ULTRASTRUCTURE AND DIFFERENTIATION OF *HYDRODICTYON RETICULATUM*

II.* FORMATION OF ZOOIDS WITHIN THE COENOBIUM

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Abstract

A summary of the life cycle of *H. reticulatum* is given here in the second of a series of papers on an ultrastructural study of the development and differentiation of the various stages in the life cycle. The formation of zooids by the coenobia is then discussed in detail. After the fragmentation of the chloroplast and disintegration of the pyrenoids the cytoplasm cleaves: firstly, to form the vacuolar envelope, a thin cytoplasmic layer that separates the vacuole from the rest of the cytoplasm; secondly, to form uninucleate fragments of the cytoplasm each of which later develops a pair of flagella. Observations on the cytoplasmic cleavage and the role of microtubules in the cleavage are related to similar events in other algae. The function of the vacuolar envelope and the golgi apparatus, and the disintegration of the pyrenoids are also discussed.

I. INTRODUCTION

We have already described (Marchant and Pickett-Heaps 1970) mitosis in the coenobia of the freshwater alga *Hydrodictyon reticulatum* and now we present a study of the differentiation of the coenobial cytoplasm into uninucleate, biflagellate zooids. We consider it appropriate to include here a diagrammatical summary of the life cycle of *H. reticulatum* (Fig. 1), illustrated with representative light micrographs (Figs. 2–8),§ to ensure coherence between this and subsequent papers. The life cycle is extensively discussed in Pocock's (1960) classic paper and is compared with that of *Pediastrum simplex* by Davis (1967).

It has long been known that the coenobia of *H. reticulatum* are capable of producing two distinct classes of zooids—either small gametes or larger, net-forming zooids. Pocock (1960) reported that the latter are also capable of behaviour other than simply forming daughter-nets. The formation of all zooids, irrespective of their fate, is essentially identical and is described below. The subtle structural differences between gametes and net-forming zooids are irrelevant here and will be discussed later (Marchant and Pickett-Heaps, unpublished data).

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§ The following abbreviations are used in Figures 2–45: *c*, centriole complex; *ch*, chloroplast; *ci*, cytoplasmic intrusion; *cv*, contractile vacuole; *f*, cleavage fissure; *g*, golgi body; *m*, mitochondria; *n*, nucleus; *p*, pyrenoid; *s*, starch granule; *t*, microtubules; *v*, vacuole; *ve*, vacuolar envelope; *w*, cell wall; *wp*, wall peg; *z*, zooid. Figures 2–16 are light micrographs of living material and 17–22 are light micrographs of fixed material. All the remaining figures, excepting Figure 36, are electron micrographs.

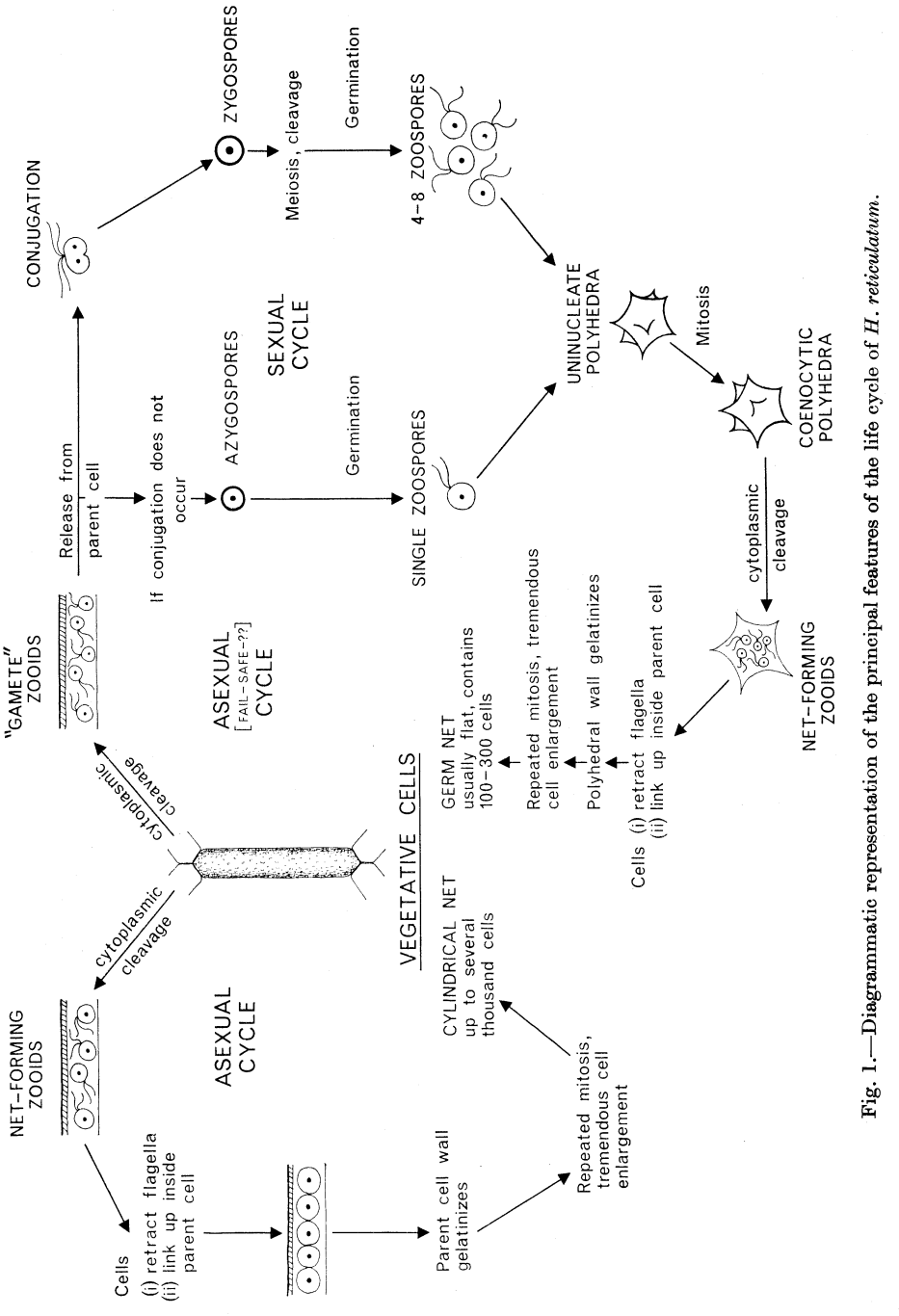


Fig. 1.—Diagrammatic representation of the principal features of the life cycle of *H. reticulatum*.

Fig. 5.—Polyhedra being formed from zoospores, produced but not released by germination of the zygospore (azygospore?). Phase-contrast. ×1170.

Fig. 6.—Polyhedron of *H. reticulatum*. Nomarski optics. ×450.

Fig. 7.—Zooids being produced within a polyhedron. Phase-contrast. ×450.

Fig. 8.—A flat germ net released from a polyhedron. ×90.

Fig. 9.—Part of the fenestrated, continuous chloroplast of a well-nourished coenobium of *H. reticulatum*. Note the telophase nuclei (arrows). Phase-contrast. ×1170.

