

Mycelial Phase of *Paracoccidioides brasiliensis* and *Blastomyces dermatitidis*: an Electron Microscope Study

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A comparative study of the mycelial phase of *Paracoccidioides brasiliensis* and *Blastomyces dermatitidis* reveals that both fungi are very much alike, containing multiple nuclei and nuclear pores, mitochondria, ribosomes, scarce endoplasmic reticulum, intracytoplasmic membrane systems, glycogen, and vacuoles. Shadowed cell walls show fine fibrillar surfaces that contrast with those in the yeast phase. The intracytoplasmic membrane system is continuous with the plasma membrane and is similar to bacterial mesosomes. Granules with light cores and dark rims are observed in the plasma membrane. Live hyphae growing inside a dead hypha are found much more frequently in immersed cultures than in solid-medium cultures, suggesting that breakage of the hypha could elicit this phenomenon.

Ultrastructural studies of the yeast phase of *Paracoccidioides brasiliensis* (4, 5) and *Blastomyces dermatitidis* (9) have been published, as well as electron-microscope studies on other septate fungi belonging to the *Ascomycetes* (22), *Basidiomycetes* (3, 25), and *Deuteromycetes* (27). However, the ultrastructure of the mycelial phase of dimorphic human pathogenic fungi, such as *P. brasiliensis* and *B. dermatitidis*, has not been described. We deemed it important to carry out electron microscopy in this field, since observations with the light microscope are morphologically insufficient for the research we are performing on the cell wall of these fungi. Research on the conformation of the septum of hyphae was also undertaken, because this could give us a clue toward clarifying the taxonomic position of *P. brasiliensis*.

MATERIALS AND METHODS

To obtain the mycelial phase of *P. brasiliensis* (IVIC-Pb9) and *B. dermatitidis* (IVIC-Bd5), the yeast phase of these fungi, grown at 37 C for 3 days, was inoculated into a GGY medium (glycine, 1%; glucose, 2%; yeast extract, 0.2%; adjusted to pH 7.2 to 7.4 with KOH) and grown at 22 C in 250-ml Erlenmeyer flasks which were placed in a reciprocal shaker at a speed of 100 oscillations/min with a stroke amplitude of about 5 cm. After cultivation from 5 to 18 days, the cells were harvested by centrifugation. Mycosel Agar (Difco) was used as a solid medium.

Samples from both the solid and liquid media were fixed in 2% glutaraldehyde with 0.1 M phosphate buffer (pH 7.2) for 24 hr, postfixed in 1% osmium

tetroxide in the same phosphate buffer for 3 hr, dehydrated in a graded series of ethyl alcohol, and embedded in Maraglas (12). Other samples were fixed in potassium permanganate (18) for 30 min and embedded in the same manner. Ultrathin sections were cut with a MT-2 Porter Blum microtome with diamond knives. The electron microscopes used were Hitachi HU-11B and JEOL JEM-7A. For studies with a light microscope, sections were obtained from material embedded in plastic and paraffin and stained with Gomori's modified (13) methenamine silver technique.

To obtain isolated cell walls, hyphae, previously fixed in glutaraldehyde for 24 hr, were disrupted in a French press 20 to 25 times. This material was then homogenized in a Waring Blendor for 10 min followed by ultrasonic disruption for 20 min, using a Branson Sonifier. Successive centrifugations at 20 g were performed. The supernatant fluids were further centrifuged at $12,100 \times g$ for 15 min. The sediment was suspended twice in 60% sucrose and centrifuged at $5,900 \times g$ for 30 min. Material from the sediment was placed on carbon-coated collodion membranes and shadowed with nichrome at a 30° angle.

RESULTS

Unless otherwise specified, the description that follows pertains to both fungi.

Fungi in liquid medium grow as small globular stromatic pellets, centrifugally and radially. The pellets from *B. dermatitidis* are round or oval, whitish, cotton-like, and fluffy. In *P. brasiliensis*, they are smaller, light brown, and round. "Filaments" are dispersed throughout the liquid

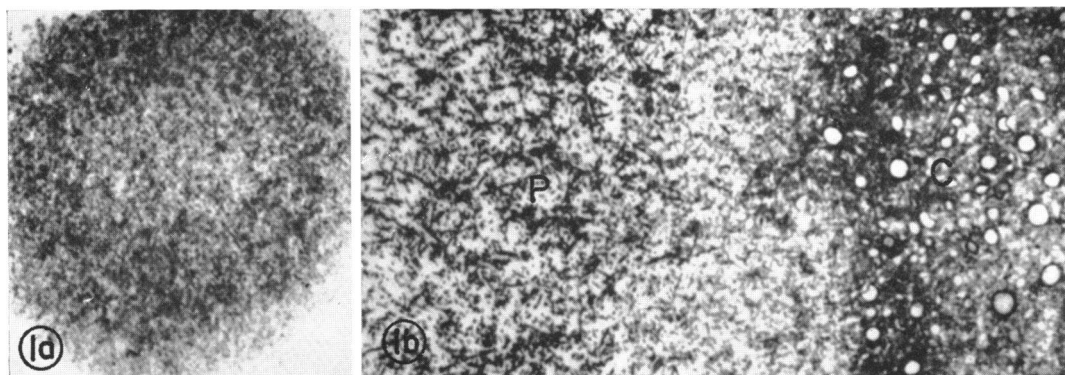


FIG. 1. Pellet from cultures of *Paracoccidioides brasiliensis*. (a) Low magnification of an 18-day-old pellet; the center is clearer than the periphery. (b) Higher magnification of the same pellet; at the periphery (P), loosely packed hyphae can be seen. Center (C), tightly packed hyphae with round bodies interpreted as chlamydo spores (sp). (a) $\times 17$; (b) $\times 19$.

medium, being more abundant in *P. brasiliensis* than in *B. dermatitidis*.

Sections of an 18-day-old pellet show that the pellet is composed of a central zone formed by tightly packed hyphae most of which are empty, and an outer zone made of slender, strongly stained hyphae (Fig. 1a, b).

Light microscope observations of *B. dermatitidis* grown in solid medium were very similar to those of *P. brasiliensis* (6).

