THE ULTRASTRUCTURE AND TAXONOMY OF PROTOSIPHON BOTRYOIDES (KUTZ.) KLEBS.

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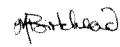
A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, for the degree of Master of Science.

DECLARATION.

I declare that this dissertation is my own unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



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ABSTRACT.

Protosiphon botryoides (Kutz.) Klebs is a unicellular, green, coenocytic, soil alga. The ultrastructure of the vegetative and asexual life history stages of *Protosiphon botryoides* are described, namely the adult sac, zoosporogenesis and zoospores, and two types of resting cell - hypnospores and coenocysts. Although comparative data on resting cell ultrastructure is limited, *Protosiphon*'s coenocysts appear similar to those of other chlorophyll a- and b-containing algae. They do however, possess concentric layers of lipocarotenoids, which are not present in the hypnospores, and have not been found in any other resting cell. Attempts to observe mitosis and sexual reproductive ultrastructure were unsuccessful.

The ultrastructure of *Protosiphon* was studied in order to solve the disputed ordinal affinity of this alga, which is referred either to the Chlorococcales (Chlorophyceae) or to the Siphonales (Ulvophyceae). Chlorophycean attributes include : biflagellate zoospores that exhibit clockwise basal body rotation with a 2-4-2-4 microtubular root system; zoosporogenesis involving cleavage furrows that are guided by a phycoplast; and details of vegetative adult cell cytology. As a Chlorophycean alga, *Protosiphon* can therefore be included in the Chlorococcales, although despite widespread rejection of the systematic schemes of Ettl and Komarek, this study suggests that the Protosiphonales Ettl and Komarek (Chlorophyceae) is a more natural classification.

1. INTRODUCTION.

1.1 AN INTRODUCTION TO PROTOSIPHON BOTRYOIDES.

The original description of *Protosiphon* was made by Klebs (1896) and is freely translated (Thomas 1968) as follows :

> Initially the cells are spherical, later tubular, botryoidal at maturity, consisting of a green, spherical part above the ground and a long, usually unbranched, colourless rhizoid; maximum length, 1.2 - 1.4 mm. Mature cells have a parietal, reticulate, chloroplast, starch-producing pyrenoids and stroma starch, numerous small nuclei distributed through the protoplasm, and a large central vacuole.
>
> Each cell is capable of division : small spherical cells by successive bipartition and tubular sacs by separation of branches from the parent thalli. Under adverse conditions such as desiccation, strong sunlight, or strong hygroscopic salt solutions, the protoplast produces many dormant spores which turn red and endure drying.
>
> Each vegetative cell and spore is capable of producing swarm cells when transferred to water, or when removed from light, if it has been previously grown in nutrient solution. The swarm cells are small, light-sensitive, have two flagella, an eyespot, and contractile vacuoles; copulate in water, in light at temperatures from 1 - 24°C, producing stellate zygotes. In a nutritive solution in the dark or generally at a temperature from 25 - 27°C, the swarm cells become quiescent without copulation and form smooth cells which are immediately capable of growth.

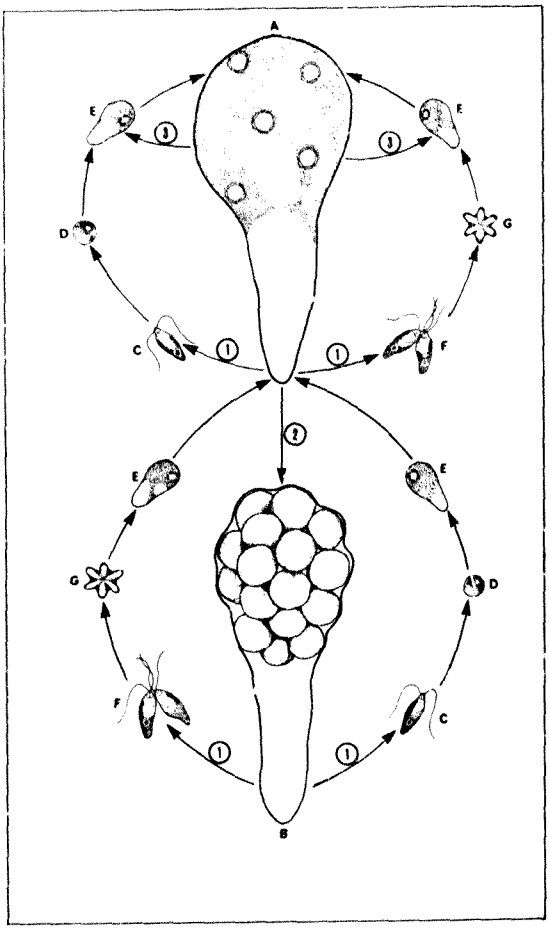

Figure 1. The life cycle of *Protosiphon botryoides*. A - vegetative siphon; B - coenocysts within parental siphon wall; C - zoospore; D - settled zoospore; E - young siphon; F - gametes; G - zygote; 1 - submersion in liquid; 2 - environmental stress, particularly desiccation and nutrient deficiency, 3 - vegetative reproduction via budding.

The alga lives in moist soil, frequently associated with *Botrydium*; it flourishes as well in nutrient solution.

The life cycle (Text figure 1) as elucidated by Klebs (1896), was confirmed and expanded by Bold (1933), Nayal (1933), Maher (1946, 1947a, 1947b), and Thomas (1968). Mature coenocytes reproduce both asexually and sexually. Asexual reproduction involves :

1. budding by septation

2. zoospore formation by progressive cleavage of the parent protoplast. Zoospores are naked and therefore vary in shape, although typically becoming spherical when motility ceases.

3. aplanospore formation. Unreleased zoospores develop into new thalli, eventually rupturing the parent wall.

4. coenocyst (resting spore) production, by

   a. condensation of all or part of the protoplast

   b. equal or unequal cleavage of the protoplast by centripetal furrowing.

   Coenocysts range in colour from green to orange to red, and may develop into thalli if moistened, or form zoospores if submerged.

Sexual reproduction has only been reported in some isolates, (for example, Thomas (1968) found zygotes in only four of the 32 isolates he studied). It involves lateral or apical fusion of isogametes indistinguishable from zoospores, and may be homothallic (Carter 1926; Bold 1933; Maher 1947b) or heterothallic (Nayal 1933; Moewus 1935; Tilden 1937). The resultant

zygotes are stellate or variously lobed, and depending on environmental conditions, they may become dormant or may germinate immmediately to give rise to a vegetative siphon. There is no evidence that meiosis occurs on zygote germination, although Moewus (1935) suggested, without verification, that the siphon is haploid and that zygote germination involves a reduction division. However, both Maher (1947b) and particularly Gowans (1976), have disproved large portions of Moewus's work - and Gowans concludes (Gowans 1976, p327) "In view of the considerable amount of evidence presented and summarized above, it is considered advisable to discount any and all of the published work of Franz Moewus unless such results have been repeated and confirmed independently."

Other publications on *Protosiphon* have not been subjected to such stringent criticism, and will be briefly reviewed.

Much of the published literature pertaining to *Protosiphon* is concerned with the effect of environmental variables on the stages of the life cycle. The optimum conditions for both vegetative growth and reproduction in a number of different isolates, were determined by Maher (1946, 1947a, 1947b) in a series of comprehensive experiments. Later studies concentrated on factors influencing zoospore production and release, such as alkaline earth elements (O'Kelley and Herndon 1961), calcium and strontium (O'Kelley and Herndon 1959; O'Kelley 1965), nitrogen and sulphur (O'Kelley and Deason 1962), light cycles (Stewart and O'Kelley 1966) and light intensity and wavelength (Durant *et al*. 1968). The most important findings of these studies were

1. light inhibits production of zoospores

2. replacement of the dark period of the light : dark cycle with red light promotes zoosporogenesis

3. synchronised zoospore production is facilitated by stirring the cultures in a 36:12 light : dark cycle

4. the optimum temperature for rapid and maximum zoospore production is 25 - 27°C.

5. zoospore production exhibits short wavelength inhibition and long wavelength stimulation.

This last characteristic prompted research into *Protosiphon*'s pigment system. Thomas *et al.* (1975) described a photoreversible system separable into two fractions, one of which contains flavin as a blue light photorecepter. Research continued in the further characterization of *Protosiphon*'s flavoproteins and their functioning (Ross *et al.* 1978). The carotenoid pigments (Strain 1951; Kleinig and Czygan 1969), and carotenoproteins (Berkaloff 1977) of *Protosiphon* have also been analysed, and their possible formation and metabolism described at the ultrastructural level (Berkaloff 1967, 1975).

Berkaloff (1967) has also studied the development of the adult siphon from the 'chlorococcal' stage (which has no discernible longitudinal axis), and has briefly described the mature cell as it appears at the electron microscope (EM) level. The physiological and ultrastructural changes resulting from the addition of streptomycine to culture media have been documented (Berkaloff and Jupin 1974), while other physiological data include :

- temperature and salinity tolerance of viable coenocysts (Nayal 1933)

- a comparison of the physiology and isozyme composition of 32 different isolates (Thomas 1968, 1971; Thomas and Brown 1970).

Thomas (1968) also found that there was no consistent correlation between physiological and morphological variation, so the cause of the variation in adult sac morphology, a widely discussed phenomenon (Bold 1933; Iyengar 1933; Ghose and Randhawa 1933; Maher 1947b; Thomas 1971; De Silva 1975), remains unknown.

1.2 THE TAXONOMY OF PROTOSIPHON BOTRYOIDES.

The generic name reflects the similarity of the sac-like adult cell to the coenocytic cells of the siphonous algae, despite the smaller dimensions of *Protosiphon* (Klebs 1896). The specific epithet refers not only to clustered coenocysts, but is a reminder of the initial confusion of *Protosiphon* with the superficially similar xanthophyte *Botrydium* (Rostafinski and Woronin 1877). There is only one species of *Protosiphon*, although the remarkable variation in sac morphology has prompted some authors to suggest that a number of varieties, or even more species, actually exist (Iyengar 1933; Nayal 1933; Thomas 1968). Thomas (1968) did attempt to describe ten new species based on a combination of factors (morphology, physiology, immunocytochemistry, isozyme analysis), but the variation within and the overlap between 'species' was so extensive, that no descriptions were ever published. However, the main taxonomic controversy surrounding *Protosiphon* does not exist at the species level, but at the ordinal level.

Protosiphon botryoides (Protosiphonaceae) is usually placed in either the Siphonales (West 1916; Setchell and Gardner 1920; Fritsch 1935; Bold 1933; Tilden 1937; Iyengar 1951; Bourrelly 1966; Puiseux-Dao 1966; Gowans 1976;

Ettl 1980)[1] or the Chlorococcales (West and Fritsch 1927; Smith 1950; Strain 1951; Prescott 1969; Thomas 1968; Chapman and Chapman 1973; Deason 1984; Bold and Wynne 1985). The only exceptions are Ettl and Komarek (1982) who created the Protosiphonales with four families- Protosiphonaceae, Hydrodictyaceae, Characiosiphonaceae, Rhopalosolenaceae. However, this order, (which encompasses all multinucleate algae with a central vacuole, that reproduce asexually by naked zoospores/aplanospores, and that do not contain siphonein or siphonoxan.), has not been recognised by other taxonomists (Deason 1984; Round 1984; Mattox and Stewart 1984; Bold and Wynne 1985).

Whether or not *Protosiphon* is included in the Chlorococcales or the Siphonales, depends on the way in which these orders are defined. In a number of systems based on vegetative and reproductive morphology (eg: Bourrelly 1966; Ettl 1980; Prescott 1969), *Protosiphon* slots into either order equally well, and the placing of the Protosiphonaceae appears to be a matter of choice dependent on scientific prejudices. For example, Prescott's Siphonales and Ettl's Bryopsidales are defined using the same features, and yet Prescott places *Protosiphon* in the Chlorococcales and Ettl places it in the siphonous grouping. Bias is especially evident in Fritsch's scheme (1935), where *Protosiphon* conforms with the characterization of the Chlorococcales and yet is placed in the Siphonales, despite

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[1] In this study the term 'Siphonales' corresponds to the Siphonales of Smith (1955); Bryopsidales of Ettl (1980) and Hillis-Colinvaux (1984); Caulerpales of O'Kelly and Floyd (1984a) and Bold and Wynne (1985); Codiales of Lee (1980); and combined Siphonales and Derbesiales of Prescott (1969) and Chapman and Chapman (1973). It is not synonymous with the Siphonales of Fritsch (1935) which is an extremely broad usage encompassing all coenocytic siphonous algae.

the fact that it does not have numerous discoidal chloroplasts - one of the distinguishing features used by Fritsch (1935) for the Siphonales.

These classifications clearly reflect Smith's comment (1933, p523) that "the simplest of the Siphonales integrate with the coenocytic Chlorococcales, and it is a debatable question whether such coenocytic algae as *Protosiphon* and *Codium* belong to the Chlorococcales or to the Siphonales." The problem is exacerbated by the polyphyletic origins of the Chlorococcales, which have caused a number of taxonomists to describe this order as "an artificial group of organisms" (Bourrelly 1966, p125 translated), "a diversified and artificial assemblage " (Prescott 1969, p56), "unnatural" (Fritsch 1935, p178); and including relationships that are "extremely vague" (Chapman and Chapman 1973, p50) and families that "are more or less artificial" (Smith 1950, p222).

In systems that include details on pigment composition, there is more consistency in *Protosiphon*'s position. Strain (1951) first described the presence of two xanthophylls, siphonoxanthin and an ester siphonein, in four members of the Siphonales. *Protosiphon* contains neither pigment and was placed in the Chlorococcales (Strain 1951). Smith (1950) mentions this information when placing *Protosiphon* in the Chlorococcales, but in failing to include it in the definition of the Siphonales, he makes this order an equally possible repository for the Protosiphonaceae. However, the use of this pigment criterion results in *Protosiphon* being firmly placed in the Chlorococcales by Strain (1951), Smith (1955), Chapman and Chapman (1973), Lee (1980), and Bold and Wynne (1985). Bold and Wynne (1985) and Smith (1955) mention both xanthophylls in their ordinal descriptions of the Siphonales, but Chapman and Chapman (1973) and Lee (1980) are more correct in stipulating the presence of siphonein alone, as *Dichotomosiphon* does not contain siphonoxanthin (Kleinig 1969). In addition, there is also a single species of *Caulerpa*, *C. filiformis*, in which both pigments are lacking (Goodwin 1974). So the use of the presence or

absence of siphonein/siphonoxanthin to include or exclude algae from the Siphonales, would appear to have limitations - especially as these xanthophylls are not restricted to the Siphonales (Bold and Wynne 1985).

The methods conventionally used to classify *Protosiphon* at the ordinal level have therefore failed to solve the problem. The morphological criteria selected are largely arbitrary, and differ from one taxonomist to the next; pigment composition is not without exception; the freshwater habitat of *Protosiphon* is of no value in excluding this alga from the Siphonales, as not all members of the Siphonales are marine (Bold and Wynne 1985); and life history cannot be used, as Chapman (1964) has done, as there is no irrefutable evidence concerning the site of meiosis or the ploidy of the cells. One of the aims of the present study was therefore to determine the ordinal status of *Protosiphon*, using ultrastructural characteristics that have recently been shown to have taxonomic significance (Pickett-Heaps 1975; Mattox and Stewart 1984).

1.3 ULTRASTRUCTURE AND GREEN ALGAL TAXONOMY.

The current interest in green algal phylogeny was triggered by the detailed and extensive ultrastructural work by Pickett-Heaps and his colleagues, on cell division in the Charales, Oedogoniales, Zygnematales, and Chlorococcales (Pickett-Heaps 1969 - 1975; Pickett-Heaps and Fowke 1969, 1970; Marchant and Pickett-Heaps 1973; Marchant *et al.* 1973). These studies revealed the diversity in structural detail of cell division, and led to the suggestion (Pickett-Heaps and Marchant 1972) that there are two main evolutionary lines in the green algae : the first characterised by closed mitotic spindles and phycoplast formation during cytokinesis; the second having persistent interzonal mitotic spindles and a

cytokinetic phragmoplast as in higher plants. The first group, Chlorophyceae, initially encompassed the Volvocales, Tetrasporales, Chlorococcales, Oedogoniales, and some Ulotrichales. The second group, Charophyceae, initially included the Zygnematales (Conjugales), Charales, Coleochaetaceae, and *Klebsormidium*, and probably gave rise to higher plants (Manton 1965, Stewart and Mattox 1975, 1978; Mattox and Stewart 1984; Melkonian 1982a; Moestrup 1978).

The existence of the two evolutionary lines based on cell division features, was supported by the results of further research by Pickett-Heaps (Pickett-Heaps 1975), and Floyd, Stewart, Mattox and colleagues (Floyd *et al.* 1972; Stewart *et al.* 1972,1973; Mattox and Stewart 1973; Birbeck *et al.* 1974; Stewart and Mattox 1975). Firstly, with the localization of glycolate oxidase in the Charophyceae but not in the Chlorophyceae; but more importantly, by the distinction of two different types of motile cell. The Charophyceae produce biflagellate motile cells with laterally inserted flagella, where the flagellar basal bodies are associated with a multilayered structure. The Chlorophyceae produce cells with two or more anteriorly inserted flagella, where the basal bodies are associated with four (or more in stephanokonts), cruciately-arranged microtubular roots.

Inevitably, as more studies were completed on the flagellar apparatus/cell division of different algae, examples were found that fitted into neither of the two groups. This resulted in the erection of additional classes (see Mattox and Stewart 1984), of which the Ulvophyceae is the largest and the most clearly defined. Mattox and Stewart (Mattox and Stewart 1977; Stewart and Mattox 1978) formed this class because certain algae had a cruciate flagellar root system but no phycoplast, cytokinesis involving a precocious furrow only. Further characterization of this class was due largely to the efforts of Floyd, O'Kelly, and co-workers (Floyd 1981; Floyd and O'Kelly 1984; Greuel *et al.* 1982; Hoops *et al.* 1982, Taylor *et al.* 1982, 1985; Stuessy *et al.* 1983; O'Kelly and

Table 1. A comparison of the two green algal classes Chlorophyceae and Ulvophyceae (Data drawn from Irvine and John 1984, chapters 2,3,4,10).

	CHLOROPHYCEAE	ULVOPHYCEAE
Cell division.	1)interzonal spindle collapses at telophase and a phycoplast develops associated with a furrow or cell plate. 2)centrioles at, or lateral to spindle poles. 3)always shortening of chromosome-to-mitotic spindle pole microtubules, during anaphase.	1)persistent interzonal spindle; precocious cytokinetic furrow. 2)centrioles lateral to spindle poles. 3)often very little shortening of chromosome-to-mitotic spindle pole microtubules during anaphase.
Motile cells.	1)thecate or naked. 2)radial or near-radial external symmetry. 3)bi-, quadri-, or multi-flagellate. 4)cruciate root system. 5)flagella apically inserted. 6)clockwise basal body orientation with 2 smaller microtubular roots extending in a line across the basal bodies 2 larger roots offset on the outer basal body surfaces. 7)flagellar hairpoint from 1 - 2µm. 8)flagellar stellate structure has a distal : proximal length ratio of 2-3:1. The transverse septum, usually attached only to the distal portion, extends to the flagellar membrane and may be centrally dilated. 9)rhizoplasts may be present in flagellates, never in swarmers.	1)scaly or naked. 2)near-radial external symmetry. 3)bi-, quadri-, or multi-flagellate. 4)cruciate root system. 5)flagella apically inserted. 6)anticlockwise basal body orientation with 2 larger microtubular roots extending in a line across the basal bodies 2 smaller roots offset on the outer basal body surfaces. 7)flagellar hairpoint usually 2µm. 8)flagellar stellate structure has a distal : proximal length ratio of 1-2:1, where the transverse septum is associated either with the proximal part or with neither proximal nor distal parts; or the stellate structure is very elongated and is either continuous with no transverse septum, or divided into proximal and distal parts with no septum. 9)rhizoplasts often present.
Habitat.	1)predominantly freshwater.	1)predominantly marine (but some freshwater forms).
Life history.	1)sexual reproduction nearly always involves production of a dormant zygote and zygotic meiosis.	1)sporic meiosis common and dormant zygotes not known.
Organization.	1)flagellate,coccoid,colonial, sarcinoid, filamentous, parenchymatous.	1)sarcinoid, filamentous, parenchymatous, thalloid, coenocytic, siphonous.
Orders.	Chlamydomonadales, Volvocales, Chlorococcales, Chaetophorales, Oedogoniales, Sphaeropleales, Chlorosarcinales	Ulotrichales, Ulvales, Siphonocladales, Siphonales, Dasycladales.

Floyd 1983; 1984a; 1984b; O'Kelly *et al.* 1984; Floyd *et al.* 1985), and enabled a detailed comparison to be made between the Ulvophyceae and the Chlorophyceae (Table 1).

A point of major significance is that the Chlorococcales are placed in the Chlorophyceae, while the Siphonales are positioned in the Ulvophyceae. This ordinal classification rests largely on mitotic and cytokinetic evidence for the Chlorococcales (provided mainly by Pickett-Heaps, but also Deason and O'Kelley 1979), and on motile cell features for the Siphonales (Burr and West 1970; Moestrup and Hoffman 1975; Hori 1977; Gori 1979; Roberts *et al.* 1981, 1982, 1984; Melkonian 1980b, 1981; Greuel *et al.* 1982). There is only one complete description of a Chlorococcalean flagellar apparatus, that of *Golenkinia* (Moestrup 1972), which is structurally unique among the green algae in that it has no transition stellate pattern, a 9+1 axoneme microtubule arrangement, and no connecting fibres. As *Golenkinia* differs from all other green algae, it is unlikely to be representative of the Chlorococcales. In contrast, the two solitary descriptions of cell division in the Siphonales (Burr and West 1970; Hori 1981), are similar and conform to the Ulvophycean description. The overall affinity of each order, however, is unquestioned.

The aim of the present study is to use this ultrastuctural classification to provide a solution to the ordinal position of *Protosiphon*, as the ultrastructural features of the Chlorococcales (Chlorophyceae) and the Siphonales (Ulvophyceae) are different and well-defined. A study, sufficiently detailed to provide the necessary information for application to the present phylogenetic scheme, would also provide the descriptive data that are still lacking in many green algal orders - relating not only to taxonomically important structure, but also to vegetative and resting life-history stages.

2. MATERIALS AND METHODS.

2.1 SOIL SAMPLES.

Over thirty soil samples were taken from dry pans in the Transvaal East Rand and the Namib - Naukluft National Park (Namibia). The four samples containing *Protosiphon* that were selected for this study were :

1. sample 13 - an extensive undulating pan in the Shangri La Agricultural Holdings, Benoni (28° 24'; 26° 05'). Soil was a yellow clay, and had bear recently disturbed as there was little higher plant vegetation, and numerous mounds of overturned earth.

2. sample 26 - a shallow pan adjacent to the Van Ryn golfcourse in Benoni (28° 21';26° 10'). Soil was friable and richly organic, and the pan was covered by grass and small herbacious plants.

3. sample 33 - a pan on the east side of the road leading south along the Gorob mine air strip (23° 30'; 15° 20'). Finely granular soil with a well - developed layer of surface litter and moribund material.

4. sample 36 - a tree-lined large pan, 12 kilometres past the Gobabeb turnoff near Aruvlei, on the main Walvis-Windhoek road (23° 30'; 15° 00'). This pan also had a litter layer of several centimetres in depth, and the soil was organic.

2.2 ISOLATION.

Five grams of each soil sample were placed in each of 3 Erhlenmeyer flasks containing 50-75ml modified BBMS (see section 2.3 Culturing). Flasks were stoppered with a cotton-wool bung and tinfoil. Well-mixed, 1ml aliquots were pipetted from each flask onto an agar plate (1.5% technical agar in modified BBMS), which was then sealed with a strip of parafilm. All plates and flasks were placed in a Labex growth chamber (Labcon model LTGC) with either a 12 : 12 light : dark cycle at 24°C, or a 10 : 14 light : dark cycle at 17°C. Light intensity was 200-230$\mu E.m^{-1}.s^{-1}$. As soon as *Protosiphon* cells could be distinguished, they were picked up using a micropipette, and transferred to a sterile agar plate. Repeated transfers over several weeks secured unialgal cultures with few bacteria. Three isolates were obtained in this way : isolates 1 (sample 13), 2 (sample 26), and 3 (sample 36). A second Namibian isolate, isolate 4 (sample 33), was kindly provided by Dr. J. Buzer, who also made all her other Namibian soil samples freely available.

Zeiss standard microscopes, both light and dissecting, were used during isolating. All photomicrographs were taken using a Zeiss standard with a camera attachment, excepting figures 1, 43, and 105 which were taken on a Zeiss photomicroscope.

All glassware used was acid-washed (10% HCl), repeatedly rinsed in distilled water, sealed, and autoclaved.

