

STUDIES OF THE ULTRASTRUCTURE AND RIBOSOMAL ARRANGEMENTS OF THE PLEUROPNEUMONIA-LIKE ORGANISM A5969

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ABSTRACT

Thin-section electron microscopy, together with isolation of cellular organelles by differential centrifugation and chemical analysis, has been used to investigate the ultrastructure of the avian pleuropneumonia-like organism A5969. Each cell (approximate diameter 5500 Å) was surrounded by a 150 Å plasma membrane. In the center of the cell was an unbounded area, granular in appearance and containing the cell's DNA. The periphery of the cell contained granules of several different sizes and densities. The most dense particles (150 Å) corresponded to the 78S ribosomes. These particles exhibited two predominant arrangements: (a) sometimes they showed cubic packing; (b) most arrays, however, were consistent with cylindrical arrangements of approximately 50 particles. Bundles of up to 18 arrays were observed. Structured blebs have been found protruding from the surface of log phase cells.

INTRODUCTION

In the present studies, reported briefly elsewhere (Maniloff, Barnett, and Morowitz, 1963), we have undertaken a morphological examination of the pleuropneumonia-like organism A5969 (*Mycoplasma gallisepticum*). The principal method employed was thin-section electron microscopy. Because the cells are small and relatively simple, it has been possible to examine all of the fine structural details within the resolution of the methods employed. These consist of plasma membranes, nuclear areas, ribosomes, ribosomal arrays, and surface blebs. There appear to be no other structures in the cell which are larger than 100 Å in the smallest dimension.

MATERIALS AND METHODS

The organism used in this study was the A5969 strain of the avian pleuropneumonia-like organism (PPLO), *Mycoplasma gallisepticum* (Tourtellotte and Jacobs, 1960). It was grown on medium prepared as follows: 20 gm tryptose (Difco Laboratories Inc., Detroit), 5 gm NaCl, 5 gm Tris, and 1000 ml H₂O. The pH was adjusted to 8.0 with HCl, and the broth was autoclaved, supplemented with 50 ml of sterile 20 per cent glucose and 10 ml Difco PPLO serum fraction, and inoculated. A 4.5 ml inoculum from a 24 hour culture was used per 2-liter culture. The cells were harvested in the exponential or the early stationary phase by centrifugation for 10 minutes at 14,350 g.

For ultrastructure studies, the pellets from a 2-liter culture were transferred to glass weighing bottles and

fixed. The fixation was done either for 2 hours in a 1 per cent osmium tetroxide solution, in pH 7.4 Veronal-acetate containing added sucrose (Caulfield, 1957), or for 1 hour in a 6.25 per cent solution of glutaraldehyde in 0.1 M sodium cacodylate, final pH 7.2 (Sabatini, Bensch, and Barnett, 1963). Glutaraldehyde fixation was followed by an overnight wash in cold 0.1 M cacodylate, and in some instances this was followed by a second fixation in osmium tetroxide. After fixation, the pellets were cut into small blocks and dehydrated in increasing concentrations of ethanol as follows: 5 minutes each in 25, 50, and 70 per cent ethanol; 10 minutes each in 80, 90, and 95 per cent ethanol and two 15-minute washes in 100 per cent ethanol. This was followed by two thirty-minute washes in propylene oxide. Each block was left overnight in a mixture of equal parts of propylene oxide and embedding mixture. Each was then embedded in a mixture of 5 ml Epon (Shell Chemical Co., New York) mixture A, 5 ml Epon mixture B, and 0.133 ml accelerator (Luft, 1961).

The blocks were then sectioned on a Porter-Blum microtome with glass knives. Sections giving pale gold or silver first order interference colors were picked up on either Formvar-coated 200-mesh copper grids or bare 400-, 500-, or 1000-mesh copper grids. Sections more stable in the electron beam, and with greater contrast, were achieved with the use of uncoated grids. The grids with mounted sections were then stained either in saturated uranyl acetate solution in 50 per cent ethanol for 15 or 30 minutes, or in an aqueous solution of lead hydroxide for 15 or 30 minutes (Karnovsky, 1961). After staining and washing, the grids were examined with an Akashi Tronscope-50 electron microscope.

For examination of subcellular fractions, 10-minute sonicates (using a Mullard Ultrasonic 1000 Watt Generator, Type E-7590A) were prepared from fresh log phase pellets resuspended in 0.01 M Tris-0.004 M succinate-0.01 M magnesium buffer, pH 7.4. Fractionation was done by differential centrifugation as follows: 10 minutes at 6,600 g, 15 minutes at

14,350 g, and 2 hours at 92,660 g. Part of each pellet was fixed in glutaraldehyde and prepared for microscopy as described above. The nucleic acid was extracted from the remainder of each pellet by putting the pellets, in 5 per cent trichloroacetic acid, into a boiling water bath for 10 minutes. The precipitate was removed by centrifugation and the supernatant analyzed for DNA by a modified indole method (Keck, 1956).

RESULTS

In all the micrographs examined, the ultrastructural details were similar, even with different preparative methods. Unstained sections of fixed material also revealed the same structures, but lacked differentiating densities.

Each cell, about 5500 Å in diameter (Figs. 1*a*, 2, and 3), was surrounded by a membrane 150 Å thick consisting of two dense lines, about 40 Å each, separated by a less dense area, about 75 Å. The outer dense line of this membrane was not equivalent to the basement membrane as seen in the cells of many metazoa. Unlike basement membranes (Fawcett, 1962; Rhodin, 1955), both dense lines at the PPLO surface appeared identical under all preparative procedures. In the center part of the cell was an unbounded area, granular in appearance, that did not stain densely with lead, but reacted strongly with uranyl acetate and was presumably the nuclear material (Figs. 1*a* and 2*a*).

