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Comparison of the Ultrastructure of Several Rickettsiae, Ornithosis Virus, and *Mycoplasma* in Tissue Culture

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ABSTRACT

ANDERSON, DOUGLAS R. (National Cancer Institute, Bethesda, Md.), HOPE E. HOPPS, MICHAEL F. BARILE, AND BARBARA C. BERNHEIM. Comparison of the ultrastructure of several rickettsiae, ornithosis virus, and *Mycoplasma* in tissue culture. J. Bacteriol. **90**:1387–1404. 1965.—In an effort to make a valid comparison of the ultrastructure of several intracellular parasites, selected agents were propagated under identical conditions in a single type of tissue culture cell; such infected preparations were processed for examination by electron microscopy by use of a standardized procedure for fixation and embedding. The organisms studied were: the Breinl and E strains of epidemic typhus, *Rickettsia prowazeki*; the Bitterroot strain of *R. rickettsii*; the Karp strain of *R. tsutsugamushi* (*R. orientalis*); *R. sennetsu;* the P-4 strain of ornithosis virus; and the HEp-2 strain of *Mycoplasma hominis* type I. Each of the rickettsial species examined had a cell wall and a plasma membrane, and contained ribosomes and deoxyribonucleic acid (DNA) in a ground substance. However, certain differences were noted. Both strains of *R. prowazeki* contained numerous intracytoplasmic electron-lucent spherical structures (4 to 10 mμ), not previously described. *R. sennetsu*, unlike the other rickettsiae, was not free in the host cytoplasm but was always enclosed in a vacuole. *R. rickettsii* was observed intranuclearly and in digestive organelles of the host cell as well as in the cytoplasm. Cells infected with ornithosis virus contained several forms representing the stages in its life cycle. The "initial bodies," made up of ribosomes and DNA strands, were morphologically similar to the rickettsiae. In cultures infected with *M. hominis*, most of the cells became large and multinucleate. Although the *Mycoplasma* organisms were readily cultivated from these cultures, only a few could be found in the electron microscope preparations. These organisms were extracellular and lacked a cell wall, being bound only by a unit membrane. Again, the internal components were ribosomes and DNA strands. Under the uniform preparative conditions employed here, the three groups of organisms were morphologically distinguishable from one another.

An increasing body of biochemical and biophysical evidence clearly delineates the larger viruses, i.e., the psittacosis-lymphogranuloma-trachoma (PLT) group, from the true viruses (Moulder, 1964) and points up their closer resemblance to rickettsiae and bacteria. In addition, during the course of studies on the ultrastructure of *Mycoplasma* (Anderson and Barile, 1965), it was noted that some forms of this organism, PLT particles, and rickettsial organisms resembled one another. This similarity was of particular interest when it was observed that *Mycoplasma* can occur in the intracellular position (Anderson and Manaker, J. Natl. Cancer Inst., *in press*). However, since different preparative techniques can result in significantly different appearances of intracellular organisms (Mitsui, Fujimoto, and Kajima 1964; Armstrong and Reed, 1964), one must use extreme caution in comparing the ultrastructure of organisms derived under one set of conditions with those prepared under different biological and physical conditions. It is the purpose of the present report to describe and compare the ultrastructure of

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several organisms representative of rickettsiae, PLT, and *Mycoplasma*. In the studies described here, the organisms were propagated in the same type of tissue culture cells under essentially identical conditions, and such cells were then subjected to standardized fixation and embedding procedures prior to examination by electron microscopy.

MATERIALS AND METHODS

Cell line. The BS-C-1 line of Cercopithecus kidney cells, known to be *Mycoplasma*-free, was employed. Detailed information concerning its origin, propagation, and maintenance is given elsewhere (Hopps et al., 1963). In the current work, stock cultures of BS-C-1 cells in the 300th to 330th passage level were grown in the stationary state in 8-oz milk dilution bottles at 35 C in an antibiotic-free medium consisting of M 199 (Morgan, Morton, and Parker, 1950) with 20% fetal bovine serum (noninactivated) and 0.1% Yeastolate (Difco). The medium was prepared in 2- to 3-liter batches, sterilized by Millipore filtration, and dispensed in 100-ml amounts. Samples of each batch of medium were tested for sterility, and no lot was used that had not been under test for at least 2 weeks.

At the high passage levels mentioned above, the BS-C-1 cells multiplied rapidly, and subcultures to new vessels were usually made at 4-day intervals. At the time of subculture, the cell monolayer was subjected to trypsinization (Hopps et al., 1963), and the resultant cell suspension was made up to 12.0 ml; one-half this volume was transferred to a new vessel, and each bottle then received an equal volume of medium. Such cultures contained approximately 2×10^5 cells per milliliter. For work with the various infectious agents employed, cultures were also prepared in 2- and 32-oz prescription bottles, according to the volume of infected-cell suspension desired.

Rickettsial, viral, and Mycoplasma strains. The following *Mycoplasma*-free rickettsial agents were employed: egg-adapted lines of the Karp strain of *Rickettsia tsutsugamushi* (*R. orientalis*), the agent of scrub typhus (Rights, Smadel, and Jackson, 1948); the Breinl (virulent) and E (avirulent) strains of epidemic typhus, *R. prowazeki*; the Bitterroot strain of *R. rickettsii*, the agent of Rocky Mountain spotted fever; and a strain of *R. sennetsu* (Misao and Kobayashi, 1955; Misao, Kobayashi, and Shirakawa, 1957) originally obtained from M. Kitaoka of the National Institute of Health of Japan. The last-mentioned organism has been tentatively placed in the rickettsial group, but its exact taxonomic position is not clear. Although *R. sennetsu* exhibits certain rickettsialike properties, no serological relationships to other rickettsial agents has been established.

In addition, two other organisms were studied: the P-4 strain of ornithosis, originally described by Smadel, Wall, and Gregg (1943) and the HEp-2 strain of *M. hominis* type I [a species of pleuropneumonia-like organism (PPLO)] originally isolated by Barile as a contaminant in the HEp-2 tissue culture cell line derived from a human epidermoid carcinoma (Moore, Sabachewsky, and Toolan, 1955). The ultrastructure of the HEp-2 strain of *Mycoplasma* in broth cultures was reported previously (Anderson and Barile, 1965).

Infection of tissue cultures. To insure optimal cell preparations for electron microscopic studies, i.e., preparations in which nearly all of the cells were heavily infected, high-titer seed stocks of each agent were employed. In the case of the rickettsial strains and ornithosis virus, each seed pool, of either yolk sac or tissue culture origin, contained 10^6 to 10^7 infectious units per milliliter, as determined by titration in mice or embryonated eggs. The material was diluted 1:10 in tissue culture fluid and, after removing the medium from each of the culture vessels, the inoculum was added directly to the cell monolayer, the volume added being dependent on the size of the culture used. Thus, for 2-, 8-, and 32-oz vessels, 1.0-, 3.0-, and 10.0-ml volumes, respectively, were employed. The cultures were then incubated for 2 hr at 35 C to permit penetration of the organisms. After this time, the inoculum was removed, and fresh medium was added. Cultures infected with *M. hominis* were treated similarly; the inoculum used was either a broth culture or tissue culture fluid harvested from infected BS-C-1 cultures and stored at −70 C prior to use. In each of the experiments, noninfected control cultures were handled in an analogous manner, substituting tissue culture medium for the infectious inoculum.

Sufficient numbers of infected cultures were included so that the extent of the rickettsial or viral infection could be estimated at 2- to 3-day intervals. The *Mycoplasma*-infected cultures were monitored by inoculating agar plates (Barile, Yaguchi, and Eveland, 1958) with samples (0.1 ml) of tissue culture fluid. Proliferation of the rickettsial and ornithosis agents was determined by direct microscopic observation of Giemsa-stained preparations. For this purpose, 1.0-ml amounts of trypsinized cell suspensions were centrifuged at 1,000 rev/min for 5 min, and the sediment was suspended in a small drop of residual fluid. A small drop of the suspension was then transferred to a slide, smeared as for blood films, and stained by the method of Giemsa. One hundred well-stained cells were examined to determine the percentage of cells invaded and the degree of the infection. When it was evident that 80 to 90% of the cells were infected, the cultures were considered suitable for observation by electron microscopy.

Electron microscopy. A 5% phosphate-buffered glutaraldehyde fixative (Sabatini, Bensch, and Barrnett, 1963) was prepared by mixing one part of 25% glutaraldehyde, which had been stored over coconut charcoal, with four parts of Millonig's (1961, 1962) phosphate buffer containing glucose and calcium chloride. Millonig's buffer was prepared by mixing 41.5 ml of 2.26% $NaH_2PO_4 \cdot H_2O$

with 8.5 ml of 2.52% NaOH; 5 ml were removed for *p*H determination, and, if the *p*H was 7.3, the remaining 45 ml were mixed with 5 ml of 5.4% glucose and 0.25 ml of 1% $CaCl_2$.

Most cultures were harvested by scraping the cells from the glass into the growth medium. The cells were centrifuged at low speed into a loose pellet, the supernatant fraction was discarded, and the glutaraldehyde fixative was gently placed over the cell pellet. After 10 to 15 min, the pellet was gently broken into small pieces and left for another 10 to 15 min in fixative.

A few cultures were harvested by removing the growth medium and replacing it with glutaraldehyde fixative. After 10 to 15 min, the cells were scraped into the glutaraldehyde, poured into a centrifuge tube, and centrifuged into a pellet. The supernatant fraction was discarded, and the sedimented cells were mixed with an equal volume of warm 4% agar. After the mixture of cells and agar had cooled, it was cut into small pieces before the subsequent steps in handling.

