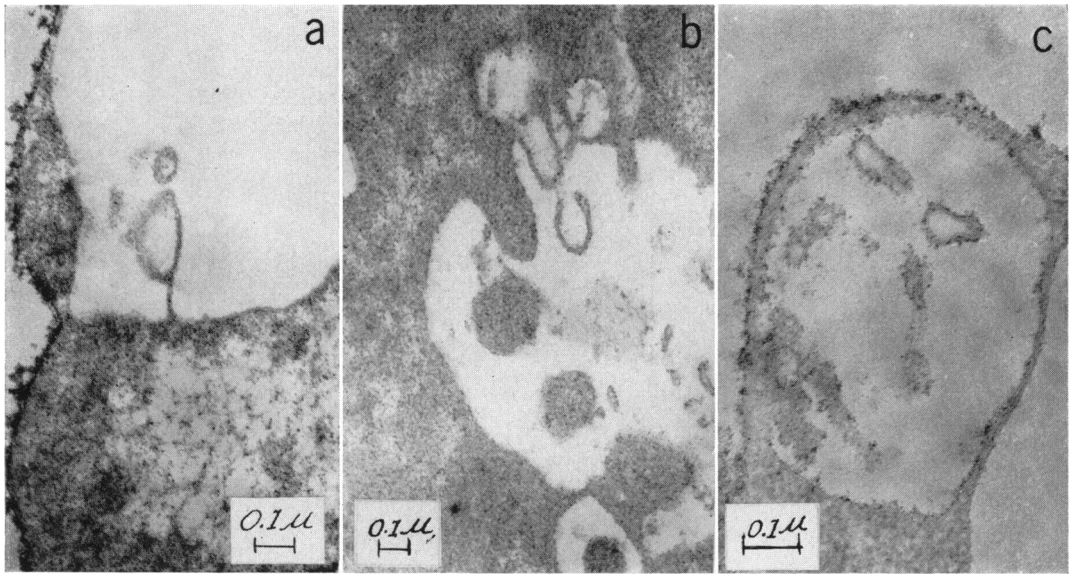

FIG. 10. *Sections of L forms showing protrusions of cytoplasm into vacuoles. (a) Proteus L VI, × 53,000, (b) Corynebacterium L form, × 34,000, (c) Corynebacterium L form, × 71,000.*

