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Phase-Contrast and Electron Microscopy of Murine Strains of *Mycoplasma*

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ABSTRACT

NELSON, JOHN B. (The Rockefeller University, New York, N.Y.), AND MICHAEL J. LYONS. Phase-contrast and electron microscopy of murine strains of *Mycoplasma*. J. Bacteriol. **90**:1750–1763. 1965.—Two strains of *Mycoplasma pulmonis* (associated with infectious catarrh) on examination in fluid culture (20% horse serum-bouillon) by phase microscopy were highly pleomorphic, with many bacilliform elements and fewer coccoid ones. Motility, characterized by gliding of rods and spinning of spherical forms, was observed through the 9th subculture of one strain and the 15th of the second. Motile elements were not seen in later transfers and pleomorphism was reduced. One strain of *M. neurolyticum* (associated with conjunctivitis and encephalitis) was much less pleomorphic and showed neither bacilliform elements nor motility at any time. When examined by negative-contrast electron microscopy, organisms of this strain were found to have an average diameter of 0.7 μ and to possess a concentrated peripheral layer of cytoplasm and a central mass which may represent the cells' nuclear equivalent. The latter feature was not prominent in spherical forms of *M. pulmonis*. These cells, when observed after 48 hr of culture, showed evidence of the generation of new progeny cells in their central area. The filamentous or bacilliform cells of *M. pulmonis* were frequently serpentine in appearance, 2.0 to 3.0 μ in length and 80 to 250 mμ in width. They appeared to generate new cells from terminal buds from which outpouchings initially developed. Older cells, in the stationary phase, showed evidence of undergoing multipolar germination. Microtubules, about 60 mμ wide, were found in association with most filamentous cells from 48-hr cultures; fragments of membrane, studded with closely packed ribosomelike particles, were also found. There was no evidence of flagella or any specialized structure that could account for the observed motility of the organisms.

The comparative morphology of strains of *Mycoplasma*, the pleuropneumonia-like organisms (PPLO), is of considerable value in their identification and classification. Pleomorphism is a characteristic feature but is by no means marked for all species, as sometimes stated. It is known that *M. pulmonis*, indigenous to mice and rats, may show elements with true motility in unfixed preparations from fluid cultures. Motility is emphasized here, as it is commonly disregarded and seems not to have been looked for in suitable cultures of other species. The present study is concerned with the morphological aspects of two murine species as observed by phase-contrast and electron microscopy.

The method of thin-section electron microscopy has been employed by a number of investigators to study the ultrastructure of *Mycoplasma* species (Edwards and Fogh, 1960; Freundt, 1960; Sharp, 1960; Ruys and van Iterson, 1961; Domermuth et al., 1964; Maniloff, Morowitz, and Barrnett, 1965*a*, *b*). Such studies have indicated that organisms of all species examined possess a limiting membrane, 70 to 150 A wide, ribosomal particles, sometimes in patterned arrays, and strands or masses of nuclear material.

Early electron microscopic studies of the organisms as whole mounts were made by Weiss (1944), and the organisms were more fully studied by Smith, Hillier, and Mudd (1948). The species used were of human origin. In these studies, the extent to which the morphology of the cells had been altered by lack of control of their osmotic environment during specimen preparation is not known. Noteworthy, however, was the description of both round and bacillary or filamentous forms. Klieneberger-Nobel (1962) made similar observations in a study of metal-shadowed preparations of caprine PPLO.

The development of the negative-contrast procedure (Brenner and Horne, 1959), with its dramatic possibilities for enhanced visibility of structure, suggested that, thereby, new aspects of the morphology of *Mycoplasma* cells and their

replicative elements might be demonstrable. This forms the basis for a major part of the study reported herein. Since the time of completion of the present study, a report on the ultrastructure of *M. hominis*, by Anderson and Barile (1965), has appeared, in which the methods of thin sectioning and negative staining were employed.

MATERIALS AND METHODS

Source of cultures. Two strains of *M. pulmonis* (S and B) were used. This species is etiologically associated with infectious catarrh of mice and rats and has been under observation in our laboratory for many years (Nelson, 1937, 1940). It was originally described as a coccobacilliform body and later as a PPLO of catarrhal type. The S strain was isolated in 1965 from the middle ear of an experimentally infected mouse. We have maintained infectious catarrh for 5 years in specific pathogen-free (SPF) Swiss weanlings by nasal passage. The B strain was also recovered from middle ear exudate. It was originally isolated in 1963 from a naturally infected mouse of outside source, and was stored in a frozen state. The second species was formerly termed a murine PPLO of conjunctival type but was recently identified by Tully (1965) as *M. neurolyticum*. It is maintained in SPF mice of the Princeton strain by contact passage (Nelson, 1950). The present culture was obtained from conjunctival washings.

Media. The basic media were heart infusion agar and bouillon (*p*H 8.0) prepared at the Rockefeller University. Serum from a horse, kept only for bleeding, was added in a concentration of 20% to both media shortly before use. The final *p*H was 7.4 to 7.6. Primary isolations were made from serum-agar containing 2,500 units of penicillin. The plates were inoculated after chilling in the refrigerator and were sealed with Scotch Tape prior to incubation at 37 C. Colonies were transferred to serum-bouillon without penicillin. Subcultures were made in the fluid media at intervals of 2 to 3 days and less often on antibiotic-free serum-agar.

Conventional microscopy. Serum-bouillon cultures were examined with a bright-field phase-contrast microscope at a magnification of 1,000 times. Cultures regularly used were 1 and 2 days old; 3-day-old cultures were used on occasion. A cover slip was placed over a drop of culture on a well-cleaned slide and was sealed with high-viscosity immersion oil to reduce streaming and prevent drying. Serum-agar plates were inspected in an inverted position by direct light at a magnification of 100 times.

Negative staining procedure. Drops of media containing the cells in suspension were placed on Formvar membrane-covered 300-mesh copper grids. After 15 sec, the excess liquid was drawn off by touching the side of the grid with filter paper. Immediately after, a drop of 2% sodium phosphotungstate solution (*p*H 7.0) was applied to the grid and the excess liquid was drawn off. The grids were air-dried and examined in the Siemens Elmiskop I electron microscope at an instrumental magnification of 20,000.

For the thin-section procedure, the cells were fixed in suspension with 1% glutaraldehyde, buffered to *p*H 7.3 with 0.067 M phosphate buffer for 30 min. The cells were then centrifuged into a pellet, rinsed with buffer, and postfixed with 1% osmic acid in 0.067 M phosphate buffer (*p*H 7.3), following which they were dehydrated, embedded in Epon in the conventional manner, and sectioned with a Porter-Blum microtome.