Upon completion of glutaraldehyde fixation, the pieces of cell pellet or agar-embedded cells were placed in 1% osmium tetroxide in Millonig's buffer. After 1 to 2 hr in osmium fixative, the specimens were rapidly dehydrated in a graded series of ethyl alcohol.

Epon-Araldite embedding mixture no. 1 of Mollenhauer (1964) was used. The stock mixture consisted of 25 ml of Epon 812, 55 to 60 ml of dodecenyl succinic anhydride (DDSA), 15 to 20 ml of Araldite 6005, and 4 ml of dibutyl phthalate. Just before use, one drop of 2,4,6-tri(dimethyl-aminomethyl) phenol (DMP-30) was added (by means of a disposable Pasteur capillary pipette) for each 1 ml of stock mixture. To accomplish infiltration, the specimens were cleared of ethyl alcohol in two 15-min changes of propylene oxide and were left overnight in a 1:1 mixture of propylene oxide and the embedding mixture. The specimen pieces were carefully blotted on filter paper and flat embedded. Polymerization was carried out at 80 C overnight or longer.

Sections giving a gray-to-silver interference color were cut and picked up on uncoated copper grids or 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). The specimens were examined and photographed with an RCA electron microscope, model EMU-3F.

RESULTS

R. prowazeki. Examination of cells infected with the Breinl and E strains revealed a striking difference in numbers of rickettsiae present in cultures infected with approximately the same number of organisms and maintained for similar periods of time; growth of the virulent Breinl strain was much more luxuriant than that of the avirulent E strain. Generally, the morphology of the two types was otherwise indistinguishable. Although some organisms were found extracellularly, the majority were distributed in the cell cytoplasm (Fig. 1 to 4) and were not enclosed in vacuoles. Occasionally, channels of endoplasmic reticulum were molded around the organism giving the illusion of a vacuole (Fig. 4).

Both intra- and extracellular organisms were bounded by a plasma membrane and a cell wall, each of which was a tri-layered structure (two dense layers separated by a pale layer). These structures tended to be rippled, which may in part represent shrinkage during the preparative procedures. The plasma membrane was sometimes invaginated, forming small pockets as observed by Ito and Vinson (1965) in studies with *R. quintana*. The individual rickettsiae were 0.3 to 0.5 μ in diameter, and when sectioned longitudinally they were of varying lengths, some as long as 4 μ (Fig. 1). The plasma membrane and cell walls of some of the larger organisms contained transverse constriction furrows characteristic of rickettsiae undergoing binary fission. Several sections revealed chains of rickettsiae (Fig. 2). Large, pleomorphic forms were also encountered; these were extremely irregular in shape but possessed the same structural components (Fig. 3).

Internally dense ribosomelike granules were randomly dispersed in a ground substance of intermediate density. Fine deoxyribonucleic acid (DNA)-like strands were seen in irregular open spaces in the ground substance, but well-defined nuclear areas were rarely observed. Unlike the other organisms studied, the cytoplasm of *R. prowazeki* contained numerous electron-lucent spherical structures, 4 to 10 mμ in diameter, which sometimes appeared to be bounded by a thin black line (Fig. 1, arrows).

R. ricketsii. The structure of *R. ricketsii* (Fig. 5) was similar to that of *R. prowazeki.* However, the organisms were shorter (2 μ), were not observed in chains, and lacked the lucent spherical structures seen in the typhus rickettsiae. In addition to the usual cytoplasmic location, a few of these organisms were observed in the nucleus (Fig. 6), unlike any of the other agents examined in this study. Occasionally, degenerated organisms could also be identified in dense vacuoles in the host-cell cytoplasm; these structures were presumed to be digestive organelles (Fig. 7).

R. tsutsugamushi. This species appeared to be somewhat shorter (1.5 μ) than either of the organisms described above, and often the DNA strands appeared to be localized in a discrete nuclear area (Fig. 8). In all other respects, its morphology was similar to that of the other rickettsiae examined.

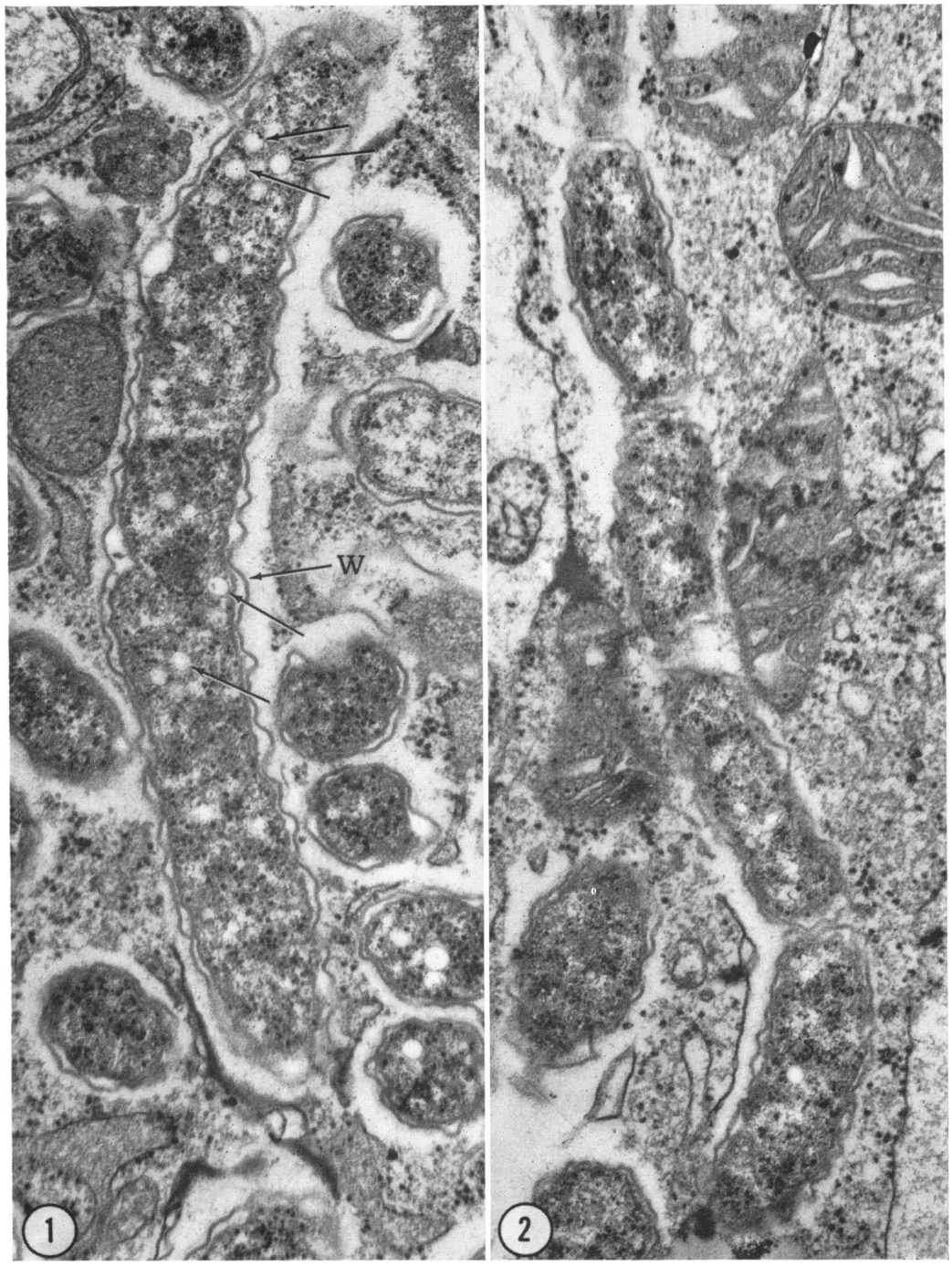


FIG. 1. *Longitudinal section through Rickettsia prowazeki which is 4 μ long. Several cross sections are also included in the field. The rippled cell wall (W) is obvious, and the underlying plasma membrane which bounds the rickettsial cytoplasm is easily seen in several places. The internal material consists of ribosomes and DNA strands, and this species also has electron-lucent spherical structures, some of which are indicated with arrows.* × *45,000.*

FIG. 2. *Section through a chain of short rod-shaped Rickettsia prowazeki.* × *45,000.*

