A CYTOLOGICAL STUDY OF *COCCIDIOIDES IMMITIS* BY ELECTRON MICROSCOPY[1, 2]

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The diphasic fungus, *Coccidioides immitis*, has been subjected to several cytological and histochemical studies (Fonseca, 1928; Ciferri, 1932; Baker, Mrak and Smith, 1943; Negroni, 1949, 1953) in an effort to classify the organism and to establish criteria for its identification. These contributions have served to elucidate many of the morphological features originally described by Posadas (1892) and Wernicke (1892) in tissue, and by Ophuls and Moffitt (1900) and Ophuls (1905) in culture.

In the present study a cytological investigation, by means of thin sections of the fungus, has been made by electron microscopy. The study has included both the tissue (parasitic) phase consisting of endospores and sporangia, frequently referred to as spherules, and the cultural (saprophytic) phase in which arthrospores and chlamydospores are produced from mycelium. A comparison of the morphology of certain cell components in the two phases is made and a description of some developmental details of septa and cleavage furrows is given.

MATERIALS AND METHODS

The organism used was isolated from a human case of coccidioidomycosis in 1952. The morphology of the organism in culture and in infected animals is typical of *C. immitis*.

Material for study of the tissue phase was obtained by intracerebral inoculation of male white mice. Inocula consisted of saline suspensions of spherules. The infected brains were minced with scalpels and suspended in saline. After being washed with saline the material was suspended in 0.25 per cent trypsin at pH 8.5 and incubated at 37 C. Fresh trypsin was added to the sediment obtained by low speed centrifugation at 15-min intervals. After 7 to 10 changes of trypsin the sediment was found by microscopic examination to contain a large number of spherules and a small quantity of amorphous tissue debris. This sediment was washed 3 times with sterile distilled water in preparation for fixation.

Culture phase organisms were obtained from liquid medium of the following composition: NH_4Cl, 1 per cent; Na_2HPO_4, 0.4 per cent; KH_2PO_4, 0.5 per cent; $MgCl_2$, 0.01 per cent; glucose, 2 per cent. Cultures were incubated at 37 C. The organisms from 3- to 5-day cultures were washed with sterile distilled water prior to fixation.

Fixation. Maintenance of structural detail in the organisms was found to be best after prolonged fixation. Fixation of the culture phase for 18 to 36 hr and 36-hr fixation of the tissue phase in 1 per cent osmium tetroxide buffered at pH 7.4 with veronal-acetate buffer (Palade, 1952) was found to be satisfactory. Dehydration of the material through graded alcohols preceded embedding in methacrylate (4 parts *n*-butyl and 1 part methyl methacrylate). Most of the material was polymerized at 45 C, but polymerization at 80 C (Borysko, 1955) produced more uniform results and better contrast.

The mycelial phase organisms reduced osmium tetroxide very little and contrast was consequently very low. To improve contrast, 0.001 M phosphomolybdic acid or phosphotungstic acid in 95 per cent alcohol was used for 30 min during the dehydration procedure.

Sections were cut with a Porter Blum microtome (Ivan Sorvall Inc., P.O. Box 230, Pearl Street, Norwalk, Conn.) using glass knives (Latta and Hartman, 1950). Methacrylate was not removed from the sections. Examination of the sections on Formvar (Shawinigan Products Corporation, Empire State Building, New York 1, N. Y.) coated copper grids was made with an

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[2] Portion of a dissertation presented by the senior author as partial fulfillment of the requirements for the degree Doctor of Philosophy at the University of Washington.

RCA model EMU-2b electron microscope equipped with self-biased gun and limiting aperture in the objective pole piece to improve contrast.

RESULTS

Mycelial Phase

Nucleus. In figure 1 the somewhat lobulate nucleus in a hypha of a 4-day culture can be seen. The matrix of the nucleus is finely granular and homogeneous. It is bounded by a double membrane measuring about 25 mμ. This membrane can be seen to be interrupted at intervals by pores measuring about 50 mμ. In outline the nuclei vary from nearly spherical to extremely lobulate. The diameter ranges from 0.7 to 1.7 μ.

Occasionally finely granular material may be found outside the nucleus. This material is of similar electron density and particle size as granules found within the nucleus. This type of material can be seen in figure 2 enclosed within the lobes of the nucleus. A similar phenomenon has been described by DeRobertis (1954) in nerve cells of invertebrates. In these latter cells material of increased density was shown to be present on each side of the nuclear membrane as though in passage across the membrane.

Nucleoli are present in many of the nuclei. They were identified by their increased density, relative to the nuclear matrix, and usually coarser granulation. No limiting membrane was observed to enclose the nucleoli (figure 3).

Mitochondria were frequently seen lying adjacent to nuclei (figure 4). Points of apparent contact between these two structures were occasionally seen.

Mitochondria. Double membranes can sometimes be seen to surround mitochondria (figure 8) and the cristae (figure 9) to project from the inner membrane. These findings correspond to those reported for mitochondria in other cells (Sjostrand and Rhodin, 1953; Palade, 1953). Some mitochondria of *C. immitis* show a discontinuous surface and a matrix interrupted by canals or tubes. The cristae of these organelles have expanded (figures 10–12).

Powers *et al.* (1955) have described canals in the mitochondria of *Paramecium* from which the methacrylate was removed with toluene. Hillier and Gettner (1950) and Sjostrand (1953) have shown that toluene extraction of the tissue may produce a distortion of the structural picture. Therefore, material prepared by this method cannot be readily compared with that from which methacrylate has not been removed. Methacrylate was not removed from sections of *C. immitis*, but distortion of structure may have resulted from the prolonged fixation. Nevertheless, even if the open cristae observed in *C. immitis* are the result of swelling of the mitochondria, the potential of these structures for expansion is apparent.

Mitochondria in the hyphal phase of the organisms are frequently elongate or filamentous.

Lipid granules. The phenomenon of the increase of lipid in aging tissue cells is well known (Hanks, 1948; DeRobertis *et al.*, 1953). Stages in the development of cytoplasmic granules believed to be lipid are shown in figures 13, 14, 15 and 16. They are identified as lipid because of their intense osmiophilia and because of the parallel development with granules staining with Sudan black B (Chiffelle and Putt, 1951). The granules measure 0.4 to 0.8 μ. They appear to develop from masses of material separated from the rest of the cytoplasm by a double-layered ring consisting of a light area inside a dense area (figures 13 and 14). The lower mass in figure 14 and the masses in figure 13 show that the light ring is the area in which the central mass is pulling away from the outer dense layer. Slender processes still connect the central mass to the outer dense ring. The mass in the tip of the hypha shown in figure 14 is largely detached from the outer ring and is seen to be located in a vacuole. In figure 15 a hyphal cell containing numerous dense granules is shown. The electron opacity of the granules increased as they became more compact. Most of them lie free in a vacuole. Similar granules in an arthrospore are shown in figure 16.

