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ELECTRON MICROSCOPY OF *COWDRIA (RICKETTSIA) RUMINANTIUM* (COWDRY, 1926) IN THE ENDOTHELIAL CELLS OF THE VERTEBRATE HOST

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Abstract

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The ultrastructure of *Cowdria ruminantium* was studied in thin sections of choroid plexus from experimentally infected sheep. Glutaraldehyde fixation and osmium tetroxide postfixation were used. The organism developed within the confines of a membrane-bound vacuole in the cytoplasm of endothelial cells. Four different forms, or particles, of the organism could be identified: small, intermediate, large and very large or giant forms. The various forms differed not only in size but also in the appearance and distribution of the nucleoid and "cytoplasmic" material within their inner structure. Within any one vacuole, in the host cell cytoplasm, the organisms were of the same form or type. Multiplication of the organism took place mainly by binary fission of the small and intermediate forms. Infrequent evidence was found that reproduction may also take place by the process of multiple budding and endosporulation. Small forms of the organism were embedded in a well developed matrix. Small and large forms were seen extracellularly, lying free in the lumen of blood vessels. Rarely small forms were seen in vacuoles in the cytoplasm of monocytes. The taxonomic position of *C. ruminantium* is discussed.

Introduction

Cowdry (1926a) described the morphology of a Gram negative organism in the endothelial cells of experimentally produced cases of heartwater in ruminants. He named this organism *Rickettsia ruminantium*. An identical organism was also found by him (Cowdry, 1926b) in the intestinal epithelial cells of the tick *Amblyomma hebraeum* Koch, 1844. His description of the morphology of *R. ruminantium* was based on its appearance in paraffin sections from the vertebrate and invertebrate host tissues, and in smears made from the alimentary tract of the latter. The organisms were seen as very uniform coccus-shaped bodies from 0.2 to 0.5 microns in diameter. They occurred in dense masses, varying from a few individuals up to several hundred, in the cytoplasm of infected cells. In a single clump the organisms were of the same size.

Jackson (1931) and Jackson & Neitz (1932) used intimal smears from large blood vessels, stained with Giemsa's method, for observations on the morphology of this organism. Apart from a considerable variation in the size of the clumps or colonies they also noted a variation in the size of the individual organisms. Jackson & Neitz (1932) divided the organisms into small (coccoid), medium and large forms with intermediate stages between them. They pointed out that the contrast between the small and large forms is very striking and that pleomorphism is present. Larger forms assume a variety of irregular shapes varying from coccoid to ring, horseshoe and bacillary.

Rake, Alexander & Hamre (1945) questioned the validity of the classification of the causative agent of heartwater under the name *R. ruminantium*. These authors stated that this organism, while not distinctly either a *Rickettsia* or a member of the lymphogranuloma-psittacosis group, probably is related to both. They argued that it is entirely dissimilar from other *Rickettsiae* morphologically and that it was proven susceptible to sulphonamide chemotherapy (Neitz, 1940). No antigenic relationship between *R. ruminantium* and the lymphogranuloma-psittacosis group of organisms could, however, be demonstrated by them.

Both Moshkovski and Bengston (according to Haig, 1955) suggested that the agent of heartwater be named *Cowdria ruminantium*. With this Coles (1953) concurred.

This study concerns the ultrastructure of *C. ruminantium* in the endothelial cells of the vertebrate host.

Material and Methods

Two two-tooth Merino sheep, obtained from a heartwaterfree area, were used. Five millilitres of blood from an untreated natural case of heartwater in a sheep from the Rustenburg area, collected during the febrile reaction, were injected intravenously into each of these sheep. They were stabled and daily rectal temperatures were recorded. After the peak of the temperature reaction was reached and after the onset of symptoms the sheep were killed by electrocution. The cranium was immediately opened and the brain removed. Small pieces from the choroid plexus were dissected out and were then minced in a pool of fixative.

Specimens were fixed in 4 per cent glutaraldehyde in Millonig's buffer (Millonig, 1961) at pH 7.2 to 7.4, postfixed in buffered 2 per cent osmium tetroxide, dehydrated and embedded in Araldite* (Luft, 1961). From the Araldite blocks sections 1 to 2 μ thick were cut, stained with toluidine blue pyronin (Ito & Winchester, 1963) and examined under a light microscope. Suitable blocks were selected for ultrathin sectioning. The thin sections for electron microscopy were stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963).

Smears were prepared from the hippocampus of each brain, according to the method described by Purchase (1945), stained by the May-Grünwald-Giemsa method and examined by light microscopy.

Results

Light Microscopy

May-Grünwald-Giemsa stained hippocampal smears

Considerable variation occurred in the size of the organisms and in the size of the clumps or colonies in which they appeared. The organisms also varied in their reaction to the May-Grünwald-Giemsa staining method. The following different staining reactions and colonies could be distinguished:–

1. Small organisms which stained a reddish purple and occurred in large colonies [Plate 1 (1)].

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2. Medium sized organisms which stained a dark blue to purplish blue and occurred in large colonies [Plate 1 (2)].
3. Large organisms which stained a pale blue and occurred in small colonies [Plate 1 (3)].
4. Very large organisms which stained a pale blue or purplish blue and occurred in small colonies [Plate 1 (4)].

A sharp delineation between the different sized organisms was not always possible as intermediate forms between them occurred. This was also true for the staining characteristics with the May-Grünwald-Giemsa staining method. Except in the colonies containing very large forms, where a great variation in size of the individual organisms was noted, the organisms within each colony appeared to be fairly uniform in size.

The most frequent shape of the organisms was coccoid. However, various pleomorphic forms were seen, especially in colonies containing very large organisms and to a lesser degree in colonies consisting of small and medium organisms. The pleomorphic forms observed were horseshoe, ring and bacillary shaped. Colonies consisting of large organisms usually only contained coccoid forms.

Araldite sections stained with toluidine blue pyronin

Examination of the Araldite sections confirmed the observations described above for the hippocampal smears. The various morphological forms of the organism could be distinguished readily [Plate 1 (5, 6, 7 and 8)]. Structures resembling the small organisms were occasionally encountered extracellularly in the lumens of the vessels [Plate 1 (5)]. In colonies of the small, and in some instances of the medium sized organisms, the material in which they were suspended, the matrix, stained a faint blue with the toluidine blue pyronin staining method [Plate 1 (5 and 6)]. This staining of the matrix was not seen in colonies of the larger forms [Plate 1 (7 and 8)]. The difference in appearance between a colony in which the matrix is stained and one in which it is not stained is well illustrated in Plate 1 (6). In the latter the matrix appears as a clear translucent substance.

Electron Microscopy

Three distinct types of organisms could be recognised: small (0.49 μ in diameter), large (1.04 μ in diameter) and very large or giant (up to 2.7 μ in diameter). A fourth, less distinct form, apparently intermediate between the small and large forms (0.76 μ in diameter), was also present. Extreme pleomorphism made exact measurement difficult. The above values are averages of organisms measured in different colonies taken at random from each type. Although there is a very striking difference in size between the small and larger forms it is not obvious between the small and intermediate forms [Plate 2 (9 to 12)].