2.3 CULTURING.

Maher (1947a) determined the optimum conditions for vegetative growth as a 10 : 14 light : dark cycle at 17°C, in BBM with a pH between 6 and 7, solidified by 0.5-1.5% agar. Siphons from all 4 isolates were grown under these conditions with some success, but in order to obtain luxuriant growth, a number of additional culture media was tested.

Siphons were harvested by centrifugation (3000rpm for 10min.). 0.5ml was either added to a sterile, acid-washed flask containing 50ml of one of six media, or spread over a 1.2% agar plate made with one of the following six media :

1. Pocock (Pocock 1937) - designed specifically for South African soil algae.

2. Modified Pocock - modified by the addition of 1 part soil-water (McLachlan 1979) and 1 part Alga-Gro (Carolina Biological Supply Company) to 1 part Pocock. (Recommended by Hoffman 1984).

3. Lewin (Buzer, pers. comm., see Appendix 1).

4. Chu no. 10 (Chu 1942).

5. BBMS - with each litre of BBM (Nichols and Bold 1965) containing 60ml soil-water.

6. Modified BBMS - BBMS with 3 times $NaNO_3$ (recommended by Brown and Bold 1964) and .0 times soil-water (as *Protosiphon* is a soil alga).

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All cultures were made in triplicate, and incubated under optimum conditions (Maher 1947a) for 4 weeks. Modified BBMS and modified Pocock promoted excellent growth, BBMS and Lewin supported adequate growth, while cultures in Pocock and Chu no. 10 showed negligible growth. The two modified culture media were therefore selected for all subsequent culturing.

The following growth conditions were imposed in order to determine whether different siphon forms are environmentally induced : 0.5ml centrifugate was transferred onto agar plates (1.5% in modified BBMS). Both Thomas (1968) and Maher (1947a) have suggested that cell density may be important in determining siphon shape, so the centrifugate was

1. spread thickly using a glass slide.

2. resuspended in 1ml modified BBMS and pipetted over the plate.

3. resuspended in 5ml modified BBMS and pipetted over the plate.

A replicate of each of the different cell density plates was then grown in each of the following :

10 : 14 light : dark at 17°C and 230$\mu E.m^{-1}.s^{-1}$.
10 : 14 light : dark at 17°C and 60$\mu E.m^{-1}.s^{-1}$.
10 : 14 light : dark at 25°C and 230$\mu E.m^{-1}.s^{-1}$.
10 : 14 light : dark at 25°C and 60$\mu E.m^{-1}.s^{-1}$.
12 : 12 light : dark at 17°C and 230$\mu E.m^{-1}.s^{-1}$.
12 : 12 light : dark at 17°C and 60$\mu E.m^{-1}.s^{-1}$.
12 : 12 light : dark at 25°C and 230$\mu E.m^{-1}.s^{-1}$.
12 : 12 light : dark at 25°C and 60$\mu E.m^{-1}.s^{-1}$.

Observations were made over 5 weeks. Siphons of different forms were isolated, placed on agar plates, and induced to form zoospores. The siphons resulting from zoospore germination were observed after 4 weeks growth, in the 8 different conditions listed above.

Contamination problems arose with a malfunctioning autoclave, and attempts to purify cultures involved

1. repeated rinsing (Brown and Bischoff 1962)

2. a combination of a number of physical processes including rinsing, ultrasonication, and atomiser inoculation (Wiedeman *et al.* 1964)

3. addition of antibiotics (Cuillard 1979)

None of these was completely successful, and cultures had to be re-isolated from the soil samples.

2.4 ZOOSPORE PRODUCTION.

The method used to obtain zoospores was based on the findings of Stewart and O'Kelley (1966), and Durant *et al.* (1968). However, the use of red light was not possible with the equipment available, and prolonged stirring of the cultures caused morphological abnormalities and so was discontinued. After 36 hours continuous light, large quantities of cells were harvested by centrifugation, and 12ml pellets were resuspended in 75ml fresh modified BBMS or modified Pocock. These thick cultures were then left in the dark at 26°C. Although zoospore production generally began 4 - 5 hours after the onset of the dark period, and peaked from 2 hours before to 1 hour after the light period began, synchrony of zoospore production was never good. Isolate 1 produced more zoospores in better synchrony than the other 3 isolates, and so most zoospores collected for EM study were of sample 13 origin.

Comparison of zoospore production by mature siphons, young coenocysts, and old red coenocysts, was also made.

2.5 GAMETE PRODUCTION.

If *Protosiphon*'s zoospores are facultative gametes as stated by Bold (1933) and Thomas (1968), then it would seem probable that some environmental factor induces conjugation. The only factor not common to both types of motile cell production, is the gamete requirement for culture media with a reduced nutrient status (Maher 1947b). As zygotes appeared in isolate 2 after the very first zoospore production trial run, siphons from this isolate were collected and placed in distilled water at 26°C in the dark. Motile cells were produced but no conjugation occurred. Cultures were observed during motile cell activity, and for 4 days after motile cell production, as zygotes are only distinguishable 2 - 3 days after formation (Maher 1947b; Bold 1933). The initial zoospore production experiment was repeated (so N-rich medium replaced the distilled water), but again only zoospores were seen.

Then followed an intensive series of experiments designed to induce gamete formation and fusion. All environmental factors known to affect sexual reproduction in green algae were considered : N - concentrations, temperature, light duration and intensity, age of the culture, and the particular isolate (Thomas 1968; Trainor 1970; Dring 1974. O'Kelley 1983, 1984; Tiftickjian and Rayburn 1986). *Protosiphon* cells of different ages from all 4 isolates (growing in modified BBMS at 17°C; 10 : 14 light : dark; 230$\mu E.m^{-1}.s^{-1}$) were harvested and placed in a variety of culture media. The freshly inoculated flasks were then incubated at different

Table 2. The combinations of different variables used to induce sexual reproduction.

VARIABLE.	COMBINATION.
Isolate	
Culture age (weeks)	
Culture medium	Modified BBMS; BBM-N (no $NaNO_3$): BBM (no soil extract); Alga-grc; Distilled water;modified Pocock; Lewin.
Temperature	17°C; 26°C; room temperature
Light : dark cycle after fresh medium	12 : 12, 24 hours darkness, then 10 : 14; 24 hours light, 14 hours dark, then 10 : 14.

temperatures and light : dark cycles. The different variable combinations are presented in Table 2.

Only isolate 2 was used in experiments involving shock temperature changes, and light intensity. Four week old cultures were placed in distilled water, BBM-N, or modified BBMS, and

1. subjected to 4 hours at 30°C before being returned to 17°C darkness

2. placed in darkness, or low light intensities (60μE.m^{-1}.s^{-1}), or high light intensities (230μE.m^{-1}.s^{-1}), at 17°C on a 10:14 cycle.

If *Protosiphon* is heterothallic (Nayal 1933; Tilden 1937), then it is possible that one strain of gamete (ie: plus or minus) had been lost during isolation and subsequent subculturing. A new isolate was made from sample 26, and subjected to the series of environmental stimuli alone, and mixed with the original solate 2. Mixtures of all 5 isolates, or paired isolate combinations, were also used. The reisolation of a sample 26 culture precluded the possibility of loss of sexuality due to culture fatigue (Trainor 1985), and the soil-water contained in some of the media was a mixture of soil extracts (from red clay, loam, organically-rich sandy soil), as differences in the composition of soils used to obtain soil-water may be important (Trainor 1970; Moestrup and Hoffman 1975). Two isolates from the Texas culture collection, UTEX 46 and 461, which were known to reproduce sexually 20 years ago (Thomas 1968), were also subjected to gametogenesis induction.

Not a single zygote was produced in any of the 1460 experimental cultures. One possible explanation is that sexual reproduction in *Protosiphon* is controlled by an endogenous rhythm (Dring 1974). It is unlikely that the rhythm is a lunar one, as *Protosiphon* is a freshwater soil alga and not subject to tidal changes. Also, the only zygotes seen were (unwittingly)

produced during the full moon of October 1985, and although a number of later experiments coincided with this lunar phase, no zygotes were subsequently formed. Attempts to obtain zygotes were carried out in October 1986 and 1987 to no avail, so the rhythm is probably not seasonal. It may be based on a set interval of days as in *Derbesia* (Page and Sweeney 1968), but it is far more difficult to determine this in a unicellular alga that no longer exists once motile cells are produced, than in an alga that can periodically delimit many gametangia by septation. The problem of inducing gamete formation and fusion in *Protosiphon* is not unique, as other authors (eg: Ellis and Machlis 1968; Hori and Enomoto 1978a; O'Kelly and Yarish 1980; Trainor 1985; Bold and Wynne 1985; Berger-Parrot *et al.* 1986) have mentioned difficulty in obtaining a particular motile phase in other green algae.

2.6 CYST PRODUCTION.

Cultures of all 4 isolates were subcultured into both modified BBMS and modified Pocock, either in 100ml sterile Erlenmeyer flasks, or on plates solidified with 1.5% technical agar. To facilitate water loss, neither the flasks nor the agar plates were tightly sealed. Culture replicates were left on a window-ledge in full sun, or incubated at 26°C, 230μE.m^{-2}.s^{-1}, 12:12 light : dark cycle. Observations were made twice weekly over a period of 4 months. In order to reduce the time period over which coenocysts developed, and to perhaps induce some degree of developmental synchrony all isolates were also grown in N-deficient BBM, liquid and solid cultures.

2.7 NUCLEAR DIVISION.

Considering the time and the expense involved in EM, light microscopy (LM) was used in an attempt to pinpoint both the life-history stage and the actual time in the diurnal cycle, during which mitosis occurs. A number of different nuclear stains were tested on siphons of 3, 7, 14, and 21 days of age :

1. *Iron alum-acetocarmine* stain designed specifically for green algae (Godward 1948,1966)

2. Giemsa staining techniques (Knox-Davies 1966,1967) and some modifications (Hrushovetz 1956; Duncan and Gaibraith 1973).

The lack of success encountered was not due to failure of the stains, but to the very small size of the nuclei - which Puiseux-Dao (1966) acknowledges as a major drawback in studying multinucleate siphonous cells. Best staining was obtained with Godward's method (Godward 1966) employing a 3:1 95% alcohol : glacial acetic acid fixative, and mordanting in a 1:10 iron alum dilution. But even so, the nuclei appeared as minute scattered dots. Therefore only EM could be used to investigate nuclear division.

This process reportedly occurs just prior to zoospore production in *Protosiphon* (Bold 1933; and implied by Puiseux-Dao 1966) and many other algae (Deason and O'Kelley 1979; Bold and Wynne 1985), so the probability of finding mitotic stages during zoosporogenesis seemed high. Zoosporogenesis was induced as before (section 2.4) using liquid cultures of all 4 isolates of various ages :

- isolate 1 - 3, 8 weeks

- isolate 2 - 3, 4, 6, 9 weeks
- isolate 3 - 6 weeks
- isolate 4 - 3, 8, 50 weeks

Subsamples for EM fixation were taken from each flask, beginning 1.5 hours after the onset of the dark cycle and continuing at 0.5 hour intervals for 5 - 7 hours. After viewing sections made of each isolate of different ages, at each time interval during the dark hours, not a single dividing nucleus had been found. The possibility that nuclear division is extremely rapid, and was occurring in the 0.5 hour intervals between fixations, cannot be ignored - but zoospore production is never synchronous, even by cells in the same culture vessel, and all the nuclei within a cell do not divide at the same time (Bold 1933). It is therefore quite incredible that a nuclear division, however rapid, never coincided with a fixation time in over 100 different samples, of which at least 2 grids were made of each.

A different approach was then adopted. On germination, uninucleate settled zoospores give rise to multinucleate siphons. Nuclear divisions must therefore occur during germination, which is first visible as cell elongation occurring some 3 days after settling. It seems probable that nuclear division accompanies this cell elongation in *Protosiphon*, as it does in *Hydrodictyon* (Marchant and Pickett-Heaps 1972). Vast quantities of zoospores were induced from cultures of all isolates, collected at the beginning of the light period, mixed together, gently centrifuged (2000rpm for 12 min), resuspended in fresh medium, and placed in a series of sterile glass test tubes. One set was kept at 17°C, and the other at 26°C, both on a 12:12 cycle, and 230$\mu E.m^{-1}.s^{-1}$. Samples were fixed over 4 days at 6 hour intervals : at the beginning of the light cycle, beginning of dark cycle, and 6 hours into the dark cycle. No samples were fixed

during the middle of the light period for practical reasons - and nuclear division commonly occurs during the hours of darkness (Godward 1966). The 6 or 12 hour interval during which division occurred could therefore be determined, and fixations could then be made at 15min. intervals during this period in a subsequent experiment.

After 4 days, however, none of the nuclei had divided, as all cells were still uninucleate. No elongation had occurred, and it is probable that normal development was disrupted by submersion of the cells in test tubes which have such a small air : liquid interface. (Test tubes were used to facilitate the ease of fixation in the middle of the dark period, and of harvesting of cells - this being important especially in the latter stages of the experiment when the numerous samples were all at different stages of EM preparation and timing was crucial).

No further attempts were made to obtain mitotic stages, but if such attempts were continued, then the suggested procedure would be to plate out the zoospores before germination, as elongation invariably occurs on a solid substrate.

2.8 ELECTRON MICROSCOPY.

2.8.1 FIXATION AND EMBEDDING.

ZOOSPORES.

Zoospores were collected from the meniscus at the beginning of the light period. Great care was taken not to include adult cells in the pipetted sample, as these created fixation problems. 9ml of zoospores were fixed in one of seven tested methods.

- A - 1ml 25% glutaraldehyde
- B - 1ml 25% glutaraldehyde with 0.025g tannic acid (Cotta-Pereira *et al.* 1976)
- C - 0.5ml 25% glutaraldehyde dropwise over 30min. Further 0.5ml 25% glutaraldehyde for fixation times exceeding 30min.
- D - 0.5ml 25% glutaraldehyde with 0.025g tannic acid added dropwise over 30min. Further 0.5ml 25% glutarladehyde with 0.025g tannic acid for fixation periods exceeding 30min.
- E - 1ml 10% glutaraldehyde (diluted in culture medium).
- F - paraformaldehyde-glutaraldehyde fixative (Karnovsky 1965) modified for delicate algal cells (Appendix 2)

- G - simultaneous glutaraldehyde-osmium tetroxide fixation (Franke *et al.* 1969)

A replicate of fixations A-F was fixed for 0.5, 1, or 4 hours, at room temperature. Fixation G was fixed for 0.5 or 1 hour at 5°C. All samples were then centrifuged at 3000rpm for 10mins to obtain a pellet. The pellet was either processed (C,E,G), or solidified in a drop of warm agar (A-F) : 1g oxoid no. 3 or purified agar in 20ml distilled water or filtered culture medium (0.2µm filter poresize). Once gelled, the agar was cut into blocks. Samples were rinsed 6 times at 15min. intervals in 0.1M phosphate buffer pH 7.3 (Sorenson in Hayat 1981) - A-F, or in 0.1M sodium cacodylate buffer pH 7.2 (Hayat 1981) - A,C,G.

Postfixation utilized 1% osmium tetroxide made up in the same buffer used in rinsing, (ie: either phosphate or cacodylate buffer). Postfixation times of 1 (A-F) or 2 (C,D,G) hours were tested at room temperature (A-F) or 5°C(G). Buffered rinses (6 x 15min.) preceded dehydration at 10min. intervals, through either an alcohol or an acetone series : 10%, 25%, 50%, 75%, 90%, 95%, 2 x 100%. Spurr's resin (Spurr 1969) was used for embedding, with a series of resin : alcohol changes from 1:3 (6 hours), 1:1 (12 hours), 3:1 (12 hours), pure resin (24 hours with 2 changes). Polymerised at 70°C for 8 hours.

Of these fixations, A and B produced a high percentage of plasmolysed cells, and in G no cellular detail was visible because of the black precipitate caused by reduced osmium compounds. Addition of tannic acid did not noticeably improve membrane or microtubule preservation so the added expense is not considered worthwhile. Therefore the 3 fixations that were repeatedly used were C, E, and F, which all yielded equally good fixation. Although some plasmolysed cells occurred in fix C (only some 5%), fixation was good, particularly after 4 hours. A 4 hour fixation time was essential in fix E, but in fix F a 1 hour fixation time was sufficient.

No difference was observed between pellets processed with or without agar, and the ease with which subsequent preparation is carried out when samples are in agarised blocks, strongly supports the use of agar. (Zoospore pellets never compact tightly and have to be repeatedly centrifuged in order not to lose the sample). The use of water or culture medium, and oxoid no. 3 or purified agar, had no effect on the final image. The phosphate and cacodylate buffers were equally efficient, so the phosphate buffer was selected because it is cheaper. No observable difference resulting from different postfixation times was evident (so 1 hour was used), and dehydration in alcohol was as effective as that in acetone (so either was used).

SIPHONS.

Siphons were harvested from liquid cultures by centrifuging (3000rpm for 10min.), and from agar plates by gentle scraping with micropipettes. All siphons were resuspended in 9ml fresh medium. The walled siphons lack the frailty of the naked zoospores, so the more gentle fixations (C-E above) were not used, (nor was the unsuccessful fixation G). Only fixations A, B, and F were tried, of which A and B were the most successful, although equally so. Fixation times of 2, 4, 6, 12, 24, and 36 hours were tested, of which only 24 and 36 hours were adequate.

All siphons were solidified in agar blocks (as in zoospore preparation) and rinsed with 0.1M phosphate buffer pH 7.3, 6 times at 20min. intervals. Postfixation in either 1% or 2% osmium tetroxide for 1, 2, or 4 hours was tried, with the best results obtained after 2 hours in 1% osmium tetroxide. Rinsing again involved 6 buffer changes at 20min. intervals. Dehydration intervals were also of 20min. duration, as 15min. dehydration

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periods were inadequate. Either an acetone or an alcohol series was employed. Resin infiltration changes were carried out every 24 hours (with 2 changes of pure resin over 48 hours). Polymerisation was at 70°C for 8 hours.

CYSTS.

The thick wall that characterizes resistant cells (Col 1983) makes ultrastructural studies exceedingly difficult because of poor fixative penetration and resin infiltration. Attempts to obtain well-preserved cysts of *Protosiphon* involved :

1. fixation with 2.5% or 5% glutaraldehyde for 6, 12, 24, 36, and 48 hours.

2. rinsing in 0.1M phosphate buffer pH 7.3, six times at 30min. intervals, or overnight (preceded and followed by 3 x 20min. changes).

3. postfixation with 1% or 2% osmium tetroxide for 1, 2, or 4 hours.

4. buffer rinsing, 6 x 30min., or overnight (preceded and followed by 3 x 20min. buffer changes).

5. dehydration in acetone or alcohol series at 0.5 or 1 hour intervals.

6. Spurr's resin changes were made every 12 or 24 hours, with infiltration occuring normally or under a vacuum.

The recommended procedure for cyst preparation is 2.5% glutaraldehyde for 48 hours, buffer rinsing overnight, postfixation in 1% osmium tetroxide for 2 hours, overnight buffer rinsing, dehydration at 1 hour intervals, vacuum infiltration with resin changes every 24 hours.

2.8.2 SECTIONING, STAINING, AND VIEWING.

Blocks were sectioned on a Reichert OMU2 ultramicrotome, using glass knives made with an LKB knifemaker. Silver to silver-grey sections were picked up onto copper grids (400 mesh or 0.25% formvar-coated slot grids). Sections were stained for 20-25min. in saturated uranyl acetate, followed by 10min. in lead citrate (Reynolds 1963). Whole zoospore mounts were negatively stained with osmic fumes for 40sec. Sections were viewed on a JEM 100S electron microscope at 80kV.

3 OBSERVATIONS.

3.1 SIPHONS.

3.1.1 SIPHON SHAPE.

All adult siphons grown on agar are immediately recognizable by their sac-like shape which is divided into an upper chlorophyllous region and a basal non-chlorophyllous rhizoidal region (Fig. 1). Siphons grown in liquid infrequently lack this polarity, and the chloroplast extends the length of the cell (Figs. 2,3). This may have been caused by stirring the cultures and so disrupting the normal geo- and phototropic growth of the siphons - such trophic growth occurring in other algae (Bean 1977; Jacobs and Olson 1980; Lembi 1980). Siphons grown continuously in liquid medium also tend to form rounded cells which although coenocytic, have a smaller central vacuole and slightly condensed cytoplasm (Fig. 3). These spheroidal vegetative cells, which differ from coenocysts in their unthickened wall and cytoplasmic vacuolation, were also common in the liquid cultures of Iyengar (1933), Maher (1947a), and Thomas (1968).

None of the four isolates ever produce buds/branches from the upper portion of the siphon, which confirms the conclusion drawn by Maher (1947b) and by Thomas (1971) that this branching ability is dependent on the isolate.

Table 3. Sizes of 4 week old siphons from 4 isolates cultured on 1.5% agar.

ISOLATE	MAX. LENGTH (µm)	AVER. LENGTH (µm)	MAX. BREADTH (µm)
1	1253	1134	143
2	1212	1185	165
	1106	972	383
3	1195	1007	177
4	1291	1218	132
	1150	966	419

3.1.2 SIPHON SIZE.

The size of the adult siphon of the different isolates varies slightly, but not sufficiently for distinction to be made between the isolates because of the overlap in size range (Table 3). A similar overlap occurs in most of the isolates studied by Thomas (1968), but the two studies are not comparable as he measured the siphons two weeks after inoculation ie: before the exponential phase of growth was complete. The siphon sizes are accordingly small, approximately one-third of all other described siphons (Nayal 1933; Maher 1946; De Silva 1975).

Isolates 2 and 4 exhibited two very different siphon sizes, 'giants' (max. 1130 x 419µm; Fig. 4) and 'normals' (max. 1291 x 132µm; Fig. 5). Both cell types could develop from an inoculum consisting only of normal cells, with the unusually large cells becoming distinguishable after 8-10 days, due as much to their extensive bright green chloroplasts and ovoid shape, as to their size (Fig. 6). The zoospores produced by an adult giant cell could develop either into normal cells or into giant cells, under similar environmental conditions. Control of which cell type would develop after inoculation could not be environmentally effected, despite the importance of culture conditions on variation in morphology (Maher 1947a; Thomas 1971). One alternative was that developmental control was genetic, an idea supported by the fact that giant cells were found initially only in isolate 2, the only isolate in which zygotes ever occurred. It was therefore surmised that giant cells were diploids or polyploids, as the greater chromosome number of plant polyploids is usually reflected in larger cell size (Burns 1980). A major problem with the question of heteroploidy however, is the presence of the exceptionally large unicells in isolate 4 which was never seen to reproduce sexually.

However, comparison of giant and normal cell sizes with the dimensions given by Nayal (1933), suggests that normal cells are actually *Protosiphon botryoides* var. *deserti* (Nayal) which are typically long and slender with an approximate ratio of 1-1.5:10 breadth : length. Giant cells fall into the maximum size reported by Klebs (1896) for *Protosiphon botryoides*, which has an approximate ratio of 1:3 breadth : length. In 21 of 32 isolates, Thomas(1968) describes long slender sacs projecting individually or in columns above the algal mass, but he does not include these in the isolate sizes given, and although ascribing these forms to inoculation technique, provides inconclusive evidence for this. These 2 growth forms require further study.

3.1.3 SIPHON STRUCTURE.

The descriptions of siphon structure at the LM level given by Bold (1933) and Nayal (1933) are excellent, and no further information can be added by the present study. At the EM level however, there are a number of details apparently omitted by Berkaloff (1967) in her study of the ultrastructure of the adult siphon. No ultrastructural differences were found between cells of different isolates.

WALL.

The siphon is surrounded by a cell wall that increases in thickness with age. Walls of siphons 2-3 weeks old vary from 0.2-0.6µm in thickness, though difficulty in such measurements exists due to the fact that the

walls slough off layers (Fig. 7), resulting in a very loosely connected fibrillar layer that may be completely (Fig. 8) or partially (Fig. 9) separated from the layers below. Its presence in many sections makes it difficult to determine whether a number of adjacent walls, or only the most recently formed wall, is being measured. A similar shedding of the outer wall layers also occurs in *Boergesenia* (Mizuta and Wada 1981), *Scenedesmus* (Wellburn *et al.* 1980, fig. 20 p51), and *Enteromorpha* (McArthur and Moss 1977), in which this process prevents persistence of epiphytes. The irregularly lamellated wall is composed of microfibrils (10nm diameter) embedded in an amorphous, slightly granular substance (Figs. 10,11). The microfibrils, which are scattered singly or in clusters (Fig. 11), are randomly arranged throughout the ground substance - as are the wall cellulose fibrils of members of the Chlorococcales, Chaetophorales, and Ulotrichales (Dawes 1966; Dodge 1973). This structural similarity, together with biochemical evidence demonstrating the presence of only cellulose fibrils in *Protosiphon*'s wall (Deason 1983), make it probable that the microfibrils seen in *Protosiphon*'s sectioned wall material, are composed of cellulose.

