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ULTRASTRUCTURAL OBSERVATIONS ON ENCEPHALITOZOON CUNICULI LEVADITI, NICOLAU ET SCHOEN, 1923, FROM MOUSE PERITONEAL MACROPHAGES

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Abstract. The life cycle of the microsporidian parasite *Encephalitozoon cuniculi* in mouse peritoneal macrophages has been studied with the electron microscope and details of the schizogonic and sporogonic phases are presented. Two embedding media, araldite and glycol methacrylate, were used and the preservation of different structures in these media is compared. A parasitophorous vacuole is formed as a space between dividing schizonts and the host cell membrane. The sporont wall forms initially as transverse bands of electron-dense material which fuse over the whole external surface to form a triple-layered wall, the outer two layers of which give rise to the exospore and endospore layers of the mature spore wall. The polar filament is formed by a reticulum of filament-forming substance around which are formed the concentric rings of the double membraned lamellae of the polaroplast.

Considerable interest has been shown over the last few years in the microsporidian parasite *Encephalitozoon cuniculi*, Levaditi, Nicolau and Schoen 1923, not least because of its importance as a latent infection in laboratory rodents when it may complicate the interpretation of experimental results. There has been much speculation as to its taxonomic position, but this has now been elucidated by electron microscopy.

Oval spores present in the brains of rabbits suffering with latent encephalitis were first reported by Wright and Craighead in 1922. Levaditi, Nicolau and Schoen described and named the parasite *Encephalitozoon cuniculi* (1923a), and considered it to be a microsporidian (1923b), though they did not demonstrate the polar filament characteristic of the group. In a review article (1924) they described their successful passages of the parasite into laboratory rodents and dogs.

Nelson (1962) working on a latent infection in laboratory mice finally confirmed the microsporidian nature of the parasite by demonstrating the presence of a polar filament extruded from the spore. Lainson et al. 1964 confirmed the presence of the coiled polar filament within the spore by electron microscopy. Weiser (1964) and Lainson et al. (1964) independently concluded that *Encephalitozoon* had no validity as a genus and was merely a junior synonym of the genus *Nosema* Nägeli.

The parasite was referred to as *Nosema cuniculi* until Cali (1970) showed that *Nosema bombycis*, the type species of the genus *Nosema*, had nuclei in a diplokaryon arrangement throughout the life cycle. No such arrangement was observed in *N. cuniculi*. She used this as a basis for separating the two parasites into different genera and proposed that *Encephalitozoon* be reinstated as a genus in the family Nosematidae.

Sprague and Vernick (1971) gave further evidence of the unpaired state of the

nuclei and showed that sporulation was disporoblastic. They gave comparative definitions for the genera *Nosema*, *Encephalitozoon* and *Glugea*. By their definitions *Encephalitozoon* differs from *Nosema* in having unpaired nuclei throughout most of the life cycle and in the development taking place within a sporogony vacuole in the host cell. It differs from *Glugea* in that it does not stimulate the production of a cell hypertrophy tumour.

There have been a number of electron microscope studies of *Encephalitozoon* but it was not until 1971 that Sprague and Vernick published material which showed details of the life cycle. They demonstrated the complete development from schizont to spore within a vacuole in the host cell cytoplasm. The thin-walled, rounded schizonts were peripherally situated, often partially embedded in the host cell cytoplasm. Schizogony occurred while adhering to the vacuole wall and the resulting stages became sporonts free in the vacuole. The elongate sporonts were recognised by a thickened membrane. Sporogony resulted in two uninucleate sporoblasts. In the sporoblasts flattened sacs of endoplasmic reticulum were visible and, in later stages, the developing polar filament, a complex system of cytoplasmic membranes and the layers of the wall were also present. Petri and Schiødt (1966) observed the membranes of the polaroplast as a system of roughly parallel membranes, which were arranged concentrically, at times occurring in groups of three. The spore was described as an ovoid body, often comma shaped in longitudinal section, with not more than 5 coils of the polar filament curving in an oblique spiral. The anterior straight part of the filament was closely associated with the membranes of the polaroplast whilst the coils surrounded a posterior vacuole.

MATERIAL AND METHODS

The parasite was maintained in a Swiss strain of laboratory mice by a two-weekly passage of 0.05 ml ascitic fluid. The original inoculum was obtained from Dr. J. B. Nelson of the Rockefeller University, New York. 2 ml of ascitic fluid were aspirated from a mouse infected 14 days previously. The fluid was fixed in 3% glutaraldehyde in sodium cacodylate buffer pH 7.2 and post-fixed in Dalton's chrome osmium. The ascites contained a quantity of blood and consequently the material coagulated to form a pellet. This pellet was stained with uranyl acetate in 10% formalin, dehydrated in alcohols, cleared in propylene oxide, and embedded in araldite.

A second preparation was prepared four months and eight passages later from a mouse similarly infected for 14 days. This material was fixed in glutaraldehyde, post-fixed in chrome osmium and stained with uranyl acetate. However, the material contained little blood and after the post-fixation it was centrifuged and the cellular material embedded in 2% agar for ease of handling. The material was dehydrated and embedded in glycol methacrylate after the method of Leduc and Bernhard (1967).

All sections were cut on an LKB Ultratome III, stained with uranyl acetate and Reynold's lead citrate and examined on a Phillips EM 300.

OBSERVATIONS

The parasites were easily detectable in peritoneal macrophages in infections of 14 days' duration (Plate I, Figs. 1 and 2). A maximum of 10% of macrophages was infected. The parasites were present in the host cell cytoplasm within a membrane-limited vacuole containing an electron-lucid substance. The host cell nucleus had been pushed to one side of the cell and the cytoplasm contained many vacuoles. The outline of the araldite embedded macrophages was very irregular with many indentations and folds. All stages of the life cycle from schizonts to spores could be observed around or in one vacuole.

Essential differences in the preservation of the parasite were observed after embedding in the two media. Unit membranes were usually not visible in the glycol methacrylate (G.M.A.) embedded material although their position could be deduced from the presence of an electron-lucid space at sites of endoplasmic reticulum and nuclear envelopes (Plate II, Figs. 3 and 4). However, other features of the sporoblasts and spores were well preserved in G.M.A. relative to araldite. In araldite, sporoblasts had a crenulated outline and the spores were extremely electron-dense and often did not maintain their integrity or show structural details (Plate I, Fig. 1). The cytoplasm of spores in G.M.A. showed a slight degree of shrinkage away from the spore coat.

Uninucleate schizonts were observed adhering to the side of the vacuole (Plate I, Fig. 1) and were often partially or wholly embedded in the host cell cytoplasm (Plate II, Figs. 1 and 2). Parasites which lay in the host cell cytoplasm were completely surrounded by a host cell membrane closely applied to the parasite's plasmalemma (Plate II, Fig. 1 arrowed). In parasites lying at the edge of a vacuole this host membrane was continuous with the vacuole membrane. With the G.M.A. material no membranes were preserved but partially embedded schizonts could be distinguished from the host cell cytoplasm by their more granular consistency (Plate II, Fig. 4).

