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ULTRASTRUCTURAL CHANGES DURING SPORANGIUM FORMATION AND ZOOSPORE DIFFERENTIATION IN BLASTOCLADIELLA EMERSONII[1]

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ABSTRACT

Samples from synchronized cultures of *Blastocladiella emersonii* were examined by electron microscopy from the late log phase to the completion of zoospore differentiation. Log-phase plants contain the usual cytoplasmic organelles but also have an unusual system of large tubules ca. 45 mμ diam that ramify in organized bundles throughout the protoplast. After induction, zoosporangium differentiation requires a 2-hr period in which the nuclei divide, a cross wall forms to separate the basal rhizoid region, and an apical papilla is produced. Nuclear division in *B. emersonii* is intranuclear with a typical microtubular spindle apparatus and paired, unequal, extranuclear centrioles at each pole. The papilla is formed by a process of localized cell wall breakdown and deposition of the papilla material by secretory granules. Differentiation of zoospores begins when one of the two centrioles associated with each nucleus elongates to form a basal body. The flagella fibers arise from the basal body and elongate into an expanding vesicle formed by the fusion of small secondary vesicles. The cleavage planes are formed by fusion of vesicles similar to those associated with flagellum initiation. When cleavage is complete, each sporangium contains ca. 250–260 uninucleate spore units with their flagella lying in the cleavage planes. Probable fusion of mitochondria to produce the single mitochondrion of the zoospore occurs after cleavage; the mitochondrion does not take its position around the basal body and rootlets until just before zoospore release. The ribosomal nuclear cap is organized and enclosed by a membrane formed through fusion of many small vesicles during a short period near the end of differentiation.

THE GENERAL details of fungal fine structure have been well covered in recent reviews (Hawker, 1965; Moore, 1965; Bracker, 1967). Relatively little work has been reported, however, on the lower aquatic fungi, which, in contrast to the higher groups, can usually be fixed well with osmium tetroxide alone, or in combination with glutaraldehyde. Two published research papers on aquatics have represented systematic studies of development; the excellent reports on gamete differentiation in *Allomyces macrogynus* by Blondel and Turian (1960), and flagella development in gametangia of *A. arbuscula* by Renaud and Swift (1964). Zoospore differentiation in *A. javanicus* has been reported in summary form by Moore (1964b, c; 1965), but the micrographs

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have not yet been published. So far as we are aware, the initial fine structural events through which log-phase plants are converted to zoosporangia (or gametangia) have not been described.

The basic ultrastructural organization of the zoospore produced by *Blastocladiella emersonii* Cantino and Hyatt was originally described by Cantino et al. (1963), and this work was extended by the isolation and characterization of the distinctive nuclear cap by Lovett (1963). Some additional structural details have recently been reported by Reichle and Fuller (1967). As part of a continuing study of the biochemical basis for development in this fungus, we have also examined the ultrastructural changes that accompany the formation and germination of *B. emersonii* zoospores in synchronized cultures. In this paper we discuss the process of sporangium formation and zoospore differentiation in post-exponential phase plants.

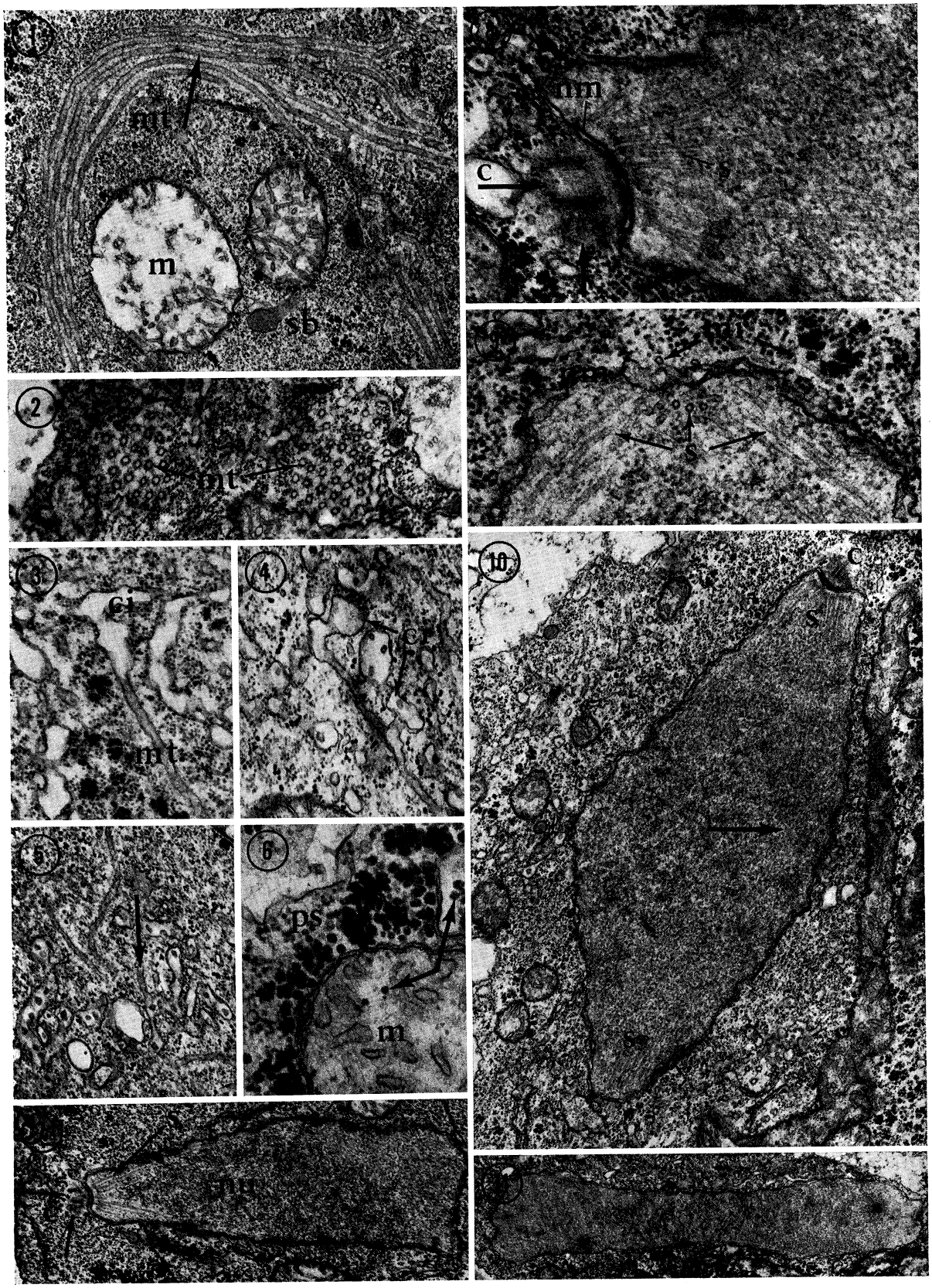
MATERIALS AND METHODS—The procedures used for growing *B. emersonii*, and for the induction of synchronous zoospore differentiation have been described elsewhere (Murphy and Lovett, 1966; Lovett, in press). Most of the micrographs to follow were prepared from a single culture with an estimated synchrony of 90%. A second culture with 95% synchrony was used to obtain material during the exponential growth phase. With the latter, samples were removed for fixation at 30-min intervals between 12 and 14 hr. For the differentiation sequence in the first culture, similar samples were removed every 15 min from 16 to 17 hr, and then every 12 min until zoospores were released. Beginning at 16 hr, 1 ml of culture was also removed and fixed every 30 min for the estimation of culture synchrony.

For electron microscopy 5–7 ml-samples were immediately pipetted into a test tube containing 1 ml of cold 2% glutaraldehyde in *p*H 7.8 veronal acetate buffer (0.018 M sodium veronal, 0.018 M sodium acetate) to prefix. After a 3–4-min centrifugation, the supernatant was decanted and the thin pellet of plants fixed for 1 hr in the 2% buffered glutaraldehyde (Chrispeels and Vatter, 1963). The glutaraldehyde was followed by a 1-hr post-fixation in 1% osmium tetroxide in the same *p*H 7.8 buffer containing 0.001 M $CaCl_2$. After fixation the pellets were dehydrated by passage through a graded series of ethanol solutions containing 1% $MgCl_2$ and finally absolute ethanol. The ethanol was replaced by propylene oxide, and the dehydrated material was embedded in a plastic mixture consisting of 15 ml Araldite 6005, 25 ml Epon 812, 55 ml dodecyl succinic anhydride, 3 ml dibutyl phthalate, and 1.5 ml 2,4,5-tri (dimethylaminomethyl) phenol (DMP-30) (Mollenhauer, 1964). All steps except the infiltration and embedding were conducted at 2–4 C. Sections were cut with glass knives on a Cambridge-Huxley ultramicrotome. Gold and silver sections were mounted on collodion-covered grids and the sections stained for 10–15 min in an alkaline solution of lead citrate (Reynolds, 1963). The sections were examined and photographed in a Philips EM-200 electron microscope. Figures 50, 51, 53, 54 were prepared from material embedded in Vestopal and were not stained.

