Onderstepoort J. vet. Res. 42 (1). 1–14 (1975)

ELECTRON MICROSCOPY OF *COWDRIA RUMINANTIUM* INFECTED RETICULO-ENDOTHELIAL CELLS OF THE MAMMALIAN HOST

J. L. DU PLESSIS, Veterinary Research Institute, Onderstepoort

ABSTRACT

DU PLESSIS, J. L., 1975. Electron microscopy of *Cowdria ruminantium* infected reticulo-endothelial cells of the mammalian host. *Onderstepoort J. vet. Res.* 42 (1), 1–14 (1975).

An electron microscopic study of structures suspected to represent a possible developmental cycle of *Cowdria ruminantium* in reticulo-endothelial cells of mice and ruminants is reported. After infection dense bodies increase in size and undergo division to form fragmented dense bodies. These in turn apparently sub-divide and become organized to give rise to mature "organisms". In none of these structures do limiting membranes separate the parasitic inclusions from the host cell cytoplasm. Present observations suggest that growth of the organism in reticulo-endothelial cells differs from that of chlamydial and rickettsial agents and somewhat resembles the replication of some viruses. Developmental stages observed after infection of ruminants with the Ball 3 strain of the heartwater agent are indistinguishable from those seen with the mouse adapted strain. These observations support the hypothesis that *C. ruminantium* released from reticulo-endothelial cells subsequently penetrates endothelial cells where further multiplication by binary fission occurs.

INTRODUCTION

Cowdria ruminantium (Cowdry, 1926) has been demonstrated by means of histological sections in endothelial cells of various organs in domestic ruminants suffering from heartwater (Cowdry, 1926) and in endothelial cells of various veins (Jackson, 1931).

More recently this parasite has been described in lymph node reticulo-endothelial (RE) cells in sheep (Du Plessis, 1970) and in peritoneal macrophages of mice (unpublished data) infected with two presumably different strains of *C. ruminantium* and it was postulated that this parasite initially replicates in RE cells from which the organisms are released to subsequently enter vascular endothelial cells. Possible developmental stages of the parasite were observed by light microscopy in both lymph node RE cells and mouse peritoneal macrophages. Strongly basophilic, rounded, homogeneous inclusion bodies which were first seen, subsequently subdivide to give rise to what appear to be colonies of mature organisms.

In a thorough and well-documented electron microscopic study of this parasite in endothelial cells of the ovine choroid plexus, Pienaar (1970) described small, intermediate, large and giant forms of the organism and concluded that replication of *C. ruminantium* in endothelial cells took place principally by binary fission.

This study is an attempt to elucidate by means of electron microscopy the observations made in infected RE cells by light microscopy as previously reported (Du Plessis, 1970).

MATERIALS AND METHODS

Mouse tissues examined included liver, spleen, myocardium and peritoneal cells from mice inoculated intraperitoneally with infective emulsified liver or heart obtained from mice reacting to the mouse adapted strain of *C. ruminantium* isolated in mice from a goat (Du Plessis & Kumm, 1971). The organ tissues were collected from the mice on the day prior to the development of clinical signs and also while manifesting symptoms.

Peritoneal macrophages were obtained from 4-week-old mice infected intraperitoneally with myocardium from mice reacting to the mouse adapted strain. The hearts had been emulsified in buffered lactose peptone and stored at —80 °C. Mice were sacrificed at 12, 24, 48, 72, 96 and 120 hours and when showing the first symptoms after 9 to 12 days. The peritoneal cells were collected in Hanks' solution, fixed by the addition of a volume of 4% glutaraldehyde, concentrated in a pellet by centrifuging at 1 000*g* for 15 min and processed as described below.

The ruminant tissues examined were mesenteric lymph nodes from several Merino sheep and a 6–month–old Jersey calf artificially inoculated with virulent blood drawn at the height of the temperature reaction from a sheep infected with the Ball 3 strain of *C. ruminantium*. The specimens were collected from the sheep and calf after they had shown a temperature reaction for several days. Mesenteric lymph nodes from an ox exhibiting marked clinical signs of heartwater were also examined.

As controls, sheep lymph node and mouse liver were collected from a sheep and mouse susceptible to heartwater. To obtain extensive hepatocellular necrosis and phagocytosis of necrotic cells and cell debris by Kupffer cells, 3-week-old mice were inoculated intraperitoneally with virulent Rift Valley fever virus and liver specimens collected prior to death.

The tissues and cells were fixed in 4% glutaraldehyde in Millonig's buffer (Millonig, 1961) at pH 7,2–7,4, post fixed in buffered 2% osmium tetroxide and dehydrated. The tissues were embedded in Araldite and the peritoneal cells in Epon. In the case of the tissues, sections 1–2 μm thick were cut from the Araldite blocks, stained with toluidine blue pyronin (Ito & Winchester, 1963) and after examination 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).

RESULTS

Mouse peritoneal macrophages

In mouse peritoneal macrophages collected 12 h after inoculation structures suspected to be heartwater organisms are present singly within vacuoles and usually surrounded by several membranous structures (Fig. 1 & 2). The double unit membranes of the organisms are poorly distinguishable and the inner structure poorly defined.

At 24 hours a structure suspected to be the earliest detectable parasitic inclusion appears as a finely granular spherical body of high electron density (Fig. 3, arrow). This body does not have distinct borders and its granules become less densely packed at its junction with the cytoplasm of the host cell.

Received 30 October 1974—Editor

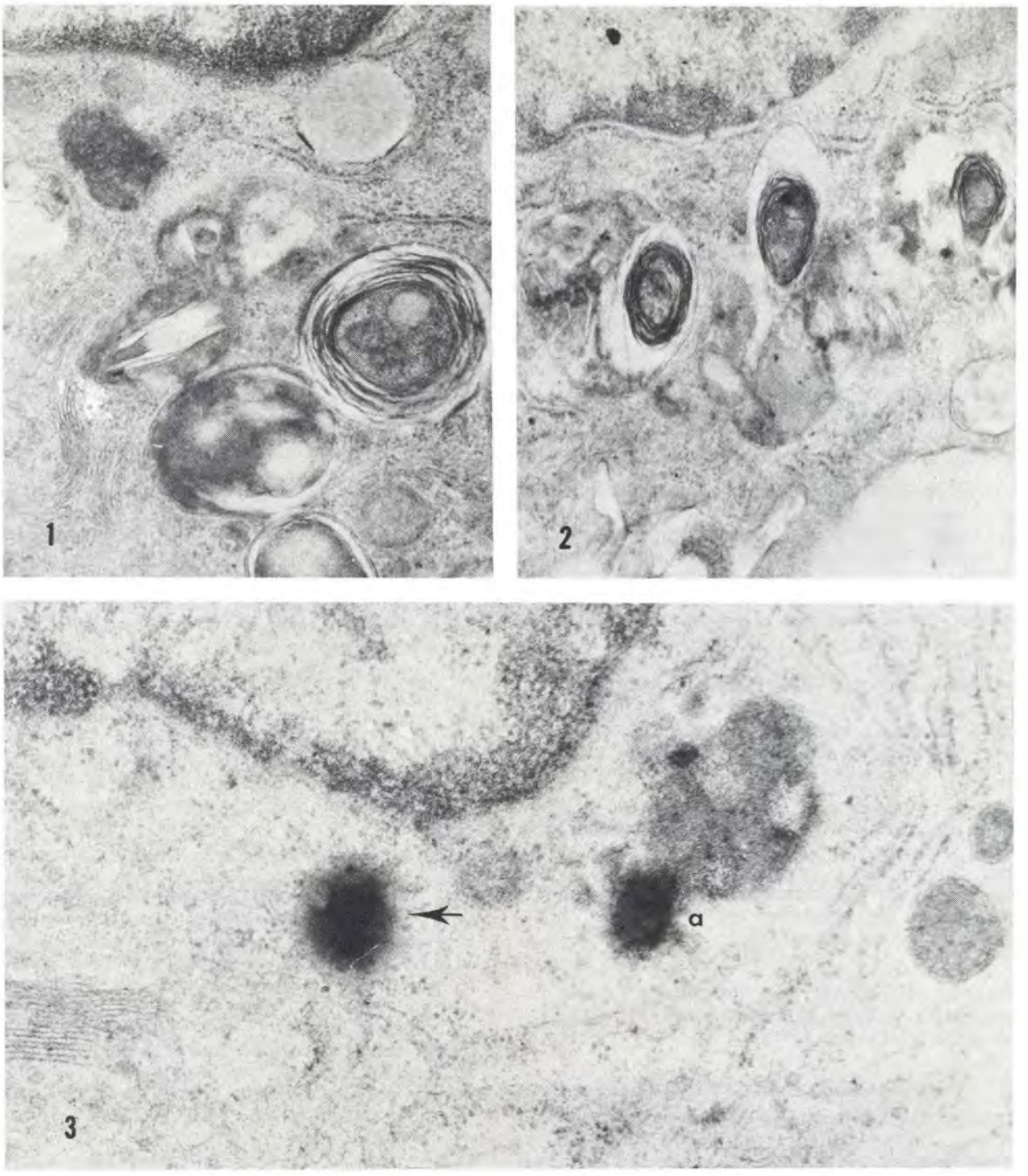


FIG. 1 & 2 Phagocytosed organisms in peritoneal macrophages collected 12 h after infection. Lamellar structures around the organisms which appear to lie in vacuoles are clearly visible in Fig. 1 & 2. The outer unit membrane of the organism at the bottom of Fig. 1 is separated from the inner membrane and in close opposition to the cytoplasm. ×28400

FIG. 3 Early dense body in peritoneal macrophage (arrow) collected at 24 h. Note intimate contact with the host cell cytoplasm and the dense inner structure compared to a membrane-bound phagosome on extreme right. Body (a) is in all probability also an early dense body. ×49800

It is approximately 0,38 μm in size. It is not possible at this stage to demonstrate an intermediate stage between these bodies and the structures detected at 12 hours but they may represent degenerating phagocytosed organisms.

