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Morphology, Ultrastructure, and Bacteriophage Infection of the Helical Mycoplasma-Like Organism (*Spiroplasma citri* gen. nov., sp. nov.) Cultured from "Stubborn" Disease of Citrus

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The mycoplasma-like organism *Spiroplasma citri* gen. nov., sp. nov., isolated from citrus infected with "Stubborn" disease and carried in serial cultures in several media, was examined by dark-field microscopy and electron microscopy of negatively-stained and shadowed preparations and of sections. It grows as motile, helical filaments in liquid, but as nonmotile, nonhelical filaments and round bodies in agar cultures. Helicity and motility are lost in old broth cultures and upon addition of a variety of negative stains, fixatives, and other solutions. No organelles accounting for motility are present, but a layer of surface projections is present on the surface of the single, bounding membrane. The mycoplasma produces a tailed, type B bacteriophage which appear to attach to the outer layer. Helical filaments are preserved in ammonium molybdate, but not in sodium phosphotungstate, and by fixation in Formalin or glutaraldehyde made up in medium, but not by osmium nor by glutaraldehyde in cacodylate buffer. This mycoplasma appears similar to the noncultured helical microorganism in corn stunt-diseased tissues and is probably a representative of a new group of mycoplasmas which are in possession of surface projections, rotary motility, and bacteriophage infection.

Mycoplasma-like bodies, seen by electron microscopy of sections, have been reported in diseased tissues of higher plants and in insect vectors (12, 13, 22-24, 32, 41, 44, 45), as well as in sections of material from primary cultures from corn stunt disease (8) and from passaged cultures originating from "Stubborn" disease of citrus (41). Examination by negative staining methods seems to have been restricted to diseased tissues or primary cultures of corn stunt agent (8, 13, 22) and showed mycoplasma-like bodies that were sometimes round, but predominantly filamentous. Recently, phase microscopy revealed helical filaments in juice from corn stunt-infected plants (13). The helical nature was not verified by negative staining but was suggested in occasional sections and freeze-etch preparations examined by electron microscopy. There are no reports of examination of cultured corn stunt agent by optical microscopy and no information obtained by other methods that suggests a helical nature of the organisms in such cultures. Neither are there reports of optical microscopy or of electron microscopy of preparations, other than sections, on other plant mycoplasmas.

We have examined, by both optical and electron microscopy, multi-passaged strains of mycoplasmas cultured (41) from Stubborn disease of citrus. These microorganisms, now classified as *Spiroplasma citri* gen. nov., sp. nov. (P. Saglio et al., Int. J. Syst. Bacteriol., in press), occur as motile helical filaments in liquid (but not in agar) cultures and are infected by a tailed bacteriophage. Details of morphology and ultrastructure are presented.

MATERIALS AND METHODS

Mycoplasmas. Strains Morocco (Maroc R8A2) and California (California 189), isolated from Stubborn disease and passed 21 times in his medium, were supplied by J. M. Bové (41). Observations on both strains were similar. For the most part, descriptions

and photographs in this paper were made on the Morocco strain.

Media and culture methods. Compositions and details are presented elsewhere (P. Saglio et al., in press). For the most part, we employed the sorbitol-containing complete medium (SMC; reference 41). Other media were horse serum-containing medium (HS) and medium containing 1% serum fraction (SF).

Optical microscopy. Observations and photographs were made with a Zeiss Universal microscope by using phase or dark-field optics. The dark-field photographs were made at shutter speeds of 1 to 2 s on Kodak Tri-X Pan film rated at EI 1000; or at shutter speeds of 3 to 5 sec on Kodak Panatomic-X film rated at EI 200. These were developed for 4 min or 2.5 min, respectively, at 68 F (20 C) in Acufine (Baumann Photochemical Co., Chicago). Very thin cover-slip preparations, unsealed and often allowed to dry partially, were required for successful photographs.

Electron microscopy. Negative stains employed were 2 and 4% sodium phosphotungstate (PTA; pH 7.0) or ammonium molybdate (AMB; pH 7.1–7.4) in concentrations of 2, 3, and 6%. Drops of broth cultures or their concentrated sediments (fresh or fixed), or agar-grown colonies minced in broth were placed on Formvar-coated copper grids for 1 min. After removal of excess fluid by touching to filter paper, the wet residues were immediately covered with the stain for 10 to 20 s which was then withdrawn in the same fashion, and the grid was air-dried before examination.

In preparation for sectioning, we first employed primary fixation in 2.5% glutaraldehyde (final concentration) made up in cacodylate buffer as previously described (37). Following two washes in sucrose-phosphate buffer, secondary fixation was in 1% osmium tetroxide in Veronal-acetate buffer, followed by treatments with uranyl acetate and embedment in Epon 812 as described (37). This procedure failed to preserve helices, and we subsequently used concentrations of either 20% Formalin or 6.7% glutaraldehyde made up in SMC, which were then added to equal volumes of cultures in SMC to give final concentrations of 10 and 3.35%, respectively. Fixation was for 2 to 4 h at room temperature followed by two washes in the Veronal-acetate buffer, pH 7.2. Secondary fixation was in 1% osmium tetroxide in the Veronal buffer overnight at room temperature, with subsequent treatments and embedment as described previously (37).

Measurement of osmolalities. Osmolar concentrations were measured by the freezing-point method, using a model 68-3L osmometer (Advanced Instruments, Inc., Needham Heights, Mass.).

Digitonin. In some experiments, concentrations of digitonin of 30 μg/ml, or greater, (which were found to be growth-inhibitory [P. Saglio et al., in press]) were employed before electron microscopy examination of mycoplasmas.

RESULTS

Optical microscopy. In SMC cultures examined between 1 and 2 days after inoculation, short, helical filaments and small, round bodies were seen by dark-field microscopy. Between 2 and 4 days, the numbers and lengths of helical filaments increased. Such filaments appeared relatively straight and moderately rigid (Fig. 1a–d). Filament lengths usually ranged from 2 to 4 μm but could be much longer in older cultures. Cultures of this age usually represented the logarithmic phase of growth, as determined by count of colony-forming units (P. Saglio et al., in press), but the time of attainment varied by a day or two according to conditions and the medium used. In post-log cultures, the helical filaments reached maximal length, and there was increasing aggregation of helices extending radially from central masses of poorly-defined bodies (Fig. 1e–h). This phenomenon was always present to some extent, but the numbers and lengths of the filaments, as well as the size of the aggregates, increased with time. As incubation continued beyond 6 days, the elongated filaments showed a progressive loss of helicity and motility, there was increasing fragmentation and distortion, and many round or irregular bodies of moderate size appeared. This appearance is better seen in negatively stained preparation by electron microscopy (see Fig. 9). However, some nonhelical filaments with bright "blebs," or small nonfilamentous bodies, could be seen in cultures of all ages (arrows, Fig. 1a, b). Identical observations were made by phase microscopy but are not shown here in photographs.

The helical filaments showed two types of motility. One was an apparent rapid rotary or "screw" motion which could reverse and lead to a minimal back-and-forth progress as in some spirochaetes. Differentiation between rotation of a helix and a flat, wave form as described for *Treponema pallidum* (11) was not attempted, but electron microscope evidence presented below indicates that a true helix was present. The second motion seen was a slow undulation and bending of filaments, sometimes with a slight rotary component, but not leading to change of position of the filament. As determined by electron microscopy (see below), the organisms possesses no flagella, axial filaments, or other organelles that might account for its motility.

The helical filaments in living preparations could be disrupted by pressure on the cover slip into shorter filaments, irregular forms, and small bodies. In drying preparations, or when filaments became closely adherent to the cover slip, motion was lost and the amplitude and "tightness" of helices decreased. Addition to

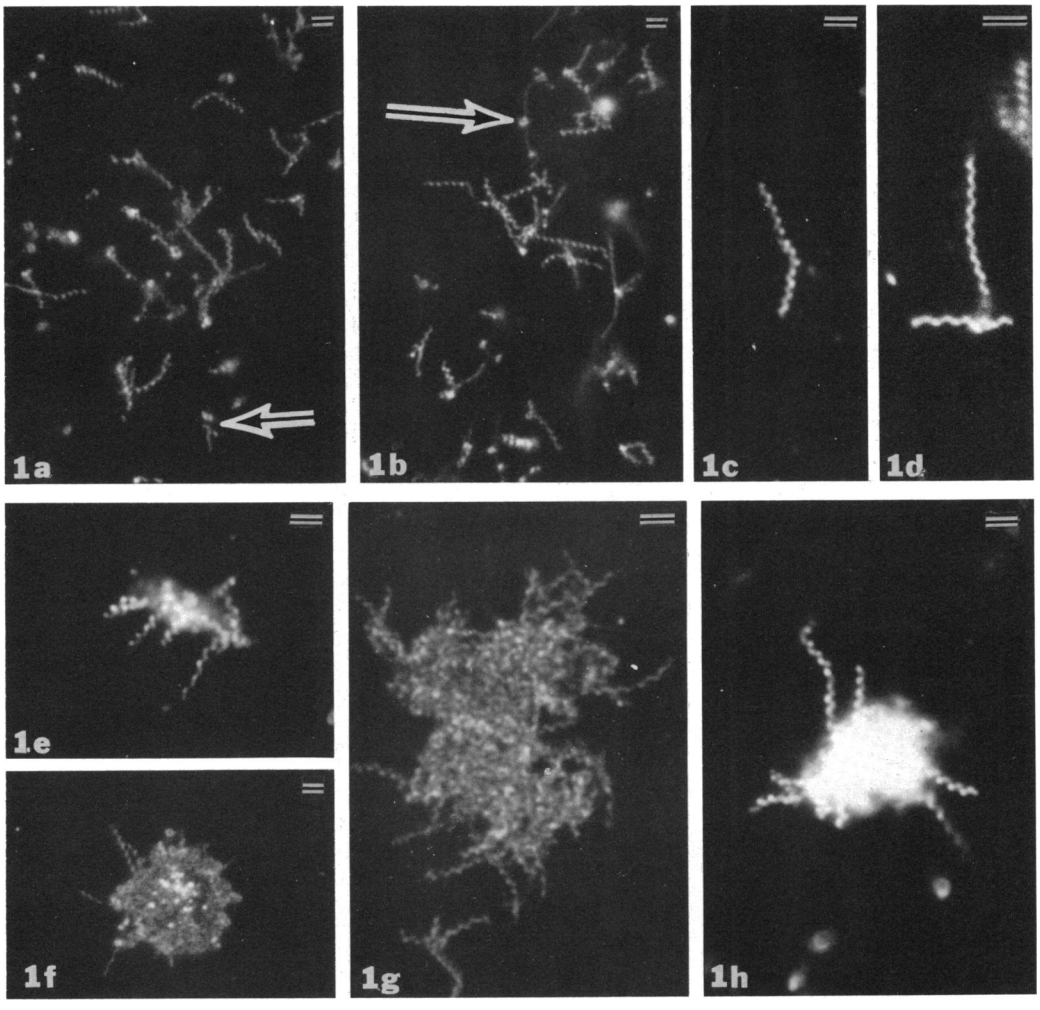

FIG. 1. *Dark-field appearance of Morocco strain showing helical filaments. (a and b) Log-phase (2-day) culture in SMC. Arrows indicate occasional nonhelical filaments with blebs and nonfilamentous bodies; ×2,800. (c and d) Individual helical filaments: (c) ×4,400, fixed in glutaraldehyde (d) ×6,000, unfixed. (e, f, g, and h) Examples of aggregates in older cultures: ×4,400, fixed; ×2,800, unfixed; ×4,400, fixed; and ×4,400, fixed, respectively. Bar, 1.0 μm.*