Cytoplasmic membrane and septa. Three stages in the development of septa are shown in figures 5, 6 and 7. Intimately associated with all these septa are dense bodies measuring 0.1 to 0.4 μ. These bodies are very opaque to the electron beam, apparently homogeneous in consistency, and lack a limiting membrane. They may consist largely of lipid, but the data obtained with the light microscope do not clearly support this view. Many of the septal granules are of a size below the resolution of the light microscope and thus could be observed only in groups. In addi-

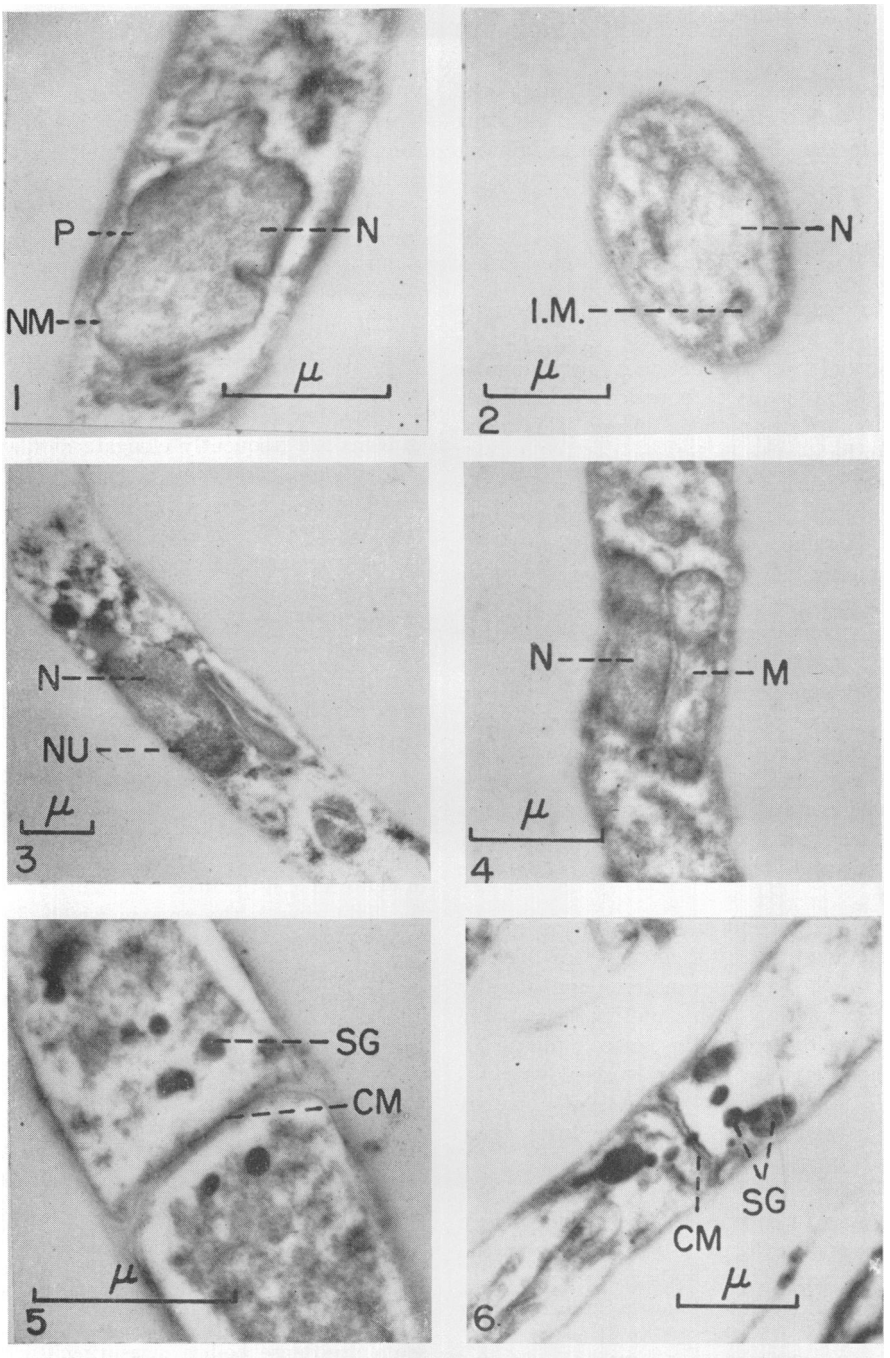

Figure 1. Four-day culture, hyphal nucleus with pores. N—nucleus, NM—nuclear membrane, P—pore.

Figure 2. Four-day culture, lobulate nucleus of hypha. N—nucleus, IM—inclusion material.

Figure 3. Hypha of 4-day culture, lobulate nucleus with nucleolus. N—nucleus, NU—nucleolus.

Figure 4. Hypha of 4-day culture, contiguous nucleus and mitochondrion. N—nucleus, M—mitochondrion.

Figure 5. Hypha of 4-day culture, early septum formation, septal granules. CM—cytoplasmic membrane, SG—septal granules.

Figure 6. Hypha of 4-day culture, later development of septum, two types of septal granules. CM—cytoplasmic membrane, SG—septal granules.

tion, both lipid and glycogen granules have been found associated with the septum. Septa, however, have been shown with the periodic acid-Schiff stain to contain material removable with lipid solvents.

Peripheral bodies associated with wall formation were found in *Bacillus cereus* (Chapman and Hillier, 1953). These bodies, as shown in the electron micrographs, resemble vacuoles from which a granule may have been lost in the process of preparation. If indeed, granules do normally occupy the space of the peripheral bodies, such granules are probably homologous with the septal granules observed in *C. immitis.*

The formation of a septum appears to begin with the division of the cytoplasm by the cytoplasmic membrane (figure 5). The septal wall is first laid down at the sides, near the lateral wall, then the membrane splits and lays down wall material between the two resulting membranes (figures 6 and 7).

During the stages of septal development when wall is being laid down, septal granules can be found on each side of the septum. Occasionally the septal granules appear to overlie the septum (figure 6). The septa of older cells lack these granules. In figure 16 the very thick septum of an arthrospore is shown divided by a dense layer, probably an abscission layer.

The cytoplasmic membrane can be seen to be convoluted slightly at the septum in figure 7 and more extensively along the wall. These projections of the membrane into the cytoplasm are also evident in figures 8, 9 and 10. Occasionally the invaginations appear contiguous to cytoplasmic organelles, nuclei and mitochondria. However, the cytoplasmic membrane is not consistently convoluted in all cells. Some cells are bounded by a smooth membrane. The state of the cytoplasmic membrane is not obviously correlated with other cellular phenomena observed in electron micrographs, but may be related to metabolic activity of the cell.