In peritoneal macrophages collected 72–96 h after infection, greatly enlarged inclusions were observed, attaining a size of 2–4 μm (Fig. 4). They also possess a dense, finely granular structure and are distinctly not within the confines of a limiting membrane. These dense bodies are usually homogeneous although some exhibit poorly demarcated areas of reduced electron density. Cellular endoplasmic reticulum and mitochondria are usually in close apposition to the inclusion body. These bodies are often seen in a juxta-nuclear position (Fig. 4).

What seems to be the following stage of development was observed in cells collected 96 h after inoculation. The inclusion bodies attain their maximum size which varies and may reach 5–6 μm. They lose their uniformity and apparently undergo cleavage (Fig. 5). Several fragmented masses of material (Fig. 5a) with the same finely granular structure as that of the dense body are seen in the cells. The fragmented bodies have irregular, poorly defined edges and are connected in places by narrow bridges (Fig. 5b).

Fragments of the original dense bodies closely associated with particles (Fig. 5c) suspected to be developing heartwater "organisms" are demonstrable in the cytoplasm at this stage. Small fragments of finely granular material varying in density, size and shape appear to become separated from the larger fragments and organized into smaller bodies or subunits with indistinct outlines and irregular shapes (Fig. 5d). In places double unit membranes can be distinguished among these structures (Fig. 5, arrow).

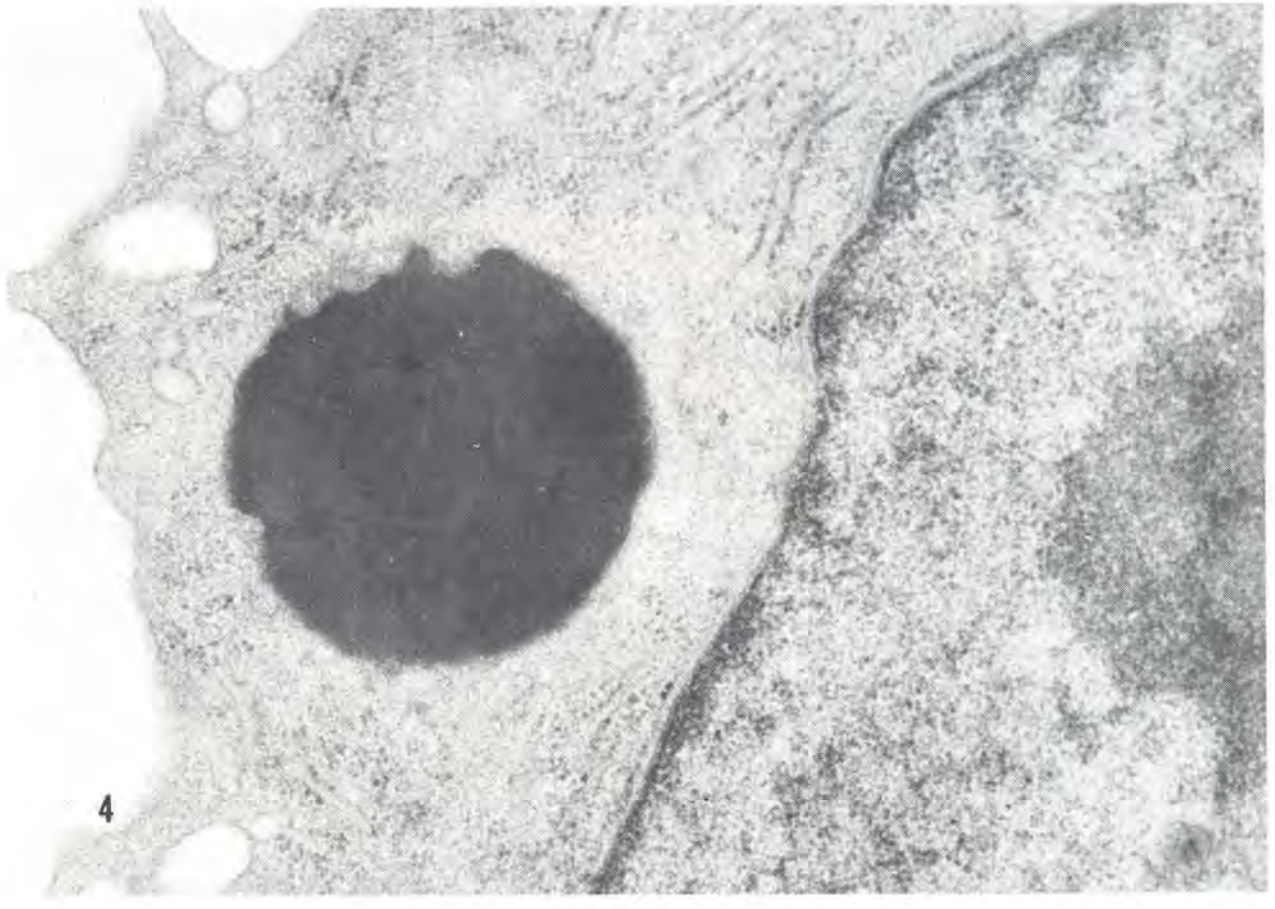


FIG. 4 Growing dense body in a peritoneal macrophage collected at 48 h. Note the sharp contrast between the inclusion and the cell nucleus and the definite absence of a limiting membrane. ×42700

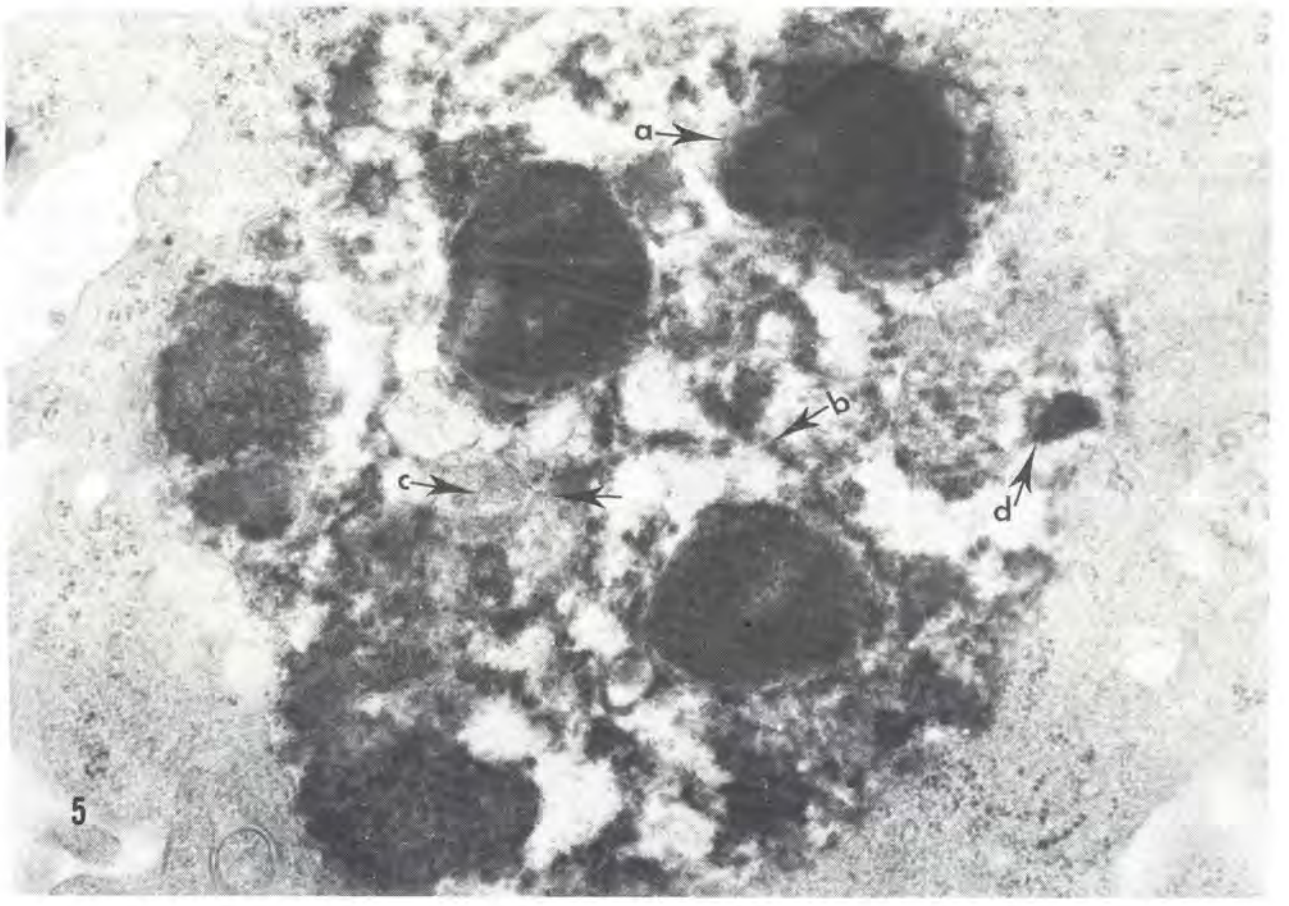


FIG. 5 Fragmented inclusion, in intimate contact with cell cytoplasm, consisting of "developing organisms" between undivided fragments, some of which have the same structure and density as the bodies in Fig. 3 & 4. The granularity of the two fragments on the left is distinctly coarser. Developing membranes (arrow) impart vague outlines to "developing organisms" which seem to have electron dense material chiefly confined to their circumference. Mouse peritoneal macrophage collected at 72 h post infection. ×38400

Subsequently the subunits within the fragmented inclusion body become more distinct (Fig. 6 & 7) and are separated from each other by double unit membranes (Fig. 6, arrow). The electron dense material is more or less evenly distributed throughout the individual subunits, which now begin to resemble mature "organisms" (Fig. 7). Roughly spherical areas of the inclusion still in the process of division (Fig. 7, arrow) resemble the earlier stage pictured in Fig. 6.

