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THE ULTRASTRUCTURE OF MULTILAYERED STRUCTURES ASSOCIATED WITH FLAGELLAR BASES IN MOTILE CELLS OF *TRENTEPOHLIA AUREA*[1]

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SUMMARY

An ultrastructural study of motile cell development in the green alga Trentepohlia aurea *has revealed the presence of multilayered structures (MLS) associated with flagellar bases. These MLS are ultrastructurally similar to MLS described in pteridophyte and bryophyte sperm and in the zoospore of the green algae* Coleochaete *and* Klebsormidium. *However, 2 MLS are found in each biflagellate motile cell of* T. aurea, *while other previously described MLS occur singly in biflagellate motile cells. In addition, the MLS of* T. aurea *consist of fewer microtubules and are structurally simpler than most other MLS described. The MLS of* Trentepohlia *may represent a stage in the evolutionary development of the MLS of land plants. The presence or absence of the MLS in motile cells of green algae may be a useful character in phylogenetic studies.*

[1] *Received May 15, 1974; revised August 15, 1974.*
[2] Partially supported by an NSF traineeship awarded through the Rackham School of Graduate Studies, University of Michigan, Ann Arbor, Michigan.

INTRODUCTION

In recent years information derived from ultrastructural studies has enabled phycologists to construct new theories about evolutionary lines in the green algae. For instance, the fine structure of the

mitotic apparatus has been used to separate the Chlorophyceae into 2 basic groups, one of which may have given rise to the ancestors of the earliest land plants (*20,25*). In addition, ultrastructural histochemistry has shown that some green algae possess peroxisomes similar to those found in land plants (*10,24*). Biochemical investigations have confirmed the existence of 2 lines of evolutionary development in the Chlorophyceae based on characteristics of glycolate metabolism (*6*). The biochemical and histochemical data give rise to the same phylogenetic conclusions as those derived from consideration of the fine structure of the mitotic process (*6,20*).

Ultrastructural features of the motile cells of green algae have also provided phylogenetic information. McBride (*14*) first noted that the zoospores of *Coleochaete* possess an unusual multilayered structure at the flagella base and compared it to the multilayered structure (MLS) described in bryophyte sperm. Pickett-Heaps & Marchant (*20*) have confirmed the presence of the MLS in the zoospore of *Coleochaete*, and have described a MLS in the zoospore of *Klebsormidium* (*11*). MLS have also been observed in pteridophyte and cycad sperm (*1,5,16*).

During an investigation of motile cell development in the green alga *Trentepohlia aurea*, structures which were morphologically similar to the MLS of bryophytes were observed near flagellar bases. Some ultrastructural features of the MLS of *T. aurea* resemble the MLS reported in other algae and bryophytes, but other features differ somewhat. The MLS of *T. aurea* are described and compared with other MLS. The phylogenetic significance of the MLS in *Trentepohlia* is discussed.

MATERIALS AND METHODS

A unialgal culture of *Trentepohlia aurea* originally obtained from the Indiana Culture Collection (ICC No. 429) was maintained in soil-water medium at 18 C on a 16–8-hr light/dark cycle. Sporangial development was induced by transferring clumps of filaments to the surface of Difco Algae Broth solidified with 1.5% agar in Petri dishes. The medium was supplemented with 0.1% micronutrient solution (*23*) and 5% soil-water supernatant. The Petri dishes were placed in a culture chamber at 18 C on an 8–16- or 12–12-hr light/dark cycle under cool-white lights at a light intensity of about 6.0×10^3 ergs/(cm^2)(sec) for 3 weeks. For electron microscopy, clumps of filaments were fixed in 2% glutaraldehyde at room temperature for 1 hr, rinsed with 0.01 M PO_4 buffer at pH 7.2, and postfixed with 1% OsO_4 in the same buffer. The filaments were then "en block" stained for 15 min with 2% aqueous uranyl acetate buffered with veronal acetate at pH 5.0. After embedding in agar blocks, the filaments were slowly dehydrated with alcohol and acetone and then embedded in Spurr's low-viscosity resin (*22*). Thin sections were cut with a DuPont diamond knife and stained with uranyl acetate and lead citrate before examination with a Zeiss EM-9S-2 electron microscope.

For scanning EM of sporangia, plants were not critical-point dried before examination. Osmium-fixed motile cells were collected and dehydrated on a Gelman Metricel DM 800 millipore filter. The cells were critical-point dried before examination with a JEOL model JSM-U3 scanning EM.

OBSERVATIONS

Using the induction procedures described above, up to 25% of the vegetative cells at the base of a clump of filaments could be transformed into thick-walled, urn-shaped sporangia (Fig. 1, 2) containing many potentially motile cells (Fig. 3). Both the day-length and the intensity of the light appear to be important in induction of this morphogenetic process, and these light effects are being investigated further. If filaments with mature sporangia are transferred to either a dilute (10^{-2} M) PO_4 buffer or distilled water, zoospores are discharged within 3–5 min into a vesicle which rapidly breaks down, releasing the naked motile cells. However, motile cell discharge is inhibited by the presence of higher concentrations of ions in the water.

Some confusion exists as to the exact nature of motile cells in *Trentepohlia aurea*. Fritsch (*7*, p. 276) stated that both stalked and sessile sporangia occur on plants growing in nature. The stalked sporangia produce bi- or quadriflagellate motile cells which reportedly germinate directly. Sessile sporangia produce biflagellate motile cells which may germinate directly, but have been observed to behave as isogametes (*7*). The life cycle of *Trentepohlia* has not been studied with modern techniques. In our cultures we found only the equivalent of sessile sporangia. In order to clarify the nature of the motile cells from these sporangia, freshly discharged populations were placed in Rose chambers or hanging drop preparations and observed continuously for several hours and intermittently for several weeks. In no case were cell fusions observed. After several weeks, the settled cells had grown into several-celled germlings. Under our culture conditions, the motile cells act as zoospores. Further studies of the life cycle of *T. aurea* are being made.

Ultrastructural observations were made on both released and unreleased cells. These motile cells possess 2 equal flagella which show the typical 9 + 2 arrangement of axonemal microtubules (Fig. 5). However, the flagella are unusual in that they possess 2 lateral keels (Fig. 5, 6). The keels are extensions of the flagellar matrix and membranes. Two additional microtubulelike fibers run longitudinally through the flagellar keels (*arrows*, Fig. 5, 6). Since most cross sections of the flagella show keels, it is probable that the keels extend throughout most of the length of the flagella, but the point of origin of the extra tubules has not been determined. Presumably the extra microtubules act to support the keels, while the keels themselves may increase swimming efficiency.

Inside the cell, each flagellum passes through a transition zone (Fig. 4) to the basal body region (Fig. 8, 17). Each flagellar base is capped with an amorphous dark-staining material (Fig. 9). This material appears to curve slightly downward and merge into the microtubular layer of the MLS (*arrow*, Fig.

8). The flagella seem to diverge at a rather large angle from each other (Fig. 11, 12).

Following the terminology of Carothers & Kreitner (*3*), we will use the term multilayered structure (MLS), which is composed of the spline and the layers (MLS 1, 2, 3, etc.). The first layer of the MLS consists of a row of 6–8 adherent microtubules (*arrow*, Fig. 15). This microtubular band extends just under the plasmalemma toward the posterior of the cell (Fig. 7, 16). It probably corresponds to the spline region of the bryophyte MLS. Along most of its length, the spline is closely associated with the outer membrane of an elongated mitochondrion (Fig. 13). The location of the distal end of the spline is hard to determine, but the spline obviously extends quite far down into the cell (Fig. 16).

