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STUDY OF THE MARINE PHYTOFLAGELLATE
✓
Pyramimonas pseudoparkeae PIENAAR et ✓
AKEN
✓
(PRASINOPHYCEAE) /



by

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Mark Ernest Aken.

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VOLUME ONE

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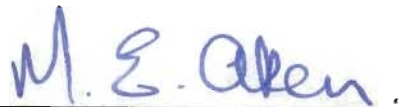
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TO MY PARENTS

P R E F A C E

The experimental work described in this thesis was carried out in the Department of Botany, University of Natal, Pietermaritzburg, from January 1979 to May 1985, under the supervision of Professor R.N. Pienaar.

These studies represent original work by the author and have not been submitted in any form to another University. Where use was made of the work of others it has been duly acknowledged in the text.



Mark Ernest Aken

May, 1985

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ABSTRACT

The morphology and structure of *P. pseudoparkeae* is described in detail. The alga resembles other species in the genus but is most closely related to *P. parkeae* being separated from it by differences in scale structure. Important taxonomic (phylogenetic) characteristics of *P. pseudoparkeae* include the possession of four flagella, trichocysts, a complete covering of three scale types on the cell body, and a 3-over-1 arrangement of the basal bodies.

The alga was grown successfully in a number of enriched and artificial seawater media. The alga grew well in a salinity of 35‰ but it is euryhaline and tolerated salinity levels ranging from 10 - 70‰. The relative growth rate (k') of the alga was significantly increased by raising the light intensity from 50 to 100 or 150 $\mu\text{Em}^{-2}\text{s}^{-1}$. At higher light intensities (200 and 300 $\mu\text{Em}^{-2}\text{s}^{-1}$) k' was reduced, probably through photoinhibition.

The alga grew well at 20 and 25°C but could not tolerate a temperature of 30°C. The growth studies indicated that optimal growth (determined by the highest relative growth rate) was achieved in PES medium at a salinity of 35‰, a light intensity of 100 - 150 $\mu\text{Em}^{-2}\text{s}^{-1}$, and a temperature of 20 - 25°C. Under these conditions the mean doubling time (G) of the cells was 26 h. Scale structure in *P. pseudoparkeae* remained constant in the different seawater media used and under a range of salinity, temperature and light intensity.

P. pseudoparkeae could not be grown axenically and was shown to have an absolute requirement for bacteria in culture. This bacteria/algal relationship is believed to be mutualistic because the alga also promoted the growth of the bacteria. The nature of the growth promoting factors involved are not known.

Cell division in *P. pseudoparkeae* was similar to that described for other species in the genus. The cells remained motile throughout the cell division cycle and they divided preferentially during the dark. Cultures of the alga could at best be partially synchronized under optimal growth conditions because the shortest mean doubling time obtained was 26 h; i.e. two hours longer than the 24 h period in a 16h:8h synchrony induction photoregime.

Cell division began with the division of the chloroplast followed by the replication of the flagellar basal bodies, dictyosomes and nucleus (in that order). Mitosis is characterized by an open spindle. Spindle microtubules, which are derived from the rhizoplast, are absent at telophase and no phycoplast develops. Cell division is completed within 90 min.

All scale types covering the alga were produced continuously by the two dictyosomes within the cell. Scale morphogenesis was shown to be a rapid process with scales being completely formed within 10,5 min. This is the time taken for a single cisterna to pass through the dictyosome (comprising 20 cisternae). Flagellar scales were stored in a scale reservoir which was always connected with the flagellar pit via a duct. These scales were released when four new flagella developed from the replicated basal bodies. A compound microtubular rootlet was always associated with the duct of the scale reservoir. Body scales moved in vesicles from the dictyosomes directly to the plasmalemma at the base of the flagellar pit where they were released by reverse pinocytosis.

The scales of *P. pseudoparkeae* were shown to be pectinaceous in nature being predominantly composed of polysaccharide and containing a small amount (4%) of protein. TLC separation of sugar residues in acid hydrolysates of scales showed that the latter were composed of neutral sugars galactose, arabinose, xylose, rhamnose and a trace of fructose. Galacturonic acid is also thought to be a major constituent of the scales because they were digested with pectinase. The scale polysaccharide is sulphated. Aspartic and glutamic acid were major amino acid residues detected on scale hydrolysates analysed on an automatic amino acid analyser. The polyanionic nature of the scales is thought to underly the mechanism of external self-assembly of the scale-boundary, and to contribute to the maintenance of water and salt balance in the cell.

P. pseudoparkeae reproduces asexually by binary fission or by producing non-motile, thick-walled cysts. Cysts developed spontaneously in cultures so that the stimuli causing encystment are not known. In fresh medium mature cysts released four motile cells which regenerated the motile phase. The cyst wall is bilayered. The outlet layer of the cyst wall has the properties of sporopollenin while the inner layer of the cyst wall has the same staining properties as scales and is possibly of similar composition. EDX analyses of the cyst wall showed that it is rich in calcium and sulphur.

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VOLUME II

PLATES AND PLATE ANNOTATIONS

CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION

The introduction has, for convenience, been divided into three components. The first component introduces the class Prasinophyceae, and includes an outline of the general characteristics of the class and presents some of the controversies concerning the recognition of the class. This component also contains a more detailed literature review of the class presented under the headings morphology and structure, physiology and biochemistry, and life-histories. It is important to mention here that the short prelude on the general characteristics of the class is intended to be brief, and for this reason specific examples and references have been omitted. Comments made here are substantiated in the literature review.

The second component of the introduction has been given to a review of the genus *Pyramimonas* SCHMARDA. The information presented here could have been included in the general literature review, but because it is relevant to the present study it appears separately.

The introduction is concluded with a component on the aims of the present study and the reasons for conducting this investigation. In this regard, the results of an earlier investigation on the experimental organism are outlined because they provided a foundation on which some of the aims of the present study were based.

1.2 THE CLASS PRASINOPHYCEAE

1.2.1 General characteristics of the class

The class Prasinophyceae comprises a group of unicellular, predominantly flagellate, green algae that are common in marine, brackish and freshwater habitats. They are most abundant in temperate waters in both hemispheres, but some inhabit colder waters. Two members of the class are cryophilic and are found in sea-ice in the Arctic and Antarctic.

Prasinophyceae possess major photosynthetic pigments, chlorophylls a and b and most green algal carotenoids. They store starch as a carbohydrate reserve product and in this respect they are similar to other green algae, being classified with them in the division Chlorophyta. Despite these similarities, members of the Prasinophyceae are separated from other green algae by a number of micromorphological and ultrastructural characteristics. The most important of these is that flagellate members of the class invariably possess a covering of unmineralized organic scales on their flagella. Most species also have a covering of scales on the cell surface. In this respect the Prasinophyceae are thought to be primitive green algae, with the body-scale boundary possibly representing the precursor to the cell wall which is present in the more advanced green algae. These organic scales, which take on a variety of architectural forms, are often beautifully constructed and are so small that they can only be seen with the aid of an electron microscope. This instrument is an essential tool for studying members of the class. The structure of the scales, and their position on the flagellar and cell surfaces, is thought to be consistent for a particular species and thus provides a useful taxonomic criterion to delineate species.

With respect to their morphology, members of this class form a heterogeneous group. They are microscopic green algae ranging in size from 1,0 μm to 30 μm in length. Some phycoma stages, however, are larger (800 μm in diameter) and can be seen with the naked eye. Motile cells possess one to eight flagella which may be inserted anteriorly, laterally or posteriorly in a depression in the cell surface. Cells may be bilaterally or radially symmetrical when viewed along the apical axis. Bilaterally symmetrical types are sometimes flattened and characteristically have one or two flagella. Biflagellate cells usually have heterokont flagella which show marked heterodynamic activity. Cells with radial symmetry have four or eight isokont flagella exhibiting homodynamic activity.

Prasinophyte cells have a typical eukaryotic internal structure that is best observed by electron microscopy. However, light microscopy reveals some of the larger organelles within the cells. These include a single parietal chloroplast and a nucleus that is usually located near the region of flagellar insertion. The chloroplast, which is often lobed and closely follows the contours of the cell, contains an eyespot and a posteriorly situated pyrenoid. Starch grains usually form a sheath around the pyrenoid but they

may occupy other positions within the chloroplast. Two organelles that may be seen in the larger members of this class are the vacuole and the striated rootlet. Trichocysts, if present, can also be seen with the aid of the light microscope.

Dictyosomes, mitochondria, microbodies and other organelles are best observed at the ultrastructural level.

Non-motile forms are found in the class. These often alternate with motile cells in an asexual reproductive cycle. Non-motile stages usually possess a cell wall but motile stages are usually covered with scales. There are, however, a few exceptions to this general rule. One genus possessing a theca in the motile stage has been assigned to the class; so have two genera that contain "naked" monads, where the plasmalemma represents the boundary of the cell.

Sexual reproduction is not known in the class.

1.2.2 Controversies over the recognition of the class

In 1941, Chadeffaud (1941), using only the light microscope, recognised some members of the class Prasinophyceae and distinguished them from other green algae. These "Prasimates" as he called them included the genera *Pyramimonas* (as *Pyramidomonas*), *Prasinocladus* KUCKUCK, *Platymonas* G.S. WEST and *Chlorodendron* SENN. [*Prasinocladus* and *Platymonas* have recently been placed into the genus *Tetraselmis* STEIN (Hori *et al.*, 1982, 1983); *Chlorodendron* is a later synonym of *Prasinocladus* (Silva, 1980)].

Chadeffaud separated these "Prasimates" from other green algae because they exhibited unique morphological and structural characteristics, namely the presence of a depression in the cell body at the region of flagellar insertion, the presence of muciferous bodies or trichocysts, and the presence of parabasal bodies (thought to be dictyosomes of the Golgi apparatus) near the flagellar pit. It was only in 1962, in Christensen's analysis of the green algae (Christensen, 1962), that additional ultrastructural characteristics were revealed which apparently supported the separation of the "Prasimates" from the main body of green algae. These "Prasimates", which possessed at least

one of the characteristics noted by Chadeffaud, invariably had an external covering of organic scales. Christensen placed these algae in the class Prasinophyceae, including in the class those pyramidal and globular cell types in which scale-covered motile cells were known. Although he separated the dorsi-ventrally flattened types bearing fine flagellar hairs into a second class, the Loxophyceae, authors of subsequent publications generally do not recognize this class as being separate from the Prasinophyceae (Bourrelly, 1970; Parke and Green, 1976). Moestrup (1982), however, has reinstated the class Loxophyceae and separates it from the Prasinophyceae. He includes in the Loxophyceae those genera which possess very fine non-tubular hairs on their flagella namely, *Monomastix* SCHERFFEL and *Pedinomonas* KORSHIKOV (and perhaps *Mantoniella* DESIKACHARY). Members of the Prasinophyceae characteristically bear complex tubular hairs, often referred to as hairscales (Moestrup and Thomsen, 1974), on their flagella.

Since its inception, the class Prasinophyceae has evoked varied responses amongst phycologists. Where some authors accept the class, others have rejected it. This controversy stems primarily from the discovery of other scale-covered monads within the division Chlorophyta. The zoospores of *Pseudendoclonium basiliense* VISCHER and *Trichosarcina polymorpha* NICHOLS *et* BOLD (both belonging to the class Ulvophyceae), possess a scale-covering (Mattox and Stewart, 1973), as do the spermatozoids of two advanced genera in the class Charophyceae, namely *Nitella* AGARDH (Turner, 1968) and *Chara* VAILLANT (Pickett-Heaps, 1968; Moestrup, 1970). Scales have also been found on the zoospores of the charophycean alga *Coleochaete* BREBISSON (McBride, 1968, Pickett-Heaps and Marchant, 1972).

In view of these discoveries, Stewart and coworkers (Stewart *et al.*, 1974) concluded that a characteristic such as the presence or absence of a scale-covering is insufficient to define a separate assemblage of green algae, and they therefore do not recognize the class Prasinophyceae. Stewart and Mattox (1975a, 1975b) agree with Manton (1965) that the "prasinophytes" are primitive green monads, but they add that they do not form a natural group because they also have diverse mechanisms of mitosis and cytokinesis. They draw attention to the differences in the appearance of the telophase spindle of the two prasinophytes *Tetraselmis subcordiformis* (WILLE) HAZEN and *Pedinomonas minor* KORSHIKOV. *Tetraselmis* has a collapsing interzonal spindle at telophase and cytokinesis is effected by a phycoplast (Stewart

et al., 1974). The phycoplast is a system of microtubules that develops between daughter nuclei and which lies parallel to the plane of division. *Pedinomonas* by contrast, has a persistent interzonal spindle at telophase and does not develop a phycoplast (Pickett-Heaps and Ott, 1974).

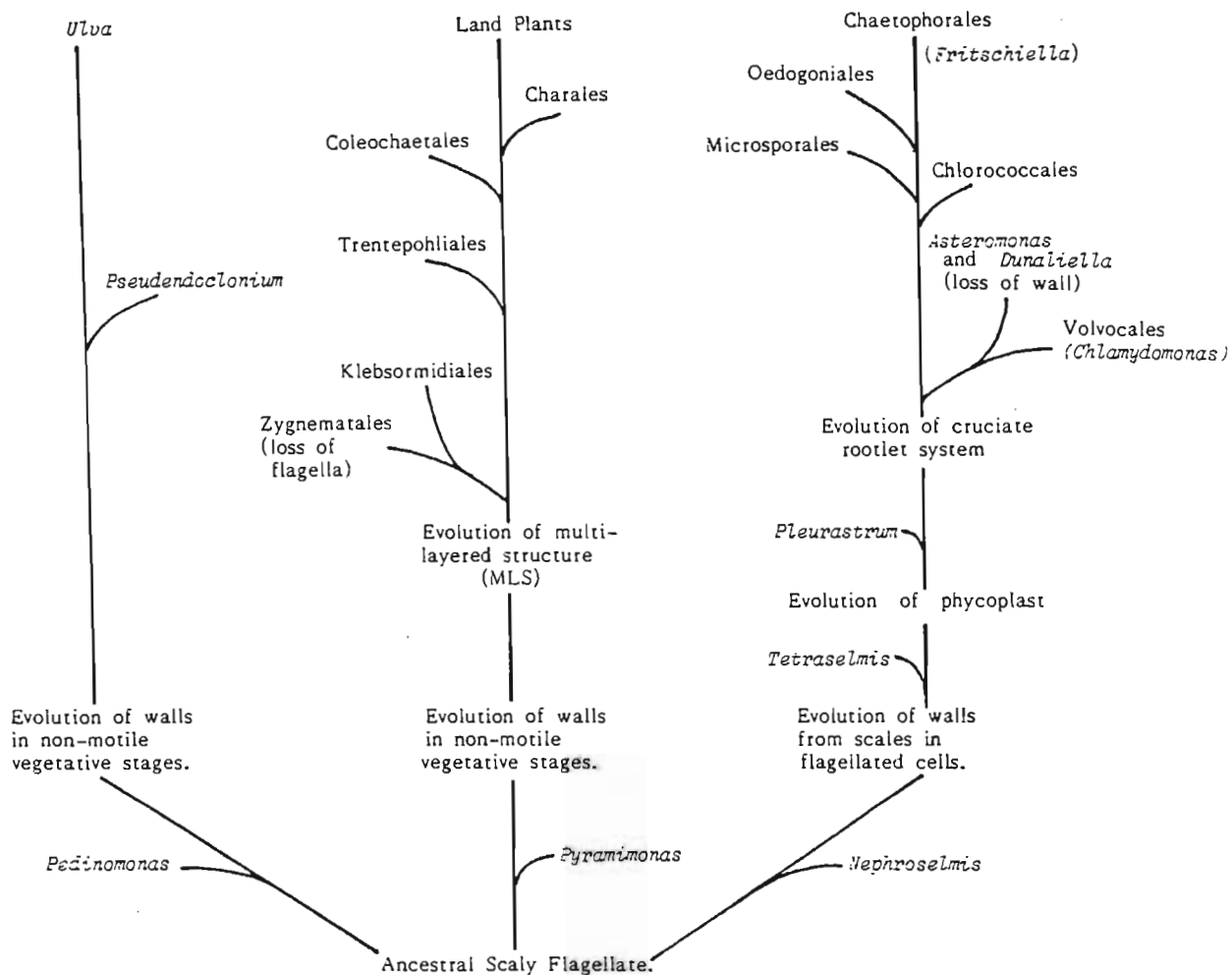
Based on their observations of the mitotic spindle at telophase, Stewart and Mattox (1975a) recognized two classes in the division Chlorophyta, namely the Chlorophyceae and Charophyceae. These two classes reflect divergent evolutionary lines. Members of the former class are characterized by having a collapsing spindle and the development of a phycoplast while members of the second class are characterized by having a persistent interzonal spindle at telophase. It is interesting to note that the persistent interzonal spindle is exhibited by all bryophytes and tracheophytes and that these plants are thought to have evolved from a charophycean progenitor (Pickett-Heaps and Marchant, 1972; Stewart and Mattox, 1975a, 1978; Mattox and Stewart, 1977; Pickett-Heaps, 1979).

Of interest here is that *Tetraselmis* and *Pedinomonas*, both thought to be related prasinophytes, can be placed into two divergent phylogenetic lines within the green algae. On this basis Stewart and Mattox (1975a, 1975b) suggested that the prasinophytes should be separated into existing classes in the division Chlorophyta. This they did in a subsequent publication (Stewart and Mattox, 1978) where *Tetraselmis* (as *Platymonas* - see Melkonian, 1979a, and Norris *et al.*, 1980 for correct name) was placed in the class Chlorophyceae (see Text Fig. 1.1). *Pedinomonas*, however, was not placed in the class Charophyceae as expected but was placed in a third class erected by Stewart and Mattox (1978), namely the class Ulvophyceae. This class contains *Ulva* (L.) THURET and its relatives and is thought to represent a third phyletic line in the green algae. The unique features of the Ulvophyceae are that their motile propagules do not have a multilayered structure (MLS) associated with the flagellar basal bodies (as do motile charophycean cells) and there is a collapsing spindle but no development of a phycoplast during telophase (the phycoplast is characteristic of chlorophycean algae). *Pedinomonas* was assigned to the class Ulvophyceae because it does not exhibit a MLS nor phycoplastic cytokinesis and because it bears one layer of body scales which resemble those found on the zoospores of some ulvophycean algae.

ULVOPHYCEAE
 (Phycoplast and multi-layered structure absent).

CHAROPHYCEAE
 (Persistent interzonal spindle at telophase; motile cells possess a multilayered structure; phragmoplast present; enzyme glycolate oxidase present).

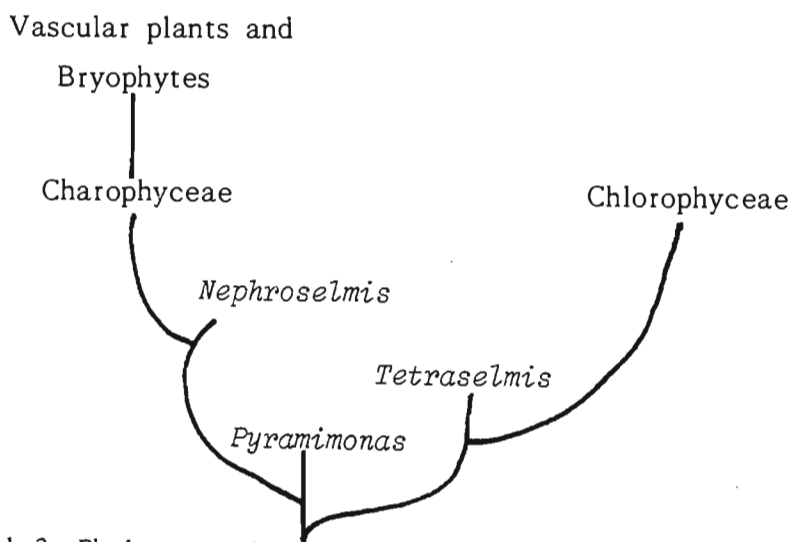
CHLOROPHYCEAE
 (Collapsing interzonal spindle at telophase; phycoplast present, enzyme glycolate dehydrogenase present).



Text Fig. 1.1: Green algal phylogeny as proposed by Stewart and Mattox (modified from Stewart and Mattox, 1978).

Stewart and Mattox (1978) included two other prasinophytes in their phyletic scheme, namely *Nephroselmis* STEIN (as *Heteromastix* KORSHIKOV - see Moestrup and Ettl, 1979 for nomenclatural priority) and *Pyramimonas*. *Pyramimonas* was placed in the class Charophyceae because the alga has an interzonal spindle that persists until late telophase. In spite of this, it is surprising that Stewart and Mattox recognize *Pyramimonas* as a member of this class because the alga does not possess a MLS and it does not contain the enzyme glycolate oxidase which is characteristic of charophycean algae and also the land plants (Fredrick *et al.*, 1973). Instead, *Pyramimonas* possesses the enzyme glycolate dehydrogenase (Floyd and Salisbury, 1977) and is therefore more closely allied with members of the Chlorophyceae. *Nephroselmis* was assigned to the class Chlorophyceae because the alga has flagellar scales that are similar to those of *Tetraselmis*. In spite of this the placement of *Nephroselmis* in the Chlorophyceae appears incongruous especially as the alga was shown by Mattox and Stewart (1977) to have a persistent interzonal spindle at telophase and no pyrenoplast (i.e. characteristics of the Charophyceae).

Moestrup and Ettl (1979) have expressed the opinion that *Nephroselmis* resembles the Charophyceae and not the Chlorophyceae with respect to the appearance of the spindle at telophase and they conclude that the alga represents a group of prasinophycean flagellates that are ancestral to the Charophyceae and hence the bryophytes and vascular plants (See Text Fig. 1.2).



Text Fig. 1.2: Phylogeny of the green algae according to Moestrup and Ettl (redrawn after Moestrup and Ettl, 1979).

Norris (1980), in his review of the Prasinophyceae, rejects Stewart and Mattox's proposal that prasinophytes should be assigned to existing classes in the division Chlorophyta. He gives convincing arguments to support his view that the class Prasinophyceae comprises a natural group of green algae. He argues that the diversity in form, ultrastructure, and mechanisms of mitosis and cytokinesis, occurring in the class, could have evolved within the group and can be explained by his hypothesis that the more advanced symmetrical prasinophytes are derived from more primitive asymmetrical types. By inference unflagellate and biflagellate species evolved to give rise to quadriflagellate species. Norris supports his hypothesis with the observations outlined below.

The evolutionary lineage from one layer of scales to two or more layers begins in biflagellate species. Species having only one type of scale (a primitive feature) are biflagellate. Uniflagellate and biflagellate species have primitive pigment systems when compared with quadriflagellate species. A progressive series of cytokinesis from sequential division of organelles prior to cytokinesis (a primitive feature) to synchronization of organelle division immediately before mitosis and cytokinesis (an advanced feature) can be seen in biflagellate and quadriflagellate species respectively. A precocious furrowing type of cytokinesis and late separation of chromatin by cytokinesis is a primitive phenomenon and occurs only in biflagellate species. Advanced quadriflagellate species have complex cell structures (e.g. trichocysts, scale reservoirs, muciferous bodies and large vacuoles) that are probably not basically important to the cell and would be expected in more highly evolved species. These points are discussed in more detail later.

Although it is generally accepted that the ultimate progenitors of the green algae, bryophytes and tracheophytes, were asymmetrical flagellates (Manton, 1965; Taylor, 1976; Stewart and Mattox, 1980; Melkonian, 1982a), some authors suggest that asymmetry in a few extant prasinophytes (and in the spermatozooids and zoospores of some charophycean and ulvophycean algae) may have arisen through "halving" of a symmetrical *Pyramimonas*-like ancestor (Chadefaud, 1977; Stewart and Mattox, 1978; Moestrup and Ettl, 1979). In this respect, Moestrup and Ettl (1979) believe that *Nephroselmis* and *Mesostigma* LAUTERBORN are modified biflagellate forms that could be derived from a *Pyramimonas*-like cell through lateral compression in the former genus and through dorsi-ventral compression in the latter genus.

Using *Nephroselmis olivacea* STEIN as an example, Moestrup and Ettl (1979) express the view that the alga is more advanced than *Pyramimonas* because it possesses a complex scale-covering comprising four layers of different scales on both the cell body and flagellar surfaces. *Pyramimonas*, by contrast, has three layers of different scales in corresponding positions. Furthermore, *Nephroselmis* is thought to be more closely related to the Charophyceae than *Pyramimonas* because some features of the flagellar root system indicate that the alga may represent an intermediate form between the cruciate root system seen in *Pyramimonas* and the unilateral root system of motile charophycean cells. *Nephroselmis* has a peculiar microtubular root system composed of three roots with the microtubules arranged in a 10-4-3 pattern : the root containing ten microtubules has the latter arranged in a multilayered pattern and therefore resembles the MLS seen in motile charophycean cells. A MLS has also been demonstrated for *Mesostigma* thus further supporting the hypothesis that some prasinophytes are closely allied to members of the Charophyceae (Rogers *et. al.*, 1981).

Moestrup and Ettl (1979) and Norris (1980), while holding different views concerning phylogeny within the Prasinophyceae, both recognize the class as one separate from the classes Chlorophyceae, Charophyceae and Ulvophyceae *sensu* Stewart and Mattox (1978).

The controversial position of the class Prasinophyceae, is evident by the fact that it has only recently been formally described (Silva, 1980), some 18 years after it was first proposed by Christensen (1962) and approximately 40 years after some members of the class were recognized by Chadeffaud (1941).

For the purpose of this dissertation the writer accepts the class Prasinophyceae. A detailed discussion of the taxonomy of the class is presented in Chapter Eight.

1.2.3 Morphology and structure

In this review only those well known genera that are generally accepted as members of the class Prasinophyceae are discussed. Many other genera have been allied with the Prasinophyceae (see Norris, 1980) but they have

been omitted here because their inclusion in the class is questionable.

Members of the class Prasinophyceae are morphologically and structurally diverse. Table 1.1 has been included to summarize the diversity exhibited by flagellate members of the class. The table includes information on cell symmetry, flagellar number and insertion, and the nature of the scale-covering. (It is important to mention that cell symmetry is described as seen with the light microscope - electron microscopy reveals that all genera are in reality asymmetrical).

The number of flagella may vary from one in the genera *Micromonas* MANTON *et* PARKE, *Pedinomonas* and *Monomastix* to eight in *Pyramimonas amyliifera* CONRAD (Manton, 1966a). Prasinophytes more often possess two or four flagella. With the exception of *Pyramimonas amyliifera*, members of the genera *Pyramimonas* and *Tetraselmis* are quadriflagellate. The remaining genera in the class contain biflagellate species (see Table 1.1).

Prasinophytes exhibit a wide range in cell size. *Micromonas* is the smallest known unicellular eukaryote and has a length of only 1.0 μm (Manton and Parke, 1960). Some species of *Pyramimonas* are large measuring 30 μm to 48 μm in length (Belcher, 1969a; Inouye *et al.*, 1984), although the non-motile phycoma stage of *Halosphaera viridis* SCHMITZ may be 800 μm in diameter (Parke and den Hartog-Adams, 1965). According to the phytoplankton size categories given by Boney (1975), prasinophytes would thus form part of the ultra-, nano-, and microplankton.

Many of the external characteristics of the Prasinophyceae become apparent at the ultrastructural level. One of the most important characteristics is the possession of a scale-covering in flagellate cells. The occurrence and arrangement of these scales on the flagellar and cell surfaces varies between genera (see Table 1.1). Scales occurring in different layers in the scale-boundary invariably have different architectural shapes. These may be oval, hexagonal, square, subrhomboidal etc. in outline, with various modifications such as having adnate spines, raised nodules or pores. Some scales may be quite ornate, exhibiting delicate sculpturing. Smaller scales are usually found adjacent to the plasmalemma, with successively larger scales occurring in the more distal layers. The number of scale-layers and

TABLE 1.1
MORPHOLOGICAL DIVERSITY IN FLAGELLATE MEMBERS OF THE PRASINOPHYCEAE

GENUS	CELL SYMMETRY	NO. OF FLAGELLA	FLAGELLAR INSERTION	FLAGELLAR SCALES	BODY SCALES	REFERENCES
<i>Micromonas</i> MANTON et PARKE	asymmetrical	one - with bi-partite condition	lateral - flagellum directed posteriorly	absent	absent	Manton, 1959; Manton and Parke, 1960.
<i>Pedinomonas</i> KORSIIKOV	asymmetrical	one - with bi-partite condition, but two basal bodies	postero-lateral - flagellum directed posteriorly.	absent but fine hairs arranged in two rows on flagellum	usually absent but one scale type in <i>P. major</i>	Ettl, 1964; Manton and Parke, 1960; Belcher, 1968b; Pickett-Henps and Ott, 1974; Sweeney, 1976; Cachon and Caram, 1979.
<i>Nonomastix</i> SCHIERFFEL	asymmetrical	one - with bi-partite condition but two basal bodies.	anterior-lateral, flagellum directed posteriorly	absent	one scale type	Belcher, 1965; Manton, 1970.
<i>Mantoniella</i> DESIKACHARY	asymmetrical	two - one extremely reduced	anterio-lateral, flagellum directed posteriorly	one scale type plus hairscales	one scale type	Manton and Parke, 1960; Desikachary, 1972; Barlow and Cattoico, 1980.
<i>Dolichomastix</i> MANTON	asymmetrical	two - subequal to equal	lateral	one scale type plus hairscales	one scale type	Manton, 1977
<i>Hamiella</i> MOESTRUP	asymmetrical	two subequal	as above but anterior flagellum longer	one scale type - plus hairscales	one scale type	Parke and Rayns, 1964; Leadbeater, 1974; Moestrup, 1984.
<i>Pseudoscourfieldia</i> MANTON	asymmetrical	two - unequal	apical/posterior - flagella directed posteriorly	two scale types - plus hairscales	two scale types	Manton, 1975; Norris, 1980.
<i>Neostigma</i> LAUTERBORN	asymmetrical	two - subequal	lateral - directed slightly anteriorly	two scale type - no hairscales	three scale types	Manton and Ettl, 1965; Rogers et al., 1981.
<i>Nephroselmis</i> STEIN	asymmetrical	two - unequal	lateral-short flagellum directed anteriorly, other trailing	two or three scale types - plus hairscales	two - four scale types	Manton et al., 1965; Ettl, 1967a; Mattox and Stewart, 1977; Moestrup and Ettl, 1979.
<i>Prasinochloris</i> BELCHER	symmetrical	Four - equal	apical/anterior	no information	no information	Belcher, 1966.
<i>Pyramimonas</i> SCHIMARDA	symmetrical	usually four - equal, but eight in <i>P. amyliifera</i>	apical/anterior - flagella directed posteriorly	three scale types - plus hairscales	usually three types of scale. Occasionally two	Manton et al., 1963; Manton, 1966, 1968, 1969; Swale and Belcher, 1968; Belcher, 1969; Moestrup and Thomsen, 1974; Norris and Pearson, 1975; Pennick and Clarke, 1976; Pennick et al., 1976; Pennick, 1977a, 1978, 1982a, 1982b, 1983; Norris and Piennar, 1978; Pennick et al., 1978; Piennar, 1978; McFadden et al., 1982; Pennick and Cann, 1982; Inouye et al., 1983, 1984.
<i>Tetraselmis</i> STEIN	symmetrical	four - equal	apical/anterior flagella directed posteriorly	two scale types - plus hairscales	thecate, with curled hairs in flagellar pit	Proskauer, 1950; Manton and Parke, 1963; Parke and Manton, 1965, 1967; McLachlan and Parke, 1967; Hori and Chihara, 1974a, 1974b; Norris et al., 1980; Hori et al., 1982, 1983.

the structure of individual scale types is thought to be species specific, thus providing a useful criterion on which species may be separated (Norris and Pienaar, 1978).

Although the presence of a scale-covering is a characteristic of the Prasinophyceae, it is not unique to the class. As mentioned in the preceding section, scale-bearing gametes and zoospores have been discovered in advanced green algae belonging to the classes Charophyceae and Ulvophyceae.

Scale-covered flagellates are also known from the classes Dinophyceae, Chrysophyceae, Prymnesiophyceae and Cryptophyceae.

The two dinoflagellates *Oxyrrhis marina* DUJARDIN and *Heterocapsa triquetra* (EHRENBERG) STEIN possess a scale-covering external to the thecal complex (Clarke and Pennick, 1972, 1976; Pennick and Clarke, 1977; Morrill and Loeblich, 1981).

In the Chrysophyceae scales are found, for example, on *Paraphysomonas* DE SAEDELEER (Manton and Leedale, 1961a; Moestrup, 1979), *Sphaleromantis* PASCHER (Harris 1963; Manton and Harris, 1966; Pienaar, 1976a), *Chryso-sphaerella* LAUTERBORN (Bradley, 1964; Kristiansen, 1969; Asmund, 1973; Moestrup, 1979), *Mallomonas* PERTY (Harris, 1970), *Synura* EHRENBERG (Takahashi, 1972, 1975, 1978; Peterfi and Momeu, 1977; Hibberd, 1978a), and *Syncrypta* EHRENBERG (Clarke and Pennick, 1975). Of these, *Sphaleromantis* and *Synura* possess both body and flagellar scales but the remainder have body scales only. These scales may be purely organic or they may be silicified. Scales of the latter variety adorn *Synura* (Schnepf and Deichgräber, 1969; Klaveness and Guillard, 1975; Lee, 1978), *Mallomonas* (Wujek and Kristiansen, 1978) and *Paraphysomonas* (Lee, 1978; Moestrup, 1979).

Scale-bearing algae in the class Prymnesiophyceae may be separated into two groups depending on the chemical nature of the scales. The first group possesses organic scales only and the second group often possess, in addition to organic scales, an outer covering of scales that have CaCO₃ crystals deposited on an organic basal plate scale. In the latter group, the mineralized scales are called coccoliths. All prymnesiophytes bear body scales only. Examples of genera possessing organic scales are *Chrysochromulina*

LACKEY (Parke *et al.*, 1955, 1956, 1958, 1959; Manton and Leedale, 1961b; Parke and Manton, 1962; Manton 1966b, 1967a, 1972a, 1972b; Leadbeater and Manton, 1969a, 1969b; Green and Leadbeater, 1972; Leadbeater, 1972; Pienaar, 1975a; Pienaar and Norris, 1979), *Prymnesium* MASSART (Manton, 1966c; Manton and Leedale, 1963; Pienaar, 1980a), *Isochrysis* PARKE (Parke, 1971; Green and Pienaar, 1977), *Platychrysis* GEITLER (Chretiennot, 1973), *Dicrateria* PARKE (Green and Pienaar, 1977) and *Imantonia* REYNOLDS (Green and Pienaar, 1977). Two well known genera bearing coccoliths are *Coccolithus* SCHWARZ (Parke and Adams, 1960; Manton and Leedale, 1969) and *Hymenomonas* STEIN (Manton and Leedale, 1969; Manton and Peterfi, 1969; Pienaar, 1969a, 1969b, 1970, 1976b, 1976c).

In a recent publication, Santore (1983) has shown that two members of the class Cryptophyceae possess a covering of minute scales. In *Chroomonas* HANSGIRG these scales are restricted to the flagellar surfaces, but in *Cryptomonas* EHRENBERG they occur on the cell only. The scales in both organisms are identical.

Although the flagellates mentioned above bear scales, they are separated from the Prasinophyceae on a number of more important taxonomic characteristics e.g. differences in pigment systems, reserve products etc. (see Bold and Wynne, 1978, pp. 18-19 and Lee, 1980 pp. 8-9).

Particularly interesting is that scales have also been found on a number of protozoans, e.g. *Paramoeba* SCHAUDINN (Grell and Bennitz, 1966; Pennick and Goodfellow, 1975), *Vannella simplex* WOLFARTH-BOTTERMANN (Hausmann and Stockem, 1972), *Mayorella riparia* PAGE (Pennick and Goodfellow, 1975) and *Cochliopodium* HERTWIG *et* LESSER (Bark, 1973).

The uniflagellate prasinophytes *Micromonas pusilla* (BUTCHER) MANTON *et* PARKE (Manton and Parke, 1960), *Pedinomonas minor* (Ettl, 1964; Pickett-Heaps and Ott, 1974) and *P. tuberculata* (VISCHER) GAMS (Manton and Parke, 1960) notably lack a scale-covering. *Pedinomonas major* KORSHIKOV (Belcher, 1968b) is unusual in the genus because it possesses one layer of body scales, but like *P. minor* and *P. tuberculata* it has two rows of very thin hairs on the flagellum. These hairs are very different from the thick tubular hairs which are typically found associated with scales on prasinophyte flagella (Moestrup, 1982). Thick flagellar hairs have their

origin in the dictyosome and have been referred to as hairscales (Moestrup and Thomsen, 1974). *Mesostigma* is exceptional in that it possesses flagellar scales but lacks hairscales (Manton and Ettl, 1965).

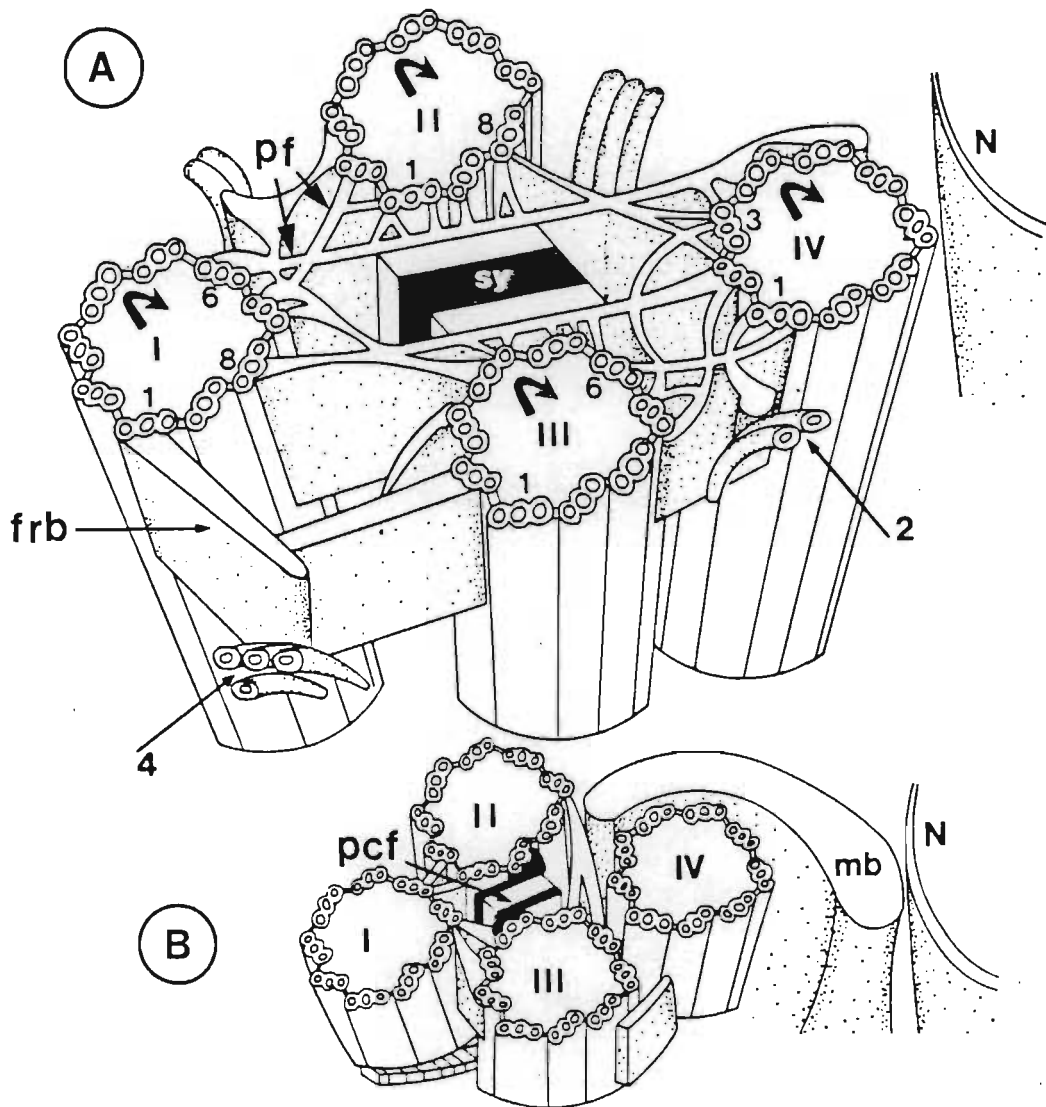
In addition to hairscales, flagellar scales may be present in one, two or even three layers (see Table 1.1).

Where body scales are present on the cell, they too usually occur in one to three layers. Genera possessing three body scale layers are thought to be more advanced than those with only one scale-layer (Norris, 1980). *Nephroselmis olivacea* (synonyms: *Heteromastix angulata* KORSHIKOV; *Bipedinomonas angulata* (KORSHIKOV) NORRIS - see Moestrup and Ettl, 1979 and Norris, 1980 respectively) is unusual in that it has four layers of body scales. As discussed previously, Moestrup and Ettl (1979) believe that this is an advanced prasinophyte closely related to members of the class Charophyceae.

Members of the genus *Tetraselmis* are unique in the Prasinophyceae as they possess a rigid theca composed of fused stellate particles (Manton and Parke, 1965). According to the phylogenetic scheme proposed by Norris (1980), thecate forms are the most advanced in the class.

The internal structure of prasinophyte cells is best revealed by electron microscopy. The major organelles visible with the light microscope have already been mentioned in Section 1.2.1 and will not be repeated here. Prasinophyte flagella have the typical 9+2 microtubular arrangement of the axoneme which was first described by Manton (1952). The flagella usually terminate abruptly and have a broadly rounded tip, but exceptions are seen in *Micromonas pusilla* (Manton and Parke, 1960), *Pedinomonas minor* (Ettl, 1964), *P. major* (Belcher, 1968b) and *Monomastix* sp. (Manton, 1970), where the flagella have a bipartite condition with the distal portion being narrower than the rest. This "hair point" results from the central pair of microtubules being longer than the peripheral microtubules of the axoneme.

Associated with the emergent flagella is the basal body system or kinetosome. The kinetosome, described in detail for *Pyramimonas* by Norris and Pearson (1975) and Melkonian (1981) - see Text fig. 1.3 - consists of the



Text Fig. 1.3. The flagellar basal body system of *Pyramimonas obovata* (reproduced from Melkonian, 1981). Fig. A shows four basal bodies (I - IV) which are connected by pericentriolar fibres (pf). Basal bodies II and III are connected by a large banded structure called the synistosome (sy). A fibrillar band (frb) connects basal bodies I and III. Four microtubular rootlets emerge from between the basal bodies in a 4-2-4-2 pattern

Fig. B. is a reconstruction of the proximal portion of the basal body system showing the association of the microbody (mb) with the basal bodies. The nucleus (N) is closely associated with the microbody. A proximal connecting fibre (pcf) lies between basal bodies II and III.

basal bodies (or kinetoplasts) with their associated transition region to the flagella proper, the synistosome, the rhizoplast, microtubular rootlets and often a microbody.

Because of the confusion that has surrounded the terminology of basal body-associated fibrous structures, Melkonian (1980) has proposed an unambiguous classification for these structures. He has suggested that the synistosome and other fibrous structures connecting different basal bodies should be called "connecting fibres". He has adopted the terminology of Micallef and Gayral (1972) for striated fibres. He suggests that rhizoplasts should be called System II fibres whilst other striated structures associated with the microtubular rootlets should be called System I fibres.

The term "rhizoplast" has not been unequivocally defined and has, in the past, been used for functionally different striated structures. The rhizoplast is currently viewed as a striated fibre that arises at the basal bodies and extends into the cell where it is associated with the nucleus (Moestrup, 1982).

With the exception of *Mesostigma viride* LAUTERBORN (Manton and Ettl, 1965) and *Mantoniella squamata* (MANTON *et* PARKE) DESIKACHARY (Manton and Parke, 1960), all prasinophytes possess a single rhizoplast (System II fibre). *Tetraselmis* is unusual, however, as it possesses two rhizoplasts, one associated with each pair of basal bodies (Melkonian, 1980).

The rhizoplast is a contractile organelle that may function in the initiation of power and recovery strokes of the flagella and it may absorb and distribute stress generated by flagellar movement. It could also serve to anchor major organelles like the nucleus and chloroplast within the cell (Salisbury and Floyd, 1978a). Stewart *et al.*, (1974) have shown that the System II fibres of *Tetraselmis subcordiformis* form a granular mass from which spindle microtubules arise during nuclear division.

Microtubular rootlets, which arise near the basal body apparatus, follow the contours of the cell and terminate toward the posterior part of the cell (Norris and Pearson, 1975). These rootlets do not penetrate deeply into the protoplast but lie close to the plasmalemma where, with additional micro-

tubules, they are thought to provide a support system that maintains cell shape in the absence of a cell wall. Quadriflagellate prasinophytes always possess four microtubular rootlets arranged in a $x-2-x-2$ pattern ($x=4$). These arise from the basal body apparatus in a cruciate pattern (refer to Text Fig. 1.3). The uniflagellate prasinophyte *Pedinomonas* is unusual in that it also possesses four microtubular rootlets arranged in a $x-2-x-2$ pattern, but here $x=3$ (Moestrup, 1982). Norris (1980) suggests that this genus probably has a complicated phylogeny and may be a derived form.

The flagellar root system has been studied in detail in two biflagellate prasinophytes. *Nephroselmis* has an unusual microtubular root system comprising three roots which have the microtubules arranged in a 10-4-3 pattern (Moestrup and Ettl, 1979). The microtubules in the larger root are multi-layered like the MLS of the Charophyceae. *Mesostigma* also has an unusual arrangement of the microtubular root system. This alga has two microtubular rootlets (both 5-7 microtubules) each being associated with a MLS-like component near to the basal bodies (Rogers *et al.*, 1981).

Excellent papers on the occurrence and nature of microtubular roots and System I and System II fibres in the green algae and plants have been given by Moestrup (1978, 1982) and Melkonian (1979b, 1980, 1982a).

Prasinophyte chloroplasts, which are similar to those in other green algae, contain numerous thylakoids that commonly form stacks of two to four thylakoids (Dodge, 1973). The pyrenoid is usually situated in the chloroplast in a position opposite the flagellar bases and has certain characteristics distinctive of the group. It is usually embedded in the chloroplast and is often traversed by thylakoids as in *Pyramimonas* (Moestrup and Thomsen, 1974; Norris and Pearson, 1975), or it is penetrated by one to several branched or anastomosing canaliculi containing cytoplasm or nucleoplasm as in *Tetrasselmis* (Parke and Manton, 1967; McLachlan and Parke, 1967). In the latter genus the nature of the invaginations has proved to be a useful taxonomic character in separating subgenera (Hori *et al.*, 1982). *Mantoniella squamata* and *Micromonas pusilla* are unusual as their pyrenoids have a solid core (Manton and Parke, 1960). Prasinophytes have one to several intraplasmidial eyespots that lie just beneath the chloroplast envelope adjacent to the plasmalemma. This eyespot consists of a variable

number of layers of osmiophilic lipid globules that accumulate carotenoids to give the organelle its orange colour when seen with the light microscope. Some species of *Tetraselmis* and *Nephroselmis* lack an eyespot (Norris *et al.*, 1980; Inouye and Pienaar, 1984).

The nucleus in prasinophyte cells, like the chloroplast, is surrounded by a double unit membrane and differs little from nuclei in other green algae. It is usually laterally positioned in uni-, and biflagellate species but occupies an anterio-lateral, centro-lateral or central position in quadriflagellate species (Norris, 1980).

A single unbranched mitochondrion has been found in the smaller prasinophytes belonging to the genera *Micromonas*, *Mantoniella*, *Mesostigma*, *Monomastix*, *Pedinomonas* and *Pseudoscourfieldia* MANTON (Norris, 1980). Larger members of the class are thought to have several mitochondria in each cell. A recent study on *Pyramimonas gelidicola* McFADDEN, MOESTRUP *et* WETHERBEE (McFadden and Wetherbee, 1982a) has however, cast doubt on the previous statement since these authors have shown that this species has one large reticulated mitochondrion. They add that the presence of a single mitochondrion may be characteristic of all prasinophyte cells, but this still needs to be confirmed. The mitochondrion occasionally occupies a central position in the cell but more commonly its lobes are found in close association with the chloroplast envelope. The cells of *Tetraselmis convolutae* (PARKE *et* MANTON) NORRIS, HORI *et* CHIHARA (Parke and Manton, 1967), and the zoospores of *Halosphaera* SCHMITZ (Manton *et al.*, 1963) are unusual in that they have mitochondria/mitochondrial lobes present between the outer chloroplast envelope and plasmalemma. Mitochondrial cristae in the Prasinophyceae are flattened (Stewart and Mattox, 1980).

The Golgi apparatus in flagellate members of the class has received much attention because of the role it plays in the production of scales which are so characteristic of members of this class. Dictyosomes of the Golgi apparatus are large and occupy a position near the basal body complex. The number of dictyosomes in a cell is usually constant for a particular species. A single dictyosome is common in most uni-, and biflagellate species and two to several dictyosomes are common in quadriflagellate species (Norris, 1980). Norris (1980) has suggested that Prasinophyceae having the forming face of the dictyosomes close to the nuclear envelope may be more primitive than

species in which a well developed peridictyosomal endoplasmic reticulum exists. This extensive endoplasmic reticulum (ER) would allow the dictyosomes to be positioned further away from the nucleus. Dictyosomal cisternae are thought to be derived from periplastidial ER (Norris and Pearson, 1975) and perinuclear ER (Norris and Pearson, 1975; Moestrup and Walne, 1979).

The scale reservoir is an interesting organelle that is found in members belonging to the genus *Pyramimonas* (see Section 1.3). It is bounded by a unit membrane and is confluent with the plasmalemma via a duct in the flagellar pit. As its name implies the organelle accumulates flagellar scales, and sometimes body scales, which have been produced in the dictyosomes (Manton, 1966a; Norris and Pearson, 1975). It has been suggested that scales are stored in the scale reservoir until being released at the time of cell division. A scale reservoir is not present in unflagellate and biflagellate species.

The trichocyst is an organelle with restricted occurrence in the class and has only been found in *Monomastix* (Belcher, 1965; Manton, 1970) and some species of *Pyramimonas*. Trichocysts are ejectile organelles that are found in membrane bound vesicles just beneath the plasmalemma. Prior to ejection they form a tightly coiled ribbon, but when discharged they appear as a hollow tube which tapers at both ends. Prasinophycean trichocysts are similar to the ejectosomes of the cryptomonads (Manton, 1969) but do not have the characteristic distal modification (Dodge, 1973). Morral and Greenwood (1980) have also shown that the trichocysts in these two groups have a different periodic substructure. The function of the trichocysts is not known. Extensive organelles of different structure and unknown function are present in the genus *Mamiella* (Moestrup, 1984).

1.2.4 Physiology and biochemistry

Two physiological processes that have been studied in detail in the class are the role of the dictyosomes in scale production, and the mechanisms of mitosis and cytokinesis.

Many publications have revealed that prasinophycean scales are formed within dictyosomal cisternae (Manton *et al.*, 1963, 1965; Parke and Rayns, 1964; Manton and Ettl, 1965; Manton and Parke, 1965; Manton, 1966a, 1968, 1970, 1975; Moestrup and Thomsen, 1974; Norris and Pearson, 1975;

Moestrup and Ettl, 1979; Moestrup and Walne, 1979; Barlow and Cattolico, 1980; Aken and Pienaar, 1981a). Progressive stages in scale formation have been seen in the dictyosome, with nascent scales occurring in cisternae at the forming face of the dictyosome, these developing to appear as fully formed scales at the mature face of the dictyosome. Complete scales are carried from the dictyosome in vesicles formed by the abstriction of mature cisternae. Cisternae dissipated in this way are replaced at the forming face of the dictyosome by peridictyosomal ER.

In *Pyramimonas*, where seven types of scales (including hairscales) are produced, more than one scale type is formed in a single cisternae at any one time (Manton, 1966a; Norris and Pearson, 1975; Moestrup and Walne, 1979). Larger scales are produced near the centre of the cisternae and smaller scales are produced toward the periphery. There is some intradictyosomal orientation of these scales with large flagellar and body scales always having their distal surfaces oriented toward the forming face of the dictyosome. Smaller scales usually do not have this fixed orientation within the cisternae. By contrast, all scale types produced by *Mesostigma* (Manton and Ettl, 1965) lack any fixed orientation within the dictyosome.

Mature scales may be transported singly from the Golgi apparatus to the cell surface or they may be temporarily stored, in some species, within a scale reservoir. Uni-, and biflagellate species usually release their scales directly onto the cell surface by exocytosis (reverse pinocytosis).

There is some evidence to suggest that scale production is intimately linked with the cell division cycle and the time of day, with the dictyosome producing different scale types at different times (Manton, 1966a; Pienaar and Pearson, 1976; Norris and Pienaar, 1978). Scale production has been studied in greatest detail in the genus *Pyramimonas* and is discussed further in Section 1.3.

The chemical composition of prasinophyte scales has not been elucidated. Lewin (1958) however, has shown that the theca of *Tetraselmis subcordiformis*, which consists of coalesced scale-like particles (Manton and Parke, 1965), is carbohydrate in nature and is composed mainly of uronic acid and galactose with traces of arabinose and amino acids. Gooday (1971), working on another

species in the genus, namely *Tetraselmis tetrathele* (WEST) BUTCHER, has shown the theca to be pectin-like with galactose, galacturonic acid and arabinose forming the major components. The theca of *T. tetrathele* may also contain calcium as the calcium salt of galacturonic acid (Manton *et al.*, 1973).

The nature of the cell boundary in non-motile representatives of the class has not been studied in detail. The phycoma stage of *Halosphaera* has a thick bilayered cell wall in which the inner layer is pectin-like; the outer layer is thought to contain material similar to sporopollenin (Parke and den Hartog-Adams, 1965). Boalch and Parke (1971) report that this outer cell wall may contain siliceous particles.

The phycoma stage of *Pachysphaera* OSTENFELD is similar to that of *Halosphaera*, with the inner wall being pectinaceous (Parke, 1966), but with the outer wall being composed of a complex lipoidal substance (Wall, 1962). Jux (1969), however, has reported that the inner wall of *Pachysphaera marshalliae* PARKE is cellulosic. Parke and coworkers (Parke *et al.*, 1978) have demonstrated that the inner wall of the phycoma stage of *Pterosperma* POUCHET, like that of *Pachysphaera* and *Halosphaera*, is pectinaceous, but that the outer wall, of unknown composition, stains yellow with iodine.

Mechanisms of mitosis and cytokinesis in the green algae have recently become the subject of much investigation and discussion. Mitosis and cytokinesis are conservative physiological processes and as such have proved to be useful taxonomic characters in attempting to elucidate phylogenetic links between primitive and advanced green algae, and between these primitive plants and the land plants. In this regard, Stewart and Mattox (1975a, 1978), drawing on the findings of Pickett-Heaps and Ott (1974) and their own observations, have separated the green algae into three classes. This separation was based primarily on the appearance of nuclear spindle at telophase. This "cytological classification" was proposed following the comments of Peterfi and Manton (1968) and Maiwald (1971) when they suggested that characters visible with the light microscope, or even the electron microscope, in interphase cells might be insufficient for a proper evaluation of the taxonomic position of unicellular green algae. This new system of classification based on the appearance of the mitotic spindle at telophase is widely accepted

amongst phycologists today.

As a result of the "cytological classification" some members of the class Prasinophyceae, because they possess different mechanisms of mitosis and cytokinesis, were placed into different classes in the division Chlorophyta (Stewart and Mattox, 1978). As outlined in Section 1.2.2, this has been a controversial issue and was opposed by Norris (1980) who believes that the prasinophytes form a natural group of primitive green algae. Variations in the mechanisms of mitosis and cytokinesis in the Prasinophyceae are discussed below.

Most members of the Prasinophyceae remain motile throughout the cell division process. Only one exception is seen in the thecate genus *Tetraselmis*, where the flagella are lost for long periods during which the cell divides. In this respect *Tetraselmis* resembles most flagellate chlorophycean algae in the order Volvocales as they too shed their flagella during cell division. Three wall-less volvocalean genera, *Asteromonas* ARTARI (Floyd, 1978), *Dunaliella* TEODORESCO (Marano, 1976), and *Hafniomonas* Ettl et MOESTRUP (1980), are notable exceptions as they retain their flagella during cell division.

It is important to mention here that the term "cell division" is used henceforth to include both mitotic and cytokinetic processes. The term "cell division cycle" refers to the period from one cell division to the next.

Cell division has been studied at the ultrastructural level in *Pedinomonas minor* (Pickett-Heaps and Ott, 1974), *Tetraselmis subcordiformis* (Stewart et al., 1974), *Pyramimonas parkeae* NORRIS et PEARSON (1975), *Mantoniella squamata* (Barlow, 1977), *Nephroselmis olivacea* (Mattox and Stewart, 1977), and *Pyramimonas amyliifera* (Woods and Triemer, 1981). The most primitive system of cell division is seen in *Mantoniella*. Preparation for division begins early in the cell division cycle with sequential replication of the eyespot, Golgi apparatus, basal bodies, pyrenoid, and chloroplast, respectively. The nucleus is the last organelle to divide. *Mantoniella* has a closed spindle and daughter nuclei formed during mitosis are separated by furrowing of the cell at cytokinesis. The closed spindle is regarded as a primitive feature (Johnson and Porter, 1968; Leedale, 1970; Pickett-Heaps, 1972, 1975b).

Pedinomonas also has a closed spindle but the daughter nuclei remain far apart at telophase because of the presence of a persistent interzonal spindle. Separation of the daughter nuclei is therefore not dependent on furrowing as in *Mantoniella*. The presence of an intact nuclear envelope and persistent interzonal spindle led Pickett-Heaps and Ott (1974) to suggest that these two features are primitive characteristics.

The cell division cycle of *Nephroselmis* is comparable with that of *Mantoniella* in that the sequence of organelle replication is similar. Mitosis in both genera is characterised by a persistent interzonal spindle but *Nephroselmis* is thought to be more advanced because it has an open spindle. During interphase *Nephroselmis* stores many scales in vesicles in the cytoplasm. Norris (1980) believes that this is an advanced feature because in *Mantoniella* the scales are transported singly to the plasmalemma.

The cell division cycle in *Pyramimonas* shows a number of advanced features not seen in the genera discussed above. There is extensive interphase preparation for cell division as seen in the formation of a large scale reservoir. There is a synchronization of organelle replication so that they divide in a relatively short time just prior to cell division. The mitotic spindle in *Pyramimonas* is open and daughter nuclei separate before cytokinesis is complete. A persistent interzonal spindle is apparent during anaphase but this disappears by late telophase. Cell division in *Pyramimonas* is later discussed in more detail.

The thecate genus *Tetraselmis* is unique within the class in that the spindle microtubules collapse at late anaphase and a phycoplast develops at telophase. The phycoplast, which plays a role in cell plate formation, is characteristic of green algae belonging to the class Chlorophyceae. *Tetraselmis* is therefore an advanced genus which may represent a phylogenetic link between the Chlorophyceae and Prasinophyceae. Replication of cell organelles in *Tetraselmis* is synchronized to occur close to the time of cell division. This genus has a vesiculate nuclear envelope during mitosis.

The starch reserve product in the class Prasinophyceae is thought to be different from that found in the Chlorophyceae, because it does not stain the typical blue-black colour with iodine, but stains a reddish-purple or brown

colour. Salisbury and Floyd (1978b) have shown that the starch grains in *Mantoniella squamata* are composed primarily of amylose. However, in the advanced genus *Tetraselmis*, the starch reserve product is similar to that found in other green algae (Suzuki, 1974). In a recent paper Bressler and Meeuse (1984), using spectrophotometry, β -amylase and X-ray diffraction, have confirmed that the reserve product produced by *Pyramimonas* and *Tetraselmis* is true starch.

Members of the Prasinophyceae excrete the carbohydrate mannitol as their chief soluble photosynthate (Hellebust 1965, 1974; Craigie *et al.*, 1967; Kirst, 1980). It is interesting to note that this compound is rarely found in other green algae although it is common in the class Phaeophyceae (Craigie, 1974).

Pigment composition has been determined for many prasinophycean cells (Ricketts, 1966a, 1966b, 1967, 1970, 1971). Ricketts' findings show that prasinophycean pigments are similar to those found in other green algae. All members of the class have chlorophylls a and b, and α and β -carotene, but many have in addition to these, the xanthophylls lutein, zeaxanthin, violaxanthin, neoxanthin and xanthophyll a and b. *Micromonas* and *Mantoniella* lack lutein, but have in its place the unusual xanthophyll micro-noine. *Nephroselmis* and *Pterosperma* are also characterized by the absence of lutein, and have instead siphonaxanthin and siphonein.

It is noteworthy that not all prasinophytes are photoautotrophic; some are auxotrophic and require vitamins for growth (Ricketts, 1974). Furthermore, some species cannot be grown in axenic culture (Ricketts, 1974), and others show enhanced growth in the presence of stimulating substances released through bacterial hydrolysis of agar (Ukeles and Bishop, 1975).

1.2.5 Life-histories

Relatively little is known about the life-histories of the Prasinophyceae. Those reported invariably describe the asexual reproductive cycle of the organism. Although sexual reproduction has been reported for *Nephroselmis* (Korshikov, 1923), *Pyramimonas reticulata* KORSHIKOV (Ettl, 1965) and *Pedinomonas* (Ettl, 1967b), it has never been clearly demonstrated in these taxa.

Pyramimonas reticulata is no longer recognized as a member of the class Prasinophyceae and has recently been placed in the order Volvocales (class Chlorophyceae) as *Hafniomonas reticulata* (KORSCHIKOFF) Ettl *et* MOESTRUP (Ettl and Moestrup, 1980)

Reports on asexual reproductive cycles generally describe the alternation between a motile phase and a non-motile phase. The motile cell always possesses a scale-covering. Where the non-motile phase predominates, for example in *Halosphaera* (Parke and den Hartog-Adams, 1965; Boalch and Mommaerts, 1969), *Pachysphaera* (Parke, 1966), and *Pterosperma* (Parke, *et al.*, 1978), this large planktonic phycocyst stage produces small motile zoospores as the alternate life-cycle stage. In *Halosphaera* these zoospores closely resemble *Pyramimonas* and they have been referred to as the *Pyramimonas*-like stage of *Halosphaera* (Parke and Adams, 1961; Manton *et al.*, 1963; Pennick, 1977b). *Pachysphaera* and *Pterosperma* also produce quadri-flagellate zoospores but these do not resemble *Pyramimonas* (Parke, 1966; Leadbeater, 1974; Parke *et al.*, 1978). It is interesting to note that the zoospores produced by the three non-motile genera mentioned above, reproduce by primary fission and can be maintained in culture for extended periods.

In general, where the motile phase predominates, the alternate life-cycle stage is a cyst or palmella. A palmella contains one or more stationary cells which are embedded in a wide colourless mucilaginous matrix. A cyst is usually spherical in shape and has a firm wall which may be smooth or variously sculptured. Belcher (1966) described the palmella stage of *Prasinocloris sessilis* BELCHER and reported in the same publication that six species of *Pyramimonas* are known to form palmella and four are known to produce cysts.

Species belonging to the thecate genus *Tetraselmis* have very similar motile stages but they have non-motile stages that are quite different (Proskauer, 1950; Chihara, 1963; Parke and Manton, 1965). For example, the motile cells of *Tetraselmis cordiformis* (CARTER) STEIN shed their flagella and remain essentially unchanged in the non-motile phase, but in *Tetraselmis ascus* (PROSKAUER) NORRIS, HORI *et* CHIHARA the non-motile stage is sessile and stalked. Some species in the genus produce a thick-walled cyst in the non-motile phase (McLachlan and Parke, 1967; Parke and

Manton, 1967; Tanoue and Aruga, 1975). Norris and coworkers (Norris *et al.*, 1980) have summarized the complex life-history of *Tetraselmis*.

Symbiotic associations between members of the Prasinophyceae and other organisms are known. *Pedinomonas noctilucae* (SUBRAHMANYAN) SWEENEY has been found in the dinoflagellate *Noctiluca miliaris* SURIRAY (Sweeney, 1976), and *Pedinomonas symbiotica* CACHON *et* CARAM is a symbiont in the radiolarian *Thallassolampe margarodes* HAECKEL (Cachon and Caram, 1979). Both species, which are only known as symbionts within these organisms, retain their single flagellum during the association. By contrast, *Tetraselmis convolutae*, the symbiont in the marine flatworm *Convoluta roscoffensis* GRAFF (Parke and Manton, 1967; Gooday, 1970; McFarlane, 1982), loses its flagella and its theca during this association. In the alternate free-living stage, however, the cell produces four flagella and appears similar to other species within the genus.

1.3 THE GENUS *Pyramimonas*

Pyramimonas is the most common and probably the most studied genus in the Prasinophyceae, and is often considered typical for the class. Belcher (1966) lists some sixty species in the genus, of which slightly less than half are from marine and brackish habitats. More recently a number of new species have been described and these include *Pyramimonas virginica* PENNICK (Pennick, 1977a), *P. parkeae* (Norris and Pearson, 1975), *P. gelidicola* (McFadden *et al.*, 1982), *Pyramimonas cirolanae* PENNICK (Pennick, 1982a), *Pyramimonas occidentalis* PENNICK (Pennick, 1982b), *Pyramimonas gorlestonae* PENNICK *et* CANN (Pennick and Cann, 1982), *Pyramimonas lunata* INOUE, HORI *et* CHIHARA (Inouye *et al.*, 1983), *Pyramimonas spinifera* PENNICK (Pennick, 1983), and *Pyramimonas pseudoparkeae* PIENAAR *et* AKEN (Pienaar and Aken, 1985). The genus is one of the more advanced in the class (Norris, 1980), and is an important primary producer within the nanoplankton (Butcher, 1959; Throndsen, 1969; Moestrup and Thomsen, 1974).

The type species for the genus is *Pyramimonas tetra-rhynchus* SCHMARDA, a freshwater alga that was first described in 1850 (Schmarda, 1850).

Pyramimonas is a motile, unicellular alga that is ovoid to obpyriform in shape. The cells are radially symmetrical when viewed along the apical axis

and they have a deep, anterior flagellar pit. With the exception of *P. amyliifera* (which has eight flagella - Manton, 1966a) all species are quadriflagellate. The flagella are isokont and exhibit homodynamic movement.

Pyramimonas superficially resembles three other genera of green algae which contain quadriflagellate members. These are *Tetraselmis* (class Prasinophyceae), and *Carteria* DIESING and *Hafniomonas* (class Chlorophyceae, order Volvocales). *Tetraselmis* is easily distinguished from *Pyramimonas* because it possesses a relatively thick theca which can be seen with the light microscope (Norris *et al.*, 1980). *Pyramimonas*, by contrast, appears naked. *Carteria* differs from *Pyramimonas* in that the flagella do not emerge from a deep depression in the cell surface, and often the reverse is true because the flagella are usually borne on an anterior papilla (Butcher, 1959). *Hafniomonas*, a relatively new genus (Ettl and Moestrup, 1980), most closely resembles *Pyramimonas* and was placed in the latter genus for many years (Belcher, 1968a). It can only be separated from *Pyramimonas* on differences observed at the ultrastructural level. *Hafniomonas*, for example, does not have a scale-covering nor rhizoplasts which are typical for *Pyramimonas*. This is one example of the usefulness of the electron microscope in separating otherwise similar organisms.

Species belonging to the genus *Pyramimonas* exhibit a wide range in cell size, with *P. virginica* (Pennick, 1977a) having a length of only 2,7 - 3,5 μm and *P. tetrarhynchus* (Belcher, 1969a) at the other extreme, having a length of 27 μm . *P. longicauda* VAN MEEL, however, has a long cytoplasmic "tail" and may be 48 μm in length (Inouye *et al.*, 1984).

All species studied with the electron microscope have been shown to possess flagella that are covered with two layers of different scale types. Moreover, the flagella always bear complex tubular hairscales. The cell surface is usually covered with three layers of scales, each layer being composed of a different scale type. *Pyramimonas virginica* is the only species described with two types of body scale arranged in two layers on the cell surface (Pennick, 1977a).

Norris and Pienaar (1978) have referred to the scale types on *Pyramimonas* as Type 1 and Type 2 flagellar scales, and Type 1, Type 2 and Type 3 body

scales, according to the position they occupy on the flagellar and cell surfaces, respectively. Type 1 scales occur adjacent to the plasmalemma and Types 2 and 3 scales are positioned in successively distal layers.

Type 1 flagellar scales are small pentagonal scales (\pm 50 nm in diameter) possessing a central boss. Larger Type 2 flagellar scales (\pm 250 nm in length) have a characteristic adnate spine and are often referred to as limuloid scales because they resemble, in outline, the shell of the king crab *Limulus* LATRIELLE.

Type 1 body scales are approximately the same size as the Type 1 flagellar scales, but are square in outline. Type 2 body scales are larger square scales (with side of \pm 250 nm) occurring in a layer distal to the Type 1 scales. In *P. virginica* (Pennick, 1977a), which has only two layers of body scales, the outer or Type 2 body scale layer is composed of basket-shaped scales. Other species have a third layer of scales, the Type 3 scales which are crown-like and have been referred to as coronate scales. Type 3 scales have approximately the same base dimensions as the Type 2 scales but they are much taller and lattice-like. Interspecific variations in scale structure are most pronounced in Type 2 flagellar scales and Types 2 and 3 body scales (Norris and Pienaar, 1978).

Several marine species within the genus, namely *Pyramimonas orientalis* BUTCHER (Moestrup and Thomsen, 1974; Pennick *et al.*, 1978), *Pyramimonas grossii* PARKE (Pennick and Clarke, 1976), *Pyramimonas obovata* CARTER (Pennick *et al.*, 1976), *Pyramimonas* aff. *plurioculata* BUTCHER (Norris and Pienaar, 1978), *P. occidentalis* (Pennick, 1982b) and *P. gorlestonae* (Pennick and Cann, 1982) are unusual in that they do not have all three types of body scales covering the whole cell surface. *P. grossii* has a complete covering of Type 1 and 2 scales, with the Type 3 scale being restricted to the flagellar pit region. In the other species mentioned above, the Type 1 body scales are restricted to the flagellar pit and Types 2 and 3 body scales cover the remainder of the cell surface.

The cryophilic species *P. gelidicola* (McFadden *et al.*, 1982) is unusual in that the Type 1 body scale layer is absent but this is replaced by

small "foot-print" scales which occupy positions between the Type 2 body scales (McFadden and Wetherbee, 1982b) *P. gelidicola* was isolated from sea-ice in the Antarctic and is one of two cryophilic members of the genus. The second species, *Pyramimonas nansenii* BRAARUD, is a Northern Hemisphere species that was described from the pack ice of Eastern Greenland (Braarud, 1935). This species has not been studied at the ultrastructural level.

At the level of the light microscope, *Pyramimonas* is characterized by a large, posterior chloroplast which has four parietal lobes extending anteriorly into the four lobes of the cell body. (*Pyramimonas amyliifera*, however, has eight chloroplast lobes projecting anteriorly into the eight lobes of the cell). The chloroplast contains an eyespot in one of the lobes, and a posterior pyrenoid which is surrounded by starch grains. A nucleus usually occupies an antero-lateral position in one of the cell lobes and is usually adjacent to the flagellar pit.

Electron microscopical examination of the genus reveals that the thylakoids of the chloroplast may occur singly, or they may be juxtaposed forming stacks comprising up to five thylakoids. The pyrenoid is usually traversed by single thylakoids.

The complex basal body system or kinetosome, is found in all species and is located beneath the flagellar pit (see Text Fig. 1.3). The basal body system has been described in detail for three species in the genus, namely *P. parkeae* (Norris and Pearson, 1975), *P. obovata* (Melkonian, 1981), and *P. gelidicola* (McFadden and Wetherbee, 1984). Adjacent to the kinetosome are usually two dictyosomes which produce all scale types. A scale reservoir and vacuole occupy a more or less central position in the cell. Freshwater species possess a contractile vacuole (Butcher, 1959; Belcher, 1969a).

Trichocysts are found in only six species, namely *P. grossii* (Manton, 1969), *P. parkeae* (Norris and Pearson, 1975), *P. pseudoparkeae*¹ (Pienaar

¹ *P. pseudoparkeae* was referred to as *P. aff. parkeae* by Pienaar and Pearson (1976).

and Pearson, 1976; Pienaar and Aken, 1985), *P. virginica* (Pennick, 1977a), *P. cirolanae* (Pennick, 1982a), and *P. lunata* (Inouye *et al.*, 1983).

Moestrup and Walne (1979) in their study on *P. tetrarhynchus* have reported that the dictyosomes are capable of producing at least four and probably all six scale types in a single cisterna. Type 2 flagellar scales, Types 2 and 3 body scales and hairscales are formed in the central region of a cisterna, and Type 1 flagellar and body scales are formed in peripheral regions. Aken and Pienaar (1981a) have confirmed that all scale types may be produced in a single cisterna in *P. pseudoparkeae*.

There is some evidence to suggest that scale production may be closely linked to stages in the cell division cycle and the time of day (Pienaar and Pearson, 1976; Norris and Pienaar, 1978). Pienaar and Pearson (1976) have reported that cells of *P. pseudoparkeae* fixed shortly after cytokinesis have only flagellar scales in their dictyosomes and scale reservoir, and only after the cells have been exposed to long periods of light, do body scales appear in these organelles.

Cell division has been studied in detail in two species in the genus. Pearson and Norris (1975) have described cell division in *P. parkeae*. The onset of cell division is marked by the division of the chloroplast and elongation of the microbody. This is followed by the sequential replication of the basal bodies, Golgi apparatus and other components of the basal body complex. The two basal body complexes move to opposite poles of the nucleus which at this stage occupies a central position in the cell. Mitosis (karyokinesis) begins with dispersion of the nuclear envelope during prophase. The cell therefore has an open spindle. Metaphase is characterised by a central plate of chromatin. Microtubules connect this chromosome plate with the striated rootlets of the two newly formed basal body complexes. Some microtubules extend from pole to pole while others extend from pole to chromosome-plate. Stages of anaphase, which is believed to be a transient phase, have not been reported for *P. parkeae*. At telophase the nuclear envelope reforms by enfolding of the endoplasmic reticulum complex which surrounds the chromatin. The nature of the telophase spindle was not demonstrated in *P. parkeae*. Cytokinesis is effected by furrowing and by the fusion of cytoplasmic vesicles in the plane of fission. *Pyramimonas* remains

motile throughout the cell division cycle.

More recently, Woods and Triemer (1981) have described cell division in the octaflagellate member of the genus, namely *P. amyliifera*. Cell division events in this species are identical to those described for *P. parkeae*. Woods and Triemer (1981) however, described two interesting features which complement the study made by Pearson and Norris (1975). Kinetochores were identified on the chromosomes at metaphase and the pole-to-chromosome microtubules were attached to the chromosomes at these structures. Furthermore, a persistent interzonal spindle was seen at early telophase but this disappeared at late telophase.

Sexual reproduction has been reported for only one species in the genus, namely *P. reticulata* (Ettl, 1965). Ettl and Moestrup (1980) have shown that this species, renamed *Hafniomonas reticulata*, correctly belongs in the class Chlorophyceae rather than the Prasinophyceae. *Pyramimonas* is therefore not known to reproduce sexually. Some members of the genus do reproduce asexually by the formation of cysts or palmella stages (Belcher, 1966). *Pyramimonas grossii* and *P. tetrarhynchus* reproduce asexually by cysts or palmella while *P. amyliifera* produces encysted cells only. Palmella stages are known for *Pyramimonas inconstans* HODGETTS, *Pyramimonas octostriata* PASCHER and *Pyramimonas quadricauda* PASCHER.

In more recent publications, Hargraves and Gardiner (1980) and Aken and Pienaar (1981b), have reported on the life-cycles of *P. amyliifera* and *P. pseudoparkeae*¹, respectively. Both species produce cysts that remain viable for extended periods. Hargraves and Gardiner have observed a single cyst containing motile cells but they are not certain if these motile cells are identical to *P. amyliifera*, because bi-, and quadriflagellate cells were found in their cultures. Aken and Pienaar have, however, demonstrated that the cysts of *P. pseudoparkeae*, when placed in fresh medium release four motile cells identical to the motile parent cell. These life-cycles are thought to be asexual cycles because no fusion of cells has been observed in cultures forming cysts.

¹ Aken and Pienaar (1981b), referred to this species as *P. parkeae*.

1.4 AIMS OF THE PRESENT STUDY

The present study evolved from an earlier study conducted by the writer (Aken, 1978). It may be fitting to discuss some of the results of this study because they provided a foundation on which at least some of the objectives of the present study were based. The aim of the preliminary study was to investigate the process of scale production in *Pyramimonas pseudoparkeae*. Scale production had not been investigated in detail in the genus at the time although some workers (Manton, 1966a; Pienaar and Pearson, 1976; Norris and Pienaar, 1978) had reported that the dictyosomes may produce different scale types at different stages in the cell division cycle, and that scale production may be a periodic process rather than a continuous one. The purpose of the preliminary study was, therefore, to investigate functional changes occurring in the dictyosomal system during the cell division cycle. This study was, unfortunately, hampered by problems concerning to the growth of the organism and the conclusions drawn were only tentative. It became clear that the growth requirements of the experimental organism should be properly understood before a successful study on scale production could be conducted.

To appreciate the importance of growth in a physiological study of this nature, it is necessary to understand the need for working with synchronous cultures. A well synchronized culture can, to all intents and purposes, be regarded as a "single cell" with regard to its physiological state at any one time. This characteristic is invaluable when working with small organisms because observations made on isolated cells, which have been harvested and prepared for electron microscopy, can be regarded, with reasonable certainty, as being representative of the culture as a whole at the time of sampling. Synchronous cultures have two important prerequisites; firstly, the cells in culture must be actively growing (exponential growth) so that the mean generation time of all cells is uniform; secondly, cell divisions need to be synchronized (phased). In algae, synchrony can be induced by using a number of stimuli, among these different photoperiods. To obtain well synchronized cultures it is essential that the period of the induction photoregime should match the mean generation time of the cells. In the writer's preliminary study the computed mean generation time of the cells in culture was 33 h. These cells were grown in Provasoli's Enriched Seawater Medium at a temperature of 18°C and a synchrony induction photoregime of 16 h light : 8 h

dark (16h:8h) with a light intensity of $50 \mu\text{E m}^{-2}\text{s}^{-1}$). A mean generation time of 33 h did not match the 24 h period of the photoregime and thus impaired synchrony. It was necessary therefore to improve the growth rate of the organism to bring the generation time closer to the period of the light/dark cycle.

The present investigation began, therefore, with a study on the growth of *P. pseudoparkeae*, in which the organism was grown in different nutrient media and under a range of different culture conditions of salinity, light intensity and temperature. Although the primary aim of this study was to improve the growth rate of the organism, the study would also provide additional information on the biology of the alga. Firstly, there has been much controversy over the taxonomic weighting of scale characteristics used in delineating different species. This controversy exists because no studies have been conducted to test if scale morphology is constant under different environmental conditions. The wide range of culture conditions employed in this growth study would provide an opportunity to assess the reliability of scale form as a useful taxonomic criterion.

Secondly, the wide range in culture conditions would give some indication of the general growth requirements of the organism and of its tolerance to extreme conditions. Finally, it was hoped that the range in culture parameters would provide conditions conducive to the induction of life cycle stages. In an earlier study on the organism a few cysts were observed on occasions. Because knowledge on the reproductive biology of the genus is scant, it was hoped that an investigation on the life-cycle of *P. pseudoparkeae* could also be undertaken.

A second major objective of the present study, and a corollary of the growth study, was an investigation of the possible functional changes in the dictyosome with regard to scale production. This study, conducted during the cell division cycle, would provide an opportunity for a concomitant study on other events relating to cell division, viz. mitosis and cytokinesis, which have not been documented for this species.

Another objective of the present study was to determine the chemical composition of the scales which cover *P. pseudoparkeae*. To date there is

no information on the chemical composition of scales of *Pyramimonas* and it was felt that the results might contribute to an understanding of cell boundaries in these primitive organisms and may also allow some interpretations on phylogenetic links between representatives of the genus and the more advanced green algae. It was also hoped that a knowledge of scale chemistry would suggest some functions of scales.

A further aim of this study was to record, in detail, the micromorphology and ultrastructure of *P. pseudoparkeae*. This aspect of the work formed, in part, a basis on which the organism was described (Pienaar and Aken, 1985).

It is hoped that the work presented in this dissertation will contribute to our knowledge of the genus *Pyramimonas*. Furthermore, this study forms part of a broader study being conducted on the marine phytoplankton of Natal.

CHAPTER TWO

MORPHOLOGY AND STRUCTURE

2.1 INTRODUCTION

The morphology and structure of *Pyramimonas pseudoparkeae* is described below. The observations presented here were made on interphase cells taken from actively growing cultures i.e. cultures in the exponential phase of growth. The morphology and structure of cells seen in other stages of the cell division cycle and in the alternate cyst-forming reproductive cycle is presented in Chapters Five and Seven, respectively. The information presented here augments the observations made by Pienaar and Pearson (1976), and provided, in part, a basis on which the species was described (Pienaar and Aken, 1985).

2.2 MATERIALS AND METHODS

2.2.1. The experimental organism

Pyramimonas pseudoparkeae was isolated in 1975 from a tidal pool sample collected by Professor R.N. Pienaar at Oudekraal on the Cape Peninsula. The organism, first referred to as *P. aff. parkeae* (Pienaar and Pearson, 1976) because of its close resemblance to *P. parkeae* (Norris and Pearson, 1975), is maintained in unialgal culture in the Department of Botany, University of Natal, Pietermaritzburg. The alga is grown in Provasoli's Enriched Seawater Medium (PES) (for recipe see Appendix A, Table I) at a temperature of 20°C, and is subjected to a 16h light:8h dark (16h:8h) illumination cycle. The light intensity, measured during the photoperiod, is $100 \mu\text{E m}^{-2} \text{ s}^{-1}$. In the following pages the culture conditions described above will be referred to as standard culture conditions.

For the purpose of the present study it was decided that a clonal culture of the alga should be used for all experiments. Since a clonal culture is one that is grown from a single cell, the population could be regarded as being genotypically homogeneous. As population variation is greatly reduced in clonal cultures, the results obtained between successive

growth experiments would be more comparable.

A clonal culture was obtained by isolating a single cell from a maintenance culture of the organism. This was achieved by diluting a small volume of the culture in PES medium so that a single cell could be followed and isolated using a flame-drawn micropipette. A Kyowa dissecting microscope (x 40 magnification) was used to facilitate this manipulation. The isolated cell was placed into a test tube containing 10 ml PES medium and was allowed to grow under standard culture conditions. This culture, which was maintained by subculturing at fortnightly intervals, served as the source of experimental material to be used in all subsequent studies.

The writer has isolated *P. pseudoparkeae* from tidal pool samples collected at different localities on the Natal south coast, viz. Amanzimtoti, Park Rynie, Sezela, Mtwalume, Hibberdene, Umzumbe, Sea Park and Palm Beach. These records extend the range of the species which had previously only been recorded from the Cape Peninsula. It has always been found as part of the tidal pool flora at all the localities where it has been collected. The alga's northerly range into more tropical waters on the Southern African east coast, and its distribution on the cooler west coast, is at present unknown.

The isolates from Natal are maintained in clonal culture alongside the Oudekraal isolate. The Mtwalume isolate was used together with the Oudekraal isolate in a comparative study on growth in axenic culture (see Section 4.3.4). In all other studies the Oudekraal isolate was used.

2.2.2 Light microscopy

A Zeiss photomicroscope with bright field, Nomarski and phase contrast optics was used in most light microscope studies. These studies included observations on the process of cell division, the development of life-cycle stages, the determination of chromosome numbers and measurements of cell sizes. In addition, the light microscope was frequently used to check for algal contaminants in all cultures, and to check for bacterial contamination in axenic cultures.

Specimens were photographed with the aid of a Zeiss photomicroscope using either black and white Agfapan Professional film (25 ASA) or Kodachrome colour film (64 ASA).

Algal growth in axenic cultures was monitored on an Olympus CK-BI inverted microscope. Cells in culture could be viewed directly through the base of the culture vessel thus avoiding bacterial contamination which could result if the flask were opened to withdraw a sample.

2.2.3 Electron Microscopy

Heavy metal shadowing

Heavy metal shadowing proved to be a valuable technique for studying scale morphology in *P. pseudoparkeae*.