wet mounts, or to cultures, of glutaraldehyde fixative in cacodylate buffer, ammonium acetate (1%, pH 2), PTA (2 and 4%, pH 7.0), sucrose up to 85%, sucrose-phosphate buffer (pH 7.2), and deionized water all caused loss of helicity, shortening and distortion of filaments, and the appearance of small bodies, in varying degrees. Helical filaments were preserved in AMB (especially in 6%), and in either glutaraldehyde (final concentration 3.35%) or Formalin (final concentration 10%) added directly to the medium without buffers or made up in the medium before addition. Subsequent osmication did not alter the helices, although osmium alone caused immediate disruption of all elements, as did digitonin when added to living cultures.

In preparations made by mincing agar growth (colonies) in SMC, helices were never seen; only rounded bodies and nonhelical filaments occurred.

Electron microscopy: negative staining. The 2% PTA, which was used initially, showed nonhelical filaments with bulbous regions, or filaments apparently originating from round bulbous bodies, in log-phase cultures in SMC (Fig. 2a). The appearance was identical to that shown by the same technique by Chen and Granados (8) for the corn stunt mycoplasma. Branching of filaments was present. In old

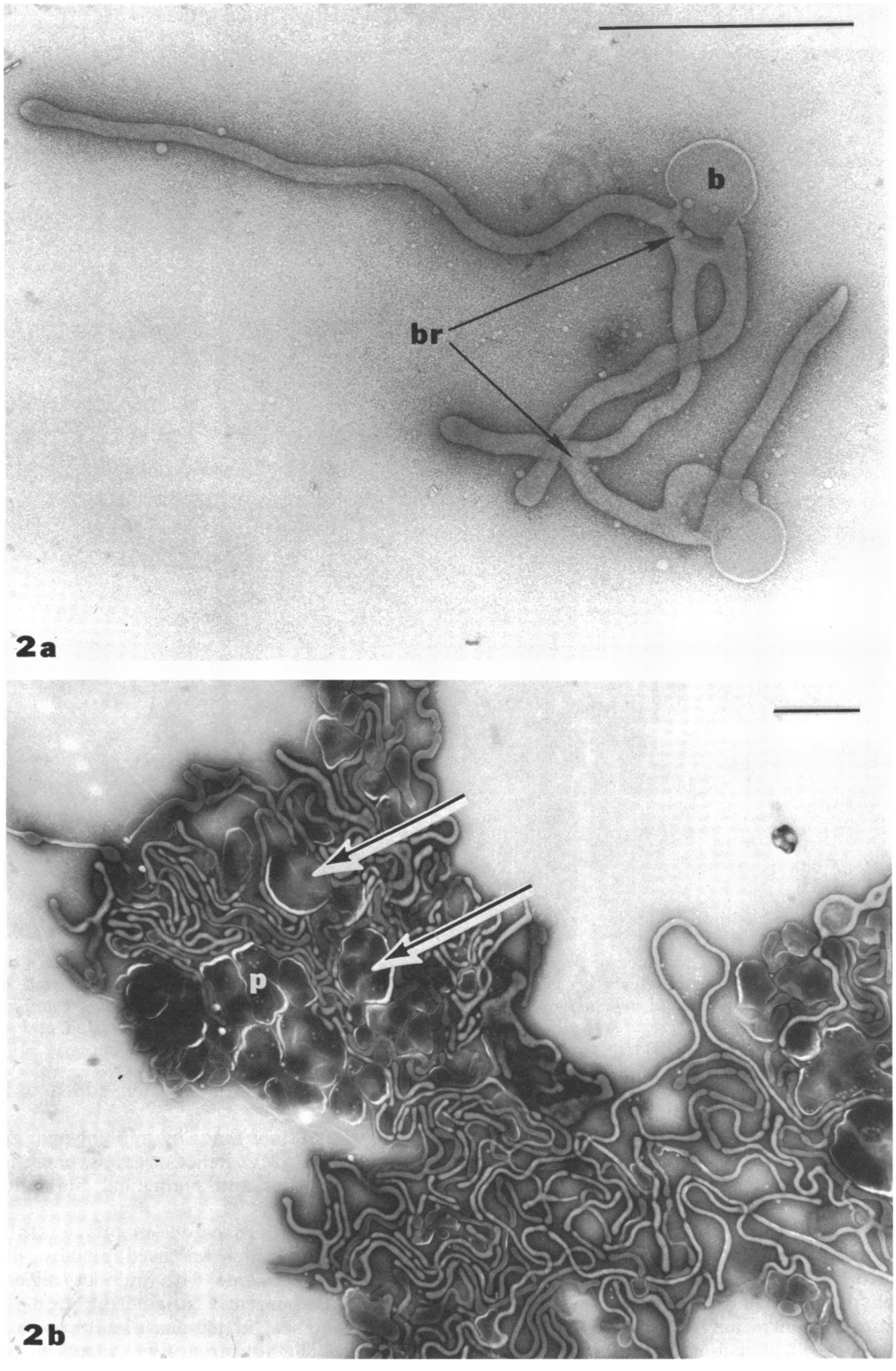

FIG. 2. *Electron micrographs of PTA-stained preparations of Morocco strain. (a) Two-day culture in SMC. Note "blebs" or "main bodies" (b) and branching (br); 2% PTA, ×37,400. (b) Seven-day culture. Note long filaments and (arrows) rounded or flattened bodies. The small bright bodies (p) are bacteriophage heads; 4% PTA, ×12,600. Bar, 1.0 μm.*

cultures (7 days), more round and flattened bodies (arrows, Fig. 2b) were seen, as had been also noted in aging primary cultures from corn stunt disease (8). In addition, the filaments seen were very long, distorted, and more easily flattened and ruptured (Fig. 2b).

However, examination at higher magnification and resolution showed features not described in the corn stunt mycoplasma. Most obviously, a "classic" tailed bacteriophage was common in the preparations, especially in the Morocco strain. The complete phage was seen free in clusters, or attached to filaments, but was most commonly found attached to large bodies (Fig. 3a). In these latter, an even more common feature was the presence of incomplete phage heads (Fig. 3b), although they were also seen occasionally within filaments. Similar intracellular empty heads have been reported in coliphage T5 (47). Rare instances of liberated mixtures of incomplete heads, tails, some complete phage, and probably polyhead tubules (15, 27, 43) were seen (Fig. 4a). The phage head was usually hexagonal in profile (Fig. 3a) and is probably an octahedron (5); it measured 47 to 50 nm in greatest diameter. Incomplete and empty heads, which appeared spherical, were only 40 to 44 nm in diameter. Individual capsomeres comprising polyhead tubules were approximately 7.4 to 8.0 nm wide with a central hole of approximately 4 nm in diameter (Fig. 4b); the dimensions are similar to those described in polyhead tubules of coliphage T4 (27). The phage tail was unsheathed, apparently noncontractile, and showed 20 to 25 cross-striations. It measured 75 to 85 nm in length and 8 nm wide; a slightly wider base plate was present from which an undefined number of short filaments, or spikes, appeared to originate (Fig. 4c, 5).

The phage was seen in the original cultures passed 21 times in SMC. Subcultures at the Laboratory of Microbiology, Bethesda, Md., in the same medium continued to show abundant phage for about 10 passages. The strains were then cloned and 50 more passages were made; subcultures to other media (HS, SF) were also initiated. In the later passages, phage cannot now be consistently detected by electron microscopy of negatively stained, pelleted mycoplasma or of culture supernatant fluids, and preliminary attempts at induction with mitomycin C have been unsuccessful. However, a rare section shows phage heads within filaments. Phage is still found abundantly in the original material and in early subcultures therefrom.

Negative staining revealed, in addition to phage, a delicate outer layer, or "nap," consisting of short projections which often appeared periodic and to which the phage appeared to attach (Fig. 4c, 5). It was best seen on flattened organisms and measured approximately 5 to 7 nm in width. In disrupting filaments from aging cultures, a protoplasmic cylinder extending free of encompassing layers and maintaining the filamentous shape was often seen (Fig. 6a). In some preparations, the outer nap and next innermost layer (assumed to be membrane) were missing in some regions of the filaments, and a periodic layer apparently surrounding the cytoplasm was seen (Fig. 6b). A similar separation was not uncommon at bends in filaments (Fig. 6c) and in the empty broken filaments which resulted from treatment with growth-inhibiting concentrations of digitonin (Fig. 6d).

On large, round or irregular bodies, a "barred" periodicity of units of approximately 4 nm in width with a center-to-center distance of approximately 5 nm was often seen in random arrangements (Fig. 7a, c). It was also commonly seen on disrupting filaments in old cultures (Fig. 7b) and on filaments disrupted with digitonin (arrow, Fig. 6d). It was seen by AMB and PTA staining and in cultures apparently free of phage as well as in those obviously phage-infected. The nature of significance of these arrangements is unknown, but in appearance and dimensions they resemble the stacked disks formed under some conditions by lipids or lipoproteins (16, 18). Striated arrangements of somewhat similar appearance, but of different dimensions and always in single long structures, have been recently observed in the Y strain of *Mycoplasma mycoides* by Rodwell (40); their function is also unknown.