RESULTS

General cultural characteristics. The two species of mycoplasma produced a very slight diffuse turbidity in serum-bouillon on initial transfer. The degree of turbidity was not increased by incubation beyond the 3rd day, but noticeably increased on continued subculture. The 215th transfer of an earlier strain of *M. pulmonis* resembled a well-diluted culture of *Escherichia coli*. The turbidity of *M. neurolyticum* was regularly somewhat greater on transfer than was that of the catarrhal organisms.

Each of the three *Mycoplasma* species produced a distinctive growth on serum-agar. The colonies of the S strain of *M. pulmonis* were delicate, in some instances dew-drop, but more commonly with vacuoles, striations, and fine granules. Those of the B strain were uniformly granular with the granules extending to the margin. The colonies of *M. neurolyticum* were also granular, but the granules were coarser and tended towards a radial arrangement. After the 4th or 5th day of incubation, the colony differences were less obvious. It is recognized that colony appearances of the same species may vary with the source of the medium. The above characteristics were constant with different lots of our media.

The two strains of *M. pulmonis* were highly pleomorphic on examination by phase microscopy. In early transfers the morphological elements varied widely in shape and size. Straight and curved rods, pear-shaped forms, drum-stick and dumbbell forms, smooth spheres, and spheres with a single projecting spicule were commonly seen. Small aggregates were also present but were not numerous. *M. neurolyticum* was much less pleomorphic and showed chiefly spherical forms of varying size. Some were discrete and others aggregated. A few were joined by a slender filament. True bacillary forms were not present.

Motility. Motile elements in fluid cultures of PPLO were seemingly first observed by Andrewes and Welsh (1946). Their organism, presumably a strain of *M. pulmonis*, was isolated from the pneumonic lungs of a mouse. Flagella were not

demonstrated, but some rods showed a passing wave of thickening while in motion. Motility was detected through the sixth subculture but was permanently lost in later transfers. Their observations were made by dark-field illumination.

Elements with true motility were regularly observed at 24 and 48 hr in the early transfers of our two strains of *M. pulmonis*. They were less readily found at 72 hr. Straight rods and drumstick forms, termed gliders, moved rapidly with frequent reversal of direction. Curved forms moved in an orbit. Spheres with a marking projection, termed spinners, rotated constantly but without reversal. The number of motile elements varied considerably from field to field, but was usually less than the stationary forms or ones showing only Brownian movement. Motile organisms were never observed at any time interval in cultures of *M. neurolyticum*.

Motility and also pleomorphism decreased with continued subculture of *M. pulmonis*. The S strain showed no motile elements after the 15th transfer and the B strain after the 9th. Bacillary forms were much less numerous in the later transfers and coccoid elements predominated. Aggregates were more numerous than in the early transfers. *M. neurolyticum* showed no significant changes on continual subculture.

Electron microscopy of M. pulmonis. At the electron-microscopic level, negligible differences were found between strains B and S. Spherical and filamentous forms were encountered in each, with variations of these basic forms being slightly more prevalent in strain B.

The organisms depicted in Fig. 1 were from a 48-hr culture of strain B which had been transferred four times. The spherical cells have diameters ranging from 650 to 800 mμ (average, 730 mμ). They appear to be disc-shaped and to possess a concentrated peripheral layer of cytoplasm. The cells in the lower part of the figure have tail-like extensions. As if to compensate for this distortion, the diameter of the head portion of the cells is found to be reduced to about 500 mμ. When the tail-like extension exceeds 1 μ in length, the cells have a drum-stick appearance. In the central areas of the cells, membranous elements are present. From the cell on the lower left of the figure, a new daughter cell has apparently emerged. This immature cell has three peripheral masses connected by an annular element which is concentric with the limiting membrane. The circular outline of the point of attachment of the daughter to the parent cell is also visible.

The filamentous cell shown in Fig. 2a was derived also from a 48-hr culture of the B strain, which had been four times transferred. This typical filamentous cell is about 2.7 μ in length and ranges from 80 to 250 mμ in width. A noteworthy feature is the short, detached tubular structure (m) with its cylindrical array of ribosomelike granules, 150 A in diameter. Fragments of membrane with more granules attached are apparent in the upper part of the figure; these are probably derived from a disrupted cell, as is also the membrane fragment studded with closely packed ribosomelike particles, which is shown in the inset at lower right of the figure. The terminal bulbous outpouching of the organism shows a granule (g) towards its center, and the faint outline of peripherally distributed material. The outpouching arises from a dense terminal bud (b), with which it is aligned in an axis at right angles to that of the proximal part of the organism. This is also apparent in Fig. 2b, in which, possibly, an earlier stage of the process is shown. Here, the structural elements of the pouch are apparently condensed. Figure 3a shows two immature cells. These apparently represent the outcome of what now appears to be a germination process, two early stages of which were depicted in Fig. 2b and 2a. In Fig. 3a, the internally situated granules are larger, the inside aspect of the limiting membrane is more dense (whiter shade), and granular areas make their appearance within the cells. The thick protrusions on the left side of the cells could conceivably become more filamentous as maturation proceeds.

The microtubules, about 60 mμ in diameter, shown in Fig. 3b and 3c are similar to that shown in Fig. 4, which appears to be comparatively undeveloped with regard to internal structure. The microtubule shown in Fig. 3b appears to have become detached from its parent body and shows evidence of granulation (arrow) along its limiting membrane; toward its distal end, strands of material appear to lie in the lumen. The microtubule in Fig. 4 has its origin in a circular body, about 120 mμ in diameter, which has a second circular element (diameter, 80 mμ) within, both with orifices. The appearance of the orifice of the outer element suggests a circular array of closely packed granules.

Cells derived from a 48-hr culture of strain S that had been transferred 204 times are shown in Fig. 5a and 5b. On the left side of Fig. 5a, the terminal elongated structure shows evidence of segregation and segmentation. In the wide part of the cell, there is evidence of the presence of what is possibly the cell's nuclear equivalent; this is much more in evidence in Fig. 5b, where it appears as an oval-shaped, disclike body, 160 by 110 mμ, containing fine concentrically arranged rings (arrow). It is surrounded in part by a horseshoe-shaped element 25 mμ thick.