A small drop of a cell suspension was pipetted onto a 3 mm copper viewing grid which had previously been coated with a thin plastic support film. The plastic used was either 0,2% Formvar in chloroform or 2% Collodion in amyl acetate. The Formvar support film was prepared by dipping a clean microscope slide into a Coplin jar containing the Formvar solution, and then allowing the chloroform to evaporate. The edges of the slide were scored with a sharp blade and the plastic film was removed by slowly immersing the slide (held at an angle of 45°) into distilled water - the surface of which had been cleaned to removed dust particles. Copper viewing grids were placed onto the plastic film which was floating on the surface of the water and a clean microscope slide was used to remove the grids and film.

Where Collodion was used, grids were placed on a piece of stainless-steel wire-mesh resting on the base of a Buchner funnel (6 cm diam.) which contained distilled water. Two drops of Collodion solution were pipetted onto the surface of the water and the amyl acetate was allowed to evaporate. The residual film of Collodion was lowered onto the grids by releasing the pressure in a flask beneath the Buchner funnel.

Cells that had been placed onto coated grids were fixed in osmium tetroxide (Os O_4) vapours for thirty seconds and then allowed to settle onto the support film for 5 min. Excess liquid was gently drawn off using dental absorbent points or fine pointed filter paper wedges. The grids were air dried for 10 min and then rinsed twice with distilled water to remove any crystallized salt. After being air dried in dust free conditions, the grids were shadowed with gold/palladium, from an angle of 30° , in a Hitachi US 3B vacuum evaporator.

Preparation of material for ultramicrotomy (modified after Reimann *et al.* 1980.)

Initially, various fixation and embedding routines were conducted in an attempt to ascertain which procedure would provide the best ultrastructural preservation for *P. pseudoparkeae*. The one routine which gave repeatable and acceptable results is outlined below. It is important to note, however, that the quality of organelle preservation varied depending on the time of day that the cells were processed. This may be a manifestation of the changing physiological state of the organism at different times in the cell division cycle.

Fixation

Cells were fixed in 2,5% glutaraldehyde in 0,1 M sodium cacodylate buffer in seawater.

Preparation of fixative:

10 ml EM grade glutaraldehyde (25% soln.) and 1,6g sodium cacodylate (CH_3)(AsO_2 , Na). $3\text{H}_2\text{O}$ was dissolved in 7,0 ml autoclaved seawater. The pH of the fixative was adjusted to pH 7,4 before being made to a final volume of 20 ml with seawater. 2,0 ml of this concentrated fixative (12,5% glutaraldehyde in 0,5 M cacodylate buffer in seawater) was added

to 8,0 ml of the algal culture to give the required fixative concentration.

Only freshly prepared fixative was used.

After adding the fixative, the culture was gently mixed and stored at 4°C. The fixation period varied between 2 and 4 hours.

Fixed cells were centrifuged at a relative centrifugal force (r.c.f.) of 100 x g for 5 min to give a concentrated pellet of cells. This was washed (2 x 30 min washes) in 0,1 M cacodylate buffer (pH 7.4) at 4°C to remove all traces of glutaraldehyde.

Preparation of buffer wash:

A stock solution of 0,2 M cacodylate buffer was made by dissolving 32 g sodium cacodylate in seawater to give a final volume of 1l. Equal parts of buffer and seawater were mixed to give a 0,1 M cacodylate buffer solution.

Post fixation

The washed cell pellet was post-fixed in 2% OsO₄ in 0,1 M cacodylate buffer for 1h at 4°C.

Preparation of post-fixative:

1,0 g crystalline OsO₄ (EM Scope) was dissolved in 25 ml distilled water to give a 4% solution of OsO₄. To obtain the correct concentration of post-fixative and buffer, equal volumes of 4% OsO₄ and 0,2 M cacodylate buffer were mixed before being added to the cell pellet.

Following post-fixation the cell pellet was again washed (3 x 20 min washes) in 0,1 M buffer at 4°C.

Dehydration

The cell pellet was dehydrated in a graded alcohol series outlined below:

i)	10% aqueous ethanol	10 min	}	4°C
ii)	30% " "	"		
iii)	50% " "	"		
iv)	70% " "	"	}	room temperature
v)	90% " "	"		
vi)	100% ethanol	2 x 30 min		

Embedding and Polymerization

The dehydrated cell pellet was impregnated with low viscosity Spurr's resin (see Table 2.1) by passing it through increasing concentrations of resin as shown below:

i)	3 parts ethanol : 1 part Spurr's resin	1h
ii)	1 part " : 1 part " "	1h
iii)	1 part " : 3 parts " "	2h
iv)	100% Spurr's resin (two changes - 6h each).	

TABLE 2.1

Spurr's resin, its composition and preparation (Spurr, 1969).

Vinylcyclohexane Dioxide (VCD or ERL 4206)	10g
Diglycidyl Ether of Polypropylene glycol (DER 736)	6g
Nonenyl Succinic Anhydride (NSA)	26g
Dimethylaminoethanol (DMAE)	0,4g
DMAE is a catalyst and should be added after the other three constituents have been thoroughly mixed.	

Spurr's resin containing the cell pellet was poured into a shallow aluminium dish and polymerized at 70°C for 16h. A container of silica gel was placed in the oven to absorb any moisture that might inhibit correct polymerization.

Sectioning

Cell pellets were cut from the cured resin wafer and glued onto resin stubs with araldite adhesive. Glass knives used for trimming and sectioning were made on a LKB knifemaker. Ultrathin sections were cut on a LKB or Reichert-Jung Ultracut OMU 4 ultramicrotome using glass or diamond knives.

Thin gold sections were floated onto water, expanded with chloroform vapours, and then picked up on 200 or 300 mesh copper grids.

Staining

Sections were stained for 20 min in 2% aqueous uranyl acetate followed by 15 min in lead citrate. The recipes for these stains are given below.

2% uranyl acetate stain:

1.0 g uranyl acetate was placed in a 50 ml volumetric flask and dissolved in distilled water and 1.0 ml 95% ethanol to give a final volume of 50 ml. The stain, which is light sensitive, was stored in a light tight flask. Fresh stain was made when needed.

Lead citrate stain (Reynolds, 1963):

1.33 g lead nitrate $\text{Pb}(\text{NO}_3)_2$ and 1.76 g sodium citrate $(\text{Na}_3(\text{C}_6\text{H}_5\text{O}_7))$. $2\text{H}_2\text{O}$ was dissolved in 30 ml cool, but freshly boiled, distilled water in a 50 ml volumetric flask. The latter was stoppered and shaken vigorously for 1 min, and agitated intermittently

for the next 30 min. The cloudy solution was cleared by adding 8.0 ml 1 N NaOH and then made up to 50 ml with distilled water. This stain is sensitive to carbon dioxide and was therefore tightly stoppered and stored at 4°C. Fresh stain was made every six months.

Small drops of uranyl acetate stain were pipetted onto a strip of dental wax placed within a Petri dish. Grids were floated, section side downward, on these drops and the whole assembly was covered with a light tight box.

Excess uranyl acetate was washed off the grids using distilled water. The grids were then similarly stained with lead citrate in a separate Petri dish. A small receptacle containing moistened NaOH pellets was placed in the Petri dish to absorb free CO₂. Grids were given a final wash with distilled water and left to dry in a dust free container.

Viewing

Heavy metal shadowed preparations and ultrathin sections were viewed on either a Hitachi HU 11E 1 or Jeol JEM 100CX transmission electron microscope. These were operated with an accelerating voltage of 50 kV or 80 kV respectively.

Preparation of material for scanning electron microscopy

Cells of *P. pseudoparkeae* were prepared for scanning electron microscopy using a modification of the method present by Pickett-Heaps (1980).

- i) Cells were fixed at room temperature for 30 min in 1% OsO₄ made up in the cell suspension.
- ii) Cells were concentrated by gentle centrifugation (r.c.f. = 200 xg) and given two washes with distilled water.

- iii) Washed cells were then dehydrated in a graded ethanol series as shown below:

10% ethanol	5 min
25% "	"
50% "	"
75% "	"
100% "	two changes 10 min each

- iv) Dehydrated cells were impregnated with amyl acetate by passing them through solutions containing 25%, 50% and 75% amyl acetate in ethanol (5 min per step). After giving the cells two changes in 100% amyl acetate (10 min each) they were placed into a perforated BEEM capsule (Marchant, 1973) and critical point dried in a Hitachi HPC-2 critical point drier. Care was taken to ensure that the cells were always covered by the solvents used so that they would not be distorted by exposure to air.

- v) Dried cells were mounted on double-sided Scotch tape on a metal stub and were omnidirectionally shadowed on a Polaron E 5100 sputter coating unit.

Cells were viewed in a Jeol JSM T200 scanning electron microscope using an accelerating voltage of 25 kV.

2.3 RESULTS

2.3.1 Light microscope observations

P. pseudoparkeae is a unicellular quadriflagellate green alga. The cells are ovoid to obpyriform in shape and have a deep anterior flagellar pit from which the flagella emerge (Pl. 2.1, figs. 1 and 2). The cells are microscopic and measure $14,27 \mu\text{m}$ ($\pm 0,41 \mu\text{m}$; $p = 0,05$) in length and $10,63 \mu\text{m}$ ($\pm 0,26 \mu\text{m}$; $p = 0,05$) in width at their broadest point. (These values were computed from size measurements made on 100 cells; see Appendix B, Table I).

In lateral view the cell has a large parietal chloroplast which contains a posteriorly situated pyrenoid and an eyespot (Pl. 2.1, figs. 1 and 2). The chloroplast has four lobes that extend anteriorly into the four lobes of the cell body. The four-lobed nature of the chloroplast is best seen in osmotically lysed cells (Pl. 2.1, fig. 7). Cells of *P. pseudoparkeae* often settle on their recurved flagella so that they present the posterior end of the cell toward the observer. Although this is a transient position, lasting a few seconds to several minutes, the cells remain firmly attached to the microscope slide and are difficult to dislodge. This settling behaviour, initiated usually by one cell, appears to evoke a settling response in others so that a tight grouping of cells results (Pl. 2.1, fig. 5).

By focussing at different levels on a settled cell the flagella and trichocysts are clearly visible. Where the cell is attached to the microscope slide, the four flagella are seen emerging from the cell in a cruciate pattern (Pl. 2.1, fig. 3). By focussing further into the cell the flagellar profiles can be seen within the flagellar pit (Pl. 2.1, fig. 4). Because the flagella follow the contours of the cell when they leave the flagellar pit (Pl. 2.1, figs. 1 and 2) they are often seen both within the flagellar pit and also on the outside of the cell between the four lobes of the cell body (Pl. 2.1, fig. 4).

Numerous trichocysts are found in the anterior part of the cell (Pl. 2.1, figs. 1 and 2) where they surround the flagellar pit (Pl. 2.1, fig. 4). They also occur laterally between the chloroplast lobes (Pl. 2.1, fig. 1). Ejected trichocysts are seen as fine threads (Pl. 2.1, fig. 7). Both chemical and physical stimuli cause the trichocysts to be ejected.

In Feulgen stained cells the median-lateral position of the nucleus is evident (Pl. 2.1, fig. 6). These preparations also reveal the lamellate structure of the pyrenoid (Pl. 2.1, fig. 8).

In cells fixed in glutaraldehyde and OsO_4 the densely staining flagellar basal body apparatus is clearly visible at the base of the deep flagellar pit (Pl. 2.1, fig. 9). In some cells the rhizoplast can be seen extending from the basal bodies toward the pyrenoid (Pl. 2.1, fig. 10).

Cells of *P. pseudoparkeae* swim in a characteristic manner. They swim rapidly (ca. $466 \mu\text{m s}^{-1}$) along a straight path while continuously rotating about their axis. They only change direction when they encounter an obstacle. Many unsuccessful attempts were made to observe the coordination of flagellar movement in quietened cells. However, each flagellum was seen to beat in a "breast stroke" pattern like that described for *Chlamydomonas* (Ringo, 1967).

The settling behaviour of cells observed in the laboratory was also seen in populations of wild material. Cells collected in test tubes settled in discrete patches ($\pm 2,0$ mm in diameter) on the sides of the container. It is interesting that settling occurred during periods corresponding to the time of high tide. The cells remained attached for longer periods (± 2 h) than observed in the laboratory. This rhythmic settling behaviour in the wild material disappeared after two days.

2.3.2 Electron microscope observations

External morphology and the scale-boundary

The external morphology of *P. pseudoparkeae* is best seen in three dimensional scanning electron micrographs (Pl. 2.2). The cells are pyramidal in shape and have four prominent anterior cell lobes (Pl. 2.2, fig. 1). Four relatively thick isokont flagella emerge from a deep anterior flagellar pit (Pl. 2.2, fig. 2). Although the cells appear naked under the light microscope, the dense scale-covering (Pl. 2.2, figs. 1-3) is clearly visible using the scanning electron microscope (SEM).

The resolving power of the transmission electron microscope (TEM) is much greater than that of the SEM with the result that much more detail on the external morphology of the cell can be obtained by viewing heavy metal shadowed specimens in a TEM. In shadowed material the flagella, which appeared smooth in the SEM, are seen to bear a dense covering of scales (Pl. 2.3, figs. 1 and 2). The flagella characteristically terminate in a bluntly rounded tip.

The cells possess seven different types of scales. Five of these are seen in the shadowed preparation in Pl. 2.3, fig. 2. Although most members of the genus are reported to possess six scale types, Pienaar and Aken (1985) have shown that *P. pseudoparkeae*, and probably other species too, has seven different scale types. Two types of small square scales, previously thought to be identical, have been recognized; one occurs on the flagellar surfaces and has a pronounced central nodule - the other scale type is found on the cell surface and is similar except that it does not have the pronounced central nodule. These two scale types are discussed in context below. For convenience an abbreviated and emended form of the scale terminology proposed by Norris and Pienaar (1978) is used in the following discussion. The Type 1, Type 2 and Type 3 body scales will be referred to as B_1 , B_2 and B_3 scales respectively. Likewise the Type 1 flagellar scale is called the F_1 scale. However, because a second new underlayer scale is present on the flagella this is designated as the F_2 scale. The Type 2 scale (limuloid scale) of Norris and Pienaar (1978) is then the F_3 scale. The hairscale, a name coined by Moestrup and Thomsen (1974) for the fourth type of flagellar scale is here designated as the F_4 scale.

The cell body bears three superimposed layers of scales, each layer being composed of a different scale type (Pl. 2.4, fig. 1). Small square scales, the B_1 scales, lie adjacent to the plasmalemma. Overlying these are larger square scales, the B_2 scales. Occupying a distal position in the scale-boundary are large crown-like scales, the B_3 scales. The three types of body scale form a scale-boundary which covers the whole cell body.

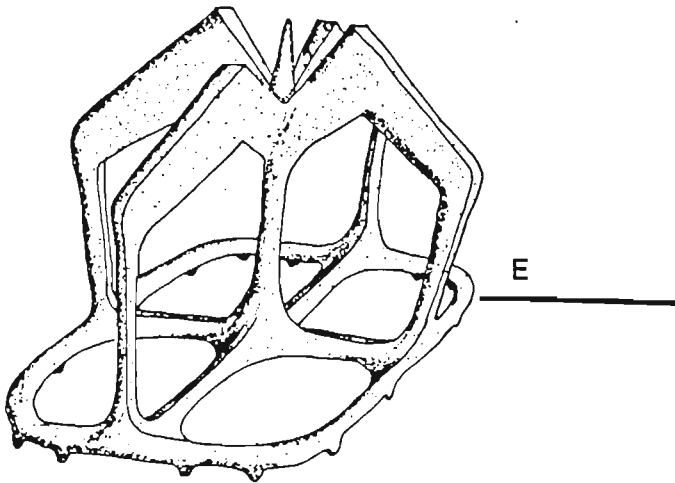
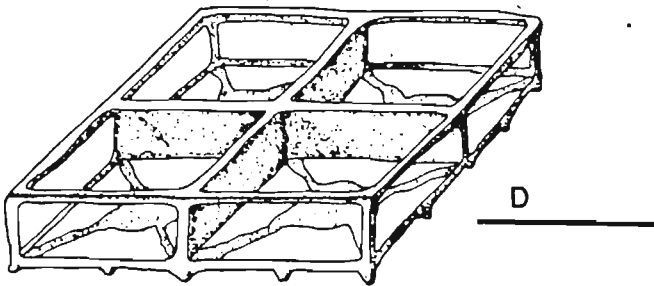
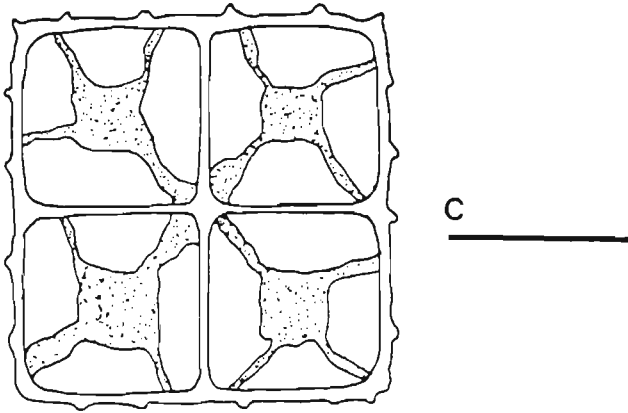
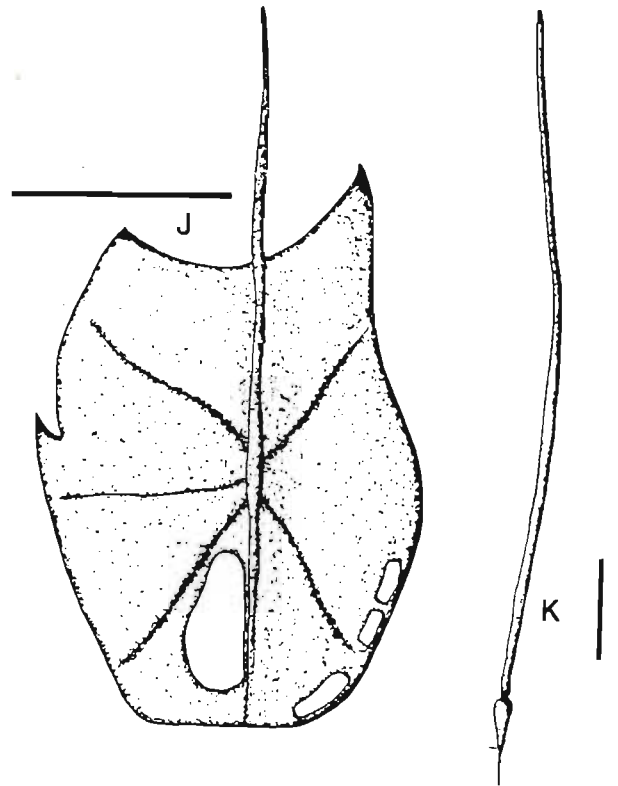
The flagella possess two superimposed layers of scales (Pl. 2.4, fig. 2). Small pentagonal scales, the F_1 scales lie adjacent to the plasmalemma, and cover most of the surface of each flagellum (Pl. 2.4, fig. 4). Small square scales (F_2 scales) also lie adjacent to the plasmalemma but these are positioned in pairs to form two longitudinal rows on opposite sides of the flagellum (Pl. 2.5, fig. 2). The F_2 scales are positioned in the scale boundary at a slightly lower level than the F_1 scales (Pl. 2.5, fig. 4). The F_3 scales cover the F_1 and F_2 scales (Pl. 2.4, figs. 2 and 4; Pl. 2.5, figs. 2 - 4). The F_3 scales are arranged in an imbricate manner with the dorsal spines of a row of scales being aligned (Pl. 2.4, fig. 3). The F_4

scales (hairscales), which are arranged along the length of the flagellum in two opposite rows, arise from between the pairs of F_2 scales (Pl. 2.5, figs. 3 and 4).

Following is a detailed description of the different scale types found on *P. pseudoparkeae*. Reconstructions of these scales are presented in Text fig. 2.1. The B_1 scales are square in plan view with each side measuring 37 nm (Pl. 2.4, fig. 5; Text fig. 2.1, A). The scales are box-like with the rim measuring 30 nm in height and they possess a central thickened region in the base of the scale (Pl. 2.4, fig. 1; Text fig. 2.1, B).

The B_2 scales, like the B_1 scales are square in outline but they are much larger, having sides measuring 198 nm (Pl. 2.4, figs. 5 and 6; Text fig. 2.1, C). These scales are also box-like with a rim measuring 30 nm in height (Pl. 2.4, fig. 1; Text fig. 2.1, D). Unlike the B_1 scales the B_2 scales have large windows in the rim of the scale. In plan view the B_2 scales are divided into four quadrats by four horizontal bars which arise midway along each side of the scale and fuse in the centre. In addition, the B_2 scales possess a proximal substructure which forms an incomplete base to the scale. This is noticeably more electron translucent than the rim and cross-members described above and may vary to some extent in old cells in ageing cultures. This substructure may assume the shape of a square in each of the four fields of the scale and is attached to the centre of the scale and the border with similar material (Text fig. 2.1, C). More often this substructure appears knee-shaped and radiates from the centre of the scale and fuses with the border of the scale (Pl. 2.3, fig. 2). The proximal boundary of the scale bears peg-like projections.

The B_3 scales are lattice-like structures having a roughly square base but with the corners more rounded than the B_2 scales (Pl. 2.4, fig. 6; Text fig. 2.1, E). The sides of the scale are 186 nm in length. Like the B_2 scales, these scales have peg-like projections on their proximal boundary and they possess four horizontal bars which fuse at the centre of the scale. Where these horizontal bars join the border of the scale, four uprights emerge. In addition a fifth upright arises from the centre of the scale where the four horizontal bars fuse. The four outer uprights arch to join the fifth upright at a position just below its apex thus giving the scale a crown-like

BODY SCALESFLAGELLAR SCALES

Text Fig. 2.1 A reconstruction of the different types of scales adorning *P. pseudoparkeae*.

Body scales

- A. and B = B₁ scales
 C and D = B₂ scales
 E = B₃ scales

Flagellar scales

- F and G = F₁ scales
 H and I = F₂ scales
 J = F₃ scales
 K = F₄ scale

Scale bars = 100 nm

appearance. The B_3 scales measure 220 nm in height.

The F_1 and F_2 scales are small scales comparable in size with the B_1 scales. The F_1 scales are pentagonal in outline (Pl. 2.4, fig. 4; Text fig. 2.1, F) and possess a central boss which extends above the rim of the scale (Pl. 2.4, fig. 2; Text fig. 2.1, G). This rim measures only 12 nm in height. The F_2 scale is square in outline (Pl. 2.5, fig. 2; Text fig. 2.1, H) and has the same base dimensions as the B_1 scale.

The F_2 scale however has a rim and central boss comparable in size with the F_1 scale (Pl. 2.5, fig. 4; Text fig. 2.1, I).

The F_3 scales are large, asymmetrical plate-like scales (250 nm in length and 175 nm wide) that have a raised adnate spine on their distal surface (Pl. 2.4, figs. 2 and 3; Pl. 2.5, fig. 4; Text fig. 2.1, J). This spine runs along the length of the scale and protrudes beyond the scale (for 75 nm) toward the free end of the flagellum. The opposite, truncated end of scale has a conspicuous aperture lying adjacent to the spine. Smaller apertures are often present near the edge of the scale on the opposite side of the spine. The F_3 scales have a characteristic notch midway along their length and on the same side as the large aperture. A number of slightly thickened spokes radiate from the centre of the scale.

The F_4 scales or hairscales are approximately 900 nm in length and are distinctly tripartite, having a tapered head, short neck and a long tubular shaft (Pl. 2.3, fig. 2; Pl. 2.5, figs. 3 and 4; Text fig. 2.1, K).

The scale-boundary of *P. pseudoparkeae* is fascinating because the scales always occupy constant positions relative to the plasmalemma. The different scale types are not only confined to a particular scale layer, but they are also precisely arranged with respect to the scales in an adjacent layer. This orientation of the scales is revealed in cells which have been fixed with minimal distortion of the scale-boundary (Pl. 2.4, figs. 1, 5 and 6). The mechanism or mechanisms underlying this precise arrangement of the scales is not known.

The cell depicted in Pl. 2.6 (fig. 1) has a complete scale-covering in

which all three body scales are represented in the scale-boundary. Scales are however, notably absent at the base of the flagellar pit. The B_1 scales are tightly packed forming an almost continuous sheet adjacent to the plasmalemma. The B_2 scales which occupy an intermediate position in the scale-boundary are arranged so as to cover sixteen B_1 scales. A section through the scale-boundary (Pl. 2.4, fig. 1) shows four B_1 scales arranged beneath and across the width of the B_2 scales. In plan view (Pl. 2.4, fig. 5), sixteen B_1 scales can be seen beneath each B_2 scale, four B_1 scales occupying a position beneath each of the four quadrats of the B_2 scale. Both the peg-like structures and the substructure material on the proximal surface of the B_2 scales may function to provide a physical basis or template which determines the orientation of the scales in these two layers. The distal B_3 scales are arranged with the centre of the scale lying over the point where four adjacent B_2 scales abut (Pl. 2.4, fig. 6). The bases of the B_3 scales then overlie one quadrat of each of the four abutting B_2 scales. The peg-like structures on the base of the B_3 scales may serve to orientate and secure scales in the intermediate and distal layers.

The F_1 scales are arranged in rows that run at approximately 45° to the long axis of the flagellum (Pl. 2.4, fig. 4). As mentioned above, this spiral arrangement of the F_1 scales is interrupted by two longitudinal rows of F_2 scales on opposite sides of the flagellum (Pl. 2.5, fig. 2). The F_3 scales, which cover the F_1 and F_2 scales are arranged in an imbricate pattern. The truncated region of the F_3 scale tucks underneath the free spine of a scale lying in front of it (Pl. 2.4, fig. 3). In this way the raised spines are aligned in rows along the length of the flagellum (Pl. 2.4, fig. 3). The F_4 scales always arise from between paired F_2 scales on opposite sides of the flagellum (Pl. 2.5, figs. 3 and 4). The F_4 scale is attached to the flagellum by its tapered head.

Many of the micrographs which follow depict cells which appear ostensibly naked. The absence of a scale-boundary is an artefact produced during fixation. A dislodged scale-boundary is shown in Pl. 2.5, fig. 1. It is interesting to note that the boundary is often displaced as a unit suggesting that there is some cohesion between the scales.

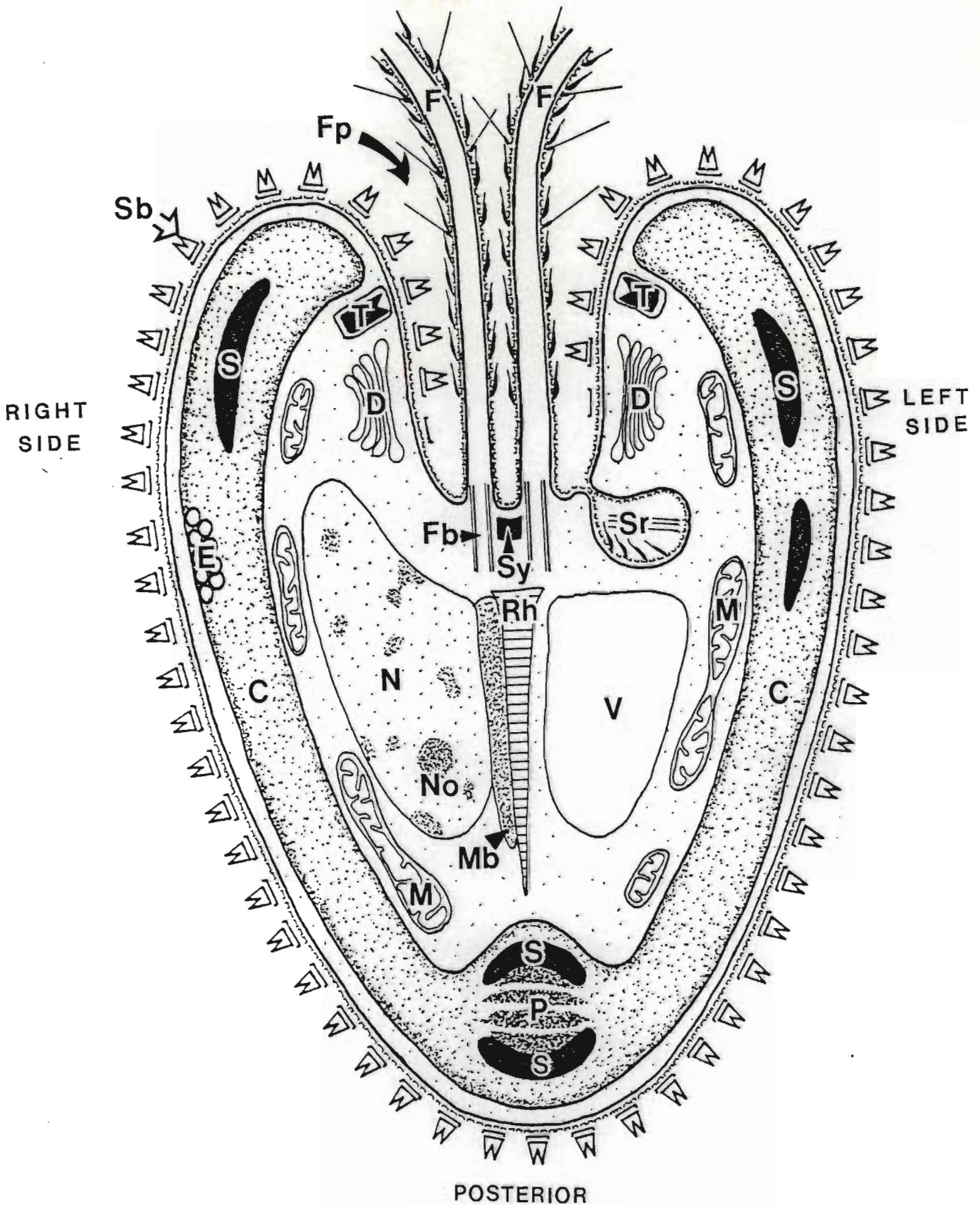
Internal organization

It is the writer's intention to present here a detailed account of the position of different organelles within the cell. Particular attention has been paid to the orientation of organelles with respect to one another. The detailed structure of individual organelles is discussed fully under subsequent headings.

The internal organization of the cell as seen in both longitudinal and transverse section is depicted in Plate 2.6 (figs. 1 and 2) and Plate 2.7 (figs. 1 and 2) respectively. A diagrammatic representation of a median longitudinal section through the alga is furnished in Text fig. 2.2.

While appearing to have radial symmetry when viewed along the apical axis under the light microscope, *Pyramimonas* is in reality an asymmetrical cell. The orientation of organelles is constant so that a left and right, and dorsal and ventral side can be recognized. The terminology proposed by Melkonian (1981) for describing cell symmetry in *P. obovata* is adopted here. Briefly, he has proposed that when the cell is viewed from its anterior end the side containing the nucleus is called the right side (the opposite side containing the scale reservoir being the left side) (Pl. 2.6, fig. 2; Pl. 2.7, figs. 1 and 2). In transverse section the duct leading from the scale reservoir into the flagellar pit is always seen to be displaced (Pl. 2.7, fig. 1) so that it is associated with a cytoplasmic spur. This spur is taken to point toward the ventral surface. The opposite side is the dorsal surface.

The protoplast is bounded by a single unit membrane called the plasmalemma. The scale-boundary described earlier lies external to and adjacent to the plasmalemma. The large parietal chloroplast contains a posterior pyrenoid and an eyespot which is situated close to the plasmalemma in a median lateral position in one of the chloroplast lobes (Pl. 2.6, fig. 1; Text fig. 2.2). In the anterior part of the cell the four lobes of the chloroplast are visible in the lobes of the cell body (Pl. 2.7, fig. 2). The pyrenoid is embedded in the chloroplast and is traversed by single thylakoids. It is usually surrounded by two cup-shaped starch grains, one lying anterior



Text Fig. 2.2 A diagrammatic representation of the structure of *P. pseudoparkeae*.

- | | | |
|-------------------|---------------------------|----------------------|
| C = chloroplast | D = dictyosome | E = eyespot |
| F = flagellum | Fb = flagellar basal body | Fp = flagellar pit |
| M = mitochondrion | Mb = microbody | N = nucleus |
| No = nucleolus | P = pyrenoid | Rh = rhizoplast |
| S = starch grain | Sb = scale boundary | Sr = scale reservoir |
| Sy = synistosome | T = trichocyst | V = vacuole |

Note; the two dictyosomes would usually only be present in the cell lobes that would lie above and below the page.

to the pyrenoid and the second (usually larger) lying posterior to the organelle (Pl. 2.6, fig. 2; Text fig. 2.2). Starch grains may also be found in different places in the chloroplast lobes (Pl. 2.6, fig. 2; Pl. 2.7, fig. 2; Text fig. 2.2). The nucleus occupies a median lateral position in the right side of the cell and lies close to the rhizoplast (System II fibres) which arise at the flagellar basal bodies and extend toward the posterior part of the cell (Pl. 2.6, fig. 2; Text fig. 2.2). The nucleus usually has an anterior lobe which extends anteriorly into one of the lobes of the cell. The flagellar basal bodies lie beneath the base of the flagellar pit and are associated with the emergent flagella. A large vacuole lies adjacent to the rhizoplast in the left side of the cell (Pl. 2.6, fig. 2; Text fig. 2.2). A scale reservoir is found in the same longitudinal plane as the vacuole but occupies an anterior position in one of the cell lobes (Pl. 2.6, fig. 2; Text fig. 2.2). The scale reservoir is connected to the base of the flagellar pit by a short duct so that the membrane of the scale reservoir is confluent with the plasmalemma (Pl. 2.6, fig. 2; Pl. 2.7, fig. 1; Text fig. 2.2). The scale reservoir and the anterior lobe of the nucleus lie adjacent to, and on opposite sides of, the flagellar pit, i.e. in the left and right sides of the cell, respectively. The two dictyosomes which comprise the Golgi apparatus are also found in the anterior part of the cell on opposite sides of the flagellar pit (Pl. 2.6, fig. 1; Pl. 2.7, figs. 1 and 2). They occupy the anterior part of the dorsal and ventral cell lobes. The dictyosomes always have their mature faces orientated toward the flagellar pit. This is evident by the presence of mature scale profiles in cisternae closest to the flagellar pit.

Trichocysts are present in the anterior region of the cell and surround the opening of the flagellar pit (Pl. 2.6, figs. 1 and 2; Text fig. 2.2). Trichocysts also occur in the longitudinal grooves of the cell between the chloroplast lobes (Pl. 2.7, fig. 2). Numerous mitochondrial profiles are present close to the centripetal surfaces of the chloroplast (Pl. 2.6, fig. 2; Text fig. 2.2). Fewer profiles are seen in other regions of the cytoplasm.

The position of organelles within interphase cells is remarkably constant and only changes when the cell prepares for, and undergoes, cell division.

Flagellar structure

The flagella are specialized protrusions of the protoplast and possess a complex internal structure. They are surrounded by a unit membrane that is continuous with the plasmalemma.

Structurally the flagellum may be divided into three regions, viz. the flagellar basal body region, the transition region and the axonematal region. (Pl. 2.8, fig. 1). The term "axonematal region" is proposed here in preference to the phrase "free part of the flagellum" (Dodge, 1973), because the transition region often extends into the emergent flagellum. The flagella of *P. pseudoparkeae* are structurally similar to those described for other green algae. They do, however, possess some unique features which will be emphasized below.

For much of its length the emergent flagellum (approximately 260 nm in diameter) has the typical 9 + 2 arrangement of microtubules that is characteristic of the flagellar axoneme of most eukaryotic organisms. The axoneme is composed of an outer ring (160 nm in diameter) of nine pairs of microtubules (doublets) and two free, central microtubules (Pl. 2.8, fig. 2). In the proximal part of the axonemal region of the flagellum there is an unusual cylindrical structure which lies inside (and close to) the ring of doublet microtubules. This cylinder begins where the central pair of microtubules arise and extends for 200 - 300 nm toward the distal end of the flagellum (Pl. 2.8, fig. 1). In transverse section the structure is seen as a continuous ring (95 nm in diameter) just inside the ring of doublet microtubules (Pl. 2.8, fig. 3). This structure is reminiscent of the transitional helix (TH) which has been described for motile members of the classes Chrysophyceae (Pienaar, 1980b) Xanthophyceae (Hibberd, 1980a) and Eustigmatophyceae (Hibberd, 1980b). In *P. pseudoparkeae*, it is very difficult to determine if this cylindrical structure is a coiled fibre (like the transitional helix) or a true cylinder. If it is a coiled fibre, it differs from the TH with respect to the tightness of the coil (being more compact giving the appearance of a cylinder) and to the length of the structure (being longer than the TH which comprises between one and eight gyres in the classes mentioned above).

The transition region of the flagellum is approximately 230 nm in length, and extends from the point where the central tubules of the free flagellum arise to the point where the outer doublet microtubules meet the triplet microtubules of the basal body (Pl. 2.8, fig. 1). The transition region occurs in the proximal part of the emergent flagellum and meets the basal body at a point just below the plasmalemma at the base of the flagellar pit. The distal part of the transition region has an outer ring of doublet microtubules which are confluent with those of the free flagellar, but the region inside this is electron translucent, and devoid of microtubules (Pl. 2.8, figs. 1 and 4). The most conspicuous structure in the transition region is the stellate structure which appears cylindrical in longitudinal section (100 nm in length and 50 nm in diameter), and is composed of fibrillar material which describes a stellate pattern when viewed in transverse section (Pl. 2.8, figs. 1 and 5). In transverse section the fibrillar strands are arranged in the form of three superimposed equilateral triangles which are displaced by 40° to one another so that the corners of each triangle attach to every third doublet. Adjacent doublets in the transition region are connected by a short fibrillar strand. *P. pseudoparkeae* does not have a well defined basal disc so that the stellate structure, when seen in longitudinal section, does not have the typical H-shape that is characteristic of many green algae. Although the basal disc is ill-defined and apparently absent in some sections, a diffuse plate is sometimes present at the distal end of the stellate structure (refer to Pl. 2.10, fig. 2). If this diffuse plate is a remnant of the basal disc then the stellate structure of *P. pseudoparkeae* lacks the distal moiety which is seen in the typical H-shaped stellate structure (Dodge, 1973). The absence of this moiety may be manifest by the electron translucent nature of the cytoplasm in the centre of the axoneme at the distal end of the transition region (Pl. 2.8, fig. 1).

The flagellar basal bodies are approximately 400 nm in length and 170 nm in diameter (Pl. 2.8, fig. 1). At the distal end of the basal body an additional microtubule is added to each of the peripheral doublets to form "triplets". These triplets become reorientated so that they lie at an angle of 40° to a tangent of the circle described by the nine triplets (Pl. 2.8, fig. 6). When viewed in transverse section from the anterior end of the cell, these triplets are orientated so that the centripetal microtubule of each triplet lies to the left of a fibrillar strand which extends from the

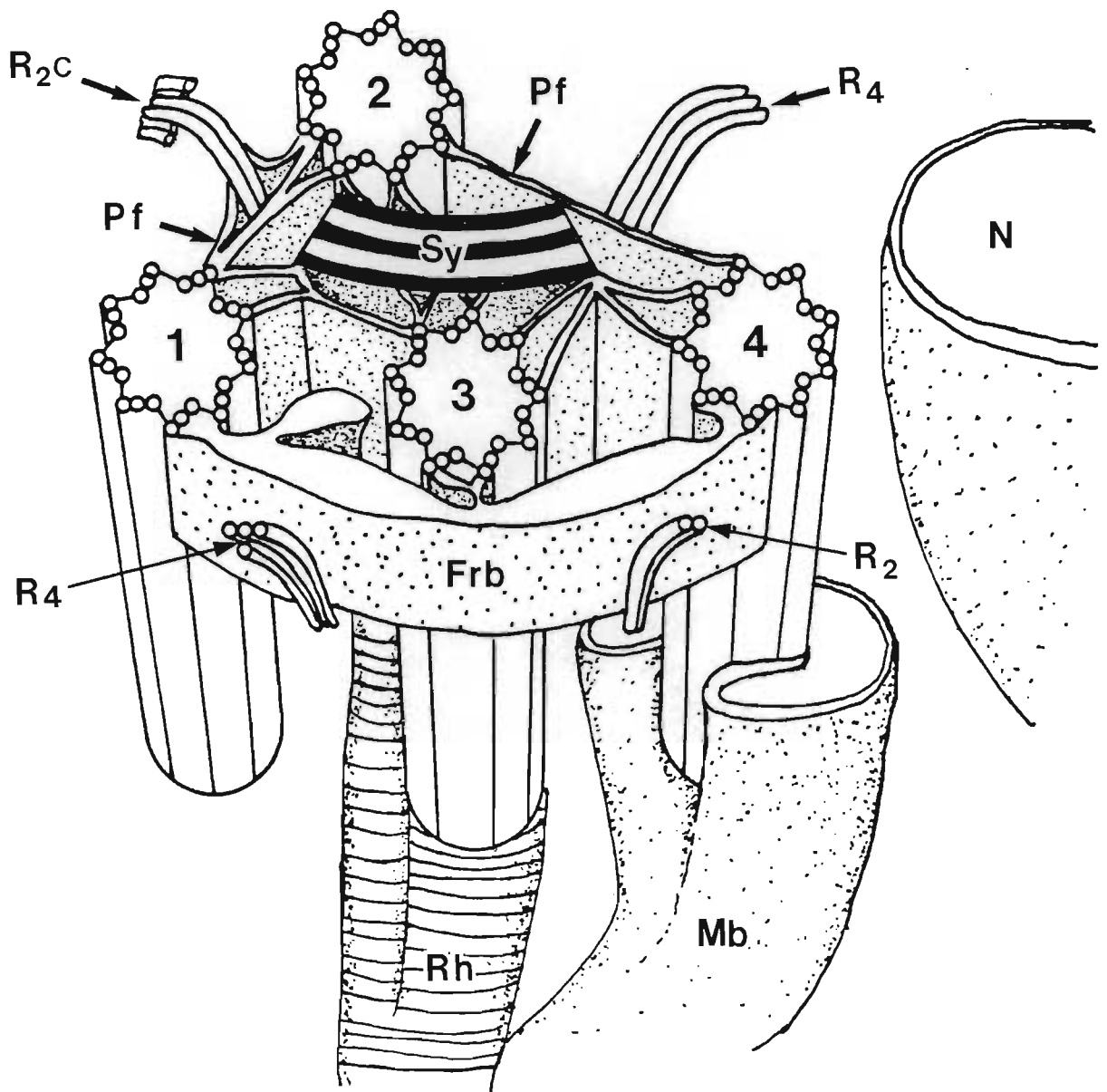
centre microtubule of each triplet. The radial fibrillar strands are slightly displaced to form a narrow cylinder in the centre of the basal body (Pl. 2.8, figs. 1 and 6). At the proximal end of the basal body the radial strands are absent and only the ring of triplets remains (Pl. 2.8, figs. 1 and 7). A short fibrillar strand connects adjacent triplets throughout the length of the basal body.

Flagellar roots and connecting fibres.

The flagellar basal bodies are held together by an elaborate system of connecting fibres and are anchored within the cell by two types of roots, namely microtubular rootlets and a striated rootlet (rhizoplast or system II fibre). Since the orientation of the basal bodies is constant with respect to other organelles in the cell, they can be numbered. The numbering system proposed by Melkonian (1981) will be used here. When seen in transverse section from a position anterior to the plane of section the basal body adjacent to the nucleus (in right side of cell) is numbered 4. Moving in a clockwise direction the other basal bodies are numbered 3, 1 and 2, respectively (Pl. 2.8, fig. 10; Text fig. 2.3). Connecting fibres brace the basal bodies so that their proximal ends are closer together than their distal ends (Pl. 2.8, fig. 8).

In *P. pseudoparkeae* there are five different types of connecting fibres associated with the basal bodies, namely the synistosome (distal connecting fibre), the proximal connecting fibre, the pericentriolar fibres, the fibrillar band and the basal connecting band. The basal connecting band connects the proximal ends of basal bodies 2 and 3 (see Text fig. 2.4) and it may be seen below the proximal connecting fibre in Pl. 2.8, fig. 1.

The synistosome is a large curved structure that lies between basal bodies 2 and 3 (Pl. 2.8, figs. 10 and 11; Text fig. 2.3). It is situated toward the distal end of the basal body complex (Pl. 2.8, fig. 1) and is approximately 200 nm in height when seen in longitudinal section. In transverse section the synistosome measures 310 nm along the curved axis and is 120 nm wide. The convex and concave surfaces of the synistosome are composed of electron dense material. A band of similar material lies in the centre of the synistosome (Pl. 2.8, fig. 10). Approximately 27



Text Fig. 2.3 A three dimensional reconstruction of the flagellar basal body apparatus of *P. pseudoparkeae*.

- 1 - 4 = flagellar basal bodies
- Frb = fibrillar band
- Mb = microbody
- N = nucleus
- Pf = pericentriolar fibres
- Rh = rhizoplast
- Sy = synistosome
- R_2 and R_4 = microtubular roots
- R_2 = two microtubules
- R_4 = four microtubules
- R_2c = compound R_2 root

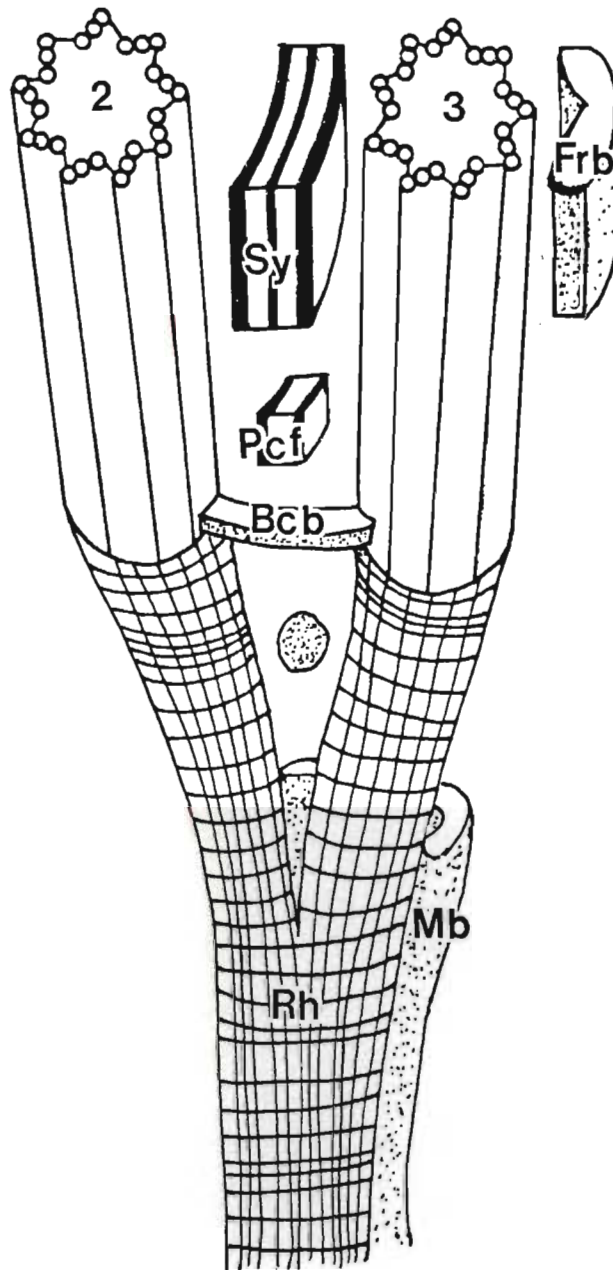
small fibrils connect the central band with the outer bands (Pl. 2.8, fig. 11).

An additional group of connecting fibres, the pericentriolar fibres form an electron dense meshwork of anastomosing fibres which join adjacent basal bodies and connect these with the synistosome (Pl. 2.8, fig. 10; Text fig. 2.3). A connecting fibre called the fibrillar band is connected to and joins basal bodies 1, 3 and 4 (Pl. 2.8, fig. 10; Pl. 2.9, fig. 6; Text fig. 2.3).

At the proximal end of the basal body apparatus there is a proximal connecting fibre resembling the synistosome (Pl. 2.8, fig. 1; Text fig. 2.4). This fibre lacks the central band of dense material.

Four microtubular rootlets emanate from the basal body apparatus in a cruciate pattern (Pl. 2.8, fig. 9; Text fig. 2.3). These roots are attached, proximally, to the pericentriolar fibres between basal bodies 1 and 2, and 2 and 4; and to the fibrillar band between basal bodies 1 and 3, and 3 and 4. The microtubular rootlets are arranged in a 4-2-4-2 pattern. This formula describes the number of microtubules represented in each of the four roots proceeding in a clockwise direction from the root between basal bodies 2 and 4. The roots containing four microtubules (R_4 roots) have the latter arranged in a three over one pattern (Pl. 2.9, fig. 1). The two microtubules in R_2 roots lie side by side (Pl. 2.9, fig. 2).

The R_2 root which arises between basal bodies 1 and 2 is unique in that it gains a number of additional microtubules (usually four) soon after leaving the basal body complex (Pl. 2.9, fig. 6). This compound root bifurcates toward its distal end (Pl. 2.9, fig. 4) where it surrounds the aperture of the duct leading to the scale reservoir (Pl. 2.9, figs. 5 and 6). The possible role of this root is discussed in Chapter Five. The R_2 root lying opposite the compound R_2 root described above does not acquire additional microtubules and remains a simple R_2 root (Pl. 2.9, figs. 5 and 6). The microtubular roots have also been called ascending rootlets because they extend anteriorly where they pass just beneath the plasmalemma surrounding the flagellar pit (Pl. 2.10, fig. 2). These roots terminate at a point anterior to, and approximately 1.0 μm from, the base of the flagellar pit. There is a small overlap of the ascending microtubules with the cytoskeletal microtubules.



Text Fig. 2.4 A diagram showing the position of connecting fibres between basal bodies 2 and 3 (pericentriolar fibres omitted).

- | | | |
|---------|---|---------------------------------------|
| 2 and 3 | = | flagellar basal bodies |
| Bcb | = | basal connecting band |
| Frb | = | fibrillar band (cropped) |
| Mb | = | microbody (cropped) |
| Pcf | = | proximal connecting fibre |
| Rh | = | rhizoplast |
| Sy | = | synistosome (distal connecting fibre) |

The rhizoplast arises at the proximal end of the basal body complex (Pl. 2.10, fig. 1) and extends posteriorly and bifurcates to lie over the chloroplast in the region of the pyrenoid (Pl. 2.10, fig. 5). The rhizoplast also bifurcates near the basal bodies to give rise to two trunks that are attached to basal bodies 2 and 3 (Pl. 2.10, fig. 3; Text fig. 2.4). The rhizoplast is composed of many fine filaments (approximately 12 nm in diameter) that are interrupted at intervals by cross-striations. The distance between cross-striations may vary from 24 nm to 140 nm.

The rhizoplast is contiguous with the microbody throughout much of its length and these two organelles always lie adjacent to, and along a ridge of the nucleus (Pl. 2.10, figs. 1, 4 and 5). In the region of this rhizoplast-microbody-nucleus complex, the nuclear envelope is interrupted by numerous pores (Pl. 2.10, fig. 1). The microbody extends into the basal body complex where it lies between basal bodies 2 and 4 (Pl. 2.9, fig. 3).

Cytoskeletal microtubules

A well developed system of microtubules lies just beneath the plasma-lemma in most regions of the cell and comprises the microtubular cytoskeleton. These microtubules arise approximately 500 nm from the base of the flagellar pit and extend anteriorly to line the flagellar pit (Pl. 2.10, fig. 2; Pl. 2.11, fig. 1). The base of the flagellar pit is not lined with microtubules. A transverse section through the flagellar pit reveals that the cytoskeleton is composed of 97 closely spaced microtubules in this region (Pl. 2.11, fig. 1). Where the cytoskeletal microtubules arise there is an overlap (approximately 100 nm) of these with the ascending microtubular roots which emanate from the basal body apparatus (Pl. 2.10, fig. 2). At the opening of the flagellar pit the cytoskeletal microtubules curve to follow the contours of the cell. The posterior extent of the microtubules is not known with certainty but they do extend to a position opposite the pyrenoid.

The cytoskeletal microtubules lying outside the flagellar pit are more widely spaced. These microtubules may occur singly (Pl. 2.11, figs. 2 and 4) or in pairs (Pl. 2.11, figs. 3 and 5) and are accommodated in longitudinal

furrows on the outer surface of the chloroplast. The grooves separating the four anterior cell lobes are maintained by cytoskeletal microtubules that are concentrated in this region (Pl. 2.11, fig. 5).

The microtubular cytoskeleton appears to provide a cytoskeletal support which maintains cell shape in the absence of a cell wall.

Chloroplast eyespot and pyrenoid

The chloroplast is bounded by a double unit membrane and contains many lamellae that are arranged in the stroma in a position parallel to the surface of the cell. The lamellae are composed of between two and seven closely appressed thylakoids (Pl. 2.12, figs. 1 and 2). Despite this variability in thylakoid number the lamellae are more commonly composed of two thylakoids each approximately 12 nm thick. Where the lamellae approach the chloroplast envelope at right angles, for example on the radial surfaces of the chloroplast lobes, they terminate abruptly (Pl. 2.12, fig. 1). The chloroplast does not have girdle lamellae and is not surrounded by a sheath of endoplasmic reticulum. As mentioned above the chloroplast envelope is corrugated where it lies adjacent to the plasmalemma (Pl. 2.11, figs. 4 and 5) and accommodates the cytoskeletal microtubules.

An intraplastidial eyespot is situated in one of the chloroplast lobes in a position adjacent to the plasmalemma (Pl. 2.12, figs. 2 and 3). In plan view the organelle is roughly circular in outline and measures approximately 1.0 μm in diameter (but may be up to 2.0 μm in diameter). It is composed of two to three layers of carotenoid globules which are tightly packed so that they appear hexagonal in outline (Pl. 2.12, fig. 4). These globules are on average 100 nm in diameter. The eyespot is usually situated beneath a concave depression in the plastid (Pl. 2.12, fig. 3). The plasmalemma, which follows the contours of this depression, retains its scale-covering. The eyespot of *P. pseudoparkeae* would be representative of a Type A eyespot (Dodge, 1973) because it is not obviously associated with the flagellar apparatus.

The pyrenoid is embedded in the chloroplast matrix at the posterior end of the cell. It is roughly lens-shaped and is capped on the anterior and

posterior surfaces by two cup-shaped starch grains (Pl. 2.12, fig. 5). The pyrenoid is traversed by single thylakoids which lie parallel to one another and give the organelle its lamellate appearance. These thylakoids, which enter the pyrenoid laterally, are evenly spaced and are approximately 200 nm apart. The pyrenoid thylakoids do not always traverse the organelle as flat transverse bands but sometimes as concave bands. A transverse section of a pyrenoid with concave bands shows the pyrenoid lamellae as concentric rings (Pl. 2.12, fig. 6). The pyrenoid of *P. pseudoparkeae* is classified as a Type E pyrenoid because it is embedded in a starch containing chloroplast (Terminology of Dodge, 1973).

The mitochondrion

Almost any section through the cell reveals numerous mitochondrial profiles. These are concentrated on the centripetal surfaces of the chloroplast lobes where they are closely appressed to the chloroplast envelope (Refer to Pl. 2.6). Fewer profiles are seen in other parts of the cytoplasm. Serial sections through the cell show that these profiles are all interconnected suggesting that it may have a single large reticulate mitochondrion (Pl. 2.12, figs. 7 and 8). The organelle is bounded by a double unit membrane. The inner membrane of the mitochondrial envelope produces flattened sac-like cristae that lie in the mitochondrial stroma (Pl. 2.13, fig. 1). The mitochondrial profiles in some cells have very long cristae which lie parallel to the chloroplast lamellae (Pl. 2.13, fig. 2). Mitochondria having this arrangement of the cristae always have a dense stroma.

Trichocysts

The trichocyst is contained in a membrane bound chamber which lies just beneath, and at right angles to, the plasmalemma (Pl. 2.13, fig. 3). Trichocysts are found near the opening of the flagellar pit and between the chloroplast lobes (refer to Pl. 2.6 and 2.7). The intact trichocyst resembles a thick-walled cylinder and is approximately 350 nm in diameter and 530 nm in height (Pl. 2.13, fig. 3). The "cylinder wall" is composed of a tightly coiled electron dense ribbon which is, for most of its length, approximately as wide as the trichocyst cylinder is high. This ribbon which has between 20 and 30 coils, is very thin being comparable in thickness

with the plasmalemma (Pl. 2.13, fig. 5). The lumen of the trichocyst contains many small vesicles each approximately 35 nm in diameter (Pl. 2.13, fig. 3).

Trichocysts are often released during fixation. Since there are no contractile structures surrounding the trichocyst, it is probable that the mechanism of release is one involving an increased hydrostatic pressure in the trichocyst chamber. It is difficult to imagine how the open-ended trichocyst depicted in Pl. 2.13, fig. 3, could extend even with an increase in hydrostatic pressure in the chamber. A proposal for the mechanism of trichocyst release is given below. This proposal is made after observing the trichocyst at different stages of development.

Immature trichocysts are formed *in situ* and are easily recognized because they have relatively few coils to the ribbon (see Pl. 5.5, fig. 1). Development of the trichocyst occurs through an increase in ribbon length on the inside of the coil. The material incorporated into the ribbon comes from small electron dense vesicles which bud off from the cytoplasmic papillae at the base of the trichocyst chamber (Pl. 2.13, fig. 3). These migrate into the lumen of the trichocyst where they fuse with the coil (Pl. 2.13, fig. 4). The trichocyst remains an open ended cylinder while the ribbon grows in length.

The final stage in the development of the trichocyst appears to be marked by the production of a large vesicle which buds off from the cytoplasmic papilla (Pl. 2.14, fig. 1). This vesicle migrates through the lumen to the distal end of the trichocyst where it "plugs" the top of the cylinder (Pl. 2.14, fig. 2). Many smaller vesicles remain in the lumen. While the function of these in the mature trichocyst is obscure, they may contain dissolved solutes that increase the osmotic pressure within the trichocyst chamber. The trichocyst depicted in Pl. 2.14, fig. 2 is considered to be a mature trichocyst that could be ejected. The distal part of the trichocyst cylinder is "plugged" so that an increase in hydrostatic pressure within the trichocyst chamber would cause the inner coils to be extended.

In the laboratory the stimuli causing the trichocyst to be ejected are either physical (e.g. pressure of a coverslip) or chemical (e.g. fixation in

glutaraldehyde). These stimuli may cause a sudden increased turgor within the trichocyst chamber causing the organelle to be ejected. When ejected the trichocyst extends through a telescoping action which begins with the inner coils (Pl. 2.14, figs. 3 and 4). The ejected trichocyst is tubular in section (Pl. 2.14, fig. 7) and there is an overlap of the coils which prevents fluid from escaping from between the coils (Pl. 2.14, figs. 5 and 6).

When the trichocyst is released it breaks through the plasmalemma and the latter appears to form a tight collar around the extending coil (Pl. 2.14, fig. 3). This may ensure that the hydrostatic pressure is maintained in the trichocyst chamber while the organelle is extending.

The ejected trichocyst is released from the cell as a complete unit leaving only the chamber membrane which fuses with, and is incorporated into, the plasmalemma (Pl. 2.14, fig. 8).

Endoplasmic reticulum, Golgi apparatus and scale reservoir.

The endoplasmic reticulum (ER) in *P. pseudoparkeae* forms a complex internal membrane system. It may be tubular or sheet-like and consists of an electron translucent lacuna that is bounded on all sides by a unit membrane. The lacuna is usually uniform in width (approximately 115 nm) so that when seen in section the lacuna lies between two unit membranes which run parallel to one another.

The ER is confluent with the nuclear envelope and arises from the nucleus as two "horns" (Pl. 2.15, fig. 1). The horns give rise to a system of peripheral ER that extends, usually without interruption, to the forming face of both the dorsal and ventral dictyosomes. This peripheral ER is not tubular as it appears in transverse section, but takes the form of a large curved sheet situated close to the inner surface of the chloroplast lobes (Pl. 2.15, fig. 2). In this position the peripheral ER seems to hold the reticulate mitochondrion against the chloroplast lobes. The peripheral ER cylinder is more reticulate in the left half of the cell after it passes the dictyosomes.

At the anterior end of the cell the peripheral ER follows the contours of the cell and extends along the sides of the flagellar pit in a position beneath the cytoskeletal microtubules (Pl. 2.15, fig. 2). This ER, referred to here as the flagellar pit ER, forms a reticulate cylinder around the flagellar pit (Pl. 2.11, fig. 1) and extends to within 1,0 μm of the pit base. The flagellar pit ER overlaps with the ascending microtubular rootlets, but this overlap is small so that most transverse sections through the pit region showing the flagellar pit ER will not show profiles of the microtubular rootlets. Where the anterior lobe of the nucleus lies adjacent to the flagellar pit, the nuclear envelope and flagellar pit ER are confluent (Pl. 2.15, fig. 1). The rest of the cytoplasm has a relatively sparse network of tubular ER.

Where the peripheral ER system passes close to the forming face of the two dictyosomes it becomes highly reticulate and fragments to form small vesicles which fuse to form the dictyosomal cisternae (Pl. 2.15, fig. 3). Each dictyosome is comprised of approximately twenty cisternae that are flattened sac-like structures appearing more or less circular in plan view. In transverse section the cisternae resemble the ER in that they have a narrow lacuna that is bounded by a unit membrane. Near the forming face of the dictyosome the lacunae are narrow but they become progressively more dilated as the mature face of the dictyosome is approached. The peripheral regions of all cisternae are usually more dilated than the rest.

Any section through a dictyosome shows scales in various stages of construction with nascent scales being present in cisternae near the forming face and mature scales being released in vesicles at the mature face. The role of the dictyosomes in scale production is discussed in detail in Chapter Five.

The scale reservoir, as the name implies, stores scales which are produced by the dictyosomes (Pl. 2.16, fig. 1). This organelle resembles the vacuole in that it is bounded by a unit membrane but differs from it because it is connected to the flagellar pit via a short duct. The membrane of the scale reservoir is confluent with the plasmalemma through this duct so that the scales stored within the scale reservoir are in reality "outside" the cell. The reservoir duct enters the flagellar pit asymmetrically

to one side of a cytoplasmic spur which separates the scale reservoir from the flagellar pit.

It is significant to note that the scale reservoir stores predominantly flagellar scales. The scales appear to be organized within the scale reservoir with F_1 and F_2 scales lying close to the membrane (these are overlain by the F_3 scales). The hairscales (F_4 scales) have their tapered head situated amongst the other scales while their shafts project toward the centre of the reservoir (Pl. 2.16, figs. 1 and 2).

Associated with the scale reservoir is a well developed duct system that apparently increases the surface area of the reservoir. These ducts are circular in transverse section and contain small flagellar scales only (Pl. 2.16, fig. 2).

The Nucleus

The nucleus in *P. pseudoparkeae* is identical to that found in other eukaryotic organisms and is bounded by a double unit membrane that is confluent with the endoplasmic reticulum system (Pl. 2.15, fig. 1). The nuclear envelope characteristically has numerous nuclear pores where it is in close association with the rhizoplast and microbody (refer to Pl. 2.10, fig. 1). It is not unusual to see a nucleus with two nucleoli (Pl. 2.16, fig. 3). The appearance and position of the nucleus is constant in interphase cells and only changes when the cell prepares for cell division. (see Chapter Five).

2.4 DISCUSSION

The morphology and structure of *P. pseudoparkeae* is discussed here with reference primarily to other species within the genus. Where appropriate some comments are made on phylogenetic trends within the genus, but this topic is discussed more fully in Chapter Eight where a proposed phylogeny for species in the genus is given. Those unique features that support the separation of *P. pseudoparkeae* from *P. parkeae* are given special attention in this discussion.

When viewed under the light microscope *P. pseudoparkeae* is morphologically and structurally similar to other species within the genus i.e. the cells are roughly ovoid to pyramidal in shape and have four flagella that are inserted anteriorly in a deep flagellar pit. *P. amyliifera* (Pennick, 1978) and *P. octopus* sp. ined (see Moestrup, 1982) are exceptions in that they both possess eight flagella and are thus easily distinguished from other species. Most members of the genus have a conspicuous parietal chloroplast that contains one eyespot in one of the lobes. *P. gelidicola* and *P. longicauda* are apparently exceptions because they have two intraplastidial eyespots (McFadden *et al.*, 1982; Inouye *et al.*, 1984). While the presence or absence of an eyespot may be significant, it is the writer's opinion that the number of eyespots in a chloroplast is not of taxonomic importance in the genus.

Many cells of *P. pseudoparkeae* have been seen with two (and more) eyespots but these only occur in cells in ageing cultures. These cells always have large deposits of starch and lipid material. Some micrographs presented by McFadden *et al.* (1982 - Figs. 1, 5 and 6) and Inouye *et al.* (1984 - Fig. 13) show cells that have large lipid globules suggesting that the cells are old. For *P. longicauda* this view is supported by the authors' comment that the cells were not actively growing when fixed (Inouye *et al.*, 1984). Throughout the present work many examples are cited where characteristics of the cell change in ageing cultures. These observations emphasize the need to work with actively growing cultures when describing the structure of a cell, particularly if it is a new species.

The trichocysts of *P. pseudoparkeae* are clearly visible under the light microscope and set it apart from most species. Only five other species possess trichocysts, viz. *P. grossii* (Manton, 1969; Pennick and Clarke, 1976), *P. parkeae* (Norris and Pearson, 1975), *P. virginica* (Pennick, 1977a), *P. cirolanae* (Pennick, 1982a), and *P. lunata* (Inouye *et al.*, 1983). *P. grossii*, *P. virginica* and *P. cirolanae* are small species (<9µm) and are clearly different from *P. pseudoparkeae*. *P. lunata* is a larger species like *P. pseudoparkeae* but has a distinctive lunate shape in lateral view. Of the trichocyst bearing species, *P. pseudoparkeae* and *P. parkeae* are impossible to distinguish under the light microscope.

In the name *P. pseudoparkeae*, the specific epithet conveys the similarity between this species and the older species, *P. parkeae*. The similarity in gross morphology between the two species also underlies similarities in ultrastructure. The internal organization of both cells is identical. It is only differences in scale structure that support the separation of these two species (see Table 2.2). Differences in scale structure are most pronounced in the B_2 scales although small differences are evident in the F_3 (limuloid) scales. The B_1 , B_3 , F_1 , F_2 and F_4 scales are identical.

The B_2 scales of *P. pseudoparkeae* have four prominent horizontal bars which arise midway along each side of the scale and fuse at the centre i.e. giving a cruciate pattern. Furthermore the rim of the scale is lattice-like having large "windows". By contrast the B_2 scales of *P. parkeae* do not have a cruciate pattern nor lattice-like rims, but rather bear many pores. The F_3 scales of *P. pseudoparkeae* are entire (except for the proximal apertures) and have only one lateral notch. This scale in *P. parkeae* has two lateral notches and is interrupted with many pores like the B_2 scales.



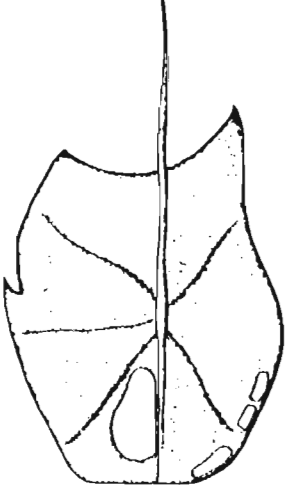


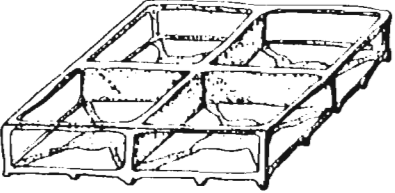
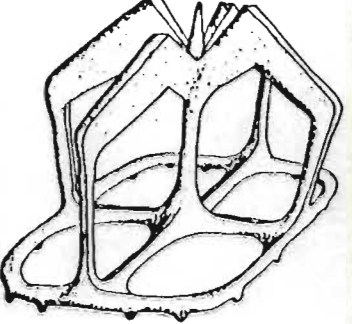

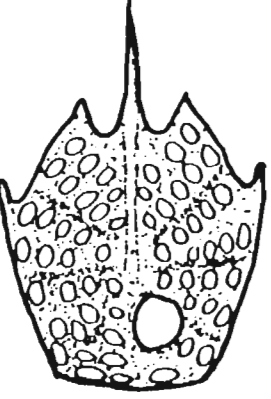

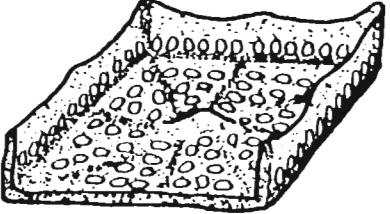
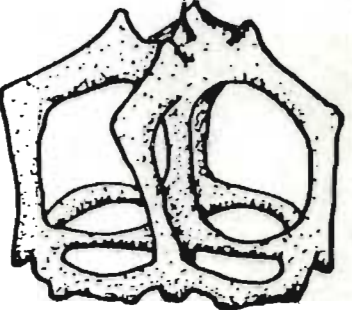
It is interesting that the prominent cruciate pattern seen in the B_2 scales of *P. pseudoparkeae* has been found in the same scale in only one other species, namely, *P. cirolanae* (Pennick, 1982a). Two additional features which emphasize the affinity between these two species are that they both possess trichocysts and have the B_1 scale-layer covering the whole cell surface. They are, however, separated by a large difference in size.

In comparison with the overall structural similarity between *P. pseudoparkeae* and *P. parkeae*, the differences in scale structure noted in Table 2.2 may appear trivial. This statement does not only apply to these two species but also to some of the smaller species. *P. obovata* (Pennick *et al.*, 1976) and *P. gorlestonae* (Pennick and Cann, 1982), for example, look identical under the light microscope and are only separated on the basis of small differences in scale structure.

At this point it may be appropriate to discuss the expediency of using scale structure as a diagnostic taxonomic character. The importance of scale structure for identifying and separating different species of

TABLE 2.2

A comparison of scale structure between *P. pseudoparkeae* and *P. parkeae*. Scales for *P. parkeae* redrawn after Norris and Pearson, 1975 (Drawings not to scale).

		FLAGELLAR SCALES				BODY SCALES		
		F ₁	F ₂	F ₃	F ₄	B ₁	B ₂	B ₃
P. PSEUDOPARKEAE								
			not shown		not shown			

Pyramimonas has been gaining support in recent years (Norris and Pienaar, 1978; Pennick *et al.*, 1978; Melkonian and Robenek, 1981). The most pronounced interspecific differences in scale structure have been observed in the B₂ and B₃ scales. Although these scales appear, therefore, to be potentially useful taxonomic characters for separating species, the diagnostic importance of B₃ scales has recently been disputed (Melkonian and Robenek, 1981) because they exhibit large variations between strains within a single species (Pennick *et al.*, 1978). The structure of B₂ scales is usually more consistent within species and they are thought to be more reliable diagnostic characters (Norris and Pienaar, 1978; Melkonian and Robenek, 1981). The B₁ scales of *Pyramimonas* have not received much attention in the past but Melkonian and Robenek (1981) have shown that they are structurally different in some species and may be taxonomically important.

Despite the emphasis placed on differences in scale structure in separating species in the genus, no studies have been conducted to test the constancy of scale structure under different environmental conditions. One of the aims of the growth studies presented in Chapter Three was to investigate the effects of a range of environmental conditions on scale structure. The results of this study are outlined here because they are relevant to this discussion. Scale structure in *P. pseudoparkeae* was remarkably constant in the salinity range 10‰ - 70‰, and at temperatures from 18°C to 30°C. Light intensities from 50 - 300 $\mu\text{E m}^{-2} \text{ s}^{-1}$ did not affect scale structure. It is important that the cells observed were in actively growing cultures. Deformed scales were only seen in old cultures (see Section 7.3.7). These aberrant scale types found on *P. pseudoparkeae* never resembled the scales found on *P. parkeae* or any other species. (The presence of deformed scales on old cells again demonstrates that actively growing cultures should be used when describing the micromorphology and ultrastructure of scale-bearing algae).

The results given above suggest that differences in scale structure between species are not environmentally induced phenotypic changes but rather that scale structure is a conservative character which reflects the genotype of the alga. If this is true then organisms with different scale types represent different genotypes. This presents an interesting problem that has direct bearing on the taxonomy of *Pyramimonas*; to what extent do genotypic differences (manifest by differences in scale morphology)

represent genetic variation within a species and what degree of genotypic divergence is required to warrant recognition of a new species? This question is addressed in Chapter Eight where possible solutions to the problem are proposed. It should be mentioned that the writer believes that the deformed scales in senescent cells of *P. pseudoparkeae* result from an aberration in the genetic constitution of the cells. Senescent cells are often pleomorphic and have two or more nuclei.

Pienaar and Aken (1985) were at first hesitant in describing *P. pseudoparkeae* as a new species because they were not satisfied that the differences in scale structure between this species and *P. parkeae* were valid for a separation at the species level. However, because the scales of *P. pseudoparkeae* are consistently different from those of *P. parkeae* under a range of environmental conditions, the alga was described as a new species (Pienaar and Aken, 1985). Further support for this separation comes from a comparative growth study using the two species (R.N. Pienaar and R.E. Norris, pers. comm.¹; Pienaar and Aken, 1985). Both species have different specific growth rates and different tolerances to temperature and salinity. The two organisms, therefore, have different physiological requirements which further suggests that there is an underlying genetic difference justifying their separation at species level.

The structure of the flagella of *P. pseudoparkeae* is identical to that described for other species in the genus. This is not surprising since the structure of the flagellum is remarkably constant among all flagellates (Cavalier-Smith, 1978). Differences in the structure of the flagellum are seen primarily in the transition region (Hibberd, 1979; Moestrup, 1982), and in this regard Manton (1965) was the first to realise the potential taxonomic value of small variations in flagellar structure in distinguishing different groups of plants.

The flagellar transition region in *P. pseudoparkeae* exhibits some departure from the "typical" flagellum described for *Chlamydomonas* EHRENBERG (Ringo, 1967). Most obvious is that *P. pseudoparkeae* does not have a well defined basal plate nor does it have the distal portion of the H-shaped structure in the transition region. Furthermore, the alga

¹ R.N. Pienaar and R.E. Norris, Department of Botany, University of Natal, Pietermaritzburg, Republic of South Africa.

possesses a transitional helix (TH)-like structure at the proximal end of the axonemal region of the flagellum.

If *Pyramimonas* represents an ancestral form which gave rise to chlorophycean algae (Moestrup and Ettl, 1979) then the absence of the distal portion of the H-shaped structure in the transition region in *Pyramimonas* would suggest that it evolved later in the flagella of chlorophycean algae. Its absence in other diverse groups of algae (see Moestrup, 1982) may suggest that this is a primitive condition.

Detailed observations on the TH-like structure of *P. pseudoparkeae* have failed to show that this structure is a coiled fibre. If it is coiled, then the gyres are so closely appressed that they are indistinguishable. By contrast the TH occurring in members of the classes Chrysophyceae, Xanthophyceae and Eustigmatophyceae (Hibberd, 1978b, 1979, 1980a, 1980b; Pienaar, 1980b) has loose coils that are easily seen. If the TH-like structure of *Pyramimonas* is homologous with the TH reported for chromophyte algae, then this will raise some interesting questions concerning the phylogeny of these flagellate algae. It would suggest that at least some members of the class Prasinophyceae have a common ancestry with supposedly remote chromophyte algae.

A TH-like structure has been reported for four species of *Pyramimonas*, namely, *P. orientalis* (Moestrup and Thomsen, 1974), *P. parkeae* (Norris and Pearson, 1975), *P. gelidicola* (McFadden *et al.*, 1982) and *P. pseudoparkeae* (Pienaar and Aken, 1985). This structure is also present in *P. obovata*, Plymouth strain No. 250 (Aken, unpublished) and may well occur in all species in the genus, but this needs to be confirmed. It is surprising that Melkonian (1981), in his detailed study of the flagellar apparatus of the same strain of *P. obovata*, did not report a TH-like structure. This structure is not visible in his micrographs but this could be explained by the plane of sectioning.

A TH-like structure is absent in *Nephroselmis* (Moestrup and Ettl, 1979) and has not been reported in other biflagellate prasinophytes. It has not been found in any chlorophycean, charophycean or ulvophycean algae.

The flagellar basal body apparatus is identical in *P. pseudoparkeae* and *P. parkeae*. When viewed in transverse section the basal bodies are arranged in a 3 over 1 pattern (refer to Text fig. 2.3). This arrangement of the basal bodies is found in a restricted group in the genus.

A second type of basal body arrangement, referred to as the rhombic pattern (refer to Text fig. 1.3) is known in some species (Moestrup and Thomsen, 1974; Melkonian, 1981; Inouye *et al.*, 1984; McFadden and Wetherbee, 1984). The two types of basal body pattern can be used to separate members of the genus into two distinct groups. Those species possessing the 3 over 1 pattern always have a curved synistosome (refer to Text fig. 2.3), whereas species with the rhombic pattern usually have a straight synistosome. It appears that species with a curved synistosome invariably possess trichocysts and have a complete covering of B_1 body scales. Species having a straight synistosome always lack trichocysts and usually do not have a complete covering of B_1 scales. A proposed phylogeny for the genus based, in part, on the appearance of the basal body apparatus is presented in Chapter Eight.

P. pseudoparkeae possesses one rhizoplast (striated rootlet, System II fibre) and in this respect resembles all other species in the genus. With the exception of *Tetraselmis*, which has two massive rhizoplasts, all other genera in the class Prasinophyceae also have one rhizoplast (Melkonian, 1980). The rhizoplast is invariably closely associated with the nucleus and occasionally the microbody too. Although the rhizoplast was thought to be a prasinophyte characteristic it has been found in some chlorophycean and ulvophycean algae (Melkonian, 1980). The rhizoplast is absent in the motile propagules of charophycean alga and land plants. Moestrup (1982) in his review of flagellar structure, has shown that algae from diverse classes possess a striated rootlet similar to the rhizoplast. Whether these are homologous with green algal rhizoplasts or not needs to be confirmed. *P. pseudoparkeae* does not have System I striated fibres associated with the ascending microtubular rootlets. These are usually restricted to members of the Chlorophyceae and Ulvophyceae (Melkonian, 1980).

P. pseudoparkeae has a 4-2-4-2 cruciate arrangement of microtubules in the ascending or superficial microtubular rootlets. This x-2-x-2 pattern

is common in motile Chlorophyceae, Ulvophyceae and Prasinophyceae (Melkonian, 1982a). The microtubular root system of *P. pseudoparkeae* is unusual because one root containing two microtubules becomes more complex after leaving the basal bodies. This root, referred to as a compound root because it acquires four additional microtubules, is always associated with the duct of the scale reservoir. The possible function of this root is discussed with reference to scale production in Chapter Five. A compound root such as this has not been found in any other species in the genus.

The chloroplast of *P. pseudoparkeae* is essentially similar to those of other species. Variations are only seen with respect to the manner in which thylakoids enter the pyrenoid. *P. pseudoparkeae*, *P. parkeae* (Norris and Pearson, 1975), *P. grossii* (Pennick and Clarke, 1976) and *P. cirolanae* (Pennick, 1982a) all have the thylakoids entering the pyrenoid laterally. Smaller members lacking trichocysts usually have the thylakoids entering the pyrenoid anteriorly. *P. occidentalis* (Pennick, 1982b) is unusual because it has a pyrenoid devoid of thylakoids. Usually only one pyrenoid is found in each cell but *Pyramimonas cruciata* CONRAD *et* KUFFERATH has four (Inouye *et al.*, 1983).

CHAPTER THREE

XENIC CULTURE STUDIES

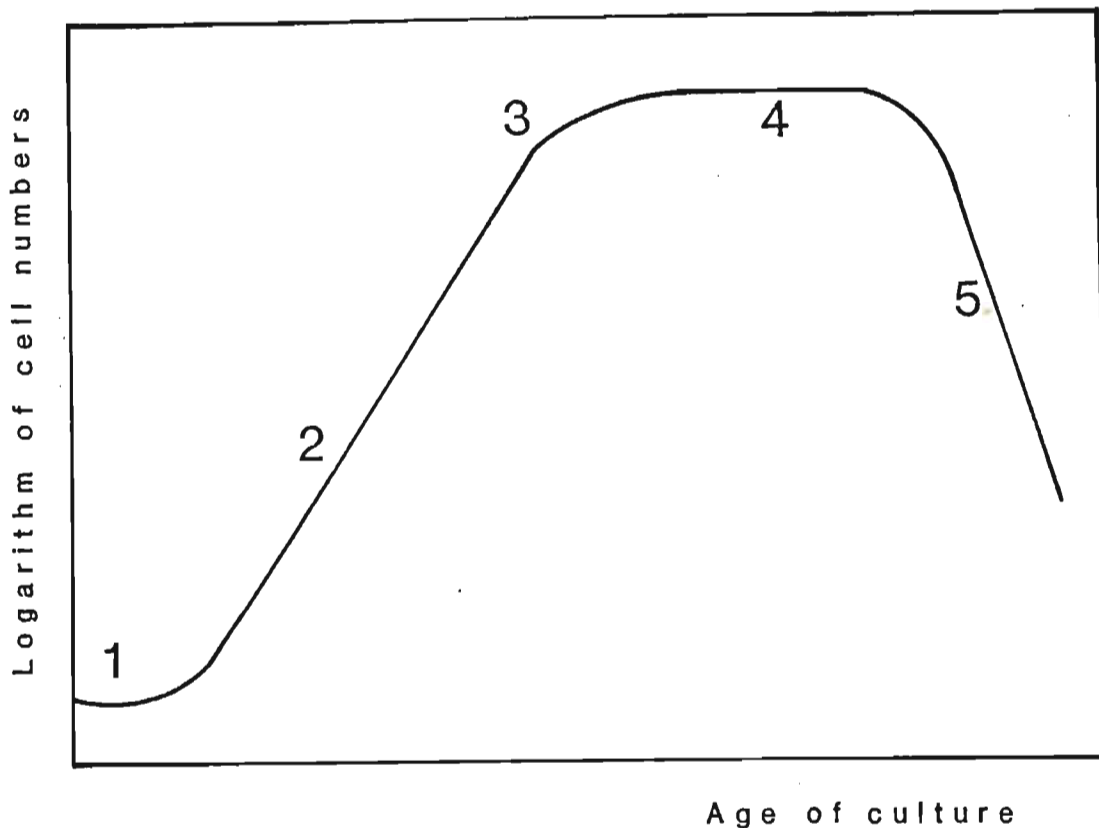
3.1 INTRODUCTION

This study was conducted with four objectives in mind. Most important was to attempt to improve the growth rate of *P. pseudoparkeae* so that good synchronous cultures could be obtained. An earlier study on the growth of the alga (Aken, 1978) revealed that cultures of the organism had a mean generation time of $\pm 33\text{h}$ when grown in PES medium at $18\text{ }^{\circ}\text{C}$, and at a light intensity of $50\ \mu\text{E m}^{-2}\text{ s}^{-1}$ measured during the light period in a 16h:8h photoregime. The generation time did not match the 24h period of the photoinduction cycle so that synchronous cell division was not observed. Synchronous cultures were needed for observations on cell division and scale production during the cell division cycle. A second objective of this study was to grow the alga in a number of enriched and artificial seawater media to be able to select one which could be used in all subsequent experiments. Artificial media were included in this experiment not only because they have a complex composition and could contain components enhancing growth, but also to forestall the need of repeating similar growth experiments should the need for a defined medium arise later in the study.

Two further objectives of this study were to test if scale structure remained constant under different culture conditions, and also to test if this range in culture conditions would provide a stimulus for the initiation of life-cycle stages.

The term xenic culture is used here to refer to a unialgal, clonal culture of *P. pseudoparkeae* which contains a natural population of bacteria.

All xenic culture experiments were conducted with cultures of limited volume i.e. batch cultures. Algal growth in batch cultures follows a sigmoid growth curve (Text fig. 3.1).



Text Fig. 3.1. The characteristic pattern of growth shown by a unicellular alga in a culture of limited volume. 1) lag phase, 2) exponential phase, 3) phase of declining relative growth rate, 4) stationary phase and 5) death phase (after Fogg, 1966).

The following discussion of the sigmoid growth curve is taken from Fogg (1966). The lag phase in cultures may be apparent or real. An apparent lag phase is produced when some of the cells inoculated into a culture are not viable. If all cells in an inoculum are viable a real lag phase may be caused by cells that are not in a condition to divide. If a parent culture, for example, is old, enzymes may have been inactivated and the concentrations of metabolites may have decreased to levels insufficient for cell division. A period of reconstitution is usually necessary before active growth will begin.