Failure to preserve the helices in PTA preparations, which produced only suggestive undulations at best, led to the trial of AMB because of its reported superior preservation of membrane-bounded bodies (33). The use of 2% AMB preserved helices, although not in the numbers seen in wet mounts by dark-field microscopy. Following the recent reemphasis by Lemcke (28) on the importance of isotonicity in fixation of mycoplasmas, we prepared solutions of negative staining compounds and of fixatives calculated to approximate the osmolalities of the media used. Osmolar concentrations of these and of other solutions and media were measured (Table 1). The low osmolality of PTA may explain its failure to preserve helices, whereas the closer approximation of 2% AMB to the osmolality of the media may account for its partial success. Increasing concentration of AMB (3 and 6%) preserved increasing numbers

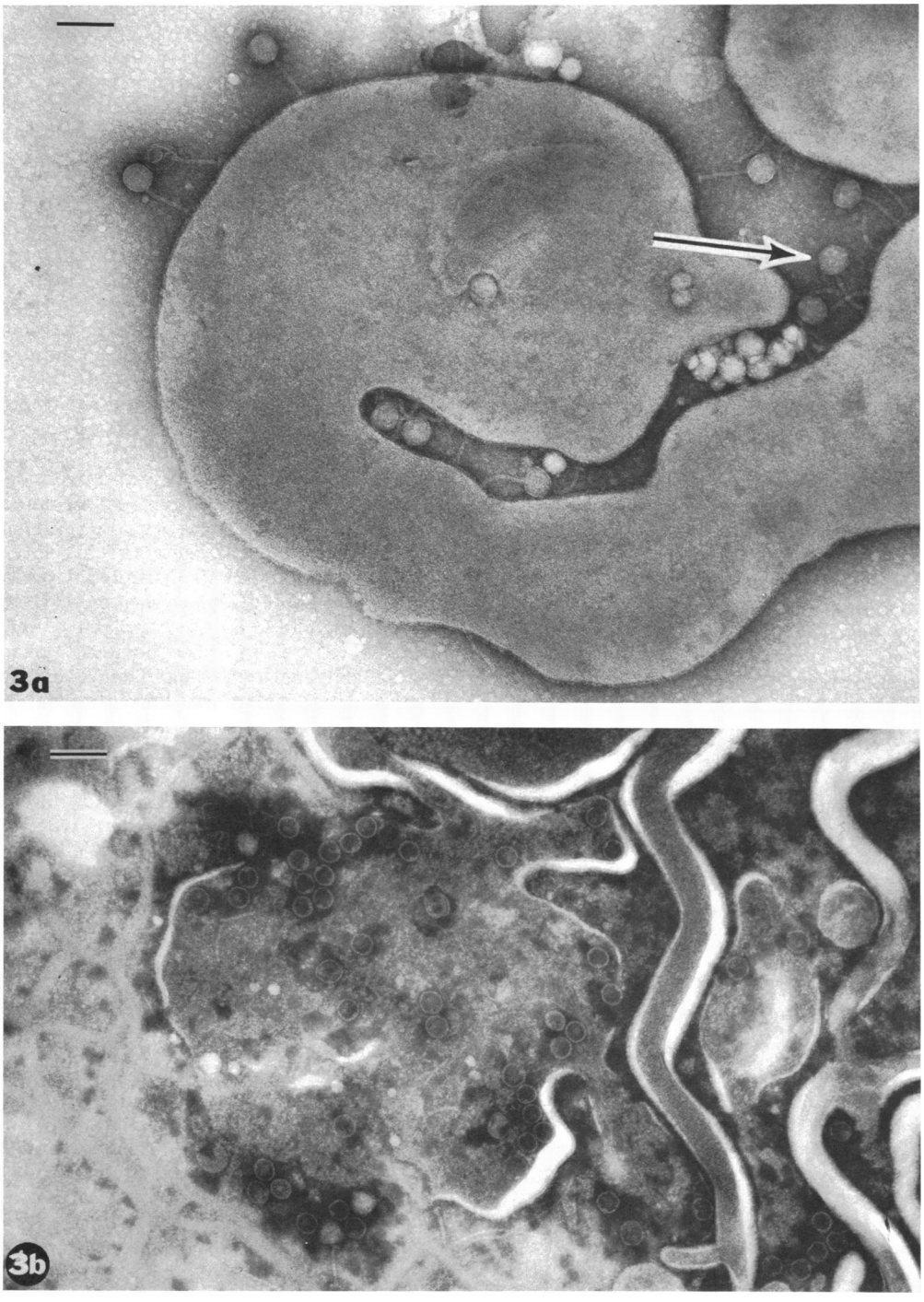

FIG. 3. *Electron micrographs of bacteriophage from Morocco strain negatively stained. (a) Phage attached to large, flattened body. Note hexagonal profile of head (arrow); 2% AMB; ×85,000. Bar = 0.1 μm. (b) Incomplete phage heads within rupturing large body; 4% PTA; ×80,000. Bar, 0.1 μm.*

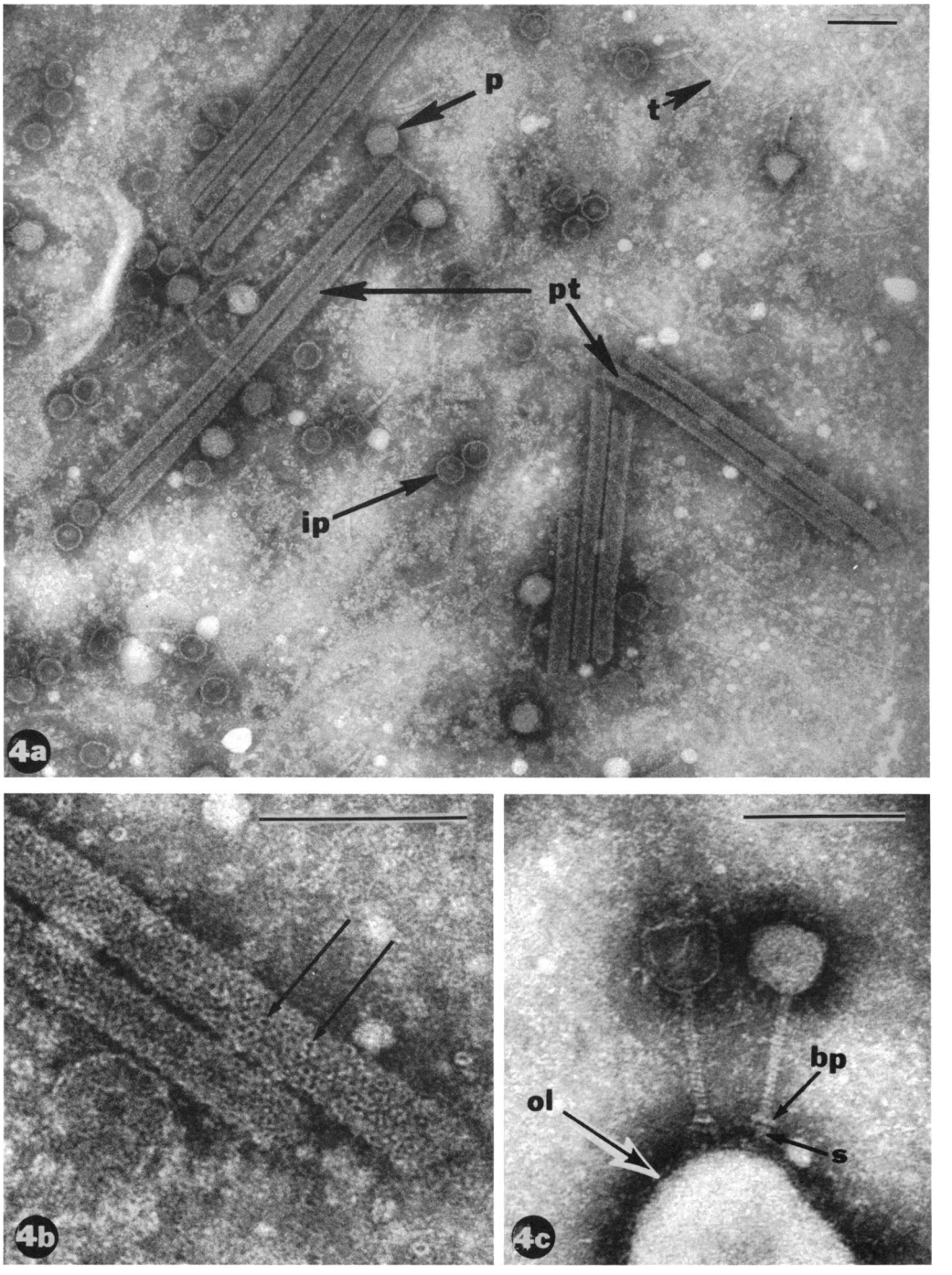

FIG. 4. *Phage from Morocco strain negatively stained. (a) Liberated mixture of incomplete heads (ip), complete phage (p), tails (t), and polyhead tubules (pt); 4% PTA; ×100,000. (b) Detail of polyhead tubule showing individual capsomeres (arrows); 4% PTA; ×300,000. (c) Phage attached to outer layer (ol) of mycoplasma. Note cross-striations of tail, base plate (bp), and short filaments or spikes (s) extending therefrom; 4% PTA; ×231,000. Bar, 0.1 μm.*