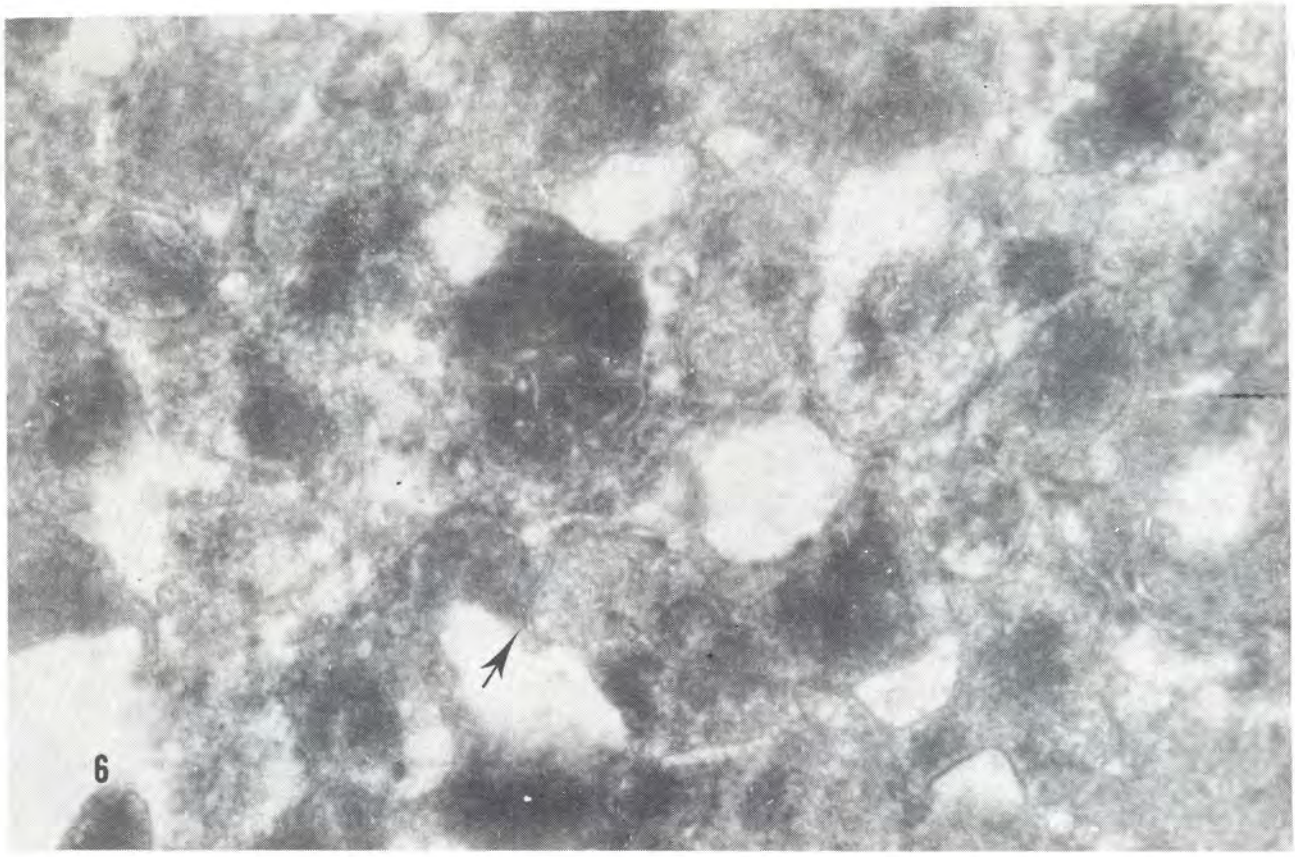
In what appears to be the final stage, the structures resembling mature "organisms" are no longer in close contact and in the absence of a limiting membrane surrounding the inclusion body they are intermixed with host cell organelles (Fig. 8 & 9) and finely fibrillar structures (Fig. 8a). Swollen smooth endoplastic reticulum (Fig. 8 & 9, arrows), which indicates impending death of the host cell, can also be seen. The organisms are uniformly round, have a double unit membrane and are 0,41–0,43 μm in size. In some, a finely granular electron dense material is evenly distributed between electron lucent areas (Fig. 9a), whereas in others the electron dense material is more scanty and inclined to be concentrated in the centre (Fig. 9b). Peritoneal macrophages harbouring fully developed organisms are prevalent at 96 h after inoculation.

At 120 h after infection exceptionally few peritoneal cells are parasitized and only a few cells with early dense bodies are demonstrable.

Mouse liver

In Kupffer cells structures suspected to be inclusions consisting of disintegrating fragmented bodies and developing "organisms" (Fig. 10, 11, 12, 13 & 25) closely resemble those described in peritoneal macrophages. They sometimes fill a large part of the cytoplasm (Fig. 25). The "organisms" in different cells closely resemble each other. They vary in size between 0,29 and 0,3 μm. Some have electron dense material occupying their whole structure (Fig. 12a), while others have loosely arranged finely granular electron dense material around the circumference or irregularly distributed.

A careful search in many sections of the liver of several mice killed *in extremis* as well as on the day prior to the onset of symptoms, revealed a moderate number of Kupffer cells harbouring the structures described above (Fig. 10 & 11). Rarely membrane-lined colonies of fully developed organisms are detected in cells suspected to be sinusoidal endothelial cells (Fig. 14 & 15). In these colonies the organisms with double unit membranes are larger (0,7–0,8 μm) than those in Kupffer cells and peritoneal macrophages, irregular in outline, round to ovoid in shape, uniform in size and have more or less evenly distributed electron dense and electron pale material. There is virtually no ground substance between the organisms.