During the exponential growth phase, dividing cells produce more cells which are themselves capable of growth so that the actual growth rate accelerates continuously. This type of geometric growth is represented by the following expression:

$$N = N_0 e^{kt}$$

where

N = the cell number after a period of time t

N_0 = the cell number at time zero

e = the base of natural logarithms

k = the relative growth constant

t = the time interval during which N_0 increase to N

the relative growth constant (k) can be expressed as:

$$k = \frac{\log_e N - \log_e N_0}{t}$$

or alternatively it can be expressed to the base 10 as:

$$k' = \frac{\log_{10} N - \log_{10} N_0}{t}$$

From this the mean doubling time (G) of a culture can be determined as:

$$G = \frac{0,301}{k'}$$

G represents the number of days per division. G multiplied by 24 gives the number of hours per division.

The mean doubling time is equivalent to the mean generation time of the cells in a culture.

For a given species the relative growth constant is a function of temperature, light intensity and other environmental factors.

In cultures of limited volume exponential growth must cease when some factor becomes growth rate limiting. Exhaustion of nutrients in batch cultures commonly limits exponential growth but other factors such as the rate of diffusion of CO_2 into the culture from the air may become limiting unless the cultures are aerated. Other factors limiting exponential growth include, an alteration of pH by the preferential absorption of particular constituents of the medium, reduction in light intensity by self-shading, and autoinhibition. The duration of the period of declining relative growth depends on the nature of the limiting factor. Nutrient exhaustion or

autoinhibition usually result in an abrupt transition from the exponential to the stationary phase but if light is limiting a prolonged phase of linear growth may intervene.

The final yield attained in the stationary phase also depends on the nature of the limiting factor. If a nutrient is limiting it is to be expected that the yield will be proportional to the initial concentration of the nutrient. If autoinhibition has occurred, growth usually ceases when a particular concentration of cells is reached.

The onset of the death phase varies according to the species and the conditions of culture.

3.2 MATERIALS AND METHODS

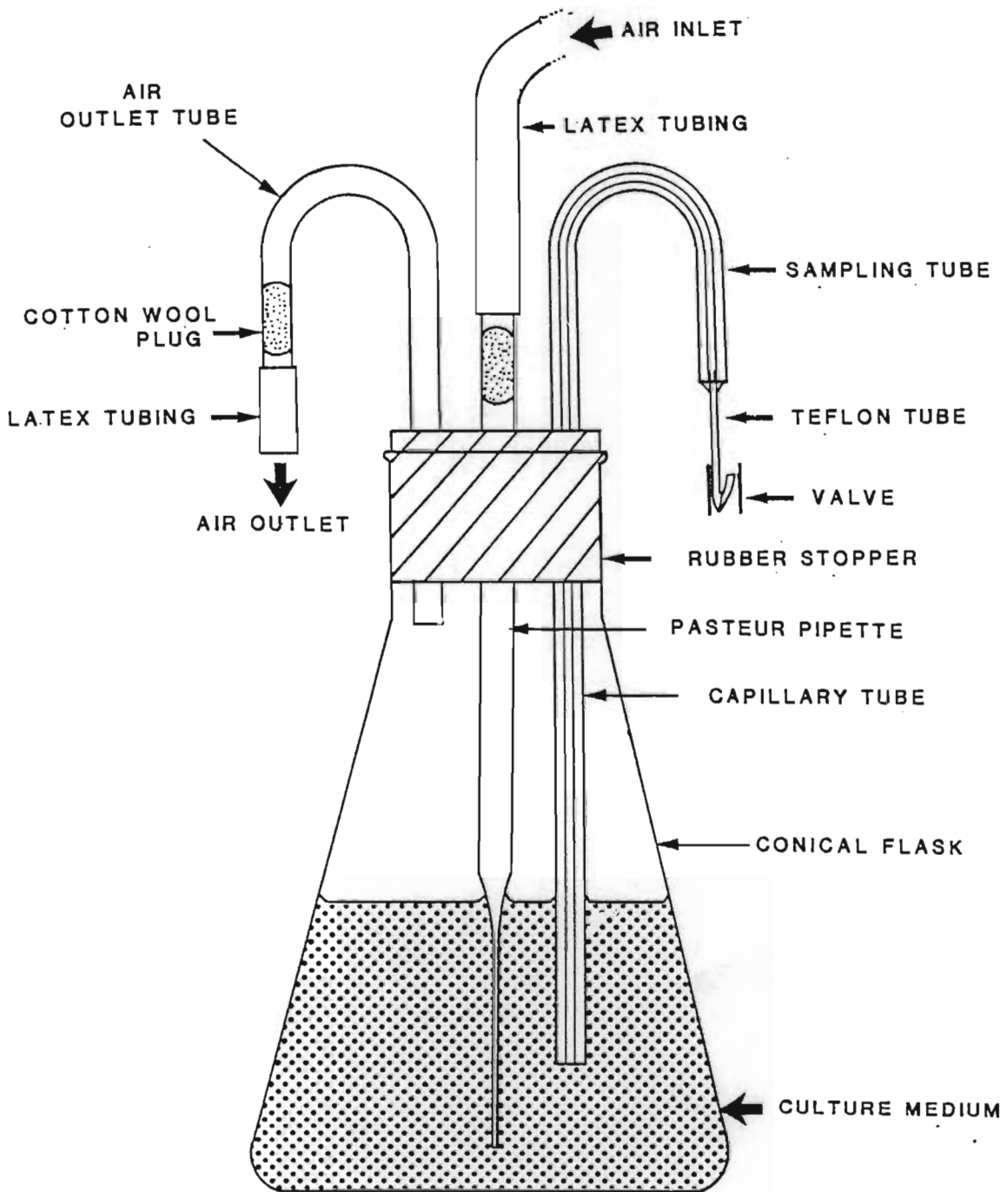
3.2.1 Culture apparatus

The culture apparatus used in all xenic culture experiments is described below.

Cells were cultured in 150 ml enriched or artificial seawater medium contained in 250 ml conical flasks. The culture vessel (see Text fig. 3.2) was sealed with a silicone rubber stopper which supported gas inlet and outlet tubes for aeration. The rubber stopper also supported a glass sampling tube.

Before commencing each growth experiment, washed culture vessels were sterilized to prevent contamination by other algae or foreign bacteria and fungi. Culture flasks containing the seawater medium were capped with aluminium foil and sterilized in an autoclave (10 min/1 bar/ 121 °C). The rubber stopper assembly was sterilized separately by being submerged in 95% ethanol for 10 min.

Sterilized culture media were allowed to cool and equilibrate under the desired culture conditions for at least 12h before being inoculated with cells. Inoculation and sealing of the culture flasks was performed under sterile conditions on a laminar flow bench. Three replicate culture vessels were used for each treatment under investigation.



Text fig. 3.2 Culture apparatus used in xenic growth experiments.

All cultures were aerated with a mixture of 2% CO₂ in air at a flow rate of 300 ml min⁻¹. Aeration served two functions; one was to keep the cells in suspension to reduce the inhibitory effects of self-shading, and the second was to provide sufficient CO₂ for the cells. The CO₂:air mixture, originating from a pressurized cylinder and compressor respectively, was passed through activated charcoal (to remove gaseous impurities), glass fibre wool (to trap dislodged charcoal particles), concentrated (33%) H₂SO₄ (to sterilize the gas), and distilled water (to moisten the gas) before being bubbled through the culture medium.

Samples were withdrawn from the culture apparatus (see Text fig. 3.2) by closing the latex sleeve on the air outlet tube and simultaneously opening the valve on the sampling tube. This produced an increased air-pressure above the culture medium causing the latter to be forced through the capillary sampling tube. When sufficient medium had been collected the valve was closed and the latex sleeve on the air outlet tube was opened. It is important to note that a small volume of the culture was held within the capillary tube between successive samplings. Encysted cells developed on the inner walls of the sampling tube and it is believed that conditions within the capillary tube triggered encystment. The life-cycle of *P. pseudoparkeae* is discussed in Chapter Seven.

3.2.2 Growth media

Seven growth media were used in the xenic growth experiments. These are given in Table 3.1

TABLE 3.1

Enriched and artificial seawater media used in the xenic culture studies

Medium	Source reference
<u>Enriched Seawater Media</u>	
Provasoli's enriched seawater-PES	Provasoli, 1968
Erdschreiber " "	Gross, 1937
F/2 " "	Guillard and Ryther, 1962
SWM " "	McLachlan, 1964

continued...

Artificial Seawater Media			
ASP-2	artificial	seawater	Provasoli <i>et al.</i> , 1957.
ASP-6	"	"	Provasoli <i>et al.</i> , 1957
Müller	"	"	Müller, 1962

These media, all of which have been used successfully for the cultivation of different unicellular marine algae, were chosen because they have different chemical constituents and because they vary with respect to the concentration of major salts, trace metals and vitamins which they contain. The recipes for these media, taken from McLachlan (1973), are given in Appendix A. High quality Analar chemicals were used in the preparation of all stock solutions. For convenience each medium was made up as three separate stock solutions, namely Stock 1 - Major salts

Stock 2 - Trace Metals

Stock 3 - Vitamins

In the enriched seawater media, aliquots of Stocks 1-3 were added to "standardized" seawater (see below), but in the artificial seawater media, Stock 1 formed the substitute seawater to which Stocks 2 and 3 were added.

Stock solutions were filter sterilized by passing them through a 0,45 μm Millipore membrane. The high concentrations of additives in these stock solutions cause precipitation if the solutions are autoclaved. The latter method of sterilization was therefore avoided. All stock solutions were stored in Pyrex glass bottles at 4°C until needed.

Seawater used in the enriched seawater media was either obtained from the Oceanographic Research Institute, Durban, or it was collected from various sites on the Natal coast. In the laboratory this was treated by adding 2,0g activated charcoal per litre of seawater. This mixture was agitated for 2h and then double filtered through Whatman's No. 1 filter paper. The charcoal reacts with the seawater and removes vitamins and other organic compounds that might be biologically active (Lewin and Lewin, 1960; Ryther and Guillard, 1962). Seawater treated with activated

charcoal is referred to as "standardized" seawater.

Seawater was heat sterilized by autoclaving for 30 min (1 bar/121°C). To prevent a precipitate forming, the seawater was reduced by diluting 900 ml charcoal treated seawater with 100 ml distilled water prior to autoclaving.

Sterilized seawater was stored in Pyrex glass bottles at 20°C until needed.

The required concentrations of nutrients from the stock solutions were then added to the seawater as required (see Appendix A).

3.2.3 Culture conditions

In all experiments, cells from actively growing stock cultures were inoculated into the experimental flasks. To prevent contamination, inoculation was performed under aseptic conditions on a laminar flow bench. A constant inoculum density was essential to obtain good correlation between three replicate cultures used for each treatment, and also to provide a sound basis on which results obtained between treatments, and in successive experiments, could be compared. Cell densities were determined daily from cell counts obtained on a Coulter Counter. Each growth experiment was conducted for 14 days. In one experiment cell density was determined every second day for 10 days.

The culture conditions used in three xenic culture experiments are described below.

Enriched/artificial seawater experiment

P. pseudoparkeae was grown in the seven different seawater media described in Table 3.1. All cultures were incubated in a "Convion" constant environment chamber at a temperature of 20°C and a light intensity of 100 $\mu\text{E m}^{-2}\text{s}^{-1}$. All cultures were subjected to a 16h:8h photoregime. Light intensity or Photosynthetic Photon Flux Density (PPFD) was measured with a LI-COR 185 B quantum radiometer-photometer fitted with a LI 192 SB sensor.

Salinity/Light intensity experiment

Cells were cultured in PES medium at a constant temperature of 20°C in a 16h:8h photoregime.

The different salinities, in parts per thousand ‰, in which the cells were grown were 10‰ 20‰ 35‰ 50‰ 70‰.

Cells grown in each of these salinities were cultured at three different light intensities, namely 200, 100 and 50 $\mu\text{E m}^{-2} \text{s}^{-1}$.

The desired light intensities were obtained by adjusting the shelf height in the growth chamber.

Salinities below that of normal seawater (35‰) were obtained by diluting natural seawater with distilled water. Higher salinities were obtained by allowing seawater to evaporate thus concentrating the natural salts. A Goldberg T/C refractometer was used to measure salinity. PES stock solutions were added to the seawater only after the required salinity had been obtained. All media were filter sterilized.

Temperature/Light intensity experiment

Cells were cultured in PES medium, the culture vessels being placed in two constant environment chambers - one set at 25°C and the other set at 30°C. In each experimental chamber cultures were exposed to two light intensities, namely 150 and 300 $\mu\text{E m}^{-2} \text{s}^{-1}$, and exposed to a 16h:8h photoregime.

3.2.4 Cell counts, growth rates and generation times

Cell counts

Cell densities in all cultures were computed from cell counts obtained using a Coulter Counter, Model ZB₁ (Coulter Electronics Inc., Florida, USA).

A 2,0 ml sample was withdrawn from a culture and diluted with 18 ml electrolyte (Isoton) supplied by Coulter Electronics. This electrolyte has the same ionic balance as seawater and prevents changes in the shape and volume of cells. The dilution ratio used proved to be suitable because it gave cell counts within the instrument's count range even when cultures reached their maximum yield. The diluted sample was well mixed and placed into the instrument which was fitted with a 100 μm aperture tube and calibrated according to the manufacturer's instructions.

As only 0,5 ml of the cell suspension was counted by the instrument and because the culture was diluted by a factor of ten, the cell count values obtained needed to be corrected by multiplying by 20 to obtain the correct cell density as cell number per millilitre (cell no. ml^{-1}). To avoid an overestimation of cell density, the mean of three background counts for pure Isoton was subtracted from each count recorded by the instrument.

All cell counts over 10 000 were corrected using a Coincidence Correction Table for 100 μm aperture, provided in the manufacturer's manual.

Three cell counts were obtained for each sample, the suspension being well mixed between successive counts to prevent cells from settling out.

All cell counts were converted to logarithmic values before growth curves were plotted to show changes in cell density (expressed as log cell no. ml^{-1}) with time (expressed in days). The cell density value plotted for each daily sampling represented the mean log value for three replicate cultures. The vertical bars placed over each mean represent the 95% confidence limits of the mean and give an indication of the variation in cell counts between replicate cultures.

Data for each growth treatment were subjected to an analysis of variance and comparisons between cell densities recorded at different times were made using the method of least significant difference - LSD (Parker, 1979).

Growth rate and mean doubling time

After plotting the growth of *P. pseudoparkeae* in different seawater media and under a range of environmental conditions, the period of exponential growth was assessed visually as that portion of the growth curve that was linear. A linear regression analysis was used to place a straight line through the cell count data for each replicate culture during the period of exponential growth. The relative growth rates (k') and mean doubling times (G) for the cells were computed from the linear regression equation computed for each of the replicate cultures. The relative growth rates and mean doubling times were calculated from the equations given by Fogg (1966 - see Introduction to this Chapter).

These data (k' and G) were subjected to a one-way analysis of variance (Parker, 1979) and comparisons between means for different treatments were made using the method of LSD.

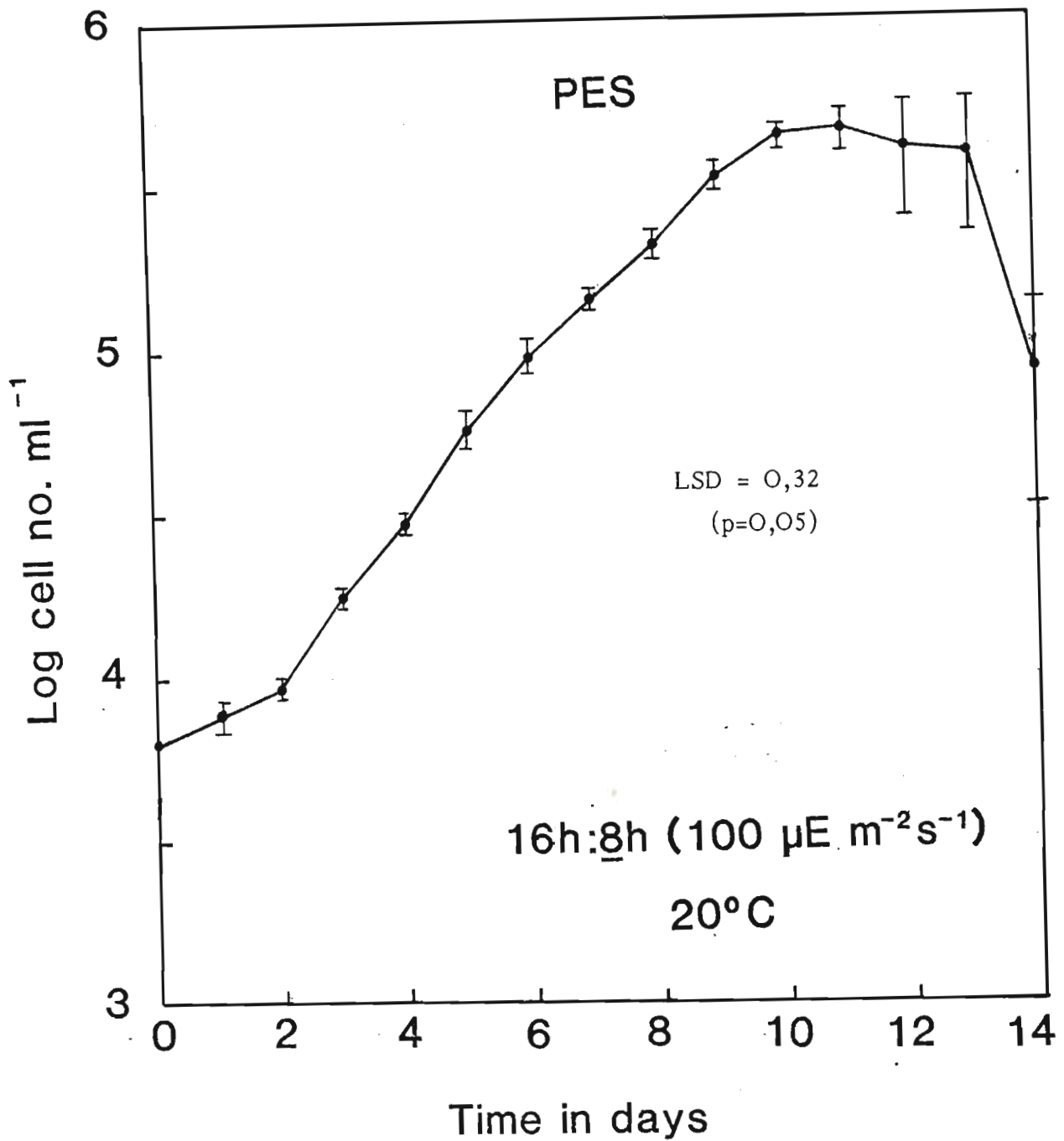
Where a linear regression equation is given for the exponential growth phase, this was computed from the means of three replicate cultures.

In one experiment where three replicate cultures were grown at each of two light intensities, k' and G were compared using the t-test (Parker, 1973).

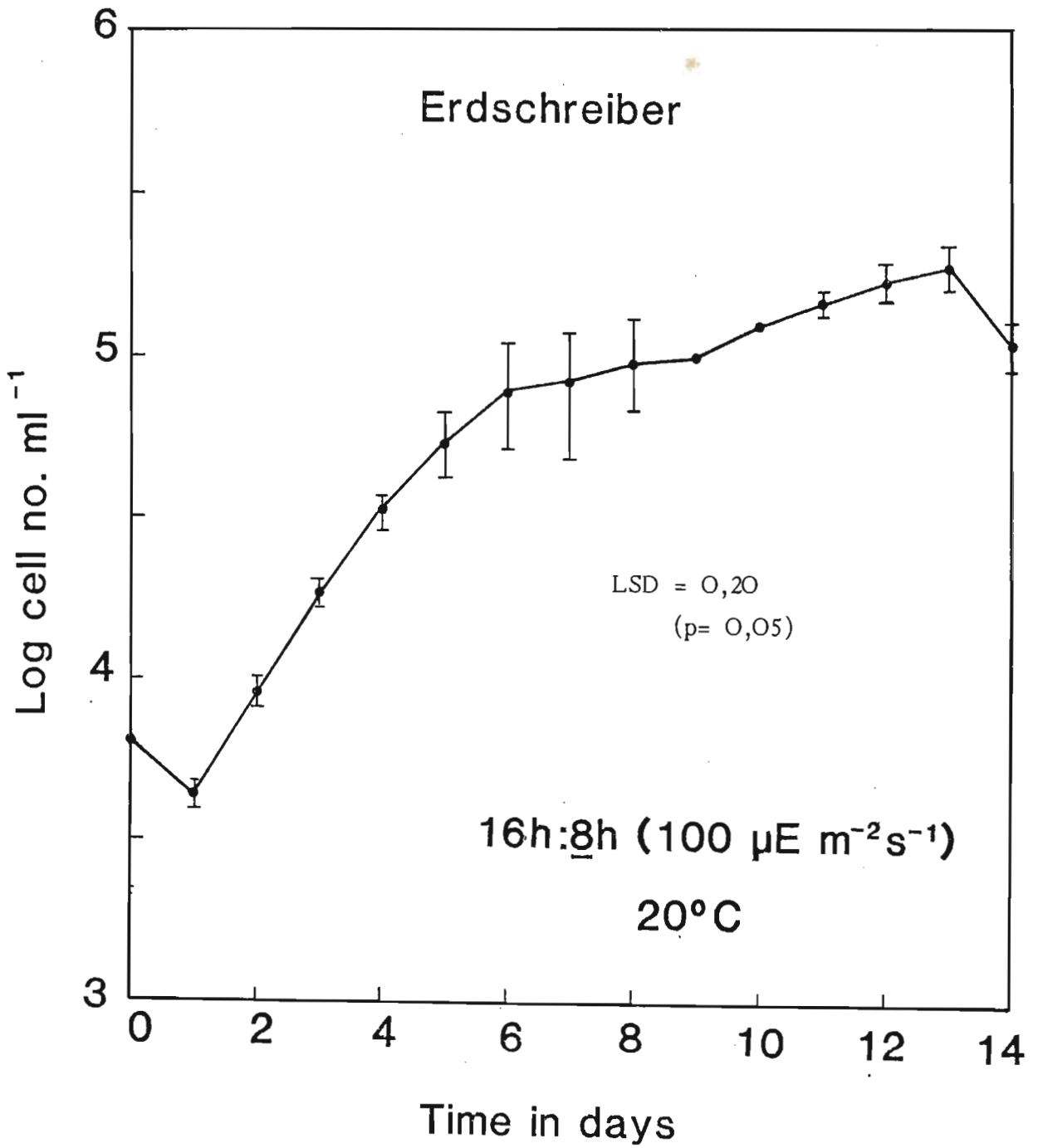
3.3 RESULTS

3.3.1 Enriched/artificial seawater experiment

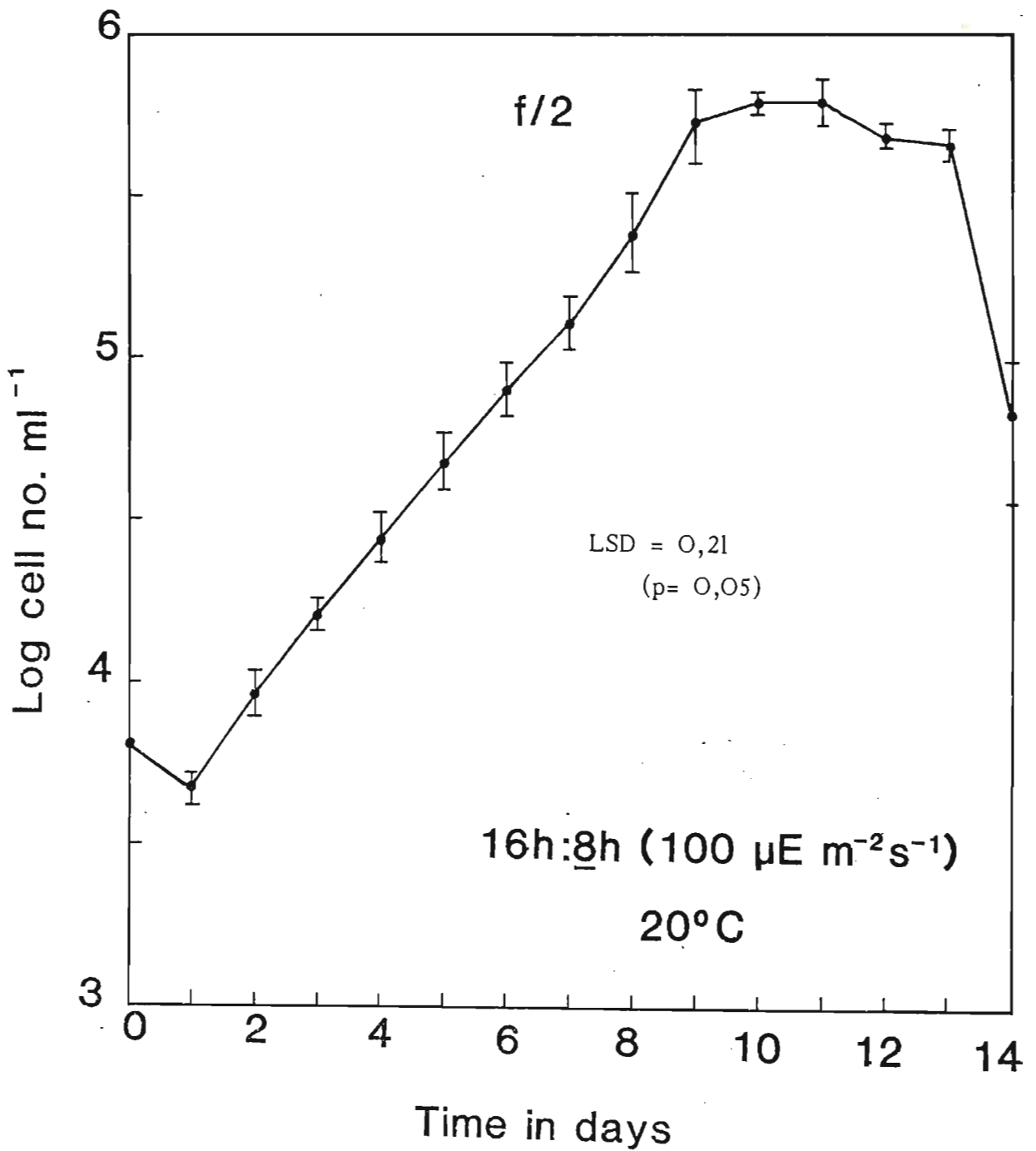
P. pseudoparkeae grew well in all seven enriched and artificial seawater media. The growth of the alga in these media is shown in the growth curves in Text figs. 3.3 - 3.9. All growth curves had the typical sigmoid shaped expected of batch cultures. Each curve is an average curve for three replicate cultures. The 95% confidence limits attached to each of the points along the curves give an indication of the variability in cell densities between replicate cultures at that time. Generally the differences in cell densities between replicate cultures were more pronounced as the cultures entered the stationary phase of growth. The



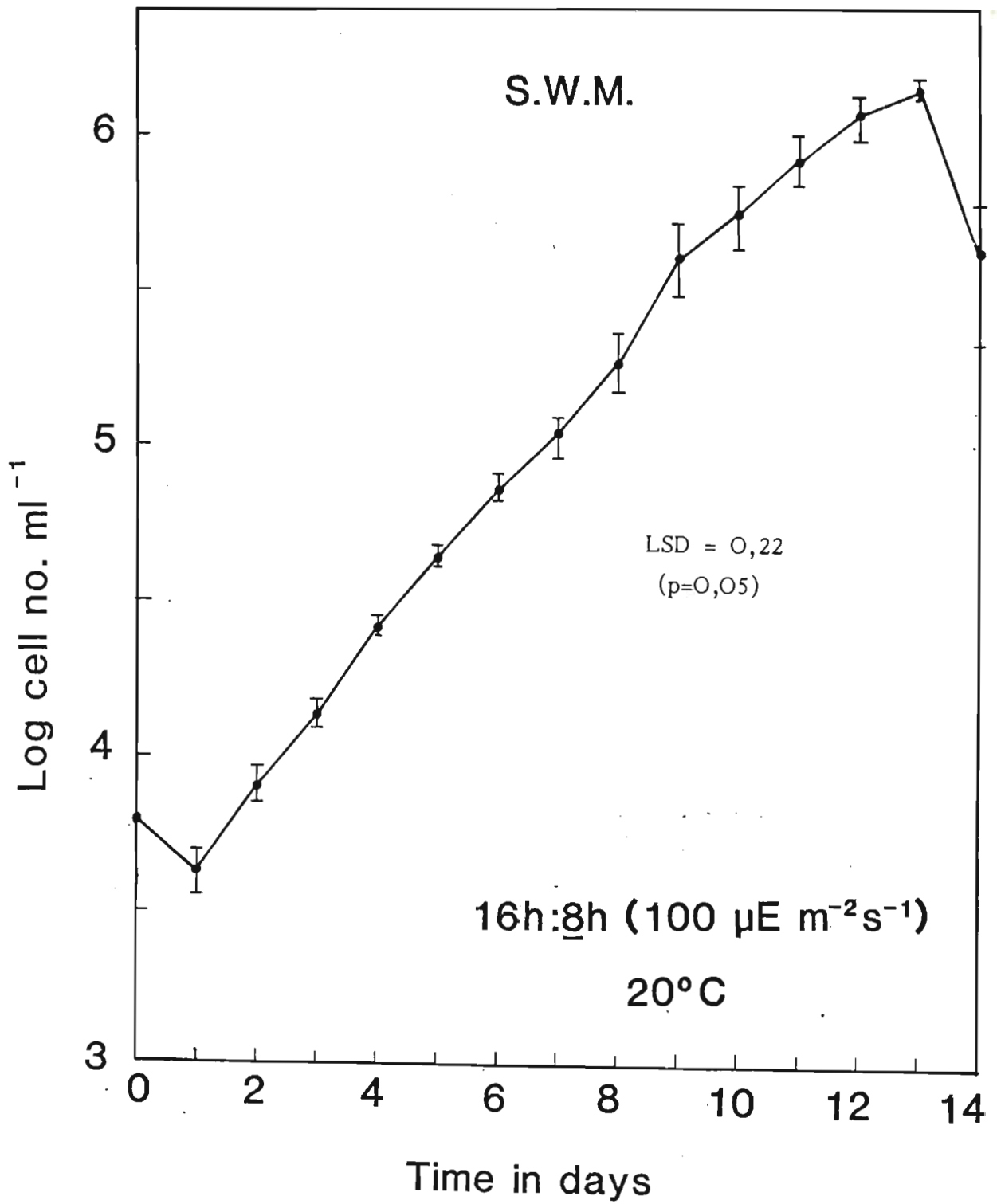
Text Fig. 3.3 The growth of *P. pseudoparkeae* in PES medium. The vertical bars represent the 95% confidence limits of the means for three replicate cultures (LSD = 0,32 log units).



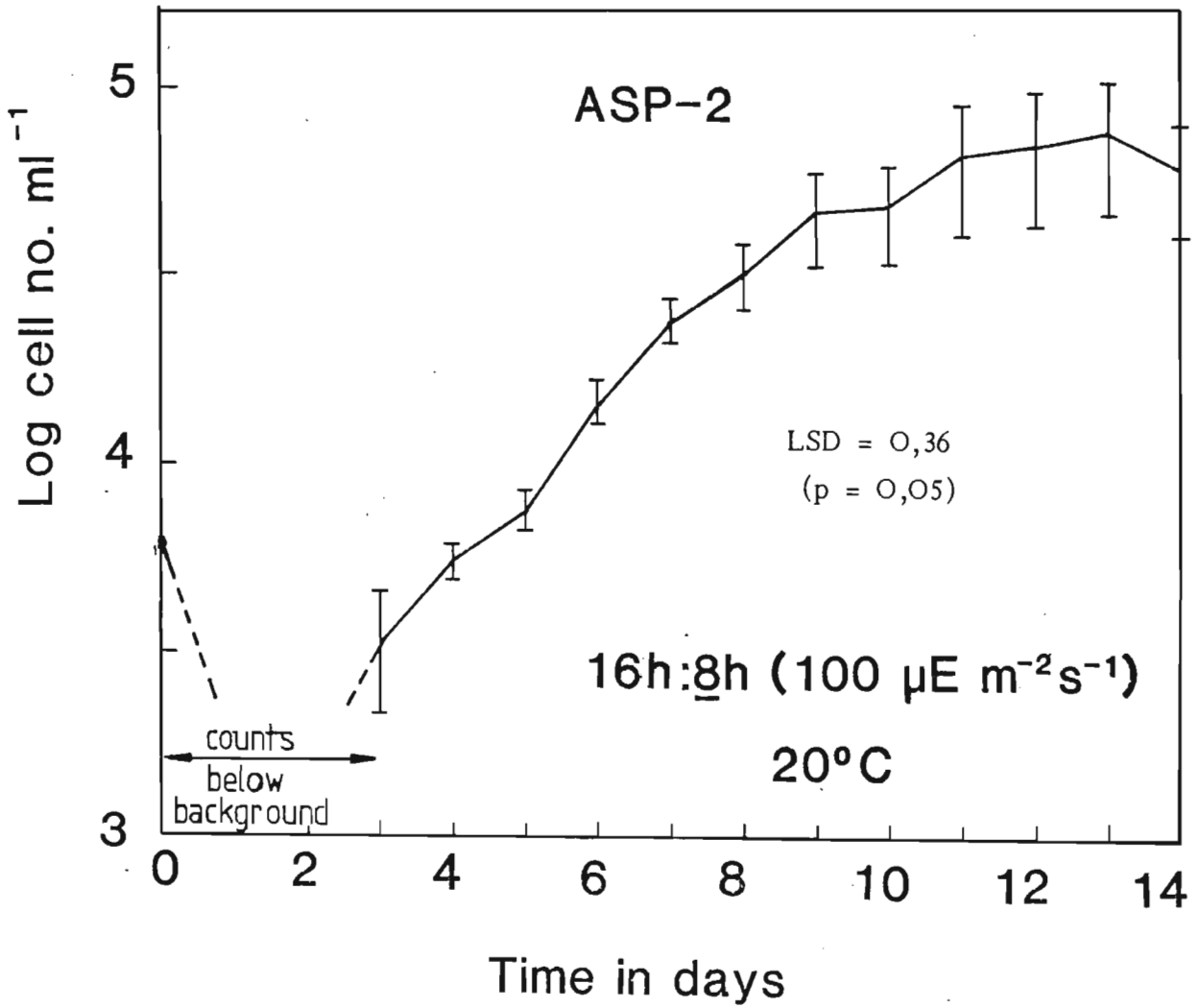
Text Fig. 3.4 The growth of *P. pseudoparkeae* in Erdschreiber medium.
(Vertical bars = 95% confidence limits).



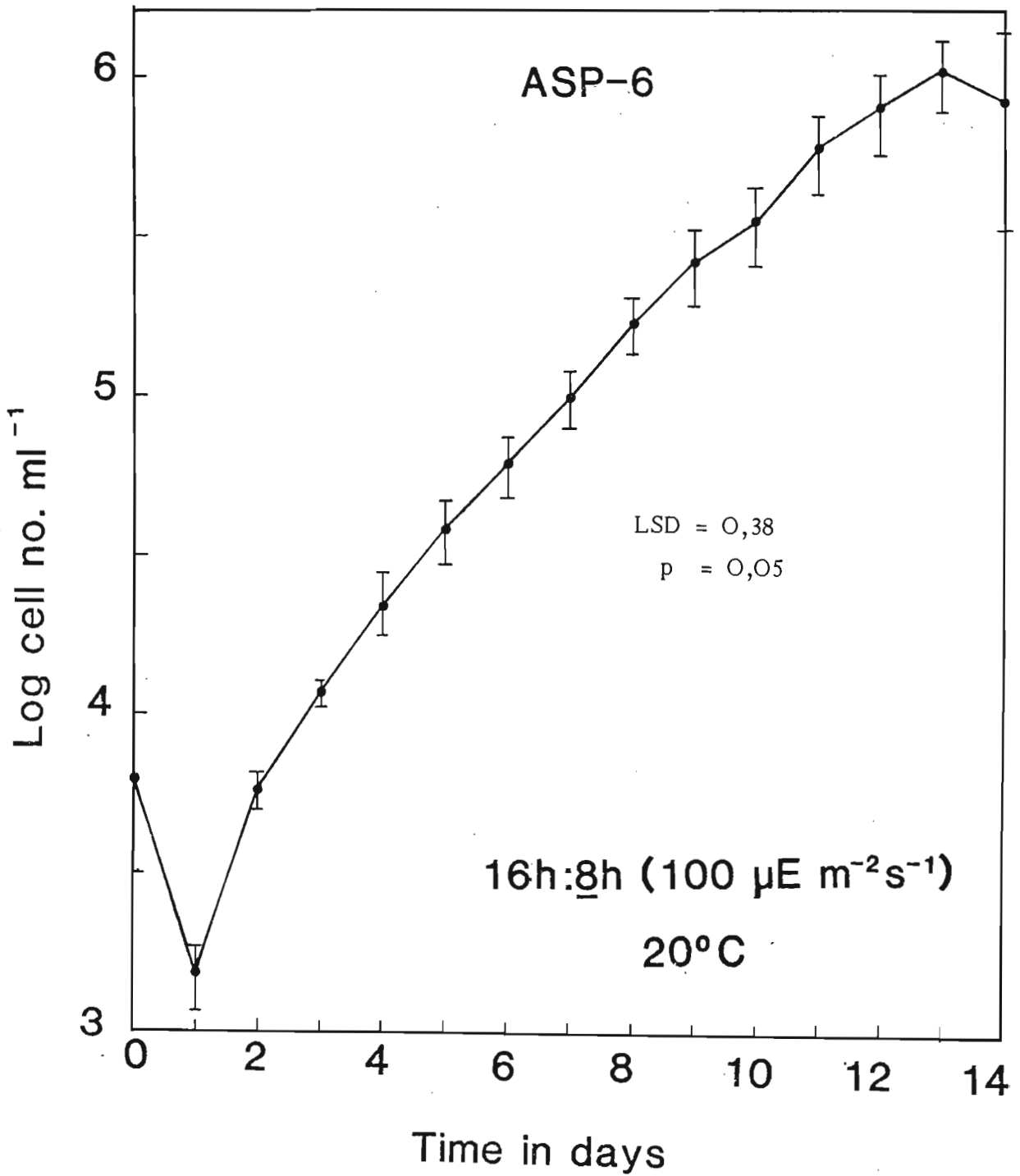
Text Fig. 3.5 The growth of *P. pseudoparkeae* in f/2 medium.
(Vertical bars = 95% confidence limits).



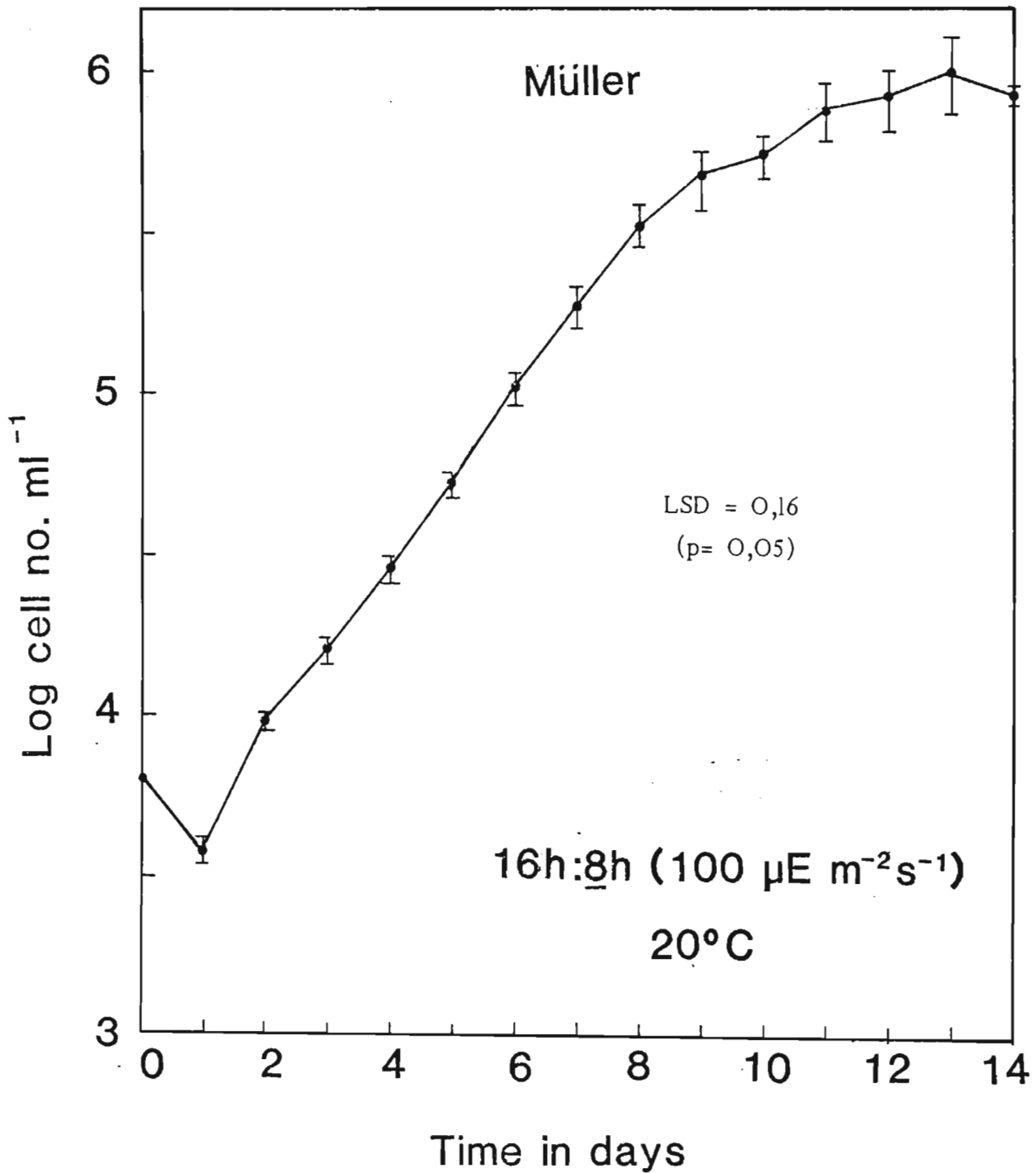
Text Fig. 3.6 The growth of *P. pseudoparkeae* in SWM medium.
(Vertical bars = 95% confidence limits).



Text Fig. 3.7 The growth of *P. pseudoparkeae* in ASP-2 medium.
(Vertical bars = 95% confidence limits).



Text Fig. 3.8 The growth of *P. pseudoparkeae* in ASP-6 medium.
(Vertical bars = 95% confidence limits).



Text Fig. 3.9 The growth of *P. pseudoparkeae* in Müller medium. (Vertical bars = 95% confidence limits).

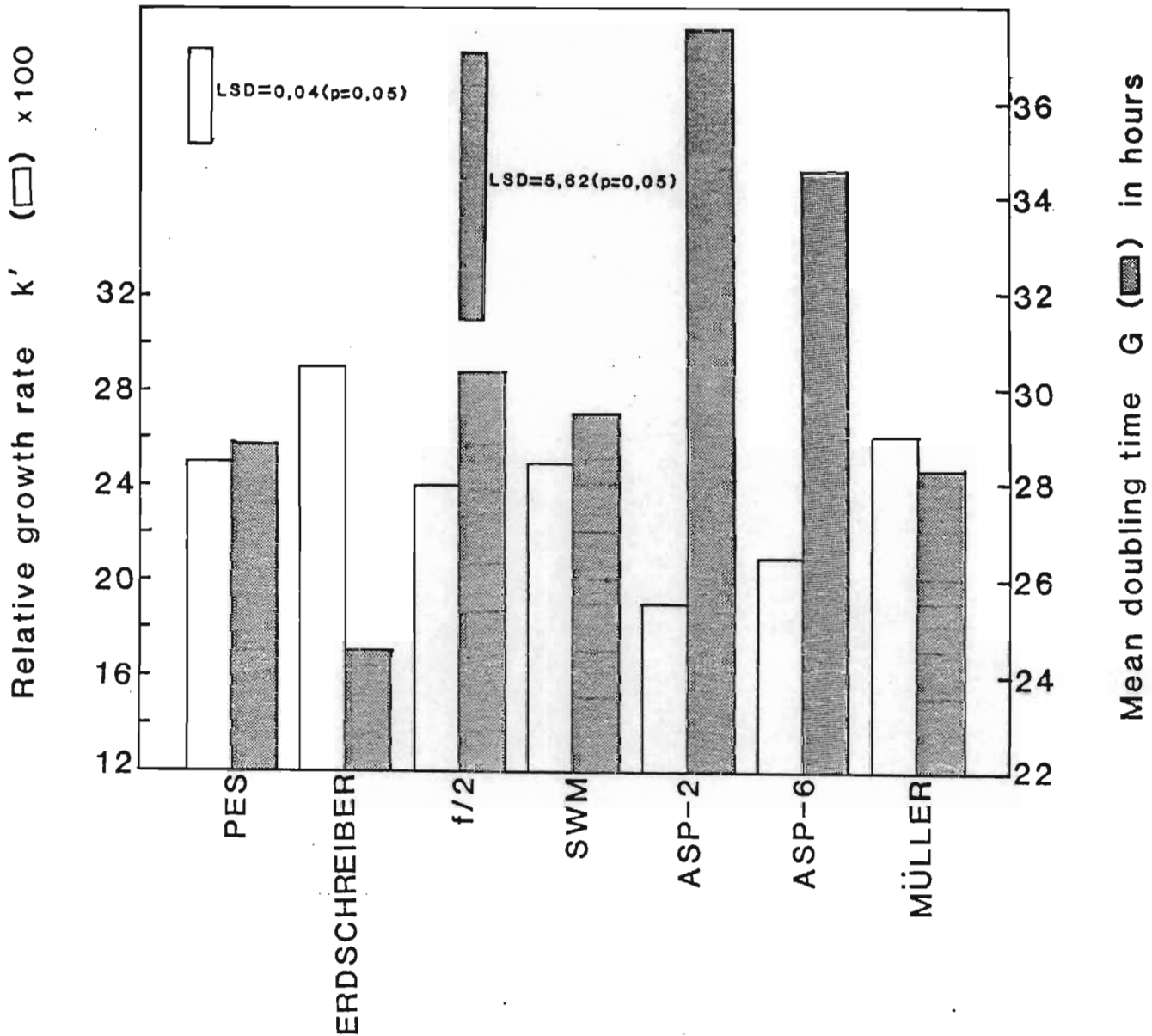
LSD value computed for each curve can be used to compare any two points (means) along the growth curve. Means differing by more than the LSD are regarded as being significantly different. The daily cell counts obtained for replicate cultures grown in the seven seawater media are given in Appendix C, Table I.

The relative growth rates (k') and mean doubling times (G) of cells cultured in seven different seawater media are summarized in the histogram in Text fig. 3.10.

The k' and G values for replicate cultures in each of the seawater media are given in Appendix C, Table II. Information on the start and duration of exponential growth, the linear regression equation describing exponential growth, and the mean values of k' and G for cells in the seven media are given in Appendix C, Table III. The pH and salinity measured in the different media both before and after autoclaving is given in Table IV (Appendix C).

In Text fig. 3.10 the relative growth rate was highest in Erdschreiber medium ($k' = 0,29$) but this was not significantly different from the lower k' values obtained for PES ($k' = 0,25$), SWM ($k' = 0,25$) and Müller medium ($k' = 0,26$). The relative growth rate in f/2 medium ($k' = 0,24$) was significantly lower than that measured in Erdschreiber medium but was not significantly different from that measured in PES, SWM and Müller media. The k' values measured in ASP-2 ($k' = 0,19$) and ASP-6 media ($k' = 0,21$) were not significantly different but were significantly lower than that measured in all other media.

The shortest mean doubling time of 24,54h was obtained in Erdschreiber medium while the longest mean doubling time of 37,67h was obtained in ASP-2 medium (see Text fig. 3.10). Despite the apparently large differences in the mean doubling time of cells in the different media, the large LSD value indicated that the small G value obtained in Erdschreiber medium was not significantly different from that obtained in PES, SWM and Müller medium (a range in G from 24,54h - 29,40h). Only f/2, ASP-2 and ASP-6 media had significantly larger values of G compared with Erdschreiber medium.



Text Fig. 3.10 A histogram showing the relative growth rates (k'), and mean doubling times (G), of *P. pseudoparkeae* in seven different seawater media.

There was no significant difference in the mean doubling time of cells in ASP-2 and ASP-6 media. The G values obtained for ASP-6, f/2 and SWM media were also not significantly different.

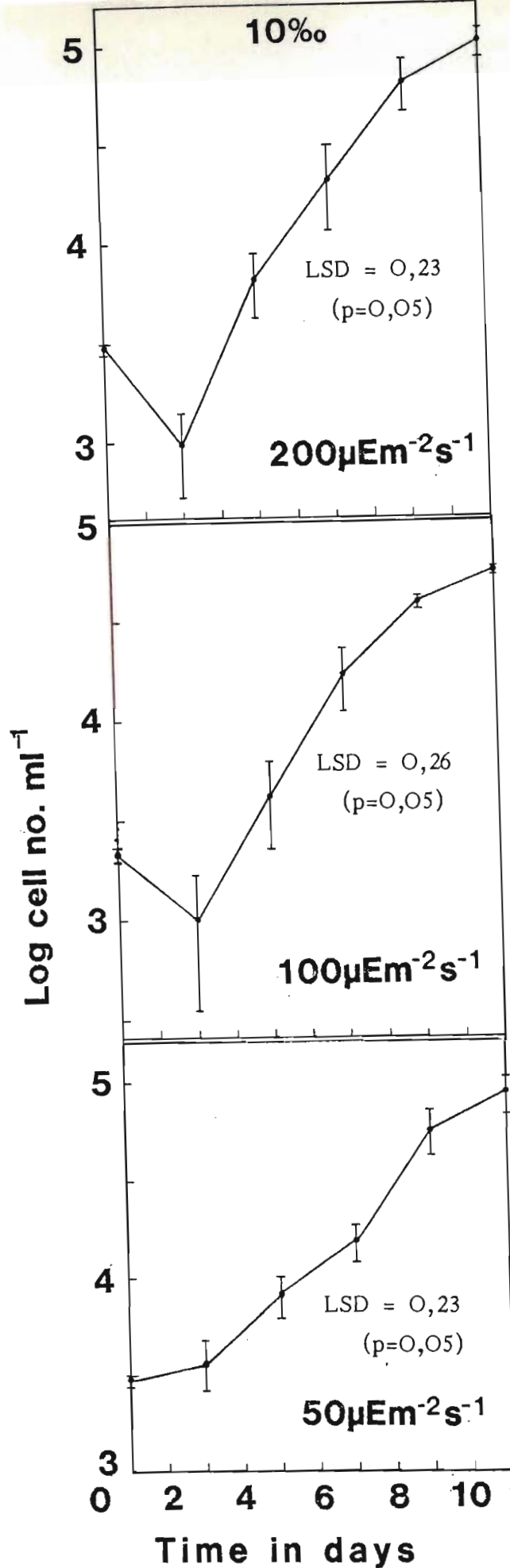
It will be recalled that one of the objectives of the present study was to select a suitable medium in which to culture *P. pseudoparkeae* and also to determine which culture conditions would bring the mean doubling time of cells close to 24h (i.e. the period of the 24h photoregime - 16h:8h). The shortest mean doubling time of 24,54h in Erdschreiber medium was not significantly different from that obtained in PES, SWM or Müller medium (giving a range in G from 25,54h to 29,40h). This meant that any of these media would be suitable for subsequent experiments. The ease with which PES medium is prepared made this the medium of choice for subsequent experiments. It is clear from the long generation times that parameters other than the type of medium used would need to be varied to shorten the mean doubling time.

All cultures used in the enriched and artificial seawater experiment produced non-motile encysted cells. It is believed that cells trapped in the capillary sampling tube of the culture apparatus (see Text. fig. 3.2) between daily samplings were induced to encyst. Many cells adhered to the inner surface of the sampling tube where they encysted. Other cysts, which collected on the bottom of the culture vessels, are thought to have developed from induced motile cells that had migrated out of the sampling tube. The life-cycle of *P. pseudoparkeae* is discussed in detail in Chapter Seven.

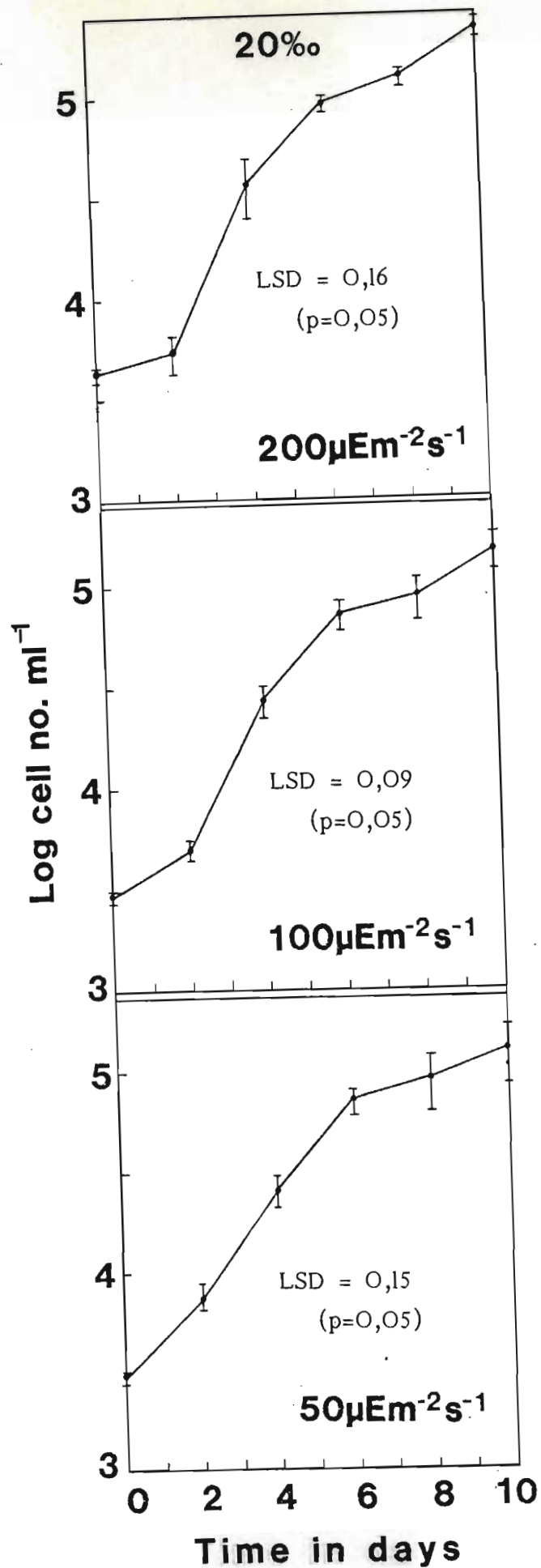
Electron microscopy of Au/Pd shadowed cells taken from the seven different seawater media revealed that scale structure remained constant. The various chemical compositions of the different seawater media therefore did not affect scale structure.

3.3.2 Salinity/light intensity experiment

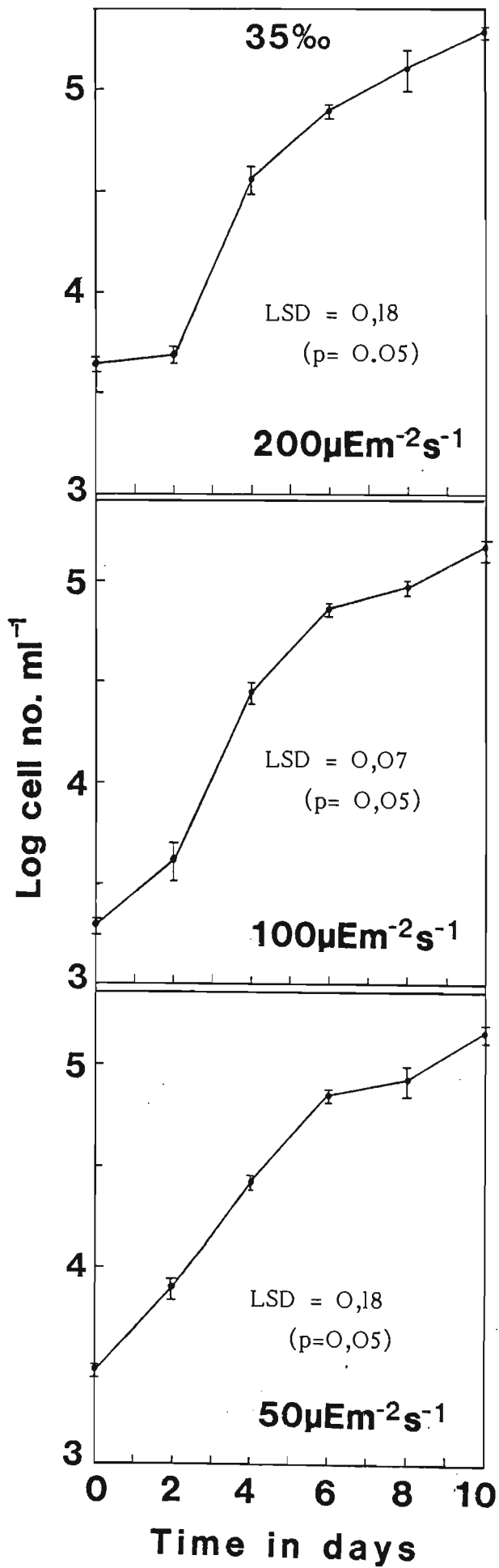
The growth of *P. pseudoparkeae* in five different salinities (10, 20, 35, 50 and 70‰) and three different light intensities (200, 100, 50 $\mu\text{E m}^{-2} \text{s}^{-1}$) is shown in the growth curves in Text figs. 3.11 - 3.15.



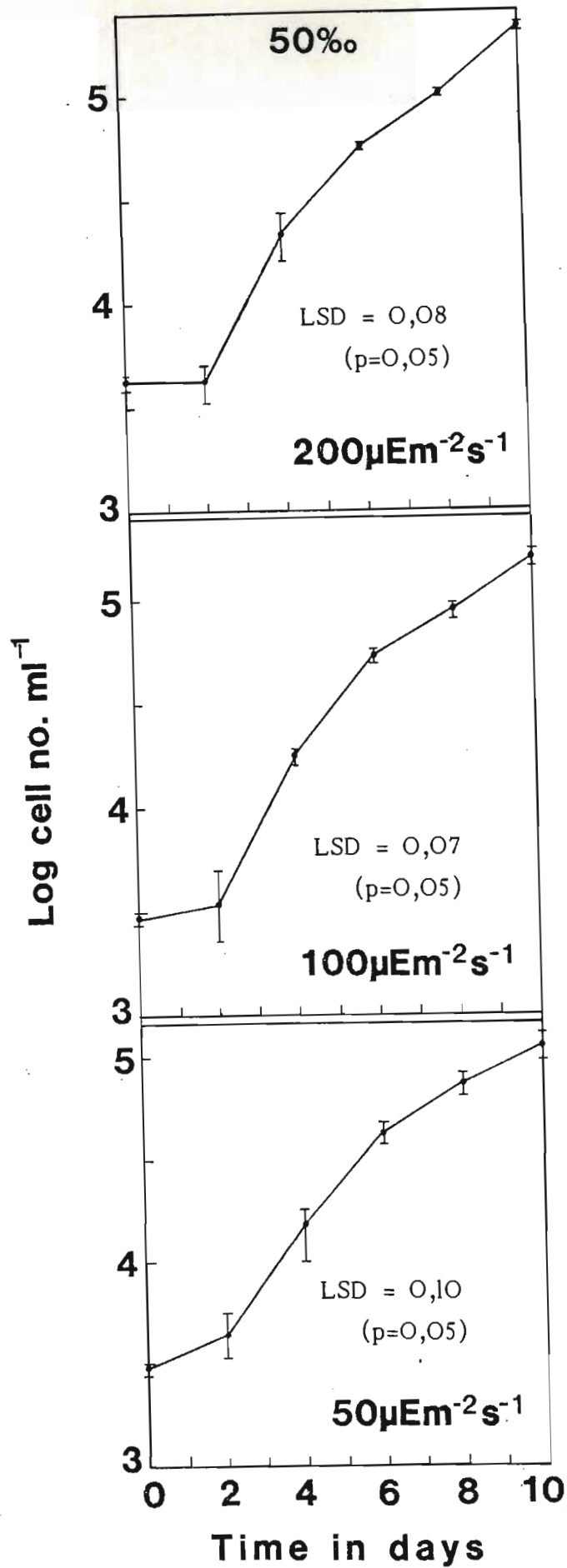
Text Fig. 3.11 The growth of *P. pseudoparkeae* in PES medium at three different light intensities with the salinity adjusted to 10‰. (Vertical bars = 95% confidence limits).



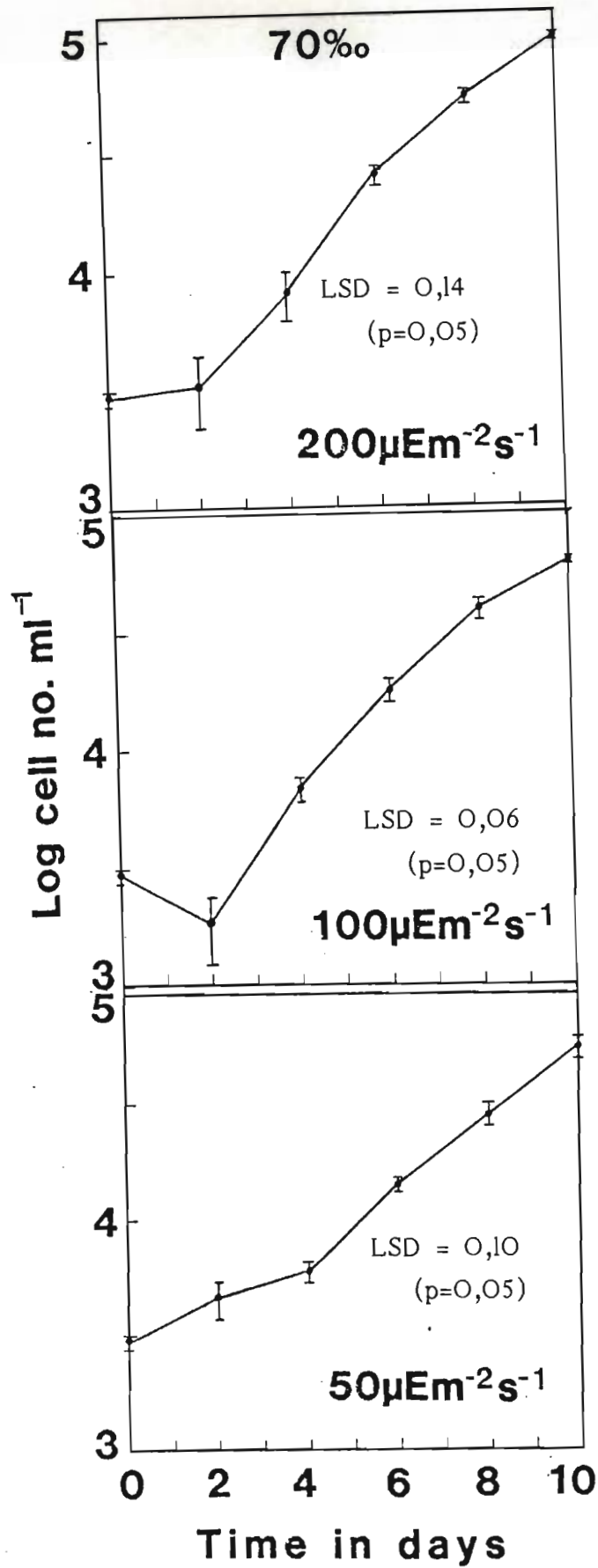
Text Fig. 3.12 The growth of *P. pseudoparkeae* in PES medium at three different light intensities with the salinity adjusted to 20‰. (Vertical bars = 95% confidence limits).



Text Fig. 3.13 The growth of *P. pseudoparkeae* in PES medium at three different light intensities in a salinity of 35‰. (Vertical bars = 95% confidence limits).



Text Fig. 3.14 The growth of *P. pseudoparkeae* in PES medium at three different light intensities with the salinity adjusted to 50‰. (Vertical bars = 95% confidence limits).



Text Fig. 3.15 The growth of *P. pseudoparkeae* in PES medium at three different light intensities with the salinity adjusted to 70‰. (Vertical bars = 95% confidence limits).

P. pseudoparkeae grew surprisingly well in all salinities at the three different light intensities.

The cell count data for this experiment are given in Appendix C Table V and the relative growth rates and mean doubling times for cells in replicate cultures grown in the different salinities and under the different light intensities are shown in Table VI in Appendix C. Table VII in Appendix C shows the start and duration of exponential growth and gives the regression equation representing the exponential growth phase; the table also includes average values for the relative growth rates and mean doubling times of cells grown under the different culture conditions; means can be compared using the LSD values given in Tables VIII and IX (Appendix C).

In referring to the growth curves in Text figs. 3.12 - 3.14 (i.e. 20, 35 and 50‰) it will be seen that there is no clear linear component representing the period of exponential growth at the two highest light intensities. This is undoubtedly the result of making too few samplings during the growth study. Daily samplings would probably have shown the linear component of the curves more clearly. Where the cells were grown at $50 \mu\text{E m}^{-2} \text{s}^{-1}$ the curves are more "linear" than at higher light intensities and the lag phase is not as pronounced as that seen in cultures at higher light intensity. To avoid subjectivity in choosing the start and period of exponential growth at the two higher light intensities in salinities 20‰ and 35‰, the start and duration of exponential growth was taken to be the same as that observed in $50 \mu\text{E m}^{-2} \text{s}^{-1}$ (see Table VII, Appendix C). In a salinity of 50‰, exponential growth was taken to have occurred throughout the 10 day growth period.

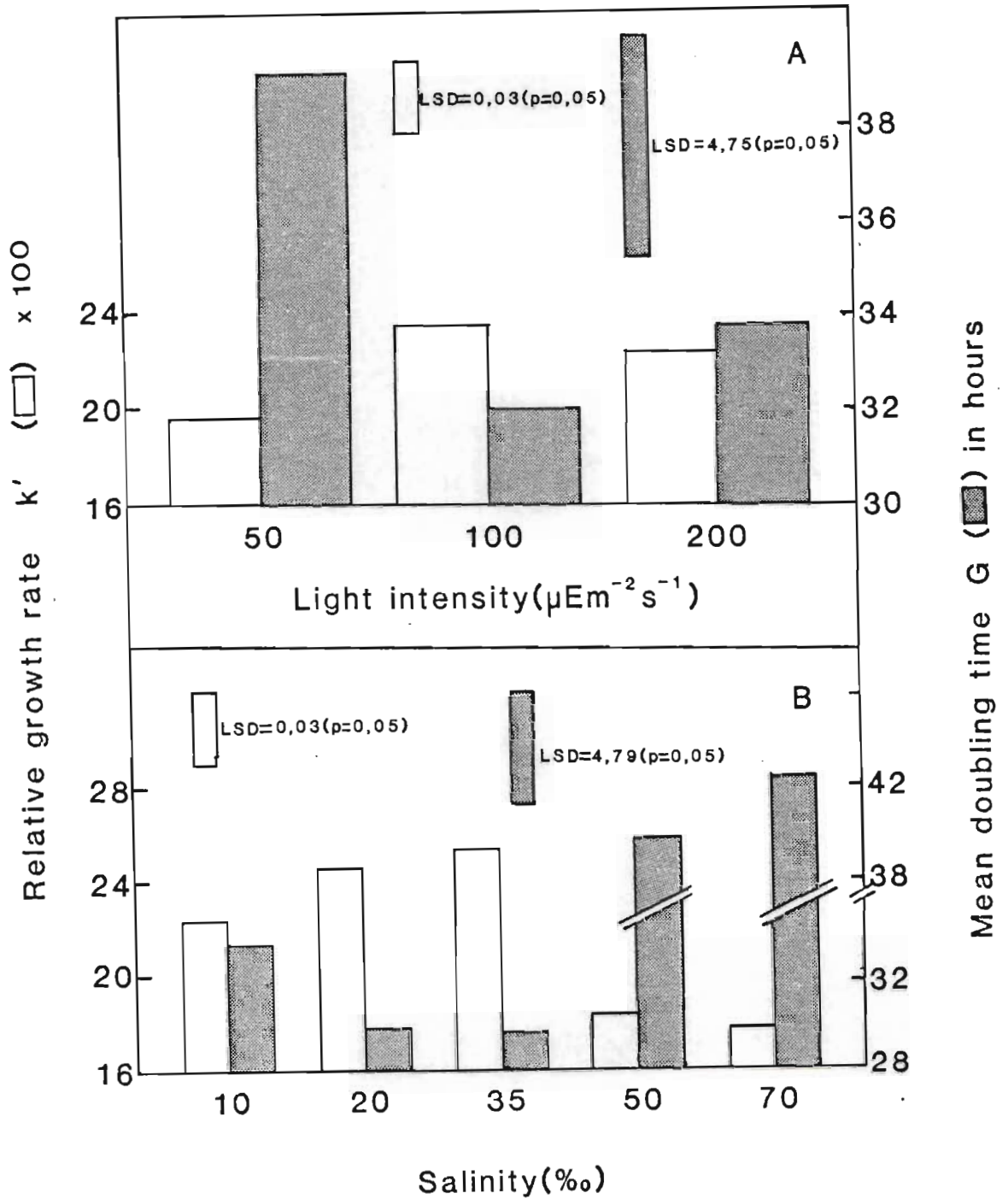
At salinities 10‰ and 70‰ the lag phase is evident at all light intensities. What is particularly interesting is that not all cells inoculated into these salinities were able to grow because exponential growth proceeds from a lower cell density than the inoculum density. In these cultures the period of exponential growth is taken from Day 2 - Day 10 (see Table VII, Appendix C).

The relative growth rates and mean doubling times of cells grown at three different light intensities and in five different salinities are summarized in the histograms in Text fig. 3.16. It must be emphasized here that the light intensity data presented in Text fig. 3.16 represents pooled salinity data. This means that comparisons between light intensity are made on pooled values of k' and G for the five different salinity treatments. Similarly, the salinity data represents pooled light intensity data. The mean values for relative growth rate and mean doubling time of cells for each permutation of light intensity and salinity are presented in Table VII; comparisons between these data can be made using the LSD values given in Tables VIII and IX. (Appendix C).

Text fig. 3.16 shows that raising the light intensity from 50 to 100 $\mu\text{E m}^{-2} \text{s}^{-1}$ significantly increased the relative growth rate of the alga and significantly reduced the mean doubling time of the cells. At 200 $\mu\text{E m}^{-2} \text{s}^{-1}$ the relative growth rate was reduced but it was not significantly lower than that obtained at 100 $\mu\text{E m}^{-2} \text{s}^{-1}$, nor was it significantly higher than that obtained at 50 $\mu\text{E m}^{-2} \text{s}^{-1}$. There was no significant difference in G at the two higher light intensities.

In Text fig. 3.16 the relative growth rate appeared to increase with increasing salinity from 10 - 35‰ but the k' values in these salinities were not significantly different. Relative growth rates were significantly lower in salinities 50‰ and 70‰ than at other salinities. The mean doubling times were progressively shortened by raising the salinity from 10 - 35‰ but the differences observed were not significant. In salinities 50‰ and 70‰ G was significantly longer than that computed for lower salinities.

In summarizing these results it can be said that the highest relative growth rate and shortest mean doubling time was obtained at a light intensity of 100 $\mu\text{E m}^{-2} \text{s}^{-1}$ and a salinity of 35‰. This interpretation, made on pooled data, is confirmed by looking at Table VII (Appendix C) where the highest growth rate ($k' = 0,28$) and shortest mean doubling time ($G = 25,8\text{h}$) was obtained at 100 $\mu\text{E m}^{-2} \text{s}^{-1}$ and a salinity of 35‰.



Text Fig. 3.16 Two histograms showing the relative growth rates (k'), and mean doubling times (G), of *P. pseudoparkeae* at three different light intensities (A), and in five different salinities (B).

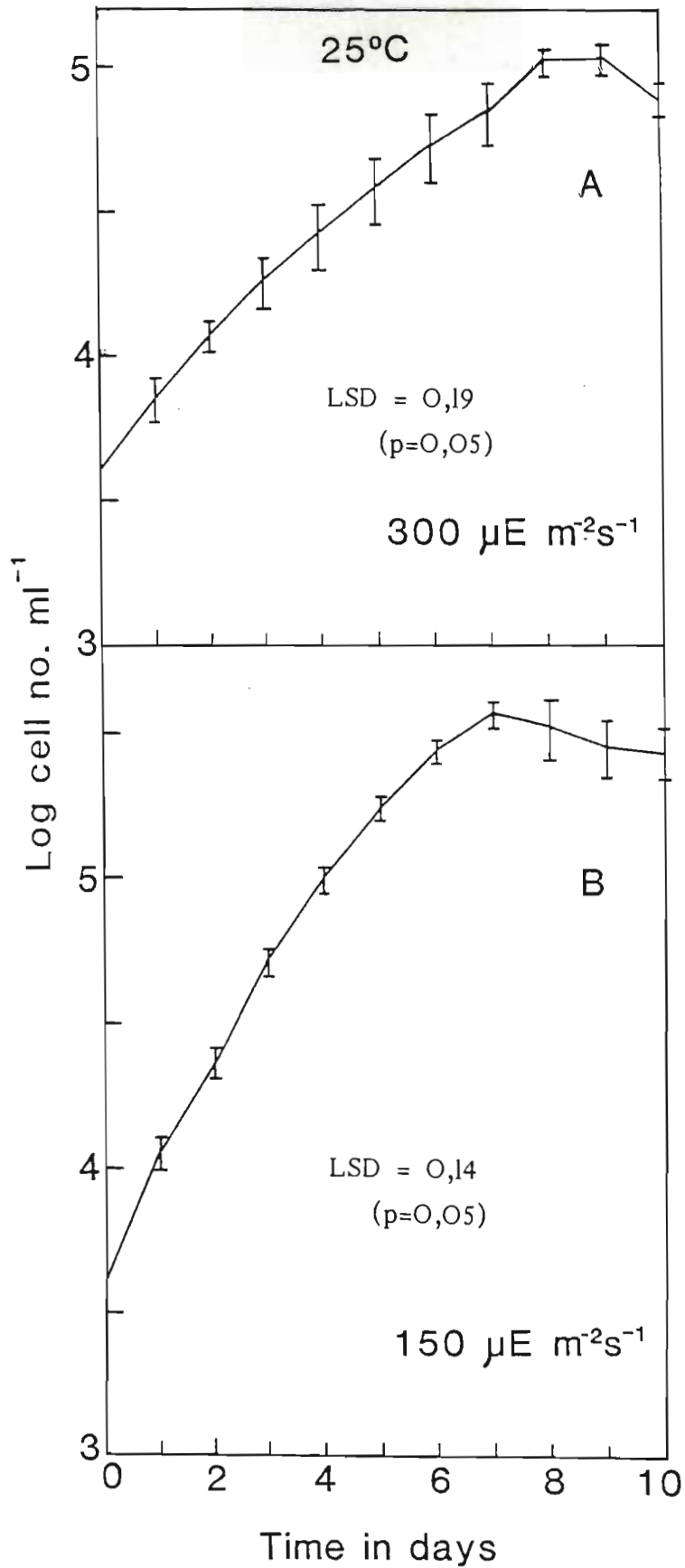
Electron microscopical examinations made on Au/Pd shadowed cells from all cultures in the salinity/light intensity experiment showed that scale structure remains constant and is not affected by changes in salinity or light intensity.

3.3.3 Temperature/light intensity experiment

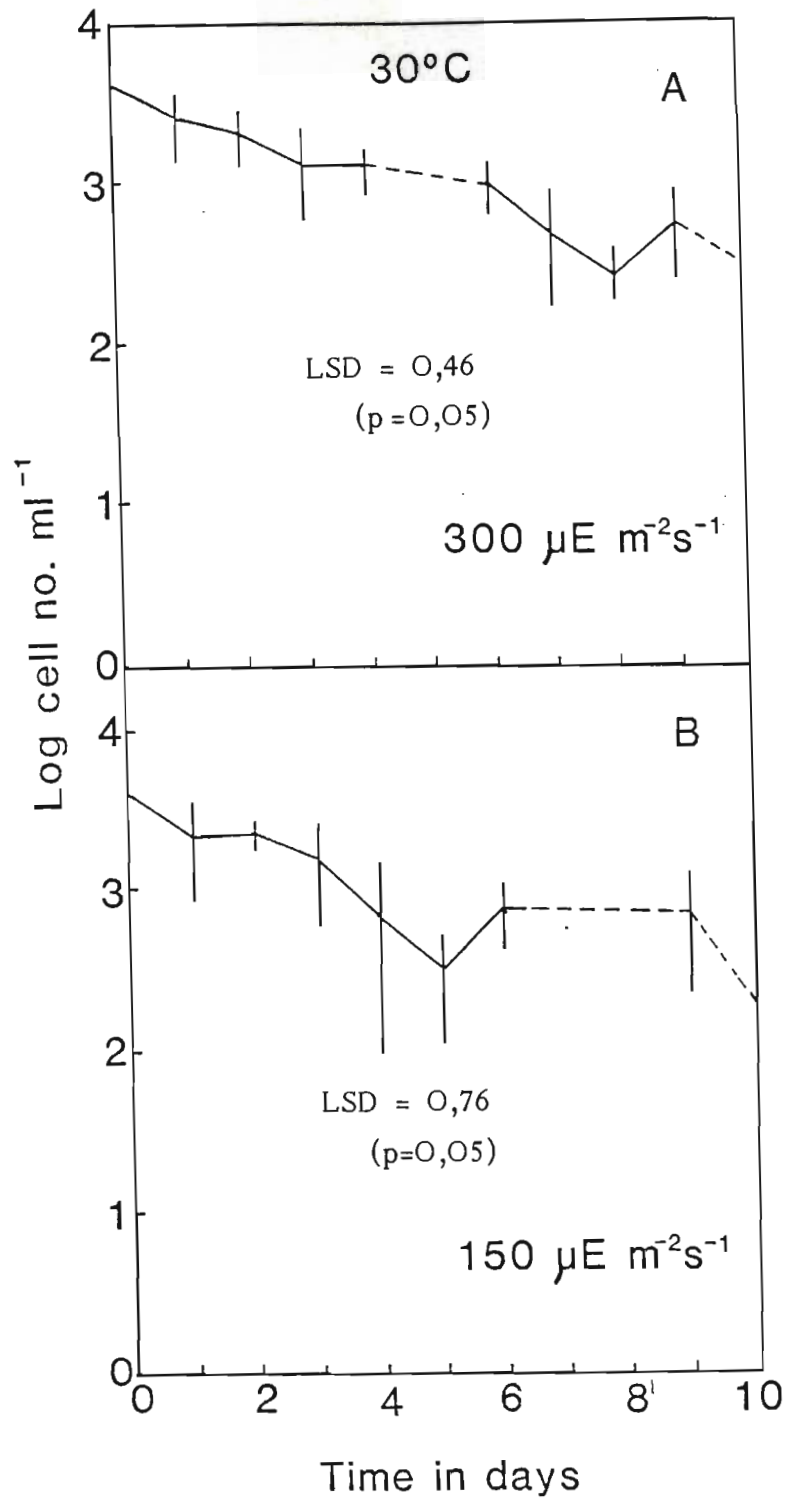
The growth of *P. pseudoparkeae* at 25°C and 30°C at two different light intensities (300 and 150 $\mu\text{E m}^{-2}\text{s}^{-1}$) is shown in Text figs. 3.17 and 3.18) respectively. The cell count data for this experiment are given in Table X in Appendix C. The relative growth rates and mean doubling times for cells in replicate cultures under the different culture conditions are given in Table XI in Appendix C. Table XII (Appendix C) shows the start and duration of exponential growth and gives the regression equation representing the exponential growth phase; this table also includes average k' and G values for cells grown under the different culture conditions.

The relative growth rate and mean doubling time results for this experiment are summarized in the histogram in Text fig. 3.19. Most striking is that the higher temperature (30°C) had a deleterious effect on *P. pseudoparkeae* with the result that the cultures had a negative growth rate. A regression line placed through the cell density data recorded during the decline in cell numbers revealed that the cultures were reduced to half the cell density every 66,21h at a light intensity of 300 $\mu\text{E m}^{-2}\text{s}^{-1}$ and every 49,65h at 150 $\mu\text{E m}^{-2}\text{s}^{-1}$. Comparisons made between the k' or G values at 30°C at both light intensities were made using the t-test; the computed t values presented in Text fig. 3.19 (with the relevant degrees of freedom) indicate that there is no significant difference in k' and G between the two light intensities at 30°C.

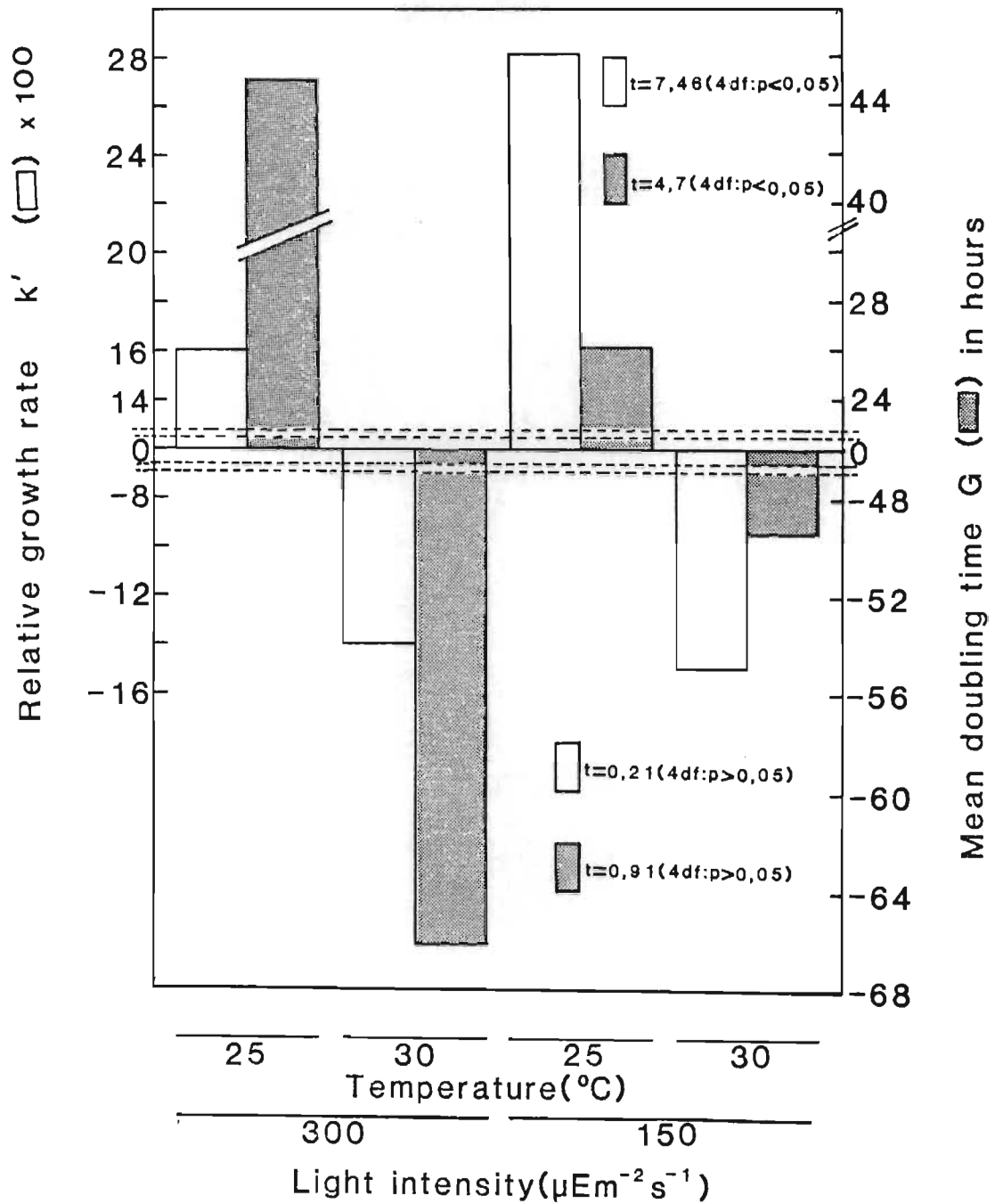
At 25°C the alga grew best at 150 $\mu\text{E m}^{-2}\text{s}^{-1}$ with a relative growth rate of 0,28 and a mean doubling time of 26,11h. At 300 $\mu\text{E m}^{-2}\text{s}^{-1}$ the relative growth rate was significantly lower and the mean doubling time was significantly longer. These data were compared using the t-test; the computed t values for a comparison of k' and G between two light intensities at 25°C are presented in Text fig. 3.19 with the relevant degrees of freedom.



