ULTRASTRUCTURE AND DIFFERENTIATION OF HYDRODICTYON RETICULATUM AND SOME OTHER RELATED ALGAE

by

Harvey J. Marchant

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Although Dr. J. D. Pickett-Heaps is coauthor of publications included as part of this dissertation, all the work described here is my own.

Harvey J Marchant.

Harvey Marchant

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CHAPTER 1

INTRODUCTION

Algae appear to have been often overlooked by cell biologists as tools for studying basic cellular processes, with such notable exceptions as the work on Acetabularia (see Hämmerling, 1963; Brachet and Bonotto, 1970; Puiseux-Dao, 1970, for reviews), studies on ion transport (see the reviews of Briggs, Hope and Robertson, 1961, and Dainty, 1962) and photosynthesis (Rabinowich and Govindjee, 1969). This neglect has been partially rectified in the last few years with, for example, a recent upsurge of interest in algal ultrastructure. However, much of this electron microscopy has been devoted to investigations of the structure of specific organelles or the morphology of algae at a particular stage of differentiation in their life cycle, while little attention has been given to morphogenetic studies. This neglect is somewhat surprising considering how actively studies on morphology and development were pursued at the end of the last and the beginning of this century. I suspect ~~that~~ the principal reasons for the lack of activity in this field ~~to be~~ are two~~fold~~. First, light microscopists, because of the physical limitations of their instruments, have been unable to detect the subtle differences between related cellular processes in various algae, particularly mitosis and cytokinesis. As a consequence, the attitude has arisen that these processes are all rather similar to one another, and to those in higher plants (e.g. references in the literature to phragmoplasts in algae), a feeling that has never been completely lost. This attitude was recently exemplified when a phycologist reviewing a manuscript for publication, commented that "cellular organization, mitosis and cytokinesis in Klebsormidium (the subject of the paper being reviewed) is like 100 green algae already described in

great detail." In their rebuttal, the authors commented that such a statement is simply absurd; the number of detailed descriptions of mitosis and cytokinesis in algae are very few, and so far have illustrated a great diversity in these cellular processes. Secondly, most algal cells are notoriously difficult to prepare for electron microscopy and frequently special precautions have to be taken to avoid gross disruption of the cells. New methods and variations of existing techniques must often be devised to handle such delicate cells, a laborious and often highly frustrating business.

The predominance of observations on animal, and in particular mammalian, cells has, in some cases, led to an acceptance that these represent what occurs in other organisms. Perhaps the best recent example of how studies on animal cells have dominated biological thinking is the widely held view that the centrioles are involved in the formation and functioning of the mitotic spindle. Work on fungi, protozoa and algae have demonstrated the implausability and limitations of many such theories concerning the centriole. Since the publication of two papers (Pickett-Heaps, 1969, 1971) seriously questioning an active role of the centriole in the mitotic apparatus, there has appeared a report of a spindle in an animal cell lacking centrioles (Szollosi and Calarco, 1971).

Frederick (1970) has aptly referred to the algae as "a group of organisms, fairly easy to propagate and manipulate, of simple body plan, with an amazing and widespread diversity and sprinkled with just enough morphological and evolutionary "enigmas" to whet the experimental

appetite." The great diversity in life cycles of the algae and their morphology has been well documented by early light microscopists (see Fritsch, 1935; Smith, 1955). I see the value of ultrastructural work on algae elucidating not only how algae themselves accomplish various feats of morphogenesis, but also the more basic problems of the function and interaction of cellular components. An understanding of ultrastructural events occurring in differentiating cells is a valuable adjunct to other experimental work undertaken to investigate the control of morphogenesis.

Since so few algae have been investigated in detail with the electron microscope, the approach in this laboratory has been largely exploratory. We have been able to study algae selected more or less at random, often collected when found growing vigorously in the wild. Such a seemingly haphazard method of study does however have its virtues. Investigations of developmental processes in widely divergent genera of green algae and comparisons of their various activities, particularly mitosis and cytokinesis, have proved most rewarding (Pickett-Heaps, 1972a). My own introduction to Hydrodictyon reticulatum was also the result of a chance finding of a specimen in the wild. The other algae used in this study, Chlorella and Pediastrum, are both members of the Chlorococcales and were selected for comparison with H. reticulatum.

The water net, H. reticulatum, is a most distinctive alga, principally because of its complex life cycle and method of asexual reproduction, which enables it to produce enormous numbers of characteristic cylindrical networks of cells in a relatively short time. It is thought

that the first written record of a specific alga is in ancient Chinese literature and could refer to Hydrodictyon (Porterfield, 1922). Such early recognition is not altogether surprising, considering the unique net-like arrangement of cells in this alga, and the size attained by these nets under optimal conditions, over 30 inches long in some cases (Pocock, 1960). Each "mesh" of a net is bounded by multinucleate cells; each cell may be over a centimetre long and is usually joined to two others at each end. While much has been written about the form of the vegetative cylindrical net and its development from motile zoospores (zooids), there have been only few attempts to propose mechanisms explaining the patterned aggregation of these zooids.

The life cycle of H. reticulatum consists of alternating motile and sessile stages, uninucleate motile zoospores usually being derived by cleavage and differentiation of sessile coenocytes (Fig. 1). Besides the cylindrical vegetative net, H. reticulatum also can produce flat "germ" nets elsewhere in its life cycle. The vegetative form of Pediastrum, a close relative of Hydrodictyon, is a planar colony of cells which apparently develops in a similar way to the flat nets of Hydrodictyon.

One of the central problems in developmental biology concerns the movement of cells and their aggregation and adhesion to form ordered multicellular systems, or as Trinkaus (1969) puts it, "how one moves from the cellular level to the level of the organism." The vast literature on embryology does little to clarify how this transition is achieved. Research on aggregation and adhesion of cells has been almost

solely within the realm of animal embryology and approached principally by studying multicellular systems *in toto*, rather than tackling the problem at the cellular level. Such an approach has lead to many morphogenetic movements in embryos (e.g. gastrulation and neurulation) being well known, but has not provided a clear understanding of what these movements entail at the cellular and subcellular level. *Hydrodictyon* and *Pediastrum* provide interesting examples from the plant kingdom of freely motile cells forming patterned aggregates. Aggregation of zoospores in both these algae has been discussed by Bonner (1952) as examples of morphogenetic movements in plant cells. He makes a distinction between the mechanism of formation of cylindrical and flat nets of *Hydrodictyon*. The former he describes as the uncontrolled morphogenetic movement of zoospores plastering themselves on the inside of their parental cell wall and adhering to form a net. He views the aggregation of zoospores to form flat nets as a movement controlled by the zoospores themselves and not requiring a mould to shape the net. It is now clear that Bonner's description of the formation of cylindrical nets is inaccurate. Here I contend that the mechanism of aggregation of zooids, whether to form cylindrical of flat nets of *Hydrodictyon* or flat colonies of *Pediastrum* is basically similar. Hopefully, an understanding of colony and net formation in *Pediastrum* and *Hydrodictyon* may be relevant to our understanding of the development of tissues in higher organisms, even though these aggregating algal cells are all essentially identical and not differentiated as are those of higher organisms.

The life cycle of *H. reticulatum* provides the theme linking the chapters of this thesis together; although other algae are dealt with,

the work on them relates directly to Hydrodictyon. Fig. 1 shows how the life cycle has been subdivided and its various facets dealt with as separate chapters. As some of the material presented here has already been published or accepted for publication, it was decided to include it in its published form or as manuscripts. For uniformity and convenience, the other chapters are presented in a similar format. Consequently, the photographs that follow each chapter are not numbered sequentially throughout the thesis. Instead, figure numbers in each chapter refer specifically to the figures found at the end of that particular chapter. References to figures in other chapters are prefixed with the number of the appropriate chapter. Regrettably, some of the abbreviations used on the figures may mean different things in different chapters. To prevent any confusion, a list of abbreviations used on figures precedes the photographic plates of each chapter.

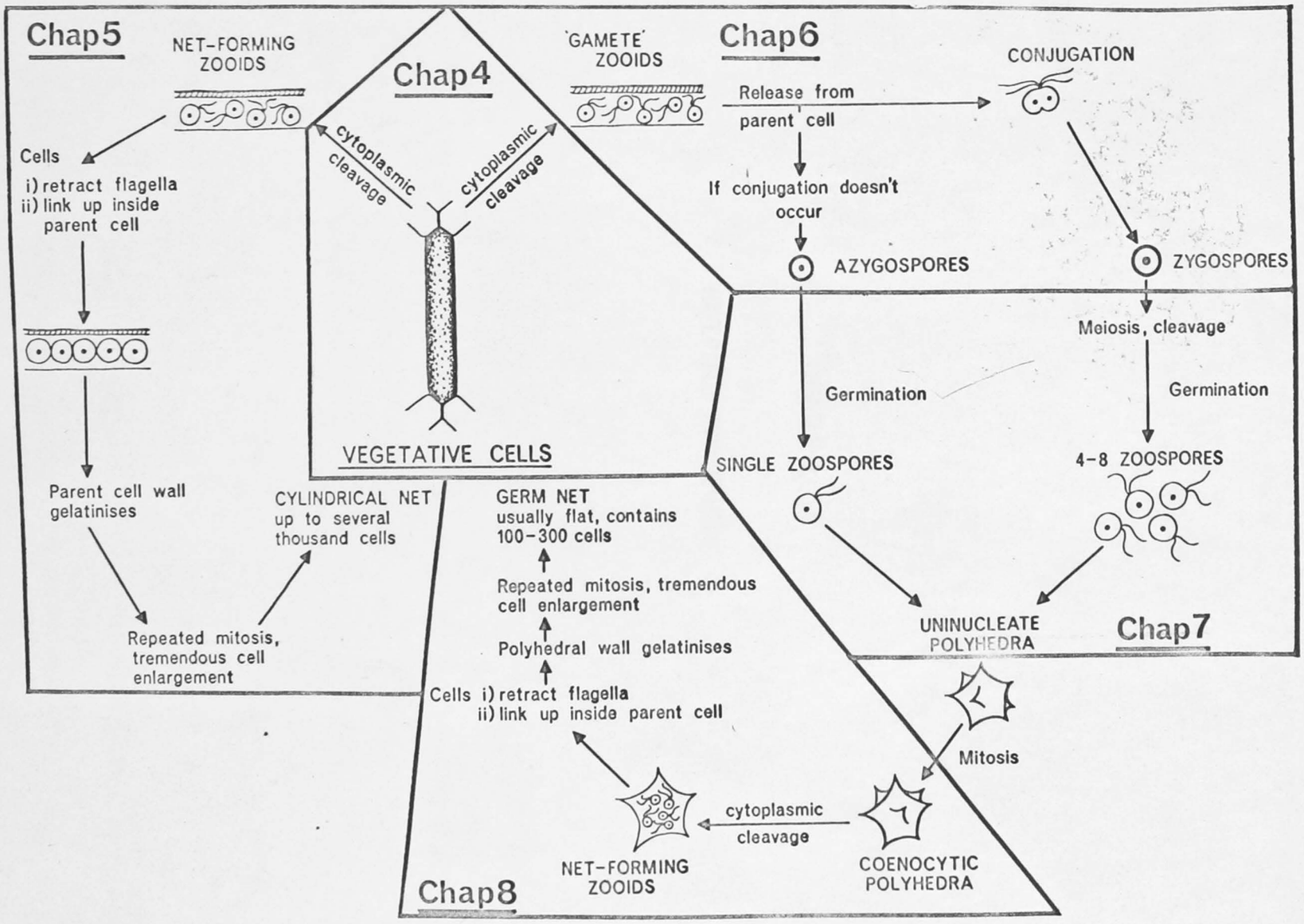

Fig. 1 Subdivisions of the life cycle of H. reticulatum dealt with in various chapters.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS.

Two strains of *Hydrodictyon reticulatum* (L.) Lagerh. were used in this investigation. One, the "Australian" strain, was collected from Coppins Crossing on the Molonglo River, A.C.T., Australia, on the 28th November, 1969. The Molonglo River is a fresh-water stream subject to periodic flooding and Coppins Crossing is about 1 mile downstream from the effluent outlet of the City of Canberra's sewage treatment works. I was unable to find *H. reticulatum* elsewhere along this river despite frequent searching throughout the year. My identification of this alga was kindly confirmed by Dr. M. A. Pocock and a specimen of it has been deposited in the Culture Collection of Algae and Protozoa, Cambridge, England. The other, "Cambridge" strain (Catalogue No. LB 236/1a), was a gift from Mr. E. A. George, Director of the Culture Collection of Algae and Protozoa, Cambridge, England. The only apparent difference between the two strains was that the Australian strain reproduced sexually more frequently than the one from Cambridge, although they were grown under identical conditions.

Pediastrum boryanum (Turp.) Menegh. (Catalogue No. LB 471) and *Chlorella pyrenoidosa* Chick (Catalogue No. LB 972) were supplied by the Indiana Culture Collection, and a mixed culture of *Pediastrum*, provisionally identified as *P. boryanum* and *P. aspersa*, was donated by Dr. I. Stevenson from a collection of algae and protozoa he made at Lake Jolimont, Western Australia.

2.2 ALGAL CULTURE

2.2.1. Culture Media

All the algae were grown in transparent, disposable "Falcon" brand Petri dishes using a variety of media. The most satisfactory were Juller's liquid medium with added soil decoction (Pocock, 1960: p. 179), "Pocock's medium," or our modification of it (Table 2.1). Also used were modifications of both Hill's (Table 2.2) (see Pickett-Heaps, 1970) or Parker, Preston and Fogg's (1963) media (Table 2.3).

TABLE 2.1: POCOCK'S MEDIUM

Compound	Stock Solns. (g/100 ml.)	Standard (ml./l)	Modified (ml./l)
KNO_3	5	.33	.33
$MgSO_4$	5	.33	.33
K_2HPO_4	5	.33	.33
K_2CO_3	7	.33	.33
$CaNO_3$	20	.33	.33
$FeCl_3$	1	.33	.20
Soil Decoction	See below	100	100
Hill's Trace Element Solution			15
B_{12} soln.	1.0 µg/ml.		2.5
Thiamin			speck
Biotin			speck

Check pH 6.7-7.0

Add vitamins after soln. has been boiled.

Soil decoction:

The soil decoction was prepared by heating approximately equal volumes of soil, rich in organic matter, and tap water in a boiling water bath for about an hour. The mixture was left to cool and settle overnight. The supernatant was decanted and filtered.

TABLE 2.2: HILL'S MEDIUM

Compound	Stock Solns.	Standard (ml./l)	Modified (ml./l)
$CaCl_2$	0.1M	5.0	1.0
$MgSO_4$	0.1M	5.0	5.0
KNO_3	1.0M	2.5	2.5
KH_2PO_4	1.0M	2.5	2.5
Trace Elements	See below	20.0	20.0
B_{12}	1.0µg/ml.	2.5	2.5
Thiamin			speck
Biotin			speck

Boil and cool before adding vitamins.

Bring to pH - 6.5-7.0 with KOH.

TRACE ELEMENT STOCK SOLUTION

Compound	Stock Soln. (g/100 ml.)	Element
$Na_2B_4O_7.10H_2O$	4.77	B
$CuSo_4.5H_2O$	0.25	Cu
$CoCl_2.6H_2O$	0.238	Co
$MnCl_2.4H_2O$	0.198	Mn
$ZnSo_4.7H_2O$	0.287	Zn
$(NH_4)_6Mo_7O_{24}.9H_2O$	0.176	Mo
$FeSo_4.7H_2O$	0.278	Fe
$N(CH_2COOH)_3$	1.91	NTA (Nitrilo triacetic acid)

Adjust to pH 2 with 10N H_2SO_4.

TABLE 2.3: PARKER, PRESTON AND FOGG'S MEDIUM

Macronutrient stock solutions

$NaNO_3$	17.0 g/100 ml. distilled water
$K_2H\ PO_4$	2.0 g/100 ml. distilled water
$MgSO_4.7H_2O$	2.0 g/100 ml. distilled water
$CaCl_2$ (anhydrous)	1.0 g/100 ml. distilled water

Micronutrient stock solutions

(a) EDTA (5.00g) + KOH (3.10g) per 100 ml. distilled water

(b) $FeSO_4.7H_2O$ (0.50g) + 0.1 ml. conc. H_2SO_4 per 100 ml. distilled water

(c) H_3BO_3 0.15g/100 ml. distilled water

(d) $ZnSO_4.7H_2O$ (0.0882g)
$MnCl_2.4H_2O$ (0.0144g)
$CuSO_4.5H_2O$ (0.0157g)
MoO_3 (0.0071g)
$Co(NO_3)_2.6H_2O$ (0.0049g)
+ 0.1 ml. conc. H_2SO_4
} combined in 100 ml. distilled water

Add 10 ml. of each macronutrient stock per litre distilled water

Add 1.0 ml. of each micronutrient stock per litre distilled water

After sterilization add 1 ml. of 10 µg/ml. Vitamin B_{12}

2.2.2. Growth Conditions

The cultures were maintained at 20-24° C with a 15hr./9hr. light/dark cycle, lighting being provided by "daylight," "cool white" or "plant growth" fluorescent tubes. In Canberra, Australia, where most of the ultrastructural studies on the life cycle of Hydrodictyon were done, cultures were covered with pieces of white cardboard and illuminated from underneath by two 40W fluorescent tubes. In Boulder, U.S.A., where most of the experimental work was carried out and the other algae investigated, the culture dishes stood on white shelving and were illuminated from above on the same light/dark cycle and at the same temperature used in Canberra. No difference in either the rate of growth or reproductive behaviour of the algae was detected between these two methods of illumination.

Cultures of the algae were also maintained in 250 ml. Erlenmeyer flasks containing Pocock's medium, through which was bubbled 1% CO_2 in air. Bubbled cultures grew more vigorously than unbubbled ones; when grown this way, coenobia of H. reticulatum invariably produced gametes instead of reproducing asexually by forming daughter nets. For prolonged observations on individual cells or colonies, the algae were grown in Petri dishes on 1% agar made up in Pocock's medium.

Axenic cultures of H. reticulatum were prepared by vigorously agitating isolated individual coenobia in 6 or 8 changes of sterile culture medium before placing them in either autoclaved or filter-sterilized medium. Samples of medium from these cultures were examined daily by phase-contrast microscopy to detect contaminating bacteria,

protozoa, fungi and other algae. However, as Hydrodictyon appeared to grow more vigorously in cultures contaminated with various protists, axenic cultures were not used to provide material for either electron microscopy or most of the experimental work. This alga was subcultured by simply transferring young nets to Petri dishes containing fresh media.

Pediastrum and Chlorella were subcultured by pipetting 1 or 2 ml. of a suspension of them into fresh media.

2.3 PREPARATION OF H. RETICULATUM FOR ELECTRON MICROSCOPY.

2.3.1. Problems encountered with coenocytic cells.

When conventional procedures were used to prepare coenocytes of H. reticulatum for electron microscopy, collapse of the central vacuole in all cells, accompanied by severe disruption of their cytoplasm, was inevitable. Considerable time was spent trying to overcome this problem by varying the dehydration and embedding schedules, examining cells by light microscopy at every stage of processing to ascertain when collapse occurred. The problem of collapse and distortion was solved by simply cutting fixed coenocytes into 2 or 3 segments before dehydration.

2.3.2. Fixation.

Coenobia were routinely fixed for 30 minutes at room temperature in 1% "Serva" glutaraldehyde (Sabatini, Bensch and Barnett, 1963; Hopwood, 1969) made up in the growth medium (pH 6.8-7.0), then transferred to 3% glutaraldehyde in the same medium for two hours. They

were then washed in about 5 changes of culture medium before being cut into 2 or 3 pieces and embedded in warm, liquid 1% "Difco" agar. When the agar had solidified, small pieces of it containing segments of coenobia were post-fixed in 1% osmium tetroxide, again made up in the growth medium, at 0° C overnight or else for 1 hour at room temperature, the latter proving more satisfactory. Sometimes cutting of the cells and embedding in agar were delayed until after post-fixation. Numerous variations of this basic routine were tried; for example, the 3% glutaraldehyde step was often omitted. In my experience, cells fixed simultaneously in glutaraldehyde and osmium tetroxide (Franke, Krein and Brown, 1969) never appeared to be as well preserved as those fixed sequentially.

Differentiating zygospores, azygospores and polyhedra were individually selected from cultures with a fine Pasteur pipette, fixed as described above, and then embedded in agar, either in the bottom of a conical centrifuge tube or after being dispersed on a "Millipore" filter which would dissolve during subsequent dehydration.

Gametes liberated from their parental cell into the culture medium were collected by gentle centrifugation, fixed and recentrifuged before being embedded in agar, still in the centrifuge tube. Such specimens however, never appeared as well preserved as gametes from the same culture which had been fixed (without centrifugation) whilst trapped within their parental cell wall.

2.3.3. Dehydration and embedding.

In early work, I initially dehydrated the cells embedded in agar with absolute methyl cellosolve at 0° C, by increasing the concentration in 5% increments every 10 minutes to 100%, followed by 3 changes of absolute methyl cellosolve over 4-8 hours. Then I replaced the methyl cellosolve with cold ethanol added in 10% increments every 10 minutes, and the specimens were left overnight in cold absolute ethanol which was changed at least 3 times (Feder and O'Brien, 1968). However, as no difference in the quality of preservation of the cells could be detected if methyl cellosolve was omitted from the schedule, I discontinued using it.

When methyl cellosolve was not used, ethanol was added more slowly. Usually 4 hours was taken to reach absolute ethanol when dehydrating cut coenobia and longer (up to 8 hours), when dehydrating spores and polyhedra. As before, cells were then left in absolute ethanol overnight. The ethanol was subsequently replaced by cold propylene oxide whose concentration was increased dropwise to 100% over 8 hours, whereupon the cells were soaked in it overnight. After the material in propylene oxide had been brought to room temperature, the propylene oxide was changed twice before the cells were embedded in epoxy resin. On some occasions, I dehydrated in acetone so that propylene oxide, a highly noxious chemical, could be omitted from the schedule. I often found this procedure to be unsatisfactory when dehydrating agar-embedded material, as the embedded specimens were often too soft for easy sectioning after polymerization. I attributed this softness to residual

acetone in the agar that had not been completely replaced by the resin mixture. There are probably two advantages in using propylene oxide in the dehydrating schedule: it is highly volatile which ensures its easy evaporation from the resin mixture and also small residual amounts of this solvent are thought to be incorporated directly into the epoxy polymer.

A mixture of Araldite resin in the appropriate solvent (propylene oxide or acetone) (1:1 v/v) was added dropwise over 6 hours to specimens in the solvent until there was judged to be slightly more than enough resin to cover the specimens after all the solvent had been evaporated off. This slow evaporation of propylene oxide or acetone lasted 1-2 days, after which the material was transferred to fresh resin in plastic "Peel-a-way" embedding dishes and left to stand for a day before the resin containing the specimens was polymerized *in vacuo* at 60° C for 24-36 hours.

When Spurr's (1969) low viscosity embedding medium became available, it was used instead of Araldite. The embedding schedule used was the same, except that polymerization was at 70° C and not under vacuum. Although Spurr's medium gave generally superior results to Araldite (e.g. cell walls were thoroughly embedded and sections did not tear along them in the electron beam), on a few occasions, it inexplicably produced blocks that were either too soft or too brittle to section satisfactorily.

2.3.4. Sectioning and staining.

Sections were cut with a Ge-Fe-Ri diamond knife on a Reichert Om U2 ultramicrotome. Thin sections with a silver interference colour, 60-90 mμ thick (Peachey, 1958), were mounted on either naked or formvar/carbon-coated copper grids. The sections were then stained by floating the grids, first on a drop of either fresh, saturated uranyl acetate in 50% ethanol or 2% aqueous uranyl acetate for 10-30 minutes. After washing with "Millipore" filtered distilled water, the grids were next floated on a drop of lead citrate solution (Venable and Coggeshall, 1965, or Reynolds, 1963) for 1-10 minutes before washing with 0.02M NaOH and distilled water. These stains were generally used at room temperature but if staining was carried out at 70° C (Locke and Krishnan, 1971), the image of many specimens was cleaner and enhanced by higher contrast. Stained sections were examined in either an Hitachi HU-11E, or Phillips EM 300 electron microscope. Photomicrographs were taken on either Ilford N50 plates, Kodak Electron Image plates or Dupont Process Film.

2.4 PREPARATION OF OTHER ALGAE FOR ELECTRON MICROSCOPY.

The unicellular alga Chlorella was collected for fixation in centrifuge tubes at times of the day when I had detected by light microscopy that it was undergoing mitosis and cytokinesis. Chlorella was initially fixed with 1% glutaraldehyde in the growth medium for 30 minutes at room temperature. The concentration of glutaraldehyde was then increased to 3% and fixation continued for another 1-2 hours.

The cells were then pelleted and washed with growth medium before post-fixation for 30-60 minutes at room temperature in 1% osmium tetroxide, again made up in growth medium. The pellets were washed in distilled water and dehydrated slowly at 0° C with acetone. After dehydration, the pelleted cells were embedded in Spurr's resin in the same way as Hydrodictyon, the only difference being that these unicells were concentrated by centrifugation in "Beem" capsules containing pure resin before polymerization.

Pediastrum was handled in two ways. Colonies were either individually selected and transferred from one solution to the next, from fixation to embedding with Pasteur pipettes, a long and tedious process, or else they were collected and processed in a centrifuge tube as was Chlorella. When these cells had been finally embedded in liquid resin, they were lightly compressed between two glass microscope slides coated with polytetrafluorethylene (P.T.F.E., Teflon). Then after polymerization, the thin layer of resin containing the cells was peeled from the slides. Differentiating colonies were selected under the microscope, cut out of the thin wafers of resin and glued on to resin stubs for sectioning.

2.5 LIGHT MICROSCOPY.

Routinely, sections with a blue-green interference colour (200-300 mμ thick) were cut from the same material that was being sectioned for electron microscopy. These sections were transferred to a drop of water on glass microscope slides and dried down. They were stained with 1%

Toluidine Blue in a solution of 1% borax by heating a drop of stain over the section for 15 seconds and then washing the slide well with distilled water. The stained sections were examined with a Zeiss Universal microscope and photographed with planapochromatic objective lenses on Kodak Panatomic-X film which was developed in Kodak Microdol-X developer, diluted 1:3.

All stages of the life cycle of H. reticulatum and asexual reproduction in Pediastrum and Chlorella have been extensively studied in vivo with the light microscope and photographed using both phase contrast and Nomarski differential interference optics. A Zeiss microflash device was used to photograph motile cells.

2.6 PREPARATION OF MATERIAL FOR SCANNING ELECTRON MICROSCOPY.

Cells at various stages in the life cycle of H. reticulatum and colonies of Pediastrum boryanum were examined with the scanning electron microscope (SEM). Numerous techniques for preparing fragile biological material for the SEM have been proposed (Echlin, 1968; Arenberg, Marovitz and MacKenzie, 1970). The technique of critical point drying with carbon dioxide was introduced by Anderson (1951) and as it had been used successfully to preserve ciliate protozoa (Horridge and Tamm, 1969) and delicate tissue culture cells (K. R. Porter, personal communication), I decided to try it on my material. Algal cells were collected on either "Solvinert" or "Mitex" Millipore filters (neither of which could be dissolved by the reagents used to dehydrate the cells) and fixed with glutaraldehyde and osmium tetroxide as described above

(section 2.3.2). After post-fixation, the cells adhering to the filter were washed in distilled water and dehydrated with ethanol, whose concentration was increased by 10% every 20-30 minutes. From 100% ethanol, the cells were transferred via 25%, 50%, 75% dilutions of n-amyl acetate in ethanol to 100% n-amyl acetate over a total period of about one hour. The "Millipore" filter on which the cells had adhered was then mounted in a specially constructed polypropylene cell designed to fit in a carbon dioxide (CO_2) critical point drying apparatus. In this apparatus, the n-amyl acetate in the specimen was gradually replaced with liquid CO_2 under high pressure. Subsequent heating of the specimen chamber of the apparatus raises the temperature of the CO_2 to above its critical point whereupon it loses its ability to co-exist in two phases, at which point all surface tension forces vanish. When the pressure in the apparatus is released slowly, gaseous CO_2 escapes to leave a dry preparation. This ingenious procedure avoids the disruption of delicate specimens caused by surface tension effects associated with the phase boundary between liquid (or solid) and gas. The "Millipore" filter bearing the dry specimens was glued to an aluminum stub with double sided adhesive tape and then omnidirectionally shadowed with carbon and gold in a "Denton" vacuum evaporator. The carbon coating ensures electrical conductivity of the specimen's surface; the gold provides a good source of secondary electrons which are emitted upon excitation by the scanning beam. The preparations were viewed in a Cambridge Stereoscan scanning electron microscope and photomicrographs taken on Polaroid P/N 55 Positive/Negative film.

2.7 COLCHICINE EXPERIMENTS.

Colchicine is known to prevent polymerization of microtubular subunits at relatively low concentrations, and to depolymerize microtubules at higher concentrations (Inoué and Sato, 1967). This drug has been widely used on both plant and animal cells to experimentally disrupt microtubular systems to investigate the role of these organelles in various cellular activities (see the reviews of Porter, 1966; Newcomb, 1969; Pickett-Heaps, 1972b). Colchicine was used here to investigate two aspects of differentiation in Hydrodictyon reticulatum and Pediastrum boryanum; cleavage of the coenobial cytoplasm to form zooids, and the subsequent aggregation of these zooids as nets or colonies.

Coenocytes of both H. reticulatum and P. boryanum about to produce zooids were treated with either 0.1%, 0.2% or 0.4% colchicine (w/v) (Sigma or BDH), freshly made up in the culture medium. After various times of exposure to colchicine, the cells were examined by light and electron microscopy. Also, coenocytes of these algae at the "pavement stage" of differentiation (i.e. where cytoplasmic cleavage is virtually complete; see Chapter 4) were treated similarly. Cultures being exposed to colchicine were placed in the dark or a dimly lit place to minimize photochemical deactivation of colchicine (Egsti and Dustin, 1955; Chapman, Smith and King, 1963; Aronson and Inoué, 1970).

2.8 ATTEMPTED INTRACELLULAR LOCALIZATION OF SODIUM IONS WITH POTASSIUM PYROANTIMONATE.

Despite the amount of physiological data on the movement of ions into and out of cells, little is known about which components of the cytoplasm are involved in ion transport. Giant algal cells such as Nitella (McRobbie, 1969), Acetabularia (Saddler, 1970) and Hydrodictyon africanum (Canny, personal communication; Raven, 1967) are popular organisms for the study of ionic flux. McRobbie (1970) showed that the movement of ions into the vacuole of Nitella was quantized and concluded on the basis of ultrastructural studies (Costerton and McRobbie, 1970), that vesicles of endoplasmic reticulum were involved in ion transport. West and Pitman (1967), studying the problem in the marine green alga Ulva lactuca, suggested that golgi vesicles may transport ions. In an attempt to resolve which components of the cytoplasm are involved in ion transport, I used potassium pyroantimonate which has been employed for the intercellular localization of sodium and other cations at the ultrastructural level in such diverse tissue as avian salt glands (Komnick and Komnick, 1963), cornea (Kaye, Cole and Donn, 1965), kidney (Bulger, 1969) and intercellular junctions in lamellibranch gill epithelium (Satir and Gilula, 1970). This technique, introduced by Komnick (1962), depends on the precipitation of insoluble, electron-dense sodium pyroantimonate or the pyroanimonate salt of some other cations (Klein, Yen and Thureson-Klein, 1972). However it is uncertain which pyroantimonate salts are precipitated, but there is an impressive amount of data to suggest that the

precipitate is mostly sodium pyroantimonate. This evidence includes:

1. The loss of radioactive sodium ions from muscle fixed in the presence of potassium pyroantimonate was considerably less than that in muscle fixed in the absence of potassium pyroantimonate (Zadunaisky, 1966).
2. Lande and Martin (1969) found that most of the precipitate was sodium pyroantimonate by electron microprobe analysis.

Coenobia of H. reticulatum to be treated with potassium pyroantimonate were placed in deionized water, which was changed often, for 2-24 hours before being immersed for 20 minutes in one of the following solutions:

1. deionized water
2. 0.4M NaCl
3. 0.4M KCl
4. 0.4M NaCl + 0.2mM oubain
5. 0.4M KCl + 0.2mM oubain
6. 2% potassium pyroantimonate in deionized water.

The pyroantimonate solution was prepared by boiling 2% (w/v) potassium pyroantimonate in deionized water for 3 minutes, cooling it quickly and centrifuging for 5 minutes (Tandler, Libanati and Sanchis, 1970).

Oubain specifically inhibits sodium-potassium transport across membranes (see Kaye, Cole and Donn, 1965). Comparison of the distribution of pyroantimonate precipitate in oubain tested "salt pulsed" material with that which had not been oubain treated should reveal the localization of sodium ions introduced during pretreatment, distinct from sodium ions retained by the cells during the washing in deionized water before the pretreatment.

All pretreated cells were fixed for 30 minutes at room temperature in either unbuffered 1% osmium tetroxide and 1% potassium pyroantimonate or a mixture of 1.5% potassium pyroantimonate, 1% osmium tetroxide and 2% glutaraldehyde. Neither method of fixation was entirely satisfactory. The cytoplasm of cells fixed in the absence of glutaraldehyde was disrupted. When glutaraldehyde was used, various organelles such as nuclei, chloroplasts and mitochondria remained intact and contained a precipitate of pyroantimonate, but the rest of the cytoplasm was not adequately preserved to conclude anything about the distribution of precipitate there.

2.9 TECHNIQUES USED TO INVESTIGATE THE NATURE OF THE ADHESIVE BETWEEN VEGETATIVE CELLS OF *H. RETICULATUM*.

2.9.1. Treatments with various enzymes.

Aggregating zooids of *H. reticulatum* as well as nets of various ages were treated with numerous enzymes to investigate the nature of the intercellular adhesive. The enzymes used, their concentrations, as well as the buffers they were made up in, are tabulated in Chapter 11. Nets of known ages were incubated in the enzyme solutions and the efficacy of each enzyme in attacking the intercellular adhesive was assessed by the relative ease with which treated nets could be teased apart with dissecting needles after a timed exposure to the enzymes. In later experiments, the time taken for both treated and untreated nets to disaggregate upon sonication was used to assess the action of various enzymes on the intercellular adhesive. For these latter

experiments, nets of various ages were incubated in 10 ml. of enzyme solution for specific periods of time, and then sonicated in a "Technic International" sonicator. Disaggregated cells were checked by light microscopy to determine whether they had parted at intercellular junctions or if the cells themselves had been ruptured.

2.9.2. EDTA.

The chelating agent ethylene diaminotetra-acetic acid (EDTA) was used at various concentrations to see if it had any effect on the adhesive joining vegetative cells of H. reticulatum. Northcote, Goulding and Horne (1960) speculated that the sites of contact between cells of H. africanum could contain pectic substances. Removal of Ca^{++} ions with EDTA from the pectic gel should convert it to the sol form which could weaken or possibly remove the intercellular adhesive.

Nets of various ages treated with disodium EDTA at concentrations ranging from 0.001 to 0.1M at pH 6.7-7.0 were all killed within 12 hours and the intercellular adhesive was not detectably affected.

2.9.3. Cytochemistry.

2.9.3.1. Staining for polysaccharides and disulphide bonds with silver hexamine.

Gomori's (1946) silver methenamine reagent has been used by several investigators for the ultrastructural localization of both polysaccharides and disulphide groups in plant and animal tissue (Swift, 1966; Rambourg and Leblond, 1967a; Pickett-Heaps, 1967, 1968; Colvin and

Leppard, 1971). Using this method, metallic silver is deposited following reduction of silver complexed in an alkaline hexamine solution, by aldehyde, disulphide and some other groups, principally ethylenic linkages (Rambourg, 1971). By pretreating sectioned material with various oxidizing and reducing agents, it is possible to introduce or block aldehyde groups and reduce and alkylate disulphide bonds. For example, peroxidation of certain polysaccharides produces aldehyde groups (the basis of the classic Periodic acid/Schiff's reaction of light microscopy).

Cells of H. reticulatum to be used for cytochemistry were fixed and embedded as outlined earlier. Some nets of H. reticulatum were simply fixed in glutaraldehyde alone, without post-fixation in osmium tetroxide. "Silver-gold" sections of the epoxy-embedded cells were collected on either the surface of a drop of water held in the centre of polythene washers 5 mm. in diameter with a hole 3 mm. in diameter, or else on palladium grids. Palladium, unlike copper, does not react with the silver hexamine reagent. The washers were punched from polythene sheets and flamed very briefly before use. Sections were trapped on the drop of liquid held in the hole of the washer and, provided care was taken, could be transferred from one solution to another as required. Batches of adjacent sections cut through an intercellular junction were subjected to combinations of the following treatments.

1. Periodate oxidation.

Batches of sections in washers were floated on 1% periodic acid for 30 minutes at room temperature, after which they were washed by

floating them on about 5 changes of distilled water for a total time of about 4-16 hours. As for the periodic acid/Schiff's reaction, periodate oxidation converts 1,2 glycol (and certain other) groups in some polysaccharides to aldehyde groups which can react with the silver hexamine reagent.

2. Reduction and/or alkylation of disulphide bonds.

Benzyl thiol (α-toluenethiol) was recommended by McLaren (1962) for reducing cystine residues in keratin to cysteine. Iodoacetate alkylates the reduced cystine forming s-carboxy methyl cysteine as follows:

$$\text{Cys S-S Cys} \xrightarrow{\text{benzyl thiol}} \text{2Cys SH} \xrightarrow{\text{iodoacetate}} \text{2Cys S-CH}_2\text{COOH}$$

Sections of coenocytes, fixed with both glutaraldehyde and osmium as well as those fixed only in glutaraldehyde and mounted on palladium grids, were immersed in 0.3M benzyl thiol in 30% n-propanol for 90 minutes at room temperature, then washed for a total time of 1 hour in 3 changes of 30% n-propanol. Previously untreated sections as well as those whose disulphide bonds had been reduced with benzyl thiol were transferred to an iodoacetate solution at pH 8 and incubated for 90 minutes at 60° C, then thoroughly washed, first with 50% n-propanol and then with distilled water. The iodoacetate reagent was made up by dissolving 1.86 gm. of iodoacetic acid and 1.235 gm. of boric acid in 40 ml. of distilled water adjusting the pH to 8.0 with 1M KOH, diluting to 50 ml. with distilled water, then adding 50 ml. of n-propanol.

Palladium grids were used for these treatments because sections were invariably lost from the drops of liquid held in plastic washers

when transferring the washers from n-propanol to water. Floating sections always appeared considerably cleaner than those sections collected on the palladium grids.

3. Untreated Material.

Some sections remained untreated, i.e., they were kept floating on the surface of distilled water while the others were being pretreated.

Sections of material fixed with glutaraldehyde alone, in which disulphide bonds had been reduced and alkylated with benzyl thiol and iodoacetate, were completely unreactive to silver hexamine while silver was deposited on untreated sections of the same material (see Chapter 11). As all reactivity could be blocked by reducing and masking disulphide bonds, it seemed likely that all the silver deposited in untreated sections was precipitated by disulphide bonds and not be aldehyde groups, either native or introduced by glutaraldehyde fixation. Hence I did not think it necessary to pretreat sections with bisulphite or dimedone, reagents which block such aldehyde groups (Pickett-Heaps, 1967).

4. Silver hexamine staining.

Sections that had been pretreated by either oxidation or reduction and/or alkylation, as well as untreated material, were floated on, or immersed in, freshly prepared silver hexamine reagent for 20-45 minutes at 60° C. This reagent was made up by mixing 5 ml. of 5% silver nitrate, 45 ml. of 3% hexamine and 5 ml. of 2% sodium borate (Rambourg and Leblond, 1967b).

5. Clearing.

After treatment with silver hexamine, all sections were washed with 5% thiosulphate for 10 minutes at room temperature to extract any covalently bound silver. Sections were then washed in distilled water and those floating on a drop within a washer were mounted on formvar-coated grids.

2.9.3.2. Periodic Acid/Schiff's staining for polysaccharides.

Schiff's reagent is widely used for the histochemical localization of polysaccharides (Feder and O'Brien, 1968). In this work, I mounted "blue-green" sections of coenobia of H. reticulatum on clean glass slides (Section 2.5). Some of these sections were immersed in 1% periodic acid for 5-10 minutes, rinsed thoroughly in running tap water, and then immersed in Schiff's reagent, along with other slides, untreated with periodic acid. After 30 minutes exposure to Schiff's reagent, all the slides were washed in two changes of freshly prepared metabisulphite solution (see below), rinsed for 5 minutes in running water and dried.

Although Schiff's reagent is commercially available, I preferred to make it up myself, following the recipe given in the class notes for "Plant Histology; Techniques in use at Harvard, July 1965" by Ned Feder. The method used can be summarized as follows:

1 gm. of basic fuschin was dissolved in 100 ml. of freshly boiled distilled water. This solution was then cooled to 60° C and filtered. 2 gm. of potassium metabisulphite and 20 ml. of 1N

HCl were added to the filtrate which was stoppered and stored in the dark for 18-24 hours. 300 mgm. of activated charcoal was added to the solution, which was shaken vigorously for a minute and then filtered. The filtrate was stored in the dark at 0-5° C.

The metabisulphite rinse was made up fresh every time the Schiff's reagent was used, by adding 5 ml. of 10% potassium metabisulphite to 5 ml. of 1N HCl and 90 ml. of distilled water.

2.9.3.3. Ruthenium red.

Ruthenium red has been widely used in electron microscopical cytochemistry to locate acid mucopolysaccharides in a variety of animal cells. Most early attempts utilized this stain added to the fixative, but the problems arising from poor penetration of the tissue by the stain and its reduction during subsequent processing were never completely resolved (see however, the recent papers by Luft, 1971a,b, and Szubzinska and Luft, 1971). Kobasi and Asboe-Hansen (1971) have recently stained Epon-embedded thin sections with ruthenium red and have claimed a high degree of specificity in staining mucopolysaccharides in mast-cell granules.

As part of my investigation of the nature and source of the intercellular adhesive, I employed Kobasi and Asboe-Hansen's technique. Thin sections of recently aggregated zooids, mounted on either naked or formvar-coated copper grids, were floated on drops of freshly made up 0.5% ruthenium red in 0.1M NH_4OH for 30-60 minutes, and were then

washed with 0.1M NH_4OH and distilled water. However, no staining of the adhesive or any other part of the zooids was detectable.

CHAPTER 3

MITOSIS IN THE COENOBIUM

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ULTRASTRUCTURE AND DIFFERENTIATION OF *HYDRODICTYON RETICULATUM*

I. MITOSIS IN THE COENOBIUM

By H. J. Marchant* and J. D. Pickett-Heaps*†

[*Manuscript received June* 19, 1970]

Summary

Newly developed processing techniques have made possible an ultrastructural study of mitosis in the coenocytic green alga, *H. reticulatum*. Centrioles, apparently persistent in the coenobia, replicated and migrated to the poles of the prophase spindle as microtubules appeared between them. A perinuclear envelope of endoplasmic reticulum enclosed the nucleus, centrioles, and cytoplasmic microtubules around the nucleus at prophase; it remained virtually intact until telophase. The nuclear envelope was deeply invaginated at the poles during prometaphase before the formation of large polar fenestrae. Through these openings extranuclear microtubules invaded the nucleus. Separation of the chromosomes was accompanied by considerable elongation of the spindle within a nuclear envelope that remained intact except for the polar fenestrae. Nuclear division in this alga is compared with that in various other organisms.

I. Introduction

Very few accounts of the ultrastructure of coenocytic algal cells have been published, no doubt due to the formidable problems of avoiding the gross damage and collapse in these large, highly vacuolate cells that follows standard preparative methods for electron microscopy. Recently, considerable development of techniques has allowed examination for delicate filamentous algae such as *Spirogyra* (Fowke and Pickett-Heaps 1969*a*, 1969*b*) and *Oedogonium* (Pickett-Heaps and Fowke 1969, 1970*a*, 1970*b*). These techniques have now been further refined to yield adequate preservation of large coenocytic cells.

The very large vegetative cells of the water net, *Hydrodictyon reticulatum*, are coenocytic and cylindrical in shape with a very thin layer of cytoplasm lining the cell wall and enclosing the large central vacuole. Cells are linked together to form extensive nets (Fig. 1). To reproduce both sexually and asexually the cytoplasm of the coenobia of this alga cleaves to form a multitude of tiny uninucleate zooids. This cytoplasmic cleavage and the various fates of the zooids will be dealt with in subsequent papers. For an extensive discussion of the life cycle of *H. reticulatum* Pocock's (1960) excellent paper should be consulted.

* Research School of Biological Sciences, Australian National University, P.O. Box 475, Canberra City, A.C.T. 2601.

† Present address: Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80302, U.S.A.

Mitosis in *H. reticulatum* has been described by a number of light microscopists, most recently by Proskauer (1952); some ultrastructural observations on the early stages of mitosis in *H. africanum* have been reported by Northcote (1968). This paper will concentrate on details of nuclear division beyond the limit of resolution of the light microscope, and the results will be related to studies on mitosis in other organisms.

II. Materials and Methods

(a) *Materials*

*H. reticulatum** was collected from Coppins Crossing on the Molonglo River, A.C.T. It was maintained in culture using Hill's medium (details given by Pickett-Heaps 1970). The alga was grown in Petri dishes at 20°C with a 15 hr/9 hr light/dark cycle, the lighting being provided from underneath by two 40W daylight fluorescent tubes. For fixation, cells were taken from cultures contaminated with various protists, as *H. reticulatum* appeared to grow more vigorously in these conditions than in axenic culture.

(b) *Methods*

(i) *Fixation.*—Cells were fixed in 1% glutaraldehyde in Hill's medium (pH 6·8) at room temperature for 30 min. They were transferred to 3% glutaraldehyde in the same medium for 2 hr. After five washings in Hill's medium over 30 min, they were post-fixed overnight at 0°C in 1% OsO_4, again in Hill's medium. They were then washed in cold distilled water and each cell cut into two or three pieces which were embedded in 1% agar.

(ii) *Dehydration and Embedding.*—Cells, in agar, were run to absolute methyl cellosolve by 5% increments added every 10 min at 0°C. After three changes of absolute methyl cellosolve over 4–8 hr, the cells were run to absolute ethanol by 10% increments, made every 10 min, and left in the cold overnight with at least three changes of ethanol. Cold propylene oxide was added dropwise to the material in ethanol over 6 hr until the concentration of propylene oxide was about 70%. The material was then transferred to 100% propylene oxide and left in the cold overnight, brought to room temperature, and washed twice with propylene oxide. A mixture of Araldite and propylene oxide (1:1 v/v) was added dropwise until there was enough resin to cover the cells when all the propylene oxide was evaporated off. The material was placed in fresh resin after 2 days of slow evaporation of the propylene oxide. This Araldite was polymerized *in vacuo* at 60°C for 36–48 hr.

(iii) *Sectioning and Staining.*—Sections were mounted on coated grids and stained with uranyl acetate and lead citrate (Venable and Coggeshall 1965) for examination in an Hitachi HU-IIE electron microscope. Thicker sections were mounted on glass slides. stained with toluidine blue (Pickett-Heaps and Fowke 1969), and examined with a Zeiss Universal microscope.

(iv) *Living Material.*—Living cells mounted in the culture medium were photographed using both phase-contrast and Nomarski differential interference-contrast optics.

III. Observations

(a) *Light Microscopy of Living Material*

The small nuclei (3–5 μm diameter) were often obscured by the overlying chloroplast(s), making detailed observation of mitosis *in vivo* of little value. Figures 2–7† show adjacent nuclei undergoing mitosis synchronously. The prominent

* See Acknowledgments, p. 1185.

† The following abbreviations are used on Figures 2–28: *c*, centriole complex; *ch*, chromosome; *g*, golgi body; *k*, kinetochore; *n*, nucleus; *nc*, nucleolus; *nm*, nuclear membrane; *p*, pyrenoid; *pf*, polar fenestra; *rn*, reforming nucleus; *t*, microtubules; *w*, cell wall.