Aspects of cellular morphology observed in

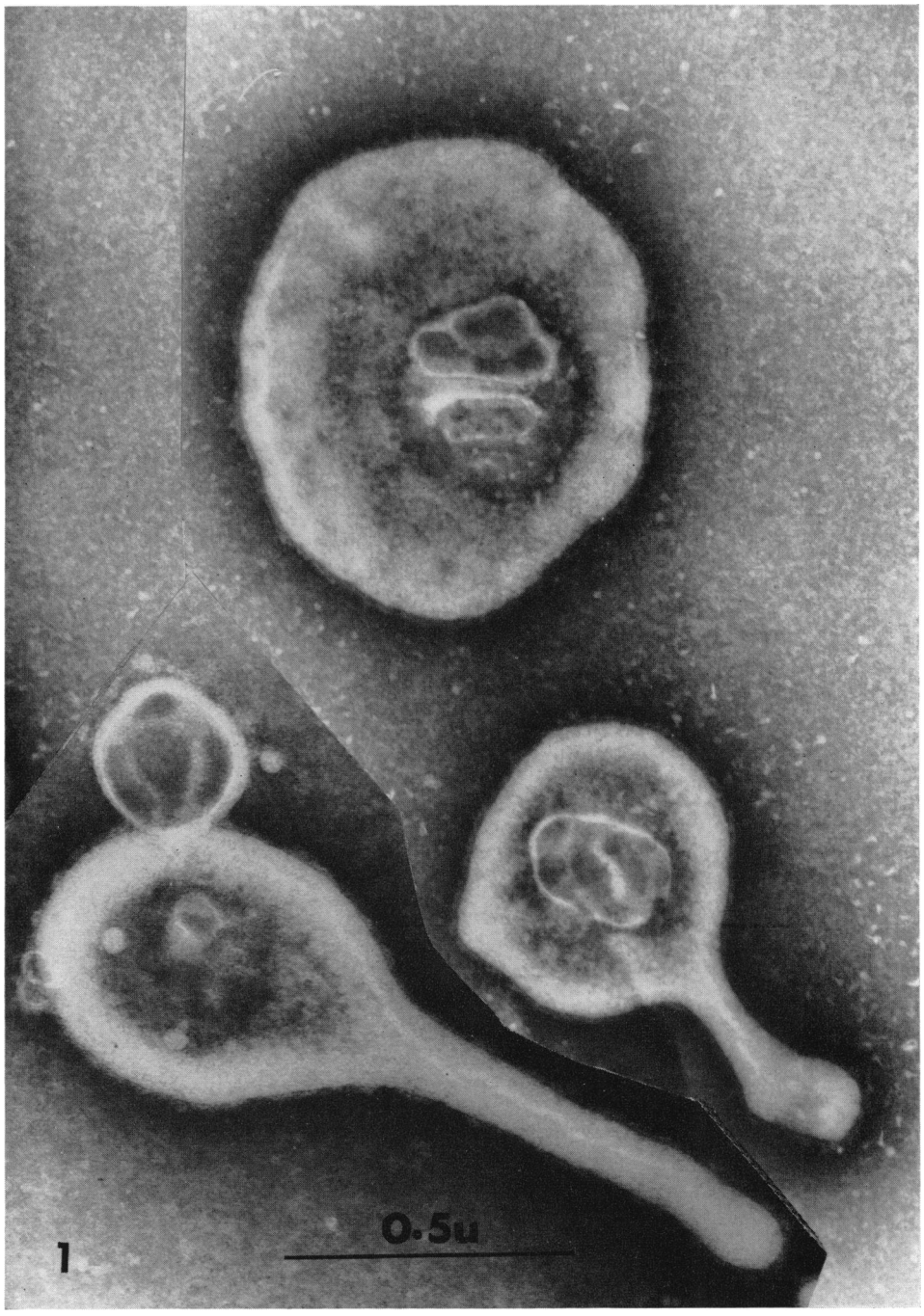


FIG. 1. *Three cells from a 48-hr culture of Mycoplasma pulmonis (B strain), two of which (lower part of figure) have tail-like extensions. Little significant fine structure is discernible in the peripherally distributed cytoplasm. A new immature cell has apparently emerged from the central area of the cell on lower left of figure. The central areas of the other two cells contain membranous structures which may represent stages in the formation of new progeny cells.* × *80,000.*

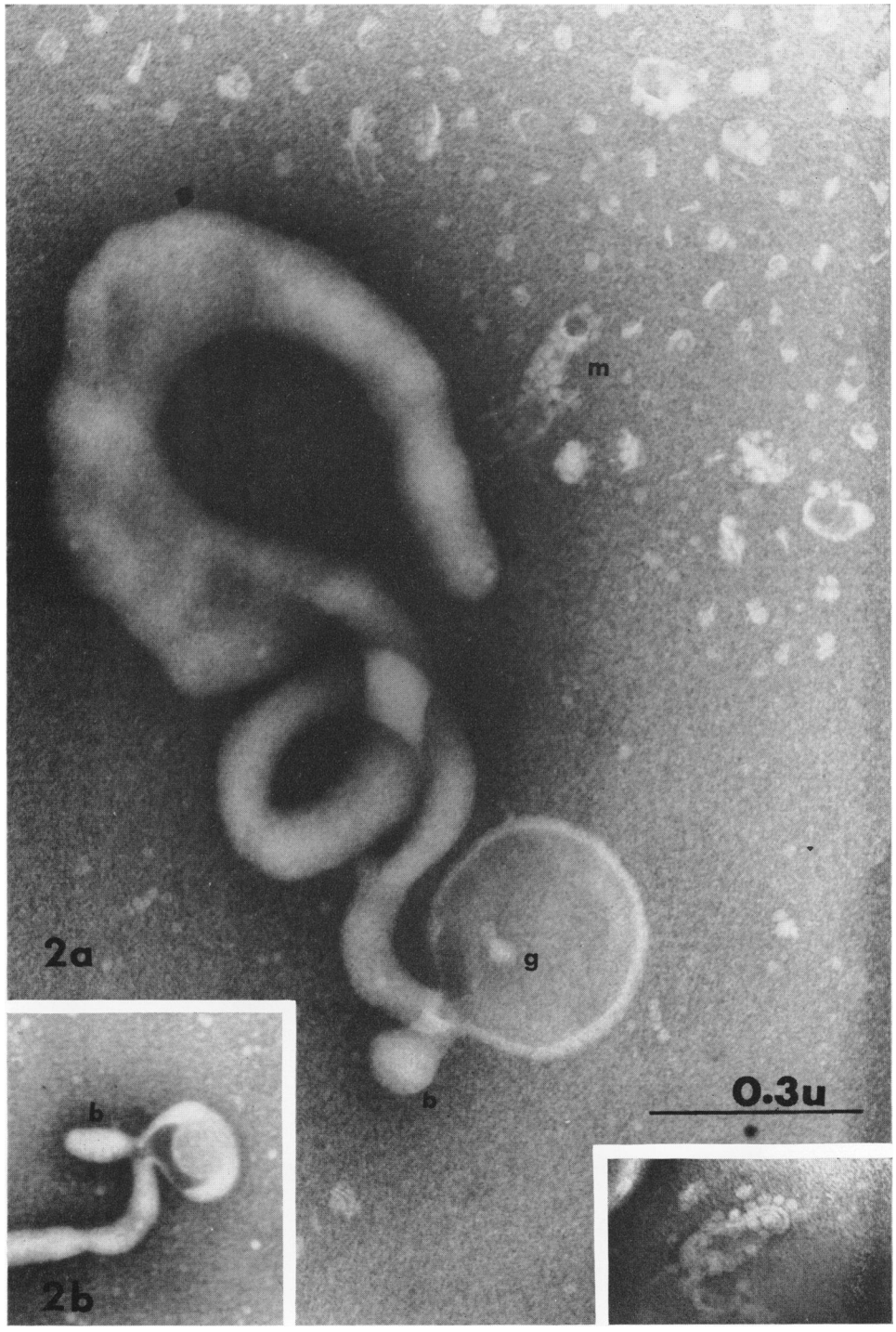


FIG. 2a. *Serpentine cell from 48-hr culture of Mycoplasma pulmonis (B strain). Short microtubular structure (m) with ribosomelike granules attached lies in close proximity to cell. Similar granules, some attached to fragments of membrane, are distributed in field. In the inset on lower right of figure, membranous fragment appears studded with closely packed granules. The terminal outpouching, with centrally located granule (g), has its origin in the terminal bulb (b).*