Text Fig. 3.17 The growth of *P. pseudoparkeae* in PES medium at 25°C and at two different light intensities. (Vertical bars = 95% confidence limits).



Text Fig. 3.18 The "growth" of *P. pseudoparkeae* in PES medium at 30°C and at two different light intensities. (Vertical bars = 95% confidence limits).



Text Fig. 3.19 A histogram showing the relative growth rates (k') and mean doubling times (G) of *P. pseudoparkeae* at two different temperatures and two different light intensities.

Observations on heavy metal shadowed cells taken from the different cultures in the temperature/light intensity study revealed that scale structure remained constant. Even at 30°C, which was deleterious to the growth of the cells, the scales remained unchanged.

A few encysted cells were observed on the bottom of all culture vessels and they were used for studies on the life-cycle of the alga.

3.4 DISCUSSION

The results of the xenic growth studies revealed some interesting facts about the growth of *P. pseudoparkeae*.

The growth experiment using enriched and artificial seawater media indicated that the alga is persistent and will grow well in any of the media used. PES medium was chosen to be used in all subsequent studies for three reasons. Firstly, the medium is relatively easy to prepare and does not readily form a precipitate when autoclaved. Two enriched seawater media (f/2 and SWM medium) and all three artificial seawater media (ASP-2, ASP-6 and Müller medium) formed a heavy precipitate when autoclaved and should rather be filter sterilized. The high concentrations of salts in these media are undoubtedly responsible for the precipitates formed during autoclaving. The second reason why PES medium was chosen was because the medium supported a relatively small bacterial population. The other media, which contained higher concentrations of organic additives, supported a large bacterial fauna. Though the bacteria did not obviously affect the growth of the alga they did lyse young encysting cells on the bottom of the culture vessels. Because these cells were required for developmental studies on the life-cycle of the alga (Chapter Seven) the media which supported large bacterial populations were rejected in favour of PES medium. The third reason for choosing the latter medium was because it gave one of the highest growth rates and consequently one of the shortest doubling times for *P. pseudoparkeae*.

Although *P. pseudoparkeae* was able to grow in all seven seawater media, ASP-2 and ASP-6 media gave lower relative growth rates.

Growth of the alga in Müller medium was as good as that obtained in enriched seawater media and would be a good medium to use if a study demanded the use of a defined medium.

P. pseudoparkeae is extremely euryhaline and was able to grow successfully in salinities ranging from 10 to 70‰. This is not unexpected because the alga inhabits tidal pools and would have to tolerate high salinities especially where the tidal pool is only flushed by spring tides and would be subjected to an increase in salinity caused by evaporation. Conversely the salinity in the tidal pools could be rapidly reduced by dilution with rainwater during cloud bursts.

P. pseudoparkeae is able to tolerate lower salinities (10, 20 and 35‰) better than it tolerates higher salinities. Relative growth rates of the alga in the lower salinities are significantly higher than at 50 or 70‰.

Increases in light intensity from 50 to 100 $\mu\text{E m}^{-2}\text{s}^{-1}$ significantly increased the growth rate. Further increase in the light intensity (200 $\mu\text{E m}^{-2}\text{s}^{-1}$) reduced the growth rate slightly though this was not significantly different from that obtained at 100 $\mu\text{E m}^{-2}\text{s}^{-1}$. It is possible that light saturation for *P. pseudoparkeae* occurs at about 100 $\mu\text{E m}^{-2}\text{s}^{-1}$ and that at 200 $\mu\text{E m}^{-2}\text{s}^{-1}$ some photoinhibition comes into play.

If the growth rates in PES medium at a salinity of 35‰ are compared at light intensities of 50, 100, 150, 200 and 300 $\mu\text{E m}^{-2}\text{s}^{-1}$ (see Tables VII and XII in Appendix C - and disregarding temperature) it can be seen that light intensities of 100 and 150 $\mu\text{E m}^{-2}\text{s}^{-1}$ give the fastest growth rates. Increasing the light intensity from 200 to 300 $\mu\text{E m}^{-2}\text{s}^{-1}$ results in a progressive reduction in the growth rate suggesting that photoinhibition occurs at the higher light intensities.

In looking at the cell count data for the salinity/light intensity experiment in Table V in Appendix C it will be seen that replication only truly exists for salinity and not light intensity. For this reason these data could not be analysed using a two-way analysis of variance to determine if there is an interaction effect between salinity and light intensity. If

light intensity had been replicated with similar results to that shown in Table V, a preliminary two-way analysis of variance indicated that there is a significant interaction effect between salinity and light intensity. This is an interesting phenomenon that should be investigated in future growth studies with *P. pseudoparkeae*,

The temperature/light intensity study revealed that *P. pseudoparkeae* was unable to tolerate a temperature of 30°C despite the fact that the alga grew particularly well at 25°C. This upper temperature tolerance is approximately 10°C lower than the upper temperature tolerance determined for a related prasinophyte, *Tetraselmis* (Ukeles, 1976).

The relative growth rate of *P. pseudoparkeae* at 25°C was significantly higher at a light intensity of 150 $\mu\text{E m}^{-2}\text{s}^{-1}$ ($k' = 0,28$) than at 300 $\mu\text{E m}^{-2}\text{s}^{-1}$ and is identical to that obtained in the salinity/light intensity study at 35‰, 20°C and a light intensity of 100 $\mu\text{E m}^{-2}\text{s}^{-1}$ (see Tables VII and XII, Appendix C).

In terms of selecting culture conditions which give the fastest relative growth rate and shortest mean doubling time for *P. pseudoparkeae* the growth studies indicated that cells cultured in PES medium at a salinity of 35‰, a light intensity between 100-150 $\mu\text{E m}^{-2}\text{s}^{-1}$ and a temperature between 20 - 25°C, grew optimally. The mean generation time of cells under these conditions was ca 26h which was two hours longer than 24h period of the 16h:8h photoregime. The reduction in the mean doubling time from 33h (Aken, 1978) to 26h would significantly improve cell synchrony in a 24h L:D cycle but because there is a two hour discrepancy (between 24h and 26h) total synchrony could not be expected (see Chapter Five).

CHAPTER FOUR

AXENIC CULTURE STUDIES

4.1 INTRODUCTION

One of the major objectives of the writer's studies on *P. pseudoparkeae* was to determine the chemical composition of the scales which cover the organism (see Chapter Six). It was felt that the study would best be conducted with axenic (bacteria-free) cultures of the alga so that scale fractions would not be contaminated with bacteria or bacterial debris. However, all attempts to obtain axenic cultures of *P. pseudoparkeae* failed. These results are presented in this chapter along with the results of other experiments designed to attempt to stimulate algal growth in axenic culture.

Because *P. pseudoparkeae* could not be cultured axenically an alternative method for separating bacteria from the scale fractions had to be used. As described in Chapter Six this was achieved using differential centrifugation.

The term axenic culture is often used synonymously with the term pure culture (Aaronson, 1970). Because the literature contains several contradictory concepts and usages of the term "pure culture", a brief review of the use of the term is presented below to clarify any misconceptions.

Traditionally the term pure culture was used by bacteriologists to signify a culture containing only one species of bacterium free from all other living micro-organisms (Bold, 1942; Aaronson, 1970). Orskov (1922) however used the term pure culture in a more restricted sense to describe bacterial cultures that were derived from a single cell i.e. clonal cultures.

In extending the term pure culture to include algal cultures some confusion has arisen. Tischutkin (1897), who was one of the first workers to use agar in the cultivation of algae, reported that he isolated 18 genera into pure culture. However later in his paper he mentioned that bacteria were present in his cultures. It is clear that his cultures were unialgal cultures rather than pure cultures. Smith (1914) first suggested the term unialgal culture and clearly distinguished it from a pure or axenic culture.

A unialgal culture contains a single species of alga in the presence of bacteria but the pure culture contains only one species of alga free from all other organisms.

Pringsheim (1926) recognized six types of cultures, one of which is the pure or axenic culture; these are a) Conservation or Preservation cultures in which the organisms can be maintained in a condition similar to that in which they are found in nature; b) Crude or Gross cultures which one observes over a long period of time noting the various organisms which appear; c) Accumulation or enrichment cultures where the medium favours the development of one group; d) Species-pure culture containing a single species in such preponderance that it cannot be confused with any other organisms present (this is a looser definition of Smith's (1914) unialgal culture); e) Absolute pure cultures (axenic cultures) in which individuals from only one species are present and are free from all other organisms; f) Mono- or clonal cultures descended from a single cell. Bold (1942) broadens the latter definition to include colonial organisms started from a single colony.

In the present study the writer uses the term "axenic culture" to denote a unialgal clonal culture of *P. pseudoparkeae* which is free from all other micro-organisms. By definition the axenic culture is necessarily unialgal and free from other micro-organisms but from the discussion above it is clear that the axenic culture does not need to be a clonal culture.

Because of the nature of many of the experiments described in this chapter the results are largely qualitative. Where possible, for example in two ancillary growth experiments, the results are quantified.

4.2 MATERIALS AND METHODS

All equipment, including culture vessels, growth media etc., used in the experiments described below were sterilized in an autoclave (30 min/1 bar/121°C). All culture manipulations were performed under sterile conditions on a laminar flow bench).

4.2.1 Methods used to obtain bacteria-free cultures

Antibiotic treatments (after Guillard, 1973).

A 1,0 ml aliquot of a moderately dense, actively growing xenic culture was inoculated into each of 5 x 125 ml Erlenmeyer flasks containing 50 ml PES medium. The antibiotic mix given below was added to these treatment flasks in the volumes given in Table 4.1.

Antibiotic mix : 100 mg Penicillin - G.
50 mg Streptomycin sulphate
10 ml distilled water

The antibiotics, obtained from Sigma Laboratories, USA, were dissolved separately in a small volume of distilled water. These were combined to give a final volume of 10 ml.

TABLE 4.1

Volume of antibiotic mix added to xenic cultures.

Treatment no.	1 (control)	2	3	4	5
Volume of anti-biotic mix added to 50 ml culture (values in ml).	0	0,25	0,5	1,0	2,0

In some cases chloramphenicol was added to the antibiotic mix (20 mg per 10 ml). This was dissolved in 1,0 ml 95% ethanol before being combined with the other antibiotics. The antibiotic mix was filter sterilized through a 0,45 μ m Millipore filter before being added to the cultures.

The final concentrations of antibiotics to which the algae and bacteria were exposed are given in Table 4.2.

TABLE 4.2.

Final concentrations of antibiotics to which the organisms were exposed
(values in mg ℓ^{-1}).

Treatment no. Antibiotic	1 (control)	2	3	4	5
Penicillin	0	50	100	200	400
Streptomycin	0	25	50	100	200
Chloramphenicol	0	10	20	40	80

After approximately 15 h, three aliquots (0,05 ml each) were removed from each treatment flask and placed into three replicate test-tubes containing 10 ml PES. This subculturing was repeated after 48 h. The cultures were incubated under standard culture conditions for 15 days.

During both the 15 h and 48 h transfer periods the treatment cultures were tested for purity by streaking a small volume of the culture onto Zobells 2216e medium (see Table 4.3) contained in Petri dishes. This was repeated in triplicate for each treatment. The nutrient agar plates were incubated in the dark at 20°C for 3 days. The plates were checked for evidence of bacterial growth and the effectiveness of the antibiotic treatment was assessed and recorded.

TABLE 4.3.

Chemical composition and preparation of Zobell's 2216e medium for sterility tests (Aaronson, 1970).

Peptone	0,5 g
Yeast Extract	0,1 g
FePO ₄	0,01 g
Agar	1,5 g
Aged seawater to	100 ml
pH	7,5 - 7,8
The mixture was autoclaved (1 bar/121°C/30 min) and allowed to cool to 35°C before being poured into sterile Petri dishes and allowed to solidify.	

After 15 days, algal growth in all subcultures was recorded. Cultures were also checked for bacterial contamination using phase contrast microscopy.

Single Cell Isolation (modified after Hoshaw and Rosowski, 1973).

A 10 ml sample, taken from an actively growing culture of *P. pseudoparkeae*, was centrifuged at 100xg for 5 min to form a loose cell pellet. This pellet was drawn into a sterile pipette, resuspended in sterile PES medium contained in a second centrifuge tube, and centrifuged again. This washing process, which was repeated six times, served to reduce the relative number of bacteria in each successive algal pellet. Bacteria, which sediment more slowly than the algal cells under increased gravity, remained in the supernatant and were discarded.

A drop of the washed algal suspension was pipetted onto the centre of a Petri dish and six drops of sterile PES medium were pipetted around this. With the aid of a dissecting microscope single cells were isolated with a micropipette. Cells were washed in each of the additional drops of sterile medium before being transferred to test-tubes also containing sterile PES medium. The tip of the micropipette, containing a single cell, was broken off beneath the surface of the medium. This ensured that the cell entered the medium.

Algal growth was recorded after 15 days. Possible bacterial contamination was checked using phase contrast microscopy and by plating onto Zobell's nutrient agar.

4.2.2 Experiments conducted to enhance algal growth

Comparison of growth in autoclaved and filter-sterilized culture media.

To test the effect of these two sterilizing procedures on algal growth, 1,2 l PES medium was separated into 2 x 600 ml samples. One sample was autoclaved (10 min/1 bar/121°C) and subsequently decanted into 6 x 250 ml conical flasks to give 100 ml of medium per flask. The second sample was filter-sterilized by passing it through a 0,45 µm Millipore filter. This was

similarly decanted into six flasks. A total of twelve flasks gave three replicate cultures for each of the four treatments outlined in Table 4.4.

TABLE 4.4.

Two methods of sterilization of PES medium used in a comparative growth study.

Type of sterilization	Type of inoculum
a) Autoclaved PES medium	xenic (control)
b) Autoclaved PES medium	axenic
c) Filter-sterilized PES medium	xenic (control)
d) Filter-sterilized PES medium	axenic

Axenic and xenic cell suspensions were incubated into the flasks at a density of ± 2200 cells mL^{-1} . Axenic cells were obtained using antibiotics. These cells were washed with sterile PES medium to prevent any carry over of antibiotics into the growth flasks.

Cultures were incubated under standard culture conditions for 14 days. Cell densities computed from cell counts obtained with a Coulter Counter were determined every second day for the duration of the growth study.

Enriched and artificial culture media.

Unsuccessful attempts to grow *P. pseudoparkeae* in axenic culture in PES medium suggested that PES medium did not satisfy the growth requirements of the alga. For this reason an attempt was made to culture the alga in a number of different seawater media which were more complex than PES medium.

Small axenic inocula of 0.05 ml obtained after treatment with antibiotics (Treatment 5 concentration of antibiotics - See Table 4.2) were placed into 50 ml Erlenmeyer flasks each containing 20 ml of one of the seven growth media given in Table 4.5. (For preparation see Appendix A). Three replicate flasks, each stoppered with a cotton wool plug and capped

with aluminium foil, were used in all treatments. A control was run in which a xenic inoculum was added to one medium.

TABLE 4.5.

Enriched and artificial seawater media used to enhance algal growth in axenic cultures

Medium	Inoculum
<u>Enriched Seawater media</u>	
a) PES medium	+ xenic inoculum (control)
b) PES medium	+ axenic inoculum
c) Erdschreiber medium	" "
d) f / 2 medium	" "
e) SWM medium	" "
<u>Artificial Seawater media</u>	
f) ASP-2 medium	+ axenic inoculum
g) ASP-6 medium	" "
h) Müller medium	" "

The cultures were incubated under standard culture conditions and algal growth and possible bacterial contamination was checked and recorded after 10 days.

Fortified media

Failure to enhance growth in the complex media outlined in Table 4.5 indicated that the growth requirements of the alga were still not being satisfied and for this reason PES medium was fortified with a number of additives as outlined in Table 4.6. An axenic cell suspension (0,05 ml) was inoculated into 20 ml fortified PES medium contained in 50 ml Erlenmeyer flasks. The axenic cells were obtained after treating a xenic culture with antibiotics (Treatment 5 - see Table 4.2) for 48 h and washing these in sterile medium to remove any residual antibiotic. Three replicate cultures were used for each treatment. The additives or

supplementary nutrients were added to the medium in concentrations recommended by Guillard (1973).

TABLE 4.6.
Fortified PES media

PES medium and additive	Concentration of additive ($g\ell^{-1}$)
PES (control)	
PES + Peptone (Oxoid No. L37)	0,5
PES + Casamino acids - vitamin free (Difco O 288-61-2)	0,5
PES + Yeast extract (Difco O127O17)	0,5
PES + Liver infusion (Oxoid L25)	0,5
PES + Glycerol (BDH Analar)	2,0
PES + Glucose (BDH Analar)	2,0
PES + D-Galactose (BDH Biochemical)	2,0
PES + Pyruvic acid (Sigma P2256)	1,0
	$mg\ell^{-1}$
PES + Gibberillic acid (GA_3)	100
PES + Kinetin	100
PES + Indole acetic acid (IAA)	100

Cultures were incubated at 20°C in a 16h:8h photoregime (light intensity = $100 \mu E m^{-2} s^{-1}$) i.e. standard culture conditions. Cultures were checked for signs of algal growth after 10 days.

Experiment using filtrates from xenic cultures.

This experiment was conducted to test if *P. pseudoparkeae* could be grown axenically in the filtrate obtained from a xenic culture. It was felt that this "conditioned" medium might contain the "factor" needed for axenic growth.

An actively growing xenic culture of the experimental organism was filtered through a 0,45 μm Millipore filter to remove algae and bacteria. PES nutrients were added to 20 ml of the filter-sterilized filtrates and an axenic cell suspension (0,05 ml) was inoculated with the medium. This was repeated using autoclaved filtrate. Both tests were repeated in triplicate.

Cultures were incubated under standard culture conditions and growth was recorded after 10 days.

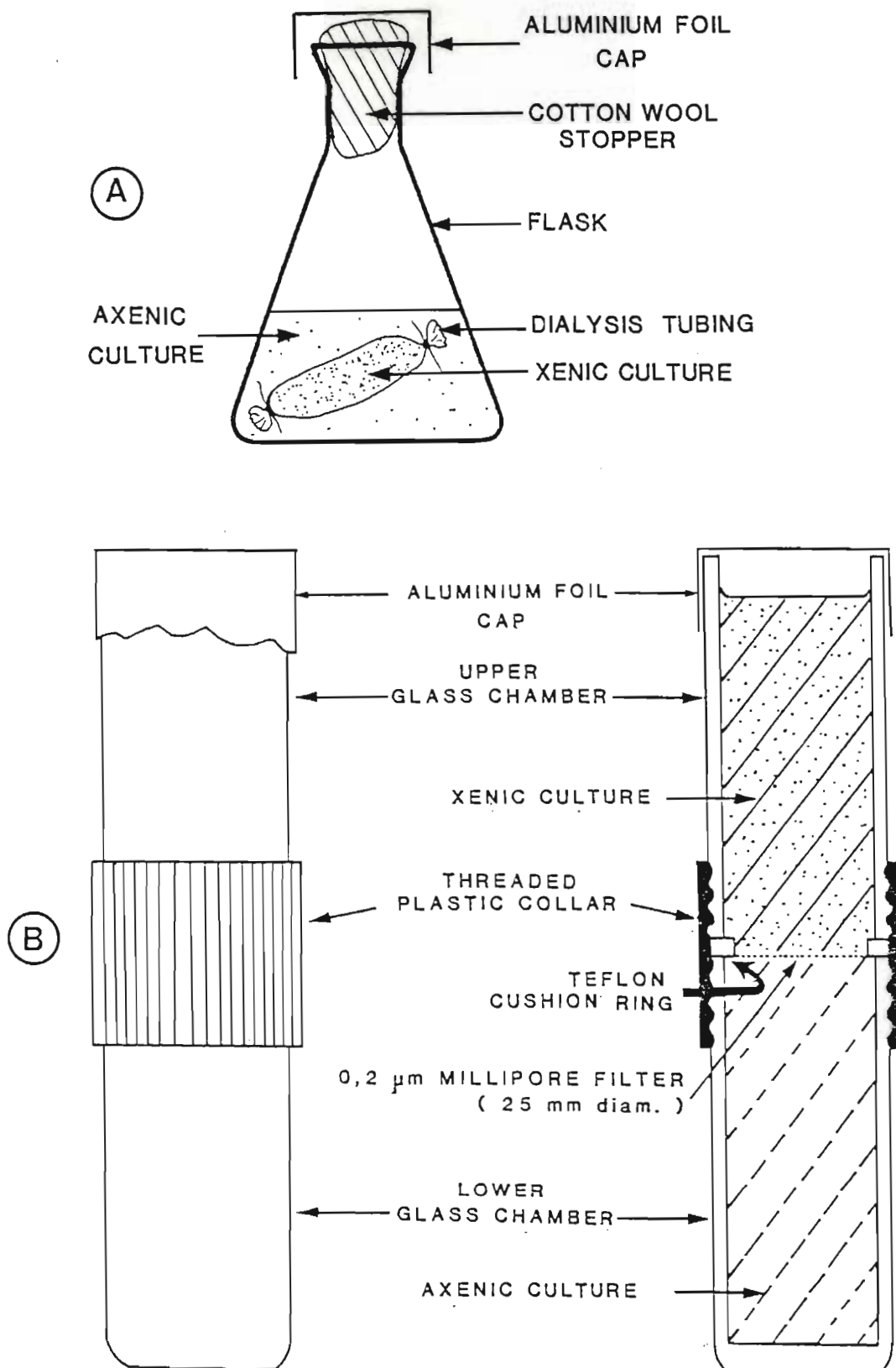
4. 2. 3 Partition culture experiments

A 10 ml sample of a xenic culture was pipetted into a length of sterile dialysis tubing which had been sealed at one end. Care was taken to ensure that the outer surface of the dialysis tubing was not contaminated with the xenic culture. The opposite end of the tubing was also sealed and the contained xenic culture was placed into 50 ml PES medium in a 150 ml Erlenmeyer flask (Text fig. 4.1, A).

Axenic cells were inoculated into the sterile medium surrounding the xenic culture.

A second partition-culture experiment was run concurrently. An axenic cell suspension (0,05 ml) was inoculated into 20 ml sterile PES medium in the lower half of a partition-culture apparatus (Text fig. 4.1 B). This was covered with a 0,2 μm Millipore filter which was secured in position by a plastic screw collar threaded at both ends. The meniscus of the medium in the axenic chamber was in contact with the membrane. The second half of the partition-culture apparatus was then screwed into position above the membrane and was filled with 20 ml of xenic culture. The upper chamber was capped with aluminium foil to prevent contamination.

Three replicate tests were performed with both partition culture systems. Growth was recorded after 14 days.



Text fig. 4.1 Two types of partition culture systems. A xenic culture was separated from an axenic culture by dialysis membrane (A) or a 0,2 μm Millipore filter (B).

4.2.4 Growth experiment using two isolates of *P. pseudoparkeae*

Because all efforts to grow *P. pseudoparkeae* (Oudekraal isolate) in axenic culture had failed, it was decided to test if another isolate of the species (Mtwalume isolate) showed a similar response when grown in bacteria-free culture. It was felt that the original clonal culture of *P. pseudoparkeae* may have undergone a genetic shift thus making it dependent on the bacterial fauna for normal growth.

Axenic inocula of both isolates were obtained using antibiotics. A monospecific population of bacteria used in this experiment was isolated by plating 0,01 ml diluted xenic culture onto Zobell's 2216e nutrient agar (Aaronson, 1970). The bacterium was identified as a species of *Pseudomonas* MIGULA.¹

All isolates were cultured in 125 ml Erlenmeyer flasks in 20 ml sterile PES medium as shown in Table 4.7.

TABLE 4.7.
Different combinations of isolates grown in PES
medium

Medium	Algal inoculum	Bacterial inoculum
PES	+ axenic Oudekraal clone	+ <i>Pseudomonas</i> (Control 1)
PES	+ axenic Mtwalume clone	+ <i>Pseudomonas</i> (Control 2)
PES	+ axenic Oudekraal clone	
PES	+ axenic Mtwalume clone	
PES		+ <i>Pseudomonas</i> only.

¹ *Pseudomonas* sp. was identified by Dr F.M. Wallis, Department of Microbiology and Plant Pathology, University of Natal, Pietermaritzburg, Republic of South Africa.

Three replicate cultures were used for each treatment and these were incubated under standard culture conditions.

Both algal density and bacterial density was determined at the start of the experiment and again after 12 days. Bacterial density was determined by plating 0,1 ml diluted culture medium onto Zobell's nutrient agar contained in a Petri dish. After 3 days the number of bacterial colonies on each of three replicate plates used, were counted and averaged. This value was multiplied by the dilution factor and by 10 to give bacterial density as cells per millilitre. Algal density was determined by counting the number of cells in a known volume of culture medium. Cells were fixed in 1% glutaraldehyde and the sample was placed in a settling chamber and left to stand overnight. Cells which had settled on the base of the chamber were counted using a Zeiss inverted plankton microscope.

4.3 RESULTS

4.3.1 Results of axenization experiments

Antibiotic Experiments

Five separate antibiotic experiments were undertaken to attempt to obtain axenic cultures of *P. pseudoparkeae*. Though not always successful, for reasons explained below, the method did produce axenic cell suspensions but these could not be maintained and the cells eventually died.

The results of the antibiotic experiments are given below.

Antibiotic Experiment I

The results of the first antibiotic experiment are summarized in Table 4.8.

Fifteen hours after adding the antibiotic mix (containing penicillin and streptomycin) algal cells were actively swimming in all treatment flasks and showed no signs of stress due to the presence of the antibiotic. The nutrient agar sterility test showed that the 15h exposure to the antibiotics at all concentrations had not been effective in killing bacteria in

TABLE 4.8
Results of Antibiotic Experiment I
(See Table 4.2 for concentrations of antibiotics
used in different treatments).

Treatment no.		1 (control)	2	3	4	5
Replicates		a b c	a b c	a b c	a b c	a b c
15h exposure to antibiotics	Algal motility after 15 h	cells active	cells active	cells active	cells active	cells active
	Sterility test after 15 h	+ + +	+ + +	+ + +	+ + +	+ + +
	Algal growth after 15 days	√ √ √	√ √ √	√ √ √	√ √ √	√ √ √
46h exposure to antibiotics	Algal motility after 46h	cells active	cells active	cells active	cells active	cells active
	Sterility test after 46h	+ + +	+ + +	+ + +	O + +	O O O
	Algal growth after 15 days	√ √ √	√ √ √	√ √ √	√ √ √	√ √ √

Legend: + = bacteria present
 O = bacteria absent
 √ = algal growth observed
 - = no algal growth

the cultures. The subcultures made from each of the treatment flasks were axenic and dense algal cultures were observed after 15 days.

Forty six hours after adding the antibiotic mix to the cultures the algal cells were still actively swimming in all treatment flasks. The sterility test done at this time showed that the bacteria had been killed in Treatment 5 flasks which had the highest concentration of antibiotics. Lower concentrations of the antibiotic mix did not kill all the bacteria. Dense subcultures of the alga were obtained from all treatments after 15 days.

Because bacteria had been removed from Treatment 5 flasks after 46h the dense subcultures from this treatment were expected to be axenic. However, closer scrutiny of the "axenic" culture revealed that it (and other cultures) contained a contaminating marine ascomycete. This organism was extremely small and was not detected on the nutrient agar plates. The source culture and subcultures used in this experiment had to be discarded and a second clonal culture of *P. pseudoparkeae* was used for subsequent experiments. The significant observation made here was that *P. pseudoparkeae* was able to grow vigorously with the yeast even when bacteria had been removed from the culture.

Antibiotic Experiment II

The results of this experiment are summarized in Table 4.9. The antibiotic mix used in this experiment also contained penicillin and streptomycin.

Fifteen hours after the addition of the antibiotic mix the algal cells in all treatment flasks were actively swimming. The nutrient agar sterility test showed that the 15 h exposure to antibiotics at all concentrations did not kill bacteria in the cultures. A similar result was obtained in Antibiotic Experiment I. An important departure is seen in the second experiment in that none of the algae subcultured from the treatment flasks and, above all, the control flask grew.

Similarly none of the subcultures inoculated from the treatment flasks after 48h became established. The nutrient agar sterility test made at this time showed that subcultures inoculated from flasks in Treatments 4 and 5 were potentially axenic.

TABLE 4.9
Results of Antibiotic Experiment II
(see Table 4.2 for concentrations of antibiotics
used in different treatments).

Treatment no.		1 (control)	2	3	4	5
Replicates		a b c	a b c	a b c	a b c	a b c
15 h exposure to antibiotics	Algal motility after 15 h	cells active	cells active	cells active	cells active	cells active
	Sterility test after 15h	+ + +	+ + +	+ + +	+ + +	+ + +
	Algal growth after 15 days	- - -	- - -	- - -	- - -	- - -
48h exposure to antibiotics	Algal motility after 48 h	cells active	cells active	cells active	cells not as active	cells swimming slowly
	Sterility test after 48 h	+ + +	+ + +	+ O +	O O O	O O O
	Algal growth after 15 days	- - -	- - -	- - -	- - -	- - -

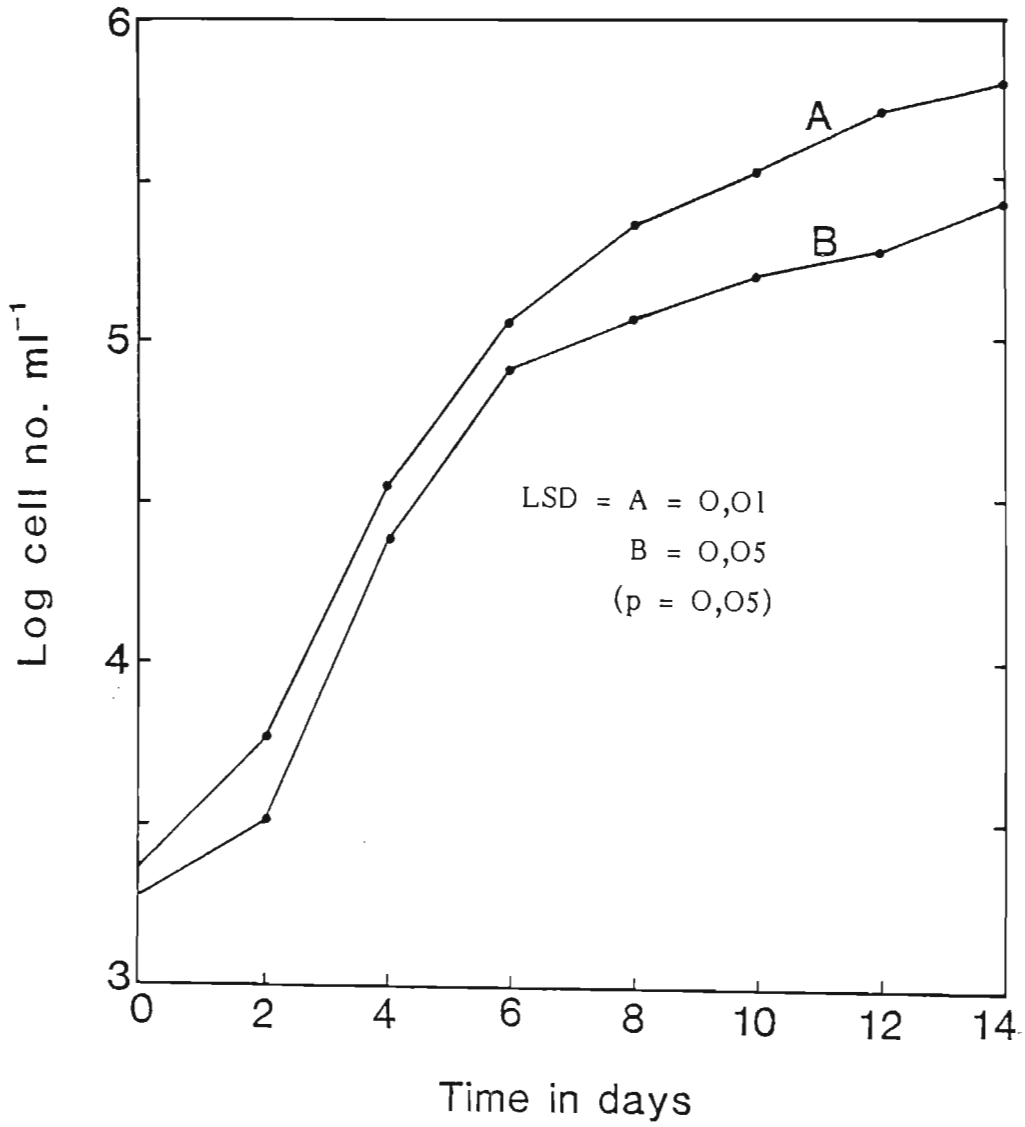
Legend + = bacteria present
O = bacteria absent
√ = algal growth observed
- = no algal growth

The absence of algal growth in all subcultures and especially those inoculated from the control caused concern because the control treatment flask which was kept for two weeks had a dense population of cells after this period. It was clear that medium into which cells were subcultured was preventing growth. When the test tubes were checked closely a layer of white precipitate, not seen initially, was found on the bottom of the tubes. A precipitate frequently forms in marine media when they are autoclaved and it could be that essential nutrients required for algal growth precipitated out and were not available to the algae in the subcultures.

To test if autoclaving affected the potential of PES medium to support algal growth, *P. pseudoparkeae* was grown in autoclaved PES medium and filter-sterilized PES medium as outlined in Section 4.2.2 above. The axenic inocula were obtained by exposing a xenic culture of the alga to the highest concentration of antibiotic (Treatment 5 - see Table 4.2) for 48h. This concentration of antibiotics had been shown to kill the bacteria (see Tables 4.8 and 4.9) after 48 h.

Axenic cells did not grow in either the autoclaved PES medium or the filter-sterilized PES medium. However, where a xenic inoculum was used the cells grew well in both media (Text fig. 4.2). The growth curves for both media are similar but because the inoculum densities were different in both media (viz. 2512 cells mL^{-1} in filter sterilized PES, and 1995 cells mL^{-1} in autoclaved PES) the yields after 14 days are not directly comparable because the cultures had not reached the stationary phase of growth i.e. the point where cell concentration would be a function of nutrient availability (as affected by the sterilizing procedures) and not inoculum density.

Because the inoculum density in the autoclaved PES medium was 34% smaller than that in the filter sterilized PES medium, a 34% difference in yield could be expected after 14 days if the cultures were behaving similarly. However after this period cell densities were 57% lower in autoclaved PES medium compared with filter sterilized PES medium (564086 cells mL^{-1} and 242488 cells mL^{-1} respectively). If 34% of this difference could be attributed to a difference in the inoculum strength then it can be concluded that the potential of PES medium to support algal growth is reduced by 23% after autoclaving.



Text Fig. 4.2 Two growth curves for *P. pseudoparkeae* grown in filter-sterilized PES medium (A) and autoclaved PES medium (B).

Though this observation was interesting it did not explain why the subcultures in Antibiotic Experiment II did not grow. It could be that the smaller volume of PES medium in the subculture test tubes was more drastically affected by the autoclaving (30 min/1 bar/121°C) causing the precipitate to form. Such a precipitate was not observed in the autoclaved media (10 min/1 bar/121°C) used in the growth study described above.

Antibiotic Experiment III

A third antibiotic experiment was carried out with PES medium that had been autoclaved for 10 min. Nutrient agar plates inoculated with this autoclaved medium were clean and did not reveal any bacterial contaminants. This indicated that a 10 min autoclaving period was sufficient to sterilized the medium. The results of this antibiotic experiment are given in Table 4.10. The antibiotic mix in this experiment contained penicillin and streptomycin.

The results obtained in this experiment were unexpected because the antibiotics were not successful in killing the bacteria in any of the cultures. Furthermore all subcultures made from the treatment flasks grew well. When the treatment cultures were examined closely it was found that the bacterial populations in all cultures were monospecific. The bacterium was identified as *Pseudomonas* sp¹. This bacterium is a motile gram negative bacillus and probably represented an antibiotic-resistant mutant. It is not clear how this mutant arose, but because it was found in all cultures it must have been present in the stock culture. Significantly the Treatment 1 flasks contained a second bacterium, *Caulobacter* HENRICI *et* JOHNSON² but this was not found in the treatment flasks containing antibiotics. It was apparently killed even at the lowest concentrations of antibiotics.

Antibiotic Experiment IV

A fourth antibiotic experiment was conducted in which the broad spectrum antibiotic chloramphenicol was added to the antibiotic mix to

¹ *Pseudomonas* sp. was identified by Dr F.M. Wallis, and

² *Caulobacter* sp. was identified by Mrs L. Brown. Both persons are employed in the Department of Microbiology and Plant Pathology, University of Natal, Pietermaritzburg, Republic of South Africa.

TABLE 4.10
 Results of Antibiotic Experiment III
 (see Table 4.2 for concentrations of antibiotics
 used in different treatments).

Treatment no.		1 (control)	2	3	4	5
Replicates		a b c	a b c	a b c	a b c	a b c
18h exposure to antibiotics	Algal motility after 18h	cells active	cells active	cells active	cells active	cells active
	Sterility test after 18h	+ + +	+ + +	+ + +	+ + +	+ + +
	Algal growth after 15 days	√ √ √	√ √ √	√ √ √	√ √ √	√ √ √
50h exposure to antibiotics	Algal motility after 50h	cells active	cells active	cells active	cells active	cells active
	Sterility test after 50h	+ + +	+ + +	+ + +	+ + +	+ + +
	Algal growth after 15 days	√ √ √	√ √ √	√ √ √	√ √ √	√ √ √

Legend + = bacteria present
 O = bacteria absent
 √ = algal growth observed
 - = no algal growth

attempt to kill the resistant pseudomonad.

The results of this experiment are given in Table 4.11. The table shows that the pseudomonad was killed in Treatment 5 after 15h and in Treatments 4 and 5 after 48 h. The subcultures made from these treatment flasks were potentially axenic cultures but as the results show there was no algal growth in the subcultures made from these flasks. Where the bacterial numbers had been reduced significantly e.g. in the Treatment 4 flask after 15 h and the Treatment 3 flask after 48h algal growth in the subcultures from these flasks was poor. The reason for this is not known though one of three possible explanations can be given. Firstly the carry over of antibiotics from the treatment flasks to the subcultures may have had a bacteriostatic effect in checking the growth of bacteria which consequently affected algal growth. Alternatively the carry over of antibiotics may have checked algal growth directly. Finally the antibiotic treatment may have had a permanent debilitating effect on the alga. The latter is not thought to be true; if cells taken from the subcultures exhibiting poor growth were washed and placed into fresh medium they grew well. These cultures were xenic. It appears therefore that the carry over of antibiotics into the subcultures was affecting algal growth either directly or by inhibiting bacterial growth which in turn affected algal growth.

Single cell isolation

To avoid using antibiotics, which were affecting algal growth directly or indirectly, single cells were isolated under sterile conditions as outlined in Section 4.2.1.

Of twenty cells isolated in this manner none grew. This was surprising because the method had been used successfully to obtain xenic clonal cultures of *P. pseudoparkeae*. It became clear that the PES medium alone was not satisfying the growth requirements of *P. pseudoparkeae* and that the bacteria were perhaps supplying an organic compound, or at least modifying the medium in some way, so as to enhance algal growth.

TABLE 4.11
 Results of Antibiotic Experiment IV
 (see Table 4.2 for concentrations of antibiotics
 used in different treatments)

Treatment no.		1 (control)	2	3	4	5
Replicates		a b c	a b c	a b c	a b c	a b c
15h exposure to antibiotics	Algal motility after 15h	cells active	cells active	cells active	cells not as active	cells swimming slowly
	Sterility test after 15h	+ + +	+ + +	+ + +	+ O O	O O O
	Algal growth after 15 days	√ √ √	√ √ √	√ √ √	✗ ✗ ✗	- - -
48h exposure to antibiotics	Algal motility after 48h	cells active	cells active	cells not as active	cells swimming slowly	many cells settled
	Sterility test after 48h	+ + +	+ + +	O O +	O O O	O O O
	Algal growth after 15 days	√ √ √	√ √ √	✗ ✗ ✗	- - -	- - -

Legend + = bacteria present
 O = bacteria absent
 √ = algal growth observed
 - = no algal growth
 ✗ = poor algal growth

4.3.2 Attempts to enhance algal growth

Enriched and Artificial culture media

To attempt to get *P. pseudoparkeae* to grow in axenic culture, an axenic suspension of cells was inoculated into the different growth media given in Table 4.5. Some of these media are more complex than the PES medium and contain a greater variety of organic constituents (see Appendix A). It was hoped that one of these more complex media would allow *P. pseudoparkeae* to grow under axenic conditions.

None of the axenic inocula placed into these media grew. This suggested that they did not meet the growth requirements of *P. pseudoparkeae* in the absence of the bacteria. The axenic inoculum placed into PES medium grew well and a dense culture of cells was observed after 10 days.

Fortified media

Because the enriched and artificial seawater media did not support algal growth under axenic conditions it was decided to fortify the PES medium as outlined in Table 4.6. The reasons for choosing the additives shown here are discussed in Section 4.4.

This experiment was performed twice because bacterial contamination during the first experiment upset the results. Nevertheless a brief description of these results are given because some interesting observations were made.

In the first experiment algal growth was obvious in all the cultures after the third day. However at the end of the fourth day all the cultures, except those containing hormones, became cloudy as a contaminating bacterial population increased rapidly. By the sixth day no algal cells were present in the cultures. It appeared that the high density of bacteria supported by the fortified media inhibited algal growth after the fourth day. Cultures containing GA₃ and IAA were pale green in colour after 10 days indicating that the algae had grown in these cultures. Those cultures containing kinetin were especially dense and bright green in colour. This

apparently luxuriant growth had not been observed previously in any culture. The cultures containing hormones were also xenic.

When the experiment was repeated using fresh axenic cell inocula none of the cultures had established after 10 days.

Filtrates from xenic cultures

Having failed to grow *P. pseudoparkeae* axenically in complex seawater media or in PES medium fortified with a variety of organic additives it was decided to test if the alga would grow axenically in sterile filtrates obtained from xenic cultures. The method used is outlined in Section 4.2.2.

The rationale behind this experiment was that the filtrate from a xenic culture should be "conditioned" and that if the bacteria were producing a compound (or compounds) needed by the alga then this should be free in the medium. If an axenic suspension of cells was inoculated into this it should then grow.

Axenic cells inoculated into both the filter-sterilized filtrate and autoclaved filtrate however failed to grow. This result was initially disconcerting because it suggested that the bacteria were perhaps not producing a growth promoting compound which the alga was utilizing. However an alternative explanation was that there was no excess compound in the medium because this was utilized rapidly by the algae. To test the latter, two partition culture experiments were designed.

4.3.3 Partition cultures

The materials and methods used in the partition culture experiments are given in Section 4.2.3.

Where axenic cells were inoculated into flasks containing a xenic culture sealed within dialysis tubing (Text fig. 4.1, A) no growth was observed in the "axenic compartment". However in a second experiment where an axenic suspension was separated from a xenic culture by a 0,2 μm Millipore membrane, in the apparatus shown in Text fig. 4.1, B,

good growth was observed in the axenic compartment. After 14 days the suspension of cells from the axenic culture was plated onto nutrient agar and viewed using phase contrast microscopy to check for contaminants. The agar plates were clean indicating that the culture was axenic. No bacteria were seen using phase contrast optics. This was the first occasion on which *P. pseudoparkeae* was grown successfully in axenic culture. It was obvious that some factor passing from the xenic culture through the 0,2 μm Millipore membrane (but not through dialysis tubing) enhanced algal growth under axenic conditions.

4.3.4 A comparative growth study using two isolates of *P. pseudoparkeae*

Because all the axenic experiments described above were conducted with the Oudekraal isolate of *P. pseudoparkeae* it was decided to test if another, geographically separate, isolate of the alga also depended on the bacteria for growth. The second isolate used was one collected at Mtwalume on the Natal south coast. The methods used in this experiment are outlined in Section 4.2.4.

The results of the experiment are summarized in Table 4.12.

Most important is that neither the Oudekraal isolate nor the Mtwalume isolate grew in axenic culture. However, where *Pseudomonas* was added to cultures inoculated with an axenic algal suspension, both algal isolates grew well. In the experiment the Mtwalume isolate grew more rapidly than the Oudekraal isolate. After 12 days the cell densities in the Oudekraal isolate cultures were 60% lower than those measured in the Mtwalume isolate cultures. At least 40% of the difference can be attributed to differences in inoculum strength. The final cell densities measured in the Oudekraal cultures were therefore approximately 20% lower than those measured in the Mtwalume cultures. The reason for this difference in yield between the two isolates is not known.

Another interesting observation to come from this experiment was that the growth of *Pseudomonas* was significantly enhanced in the presence of the alga. Although a significant increase in the cell density of *Pseudomonas* was observed in PES medium alone after 12 days, a much greater yield was obtained where the bacteria and algae were combined in the PES medium.

TABLE 4.12

A comparison of growth between two isolates of *P. pseudoparkeae* in xenic and axenic culture (Values in cell no. mL^{-1} : Figures in parentheses represent the 95% confidence limits of the means for three replicate cultures).

Isolate/s	Day 0	Day 12
<i>P. pseudoparkeae</i> (Oudekraal isolate) - axenic	18(5)	-
<i>P. pseudoparkeae</i> (Mtwalume isolate) - axenic	30(3,7)	-
<i>P. pseudoparkeae</i> (Oudekraal isolate) plus <i>Pseudomonas</i> sp.	18(5) $2,3 \times 10^4$ (5059)	900 (60) $> 340 \times 10^6$
<i>P. pseudoparkeae</i> (Mtwalume isolate) plus <i>Pseudomonas</i> sp.	30(3,7) $2,3 \times 10^4$ (5059)	2225 (248) $> 340 \times 10^6$
<i>Pseudomonas</i> sp.	$2,3 \times 10^4$ (5059)	$1,47 \times 10^6$ (75 882)

4.4 DISCUSSION

The results described above reveal some interesting facts about the relationship between *P. pseudoparkeae* and bacteria in culture. The single most important fact is that *P. pseudoparkeae* cannot be grown or maintained in axenic culture and requires a bacterial population to grow normally.

Antibiotics successfully killed bacteria in cultures of *P. pseudoparkeae* and did not appear to have any permanent debilitating effect on the alga. The latter was deduced from two observations. Where the antibiotic-resistant *Pseudomonas* was not killed by antibiotics (streptomycin and penicillin) the alga grew well even after being exposed to Treatment 5 concentrations of antibiotics (refer to Table 4.10) for 50h. Secondly an axenic suspension of *P. pseudoparkeae* (obtained after treatment with antibiotics - penicillin, streptomycin and chloramphenicol) could be grown successfully in subcultures from Treatment 5 flasks only if bacteria were inoculated into the axenic suspension of cells. If these cells were kept under axenic conditions they failed to grow.

Two interesting points emerging from these observations were that the alga was not affected by being exposed to the highest concentration of antibiotics, and that the alga apparently had an absolute requirement for the bacteria before it was able to grow. If the antibiotics were having a permanent effect on the alga it would not have grown when the cultures were bacterized.

However where the alga was exposed to antibiotics for extended periods (ca 3 - 5 days), for example in Treatment 5 flasks that were kept for this period, the algal cells appeared to bleach and eventually died. Bleaching and death occurred more rapidly where the concentration of antibiotics was highest. To understand the process of bleaching it is necessary to examine the mode of action of the antibiotics used. The effect of these on bacterial and algal cells is also discussed below.

In bacteria, penicillin, streptomycin and chloramphenicol halt bacterial growth by operating on difference systems within the cell (Hammond and Lambert, 1978). Penicillin specifically inhibits the crosslinking of linear peptidoglycan strands in the cell walls of Gram-positive and Gram-negative

bacteria. At lethal concentrations the cells lyse. Streptomycin reacts only with bacterial ribosomes (70s ribosomes) and specifically with the smaller (30s) subunit of the ribosome.

The binding of the antibiotic distorts the ribosome so preventing normal interaction between the codon of mRNA and the anticodon of tRNA. This causes miscoding resulting in the production of nonsense proteins which effectively halts normal protein synthesis. Chloramphenicol acts upon the larger (50s) subunits of bacterial ribosomes and inhibits peptide bond formation between amino acids so also effectively blocking protein synthesis.

In eukaryotic algal cells those antibiotics having the ribosomes as their target sites (streptomycin and chloramphenicol) cause most damage. These antibiotics act only on the 70s ribosomes in the chloroplast and mitochondria and do not affect the 80s ribosomes in the cytoplasm of eukaryotic cells (Rodriguez-Lopez and Vasquez, 1968). These authors have shown that antibiotics, specific for 70s ribosomes, cause bleaching in *Chlorella* BEIJERINCK by affecting protein synthesis in 70s plastidial ribosomes. Bleaching in *P. pseudoparkeae* after prolonged exposure to antibiotics may have been caused by the effects of streptomycin and chloramphenicol on 70s ribosomes in the chloroplast. There are relatively few reports on the effects of antibiotics on the growth of algal cells. Spencer (1952) has shown that streptomycin inhibits growth in the diatom *Nitzschia* HASSALL. Chloramphenicol inhibits mitochondrial protein synthesis in *Polytomella* ARAGAO and thus inhibits its growth (Lloyd *et al.*, 1970).

The failure of *P. pseudoparkeae* to grow in axenic culture suggested that the bacteria modified the medium in some way to promote algal growth. Ukeles and Bishop (1975) conducted some elegant experiments in which they showed that the growth of an unknown species of *Pyramimonas* was enhanced by the bacteria *Escherichia coli* (MIGULA) CASTELLANI *et* CHALMERS and *Vibrio anguillarum* BERGMAN. They showed that the growth of *Pyramimonas* on agar plates was enhanced where bacterized filter paper discs were placed on the gel. They concluded that growth stimulation resulted from the digestion of agar by viable bacteria with the release of small molecules, either sugars or acids, that were utilized by the alga. Though the results of Ukeles and Bishop (1975) and the results of the present study show that algal growth is enhanced by bacteria

there are two fundamental differences in the two studies. The *Pyramimonas* species used by Ukeles and Bishop (1975) could be grown axenically whereas *P. pseudoparkeae* could not. Secondly the source of nutrients promoting growth in *Pyramimonas* sp. were supposedly derived from the hydrolysis of agar. This substrate was not present in the liquid cultures of *P. pseudoparkeae* and it raises the question as to whether the same or different compounds promote growth in *P. pseudoparkeae*.

A point not overlooked by Ukeles and Bishop (1975) was that the stimulatory effects of the bacteria on the alga may have resulted from bacterial induced changes in the physical-chemical conditions (pH, CO₂) in the region of the charged filter paper discs. The results of the present study indicate that these factors are probably not of primary importance in stimulating algal growth in bacteria-free cultures of *P. pseudoparkeae* because the alga could not be grown axenically when bacteria-free cells were separated from a xenic culture by dialysis tubing. Ions and gases would move freely through this membrane and if they were promoting growth this would have been observed. Lange (1971) has, however, demonstrated that CO₂ released by bacteria may stimulate algal growth.

Two terms that should be introduced here because they are useful in describing bacterial-algal relationships are "phycosphere" and "bactosphere". The term phycosphere was proposed by Bell and Mitchell (1972) and refers to a zone of undefined area extending outward from an algal cell or colony which contains extracellular products that stimulate bacterial growth. Ukeles and Bishop (1975) proposed a parallel term, the bactosphere, describing the zone of enrichment around bacterial cells or populations that enhances the growth of or attracts other micro-organisms.

P. pseudoparkeae could not be grown axenically in a number of complex enriched and artificial seawater media. This indicated that the media were not satisfying the growth requirements of the alga and that the constituent nutrients in the media could not substitute for the bactosphere. Similarly fortified PES media with complex organic additives did not satisfy the growth requirement of the alga in axenic culture. This was surprising because the extracts contained many organic constituents,

e.g. amino acids (peptone, casamino acids, liver extract), vitamins (peptone, liver and yeast extract), and purines and pyrimidines (liver extract). Glycerol, glucose and galactose were added to the PES medium because they may stimulate growth in some unicellular green algae (Bold, 1942). Pyruvic acid was added to the PES medium because this compound is known to stimulate growth of numerous species of marine phytoplankton (Ukeles and Rose, unpublished, in Ukeles and Bishop, 1975). None of the hormones added to the PES medium enhanced algal growth in axenic culture. There are a few often contradictory reports in the literature of the effect of exogenously applied hormones on the growth of algae. Evans and Sorokin (1971) showed that GA₃ enhanced the growth of *Chlorella* at a concentration of 200 ppm but Lien *et al.* (1971) showed that neither GA₃ nor IAA had any stimulatory effect on the growth of *Chlorella*. IAA is known to promote growth in *Nostoc* VAUCHER (Ahmad and Winter, 1968; Ahmad, 1971) and *Exuviaella* CIENKOWSKI (Iwasaki, 1971). Especially interesting is that *Nostoc* grows well in the presence of *Caulobacter* and poorly without; 1 or 2 drops of a 1 mg l⁻¹ solution of IAA replaced the bacterial effect (Bunt, 1961). Moewus (1959) has shown that kinetin increases the rate of cell division in *Polytoma* EHRENBERG.

The inability of *P. pseudoparkeae* to grow in enriched or artificial media, or fortified PES media, suggested that the growth promoting "substance" in the bactosphere was not being supplied by these media. Alternatively if it was supplied it was perhaps present in concentrations that did not stimulate growth. That the alga could not be grown axenically in the sterile filtrates from xenic cultures indicated that the growth promoting substance in the bactosphere was not present in excess in the medium or that it was labile and lost its growth promoting capacity in a short period of time. If the former is true then it may be possible that the growth of *P. pseudoparkeae* is limited by the production of this substance in the bactosphere.

In the comparative study of growth between two isolates of *P. pseudoparkeae* some interesting facts emerged. Neither the Oudekraal nor the Mtwalume isolate could be grown axenically. This indicates that the dependence on the bacteria is characteristic for the species. Another interesting observation is that bacterial growth was enhanced in the presence of the alga. It appears therefore that there is a mutualistic relationship

between *P. pseudoparkeae* and *Pseudomonas*. The writer does not believe that this is a species specific relationship because growth of *P. pseudoparkeae* is also promoted by *Caulobacter*, an unidentified non-motile bacillus and also a marine ascomycete. It is not known if other marine bacteria stimulate the growth of *P. pseudoparkeae*. This should be tested in future studies.

The utilization of photosynthetically derived extracellular products of algae by bacteria has been established in the laboratory as well as in the field (Bell and Mitchell, 1972; Nalewajko and Lean, 1972; Larsson and Hagström, 1979; Blaauboer *et al.*, 1982; Cole *et al.*, 1982; Kogure *et al.*, 1982; Chrost and Faust, 1983). Some workers studying wastewaters have reported that bacteria may stimulate algal growth (Ludwig *et al.*, 1951; Humenik and Hanna, 1971) though this has never been conclusively proved and is questioned by some authors who believe that bacterial-algal relationships are antagonistic and bacteria inhibit algal growth in wastewater ponds (Dor and Benzion, 1980; Toerin *et al.*, 1984). Contrary to this the results of the present study indicate that bacterial-algal relationships may be mutualistic or synergistic. A mutualistic relationship has also been found between *Navicula* BORY and *Flavobacterium* BERGEY, HARRISON, BREED, HAMMER *et* HUNTON (Jolley and Jones, 1977).

In the writer's opinion it is unsound to generalize on the relationships between bacteria and algae because a whole spectrum of relationships may exist under different conditions. At one extreme the bacterial-algal relationship may be antagonistic favouring the bacteria. This is especially true in nutrient "overloaded" or eutrophic systems e.g. in wastewaters. Under these conditions algal growth may be inhibited by bacteria (Shilo, 1970; Berland *et al.*, 1972; Rhee, 1972; Reim *et al.*, 1974; Coughlan, 1977; Berger *et al.*, 1979; Dor and Benzion, 1980; Toerien *et al.*, 1984). This inhibition is thought to be the result of a number of possible causes. Some bacteria may lyse the cell walls of algal cells or they may release toxic substances that inhibit algal growth. Furthermore algae may not compete as effectively as bacteria for certain nutrients. An interesting observation made in the present study was that the growth of *P. pseudoparkeae* was inhibited by bacterial contaminants present in fortified PES media. It appears therefore that the mutualistic bacterial-algal relationship in PES may become antagonistic, favouring the bacterium, under nutrient rich conditions.

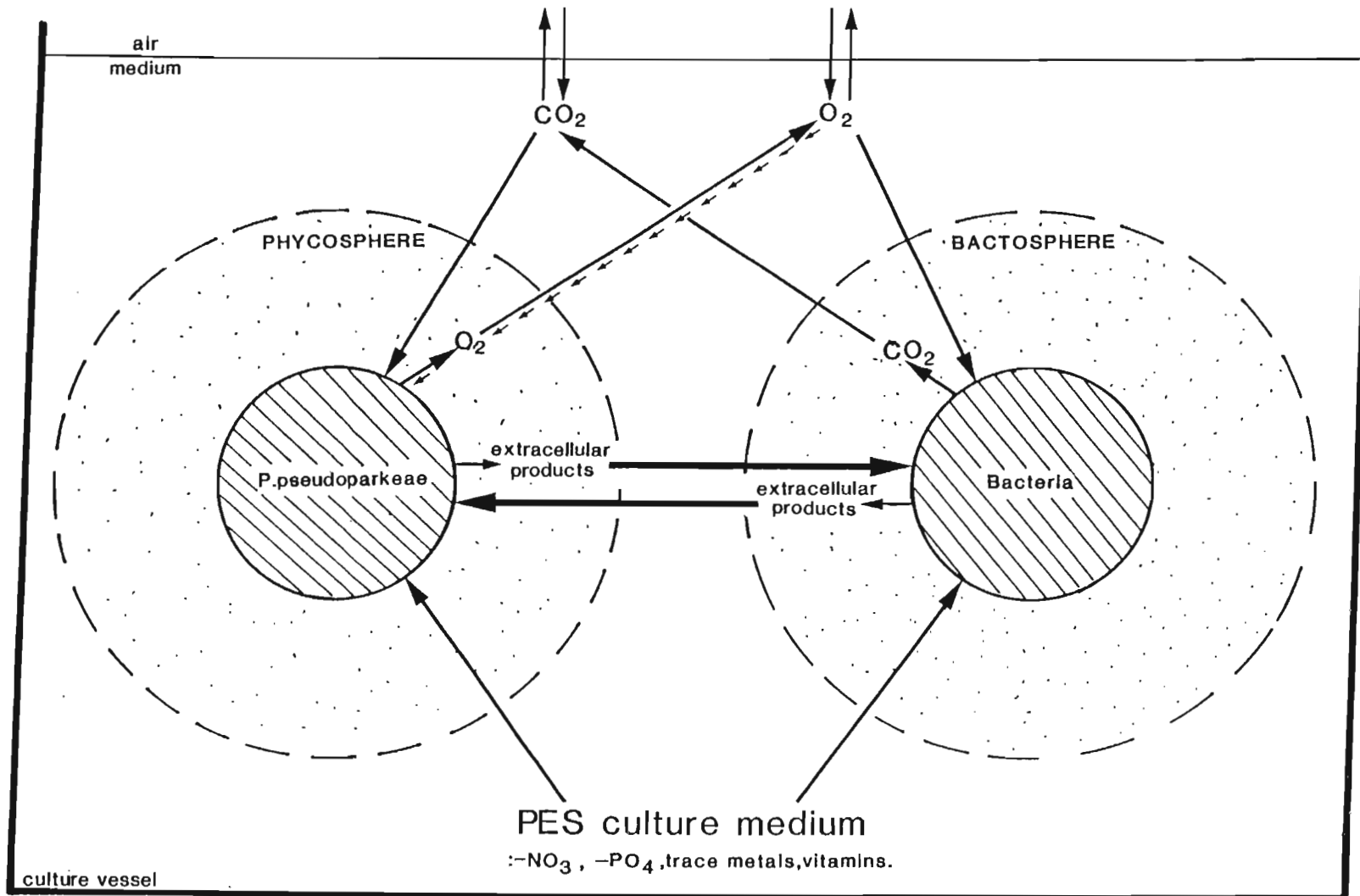
At the other end of the scale bacterial-algal relationships may be antagonistic favouring the alga. The studies of Pratt and coworkers revealed that bacterial inhibition may be caused by algal activity (Pratt and Fong, 1940; Pratt, 1942; Pratt *et al.*, 1944). Antibacterial extracellular substances are produced by *Isochrysis* (Bruce and Duff, 1967), *Pandorina* BORY (Harris, 1971) and *Phaeocystis* LAGERHEIM (Sieburth, 1959; Guillard and Hellebust, 1971). Accorinti (1964) and Vladimirova (1960) have also reported the suppression of bacterial growth by algae or their lytic products.

Synergistic relationships between bacteria and algae (present study and, Jolley and Jones, 1977) would occupy an intermediate position between the two extremes of antagonistic relationships mentioned above. Synergistic or mutualistic relationships between algae and bacteria have seldom been reported and it is obvious that future studies on this type of bacterial-algal relationship will be rewarding. Our poor knowledge of synergistic relationships between algae and bacteria may explain why so few algal species have been successfully grown in axenic culture.

The partition culture experiments conducted in the present study revealed that the property of the bactosphere to promote algal growth is transmitted through a 0,2 μm Millipore filter but not through dialysis tubing. Based on this observation it is proposed that the substance/s promoting algal growth is a large molecule. Because this substance was not present in filtrates from xenic cultures it is further proposed that it is utilized rapidly by the alga and that it may be growth limiting.

A diagrammatic model for the proposed synergistic relationship between *P. pseudoparkeae* and bacteria in culture is given in Text Fig. 4.3. The model shows the algae and bacteria in PES medium. Though the alga undoubtedly utilizes PES nutrients the present study has shown that the medium does not satisfy the alga's growth requirements in axenic culture. Bacteria also utilize PES nutrients as they were shown to increase in numbers in PES medium which contained no algal cells.

The alga is surrounded by its phycosphere which contains algal extracellular products and O_2 released as a by-product of photosynthesis. The bacterium is surrounded by a bactosphere containing bacterial extracellular products and CO_2 which is a by-product of bacterial metabolism.



Text fig. 4.3 A diagrammatic representation of the proposed synergistic relationship between *P. pseudoparkeae* and bacteria in culture (see text for explanation).

Oxygen produced by the alga during photosynthesis is released into the medium. This dissolved O₂ may escape from the culture medium at the air/medium interface or it may be utilized directly by the bacteria. Similarly CO₂ release by bacterial respiration may be used by the alga directly or it may escape as gaseous CO₂ at the air/medium interface. Some O₂ evolved by the alga, or entering the medium from the air above the medium, may be utilized by respiratory processes in the alga if the demand is not met by intracellular levels of dissolved O₂.

The present study revealed that bacterial growth is enhanced in the presence of the alga. This may be due to increased O₂ levels in the medium resulting from algal photosynthesis or the bacteria may use extracellular products excreted by the alga. Hellebust (1965) and Craigie *et al.* (1967) have shown that *Pyramimonas* may excrete as much as 20% of the total carbon assimilated during photosynthesis. Of this 62% was recovered in the neutral fraction which contained predominantly (>70%) mannitol (Hellebust, 1965). This sugar alcohol excreted by *Pyramimonas* can be metabolized by many bacteria, including *Pseudomonas* (Doelle, 1975). Both *Pseudomonas* and *Caulobacter*, two organisms known to promote the growth of *P. pseudoparkeae*, can utilize glucose and mannitol equally well as a carbon source (Aken, unpublished). It is possible that the growth of *Pseudomonas* sp. was stimulated by mannitol excreted by *P. pseudoparkeae* when the two organisms were combined in culture.

Nothing is known about the extracellular products of the bacteria *Pseudomonas* and *Caulobacter* (or the marine ascomycete) which promoted the growth of *P. pseudoparkeae*. It is proposed that one or more organic compounds excreted by the bacterium into the bactosphere are required by *P. pseudoparkeae* for normal growth. The compound stimulating growth is probably a large molecule as it does not pass through dialysis membrane but does pass through an 0,2 µm Millipore filter.

This work on the interaction between *P. pseudoparkeae* and bacteria should be followed up because it is one of the few examples of a synergistic relationship between an alga and bacteria. Though the writer has no evidence that only selected species of bacteria promote the growth of *P. pseudoparkeae*, this needs to be tested. Bacterial identifications

should be made to species level if possible because different species in the same genus often behave differently and have different metabolic pathways. This may be important in trying to identify products in the bactosphere.

To identify the compound promoting growth of the alga, bacteria should be grown in seawater enriched with mannitol. After a few days the medium should be separated into different fractions (acidic, basic and neutral) using ion-exchange chromatography and the growth promoting properties of these fractions should be tested on *P. pseudoparkeae*. When the fraction promoting growth is found, more refined chromatographic techniques could be used to isolate and purify the compound for identification.

One point that has not been mentioned previously but which deserves consideration is that *P. pseudoparkeae* may be producing an autoinhibitor which prevents its growth in axenic culture. In xenic culture the bacteria may metabolize this inhibitor thus allowing the alga to grow. Though the presence of an autoinhibitor would appear to be suicidal, having apparently little ecological or evolutionary advantage, autoinhibitors are present in the Volvocales (Harris, 1970, 1971).

The work of Harris reveals some interesting facts that seem to indicate that an autoinhibitor is not operating in the phycosphere of *P. pseudoparkeae*. The first fact is that the autoinhibitors are produced or become active only when the cultures approached the stationary phase of growth. *P. pseudoparkeae* could not be grown in axenic culture so that dense axenic cultures in stationary phase of growth were not obtained. Secondly, Harris (1970) has shown that the autoinhibitory substance produced by *Platydorina* KOFOID is not metabolized by bacteria.

It is interesting that four species of *Pyramimonas* grown by Ricketts (1974) could not be maintained in axenic culture. It is apparent that the growth requirements of species in the genus are not understood and that this is an interesting topic for future research.

The close relationship between *P. pseudoparkeae* and bacteria is not surprising because the alga thrives in tidal pools that are nutrient rich and undoubtedly have large numbers of bacteria. On the Natal coast

P. pseudoparkeae has been isolated from tidal pools containing decaying sardine bait discarded by fishermen. Local inhabitants on the Natal coast drive their livestock along stretches of the beach and where faeces are deposited in tidal pools, *P. pseudoparkeae* grows particularly well. On the Cape coasts rotting seaweeds are often found in tidal pools containing *P. pseudoparkeae*. Bacteria are also abundant in these pools and may stimulate the growth of *P. pseudoparkeae*.

CHAPTER FIVE

CELL DIVISION AND SCALE PRODUCTION

5.1 INTRODUCTION

The primary objective of the growth studies presented in Chapter Three was to improve the growth rate of *P. pseudoparkeae* for this study on cell division and scale production. An earlier attempt to study scale production during the cell division cycle (Aken, 1978) was hampered because the generation time of the cells (\pm 33h) did not match the period of the 24h photoregime (16h:8h). This precluded the possibility of obtaining good synchronous cultures so that observations made at the ultrastructural level could not be related with accuracy to the time of day or stage in the cell division cycle.

The results of the growth studies presented in Chapter Three showed that the generation time of cells of *P. pseudoparkeae* could be brought close to 24h if the cells were grown in PES medium at a salinity of 35‰, a temperature of 25°C and a photoregime of 16h:8h (light intensity $150 \mu\text{E m}^{-2}\text{s}^{-1}$).

James (1966) has reviewed the many different methods for obtaining synchronous cultures and these are outlined below. Synchronous culture can be of two types, namely induced synchronous cultures or selection synchronous cultures. In induced synchrony, synchronous cultures can be obtained by (1) temperature methods - cultures can be subjected to repeating cold-warm cycles matching the generation time. Shifts in temperature from low to high levels also induce synchronous cell division with divisions occurring in the warm period. Temperature shocks can induce synchrony if rapid pulses of suboptimal and supraoptimal temperatures are given; (2) light methods - light/dark cycles can entrain cell division; (3) nutritional methods - growth in a basal medium followed by the addition of enriched medium may induce synchrony. Starvation followed by the addition of nutrients is also used to synchronize cells; (4) gasometric methods - single or multiple cycles of bubbling nitrogen then air through cultures can induce synchrony;

(5) inhibitory methods - here an inhibitor of cell division causes cells in a specific stage of the cell division cycle to accumulate.

In selection synchrony, synchronous cultures are obtained by (1) sizing methods - using filtration or centrifugation, cells of different sizes (reflecting cell age or stage in the cell division cycle) can be obtained and used as the starting material for synchronous cultures; (2) grow-off methods - parent cells are adsorbed onto filter packs so that daughter cells (in the same stage in the cell division cycle) are released at fission to form the starting material for synchronous cultures.

To attempt to improve the synchronous division of *P. pseudoparkeae* cells were grown under different L:D cycles, with changes in temperature during the L:D cycle, and with dilution of the medium once in every cell division cycle. These methods would fall into the induced synchrony methods given by James (1966). A completely synchronized culture is one in which all the cells (or > 98%) divide simultaneously or at least within 10% of the time of the cell cycle (Lorenzen and Hesse, 1974).

The aim of the work presented in this chapter is to record cell division in *P. pseudoparkeae* at both the light and electron microscope level and to study scale production in the cell division cycle in synchronous cultures. Scale production is construed by the writer to include the processes of scale morphogenesis, scale storage and scale release.

Cell division has been studied in detail in only two species in the genus, viz. *P. parkeae* (Pearson and Norris, 1975) and *P. amyliifera* (Woods and Triemer, 1981). Scale production and scale morphogenesis has been studied previously in *P. amyliifera* (Manton, 1966a) and *P. tetrarhynchus* (Moestrup and Walne, 1979). The latter studies on scale production and morphogenesis did not include frequent observations at different stages in the cell division cycle so that possible temporal changes in dictyosome activity were not recorded. Despite this Manton (1966a) proposed that scale production is a periodic process rather than a continuous one. Pienaar and Pearson (1976) have reported that cells of *P. pseudoparkeae* fixed shortly after cytokinesis have only flagellar scales in the dictyosomes and scale reservoir and that only after the cells have been exposed to long periods of light do body

scales appear on these organelles. These observations suggest that scales are produced rhythmically. One of the aims of the present study was to test this. In their detailed study of scale morphogenesis, Moestrup and Walne (1979) computed the number of scales covering the cells of *P. tetrarhynchus* but because they had no information on the growth rate of the cells, they could not determine the rate of scale production accurately. Because the generation time of *P. pseudoparkeae* is known under controlled conditions a computation of the number of scales surrounding the alga would allow a determination of the rate of scale production. This information would be valuable because there is very little information in the literature on the turnover times of dictyosomes in the algae.

5.2 MATERIALS AND METHODS

5.2.1 Synchrony induction experiments

In this study a number of experiments were conducted in an attempt to find a suitable synchrony induction regime to synchronize cultures of *P. pseudoparkeae*. Cells were cultured in 100 ml PES medium contained in 250 ml conical flasks and were exposed to various synchrony induction cycles. Cultures were illuminated with a light intensity of $100 \mu\text{E m}^{-2}\text{s}^{-1}$, measured during the photoperiod in the different synchrony induction cycles given in Table 5.1.

TABLE 5.1
Synchrony induction cycles.

Light regime	Temperature (°C)		Additional manipulations
	Light phase	Dark phase	
14h:10h	20	20	Culture diluted to half cell density at start of 16h light period
16h:8h	20	20	
16h:8h	20	20	
16h:8h	15	25	Culture diluted to half cell density at start of 16h light period.
16h:8h	15	25	

5.2.2 Preparation of cultures for a 24h study of cell division and scale production

Culture apparatus and culture conditions

The cultures used in this study were incubated at a constant temperature of 25°C in a 16h:8h photoregime (Light intensity 150 $\mu\text{E m}^{-2} \text{s}^{-1}$). The middle of the dark period corresponded to 24h00 (midnight). Cells were cultured in PES medium as shown in Table 5.2

TABLE 5.2

Differences between four cultures used in a 24h study of cell division and scale production

Culture	Photoregime	Culture volume (ml)	Inoculum density (cell no. ml^{-1})	Remarks
A	16h:8h	1000	50 000	Cultured aerated with 2% CO_2 in air.
B	16h:8h	500	50 000	Cells cultured in 1l Feinbach flask to give a large surface area for gaseous exchange.
C	16h:8h	500	100 000	"
D	continuous light	500	100 000	"

This experiment was conducted using relatively large volume cultures to ensure that sufficient material was available for ultrastructural studies on scale production and cell division. Because preliminary synchrony experiments had shown that cell division is best synchronized using a 16h:8h photoregime accompanied by dilution of the culture to half the cell density at 24h intervals, this method was adopted in this study. Since the cultures were relatively large-volume cultures, previous experiments having been conducted with small volume cultures, it was decided to vary some culture parameters as indicated in Table 5.2. It was hoped that one set of conditions would induce good synchronous cell divisions.

Culture D was kept under constant illumination to follow the course of cell growth in the absence of the L:D synchrony induction cycle.

All cultures were allowed to grow for 2 days under the respective light regimes before the 24h study was initiated. The cultures were diluted to half their cell density at 08h00 on the third day of growth (this time being 4h into the light phase). It should be noted that the 24h study was not initiated at the start of the 16h light period because observations on growth, cell division and scale production were required both before and after the 8h dark period.

5.2.3 Sampling and treatment of samples

Samples were withdrawn from the four cultures every two hours from 08h00 to 18h00 and then every hour from 18h00 to 06h00. The period of hourly sampling included two samples taken before the onset of the dark period and two samples taken after the onset of the following light period. An additional sample was taken at 08h00, at the start of the 4th day of growth, to complete the 24h study.

Samples were treated as follows:

i) Cell counts

At each sampling the cell density of the cultures was computed from cell counts obtained on a Coulter Counter. The 95% confidence limits of the mean of three separate counts made on a single culture at each sampling were computed and attached to the growth curves plotted.

ii) Cell volume measurements

Cell volumes were determined using the Coulter Counter model ZB₁ coupled with a Coulter Channelyzer Model P₁₂₈. Both instruments were calibrated according to the manufacturer's instructions. Cell volume data stored in the Channelyzer unit were plotted using a Teleprinter interface. Cell volumes were computed from the mode value on the graph using equations given in the manufacturer's manual.

iii) Light microscopy

Permanent slides were made by polymerising resin impregnated cells (which had been fixed at each sampling) on microscope slides. These slides were used for light microscopical studies on the process of cell division and in determining the proportion of cells undergoing cell division at each sampling time.

iv) Electron microscopy

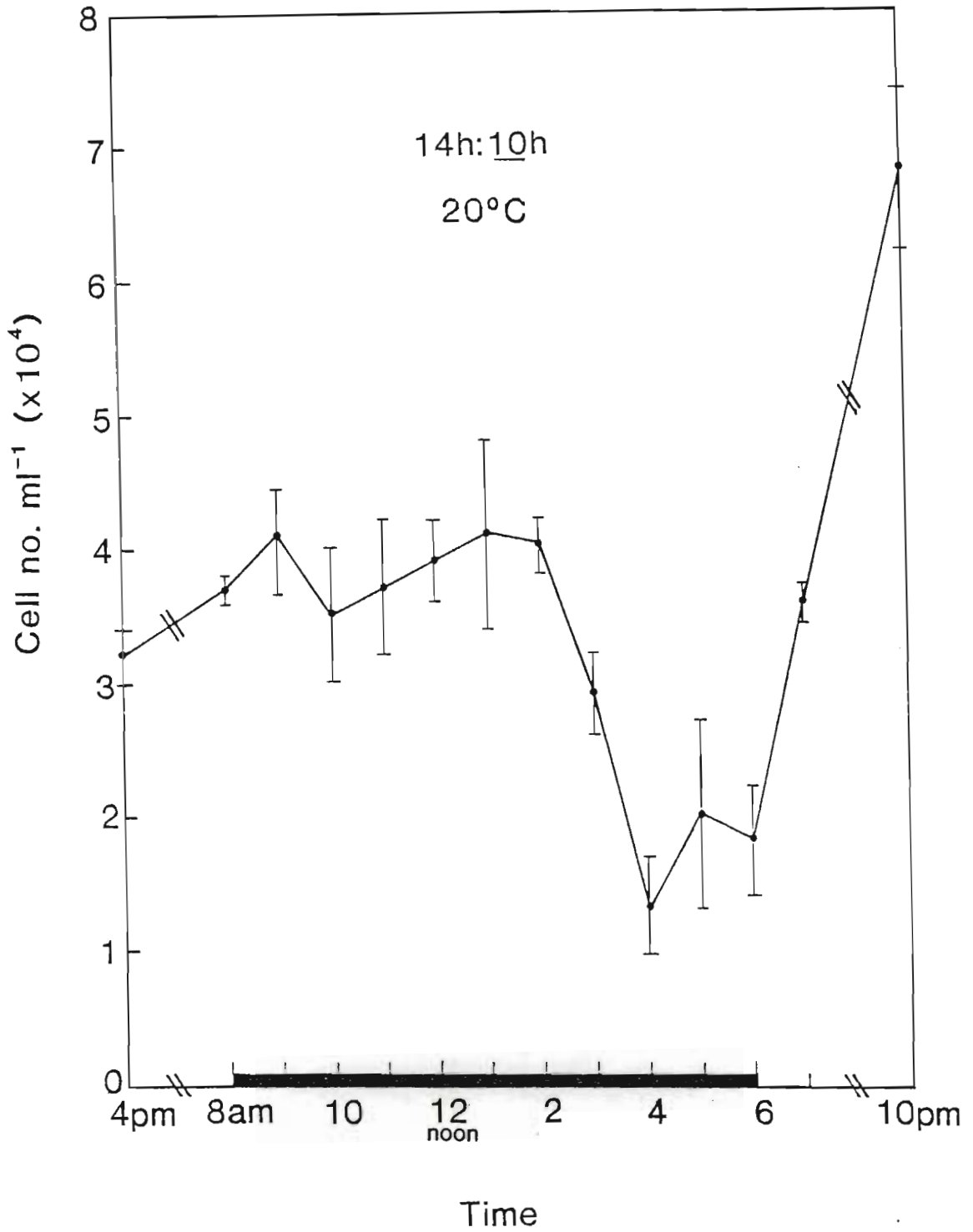
Cells harvested from cultures A, B and C at the different sampling times were processed for transmission electron microscopy. Ultrastructural observations on cell division and scale production were recorded photographically.

5.3 RESULTS

5.3.1 Synchrony induction experiments

In presenting the results of the synchrony induction experiments it is important to mention that this work was conducted before it was known that a light intensity of $150 \mu\text{E m}^{-2}\text{s}^{-1}$ and concomitant temperature of 25°C improved the growth rate of *P. pseudoparkeae* to match more closely the 24h period of the L:D cycle. The light intensity used in the synchrony induction experiments was $100 \mu\text{E m}^{-2}\text{s}^{-1}$ and the temperature was 20°C (or alternating 15°C and 25°C). Consequently the generation time of *P. pseudoparkeae* was longer than the 24h photoregime so that absolute synchrony was not expected. Cell numbers, therefore, could not be expected to double in any 24h L:D cycle.

The growth of *P. pseudoparkeae* under a 14h:10h photoregime (20°C) is shown in Text fig. 5.1. There was a small, but significant, increase in cell numbers in the 14h light period preceding the dark period depicted. Cell numbers remained constant during the first half of the dark cycle whereafter a significant decrease was recorded. When these cultures were viewed under red light small clumps of cells were seen scattered on the



Text Fig. 5.1 The growth of *P. pseudoparkeae* at 20°C under a 14h:10h photoregime. The culture was placed in a REVERSED L:D cycle so that the 10h light period began at 08h00 (the vertical bars represent the 95% confidence levels).

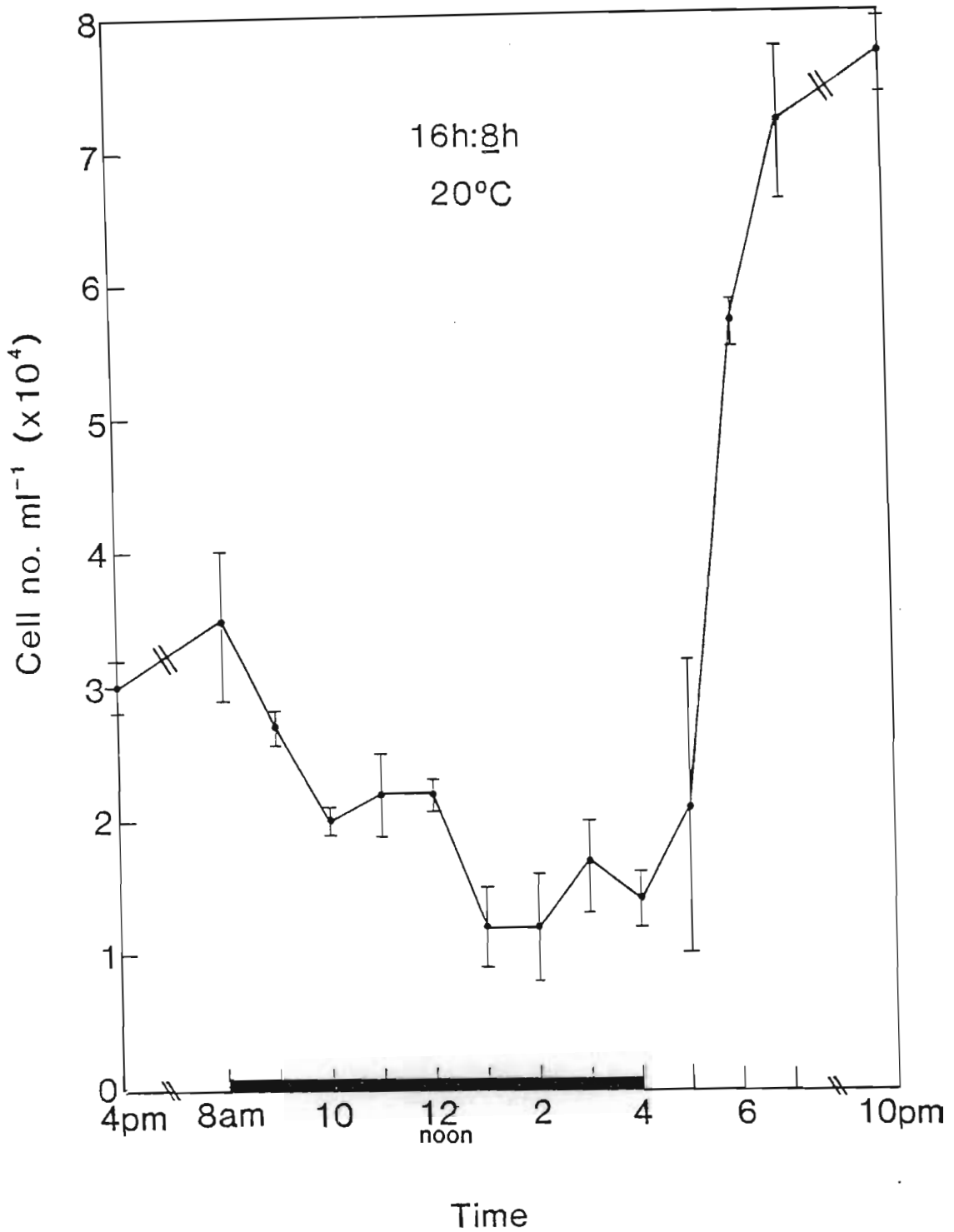
sides of the culture vessel. This settling phenomenon was unexpected because the alga usually remains motile throughout the cell division cycle. It is not known if cell division occurred in the second half of the dark period or whether it only occurred in the early part of the following light period where a significant increase in cell numbers was recorded.

The growth of *P. pseudoparkeae* at 20°C in a 16h:8h photoregime (Text fig. 5.2) was similar to that observed in the 14h:10h photoregime except that cell numbers declined throughout the dark cycle. This decrease was also caused by the settling of cells on the sides of the culture vessel. There was no significant increase in cell numbers during the 16h light period preceding the dark period. The extremely rapid increase in cell numbers after the dark period indicated that the cells had probably divided while they were settled and that the onset of the light period stimulated the cells to swim again.