It would seem that the vacuole developed initially as an extension of the narrow space between the schizont membrane and the host membrane. The extension would occur along the planes of division and separation of the daughter schizonts. Figure 4 of Plate II shows a vacuole formed between 4 schizonts. Initially the vacuole would form between 2 schizonts (Plate II, Fig. 3). In this figure infection must have resulted from invasion by 2 separate sporoplasms.

Sporonts were recognised by their thick surface covering. Stages apparently intermediate between schizonts and sporonts were observed (Plate III, Fig. 1); the parasite surface still in contact with the wall of the vacuole was bounded by a simple plasmalemma while the surface presented towards the vacuole was partially thickened. The formation of the additional layers of the sporont wall occurred either while the parasite was still attached or after it had become free in the vacuole. Mature sporonts were found only in the vacuole centre.

The electron-dense layer of the sporont wall was laid down in transverse bands as seen in sections cut tangential to the wall (Plate III, Fig. 3). In sections cut perpendicular to the wall (Plate III, Fig. 2) the bands were seen as short bars (small arrows) which joined to form a more complete covering (large arrows) as more electron-dense material was deposited.

The wall of the mature sporont was composed of three layers (Plate III, Fig. 4): an inner unit membrane (μm) bounding the cytoplasm, a middle electron-lucent layer—the future endospore (en)—and a uniformly thick outer electron-dense layer, the future exospore (ex). Thus the first stages of endospore formation occurred concurrently with exospore deposition in the sporont but completion was not achieved until spore maturation.

Although dividing sporonts were not seen, the early sporoblast was recognised by the presence of substantial amounts of endoplasmic reticulum arranged concentrically around the nucleus (Plate IV, Fig. 3). The first evidence of sporulation was the presence of a rudimentary polar filament in close association with a reticular structure (Plate IV, Fig. 1). It was considered that this structure played a part in filament formation as it was invariably found associated with the straight part of the filament at the anterior end.

The axial electron-dense core of the filament was formed first and an outer electron-dense layer was deposited around it, probably by the reticular structure (Plate IV, Figs. 1 and 2).

As seen in araldite sections (Plate IV, Fig. 3) the immature filament was composed of a double wall surrounding a ring of electron-dense material which was itself separated by a lucent ring from the central core.

The anterior region of the polar filament, still surrounded by the reticular filament-forming substance, was seen in an almost mature spore within the lamellae of the polaroplast (Plate VI, Fig. 1). These lamellae formed a series of concentric bands, alternately electron-dense and lucent, occurring usually in groups of three so that they had the appearance of two closely apposed double membranes. (Plate V, Fig. 1 and Plate VI, Fig. 1). It is unlikely that they had the typical structure of the unit membrane since they consistently occurred in a well preserved state in G.M.A. embedded material. These membranes were seen in one spore to run posteriorly in much the same direction as the polar filament (Plate V, Fig. 2) but they never surrounded the coiled part of the latter. External to the ordered membranes of the polaroplast was an area of single membranes and vesicles (Plate V, Fig. 2 and Plate VI, Fig. 1).

The appearance of the mature spore presented no structural features that have not already been described. The appearance of the polar filament was markedly different in araldite and G.M.A. embedded specimens. In araldite the layers of the polar filament of immature spores resolved into a central electron-dense core 41nm in diameter within a lucent ring and the whole enclosed by two distinct membranes (Plate VI, Fig. 2). Differentiation of the outer layer of the lucent ring as a layer of medium density just within the membranes was observed in some sections. In G.M.A. embedded material the whole was enclosed by an electron-lucid ring—the site of the membranes seen with araldite. Internal to this lucid ring was an electron-dense layer surrounding an electron-lucent layer which enclosed an electron-dense core. At times a narrow electron-dense ring could be seen within the electron-lucent layer (Plate V, Fig. 1).

Four to five coils of the polar filament were observed, although four coils occurred more frequently than five (Plate VI, Fig. 2).

DISCUSSION

The life cycle of *E. cuniculi* seen with the electron microscope is a relatively simple one, as outlined by Sprague and Vernick (1971). Uninucleate schizonts divided by binary fission and eventually by the formation of a thick wall gave rise to sporonts and migrated into the vacuole centre. Sporonts divided to give two sporoblasts and each developed into a mature spore with a polar filament, anterior polar sac and polaroplast, and posterior vacuole typical of the Microsporida.

The present study concurs with this account and indeed agrees with the interpretation of the life cycle by Lainson et al. (1964) in their light microscope investigation. However, there have been a number of light microscope studies reporting details not yet seen with the electron microscope.

Petri (1969) working with the parasite in rat ascites sarcoma cells described the occasional tetranucleate rounded form which he considered to be a part of the schizogonic cycle. He further reported bands of dividing cells and angled pairs of slim cells indicating binary division, but he made no mention of these in his electron microscope study.

Meiser, Kinzel and Jírovec (1971) working with a plasmacytoma ascites in Syrian golden hamsters observed the multiplication stages of the schizonts occurring in long chains. Vávra, Bedrník and Činátl (1972) observed two structurally distinct types of fission, followed by sporogony, in a culture of rabbit choroid plexus cells. The first cycle involved synchronous divisions of the nucleus and cytoplasm and they considered

that this was not true schizogony. The second cycle was a typical schizogony with cytokinesis delayed until the completion of nuclear division and the migration of the nuclei to opposite poles of the cells. The latter cycle often resulted in the bands or chains of cells observed by the previously mentioned authors.

These bands of cells were not observed in any electron microscope studies, most of which were concerned with parasites developing within normal tissues of the host animals. It may be that the longitudinal bands of dividing cells are more characteristic of development within tumour or tissue culture cells.

Since 1964 when Lainson et al. first demonstrated by electron microscopy the presence of the polar filament in *Encephalitozoon* isolated from rats, there have been a number of ultrastructural studies of the parasite in material from rabbits (Cali 1971), rat ascites sarcoma cells (Petri and Schiødt 1966, Petri 1969) and mice (Arison, Cassaro and Pruss 1966, Akao 1969, Sprague and Vernick 1971). Recent studies of infection in the squirrel monkey, *Saimiri sciureus* (Brown et al. 1973, Anver, King and Hunt 1973) have included electron micrographs of spores.

There appear to be no differences, apart from those arising from problems with fixation, between the organisms isolated from the various hosts, a finding which was substantiated by Montrey, Shadduck and Pakes (1973) in their in-vitro study of three isolates of *Encephalitozoon* from mice, hamsters and rabbits.