The middle and lower layers of the MLS can be observed at the anterior of the cell before the spline adjoins the elongated mitochondrion. The middle layer may be composed of more than one stratum and appears striated in both cross and longitudinal sections (Fig. 7, 9, 15). In other MLS these striations represent sections of plates oriented vertically to the plane of the microtubule band. Generally, the angle between the long axis of the plates and the microtubule band is 35–40° (*3,5,18*), but this angle appears to be 90° in *Klebsormidium* (*11*). In *Trentepohlia*, the angle has not yet been determined since the striations do not appear well defined in either cross, oblique, or longitudinal section (Fig. 15, 18, 7). The lower layer of the MLS appears as a dense, flat line in both cross and longitudinal sections (Fig. 15, 7). Therefore, it probably exists as a rectangular plate which underlies the upper layers of the MLS for only a short distance. Very tiny tubules may sometimes be discerned within the lowest layer, but they are generally obscured by the electron-dense matrix (Fig. 7).

In bryophyte sperm, the MLS is positioned directly over an apical mitochondrion (*2,4,17*). In contrast, the MLS of *T. aurea* are positioned over an anterior protrusion of the nucleus (Fig. 7, 10). Only the spline portions of the MLS are appressed to an elongated mitochondrion on each side of the nucleus. At lower levels in the cell, additional microtubules appear to join the spline, so that the spline may contain more

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NOTE: *bb*, basal body; *c*, chloroplast; *co*, flagellar collar; *fc*, flagellar cap; *km*, keel microtubules; *m*, mitochondrion; *mls*, multilayered structure; *n*, nucleus; *pd*, pigment droplets; *pl*, plasmalemma; *s*, starch; *sm*, spline microtubules; *v*, vesicles.

FIG. 1. Light micrograph of sessile sporangia on a filament of vegetative cells of *Trentepohlia aurea*. × 1430.

FIG. 2. Scanning electron micrograph of a sporangium of *T. aurea*. Note the apical pore. Also visible are vegetative cells which have collapsed. × 3220.

FIG. 3. Light micrographs of biflagellate motile cells of *T. aurea*. *B*. Double images of the flagella due to 2 lateral keels. Nomarski optics. × 1700. *C*. Scanning electron micrograph of critical-point dried motile cell. Ends of flagella have broken off. Note that the flagella emerge from the apical papilla. × 12,000.

FIG. 4. The stellate region of one of the flagella of a potentially motile cell. Note membrane-bound vesicles and the flagellar collar (*arrow*). × 130,000.

FIG. 5. Cross section of a flagellum. Note the microtubulelike fibers in the keels (*arrows*). Spline microtubules are visible in a nearby cell. × 130,000.

FIG. 6. Longitudinal section of a flagellum. Note the thinner keel microtubule (*arrow*). × 78,000.

FIG. 7. Anterior region of a motile cell. Note the multilayered structure (*arrow*). At least 3 layers (*1,2,3*) are visible. Note the dark-staining material above the first layer. Spline microtubules of the opposite MLS are visible. Note the apparently branched mitochondrion. × 104,000.

FIG. 8. Flagellar connection to the first layer of the MLS. Note the dense-staining flagellar cap (*arrow*). × 104,000.

FIG. 9. Relationship between a MLS and flagellum. MLS is probably connected to the opposite flagellum which is out of the plane of this section. This flagellum is probably connected to a MLS which is not visible. Note pigment droplets. × 91,000.

FIG. 10. MLS connected to dense material which is probably cap of a flagellum. Note relation to nucleus. × 78,000.

FIG. 11. View of the 2 flagella extending from the cell at a wide angle. Portions of both splines are visible (*arrows*). × 62,500.

FIG. 12. Portions of both splines visible (*arrows*). Note relationship of chloroplasts, nucleus, and flagellar apparatus. × 39,200.

FIG. 13. Longitudinal section of elongated mitochondrion with appressed spline microtubule layer (*arrow*). × 63,000.

FIG. 14. Cross section of mitochondria with closely associated spline microtubules (*arrows*). Other microtubules may join spline at this level. × 130,000.

FIG. 15. Cross section of MLS (*arrow*) with 7 microtubules. One basal body and the other flagellar cap can also be seen. Note shape of nucleus. × 72,000.

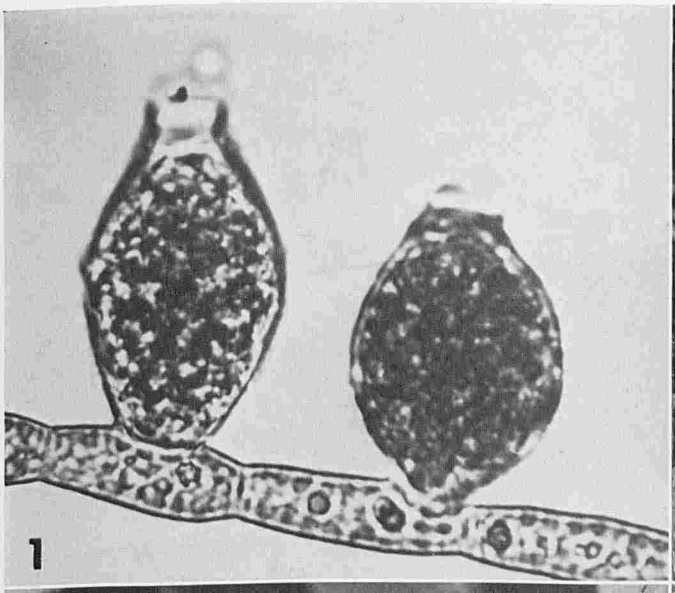
FIG. 16. Spline (*arrow*) extending far down into motile cell. Note elongated mitochondrion. The opposite spline and mitochondrion can also be seen (*arrowhead*). × 30,400.

FIG. 17. Two basal bodies lying between parts of 2 MLS (*arrows*). × 114,400.

FIG. 18. One basal body adjacent to a flagellar cap. Note the position of the MLS (*arrow*). × 104,000.

FIG. 19. Chloroplast of a vegetative cell of *T. aurea*. Note the small starch reserves and well-defined photosynthetic lamellae. × 35,200.

