ELECTRON MICROSCOPY OF CULTURED SPHERULES OF *COCCIDIOIDES IMMITIS*

ABRAHAM M. BRESLAU, THOMAS J. HENSLEY, and JOHN O. ERICKSON, Ph.D.

From General Medical Research and Radioisotope Services, Veterans Administration Hospital, and the Departments of Pathology and Radiology, University of California Medical Center, Los Angeles

ABSTRACT

Spherules of *C. immitis* have been grown *in vitro* in modified Roessler's medium under CO_2 tension and continuous cultures now maintained for over 18 months. Transformation of hyphae and development of the spherule form have been studied by thin section electron microscopy. Cells of organisms in the hyphal stage have thin (*ca.* 50 mμ), apparently structureless walls and a cytoplasmic membrane. Many nuclei, elongated mitochrondria with both transverse and longitudinal cristae, and lipid particles are present. The hyphal wall thickens and the cell transforms into spherules. A large central accumulation of electron-transparent polysaccharide appears in the spherule. The peripheral cytoplasm contains nuclei, each enclosed in a double-layered membrane, mitochondria, and small dense particles. Prior to cleavage the polysaccharide droplets are lost, while mitochrondria become small and spherical. Endospores are formed and liberated when the spherule wall breaks. These begin to grow and repeat the cleavage cycle.

INTRODUCTION

Coccidioides immitis is a dimorphic fungus which in its saprophytic form is filamentous, whereas in the mammalian host it takes the form of a spherule and reproduces by means of asexual endospores. After recovery from primary coccidioidomycosis, patients retain long-time immunity to the disease, indicating that the parasite produces satisfactory antigens. Therefore, it could be feasible to grow the spherule form *in vitro* (1) as a source of antigenic material useful for vaccines as well as in serodiagnosis. In our laboratories we have been growing *in vitro* spherules in continuous culture for long periods of time, with the current series of cultures being more than 18 months old and growing vigorously. This paper describes changes occurring in the spherule during its development as seen by thin section electron microscopy.

MATERIALS AND METHODS

The organism employed was originally obtained in 1955 from a hospital patient with disseminated coccidioidomycosis. Cultures of this organism have been grown under CO_2 tension in modified Roessler's basal synthetic medium (2, 3) enriched with vitamins (including thiamine, riboflavin, pantothenate, biotin, and nicotinamide) and amino acids (Difco casein hydrolysate). In this medium, within three weeks the hyphal forms are almost totally transformed into spherules which continue to multiply by means of asexual endospores.

Specimens for thin sectioning were fixed in 1 to 2 per cent osmium tetroxide in Palade buffer, pH 7.4 or in medium at pH 5.4. With few exceptions, all fixation was in the cold for 2 to 24 hours. A few specimens were treated with 1 per cent uranyl nitrate for 2 hours at room temperature after osmic fixation. While the quality of specimens appeared

to be better when rapid dehydration with alcohol was performed in the cold, some specimens were successfully dehydrated at room temperature with acetone. The fixed specimens were usually embedded in pure butyl methacrylate but occasionally mixtures with methyl methacrylate (10 to 20 per cent by volume) were tried, as was epon 812 resin. Polymerization was effected in the presence of luperco CDB by heat (60 to 85°C.) or by ultraviolet light. Specimens were sectioned with glass knives (4) on a motor-driven Porter-Blum microtome and collected on 20 per cent acetone-water mixture prior to mounting on carbon-coated parlodion membranes supported on 200 mesh grids. All specimens were examined and photographed in an RCA EMU-2B electron microscope equipped with a 25 to 50 μ objective aperture and various Canalco accessories.

OBSERVATIONS

Mycelium: The thin wall of the hypha of *C. immitis* (Figs. 1, 2) is not very electron opaque, seemingly double-layered, and with no other visible structural elements at this magnification. Judging from numerous sections and from whole mounts of disintegrated hyphae, the wall appears to have a total thickness of about 50 mμ. The cytoplasmic membrane has very few invaginations. In the cytoplasm are many small electron-opaque particles dispersed in a lightly staining meshwork. Numerous oval to elongated mitochondria lie in the peripheral zone of the cytoplasm. Frequently their cristae run longitudinally. Many large, irregularly shaped osmiophilic particles are always present. More centrally located are the nuclei, of which there may be many in each cell. Within the double-layered nuclear membrane are lighter staining granular nucleoplasm and a large, irregularly shaped, granular, electron-opaque nucleolus.

Usually the mycelium begins to transform into spherules (Fig. 3) when the large multinucleate hyphal cells round up, and walls thicken and become lamellar. The individual spherule wall develops beneath the hyphal wall. Cleavage of the spherules into endospores is sometimes observed to commence before the spherule is detached from the hypha to lead an independent existence. Usually, however, the spherules are independent before cleavage begins.