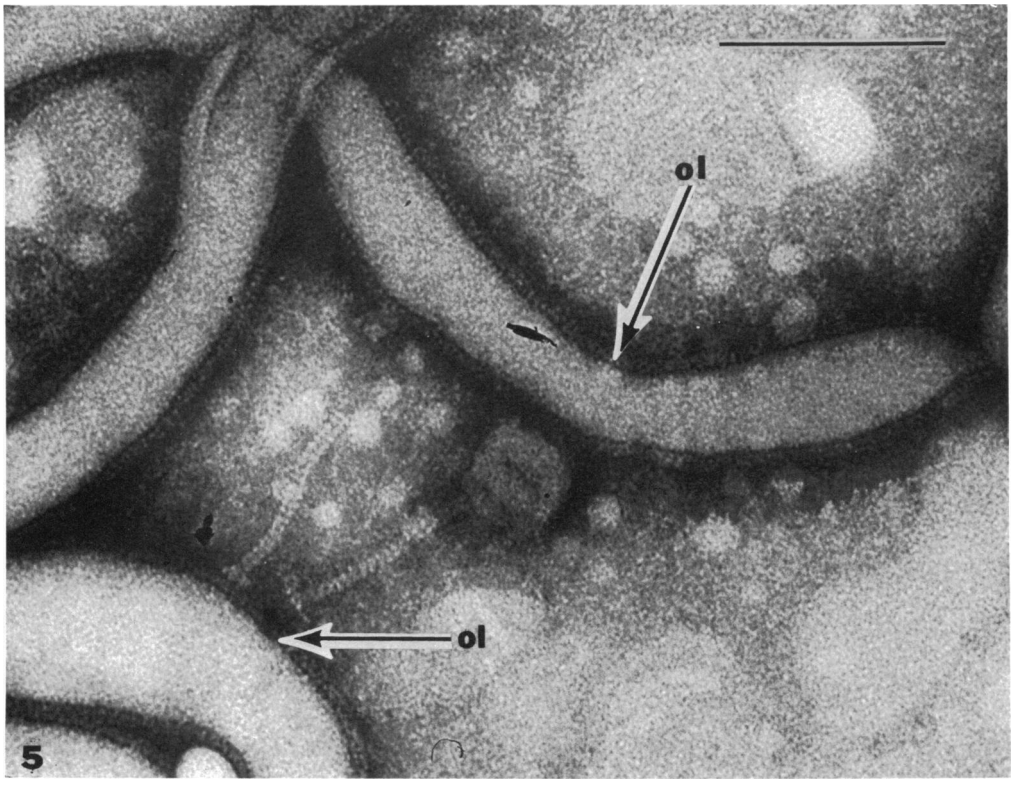

FIG. 5. *Outer layer or "nap" (ol) on filaments of Morocco strain and attached phage; 2% PTA; ×300,000. Bar, 0.1 μm.*

of helices in preparations of cultures from media of the same approximate osmolality, provided that such cultures were in log phase and thus showed maximal numbers of helical filaments by dark-field microscopy. (As discussed below, filaments in old cultures lose helical structure, and post-log cultures are not, therefore, suitable for determining the preservation of helices in negative stains or various fixatives.)

Some results of negative staining with AMB are shown in Fig. 8. Even in the best preparations, all degrees of coiling could be seen, often in different regions of the same filament. The effect appears to be, in part, a physical one, for the best helices were seen in the thickest areas of the preparations and were never seen when not well surrounded by the stain. In AMB, the filament diameters measured from 72 to 100 nm, and the widths of the helices varied from 203 to 333 nm.

A direct comparison of the effects of AMB and PTA on replicate samples from the same culture is shown in Fig. 9a and b; the failure of PTA to preserve helices is clear. In these and the preceding micrographs, it can be seen that the filaments—whether in AMB or PTA and whether helical or not—were almost invariably associated at one or both ends or elsewhere with large bodies or flattened blebs and were often branched. The appearance of "... filaments which emerge from the main body," was also seen in the corn stunt mycoplasma by Chen and Granados (8) and by Davis et al. (13) and, as they also noted, several filaments may apparently emerge from such a "main body."

Effects of culture age on morphology. By use of both dark-field microscopy of wet mounts and electron microscopy of AMB-stained preparations, we examined cultures sequentially from 1 to 14 days of incubation after inoculation. As noted above, filaments lose their helical appearance, become greatly elongated, and irregular or round bodies of various sizes appear as incubation proceeds. In preparations stained with AMB, the trend of these changes is illustrated by comparing Fig. 9a (2-day culture) with Fig. 9c and d (7- and 10-day cultures, respectively).

Agar cultures. Preparations of colonies minced in SMC, regardless of whether negatively stained with PTA or AMB, showed no helical filaments. The appearance was similar

to that shown in Fig. 2b with many round bodies and nonhelical filaments. However, only "mature colonies" grown for 4 to 8 days were examined, and the possibility of some helical forms at early stages is not ruled out.

Electron microscopy: sections. As noted above, the addition to cultures of 1% osmium tetroxide caused immediate lysis as seen by dark-field microscopy. Sections of the mycoplasma so fixed showed only membranes and phage (Fig. 10a). No suggestion of asymmetry of membranes, nor of an outer layer, was seen in these preparations.

After fixation by our usual procedure (2.5% glutaraldehyde in cacodylate buffer, followed by 1% OsO_4 in Veronal buffer), the sections

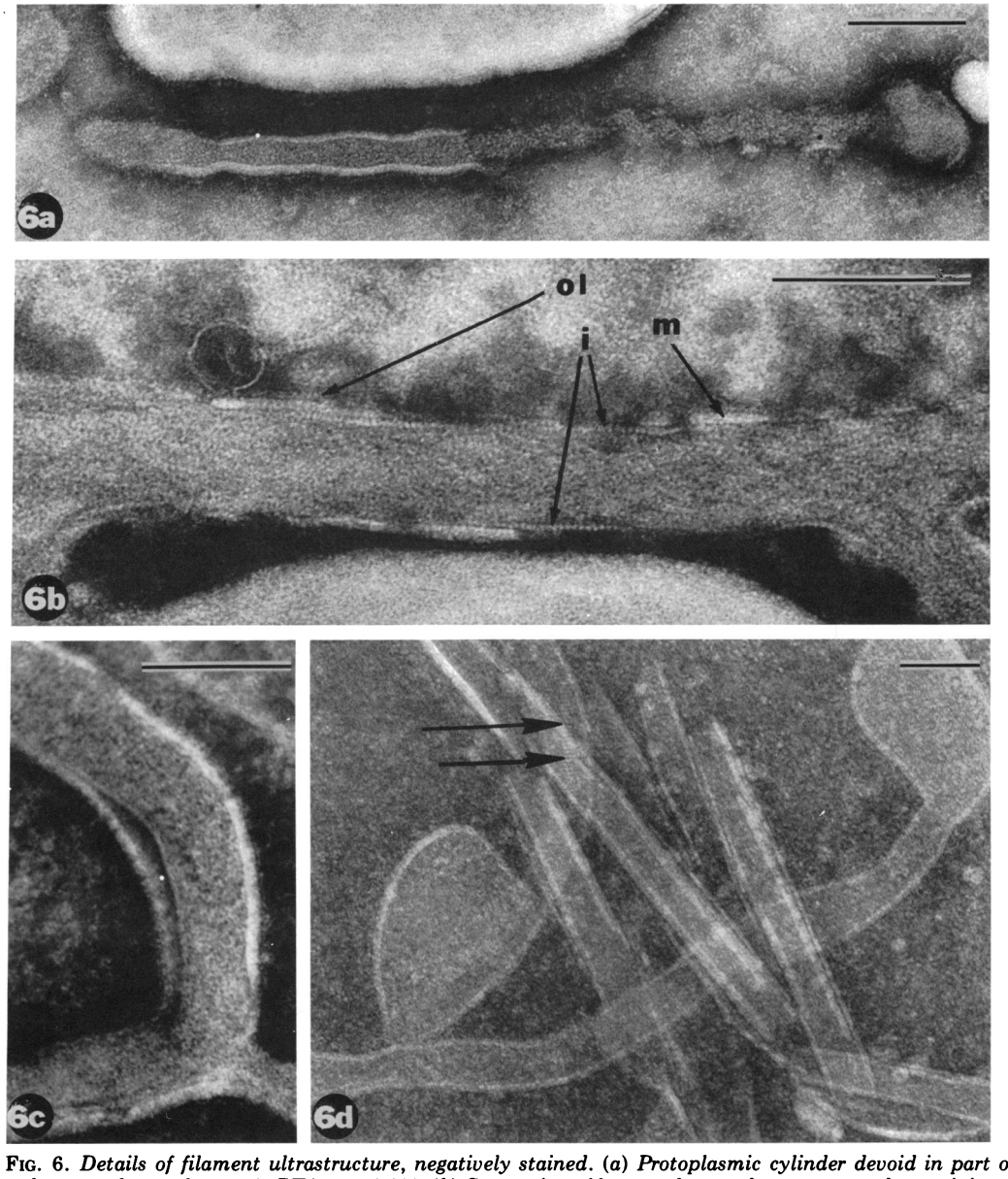

FIG. 6. *Details of filament ultrastructure, negatively stained. (a) Protoplasmic cylinder devoid in part of membrane and outer layer; 4% PTA; ×160,000. (b) Separation of layers; ol, outer layer; m, membrane; i, inner periodic layer; 4% PTA; ×268,000. (c) As in (b); 2% PTA; ×200,000. (d) Preparation after treatment with 30 μg of digitonin per ml; 3% AMB; ×115,000. Note "barred" structures (arrows). Bar, 0.1 μm.*