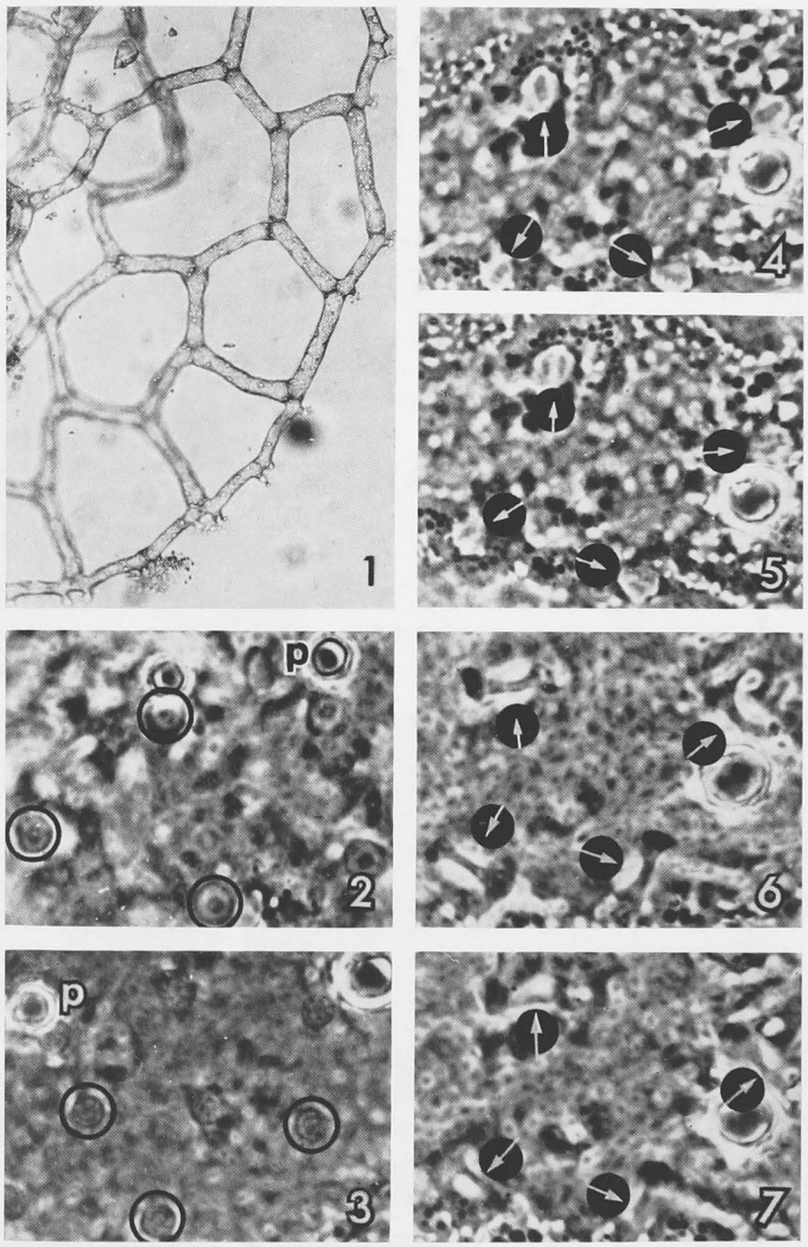
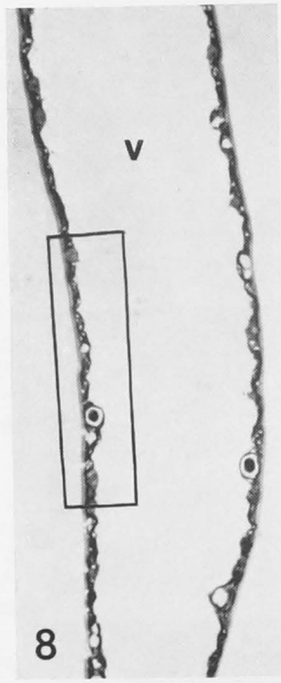


Fig. 1.—Part of a young net of *H. reticulatum*. ×110. [Figures 1–9 are light micrographs; Figures 10–28 are electron micrographs.]

Figs. 2–7. Living cells of *H. reticulatum* undergoing mitosis. In Figure 2 some interphase nuclei are ringed, and nucleoli may be observed. In Figure 3—prophase—the nucleoli have dispersed. Figures 4–7 show part of a series following mitosis in four nuclei within one cell. Arrows indicate the plane of the metaphase plate. *4*, metaphase; *5*, anaphase—early separation of chromatids; *6*, early telophase—spindle elongation may be noted; *7*, telophase. All phase-contrast. ×1400.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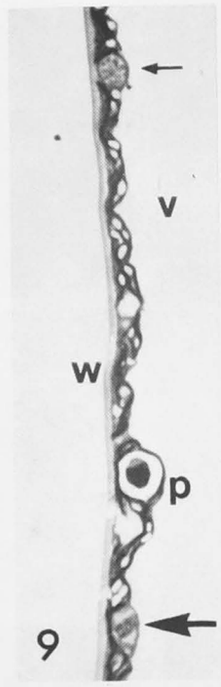
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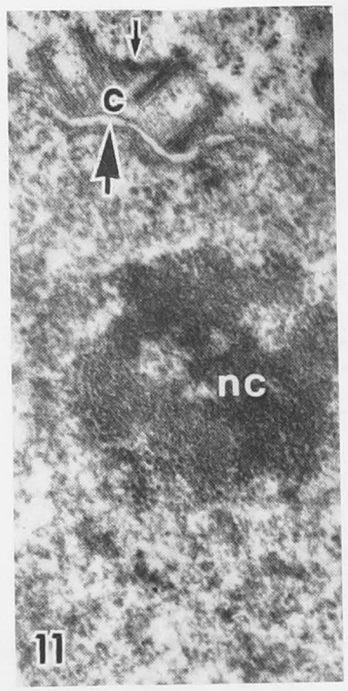
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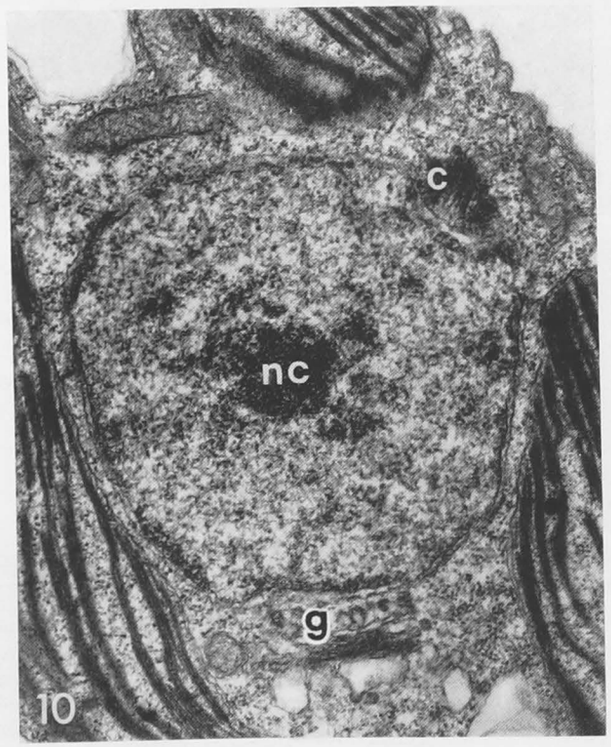
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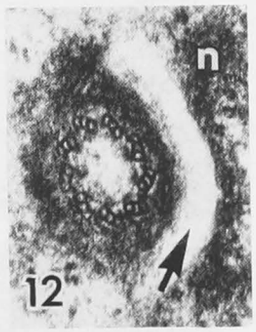
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nucleolus (Fig. 2) dispersed as the chromatin condensed at prophase (Fig. 3); a metaphase plate was clearly visible (Fig. 4) and separation of the chromosomes (Fig. 5) was accompanied by considerable elongation of the spindle (Figs. 6 and 7). The interphase to metaphase transition took about 20 min and metaphase to telophase only about 5 min. Mitotic waves, described by early workers (e.g. Timberlake 1902 and Proskauer 1952), were commonly seen in the coenobia.

(*b*) *Light Microscopy of Fixed Material*

The cells of *H. reticulatum* used were around 2 mm long and 0·2 mm in diameter, rather larger than those shown in Figure 1; unless they were cut after fixation and unless the subsequent dehydration and embedding in resin were very gradual, severe collapse was inevitable. The efficacy of our processing methods can be judged from Figures 8 and 9, which are light micrographs showing part of a longitudinally sectioned cell with its enormous central vacuole. The peripheral layer of cytoplasm was only very rarely detached from the cell wall; the tonoplast was sometimes damaged, ballooning into the vacuole.

(*c*) *Electron Microscopy*

Centrioles appeared to be persistent in the coenocytic stage of the life cycle but they have not been found in some of the uninucleate sexual stages (Marchant and Pickett-Heaps, unpublished data).

(i) *Interphase*

At interphase, the chromatin was usually diffuse (Fig. 10) but sometimes partially condensed near the nuclear envelope. The nucleolus apparently comprised two components (Fig. 11)—a dense matrix and loosely clustered granules about the size of ribosomes. Two centrioles, at right angles, lay adjacent to the nuclear envelope which was distended at this site (Figs. 10–13). A similar modification of the nuclear envelope has also been observed by Northcote (1968) in *H. africanum*. Microtubules were uncommon at interphase even near the cell wall where they might have been expected from observations on other algae and higher plants. Golgi bodies were frequently positioned with one face close to the nuclear envelope; coated vesicles often lay between the nuclear envelope and the face of the golgi body (Fig. 10).

Fig. 8.—Longitudinally sectioned cell of *H. reticulatum* showing the thin layer of cytoplasm enclosing the large central vacuole (*v*). The enclosed area is shown at higher magnification in Figure 9. ×560.

Fig. 9.—Peripheral cytoplasm of *H. reticulatum* containing two nuclei undergoing mitosis: prophase (small arrow), early anaphase (large arrow). Also note the large pyrenoid (*p*). ×1300.

Fig. 10.—Interphase nucleus with adjacent golgi body and centriole complex. ×26,000.

Fig. 11.—Part of an interphase nucleus showing the components of the nucleolus. The nuclear envelope is dilated near the centriole complex (large arrow). Note the bridge (small arrow) linking the centrioles. ×35,000.

Fig. 12.—Transverse section of a centriole complex showing the triplet structure surrounded by amorphous material. Note also the dilated nuclear envelope (arrow). ×65,000.

Fig. 13.—Centriolar replication at prophase; of the four centrioles, two are sectioned transversely and two tangentially. ×30,000.

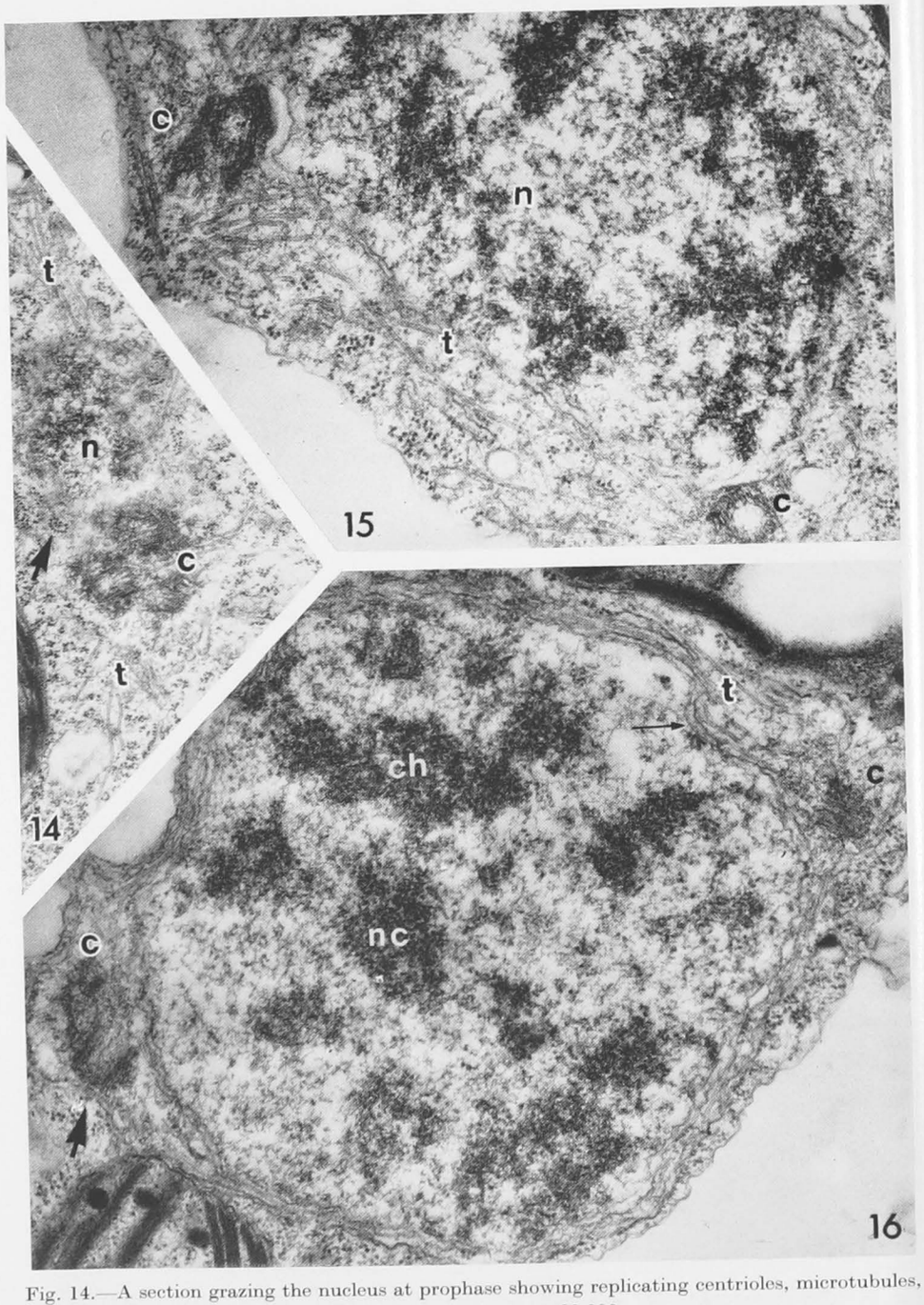

Fig. 14.—A section grazing the nucleus at prophase showing replicating centrioles, microtubules, and polysome spirals (arrow) on the nuclear envelope. ×20,000.

Fig. 15.—Separation of the centriole complexes to establish the poles of the spindle. ×30,000.

Fig. 16.—Nucleus at late prophase showing condensing chromatin, the nucleolus dispersing, and the centriole complexes at the poles. The perinuclear envelope (large arrow) encloses one centriole. Note the slight invagination of the nuclear envelope (small arrow). ×30,000.

(ii) *Preprophase*

Microtubules proliferated outside the nucleus near the centrioles. The nucleolus lost its dense matrix leaving a loose assembly of granules. The golgi bodies and nuclei separated at preprophase and remained so until late telophase.

(iii) *Prophase*

The centrioles, surrounded by amorphous material (Fig. 12), replicated (Figs. 13, 14), and the centriole complexes separated (Fig. 15) to establish the poles of the spindle, the nucleus becoming ensheathed with microtubules (Figs. 16, 17). Concurrently a layer of endoplasmic reticulum enveloped the nucleus and the surrounding layer of cytoplasm containing the centrioles and microtubules (Figs. 16, 17). Dispersion of the nucleolus rendered it almost indistinguishable from the condensing chromatin (Fig. 16).

(iv) *Prometaphase*

Ribosome-like particles were liberated into the nucleoplasm by the complete dispersion of the nucleolus (Fig. 19). Cytoplasmic microtubules began invading the nucleus through polar fenestrae (i.e. openings) forming in the nuclear envelope near the centrioles (Figs. 19, 20). Often the nuclear envelope was temporarily invaginated at the poles, presumably by these elongating microtubules (Figs. 16, 18, 19, 20).

(v) *Metaphase*

The paired chromosomes on the metaphase plate were traversed by continuous microtubules while chromosomal microtubules linked the kinetochores with the poles (Figs. 21–23). The polar fenestrae in the nuclear envelope enlarged but the perinuclear envelope of endoplasmic reticulum remained intact (Fig. 21). The centrioles usually lay in the polar fenestrae (Fig. 21) but Figure 22 shows an intercalation of the nuclear membrane between the centriole and the spindle. Elongation of the spindle started during metaphase (compare Figs. 16 and 21).

(vi) *Anaphase*

The separation of the chromosomes was accompanied by considerable elongation of the spindle (compare Figs. 4–7 with Figs. 22–25). Both nuclear and perinuclear envelopes remained essentially intact (apart from the polar fenestrae of the former) as they contracted between the separating chromosomes (Fig. 24).

(vii) *Telophase*

Mid-bodies were never found between the widely separated daughter nuclei. The perinuclear envelope dispersed and the restoration of intact nuclear envelopes preceded the disappearance of the continuous microtubules remaining between the daughter nuclei (Figs. 25, 26, 28). At late telophase, some of the centriole complexes appeared to move around the nuclear envelope until they were about 90° from the spindle axis (Fig. 27). Unlike in some other algae (*Oedogonium*, *Closterium*, *Ulva*, and other species) the post-mitotic nuclei in *H. reticulatum* did not come close together.

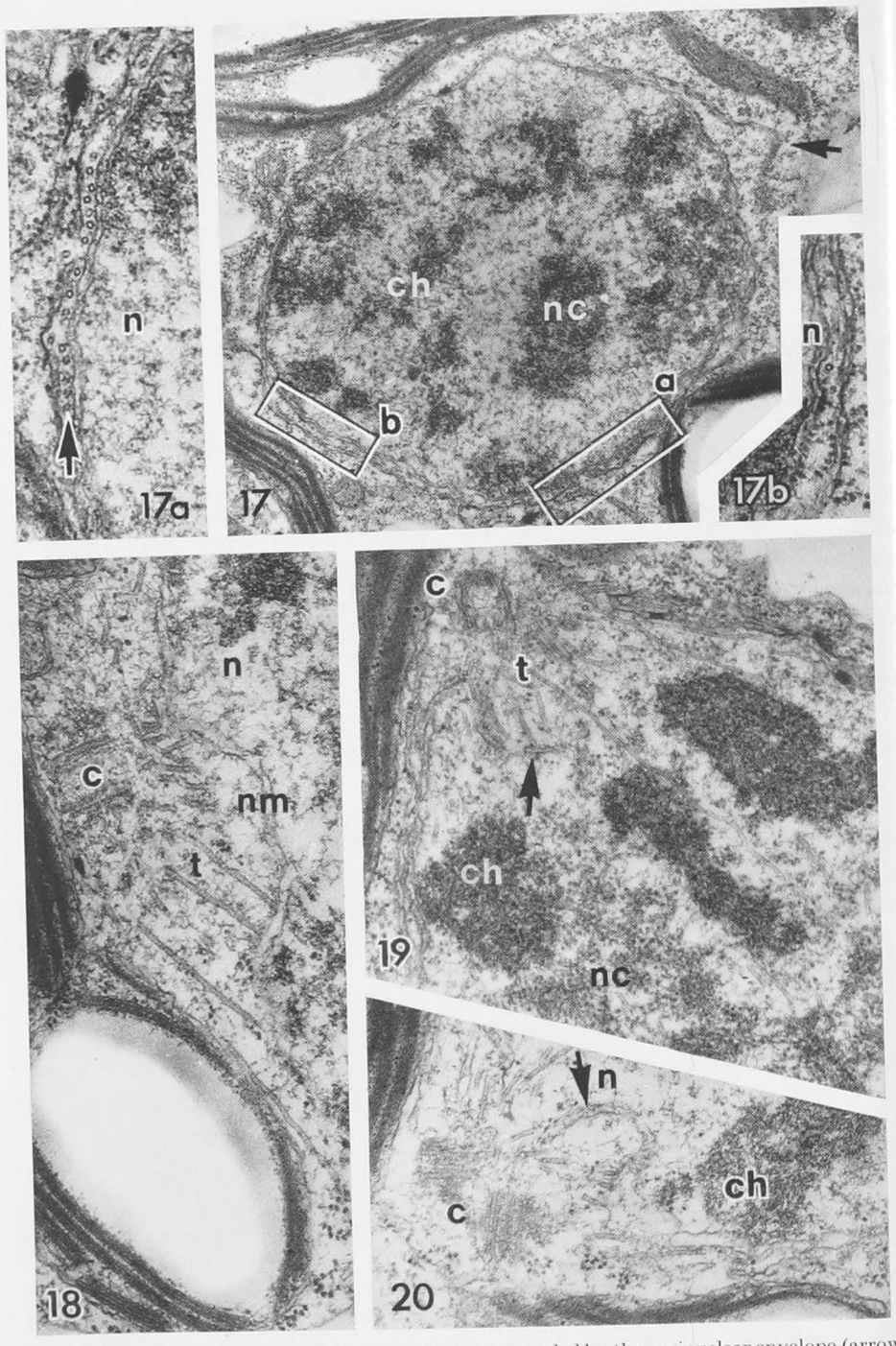

Fig. 17.—A nucleus at prophase almost completely surrounded by the perinuclear envelope (arrow). Fine detail of the enclosed areas is shown in Figures 17*a* and 17*b*. ×17,500.

Fig. 17*a*.—A band of transversely sectioned microtubules (arrow) lying between the nuclear and perinuclear envelope. ×45,000.

Fig. 17*b*.—Fine detail of the nuclear and perinuclear envelopes. ×45,000.

IV. Discussion

The dearth of published observations on the ultrastructure of highly vacuolate coenocytic cells, despite their importance in studies on ion transport, etc., probably reflects the difficulty of preserving these cells for electron microscopy. In our experience, ultrastructural information from cells damaged by conventional processing techniques can be misleading and render any discussion of the gross structure and spatial relationships between cytoplasmic components futile.

Unlike most of the algae studied in this laboratory, it was not essential to meticulously select young, actively growing cells for fixation which is usually necessary (Pickett-Heaps and Fowke 1969, 1970*a*) to avoid cytoplasmic denseness that obscures ultrastructural detail. Cells of some other stages of the life cycle of *H. reticulatum* do, however, exhibit this quality (Marchant and Pickett-Heaps, unpublished data).

In most higher plant and animal cells, the nuclear envelope fragments early in mitosis and reforms at telophase, while in many lower forms of both kingdoms the nuclear envelope remains more or less intact throughout mitosis. Both intact and open spindles are found among the algae. Those with open spindles include *Chara* (Pickett-Heaps 1967), *Prymnesium* (Manton 1964), and *Closterium* (Pickett-Heaps and Fowke 1970*c*); examples of those having closed spindles include species of *Oedogonium* (Pickett-Heaps and Fowke 1969), *Ulva* (Løvlie and Bråten 1970), *Chlamydomonas* (Johnson and Porter 1968), *Kirchneriella* (Pickett-Heaps 1970), and other species [see Pickett-Heaps (1969) for review]. During mitosis in *H. reticulatum*, the behaviour of the nuclear envelope resembles that of *Chlamydomonas* and *Kirchneriella* as it remains essentially intact except for large polar fenestrae.

We find most intriguing the formation of the perinuclear envelope at prophase and its disappearance at telophase, particularly as a similar structure has recently been discovered in *Kirchneriella* (Pickett-Heaps 1970), a related member of the Chlorococcales. This envelope cannot however be discerned from Northcote's (1968) micrographs of *H. africanum*. In *H. reticulation*, the perinuclear envelope apparently isolates the nucleus undergoing mitosis, the centrioles, and a thin layer of cytoplasm around the nucleus from the rest of the cell.

Northcote (1968, p. 183) says centrioles appear at the nuclear envelope of *H. africanum* at the beginning of prophase and he suggests that their occurrence might be significant in plant cells where no rigid cell wall is formed after mitosis. The basis of this latter hypothesis is not given and it appears refuted, for example, by work on spermatogenous cells undergoing mitosis in *Chara* (Pickett-Heaps 1968) and dividing vegetative cells of *Stigeoclonium* (Pickett-Heaps, unpublished data) which have centrioles and also cell plates forming rigid cell walls. Northcote (1968)

Fig. 18.—Prometaphase; polar invagination of the intact nuclear envelope. ×39,000.

Fig. 19.—The nuclear envelope (arrow) is deeply invaginated and fenestrated. Microtubules invading the nucleus. Note the dispersed nucleolus and the ribosome-like particles in the nucleoplasm. ×36,000.

Fig. 20.—Spindle pole with microtubules both inside and outside the nucleus. The nuclear envelope is invaginated (arrow). ×36,000.

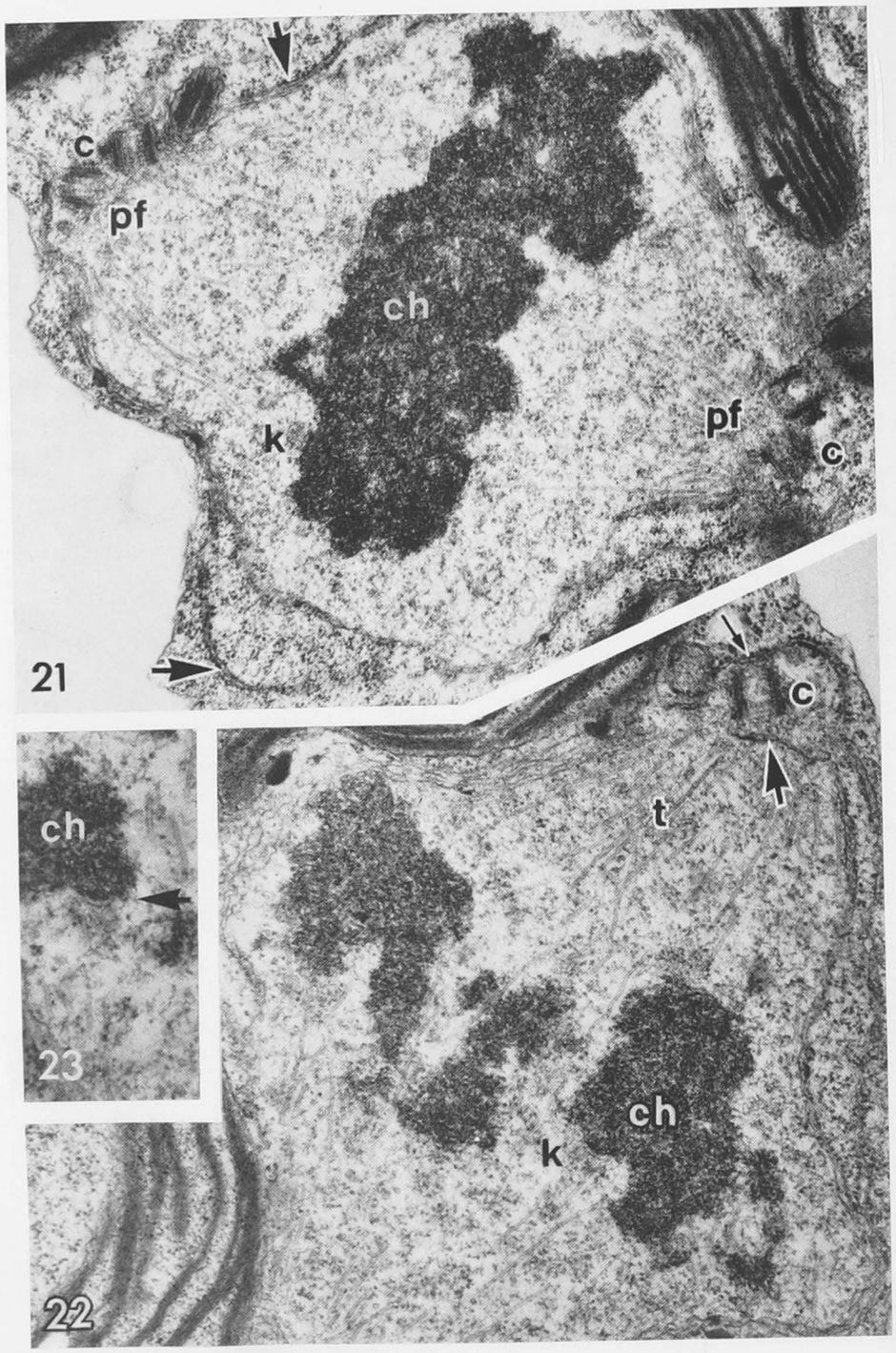

Fig. 21.—A nucleus at metaphase with centrioles lying in the polar fenestrae. The perinuclear envelope (arrows) is shown both closely pressed and separated from the nuclear envelope. ×26,000.

Fig. 22.—Centriole (*c*) between the perinuclear envelope (small arrow) and the nuclear membrane (large arrow). Note the distinct kinetochore (*k*). ×29,000.

Fig. 23.—A kinetochore at metaphase. ×39,000.

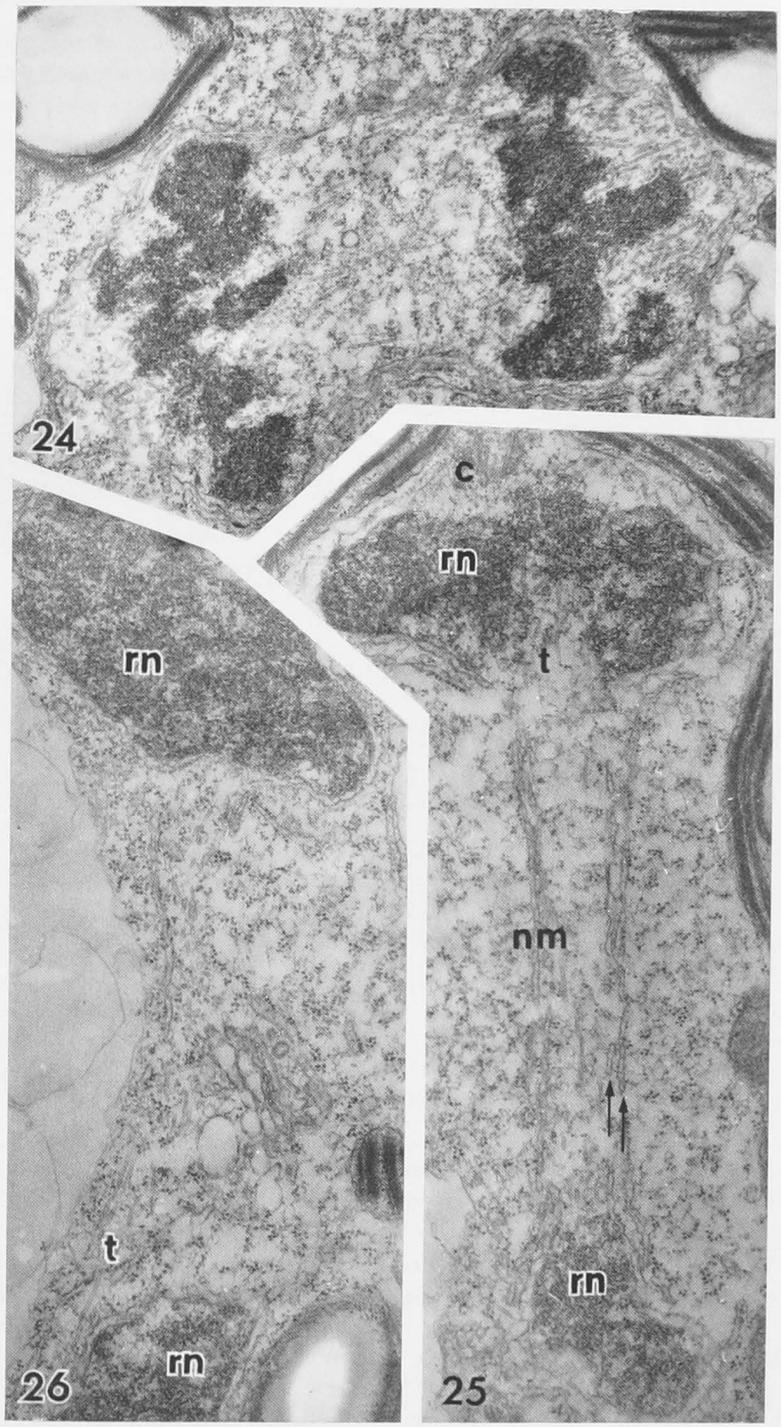

Fig. 24.—Anaphase; intact nuclear envelopes contracting between the separating chromosomes. ×20,000.

Fig. 25.—Telophase; both the nuclear and perinuclear envelopes (arrows) disintegrating. Note that the aggregated chromosomes and centriole (*c*) are still enclosed within the perinuclear envelope. Compare this figure with the light micrograph (Fig. 6) of telophase. ×25,000.

Fig. 26.—Nuclear membrane reforming around the daughter nuclei. Fragments of membrane and disorganized spindle microtubules remain. ×25,000.

similarly associates the presence of kinetochores with the absence of post-telophase wall formation; this also conflicts with the recent work on *Oedogonium* in which a 7-layered kinetochore and rigid transverse walls formed after cytokinesis are found (Pickett-Heaps and Fowke 1970*a*). In *H. reticulatum*, centrioles were apparently persistent in the coenobia which is not unexpected as the cytoplasm of the coenobia cleaves (Marchant and Pickett-Heaps, unpublished data) to form biflagellate zooids *not* at a specific stage of maturity but rather in response to changes in the external environment (Pocock 1960).

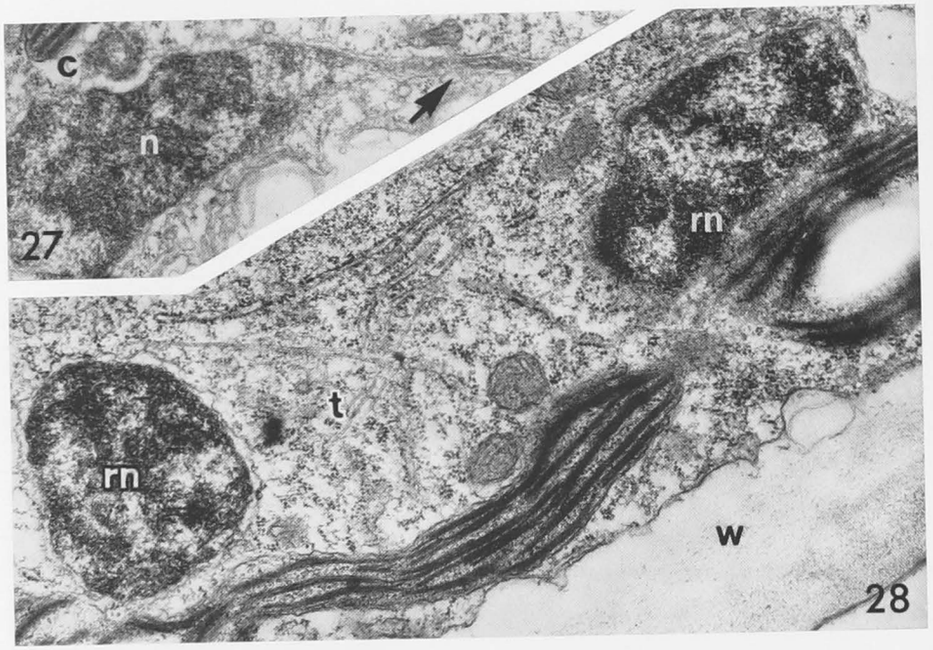

Fig. 27.—A telophase nucleus with centriole (*c*) at right angles to the axis of the spindle. Note the elongation of the nucleus (arrow). ×19,500.

Fig. 28.—Post-telophase daughter nuclei each enclosed in a single nuclear envelope. Other cytoplasmic components have invaded the space between the nuclei where some remnants of the spindle apparatus remain (cf. Fig. 26). ×21,000.

For reasons argued elsewhere (Pickett-Heaps 1969, 1970) we think it most unlikely that centrioles *per se* are involved in the actual formation of the spindle; in *H. reticulatum*, their function appears to be solely connected with some future production of flagella (see above). One rather striking piece of evidence for this non-involvement of the centriole in the spindle apparatus is shown in Figure 22 where nuclear membrane is found between the centriole and the spindle microtubules. This reminds one of various fungi which have extranuclear centrioles situated at the poles of closed spindles (Ichida and Fuller 1968; Lessie and Lovett 1968).

Figures 18, 19, and 20, showing cytoplasmic microtubules abutting the invaginated nuclear membrane, suggest that these invaginations may be caused

by elongation of the microtubules before fenestration of the nuclear membrane is complete. Similar observations on *Haemanthus* endosperm (Bajer and Molè-Bajer 1969), *Kirchneriella* (Pickett-Heaps 1970), and some animal cells (Threadgold 1967, p. 281) strongly suggest a movement of microtubules from the cytoplasm into the spindle during the early stages of mitosis.

A close, specifically orientated association between golgi bodies and interphase nuclei is known in a number of algae including species of *Ulva* (West and Pitman 1967), *Chorda* and *Giffordia* (Bouck 1965), *Kirchneriella* (Pickett-Heaps 1970), and other species. Such an association is evident in *H. reticulatum*, and, as in *Kirchneriella*, it is temporarily lost during mitosis. The activities of the golgi bodies will be discussed in detail when the differentiation of the coenobia to form zooids is described in a later paper.

V. Acknowledgments

We are most grateful to Dr. M. A. Pocock for confirming our identification of the *Hydrodictyon* species used and for other helpful advice. One of us (H.J.M.) gratefully acknowledges the receipt of a Commonwealth Post-Graduate Award.

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CHAPTER 4

FORMATION OF ZOOIDS WITHIN THE COENOBIUM

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ULTRASTRUCTURE AND DIFFERENTIATION OF *HYDRODICTYON RETICULATUM*

II.* FORMATION OF ZOOIDS WITHIN THE COENOBIUM

By H. J. Marchant†‡ and J. D. Pickett-Heaps†‡

[Manuscript received October 15, 1970]

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).

* Part I, *Aust. J. biol. Sci.*, 1970, **23**, 1173–86.

† Research School of Biological Sciences, Australian National University, P.O. Box 475, Canberra City, A.C.T. 2601.

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

§ 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.

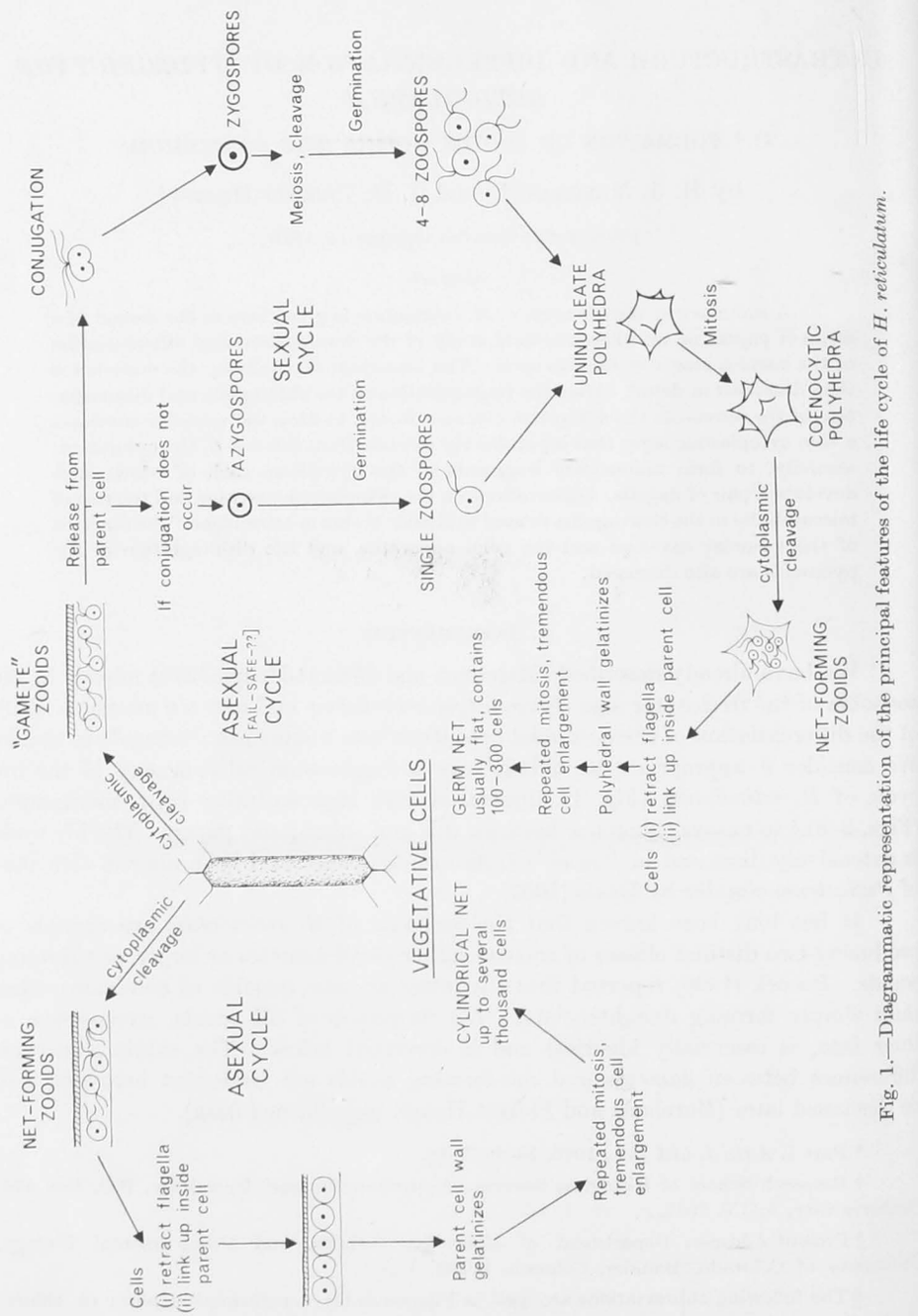

Fig. 1.—Diagrammatic representation of the principal features of the life cycle of *H. reticulatum*.

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.

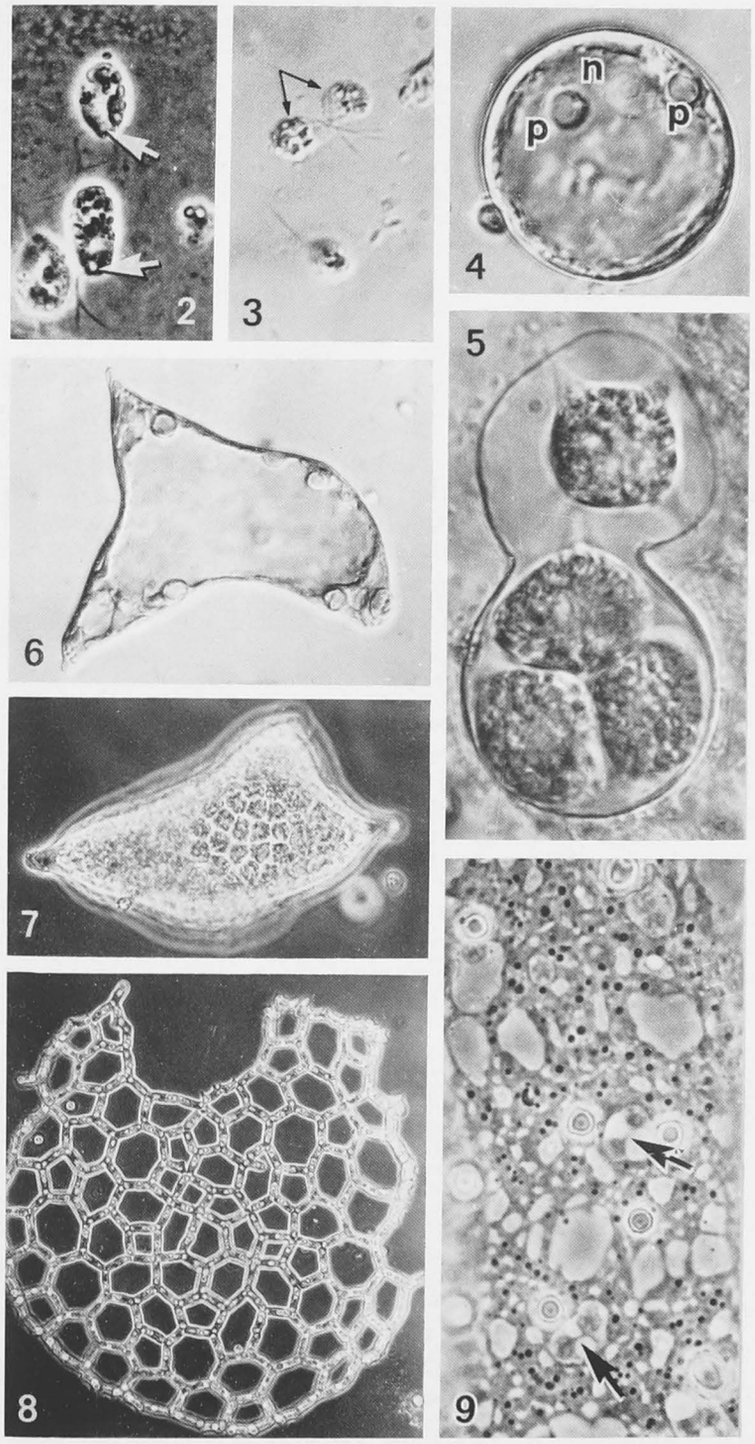

Fig. 2.—Biflagellate net-forming zooids; note contractile vacuoles (arrows). Phase-contrast. ×1170.
Fig. 3.—Conjugating "gamete" zooids (arrows); compare their size with zooids in Figure 2. Nomarski optics. ×1170.
Fig. 4.—Zygospore (azygospore?). Nomarski optics. ×1170.

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

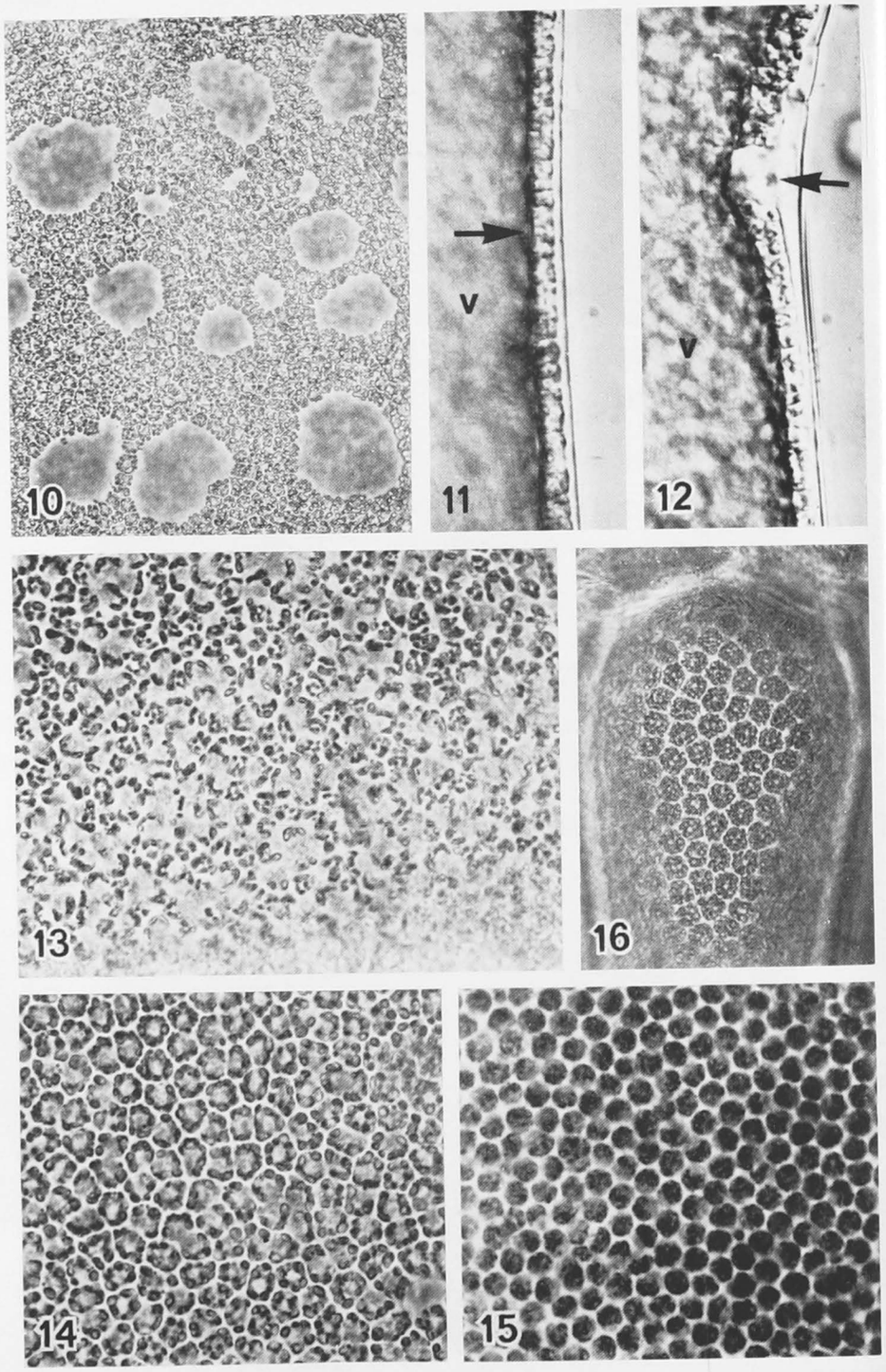

Fig. 10.—Part of a differentiating coenobium showing clear areas of the cytoplasm following partial starvation. Phase-contrast. ×500.

