# Ultrastructure of *Mycoplasma hominis*

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## ABSTRACT

ANDERSON, DOUGLAS R. (National Cancer Institute, Bethesda, Md.), AND MICHAEL F. BARILE. Ultrastructure of *Mycoplasma hominis*. J. Bacteriol. **90**:180–192. 1965.—Both thin-sectioning and negative staining were used in an electron microscopic study of the morphology of pleuropneumonia-like organism (PPLO) strain HEp-2 (*Mycoplasma hominis*, type I) grown in an artificial liquid medium. The morphology is quite variable and seems to depend, in part, on the age of the culture. The smallest form observed ("elementary body") is 80 to 100 mμ in diameter. The internal components of the larger PPLO cells (0.5 to 1 μ) are variable—some have ribosomelike granules and nuclear areas of netlike strands, and others have only irregular dense areas in a pale ground-plasm. Some of the forms have dense cytoplasmic bodies which look much like elementary bodies. Others have vacuoles which may contain structures which look like smaller organisms. Especially in older cultures, very large (10 μ) vacuolated organisms are seen, probably corresponding to the "large bodies" described by light microscopists. Filamented forms are also seen. These observations suggest several possible modes of reproduction, each perhaps operating under different cultural conditions or at different ages of the culture.

Preliminary observations indicated that mycoplasma have some forms which, by negative staining at least, look similar to the "virus-like particles" being found during electron microscopic screening of leukemic plasma (Porter et al., 1964) and tissue cultures. These observations were a cause of concern, since mycoplasma might well be present in these specimens being studied in search of virus—these organisms have been isolated from a patient with leukemia (Grace et al., 1963), and they frequently contaminate tissue cultures used for virus studies (Hayflick, Texas Rept. Biol. Med., *in press*). Therefore, a study of the morphology of mycoplasma was undertaken in hopes of being able to recognize pleuropneumonia-like organisms (PPLO) or differentiate them from virus during electron microscopic studies of leukemia and lymphoma, as well as other diseases of uncertain etiology.

A strain of mycoplasma was chosen which might be representative of tissue-culture contaminants (and perhaps also of the human strains carried by seemingly healthy laboratory personnel). It was grown in a liquid artificial medium to eliminate the possible presence of foreign matter (e.g., fragments of blood-agar plates,

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passenger virus carried in tissue culture, or disrupted tissue-culture cells) which, in the final preparations, might be mistaken for the organism. No antibiotics were in the culture medium to avoid masking contamination with other organisms, and especially to avoid the possibility of inducing bacterial L-forms in cases of undetected contamination with bacteria. Two preparative procedures were used: negative staining to visualize whole organisms, and thin-sectioning to study internal structures.

## MATERIALS AND METHODS

The organism used was PPLO strain HEp-2, originally isolated from a culture of the human epidermoid carcinoma of the skin (Fjelde) cell line. It has been serologically typed as *Mycoplasma hominis* type I (*unpublished data*), and has been used in other studies as a prototype tissue-culture strain of mycoplasma (Malizia, Barile, and Riggs, 1961; Barile, Malizia, and Riggs, 1962; Schimke and Barile, 1963*a*, *b*; Schmidt, Barile, and McGinnis, 1965).

The organism was grown in a medium consisting of the following: Brain Heart Infusion, 37 g; yeast extract, 2 g; distilled water, 850 ml; horse serum, 150 ml. Samples for negative staining or sectioning were taken from cultures after several hours to 6 days of incubation at 37 C.
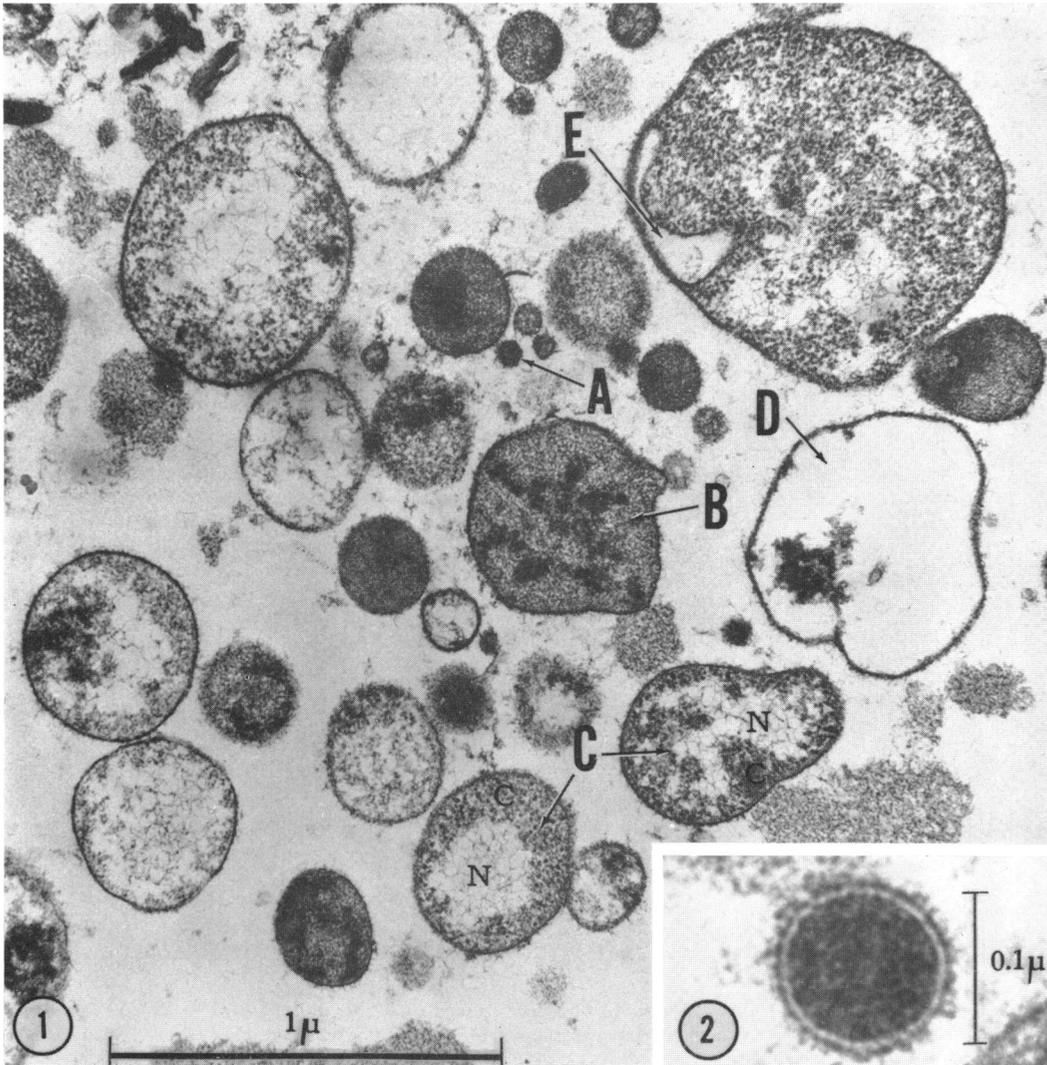
For negative staining (Brenner and Horne,