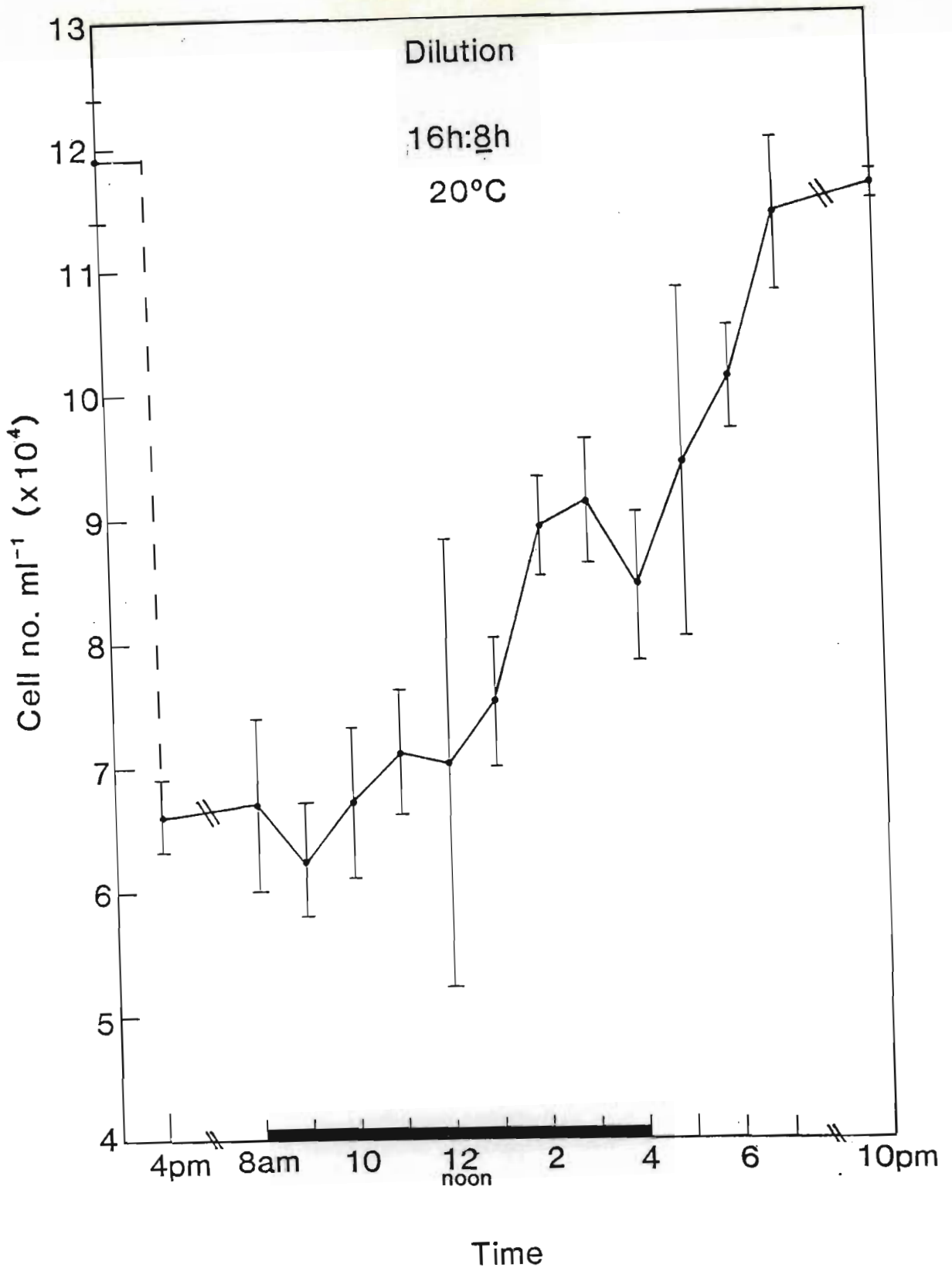
It is interesting that algal behaviour was different where a similar culture (incubated at 20°C in a 16h:8h photoregime) was diluted 16h before the dark period (Text fig. 5.3). In this culture the cells gradually increased in number over the dark period and into the following light period. There was no significant increase in cell numbers from the time of dilution to the start of the dark period. In this culture the dark phase appeared to trigger cell division.

Where *P. pseudoparkeae* was grown in a 16h:8h photoregime in which the temperature was varied with the L:D cycle (15 and 25°C, respectively) an unusual growth pattern was observed (Text fig. 5.4). Here there was a significant increase in cell numbers during the 16h light period preceding the dark period. After one hour into the dark period there was a small but significant decrease in the number of cells. This is presumed to have been caused by the settling phenomenon seen in other cultures. There was no significant increase in cell density from the beginning of the dark period to six hours into the following light period.

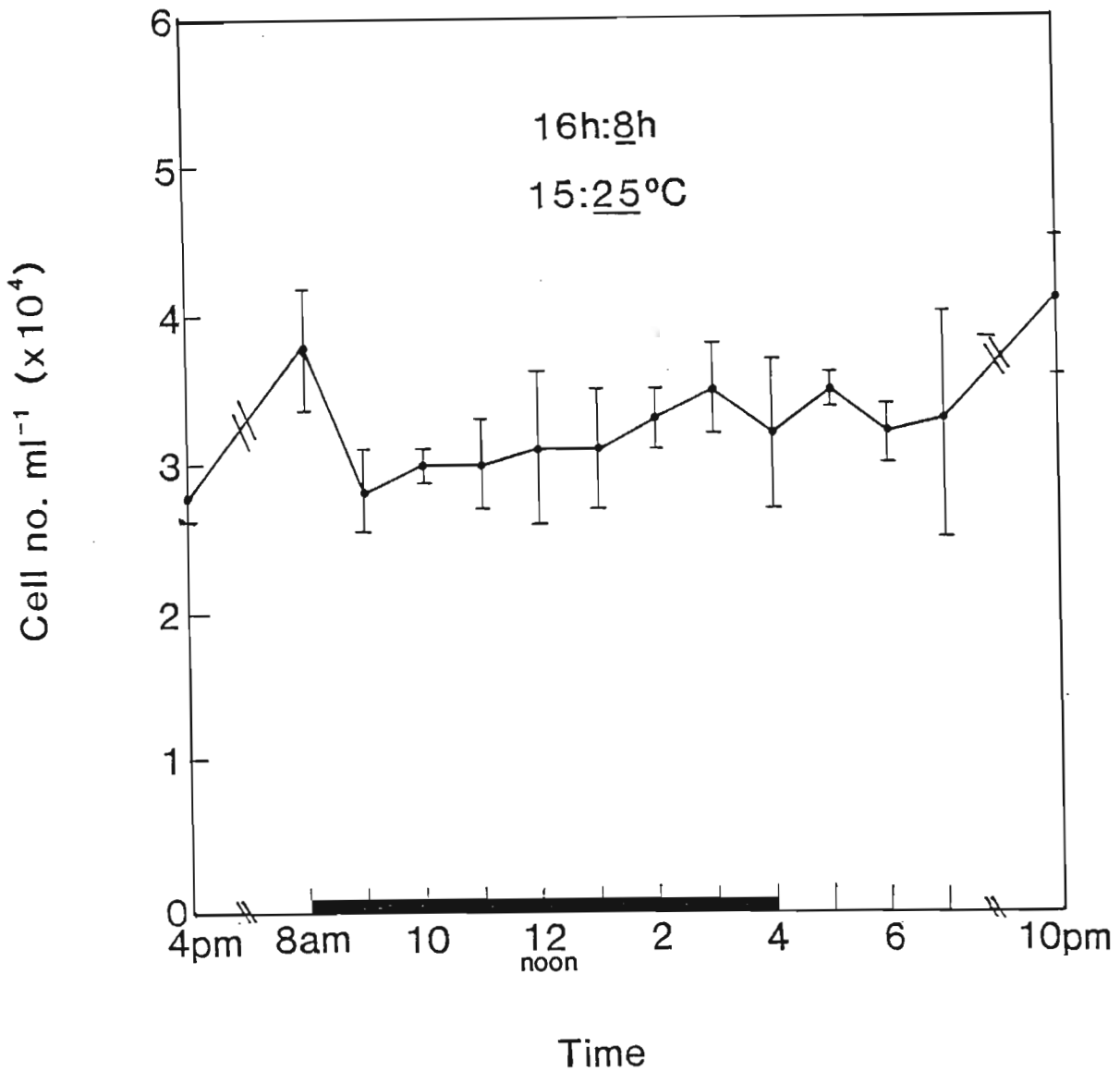
Where a similar culture (incubated in a 16h:8h photoregime with respective L:D temperatures of 15 and 25°C) was diluted at the beginning of the 16h light period the cells behaved differently (Text fig. 5.5). Though



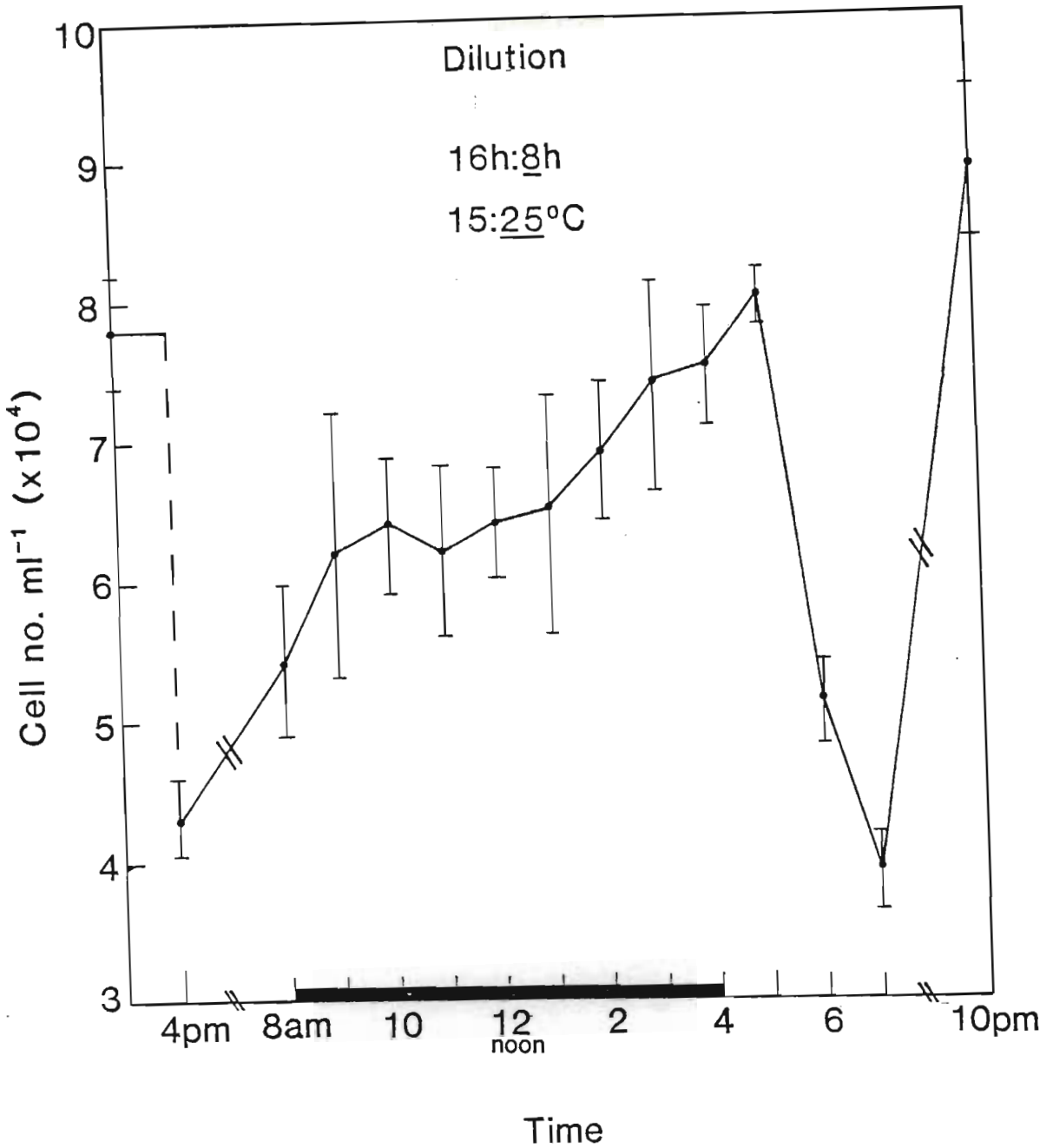
Text fig. 5.2 The growth of *P. pseudoparkeae* at 20°C under a 16h:8h photoregime, the culture was placed in a REVERSED L:D cycle so that the middle of the dark period corresponded with noon (12h00). (The vertical bars represent the 95% confidence limits).



Text fig. 5.3 The growth of *P. pseudoparkeae* at 20°C under a 16h:8h photoregime in which the culture was diluted at the start of the 16h light period. The culture was placed in a REVERSED L:D cycle so that the middle of the dark period corresponded with noon (12h00). (The vertical bars represent the 95% confidence limits).



Text fig. 5.4 The growth of *P. pseudoparkeae* under a 16h:8h photoregime with a superimposed temperature regime of 15°C and 25°C, respectively. The culture was placed in a REVERSED L:D cycle so that the middle of the dark period corresponded with noon (12h00). (The vertical bars represent the 95% confidence limits).



Text fig. 5.5 The growth of *P. pseudoparkeae* under a 16h:8h photoregime with a superimposed temperature regime of 15°C and 25°C, respectively; the culture was diluted at the start of the 16h light period. The culture was placed in a REVERSED L:D cycle where the middle of the dark period corresponded with noon (12h00). (The vertical bars reepresent the 95% confidence limits).

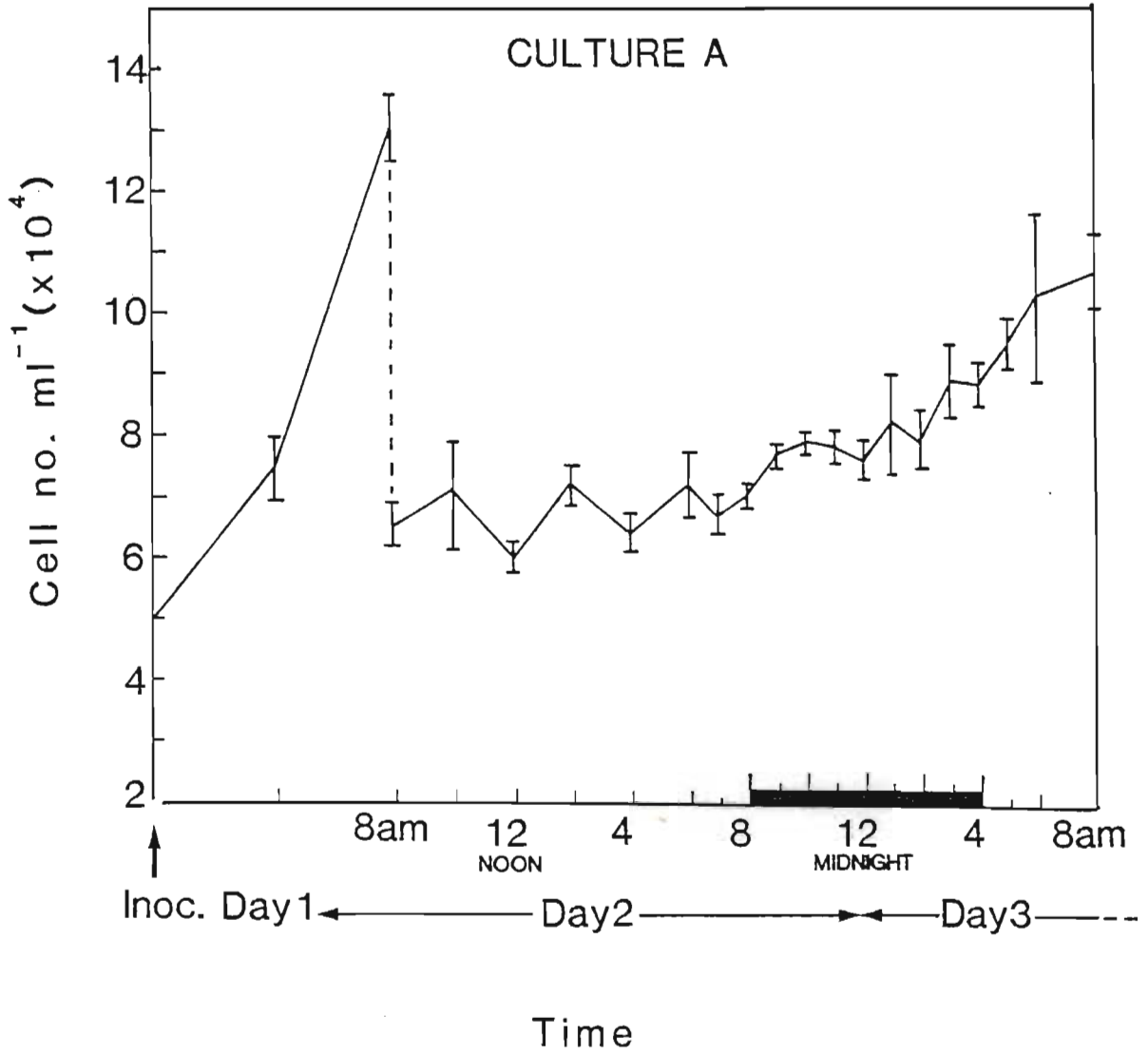
a significant increase in the number of cells was observed in the 16h light period preceding the dark period cell density increased significantly throughout the dark period. After one hour into the following light period cell density declined rapidly over a two hour period after which it increased rapidly. The lower temperature during the light phase may have caused the cells to settle out. Why the cells cultured in a similar synchrony induction regime did not settle out is difficult to explain (See Text fig. 5.4).

5.3.2 Culture characteristics during the 24h study period

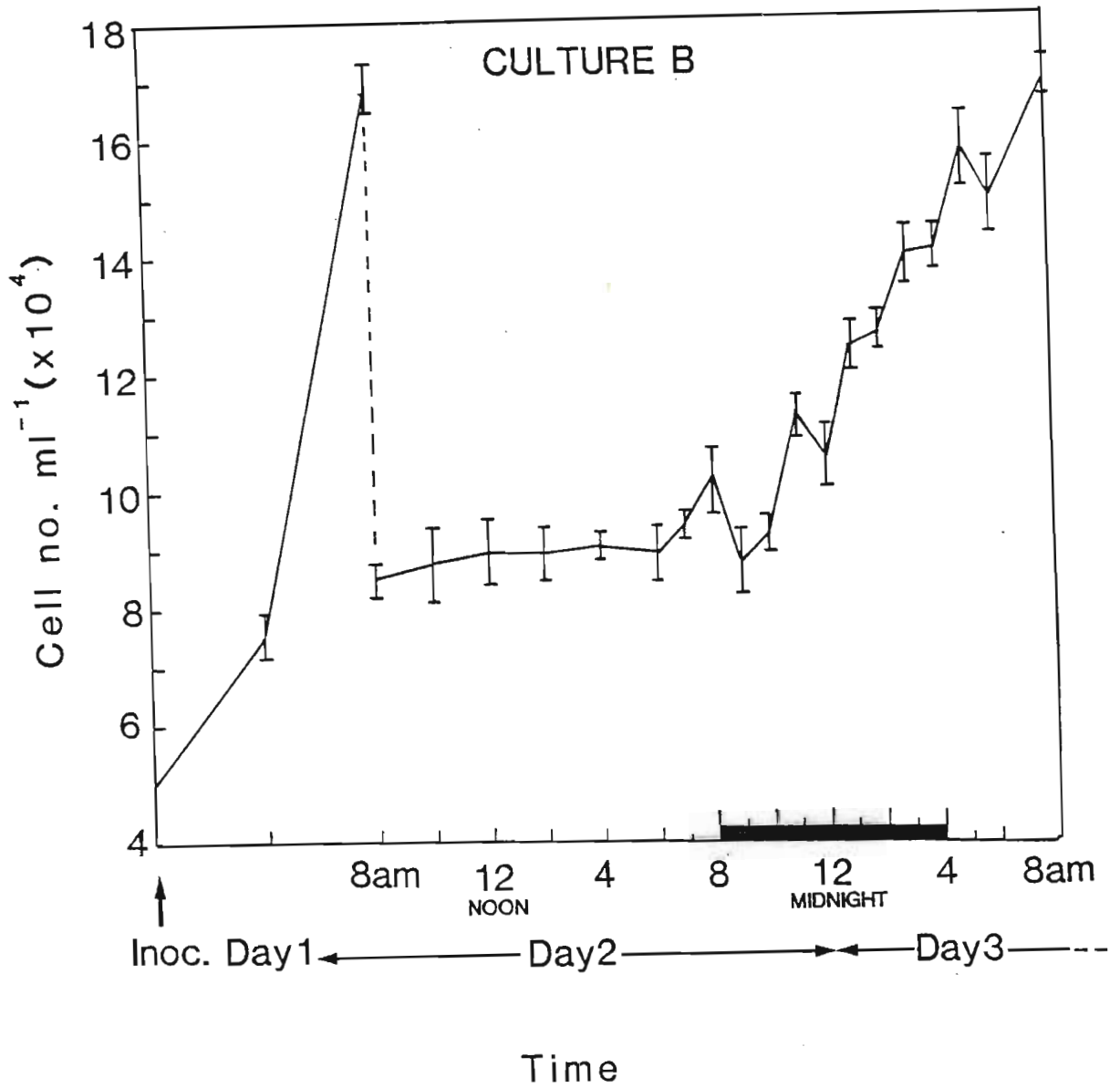
The growth of *P. pseudoparkeae* in the four cultures used in the 24h study (see Table 5.2) is shown in Text figs. 5.6 - 5.9. In Culture A (Text fig. 5.6) no significant increase in cell density was detected during the light period on Day 2. From the onset of the dark period there was a gradual increase in cell density so that significantly more cells were present in the culture at the end of the dark period than at the beginning. Cell density increased into the light period on Day 3. It is significant that the cell density did not double during the 24h study period.

In Culture B (Text fig. 5.7) there was no significant change in cell density during most of the light period on Day 2. However a small but significant increase was recorded at the start of the dark period. Cell density increased rapidly during the dark period and into the light period on Day 3. During the 24h period cell numbers doubled in Culture B indicating that the generation time of the cells had matched the period of the 24h photoregime. The rapid increase in cell numbers occurred over a period of ± 10 h so that the conditions under which Culture B was grown only induced partial synchrony.

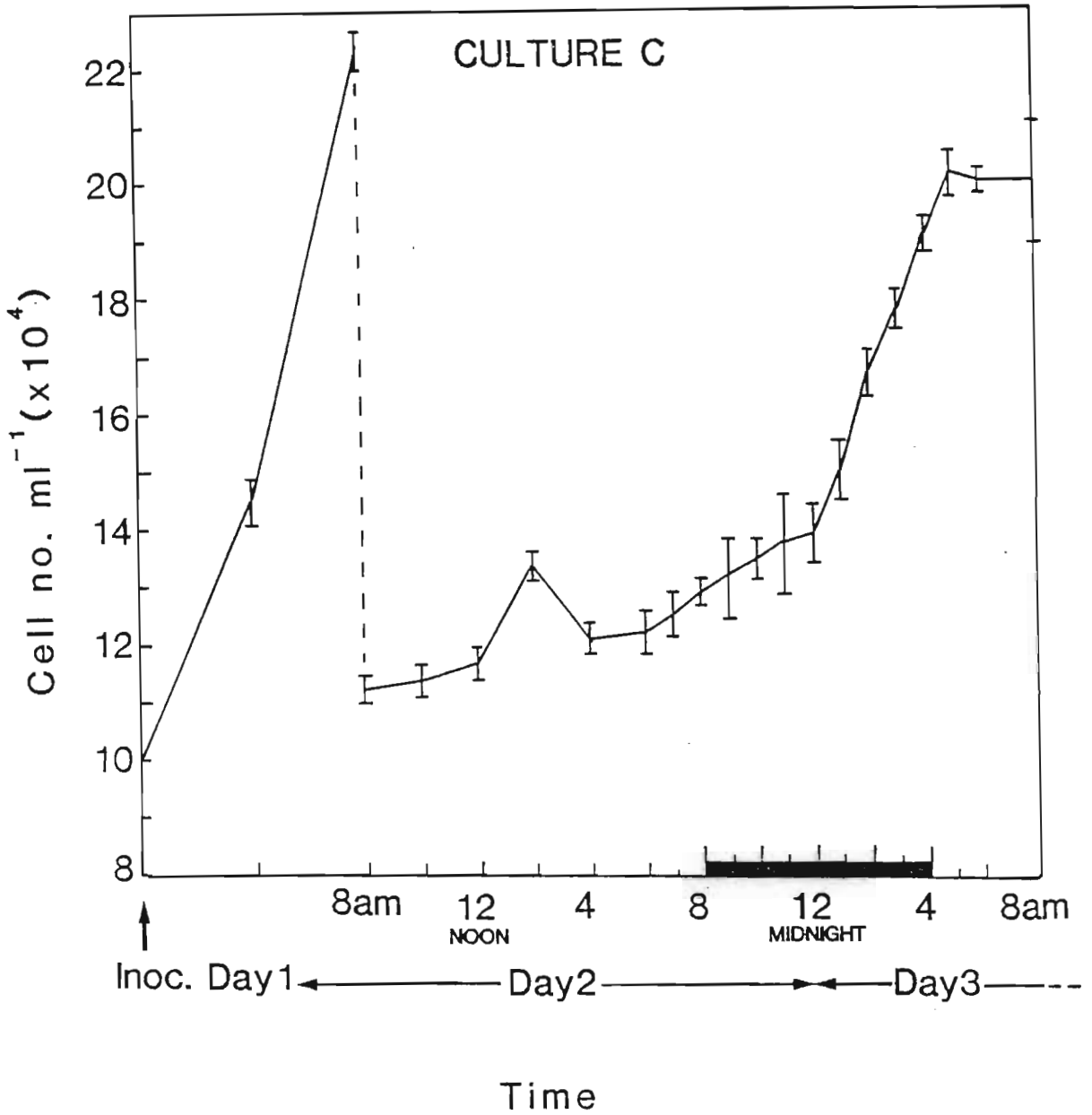
In Culture C there was a gradual increase in cell density during the light period on Day 2 (Text fig. 5.8). The single high count at 14h00 is thought to be spurious and could be attributed to human error in pipetting the incorrect volume of sample into receptacles for counting. Cell density increased gradually during the first half of the dark period but then increased rapidly during the second half of the dark period. No significant increase in cell density was recorded during the light period on Day 3.



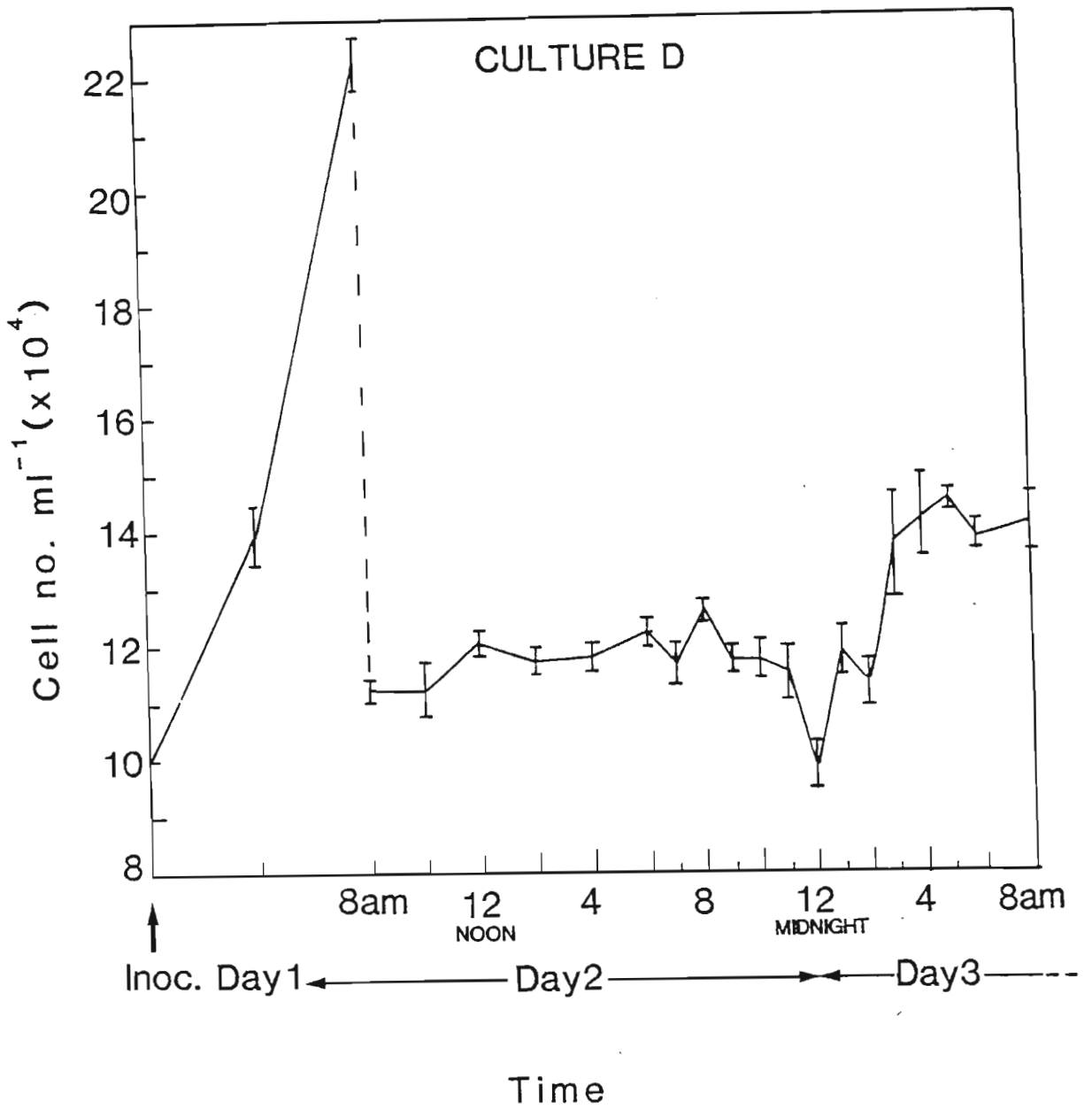
Text fig. 5.6 The growth of *P. pseudoparkeae* in Culture A.
 (16h:8h photoregime; temperature = 25°C).
 (Vertical bars represent the 95% confidence limits).



Text fig. 5.7 The growth of *P. pseudoparkeae* in Culture B.
 (16h:8h photoregime; temperature = 25°C).
 (Vertical bars represent the 95% confidence limits).



Text fig. 5.8 The growth of *P. pseudoparkeae* in Culture C.
 (16h:8h photoregime; temperature = 25°C).
 (Vertical bars represent the 95% confidence limits).



Text fig. 5.9 The growth of *P. pseudoparkeae* in Culture D.
 (Temperature = 25°C; constant illumination).
 (Vertical bars represent the 95% confidence limits).

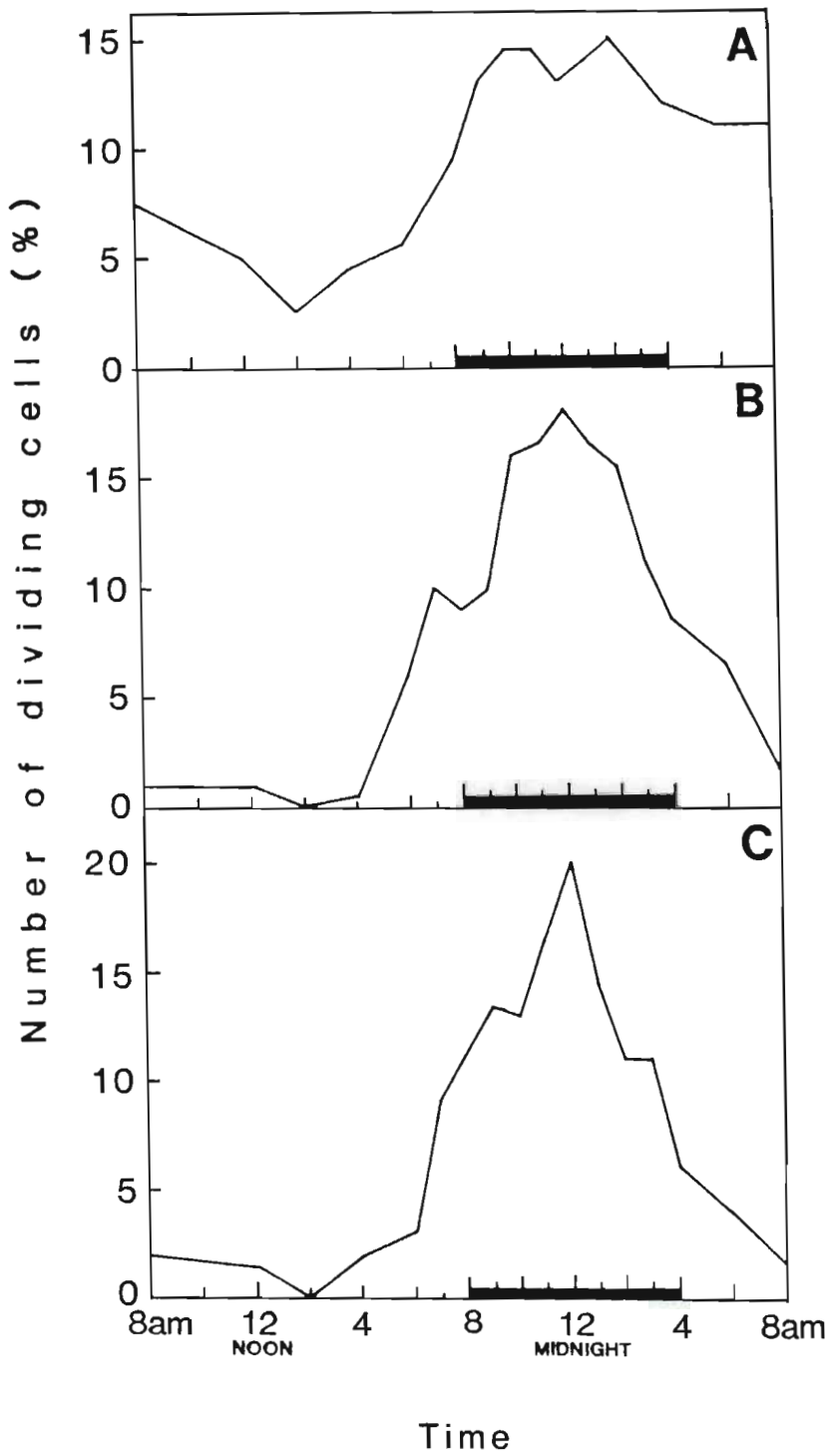
In Cultures A - C the onset of the dark period appeared to trigger cell division. In Culture D (Text fig. 5.9) where the culture was kept in continuous light after dilution no significant increase in cell density was recorded for eighteen hours (between 08h00 on Day 2 to 02h00 on Day 3). A small but significant increase in cell density was recorded after nineteen hours and this did not change significantly for the rest of the 24h study period.

It is interesting that in the absence of the dark period the cells were not stimulated to divide as vigorously as they did in the 16h:8h photoregime.

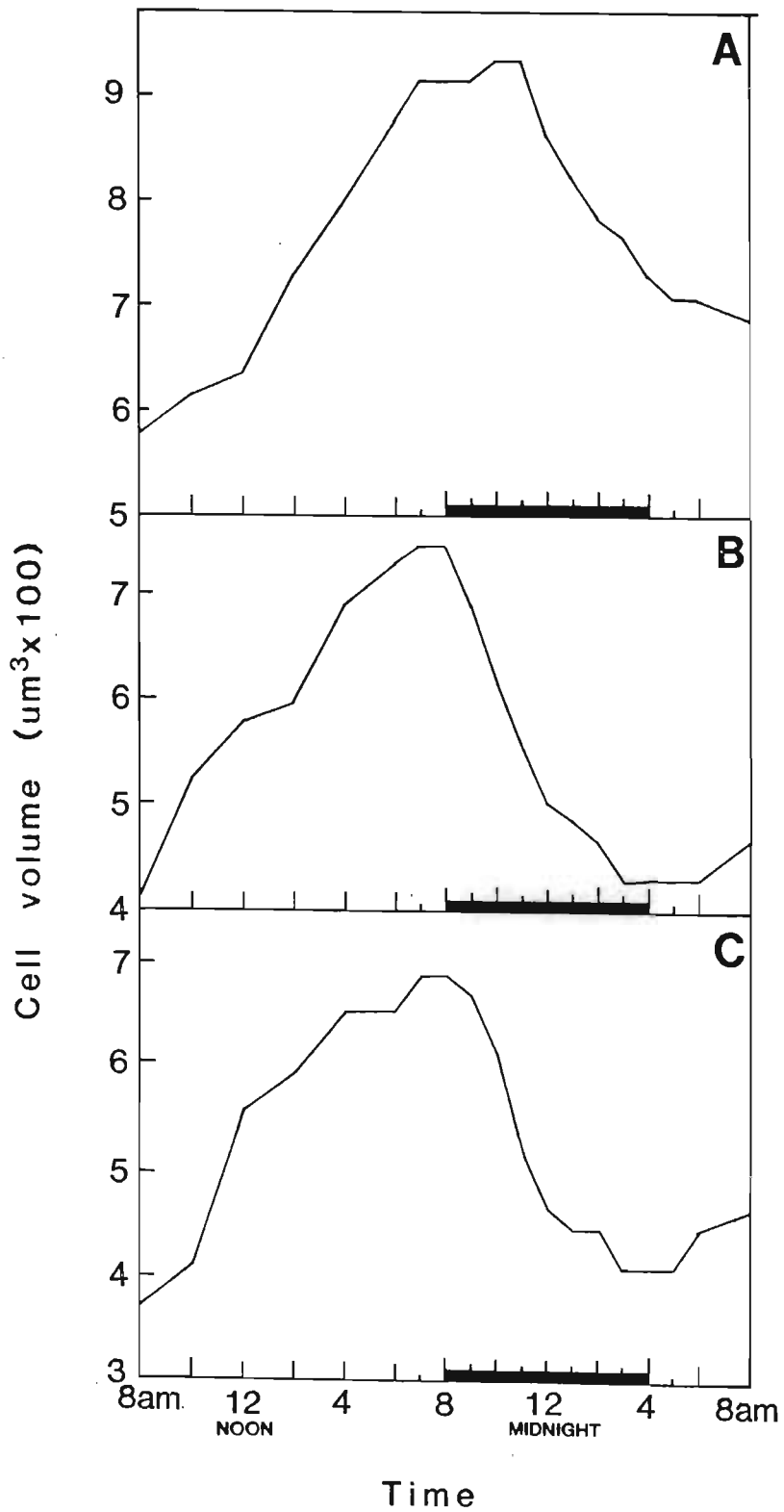
The number of dividing cells recorded in Cultures A - C at different times in the 24h study is presented in Text fig. 5.10. A similar pattern was seen in all three cultures with relatively few cells dividing during the first light period. In all cultures the maximum number of dividing cells was recorded in the middle of the dark period. If these data are compared with the growth curves in Text figs. 5.6 - 5.8 it can be seen that the most rapid increases in cell density were recorded after the maximum number of dividing cells were present. This reflects an increase in the number of daughter cells formed on completion of cytokinesis in the parent cells. In Culture A (Text fig. 5.10) the percentage of dividing cells does not decrease rapidly after the dark period as seen in Cultures B and C.

The values for the percentage of dividing cells present in Cultures A - C (Text fig. 5.10) appear to be low. These estimates are undoubtedly underestimates because early stages in cell division are easily overlooked unless the cells are orientated in the correct position. The earliest sign of impending cell division is the division of the chloroplast. If a cell is positioned so that the plane of division is parallel to the microscope slide then the division of the chloroplast is not obvious. The results presented in Text fig. 5.10 were valuable because they revealed in which samples the greatest number of dividing cells could be found for study at the ultrastructural level.

Cell volume changes recorded during the 24h study period, in Cultures A - C are presented in Text fig. 5.11. Cells in all cultures increased in cell volume during the light period and decreased after the onset of the



Text fig. 5.10 The number of dividing cells recorded at different times during the 24h study in Cultures A-C.



Text fig. 5.11 Cell volume changes recorded at different times during the 24h study in Cultures A-C.

dark period. In Culture A cell volume began to decrease three hours into the dark period whereas it decreased immediately at the onset of the dark period in Cultures B and C. Comparing these cell volume data with the data in Text Fig. 5.10 it is clear that as the percentage of dividing cells increases, cell volume decreases. This would be expected because daughter cells formed after cell division would have a smaller volume and would tend to reduce the average cell volume in the cultures. In Cultures B and C the cell volume decreased to a minimum at the end of the dark period whereafter it began to increase. The minimum cell volume measured at the end of the dark period was equivalent to that measured after dilution at the beginning of the 24h study. In Culture A the cell volume at the end of the 24h study was still decreasing and was higher than at the beginning of the study.

5.3.3 Cell division

Light microscopy

The first sign marking the onset of cell division is the elongation of an axial strand (the microbody). As the microbody elongates it extends into the chloroplast causing the chloroplast envelope to invaginate and in so doing the anterior starch grain capping the pyrenoid is cleaved (Pl. 5.1, fig. 1). As the microbody elongates further the invagination of the chloroplast envelope deepens to cleave the pyrenoid (Pl. 5.1, fig. 2).

Shortly after the division of the chloroplast the flagellar basal bodies replicate and four new flagella develop (Pl. 5.1, fig. 3). These grow in length until they are as long as the existing flagella. The appearance of the four new flagella is accompanied by a broadening of the base of the flagellar pit (Pl. 5.1, fig. 3). The basal bodies then separate and two new flagellar pits develop, each containing four flagella.

The nucleus is prominent at this stage and migrates to take up a position between the now widely separated basal bodies (Pl. 5.1, fig. 4). Accompanying the separation of the basal bodies is the appearance of a posterior cleavage furrow which separates the daughter chloroplasts in the region of the pyrenoids.

Division of the nucleus is not clear at the level of the light microscope though the anaphase stage can be discerned (Pl. 5.1, fig. 5). After the nucleus has divided an anterior cleavage furrow develops between the daughter nuclei as they enter telophase (Pl. 5.1, fig. 6). As cytokinesis progresses a "back-to-back" stage results (pl. 5.1, fig. 7). Cytokinesis is completed when the two cleavage furrows meet and two identical daughter cells separate.

Cell division in *P. pseudoparkeae* takes approximately 90 minutes from the time of the division of the chloroplast until the completion of cytokinesis.

Electron microscopy

Cell division in *P. pseudoparkeae* begins with the division of the chloroplast. The elongating microbody extends into the chloroplast causing the chloroplast envelope to invaginate anteriorly (Pl. 5.1, fig. 8; Pl. 5.2, fig. 1). A posterior invagination of the chloroplast envelope develops soon after the microbody extends into the chloroplast. Both invaginations cleave the anterior and posterior starch grains capping the pyrenoid. The mitochondrion is sometimes associated with the anterior invagination of the chloroplast envelope (Pl. 5.2, fig. 2).

Chloroplast division is completed when the anterior and posterior invaginations of the chloroplast envelope meet and fuse in the region of the pyrenoids (Pl. 5.2, fig. 3; Pl. 5.3, fig. 1). The nucleus is relatively large and occupies a central position within the cell (Pl. 5.3, fig. 1).

The flagellar basal bodies replicate soon after the chloroplast has divided (Pl. 5.3, fig. 2). The flagella grow in length until eight flagellar profiles are present in the flagellar pit (Pl. 5.3, fig. 3; Pl. 5.4, fig. 1). At this stage the cell still has two dictyosomes and the nucleus is in interphase. The scale reservoir remains connected with the flagellar pit via a duct as the base of the pit broadens and the nucleus enters preprophase (Pl. 5.4, fig. 2). The compound microtubular root (R_2c) surrounds the opening of the duct of the scale reservoir.

Numerous trichocysts are present around the flagellar pit indicating that they multiply at this stage by developing *in situ* (Pl. 5.5, fig. 1).

After the four new flagella have been produced the nucleus enters prophase and the chromatin begins to condense (Pl. 5.5, fig. 2). The dictyosomes divide during prophase (Pl. 5.5, fig. 3) so that the cells have four dictyosomes (Pl. 5.6, figs. 1 and 2). During prophase the nuclear envelope becomes progressively more vesiculate and at this stage the rhizoplast becomes microtubular and loses its characteristic cross striations (Pl. 5.5, fig. 2). The transformed rhizoplast appears to be the primary source of spindle microtubules. The base of the flagellar pit broadens as the basal bodies move apart and the nucleus moves to a position between the basal bodies as it enters metaphase. The nuclear envelope becomes more vesiculate at metaphase and soon disappears. At metaphase the chromosomal material is arranged in a metaphase plate which extends along the plane of cell division (Pl. 5.7, figs. 1 and 2; Pl. 5.8, figs. 1 and 2). Two lateral cleavage furrows develop at this stage and invaginate along the plane of cell division (Pl. 5.8, fig. 1).

Spindle microtubules radiate from the flagellar basal bodies and extend between these and the metaphase plate (Pl. 5.9, figs. 1 and 2). Although not always clear, some microtubules can be seen attached to kinetochores while others extend through the metaphase plate and are attached to the other basal body apparatus (pole-to-pole microtubules) (Pl. 5.9, fig. 2).

The chromatin mass separates at anaphase and a posterior cleavage groove develops (Pl. 5.10, fig. 2). A few microtubules are present in the interzonal region at anaphase but these disappear at telophase. Endoplasmic reticulum is seen along the anterior and posterior boundary of the anaphase "nucleus" (Pl. 5.10, fig. 2).

Anaphase and telophase stages were rarely seen and it is presumed that the separation of the chromatin and its compartmentalization into telophase nuclei is rapid. After the chromatin has separated during anaphase a cleavage furrow develops anteriorly to extend down the centre of the cell between the two flagellar pits (Pl. 5.10, fig. 1). It is interesting that the scale reservoir is still attached to one of the flagellar pits at this stage.

It is not known exactly when the second cell lobe acquires a scale reservoir but it must be during the late stages of cytokinesis because all newly formed daughter cells have the organelle. At telophase the daughter nuclei remain close to one another while the flagellar poles separate further (Pl. 5.11, fig. 1). There is no interzonal spindle or phycoplast between telophase nuclei (Pl. 5.11, fig. 2).

Cell division in *P. pseudoparkeae* is summarized diagrammatically in Text Fig. 5.12.

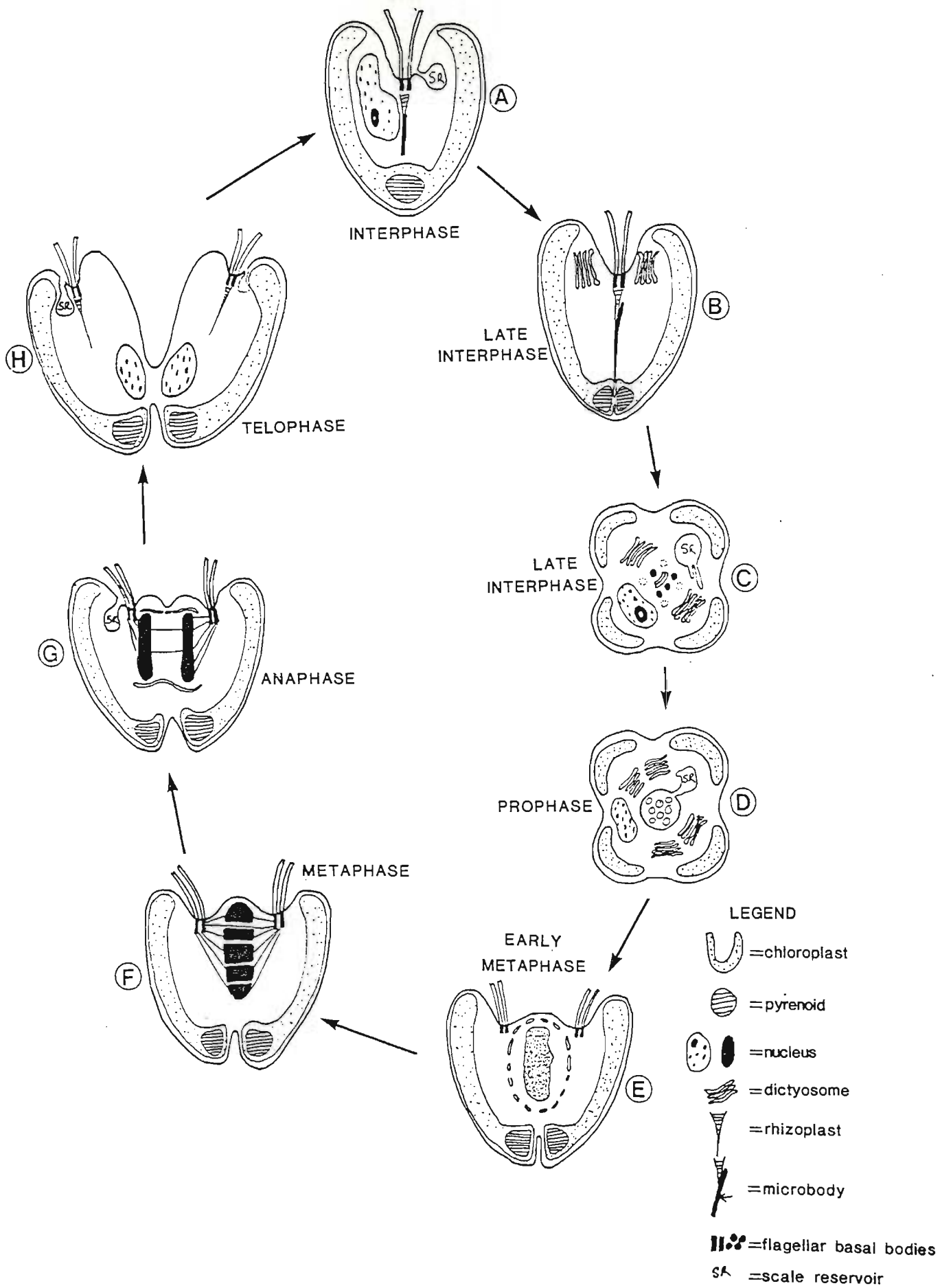
5.3.4 Scale production

One of the aims of this study on scale production was to determine if there is a rhythmic production of scales in the dictyosomes. After viewing many cells at different stages in the cell division cycle over a 24h period it was concluded that all scale types are produced continuously by both dictyosomes and that there are no apparent functional differences between the two dictyosomes. The results below therefore are not discussed with much emphasis on the stage in the cell division cycle or time in the 24h study period.

Scale production is construed by the writer to include three steps, viz. scale morphogenesis within the dictyosomes, scale storage in the scale reservoir, and scale release either directly by reverse pinocytosis at the plasmalemma or by release via the duct of the scale reservoir.

Scale morphogenesis

The two dictyosomes of *P. pseudoparkeae* exhibit marked polarity. The forming faces of the dictyosomes are closely associated with the peridictyosomal ER (refer to Pl. 2.15, figs. 1 and 2) and the cisternae of the dictyosomes are derived from this ER. The mature faces of the dictyosomes are directed toward the flagellar pit (refer to Pl. 2.15, figs. 1 and 2). In the following pages a section through the dictyosome including the forming face and mature face is termed a longitudinal section. A transverse section of the dictyosome is one taken parallel to the cisternae.



Text fig. 5.12

Each dictyosome is comprised of 20 cisternae though this number may vary between 16 and 24 cisternae per dictyosome (Pl. 5.12, fig. 1). Because of this variation, reference made to a particular cisterna (numbered 1 - 20 beginning at the forming face) is only an approximate indication of the position of the cisterna in the dictyosome. Generally the first 5 cisternae at the forming face did not contain any recognizable scale material though scale precursor molecules would undoubtedly be present in these cisternae (Pl. 5.12, fig. 1). Between cisterna 5 and cisterna 20 at the mature face of the dictyosome, scales were seen to develop progressively with more scale material being deposited on the growing scales (Pl. 5.12, figs. 1 and 2). At the mature face of the dictyosome the scales were completely formed and were released from the dictyosome in unit membrane bound vesicles (Pl. 5.12, fig. 2). A transverse section through the dictyosome at about the position of cisterna 6 shows numerous nascent scales (Pl. 5.13, fig. 1). There is some degree of organization of the scale material as soon as it is deposited or polymerized within the cisternae. A transverse section taken near the mature face of the dictyosome shows evidence of intracisternal differentiation with larger scales being produced towards the centre of the cisterna and smaller underlayer scales (B_1 , F_1 and F_2 scales) being produced in peripheral regions (Pl. 5.13, fig. 2). It is interesting that the larger scales always had a definite orientation within the dictyosomes. The B_3 , B_2 and F_3 scales always had their proximal surfaces directed toward the mature face of the dictyosomes (see B_3 , B_2 and F_3 scale profiles in Pl. 5.12, figs. 1 and 2). A longitudinal section through the dictyosome of a dividing cell shows that B_3 , B_2 , F_3 and underlayer scales may be formed within a single cisterna (Pl. 5.14, figs. 1 and 2). F_4 scales may also be formed in the same cisterna as other scales. F_4 scales are seen in the same cisterna as B_3 scales in Pl. 5.12, fig. 2. F_4 scales are usually formed towards the centre of a cisterna in groups of 3 to 6, arranged parallel to one another (Pl. 5.12, fig. 2).

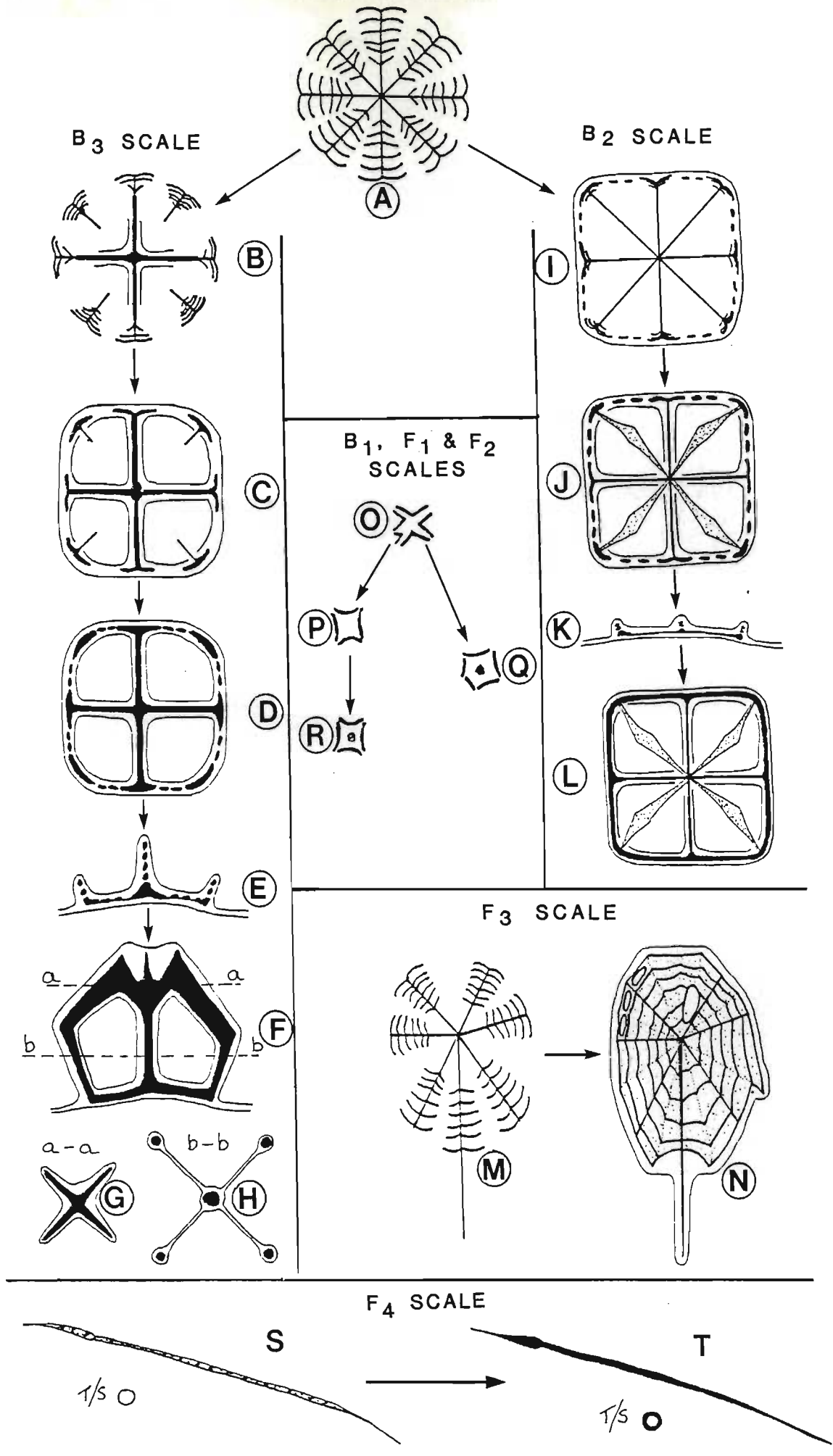
Underlayer scales did not have any fixed orientation within the dictyosomes so that the proximal surfaces of the scales were either directed toward the forming face (Pl. 5.14, fig. 3) or mature face of the organelle.

Details of the progressive morphogenesis of the different scale types within the dictyosomes are presented below.

The B_3 (and B_2) scales develop from a symmetrical scale precursor that resembles a snowflake and is termed the "snowflake structure" (Pl. 5.15, fig. 1; Text fig. 5.13A). The snowflake structure which develops in cisternae 6 or 7, is based on a symmetry of eight and has eight radiating arms of equal thickness and length. The arms bear smaller lateral appendages which are arranged pinnately along their length. The lateral appendages gradually increase in length toward the distal end of each arm. In the development of the B_3 scale the snowflake structure becomes markedly modified and provides a skeleton onto which additional scale material is deposited. When the scale has moved to the position of cisterna 10, four of the arms of the snowflake structure become thicker than the other (transient) arms to form a prominent cruciate structure (Pl. 5.15, fig. 2). Thickening of the cruciate structure apparently takes place by the incorporation of some of the pinnate appendages into the radiating arms and also by the deposition of additional scale material. As the cruciate structure emerges cisternal membrane begins to form a close association with the developing scale (Pl. 5.15, fig. 3; Text fig. 5.13B). The transient arms of the developing B_3 scale become detached proximally and shorten (Pl. 5.15, fig. 4; Text fig. 5.13B). The transient arms and their pinnate appendages condense to form the rounded corners of the B_3 scale (Pl. 5.15, fig. 5; Text figs. 5.13 C and D). Following the incorporation of transient arms into the base of the B_3 scale cisternal membrane is seen to lie close to the developing scale in all parts. It is interesting that the cisternal membrane, covering what will become the distal surface of the scale, is convoluted and follows the contours of the developing scale (Pl. 5.15, fig. 6). For convenience this membrane is termed the α cisternal membrane as it lies closer to the forming face. The membrane lying adjacent to the proximal surface of the scale is termed the β cisternal membrane. The latter membrane remains relatively flat and does not follow the contours of the scale and may therefore be functionally different.

The development of the B_3 scale up to the position of cisterna 15 is usually only two dimensional. From cisterna 16 - 19 the B_3 scale

snowflake-structure



Text fig. 5.13

develops three dimensionally to give the scale its typical coronate appearance (Pl. 5.15, figs. 7 and 8; Text fig. 5.13 E). Additional scale material is deposited in the region of the four peripheral vertical struts and the central vertical strut of the B_3 scale. Throughout the three dimensional growth of the scale the α cisternal membrane remains in close association with the scale. Transverse sections taken midway through the mature B_3 scale (Pl. 5.15, fig. 10; Text fig. 5.13 H) and through the distal region of the scale (Pl. 5.15, fig. 9; Text fig. 5.13 G) shows the intimate association between the α cisternal membrane and the scale.

When the B_3 scale reaches cisterna 20 it is completely formed (Pl. 5.15, fig. 11; Text fig. 5.13 F) and soon loses its close association with the α cisternal membrane. The cisterna at the mature face of the dictyosome becomes dilated and fragments to give rise to vesicles containing mature scales (see Pl. 5.12, fig. 2).

The B_2 scales, like the B_3 scales, develop from the symmetrical snowflake structure and therefore also have a symmetry based on eight. During the development of the B_2 scale the snowflake structure does not become as markedly modified as that giving rise to the B_3 scale. Most obvious is that the eight radiating arms are persistent in the B_2 scale. Four of the radiating arms become thickened while the other four (subordinate) arms remain less prominent (Pl. 5.15, fig. 2). At this stage the nascent B_2 and B_3 scales are indistinguishable.

Early in the development of the B_2 scale (in cisterna 10) the α cisternal membrane forms a boundary around the base of the B_2 scale (Pl. 5.15, fig. 12; Text fig. 5.13 I). The pinnate appendages of the eight radiating arms condense distally to form part of the base of the scale (Text fig. 5.13 I). When the developing B_2 scale reaches cisterna 15 the α cisternal membrane forms a close association with the developing B_2 scale in all parts (Pl. 5.15, figs. 13 and 15; Text fig. 5.13 J-L). The subordinate arms of the B_2 scale give rise to the knee-shaped substructures found within the four quadrats of the B_2 scale (Pl. 5.15, figs. 13 and 14; Text fig. 5.13 J and L). The knee-shaped structures are not bounded by the α cisternal membrane as they lie close to the β cisternal membrane at the proximal surface of the scale. At the position of cisterna 15 the

B_2 scale has developed in two dimensions only. Between cisternae 16 - 19 the scale grows three dimensionally with additional scale material being deposited to form the rim and the cross members of the scale (Pl. 5.15, fig. 6; Text fig. 5.13 K). Throughout this three dimensional growth (which is not as pronounced as in the B_3 scale) the α cisternal membrane remains in close association with the B_2 scale (Pl. 5.15, fig. 16). The B_2 scale is completely formed by the time it reaches cisterna 19. At the mature face (cisterna 20) the scale loses its close association with the α cisternal membrane and leaves the dictyosome in a vesicle cut off from the cisterna.

The F_3 or limuloid scale differs from the B_3 and B_2 scales in that it has a symmetry based on seven. The F_3 scale develops from an asymmetrical snowflake structure that appears in cisterna 6 (Pl. 5.15, fig. 17; Text fig. 5.13 M). This nascent F_3 scale occurs with the symmetrical snowflake structures giving rise to the B_3 and B_2 scales but it is easily distinguished because it possesses seven rather than eight radiating arms. One arm is longer than the others and ultimately forms the spine of the F_3 scale. All arms possess pinnately arranged lateral appendages.

The development of the F_3 scale is unusual because the pinnate appendages do not condense in the distal parts of the arms but rather they anastomose with corresponding appendages on adjacent arms to form a "spider web" structure (Pl. 5.15, fig. 18; Text fig. 5.13 N). This fusion of the pinnate appendages is completed by the time the scale is found in cisterna 12 in the dictyosome. At this stage the α cisternal membrane lies close to the developing scale. Between cisternae 13 - 19 additional scale material is deposited on the F_3 scale to fill in the spaces between the radiating arms and fused pinnate appendages. Except for the characteristic pores of the F_3 scale the scale develops into a solid plate (Pl. 5.15, fig. 19). The F_3 scale develops three dimensionally along one axis to give rise to the raised spine characteristic of the F_3 scale (Pl. 5.15, fig. 20).

The morphogenesis of underlayer scales (B_1 , F_1 and F_2 scales) is more difficult to interpret because they are so small. It appears that all underlayer scales develop from a scale precursor containing five components (Pl. 5.16, fig. 1; Text fig. 5.13, O-R). Underlayer scales develop in

peripheral regions of the dictyosome cisternae. Small ER-derived vesicles with dense contents fuse with the cisternae at the periphery of the cisternae and may contain precursor material for the underlayer scales (Pl. 5.16, fig. 1). During the development of the B_1 scale the two smaller components of the scale precursor appear to fuse to form one side of the B_1 scale. The remaining three components form the other three sides of the square B_1 scale (Text fig. 5.13 O and P). The F_2 scale, which is also square in plan view, develops in the same way as the B_1 scale. The characteristic raised central nodule of the F_2 scale develops after the sides of the scale have fused (Text fig. 5.13 O and R). It appears to be formed by the deposition of additional scale material and not from any of the components of the scale precursor. During the development of the F_1 scale each of the five components of the scale precursor form one side of the pentagonal F_1 scale (Text fig. 5.13 O and Q). The raised central spine of the F_1 scale develops like that described above for the F_2 scale.

The F_4 scale is not as complex as other scales and its development appears to be relatively simple. The scale develops from a linear strand of scale material that subsequently differentiates to form a hollow tube which has a thin wall (Pl. 5.16, fig. 2; Text fig. 5.13 S). Undifferentiated F_4 scales only appear in cisterna 10 and these develop quickly so that the scale is completed in cisterna 15. As the F_4 scale matures additional material is added to the wall so that this thickens. The scale remains tubular. The completed F_4 scales have a characteristic tapered head, short neck and long shaft and are usually seen arranged parallel to one another in the cisternae (Pl. 5.16, figs. 2 and 3, Text fig. 5.13 T).

Scale storage

In actively growing cultures of *P. pseudoparkeae* only flagellar scales are stored. Body scales move directly from the dictyosome to the plasmalemma. Flagellar scales migrate in vesicles from the mature face of the dictyosomes to the scale reservoir where they are released into the reservoir by reverse pinocytosis. The vesicle membrane appears to fuse with the membrane of the scale reservoir to increase its surface area. The scale reservoir is connected with the flagellar pit via a short duct so that the membrane of the scale reservoir is continuous with the

plasmalemma. The scales within the scale reservoir are therefore in reality outside of the cell. The scale reservoir is connected with the flagellar pit throughout the cell division cycle. The duct is persistent in interphase cells (refer to Pl. 2.6, fig. 2; Pl. 2.7, fig. 1; Pl. 2.16, fig. 1), in preprophase cells (refer to Pl. 5.6, fig. 2) and in cells undergoing cytokinesis (refer to Pl. 5.10, fig. 1; Pl. 5.11, fig. 1).

Although the scale reservoir contains only flagellar scales in actively growing cells, the writer has refrained from calling the organelle a flagellar scale reservoir (as in *P. amyliifera* - Manton 1966a) because the scale reservoir can accumulate body scales when cells age or when the cells prepare for encystment (see Chapter Seven).

The duct of the scale reservoir appears to be held open by a compound microtubular rootlet (R_2c) which emanates from between flagellar basal bodies 1 and 2 and bifurcates to surround the duct (refer to Pl. 2.9, figs. 4-6). A section through the scale reservoir duct shows that a thin layer of fibrillar material emanates from the R_2c rootlet which surrounds the duct (Pl. 5.16, fig. 4). This fibrillar material extends for a short distance just beneath the plasmalemma and along the membrane of the scale reservoir.

The scale reservoir increases in surface area throughout interphase so that just prior to the formation of the four new flagella a well developed duct complex is associated with the scale reservoir (Pl. 5.17, figs. 1 and 2; refer to Pl. 2.16, fig. 2). This duct complex usually contains flagellar underlayer scales only.

Flagellar scales are organized within the scale reservoir (Pl. 5.17, fig. 2). The underlayer scales (F_1 and F_2 scales) lie adjacent to the membrane of the scale reservoir and are overlain by the F_3 scale (Pl. 5.17, figs. 2 and 3). The F_3 scales are attached to the underlayer scales at their truncated end and have their spine projecting toward the centre of the reservoir. The F_4 scales have their tapered heads inserted between the underlayer scales and F_3 scales while their shafts extend towards the centre of the reservoir.

Scale release

The body scales of *P. pseudoparkeae* are carried in vesicles from the dictyosomes directly to the plasmalemma at the base of the flagellar pit (Pl. 5.18, figs. 1 and 2). The scales which are contained in membrane bound vesicles are released from the cell by reverse pinocytosis. The membrane of the scale vesicles is incorporated into the plasmalemma as the scales are released (Pl. 5.18, fig. 2). Body scales are carried singly or in groups to the cell surface. The body scales do not appear to be organized within the scale vesicles so it is clear that these scales are organized externally to form the scale-boundary. It is proposed (in Chapter Six) that the mechanism underlying the external self-assembly of the scale-boundary is one based on charge density differences between different scale types. Furthermore the scales may also have different patterns of surface charges which cause them to be arranged in their characteristic order on the cell surface.

Flagellar scales are released from the scale reservoir as the four new flagella are formed when the cell prepares for cell division. It is proposed that the membrane of the scale reservoir (with the associated scales) coats the flagella as they grow so that the membrane of the scale reservoir becomes the membrane covering the flagella. This is suggested because the surface area of the scale reservoir is markedly reduced after the four new flagella are completely formed (refer to Pl. 5.6, fig. 2). The extensive duct complex is absent in cells having eight flagella. It is obvious that some reorganization of scales must take place on the flagellar surfaces. The F_3 scales which were arranged perpendicular to the membrane in the scale reservoir lie flat against the underlayer scales on the flagella. The F_4 scales too must be reorganized on the flagellar surfaces to take up their characteristic distichous position along each flagellum. In the scale reservoir they were stored in dense groups.

The rate of scale production

The rate of scale production has not been computed previously for *Pyramimonas*. Two prerequisites for such a computation are needed; these are a knowledge of the total number of scales covering the cell surfaces

and a knowledge of the generation time of the cells. During the cell division cycle sufficient numbers of scales are produced to cover what is in essence a completely new cell. If the total surface area of a cell is known, along with the density of different scale types in different parts of the cell, then the total number of scales can be calculated. Growth studies have shown that under ideal conditions *P. pseudoparkeae* has a generation time of 24 h. Knowing the total number of scales on the cell and the generation time, the rate of scale production can be computed. The results of the surface area computations and determinations of scale densities are presented below.

The total surface area of a cell was computed from the diagram in Text fig. 5.14.

Surface area determinations:

Surface area of the cell body	=	503 μm^2
Surface area of the four flagella	=	<u>63 μm^2</u>
Total surface area of cell	=	566 μm^2

Scale density determinations:

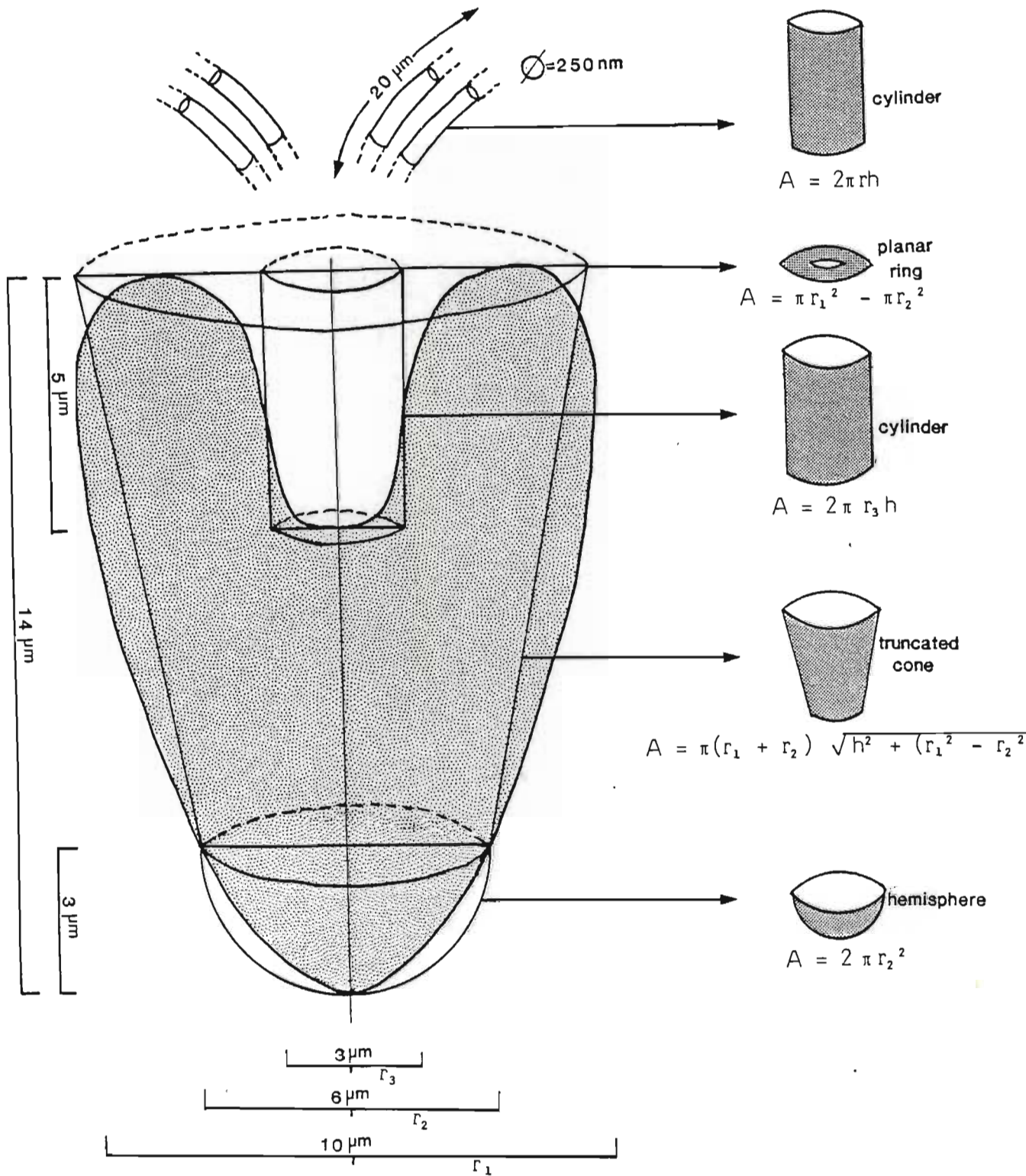
Body scales

B_1	-	480 scales per μm^2 x 503 μm^2	=	241 440 scales
B_2	-	30 scales per μm^2 x 503 μm^2	=	15 090 scales
B_3	-	30 scales per μm^2 x 503 μm^2	=	<u>15 090 scales</u>
Total number of body scales			=	271 620 scales

Flagellar scales

F_1 and F_2	-	428 scales per μm^2 x 63 μm^2	=	26 964 scales
F_3	-	33 scales per μm^2 x 63 μm^2	=	2 079 scales
F_4 scales	-	227 per flagellum (x 4)	=	<u>908</u>
Total number of flagellar scales			=	29 951 scales

Total number of body and flagellar scales = 301 571 scales



Text fig. 5.14 Diagram used to calculate the surface area of *P. pseudoparkeae*. The shaded areas of the geometric figures on the right represent the surface areas computed.

P. pseudoparkeae therefore produces 301 571 scales in 24 h (the shortest generation time obtained in some cultures). This is equivalent to 12 565 scales per hour or 209 scales per minute. Because *P. pseudoparkeae* has two dictyosomes that are continuously producing scales, each dictyosome would produce approximately 10⁴ scales per minute.

If it is estimated that each dictyosomal cisterna contains ca 52 scales (see scales in Pl. 5.13, fig. 2) and that the dictyosome comprises 20 cisternae, then one cisterna would be released from the dictyosome every 30 seconds. Thus a single cisterna would take 10 minutes to move from the forming face of the dictyosome to the mature face. Scale morphogenesis is therefore rapid with scales being constructed in approximately 10 minutes.

The above computation does not take into account the fact that the cell contains four dictyosomes for one hour prior to completion of cytokinesis. To obtain a more realistic figure for the rate of scale production the contribution of the two additional dictyosomes must be taken into account.

If a total of 301 571 scales are produced by *P. pseudoparkeae* in 24 h then over 23 h the two dictyosomes will produce 277445,32 scales or 6031,42 scales per dictyosome per hour. At the same rate of production four dictyosomes would produce 24125,68 scales in one hour. Adding 277445,32 and 24125,68 gives 301571 i.e. the total number of scales produced in 24 h.

If 6031,42 scales are produced per dictyosome in one hour then 100,52 scales are produced in one minute. Taking the previous estimate of 52 scales per cisterna then one cisterna would be released from the dictyosome every 31 seconds. If the dictyosome comprises 20 cisternae then it takes 10,3 minutes for a cisterna to move from the forming face to the mature face of the dictyosome. This would be approximately the time taken for a scale to be completely formed in the dictyosome. This value is not very different from that computed above where the productive potential of four dictyosomes for one hour of the cell division cycle was not taken into account.

The above calculations were made using the shortest mean doubling time (24h) obtained in some cultures. More often the cultures had a mean doubling time of 26h. Even with this increased generation time one cisterna would be released from the dictyosome every 34 seconds which means that the dictyosome turnover time (assuming 20 cisternae) is 11,3 minutes.

5.4 DISCUSSION

The preliminary synchrony induction experiments conducted to synchronize cultures of *P. pseudoparkeae* were not as successful as hoped because the cells did not always divide synchronously and in some cultures the cells settled out during the dark period. In other cultures the cells did not double during the 24h photoregime. These observations could be attributed to three possible factors. Firstly, the cultures were incubated in a reversed L:D cycle which may have worked against a natural or endogenous rhythm in the cells thus impairing synchrony. Furthermore, the stock culture used to provide the inocula for the experimental cultures was placed in a reversed L:D cycle for only two days before the experiment commenced. This period may have been too short to entrain the cells to divide synchronously in the new, phase-shifted (reversed) photoregime.

As mentioned previously the other factor that could account for the fact that the cells did not double in number during the 24h photoregime was that they were not grown under conditions giving a generation time equivalent to the period of the photoregime (20°C or 15–25°C with a light intensity of $100 \mu\text{E m}^{-2} \text{s}^{-1}$). Under these conditions the cells would have had a generation time of ca. 33h which would not have matched the period (24h) of the L:D cycles used. This would also result in a loss of synchrony.

It was interesting that the cells grown in two of the reversed L:D cycles (14h:10h and 16h:8h at 20°C) settled out during the dark period. This was unexpected because *P. pseudoparkeae* usually remains motile throughout the cell division cycle, at least in laboratory cultures. As mentioned above this behaviour could possibly be attributed to an antagonistic interaction between an entrained or endogenous rhythm and the imposed (phase-shifted) rhythms of the reversed L:D cycle. Despite the fact that *P. pseudoparkeae*

remains motile throughout the cell division cycle in the laboratory, settling must be an important part of the behaviour of the cells in the natural environment. To avoid being washed out of their tidal pool habitat cells would have to settle out in or on the substratum of the tidal pool. Settling would probably be a more frequent occurrence in cells inhabiting tidal pools in the lower littoral where neap tides would flush the pools at every high tide. Higher up the littoral settling may be less frequent perhaps occurring only during the periods of spring tides. Studies on the settling behaviour of *P. pseudoparkeae* in the field would provide interesting data on the autecology of the alga. It would be especially interesting to know how the settling behaviour is phased with the cell division cycle and if the latter is phased with a tidal cycle.

Grant and Vadas (1976) have reported that *Tetraselmis* shows a diurnal settling rhythm which is phased with the cell division cycle. Toward the end of the light phase, in a 12h:12h photoregime, the cells settle out and lose their flagella. Cell division occurs during the dark phase.

The most promising synchrony induction regime used was that in which the cells were grown in a 16h:8h photoregime with the culture diluted to half cell density at the start of the light period. By diluting the culture the settling behaviour of the cells in the dark was counteracted. Temperature shifts during the L:D cycle did not appear to enhance synchronous cell divisions.

Synchrony has not been studied in many prasinophycean alga. To the writer's knowledge synchronous cell division has only been studied in *Tetraselmis* (Grant and Vadas, 1976; Ricketts, 1977). This alga could be brought into synchronous division in a 14h:10h or 12h:12h photoregime at 20°C. Ricketts (1977) showed that synchrony was improved where the cultures were diluted to half the cell concentration once every 24h.

The cultures used in the 24h study on cell division and scale production showed some interesting trends. Cells in Cultures A-C, which were exposed to a 16h:8h photoregime, divided preferentially during the dark phase. Cell numbers remained more or less constant during the preceding light period. Cells in Culture D, which was kept in continuous

light, divided approximately four hours after the cells in the L:D cycle. It was especially interesting to note that relatively few cells (about 25%) in Culture D divided during the 24h period. These observations suggest that under continuous light *P. pseudoparkeae* may have a natural (endogenous?) cell division cycle longer than 24h and that cell divisions are entrained to a shorter period in a 16h:8h photoregime where the dark phase triggers cell division.

Microalgae can generally be separated into two groups according to the timing of cell division in L:D cycles (Nelson and Brand, 1979). Diatoms and dinoflagellates tend to divide during the light phase while other taxa divide preferentially during the dark phase. *P. pseudoparkeae* belongs in the latter group.

In Culture B the cell numbers doubled during the 24h study period indicating that the mean generation time (24h) matched the period of the 24h L:D cycle. In Cultures A and C the cell numbers did not double during the 24h study period. This could be attributed to self-shading in both cultures. In culture C the high concentration of cells may have caused self-shading even in the relatively shallow Feinbach flask. In Culture A, which had approximately half the concentration of cells in Culture C, self-shading could have resulted from the larger culture volume and type of culture vessel used. The 1l culture was contained in a conical flask so that cells in the centre of the culture may have received less light. Although the cultures were mixed (by aerating) the average irradiance received by cells during the study period may have been lower than that experienced by cells contained in the Feinbach flasks.

Cell division in *P. pseudoparkeae* is essentially identical to that described for *P. parkeae* (Pearson and Norris, 1975) and *P. amyliifera* (Woods and Triemer, 1981). In all three species the chloroplast divides first. This is closely followed by the replication of the flagellar basal bodies and dictyosomes. Mitosis is characterized by an open spindle in which the flagellar basal bodies act as poles. In *P. pseudoparkeae* the rhizoplast is transformed into spindle microtubules but in *P. amyliifera* and *P. parkeae* this organelle is reported to be persistent during mitosis. Microtubules may extend from pole to pole or they extend from the poles to chromosomes where they are attached at the kinetochores.

P. parkeae and *P. amyliifera* have a persistent interzonal spindle in early telophase but this disappears by late telophase. In *P. pseudoparkeae* an interzonal spindle was seen during anaphase but because no stages of early telophase were seen it is not clear when these disappear. They are not present at late telophase. Cytokinesis is effected by anterior and posterior, as well as lateral cleavage furrows.

Cell division has been studied in three other genera in the Prasinophyceae, namely, *Pedinomonas* (Pickett-Heaps and Ott, 1974), *Nephroselmis* (Mattox and Stewart, 1977) and *Tetraselmis* (Stewart *et al.*, 1974).

In *Nephroselmis* and *Tetraselmis* the nuclear envelope disperses during mitosis so that an open spindle is formed. In this respect the two genera resemble *Pyramimonas*. *Pedinomonas* is unusual in that it has a closed spindle. This condition is thought to be more primitive than the open spindle (Pickett-Heaps and Ott, 1974).

The rhizoplast disappears by metaphase in *Pedinomonas*, *Nephroselmis*, *Tetraselmis* and *P. pseudoparkeae*. The present work supports the view of Stewart and coworkers (Stewart *et al.*, 1974) that the rhizoplast is involved in spindle formation. In this regard it is difficult to explain why the rhizoplast is persistent in *P. parkeae* and *P. amyliifera*. The organelle becomes somewhat reduced in these species so that it may contribute in part to the spindle microtubules.

Pedinomonas, *Nephroselmis* and *Pyramimonas* have a persistent spindle at telophase which allies these species with the Charophyceae and Ulvophyceae. *Tetraselmis* is unusual in that it has a collapsing spindle at telophase and the cell develops a phycoplast between the telophase nuclei. The presence of a phycoplast allies *Tetraselmis* with the Chlorophyceae.

Scale production has been studied in detail in only two other species in the Prasinophyceae: both belong to the genus *Pyramimonas*. In 1966 Manton (1966a) described scale production in *P. amyliifera* and more recently Moestrup and Walne (1979) have studied scale morphogenesis in *P. tetrarhynchus*.