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

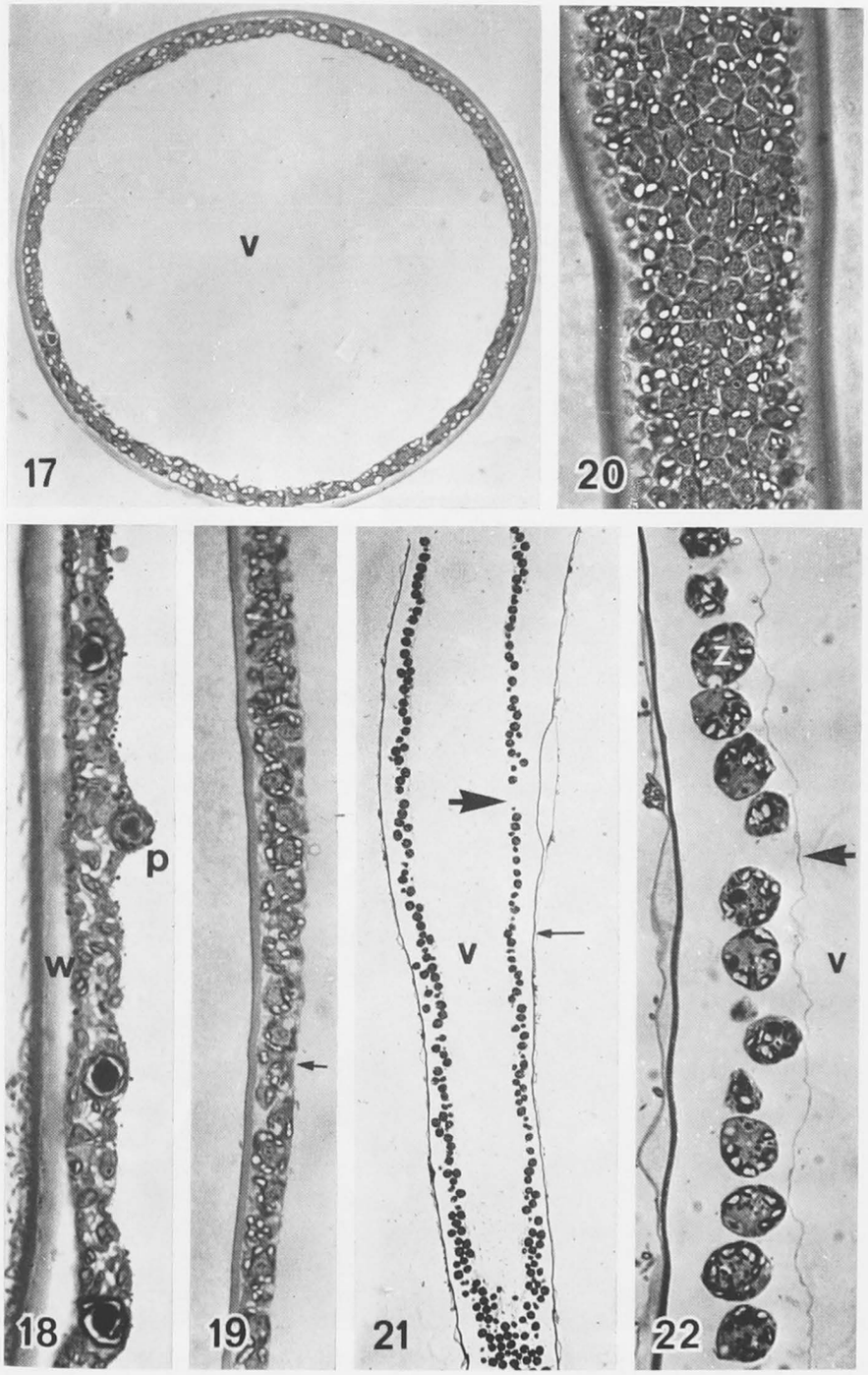

Fig. 17.—Transverse section of a coenobium of *H. reticulatum* showing the thin peripheral cytoplasm surrounding the immense central vacuole. ×750.

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

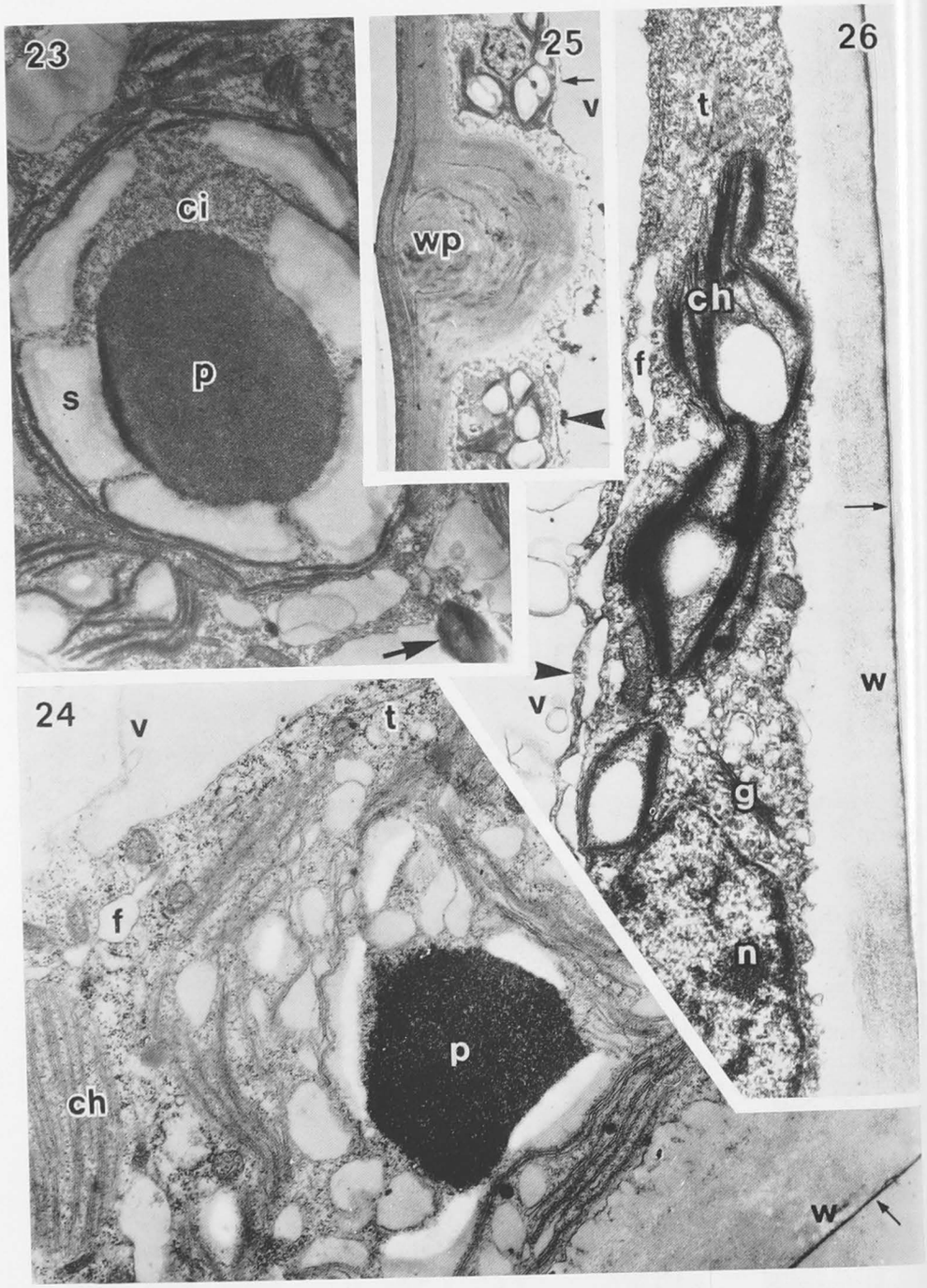

Fig. 23.—Pyrenoid with a cytoplasmic intrusion and surrounding starch plates at an early stage of differentiation. Note the densely staining body (arrow) on the tonoplast. ×10,000.

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.

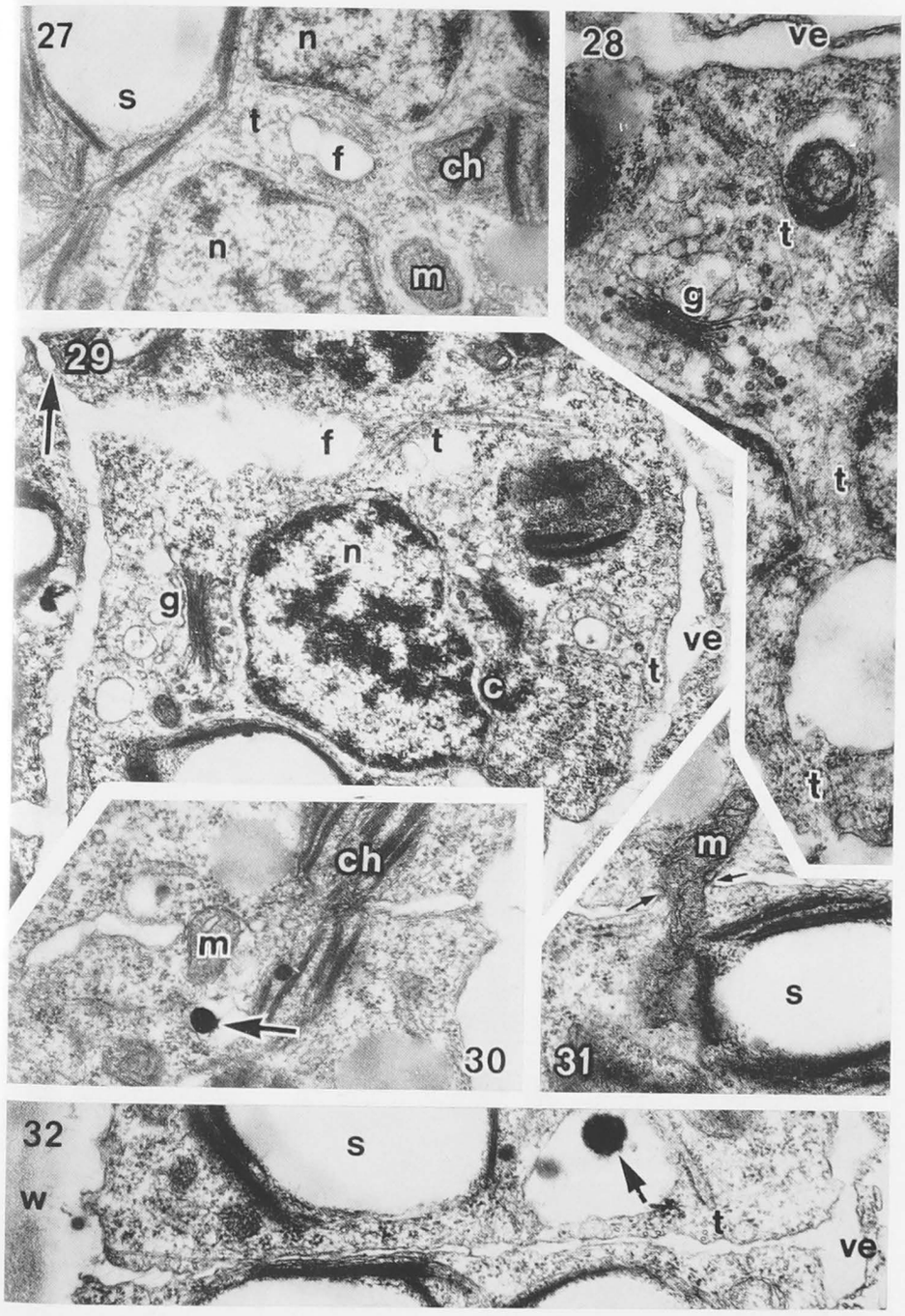

Fig. 27.—Early radial cleavage of the cytoplasm; note the developing fissure (*f*) surrounded by microtubules. ×29,000.

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.

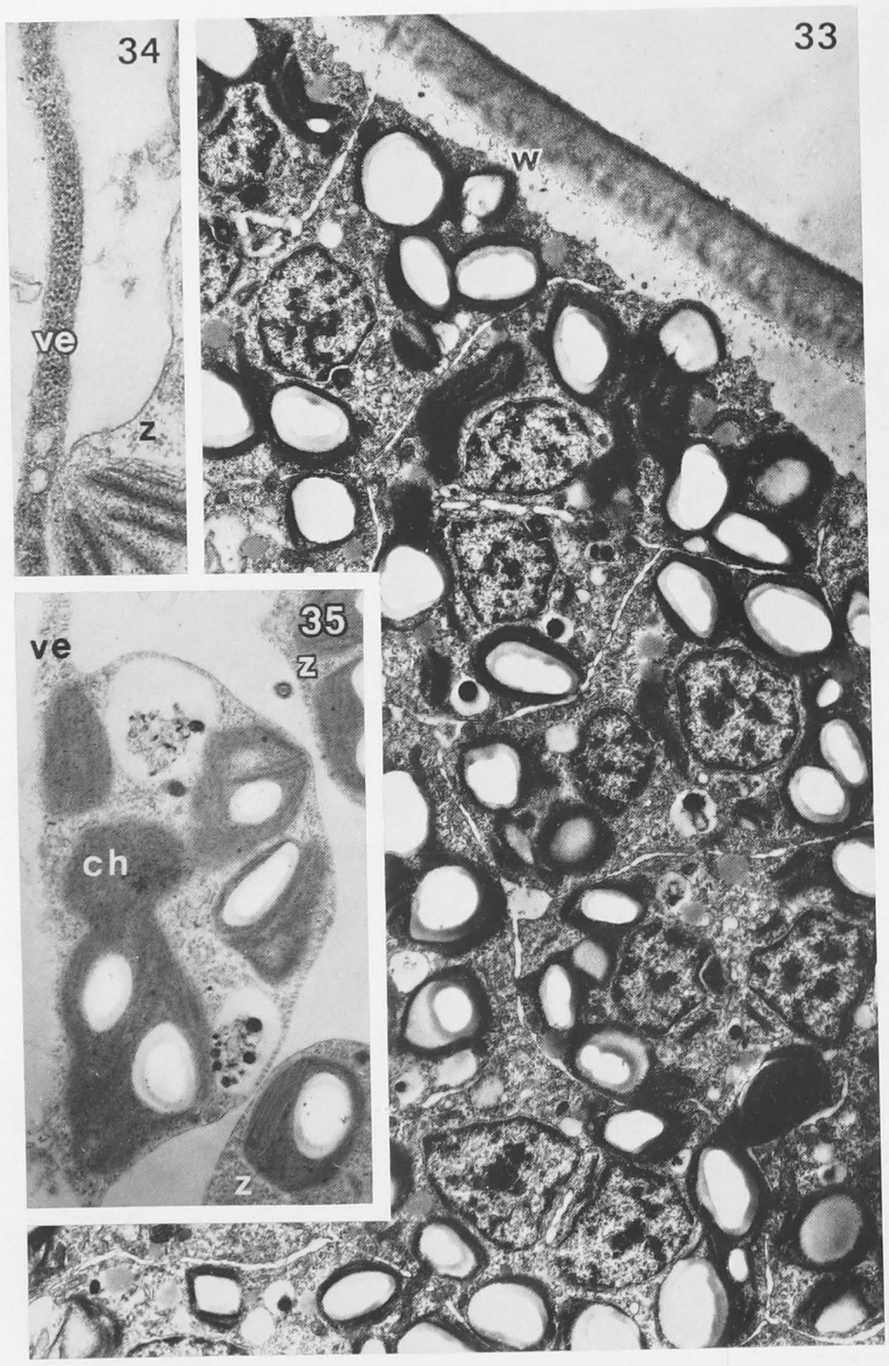

Fig. 33.—Low magnification micrograph of a tangentially sectioned coenobium approaching the "pavement" stage (cf. Figs. 14, 15, 20). ×8100.

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*.

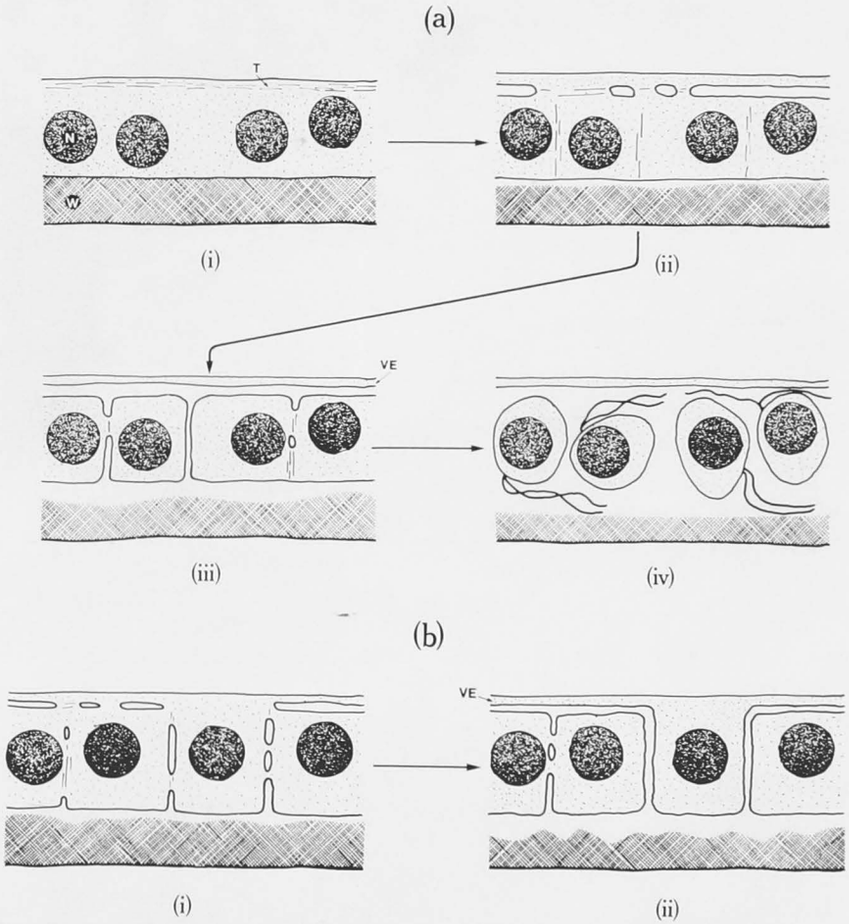

Fig. 36.—(*a*) Diagrammatic representation of the principal events in the formation of zooids from the coenobial cytoplasm: (i) microtubules (*T*) appear near the tonoplast; (ii) formation of the vacuolar envelope; (iii) radial cleavage of the cytoplasm within an intact vacuolar envelope (*VE*); (iv) 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.

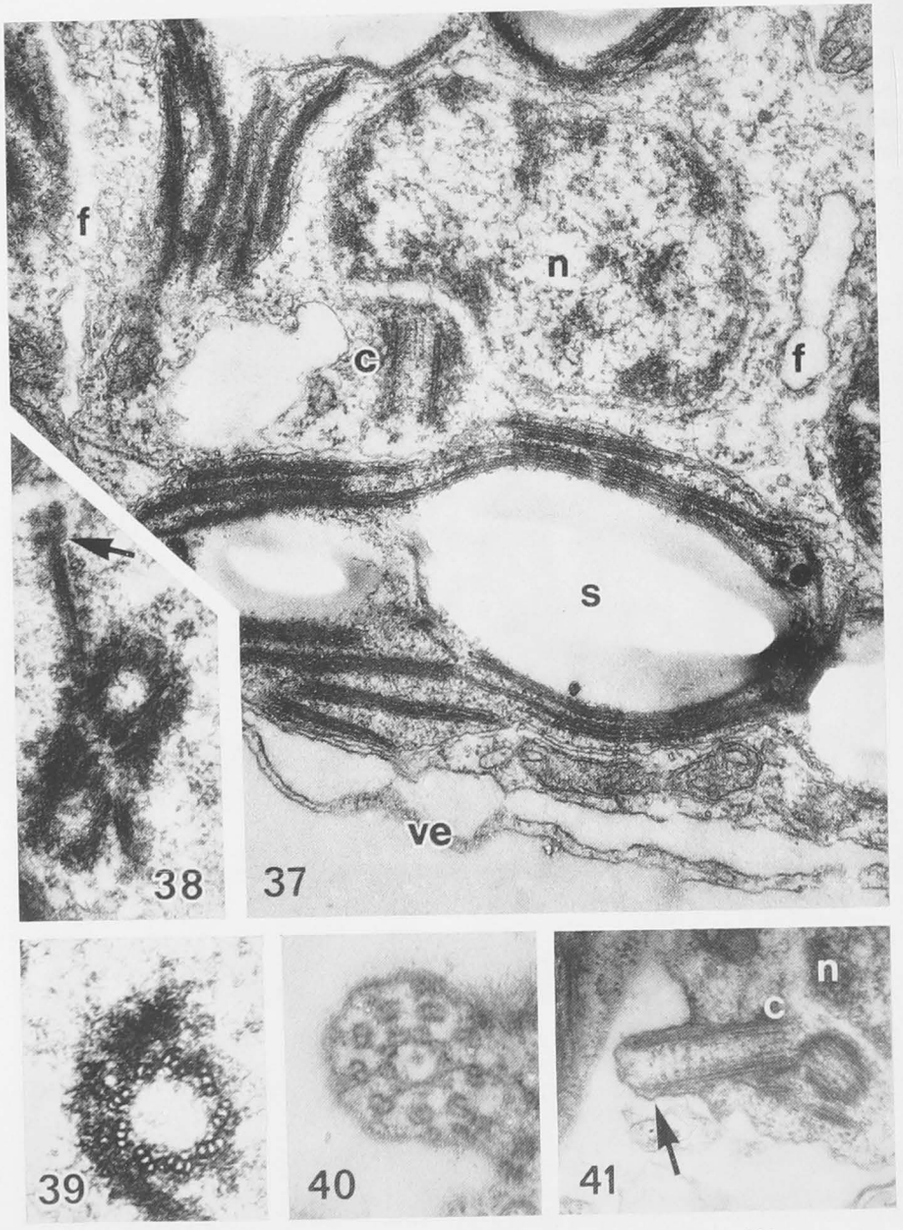

Fig. 37.—Elongating centriole at an advanced stage of radial cleavage. ×39,000.

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.

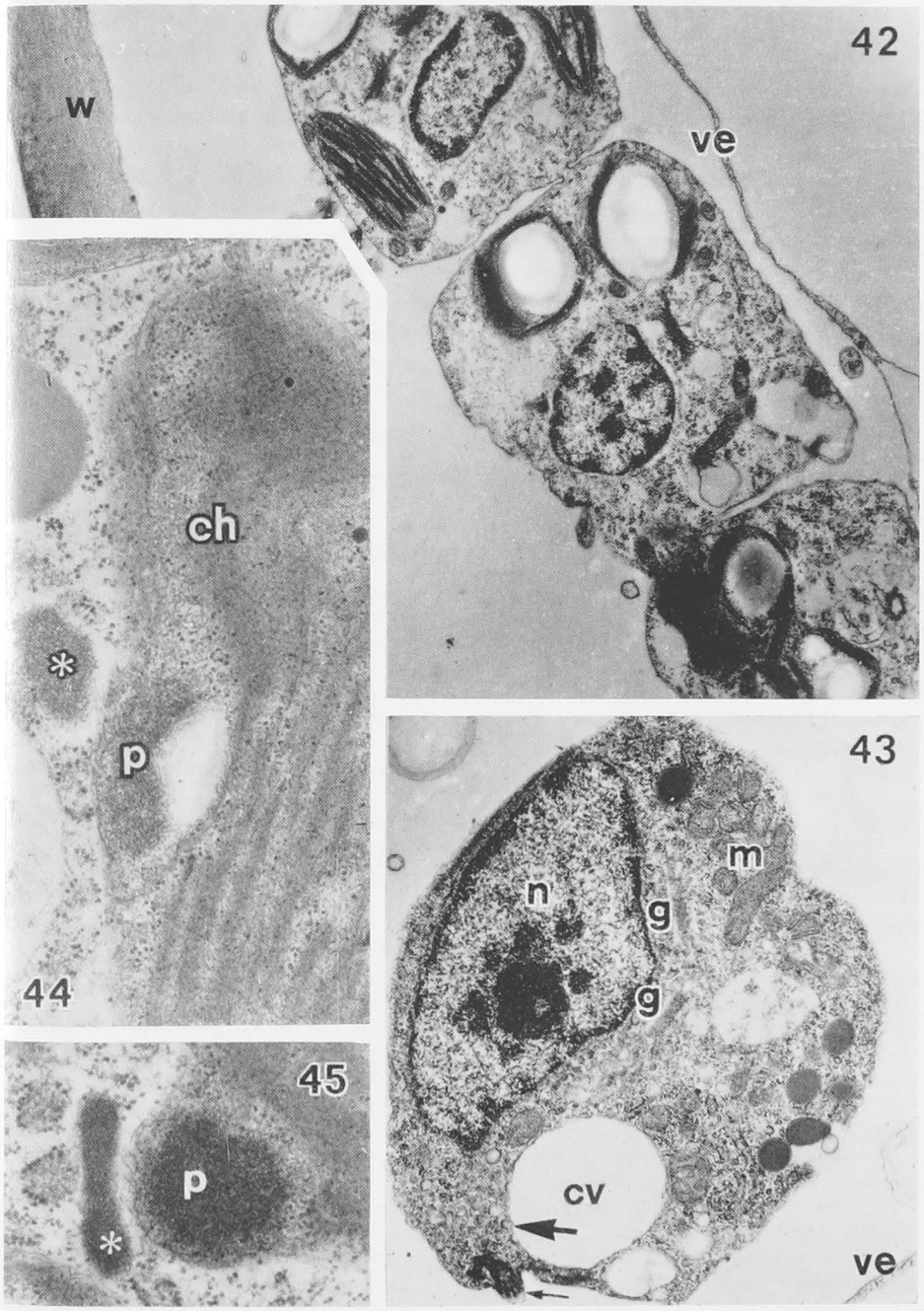

Fig. 42.—Zooids before separation. Note both the cell wall and vacuolar envelope. ×11,000.

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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CHAPTER 5

FORMATION OF THE VEGETATIVE DAUGHTER NET

ULTRASTRUCTURE AND DIFFERENTIATION OF *HYDRODICTYON RETICULATUM*.

III. FORMATION OF THE VEGETATIVE DAUGHTER NET

by

H.J. Marchant[1,2] and J.D. Pickett-Heaps[2]

1. Research School of Biological Sciences
Australian National University
Box 475, Canberra City ACT 2601

2. Present address:
Department of Molecular, Cellular and Developmental Biology
University of Colorado
Boulder, Colorado 80302 USA

Received:

Running title: Daughter net formation in *H. reticulatum*

SUMMARY

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 linking with usually 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.

INTRODUCTION

During both asexual and sexual phases of the life cycle of H. 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.

MATERIALS AND METHODS

In this investigation we used a culture of H. reticulatum, a gift from the Curator of the Culture Collection 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.

OBSERVATIONS

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; Hoffman, 1970; Pitelka, 1969). 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 pulsated 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.

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, 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 paralyzed 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).

Fig. 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).

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.

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 net of H. reticulatum: Marchant and Pickett-Heaps, see Chapter 8; 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 possibly 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 (as described in Millington and Gawlik's 1970 paper) in terms 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) in 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, in preparation and Chapter 11). Colchicine depolymerizes microtubules, whose role in maintaining or altering the shape of cells has been widely documented (see reviews of Newcomb, 1969; Porter, 1966; Pickett-Heaps, 1972). Our colchicine experiments strongly suggest that bands of peripheral

microtubules, so characteristic of colony-forming zooids, are indeed important in the ordered linking of the zooids.

An amorphous material appears in the intercellular space between flattened adjacent zooids but apparently not elsewhere over their surface. Similar material is also detected between adjacent cells of Pediastrum before secretion of the wall (Hawkins and Leedale, 1971; Gawlik and Millington, 1969). 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 is presently being investigated.

Hawkins and Leedale (1971) correlate the appearance of the outer

membrane-like layer of the wall with the irrevocable gluing 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 reinhardi are shed at mitosis (Johnson and Porter, 1968), as are those of zoospores of Oedogonium as these cells settle and become germlings (Pickett-Heaps, 1971). 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). 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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ABBREVIATIONS USED IN FIGURES

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

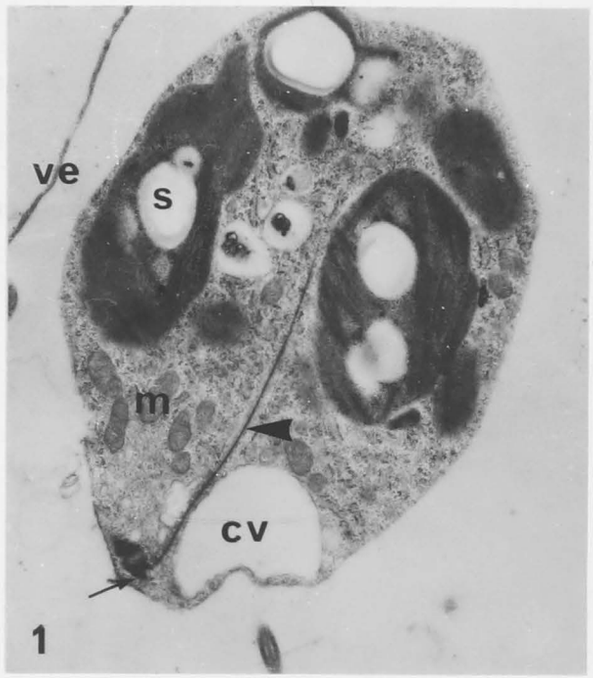
Fig. 1 Electron micrograph of a net forming zooid, the nucleus is out of the plane of the section. Note the vacuolar envelope (ve) and the rootlet microtubules (arrowhead) emanating from the basal body region (arrow). X 11,000.

Fig. 2 Living net-forming zooid showing contractile vacuoles (arrowhead). Phase contrast, X 1,300.

Fig. 3 The same zooid as shown in Fig. 2 photographed as one of the contractile vacuoles is discharging (arrowhead). Phase contrast, X 1,300.

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). X 32,000.

Fig. 5 Interconnection between the cores of basal bodies (arrowhead). Note also the four bands of rootlet microtubules (arrows). X 53,000.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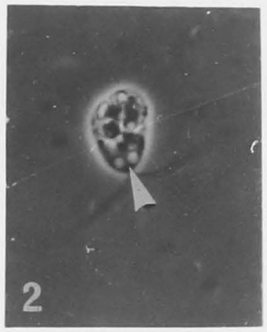
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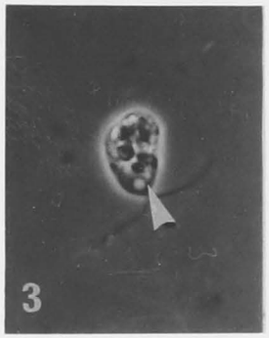
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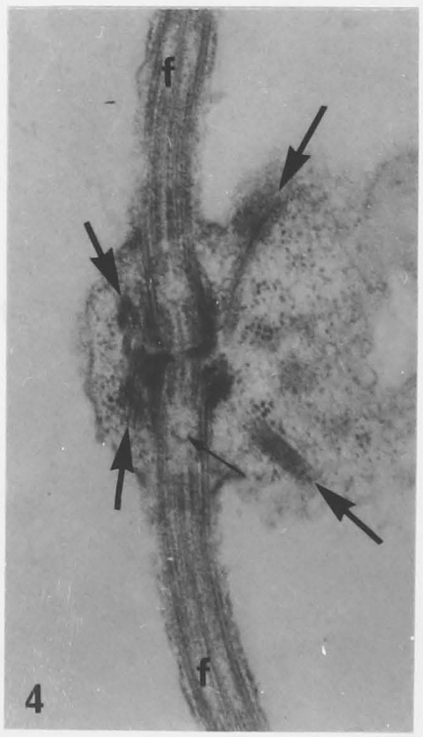
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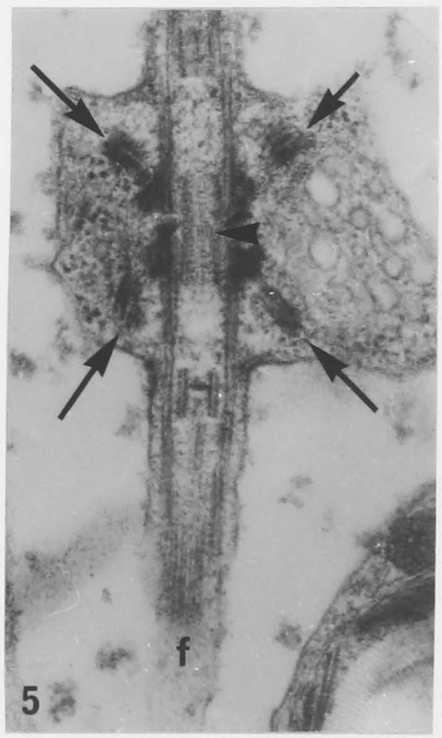
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Fig. 6 Bridge interconnecting basal bodies (arrow). X 35,000.

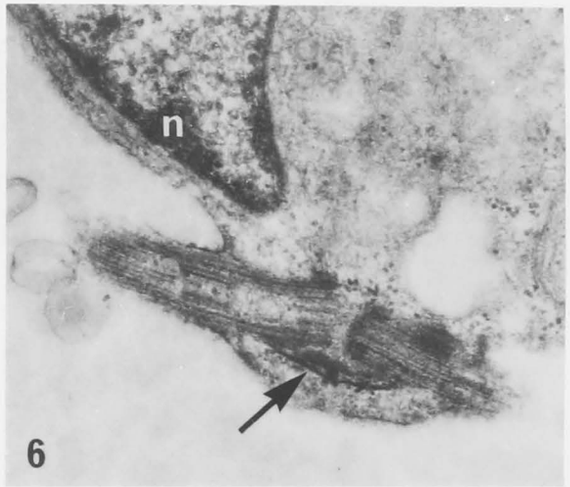
Fig. 7 Two bands of rootlet microtubules, one cut transversely (arrowhead) the other longitudinally, arising from near bridge between basal bodies. X 42,000.

Fig. 8 Rootlet microtubules sectioned transversely. X 53,000.

Fig. 9 Different configuration of rootlet microtubules to that shown in Fig. 8. X 52,000.

Fig. 10 Microtubules surrounded by diffuse material appressed to and apparently indenting (arrowhead), the chloroplast membrane. X 53,000.

Fig. 11 Longitudinally sectioned microtubules adjacent to the chloroplast. X 35,000.



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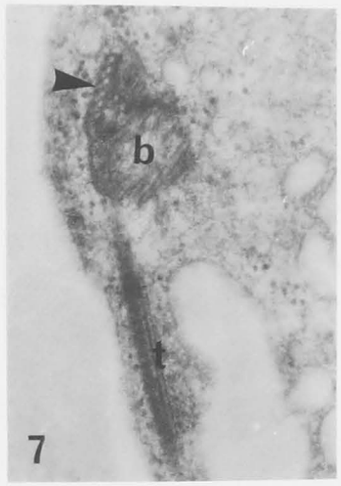

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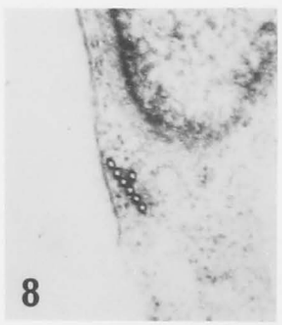

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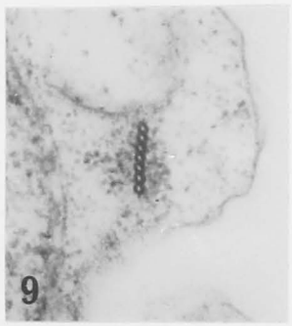

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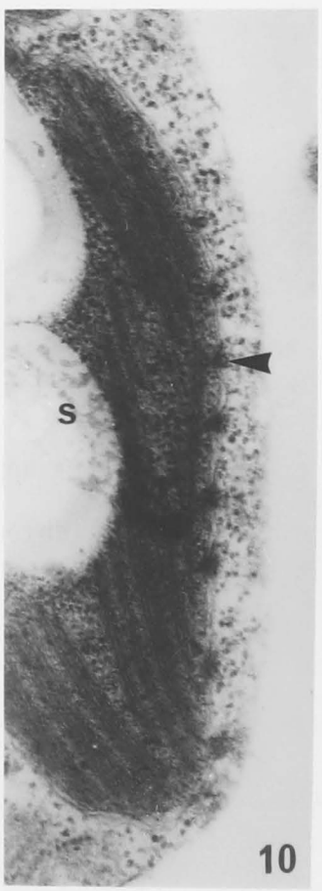

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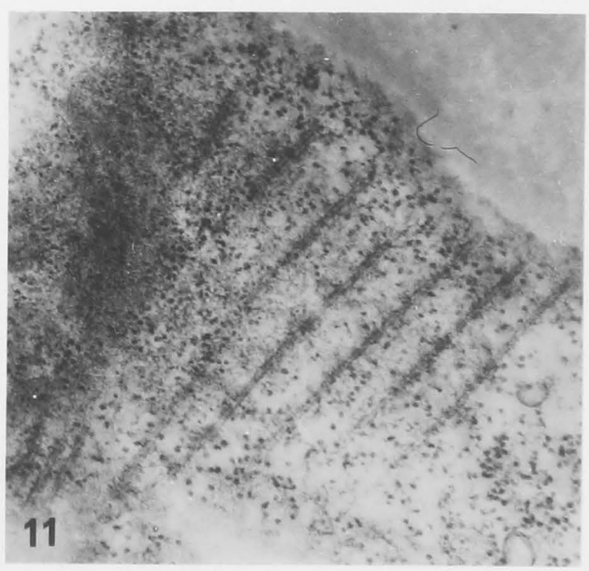

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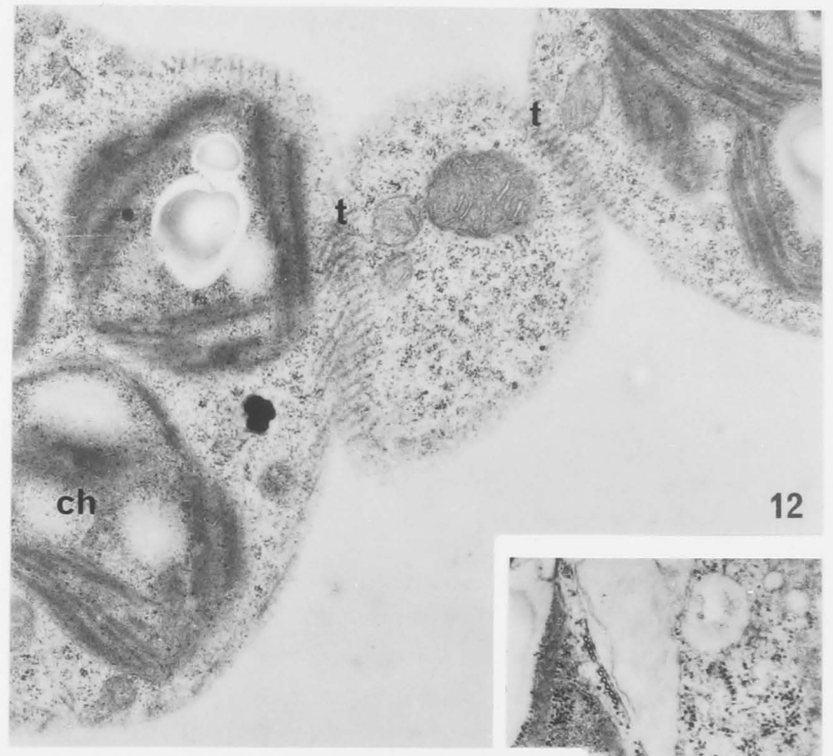
Fig. 12 Three zooids in contact. Note the arrays of peripheral microtubules underlying their plasmalemmae where they are in contact but the relatively few microtubules elsewhere. X 31,000.

Fig. 13 Periphery of two net-forming zooids showing a band of peripheral microtubules in one but lacking in the other (cf. Fig. 16). X. 27,000.

Fig. 14 Zooids connected in vivo by fine cytoplasmic strands (arrowheads). Phase contrast, X 1,300.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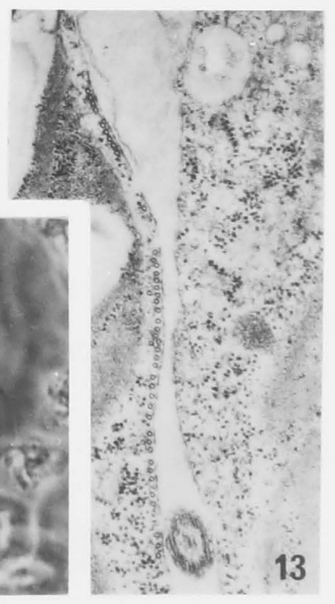
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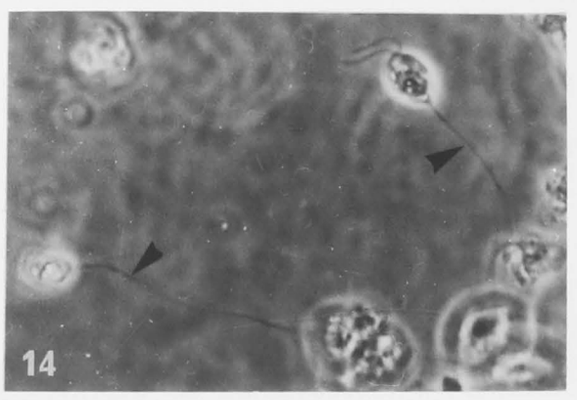
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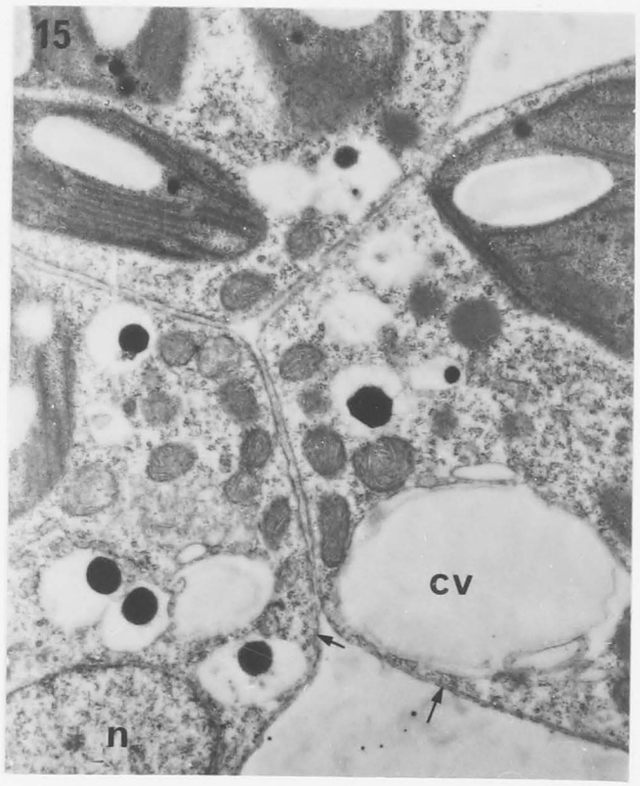
Fig. 15 Adhering zooids sectioned nearly perpendicular to those in Fig. 12. Note the longitudinally sectioned microtubules (arrows) and the amorphous "adhesive" only in the flattened intercellular spaces. X 13,000.

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

Fig. 17 Section grazing a band of peripheral microtubules.

Fig. 18 Light micrograph of an early stage in net formation. The parental coenobia were starved during growth. Note the rhomboidal outline of the packed zooids. Phase contrast, X 1,300.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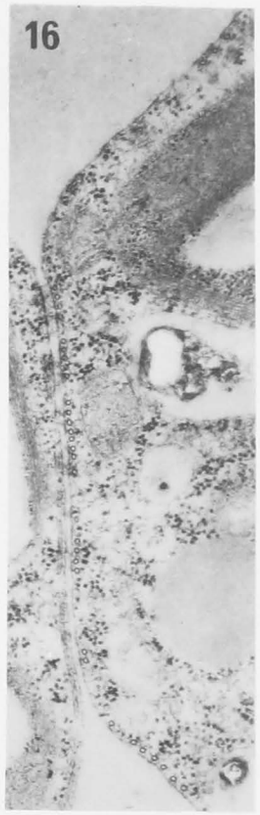
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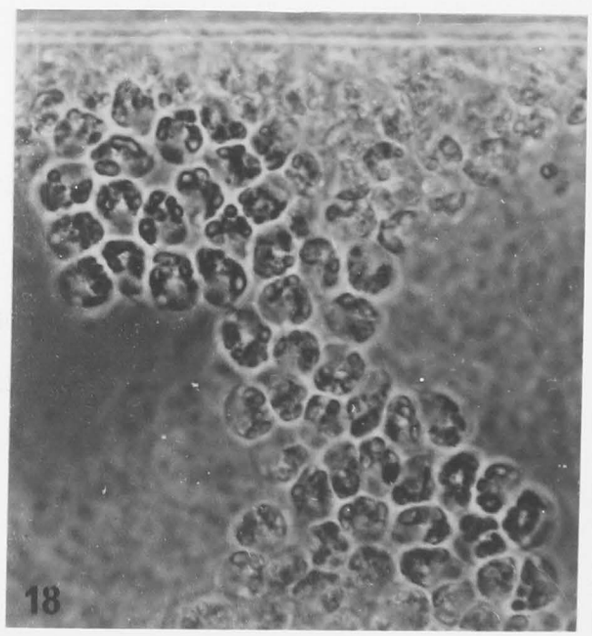
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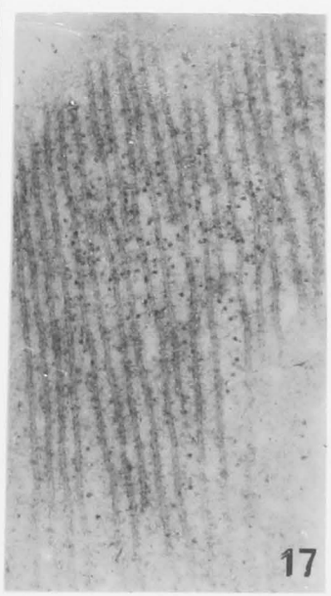
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Figs. 19-21 Stages in the retraction of flagella, about 10 seconds between each exposure. All phase contrast, X 2,400.

Fig. 19 Flagellum "paralyzed" and shortening.

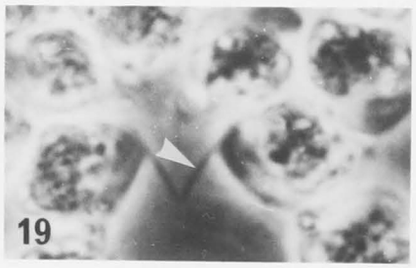
Fig. 20 Shortening and bending towards zooid. Note that the flagellum on the zooid to the left has disappeared.

Fig. 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 Fig. 23. Note the microtubules at the site of contact between zooids (arrow). X 13,000.

Fig. 23 Detail of enclosed area in Fig. 22. Flagellar microtubules in cytoplasm, basal body (arrow) apparently disintegrating. X 27,000.

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, X 1,300.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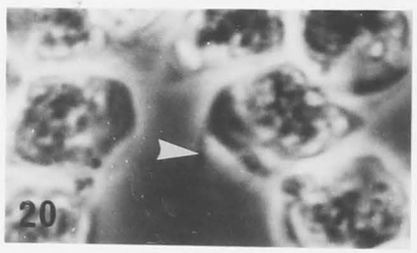
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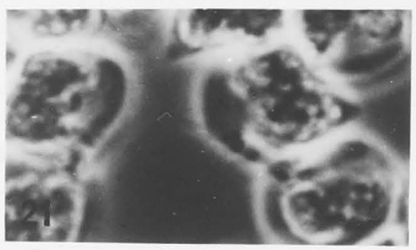
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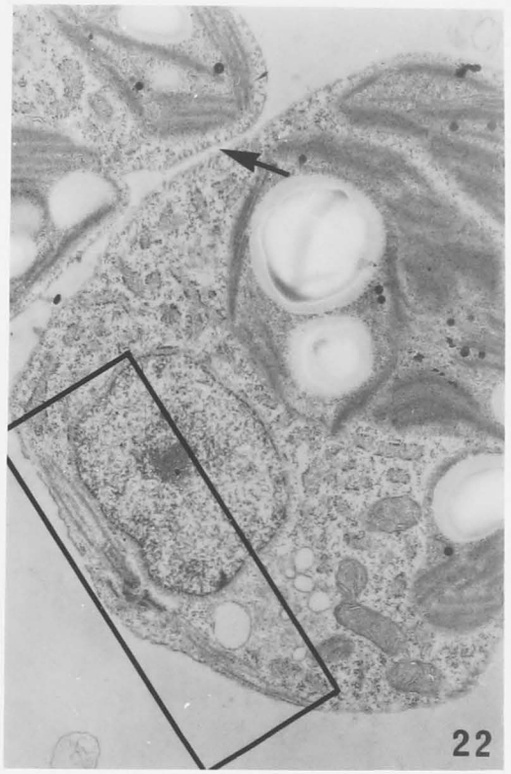
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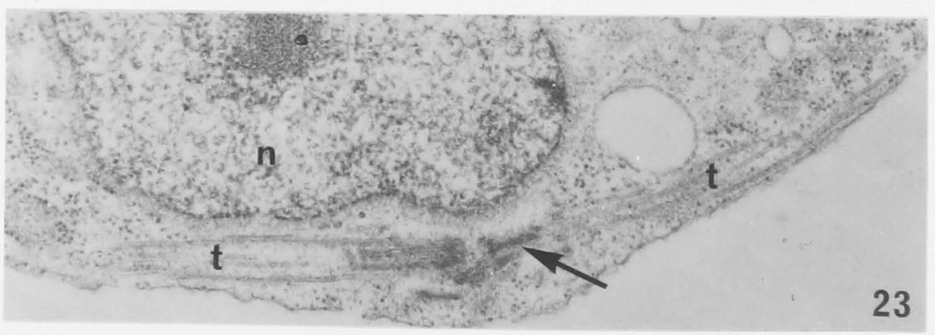
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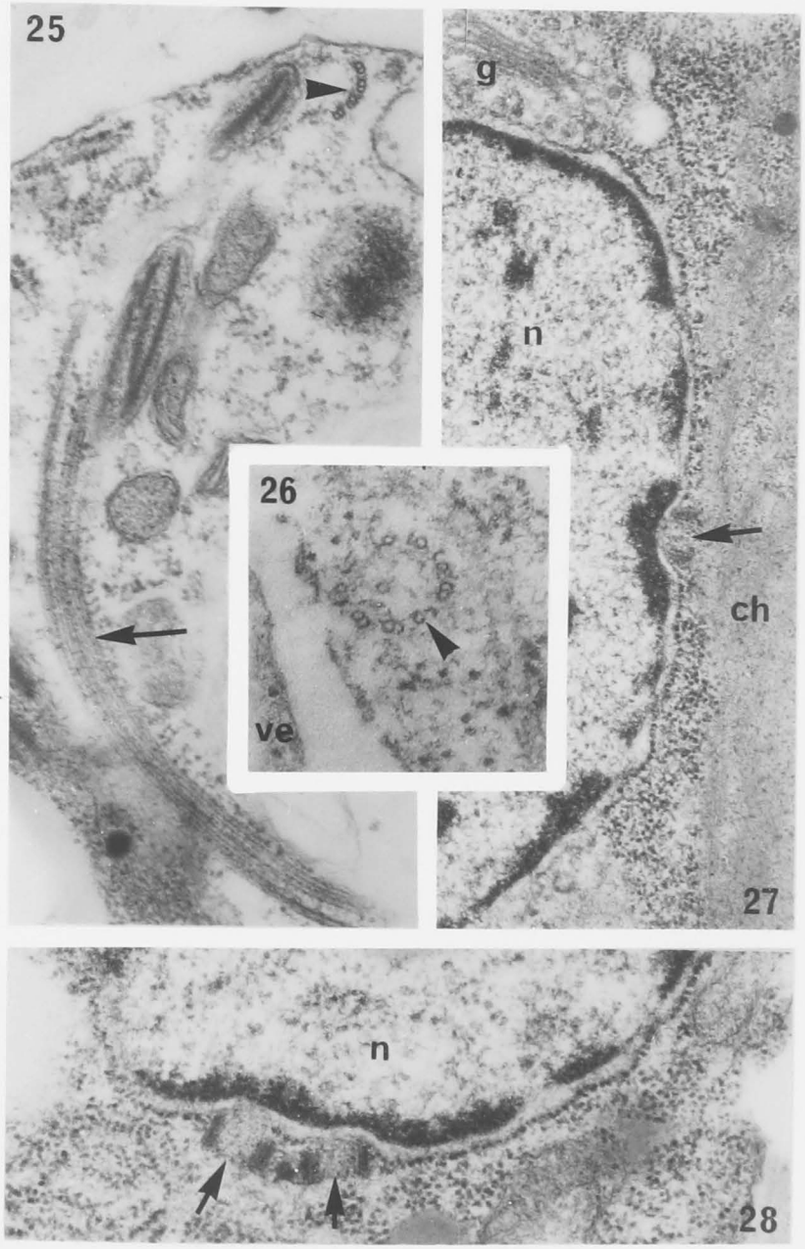
Fig. 25 Flagellar microtubules lying within the cytoplasm of a net-forming zooid (arrow). Note the disintegrating microtubules (arrowhead). X 45,000.

