Micromorphology of *Cryptococcus neoformans*¹

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Fine details of the internal and external morphology of *Cryptococcus neoformans* as seen in ultrathin sections are described and illustrated with electron micrographs. The capsule characteristic of this species contained microfibrils (30 to 40 A in diameter) that appeared to radiate from the cell wall and to coil and intertwine in various directions. These thin, uniformly structured, electron-dense filaments are believed to represent complex polysaccharide molecules. The internal morphology of *C. neoformans* was in many ways similar to that of yeasts studied by other authors. The cell was uninucleate with a single nucleolus. The nuclear envelope, a pair of unit membranes interrupted by pores, was typical of that found in eucaryotic organisms. Smooth endoplasmic reticulum, mitochondria, vacuoles, storage granules, and ribosomes were consistent features of the cytoplasm. In addition, *C. neoformans* presented membranous organelles derived from the plasma membrane and comparable to bacterial mesosomes and mitochondria of an annulate type.

*Cryptococcus neoformans* (Torula histolytica) is of particular interest as a lethal pathogen for man and is capable of causing widely disseminated infection, but is best known as the agent of cryptococcal meningitis (16). Occasionally it develops pseudohyphae (30), but otherwise consists, both in parasitized tissue and in artificial culture, of spherical to ovoid, budding cells surrounded by a large mucoid capsule, which is highly antigenic and probably of considerable significance in determining virulence of the microorganism for man and other mammals. A previous publication on thin-section electron microscopy of *C. neoformans* (31) omitted any mention or illustration of the capsular structure. The micrographs of Ribi and Salvin (26), who attempted to demonstrate the capsule in dried whole cells exposed to osmium vapor, revealed only a finely dispersed amorphous material. Unpublished studies by the late George A. Edwards demonstrated large cryptococcal capsules in thin sections of mouse lung and spleen, but internal structures of the yeast cell were not well preserved. The present report describes details of both the capsule and the internal structure in thin section. Observations are also made on newly disclosed structures such as ring-shaped mitochondria and mesosomes.

1 Part of this paper was presented (7) at the Sixth International Congress for Electron Microscopy, 1966, Kyoto, Japan.

**Materials and Methods**

Strains M620 and Neill 5 of *C. neoformans*, both from this laboratory’s collection (mycological collection under the care of George N. Little), were cultured on Sabouraud Dextrose Agar (Difco) or 1% Neopeptone-4% Dextrose Broth (Difco) for periods of 8 hr to 3 days at 25 to 27°C. Appropriate developmental stages of the cells were determined by light microscopy. Fixation for electron microscopy was done by one of the following methods, the cells being separated from broth by centrifugation or transferred directly from the agar surface to the fixing solutions: (i) Glutaraldehyde-osmium (29). Cells were treated for 2 hr at room temperature with 4% glutaraldehyde solution in Sörensen’s buffer (pH 6.8), then washed three times (5 min each) in the buffer (with or without addition of sucrose), and postfixed for 0.5 to 1 hr with 1% osmium tetroxide in the same buffer. In one preparation, the buffer was 0.1 N sodium cacodylate at pH 7.6. (ii) Osmium tetroxide. In Sörensen’s or Veronal acetate buffer (24) at pH 7.0 or 7.4, 1 or 2% osmium tetroxide was used for 0.5 hr at room temperature. (iii) Potassium permanganate. A 0.6% solution of potassium permanganate was used in Veronal acetate buffer at pH 7.4 to 7.6 (18), or a 0.5 to 1% solution in distilled water without pH adjustment, for 30 to 45 min. After washing in water, some samples were postfixed in 1% osmium tetroxide for 0.5 to 1 hr (20).

Fixed cells were pre-embedded in 1.5% agar, cut in tiny blocks, and dehydrated in a series of graded ethyl alcohol solutions, followed by propylene oxide. Either Epon 812 or methacrylate was used for embedding. With the former, an epoxy resin mixture, in
the proportion of four parts of A and six of B, according to Luft (19), was added gradually to the material in propylene oxide to ensure proper infiltration. Samples were shaken for 1 hr, then transferred to Epon mixture alone, and shaken overnight. The material was finally placed in gelatin or Beem capsules with a freshly prepared mixture of the resin and was polymerized at 45°C for 12 hr, then at 65 to 70°C for about 24 hr.

For samples to be embedded in methacrylate, the propylene oxide treatment was omitted, and the material was transferred directly from absolute alcohol into a mixture of this reagent with methacrylate (nine parts n-butyl and one part methyl monomers). After 30 min, the samples were transferred to the methacrylate mixture without alcohol and were shaken for 1 to 3 hr, then left in a partially polymerized methacrylate mixture of the same composition at 4°C for 1 to 4 days. Finally, the blocks were placed in gelatin capsules containing a fresh prepolymerized methacrylate mixture and were incubated at 46°C until embedding was completed.