Manton (1966a) reported that body scales were most abundant in the Golgi cisternae in day time fixations thus implying that body scale production was a rhythmic process. This observation would further imply that there is some temporal change in dictyosome functioning. Because Manton (1966a) had not observed the process of body scale liberation she suggested that the process was also rhythmic. Contrary to Manton's observations, the present study on scale production in *P. pseudoparkeae* revealed that body scales (and flagellar scales), are produced continuously over a 24h cycle by both dictyosomes. There is also a continuous liberation of body scales from the cell. Body scales are released at the base of the flagellar pit by a process of reverse pinocytosis. The membrane of the vesicles carrying body scales from the dictyosome to the cell surface fuses with the plasmalemma as the scales are released.

Other observations support the interpretation that body scale production and release is a continuous process rather than a rhythmic one. Body scales were always present in the dictyosomes at any stage in the cell division cycle. Because body scales are not stored by the cell they are undoubtedly released as they are produced. Cells of *P. pseudoparkeae* increase in volume (and therefore surface area) throughout the cell division cycle. Since no cells have been found with only a partial scale-covering it is clear that body scales are released continuously to cover new cell surfaces formed as the cell volume increases prior to division.

The scale reservoir found in *P. amyliifera* is unusual in that it has internal villi and stores exclusively flagellar scales (Manton, 1966a). The organelle has been called a flagellar scale reservoir. The scale reservoir in actively growing cells of *P. pseudoparkeae* does not have any internal villi though it also stores predominantly flagellar scales. The term flagellar scale reservoir, however, cannot be used for *P. pseudoparkeae* because the organelle may store body scales when the cells age in batch cultures.

In her work on *P. amyliifera*, Manton (1966a) did not know if the scale reservoir was a permanent organelle in the cell and if it was permanently or temporarily attached to the flagellar pit. Manton concluded that the connection between the scale reservoir and flagellar pit was

temporary because fewer cells fixed in the daytime had the scale reservoir connected with the flagellar pit. The present study showed that the scale reservoir of *P. pseudoparkeae* is a permanent feature of the cell being present, with some modification in size, throughout the cell division cycle. The organelle is always connected with the flagellar pit.

Manton's suggestion that the liberation of flagellar scales through the duct of the scale reservoir is a rhythmic process is supported by the present study. Flagellar scales that are produced continuously by the dictyosomes accumulate in the scale reservoir and are released over a short period when the four new flagella are formed during cell division.

It has been calculated that the four flagella of *P. pseudoparkeae* bear a total of 29 951 flagellar scales. If the dictyosomes were to produce these scales during the period taken the four new flagella develop (about 20 min) then they would each have to produce 7500 scales per minute. It is inconceivable that the dictyosomes could meet this demand. The evolution of the scale reservoir appears to be a logical way of overcoming the great demand for specific scale types in a relatively short period in the cell division cycle. Flagellar scales produced by the dictyosomes throughout the cell cycle can accumulate in the scale reservoir to be released only when needed.

The process by which the flagellar scales reach the flagellar surfaces is not properly understood though it is clear that the flagellar scales leave the scale reservoir via the duct leading to the flagellar pit. Because there is a marked reduction in the size (surface area) of the scale reservoir when the four new flagella are formed, it is proposed that the membrane of the scale reservoir is extruded to become the membrane surrounding the developing flagella. There is no reason to believe that the association of flagellar scales with the scale reservoir membrane is lost during the process, so that the flagella would become covered with scales (with some reorganization) as they grow in length.

The compound microtubular rootlet (R_2c) with its elaborate structure and close association with the duct of the scale reservoir must have some functional significance. Besides helping to anchor the flagellar basal

bodies within the cell, the writer believes that the R_2c root plays an important role in the rhythmic release of flagellar scales. Being microtubular the root may be contractile and could serve to draw membrane (along with scales) from the scale reservoir to cover the developing flagella. The smaller fibrils associated with the microtubules surrounding the duct of the scale reservoir may be important in determining which part of the scale reservoir membrane is extended and in which direction it moves. It is reasonable to assume that the membrane would be needed only at the base of the flagellar pit where the new flagella develop.

The morphogenesis of different scale types in *P. pseudoparkeae* is essentially identical to that described for the scales of *P. tetraarhynchus* by Moestrup and Walne (1979). In both species the B_3 and B_2 scales have a symmetry based on the number eight while the F_3 scales have a symmetry based on the number seven. The underlayer scales in both species develop from a scale precursor composed of five components. The small size of these scales, despite their apparently simple construction, makes it difficult to trace different stages in their construction. More information needs to be accumulated on the morphogenesis of the underlayer scales (B_1 , F_1 and F_2 scales).

The rate of scale production has not been determined previously for *Pyramimonas* or any other prasinophyte. Moestrup and Walne (1979), who showed that a cell of *P. tetraarhynchus* is covered with more than 370 000 scales, suggested that scales would take about 90 minutes to be formed within the dictyosomes assuming a generation time of 24h and a continuous production of scales.

This may be an underestimation of the productive potential of the Golgi apparatus because the present study has shown that scales may be completely formed within the dictyosome in a period of only 10-11 minutes. This period represents the turnover time of the dictyosomes.

CHAPTER SIX

CHEMICAL COMPOSITION OF SCALES

6.1 INTRODUCTION

Members of the class Prasinophyceae have been the subjects of many detailed structural, developmental and biochemical investigations. During the course of these studies a number of authors have realized the need for understanding the chemical composition of scales and have drawn attention to this (Moestrup and Walne, 1979; Domozych *et al.*, 1980; Melkonian and Robenek, 1981; Melkonian *et al.*, 1981; Norris, 1982b; Melkonian, 1982b). It is surprising, therefore, that the chemical composition of scales in the Prasinophyceae has not been previously studied.

The primitive phylogenetic position of the Prasinophyceae has evoked much speculation on the origin of other advanced green algae from this group of scale-bearing green algae. One topic pertinent to the present study is the view that scales were precursors to the cell wall of more advanced green algae (Norris, 1980). Manton and Parke (1965), showed that the theca of the advanced prasinophyte *Tetraselmis*, is formed by the coalescence of scale-like particles. Cell walls may have evolved through the fusion of scales in a similar manner.

Some popular, though speculative, views on the origin of the cell wall are presented below.

The acquisition of a cell wall was probably the most important event in the evolution of algae and higher plants (Swain, 1972; Mattox and Stewart, 1977; Domozych *et al.*, 1980; Melkonian *et al.*, 1981). It not only provided early plants with an osmotic control and a rigid structure which allowed the elaboration of complex multicellular forms but also altered some cytoplasmic processes secondarily. Most important were changes in the mitotic and cytokinetic processes which have had a profound effect on algal taxonomy.

In contrast with the vast amount of information published on the

chemical composition and growth of the plant cell wall, virtually nothing is known about the evolutionary origin of the cell wall.

Bell and Woodcock (1983) regard the cell wall as an evolutionary consequence of photosynthesis that developed when green plants emerged from the aquatic environment. They point out that algae in Windermere may release up to 35% of the total carbon fixed during photosynthesis. Considering this, they believe that emergent plant would have accumulated "embarrassingly large" and possibly toxic quantities of carbohydrates in the cells. Increased irradiance on land would have resulted in increased rates of carbon assimilation and this, along with the diminishing loss of carbohydrate by outward diffusion, would have increased internal carbohydrate levels. They believe that the production of cell walls, like the condensation of sugar to starch, was a way of removing fixed carbon from the general metabolism.

While the cell wall may have evolved as a consequence of photosynthesis, it is difficult to imagine that this structure arose as Bell and Woodcock (1983), propose. Their hypothesis does not explain the presence of cell walls in submerged aquatic algae which must have existed before land plants.

A more tenable hypothesis for the origin of cell walls, which takes into account the possible role of scales as precursors to cell walls in at least some green algae, was proposed by Mattox and Stewart (1977). They believe that the cell walls of the Chlorophyceae may have arisen through fusion of body scales in a prasinophycean ancestor. They suggest that this may have occurred in a manner similar to theca formation in *Tetraselmis* (Manton and Parke, 1965). To substantiate this Mattox and Stewart (1977) draw attention to the absence of scales in chlorophycean algae. They interpret this observation to mean the scales were consumed in the evolution of cell walls in the Chlorophyceae.

Mattox and Stewart (1977) do not believe that the cell walls in the classes Charophyceae and Ulvophyceae evolved from scales because the zoospores and sperm cells of members of these classes often have body scales. They believe that scales in these groups have not been altered in the evolution of the cell wall and that perhaps the cell walls in the Charophyceae and Ulvophyceae evolved in non-flagellate, coccoid or dormant stages rather than in flagellate stages.

Domozych *et al.*, (1980) have gone further to suggest that the cell walls of the latter two classes could have evolved in flagellate stages but that they developed beneath, and independently from, the body scales. This view is supported by the ontogenetic development of cell walls in *Pseudendoclonium basiliense* (Mattox and Stewart, 1973). Here the cell wall develops beneath the scales of germlings growing from scaly zoospores.

Though there have not been any previous studies on the chemical composition of scales in the Prasinophyceae, Lewin (1958) has investigated the chemical composition of the theca of *Tetraselmis*. He has shown that the theca is predominantly composed of carbohydrate with thecal hydrolysates yielding galactose, uronic acid and traces of arabinose. Gooday (1971) confirmed these observations by reporting that the theca of another species of *Tetraselmis* is pectin-like in composition with galactose, galacturonic acid and arabinose representing major hydrolysis residues. A later study by Manton and coworkers (Manton *et al.*, 1973) revealed that the theca contains significant amounts of calcium, probably existing as the calcium salt of galacturonic acid.

The primary objective of the present study was to determine for the first time the chemical composition of scales in a prasinophyte, in this case *Pyramimonas*. This knowledge would provide a chemical basis for deciding whether cell walls may have evolved from fused scales. Furthermore it was hoped that an understanding of the chemical composition of scales would provide additional information on the function of scales, and the possible nature of scale-scale and scale-membrane interactions.

6.2 MATERIALS AND METHODS

6.2.1 Mass cultures

To obtain sufficient material on which to conduct this study, *P. pseudoparkeae* was mass cultured in 16 l PES medium. Cultures were incubated at a constant temperature of 20°C in a 16h:8h photoregime (light intensity 100 $\mu\text{E m}^{-2} \text{s}^{-1}$). A mixture of 2% CO₂ in air was bubbled through the medium (at a flow rate 2l per min) to keep the cultures well mixed and to prevent CO₂-limited growth. Cultures were allowed to grow

for approximately 14 days (this time representing a stage just prior to stationary growth in mass culture) after which cells were harvested.

6.2.2. Isolation and purification of scales

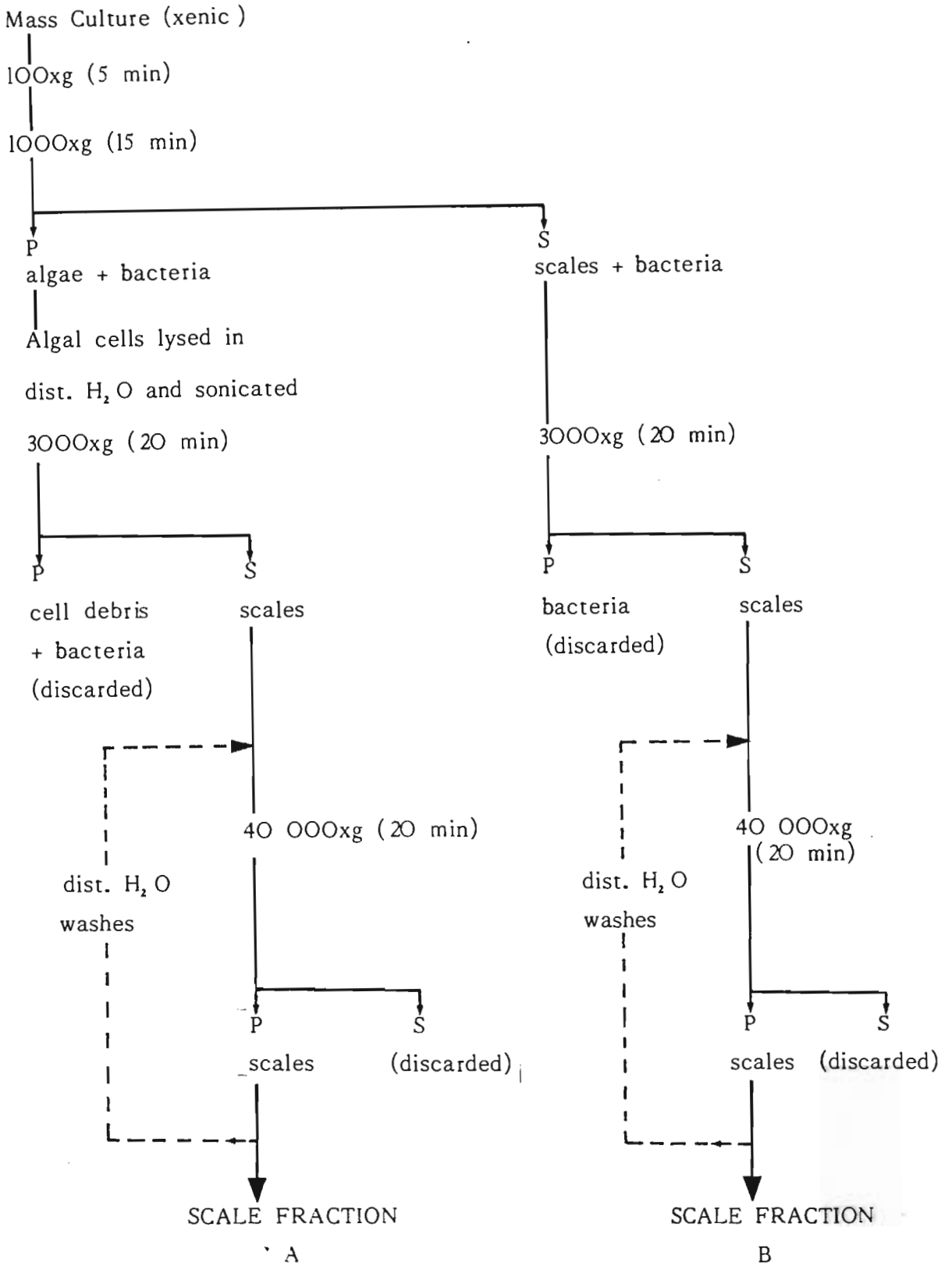
Scale fractions were obtained from mass cultures by using differential centrifugation as shown in Text fig. 6.1. The first supernatant recovered was not discarded because it contained a significant quantity of unattached scales. Algae collected in the first cell pellet were lysed in distilled water for a period of 10 min. The lysed cells were further disrupted by sonicating the cell slurry in a Dawe "Soniclean" sonicator Type 114B for 10 min (frequency $20 \text{ kc } 5^{-1}$, power out put 50W). A surfactant, Triton-X-100 (BDH product) was added to the cell slurry to aid cell disruption while sonicating. The scale fraction collected from lysed cells was called scale fraction A and the scale fraction ultimately collected from the supernatant after the first centrifugation was called scale fraction B.

It is important to mention that the mass cultures used in this experiment were xenic cultures. As outlined in Chapter Four, *P. pseudoparkeae* has an absolute requirement for bacteria to grow in culture. Because dense cultures of the alga were needed for this study on scale composition the alga had to be grown in xenic culture. Bacteria in the mass cultures were easily separated from the scale fractions by differential centrifugation. Bacteria did not lyse when placed in distilled water so scale fraction A was not contaminated with bacterial debris. The supernatant collected after the second step in centrifugation yielded predominantly scales.

Scale fractions were repeatedly washed in distilled water and differentially centrifuged to remove small amounts of contaminating debris. The purity of the scale fractions was checked by viewing heavy-metal shadowed samples in the transmission electron microscope (TEM).

6.2.3 Chemical tests and analyses

Both scale fractions were separated into two subfractions, one for a sugar analysis and the other for an amino acid analysis. A small aliquot of the scale suspension from scale fraction A was set aside for an analysis



Text Fig. 6.1 Isolation of scale fractions using differential centrifugation (P = pellet; S = supernatant)

of the general chemical nature of scales. Only fraction A scales were used because many more scales were collected in this fraction when compared with fraction B.

General chemical nature of scales

The chemical nature of the scales was determined by placing small aliquots of the scale suspension into test tubes containing 1,0 ml of the enzyme and chemical solutions given in Table 6.1.

TABLE 6.1

Enzymatic and chemical treatment of scale fraction A (modified after Green and Jennings, 1967).

Treatment solution	Incubation conditions
5% aqueous EDTA	8h at 20°C - pH adjusted to pH 7,1 with KOH.
distilled water	8h at 100°C
0,1N HCl	8h at 20°C
0,1N NaOH	
1% Cellulase (Sigma - activity 0,5-1,0 unit per mg solid)	12h at 20°C - buffered to pH 5 using McIlvaine's buffer (Hale, 1958)
1% Pectinase (Sigma - activity 1 unit per mg solid)	12h at 20°C - buffered to pH 4 using McIlvaine's buffer.

After the required incubation period a sample was withdrawn from each reaction vessel and pipetted onto a collodion-coated copper viewing grid. Preparations were allowed to settle for 10 min and were then washed with distilled water, shadowed with Au/Pd and viewed in a TEM. A photographic record of the appearance of the scales was made.

A few drops of the scale suspension were stained with 0,02% aqueous ruthenium red (Gurr, 1965). The staining reaction which is positive for pectic substances, was recorded before and after the preparation was irrigated with 5% aqueous EDTA. Scale suspensions were also stained with 0,1% (w/v) alcian blue in 0,5N acetic acid (pH 2,5) and 0,1% (w/v) alcian blue in 0,5N HCl (pH 0,5) (Crayton, 1982). Alcian blue binds with acidic polysaccharides (carboxylated or sulphated) at pH 2,5 (Ramus, 1977) and stains sulfated polysaccharides at pH 0,5 (Parker and Diboll, 1966).

Analysis of sugar residues

Subfractions from scale fractions A and B were freeze-dried and then weighed. Dried scales were resuspended in 2,0 ml 2N H₂SO₄ and transferred to 5 ml glass ampoules which were sealed under vacuum. Scales were hydrolysed for 1h in a water bath maintained at 100°C. The hydrolysate was neutralized with excess barium carbonate (BaCO₃), using methyl red as an indicator, and was subsequently centrifuged to produce a clear supernatant which contained scale residues. The BaCO₃ pellet was washed three times with distilled water and in each case the supernatants were combined. The clear hydrolysate was taken to dryness in a freeze-drier and the residues were eventually redissolved in 2,0 ml 10% isopropanol to prevent bacterial contamination.

Attempts to separate the sugar residues in the hydrolysate using Whatman's No. 1 chromatography paper proved unsuccessful because the concentrations of sugars in the hydrolysate were too low to be detected.

Thin layer chromatography (TLC) was therefore employed in all subsequent separations. The TLC plates used were Merck silica gel 60F₂₅₄ pre-coated aluminium sheets. The hydrolysate was applied to the plates as small spots, the volume varying from 100 - 250 μ l. In addition to the scale hydrolysates, 10 μ l aliquots of different reference sugar solutions were spotted onto the same chromatogram. These solutions were made up as 0,1% solutions in 10% isopropanol. The reference sugars used are given in Table 6.2.

TABLE 6.2.

Reference sugars used in Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC).

a) L-Rhamnose
b) D-Glucose
c) D-Galactose
d) D-Ribose
e) D-Xylose
f) L-Arabinose
g) D-Fructose
h) D-Mannose

The chromatograms were developed by ascending chromatography using the solvents given in Table 6.3.

TABLE 6.3.

Four solvent systems used to separate sugars on silica gel TLC plates

Solvent System	Solvent ratios	Reference
n-butanol:acetic acid: ether: water (BAEW)	9 : 6 : 3 : 1	Harborne, 1973
n-butanol:benzene:pyridine: water (BBPW)	5 : 1 : 3 : 3	Harborne, 1973
phenol/water	phenol saturated with water.	Harborne, 1973
chloroform:acetic acid: water (CAW)	6 : 7 : 1	McCready, 1970

The solvents were decanted into chromatography tanks which were sealed and allowed to equilibrate at 18°C for at least 1h before commencing

the separation. Chromatograms were developed for 4 - 5h or until the solvent front had moved 16 cm.

Sugars were detected by spraying dried chromatograms with one of three spray reagents in Table 6.4.

TABLE 6.4

Spray reagents used to detect sugars on Chromatograms

Spray reagent	Preparation	Activation and expected results
aniline hydrogen phthalate (Harborne, 1973).	9,2 ml aniline and 16g phthalic acid are dissolved in 490 ml n-butanol, 490 ml diethyl ether and 20 ml distilled water.	sprayed plates are dried and heated for 5 min at 105°C to reveal the sugars
naphthoresorcinol/ H ₃ PO ₄ (Harborne, 1973).	200 mg naphthoresorcinol is added to 100 ml n-butanol containing 10% (v/v) phosphoric acid.	sprayed plates are dried and heated for 10 min at 100°C. Sugars appear as coloured spots. Ketoses, pentoses and hexoses appear as pink, green and blue spots respectively.
naphthoresorcinol/ HCl (Bell, 1955)	100 mg naphthoresorcinol is added to 100 ml ethanol containing 20% (v/v) 2 N HCl	when sprayed plates are dried at room temperature uronic acids appear as blue spots on a rose background. When plates are heated for 5 min at 75°C uronic acids appear as blue spots, pentoses and 6-deoxy aldohexoses appear as violet-blue spots and hexuloses appear as bright red spots.

Sugar spots were ringed with pencil and the chromatograms were photographed to provide a permanent record of the separation.

With improved methods for separating sugars viz. High Pressure Liquid Chromatography (HPLC) and Gas-Liquid Chromatography (GLC), identifications made on paper or thin layer chromatograms are not always regarded as conclusive. For this reason some of the scale hydrolysate was separated using HPLC. Mr R. Berry, (Department of Biochemistry, University of Natal, Pietermaritzburg) kindly operated the chromatograph and his help is gratefully acknowledged. Reference sugars (see Table 6.2) made up as 0.1% solutions and scale hydrolysates were separated in a Varian 5000 High Pressure Liquid Chromatogram fitted with a μ Bondapak carbohydrate analysis column (Waters Associates, Milford, Massachusetts). The solvent system used was acetonitrile : water (3 : 1 v/v) and this was passed through the column at a rate of 4 ml min^{-1} . Sugars were detected on an inline Varian Refractive Index Detector. As explained later, HPLC was not able to confirm the TLC results because the detector used was not sensitive enough to detect sugars in the hydrolysate.

Some scale hydrolysate was frozen for about 12 months before the use of a GLC became available. GLC is far more sensitive than HPLC and it was hoped that scale residues would be separated and detected in the hydrolysate. The help of Professor J. van Staden (Department of Botany, University of Natal, Pietermaritzburg), who kindly did the GLC separation, is gratefully acknowledged. The separation was done in a Varian 3700 Gas Chromatograph using a Chromosorb WHP 2m glass column (I.D. = 3 mm) with a liquid phase OV-17(80/100 mesh). Trimethylsilyl derivatives of scale residues and standard sugar solutions were separated by temperature programming (linear increase in temperature from 124°C to 250°C over 21 min). Sample components were detected with a flame ionization detector and a print-out of the residues was obtained.

Analysis of amino acid residues

Scale fractions were freeze-dried, weighed, and then hydrolysed *in vacuo* in 6 N HCl for 24h at 110°C . The hydrolysate was analysed on an Automatic Amino Acid Analyser (Beckman, Model 119) using a column packed with Beckman AA15 cation exchange resin. All amino acid analyses were kindly done by Mrs E.M. Stephenson (Department of Biochemistry, University of Natal, Pietermaritzburg). Her help is gratefully acknowledged. Dr C. Dennison (also in the Department of Biochemistry) is thanked for his

help in interpreting the amino acid results.

6.3 RESULTS

The presence of bacteria in the mass cultures used in this study initially caused some concern because it was felt that they may contaminate the scale fractions. Fortunately the bacteria were not lysed when they were resuspended in distilled water and sonicated. The algal cells, because they do not have a rigid cell wall, were easily disrupted. Intact bacteria are shown amongst algal debris after sonication in Pl. 6.1, fig. 1. The bacteria, along with larger cell debris, were successfully separated from scales by differential centrifugation. Scale fraction B was relatively easily obtained because this fraction comprised "free-floating" scales that were not associated with cell debris. Scale fraction A had to be repeatedly washed before a dense scale pellet free from contaminating debris was obtained (Plate 6.1, fig. 2). A diluted sample from scale fraction A is shown in Plate 6.1, fig. 3. All seven scale types were present in the pellet but there were proportionally fewer B_1 , F_1 and F_2 scales. These small scales were often trapped with cell debris and were lost when this was removed along with the bacteria. Underlayer scales were also lost in the final step in centrifugation. Most scales pelleted at 40 000xg (20 min) without much contaminating debris. However longer times and higher r.c.f's tended to bring down microsomes which contaminated the scale pellet. Underlayer scales which are only \pm 35 nm in diameter were unfortunately not all pelleted at 40 000xg (20 min) and remained in the supernatant and were discarded with microsomes and other minute fragments.

The dried scale pellets weighed 7,3 mg for scale fraction A and 1,5 mg for scale fraction B. No attempt was made to separate the different scale types for separate chemical analyses. The small amount of scale material harvested made this an impracticable venture. Preliminary tests using discontinuous gradient centrifugation (sucrose gradient) showed that this technique could be useful to separate different scale types if sufficient scale material was available.

6.3.1 Chemical and enzyme treatments

Scales treated with EDTA, pectinase, 0,1N NaOH and 0,1N HCl and hot water were degraded to different degrees. Two scales in advanced stages of degradation were seen in EDTA and pectinase treated samples (Pl. 6.2, figs. 1 and 2, respectively). No scales were found after treatment with NaOH. Many partly digested scales were seen in HCl treated samples (Pl. 6.2, fig. 3). Scales treated with hot water were somewhat distorted, indicating that some extraction had taken place (Pl. 6.2, fig. 4). Cellulase did not affect the scales (Pl. 6.2, fig. 5).

Scales from scale fraction A readily bound alcian blue at pH 2,5 and pH 0,5 (Pl. 6.2, figs. 6 and 7 respectively). Scales also stained positively with ruthenium red (Pl. 6.2, fig. 8) but preparations quickly lost their colour when slides were irrigated with 5% EDTA. In suspension the scales tended to clump so that areas of more intense staining were visible.

6.3.2 Sugar residues in scale hydrolysates

Sugar residues detected in scale fractions A and B are shown on the chromatograms in Pls. 6.3, 6.4 and 6.5. Some solvent systems separated the sugars better than others. The solvent system BBPW was not useful because it did not separate the sugars (reference and hydrolysate sugars) as distinct spots on the chromatogram (Pl. 6.3, fig. 1). The sugars were poorly separated so that no accurate Rf values could be obtained. Poor separation was also a problem in chromatograms developed in phenol/H₂O (Pl. 6.3, fig. 2). In this case the sugars were not streaked but the problem lay in the short distances moved by the sugars. Their greater solubility in water rather than phenol accounted for the relatively low Rf values of these sugars (see Table 6.5).

Four sugars residues were present in both scale hydrolysates. They could not be identified because they often co-chromatographed (or very nearly so) with more than one reference sugar. The spray reagent naphthoresorcinol/H₃PO₄ was somehow affected by phenol and did not give the expected colour reactions with different sugars. All hexoses and fructoses were coloured purple with only fructose appearing as a brown spot.

TABLE 6.5

Rf values and colour reactions for sugars separated in phenol/H₂O solvent system and sprayed with naphthoresorcinol/H₃PO₄

	Sugar	Rf values (x 100)	Colour with naphthores- orcinol
Reference sugars	Rhamnose	32	purple
	Glucose	13	"
	Galactose	19	"
	Ribose	21	"
	Xylose	14	"
	Arabinose	22	"
	Fructose	9	brown
	Mannose	18	purple
Scale Fraction A	4 rhamnose?	29	purple
	3 arabinose?	23	"
	2 galactose? ribose? or mannose?	19	"
	1 xylose? or glucose?	12	"
Scale Fraction B	4 rhamnose?	29	"
	3 arabinose?	23	"
	2 galactose? ribose? or mannose?	20	"
	1 xylose? or glucose?	15	"

Note: The numbers preceding the sugars in scale fractions A and B in this and subsequent tables refers to the spots seen on the chromatograms (1 is the most polar spot while 4 is the least polar spot).

Chromatograms developed in CAW solvent system also revealed the presence of four sugar residues in the scale hydrolysates (Pl. 6.4, fig. 1). Although the residues appeared to be better separated using this solvent system a few problems were encountered. Some reference sugars, particularly galactose and glucose but also arabinose, fructose and mannose, were not well separated. The Rf values of sugars separated in this solvent system are given in Table 6.6.

TABLE 6.6.

Rf values for sugars separated in CAW solvent system

	Sugar	Rf value (x100)
Reference sugars	Rhamnose	44
	Glucose	23
	Galactose	22
	Ribose	38
	Xylose	34
	Arabinose	31
	Fructose	29
	Mannose	29
Scale Fraction A	4 rhamnose?	43
	3 xylose?	35
	2 arabinose?	31
	1 glucose? galactose?	23
Scale Fraction B	4 rhamnose?	43
	3 xylose?	35
	2 arabinose?	31
	1 glucose? galactose?	23

The poor separation of the sugars made identification difficult and only tentative names are given for hydrolysate residues in Table 6.6.

Because the chromatogram was sprayed with aniline hydrogen phthalate all sugars appeared as brown spots so that in this case colour could not be used to aid identification.

In general practice sugars are separated by descending chromatography on paper. Using this method the chromatogram can be developed for long periods (+ 24h) so that the solvent front moves off the paper to give a better separation of the relatively polar sugar residues. This method of separation was attempted with the scale hydrolysates but it proved to be unsuccessful. The sugar residues in the developed chromatogram were present at concentrations too low to be detected with the spray reagents used.

Sugar residues were most successfully separated on plates developed in the BAEW solvent system. The chromatogram on which scale hydrolysate residues were ultimately identified is given in Pl. 6.4 (fig. 2). The R_f values and colour reactions of all sugars on this plate are given in Table 6.7.

The BAEW solvent system separated glucose and galactose sufficiently to show clearly that the most polar spot (spot 1) in both hydrolysates was galactose. The least polar residue on both scale hydrolysates (spot 4) was identified as rhamnose. Although this residue ran a little slowly on this chromatogram it did co-chromatograph with the reference sugar in the CAW solvent system and in the BAEW solvent system when the latter had been developed twice in the same dimension (see Pl. 6.4, fig. 1 and Pl. 6.5, fig.1, respectively). Both rhamnose and galactose were coloured bright blue with naphthoresorcinol confirming that they were hexoses sugars. The remaining two spots were distinctly green in colour and were therefore pentose sugars. Spot 3 was well separated from other sugars and co-chromatographed with xylose. Spot 2 was identified as arabinose because it was coloured green and because it co-chromatographed with this reference sugar. It should be emphasized, however, that the green colour of the spot may only indicate that arabinose predominates. Other sugars with similar R_f values (e.g. glucose and fructose) could be masked beneath the arabinose spot.

TABLE 6.7

Rf values and colour reactions for sugars separated in BAEW solvent system and sprayed with naphthoresorcinol/H₃PO₄

	Sugar	Rf values (x100)	Colour with naphthore- sorcinol
Reference sugars	Rhamnose	62	blue
	Glucose	37	blue
	Galactose	32	blue
	Ribose	44	green
	Xylose	51	green
	Arabinose	39	green
	Fructose	37	brown
	Mannose	44	blue
Scale Fraction A	4 rhamnose	59	blue
	3 xylose	51	green
	2 arabinose	40	green
	1 galactose	33	blue
Scale Fraction B	4 rhamnose	59	blue
	3 xylose	51	green
	2 arabinose	40	green
	1 galactose	33	blue

The chromatogram depicted in Pl. 6.5, fig. 1 has already been alluded to and shows sugars also separated in the BAEW solvent system. Whereas the previous chromatogram was developed only once, this plate was developed twice. This was done in an attempt to obtain more discrete sugar spots and to improve separation. Unfortunately redeveloping the plate did neither. The only advantage gained was that the least polar spot (spot 4) of the scale hydrolysates co-chromatographed with, and was identified as rhamnose. (see Table 6.8).

TABLE 6.8

Rf values for sugars separated in BAEW solvent system. Plate developed twice in the same direction.

	Sugar	Rf value(x100)
Reference sugars	Rhamnose	75
	Glucose	56
	Galactose	49
	Ribose	62
	Xylose	66
	Arabinose	57
	Fructose	55
	Mannose	63
Scale Fraction A	4 rhamnose	76
	3 xylose	68
	2 arabinose	59
	1 ? not clear	53
Scale Fraction B	4 rhamnose	75
	3 xylose	66
	2 arabinose	58
	1 ? not clear	52

In all other plates rhamnose ran slower than the standard. Spot 1, which had been previously identified as galactose, has an Rf value between that of glucose and galactose. It is difficult to determine why the hydrolysate residues in this plate ran faster than the reference sugars. Perhaps the greater concentration of sugars in the standards retarded their movement. Similarly the slower movement of sugars in the scale fraction B hydrolysate compared with that for scale fraction A may be a result of heavy loading at the origin. Because the concentration of sugars was low in the fraction B hydrolysate much more of the hydrolysate was applied to the plate.

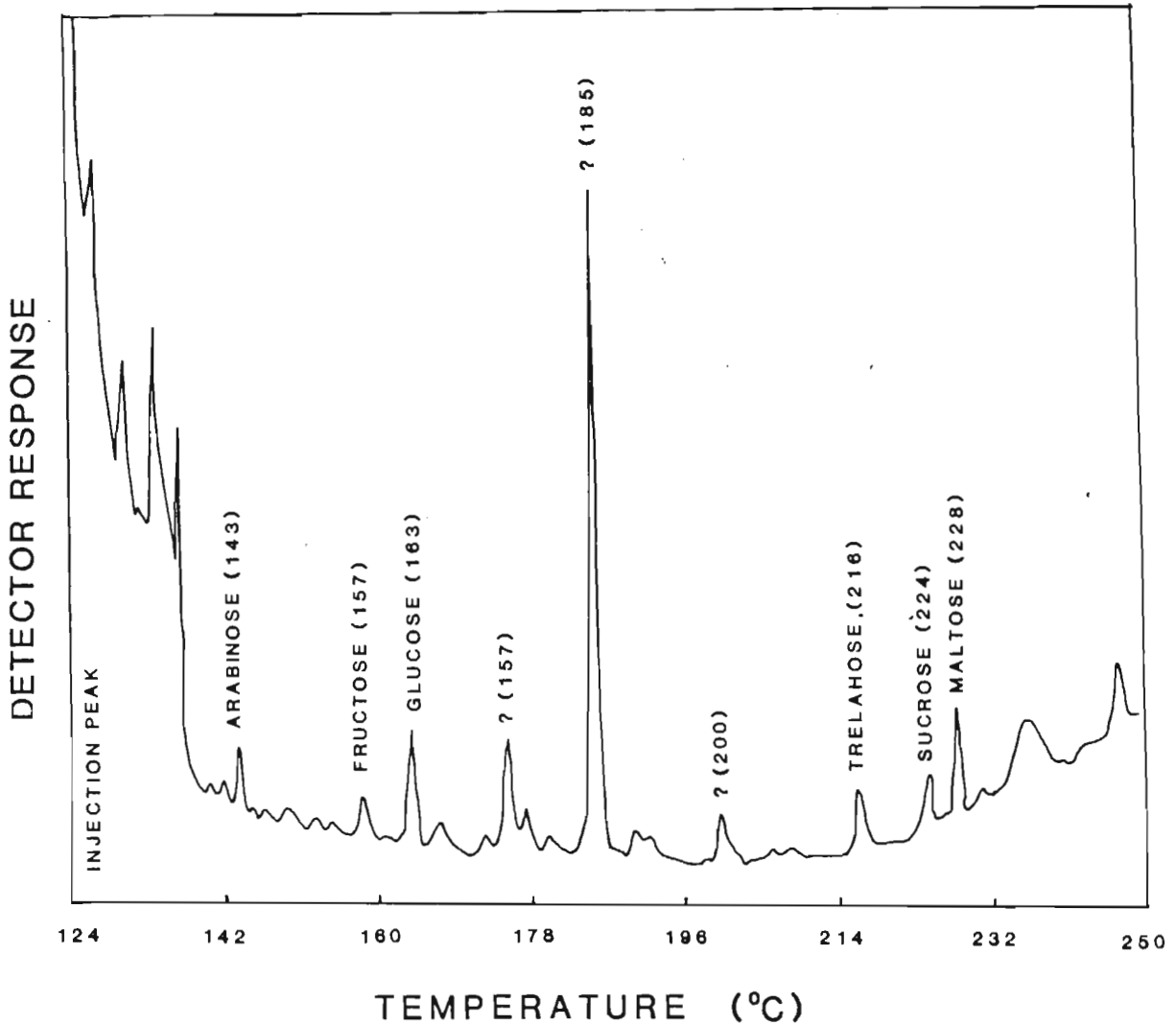
In summary, the scale hydrolysates contained four residues that were identified as rhamnose, xylose, arabinose and galactose. It was surprising however, that no uronic acid was detected on the chromatograms. Plates sprayed with naphthoresorcinol/HCl (Bell, 1955) showed no colour development when left to dry out at room temperature. The fact that scales were digested with pectinase suggested that they contained a polygalacturonan that should have been hydrolysed to give uronic acid residues on the chromatograms. It is important to mention here that not all the scale material was digested in 2 N H₂SO₄. A gelatinous, straw-coloured residue remained in the hydrolysis vessels and this may have represented the unhydrolysed polygalacturonan. The acidic nature of this residue was confirmed by its ability to bind alcian blue at pH 2,5 (Pl. 6.2, fig. 9). Pectinase could not digest the residue but it is possible that the compound had undergone structural change during hydrolysis. The residue also bound alcian blue at pH 0,5 suggesting that sulphate groups were present (Pl. 6.2, fig. 10). The residue was extremely acid resistant and was not visibly affected by further hydrolysis (5N H₂SO₄ for 1h at 100°C). After this treatment the Scale Fraction B hydrolysate contained a trace of fructose (Pl. 6.5, fig. 2). On the chromatogram the spot was coloured brown and co-chromatographed with fructose (both having an R_f value of 0,35). A heavy application of Scale Fraction A hydrolysate resulted in a poor separation of the sugars and retarded their movement. Galactose and arabinose were not discrete spots and their intense colour may be masking fructose in that hydrolysate.

Attempts to confirm the TLC identification of sugars using HPLC were unsuccessful. The Refractive Index Detector was too insensitive to detect hydrolysate sugars or the standard sugars (0,1% solutions) even when a 100 µl sample was injected onto the column. Mr. Berry¹, who operated the HPLC, has informed the writer that in subsequent tests he determined that the detector used could only resolve sugars when they were made up as 2% solutions and when 100 µl of the sample was applied to the column.

The separation of sugars using GLC was more successful than HPLC and thus is undoubtedly the better technique. A chromatograph of the

¹ Mr R. Berry (Department of Biochemistry, University of Natal, Pietermaritzburg).

TMS derivatives in the scale fraction A hydrolysate is shown in Text fig. 6. 2.



Text fig. 6.2. A GLC chromatogram of the TMS derivatives of sugars in the hydrolysate from scale fraction A. (Numbers in brackets represent the temperature in °C at which the derivatives came off the column).

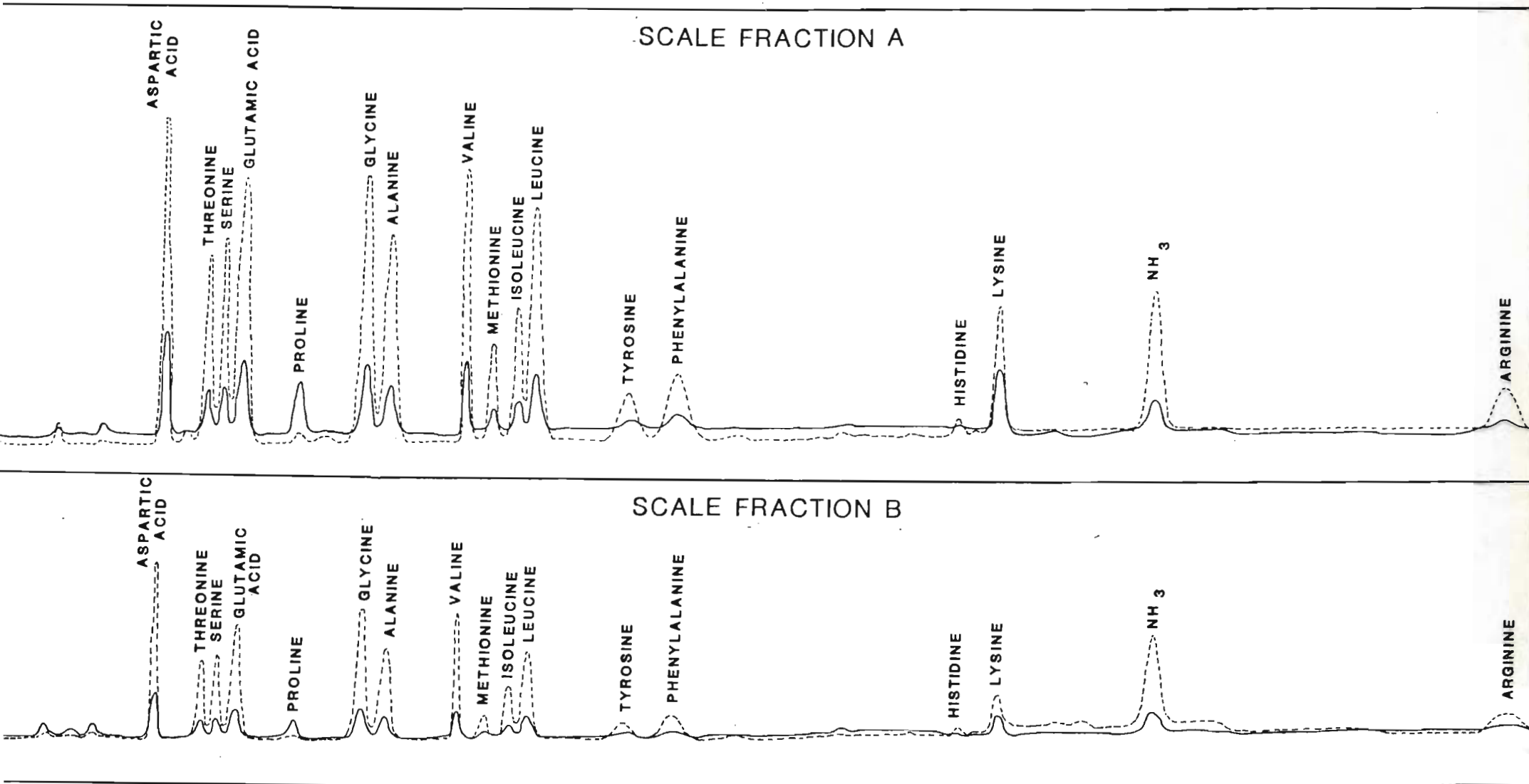
The chromatogram depicted in Text fig. 6.2 has been included here even though it does not substantiate the results obtained using TLC. It was included to show that TMS derivatives of sugars can be successfully separated using temperature programming on a GLC. The reason that these results do not support the TLC results may be because the hydrolysate was old. It was stored for 12 months before the GLC became available and in this time the sugars may have undergone some structural change. The

results are treated with suspicion because the three disaccharide sugars trehalose, sucrose and maltose should not have been present in the hydrolysate. The glycosidic bonds between the sugar residues comprising the disaccharides would have been broken during acid hydrolysis.

Although the hydrolysate was frozen during storage it was kept with the Ba SO₄ precipitate formed when the H₂ SO₄ was neutralized with Ba CO₃. This may have changed the pH balance of the hydrolysate and affected the sugar residues. In dilute alkali, for example, glucose undergoes enolization to produce an equilibrium mixture containing glucose, fructose and mannose. This reaction is known as the Lobry de Bruyn - von Ekenstein transformation (Conn and Stumpf, 1972). At higher concentrations of alkali the monosaccharides are unstable and undergo oxidation, degradation or polymerization. By contrast monosaccharides are generally stable in dilute mineral acids. If the hydrolysate was alkaline, this may have produced the anomalous peaks on the chromatogram. It is interesting that arabinose and fructose were detected in the GLC chromatogram and on TLC plates. This may be confirmation that the two sugars are present in the scale carbohydrate. Rhamnose, which elutes at a lower temperature than arabinose may be lost in the shoulder of the injection peak. The presence of glucose on the chromatogram is difficult to explain. Xylose and galactose were not detected. The relatively large peaks eluting at 157, 185 and 200°C were not identified.

6.3.3 Amino acid residues in scale hydrolysates

The amino acid residues detected in the scale hydrolysates are shown in the amino acid analyser chromatograms in Text fig. 6.3. These data are summarized and quantified in Table 6.9. A total of sixteen amino acid residues were present in both hydrolysates. Glutamic acid and aspartic acid were the dominant residues. The proportions of all residues were similar in both scale fractions although values for scale fraction B were slightly lower than those obtained for scale fraction A. Without replicate analysis it is impossible to know if these differences are significant. The absence of non-protein amino acids (e.g. citrulline and ornithine) suggests that the amino acid residues detected in the scale hydrolysates were derived from scale protein. The sum of the residues in each scale fraction gives an approximate value for the total protein content of the scales.



Text Fig. 6.3 Automatic amino acid analyzer chromatograms showing the amino acid residues present in Scale Fraction A and Scale Fraction B. Ninhydrin reaction products were read in two colorimeters set at 440 nm (solid line) and 570 nm (broken line).

TABLE 6.9

Scale fractions A and B amino acid residues detected with the automatic amino acid analyser. (Values expressed as percentages of sample dry weight).

Amino acid residue	Scale Fraction A	Scale Fraction B
Aspartic acid	0,52	0,40
Threonine	0,27	0,19
Serine	0,24	0,17
Glutamic acid	0,50	0,33
Proline	0,19	0,10
Glycine	0,22	0,16
Alanine	0,28	0,18
Valine	0,38	0,27
Methionine	0,10	0,05
Isoleucine	0,19	0,14
Leucine	0,38	0,21
Tyrosine	0,19	0,10
Phenylalanine	0,27	0,16
Histidine	0,08	0,02
Lysine	0,23	0,09
Arginine	0,22	0,12
Estimated protein content (sum of residues)	4,50%	3,19%

6.4 DISCUSSION

The scales of *P. pseudoparkeae* are not cellulosic in composition because they were not digested with the enzyme cellulase. Rather they appear to be pectinaceous in composition having many of the characteristics of plant pectins. The results supporting this interpretation are presented below.

The observation that scales were digested with pectinase suggests that they are composed, at least in part, of a polygalacturonide. Pectinase, properly known as Poly-(1,4- α -D-galacturonide) - glycanohydrolase, hydrolyses α -1,4 glycosidic linkages between D-galacturonic acid residues in pectins (Aspinall, 1970). Pectins are generally heteropolysaccharides and pure galacturonans seldom occur in nature (Aspinall, 1980). They often have a number of structural variations superimposed on the D-galacturonan chains; they may have rhamnopyranose residues inserted between galacturonan blocks; they may have other neutral sugars attached to the galacturonan in sidechains; the galacturonic acid residues may be esterified; and acetylation of hydroxyl groups may occur.

The neutral sugars detected in the scale hydrolysate were easily hydrolysed and it is likely that they were attached as sidechains to the galacturonan. These neutral sugars, viz. galactose, arabinose, xylose, rhamnose and fructose (trace), are common constituents of plant pectins (Aspinall, 1970; Cook and Stoddart, 1973) and their presence in the hydrolysates supports the view that scales are pectin-like in composition.

Although galacturonic acid was not present in the hydrolysates its presence can be inferred on two accounts. The first, already mentioned, is that the scale material was digested with galacturonidase. Secondly the scales bound alcian blue at pH 2,5. At this pH the stain is specific for carboxylated and/or sulphated acidic polysaccharides (Parker and Diboll, 1966; Aminoff *et al.*, 1970; Crayton, 1982). The ability of scales to bind alcian blue at pH 0,5 is interesting because it confirms that sulphate esters are present in the scale carbohydrate. At this very low pH carboxyl groups do not dissociate so that the stain complexes exclusively with sulphate groups. The latter are presumed to be associated with the carbohydrates rather than the protein component of the scales because sulphate esters of proteins are seldom free to react, and if they do, no appreciable colouration is produced under histochemical conditions (Aminoff *et al.*, 1970).

The absence of uronic acid on the chromatograms indicates that the compound withstood acid hydrolysis (even in 5N H₂SO₄ for 1h at 100°C). The difficulty attending hydrolysis of uronic acids has often been reported

(Bell, 1955; O'Colla, 1962; Adams, 1965; Albersheim, 1965; Percival and McDowell, 1967; Sharon, 1975). Because the residue bound alcian blue at pH 0,5 it must contain sulphate groups. It is tempting to speculate that the sulphate groups in unhydrolysed scales are associated with the acidic component (galacturonan) but it is possible that sulphuric acid contributed sulphate during hydrolysis. One observation that may support the view that galacturonic acid polymers bear sulphate esters is that the scales were digested with NaOH. Aspinall (1970) reports that pectins having esterified galacturonic acid residues are degraded on treatment with alkali. The acid resistant polymer apparently had undergone structural change during hydrolysis because pectinase did not digest this material. Percival and McDowell (1967) in their treatment of sulphated acidic polysaccharides have shown that the mucilages of *Ulva* and *Enteromorpha* LINK have sulphate esters on rhamnose. The possible association of sulphate with rhamnose and the possible presence of this sugar in the internal galacturonan chains in scales should be investigated.

Another property of scales which suggests that they are pectin-like in composition is that they were solubilized in EDTA. This chelating agent is used to extract pectin from plant tissue (Aspinall, 1973). Pectins characteristically contain divalent cations (usually calcium) which forms complexes with the carboxyl and hydroxyl groups of galacturonic acid residues. These cations act as transverse linkages joining adjacent polymers of galacturonic acid (Stace, 1971; Smith, 1977). In the presence of EDTA the divalent cations are removed thus breaking the ionic bridges between chains and rendering the pectin soluble (Hall *et al.*, 1974). Scales treated with EDTA may have been solubilized in this way.

The divalent cation present in scales is believed to be calcium. Though its presence has not been demonstrated in scales, the ion is present in the cyst wall of *P. pseudoparkeae* (see Section 7.3.6). The cyst wall (inner layer) and scales have similar staining properties (both bind alcian blue at pH 0,5 and pH 2,5) and they are probably both composed of a sulphated acidic polysaccharide. It is unlikely that two different cations would be used to stabilize galacturonan polymers in the two types of cell boundaries and it is for this reason that calcium ions are believed to be the cations present in scales. The theca of the advanced prasinophyte

Tetraselmis is not degraded in EDTA (Gooday, 1971). This is surprising because scales and thecae are similar in composition (discussed later). Perhaps the theca contains components that are more strongly polymerized.

Scales treated with boiling water were markedly distorted thus indicating that some extraction of scale material had taken place. Cook and Stoddart (1973) recognize three pectin fractions that make up what they call 'whole pectin'. These are Type 1 pectinic acid, Type 2 pectinic acid and an arabinogalactan. Type 1 and Type 2 pectinic acid are extracted in cold and hot water respectively. Type 1 pectinic acid yields traces of rhamnose and xylose on hydrolysis but yields predominantly galacturonic acid after treatment with pectinase. Interestingly the Type 2 pectinic acid yields similar residues to scales. On hydrolysis Type 2 pectinic acid yields arabinose, galactose, rhamnose and traces of other sugars. Treated with pectinase this fraction yields largely galacturonic acid. Because scales were digested with hot water further suggests that they are pectin-like resembling Type 2 pectinic acid. Arabinogalactans yield only arabinose and glucose on hydrolysis. Because the scales were partly soluble in hot water it is possible that some of the uronic acid residues are methylated (Percival and McDowell, 1967).

The observations made here on the sugar components of the scales of *P. pseudoparkeae* were purely qualitative. The objectives of any future studies on the carbohydrate component of scales should be to quantify and verify the presence of the different sugars using a more sophisticated technique than TLC. Preliminary tests using the GLC indicate that these sugars may best be separated as TMS derivatives using gas-liquid chromatography.

The sugar residues detected in scale hydrolysates are remarkably similar to those detected in thecal hydrolysates of *Tetraselmis* (Lewin, 1958; Gooday, 1971). Besides galactose, galacturonic acid, arabinose and rhamnose, only xylose and fructose (trace) are additional sugars in scales. This observation provides chemical evidence that the scale-boundary of *Pyramimonas* and the theca of *Tetraselmis* may be homologous structures and that the theca may well have evolved through the fusion of scales as Mattox and Stewart (1977) propose. Manton and Parke (1965) first showed

that the theca of *Tetraselmis* is formed extracellularly by the coalescence of small stellate scale-like particles. To suggest that the fusion of scales may have given rise to the cell wall of more advanced green algae is premature. Much more work needs to be done on scales, especially in other prasinophytes, before the role of the scale-boundary in the evolution of the cell wall is understood. At present the logic of Mattox and Stewart (1977) and Domzych *et al.* (1980) must prevail. This logic states that scale covered monads are absent only in the Chlorophyceae and it is likely that scales were consumed in the production of the cell wall in this group. What makes this idea even more attractive is that a fusion of scales is seen in an organism like *Tetraselmis* which occupies an intermediate position between the Chlorophyceae and Prasinophyceae. The alga has flagellar scales (prasinophycean characteristics) but it also produces a phycoplast (chlorophycean characteristic) during cell division.

The amino acid residues detected in the scale hydrolysates are believed to originate from protein in the scales. Although not substantiated here, Domzych *et al.* (1980) and Melkonian *et al.* (1983) have reported that protein is present in prasinophyte scales. Since both scale fractions A and B contained comparable amounts of protein (4,5% and 3,19% respectively), with similar proportions of the different amino acid residues, it is probable that this is a close approximation of the protein content of scales. It is unlikely that the protein detected in scale fraction B could be attributed to protein contamination because this fraction contained "free-floating" scales that were not associated with a cell slurry. The slightly higher protein content in scale fraction A may be attributed to contamination but without replicate analyses it is not possible to determine if this difference is significant.

The nature of the protein/carbohydrate complex in scales still needs to be investigated. If the two components can be shown to be intimately linked then the material comprising the scales would be a glycoprotein and not a true pectic substance as suggested above. In all probability the scales will be shown to be composed of glycoprotein but the writer has refrained from using the term because there is no evidence to show that the carbohydrate and protein components are covalently linked. By definition a glycoprotein is a protein which contains glycosidic substituents, that is to say, the amino acid and sugar residues are covalently linked to each other

(Montgomery, 1970; Lamport, 1980). The low concentration of protein in scales may indicate that the scale material is a highly glycosylated glycoprotein, preferably termed a proteoglycan (Sharon, 1975; Lamport, 1980). Melkonian *et al.*, (1983) have reported that the flagellar scales of *Tetraselmis* are glycoprotein in composition. If body scales in the Prasinophyceae are glycoproteinaceous then it is reasonable to speculate that the cell walls of those chlorophycean algae containing glycoprotein (e.g. *Chlamydomonas*, Roberts *et al.*, 1972) may have evolved, as Mattox and Stewart (1977) propose, from the fusion of scales. The composition of scales in other prasinophytes should be investigated for comparative purposes. The abundance of hydroxyproline in the cell wall of *Chlamydomonas* emphasizes the need to determine if this amino acid is present in prasinophyte scales. Hydroxyproline has a limited distribution in nature and its occurrence may have significant taxonomic implications in the algae (Conn and Stumpf, 1972; Lewin, 1974). For example, Gotelli and Cleland (1968) showed that hydroxyproline was absent in all red algae tested and was rare in the cyanobacteria. In the brown algae it is chiefly found in the cytoplasm but in the green algae it is invariably found concentrated in the cell walls.

In the unicellular members of the Chlorophyceae the composition of the cell wall has been studied in detail in *Chlamydomonas* and *Chlorella*. The cell wall of *Chlamydomonas* is non-cellulosic and is composed of a crystalline glycoprotein matrix of which 30% is protein (Roberts *et al.*, 1972). As mentioned above, the most abundant amino acid residue is hydroxyproline which accounts for 3.6% of the cell wall protein. The sugar residues include arabinose and galactose with trace amounts of mannose. The biochemical composition of the cell walls of other volvocacean monads is similar to that of *Chlamydomonas* (Roberts, 1974).

The cell wall of *Chlorella* contains an acidic polysaccharide which yields rhamnose, arabinose, xylose, mannose, galactose and glucuronic acid on complete hydrolysis (White and Barber, 1972). More recently, Takeda and Hirokawa (1984) have found that the cell wall of *Chlorella* also contains glucose and five other (unidentified) sugar residues. They showed that the cell wall also contains many different amino acids. Most significant was that they revealed that the composition and concentration of different sugar and amino acid residues differed between strains of *Chlorella*. This did not

change during the cell cycle and they believe that differences in cell wall composition could be a good index for identifying different species and strains. Considering this, it is possible that there may be differences in the chemical composition of scales between species in the genus *Pyramimonas* and certainly between genera in the Prasinophyceae. This again emphasizes the need to investigate scale chemistry in other members in the class.

The basic knowledge of scale composition gleaned from the present study allows a few hypotheses to be made on the possible mechanism(s) underlying the external self-assembly of the scale-boundary. As shown in Chapter Two, scales released from the cell always occupy precise positions in the scale-boundary and often show definite orientation; this is most noticeable in the limuloid flagellar scale for example. The uronic acid residues in scales and sulphate groups (which may be sulphate esters on uronic acid) undoubtedly impart a high negative charge to scales.

Two mechanisms are proposed here to explain how different scale types arrive at their respective positions in the scale-boundary. The first is that different scale types may possess different charge densities resulting in a shuffling of scales in the scale boundary according to a charge density gradient from underlayer scales to the outermost scale layer. This hypothesis requires that different scale types have different compositions (perhaps only slightly so) imparting a different charge density. This needs to be investigated in future studies. Until now there have been no suitable methods to separate different scale types. Although density gradient centrifugation could work, it is proposed that electrophoretic or ion-exchange techniques will be most useful.

The second mechanism, which could be working in conjunction with the first, is that scales may possess characteristic patterns of surface charges, providing a "lock and key" mechanism for the orientation and positioning of scales in the scale-boundary. In this respect the role of protein in the external assembly of the boundary should not be overlooked. It may be no coincidence that the predominant amino acids, aspartic and glutamic acid, are ionized over wide pH ranges so that reactive groups on these residues could be available for ionic bonding.

Tannic acid, known for its protein binding capacity (Swain, 1965; Schanderl, 1970), has been shown to stabilize scales in the scale-boundary of *Pyramimonas* (Melkonian and Robenek, 1981; Melkonian *et al.*, 1981). This would indicate that protein is perhaps playing a role in securing scales, especially so in linking the underlayer scales to the plasmalemma in the "basal scale plate" region.

Other scales in the scale-boundary may be secured by ionic bonds involving divalent cations. Divalent cations, already shown here to play a role in maintaining scale structure, may also be involved in ionic cross-linkages between scales. Melkonian (1982b) has shown that the presence of rod-shaped scales on the flagella of *Tetraselmis* is dependent on the presence of divalent cations (especially calcium) in the culture medium. Significantly, tannic acid has no effect in stabilizing rod-shaped scales in the scale-boundary, indicating that calcium ions rather than proteins are perhaps securing these scales in position. However, Melkonian (1982b) notes that glutaraldehyde is known to cause significant modifications to the molecular structure of proteins and he proposes that glutaraldehyde specifically interferes with calcium-binding sites (proteins?) which probably link individual rod-shaped scales to one another and to the underlayer scales. His results however apparently contradict this hypothesis because the rod-shaped scales were not stabilized in the scale-boundary using tannic acid.

The underlayer scales in *Tetraselmis* were not as sensitive as rod scales to a decrease in calcium ion concentration and fixation procedures, and were not lost as easily. This suggests that in *Tetraselmis* the different scale types have different chemical compositions. This work should be extended to the genus *Pyramimonas* and emphasizes the need to determine the chemical composition of individual scale types.

While not much is known about the functional significance of scales in green algae, Melkonian *et al.* (1981) have suggested that they may have a protective function and may play a role in divalent cation metabolism either providing an extracellular store of calcium or enabling cells to survive in a calcium-rich environment.

Building on the latter it is proposed that scales, by virtue of their polyanionic nature, contribute to the maintenance of the water and salt balance in cells. Acidic polysaccharides, rich in carboxyl and sulphate groups, are known to have ion exchange properties that may be important in regulating the water and salt balance of cells (Cook and Stoddart, 1973; Sharon, 1975) especially in organisms living in a saline medium (Percival and McDowell, 1967). Melkonian *et al.* (1981) have suggested that flagellar scales might play a role in sexual recognition but this proposal is premature because sexual reproduction is not known to occur in the class Prasinophyceae. Melkonian and coworkers (*op. cit.*) have noted that scales may play a role in the development of flagellar "stickiness" and that their dissolution might be under active control of the cell. This "stickiness" of the flagella has been observed in *P. pseudoparkeae* but this phenomenon is believed to have an ecological function in allowing cells to adhere to sand particles or hard substrata when the incoming tide would otherwise remove them from their tidal pool habitat. The writer has observed that populations of *P. pseudoparkeae* are found approximately 4 cm below the surface of sand in tidal pools that have recently been exposed on a receding tide.

CHAPTER SEVEN

LIFE — HISTORY STUDIES

7.1 INTRODUCTION

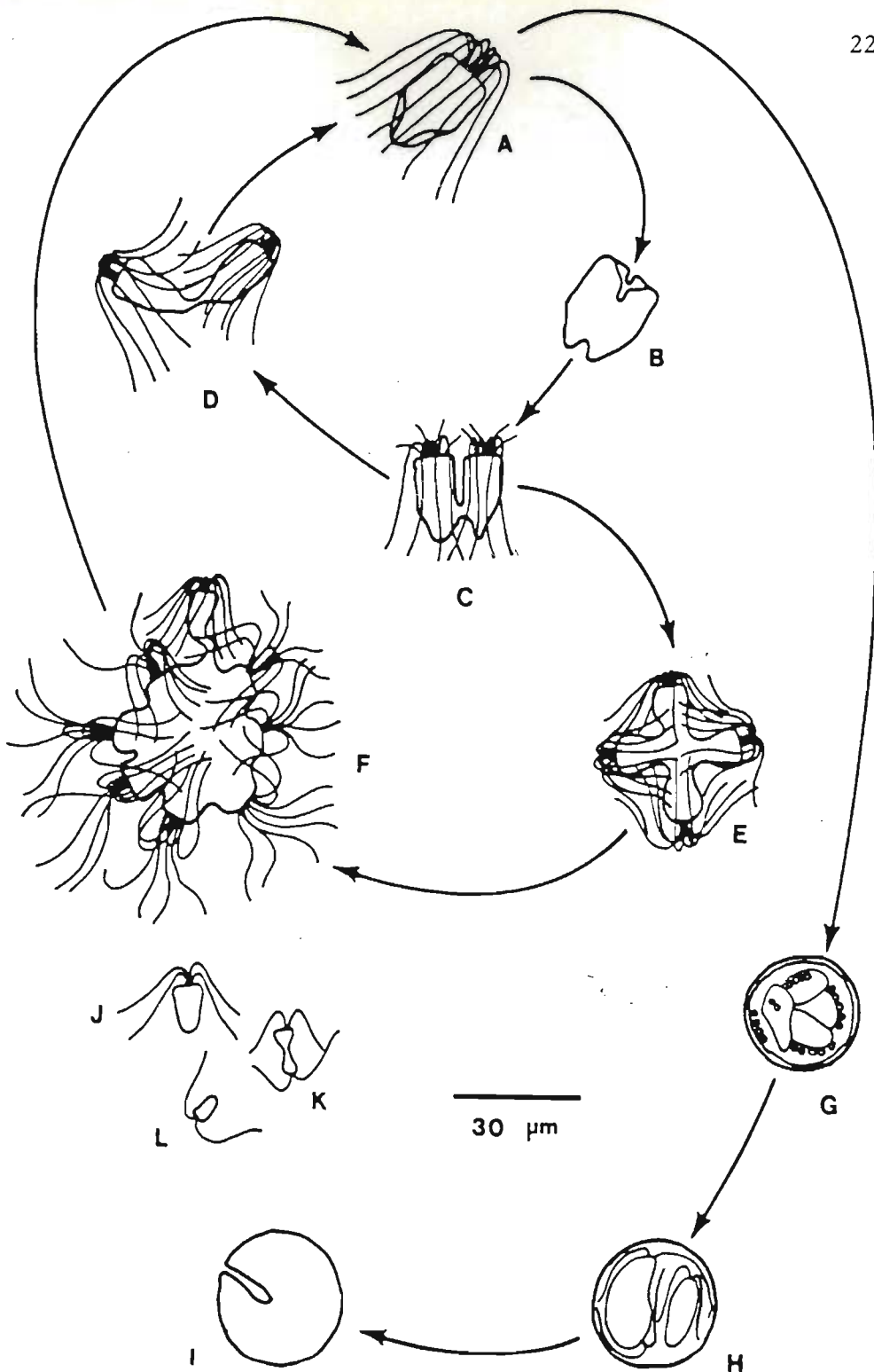
Very little is known about the life-histories of algae assigned to the class Prasinophyceae. Norris (1980) has summarized what little is known and he has called for contributions to be made on this aspect of the biology of prasinophytes.

Though some genera are reported to undergo sexual reproduction, this has never been clearly demonstrated and it is generally accepted that sexual reproduction does not occur in the class Prasinophyceae. Some life-cycle stages of a few members of the class have been described (see Section 1.2.5) but relatively few reports describe the complete life-cycle of the organisms.

Pertinent to the present study is that Hargraves and Gardiner (1980) have reported on some life-cycle stages of the octoflagellate species *Pyramimonas amyliifera* (see Text fig. 7.1). They have shown that the cells become non-motile and encyst. Though they reported seeing empty cyst casings in their cultures they did not observe the process of excystment. Biflagellate and quadriflagellate cells were present in their cultures and they suggested that these may represent the products of cyst germination. *P. amyliifera* also reproduces by binary fission and Hargraves and Gardiner (1980) indicated that pleomorphic cells may also regenerate the octoflagellate stage (Text fig. 7.1). Woods and Triemer (1981) have described the ultrastructural aspects of cell division in *P. amyliifera*.

The present study was undertaken to document the life-history of *P. pseudoparkeae* at the light microscope level and to obtain as much information as possible on the ultrastructure of the different life-cycle stages. Further information on the cells was obtained through karyological and histochemical studies and energy dispersive X-ray analyses.

A report on the reproduction of *P. pseudoparkeae* (Aken and Pienaar, 1981b) was based, in part, on this study.



Text fig. 7.1. The possible life-history of *Pyramimonas amyliifera* (taken from Hargraves and Gardiner, 1980).

A = octoflagellate; B, C and D = early, later and late stages in binary fission; E = quadrilobate cell; F = octolobate cell with top lobe separating from the rest of the mass; G = cyst with large starch grains; H = cyst containing swimmers; I = empty cyst; J = quadriflagellate; K = quadriflagellate dividing or biflagellates fusing; L = biflagellate.

7.2 MATERIALS AND METHODS

7.2.1 Cyst induction experiments

Most often encysted cells of *P. pseudoparkeae* developed spontaneously in ageing cultures. However during the xenic growth studies described in Chapter Three some cells in exponential cultures became cysts. These non-motile cells occurred in those cultures which were grown in the culture vessel shown in Text fig. 3.2.

In a subsequent trial experiment cells were grown in the same culture vessel and also in flasks without the rubber stopper assembly. The latter vessels were sealed with cotton wool and aerated. Those cultures with the rubber stopper assembly were "sampled" daily to ensure that a fresh suspension of cells was drawn into the capillary sampling tube. Encysted stages were again found in the vessels with the rubber stopper assembly. No cysts were found in the cultures sealed with cotton wool plugs. It was clear therefore that the cells trapped within the sampling tube between successive samplings were induced to encyst. This method however produced insufficient encysted material on which to conduct further studies.

If conditions inducing encystment in the sampling tube could be simulated, then large numbers of cells could be induced to encyst. This would provide sufficient material for detailed electron microscopical studies on the life-cycle stages of the organism.

Of many possible factors that could induce encystment in the sampling tube, it was decided to test the effects of different nutrient levels, reduced CO₂ levels, and a range of pH levels in the medium.

Modified PES growth medium

Cells were cultured in 100 ml modified PES medium in 250 ml Erlenmeyer flasks as outlined in Table 7.1. Cells were washed in seawater using centrifugation and inoculated into the medium at a density of 3000 cells ml⁻¹.

TABLE 7.1

Modifications made to PES medium for cyst induction

- | | |
|----|---------------------------------------|
| a) | Seawater + PES major salts (Stock 1) |
| b) | Seawater + PES trace metals (Stock 2) |
| c) | Seawater + PES vitamins (Stock 3) |
| d) | PES medium - major salts |
| e) | PES medium - trace metals |
| f) | PES medium - vitamins |
| g) | Seawater only |

Three replicate cultures were used in each treatment. The cultures were incubated under standard culture conditions for 14 days. The cultures were checked daily for the presence of encysted stages. Cell densities were determined from cell counts taken at the termination of the experiment.

Reduced CO₂ levels

In this experiment cells were grown in 100 ml PES medium contained in a 250 ml Erlenmeyer flask which was sealed with a rubber stopper. The air above the culture medium was rendered free of CO₂ by suspending a vial containing 2N KOH within the flask. A piece of fluted filter paper was placed into the vial to improve the efficiency of CO₂ absorption. The alkaline solution was replaced every two days.

The KOH solution would absorb gaseous CO₂ above the culture medium and this would promote a CO₂ diffusion gradient from the culture to the air above the medium. It was hoped that reduced CO₂ levels in the medium would induce the cells to encyst.

Adjusted pH levels

Cells were cultured in 100 ml PES medium with the pH adjusted to give a range of pH5, pH6, pH7, pH8, pH9 and pH10. Cultures, which were incubated under standard culture conditions for 14 days, were checked regularly to see if cysts were present.

7.2.2 Karyological studies

In an attempt to elucidate whether cysts were asexual stages in the life-cycle of *P. pseudoparkeae* or whether they arose through fusion of motile cells, chromosome numbers were determined for the different life-cycle stages.

Three chromosome staining methods were used, namely, the Aceto-Basic Fuchsin method (Gurr, 1965), the Iron Alum Acetocarmine method (Goodwood, 1948; 1966), and the Feulgen stain for DNA (modified after Gurr, 1965). The latter proved to be the most successful technique and is outlined below:

Solution A: Schiff's reagent; prepared as follows:

0,5 g Basic Fuchsin was dissolved in 100 ml distilled water which had been boiled and cooled to 70°C. 0,5 g Potassium metabisulphite and 10 ml 1N HCl were added to this solution when the temperature had dropped to 25°C. The solution, which was left to stand overnight, was decolourized with 1,0 g activated charcoal. The latter was removed from the stain after 30 min by filtering through Whatman's No. 1 filter paper. The resulting colourless stain was stored in a tightly-stoppered dark bottle in a refrigerator.

Solution B: Sulphite Water

0,5 g Potassium metabisulphite and 5 ml 1N HCl were added to 90 ml distilled water. This solution was made fresh immediately before use.

Solution C: Counter stain.

0,1 g Fast green FCF was dissolved in 100 ml distilled water.

Technique:

A 10 ml sample of a culture containing cells in various stages of the life-cycle was placed into a centrifuge tube and centrifuged at 100xg for 5 min to form a cell pellet. Cells were kept in the centrifuge tube during subsequent steps.

Cells were:

- 1) Fixed in Farmer's fluid (acetic acid : 95% ethanol; 1:3 v/v) for 10 min (Chayen *et al.*, 1969).
- 2) Rinsed briefly in 1N HCl
- 3) Immersed in 1N HCl for 5 min at 60°C
- 4) Rinsed briefly in cold 1N HCl
- 5) Washed in distilled water
- 6) Immersed in Solution A for 30 min
- 7) Rinsed in three changes of Solution B
- 8) Washed in distilled water
- 9) Counterstained in Solution C for 30 seconds

Chromosomes were viewed in wet preparations with the light microscope.

7.2.3 Histochemistry

The following histochemical tests were used during this investigation on various life-cycle stages of *P. pseudoparkeae*.

Gram's iodine test for starch (after Gurr, 1965).

Cells fixed in Farmer's fluid were mounted on a microscope slide and the preparation was irrigated with Gram's iodine, prepared as follows:

2.0 g Potassium iodide and 1.0 g iodine were dissolved separately in 5 ml distilled water. The two solutions were combined and the volume made to 300 ml with distilled water.

Result: Starch stains blue

Periodic acid - Schiff (PAS) reaction for carbohydrates (modified after Chayen *et al.*, 1969).

To determine if the cyst wall of *P. pseudoparkeae* was carbohydrate in nature, cysts were subjected to the PAS reaction. All steps in the procedure were conducted with cysts contained in a centrifuge tube. After each step cysts could be centrifuged to form a pellet and the supernatant could be poured off with ease.

- Cysts were: 1) Fixed in Farmer's fluid for 5 min
- 2) Washed in 70% ethanol
- 3) Immersed in periodate solution for 5 min at room temperature. Periodate solution was made up as follows:
- 400 mg periodic acid was dissolved in 15 ml distilled water, and 135 mg sodium acetate (crystalline, trihydrate) was dissolved in 35 ml absolute ethanol. The two solutions were combined. Periodate solution was prepared just before use.
- 4) Washed in 70% ethanol
- 5) Immersed for 5 min in a reducing rinse, prepared as follows:
- 1.0 g Potassium iodide and 1.0 g sodium thiosulphate were dissolved in 20 ml distilled water. 30 ml Ethanol and 0.5 ml 2N HCl were added to this solution with constant stirring.
- 6) Washed in 70% ethanol
- 7) Immersed in Schiff's reagent for 30 min (for preparation see Section 7.2.2 above).
- 8) Washed in three changes of sulphite water (see Section 7.2.2 above).

The results of the staining reaction were viewed in wet mount preparations with the light microscope.

An acetylation control was run to test if a positive reaction was due to carbohydrate. Fixed cysts were incubated in the acetylation solution (mixture of 16 ml acetic anhydride and 24 ml dry pyridine) at 60°C for 18h. After the cells had been washed in distilled water, the PAS test was continued (step 2 above).

Result: Structures staining red or purple-red, i.e. giving a positive PAS reaction, are probably carbohydrate in nature. If these structures are PAS negative after acetylation then their carbohydrate nature is confirmed.

Gram's iodine/sulphuric acid test for cellulose (after Gurr, 1965)

This test was undertaken to determine if the cell walls of the cysts were cellulosic. Fixed cells were stained with Gram's iodine on a microscope slide. The cells were covered with a coverslip and the preparation was irrigated with 75% sulphuric acid. Regions of blue colouration were noted both before and after acid hydrolysis.

Results: Prior to hydrolysis starch is stained blue with Gram's iodine. Cellulose does not produce this characteristic colour reaction. On hydrolysis cellulose structures swell and the resulting hydrocellulose product stains blue.

Alcian blue stain for acidic mucopolysaccharides (after Crayton, 1982)

Fixed or living cysts were stained with 0,1 % alcian blue at pH 2,5 (100 mg alcian blue 8GX added to 100 ml 0,5 N acetic acid) or pH 0,5 (100 mg alcian blue 8GX added to 100 ml 0,5 N HCl).

Results: At pH 2,5 alcian blue binds with acidic polysaccharides (sulphated and/or carboxylated) but at pH 0,5 it binds exclusively with sulphated polysaccharides.

Ruthenium red stain for pectic substances (after Gurr, 1965)

Living cysts were stained in 0,02% aqueous Ruthenium red for 30 min and viewed under the light microscope.

Results: Pectic substances stain red.

The site of ruthenium red binding in the cyst was determined at the ultrastructural level using an *en bloc* staining procedure modified from Trelease (1980). The method is outlined below.

<u>Solution A:</u> glutaraldehyde (5% in seawater)	5,0 ml
0,4 M sodium cacodylate (pH 7,2 in seawater)	2,5 ml
ruthenium red (200 mg in 50 ml seawater)	2,5 ml

<u>Solution B:</u> aqueous osmium tetroxide (4%)	5,0 ml
0,4 M sodium cacodylate (pH7,2 in seawater)	2,5 ml
ruthenium red (200 mg in 50 ml seawater)	2,5 ml

Cysts were prepared for ultrathin section viewing as outlined in Section 2.2.3, except that Solution A was substituted for the primary fixative (1h fixation) and Solution B was substituted for the post-fixative (3h fixation).

Result: Ruthenium red binds to acid polysaccharides and interacts with osmium tetroxide at the site of binding to produce an electron dense image.

Acetolysis test for sporopollenin (Atkinson *et al.*, 1972).

To test if the cell wall of *P. pseudoparkeae* contained sporopollénin, cysts were subjected to acetolysis : cysts were suspended in glacial acetic acid for 5 min and then transferred to the acetolysis solution (9 parts acetic anhydride plus 1 part conc. sulphuric acid) equilibrated at 95°C in a hot water bath. Acetolysis was allowed to proceed for 10 min and the remaining cell material was washed in glacial acetic acid and viewed under the light microscope.

Result: Sporopollenin, which is the only known organic constituent of plant cell walls to withstand acetolysis, remains.

Sudan black B stain for lipids (Chayen *et al.*, 1969)

Living cysts were stained with Sudan black B (saturated solution in 70% ethanol) for 30 min.

Result: All lipids (other than steroids) stain up grey to blue black in colour.

Gomori reaction for acid phosphatase (after Trelease, 1980)

To localize the sites of acid phosphatase activity in different life-cycle stages of the experimental organism, cells were examined at the ultrastructural level after being subjected to the Gomori procedure

(Gomori, 1952), outlined below.

- Cells were; 1) fixed in 2,5% glutaraldehyde in 0,05 M cacodylate buffer for 1 h
- 2) preincubated in 0,05 M sodium acetate buffer (pH 5,0) for 20 min at room temperature
- 3) incubated in the Gomori reaction mixture in a corked vial for 40 min at 37°C

Preparation of Gomori reaction mixture:

Sodium- β -glycerophosphate	30 mg
0,05 M sodium acetate buffer, pH 5,0	11 ml
0,36 M lead citrate	0,1 ml

The constituents were added in the order indicated.

To prevent the formation of lead carbonate, lead nitrate was dissolved in distilled water (previously boiled) and this solution was added dropwise with gentle stirring to the buffered substrate.

- 4) rinsed in distilled water for 5 min and immersed in a 1% (v/v) aqueous acetic acid solution for 5 min, and then rinsed again in distilled water
- 5) post-fixed and prepared for ultramicrotomy in the manner described in Section 2.2.3.

Two controls to the reaction were included. In one control the substrate, sodium- β -glycerophosphate, was omitted from the Gomori reaction medium and secondly an inhibitor, sodium fluoride (0,1 M solution) was added to the Gomori reaction medium just prior to the addition of the lead nitrate solution. The two control mixtures were substituted for step 3 above, the remaining steps being identical.

Result: Sites of acid phosphatase activity are indicated by a precipitate of lead which appears electron dense in the sectioned material.

7.2.4 Energy Dispersive X-ray (EDX) analysis of the cyst wall

This analysis was undertaken to investigate if inorganic elements were present in the cyst wall of *P. pseudoparkeae*.

Cysts were prepared for ultrathin section viewing in the manner described in Section 2.2.3. A second sample of cysts was similarly prepared except that post-fixation in osmium tetroxide was omitted from the routine.

Thick sections (0,5 - 1,0 μm sections) of the polymerized material were placed onto copper viewing grids. Unstained cells were viewed in a Jeol 35 scanning electron microscope fitted with a KEVEX electron dispersive X-ray analyser.

The inorganic elements present in the cyst wall were recorded from "spot" analyses focussed on this structure.

This work was undertaken in the Electron Microscope Unit, University of Natal, Durban. The analyses were performed by Mr P. Evers to whom I am greatly indebted.

7.2.5 Variations in scale structure

Scale structure in actively growing cultures was extremely constant but some deformed scales were found associated with ageing (senescing) cultures. To obtain a record of the variations in scale structure a suspension of cells from an ageing culture was placed on a copper viewing grid coated with collodion. Cells were killed with O_5O_4 vapours and allowed to settle for 10 min. Excess medium was drawn off the grids and the cells were washed with distilled water to remove any residual salt. The grids were shadowed with Au/Pd and viewed in a TEM. Variant scale forms were also detected in sectioned material embedded in Spurr's resin.

7.3 RESULTS

7.3.1 Cyst induction

No encysted cells were seen in cultures in which the PES medium was modified or in which the CO₂ levels were reduced. Increased pH levels in the culture medium however induced some cells to encyst. At pH9 and pH 10 a few encysted cells were present on the base of the culture flasks. Unfortunately the high pH levels caused dissolved salts to precipitate so that large angular crystals obscured the cysts so that no quantitative data could be obtained on the number of cysts formed. Attempts to separate the cysts from the precipitated salts were unsuccessful because the cells had a "sticky" surface property that allowed them to adhere tenaciously to the precipitate or to the base of the culture flask. They could not be pipetted directly off the base of the flasks but had to first be dislodged with a fine paint brush. Even on a clean microscope slide the cysts became firmly attached.

Despite the high density of motile cells in the cultures with raised pH levels (inoculated at 3000 cells mL⁻¹) a relatively small proportion of these became cysts. Proportionally greater numbers of cysts were encountered during the xenic growth studies (Chapter Three) where the culture flasks were sealed with a rubber stopper assembly with a capillary sampling tube (see Text fig. 3.2.). The conditions within the capillary sampling tube apparently provided a greater stimulus for cyst induction than the raised pH levels in this study.

Because cells were inoculated into the modified PES media at a constant cell density (3000 cells mL⁻¹) the cell densities measured at the end of the experiment can be compared. This would give an indication of the ability of the alga to grow in the different culture media. No cell counts were made during the course of the study so that cell densities at different times during exponential growth are not known. This information would have been valuable because the growth rates of the alga under the different treatments could have been compared. It must be emphasized though that the primary objective of this study was to test if different modifications to the PES medium induced encystment.

The final cell densities of cells grown in modified PES media are shown in Text fig. 7.2. High cell densities were reached only in those cultures which contained PES major salts (NaNO_3 and $\text{Na-}\beta$ -glycerophosphate). Where PES trace metals or vitamins were added to seawater the cell densities after 14 days were significantly lower. In cultures that had only PES vitamins added to seawater the final cell density was significantly lower than those cultures containing PES trace metals only. Where PES trace metals and PES vitamins were combined (i.e. in PES - major salts) the cell density reached was not significantly different from that obtained where vitamins or trace metals alone were added to seawater.

In cultures where trace metals only were excluded from the PES medium the final cell density reached after 14 days was not significantly different from cultures that had only PES major salts added to seawater. Where vitamins only were omitted from the PES medium the cell density reached was high, as mentioned above, but was significantly lower than that in other cultures containing PES major salts.

Cells grew surprisingly well in seawater alone and the final cell density was not significantly lower than that in cultures containing trace metals or vitamins only or trace metals and vitamins in combination.