(1) Young Spherule or Early Growth Stage: In the early stage of development (Fig. 4) the thick wall stains poorly with osmium tetroxide. In most sections of these spherules the thickness of the wall varied between 200 and 600 mμ. It should, however, be emphasized that at

KEY TO ABBREVIATIONS

CEM, central membranes
Ch, chromosome
CM, cytoplasmic membrane
CP, cleavage plane
CS, metaplastic central substance
CW, spherule wall
EW, endospore wall
L, lipid particle
M, mitochondrion
N, nucleus
Nu, nucleolus
V, vesicle

FIGURE 1

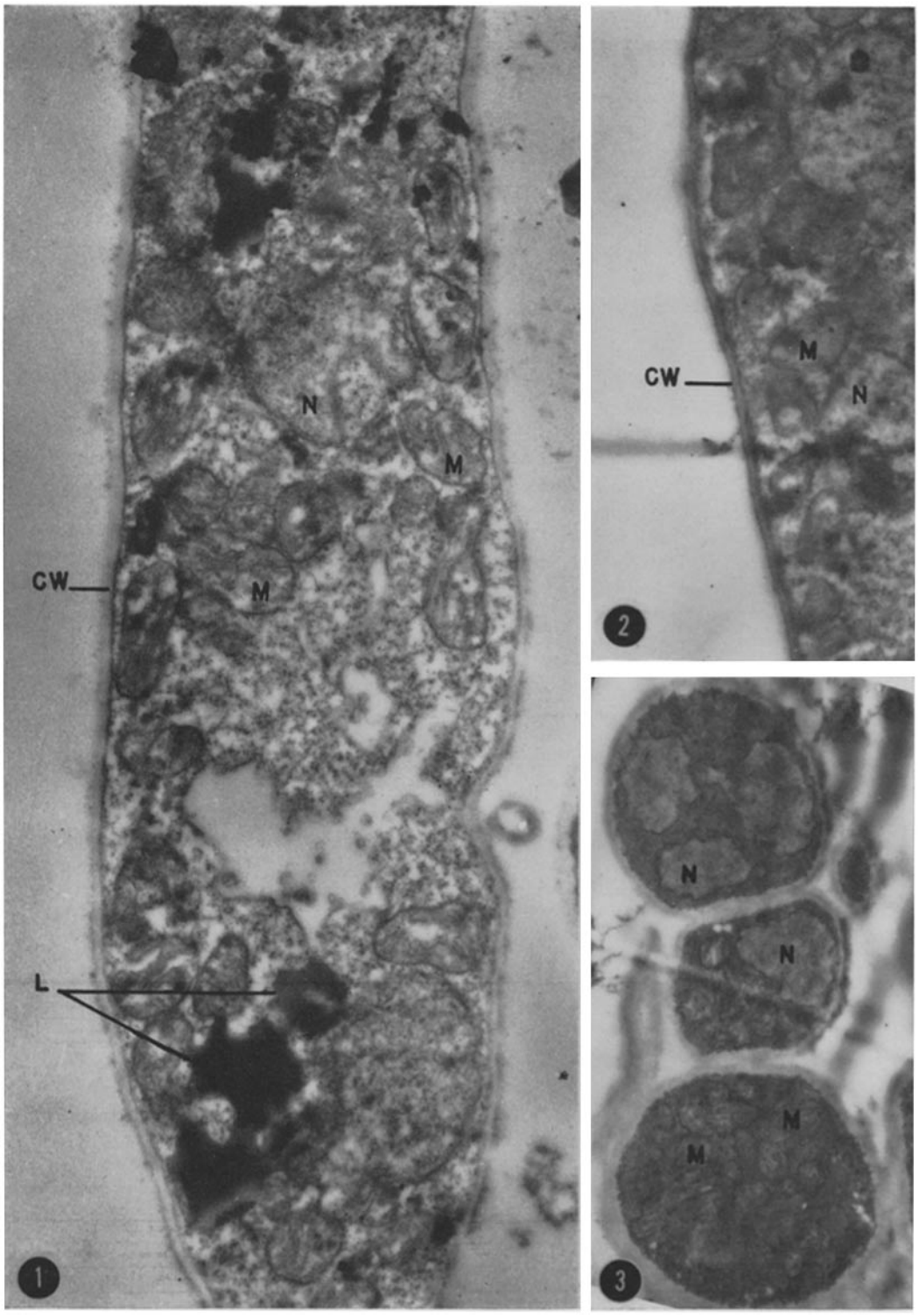
A section through a hypha, showing the thin, structureless cell wall, nuclei, mitochrondria, and lipid particles. The dark contaminating particles at the top of the picture are obvious. × 29,000.

FIGURE 2

A thicker section showing the apparent double nature of the outer wall of the hyphal form. × 22,000.

FIGURE 3

A stage in the most common form of transformation observed in *C. immitis* cultures. The hypha has developed thick walls, divided into segments, and each segment is becoming spherical in shape. Note the numerous mitochondria and nuclei in each segment. Embedded in epon. × 11,500.


N
M
CW
M
L
1
CW
M
N
2
N
N
M
M
3

present it is hazardous to assign precise values to wall thickness, in the light of repeated observations during the course of this work that the preparative procedures appear to alter the integrity of the wall structure. In those specimens where the wall is disrupted by the experimental procedures it is definitely lamellar in appearance. The bulk of the wall consists of relatively non-osmiophilic material interspersed by dark thin lines of material. The spherule wall thickens as the cell matures. No external osmiophilic layer is seen in the cultured spherules.