FIG. 1. *Culture of Mycoplasma hominis showing a variety of forms. At (A) is a small dense form ("elementary body")* ≈ *100 mμ in diameter. One type of the large forms (B) has a finely granular protoplasm divided into light and dark areas. A second major type (C) has its protoplasm divided into a central nuclear area (N) of netlike strands and a cytoplasm (C) containing ribosomelike granules. The internal material in several of the large forms has a watery appearance, and sometimes only an empty plasma membrane is seen (D). One of the organisms in this field has a membrane-bound vacuole (E) at its periphery. ✕ 53,000.*

FIG. 2. *Higher magnification of a small dense form ("elementary body")* ≈ *100 mμ in diameter, similar to (A) in Fig. 1. The lucent rim at the periphery is the space between the leaflets of a unit membrane, the inner leaflet being obscured by the dense internal material. ✕ 200,000.*

1959), it was usually necessary to concentrate the organisms. For this purpose, samples (4 to 10 ml) of the cultures were centrifuged at 27,000 ✕ $g$ (20,000 rev/min, no. 40 Spinco head) for 0.5 hr, and the pellets were suspended in several drops of supernatant fluid. One part of the culture, or of the concentrated suspension of organisms, was mixed with two parts of 2% phosphotungstic acid which had been adjusted to $p$H 5 with 5 N potas-

sium hydroxide. One drop of the mixture was placed on a collodion-coated copper grid covered with carbon. Excess fluid was drained by touching the edge of the grid to filter paper, and the grid was allowed to dry before being examined. Addition of bovine serum albumin to the phosphotungstic acid as a spreading agent was not found to be necessary, presumably because the protein of the culture medium served this purpose. However,
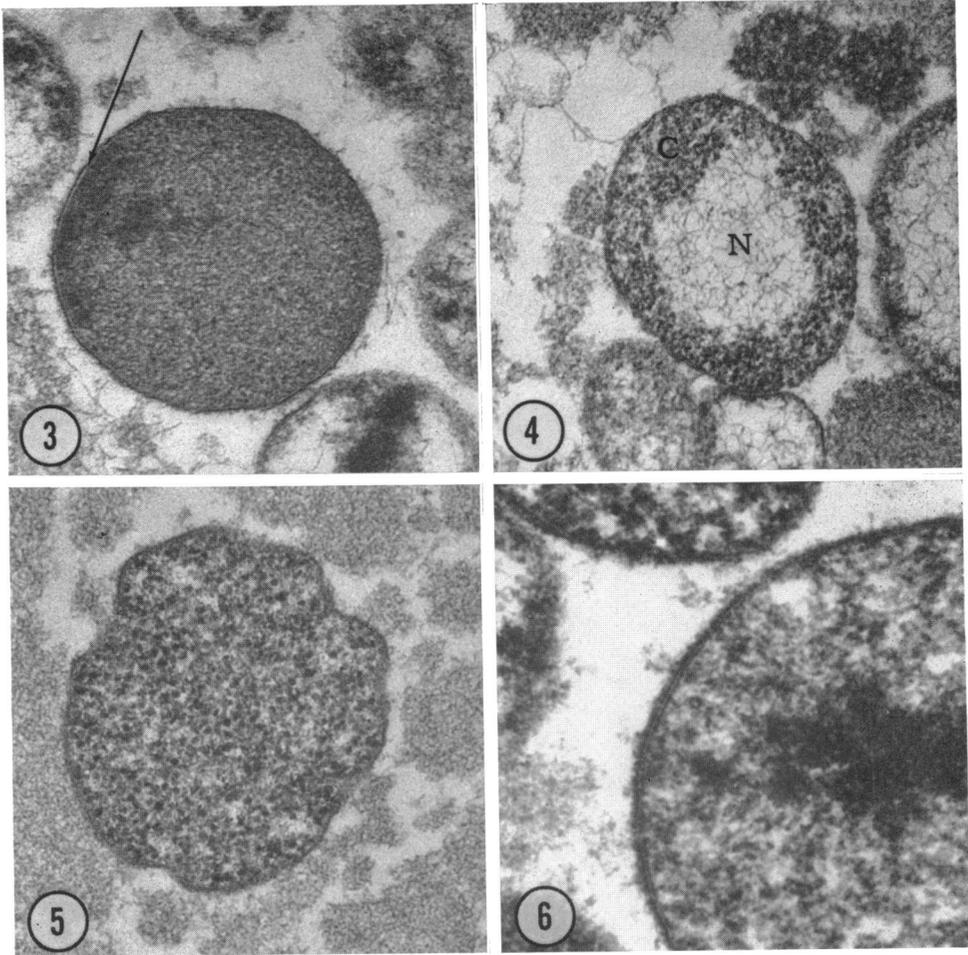
FIG. 3. *An organism with finely granular protoplasm divided into light and dark zones. Where the dense material (nucleic acid?) touches the plasma membrane (arrow), the inner leaflet of the unit membrane is obscured, and the lucent space between the leaflets is accentuated.* × 70,000.

FIG. 4. *Another form of the mycoplasma strain HEp-2 consisting of a nuclear area of netlike strands (N) and a cytoplasm (C) containing ribosomelike granules.* × 70,000.

FIG. 5. *An organism of somewhat irregular shape with ribosomelike granules, but lacking a nuclear area.* × 70,000.

FIG. 6. *Structure of the unit membrane, with flocculent amorphous material adherent to the outside surface.* × 110,000.

addition of sucrose to a concentration of 0.05% seemed to help the spreading properties of the preparation.

Pellets like those suspended for negative staining were made for sectioning. "Prefixing" by adding fixative before centrifugation did not seem to have any advantage over fixing the pellets that were not prefixed. After a trial of a variety of buffers and fixatives, three reagents were adopted: (i) 5% glutaraldehyde fixative (Sabatini, Bensch, and Barrnett, 1963), made by adjusting 20 ml of commercial 25% glutaraldehyde which has been stored over coconut charcoal to pH 7.4 with 0.2 M sodium