RESULTS—The differentiation induced by the change in medium takes place in two distinct phases, the presporulation modification of the plant to form a multinucleate zoosporangium and the subsequent formation of the individually organized uninucleate zoospores. The results will deal with three separate stages: the structure of the cytoplasm in log-phase plants before 15½ hr, sporangium formation between 16 and 18 hr, and zoospore differentiation from 18 to 19 hr.

Log-phase plants—The cytoplasm of growing plants contains many nuclei with large granular nucleoli; numerous mitochondria; occasional, dense and amorphous bodies which appear to be lipid globules; more numerous, smaller, irregularly shaped granules with moderately dense granular contents surrounded by a single-layered membrane (*sb*, Fig. 1); large numbers of polysaccharide granules; and densely packed ribosomes (Fig. 1). A moderate amount of irregularly organized endoplasmic reticulum occurs and appears mainly to be of the smooth type with little conspicuous association with ribosomes. Many vacuoles are present but vary in number and size from plant to plant. The cytoplasm in the basal area near the rhizoids usually has a rather ragged appearance because of a higher concentration of small vacuoles and a paucity of ribosomes (Fig. 12). The latter condition, plus unusually long mitochondria, is also characteristic of the anucleate rhizoids. The polysaccharide granules occur both as smaller β-particles (Revel, 1964) of 250–300 A diam and as aggregates of these in rosettes or α-particles (Fig. 6). The polysaccharide granules are frequently clustered around mitochondria and occasionally occur in very large aggregates; particles which resemble the smaller β-particle are also found in some mitochondria (Fig. 6).

A striking feature of the cytoplasm in growing plants is the presence of large numbers of tubular structures. Most of these are found in organized bundles that wind and branch throughout the cytoplasm (Fig. 1, 2). The individual tubules have a diam of approximately 45 mμ and appear to be membranous structures with some additional fine material attached to the outer surface. In some longitudinal sections the lumen of the tubules has a distinctly higher density than the immediately surrounding area within the bundle. Because of their large size relative to microtubules,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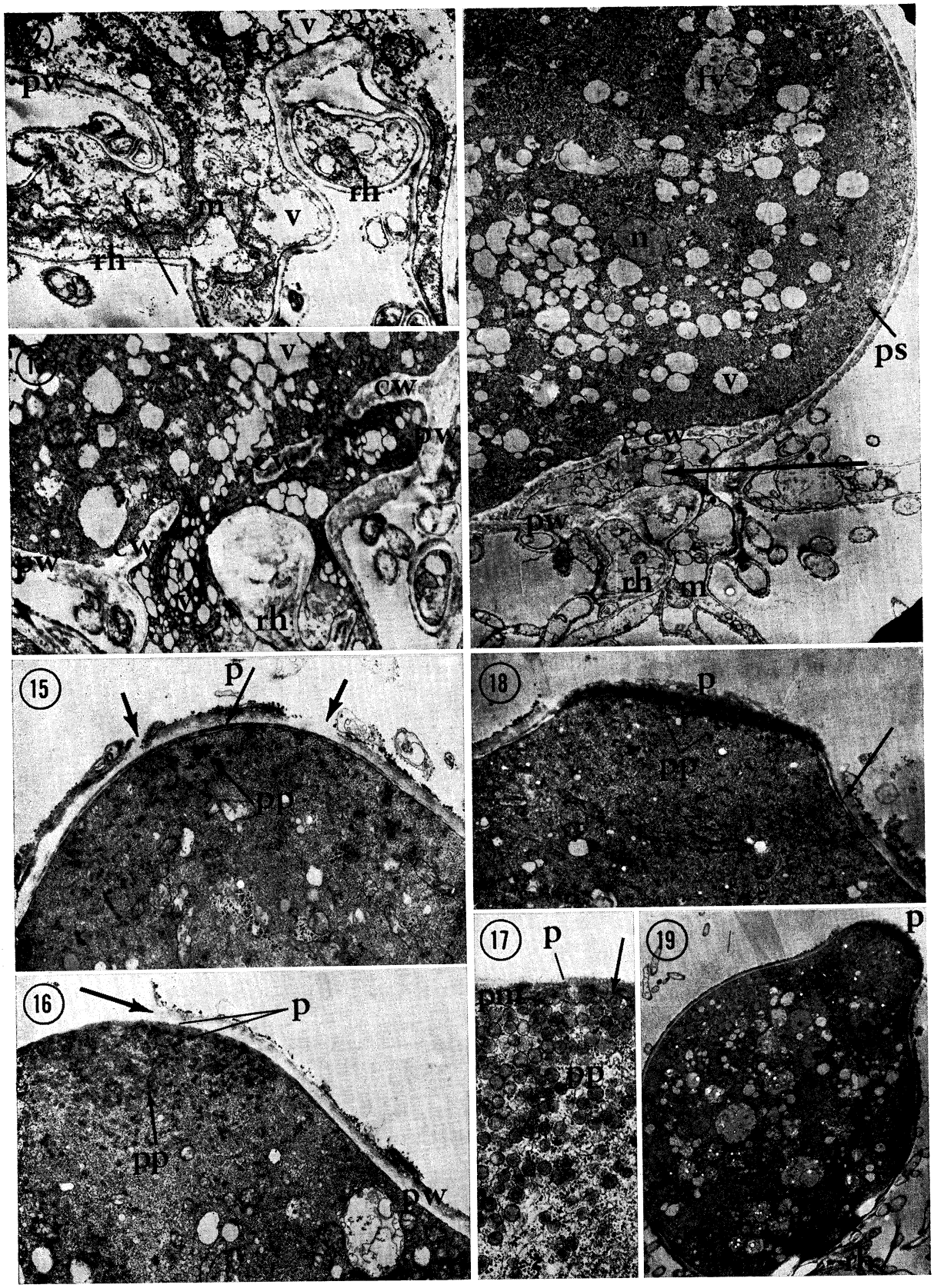
we will refer to them as "macrotubules." The well-organized bundles of macrotubules are not surrounded by a limiting membrane but are nearly devoid of ribosomes or other particles between the constituent tubules. Cross sections of bundles (Fig. 2) show a regular (ca. 115 mμ center to center) spacing with each inner macrotubule lying in the center of a hexagonal array. The number of tubules per group ranges from as few as 2 or 3 to 30 or more. The bundles of macrotubules show considerable irregular branching as they ramify through the cytoplasm and give the impression of forming a more or less continuous network. Occasional sections have shown macrotubules terminating in irregular vesicles or cisternae (Fig. 3, 4, 5). The bundles gradually lose their organization and the macrotubules disappear during the subsequent differentiation of the sporangium and spores.

Sporangium formation—Immediately after the change of medium at 16 hr, the cytoplasm of the plants appears similar to that during the growth phase. At approximately 16½ hr a round of nuclear division starts, and the formation of a cross wall to separate the sporangium from the rhizoids begins.

Nuclear division—Somatic nuclear division takes place within the intact nuclear membrane and the granular nucleolar material persists in a diffuse state throughout the division (Fig. 7, 10, 11). Paired centrioles are found at each pole of the dividing nucleus with their axes oriented at right angles to each other (Fig. 7, 8). The larger centriole lies outside the nuclear membrane in the center of a depression directly opposite the apex of the endonuclear spindle apparatus. The second small centriole is always found at right angles and to one side of the longer one. The spindle fibers appear to arise just within the nuclear membrane (Fig. 7–10). In some sections they can be traced beyond the center of the dividing nucleus (Fig. 10) where they may cross fibers from the opposite pole. The fibers are microtubules with a circular cross-sectional profile and a diam of ca. 20 mμ (Fig. 9). Separation of the daughter nuclei occurs by considerable elongation followed by fusion of the nuclear membrane in the isthmus. Figure 11 illustrates an intermediate stage of elongation with the typical dumbbell shape, which can also be observed in the light microscope. The nucleoli immediately re-form in the daughter nuclei. The nucleus in Fig. 7 from a 13-hr log-phase plant is included to show the distinct particulate nucleolar material and to demonstrate the presence of the paired centrioles in growing plants. All of the nuclei in a plant divide in a nearly synchronous fashion between 16½ and 17½ hr to yield the final average number of ca. 250–260 per plant (Murphy and Lovett, 1966).

Septation—Cross wall formation at the base of the plant begins soon after the start of nuclear division and is also complete by about 17½ hr. This involves two separate processes, the loss of cytoplasmic material from the region of the anucleate rhizoids and the deposition of the cross wall itself. Actual movement of the cytoplasmic material is difficult to document. There is, however, a distinct change in the progression from the low density of ribosomes and other particles in the rhizoids of the log-phase plant (Fig. 12) to the complex, crowded situation in the area of rhizoid attachment during cross wall formation (Fig. 13) and the essentially empty condition of the rhizoids after the septum is complete (Fig. 14). In the formation of the basal cross wall the wall material is deposited first on the outer plant wall and then progressively inward (Fig. 13). The closure of the cross wall results in the separation of the upper multinucleate sporangium and a small basal "cell" with contiguous rhizoids (Fig. 14). Although the plasma membrane persists, the rhizoids contain little but vacuoles and a few mitochondria. Large numbers of vacuoles also appear during sporangium differentiation and give the cytoplasm a distinctly coarse appearance (Fig. 14).