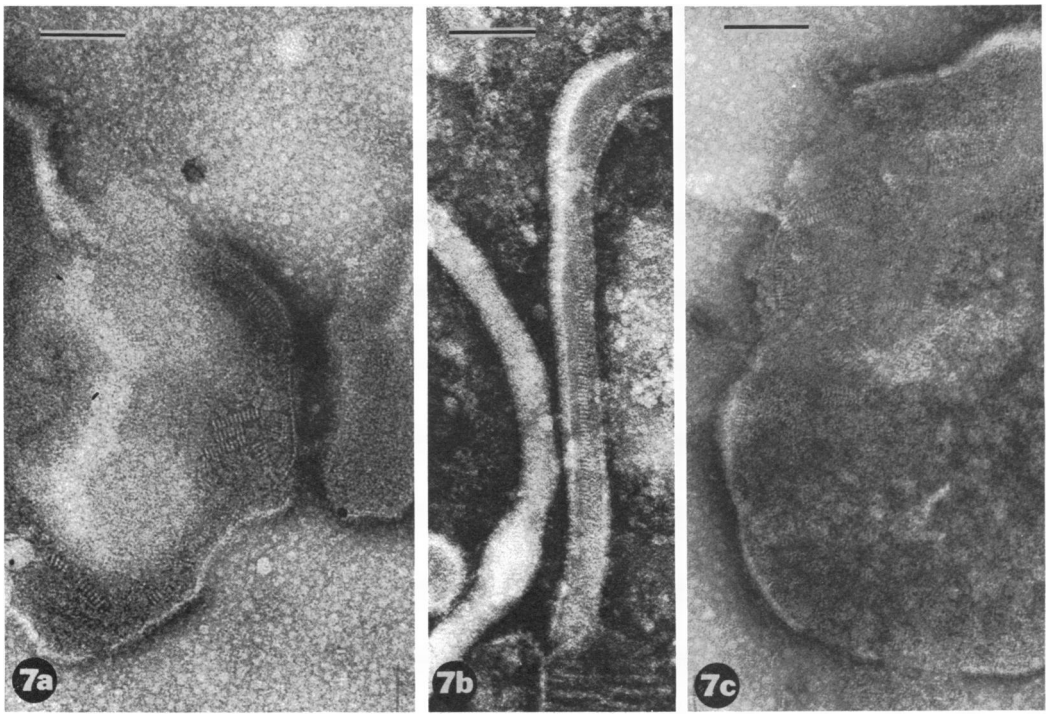

FIG. 7. *"Barred" or striated arrangements seen by negative staining on large flattened bodies (a, c) or filaments (b). All in 2% AMB. (a) ×120,750; (b) ×120,350; (c) ×120,000. Bar, 0.1 μm.*

showed well-preserved filaments and rounded forms, but no suggestion of helices (Fig. 10b). The finding is in conformity with the immediate loss of helicity seen by dark field after addition of this glutaraldehyde fixative mixture. In cultures from early passages, from which the preparations shown in Fig. 10b and 11 were made, incomplete and complete phage heads were common intracellularly. The organisms were bounded by a single membrane, but it often appeared asymmetric or with a vaguely defined outer layer (Fig. 11), as shown in sections of some other mycoplasmas (14). Occasionally, a hint of periodicity was seen but was not constant, and the layer was difficult to delineate from precipitated debris.

We subsequently experimented with fixation by various percentages and osmolalities of glutaraldehyde (Table 1) and with Formalin. The initial criterion was preservation of helices as monitored by dark-field microscopy. Success was achieved with final concentrations of 3.35% glutaraldehyde or 10% Formalin when either was made up in the culture medium (SMC) to twice the concentration desired, the pH was adjusted to neutrality, and the mixture was added to an equal volume of log-phase culture. Subsequent osmication (1% in Veronal buffer) did not alter the helices. Cultures fixed in glutaraldehyde or Formalin in this fashion, and with or without secondary fixation in osmium,

TABLE 1. *Osmolar concentrations and pH of culture media and solutions used*

Material	Osmolar concn (mosmol/kg)	pH
Complete Bové medium (SMC)	809 (809)[a]	7.8 (5.5)[a]
Horse serum medium (HS)	374	7.8
1% Serum fraction medium (SF)	408 (400)[a]	7.8
2% Sodium phosphotungstate	54	7.0
4% Sodium phosphotungstate + 0.004% sucrose	105	7.0
2% Ammonium molybdate	331	7.2
3% Ammonium molybdate	421	7.4
6% Ammonium molybdate	776	7.1
2.5% Glutaraldehyde in 0.02 M cacodylate	304	6.9
3.35% Glutaraldehyde in 0.02 M cacodylate	420	6.9
6.7% Glutaraldehyde in 0.02 M cacodylate	851	6.9
6.7% Glutaraldehyde in SMC	1,470	6.9
Sucrose (0.2 M)-phosphate (0.1 M) buffer	432	7.2
Sucrose (0.4 M)-phosphate (0.1 M) buffer	557	7.2
Veronal-acetate buffer	403	7.0

[a] After log-phase growth of Stubborn mycoplasma.

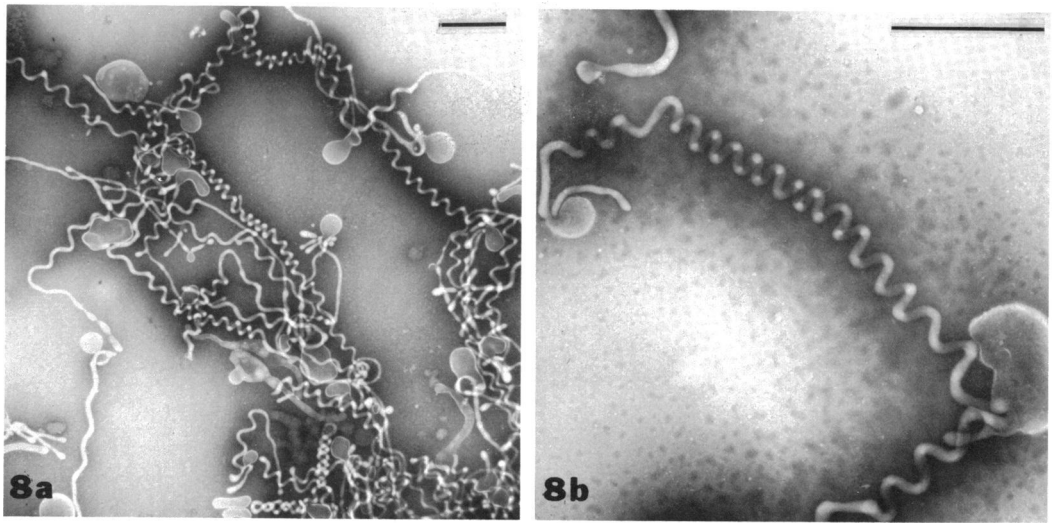

FIG. 8. *Helical filaments preserved by negative staining with AMB. (a) Two-day culture in SMC; 6% AMB; ×8,400. (b) Detail of filament from 2-day culture in SMC, showing blebs; 6% AMB; ×20,700. Bars, 1.0 μm.*

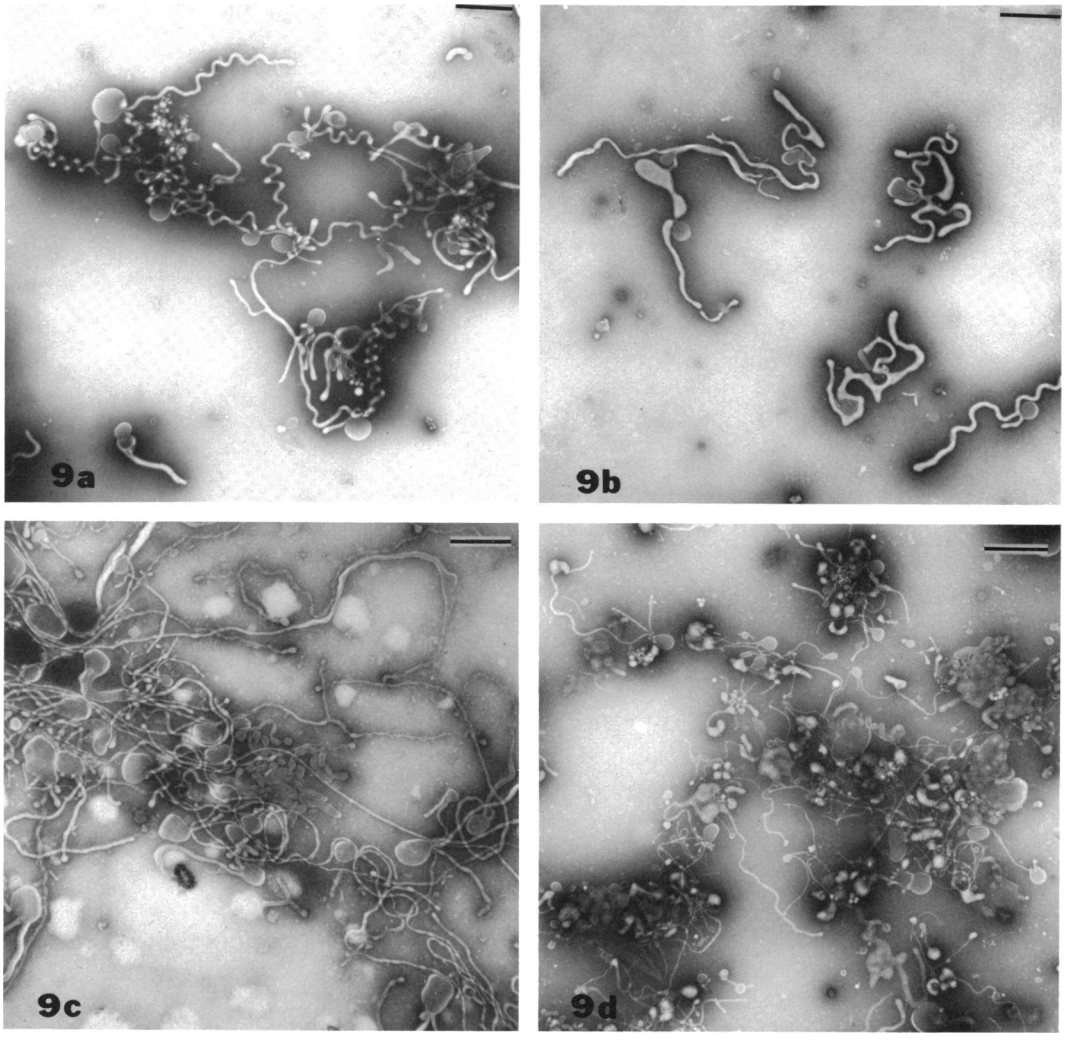

FIG. 9. *Comparison of 2-day Morocco cultures in SMC, negatively stained with 3% AMB (a) or with 2% PTA (b), ×7,700. Note extensive flattening and lack of helices in the latter. Compare also the better preservation of helices in 6% AMB in Fig. 9. (c and d) Effects of culture age. (c) Morocco strain, 7-day culture in SMC; 3% AMB; ×7,700. (d) Same, 10-day culture; 3% AMB; ×7,700. Bar, 1.0 μm.*