cacodylate (about 20 to 35 ml), and adding sufficient water to make 100 ml of fixative (de Thé, personal communication of method learned from S. J. Holt); (ii) chrome-osmium fixative of Dalton (1955), modified to contain 0.1% calcium chloride and 0.8% sodium chloride in the final fixative; (iii) a final rinsing solution, freshly prepared by dissolving 0.05 g of uranyl acetate in 10 ml of 10% formalin which has been stored over calcium carbonate crystals. [The lower pH and very long periods of fixation found optimal for bacteria by Ryter et al. (1958) were not tried, even though better preservation might have been obtained, especially
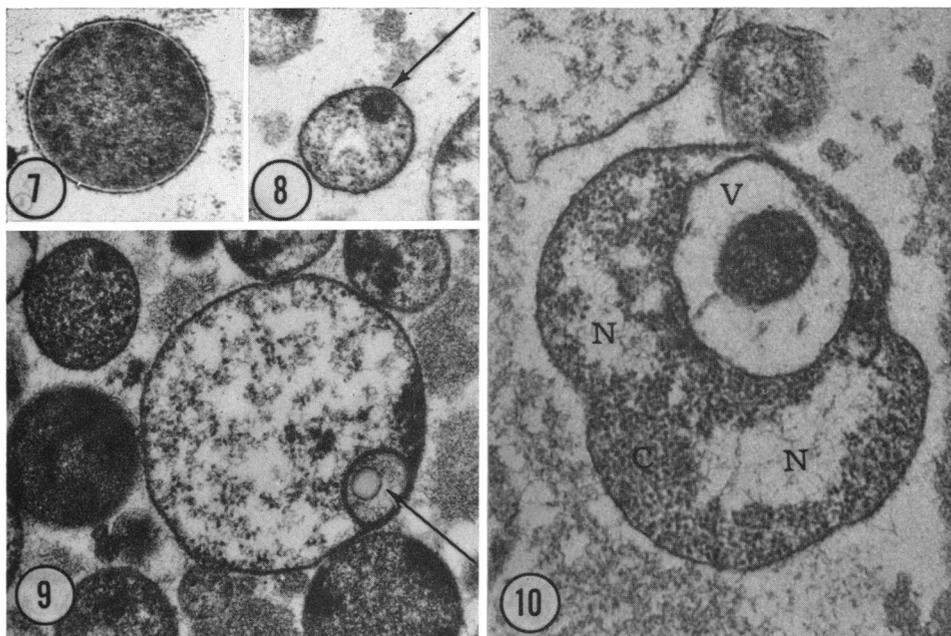
FIG. 7. *A form transitional between the small dense form ("elementary body") and the larger form. In this organism, the dense material is clumped into aggregates ≈ 100 A in diameter.* × *75,000.*

FIG. 8. *An organism containing a dense body (≈ 100 mμ in diameter) limited by a unit membrane (arrow). As is typical, the dense body is at the periphery of the organism, and it has the same appearance as the "elementary body."* × *60,000.*

FIG. 9. *An organism with a different type of membrane-bound cytoplasmic body (arrow) at its periphery. The cytoplasmic body has a lucent area which is not defined by a membrane.* × *42,000.*

FIG. 10. *Vacuole (V) enclosing a structure with components similar to the cytoplasm of the organism which contains it. The organism contains an irregular nuclear area (N) and ribosomelike granules in the cytoplasm (C).* × *60,000.*

in the nuclear region. The reason is that fixatives and schedules were chosen which gave adequate results and also seemed likely to be adaptable for use in future studies of PPLO-infected tissue cultures or cytochemical studies. Any such future studies could then be compared with this study without concern about differences in preparative techniques.]

These reagents were used on the pellets according to two schedules which gave comparable results: (i) 0.5 hr or more in glutaraldehyde, 1 to 2 hr in osmium, and 2 to 3 hr in rinsing solution of uranyl acetate in formalin; (ii) 1 to 2 hr in osmium followed by 2 to 3 hr in rinsing solution of uranyl acetate in formalin.

After fixation, the pellets were dehydrated rapidly in a graded series of ethyl alcohol.

Epon-Araldite embedding mixture no. 1 of Mollenhauer (1964) was used. To accomplish infiltration, the pellets were cleared of ethyl alcohol in two 15-min changes of propylene oxide and placed for 1 hr to overnight in a 1:1 mixture of propylene oxide and embedding mixture. Pieces of the pellet were carefully drained on filter paper and flat-embedded. Polymerization was carried

out at 80 C overnight or longer. [With the Epon-Araldite mixture, flat embedding seems to give better preservation of structure and more consistently gives good cutting properties than embedding in capsules (Dalton, *personal communication*). For this purpose, shallow, disc-shaped dishes (internal diameter, 1.5 cm; internal depth, ca. 3 mm) are made by cutting the tops off no. 3 polyethylene hollow stoppers. Several pieces of the same specimen are embedded along the periphery of the same mold, and a paper label written with soft lead can be placed in the center. The entire disc-shaped block which results can be held in a vise-type specimen holder available for LKB ultrotomes, and, by successive repositioning of the block in the holder, any number of the embedded pieces can be sectioned.]

Sections giving a gray-to-silver interference color were cut and picked up on collodion-coated grids covered with carbon. They were doubly stained—first for 15 min with 50% ethyl alcohol saturated with uranyl acetate (Gibbons and Grimstone, 1960), and then for 15 min with lead citrate (Reynolds, 1963).
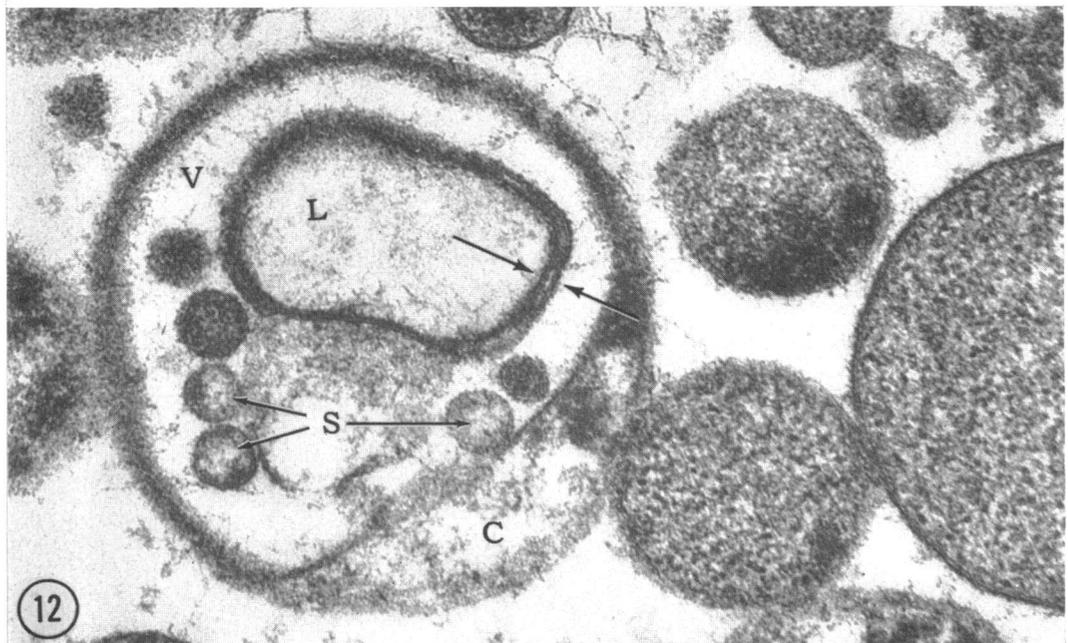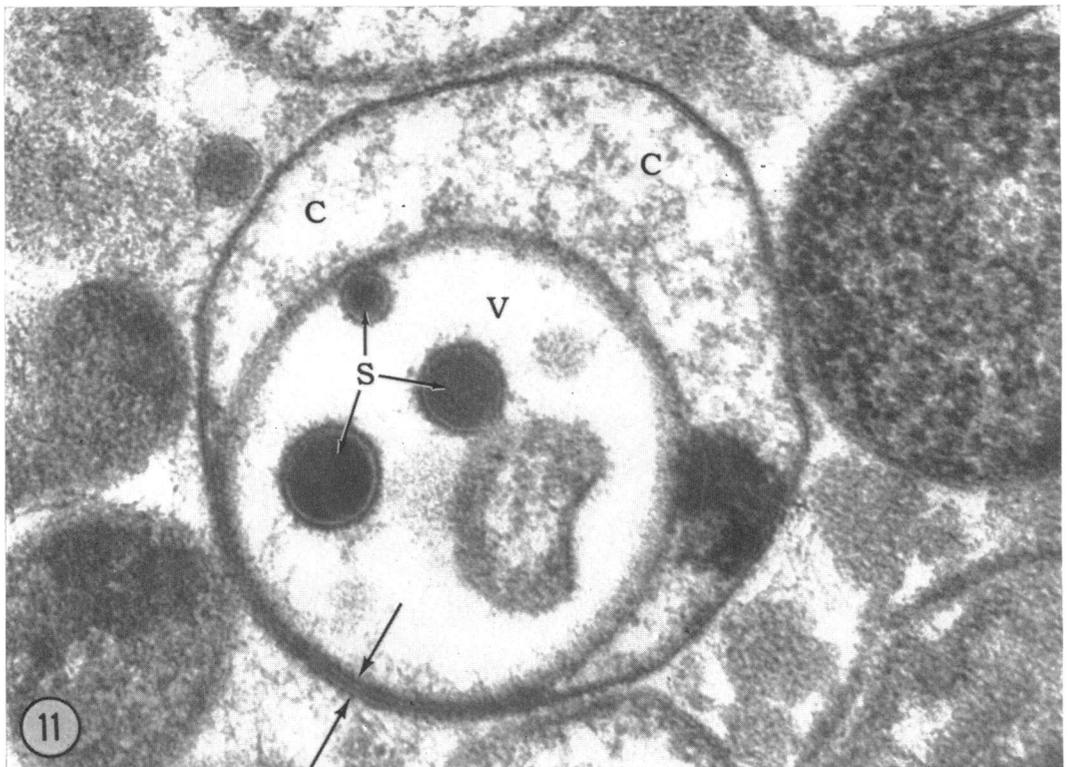
Both the negatively stained and the sectioned