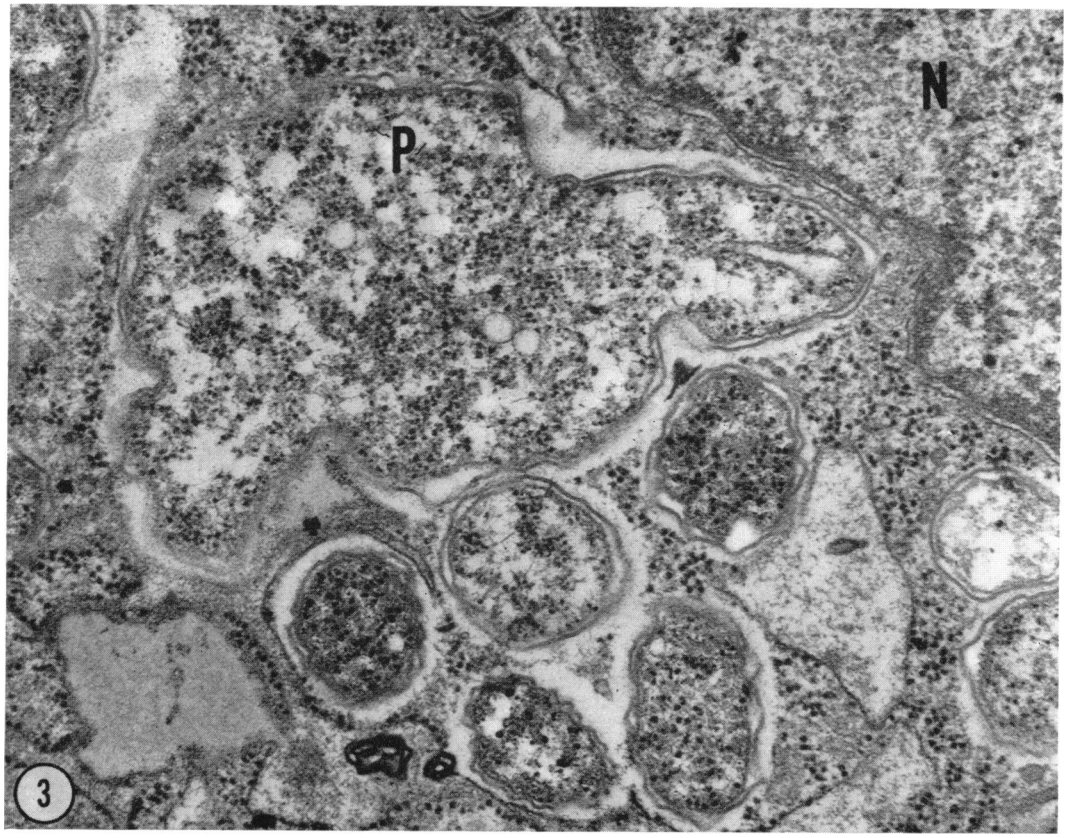


FIG. 3. *"Pleomorphic form" (P) of Rickettsia prowazeki. It is large and irregular in shape, but its structural components are the same as in the more usual rod-shaped organisms. Cross sections of the usual form of rickettsiae are also included in the field, as is a portion of the host-cell nucleus (N). × 45,000.*

R. sennetsu. At first examination, these organisms appeared to be free in the host-cell cytoplasm, as did the other rickettsiae examined. However, closer scrutiny revealed that the organisms, singly or in groups, were contained in a membrane-lined vacuole (Fig. 9 and 10). When a fixative other than glutaraldehyde was employed, i.e., Dalton's (1955) chrome-osmium, the vacuoles became distended and the intravacuolar position of the rickettsiae became obvious. Occasionally, such distended vacuoles were also seen in glutaraldehyde-fixed material (Fig. 11) and closely resembled an "initial body" vacuole of ornithosis virus (see Fig. 17).

The diameter of many organisms was 0.5 to 1 μ (Fig. 9 to 11), but their extreme pleomorphism made measurement difficult and some were several microns in diameter. Some round and rod-shaped organisms were seen (Fig. 9 to 11), but many were extremely irregular in shape as illustrated in Fig. 14.

The arrangement of the organisms in vacuoles is shown in Fig. 10 to 13. Some vacuoles contained a single rickettsia (Fig. 10) or a pair of rod-shaped bodies lying end to end, and others contained groups of rickettsiae (Fig. 11 to 13). Elongated organisms were seen closely aligned,

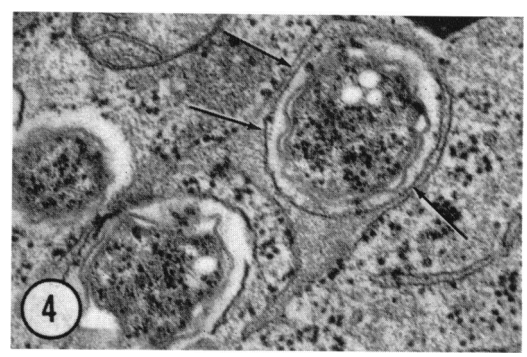


FIG. 4. *Rickettsia prowazeki. A cisterna of the endoplasmic reticulum (arrows) of the host cell appears to be wrapped around the organism and gives the illusion of enclosing it in a vacuole. × 45,000.*

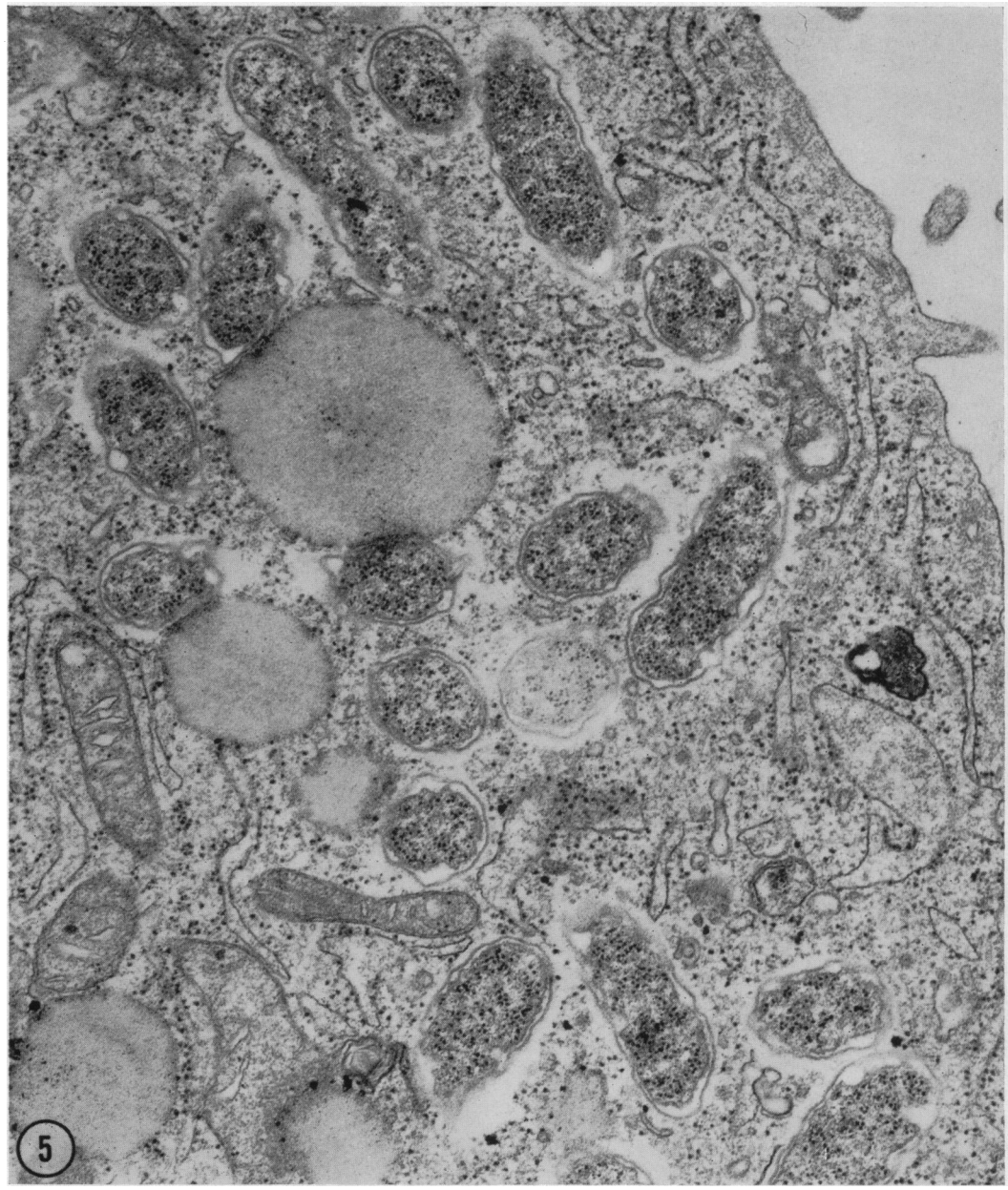


FIG. 5. *Rickettsia rickettsii distributed among the organelles of the host-cell cytoplasm. Note that the host-cell cytoplasmic ground substance frequently extends up to the rickettsial cell wall. At other places, a lucent space separates the cell wall from the host-cell cytoplasmic ground substance, and this space is presumed to be artifact.* × *45,000.*

side by side (Fig. 13), or were sometimes tightly wrapped around one another (Fig. 12).

Each organism was bounded by a rippled tri-layered structure (the cell wall) and a tri-layered plasma membrane (Fig. 10). The plasma membrane was frequently not well demonstrated. In this circumstance, the close juxtaposition of the host membrane which lines the vacuole could be mistaken for the cell wall, and the cell wall could be mistaken for the plasma membrane, explaining

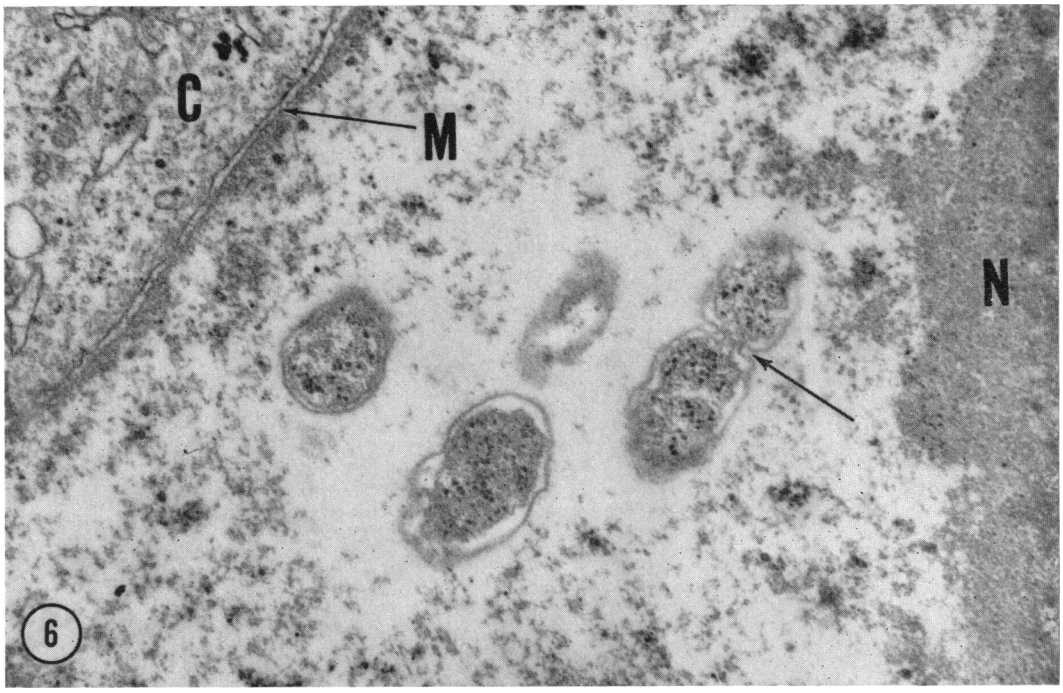


FIG. 6. *Rickettsia rickettsii within the nucleus. The host-cell cytoplasm (C), nuclear membrane (M), and nucleolus (N) are indicated. One of the rickettsia is caught in the process of division (arrow).* × *45,000.*

the initial impression that the organisms were free in the host cytoplasm. The organism in the left half of Fig. 13 illustrates this point.