Tissue Phase Organisms

Nuclei. Tissue phase nuclei resemble nuclei of the mycelial phase. Like hyphal nuclei they are limited by a double membrane with occasional pores measuring about 50 mμ (figure 20). The diameter of the nuclei in this phase ranges from 1.5 to 2.0 μ. The outline may be fairly regular (figure 20) or exhibit minute convolutions (figures 21 and 22). Nucleoli of coarser granulation, nearly always seen in contact with the nuclear membrane, were often found (figures 20–22). Their observed dimensions are from 0.3 to 0.8 μ. In addition, intranuclear granules (figure 20) of the same density and size as the particles of the cytoplasmic matrix, but not so well organized as the nucleolus, were sometimes found near the nuclear membrane. This material appears similar to that noted in culture phase nuclei, outside the nuclear membrane. Frequently very opaque granules measuring 15 to 60 mμ were seen within the nucleus (figures 21 and 22).

Sporangia in cleavage contain varying numbers of nuclei within each segment. This variation is found also to occur in mature endospores ready for release (figure 24). Baker *et al.* (1943) found a variation in nuclear number in endospores at liberation, in their strains of *C. immitis.* Ciferri (1932) reported uninucleate endospores were released from the sporangium of the strains he studied. Strains may be found to differ in this respect, but some evidence would suggest that the number of nuclei per endospore may be the result of the environment. In bovine coccidioidomycosis, daughter sporangia were frequently found by Dickson (1937). These consist of endospores in which cleavage and spore formation has occurred before release of the endospore from the mother sporangium. Cleavage appears to be inhibited in these sporangia, for only a few endospores are found within them. It appears that the host resistance results in the development of a very thick sporangial wall which prevents release of endospores, but the endospores continue to develop: the nuclei divide, and, in some, cleavage and spore formation occur.

Whereas sporangia in fertile hen's eggs appear to be unrestrained in development, attain an enormous size, some of 200 μ, and contain large numbers of endospores (Burke, 1950), they have thin walls and small endospores which are probably released soon after formation, before nuclear division in the spores has occurred.

Mitochondria. Mitochondria were often observed close to the nucleus and appeared sometimes to be in contact with it (figure 25). Like their counterparts in the mycelial phase, the cristae appeared to open and become continuous with the cytoplasm (figures 25–27). Mitochondria

in spherules containing no nucleus on the plane of the section are usually clustered at one side of the cell, separated from the larger granule area of the cytoplasm.

Mitochondria in tissue phase organisms tend to be spherical; filamentous forms were not often observed. Since it has been observed that mitochondrial morphology tends to correlate with cell type under relatively constant conditions (Porter *et al.* 1945), the difference in morphology of the mitochondria of the two phases of this organism may be significant.

Endoplasmic reticulum. Some areas within the cytoplasm of sporangia show double-membrane strands of vesicles (figure 28). These strands appear to correspond to the endoplasmic reticulum described by Porter (1954). This component has not been observed in the mycelial phase of *C. immitis.*

Cleavage furrows. The cytoplasmic membrane of the sporangia and endospores can be seen to remain with the cell wall (figure 23) on rupture of the wall during polymerization of the plastic embedding material. It was not observed in convolutions like those of the mycelial phase, but due to the frequency of rupture of the wall it was not possible to make as many observations on the intact wall of this phase.

The cytoplasmic membrane is intimately associated with the cleavage furrows (figures 29–31). It appears that the membrane precedes the walls in segmenting the cytoplasm (figure 29). The membrane is therefore believed to be concerned in the deposition of the cross walls. Preston (1951) expressed a similar view with regard to the formation of walls of the alga, *Valonia.* This alga was not studied in thin sections but rather in layers stripped from the wall.

In sections of *C. immitis* cleavage furrows can be seen to originate at the cell wall (figures 30 and 31). The cytoplasmic membrane divides the cytoplasm in advance of the wall material. The membrane then appears to split and deposit wall material between the two layers. The furrows are thicker nearer the cell wall (figures 29–31). They branch as they proceed toward the center of the cell before the cytoplasm at the center of the sporangium has yet been divided (figures 29 and 30). No granules comparable to those associated with septum formation have been seen in cleaving spherules. Many mitochondria are present near the furrows (figure 31).

The rupture of cleavage furrows occurs between two layers of the cell wall prior to release of spores (figure 32). The cell wall can be seen to be double at points of breakage while still single within the cleaving spore.

Cell wall. The cell wall proper has been shown to consist of chitin in a protein matrix (Tarbet and Breslau, 1953). X-ray diffraction diagrams of the water insoluble polysaccharide fraction of the wall show no lines for cellulose (Blank and Burke, 1954). In some electron micrographs of *C. immitis* the wall can be seen to be fibrillar (figure 23) where the layers of the cell wall have separated.

The outer layer of the spherules, sometimes referred to as the capsule, was shown by Tarbet and Breslau to contain mucopolysaccharide. This layer is shown in figures 29 and 30 and in higher magnification in figures 18 and 19. Unlike the chitinous wall it is osmiophilic. The organized projections arising from it, the clubs or spines, sometimes attain sufficient size to be visible with the light microscope and can then be shown with Sudan black B to contain lipid. In figure 17 it can

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Figure 7. Hypha of 4-day culture, later development of septum, septal granules and convoluted cytoplasmic membrane. CM—cytoplasmic membrane, SG—septal granules, SW—septal wall.

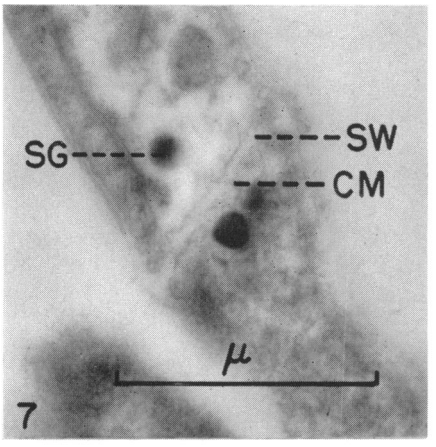
Figure 8. Hypha of 4-day culture, mitochondrion with double membrane, convoluted cytoplasmic membrane. M—mitochondrion, MM—mitochondrial membrane, CR—crista, CM—cytoplasmic membrane.

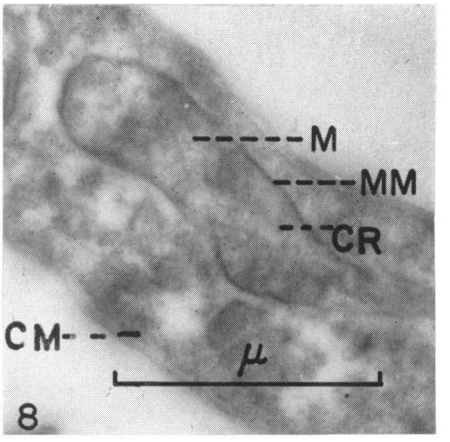
Figure 9. Hypha of 4-day culture, mitochondrion with closed cristae. M—mitochondrion, CR—crista, CM—cytoplasmic membrane.