This electron microscopy study on *E. cuniculi* concurred with all previous studies in showing that the parasite was uninucleate throughout most of the life cycle and that, when two nuclei were present immediately following nuclear division, they were not adjoined and did not behave as a diplokaryon.

The cytoplasm of the schizonts and the sporogonic stages compared with that of the host cells, had a more granular appearance, due to an abundance of ribosomes, and, in common with other microsporidian genera (Lom and Corliss 1967), there were no mitochondria. Endoplasmic reticulum with its associated ribosomes was present in all stages but was more abundant in the sporoblasts. The presence of endoplasmic reticulum in G.M.A. embedded material could only be deduced by electron-lucid bands in the cytoplasm.

The nucleus was typical for the Microsporida; the nucleoplasm had a similar electron-density to the cytoplasm and was often defined in the cell only by the nuclear envelope. When G.M.A. was used, nuclear material was often difficult to distinguish as the envelope was poorly preserved.

Studies by Ishihara (1968) and Weidner (quoted by Trager 1974) showed that the sporoplasm lay directly in the host cell cytoplasm after injection through the polar filament. Vávra (1973) stated that typically in the vegetative cycle of the microsporidia the parasite was separated from the host cell cytoplasm only by the surface membrane of the parasite and a host cell membrane was formed during the sporulation sequence. The present study showed that a unit membrane of host cell origin developed externally to the surface membrane of the schizont and later became the membrane of the vacuole. A similar membrane of host cell origin formed in response to invasion by merozoites of *Eimeria tenella* (Scholtyseck 1969) and *Toxoplasma* (Trager 1974) and also formed the limiting membrane of the parasitophorous vacuole. By comparison with this development in coccidians it is suggested that the vacuole in which *E. cuniculi* developed should be termed a parasitophorous vacuole.

Trager (1974) stated also that the amastigotes of *Leishmania donovani* in hamster spleen cells were enveloped by a host cell membrane. However, in contrast to *E. cuniculi*, when the parasites divided each daughter cell became directly surrounded by its individual outer membrane and no vacuole was formed.

Schizogony, in this study and in that of Sprague and Vernick (1971), occurred in

a similar manner to that described in the initial stages of the second cycle of division of Vávra et al. (1971). Karyokinesis was completed before the outset of cytokinesis, the latter occurring in a plane transverse to the longitudinal axis. Schizogony continued alongside sporulation, so that all stages were present in a single host cell.

In the formation of the sporonts transverse bands of electron-dense material were laid down external to the plasmalemma. The bands later merged to form a continuous layer over the whole cell. This layer was separated from the plasmalemma by an electron-lucent layer believed to be the future endospore of the mature spore. Cali (1970), maintained that the thickenning of the parasite cell membrane was the one morphological change that indicated the initiation of the sporogonic phase and the cell's commitment to spore formation. She showed this thickening as a deposition of electron-dense material on the exterior of the plasmalemma of *Nosema bombycis* and *N. apis*. The present study shows clearly that in *E. cuniculi* the layers of the spore wall were also laid down external to the plasmalemma. The three surface layers of the sporont, consisting of plasmalemma, endospore and exospore, bear a marked resemblance of the dark, light, dark layers seen by Sprague and Vernick (1971) at the surface of sporonts. Lom and Corliss (1967) noted a thickening of the sporont wall in *Pleistophora hypessobryconis*, but in this case there were only two layers—the inner plasmalemma and an outer, thick homogenous wall. A similar wall of only 2 layers was shown by Canning and Sinden (1973) in *Nosema algerae*. The latter authors further showed that the deposition of the outer electron-dense layer was a more complex process than that of *Encephalitozoon*. In *N. algerae* the electron-dense exospore material was present in the schizont beneath and between a series of transverse tubules. In the formation of the sporont wall the exospore material passed between the tubules to form a continuous layer lying close to the plasmalemma. The electron-lucent endospore was not secreted in *N. algerae* until the sporoblast stage, whereas in *E. cuniculi* the rudiments were laid down concurrently with the exospore.

Early sporoblasts were distinguished from the sporont by the presence of a reticular structure and the rudiments of the developing polar filament. These structures were present in the material of Petri and Schiødt (1966) within a sporoblast, which they did not recognise as such but which has the three layered wall structure typical of the sporont and early sporoblast. The universal association of the reticular structure with sections of developing polar filament strongly suggested that at least the anterior straight part of the filament was formed from this structure. The polar filament was laid down as a central core surrounded by an electron-lucent area with radiating strands. The outer electron-dense layer was deposited around this by the filament forming substance. Canning and Sinden (1973) described a similar process of filament formation in *N. algerae* but the development described by Lom and Corliss (1967) in *P. hypessobryconis* was radically different. They showed that the filament formed from a number of vesicles which appeared in positions where the coils of the polar filament would develop. The walls of the vesicles gave rise to the outer wall of the filament. Vávra (1965) believed that the polar filament differentiated from a large and irregular cytoplasmic canal which itself arose by the coalescence of cytoplasmic Golgi-type vesicles.

The structure of the mature polar filament was seen by Vávra, Joyon and de Puytorac (1966) in many microsporidian genera to possess a fundamental structure of concentric electron-dense and lucent bands. Typically there was an electron-dense core, a dark outer layer with an intervening electron-lucent layer, and a thin outer electron-lucent layer bounded by a unit membrane. In the present study the polar filament was bounded by a double membrane which was seen as an electron-lucid ring in G.M.A. embedded material. Internal to the membranes was an electron-dense layer—appearing as a more distinct and darker layer in G.M.A. embedded material—

an intervening electron-lucent area and a dense core. In the G.M.A. material an electron-dense ring was at times seen within the electron-lucent area. Lom (1972) referred to a similar ring as the middle osmiophilic layer, which Akao (1969) had tentatively named an inner sheath. This ring could not be here interpreted as the numerous spirally wound fibrils seen by Vávra (1965), Lom and Corliss (1967) and Akao (1969). Akao further reported a minute electron-dense fibril in the centre of the electron-dense core, a structure not shown by other authors and not seen in the present study.

The polaroplast of *E. cuniculi* appeared as a series of triple membranes arranged concentrically around the polar filament and the reticular filament-forming substance. These triple membranes were previously described by Petri (1969) but the layer of single membranes external to them are newly described. The membranes of the polaroplast were seen in *P. hyphessobryconis* as irregular lamellae and in a *Glugea* sp. as vesicles (Lom and Vávra 1963). However, Canning and Sinden (1973) showed a polaroplast in *N. algerae* with a similar organisation of double-membraned or triple-layered lamellae, whilst Lom and Corliss (1967) observed that the irregular laminae of *P. hyphessobryconis* always separated into 3 electron-dense membranes. Canning and Sinden (1973) postulated that these membranes were derived from Golgi vesicles. They further suggested that the membranes of the polaroplast were arranged in stacks following the contours of the polar cap and with a central space accommodating the polar filament. A similar configuration was postulated by Weidner (1972) in *Nosema michaelis*. In the present study insufficient material was available to speculate on the configuration of the polaroplast in *E. cuniculi*.