Cell wall. The cell wall thickness measures from 80 to 150 $m\mu$, depending on the region of the hypha; e.g., at the septum implantation, it is thicker than in any other place. Occasionally, cell walls display thin, inner and outer electron-dense layers and a middle, broad low-dense layer (Fig. 2c). Others show three layers, with the middle layer being more electron-dense than the other two (Fig. 2b). Most frequently, cell walls are homogeneous (Fig. 2a) without any distinct layering. In osmium or permanganate fixation, the cell walls possess similar characteristics.

After shadowing with nichrome, the outer surface of the cell wall has a fibrillar appearance and the inner surface shows a delicate fibrillar pattern (Fig. 3).

Septum. The so-called septum is between 110 and 150 $m\mu$ thick and may be considered a sort of deep invagination of the innermost layer of the cell wall. This innermost layer is composed of electron-dense granules embedded in a homogeneous low-density material (Fig. 2c, 5, and 6). At the site of the septum implantation, there is an increase in the diameter of the hypha caused by bulging of the cell wall at this point (Fig. 5). Remnants of the intracytoplasmic membrane system (ICMS) are embedded in the cell wall (Fig. 6). The septum ingrows from the cell wall

towards the middle of the hypha, leaving a small pore at the center of the mature septum. Its base is broad and faces the cell wall. The developing wall is at all stages surrounded by the invaginated plasma membrane even, when the septum reaches maturity. When the section is not made at the level of the pore, the septum appears to be continuous and one or two layers are visible (Fig. 6), but never as clearly outlined as in other fungi (3).

Plasma membrane and intracytoplasmic membrane system (ICMS). The cell membrane proper (plasma membrane) is continuous from cell to cell through the pore. Its structure differed according to the sectioning angle. Sometimes a double membrane, composed of an outer, wide, electron-dense layer, a low-density middle space, and a very narrow inner electron-dense layer, is seen. Attached to the outer layer of the plasma membrane, the electron inner layer of the cell wall is visible (Fig. 2c). More frequently, only a wide electron-dense layer is observed closely attached to the cell wall in one side and to the cytoplasm in the other side (Fig. 2a).

When the plasma membrane presents small invaginations, a three-layered structure is clearly seen (Fig. 9a): two of these layers, 3 $m\mu$ each, are electron-dense and separated by an electron-transparent layer measuring approximately 3 $m\mu$ (Fig. 9b). At a higher magnification, the middle layer is clearly outlined, but the other two layers display an irregular outer surface in which the presence of granules (with light cores and dark rims) is suggested (Fig. 9b).

The invaginations of the plasma membrane are interpreted as the beginning of the ICMS. This membranous system undergoes additional invaginations forming multivesicular or lamellar structures (Fig. 10a, b) that are interpreted as

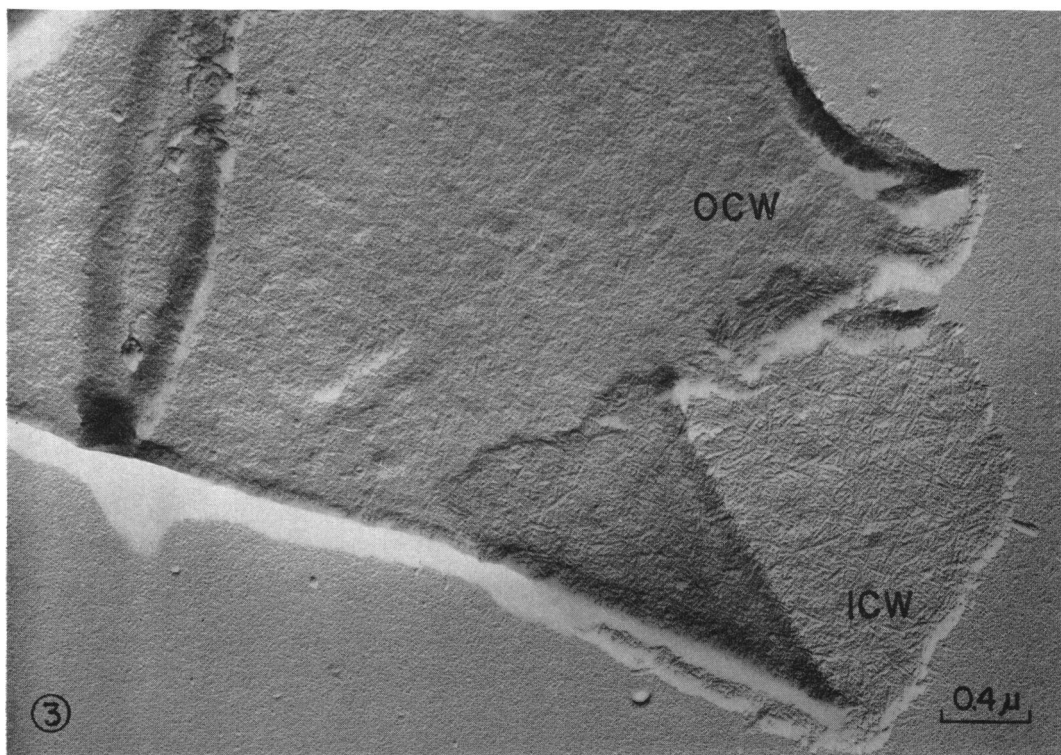
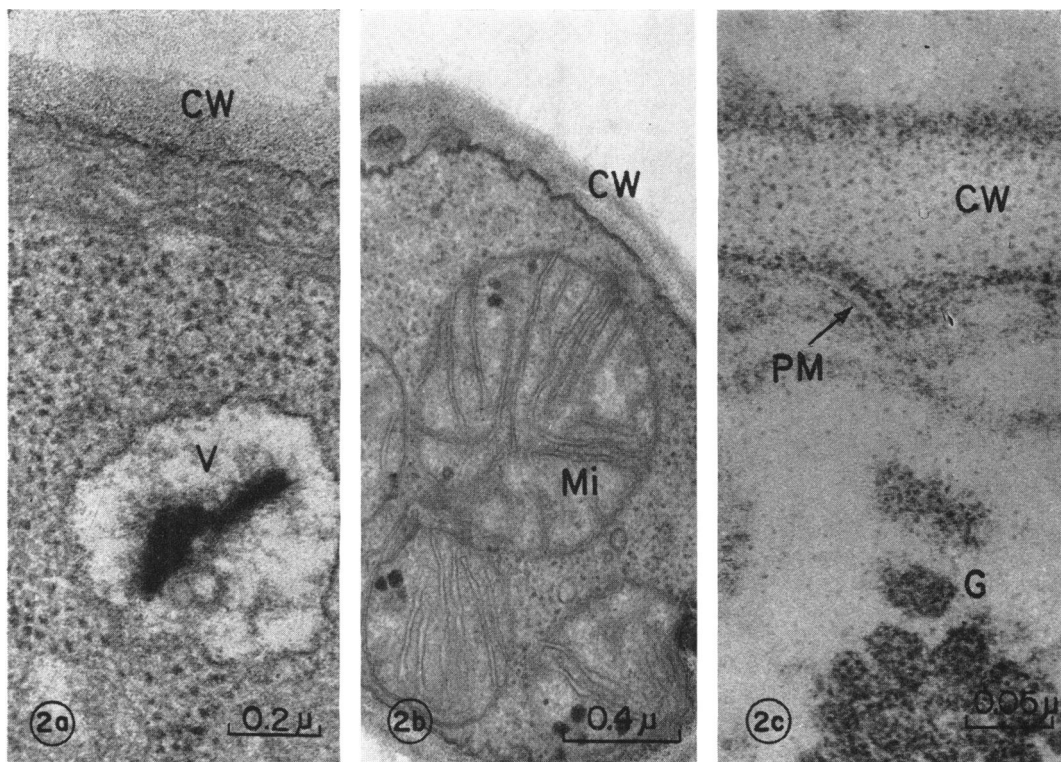