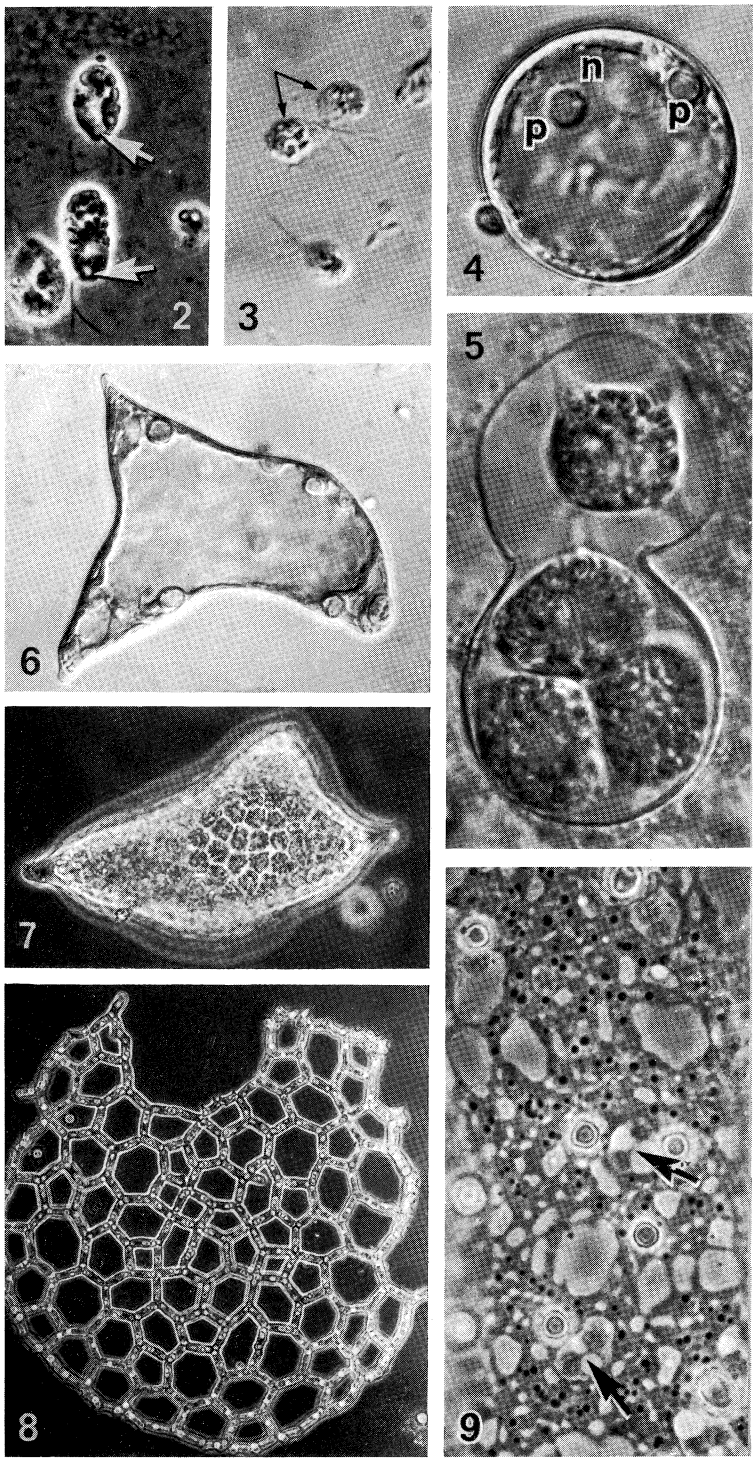
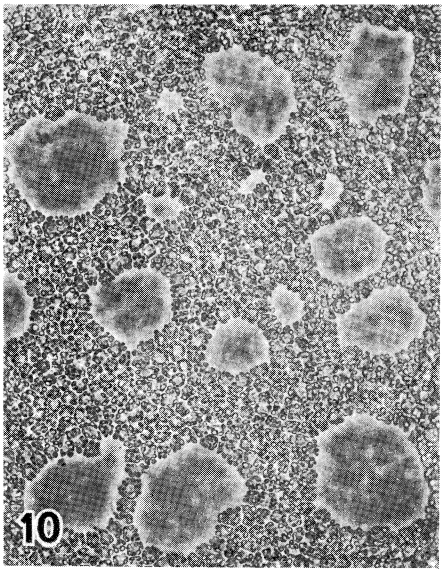
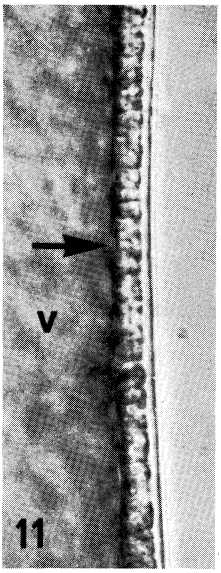
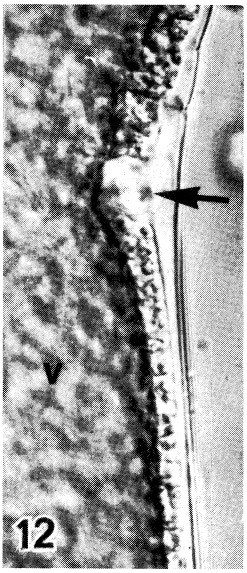
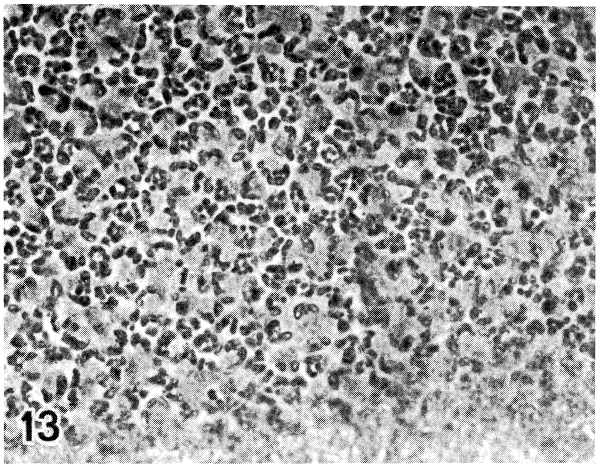


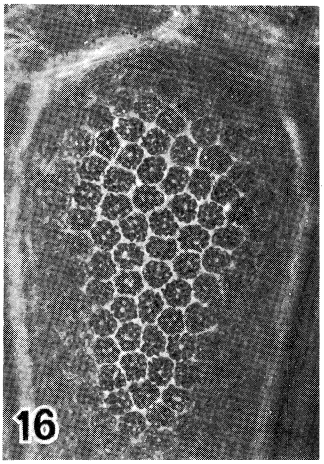
Fig. 2.—Biflagellate net-forming zooids; note contractile vacuoles (arrows). Phase-contrast. ×1170.
Fig. 3.—Conjugating "gamete" zooids (arrows); compare their size with zooids in Figure 2. Nomarski optics. ×1170.
Fig. 4.—Zygospore (azygospore?). Nomarski optics. ×1170.

