Electron Microscopy of *Neurospora crassa* Mycelia*

By A. J. SHATKIN and E. L. TATUM, Ph.D.

(*From The Rockefeller Institute*)

ABSTRACT

The fine structure of vegetative mycelia of the filamentous Ascomycete, *Neurospora crassa*, has been investigated by the standard techniques of electron microscopy. Addition of uranyl nitrate to the methacrylate-embedding medium minimized disruption of the specimens—an accident often observed in the preparation of microbial material.

This report describes the presence of a chitinous polysaccharide wall containing fine fibrils embedded in a homogeneous matrix. A sinuate plasma membrane lies adjacent to the inner wall surface. This membrane is often closely associated with the endoplasmic reticulum of the cytoplasm. Numerous mitochondria of the classical type, and dense particles of 10 mμ diameter occur throughout the cytoplasm. The nuclear region is surrounded by a double membrane with pore openings. Associated with the nuclear envelope is a dense area, the nucleolus.

The significance of these observations and their relationship to other forms is discussed.

INTRODUCTION

The extensive genetic and biochemical knowledge of the bread mold, *Neurospora crassa*, justifies an investigation of the fine structure of the organism. The gross morphological features of various stages in the life cycle of the mold cell have been examined previously (1–4). This report describes the electron microscopical structure of the wild type *N. crassa* mycelium and the techniques used in the investigation.

Materials and Methods

Stock cultures were maintained on a complete agar medium. Conidia were transferred to 20 ml. liquid minimal medium (5) plus 1 per cent sucrose in 125 ml. Erlenmeyer flasks and grown for 15 to 20 hours with slow shaking at 25°C. Fixation of mycelial mats was performed in 1 per cent OsO₄ buffered with veronal acetate (pH 7.4) (6) for 2 hours at 0–4°C. After dehydration in a series of acetone solutions of increasing strength, the mycelia were placed in 100 per cent acetone for 1 hour. They were then soaked for 40 minutes in a mixture of 1 part acetone to 1 part *n*-butyl/methyl methacrylate (4:1 *v./v.*) monomer. Finally the tissue was immersed for 1½ hours in the methacrylate monomer containing 0.035 per cent uranyl nitrate (7) and 2 per cent luperco catalyst and embedded in the latter mixture in gelatin capsules (8). Polymerization was allowed to proceed at 45°C. for 12 to 24 hours.

Sections 50 to 100 mμ thick were cut with a glass knife on the Porter-Blum microtome, and micrographs obtained with the modified EMU-2c RCA electron microscope.

The usefulness of uranyl nitrate for preventing polymerization damage due to the explosion phenomenon should be emphasized. Several other techniques were employed with considerably less success. These included addition of sucrose to the fixative; use of KMnO₄ as a fixative (9), formalin, and combinations of these with OsO₄; buffer variations; soaking in monomer and in partially prepolymerized methacrylate (10); removal of air dissolved in the methacrylate (11); higher temperatures (12); and ultraviolet light for polymerization. Although epoxy (13) and polyester (14) resins did not cause rupture of the cell or cell components, they were less convenient to use.

OBSERVATIONS

The growth of *N. crassa* is known to be associated with cytoplasmic streaming toward an ad-

vancing mycelial tip, the site of new cell wall formation. Incomplete septa are formed at intervals within the growing hypha, resulting in a coenocytic condition. Wild type hyphal cells average 3 to 4 μ in diameter and may attain a length of several μ.

The fine structure of the following components: cell wall and septa, cell membrane, nuclei, mitochondria, endoplasmic reticulum, and various cytoplasmic inclusions was studied in thin sections of *Neurospora crassa* (Fig. 1).

Cell Wall.—Extensive chemical and structural studies of *N. crassa* cell wall have not been made. Such information as is available indicates that chitin is a significant constituent (15, 16). *N. crassa* cell walls are strongly positive with the periodic acid-Schiff test for polysaccharides and the chitosan test for chitin.

Electron images showed that the outer surface of the cell wall consists of a fibriform meshwork of great density (Figs. 1, 11). That these dense fibers appear to aggregate and slough off the wall surface is evidenced by the occurrence of fibrous material at distances from the cell. In a few instances internal structure in the wall was observed. Here a network of fibrils, finer and less dense than those on the outer surface, was discernible in the area where the wall usually appeared to be structureless (Fig. 2).

Attempts to stain the osmium-fixed cell wall for electron microscopy with dilute solutions of lanthanum nitrate (17), lead hydroxide (18), uranyl acetate, or phosphotungstic acid (19) were unsuccessful. However, prolonged exposure of sections to solutions of ruthenium red ($1:10^4$ w./v.) appears to increase the cell wall density.

Septa.—Light microscopic examination of hyphae before fixation often reveals more dense regions at the outer surface of the cell wall adjacent to the septa. In the electron microscope these thickenings seem to be due to increased deposition of the fibriform meshwork (Fig. 3). This is understandable when it is realized that septum formation occurs by a process of cell wall invagination and fusion. Cell wall material is laid down at the internal perimeter of the newly formed pore, the diameter of which decreases to ~ 0.2 μ, thus still permitting intercellular streaming of both cytoplasm and nuclei. In old age or when cell damage occurs, cytoplasmic continuity is interrupted by the formation of a septal pore plug (Fig. 7). The plug is composed of two bodies 60 mμ thick separated by two light layers and a central dense one, each 20 mμ thick.

Cell Membrane.—Immediately within the cell

wall lies a highly convoluted plasma membrane. This plasmalemma consists of two distinct dark layers separated by a region of lesser density, total thickness ~ 7 mμ (Fig. 4). Numerous infoldings occur at the membrane surface (Figs. 4, 11). The cell membrane may form invagination pockets which appear in sections as ellipsoid and circular profiles of varying sizes (20).

Reticular System.—In addition to the profiles of vesicles, the peripheral regions of the cell often contain long, smooth double-membrane elements. Within the cytoplasmic interior there are short tubules or vesicles of irregular profile. It is assumed that all these represent components in section of a branched, three-dimensional network of flattened vesicles and tubules. This system is homologous to the endoplasmic reticulum (21), previously described in both plant and animal cells. The reticulum divides the cytoplasm into two phases, the extravesicular one more dense and less homogeneous than the membrane-enclosed one. The variability in concentration and density of the endoplasmic reticulum suggest the possibility that its appearance may reflect its functional activity in the hypha (Fig. 5).