Fig. 26 Higher magnification of disintegrating flagellar microtubules (arrowhead). X 70,000.

Fig. 27 Centriole forming de novo on the nuclear envelope (arrow). X 37,000.

Fig. 28 Two centrioles forming de novo on the nuclear envelope (arrows). X 53,000.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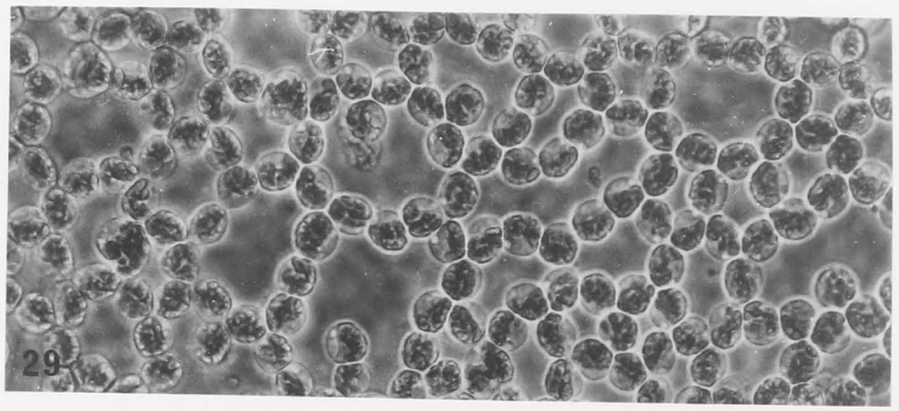
Figs. 29-31. Light micrographs of stages in the development of the daughter net. Irregularities in the pattern of the net are largely caused by compression by the cover slip. All phase contrast, X 600.

Fig. 29 Zooids at the time of flagellar retraction.

Fig. 30 Elongation of the adhering zooids.

Fig. 31 Vacuoles appearing at each end of the linked cells (arrowheads).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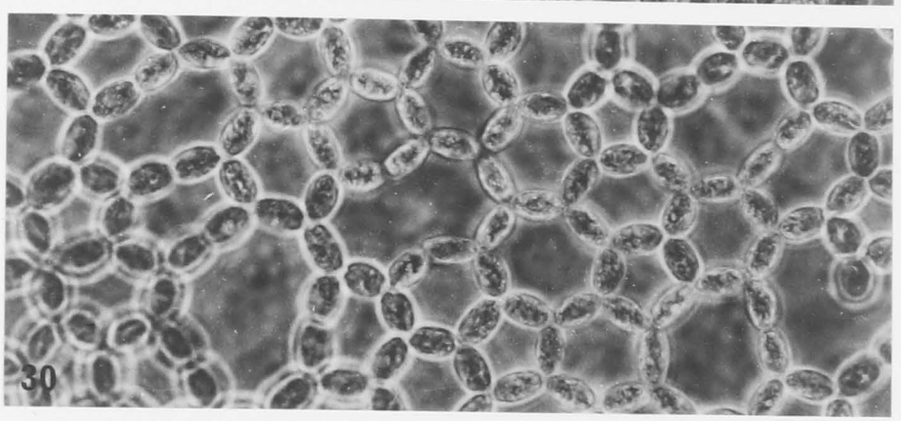
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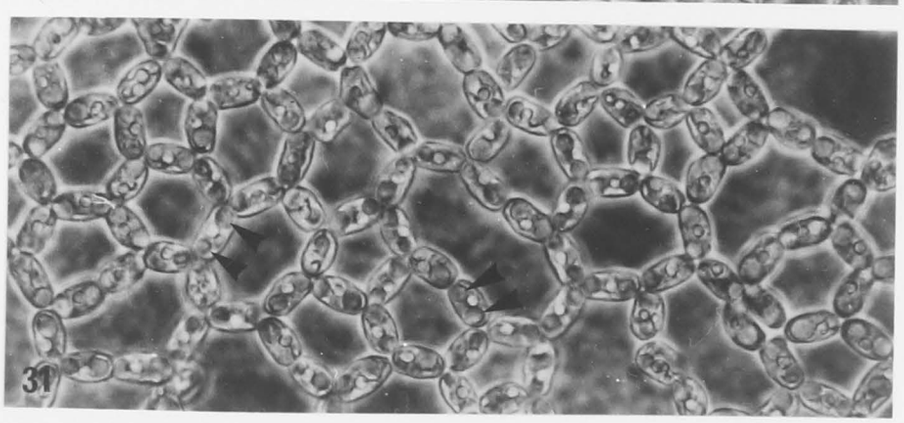
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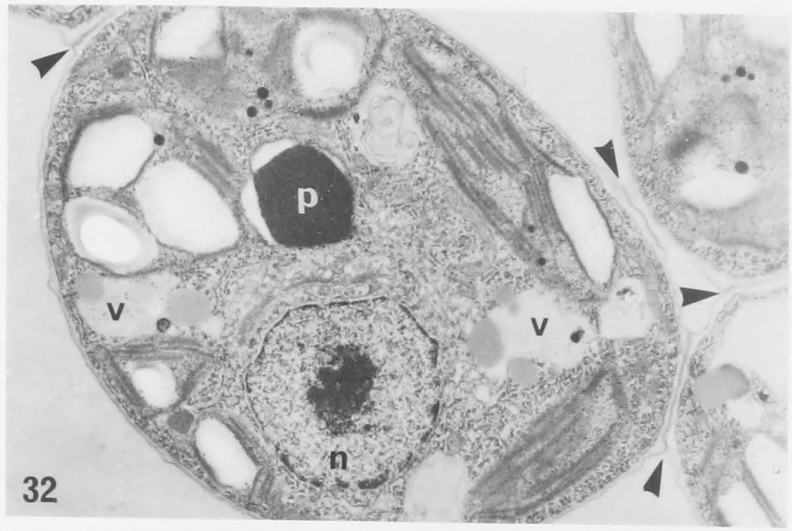
Fig. 32 Young cell of a daughter net at the time of deposition of the inner layer of wall. Note the junction with three other cells (arrowheads), the developing vacuoles and pyrenoid. X 9,000.

Fig. 33 The wall between two recently adhered cells. Note the thin "membrane-like" outer layer and the more massive inner layer. X 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. Fig. 33). X 29,000.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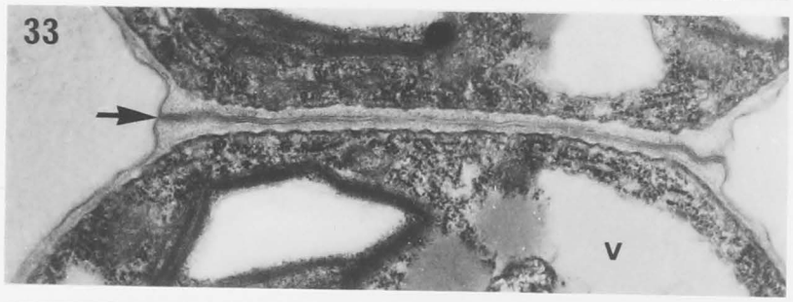
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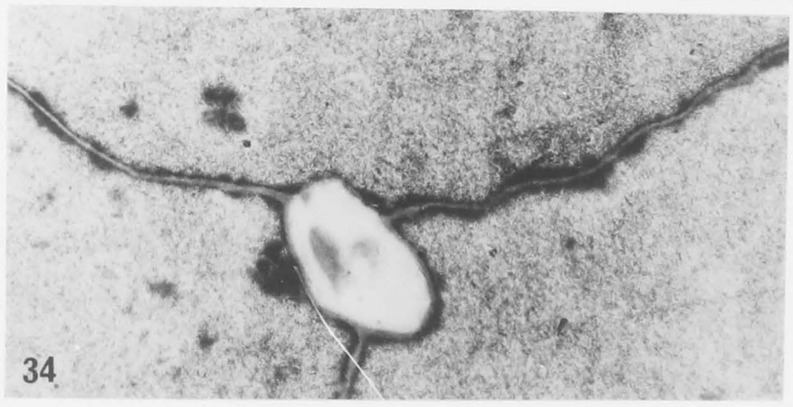
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CHAPTER 6

CONJUGATION OF GAMETES AND THE DEVELOPMENT OF ZYGOSPORES AND AZYGOSPORES

ULTRASTRUCTURE AND DIFFERENTIATION OF *HYDRODICTYON RETICULATUM*.

IV. CONJUGATION OF GAMETES AND THE DEVELOPMENT OF ZYGOSPORES AND AZYGOSPORES

by

H.J. Marchant[1,2] and J.D. Pickett-Heaps[2]

1. Research School of Biological Sciences
Australian National University
Box 475, Canberra City, ACT 2601

2. Present address:
Department of Molecular, Cellular and Developmental Biology
University of Colorado
Boulder, Colorado 80302 USA

Received:

Running Title: Fertilization in *H. reticulatum*

SUMMARY

In H. reticulatum, gametes differed from other zooids principally in that some of them bore an electron dense apical cap between their flagella. Conjugation did not take place until the walls of the coenobia, in which the gametes developed, ruptured; it often did not occur even among the liberated gametes. Only in zooids collected from cultures in which conjugation was evident, were these apical caps extended as fertilization tubules. Fertilization took place by the fusion of the tip of this tubule on one gamete with the membrane between the flagella of another which apparently lacked a fertilization tubule. Subsequent lateral fusion of the united zooids produced quadriflagellate zygotes which were active for only a short time before flagellar retraction and cell wall deposition. Karyogamy usually preceded wall secretion. Gametes failing to conjugate often laid down a wall to form azygotes.

INTRODUCTION

In an earlier paper (Marchant and Pickett-Heaps, 1971) we discussed the differentiation of the coenobial cytoplasm of Hydrodictyon reticulatum to form uninucleate, biflagellate zooids. We have already described the structure of the net-forming zooids and how they link together to form nets (Marchant and Pickett-Heaps, 1972). In this paper we first describe the gamete zooids and indicate structural differences between them and net-forming zooids, and then discuss conjugation and the development of zygospores, and also azygospores from those gametes failing to conjugate. A diagrammatic representation of the life cycle of H. reticulatum has been given earlier (Marchant and Pickett-Heaps, 1971).

Among the few algae in which fertilization has been studied ultrastructurally are Prasiola stipitata (Manton and Friedmann, 1960), Chlamydomonas moewusii (Brown, Johnson and Bold, 1968) and Chlamydomonas reinhardi (Friedmann, Colwin and Colwin, 1968). Conjugation in H. reticulatum, while superficially resembling that in C. reinhardi, also has many distinctive features which will be discussed and compared with fertilization in the algae listed above and other organisms.

MATERIALS AND METHODS

Both the Australian and Cambridge strains of H. reticulatum were cultured and prepared for electron microscopy as already described (Marchant and Pickett-Heaps, 1970, 1971).

OBSERVATIONS

Structure of the gametic zooids.

The obvious visible differences between the two types of zooids can be conveniently set out as follows:

1. Gametes measure around 4μm x 6μm while net-forming zooids are about 6μm x 8μm, although in our experience these values can vary widely.
2. Gametes contain more lipid and starch (Figs. 9, 20, 21; cf. Figs. 1, 22, Marchant and Pickett-Heaps, 1972) than net-forming zooids.
3. The angle between their flagellar bases is usually closer to 90° (Figs. 13, 19) than the 180° of the net-forming zooids (cf. Figs. 3, 5, 6, Marchant and Pickett-Heaps, 1972).
4. A most important structural feature unique to gametes and never found in net-forming zooids, is the electron-dense "apical cap" (Fig. 7); this was first visible during radial cleavage of the parental coenobial cytoplasm (Figs. 11, 12). We have not determined the proportion of gametes bearing these apical caps, whose structure will be described later.

Sections revealed eye-spots in some gametes (Figs. 7, 9, 10). As in net-forming zooids, four bands of rootlet microtubules arose from near the bridge between the basal bodies (Figs. 7, 15). Gametes have fewer peripheral microtubules than net-forming zooids except when forming azygospores and lack completely the localized arrays of peripheral microtubules, so characteristic of net-forming zooids (Marchant

and Pickett-Heaps, 1972). In other respects gametes and net-forming zooids appear similar.

Behavior of the gametes.

Under our cultural conditions, both strains of H. reticulatum produced gametes only infrequently and often these failed to conjugate. On numerous occasions we have watched gametes released from the parental cell swarming in clumps for hours without conjugating. Such gametes appeared ultrastructurally identical with those selected from cultures undergoing conjugation, except for certain changes in the apical cap (see later). These gametes which failed to conjugate either laid down a wall to become azygotes, or disintegrated. When copulation did occur, it always followed rupture of the parental cell wall, whereupon gametes retained inside, as well as those released, conjugated. We have never observed conjugation among gametes within an intact parental wall.

Conjugation.

The vigorous movements of gametes hampered observations on conjugation in vivo; however, initial contact of the zooids was seen to be made adjacent to their flagella (Fig. 1). Lateral fusion (Figs. 2, 3) followed giving rise to quadriflagellate zygotes (Figs. 4, 5) which remained active for only a few minutes before the flagella were withdrawn as in net-forming zooids (Marchant and Pickett-Heaps, 1972) and the wall deposited (Fig. 6).

Using electron microscopy, the apical caps of gametes from cultures undergoing conjugation were seen to be elongate (Figs. 15, 16), a

phenomenon never encountered in gametes from non-conjugating cultures (Fig. 14). We will hereafter refer to an extended apical cap as a "fertilization tubule" (cf. Friedmann, Colwin and Colwin, 1968). The structure of the unextended apical cap was hard to discern, but it may be layered (Fig. 8). Upon elongation, an electron-dense "basal" ring (Figs. 16, 17) was regularly seen under the plasmalemma of the fertilization tubule which also bore other electron-dense areas along its length (Fig. 16). Only one basal ring could be seen in longitudinally sectioned, paired cells immediately following conjugation (Figs. 18, 19).

Following contact and fusion of the membranes, the zooids "jackknifed" and coalesced laterally, it would seem, along one of the bands of rootlet microtubules (Fig. 20). We have often observed a contractile vacuole between laterally fusing gametes (Fig. 21).

Development of zygospores and azygospores.

Some but not all gametes which failed to conjugate, retracted their flagella and secreted a wall to form azygospores. The structure of this wall differed from that of coenobia in that the inner layer, not the outer, was thin and densely staining (Fig. 24).

Soon after syngamy the nuclei appeared simply to move together and coalesce (Figs. 22, 23, 25). Fig. 24 shows two nucleolar areas in a large, bilobed nucleus, the only time during the life cycle we have ever seen nuclei containing more than one nucleolus. Binucleate spores (Fig. 25) were sometimes encountered; we cannot decide whether these are zygospores containing unfused nuclei or whether they represent

abnormal azygospores derived from binucleate zooids resulting from incompleted cleavage of the coenobial cytoplasm.

Depending on the conditions of growth the spores, both zygotes and azygotes, may remain dormant for some time especially in old cultures. If placed in fresh media they soon germinated forming zoospores which later differentiated into the characteristic polyhedra (see Marchant and Pickett-Heaps, 1971: Figs. 1 and 6; also unpublished data).

DISCUSSION

Mainx (1931) gives a comprehensive account of conjugation, concentrating on the aggregation and precopulatory behavior of Hydrodictyon gametes. Pocock (1960) stresses the plasticity of zooid behavior in H. reticulatum; she maintains that net-forming zooids may alternately form asexual resting spores or even function as gametes; she further states that if they fail to conjugate, the smaller gamete-zooids give rise to azygospores but never to a net (Pocock, 1960: p. 280). We have demonstrated ultrastructural differences between gametes and net-forming zooids, the most significant being the possession of the apical cap by some gametes, a structure never found in net-forming zooids. Initial contact between conjugating gametes is achieved through this structure. Presumably, if "net-forming" zooids do conjugate as Pocock says, they would have to acquire apical caps during their development.

We do not know what induces coenobia to produce gametes instead of net-forming zooids. Whatever this stimulus, it apparently differs from that which much more rarely induces the gametes to conjugate. Non-conjugating gametes appear ultrastructurally identical with those from conjugating cultures except that their apical caps are not extended.

In our experience, conjugation always closely follows the rupture of the parental cell wall and liberation of some gametes; those gametes retained inside it also conjugate. Pocock (1960: p. 239) reports that conjugation sometimes took place before gametes were released; she does not specify, however, whether the parental wall was unbroken or not on these occasions.

Gametes from conjugating cultures never exhibited extended apical caps before the wall was ruptured. Neither were the apical caps ever extended in non-conjugating gametes, whether they were released or not. This obviously suggests that the extension is prerequisite for conjugation. Light microscopists have reported fine cytoplasmic connections between conjugating gametes of various algae including Chlamydomonas chlamydogama (Bold, 1949), Oedogonium cardiacum (Hoffman, 1961), Astrephomene gubernaculifera and Volvulina steinii (Stein, 1958), and the fungus Blastocladiella (Whiffen, 1942). In Chlamydomonas reinhardi (Friedmann, Colwin and Colwin, 1968) an elaborate "fertilization tubule" on one gamete of each conjugating pair, establishes initial contact. In Chlamydomonas moewusii however, "long strands of seemingly amorphous material emerged from flagellar tips of sexually active cells" which made initial contact between conjugants before cytoplasmic fusion by means of "plasma papillae" (Brown, Johnson and Bold, 1968). Gametes of C. moewusii merge "head on", unlike those of C. reinhardi and H. reticulatum in which recently conjugated gametes merge by lateral fusion ("jack knifing"). While it is not reported precisely when these fertilization tubules, cytoplasmic extensions, or amorphous extensions of the flagella and plasma papillae arise, they appear to be formed shortly before fertilization. These events may be analogous to the morphological changes induced in sperm of various animals by the female before fertilization is possible, for example, "capacitation" of mammalian sperm (Austin, 1965) and "maturation" in sperm of some nematodes (Sommerville and Weinstein, 1964; Marchant, unpublished data). Friedman (1962) argues that in both algal and animal fertilization,

fusion of the plasma membranes starts at a predetermined site on the male gamete, giving as examples the flagellar tip of Prasiola (Manton and Friedmann, 1960) and the acrosome filament of animals including Hydroides (Colwin and Colwin, 1961) and the rat (Szollosi and Ris, 1961). Recent work on animals (Austin, 1968) and algae (see earlier references) including this investigation, supports Friedmann's basic contention: furthermore, as in the sperm of many animals, some algal and possibly fungal gametes may have to undergo structural modification (e.g. extension of the fertilization tubule) induced by an external stimulus (the presence of the opposite "sex" or mating type?) before fertilization.

Previously, the gametes of H. reticulatum have been regarded as isogametes (i.e. all morphologically identical), but we have found ultrastructural dissimilarities between conjugating gametes (namely the presence or absence of an apical cap) as is found in various "isogamous" species of Chlamydomonas (Förster and Wiese, 1955; Förster, Wiese and Braunitzer, 1956; Friedmann, Colwin and Colwin, 1968; and Lewin, 1950, 1952, 1954). We stress that conjugation can take place between gametes from the same coenobium (Fritsch, 1935), the many nuclei of which arise from repeated mitoses of a single haploid nucleus, raising the interesting problem ~~concerning~~ of how the two different mating types could be formed.

ACKNOWLEDGEMENT

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ABBREVIATIONS USED IN FIGURES

b - bridge between basal bodies

ch - chloroplast

cv - contractile vacuole

f - flagellum

g - golgi body

l - lipid droplet

m - mitochondrion

n - nucleus

nc - nucleolus

p - pyrenoid

s - starch grain

t - microtubules

Figs. 1-5 Light micrographs of living material. Nomarski optics, X 1,200.

Fig. 1 Contact of gametes at their flagellar end.

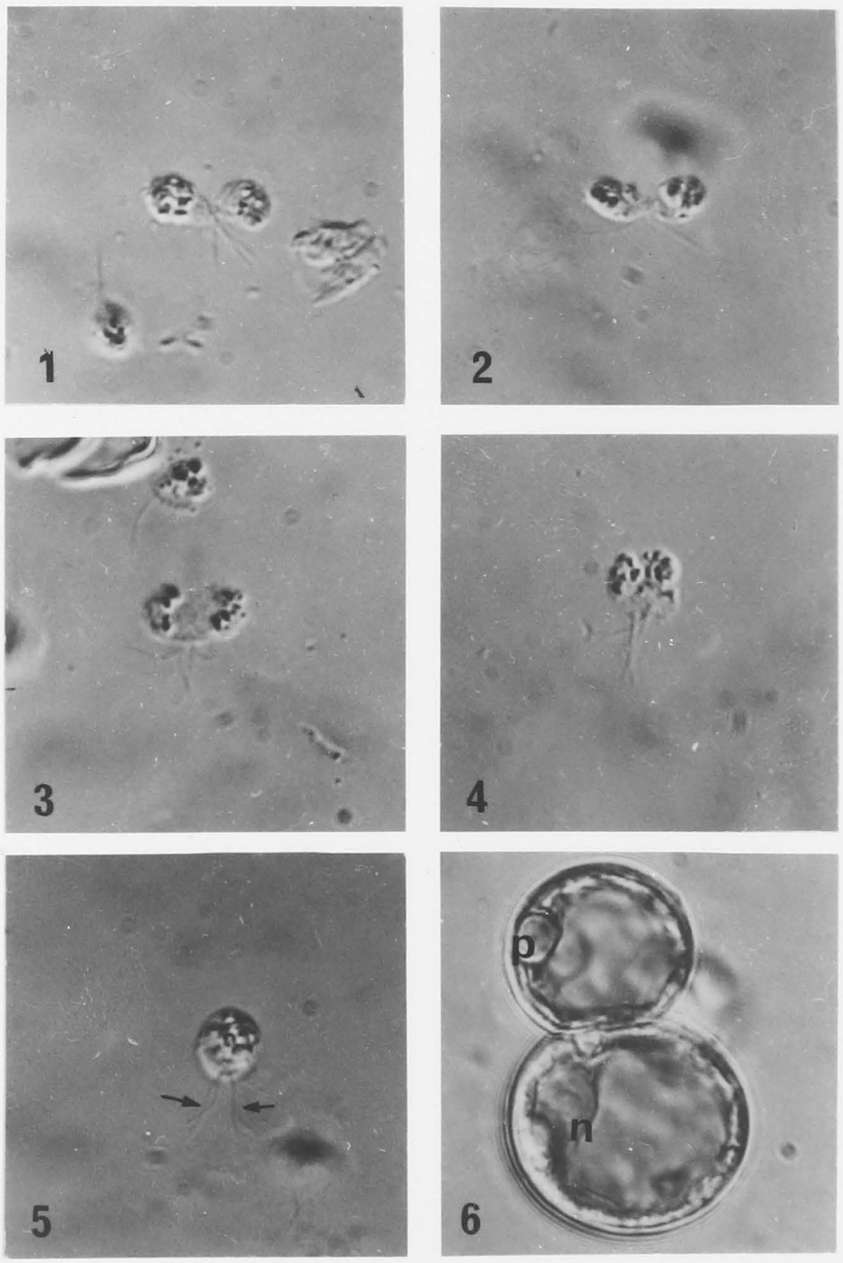
Fig. 2 Commencement of lateral fusion (jack-knifing).

Fig. 3 Lateral fusion of gametes.

Fig. 4 Lateral fusion completed.

Fig. 5 Zygote rounding up. Note the two pairs of flagella (arrows).

Fig. 6 Walled zygotes. Nomarski optics, X 500.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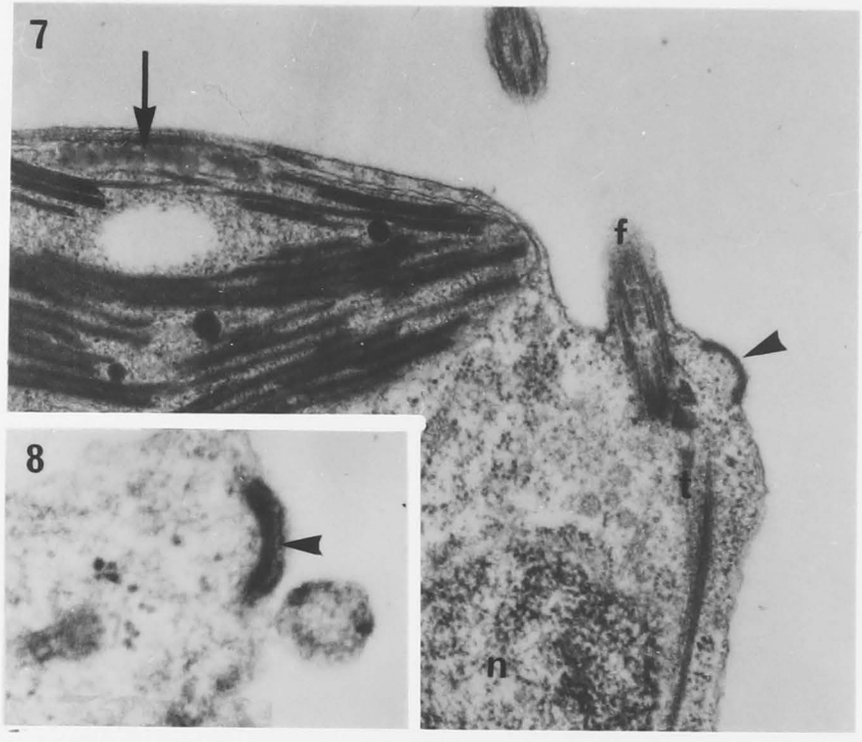
Figs. 7-26 Electron micrographs.

Fig. 7 Flagellar end of a gamete showing the apical cap (arrowhead). Note the eyespot (arrow) and microtubules near the basal body. X 38,000.

Fig. 8 An apical cap at high magnification. Note its layered structure. X 71,000.

Fig. 9 Section of gamete showing extensive starch deposits, remnant pyrenoid and eyespot within chloroplast. Note also the golgi bodies adjacent to the nucleus. The enclosed area is shown at higher magnification in Fig. 10. X, 14,500.

Fig. 10 Details of eyespot lying under the outer membrane of the chloroplast. X 29,000.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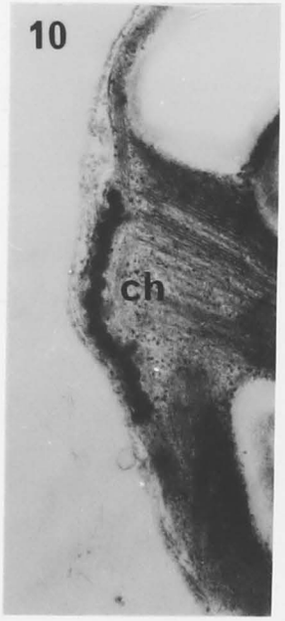
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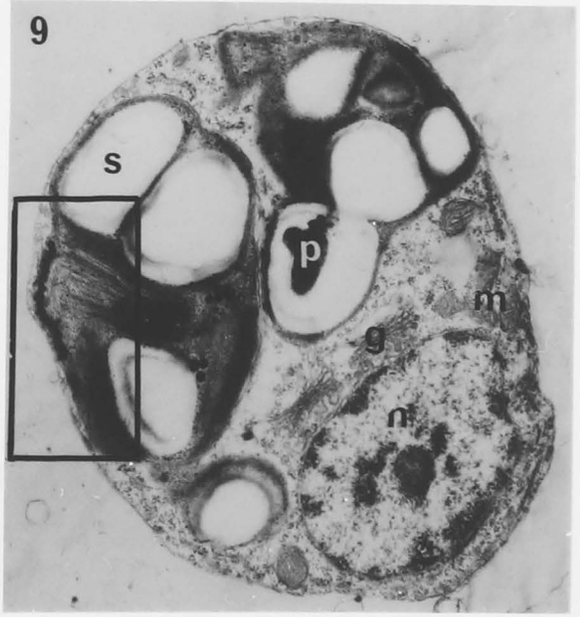
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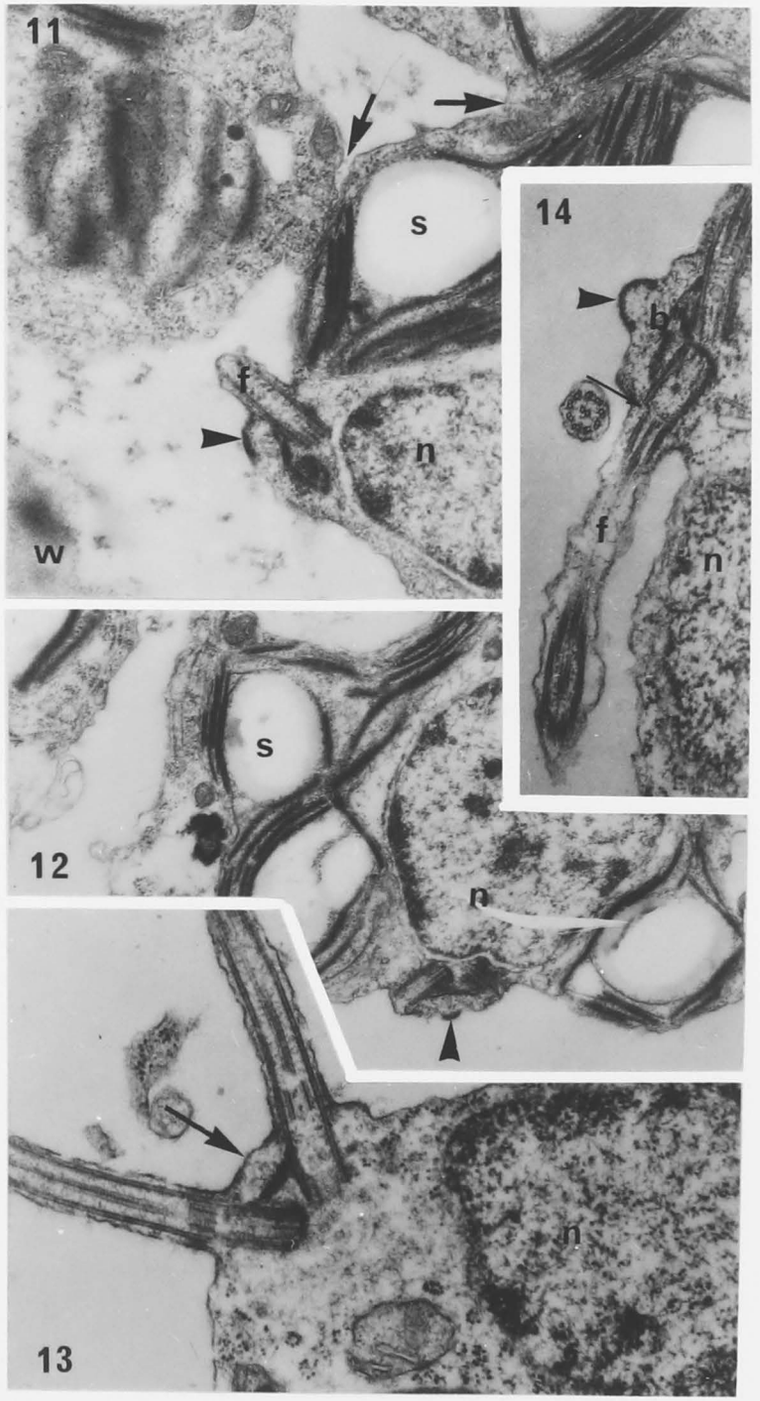
Fig. 11 Late stage in the cleavage of coenobial cytoplasm to produce gametes. Note the apical cap along side the elongating flagellum and the incomplete cleavages (arrows). X 26,500.

Fig. 12 Coenobial cytoplasm forming gametes, flagella not elongated. Note the apical cap between the basal bodies linked by a bridge. X 20,500.

Fig. 13 Flagellar end of a gamete lacking an apical cap. Note the bridge between the basal bodies. X 28,500.

Fig. 14 Unextended apical cap (arrowhead) on gamete in culture in which conjugation is not taking place. The flagella are being retracted. Note the wrinkled flagellar membrane and the disjunction between the outer flagellar tubules and those of the basal body (arrow). X 36,500.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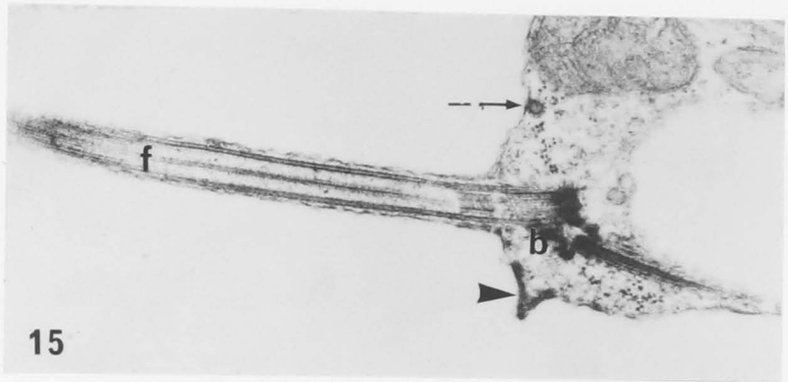
Figs. 15-17. Stages in the elongation of the apical cap to form a fertilization tubule.

Fig. 15 Note the densely staining material within the apical cap (arrowhead), the rootlet microtubules arising from the bridge between the basal bodies and a coated vesicle on the plasmalemma (arrow). X 28,500.

Fig. 16 Densely staining material lining the developing fertilization tubule. Basal ring (small arrow) becoming obvious. X 38,500.

Fig. 17 Fertilization tubule with basal ring (small arrows). X 38,000.

Fig. 18 Tangential section through a developing fertilization tubule showing basal ring (arrow). Note the centriole (arrowhead). X 61,000.



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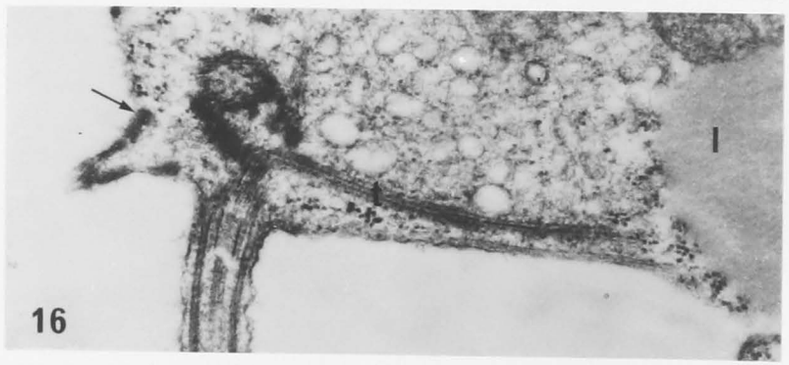

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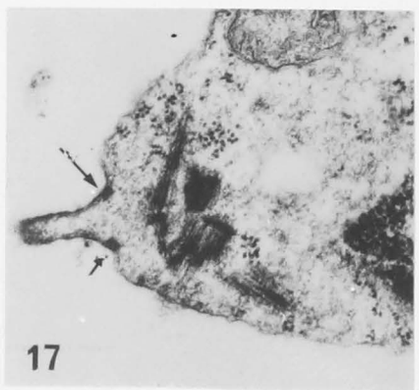

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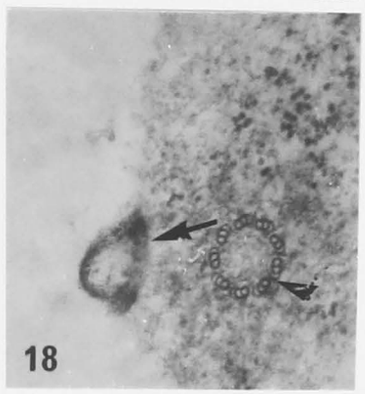

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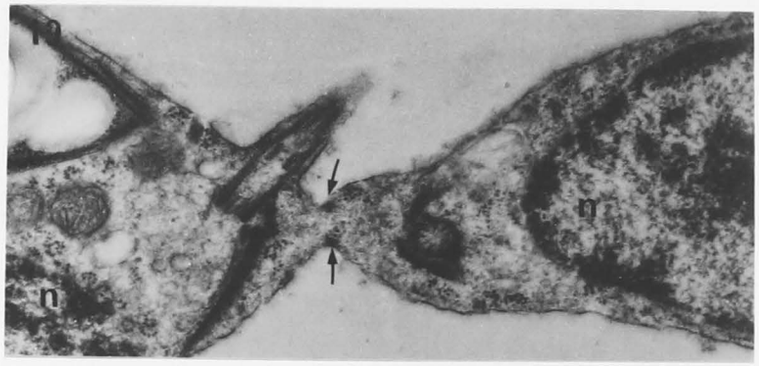
Fig. 19 Union of gametes membranes via the fertilization tubule. Note the basal ring (arrows). X 24,500.

Fig. 20 Higher magnification of site of gametic union. X 43,000.

Fig. 21 Lateral fusion of gametes along a band of rootlet microtubules. X 15,000.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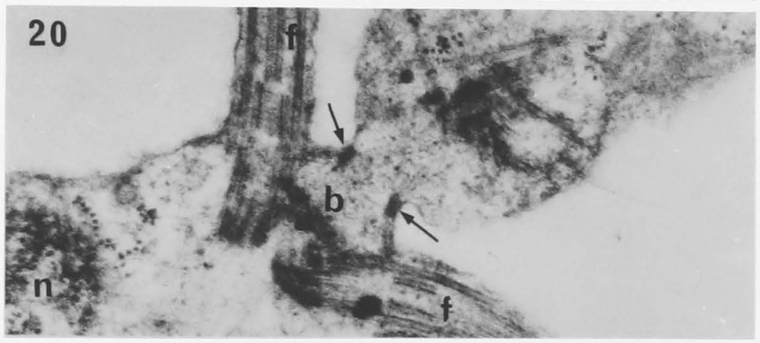
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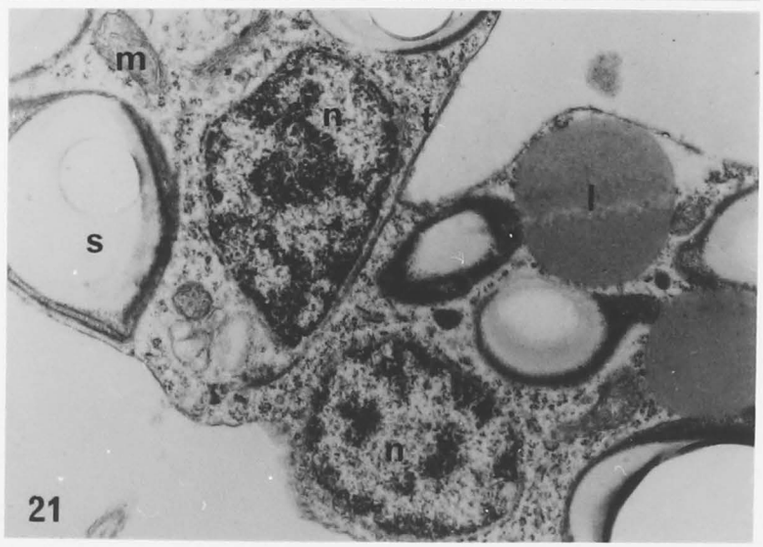
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Fig. 22 Contractile vacuole between laterally fusing gametes. Note the remnant basal ring (arrow) between the basal bodies. X 20,000.

Fig. 23 Nuclear envelopes of newly formed zygote in contact (arrowhead). X 32,500.

Fig. 24 Bilobed zygotic nucleus from fusion of gametic nuclei. Note the two nucleolar regions. X 17,000.

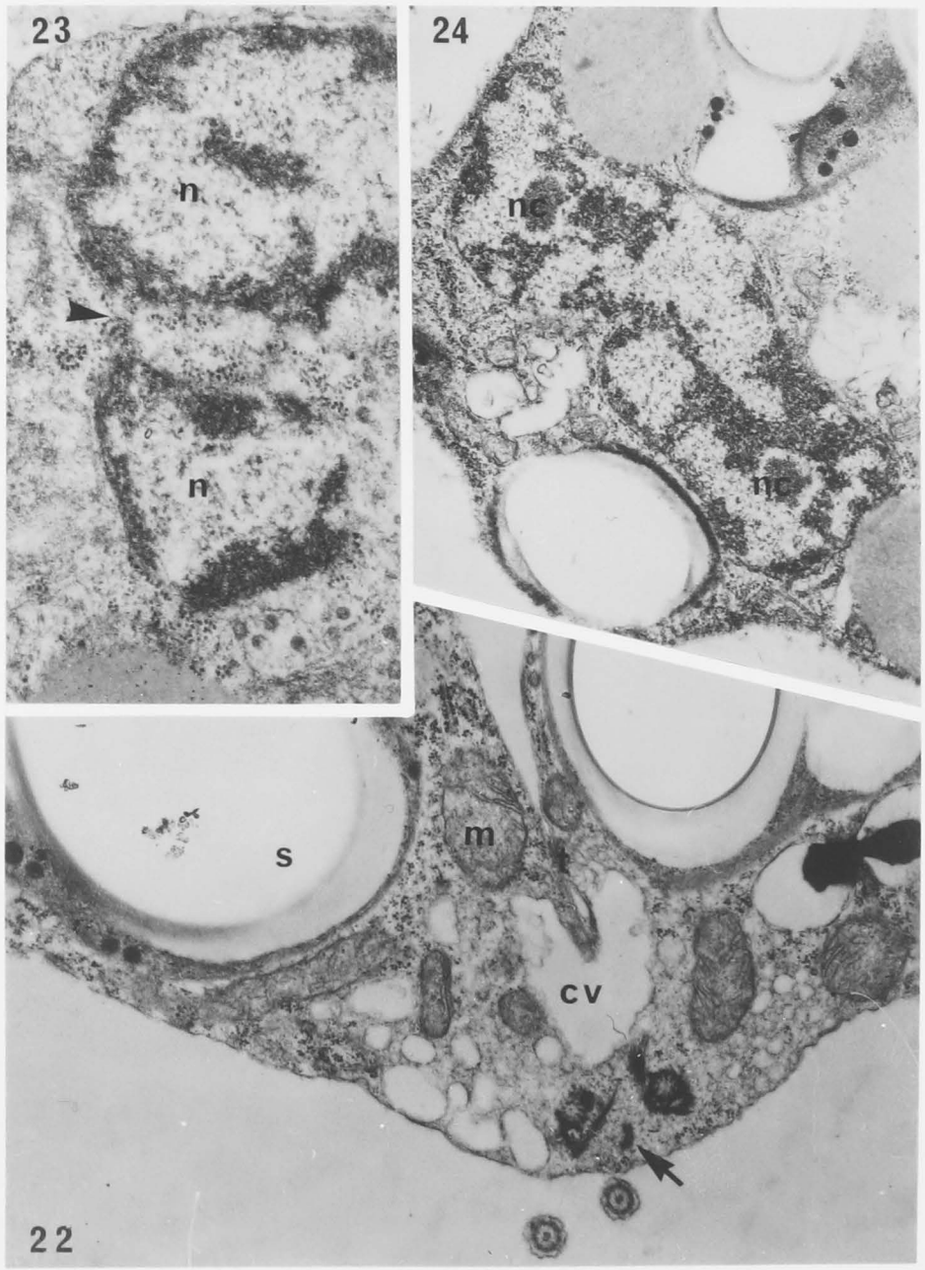
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Fig. 25 Binucleate walled spore, either a zygote in which the gamete nuclei failed to unite or an azygote formed from a binucleate gamete. Note the bilayered wall, with the densely staining inner layer. X 15,000.

Fig. 26 Walled zygospore. X 15,000.

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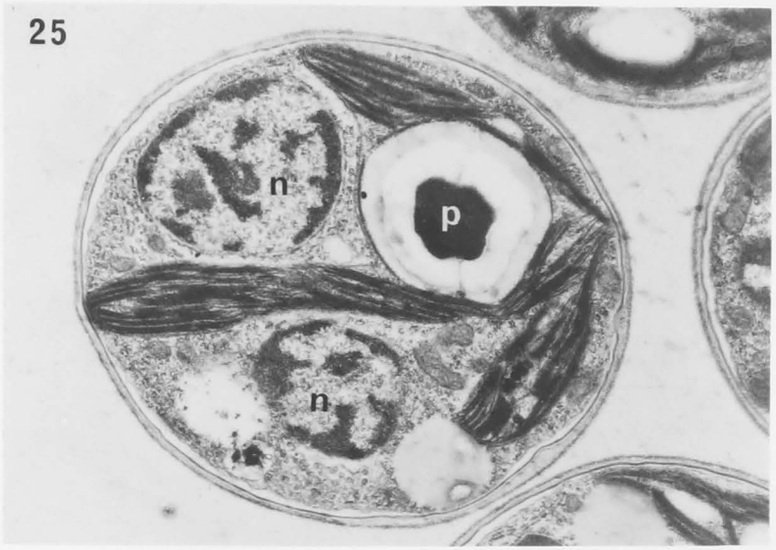

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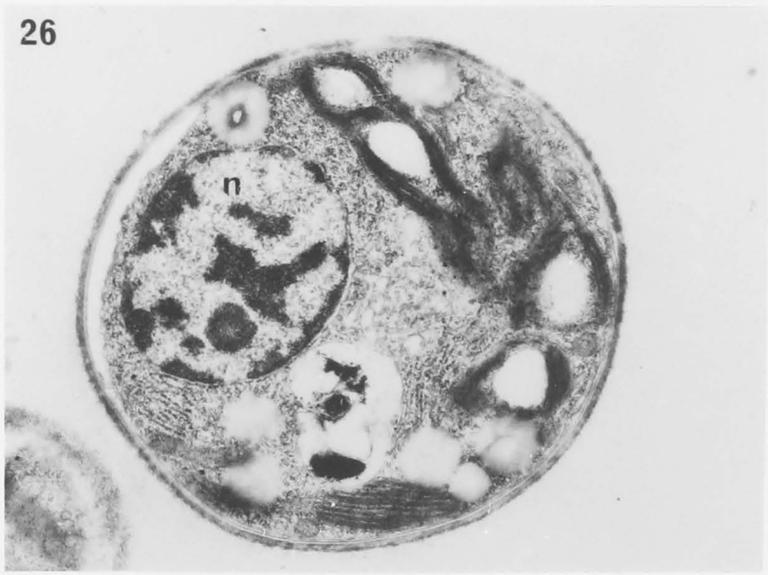

CHAPTER 7

DEVELOPMENT OF POLYHEDRA

SUMMARY

The fine structure of mature zygospores and azygospores of H. reticulatum is briefly discussed before their differentiation to form biflagellate zoospores is described. These large zoospores are liberated through a rupture in the parental wall and after a brief period of feeble motility, retract their flagella and secrete a cell wall themselves. This wall is deposited unevenly, producing the characteristic spines of polyhedra.

INTRODUCTION

In a previous paper (Marchant and Pickett-Heaps, 1972b), we discussed conjugation of the gametes of H. reticulatum and the subsequent development of thick-walled zygospores. We also mentioned that some of the gametes which fail to conjugate, retract their flagella and secrete a wall to become azygospores. This paper describes the ultrastructure of mature spores, their germination to form zoospores and subsequent deposition of a wall by the zoospores, after a brief period of motility, to produce polyhedra. Differentiation of these cells will be related to similar processes elsewhere in the life cycle of this alga and in other organisms. The position of polyhedra in the life cycle of H. reticulatum is illustrated in an earlier paper in this series (Marchant and Pickett-Heaps, 1971).

