

ULTRASTRUCTURE AND DIFFERENTIATION OF *HYDRODICTYON RETICULATUM*

III.* FORMATION OF THE VEGETATIVE DAUGHTER NET

By H. J. MARCHANT†† and J. D. PICKETT-HEAPS‡

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Abstract

Vegetative zooids of *H. reticulatum*, produced by cleavage of parental coenobial cytoplasm, linked together within their parental cell walls to form cylindrical nets characteristic of this alga. A conspicuous feature of net-forming zooids were bands of microtubules underlying the plasmalemma. An active role is proposed for these microtubules in the ordered linking of the zooids. Amorphous material, presumably adhesive, was seen only in intercellular spaces between aggregating zooids. Following adhesion of the zooids, each one usually linking with four others, their flagella were retracted and both flagellar microtubules and basal bodies disintegrated. Centrioles arose *de novo* on the nuclear envelope of each cell at the time of deposition of a bilayered wall.

I. INTRODUCTION

During both asexual and sexual phases of the life cycle of *Hydrodictyon reticulatum*, uninucleate, biflagellate zooids are produced following cytoplasmic cleavage of coenocytic parental cells. Vegetative zooids link together within the parental cell wall to form cylindrical nets; in contrast, smaller "germ" nets, often flat, are formed from similar vegetative zooids produced within polyhedra late in the sexual cycle. A diagrammatic summary of the life cycle of *H. reticulatum* is included in Marchant and Pickett-Heaps' (1971) paper on the development of zooids. We describe here the structure of net-forming zooids produced by the cylindrical coenobia, their linking together to form cylindrical daughter nets, and aspects of the early development of the net. The formation of the germ net will be considered later.

Not unexpectedly, daughter-net formation has received considerable attention from light microscopists. Pocock (1960) reviews comprehensively the earlier work as well as contributing new and valuable observations, stressing in particular the role of the vacuolar envelope in this unique process. Recently, Hawkins and Leedale (1971) described the ultrastructure of zoospores (zooids) of *H. reticulatum* and various species of *Pediastrum*. However, because they used osmium fixation alone, some details of the structure and events in the aggregation of the zooids were not observed.

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† Research School of Biological Sciences, Australian National University, Canberra, A.C.T.; present address: Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80302, U.S.A.

‡ Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80302, U.S.A.

II. MATERIALS AND METHODS

In this investigation we used a culture of *H. reticulatum*, a gift from the Curator of the Culture Centre of Algae and Protozoa, Cambridge, England, as well as an Australian strain of the same species (Marchant and Pickett-Heaps 1970). Both were maintained in Juller's liquid medium with soil extract added (see Pocock 1960, p. 179) under the lighting conditions detailed in Marchant and Pickett-Heaps (1970).

Fixation of differentiating cells in glutaraldehyde followed by osmium tetroxide and their subsequent processing for electron microscopy were the same as described previously (Marchant and Pickett-Heaps 1970, 1971). A Zeiss microflash was used on a Zeiss Universal microscope for photomicrography of living cells.

III. OBSERVATIONS

(a) Structure of the Net-forming Zooids

Basal bodies of the biflagellate zooids sometimes appeared at right angles to one another but more often their axes lay nearly 180° apart (Figs. 4, 5, 6).^{*} In some sections the cores of the basal bodies appeared interconnected (Fig. 5) and they were also externally linked by an ill-defined bridge (Figs. 6, 7), not a striated fibre as found between basal bodies in some other algae and protozoa (Ringo 1967; Pitelka 1969; Hoffman 1970). From near this bridge emanated four bands of 5–8 rootlet microtubules lying close to the plasmalemma (Figs. 1, 4, 5, 7, 8, 9). Living zooids had a pair of contractile vacuoles which pulsed alternately (Figs. 2, 3). The endoplasmic reticulum often appeared markedly hypertrophied (Figs. 1, 22), but this may be artifactual. The disposition and structure of the chloroplast(s) has been described by Hawkins and Leedale (1971). In some sections, a distinctive group of 5–8 microtubules, each surrounded by diffuse material, lay near the outer membrane of the chloroplast (Figs. 10, 11); these appeared similar to rootlet microtubules that might have separated. Elsewhere, numerous other parallel microtubules were deployed in localized arrays around the cell's periphery (Figs. 12, 13, 15, 16, 17). Their significance will be described below.

(b) Formation of the Daughter Net

Since we agree substantially with Pocock's (1960) account of net formation *in vivo*, only a brief résumé of this aspect precedes our ultrastructural observations. Normally, newly formed zooids were confined to the small volume between the vacuolar envelope and parental wall, where they jostled more and more vigorously,

^{*} Abbreviations used in Figures 1–34 are as follows: *b*, basal body; *ch*, chloroplast; *cv*, contractile vacuole; *f*, flagellum; *g*, golgi body; *m*, mitochondria; *n*, nucleus; *p*, pyrenoid; *s*, starch granule; *t*, microtubules; *v*, vacuole; *ve*, vacuolar envelope.

Fig. 2.—Living net-forming zooid showing contractile vacuoles (arrowhead). Phase contrast. $\times 1300$.

Fig. 3.—The same zooid as shown in Figure 2 photographed as one of the contractile vacuoles is discharging (arrowhead). Phase contrast. $\times 1300$.

Fig. 4.—Flagellar end of a net-forming zooid showing the four bands of rootlet microtubules (arrows) and vesicles within the basal bodies (small arrow). $\times 32,000$.

Fig. 5.—Interconnection between the cores of basal bodies (arrowhead). Note also the four bands of rootlet microtubules (arrows). $\times 53,000$.