The internal structure of the *R. sennetsu* agent was made up of rather prominent ribosomes and irregular pale patches containing DNA strands. In some organisms, the ground substance was electron-dense, and, when small, such organisms looked quite like the elementary bodies of ornithosis except that they lacked the eccentric condensation of dense homogeneous material (Fig.15). Pocket-like invaginations of the plasma membrane were seen which in other planes of section looked like small vacuoles.

Ornithosis virus. The various stages of the reproductive cycle of ornithosis were identified. Polygonal "giant bodies," 1 to 2 μ in diameter, were tightly packed in a membrane-lined inclusion in the host-cell cytoplasm, with no space between the organisms or between the organisms and the host membrane lining the inclusion vacuole (Fig. 16). Although the tight packing of the organisms made identification of the individual membranes difficult, each giant body seemed to be bounded by a tri-layered membrane (cell wall) and a second ill-defined membrane just beneath the outer membrane. The internal material consisted of a milieu of strands (presumably DNA) and granules 100 to 130 A in diameter (presumably ribosomes).

The "initial bodies" or "large bodies" were also irregular in shape, but smaller (0.45 to 1 μ). They were not tightly packed, but were separated from one another within the membrane-lined vacuole (Fig. 17). Each initial body was bounded by a tri-layered membrane (cell wall), and, again, there was a second membrane just beneath the outer membrane (Fig. 18). When the internal material was slightly retracted from the outer membrane (cell wall), the membrane was most easily seen as a layer of increased density which bounded the retracted internal material. The internal structure was similar to that of the "giant bodies," but in some organisms the DNA strands were more prominent and seemed to form a discrete central nuclear area (Fig. 18).

Stages between the initial bodies and the "elementary bodies" (the latter are described in the next paragraph) have been called "intermediate forms" (Fig. 19). These possessed central condensations of dense material in close association with the DNA strands. Usually, the central dense material was surrounded by a pale zone containing the DNA strands, which in turn was surrounded by a moderately dense cytoplasm containing ribosomes. As with the other

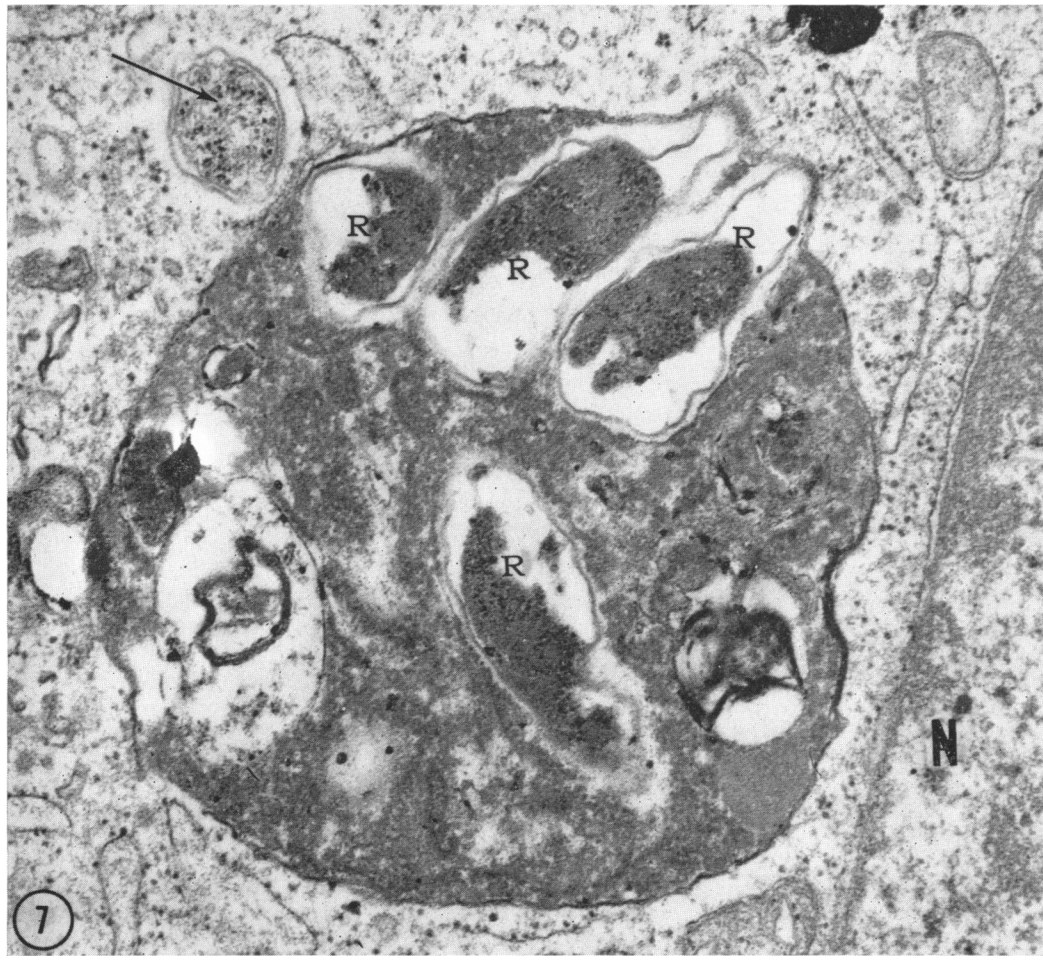


FIG. 7. *Degenerate Rickettsia rickettsii (R) within a digestive organelle of the host cell. A healthy rickettsia is in the host cell cytoplasm (arrow). A portion of the host-cell nucleus (N) is indicated.* × *45,000.*

stages, an ill-defined membrane was seen just beneath the tri-layered structure (cell wall) which bounded the whole organism.

Although the vacuoles in the host-cell cytoplasm sometimes contained only giant bodies or initial bodies, vacuoles containing "elementary bodies" (or "mature particles") always contained other forms as well (Fig. 20). The internal material of the elementary bodies was dense and usually retracted from the limiting membrane (Fig. 21). The central density of the intermediate form was now eccentric. It was usually homogeneous, but sometimes had the appearance of a closely packed tuft of fibrils. The cytoplasm consisted of a tag of very dense ground substance containing ribosomes. The ribosomes were more prominent than in the other stages because: (i) they were more concentrated; (ii) they were more densely stained; and (iii) they were slightly larger (100 to 150 A).

Dumbbell shapes indicating division were observed in the elementary bodies (Fig. 24), as well as in the intermediate forms (Fig. 23) and the initial bodies (Fig. 22). Rarely, as also reported by Higashi (1965), initial bodies seemed to contain smaller initial bodies (Fig. 25); however, the significance of this arrangement in terms of the reproductive cycle is not known.

Mycoplasma. *Mycoplasma* were easily recovered on agar plates from the infected BS-C-1 cultures, but only a few organisms were identified in seven separate cultures examined by electron microscopy. The organisms were extracellular and irregular in shape, sometimes in the form of an