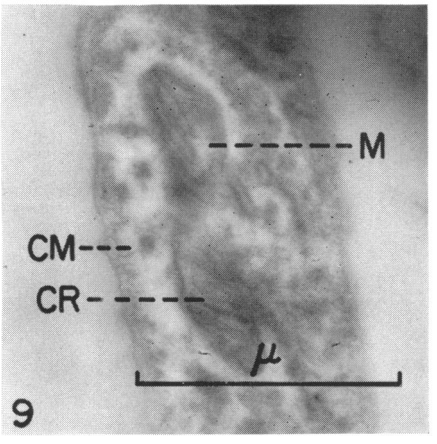
Figure 10. Hypha of 4-day culture, mitochondrion with open cristae. M—mitochondrion, CR—crista.

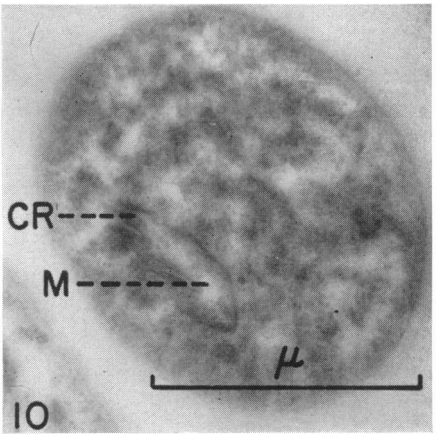
Figure 11. Hypha of 4-day culture, mitochondrion with open cristae. M—mitochondrion, CR—crista.

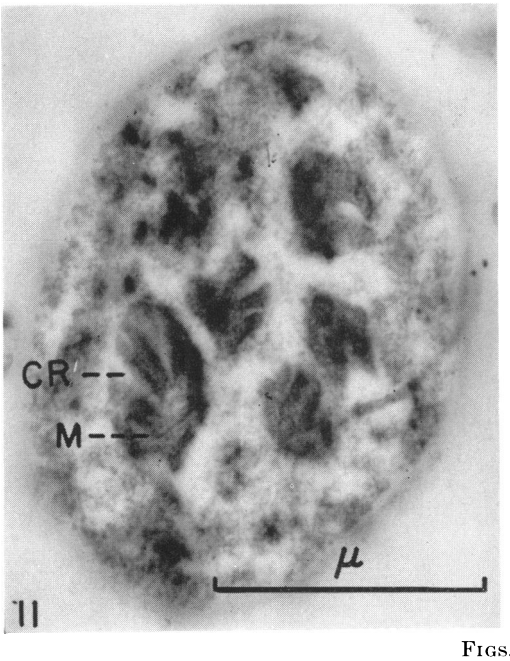
Figure 12. Hypha of 4-day culture, mitochondrion with open cristae. M—mitochondrion, CR—crista.