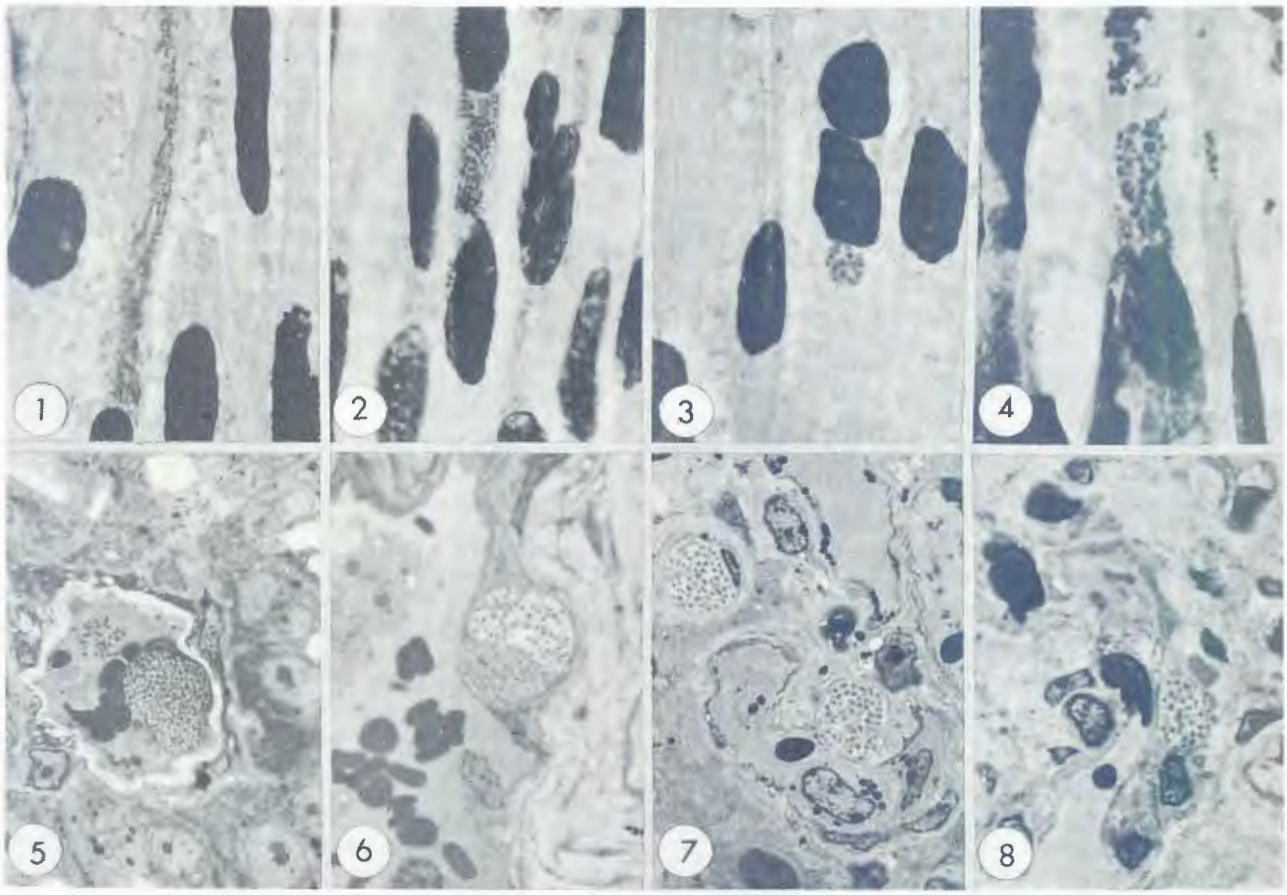

PLATE 1 Photomicrographs of *C. ruminantium* in hippocampal smears (1 to 4) stained by the May-Grünwald-Giemsa method and in Araldite sections (5 to 8), thickness circa 1.5 μ and stained toluidine blue pyronin. × 1,200. 1. Small forms. Note large size of the colony. 2. Intermediate forms. 3. Large forms. Organisms are in close proximity to each other and the colony is small. 4. Very large or giant forms. Extreme variation in size and shape can be seen. Smaller organisms are present in the same colony with the very large ones. 5. Small forms within an endothelial cell. Extracellular small forms can be seen in the lumen of the vessel, top left. 6. Two adjoining colonies of intermediate forms. Note the difference in staining of the matrices. 7. Two colonies of large forms. 8. Colony containing very large forms.

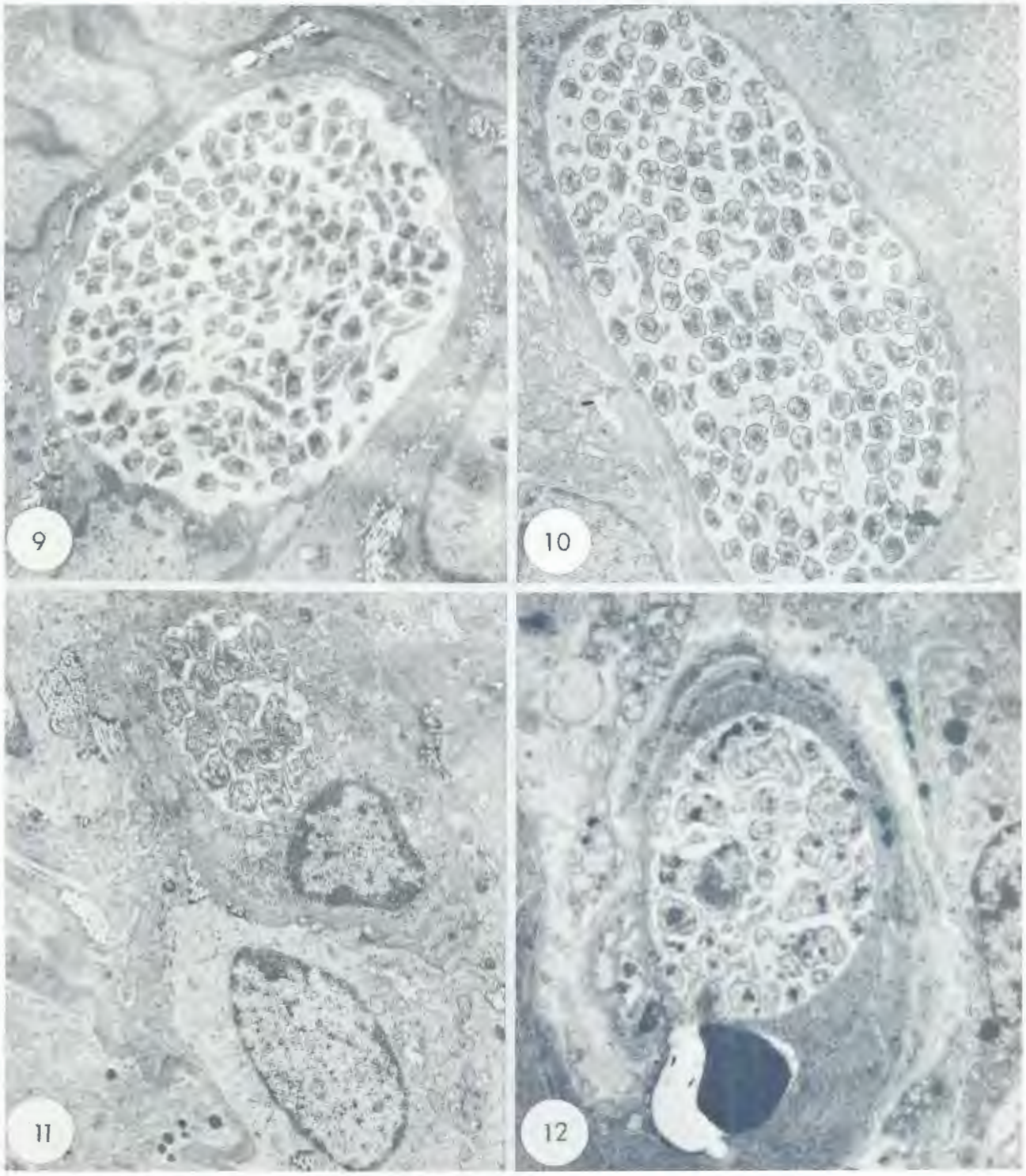

PLATE 2 9. Small forms. Pronounced pleomorphism is present. Coccoid, bacillary and filamentous shapes can be seen. × 7,250. 10. Intermediate forms. × 7,250. 11. Large forms × 7,250. 12. Very large bizarre forms. × 7,250. The small and intermediate forms occur in large colonies while the two larger forms are usually present in smaller colonies.