Dense particles of ~ 10 mμ diameter are found dispersed throughout the cytoplasm (Figs. 6, 11). These are infrequently associated with the outer surfaces of vesicles of the cytoplasmic interior. *N. crassa* is intensely basophilic and treatment with ribonuclease abolishes the staining reaction (22). The dense particles probably correspond to ribonucleoprotein particles described in animal, plant, and bacterial cells and considered to be involved in protein synthesis (23).

Mitochondria.—In structural detail, *N. crassa* mitochondria resemble those described for many organisms (24). They appear in section as elongated, circular, and irregular profiles (Fig. 6). In three dimensions they seem to be highly convoluted rods of average dimensions 0.2 to 0.3 μ by 1 to 2 μ (Fig. 6). A region of low density separates a continuous outer membrane from an inner one. The double unit has an average thickness of ~ 14 mμ. Infoldings in the inner membrane give rise to parallel internal ridges, the "cristae mitochondriales" (25). These usually lie perpendicular to the long axis of the rod-shaped organelles, but occasionally are parallel as in yeast (26). Anastomosis of some cristae does occur, but the organelle is apparently not subdivided into compartments. This structural arrangement allows increased membrane surface contact with the entire intramitochondrial matrix. The matrix contains dense granules 30 to

40 mμ in diameter and similar to those described in liver cells by Palade (24). The development of new mitochondria from pre-existing ones, rather than de novo synthesis in the hypha, is suggested by the presence of branching forms of these organelles.

The Nucleus.—Several oval, Feulgen-positive bodies 1 to 2 μ in diameter, the nuclei, occur within each cell (Figs. 1, 4, 8, 10). In transverse section the nucleus is a region bounded by a 100 A wide envelope consisting of two apposed membranes separated by a clear space. At varying intervals the nucleolemma is interrupted by 40 to 70 mμ pores which appear to be unobstructed continuities between the nucleo- and cytoplasm. Connections between the nuclear membrane and the endoplasmic reticulum have been seen occasionally.

The nuclear matrix consists of irregular regions of dense material surrounded by lighter space. Close examination of the network reveals the presence of fine, coiled fibrils (Fig. 8). In some cells the nuclei may be ellipsoid or have pseudopodal extensions. During streaming, the nucleus has a remarkable capacity for reversible deformation and is able to squeeze through septal pores one-fifth its diameter (Fig. 10).

Nucleolus.—Dense particles, ~3 mμ in diameter, surrounding an island of low density comprise a region within the nucleus which probably corresponds to the nucleolus (Fig. 9). It may occupy up to 20 per cent of the nuclear volume and is invariably attached to or associated with the nuclear envelope. La Fontaine has described a similar situation in the nucleoli of *Allium cepa* and *Vicia faba* (27).

Cytoplasmic Inclusion Bodies.—Electron opaque bodies ~1 μ in length appear in ultrathin sections (Fig. 11). These dense hexagonal crystalloids possess no apparent internal structure. The presence of a limiting membrane in these bodies is not clear. Other small particles, probably lipid or polysaccharide storage granules, are scattered in the cytoplasm.

DISCUSSION

Technical Aspects.—The investigation of the fine structure of microorganisms has been hampered by the phenomenon of cellular explosion produced during methacrylate polymerization. Concentrations of less than 0.05 per cent uranyl nitrate in the methacrylate monomer reduced polymerization damage in *N. crassa* mycelia to negligible proportions. This was reported originally by Ward for frog red blood cells and snail spermatids (7). Fur-

ther observations made in the laboratories of Porter and Palade indicate that uranyl nitrate improves the preservation of the fine structure of many cell types including onion root tips, algae, insect tissue, and myelin sheaths in mammalian nerve cells. These findings demonstrate the general usefulness of uranyl nitrate in methacrylate embedding.

Morphological Aspects.—Several points of interest concerning the fine structure of *N. crassa* merit further discussion. Although the structural nature of the chitinous polysaccharide cell wall has not been completely elucidated, the evidence indicates that it consists of a network of fine, dense fibrils embedded in a homogeneous matrix. The orientation of the fibrillar material has not been investigated. The technique of shadowing the cell wall surface with heavy metals combined with various chemical extraction procedures is being applied for a more complete analysis.

The morphological cell membrane, a region of two dense lines separated by a lighter space, probably corresponds to the physiological cell border. As described for yeast spores (28) and higher plant cells (29), the invaginations in the cell membrane of *N. crassa* may be correlated with cellular activity. These findings suggest that nutrients enter the cytoplasm, after passing through the wall, by cell membrane activity resembling pinocytosis.

The observation of continuity between the endoplasmic reticulum and nuclear membrane and the close relationship between the reticulum and cell membrane agrees with the findings in conidiophores of the deuteromycete, *Stilbum zacalox-anthum* Moore (32), in maize root cells (30), and in *Chrysanthemum segetum* (31). Connections between the nucleus and cytoplasm through nuclear pores have been described for many cell types (33) including the fungi, *Coccidioides immitis* (34) and *Allomyces macrogynus* (35). That such nucleo-cytoplasmic communications are probably significant in genetic control of cellular metabolism is obvious.

The mitochondria of *N. crassa* appear to offer no striking differences from those previously described in other organisms (36, 37). Infrequent mitochondrial figures in which discontinuities in the outer membrane occur are probably polymerization artifacts.

The relationship between cell type and numbers of free vs. membrane-bound ribonucleoprotein particles has been pointed out by Palade (38). Vegetative wild type *N. crassa* as studied here are actively synthesizing cellular constituents and con-

tain most of the dense particles free in the cytoplasm. This agrees with the findings in embryonic and other rapidly proliferating cells of both animal and plant origin (31).