FIG. 2. (a) *Blastomyces dermatitidis* exhibits a homogeneous cell wall (CW); vacuoles (V) with electron-dense material inside. (b) *B. dermatitidis* displays a three-layered cell wall; mitochondria (Mi) shows dense granules (c) Cell wall (CW) of *Paracoccidioides brasiliensis* composed of a thin electron-dense layer and an inner electron-lucent layer. Attached to the inner layer of the cell wall is the plasma membrane (PM) which shows a wide, dense, outer layer, a middle low-density space, and an inner, narrow, dense layer. G, glycogen. (a) $\times 60,000$; (b) $\times 37,500$; (c) $\times 232,000$.

FIG. 3. Shadowed isolated cell wall of *Blastomyces dermatitidis*. Cell wall is broken and turns up, showing an inner fibrillar surface (ICW). Outer surface (OCW) is less fibrillar than the inner surface. Septum appears as an annular bulged structure. $\times 29,000$.

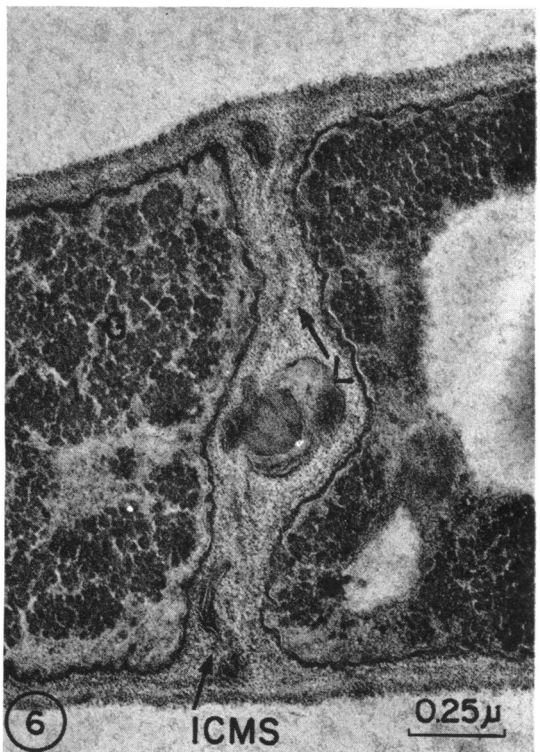
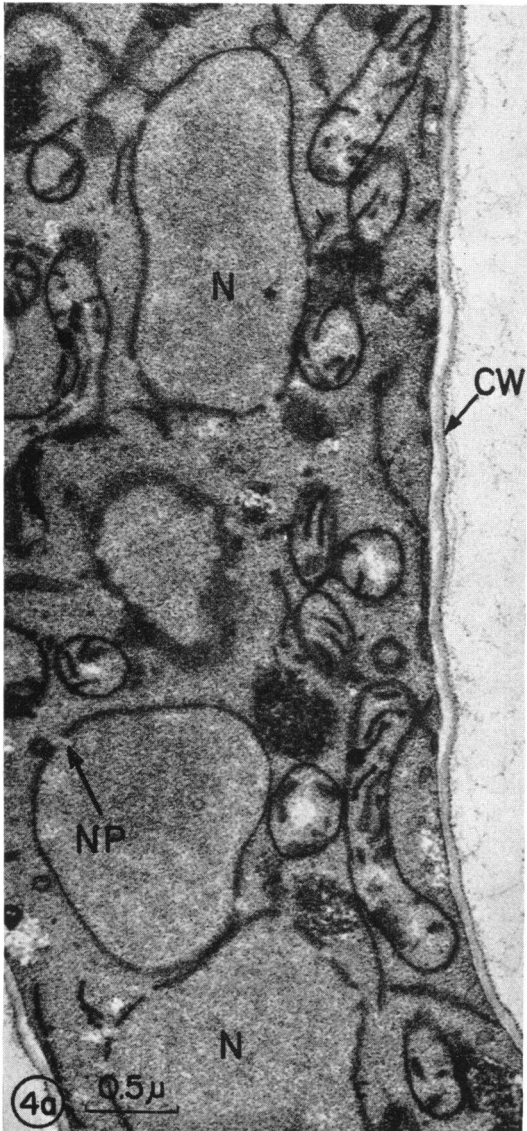
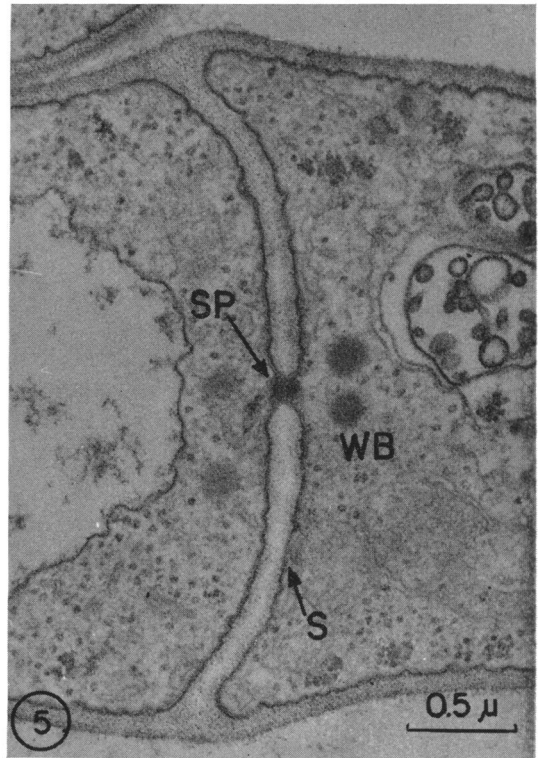
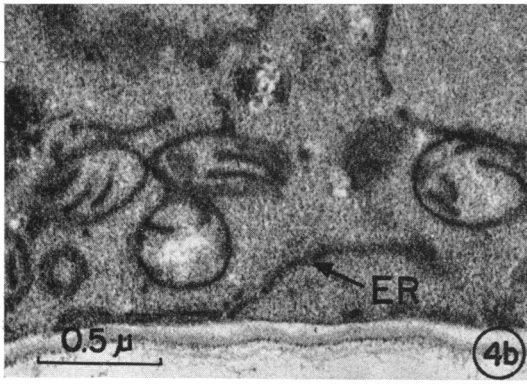