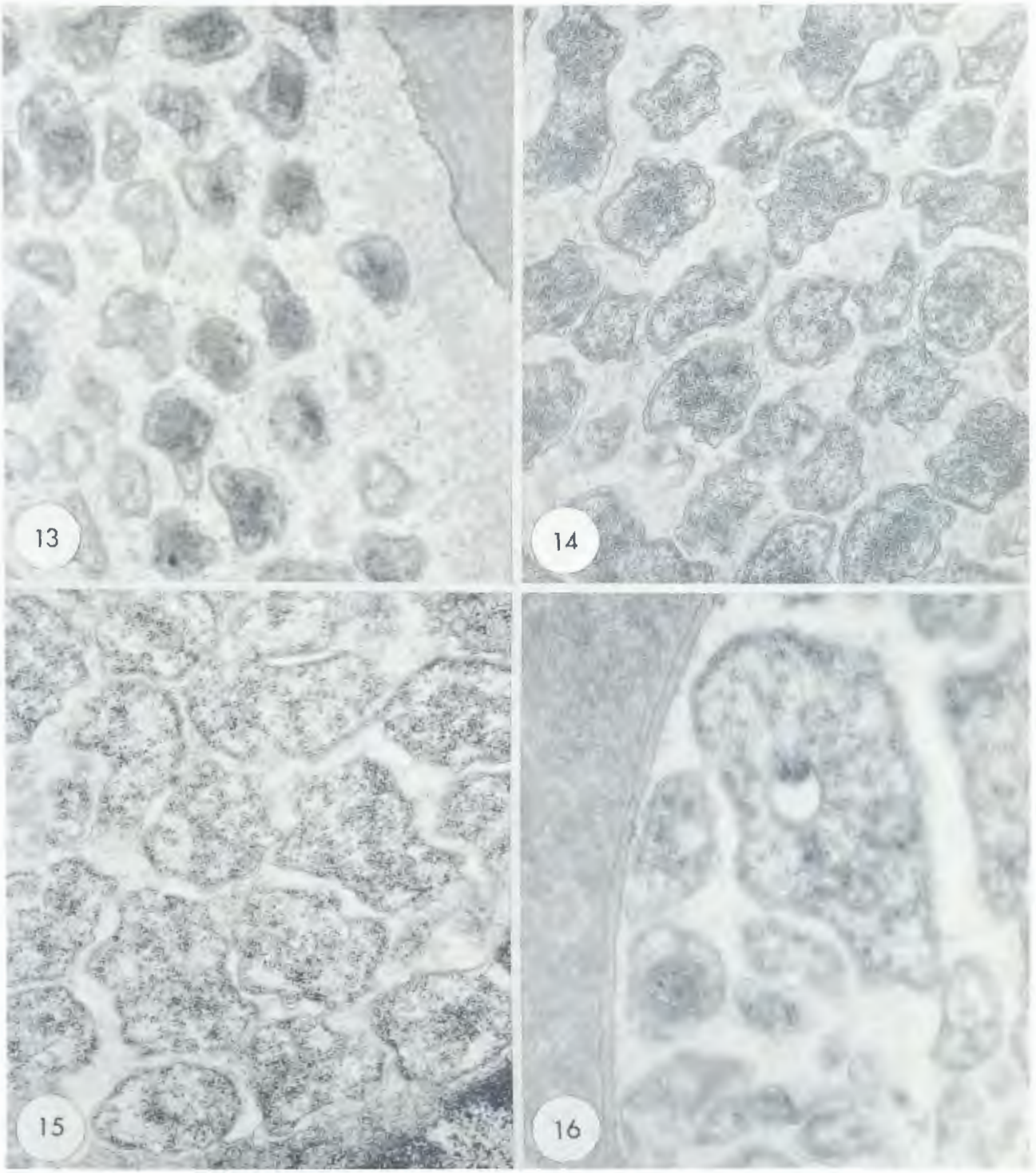

PLATE 3 13. Small forms. Organisms are loosely arranged. A well developed matrix is present which is condensed at the periphery of the cytoplasmic vacuole. The cytoplasm of the host cell has been completely displaced (top right-hand corner) and the vacuole with the organisms is only contained by the cell wall of the endothelial cell. This may indicate that rupture of the cell may take place in this region. × 20,000. 14. Intermediate forms. Organisms are closer spaced. The "cytoplasm" is less dense compared to that of the small forms. The matrix is not as well developed, although still fairly obvious. × 20,000. 15. Large forms. Organisms are much closer packed and have a polygonal appearance. "Cytoplasm" is less dense than that of the intermediate forms. Nucleoid and "cytoplasmic" material are evenly distributed throughout the inner structure of the organisms. × 20,000. 16. Bizarre giant forms. Note the organism in top half of picture containing a vacuole lined by a double membrane with a dense body to one side of the vacuole. Below the vacuole is a circular double membrane and to the right-hand side of the vacuole an area where the inner structure seems rarefied. × 20,000.