MATERIALS AND METHODS

The Australian strain of H. reticulatum (Marchant and Pickett-Heaps, 1970) was used for the work described here. Differentiating spores, zoospores and polyhedra were cultured and processed for transmission electron microscopy as previously described (Marchant and Pickett-Heaps, 1970, 1971, 1972a). Some cells, fixed in glutaraldehyde and osmium tetroxide as usual, were collected on solvent resistant "Millipore" filters, dehydrated in ethanol and n-amyl acetate and dried in a CO_2-critical point drying apparatus (Anderson, 1951; Horridge and Tamm, 1969). These dried specimens were omnidirectionally shadowed with carbon and gold and examined in a Cambridge Stereoscan scanning electron microscope.

RESULTS

Of all stages in the life cycle of H. reticulatum, zygospores, azygospores, zoospores and polyhedra derived from the latter have proved the most difficult to preserve satisfactorily for electron microscopy, the main reasons being:

1. Zygotes accumulated lipid droplets which came to occupy most of the cytoplasm (Figs. 4, 5, 6). This lipid was difficult to fix and embed, and structures surrounding it were rarely adequately preserved and usually disintegrated when being sectioned (Fig. 5).

2. Zoospores were extremely fragile, being easily disrupted by mechanical damage and slight changes in osmotic pressure. In addition, they were only active for a short period of time after release from the spore, and were thus difficult to collect. They, too, contained much lipid (Fig. 7).

3. Polyhedra initially contained lipid droplets (Figs. 20, 21). As they matured they became vacuolate, but the individual cells were too small to cut into segments, the procedure we adopted to obviate disruption during processing of cylindrical coenobia (Marchant and Pickett-Heaps, 1970). Hence it was difficult to avoid collapse when processing polyhedra for electron microscopy.

Mature zygospores and azygospores.

Zygospores were usually considerably larger than azygospores (Fig. 2), although the size of both varied widely. As these resistant spores

aged, they lost their green colour and became orange and could be stored dry for several weeks. No attempt was made however, to investigate how long they could survive desiccation. Both zygospores and azygospores had a thick bilayered wall (Figs. 4, 5), similar to that of cylindrical coenobia; sometimes however, the layering of the wall appeared reversed, i.e., they had a thin inner layer (Fig. 25, Marchant and Pickett-Heaps, 1972b). The spores were often attached to one another in irregular aggregates, or to the substrate, by their outer layer of cell wall (Fig. 5). What induces their aggregation is unknown. That zygotes and azygotes are negatively phototactic before losing their motility and secreting a wall is illustrated in Fig. 3. Here, spores had aggregated and adhered to one another and to the bottom of a Petri dish in a pattern corresponding to the date written with a black felt pen on the top of another Petri dish immediately subjacent to the dish containing these spores.

Developing zoospores.

Whereas zygospores cleaved to form usually four zoospores on germination, azygospores generally only produced a single zoospore. Meiosis was not observed in our material. Cleavage furrows (Fig. 2, cf. Fig. 1) in the zygotic cytoplasm grew along bands of phycoplast (Pickett-Heaps, 1972a) microtubules (Fig. 9). Following cytoplasmic cleavage, part of the outer layer of zygotic wall expanded (Figs. 6, 7, 8, 13), and the zoospores moved into this space before being liberated through a rupture in the distended wall. If this distended zygotic wall failed to rupture, the trapped zoospores completed their transformation to

polyhedra within it (Fig. 21; also see Fig. 5 in Marchant and Pickett-Heaps, 1971).

These zoospores bore a pair of flagella (Fig. 10) and were only weakly motile. Occasionally quadriflagellate zoospores (Figs. 11, 12) were observed. In culture, zoospores appeared to lack any phototactic response and rarely moved far from where they were released.

Formation of polyhedra from zoospores.

After a period of motility which lasted only a few minutes, zoospores retracted their flagella and secreted a cell wall (Figs. 14-19). Wall formation was extremely rapid and not uniform over the surface of the zoospores, so that the characteristic projections of polyhedra (Fig. 22) soon became obvious. The cytoplasm of young polyhedra contained lipid droplets (Figs. 20, 21) which disappeared as the polyhedra aged.

DISCUSSION

Proskauer (1952) reported that meiosis in H. reticulatum, as expected, precedes germination of the diploid zygotes and so restores the haploid condition of the rest of the life cycle. Our attempts to confirm his observations have been thwarted: first, fragmentation of the zygotic chloroplast completely obscures the nucleus shortly before the presumed meiotic division and differentiation of the zygote (cf. Figs. 1, 2). Secondly, we have been unable to fix and embed the cytoplasm of mature zygotes satisfactorily for electron microscopy. These zygotes frequently disintegrated when sectioned, and when they were adequately fixed and embedded, they appeared dense and ultrastructural detail was obscured.

Zygospores often attach to the bottom of culture dishes or some other substrate within the medium, or may stick to one another by their outer layer of wall. Many become attached to the underside of the surface film of the culture medium. We do not know whether motile zygotes are weakly mutually attracted to each other or whether their aggregation is a result of being negatively phototactic; Fig. 3 suggests the latter is more likely.

The motility of the large zoospores released by zygospores and azygospores appears not sufficient to constitute an important factor in the dispersal of this alga. Polyhedra however are planktonic, their characteristic spiny processes and their lipid content presumably aiding in floating, and it is these cells, we suspect, which are carried by water currents to disperse the alga in nature. The bristles and spines

on Pediastrum (see Chapter 10) and Scendesmus (Trainor and Burg, 1965) have been considered adaptations to a planktonic existence (Fritsch, 1935); however, Trainor and Burg suggest that they principally serve in spacing out the colonies, thus reducing overcrowding and shading of one another. No bristles were evident on polyhedra of H. reticulatum which was somewhat surprising since they occur on the polyhedra of its close relative, Pediastrum, (Davis, 1967) as well as on the vegetative cells of that alga (see Chapter 10).

Polyhedra can also be considered another resistant stage in the life cycle; although not being able to withstand desiccation to the same extent as zygospores or azygospores, they will survive drying that would kill cylindrical coenobia. Other members of the Chlorococcales have a polyhedral stage in their life cycle (e.g. Pediastrum; Davis, 1967) while the vegetative cells of Tetraedron (Pickett-Heaps, 1972c) are polyhedral in shape. In fact, some early phycologists apparently considered polyhedra of Hydrodictyon and Pediastrum to be species of Tetraedron. There are numerous similarities, not only in their gross morphology, but also at the ultrastructural level between these three genera. Vegetative cells of Tetraedron are uninucleate when first formed and become coenocytic by repeated mitoses; its mitotic nuclei are enclosed by a perinuclear envelope and cleavage of the multinucleate cytoplasm utilizes phycoplast microtubules (Pickett-Heaps, 1972c); all these phenomena are observed in both Hydrodictyon and Pediastrum (Marchant and Pickett-Heaps, 1970, 1971 and unpublished data). However the cells produced by cleavage of Tetraedron are usually non-motile autospores. Only very rarely are motile zoospores formed (Davis, 1966;

Starr, 1954), the usual product of cleavage in polyhedra of Hydrodictyon (Pocock, 1960; Marchant and Pickett-Heaps, 1972d) and Pediastrum (Davis, 1967).

ABBREVIATIONS USED IN FIGURES

l - lipid droplet

n - nucleus

p - pyrenoid

v - vacuole

w - cell wall

Fig. 1 Zygotes of H. reticulatum. Note the nuclei and pyrenoids. Nomarski optics. X 1,100.

Fig. 2 Zygote cleaving to form zoospores. The chloroplast obscures other cytoplasmic components. The smaller spore is probably an azygospore. Nomarski optics. X 1,100.

Fig. 3 Pattern of settling of zygotes and azygotes corresponding to writing (19/5) on the top of another petri dish which was subjacent to the dish in which the spores had settled. X 1.6.

Fig. 4 Part of the wall and cytoplasm of a zygospore. Note the lipid droplets and poorly preserved chloroplasts. X 20,000.


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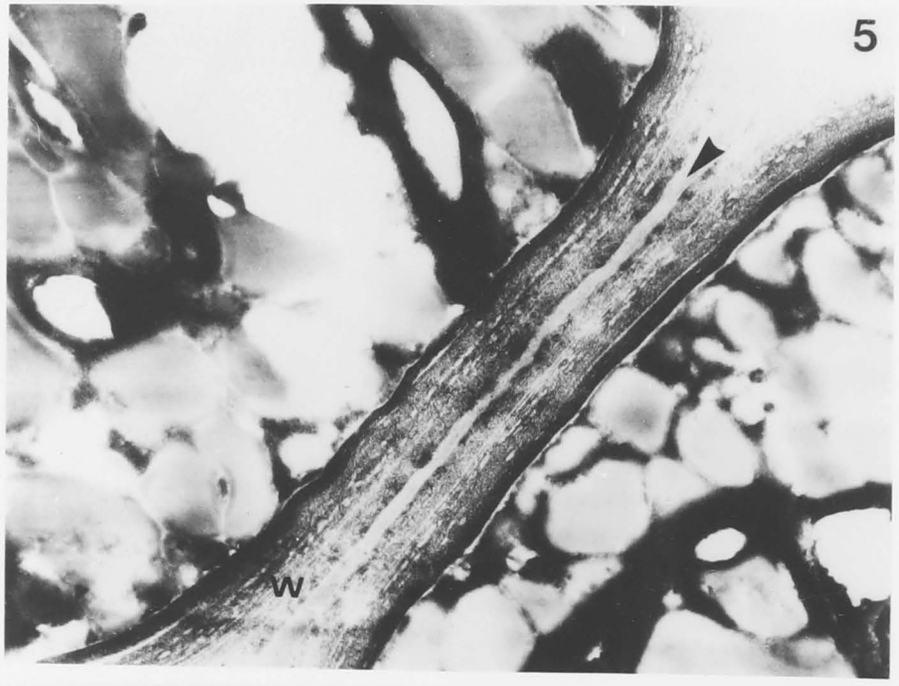
Fig. 5 Zygospores adhering to one another by their outer layer of wall (arrowhead). Note the basic similarity in appearance of this junction with that between mature vegetative cells. X 20,000.

Fig. 6 Initial cleavage of zygospores to produce zoospores. Note the wall weakening and the preponderance of lipid droplets in the cytoplasm. X 4,700.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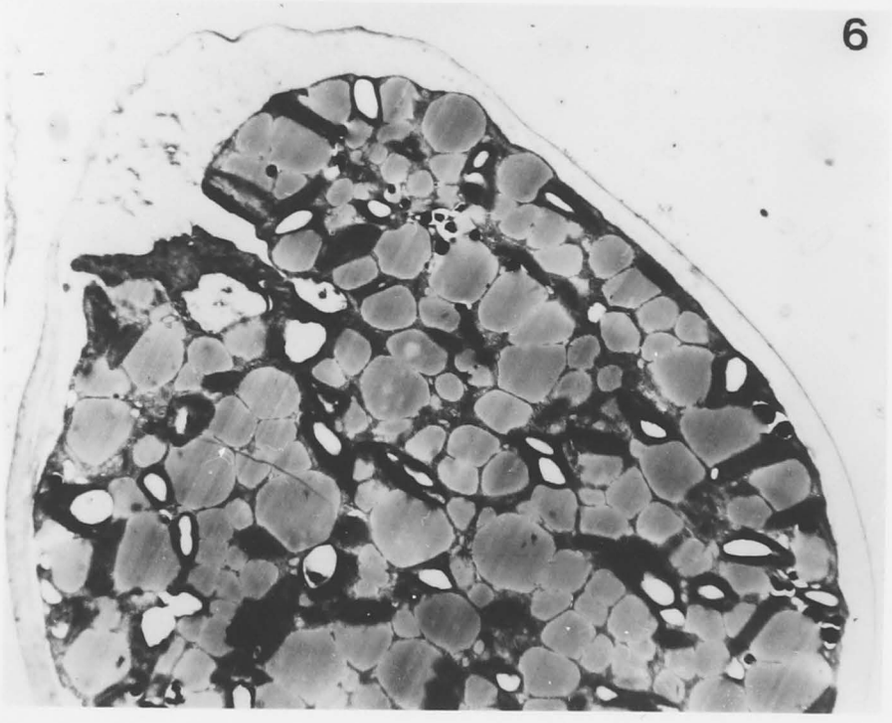
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Fig. 7 Zoospores formed by cytoplasmic cleavage of zygospores. At the wall weakening the inner layer of wall has disappeared and the thin outer layer (arrowhead) is becoming greatly distended. Note the transverse section of a flagellum (arrow). X 6,300.

Fig. 8 Light micrograph of adjacent section to Fig. 7, showing the distension of the outer layer of wall and the other zoospores. X 1,200.

Fig. 9 Cleavage of zygotic cytoplasm. Note the microtubules (arrow) adjacent to the developing cleavage furrow. X 36,000.

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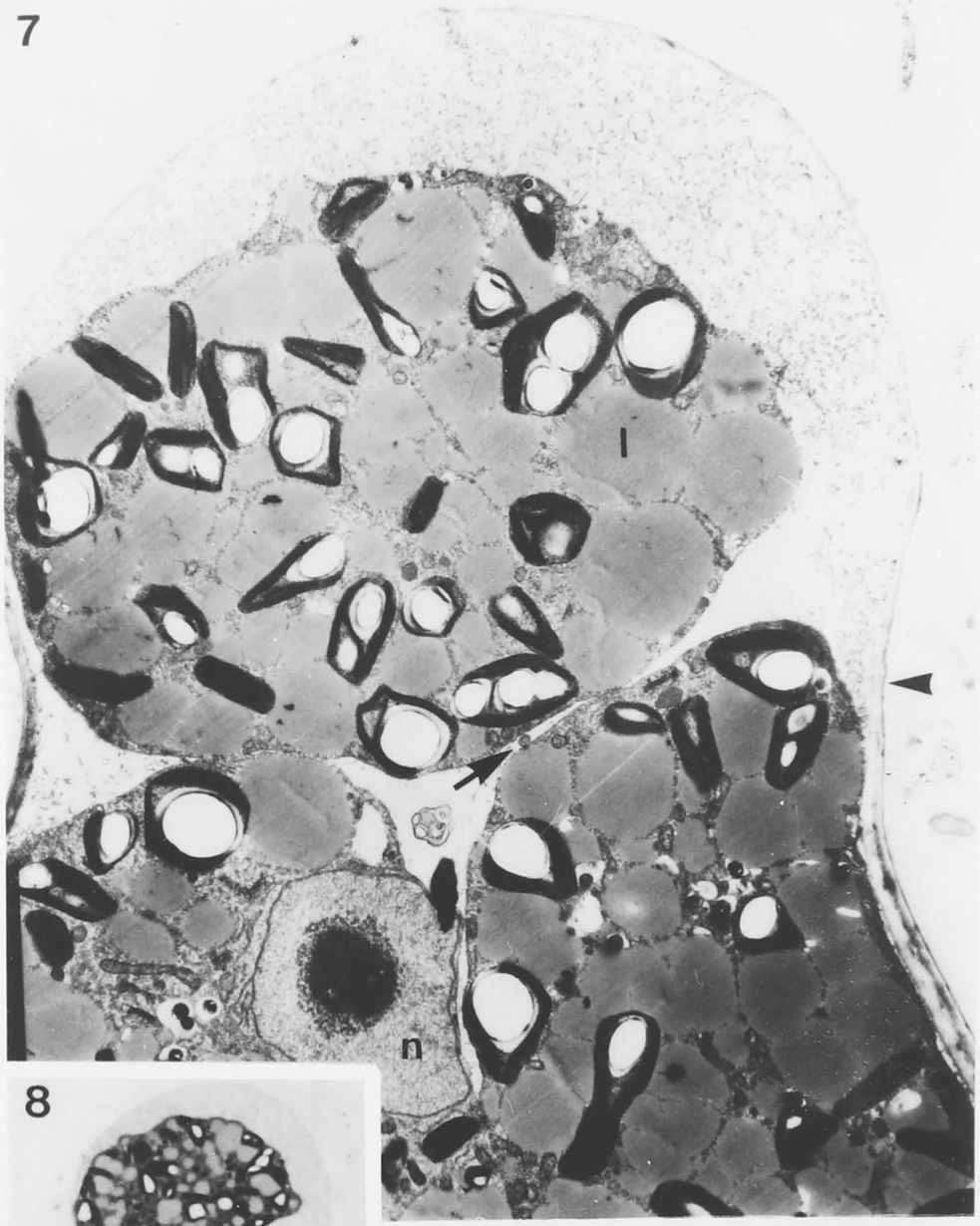

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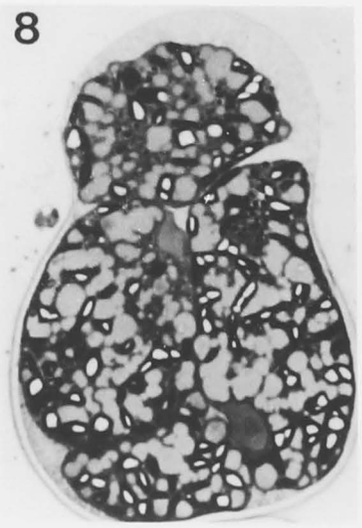

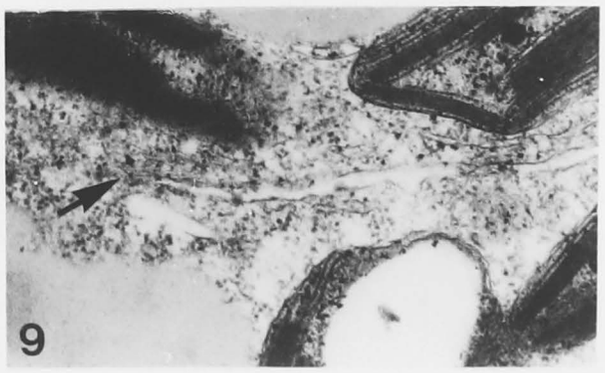

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Fig. 10 Scanning electron micrograph of a zoospore showing the flagella and distortion of the plasmalemma by lipid droplets. X 2,300.

Fig. 11 Quadriflagellate zoospore (cf. Fig. 10). Attachment of flagella shown at higher magnification in Fig. 12. X 2,300.

Fig. 12 Scanning electron micrograph of part of a quadriflagellate zoospore. Note the projections on the zoospore from which the flagella arise. X 12,000.

Fig. 13 Four zoospores within a highly distended zygotic wall. X 1,300.


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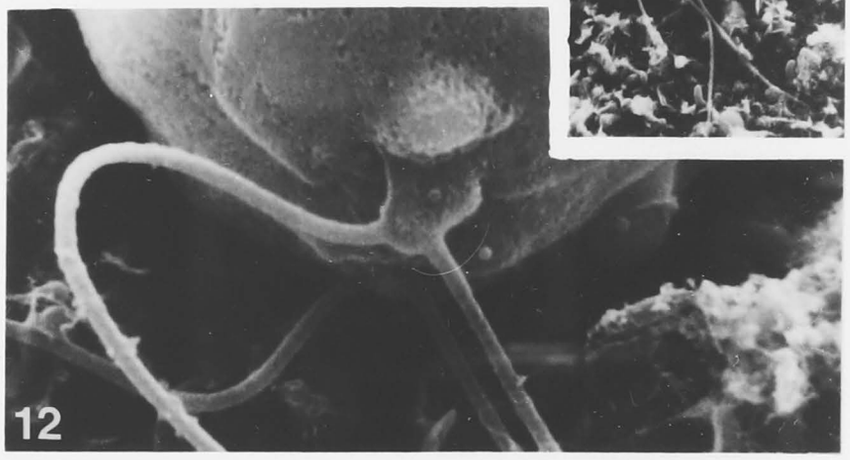
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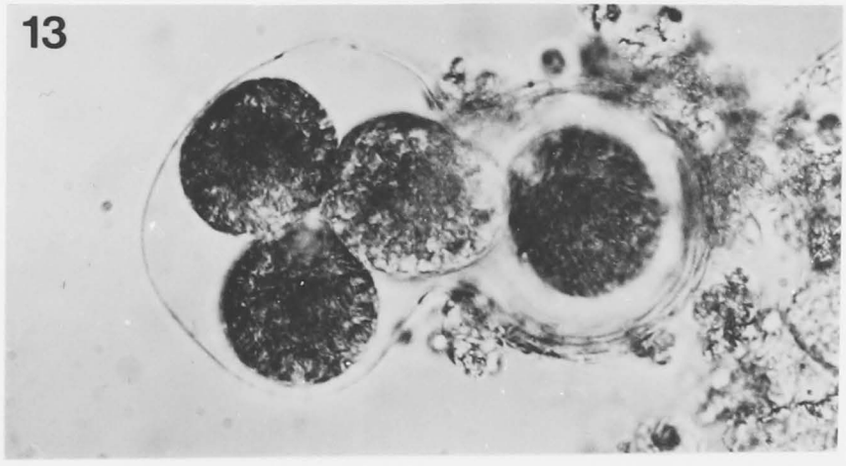
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Figs. 14-19 Series of micrographs illustrating a zoospore retracting its flagella and developing the first projection to become a polyhedron. All phase contrast. X 1,100

Fig. 14 Actively swimming zoospore. Arrow indicates the attachment of flagella.

Fig. 15 Flagellar shortening and commencement of elongation of projection.

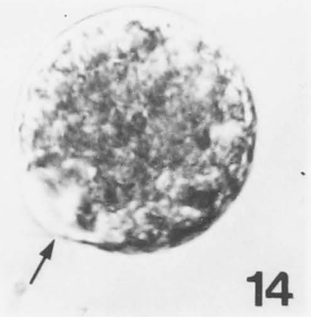
Fig. 16 Flagella much shortened.

Fig. 17 Considerable elongation of projection and flagella almost completely retracted.

Fig. 18 Flagella completely retracted.

Fig. 19 Extension of first projection nearly complete.

Fig. 20 Electron micrograph of part of a young polyhedron showing the remnant lipid droplets in the cytoplasm. X 12,000.



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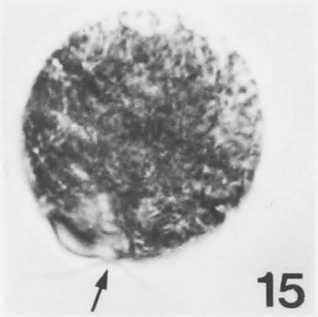

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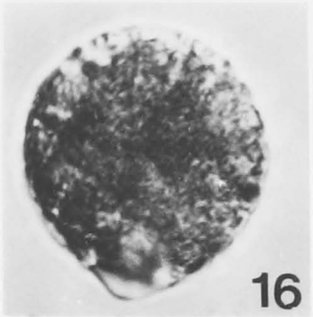

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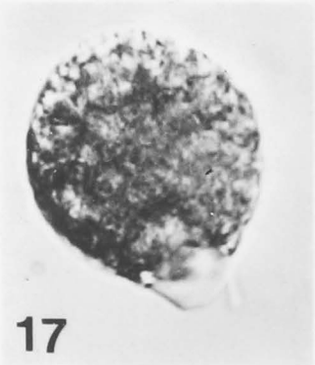

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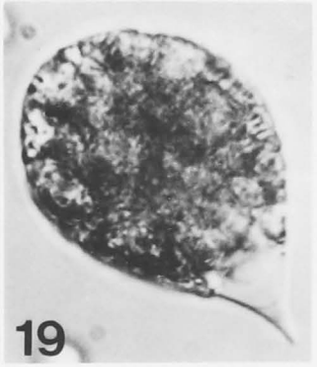

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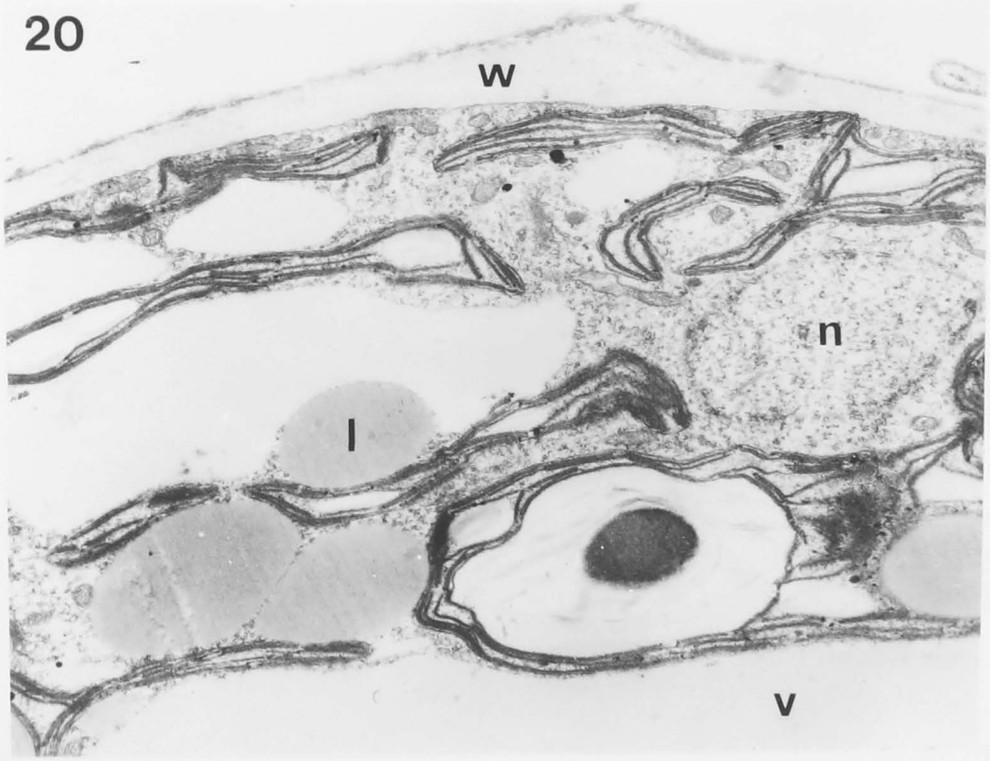
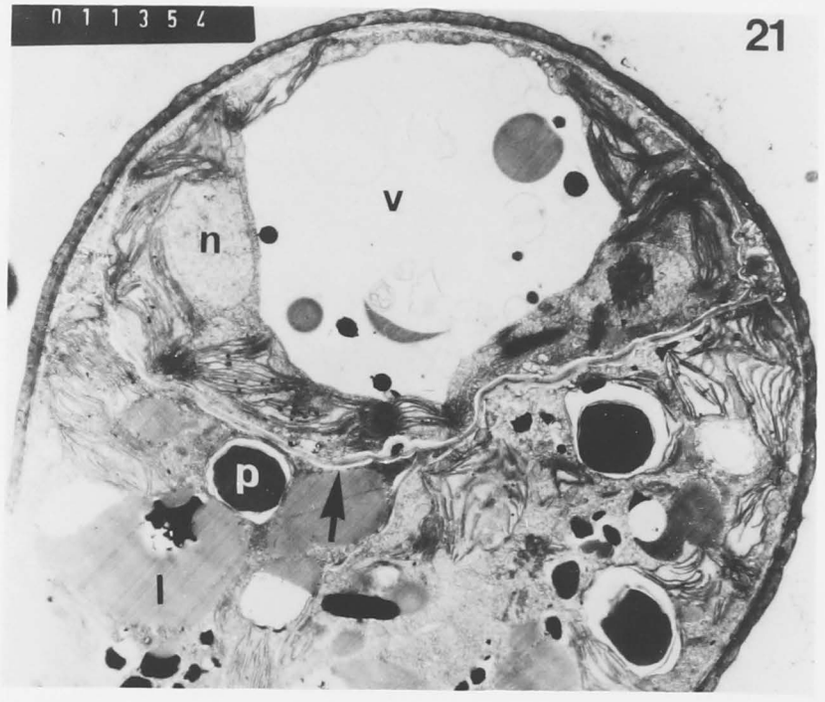


Fig. 21 Irregular polyhedra produced by zoospores which had not been released from the zygotic wall. Note the new polyhedral wall (arrow). X 8,600.

Fig. 22 Scanning electron micrograph of a mature polyhedron. X 2,300.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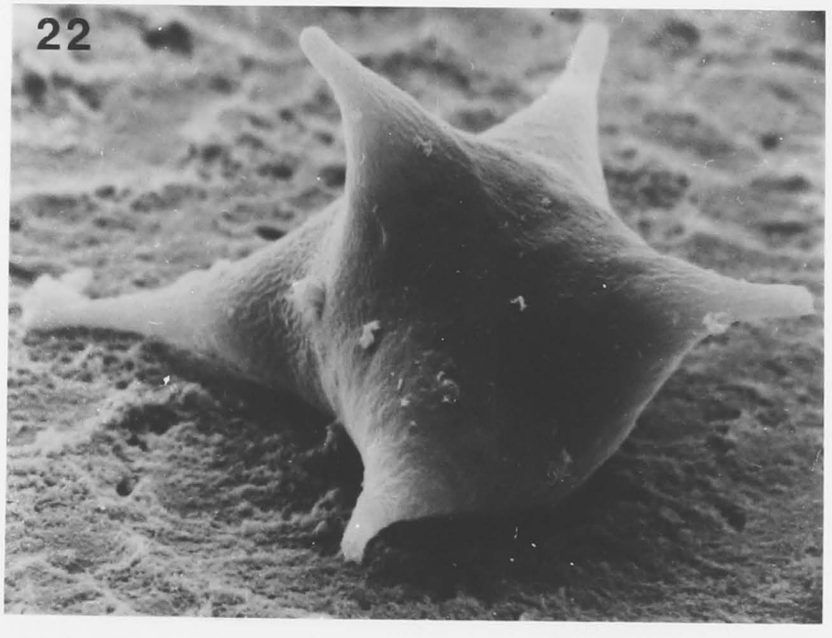
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CHAPTER 8

FORMATION OF THE GERM NET

SUMMARY

Uninucleate, biflagellate, net-forming zooids arise by cleavage of the multinucleate cytoplasm of polyhedra. These zooids appeared indistinguishable from net-forming zooids produced by cylindrical coenocytes. Whereas zooids derived from cylindrical cells aggregated within their parental cell wall to form cylindrical nets, zooids produced by polyhedra swarmed within a spheroidal vesicle, probably derived from the inner layer of polyhedral wall, and aggregated usually as a flat net, similar to nets of other species of Hydrodictyon and vegetative colonies of Pediastrum. Bands of peripheral microtubules underlaid the initial sites of contact of aggregating zooids; the role of these microtubules, which were generally oriented in the plane of the developing net, and other aspects of patterned cellular aggregation are discussed.

INTRODUCTION

Twice during the life cycle of H. reticulatum, uninucleate, biflagellate zooids are produced by cytoplasmic cleavage of coenocytic cells and these later link up to form nets. Earlier (Marchant and Pickett-Heaps, 1972a), we described how the zooids aggregated within the wall of vegetative cells giving rise to cylindrical daughter nets which are characteristic of asexual reproduction in this alga. We now briefly discuss the development of zooids from coenocytic polyhedra before describing their linking up to form germ nets. The life cycle of H. reticulatum is diagrammatically summarized in a previous paper (Marchant and Pickett-Heaps, 1971) where differentiation of zooids from cylindrical coenocytes is considered.

We have found, contrary to Pocock's (1960) observations, that germ nets of H. reticulatum are predominantly flat, similar to nets of H. patenaeforme and H. africanum and vegetative colonies of Pediastrum, a closely related member of the Chlorococcales. Similarities between formation of both flat and cylindrical nets of Hydrodictyon and colonies of Pediastrum are discussed.

MATERIALS AND METHODS

Both Australian and Cambridge strains of *Hydrodictyon reticulatum* were used in this investigation. Selected material was prepared for transmission and scanning electron microscopy as previously described (Marchant and Pickett-Heaps, 1970, 1971, 1972c).

OBSERVATIONS

Formation of zooids from polyhedral cytoplasm.

Development of polyhedra (Fig. 1) from zoospores released from either zygospores or azygospores has already been discussed (Marchant and Pickett-Heaps, 1972c). Cleavage and differentiation of the multinucleate polyhedral cytoplasm to form zooids closely resembled the analogous process in cylindrical coenobia (Marchant and Pickett-Heaps, 1971). First, if a vacuole was present (see below) the tonoplast was cleaved from the cytoplasm to form a vacuolar envelope (Fig. 5) and then the rest of the cytoplasm was progressively cleaved along bands of phycoplast microtubules (Fig. 3) into uninucleate fragments (Fig. 2). Very often however, at the time of cytoplasmic cleavage, the vacuole had disappeared; even if it remained, it was most often small and inconspicuous (Fig. 5) and its vacuolar envelope was never seen to play any significant part in the subsequent formation of germ nets (in contrast to its role in the development of cylindrical daughter nets--Marchant and Pickett-Heaps, 1972a). Concurrent with cytoplasmic cleavage, a vesicle was formed enclosing the zooids. The origin of this vesicle is unclear, it was probably derived from the inner layer of polyhedral wall (Figs. 8, 9) but it may be a secretion from the developing zooids themselves (Fig. 7).

By the time zooids extended their flagella (Fig. 4) from basal bodies surrounded by amorphous material (Fig. 6), the polyhedral wall was only a fraction of its original thickness. The vesicle containing the highly active zooids ballooned (Fig. 10) through a rupture in the

outer layer of the polyhedral wall which often remained as a crumpled "ghost" attached to the vesicle (Fig. 11). If a polyhedron had matured rapidly, a vesicle was often not released; instead, the entire polyhedral wall became highly distended and within it, the zooids swarmed.

Structure of the zooids.

We have been unable to detect any structural difference between net-forming zooids derived from either polyhedral cytoplasm or produced by cylindrical coenocytes (Marchant and Pickett-Heaps, 1971, 1972a). Characteristic of germ-net-forming zooids, as well as those that form daughter nets, were bands of peripheral microtubules (Fig. 12).

Formation of the germ net.

Under our growth conditions, we found that both Australian and Cambridge strains of H. reticulatum produced germ nets that were predominantly flat. Of 831 germ nets of the Australian strain examined, 81% were flat, while 65% of 368 nets of the Cambridge strain were flat (Table 8.1). The number of cells per net at the time of its formation, irrespective to its shape, was usually 256 (2^8) or slightly fewer.

Zooids swarmed vigorously within the spheroidal vesicle and jostled together as they arranged themselves in a planar configuration. If the vesicle in which they swarmed did not expand sufficiently to accommodate them arranged in a plane, they aggregated as a tangled mass or rarely as a hollow ball of cells.

These zooids, like those forming a cylindrical net, changed shape

TABLE 8.1

PROPORTION OF FLAT AND IRREGULAR GERM NETS IN CULTURESOF TWO STRAINS OF *H. RETICULATUM*

Strain	Date	Shape of nets				Total
		Flat		Irregular		
		No.	(%)	No.	(%)	
Australian	23-9-70	73	(64.5)	40	(33.5)	113
	25-9-70	10	(47.5)	11	(52.5)	21
	6-10-70	86	(82.5)	18	(17.5)	104
	1-12-70	125	(77.6)	36	(22.4)	161
	29-1-71	88	(84)	17	(16)	105
	26-5-71	94	(90.4)	10	(9.6)	104
	21-6-71	100	(81.3)	23	(18.7)	123
	23-7-71	95	(95)	5	(5)	100
Total		671	(80.7)	160	(19.3)	831
Cambridge	21-6-71	91	(57.9)	66	(42.1)	157
	9-8-71	90	(75.5)	29	(24.4)	119
	15-8-71	60	(65.2)	32	(34.8)	92
Total		241	(65.5)	127	(34.5)	368

from being initially oval to rhomboidal in outline before aggregation. As in development of cylindrical nets, sites on the plasmalemma of zooids which made contact with others were underlain by microtubules (Figs. 13, 14, 15) and amorphous material appeared on the plasmalemma of the zooids only between sites of contact with others (Figs. 14, 15). The bands of peripheral microtubules were usually found lying in the plane of the developing net (Fig. 14). Each zooid linked with usually four others, two at each end (Figs. 19, 20, 22, 26, 27, 29). Zooids on the outside of a flat net and to a lesser extent, inner cells which had only joined to two others (one at each end), often developed a pair of "horn-like" projections (Figs. 20, 21, 23, 25, 29) only on their "chloroplast side" as distinct from their "nuclear side" (cf. Figs. 20, 22).

The tips of these horns appeared different to other parts of the wall following wall deposition; these sites however did resemble "half" an intercellular junction (cf. Figs. 24, 25). Sometimes flat nets formed with a hole near their centre (Figs. 26, 29).

Development of the germ net.

Retraction of flagella (Fig. 16), disintegration of flagellar microtubules and basal bodies (Fig. 17), secretion of the bilayered cell wall (Fig. 24), cytoplasmic reorganization of newly adhered cells (Fig. 28) and their apparent development of centrioles de novo (Fig. 18) were identical with that in cells of cylindrical nets (Marchant and Pickett-Heaps, 1972a).

Often the vesicles (Figs. 19, 20) in which germ nets formed, remained intact for some time; it is not known what caused their subsequent dissolution allowing the escape of young nets (Figs. 26, 29). Flat germ nets seldom remained completely planar. Cells of the net usually grow at slightly differing rates, often the outer ones developing more quickly than those near the centre of the colony. Hence, flat germ nets often became saucer shaped and eventually a tangled mass of coenocytes, which when mature, generally reproduced asexually by forming cylindrical nets. Rarely, mature daughter and germ nets disaggregated as do nets of H. africanum (Pocock, 1960). What causes this separation of the coenocytes is unknown.

DISCUSSION

The form of the germ net of H. reticulatum has been a point of contention for over a hundred years. The argument is discussed by Pocock (1960: p. 309 et seq.). According to Pringsheim (1861), and more recently Pocock, the normal form of the germ net is a hollow sac, and that flat germ nets are atypical. Mainx (1931) however maintained that the germ nets are usually flat, which certainly has been the case in our experience. Pocock (1960: p. 251) found that the maximum proportion of flat nets in her South African strain of H. reticulatum was 25%, and only slightly higher in the Cambridge strain, compared with our values of 81% and 65% for the frequency of flat nets in the Australian and Cambridge strains respectively. In the other species, H. africanum and H. patenaeforme, flat germ nets are typical (Pocock, 1937; 1960). We observed that irregular germ nets of H. reticulatum or those in the form of a hollow sac most often resulted from the vesicle, in which the zooids swarm, not enlarging sufficiently to accomodate the zooids arranged in a single plane. Pocock (1960: p. 310) found a higher proportion of flat nets in uncrowded, healthy cultures of H. patenaeforme than in cultures which were contaminated or otherwise unhealthy; we found the same in our cultures of H. reticulatum.

Pocock (1937) proposed H. patenaeforme as a separate species because asexual reproduction in it is unknown and because it has flat germ nets, unlike H. reticulatum which can reproduce asexually forming cylindrical daughter nets. However, in our cultures, flat germ nets of H. reticulatum are common and there is no doubt that our material

is H. reticulatum as the coenocytes comprising these flat nets usually go on to reproduce asexually, forming cylindrical daughter nets. Assuming that germ nets of H. reticulatum are normally flat under optimal conditions like those of H. patenaeforme (even though germ nets of the latter reportedly contain more cells), then the only distinction between the two species is that asexual reproduction has never been observed in H. patenaeforme. Prolonged observations of both Australian and Cambridge strains of H. reticulatum, cultured under identical conditions, have shown that the Cambridge strain reproduces asexually much less frequently than the Australian strain. In the light of this variability between strains of the same species, it seems likely that H. patenaeforme could be, as Pocock cautioned, a variety of H. reticulatum for which asexual reproduction is unknown.

How do zooids of H. reticulatum aggregate to form either cylindrical daughter or flat germ nets? While numerous authors have described these processes, particularly daughter net formation and the apparently similar process of colony formation in Pediastrum, only a very few have proposed mechanisms to explain these phenomena. Neither Pocock (1960), Hawkins and Leedale (1971) or ourselves (Marchant and Pickett-Heaps, 1971, 1972a) have found any evidence to support the notion that zooids of H. reticulatum are permanently connected to the vacuolar envelope (Moewus, 1948) or to one another by cytoplasmic strands, the contraction of which pulls the zooids together (Klebs, 1891). Both Hawkins and Leedale (1971) and our own observations also refute Davis' (1964) hypothesis of colony formation in Pediastrum (see Chapter 10).

Observations that colonies of Pediastrum (Hawkins and Leedale, 1971) and germ nets of H. reticulatum develop in a spheroidal vesicle suggest that net-forming zooids of H. reticulatum and colony-forming zooids of Pediastrum may possess an innate capacity to aggregate in a planar configuration. That fragments of net may develop free in the culture medium if the parental wall or vesicle is damaged (Pocock, 1960; Marchant and Pickett-Heaps, 1972a) further strengthens this conclusion.

Unlike Hawkins and Leedale (1971) who propose that "cells of both Pediastrum and Hydrodictyon are potentially capable of sticking together over all their surfaces," we suspect that there are specific sites on the plasmalemma of zooids that can adhere to other zooids. Hawkins and Leedale also consider that changes in shape of the zoospores determine the pattern of aggregation. Such changes in shape however do not explain why, if the zoospores can adhere anywhere over their surface, the resulting colonies are planar and not chains, stacks or clumped together in irregular patterns. These authors correlate secretion of the outer "membrane-like" layer of cell wall with the irreversible glueing together of zoospores. However, since wall formation follows retraction of flagella, what then holds the cells together during flagellar retraction when there is no wall around them? We believe that the zooids are linked initially (and probably not very firmly) by an amorphous material located only between flattened sites of the plasmalemma underlain by localized bands of microtubules. Subsequent secretion of the outer layer of wall provides more permanent glueing of the cells together. Zooids not adhering initially may link with others later, at the time of wall secretion, forming abnormal junctions.

Peripheral cells in colonies of Pediastrum usually bear conspicuous horns. They may also develop on other cells of the colony if they fail to make contact with cells on their outer, "chloroplast side." Development of horns on inner cells of a colony can occur naturally or if a forming colony is mechanically disrupted (see Chapter 11). It appears that all colony-forming zooids of P. boryanum have an innate tendency to produce horns, but their production is usually suppressed, in all but the outer cells, by contact with other cells in the developing colony (Chapter 10). Horns also develop on some cells in germ nets of H. reticulatum. We agree with Pocock's (1960: p. 306) conclusion that these horns probably represent unsatisfied contact sites as the tips of these horns clearly resemble "half" an intercellular junction shortly after these cells have secreted wall material. The other proposed contact sites on the "nuclear side" of zooids do not produce horns.

Deployment of these proposed receptive sites on the plasmalemma of zooids and their relationship to bands of peripheral microtubules will be discussed in a later paper where a mechanism to explain development of planar colonies of both Pediastrum and Hydrodictyon is suggested.

ABBREVIATIONS USED IN FIGURES

ch - chloroplast

f - flagellum

g - polyhedral ghost

iw - inner layer of wall

m - mitochondrion

n - nucleus

o - outer layer of wall

p - pyrenoid

t - microtubules

v - vacuole

w - cell wall

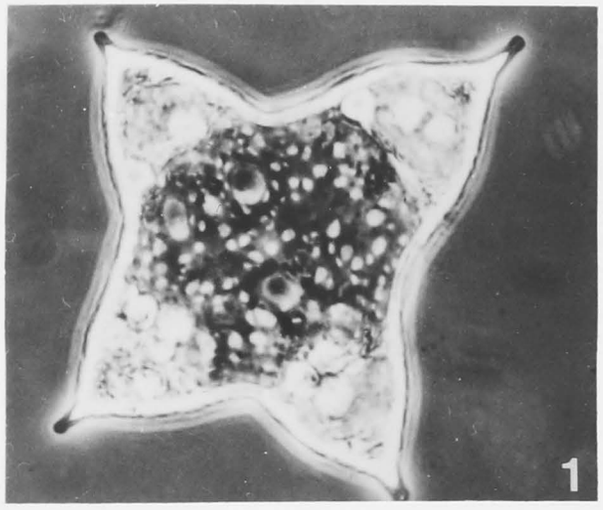
z - zooids

Fig. 1 Polyhedron of H. reticulatum. The darker area in its centre represents the position of vacuole. Phase contrast. X 330.

Fig. 2 Cleavage of polyhedral cytoplasm into uninucleate fragments. An adjacent section to the enclosed area is shown at higher magnification in Fig. 4. X 4,400.

Fig. 3 Microtubules flanking a cleavage of the polyhedral cytoplasm. X 54,000.

Fig. 4 Elongating flagellum on a developing net-forming zooid within a polyhedron. X 29,000.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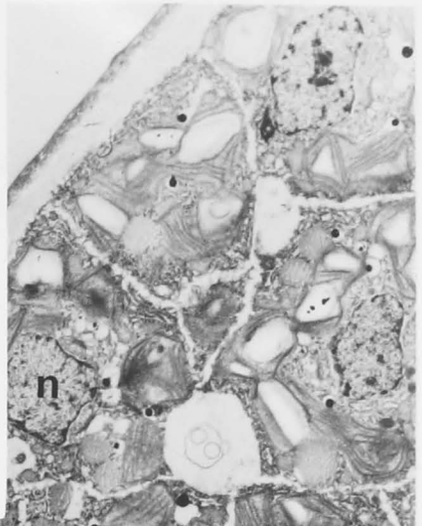
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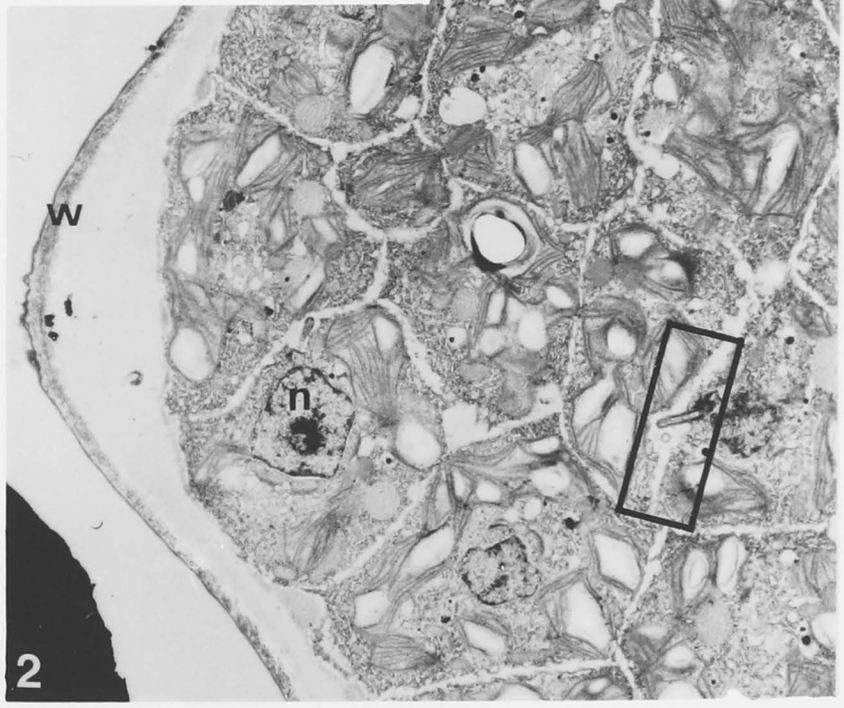
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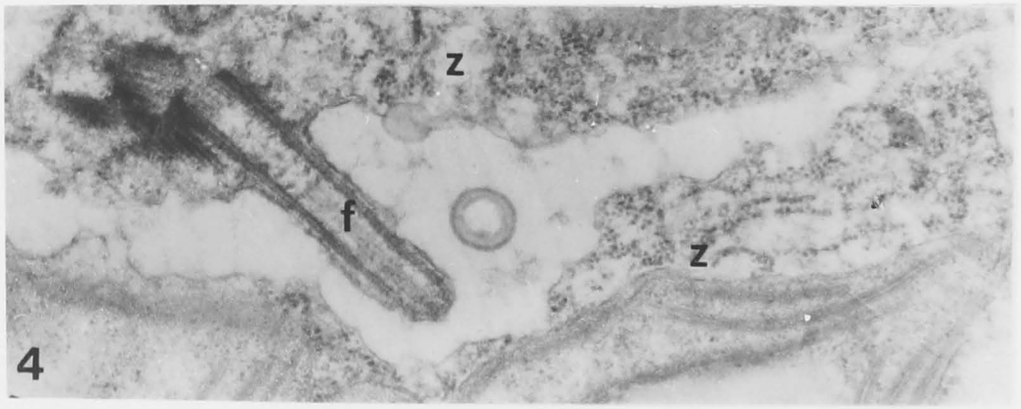
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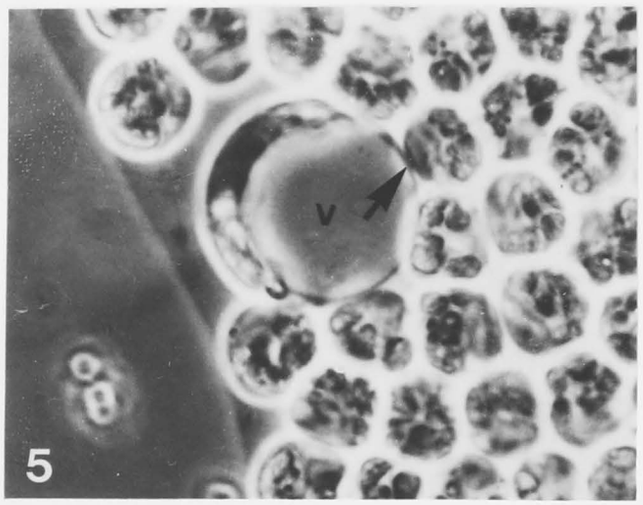
Fig. 5 Vacuole enclosed by a vacuolar envelope (arrow) surrounded by net-forming zooids. The vacuole is only a fraction of the size of that shown in Fig. 1. Phase contrast. X 1,100.