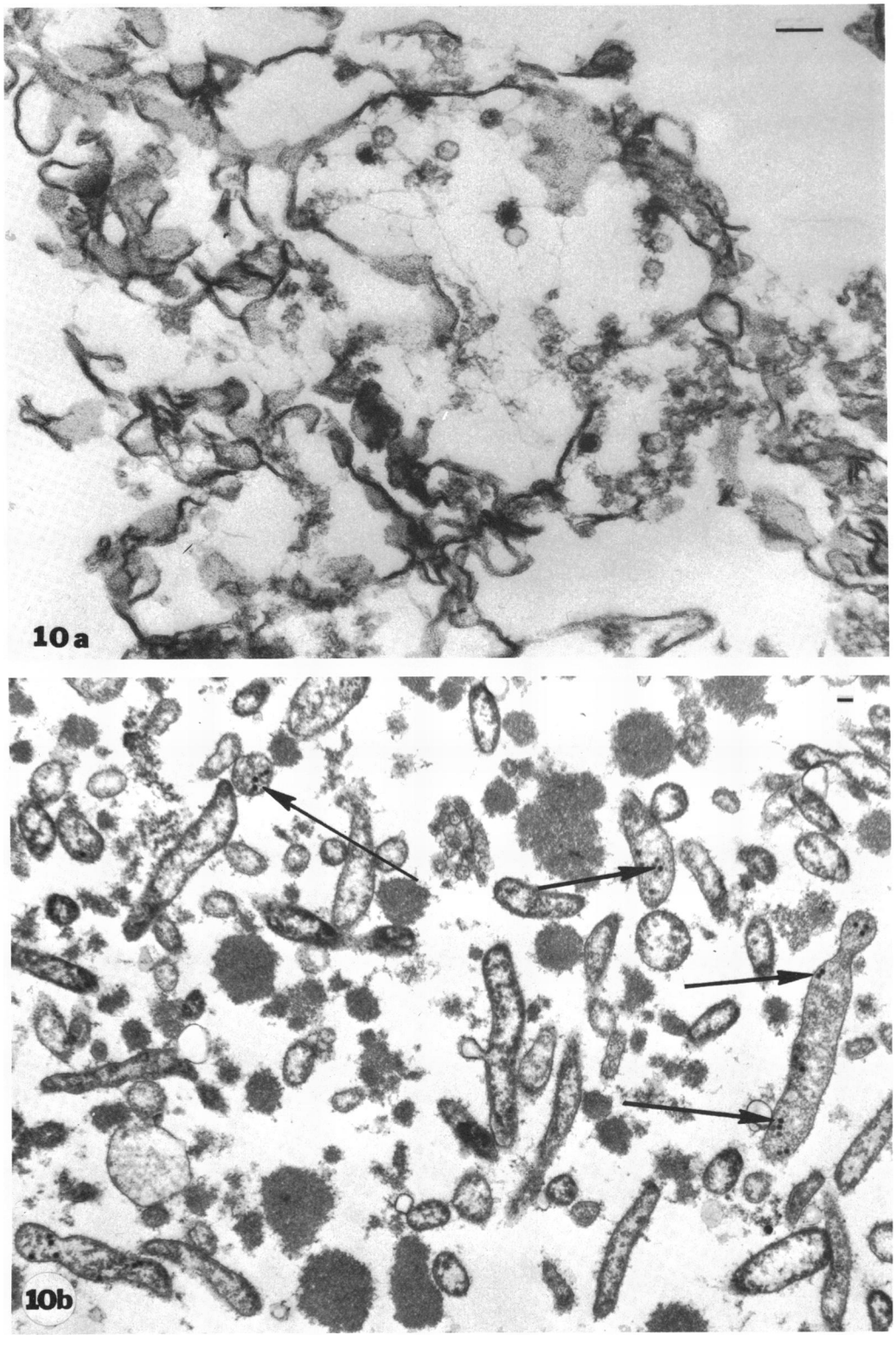

FIG. 10. *Ultrathin section of Morocco strain, 5-day growth in SMC. (a) Fixed in 1% osmium. Note lack of complete organisms; only membranes and phage remain; ×72,000. (b) Fixed in 2.5% glutaraldehyde in cacodylate buffer, followed by 1% osmium. Arrows indicate intracellular phage heads; ×21,000. Bar, 0.1 μm.*

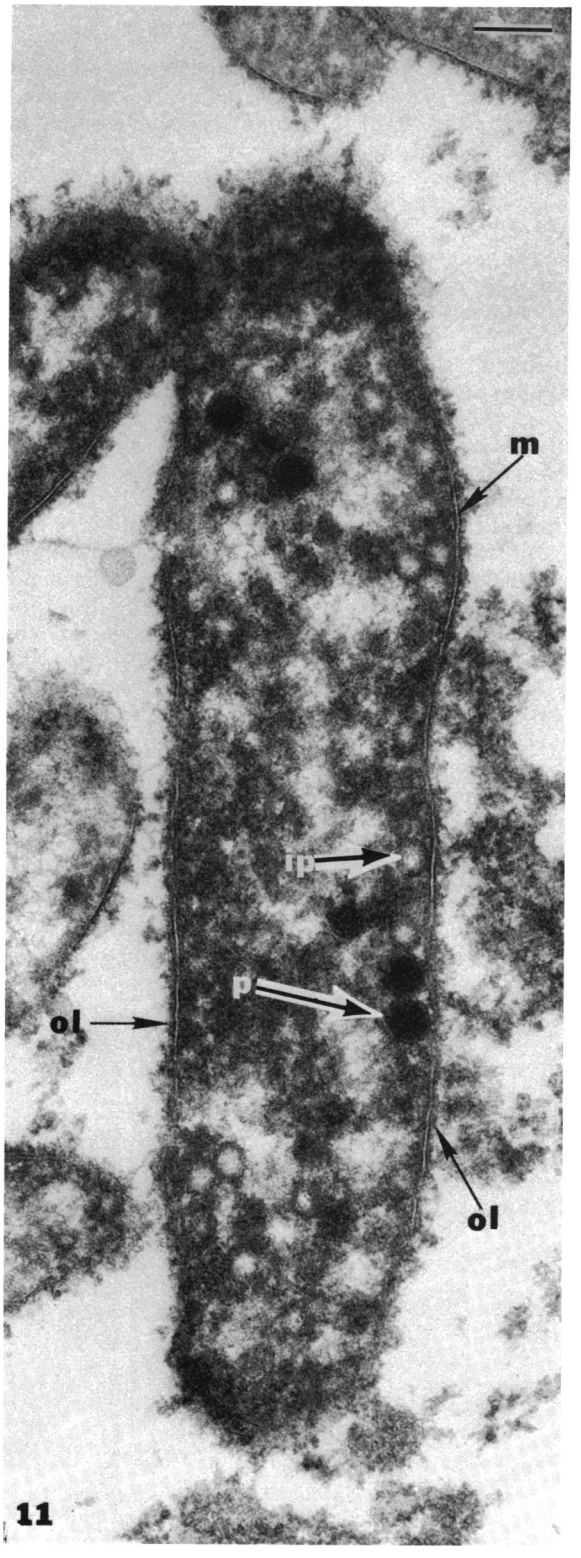

FIG. 11. *Ultrathin section of Morocco strain, 5-day growth in SMC. Fixation as in Fig. 10b. Note complete (p) and incomplete (ip) phage heads, membrane (m), and outer layer (ol).* ×*103,500. Bar, 0.1 μm.*

were processed further and embedded. Replicate samples, prior to embedding, were examined by negative staining (Fig. 12a) by scanning electron microscopy (Fig. 12b) and in the transmission electron microscope after direct metal shadowing (Fig. 12c). The figures show that the helices were retained, although the pitch or "tightness" of the coils was usually less than that seen in the best AMB-stained preparations made from unfixed specimens (Fig. 8).

Sections from the embedments showed good helices in thick (1 μm) sections after fixation in glutaraldehyde-SMC (Fig. 13a), but direct comparison with the same culture fixed in glutaraldehyde-cacodylate emphasized the failure of this fixative mixture to preserve shape and size (Fig. 13b). Ultrathin sections of the same blocks demonstrate more strikingly the disparities in filament diameters and internal densities of the mycoplasma fixed by the two different solutions. The helical forms (glutaraldehyde-SMC) were quite uniform, ranging from 104 to 170 nm in diameter (averaging 120 nm), and were moderately dense (Fig. 14a). On the other hand, diameters of filaments fixed in glutaraldehyde-cacodylate ranged widely from 90 to more than 250 nm (averaging about 200 nm); they were less dense, with apparently "expanded" nucleoplasm, and more variable in shape (Fig. 14b). The deoxyribonucleic acid strands appeared coarser and more compact in the helics (Fig. 14a) than in the nonhelical filaments (Fig. 14b); they and the surrounding "nucleoplasm" appeared rather evenly distributed throughout the filaments and other bodies, and there was no evidence of discrete nucleoids as in bacteria nor of any regular cell partition suggesting a mode of cell division. Though not shown here, branching was commonly seen as a continuation of both cytoplasm and membrane. In both types of filament, dense ribosomes of approximately 17 nm in diameter were present. In addition, in these preparations (which received a wash in Veronal buffer before osmication) there was no clear evidence of asymmetric membranes nor of outer layers such as suggested in glutaraldehyde-cacodylate-fixed preparations washed in sucrose-phosphate buffer before osmication (Fig. 11). After either fixation shown in Fig. 14, the "triple-track" membrane profiles measured from 6.7 to 8.7 nm in width but were most commonly 7.2 nm wide.