This report demonstrates the feasibility of applying the technique of electron microscopy to the examination of *N. crassa*. An extension of these techniques to a study of several problems of biochemical and genetic interest in this organism would seem to offer promising possibilities. Such problems include investigations of the *poky* strains (39) and the environmentally induced (40, 41) and genetic colonial forms (42).

BIBLIOGRAPHY

1. Buller, A. H. R., Researches on Fungi, London, Longmans, Green and Company, 7 vols., 1909–1950.
2. Dodge, B. O., *J. Agric. Research*, 1927, **34**, 1019.
3. McClintock, B., *Am. J. Bot.*, 1945, **32**, 671.
4. Bakerspiegel, A., *Am. J. Bot.*, 1959, **46**, 180.
5. Vogel, H. J., and Bonner, D. M., *Microbial Genetics Bull.*, 1956, **13**, 43.
6. Palade, G. E., *J. Exp. Med.*, 1952, **95**, 285.
7. Ward, R. T., *J. Histochem. and Cytochem.*, 1958, **6**, 398.
8. Newman, S. B., Borysko, E., and Swerdlow, M., *J. Research Nat. Bur. Standards*, 1949, **43**, 183.
9. Luft, J. H., *J. Biophysic. and Biochem. Cytol.*, 1957, **2**, 799.
10. Borysko, E., and Sapra nauskas, P., *Bull. Johns Hopkins Hosp.*, 1954, **95**, 68.
11. Moore, D. H., and Grimley, P. M., *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 255.
12. Borysko, E., *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, No. 4, suppl., 3.
13. Glauert, A. M., Rogers, G. E., and Glauert, R. H., *Nature*, 1956, **178**, 803.
14. Ryter, A., and Kellenberger, E., *J. Ultrastruct. Research*, 1958, **2**, 200.
15. Blumenthal, H. J., and Roseman, S., *J. Bact.*, 1957, **74**, 222.
16. Glaser, L., and Brown, D. H., *J. Biol. Chem.*, 1957, **228**, 728.
17. Mayall, B. H., and Robinow, C., *J. Appl. Bact.*, 1957, **20**, 333.
18. Watson, M. L., *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 475.
19. Watson, M. L., *J. Biophysic. and Biochem Cytol.*, 1958, **4**, 727.
20. Girbardt, M., *Arch. mikrobiol.*, 1958, **28**, 255.
21. Porter, K. R., Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1945, **81**, 233.
22. Tsuda, S., personal communication.
23. Microsomal Particles and Protein Synthesis, (R. B. Roberts, editor), New York, Pergamon Press, 1958.
24. Palade, G. E., *in* Enzymes: Units of Biological Structure and Function, New York, Academic Press, Inc., 1956, 185.
25. Palade, G. E., *Anat. Rec.*, 1952, **114**, 427.
26. Agar, H. D., and Douglas, H. C., *J. Bact.*, 1957, **73**, 365.
27. La Fontaine, J. G., *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 229.
28. Hashimoto, T., Conti, S. F., and Naylor, H. B., *J. Bact.*, 1958, **76**, 406.
29. Buvat, M. R., and Lance, A., *Compt. rend. Acad. sc.*, 1957, **23**, 2083.
30. Whaley, W. G., Mollenhauer, H. H., and Kephart, J. E., *J. Biophysic. and Biochem. Cytol.*, 1959, **5**, 501.
31. Lance, A., *Compt. rend. Acad. sc.*, 1957, **3**, 352.
32. McAlear, J. H., and Edwards, G. A., *Exp. Cell Research*, 1959, **16**, 689.
33. Watson, M.L., *J. Biophysic. and Biochem. Cytol.*, 1955, **1**, 257.
34. O'Hern, E. M., and Henry, B. S., *J. Bact.*, 1956, **72**, 632.
35. Turian, G., and Kellenberger, E., *Exp. Cell Research*, 1956, **11**, 417.
36. Palade, G. E., *J. Histochem. and Cytochem.*, 1953, **1**, 188.
37. Tsuda, S., *Indian Phytopath.*, 1955, **8**, 83.
38. Palade, G. E., *in* Microsomal Particles and Protein Synthesis, (R. B. Roberts, editor), New York, Pergamon Press, 1958, 36.
39. Haskins, F. A., Tissieres, A., Mitchell, H. K., and Mitchell, M. B., *J. Biol. Chem.*, 1953, **200**, 819.
40. Tatum, E. L., Barratt, R. W., and Cutter, V. M., *Science*, 1949, **109**, 509.
41. Shatkin, A. J., *Tr. New York Acad. Sc.*, 1959, **21**, 446.
42. Barratt, R. W., and Garnjobst, L., *Genetics*, 1949, **34**, 351.