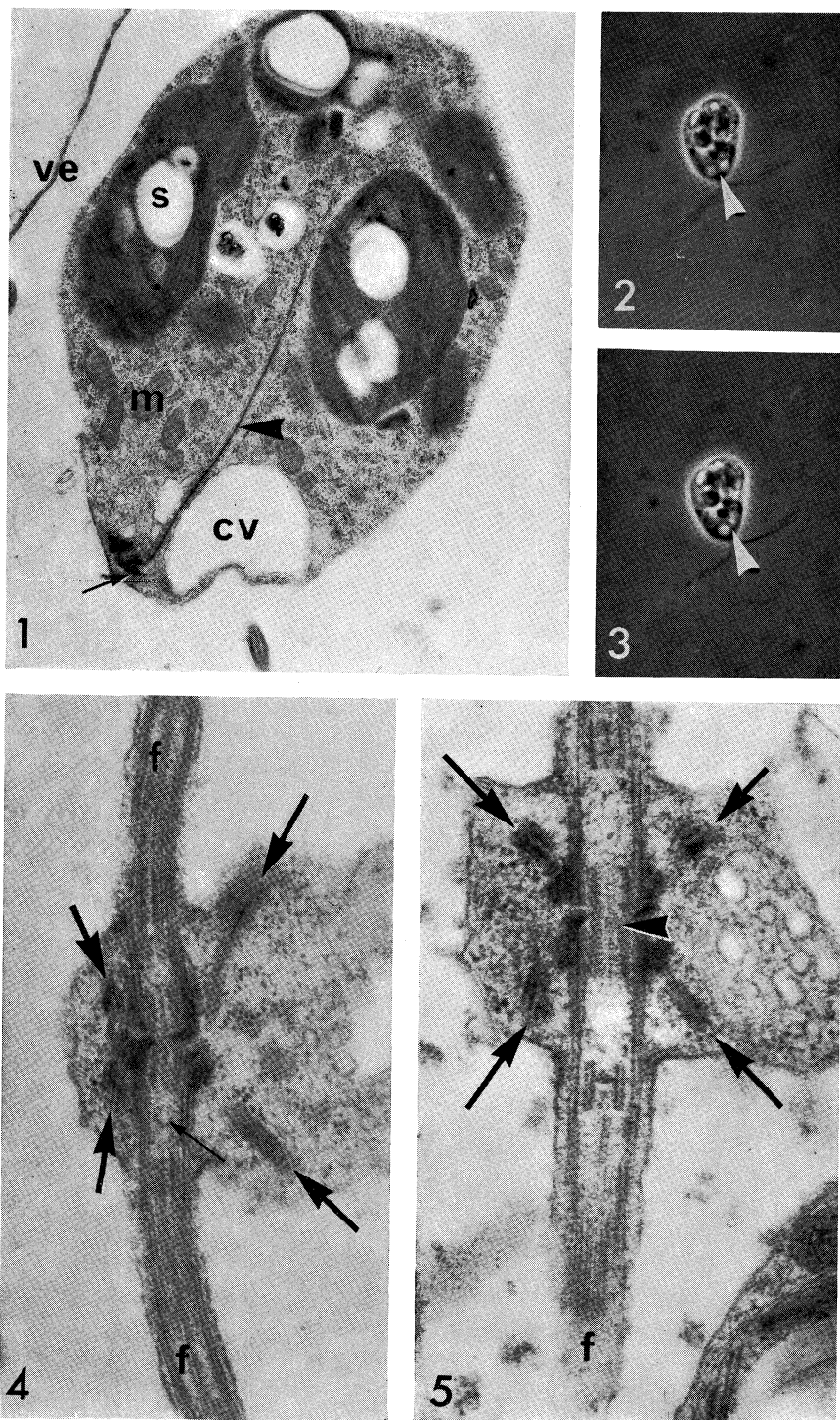


Fig. 1.—Electron micrograph of a net-forming zooid; the nucleus is out of the plane of the section. Note the vacuolar envelope (*ve*) and the rootlet microtubules (arrowhead) emanating from the basal body region (arrow). $\times 11,000$.