A matter of additional interest was the irregular presence of a layer internal to and abutting the cytoplasmic membrane (Fig. 15). This layer, about 6.7 nm wide, appeared somewhat membrane-like but had obvious internal content occasionally suggesting cross-striations. It varied in length, was not seen in every cell sectioned, and was discontinous when seen. Rarely, it appeared double. Although most common in cultures obviously producing phage, it could be seen in cells not containing phage in the plane sectioned. It could also be seen occasionally in helices from cultures in which no phage was detected microscopically (Fig. 14a). Whether it represents a phage assembly product

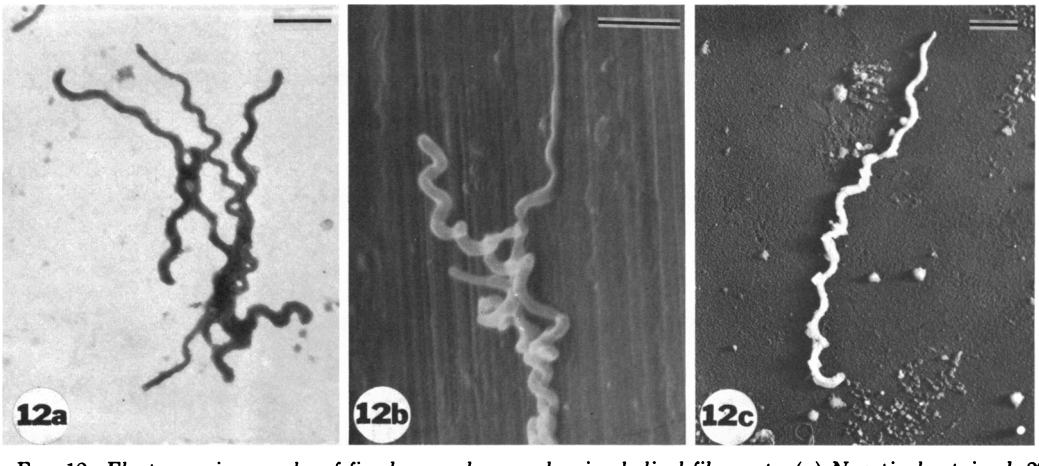

FIG. 12. *Electron micrographs of fixed mycoplasma, showing helical filaments. (a) Negatively stained; 2% AMB. Fixed in 3.35% glutaraldehyde in SMC followed by 1% osmium, and washed in Veronal buffer and deionized water; ×8,500. (b) Scanning electron micrograph. Fixed in 10% Formalin in SMC, followed by 1% osmium, and washed in Veronal buffer and deionized water; ×12,100. (c) Directly shadowed with platinum-palladium. Fixed and treated as in (a); ×6,650. Bar, 1.0 μm.*

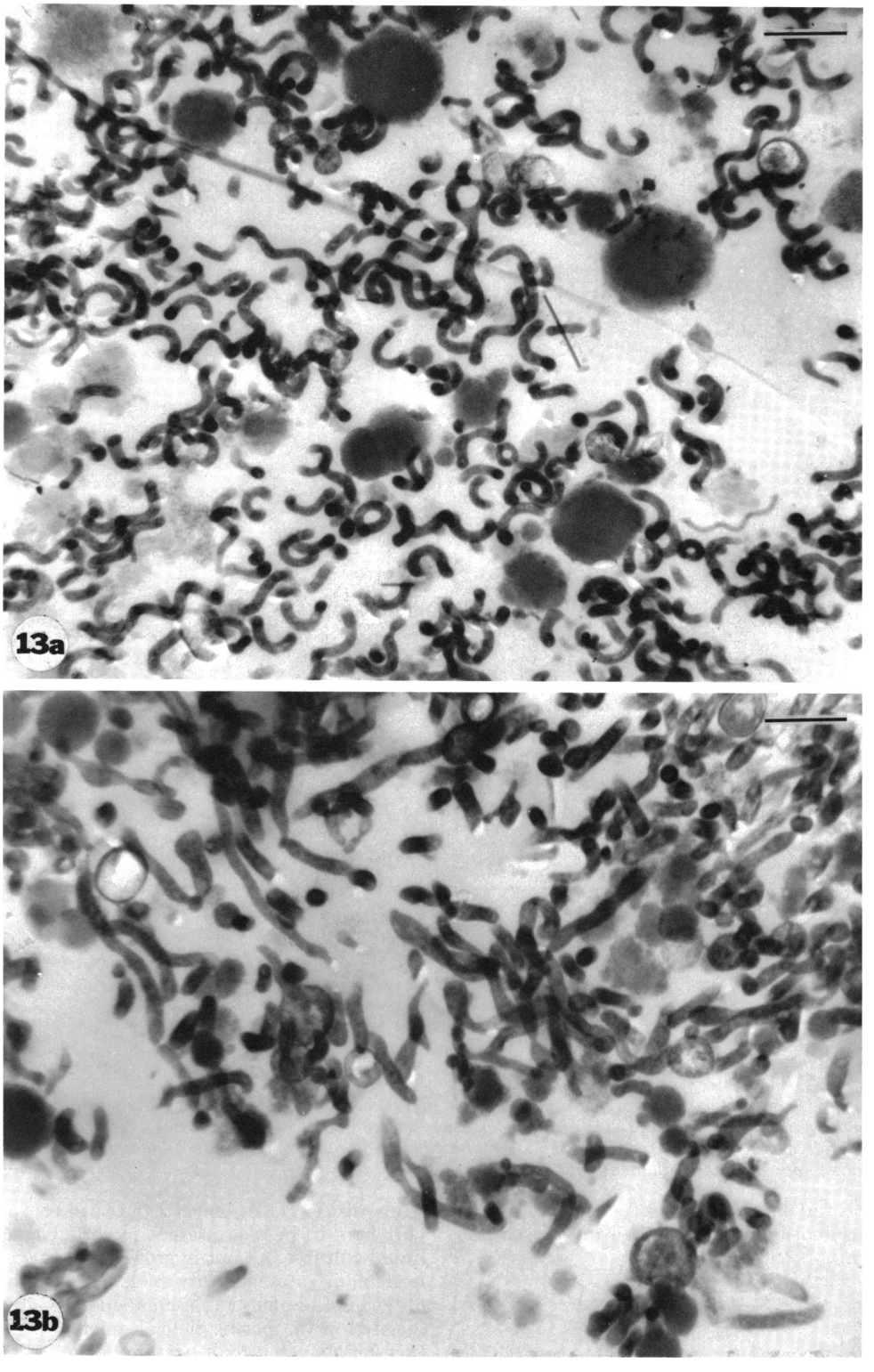

FIG. 13. *Electron micrographs of thick (1.0 μm) sections of Morocco strain, 2-day culture in SMC. (a) Fixed as in Fig. 12a. Note narrow uniform filaments with helical configurations; ×11,900. (b) Fixed in 3.35% glutaraldehyde in cacodylate buffer followed by 1% osmium. Note wide, variable, nonhelical filaments and some round bodies; ×11,900. Bar, 1.0 μm.*

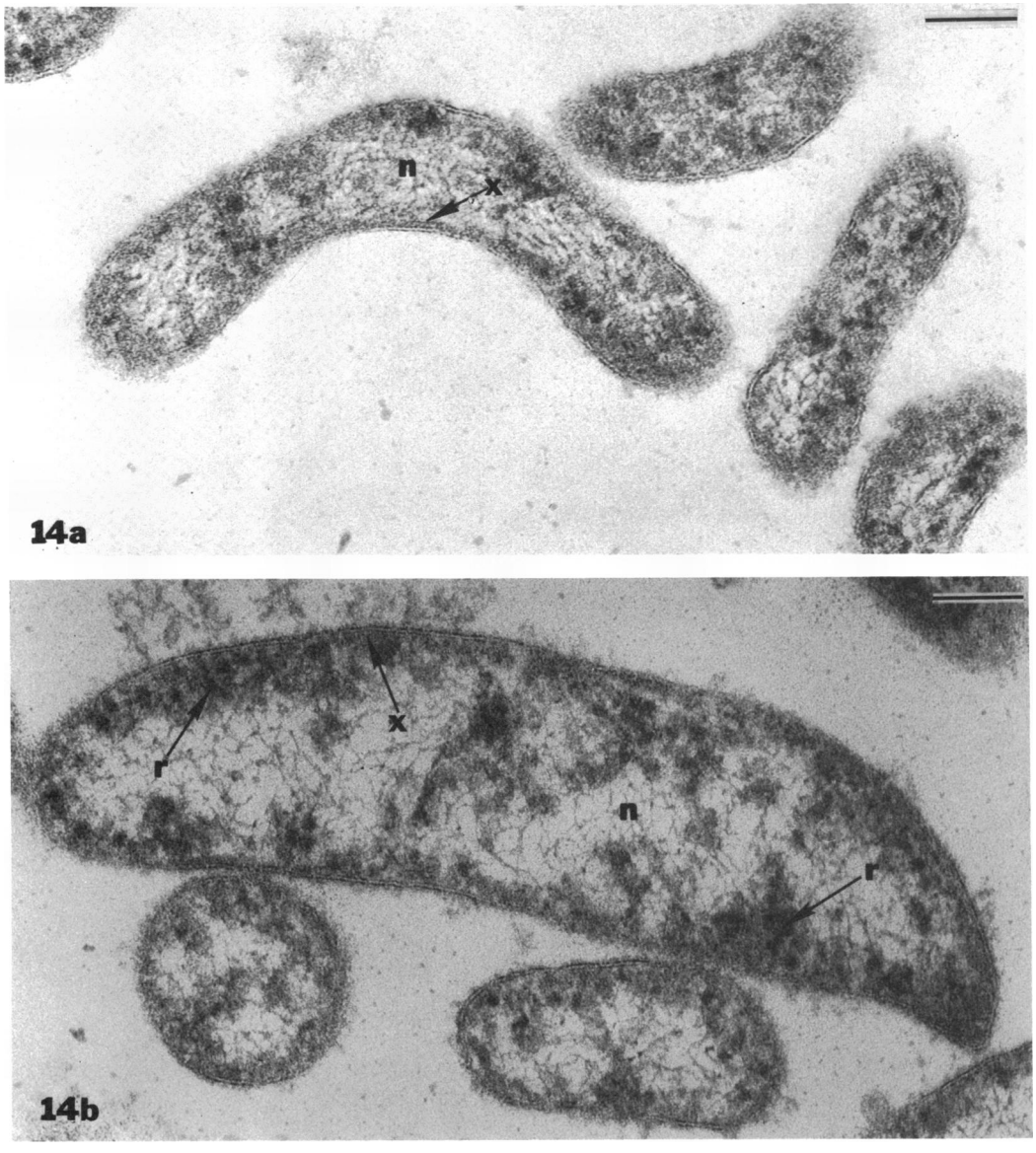

FIG. 14. *Ultrathin sections of preparations shown in Fig. 13, at higher magnification (×120,000). (a) Primary fixation by glutaraldehyde in SMC. (b) Primary fixation by glutaraldehyde in cacodylate. n, Deoxyribonucleic acid strands of nuclear material; x, inner layer abutting membrane; r, ribosomes. Note greater width and "expanded" appearance of filament. Bar, 0.1 μm.*

such as tail or is a unique structural component of this motile mycoplasma is at present unknown.

