ON THE EVOLUTION OF CHLOROPLASTS FROM EUKARYOTIC ENDOSYMBIONTS: A STUDY OF *GLENODINIUM FOLIACEUM* (DINOPHYCEAE)

by

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Thesis

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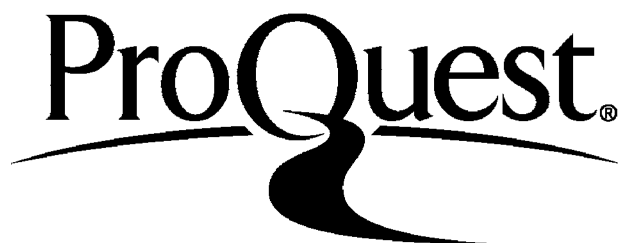

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To

My Mother and Father

ABSTRACT

A study of the marine dinoflagellate *Glenodinium foliaceum* is presented which attempts to provide information on the process of host-endosymbiont integration in a unicellular system that may be of relevance to current hypotheses on chloroplast evolution.

An experimental ultrastructural review of *G. foliaceum* supports the proposal that the chloroplasts and supernumerary nucleus belong to a morphologically reduced, endosymbiotic chromophyte. DAPI staining shows that the endosymbiont nucleus is fragmented in some cells and appears to be randomly divided by the host's cleavage furrow at cytokinesis. A quantification of the intensity of mithramycin fluorescence from single cells by flow cytometry suggests that both nuclei synthesize their DNA in synchrony.

Protocols are outlined for isolating the chloroplasts and dinoflagellate nucleus from *G. foliaceum* and a method is given for fractionating whole cell lysates to prepare chloroplast, host nuclear, and endosymbiont nuclear DNAs. It is estimated that the symbiont and dinoflagellate nuclei contain about 34 pg and 40 pg of DNA respectively. There is no evidence of amplified gene-sized DNA molecules in the symbiont nucleus. The results of preliminary reassociation experiments show only the presence of highly repeated DNA and sequences of intermediate repetitiveness in total cell DNA, which together comprise about half the genome. The chloroplast DNA is shown to have a molecular weight of approximately 103 kb by restriction endonuclease analysis but an attempt to localize the *rbc* S gene in either the chloroplast or nuclear DNAs using a cloned pea DNA probe proved unsuccessful.

Two other anomalously pigmented dinoflagellates, *Gyrodinium aureolum* and *Gymnodinium galatheanum*, are demonstrated to lack endosymbionts by electron and fluorescence microscopy, but the atypical ultrastructural or cytological characters of their chloroplasts are considered to indicate an endosymbiotic origin. The possibility that *Glenodinium foliaceum* could evolve into a similar uninucleate species is discussed in relation to the conservation of genetic information for nuclear-encoded, chloroplast proteins.

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Abbreviations

ATA	aurintricarboxylic acid
BAM	basic antibiotic mixture
BSA	bovine serum albumin
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
HAP	hydroxylapatite
kb	kilobase pair
MIC	minimum inhibitory concentration
NIC	Nomarski interference contrast
PB	sodium phosphate buffer pH 7.0
RNA	ribonucleic acid
RNase	ribonuclease
Sarkosyl	sodium dodecyl sarcosine
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
Tris	tris(hydroxymethyl)aminomethane

Maximum relative centrifugal forces are always quoted and are expressed relative to the acceleration due to gravity 'g'.

CHAPTER 1

INTRODUCTION

1.1. THE SERIAL ENDOSYMBIOSIS THEORY.

It was just over 100 years ago that Schimper (1883) first suggested that chloroplasts may have evolved from endosymbiotic cyanobacteria. Subsequently a symbiotic origin for mitochondria was also proposed (Altman 1890) and later Mereschkowsky (1910) developed these ideas in his "Theory of Symbiogenesis". However, this hypothesis was generally rejected in favour of the more traditional view that eukaryotic organelles had evolved autogeneously and gradually. Nevertheless, by the mid-1960's enough structural and biochemical information had accumulated, such as the discovery of DNA in chloroplasts and mitochondria (Ris and Plaut, 1962; Nass *et al*., 1965), for it to be considered useful to reformulate a modern endosymbiotic hypothesis (Echlin, 1966; Sagan, 1967; Margulis, 1970). During the 1970's the hypothesis began to win wide acceptance so that by the time Margulis published her second synthesis on the processes and mechanisms of cell evolution (Margulis, 1981), the concept of endosymbiosis had become the new tradition rather than the radical alternative.