The cytoplasmic membrane shows only slight invagination. The cytoplasm contains many small dense particles enclosed in, or attached to, a less osmiophilic meshwork. Numerous mitochondria, more or less spherical in shape, are seen. Lipid particles are not nearly so numerous as in the hyphal form. In the center of the spherule, a membrane-like structure develops in proximity to a number of vesicles of circular cross-section (Fig. 4). In the nucleus one finds nucleoplasm enclosed by a nuclear envelope which is composed of two membranes. Frequently, the dense, granular nucleolus is in close association with, if not attached to, the nuclear membrane. Although chromosomes are seldom observed during the course of this work, occasionally in uranyl nitrate-treated specimens we do see structures resembling chromosomes in prophase (Fig. 5). In similarly prepared sections, some fragments of membranes frequently almost paralleling the cytoplasmic membrane are present.

(*2*) *Precleavage Spherule:* The spherule continues to grow and the nuclei multiply. As seen in Fig. 6, the nuclei often show marked irregularity in shape. The single nucleolus of each nucleus is eccentrically located and very conspicuous. The mitochondria tend to lengthen and show signs of fission. Restricted to peripheral cytoplasm one finds small, dense particles. Some membranes (endoplasmic reticulum) appear but are by no means abundant in the cytoplasm at any stage under our experimental conditions. An accumulation of electron-translucent droplets appears enclosed in a cytoplasmic meshwork in the center of the spherule. No membrane separates this zone from the peripheral cytoplasm.

(*3*) *Cleavage and Endosporulation:* The central metaplastic substance disappears. Deposition of the septa (Fig. 7) follows the invaginating cytoplasmic membrane. Cytokinesis continues and results in small angular masses of cytoplasm containing many small dense particles, small spherical mitochondria, and one or two nuclei (Fig. 8). Finally, each rounding endospore is enclosed in a thin wall (Fig. 9) which gradually thickens prior to liberation when the sporangial wall breaks.

DISCUSSION

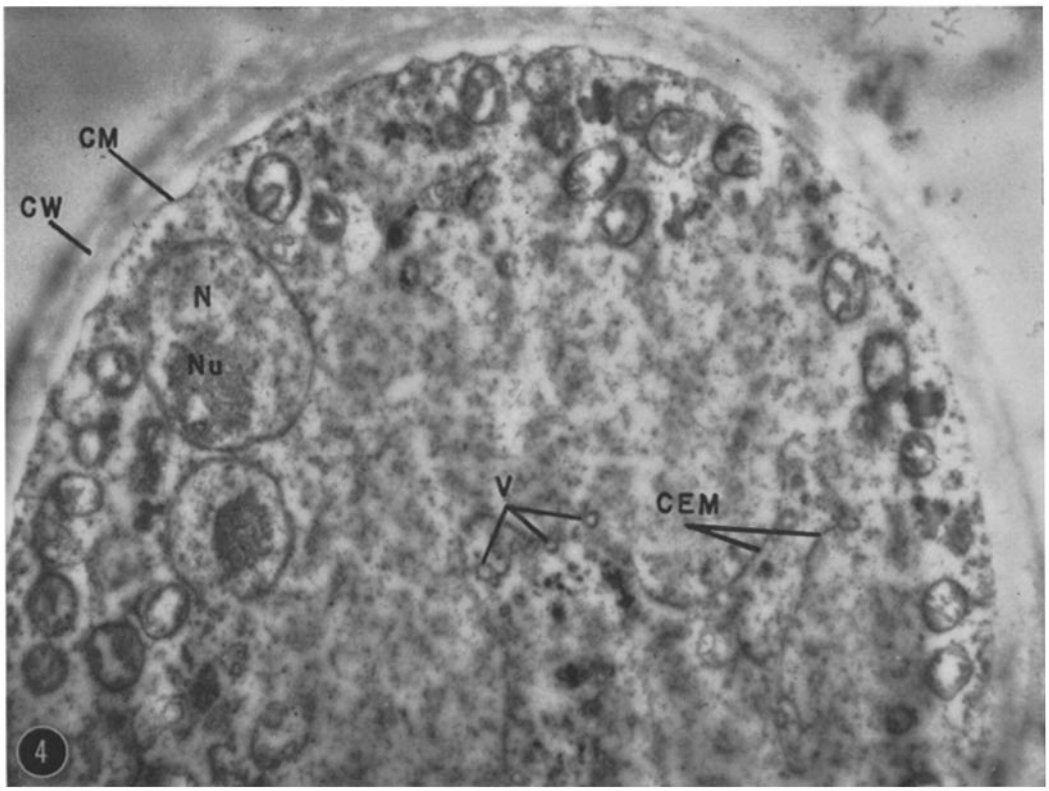
The walls of the hyphae of *C. immitis* are similar superficially to the walls of other filamentous fungi, for example, *Allomyces macrogynus* (5) and *Neurospora crassa* (6). But, from our histochemical studies and from biochemical studies (7) we know that in addition to chitin there is another complex polysaccharide and that it is not glucan. The evidence that the wall has a double layer of osmiophilic material is not completely convincing. Rather, there seem to be an outer dense layer and an inner less osmiophilic layer. In yeast (8) the application of a histochemical test indicates that the outer portion of the wall contains mannan and the inner layer contains glucan. In