Fig. 6 Transverse section of basal body in a net-forming zooid (cf. Fig. 16). Note the tangentially sectioned rootlet microtubules and the amorphous material surrounding the basal body. X 100,000.

Fig. 7 Envelope (arrow) containing net-forming zooids within the parental polyhedral wall (w). X 27,000.

Fig. 8 Separation of inner and outer layers of the polyhedral wall. Note that the outer layer of wall has ruptured. The inner layer has been severely extracted during processing. X 22,000.

Fig. 9 The inner layer of polyhedral wall which envelopes the net-forming zooids has separated and burst through the outer layer. X 16,000.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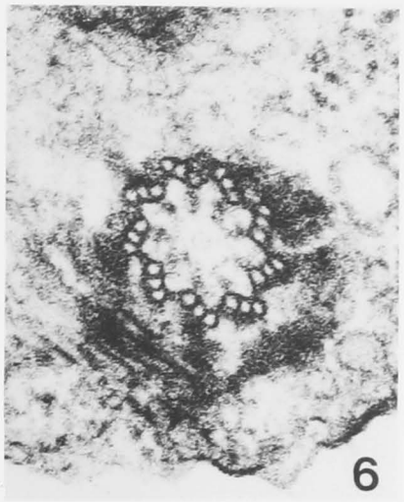
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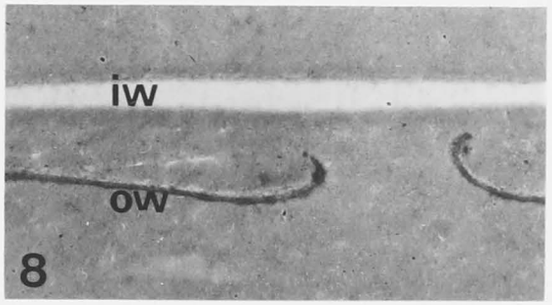
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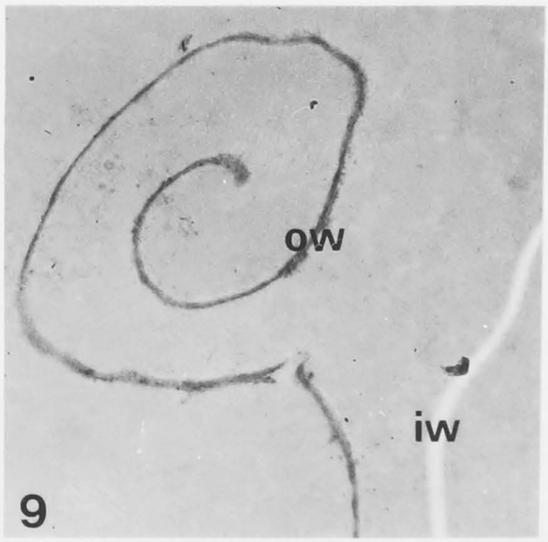
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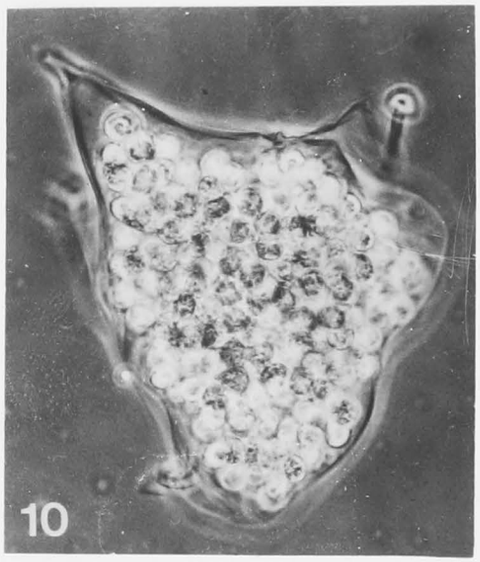
Fig. 10 Mass of net-forming zooids swarming into the vesicle released through the outer layer of polyhedral wall. Phase contrast. X 410.

Fig. 11 Zooids swarming within expanded vesicle (arrow) attached to the remnant polyhedral wall (polyhedral ghost). Phase contrast. X 200.

Fig. 12 Band of peripheral microtubules in net-forming zooid. X 42,000.

Fig. 13 Zooids in apposition; note the microtubules in each zooid and their apparent lining up. X 32,000.



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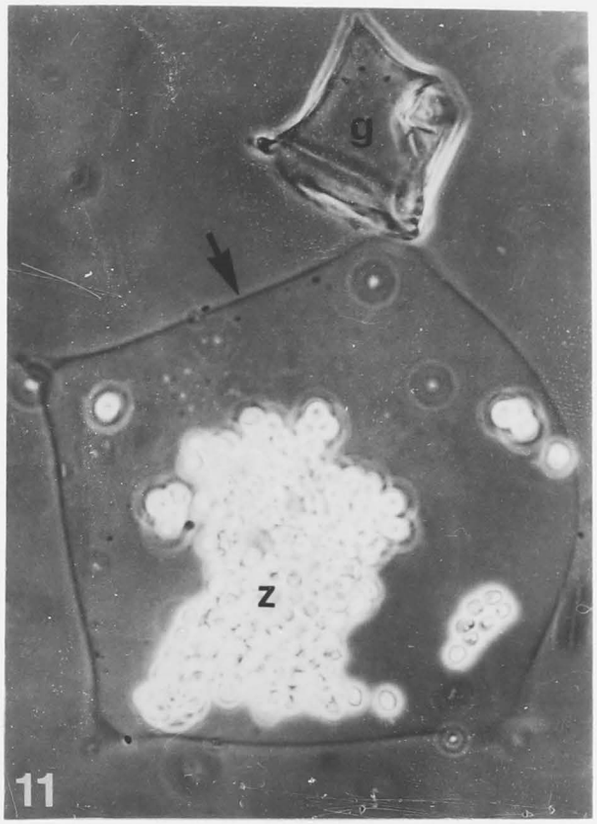

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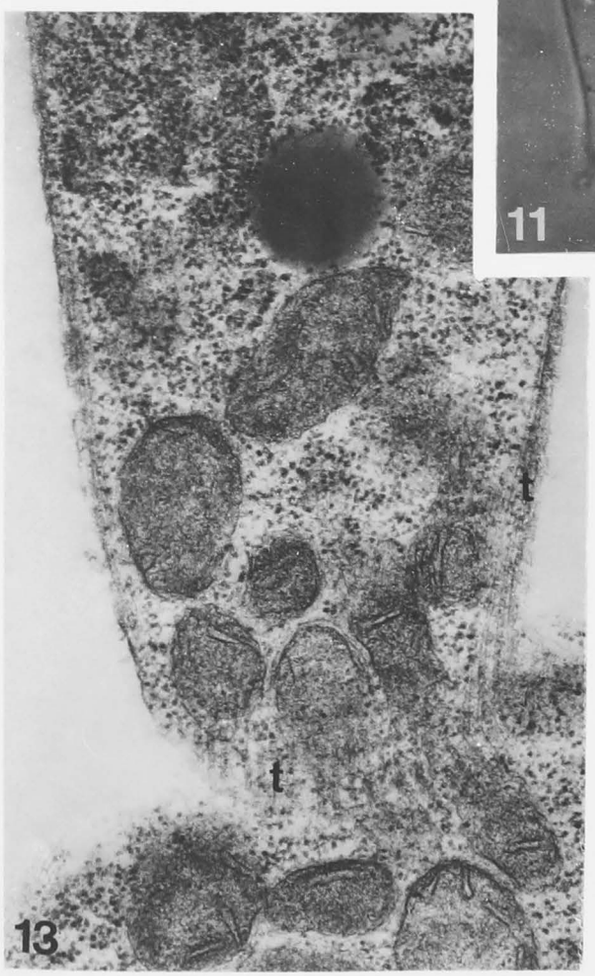

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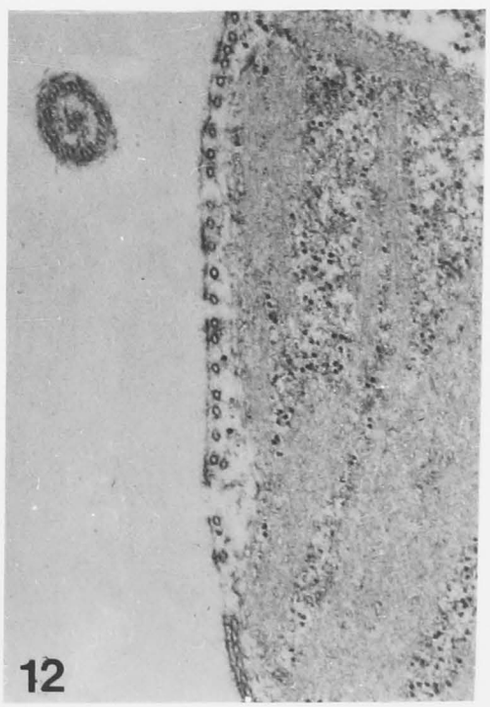

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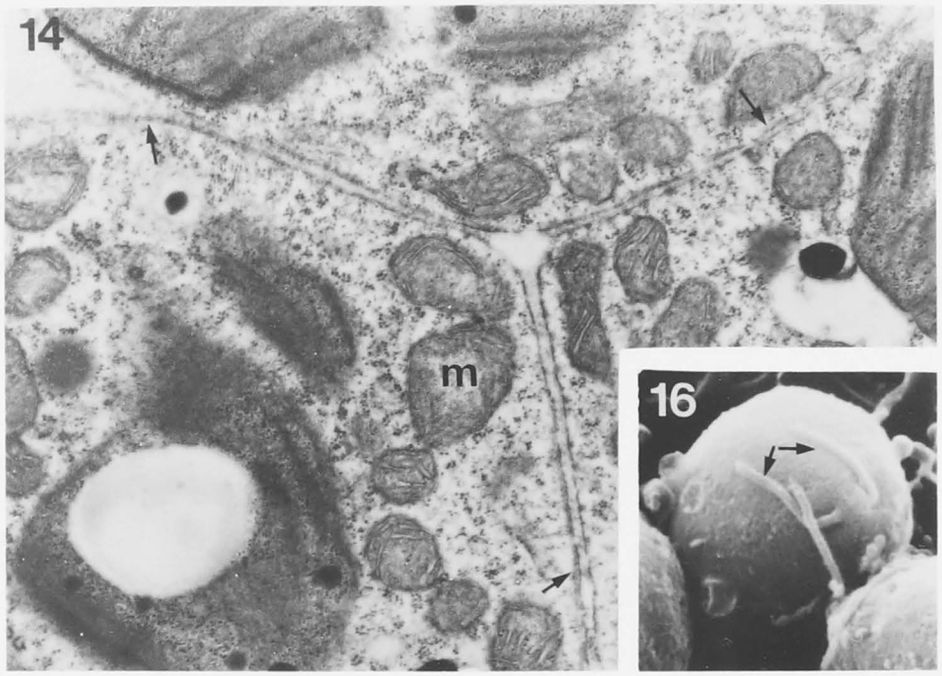
Fig. 14 Section cut in the plane of a developing net through three adhering zooids; note the longitudinally sectioned microtubules (arrows) underlying the flattened sites of contact and the amorphous material apparently only between these sites. X 17,000

Fig. 15 Contact between zooids, note the transversely sectioned microtubules and amorphous intercellular material. X 29,000.

Fig. 16 Scanning electron micrograph of a net-forming zooid retracting its flagella (arrows). Note the shortness of the flagella and that they are lying against the membrane of the zooids. X 6,400.

Fig. 17 Basal body of a net-forming zooid apparently disintegrating following flagellar retraction (cf. Fig. 6). X 59,000.

Fig. 18 Centriole (arrow) forming <u>de novo</u> at the nuclear envelope. X 46,000.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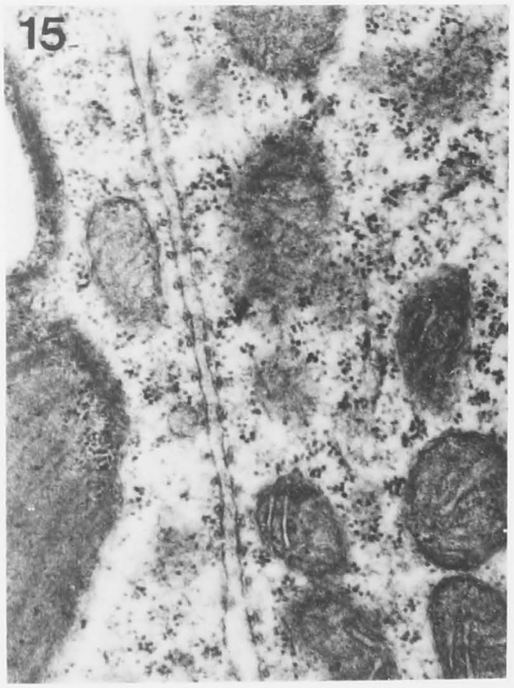
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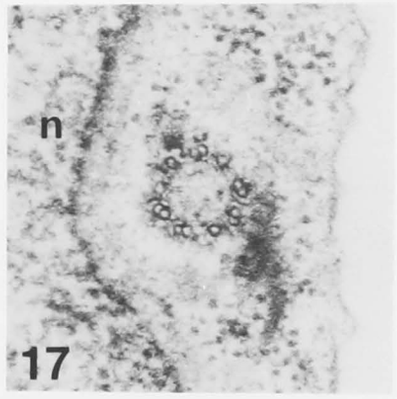
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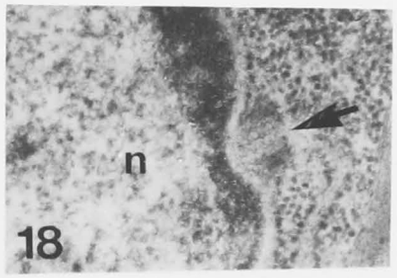
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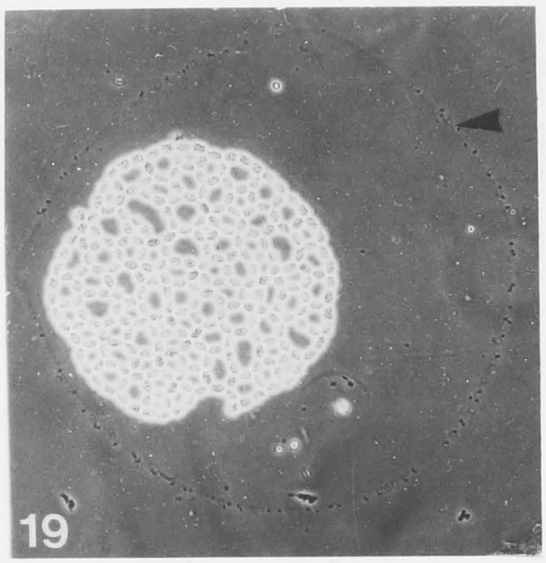
Fig. 19 Newly formed flat germ net contained by parental vesicle (arrowhead). Phase contrast. X 420.

Fig. 20 Part of a newly formed flat net still enclosed by parental vesicle (arrowhead) to which contaminating bacteria are attached. Note the projections on some of the outer cells (arrows). Nomarski optics. X 1,250.

Fig. 21 Scanning micrograph of part of the periphery of a flat germ net. Note the projections on the outer cells (arrowhead). X 2,000.

Fig. 22 Recently aggregated cells of a flat germ net fixed shortly after commencement of wall secretion. Note that each cell has linked with two others at each end. Also note the symmetry of each cell and the distinction between "chloroplast" and "nuclear" side. A developing centriole in one of the cells is indicated. X 5,000.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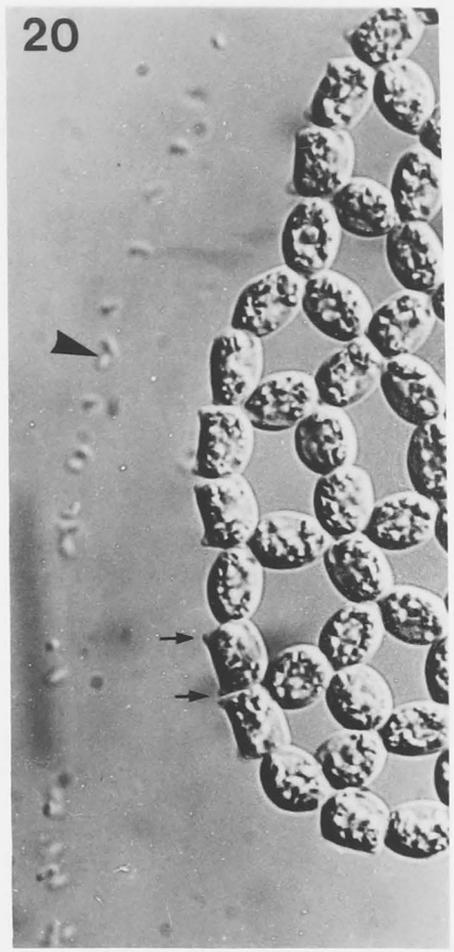
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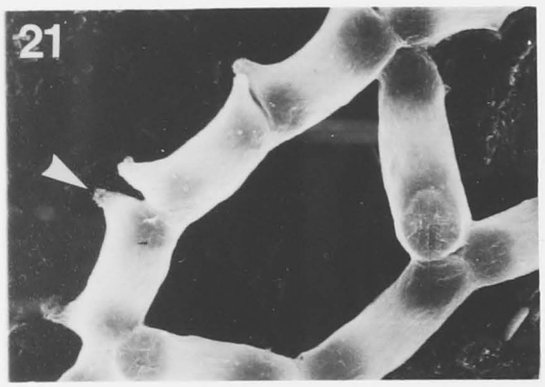
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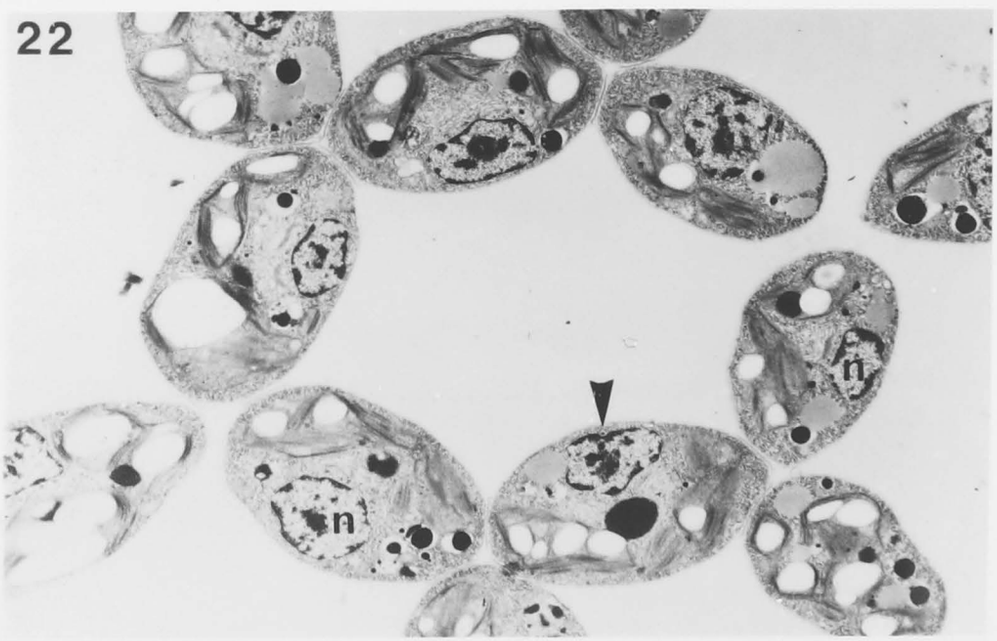

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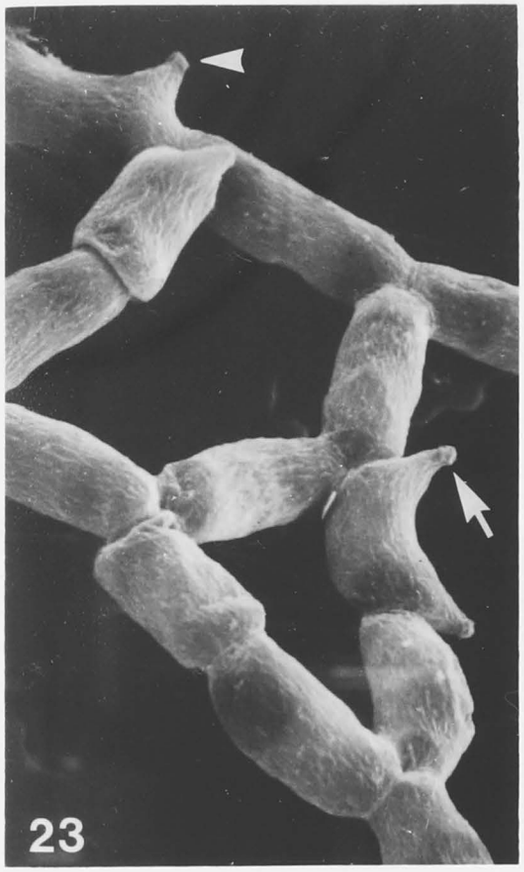
Fig. 23 Scanning electron micrograph of part of an irregular germ net showing projections both on a peripheral cell (arrowhead) and on internal cells (arrow). X 2,500.

Fig. 24 Junction between cells of a flat germ net sectioned in the plane of the net. Note the appearance of the intercellular junctions. X 34,000.

Fig. 25 Projection on the outer cell of a flat germ net sectioned in the plane of the net. Note the similarity in appearance of the tip of the projection with "half" an intercellular junction shown in Fig. 24. X 36,000.



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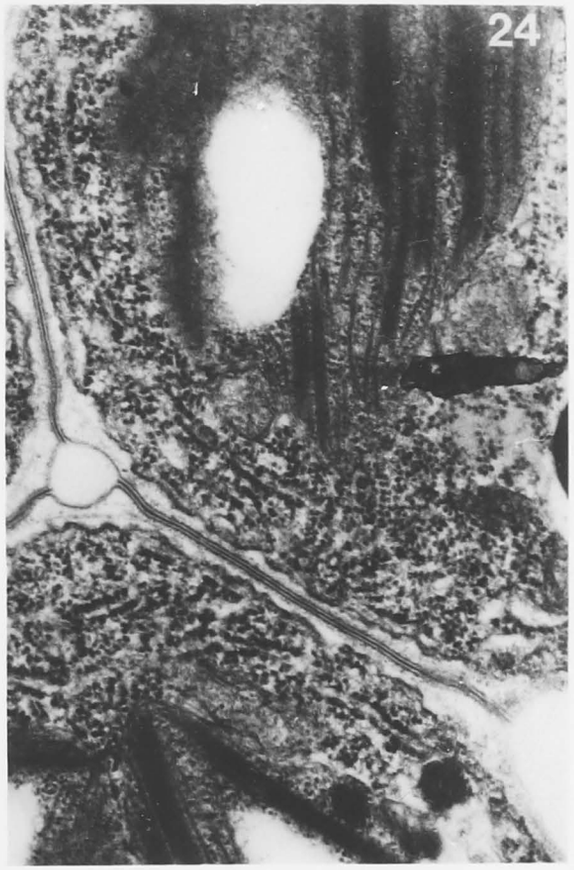

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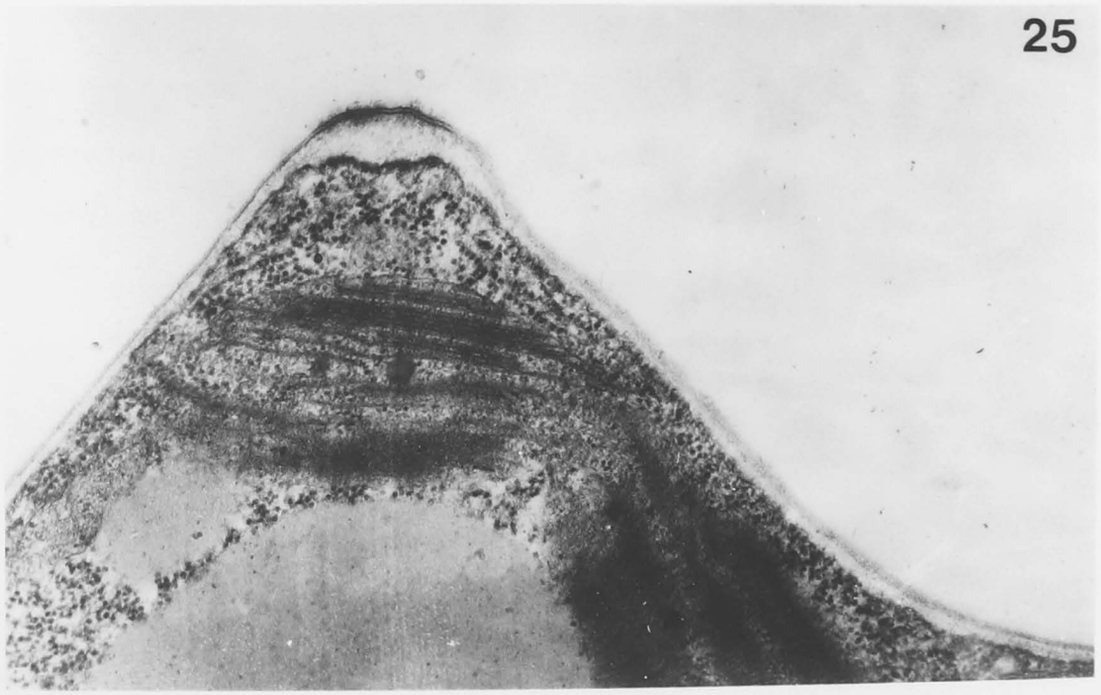

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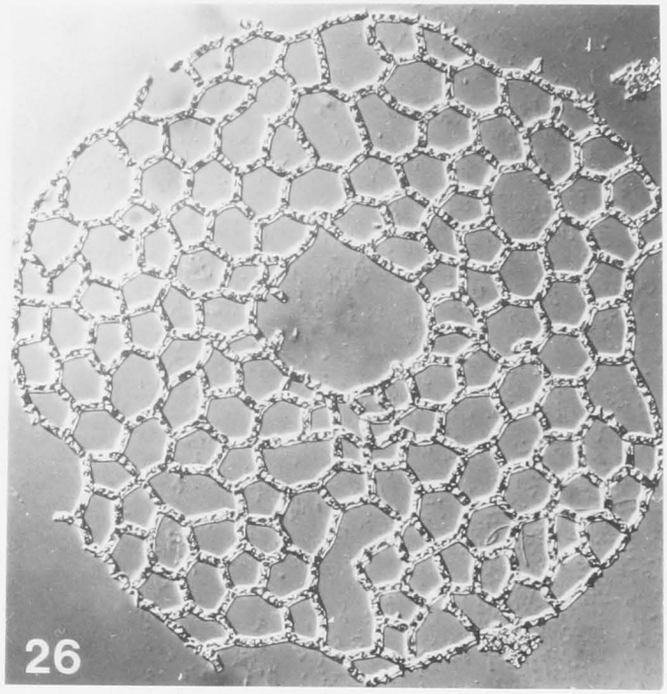
Fig. 26 Flat germ net free from the vesicle in which it formed. While most meshes of the net are hexagonal, there are many of other shapes. Note also the hole near the centre, a relatively common feature of flat germ nets. Nomarski optics. X 400.

Fig. 27 Part of a flat net; the cells are of the same age as those in Fig. 25 (cf. Figs. 20, 22 and 28). Nomarski optics. X 870.

Fig. 28 Elongating cells of a recently formed flat net. Note the position of the chloroplast containing the pyrenoid and the vacuoles. The nucleus is out of the plane of the section (cf. Fig. 22). X 13,000.



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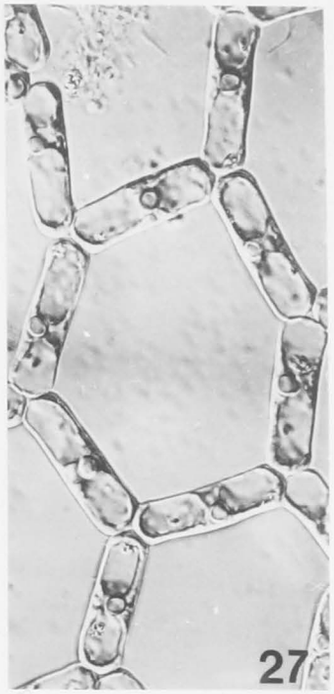

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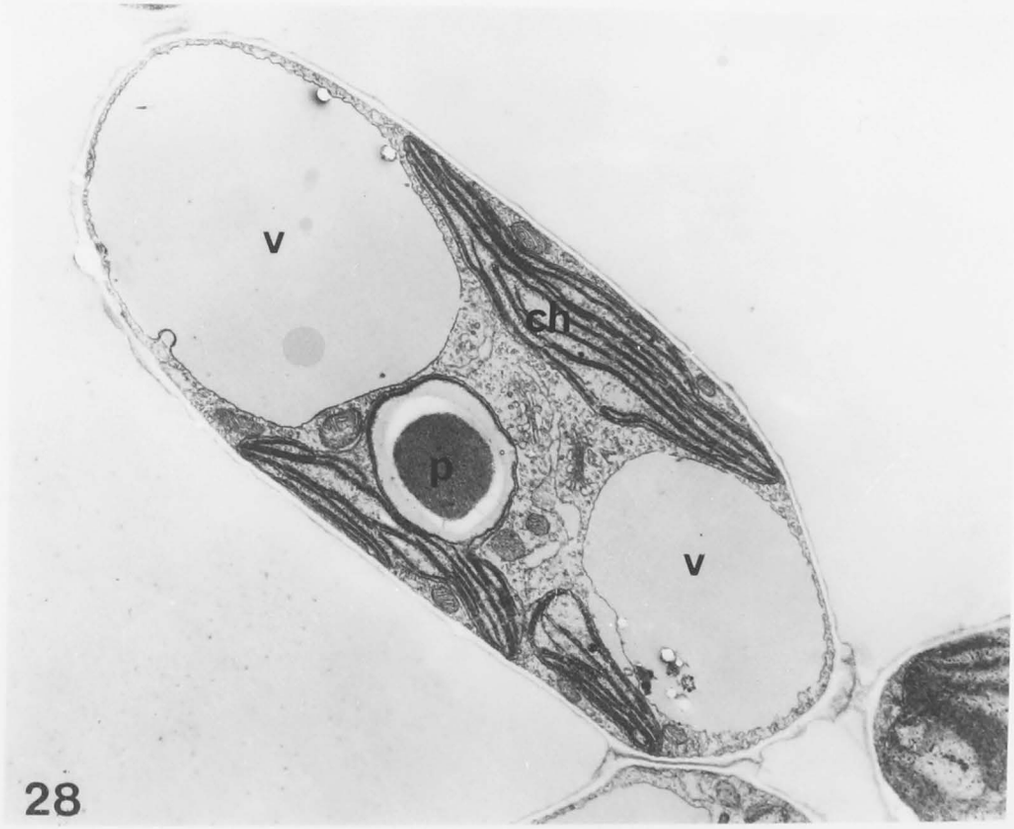

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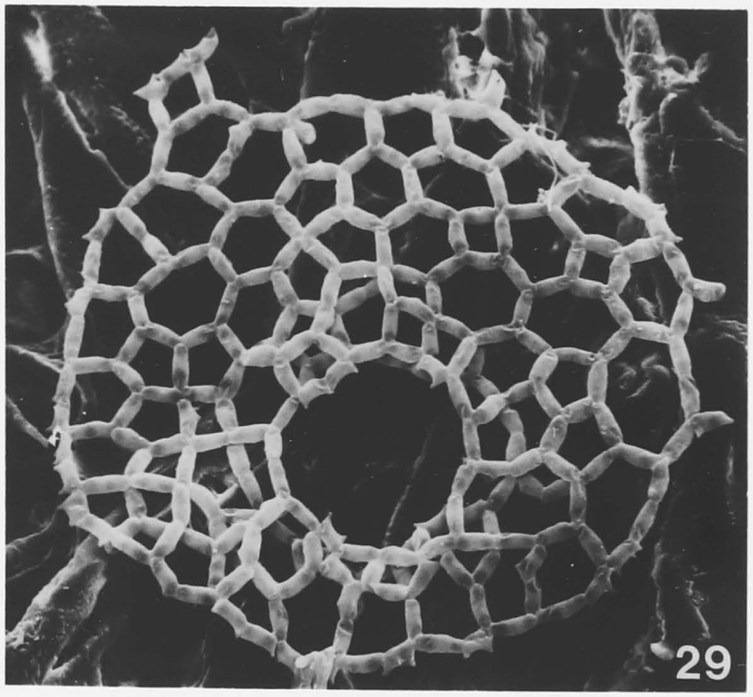
Fig. 29 Scanning electron micrograph of an irregular germ net. Note the double layer of cells around the central hole and horns on some peripheral and internal cells. X 490.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CHAPTER 9

MITOSIS AND AUTOSPORE FORMATION IN *CHLORELLA PYRENOIDOSA*

INTRODUCTION

Chlorella, as well as being widely used in biochemical studies, has received considerable attention from electron microscopists investigating cell wall synthesis (e.g. Staehelin, 1966; Bisalputra, Ashton and Weier, 1966; Sassen, van Eyden-Emons, Lamers and Wanka, 1970) and its life cycle (Murakami, Monimura and Takamiya, 1963; Soeder, 1964, 1965; Wanka and Mulders, 1967). Most early workers fixed this unicellular green alga with potassium permanganate ($KMnO_4$), which, while preserving membranes, destroys many other cytoplasmic components. Although the majority of these investigators were working with synchronized cultures and studying that part of the life cycle when nuclei divide and autospores develop, none reported finding mitotic nuclei, which should be recognizable by their shape, even in $KMnO_4$-fixed material. Wanka and Mulders (1967) described nuclear division as being amitotic and Wanka (1968) concluded that "nuclei duplicate by elongation and subsequent separation into two without disruption of the nuclear envelope and formation of a spindle apparatus and phragmoplast." This seems a risky deduction based on very inadequate evidence in a paper on the effect of colchicine on cell division in Chlorella. My disbelief of this conclusion, and my wish to investigate mitosis in a "simple" member of the Chlorococcales for comparison with Hydrodictyon, and other "higher" Chlorococcales, led to my study of Chlorella. My interest was further stimulated by a recent report (Atkinson, Gunning, John and McCullough, 1971) that centrioles were present during all stages of the life cycle following glutaraldehyde fixation of Chlorella (strain 211-8p) while I, working with strain

211-8b, was unable to find these organelles. The significance of this difference will be discussed.

MATERIALS AND METHODS

These are given in Chapter 2.

OBSERVATIONS

An interphase cell of C. pyrenoidosa is shown in Fig. 1. The single nucleus lay near the centre of the spheroidal cell and was flanked by a golgi body and a cup-shaped chloroplast. Within the chloroplast, the pyrenoid was traversed by a single thylakoid and surrounded by starch (Figs. 1, 2, 5). Under the conditions of growth I used, this alga reproduced asexually by forming four or sometimes eight autospores. Formation of four autospores from a single parental cell is described here; for convenience, this process can be divided into five phases:

1. Primary mitosis
2. Primary cleavage
3. Secondary mitosis
4. Secondary cleavage
5. Wall formation.

Reduction in size and subsequent division of the pyrenoid, loss of much of its surrounding starch and division of the chloroplast into two (Figs. 2, 3, 4) usually preceded primary mitosis. Following constriction (Fig. 4) and cleavage of the chloroplast, an extension of the nucleus nearly touched the plasmalemma on the side of the cell opposite to the cleavage in the chloroplast (Fig. 2). Microtubules appeared between the plasmalemma and this extension of the nucleus (Fig. 2). No perinuclear envelope or centrioles were evident during primary mitosis and the nuclear envelope remained largely intact except for conspicuous polar fenestrae (Fig. 3). Development of the primary cleavage shortly

followed the primary mitotic division. Whether this cleavage arose by an infurrowing of the plasmalemma or by the accumulation and fusion of vesicles or a combination of both is unknown. Some micrographs suggest that cleavages developed along arrays of phycoplast microtubules (Fig. 3). Prominent golgi bodies flanked the primary cleavage in which granular material appeared (Fig. 5). Synchronous secondary mitoses and cleavages followed shortly after primary cleavage; division of the chloroplast, as before, preceded the secondary mitoses. Metaphase plates of the secondary mitoses were at right angles to that of the primary division; again no centrioles or perinuclear envelopes were evident (Fig. 6).

Deposition of wall by the four daughter cells (Figs. 7, 8) preceded rupture of the parental wall and release of daughter autospores (Fig. 9).

DISCUSSION

Chlorella can be grown in culture in which at least 90% of the cells are synchronized. The time course of this alga's development has been established by direct light and electron microscopy (Murakami, Monimura and Takamiya, 1963). Colchicine sensitive stages of its life cycle have also been determined (Wanka, 1965). These authors have shown that division occurs between 14½ hours and 18½ hours after the start of the light cycle of a 15 hour light-9 hour dark day. The time taken for each division of both nucleus and cytoplasm is about 35 minutes, synthesis of all the DNA required for all divisions apparently being completed before the first mitosis (e.g. nuclei of cells that will produce 16 autospores contain 16 times the amount of DNA found in young daughter autospores just before the first mitotic division--Wanka, 1965; Wanka and Mulders, 1967). It would be interesting to know whether such an accumulation of DNA (unknown, to my knowledge, in higher organisms) is common among other organisms that reproduce by rapid successive mitoses and cell cleavages [e.g. some other members of the Chlorococcales such as Chlorococcum (Bold, 1930) and Kirchneriella (Pickett-Heaps, 1970)]. Other members of this algal group (e.g. Hydrodictyon: Marchant and Pickett-Heaps, 1970; Tetraedron: Pickett-Heaps, 1972c and Pediastrum: see Chapter 10) become multinucleate before cytoplasmic cleavage. Wanka (1965) did not investigate whether the amount of DNA produced is reduced accordingly if 4 or 8 autospores are formed from a single parental cell. Presumably it would, to prevent the cells from remaining polyploid.

Wanka and Mulders (1967) described cells of Chlorella 211-8b, that

they had fixed with permanganate, as dividing amitotically. My observation on the same strain of this alga, fixed with glutaraldehyde, revealed mitotic spindles. Either we were investigating different organisms, both with the same designation, or they interpreted their results without considering that these observations were made on cells fixed in a way known to destroy spindles. These authors also describe "a cytoplasmic membrane surrounding the nucleus almost completely...to be typical of nuclear division." A similar structure, the perinuclear envelope, surrounds mitotic nuclei of Hydrodictyon and numerous other members of the Chlorococcales (see Chapter 10). My not finding a perinuclear envelope in apparently the same strain that they examined makes me suspect that the strains we were investigating were in fact different.

The mechanism of cytoplasmic cleavage in C. pyrenoidosa is unclear. Wanka (1968) and Bisalputra, Ashton and Weier (1966) found small vesicles associated with developing cleavages in Chlorella; so did Sassen, van Eyden-Emons, Lamers and Wanka (1970) in freeze-etch preparations of C. pyrenoidosa. They concluded that fusion of these vesicles produces the cleavage furrow. However, I find their evidence unconvincing, since nowhere do any of these workers illustrate the small vesicles actually being incorporated into a developing cleavage. Soeder (1965), on the other hand, considers that the cleavages form by infurrowing of the plasmalemma. My observations give some support to Soeder's conclusion although the possibility of fusion of vesicles forming the developing cleavage cannot be eliminated. I suspect that both mechanisms may be acting both in this alga and among other members of the Chlorococcales (Pickett-Heaps, 1972a). In Hydrodictyon, radial cleavage following

formation of the vacuolar envelope may involve in part centripetal growth of the plasmalemma; however, the vacuolar envelope could not develop in this way; in its formation, fusion of vesicles or ramification of a self generating furrow would have to be utilized (Marchant and Pickett-Heaps, 1971). Formation of a vacuolar envelope is of particular interest, as it provides an example of a cytokinetic apparatus being modified during evolution so that it is used, not for internuclear cleavage and formation of uninucleate cells, but for another process not directly involved with internuclear cleavage. Spheroplea is an alga quite unrelated to Hydrodictyon, in which multinucleate cytoplasm also cleaves to form uninucleate gametes (Fritsch, 1935). It also utilizes a vacuolar envelope to isolate the tonoplast from subsequent internuclear cleavages (Ø. Moestrup, personal communication).

Members of the Chlorococcales reproduce either by flagellated zoospores (zooids) or by non-flagellate aplanospores or autospores. Those that regularly produce flagellated cells have persistent centrioles (e.g. Hydrodictyon and Pediastrum). Scenedesmus appears to have persistent centrioles (Pickett-Heaps, unpublished data) but only produces flagellate cells under exceptional conditions (Trainor, 1963); this also is the case for Tetraedron (Starr, 1954; Davis, 1966; Pickett-Heaps, 1972c). Persistent centrioles have not been found in Chlorococcum (Marchant, unpublished data), which, like Scenedesmus, only produce flagellate cells under some conditions (Bold, 1930). Rudimentary or degenerate centrioles are present at the poles of mitotic spindles but probably do not persist through interphase in Kirchneriella (Pickett-Heaps, 1970), an alga thought to lack a motile stage. I was naturally

intrigued to see Atkinson, Gunning, John and McCullough's (1971) report of persistent centrioles in Chlorella, while I was unable to find them in a different strain of this non-motile unicell. Chlorella to my knowledge is the only organism in which ~~two~~ strains have been found with and without centrioles.

What are the possible functions of centrioles in non-motile algal forms? First, just as Scenedesmus has been found to produce motile cells under unusual conditions, so perhaps "non-motile" genera which contain centrioles may form flagellated cells under some conditions which have not yet been discovered. I prefer the view that these centrioles are now non-functional relics indicating that, sometime in the past, these algae had a motile stage in their life cycle. Atkinson et al. suggest that a way to investigate the role of centrioles in Chlorella would be to block their duplication with mercaptoethanol. Pickett-Heaps (1969, 1971) has argued that centrioles are a "highly conspicuous, structured form of a microtubule organizing center (MTOC) and the role of centrioles is to act as a template for flagellar development." If Pickett-Heaps' view is correct, the suggestion of using mercaptoethanol to investigate the function of centrioles is probably not likely to succeed, because as well as disrupting duplication of centrioles per se, mercaptoethanol would most likely prevent duplication and function of the MTOC.

Atkinson, Gunning, John and McCullough (1971) suggest that in Chlorella, centrioles act as MTOCs. Since the strain of Chlorella I used lacked centrioles, MTOCs in Chlorella obviously do not have to be

structured as centrioles. The reason that the polar MTOCs contain centrioles in Atkinson et al.'s material is unknown, but it cannot be related to the structure and function of the mitotic apparatus. Hopefully, our intended collaboration with this group will reveal the function (if any) of centrioles in these non-motile algae.

ABBREVIATIONS USED IN FIGURES

g - golgi body

m - mitochondrion

n - nucleus

p - pyrenoid

pf - polar fenestra

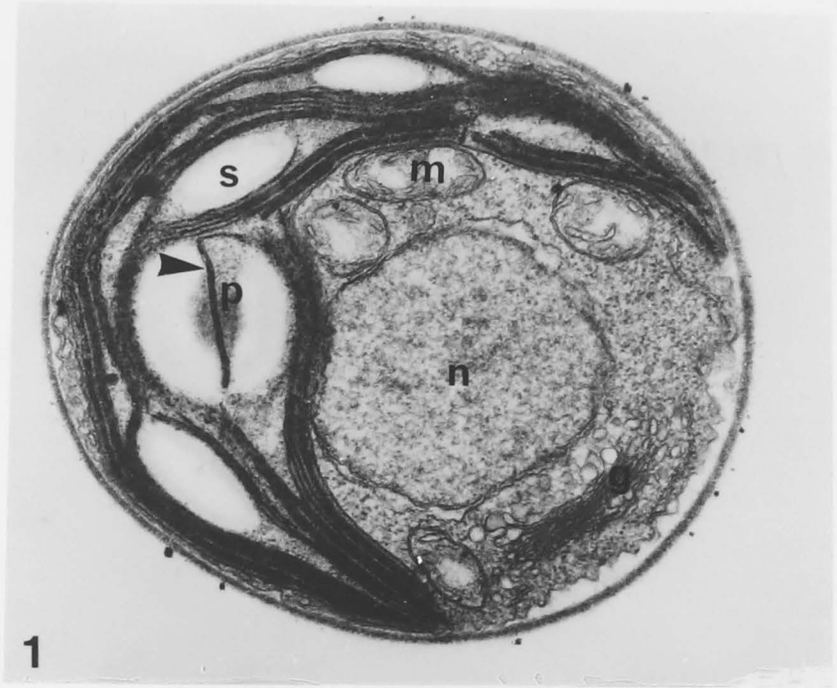
s - starch

v - vacuole

w - cell wall

Fig. 1 Interphase cell of Chlorella showing the position of the nucleus, golgi body and chloroplast. The pyrenoid is surrounded by starch and traversed by a single thylakoid (arrowhead). X 34,000.

Fig. 2 Cleavage of the chloroplast and extension of the nucleus before primary mitosis. Note the microtubules near the extended nucleus (large arrow) and presumed microbodies (small arrows). The pyrenoid had divided and the golgi vesicles appear hypertrophied. X 37,000.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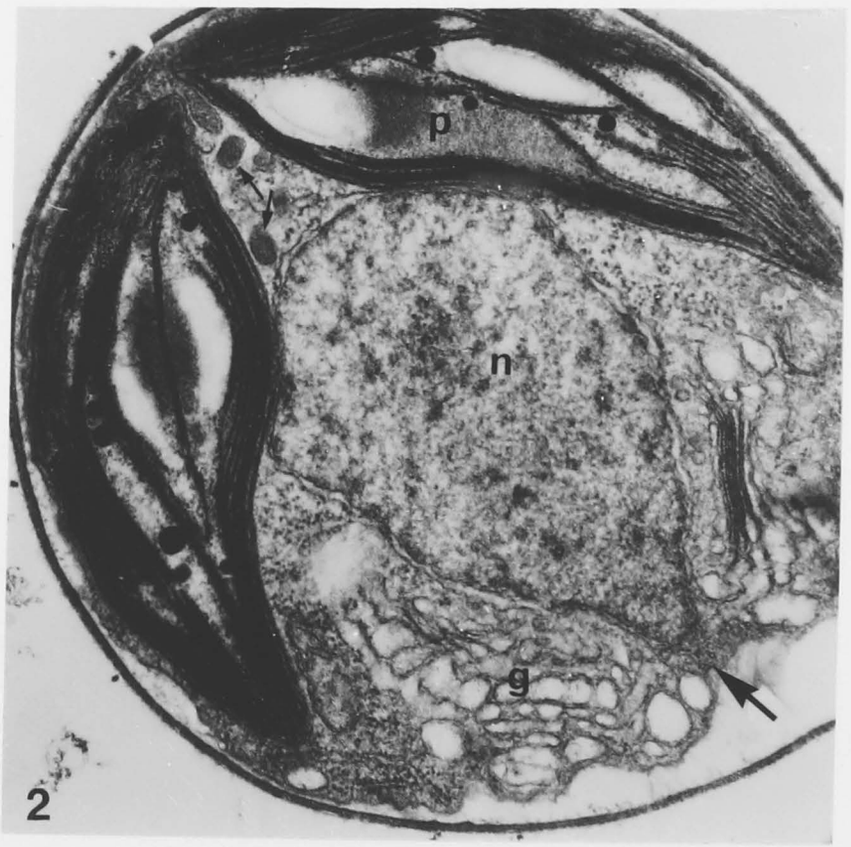
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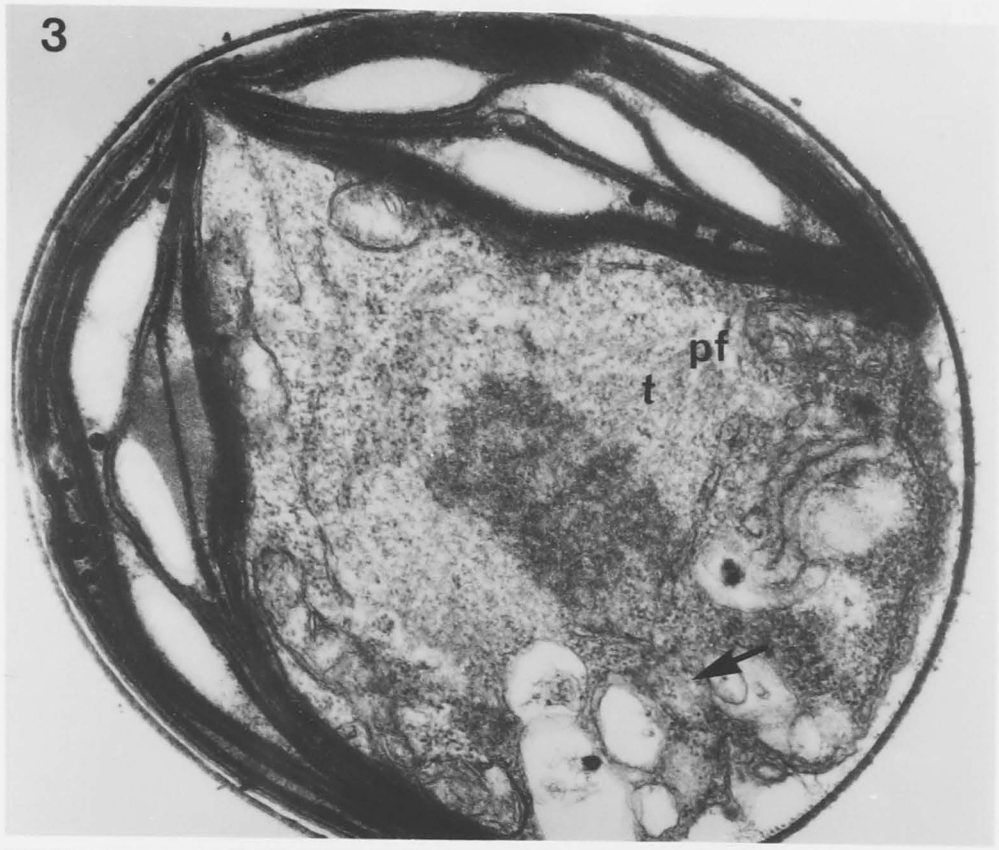
Fig. 3 Metaphase of primary mitosis. No centrioles are evident in the polar fenestrae. Microtubules (arrow) "predict" the path of the primary cleavage. X 37,000.