EXPLANATION OF PLATES

PLATE 210

FIG. 1. Micrograph of a longitudinal section through two *N. crassa* cells partially separated by a septum (s). The cell wall (*w*) which encloses the cell membrane (*cm*) consists of a dense, frayed fibrous coat (*f*) on the outer surface of an apparently homogeneous material of low density. Note the accumulation of mitochondria (*m*) adjacent to and apparently streaming through the septal pore (*p*). Close to the envelope (*nm*) of the nucleus (*N*) is the nucleolus (*Nu*). The cytoplasm contains a particulate component (P_1) and numerous tubules and vesicles (*er*). × 47,000.
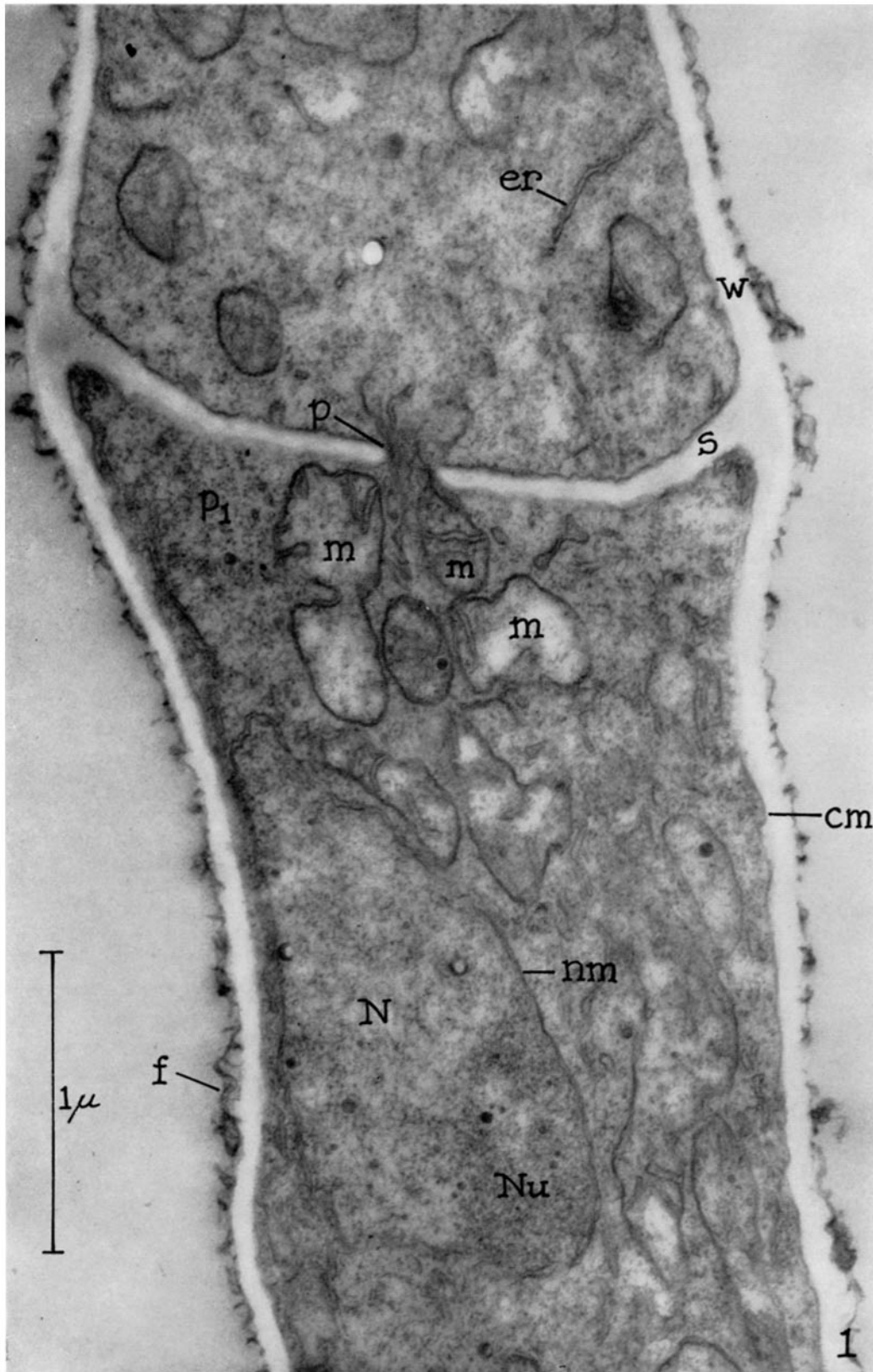
PLATE 211

FIG. 2. Image of hypha of mutant B110 in which a fine network of fibers (*f*) is evident in the usually homogeneous cell wall. Continuity between this and the dense frayed outer surface of the wall may be seen at arrows. Note the extensive sloughing of the outer wall surface. × 43,000.