Fig. 1–11.—Fig. 1. A longitudinal section through a bundle of macrotubules (mt) at 13 hr. m, mitochondria; sb, side-body granules, ×18,800.—Fig. 2. Cross-sections of macrotubular bundles. Each bundle contains ca. 30 macrotubules (mt). 16 hr, ×23,000.—Fig. 3–5. Macrotubules connected to cisternae.—Fig. 3. A tubule (mt) terminating in an empty cisternum (ci). 16 hr 15 min, ×65,700.—Fig. 4. A macrotubule connected to two cisternae (ci), one with small dense granules. 18 hr, ×73,800.—Fig. 5. An irregular cisternum with granules connected to a macrotubule (arrow). 19 hr, ×22,700.—Fig. 6. Intracellular polysaccharide. A cluster of polysaccharide (ps) granules next to a mitochondrion (m). Similar particles are in the mitochondria (arrows). 18 hr, ×58,000.—Fig. 7. Nuclear division in a growth-phase plant. One-half of a mid-division nucleus with a well-organized spindle (s), two unequal centrioles (c, arrows), and prominent granular nucleolar (nu) material. A small granule lies at the base of the larger centriole. 13 hr, ×16,100.—Fig. 8. Paired extranuclear centrioles. Nuclear division during sporangium formation to show the paired centrioles (arrows) and intranuclear spindle fibers (s); nm, nuclear membrane. 17 hr, ×52,800.—Fig. 9. Microtubular spindle fibers. The arrows indicate spindle fibers (s) cut in longitudinal and cross-section. Extranuclear microtubules (mi) may represent rudimentary astral rays. nm, nuclear membrane. 17 hr 24 min, ×60,800.—Fig. 10. Long section of dividing nucleus. Note the intact nuclear membrane, part of one centriole (c), numerous spindle fibers (s) at each pole, and the fibers which cross (arrow) in the center of the nucleus. 17 hr 24 min, ×17,600.—Fig. 11. Nuclear elongation before separation of daughter nuclei. 17 hr, ×7,500.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Papilla formation—Each plant forms a terminal discharge papilla between the 17th and 18th hour. At the early stage shown in Fig. 15 there is some evidence of plant wall breakdown at two points. At the same time a thin layer of denser material can be seen forming within the wall, just outside the plasma membrane. Within the cytoplasm below the plasma membrane numerous membrane-enclosed granules of ca. 150–300 mμ diam can be seen to contain material of identical appearance. At a later stage part of the wall has completely disappeared, the band of dense "papilla material" has increased in thickness, and the cytoplasmic granules are present in great numbers (Fig. 16, 17). Continued dissolution of the wall and additional deposition of papilla material result in the mature lens-shaped papilla (Fig. 18, 19), after which the cytoplasmic granules disappear. Maturation of the papilla completes the differentiation of the zoosporangium.

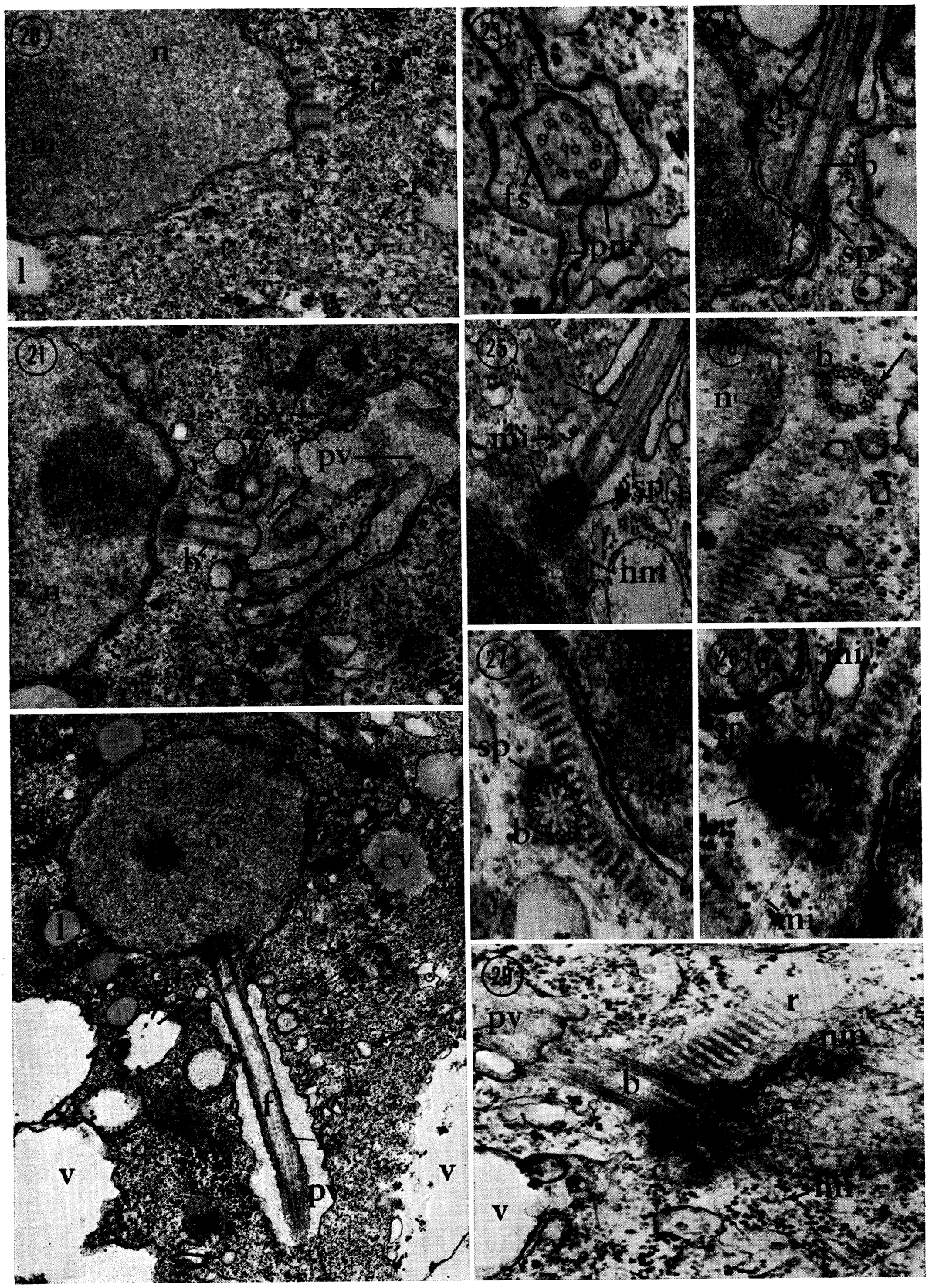
Zoospore differentiation—In the mature sporangium at 18 hr the nuclei are surrounded by numerous spherical, refractile, lipid globules, in a condition described by Blondel and Turian (1960) as the "lipid crown" in *Allomyces* gametangia (Fig. 30). Unlike *Allomyces*, a second type of granule, the unidentified *sb* granules present in the log phase, also surround the nuclei at this time (Fig. 22).

Flagellum formation—The section in Fig. 20 shows a lipid crown stage nucleus with two unequal centrioles lying in shallow depressions of the nuclear membrane. The smaller centriole (ca. 85 mμ long) does not change in size or structure during spore differentiation. The larger centriole (ca. 150 mμ long) elongates to become the basal body (ca. 450–500 mμ long by 190 mμ diam) which subsequently gives rise to the fibers of the zoospore flagellum (Fig. 21). When the basal body begins to produce flagellum fibers from its distal end, a new type of vesicle appears in the nearby cytoplasm. These vesicles, easily recognized by their finely reticulate contents (Renaud and Swift, 1964), fuse to enclose the flagellar fibers as they elongate. The result is a continuously expanding vesicle (Fig. 21, 22). The inner unit membrane of this vesicle (the "primary flagellar vesicle" of Renaud and Swift, 1964) forms the flagellar sheath while the outer membrane becomes part of the plasma membrane of the zoospore. Because they take an extensive and irregular path through the cytoplasm, the actual time which elapses between the initiation and completion of the flagella is difficult to determine. The process seems to be nearly complete at an early stage of cytoplasmic cleavage and the flagella are first found in independent channels running through the relatively undifferentiated cytoplasm (Fig. 30, 31).

Evidence of the flagellar rootlets (Cantino et al., 1963) can be found at an early stage of flagellum formation (Fig. 21), and it seems probable that they are organized soon after the elongation of the centriole to form the basal body. They consist of parallel plates or wafers spaced ca. 35–40 mμ apart; each plate is composed of two outer dense regions separated by an area of low density (Fig. 29).

Within the outer sheath of the flagellum the axoneme (Fig. 23) consists of the nine outer doublet fibers surrounding two single central fibers. Each of the central fibers bears a pair of somewhat tenuous projections on opposite sides. At the base of the axoneme the outer fibers continue through the basal plate region and are continuous with the fibers of the basal body. The central fibers terminate at the basal plate area (Fig. 24, 25). At or just below the basal plate the outer fibers are connected to each other by a curving filament with the same width as the dense "walls" of the fiber subunits themselves (Fig. 26). These may represent the distal (from the nuclear membrane) portions of the third and outer subunit fiber of the nine triplets that form the wall of the basal body just below this region (Fig. 27).