The wall lamellations result from alternating areas of loosely arranged microfibrils with areas of compacted microfibrillar material. During sloughing, the walls split between the the stronger, compacted regions (Fig. 9), and as the upper layers are torn away, the underlying fibrils become disorganized. The granular substance is no longer apparent (Figs. 8,9), presumably because it is mucilagenous (Mackie and Preston 1974) and is rapidly dissolved.

Protosiphon's wall does not resemble that of any member of the Siphonales which, with the exception of *Caulerpa* (Mishra 1969), all have a cuticle and a granular substructure exhibiting little or no layering (Burr and West 1970; Turner and Friedmann 1974; Borowitzka and Larkum 1977; Columbo 1978; Roth and Friedmann 1987). It is similar to that of the

Siphonocladales (Frei and Preston 1961; Hori and Enomoto 1978a, 1978b; Pearlmutter and Lembi 1980; Mizuta and Wada 1981), *Enteromorpha* (McArthur and Moss 1977), and *Acrosiphonia* (Hudson and Waaland 1974), but the layering is not as regular as in these Ulvophycean algae, and the matrix is more granular. Comparison of *Protosiphon*'s wall with members of the Chlorophyceae is more difficult, as some of the Chlorophycean orders do not exhibit the structural coherency that is so pronounced at the ordinal level within the Ulvophyceae. The walls of Chlorococcalean algae may therefore be very regularly layered with a granular matrix as in *Coelastrum* (Morris *et al*. 1973) and *Oocystis* (Robinson and Preston 1972), or amorphous with no layering as in *Pectodictyon* (Lang *et al*. 1987). Some Chlorococcales have an outer trilaminar wall layer resembling a sporopollenin layer (Marchant 1977; Staehelin and Pickett-Heaps 1975; Pickett-Heaps 1975), but this may be absent in other genera and species (Atkinson *et al*. 1972). Despite this variation, *Protosiphon*'s wall most closely resembles the walls of two Chlorococcalean genera, viz. *Tetraedron* (Pickett-Heaps 1975, fig. 3.34, p93) and *Characiosiphon* (Stewart *et al*. 1978), and it is also very similar to that of *Microspora* (Pickett-Heaps 1975, fig. 4.50, p203). All observations confirm Deason's report (1983) of the absence of a crystalline lattice component which typically occurs in members of the Volvocales (Roberts *et al*. 1972; Fulton 1978; Domozych *et al*. 1981a), but which has been found in some genera of the Chlorococcales (Miller 1978; Deason 1983).

GENERAL ARRANGEMENT OF THE CYTOPLASM.

The internal structure of the siphon is typically coenocytic, with a large central vacuole surrounded by a peripheral layer of multinucleate protoplasm (Figs. 12,13,15,16). In most sections this layer is not

confluent with the wall, a situation that is also found in *Characiosiphon* (Stewart *et al.* 1978). In the rhizoidal region the cytoplasmic layer is extremely thin and usually contains only scattered ribosomes and short endoplasmic reticulum (ER) fragments (Fig. 17), but progressively towards the aerial portion of the siphon, more organelles appear in the peripheral cytoplasm. Initially only chloroplast profiles occur (Fig. 18), but as the band of cytoplasm widens, mitochondria, nuclei, polysomes, and an extensive endomembrane system become apparent (Figs. 19,20). Lenticular starch grains also appear in the chloroplast at this stage (Fig. 20), but pyrenoids only develop in the upper third of the siphon. Most other coenocytes show a similar apical predominance of organelles and cytoplasm (Dawes and Rhamstine 1967; Burr and West 1970; Palandri 1972; Columbo 1978; Stewart *et al.* 1978).

CENTRAL VACUOLE.

In young siphons, a number of large central vesicles are separated by thin cytoplasmic threads (Figs. 13,14,15,16), which may contain portions of the reticulate chloroplast (Fig. 13). These vesicles arise by a series of fusions of smaller vesicles (Figs. 14,15,16), and disruption of the cytoplasmic strands between them results in the formation of the large central vacuole typical of the adult siphon. This vacuole is identical to that of *Characiosiphon* (Stewart *et al.* 1978) and *Hydrodictyon* (Marchant and Pickett-Heaps 1971), but differs from that of the Siphonales in that neither an extensive 'vacuome' (terminology of Friedmann and Roth 1977) nor a granular ground substance within the central vacuole, is present. The spherical bodies typical of the Halimedineae (Sabnis 1969; Palandri 1972; Turner and Friedmann 1974; Columbo 1978; Roth and Friedmann 1987), are also absent. *Protosiphon*'s central vacuole is also dissimilar to

those of the Siphonocladales as it lacks the distinct vacuolar layer beneath the cell wall (Scott and Bullock 1976; Hori and Enomoto 1978a, 1978b).

In mature siphons that have reached the stationary phase of growth, some of the smaller vesicles appear to develop from the digestion of membrane-bound osmiophilic spheres (1.0-2.5μm) scattered throughout the cytoplasm (Figs. 21, 22). Intermediate stages of digestion/dissolution resemble polyphosphate granules (Fig. 23), and stain positively with methylene blue-H_2SO_4 (Fuhs 1973), which suggests that polyphosphate bodies are metabolized once phosphorous has become depleted in the culture medium.

CHLOROPLAST.

The extensive chloroplast is reticulate, anastomosing throughout the aerial portion of the siphon (Fig.16). Its ultrastructure varies with the stage in the life cycle, but in the adult siphon it is composed of numerous closely appressed thylakoids which, for the most part, form pseudograna. The term 'pseudograna' was first used by Lembi and Lang (1965) in describing *Carteria*'s chloroplast, but formation of these grana, which do not possess complete discs as defined by Weier *et al.* (1963), was elucidated by Wilsenach (1963). The pseudogranal lamellae are double membranes formed by the invagination and folding of the outer single membrane of the thylakoid at one end. These foldings can become most intricate and interdigitate with those of other thylakoids, resulting in the lamellae of the pseudograna being variously arranged (Figs. 24,25). The pseudograna are connected by intergranal thylakoids which

traverse the finely granular chloroplast matrix singly or in pairs - an arrangement characteristic of the Chlorophyta (Gibbs 1970).

Plastid structure within the green algae is remarkably uniform, with only the Halimedineae displaying both heteroplasty and concentric lamellar structures (Hillis-Colinvaux 1984). *Protosiphon*'s chloroplast is remarkable only in its extensive reticulation, a feature shared with *Hydrodictyon* alone (Bold and Wynne 1985), as all other coenocytic algae possess numerous small plastids or peripheral plastids with reduced reticulation (Chlorococcales - Stewart *et al.* 1978; Sphaeropleales - Cáceres and Robinson 1980; Siphonales - Dawes 1969; Burr and West 1970; Borowitzka and Larkum 1977; Turner and Friedmann 1974; Colombo 1978, Roth and Friedmann 1987; Moestrup and Hoffman 1973; Siphonocladales - McDonald and Pickett-Heaps 1976; Hori and Enomoto 1978a,1978b).

CHLOROPLAST INCLUSIONS.

The chloroplast contains a variable number of plastoglobuli and starch grains (Figs. 24,25,26), and from eight to twenty pyrenoids are also found in the chloroplast of each mature siphon.

Protosiphon's pyrenoid is typically Chlorophycean (Dodge 1973) in that it is internal within the chloroplast and has a densely granular matrix surrounded by a starch sheath (Figs. 26,27). It is polypyramidal with numerous intrapyrenoidal lamellae, more numerous than for most other green algal pyrenoids of this type, with the exceptions of *Chlorococcum polymorphum* (Brown and McLean 1969, fig. 4, p115), *Carteria* sp. (Lembi and Lang 1965, fig. 5, p467), *Closterium littorale* and *Zygnema*

sp.(Pickett-Heaps 1975, fig. 6.70 p394, and fig. 6.41 p 379) - all of which belong to the Chlorophyceae. Though members of the Acrosiphoniales, viz. *Urospora* sp. (Berger-Perrot and Thomas 1982), *Urospora penicilliformis* (Hori and Ueda 1975), *Acrosiphonia* (Hudson and Waaland 1974) and *Spongomorpha* (Hori and Ueda 1967), have many inclusions in the pyrenoid matrix, these are branched tubular structures quite unlike the unbranched convoluted lamellae of *Protosiphon*, which closely resemble distended chloroplast thylakoids. All other Ulvophycean pyrenoids are relatively simple, with few intrapyrenoidal lamellae (Hori and Ueda 1975).

The pyrenoid thylakoids extend from the chloroplast, between the starch grains, and into the pyrenoid matrix. Pads of chloroplast stroma and thylakoids, from which pyrenoidal thylakoids arise, often appear beneath the starch sheath (Figs. 26,30,31,32,33). In *Ulothrix albicans* thylakoids do occur beneath the starch grains (Lokhorst and Star 1980), but these are single thin thylakoids which do not extend into the matrix. The only other reported case of subsheath thylakoids is during starch production in *Scenedesmus quadricauda* (Bisalputra and Weier 1964), where the chloroplast lamellae extend across and beneath the starch grains in order to separate them from the pyrenoid matrix. These chloroplast lamellae never extend beneath the sheath unless at least one starch grain of the new sheath has been formed internal to the existing sheath. This is not the case in *Protosiphon*, where the subsheath thylakoid pads occur when the sheath encircles the matrix without any inwardly projecting grains. *Protosiphon*'s pyrenoid therefore appears to be quite distinctive, but the dissimilarity between *Scenedesmus* and *Protosiphon* may simply be due to the fact that *Scenedesmus* has no intrapyrenoidal lamellae whereas *Protosiphon* does, and this difference is reflected during starch synthesis. The process of starch synthesis in *Protosiphon* appears similar to that described by Reid (1931) for *Chlorococcum infusionum*, Bisalputra and Weier (1964) for *Scenedesmus quadricauda*, Eloranta (1979) for

Monoraphidium griffithii, and Griffiths ('970). The pyrenoid starch sheath is fissured and discontinuous, and composed of large starch grains (1.4 x 4.7µm in cross-section). Lenticular grains occur around the pyrenoid in concentric layers, having been separated after formation at the pyrenoid, by intrusion of the chloroplast lamellae. Stages in this centripetal separation are evidenced by connections between starch grains in adjacent layers (Figs. 16,26).

The intrapyrenoidal lamellae appear in most sections, to consist of a single thylakoid, but in some cases consist of 2 appressed thylakoids whose central lamella does not run parallel to the 2 outer layers, but undulates (Fig. 28). A similar sinuosity occurs in the bithylakoid pyrenoid lamellae of *Chlamydomonas moewusii* (Gibbs 1962) and *Tetracystis isobilateralis* (Brown and Bold 1964). Once the thylakoids enter the pyrenoid matrix they become expanded up to 5 times their normal diameter (Fig.29), and although it is possible that tangential sectioning of 2 thylakoids may result in a pyrenoid with apparently very distended single thylakoids, this intrapyrenoidal lamellar expansion also occurs in other Chlorophycean pyrenoids (Gibbs 1962; Griffiths 1970).

There is however, one feature of *Protosiphon*'s pyrenoid that is distinctive, bearing no resemblance to any published micrographs. In the centre of each pyrenoid, the convoluted thylakoids aggregate forming a layer around a pyrenoid core. This core is composed of a granular matrix, finer than the chloroplast stroma, which becomes increasingly osmiophilic or denser towards the centre (Figs. 32,33). In the middle is an electron translucent sphere which may be either hollow or composed of starch (though this is pure speculation, and studies on pyrenoid functioning may provide some information about the nature of this core). This structure is definitely in the centre of the pyrenoid (and not a section through an irregularly shaped pyrenoid with an interiorly projecting starch grain), as a number of these 'bull's-eye' pyrenoids were seen; the

pyrenoids never have interiorly projecting starch grains; their shape is always circular or slightly ovoid, and 'bull's-eye' pyrenoids consistently have the greatest diameter (5µm across the widest point of the proteinaceous matrix).

NUCLEUS AND ENDOMEMBRANE SYSTEM.

The interphase nuclei are spheroidal to ellipsoidal (1.7 x 1.9µm - 1.6 x 2.6µm), and are perforated by nuclear pores 60nm in diameter (Figs. 34,37). A granular nucleolus occupies the centre of each nucleus, and in some cases has a lightly staining, finely granular portion (Fig. 34), a feature typifying the macrosegregated nucleoli of certain siphonous green algae (Roth and Friedmann 1980). Heterochromatin may be scattered through the nucleus, but is more common as condensed aggregates along areas of the inner nuclear membrane (Figs. 35, 37). The outer membrane of the nuclear envelope is frequently studded with ribosomes, and in some sections is seen to be continuous with long RER cisternae (Figs. 35,36).

ER cisternal lengths occur throughout the adult siphon, frequently lining the plasmalemma (Figs. 16,19) as in the coenocytic *Sphaeroplea* (Caceres and Robinson 1980), but never forming the continuous 'paramural' ER of *Pectodictyon* (Lang *et al.* 1987). A dictyosome of 5-7 cisternae is associated with each nucleus, numerous small vesicles being found between the nuclear membrane and the forming face of the dictyosome (Figs. 35,36). Nucleus-Golgi associations are common in members of the Chlorococcales (Moner and Chapman 1960; Bisalputra and Weier 1964; Griffiths and Griffiths 1969; Morris *et al.* 1973; Marchant and Pickett-Heaps 1971; Marchant 1974a, 1977) and have been reported in 1 genus of the Siphonocladales (Hori and Enomoto 1978b). The ER-Golgi associations that

are characteristic of all members of the Siphonales (Sabnis 1969; Barr and West 1970; Moestrup and Hoffman 1973; Borowitzka and Larkum 1977), also occur in a variety of Chlorophycean algae : *Sphaeroplea* (Cacéres and Robinson 1980); Chlorococcales (Brown and Bold 1964; Deason 1965); and all the Volvocales (see for example Goodenough 1970; Pickett-Heaps 1975; Porcella and Waine 1980). There is therefore little taxonomic value in the origin of the dictyosomal cisternae at the class level, although some orders (eg. Volvocales and Siphonales) exhibit remarkable consistency among their members - so perhaps this feature may be of value at the ordinal level. As is the case in wall ultrastructure however, the Chlorococcales display marked variation in their dictyosomal origins, which again demonstrates the heterogeneity of this order.

MICROBODIES AND MITOCHONDRIA.

Peroxisome-microbody associations are common in a number of green algae (Stewart *et al.* 1972; Silverberg 1975; Roth and Friedmann 1980) but do not occur in *Protosiphon* although microbodies are present. These are generally round, though slightly elongate forms which tend towards a dumbell-shape, are also found (Figs. 16,38,39). The small size of *Protosiphon*'s microbodies (min. diameter = 0.2µm) is matched only by *Botryosphaerium pulchellum* (Silverberg 1975), though the maximum length (1µm) falls into the average size range given by Silverberg (1975). The matrix is finely granular, of moderate electron opacity, and contains no crystalline or nucleoid inclusions.

The microbodies are consistently found in close association with the chloroplast and mitochondria. This proximity may be physiologically necessary, as in higher plants photorespiration entails the transfer of

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glycolate from the chloroplast to the microbody where it is metabolised into glycine. This is then converted into serine by a mitochondrial enzyme, and the serine on re-entering the microbody, is converted into glycerate (Frederich *et al.* 1975). In green algae, several species are known to produce glycolate and to possess a functional glycolate pathway (Merrett and Lord 1973), but there is no consensus that the enzymes of this pathway are housed in the microbody, and variation in the type of glycolate-oxidizing enzyme present in different green algae has contributed to this controversy (Nelson and Tolbert 1970).

Protosiphon's microbodies are usually found in conjunction with ER (Figs. 38,39), which supports the current theory that the ER plays an ontogenetic role in microbody development (Vigil 1970; Frederich *et al.* 1975).

Although some algae have only 1 microbody per cell (eg: *Klebsormidium*, Floyd et al. 1972), Silverberg (1975) describes a number of algae with multiple microbodies, including *Protosiphon*. The present study corroborates this, but the 1:10 ratio of microbodies to mitochondria that Silverberg (1975) obtained from the number of profiles per cell section, far exceeds the numbers observed here. Although growth conditions, culture medium, and stage of development affect the abundance and prominence of microbodies in *Euglena* (Graves *et al.* 1971), Silverberg (1975) maintains that only the enzyme composition and activity of microbodies are affected by these factors. These factors could not therefore account for the disparity in number of profiles in Silverberg's (1975) study and the present one. In any case, environmental conditions of the two studies were the same, and all life-history stages were examined in the present investigation, with the highest profile ratio (1:23) being observed in regreening coenocysts.

In any section numerous mitochondrial profiles are present, varying in shape from circular to dumbell to elongate (Figs. 16,38,39,40). The av-

erage width varies from 0.37-0.5µm. Although reticulation does occur in *Protosiphon*'s mitochondria (Fig. 40), it is impossible to determine the number of mitochondria present within a siphon without serial sectioning, as some unicellular algae have a single branched mitochondrion (eg: *Chlorella fusca*, Atkinson *et al.* 1974), others have 3 different kinds of mitochondria during all life cycle stages (eg: *Polytoma papillatum*, Gaffal and Schneider 1980), while in others many small compact mitochondria grow, ramify, and interfuse during growth (*Chlorococcum infusionum*, Chida and Ueda 1986).

3.2 CYSTS.

3.2.1 TECHNICAL PROBLEMS RELATED TO COENOCYST OBSERVATIONS.

A major difficulty in attempting to describe resting cell ultrastructure is caused by the condensation and compaction of the cell contents, resulting in indistinct membranes and organelles (Fowke and Pickett-Heaps 1971; Cavalier-Smith 1976; Sicko-Goad 1986). This obscurity of detail is evident in *Protosiphon*'s smaller cysts (section 3.2.3) but not in the larger coenocysts (section 3.2.4).

When sectioned and viewed, mature coenocysts contained structures undescribed in the literature (section 3.2.4). In order to determine the origin and formation of these structures, an attempt was made to obtain a developmental sequence of coenocyst maturation, but a second technical difficulty was encountered : the unpredictability of cyst development.

Even in a clonal culture some cysts matured (changed colour) weeks before others, so a study of progressive development was not practical. This lack of temporal organization also foiled an attempted developmental study on *Acetabularia* cysts by Woodcock and Miller (1973). Once the cysts have become orange they all appear similar at the LM level, and yet some are no longer viable while others, from the same culture vessel, are maintaining their state of suspended animation and will germinate if placed in fresh medium. This means that it is impossible to predict whether the cyst sectioned for TEM is viable or not. Gaff *et al.*(1976) maintain that loss of nuclear integrity is synonymous with loss of viability, so in this study of *Protosiphon*, only cysts with double-membrane-bound nuclei are described.

The [illegible]ical problems related to ultrastructural studies on resistant cell[illegible] reflected in the paucity of publications on the subject. Comparisons made between *Protosiphon*'s cysts and those of other algae, do not therefore necessarily indicate taxonomic affinities.

3.2.2 CYST PRODUCTION.

Cysts occur in both modified BBMS and modified Pringsheim liquid and plate cultures, 5 weeks after inoculation, but it takes at least 9 weeks before all cells encyst. Cells in liquid culture change colour at a slower rate than those on agar, frequently remaining green for over 4 months despite possessing a thickened wall and exceedingly dense cytoplasm (Fig. 41). All cysts on solid media are dull orange - bright red after 12 weeks, irrespective of the isolate. Cyst production in BBM-N is more rapid than in N-containing media (first cysts appear after 3.5 weeks), but encystment within [illegible]ulture still extends over 1 month. Thomas (1968) also describes

wide temporal variation in the development of both cysts and secondary carotenoids.

All observations on cyst formation confirm those of Bold (1933), Nayal (1933), and Thomas (1968). Cysts develop

1. by condensation of the entire protoplast to form a single coenocyst (Figs. 41,42).

2. by progressive cleavage of the cytoplasm into a number of walled portions (Figs. 42,43). Cleavage is initially centripetal but the resultant fragments are often bipartitioned equally or unequally, at 90° to the initial furrow (Fig. 43).

3. occassionally the contents of a siphon condense to form a number of spatially separated cysts, (Fig. 44), with the intervening residual cytoplasm being non-functional and eventually disintegrating (Fig. 45).

4. when zoospores unable to escape from the lumen of the siphon, settle within the parent wall and subsequently encyst (Figs. 46,47). So the centripetal furrowing and the cyst formation are temporally separated by a period of motility. These cysts are therefore hypnospores (terminology of Coleman 1983), and they differ from coenocysts in ultrastructure and in size - as the tight packing of the newly settled zoospores prevents expansion, resulting in cysts not much bigger than zoospores (Figs. 47,48).

3.2.3 HYPNOSPORES.

The use of the term 'hypnospores' is validated not only by LM observation, but also by two ultrastructural details :

1. the size of the encysted cells is slightly smaller than that of the zoospores, with hypnospores being 4.6 x 5.5µm on average, compared to 7 x 3µm in the motile stages. The decrease in size reflects the rounding up of the spindle-shaped zoospores, as well as the dehydration and dense packaging of the cell contents.

2. all hypnospores contain a single nucleus - as do newly-settled zoospores.

In contrast, coenocysts are large (average 20 x 23µm) and multinucleate, being formed by cleavage of the multinucleate cytoplasm of adult siphons.

ULTRASTRUCTURE OF THE HYPNOSPORES.

The contents of the mature hypnospores are so compacted that it is extremely difficult to positively identify any organelles other than the nucleus and the occassional mitochondrial profile (Figs. 49,50). Younger hypnospores with less dense contents contain a few vesicles and some membranous tubules, but the cytoplasm is already very granular (Fig. 49). Mature hypnospores do not contain vesicles or tubules, and the roughly spherical nucleus evident in earlier stages becomes irregularly lobed (Fig. 50), this nuclear lobing being common in resting cells (*Gomez et al.* 1974; Wolf and Cox 1981; Santos and Mesquita 1984). No Golgi apparatus

or recognizable chloroplasts are ever seen in hypnospores of *Protosiphon* or *Chlamydomonas reinhardii* (Cavalier-Smith 1976), although reduced chloroplasts do occur in the cysts of *Botryococcus braunii* (Wolf and Cox 1981) and the hypnozygotes of *Chlamydomonas moewusii* (Brown *et al.* 1968).

A peripheral layer of uniform lipocarotenoid globules (0.25µm diam.) develops. Its position presumably relates to the ability of carotenoids to absorb visible light, and so protect the viable organelles against photosensitized oxidation (Goodwin 1980). In older hypnospores, larger globules (0.6 - 1.0µm) also occur within the cell (Fig. 50), but neither peripheral nor intracellular droplets appear to be membrane bound. Although the accumulation of storage products in resistant cells is standard (Coleman 1983), only the zygotes of *Closterium ehrenbergii* exhibit a similar lipid globule orientation (Ueda *et al.* 1985) - though this similarity is less likely to demonstrate a *Protosiphon* - *Closterium* link, than to reflect the dearth of information related to resistant cell ultrastructure.

Though more elaborate than the coenocyst wall (section 3.2.4), the hypnospore wall has a constant structure. It has five well-defined layers, but because the outermost layer cannot be distinguished in younger hypnospores it is possible that this layer is formed by disintegration of the underlying layer, as many hypnospores and hypnozygotes do have an outer layer that dissolves into the surrounding medium (Cavalier-Smith 1976; Coleman 1983; Noguchi and Ueda 1985). Younger hypnospore walls also lack the characteristic spines of mature cyst walls (compare Fig. 49 and Fig. 50).

- The outermost wall layer is thin and electron-transparent, though the boundary is occassionally speckled by extra-cellular deposits (Fig. 51).

- the adjacent layer is bounded by a compacted zone of wall material which is composed of fine fibrils. These fibrils are clearest in the basal areas of the spines, where the second layer is most expanded (Fig. 51). Spine position often reflects corrugations of the 3 inner wall zones and the plasmalemma. This layer is analogous to the alveolate layer of *Chlamydomonas* zygotes (Cavalier-Smith 1976).

- the third layer of the hypnospore wall is composed of a tri-laminar sheath (TLS) (Figs. 51,52), which is sometimes doubled, and occassionally folded back over itself to form small 'cushions' (Fig. 52). The TLS of *Spongiochloris* akinetes exhibits similar conformations (McLean 1968).

- beneath the third wall layer is the most electron dense portion of the wall, a narrow strip (50nm) of extremely fine granular material (Figs. 51,52)

- the fifth wall layer, adjacent to the plasmalemma, is a second TLS (Figs. 51,52). This TLS, like those of the cyst walls of *Polytoma agilis* (Brown *et al.* 1976) and *Botryococcus braunii* (Wolf and Cox 1981), is never doubled or convoluted - and is very difficult to see in sections in which the plasmalemma is still confluent with the wall (Fig. 51).