The rest of the cell contained granules of several different sizes and densities. With either lead hydroxide or uranyl acetate, the most densely stained particles (approximately 150 Å) probably corresponded to the ribosomes which had been identified in cell homogenates. The dense particles exhibited two predominant arrangements, other than appearing as individual ribosomes

Abbreviations for Figures

m, plasma membrane

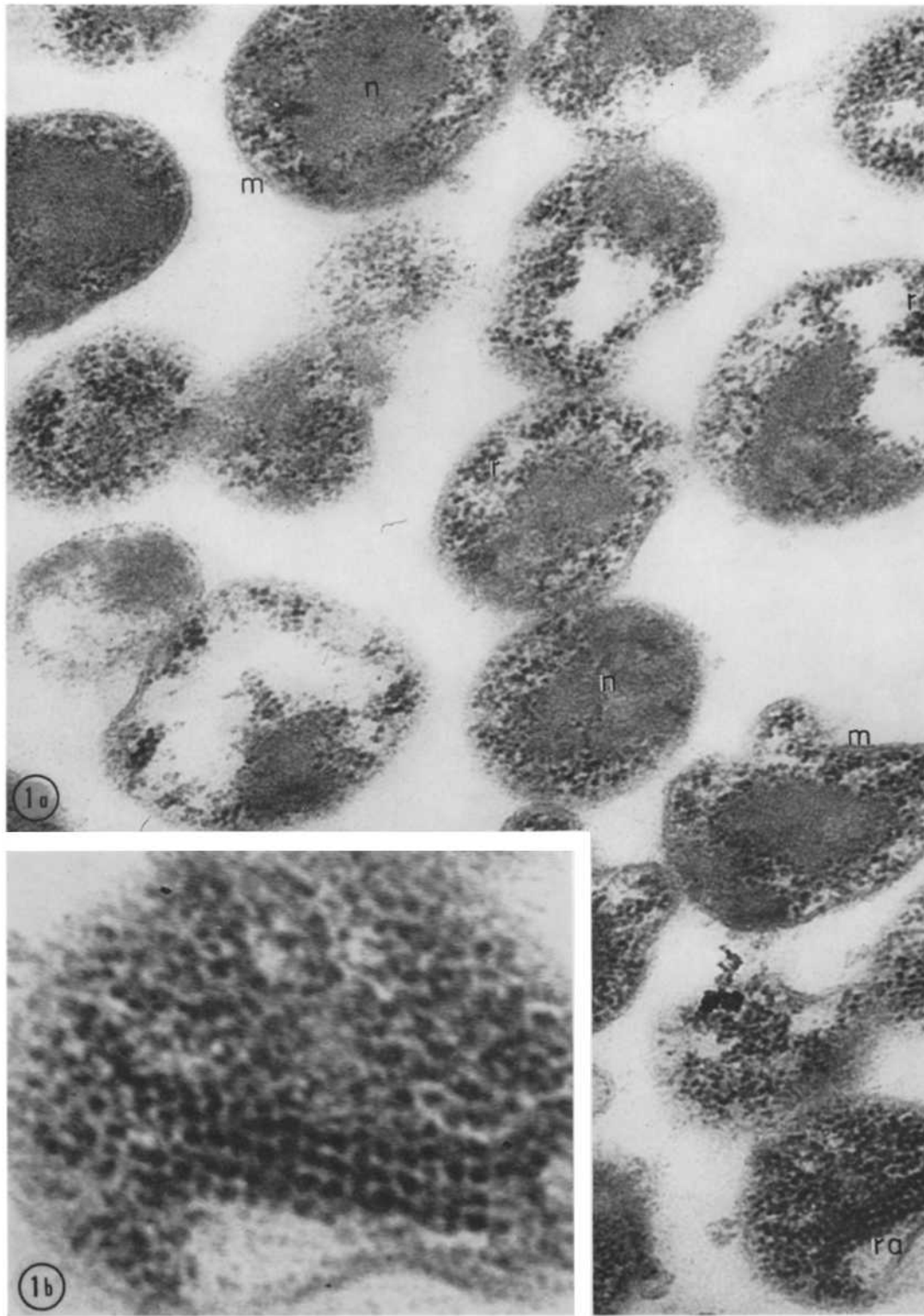
n, nuclear area

r, randomly oriented ribosomes

ra, ribosome arrangements

b, bleb

FIGURE 1 Stationary phase cells (PPLO A5969) fixed in glutaraldehyde and osmium tetroxide and stained with lead hydroxide. In Fig. 1*a*, Fine structural elements are clearly depicted and include: plasma membranes, nuclear areas, ribosomes and ribosomal arrays. The cell at the lower right shows cubic packing of ribosomes, 68,000 ×. Fig. 1*b* shows enlargement of ribosomes in cubic packing of cell in lower right of Fig. 1*a*. 176,000 × Fig. 1*a*, × 68,000; Fig. 1*b*, × 176,000.