The Serial Endosymbiosis Theory (Taylor, 1974) basically proposes that some eukaryotic cell organelles primarily originated through sequential endosymbiotic associations with different prokaryotes. The support for this hypothesis has come from two

main sources: the study of modern intracellular symbioses has provided models that illustrate possible evolutionary events (Whatley *et al.*, 1979; Margulis, 1981); and the development of rapid protein and nucleic acid sequencing techniques has enabled molecular phylogenies to be generated and these have emphasized the prokaryotic nature of mitochondria and chloroplasts (Buetow, 1976; Dayhoff and Schwartz, 1981; George *et al.*, 1983; Küntzel *et al.*, 1983; Spencer *et al.*, 1983; Wolters and Erdmann, 1984). However, none of this evidence is unequivocal (Uzzell and Spolsky, 1981; Gray and Doolittle, 1982) particularly in the case of mitochondria (Cavalier-Smith, 1983), and it can be argued that the molecular data equally supports autogenous hypotheses (e.g. Raff and Mahler, 1972; Uzzell and Spolsky, 1974; Cavalier-Smith, 1981a; Keynani, 1983). Nevertheless the endosymbiotic hypothesis provides a firm foundation on which to base current ideas about the organization and functioning of the eukaryotic cell (Sitte, 1983; Whatley and Whatley, 1984).

1.2. CHLOROPLAST ORIGINS AND EVOLUTION.

1.2.1. Hypotheses on the origin and evolution of chloroplasts.

Amongst the organelles for which an endosymbiotic origin has been proposed, the evidence supporting such an origin for chloroplasts is the most persuasive (Cavalier-Smith, 1981). The diversity of chloroplast types strongly suggests that at least some were acquired endosymbiotically (Gray and Doolittle, 1982), although there is little agreement on the precise details of the endosymbiotic events which might have generated this diversity and numerous theories have been proposed (e.g. Raven, 1970; Lee 1972; 1977; Gibbs, 1978; 1981a; Dodge, 1979; Whatley *et al.*,

1979; Whatley and Whatley, 1981; Cavalier-Smith, 1982b; McQuade, 1983).

The double-membrane bound chloroplasts of green algae, higher plants and red algae are generally regarded as the direct result of a primary endosymbiotic association with a photosynthetic prokaryote: the inner chloroplast membrane being thought to be derived from the symbiont's plasma membrane, and the outer chloroplast membrane is assumed to be homologous with a phagosome membrane. However, Cavalier-Smith (1982b) has argued that the uptake of a gram-negative prokaryote would produce a plastid with three enveloping membranes, as in dinoflagellates and euglenoids, and that the third, middle membrane corresponds to the 'outer membrane' of the symbiont's wall. To account for the variety of accessory pigments present in algal chloroplasts it has been assumed that these either evolved in the ancestral free-living prokaryotes before endosymbiosis, and therefore a number of primary endosymbiotic events are thought to have occurred (Raven, 1970); or during the conversion of a cyanophyte-like prokaryote into a chloroplast after a single endosymbiosis (Cavalier-Smith, 1982b; McQuade, 1983); or a combination of these two alternatives (Dodge, 1979; Whatley and Whatley, 1981).

At the time Raven (1970) proposed his scheme for the multiple origin of chloroplasts, the only chlorophyll a containing prokaryotes known were cyanobacteria, which are similar to red algal chloroplasts in having phycobilins as accessory pigments. Subsequently, the discovery of Prochloron, a prokaryote possessing both the "green plant" chlorophylls a and b (Lewin, 1976; 1983), lent support to the idea of multiple symbioses, although Prochloron could well be more closely related to cyanobacteria than to green plant chloroplasts (van Valen, 1982). The recent report (Margulis, 1985) of a novel bacterial chlorophyll in Heliobacterium, which is very similar to chlorophyll c, the accessory pigment in all other algae, could further suggest that

the pigmentation of photosynthetic prokaryotes might have once paralleled that of present-day chloroplasts. However, the resolution of the multiple origin versus a single origin conflict can only come from more detailed and extensive molecular comparisons.

With the exception of dinoflagellates, all algae which contain chlorophyll c possess chloroplasts that are surrounded by four membranes: two chloroplast envelope membranes and two membranes of chloroplast endoplasmic reticulum (ER) (Gibbs, 1981b). Following the discovery in cryptophytes of a small, nucleus-like body (the nucleomorph) in the cytoplasm between these two sets of membranes (Greenwood, 1974), it was suggested that the chloroplasts of both cryptophytes and chromophytes (*sensu* Cavalier-Smith, 1981b) were derived from reduced eukaryotic endosymbionts (Greenwood *et al.*, 1977; Lee, 1977). The idea has subsequently been elaborated by other authors (Dodge, 1979; Whatley *et al.*, 1979; Gillot and Gibbs, 1980; Gibbs, 1981a; Whatley and Whatley, 1981; Cavalier-Smith, 1982b; McQuade, 1983) and strengthened by the recent demonstration of DNA in the cryptophyte nucleomorph (Ludwig and Gibbs, 1985). Also, a freeze-fracture study of chloroplast ER has shown that the membranes have the polarity expected if the inner and outer chloroplast ER membranes were derived, respectively, from the plasmalemma of the eukaryotic symbiont and the phagosome membrane of the host, as the hypothesis predicts (Lefort-Tran, 1983). Clearly if the chloroplasts of cryptophytes and chromophytes did originate from endosymbiotic associations with eukaryotes, then the symbiont has undergone extensive reductional integration at both the morphological and genetic level.

