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Morphology and Ultrastructure of Human T-Mycoplasmas

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Four serologically distinct human T-mycoplasmas grown in liquid medium were studied in the electron microscope after ultrathin sectioning and negative staining. The morphology and ultrastructure of these strains was found to be essentially identical to that of other mycoplasmas; i.e., mainly spherical or ovoid cells were observed, but also short rod-shaped cells and filamentous, partly branched forms were noted. The cells were found to be enveloped by a triple-layered membrane, on the outer surface of which an electron-dense layer consisting of radiating hairlike structures was consistently present. In addition to ribosomes, now and then arranged in a regular geometric pattern, the ultrathin sections reveal vacuole-like structures in the interior of the cells.

Although considerable effort has been devoted to clarifying the morphology and ultrastructure of the classical large-colony-forming mycoplasmas (2, 9, 10) little information is available on the ultrastructure of the T-mycoplasmas (16, 20, 21).

This paper presents the results of electron microscopy of thin-sectioned and negatively stained material of four serotypes of human T-mycoplasmas.

MATERIALS AND METHODS

Mycoplasma strains. The organisms used were T-mycoplasma strains no. 23, no. 58, Pirillo, and Cook, all of them obtained from D. K. Ford (Univ. of British Columbia, Vancouver, B.C., Canada).

Growth medium. This was a modified Shepard medium (19) consisting of Trypticase soy broth (TSB; Baltimore Biological Laboratory) supplemented with 2.5% PPLO serum fraction (Difco), 0.04% urea, 0.002% phenol red, and 2,000 units of sodium penicillin per ml (SPU medium). The replacement of whole horse serum with the PPLO serum fraction proved essential for avoiding the accumulation of extracellular material which otherwise tended to obscure the electron micrographs, as seen in Fig. 2. A further substantial clarification of the medium was accomplished by sedimentation, prior to inoculation, in a Sorvall high-speed (RC2b) centrifuge at 20,000 rev/min for 1 hr, using a continuous-flow system followed by filtration through a Zeiss filter (EK 5).

Growth and harvest of cells. For production of material for ultrathin sectioning, SPU medium in volumes of 5 liters and contained in 6-liter Erlenmeyer flasks was inoculated with 300 ml of a fresh log-phase culture. For negative staining, 300 ml of the SPU medium contained in 500-ml flasks was inoculated with 15 ml of a log-phase culture. After 10 to 14 hr of incubation at 37 C, i.e., in the late log phase, growth was stopped by cooling to 5 to 10 C, and harvest was made in a Sorvall RC2b centrifuge at 16,000 rev/min using a continuous-flow system with a flow rate of 40 ml/min.

Preparation of specimens: (i) sectioned material. The pellet resulting from centrifugation was suspended in 40 ml of precentrifuged and filtered TSB. Dropwise and with vigorous stirring, 1 ml of 12.5% glutaraldehyde (GA) in Veronal acetate (VA) buffer, *p*H 6.1, was added to yield a 0.3% concentration of GA in the suspension which was immediately centrifuged again at 16,000 rev/min for 1 hr; the medium was then discarded, and the pellet was warmed to 45 C prior to addition of and gentle mixing with three to five drops of warm (45 C) melted agar (1.5% Noble agar [Difco] in VA buffer, *p*H 6.1). After solidification, small agar blocks (about 1 mm^3) with cells embedded were cut and fixed for 1 hr at room temperature (20-22 C) in 3% GA in VA buffer to which was added one part of YAP medium (yeast extract-sodium acetate-peptone medium [0.3%, 0.05%, and 0.3%, respectively; Difco products]) to ten parts of fixative (v/v). After this fixation, the agar blocks were rinsed twice with 0.2 M sucrose in VA buffer, *p*H 6.1, and stored overnight at 4 C. The blocks were then rinsed twice in pure VA buffer and then fixed overnight (17) at room temperature (20-22 C) in 1% VA-buffered OsO_4 to which was added one part of YAP medium to ten parts of fixative. After a brief wash in VA buffer and a 1-hr treatment at 20 to 22 C with 2% uranylacetate in the same buffer, the agar blocks were dehydrated in 70%, 96%, and 100% alcohol, briefly treated with propylene

oxide, and finally embedded in Vestopal-W (18). Sections of the hardened blocks were obtained with an LKB Ultrome-III microtome and post-stained with magnesium uranyl acetate (7) and lead citrate (15).