FIG. 20. Chloroplast of mature motile cell. Note disorganization of photosynthetic lamellae (*arrow*) and accumulation of starch and lipid. × 78,000.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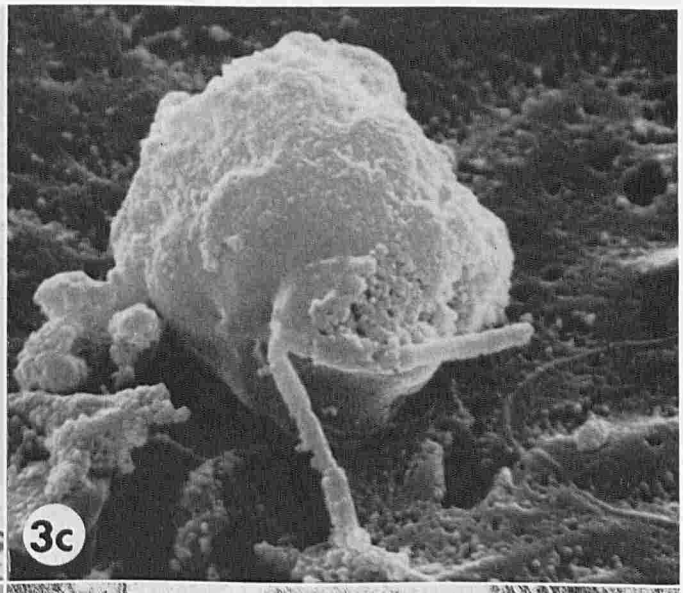
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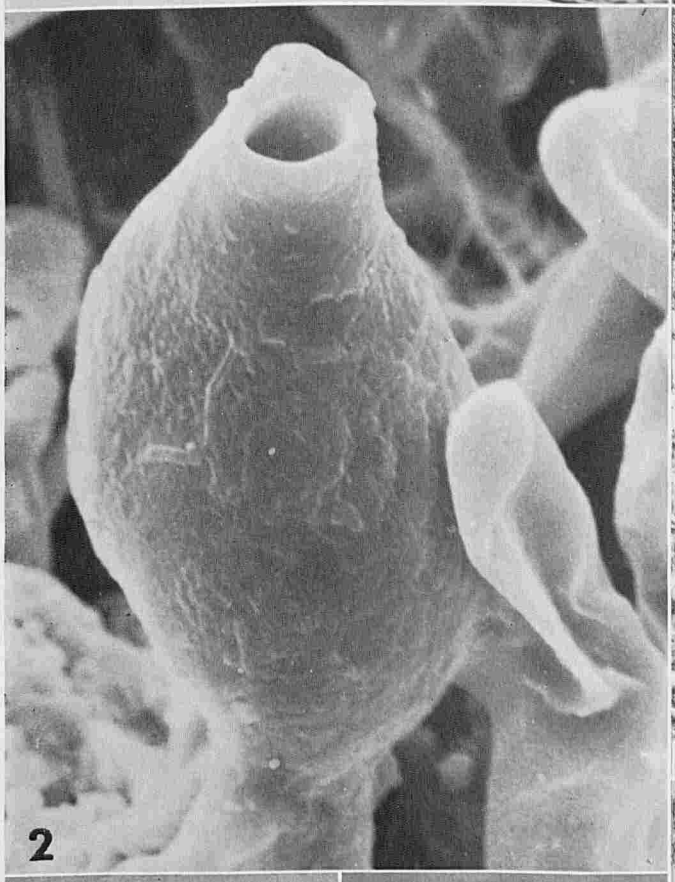
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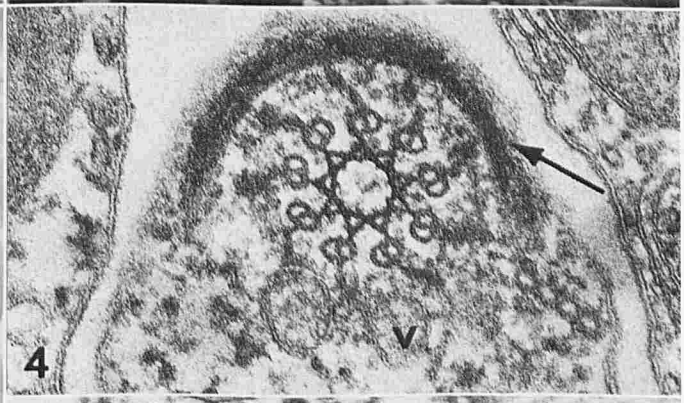
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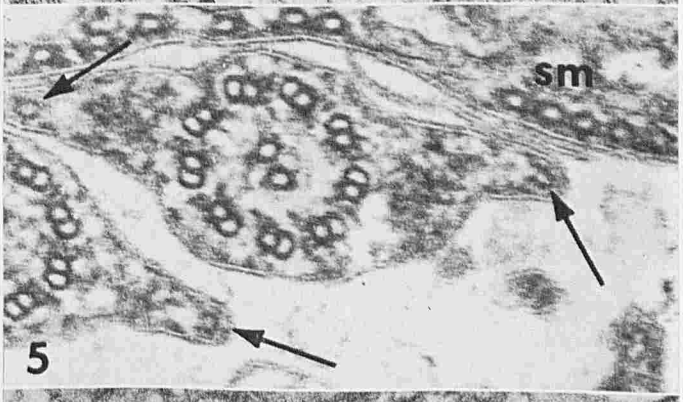
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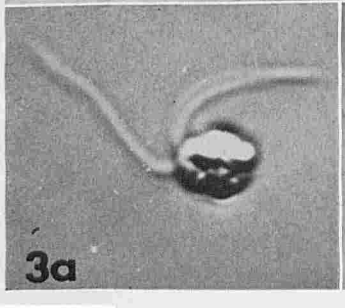
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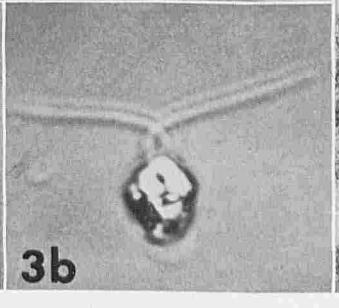
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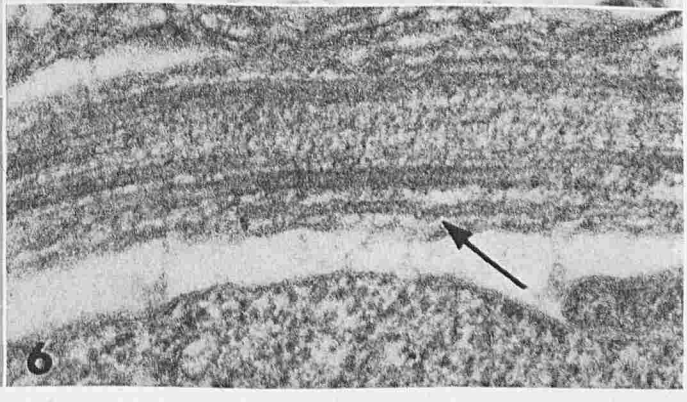
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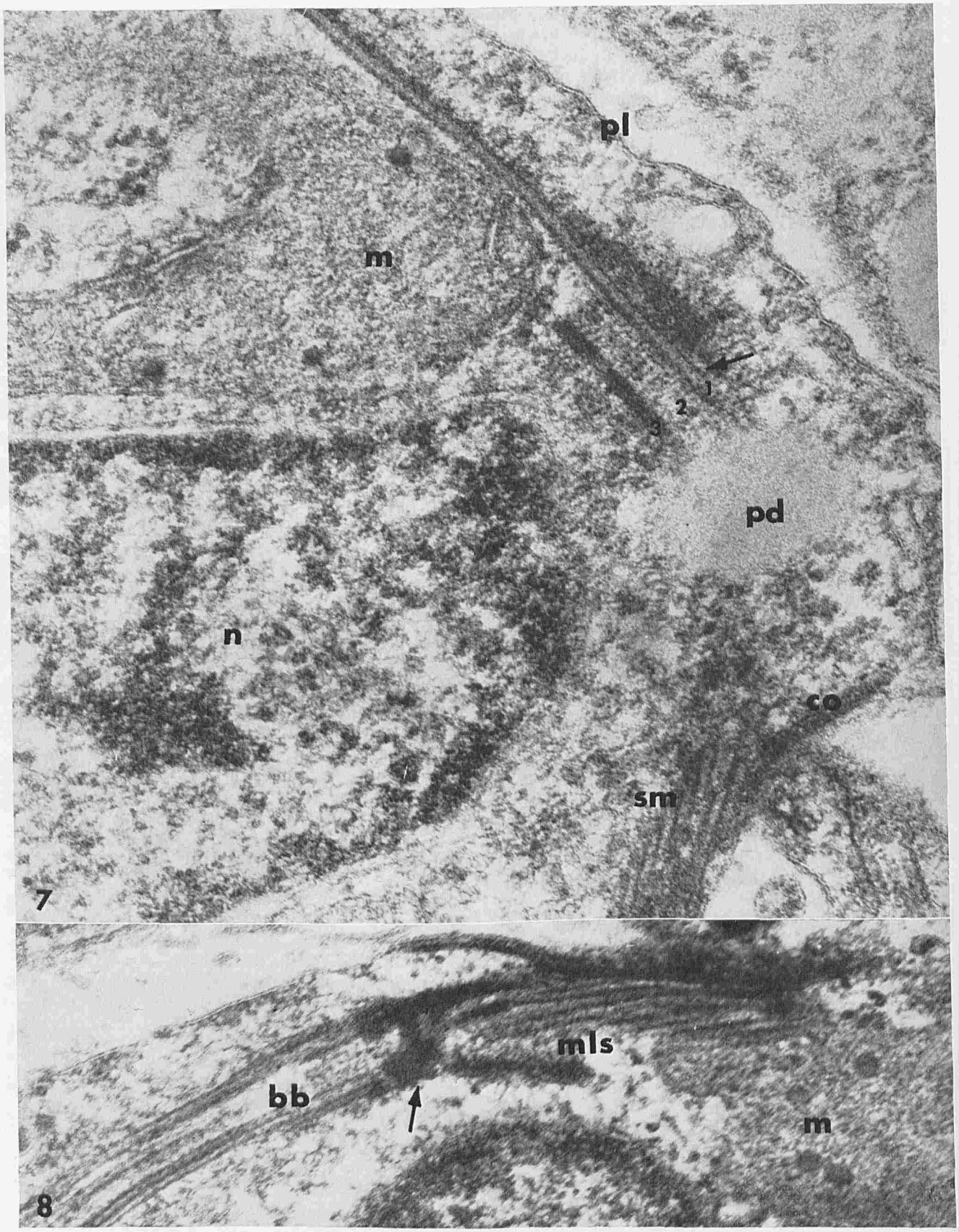
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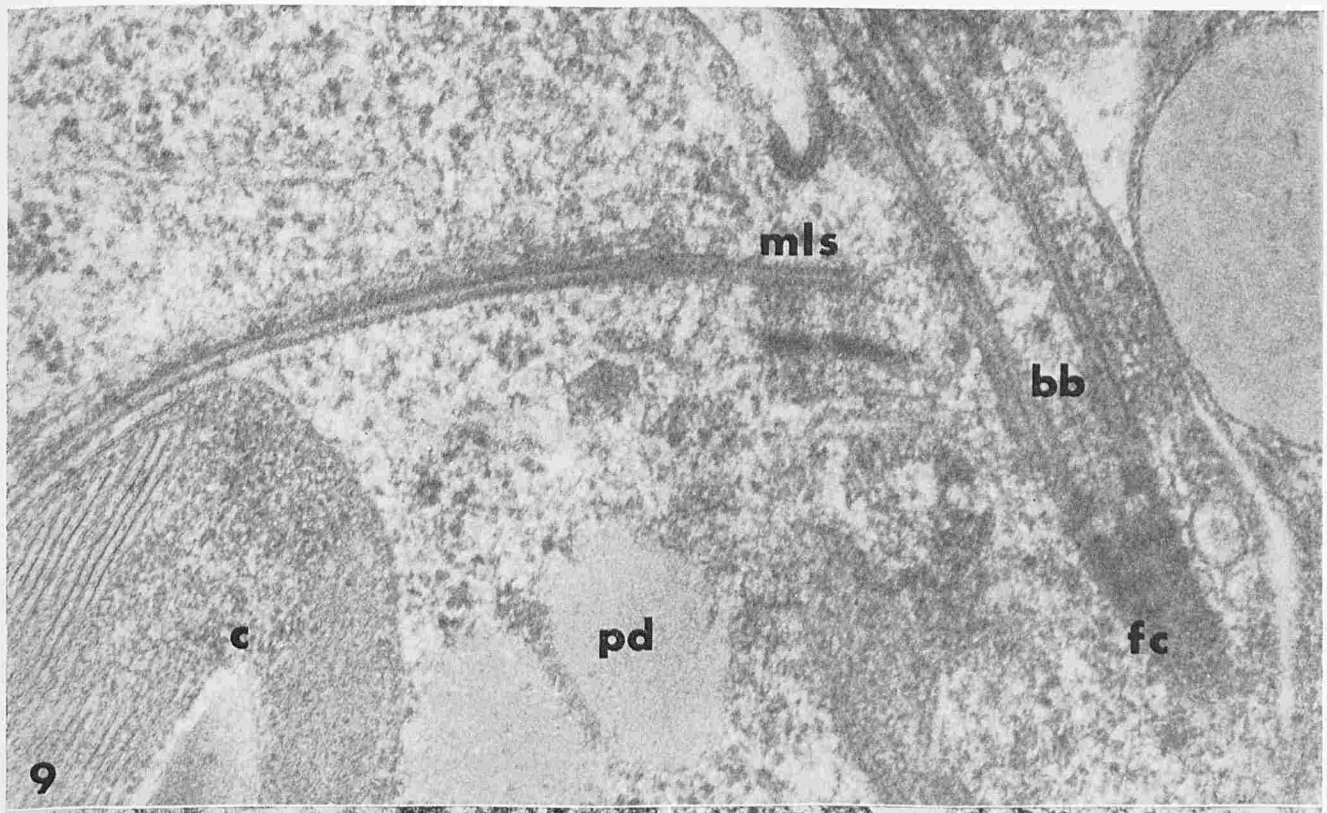