DISCUSSION

Spiroplasma citri demonstrates several features not commonly associated with mycoplasmas. (i) It can occur as a helical filament like the mycoplasma from infected tissues of corn stunt disease (13)—an attribute which we report here to be also present in serially passed liquid cultures but not in growth from agar. (ii) It demonstrates a rotary motility despite the absence of demonstrable organelles usually associated with motile, helical microorganisms. (iii) It is infected by a bacteriophage of classic type B morphology (5)—a phenomenon previously unknown among mycoplasmas in which only a few viruses of entirely diffrent mor-

phology have been described (19-21, 29, 36). (iv) It possesses a layer of surface projections outside of and adherent to the cytoplasmic membrane, as well as some unique structures irregularly abutting the inner side of membrane. Nevertheless, it is a membrane-bounded microorganism without a definite cell wall and, by additional criteria (P. Saglio et al., in press), appears to meet the definition of a mycoplasma.

Except for the corn stunt agent (13), no other helical mycoplasmas have been described previously. However, the so called "SR-spirochete" of *Drosophila* spp. appears closely similar in morphology by dark-field microscopy and negative staining and, furthermore, produces a virus (phage) intracellularly (35). The interrelationships of these microorganisms await clarification. On the other hand, nonhelical filamentous mycoplasmas, or filamentous stages in their growth, have been commonly reported among known species of *Mycoplasma* or *Acholeplasma* (2, 4, 10, 17, 25, 26, 30, 31, 39). These descriptions, which usually include note of associated round bodies, ring forms, and beaded filaments, differ little from the PTA-stained appearance of the corn stunt (8, 13) or Stubborn disease mycoplasmas. However, the effects of different negative stains and fixatives, freezing, drying, and other preparative techniques on morphology have not been well explored. For example, it is clear from our experience that the helical nature of the Stubborn mycoplasma would not have been verified by electron microscopy if only PTA and standard methods of glutaraldehyde-osmium fixation had been used. On the other hand, one may wonder why more mycoplasmas (especially those of plants) have not been examined by readily available methods such as dark-field microscopy or negative staining with a variety of heavy-metal-containing compounds. One deterrent to widespread use of the latter is undoubtedly the problem of mycoplasma-like artifacts in host materials, as emphasized in plants by Wolanski and Maramorosch (46). This difficulty can be overcome only when, as in the present instance, the suspect microorganism can be serially propagated in artificial media—a requirement which, in any event, is a requisite for definitive characterization.

The capacity of *S. citri* for independent motion is, as yet, unexplained. The observations to date suggest an inherent, but fragile, contractile mechanism that requires optimal physiological and physical conditions since the organism has no organelles, loses both motility and helicity in aging broth cultures, and fails to demonstrate either in agar cultures. Other mycoplasmas have been reported to be motile, including *M. pulmonis* (34) and *M. pneumoniae* and *M. gallisepticum* (W. Bredt, Summaries of Int. Mycoplasma Symp., Mainz, 6 to 7 Sept., 1971; in Med. Microbiol. Immunol. **157**:169-180, 1972). Except for an additional spinning of coccoid bodies in *M. pulmonis* (34), the type of motion reported seems to be a gliding one which requires surface adherence (S. Razin, Advan. Microbial. Physiol., in press) and which is, therefore, entirely different from that seen in the free-floating helical *Spiroplasma*. It may be of some significance that special structures or surface projections, or both, have been well demonstrated only in these motile mycoplasmas.

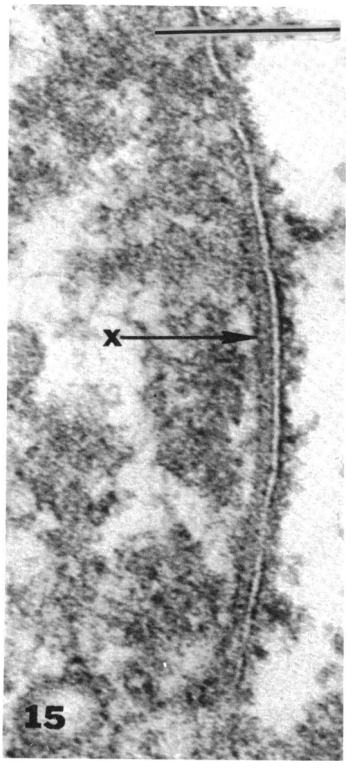

FIG. 15. *Example of inner layer (x) abutting membrane;* ×*240,000. Bar, 0.1 μm.*

The finding of a classic tailed bacteriophage attaching to and developing within *S. citri* was totally unexpected, is completely new, and raises taxonomic and other questions which remain unanswered until susceptible indicator strains are found and high phage yields are obtained and studied. We do not know, for example, whether the phage is present in a state of lysogeny or the condition is one of a carrier state or pseudolysogeny. The presence of many empty heads and, occasionally, polyheads (15,

27, 43) suggests a partial state of defective lysogeny. It any event, it now appears that we must revise previous ideas that classic or tailed bacteriophages cannot infect mycoplasma or similar microorganisms—usually called wall-less—which do not possess cell walls similar in all respects to those of known bacteria. Presently characterized mycoplasma viruses are tail-less, and either bullet-shaped and nonenveloped (19, 29) or spherical and enveloped (20). All of these infect only strains of *A. laidlawii* as the indicator. The nature of rod-shaped particles associated with clover dwarf agent (36) is unclear, and the morphology of "phage" produced by the "SR-spirochetes" (35) has not been clearly shown.

The surface projections or outer layer of this helical mycoplasma, while perhaps dubious and erratically present in sections after different fixations, were clearly demonstrated on flattened filaments by negative staining. They resemble those seen by negative staining on *M. pulmonis* (36, 42), *M. gallisepticum* (1, 9), and human T-strain mycoplasmas (3). In sections, suggestive asymmetry of membranes, or outer layers, have been pictured on *M. pulmonis* (14), *M. gallisepticum* (1), T-strains (3), and *M. canis*, *M. gallinarum*, and *M. hyorhinis* (14). The nature and possible relation to motility or other functions of these surface structures is unknown; and we must now add the question of possible participation in reception of phage or other viruses.

A most interesting question is that of acquisition and maintenance of the helical shape. Its presence in all broth cultures (SMC, HS, SF) but absence in agar cultures (colonies) made with the same media suggests a physical factor; but its loss in aging broth cultures indicates additional influences, which may include low pH, exhaustion of essential nutrilites, and changes in osmolality. Failure to preserve helices in PTA, (54–105 mosmol/kg) and best preservation in 6% AMB (851 mosmol/kg) (Table 1) suggests the importance of tonicity, and the success of glutaraldehyde or Formalin when made up in the medium of high osmolality seems to be further indication of this effect. However, the deleterious effect of cacodylate buffer is unexplained, and further investigations of the effects of metabolic poisons, various ions, ionic strength, and the like, are required. The presence of helical filaments of the corn stunt agent in material from diseased plants (13) suggests that *S. citri* may also occur in this form in diseased tissues, and this appears to be so (Fig. 16). It is also possible that the age or duration of the infection within a given group of

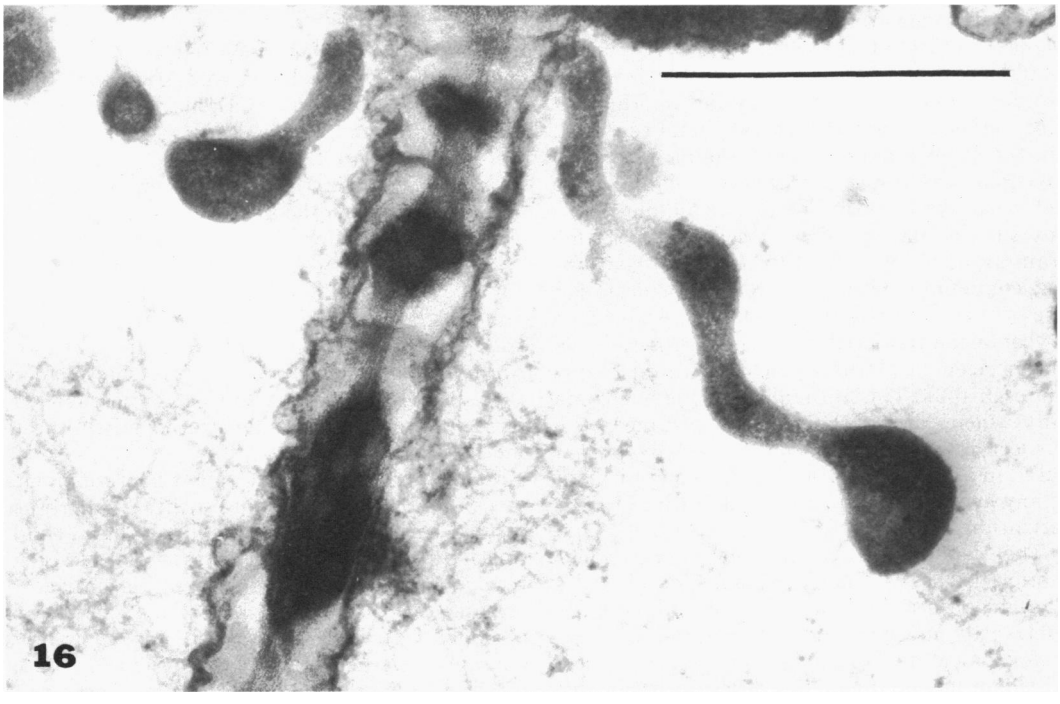

FIG. 16. *Helical organism in sieve tube of Madam Vinous sweet orange seedling infected with Stubborn (California Strain 189) and growth at 32 C;* ×45,000. *Bar, 1.0 μm. (Electron micrograph by J. M. Bové.)*

plant cells may influence the morphology of the mycoplasma, as was shown in broth cultures—a possibility that could account for failure to detect helices at given times of examination.

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