(ii) Negatively stained material. Pellets of cells grown and centrifuged as described above were suspended either in pure, precentrifuged and filtered TSB medium or in the same medium to which was added glutaraldehyde to a concentration of 0.3%. In a few experiments some pellets were suspended directly without fixation in a volatile buffer (1% ammonium acetate adjusted to *p*H 7-7.2 with ammonium hydroxide).

Negative staining was performed with 1% ammonium molybdate (adjusted to *p*H 7-7.2 with ammonium hydroxide) by first floating carbon-reinforced Formvar-coated grids on drops of suspended cells on dental wax and then removing most of the liquid on the grid surface by touching the edge of the grid with a piece of filter paper. The actual staining was now done by repeating this procedure after floating the same grids on drops of the staining solution.

Electron microscopy. Sectioned as well as negatively stained material was examined with a Philips EM200 or EM300 electron microscope. Exposures were made on Eastman Kodak fine-grain release positive film, type 5302.

RESULTS

Sectioned specimens. In thin sections (Fig. 1-4), most of the cells are sperhical or ovoid, although short rod-shaped or filamentous structures are also seen.

The size of the round cells ranges from 140 nm to about 850 nm. The filamentous cells can reach a length of approximately 2 μm, and their width varies from 50 to 300 nm. All cells are surrounded by a triple-layered membrane (Fig. 1 and 3), the minimal thickness of which is 8 to 9 nm for all strains examined. The outer surface of the limiting membrane is consistently found to possess an electron-dense layer of 9 to 11 nm thickness, sometimes presenting a well defined pattern of dense hairlike structures radiating from the cell surface (Fig. 1 and 3).

The appearance of the interior of the cells varies with their size, the smaller cells being more electron-dense and homogeneously filled with cytoplasmic material than the bigger ones (Fig. 1 and 2). The cytoplasm of the smaller cells is crowded with ribosomes that are either randomly distributed or partly arranged in closely square-packed groups of four (Fig. 4) which are themselves sometimes arranged to form corn-cob-like patterns (Fig. 3). This phenomenon was found in all four strains (serotypes) examined. In larger cells containing only sparse amounts of cytoplasm the ribosomes are largely located in the peripheral part. Membrane-bounded vacuole-like structures are sometimes seen in the interior of large swollen cells (Fig. 2). The triple-layered limiting membrane of these vacuoles is always of the same thickness as the cell membrane and is furthermore lined, at the interior surface, with an extra layer of exactly the same thickness and structure as the surface layer of this membrane (see Discussion). The central area of medium-sized cells is often partly occupied by delicate filaments (Fig. 1) that in other mycoplasmas have been identified, by cytochemical methods, as deoxyribonucleic acid strands (12).

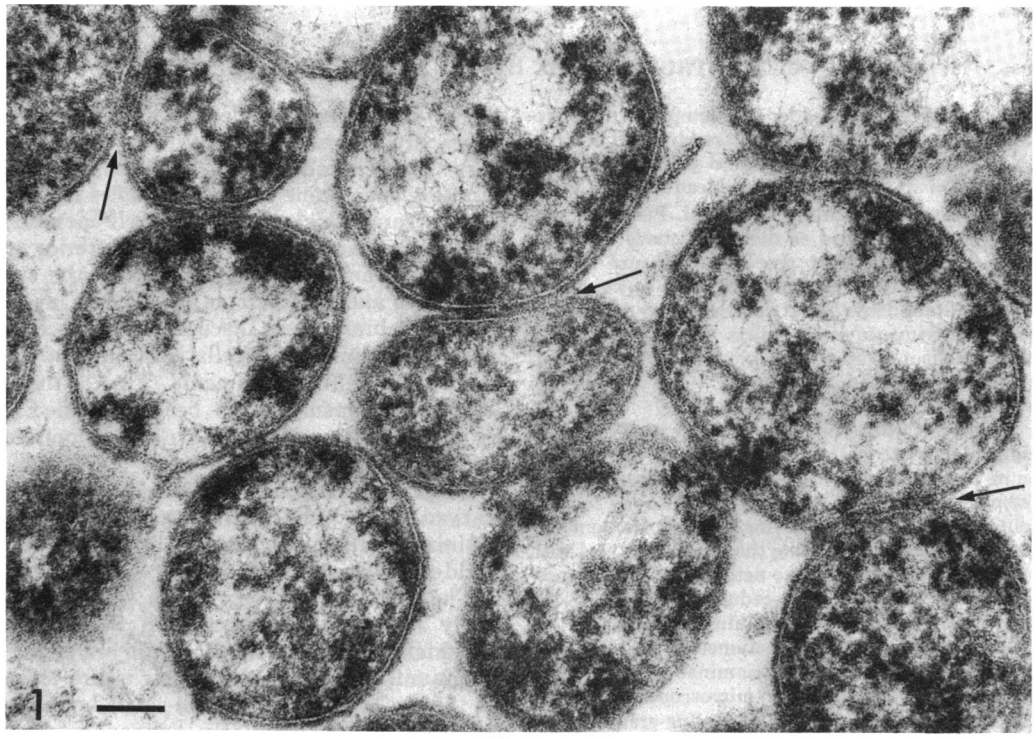
Negatively stained specimens. Among the various stains used in this study for negative staining, ammonium molybdate turned out to yield the best results.