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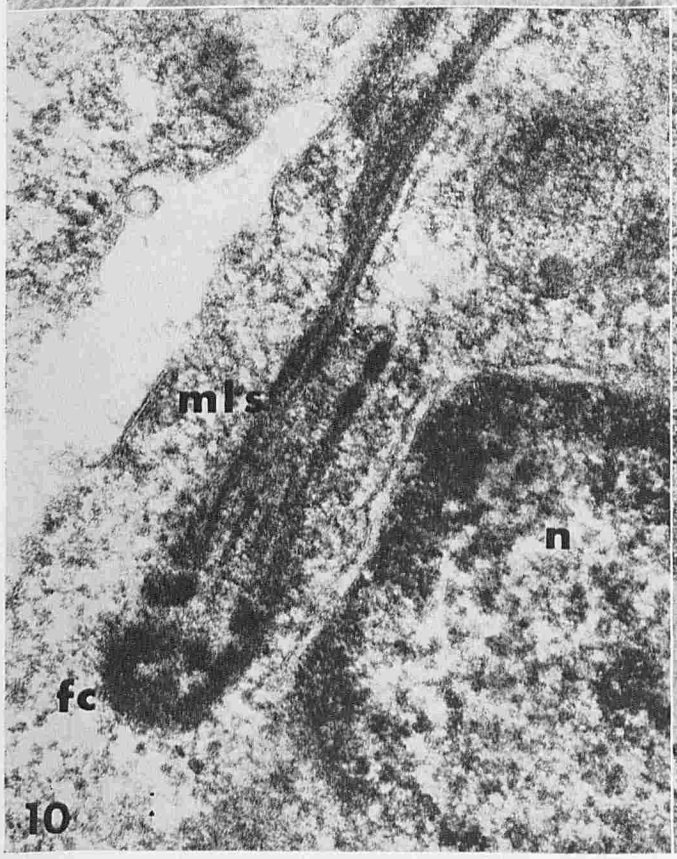