FIG. 4. (a) Hyphae of *Paracoccidioides brasiliensis*. Outer layer of the cell wall (CW) is denser than the inner layer. Several nuclei (N) with multiple nuclear pores (NP). (b) Endoplasmic reticulum (ER) in continuity with the plasma membrane. (a) $\times 24,000$, (b) $\times 32,000$. $KMnO_4$ fixation.

FIG. 5. Septum in *Paracoccidioides brasiliensis*. At the site of the septum (S) implantation, the diameter of the hypha increases. Plasma membrane is continuous through the septum's pore which is obliterated by a septal plug (SP). Four Woronin bodies (WB) near the septum. V, vacuole. $\times 28,500$.

FIG. 6. Septum in *Blastomyces dermatitidis*. At the center of the septum is a dense material; at the sides are remnants of the intracytoplasmic membrane system (ICMS). Two more dense layers (L) are lengthwise of the septum. Abundant glycogen (G). $\times 50,000$.

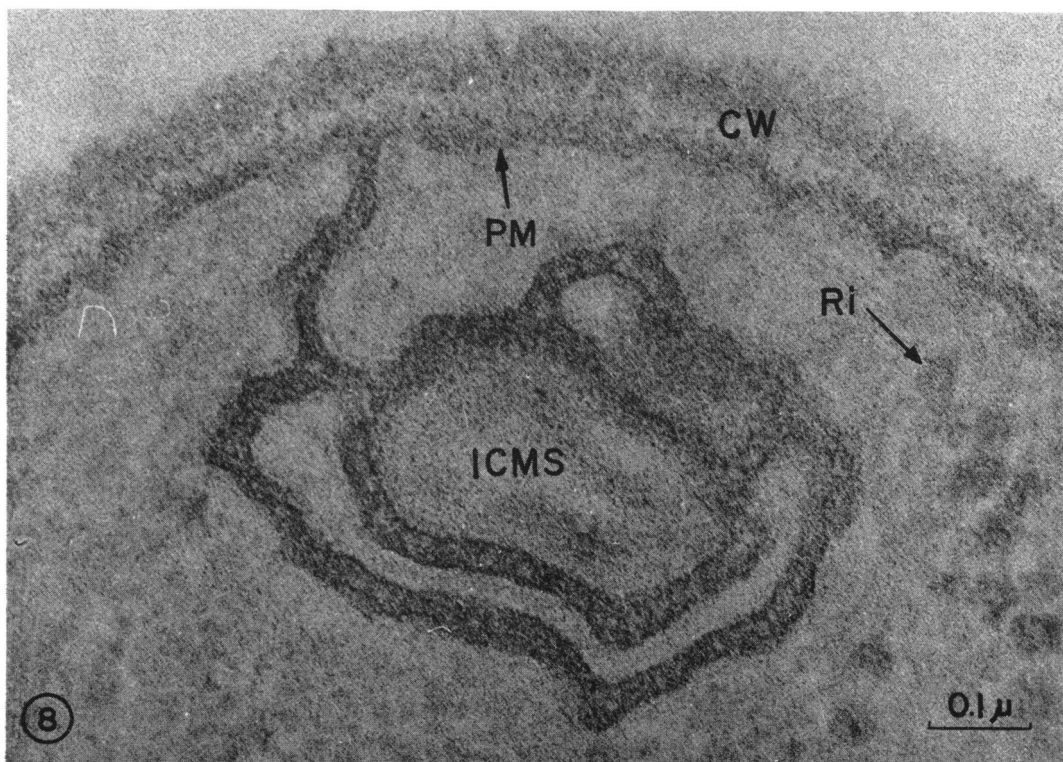
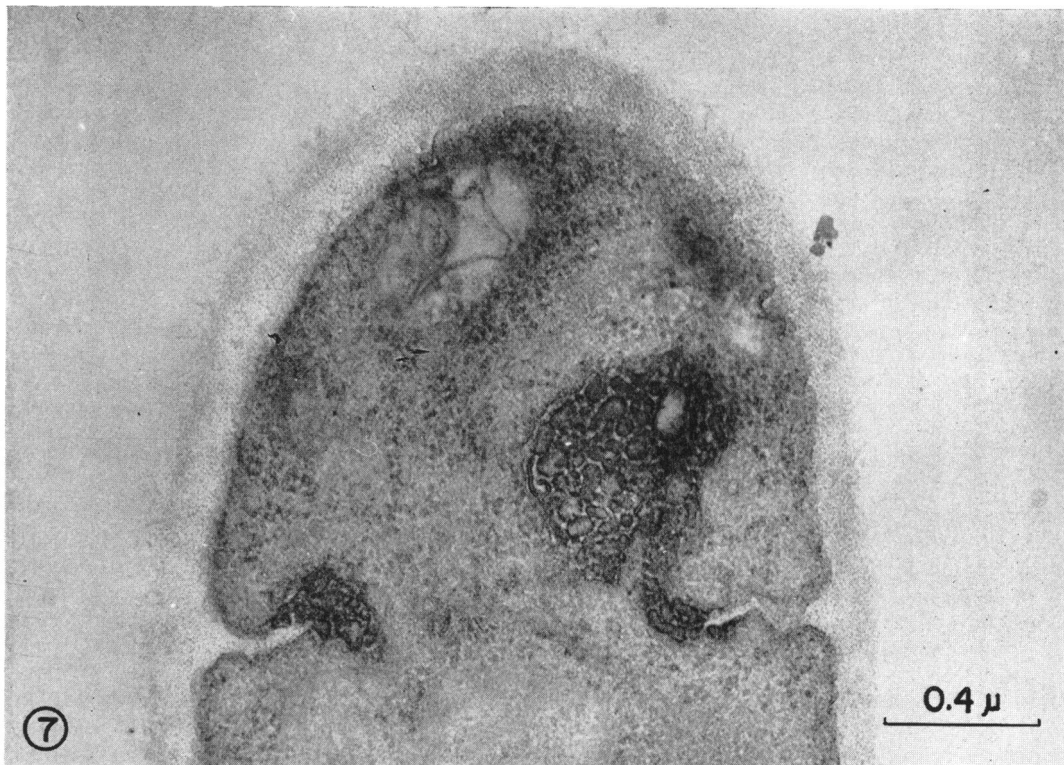