The gross morphology of the cells is essentially identical with that seen in sectioned material (Fig. 5-7). The filaments are either of fairly uniform width along their entire length, or they possess terminal club-shaped swellings together with irregularly spaced swellings along their length (Fig. 5 and 6). Some filaments present branching (Fig. 6).

The existence of an additional outer layer showing a radiating hairlike structure on the surface of the limiting membrane is confirmed by negative staining (Fig. 5-7). Centrally located in the terminal swelling of one of the filaments (Fig. 7) is seen a well defined circular structure, about 200 nm in diameter, the possible interpretation of which will be discussed below.

DISCUSSION

The general morphology and dimensions as well as the ultrastructural details of the T-mycoplasmas examined in this study are in overall agreement with the basic structural characteristics of the large-colony-forming mycoplasmas. Suggestive evidence for the existence of a superficial extra-membraneous layer, tentatively interpreted as a capsular substance, has been reported for a great variety of *Mycoplasma* species (6). More or less distinct surface projections resembling the hairlike radiating structures seen in this study have been observed in only a few *Mycoplasma* species, viz., *M. gallisepticum* (5) and *M. pulmonis* (10). Their occurrence in T-mycoplasmas was reported also by Williams (21). It remains to be determined whether such surface structures are confined to a few species only, or whether the failure to detect them in other mycoplasmas examined is due to damage re-



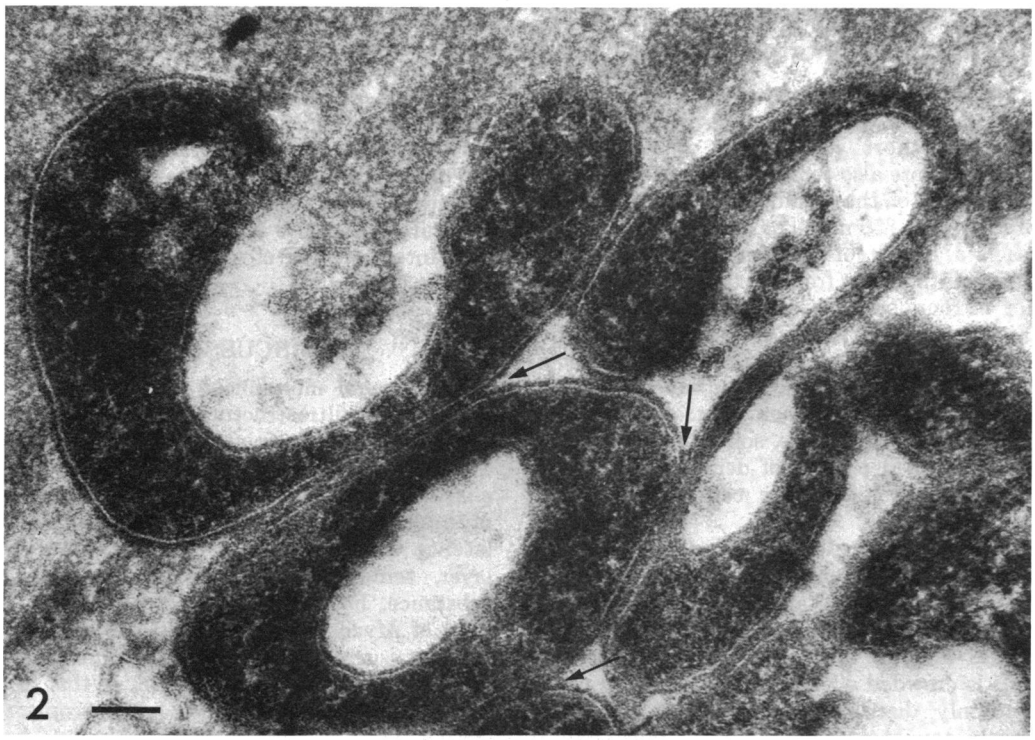
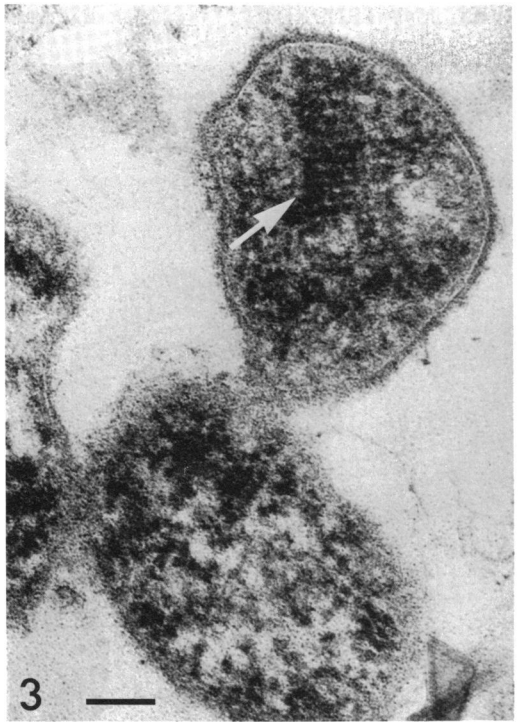