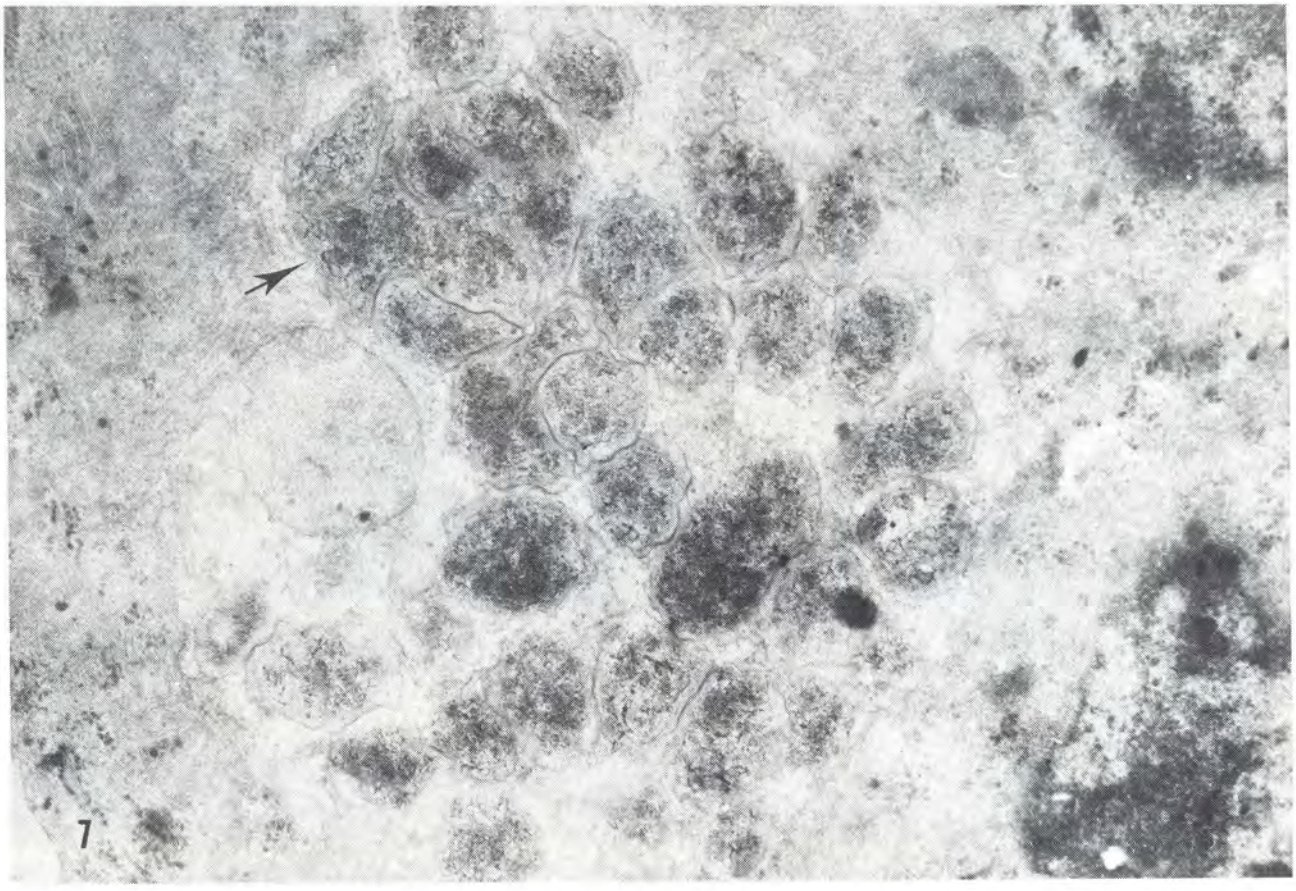
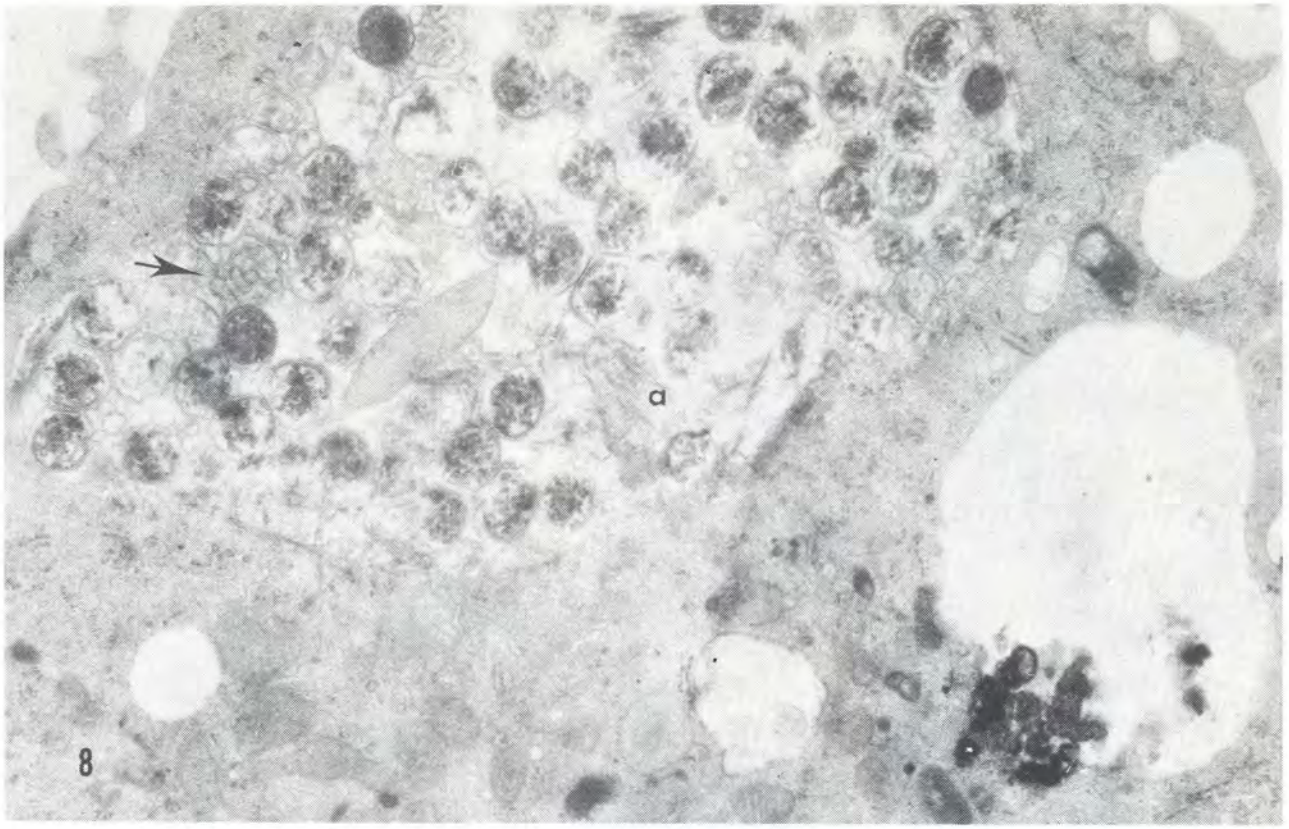


FIG. 6 Division between "developing organisms" more clearly defined (arrow) than in Fig. 5. A mosaic of closely packed organisms becomes visible. Peritoneal cell collected at 96 h post infection. ×42700

FIG. 7 Colony of what appears to be almost fully developed organisms in intimate contact with cytoplasm of a peritoneal macrophage collected 96 h after infection. The "organisms" are densely packed but clearly distinguishable, except at the top left (arrow) where a roughly spherical group of structures is reminiscent of the pattern seen in Fig. 6. ×34200



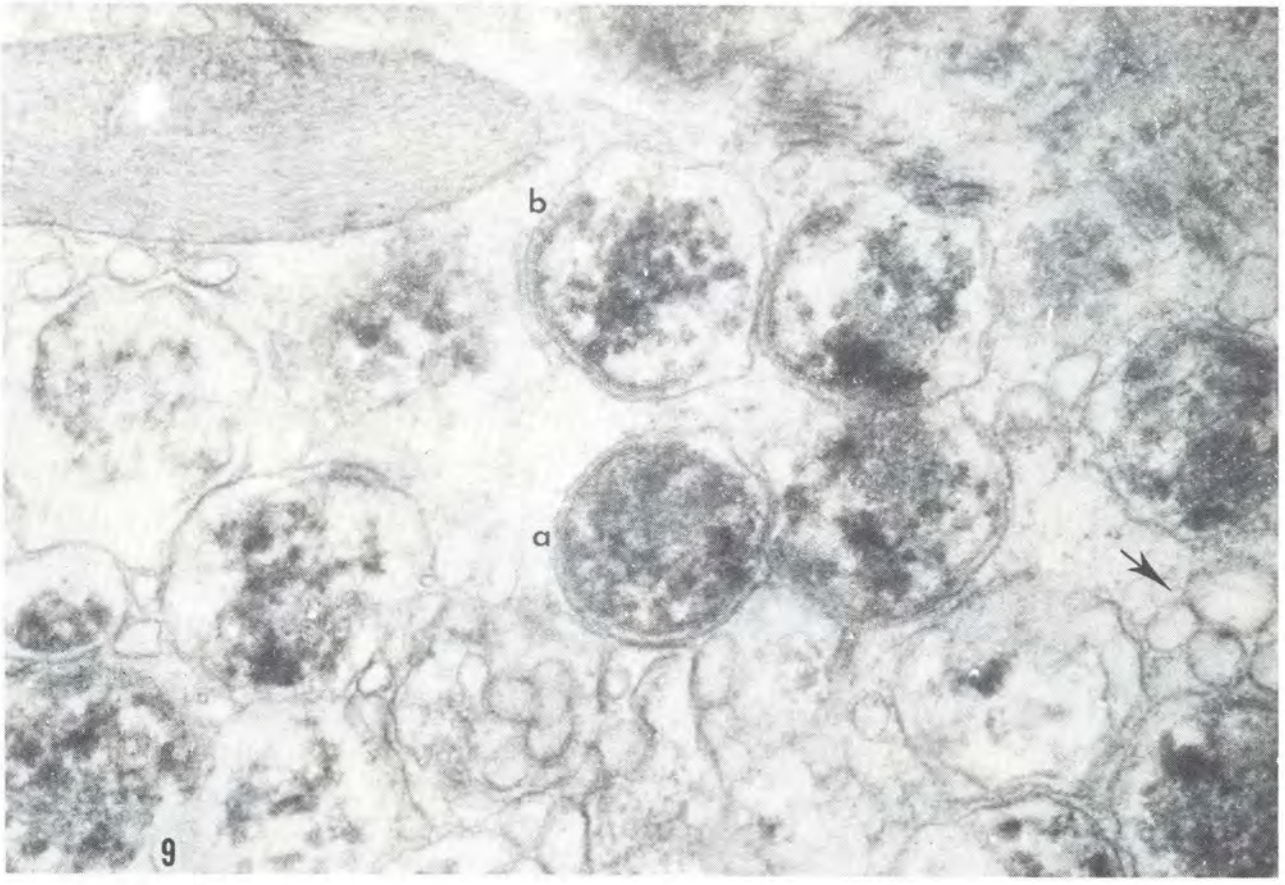


FIG. 8 Fully developed "organisms" loosely arranged in a mouse peritoneal cell at 96 h post infection. In the absence of a limiting membrane "organisms" infiltrated between swollen endoplastic reticulum (arrow) and unidentifiable fibrillar material (a), presumably of cell origin. Note the regular round shape and size of the "organisms" as well as the even distribution of electron dense and electron lucent material in them. ×18900

FIG. 9 Higher magnification of colony in Fig. 8 to show the double unit membrane (a & b), swollen endoplastic reticulum (arrow) and finer detail of distribution of electron dense and lucent material in each "organism". ×72000