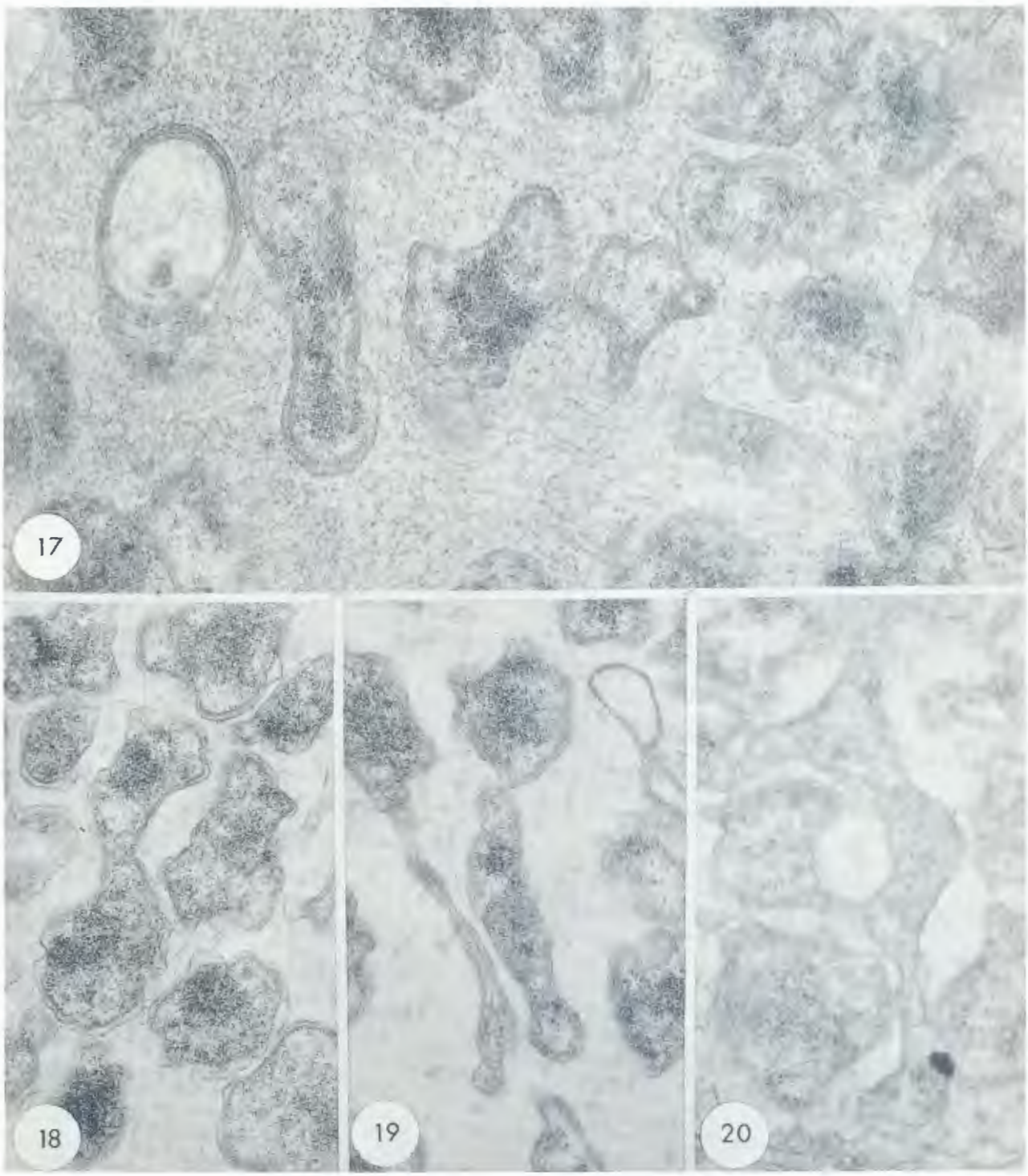

PLATE 4 17. Small forms. The matrix can be seen to consist of numerous fine fibrils arranged in denser masses in places. Numerous small electron-dense dust-like granules are also present. One organism on the left-hand side contains a large vacuole. The double unit membranes of the organism and that of the vacuole are in close apposition to each other over a large area. A dense body is present inside the vacuole. × 32,000. 18. An intermediate organism undergoing binary fission by the formation of a circumferential furrow. × 24,000. 19. Further intermediate organisms in process of dividing. The circumferential furrow has deepened and some of the daughter organisms have elongated into filamentous structures. × 24,000. 20. A large form of the organism showing the formation of circumferential furrows in various stages of development at various points. × 20,000.

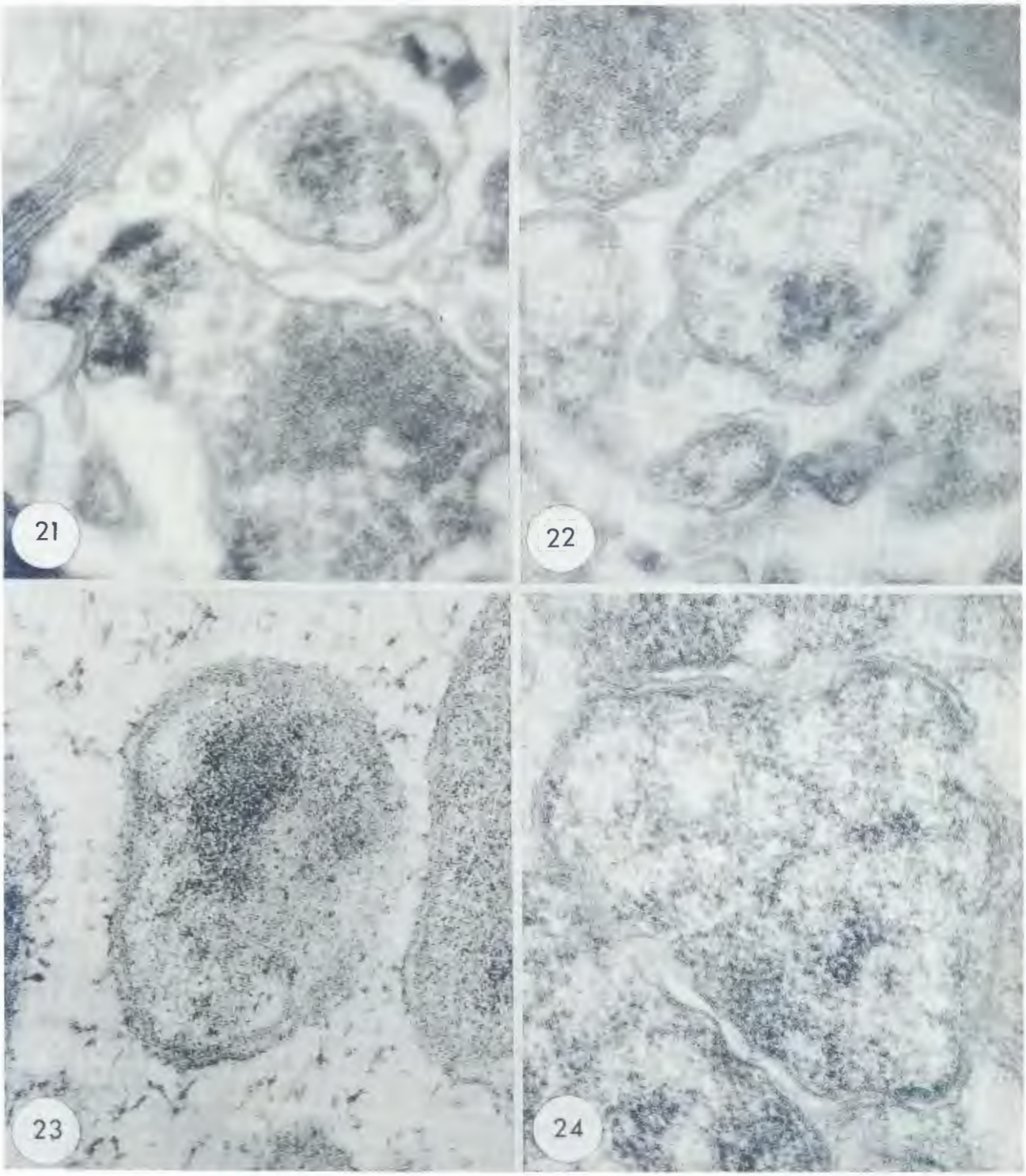

PLATE 5 21. Giant forms. The organism at the top is surrounded by an additional double unit membrane containing "cytoplasmic" and nucleoid material on one side. The outer unit membrane of the bizarre organism at the bottom can be seen to form bleb-like outpouchings. × 26,500. 22. Very large organism with outpouching of outer unit membrane. "Cytoplasm" is condensed to one area of the inner structure. × 32,000. 23. High magnification of a small organism. Double unit membrane can be clearly seen. Nucleoid material present as electron-pale areas at periphery of organism. "Cytoplasm" consists of area of intermediate electron-density with dark staining ribosomes embedded to one side of it. × 100,000. 24. High magnification of a large organism. The nucleoid and "cytoplasmic" material are evenly spread out in the inner structure. Fine fibrils are present in the areas of nucleoid material. Note the close proximity of the organisms to each other. × 100,000.