FIG. 7. ICMS in *Blastomyces dermatitidis*. The ICMS is attached to the tip of the septum in formation (S). $\times 51,000$.

FIG. 8. ICMS in *Paracoccidioides brasiliensis*. Note continuity of the plasma membrane (PM) with the ICMS. CW, cell wall; Ri, ribosomes. $\times 137,000$.

tubular infoldings of the plasma membrane seen in different sectioning angles. The ICMS is enclosed by the innermost layer of the cell membrane (Fig. 10a, b) and is attached to the tip of the forming septum (Fig. 7).

In a well-developed ICMS, the outermost layer of the plasma membrane is attached to a material that resembles the inner layer of the cell wall (Fig. 9a, b). Once more, blurring of the plasma membrane was observed at the place in which the cell wall-like material is formed. Since this cell wall-like material was observed when the plasma membrane invaginated, it is believed that there is a continuity between the cell wall and this material. The ICMS was frequently seen surrounded by glycogen granules. These granules were seldom in direct contact with the ICMS; instead, a clearly delimited space was observed.

Mitochondria. Mitochondria are spherical or elongated and their long axis parallels the long axis of the hypha. They show a moderately dense matrix which frequently contains dense granules (Fig. 2b). In young hyphae, mitochondria exhibit few cristae extending deeply into the central matrix (Fig. 2b). The membrane that forms the mitochondria is different from the plasma membrane which is more electron-dense (Fig. 2a, b).

Nuclei. Nuclei generally elongate and extend toward the bifurcation of the hypha. In some transverse sections, the nucleus occupies most of the hypha; in others, the hypha is often multinuclear (Fig. 4a). In material fixed with KMnO_4 , multiple nuclear pores are seen (Fig. 4).

Endoplasmic reticulum and ribosomes. The term endoplasmic reticulum has been generally used to describe the endoplasmic membranous system in fungal cells (14). In the cytoplasmic matrix, membranes are scarce and sometimes seem to be the continuation of the nuclear membrane (Fig. 4). Neither the Golgi apparatus nor "cortical membranes," like the ones that appear in other fungi and plant cells (14), were identified.

In young hyphae, electron-dense granules of 160 to 200 Å in diameter are regularly distributed throughout the cytoplasm (Fig. 10a). These ribosomes are seldom attached to the scarce membranes of the cytoplasm (Fig. 2a, and b), and it is difficult to identify them in the old cells. There is no morphological relationship between the ICMS and the ribosomes (Fig. 10a).

Although the cytoplasmic ground substances seem to be granular, caution must be taken in this interpretation, since these granules may be artifacts caused by fixatives.

Other cytoplasmic components. Large and small vacuoles, with an irregular outer contour, are observed only in mature hyphae. Sometimes these vacuoles almost occupy the entire space between

two septa. They are surrounded by a jagged electron-dense, one-layer membrane (Fig. 2a, 5, 11, and 13). The large vacuoles generally contain an electron-lucent, flocculent-like material in which some electron-dense particles are embedded. Some of the small vacuoles are entirely filled with a central body of high electron opacity (Fig. 2a and 15). Frequently, the ICMS invaginates into a vacuole, or a vacuole invaginates into another vacuole (Fig. 11), giving the appearance of a double membrane. Glycogen (Carbonell, unpublished data) is observed in young and mature hyphae and has a tendency to cluster instead of spreading throughout the cytoplasm (Fig. 6, 12, and 15). At a higher magnification, the glycogen granules exhibit a rosette-like structure in which granules are electron-dense, and the zone that surrounds them is electron-lucent (Fig. 2c).

The round-cell structures seen in *P. brasiliensis* by light microscopy (Fig. 1b; reference 6) are identified as chlamydospores (11) when observed with the electron microscope. They measure from 5 to 15 μ , and their cell wall is approximately 160 to 200 $m\mu$ thick. Inside the chlamydospores, the same organelles that were observed in the hyphae were seen: glycogen, ribosomes, and vacuoles (Fig. 11). Electron-opaque bodies (Woronin bodies) are seen associated with septa and especially with septal pores (Fig. 5). These bodies are oval or round and measure from 140 to 180 $m\mu$ in *P. brasiliensis* and from 160 to 200 $m\mu$ in *B. dermatitidis*. They are found at each side of the septum in numbers of one to four (Fig. 5). The visible number of bodies and the difference in density depends on the way the sectioning angle passes through the pore and its adjacent areas. With the light microscope, it is difficult to identify them in either fungus.