FIG. 2b. *Portion of another cell with terminal bulb and outpouching which appears condensed relative to that in Fig. 2a, and which possibly represents an earlier stage in development.* × *100,000.*

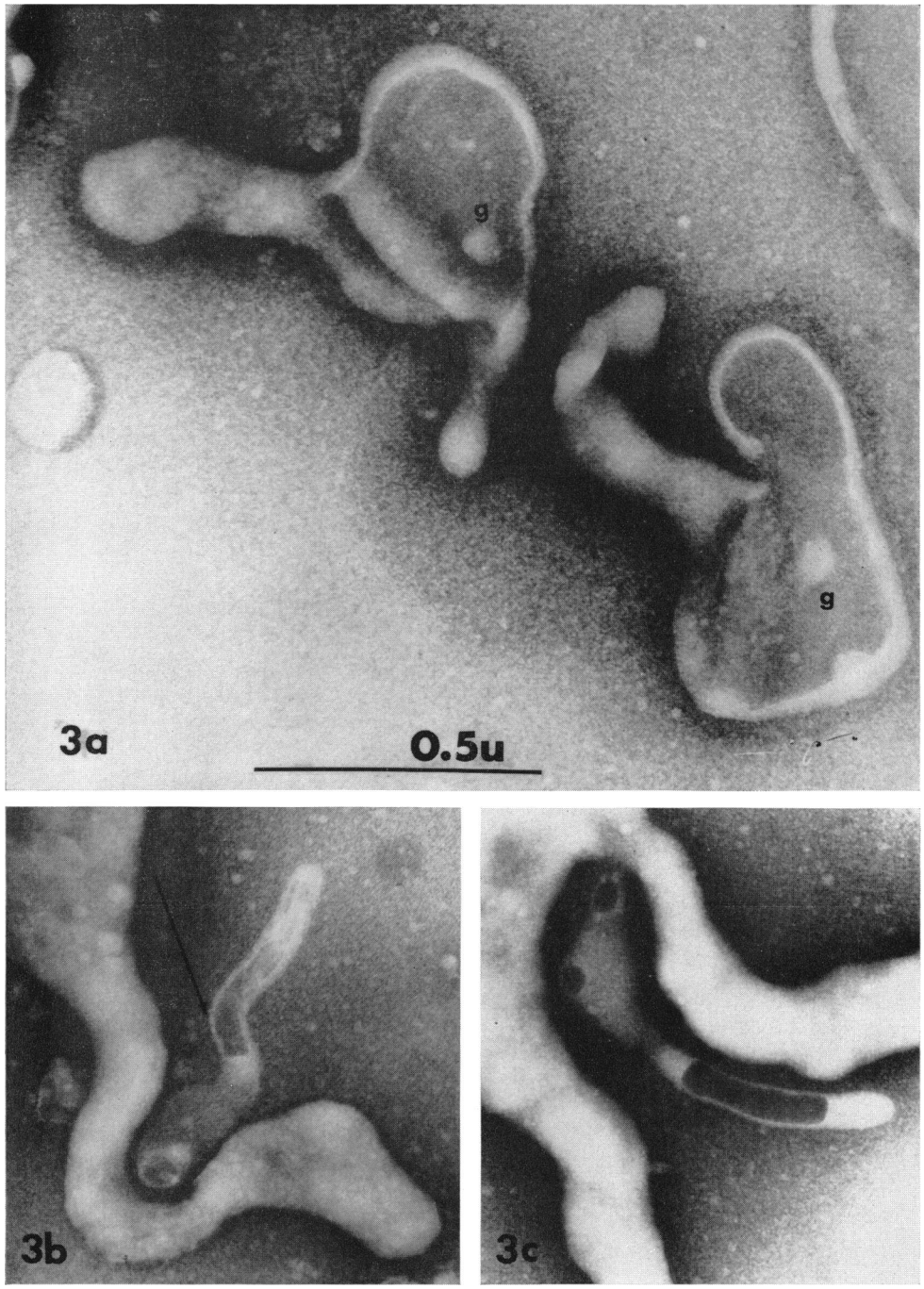


FIG. 3a. *Two cells, possibly immature, from a 48-hr culture of Mycoplasma pulmonis (B strain), which may have resulted from a process of development involving terminal outpouching of which two earlier stages were presented in Fig. 2b and 2a, consecutively. Granules (g) are apparent within the cells.*

FIG. 3b and 3c. *Two microtubules associated with filamentous cells of Mycoplasma pulmonis from 48-hr cultures. The proximal parts are distended. The microtubule in 3b, which is detached from its parent cell, shows evidence of globular subunits in its limiting membrane (arrow), and fibrous material appears to lie in the lumen. Two orifices are apparent in the proximal region of the microtubule in 3c.* × *80,000.*