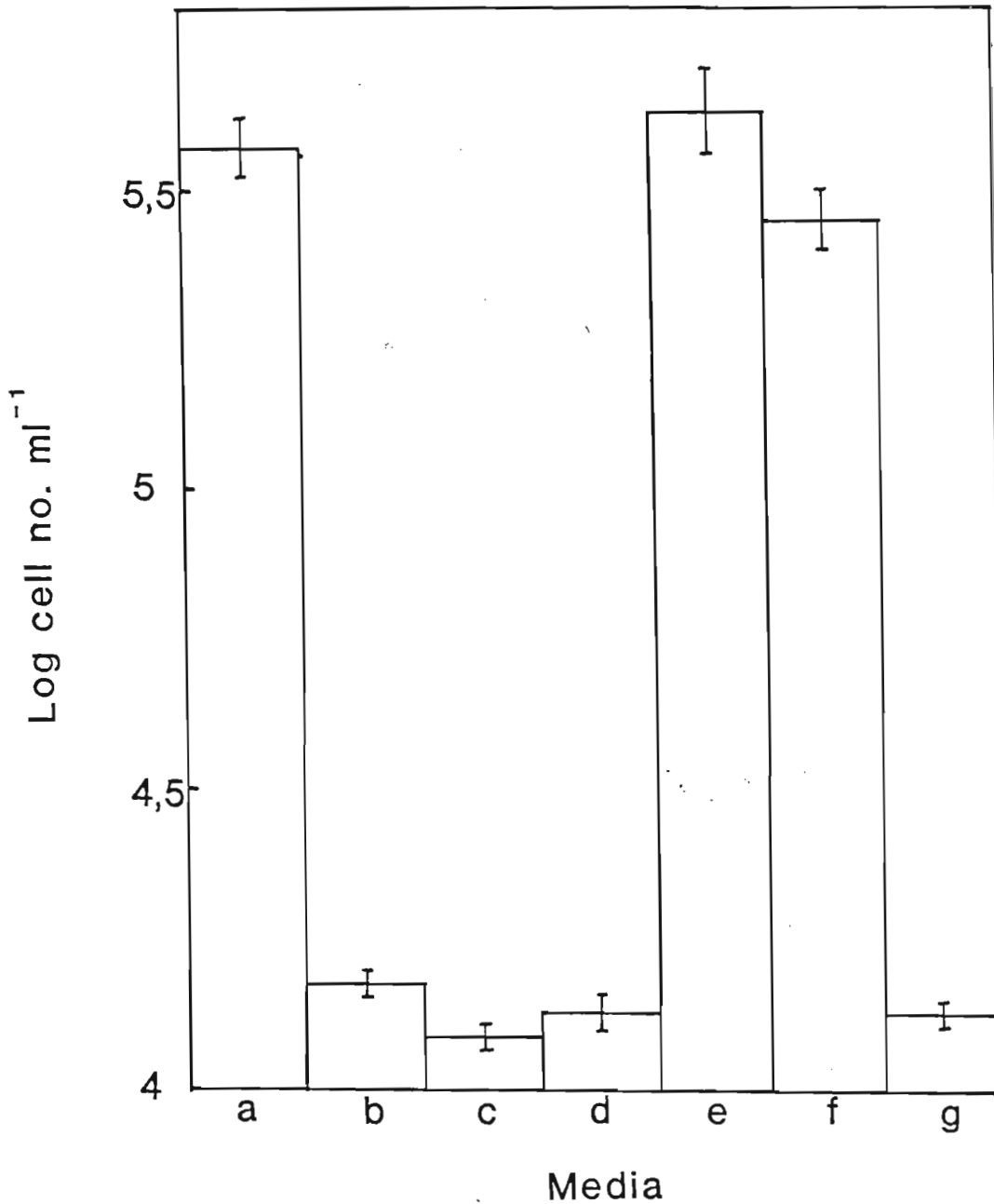
Cells in all cultures multiplied so that after 14 days the cell densities reached were significantly greater than the inoculation density at the start of the experiment.

7.3.2 Light microscopy

Life-cycle stages of *P. pseudoparkeae* were observed in ageing cultures i.e. cultures that had reached the stationary phase of growth. In batch culture the stationary phase of growth was reached after approximately 14 days.

Types of motile cells present in ageing cultures

In ageing cultures two types of cells were clearly visible. Smaller cells called t-cells (typical cells) were found growing with much larger



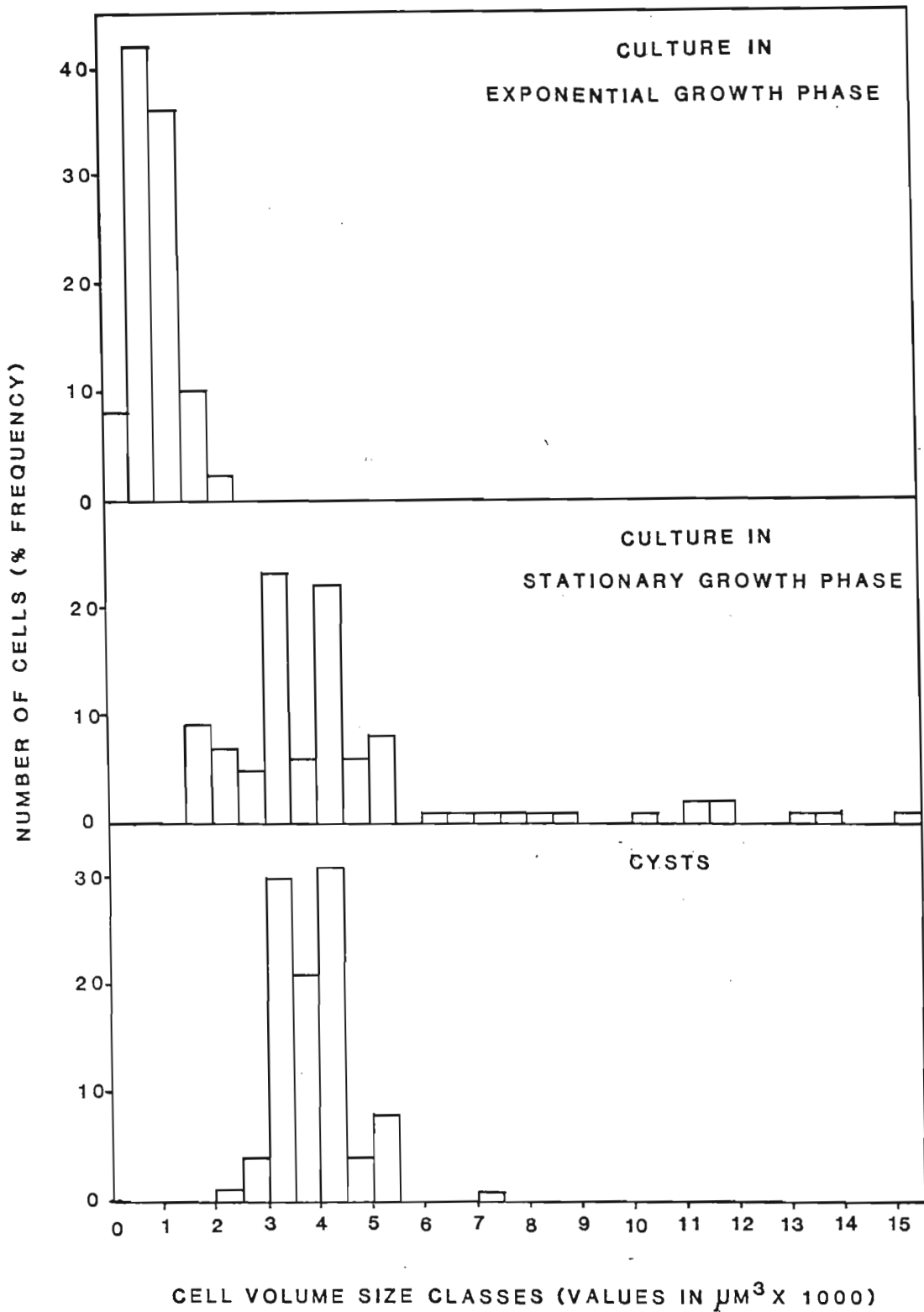
Text fig. 7.2 A bar graph showing cell densities after 14 days of growth in modified PES. media. (Cultures were inoculated at a cell density of 3000 cells ml⁻¹; log cell no. ml⁻¹ = 3,47 ± 0,02 with p = 0,05). The 95% confidence limits of the mean computed from three cell counts made on each of three replicate cultures is shown at the top of each bar.

- a = Seawater + PES major salts (Stock 1)
- b = Seawater + PES trace metals (Stock 2)
- c = Seawater + PES vitamins (Stock 3)
- d = PES medium - major salts
- e = PES medium - trace metals
- f = PES medium - vitamins
- g = Seawater only.

l-cells (large cells). These two types of cells are shown in Pl. 7.1, fig. 1. The l-cells are pyramidal in shape and bear four flagella at the anterior end (Pl. 7.1, fig. 2). Besides the large size difference between t-cells and l-cells, the latter are distinctive in that they have large reserves of starch and lipid material. Scattered lipid globules can be seen in the cytoplasm in the l-cell shown in Pl. 7.1, fig. 1. Four large starch grains are present in each of the chloroplast lobes (Pl. 7.1, fig. 3). L-cells may possess one to four (occasionally more) eyespots (Pl. 7.1, figs. 4 and 5). The nature of the storage products was determined histochemically (see Section 7.3.5 below).

The average cell dimensions in ageing cultures were significantly greater than those determined for cells in exponentially growing cultures. Cell size measurements made on 100 cells in a culture in the stationary phase of growth (see Appendix B; Table II) showed that the average cell length was $22,54 \mu\text{m}$ ($\pm 0,8 \mu\text{m}$; $p = 0,05$) and the average width was $16,68 \mu\text{m}$ ($\pm 0,56 \mu\text{m}$; $p = 0,05$). In an actively growing culture the cells had a length of $14,27 \mu\text{m}$ and a width of $10,63 \mu\text{m}$. Though this difference is not initially striking, it is interesting to compare the average cell volume of cells in the different cultures.

In an actively growing culture the average cell volume (computed from data in Appendix B; Table I) was $1064 \mu\text{m}^3$ ($\pm 78 \mu\text{m}^3$, $p = 0,05$). The average cell volume in an ageing culture was four times greater being $4323 \mu\text{m}^3$ ($\pm 515 \mu\text{m}^3$; $p = 0,05$; computed from data in Appendix B; Table II). This latter sample of cells contained a number of very large, probably pleomorphic, cells so that the computed mean was probably not the true mean for non-pleomorphic cells. The cell volumes of cells in an actively growing culture, in an ageing culture, and a population of cysts are depicted in Text fig. 7.3. For each sample of cells the cell volumes have been broken into size categories to show the percentage frequency in each size class. For the present the cell volumes of encysted cells can be ignored. In the actively growing culture the highest percentage frequency was seen in cells with a cell volume lying between $500 - 999 \mu\text{m}^3$. No cells in the ageing culture fell within this cell volume category. The relatively small number of size classes in the actively growing culture reflects that cell size or volume was remarkably constant in these cells.



Text fig. 7.3. Bar graphs showing cell volume distributions :

- cells in actively growing cultures (A);
- cells in a culture in the stationary growth phase (B);
- a sample of encysted cells (C).

Each bar width represents a cell volume class of $499 \mu\text{m}^3$ in a series beginning $0-499 \mu\text{m}^3$, $500 \mu\text{m}^3 - 999 \mu\text{m}^3$, $1000 - 1499 \mu\text{m}^3$, etc.

This is confirmed by the small 95% confidence limits of the mean of the cell volume measurements of these cells. In the ageing culture a small proportion of the cells had very large cell volumes (between 10000 μm^3 - 15500 μm^3 ; see Text fig. 7.3). It is unlikely that these cells would give rise to cysts because a glance at the cell volumes of cysts shows that most had a cell volume below 5500 μm^3 . If those cells with cell volumes greater than 10000 μm^3 are excluded from the sample in the ageing culture then the computed mean ($3735 \mu\text{m}^3 \pm 298 \mu\text{m}^3$; $p = 0,05$) matches the mode of the sample more accurately.

In the ageing culture it can be seen (in Text fig. 7.3) that there was a relatively low percentage frequency of cells in the two smallest cell volume classes. These cells are recognised as t-cells. They resembled the cells in the actively growing culture because they fell within the cell volume range of these cells, and because they did not contain any lipid reserve material. Furthermore, the t-cells swam rapidly like those cells in the actively growing culture. The l-cells are recognized as those cells having a cell volume greater than 3000 μm^3 . Motility in l-cells was always sluggish. The l-cells were present in greater numbers than t-cells so that approximately half of the cells in the sample had a cell volume lying between 3000 μm^3 - 4500 μm^3 . It is probably this disproportion in numbers that emphasizes the difference between t-cells and l-cells despite the fact very few cells of intermediate size were present. As will be discussed below, the l-cells may reproduce by binary fission so that the cells measured in the size class range 6000 μm^3 - 9000 μm^3 may have represented cells undergoing division.

Cell division in l-cells

The l-cells occasionally divided by binary fission in a manner similar to that described for t-cells in actively growing cultures in Chapter Five. The first sign of cell division was marked by the division of the chloroplast (Pl. 7.1, fig. 6) which was accompanied by a small cleavage groove at the posterior end. Subsequently the cell produced four additional flagella which increased in length until they were as long as the existing flagella (Pl. 7.1, figs. 7, 8 and 9). The replicated flagellar basal bodies

separated as an anterior cleavage furrow developed (Pl. 7.1, fig. 10, Pl. 7.2, figs. 1 and 2). The separation of the flagellar poles was not as rapid as in t-cells. When seen in apical view the dividing cells were somewhat rectangular in shape being extended in a plane at right angles to the plane of division (Pl. 7.2, fig. 3). Four starch grains were clearly visible in chloroplast lobes. As cytokinesis progressed the flagellar poles became widely separated (Pl. 7.2, figs. 4 and 5) and eventually two quadriflagellate daughter l-cells were produced. These cells were asymmetrical having two large starch grains (Pl. 7.2, fig. 6). Soon two additional starch grains were formed in the two new lobes of the chloroplast. These cells sometimes divided again or they became non-motile and encysted.

L-cells often had impaired (incomplete) cytokinesis so that pleomorphic cells resulted. Where cytokinesis was inhibited in early stages the flagellar poles did not separate (Pl. 7.2, fig. 7). Although a longitudinal cleavage line was apparent in these cells they never separated. In other cells cytokinesis progressed more rapidly at the posterior end but again these cells did not separate (Pl. 7.2, fig. 8). Cytokinesis was sometimes arrested only at a late stage so that cells remained in a permanent "back-to-back" stage (Pl. 7.2, fig. 9). Often one of the lobes of the cell was smaller than the other (Pl. 7.2, fig. 10 and 11). The larger lobe sometimes divided. Again cytokinesis was usually incomplete so that a pleomorphic cell with three lobes was formed (Pl. 7.2, fig. 12). Each lobe of the cell had four flagella in a flagellar pit. As many as four such lobes have been seen in pleomorphic cells. On one occasion the detachment of one of the lobes of a pleomorph was observed. It is not known if this cell is able to regenerate the t-cell phase.

Cyst formation

Only l-cells with large reserves of starch and lipid developed to form cysts. The onset of encystment was marked by the rounding off of the cells and the reduction in the length of the flagella (Pl. 7.3, figs. 1 and 2). These cells eventually resorbed their flagella (Pl. 7.3, fig. 3) and either settled out on the bottom of the culture vessel or adhered to the sides of the vessel at the air/medium interface. The first cysts usually

appeared in cultures that were approximately 14 days old.

The cyst had between one and four (occasionally more) eyespots. As the cysts matured they deposited a thick cell wall (Pl. 7.3, figs. 4 and 5). If the cyst was seen lying in the correct position the four large starch grains that were characteristic of the l-cell could be seen in the chloroplast lobes (Pl. 7.3, fig. 6). As the cysts matured one or two additional large starch grains were formed.

The mature cyst often had a "fuzzy" outline which at first sight gave the cyst wall the appearance of having spiny projections (Pl. 7.3, fig. 7). This fuzzy layer was in fact a dense coating of rod-shaped bacilli that were attached at one end to the cyst wall.

Encystment was not synchronous. Although the first cysts appeared in the cultures after 14 days, they continued to form for another 60 days.

A comparison of the cell volumes of l-cells and cysts (Text, fig. 7.3) shows that the l-cells formed cysts directly without an increase in cell volume. The computed mean cell volumes of l-cells and cysts were not significantly different ($p = 0,05$). The average diameter of cysts determined from 100 cells (See Appendix B, Table III) was $19,26 \mu\text{m}$ ($\pm 0,27 \mu\text{m}$; $p = 0,05$).

Excystment

Cysts remaining in old cultures seldom germinated. If they did the motile cells produced were smaller than usual and they were almost colourless. It is not known if these cells remained viable after they were released into the medium.

When cysts were placed into fresh PES medium they began excysting after 10 days. Excystment was not synchronous and some cysts only germinated after 30 days. It is noteworthy that in any population of cysts by far the majority did not germinate. The stimulus resulting in excystment is not known. While fresh medium induced germination in some

cells, others remained dormant. It may be that these cells had not reached physiological maturity or that the culture requirements for germination were not met by the addition of fresh medium alone.

Cysts were extremely resistant and remained viable after being placed in a refrigerator in the dark at 4°C for 30 days. Furthermore cysts placed into an evaporating dish remained viable after the salts in the medium had reached a concentration of 160% as determined with a refractometer.

The first sign of cyst germination was seen in the movement of the protoplast away from one pole of the cyst (Pl. 7.3, fig. 8). The chloroplast divided soon afterwards (Pl. 7.3, fig. 9) and this was followed by the first division of the protoplast (Pl. 7.3, fig. 10). The two daughter cells formed were non-motile.

The cyst wall then ruptured and the daughter cells were extruded from the cyst in what will be called the parental sac (Pl. 7.3, fig. 11). This parental sac appeared to be membranous but it is not known if the sac represents the plasmalemma of the parental cell or if it is a delaminated sphere from the inner layer of the cyst wall. As will be shown in Section 7.3.5 below, the inner layer of the cyst wall remained intact after the contents were extruded so that the parental sac may represent the parental plasmalemma. If this is true then each daughter protoplast would have to synthesize its plasmalemma *de nova*.

After being released from the cyst casing the parental sac rapidly increased in volume (Pl. 7.4, fig. 1). The daughter cells differentiated to produce two motile cells each possessing a flagellar pit from which four flagella emerged (Pl. 7.4, fig. 2). These cells orientated themselves in such a way that they came to lie at right angles to one another. Both underwent one further division. These divisions were not synchronous because one cell divided before the other (Pl. 7.4, fig. 3). After the first cell had divided the parental sac contained two small cells and one larger cell (Pl. 7.4, figs. 4 and 5). The division of the second, larger daughter cell followed resulting in four motile cells within the parental sac (Pl. 7.4, fig. 6). These motile cells resembled the t-cells.

Most often the parental sac contained only four motile cells that were released into the culture medium when the parental sac ruptured. Five or six motile cells have been seen in the parental sac but this was a rare occurrence (Pl. 7.4, figs. 7 and 8).

Single cysts were isolated and placed into fresh PES medium. These germinated to produce four t-cells that multiplied by repeated bipartition to form dense cultures of t-cells.

Other observations

It is interesting that most cysts developed on or in an acidic mucopolysaccharide film found on the base of the culture flasks in ageing cultures (Pl. 7.4, fig. 9). Numerous bacteria were present in this film and the latter is thought to have been secreted by them. Motile t-cells and l-cells often settled on this material (Pl. 7.4, fig. 10). This behaviour in at least the l-cells may be important in selecting the site for the development of the cyst.

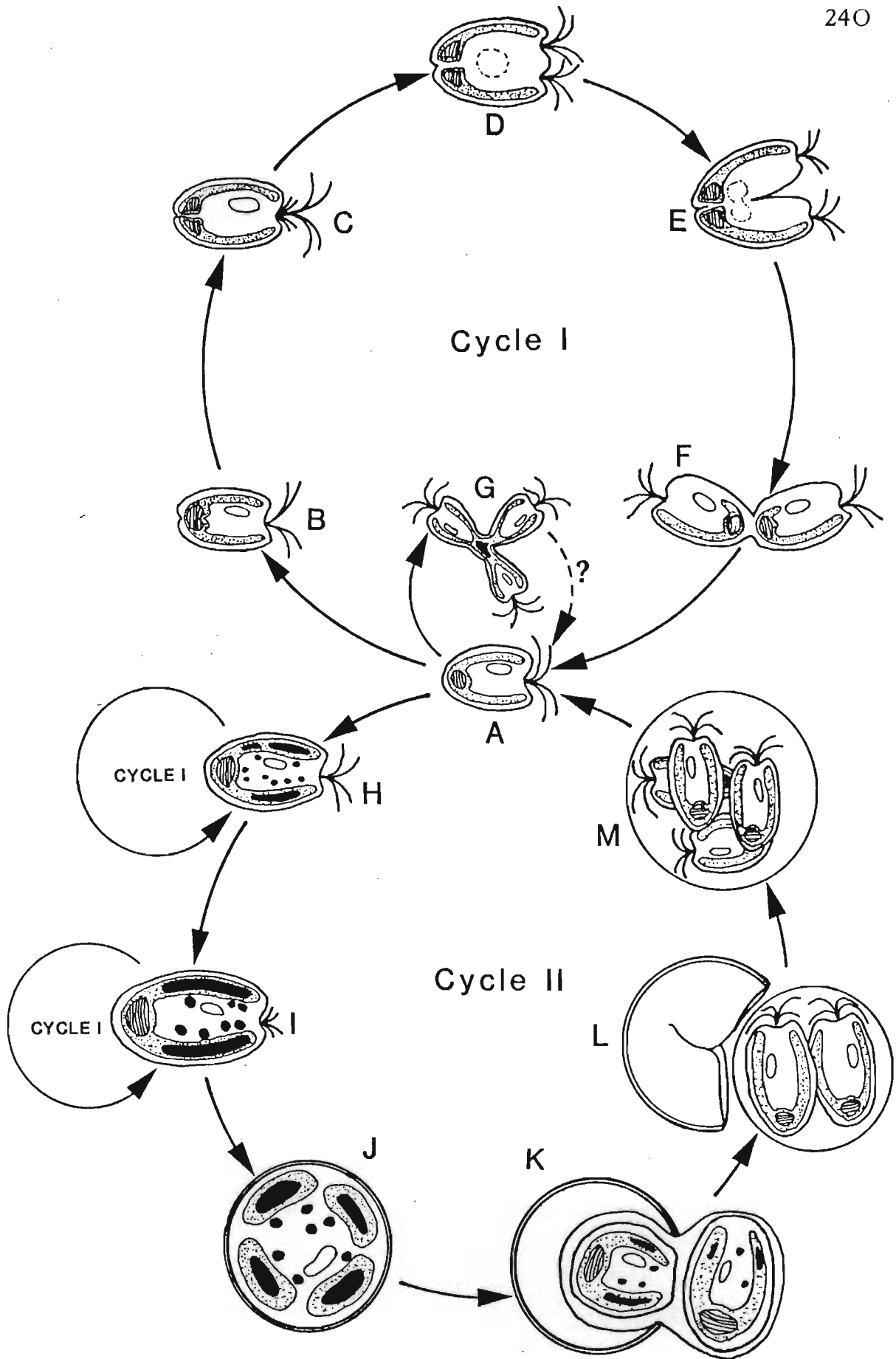
Besides the cyst, a second type of non-motile cell of *P. pseudoparkeae* was found in one culture in the stationary phase of growth. These cells, which occurred in groups, were irregular in outline and had a large vacuole (Pl. 7.4, fig. 11). Under the pressure of a coverslip the cells "flowed" in an amoeboid fashion indicating that the cells had different sol-gel properties than motile cells. The latter always burst under the pressure of a coverslip. The role of these cells in the life-cycle is not understood.

The observations reported above are summarized in Text fig. 7.4 in the form of a diagrammatic life-history of *P. pseudoparkeae*.

7.3.3 Electron microscopy

The ultrastructure of t-cells was identical to that described for actively growing cells in Chapter Two and will not be repeated here.

As seen in longitudinal and transverse section the l-cell had the same complement of organelles reported for t-cells. (Pl. 7.5, figs. 1 and 2;



Text fig. 7.4 The life-history of *Pyramimonas pseudoparkeae*.

Pl. 7.6, fig. 1). The l-cells differed in that they were larger and had many lipid globules in the cytoplasm and large starch grains in the chloroplast. In l-cells the scale reservoir remained connected with the flagellar pit via a duct. The compound microtubular rootlet was seen to surround the duct of the scale reservoir (Pl. 7.6, figs. 1 - 3). The striking size difference between t-cells and l-cells is evident in Pl. 7.7, fig. 1. The scale-boundary in l-cells was identical to that described for actively growing cells in Chapter Two.

The development of lipid globules in l-cells is interesting and began with the inclusion of a number of small electron translucent spheroids in what was to become the boundary surrounding the lipid globule (Pl. 7.7, figs 2 and 3). When these translucent spheroids fused some polymerization must have occurred as the product formed was osmiophilic indicating that lipidoidal material was present (Pl. 7.7, fig. 4). Further fusion of the vesicles resulted in an almost mature lipid globule (Pl. 7.7, fig. 5). Mature lipid globules were sometimes surrounded by endoplasmic reticulum (Pl. 7.7, fig. 6), but the significance of this observation is not known.

Cell division in l-cells began with the division of the chloroplast as in t-cells (Pl. 7.8, fig. 1). When this was completed the base of the flagellar pit broadened and the basal bodies replicated (Pl. 7.8, fig. 1). Four new flagella were formed so that a transverse section through the flagellar pit showed that the cell had eight flagella (Pl. 7.8, fig. 2). As cytokinesis proceeded the two sets of basal bodies separated so that the cell was joined only near the posterior region (Pl. 7.9, fig. 1). Below the level of the two flagellar pits the nucleus divided and the two daughter nuclei came to lie close to one another (Pl. 7.9, fig. 2). No interzonal spindle or phycoplast was seen between the telophase nuclei (Pl. 7.9, fig. 3).

When l-cells retracted their flagella and became non-motile the cell rounded off and lost its characteristic flagellar pit (Pl. 7.10, fig. 1). At this stage the scale reservoir contained a large number of body scales (Pl. 7.10, fig. 2) and was separated from the vacuole by a thin cytoplasmic partition. As the non-motile cell matured the vacuole accumulated membranous inclusions and scales. At a later stage many vesicles were

present in the cytoplasm and they contained scales, trichocysts and membrane fragments (Pl. 7.10, fig. 3). At this stage it was difficult to decide which of the vesicles was the scale reservoir and which was the vacuole. Scales and other inclusions in the vesicles appeared to be degraded. The Gomori test was done to determine if the vesicles contained enzymes, specifically acid hydrolases (see Section 7.3.5 below), that may be used to hydrolyse the contents of the vesicles.

As the non-motile l-cell rounded off further it began depositing a cell wall beneath the scale-boundary (Pl. 7.11, figs. 1 and 2). This cell could be referred to as an immature cyst. The scale-boundary of the immature cyst was soon lost. This may have occurred because the scales were no longer closely associated with the plasmalemma. Scales may also have been removed by bacteria which sometimes attached to the cyst wall (Pl. 7.11, fig. 3). The bacteria seemed to digest the scales and often bacteria were seen with scales adhering to them (Pl. 7.11, fig. 4).

The Golgi apparatus of the immature cyst, which consisted of two dictyosomes in the l-cell, became more extensive and many smaller dictyosomes were found in the cytoplasm. These were usually situated near the plasmalemma and were associated with a peripheral network of endoplasmic reticulum (Pl. 7.12, figs. 1-3). In this position the ER and dictyosomes were probably involved in the deposition of the cell wall.

The immature cyst first deposited an electron translucent wall which was between 50 nm and 100 nm in thickness (Pl. 7.12, fig. 1). As the cyst matured a second, electron dense, layer was added to the cell wall (Pl. 7.12, figs. 2 and 3). The peripheral ER and dictyosomes were found near the plasmalemma throughout the period of cell wall growth. During the deposition of the inner layer of the cyst wall, vesicles containing electron dense material were found at the mature face of the dictyosome (Pl. 7.12, figs. 2 and 3). It is possible that this material was eventually released from the cell to form the inner layer of the cyst wall. It is surprising that the mature face of the dictyosome depicted in Pl. 7.12, fig. 2, was directed toward the centre of the cell. The position of the dictyosome may be dictated by the position of the endoplasmic reticulum which is always found around the periphery of the cell. One dictyosome

was seen to be inclined to the cell surface so that vesicles containing what appears to be wall material had a shorter path to the plasmalemma (Pl. 7.12, fig. 3).

The inner layer of the cyst wall continued to grow as the cyst matured with electron dense material being added to the wall by apposition. Occasionally fixation caused the innermost layer of wall material to separate from the wall (Pl. 7.12, fig. 4). This lamellate structure of the wall supports the view that cell wall material is deposited by apposition. The inner layer of the cyst wall was observed to reach a thickness of 400 nm. Although this layer was usually uniformly thick it appeared to be strengthened with nodules or ribs (Pl. 7.12, fig. 5). The outer layer of the cyst wall sometimes had sparse filiform projections (Pl. 7.12, fig. 5).

The mature cyst was spherical and lipid and starch storage material were abundant in the cell (Pl. 7.13, fig. 1). The contents of the cyst were difficult to preserve and the cells always plasmolysed to some extent during processing for electron microscopy. This may indicate that encysted cells had an osmotic potential different from that in t-cells and l-cells.

The cyst had a single nucleus (Pl. 7.13, fig. 1) which was associated with the rhizoplast and microbody (Pl. 7.13, fig. 2). Flagellar basal bodies persisted in the encysted cells (Pl. 7.13, fig. 3).

On one occasion both mature and immature cysts that were settled on the bottom of a culture flask migrated to the air/medium interface. This happened when the air supply apparatus used to aerate the cultures was turned off. When these cysts were processed for electron microscopy they were seen to have dilated thylakoids in the chloroplast (Pl. 7.14, figs. 1-3). This unusual appearance of the chloroplast was not seen on any other occasion and it is not known if this was an artefact of fixation or if the dilations somehow imparted some buoyancy to the cells.

As mentioned above, cysts did not germinate synchronously when placed in fresh medium. Usually the majority did not germinate. This made it very difficult to obtain an ultrastructural record of excystment. Two cysts were seen in an early stage of germination (Pl. 7.14, fig. 1;

Pl. 7.15, fig. 1). In one cyst the chloroplast had begun to divide as two invaginations of the chloroplast envelope were seen to grow toward each other in the region of the pyrenoid (Pl. 7.15, figs. 1 and 2). In this cell a massive microbody was closely associated with the dividing chloroplast in the region of the pyrenoid (Pl. 7.15, fig. 2). In the cell shown in Pl. 7.14, fig. 1, the chloroplast had divided.

Large pleomorphic cells were common in ageing cultures. These motile cells had aberrant mechanisms of mitosis and cytokinesis. In l-cells the nucleus usually divided after cytokinesis had progressed to a point where the flagellar poles of the prospective daughter cells were well separated. However the pleomorph depicted in Pl. 7.16, fig. 1, had only a single flagellar pit but contained two nuclei lying close to one another. Though the chloroplast had divided, cytokinesis was arrested. In some cells cytokinesis progressed normally at first, so that the flagellar poles became separated, but shortly after the formation of an anterior cleavage groove cytokinesis stopped (Pl. 7.16, fig. 2). This cell had two telophase nuclei that were not associated with a persistent telophase spindle (Pl. 7.16, fig. 3).

In some pleomorphic cells the separation of the daughter cells was prevented when they remained attached posteriorly in the region of the pyrenoid (Pl. 7.17, fig. 1). One lobe of the pleomorph was usually larger than the other and this also divided occasionally (Pl. 7.17, fig. 2) so that the resulting pleomorph had three flagellar poles (Pl. 7.17, fig. 3). Pleomorphs usually had one nucleus in each of the lobes of the cell.

One exceptionally large pleomorphic cell was seen with a large deformed microbody that coiled around the rhizoplast (Pl. 7.18, figs. 1 and 2).

7.3.4 Karyological studies

No fusion of t-cells or any other cells was observed in cyst-forming cultures and for this reason it is believed that *P. pseudoparkeae* reproduces only asexually.

Two observations however appeared to suggest that l-cells could be derived from a fusion of t-cells and thus made a determination of the chromosome numbers in these cells essential. The first observation was that when two t-cells came to lie side by side they often adhered to one another. These cells always separated after a time but the behaviour suggested that the cells have the potential to fuse. The second observation was that the cell volume of l-cells was approximately twice that of t-cells and that l-cells could have resulted from an undetected fusion.

Although the Feulgen reaction for DNA clearly revealed the nucleus in all cells stained in this manner, chromosomes were rarely seen and especially so in l-cells. The reason for this was that the nucleus had to be undergoing mitosis before the chromosomes had condensed sufficiently to be seen. Because l-cells divided infrequently metaphase nuclei with well defined chromosomes were difficult to find. Furthermore, the starch and lipid reserves, which were abundant in these cells, often obscured the chromosomes.

Approximately 13 (\pm 1) chromosomes were counted in t-cells (Pl. 7.19, figs. 1-3). The chromosomes depicted here had divided so that paired chromatids were evident in the preparation.

In l-cells the chromosome number was also estimated to be 13 (\pm 1) chromosomes (Pl. 7.19, figs. 5-7). The l-cell from which the chromosome count was made is shown in Pl. 7.19, fig. 4. In this micrograph a second l-cell of comparable size, but with much more reserve material is shown. The storage material in some cells was occasionally hydrolysed in the preparatory steps when the cells were placed in 1 N HCl at 60°C for 5 min.

No chromosomes were seen in cysts. Cysts had a single nucleus which was always seen in the interphase stage. Chromosomes would only have been evident in germinating cysts when the nucleus prepared for division. Because this occurred infrequently prophase and metaphase stages were not observed.

The results of the chromosome counts confirmed that t-cells and l-cells have the same ploidy and that *P. pseudoparkeae* reproduces asexually. Because motile cells released from the germinating cyst did not fuse, and because they fell within the size range of t-cells, it is improbable that *P. pseudoparkeae* has a gametic life cycle with motile products of cyst germination representing the products of a reduction division.

7.3.5 Histochemistry

The chemical nature of both the cyst wall and storage products in cysts and l-cells was determined using a number of histochemical tests. At the ultrastructural level the sites of acid phosphatase activity in t-cells and l-cells was investigated using the Gomori reaction. The site of ruthenium red binding in the cyst wall was confirmed in ultrathin sections.

Whereas t-cells and l-cells always stained rapidly, living or dead cysts with an intact wall were difficult to stain. To obtain any satisfactory staining reaction the cysts had to be placed in the stains for long periods. However, where the cyst wall was ruptured, either physically or during excystment, staining was rapid. The fortuitous delamination of the cyst wall in some preparations revealed that only the inner layer of the cyst wall reacted with the stains used. The outer layer, which obviously impeded the entry of stains into the cell, was apparently inert.

In contrast with the t-cells, l-cells contained large reserves of starch that stained purplish brown with Gram's iodine (Pl. 7.20, fig. 1). Four elongate starch grains were usually found in each of the four chloroplast lobes (Pl. 7.20, figs. 2 and 3).

The cyst wall stained with ruthenium red (Pl. 7.20, fig. 4) and also bound alcian blue at pH 2,5 and pH 0,5 (Pl. 7.20, figs. 5 and 6, respectively). A positive staining reaction with these stains suggested that the cyst wall is composed of an acidic polysaccharide. The ability of the cyst wall to bind alcian blue at pH 0,5 is indicative of the presence of sulphated groups in the polysaccharide. In some preparations the cyst wall delaminated at the interface between the outer and inner layers of the

wall (Pl. 7.20, fig. 5). These preparations revealed that only the inner layer of the cyst wall was reacting with the stains and was composed of acidic polysaccharide material. Where cysts were stained with Gram's iodine the large starch grains within the cyst were evident (Pl. 7.20, fig. 7). The cell wall of the cyst stained a pale straw colour with iodine but this did not swell or assume a blue colouration when the preparation was irrigated with sulphuric acid. This indicated that the cyst wall was not cellulosic in composition.

Where cysts were stained with sudan black B the large lipid globules in the cytoplasm stained black (Pl. 7.20, fig. 8). The cyst wall stained a pale grey with this stain indicating that some lipoidal material was present in the wall. The lipoidal nature of the inner layer of the cyst wall was confirmed by its osmiophilic property as seen in thin sections.

At the ultrastructural level it was confirmed that the inner layer of the cyst wall is the site of ruthenium red binding. The inner layer of the cyst wall appeared much more electron dense after staining with ruthenium red (Pl. 7.20, fig. 10). In a control test where ruthenium red was omitted from the fixative the inner layer of the wall was not as densely stained (Pl. 7.20, fig. 9).

The cell walls of cysts did not react with Schiff's reagent and are therefore PAS negative (Pl. 7.20, fig. 11) This result was surprising because the cyst wall (inner layer) was shown histochemically to contain an acidic polysaccharide. The PAS reaction is a standard test for localizing carbohydrate material. That starch grains in the cyst were coloured pink indicated that the test was successful.

The outer layer of the cyst wall was resistant to the acetolysis treatment (Pl. 7.20, fig. 12). Both the contents of the cyst and the inner layer of the cell wall were digested. The latter was confirmed when walls remaining after acetolysis failed to bind alcian blue. The resistant outer layer of the cyst wall is believed to be composed of sporopollenin because this is the only organic substance known to withstand acetolysis for more than a few minutes.

The Gomori reaction showed that in t-cells the cisternae near the forming face of the dictyosome had most acid phosphatase activity (Pl. 7.21, figs. 1 and 2). Acid phosphatase activity was also detected in the outer region of the chloroplast and in the mitochondrion (Pl. 7.21, figs. 3 and 4). The site of phosphatase activity was determined by the dense precipitate of lead phosphate occurring in these regions. A low density of lead phosphate precipitate was evident in the nucleus and other parts of the cytoplasm (Pl. 7.21, fig. 1).

In mature l-cells acid phosphatase activity was also detected in the dictyosomes and outer regions of the chloroplast but it was especially evident in large lysosomal vesicles which contained a large amount of Gomori reaction product (Pl. 7.22, figs. 1 and 2). The origin of the lysosomal vesicles is not known with certainty. They appear to develop from the vacuole, scale reservoir or other cytoplasmic vesicles. Occasionally one or more type of vesicle may fuse to give rise to the lysosomal vesicle.

In l-cells the scale reservoir (and vacuole) was usually packed with flagellar and body scales. The Gomori reaction showed that there was acid phosphatase activity in both these organelles (Pl. 7.22, fig. 3; Pl. 7.23, fig. 1). The Gomori reaction product was seen to be deposited on the scales. This observation together with the poorly defined structure of the scales indicated that they were being subjected to enzymic degradation. It is believed that the hydrolysis products of enzyme activity on scales (and other components, e.g. trichocysts and membrane fragments) may be used during the deposition of the cyst wall as the l-cell develops into the cyst. Dense deposits of Gomori reaction product were sometimes seen in the scale reservoir indicating that this organelle develops into a lysosomal vesicle (Pl. 7.23, fig. 2).

Two controls to the Gomori test were run. Where the Na- β -glycerophosphate substrate was omitted from the Gomori reaction medium acid phosphatase activity was evident in cisternae at the forming face of the dictyosome, and also in the chloroplast and scale reservoir (Pl. 7.23, fig. 3). For a lead phosphate precipitate to have been detected in these organelles it is suggested that a phosphate substrate was present at these sites. A relatively small amount of substrate may have been present because

smaller deposits of lead phosphate precipitate were detected. In the second control where cells were treated with the enzyme inhibitor sodium fluoride, no acid phosphatase activity was detected in the cells (Pl. 7.23, fig. 4).

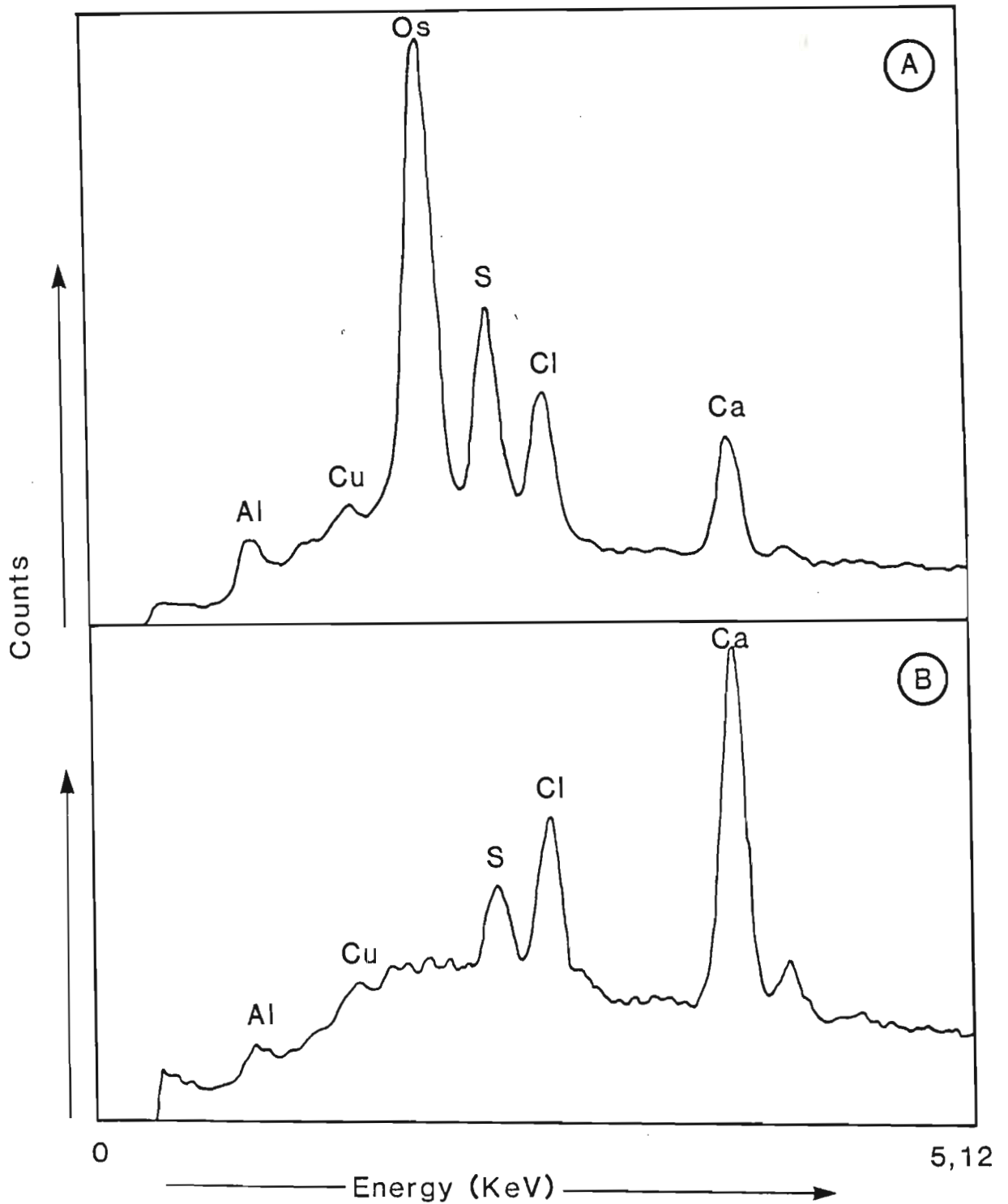
7.3.6 Energy dispersive X-ray (EDX) analysis of the cyst wall

The elemental composition of the cyst wall of *P. pseudoparkeae* was determined by EDX analyses. Two elemental spectra were obtained; one from cells that had been fixed in glutaraldehyde and post-fixed in osmium tetroxide, and one from cells that had been fixed in glutaraldehyde only (see Text fig, 7.5).

Where the cells were post-fixed in O_5O_4 , the element osmium formed a massive peak in the spectrum. This peak was expected because the cyst wall (inner layer) was known to be osmiophilic. An osmium peak was not present in the cell walls of cells fixed in glutaraldehyde only. It is therefore not a natural component of the cyst wall and its presence in the other spectrum can be ignored. Aluminium and copper were present in both spectra but they can be disregarded because they represent contamination from the copper viewing grid. Similarly chlorine can be ignored because this element is present in the Spurr's resin in which the cysts were embedded. Most important in both spectra are the peaks for sulphur and calcium. Line scans made across the cyst in the sulphur and calcium windows showed that these elements were concentrated in the cyst wall (Pl. 7.24, figs. 1 and 2, respectively). The detection of sulphur in the cell wall confirmed the results of alcian blue staining at pH 5. At this low pH the stain binds exclusively with sulphate groups.

7.3.7 Variations in the structure of scales

One of the objectives of the growth studies presented in Chapter Three was to test if scale structure varied under different environmental conditions. These studies revealed that scale structure was remarkably constant under all conditions tested. Only in ageing cultures were deformed scales observed. Information on variations in scale structure is presented here in Chapter Seven because ageing cultures contained life-cycle stages and cells bearing deformed scales.



Text fig. 7.5. The elemental composition of the cyst wall of *P. pseudoparkeae* determined by Energy Dispersive X-ray analysis.

Spectrum A - cells fixed in glutaraldehyde and post-fixed in O_3O_4 .

Spectrum B - cells fixed in glutaraldehyde only.

Deformed scales were usually found on pleomorphic cells which included very large l-cells. Deformed scales were produced within the dictyosomal cisternae and did not change once they were released from the cell. Variations in the structure of scales were most apparent in B₃ and B₂ scales. A selection of micrographs depicting the types of variations occurring in these scales is shown in Plate 7.25, figs. 1-6, 10). The deformed B₃ scales usually contained more scale material than normal B₃ scales and did not have the typical coronate structure. A variant type of B₃ scale with projections on the corners of the scale is seen in Pl. 7.25, fig. 1). Most often deviations from the typical B₃ scale were seen in the acquisition of additional radiating struts on the base of the scale (Pl. 7.25, figs. 2 and 3). Additional scale material was usually added to the base of the scale making it more plate-like (Pl. 7.25, figs. 4-6). In the B₂ scales the pattern of the material forming the substructures in the four quadrats of the scale varied to different degrees (Pl. 7.25, fig. 10). In some scales it was almost lost and in others an elaborate pattern developed. The four prominent cross-bars on the B₂ scale seldom changed, but if they did they appeared to be more delicate than usual. One B₂ scale (Pl. 7.25, fig. 10) was seen with one cross member missing.

The structure of the F₃ flagellar scales was more consistent than the B₃ and B₂ body scales. Minor variations were seen where the truncated end of the F₃ scale became prominent and angular (Pl. 7.25, fig. 7). In this scale additional scale material was added to fill the large apertures usually lying adjacent to the raised spine. In other scales a second tail-like proliferation was formed which ran parallel to the free spine. (Pl. 7.25, fig. 8). The raised spine also extended beyond the truncated end of the scale in some cases (Pl. 7.25, fig. 9).

B₁, F₁, F₂ and F₄ scales were not seen to vary even on pleomorphic cells.

Despite the variations in scale structure observed above, none of the deformed scales resembled those of any other species of *Pyramimonas*. This study, nevertheless, demonstrates that scale structure can vary in old or pleomorphic cells and it emphasizes the need to work with actively growing

cultures when describing the scales found on members of this genus, especially if the alga is considered to be a new species.

7.3.8 Virus and virus-like inclusions in cells

Virus and virus-like particles (VLP's) were seen in l-cells on three occasions. Cells containing the virus or VLP's were always abnormal and probably moribund.

An l-cell containing well defined virus particles is shown in Pl. 7.26, fig. 1. The virus particles were scattered in the cytoplasm and were not present in other organelles. The cytoplasm had a lower concentration of ribosomes causing it to be paler than usual. The l-cell was also unusual in that the chloroplast contained only two starch grains on one side. A large scale reservoir was present in the cell as were a few trichocysts and a nucleus. Dictyosomes were not apparent in the cell though scattered ER was evident. One flagellum in the flagellar pit was unusual in that it lacked the typical structure of the axoneme. The nucleus in infected cells was apparently enclosed within dilated segments of ER (Pl. 7.26, fig. 2). The virus particles themselves were hexagonal in outline in any plane of section and they did not have any appendages (Pl. 7.26, fig. 3). This suggested that the virus had the form of an icosahedron. Each virus particle was approximately 200 nm in diameter at the widest point. The virus capsid was tri-lamellate and was approximately 17 nm thick. Some particles had a dense filamentous core while others had a lighter core surrounded by a dense capsid. The latter particles may have released their contents. A region of low electron density was usually seen surrounding particles with a dense core.

A second type of VLP (approximately 60 nm in diameter) was seen in an extremely aberrant cell in an ageing culture. The VLP's, which were electron dense, were concentrated in the nucleus although some were present in vesicles in the cytoplasm (Pl. 7.27, fig. 1). The chloroplast in this cell was unusual. The lobe containing the pyrenoid had a dense stroma containing sinuous thylakoids curved around what appeared to be scattered carotenoid globules. A similar arrangement of the thylakoids

and free carotenoid globules was seen in the chloroplast lobe containing the eyespot. The chloroplast stroma in this lobe was less dense than that in the other lobe. Trichocysts, mitochondria and a vacuole were the only other organelles present in the cell. The VLP's appeared to be eroding the nucleic acids in the nucleus as nucleoplasm in the vicinity of the VLP's was less dense than in other parts of the organelle (Pl. 7.27, fig. 2).

A third "virus-like inclusion" was seen in the posterior region of one l-cell (Pl. 7.27, figs. 3 and 4). This inclusion contained many regularly arranged units and resembled the crystalline particle of the tobacco mosaic virus. Each unit was approximately 50 nm in diameter and had a central lighter region. The exact nature of this inclusion is uncertain as it also resembles the tubular structures involved in crystal formation in *Pleonosporium* (NAEG). HAUCK. (Kugrens, 1983).

7.4 DISCUSSION

The life-cycle of *P. pseudoparkeae* presented above is the first record of a complete asexual life-cycle in the genus. Hargraves and Gardiner (1980) have reported on some stages in the life-history of *P. amyliifera* but the complete life-cycle of the organism was not documented. It appears from the information presented by Hargraves and Gardiner (1980) that *P. amyliifera* will be shown to have a life-cycle similar to that of *P. pseudoparkeae*. It is surprising that the life-cycle of *Pyramimonas* has not been elucidated before because cysts have been known in the genus for a long time. Almost a century ago *P. tetrarhynchus* was known to produce spherical cysts (20-30 μm in diameter) that may be smooth or bear irregularly arranged spines (Dangeard, 1889; Dill, 1895; Korshikov, 1938; Belcher, 1969a). The cysts of *P. amyliifera* are usually between 18 - 24 μm in diameter (Belcher, 1966) though those figured by Hargraves and Gardiner (1980) were computed to be only 16 μm in diameter. Besides *P. pseudoparkeae*, *P. grossii* is the only other species known to produce cysts (Parke, 1949). These are small being only 6 - 7 μm in diameter. Cysts have also been reported for *P. reticulata* (Korshikov, 1925; Lund and Scott, 1952) but this species has been moved to the Chlorophyceae as *Hafniomonas reticulata* (Ettl and Moestrup, 1980).

Some species of *Pyramimonas* produce palmella stages, viz. *P. grossii* (Parke, 1949), *P. inconstans* (Hodgetts, 1920), *P. octostriata* and *P. quadricauda* (Pascher, 1932) and *P. tetrarhynchus* (Pascher, 1927), as an alternate life-cycle stage. The amoeboid cells of *P. pseudoparkeae* may represent a palmella stage.

Other genera in the Prasinophyceae are known to produce cysts. These include *Nephroselmis*, *Monomastix* and *Pedinomonas* (Belcher, 1970) and *Tetraselmis* (Kobara and Hori, 1975; Tanoue and Aruga, 1975; Norris *et al.*, 1980).

The factors inducing encystment in *P. pseudoparkeae* are not understood. Cysts developed spontaneously in ageing cultures or when grown in the culture vessel depicted in Text, fig. 3.2. It is noteworthy that all other reports of cysts in prasinophycean algae mention that this life-cycle stage was found only in old cultures. It appears therefore that some factor or factors prevailing in old cultures provide the stimulus for encystment. Depletion of nutrients in old cultures may be an obvious stimulus but this has not been proven. In the present study different manipulations of the PES medium did not induce the cells to encyst. Even where washed cells were transferred to seawater no cysts were found. From these preliminary studies it is apparent that low nutrient levels alone do not provide the stimulus for encystment.

Where cysts were formed in the culture vessels containing a capillary sampling tube, a factor other than nutrient concentration must be responsible for stimulating cyst formation. Cells confined to the sampling tube would not deplete the nutrients more rapidly than cells in the surrounding culture because the number of cells per unit volume remains the same. Because cells in the surrounding culture medium were growing exponentially indicates that nutrients were not limiting. Reduced CO₂ levels in the sampling tube may have caused encystment. Cells in the surrounding medium were aerated with 2% CO₂ in air so that once the cells were isolated in the sampling tube this source of CO₂ would not be available to them. Where the CO₂ levels were reduced subsequently in larger cultures no cysts were found. A second factor that could have

induced cells to encyst in the sampling tube, and one that may be linked with CO₂ availability, is pH. The pH levels within the confines of the sampling tube would undoubtedly increase when the cells were photosynthesizing during the light phase. In the surrounding medium the rise in pH would be retarded or negated by the carbonic acid input from the CO₂/air mixture. Where cells were subjected to a range in pH, in a subsequent test, some cells growing at high pH (pH9 and pH10) were induced to encyst. The pH therefore appears to be an important factor for cyst induction but since relatively few cells encysted it is clear that pH is not the only factor responsible.

Another factor that may play a role in cyst formation is the extracellular production of organic substances. Old cultures always have a milky appearance indicating that extracellular products are in high concentration in the cultures. The direct role of these products in stimulating encystment and their possible role in changing the quality of light in cultures should be tested.

Tanoue and Aruga (1975), in their study of *Tetraselmis*, are the only workers who have attempted to determine what factors influence cyst production in the Prasinophyceae. They have shown that by changing the pH of the medium of a culture in the stationary phase of growth, they were able to induce many cells to encyst. This occurred when the pH (pH7,6) was either lowered to pH6 or raised to pH9,5. Surprisingly, few cysts were formed between pH7 and pH8.

The cysts of *P. pseudoparkeae* appear to be resistant resting stages because they remain viable in old cultures for up to four months. They also germinate in fresh medium after the medium in which they were formed was allowed to concentrate to give a salinity reading of 160‰ in a refractometer.

Belcher (1970) tested the resistance of prasinophycean cysts to desiccation and heat. She found that *Nephroselmis*, *Monomastix* and *Pedinomonas* cysts were resistant to desiccation and germinated after being kept in a desiccator for 1 week. Cysts of *Pyramimonas* did not germinate under the same conditions. Where cysts were subjected to elevated

temperatures *Nephroselmis* cysts germinated after being heated to 60°C for 1 hour. The cysts of *Monomastix* and *Pedinomonas* were very much more heat resistant and germinated after being heated for 1 hour at 100°C and 90°C respectively. In this experiment the cysts of *Pyramimonas* did not germinate after being kept at temperatures in the range 20°C - 100°C.

In *Tetraselmis*, cysts that were exposed to air for half an hour or washed repeatedly in fresh medium germinated well (90% germination) after 48h when placed into fresh medium. This almost synchronous germination of cysts was not observed in *P. pseudoparkeae*.

The process of excystment in *P. pseudoparkeae* is similar to that described for *Tetraselmis* (Kobara and Hori, 1975; Tanoue and Aruga, 1975; Norris *et al.*, 1980). The only difference is that the contents of the cyst of *P. pseudoparkeae* are extruded when only two non-motile daughter cells are formed. In *Tetraselmis* four motile cells are produced within the cyst before they are released in a membranous sac. In *P. pseudoparkeae* the cells only become motile when the parental sac is extruded from the cyst casing. One further division of each daughter cell produces four zoospores. It is interesting that both genera produce four zoospores that are released into the medium when the parental sac ruptures.

Very little is known about the ultrastructure of cysts in the Prasinophyceae. Norris *et al.* (1980) have published a micrograph of the cyst wall of *Tetraselmis*. The wall bears sparsely arranged "spines" on its surface and in this respect it resembles those found on the cyst of *P. pseudoparkeae*. The cyst wall in both organisms is bilayered but in *Tetraselmis* the outer wall is electron dense and the inner wall is electron translucent. This arrangement is reversed in *P. pseudoparkeae*.

Very little is known about the composition of the cell walls of non-motile prasinophycean algae. The phycoma stages of *Halosphaera*, *Pachysphaera* and *Pterosperma* also have a bilayered cell wall. In *Halosphaera* the inner layer is pectin-like while the outer layer is thought to contain material similar to sporopollenin (Parke and den Hartog-Adams, (1965). The composition of the cyst wall of *P. pseudoparkeae* is apparently

identical to that of the phycoma wall of *Halosphaera*. The outer layer of the cyst wall of *P. pseudoparkeae* was resistant to acetolysis and is therefore believed to be sporopollenin. This material is "the only known organic constituent of plant cell walls that withstands acetolysis for more than a few minutes....." (Atkinson *et al.*, 1972). The inner layer of the cyst wall is believed to be pectinaceous because it stains positively with ruthenium red. The term "pectinaceous" is a rather loose term and the results of alcian blue staining suggest that the inner layer of the cyst wall is composed of a sulphated acidic polysaccharide. An EDX analysis of the cyst wall of *P. pseudoparkeae* has shown that it contains significant amounts of calcium and sulphur. It is not known if these elements are present in the cell wall of *Halosphaera*. Boalch and Parke (1971) have suggested that the outer layer of the cell wall of *Halosphaera* may contain siliceous particles. Silicon was not detected in the cell wall of *P. pseudoparkeae* during the EDX analysis indicating that siliceous particles were not present in the cyst wall.

The composition of the cell walls of the phycoma of *Pterosperma* and *Pachysphaera* is similar to that in *Pyramimonas* and *Halosphaera* in that an inner layer of pectic material is surrounded by an outer layer of sporopollenin (Wall, 1962; Boalch and Parke, 1971; Parke *et al.*, 1978). Sporopollenin is probably more widely distributed in the Prasinophyceae than generally recognized. Its presence in the fossil prasinophyte *Tasmanites* NEWTON (Brooks, 1971) indicates that the compound has had a long history in the class.

The staining reaction of a pellet of scales is similar to that observed for the inner layer of the cyst wall. Both boundaries bind alcian blue at pH2,5 and pH0,5 and they are probably both composed of a sulphated acidic polysaccharide. It may be significant that scales appear to be digested within cytoplasmic vesicles (lysosomal vesicles) in the immature cyst. This is especially true when the inner layer of the cyst wall begins to be deposited. It may be that the scale reservoir and other vesicles containing scales and organelle fragments act as lysosomes by digesting scales and releasing the products for reutilization in building the cyst wall. The results of the Gomori test demonstrated that acid phosphatase activity was present in the scale reservoir and other vesicles in the immature cyst.

Although not shown in the work on scale composition, the scales probably bear phosphate groups. Lead phosphate precipitates were superimposed on scales in the lysosomal vesicles indicating that phosphatases were acting on scales. The significance of the Gomori test is that it showed that scales and other organelle fragments may be subjected to enzymic degradation in l-cells and immature cysts. Enzymes other than acid phosphatases are probably present in the lysosomal vesicles and may be more abundant but this is difficult to test histochemically.

Phosphatase activity was also detected in the chloroplasts and in cisternae near the forming face of the dictyosome. The presence of lead phosphate precipitate in the chloroplast is in the writer's opinion not surprising because three phosphatase enzymes involved in glycolate biosynthesis may have acted on Na- β -glycerophosphate in the Gomori reaction medium. These enzymes are P-glycolate phosphatase, P-glycerate phosphatase and 3-Phosphoglycerate phosphatase (Tolbert, 1974).

The significance of phosphatase activity in the cisternae and on the scales at the forming face of the dictyosome is not understood. It is interesting that these cisternae contain the nascent scales consisting of a basic frame-work onto which more scale material is deposited in more mature cisternae. It is speculated that the precursors of molecules deposited to form the nascent scale may have phosphate groups.

The asexual life-histories of phycomate genera in the Prasinophyceae are well documented. All these life cycles have two-distinct alternate phases, one being the quadriflagellate motile phase and the other being the pelagic non-motile cyst (phycoma) phase. The life-cycle of *P. pseudoparkeae* is essentially similar except that the motile phase predominates in the life-cycle of the organism. In *Pachysphaera*, *Pterosperma* and *Halosphaera* the pelagic phycoma is most often seen in wild material. This may be because this stage is larger and more conspicuous than the motile phase. The phycomata of *Pterosperma* vary between 14 - 230 μm in diameter depending on the species (Parke *et al.*, 1978) while those of *Pachysphaera* may be between 95 - 175 μm in diameter (Parke, 1966). The phycomata of different species of *Halosphaera* are usually much larger being between

190 - 550 μm in diameter (Boalch and Mommaerts, 1969). The cysts of *P.pseudoparkeae* are generally smaller (19 μm in diameter) and only one small species of *Pterosperma* has a cyst of comparable size (Parke *et al.*, 1978).

Despite the similarities in the composition of the cyst wall in the above genera, structural differences in the wall can be used to separate some genera. *Pterosperma* for example is the only genus to have the outer wall elaborated to produce wing-like structures called an alae (Parke *et al.*, 1978). Both *Halosphaera* and *Pachysphaera* have punctate cell walls though some species of *Halosphaera* have smooth cysts (Parke, 1966; Boalch and Mommaerts, 1969). These two genera can be more clearly separated if their motile cells are compared. The motile cells of *Pachysphaera* are quadriflagellate having four long flagella arising from an asymmetrical cell (Parke, 1966). In *Halosphaera* the motile cells are symmetrical and resemble the motile cells of *Pyramimonas* (Manton, *et al.*, 1963; Boalch and Mommaerts, 1969). Furthermore, the types of scales found on the motile cells of *Halosphaera* resemble those of *Pyramimonas* (Parke and Adams, 1961; Manton *et al.*, 1963; Pennick, 1977b) but are quite different from those of *Pachysphaera* and *Pterosperma* (Parke *et al.*, 1978).

The similarity in the structure of the motile cells of *Pyramimonas* and *Halosphaera* has led some authors to refer to the latter as the *Pyramimonas*-like motile stage of *Halosphaera* (Parke and Adams, 1961; Manton *et al.*, 1963; Pennick, 1977b). This, along with the fact that the two genera have identical cell walls, casts doubts on the validity of a separation at the generic level. In the writer's opinion the only factor favouring the separation is one concerning the development of motile cells within the cysts. In *P.pseudoparkeae* the cyst contents differentiate to produce four motile cells whereas in *Halosphaera* many more (64 - 512 cells) are produced (Parke and den Hartog-Adams, 1965). *Pachysphaera* and *Pterosperma* also release large numbers of swarmers (Parke, 1966; Parke *et al.*, 1978). It is interesting that the swarmers of *Pterosperma* are released rhythmically in a lunar cycle (Parke *et al.*, 1978).

Cyst formation in *P. pseudoparkeae* differs from that in *Halosphaera*, *Pachysphaera* and *Pterosperma* in that the non-motile cell does not increase in size during its development. The cell volume data in Text fig. 7.3, shows that l-cells and mature cysts have comparable cell volumes. By contrast all phycomata gradually increase in volume until they are mature (Parke and den Hartog-Adams, 1965; Parke, 1966; Parke *et al.*, 1978). The origin of the parental sac that is released from the cyst prior to the liberation of motiles needs to be investigated. Parke and coworkers consider the parental sac to be the released inner layer of the phycoma wall (Parke and den Hartog-Adams, 1965; Parke, 1966; Parke *et al.*, 1978). However in *P. pseudoparkeae* the inner component of the cyst wall remained intact (shown by alcian blue staining) after the parental sac had been extruded from the cyst casing.

Scale structure in *P. pseudoparkeae* appears to be remarkably constant over a wide range of environmental conditions. Aberrant scales were only seen on abnormally large l-cells or pleomorphic multilobate cells in senescent cultures. Scale variations were most common in B₃, B₂ and F₃ scales in that order. It may be significant that aberrant scales were only found on pleomorphic cells in which cytokinesis was arrested or inhibited to varying degrees. Incomplete cytokinesis is not uncommon in the algae. A number of mutant strains of *Chlamydomonas* have been isolated in which a high percentage of the cells fail to complete cell divisions (Lewin, 1953; Wetherell and Krauss, 1957; Gowans, 1960). It is possible that pleomorphic cells of *P. pseudoparkeae* are mutant forms. If so, it is noteworthy that aberrant scale forms are found on cells with incomplete cytokinesis. This may be a manifestation of pleotrophy where the gene controlling cytokinesis may also control scale form to some extent. Alternatively the two variant characters may be the result of an epistatic interaction between genes at different loci. The structure of other scales (B₁, F₁, F₂ and F₄ scales) does not vary on pleomorphic cells and it is possible that this conservatism is a result of polygenic control of the characters. This may explain why these scales are similar in most species in the genus and even similar to those in other genera in the class.

It would be interesting to know if pleomorphic cells remain aberrant under suitable growth conditions in fresh culture medium and if cells released from the pleomorph revert to the normal t-cell or l-cell. Lewin (1954) has shown for example that mutant strains of *Chlamydomonas* with partial paralysis of the flagella may revert to the wild-type motility.

McFadden *et al.*, (1982) have shown that the F₃ (limuloid) scales of *Pyramimonas gelidicola* may vary in their perforation pattern. It has been pointed out before that the cells figured by McFadden *et al.* (1982) have some characteristics of l-cells. These include an abundance of lipid globules in the cytoplasm, a large starch reserve, and more than one eyespot (reported to be two in *P. gelidicola*). It is not clear whether the cells were actively growing or if they represent l-cells. If the latter is true then it may be possible that some cells in their cultures were pleomorphs with aberrant scale forms. Variations in scale structure in *P. gelidicola* need to be confirmed in actively growing cultures of the organism.

Virus-like particles (VLP's) seen in *P. pseudoparkeae* have also been reported in two other species in the genus. Two hexagonal-shaped viruses have been discovered in *P. orientalis* (Moestrup and Thomsen, 1974). They differed only in size with the smaller particle measuring 35 - 60 nm in diameter and the larger particle being three times as large. The hexagonal-shaped virus in *P. pseudoparkeae* is approximately the same size as the large virus found in *P. orientalis*. The six sided appearance of these particles suggests that they have an icosahedron structure. *P. tetraerhynchus* is also known to be infected with a virus (Belcher, 1969a) but the shape of the particle is not known.

Viruses have also been found in other genera in the Prasinophyceae. An intranuclear VLP has been found in *Tetraselmis* (Pearson and Norris, 1974). This VLP is hexagonal in outline when sectioned and is between 51 - 57,5 nm in diameter. These VLP's are approximately the same size as the smaller VLP's described for *P. orientalis* and both are characteristically arranged in parallel rows. Melkonian (1982c) has described a virus in *Mestostigma*. The virus, which is 130 nm in diameter, has an icosahedron structure and is similar to that found in

P. pseudoparkeae. In both algae the virus is present in the cytoplasm. The smaller VLP's seen in *P. pseudoparkeae* do not have an icosahedral structure. Similar VLP's have not been reported in the Prasinophyceae.

CHAPTER EIGHT
GENERAL DISCUSSION
AND
CONCLUSION

STRUCTURE, MORPHOLOGY AND PHYLOGENY

The present study revealed that *P. pseudoparkeae* most closely resembles *P. parkeae* (Norris and Pearson, 1975). The two species can only be separated at the ultrastructural level on small differences in scale structure. The electron microscope is thus an essential tool for studying members of the genus. Although the differences in scale structure may appear to be trivial for separating the two taxa at species level, Pienaar and Aken (1985) described *P. pseudoparkeae* as a new species because it exhibits different specific growth rates, and different temperature and salinity tolerances (Pienaar and Norris, unpublished, pers. comm.¹). The English diagnosis for *P. pseudoparkeae* is presented in Appendix D.

Other species in the genus, (particularly *P. orientalis* and its allies - Pennick *et al.*, 1978), are also separated on minor differences in scale structure, but unfortunately these separations are not supported by comparative growth studies. A difference in scale structure is therefore the primary means of separating some species despite the lack of information on the reliability of scale structure as a taxonomic character.

The present study has revealed that scale structure remains constant under a wide range of culture conditions and is probably genetically controlled and not subject to phenotypic changes. Scale structure can therefore be considered to be a conservative taxonomic character reflecting the genotype of the species. Two problems which need to be resolved, however, are (i) to what extent do differences in the genotype (reflected by differences in scale structure) represent genetic variation within a species, and (ii) what degree of genetic distinctness is required to separate taxa at species level.

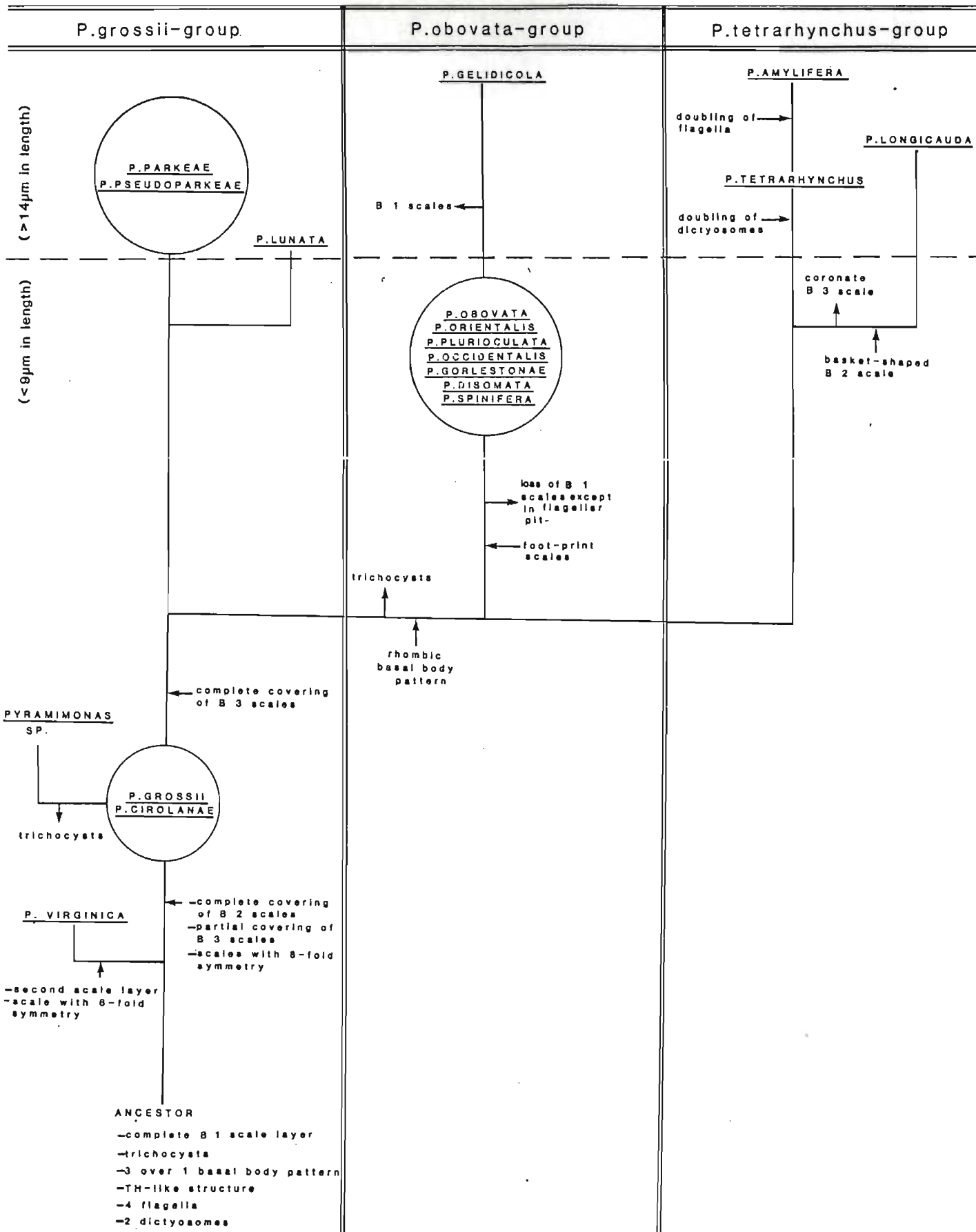
¹ R.N. Pienaar and R.E. Norris, Department of Botany, University of Natal, Pietermaritzburg, Natal, Republic of South Africa.

Because sexual reproduction is not known for *Pyramimonas* (or any members of the Prasinophyceae) it is not possible to apply the biological species concept (Mayr, 1970) in trying to identify species. Of necessity the morphological species concept provides the only means of recognizing species and here morphological distinctness is the decisive criterion of species rank (Mayr, 1970). "Species characters" are used to distinguish one species from another, and they must fulfil two requirements; they must be diagnostic (i.e. be able to differentiate one species from another) and they must be reasonably constant (i.e. non-variable). If scale structure is taken as a species character it fulfills both attributes so that differences in scale structure could be used to separate species.

The writer has one objection to this approach with respect to our current state of knowledge on the genus *Pyramimonas*. Most studies have been conducted with clonal cultures so that variation in characters can be expected to be small. Thus, although scale structure may be genetically fixed, it may vary within populations of species where a large gene pool exists. Many isolates related to *P. orientalis*, for example, possess scales with minor differences in their structure (Pennick *et al.*, 1978) and they may all be morphological variants of a single species. The writer has isolated *P. pseudoparkeae* at various sites along the Natal South coast and has found that scale structure is identical in these isolates. Inouye *et al.* (1983) have also reported that three strains of *P. grossii* isolated from different localities in Japan have identical scale structure. Similar information needs to be gathered on other species before the true value of scale structure as a taxonomic character can be assessed. Besides morphological and structural characteristics, other information on the ecology, physiology, biochemistry, behaviour and reproductive biology of species needs to be collected to aid the delimitation of species.

At present there is sufficient morphological and structural information available on some species of *Pyramimonas* to allow the writer to propose a tentative phylogenetic scheme for the genus.

In the writer's opinion four characters appear to be conservative and can be used to separate *Pyramimonas* into at least three groups (see Text fig. 8.1). These four characters are, 1) the presence or absence of trichocysts, 2) the arrangement of the basal bodies, 3) the extent of the B₁ scale



Text Fig. 8.1. A proposed phylogeny for the better-known species

covering, and 4) the presence or absence of foot-print scales. Other characteristics such as the number, type and arrangement of scales in the scale-boundary, the manner in which thylakoids enter and traverse the pyrenoid, the number of flagella and dictyosomes, and the presence or absence of a transitional helix-like structure are useful in separating species and for drawing phylogenetic links between species in a group. In Text fig. 8.1 three distinct phylogenetic groups are shown viz. *P. grossii*-; *P. obovata*-; and *P. tetra-rhynchus*-groups). Within each group closely related species are positioned within circles which are referred to as species clusters. The species clusters are typified by the first species name appearing in any cluster.

Trichocysts are found in only six species in the genus namely *P. grossii* (Manton, 1969), *P. parkeae* (Norris and Pearson, 1975), *P. virginica* (Pennick, 1977a), *P. cirolanae* (Pennick, 1982a), *P. lunata* (Inouye *et al.*, 1983) and *P. pseudoparkeae* (Pienaar and Aken, 1985). It is interesting that those species which have been studied in detail (*P. parkeae*, *P. lunata* and *P. pseudoparkeae*) also have the flagellar basal bodies arranged in a 3 over 1 pattern and have a complete covering of B_1 scales around the cell. The presence of trichocysts, a 3 over 1 pattern of the basal bodies and a complete covering of B_1 scales are strongly correlated characters and are diagnostic for species assigned to the *P. grossii* group (see Text fig. 8.1). Of the smaller trichocyst bearing species *P. grossii* (Manton, 1969) is known to have a 3 over 1 arrangement of the flagellar basal bodies (see Inouye *et al.*, 1983) and a complete covering of B_1 scales (Pennick and Clarke, 1976). This species is therefore closely allied with the larger trichocysts bearing species. It is the writer's belief that *P. virginica* (Pennick, 1977a) and *P. cirolanae* (Pennick, 1982a), the remaining small species bearing trichocysts and a complete covering of B_1 scales, will be shown to have a 3 over 1 arrangement of the basal bodies.

All trichocyst-bearing species mentioned above are assigned to the *P. grossii*-group and are typified by this species because it is the first described species in the group known to have the three fundamental characteristics of the group. One species which has not been mentioned hitherto, and which is designated *Pyramimonas* sp. in Text fig. 8.1, is

unusual because it lacks trichocysts (R.N. Pienaar, pers. comm.¹) This species does however have a complete covering of B_1 scales and a 3 over 1 arrangement of the flagellar basal bodies and should be retained in the *P. grossii*-group.

Having established the species belonging to the *P. grossii*-group it is possible to draw some tentative conclusions about their relationships along phyletic lines.

Before this can be done it is necessary to discuss which characters or character conditions are thought to be primitive and which are thought to be advanced. Those characters construed by the writer to be phylogenetically important have already been mentioned, viz. presence or absence of trichocysts, nature of the B_1 scale covering, the arrangement of basal bodies, and the presence or absence of foot-print scales.

The B_1 scales of *Pyramimonas* are similar and sometimes identical to the underlayer scales on motile cells of many genera in the Chlorophyta. Moestrup (1984) has reported that nine genera of prasinophycean algae (including *Pyramimonas*) possess square or diamond-shaped underlayer scales. Similar underlayer scales are also found on motile cells of charophycean algae (McBride, 1968; Pickett-Heaps, 1968; Turner, 1968; Moestrup, 1970, 1974; Sluiman, 1983) and ulvophycean algae (Mattox and Stewart, 1973; Moestrup, 1978). Scales are not known in the class Chlorophyceae.

This widespread occurrence of small underlayer scales suggests that they had evolved in a remote ancestor which gave rise to the Prasinophyceae, Charophyceae and Ulvophyceae. What is pertinent to the argument being formulated here is that where the underlayer scales occur they invariably cover the whole surface of the cell body. The presence of a complete covering of B_1 scales in *Pyramimonas* is therefore interpreted to be a primitive condition and that a reduction in the number of B_1 scales to form a partial covering (as in the *P. obovata*-group - see Text fig. 8.1) represents a derived condition.

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One additional characteristic which supports this interpretation is that those species having a complete covering of B_1 scales also possess trichocysts. As Stewart and Mattox (1980) point out, the presence of trichocysts is an animal-like characteristic. This would suggest that members of the *P. grossii*-group are more closely related to a zooflagellate ancestor than other members of the genus. By implication the *P. grossii*-group is believed to be the most primitive group in the genus. If the presence of a complete covering of B_1 scales and the presence of trichocysts indicate a primitive condition (as formulated above) then the presence of a 3 over 1 arrangement of the flagellar basal bodies can also be regarded as a primitive condition because the three characters are strongly correlated.

To return to the phylogenetic relationship of species within the *P. grossii*-group it is proposed that species in the *P. grossii*-cluster (circle) are more primitive than, and probably gave rise to, species in the *P. parkeae*-cluster. Following Norris' (1980) hypothesis that an increase in the number of scales in the scale-boundary reflects an evolutionary advancement, the incomplete covering of B_3 scales seen in species in the *P. grossii*-cluster is interpreted as a primitive condition (compared with a complete covering of B_3 scales in the *P. parkeae*-cluster). The appearance of many thylakoids in the pyrenoid of species in the *P. parkeae*-cluster may also be interpreted as being more advanced than the condition seen in species of the *P. grossii*-cluster where a single thylakoid traverses the pyrenoid. *Pyramimonas* sp. (Text fig. 8.1) is undoubtedly derived from the *P. grossii*-cluster because it is similar to these species except that it has lost its trichocysts.

P. virginica is an unusual member of the *P. grossii*-group because it has only two layers of body scales (a primitive condition) where the outer body scale has a symmetry based on six. All other species in the genus have the B_2 and B_3 scales based on a symmetry of eight. *P. lunata* is undoubtedly related to other members of the *P. grossii*-group because it has the three fundamental characteristics of the group. *P. lunata* most closely resembles species in the *P. parkeae*-cluster but can be separated from them on three characteristics. *P. lunata* apparently does not have a transitional helix-like (TH-like) structure in the flagella (Inouye *et al.*, 1983), it has the thylakoids entering the pyrenoid anteriorly, and the B_3

scales have the peripheral upright struts diagonally arranged and attached to the corners of the scale (termed here the B₃d pattern). By contrast species in the *P. parkeae*-cluster possess a TH-like structure, have the thylakoids entering the pyrenoid laterally, and the B₃ scales have the peripheral upright struts attached to the centre of each side of the scale (termed here the B₃c pattern). The characteristics of *P. lunata* suggest that it evolved along a separate line as indicated in Text fig. 8.1.

A second group of phylogenetically related species is recognized in the genus *Pyramimonas* and is referred to as the *P. obovata*-group (see Text fig. 8.1). Members of this group are characterized by having the flagellar basal bodies arranged in a rhombic pattern, by having the B₁ scales restricted to the flagellar pit, and by having foot-print scales. Three species in the *P. obovata*-group are known to have all of the abovementioned characteristics. They are *P. obovata* (Pennick *et al.*, 1976; Melkonian, 1981; McFadden unpublished - see McFadden and Wetherbee, 1984; own observations), *P. orientalis* (Moestrup and Thomsen, 1974; Pennick *et al.*, 1978; Norris and Pienaar, 1978; McFadden unpublished - see McFadden and Wetherbee, 1984), and *P. plurioculata* (Norris and Pienaar, 1978; R.N. Pienaar pers. comm.¹). The group is typified by *P. obovata* because it is the first described species known to have all the characteristics of the group. Other small species that are placed in the *P. obovata*-group (see *P. obovata* species cluster in Text fig. 8.1) all have the B₁ scales restricted to the flagellar pit. Although none of these species have been studied in detail the writer proposes that they will be shown to have a rhombic arrangement of the flagellar basal bodies. Another characteristic which suggests that they form a cohesive group and should be assigned to the *P. obovata*-group is that they possess foot-print scales. Although the presence of these scales has not often been reported in the literature they are evident in published micrographs of *P. disomata* (Norris and Pienaar, 1978 - fig. 18), *P. occidentalis* (Pennick, 1982b - fig. 18), and *P. gorlestonae* (Pennick and Cann, 1982 - figs. 11 and 13). The presence of foot-print scales has yet to be confirmed for *P. spinifera* (Pennick, 1983).

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The rhombic basal body pattern is believed to be derived from the 3 over 1 pattern. Reasons for considering the 3 over 1 pattern to be more primitive have already been stated. One possibility that has not been entertained is that species having the rhombic pattern of basal body arrangement have had an independent origin. The writer considers this unlikely because other characteristics suggest that all species in the genus had a common ancestry. Universal characteristics such as the shape of the cells, the number of flagella, the nature of the underlayer scales, the fundamental similarity in symmetry and structure of the B_2 and B_3 scales, and general internal organization support the view that the genus is monophyletic. Another feature which argues in favour of a common ancestor for all species in the genus is that some members in each of the three phyletic groups have a TH-like structure. It is unlikely that this unusual structure would have been acquired more than once in the evolutionary history of the genus [see Hibberd's (1979) discussion of the TH in chromophyte algae].

Within the *P. obovata*-group an assemblage of closely related species is evident and these are assigned to the *P. obovata*-cluster. Although Pennick (1984) has separated these species into two "clusters" based on small differences in the structure of the B_2 scale, the writer believes that they should be considered as a single "cluster" until the variation and constancy of scale structure has been investigated in natural populations. A TH-like structure is present in at least three species in the *P. obovata* group and may be present in all species assigned to the group. Those species possessing a TH-like structure are *P. orientalis* (Moestrup and Thomsen, 1974), *P. gelidicola* (McFadden *et al.*, 1982), and *P. obovata* (personal observation).

P. gelidicola is thought to be derived from the *P. obovata* species cluster through the loss of B_1 scales.

A third group of less well defined species is assigned to the *P. tetrarhynchus*-group. The group is arbitrarily typified by *P. tetrarhynchus* because it is the first described member of the group (see Text fig. 8.1). Species in this group are characterised by having a rhombic basal body pattern and a complete covering of B_1 scales. The presence of a rhombic basal body pattern and absence of trichocysts in the *P. tetrarhynchus*-

and *P. obovata*-groups indicates that they are closely related groups that diverged after the acquisition of this type of basal body system. Another characteristic which perhaps supports this view is that *P. tetraarhynchus* and *P. amyliifera* have similar B₁ scales which are significantly smaller than the B₁ scales in two members of the *P. grossii*-group, viz. *P. parkeae* and *P. grossii* (Melkonian and Robenek, 1981).

P. tetraarhynchus is thought to be derived from the *P. grossii*-group by the loss of trichocysts, rearrangement of basal bodies to the rhombic pattern, and by a doubling of the number of dictyosomes. *P. amyliifera* could be derived from *P. tetraarhynchus* simply by a doubling of the number of flagella. This condition could have arisen if a cell failed to undergo cytokinesis after replication of the basal bodies. *P. longicauda* is unusual because it does not have a typical coronate B₃ scale (Inouye *et al.*, 1984). Instead the outer scale in this species resembles the B₂ scale of *P. tetraarhynchus* and *P. amyliifera*. It would appear that the typical coronate B₃ scale has been lost in *P. longicauda* and a novel scale type ("basket scale") evolved to occupy an intermediate position in the scale boundary. Similar basket-shaped scales are seen in *Mesostigma* (Manton and Ettl, 1965). The acquisition of the basket scale and the possession of a straight synistosome (always associated with the rhombic basal body pattern) in *P. longicauda* suggests that *Mesostigma* may have evolved from this type of cell by "halving". This interpretation was considered by Chadeaud (1977), Stewart and Mattox (1978) and Moestrup and Ettl (1979).