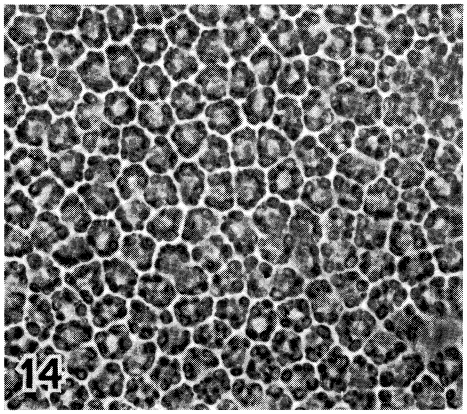












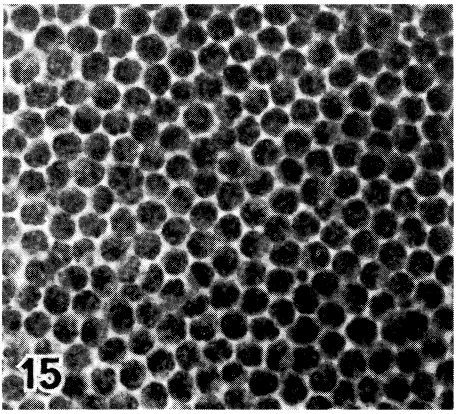


Fig. 10.—Part of a differentiating coenobium showing clear areas of the cytoplasm following partial starvation. Phase-contrast. $\times 500$.

Fig. 11.—An "optical section" through a differentiating coenobium showing the vacuolar envelope (arrow). Nomarski optics. $\times 1000$.

II. Materials and Methods

H. reticulatum was cultured as described previously (Marchant and Pickett-Heaps 1970). Production of net-forming zooids was induced by transferring coenobia into fresh growth medium; from these cultures cells were selected for fixation at various stages of differentiation. Induction of "gamete" zooids was considerably more difficult, their production being an infrequent response to adverse cultural conditions (e.g. lower light intensity, exhausted culture media); these cells were also selected for fixation at appropriate developmental stages.

Preparation of the material for electron microscopy was the same as described previously except that methyl cellosolve was often omitted from the dehydration schedule without any deleterious effects and Spurr's (1969) low-viscosity resin was often used for embedding instead of Araldite.

III. Observations

(*a*) *Living Material*

Chloroplasts containing prominent pyrenoids and starch grains often obscure the underlying nuclei of undifferentiated coenobia (Marchant and Pickett-Heaps 1970). Our observations support Pocock's (1960) contention that the chloroplasts of well-nourished coenobia are single fenestrated cylinders (Fig. 9); starvation or other adverse cultural conditions lead to their fragmentation.

An early sign of imminent formation of zooids is the disappearance of the pyrenoids coinciding with an accumulation of starch grains in the chloroplasts, the colour of which intensifies markedly as they fragment (Fig. 13; see also Fig. 20) and apparently thicken. Regularly spaced nuclei each become surrounded by the fragments of chloroplast. If the coenobia had previously been starved, large clear areas develop in the cytoplasm (Fig. 10).

The vacuolar envelope, discernible *in vivo* best with Nomarski differential interference–contrast optics (Fig. 11; cf. Figs. 19 and 26) is now formed enclosing the entire vacuole. The term "vacuolar membrane" used by Pocock (1960) for this important structure is inappropriate in our following ultrastructural description. Once the vacuolar envelope is formed, progressive cleavage of the cytoplasm into smaller and smaller segments gives rise to the characteristic "pavement stage" (Fig. 14) each unit of which eventually becomes uninucleate and then extends a pair of flagella, becoming motile (Figs. 2 and 3), often after a delay of some hours; the speed of development appears dependent on the light regime in which the alga was cultured. The difference in size between net-forming zooids and gametes is reflected in the size of their pavement units (cf. Figs. 14 and 16). Mature zooids (Figs. 2 and 3) are extremely active with the beating of the flagella and pulsation of the paired contractile vacuoles clearly visible. Zooids released from the parental cell on microscope slides often adhere to the glass by the tips of their flagella.

Fig. 12.—A wall peg (arrow) penetrating the cytoplasm of a coenobium. Nomarski optics. × 1000.

Figs. 13–15.—Stages in the formation of "gamete" zooids within a coenobium. All phase-contrast. × 1000.

Fig. 13.—Fragmentation of the chloroplast (cf. Fig. 9).

Fig. 14.—The "pavement" stage, in the differentiation of "gamete" zooids, cytoplasmic cleavage completed.

Fig. 15.—Rounded zooids just starting to move about within the parental cell wall.

Fig. 16.—The "pavement" stage in the differentiation of net-forming zooids within a small coenobium (cf. Fig. 14, at twice the magnification). Phase-contrast. × 500.

(*b*) *Fixed Material*

(i) *Light Microscopy*

Transverse sections of coenobia (Fig. 17) show the large central vacuole surrounded by a thin cytoplasmic layer lining the cell wall; they also demonstrate the quality of the preservation achieved with this alga. The other light micrographs demonstrate the various gross cytoplasmic changes which occur during differentiation. Distintegration of the pyrenoids, fragmentation of the chloroplast (Fig. 18), and formation of the vacuolar envelope can be seen. Radial cleavage of the cytoplasm (Figs. 19 and 20) then forms zooids confined between the partly gelatinized cell wall and the vacuolar envelope (Figs. 21 and 22) and not between the "vacuolar membrane" and an "outer protoplasmic membrane" as described by Pocock (1960, pp. 228, 296, and fig. 5). We have never seen any evidence for this outer membrane and cannot suggest what Pocock may have seen.

(ii) *Electron Microscopy*

(1) *Undifferentiated Cytoplasm of the Coenobia.*—We will not give a comprehensive description of the ultrastructure of the coenobia of *H. reticulatum*, concentrating instead on those organelles directly involved in the formation of zooids. The cell wall, which contains at least two layers (Figs. 24–26), usually becomes appreciably thinner, apparently by dissolution of the inner layers during the formation of the zooids and has often completely disappeared as the cells of the daughter net become cylindrical (Marchant and Pickett-Heaps, unpublished data). Wall pegs, contorted laminations of the inner layers of the wall, occur moderately frequently in old coenobia of this strain of *H. reticulatum* (Fig. 25, and the light micrograph Fig. 12). Their distribution among the various species of *Hydrodictyon* is discussed by Pocock (1937, 1960, p. 295 *et seq.*).

Small coated vesicles lie between the nuclear envelopes (or less frequently the endoplasmic reticulum) and one face of the golgi bodies throughout differentiation (Figs. 26, 28, 29, 33, 42, 43). Mitochondria, characteristically long and thin, usually lie circumferentially about the cell. The disposition of the centrioles, persistent in the coenobia but not in all stages of the life cycle, has been discussed previously (Marchant and Pickett-Heaps 1970).

(2) *Initial Stages of Differentiation.*—Cytoplasmic differentiation is heralded by the appearance of randomly dispersed microtubules, predominantly near the tonoplast, and increasing numbers of small vacuoles. The cytoplasmic intrusions (Fig. 23) in the pyrenoids become increasingly prominent as the starch plates disintegrate (Fig. 24); concurrently, stromal starch increases markedly. Densely staining bodies coalesce on the tonoplast (Fig. 23) and are apparently released into the vacuole where they later disperse (Fig. 25). Their origin and function are unknown.

Fig. 19.—Formation of zooids following the creation of the vacuolar envelope (arrow). × 1000.

Fig. 20.—Tangential section of a differentiating coenobium at the "pavement stage" (cf. Fig. 14). × 1000.

Fig. 21.—Longitudinally-sectioned coenobium with mature zooids between the cell wall (small arrow) and the continuous vacuolar envelope (large arrow). × 160.

Fig. 22.—Similar to Figure 21 but at higher magnification, showing the zooids and vacuolar envelope (arrow). × 1000.