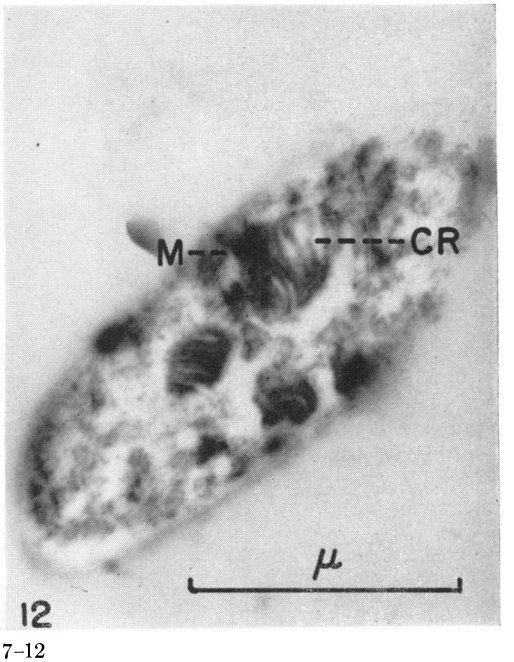












FIGS. 7–12

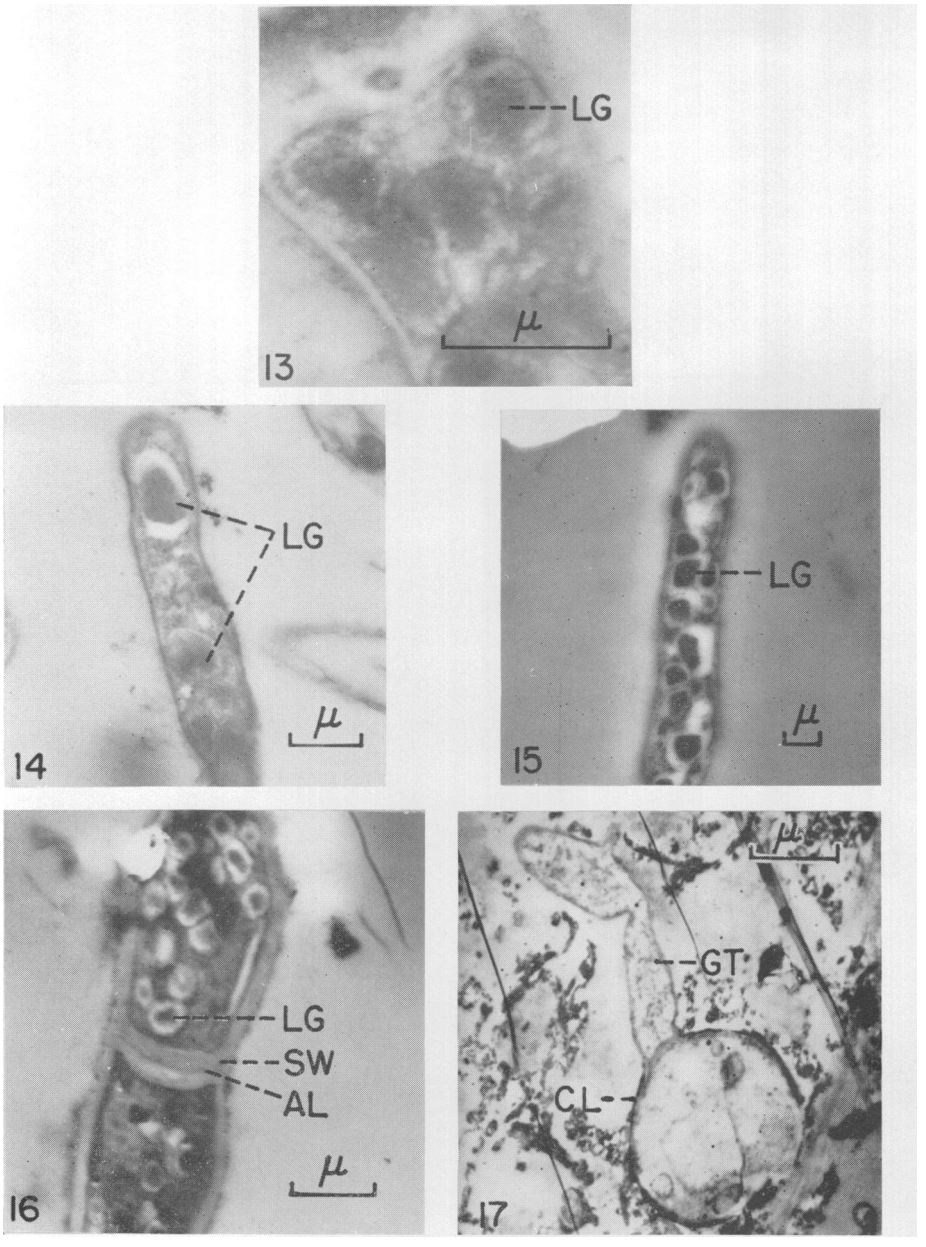


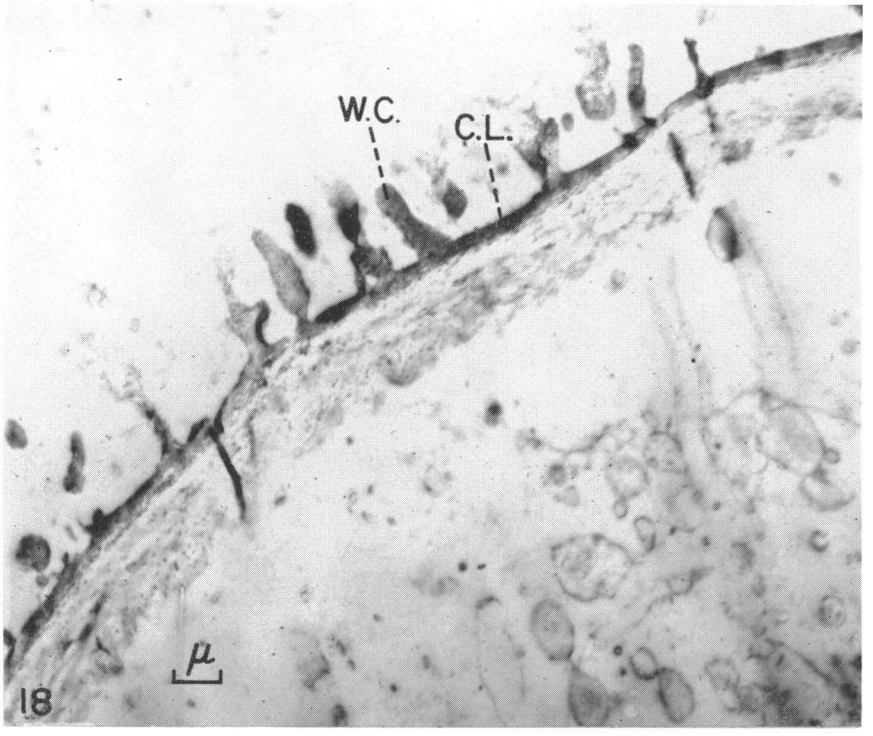
Figure 13. Hypha of 20-day culture, early development of lipid granules. LG—Lipid granule.

Figure 14. Hypha of 20-day culture, later development of lipid granules. LG—lipid granule.

Figure 15. Hypha of 20-day culture, later development of lipid granules. LG—lipid granule.

Figure 16. Twenty-day culture, arthrospore with lipid granules, thick cell wall and abscission layer in septum. LG—lipid granule, SW—septal wall, AL—abscission layer.

Figure 17. Germinating tissue spherule, capsule layer absent on germ tube. GT—germ tube, CL—capsule layer.



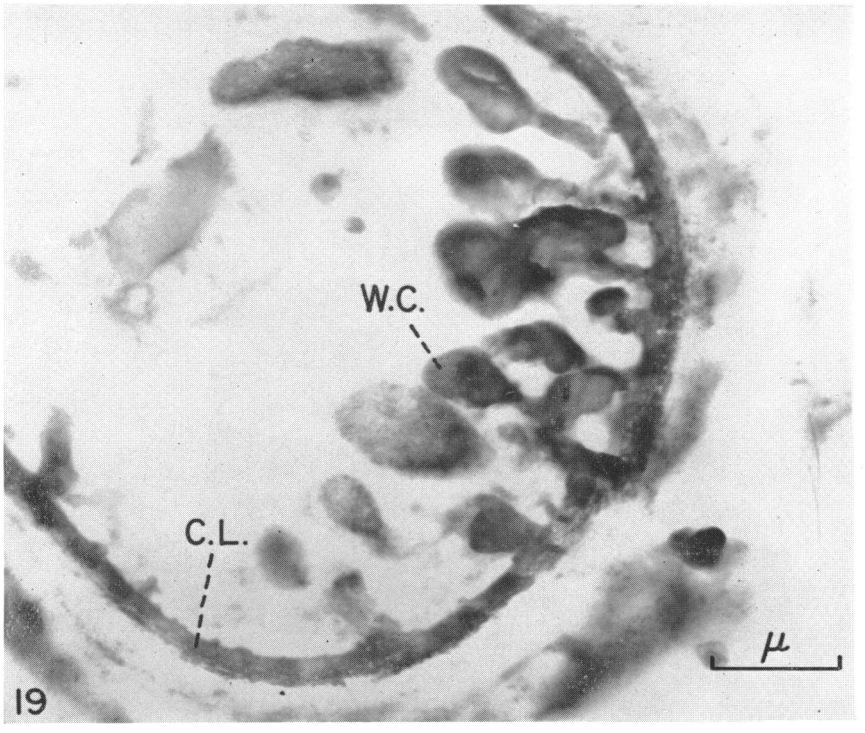


Figure 18. Tissue spherule, capsule layer, wall clubs. CL—capsule layer, WC—wall clubs.
Figure 19. Tissue spherule, capsule layer, wall clubs. CL—capsule layer, WC—wall clubs.