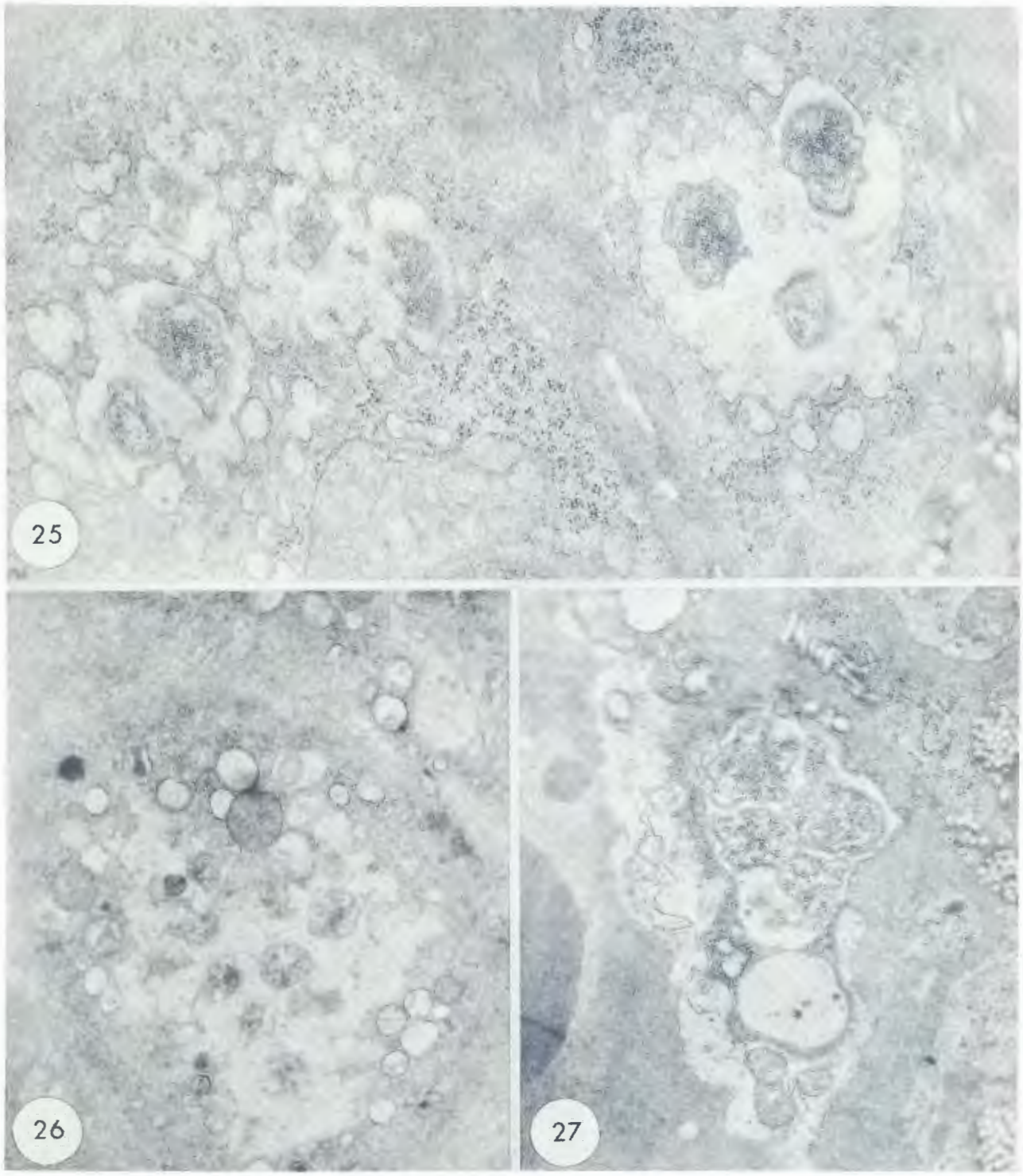

PLATE 6 25. Formation of the vacuole containing the organisms. On the left, small organisms are present within small vacuoles. Numerous small vesicles surround the vacuoles containing the organisms. Some of the vesicles are in the process of bursting into the larger vacuoles. On the right, the vacuole with organisms is larger and fewer vesicles are present around it. × 30,000. 26. Cytopathological changes in an endothelial cell containing small organisms. Dilatation of rough-surfaced, and smooth endoplasmic reticulum around the vacuole with organisms can be seen. × 20,700. 27. Endothelial cell on right with large organisms. The lower organism is undergoing binary fission. Dilatation of smooth and rough-surfaced endoplasmic reticulum is present in this cell. The uninfected endothelial cell on the left, overlapping the one with the parasites, is severely damaged. No normal organelles are present in its cytoplasm which is swollen and decreased in density. A portion of an erythrocyte in the lumen of the vessel can be seen on the extreme left. × 15,200.

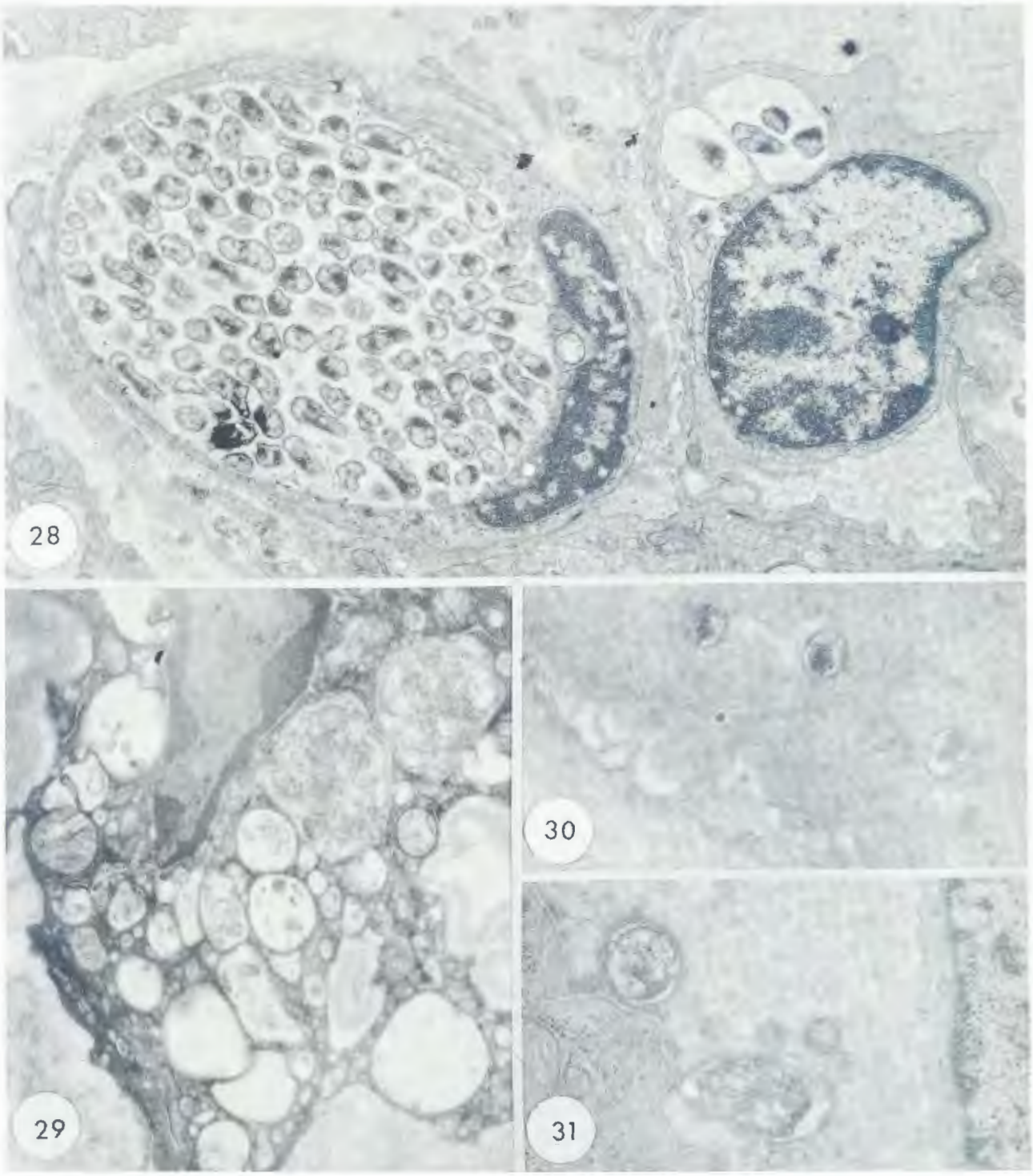

PLATE 7 28. Colony of small organisms. The cytoplasm of the host cell can be seen as a thin rim surrounding the vacuole containing the organisms. The nucleus is pushed to one side. The distended endothelial cell completely obstructs the lumen of the capillary. In an adjoining capillary, to the right, a monocyte is present with small organisms in vacuoles in its cytoplasm. × 10,000. 29. An endothelial cell showing marked cytopathological changes. The ground substance of the cytoplasm has an increased density. Numerous vacuoles are present in the cytoplasm. Mitochondria are swollen with loss of cristae. The chromatin is clumped into dense masses on the periphery of the nucleus. Two large organisms can be seen towards the right of the nucleus. × 17,250. 30. Three small organisms lying extracellularly in the lumen of a capillary. × 17,250. 31. Two large organisms, extracellularly in the lumen of a vessel. × 15,000.