FIG. 1. *Thin section of T-mycoplasma (strain Pirillo), 14-hr culture. Ovoid and spherical cells surrounded by a triple-layered membrane, on the outer surface of which an electron-dense layer is seen. Note zones of contact between neighbor cells (arrows). Bar on this and following micrographs represents 100 nm.*

FIG. 2. *Thin section of T-mycoplasma (strain Pirillo), 14-hr culture. Filamentous cells together with an ovoid cell, all limited by a triple-layered membrane coated on the outer surface with an electron-dense layer. Note zones of contact between neighbor cells (arrows). The ovoid cell contains a vacuole also limited by a triple-layered membrane, which on the side facing the vacuole lumen carries an electron-dense layer similar to that on the cell surface. The extracellular material is clearly distinguishable from the outer surface layer consisting of radiating hairlike structures.*



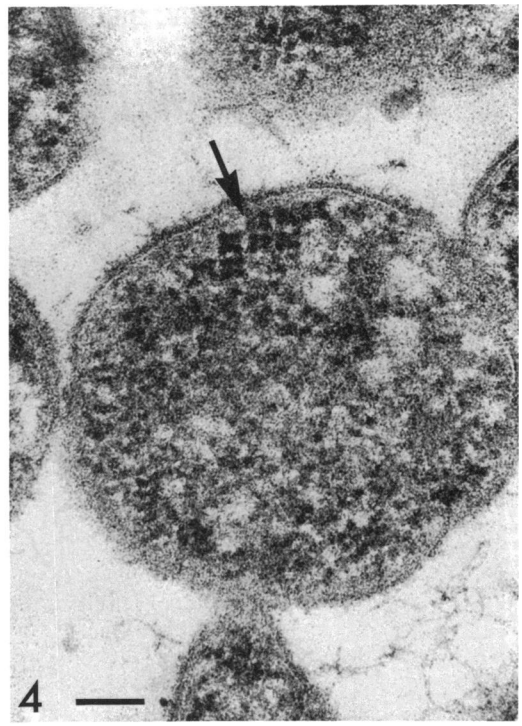


FIG. 3. *Thin section of T-mycoplasma (strain Cook), 12-hr culture. Ribosomes partly arranged in a regular geometrical pattern (arrow).*

FIG. 4. *Thin section of T-mycoplasma (strain Cook), 12-hr culture. Ribosomes arranged in closely square-packed groups of four located in the periphery of the cytoplasm (arrow).*