FIG. 3. Micrograph of hypha in the process of septum formation. The fibriform meshwork is more extensive at the sites of cell wall invagination. × 21,000.
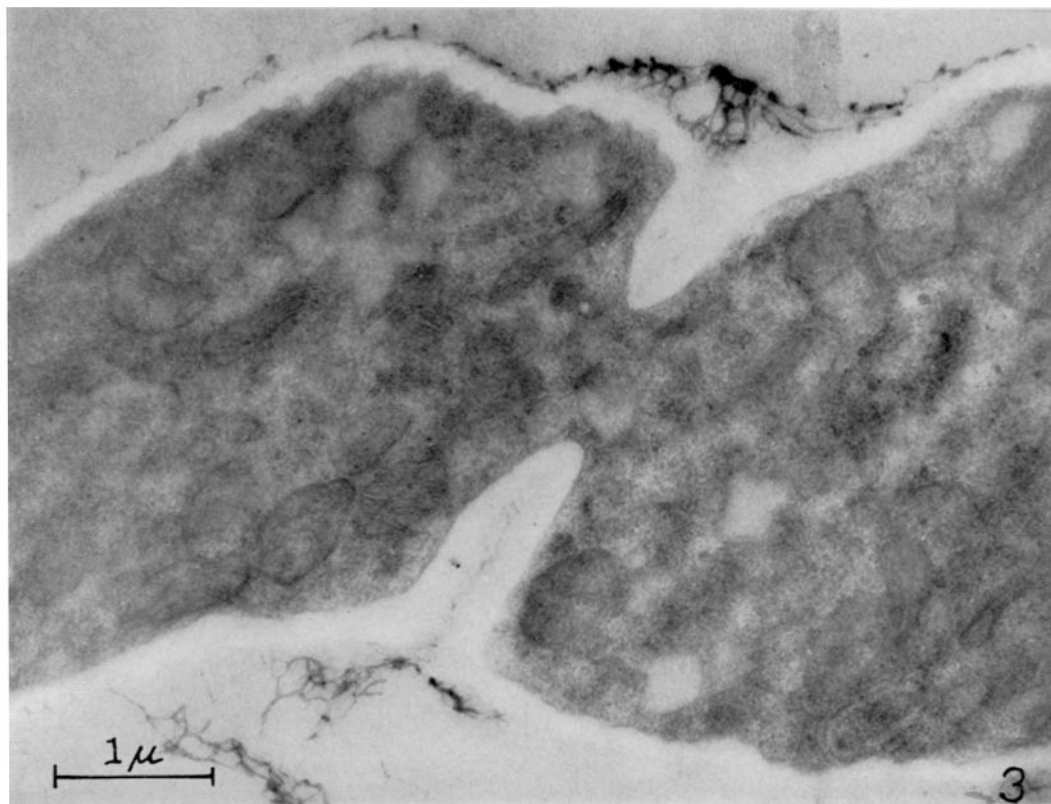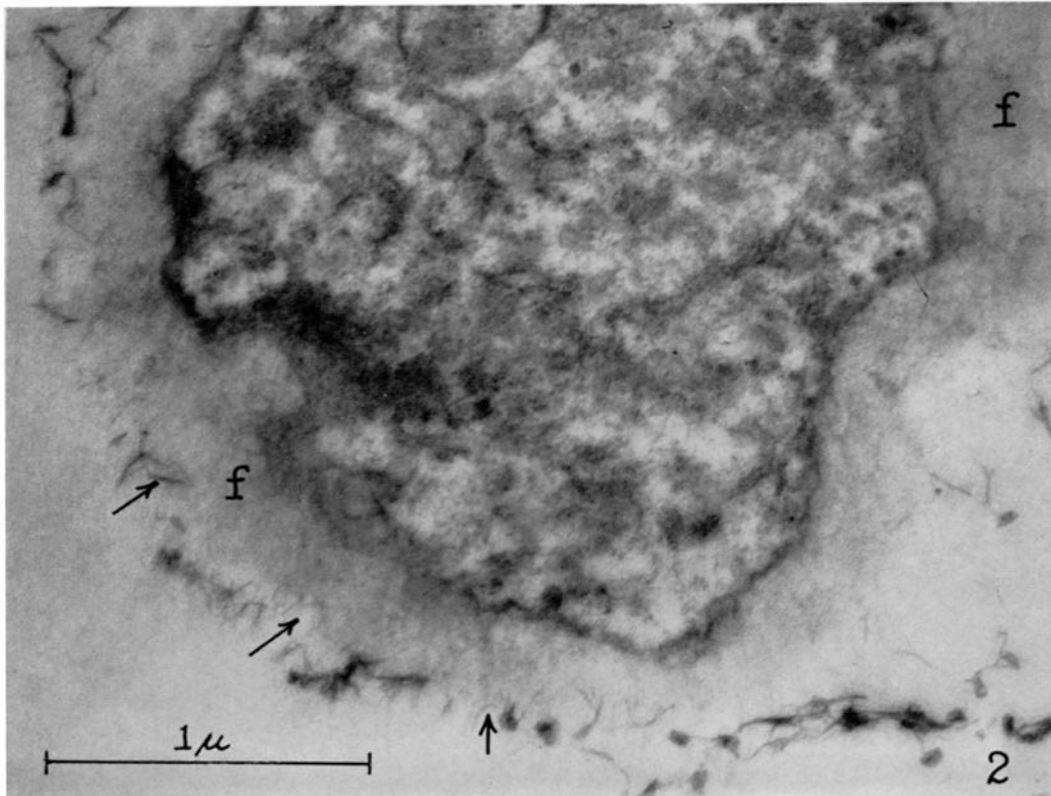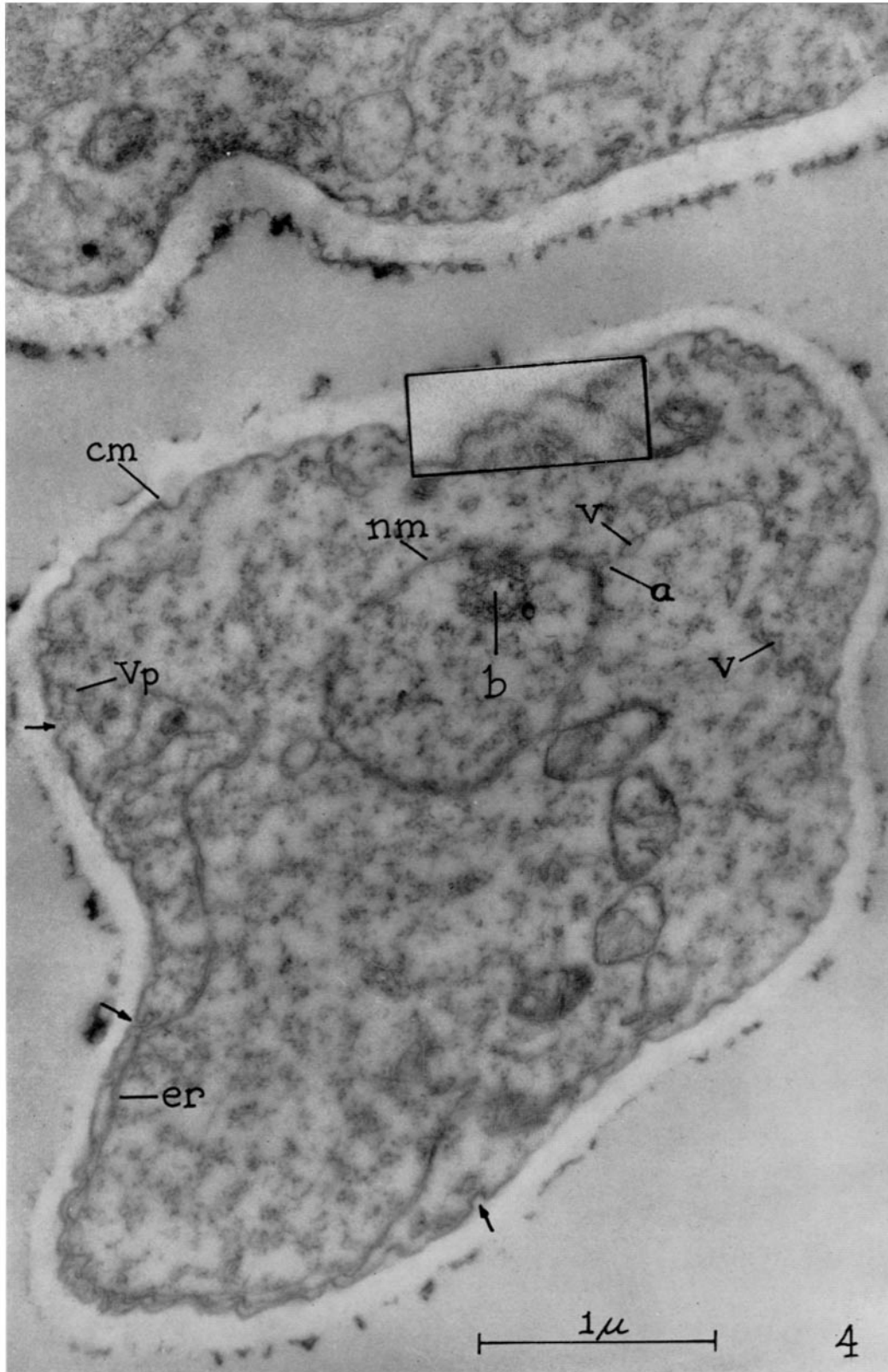
(Shatkin and Tatum: *Neurospora crassa* mycelia)