Ultrathin sections were cut with diamond knives in either an LKB or Porter-Blum microtome, picked up on copper grids without supporting film, and stained with uranyl acetate (alcoholic solution), followed by lead citrate (25). Electron micrographs were taken on Kodak electron image plates with a Siemens Elmiskop I or IA electron microscope.

RESULTS

The cells of *C. neoformans* generally appeared round to oval in section (Fig. 1, 4, and 10). As determined by light microscopic measurements of both strains used in this study, round cells ranged in diameter from 2 to 8 μ, excluding the capsule, and the average long and short axes of oval cells were 5 and 4 μ, respectively. Most of the details of the fine internal structure resembled those reported in yeast cells of other species (1, 4-6, 8, 12, 21, 28, 32).

Cell envelope. The cell envelope comprises the plasma membrane, the cell wall, and the mucilaginous capsule.

Plasma membrane. The outer limit of the cytoplasm is the plasma membrane (Fig. 1, 5, and 10), which, as in other fungal cells, has the characteristics of a "unit membrane" (for references, see Moore (22) and Robinow (27)). It appears in cross section as two dense layers, each 25 to 30 A in width, separated by a lighter zone of about the same thickness. It is a convoluted or undulating structure appressed to the inner border of the cell wall (Fig. 1 and 5). At certain points, however, the membrane invaginates into the peripheral cytoplasm to form small pockets (Fig. 5) or more complex organelles (Fig. 15-18), often resembling bacterial membranous systems that have been called mesosomes (10) or plasmalemmosomes (9). The plasma membrane and its derivatives in *C. neoformans* are seen clearly in micrographs of cells fixed with glutaraldehyde and then with osmium (Fig. 5 and 15-18). With potassium permanganate fixation (Fig. 2-4), it is not readily shown, but it may be delineated by means of its light middle layer, which usually remains unstained (32).

Cell wall. External to the plasma membrane is a cell wall (Fig. 1-6 and 10-12) of medium to high electron opacity and variable width (480 to 860 A). In most of our micrographs, there is also a clearly visible zone, transparent to electrons, located between the cell wall proper and the capsule (Fig. 1, 6, and 10). This light zone, or white rim (wr) as it is labeled in Fig. 10, has an average width of 400 A as determined from numerous micrographs.

Capsule. Investing the cell wall of *C. neoformans* as seen in light microscopy is a mucoid capsule, the width of which varies with cell age and strain but sometimes reaches a diameter greater than that of the cell itself. Strain M620 characteristically develops a large capsule (ca. 3.5-μ thick), whereas that of Neill 5 is smaller and inconspicuous. Electron microscopy confirms this difference, although, despite careful preparation of the material, considerable loss of the capsular substance occurs. Well-preserved capsules are found in only a few micrographs (Fig. 6, 10, 11, 13, and 14), and even in these instances the peripheral portion has been lost (compare width of capsule, about 0.5 μ in electron micrographs, with that observed by light microscopy). In some micrographs (Fig. 13 and 14), this capsular material is shown to be composed of thin, dense microfibrils (30 to 40 A in diameter) which apparently are long, coiled, and intertwining, although often seen as dots or in varying lengths depending upon the plane of sectioning.

Those cells with the least capsular material (Fig. 1, 4, 5, 8 and 9) display the best preservation of intracytoplasmic structures (contrast with more heavily encapsulated cells in Fig. 6 and 10), suggesting that removal of the capsule during cell fixation and embedding facilitates penetration of the reagents. The effect of permanganate, which apparently eliminates most of the capsular components and produces good fixation of internal membranous organelles (Fig. 2-4), seems to support this interpretation.

Nucleus. The cells of *C. neoformans* are uninucleate, with a single, granular nucleolus usually located peripherally at a lobe (Fig. 1). The nucleus is irregular in shape and varies in size, the average of its longer and shorter dimensions being 1.5 and 1.2 μ, respectively. The nuclear
Fig. 1. Cell showing cell wall (cw), plasma membrane (pm), and nucleus limited by nuclear membrane (nm) and containing a peripherally located nucleolus (nc). In the cytoplasm ribosomes (r), mitochondria (m), profiles of the endoplasmic reticulum (er), vacuole (v), and storage granules (g) are indicated. Glutaraldehyde-osmium fixation. Epon. × 40,000.

The envelope (Fig. 1–4) is a double unit membrane spaced by the nuclear cisterna, such as that reported for other yeasts (28, 32). It also has discontinuities or pores (Fig. 2), and, as in other fungi (20), is continuous with membranes of the endoplasmic reticulum (Fig. 3). In cells fixed with either or both glutaraldehyde and osmium, the nucleoplasm shows uniformly distributed granular and filamentous material, whereas in nuclei fixed with permanganate there are areas of varying electron opacity. This has been noted in Saccharomyces (32) and several other fungal species [see review by Moore (22)].