It was shown in this study and that of Canning and Sinden (1973) that the structure of the polaroplast membranes was not that of typical unit membranes as evidenced by their universal preservation in glycol methacrylate.

Vávra (1965) stated that the end of spore development was marked by the appearance of a thick electron-transparent layer under the external membrane of the young spore. Sprague and Vernick (1968) suggested that the endospore occupied the space between the two layers of the plasmalemma, but in *E. cuniculi* it was clearly seen as a layer external to the plasmalemma and itself bounded by the electron-dense exospore. The formation of the spore coat was characterised by a thickening of this middle, endospore layer. In *N. algerae* (Canning and Sinden 1973) and *P. hyphessobryconis* (Lom and Corliss 1967) the endospore was first seen as a layer interpolated between the plasmalemma and electron-dense exospore material during sporulation and later increased in thickness.

Electron microscope studies of microsporidian spores suggest that there is a fairly constant number of polar filament coils for a given species. In all studies of *Encephalitozoon*, whether from rodent, rabbit or primate sources, there were consistently 4—5 coils, although the present author found 4 coils to occur more frequently than 5. The coils lay at an angle roughly 45° to the longitudinal axis.

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НАБЛЮДЕНИЕ НАД УЛЬТРАСТРУКТУРОЙ МИКРОСПОРИДИИ *ENCEPHALITOZOON CUNICULI* LEVADITI, NICOLAU ET SCHOEN, 1923, ИЗ МЫШИНЫХ ПЕРИТОНЕАЛЬНЫХ МАКРОФАГОВ

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Резюме. С помощью электронной микроскопии изучен жизненный цикл паразитической микроспоридии *Encephalitozoon cuniculi*, обнаруженной в мышиных перитонеальных макрофагах и подробно представлены схизогонные и спорогонные стадии. Для заливки были использованы две среды: аралдит и гликоль метакрилат; дано сравнение разных структур, заключенных в этих средах. Между шизонтами и мембраной хозяинной клетки образуется в виде пространства паразитофорная вакуоль. Стенка спороита формируется сначала как поперечные полосы электронно-плотного материала, которые сливаются по целой внешней поверхности, образуя трехслойную стенку; внешних два слоя дают начало экзоспоровой и эндоспоровой слоям зрелой стенки споры. Полярное волокно образуется путем сети волокнистой субстанции, вокруг которой формируются концентрические кольца пластинок с двойной мембраной, представляющие собою поларопласт.

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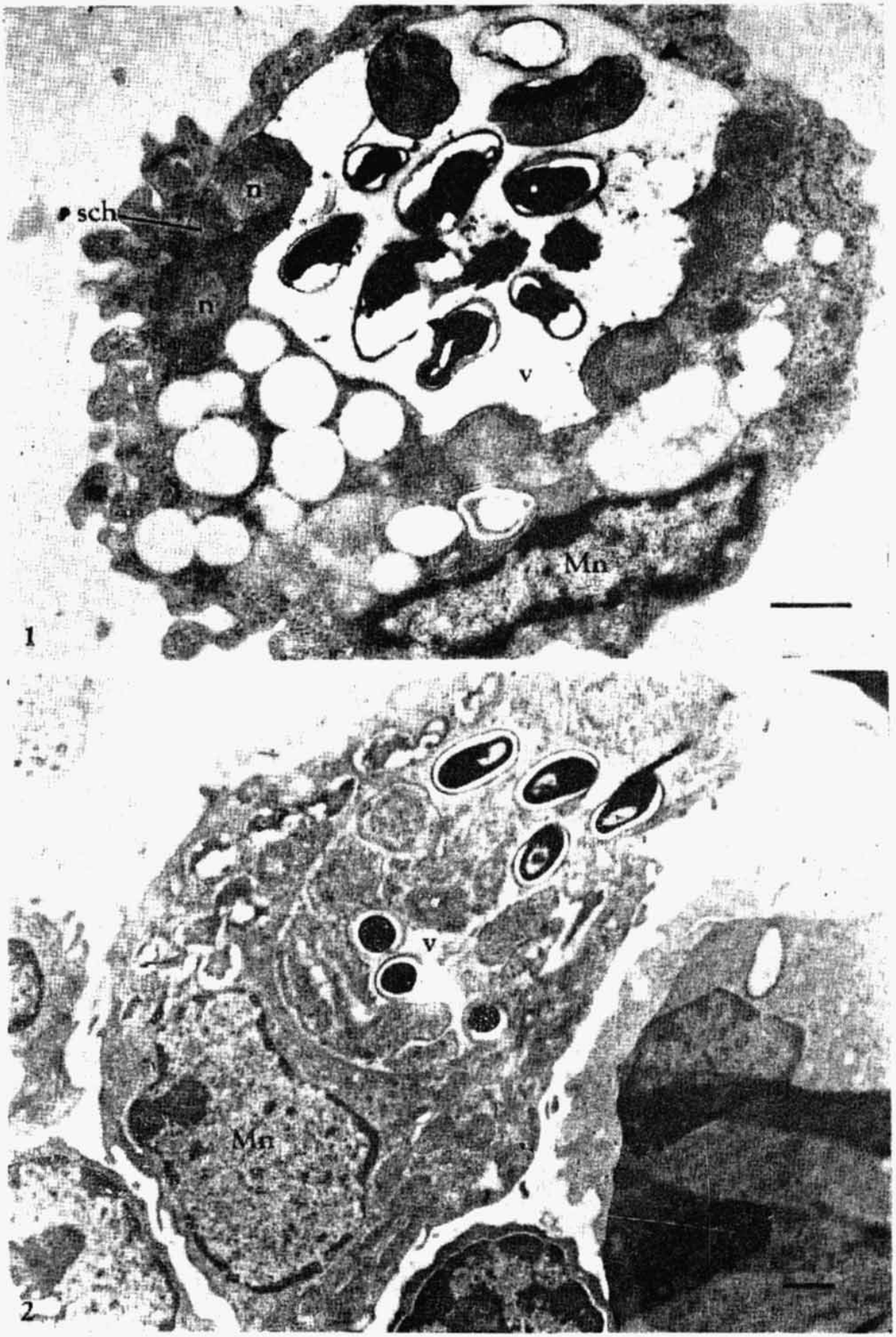


Fig. 1. Araldite-embedded macrophage showing the parasites in or around the periphery of the vacuole (v.) A dividing schizont (sch) with two nuclei (n) can be seen. Mn = macrophage nucleus. (scale = 2 μm)