Fig. 12–19.—Fig. 12. The basal area of a growth-phase plant. Large areas of empty-looking cytoplasm (arrow) occur in the base of the plant and rhizoids (rh). 13 hr 30 min, ×5,600.—Fig. 13. Cross wall formation. Cross wall (cw) deposition is occurring from the plant wall (pw) inward. Note the dense cytoplasm in the cross wall region, the row of vacuoles (v) through the aperture, and the particle-free appearance of the rhizoids (rh) below. 17 hr, ×5,900.—Fig. 14. A nearly septate plant. The cross wall (cw) is complete except for a fine central pore; the basal "cell" (arrow) and rhizoids contain mainly vacuoles and a few mitochondria (m). Numerous transparent vacuoles (v) and fewer "filled" vacuoles (fv) are present in the sporangium. n, nucleus; ps, clustered polysaccharide near the wall. 16 hr 45 min, ×3,240.—Fig. 15. Early papilla formation. Plant wall breakdown is apparent (large arrows) and a thin layer of papilla material (p) has formed within the outer wall. Secretory granules (pp) are present just below. 17 hr 48 min, ×5,900.—Fig. 16. Mid-papilla formation. Part of the plant wall (pw) has disappeared and the middle layers appear degraded (large arrow). The papilla layer (p) has become four times thicker, and many secretory granules (pp) are present. 17 hr 48 min, ×5,900.—Fig. 17. Papilla deposition. The membrane-enclosed secretory granules (pp) are clustered below the plasma membrane (pm). The contents of one granule lie outside the membrane (arrow), just below the papilla material (p). 17 hr 48 min, ×16,800.—Fig. 18. Late papilla formation. The plant wall has been replaced by a thick layer of papilla material (p) which tapers into the wall on either side (arrow); fewer secretory granules (pp) are present. 17 hr 48 min, ×4,500.—Fig. 19. A mature papilla-stage zoosporangial plant. A multinucleate plant with an apical papilla (p) and the basal rhizoids (rh) separated by a cross wall (not shown). 18 hr, ×1,400.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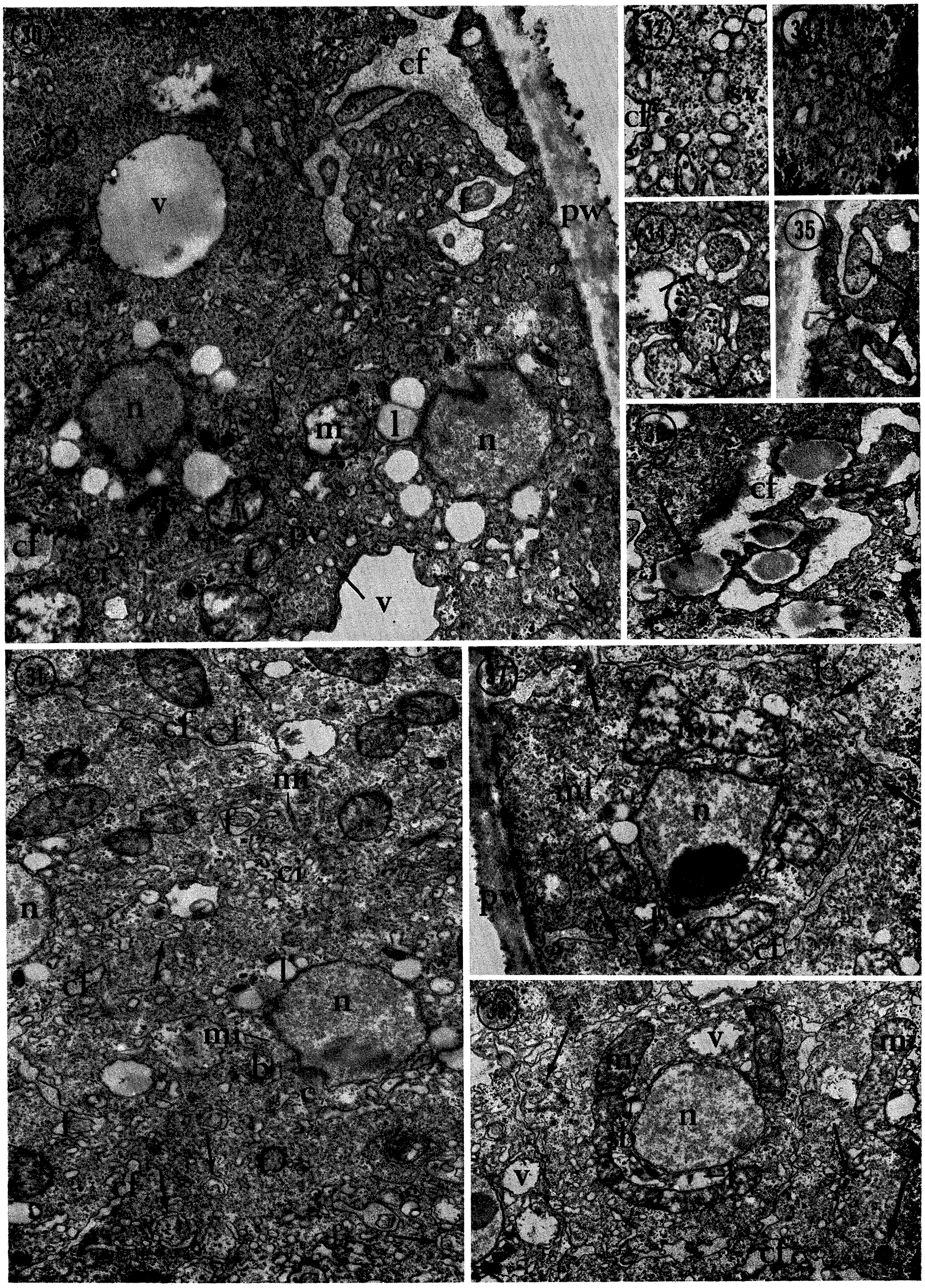
In the proximal region of the basal body each of the triplets in the typical pin-wheel pattern is connected to the next set and to the central region by fine strands. The interior at this level contains a very slender strand surrounded by two sheaths of fine strands. An acentrically located band of dense amorphous material also lies to one side of the basal body (Fig. 27). At the proximal end of the basal body this material is prominent and takes the shape of a distinct crescent (Fig. 28). The same structure is evident as a spur in longitudinal sections (Fig. 24, 53); also, at this level a small dense body is associated with each of the nine outer fiber assemblies (Fig. 28). The proximal end of the basal body lies very close to the nuclear membrane. In addition to forming the flagellar fibers from its distal end, it now seems likely that a series of microtubules arise from its proximal end and lie next to the nuclear membrane of the zoospore (Fig. 25, 29, 47, 52, 53). The second, smaller centriole remains closely adjacent to the functional basal body at an angle of ca. 60° (Fig. 29).

Cleavage—Soon after the beginning of flagella formation it is possible to find early stages of "cleavage furrow" formation (Fig. 22, 30). This process, too, involves the fusion of many small vesicles and it is impossible to distinguish between the flagellar vesicles and cleavage vesicles in areas where both processes are occurring simultaneously. Concomitant with the appearance of the small "secondary" cleavage vesicles in large numbers, numerous irregular, and often interconnected cisternae containing dense particles become prominent in the same regions (Fig. 30, 31, 32). Secondary cleavage vesicle fusion results in progressive expansion of the "primary" cleavage furrows and it appears that this activity is simultaneously initiated at many points. Occasionally vesicles can be found in somewhat linear arrangements (Fig. 32) over a short distance. They more often occur in less orderly clusters and fuse in irregular ring-shaped patterns lying roughly in the plane of the developing cleavage furrow (Fig. 31, 33, 34). The frequent occurrence of cytoplasmic peninsulas surrounded by U-shaped areas of cleavage vesicle (Fig. 35) suggests that many of the rings may in fact be short cylinders; if so, the closure and interconnection of the rings may be irregular and only gradually assume the form of a regular furrow. The section in Fig. 36 is taken through a developing cleavage furrow parallel to the plane of expansion and shows this irregularity distinctly. The cleavage furrows also fuse with the earlier-formed vesicles surrounding the flagella with the result that these finally lie within the cleavage furrows and outside of the uninucleate blocks of cytoplasm delineated by the membrane system.

Cleavage is completed between 18 hr 40 min and 18 hr 50 min. At this stage each nucleus lies near the center of the spore initial surrounded by the lipid and unidentified *sb* granules (Fig. 37, 38). The mitochondria lie outside the granules, and their profiles often suggest that they have begun to fuse (Fig. 38).

An additional feature evident at the end of cleavage is the appearance of a new type of dense granule in irregular cisternae. These intracisternal granules are usually in restricted regions and largely fill the vesicles which contain them (Fig. 38, 39). At first the cisternae contain one to a few particles, but their appearance is almost immediately followed by the accumulation of larger, roughly spherical vesicles that contain many granules of similar size and appearance (Fig. 38, 39, 40). At about the same time vesicles of like size are found in which the granular contents have assumed a highly ordered ellipsoidal-hemispherical pattern (Fig. 41, 42). These are followed by the appearance of the fully formed vesicle-enclosed, cup-shaped granules of the mature spore (Cantino et al., 1963) (Fig. 43, 46, 50). As many as eight such granules have been counted in a single section from a zoospore (Fig. 51).