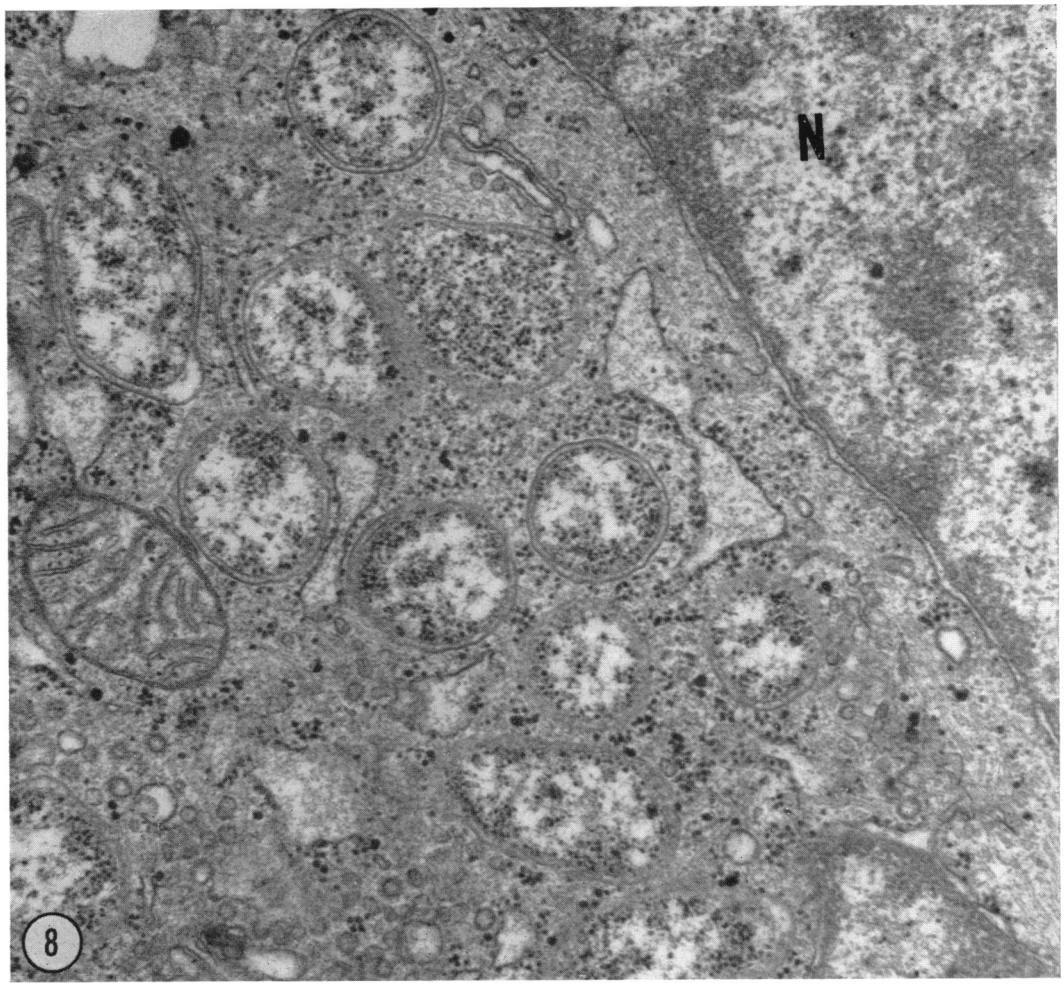


FIG. 8. *Rickettsia tsutsugamushi within the host-cell cytoplasm. The cell wall and plasma membrane are easily seen on several of the organisms. The pale areas containing fine DNA strands (nuclear zones) are somewhat more prominent in this species than in the others. The host-cell cytoplasmic ground substance extends up to the cell wall of the organisms. A portion of the host-cell nucleus (N) is included in the field.* × *45,000.*

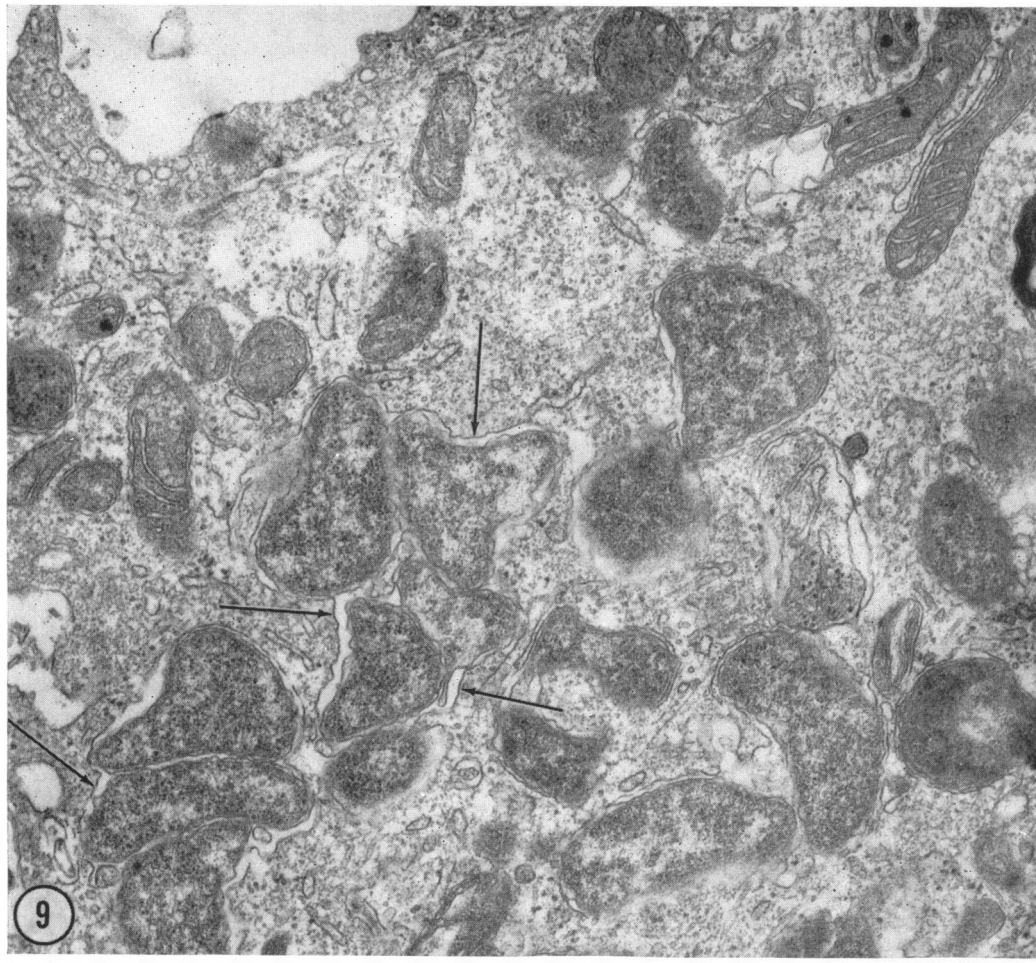
elongated filament (Fig. 26). They lacked a cell wall, being bounded only by a unit membrane. Internal material consisted mainly of ribosomes in a gray ground-substance, but strands of DNA were seen either collected together as a nuclear zone or randomly intermingled with the cytoplasmic components.

Despite the paucity of *Mycoplasma* observed in the fixed preparations, there was a marked effect of this agent on the morphology of the BS-C-1 cells growing in culture. Examination of Giemsa-stained cover-slip preparations of the cells 7 to 21 days after infection with *Mycoplasma* revealed that there were many fewer BS-C-1 cells present than in the uninfected control cultures. Moreover, many of these were multinucleate giant cells.

DISCUSSION

With the uniform conditions employed in these studies, the rickettsial, PLT, and *Mycoplasma* organisms were readily distinguishable from one another. However, it should be reemphasized that under less rigid conditions certain pleomorphic forms of any of the agents might be confused with one another.

The rickettsiae examined in these studies differ from one another in several respects, such



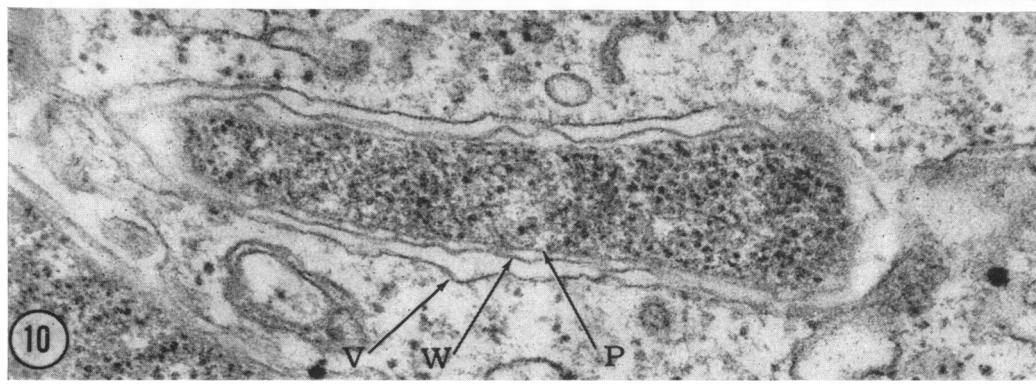


FIG. 9. *Rickettsia sennetsu within vacuoles in the host-cell cytoplasm. The membrane of the vacuoles can be seen in several places (arrows).* × *31,000.*

FIG. 10. *Higher magnification of a vacuole containing an elongated Rickettsia sennetsu. The membrane of the vacuole (V), the cell wall (W), and the plasma membrane (P) are indicated.* × *70,000.*