Woronin bodies are formed by a granular electron-dense material which is sometimes denser at the periphery. The pore is often obliterated by one round or elongated body that fits into it (Fig. 5), and which has the same characteristics of the Woronin bodies already described. Blurring of the plasma membrane is observed at the site in which the septal plug comes in contact with the pore (Fig. 5).

Intrahyphal hyphae. Methenamine silver staining of cultures grown in liquid medium revealed septa within the hyphae located in the center and at the periphery of the pellets. They are more frequent in cultures of liquid medium than in cultures of solid medium. At a higher magnification, hyphae sectioned at different angles are observed within a hypha. Longitudinal and transversal sections show hyphae within a hypha distributed at random (Fig. 12).

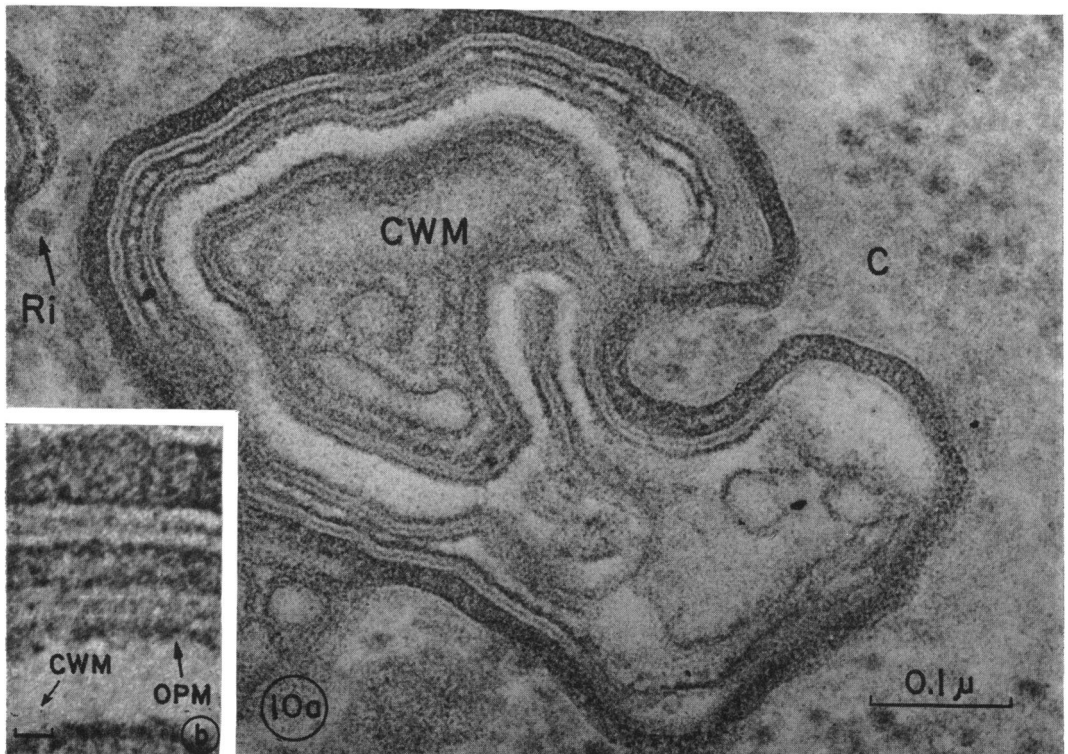
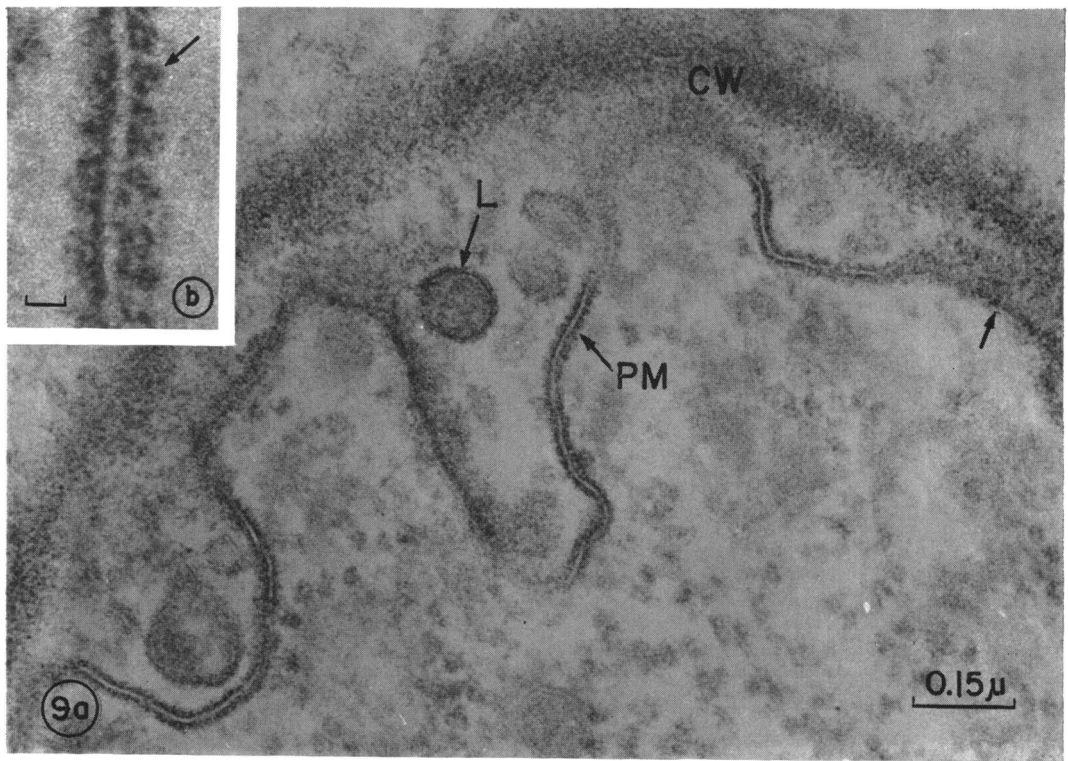


FIG. 9. Plasma membrane in *Paracoccidioides brasiliensis*. (a) Plasma membrane (PM) shows a clear middle layer with two dense layers along its side. Blurring of the plasma membrane is seen at the site in which the cell wall-like material adheres to the plasma membrane (arrow). Inside the invaginated plasma membrane (PM) are lomasomes (L). (b) Higher magnification of the plasma membrane shows units (arrow) composed of a clear inner zone and an outer dark rim. (a) $\times 90,000$; (b) $\times 355,000$.