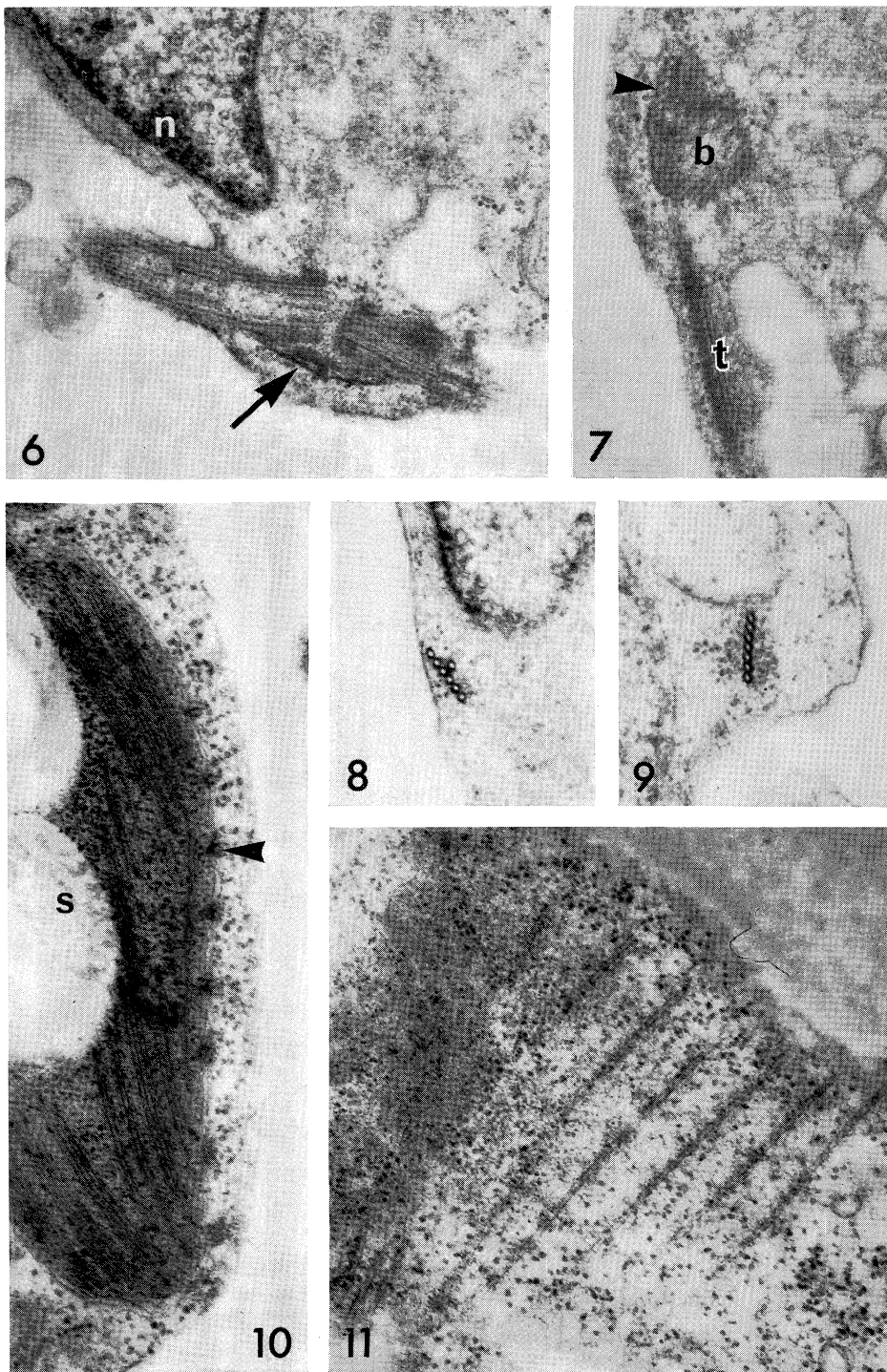


Fig. 6.—Bridge interconnecting basal bodies (arrow). $\times 35,000$.

Fig. 7.—Two bands of rootlet microtubules, one cut transversely (arrowhead) the other longitudinally, arising from near bridge between basal bodies. $\times 42,000$.

Fig. 8.—Rootlet microtubules sectioned transversely. $\times 53,000$.

Fig. 9.—Different configuration of rootlet microtubules to that shown in Figure 8. $\times 52,000$.

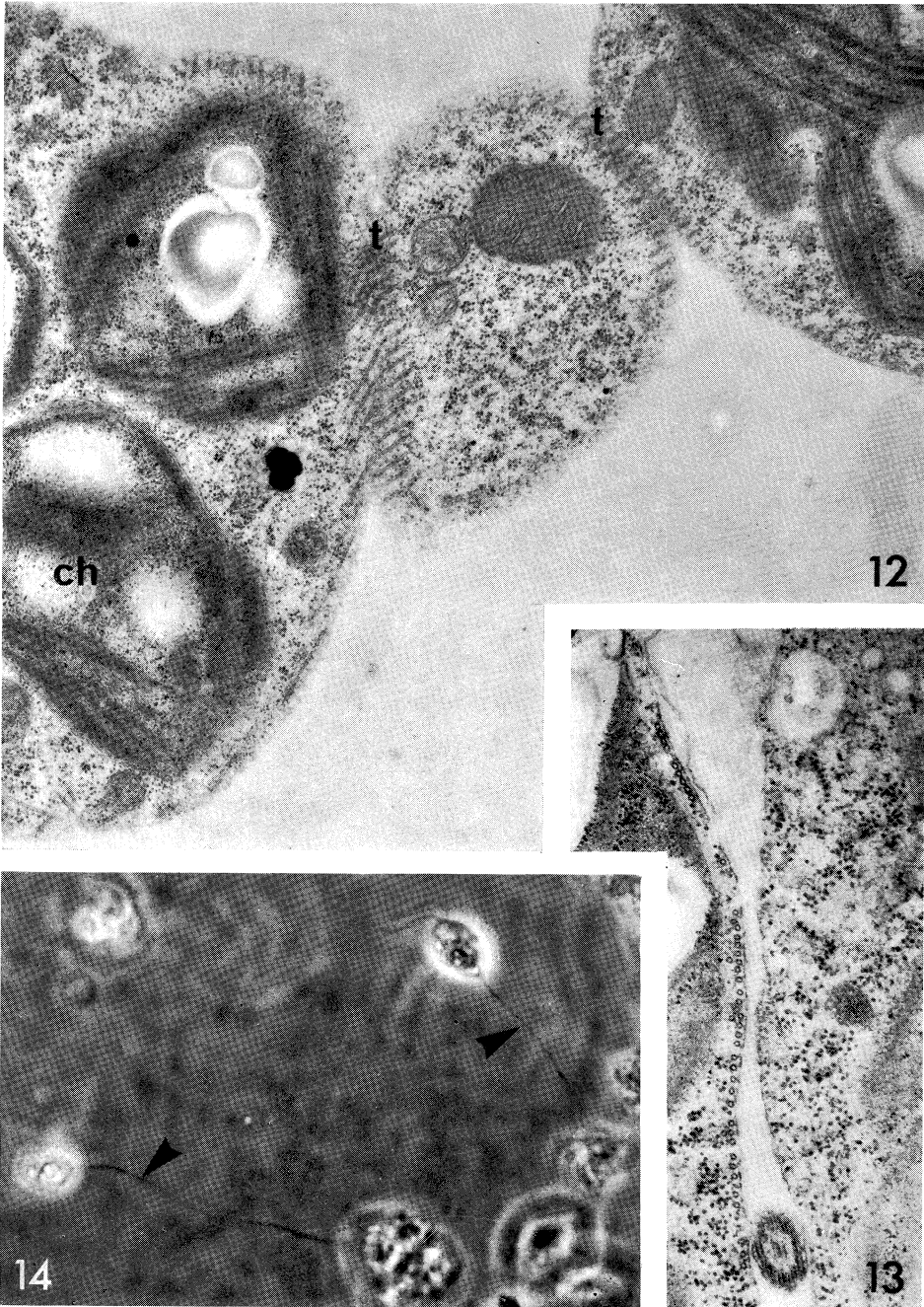


Fig. 12.—Three zooids in contact. Note the arrays of peripheral microtubules underlying their plasmalemmas in contact but the relatively few microtubules elsewhere. $\times 31,000$.

Fig. 13.—Periphery of two net-forming zooids showing a band of peripheral microtubules in one but lacking in the other—cf. Figure 16. $\times 27,000$.

Fig. 14.—Zooids connected *in vivo* by fine cytoplasmic strands (arrowheads). Phase contrast. $\times 1300$.

Fig. 10.—Microtubules surrounded by diffuse material appressed and apparently indenting (arrowhead) the chloroplast membrane. $\times 53,000$.

Fig. 11.—Longitudinally sectioned microtubules adjacent to the chloroplast. $\times 35,000$.