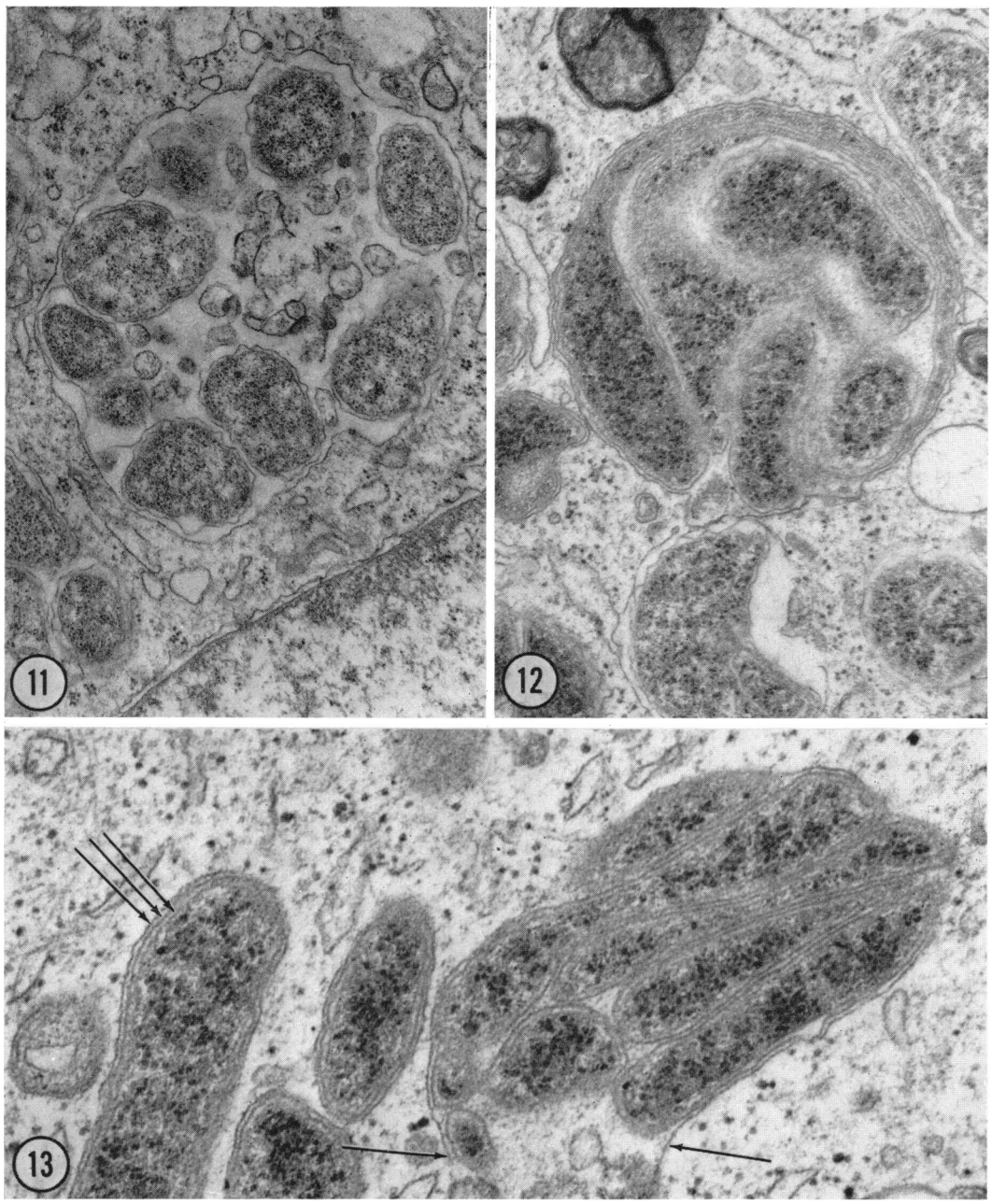


FIG. 11. *Rickettsia sennetsu in a distended vacuole. In this field, the fact that the organisms are enclosed in a membrane-lined vacuole is clearly demonstrated. Note that each organism is bounded by a cell wall and a plasma membrane. The overall appearance is quite like the initial bodies of ornithosis.* × *30,000.*

FIG. 12. *Rickettsia sennetsu twisted around each other. They are enclosed in a membrane-lined space (vacuole) in the host cytoplasm. The closely applied membrane of the vacuole can be followed around the entire circumference.* × *30,000.*

FIG. 13. *Rickettsia sennetsu in host-cell cytoplasm. In the right half of the field, several organisms are tightly packed side by side in a vacuole. The membrane of the vacuole can be seen at the arrows. In the left half of the field are several organisms which look much like the other species of rickettsiae, but careful examination shows the presence of three structures (arrows) bounding the organism—the membrane which lines the vacuole, the cell wall, and the plasma membrane.* × *50,000.*

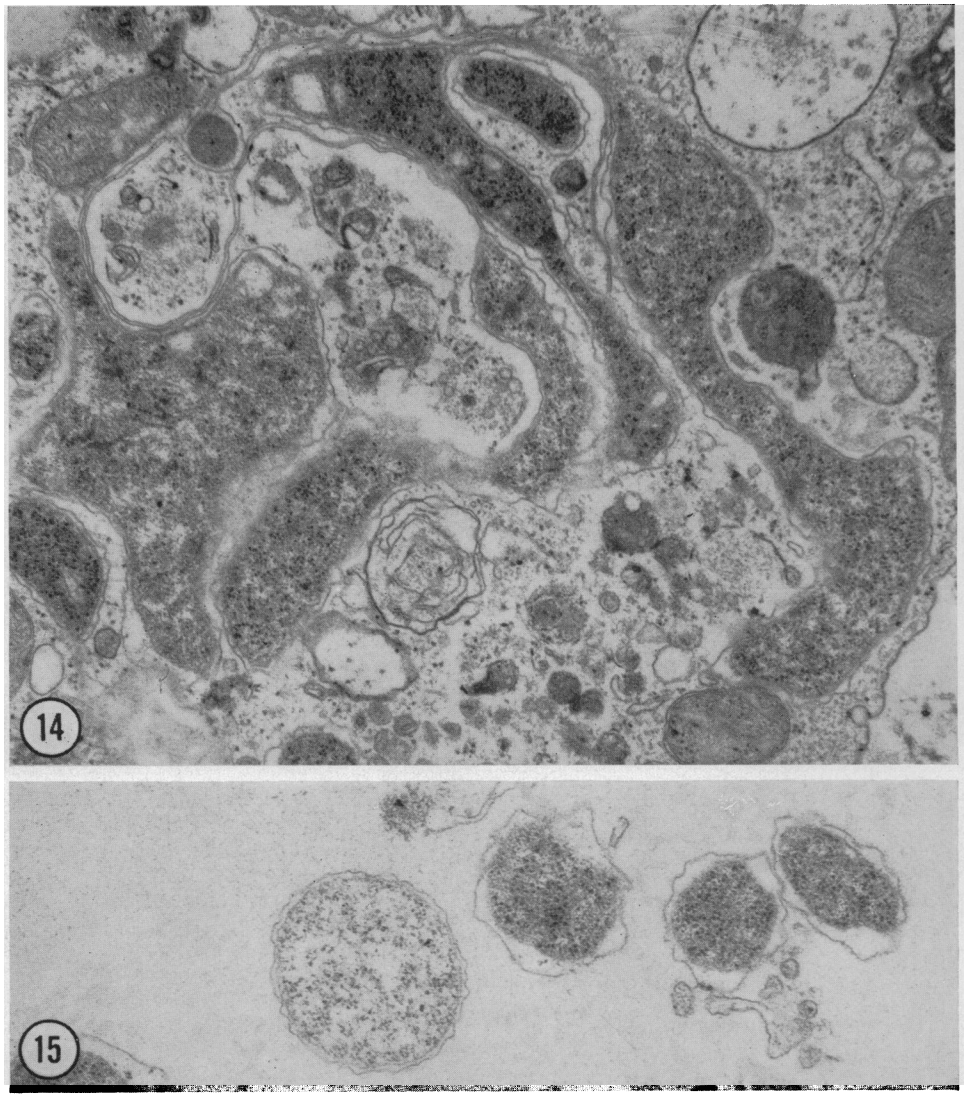


FIG. 14. *Rickettsia sennetsu with an irregular shape.* × *30,000.*

FIG. 15. *Rickettsia sennetsu. The three organisms in the right half of the field are small (0.5 to 0.6 μ) and have condensed cytoplasm with prominent ribosomes. Except for the absence of an eccentric dense homogeneous mass, these look similar to the "elementary bodies" of ornithosis. The organism just to the left of the center is quite similar to the "initial bodies" of ornithosis.* × *30,000.*

as length, arrangement in chains (*R. prowazeki*), the presence of lucent cytoplasmic spheres (*R. prowazeki*), and location in the host cell (*R. rickettsii*). However, with the exception of *R. sennetsu*, all of the rickettsiae exhibited the same basic morphology. The organisms were free in the cytoplasm of the host cell, were usually cylindrical in shape, were bounded by a cell wall and plasma membrane, and each contained ribosomes and DNA strands. These findings are in agreement with previously published electron micrographs of thin-sectioned *R. mooseri* (Wissig et al., 1956), *R. tsutsugamushi* (Schaechter et al., 1957*b*), and *R. quintana* (Ito and Vinson, 1965).

Tanaka and Hanaoka (1961) described the intravacuolar position of *R. sennetsu* and suggested that all rickettsiae are similarly located. They observed that the sennetsu rickettsia was