PLATE 212

FIG. 4. Active cell as seen in cross-section. The cell membrane (*cm*) appears as two dense layers enclosing a light space (see enlargement). Many invaginations occur in the cell membrane (arrows). Profiles of vesicles (*v*), some with 10 mμ particles attached to the outer surface (*Vp*), are distributed throughout the cytoplasm. Paralleling a portion of the plasmalemma is a long profile of the endoplasmic reticulum (*er*). The nuclear membrane (*nm*) is continuous with a small part of the endoplasmic reticulum at *a*. Within the dense nucleolus is a region of lower density (*b*). $\times$ 37,000.

PLATE 213

FIG. 5. Image of longitudinal section through a cell with extensive endoplasmic reticulum. Interconnecting channels of reticular elements (er) between cytoplasmic arms (C) form a highly branched system which extends from the surface of the cell (a) to the cytoplasmic interior (b). $\times$ 40,000.
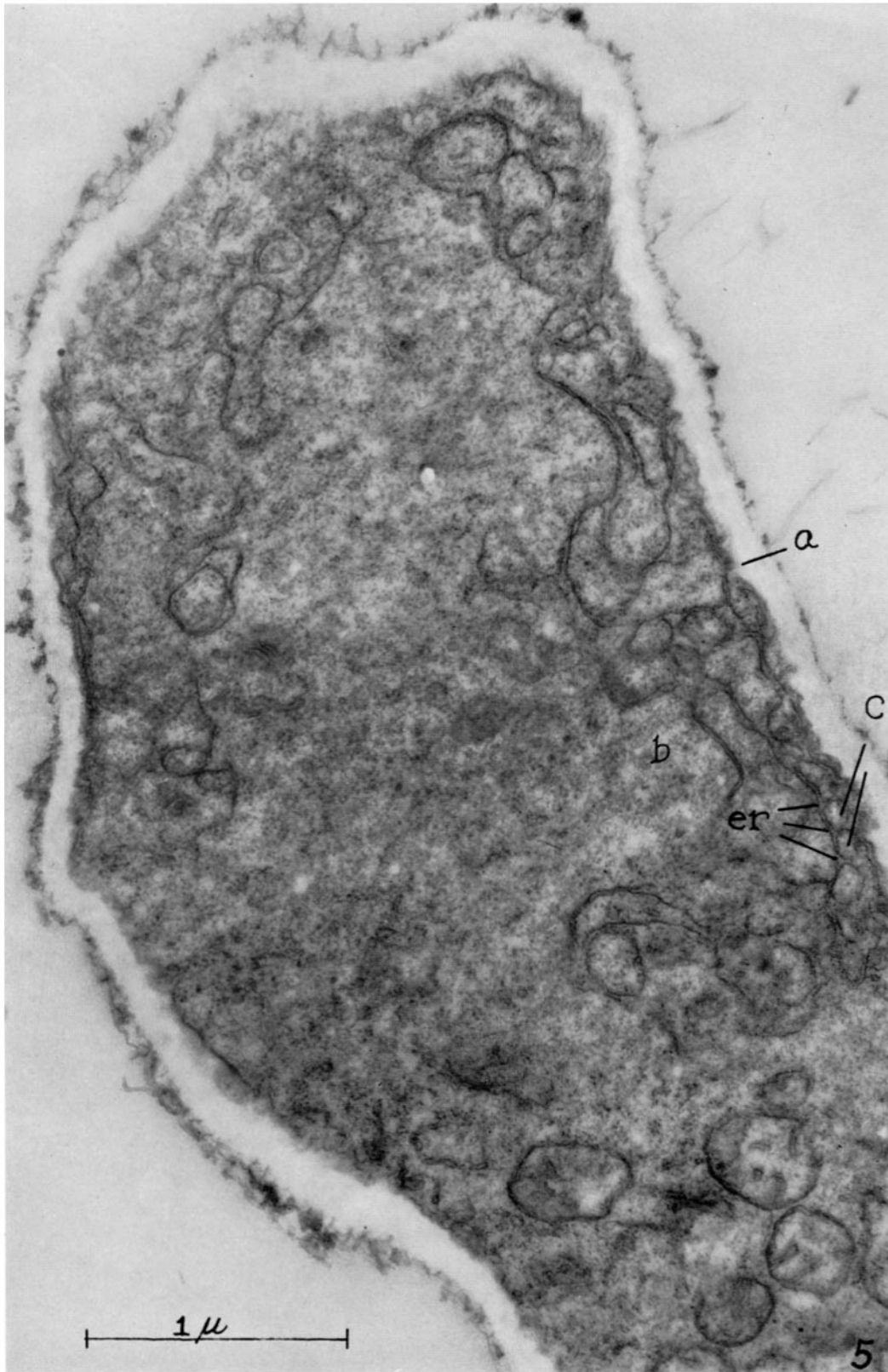
(Shatkin and Tatum: *Neurospora crassa* mycelia)

PLATE 214

FIG. 6. Cross-section through hypha with numerous mitochondria (*m*). The cristae may be discerned as continuous with the inner membrane of the mitochondria (see inset). Within the mitochondrial matrix are dense granules (*g*). The cytoplasm is densely populated with a free particulate component (P_1). $\times$ 42,000.