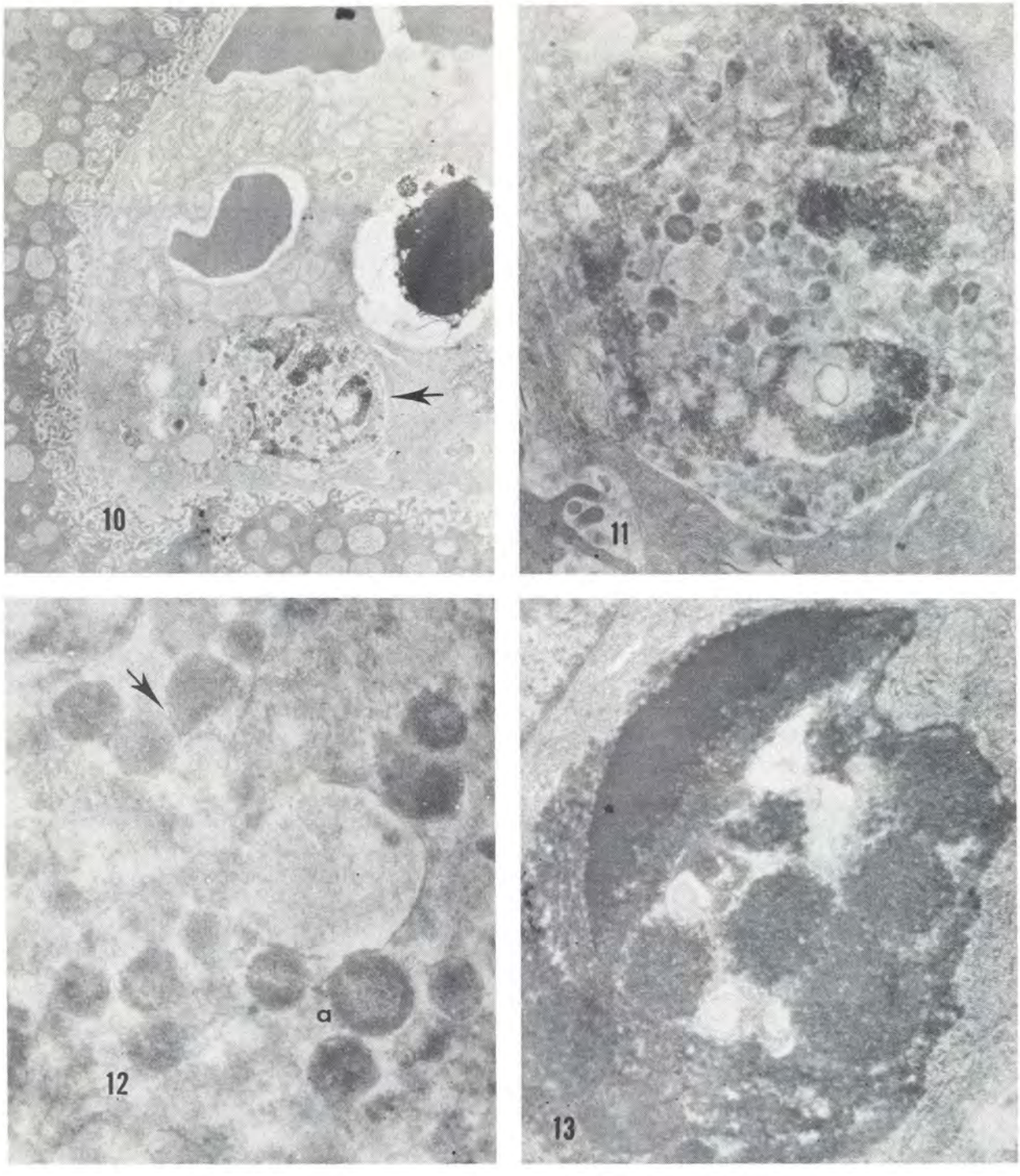


FIG. 10 Inclusion in a Kupffer cell (arrow). ×5800. Fig. 11 (×15 000) and 12 (×44 000) are higher magnifications of Fig. 10
FIG. 13 Fragmented body in the cytoplasm of a mouse Kupffer cell

Mouse heart

In sections of membrane-bound colonies of *C. ruminantium* in myocardial capillary endothelial cells (Fig. 16 & 17), the organisms in shape closely resemble those described in liver sinosoidal endothelial cells. However, they contain more abundant electron dense material and lie in a matrix of dense reticular to fibrillar network. In some colonies the organisms appear to be uniform in size (Fig. 17), whereas in others large (0,9 μm), (Fig. 16, arrow), intermediate and small (0,5 μm) forms are seen in the same colony (Fig. 16).

Mouse spleen

Solid dense bodies suspected to be parasitic inclusions which were detected in splenic histiocytes of infected mice (Fig. 18 & 19) are indistinguishable from those described in mouse peritoneal macrophages. Only rarely do they appear to be within a vacuole (Fig. 18), which, however, is not membrane-bound and not clearly distinguished from the cytoplasm.

Lymph nodes of sheep and cattle

Structures suspected to be parasitic inclusions, comprising solid dense bodies and those undergoing cleavage, observed in the lymph nodes of sheep and cattle infected with the Ball 3 strain of *C. ruminantium* (Fig. 20–24), are indistinguishable from those described in the macrophages and Kupffer cells of mice infected with the mouse adapted strain. They are densely granular in structure and are devoid of limiting membranes.