FIG. 11. *An organism with a vacuole* (V) *containing small dense structures* (S) *having the same appearance as the "elementary bodies." In contrast to Fig. 10, the cytoplasm* (C) *of this organism appears empty. The vacuole is usually at one edge of the organism, and, at the contact points, the plasma membrane and the membrane of the vacuole fuse to form a trilayered compound membrane* (arrows). × 110,000.

FIG. 12. *Another organism containing a vacuole* (V). *Several small forms are in the vacuole, and some* (S) *are pale, in contrast to those of Fig. 11. Also, in the vacuole is a larger structure* (L) *which, itself, is vacuolated. The unit membranes of this large structure and its vacuole are seen between the arrows. The cytoplasm* (C) *of the vacuolated organism has a degenerate appearance, whereas the organism at the right has a healthy-looking, well-preserved cytoplasm.* × 80,000.
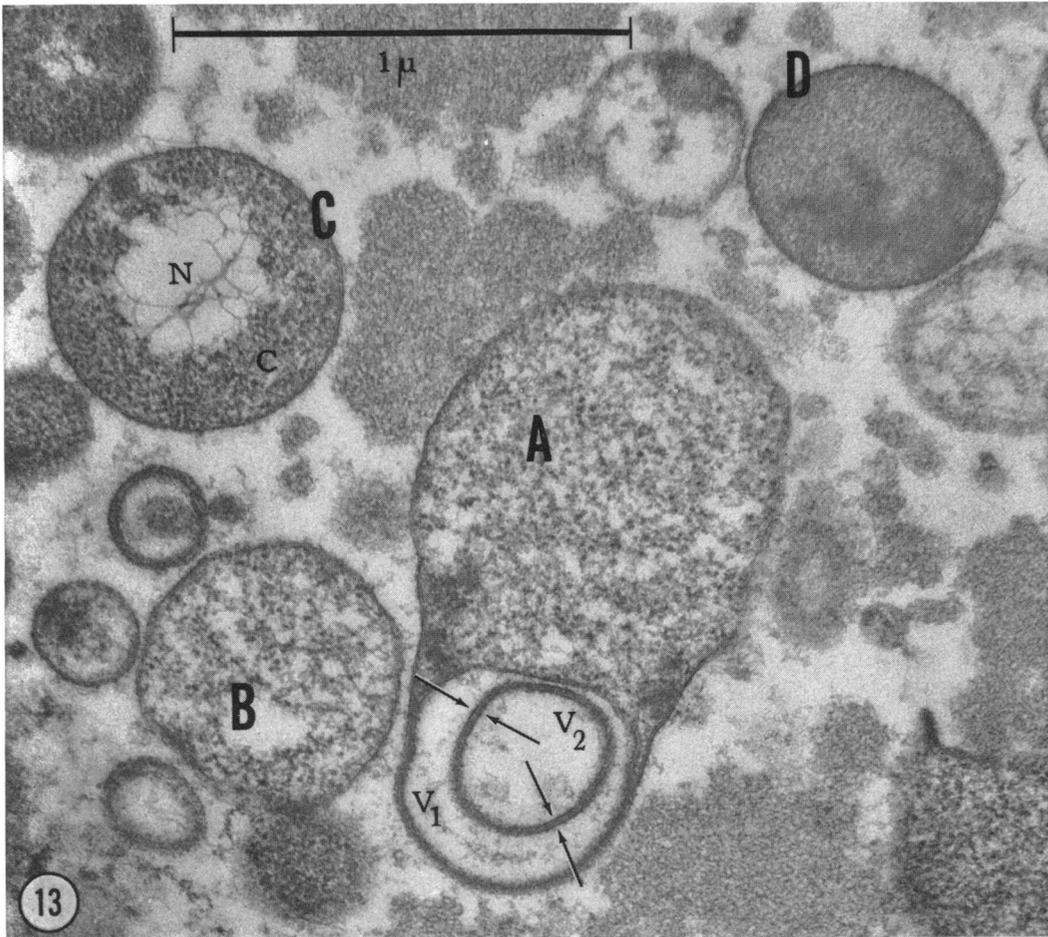
184

FIG. 13. *A field containing various forms of mycoplasma. At one end of organism A is a vacuole ($V_1$) whose only content (in this plane of section) is a membrane-bound organism completely filled with a vacuole ($V_2$). The latter's vacuolar and limiting membranes are fused into a trilayered compound membrane (between arrows). Although not to the extent illustrated in Fig. 11 and 12, the cytoplasm of organism A, as well as the one next to it (B), has a watery appearance. Organisms C and D do not have this degenerate appearance, suggesting that the appearance of A and B is not merely a result of inadequate fixation. Organism C is the type which contains a nuclear area (N) and a cytoplasm (C) containing ribosomelike granules. Organism D is the finely granular type with light and dark areas. $\times$ 60,000.*

specimens were examined and photographed with RCA electron microscopes, models EMU-3F and EMU-3G.

### RESULTS

*Sectioned material.* A variety of forms were found in each culture. The proportion present of each form was variable, but, from this study, it was not possible to determine the factors that influence which morphological forms predominate.