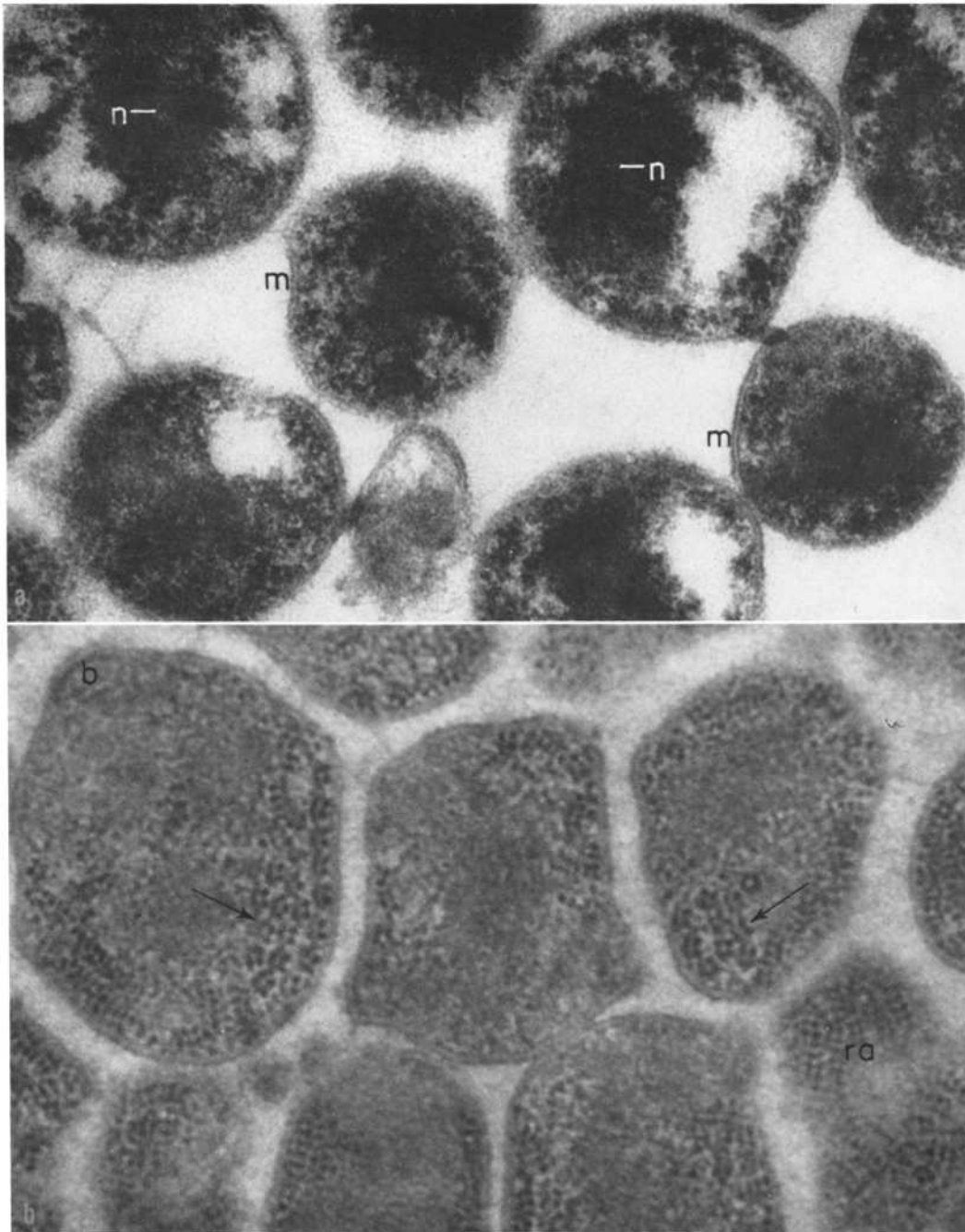


FIGURE 2 Cells fixed in glutaraldehyde and stained with uranyl acetate. Fig. 2 *a* shows section of stationary phase cells showing lack of ribosomal structures, relative to (*b*). The nuclear areas are intensely stained. Fig. 2 *b* shows log phase cells with complex and abundant arrangements of ribosomes. Cylinders, apparently cut perpendicular to the long axis (arrows), have hollow cores and four or five ribosomes, only one or two of which is in focus, in each cross-section. An elliptical bleb can be seen in one of the cells in this field. $\times 68,000$.