Fig. 20–29.—Fig. 20. Paired centrioles at the lipid crown stage. The two centrioles (c) lie side by side. n, nuclear membrane; nu, nucleolus; l, lipid droplet; er, rough reticulum. 17 hr 24 min, ×31,600.—Fig. 21. Early flagella formation. The basal body (b) is producing the flagella fibers (not shown) out into the primary flagellar vesicle (pv) formed by the small secondary vesicles (sv). r, traces of a rootlet. 18 hr, ×23,800.—Fig. 22. Mid-flagellum formation. Numerous small secondary vesicles (arrows) fuse with the enlarging primary flagella vesicle (pv) and cleavage vesicles (cv). f, flagellum; fs, flagellar sheath; l, lipid; sb, sidebody granules. 19 hr, ×16,900.—Fig. 23. Cross-section of a flagellum. The axoneme of the flagellum surrounded by a unit membrane, the flagellar sheath (fs), lies in a cleavage furrow (cf) between the plasma membranes (pm) of adjacent zoospore units. 19 hr, ×53,000.—Fig. 24, 25. Longitudinal sections of the basal body and spur.—Fig. 24. Shows the flagellum (f), basal body (b), spur (sp), and a small granule (arrow) at the proximal end of the basal body. bp, basal plate. 19 hr, ×32,300.—Fig. 25. A section with the spur (sp) and a microtubule (mi) close to the nuclear membrane (nm). Arrow, central flagella fibers. 18 hr 12 min, ×31,600.—Fig. 26–28. Cross sections of the basal body.—Fig. 26. The distal region of the basal body (b) contains nine outer doublet fibers interconnected by curved elements (arrow) and no central fibers. 19 hr, ×50,500.—Fig. 27. The proximal part of the basal body (b). Two of the rootlets (r) appear to connect with it. sp, trace of spur. 18 hr 12 min, ×54,300.—Fig. 28. The proximal end of the basal body with a dense body (arrow) next to each outer fiber, the acentric spur (sp), rootlet (r), and microtubules (mi). 18 hr 12 min, ×57,100.—Fig. 29. Basal body and small centriole. An early stage of flagellum formation. The small centriole (c) lies close to the basal body (b). Microtubules (mi) extend back along the nuclear membrane (nm). pv, primary vesicle; r, rootlet. 17 hr 12 min, × 54,600.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Nuclear cap formation—The earliest evidence of a change in ribosome distribution is seen when the ribosomes begin to aggregate in small irregular clumps near the end of cleavage or at about 18 hr 40 min (Fig. 38, 39). The clumps are, however, still spread throughout the cytoplasm. The obvious accumulation of ribosomes around the nucleus can be seen between 18 hr 40 min and 18 hr 50 min (Fig. 44) and is complete by 19 hr (Fig. 45). In this process the ribosomes rapidly become packed in the space surrounding the anterior two-thirds of the nucleus with the thickest accumulation directly opposite the position of the basal body. The removal of the ribosomes leaves the peripheral cytoplasm with a distinctly "empty" appearance. Even before the ribosomes are completely packed in the cap region, small vesicles begin to appear around the periphery of the aggregate (Fig. 45). By the time the particles are tightly packed, the vesicles nearly surround the cap region (Fig. 46) and have begun to fuse in an anastomosing network to form the outer nuclear cap membrane (Fig. 48, 49). This finally results in an uninterrupted double-layered membrane which surrounds the entire ribosomal nuclear cap and all but a small part of the posterior end of the nucleus (Fig. 50, 51).

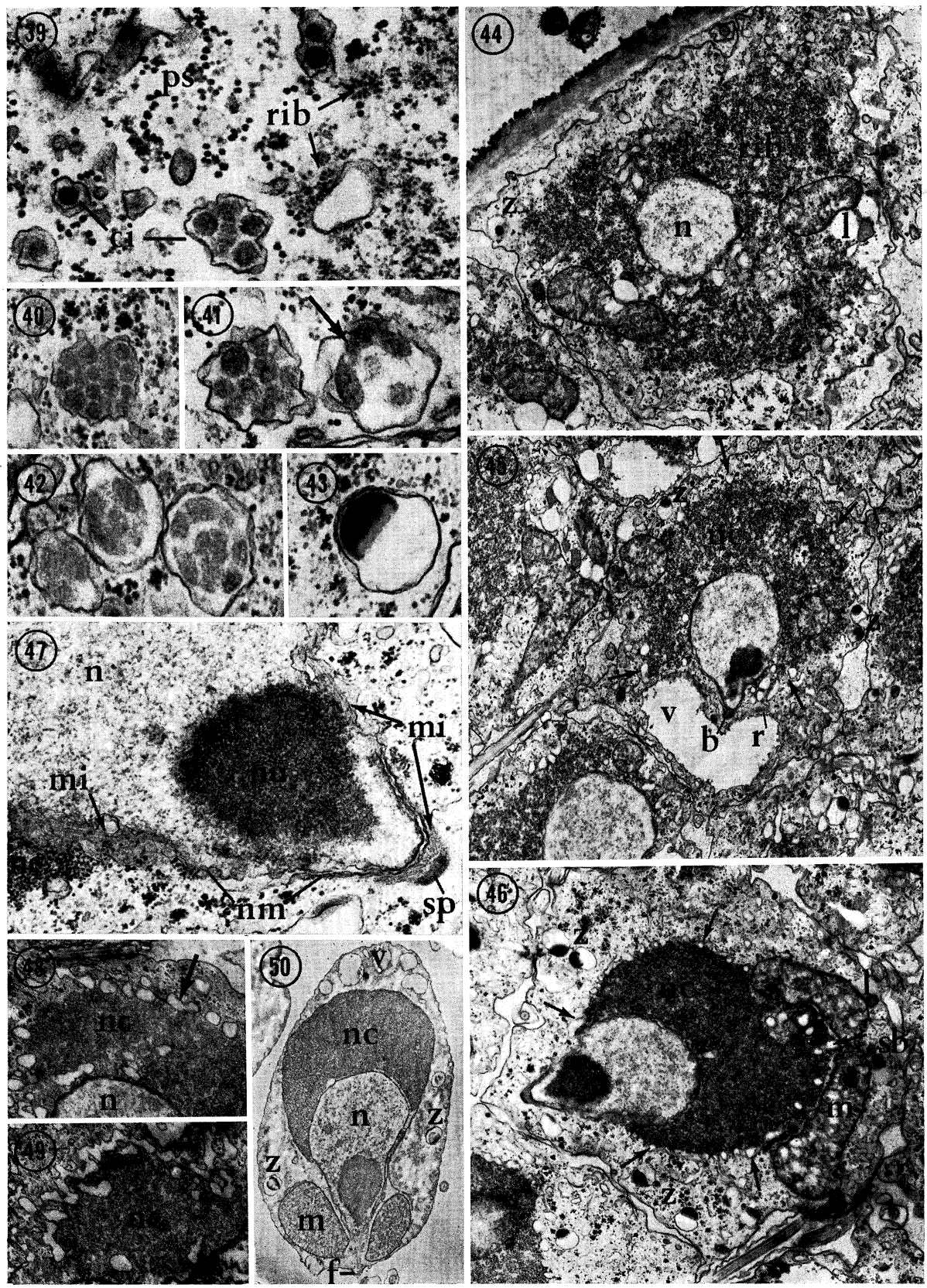
During cap formation the presumed fusion of mitochondria has also been completed, and the resulting single large mitochondrion lies to one side of the cell. The lipid and *sb* granules now lie against the outer side of the mitochondrion (Fig. 46), and they retain this relationship in the mature spore where the aggregate has long been known as the "sidebody." In Fig. 47 the microtubules that extend from the basal body region up along the nuclear membrane are clearly evident. The mitochondrion is not yet found surrounding the basal body and rootlets even at this late stage.

In a liquid environment the zoospores are released by dissolution of the papilla as soon as their differentiation is complete; in our system this is between 19 and 19¼ hr. The process of spore differentiation occurs during the final hour, although the entire sequence including sporangium differentiation requires 3 hr. During this hour, the undifferentiated coenocytic protoplast is converted into some 250–260 uninucleate, highly organized zoospores each ca. $7 \times 9\ \mu$, with a $20\ \mu$ flagellum whose basal body and rootlets are surrounded by a portion of the acentrically located mitochondrion (Fig. 50, 53, 55). The mitochondrion extends forward with the localized "sidebody" granules lying between it and the flexible plasma membrane (Fig. 51). The nucleus is nearly surrounded by the massive ribosomal nuclear cap (Fig. 50, 51), and it contains a compact nucleolus lying adjacent to the basal body (Fig. 50, 53). Microtubules extend from the area of the basal body up along the nucleus (Fig. 52, 53). The ribosome-free cytoplasm contains polysaccharide particles; the cup-shaped granules in small vesicles (Fig. 50, 51); and a few small, apical vacuoles; some irregular smooth vesicles; and a very limited amount of smooth endoplasmic reticulum. The last extends from the nuclear or nuclear cap membrane outward and around the mitochondrion and "sidebody" granules in a somewhat irregular fashion (Fig. 50, 51, 54).

DISCUSSION—In the diagram of Fig. 56 we have summarized the sequence of important changes that occur during sporangium formation and subsequent zoospore differentiation. Because the differentiating culture was only 90% synchronized, random samples at specific times always included plants at somewhat different stages. The times given for Fig. 56 and in the text represent our best estimate of the situation after examining numerous sections of plants at each sample time. This sampling problem limits the precision of the time estimates for specific events but does not alter their sequence.