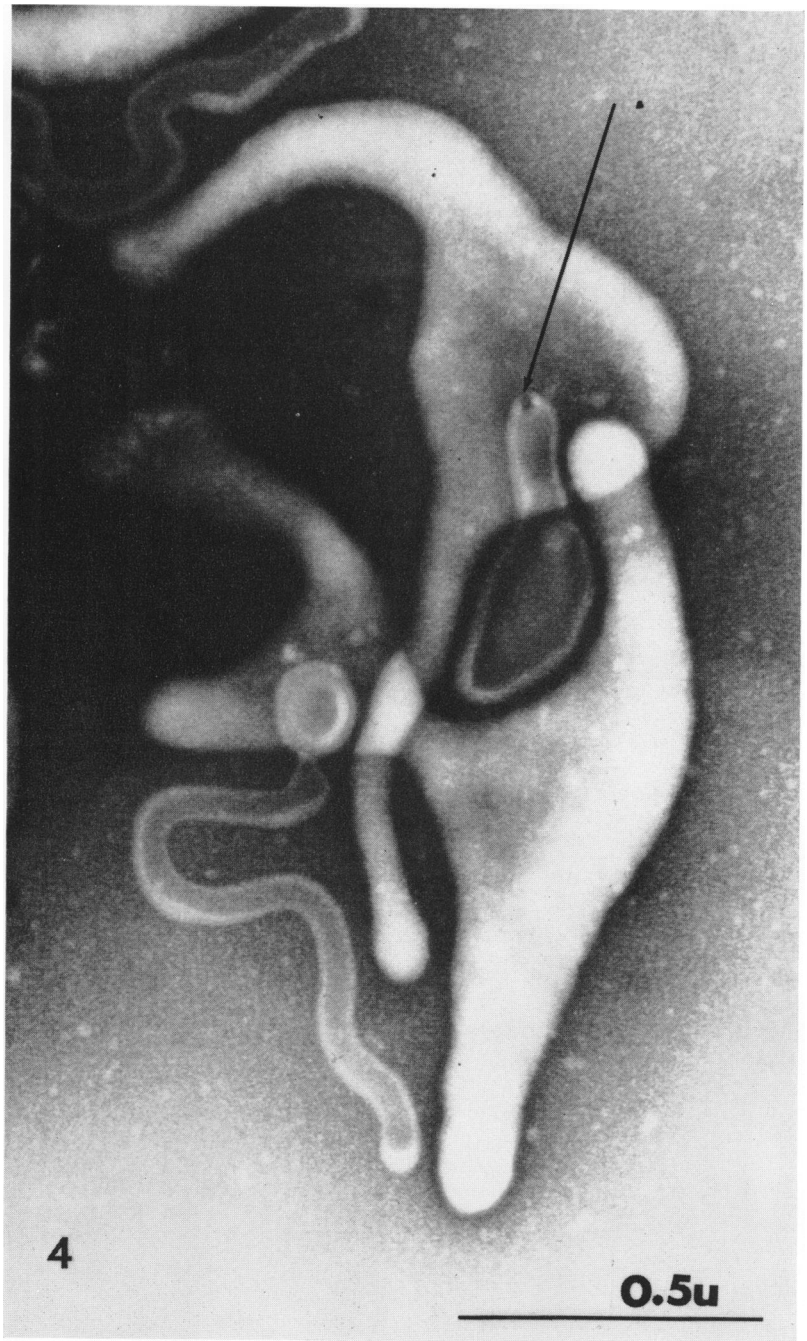
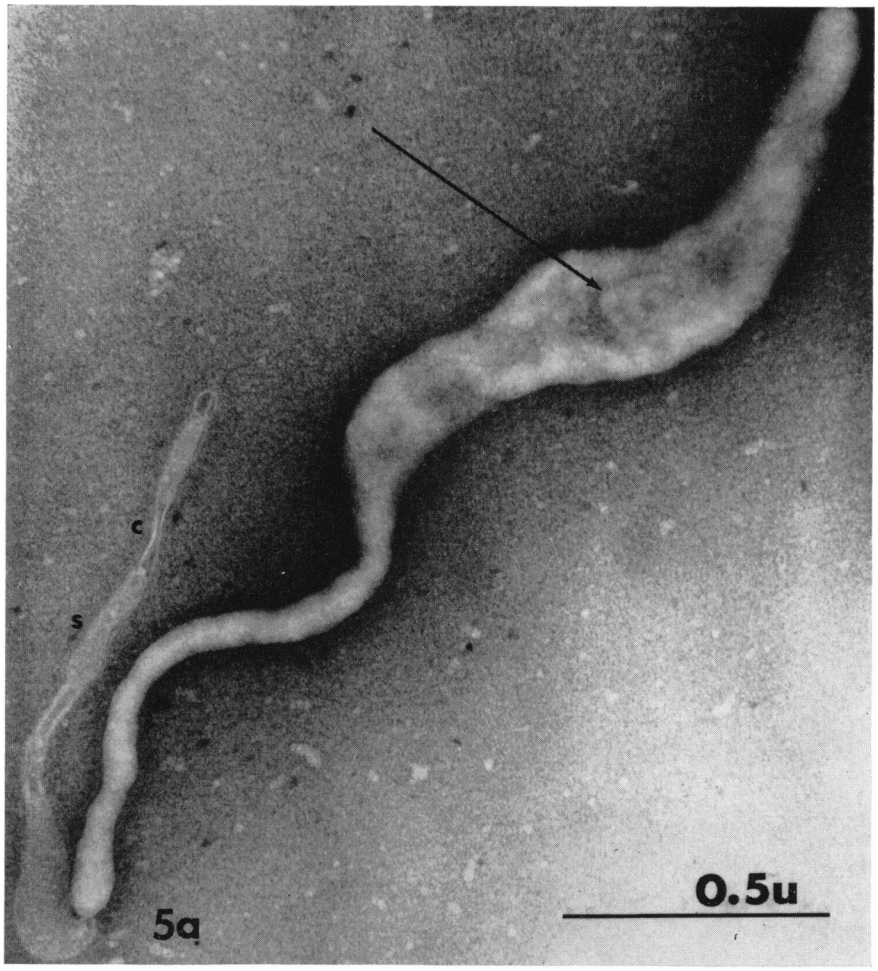


FIG. 4. *Two cells from a 48-hr culture of Mycoplasma pulmonis overlap at two areas. At the upper left, part of another cell with an associated microtubule is apparent. Associated with the lower cell is a microtubule which has its origin in a structure composed of two concentric circular elements which show evidence of possessing granular subunits. At the origin of the pouchlike structure associated with the upper cell, a small pore is observed (arrow); the inner contents of the pouch are outlined faintly.* × *80,000.*



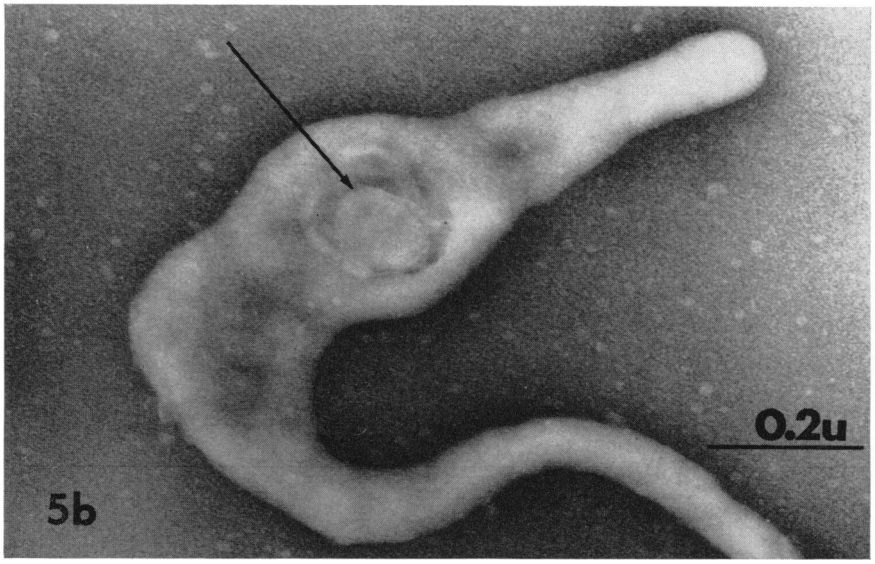


FIG. 5a. *Filamentous cell from a 48-hr culture of Mycoplasma pulmonis (S strain). The tubular structure which is attached to the lower end of cell shows segregation of a component into four discrete masses (s). A constriction is observed (c) which suggests that segmentation may be occurring. The outline of a complex inclusion body which may represent the cell's nuclear equivalent is arrowed.* × *70,000.*