Fig. 4 Constriction of the chloroplast before its cleavage. X 37,000.

Fig. 5 Primary cleavage of the cytoplasm nearly complete. X 27,000.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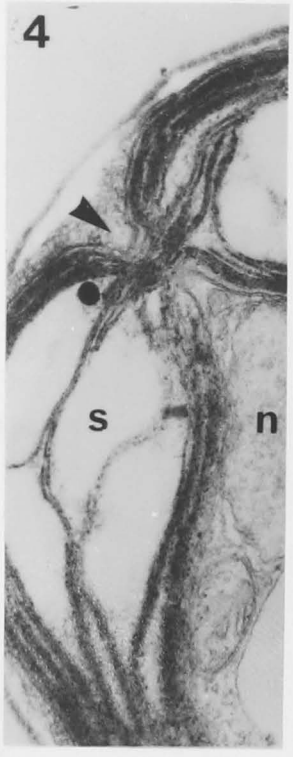
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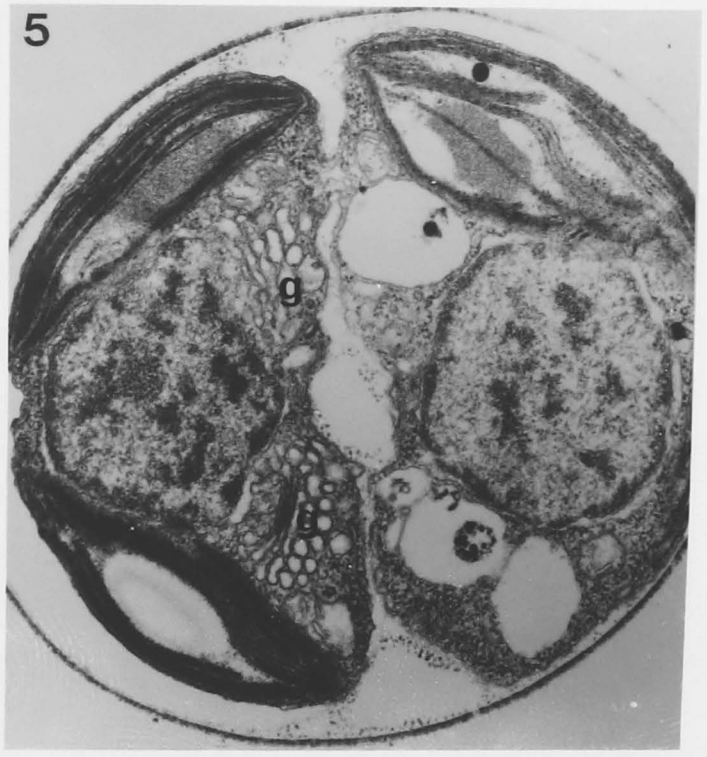
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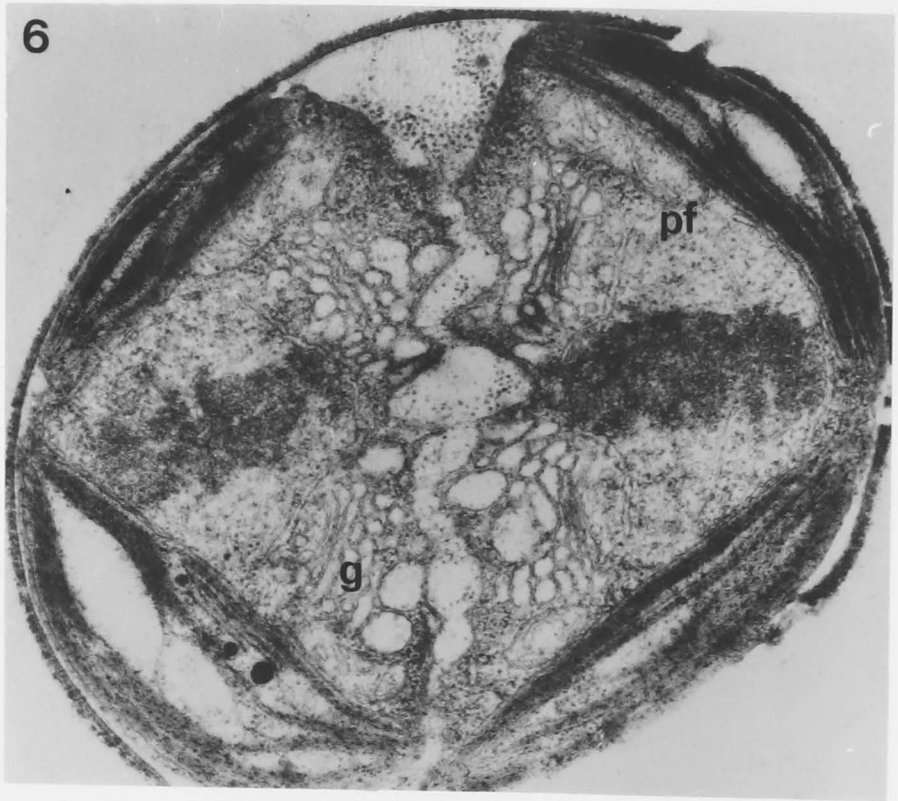
Fig. 6 Metaphase of the second mitosis. Note the numerous large golgi vesicles and the granular material in the primary cleavage. X 36,000.

Fig. 7 Completion of the secondary cleavage. Note the material surrounding the new autospores. X 32,000.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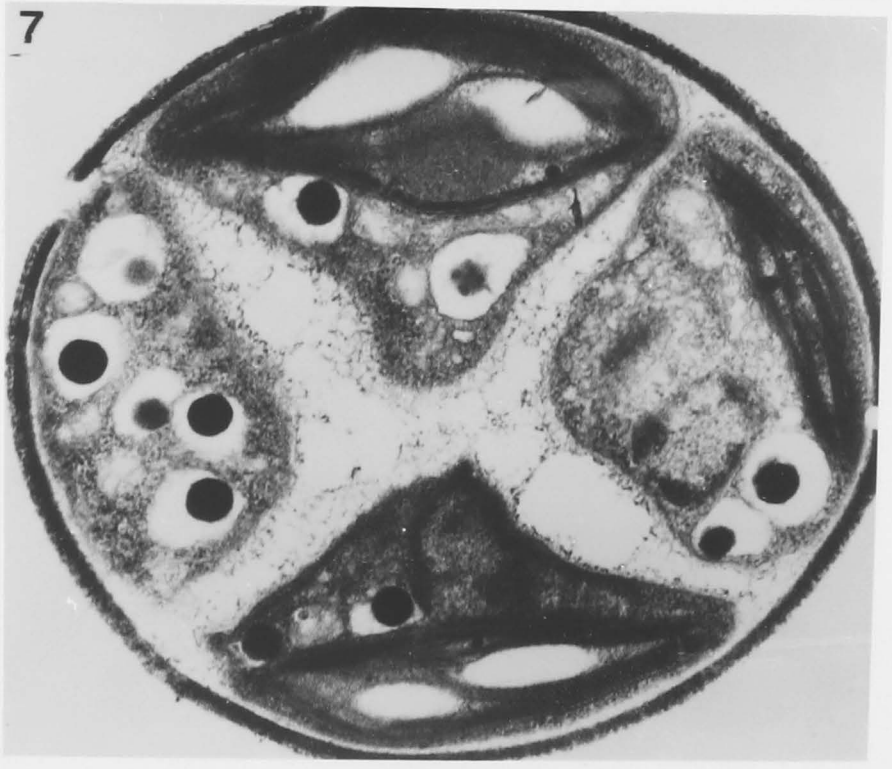
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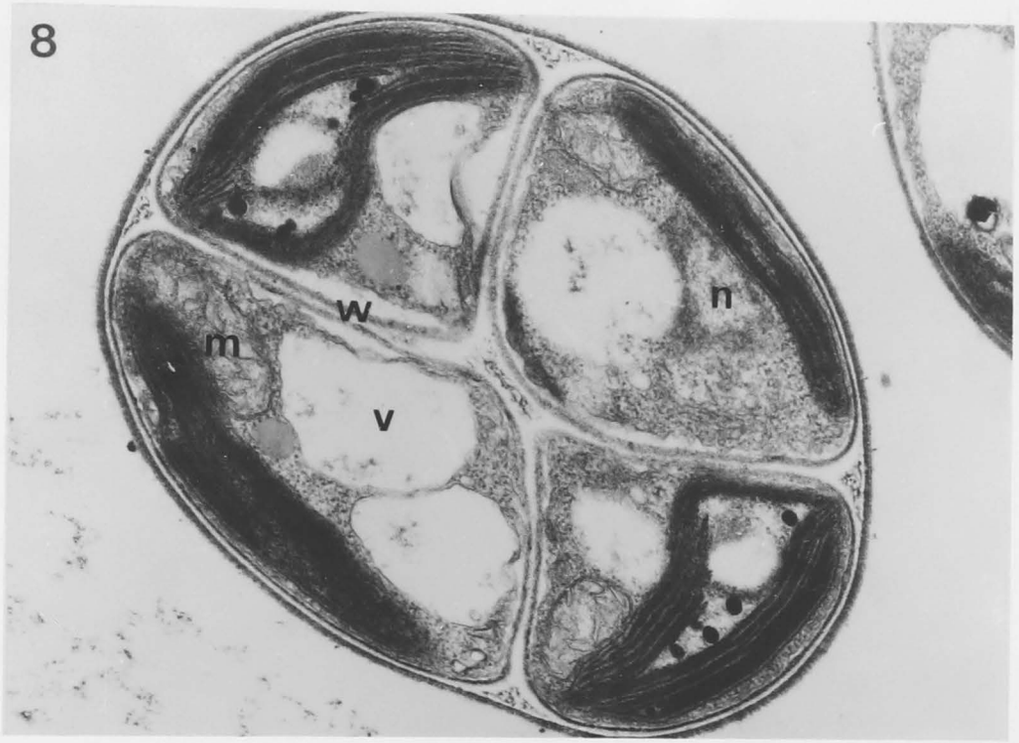
Fig. 8 Formation of wall by the autospores and the disappearance of most of the granular and amorphous material within the parental wall. X 30,000.

Fig. 9 Release of autospores from the parental cell. X 18,000.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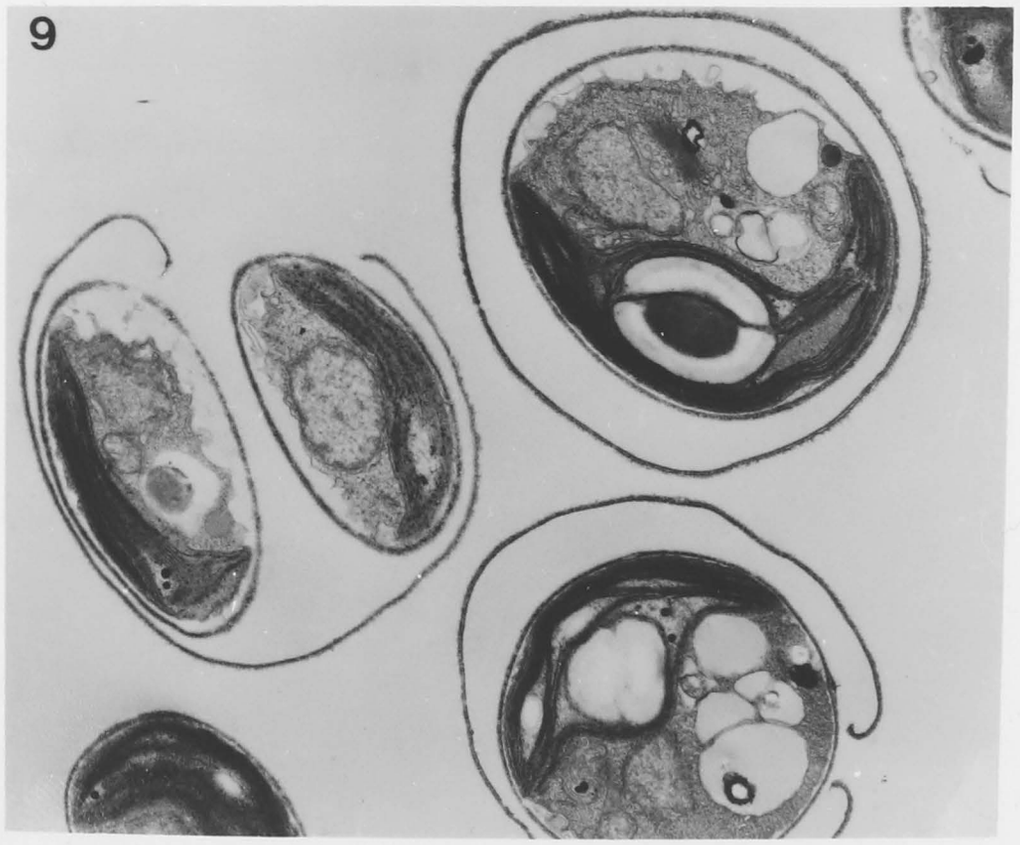
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CHAPTER 10

MITOSIS, CYTOKINESIS AND COLONY FORMATION IN *PEDIASTRUM BORYANUM*

INTRODUCTION

Pediastrum is a close relative of Hydrodictyon, both being members of the Hydrodictyaceae (Fritsch, 1935). Uninucleate, biflagellate zoospores (zooids) of Pediastrum, like those of Hydrodictyon, arise by cleavage of multinucleate parental cells and they later link up with one another forming multicellular colonies. Despite considerable interest by microscopists in both the formation of colonies in Pediastrum and nets by Hydrodictyon, no satisfactory explanation of their mechanisms of cellular aggregation has been forthcoming. Even the possibility that both net and colony formation in these two genera could be similar has only recently been seriously suggested (Hawkins and Leedale, 1971). Mitosis, cytokinesis and colony formation in Pediastrum were investigated here for comparison with these processes which have been recently studied ultrastructurally in H. reticulatum (Marchant and Pickett-Heaps, 1970, 1971, 1972a,d).

MATERIALS AND METHODS

See Chapter 2.

OBSERVATIONS

Colonies of Pediastrum are most distinctive, both because of their cog or gearwheel shape (Fig. 1) and characteristic wall decoration (Figs. 1, 2). Fig. 2 also shows spines, unfortunately distorted by processing, projecting from the peripheral horns. Each cell in a colony of Pediastrum was initially uninucleate, repeated mitoses produced 2^n nuclei (where n = 1-7) in each cell. P. boryanum usually gave rise to 16 or 32 nuclei under my growth conditions (Figs. 3, 6) before cytokinesis. Mitosis in P. boryanum appeared virtually identical with that in Hydrodictyon (see Chapter 3). Figs. 4 and 5 show mitotic nuclei enclosed by one or two perinuclear envelopes of endoplasmic reticulum, which were absent from interphase nuclei (Fig. 7). Within this envelope, the nuclear membrane remained largely intact throughout mitosis except for polar fenestrae. Centrioles were persistent in the cells (Figs. 3, 5, 6, 10, 12) and as in Hydrodictyon, mitotic divisions were synchronized.

Lipid droplets were common in the cytoplasm (Figs. 3, 6, 7, 12), unlike vegetative cells of H. reticulatum cultured under identical conditions. Interphase nuclei often had conspicuous protuberances (Fig. 9) which often projected a considerable distance into the cytoplasm. Golgi bodies generally appeared associated with the nuclear envelope (Figs. 7, 8). Vesicles between golgi and nucleus presumably arose from the nuclear envelope (Fig. 7); other large vesicles, containing opaque material, were often seen on the golgi's maturing face (Fig. 8).

Although the whole process has not been studied in detail

ultrastructurally, cytoplasmic cleavage of P. boryanum to form zoospores appeared similar to the cleavage described earlier in cylindrical coenobia, zygospores and polyhedra of H. reticulatum. Arrays of phycoplast microtubules predicted the paths of cleavages (Fig. 11). Unlike H. reticulatum but similar to Kirchneriella (Pickett-Heaps, 1970) and Tetraedron (Pickett-Heaps, 1972c), these microtubules often appeared associated with centriole complexes (Fig. 10). When multinucleate cytoplasm had differentiated to uninucleate, biflagellate zooids (Fig. 12), a rupture developed in the ornamented outer layer of cell wall (Fig. 13), through which the inner layer of wall ballooned, forming a lenticular vesicle in which the zooids swarmed (Figs. 17, 18). Sections through the transition region between basal bodies and flagella of zooids revealed the stellate pattern found in H. reticulatum (Marchant and Pickett-Heaps, 1971) and various other motile plant cells (Ringo, 1967). Zooids of P. boryanum had peripheral bands of microtubules (Figs. 15, 16) which, when the zooids aggregated, underlaid the plasmalemma at the sites of contact with others (see Millington and Gawlik, 1970). The 16 or 32 zooids then aggregated in a single plane, usually arranged in concentric circles of 5 and 10 cells around a central cell, before retracting their flagella and secreting a wall (Fig. 18). Figs. 19-22 illustrate patterns of adhesion when only four or eight zooids developed from the parental cytoplasm.

DISCUSSION

A perinuclear envelope has been found enclosing mitotic nuclei of Pediastrum, Hydrodictyon (Marchant and Pickett-Heaps, 1970) and also Kirchneriella (Pickett-Heaps, 1970), a relative of Hydrodictyon. Since then, other members of the Chlorococcales have been examined; mitotic nuclei of Tetraedron (Pickett-Heaps, 1972c) were found to possess a perinuclear envelope, while those of Scenedesmus and Ankistrodesmus probably lack this structure (Pickett-Heaps, unpublished data). A perinuclear envelope was found in Chlorella by Wanka and Mulders (1967), but my investigation of the same strain failed to reveal it (see Chapter 9). To my knowledge, the few members of the Chlorococcales mentioned above are the only organisms in which such a structure has been found.* The function of this perinuclear envelope, which apparently isolates mitotic nuclei from the rest of the cytoplasm is unknown.

Characteristic of colonies of P. boryanum are conspicuous horns on the peripheral cells. If developing colonies are mechanically disrupted as the zoospores are becoming stationary, all the disaggregated cells develop horns (see Chapter 11). These horns only develop on one side of the zoospores, defined as the "chloroplast side" (Davis, 1964; Hawkins and Leedale, 1971). Some cells also develop another pair of projections. These projections on the opposite, "nuclear side" of the

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* Very recently a perinuclear envelope has been reported to enclose mitotic nuclei of Membranoptera, a member of the Rhodophyta (McDonald, 1973, in press).

zoospores are rounded and not nearly as prominent as the horns mentioned above. Cells possessing this second pair of projections develop a roughly H-shaped outline (Fig. 18). All zoospores appear to have the capacity to produce horns; however, this potential is often not expressed, as formation of horns is inhibited by contact of sites that produce the horns with other cells. Although cells of some colonies appear to make contact with others all around their periphery, other individual cells often only have four intercellular sites of contact. These regions are almost invariably sites where horns would have been produced if the cells had failed to make contact with others.

There have been numerous descriptions of the structure of colonies of Pediastrum but very few attempts to explain the patterned aggregation of zoospores to form these colonies; such is also the case in Hydrodictyon. Davis (1964) proposed that flat colonies of P. boryanum arise because the lenticular vesicle in which zoospores aggregate is rigid and of such a shape as to mould adhering zoospores into a planar configuration. His hypothesis requires that the lenticular vesicle be only slightly thicker than the diameter of individual zoospores. Both Hawkins and Leedale's (1971) and my own observations reveal that planar colonies of this alga develop in vesicles whose dimensions are considerably greater than those required by Davis' hypothesis.

It seems likely that colony formation in Pediastrum, like germ net formation of H. reticulatum (Chapter 8), is the result of a morphogenetic movement controlled entirely by the zoospores themselves. As discussed earlier (Chapter 8), I suspect that there are specific sites on the

surface of zoospores of both H. reticulatum and P. boryanum which can join with others, and that zoospores are not "potentially capable of sticking together over all their surface" as Hawkins and Leedale (1971) propose.

Zoospores aggregate when forming colonies of P. boryanum in such a way that their chloroplast and subsequently their horns are oriented towards the outside of the colony. Similarly, when only two or four zoospores aggregate, there is a tendency for them to adhere by their "nuclear sides" rather than via their horns (Fig. 19, 20), suggesting that there may be a difference between the proposed adhesive sites in the readiness with which they will adhere to other.

If the hypothesis that there are four contact sites on each aggregating zoospore of P. boryanum is correct and holds for other species of Pediastrum, there could not be a direct correspondence between the number of horns and rounded projections and contact sites in some species, as some of these produce one or four horns per cell (Fritsch, 1935).

All the members of the family Hydrodictyaceae form colonies following aggregation of biflagellate zoospores. Besides Hydrodictyon and Pediastrum, which have been studied in detail here, colonies of Sorastrum contain 8-64 cells arranged in a sphere. Each cell is on a rigid stalk which is interconnected with other stalks near the centre of the colony. Each cell therefore apparently has only a single contact site although this site may be a contact with numerous other cells.

The other member of the Hydrodictyaceae, Eurastropsis, exists as colonies containing only two cells. These colonies apparently develop in much the same way as those of Pediastrum, i.e., the multinucleate cytoplasm of cells cleaves to form many biflagellate, uninucleate zooids which swarm in a vesicle derived from the parental cell wall. Unlike zoospores of Pediastrum however, zoospores of Eurastropsis do not link up to form a planar colony, instead they aggregate in pairs and develop two horns on each cell. Presumably, zoospores of Eurastropsis have fewer contact sites on their surface (possibly only one or two) than zoospores of Pediastrum. Further study is proposed to investigate the number and distribution of contact sites on zoospores of these algae.

ABBREVIATIONS USED IN FIGURES

c - centriole

g - golgi body

l - lipid droplet

n - nucleus

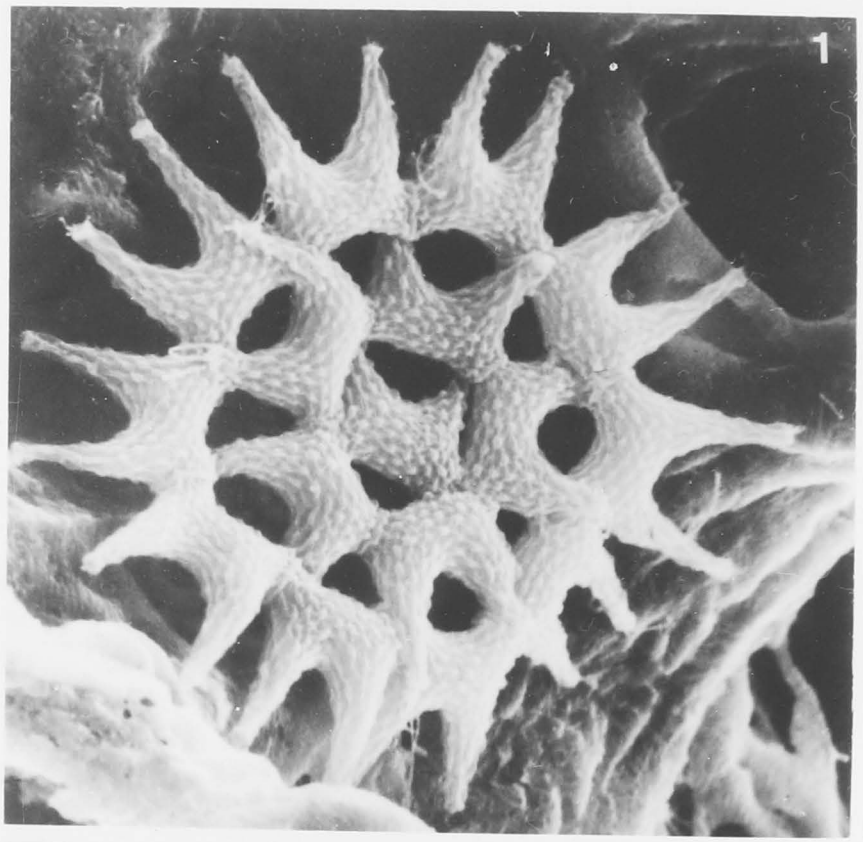
np - nuclear pore

p - pyrenoid

t - microtubules

Fig. 1 Scanning electron micrograph of a colony of P. boryanum showing the patterned decoration of the cell walls, the 1, 5, 10 arrangement of cells in concentric circles, and horns, not only on the peripheral cell, but on inner ones that have not made contact with those towards the outside of the colony. X 3,600.

Fig. 2 Spines, somewhat distorted by processing, arising from the tips of the horns on peripheral cells. Note also detail of the decorated cell wall. X 5,700.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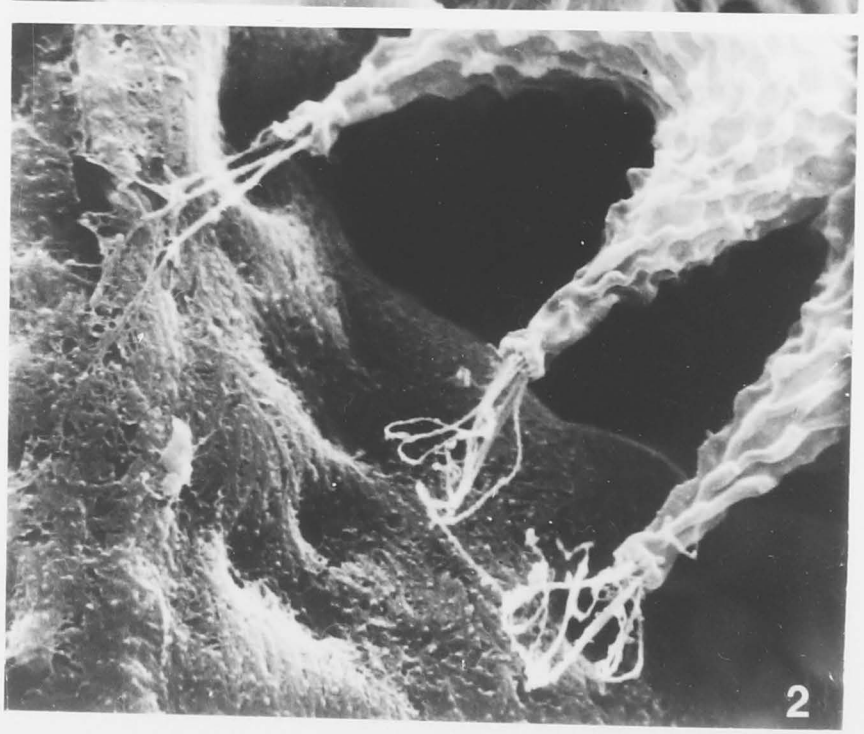
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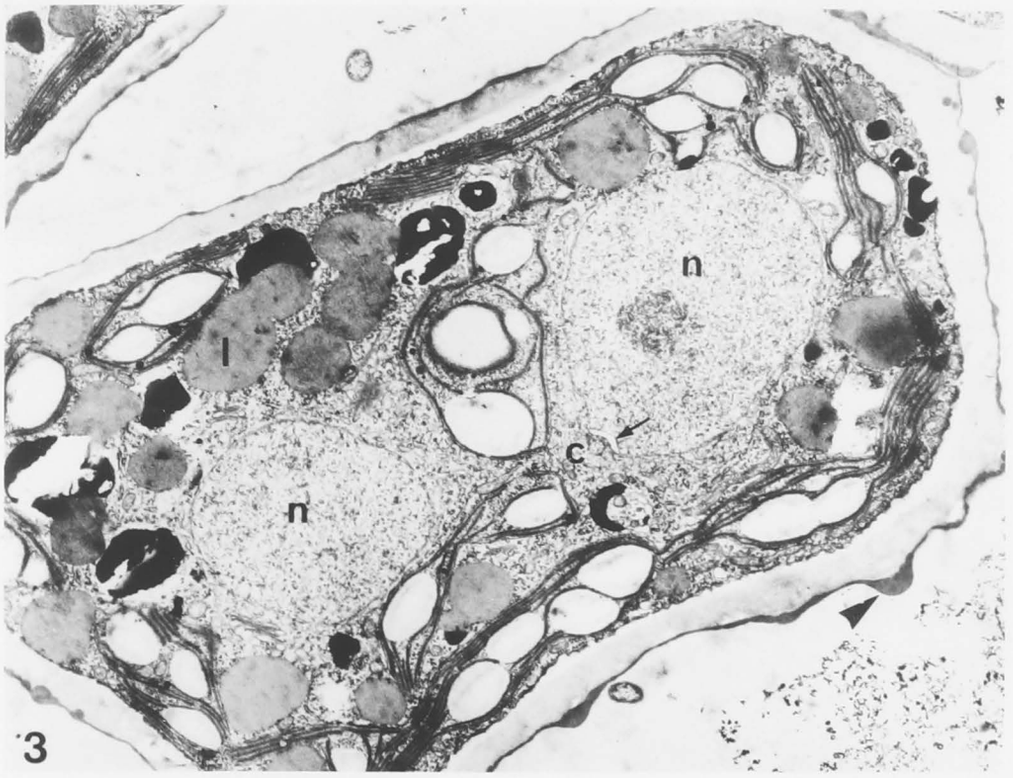
Fig. 3 Binucleate cell of P. boryanum showing lipid droplets in the cytoplasm and the bilayered cell wall. Note the dilation of the nuclear envelope (arrow) near the centriole and blebs on the outer layer of the cell wall (arrowhead) which are wall decoration. X 12,000.

Fig. 4 Obliquely sectioned nucleus at metaphase showing the perinuclear envelopes of endoplasmic reticulum (arrowheads). X 35,000.

Fig. 5 Nucleus at anaphase showing centrioles and perinuclear envelope. X 27,000.



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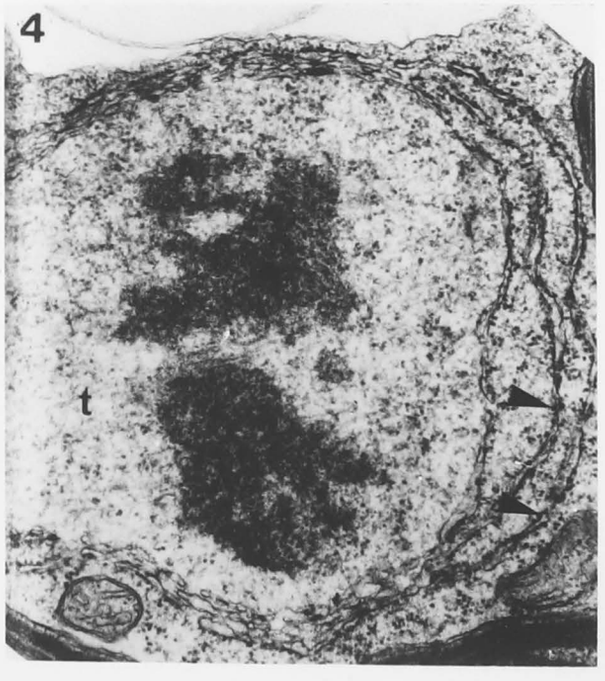

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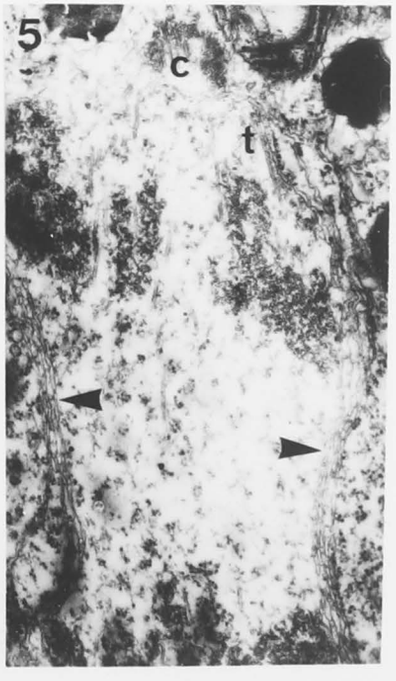

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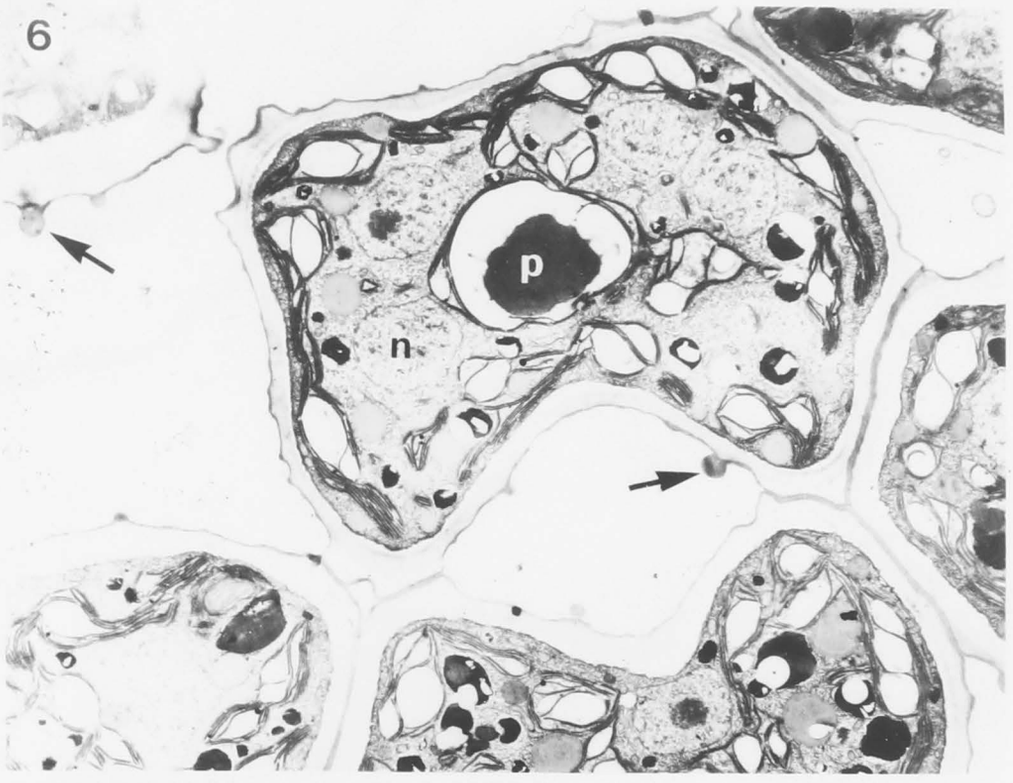
Fig. 6 Multinucleate cell in a colony of Pediastrum. Note the pyrenoid in the centre of the cell, the intercellular junctions and blebs (arrows) of the outer layer of wall which comprise the wall decoration (cf. Figs. 10 and 11). X 9,900.

Fig. 7 Interphase nucleus of P. boryanum with adjacent golgi body and vesicles possibly arising from the nuclear envelope (arrow). No perinuclear envelope is present. X 28,000.

Fig. 8 Golgi body apparently giving rise to vesicles containing amorphous material (arrow). X 43,000.

Fig. 9 Projection into the cytoplasm of an interphase nucleus. X 43,000.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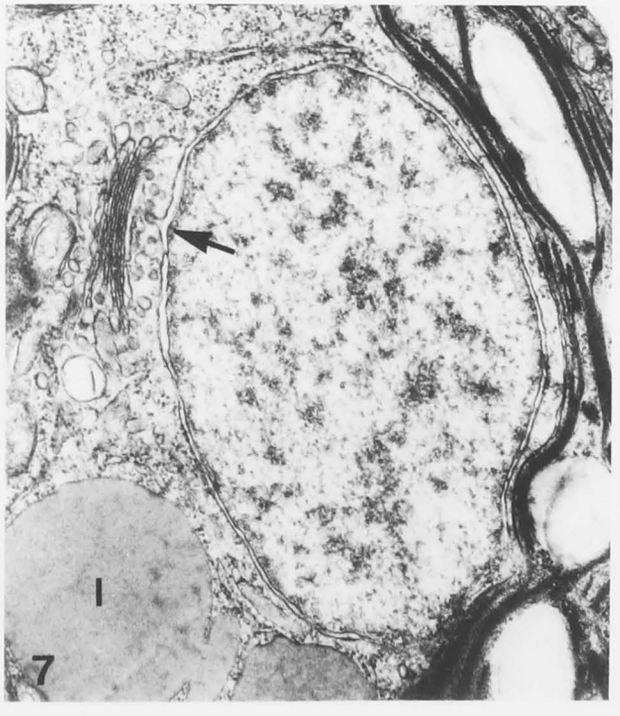
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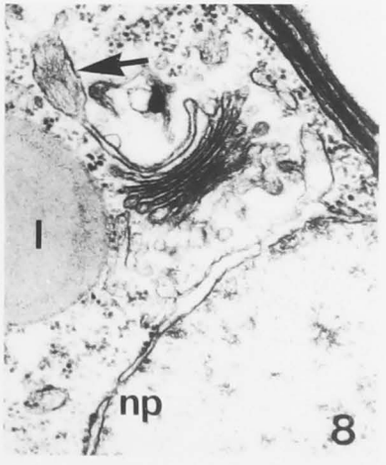
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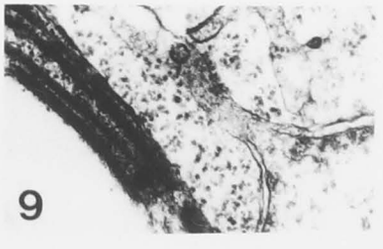
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Fig. 10 Centrioles adjacent to dilations (arrowhead) of nuclear envelopes in a cell about to undergo cytoplasmic cleavage. Phycoplast microtubules are often found associated with centrioles. X 31,000.

Fig. 11 Accumulation of vesicles to form a phycoplast passing near a nucleus (n). X 45,000.

Fig. 12 Zoospores formed by cytoplasmic cleavage within parental cell wall. Note the flagella and basal bodies (arrow) and that most of the inner layer of wall has disappeared but the outer layer is intact (cf. Figs. 12 and 15). X 8,100.

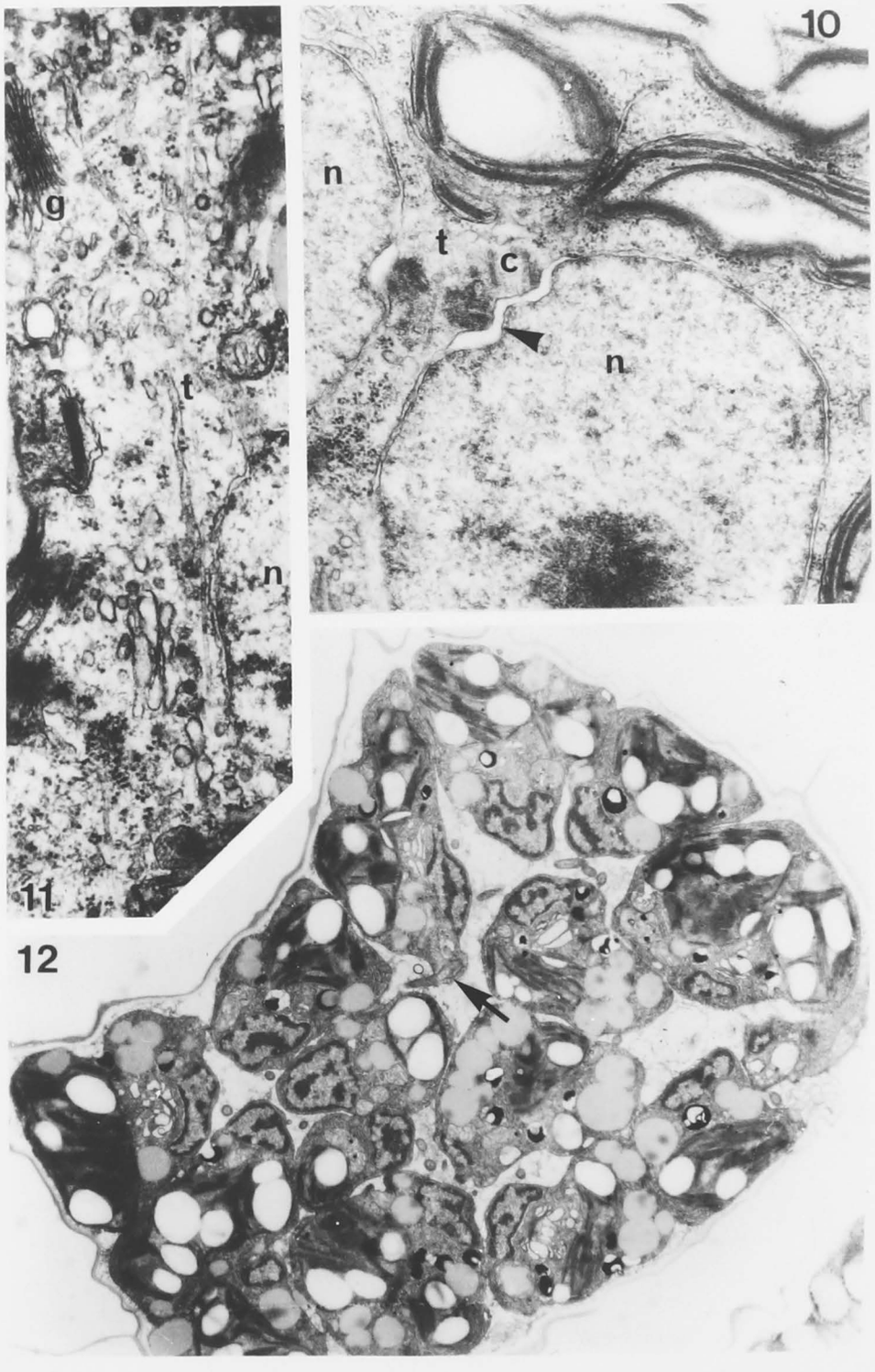
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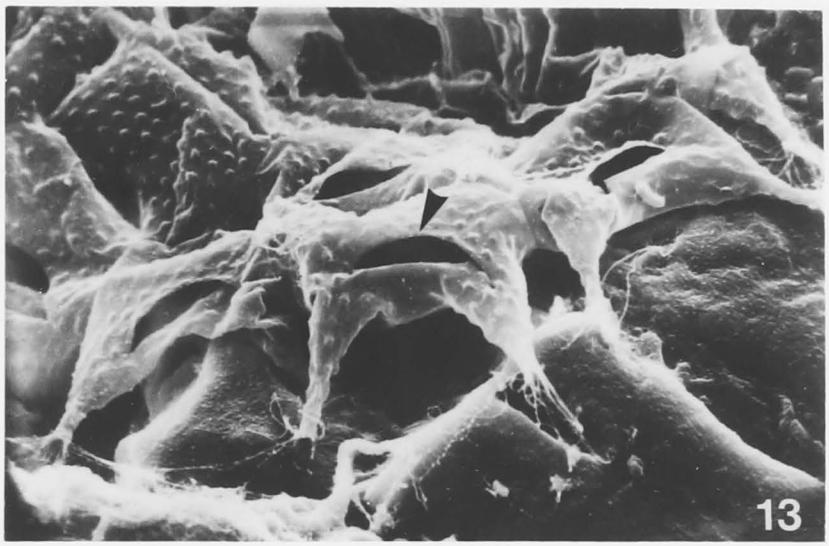
Fig. 13 Scanning electron micrograph of part of a colony of *Pediastrum* showing ruptures in the outer layer of wall (arrowhead) through which the vesicle containing zoospores is released. X 2,000.

Fig. 14 Transverse section through the transition region between basal body and flagellum showing the stellate pattern. X 160,000.

Fig. 15 Peripheral microtubules in a zoospore of *Pediastrum*. X 36,000.

Fig. 16 Peripheral microtubules in two zoospores, fixed when they were seen to be aggregating. X 48,000.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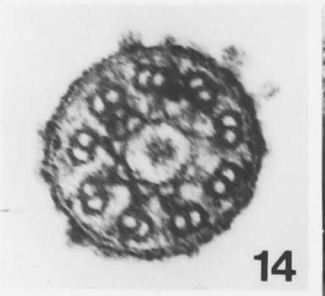
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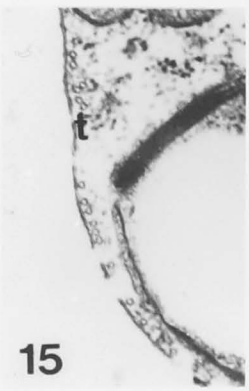
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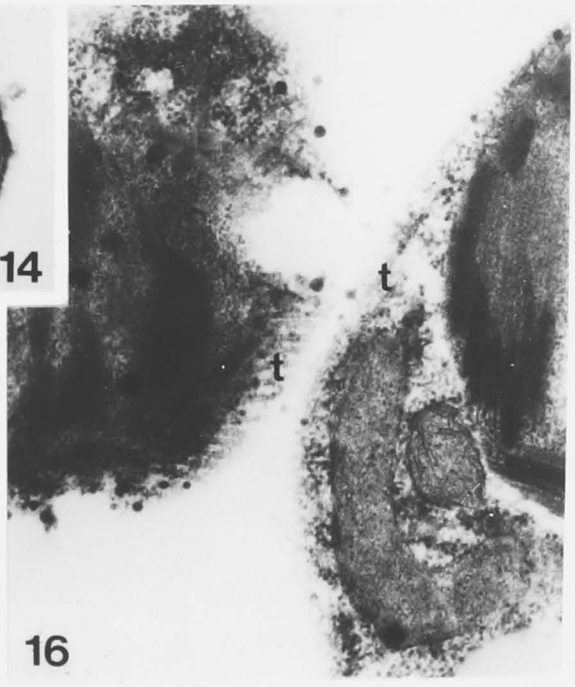
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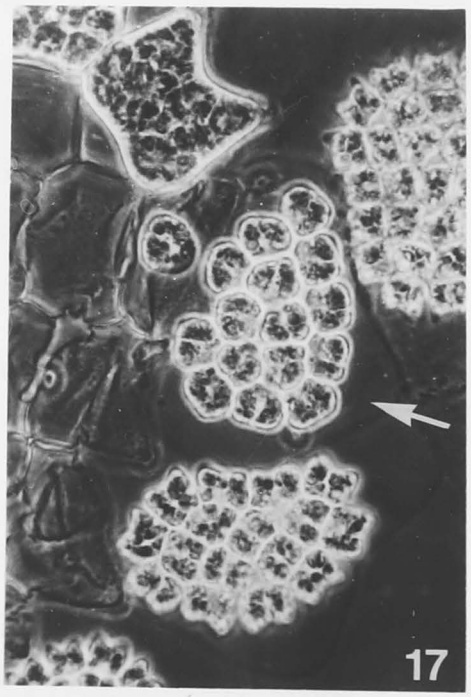
Fig. 17 Periphery of a colony of P. boryanum producing daughter colonies. note the empty cells in which the cytoplasm has cleaved. Some vesicles contain swarming zoospores (arrow), others with very young, distorted colonies. Phase contrast. X 480.

Fig. 18 A very young colony with well developed horns still within the parental vesicle. Phase contrast. X 480.