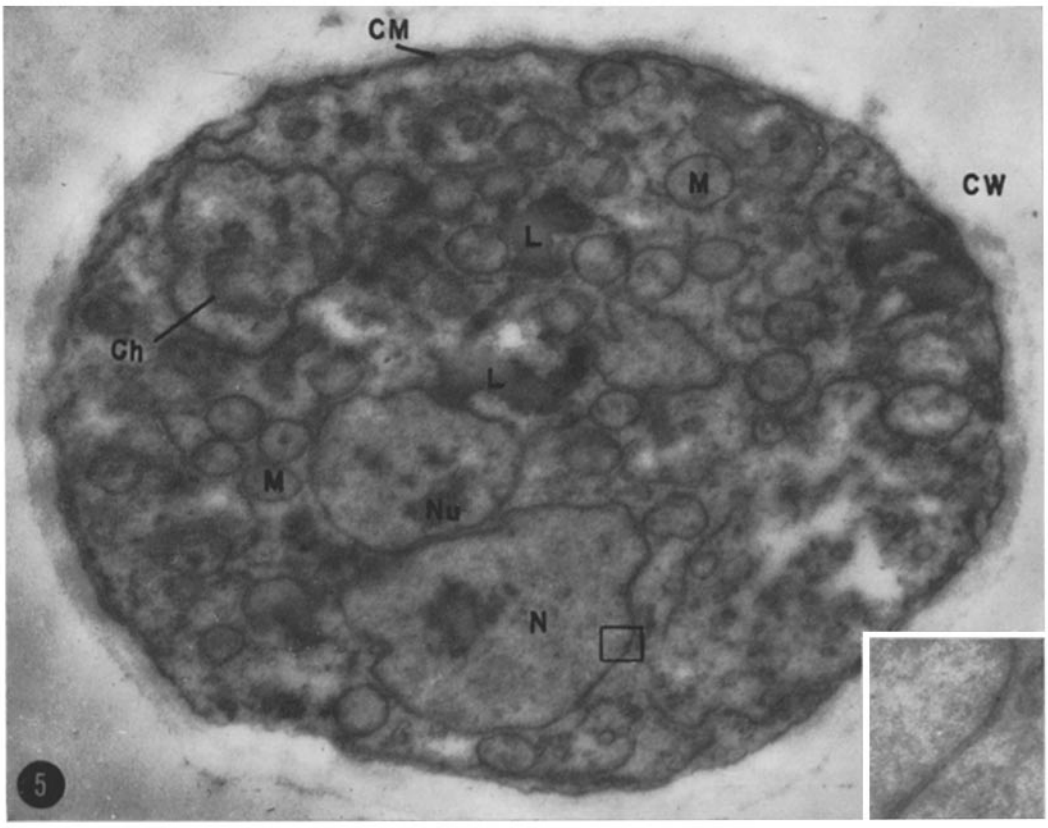
FIGURE 4

A cultured spherule in early growth stage. Note the peripheral distribution of nuclei and spherical mitochondria, and the centrally located membranes with associated vesicles. × 11,500.

FIGURE 5

Tangential section through a cultured spherule in growth stage. The mitochondria are nearly all spherical, the nuclei are irregular in shape and contain what might be chromosomes in prophase. Characteristically (as in Fig. 4) the cell wall is thick, yet scarcely discernible under our conditions. This specimen was treated with uranyl nitrate. × 11,500. The inset, an enlargement of the indicated area, shows the double membrane of the nucleus. × 59,000.


CM
CW
N
Nu
V
CEM
4


CM
M
CW
L
Ch
L
M
Nu
N
5

C. immitis the outer layer may very well contain lipopolysaccharide or lipoprotein (9). The inner osmiophilic layer seen in Fig. 2 probably represents the cytoplasmic membrane.

Particulate storage polysaccharide is not present in the cytoplasm of the hyphal cell. Nor do we find dark granules associated with septal formation as described by O'Hern and Henry (10). The large dark masses (*L* in Fig. 1) probably represent lipid which is demonstrable by staining with Sudan black or oil red O. Hyphae pretreated with fat solvents do not give this staining reaction.

As in the thallus of *A. macrogynus* (5), the mitochondria seen in hyphae of *C. immitis* are elongate rather than oval. Their cristae are not tubular as reported in protozoa such as Paramecium (11) or in the basidiomycete *Polystictus versicolor* (12).

In addition to the more usual type of transformation of hyphae into spherules described under Observations, by bright light microscopy we observe a more uncommon type of transformation. By consecutive transverse divisions, the hyphal cell divides into a number of short segments. Subsequent longitudinal division produces a large number of minute cells with one or two nuclei each. These minute cells round up and separate to produce a cluster of endospore-like structures which grow and develop into mature spherules.