Cytoplasm. Numerous membranous organelles and ribosomes (ribonucleic acid particles) are found in the cytoplasm of C. neoformans. The latter are well preserved by osmium fixation with or without glutaraldehyde, and appear as free particles (140 to 170 Å in diameter) scattered throughout the cytoplasm (Fig. 1, 5, and 7–9) and sometimes inside mitochondria (Fig. 8 and 9). Profiles of the endoplasmic reticulum (Fig. 3–5...
FIG. 2. Portion of cell demonstrating nucleus (n) with nuclear membrane (nm), nuclear pores (arrows), and part of a mitochondrion (m). Permanganate in water. Epon. × 40,000.

FIG. 3. Portion of cell showing continuity (arrows) of nuclear membrane (nm) with endoplasmic reticulum (er). Permanganate in Veronal acetate buffer. Epon. × 60,000.

FIG. 4. Cells limited by their cell walls (cw) displaying nuclei and their membranes (nm), mitochondria (m), endoplasmic reticulum (er), and vacuole (v). Permanganate in Veronal acetate buffer. Epon. × 30,000.
FIG. 5. Peripheral portion of a cell showing cell wall (cw) and tripartite plasma membrane (pm) with invaginations (arrows). Profiles of the endoplasmic reticulum (er) are also seen. Glutaraldehyde-osmium fixation. Epon. × 80,000.

FIG. 6. Part of cell enveloped by the capsule (ca) and the cell wall (cw). A halo is seen between these structures. In the granular cytoplasm (cy), mitochondria (m) and storage granules (g) are present. Osmium in Sörensen's buffer. Epon. × 60,000.

FIG. 7. Section including a ring-shaped (m) and three smaller mitochondria (1, 2, 3). Portion of a nucleus (n), ribosomes (r), and vacuole (v) are represented. Glutaraldehyde-osmium fixation. Epon. × 50,000.
FIG. 8. Ring-shaped mitochondrion (m) with a branch (b). Dense dots in the cytoplasm are ribosomes, some of which are also seen inside the mitochondrion (arrows). Parts of two vacuoles (v) limited by single unit membranes are in evidence. Glutaraldehyde-osmium fixation. Epon. $\times 60,000$.

FIG. 9. Peripheral part of a cell showing profiles of the endoplasmic reticulum (er), some of which lie parallel to the plasma membrane, and a large mitochondrion (m) with numerous cristae. Note ribosomes (arrows) inside the mitochondrion. Glutaraldehyde-osmium. Epon. $\times 60,000$. 

Figures 8 and 9 illustrate the micromorphology of C. neoformans.
FIG. 10. Cells of different ages readily distinguished by the thickness of their cell walls (cw) and capsules (ca). The younger cell (left) seems better preserved, and shows nucleus (n), profiles of the endoplasmic reticulum (er), and a few mitochondria. Plasma membrane (pm) and mitochondria (m) are indicated in the older cell. Note in both cells the halo or white rim (wr) between the wall and the capsule. Osmium in Sörensen's buffer. Epon. × 20,000.

FIG. 11. Cell wall (cw) and capsule (ca) in an old cell. Note absence of halo or white rim. Osmium in Veronal acetate. Methacrylate. × 80,000.

FIG. 12. Part of a degenerating cell with wall (cw) separated from the plasma membrane (pm) which is retracted with the cytoplasm (cy). Note that the capsular material is clumped but still adherent to the wall. Osmium in Veronal acetate. Epon. × 40,000.
Fig. 13 and 14. Sections grazing the cell wall (cw) and the capsule (ca) with filaments (f). Note also filaments (arrows) in the lighter zone between cell wall and capsule. Osmium in Sörensen’s buffer. Epon. Fig. 13, × 240,000. Fig. 14, × 80,000.
and 9) are scarce and generally free from ribosomal particles, i.e., the smooth endoplasmic reticulum seems to be the predominant type. As mentioned above, these organelles may connect with the nuclear membrane and also with the plasma membrane (Fig. 3). Tubular elements of this type are frequently found parallel with the plasma membrane (Fig. 4 and 9) or with each other (Fig. 9 and 10). Mitochondria are of various types and shapes (Fig. 1, 4, 8, and 9); as is true of other fungi (14, 22), they show the characteristic outer and inner membranes, the latter projecting internally to form cristae. These projections appear somewhat dilated in many micrographs (Fig. 9), probably on account of the procedure employed in their fixation. When cacodylate buffer is used instead of phosphate or Veronal acetate (unpublished observations), the cristae are not so open and, in general, the whole mitochondrion appears less swollen. However,
with osmium tetroxide in cacodylate buffer, the ground cytoplasm and its organelles appear darkened in a uniform manner, making it very difficult to discern structural details. Noteworthy in the present report are the ring-shaped mitochondria (Fig. 7 and 8), which are frequently found in young cells and sometimes branch (Fig. 8). In some sections (not illustrated here for lack of space), long and tortuous mitochondria were observed, so that what seem to be separate structures in many of the micrographs may represent parts of a single organelle. As indicated in Fig. 8 and 9, mitochondria may enclose particles similar to ribosomes, an observation made also by Luck (17) in *Neurospora crassa*.