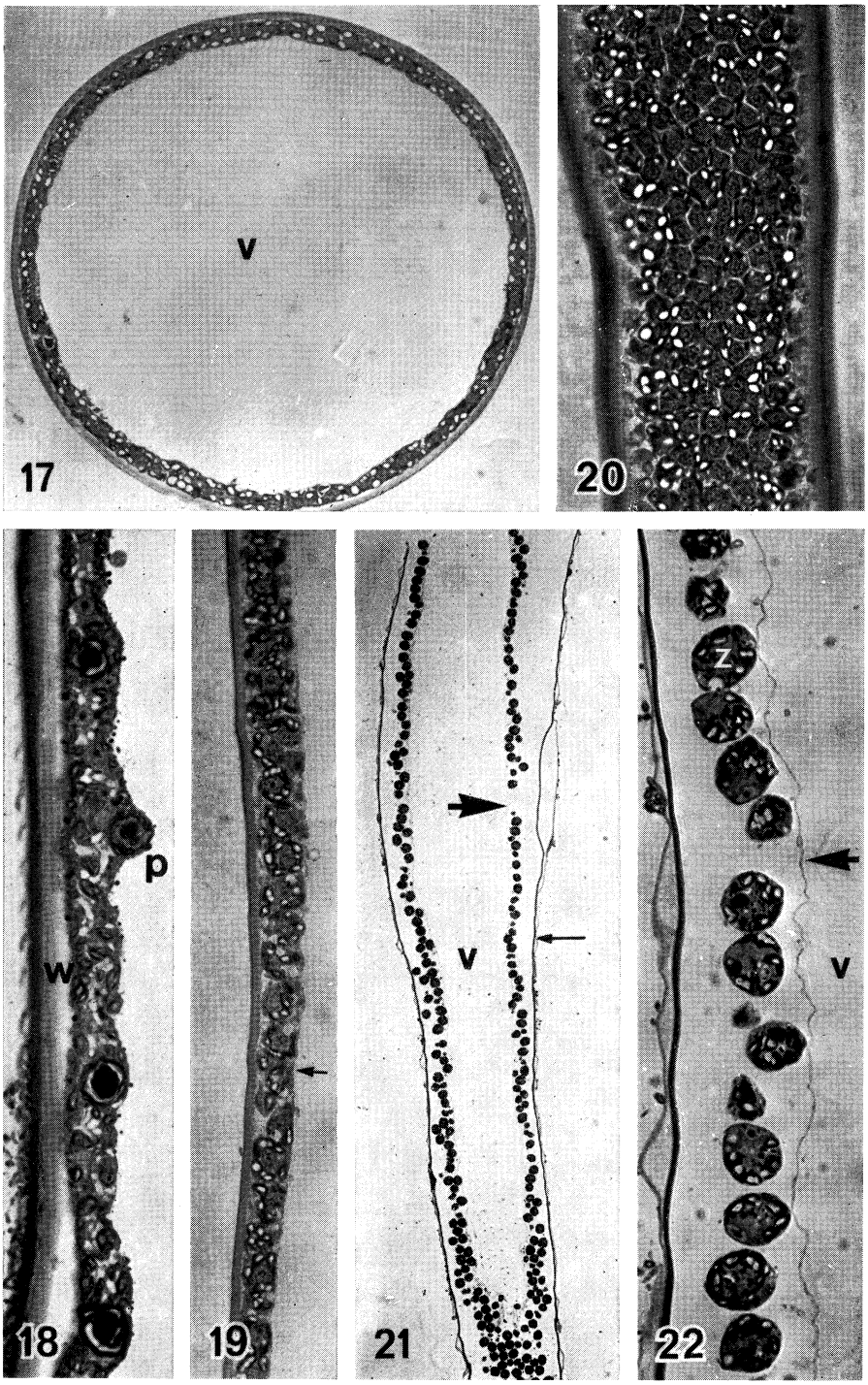


Fig. 17.—Transverse section of a coenobium of *H. reticulatum* showing the thin peripheral cytoplasm surrounding the immense central vacuole. ×750.

Fig. 18.—Early stage of cytoplasmic differentiation showing fragmentation of the chloroplast and the disintegration of the pyrenoid (*p*). ×1000.

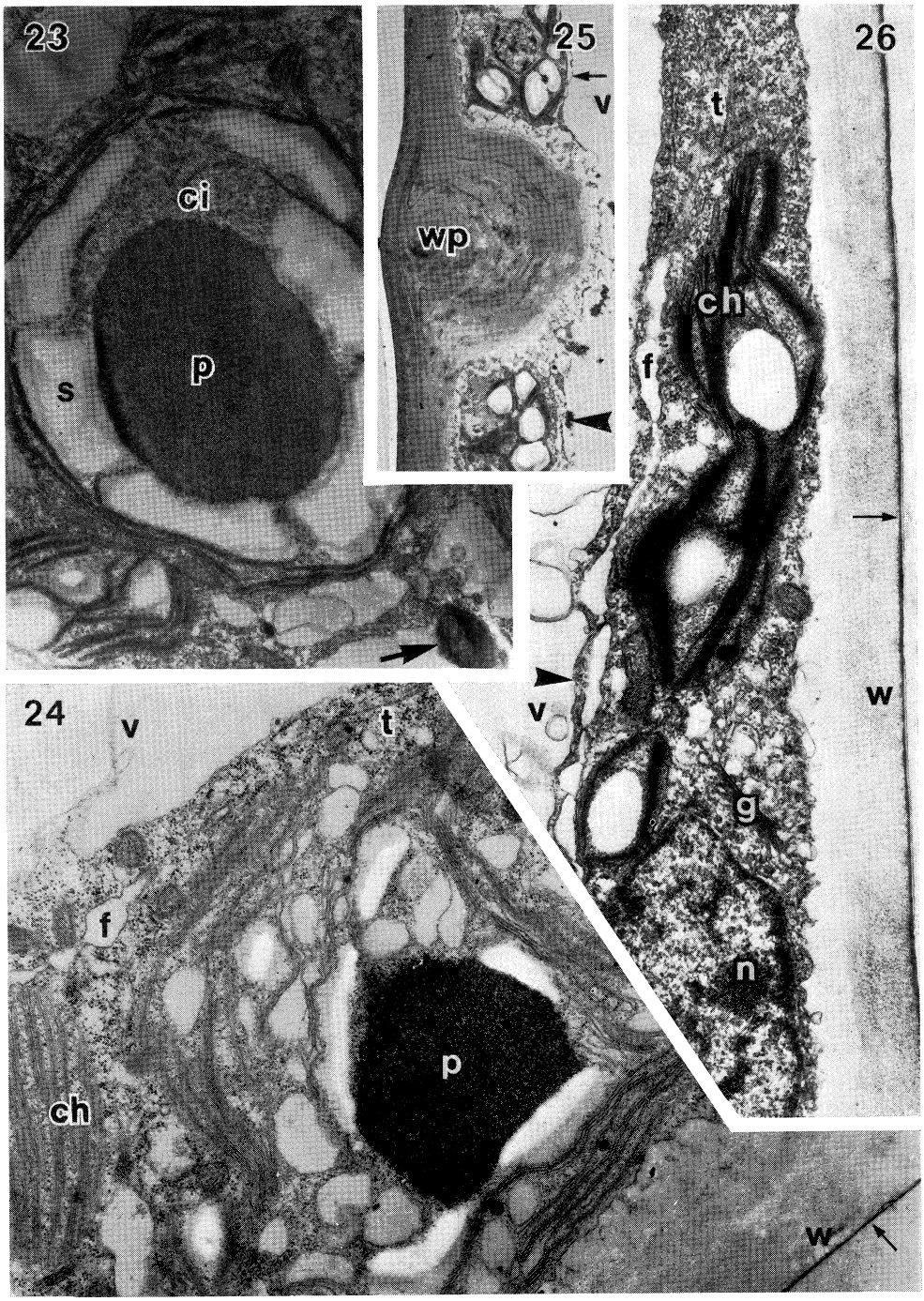


Fig. 23.—Pyrenoid with a cytoplasmic intrusion and surrounding starch plates at an early stage of differentiation. Note the densely staining body (arrow) on the tonoplast. ×10,000.