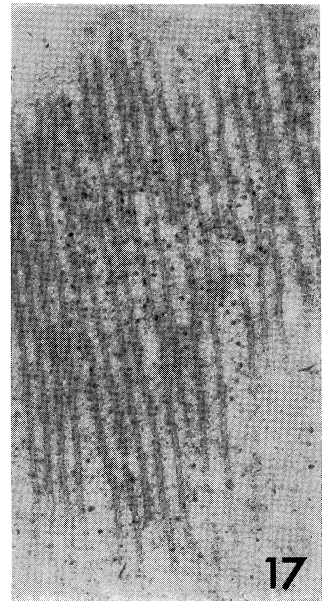
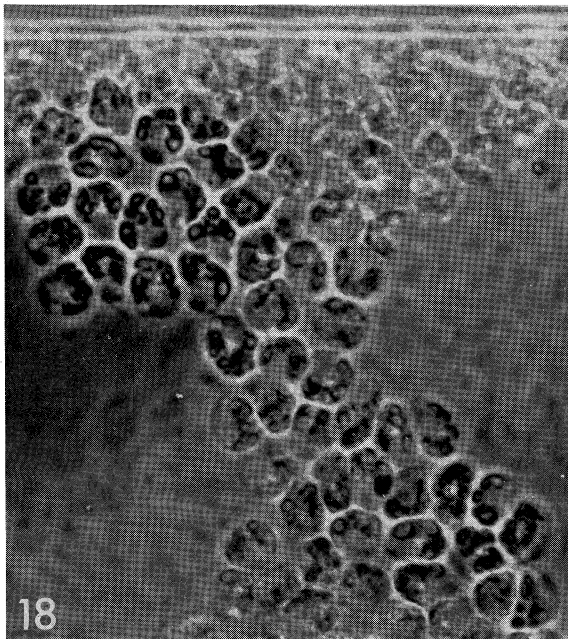
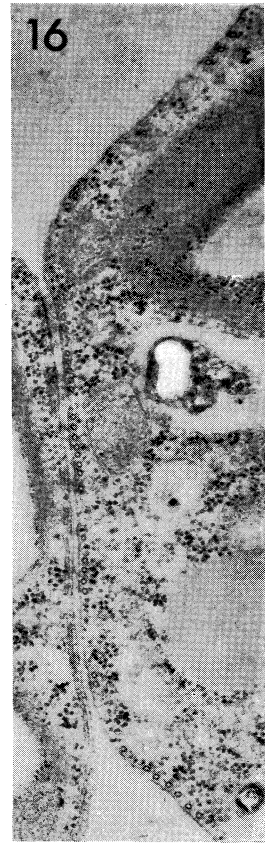
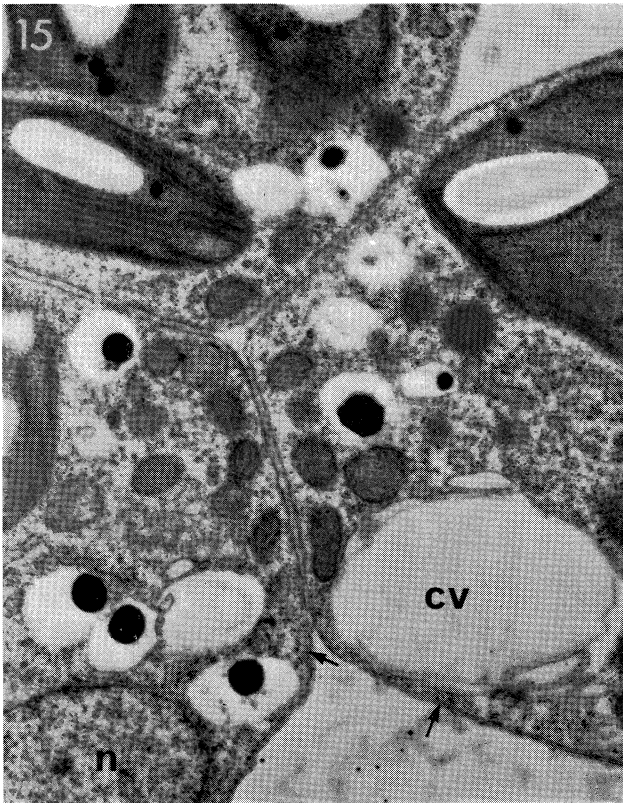


Fig. 15.—Adhering zooids sectioned nearly perpendicular to those in Figure 12. Note the longitudinally sectioned microtubules (arrows) and the amorphous “adhesive” only in the flattened intercellular spaces. $\times 13,000$.

rendering observations *in vivo* difficult; this activity soon diminished, however, as each zooid began to link with, usually, four others. The vacuolar envelope is of paramount importance in determining the overall shape of the forming daughter net; if this envelope developed abnormally or was damaged, irregular nets resulted. Most strikingly the zooids changed their shape; their outline, initially oval, became rhomboidal (Figs. 18, 20, 22, 29; cf. Figs. 1, 2) as they aggregated.

Electron microscopy showed that linking together of the closely packed zooids began with the apposition of those areas of their plasmalemmas under which lay the localized bands of microtubules mentioned above (Figs. 12, 16). Such microtubular arrays were also present in zooids before aggregation (see earlier, and Fig. 13). The zooids were generally flattened at these sites, where amorphous material appeared in the intercellular space (Figs. 15, 16). As the zooids aggregated, their flagella were retracted. Flagella of living zooids were seen to straighten (Fig. 19) and apparently become paralysed before shortening to about one-half to one-third of their original length (Fig. 20); they then folded back along the cell and disappeared (Fig. 21). Electron microscopy revealed flagellar microtubules lacking a membrane within the zooid's cytoplasm (Figs. 22, 23), suggesting that flagella had been assimilated by fusion of their membranes with the plasmalemma. Following retraction, both the flagellar microtubules and apparently the basal bodies disintegrated (Figs. 23, 25, 26).

Figure 14 illustrates the rare occurrence of living zooids interconnected by fine cytoplasmic threads, which parted before the zooids aggregated to form daughter nets. Sometimes during net formation, the parental cell wall ruptured liberating zooids into the culture medium. These zooids usually disintegrated but occasionally they aggregated to form small irregular nets (Pocock 1960, p. 235) or adhered laterally to form chains (Fig. 24.)