Storage granules (Fig. 1 and 6) are interpreted as containing lipid, since lipid can be demonstrated by light microscopy. They may be similar to poly-β-hydroxybutyrate bodies of bacteria (see Discussion). Vacuoles (Fig. 1 and 8) are also common in *C. neoformans* and are limited by a single unit membrane (Fig. 8). Golgi membranes have not been found in any of our preparations.

**Cell division.** As a yeast, *C. neoformans* multiplies by means of a budding process, i.e., by outgrowth of a small area of the peripheral cytoplasm with its plasma membrane and the adjacent younger part of the cell wall. This process has been studied and will be illustrated elsewhere. In general, it is similar to that of other yeasts (8, 12).

**Discussion**

In this study, *C. neoformans* was found to possess ultrastructural characteristics of both eu- and prokaryotic cells. [Protocaryotic used is advisedly, in preference to procaryotic (11).] Among the former are the nuclear envelope, the nucleolus, mitochondria of various shapes and sizes, endoplasmic reticulum, lipid bodies, and vacuoles, all of which have been described in pathogenic yeast-like organisms (2, 3, 6, 8) and other fungi (14, 22). Ribosomal particles were abundant in the cytoplasm and were also found inside mitochondria, but Golgi membranes were absent. Mesosome-like structures—membranous organelles known to exist in protocaryotic cells such as bacteria (9, 10)—were frequently noted at the peripheral cytoplasm of *C. neoformans*. These organelles are of varied size and appearance (Edwards, *in preparation*), but their function and significance remain to be ascertained. In an investigation of *Geotrichum candidum*, Hashimoto and Yoshida (13) also found structures similar to mesosomes of bacteria and associated them with glycogen synthesis. Their illustrations show membranous organelles largely vacuolized and containing granules interpreted by the authors as glycogen particles. We found no evidence of glycogen in our fungus. Vacuoles were present in *C. neoformans* and are limited by a single unit membrane of the type frequently described for fungi, as, for example, in *Saccharomyces cerevisiae* (32). Occasionally, dense material was seen inside the vacuoles, but it never was in the form of the regular aggregates characteristic of the fine structure of glycogen. In some cases, there were also connections between the plasma membrane and vacuoles, but whether the latter were derived from mesosomes was not ascertained. We did find in the cytoplasm of this fungus large granules which are similar in appearance to poly-β-hydroxybutyrate bodies of bacteria. They show dense electron opacity after osmium fixation and low density in material fixed by permanganate. Preliminary results of chemical analyses by Robert P. Mahoney (Skidmore College, Saratoga Springs, N. Y.) suggest the possibility that this lipid is stored in *C. neoformans*; if poly-β-hydroxybutyrate is confirmed, its presence would represent another protocaryotic trait in our fungus.

Mesosome-like organelles (10, 33), or plasmakinetic vesicles (9) may be common among the fungi. Similar structures were described in a basidiomycete (15). It is possible that the fungal structures named lomasomes by Moore and McAlear (22, 23) are derived from the plasma membrane, as are the mesosomes (10) of bacteria and of *C. neoformans*, but the significance of these organelles in the fungal cell remains obscure.

The use of glutaraldehyde followed by that of osmium tetroxide (29), a procedure applied by others to the fixation of fungi (15, 28), resulted in excellent preservation of some of the fine cytoplasmic details of *C. neoformans*, as well as of the nucleolus, and in the particularly clear demonstration of ribosomes. In contrast, when permanganate was used as the fixative, ribosomes and nucleoli were not preserved at all, a fact that is consistent with reports in the literature (22, 32). However, since the latter method caused the ground cytoplasm to appear less dense, owing to absence of the ribosomal particles, it permitted the membranous system to be seen more distinctly.

It is not clear whether the halo (white rim in Fig. 10) is part of the cell wall, part of the capsule, or possibly an artifact resulting from separation of capsule from wall during Epon polymerization (it does not appear in the methacrylate section, Fig. 11). However, its uniform width and sparse content of microfibrils (Fig. 13 and 14) suggest that it is a zone of capsular polysaccha-

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ride synthesis, the macromolecules undergoing condensation peripherally to form the denser sheath.

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