The hypnospore wall of *Protosiphon* is remarkably similar to the zygote wall of *Chlamydomonas reinhardii* (Cavalier-Smith 1976), differing in only three aspects.

1. the relative sizes of the third, fourth, and fifth wall layers (max. difference only a matter of 25nm).

2. the absence of a pale grey, homogeneous layer next to the plasmalemma in *Protosiphon*, although this layer was not present in *Chlamydomonas* zygotes matured in liquid culture.

3. presence of hypnospore spines.

Spine formation in *Protosiphon* differs from that in *Chlamydomonas moewusii* zygotes (Brown *et al*. 1968) and *Polytomella agilis* cysts (Brown *et al*. 1976) in that only the alveolate wall layer is involved in *Protosiphon*, whereas all wall layers excepting that adjacent to the plasmalemma, are components of the spines in the other two species.

3.2.4 COENOCYSTS.

SIZE AND SHAPE.

Adult siphons consist of an inflated chlorophyllous region which tapers down to a relatively narrow rhizoidal region. Transverse cleavage across the siphon's length results in a number of larger coenocysts being produced apically, while those found in the rhizoidal region are relatively smaller. So although the majority of the cysts fall within the range of 13 x 22µm - 29 x 33µm, cysts as small as 9 x 6µm, and as large as 56 x 42µm, are encountered. These sizes concur with the averages given by Nayal (1933) and Thomas (1968), although the maximum cyst size of 60µm reported by Thomas (1968) was never seen. Coenocyst shape varies from roughly spherical to elongate ovoid.

ULTRASTRUCTURE.

WALLS.

Coenocyst walls vary in thickness from 1-18µm, though generally they range from 2-4µm, with the thickest walls still enclosing cytoplasm (ie: penetrated by fixative), being 10µm. Each wall is constituted by a variable number of lamellations (Figs. 53,54,56), as in the cysts of *Chlamydomyxa montana* (Pearlmutter and Timpano 1984), the oospores of *Bulbochaete hiloensis* (Pickett-Heaps 1975), and the central zones of zygote walls of *Spirogyra verruculosa* (Ogawa 1982). Younger wall layers are composed of material that is compacted at the edges of each layer to give a banding effect similar to, but more pronounced than, the adult siphon wall (Fig.53). Older wall layers become increasingly disrupted and expanded (aptly described by Barkaloff (1967) as 'gonflé'), and are therefore less electron dense, frequently graduating into loosely arranged fibrillar material that dissolves into the surrounding medium (Figs. 54,55). The mature zygote walls of *Closterium ehrenbergii* possess similarly layered walls with increasing density towards the plasmalemma (Noguchi and Ueda 1985). These zygote walls also resemble some of *Protosiphon*'s thicker coenocyst walls in that the outer layer is firmly bound (Fig.56). It is possible that these walls are thicker because they are not continually dissolved away. Although the outermost wall layer may be seen to be composed of fine fibrils (Figs. 53,55), the wall material usually appears amorphous. There is no regularity in the wall stratification, and plugs of convoluted material often occur between the layers of thicker walls (Figs. 57,58). McLean and Pessoney (1971) describe similar excrescences in the akinete walls of *Zygnema*.

PROTOPLASM - GENERAL APPEARANCE.

Berkaloff (1975) described the contents of mature coenocysts as having a definite orientation, with the chloroplast confined to one side, the nuclei, mitochondria and Golgi centrally positioned, and the other pole being filled with the numerous lipocarotenoid globules (LCGs) that give the coenocysts their orange colour. Neither earlier work (Berkaloff 1967) nor the present study confirm this rigid orientation. Some coenocyst cross-sections present cells that could be roughly divided into a LCG- and nucleus-containing region, and a chloroplast- and lipid-containing region (Fig. 59), but such micrographs display too great a variability to support the description of such an inflexible organelle positioning.

No such orientation is ever seen in longitudinally sectioned cells (Fig. 60), which show no regularity in the positioning of the compacted chloroplast portions , nuclei, lipid droplets, or LCGs. Varying degrees of plasmolysis may occur, which reflect a natural state of dehydration and not an artefact, as in many coenocysts the plasmalemma lines the cyst wall (Figs. 59,72). When shrinkage of cell contents has resulted in plasmolysis, vesicles and membranous fragments are found in the intervening space between the wall and the cell membrane (Figs.60,65).

CYTOPLASMIC VESICLES.

One of the most striking features of coenocyst ultrastructure is the amount of vesiculation that occurs in the coarsely granular cytoplasm. Many of the vesicles contain recognizable storage/waste products such as LCGs (Figs. 61,65,67,68) or crystals (Figs. 62,66,68,70), while others

contain remnants of organelles or membranous fragments (Figs. 63,64). Autophagic-like vacuoles with inclusions resembling multivesicular bodies are also present (Fig. 64).

There is a tendency for the vesicles to aggregate, forming clusters with slightly angular tonoplasts due to the mutual compression (Figs. 63,64). Some of the vacuoles fuse with the plasmalemma while others appear to have been exocytosed (Fig. 65). This vesiculation, also described in *Protosiphon* by Berkaloff (1975), is common in old or encysted cells (Brown *et al.* 1968; Bibby and Dodge 1972; Woodcock and Miller 1973; Gomez *et al.* 1974; Wolf and Cox 1981; Ogawa 1982; Santos and Mesquita 1984; Sicko-Goad 1986) and means that the isolated substances can be degraded by autolysis and subsequently recycled in the metabolic pool for re-utilization (Swift and Hruban 1964).

NUCLEI AND ASSOCIATED ORGANELLES

As coenocysts lack the protoplasmic compaction characteristic of the hypnospores, the nuclei are not compressed and lobed, but resemble those of the vegetative siphon, with a size range from (1.6 x 1.75µm) - 1.9 x 2.3µm - (1.5 x 3.2µm), and a double membraned envelope that is pored (Fig. 66). A central nucleolus is usually present and in many cases has a lighter core (Figs. 66,67). Varying amounts of scattered heterochromatin occur, some of which condense as peripheral aggregates on the inner envelope membrane (Figs. 67,68), though not to the extent observed in siphon nuclei. A few ribosomes are attached to the outer nuclear membrane, but an extensive nucleus-associated RER is absent, with only short RER fragments visible in the vicinity of the nuclei and scattered through the cytoplasm (Figs. 66,69,70). The ER that lines the plasmalemma of adult

siphons persists during c formation, but with reduced cisternal length. Dictyosomes are st of only 3-5 cisternae, and have no conspicuous secretic cles (ie: large vesicles with recognizable contents). Such Golgi (Figs. 69,70) are termed 'quiescent' (Mollenhauer and Morré 1966), and are typical of resting cells (Brown *et al.* 1968; McLean 1968; Woodcock and Miller 1973; Wolf and Cox 1981; Coleman 1983).

MITOCHONDRIA AND MICROBODIES.

Mitochondrial profiles are almost all circular, and the elongated shapes of the siphonous stage are seldom present. The partially swollen cristae that occur in some mitochondrial sections (Fig. 71), may be related to loss of coenocyst ability to germinate (Woodcock and Miller 1973).

The identification of microbodies in coenocysts is difficult because of the numerous degenerating organelle fragments which have become very granular after losing their internal membranes. The microbodies that do occur (Figs. 70,71,72) are most frequently found adjacent to lipocarotenoid deposits, which implies that they function as glyoxysomes. These organelles are also found in association with lipid deposits in ageing cells of *Euglena* (Gomez *et al.* 1974), resting cells of *Botryococcus* (Wolf and Cox 1981), zygotes of *Closterium* (Ueda *et al.* 1985), and were reported by Berkaloff (1975) in *Protosiphon*.

CHLOROPLAST.

The reticulations of the adult siphon's chloroplast are lost, and in younger coenocysts the chloroplast consists of compacted thylakoid masses containing starch, lipid, and plastoglobuli (Figs. 59,60,61). The chloroplast no longer occupies the peripheral position evident in the siphon, and many cytoplasmic inclusions and organelles may be found between the chloroplast masses and the plasmalemma (Figs. 60,70,72). Pyrenoids are occassionally present in younger coenocysts, but the surrounding starch sheath disappears,and the pyrenoid matrix with its convoluted intrapyrenoidal thylakoids becomes indistinguishable from the rest of the condensed chloroplast. As a result, no pyrenoids are ever seen in mature coenocysts, an observation also made by Berkaloff (1975). In the mature coenocysts, the condensed chloroplast may remain for some time, but gradually the intergranal thylakoids disappear (Fig. 73) and the pseudograna are pinched off to form independent units surrounded by a double membrane (Figs. 70,72,74,75). Similar individual granal packets are present in the zygotes of *Chlamydomonas mcewusii* (Brown *et al.* 1968), in resting cells of *Botryococcus* (Wolf and Cox 1981), in senescent cells of *Coelastrum* (Chan 1978), and in the akinetes of *Zygnema* (McLean and Pessoney (1971). Whatley (1978) describes chloroplast senescense in higher plants as involving loss of stromal thylakoids and persistence of grana, and Drawert and Mix (1961) demonstrated a relationship between individual pseudogranal formation and metabolic disorder (induced by antibiotics) in *Micrasterias*. So it is likely that the chloroplasts of the above five species of resting cells are degenerating. In *Protosiphon* some portions of the chloroplast appear to undergo senescence even before the pseudograna become separated (Figs. 76,77,78). The thylakoids, particularly those of the intergranal regions, become swollen and increasingly convoluted, giving the chloroplast a somewhat rococo appearance (Fig. 78). Ageing chloroplasts of *Euglena granulata* (Palisano and Walne 1972)

contain unravelled thylakoid structures almost identical to that in figure 77. Slightly distended thylakoids also twirl within the chloroplast of *Chlamydomonas* zygotes (Brown *et al.* 1968), and a more regular, quasi-crystalline, lamellar lattice of unravelled and swollen thylakoids forms in old cells of *Zygnema* (McLean and Pessoney 1970).

Intraplastidial starch grains do occur, but with increasing rarity due to chloroplast fragmentation. The number of cytoplasmic starch grains therefore increases, these storage grains initially being associated with some thylakoid remnants which later disappear (Figs. 69,70,71,72). The increase in starch content is a common phenomenon in resistant cells, occurring for example, in *Acetabularia* cysts (Woodcock and Miller 1973), *Chlamydomonas moewusii* zygotes (Brown *et al.* 1968), *Spongiochloris* cysts (McLean 1968), *Chlorella* cysts (Guerin-Dumartrait *et al.* 1970), *Zygnema* akinetes (McLean and Pessoney 1971), and in *Chlamydomonas reinhardii* zygotes, where the chloropast consists simply of strands of envelope connecting the numerous starch grains (Cavalier-Smith 1976).

Similarly, large accumulations of lipid are characteristic of resistant cells (Coleman 1983), and in *Protosiphon* the lipid is complexed with the carotenoid pigments (Kleinig and Czygan 1969; Berkaloff 1977). Berkaloff (1967, 1975) maintains that the carotenoid-containing lipoidal substances occur mainly as free globules in the cytoplasm (LCGs), lacking membranous boundaries and appearing diffuse, with only the occassional irregular profile present usually in contact with a chloroplast envelope. In the present study, these spherical electron-dense bodies of variable size are present in all coenocysts, but they often appear to be membrane-bound (Figs. 61,65,67,68,72). They are also associated with one of the most intriguing features of coenocysts, which was never reported by Berkaloff (1967, 1975, 1977; Berkaloff and Jupin 1974). Adjacent to the coenocyst wall in virtually all coenocysts, the irregular lipocarotenoid material is arranged in long, roughly parallel strings, often with a 'beaded' ap-

pearance (Figs. 60,61,71,72,75,76). These linear arrays, which can have up to 10 constituent layers, are never centrally positioned in the coenocysts. The sausage-like structures along a single strand are joined by thin electron-dense lines that resemble the half-unit membrane of spherosomes (Yatsu and Jacks 1972). The continuity of the hemimembrane, evident in rare sections, and the whorled appearance of many arrays, reveal the concentric arrangement of these 'sausage' strings (Figs. 72,76).

The formation of these structures is an unresolved problem, though consideration of the theories concerning secondary carotenoid formation in *Haematococcus*, can provide a possible answer. The extraplastidial astaxanthin that accumulates with age in cells of *Haematococcus* is believed to be formed

1. *de novo* from an exogenous carbohydrate source (Donkin 1976)

2. in association with the ER (Lang 1968; Santos and Mesquita 1984). This is based on the observation of presumed astaxanthin within ER cisternae, but production of carotenoid globules from the ER is assumed. However, support for this theory may be found in pollen wall development of *Zumia* (Zavada 1983). Tubular ER profiles filled with dark material develop in tapetal cells when acid phosphatase levels are highest - and it is believed that this enzyme plays a role in esterification of isopentenyl pyrophosphate, a precursor molecule involved in carotenoid synthesis.

3. in the Golgi body (Galil *et al.* 1966).

4. from the products of lipophanerosis of the chloroplasts (Spurr 1970).

In *Protosiphon*'s coenocysts the lipocarotenoids are never seen inside, or associated with, ER- or Golgi cisternae, and although the production

of spherical globules from these sources seems feasible, it is difficult to conceive how the reduced quantities of ER present in coenocysts, or the inactive and infrequent dictyosomes of the resting cells, could produce such extensive concentric strands. However, some micrographs indicate that Sprey's theory of carotenoid production (1970) warrants further consideration. 'Strings' of lipocarotenoids which are not heavily beaded, are found around chloroplast fragments and starch grains, and it is often difficult to distinguish between the hemimembrane on which the lipocarotenoid occurs, and the envelope of the chloroplast or the starch grain (Figs. 61,65,69,70,71,72,76). Carotene aggregations develop on the thylakoid membranes of some higher plant chromoplasts (Harris and Spurr 1969), but in some coenocysts the hemimembrane appears in fact, to be the outer membrane of the chloroplast envelope. The material constituting the chloroplast may be being broken down and deposited around the chloroplast remnant (whether thylakoidal or starch in nature), and as each encircling hemimembrane accumulates sufficient material, it either expands away from the chloroplast remnant's inner membrane, or the chloroplast remnant shrinks. Formation of successive layers would result in the concentric configurations that are evident in some micrographs. This process would also explain :

- the disappearance of the thylakoids around the starch grains released by chloroplast fragmentation.

- the positioning of the lipocarotenoid sheets adjacent to the cell wall. In siphons, from which the coenocysts form, the reticulate chloroplast lines the cell wall. Lipoplasmorosis of the larger chloroplast portions present in the coenocysts, would result in multilayered lipocarotenoids in the position of the former chloroplast. The smaller, irregular, electron-dense patches that occur centrally, are formed around the smaller chloroplast remnants that occur in this portion of both the coenocysts and of the original

siphon. The cytoplasmic packing may be denser centrally (after loss of the central vacuole), and not permit the formation of numerous concentric layers. These non-linear irregular patches do not appear to be associated with a half-unit membrane (Figs. 65,69), but as Yatsu and Jacks (1972) pointed out, the lipoidal nonpolar surface of any half-unit membrane is in contact with the internal storage lipid, and since both oil and membrane are osmiophilic, a distinct delimiting membrane is difficult to discern after osmium tetroxide postfixation.

- the presence of traces of chlorophyll and of galactolipids in the lipocarotenoids of *Protosiphon* (Berkaloff 1977), two substances which are only found within the chloroplasts of other algae.

The spherical, membrane-bound globules may be formed either by budding-off from denser regions of the concentric layers, or by final condensation of the last remnants of a chloroplast fragment (suggested by their central position within a lipocarotenoid boundary) - though it seems improbable that these two mechanisms could produce the numbers of spherical profiles seen. Of course both structures ('sausages' and globules) need not be produced by the same mechanism.

A possible, but less satisfactory explanation for the 'sausage'-strand phenomenon involves lipocarotenoid digestion. Digestion of both spherosomes (Wanner and Theimer 1978) and LCGs (Berkaloff 1975) begins centrally and extends towards the edges, creating annular structures with cytoplasm centrally and the remains of electron-dense lipidic substances around the periphery (Fig. 61). A similar process of lipocarotenoid digestion may take place in planar sheets in *Protosiphon*, where although the profiles are not circular they have the same structure. This does not, however, explain the concentric formation, and also necessitates the formation of sheets of lipocarotenoids prior to digestion. These were

never seen, but their existence cannot be refuted until a complete developmental investigation is carried out.

It is important to realise that in suggesting a lipophanerotic - outer chloroplast membrane mode of concentric layer formation, no biochemical analyses were made on the constituent material, so the assumption that these layers and the LCGs are composed of the same or similar substances, is based purely on their ultrastructure. Both have the same electron density and the same 'floury' texture, and if layers of material exist just below a cyst wall, it would be logical if they were composed, at least partially, of carotenoids, given the photoprotective function of these pigments.

OTHER CHROMOPLAST-LIKE COENOCYSTIC FEATURES.

The process of lipophanerosis has only been studied in higher plants during the conversion of chloroplasts to chromoplasts (Harris and Spurr 1969; Whatley 1978). *Protosiphon*'s coenocysts contain a number of other features characteristic of chromoplasts, in addition to the production of large amounts of carotenoid pigments, loss of typical thylakoid structure, and chlorophyll breakdown (Berkaloff 1975). These features are

1. one or more thylakoid plexes

2. crystals (probably carotenoid crystals)

3. concentric thylakoid lamellae. Though not observed in the present study, they have been described for *Protosiphon* by Berkaloff (1967), and have also been seen in other algal cells in which chlorophyll

concentrations are declining (Brown and Bold 1964; Walne 1967; Chan 1978).

Thylakoid plexes.

These arise from disorganisation of the intergranal thylakoids and are composed of irregularly branching tubules of intergranal thylakoid origin (Spurr and Harris 1968). A number of structures answering to this description are present in a few coenocysts, and have also been found in stationary phase cells of *Anabaena* (Lang and Rae 1967) and cysts of *Acetabularia wettsteinii* (Godward *et al.* 1979). The thylakoid plexes of *Protosiphon* arise in the degenerating chloroplasts between the pseudograna that are in the process of being isolated (Fig. 79). The constituent tubules (30nm diameter) form honey comb-like cells which may be four, five, or six sided, though some of the tubules have extensions that encircle isolated pseudograna or starch grains (Figs. 80,81). It is these elongated tubules that differentiate the plexes from the tubular reticulum that forms the distal cisterna of many dictyosomes (Menge and Kiermayer 1977).

Although superficially similar to the prolamellar bodies formed in mutants of *Chlorella* (Bryan *et al.* 1967; Budd *et al.* 1969), *Chlamydomonas* (Friedberg *et al.* 1971), *Euglena* (Ben-Shaul *et al.* 1963; Salvador *et al.* 1971) and *Scenedesmus* (Wellburn *et al.* 1980), and in *Chara* (Pickett-Heaps 1975) and higher plant proplastids/etioplasts (Whatley 1978), the thylakoid plexes cannot be considered as prolamellar bodies because

1 they develop from the thylakoids in cells in which lamellae already exist (Figs. 79,80,81), and are not derived from perforated lamellar sheets (Henry 1979).

2. they lack the crystalline regularity of prolamellar bodies and so conform to neither the cubic model (Gunning 1965) nor the hexagonal model (Weier and Brown 1970; Ikeda and Toyama 1986) of lattice structure.

Crystals.

In mature coenocysts, crystallization of the lipocarotenoids occurs within the spherical globules. As the crystal size and number increases, the amount of electron-dense material within the membrane decreases, but never vanishes completely (Figs. 66,68,69,70). It is therefore possible that only part of the lipid-pigment complex is crystallizing out. Carotenoid crystals are usually associated with starch grains during their formation in higher plant chromoplasts (Frey-Wyssling and Schweigler 1965; Muhlethaler 1971), but occassionally form within vesicles without a starch association (Ben-Shaul and Klein 1965). Crystal shape varies but is generally needle-like or of flat platelets which may be superimposed to form step-like layers (Ben-Shaul and Klein 1965). These shapes are visible in some of the membrane-bound bodies in coenocysts (Figs. 66,69,70). The possibility also exists that the lipid portion of the lipocarotenoid complex is crystallizing, as Marcenko (1978) describes the formation of crystalline wax bodies in aged *Euglena* cells. These crystals form when the accumulation of lipid reaches saturation point, and consist of aggregations of plates of varying thicknesses often found in association with osmiophilic blobs. An obvious structural similarity

therefore exists between these *crystalloid bodies* and those of *Protosiphon*, but without histochemical studies the crystals cannot be conclusively identified. If formation within a vesicle is of primary importance, then they are more likely carotenoid than lipid in nature, as the wax bodies in *Euglena* form freely within the cytoplasm (Marcenko 1978). Also the presence of small lipid droplets inside some crystal-containing vesicles (Fig. 82) suggests that it is the carotenoids that are crystallizing.

A second type of crystal also forms in mature coenocysts, but it is not related to LCGs and is far less frequent than the first type. It too forms within a membrane, but consists of an aggregation of slender black spines which crystallize out from a large, highly electron-dense, spherical vesicle (0.7 x 0.9µm). The druse-like formation of the crystals (Fig. 62) recalls that of certain calcium oxalate crystals (Franceschi and Horner 1980) which are also formed within membranes or vacuoles (Frey-Wyssling 1981). *Calcium oxalate crystals* are found in some siphonous green algae (Dawes 1969; Friedmann *et al*. 1972; Borowitzka *et al*. 1974), but calcium oxalate sublimates when subjected to the electron beam after TEM preparation (Dawes 1969; Friedmann *et al*. 1972; Borowitzka *et al*. 1974). All other algal crystalline formations (Barton 1967; Bibby and Dodge 1972; Pokorny and Gold 1973; Pearlmutter and Timpano 1984) are composed of non-aggregated electron translucent crystals. Franke (1962) states that many algae, particularly freshwater algae, will deposit crystallized *calcium*, *potassium*, or phophorous salts temporarily under certain conditions including nutrient stress and ageing, so it is possible that the black crystals are composed of one or more of these three elements.

3.3 ZOOSPOROGENESIS.

The stages of *Protosiphon*'s life cycle are notable for their lack of synchrony within a culture (Maher 1947b), and the process of zoosporogenesis is no exception. Coenocysts of the same age, from the same culture vessel, that appear similar under the LM can produce zoospores up to 10 hours apart, while mature siphons can exhibit 4 hour differences in product intervals. So once again a complete sequence of ultrastructural changes proved difficult to obtain.

3.3.1 COENOCYST REGREENING.

Zoospore production by young coenocysts (5 weeks) and by siphons, is more rapid than that by orange-red coenocysts, as these cells regreen at least partially before progressive cleavage - an observation supported by ultrastructural studies. Bold (1933) found that zoospore production by red coenocysts was most rapid, but in all zoosporogenic experiments in this study, red coenocyst production of motile cells lagged by 1.5 - 6.5 hours behind green cells of the same isolate, under the same conditions. Production of gametes from old coenocysts is also slower than that from younger cells (Maher 1947b). The major ultrastructural changes that occur during coenocyst regreening prior to zoosporogenesis involve

1. metabolism of carotenoid globules and irregular carotenoid deposits, a process outlined above and described in detail by Beikaloff (1975). The membrane-bound lipocarotenoids are also digested, leaving numerous residual vesicles (Fig. 83).

2. synthesis of chloroplast membranes. The isolated *pseudograna of ma*ture coenocysts become linked by newly formed intergranal thylakoids and by a connecting chloroplast envelope (Fig. 84). The developing thylakoid systems which are generally associated with one or more *starch grains, frequently* exhibit a triangular arrangement with the enclosed area containing a number of tubular structures (Fig. 85). Smaller numbers of these tubules a so occur in slightly older, but still immature, plastid portions (Fig. 86). *Tubular elements*, circular in cross-section and of a similar magnitude to those in *Protosiphon*, have been observed during plastid development in several green algae, eg: *Volvox* and *Chara* (Pickett-Heaps 1968), *Oedogonium* (Hoffman 1967), *Ulva* (Lovlie and Bråten 1970), and *Closterium* (Pickett-Heaps and Fowke 1970). These tubules are believed to play a role in chloroplast movement during growth or division (Muhlethaler 1971), or perhaps to *have some* other structural or even chemical function (Moestrup and Hoffman 1973).

3. reconstitution of the pyrenoids (Fig. 84). *It could not be ascertained* whether these arose *de novo* or from pyrenoidal remnants, although both production mechanisms may occur within a species (Ueda 1963; Hoffman 1968; Archibald *et al*. 1970).