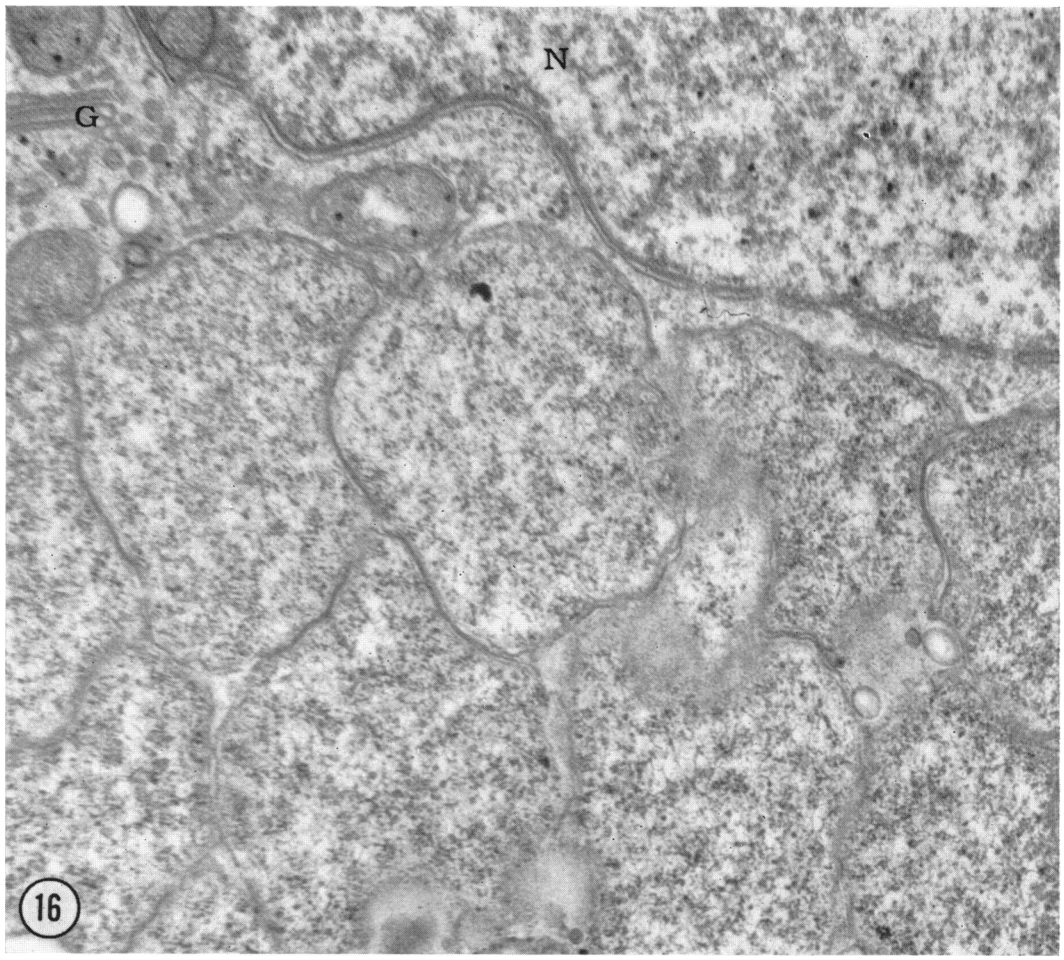


FIG. 16. *Tightly packed polygonal "giant bodies" of ornithosis, each bounded by a cell wall. The internal content is a mixture of ribosomes and DNA strands. They are crowded in a membrane-lined inclusion in the host-cell cytoplasm. The host-cell nucleus (N) and Golgi zone (G) are indicated.* × *45,000.*

bounded by a cell wall and enclosed by a membrane of host-cell origin. Since the techniques then available apparently did not permit visualization of the plasma membrane, the authors concluded that the two structures (plasma membrane and cell wall) which had been reported to bound other rickettsiae were, in fact, the cell wall of the rickettsia and the vacuolar membrane of the host cell. However, in this study and that of Ito and Vinson (1965), both extracellular and intracellular organisms are enclosed by only two structures (the cell wall and the plasma membrane), except for *R. sennetsu* which has an additional enclosing membrane of host-cell origin. Thus, it is clear that only *R. sennetsu* is enclosed in a vacuole.

The sennetsu agent differed from the other rickettsiae in other respects: the organisms were pleomorphic and often tightly packed or wrapped around each other in a tight ball. It is apparent that the morphology of *R. sennetsu* and its relationship to the host cell are strikingly different from the other rickettsiae. On the other hand, no evidence of a life cycle was observed, and, as pointed out by Tanaka and Hanaoka (1961), there is little morphological support for the hypothesis that *R. sennetsu* may be related to the PLT group.

The thin-sectioning techniques used in electron microscopy eliminate the difficulty encountered in Giemsa-stained whole-cell preparations of distinguishing the intranuclear organisms from those

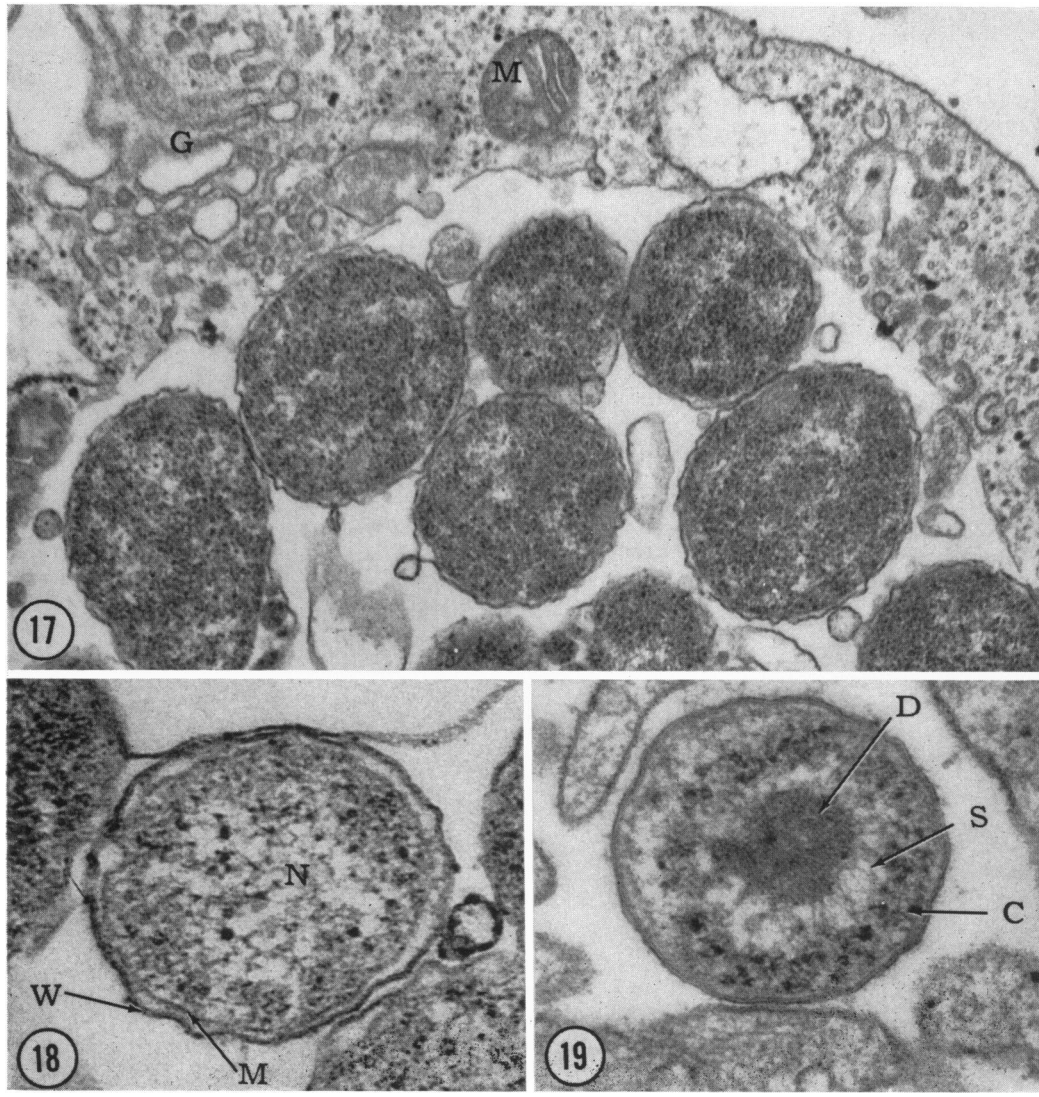


FIG. 17. *"Initial bodies" of ornithosis. These are smaller than the "giant bodies" shown in Fig. 16, and not so tightly packed. They are confined to a membrane-lined space in the host-cell cytoplasm. The host-cell Golgi apparatus (G) and a mitochondrion (M) are indicated.* × *45,000.*

FIG. 18. *Higher magnification of an "initial body' of ornithosis. Note the cell wall (W) and the cell membrane (M). In this initial body, the DNA strands are concentrated centrally to form a nuclear area (N) while the cytoplasmic components have migrated peripherally.* × *90,000.*

FIG. 19. *"Intermediate form" of ornithosis. Homogeneous dense material (D) has formed within a central area of DNA strands (S), and the peripheral cytoplasmic components (C) include prominent ribosomes.* × *90,000.*

in the cytoplasm which are merely near or on the nucleus. Thus, the observations of Schaechter, Bozeman, and Smadel (1957*a*) describing intranuclear *R. rickettsii* were confirmed here. However, as these authors observed, most of the organisms present were in the host-cell cytoplasm. These observations are in contrast to those of Pinkerton and Hass (1932), who reported that the majority of spotted fever rickettsiae were in the nucleus, and few were in the cytoplasm. The