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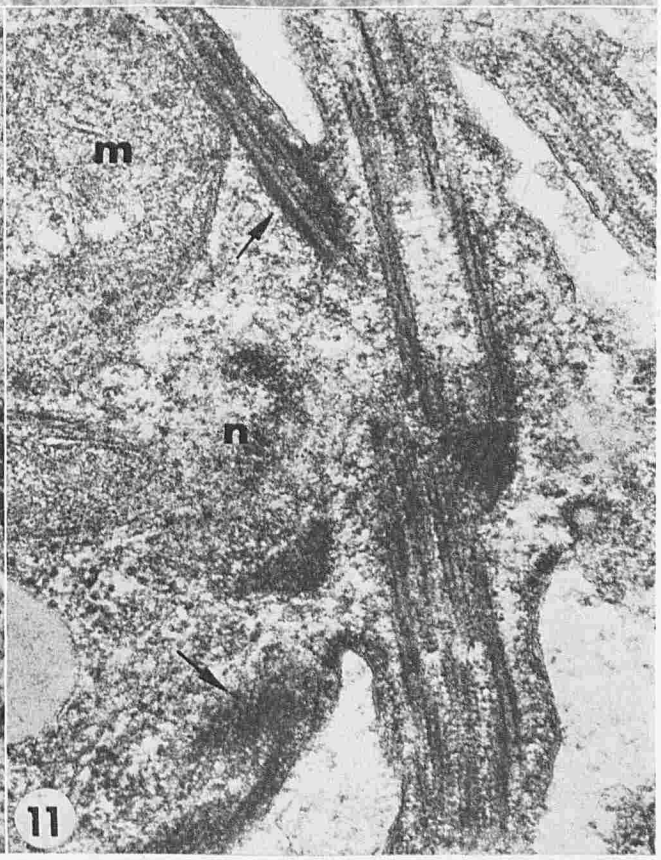