Fig. 24.—A disintegrating pyrenoid surrounded by very little starch (cf. Fig. 23). Note the cleavage fissure (*f*) forming the vacuolar envelope and the thin outer layer (arrow) and thicker inner layer of the wall (*w*) ×18,000.

(3) *Formation of the Vacuolar Envelope.*—Proliferating cytoplasmic microtubules become oriented predominently parallel to the long axis of the cell, close to the tonoplast (Fig. 36*a*). Vesicles appearing among these microtubules seem to coalesce, thus giving rise to an extending cleavage fissure (see below and Fig. 36*a*) that isolates a thin, continuous layer of cytoplasm, the vacuolar envelope, which eventually partitions the bulk of the cytoplasm from the vacuole (Fig. 36*a*). This envelope mostly contains only very small organelles, e.g. ribosomes, microtubules, etc. (Figs. 26, 28, 29, 34).

(4) *Radial Cleavage of the Cytoplasm.*—As the vacuolar envelope forms, more microtubules, generally oriented radially, now appear between the nuclei (Fig. 28). Among these microtubules vesicles again appear (Fig. 27), elongating and condensing with one another and with growing invaginations of the plasmalemma (Figs. 29, 36*a*). Thus the cytoplasm is cleaved into progressively smaller units (Figs. 32, 33, 42). The sources of the vesicles remains unclear; indeed the vesicles could represent profiles of continuously ramifying cleavages.

As expected, cellular organelles, particularly chloroplasts and mitochondria, often lie across the path of cleavage fissures. These organelles are severed by some unknown mechanism after the fissure has encircled them (Figs. 30, 31). Rarely, radial cleavage precedes the completion of the vacuolar envelope in localized regions (Fig 36*b*). This results in the vacuolar envelope containing some inclusions of large organelles, e.g. nuclei, chloroplasts, etc. (Fig. 35).

(5) *Centriolar Morphogenesis and Flagella Development.*—Radial cleavage is accompanied by elongation of the centrioles (Fig. 37) and modification of their surrounding amorphous material (Figs. 38, 39). The extension of the flagella (Fig. 41) usually starts at the uninucleate pavement stage. Transverse sections through the transition region between basal bodies and flagella (Fig. 40) reveal the stellate pattern reported in some other motile plant cells (Ringo 1967). Four bands of microtubules are directed anteriorly in the mature zooid; these arise from the amorphous component of the centriole complex and are surrounded by "fluffy" granules (Fig. 38) when elongating.

(6) *Mature Zooids.*—The structure of mature zooids will be discussed in detail later; however, a few general points are best mentioned here. Each zooid has a pair of contractile vacuoles into which small vesicles apparently discharge (Fig. 43). All zooids have what appears to be a small residual pyrenoid near to which, in net-forming zooids but not gametes, is another body of very similar texture (Figs. 44, 45). We are unable to confirm the observations of various early microscopists (see Fritsch 1935 and Pocock 1960, p. 301 *et seq.*, for references) that net-forming zooids are permanently connected by cytoplasmic threads, either to the vacuolar envelope or to one another.

Fig. 25.—Contorted inner layer of the cell wall in a wall peg. The vacuolar envelope (small arrow) is fully formed and densely staining granules are dispersing (arrowhead) into the vacuole. ×4050.

Fig. 26.—Developing cleavage fissure with microtubules forming the vacuolar envelope. Note wall layers, outer (arrow), inner (*w*). ×26,000.

IV. Discussion

Early microscopists (e.g. Braun 1853, p. 261 *et seq.*) recognized that two kinds of zooids were formed by cytoplasmic cleavage of *H. reticulatum*: the smaller gametes, "microgonidia", and large net-forming zooids, "macrogonidia". Pocock (1960) found that the larger zooids were also capable of conjugating or forming azygotes (this alternate behaviour of the zooids is not included in Fig. 1). She concludes that these macrogonidia are all identical and differ only in behaviour, citing cases of coenobia being ruptured while forming daughter nets and producing both spores and elongate coenocytes at the dislocation; she does not, however, mention any instance of zooids from such coenobia conjugating. Evidence will be presented later (Marchant and Pickett-Heaps, unpublished data) of structural differences between net-forming zooids and gametes. Pocock's and our own work suggests that the formation of azygospores could be a "fail-safe" mechanism whereby gametes failing to conjugate or net-forming zooids failing to link up before a certain stage of development both form walled, resistant spores.

Microtubules, predicting the paths of the cleavages which form the vacuolar envelope and the uninucleate cytoplasmic fragments, appeared more numerous during the development of gametes than during development of net-forming zooids. The distribution of these microtubules was not discernibly influenced by that of centrioles or any other recognizable organelle.

Cytoplasmic cleavage in *H. reticulatum* is very extensive and differs somewhat from cleavage in other algae. The cytoplasm of *Kirchneriella*, a related member of the Chlorococcales, is cleaved by a similar cytokinetic apparatus utilizing transversely oriented microtubules and growing cleavage fissures (Pickett-Heaps 1970) to form four autospores following two mitotic divisions. In contrast to *H. reticulatum*, however, the centrioles always moved into a characteristic position near the cleavage furrows. Arrays of microtubules and cytokinetic fissures similar to those in *Hydrodictyon* have been recorded in *Scenedesmus*, *Ankistrodesmus*, and *Tetraëdron* (Pickett-Heaps, unpublished data). In other algae too, transverse microtubules appear between post-mitotic nuclei, having various and differing involvements in cytokinesis, e.g. *Chlamydomonas* (Johnson and Porter 1968); *Oedogonium* (Pickett-Heaps and Fowke, 1969); *Closterium* (Pickett-Heaps and Fowke 1970); and *Stigeoclonium* (Pickett-Heaps, unpublished data). The significance of these variations of cytokinesis is discussed by Pickett-Heaps (1969). In still other algae, cleavage furrows are not associated with microtubules, e.g. *Ulva mutabilis* (Løvlie and Bråten, 1968, 1970). As in *Kirchneriella* (Pickett-Heaps 1970) the source of the cleavage membrane in *H. reticulatum* is obscure; the radial cleavages may well be formed from ingrowing ramifications of the plasmalemma which could appear as vesicles in thin sections. However, such a hypothesis cannot explain how the vacuolar envelope is cleaved off.

The vacuolar envelope is most interesting, and so far a unique structure in algae. Its formation suggests that the tonoplast is not compatible with the plasmalemma; i.e. if the cytoplasm simply cleaved up, part of the zooids' surface would

Fig. 30.—Division of a chloroplast straddling the developing cleavage. Note the vacuole containing a dense granule (arrow). ×26,000.

Fig. 31.—Mitochondrion lying across a developing cleavage (arrows). ×26,000.

Fig. 32.—Completed radial cleavage with associated microtubules. Note the dense granule (arrow) in the vacuole. ×29,000.