Nuclear division—When examined by light microscopy, the nuclei of *B. emersonii* appear to divide by a process of elongation and constriction, with the elongation of the persistent nucleolus a characteristic feature. Nuclei undergoing division

Fig. 30–38.—Fig. 30. Early cleavage. Small portions of cleavage furrow (cf) are present at the upper right and lower left. Numerous cisternae with granules (ci) are present, as well as clusters of secondary cleavage vesicles (arrows); one flagellum (f) lies in a cleavage furrow, others still lie in the primary vesicles (pv). 18 hr 12 min, × 15,300.—Fig. 31. Mid-cleavage. Several sections of cleavage furrow (cf) are complete but not yet interconnected, while ring-shaped patterns of secondary vesicles (arrows) are frequent. Some disorganized macrotubules (mt) remain. b, basal body; c, centriole; ci, cisternae; mi, microtubules. 18 hr, × 12,300.—Fig. 32–36. Cleavage vesicles.—Fig. 32. Several secondary vesicles (sv) and cisternae (ci) with granules next to a developing cleavage furrow (cf). 18 hr 12 min, × 27,700.—Fig. 33. Ring-shaped pattern of fusing (arrow) secondary vesicles. 19 hr, × 22,000.—Fig. 34. Four adjacent rings (arrows) in the same plane. 18 hr 12 min, × 17,700.—Fig. 35. Peninsulas of cytoplasm (arrows) surrounded by cleavage plane vesicles. 18 hr 12 min, × 12,300.—Fig. 36. A section through the plane of cleavage (cf) showing its irregularity and surface views of the membrane (arrow), the plasma membrane of the spore. 19 hr, × 13,200.—Fig. 37. Late cleavage. Cleavage furrow (cf) formation is nearly complete with only small connections (arrows) between adjacent cell units; mitochondria (m) surround the nucleus (n); b, basal body; mi, microtubules; pw, plant wall. 18 hr 12 min, × 9,590.—Fig. 38. Cleavage complete. Mitochondrial (m) fusion appears to be in process around the outside of the sidebody (sb) and lipid (l) granules; note the dense intracisternal granules (small arrows) and the large vesicles with several similar granules (large arrow). 19 hr 24 min, × 7,160.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of this type (termed karyochorisis by Moore, 1964a, 1965) have been classified as directly dividing nuclei by Robinow and Bakerspigel (1965). Turian and Cantino (1960) examined dividing nuclei in young *Blastocladiella* plants by conventional cytological methods. They correctly interpreted the division as intranuclear and suggested that the elongating nucleolus served to separate the chromatic material. Despite prior ribonuclease digestion to remove the large nucleolus, it was difficult for these workers to resolve individual chromosomes because of their very small size and crowded position between the nucleolus and nuclear membrane. They did not observe typical metaphase figures. Despite these limitations, the sequential positions of the irregular chromatic material were certainly consistent with the typical stages of mitosis. The results of Turian and Cantino (1960) together with the micrographs of Fig. 7–11 indicate that vegetative nuclear division in *B. emersonii* is mitotic in most of its fundamental aspects, particularly in the presence of polar centrioles and a well-organized, microtubular spindle. Its unusual appearance in the light microscope can mainly be attributed to the continuous presence of the nucleolus and nuclear membrane. Nuclear division in the related genus *Allomyces* has also been interpreted as mitotic (Robinow and Bakerspigel, 1965). The advent of glutaraldehyde fixation, which preserves microtubules and centrioles, has led to a few other reports, though few published micrographs, of polar centrioles and/or spindles in fungi (Berlin and Bowen, 1965; Fuller and Reichle, 1965; Robinow and Bakerspigel, 1965; Robinow and Marak, 1966).

The problem of centriole replication from pre-existing organelles versus de novo synthesis has been discussed thoroughly by Renaud and Swift (1964), and Mizukami and Gall (1966). The presence of the two unequal centrioles next to the nuclei at all stages examined certainly indicates that in *B. emersonii* a pair exists with each nucleus throughout the life cycle.

Papilla formation—The apical papilla results from a combined process of cell wall breakdown and deposition. Because large numbers of membrane-enclosed particles of similar density appear in the area during this process, we believe that these serve as secretory granules to transport the papillar substance. Skucas (1966) has provided cytochemical evidence for pectic materials in the papillae of *Allomyces* zoosporangia, and it is probable that the papillae of these closely related genera have a similar composition. When fixed with $KMnO_4$ the papillae of *Allomyces* appear homogeneous in electron micrographs (Skucas, 1966), as do those of *Phytophthora erythroseptica* (Chapman and Vujicic, 1965) and *Rhizophlyctis rosea* (Chambers and Willoughby, 1964), but the development of the papillae has not been described.

Flagellum formation and cleavage—Renaud and Swift (1964) described flagellum formation by one of the two centrioles associated with each nucleus in gametangia of *Allomyces arbuscula*. Because they found only one centriole per nucleus in the undifferentiated hyphae they were not sure if two existed before the gametangial stage. No equivalent to the "rootlets" associated with the basal body of *B. emersonii* zoospores has been found in *Allomyces* gametes. With these exceptions our results generally agree with those of Renaud and Swift (1964).

Renaud and Swift (1964) concluded that the plasma membrane was the source of the secondary vesicles which fuse to form the primary vesicles of the elongating flagella. This was based upon an occasional continuity between the primary vesicle and plasma membrane and a similarity in the staining properties of the developing flagellar sheath and plasma membrane. However, the similarity in staining properties of the flagellar sheath, vesicles, and plasma membrane would be expected since the plant plasma membrane becomes part of the membrane of the spores formed in the blocks of cytoplasm adjacent to it

Fig. 39–50.—Fig. 39–43. Formation of vesicle-enclosed granules.—Fig. 39. Cytoplasm at the end of cleavage with clumped ribosomes (rib), granules in irregular cisternae (ci), and scattered polysaccharide (ps). 18 hr 24 min, × 41,600.—Fig. 40. Clustered granules in a vesicle. 19 hr 24 min, × 31,300.—Fig. 41. A vesicle with granules next to one where the granules are becoming organized (arrow). 18 hr 24 min, × 41,600.—Fig. 42. Three adjacent vesicles with well-ordered, spheroidal aggregates. 19 hr 24 min, × 40,400.—Fig. 43. A complete cup-shaped granule in a vesicle; the substituent particles are no longer distinguishable. 19 hr 12 min, × 43,900.—Fig. 44. Ribosome aggregation to form the nuclear cap. Irregular clumps of ribosomes (rib) have become loosely clustered around the pre-spore nucleus (n) leaving the outer areas free of ribosomes. l, lipid; z, vesicle-enclosed granule. 19 hr 24 min, × 9,900.—Fig. 45. Mid-cap formation. The ribosomes have assumed the shape of the zoospore nuclear cap (nc) opposite the basal body (b), but are not yet fully packed together; arrows indicate the peripheral vesicles; the vacuoles prominent at this stage later disappear; r, rootlet; z, granules. 19 hr 24 min, × 7,500.—Fig. 46. Late cap formation. A nearly continuous series of fusing vesicles (arrow) enclose the tightly packed nuclear cap (nc) ribosomes. 19 hr 12 min, × 9,900.—Fig. 47. Enlargement of the nucleus in Fig. 46. Note the spur (sp) of the basal body region and the numerous microtubules (mi) which extend from this region (arrows) up along the nuclear membrane (nm); nc, nuclear cap, × 32,200.—Fig. 48–49. Nuclear cap membrane formation. Two areas from the same micrograph show the rapid fusion of the numerous vesicles (Fig. 48, arrow) which appear around the ribosome aggregate to form a continuous membrane (Fig. 49). 19 hr 24 min, × 11,300.—Fig. 50. Discharged zoospore. A longitudinal section of a spore to show the relationship of the nuclear cap (nc), nucleus (n), flagellum (f), and the mitochondrion (m) which surrounds the basal body. v, apical vacuoles; z, vesicle-enclosed granules, ca. × 6,200.