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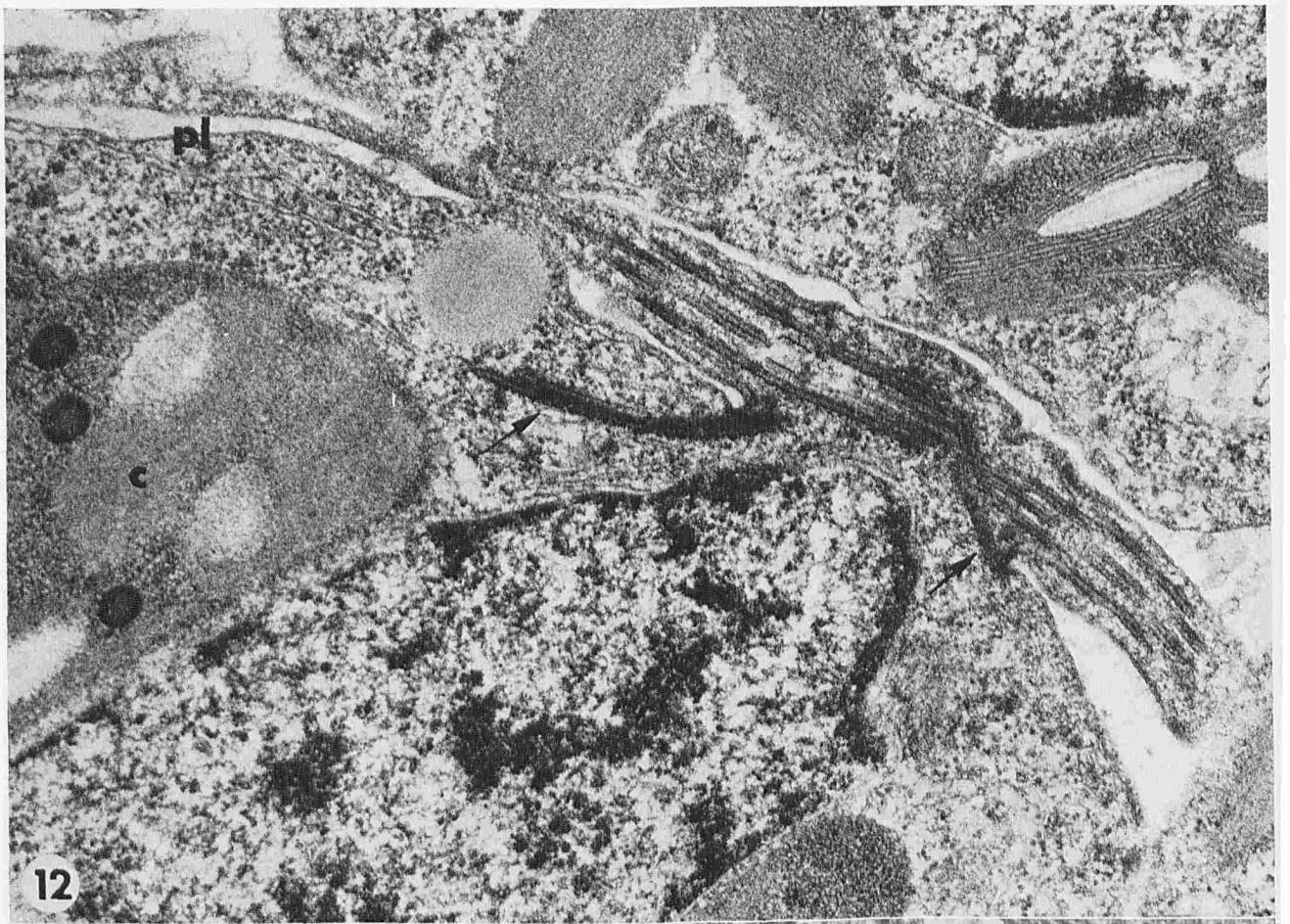
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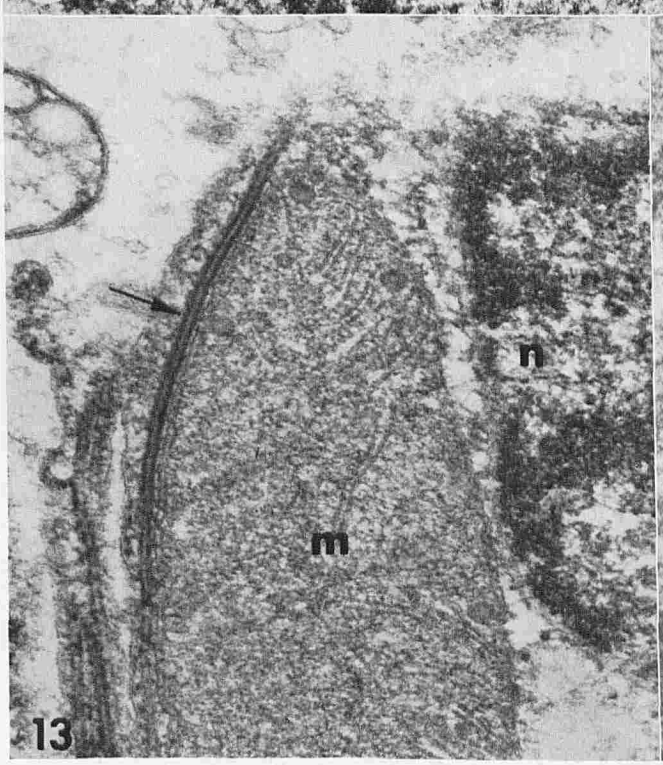
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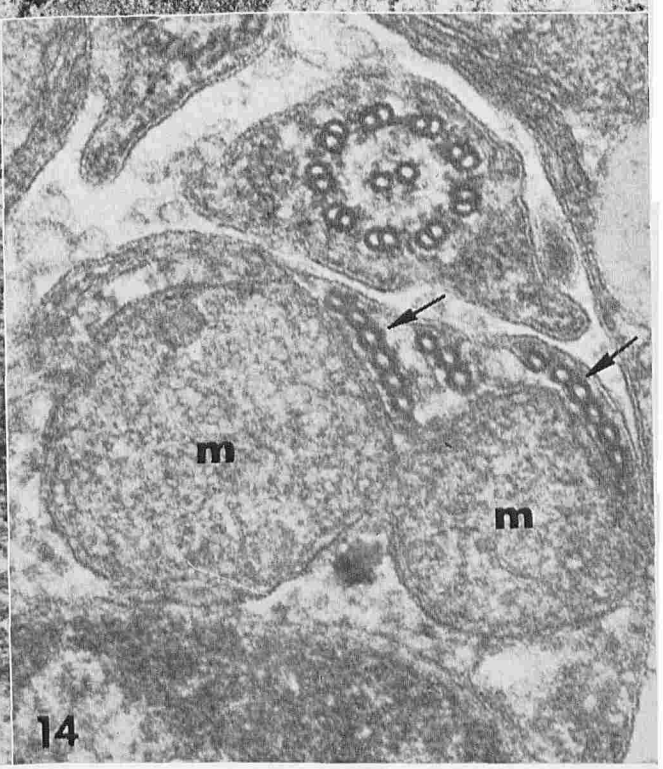
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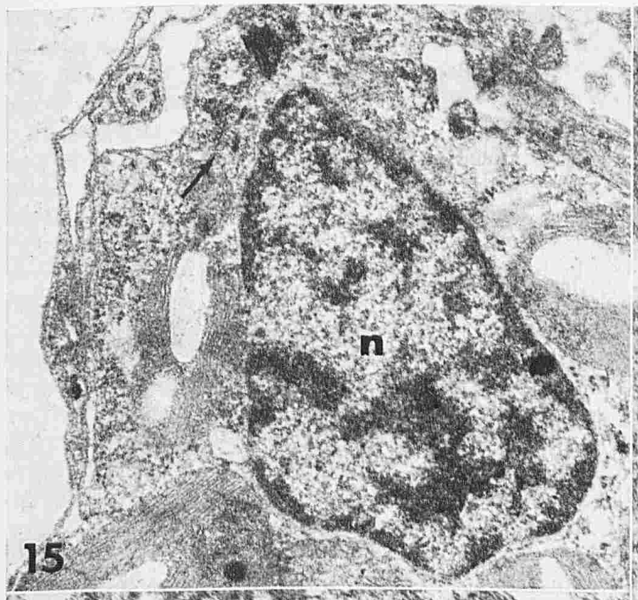
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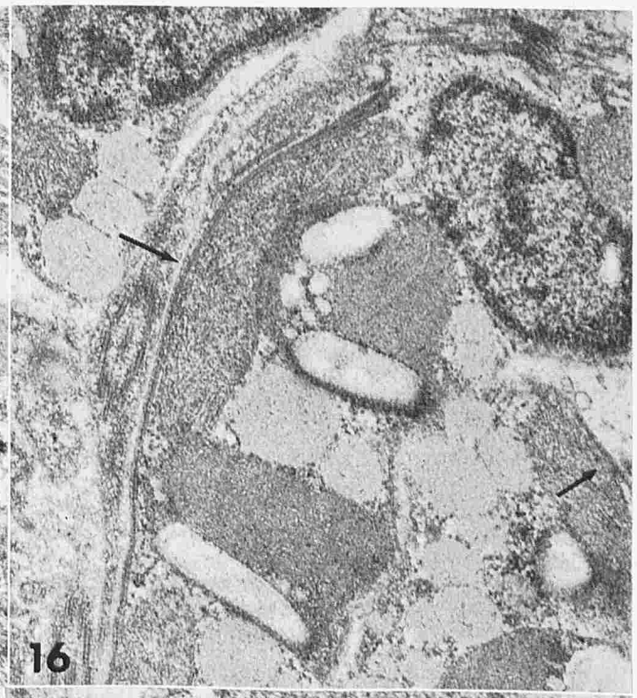
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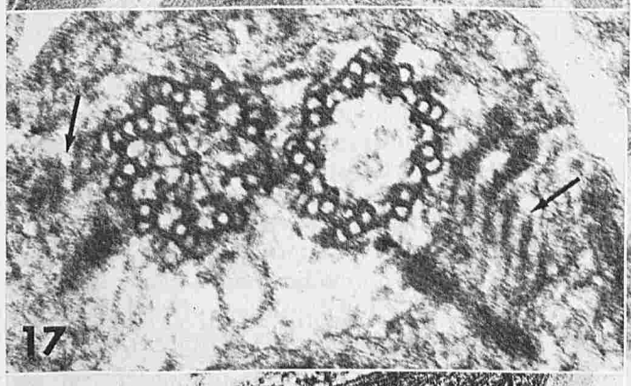
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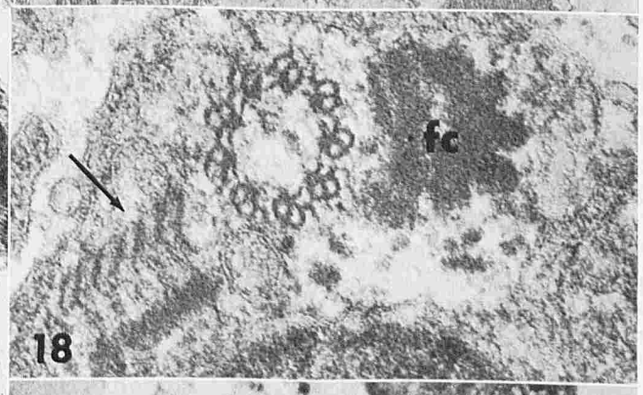
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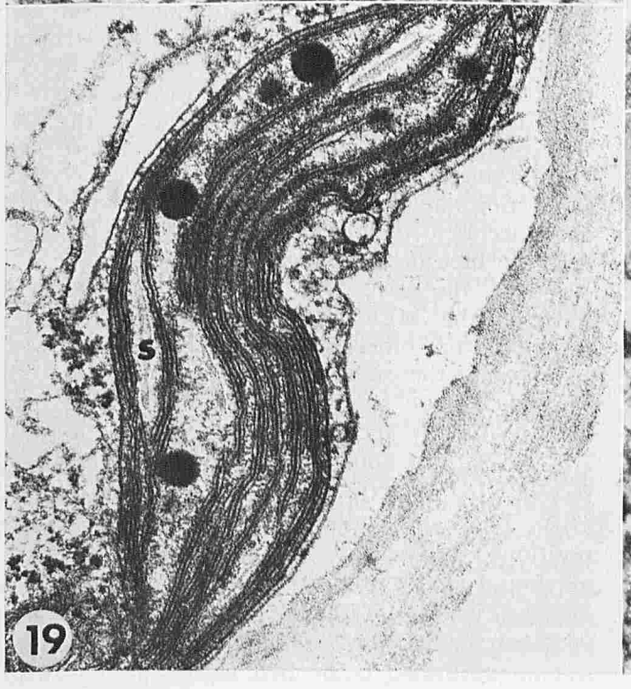
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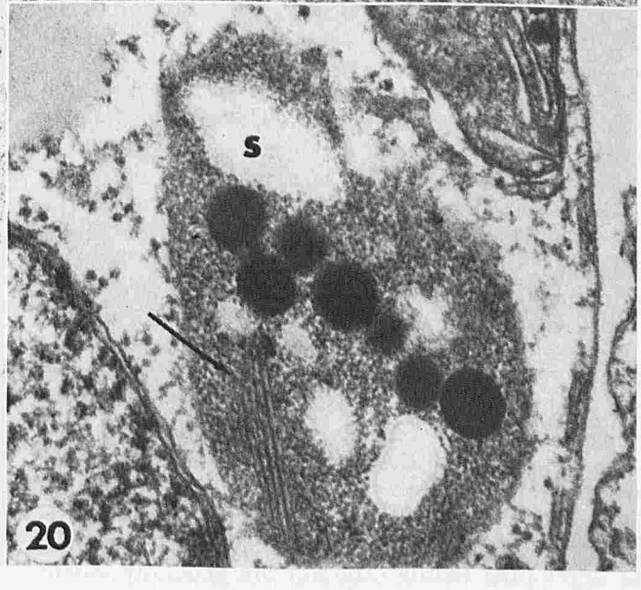
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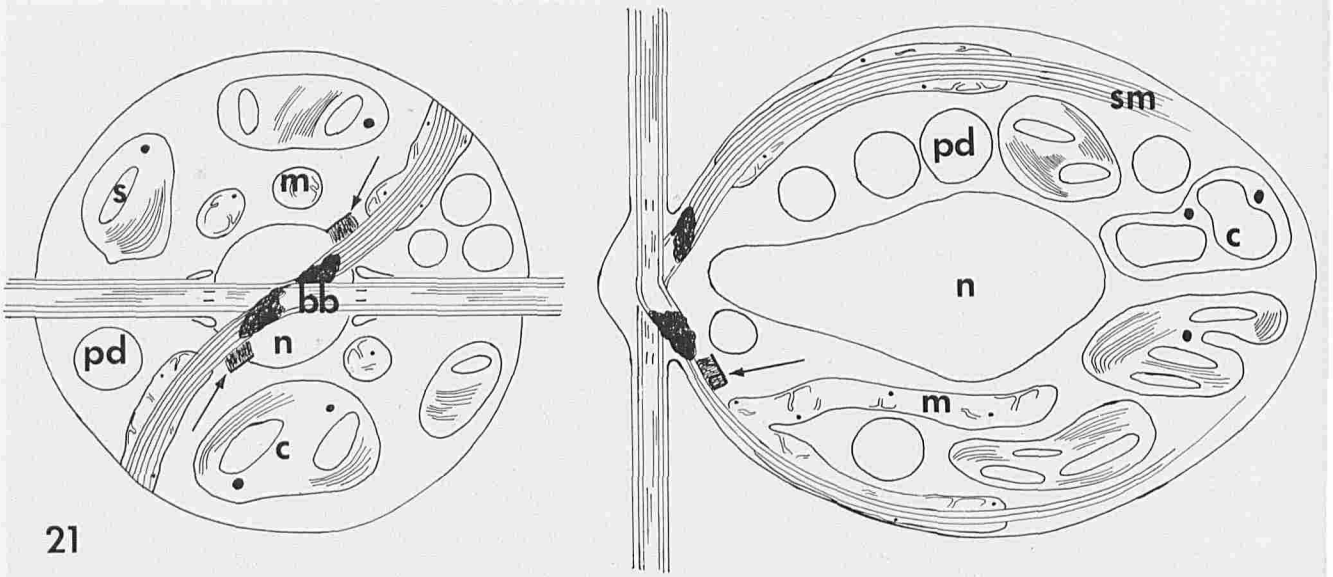