FIG. 5b. *Part of another organism from the same culture is shown. Here the complex inclusion body is more obvious. Around the periphery of the oval-shaped component (which is of a size range with the classical elementary bodies), fine filaments appear to be wound (arrow).* × *100,000.*

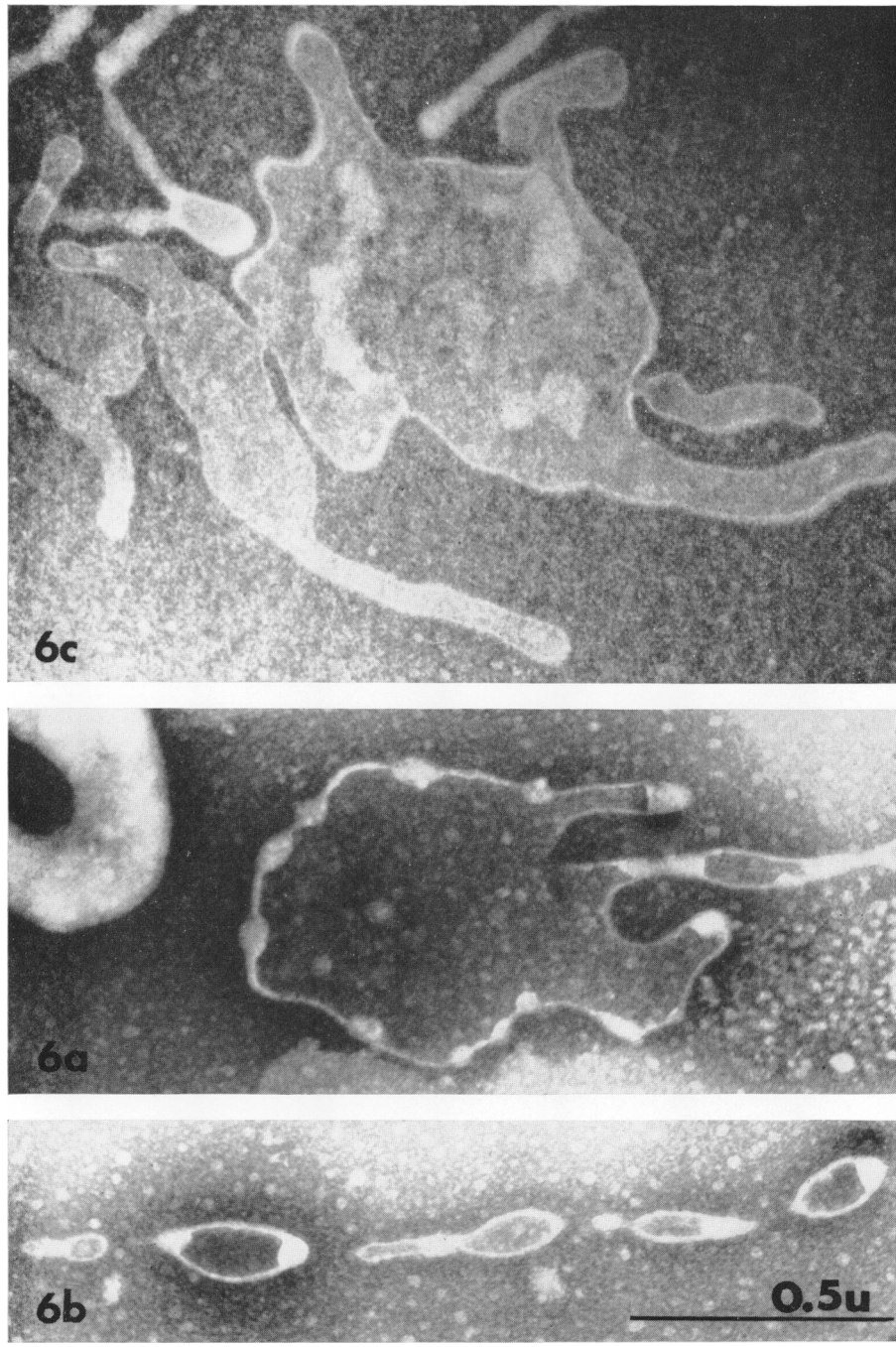


FIG. 6a. *Pleomorphic cell frequently observed in old (72 hr) cultures of Mycoplasma pulmonis. Cell appears void of internal components; the limiting membrane is nodular. Elongated process at right of figure appears to be segmenting. Figure 6b, with its row of separated particles, depicts the continuation of this elongated process. Figure 6c shows a less commonly encountered pleomorphic cell from a young culture of M. pulmonis. Clumps of material are randomly distributed within the cytoplasm. The limiting membrane is without nodular deposits.* × *80,000.*