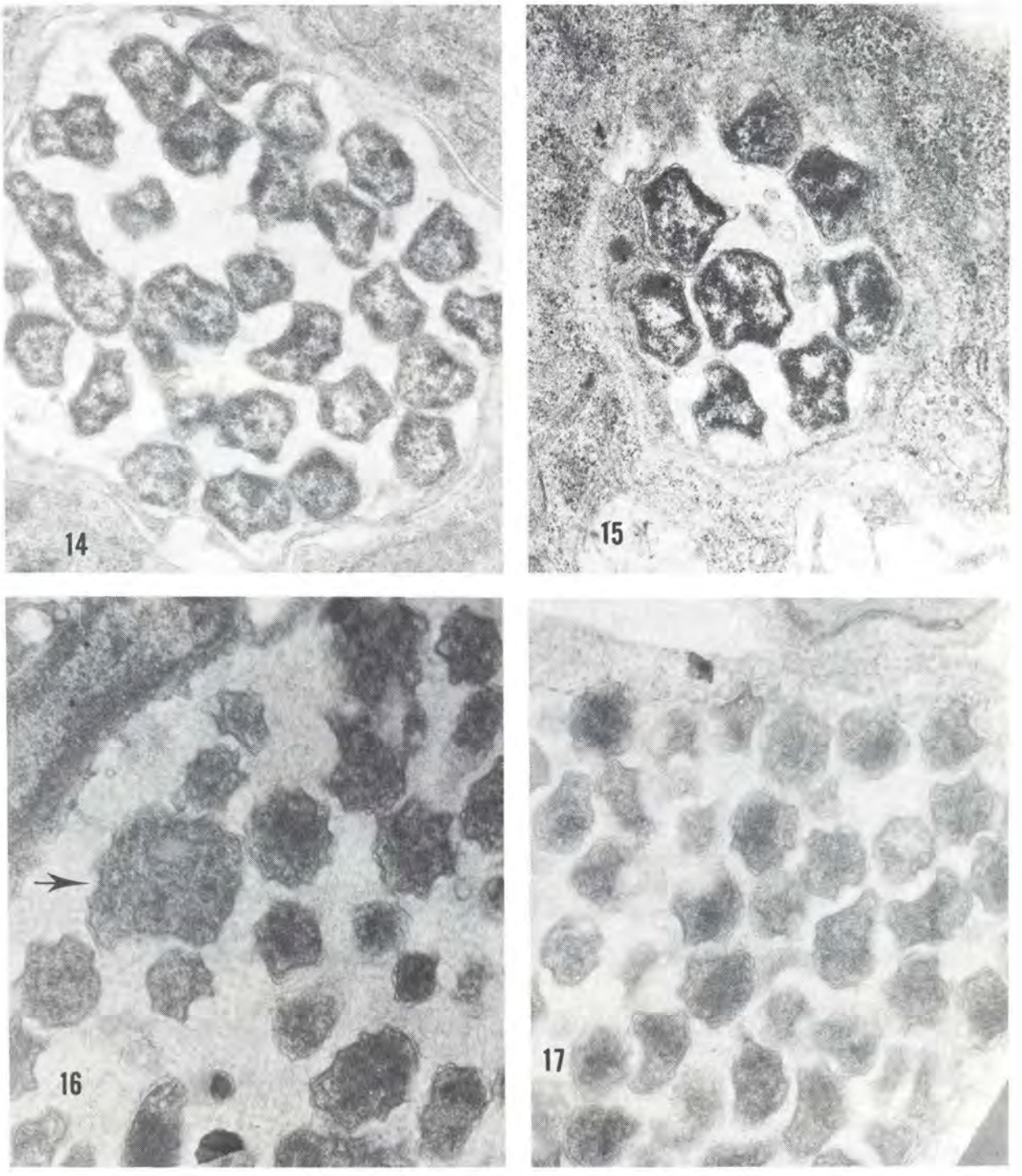


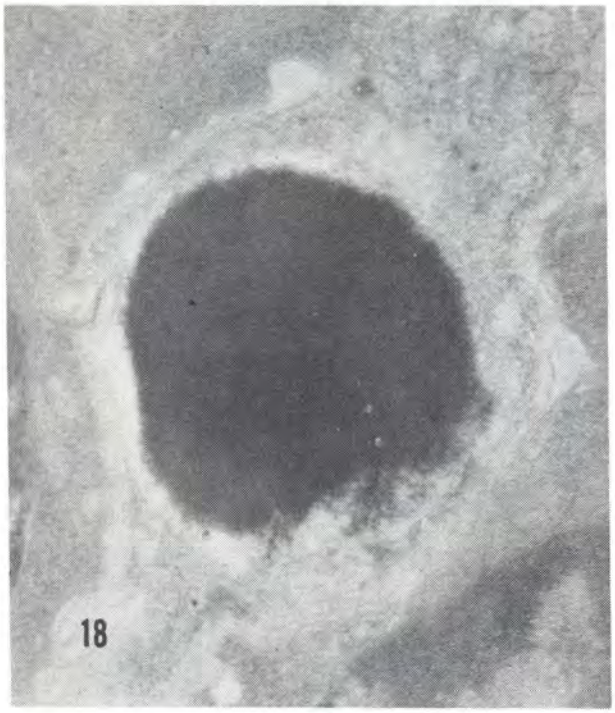
FIG. 14–17 *C. ruminantium* in mouse endothelial cells of liver sinusoids (Fig. 14 & 15) and myocard capillaries (Fig. 16 & 17). Note the uniformity in size, shape and distribution of electron dense and lucent material of the organisms in the sinusoidal endothelial cells. Fig. 14, ×20 000 and Fig. 15, ×24 000

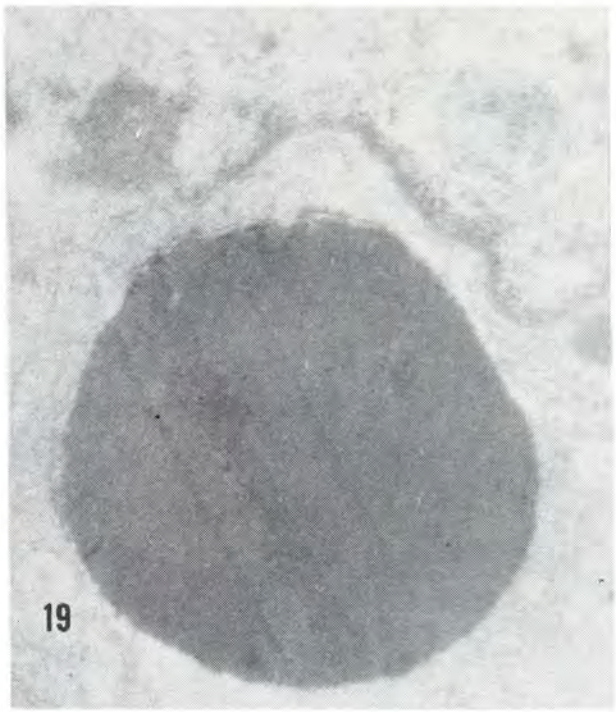
FIG. 16 Myocardial capillary endothelial cell. Note the pleomorphism in size of the organisms and the dense fibrillar matrix between the cells ×22 000

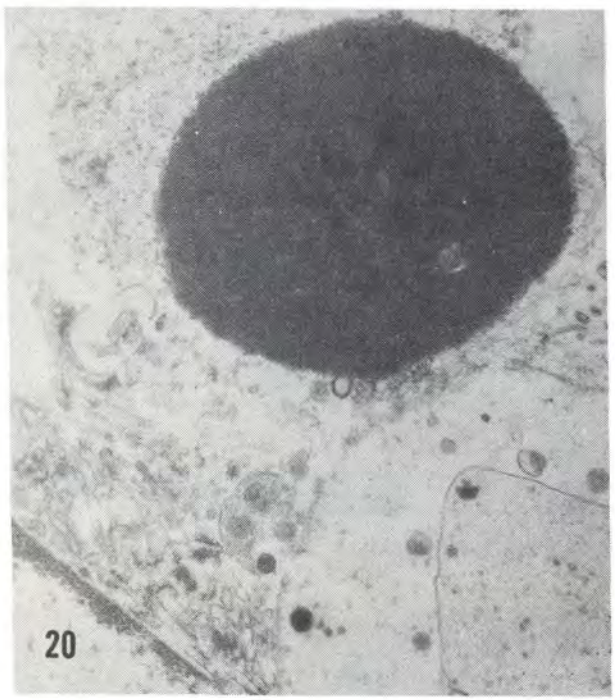
FIG. 17 The uniformity in size and shape and the preponderance of electron dense material in this photo is noteworthy. ×19 000

Controls

In the Kupffer cells of control mice infected with Rift Valley fever virus, phagocytosed cell debris are seen as ill-defined structures containing vacuoles and indistinct membranes against a dull amorphous background (Fig. 26 & 27). Furthermore, these structures are surrounded by a distinct membrane (Fig. 27) and are indistinguishable from phagosomes. In lymph node RE cells of heartwater-susceptible control sheep, membrane-bound phagosomes contain electron dense structures-of irregular shape and granularity on an amorphous moderately electron dense background (Fig. 28). Phagocytosed cell debris in peritoneal macrophages lack definition and usually lie in a large membrane lined vacuole (Fig. 29).







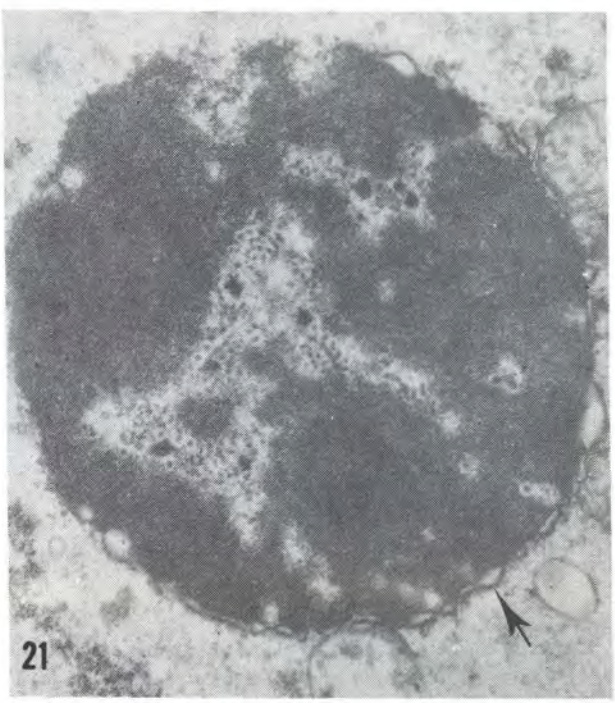
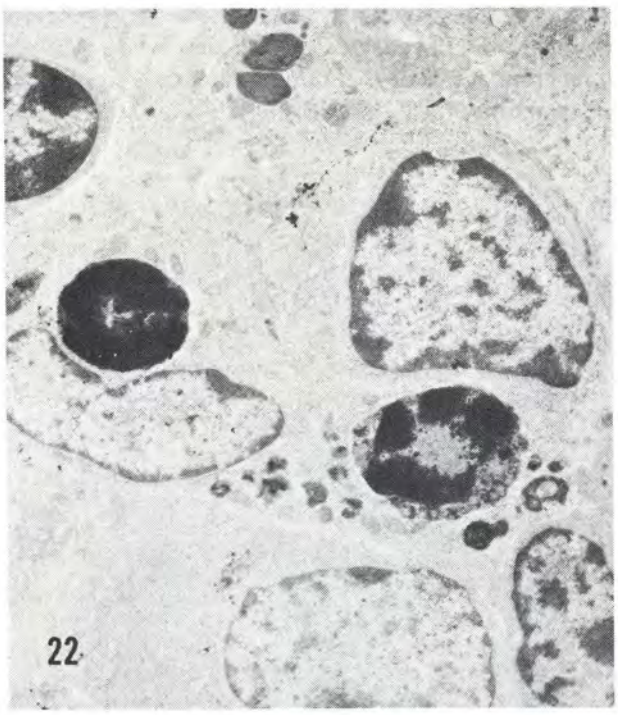
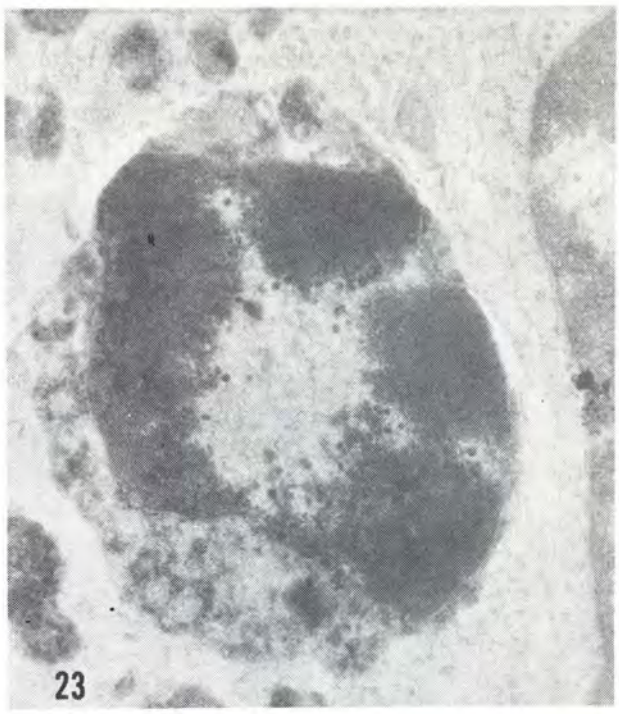