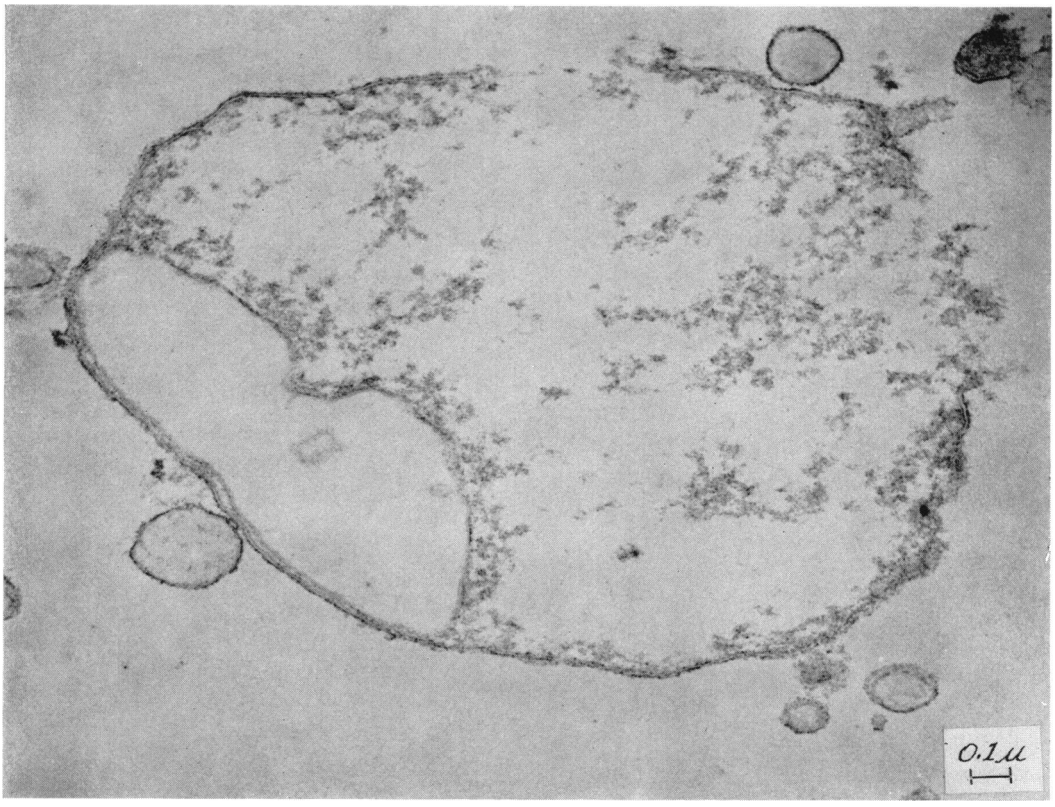

FIG. 11. *Section of Proteus L form, strain L D52, grown in Abrams' semisynthetic medium. The organism has undergone extensive lysis. Note indications of rupture of peripheral membrane. × 48,000.*