FIG. 10. Intracytoplasmic membrane system in *Paracoccidioides brasiliensis*. (a) Dense broad layer is formed by invagination of the plasma membrane. Cell wall-like material (CWM) is always inside the ICMS and in contact with the outer layer of the plasma membrane, and never in direct contact with the cytoplasm. Ri, ribosomes; C, cytoplasm. (b) Higher magnification of the layered ICMS. OPM, outer layer of the plasma membrane. (a) $\times 182,000$. (b) $\times 497,000$.

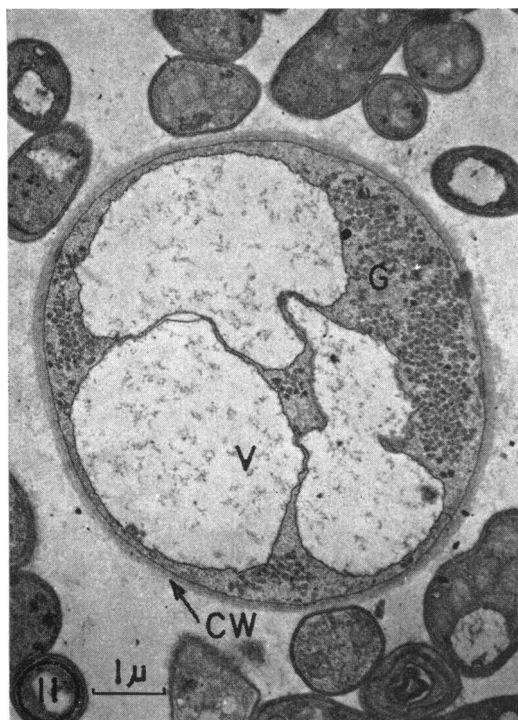


FIG. 11. *Chlamydozooids* in *Paracoccidioides brasiliensis*. The cell wall (CW) is wider in the chlamydozooids than in the hyphae. Observe the invagination of a vacuole (V) into a vacuole. G, glycogen. $\times 9,600$.

Some of the intrahyphal hyphae display an electron-lucent cell wall (Fig. 12) which is better seen with KMnO_4 . Inside the intrahyphal hyphae, septa, nuclei, mitochondria, endoplasmic reticulum, empty or lipid vacuoles, glycogen, and ICMS can be identified (Fig. 13–15). In the space left between the intrahyphal hyphae and the hypha, the following can be seen: glycogen, mitochondria with few cristae, ICMS, a membrane that resembles the endoplasmic reticulum, and few septa (Fig. 13–15). Nuclei were never observed and ribosomes only rarely. Between the plasma membrane and the intrahyphal hyphae, remnants of the ICMS form, which, if seen in three dimensions, could be a peripheral net (Fig. 14).

In some hyphae, the tip is open and a new hypha seems to squeeze through the opening (Fig. 15). In others, the opening is larger, and cytoplasmic structures can be seen outside the hyphae (Fig. 14).

DISCUSSION

The cell wall is thinner in the mycelial phase than in the yeast phase. In addition, the clear fibrillar pattern of the outer layer of the yeast phase contrasts with the finer fibrillar appearance of the mycelial phase. The inner layer is homogeneous in the yeast (7) and finely fibrillar in the mycelia. Although differences were observed in the cell wall of the yeast phase of both fungi, it was not so in the mycelia. The interpretation of

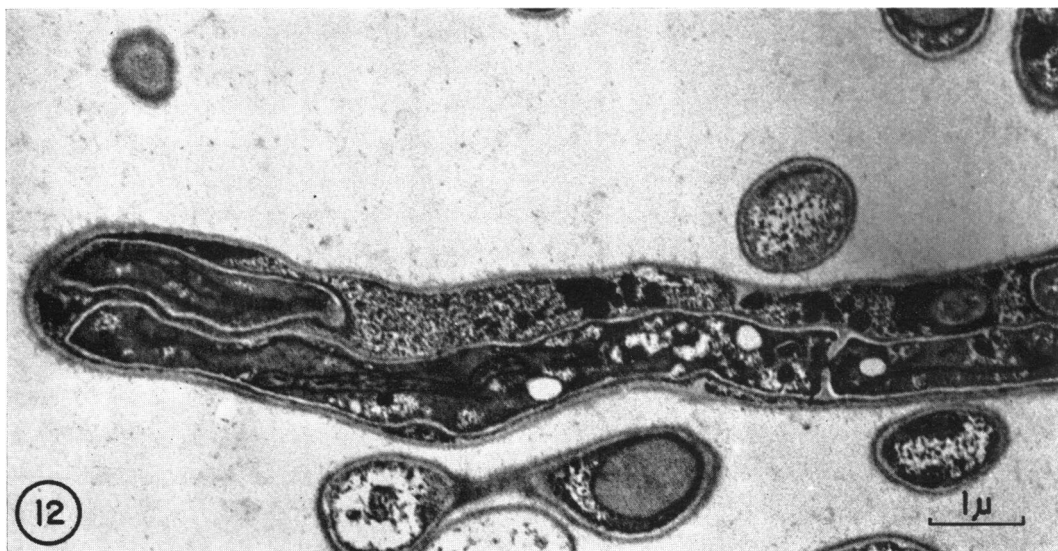


FIG. 12. Intrahyphal hyphae in *Paracoccidioides brasiliensis*. Longitudinal section of a dead hypha filled with live hyphae distributed at random. Clear halo delimits the live hyphae. KMnO_4 fixation. $\times 12,000$.