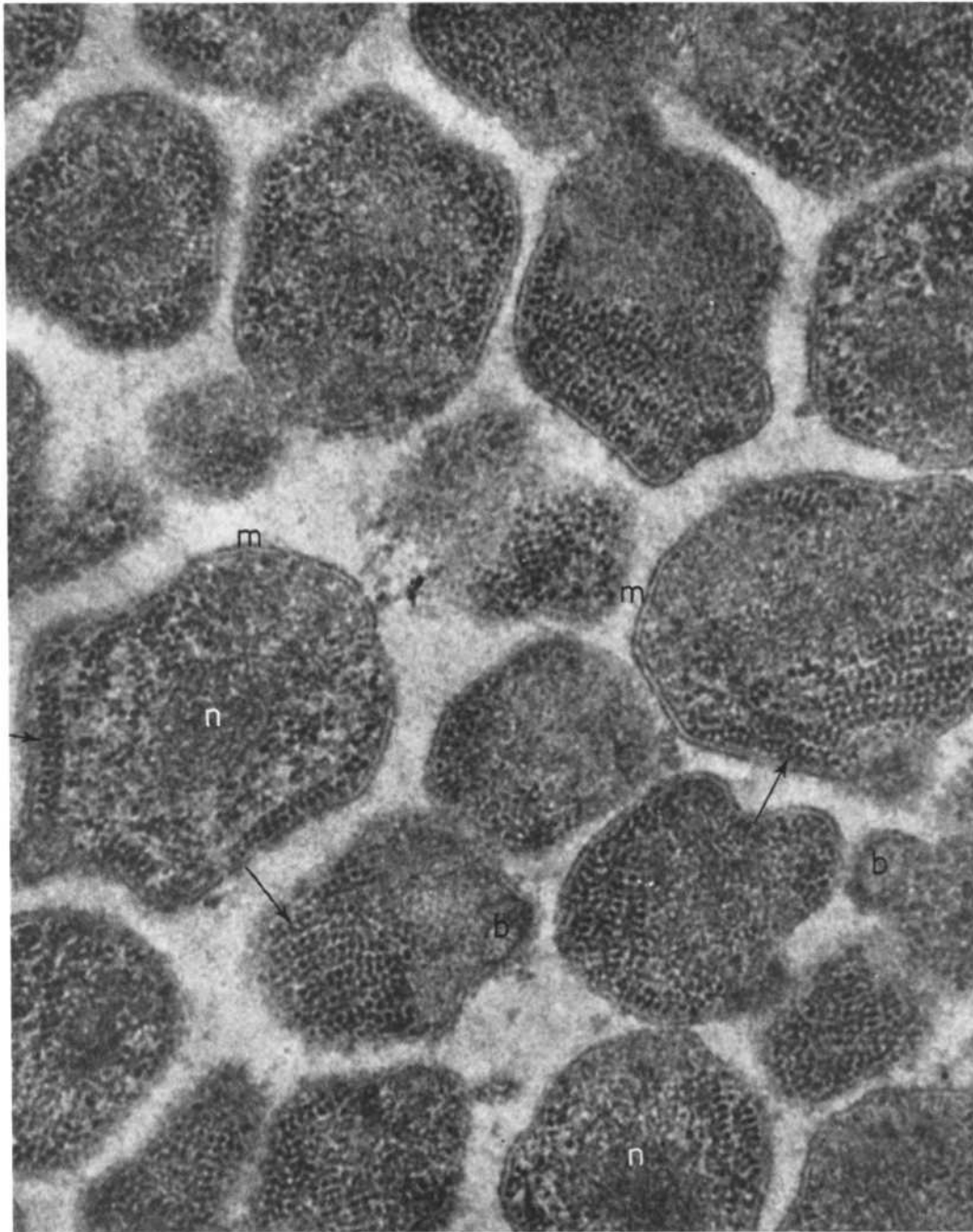


FIGURE 3 Log phase cells fixed in glutaraldehyde and stained with uranyl acetate. Cells show the ultrastructure described in the text. Cylindrical arrangements of ribosomes (arrows) can be seen packing the cytoplasm of the cells. In several places a longitudinal strand of less dense material appears to connect adjacent ribosomes. Several stages of bleb formation can be seen. $\times 68,000$.