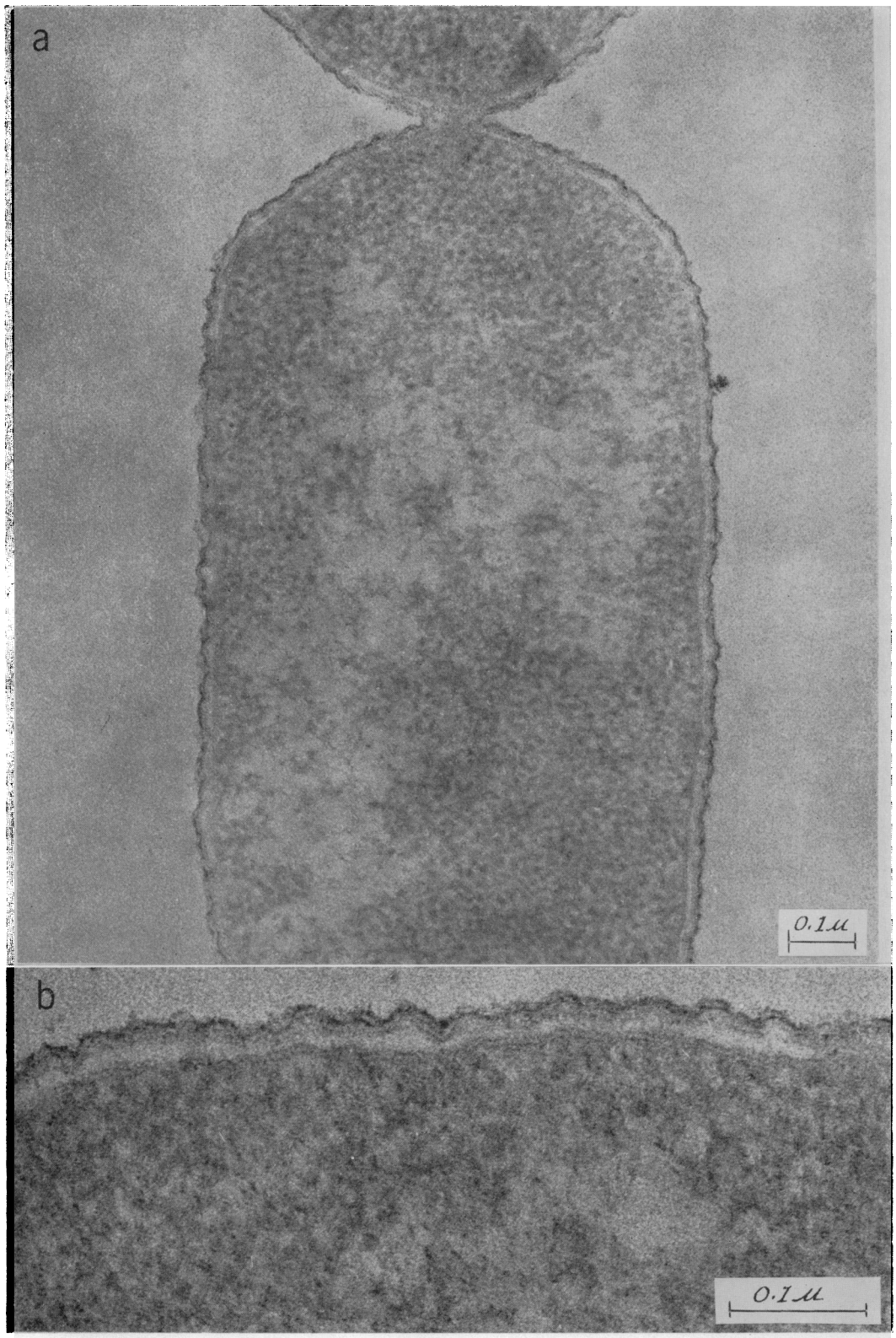

FIG. 12. *Sections of normal cells of Proteus mirabilis, D52. (a) Part of cell chain. × 100,000. (b) Part of surface region showing cell wall and cytoplasmic membrane. The former is multilayered and the latter has the appearance of a unit membrane. × 210,000.*

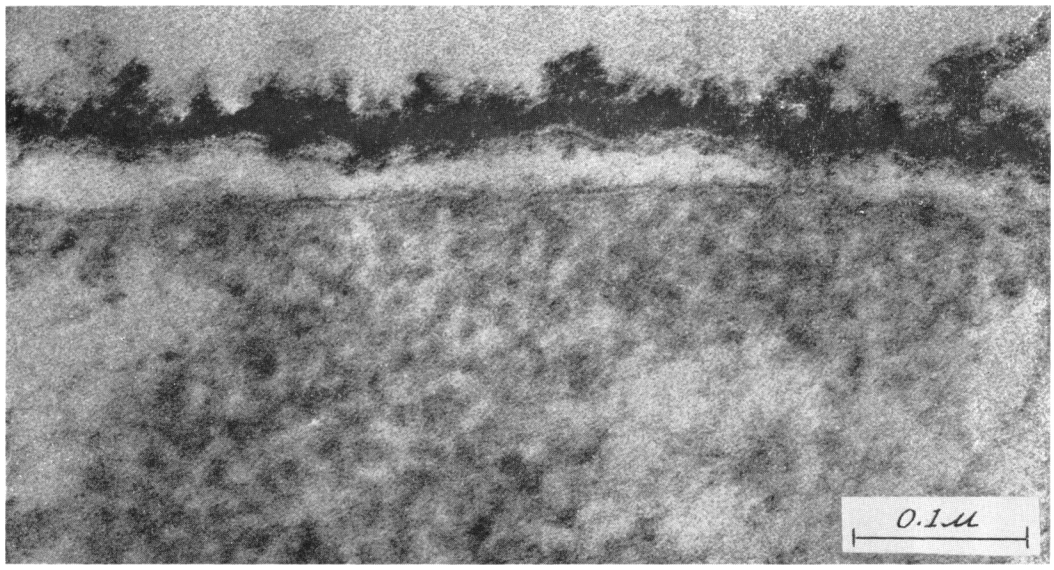

FIG. 13. *Section of surface layer of normal cells of Proteus mirabilis 9. Note outer dark material probably precipitated during fixation.* × *230,000.*