(c) *Early Development of the Net*

Once the cells had adhered to one another they first secreted a thin, densely staining, "membrane-like" layer of wall over their entire surface while microtubules remained near the plasmalemma (Fig. 15). This membrane-like appearance of the outer layer of wall did not persist, however, as the cells aged (Fig. 34). Microtubules were no longer evident, however, once the secretion of the thicker, fibrillar inner layer had begun (Figs. 32, 33). Shortly after the start of wall deposition, each cell in the new net underwent considerable internal reorganization. The pyrenoid and surrounding starch grains soon reformed (Fig. 32). After the retraction and disintegration of flagellar microtubules and presumably the basal bodies, new centrioles developed, apparently *de novo*, on the nuclear envelope (Figs. 27, 28). These were very short and hardly recognizable at first but soon elongated. Vacuoles appeared initially at either end of the growing cells (Figs. 31, 32; cf. Fig. 30), later fusing to form a large single vacuole enclosed by cytoplasm.

Fig. 16.—Flattened sites of two zooids in contact which may have linked abnormally. Note the microtubules are sectioned longitudinally in one and transversely in the other. Also note the "adhesive" only in the intercellular space. $\times 27,000$.

Fig. 17.—Section grazing a band of peripheral microtubules. $\times 35,000$.

Fig. 18.—Light micrograph of an early stage in net formation. The parental coenobia was starved during growth. Note the rhomboidal outline of the packed zooids. Phase contrast. $\times 1300$.

IV. DISCUSSION

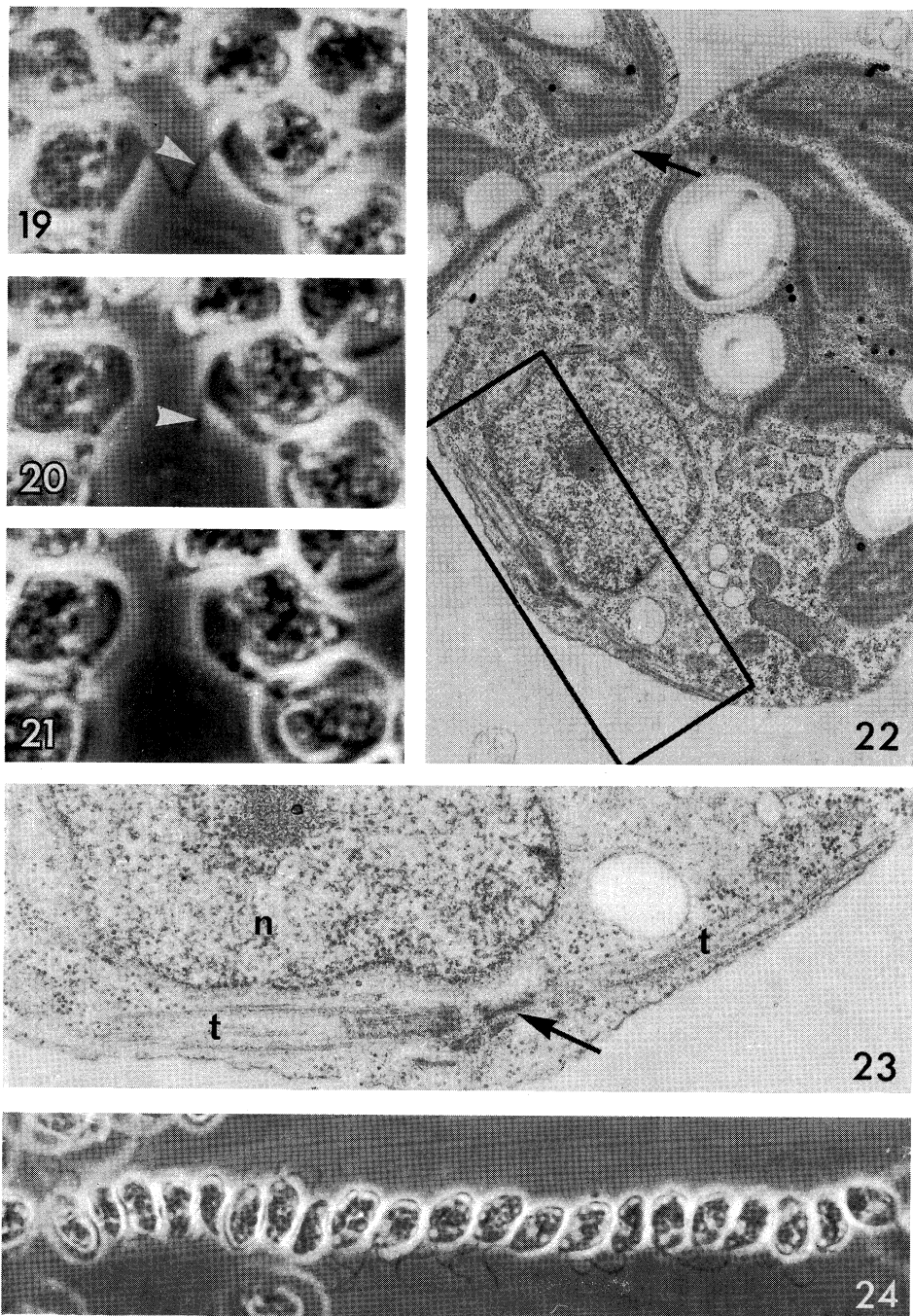
Earlier (Marchant and Pickett-Heaps 1971), we described how the vacuolar envelope cleaves from the coenobial cytoplasm before the zooids differentiate and we briefly mentioned its importance in determining the form of the daughter net. There has been considerable confusion in the past as to its precise role in this intriguing phenomenon; many early microscopists thought that the zooids were permanently connected either to one another or to the vacuolar envelope by fine cytoplasmic threads (see Fritsch 1935 and Pocock 1960 for references). On occasions we also have seen zooids connected by slender cytoplasmic strands; these appear to result from incomplete cleavage of the parental cytoplasm and play no part in the orderly linking of the zooids. As Hawkins and Leedale (1971) suggest "a planar orientation is a feature of colony forming zoospores" in both *Pediastrum* and *Hydrodictyon*. We agree with Pocock (1960) that when cylindrical daughter nets form in *H. reticulatum*, the vacuolar envelope acts as a mould around which swarm a single layer of zooids apparently mutually attracted to one another.