The smallest form encountered (Fig. 1A and 2) is round and 80 to 100 m$\mu$ in diameter. It is very electron-dense, except for an electron-lucent area just beneath its periphery which represents the space between the leaflets of a unit membrane, the inner leaflet being obscured by the dense internal material.

Larger forms (400 to 1,000 m$\mu$) possess a variety of internal structures. Three main types might be distinguished. One type (Fig. 1B, 3, and 13D), always in the minority, is made up of very dense areas superimposed on a finely-granular ground substance of intermediate density. A second type (Fig. 1C, 4, 10, and 13C) has zones of netlike strands presumed to be nuclear areas. These
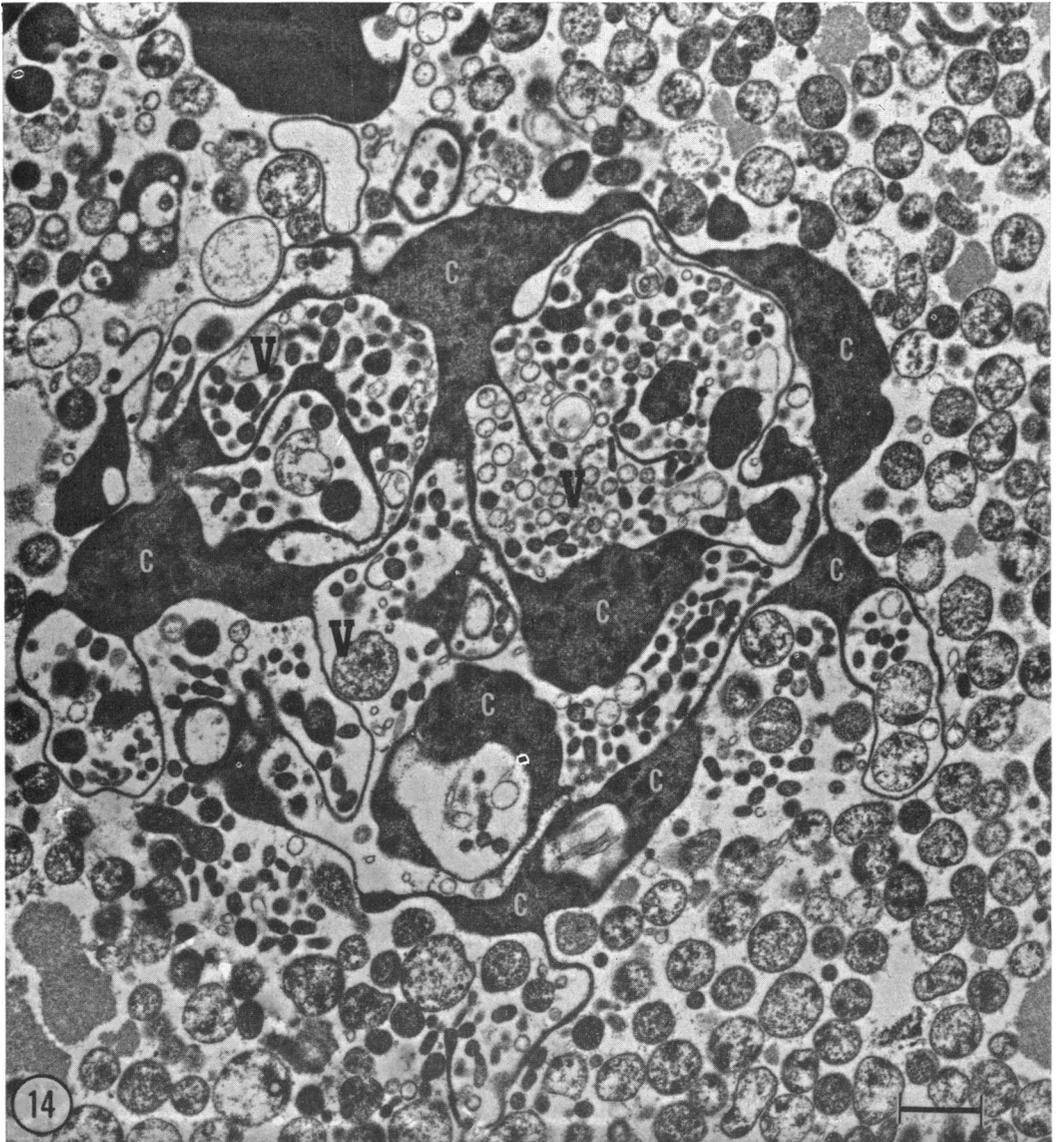
FIG. 14. *A very large organism with dense cytoplasm (C) and extensive vacuolar system (V) containing many smaller organisms of various kinds. The marker in the lower right corner indicates (1 μ.) × 10,000.*

areas are usually central and ovoid, but may be irregular in shape. The boundary between these areas and the cytoplasmic areas is discrete, but not marked by a membrane. The cytoplasm in this type consists of ribosomelike granules approximately 100 A in size in a ground substance of intermediate density. This form dominates some samples, notably the younger cultures.

A third type (Fig. 5) contains ribosomelike granules in a pale, ground substance, but no areas of netlike strands. In some cases, of course,

the apparent lack of the presumed nuclear area is a result of an unfortunate plane of section. However, it is clear that some organisms lack them altogether. Such forms are quite often midplane sections (as indicated by appearance of the membrane), and, in addition, they are seen more frequently than the plane of section phenomenon could explain, even being the most predominant form in some samples.

Some organisms contain other combinations of the internal components described, but the three

types described make up the majority of the large forms.

Organisms in a spectrum of sizes between the smallest and the larger forms have dense internal material. In some, it is dimly seen that the density is somewhat inhomogeneous, with hazy aggregates in the order of magnitude of 100 A (Fig. 7). In others, however, as their size approaches that of the large forms, the cytoplasm divides into homogeneous zones of dense and somewhat paler zones, approaching the appearance of the first type of large organism (Fig. 1B, 3, and 13D).