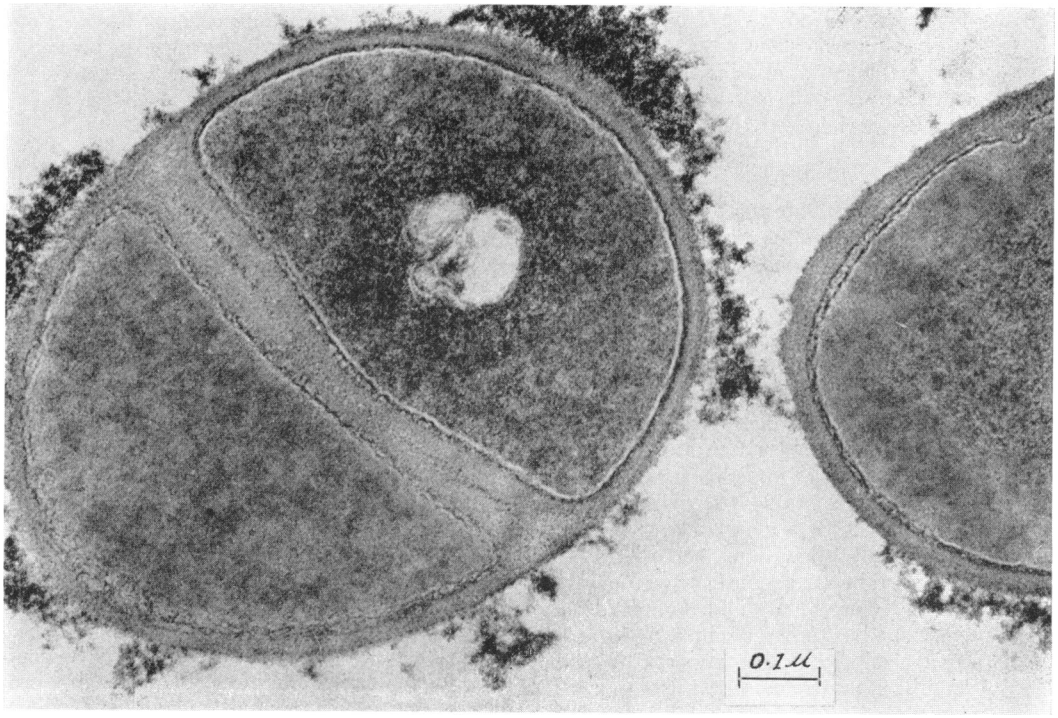

FIG. 14. *Section of normal Staphylococcus cell. Note mesosome and thick surface layer.* × *100,000.*

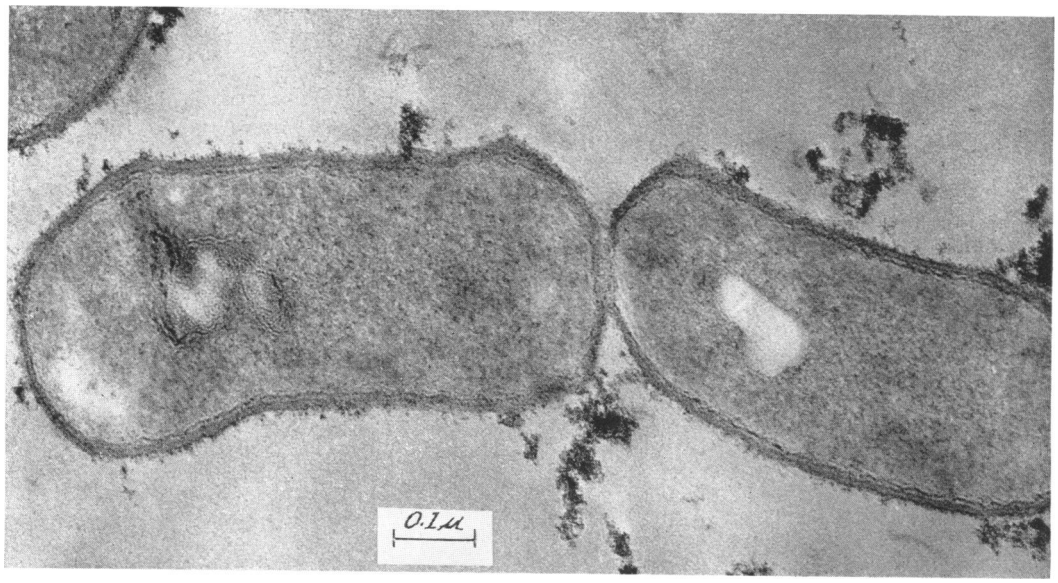

FIG. 15. *Section of normal Corynebacterium cell. Note multilayered surface region and mesosome connected with cell surface.* × *100,000.*

Staphylococcus and *Corynebacterium* cells could not be clearly differentiated. In these organisms, the cytoplasm and the nuclear regions appeared more compact than in *Proteus* (Fig. 14 and 15). The granular appearance of the staphylococcal cytoplasm was especially striking (Fig. 14).

DISCUSSION

Perhaps the most noteworthy result of the present investigation is the demonstration of a morphological similarity among the L forms studied. This is in contrast to the obvious structural differences existing among their parent bacteria. Moreover, striking physiological and chemical differences exist among these L forms. Thus, the *Staphylococcus* and *Corynebacterium* L forms require a medium of rather high osmotic pressure for growth, a requirement not exhibited by the *Proteus* L forms (Dienes and Sharp, 1956). The first two organisms contain little or no catalase and cytochromes, enzymes found in large amounts in the last (Weibull and Gyllang, 1965). One *Proteus* L form, strain L 9, contains considerable amounts of a typical cell-wall component, diaminopimelic acid (DAP) (Weibull, 1958); the others, only trace amounts (Morrison and Weibull, 1962). Strain L 9, moreover, is of the so-called C type (Kandler and Zehender, 1957); strains L VI and L D52 are of the 3 A type (Dienes and Weinberger, 1951). (The 3 A strains initially require serum for growth and initially form small colonies on agar. They seldom revert to the bacillary form. The 3 B strains form large colonies on agar, do not require serum for growth, and revert to the bacillary form when grown on penicillin-free media. The C type is similar to the 3 B type except that strains of this type do not revert to the bacillary form.)