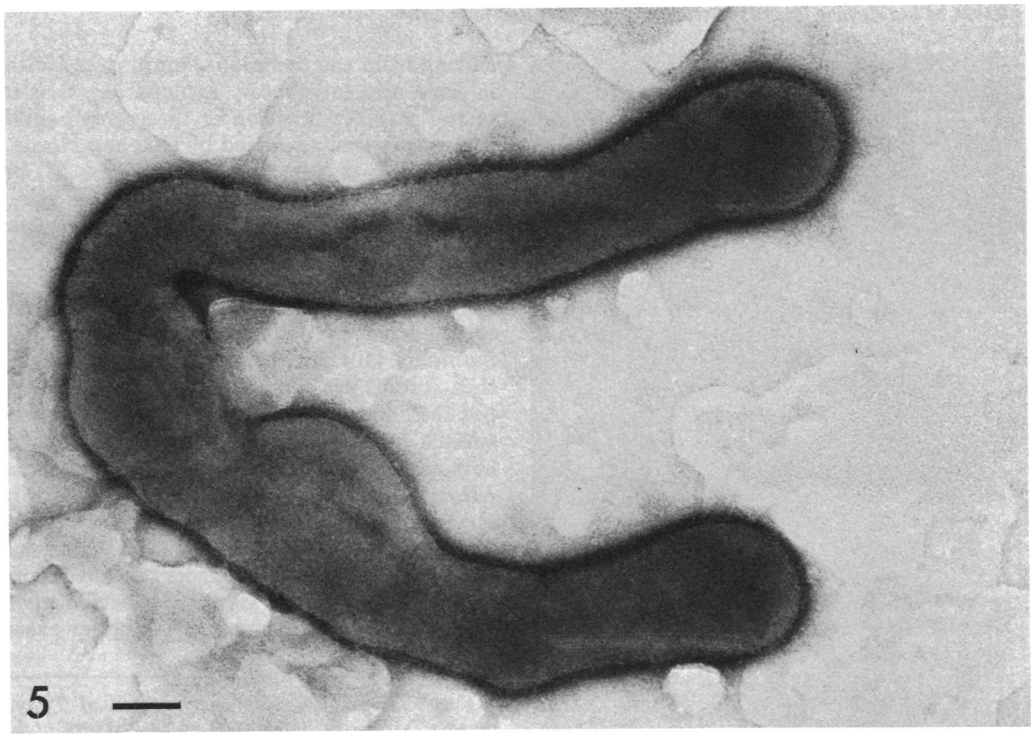


FIG. 5. *Negatively stained specimen of T-mycoplasma (strain 58), 12-hr culture. Filamentous cell with terminal club-shaped swellings. The surface layer seems to consist of radiating structures.*

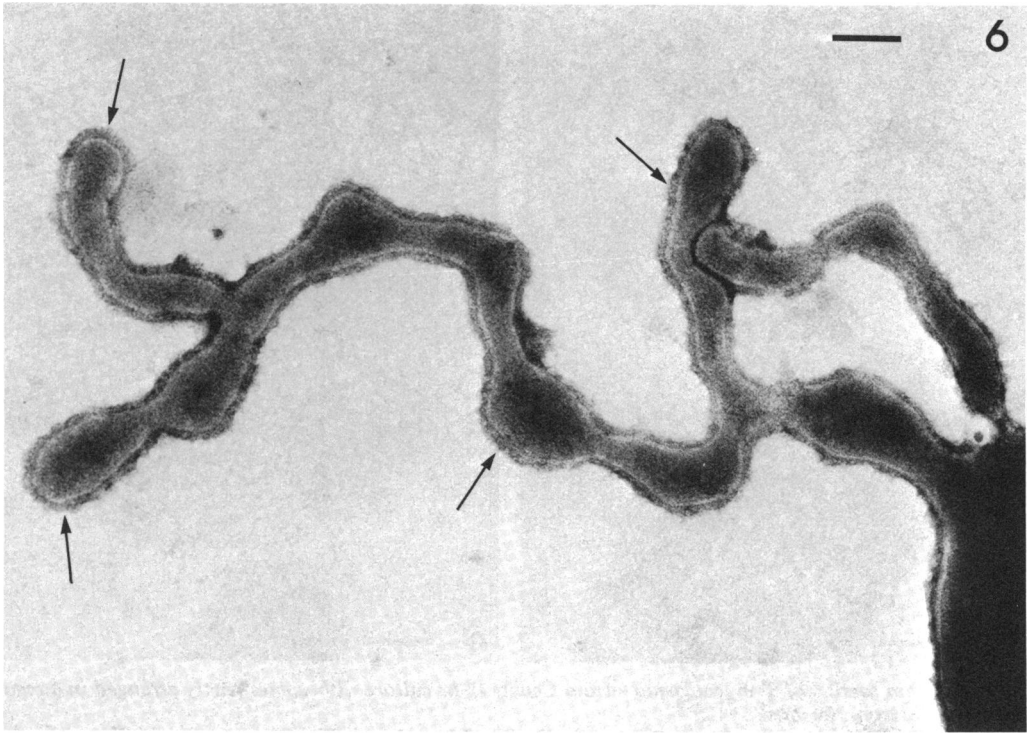
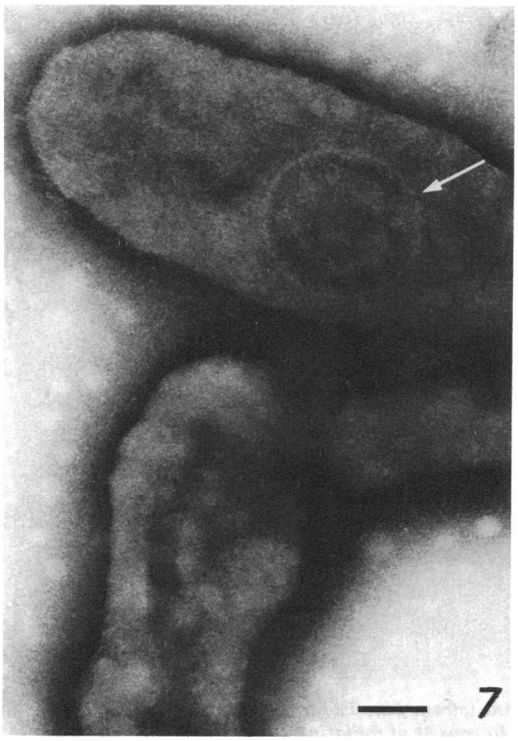