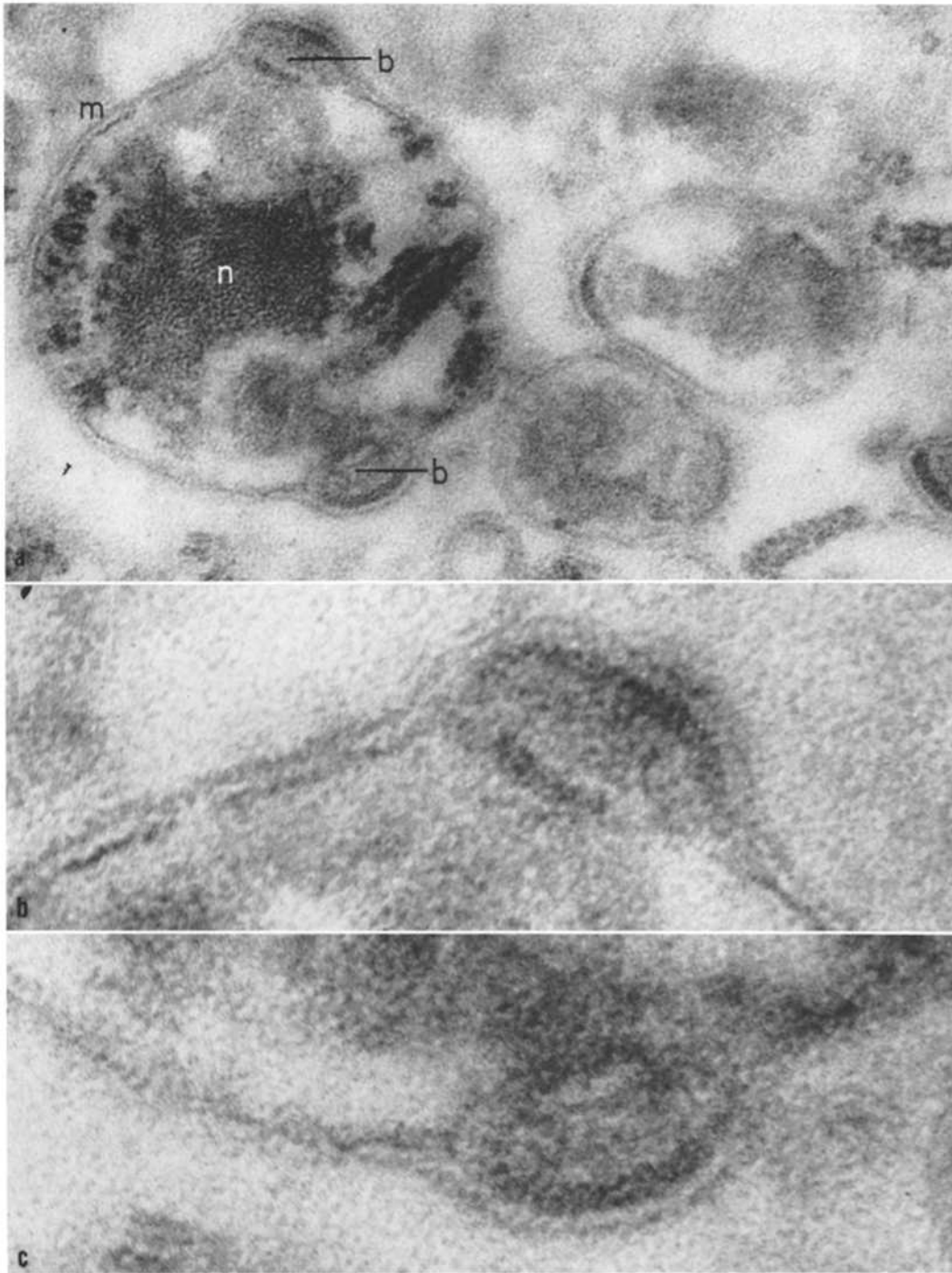


FIGURE 4 A portion of the low-speed cellular fraction fixed in glutaraldehyde and stained with uranyl acetate is shown in Fig. 4 *a*. The blebs showed the characteristic morphology. 102,000 \times . Figs. 4 *b* and 4 *c* show enlargements of the upper and lower blebs, respectively, of Fig. 4 *a*. \times 264,000.

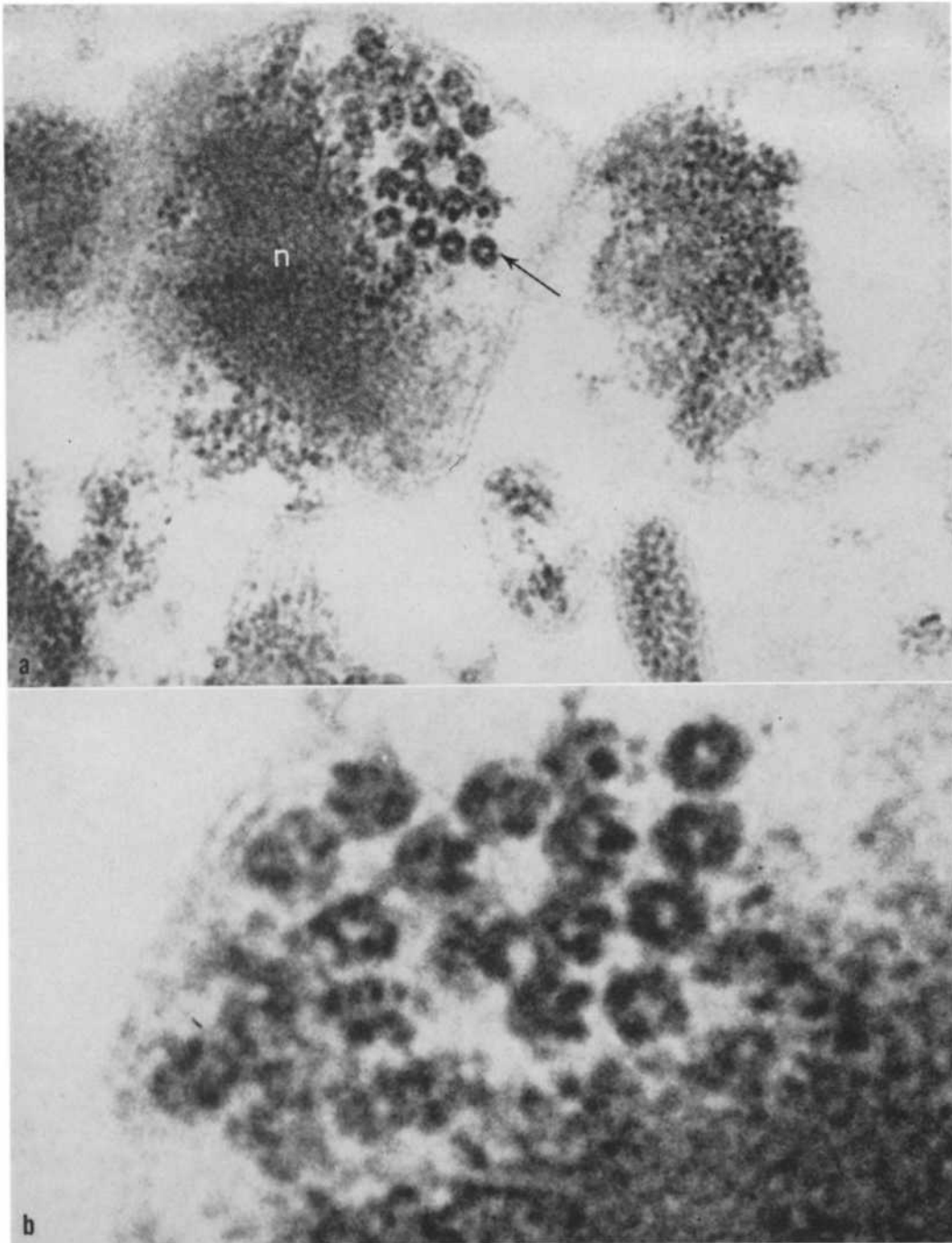


FIGURE 5 Figure 5 *a* shows low-speed fraction fixed in glutaraldehyde and stained with uranyl acetate. The section appears to be perpendicular to the cylinder axis and shows cross-sections of the ribosomal cylinders (arrow). 102,000 \times . Fig. 5 *b* is an enlargement of cylinder cross-sections in Fig. 5 *a*. Fig. 5 *a*, \times 102,000; Fig. 5 *b*, \times 264,000.