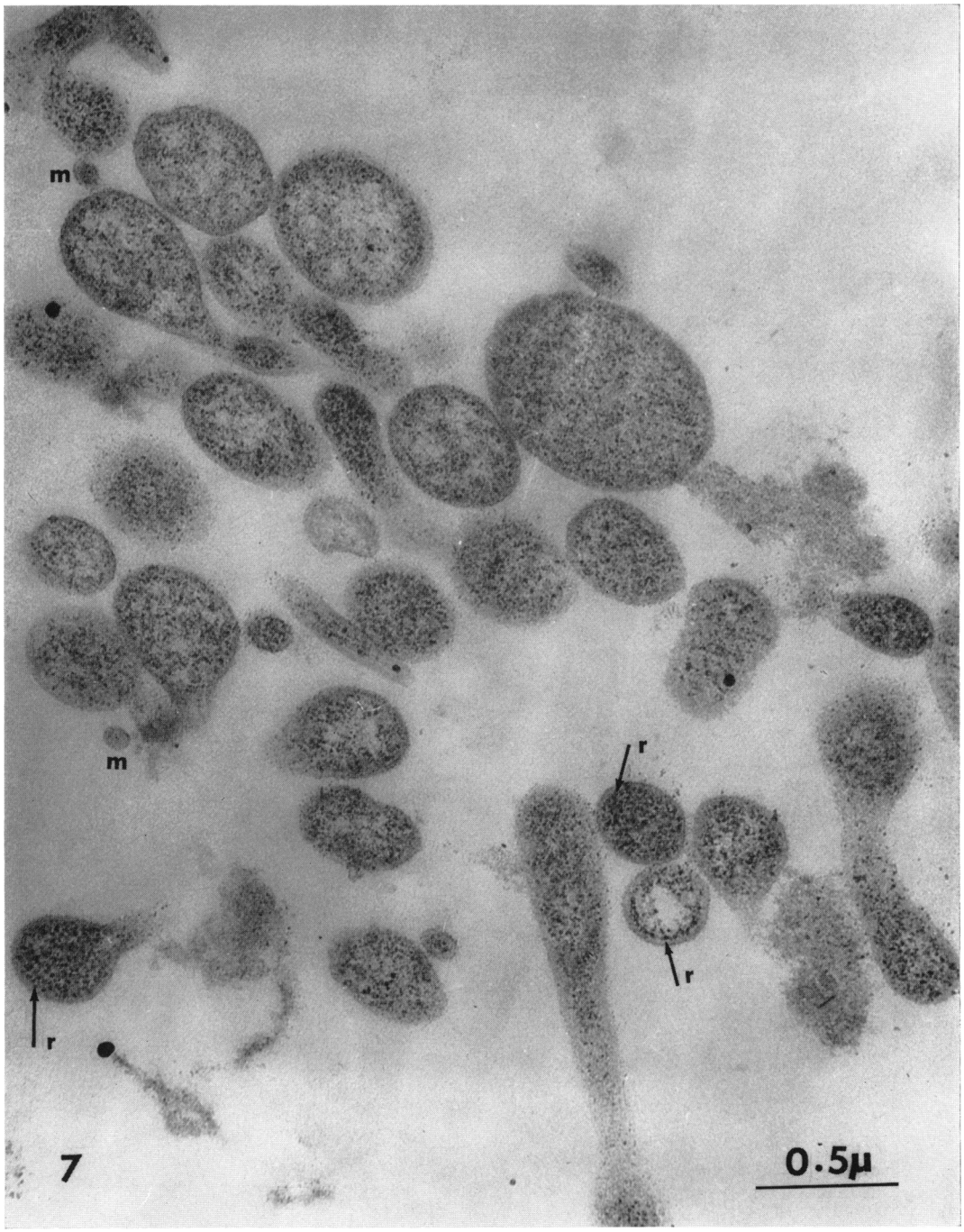


FIG. 7. *Fixed thin section through pellet of Mycoplasma pulmonis cells derived from a 48-hr culture. Both spherical and filamentous cells are present. Many cells appear to have tail-like extensions. There are no obvious nuclear masses within cells, but arrays of ribosomelike particles are in evidence (r). Small structures (m) are in the size range of the microtubules in transverse section.* × *42,000.*

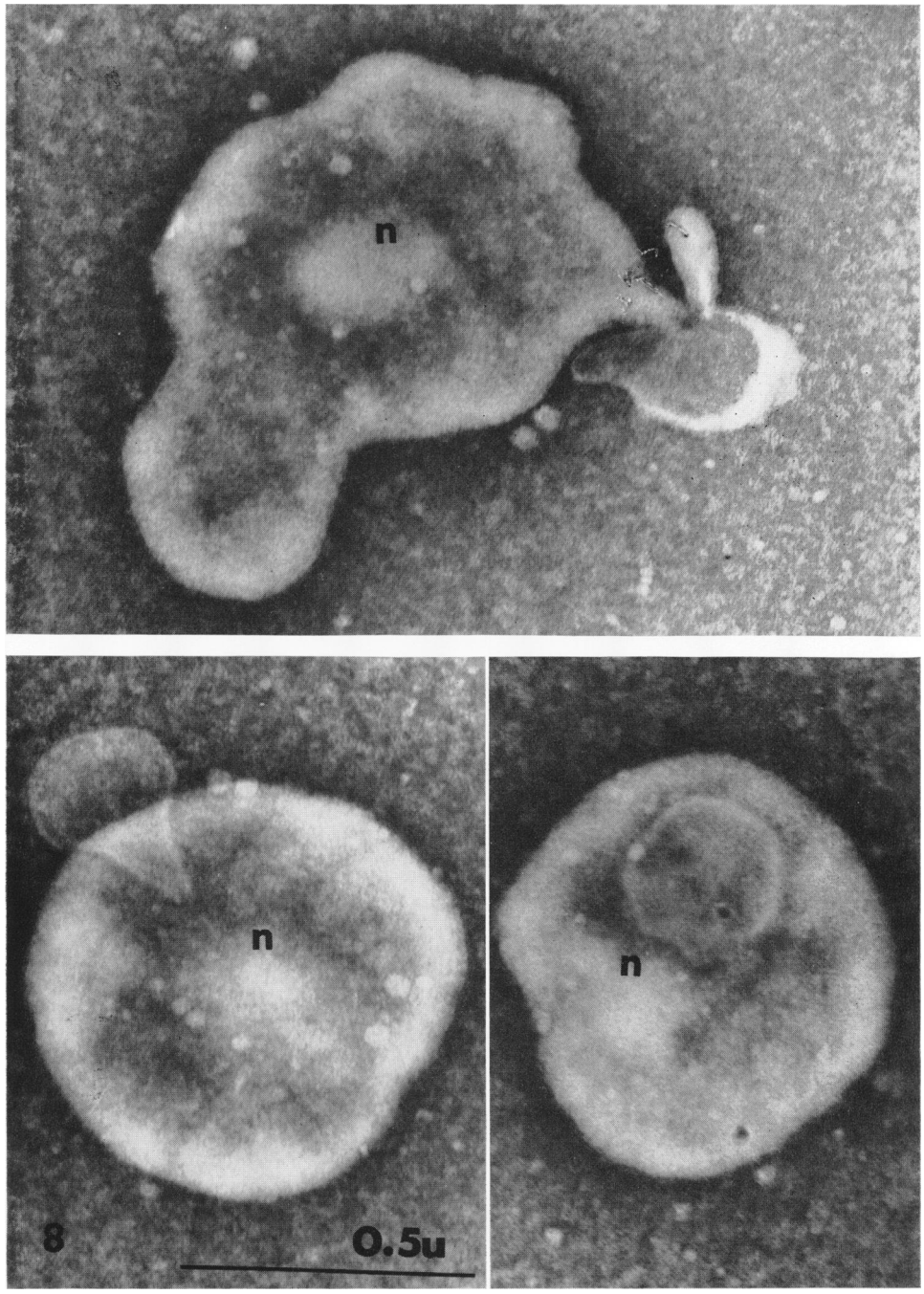


FIG. 8. *Three cells from a 48-hr culture of Mycoplasma neurolyticum, showing different aspects of cellular outpouching. The cells have a nonuniform peripheral layer of cytoplasm and a "nuclear" mass towards the cell center (n).*

72-hr cultures of strain S are presented in Fig. 6a. The flat pleomorphic cell was typical of those found in such older cultures. There appears to be little evidence of cytoplasm, but small aggregates, 30 to 50 mμ across, are laid down at the limiting membrane, giving it a nodular appearance. The cell has an elongated process (at right of figure) which appears to be segmenting into a number of oval-shaped or elongated membranous sacs, each containing one or two of the dense aggregates. Figure 6b represents an extension of Fig. 6a. A similarly pleomorphic cell from a younger culture (48 hr) of strain S is shown in Fig. 6c, with which the older, more degenerate cell may be compared. The younger cell shows clumps of aggregated material within cytoplasm, and the limiting membrane is free from nodular deposits.