The development of the cultured spherule is quite similar to that of the parasitic spherule described by Tarbet *et al.* (13) under light microscopy. There are, however, two differences. The cultured spherule does not develop a true vacuole in the cleavage stage. Also, the cultured spherule does not become enclosed in a distinct layer of phospholipid. This outer phospholipid layer was identified by Tarbet and Breslau (14) in the parasitic spherule. Its appearance is associated with the change in host response from polymorphonuclear neutrophil to monocytic reaction. This layer was also described by O'Hern and Henry (10) in thin section electron microscopy of the parasitic spherules in infected mouse brain, and was confirmed in our laboratory. Its origin is unknown and it may be produced either by the host in response to the parasite or by the parasite in response to the surrounding host tissue. Neither the cultured nor parasitic yeast-like forms of *Histoplasma capsulatum* (15, 16) possess such an external phospholipid layer.

Architecturally, the wall of the spherule is more complex than the wall of the hypha. When the former is disrupted (Fig. 6) in processing, which is advantageous in this case, the lamellation is clearly seen. Generally it is impossible to make out more structural details due to the electron transparency of the polysaccharide of which it is composed. From our histochemical studies, which will be reported elsewhere (17), we conclude that a structural polysaccharide is present in addition to chitin and that it is not glucan. Although there may be qualitative chemical differences between the walls of the spherule and those of the mycelium, they have not been demonstrated as yet. In Fig. 9 the translucent lamellae are seen to be enclosed between thin osmiophilic layers. The latter may contain lipopolysaccharide or lipoprotein, judging from their staining reaction.

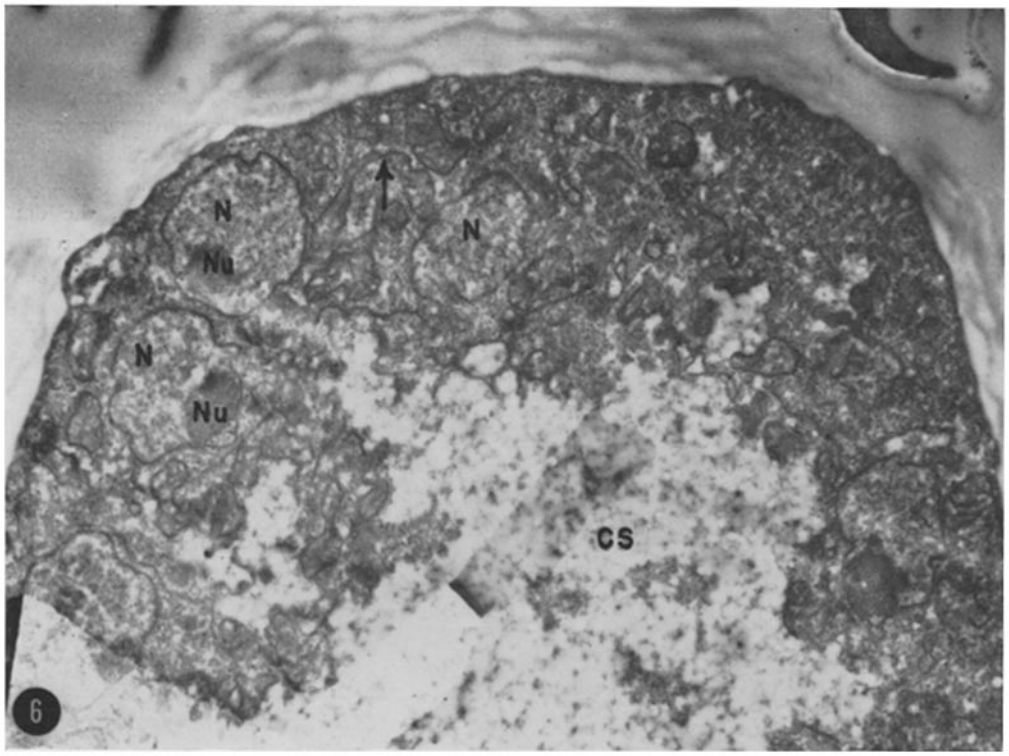
Beneath the wall is the electron-opaque cytoplasmic membrane. Unlike *H. capsulatum* (15)