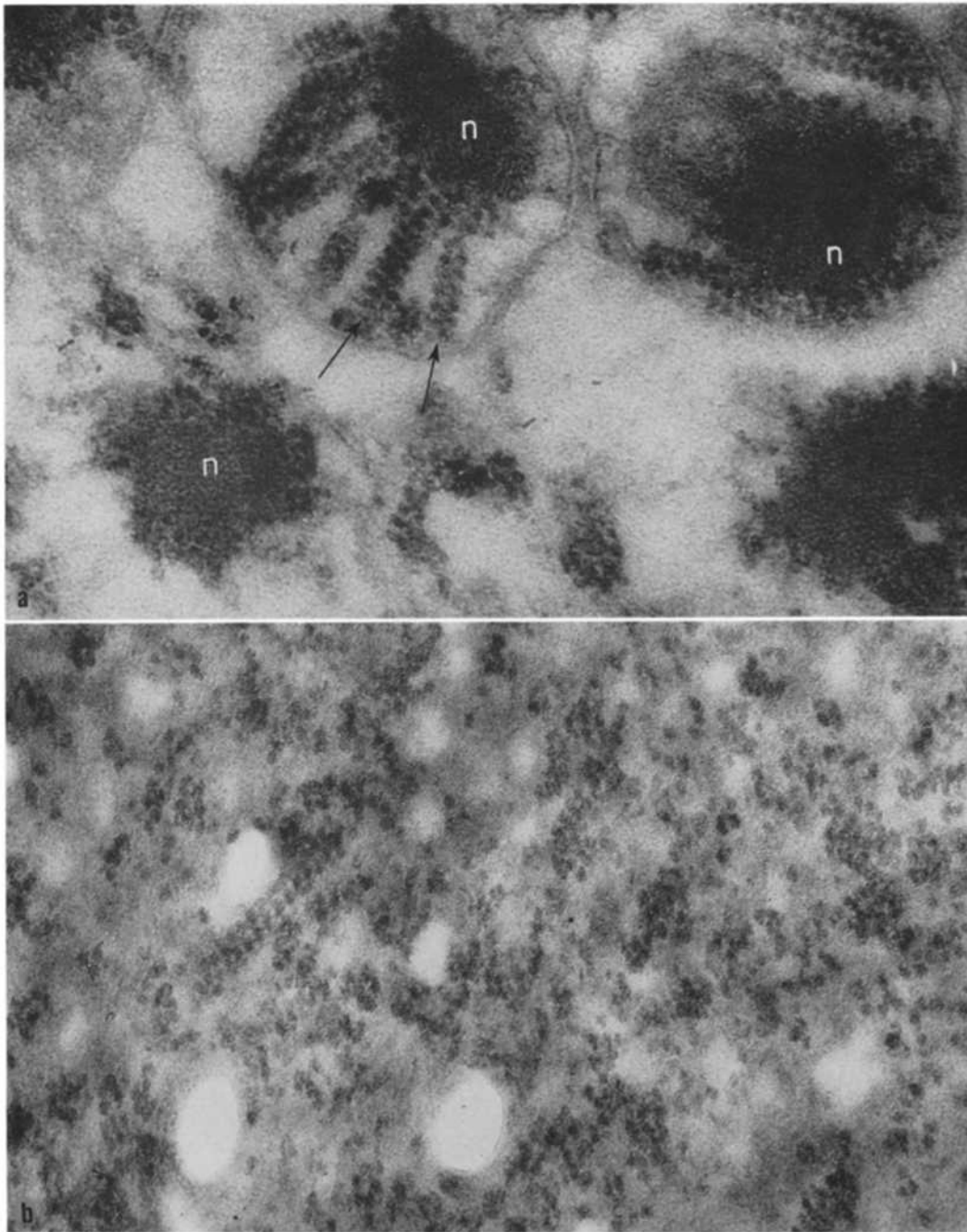


FIGURE 6 The fractions fixed in glutaraldehyde and stained with uranyl acetate. Low-speed fraction showing cylindrical, perhaps helical, arrangements of ribosomes (arrows), and discrete bodies of nuclear material with adherent ribosomes. Fig. 6 b depicts high-speed fraction showing single ribosomes and small linear arrangements of ribosomes. $\times 102,000$.