Colonies or groups of the organisms were always located in a vacuole, or compartment, in the cytoplasm of endothelial cells. This vacuole was clearly demarcated by a membrane very similar, if not identical, to the cell membrane from the organelles of the cytoplasm of the host cell. Organisms were not seen intracellularly, i.e. in direct contact with the cytoplasm of the host cell. Often the host cell was greatly enlarged by the vacuole containing the organisms. In these cases the cytoplasm was pushed out as a thin rim around the periphery of the vacuole. Often, too, the nucleus was eccentrically situated and crescent-shaped [Plate 7 (28)].

Small and intermediate forms of the organism were usually widely spaced within the vacuole [Plate 2 (9 and 10) and Plate 3 (13 and 14)]. In contrast, the large forms were closer together [Plate 2 (11)] and often very densely packed [Plate 5 (24)]. The very large forms were again loosely arranged in the cytoplasmic vacuole [Plate 2 (12)].

Usually only organisms of the same form or type were found within a particular vacuole. Small organisms were never found together with large ones. This, however, was not always the case for the small and intermediate forms. In these instances the organisms in any given colony were predominantly of one type or the other. Often, however, colonies were encountered containing a few organisms with characteristics of the other type. Extreme variation in size was often seen in colonies containing mostly giant forms. The colonies containing giant cells usually included some smaller organisms. However, the latter, despite their size, structurally resembled the very large forms rather than the small forms mentioned previously (*vide infra*) [Plate 2 (12)].

The small and intermediate forms usually looked coccoid in shape under the light microscope. At higher magnifications, though, they were seen to be very pleomorphic and coccoid, oval, bacillary, rod-shaped or filamentous and horseshoe-shaped forms could often be observed [Plate 2 (9 and 10)]. On the other hand the large forms showed much less diversity morphologically, being constantly polygonal in shape [Plate 2 (11) and Plate 3 (15)] while the giant forms were bizarre in appearance [Plate 2 (12)].

Each organism was enveloped by two unit membranes [Plate 5 (23 and 24)] comprising an outer wall which bounded the whole organism and an inner wall around the inner structure. A narrow electron-pale space separated the two unit membranes. The outer membrane was clearly defined and often had a rippled appearance [Plate 3 (14 and 15) and Plate 5 (24)]. The inner structure consisted of electron-dense and electron-pale areas. The electron-dense areas consisted of a ground substance of intermediate electron-density which contained numerous small granules similar to the ribosome-like granules of bacteria, rickettsiae and the psittacosis-lymphogranuloma-trachoma (PLT) agents. In the electron-pale areas an indistinct network of fine fibrils was present. In the organisms mentioned above similar fibrils are believed to be composed of de-oxyribonucleic acid (DNA) strands (Anderson, Hopps, Barile & Bernheim, 1965; Fuhs, 1965). The electron-dense and electron-pale areas of the inner structure were not well delineated from each other [Plate 5 (23 and 24)]. According to the terminology used for bacteria, the electron-dense area is called the "cytoplasm" and the electron-pale area the "nucleoid".

In the small organisms the "cytoplasm" was condensed or localized roughly in one area of the inner structure [Plate 3 (13) and Plate 5 (23)]. It consisted of an area of intermediate density or ground substance, with numerous closely packed ribosomes, staining very intensely, around its periphery. Sometimes the ribosomes were arranged like a tag on one side of the ground substance [Plate 5 (23)]. The "cytoplasm" was dense in appearance and either centrally or, more commonly, eccentrically situated in the inner structure.

The "cytoplasm" and nucleoid material were evenly distributed throughout the inner structure of the large organisms [Plate 3 (15) and Plate 5 (24)]. The areas of "cytoplasm" did not stain as intensely as did those of the small organisms. In the very large organisms the "cytoplasm" was seen as well defined areas of ground substance, of varying size, scattered throughout the inner structure with the ribosomes evenly distributed in them [Plate 3 (16) and Plate 5 (21 and 22)]. These areas of "cytoplasm" sometimes coalesced to form very much larger areas [Plate 5 (21)]. In the intermediate-sized organisms the "cytoplasm" occupied the greater part of the inner structure and was less electron-dense than that of the small organisms, but more so than that of the large organisms. The ribosomes were evenly distributed in the ground substance [Plate 3 (14) and Plate 4 (18 and 19)].

Some of the very large organisms had an extra layer of double unit membranes surrounding them. In some instances, areas of "cytoplasm" and nucleoid material were enclosed in this extra outer membrane [Plate 5 (21)]. In many of these very large organisms the outer unit membrane bulged out in places to form bleb-like structures [Plate 5 (21 and 22)].

Numerous fine fibrils, with which many small electron-dense dust-like granules were associated [Plate 4 (17)], were present between the individual elements in most of the colonies of small organisms. In places these fibrils formed denser masses or were concentrated around the periphery of the cytoplasmic vacuole [Plate 3 (13)]. In colonies of the intermediate form the fibrils were not as clearly defined and were fewer in number [Plate 3 (14)], while in colonies containing the larger forms they were very rare [Plate 3 (15 and 16)].

Forms whose shape suggested that they were undergoing binary fission were often encountered in colonies of the small and intermediate types of organism. This was seen as a circumferential furrow, roughly in the middle of the organism [Plate 4 (18)], which apparently deepened and by constriction resulted in the formation of two separate organisms. No definite crosswall was seen between two conjoined daughter cells such as occurs in bacterial cell division (Bisset, according to Moulder, 1962). A filamentous form of organism, possibly elongating prior to division, is illustrated in Plate 4 (19). Shapes indicative of binary fission were very rarely observed in the two larger types of organisms, though such a form can be seen in Plate 6 (27).

Apparently, binary fission is not the organism's only method of reproduction. Rarely forms were observed which indicated that other mechanisms may exist. Plate 4 (20) shows a large organism apparently dividing into several different-sized daughter cells. Various circumferential furrows are present at different points. In Plate 3 (16) a bizarre giant cell is shown containing a vacuole, delimited by a double unit membrane, with a dense body in it. This cell also contains two areas, one encircled by a double membrane and the other of decreased electron-density, which are probably early stages in the formation of additional vacuoles. Plate 4 (17) shows a small organism with a vacuole, also containing a dense body.

The formation of such vacuoles with dense bodies may indicate an additional mode of multiplication (*vide infra*).

Frequently single and more rarely little groups of organisms were seen lying free in the plasma in the lumen of a blood vessel. The majority were of the small type [Plate 7 (30)] but occasionally large organisms were also found free in the blood vessels [Plate 7 (31)].

Organisms in the process of entering endothelial cells were not observed. It is presumed that this process takes place by phagocytosis. Small organisms that had apparently recently entered the cytoplasm of an endothelial cell can be seen in Plate 6 (25). The small vacuoles around the organisms on the left-hand side of the electron microphotograph probably represent the initial phagocytic vacuoles. Numerous smaller vacuoles or vesicles of varying size are collected around them and some are coalescing with, or breaking into, the larger initial vacuoles containing the organisms. On the right-hand side a further step in the formation of a cytoplasmic vacuole can be seen: the vacuole containing the organisms has enlarged and fewer vesicles are present around it.