Fig. 2. G.M.A.-embedded macrophage showing the parasites within the vacuole (v.). (scale = 1 μm)

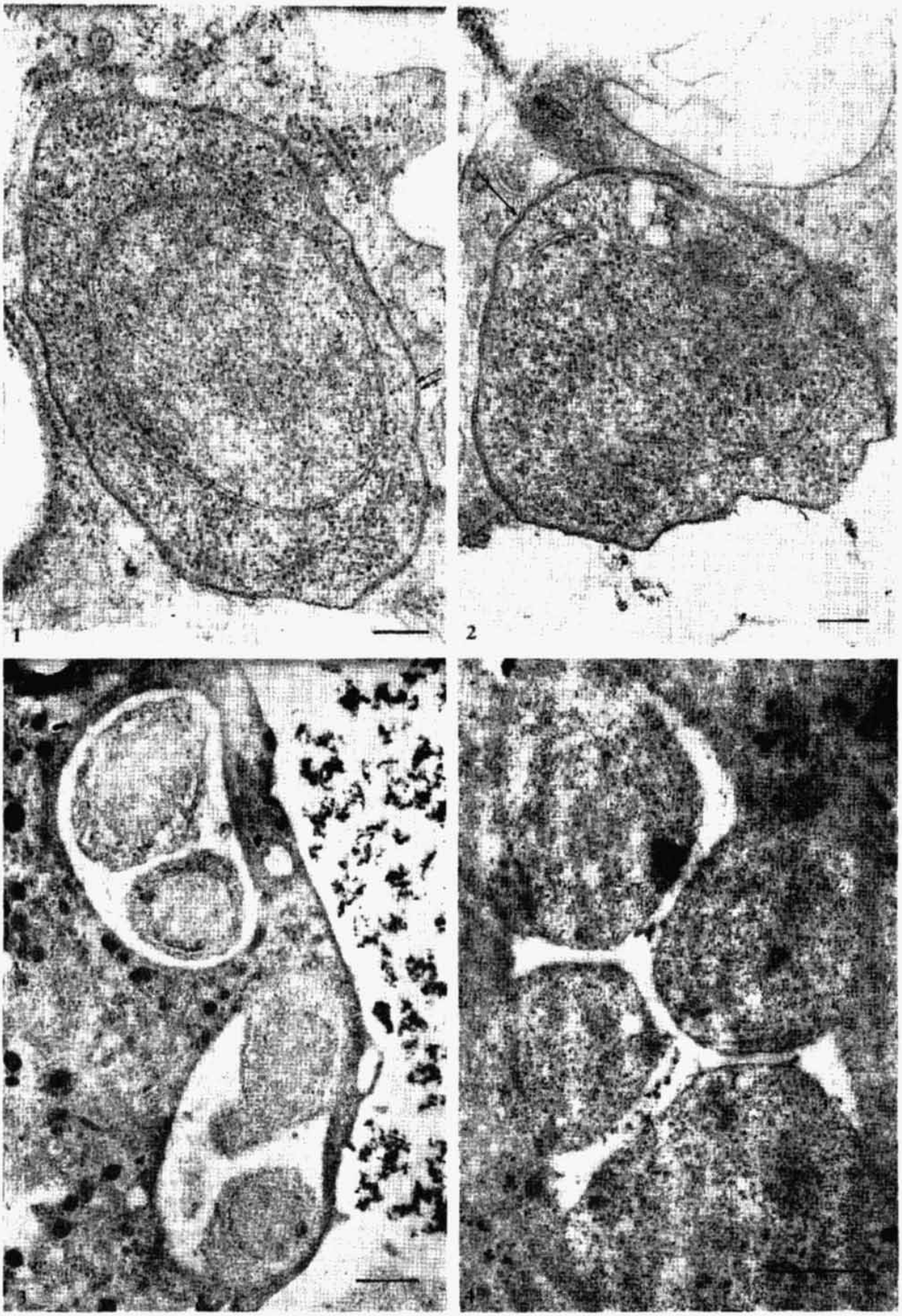


Fig. 1. Araldite-embedded schizont within the host cell cytoplasm showing the two membranes (arrowed) of host cell and parasite origin. (scale = 0.2 μm) **Fig. 2.** Araldite-embedded schizont partially embedded in host cell cytoplasm but separated from it by a host membrane (arrowed) continuous with the vacuolar membrane. (scale = 0.2 μm) **Fig. 3.** G.M.A.-embedded macrophage with infection resulting from two sporoplasms, each forming its own parasitophorous vacuole. (scale = 1 μm) **Fig. 4.** G.M.A.-embedded macrophage showing the parasitophorous vacuole forming between 4 schizonts. (scale = 1 μm)