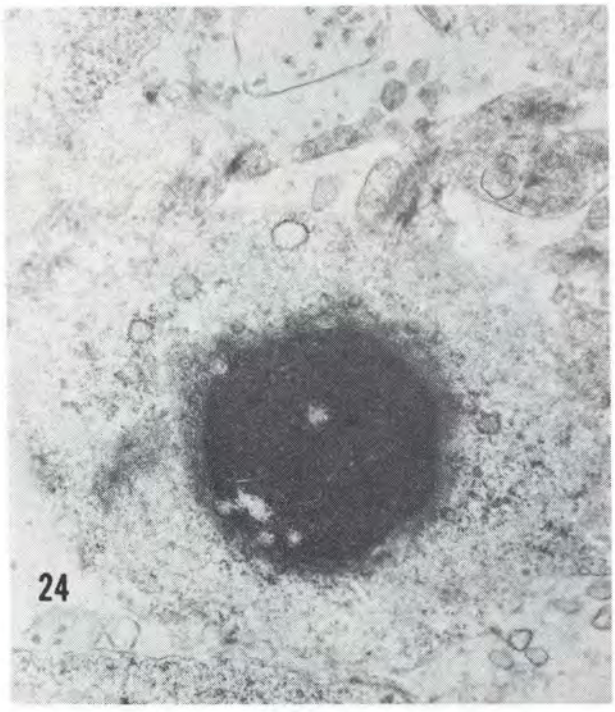
FIG. 18 & 19 Dense bodies in histiocytes of mouse spleen. ×15 000

FIG. 20 Dense bodies in histiocytes of bovine mesenteric lymph node. Note the identical appearance with the dense bodies in mouse cells infected with the mouse adapted strain. ×16 000

FIG. 21 Dense body undergoing cleavage in mesenteric lymph node of an ox. The structure resembling a membrane (arrow) may be endoplasmic reticulum in close contact with the growing parasitic inclusion. ×16 000







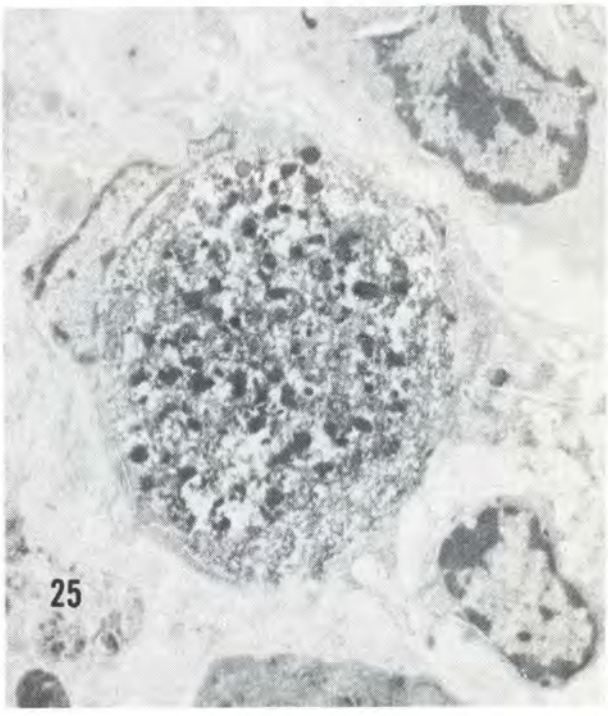


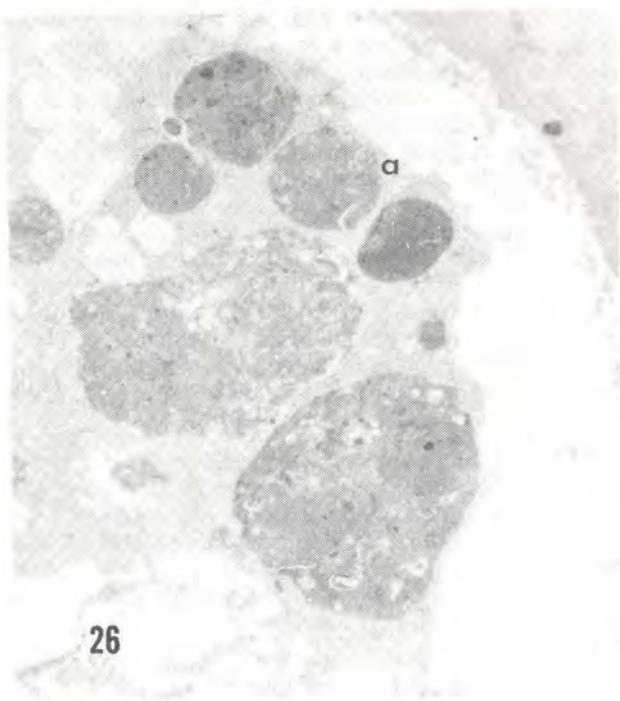
FIG. 22 & 23 Suspected parasitic inclusions in mesenteric lymph nodes of sheep

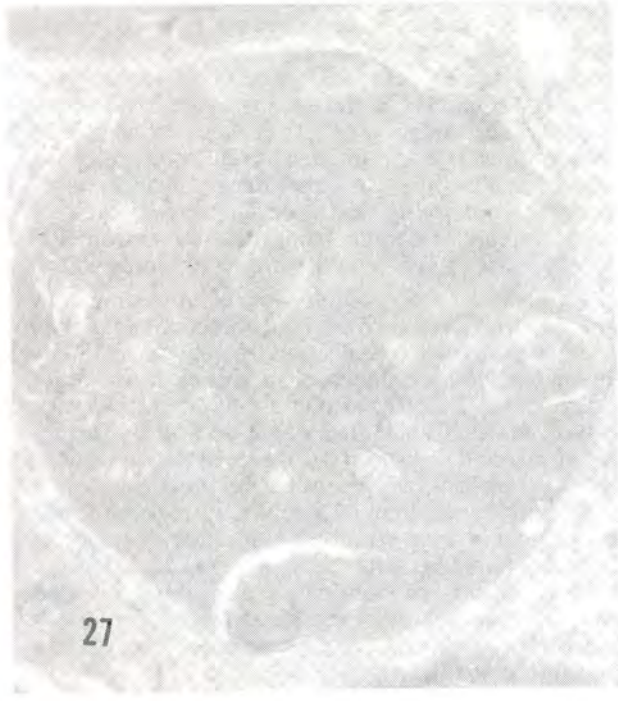
FIG. 22 Dense body (arrow) and a fragmented body (a) in 2 adjoining histiocytes. ×5 000

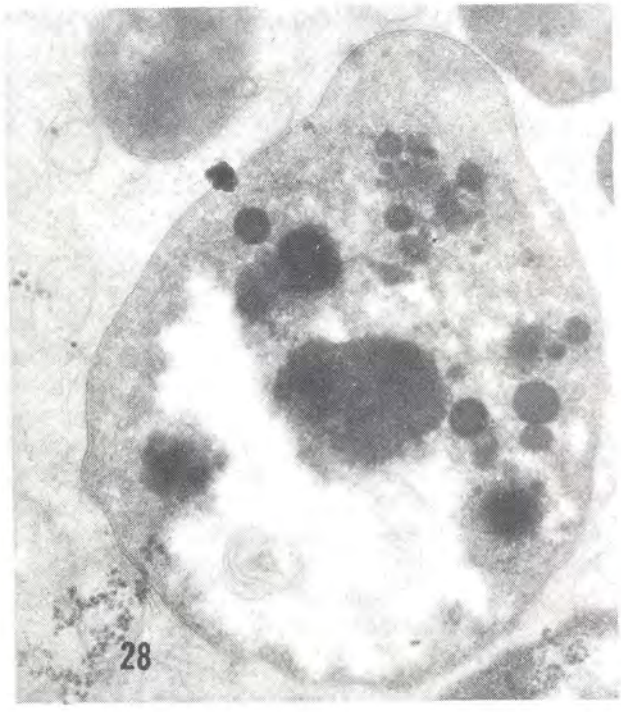
FIG. 23 Higher magnification of the fragmented body in Fig. 22. ×14 400

FIG. 24 Dense body in histiocyte of bovine mesenteric lymph node. ×16 000

FIG. 25 Kupffer cell in the liver of a mouse with a parasitic inclusion filling the entire cytoplasm. The "organisms" are at about the same stage of development as those shown in Fig. 6. ×6500