Despite the presence of large numbers of organisms within vacuoles in the cytoplasm of the endothelial cells clearly detectable signs of cell injury, apart from severe distension of the cytoplasm, were rarely noticed. Cytopathological effects are, however, obvious in the cells illustrated in Plate 6 (26) and Plate 7 (29). In the former marked ballooning of the rough-surfaced endoplasmic reticulum, and of some of the mitochondria in the vicinity of the vacuole containing the parasites, is shown. The cell in Plate 7 (29) is severely damaged and probably dead. The cytoplasmic ground substance is very dense in appearance and numerous vacuoles are present in the cytoplasm, many no doubt representing markedly swollen endoplasmic reticulum. Swelling of mitochondria, with loss of cristae and clumping of the chromatin of the nucleus into dense masses at the periphery, are evident. Two large organisms can be seen to the right of the nucleus.

More commonly, endothelial cells containing no organisms but showing severe cellular damage, were seen [Plate 6 (27)]. These cells were swollen, the cytoplasm was decreased in density and no normal organelles could be recognised in them.

Hundreds of thin sections were examined but in only two of these were single cells other than endothelial cells infected. These two cells both appeared to be monocytes and the organisms were found in vacuoles in their cytoplasm. In Plate 7 (28) such a cell can be seen lying in the lumen of a capillary.

Discussion

The ultrastructural appearance of *C. ruminantium* adds further evidence to the existence of different morphological forms of the organism, first reported by Jackson & Neitz (1932). The forms found with the electron microscope correspond to those observed with the light microscope in brain smears stained with the May-Grünwald-Giemsa method. Not only do these various forms differ in particle size but also in the pattern of the organization of the nucleoid and "cytoplasmic" material in their internal structure, in the size of the colonies in which they occur in the cytoplasm of endothelial cells and in the way they are arranged within these vacuoles.

It is not clear at this stage whether these various forms represent changes in the morphology of the organism during a growth cycle, although they are highly suggestive of such a process. Elucidation of this question will have to await methods of creating a suitable milieu for growth and multiplication of *C. ruminantium* outside the animal body.

Cowdry (1926a) found no morphological evidence of a multiplication phase other than diploformation, which indicated the likelihood of simple division. Jackson (1931) and Jackson & Neitz (1932) came to a similar conclusion. In this study convincing evidence was found that multiplication of *C. ruminantium* takes place mainly by binary fission. In addition, forms were found which indicate that other possible mechanisms of reproduction may also exist. The process illustrated in Plate 4 (20) can be regarded as multiple budding (unequal division of a single particle) while that illustrated in Plate 3 (16) and Plate 4 (17) suggests a form of endosporulation. The dense bodies within the vacuoles in the latter instance could be small cells in an early stage of their development. This method of multiplication may explain the presence of an additional double unit membrane, containing a tag of "cytoplasmic" and nucleoid material, around some forms of the organism, as shown in Plate 5 (21). The inner organism may be the initial dense body which has enlarged and in the process pushed away the inner structure of the cell in which it originated. It is of interest to note here that Mitsui, Kajima, Nishimura & Konishi (1962) illustrated particles of the trachoma agent which are apparently surrounded by an extra outer unit membrane. However, it must be pointed out that these structures, which are regarded as evidence of multiple budding and endosporulation by *C. ruminantium,* were observed very infrequently in the thin sections.

Budding has been reported in the agent of meningopneumonitis by both Higashi (according to Moulder, 1962) and Gaylord (1954). The process of endosporulation observed in *C. ruminantium* probably is analogous to the multiple endosporulation described in the agent of ornithosis (Anderson *et al.,* 1965), in the agent of trachoma (Bernkopf, Mashiah & Becker, 1962) and in the agent of meningopneumonitis (Gaylord, 1954). Higashi (according to Moulder, 1962) also observed this phenomenon in the agent of meningopneumonitis. According to Moulder (1966) the structures regarded as evidence of multiple endosporulation in the group of PLT organisms mentioned above, are found at such low frequencies that this process cannot be a quantitatively important method of multiplication.

Apart from these different modes of division of the PLT group of organisms, a number of workers also observed structureless matrices in the cytoplasm of host cells infected with the agents of meningopneumonitis and trachoma, in which new agent cells were apparently being organized by some virus-like reproductive process (Tajima, Nomura & Kubota, 1957; Bernkopf *et al.,* 1962; Higashi, Tamura & Iwanaga, 1962; Armstrong, Valentine & Fildes, 1963; Mitsui, Fujimoto & Kajima, 1964). Structures which could be interpreted as particles being formed from a viral matrix were not observed during the study of *C. ruminantium*. More recent work [Armstrong & Reed, 1964; Higashi (according to Moulder, 1966); Armstrong, 1968] has demonstrated that these matrices were artefacts resulting from the disruption of the fragile large forms during fixation and embedding of the infected cell for thin sectioning. There seems to be no convincing electron microscopical evidence in favour of such an eclipse phase for the PLT group of organisms.

In spite of the known infectivity of the blood of reacting animals, previous workers have failed to demonstrate *C. ruminantium* in the blood. Alexander (1931) suggested that the organism is firmly adherent to the

erythrocytes and leukocytes. Jackson & Neitz (1932) could not confirm this, but believed that they had seen organisms lying free in the plasma. Cowdry (1926a) described what he thought to be rupturing of endothelial cells containing organisms in their cytoplasm with the actual discharge of organisms into the blood stream. That *C. ruminantium* does indeed occur freely in the plasma *in vivo* is confirmed by the finding with the electron microscope of frequent extracellular organisms, mainly of the small form, in the lumen of blood vessels. Rupturing of the endothelial cells and discharge of the organisms were not seen but it can be assumed that this is the mechanism by which the organisms are released. Excessive thinning of the rim of cytoplasm surrounding the vacuole containing the organisms suggests that it may be the case [Plate 3 (13)].

Jackson & Neitz (1932) demonstrated colonies of *C. ruminantium* in the cytoplasm of leukocytes present in blood smears made from affected ruminants. They regarded some of these cells as being definite macrophages, while others were thought to be endothelial cells desquamated during life, or after death. With the electron microscope, small forms of *C. ruminantium* were rarely found in vacuoles in the cytoplasm of cells thought to be monocytes. As only a very small fraction of the total white cells of the blood were incidentally examined during this study, it is difficult to judge the significance of this finding.

Cowdry (1926b) noticed a matrix between the organisms which differed in staining properties from the ground substance of the host cell. The occurrence of this matrix was not constant, even in neighbouring colonies of organisms in the same section, and was most frequently found in colonies where masses of the organisms were not very tightly packed. These observations of Cowdry (1926b), were confirmed by the finding of a well developed matrix in colonies of the small form of *C. ruminantium* with the electron microscope, while colonies consisting of the larger forms contained only an ill-defined matrix. The dust-like granules present in the matrices may represent RNA. Matrices of psittacosis and ornithosis are rich in RNA (Moulder, 1962).

The growth of *C. ruminantium* within the cytoplasmic vacuole appears to have very little deleterious effect upon the host cell. On the other hand, marked cytopathic effects were obvious in many endothelial cells containing no organisms. Whether the production of a toxin by the organism at some phase of its cycle in the vertebrate host, or whether some immune mechanism forms the basis of this interesting phenomenon, are unexplored possibilities that warrant further investigation.

The results of the present study necessitate a discussion of the taxonomic position of *C. ruminantium*. Similarities with the PLT group of organisms in certain modes of multiplication have already been indicated. *C. ruminantium* develops within the confines of a membrane-bound vacuole in the cytoplasm of the host cell. This is also the locality of development in the PLT group of organisms (Moulder, 1962). In contrast, most of the Rickettsiae, with the exception of *Coxiella burnetii* (Derrick, 1939) as reported by Kokorin (1968) and *R. sennetsu,* Misao & Kobayashi, 1954, as reported by Anderson *et al.* (1965), multiply directly in the cytoplasm among the cell organelles of the host cell, after a rapid disappearance of the membrane of the initial phagocytic vacuole.