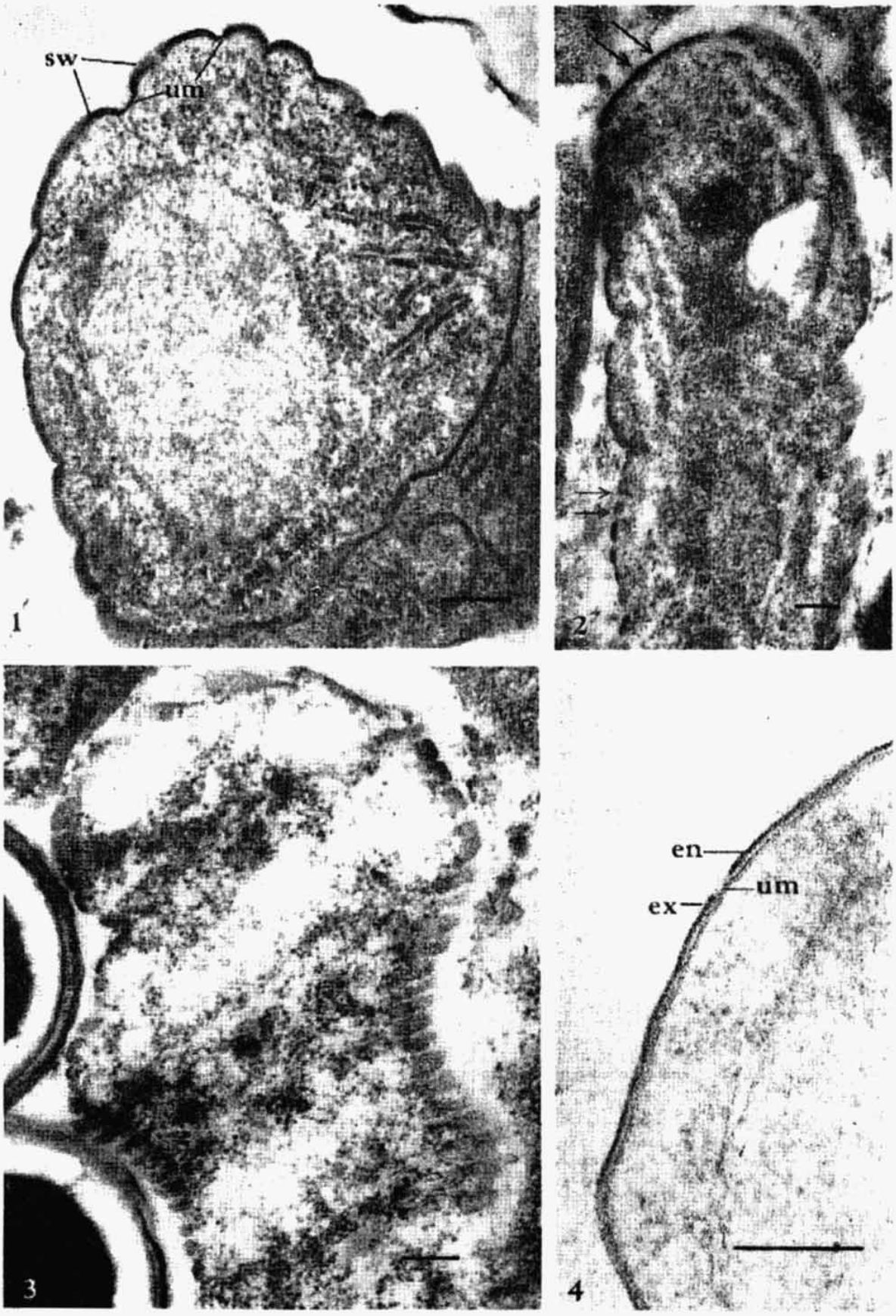
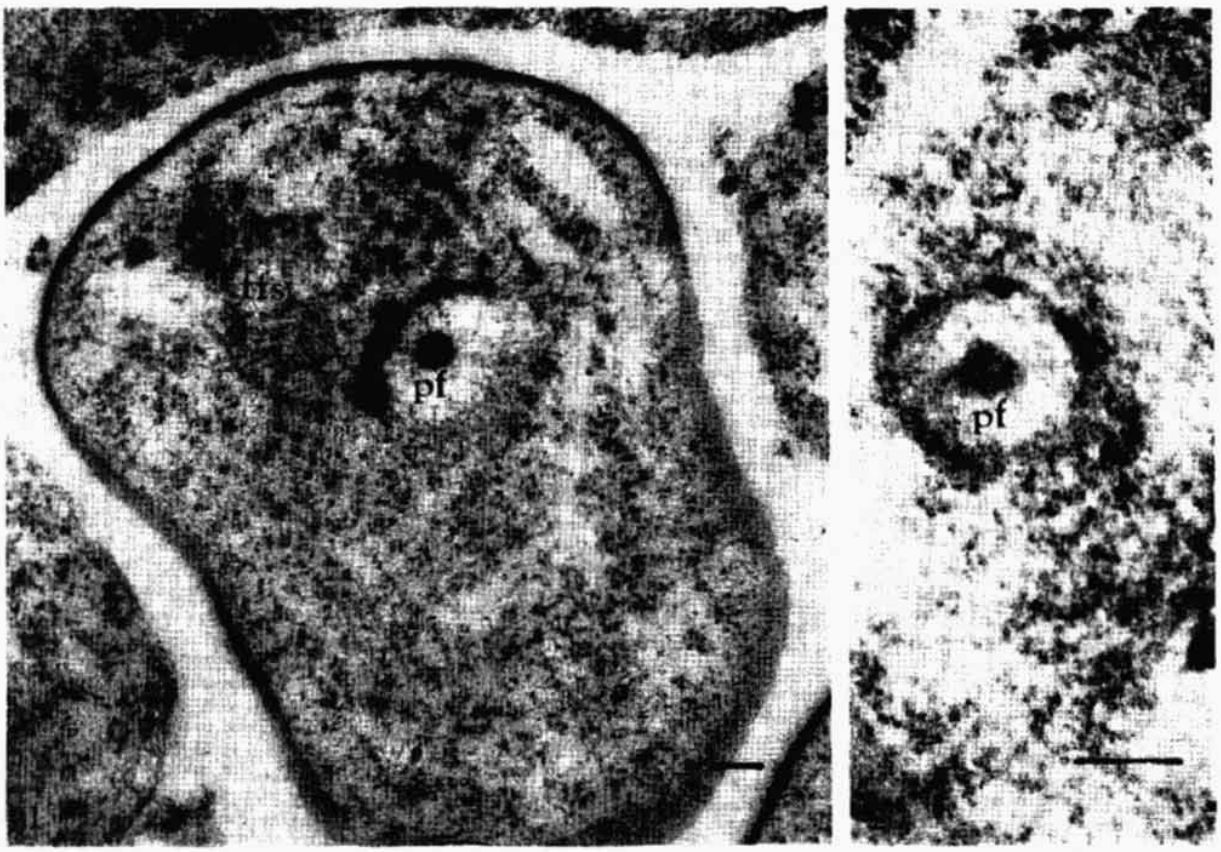


Fig. 1. Araldite-embedded parasite intermediate between a schizont and sporont. Sporont wall (sw) formed as bars of thickening laid down over the plasmalemma (μm) of the schizont. (scale = 0.2 μm) **Fig. 2.** G.M.A.-embedded sporont showing the formation of the sporont wall initially as short bars of electron-dense material (small arrows) which later merge to give a complete layer (large arrows). (scale = 0.2 μm) **Fig. 3.** G.M.A.-embedded sporont with wall cut tangentially to show the transverse bands of thickening. (scale = 0.2 μm) **Fig. 4.** Araldite-embedded sporont showing details of the three layers of the wall: unit membrane (μm), endospore (en), and exospore (ex.) (scale = 0.2 μm)