The basic mechanisms involved in the linking of zoospores appears similar whether the colonies formed are flat (e.g. germ nets of *H. reticulatum*: Marchant and Pickett-Heaps, unpublished data; colonies of *Pediastrum*: Millington and Gawlik 1970; Hawkins and Leedale 1971) or are cylindrical (e.g. the daughter net of *H. reticulatum*). Microtubules underlie the plasmalemma of zooids of both *P. boryanum* (Gawlik and Millington 1969; Millington and Gawlik 1970) and *H. reticulatum* and in both cases are particularly conspicuous at their flattened sites of contact. Such bands of peripheral microtubules, however, have never been seen underlying the plasmalemma of gametes (Marchant and Pickett-Heaps 1972). Millington and Gawlik (1970) comment on the "remarkable but not exact and possible coincidental alignment of microtubules in adjacent adherent cells" in *P. boryanum* without considering that they might be significantly involved in colony formation. Hawkins and Leedale (1971) using osmium fixation alone, did not preserve these microtubules in either *P. boryanum* or *H. reticulatum* and refer to their occurrence in *P. boryanum* (described in Millington and Gawlik's 1970 paper) in terms of "expressions of cell orientation rather than a causal mechanism in the process" (*of colony formation*). They then describe a mechanism of aggregation not requiring any involvement of microtubules.

The precise function of these peripheral microtubules, present both before as well as during the linking of zooids, is unclear; we suspect that they are involved in changing the shape of the zooids from being oval to rhomboidal in outline, and in the localized flattening at the sites of contact with other zooids. This flattening, by increasing the area of contact between the zooids, may facilitate their subsequent adhesion. Following treatment with colchicine, net-forming zooids often fail to link up and those that do form highly irregular nets comprised of generally misshapen cells (Marchant and Pickett-Heaps, unpublished data). Colchicine depolymerizes microtubules, whose role in maintaining or altering the shape of cells has been widely documented (reviews of Porter 1966, Newcomb 1969, Pickett-Heaps 1972). Our colchicine

Fig. 23.—Detail of enclosed area in Figure 22. Flagellar microtubules in cytoplasm, basal body (arrow) apparently disintegrating. $\times 24,300$.

Fig. 24.—Light micrograph of zooids released from ruptured parental cell linking up in the culture medium. Notice that they are adhering only laterally. Phase contrast. $\times 1200$.



Figs. 19–21.—Stages in the retraction of flagella about 10 seconds between each exposure. All phase contrast. All $\times 2200$. 19, Flagellum “paralysed” and shortening. 20, Shortening and banding towards zooid. Note that the flagellum on the zooid to the left has disappeared. 21, Disappearance of flagellum.

Fig. 22.—Flagellar microtubules devoid of a flagellar membrane lying within zooid’s cytoplasm. The enclosed area is shown at higher magnification in Figure 23. Note the microtubules at the site of contact between zooids (arrow). $\times 11,700$.