It is hoped that the proposed phylogeny discussed above and summarized in Text fig. 8.1 will provide a foundation for further comparative studies in the genus. It is believed that the phylogenetic relationships between species should be investigated at the molecular level. An electrophoretic study of the isoenzymes in different species of *Pyramimonas* may give some interesting information on the phylogenetic relationships between species and would be a useful approach to test the proposed phylogenetic scheme presented above. The electrophoretic separation of isoenzymes in a number of algae has proved to be useful in identifying species and for drawing conclusions on phylogenetic relationships between these (Thomas and Brown, 1970a, 1970b; Murphy and Guillard, 1970; Thomas and Delcarpio, 1971;

Murphy, 1978; Grant and Proctor, 1980; Hayhome and Pfiester, 1983; Marsden *et al.*, 1984; to mention a few).

Many interesting characteristics of species in the genus *Pyramimonas* are not understood and need to be investigated. For example, does the TH-like structure occur in all species in the genus? It has yet to be found in *P. virginica*, *P. cirolanae*, *P. lunata*, *P. occidentalis*, *P. gorlestonae*, *P. disomata*, *P. spinifera*, *P. tetrarhynchus* and *P. longicauda*. Two different forms of the coronate B₃ scale occur in *Pyramimonas* and may have what has been referred to earlier as a B₃d structure or B₃c structure. The taxonomic significance of the two forms of the B₃ scale is not understood. All species of the *P. tetrarhynchus*-group have the B₃d scale type whereas members of the *P. grossii*- and *P. obovata*-groups have both B₃d and B₃c scales. In the *P. grossii*-group only *P. lunata* has a B₃d scale (Inouye *et al.*, 1983) and in the *P. obovata*-group only *P. orientalis* has this type of scale (Moestrup and Thomsen, 1974; Norris and Pienaar, 1975; Pennick *et al.*, 1978; Pennick, 1984). Inouye *et al.* (1983, 1984) believe that the way in which the chloroplast thylakoids enter the pyrenoid, and the number of flagellar pit microtubules may be taxonomically important at species level. The phylogenetic significance of these characters is still not known. At present they do not appear to be strongly correlated with other conservative characters.

The phylogenetic significance of cell size is not known at present. It is nevertheless interesting to note that larger cells (>14 μm in length) in the *P. grossii*- and *P. obovata*-groups appear to be derived from smaller species (< 9 μm in length)(see Text fig. 8.1)

XENIC GROWTH STUDIES

The xenic growth studies provided valuable information on both the growth characteristics of *P. pseudoparkeae* and other aspects of its biology.

P. pseudoparkeae is euryhaline and grows successfully in salinities ranging from 10 - 70‰. As has been pointed out before, this ability to tolerate large ranges in salinity would be expected of a successful tidal pool organism. Very little information is available on salinity tolerances in

other species in the genus although there has been one report that *P. gelidicola* blooms in seawater at a salinity of 150‰ (see McFadden *et al.*, 1982). The present study showed that the growth rate of *P. pseudoparkeae* was not significantly different in salinities of 10-35‰ but the growth rate was significantly reduced at higher salinities (50 and 70‰). Vosjan and Siezen (1968) studied the effect of salinity on the photosynthetic rate of a marine species of *Chlamydomonas* and found that salinities between 10 - 100‰ did not affect photosynthesis. The latter species is clearly more euryhaline than *P. pseudoparkeae*.

P. pseudoparkeae grew well at a temperature of 20 - 25°C but the alga could not tolerate a constant temperature of 30°C. The upper temperature tolerance of *Tetraselmis*, a related prasinophyte, is 35°C (Ukeles, 1976). As Soeder and Stengel (1974) point out, the artificial character of laboratory studies using constant temperatures should be appreciated. One strain of *Scenedesmus* MEYEN for example, has a constant temperature maximum of 34°C in the laboratory but this alga grows without inhibition under outdoor conditions at noon peaks of 45°C (see Soeder and Stengel, 1974). The lower temperature tolerance for *P. pseudoparkeae* is not known although the alga remains viable for at least one week in the dark at 4°C.

The xenic growth studies revealed which culture conditions gave the shortest mean doubling time for *P. pseudoparkeae*. This information was important for the 24h study on cell division and scale production where synchronous cultures were needed. The shortest mean doubling time obtained for the alga was 26h; this was significantly shorter than the 33h doubling time obtained in an earlier study (Aken, 1978). A 26h doubling time would mean that 92% of the cells in culture would divide during the 24h period of the synchrony induction photoregime i.e. 16h:8h. The two hour difference between the period taken for all cells in the culture to divide and the period of the 24h photoregime would have impaired synchrony to some degree. It is undoubtedly this discrepancy that resulted in the extended period taken for synchronous cell divisions to occur in cultures used in the 24h study. Had the mean doubling time matched the 24h period of the synchrony induction photoregime, improved synchrony would have been obtained. The combined culture conditions giving the cells a mean doubling time of 26h were a salinity of 35‰, a temperature of 25°C and a light

intensity of $150 \mu\text{E m}^{-2}\text{s}^{-1}$ (measured during the light period of a 16h:8h photoregime). No attempt was made to grow the alga under continuous light because there is some evidence to suggest that L:D cycles improve the growth rate of green algae (Sorokin and Krauss, 1959).

Since the growth rate of *P. pseudoparkeae* could not be increased to give a mean doubling time closer to 24h suggests that some factor may be limiting the growth of the alga. The axenic growth studies revealed that *P. pseudoparkeae* is dependent on some substance released into the growth medium by bacteria. It is possible that limiting concentrations of this substance could have precluded the possibility of improving the growth rate. This could be tested by identifying the growth promoting substance and supplying it to *P. pseudoparkeae* at non-limiting concentrations and determining if the growth rate is improved.

In one experiment where *P. pseudoparkeae* was grown at three different light intensities the growth rate of the alga was improved by increasing the light intensity from $50 \mu\text{Em}^{-2}\text{s}^{-1}$ to $100 \mu\text{Em}^{-2}\text{s}^{-1}$. A further increase in light intensity to $200 \mu\text{Em}^{-2}\text{s}^{-1}$ resulted in a lower growth rate though this was not significantly different from that obtained at $100 \mu\text{Em}^{-2}\text{s}^{-1}$. The relationship between light intensity and rates of photosynthesis or growth show a rectangular hyperbolic function with inhibition occurring at super-saturating light intensities (Soeder and Stengel, 1974). It would appear that light saturation for *P. pseudoparkeae* occurs around $150 \mu\text{Em}^{-2}\text{s}^{-1}$. This value compares favourably with the most commonly reported light intensities giving maximum photosynthetic rates, i.e. between $60 - 110 \mu\text{Em}^{-2}\text{s}^{-1}$ (Harris, 1978).

It is important to remember that growth rates of algae under different light intensities are markedly affected by temperature (Sorokin and Krauss, 1962; Setlik *et al.*, 1969; Smayda, 1969), salinity (McCombie, 1960) and nutrient level (Maddox and Jones, 1964). It is essential therefore to quote the temperature and salinity, or at least keep these parameters constant, when investigating the effect of light intensity on algal growth. A statistical investigation of the interactions between light intensity and temperature (by two-way analysis of variance) could not be applied to the data obtained in the light intensity/salinity experiment (Chapter Three)

because there was insufficient replication in the experimental design.

The xenic growth studies revealed that scale structure in *P. pseudoparkeae* was not affected by the type of growth medium used, nor was it affected by salinity, temperature or light intensity. As mentioned before it is believed that scale structure is genetically fixed. Aberrant scales were only found in old cultures that contained pleomorphic and senescent cells. It is suggested that deformed scales are produced by cells which have some degree of genetic aberration. No previous studies have shown that scale structure remains constant under a wide variation of culture conditions.

McFadden *et al.* (1982) have reported that the limuloid scales of *P. gelidicola* have variable perforation patterns. As has been mentioned before the large deposits of lipid in *P. gelidicola*, while perhaps playing a role in allowing the cell to grow in the cold Antarctic waters, could indicate that the cells were not actively growing and may have been senescent or at least permanently stressed. It is possible that the variant scale types reported by McFadden *et al.* (1982) were produced by genetically aberrant cells in their cultures. It would be interesting to test if scale structure is constant in actively growing cultures of *P. gelidicola*.

The encysted cells of *P. pseudoparkeae* that developed spontaneously during the xenic growth studies are thought to have been induced to encyst within the sampling tube of the culture apparatus. The possible factors inducing encystment are discussed later when the life-history of *P. pseudoparkeae* is considered.

AXENIC CULTURE STUDIES

The axenic culture studies revealed that *P. pseudoparkeae* cannot be grown in axenic culture and requires a bacterial component in the culture before it will grow. When the alga and bacteria were cultured together both had a stimulatory effect on the growth of the other. While the bacteria could grow on their own, the alga had an absolute requirement for bacteria. The physiological processes involved in this mutualistic relationship are not understood. It is known that the "growth stimulator" released by the bacteria, and utilized by *P. pseudoparkeae*, is a large compound that passes through a 0,2 μm Millipore filter but not through dialysis tubing.

Very little is known about the nutritional requirements of *Pyramimonas*. Ricketts (1974) has also shown that four species of *Pyramimonas* could not be grown in axenic culture. This is also true for some species in other genera in the class Prasinophyceae (Ricketts, 1974). Ukeles and Bishop (1975) have shown that the growth of an unknown species of *Pyramimonas* is enhanced by bacteria. It is clear that the nutrition of *Pyramimonas* (and other Prasinophyceae) is an area that requires deeper investigation especially with respect to bacterial/algal relationships.

At this stage it is premature to attempt to define the nutritional characteristics of *P. pseudoparkeae*. The alga cannot be placed within any nutritional scheme such as those proposed by Lwoff (1951) and Fogg (1953). Much more information needs to be gathered on the growth requirements of the species. It is not known if *P. pseudoparkeae* is auxotrophic i.e. requires vitamins for growth. Ricketts (1974) has, however, shown that *P. obovata* requires vitamin B₁₂ and thiamine. The dependence of *P. pseudoparkeae* on bacteria in culture indicates that the alga is clearly not photo-autotrophic.

The relationships between algae and bacteria appear to vary in the intimacy of the association. Bacterial influences (beneficial or harmful) on algae may be derived from 1) free-living bacteria, 2) epiphytic bacteria, or 3) endophytic bacteria.

The bacterial/algal relationship described in the present study would fall into the first category. Examples of bacterial/algal relationships where the bacteria are free living and benefit the alga are relatively few (Ricketts, 1974; Ukeles and Bishop, 1975; Jolley and Jones, 1977). It is the writer's belief that this does not reflect that there are few bacterial/algal relationships which favour the alga as much as it reflects our paucity of knowledge of bacterial/algal associations. Free-living bacteria are also reported to inhibit algal growth, especially in wastewaters (see Toerien *et al.*, 1984).

There have been many reports of algae having epiphytic or endophytic bacteria. For the most part the nature of the bacterial/algal associations is not known and is therefore not emphasized below. Epiphytic bacteria are

known to be attached to unicellular, sometimes motile, algae in the classes Chlorophyceae (Weiss, 1983; Hamburger, 1958), Chrysophyceae (Geitler, 1948; Kogure *et al.*, 1982), and Cryptophyceae (Klaveness, 1982). Epiphytic bacteria are common components of the epiphyton on thallose green, brown and red algae (personal observations) but this association is not unexpected because the thallose forms are usually attached and provide a suitable substrate for colonization. Many attached alga however release antimicrobial substances which reduce or inhibit the growth of epiphytic bacteria (Conover and Sieburth, 1964).

Many more cases have been reported where bacteria are endophytic in algae assigned to the classes Chlorophyceae (Kochert and Olsen, 1970; Lee and Kochert, 1976), Ulvophyceae (Burr and West, 1970; Turner and Friedmann, 1974; Dawes and Lohr, 1978; Mariani Colombo, 1978), Prasinophyceae (Wujek *et al.*, 1982), Euglenophyceae (Leedale, 1969; Peterfi *et al.*, 1979; Kies 1980), Chrysophyceae (Belcher, 1969b; Hibberd, 1971; Wujek, 1978; Preisig and Hibberd, 1984), Xanthophyceae (Ott, 1979), Raphidophyceae (Heywood, 1978), Dinophyceae (Dodge, 1973; Silva, 1978; Pienaar, 1980c; Lucas, 1982), and Prymnesiophyceae (Pienaar pers. comm.¹).

To the writer's knowledge there have been no reports on endophytic bacteria in the Rhodophyceae though bacteria have been found within the cell walls of some Phaeophyceae (see Pellegrini and Pellegrini, 1982).

The widespread occurrence of bacterial/algal associations, whatever their degree of intimacy, seems to suggest that bacteria may be playing an important role in the survival of these algae. This may be especially true of endophytic bacteria. The serial endosymbiotic theory proposed by Margulis (1970) postulates that the eukaryotic cell evolved by a series of symbioses involving prokaryotic cells. The chloroplast and mitochondrion, two vital organelles in eukaryotic cells, are believed to have developed from endophytic prokaryotes. The writer finds it difficult to believe that endophytic bacteria (other than pathogenic ones - see Wujek *et al.*, 1982) have a commensal relationship with the algal host. In many cases endophytic bacteria divide simultaneously with the algal host (Preisig and Hibberd,

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1984), so that subsequent algal generations have a bacterial endophyte. This harmony of life processes and the precision with which it is maintained suggests that the alga benefits from the bacteria and vice versa i.e. the organisms have a mutualistic or symbiotic relationship. Without at least some mutual benefit the association would undoubtedly be lost in time.

It is unfortunate that so few investigations on these symbiotic relationships have been undertaken. This is an area of much needed research that may throw some light on why these relationships are maintained, if indeed they are.

There is some evidence to show that bacteria influence morphogenesis in algae. Provasoli and Pintner (1980) have shown that bacteria induce polymorphism in *Ulva lactuca* L., while Machlis (1973) has demonstrated that *Oedogonium cardiacum* WITTR only develops reproductive structures in the presence of bacteria.

CELL DIVISION

The process of cell division in *P. pseudoparkeae* is almost identical to that described for two other species in the genus viz. *P. parkeae* (Pearson and Norris, 1975) and *P. amyliifera* (Woods and Triemer, 1981). Cell division in these species has already been discussed (Section 5.4) and will not be repeated here. Instead the main features of cell division in *P. pseudoparkeae* are summarized below and discussed with reference to other algae, primarily other green algae. Cell division in *P. pseudoparkeae* has the following characteristics;

- 1) an open mitotic spindle,
- 2) spindle microtubules derived from the transformation of the rhizoplast;
- 3) basal bodies act as centrioles,
- 4) chromosomes have kinetochores to which spindle microtubules are attached,
- 5) cytokinesis effected by furrowing and does not involve a phycoplast,
- 6) a persistent interzonal spindle.

The classical separation of green algae into three morphological lines that were thought to represent evolutionary distinct groups i.e. the volvocine, siphonous and tetrasporine lines (Weier *et al.*, 1970; Raven *et al.*, 1976) has now been superseded by a new approach to green algal classification. This approach places much emphasis on the conservative nature of mitotic and cytokinetic processes. Pickett-Heaps and Marchant (1972) were the first to propose a new phylogeny for the green algae. They suggested that the green algae evolved along two separate lines where one group possessed a phycoplast (and had a collapsing interzonal spindle) and the second group lacked a phycoplast (and had a persistent interzonal spindle) during cytokinesis.

In their "cytological classification" of the green algae, Stewart and Mattox (1975a) also separated the green algae into two groups and they assigned these groups class status. The Chlorophyceae were characterized by having a "closed" mitotic spindle, a collapsing interzonal spindle at telophase and phycoplastic cytokinesis, while the Charophyceae were characterized by having an open mitotic spindle, a persistent interzonal spindle at telophase, and having cytokinesis effected by furrowing or through the development of a phragmoplast. In a subsequent publication Stewart and Mattox (1978) erected a third class, the Ulvophyceae, to include those green algae having a "closed" and persistent interzonal spindle and no phycoplast. Motile ulvophycean cells lack a MLS characteristic of the Charophyceae (refer to Text fig. 1.1).

Stewart and Mattox (1980) recognize two types of spindles in eukaryotic organisms. The first type is where the spindle microtubules always lie outside the nuclear envelope which remains intact. This is called the Type I spindle and is present in the Dinophyceae (Dodge, 1973; Steidinger and Cox, 1980). The second type, the Type II spindle, is where the spindle microtubules occur within the nucleoplasm for at least the later stages of mitosis. Two important variations in the Type II spindle are recognized. In one case the spindle microtubules develop in the cytoplasm and later penetrate the nucleoplasm. The nuclear envelope may be fenestrated or it may disperse i.e. result in an open spindle. This is referred to as the Type II a spindle. Type II b spindles are characterized by the development of the microtubules within the nucleoplasm which is always surrounded by a nuclear envelope. Type II b spindles are present

in the Euglenophyceae (Dodge, 1973; Leedale, 1958, 1968, 1970) and in the Xanthophyceae (Ott and Brown, 1972). One prasinophyte, *Pedinomonas*, has been shown to have a Type II b spindle which is entirely closed (Pickett-Heaps and Ott, 1974).

The Type IIa spindle is characteristic of most chlorophytan algae. Because of the important variations in the Type IIa spindle which led to the reclassification of green algae (as discussed above) it is clear that a more refined system to differentiate the spindle types in the Chlorophyceae, Charophyceae and Ulvophyceae will have to be developed. The "closed" spindles of the Chlorophyceae which collapse at telophase and are replaced by phycoplast microtubules should be differentiated from the "closed" spindles of the Ulvophyceae which have a persistent interzonal spindle at telophase and do not develop a phycoplast. Similarly the open and persistent spindles of the Charophyceae (and higher plants) deserve a different name. Except for *Pedinomonas*, the Prasinophyceae also have a Type IIa spindle.

Type IIa spindles, where the nuclear envelope disperses (i.e. open spindles) are known for the Cryptophyceae (Gantt, 1980; Meyer and Pienaar, 1984), Chrysophyceae (Slankis and Gibbs, 1972) and Prymnesiophyceae (Manton, 1964; Stacey and Pienaar, 1980; Hori and Inouye, 1981).

The process of mitosis has not been studied extensively in the Phaeophyceae and Rhodophyceae. In both groups of algae the nuclear envelope appears to remain intact except for polar fenestrations through which spindle microtubules pass (Dodge, 1973; Duckett and Peel, 1978). Red and brown algae therefore have a Type IIa spindle.

With regard to the origin of the different spindle types, Pickett-Heaps (1974, 1975a) and Cavalier-Smith (1978) argue that the mitotic spindle originated inside the nucleus and that intranuclear, closed spindles (Type IIb) are therefore more primitive. Stewart and Mattox (1980) do not accept this interpretation and propose that the Type I spindle is more primitive than, and gave rise to, the Type II spindle. They have two reasons for believing this. Organisms possessing a Type I spindle (e.g. Dinoflagellates) have the chromosomes attached to the nuclear envelope

and the chromosomes lack histones. These two features are characteristic of prokaryotic organisms and indicate a primitive condition.

Mattox and Stewart (1977) believe that the modification of the Type IIa spindle in the Chlorophyceae has evolved in response to a reduction in the space available for mitosis to occur. The acquisition of a "one-piece" cell wall in chlamydomonad flagellates would have severely restricted spindle elongation during division. The collapsing spindle and phycoplast are seen as an adaptation to overcome this problem. The phycoplast would ensure that the cleavage furrow formed during cytokinesis would pass accurately between two daughter nuclei whose degree of separation was restricted by the cell wall. A logical question to ask at this stage is why algae belonging to the Ulvophyceae and Charophyceae, which also have cell walls, do not have a collapsing spindle and phycoplastic cytokinesis. Mattox and Stewart (1977) have answered this by suggesting that cell walls in the latter classes evolved in non-motile coccoid or filamentous stages. These cells could gradually double in size to accommodate the spindle and division products.

In *P. pseudoparkeae* the rhizoplast is completely transformed to produce the spindle microtubules. This also occurs in *P. parkeae* and *P. amyliifera* (Pearson and Norris, 1975; Woods and Triemer, 1981) but in these species the rhizoplast is not totally transformed in the formation of the spindle. The rhizoplast of *Tetraselmis* is also involved in spindle formation (Stewart *et al.*, 1974) as in that of the chrysophyte, *Ochromonas* (Slankis and Gibbs, 1972; Bouck and Brown, 1973).

Though rhizoplasts are clearly visible in prasinophycean cells they are not generally known outside the class. There are a few reports of rhizoplasts (System II fibres) in certain species in all classes of chlorophycean alga (Melkonian, 1980). Although rhizoplasts are apparently lacking in some green algae, Stewart and Mattox (1980) suggest that they may be less highly organized and therefore unrecognizable in these species. Stewart and Mattox (1980) go further to suggest that the granular mass at the spindle poles of *Chlamydomonas* may be microtubule organizing centres consisting of the same material as rhizoplasts.

The association of spindle microtubules with the flagellar basal bodies in *P. pseudoparkeae* indicates that these structures act as centrioles.

This has been confirmed for two other species of *Pyramimonas* (Pearson and Norris, 1975; Woods and Triemer, 1981). Flagellar basal bodies are known to act as centrioles in a number of flagellate algae. Basal bodies act as the spindle poles, for example, in *Chlamydomonas* (Coss, 1974; Triemer and Brown, 1974), *Euglena* EHRENBERG (Gillott and Triemer, 1978) and *Chroomonas* (Meyer and Pienaar, 1984). The flagellar bases do not occupy the poles of the mitotic spindle in the prymnesiophytes *Prymnesium* (Manton, 1964) and *Hymenomonas* (Stacey and Pienaar, 1980) nor in the chrysophyte *Ochromonas* WYSSOTZSKI (Slankis and Gibbs, 1972).

SCALE PRODUCTION

Manton (1966a) and Moestrup and Walne (1979) have contributed much to our understanding of scale production in *Pyramimonas*. The present study on scale production in *P. pseudoparkeae* has, however, revealed some interesting facts not hitherto known. Most interesting is that there do not appear to be any functional differences between the two dictyosomes nor do the dictyosomes appear to be more active at any one stage in the cell division cycle. This contrasts with Manton's proposal (Manton, 1966a) that the possible rhythmic release of body scales indicates a rhythmic production of scales by the dictyosomes. The release of body scales in *P. pseudoparkeae* occurs continuously but is not always clearly seen. It is possibly this fact that led Manton (1966a) to propose a periodic liberation of body scales in *P. amyliifera*.

Manton (1966a) did not know if the scale reservoir was a temporary or permanent structure in *P. amyliifera* or if it was always attached to the flagellar pit via a duct. Observations made throughout the 24h cell division cycle of *P. pseudoparkeae* revealed that the scale reservoir is always present in the cells and is always open to the flagellar pit via the scale reservoir duct.

Concerning the rhythmic production of scales in *Pyramimonas* it is important to define whether this applies to the morphogenesis of scales or whether it applies to the liberation of scales. It has already been stated that scale morphogenesis does not appear to be a periodic process but rather a continuous one. There is therefore no apparent rhythmic activity in the dictyosomes. If the release of scales from the cells is considered

then there is a rhythmic liberation of both flagellar and body scales. Flagellar scales are released from the cells during a short period when the four (or eight) new flagella are formed when the cell undergoes division. This release of a large number of scales in a short period in the cell division cycle is a rhythmic phenomenon. A rhythmic release of body scales may also occur just prior to completion of cytokinesis when the cell containing four dictyosomes would be producing twice as many scales than at other times when the cell only has two dictyosomes. This increased production of scales would be expected because many more scales would be needed to cover newly formed surfaces as the cell undergoes cytokinesis.

The rate of scale morphogenesis in *P. pseudoparkeae* is extremely rapid taking about 10,5 minutes for a scale to be completely formed within the dictyosome. This is equivalent to one cisternae being released from the dictyosome every 31 seconds. Moestrup and Walne (1979) estimated that the scales of *P. tetrarhynchus* take 1 h 30 min to be formed within the dictyosomes. This is probably an underestimate of the productivity of the Golgi apparatus in *P. tetrarhynchus* and could be explained by the fact that the growth characteristics of the species were not understood by the authors.

The dictyosome turnover rate of *P. pseudoparkeae* is approximately four times as rapid as that reported for *Pleurochrysis scherffellii* PRINGSHEIM (Brown, 1969). In this prymnesiophyte, one dictyosome cisterna is released every two minutes (Brown, 1969).

In all scale bearing algae the dictyosome has been shown unequivocally to be the site of scale morphogenesis. The observations on scale production in algae have done much to elucidate the dynamics of the Golgi apparatus and show that at least in this group of plants the Golgi cisternae are mobile. Farquhar and Palade (1981) proposed that individual cisternae maintain a fixed position in the Golgi stack and that material transfer takes place by the fusion and release of vesicles at the rims of the cisternae. The earlier interpretation of Morré and Mollenhauer (1974) suggests that Golgi cisternae are produced by the transformation of ER membranes, and that these cisternae follow a steady maturation as they move from the forming face to the mature face of the Golgi apparatus (membrane-flow-differentiation hypothesis).

Farquhar and Palade (1981) in their static model of the dictyosome based their main argument against the membrane-flow-differentiation hypothesis on the observation that individual cisternae are cytochemically heterogeneous and disregard the idea that there is a continuing programmed reorganization of both cisternal membrane structure and the enclosed enzymatic reactions.

The observations on scale production in *P. pseudoparkeae* and other scale bearing algae support the hypothesis that cisternae move through the dictyosome. Scales are large, easily recognizable products and their morphogenesis can be followed within the dictyosome. The rapid rate at which the scales of *P. pseudoparkeae* and *Pleurochrysis scherffellii* are formed indicates that the Golgi apparatus is capable of rapid turnover rates which is accompanied by a rapid transformation of membranes and scale material. The fact that different scale types in *P. pseudoparkeae* can be produced in a single cisterna reveals the extent to which intracisternal differentiation can be refined. In this respect the cytological differences between different cisternae in the static cisternae model (Farquhar and Palade, 1981) are not exceptional and do not constitute a valid reason for supporting their hypothesis.

Outside the Prasinophyceae the process of scale production has been studied in detail in the chrysophytes *Paraphysomonas* (Manton and Leedale, 1961a) and *Mallomonas* (Wujek and Kristiansen, 1978), and the prymnesiophytes *Prymnesium* (Manton, 1966c), *Chrysochromulina* (Manton, 1967a, 1967b; Pienaar and Norris, 1979), *Hymenomonas* (Pienaar, 1969b, 1976c; van der Wal *et al.*, 1983) and *Pleurochrysis scherffellii* (Brown, 1969; Brown *et al.*, 1970, 1973).

The study of scale production in *Hymenomonas carterae* (Pienaar, 1976c) is especially interesting because it revealed that the dictyosome in this alga produces different scale types at different times in the cell division cycle. This rhythmic production of scales and the obvious functional changes in the dictyosome have not often been reported. The Golgi apparatus of *Pleurochrysis* also undergoes periods of differential secretory activity (Brown, 1969).

SCALE COMPOSITION

The scales of *P. pseudoparkeae* have been shown in the present study to be predominantly polysaccharide in composition. They contain approximately 4% protein. The sugar moieties comprising the scales are galacturonic acid, galactose, arabinose, xylose, rhamnose and a trace of fructose. The scales are chemically similar to plant pectins and have the staining properties of these substances. It is for this reason that the scales have been described as pectinaceous in character. If the protein component can be shown to be covalently linked to the carbohydrate component then the scales would be composed of glycoprotein. This needs to be investigated in future studies.

The scales of *P. pseudoparkeae* are almost identical in composition to the theca of *Tetraselmis* (Lewin, 1958; Gooday, 1971; Manton *et al.*, 1973) and indicates that the two cell boundaries are probably homologous structures. The theca of *Tetraselmis* probably evolved through a fusion of scales as first suggested by Mattox and Stewart (1977).

Domozych *et al.* (1980) have proposed a phylogenetic scheme for the evolution of chlorophytan algae based upon preliminary cell-wall studies and ultrastructural characteristics of mitosis and cytokinesis. This scheme is discussed below.

Domozych *et al.* (1980) propose that early in the prasinophyte line cells lacked a defined outer covering but possessed a thin skin of mucilage which was very likely proteinaceous in character. During the evolution of early photosynthetic prasinophytes a sugar (or sugars) became more abundant and thus more saccharide material may have been incorporated into the essentially proteinaceous exterior coating to form a sort of structural reinforcement. With increasing amounts of polysaccharide being added to the protein component this material could become more highly polymerized to form structural entities like scales or this material could have been used to crosslink more crystalline polymers like cellulose, when this evolved.

The Ulvophyceae are thought to have evolved from an early prasinophyte which had a predominantly proteinaceous or glycoproteinaceous exterior covering. The production of cellulose, hemicelluloses and complex

water soluble polysaccharides in the cell walls of the Ulvophyceae is thought to have evolved in non-motile stages because scaly monads are known in extant members of the class. Because these scales are present in motile members of the class the cell walls in the Ulvophyceae are thought to have evolved independently of scales. In more complex ulvophycean algae the proportion of protein in the cell wall was reduced and the polysaccharide component became more predominant.

The Chlorophyceae and Charophyceae are thought to have evolved from more complex prasinophycean cells where the extensive addition of various sugars to extracellular coatings may have caused numerous and variant scales to develop. The Chlorophyceae are thought to have developed directly from such a scaly green monad where significant glycosylation and polymerization in the scale boundary may have caused fusion of the scales into a theca. This proposal is supported by the present study. Finally the carbohydrate material is thought to have increased in quantity and form to give the typical cell walls of the Chlorophyceae. Like the Ulvophyceae the cell walls of the Charophyceae are thought to have evolved independently of scales in a non-motile phase because scale-bearing monads are known in extant members of the class. Here the glycoproteinaceous interscaly amorphous material is thought to have formed crosslinkages between cellulose polymers, when they evolved.

Scale composition has been investigated in only a few algae, viz. *Chrysochromulina* (Green and Jennings, 1967), *Pleurochrysis* (Brown *et al.*, 1969, 1970, 1973; Herth *et al.*, 1972) and *Hymenomonas* (Isenberg *et al.*, 1966; Pienaar, 1970). These three algae are prymnesiophytes and have been shown to have scales that are predominantly carbohydrate in composition. The scales of *Chrysochromulina* have been shown to be pectin-like in nature (Green and Jennings, 1967) while the scales of *Pleurochrysis* have been shown to be composed of cellulose and embedded in a gelatinous matrix in the cell wall of the alga (Brown *et al.*, 1970). The latter authors were the first to show that cellulose microfibrils can be synthesized and organized within the Golgi cisternae. The gelatinous matrix in which the scales of *Pleurochrysis* are embedded is pectic in character.

The scales of *Hymenomonas* are also predominantly carbohydrate in composition but also contain a small amount of protein (Isenberg *et al.*,

1966; Pienaar 1970). The outer scales of *Hymenomonas* are unusual because they are composed of an unmineralized base-plate onto which CaCO_3 is deposited. This scale with its CaCO_3 component is called the coccolith. It is interesting that calcification of the base-plate scale can take place in the light or dark but that calcium uptake by the cell is light dependent (Ariovich and Pienaar, 1979).

LIFE-HISTORY

The present study has shown that *P. pseudoparkeae* can reproduce asexually by binary fission or by producing non-motile cysts which release motile cells after a period of maturation. The life-cycle is believed to be asexual because cells were never seen to fuse. Furthermore t-cells and l-cells have the same chromosome number. It is unlikely that l-cells fuse before giving rise to the cyst because l-cells and cysts have the same cell volume.

It must be noted that the cultures of *P. pseudoparkeae* were clonal cultures. That sexual reproduction did not occur in these cultures indicates that either the alga does not reproduce sexually or that this process does not occur in homothallic strains. Sexual reproduction could occur in heterothallic strains (if these exist) but this still needs to be investigated.

The partially elucidated life-cycle of *P. amyliifera* (Hargraves and Gardiner, 1980) is similar to that described for *P. pseudoparkeae*. The life-cycles of other members of the Prasinophyceae are not well known and as Norris (1980) has noted, much more research is needed on this aspect of the biology of the Prasinophyceae.

The factors inducing encystment in *P. pseudoparkeae* are not understood. While temperature, salinity, light intensity, and CO_2 deficiency do not individually appear to induce the cells to encyst, high pH levels (pH 9) may be a contributing factor. Tanoue and Aruga (1975) have shown that high pH levels can induce *Tetraselmis* to encyst.

Many cysts of *P. pseudoparkeae* formed spontaneously in cultures which were grown in a culture vessel containing a capillary sampling tube (Text fig. 3.2). It is believed that cells trapped in this sampling tube were

induced to encyst and either became attached within the sampling tube or migrated back into the culture vessel where encystment was completed. A combination of factors such as a change in light quality and reduced pressure within the sampling tube, along with the other factors mentioned above, may induce the cells to encyst. The factors controlling encystment and excystment in *P. pseudoparkeae* need to be investigated in more detail in future studies.

Coleman (1983) has reported that thick-walled resting spores of chlorophytes are all formed in response to conditions of adequate photosynthesis but limiting nitrogen. This needs to be tested with *P. pseudoparkeae*. Low nutrient levels may well play a role in inducing encystment in *P. pseudoparkeae* because cysts do form in nutrient depleted medium in the stationary phase of growth.

The cyst of *P. pseudoparkeae* in Coleman's definition (Coleman, 1983) could be termed an akinete. Coleman recognizes two types of thick-walled cells in the Chlorophyta, these are akinetes, and hypnospores or hypnozygotes. The akinete is a modified vegetative cell in which the cell wall continues to thicken and may incorporate additional kinds of wall material. Hypnospores and hypnozygotes represent protoplasts that have separated from their parental wall and participated in some further activity. This may only be the formation of a sedentary zoospore or it may involve gametogenesis and fusion of gametes; these cells then deposit a new, usually very thick cell wall.

The cyst wall of *P. pseudoparkeae* is bilayered and has an outer component which is believed to be composed of sporopollenin. This, however, still needs to be confirmed using infra-red spectroscopy. Four chlorophycean algae also known to have sporopollenin in their cell walls are *Chlorella*, *Pediastrum*, *Scenedesmus* and *Trebouxia* DE PUYMALY (Brooks and Shaw, 1971; Atkinson *et al.*, 1972; Staehelin and Pickett-Heaps, 1975; König and Peveling, 1980). The spores of the charophycean alga *Chara* also have sporopollenin (Brooks and Shaw, 1971). The inner component of the cyst wall of *P. pseudoparkeae* is pectic in nature having the same staining properties as scales.

The cyst of *P. pseudoparkeae* is very similar to the phycoma of *Halosphaera*. Both cells have a bilayered cell wall which has an electron dense inner layer and an electron translucent outer layer. This similarity, along with the close structural resemblance of the motile cells in the two genera (Parke and Adams, 1961; Manton *et al.*, 1963; Pennick, 1977b) suggests that the two organisms are phylogenetically closely related and possibly should not be placed into separate orders as done by Moestrup (1984). The taxonomy of the class Prasinophyceae is discussed later.

The use of the Gomóri reaction to localize acid phosphatase in the l-cells and the cysts of *P. pseudoparkeae* showed that these enzymes are present in the Golgi cisternae near the forming face of the dictyosome and in large vesicles which contain scales. The presence of acid phosphatases in the Golgi apparatus has also been reported in *Euglena* (Brandes *et al.*, 1964; Kivik and Vesk, 1974), *Ochromonas* (Schuster *et al.*, 1968; Stoltze *et al.*, 1969), *Hymenomonas* (Pienaar, 1971), and *Dunaliella* (Eyden, 1975).

Eyden (1975) suggested that the localization of Gomori reaction product in the Golgi apparatus of *Dunaliella* implicates the organelle in lysosome formation. It is possible that this also applies to *P. pseudoparkeae* because cells containing large deposits of lead phosphate in the Golgi cisterna contain lysosome-like vesicles which also have hydrolase activity. It is believed that the scales within the lysosome-like vesicles in *P. pseudoparkeae* are broken down and repolymerized to form the inner component of the cyst wall. Pienaar (1971) has demonstrated that certain digestive vacuoles in *Hymenomonas* contain acid hydrolases that digest unmineralized scales which fail to reach the plasmalemma after a 24 h cycle of scale production.

The high concentration of Gomori reaction product in the immature cisternae in *P. pseudoparkeae* may also indicate that scale precursor molecules may be bound to phosphate.

VIRUSES

The occurrence of viruses in *P. pseudoparkeae* is not unusual as they have been shown to be present in *P. orientalis* (Moestrup and Thomsen, 1974) and a number of other Prasinophyceae (Pearson and Norris, 1974;

Pienaar, 1975b, 1976d; Mayer and Taylor, 1979; Melkonian, 1982c).

Sherman and Brown (1978) and Dodds (1979) have reviewed the occurrence of viruses in eukaryotic algae. Since these reviews a number of new reports on viral-like infection have appeared in the literature. Virus-like particles have been reported in a number of green algae (Stanker and Hoffman, 1979; Dodds and Cole, 1980; Gromov and Mamkaeva, 1981; Meints *et al.*, 1981). Virus-like particles have also been reported in the Dinophyceae (Sicko-Goad and Walker, 1979; Pienaar, 1980c), Cryptophyceae (Meyer, 1981) and Chrysophyceae (Priesig and Hibberd, 1984).

Viral particles are found in other algal groups. They are known in the Phaeophyceae (La Claire and West, 1977; Oliveira and Bisalputra, 1978), Rhodophyceae (Chapman and Lang, 1973), and Prymnesiophyceae (Pienaar, 1975b; 1976d).

The icosohedral virus particles in *P. pseudoparkeae* are approximately 180 nm diameter and resemble those (130 nm in diameter) of the related prasinophyte *Mesostigma* (Melkonian, 1982c). Melkonian (1982c) has reviewed the relationship between size and occurrence of polygonal viruses in different algal groups. Virus particles generally have a uniform diameter in different groups. These are 200 - 400 nm in diameter in the Chlorophyceae, 40 - 60 nm in diameter in the Rhodophyceae and 170 nm in the Phaeophyceae. Within the Prasinophyceae virus particles may vary between 60 nm in *Tetraselmis* (Pearson and Norris, 1974) and 200 nm in *Pyramimonas orientalis* (Moestrup and Thomsen, 1974). The virus particles of *Mesostigma* and *Micromonas* (Melkonian, 1982c; Mayer and Taylor, 1979) are 130 nm in diameter. Melkonian (1982c) has noted that the apparent heterogeneity in size of virus-like particles in the Prasinophyceae matches the ultrastructural and phylogenetic heterogeneity that is apparent in this group of green flagellates. The significance of this is still not known.

TAXONOMY OF THE CLASS PRASINOPHYCEAE

The most recent taxonomic scheme for the Prasinophyceae has been presented by Moestrup (1982, 1984).

Moestrup (1982) reinstated the class Loxophyceae (recognized by Christensen, 1962) and includes in the class, the "prasinophytes" *Monomastix*, *Pedinomonas*, *Scourfieldia* and *Micromonas*. The Loxophyceae are separated from the Prasinophyceae because they have fine non-tubular hairs on the flagella. Flagellar hairs (or hairscales) in the Prasinophyceae are thick and tubular.

Moestrup (1984) recognizes three orders in the class Prasinophyceae, namely the Mamiellales, Pyramimonadales and the Halosphaerales.

The Mamiellales is thought to be the most primitive order in which the cells lack an underlayer of square or diamond-shaped scales. Included in this order are the genera *Mamiella*, *Mantoniella* and *Dolichomastix*.

The order Pyramimonadales contains cells which possess an underlayer of square or diamond-shaped scales on the flagella and often the cell body. Genera assigned to this order are *Pyramimonas*, *Nephroselmis*, *Mesostigma*, *Prasinochloris* and *Tetraselmis*.

The order Halosphaerales contains genera which possess a phycoma but which are otherwise similar to the Pyramimonadales.

Though Moestrup's (1982, 1984) taxonomic scheme for the Prasinophyceae is practical and simple, the writer does not believe that it is entirely suitable because some of the orders do not accurately reflect phylogenetic differences between genera. For example, the close similarity between *Pyramimonas* and *Halosphaera* (both in the motile stage and non-motile cyst or phycoma stage) suggests that they are phylogenetically closely related and should be placed in the same order. Moestrup (1984) has placed these two genera into separate orders viz. the Pyramimonadales and Halosphaerales.

Norris (1982a) has presented a separate taxonomic scheme for the Prasinophyceae which includes members of the Loxophyceae *sensu* Christensen (1962) and Moestrup (1982). Norris (1982a) recognizes five orders in the class Prasinophyceae which are briefly discussed below. The species given in brackets within each order were not mentioned by Norris (1982a) but have been added after a personal communication with

R.E. Norris¹.

The Pedinomonadales is thought to contain the most primitive members of the class. The flagellate cells do not possess scales and have a single flagellum which may bear fine hairs. *Pedinomonas* (and *Micromonas*) belongs to this order.

The Monomastigales contains species which are morphologically and structurally diverse. Members of this order may have one or two flagella (always two basal bodies) and one or two layers of scales covering the cell. The scales have a more or less radiating pattern of fibrils. (The genera included in this class are *Monomastix*, *Mantoniella*, *Nephroselmis*, *Dolichomastix*, *Pseudoscourfieldia*, *Scourfieldia* and *Bipedinomonas*).

[The inclusion of *Scourfieldia* in the Prasinophyceae is doubtful (Manton, 1975). *Pseudoscourfieldia* and *Bipedinomonas* are now thought to be synonyms of *Nephroselmis* (see Moestrup, 1983).]

The order Pyramimonadales contains cells with two or four (occasionally eight) flagella and have one to several layers of scales not having a radiating pattern of fibrils. Scales in different layers have different forms. Norris (1982a) recognizes two families in this order, viz. the Pyramimonadaceae and the Halosphaeraceae. Members of both families are known to have motile and non-motile stages in their life-cycle. In the Halosphaeraceae the non-motile phase or phycoma predominates while in the Pyramimonadaceae the flagellate cell dominates the life-cycle. The genera *Pyramimonas* and *Mestostigma* are assigned to the family Pyramimonadaceae. The Halosphaeraceae is a monogeneric family containing *Halosphaera*. The motile cells of *Pyramimonas* and *Halosphaera* are almost identical. The cells are pyramidal in shape, are radially symmetrical, and have four anteriorly inserted flagella.

The Pterospermales contains two genera, *Pterosperma* and *Pachysphaera*, which have spheroidal phycomata with thick cell walls. The motile cells of these genera do not resemble the motile cells of

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Halosphaera. They are compressed and convex-concave and have four equal flagella that are laterally attached and held in a posterior position as the cell swims.

The order Chlorodendrales contains only one genus, *Tetraselmis*. The flagellate cell has four flagella and is enclosed in a theca. Scales are only found on the flagellar surfaces.

The taxonomic scheme of Norris (1982a) recognizes the phylogenetic closeness between *Pyramimonas* and *Halosphaera* and at least on this issue this scheme may be preferable to that proposed by Moestrup (1984).

It is the writer's belief that all genera having square or pentagonal underlayer scales and limuloid flagellar scales (i.e. *Pyramimonas*, *Prasinochloris?*, *Halosphaera*, *Pterosperma* and *Pachysphaera*) are phylogenetically related and should perhaps be placed in a single order with separations being made at the family level.

The placement of *Tetraselmis* in a separate order has its merits because the alga is unusual in having a theca and phycoplasmic cytokinesis.

Norris' (1982a) Monomastigales and Moestrup's (1984) Mamiellales are almost identical and seem to define a well circumscribed group. The inclusion of *Monomastix* in the Monomastigales is the only difference between the two orders. Moestrup (1984) preferred to place *Monomastix* in the Loxophyceae. Because the Loxophyceae does not appear to represent a valid class (see appraisal of class Prasinophyceae below) the inclusion of *Monomastix* in the Monomastigales appears to be best placement for the organism at this stage. The presence of a body scale with a radiating pattern of fibrils may ally *Monomastix* with other members of the Monomastigales (or Mamiellales).

Pseudoscourfieldia, *Nephroselmis* and *Mesostigma* are probably closely related and should perhaps be placed in a separate order. These genera are similar to *Pyramimonas*, *Pterosperma*, *Pachysphaera* and *Halosphaera* in that they have pentagonal underlayer scales on the

flagella and square underlayer scales on the cell body. They differ however in that they lack a limuloid flagellar scale which is replaced by a naviculoid, fusiform or rod-shaped scale.

AN APPRAISAL OF THE CLASS PRASINOPHYCEAE

The following discussion outlines those characteristics which the writer considers important in separating the Prasinophyceae from other green algae.

The class Prasinophyceae is construed by the writer to be a distinct but heterogeneous group of primitive, unicellular green algae. Motile members of the class are united in that they:

- (i) possess some form of scale-covering (except *Micromonas* which is a naked unicell — Manton, 1959; Manton and Parke, 1960),
- (ii) possess the photorespiratory enzyme glycolate dehydrogenase (Floyd and Salisbury, 1977).
- (iii) possess mannitol rather than sucrose as a photosynthetic product (Craigie *et al.*, 1967),
- (iv) do not develop a phycoplast during telophase of mitosis (Pickett-Heaps and Ott, 1974; Pearson and Norris, 1975; Mattox and Stewart, 1977; Woods and Triemer, 1981). *Tetraselmis* is an exception (Stewart *et al.*, 1974).
- (v) have their flagella inserted in a depression on the cell surface,
- (vi) often reproduce asexually, having a *unicellular* cyst or phycoma as the alternate life cycle stage (Parke and den Hartog-Adams, 1965; Parke, 1966; Belcher, 1970). The exceptions cited here are discussed below.

Each point mentioned above, if taken in isolation, does not constitute a valid reason for recognizing the Prasinophyceae. Scales, as

mentioned earlier, are found on the motile propagules of some ulvophycean and charophycean algae. The prasinophyte *Micromonas* lacks a scale-covering but can be included in the Prasinophyceae because it possesses some unusual photosynthetic pigments which are present in the more advanced members of the class (Ricketts, 1966a). Furthermore, the alga produces mannitol as a photosynthetic product and in this respect it is similar to all other prasinophytes (Craigie *et al.*, 1967). Only two green algal genera outside the Prasinophyceae produce this polyol, namely *Enteromorpha* (Lindberg, 1955) and *Cladophora* KÜTZING (Craigie *et al.*, 1966).

The photorespiratory enzyme glycolate dehydrogenase is present in members of the Prasinophyceae (and Chlorophyceae) but has not been detected in members belonging to the class Charophyceae (Frederick *et al.*, 1973). This discrepancy reveals an important evolutionary disjunction between the Prasinophyceae and Charophyceae. Members of the former class could not be included in the Charophyceae if a natural classification is desired. However, three characteristics exhibited by some prasinophytes are also seen in charophycean algae and this suggests that there may be a phylogenetic connection between the two classes. These are: (a) the presence of scales, (b) the possession of a MLS associated with the flagellar apparatus (Moestrup and Ettl, 1979; Rogers *et al.*, 1981) and (c) a persistent interzonal spindle at telophase (Stewart *et al.*, 1974; Pearson and Norris, 1975; Woods and Triemer, 1981).

Members of the Prasinophyceae are biochemically more closely related to members of the Chlorophyceae than the Charophyceae because the two former groups possess the photorespiratory enzyme glycolate dehydrogenase (Frederick *et al.*, 1973). The affinity between the two classes becomes more apparent if members of the Prasinophyceae are compared with chlorophycean representatives of the order Volvocales. Both taxa are united in that they contain unicellular motile algae (although some members of the order Volvocales are colonial). This relationship is, however, superficial because a number of characteristics can be cited which demonstrate that the Prasinophyceae and Chlorophyceae are mutually exclusive groups. Members of the Prasinophyceae can be excluded from the Chlorophyceae because (a) they do not develop a phycoplast which is characteristic of chlorophycean algae, (b) they possess a scale-covering

which is absent in members of the Chlorophyceae, (c) they have their flagella inserted in a depression in the cell (members of the order Volvocales have their flagella inserted on a papilla), and (d) they possess a pectin-like cell boundary (Lewin, 1958; Gooday, 1971; results of this work - see Chapter Six) in contrast with the glycoproteinaceous cell wall seen in members of the order Volvocales (Roberts *et al.*, 1972), and the cellulosic, mannan and xylan cell walls of other Chlorophyceae (see Mackie and Preston, 1974).

With regard to point (a) above, *Tetraselmis* is the only prasinophyte which breaks this rule. It does, however, conform with points (b) - (d) above. The alga also produces mannitol as a photosynthetic product and should therefore be grouped with the prasinophytes. Nevertheless, *Tetraselmis* undoubtedly represents a strong phylogenetic link between the Prasinophyceae and Chlorophyceae. With respect to point (c) above only one member of the order Volvocales, namely *Hafniomonas* has its flagella inserted in a depression in the cell (Ettl and Moestrup, 1980). *Hafniomonas*, like *Tetraselmis*, is another example emphasizing the close relationship between the Chlorophyceae and Prasinophyceae.

The taxonomic position of the unusual prasinophyte *Pedinomonas*, is difficult to assess because of a paucity of data on the organism. The alga was recently assigned to the class Ulvophyceae (Stewart and Mattox, 1978) because it resembled the zoospores of members of this group. *Pedinomonas* bears very fine hairs on the single flagellum and it has a cell-covering comprising one layer of body scales (Belcher, 1968b). It appears, initially, quite illogical to liken a uniflagellate prasinophyte to a quadriflagellate zoospore. However, this discrepancy is not as great as it first appears. The alga has two basal bodies - one of which is non-functional (Pickett-Heaps and Ott, 1974) and a cruciate microtubular root pattern (Moestrup, 1982). The latter would be expected in a quadriflagellate organism. As Norris (1980) explains, this alga is probably a derived form of complicated phylogeny. A significant characteristic that must be mentioned here is that *Pedinomonas* is known to reproduce asexually by producing a thick-walled cyst (Belcher, 1968b). It is difficult to imagine how a unicellular cyst could give rise to the relatively complex multicellular thalli of the Ulvophyceae without undergoing a major evolutionary change. This

fact would suggest that *Pedinomonas* should be retained in the Prasinophyceae.

At the present time there appears to be sufficient evidence in favour of recognizing the Prasinophyceae as a separate class in the division Chlorophyta. Members of the class form a heterogeneous assemblage of unicellular green algae, and it is this diversity, as Norris (1980) suggests, that may have provided a foundation on which other classes in the division Chlorophyta could have evolved.

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APPENDICES

APPENDIX A

RECIPES FOR SEAWATER MEDIA

(taken from McLachlan, 1973)

ALL STOCK SOLUTIONS ARE ADJUSTED TO pH 7,5,
 FILTER STERILIZED AND STORED AT 4°C

A. Enriched seawater media

TABLE I
Provasoli's Enriched Seawater Medium-PES

Additive	Weight	
Stock 1 - major salts		
NaNO ₃	5,61	g
Na ₂ C ₃ H ₅ (OH ₂) PO ₄ .5 H ₂ O	0,78	g
Fe. EDTA	0,26	g
Tris. buffer	7,99	g
Make to one litre with distilled water		
Stock 2 - PII trace metals		
Zn SO ₄ .7H ₂ O	230	23 mg
MnSO ₄ .4H ₂ O	163	mg
CO ₂ SO ₄ .7H ₂ O	5,0	mg
H ₃ BO ₃	1,14	g
Na ₂ EDTA	1,0	g
Fe. citrate	60	mg
Make to one litre with distilled water		
Stock 3 - vitamin mix		
Cyanocobalamin	0,8	mg
Biotin	0,4	mg
Thiamine (Aneurine).HCl	10	mg
Make to 500 ml with distilled water		

Method : To make one litre of enriched seawater, combine;

10 ml stock 1

10 ml stock 2

1,0 ml stock 3, and make to one litre with sterilized seawater.

Note: a) PES stock solutions are filter sterilized through a 0,45 Millipore membrane.

b) In preparing the P-II trace metal solution, each trace metal is chelated with Na_2EDTA before being combined. Chelation is effected by adding the trace metal and chelator (weight ratio 1:4) to a small volume of distilled water and boiling for 5 min.

c) The pH of all stock solutions are adjusted to pH 7,5 using either 1N HCl or 1N NaOH.

TABLE II
Erdschreiber Enriched Seawater Medium

Additive	Weight
Stock 1 - major salts	
NaNO_3	20 g
Na_2HPO_4	2,0 g
Make to one litre with distilled water.	
Soil extract - prepared as follows;	
Mix 1 vol. of virgin soil with 2 vol. distilled water and steam for 1 h. After cooling, the liquid is cleared by centrifugation.	

Method: To make one litre of enriched seawater, combine;

10 ml stock 1

50 ml soil extract and make to one litre with sterilized seawater.

Note: Salts in stock 1 are dissolved separately by heating in a small volume of distilled water. They are combined when cool.

TABLE III
f/2 Enriched Seawater Medium

Additive	Weight
Stock 1 - major salts	
NaNO ₃	7,48 g
NaH ₂ PO ₄ .2H ₂ O	0,57 g
Na ₂ Si O ₃ .5H ₂ O	1,45 g
Make to one litre with distilled water	
Stock 2 - f/2 trace metals	
Zn SO ₄ .7H ₂ O	2,3 mg
Mn SO ₄ .4H ₂ O	20 mg
Na ₂ MoO ₄ .2H ₂ O	0,7 mg
CoSO ₄ .7H ₂ O	1,4 mg
CuSO ₄ .5H ₂ O	1,0 mg
Fe.citrate	390 mg
Na ₂ EDTA	436 mg
Make to one litre with distilled water	
Stock 3 - Vitamin mix	
Cyanocobalamin	0,25 mg
Biotin	0,25 mg
Thiamine. HCl	50 mg
Make to 500 ml with distilled water	

Method: To make one litre of enriched seawater, combine;

10 ml stock 1

10 ml stock 2

1,0 ml stock 3, and make to one litre with sterilized seawater.

Note: f/2 trace metals are chelated with Na₂EDTA as outlined above under Provasoli's Enriched Seawater, except here the metal/chelator ratio is approx. 1:1 by weight.

TABLE IV

SWM Enriched Seawater Medium

Additive	Weight	
Stock 1 - major salts		
NaNO ₃	17,0	g
NaH ₂ PO ₄ .2H ₂ O	1,56	g
Na ₂ SiO ₃ .5H ₂ O	4,24	g
Fe EDTA	0,73	g
Glycylglycine buffer	66	g
Make to one litre with distilled water		
Stock 2 - TMS-I trace metals		
ZnSO ₄ .7H ₂ O	1,0	g
MnSO ₄ .4H ₂ O	223	mg
Na ₂ MoO ₄ .2H ₂ O	1 21	mg
CoSO ₄ .7H ₂ O	8,4	mg
CuSO ₄ .5H ₂ O	7,5	mg
Fe.citrate	70	mg
Na ₂ EDTA	1,79	g
H ₃ BO ₃	2,5	g
Make to one litre with distilled water		
Stock 3 - S ₃ vitamin mix.		
Thiamine.HCl	250	mg
Nictonic acid	50	mg
Ca.Panthothenate	50	mg
i-Inositol	2,5	g
Thymine	1,5	g
p-Aminobenzoic acid	5,0	mg

Continued overleaf

Biotin	0,5	mg
Folic acid	1,0	mg
Cyanocobalamin	0,5	mg
Make to 500 ml with distilled water		
Soil extract (for preparation see Table II above)	50	ml
Liver extract Oxoid L 25	10	mg

Method: to make one litre of enriched seawater, combine:

10 ml Stock 1
 10 ml Stock 2
 1,0 ml Stock 3
 50 ml soil extract
 10 mg liver extract,
 and make to one litre with sterilized seawater.

Note: Trace metals are chelated with Na₂EDTA (1:1 w/w). Any excess chelator is added after the trace metals are combined.

B. Artificial seawater media

TABLE V
ASP-2 Artificial Seawater Medium

Additive	Weight	
Stock 1		
NaCl	18	g
MgSO ₄ .7H ₂ O	5,0	g
KCl	604	mg
Na ₂ CO ₃	31,8	mg
CaCl ₂	278	mg
NaNO ₃	50	mg

Continued overleaf

K ₂ HPO ₄	5,0	mg
NTA	100	mg
Fe Cl ₃	1,5	mg
Na ₂ SiO ₃ .5H ₂ O	112	mg
Tris. buffer	1,0	g
Make to one litre with distilled water		
Stock 2 - PII trace metals	30	ml
See Provasoli's Enriched Seawater - see Table I above.		
Stock 3 - S ₃ vitamin mix.	1	ml
See SWM Enriched Seawater - see Table IV above.		

Method: Stock 1 represents the artificial seawater. To make one litre of ASP-2 medium combined:

30 ml Stock 2

1,0 ml Stock 3, and make to one litre with Stock 1.

Note: To prevent compounds in Stock 1 from precipitating, it is best to keep the pH slightly acidic. This can be adjusted to pH 7,5 when the medium is made up.

TABLE VI

ASP-6 Artificial Seawater Medium

Additive	Weight	
Stock 1		
NaCl	24	g
MgSO ₄ .7H ₂ O	8,0	g
KCl	701	mg
CaCl ₂ .2H ₂ O	551	mg
NaNO ₂	300	mg
Na ₂ -glycerophosphate	100	mg
Na ₂ SiO ₃ .5H ₂ O	52	mg
Tris.buffer	1,0	g
Make to one litre with distilled water		

Stock 2 - P-8 trace metals

ZnSO ₄ .7H ₂ O	221	mg
MnSO ₄ .4H ₂ O	402	mg
Na ₂ MoO ₄ .2H ₂ O	126	mg
CoSO ₄ .7H ₂ O	4,8	mg
CuSO ₄ .5H ₂ O	10	mg
Fe.citrate	1,2	g
Versonal	3,0	g
H ₃ BO ₃	1,14	g
Make to one litre with distilled water.		

Stock 3 - 8A vitamin mix

Thiamine HCl	1,0	g
Nicotinic acid	0,5	g
Ca pantothenate	0,5	g
p-Aminobenzoic acid	50	mg
i-Inositol	5,0	g
Thymine	4,0	g
Pyridoxine.HCl	0,2	g
Pyridoxamine.2HCl	0,1	g
Choline. H ₂ Citrate	2,5	g
Orotic acid	1,3	g
Biotin	2,5	mg
Folic acid	12,5	mg
Cyanocobalamin	0,25	mg
Riboflavin	25	mg
Folonic acid	1,0	mg
Make to 500 ml with distilled water		

Method: To prepare the ASP-6 artificial seawater medium, combine:

10 ml Stock 2

1,0 ml Stock 3 and make to one litre with Stock 1.

Notes: a) See note under Table V preceding,

b) Because of the complex nature of Stock 3 it is often desirable to reduce the concentration of vitamins to one-tenth that shown above. This prevents precipitation during storage. The volume of Stock 3 added to the medium should consequently be increased from 1,0 ml to 10 ml.

- c) Trace metals are chelated with Versonal (ratio 1:1). Any excess chelating agent is added to Stock 2 when trace metals are mixed.

TABLE VII
Müller's Artificial Seawater medium

Additive	Weight	
Stock 1		
NaCl	26,7	g
MgSO ₄ ·7H ₂ O	6,56	g
MgCl ₂ ·6H ₂ O	4,68	g
CaCl ₂ ·2H ₂ O	1,98	g
KCl	731	mg
NaHCO ₃	202	mg
NaNO ₃	100	mg
Na ₂ SiO ₂ ·5H ₂ O	14,8	mg
Na ₂ HPO ₄	20	mg
Make to one litre with distilled water.		
Stock 2 - M-I + M-II trace metals		
M-I		
ZnSO ₄ ·7H ₂ O	400	mg
MnSO ₄ ·4H ₂ O	85	mg
Na ₂ MoO ₄ ·2H ₂ O	20	mg
CoSO ₄ ·7H ₂ O	1,1	mg
CuSO ₄ ·5H ₂ O	0,2	mg
Fe.citrate	40	mg
Na ₂ EDTA	2,0	g
H ₃ BO ₃	2PP	mg
M-II		
NaBr	2,0	g
SrCl ₂ ·6H ₂ O	370	mg
AiCl ₃	2,8	mg
RbCl	1,6	mg
LiCl	0,6	mg
KI	2,0	mg

Stock - 3 f/2 - vitamin mix.	1	ml
see f/2 Enriched Seawater - Table III above.		

Method: To make one litre of Müller's artificial seawater medium, combine:

10 ml Stock 2

1,0 ml Stock 3, and make to one litre with Stock 1.

Note: See note under Table VI above.

APPENDIX B

CELL SIZE MEASUREMENTS

TABLE I

Cell size measurements of *P. pseudoparkeae* in an actively growing culture
(values in μm -rounded off to nearest integer)

Length x Width	Length x Width	Length x Width	Length x Width
14 x 10	16 x 12	12 x 10	11 x 8
12 x 10	13 x 10	12 x 9	18 x 12
16 x 12	11 x 8	14 x 10	15 x 11
16 x 12	14 x 10	14 x 11	17 x 13
13 x 10	14 x 11	12 x 10	14 x 10
14 x 12	12 x 8	13 x 10	15 x 11
18 x 13	15 x 11	15 x 12	14 x 11
18 x 12	13 x 10	10 x 8	16 x 11
15 x 11	12 x 8	14 x 10	13 x 10
17 x 12	18 x 12	14 x 10	14 x 9
18 x 14	12 x 9	12 x 10	12 x 9
16 x 12	12 x 10	18 x 12	16 x 11
18 x 14	12 x 10	18 x 12	14 x 11
12 x 11	14 x 11	16 x 10	19 x 12
15 x 12	12 x 10	14 x 12	15 x 11
13 x 10	15 x 12	15 x 10	16 x 12
18 x 12	14 x 11	15 x 10	14 x 10
18 x 12	15 x 12	12 x 9	15 x 11
14 x 10	15 x 12	16 x 11	10 x 8
11 x 8	14 x 11	11 x 8	15 x 11
14 x 10	12 x 10	15 x 10	14 x 10
14 x 10	16 x 12	14 x 11	13 x 10
12 x 10	16 x 11	12 x 10	14 x 10
14 x 11	16 x 12	13 x 10	13 x 10
12 x 10	15 x 11	12 x 9	17 x 12

TABLE II

Cell size measurements of *P. pseudoparkeae* in cultures in the stationary phase of growth (values in μm -rounded off to nearest integer).

Length x Width	Length x Width	Length x Width	Length x Width
25 x 17	22 x 17	17 x 12	17 x 14
31 x 26	23 x 16	17 x 12	22 x 17
20 x 17	20 x 16	20 x 16	22 x 16
25 x 17	22 x 16	23 x 17	19 x 14
19 x 14	25 x 19	19 x 14	17 x 12
25 x 17	30 x 22	23 x 16	23 x 19
34 x 23	22 x 16	22 x 17	22 x 17
23 x 19	20 x 16	20 x 14	19 x 14
25 x 19	22 x 16	22 x 14	17 x 12
20 x 15	23 x 17	20 x 16	37 x 22
22 x 15	23 x 17	34 x 26	34 x 23
26 x 22	20 x 14	25 x 16	22 x 17
22 x 15	16 x 12	22 x 17	22 x 17
25 x 21	20 x 16	22 x 17	22 x 16
22 x 17	19 x 12	22 x 16	20 x 16
30 x 23	22 x 16	20 x 16	22 x 16
17 x 12	22 x 17	20 x 14	23 x 19
25 x 20	22 x 17	23 x 16	28 x 22
22 x 17	28 x 20	23 x 16	20 x 14
22 x 15	22 x 17	22 x 16	23 x 17
25 x 19	22 x 16	25 x 19	35 x 17
23 x 17	22 x 19	25 x 19	22 x 16
19 x 14	17 x 12	19 x 12	17 x 14
22 x 17	37 x 22	23 x 17	22 x 17
22 x 16	25 x 17	22 x 17	19 x 16

TABLE III

Cell size measurements of *P. pseudoparkeae* cysts (values in μm -rounded off to nearest integer).

Diameter	Diameter	Diameter	Diameter
22	22	22	19
21	18	20	20
18	20	20	18
18	18	18	18
16	24	19	19
18	19	21	22
20	20	20	19
20	20	20	20
18	20	18	19
18	20	20	20
18	19	18	18
20	19	18	22
19	20	19	19
19	20	20	20
18	19	19	19
18	19	18	18
18	22	18	18
19	17	17	19
20	18	21	18
18	18	22	17
22	17	18	21
20	20	20	18
19	18	20	20
20	20	19	18
22	19	20	20

APPENDIX C

GROWTH DATA

TABLE I

Cell counts for *P. pseudoparkeae* grown in seven enriched and artificial seawater media. (Values in log cell no. ml⁻¹).

Day	Reps	Enriched medium				Artificial medium		
		PES	Erdschreiber	F/s	SWM	ASP-2	ASP-6	Müller
0	a	3,80	3,80	3,80	3,80	3,80	3,80	3,80
	b	3,80	3,80	3,80	3,80	3,80	3,80	3,80
	c	3,80	3,80	3,80	3,80	3,80	3,80	3,80
1	a	4,14	3,69	3,72	3,62	—	3,09	3,52
	b	3,68	3,61	3,61	3,71	—	3,24	3,60
	c	3,67	3,59	3,69	3,53	—	3,20	3,60
2	a	4,00	3,95	4,06	3,96	—	3,77	3,96
	b	3,93	4,01	3,87	3,94	—	3,73	3,98
	c	3,99	3,90	3,98	3,81	—	3,79	3,98
3	a	4,28	4,31	4,28	4,15	3,57	4,06	4,16
	b	4,23	4,24	4,15	4,18	3,61	4,03	4,24
	c	4,24	4,23	4,22	4,05	3,59	4,09	4,19
4	a	4,49	4,59	4,56	4,42	3,68	4,44	4,46
	b	4,44	4,49	4,33	4,45	3,73	4,27	4,49
	c	4,49	4,46	4,43	4,38	3,79	4,26	4,39
5	a	4,80	4,87	4,81	4,59	3,82	4,72	4,76
	b	4,67	4,57	4,56	4,65	3,92	4,55	4,76
	c	4,80	4,71	4,65	4,67	3,87	4,43	4,63
6	a	5,03	5,10	5,02	4,83	4,17	4,90	5,05
	b	4,89	4,64	4,78	4,82	4,23	4,79	5,06
	c	5,00	4,82	4,88	4,93	4,09	4,62	4,92
7	a	5,19	5,15	5,23	5,00	4,37	5,09	5,35
	b	5,11	4,69	4,99	4,94	4,45	5,02	5,29
	c	5,15	4,75	5,08	5,12	4,27	4,83	5,15
8	a	5,37	5,17	5,55	5,24	4,53	5,20	5,58
	b	5,26	4,84	5,22	5,14	4,59	5,34	5,56
	c	5,30	4,85	5,34	5,40	4,32	5,10	5,39
9	a	5,58	4,98	5,88	5,61	4,72	5,37	5,65
	b	5,45	5,00	5,58	5,37	4,78	5,56	5,78
	c	5,53	4,99	5,66	5,74	4,37	5,23	5,51
10	a	5,69	5,08	5,84	5,75	4,75	5,47	5,71
	b	5,62	5,10	5,74	5,55	4,78	5,71	5,82
	c	5,61	5,09	5,79	5,85	4,35	5,38	5,63
11	a	5,73	5,13	5,89	5,93	4,94	5,72	5,80
	b	5,67	5,21	5,71	5,77	4,91	5,93	6,00
	c	5,55	5,15	5,75	6,00	4,33	5,59	5,79
12	a	5,79	5,16	5,73	6,12	4,99	5,82	5,82
	b	5,63	5,32	5,62	5,93	4,89	6,06	6,05
	c	5,19	5,20	5,70	6,09	4,40	5,75	5,83
13	a	5,82	5,19	5,73	6,17	5,02	5,91	5,90
	b	5,57	5,36	5,59	6,10	4,95	6,18	6,16
	c	5,17	5,24	5,65	6,13	4,41	5,89	5,85
14	a	5,24	4,93	5,08	5,87	4,93	5,92	5,92
	b	4,74	5,15	4,62	5,80	4,80	6,21	5,97
	c	4,33	5,00	4,59	5,28	4,44	4,88	5,88

TABLE I I

Relative growth rates and mean doubling times of *P. pseudoparkeae* in replicate cultures in seven seawater media.

Medium		Relative growth rate (k')	Mean doubling time (G) in hours
PES	a	0,26	28,05
	b	0,24	30,10
	c	0,25	28,61
	\bar{x}	0,25	28,92
Erdschreiber	a	0,30	24,08
	b	0,29	24,63
	c	0,29	24,91
	\bar{x}	0,29	24,54
F/2	a	0,25	28,70
	b	0,23	31,41
	c	0,23	31,18
	\bar{x}	0,24	30,43
SWM	a	0,24	29,85
	b	0,22	32,54
	c	0,28	25,80
	\bar{x}	0,25	29,40
ASP-2	a	0,20	36,12
	b	0,21	34,40
	c	0,17	42,49
	\bar{x}	0,19	37,67
ASP-6	a	0,19	38,84
	b	0,26	28,00
	c	0,19	37,24
	\bar{x}	0,21	34,69
Müller	a	0,27	27,76
	b	0,26	27,43
	c	0,24	30,74
	\bar{x}	0,26	28,31

TABLE III

The relative growth rates and mean doubling times for *P. pseudoparkeae* grown in seven different seawater media. The table includes data on the start and duration of exponential growth, the computed regression equation for these data and the t-values for the regressions.

	Medium	Start of exponential growth	Period of exponential growth (days)	Computed regression equation	t	Degrees of freedom	Relative growth rate (k')	Mean doubling time (G) in hours
Enriched Seawater Media	PES	Day 2	4	$y=3,47+0,25x$	39,5	3	0,25	28,92
	Erdschreiber	Day 1	3	$y=3,36+0,29x$	30,7	2	0,29	24,54
	F/2	Day 1	6	$y=3,49+0,24x$	41,0	5	0,24	30,43
	SWM	Day 1	5	$y=3,40+0,25x$	44,0	4	0,25	29,40
Artificial Seawater Media	ASP-2	Day 3	4	$y=2,96+0,20x$	11,8	3	0,19	37,67
	ASP-6	Day 4	5	$y=3,52+0,21x$	29,8	4	0,21	34,69
	Müller	Day 2	6	$y=3,43+0,26x$	67,3	5	0,26	28,31

TABLE IV

pH and salinity measurements made on seven seawater media before
and after autoclaving

		Enriched Seawater Media				Artificial Seawater Media		
		PES	Erdschreiber	F/2	SWM	ASP-2	ASP-6	Müller
Before autoclaving	pH	8,45	8,65	8,45	8,30	7,65	7,45	7,05
	Salinity ‰	32	30	32	30	20	28	34
After autoclaving	pH	8,45	8,65	8,60	8,65	7,35	7,20	7,60
	Salinity ‰	32	30	32	30	20	28	34

TABLE V

Cell counts for *P. pseudoparkeae* grown at three different light intensities in five different salinities (Values in log cell no. ml⁻¹).

Day	Reps	200 $\mu\text{Ein}^{-2} \text{s}^{-1}$					100 $\mu\text{Ein}^{-2} \text{s}^{-1}$					50 $\mu\text{Ein}^{-2} \text{s}^{-1}$				
		10‰	20‰	35‰	50‰	70‰	10‰	20‰	35‰	50‰	70‰	10‰	20‰	35‰	50‰	70‰
0	a	3,5	3,8	3,8	3,7	3,5	3,3	3,4	3,3	3,4	3,5	3,5	3,5	3,3	3,5	3,6
	b	3,4	3,5	3,4	3,6	3,6	3,4	3,5	3,3	3,4	3,5	3,3	3,4	3,3	3,4	3,5
	c	3,7	3,6	3,8	3,6	3,3	3,4	3,4	3,3	3,4	3,5	3,5	3,5	3,7	3,4	3,5
2	a	2,9	3,7	3,7	3,6	3,5	2,6	3,7	3,7	3,5	3,3	3,6	3,9	3,9	3,8	3,7
	b	2,8	3,8	3,7	3,6	3,5	3,1	3,7	3,6	3,5	3,3	3,5	3,9	3,9	3,8	3,7
	c	3,1	3,7	3,7	3,7	3,6	3,1	3,7	3,6	3,6	3,3	3,6	3,8	3,9	3,7	3,6
4	a	3,9	4,4	4,6	4,3	4,0	3,4	4,4	4,4	4,3	3,9	4,0	4,5	4,4	4,2	3,8
	b	3,8	4,6	4,5	4,4	3,9	3,6	4,4	4,4	4,3	3,8	3,8	4,4	4,4	4,2	3,7
	c	3,7	4,6	4,5	4,3	3,8	3,7	4,5	4,5	4,2	3,9	3,9	4,4	4,4	4,1	3,8
6	a	4,5	4,9	4,9	4,7	4,4	4,1	4,8	4,9	4,7	4,3	3,9	4,9	4,9	4,7	4,2
	b	4,2	5,0	4,9	4,7	4,4	4,2	4,9	4,9	4,7	4,2	4,3	4,8	4,9	4,6	4,1
	c	4,2	5,0	4,9	4,8	4,4	4,3	4,9	4,9	4,7	4,2	4,4	4,9	4,9	4,6	4,2
8	a	4,9	5,1	5,1	5,0	4,7	4,6	4,9	4,9	4,9	4,6	4,8	5,1	4,9	4,9	4,5
	b	4,8	5,1	5,1	5,0	4,7	4,6	5,0	5,0	5,0	4,6	4,7	4,9	4,9	4,9	4,4
	c	4,7	5,1	5,2	5,0	4,7	4,6	5,0	5,0	5,0	4,6	4,7	4,9	5,0	4,8	4,4
10	a	5,1	5,3	5,3	5,3	5,0	4,7	5,1	5,2	5,2	4,9	5,0	5,2	5,2	5,1	4,8
	b	5,0	5,4	5,3	5,3	5,0	4,7	5,2	5,2	5,2	4,9	4,9	5,0	5,2	5,0	4,7
	c	5,0	5,4	5,3	5,3	5,0	4,8	5,2	5,2	5,2	4,9	4,9	5,0	5,1	5,0	4,7

TABLE VI

Relative growth rates and mean doubling times of *P. pseudoparkeae* in replicate cultures under three different light intensities and in five different salinities.

Light intensity	Salinity (‰)	Relative growth rate (k^{-1})	Mean doubling time (G) in hours
200 $\mu\text{Em}^{-2}\text{s}^{-1}$	10	a	0,27
		b	0,27
		c	0,24
		H	0,26
	20	a	0,20
		b	0,27
		c	0,26
		H	0,24
	35	a	0,21
		b	0,27
		c	0,21
		H	0,23
	50	a	0,18
		b	0,19
		c	0,18
H		0,18	
70	a	0,19	
	b	0,19	
	c	0,19	
	H	0,19	
100 $\mu\text{Em}^{-2}\text{s}^{-1}$	10	a	0,27
		b	0,21
		c	0,21
		H	0,23
	20	a	0,25
		b	0,25
		c	0,27
		H	0,25
	35	a	0,28
		b	0,28
		c	0,29
		H	0,28
	50	a	0,19
		b	0,20
		c	0,20
H		0,20	
70	a	0,20	
	b	0,20	
	c	0,20	
	H	0,20	
50 $\mu\text{Em}^{-2}\text{s}^{-1}$	10	a	0,18
		b	0,19
		c	0,17
		H	0,18
	20	a	0,24
		b	0,24
		c	0,24
		H	0,24
	35	a	0,27
		b	0,27
		c	0,19
		H	0,24
	50	a	0,17
		b	0,17
		c	0,17
H		0,17	
70	a	0,15	
	b	0,14	
	c	0,14	
	H	0,14	

TABLE VII

The relative growth rates and mean doubling times for *P. pseudoparkeae* grown at different salinities under different light intensities. The table includes data on the start and duration of exponential growth, the computed regression equation for these data and the *t* values for the regressions.

Light intensity	Salinity (‰)	Start of exponential growth	Period of exponential growth (days)	Computed regression equation	<i>t</i>	Degrees of freedom	Relative growth rate (<i>k'</i>)	Mean doubling time (G) in hours
$200\mu\text{Em}^{-2}\text{s}^{-1}$	10	Day 2	8	$y=2,61+0,26x$	8,1	3	0,26	27,87
	20	Day 0	6	$y=3,49+0,24x$	7,9	4	0,24	30,57
	35	Day 0	6	$y=3,52+0,23x$	4,3	2	0,23	32,30
	50	Day 0	10	$y=3,52+0,18x$	9,8	4	0,18	39,41
	70	Day 2	8	$y=3,18+0,19x$	17,3	3	0,19	38,71
$100\mu\text{Em}^{-2}\text{s}^{-1}$	10	Day 2	8	$y=2,62+0,23x$	7,4	3	0,23	31,59
	20	Day 0	6	$y=3,35+0,25x$	8,5	2	0,25	28,74
	35	Day 0	6	$y=3,23+0,28x$	9,2	2	0,28	25,81
	50	Day 0	10	$y=3,36+0,20x$	9,9	4	0,20	36,86
	70	Day 2	8	$y=3,00+0,20x$	14,8	3	0,20	36,74
$50\mu\text{Em}^{-2}\text{s}^{-1}$	10	Day 2	8	$y=3,20+0,18x$	14,2	3	0,18	40,56
	20	Day 0	6	$y=3,45+0,24x$	22,9	2	0,24	30,31
	35	Day 0	6	$y=3,42+0,25x$	94,5	2	0,24	30,96
	50	Day 0	10	$y=3,48+0,17x$	13,4	4	0,17	42,92
	70	Day 2	8	$y=3,32+0,14x$	11,7	3	0,14	51,64

TABLE VIII

LSD values for comparing the mean relative growth rates and mean doubling times obtained in the different salinities at each light intensity in Tables VI and VII. Asterisks (***) indicate that means are significantly different ($p = 0,05$)

Light Intensity	Salinity (%)	K'	G
200 $\mu\text{Em}^{-2}\text{s}^{-1}$	10	}	0,04 ***
	20		
	35		
	50		
	70		
100 $\mu\text{Em}^{-2}\text{s}^{-1}$	10	}	0,03 ***
	20		
	35		
	50		
	70		
50 $\mu\text{Em}^{-2}\text{s}^{-1}$	10	}	0,04 ***
	20		
	35		
	50		
	70		
			5,59 ***
			3,63 ***
			15,39 ***

TABLE IX

LSD values for comparing the mean relative growth rates and mean doubling times obtained at different light intensities at each salinity in Tables VI and VII. Asterisks (***) indicate that means are significantly different ($p = 0,05$).

Salinity	Light Intensity ($\mu\text{Em}^{-2} \text{s}^{-1}$)	K'	G
10‰	200	0,05 ***	5,70 ***
	100		
	50		
20‰	200	0,05	5,79
	100		
	50		
35‰	200	0,07	8,97
	100		
	50		
50‰	200	0,01 ***	0,94 ***
	100		
	50		
70‰	200	0,01 ***	2,32 ***
	100		
	50		

TABLE X

Cell counts for *P. pseudoparkeae* grown at two different temperatures under two different light intensities. (Values in log cell no. ml⁻¹).

Day	Reps	300 $\mu\text{Em}^{-2} \text{s}^{-1}$		150 $\mu\text{Em}^{-2} \text{s}^{-1}$	
		30°C	25°C	30°C	25°C
0	a	3,60	3,60	3,60	3,60
	b	3,60	3,60	3,60	3,60
	c	3,60	3,60	3,60	3,60
1	a	3,25	3,87	3,47	4,02
	b	3,23	3,86	3,17	4,11
	c	3,61	3,83	3,27	4,05
2	a	3,31	4,06	3,33	4,32
	b	3,15	4,13	3,42	4,41
	c	3,41	4,00	3,30	4,37
3	a	3,19	4,19	3,51	4,67
	b	3,27	4,39	2,93	4,74
	c	2,72	4,16	2,84	4,74
4	a	3,20	4,36	3,20	5,01
	b	3,14	4,58	2,36	5,01
	c	2,88	4,28	2,24	4,99
5	a	-	4,49	2,88	5,27
	b	-	4,47	2,71	5,21
	c	2,98	4,44	2,26	5,21
6	a	2,74	4,63	2,84	5,46
	b	3,00	4,89	2,94	5,41
	c	3,11	4,61	3,01	5,42
7	a	2,49	4,76	2,91	5,62
	b	2,95	4,99	-	5,53
	c	2,26	4,73	-	5,55
8	a	2,48	4,99	3,02	5,66
	b	2,43	5,08	1,43	5,39
	c	2,30	4,98	-	5,48
9	a	2,10	4,97	2,87	5,57
	b	3,04	5,03	3,15	5,28
	c	2,75	5,07	2,15	5,46
10	a	-	4,81	2,82	5,54
	b	2,66	4,84	-	5,26
	c	-	4,97	-	5,46

TABLE XI

Relative growth rates and mean doubling times of *P. pseudoparkeae* in replicate cultures under two different light intensities at two different temperatures.

Light Intensity	Temperature (°C)	Relative growth rate (k')	Mean doubling time (G) in hours
300 $\mu\text{Em}^{-2}\text{s}^{-1}$	25	a	0,15
		b	0,18
		c	0,15
		\bar{x}	0,16
	30	a	-0,09
		b	-0,09
		c	-0,23
		\bar{x}	-0,14
150 $\mu\text{Em}^{-2}\text{s}^{-1}$	25	a	0,30
		b	0,26
		c	0,27
		\bar{x}	0,28
	30	a	-0,13
		b	-0,14
		c	-0,17
		\bar{x}	-0,15

TABLE XII

The relative growth rates and mean doubling times for *P. pseudoparkeae* grown under two different light intensities at two different temperatures. The table includes data on the start and duration of exponential growth, the computed regression equation for these data and the *t* values for the regressions.

Light Intensity	Temperature (°C)	Start of exponential growth	Period of exponential growth (days)	Computed regression equation	<i>t</i>	Degrees of freedom	Relative growth rate (<i>k'</i>)	Mean doubling time (<i>G</i>) in hours
300 $\mu\text{Em}^{-2}\text{s}^{-1}$	25	Day 1	5	$y=3,72+0,16x$	14,5	4	0,16	45,13
	30	Cell numbers decline from Day 0 (decline measured over 4 days)		$y=3,55-0,14x$	5,8	3	-0,14	-66,21
150 $\mu\text{Em}^{-2}\text{s}^{-1}$	25	Day 1	5	$y=3,83+0,28x$	19,8	4	0,28	26,11
	30	Cell numbers decline from Day 0 (decline measured over 6 days)		$y=3,51-0,15x$	3,5	5	-0,15	-49,65

APPENDIX D

DIAGNOSIS OF *Pyramimonas pseudoparkeae*

(Taken from Pienaar and Aken, 1985)

Pyramimonas pseudoparkeae sp. nov.

Cells ovoid to obpyriform, flagellar end broader than posterior end. Cells taper to pointed or rounded end. Cells 14-16 μm long to 9-10 μm broad; square and lobed when seen from the anterior end. Four equal flagella emerge from flagellar pit and bend backwards over the cell. Single deeply lobed parietal chloroplast with intraplastidial eyespot. Basal pyrenoid traversed by single parallel thylakoids. Trichocysts present around flagellar pit and in longitudinal grooves. Cells swim rapidly with flagellar pole forward and rotate around their longitudinal axis. Large cells produced; size 23 μm long 17 μm wide which contain lipid and starch. These produce cysts with bilayered walls at the water/air interface. Cyst diameter 19-20 μm .

Scale covering: Scales cover the flagellar and cell surface.

Flagellar scales: 4 types. Type 1 pentagonal with raised rims and a central raised area. Size; 48 nm long axis and 42 nm short axis; Type 2 rectangular with raised rim and pronounced central spine. Found as two rows on opposite sides of flagella, size 35 nm x 24 nm; Type 3 - limuloid in shape with spine size 180 x 288 nm. Type 4 - hairscales made up of tapered head and tubular shaft. Size; length 1 μm , width 12 nm.

Body scales: 3 types, each occurring as a single complete layer surrounding the cell and situated external to the plasma-membrane. Type 1 - square with raised rim and central boss; situated closest to plasma-membrane size 35 nm x 35 nm; Type 2 large square scales with raised basket-like rim; basal region divided into quadrates with central radiating material; size - 185 nm x 172 nm x 25 nm; Type 3 - form the outer layer, coronate in shape. Size 184 nm x 128 nm.

Habitat: Marine tidal pools, on east, south and west costs of South Africa. Type material collected from Oudekraal, Cape Town on 10th November 1975 and maintained in culture at the University of Natal, Pietermaritzburg, South Africa.

Holotype. Figure 1.