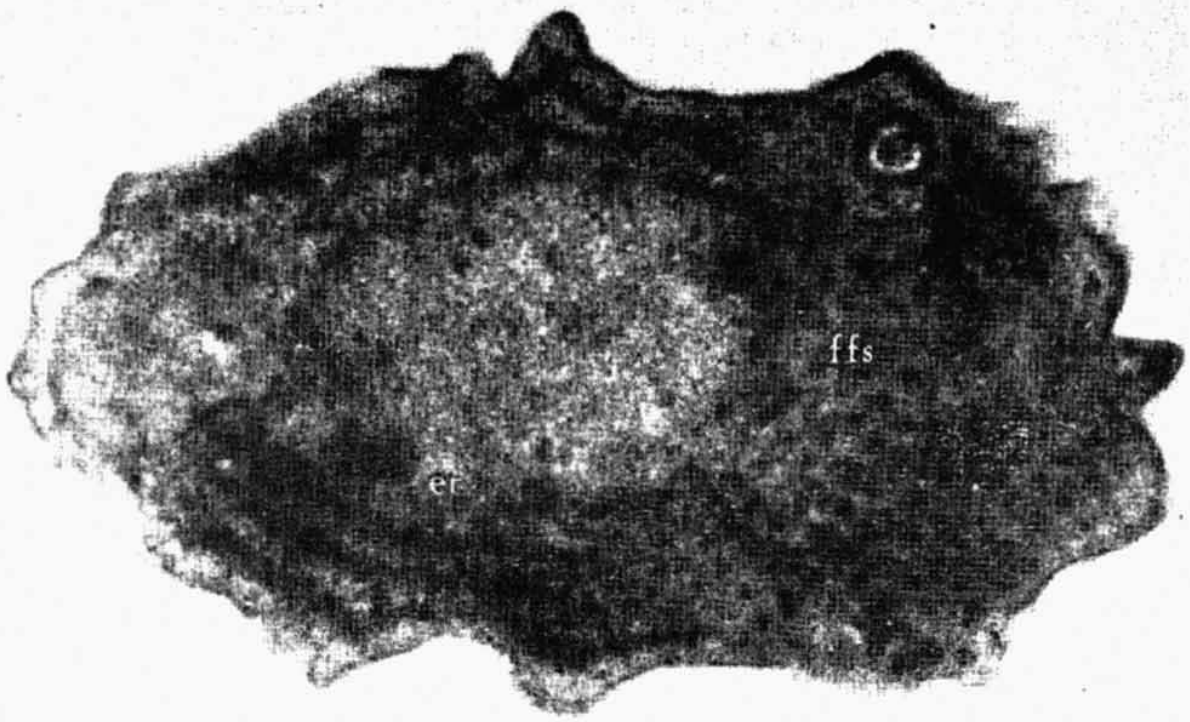


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Fig. 1. G.M.A.-embedded sporoblast showing the reticulum of filament-forming substance (f.f.s.) and the developing polar filament (p.f.). (scale = 0.2 μm) **Fig. 2.** G.M.A.-embedded sporoblast showing later stage in the development of the polar filament (p.f.). (scale = 0.2 μm) **Fig. 3.** Araldite-embedded sporoblast showing the crenulated outline, the concentric arrangement of endoplasmic reticulum (e.r.) and an immature polar filament with associated filament-forming substance (f.f.s.). (scale = 0.2 μm)

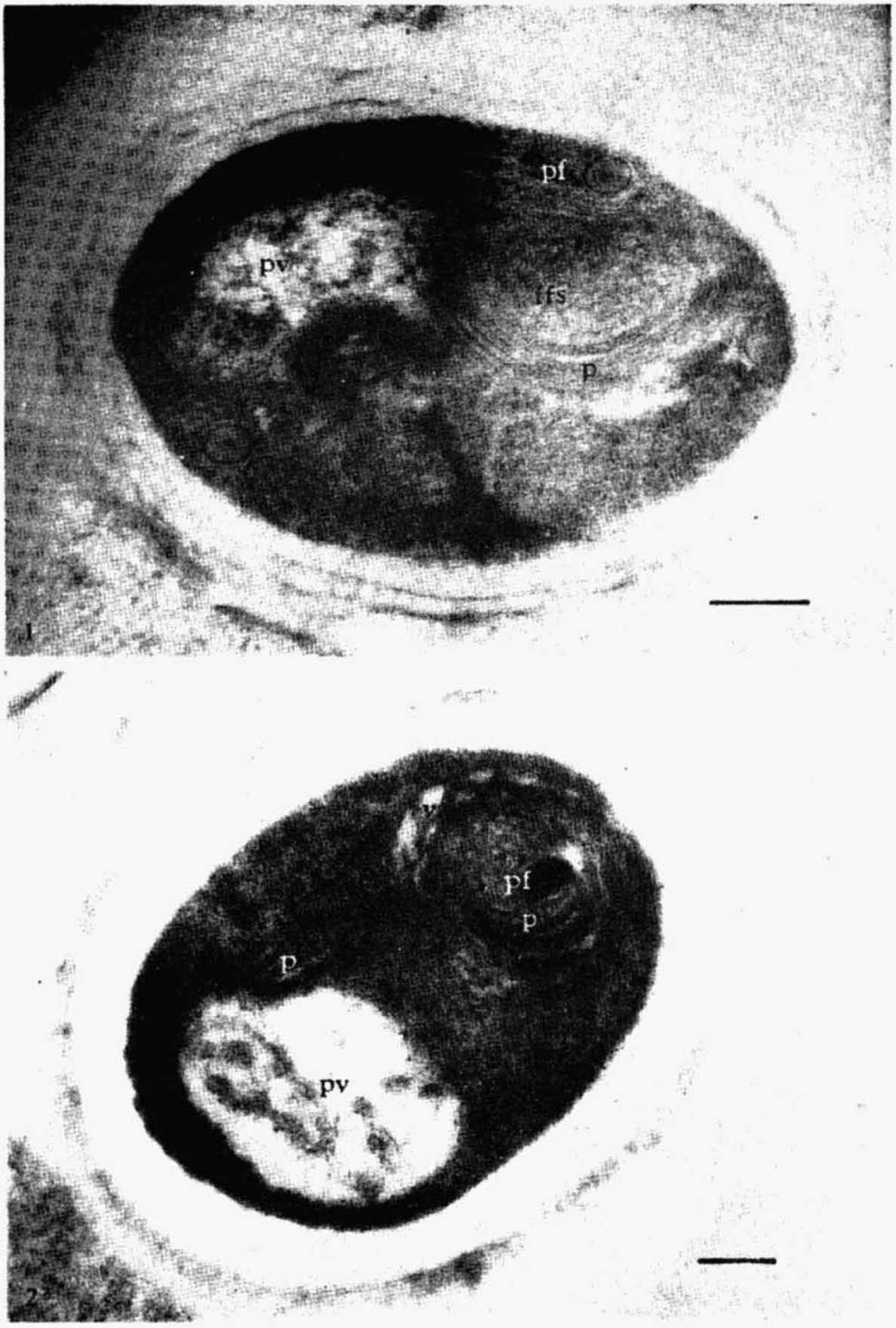
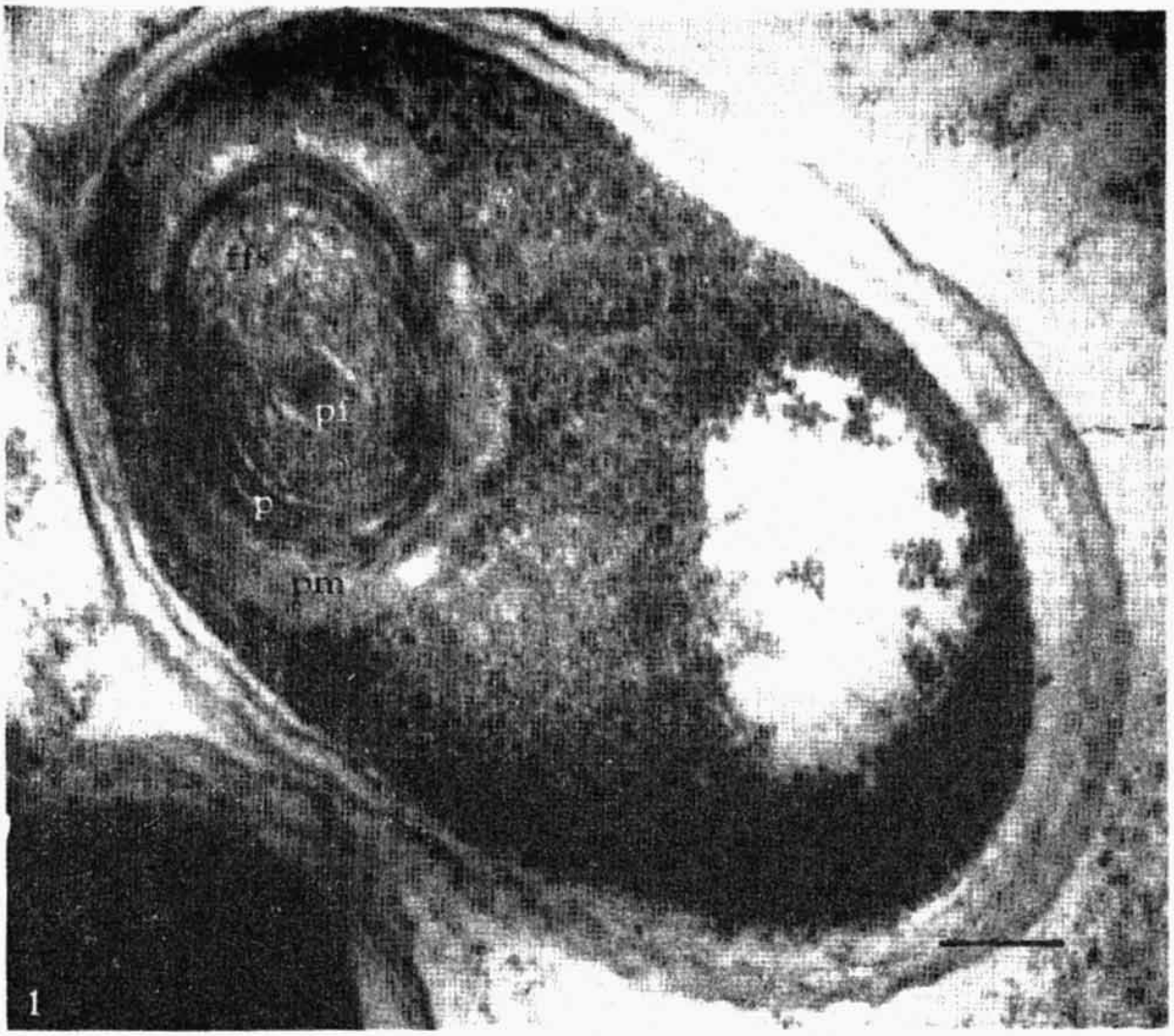