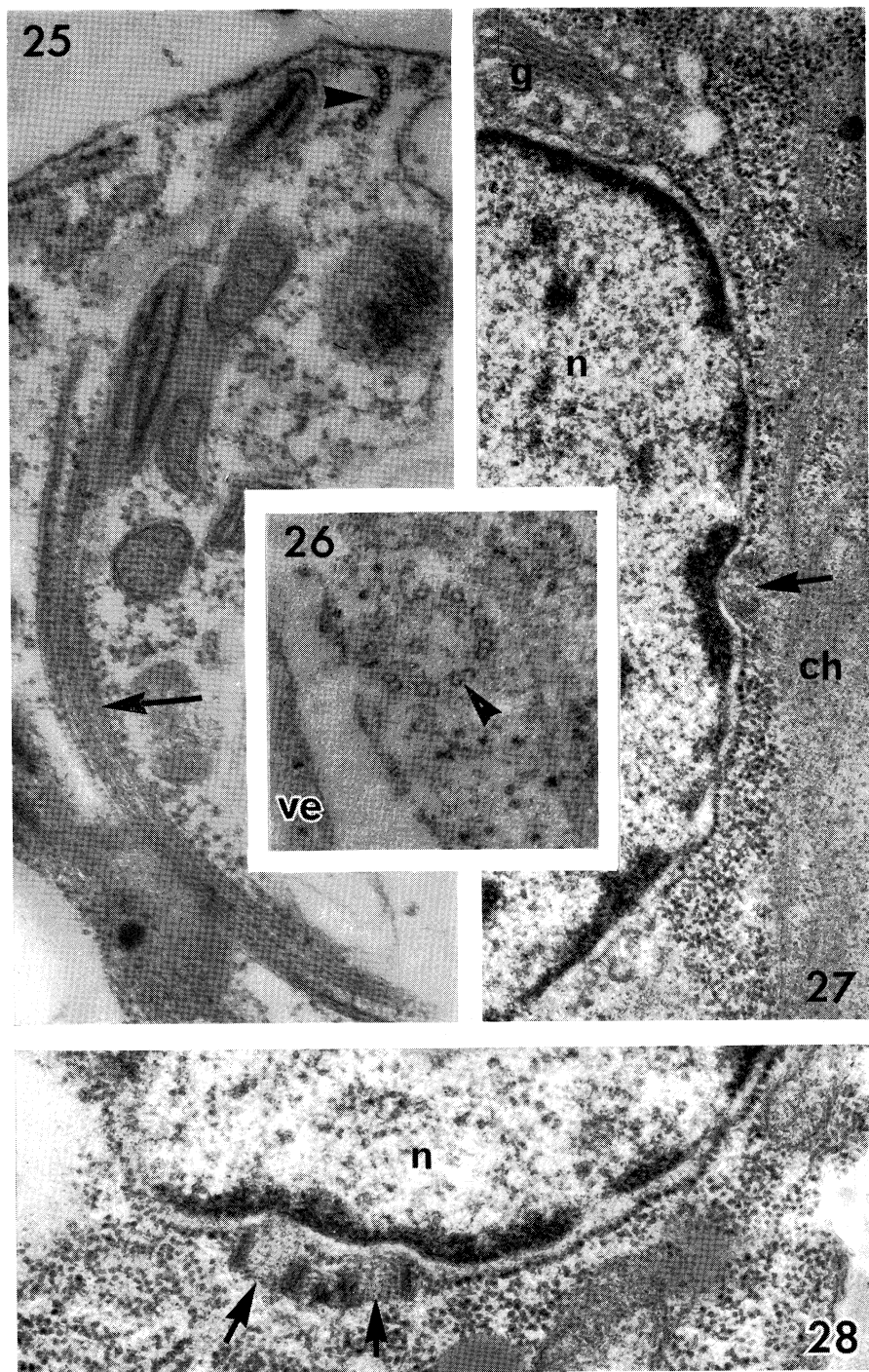


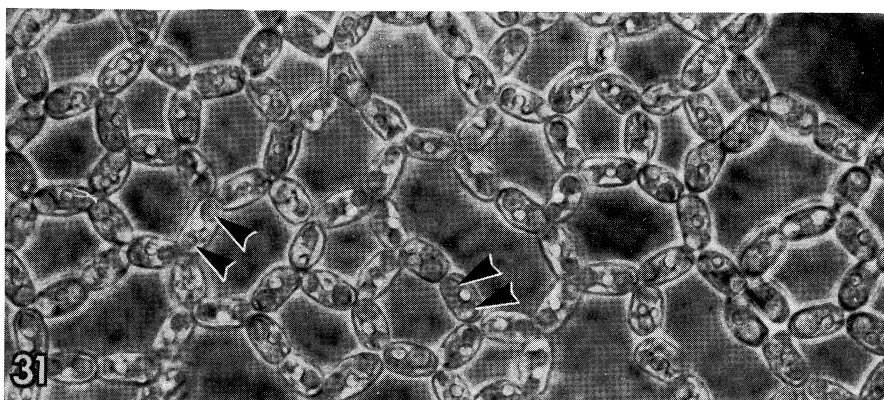
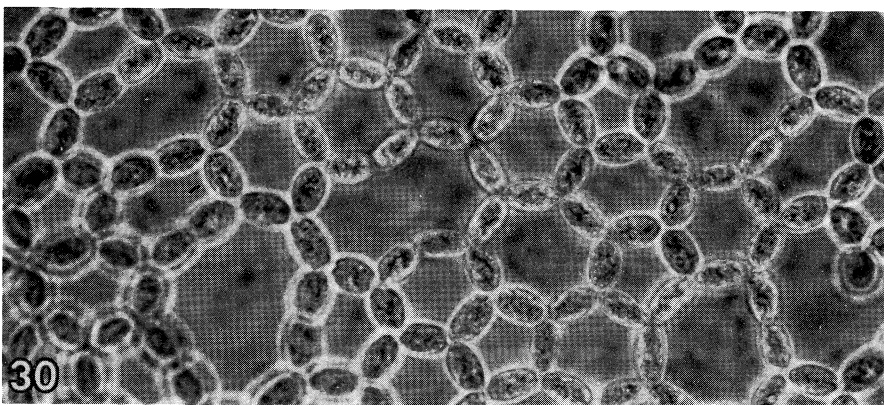
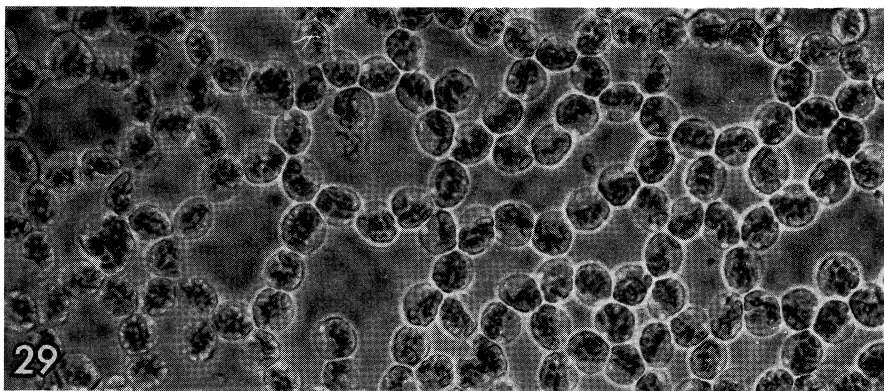
Fig. 25.—Flagellar microtubules lying within the cytoplasm of a net-forming zooid (arrow). Note the disintegrating microtubules (arrowhead). $\times 45,000$.

Fig. 26.—Higher magnification of disintegrating flagellar microtubules (arrowhead). $\times 70,000$.

Fig. 27.—Centriole forming *de novo* on the nuclear envelope (arrow). $\times 37,000$.

Fig. 28.—Two centrioles forming *de novo* on the nuclear envelope (arrows). $\times 53,000$.

experiments strongly suggest that bands of peripheral microtubules, so characteristic of colony-forming zooids, are indeed important in the ordered linking of the zooids.



Figs. 29–31.—Light micrographs of stages in the development of the daughter net. Irregularities in the pattern of the net are largely caused by compression by the cover slip. All phase contrast. All $\times 540$. 29, Zooids at the time of flagellar retraction. 30, Elongation of the adhering zooids. 31, Vacuoles appearing at each end of the linked cells (arrowheads).