FIG. 7. Medial section of hypha in which the septal pore has been closed by the formation of a dense plug. Five distinct layers compose the plug. $\times$ 48,000.
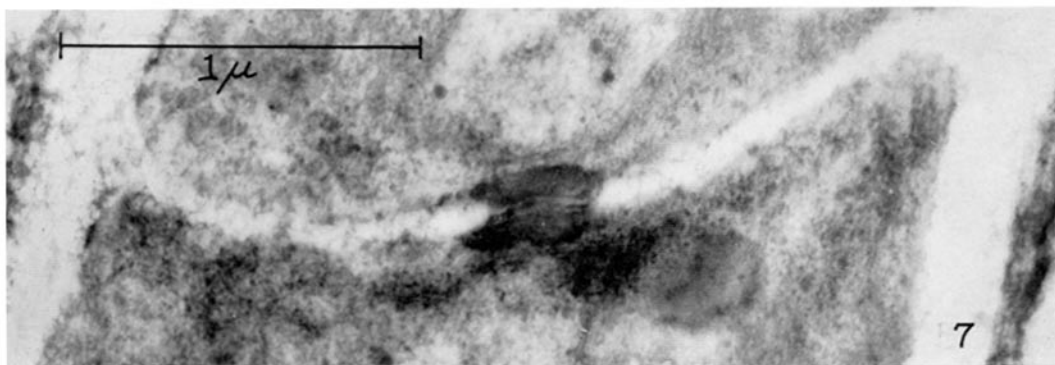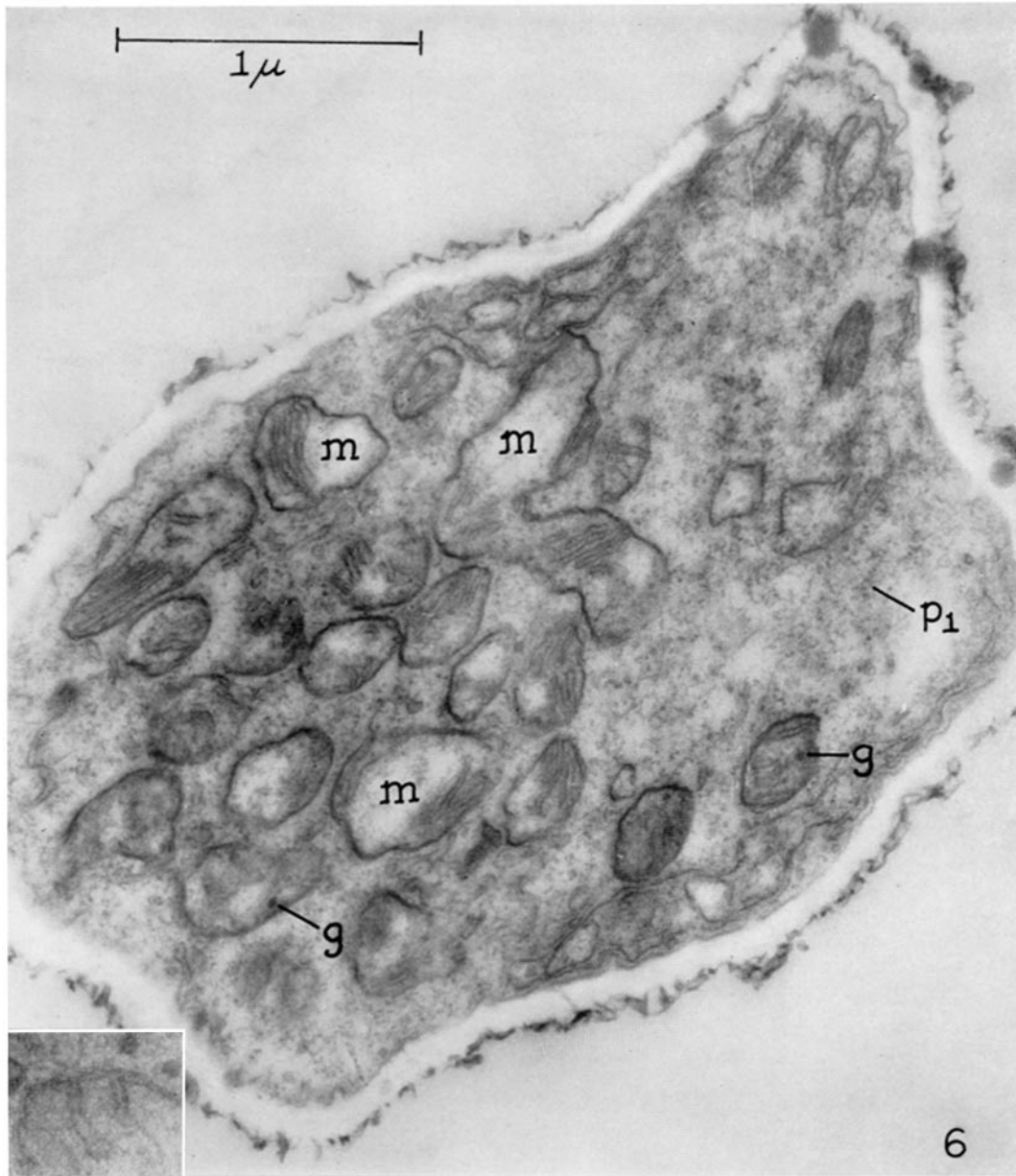
(Shatkin and Tatum: *Neurospora crassa* mycelia)

PLATE 215

FIG. 8. Micrograph of transverse section through the nuclear membrane (nm) with pores (arrows) of 40 to 70 mμ at varying intervals. Arrays of dense particles in the nucleoplasm may be traced among the areas of lower density. Note the coiled fibrils extending from a common centre, a. × 58,000.