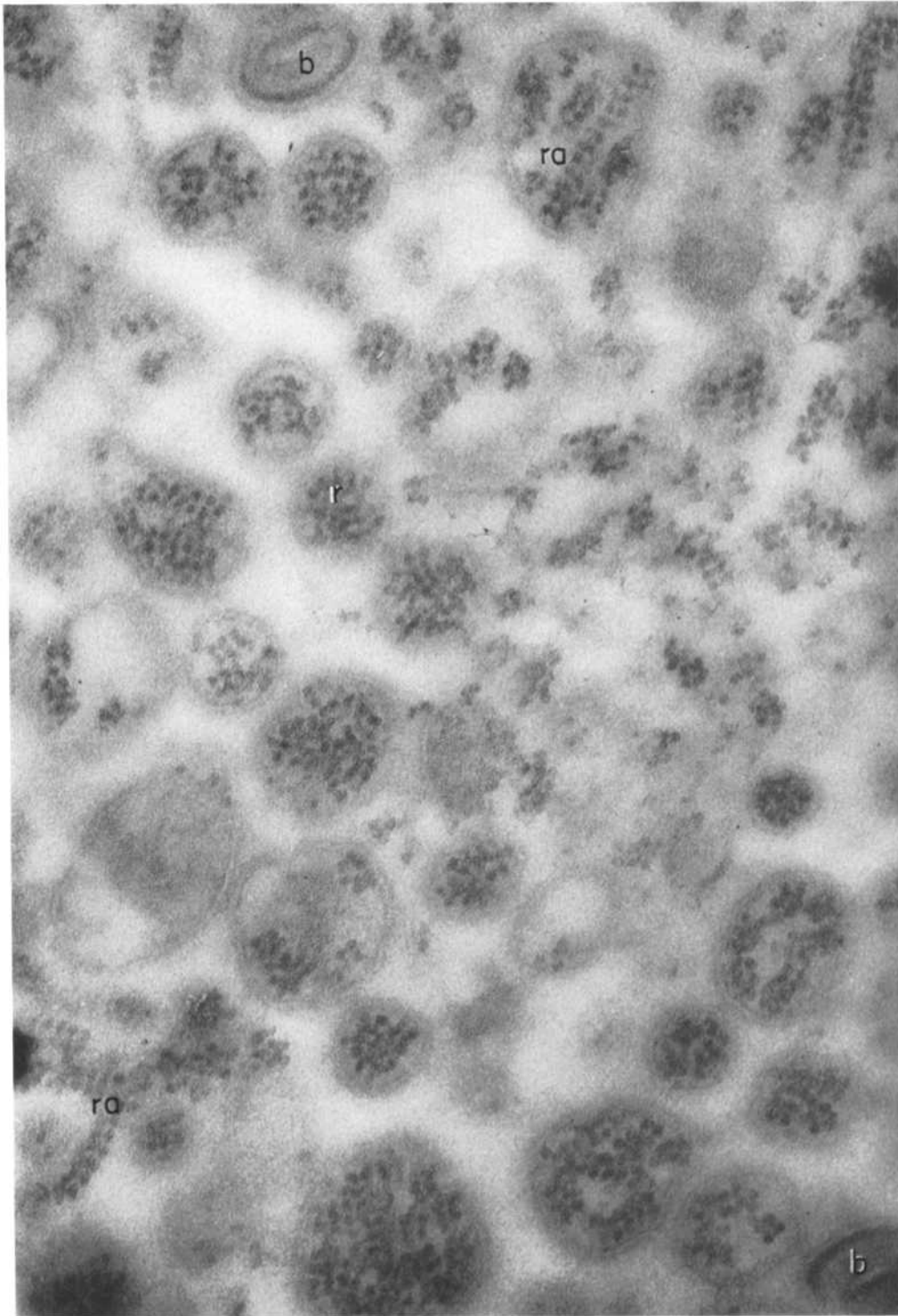


FIGURE 7 The medium-speed fraction fixed in glutaraldehyde and stained with uranyl acetate. The section shows ribosomal arrays, many of which are still within membranes, randomly arranged aggregates of ribosomes, and free blebs. $\times 102,000$.

(Fig. 1 *a*): (*a*) sometimes they showed cubic packing, typically measuring four by ten particles (Fig. 1 *b*); (*b*) most arrays of particles, however, were consistent with cylindrical arrangements, typically 1500 Å long (Figs. 3, and 6 *a*). Sections believed to be perpendicular to the cylinder axis indicated the presence of a hollow core (Figs. 2 *b* and 5). Such cross-sections also revealed four or five ribosomes in the plane of the section. These ribosomes did not appear to all be in the same plane, suggesting a staggered three-dimensional arrangement. Many more such arrays were seen in log phase cells than in stationary phase cells (Fig. 2 *b* as compared with Fig. 2 *a*). In fact, most log phase cells seemed to have the entire intracytoplasmic area packed with highly ordered ribosomal arrays. The stationary phase cells had less orderly ribosomal packing.

Cells in the exponential phase contained certain other morphological features, absent in stationary phase cells, apparently related to the replication process. Many cells contained protruding elliptical blebs, about 1600 Å by 1000 Å (Figs. 2 *b*, 3, and 4 *a*). The blebs were usually two in number and appeared at opposite ends of the cell. These protruding structures had a somewhat different appearance in different cells. In small protrusions, the contents were homogeneous (Fig. 3). When the protrusion of the bleb was more pronounced (Fig. 4), two elliptically shaped dense areas or plates were prominent. The larger of these plates was parallel to the surface of the protrusion, while the other dense region formed a convex plate between the bleb and the rest of the cell. The contents of the bleb between the two dense plates was homogeneous. In some instances, prior to the appearance of plates, the surface membrane of the cell covered the outer surface of the bleb. However, when the plates were pronounced in the bleb, this membrane appeared to split at the lateral margins of the bleb: the outer portion of the membrane covering the outer surface of the bleb, the inner portion of the membrane covering the inner surface of it (Figs. 4 *b* and *c*). This phenomenon appears to be quite different from that of the invaginations of membranes during spore formation in microorganisms (Glauert, 1962; Robinow, 1962).

The low-speed pellets from cell sonicates contained unbroken and partially extracted cells, ribosomal arrays, and discrete bodies of nuclear material with a few adherent ribosomes (Figs.