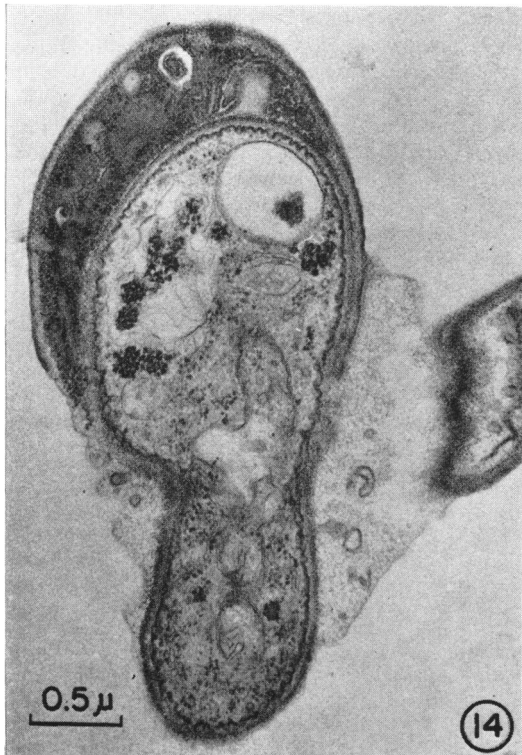
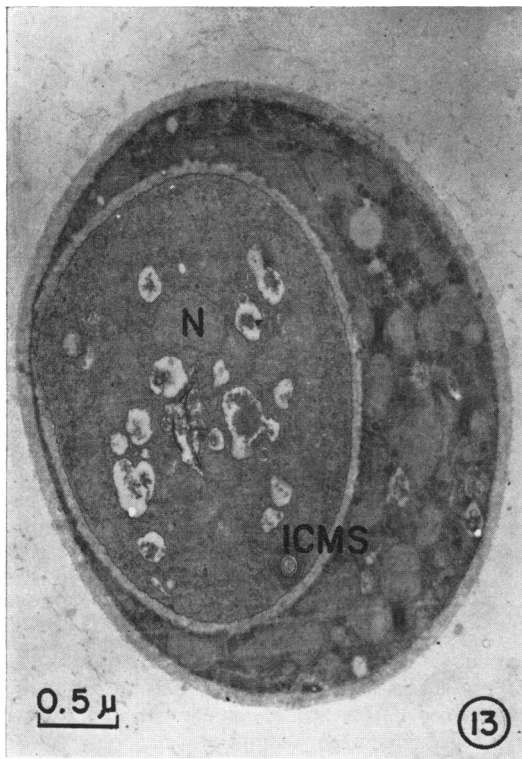


FIG. 13. Intrahyphal hyphae in *Blastomyces dermatitidis*. One live hypha is inside a dead one; in the live hypha are nuclei (N) and ICMS. Glycogen and bodies that could be mitochondrial ghosts are in the dead hypha. $\times 21,000$.

FIG. 14. Intrahyphal hyphae in *Blastomyces dermatitidis*. Cytoplasmic debris are outside the broken dead hypha. $\times 24,000$.

FIG. 15. Intrahyphal hyphae in *Paracoccidioides brasiliensis*. A live hypha entering into a dead hypha. The tip (T) of the live hypha is surrounded by a clear halo. Beginning of septum (S) formation in the live hypha. G, glycogen. $\times 26,000$.

the difference in structure of the two phases must be made cautiously. It might be that these changes correspond to gross variations in the composition of the cell wall, or to minor physical or chemical changes of a particular wall component.

It is assumed that, taxonomically, *P. brasiliensis* belongs to the family of the *Ascomycetes*, since these have a characteristic septum formation (22) which was also observed in the fungi under study. Furthermore, *B. dermatitidis* was recently classified in the family of the *Gymnoascaceae* (20).

The ICMS has been described in several human pathogenic and nonpathogenic fungi (2, 7, 9, 14, and 25). The "lomasomes" observed by Moore and McAlear (21) in several fungi could be part of the ICMS. In *Penicillium vermiculatum*, ascospore cell wall formation is closely related to lomasomes (29). In *P. brasiliensis* and *B. dermatitidis*, the cell wall material is observed attached to the plasma membrane and also to the outermost layer of the ICMS, as shown in Fig. 10 and 11. This material is never seen in direct contact with the cytoplasmic matrix, and no relation is observed between the ICMS and the nuclear membrane (19).

The term mesosome is applied in bacteria to a membranous structure that originates as an invagination of the plasma membrane which subsequently expands into the cytoplasm. The main features of mesosomes are their vesicular structures—bound by a membrane similar in appearance to the plasma membrane—and their role in cell division and septum formation (10, 15).

The ICMS in *P. brasiliensis* and *B. dermatitidis* resemble mesosomes and have similar characteristics; i.e., they originate from the plasma membrane, they form vesicles or tubules larger than those in bacteria, and they contain cell wall-like material. Furthermore, they also take part in septum formation as has been demonstrated in serial sections of mesosomes of *Bacillus megaterium* (10).

The theory that membranes from a variety of sources may be composed of subunits was strengthened by Blaise (1) in his electron microscope and X-ray diffraction study. Weir (28) recently demonstrated the presence of subunits which have light cores and dark rims. Occasionally, subunits with similar characteristics were observed in the invaginations of the plasma membrane.

Septal plugs have been described in degenerated hyphae, in hyphae undergoing degeneration, or in hyphae which have been damaged (24). In our studies, septal plugs with some adjacent Woronin bodies are frequently seen, and the

organelles on either side of the cytoplasm are generally undamaged.

The intrahyphal hyphae, which are also called intrahyphal mycelium, self-penetration (16), self-parasitism (8), endohypha (17), and "prolifera-tion interne" (23), have been observed in *Basidio-mycetes*, *Ascomycetes*, and some in *Fungi imperfecti* (8). Descriptions of these structures always associate a dead hypha with penetration and the subsequent growth of live hyphae within them. In this study, true parasitism (8) was not considered, since the work was done with pure strains.

The presence of live hyphae inside a dead one mimics the structures identified as asci and ascospores (22). These structures, that is, the perfect stage of *B. dermatitidis*, were obtained only after pairing several different strains (20). In *P. brasiliensis*, the perfect stage has not yet been obtained.

In *Neurospora crassa*, the presence of intrahyphal hyphae has been attributed to the result of the mutation which causes periodic growth (26). Sussman et al. (26) suggested that intrahyphal hyphae are probably induced by wounds or intoxication, with subsequent death of the hyphae accompanied by blockage of the septal pore. The fact that, in our material, the intrahyphal hyphae appear much more frequently in the immersed, shaken cultures than in the solid-medium cultures suggests that breakage of the hyphae could be the main factor that elicits the formation of these structures. However, the mechanism by which the live hyphae are attracted toward the dead ones, or vice versa, remains obscure.

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