4. proliferation of the endomembrane system (Figs. 83,87,88). The number of dictyosomes increases, with two dictyosomes often occupying opposite sides of *a nucleus, or occassionally* occurring in the same extranuclear area (Figs. 87,89). A number of dictyosomal replication pathways exists (Robards 1970), of which two are seen to occur in *Protosiphon*'s regreening coenocysts :

   a. breakage and unfurling of the concentrically arranged membranes of ring structures, to form mature Golgi bodies (Fig. 88). The *initial* step of this process, where rings composed of a single

cisterna are present (Maruyama 1965), is also commonly seen during the regreening process (Fig. 83).

b. vertical cleavage through a stack (Fig 89)

This Golgi replication would appear to be associated with the incipient zoosporogenesis, and not only with coenocyst regreening, as each zoospore has to contain a dictyosome adjacent to the nucleus, and many coenocyst nuclei lack this association (section 3.2.4). Large quantities of short tubular fragments, either Golgi- or SER-derived, accumulate in coenocyst cytoplasm (Fig. 83), but this membranous proliferation is confined to the regreening process and does not occur during zoosporogenesis in adult siphons.

It is important to realize that much of the increased Golgi quantity and activity described during zoosporogenesis in some green algae, eg: *Tetracystis* (Brown and Bold 1964), *Carteria* (Domozych 1987), and *Klebsormidium* (Marchant *et al.* 1973), is involved in production of wall material for the walled zoospores. This is not the case in *Protosiphon* which has wall-less zoospores, and in which Golgi quantity and activity increases in regreening coenocysts alone, in order that they contain nucleus : dictyosome ratios similar to those of the mature siphons, prior to zoosporogenesis.

3.3.2 ZOOSPORE PRODUCTION.

Bold (1933) adequately described the process of zoosporogenesis at the LM level (Fig. 100), so the following observations are additional details derived from EM studies. The main ultrastructural developments associated

with zoospore production by siphons and cysts of *Protosiphon* are as follows.

1. as in *Oedogonium* (Pickett-Heaps 1971a), incipient zoosporogenesis is signalled by the appearance of centrioles. In *Protosiphon*, these are paired and form in a concave or flattened area of the nucleus, initially roughly 90° from the dictyosome (Fig. 91), but then organelle migration occurs so that the two centrioles lie opposite the Golgi apparatus at approximately 180° (Fig. 92), the position they occupy in the zoospore. A similar centriole rotation occurs in *Klebsormidium flaccidum* (Marchant *et al.* 1973). Bold (1933) identified centriole formation during zoosporogenesis, and Berkaloff (1967) has described a nucleus-associated centriole pair in a young siphon, but it's impossible to determine from the micrograph or the methods section whether this siphon is undergoing zoosporogenesis or not. In the present study, no centrioles were ever seen in siphons or coenocysts that had not been induced to form motile cells. The absence of centrioles from parts of the life cycle is a well documented phenomenon (Cavalier-Smith 1974; Pickett-Heaps 1971b; Hori and Enomoto 1978b), but as most electron microscopists are aware, it is often easier to find a particular structure than to state with certainty that it does not occur, so it is possible that centrioles are present in vegetative stages.

2. the pyrenoids' starch sheaths are lost, and with the subsequent chloroplast constriction and splitting that is necessary in order to provide each zoospore with photosynthetic apparatus, the intrapyrenoidal thylakoids become indistinguishable (Fig. 90). A similar loss of pyrenoidal integrity has also been reported during zoosporogenesis in *Hydrodictyon* (Marchant and Pickett-Heaps 1971) and *Tetracystis* (Brown and Bold 1964), and gametogenesis in *Bryopsis* (Burr and West 1970), although pyrenoids remain intact during

zoosporogenesis in *Characiosiphon* (Stewart *et al.* 1978) and autosporogenesis in *Pseudochlorococcum* (Archibald *et al.* 1970).

3. the cleavage furrows that divide the multinucleate cytoplasm into uninucleate portions, constitute the cytokinetic apparatus of *Protosiphon*. The mitotic stages that precede cytokinesis were never seen, one of the most disappointing and irritating results of this stage, considering the evolutionary and taxonomic significance of mitotic features. No perinuclear ER envelope was ever sectioned, but migration of the centriolar complex to the internuclear equatorial cytoplasm apparently occurs, as centriole pairs may be opposed across cleavage furrows (Fig. 94). The origin of the furrows is obscure, these often appearing first as an elongated two-membraned structure near the centre of the dividing cell (Figs. 93,95,96,97), as in the coccoid green algae *Kirchneriella* and *Tetraedron* (Pickett-Heaps 1970,1973) and the coenocytic *Hydrodictyon* (Pickett-Heaps 1975). The furrows must arise by invagination of the plasmalemma, as neither ER- nor Golgi-derived vesicles appear to contribute to furrow-membrane formation in adult siphons, as they do in so many other green algae (Wanka 1968; Deason and Darden 1971; Dodge 1973; Wilson *et al.* 1973; Chan and Wong 1975; Pickett-Heaps 1975; Marchant 1977; Deason and O'Kelley 1979; Domozych 1987; Lang *et al.* 1987) excluding the Acrosiphoniales (Hudson and Waaland 1974; Lokhorst and Star 1983), *Bryopsis* (Burr and West 1970), *Cladophora* (Scott and Bullock 1976), *Dictyosphaeria* (Hori and Enomoto 1978b) and *Derbesia* (Wheeler and Page 1974). In all these coenocytic forms, septum formation is by progressive vacuolation accomplished by ingrowing, ramifying, intrusions of the bounding membrane (although figures 18 and 21 p1557 of Scott and Bullock 1976, appear to support Sluiman's theory (1984) of ER-membrane biogenesis). Due to contraction and shrinkage, *Protosiphon* cells undergoing zoosporogenesis no longer possess the central vacuole typical of siphonous coenocytes, so if

cleavage furrows do arise by membranous invagination, this process can only proceed centripetally and not centrifugally from the tonoplast as well. The occassional presence of RER adjacent to a developing furrow (Figs. 94,96) does not necessarily imply involvement in this process, but serves as an indication of the position of RER in mature zoospores - underlying the plasmalemma. A number of other algae also have non-participating RER beside the cleavage furrows, for example, *Stephanosphaera* (Domozych 1982), *Chlamydomonas* (Triemer and Brown 1974), *Carteria* (Domozych 1987), and *Chlorococcum* (Deason 1965). The possibility of ER- or Golgi-derived vesicle participation in furrow formation in coenocysts, cannot be entirely dismissed, as it is not certain whether the membranous proliferation that begins during regreening is entirely divorced from cleavage processes. Direct participation of the membranous fragments in furrow formation was however never seen.

One of the most important features of the dividing septa is the presence of microtubules along the edges of the developing furrows. These microtubules are generally transversely aligned (ie: running parallel to the fissure), although a scattering of cross-sectioned microtubules is present (Figs. 94,95). This cleavage furrow-microtubule association conforms to the definition of a phycoplast given by Lokhorst and Star (1983). In some cases it is possible that presumptive phycoplast microtubules are actually the cytoskeletal microtubules of the zoospores. However, Pickett-Heaps (1975) considers similar microtubule-furrow arrangements in *Hydrodictyon*, to constitute a phycoplast (compare Pickett-Heaps 1975. fig. 3.95 p131; with fig. 94), even though it is not as extensive or as well-developed as that in many other Chlorococcales (compare Pickett-Heaps 1975; fig. 3.10 p77, fig. 3.28 p 87; with the phycoplasts of *Protosiphon* and *Hydrodictyon*).

The furrows are frequently filled with membranous oddments, cytoplasm, and even lipocarotenoids (Figs. 93,95,96), a situation analagous to that in *Hydrodictyon* (Pickett-Heaps 1975), *Bryopsis* (Burr and West 1970), and *Urospora* (Lokhorst and Star 1983).

Contractile vacuoles develop during cleavage, although eyespots only become evident in freely-moving zoospores. Once uninucleate cytoplasmic portions have been cleaved, flagella appear between the zoospores which remain tightly packed for some time. They then begin to separate, until individual zoospores can be seen swimming within the parental cell wall (Figs. 98,99). Abnormal zoospores are common and result from incomplete cleavage in the posterior region (Fig. 101). This is a frequent malformation during zoosporogenesis in the siphonous *Dictyosphaeria* (Hori and Enomoto 1978b), which suggests that the problem may result from the difficulties involved in cleaving the large amounts of multinucleate cytoplasm of coenocytes. It is also possible that the abnormal zoospores in *Protosiphon* are formed in cells that have begun to cleave at the end of the dark period, but that are interrupted by the onset of the light hours, as light inhibits cytoplasmic cleavage in parent cells (Stewart and O'Kelley 1966).

Zoospores are released via an undifferentiated pore that develops in the thinnest wall region of the parent wall (Fig. 99). Dissolution of the pore wall region must begin during zoosporogenesis, as in *Hydrodictyon* (Marchant and Pickett-Heaps 1971), but pore position is variable, and the small size of the opening results in zoospore release being a continual process and not an explosive one. The zoospores of *Characiosiphon* (Stewart *et al.* 1978) are released in much the same way. Both *Protosiphon*'s and *Klebsormidium*'s zoospores (Marchant *et al.* 1973) leave the parent cell chloroplast-end first, presumably so that the flagella can assist in the escape by beating (Fig. 102a-d). The empty walls of parent cells are a common feature of cultures undergoing zoosporogenesis (Fig. 103), while

zoospores unable to penetrate the parent cell wall round up inside the parent and form hypnospores (section 3.2.3).

3.4 ZOOSPORES.

3.4.1 SIZE AND SHAPE.

The contortions of zoospores squeezing through pores in parent cell walls, suggest a high degree of morphological plasticity. This is confirmed by observations of freely-swimming zoospores, which display constantly changing shapes from spherical to elongate spindle-shaped to comma-shaped to ovoid (Fig. 104). This elasticity is due to the fact that the motile cells are naked, being limited only by a plasmalemma. The versatility in shape-change poses a problem when measuring zoospores, a difficulty enhanced by the production of different sized zoospores from different sized parent cells - a feature also noted by Bold (1933) in *Protosiphon* and Birbeck *et al.* (1974) in *Schizomeris*. The range in zoospore size is, therefore, wide (4.0 x 1.7µm) - 6.5 x 2.8µm - (9.1 x 3.2µm), but similar to that reported by Nayal (1933). The minimum length of 3µm given by Bold (1933) and the maximum length of 11µm described by both Bold (1933) and Thomas (1968) were not measured in the present study. The distinction made by Nayal (1933) between elongate cells ('facultative gametes') and rounded cells ('zoospores') does not concur with observations made by Bold (1933) or the present study. Bold (1933) could not distinguish between zoospores and gametes prior to fusion, and in this study neither elongate nor rounded cells could ever be induced to fuse. It is possible that all motile cells are facultative gametes and Nayal could only distinguish them

zoospores unable to penetrate the parent cell wall [illegible] inside the parent and form hypnospores (section 3.2.3).

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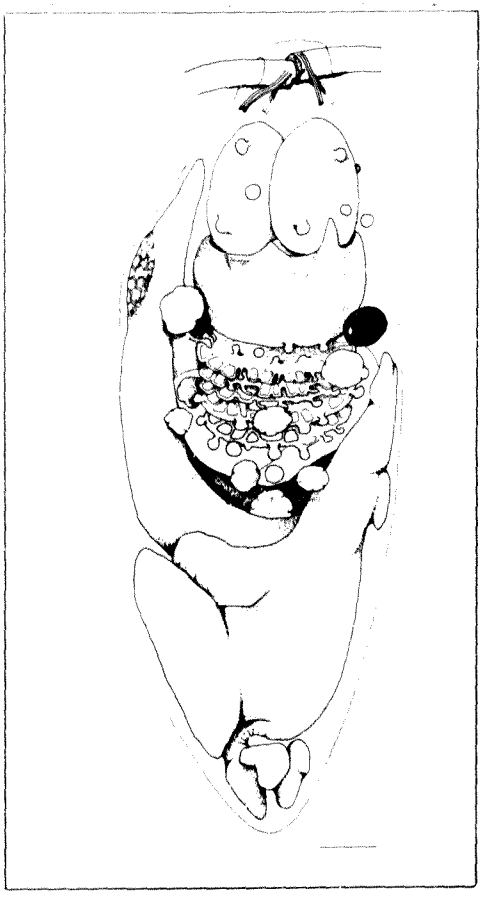

Figure 2. The general morphology of the zoospore of *Protosiphon botryoides*. Flagella are inserted at 90° to the illustrated longitudinal plane. Below them are the two contractile vacuoles, the nucleus, Golgi and central vesicles, and the cup-shaped chloroplast with the anteriorly positioned eyespot. Two microbodies (shaded black), lie at the base of the nucleus. The mitochondrial and ER profiles have not been included in the interests of accuracy. (Scale = 0.5μm).

as 'zoospores' once they became quiescent and rounded up without having fused.

Many of the shape changes are related to swimming movements, as the flagellar positions of zoospores of the same shape are frequently similar. For example, spindle-shaped zoospores with a pronounced 'neck' have flagella raised away from the body and trailing posteriorly, like two ponytails, - the effective power stroke; less elongate zoospores with only a slight apical extension generally have flagella curved backwards like a pair of brackets enclosing, but not touching, the body - the end of the power stroke; ovoid zoospores have flagella that lie flush along the plasmalemma and then curve outwards beyond the end of the zoospore - the beginning of the return stroke. Surprisingly, flagella were never seen to extend directly in front of the zoospore, and the prerequisite basal body positioning at 90°-110° (Ringo 1967) was never apparent in any micrograph.

3.4.2 ULTRASTRUCTURE.

The following ultrastructural descriptions are composite ones based on over 300 micrographs of all isolates (no distinction could be made between the zoospores of different isolates). So although details of organelle distribution are as accurate as possible, changes in zoospore shape must alter the internal organisation, at least slightly, causing minor discrepancies between generalised descriptions and individual micrographs. The lack of serial sections was also sorely felt during attempts to reconstruct the three-dimensional appearance of the zoospores. A simplified reconstruction illustrating general morphology has been drawn (Text fig. 2).

CONTRACTILE VACUOLES.

With the exception of the male gametes of *Dichotomosiphon* (Moestrup and Hoffman 1975) and the Charales (Pickett-Heaps 1975), the motile cells of all freshwater algae always contain one or more contractile vacuoles (Moestrup 1974), so the presence of two of these organelles just below the flagellar insertion in *Protosiphon*'s zoospores is not surprising (Figs. 106-110). When fully distended, the vacuoles (1.1 x 0.7µm) are delimited by the plasmalemma and a thin layer of cytoplasm, which are gradually eroded until the vacuole bursts to release its contents (Fig. 106a-c). There is no apparent synchrony in vacuole contraction, as one zoospore may contain a distended vacuole adjacent to a newly forming one (Fig. 108) but *Protosiphon* lacks the rigid alternation in vacuolar contraction exhibited by *Hydrodictyon* zoospores (Marchant and Pickett-Heaps 1972).

The vacuoles form by fusion of vesicles of various sizes (Figs. 107,109,110). These vesicles are neither coated vesicles as in some other algal contractile vacuole formation (*Cylindrocapsa* (Hoffman 1976); or *Chlamydomonas* (Weiss 1983), for example), nor are they directly derived from the Golgi as no vesicles are ever seen to migrate anteriorly past the nucleus. The contractile vacuoles usually contain small oddments of cellular material derived from

1. vesicles filled with this waste, which is released into the vacuole lumen when vesicle and vacuole fuse (Figs. 110,111).

2. multiple vesicle fusions during which the intervening cytoplasmic portions are pinched off into the resultant vacuole (Figs. 106c,107,109).

Although the two contractile vacuoles are dynamic, their position is constant, and is probably maintained by a framework composed of

1. the four microtubular roots (Figs. 107,109).

2. cytoskeletal microtubules which underlie the plasmalemma (Fig. 107)

3. the nucleus

NUCLEUS AND ENDOMEMBRANE SYSTEM.

The nucleus is roughly spherical though tending to have squared-off corners in section. Two anterior projections, from diagonally opposed corners of the nucleus, curve up around the contractile vacuoles, extending along the pathways of the 2-microtubular roots of the cell (Text fig. 2; Fig. 112). Nuclear projections towards the basal bodies are frequently seen in the motile cells of freshwater algae, for example, *Hydrodictyon* (Marchant and Pickett-Heaps 1972), *Golenkinia* (Moestrup 1972), *Dictyotomosiphon* (Moestrup and Hoffman 1975), *Characiosiphon* (Stewart *et al.* 1978), *Friedmannia* (Melkonian and Berns 1983), and *Atractomorpha* (Hoffman 1984).

Two nuclear hollows support the bases of the contractile vacuoles (Text fig. 2; Fig. 106b), and are separated by a low ridge that dips centrally,

resulting in a concave profile in BLS[2] (Figs. 113,118). Glancing sections through the nucleus confirm the presence of the median ridge and the two hollows (Fig. 114).

Although nuclear shape differs from that in other vegetative stages, structurally it is the same. A nucleolus and peripheral chromatin aggregates are always present. The outer nuclear envelope may have attached ribosomes, while nuclear pores do occur but more rarely than in siphon or coenocyst nuclei (Figs. 113,115). The structural continuity between different life history stages is also reflected in

1. the presence of a Golgi apparatus below the nucleus, where once again the outer nuclear envelope and the Golgi forming face are closely associated (Figs. 112,113,116,118). Some zoospores have two dictyosomes (Fig. 117), which may have arisen by cleavage of one long sinuous cisternal stack (Fig. 118). The position of the Golgi below the nucleus is constant in all Chlorophycean and Ulvophycean freshwater motile unicells having fixed organelle positioning (ie: excluding certain species of *Chlorosarcinopsis* -Melkonian 1977,1978), namely *Dichotomosiphon* (Moestrup and Hoffman 1975), *Characiochloris* (Lee 1974), all Sphaeropleales (Hoffman 1984; Buchheim and Hoffman 1986), *Characiosiphon* (Stewart *et al.* 1978), *Desmotetra* (Deason and Floyd 1987), *Sorastrum* (Marchant 1974a), *Uronema belkae* (Floyd *et al.* 1980), *Fritschiella* (Melkonian 1975), and *Draparnaldia* (Bakker and Lokhorst 1984). In all motile marine cells possessing a Golgi body, the dictyosomes are stacked between the nucleus and the basal bodies, for example, *Enteromorpha* (Evans and Christie 1970), *Gayralia* (Hoops

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[2] BLS - the plane of section passes longitudinally through the basal bodies, in the plane of the flagellar beat. TLS - the plane of section is at right angles to the plane of flagellar insertion.

et al. 1982), *Entocladia* (O'Kelly and Floyd 1983), *Bryopsis* (Burr and West 1970), *Ulothrix zonata* (Sluiman *et al.* 1980), *Urospora* (Kristiansen 1974), *Chaetomorpha* (Bakker and Lokhorst 1985), and *Monostroma* (O'Kelly *et al.* 1984). This remarkably rigid division in ultrastructure must be related to Golgi functioning in different environments, or the absence of contractile vacuoles in marine cells (where the Golgi presumably functions in their stead). The only exceptions, *Microthamnion* (Watson and Arnott 1973) and *Friedmannia* (Melkonian and Berns 1983), belong to the Pleurastrophyceae which exhibit an intriguing mixture of features related to both present freshwater ecology and to their marine ecological origins (Mattox and Stewart 1984)

2. extensive peripheral RER that lines the plasmalemma both anteriorly and posteriorly (Figs. 109,116,119,130). Peripheral ER is a common feature of motile cells from a variety of algal orders including the Ulvales (Evans and Christie 1970; McArthur and Moss 1977), Ulotrichales (Sluiman *et al.* 1980), Chlorococcales (Moestrup 1972; Marchant 1974a; Stewart *et al.* 1978), Chaetophorales (Melkonian 1975), and Siphonales (Roberts *et al.* 1981). The widespread occurrence of this cellular feature means that it is of little value in delimiting groups of algae.

The zoospores generally contain a number of vesicles filled, presumably, with waste material. These vesicles are clustered centrally, which suggests a dictyosomal origin (Figs. 112,116,120). Vesicles are also found amongst the chloroplast lobes in the posterior portion of the cell, an area often filled by large lipid or lipocarotenoid deposits which tear and smudge easily during sectioning (Figs. 116,121). Birbeck *et al.* (1974) and Melkonian (1975) also encountered this problem when sectioning zoospores produced by old vegetative cells. In addition to lipocarotenoids, vesicles may contain crystals (found previously in

coenocyst cytoplasm), irregular electron-dense wastes, or osmiophilic granules reminiscent of eyespot globules (Figs. 120,121,122,123). It is unlikely however, that these droplets are associated in any way with the eyespot, because they are extrachloroplastic and also occur in male gametes of *Sphaeropl* which lack eyespots (see Buchheim and Hoffman 1986 fig. 4, p178). Posterior aggregations of vesicles are common in both male gametes and zoospores (Evans and Christie 1970; Marchant and Pickett-Heaps 1972; Moestrup 1972; Marchart *et al.* 1973; Watson and Arnott 1973; Melkonian 1975, 1978; Hori and Enomoto 1978b; Floyd *et al.* 1980; Herth *et al.* 1981; O'Kelly and Floyd 1983; Roberts *et al.* 1984; Bakker and Lokhorst 1985; Greuel and Floyd 1985), and are probably an indication of the high metabolic rates of these active cells. They are frequently visible at the LM level in *Protosiphon*, as hyaline droplets at the distal ends of the zoospores (Fig. 104).

CHLOROPLAST, EYESPOT AND PYRENOID.

The mass of the chloroplast is located in the posterior region of the zoospore, but it is longitudinally lobed and fissured, and is cup-shaped, so lobe portions extend along the plasmalemma towards the anterior end of the cell (Text fig.2). One of the anterior lobes extends to the base of the contractile vacuoles, and it is in this lobe that the eyespot is embedded (Figs. 112,121,124), a characteristically Chlorophycean position (Melkonian and Robenek 1984). In structure, the eyespot is also typically Chlorophycean (Dodge 1973). It is uniseriate, being composed of a single layer of hexagonally packed carotenoid globules. These globules (max. diam. 0.1µm), which fall into the average size range given by Arnott and Brown (1967), are closely packed inside the chloroplast envelope (Fig. 124 inset), which is in turn tightly appressed against the plasmalemma,

forming a small lateral bulge. Such bulges are often formed in association with eyespots (eg: in *Fritschiella* (Melkonian 1975; and in *Uronema belkae* (Floyd *et al.* 1980). *Protosiphon*'s eyespot lies in the plane of the flagellar beat, and it is therefore never seen in TLS sections. This alignment is also found in *Chlorosarcinopsis* (Melkonian and Robenek 1980), *Tetraselmis* (Arnott and Brown 1967), and *Microthamnion* (Watson 1975), and according to Watson (1975) is indicative of a microtubular root-eyespot association. These associations involve an X-microtubular root of an X-2-X-2 system (for a review see Melkonian and Robenek 1984), and in *Protosiphon* the eyespot does lie in the path of the 4-microtubular root. No association was seen though between the structures.

The lobes of the chloroplast also contain stromal starch, infrequent plastoglobuli, and one central or two peripheral pyrenoids (Figs. 112,113,125). The assertion made by Moewus (1935) that the presence of pyrenoids in zoospores depends on the interaction of both genetic and nutritional factors, was disproved by Maher (1947b), although she too found that some zoospores had pyrenoids whilst others lacked them. EM has solved this apparent inequality. Pyrenoids develop *de novo* within each zoospore's chloroplast, and until the formation of a circular starch sheath is completed, the pyrenoids are not distinguishable at the LM level. The lack of synchrony in zoospore production exacerbates the problem, as primarily released zoospores can form pyrenoids before younger zoospores have even escaped from their parent cell. Iodine staining at this stage would therefore reveal some zoospores with pyrenoids and others without. The pyrenoids arise in a manner similar to that described in *Oedogonium*'s zoospores (Hoffman 1968), *Bulbochaete*'s zoospores (Retallack and Butler 1970), and *Volvulina steinii* (Nozaki *et al.* 1987):-The initial differentiation of a dense region within the chloroplast stroma (Fig. 123); the development of intrapyrenoidal thylakoids(Figs. 126,127); and the progressive production of a starch sheath (Figs. 128,129). In both *Protosiphon* and *Oedogonium* (Hoffman

1968), there is an association of chloroplast microtubules with the developing pyrenoids (Fig. 126). Some cells contain two pyrenoids, although this is infrequent (Fig. 125).

MITOCHONDRION AND MICROBODIES.

The diversity of descriptions of the mitochondrial apparatus of motile reproductive cells reflects the incompleteness of most studies, in that there is only one absolute mitochondrial configuration based on serial sectioning - that of *Friedmannia* zoospores (Melkonian and Berns 1983). Lack of serial sectioning in the present study also means that no complete description of the mitochondrion (ia?) can be given. However, parallels in mitochondrial structure and position exist between diverse green algal orders, suggesting that this organelle has little taxonomic value. For example, the mitochondrial apparatus of the motile cells of *Chlorosarcinopsis* (Melkonian 1977), *Atractomorpha* (Hoffman 1984), *Fritschiella* (Melkonian 1975), *Entocladia* (O'kelly and Floyd 1983), *Ulva* (Melkonian 1979), and *Derbesia* (Roberts *et al.* 1981), is remarkably similar in random sections.