FIG. 9. Portion of nucleus in which the nucleolus (Nu) is a region closely associated with the nuclear membrane (nm). Particles (P₂) of ∼3 mμ are scattered throughout the dense part of the nucleolus but do not occur in the light spaces (a). Stained 1½ hours with 1 per cent lanthanum nitrate in aqueous solution. × 103,000.
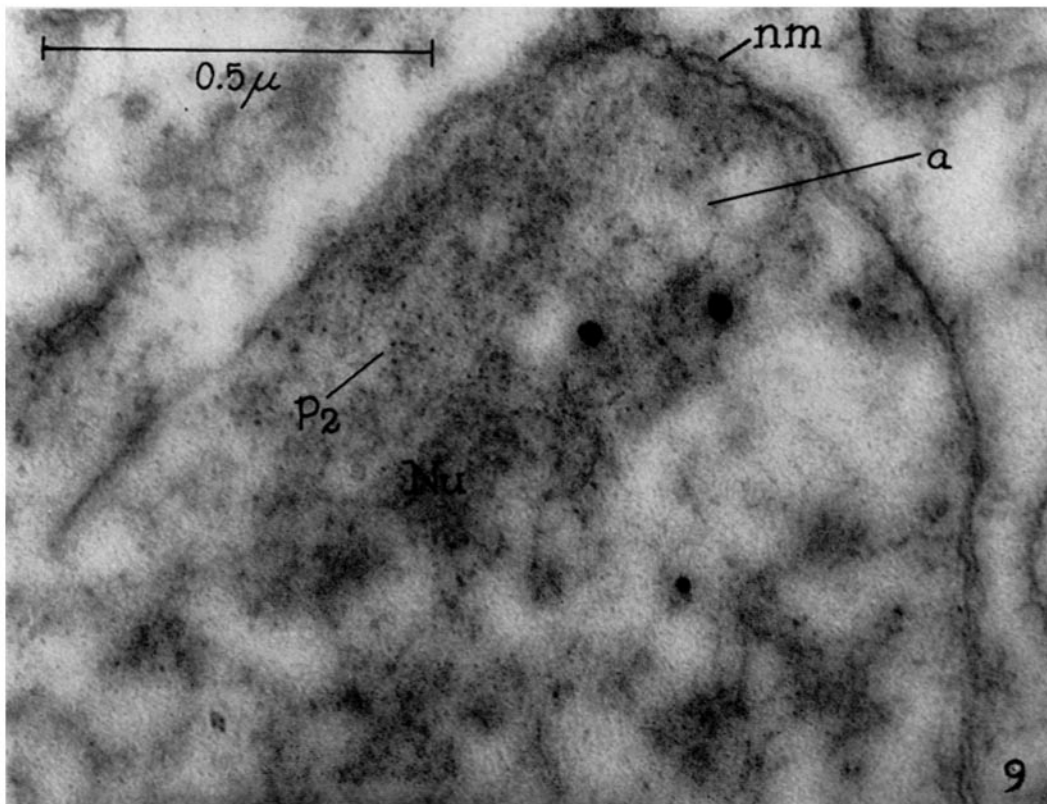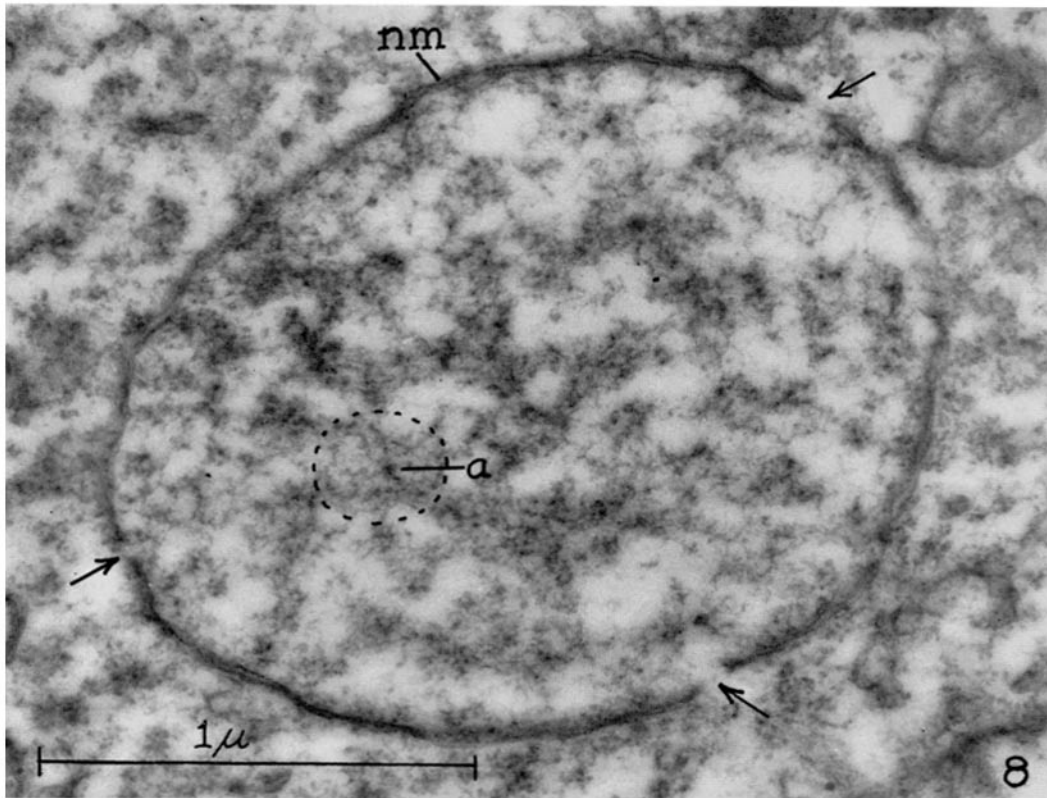
PLATE 216

FIG. 10. Medial section of hypha and pore through which the nucleus (N) presumably was in the process of streaming when the hypha was fixed. Plasticity of the nucleus is illustrated by this micrograph. × 37,000.

FIG. 11. Image of a hexagonal crystalloid cytoplasmic inclusion body (h). The periphery exhibits higher electron density than the internal areas. Some indication of a limiting membrane may be seen at a. The fibriform mesh-work (f) of the outer cell wall surface is extensive. At the cell membrane surface are invaginations which seem to be tubular (t). Cytoplasmic 10 mμ particles (P_1) are diffusely scattered. Openings in the outer mitochondrial membrane at b are probably polymerization artifacts. In an adjacent cell (c) sectioned tangentially to the cell membrane surface, folds in the plasmalemma appear as parallel layers of double membranes (dm). × 57,000.

(Shatkin and Tatum: *Neurospora crassa* mycelia)