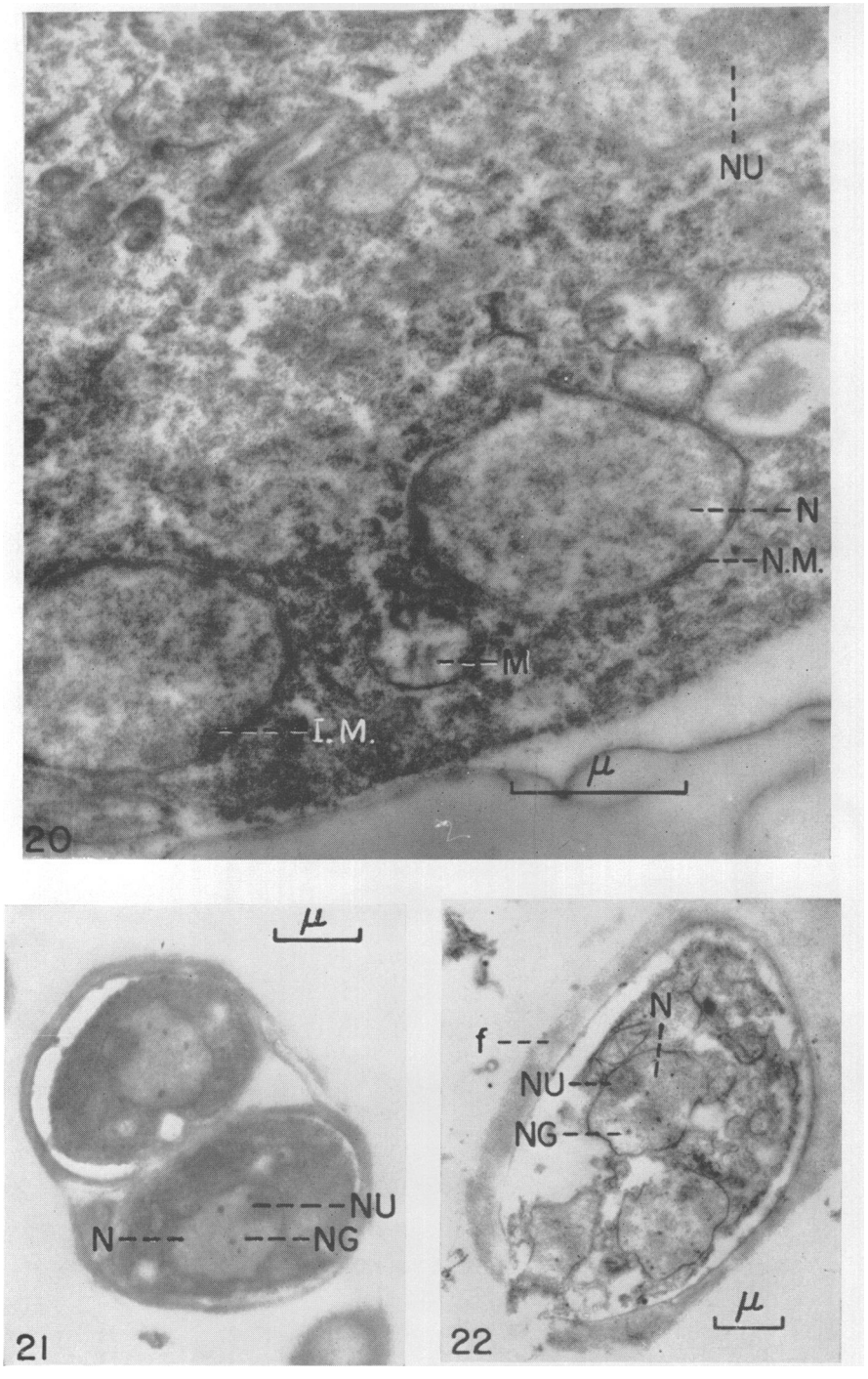


Figure 20. Tissue spherule, nuclei, nucleolus, mitochondria, intranuclear material. N—nucleus, NU—nucleolus, M—mitochondrion, IM—intranuclear material, NM—nuclear membrane.

Figure 21. Tissue spherule, endospores within sporangium. N—nucleus, NG—nuclear granules, NU—nucleolus.

Figure 22. Tissue spherule, fibrillar wall. N—nucleus, NU—nucleolus, NG—nuclear granules, F—fibril.

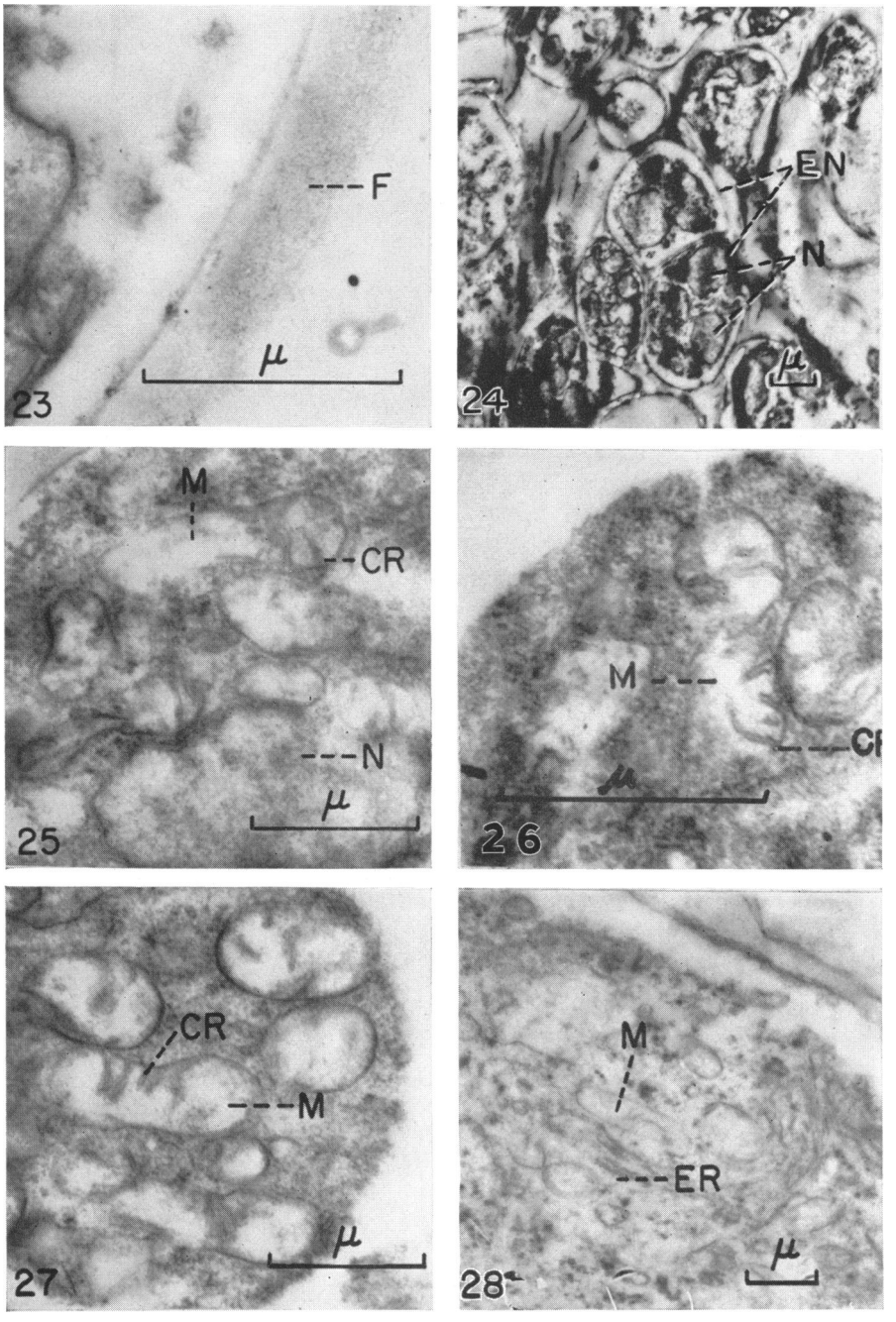


Figure 23. Tissue spherule, detail of fibrillar wall. F—fibril.