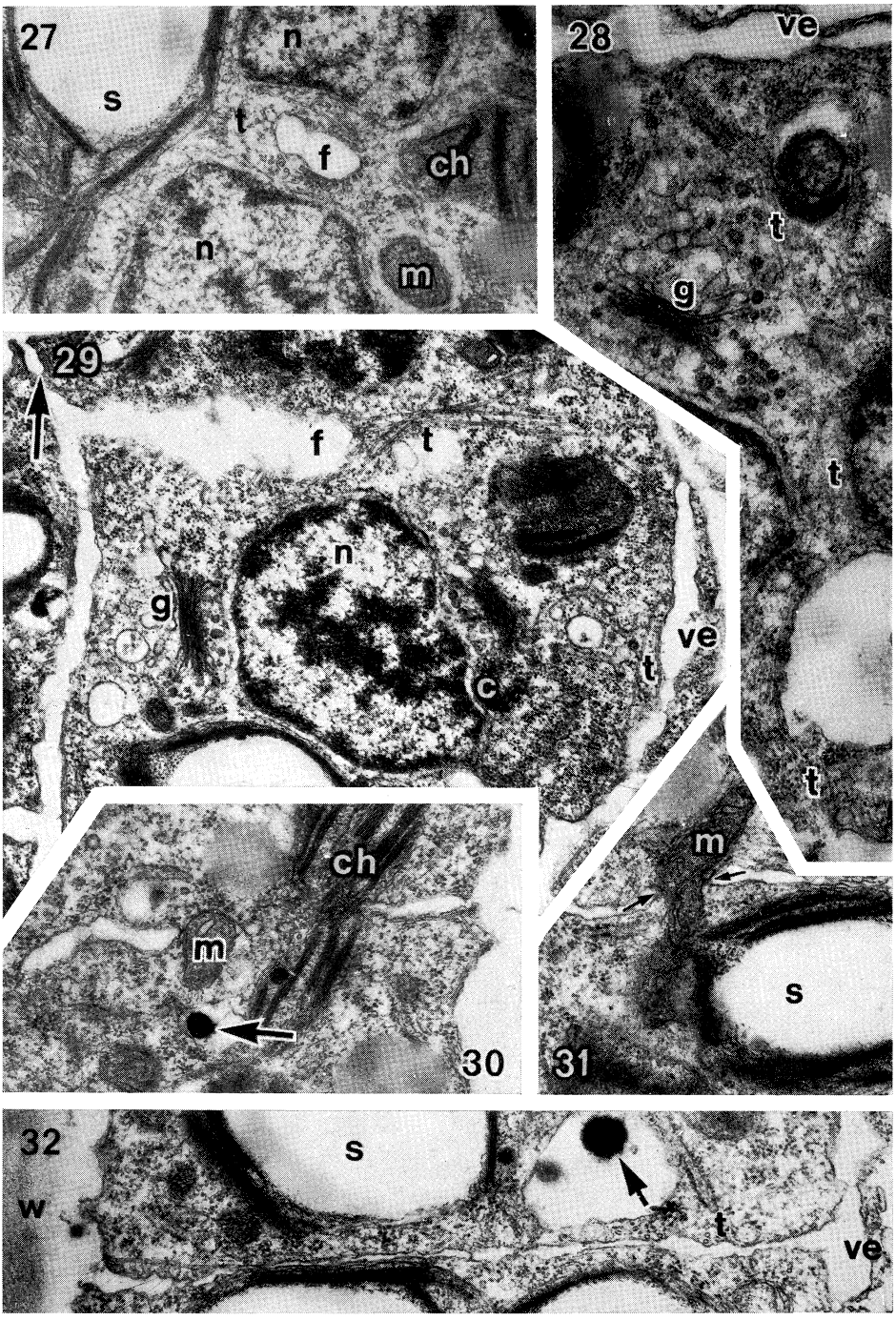


Fig. 27.—Early radial cleavage of the cytoplasm; note the developing fissure (*f*) surrounded by microtubules. ×29,000.

Fig. 28.—Microtubules "predicting" the path of a radial cleavage. ×26,000.

Fig. 29.—Simultaneous radial cleavage (*f*) and formation of the vacuolar envelope, both cleavages associated with microtubules. Note the invagination of the plasmalemma (arrow) and the undifferentiated centriole. ×20,000.

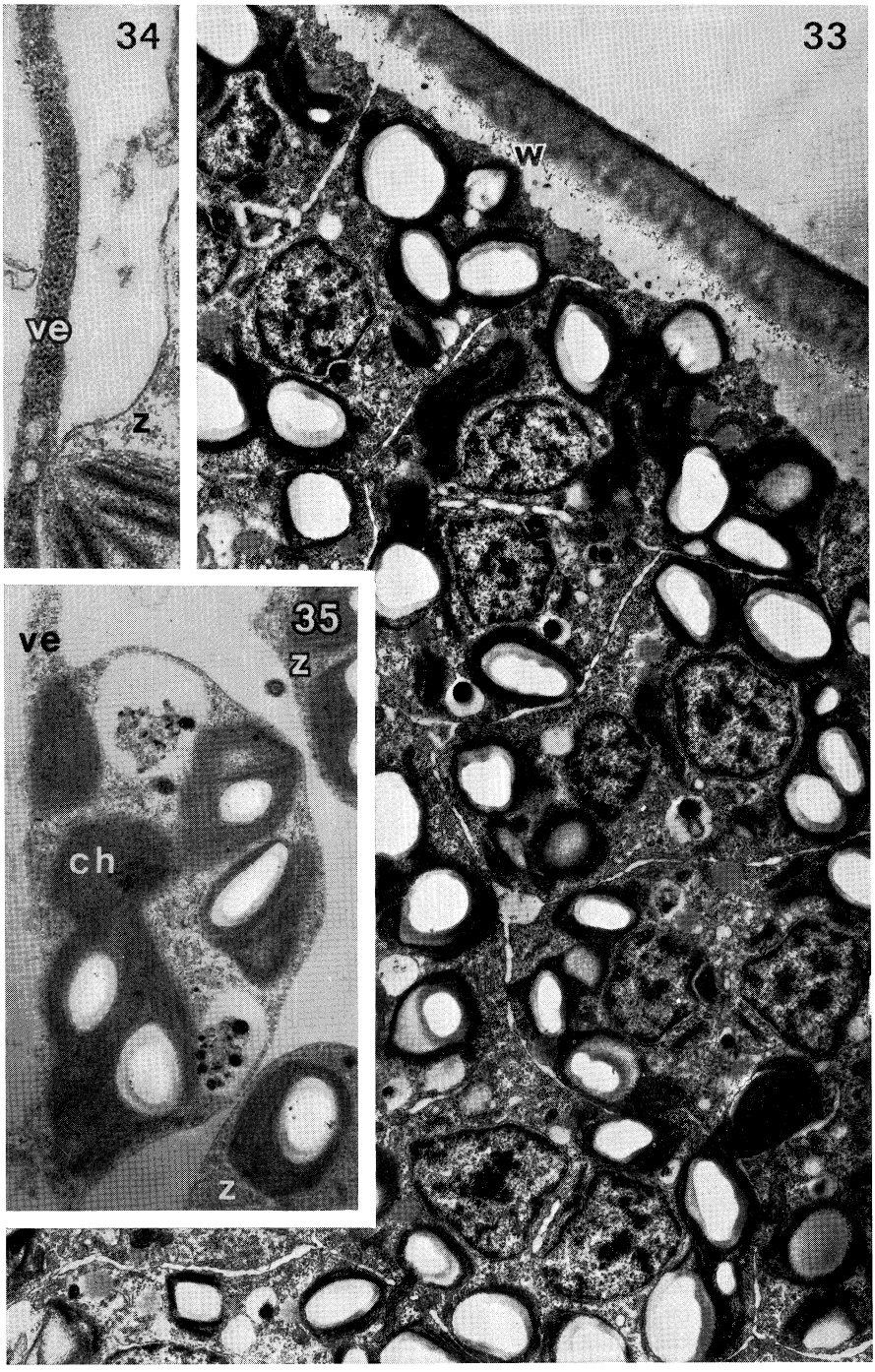


Fig. 33.—Low magnification micrograph of a tangentially sectioned coenobium approaching the "pavement" stage (cf. Figs. 14, 15, 20). ×8100.