FIG. 6. *Negatively stained specimen of T-mycoplasma (strain Pirillo), 12-hr culture. Filamentous cell showing branching and terminal club-shaped swellings and irregular swellings along the length. Hairy substructure of the surface layer is particularly well defined (arrows).*



sulting from the techniques employed. Neither is there any satisfactory explanation, thus far, of the possible function of these small spikes. The theory was advanced by Chu and Horne in their study of *M. gallisepticum* and *M. mycoides* (5) that the spikes may play a role in hemagglutination similar to that of the surface spikes of myxoviruses. *M. gallisepticum* does indeed agglutinate and adsorb red blood cells from a variety of animal species, and hemadsorption has been demonstrated also with *M. pulmonis* (10) and T-mycoplasmas (20). Further studies are required, however, to prove a possible correlation between the presence of surface projections and hemagglutinating and hemadsorbing capabilities of mycoplasmas. Alternatively, the projections may simply serve to reinforce the delicate limiting membrane of the mycoplasma cell, as suggested earlier (9). Actually, this theory would seem to receive some support from the electron micrographs of the present study. On closer exami-

FIG. 7. *Negatively stained specimen of T-mycoplasma (strain Pirillo), 12-hr culture. Parts of filamentous cells, one of which contains a circular structure subterminally (arrow).*

nation the projections seem to be rooted in the limiting membrane. It might finally be argued that the surface layer, whether presenting itself as an amorphous substance or showing some degree of organization, could merely be a preparative artifact, produced, for example, by adsorption to the cell surface of media components. However, the difference in appearance of the surface layer and that of other extracellular material found in variable amounts in the micrographs is clearly distinct (Fig. 2).

The detection in all four T-mycoplasma strains examined of the arrangement of some of the ribosomes in a regular geometric pattern deserves particular mention since similar structures have been found previously only in one or two species, viz., *M. gallisepticum* (1, 4, 6, 12, 13) and possibly *M. pulmonis* (10). The significance of the organized ribosome arrangements remains obscure. Their interpretation as polysomes (1, 4, 8, 11, 12) was recently contested by Maniloff (11) who showed that their formation in *M. gallisepticum* was unaffected by inhibitors of ribonucleic acid and protein synthesis.

Membrane-bounded "vacuoles" have been found repeatedly in mycoplasmas and have also been observed in the present study (Fig. 2). We believe that the delimitation of these structures by a triple-layered membrane, which was found to possess on its inside a superficial layer that is indistinguishable from the surface layer of the cell membrane, suggests that these vacuoles are, in some instances at least, cross cuts of membrane invaginations, rather than true cytoplasmatic vacuoles. The circular structure found in the terminal broad part of a negatively stained filamentous cell (Fig. 7) resembles the "inclusion bodies" observed by negative-contrast electron microscopy of other mycoplasmas and tentatively interpreted as nuclear equivalents. Here again a more trivial explanation may be offered inasmuch as such structures may be nothing but invaginations with accumulated staining material or possibly protrusions of the cell membrane with heavy metal salts piled up around them.

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