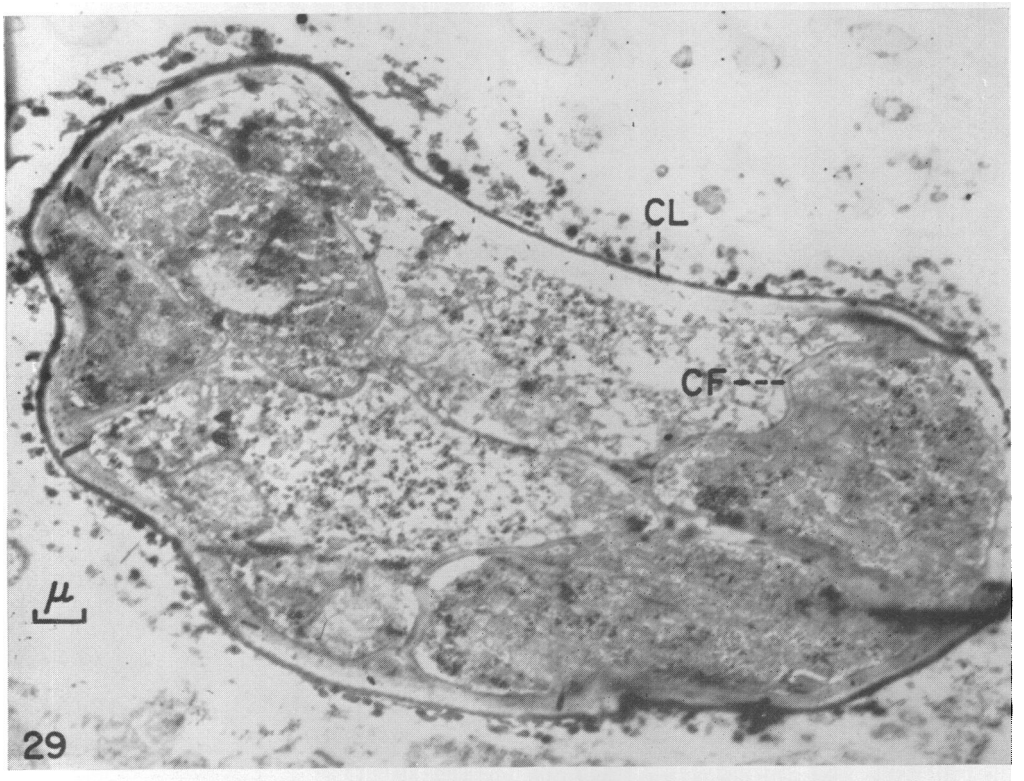
Figure 24. Portion of mature tissue sporangium with plurinucleate endospores. N—nucleus, EN—endospore.

Figure 25. Tissue spherule, mitochondria. M—mitochondrion, CR—crista, N—nucleus.

Figure 26. Tissue spherule, mitochondria. M—mitochondrion, CR—crista.

Figure 27. Tissue spherule, mitochondria. M—mitochondrion, CR—crista.

Figure 28. Portion of tissue spherule, endoplasmic reticulum, mitochondrion. ER—endoplasmic reticulum, M—mitochondrion.



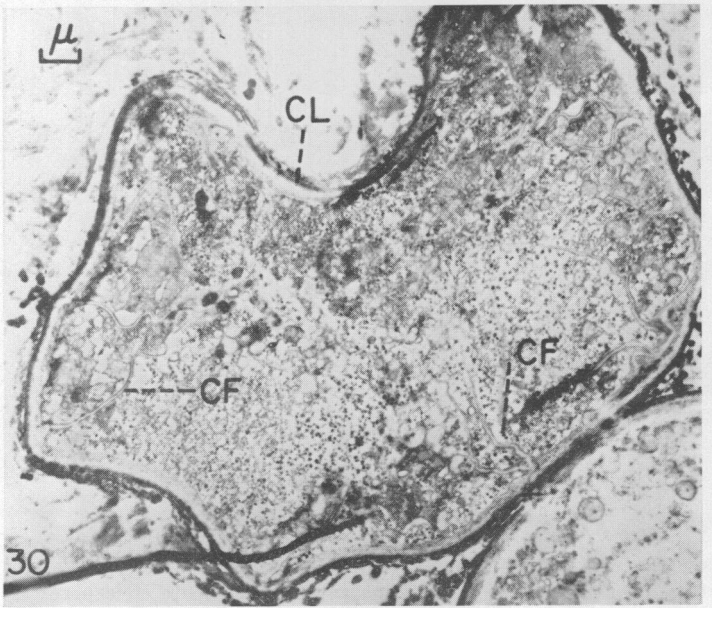
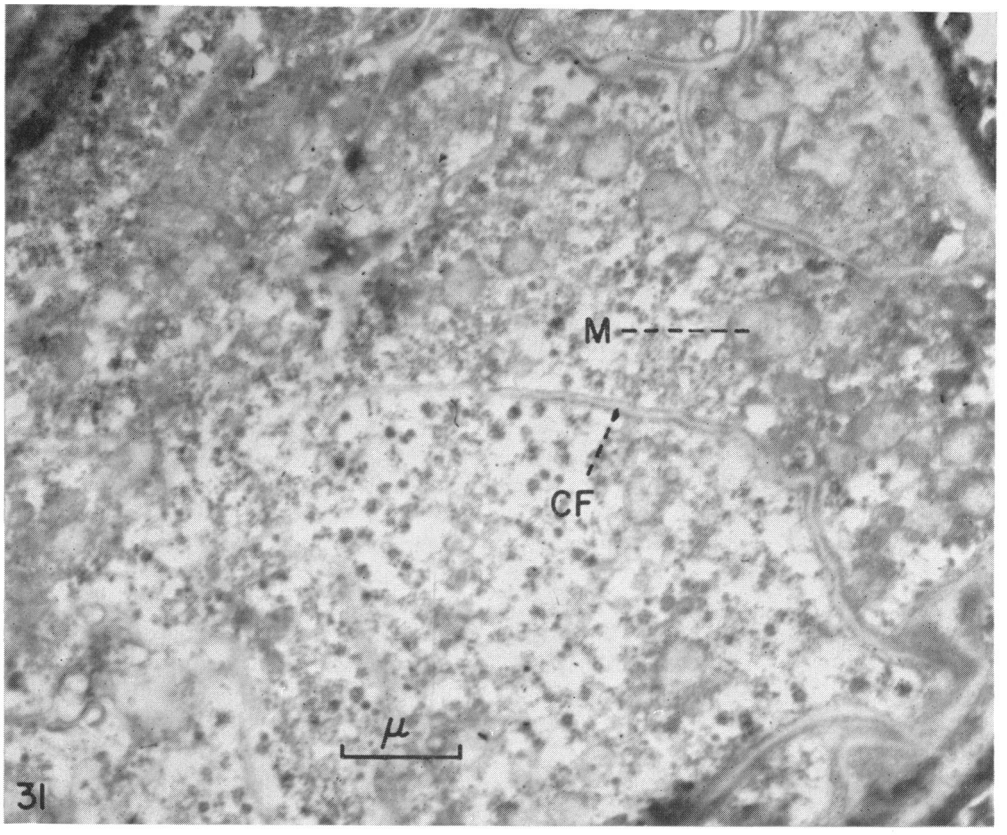


Figure 29. Tissue spherule in cleavage. CL—capsular layer, CF—cleavage furrows.
Figure 30. Tissue spherule, early cleavage. CL—capsular layer, CF—cleavage furrow.