Fig. 34.—Detail of a vacuolar envelope containing mostly ribosomes. ×34,000.

Fig. 35.—Part of an abnormal vacuolar envelope containing large cellular organelles, resulting from the vacuolar envelope being incompletely formed before radial cleavage—see Figure 36*b*. ×7500.

necessarily have been derived from the tonoplast. Instead it appears as if the cytoplasm must isolate the tonoplast from those membranes involved in cytoplasmic cleavage. Another function of the vacuolar envelope concerns the formation of the daughter net. It confines the zooids into a single-celled layer appressed to the cell wall so that when the zooids link together (Fig. 1) they automatically assume the configuration of a cylindrical monolayered net, so characteristic of *H. reticulatum*.

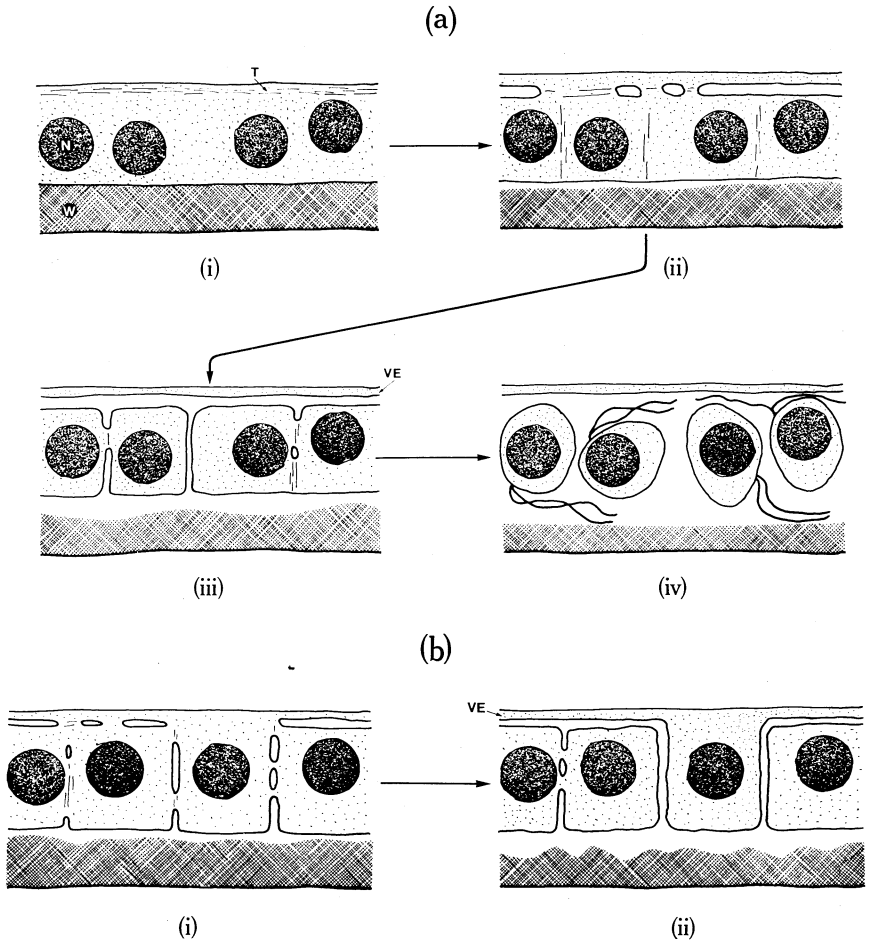


Fig. 36.—(*a*) Diagrammatic representation of the principal events in the formation of zooids from the coenobial cytoplasm: (i) microtubules (*T*) appear near the tonoplast; (ii) formation of the vacuolar envelope; (iii) radial cleavage of the cytoplasm within an intact vacuolar envelope (*VE*); (iv) mature flagellated zooids confined between the vacuolar envelope and the cell wall (*W*). (*b*) Abnormal cytoplasmic cleavage: (i) localized radial cleavage preceding the formation of the vacuolar envelope produces (ii), a vacuolar envelope that contains inclusions of large organelles.

If the vacuolar envelope is damaged, irregular nets are formed (Pocock 1960). This vacuolar envelope is essentially composed of a plasmalemma and a tonoplast bounding an extremely thin layer of cytoplasm; although generally devoid of large organelles, it remains turgid throughout radial cleavage and formation of the daughter net. It has obvious potential in studies of ion transport, etc.