A thin section representing a typical field of cells from a 48-hr culture of *M. pulmonis* strain S is shown in Fig. 7. Many of the cells appear elongated, possibly tubular, and consist of a bulbous head with a narrower tail-like extension—the latter feature only suggested in some instances due to the angle of cutting of the section. The cells show little sign of vacuolization. Arrays of densely stained ribosomelike granules are found located on the inside of the limiting membrane in cells at the lower right (r). The relatively small structures (m) that appear in close association with a number of cells may represent microtubules in transverse section.

In none of the cells examined in either of the strains was there any indication of flagella or any specialized structure that could account for the observed motility.

Electron microscopy of M. neurolyticum. Figure 8 depicts three cells which were found to be characteristic of a 48-hr culture of this species. The cells were generally spherical in appearance and had an average diameter of 0.7 μ, with a range of 0.65 to 0.75 μ. They possessed a concentrated peripheral layer of cytoplasm in which no fine structure could be distinguished with certainty. Nearer the center of the cells lay amorphous masses, 100 to 200 mμ in diameter, which may represent the cells' nuclear equivalent. Three different aspects of cellular outpouchings are apparent.

DISCUSSION

During the past 15 years, we have examined numerous rats and mice from outside colonies suspected of harboring the agents of chronic respiratory disease. *M. pulmonis* was usually recovered from the lungs, middle ears, or nasal passages of rats which snuffled and mice which chattered. The few exceptions were animals infected only with the virus of enzootic bronchiectasis (Nelson, 1963). Mycoplasmas from any given colony regularly showed uniform morphological and colonial characteristics, but ones from unrelated colonies tended to vary somewhat in these respects. Six different strains could be so distinguished. The S and B strains used here are illustrative of the colonial differences. All six strains were highly pleomorphic in horse serum-bouillon. Motility was demonstrable with five strains but was not observed in one, despite the presence of similar bacilliform elements. *M. neurolyticum* was rarely encountered in nature, and only three strains were isolated over an extended period. The three isolated showed similar characteristics and were nonmotile.

The mechanism of motility in cultures of *M. pulmonis* is still uncertain and its purpose is unknown. The absence of flagella was noted by Andrewes and Welch (1946) and was confirmed by our observations with the electron microscope. They suggested a kinship with myxobacters, which resemble mycoplasmas in their movement on solid surfaces and their lack of a restraining cell wall. Alternate waves of contraction and expansion might account for the gliding motion of bacilliform elements in the mycoplasma cultures but not for the spinning of coccoid forms.

The electron micrographs here presented were selected to demonstrate various structural features that were regularly encountered in the many hundreds of organisms examined. Not included were abnormal forms which accounted for less than 5% of the total number of cells examined.

By use of the negative-staining procedure, it was possible to distinguish one species of *Mycoplasma* from another, and to distinguish the surprisingly uniform-sized spherical cells of *M. neurolyticum*, with their centrally located nuclear mass, from the spherical forms exhibited by *M. pulmonis*, which did not appear to possess this prominent feature. As indicated by phase-contrast microscopy and by negative-contrast and thin-section electron microscopy, numerous filamentous organisms were demonstrable in 48-hr cultures of *M. pulmonis*; that they represent a significant morphological form of this species can be confidently asserted. They were not observed in *M. neurolyticum*.

Care was taken to minimize artifacts during specimen preparation; centrifugation was avoided, and the osmotic environment of the organisms was maintained up to the time of application of the layer of phosphotungstate solution and the air-drying of the specimens. It is, nevertheless, possible that elongation of the flexible filamentous cells occurs on drying, such that, for purposes of characterization, the overall length of the organisms may be of less importance than the

fact that they possess a relatively distended or broad area, which gives them a serpentine appearance when this is located toward one end. The broad part of the cell is sometimes found to contain an oval disclike inclusion in the size range of the classical elementary bodies (Fig. 5b). Sometimes outpouchings arise which may assume the form of microtubules (Fig. 4). These tubular elements, while they may vary in length from cell to cell, have a uniform diameter of about 60 mμ. They often appear to be modified as if undergoing degenerative change. The short microtubule arrowed in Fig. 2a appears to have a cylindrical array of ribosomelike granules. The identification of these remains conjectural, especially in view of the known instability of unfixed ribosomes in phosphotungstate (Huxley and Zubay, 1960). The granulation observed in the limiting membrane of the microtubules (e.g., Fig. 3b) was suggestive of the presence of globular lipid micelles, such as are observed in cholesterol-rich lipoidal structures (Lucy and Glauert, 1964). No intermediate stages were found to suggest that the microtubules develop into new cells, although this is believed to be a possibility.

The evidence for reproduction by a terminal budding process, although still scanty, was found to be relatively stronger, as the successive stages depicted in Fig. 2b, 2a, and 3a indicate. It is hardly necessary to point out the inadequacy of attempting to describe a dynamic process by means of a series of micrographs from which a pattern of development may be barely discernible.

In older cultures of *M. pulmonis*, i.e., those sampled at 72 hr, large pleomorphic cells were found to predominate. One such cell is shown in Fig. 6a. This appears to lack the recognizable internal elements present in the equally pleomorphic, possibly abnormal or involuted, cell from a 48-hr culture (Fig. 6c). The limiting membrane of the older cell is nodular, and at one point a tubule, with nodular deposits at intervals along its length, shows evidence of segmentation. From this segmenting tubule was derived the array of oval membrane-bounded particles (Fig. 6b). It is possible that the cell is undergoing multipolar germination and that the separated particles represent one form of the classical "minimal reproductive units." An electron micrograph of a similar cell from a 96-hr culture of *M. salivarium* was included in a recent paper by Domermuth et al. (1964). It appears probable that the generation of new daughter cells may be accomplished in different ways, depending on the physiological age of the cultures. A similar observation has been made regarding the species *M. hominis* by Anderson and Barile (1965).

Quite apart from the considerable interest that attaches to *Mycoplasma* species from the phylogenetic viewpoint, as the smallest of free-living organisms, additional interest obviously accrues to those species that infect the mouse, the most widely used of experimental animals. Since their presence may influence in various unrecognized ways experimental findings, the problem of their identification in biological preparations of murine origin assumes importance. In the solution of this problem, the observations contained in the present paper may find application.

ACKNOWLEDGMENTS

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