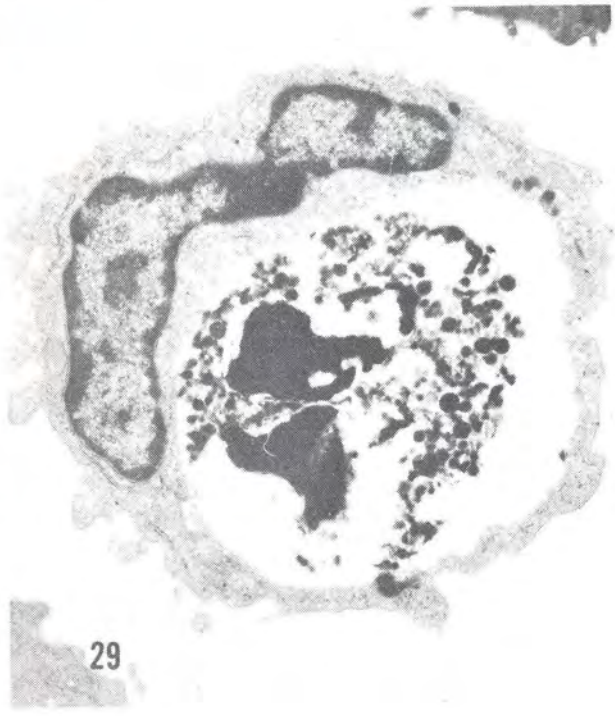


FIG. 26 to 29. Control electron photomicrographs depicting phagocytosed cell debris. Fig. 26. Phagocytosed cell debris in mouse Kupffer cell. Note the lack of definition and the fact that each portion of debris is enclosed in a membrane. ×6 500

FIG. 27 Higher magnification of one of the phagosomes in Fig. 26, to show the disorderly arrangement of membranous structures against a dull ill-defined background and a distinct phagocytic membrane. ×27 500

FIG. 28 Membrane-lined phagosome in a mesenteric lymph node RE cell of a control sheep. ×24 700

FIG. 29 Cell debris in a mouse peritoneal macrophage. Note the distinct vacuole lined by a membrane and the irregularity and absence of structure of the phagocytosed particles. ×8 000

Discussion

While the observations reported await confirmation, some preliminary conclusions are believed to be justified. The structures described in infected mouse peritoneal macrophages closely resemble those seen in mouse Kupffer cells and ruminant lymph node reticulo-endothelial cells and are suspected to represent developmental stages of *C. ruminantium*. The sequential observation of structures, interpreted as parasitic inclusions, in peritoneal macrophages suggests the possibility that a developmental cycle of *C. ruminantium* different from that in endothelial cells may exist in RE cells of ruminants and mice.

The earliest recognisable inclusion which is suspected to develop from an infective organism penetrating the host cell, is an extremely dense, finely granular body which increases in size to form larger dense bodies. These presumably undergo cleavage and give rise to fragmented dense bodies. By further sub-division and organization these bodies appear to form "organisms" which round off and develop a double unit membrane to form mature organisms. These structures may represent evolutionary stages of *C. ruminantium* because they are readily demonstrable in tissues of ruminants and mice infected with 2 different strains of the parasite and are absent in sections prepared from uninfected heartwater susceptible control animals.

The large dense bodies and particularly those undergoing fragmentation bear some superficial resemblance to phagocytosed necrotic cells and cell debris. From the electron photomicrographs, however, the differences are evident. The granular substructure of the fragmented bodies is identical to that of the preceding dense bodies, the "developing organisms" between the fragments have a distinct structure and in the absence of a limiting membrane the whole inclusion is in direct contact with the cell cytoplasm. Phagocytosed debris, on the other hand, have disorderly membranous structures in an amorphous, dull ground substance, they lack the characteristic structures of developing organisms and are contained within a distinct phagocytic membrane. Necrotic nuclear debris phagocytosed by peritoneal macrophages lie within large vacuoles bounded by a distinct membrane.

The fact that the structures suspected to be different forms of the heartwater agent as observed by electron microscopy, may be correlated with some of the parasitic stages seen with the light microscope in lymph node impression smears (Du Plessis, 1970), and mouse peritoneal macrophages (Du Plessis, unpublished data, 1974) supports the supposition that they represent developmental stages of *C. ruminantium*. In lymph node RE cells several stages of disintegrating fragmented bodies have been described in the light microscope study as well as cells with parasitic inclusions consisting of apparently fully developed organisms. Clearly defined vacuoles have proved to be phagocytic vacuoles in the electron microscopic study. They are a prominent feature of infected lymph node cells and mouse peritoneal macrophages but their relationship to the development of the parasite cannot be determined at present.

It has been suggested (Du Plessis, 1970), and these findings tend to support the hypothesis, that *C. ruminantium* may have a two-phase development in ruminants and mice. Following infection, initial development appears to be mainly but not exclusively, confined to RE cells with phagocytic abilities. The suspected parasitic stages characterized by the formation of dense bodies, their disintegration and eventual formation of mature "organisms" free in the cytoplasm of the host cell, are to be differentiated from the second phase in which membrane-bound colonies of organisms in morular form appear in the cytoplasm of vascular endothelial cells. In a newly infected animal some infective organisms probably penetrate vascular endothelial cells at the outset but it is thought that organisms liberated from RE cells are mainly responsible for the parasitization of endothelial cells. This would be in accordance with the finding that organisms suspected to be *C. ruminantium* can be demonstrated in the lymph node RE cells of infected sheep several days before typical colonies can be demonstrated in brain endothelial cells and that blood and lymph nodes are capable of transmitting the infection 1–3 days before brain capillaries are parasitized (Du Plessis, 1970).

A study of the suspected developmental stages in peritoneal macrophages collected on consecutive days, suggests that the cycle in RE cells is completed in 3–4 days, as fully developed organisms are seen as early as 72 hours after infection and newly developing dense bodies presumably of the second generation as early as 96 h after infection.

Noteworthy differences between the membrane-bound morular endothelial colonies and the suspected developmental stages described in RE cells are the absence of a limiting membrane between the parasitic inclusion and the host cell cytoplasm and the smaller and more uniform size of the organisms in the latter. The organisms occurring in membrane-bound morular colonies are pleomorphic in size, shape and inner structure in mouse heart endothelial cells and occur as small, intermediate and large forms in brain endothelial cells (Pienaar, 1970).

Should the proposed developmental cycle of *C. ruminantium* in RE cells be confirmed, this organism would occupy a unique position amongst other micro-organisms. The suggested cycle differs from that of the chlamydial agents with which it has some resemblance and with which it has been associated taxonomically (Pienaar, 1970). Chlamydial infective elementary bodies undergo reorganization within a membrane-bound vacuole in the host cell to form large reticulate forms which are not infectious and which multiply by binary fission. During a second process of re-organization small dense-centred elementary bodies arise which assume infectivity but do not divide (Storz, 1971).

The absence of a limiting membrane around colonies of suspected organisms in reticulo-endothelial cells further distinguishes *C. ruminantium* from the chlamydial agents during the development of which a membrane is found to be a distinct entity developing at the earliest stage of inclusion formation and persisting throughout the evolution of the infectious agent (Mitsui, Fujimoto & Kajima, 1964; Storz, 1971). In this respect the heartwater organism apparently resembles the rickettsias which replicate free in the cytoplasm amongst the organelles, except *Rickettsia sennetsu*, the colonies of which have an enclosing membrane of host-cell origin (Anderson, Hopps, Barile & Bernheim, 1965).

The dense bodies which have been described in this study as part of a suspected developmental cycle, show some resemblance to the structure of intracytoplasmic inclusion bodies seen in the diplorna viruses (Verwoerd, 1970). In this respect the developmental cycle of *C. ruminantium* appears to simulate that of viruses closer than any other micro-organisms larger than viruses and suggests that the taxonomic position of this organism should perhaps be reconsidered.

Some of the structures thought to represent developmental states of *C. ruminantium* in lymph node cells of animals infected with the Ball 3 strain are identical to those found in RE cells of mice infected with the mouse adapted strain. This is further proof that the organism isolated in mice from a goat (Du Plessis & Kumm, 1971) is *C. ruminantium*. Convincing evidence is the fact that the organisms described in myocard endothelial cells of mice infected with the mouse adapted strain are indistinguishable from the small and intermediate forms described in choroid plexus endothelial cells of sheep inoculated with blood obtained from a natural case of heartwater (Pienaar, 1970).

ACKNOWLEDGEMENTS

The author wishes to thank Mrs Shirley D. Ellis, Mr H. J. Els and Mr R. Watermeyer for the preparation of electron microscopic sections, Dr B. J. Erasmus for the supply of mice infected with Rift Valley fever virus and Mr W. J. Pienaar for technical assistance.

REFERENCES

ANDERSON, D. R., HOPPS, H. E., BARILE, M. F. & BERNHEIM, BARBARA C., 1965. Comparison of the ultrastructure of several rickettsiae, ornithosis virus and *Mycoplasma* in tissue culture. *J. Bact.*, 90, 1387–1404.

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

DU PLESSIS, J. L., 1970. Pathogenesis of heartwater: I. *Cowdria ruminantium* in the lymph nodes of domestic ruminants. *Onderstepoort J. vet. Res.*, 37, 89–96.

DU PLESSIS, J. L. & KUMM, N. A. L., 1971. The passage of *Cowdria ruminantium* in mice. *Jl S. Afr. vet. med. Ass.*, 42, 217–221.

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.

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

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.

PIENAAR, J. G., 1970. Electron microscopy of *Cowdria* (*Rickettsia*) *ruminantium* (Cowdry, 1926) in the endothelial cells of the vertebrate host. *Onderstepoort J. vet. Res.*, 37, 67–78.

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.

STORZ, J., 1971. Chlamydia and chlamydia-induced diseases, 1st. ed. Springfield, Illinois: Charles C. Thomas.

VERWOERD, D. W., 1970. Diplornaviruses: A newly recognized group of double-stranded RNA viruses. *Prog. med. Virol.*, 12, 192–210. (Karger, Basel, 1970).

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

Printed by and obtainable from The Government Printer, Private Bag X85, Pretoria, 0001.