FIG. 21. Schematic overhead and side views of a motile cell of *Trentepohlia*. In this interpretation of the cytoskeletal arrangement, the basal bodies overlap so that each flagellum is connected to a MLS on the opposite side of the cell. × 15,000.

microtubules than the MLS. In some sections, the mitochondria appear to be branched at the anterior of the cell (Fig. 7). One arm of the mitochondrion remains appressed to the nucleus, while the other arm extends along the periphery of the cell. Frequently, both arms of a branched mitochondrion and associated spline microtubules are cross sectioned (Fig. 14).

During development of the motile cells, the MLS begins to appear before all cell divisions are complete. The upper layer of microtubules develops first. By the time the flagella have extended from the cell, the lower layers of the MLS have begun to form.

Small membrane-bounded vesicles can often be observed near the flagella bases (Fig. 4), and similar vesicles have been observed near basal bodies of other motile cells (*19*). The dark, curved line which can often be noted at the point where the flagella begin to extend from the cell (*arrow*, Fig. 4) probably represents a portion of a flagellar collar such as is described by Ringo in *Chlamydomonas* (*21*).

Much of the lower portion of the motile cells is filled with chloroplasts. Early in motile cell development, the chloroplasts resemble those of vegetative cells and show the typical green algal membrane arrangement with little accumulation of starch and lipid (Fig. 19). Later, in motile cell development, the photosynthetic membranes appear to have undergone considerable rearrangement (*arrow*, Fig. 20). The thylakoids are tightly packed together and large starch grains have developed. No pyrenoids have been observed at any developmental stage. Eyespots are not present. Golgi bodies can be observed in developing motile cells but are generally absent from more mature cells which are ready to be released. Large droplets which are ultrastructurally similar to the astaxanthin of *Haematococcus* (*8*) are also commonly observed in the cytoplasm of the zoospores (Fig. 7, 9). Carotenoidlike pigments contained in these droplets impart an orange color to the motile cells as viewed by light microscopy.

DISCUSSION

Line drawings of a motile cell of *T. aurea* showing the relationships between the flagella, basal bodies, MLS, mitochondria, nucleus, and other cell components can be found in Fig. 21. When the structure of the MLS of *T. aurea* is compared to the MLS of bryophytes (*2,4,17*), several differences are apparent.