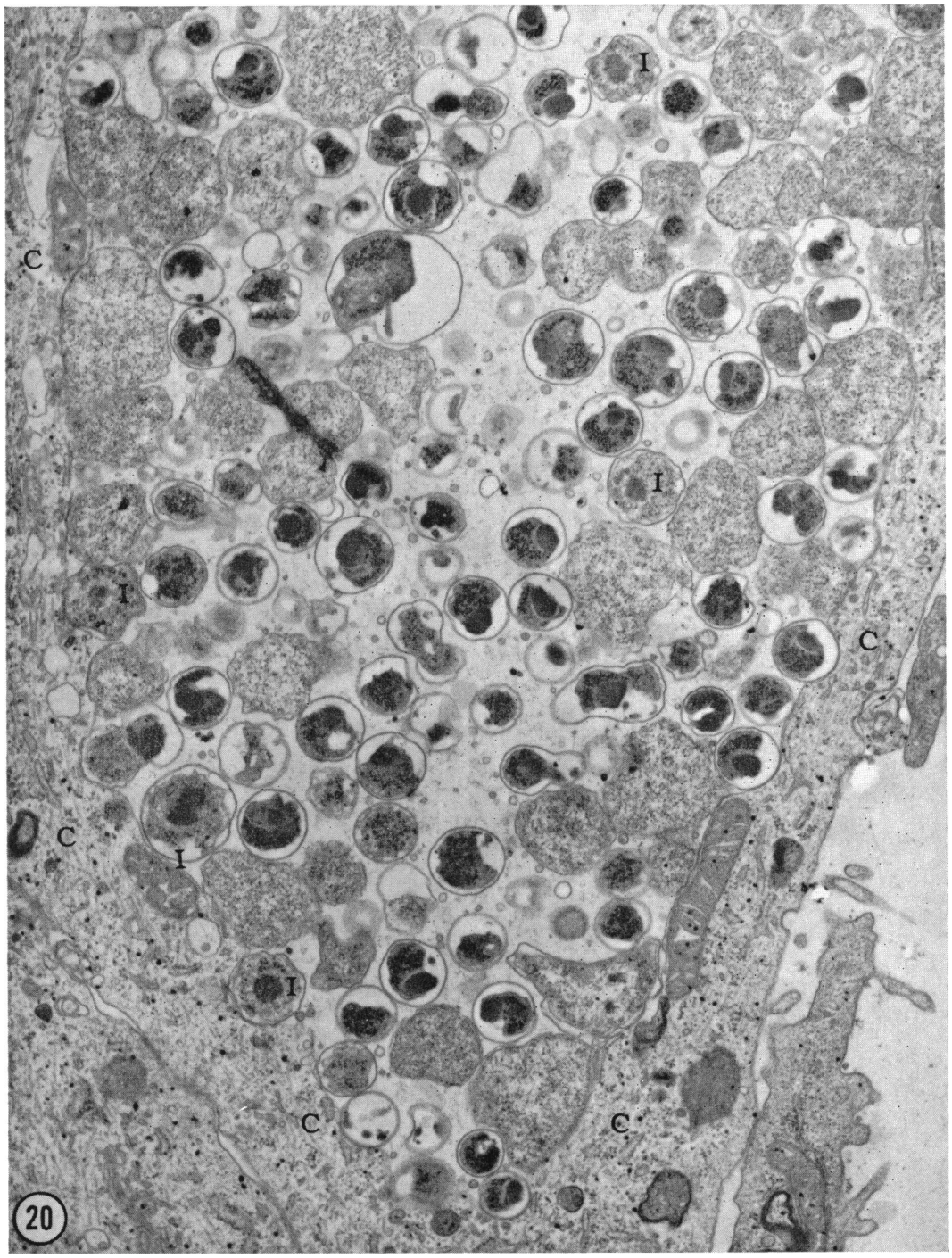


FIG. 20. *Portion of a vacuole containing ornithosis in late stages of development. Many "elementary bodies" (or "mature particles") are seen, along with a few "intermediate forms" (I) and "initial bodies." The membrane lining the vacuole is obvious in several places. The thin rim of host-cell cytoplasm (C) contains several mitochondria and other organelles.* × *15,000.*

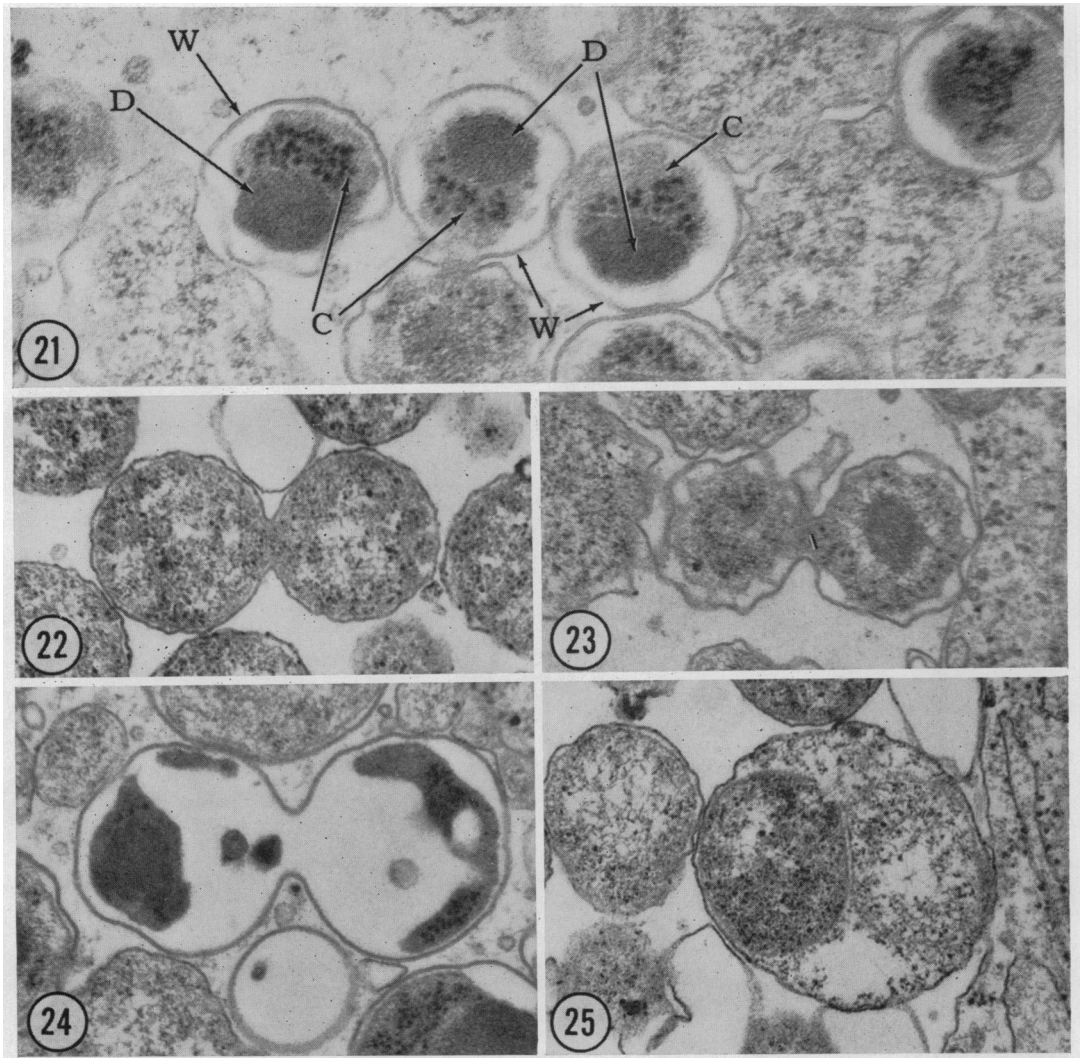


FIG. 21. *Higher magnification of three "elementary bodies" of ornithosis. The homogeneous dense material (D) is eccentric, and ribosomes are prominent in the cytoplasmic tag (C). These internal components are retracted from the cell wall (W).* × *70,000.*

FIG. 22. *Dumbbell-shaped "initial body" of ornithosis, indicating binary fission.* × *45,000.*

FIG. 23. *Dumbbell-shaped "intermediate form" of ornithosis.* × *45,000.*

FIG. 24. *Dumbbell-shaped "mature form" of ornithosis.* × *45,000.*

FIG. 25. *"Initial body" of ornithosis which seems to contain another, somewhat more dense "initial body" within.* × *45,000.*

extent to which these organisms invade the nucleus may depend on the particular host cell employed (Schaechter et al, 1957*b*).

In the most recent studies of PLT organisms (Armstrong and Reed, 1964; Higashi, 1965), a life cycle without a morphological eclipse phase has been demonstrated. Although Higashi (1965) noted binary fission of particles as late as the "intermediate form" stage, Fig. 22 to 24 indicate that division occurs in all stages, including the elementary body stage.

The cell-wall preparations of meningopneumonitis obtained by Jenkins (1960) coupled with the biochemical studies of Perkins and Allison (1963), in which muramic acid was found to be present in several members of the PLT group, are firm

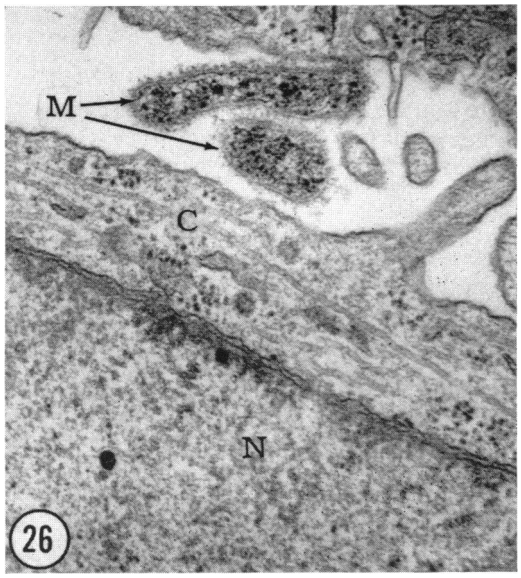


FIG. 26. *Mycoplasma hominis type I in an extracellular location (M). It is in the form of a narrow filament, bounded by a plasma membrane. There is no cell wall, but there is a flocculent material adherent to the outside of the organism. The cytoplasm (C) and nucleus (N) of the tissue culture cell are indicated.* × *45,000.*

evidence for the conclusion that the PLT group of organisms possesses cell walls analogous to those of rickettsiae and bacteria in at least some stages of the life cycle. It appears that the prominent tri-layered structure (often called the "outer limiting membrane") which bounds the ornithosis virus particles in its various stages (Fig. 16 to 25) is a true cell wall, under which there is a plasma membrane which is often poorly visualized. Only in the elementary body stage was the underlying membrane never visualized, an observation also reported by Mitsui et al. (1964). However, it seems probable that the visualized tri-layered structure of the elementary body is the cell wall even in this stage, and that the inner plasma membrane is retracted along with the internal components, and thus is not readily seen.

The presence of only a few *M. hominis* organisms in the fixed preparations of BS-C-1 cultures was unexpected, since organisms were recovered from the tissue culture fluids. Inability to see *Mycoplasma* may have resulted from a low titer of organisms in the tissue cultures. Whether any of the organisms were in the intracellular position could not be determined; careful searching of the cells did not reveal the presence of structures identifiable as *Mycoplasma*.

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