The surface layer of the L forms studied here consisted of one triple-layered structure, a unit membrane. Also, Tulasne et al. (1962) found that stable L forms possess one peripheral unit membrane. Hofschneider and Lorek (1962), however, reported the presence of two unit membranes at the periphery of stable L forms, in their study of *P. mirabilis* Kandler L. Perhaps this discrepancy could be explained in terms of strain differences. There is also the possibility that the inner integument described by Hofschneider and Lorek represents the peripheral membrane of an L body developing inside a larger L form and finally filling it up more or less completely. In this way, one could expect the presence of two unit membranes close to each other.

When comparing the surface structure of normal *Proteus* cells (Fig. 12 and 13) with that of *Proteus* L forms (Fig. 7 and 9), one would perhaps conclude that the normal cell wall is completely eliminated when the L forms are produced. The peripheral unit membrane of the L forms would thus correspond to the cytoplasmic membrane of the normal bacteria. However, this cannot be considered fully established. One of the L forms studied, strain L 9,

was found to contain considerable amounts of DAP, which was present in the trichloroacetic acid-soluble fraction of the cells (Weibull, 1958). The topical distribution of the DAP was not studied. Recently Fleck (1965) reported the presence of DAP in the membrane fraction of an L form derived from *P. mirabilis* P 18.

The multilayered structure found within the cytoplasm of some L forms (Fig. 4 and 5) appears similar to that found in myelin fixed for several hours with potassium permanganate (Robertson, 1959). In both cases, dense lines, spaced at a periodicity of about 50 A, are evident.

Ryter and Landman (1964) found that normal cells of *Bacillus subtilis* lose their mesosomes when the bacilli are converted into L forms. Our findings show that this phenomenon is not restricted to *B. subtilis*.

Hardly any vacuoles are seen in the electron micrographs of L forms published by Tulasne et al. (1962) and Ryter and Landman (1964), whereas we found vacuoles frequently in sectioned L forms. The presence of vacuoles in L forms is also mentioned in the brief reports of Marston (1964) and van Iterson, Ruys, and Botman (1964). Vacuolated L forms are also easily detected by light microscopy (Weibull, 1963). Perhaps the somewhat diverging results could be attributed to different growth conditions for the organisms studied.

The appearance of some small bodies (diameter, 0.1 to 0.2 μ) inside larger L forms (Fig. 4 and 9) is interpretable as a division process. Often the appearance of unsectioned L forms (Fig. 2) suggests that small L bodies peripherally connected with larger L forms may be capable of continued growth in size. Formerly it was shown (Weibull, 1963) that isolated L forms of a diameter less than 0.6 μ did not grow. However, these findings do not exclude the possibility that smaller L bodies inside or connected with larger L forms may grow and divide.

Extensive morphological investigations on sectioned pleuropneumonia-like organisms (PPLO) were carried out by Domermuth et al. (1964). No definite structural differences between these PPLO and the L forms we investigated can be ascertained. Thus, it seems doubtful whether structural details could be used for a general differentiation between PPLO and L forms.

The electron micrographs of normal *Proteus* cells (Fig. 12 and 13) presented in this paper indicate that the cell wall of this bacterium cannot be described as simply a unit membrane (*see* Clarke and Lilly, 1962; Weibull et al., 1965). This is in accordance with the recent studies of Reyn and Birch-Andersen (1964), de Petris (1965), and Murray, Steed, and Elson (1965) on various gram-negative eubacteria.

The electron micrographs of normal *Staphylococcus* and *Corynebacterium* cells (Fig. 14 and 15) illustrate that a clear distinction between cell wall and cytoplasmic membrane cannot be made solely on the basis of morphological studies on intact cells. Evidently, however, the bulk of the peripheral structure and the transverse septum of the *Staphylococcus* cells (Fig. 14) represents cell-wall material. Suganuma (1964) could demonstrate a triple-layered cytoplasmic membrane in cells of *S. aureus* which had been treated with warm hydrochloric acid.

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