Figs. 19-22. Colonies containing only four or eight cells. Note that each cell apparently has four contact sites, two of which, if they do not make contact, form horns. All Nomarski optics. X 1,400.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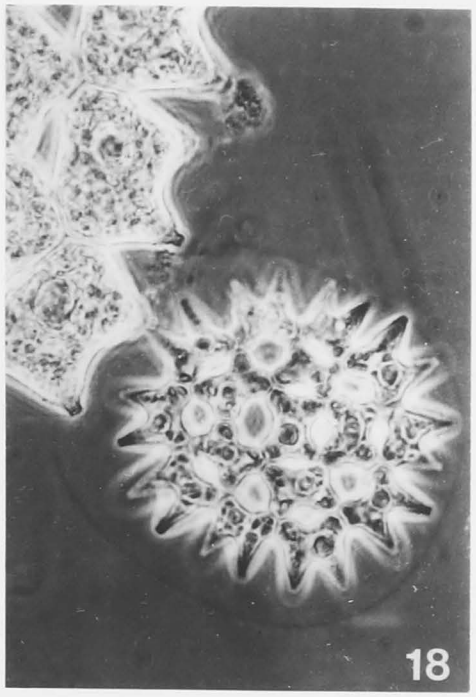
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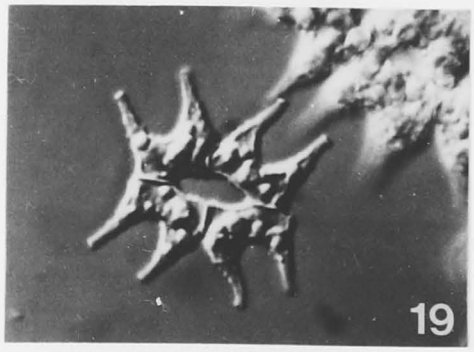
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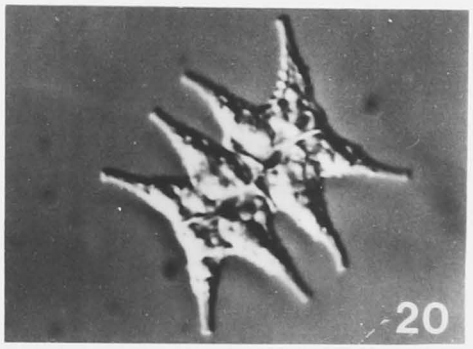
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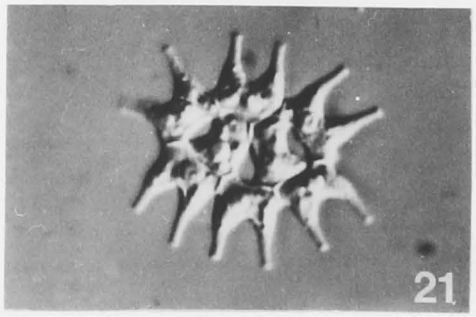
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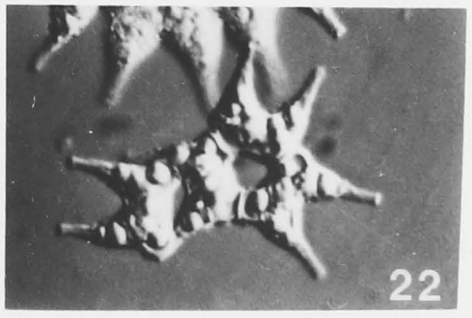
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CHAPTER II

THE EFFECT OF COLCHICINE AND VARIOUS ENZYMES ON AGGREGATION AND ADHESION OF ZOOSPORES AND VEGETATIVE CELLS OF *HYDRODICTYON RETICULATUM* AND *PEDIASTRUM BORYANUM*

INTRODUCTION

Earlier, we reported ultrastructural observations on aggregating zooids of H. reticulatum forming both vegetative, cylindrical daughter nets (Marchant and Pickett-Heaps, 1972a) and flat germ nets (Marchant and Pickett-Heaps, 1972d). A diagrammatical representation of the life cycle of this alga is included in Marchant and Pickett-Heaps' (1971) paper on development of zooids from multinucleate cytoplasm of cylindrical coenobia.

Conspicuous bands of microtubules underlie the plasmalemma of net-forming zooids, irrespective of the shape of the net they are about to form. Gametes, whose development is virtually indistinguishable from that of net-forming zooids, lack these microtubules (Marchant and Pickett-Heaps, 1972b) which we previously speculated to have an active role in the ordered linking of zooids (Marchant and Pickett-Heaps, 1972a, 1972d). Similar bands of microtubules occur in colony-forming zoospores of Pediastrum boryanum (Gawlik and Millington, 1969; Millington and Gawlik, 1970; see also Chapter 10). However Millington and Gawlik do not discuss the possibility that these microtubular bands are directly involved in colony formation. Hawkins and Leedale (1970), studying formation of daughter nets in H. reticulatum and colonies of various species of Pediastrum, did not report finding microtubules in aggregating zooids which they fixed with osmium tetroxide alone, in the cold.

This paper describes the experimental use of colchicine, to investigate the role of microtubules in the aggregation of zooids in both H. reticulatum and Pediastrum. This drug is known to prevent the

polymerization of microtubular subunits and to depolymerize existing microtubules. The allied problem of how aggregating zooids adhere to one another and whether the intercellular adhesive alters as cells age has been investigated cytochemically and by attacking the adhesive with various enzymes and the chelating agent, EDTA. On the basis of this experimental work and earlier observations, a mechanism is proposed to explain various aspects of colony formation in Hydrodictyon and Pediastrum.

MATERIALS AND METHODS

Both Australian and Cambridge strains of H. reticulatum were used in this investigation as well as Pediastrum boryanum, supplied by the Indiana Culture Collection. The methods used here are described in Chapter 2.

RESULTS

The effect of colchicine on net and colony formation.

Normal cleavage of the cytoplasm to form zooids in both Hydrodictyon and Pediastrum was inhibited by 0.2% colchicine. If the cytoplasm did cleave, the resulting pieces were most irregular, not uninucleate fragments that are produced in the absence of colchicine. Net-forming zooids of H. reticulatum very rarely adhered to form either daughter or germ nets in the presence of 0.2 or 0.4% colchicine. The few that did link up formed highly irregular aggregates of cells (Figs. 1, 2, 6). In such irregular nets, zooids often joined with two others (Fig. 1), one at each "end," in contrast to untreated control cells which usually linked with four other cells, two at each end. Neither did colchicine-treated zooids become rhomboidal in outline prior to aggregation as did untreated control cells (Fig. 3). Zoospores of P. boryanum treated with colchicine sometimes aggregated to form planar colonies but the arrangement of these cells was generally irregular (Fig. 4), not the 1,5,10 pattern of concentric rings commonly found among untreated cells (Fig. 8). Furthermore, the characteristic "horns," conspicuous on outer cells of untreated colonies (Figs. 8, 9), rarely developed on treated cells, whether they had aggregated or not (Fig. 5). Among untreated controls, horns only developed on the "chloroplast side" of zooids when these sites are not in contact with other cells (Fig. 9), such as those on the outside of a colony or inner cells that fail to make contact with those outward of them. Horns developed on all normal zooids if they had been mechanically prevented from aggregating as a colony (Fig. 7).

Treated cells of Pediastrum that had managed to form colonies, almost always disaggregated later, if left in the colchicine solution or transferred to fresh medium; individual cells became nearly spherical (Fig. 5) but subsequently could reproduce asexually in the absence of colchicine, producing normal planar colonies. Colchicine treated zooids of both H. reticulatum and P. boryanum lacked the peripheral bands of microtubules characteristic of untreated controls (see Chapters 5, 8, 10).

The action of enzymes and EDTA on the intercellular adhesive.

Nets of different ages were exposed to various enzymes at a concentration of 1 mgm/ml at 24° C for 4 hours. The relative ease with which both treated and untreated nets could be teased apart with dissecting needles is given in Table 10.1. The results of sonicating nets of different ages after one hour's exposure at 24° C to various enzymes were very variable. Light microscopy confirmed that the disaggregated nets had parted at the intercellular junctions and that the cells themselves were intact. EDTA did not detectably weaken the intercellular adhesive. Cells exposed to this chelating agent however, all died within 12 hours.

Electron microscopy of the normal and enzyme treated intercellular junctions of Hydrodicyton.

Extracellular amorphous material appeared between aggregating zooids before the flagella were retracted. When the cell wall had been secreted, the intercellular junctions had a trilaminar appearance which

was lost as the net aged (Figs. 5.33, 5.34). Of the nets that had been incubated in various enzymes for 1 hour, only those treated with trypsin or pronase showed any change in the structure of the intercellular junction. The outer wall layer of cells treated with these proteolytic enzymes was detached from the inner layer (Figs. 10, 11). α-Amylase, which weakened the intercellular bond between young cells, did not detectably alter the structure of the outer layer of cell walls; however the inner layer of wall appeared more fibrous than untreated controls, as if some component of it had been extracted (Fig. 12).

Cytochemistry of the intercellular junction of Hydrodictyon.

Sections of coenobia pretreated with periodic acid, then stained with Schiff's reagent and examined with the light microscope, revealed dense staining of the starch grains in the cytoplasm or in daughter zooids. However the inner layer of wall stained very lightly and there was no apparent reaction of the intercellular junction itself (Fig. 13). Unoxidized sections did not stain at all with Schiff's reagent.

Following staining with the silver hexamine reagent there was no detectable difference in either the density or localization of the considerable deposition of silver grains between untreated control sections and those that had been oxidized with periodate. While the amorphous material between recently aggregated zooids did not stain with the silver hexamine reagent (Fig. 14), silver grains were deposited over the "middle lamella" between cells which had recently secreted a wall (Fig. 15) and between the inner and outer layers of wall in mature cells (Figs. 16, 17). In these older cells, no staining of the middle

lamella was detectable (Figs. 16, 17). Virtually no silver was deposited on sections of material fixed only in glutaraldehyde which had been reduced and alkylated or on those which had been alkylated without reduction (Fig. 18).

TABLE 11.1

ENZYMES, BUFFERS AND THE RELATIVE EASE OF DISAGGREGATION OF TREATED VEGETATIVE NETS OF H. RETICULATUM

Enzyme	Buffer	pH	Age of treated nets		
			0-2 hrs	20-24 hrs	24+ hrs
Trypsin	0.02M Tris HCl	7.4	+++	+++	+++
Pronase	0.02M Tris HCl	7.3	+++	+++	+++
α-Amylase	unbuffered	6.8	++	+	+
β-Amylase	0.02M Acetate	4.8	±	-	-
Pectinase	0.02M Acetate	4.0	++	-	-
Cellulase	0.02M Acetate	4.5	-	-	-
β-Glucosidase	0.02M Acetate	5.3	-	-	-
Lipase	0.02M Tris HCl	7.4	-	-	-

+++ Disaggregate easily
- unaffected.

DISCUSSION

Two members of the Chlorococcales, Pediastrum and Hydrodictyon, are unique among the green algae in that both form extensive planar colonies of cells from zoospores at some stage in their life cycle. Pediastrum forms a flat colony, in which the peripheral cells bear one or more projections, giving the colony the appearance of a cog or gear-wheel. Colonies of Hydrodictyon are either flat nets or a single layer of cells arranged as a hollow cylinder (Pocock, 1960; Marchant and Pickett-Heaps, 1971, 1972a, 1972d). I found it convenient to think of the arrangement of cells in these cylindrical colonies as being essentially planar, since the curvature of the colonies is slight in relation to the size of individual zoospores.

Although much has been written about the nature of cylindrical nets of H. reticulatum (Burton, 1915; Harper, 1908; Lowe and Lloyd, 1927; Moewus, 1948; Jost, 1930) and colonies of Pediastrum (Harper, 1918; Davis, 1962; Moner, 1954; Moner and Chapman, 1960; Smith, 1916), there have been only few attempts to explain their formation, and still fewer attempts to explain the development of flat nets of Hydrodictyon. This could be partly due to confusion in the past concerning the form of the germ net of H. reticulatum, but evidence reported earlier (Marchant and Pickett-Heaps, 1972d) indicated that under optimum conditions, germ nets of this species are predominantly flat, similar to H. africanum and H. patenaeforme, and not commonly tangled or double centred as described by Pocock (1937, 1960).

Earlier (Marchant and Pickett-Heaps, 1972a,d and Chapter 10), we

considered various hypotheses that have been advanced to account for net and colony formation in Hydrodictyon and Pediastrum and discussed similarities in the development of these cellular aggregates. Some basic features are common to aggregating zoospores of Pediastrum, and zooids of H. reticulatum forming either cylindrical daughter nets or flat germ nets, and these can be set out as follows.

1. Colony-forming zooids of both algae have peripheral bands of microtubules. When zooids aggregate, their initial sites of contact are usually underlain by these microtubules, which, when forming both daughter and germ nets of H. reticulatum, are oriented predominantly in the plane of the developing net. Preliminary evidence suggests a similar orientation in colonies of Pediastrum.

2. Before aggregation, zooids change their shape, becoming rhomboidal or nearly rectangular in outline.

3. Each zooid links with usually four others, generally at predictable sites.

4. The pattern of cells in a colony is determined before flagellar retraction, which is followed by secretion of the characteristic bilayered wall.

5. Horns often develop at certain sites on zooids if these do not come in contact with other zooids.

Colchicine is known to prevent polymerization of microtubular subunits at relatively low concentrations and to depolymerize microtubules

at slightly higher concentrations (Inoué and Sato, 1967). Exposure of both Pediastrum and Hydrodictyon to this drug interferred markedly with their normal aggregation to form colonies or nets. The absence of microtubules in treated cells, the failure of these cells to change shape, and the irregular aggregates of cells they form (when they aggregate at all!) all argue strongly that the peripheral microtubules, absent in gametes of H. reticulatum (Marchant and Pickett-Heaps, 1972b), play a vital role in the patterned aggregation that gives rise to the characteristic nets and colonies of these algae. We suggest that these microtubules have a cytoskeletal function and are responsible for the change in shape of zooids to become rhomboidal in outline just before adhesion; precisely how this is achieved is not entirely clear.

Even when treated with colchicine, the few zooids that adhere do so predominantly laterally. How could this happen if the zooids were sticky over their entire surface as Hawkins and Leedale (1971) propose? Although direct evidence is scanty, it appears that at the time of aggregation the only adhesive areas of plasmalemma are those underlain by microtubules. Evidence for the existence of this adhesive is the finding of amorphous material on the surface of aggregating zooids only at their sites of contact. The "membrane-like" outer layer of cell wall which is secreted shortly after flagellar retraction and initial adhesion of the zooids is almost certainly also adhesive. If zooids are not initially arranged in nets or colonies, they then may adhere via this outer layer of wall and form abnormal junctions as this presumed second adhesive is present all over the zoospores' surface. As well as the consistent observations of amorphous material between adhering zooids,

there is a logical requirement for something to hold the zooids together from the time they aggregate in their characteristic pattern until secretion of the cell wall following retraction of their flagella, since there is a delay between their first linking as a patterned aggregate and wall secretion.

Each net- or colony-forming zooid linked with usually four others at predictable positions on its surface (i.e., laterally with respect to the nucleus), and horns develop at some of these sites if they are "unsatisfied," i.e., do not come in contact with other zooids. Tips of these horns (at least in H. reticulatum) closely resemble "half" an intercellular junction (Marchant and Pickett-Heaps, 1972d). These observations suggest the possibility of there being discrete sites on the plasmalemma of zooids which will establish initial contact with others. If these proposed receptive sites are adhesive and have an asymmetric shape, swarming zooids might arrange themselves in such a way as to have both the maximum number and greatest area of receptive sites in contact. A planar colony of cells can easily be visualized as resulting from such an aggregation. The proposed asymmetry of these sites most likely is not developed so much by the site itself but by the underlying microtubules altering the shape of the zooids. In the case of cylindrical daughter nets, the planar arrangement of cells is modified by the vacuolar envelope, giving rise to the overall cylindrical shape of the net (Marchant and Pickett-Heaps, 1972a).

Although each cell of H. reticulatum is usually connected to two others at each end, the number of attachments per end of a cell can

range between one and five, but very rarely more than three. I interpret this variability in the number of intercellular contacts being due to a single zooid occupying more than one receptive site, or else, more than one zooid attached to a single receptive site.

The ease with which nets could be disassociated with dissecting needles would appear to be a crude and subjective technique for assessing the action of enzymes on the intercellular junction. However, while this method is unsuitable for detecting slight differences in relative adhesion, it is quite sound for determining gross differences. For instance, the effect of trypsin and pronase on the junction between cells of all ages is such that they often disaggregate without any need to tease them apart. The problem of assessing the action of enzymes on the intercellular adhesive by sonicating the cells after a timed exposure to enzymes arises from the great variability in the time taken for nets of the same age, incubated in the same enzyme, to disaggregate. Until more refined experiments are done, all I can safely conclude from these enzyme experiments alone is that a proteinaceous component of the cell wall is important in keeping nets intact throughout their development. Also, the intercellular junction becomes harder to disrupt enzymatically as the net ages.

Northcote, Goulding and Horne (1960) found galacturonic acid in hydrolysates of whole cell walls of H. africanum and suggested that it was derived from pectic substances. Continuing, these authors made the analogy that "in higher plants these (pectic) materials were found in the middle lamella between adjacent cells, and in Hydrodictyon (africanum)

it might well be present in the thin circular pads which occur between neighbouring cells in the colony." If pectin occurs in intercellular junctions in H. reticulatum, we have no evidence that it has any role in intercellular adhesive as neither pectinase nor the chelating agent, EDTA,weakened the intercellular adhesive except between very young cells. Karr and Albersheim (1970) found that polysaccharides in the cell wall of higher plants are resistant to enzymic attack before treatment with a wall-modifying enzyme. If intercellular junctions in Hydrodictyon contain polysaccharides, perhaps they are similarly resistant to the action of the enzymes used. Electron microscopical examination of trypsin-treated cells revealed that the outer layer of cell wall was apparently not affected by the enzyme; however it became detached from the inner layer of wall.

Cytochemistry with the silver hexamine reagent is a useful tool of the electron microscopist. The basis of the staining reaction is that metallic silver is deposited by reduction of the alkaline silver complex by aldehyde groups (as in a Tollens test) or by disulphide bonds or ethylenic groups (Pickett-Heaps, 1967; Colvin and Leppard, 1971; Swift, 1968). Periodate oxidation of polysaccharides produced aldehyde groups while the other pre-treatment with benzyl thiol and iodoacetate reduces and carboxy methylates disulphide bonds. Deposition of silver grains on untreated sections of the intercellular junctions was unaltered by periodate oxidation and was eliminated by reduction and alkylation of cells fixed only in glutaraldehyde (osmium is capable of depositing silver). This suggests that the silver was being deposited over cysteine-containing protein. That the sites of silver deposition (Figs. 16, 17)

were the same parts of the wall that were affected by proteolytic enzymes (Figs. 10, 11) strengthens this conclusion. The absence of silver staining following reduction and alkylation indicates that the deposition of silver in the untreated controls was not produced by ethylenic linkages or aldehyde groups which may be introduced by glutaraldehyde fixation (Pickett-Heaps, 1967).

Hence it appears that the middle lamella of young intercellular junctions is proteinaceous as well as the boundary between the outer and inner layer of wall, but as the net ages protein disappears from the middle lamella and is found instead only between the outer and inner layers of the cell wall. The composition of the amorphous material between aggregating zooids and the nature of the outer layer of wall remains unknown.

ABBREVIATIONS USED IN FIGURES

iw - inner layer of wall

p - pyrenoid

s - starch

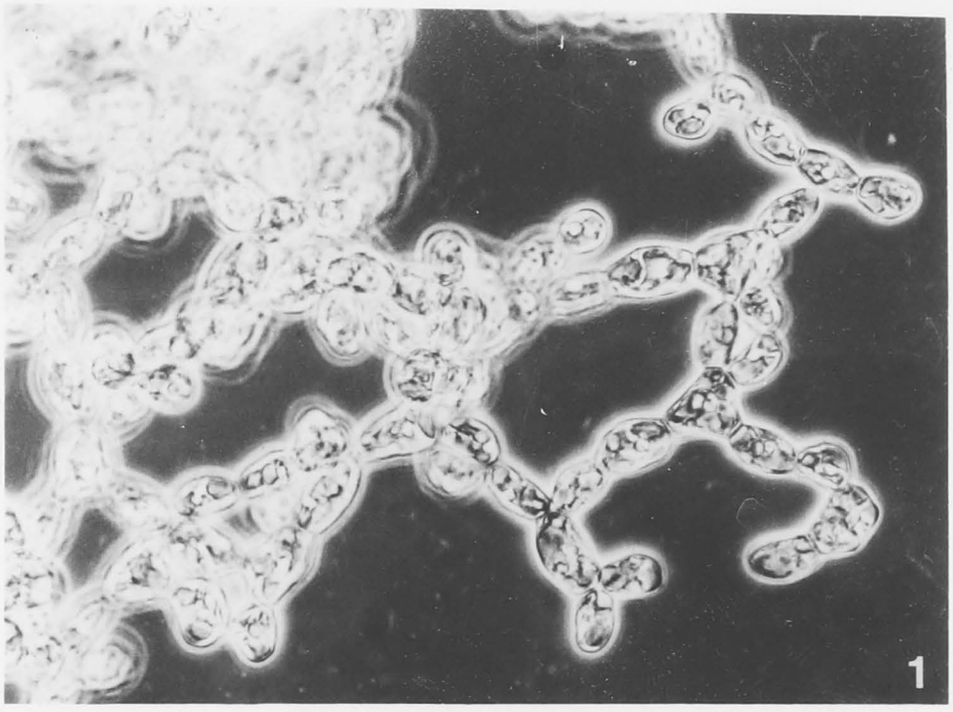
v - vacuole

Fig. 1 Part of an irregular net derived from net-forming zooids of H. reticulatum treated with colchicine. X 1,600.

Fig 2 Cells of H. reticulatum, many of which have failed to aggregate following treatment with colchicine. X 1,600.

Fig. 3 Scanning electron micrograph of net-forming zooids of H. reticulatum which had not been treated with colchicine. Note their rhomboidal outline. X 5,700.

Fig. 4 A highly irregular colony of P. boryanum which developed in the presence of colchicine (cf. Fig. 8). X 1,000.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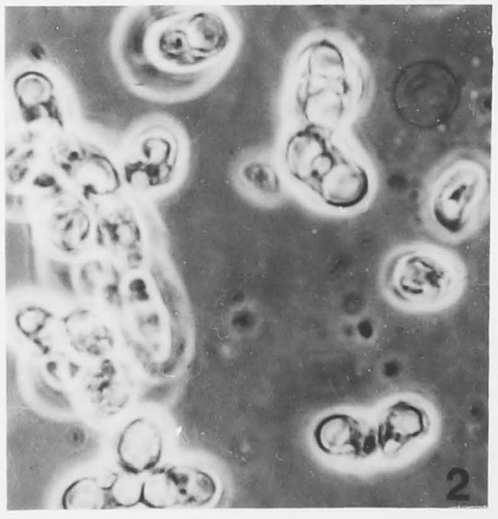
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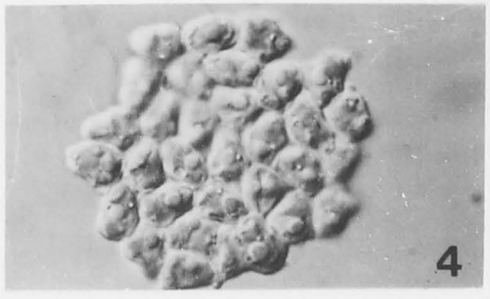
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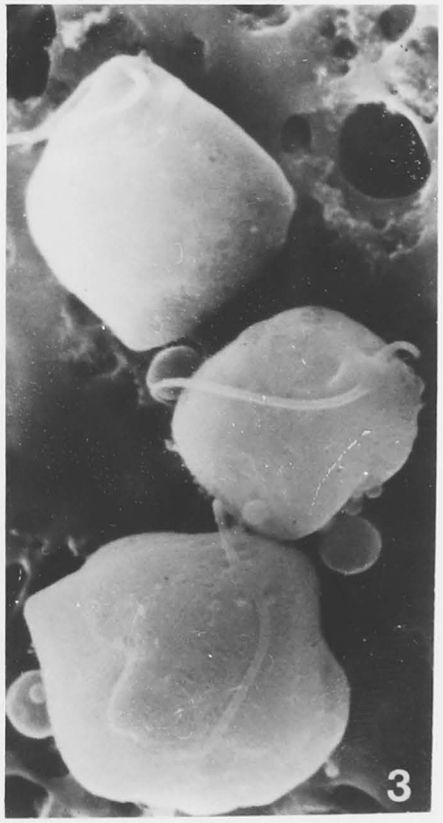
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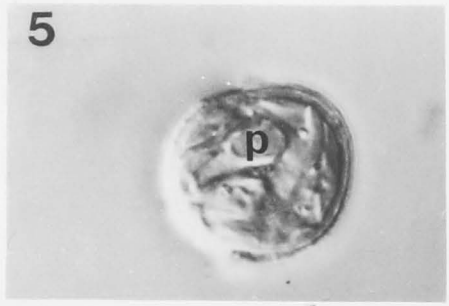
Fig. 5 A single cell of P. boryanum which was never part of a colony, the formation of which was prevented by colchicine. Note the lack of horn on this cell. X 1,600.

Fig. 6 Abnormal germ net of H. reticulatum produced from colchicine treated net-forming zooids. X 470.

Fig. 7 A vesicle containing the remnants of a colony of P. boryanum. Just as this colony was adhering normally, it was mechanically disrupted. The cells failed to re-aggregate and virtually all of them developed horns. X 1,000.

Fig. 8 A normal colony of P. boryanum showing the 1, 5, 10 arrangement of the cells. X 1,200.

Fig. 9 A naturally formed abnormal colony of P. boryanum. Note the absence of horns on the arrowed "outer" cell. X 1,200.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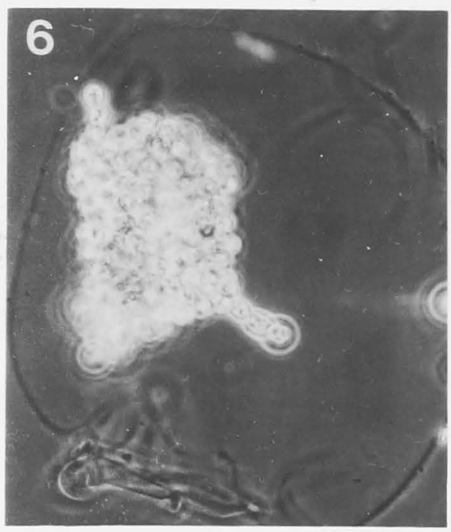
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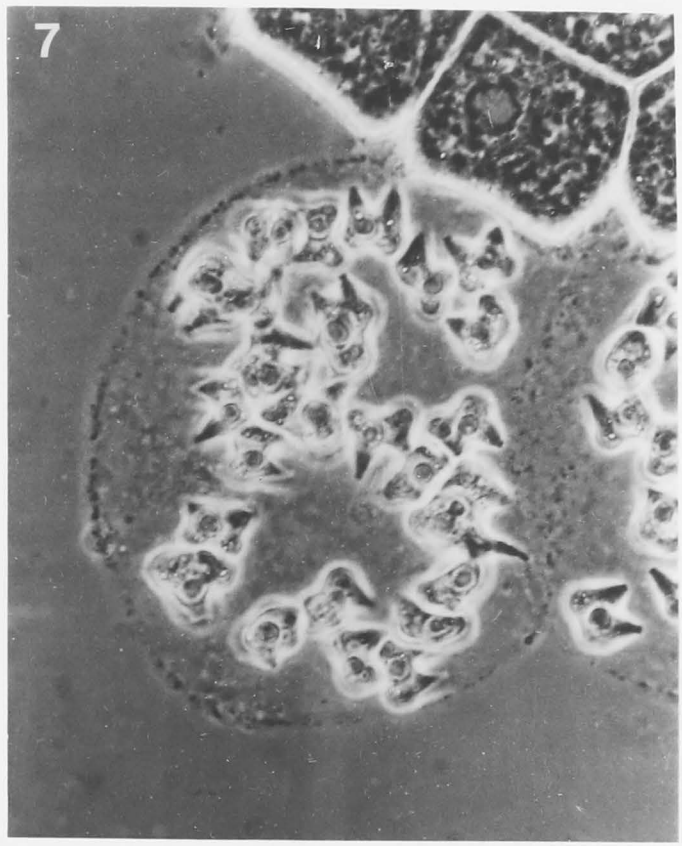
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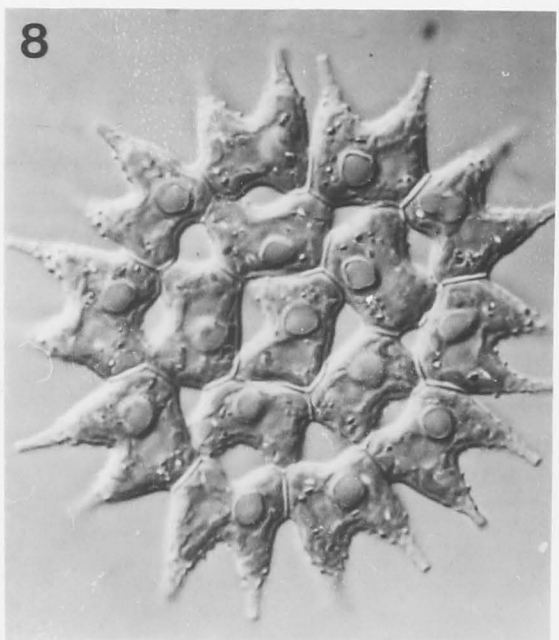
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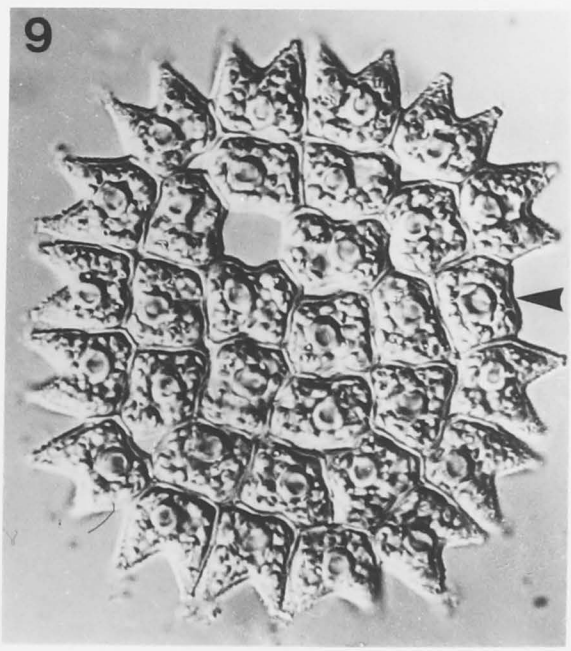
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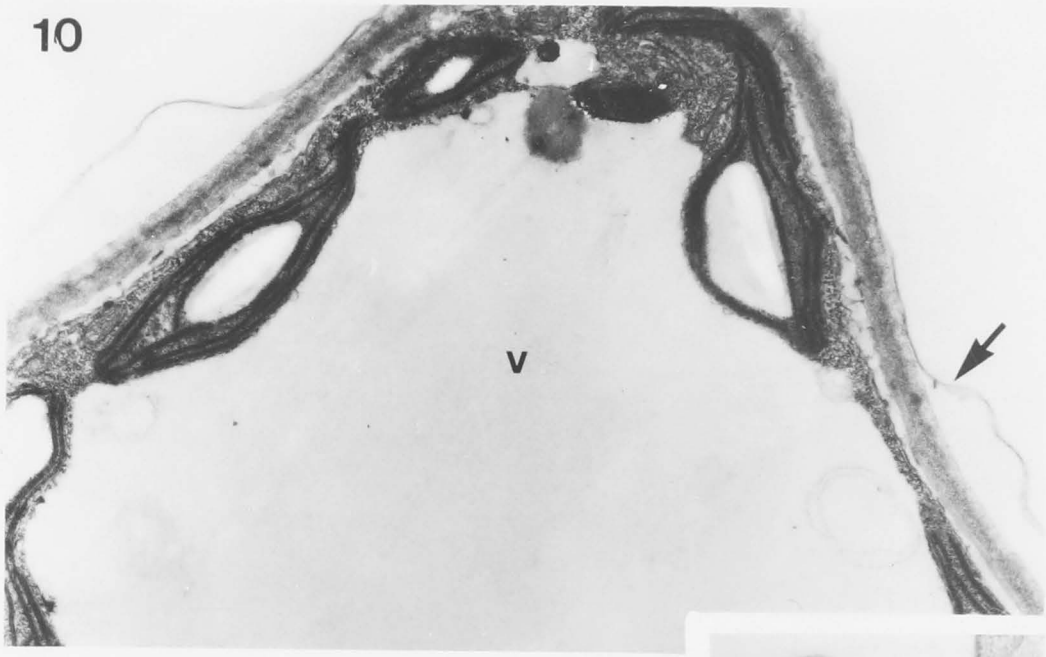
Fig. 10 Trypsin treated coenobium of H. reticulatum showing the separation of outer layer of wall (arrow) from the inner. X 15,000.

Fig. 11 Trypsin treated intercellular junction. Note that the outer layer of wall is intact but that it has become detached (arrow) from the inner layer. X 38,000.

Fig. 12 Intercellular junction of H. reticulatum treated with α-amylase. Although the junction itself is apparently unaffected, the inner layer of wall has a fibrous appearance as if some material has been extracted (cf. Figs. 10 and 11). X 44,000.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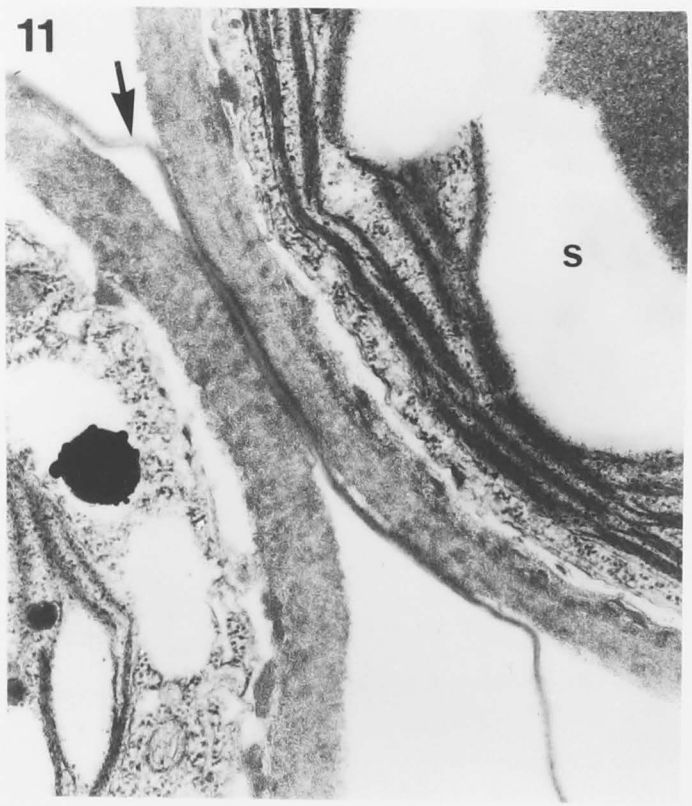
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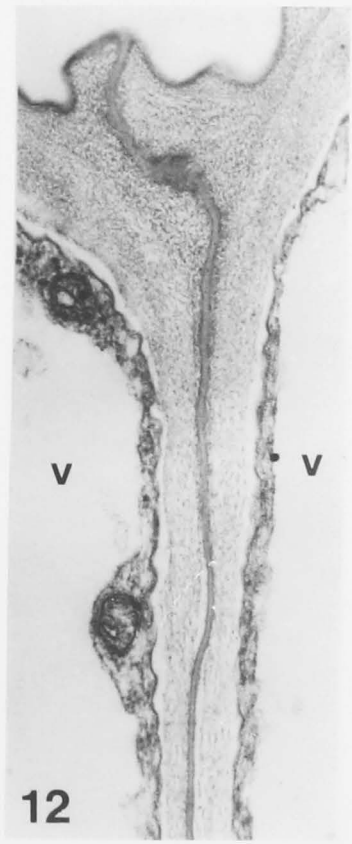
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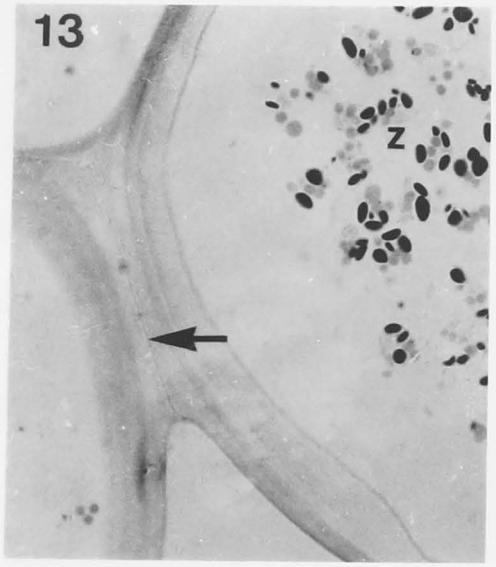
Fig. 13 Light micrograph of PAS treated section of H. reticulatum showing the strong reaction of the starch grains in zooids but no staining of the cell wall or intercellular junction (arrow). X 1,300.

Fig. 14 Two zooids in contact stained for electron microscopy with the silver hexamine reagent following peroxidation. There is no staining of the amorphous intercellular material (arrow). X 54,000.

Fig. 15 Silver hexamine staining, without prior peroxidation, of an intercellular junction showing silver deposition along the "middle lamella" of the junction (arrows). X 50,000.

Fig. 16 Junction between two older cells than illustrated in Fig. 15, stained with silver hexamine, without prior peroxidation. Here there is not staining of a "middle lamella" but a heavy deposition of silver grains between the outer and inner layers of cell wall. The deposition of silver in adjacent sections to those shown in Figs. 15 and 16, but peroxidized before treatment with the silver hexamine reagent, appeared identical to the unperoxidized sections shown here. X 37,000.

Fig. 17 Unperoxidized, silver hexamine treated junction fixed only in glutaraldehyde without osmium tetroxide post fixation. Note the staining between the outer and inner layers of wall. X 31,000.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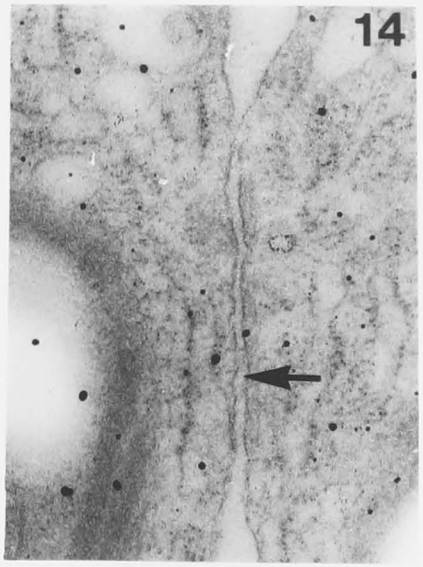
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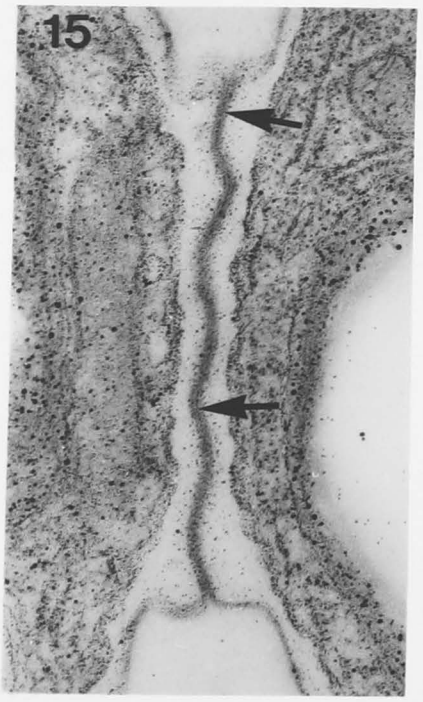
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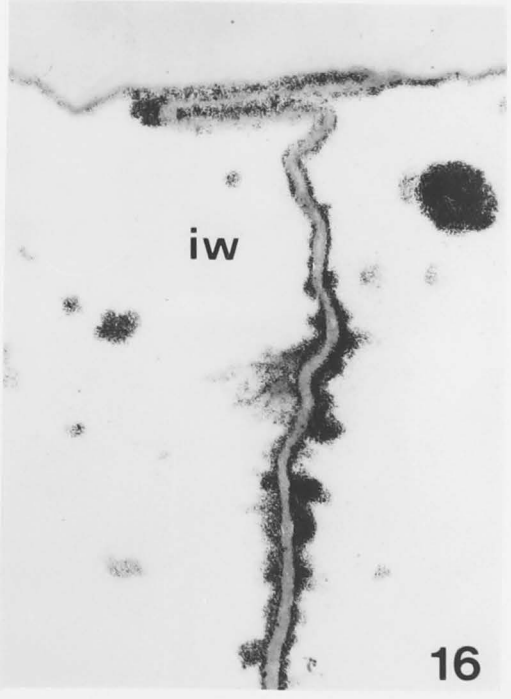
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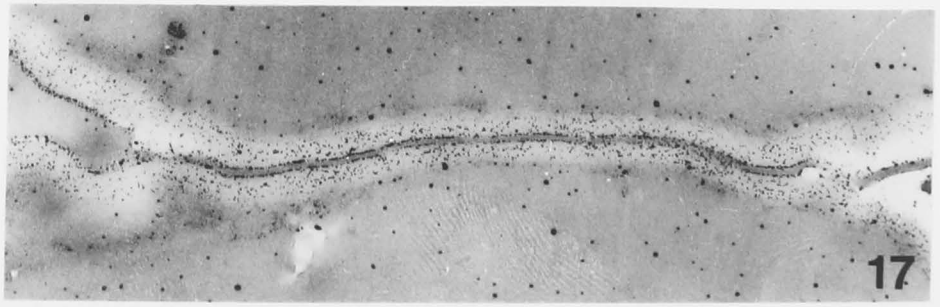
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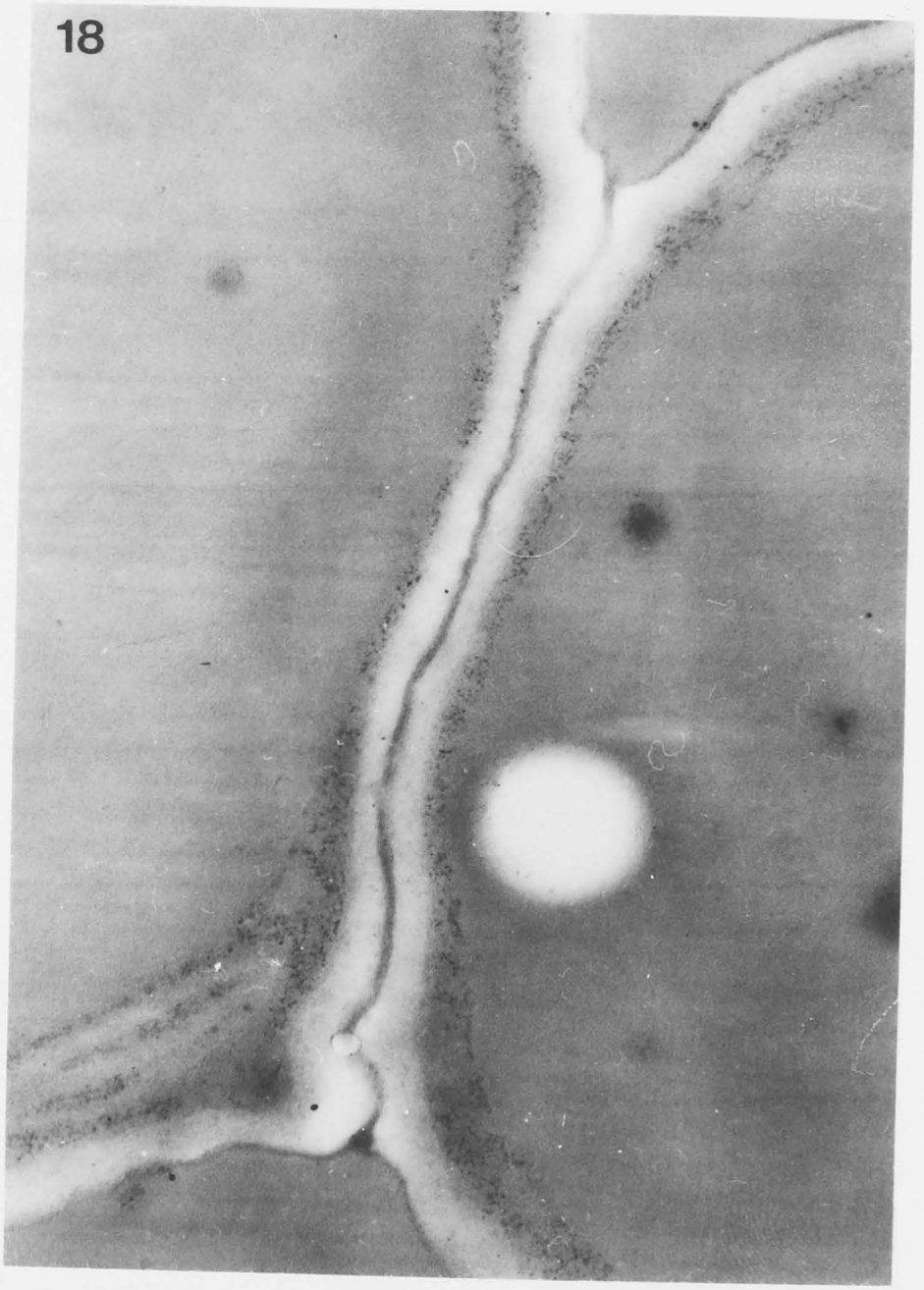
Fig. 18 Section of a silver hexamine treated intercellular junction fixed in glutaraldehyde alone and pretreated by reduction and alkylation. Note the absence of silver staining. X 47,000.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ADDENDUM

TAXONOMY OF *HYDRODICTYON RETICULATUM*

The taxonomy of the genus *Hydrodictyon* is confused. Until 1913 the genus was regarded as monotypic, the single species being *H. reticulatum*, but since then, *H. africanum* Yamanouchi (1913), *H. patenaeforme* Pocock (1937), both from South Africa, and *H. indicum* Iyengar (1928) from near Madras, India have been described, the latter species from a single collection of imperfectly preserved material.

The individual cylindrical coenobia of *H. indicum* were reportedly large (10-16 mm long and 1 mm in diameter), with numerous peg-like ingrowths of the thick cell wall. As Iyengar saw no complete nets, their form, whether cylindrical or flat, is unknown although he described them as "resembling those of *H. reticulatum*." He also reported that mature nets disaggregated as do the coenobia of *H. africanum* (Pocock, 1960).

As Pocock (1937, 1960) points out, the diagnostic value of the specific criteria Iyengar used in erecting *H. indicum* as a separate species is very small. The size of individual coenobia of *H. reticulatum* is extremely variable, depending on environmental conditions. The dimensions of coenobia of *H. indicum* lie within the recorded size range of the coenobia of *H. reticulatum*. In old cultures of *H. reticulatum*, wall-pegs are not uncommon (Marchant and Pickett-Heaps, 1971) and separation of mature coenobia has been observed (Chapter 8). What causes this disaggregation is unknown. Until *H. indicum* is rediscovered

and specific differences between it and H. reticulatum are convincingly established, it must remain at least highly suspect as a distinct species.

Pocock (1937) herself expresses doubts about the validity of considering H. patenaeforme as a distinct species. The difference between it and H. reticulatum can be summarized as follows: first, the material she described as H. patenaeforme has no vegetative reproduction, and hence does not produce a cylindrical net; secondly, its germ net is usually flat whereas, in contrast, her cultures of H. reticulatum as well as reproducing asexually, produces spherical or irregular germ nets each containing 2^9 or 2^{10} cells. However in my experience, germ nets of both Australian and Cambridge strains of H. reticulatum are most often flat, containing usually 2^8 cells (see Chapter 8). Basically then, the principal difference between H. reticulatum and Pocock's H. patenaeforme is that no asexual reproduction has been reported in the latter; in other respects, the two alga appear so similar that one suspects that H. patenaeforme could be a variety of H. reticulatum.

H. africanum differs from H. reticulatum in that its coenocytes are spheroidal or spherical and that the polyhedral stage of the life cycle is often omitted, the germ net forming directly from zoospores derived from the zygospore or azygospore (Wigglesworth, 1927-28). As with H. patenaeforme, no evidence of asexual net formation has been obtained. As well as these differences in their life cycles, the structure of the cell wall of H. africanum and H. reticulatum differs. Northcote, Goulding and Horne (1960) report that the wall of H. africanum has three layers, thin osmophilic outer and inner layers

between which is a massive fibrous layer and the wall is traversed by pores. H. reticulatum on the other hand has a bilayered wall devoid of pores (Marchant and Pickett-Heaps, 1971). There seems little doubt that H. africanum is a true species, distinct from H. reticulatum. Ultrastructural studies on the wall of H. patenaeforme would be interesting to see if it resembled that of H. reticulatum or H. africanum.

SUMMARY

The complex life cycle of the fresh-water alga Hydrodictyon reticulatum has been known to light microscopists for many years but has not been studied with the electron microscope. This thesis reports a detailed ultrastructural study of differentiation in this alga. The vegetative form of H. reticulatum is a hollow cylindrical network of cylindrical coenocytes, each cell joined to usually four others, two at each end. Cytoplasmic cleavage and differentiation produce thousands of tiny uninucleate, biflagellate zoospores which subsequently aggregate within their parental cell wall to form a new cylindrical net. Gametes also develop from the coenobial cytoplasm. Upon release from their parental cell some gametes conjugate, initial membrane fusion being achieved via a specialized structure between the flagella of one conjugant and an apparently undifferentiated site between the flagella of the other. Gametes failing to conjugate often retract their flagella and secrete a cell wall to become azygotes. Zoospores are produced following germination of both zygotes and azygotes. After a brief period of motility these uninucleate zoospores withdraw their flagella and deposit a cell wall, becoming polyhedra. Polyhedra, like cylindrical vegetative cells, become coenocytic by repeated mitoses. Cleavage of the multinucleate polyhedral cytoplasm produces uninucleate, biflagellate net-forming zoospores which aggregate within a spheroidal vesicle, derived from the polyhedral wall, to form predominently flat nets. Vegetative reproduction has been investigated in other algae related to Hydrodictyon, notably Pediastrum, which forms flat colonies of cells. Aspects of the mechanism of patterned cellular aggregation

have been further experimentally studied in these organisms.

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