4 *a*, 5 *a*, and 6 *a*). This fraction contained all the morphologically identifiable nuclear material (Fig. 6 *a*) and was found by chemical analysis to be the only fraction containing DNA.

The medium-speed pellets contained mainly ribosomal arrays (Fig. 7) sometimes still within membranes, clumps of ribosomes randomly arranged, and free blebs. The high-speed pellets were known to contain particles with uncorrected sedimentation coefficients 78S and higher (Kirk). This fraction showed only single ribosomes and small linear arrangements of ribosomes (Fig. 6 *b*).

DISCUSSION

The extremely small cell size, small total number of macromolecules, and small amount of DNA per clone-forming unit of the pleuropneumonia-like organisms affords the chance of studying simple, but complete, autonomous self-replicating systems (Morowitz and Cleverdon, 1959). The simplicity of such a small cell has led to a reasonably complete description of the organism's ultrastructure. All organelles above the macromolecule level should have been observed by the techniques used. The list of elements of the ultrastructure of *Mycoplasma gallisepticum* includes:

1. MEMBRANE: The plasma membrane, which was the only membranous structure found in the cell, has a "unit membrane" appearance (Robertson, 1957) and the 150 Å thickness is in fair agreement with the 125 Å value indicated in electrical measurements (Schwan and Morowitz, 1962). In disrupted homogenates both dense components of the membrane retained the same relationship as found in the intact cell. This finding, together with the lack of polysaccharides and diaminopimelic acid (Morowitz, *et al.* 1962), suggests a unit structure, as opposed to the surface structures found in bacteria (Kellenberger and Ryter, 1958).

2. NUCLEAR MATERIAL: The DNA appeared to be in a compact nuclear area, similar to that observed in bacteria (Glauert, 1962). The use of a specific electron-dense stain for DNA, by a modification of the indium method of Watson and Aldridge (1961), showed that the stain reacted with material in the nuclear area (Morowitz *et al.*, 1964).

3. RIBOSOMES: Although single ribosomes were seen, most of the ribosomes in log phase cells occurred in cylindrical arrangements. The ribosomes stained with uranyl acetate and lead

hydroxide, as in other organisms, and were extracted by cold 10 per cent perchloric acid during the indium stain procedure (Morowitz *et al.*, 1964). Since the geometric packing of the ribosomes in the cylinder appeared to be staggered, it is possible that the cylinder is a helix.

4. REPLICATIVE STRUCTURES: Homogeneous areas and structured blebs (apparently two per cell) were formed during the replicative process. In view of the fact that structures suggesting a continuous transition from the seemingly unstructured protuberances to structured blebs have been seen, it seems probable that these represent different stages in the development of the same structure. This aspect of the cell's ultrastructure is discussed in another paper describing further studies of the organisms' replication (Morowitz *et al.*, 1964).

It has been possible to catalogue the machinery available to an autonomous self-replicating system in a fairly short list. The small amount of ultrastructure is obviously sufficient for the replicative process. The shortness of the list also suggests that all the structure is necessary; that the size of the organism has indeed served to select for the removal of all unnecessary organelles. A small organism could be expected to function within its spatial limitations in one of two extreme ways: either it could be completely structureless with all cellular processes being carried on in a homogeneous phase, or it could be highly structured thereby imposing a high degree of organization on all of its processes. The latter choice seems to have been made in *Mycoplasma gallisepticum*—generalizations cannot be made at this time, but further studies are indicated on other small organisms.

The replicative structures seem formidable for such a small cell, as is discussed elsewhere (Morowitz *et al.*, 1964).

The ordered ribosomal structures are larger and more complex than any similar structure yet reported. Ribosomal arrays, interpreted as being helical, but differing in appearance from those reported here, have been observed in undifferentiated embryonic rat (Behnke, 1963) and frog (Waddington and Perry, 1963) tissue. Most of the cylindrical arrangements of ribosomes reported in this paper were about ten ribosomes long, and most cross-sections showed five ribosomes. Hence, the arrangements are composed of

approximately fifty ribosomes, as large as the largest polysomes thus far described (Rich *et al.*, 1963) and apparently more complicated in superstructure.

Indeed, the PPLO ribosomal arrays, when viewed in cuts perpendicular to the long axes, exhibit a clustering into bundles of linear structures. The largest bundle seen contained 18 identifiable cross-sections of ribosomal arrays. These bundles have been seen both lined up along the plasma membrane and apparently radiating from the nuclear material.

It may be significant that these arrays are most prominently seen during the replicative process, whereas they are neither as abundant, prominent, nor complex during the stationary phase. This arrangement of ribosomes might suggest that certain key protein syntheses are required at this stage, and that the packing of ribosomes represents the geometry most favorable for these syntheses and for the resulting differentiation.

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Note Added in Proof: Since this paper was submitted, a study of the ultrastructure of fifteen *Mycoplasma* species has been published (Domermuth, C. H., Nielsen, M. H., Freundt, E. A., and Birch-Anderson, A., 1964, *J. Bacteriol.*, **88**, 727, 1428). All of the strains appeared to be spherical and, of the two *M. gallisepticum* strains in this study (strains JA and W), only one (JA) showed ribosomal structures and blebs similar to those reported here. However, recent studies in our laboratory, have indicated that, in our studies reported here, alterations in cellular morphology have arisen during preparation. In particular, the cell is rod shaped, not spherical, with the bleb forming one end of the rod. A manuscript describing these studies has been submitted for publication.

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