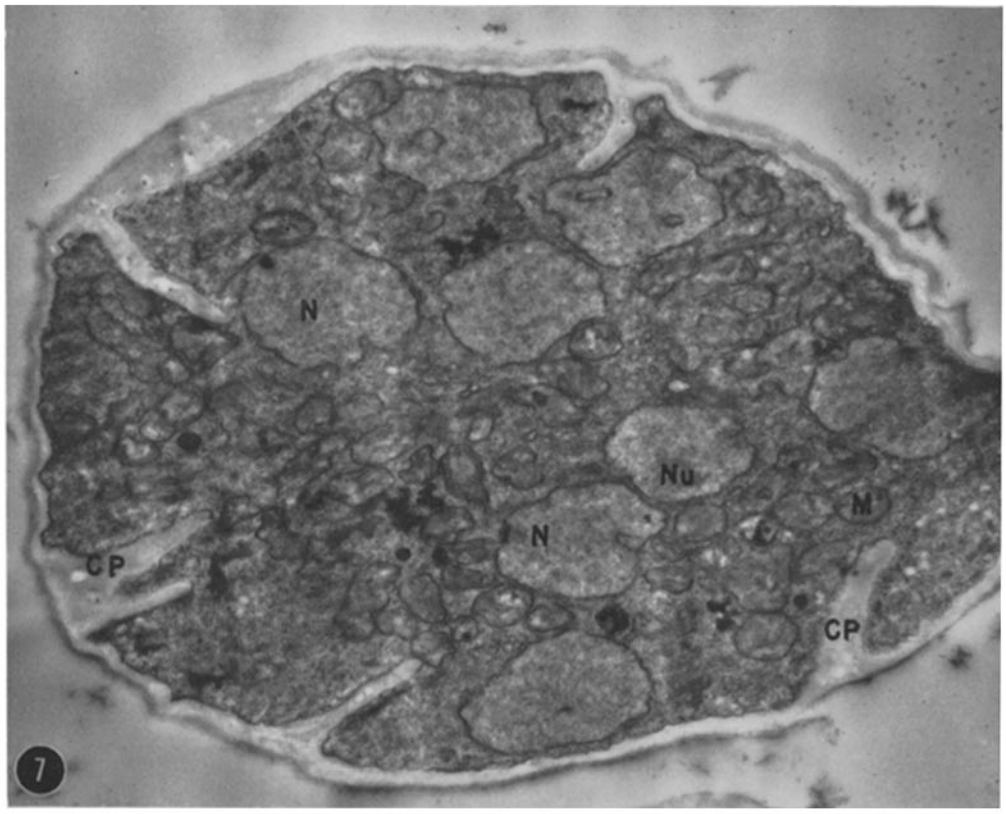
FIGURE 6

A precleavage stage showing the peripheral concentration of organelles and the central concentration of metaplastic substance. Occasionally, small dense particles can be seen in the central area, but they are chiefly concentrated in the peripheral zone. Nucleoli are near or in close association with the nuclear membrane, while the mitochondria are irregular in shape and in some instances (*arrow*) pinched areas suggest the possibility of their undergoing division. The tear in the supporting membrane is obvious. × 20,200.

FIGURE 7

A spherule in the cleavage stage. Note how the cytoplasmic membrane has invaginated, growing inward as the cleavage wall material is synthesized. In the early stages of cleavage there appears to be very little visible structure in the septa, but the lamellated appearance becomes evident as they grow and the new wall develops. × 11,500.


N
Nu
N
N
Nu
CS
6


N
Nu
M
N
CP
CP
7

and *Saccharomyces cerevisiae* (18), in which the membrane shows marked invaginations, in *C. immitis* the membrane is at best described merely as irregular. Endospores are formed in this organism by the invagination of the cytoplasmic membrane, preceding the deposition of the septal material in close association with the folded membrane. This is markedly different from ascospore formation in yeast (19), where there are no invaginations of the membrane and plasma membrane and walls form *de novo* in the cytoplasm, a short distance from the nucleus.

The numerous small dense granules of the cytoplasm probably are ribosomes. Techniques for demonstration of ribonucleic acids, *i.e.*, basic stains with and without ribonuclease treatment, indicate that RNA is present in large quantities in the cytoplasm at all stages of development. In the endospore and in the early growth stages, this substance is distributed throughout the cytoplasm, but in the precleavage stage it is largely restricted to the peripheral cytoplasm. This correlates with the distribution of small dense granules seen in ultrathin sections by electron microscopy. Blondel and Turian (5) identified ribosomes in *A. macrogynus* which undergo interesting and complex changes in quantity in relation to the sexual cycle. *C. immitis* has no sexual cycle and no such changes. In view of the large quantity of RNA present (20), it is likely that the spherule actively synthesizes protein in all stages of its developmental cycle. The protoplast of the spherule is syncytial.

Only one or two nuclei are found in the endospore. In the growing spherule they increase in number. The interphase nucleus has a single nucleolus, a condensation of electron-opaque granules containing RNA. Especially during cytokinesis the nucleus is very irregular in shape, just as if it were engaged in active movement. As in *H. capsulatum* (15), *S. cerevisiae* (21, 22), and *N. crassa* (6), among other fungi, the double nuclear membrane remains intact throughout the process of nuclear division. Yotsuyanagi (23) recently has described chromosomes in the nucleus of *S. cerevisiae* as seen by electron microscopy. Generally, we are unable to see these in *C. immitis*, but in a few specimens of cultured spherules treated with uranyl nitrate after osmic fixation, structures (Fig. 5) which seem to be portions of chromosomes in prophase are visible.

The mitochondria (Fig. 10) are similar to those of other deep fungi. Their cristae do not possess the tubular structures seen in protozoa (11) and basidiomycetes (12). The mitochondria appear to go through a cycle, appearing as spheres in the endospores, increasing in length in the growth stage and breaking up into bead-like organelles in the precleavage stage by pinching off small segments (Fig. 6).