An amorphous material appears in the intercellular space between flattened adjacent zooids but apparently not elsewhere over their surface. Similar material is

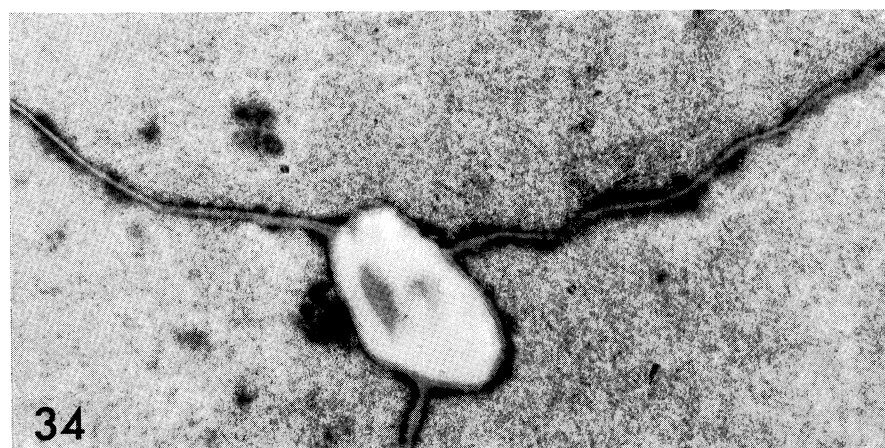
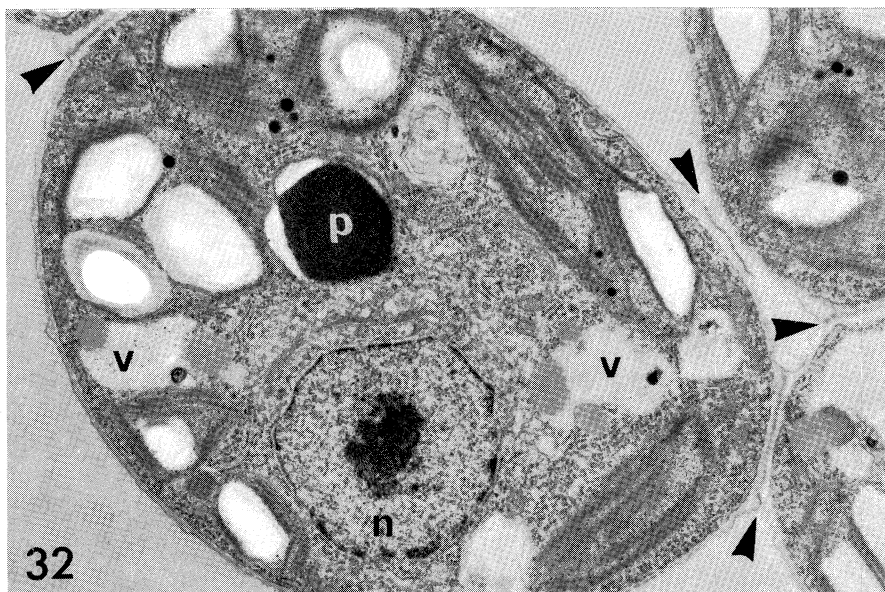


Fig. 32.—Young cell of a daughter net at the time of deposition of the inner layer of wall. Note the junction with three other cells (arrowheads), the developing vacuoles and pyrenoid. $\times 9000$.

Fig. 33.—The wall between two recently adhered cells. Note the thin “membrane-like” outer layer and the more massive inner layer. $\times 20,000$.

Fig. 34.—The cell wall at the junction of three mature cells. Note the disappearance of the “middle lamella” of the outer wall layer—cf. Figure 33. $\times 29,000$.

also detected between adjacent cells of *Pediastrum* before secretion of the wall (Gawlik and Millington 1969; Hawkins and Leedale 1971). Gawlik and Millington suggest that material in the vesicle of *Pediastrum*, in which the zoospores aggregate to form the daughter colony, may participate in the adhesion of the zoospores. Such an origin is difficult to reconcile with its apparent localization only between adhering zooids; furthermore, both Pocock's (1960) and our own observations show that net-forming zooids of *H. reticulatum* may link up free in the culture medium if the parental coenobial wall is ruptured and, presumably, such material is lost. As the net ages, the appearance of the intercellular sites of contact changes. We suspect that the amorphous material may bind the cells initially as they are regularly arranged even before flagellar retraction and secretion of the wall. Following wall deposition, the membrane-like outer layer is modified at sites of contact (by the amorphous material?) to a trilaminar structure (Fig. 33). This trilaminar appearance is lost in mature nets (Fig. 34); the cells are then attached to one another by sharing a common outer layer of wall. The nature of both the amorphous material and the outer layer of wall are presently being investigated.

Hawkins and Leedale (1971) correlate the appearance of the outer membrane-like layer of the wall with the irrevocable glueing together of the zooids. They conclude that the zooids are potentially capable of sticking together all over their surfaces, but are prevented from doing so by colony organization and changes in cell shape. The zooids, described by Hawkins and Leedale, were polyhedral following cleavage of the parental cytoplasm, becoming spherical at the commencement of swarming, and then oval or almost rectangular at the time of aggregation; these changes in shape were not explained. They said that the final shape of the zoospores was determined by the disposition of its constituent organelles, particularly the chloroplast, which presumably means that these organelles themselves must either undergo a change in shape or repositioning. Wall deposition shortly followed adhesion of the cells and flagellar retraction, first with an outer osmiophilic membrane-like wall, and later the much thicker inner layer. The structure of the wall of *H. reticulatum* differs from that in *H. africanum* (Northcote, Goulding, and Horne 1960) in that the former lacks pores and only has two layers whereas *H. africanum* has pores in a three-layered wall.

Little attention has been paid to the fate of flagella of motile plant cells on becoming sessile. The flagella of *Chlamydomonas reinhardtii* are shed at mitosis (Johnson and Porter 1968), as are those on the zoospores of *Oedogonium* as these cells settle and become germlings (Pickett-Heaps 1971b). Retraction or reabsorption of flagella occur within zoospores of the algae *Enteromorpha* (Evans and Christie 1970), *Vaucheria sessilis* (Greenwood 1959; Marchant, unpublished data), *Pediastrum* spp. (Hawkins and Leedale 1971; Marchant, unpublished data), *Stigeoclonium* (Manton 1964), and some fungi (Koch 1968; Reichle 1969). The retracted flagellar microtubules of *V. sessilis* disintegrate but the basal bodies remain intact adjacent to the nuclear envelope (Marchant, unpublished data). In germlings of *Stigeoclonium*, Manton (1964) inferred that basal bodies migrated to the nuclear envelope, but she does not mention the possible breakdown of these organelles and synthesis of new centrioles. Both basal bodies and flagellar microtubules disintegrate in *H. reticulatum*. New centrioles are apparently formed close to the nuclear envelope, as in other cells forming centrioles *de novo*, e.g. *Naegleria* (Dingle and Fulton 1966), *Chara* (Pickett-Heaps 1968), and *Oedogonium* (Pickett-Heaps 1971).

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