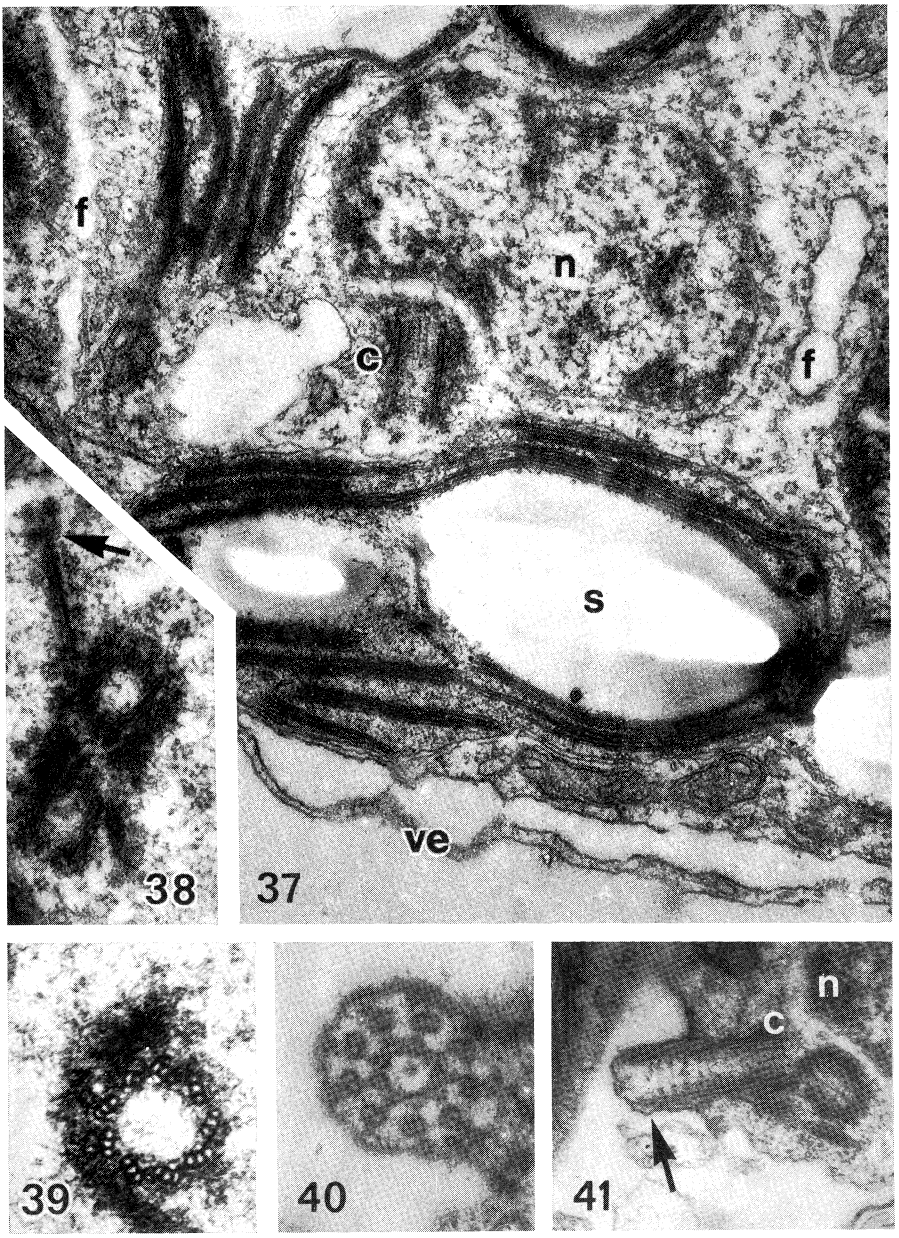


Fig. 37.—Elongating centriole at an advanced stage of radial cleavage. ×39,000.

Fig. 38.—Elongation of anteriorly directed microtubules from the amorphous material around the centrioles; note the "fluffy" granules around them, particularly at their ends (arrow). ×39,000.

Fig. 39.—Transverse section of a centriole complex showing modified amorphous material early in the formation of the anteriorly directed microtubules. ×80,000.

Fig. 40.—Transverse section of the transitional region between the basal body and the developing flagellum, showing the stellate pattern. ×85,000.

Fig. 41.—Longitudinal section of an elongating flagellum (arrow). ×39,000.

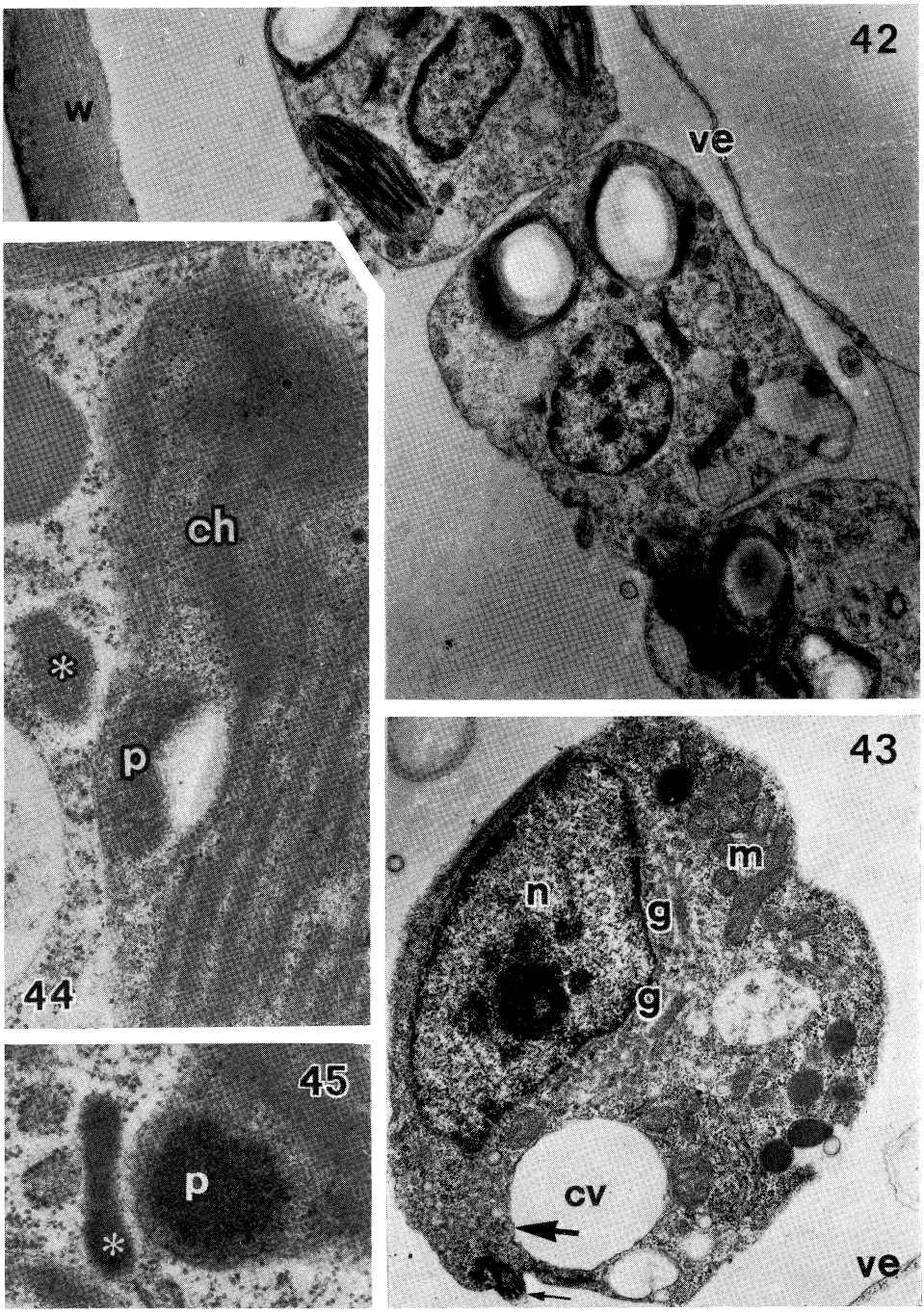


Fig. 42.—Zooids before separation. Note both the cell wall and vacuolar envelope. ×11,000.

Fig. 43.—Mature net-forming zooid with a conspicuous basal body (small arrow) and a contractile vacuole into which small vesicles are apparently discharging (large arrow). ×11,000.

Fig. 44.—Remnant pyrenoid (*p*) in a net-forming zooid with accompanying satellite structure (asterisk). ×39,000.

Fig. 45.—Remnant pyrenoid and associated body in another net-forming zooid. Note the similarity in texture of the satellite body and pyrenoid in both this and the previous figure. ×39,000.

Golgi bodies and their associated vesicles are numerous in the cytoplasm of *H. reticulatum* throughout the formation of zooids; their role, however, remains obscure. In *Ulva lactuca*, a marine alga that accumulates potassium in its vacuoles, West and Pitman (1967) speculated that the golgi bodies may be responsible for ion transport and in particular the selective flux of potassium across the cytoplasm. *Hydrodictyon* also accumulates potassium in its vacuole (Blinks and Nielsen 1939; Raven 1967) and so offers a system for the detection of differences between ion transport by the golgi apparatus and by the membranes limiting the cytoplasm.

The pyrenoids of *H. reticulatum* were previously thought to disappear completely during the formation of the zooids and to form *de novo* in both spores and cells of the daughter nets. We have found small residual pyrenoids which in the net-forming zooids are often accompanied by a satellite structure lying outside the chloroplast (Figs. 44, 45).

V. Acknowledgment

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VI. References

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