First, the connection of basal bodies to the MLS is accomplished by means of a "central core" and "triplet microtubules" in bryophyte sperms (*3*). The connection between the basal bodies and MLS of *T. aurea* seems to be an electron-dense material which caps the base of each flagellum (Fig. 8).

Second, the position of the basal bodies with respect to the MLS of *Trentepohlia* is somewhat different from other MLS described. In bryophyte sperm and the zoospores of *Klebsormidium* and *Coleochaete* the basal bodies both originate from, and lie directly above, a single MLS. As a result, cross sections of both basal bodies can often be observed lying directly above the MLS in the cells (*3,4*). The basal bodies of *T. aurea* are never seen positioned over a MLS. Sections of one or both MLS are found at the sides of cross sections of the basal bodies (Fig. 15, 17, 18).

Third, the MLS of *Trentepohlia* generally contains 6–8 microtubules in the first layer. The sperms of the bryophytes, pteridophytes, cycads, and the zoo-

spores of *Klebsormidium* and *Coleochaete* usually contain more microtubules in the MLS apparatus. The MLS of *T. aurea* is probably the simplest MLS described with respect to the number of microtubules in the first layer. However, the motile cells of this alga are smaller (5–9 μ) than most MLS-containing cells. The size of the MLS may be a function of the size of the motile cell.

Fourth, only 1 MLS per flagella pair has been described in bryophyte sperms, and other algal zoospores (cycad and pteridophyte sperms contain a very long MLS with multiple flagella) (***1,5,16***). There appear to be 2 symmetrically arranged MLS per flagellar pair in *T. aurea*. In *T. aurea* the flagella are inserted apically in a symmetrical manner (Fig. 3*C*), while the flagella of *Klebsormidium* are inserted slightly laterally (asymmetrically) (***11***). Manton (***9***) has suggested that symmetry in the flagellar apparatus may be an important phylogenetic consideration. The apparent symmetry of the *Trentepohlia* cytoskeleton may represent the primitive condition—a link with algae possessing symmetrical roots—but no MLS. Alternatively, the cytoskeleton of *Trentepohlia* may be a specialized derivative of the asymmetrical cytoskeleton. Perhaps as more green algal motile cells are examined, we will discover more about how the MLS evolved, but at present we cannot definitely say how *Trentepohlia* might fit into such an evolutionary scheme.

The sperm of 2 species of *Chara* and 1 species of *Nitella* have been studied ultrastructurally (***15, 19,26***). Although sperms of both species of *Chara* show a microtubule band which extends down into the cell, no MLS has been described. In developing sperms of *Nitella*, Turner described a microtubule band similar to that of *Chara* (***26***), but no MLS was described. However, 2 of his pictures (Fig. 21, 22) show a vague, dense, layered material between the anterior end of the microtubule band and the nucleus. Although ill-defined, this material may represent a rudimentary MLS. Alternatively, the material may represent the remains of a MLS which is in the process of being lost. The question of the homology of the microtubule band (and nearby structures) in sperms of the Charophyta to the MLS and spline of the land plants and other algae needs additional study.

The type of cell division, whether phycoplast or phragmoplast, has not yet been described for *Trentepohlia*. Plasmodesmata are present in the cell walls of *Trentepohlia* and Stewart *et al.* (***25***) believe that mitosis in *Trentepohlia* may be similar to that in *Coleochaete*, which has a phragmoplast (***10***). *Coleochaete* and *Klebsormidium* are the only other algae which have been shown to possess a definite MLS in a motile cell (***11,14,20***). Pickett-Heaps considers these 2 genera to be aligned with the group of algae which gave rise to the land plants (***20***). When the mitotic characteristics of *Trentepohlia* have been described, it is likely that this alga will be placed phylogenetically with *Coleochaete* and *Klebsormidium*.

In the past, the ultrastructure of the mitotic process has proven most useful for studies of evolutionary trends in the green algae. The presence or absence of the MLS also appears to be a useful character for phylogenetic analysis. The motile cells of many other green algae should be examined for the presence of the MLS. One ultimate goal will be to determine which of the extant green algae are descendants of the ancient progenitors which gave rise to the land plants. Another goal will be to clarify the phylogenetic relationships among the various groups of the green algae.

NOTE ADDED IN PROOF

Moestrup has recently reported the presence of a MLS in the zoospore of *Chaetosphaeridium*, and this MLS is very similar to that found in the zoospore of *Coleochaete*. (Moestrup, Ø. 1974. Ultrastructure of the scale-covered zoospores of the green alga *Chaetosphaeridium*, a possible ancestor of the higher plants and bryophytes. Biol. J. Linnean Soc. **6**:111–25.)

ACKNOWLEDGMENT

We wish to thank Dr. Rufus Thompson for some helpful discussion during the early stages of this study. We also thank Dr. Jeremy Pickett-Heaps, P. Dayanandan, and Larry Allard for help with critical-point drying techniques. P. Dayanandan took the photo in Fig. 2. and John Mardinly took the photo in Fig. 3*C*.

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J. Phycol. **11**, 96–104 (1975)

DEVELOPMENT AND GERMINATION OF AKINETES OF *APHANIZOMENON FLOS-AQUAE*[1]

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SUMMARY

Flakes of Aphanizomenon flos-aquae *collected from an ice-covered lake were found to contain all developmental stages from vegetative cells to mature akinetes. Changes during development include increase in cell size, gradual disappearance of gas vacuoles (clusters of gas vesicles), narrowing of intrathylakoidal spaces, and increase in cytoplasmic density. Development of akinetes is accompanied by proliferation of ribosomes, including polyribosomes, cyanophycin granules (structured granules), and glycogen granules. The lipid bodies of vegetative cells are reduced in size and number in mature akinetes. Akinetes may occur singly or as multiples in sequence in a filament, either terminal or intercalary. Loss of flotation by increase in cytoplasmic density permits filaments to sink and overwinter in bottom sediments. The sequence was found to be reversed during germination of akinetes. Cyanophycin granules are reduced in size and staining density in the sporelings, and very few glycogen granules are seen. Gas vesicles reappear and increase in number, and intrathylakoidal spaces become wider. These changes then would permit the sporelings to rise from the bottom and begin another season's bloom.*

[1] *Received May 15, 1974; revised August 5, 1974.*

INTRODUCTION

The filamentous blue-green alga *Aphanizomenon flos-aquae* is one of the most common sources of summer "nuisance blooms" in shallow, alkaline lakes and ponds. The vegetative cells are buoyed up by internal gas vacuoles (clusters of gas vesicles) during summer blooms, and characteristic macroscopic flakes or rafts are formed by lateral contact of parallel filaments.

Rose (***19***) described the life history of *A. flos-aquae* at the light microscope level. Although he found healthy looking flakes in open water in Iowa in December, he concluded that vegetative propagation by fragmentation of the flake is not the usual method of overwintering. He found 1 or more akinetes (blue-green algal spores) in each filament of December-harvested flakes and assumed that these specialized cells serve to initiate each new season's growth.

Changes in ultrastructure during formation of akinetes in cultured *Anabaena cylindrica* (***26***) and *Cylindrospermum* sp. (***2,13,17***) and during germination of akinetes of *Cylindrospermum* sp. (***17***) have been reported.

In our study, green flakes of *A. flos-aquae* beneath lake ice were found to contain numerous akinetes in various stages of development. Examination of these stages by transmission electron microscopy revealed