The cytoplasm of some organisms appears pale and watery in varying degrees, containing empty spaces interspersed with clumps of cytoplasmic ground substance, ribosomelike granules, remnants of dense areas, netlike strands, or the cytoplasmic bodies and vacuoles described below. In the extreme, only an empty plasma membrane is seen (Fig. 1D). In some samples, especially from older cultures, most of the organisms have this degenerate appearance. Their appearance is probably not a result of poor fixation, since they can be seen side by side with well-preserved organisms (Fig. 12 and 13).
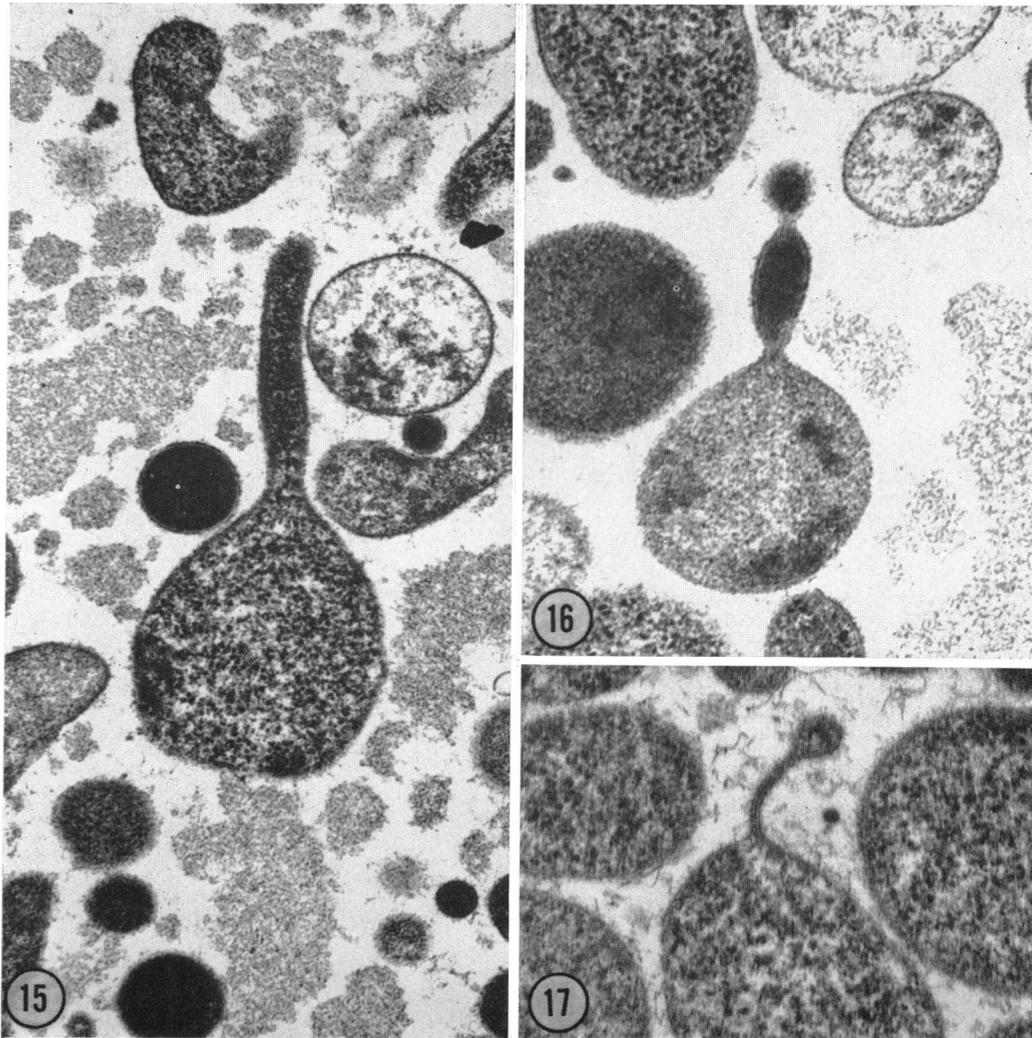


FIG. 15. *Organism with a filamentous projection having the same structure as the main body of the organism.* × 45,000.

FIG. 16. *Organism with a filamentous projection having a different structure from the main body of the organism.* × 70,000.

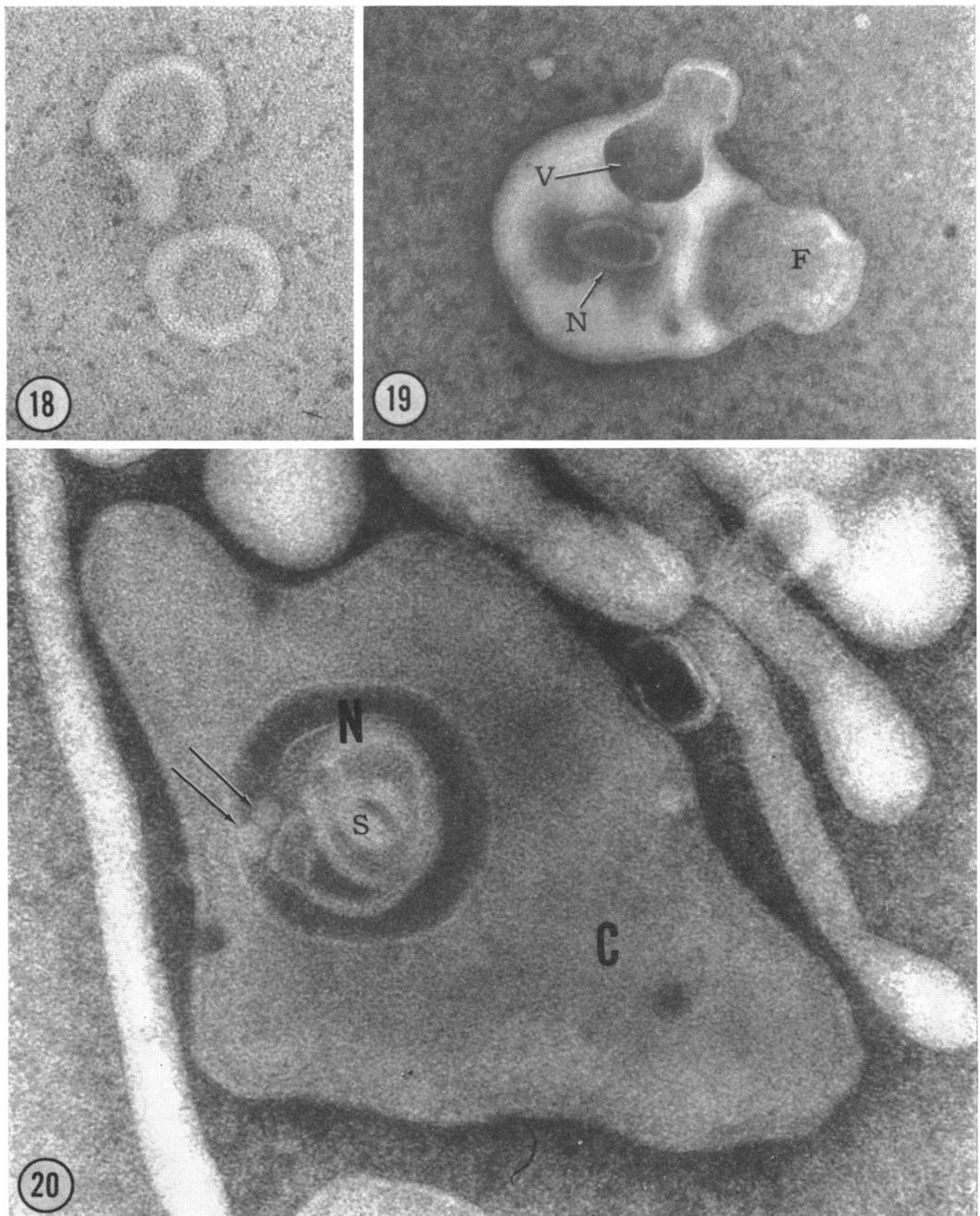FIG. 17. *Organism with projection consisting of a trilayered compound membrane.* × 70,000.

FIG. 18. *Small forms, one with a projection, in a negatively stained preparation.* × 70,000.

FIG. 19. *Larger organism seen by negative staining. Central strands are presumed to represent nuclear material (N), and the sharply defined structure (V) is suggestive of a vacuole. The dense area to the right (F) lacks a discrete border and most likely represents merely a flattening of that end of the organism.* × 45,000.