In *Protosiphon*'s zoospores the most extensive mitochondrial profiles are aligned parallel to the long axis of the cell (Figs. 120,130,131). Although occurring between the plasmalemma and chloroplast in the posterior portion of the zoospores, most of the mitochondrion is found anteriorly, in the more dynamically active half of the cell. Here the mitochondrial branches extend beyond the nucleus along the 2-microtubular root pathway, to form a simple cupping structure around the contractile vacuoles (Figs. 131,141). In so doing, *Protosiphon*'s mitochondrion resembles that of

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Friedmannia (Melkonian and Berns 1983), *Platydorina* (Taylor *et al.* 1985), and to a lesser degree *Acetabularia* (Herth *et al.* 1981).

One or two small microbodies lie below the nucleus along the edge of the centrally positioned Golgi body (Figs. 115,121,124,125). They are closely associated with the mitochondrion and the nucleus, as is the case in all microbody-containing motile cells (ie: *Chaetosphaeridium* (Meesters 1974), *Bryopsis* (Hori 1977), *Ulothrix zonata* (Sluiman *et al.* 1980), *Friedmannia* (Melkonian and Berns 1983), and members of the Sphaeropleales (Hoffman 1984; Buchheim and Hoffman 1986).

3.4.2.2 FLAGELLAR STRUCTURE.

The two isokont flagella are apically inserted in a small pyramidal mound above the contractile vacuoles. The flagella extend beyond the zoospore body, being 1.5-2 times the zoospore length (usually 11µm).

FLAGELLAR TIP.

Protosiphon's flagella are acronematic (terminology of Leflandre 1934), and possess a 2-stranded hairpoint (Figs. 132,133). In rare sections through flagellar tips, the hairpoint is seen to have a length of 1-2µm, which is typical of the Chlorophyceae. Ulvophycean hairpoints measuring 7µm (Melkonian 1984). Although negatively-stained preparations were of little value in determining hairpoint length due to cell shrinkage, they did serve to indicate that the flagellar tip and shaft surfaces are naked

(Fig. 113), this being the usual appearance of Chlorophycean flagella (Moestrup 1982), with the notable exception of *Chlamydomonas* (Ringo 1967). The A-tubules extend further up the tip than do the B-tubules, and the central microtubule cap is evident as a dense area at the very tip of the flagellum. The flagellar matrix is clearer than in the flagellar shaft (Fig. 132).

FLAGELLAR SHAFT.

This is one of the most unvarying components of the flagellar apparatus (see Moestrup 1982). In cross-section, the typical 9+2 axoneme structure is apparent, with dynein arms linking A and B tubules of adjacent doublets (Fig. 138a). The delicate α, β and γ extensions from the central microtubule pair (CMP) are clearly evident in all sections (Fig. 138a). The radial spokes and secondary fibres extending from the A tubules can occassionally be seen in cross-sections, though they are always visible in longitudinal sections, resulting in the characteristically mottled matrix of the flagellar shaft (Fig. 134). These radial spokes and their extensions to the CMP are frequently paired, with each pair lying at the same angle to the CMP (Fig. 134). No B-tubule septations occur, although this is not necessarily a Chlorophycean indication (Melkonian (1984) and O'Kelly and Floyd (1983) having rejected B-tubule septation as a purely Ulvophycean characteristic.)

TRANSITION REGION.

A stellate structure is present in the transition region but the V-shaped filaments extending from the central hub to the A-tubules are not always as clearly defined as they are in many other green algae (Moestrup 1982). Instead they tend to be C-shaped with only a small spine extending from the convex curve of each 'C' to each A-tubule (Fig. 138b). This rather unusual appearance correlates with longitudinally sectioned stellate regions which exhibit a central 'hub' with two distinct lines (the tips and the curve of each 'C') and then a very faint region between the outer dark line and the A-tubules (the 'spines') (Figs. 135,136). Sections having continuity between the innermost line and the A-tubules (Fig. 137) are glancing sections along the curve of the 'C'. It is possible though, that the crudity of *Protosiphon*'s stellate pattern is due to inadequately prepared material.

A well-defined transverse septum is attached to the distal part of the stellate region, in the manner of Chlorophycean algae (Melkonian 1984). It lacks a central dilation, and although some median longitudinal sections suggest that it is lightly striated (Fig. 136), a fortunate cross-section through the septum reveals only a roughly granular substructure (Fig. 138c). The septum divides the transition region into two components, with the distal : proximal length ratio being 2:1. This is consistent with the 'Chlorophyceae' septum type of Melkonian (1984), as is the extension of the septum to the flagellar membrane (Fig. 137). The length of the transition region is 100nm, but this alone cannot be considered a useful quantitative characteristic, because of the large amount of overlap in size ranges of different algal groupings (see Melkonian 1984).

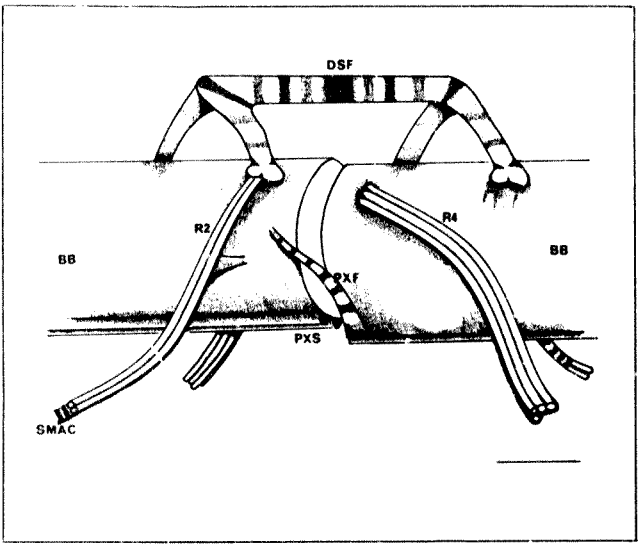

Figure 3. A reconstruction of the flagellar apparatus of the zoospores of *Protosiphon botryoides*. BB - basal body; DSF - distal striated fibre; PXF - proximal fibre; PXS - proximal sheath; R2 - two stranded microtubular root; R4 - four stranded microtubular root; SMAC - striated microtubule associated component. (Scale = 0.1µm).

BASAL BODIES AND ASSOCIATED STRUCTURES.

The two basal bodies of *Protosiphon*'s zoospores exhibit clockwise rotation with 180° rotational symmetry, and do not overlap (Figs. 139,140,142). The basal bodies are displaced by two-thirds of the width of one basal body, so that when viewed from the zoospore's anterior end, only the left one-third of basal body 1 and the right one-third of basal body 2 are in the same plane. The two-stranded microtubular roots extend into the area of basal body displacement, while X-stranded roots are restricted to the right and left sides of basal bodies 1 and 2 respectively (Figs. 139,140,142). There are no accessory basal bodies, and terminal caps are not apparent. (Text figure 3; and see appendix 3 - comments on flagellar terminology).

The internal structure of the basal bodies resembles that of most other green algae (Moestrup 1982; Melkonian 1984), displaying both triplet and cartwheel patterns (Figs. 138d,138e,135,137,141). Tangential sections at the junction of transition and basal body regions (Fig. 138d) reveal the presence of the transitional fibres described by Cavalier-Smith (1974).

The basal bodies are connected along their anterior surfaces by a broad distal fibre that is characteristically striated with the striation pattern resembling that of *Chlorosarcinopsis* (Melkonian 1978) most closely (Fig. 143). It has a central structure composed of a wide dense band subtended on each side by a narrow line. Paired striations occur on each side of this central structure, with the inner line of each pair often being darker than its associated striation (Figs. 136,143). The fine filaments that constitute the distal fibre, run along its length perpendicular to the striation bands (Figs. 143,144,145). The distal fibre appears, in median longitudinal sections, to attach to each basal body at two points : one in the region of the C-tubule appearance, the other

at the proximal end of the cartwheel formation (Figs. 136,143,146). There are no published descriptions of a distal fibre with four attachment points, although *Chlorosarcinopsis* (Melkonian 1978, fig. 13 p271) is rather similar, although tangentially sectioned. But Melkonian (1978) states that the distal fibre is attached to only the C-tubules of two triplets in each basal body, these attachments being in the same basal body cross-section (Fig. 50, p277). One possibility is that there is only one direct connection between the distal fibre and each basal body, this being the distal connection at the C-tubule origin. The proximal connection would be indirect, and formed by the ends of the microtubular roots and the associated electron dense material, as suggested by Fig. 145. This however, presupposes that each 2-stranded microtubular root approaches the inner face of a basal body and attaches to the anterior surface of that basal body, in order that the correct angle between distal fibre and inner 'foot' is obtained. Cross-sections of such an arrangement would therefore reveal THREE microtubular roots per section. This is the case in *Derbesia* (Roberts *et al.* 1981, figs. 33 and 35 p338) in which the type 1 roots extend onto the facing basal bodies. Cross sections through *Protosiphon*'s basal bodies never contain three microtubular root strands, only two roots (or occassionally one) occur per section. It would appear, then, that *Protosiphon* has a distal fibre unlike any other yet described, in that it has four attachment points, two of which are proximal.

The posterior surfaces of the basal bodies are coated by a proximal sheath, although the term 'proximal' is something of a misnomer as the sheath extends as far along the basal bodies as does the distal fibre (Figs. 135,136,137,143). In longitudinal sections, the sheath appears either as two separate subunits, one on each basal body, or as a continuous electron dense plaque underlying both basal bodies (Figs. 143,146). In basal body cross-sections, the sheath is evident as a trilobed electron dense structure, each lobe associated with one basal body triplet (Fig. 141). This is remarkably similar to the structure of the proximal sheath

('median proximal fibre') found in four *Chlorosarcinopsis* species (Gelkonian 1978). In tangential longitudinal sections, a striated band arises from each of the subunits of the proximal sheath (Figs. 137,147). These bands may correspond to the two interbasal body structures evident in horizontal sections (Figs. 139,140), although striations were not observed in this plane of section. So it appears that in *Protosiphon* the two basal bodies are subtended by a continuous proximal sheath along their posterior ends, this sheath also serving as an attachment point for two striated bands connecting the two basal bodies, ie: two proximal fibres. The resemblance of this arrangement to that found in Volvocalean algae (Ringo 1967; Greuel and Floyd 1985) is striking, despite the fact that *Protosiphon*'s basal bodies are not in a fixed V-shape position but lie at 180° to each other.

The microtubular root system is cruciate, with 2-stranded roots (R2s) alternating with roots composed of 4 microtubules (R4s). The R2s curve over the anterior proximal part of each basal body (Fig. 141) to which they are attached by electron dense material. Each R2 is also joined to its basal body by a band extending from one or two triplets on the side of the basal body above the proximal sheath (Figs. 139,147). No such bands connect the R4s to the lower curves of the basal bodies, but these roots are connected just below the basal body apices to two or possibly three triplets, via electron dense material which is visible in some horizontal sections as a small dark pad against the basal bodies (Figs. 139,141,142). A System 1 fibre (SMAC) is associated with the microtubules of each R2 (Figs. 148,150). Whether the SMACs are continuous beneath the distal fibre, as they are in *Gonium* (Greuel and Floyd 1985) and *Platydorina* (Taylor *et al.* 1985), is undetermined. Small connectives link the microtubules of the two-stranded roots to the overlying distal fibre (Figs. 145,149).

Electron dense material is found around all four roots (Figs. 150,151). The changes in position of tubule one, relative to the other three microtubules in the R4s, is consistent with other descriptions (eg: Ringo 1967; Hoops and Floyd 1982). The most anterior cross-sections display a three-over-one configuration with tubule 1 underlying tubule 3, and then migrating outwards until all four tubules are in a line (Figs. 107,151).

3.4.3 ZOOSPORE SETTLING.

In settling, *Protosiphon*'s zoospores exhibit a number of features described for *Oedogonium*'s zoospores (Pickett-Heaps 1972). In both genera the duration of the motile phase is variable, though in *Protosiphon* the zoospores generally become quiescent after 1-3 hours motility. This time interval is partly dependent on whether the zoospores encounter a substrate or not, for example swimming periods are reduced if a coverslip is placed on a drop containing active zoospores. If the zoospores fail to find a substrate they aggregate on the water surface and form a floating mat (Fig. 105). In both *Protosiphon* and *Oedogonium* (Pickett-Heaps 1972e) germination of cells in the floating mat is poor compared with cells either attached to the sides of culture vessels or placed on agar.

Three major structural changes occur on quiescence :

1. The zoospores round up, losing their elongated spindle-shape, and become spherical (Fig. 152).

2. The flagella are resorbed in a process that conforms to Bloodgood's type 4 category of flagella loss (Bloodgood 1974). The flagella lie against the cell surface, fuse with the plasmalemma, and the axoneme

enters the cytoplasm where it disintegrates (Fig. 155). This mode of flagellar resorption is found in zoospores of the Chlorococcalean algae *Hydrodictyon* (Pickett-Heaps 1975), *Pediastrum* (Marchant 1974b), and *Chlorosarcinopsis* (Melkonian and Robenek 1980); the Ulvophycean algae *Ulva* (Bråten 1971), and *Monostroma* (Jónsson and Chesnoy 1974); and the primitive Charophycean alga *Klebsormidium* (Marchant *et al.* 1973). This widespread occurrence indicates the importance of the economical reutilization of flagellar proteins.

3 A wall is formed around the spherical cells within 30 minutes. The rapidity of this process is a common phenomenon in naked motile cells (Robinson and Preston 1971; Rogalski *et al.* 1977; Robinson and Schlosser 1978; Melkonian and Robenek 1980; Domozych *et al.* 1981b; Lang *et al.* 1987), and makes if difficult to study wall ontogeny with TEM alone. At least some of the wall material is obtained from Golgi-derived vesicles (Figs. 152,153,154,156), as the dictyosomes are hypersecretory and the resultant vesicles are filled with electron dense fibres that resemble the wall microfibrils (Fig. 153). The discontinuities between the plasmalemma and the forming wall are believed to be the sites of vesicle-plasmalemma fusion (Figs. 152,154,156) - as assumed by Robinson and Preston (1971), Brown *et al.* (1976), and McArthur and Moss (1977).

The adhesion of cells to form floating mats is achieved by intermingling of wall initial material at the sites of cell-cell appression (Figs. 152,156), and the free exposure of the adhesive material of developing walls may account for the accumulation of extracellular material around the cells' peripheries (Fig. 152). Once settled, the zoospores germinate to produce the coenocytic siphons that characterize the vegetative phase of the life cycle.

4 DISCUSSION.

4.1 CLASS- AND ORDINAL AFFINITIES OF PROTOSIPHON.

Protosiphon conforms to the ultrastructural characterisitics exclusive to the Chlorophyceae, in that cytokinesis is accomplished by means of a phycoplast, and the flagellar apparatus is composed of two basal bodies which show clock-wise rotation with the correct subsequent alignment of the cruciately arranged microtubular roots. The stellate structure and the flagellar tip are also Chlorophycean in nature, and the connecting fibres resemble those of the Chlorosarcinales (Melkonian 1978) and Volvocales (eg: Ringo 1967) most closely. Details of vegetative ultrastructure affirm Chlorophycean connections, in the presence of chloroplast pseudograna, an ER-plasmalemma lining, an elaborate pyrenoid, and a zoospore eyespot embedded in an anterior chloroplast lobe, - these features only having been described in Chlorophycean algae. Vegetative differences to Siphonalean algae include the reticulate chloroplast, the wall structure, the lack of an ER-Golgi body association, and the appearance of the central vacuole. So by using the criteria of modern phylogenies, which in this case are supported by comparative pigment composition, the incorporation of *Protosiphon* into the Siphonales is clearly untenable.

Although the Protosiphonaceae are unquestionably Chlorophycean, their position in the Chlorococcales is perhaps not the most natural. The aim of phylogenetic schemes is to create monophyletic lineages, but the Chlorococcales are clearly polyphyletic in nature. Ettl and Komarek (1982) may have begun to solve the problem by splitting off coenocytic forms into the Protosiphonales. Although the erection of the

Protosiphonales on the basis of cellular morphology is acceptable (considering that Mattox and Stewart (1984) and Bold and Wynne (1985) have also used this feature in ordinal delimitation), Deason (1984) has vehemently rejected the formation of this order. However, the evidence he presents is sometimes untrue (the presumed ultrastructural similarity between *Protosiphon* and some members of the Chlorococcaceae), or based on facts taken out of context (the descriptions of isolates producing zoospores that do not become spherical on quiescence and which do not produce sacs, are certainly found in Thomas (1971), but Deason (1984) fails to mention that Thomas (1968, 1971) does not consider these isolates to belong to the genus *Protosiphon*. "Results from this present study are sufficient to question the present taxonomic status of these two isolates. More research is needed before their systematic disposition can be resolved " (Thomas 1968, p48-49).

In addition, the multinucleate nature of *Neospongiococcum prolíferum* does not make it a coenocyte, and the reported serological relationship between *Protosiphon* and some members of the Chlorococcaceae is hardly surprising, as Thomas (1969) was comparing *Protosiphon* to these coccoid greens, and to two xanthophytes (similarities are not likely across divisional boundaries!). Serological data of relevance (for example, comparison of *Protosiphon* to representatives of other Chlorophyceae orders) do not exist.

Apart from Deason's objections (1984), there is evidence that supports the grouping of the Protosiphonales. The vegetative structure of *Protosiphon* resembles that of *Hydrodictyon* (Pickett-Heaps 1975) more closely than any other alga, particularly in the reticulate chloroplast and central vacuole. *Characiosiphon*'s wall and vacuole (Stewart *et al.* 1978) are identical to those of *Protosiphon*, and the processes of zoosporogenesis and zoospore release are comparable in all three genera : similar phycoplasts are found during furrowing in both *Protosiphon* and

Protosiphonales on the basis of cellular morphology is acceptable (considering that Mattox and Stewart (1984) and Bold and Wynne (1985) have also used this feature in ordinal delimitation), Deason (1984) has vehemently rejected the formation of this order. However, the evidence he presents is sometimes untrue (the presumed ultrastructural similarity between *Protosiphon* and some members of the Chlorococcaceae), or based on facts taken out of context (the descriptions of isolates producing zoospores that do not become spherical on quiescence and which do not produce sacs, are certainly found in Thomas (1971), but Deason (1984) fails to mention that Thomas (1968, 1971) does not consider these isolates to belong to the genus *Protosiphon*. "Results from this present study are sufficient to question the present taxonomic status of these two isolates. More research is needed before their systematic disposition can be resolved." (Thomas 1968, p48-49).

In addition, the multinucleate nature of *Neospongiococcum proliferum* does not make it a coenocyte, and the reported serological relationship between *Protosiphon* and some members of the Chlorococcaceae is hardly surprising, as Thomas (1969) was comparing *Protosiphon* to these coccoid greens, and to two xanthophytes (similarities are not likely across divisional boundaries!). Serological data of relevance (for example, comparison of *Protosiphon* to representatives of other Chlorophycean orders) do not exist.

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Hydrodictyon (Marchant and Pickett-Heaps 1971), (the study on *Characiosiphon* does not include cytokinetic details); and in both *Protosiphon* and *Hydrodictyon* zoosporogenesis involves loss of pyrenoid substructure. Zoospores of all genera studied are naked, and are released singly via an undifferentiated pore formed by dissolution of the parent wall. The vegetative structure of the 'true' Chlorococcales is not coenocytic and is therefore very different from that of *Protosiphon*, *Hydrodictyon* and *Characiosiphon*. In addition, the phycoplasts of the 'true' Chlorococcales are more extensive and well-developed, and the zoospores are usually walled, infrequently naked, and are generally released *en masse* in a vesicle (Deason 1967; Bold and Wynne 1985). A comparison of flagellar ultrastructure between *Protosiphon* and either the Chlorococcales or the Protosiphonales is not possible. The few micrographs of the motile cells of *Hydrodictyon* (Pickett-Heaps 1975), *Pediastrum* (Marchant 1974b), *Sorastrum* (Marchant 1974a) and *Characiosiphon* (Stewart *et al.* 1978) reveal very little, and no Chlorococcalean flagellar studies exist (excepting *Golenkinia* (Moestrup 1972) which is probably not representative of the order, or even the class). There is also no detailed information on the Rhopalosolenaceae, and perhaps complete studies on all members of the Protosiphonales would reveal differences greater than the uniformity imposed by a coenocytic habit. But until then the Protosiphonales would appear to be a welcome initial step in the reduction in the artificiality of the Chlorococcales.

4.2 GREEN ALGAL TAXONOMY.

One of the problems encountered in taxonomic systems based on ultrastructure is that the use of TEM in taxonomy has resulted in algal groupings and affinities that are contrary to those traditionally used

in taxonomies based by necessity, on gross morphology and to a lesser extent, life-history. This has caused some phycologists to dismiss the modern phylogenies : "it seems preferable to use all this fascinating new information concerning the ultrastructure of green algal zoids to construct hypothetical evolutionary lineages rather than to disrupt the current formal system." (van den Hoek 1984, p160). The reasoning preceeding this conclusion displays a surprising misunderstanding and lack of familiarity with the dynamic field of siphonous ultrastructure. It is all too easy to use criteria initially considered to be phylogenetically important, and cite references to demonstrate their inconsistency, so providing the platform for rejection of the ultrastructural taxonomic field, despite the fact that these criteria are now acknowledged not to be class- characteristic. (Compare van den Hoek's 'ultrastructure of the zoids' (1984) with the Ulvophycean definition of O'Kelly and Floyd (1984a).

Other phycologists are not quite so disparaging, and yet still exhibit a reluctance to whole-heartedly accept ultrastructural systems. For example, Round (1984, p22) comments that "Early workers, relying on gross features, produced the best schemes possible, whereas latterly electron microscopy has become prominent and has yielded much sounder systems, marred only by lack of consideration of the gross features used by the pioneers." The current phylogenies do not ignore gross morphology, as this is still the most important feature used at the generic and specific levels (*Protosiphon* will always look like *Protosiphon*, and is still called *Protosiphon*. Only the genus *Ulothrix* has been split as a result of ultrastructural studies, and the taxonomy of this genus has always presented a problem (see Lokhorst 1978). It is largely the ordinal and class groupings that are different, and that are beginning to reflect evolutionary lineages and true affinities - a reflection based on conservative evolutionary features rather than on morphology, which is variable within a species, subject to environmental manipulation, and limited to a number

of forms that have arisen independently several times and which are therefore polyphyletic characters.

The incorporation of evolutionary tendencies in a taxonomic scheme means that the predictive value of the scheme is enhanced. The observation that a unicellular sarcinoid alga produced zoospores with laterally inserted flagella, enabled Rogers *et al.* (1980) to predict that this algal form was on the Charophycean evolutionary line and would therefore have a multilayered structure and probably a scaly covering. And despite its 'Chlorophycean' growth form, it did. Stewart and Mattox (1975) have also mentioned the value of predictability when selecting algae for biochemical studies. For example, detailed work on glycolate oxidase can be restricted to members of the Charophycean line, *without wasting resources* on studies utilizing morphologically similar algae from a *different evolutionary line* that has developed glycolate dehydroganase.

Conservative prejudice aside, there are naturally other problems associated with the present phylogenetic systems. For example : 1) One of the differences between the Chlorophyceae and the Ulvophyceae, is that in the former class the smaller microtubular roots lie in a line, with the larger roots (X roots) offset and attaching to the outer edges of the basal bodies. The reverse arrangement is true of the Ulvophyceae (Mattox and Stewart 1984). Diagrams and micrographs demonstrating this distinction in both bi- and quadriflagellate cells, are frequent. (For eg: class comparisons in Mattox and Stewart 1984 fig. 3, p35; O'Kelly and Floyd 1983 fig.29, p161; Melkonian and Berns 1983 fig. 40, p83; Ulvophyceae alone in Floyd *et al.* 1985 fig. 2, p617 (quadriflagellate) and fig 3, p618 (biflagellate); Roberts *et al.* 1982 figs. 47 and 48, p506; O'Kelly *et al.* 1984 fig. 46, p197; Berger-Perrot *et al.* 1986 fig. 54, p26; Herth *et al.* 1981 fig. 23, p266; Chlorophyceae alone - Deason and Floyd 1987 fig. 12, p191; Melkonian 1978 fig. 11, p269, Katz and McLean 1979 fig. 9, p378; O'Kelly and Floyd 1984b fig 4, p235. Only one example from each order

studied is given.) But a problem arises in one of the Chlorophycean orders. In the two studies on Chaetophoralean algae, both Floyd *et al.* (1980), and Bakker and Lokhorst (1984), have reconstructions of quadriflagellar apparatus in which one pair of basal bodies has rotated but the components of the other remain at 180° Floyd *et al.* (1985) suggest that even if only one pair of basal bodies is displaced, it should be sufficient to indicate the direction of rotation, as clockwise rotation results in L-shaped pairs of basal bodies at acute or right angles, whereas anticlockwise rotation results in L-shaped pairs of basal bodies at obtuse angles. In the reconstructions of both *Uronema belkae* (Floyd *et al.* 1980 fig. 4, p20) and *Draparnaldia* (Bakker and Lokhorst 1984 fig. 5, p264) the displaced basal bodies are drawn as though they have rotated clockwise, and yet it is the X microtubular roots that run 'into' the basal bodies, with the smaller roots offset on the outside. The mirror image of these reconstructions has anticlockwise rotation with X roots ending in a line inside the basal bodies. As basal body overlap is absent, the arrangement is not typically Ulvophycean, but rather resembles an order in the Pleurastrophyceae (see Mattox and Stewart 1984; O'Kelly and Floyd 1984b). As the basal body angles are acute, and the Chlorophycean nature of this order is undisputed, it means that in some quadriflagellate cells, only the angle between adjacent basal bodies will reveal the direction of rotation - whereas the insertion of the microtubular roots, which is the only criterion for determining the direction of rotation in biflagellate cells, is invalid.