There seems to be no definite growth cycle in rickettsial infections (Moulder, 1962), with the exception of *Coxiella burnetii* which, as reported by Rosenberg & Kordová (according to Tuomi & von Bonsdorff, 1966), has a developmental cycle involving small particles and intermediate forms. Moulder (1962) stated that probably the most striking characteristic of the PLT group as a whole is the regular sequential appearance of structurally and functionally different particle types during multiplication of these agents in infected cells. Attention has already been drawn to the fact that the different forms of *C. ruminantium* may possibly represent such structurally different particles as part of a developmental cycle (*vide supra*).

The large forms of *C. ruminantium* resemble the polygonal "giant bodies" illustrated by Anderson *et al.,* (1965) for the agent of ornithosis in size, shape, in the way they are tightly packed within the cytoplasmic vacuole and in the way the "cytoplasmic" and nucleoid material are distributed in the inner structure. There is also a close resemblance on the above-mentioned points between the "elementary body" of this agent and the small form of *C. ruminantium.*

In common with the representatives of the genus *Rickettsia, C. ruminantium* is arthropod transmitted.

The causative agent of heartwater thus seems to share certain properties with organisms of both the genus *Rickettsia* (arthropod transmission) and the PLT group (morphology). Tuomi & von Bonsdorff (1966) agreed with the inclusion of the agent of tick-borne fever in a heterogeneous family of organisms which share properties of both the genus *Rickettsia* and the PLT group, as suggested by Foggie (according to Tuomi & von Bonsdorff, 1966). Rake *et al.,* (1945) suggested such a position for the agent of heartwater, an opinion which is equally justifiable when the results of the present study are considered.

Summary

The structure of *C. ruminantium* was studied with the electron microscope. Thin sections were cut from the choroid plexus of two sheep experimentally infected by the intravenous route with 5 ml blood obtained from a natural untreated case of heartwater in a sheep. This material was fixed in glutaraldehyde and postfixed in osmium tetroxide. Four different forms, or particles of the organism, varying in size and in structure are described. The taxonomic position of *C. ruminantium* is discussed and an intermediate grouping of this organism between the genus *Rickettsia* and the psittacosis-lymphogranuloma-trachoma (PLT) agents is suggested.

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References

Alexander, R. A., 1931. Heartwater - The present state of our knowledge of the disease. *17th Rep. Dir. vet. Res. Un. S.Afr.,* 89–150.

Anderson, D. R., Hopps, H. E., Barile, M. F. & Bernheim, B. C., 1965. Comparison of the ultrastructure of several rickettsiae, ornithosis virus and *Mycoplasma* in tissue culture. *J. Bact.,* 90, 1387–1404.

Armstrong. J. A., 1968. Relation of PLT organisms to the rickettsiae, with special reference to structure and multiplication. *Acta Virol.,* 12, 15–17.

Armstrong, J. A. & Reed, S. E., 1964. Nature and origin of initial bodies in lymphogranuloma venereum. *Nature, Lond.,* 201, 371–373.

ARMSTRONG, J. A., VALENTINE, R. C. & FILDES, C. J., 1963. Structure and replication of the trachoma agent in cell cultures as shown by electron microscopy. *J. gen. Microbiol.*, 30, 59–73.

BERNKOPF, H., MASHIAH, P. & BECKER, Y., 1962. Correlation between morphological and biochemical changes and the appearance of infectivity in FL cell cultures infected with trachoma agent. *Ann. N.Y. Acad. Sci.*, 98, 62–81.

COLES, J. D. W. A., 1953. Classification of rickettsiae pathogenic to vertebrates. *Ann. N.Y. Acad. Sci.*, 56, 457–483.

COWDRY, E. V., 1926a. Cytological studies on heartwater. I. The observation of *Rickettsia ruminantium* in the tissues of infected animals. *11–12th Rep. Dir. vet. Res. Un. S.Afr.*, 161–177.

COWDRY, E. V., 1926b. Cytological studies on heartwater. II. *Rickettsia ruminantium* in the tissues of ticks capable of transmitting the disease. *11-12th Rep. Dir. vet. Res. Un. S.Afr.*, 181–196.

FUHS, G. W., 1965. Symposium on the fine structure and replication of bacteria and their parts. I. Fine structure and replication of bacterial nucleoids. *Bacteriol. Rev.*, 29, 277–293.

GAYLORD, W. H., 1954. Intracellular forms of meningopneumonitis virus. *J. exp. Med.*, 100, 575–580.

HAIG, D. A., 1955. Tickborne rickettsioses in South Africa. *Advan. in vet. Sci.*, 2, 307–325.

HIGASHI, N., TAMURA, A. & IWANAGA, M., 1962. Developmental cycle and reproductive mechanism of the meningopneumonitis virus in strain L cells. *Ann. N.Y. Acad. Sci.*, 98, 100–121.

ITO, S. & WINCHESTER, R. J., 1963. The fine structure of the gastric mucosa in the bat. *J. Cell Biol.*, 16, 541–577.

JACKSON, C., 1931. The microscopic diagnosis of heartwater: A preliminary note on the value of intima smears. *17th Rep. Dir. vet. Res. Un. S.Afr.*, 161–173.

JACKSON, C. & NEITZ, W. O., 1932. On the aetiology of heartwater. *18th Rep. Dir. vet. Res. Un. S.Afr.*, 49–70.

KOKORIN, I. N., 1968. Biological peculiarities of the development of rickettsiae. *Acta Virol.*, 12, 31–35.

LUFT, J. H., 1961. Improvements in epoxy resin embedding methods. *J. biophys. biochem. Cytol.*, 9, 409–414.

MILLONIG, G., 1961. Advantages of a phosphate buffer for osmium tetroxide solutions in fixation. *J. appl. Phys.*, 32, 1637.

MITSUI, Y., KAJIMA, M., NISHIMURA, A. & KONISHI, K., 1962. Morphology of trachoma agent in conjunctiva and chick embryo. *Ann. N.Y. Acad. Sci.*, 98, 131–144.

MITSUI, Y., FUJIMOTO, M. & KAJIMA, M., 1964. Development and morphology of trachoma agent in the yolk sac cell as revealed by electron microscopy. *Virology*, 23, 30–45.

MOULDER, J. W., 1962. The biochemistry of intracellular parasitism. Chicago: The University of Chicago Press.

MOULDER, J. W., 1966. The relation of the psittacosis group (Chlamydiae) to bacteria and viruses. *Ann. Rev. Microbiol.*, 20, 107–130.

NEITZ, W. O., 1940. Uleron in the treatment of heartwater. *Jl S.Afr. vet. med. Ass.*, 11, 15.

PURCHASE, H. S., 1945. A simple and rapid method for demonstrating *Rickettsia ruminantium* (Cowdry) in heartwater brains. *Vet. Rec.*, 57, 413–414.

RAKE, G., ALEXANDER, R. & HAMRE, DOROTHY M., 1945. The relationship of the agent of heartwater fever - *Rickettsia ruminantium. Science*, 102, 424–425.

REYNOLDS, E. S., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.*, 17, 208–212.

TAJIMA, M., NOMURA, Y. & KUBOTA, Y., 1957. Structure and development of viruses of the psittacosis-lymphogranuloma group observed in the electron microscope. *J. Bact.*, 74, 605–620.

TUOMI, J. & VON BONSDORFF, C. H., 1966. Electron microscopy of tick-borne fever agent in bovine and ovine phagocytizing leukocytes. *J. Bact.*, 92, 1478–1492.

WATSON, M. L., 1958. Staining of tissue sections of electron microscopy with heavy metals. *J. biophys. biochem. Cytol.*, 4, 475–478.

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