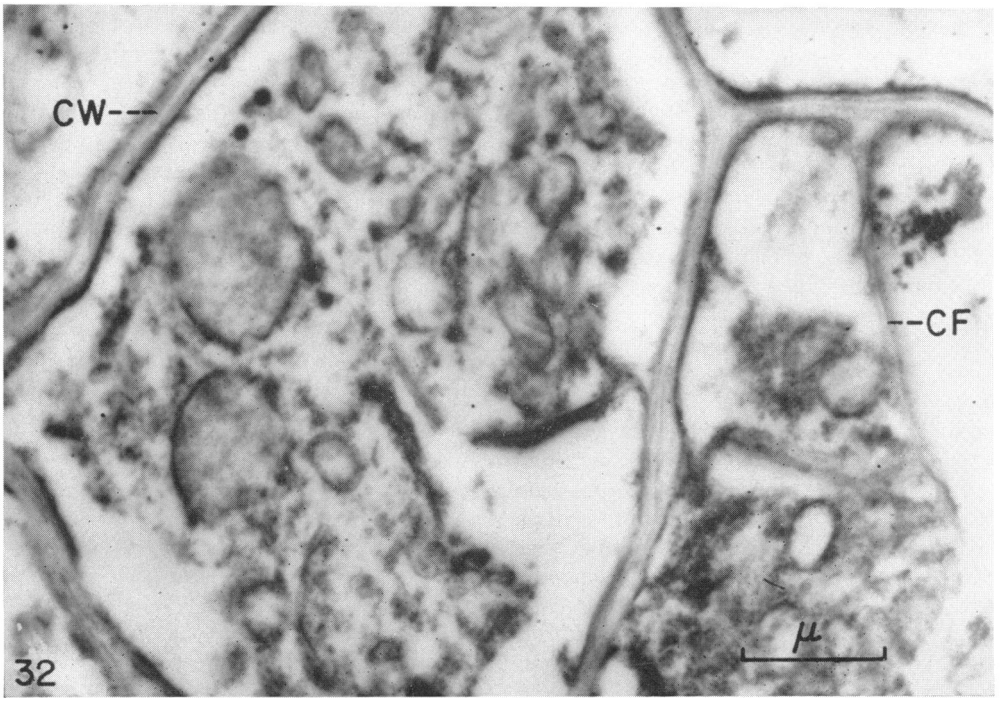


Figure 31. Tissue spherule in early cleavage, detail. M—mitochondrion, CF—cleavage furrow.
Figure 32. Tissue spherule in late cleavage. CF—cleavage furrow, CW—cell wall.

be seen that this capsular layer does not accompany the germ tube.

DISCUSSION

The structure of the nucleus of *C. immitis* appears to be similar to that of higher organisms such as mammalian tissue cells. It has a double membrane with pores and a relatively homogeneous matrix. No chromosomes were observed, but no effort was made to select rapidly growing cells. The nucleus may contain a nucleolus of greater density and sometimes coarser granulation, without a limiting membrane.

The mitochondria appear to differ from those of most cells thus far studied, in that the cristae expand into channels continuous with the cytoplasm. In *Paramecium* a similar mitochondrion has been shown in a thin section from which methacrylate had not been removed (Powers *et al.*, 1955). It may, therefore, be a general property of cristae to expand under certain conditions, or the property may be shown to be restricted to the mitochondria of a certain group of organisms.

The formation of septa in *C. immitis* differs from that shown in thin sections of *B. cereus* by Chapman and Hillier (1953). In *B. cereus* the cell wall appears to push the cytoplasmic membrane across the bacterial cell. A full-thickness wall cuts across the bacterial cell. In contrast, the cell wall trails the cytoplasmic membrane in *C. immitis* septa and cleavage furrows.

Lipid granules appear to develop by contraction of material of moderate electron density away from a vacuole wall. In the early stages of contraction the condensing material can be seen to remain attached to the vacuole wall by slender processes. After becoming free in the vacuole, this material increases in electron density.

The septal granules observed in young hyphae are opaque and amorphous like lipid granules. Lipid granules have been found near the septa of young hyphae, as well as elsewhere in these cells, and the septa have been shown to contain material removable with lipid solvents; thus it may be that septal granules are composed largely of lipid. However, granules most consistently found near septa to the exclusion of other parts of the cell have been shown to consist of glycogen. Because of the minute dimensions of septal granules and of the membrane initiating cell division, the composition of these structures cannot be precisely determined.

The cytoplasmic membrane in young cells, before the development of lipid, is found to be greatly folded. Regular convolutions into the cytoplasm can be seen. Occasionally these folds appear to contact nuclear and mitochondrial membranes. Since the convolutions are not consistently found in all cells, this phenomenon may be related to metabolic activity of the cell. However, the stretching or folding of the membrane may merely be related to the volume of the protoplast. By light microscopy it can be observed that the diameter of vegetative hyphae increases with age. Thus it may be that with increase in volume of the protoplast, the folded cytoplasmic membrane becomes stretched and flattened.

It was not possible to demonstrate nuclei and mitochondria in all the material examined. Some cells were undoubtedly sectioned at a plane devoid of these organelles, but some young cells were found too opaque to reveal much internal structure. This is believed due in part to the composition of the cell at the time of fixation and upon the fixation period. The cell composition is dependent upon the age of the cell and, to some extent, upon the composition of the medium in which it has been grown. Neither nuclei nor mitochondria were demonstrable in old cells containing large quantities of lipid. There was little contrast between nucleus and cytoplasm in some cells (figure 21).

Prolonged fixation effects solution of increasing amounts of the cytoplasmic matrix (Palade, 1952) making structural details visible. When fixation is too prolonged the organized cell components, nuclei and mitochondria, are destroyed.

ACKNOWLEDGMENT

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SUMMARY

Electron micrographs of sections of the saprophytic and parasitic phases of *Coccidioides immitis* are presented.

The nuclei are limited by a double membrane with pores and possess nucleoli in contact with the nuclear membrane.

Mitochondria are shown in close association with the nucleus and with cleavage furrows. They tend to be spherical in the sporangial

phase and elongate or filamentous in the mycelial phase. Some mitochondria possess a discontinuous membrane opening to channels formed from the cristae mitochondriales.

The cytoplasmic membrane precedes the cell wall in the formation of septa and cleavage furrows. Cross wall formation is intimately associated with the cytoplasmic membrane and with septal granules.

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