FIG. 20. *Organism showing the appearance of the nuclear area (N) and cytoplasm (C) by negative staining. The nuclear area is a sharply defined zone containing coiled strands (S) and two tiny spheres (arrows), a much more usual appearance than that shown in Fig. 19.* × 100,000.

All of the organisms are limited by a unit membrane (Robertson, 1957) consisting of two leaflets (Fig. 6). Where the dense area of the large forms touches the membrane, the inner leaf is obscured (Fig. 3, arrow). This observation, along with inspection of the series of forms transitional to the small forms, is the basis for stating above that the lucent area at the periphery of
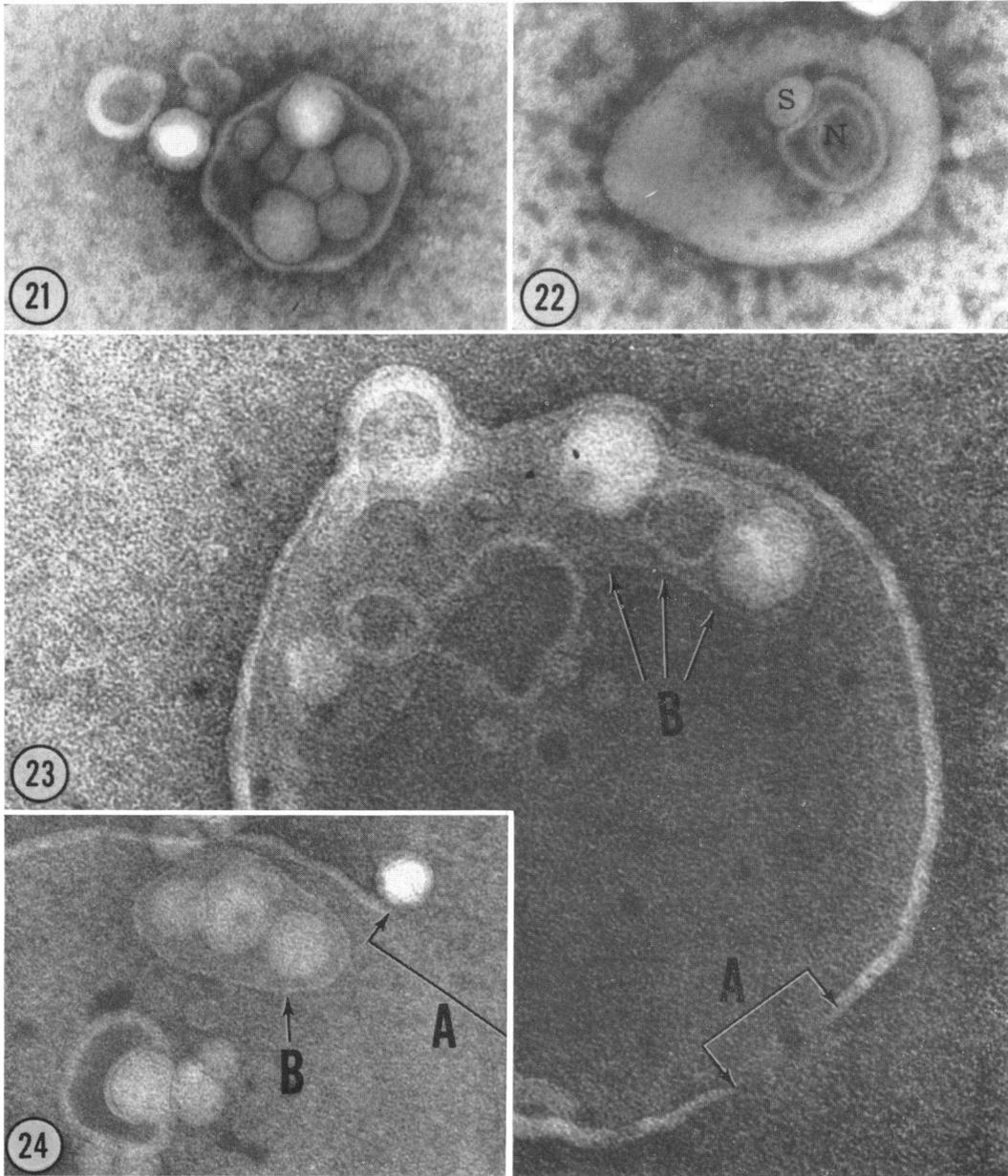


FIG. 21. *Organism filled with small lucent spheres. Negatively stained.* × *50,000.*

FIG. 22. *Organism with a lucent sphere (S) indenting the nuclear area (N). Negatively stained.* × *50,000.*

FIG. 23. *Organism with a broken membrane (A). The phosphotungstate used as a negative stain entered through the break (A) and outlined a sac (vacuole) enclosing the lucent spheres (B).* × *75,000.*

FIG. 24. *Spheres (similar to those in Fig. 23) enclosed in a vacuole (B) outlined by phosphotungstate which entered through a break (A) in the plasma membrane.* × *75,000.*

the small forms (Fig. 2) is the space between the leaflets of a limiting unit membrane. There is no cell wall, although there is an irregular layer of amorphous, flocculent, homogeneous material adherent to the outer surface (Fig. 6), similar to that found on the outer surface of cell membranes.

The cytoplasm of the large forms and of the forms transitional to the small ones may contain structures consisting of a dense core limited by a unit membrane (Fig. 8). Usually these structures are strikingly similar to the free small forms (Fig. 2), but their core is not always a solid, uniform density, and the material sometimes appears glassy rather than finely granular. There

are also similar, but larger and paler, structures which may contain a rather sharply defined lucent zone (Fig. 9).