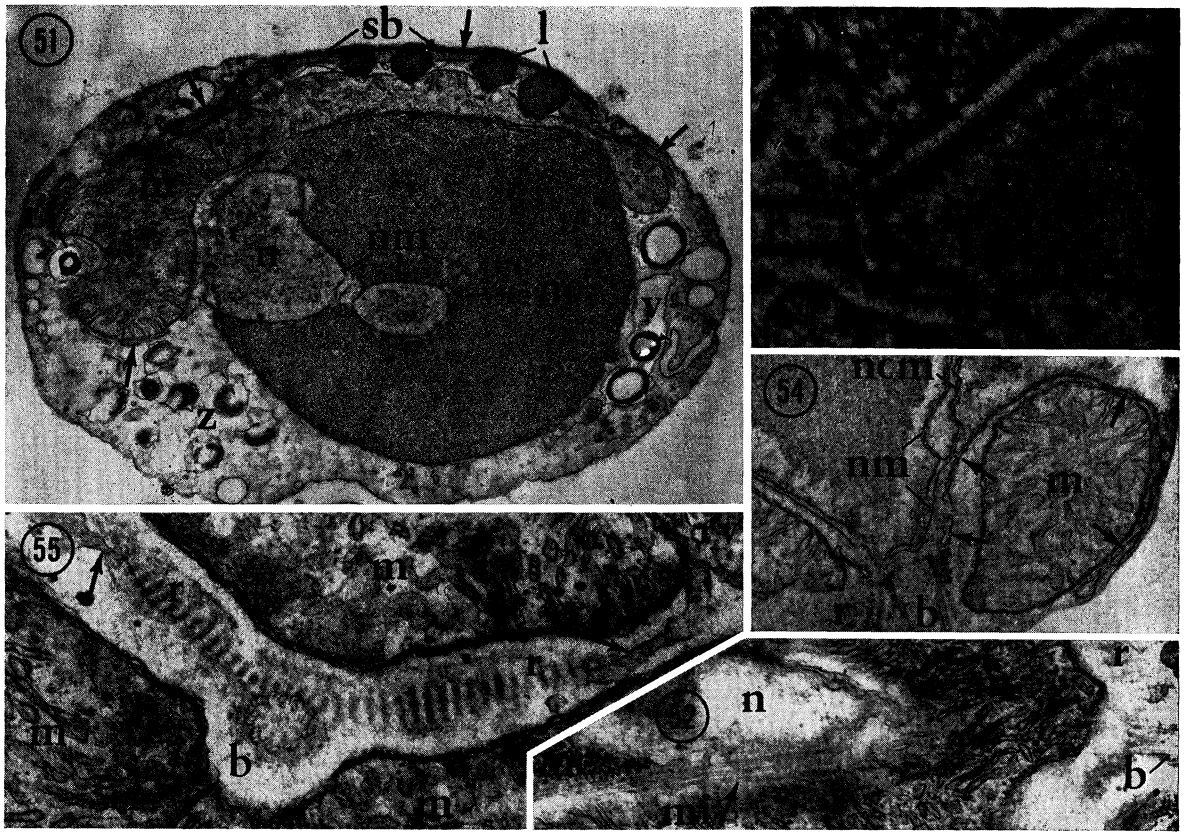


Fig. 51–55.—Zoospore structure.—Fig. 51. A section nearly tangential to the nuclear membrane (nm), to show the acentric mitochondrion (m), the associated lipid (l) and sb granules, and smooth membranes (arrows). nc, nuclear cap; v, apical vacuoles; z, cup-shaped granules; rv, vesicle at the end of a rootlet channel, ca. × 10,400.—Fig. 52. A set of three microtubules (mi) running from the basal body region (b) along the nuclear membrane. n, nucleus; m, mitochondrion; nc, nuclear cap; r, rootlet, × 31,300.—Fig. 53. A microtubule continuous with a basal body (b) fiber (arrows). sp, spur; nu, nucleolus, ca. × 37,000.—Fig. 54. Interconnections between the nuclear membrane (nm), nuclear cap membrane (ncm), and smooth membrane (arrows) that extends around the mitochondrion (m), ca. × 30,000.—Fig. 55. Cross-section through the basal body region to show the mitochondrion (m) surrounding the channels of the basal body (b) and two rootlets (r). Some smooth membrane (arrows) penetrates irregularly into the rootlet canals, × 41,800.

in *B. emersonii* and *Allomyces* (Moore, 1964c; Renaud and Swift, 1964). Renaud and Swift also suggested that the centrioles of *Allomyces* gametangia, which all lie near the cell membrane, induced the formation of the vesicles by this membrane when they became basal bodies. With *B. emersonii* such an induction seems a less compelling argument since many of the nuclei are quite removed from the plasma membrane and flagellum formation appears to be started by all the centrioles at nearly the same time. It is certain from our results that the cleavage planes are formed by fusion of vesicles which are indistinguishable from the "secondary flagellar vesicles" as defined by Renaud and Swift (1964), a point mentioned in passing by the same authors. We think that the nearly simultaneous appearance of these "flagellar-cleavage vesicles" in large numbers throughout large areas of the sporangium argues against their exclusive formation by pinching off from the plasma membrane. The large number of particle-containing cisternae that appear simultaneously could be a possible source. Blondel and Turian (1960) did not discuss the early stages in cleavage. They did show double chains of small vesicles between complete sections of cleavage planes at a late stage and suggested that the spore membranes might arise by their fusion. The cleavage planes of *R. rosea* contain material with the same finely reticulate arrangement (Chambers and Willoughby, 1964). In the $KMnO_4$-fixed sporangia of *A. javanicus* the contents of the cleavage vesicles and cleavage planes appear uniformly electron-dense (Moore, 1964c).

Flagellar apparatus—The organization of the axoneme and basal body of *B. emersonii* zoospores corresponds to the well-established organization of the same structures in other flagellated cells (Fawcett, 1961). Cantino et al. (1963) and Reichle and Fuller (1967) described the basic

organization of the basal body and associated rootlets within channels through the mitochondrion. The micrographs here show in addition the presence of dense material just outside and at the base of each outer triplet of the basal body (Fig. 28), as also occurs in *Allomyces arbuscula* (Renaud and Swift, 1964). Similar material exists in *B. emersonii* in the form of a single, large acentric spur that extends back toward the nuclear membrane (Fig. 24, 28, 53). The second (smaller) centriole next to the basal body of *Allomyces* gametes was first described by Renaud and Swift (1964) and has since been reported for *Rhizidiomyces* and *Blastocladiella* zoospores (Fuller, 1966; Reichle and Fuller, 1967).

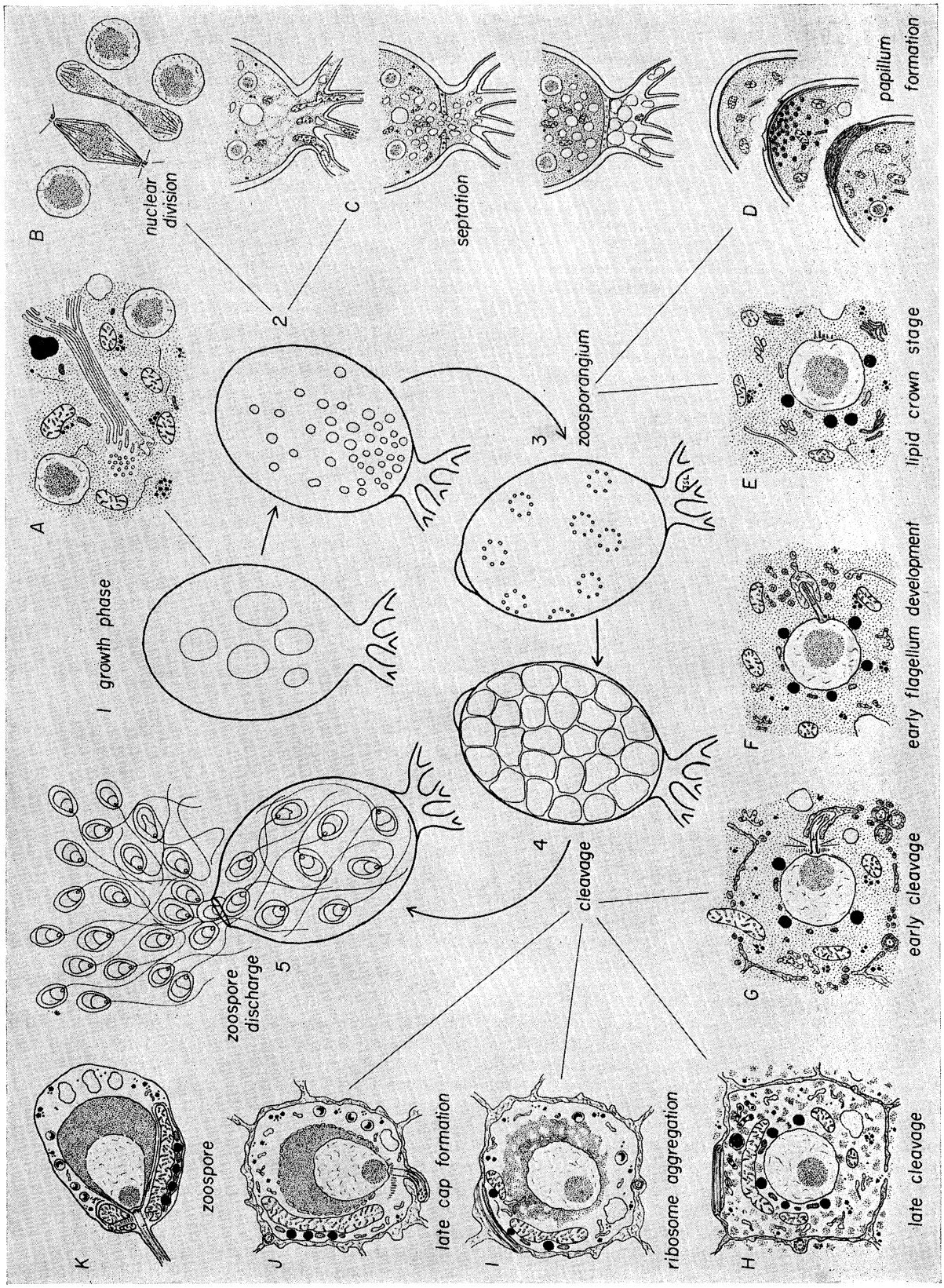
The details of the flagellar rootlets have been difficult to resolve, but the parallel array of uniformly spaced membranous discs or plates is unlike the striated rootlets of other flagellates (Fawcett, 1961), including the aquatic fungus *Monoblepharella* (Fuller, 1966). Our interpretation of the structure of *B. emersonii* rootlets differs from Fuller's description of them as "fibrils with 25 mμ striations." The exact relationship of the rootlets to the basal body remains unclear. In some sections two of the rootlets actually seem to be arms of a single continuous structure that passes close to (or is attached to) the basal body fibrils (Fig. 27, 55).