2) Jónsson (1962) grouped a number of marine filamentous algae together on the basis of their heteromorphic life histories, and perforate chloroplasts, forming the Acrosiphoniales. This order, or its members (O'Kelly and Floyd 1984a do not recognize the order and place its genera in the Ulotrichales, although they differ from Ulotrichalean algae in flagellar structure, cytokinesis, sexual life history, motile cell exit apertures, pyrenoid ultrastructure, and cell wall composition), are in-

variably referred to the Ulvophyceae (Sluiman *et al.* 1982; Floyd and O'Kelly 1984; Mattox and Stewart 1984; Lokhorst 1984). Yet a critical examination of the literature reveals a relationship with this class that is at best tenuous, and also that the Acrosiphoniales do not fit into any of the five described classes of Mattox and Stewart (1984). In both *Urospora* (Lokhorst and Star 1983) and *Acrosiphonia* (Hudson and Waaland (1974), the cytokinetic mechanism involves a reduced phycoplast. Phycoplasts are found only in the Chlorophyceae and the Pleurastrophyceae, but not in the Ulvophyceae (Mattox and Stewart 1984). The Acrosiphonealean features of mitosis are Chlorophycean and not Pleurastrophycean, so it would appear that this order should be assigned to the Chlorophyceae. The ultrastructural features of the flagellar apparatus of *Urospora penicilliformis* zoospores concur with those of the Chlorophyceae, despite the determination of Sluiman *et al.* (1982) to describe these cells as Ulvophycean. The features cited (rhizoplasts and 'capping plates') are now known not to be infallible characteristics of the Ulvophyceae (Table 1; O'Kelly and Floyd 1984a). In addition, the basal body arrangement is perfectly cruciate, a situation otherwise only found in the Chlorophycean alga *Chaetopeltis* (O'Kelly and Floyd 1984b). However, the gametes of *Acrosiphonia* and *Urospora gregaria* (Floyd and O'Kelly 1984) exhibit two features not found in the Chlorophyceae but in the Ulvophyceae : a simple terminal cap can be distinguished at the proximal end of each basal body, and the basal bodies are overlapping.

Rejection of overlapping basal bodies as an Ulvophycean characteristic (O'Kelly and Floyd 1983) is based on the fact that

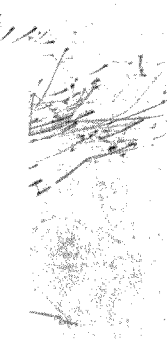
1. *Urospora* zoospores do not exhibit overlap. This surely suggests that this alga might not be Ulvophycean, rather than that over 20 Ulvophycean genera happen to exhibit this feature coincidentally

2. basal body overlap is said to occur within the Chlorophyceae in two cases, *Golenkinia* (Moestrup 1972) and a stage in the life history of the flagellate *Korschikoffia* (Melkonian 1982b). As mentioned previously, *Golenkinia*'s flagellar apparatus is quite extraordinary (and to visualize basal body overlap in figures 24 and 25, p176, requires some imagination). The second example of Chlorophycean basal body overlap comes from an abstract. There are no published micrographs available, as the complete paper referred to by O'Kelly and Floyd (1984b), 'Melkonian and Preisig 1983' was "in press" but has never been published, according to biological abstracts. In addition, O'Kelly and Floyd (1984b) have clearly demonstrated that basal body absolute orientations can easily be misinterpreted during flagellar apparatus replication (citing an example from published *Mantoniella* studies as evidence). It is therefore quite possible that the basal body overlap described during reproduction in *Korschikoffia* is a misinterpretation, a possibility reinforced by the transient nature of the phenomenon.

Even if basal body overlap is disregarded however, there is still the problem of the terminal caps and the fact that Floyd and O'Kelly (1984b) describe the basal body rotation in *Acrosiphonia* gametes and *Urospora pregaria* male gametes, as being anticlockwise. O'Kelly and Floyd (1984a) stipulate that members of the Ulvophyceae cannot be defined on the basis of flagellar ultrastructure alone, but if cell division details are incorporated then the Acrosiphoniales no longer belong in the Ulvophyceae. The Ulvophycean flagellar features preclude inclusion in the Chlorophyceae, and mitotic and zoospore descriptions are at odds with those of Pleurastrophycean algae (although cytokinetic features and gamete ultrastructure are similar to those of the Pleurastrophyceae).

This confusion merely illustrates that the present phylogeny is by no means complete, and that many more years of exciting explorative research

are necessary. The two examples above also do not invalidate the concepts on which present phylogenies are based, but instead reflect differences in interpretation and groupings of algae. Orders and classes may be redefined, incorporated, or enlarged, but their foundations will still rest on ultrastructural features. As the number of studies revealing these features has increased, correlations between ultrastructure, and habitat and life-histories have emerged (Irvine and John 1984), resulting in the most holistic approach to green algal taxonomy and phylogeny, yet conceived. Adoption of this approach was attempted in the present study, and although reproductive data are lacking, the taxonomic controversy surrounding *Protosiphon* has been solved.



5 APPENDICES.

APPENDIX 1 - LEWIN'S CULTURE MEDIUM.

To 1L distilled water, add 10ml. of each of the following stock solutions :

$Ca(NO_3)_2.4H_2O$..... 7.00g/L

K_2HPO_4 anhydrous 0.50

$MgSO_4.7H_2O$ 2.50

$FeSO_4.7H_2O$ 0.03

$MnCl_2.4H_2O$ 0.02

Na_2EDTA 0.50

Finally add 15ml soil extract.

Autoclave.

APPENDIX 2 - MODIFIED KARNOVSKY'S FIXATIVE.

Dissolve 2g paraformaldehyde in 25ml distilled water, by heating to 60-70°C and stirring. Add three drops 1N NaOH while stirring. Allow to cool, add 5ml of 25% glutaraldehyde, and make up to 50ml with 0.1M Phosphate buffer, pH 7.4. Fix for selected time interval (dependent on material), rinse in phosphate buffer, and postfix in 1% osmium tetroxide.

APPENDIX 3 - COMMENTS ON FLAGELLAR TERMINOLOGY.

1. Ringo (1967) was the first to use the term 'proximal fibres' to describe two striated bands that connected the V-shaped basal bodies in *Chlamydomonas*. In other studies on the Volvocales (Greuel and Floyd 1985; Taylor *et al.* 1985; Hoops and Floyd 1982) and in those on quadriflagellate Chaetophoralean zoospores (Melkonian 1975; Birbeck *et al.* 1974; Floyd *et al.* 1980; Bakker and Lokhorst 1984), the proximal fibres very definitely connect to adjacent basal bodies as well as to microtubular roots. However, in the Chlorosarcinales (Melkonian 1977,1978; Deason and Floyd 1987) the basal body-proximal fibre connection is proving more difficult to demonstrate than the proximal fibre-microtubular root connection. So in the Chlorosarcinales at least, the proximal fibres appear to have the same position and single function of the striated bands of the Ulvophyceae. Unless a striated fibre can be positively shown to connect adjacent basal bodies, it should not be referred to as a proximal fibre.

An interesting observation in this respect, is that in all Chlorophycean algae, proximal fibres are located on the lower surface of the basal bodies in longitudinal section. However, in the Siphonales, and perhaps Ulvales, proximal fibres occur above the basal bodies (Roberts *et al.* 1981, 1982; Hori 1977; and see Melkonian 1981 fig. 22, p362). This Ulvophycean positioning of a striated proximal connective may be related to the feeble, or even absent, striation of the distal fibre.

Members of the Siphonales have distal fibres that are unstriated (showing no median interruption), or faintly striated, which suggests inefficient functioning as an 'elastic' connective (Ringo 1967; Watson 1975). It would be interesting to determine whether the func-

tion of the distal fibre has been appropriated by the well-striated proximal fibres linking the basal bodies anteriorly, and leaving the distal fibres to become modified into components of the mating system. This is particularly evident in the female gametes of the three Siphonalean genera studied, where in both *Pseudobryopsis* (Roberts *et al.* 1982) and *Bryopsis* (Melkonian 1981), cylindrical extensions of the 'capping plate' line the microtubular roots and connect to the mating structure on the plasmalemma; and in *Derbesia* (Roberts *et al.* 1981) where the extremely complex perforated 'capping plate' extends posteriorly along the 2-stranded microtubular roots for several microns (unfortunately no information on a possible role as a mating structure).

The terminology of the distal fibre in Ulvophycean swarmers, is disputed between Floyd, O'Kelly and colleagues, and Stewart, Mattox and colleagues. Floyd et al (1985) recommend that all distal fibres be called exactly that, irrespective of striation patterns and algal evolutionary group. The r opponents in this semantic battle, reveal a bias towards unfounded differentiation between the Chlorophyceae and the Ulvophyceae. Despite the fact that every Ulvophycean order has members with striated distal fibres, these phycologists persist in calling all Ulvophycean distal fibres 'capping plates', a term first used by Melkonian (1979) in describing the apparently non-striated distal fibre of *Ulva* zoospores. Later papers (Melkonian *et al.* 1981, Roberts *et al.* 198 , 1982, 1984; Roberts 1984; Melkonian and Berns 1983) continue to refer to 'capping plates' to differentiate them from the Chlorophycean distal striated fibres, despite the fact that many of the 'plates' show central striations (eg: *Bacophora*'s distal fibre is virtually indistinguishable from that of *Sphaeroplea* - compare Roberts *et al.* 1984 figs. 7 and 12, p186; with Buchheim and Hoffman 1986 figs. 20 and 23, p182). Even the derogatory comment made by Stuessy *et al.* (1983, p256) that "capping plate configuration ...

may be a misinterpretation of poorly fixed material" had little effect on inducing conformity. A pity, as a distal fibre is a distal fibre regardless of its striation pattern.

2. Certain authors (Melkonian and Berns 1983; Bakker and Lokhorst 1984) incorrectly refer to the clockwise arrangement of Chlorophycean flagellar bases as the 1/7 o'clock position. This term was first used by Roberts *et al.* (1982) to describe the mirror image of the absolute flagellar configuration typical of the Ulvophyceae. The characteristics of basal body overlap and RX insertion into the basal bodies, are therefore inherent in the use of this term, and it should be avoided with reference to the Chlorophyceae. The clockwise and anticlockwise terminology of O'Kelly and Floyd (1983) is less ambiguous.

3. Melkonian (1980a) reviewed basal body associated structures, describing two types of connecting fibres : System 1 (or SMAC of Floyd *et al.* 1980) and System 2 (rhizoplast). System 2 fibres are defined as "fibrous roots consist(ing) of a bundle of fine filaments ... interrupted by cross-striations with a repetitive unit greater than 80nm. They are, with one exception (*Tetraselmis*) not closely associated with microtubular roots." (Melkonian 1980a p97). Despite this definition, some of the studies on the Siphonales refer to structures connecting basal bodies to microtubular roots, as 'rhizoplasts' (Roberts *et al.* 1981, 1982). The striated bands in similar positions, of members of the Ulotrichales (Sluiman *et al.* 1980; O'Kelly *et al.* 1984), Siphonocladales (Floyd *et al.* 1982), and Ulvales (O'Kelly and Floyd 1983; Stuessy *et al.* 1983; O'Kelly *et al.* 1984), are correctly not referred to as rhizoplasts, but simply as striated bands. The periodicity of such short bands is difficult to measure accurately, (if the reason for the use of 'rhizoplast' was prompted by wide striations), and there is increasing evidence that periodicities alter with flagellar functioning and are actually of little taxonomic

value (Moestrup 1978; Melkonian 1980b; Floyd *et al.* 1985; Berger-Perrot *et al.* 1986).

Another example of misuse of the term 'rhizoplast' can be found in the study by Bakker and Lokhorst (1985), where an electron dense plate associated with a microtubular root in *Chaetomorpha*, is described as an unstriated rhizoplast - by definition impossible.

4. In four species of *Chlorosarcinopsis* Melkonian (1978,1980a) described a 'median proximal fibre' found on the lower surface of basal bodies as an electron dense mass. Serial sections demonstrated that the structure splits into three distinct, roughly triangular, portions (Melkonian 1978 figs. 49 and 50, p277). Despite the fact that the structure is not fibrous, and that the only micrograph purported demonstrating that it links the two basal bodies along their lower surfaces, is not convincing (see Melkonian 1978 fig. 3, p267), Melkonian saw fit to name it a median proximal fibre. A remarkably similar structure has been described in a number of Ulvophycean orders, but in this class it is known as a 'proximal sheath' - described by O'Kelly and Floyd (1984a, p132) for the Ulvales as "subtending all basal bodies (and) composed of two equal subunits, triangular in cross-section, that narrow and finally join together proximally". The number of subunits is variable within the Ulvophyceae, with two typical of the Ulvales, one or two in the Ulotrichales, and three or none in the Siphonocladales (Floyd and O'Kelly 1984; Bakker and Lokhorst 1985). The only difference between the subunits of a 'median proximal fibre' and those of the Ulvales and Cladophoraceae, is in the height of the constituent triangles, with those of the Ulvophycean algae generally being longer than those of *Chlorosarcinopsis*. However, comparison of the reduced or single cupping proximal sheaths of other Ulvophycean basal bodies (eg: Dasycladales, some Ulotrichales) with Chlorophycean 'median proximal fibres', demonstrate that the

Chlorophycean ones extend further posteriorly than those of some Ulvophyceae. The posterior extension of proximal sheath subunits also *depends on what portion of the basal body is sectioned.* Published micrographs of Chlorophycean basal bodies indicate that

a. electron dense material underlies most basal bodies (Bakker and Lokhorst 1985 fig.11, p269; Greuel and Floyd 1985 fig.13, p363; Taylor *et al.* 1985 fig. 10, p537; Hoffman 1976 fig.52, p213; Buchheim and Hoffman 1986 fig.11, p180; Deason and Floyd 1987 fig.11, p191)

b. In some cross-sections it is triangularly extended, eg: *Cylindrocapsa* (Hoffman 1976 fig.34, p213); while in others it forms a crescent cap under the lowermost triplets, eg: *Atractomorpha* (Hoffman 1986 fig.28, p578), just as it does in members of the Ulotrichales (O'Kelly and Floyd 1984a; Floyd and O'Kelly 1984)

c. *Gayralia* (Hoops *et al.* 1982 fig.10, p154) possesses the electron dense basal body shroud of *Gonium* (Greuel and Floyd 1985 fig.11 p363) and also has two subunits in cross-section.

d. In *Desmotetra* (Deason and Floyd 1987 fig.11, p191) the proximal electron dense material serves as an attachment for the rhizoplasts, as do the proximal sheaths of certain Ulvophycean motile cells (Stuessy *et al.* 1983; O'Kelly *et al.* 1984). A similar function therefore seems probable in System 2-containing motile cells.

If there is any basis for differentiation between Ulvophycean proximal sheaths and Chlorophycean median proximal fibres, then perhaps it could be found in the fact that theoretically, the median

proximal fibres link opposing basal bodies and that proximal sheaths underlie single basal bodies. However, this linking function is not well demonstrated by Melkonian (1978) and equally convincing links can be discerned between the proximal sheaths of Ulvophycean algae sectioned in a suitable plane (see Berger-Perrot *et al.* 1986 fig.30, p23; O'Kelly *et al.* 1984 fig.3, p182; Melkonian 1980b fig.18, p156). So 'median proximal fibres' and 'proximal sheaths' cannot be distinguished on this functional basis, as there is no clearcut evidence to support such a division. Hirayama and Hori (1984) were criticized by Floyd et al (1985) for calling proximally situated electron dense material a proximal sheath in *Chaetomorpha* - as Floyd *et al.* (1985 p625) consider proximal sheaths to be "discrete electron-dense fibres subtending the proximal ends of the basal bodies". Theoretically perhaps, but the fibrous nature of proximal sheaths is not evident in any micrographs (no individual fibres are actually discernible); and in their entirety, the proximal sheaths are not 'discrete' in those Ulvophycean members having reduced sheaths or sheaths composed of a single unit, nor do the very most proximal portions of sheaths composed of two or three subunits have well-defined edges. Assuming though, that proximal sheaths conform in reality with the definition given by Floyd *et al.* (1985), then Melkonian's median proximal fibres (Melkonian 1978, 1980a) could, by definition, be proximal sheaths. Until a valid difference between the two can be demonstrated, it is suggested that all electron dense matter ensheathing the proximal basal body surface on the lower side be termed proximal sheaths, in order to emphasize the homology of cruciate root systems.

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8 FIGURES.

ABBREVIATIONS USED IN THE FIGURES.

A - axoneme

B - basal body (bodies)

C - chloroplast

CF - cleavage furrow

Cp - cytoplasm

CV - central vacuole

D - dictyosome

e - eyespot

F - distal striated fibre

H - linear formations of irregular lipocarotenoid deposits

ip - incipient pyrenoid

k1 - crystal type 1 (carotenoid)

k2 - second crystal type

L - lipocarotenoid

M - mitochondrial profile

N - nucleus

O - annular structures resulting from lipocarotenoid-globule digestion

P - pyrenoid

pg - pseudograsum

pp - polyphosphate granule

Qg - lipocarotenoid globule

Qi - irregular lipocarotenoid deposit

r2 - two-stranded microtubular root

r4 - four-stranded microtubular root

S - starch

t - triplet microtubule arrangement in basal body

Tp - thylakoid plexus

TV - contractile vacuole

U - microbody

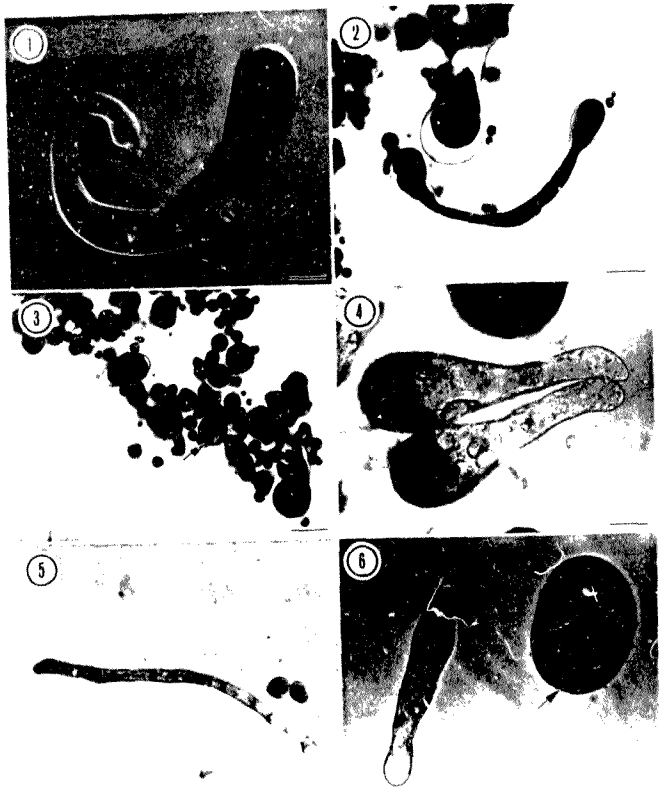
V - vesicle

W - wall

x - proximal sheath

Y - thylakoid pad

z - cartwheel region of basal body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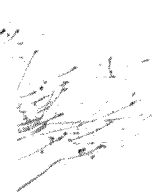

Figure 1. A young vegetative cell of *Protosiphon botryoides*, with a chlorophyllous and a rhizoidal region. (Scale = 100µm).

Figure 2. Siphons grown for extended periods in stirred liquid cultures frequently possess chloroplasts that fill the entire length of the cell. (Scale = 20µm).

Figure 3. Continual growth in liquid culture results in the production of spherical vegetative cells with vacuolate cytoplasm (arrow). (Scale = 20µm).

Figure 4. Young 'giant' cells. (Scale 100µm).

Figure 5. The elongate siphon shape present in all four isolates. (Scale 150µm).

Figure 6. A 'giant' cell (arrow) and a normal cell after eight days growth in liquid culture. (Scale = 100µm).


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Figure 7. Sloughing of wall layers (arrowheads) in an actively growing young siphon. (Scale = 1µm).

Figure 8. Wall layers may be completely separated from the underlying lamellated wall material. The granular mucilagenous substance dissolves to leave only wall fibrils. (Scale = 1µm).

Figure 9. A mature siphon in which the compacted areas of a wall layer persist (arrowheads), but the intervening material has been lost. The outermost portion appears detached from the inner area which lies adjacent to the younger wall material. (Scale = 1µm).

Figure 10. Longitudinally sectioned wall microfibrils (arrowheads). ale = 0.25µm).

Figure 11. Wall microfibrils (arrowheads) appear scattered singly or in clusters, in the granular ground material. (Scale = 0.25µm).

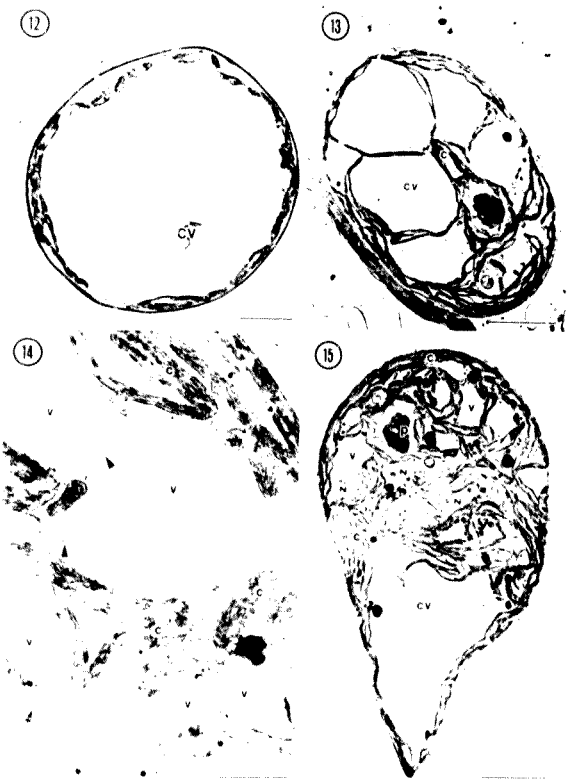
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Figure 12. A cross-section through a young siphon illustrates the large central vacuole and the thin layer of peripheral cytoplasm. (Scale = 4μm).

Figure 13. Branches of the reticulate chloroplast anastomose across the central vacuole of an adult siphon. (Scale = 4μm).

Figure 14. The central vacuole of the siphon develops by fusion (arrowheads) of numerous vesicles in the cytoplasm. Some vesicles are derived from the digestion of polyphosphate granules (arrows). (Scale = 1μm).

Figure 15. A longitudinal section through the botryoidal chlorophyllous region of a siphon. Thin cytoplasmic threads traverse the central vacuole. (Scale = 8μm).


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Figure 16. A portion of the multinucleate peripheral cytoplasm of an adult cephon. ER cisternae line the plasmalemma (arrowheads), while the reticulate chloroplast anastomoses between the cell wall and the central vacuole. Some of the vacuolar areas contain the beaded membranes typically left after polyphosphate granule digestion (arrows). (Scale = 1μm).

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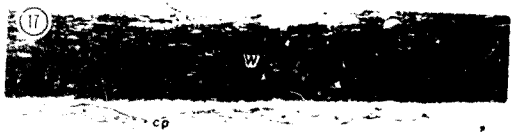

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Figures 17 - 20. A series of sections through the cytoplasm of a siphon, from the rhizoidal region (Fig. 17), up towards the chlorophyllous region (Fig. 18), which apically contains increasing numbers of organelles (Figs. 19 and 20). Note the peripheral ER lining the plasmalemma (Fig. 19 arrowheads). (Scales = Fig. 17 - 3μm; Fig. 18 - 1μm; Figs. 19 and 20 - 0.5μm).


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Figures 21 and 22. The cytoplasm of an old siphon containing complete polyphosphate granules, and granules in the process of digestion. Once digested, the granules leave a thin beaded membrane in the resulting vesicle (arrowheads). (Scales = 1µm).