The reticular membranes are never very numerous, but can be seen more readily in specimens treated with uranyl nitrate. Occasionally, membranes (Fig. 5) almost parallel to the cytoplasmic membrane are visible. We do not know the function of these membranes. The only structures seen which may correspond to the Golgi apparatus are represented by the circular cross sections (Fig. 4) associated with the central membrane-

FIGURE 8

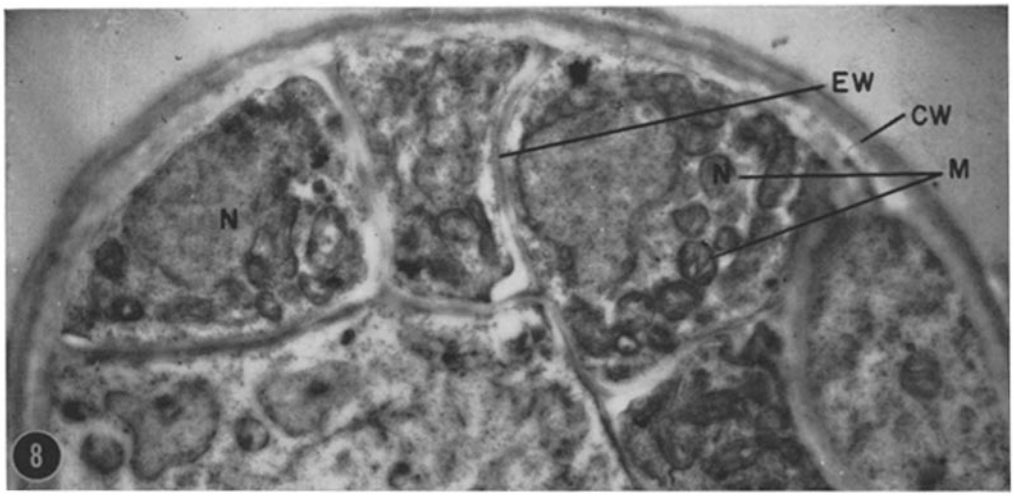
A spherule in late stages of endosporulation, probably just prior to the rounding up of the original endospores. One or more nuclei and numerous spherical mitochondria are contained in each cell. Note the lamellar structure of the wall of the sporangium and the osmiophilic substance in the septa. × 8,600.

FIGURE 9

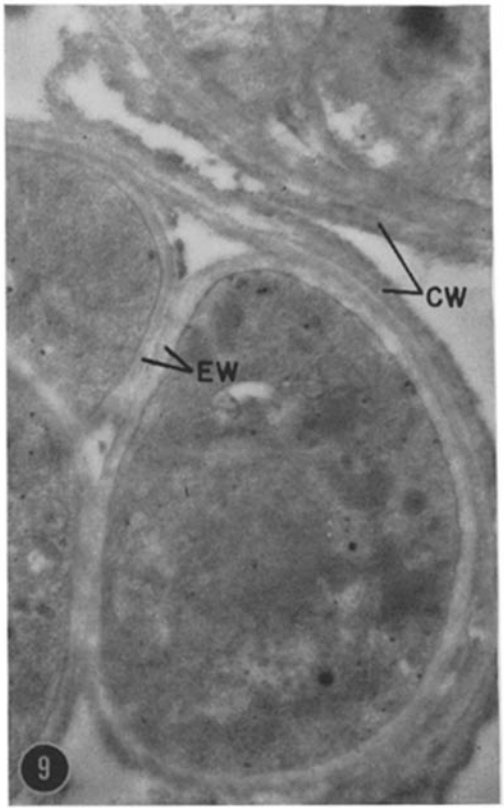
A section through the sporangial wall and two adjacent endospores. Each endospore has its own cell membrane and relatively thin wall. Note the lamellation of the outer sporangial wall. Embedded in epon. × 16,800.

FIGURE 10

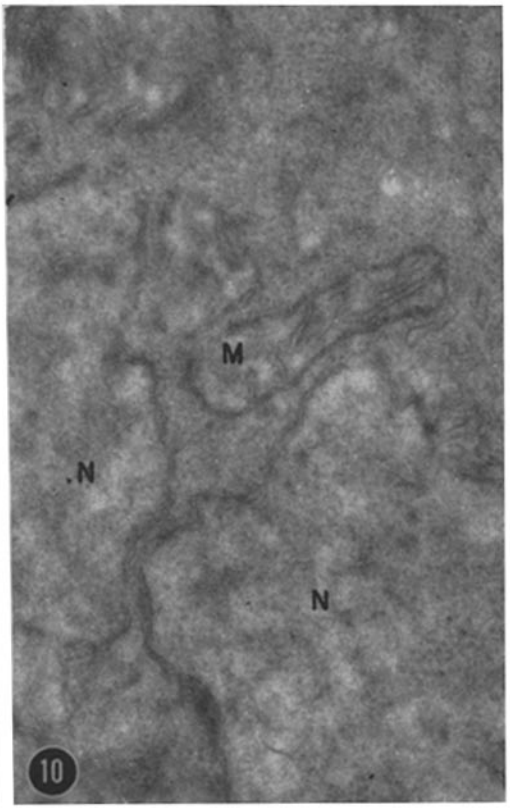
A section through the cytoplasm of a spherule showing portions of two nuclei and a mitochondrion. Note the double nuclear membrane in several areas and the cristae and double wall of the mitochondrion. × 40,000.



8



9



10

like organelle in the early growth stage of the spherule.