Microtubules—The presence of microtubules extending from the region of the basal body up along the nuclear and nuclear cap membranes was recently reported by Reichle and Fuller (1967). It now seems likely that they may continue directly from the proximal end of the outer triplet fibers of the basal body (Fig. 25, 29, 47, 53) and travel forward in groups of three (Fig. 52). During earlier stages of flagellum development microtubules are also seen to extend outward away from the basal body in various directions (Fig. 25, 28, 31). The relationship of these, if any, to those around the nucleus in the zoospore is not clear. Similar tubules are also found occasionally in other areas of the cytoplasm (Fig. 37).

Nuclear cap formation—The process of nuclear cap formation in *B. emersonii* corresponds to that described for *Allomyces macrogynus* gametes by Blondel and Turian (1960). In *Allomyces* the dense accumulation of ribosomes around the nucleus seems to occur a little earlier than in *Blastocladiella*, overlapping partly with cytoplasmic cleavage. An examination of RNA synthesis during zoospore differentiation in *B. emersonii* (Murphy and Lovett, 1966) has shown that despite extensive RNA degradation, the ribosomes of the nuclear cap are those formed during the prior growth phase. Thus two distinct processes are involved: ribosome destruction, and aggregation and migration of the ribosomes to be conserved in the cap. Our observations and those of Blondel and Turian (1960) differ sharply from the suggestion by Moore (1964b,c; 1965) that the cap of *A. javanicus* is formed from the contents of electron-dense vesicles attached to the endoplasmic reticulum. His micrographs have not been published, but it would be difficult to identify the source of the cap contents in $KMnO_4$-fixed material where the ribosomes were not preserved. The organization of the cap as a late and rapid process is consistent with our previous observation (Murphy and Lovett, 1966) that active protein synthesis, unlike RNA, persists until near the end of differentiation. The fusion of numerous small vesicles to form the nuclear cap membrane (Fig. 48, 49) was also observed in *Allomyces* (Blondel and Turian, 1960; Moore, 1965). It is normally connected to the nuclear membrane (Fig. 53, 54) although gaps occur (Fig. 50), and it may also be connected with membranes of the limited amount of smooth endoplasmic reticulum which irregularly surrounds the mitochondrion and "sidebody" granules.

We do not know the function of the numerous vesicle-enclosed cup-shaped granules found in zoospores of *B. emersonii*. It was suggested on the basis of their size and number (Cantino et al., 1963) that they could be the "gamma" particles detected by staining with the Nadi reagent (Cantino and Horenstein, 1956). Verification of this, however, must await their isolation and chemical identification.

"Sidebody" granules—It is evident from Fig. 51 that two different kinds of granules occur next to the mitochondrion in the "sidebody" complex of *Blastocladiella;* these are the unidentified *sb* particles present in the growth phase, and the spherical lipid granules that appear at the "lipid crown" stage. In several micrographs, Reichle and Fuller (1967) labeled the *sb* granules as the lipid granules and criticized Cantino et al. (1963) for failing to recognize the "electron-transparent vesicles" which they claim to lie between them. It is obvious that the "electron-transparent vesicles" of Reichle and Fuller (1967) represent the empty spaces from which the lipid was extracted during preparation. Their micrographs do demonstrate that the lipid granules are surrounded by a unit membrane, i.e., that which encloses their "electron-transparent vesicle." Although we do not yet have good sections to document the process, we believe that the mitochondrion surrounds the completely preformed basal body and rootlets of the flagellar apparatus shortly before the zoospores are released from the sporangium. It is clear, however, that the mitochondrion not only moves to surround these structures but also coalesces in such a way that the basal body and three rootlets lie in complete channels which pass directly through its body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C
septation
D
papillum formation
E
lipid crown stage
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early flagellum development
G
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ribosome aggregation
J
late cap formation
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zoosporangium
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(Fig. 50, 53, 55; Cantino et al., 1963; Reichle and Fuller, 1967).

Polysaccharide—Particles resembling animal glycogen (Revel, 1964) in size, shape, and staining reaction have also been reported for the basidiomycete *Collybia velutipes* (Foerster, Behrens, and Airth, 1965). We have isolated the polysaccharide particles from both zoospores and plants and demonstrated their morphological similarity to the intracellular α- and β-particles, as well as their glucose composition (Hall and Lovett, unpublished). There seems little doubt that they represent the storage polysaccharide extracted from *B. emersonii* by Goldstein and Cantino (1962).

Macrotubules—The highly ordered, parallel arrays of flat cisternae and interconnected tubate cisternae, which typify the plant Golgi apparatus or Golgi-dictyosome (Cunningham, Morré, and Mollenhauer, 1966), are relatively rare in fungi (Hawker, 1965; Moore, 1965). We have been unable to identify structures in *B. emersonii* that resemble a typical Golgi apparatus. On the other hand, the ubiquitous distribution of the organized macrotubular strands strongly suggests that these unusual structures have a significant function in the growing coenocytic plants. Several observations lead us to suggest that the macrotubule system in *B. emersonii* may be the functional equivalent of the Golgi apparatus: (a) it is most extensive and well organized during rapid growth when synthetic rates are maximum; (b) the tubules sometimes open directly into irregular cisternae (Fig. 3, 4, 5); (c) cisternae associated with the tubules often contain "secretory-like" granules (Fig. 4, 5); and (d) large numbers of similar irregular vesicles or cisternae with granules appear during differentiation when the macrotubule system becomes disorganized and ultimately disappears (Fig. 30, 31, 32). On the basis of this circumstantial evidence, and despite their unusual structure, it seems reasonable to suggest tentatively a secretory and/or transport function for the macrotubules.

Perhaps the main value of the work reported here is that it has provided a reasonably detailed description of the entire sporulation process, under reproducible conditions, in a single water mold. The developmental synchrony obtainable with *Blastocladiella* provides a system in which we can now temporally "isolate" specific intracellular events for much more detailed structural study as well as for the analysis of their underlying biochemical regulation.

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Fig. 56. The stages of sporangium formation and zoospore differentiation in *Blastocladiella*. The inner diagrams illustrate the appearance of plants from synchronous cultures in the light microscope. The outer series summarize the intracellular changes during differentiation. The timing of these events is as follows: (1) 15½ hr, (2) 17½ hr, (3) 18 hr, (4) 18 hr 40 min, (5) 19 hr–19 hr 15 min; (A) 15½ hr, (B) 16½–17½ hr (Fig. 8–11), (C) 16½–17½ hr (Fig. 13, 14), (D) 17–18 hr (Fig. 15–19), (E) 18 hr (Fig. 20), (F) 18 hr 10 min (Fig. 21, 22, 29), (G) 18 hr 20 min (Fig. 30, 31), (H) 18 hr 40 min (Fig. 38, 39, 40–43), (I) 18 hr 50 min (Fig. 44, 45), (J) 18 hr 50 min–19 hr (Fig. 46, 48, 49), (K) 19 hr 15 min (Fig. 50–55).

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FLORAL DEVELOPMENT AND VASCULATURE IN HYDROCLEIS NYMPHOIDES (BUTOMACEAE)[1]

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A B S T R A C T

The flower of *Hydrocleis nymphoides* consists of three sepals which arise in spiral succession, three simultaneously arising petals, numerous stamens and staminodia which arise in centrifugal order, and six carpels. A residual apex remains at maturity. The first-formed members of the androecium are stamens and the later-formed members are staminodia which develop below the stamens and which become outwardly displaced during expansion of the receptacle. The androecium is supplied by branching vascular trunk bundles. The carpels are completely open but the ventral margins are slightly conduplicately appressed basally. A single dorsal bundle provides the stigmatic area with vascular tissue, and a network of small placental bundles supplies the numerous laminar ovules. There are no clearly defined ventral bundles. It is suggested that *Hydrocleis nymphoides* is neither the most primitive nor the most advanced member of the family. A pattern of phylogenetic reduction in the androecium and receptacle is suggested for the entire family.

THE BUTOMACEAE is sometimes considered to comprise four genera: *Butomus*, *Tenagocharis*, *Hydrocleis*, and *Limnocharis*. A fifth genus, *Ostenia*, is sometimes included but according to Pedersen (1961) all of the characteristics of that genus can be found within *Hydrocleis* and he therefore suppressed the name *Ostenia*. All of the genera except *Hydrocleis* are monotypic.

Stant (1967) has studied the vegetative anatomy of the entire family and has reviewed the pertinent literature. Certain features of the structure and development of the flowers in the family have not been studied, and investigations of floral morphology and ontogeny in *Hydrocleis* have centered upon *H. nymphoides* Buch. Saunders (1929) illustrated some aspects of the anatomy of the gynoecium, and Ronte (1891) and Eber (1934) have reported some of the developmental features of the flowers. Almost nothing is known of the structure of the other species of

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