Especially in older cultures, some organisms, which may or may not be a little larger than the others, contain vacuoles (Fig. 1, 10, 11, 12, and 13). The previously described large form with pale and dark areas rarely has a vacuole, but vacuoles are not unusual in any of the other large forms. The vacuoles are typically seen at one edge of the organism. Where the unit membrane limiting the organism touches that of the vacuole, the adjacent leaflets sometimes fuse to give the appearance of a trilayered compound
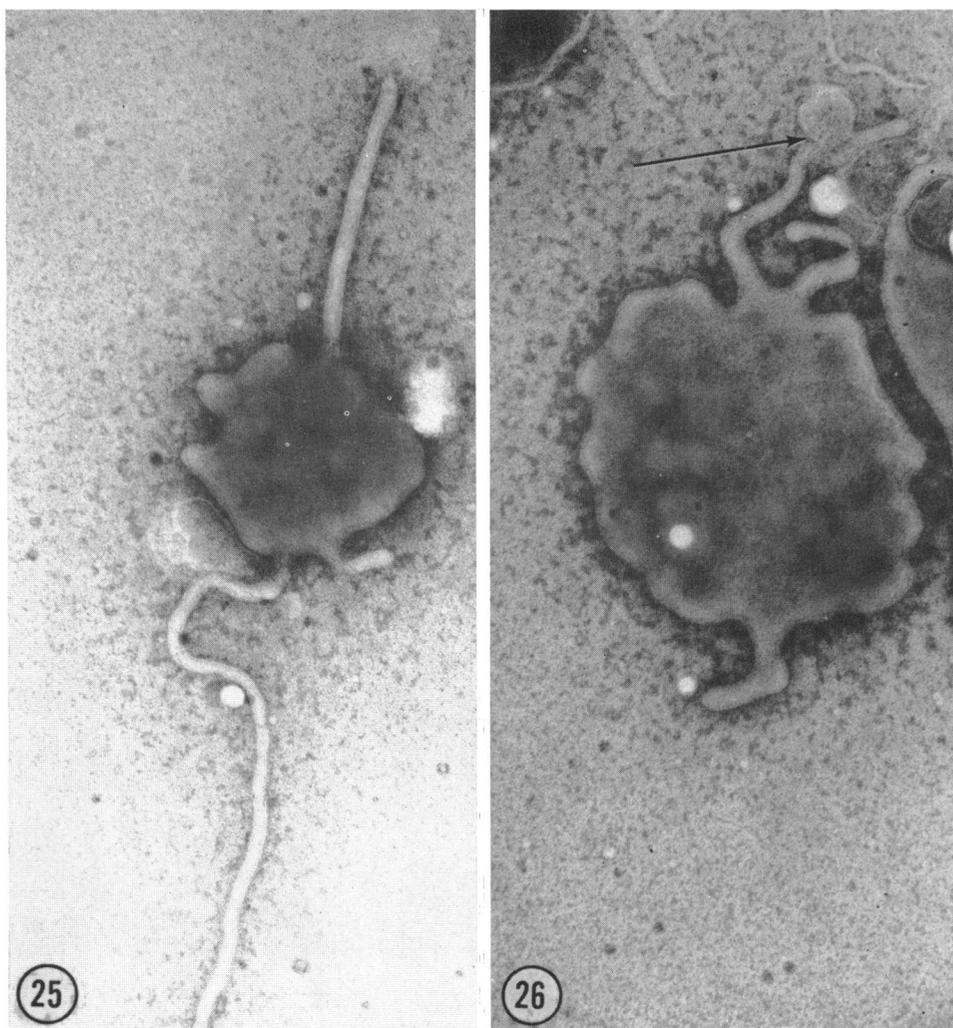



FIG. 25. *Demonstration of long filaments. Negatively stained.* × *33,000.*

FIG. 26. *Organisms with shorter filaments, one with a swelling emerging from one side (arrow). Negatively stained.* × *33,000.*

membrane (Robertson, 1958) separating the inside of the vacuole from the outside of the organism (Fig. 11). The vacuoles may appear empty, but, especially in the larger organisms, the vacuoles contain what appear to be other smaller organisms. The enclosed organism may be of the small, dense form (Fig. 11), but, more often, it is paler (Fig. 12) or is made up of a cytoplasm containing ribosomelike granules (Fig. 10). The enclosed organism may, itself, contain a vacuole (Fig. 12 and 13). Giant forms have also been observed which appear to have an extensive vacuolar system with many enclosed organisms (Fig. 14), but, in this case, it is difficult to assert that any given space is not an invagination.

Structures which might represent sections through filamentous projections from the surface of the large forms are of three types. One looks like an unspecialized projection of the organism (Fig. 15), and another has a dense core which is different from the main body of the organism (Fig. 16). A third type is merely trilayered compound membrane (Fig. 17) possibly derived from close apposition of opposing areas of the limiting membrane of the organism, the adjacent leaves of the two membranes having fused.

*Negatively stained material.* In preparations negatively stained, images thought to represent the small and intermediate forms seen in sectioned material have white rims surrounding round cores with the same intensity as the background (Fig. 18). The outer diameter of the white rim is rarely less than 150 or more than 500 m$\mu$; it may be round or irregular, sometimes giving the appearance of a stumpy projection. The dense core is always round, with a diameter between 150 and 400 m$\mu$.

Images of the larger forms are round or irregular, measuring 0.5 to 2 $\mu$. Some of them, but not all, exhibit internal structures. A typically central structure (presumed to be the nuclear area) consists of a sharply defined area containing coiled strands and usually one or two tiny white spheres (Fig. 20). Sometimes, the sharply defined dense area is not evident, and only the free strands are seen in a somewhat looser coil (Fig. 19).

Lucent spheres (diameter, 100 to 150 m$\mu$) are present within some of the large forms and may seem to fill the organism (Fig. 21). It is not unusual for a sphere to indent the presumed nuclear area (Fig. 22). When the organism's limiting membrane is not intact, and phosphotungstic acid enters through the break, at least some of the spheres can be seen enclosed in a sac or vacuole (Fig. 23 and 24). These vacuoles probably do not correspond to the empty, dense areas sometimes seen in the intact organisms which are suggestive of vacuoles (Fig. 19).

Filaments of uniform diameter are seen in varying numbers and lengths projecting from the surface of organisms in some samples (Fig. 25). These may have swellings or blebs which often emerge from one side of the filament (Fig. 26).

## DISCUSSION

Although we have presented morphological forms of mycoplasma which have not been previously described, our findings are in agreement with previously published observations of sectioned mycoplasms (Edwards and Fogh, 1960; Freundt, 1960; van Iterson and Ruys, 1960a, b; Domermuth et al., 1964; Anderson, 1964).

The mode of reproduction of these organisms remains controversial (Freundt, 1958; Klieneberger-Nobel, 1962). The small dense forms are probably the "minimal reproductive units" responsible for growth of these organisms in ultra-filtrates (Elford, 1929; Laidlaw and Elford, 1936; Klieneberger-Nobel, 1962). Micrographs such as Fig. 16 suggest that they form by segmentation or beading of filaments, as described by Freundt (1958). However, there is evidence that they may also form by budding from the surface of mycoplasma cells or into vacuoles (Anderson, 1965).

Often, in young cultures, the form with netlike strands and ribosomelike granules was the only one present. Population studies (*unpublished data*) done on some of the cultures in this study showed that the colony-forming titer was rising rapidly in these young cultures. These findings indicate that the increase in titer is not mediated by internal granules or by means of segmenting filaments, but rather that some process of fragmentation, fission, or budding is the mode of reproduction.

In the older cultures examined, the colony-forming titer was decreasing (*unpublished data*), and many of the cells had a degenerate appearance. It is in these cultures that the vacuoles containing dense bodies are seen. These findings might be interpreted to mean that the dense bodies ("minimal reproductive units") are a viable form less subject than the larger ones to the adverse conditions (accumulated toxic products or depleted nutrients, or both) of the older cultures and, thus, represent a method of protecting the viability of the culture.

The fact that, in this study, some cultures contained the "minimal reproductive unit" and other cultures of the same organism did not is interesting in the light of the observation by

Grace et al. (1963) that the size of the smallest filterable unit is different under different conditions of culture.

Thus, the observations of this study suggest the possibility that mycoplasma have several alternate modes of reproduction, and that the cultural conditions (e.g., composition of the original growth medium and the change in composition as the culture ages) influence or even determine the mode of growth and reproduction, as well as the morphology. Differences in cultural conditions may account for the confusing variation in descriptions of morphology and reproduction of PPLO, although some of the descriptive differences may also be due to differences in investigative technique or differences between the species studied, or both.

### ACKNOWLEDGMENTS

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