Figure 23. Two vesicle-bound polyphosphate granules in the process of being digested. (Scale = 0.2µm).

Figures 24 and 25. The pseudograna (arrowheads) of the reticulate chloroplast, which also contains starch and plastoglobuli. (Scale = 1µm).

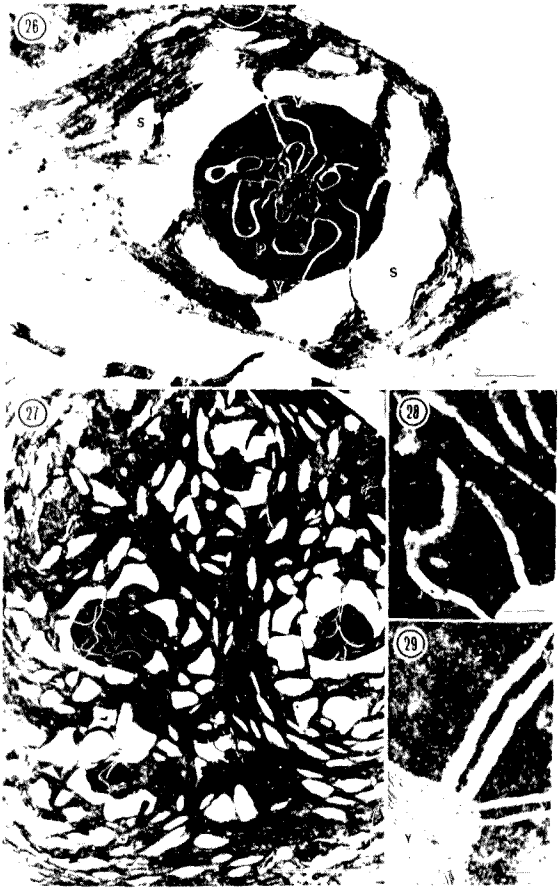
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Figure 26. A chloroplast portion containing plastoglobuli, starch, and a pyrenoid. Connections occur between adjacent layers of the starch sheath (arrowhead). (Scale = 1µm).

Figure 27. The concentric arrangement of starch grains formed by pyrenoids in the apical chlorophyllous region of an adult siphon. (Scale = 1µm).

Figure 28. An undulating thylakoid membrane within an intrapyrenoidal lamella. (Scale = 0.2µm).

Figure 29. Expanded thylakoids entering the pyrenoid matrix from a thylakoidal pad. (Scale = 0.2µm).

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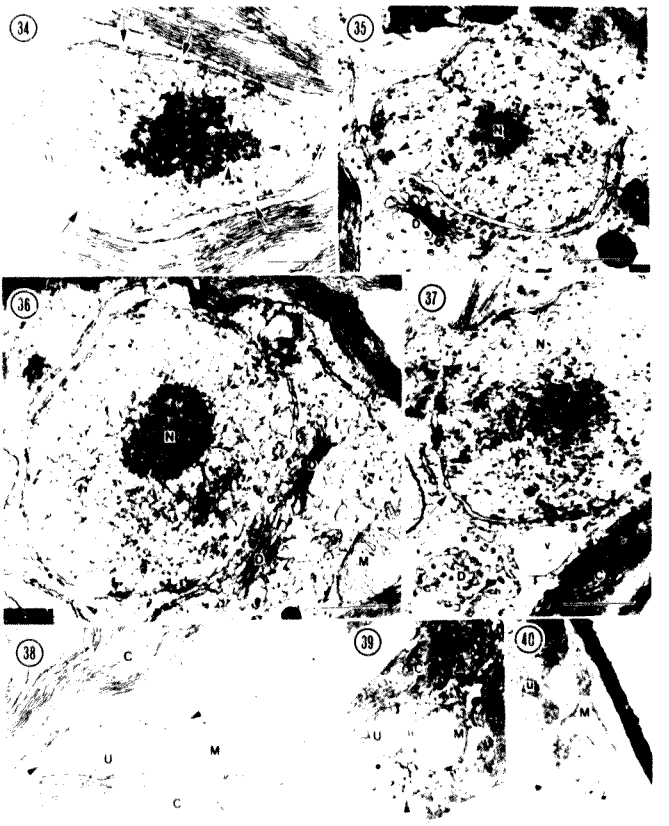
Figures 30 - 32. A series of sections through a pyrenoid : just grazing the central core structure through its enclosing thylakoids (Fig. 30); through the osmiophilic matrix underlying the central thylakoids (Fig. 31); through the centre of the core structure (Fig. 32). (Scales = 1µm).

Figure 33. The central core structure of a pyrenoid. (Scale = 0.7µm).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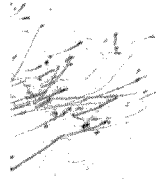

Figure **34.** *An adult siphon's nucleus with a macrosegragated nucleolus (arrowheads) and a pored envelope (arrows). (Scale = 0.5µm).*

Figure 35. Peripheral chromatin aggregates (arrowheads) are common on the inner nuclear envelope. (Scale = 0.5µm).

Figure 36. Long RER cisternae arise from the nuclear envelope (arrowheads). The forming face of the Golgi apparatus is closely associated with the outer nuclear membrane. (Scale = 0.5µm).

Figure 37. Ribosomes line the outer envelope of the nucleus, while chromatin condensates occur internally (arrowheads). Note the large pore (arrow). (Scale = 0.5µm).

Figures 38 and 39. Small microbodies are closely associated with chloroplast reticulations, mitochondrial profiles, and RER cisternae (arrowheads). (Scales = 0.2µm).

Figure 40. An annular mitochondrial profile lies next to a dumbbell-shaped microbody. (Scale = 1.5µm).

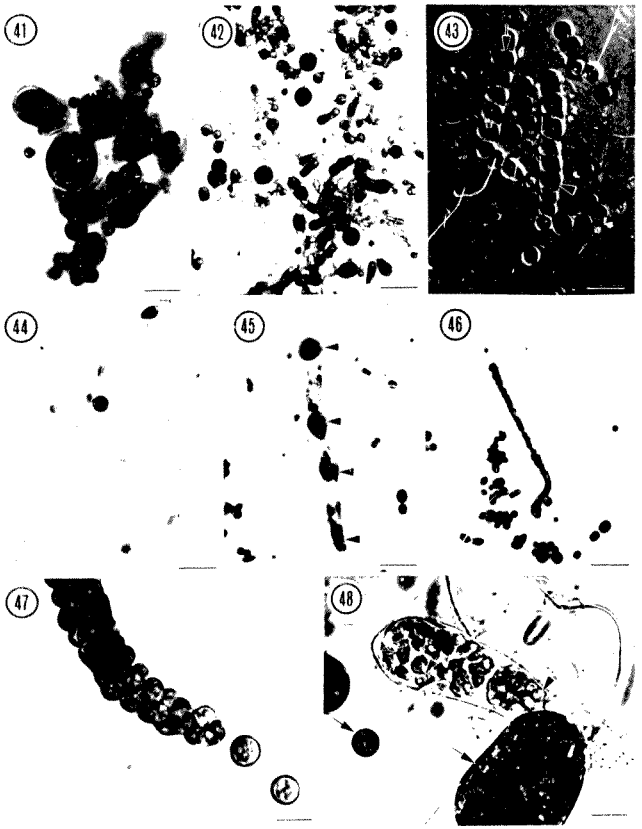
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Figure 41. A coenocyst formed by condensation of an entire siphon's protoplast. Note the thickened wall. (Scale = 15μm).

Figure 42. The bright orange cysts formed after 3 months of adverse conditions. Some cysts represent whole siphon protoplasts, while others are derived from portions of one siphon's protoplasm. (Scale = 65μm).

Figure 43. Two siphons that have undergone transverse cleavage during coenocyst formation. In some cases, the resultant cysts have been subsequently partitioned either equally (arrow) or unequally (arrowhead). (Scale = 50μm).

Figure 44. Siphons containing coenocysts that are spatially separated within the parental wall. (Scale = 70μm).

Figure 45. *Formation of spatially separated coenocysts.* The cytoplasm between the four forming cysts (arrowheads) will degenerate. (Scale = 50μm).

Figure 46. The zoospores produced within this slender siphon have encysted within the parent wall, forming small hypnospores. (Scale = 50μm).

Figure 47. Detail of a portion of a siphon that has undergone zoosporogenesis, with the resulting zoospores encysting within the siphon. The zoospores are tightly packed and will form red hypnospores. (Scale = 8μm).

Figure 48. *A comparison between the sizes of cysts formed by protoplast condensation (arrows) and those formed by entrapped zoospores (arrowheads).* (Scale = 8μm).

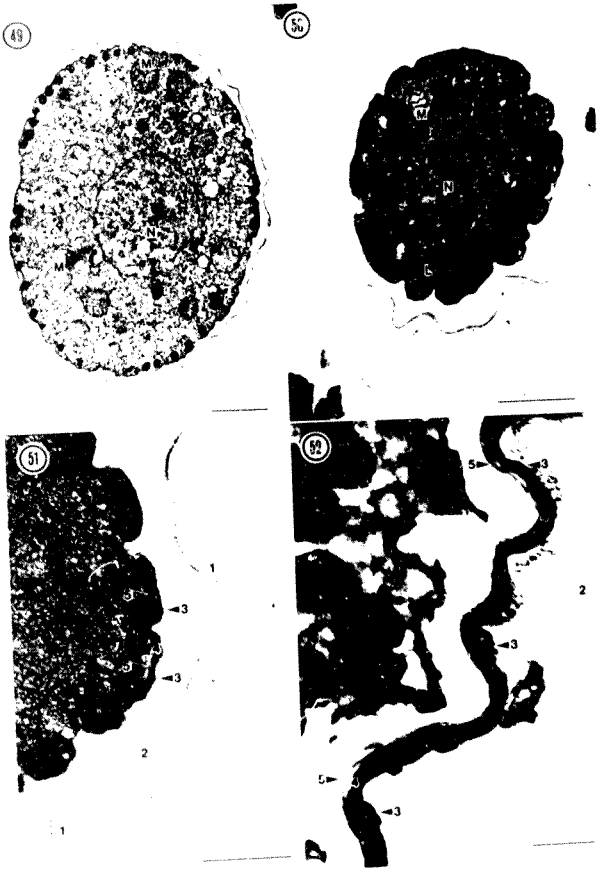
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Figure 49. A young hypnospore containing few recognizable organelles, and an incompiete, peripheral lipocarotenoid globule layer. (Scale = 1µm).

Figure 50. Compacted, mature hypnospore with spiny wall, complete lipocarotenoid layer, internal lipid droplets, and a lobed nucleus. (Scale = 1µm)

Figure 51. The five wall layers of a mature hypnospore - outermost layer (1), spine-containing fibrous layer (2), trilaminar sheath (3), electron dense layer (4), second trilaminar sheath adjacent to the plasmalemma (5). (Scale = 0.5µm)

Figure 52. A hypnospore in which che plasmalemma has torn away from the fifth wall layer (5). Note the convolutions of the trilaminar sheath comprising the third wall layer. (Scale = 0.25µm).

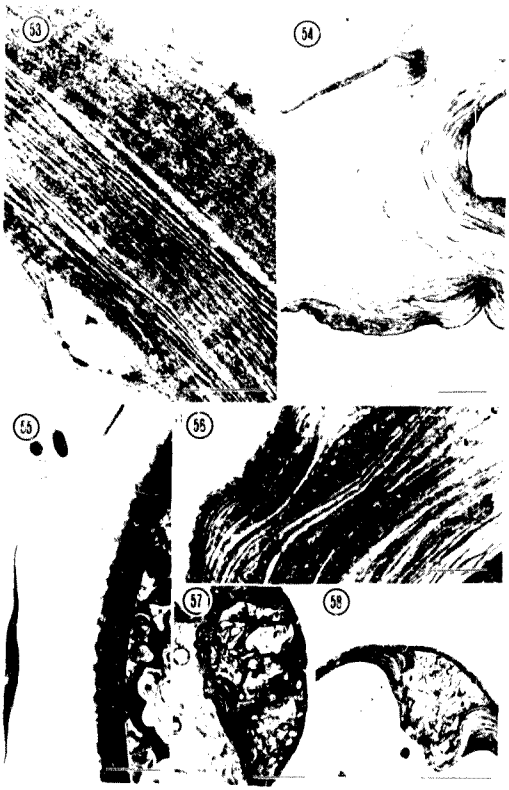
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Figure 53. The highly laminated wall of a coenocyst, the outermost layer not being compressed. (Scale = 1µm).

Figure 54. An old coenocyst with a thick, 'gonfle' wall. Lamellations are irregular and the constituent material is randomly arranged between the layers. (Scale = 3µm).

Figure 55. A coenocyst with cell contents, and an outer wall layer dissolving into the surrounding medium. (Scale = 3µm).

Figure 56. Some coenocysts have outer wall layers that are compacted and do not dissolve into the medium. Occassional layers contain peculiar convolutions. (Scale = 1µm).

Figures 57 and 58. The excrescences typical of young coenocyst walls. (Scales = 2µm).

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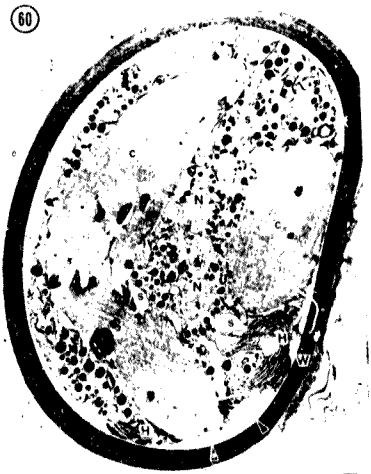

Figure 59. A young coenocyst, cross-sectioned, still with a pyrenoid and a large compacted chloroplast portion. (Scale = 4μm).

Figure 60. A longitudinally-sectioned coenocyst displaying natural dehydration, with membranous fragments (arrowheads) between the wall and the plasmalemma. Irregular lipocarotenoids are arranged in linear concentric arrays adjacent to the wall. (Scale = 4μm).

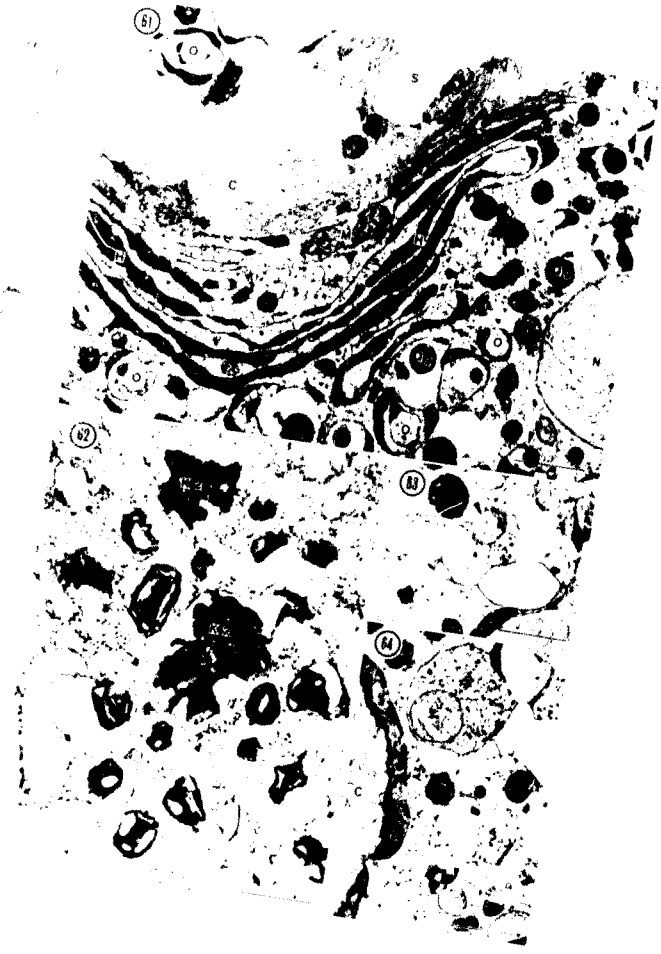
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Figure 61. Typical coenocyst cytoplasm with numerous vesicles (arrowheads), empty or containing lipocarotenoid globules. Both the annular structures resulting from lipocarotenoid globule digestion, and the linear arrays of lipocarotenoids, are present. (Scale = 1µm).

Figure 62. Vesiculated cytoplasm containing the second type of crystal (k2) found in coenocysts. (Scale = 0.5µm).

Figure 63. The remnant of an organelle (arrowhead) enclosed and surrounded by vesicles with appressed membranes. (Scale = 0.5µm).

Figure 64. Cytoplasmic vesiculation including autophagic vacuoles. (Scale = 0.5µm).

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Figure 65. The coenocyst cytoplasm is typically filled with vesicles, lipocarotenoid globules, and irregular lipocarotenoids. Some vesicles and cell contents have been exocytosed (arrowheads) and lie against the cell wall. (Scale = 0.5µm).

Figure 66. A coenocyst nucleus with a central nucleolus and a pored envelope (arrowheads). A short ER fragment (arrow) lies near the nucleus, as do both crystal types. (Scale = 0.5µm).

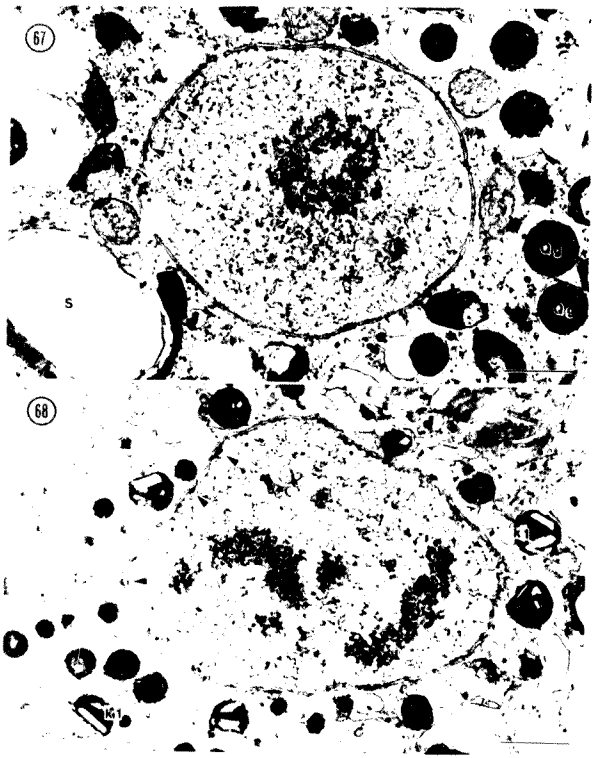
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Figure 67. A commonly-occurring type of nucleus with a cored nucleolus and peripheral chromatin aggregates (arrowheads). (Scale = 0.5µm).

Figure 68. Although dense heterochromatin is scattered through the nucleoplasm, peripheral aggregates still occur (arrowheads). (Scale = 0.5µm).

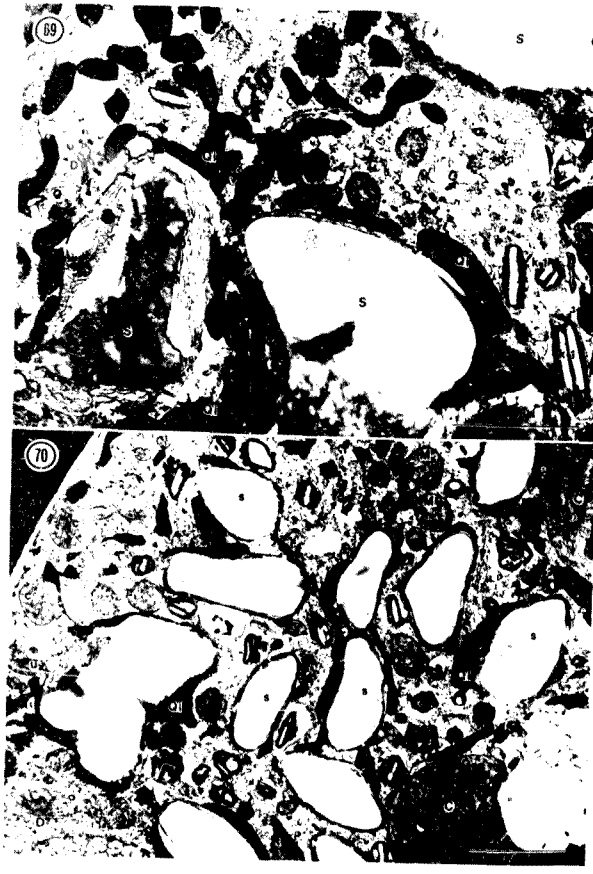
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Figure 69. A young coenocyst in which lipocarotenoid arrays are still being formed on the edges of chloroplast remnants, both thylakoidal and starch. The plate-like structure of type 1 crystals is clearly evident. A reduced dictyosome and a short RER profile occur. (Scale = 1µm).

Figure 70. Irregular lipocarotenoids surrounding isolated starch grains. Numerous type 1 crystals occur in vacuoles scattered throughout the cytoplasm. (Scale = 1µm).

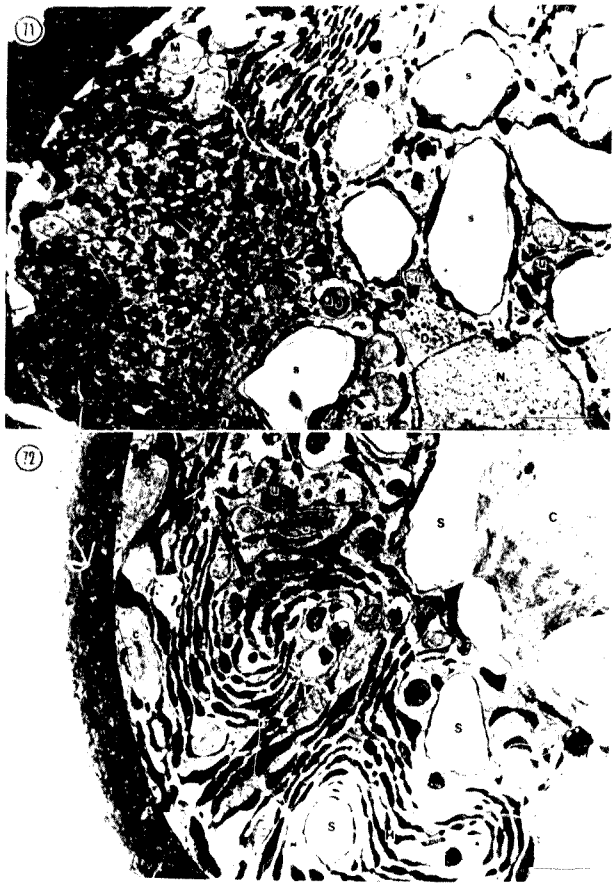
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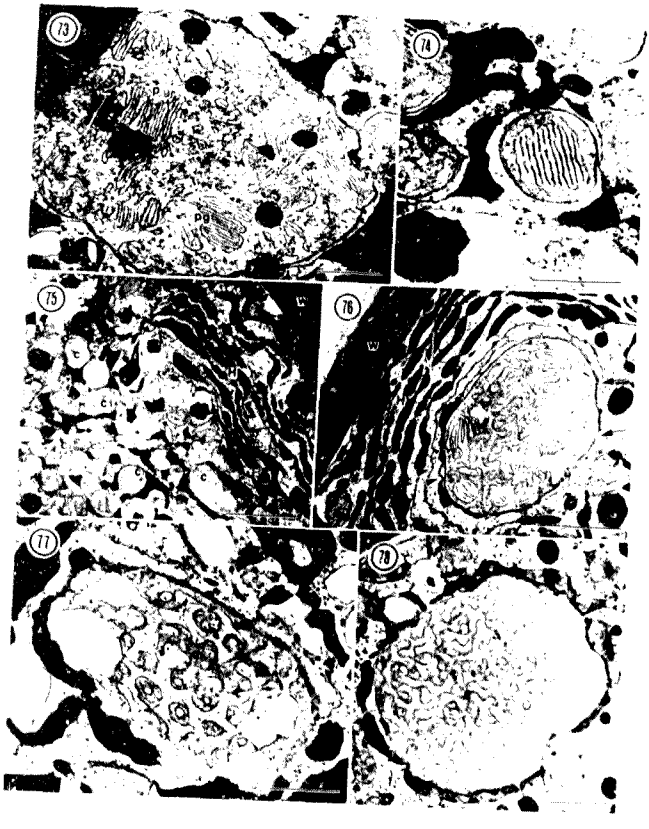
Figure 71. Beaded linear arrays of lipocarotenoids lining the coenocyst wall, and extending from an irregular lipocarotenoid mass. (Scale = 1µm).

Figure 72. The concentric paths of the lipocarotenoid - beaded hemimembranes occur between the wall and the large chloroplast mass. (Scale = 1µm).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