Fig. 1. G.M.A.-embedded immature spore. f.f.s. — filament forming substance, p — membranes of the polaroplast, p.f. — polar filament, p.v. posterior vacuole. (scale = 0.2 μm) **Fig. 2.** G.M.A.-embedded immature spore showing anterior part of polar filament (p. f.) surrounded by triple membranes of polaroplast (p) and an external layer of vesicles (v.) (scale = 0.2 μm)



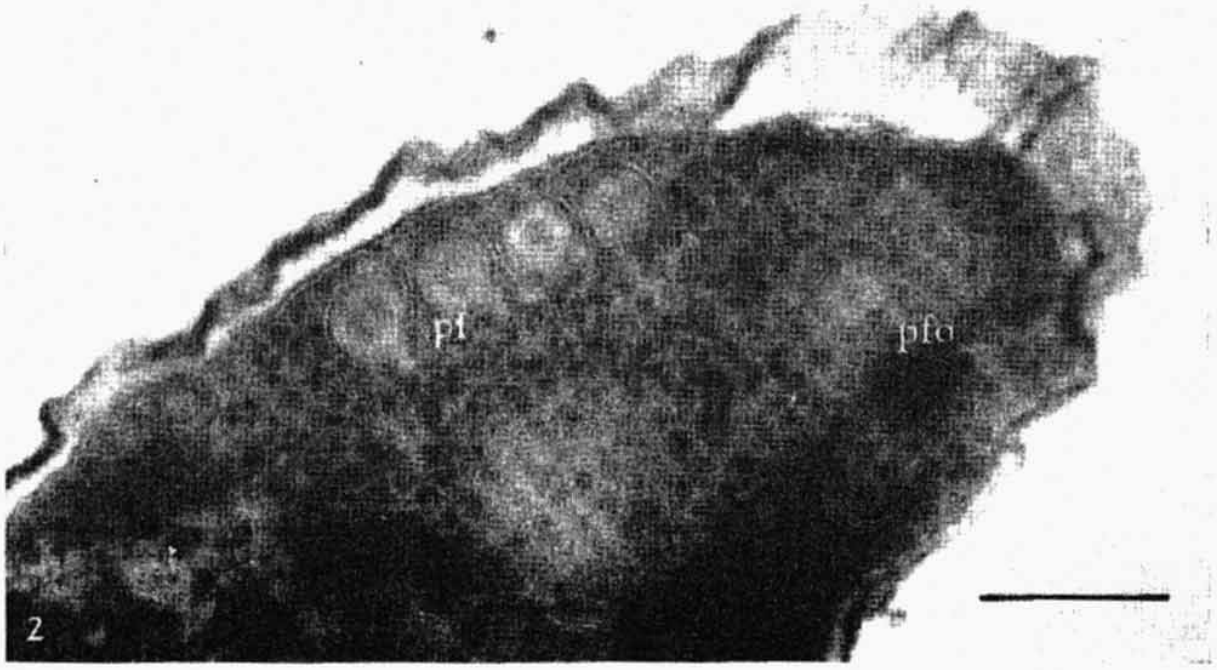


Fig. 1. G.M.A.-embedded nearly mature spore showing anterior part of filament (p.f.) surrounded by filament-forming substance (f.f.s.), trilamellar membranes of polaroplast (p) and outer layer of single membranes (pm). (scale = 0.2 μm)

Fig. 2. Araldite-embedded spore showing 4 sections of the polar filament (p.f.) and a 5th section cut obliquely (p.f.o.). (scale = 0.2 μm)