No particulate storage polysaccharide appears in the endospore and early growth stage. In the precleavage stage (Fig. 7) we find a large number of electron-translucent droplets enclosed in a thin cytoplasmic meshwork in the center of the cultivated spherule. This area is deficient in ribosomes and is not enclosed in any visible membrane. Yeast stores glucan, but in *C. immitis*, droplets behave histochemically like polysaccharides other than either glucan or glycogen. Both acid and neutral polysaccharides are present and the acid polysaccharides are carboxylated but not sulfated. Whether these neutral and acid polysaccharides possess antigenic properties is at present unknown. The heteropolysaccharides of the spherule wall, on the other hand, induce immunity in mice and bind complement. Additional immunochemical studies should supply information on the nature of the different antigens produced by the cultured spherule and their location in the organism.

This study was supported by partial financial assistance from the California Tuberculosis and Health Association and by Grant C-1177 from the United States Public Health Service.
Received for publication, August 21, 1960.

REFERENCES

1. LUBARSKY, R., and PLUNKETT, O. A., In vitro production of the spherule phase of *Coccidioides immitis*, *J. Bact.*, 1955, **70**, 182.
2. ROESSLER, W. G., HERBST, E. J., MCCULLOUGH, W. G., MILLS, R. C., and BREWER, C. R., Studies with *Coccidioides immitis*. I. Submerged growth in liquid mediums, *J. Infect. Dis.*, 1946, **79**, 12.
3. CONVERSE, J. L., Growth of spherules of *Coccidioides immitis* in a chemically defined liquid medium, *Proc. Soc. Exp. Biol. and Med.*, 1955, **90**, 709.
4. LATTA, H., and HARTMANN, J. F., Use of a glass edge in thin sectioning for electron microscopy, *Proc. Soc. Exp. Biol. and Med.*, 1950, **74**, 436.
5. BLONDEL, B., and TURIAN, G., Relation between basophilia and fine structure of cytoplasm in the fungus *Allomyces macrogynus* Em, *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 127.
6. SHATKIN, A. J. and TATUM, E. L., Electron microscopy of *Neurospora crassa* mycelia, *J. Biophysic. and Biochem. Cytol.*, 1959, **6**, 423.
7. MCNALL, E. G., SORENSON, L. J., NEWCOMER, V. D., and STERNBERG, T. H., The role of specific antibodies and properdin in coccidioidomycosis, *J. Invest. Dermatol.*, 1960, **34**, 213.
8. MUNDKUR, B., Electron microscopal studies of frozen-dried yeast. Localization of polysaccharides, *Exp. Cell Res.*, 1960, **20**, 28.
9. ERICKSON, J. O., and BRESLAU, A. M., Electron microscopy of *Coccidioides immitis*, *Fed. Proc.*, 1960, **19**, 243.
10. O'HERN, E. M., and HENRY, B. S., A cytological study of *Coccidioides immitis* by electron microscopy, *J. Bact.*, 1956, **72**, 632.
11. SEDAR, A. W., and RUDZINSKA, M. A., Mitochondria of protozoa, *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, No. 4, suppl., 331.
12. GIRBARDT, M., Über die Substruktur von *Polystictus versicolor L.*, *Arch. Mikrobiol.*, 1958, **28**, 255.
13. TARBET, J. E., WRIGHT, E. T., and NEWCOMER, V. D., Experimental coccidioidal granuloma. Developmental stages of sporangia in mice, *Am. J. Path.*, 1952, **28**, 901.
14. TARBET, J. E., and BRESLAU, A. M., Histochemical investigation of the spherule of *Coccidioides immitis* in relation to host reaction, *J. Infect. Dis.*, 1953, **92**, 183.
15. EDWARDS, M. R., HAZEN, E. L., and EDWARDS, G. A., The fine structure of the yeast-like cells of *Histoplasma* in culture, *J. Gen. Microbiol.*, 1959, **20**, 496.
16. EDWARDS, G. A., EDWARDS, M. R., and HAZEN, E. L., Electron microscopic study of *Histoplasma* in mouse spleen, *J. Bact.*, 1959, **77**, 429.
17. BRESLAU, A. M., Polysaccharides in microorganisms, in Graumann, W., and Neumann, K., eds., Handbuch der Histochemie, Stuttgart, Fischer Verlag, in press, Band 2, Teil. 1.
18. AGAR, H. D., and DOUGLAS, H. C., Studies on the cytological structure of yeast: Electron microscopy of thin sections, *J. Bact.*, 1957, **73**, 365.
19. HASHIMOTO, T., GERHARDT, P., CONTI, S. F., and NAYLOR, H. B., Studies on the fine structure of microorganisms. V. Morphogenesis of nuclear and membrane structures during ascospore formation in yeast, *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 305.
20. BRACHET, J., Ribonucleic acids and synthesis of cellular proteins, *Nature*, 1960, **186**, 194.

21. HASHIMOTO, T., CONTI, S. F., and NAYLOR, H. B., Fine structure of microorganisms. IV. Observations on budding *Saccharomyces cerevisiae* by light and electron microscopy, *J. Bact.*, 1959, **77**, 344.
22. YOTSUYANAGI, Y., Étude au microscope électronique des coupes ultra-fines de la levure, *Compt. rend. Acad. sc.*, 1959, **248**, 274.
23. YOTSUYANAGI, Y., Mise en évidence au microscope électronique des chromosomes de la levure par une coloration specifique, *Compt. rend. Acad. sc.*, 1960, **250**, 1522.