Gibbs has further argued that the three-membrane bound chloroplasts of euglenophytes and dinoflagellates could also have evolved from endosymbiotic eukaryotes by the loss of the outermost vacuolar membrane (Gibbs, 1978; 1981a). However, Whatley favours a scheme in which these two groups acquired their chloro-

plasts via the capture of isolated organelles (Whatley *et al.*, 1979, Whatley and Whatley, 1981) and, as explained earlier, Cavalier-Smith (1982b) considers that three-membraned chloroplasts were produced directly from the primary endosymbiosis with a prokaryote.

Thus, whilst it may never be possible to experimentally determine the precise ancestry of chloroplasts, the process of host-symbiont integration in living associations can be studied to gain some information on how, rather than from what, chloroplasts evolved. It is with this aspect of chloroplast evolution that the present thesis is concerned.

1.2.2. Modern cryptic endosymbioses and *Glenodinium foliaceum*.

There are numerous examples of intracellular symbioses (see Margulis, 1976; 1981; Jeon, 1983). In a few of these the process of host-symbiont integration has proceded to the extent that the exact identity of the symbiont is no longer readily apparent. These can be loosely termed "cryptic endosymbioses" and fortunately some such associations have been discovered which possibly illustrate intermediate stages in the evolution of chloroplast from both prokaryotic and eukaryotic endosymbionts.

The cryptic prokaryotic endosymbionts termed cyanelles have been extensively investigated (see Trench, 1982). They are found in several protists of uncertain taxonomic affinity and are regarded as being endosymbiotic cyanobacteria on the basis of ultrastructural and biochemical similarities. The cyanelles of *Cyanophora paradoxa* possess a reduced peptidoglycan wall, confirming their cyanobacterial nature, but no wall has been demonstrated in *Glaucocystis nostochinearum* or *Cyanidium caldarium* and these cyanelles could be considered as *de facto* red algal chloroplasts (Seckbach *et al.*, 1983). More significantly

than this morphological reduction is the genetic integration of the cyanelles with their hosts. The cyanelles of C. paradoxa, for example, have only 10% of the genetic capacity of cyanobacteria (Jaynes and Vernon, 1982). Therefore, cyanelles appear to demonstrate that chloroplast-like organelles can originate from endosymbioses with prokaryotes.

Cryptic eukaryotic endosymbionts have been studied in somewhat less detail. They have only been detected in two groups of protists; ciliates and dinoflagellates. The ciliate Mesodinium rubrum contains numerous cryptophyte-like chloroplasts, each associated with a nucleomorph and mitochondria, in ribosome-dense cytoplasmic compartments delimited by a single membrane (Taylor et al., 1969; 1971; Hibberd, 1977; Oakley and Taylor, 1978). In some populations of the organism, these "chloroplast-mitochondrial complexes" are interconnected and joined to a cytoplasmic unit containing a nucleus (Hibberd, 1977), but in other populations, some or all of the chloroplast complexes exist as discrete entities separate from the supernumerary nucleus (Oakley and Taylor, 1978). The interpretation of this ultrastructural data is that M. rubrum once captured a cryptophyte which subsequently proliferated and became fragmented in its intracellular environment. The host phagosome membrane is assumed to have been lost during this process so that the symbiont now lies free in the cytoplasm.

A similar endosymbiotic cryptophyte has been found in the dinoflagellate Gymnodinium acidotum (Wilcox and Wedemayer, 1984) and two other dinoflagellate, Peridinium balticum and Glenodinium foliaceum, are also thought to possess cryptic eukaryotic endosymbionts but with chromophyte affinities (Tomas and Cox, 1973; Jeffrey and Vesk, 1976). Dinoflagellates are a large and diverse group of predominantly unicellular protists containing both photosynthetic and heterotrophic species (Taylor, 1980; Loeblich, 1982; 1984; Dodge, 1983b). They seem particularly adept at

forming symbiotic associations and casual reports of unusually pigmented forms (see Dodge, 1983b) may indicate that many more than the three species mentioned above harbour autotrophic endosymbionts. A study of these anomalously pigmented dinoflagellates could therefore offer insights into various aspects of chloroplast evolution, in particular the process of partner integration in those species, such as _G. foliaceum_, that are known to contain eukaryotic endosymbionts.

Glenodinium foliaceum is a brackish water dinoflagellate that frequently occurs in large numbers around the British Isles (Dodge, 1982; Pybus _et al_., 1984). Unlike _Gymnodinium acidotum_ and the ciliate _Mesodinium rubrum_, it can readily be cultured in the laboratory. Thus, a detailed study of _G. foliaceum_ and its cryptic endosymbiont was undertaken in an attempt to obtain some experimental data relevant to hypotheses on the evolution of chloroplasts from eukaryotic endosymbionts.

CHAPTER 2

MATERIALS AND EXPERIMENTAL METHODS

2.1. ALGAE AND CULTURE CONDITIONS.

2.1.1. Algae.

Glenodinium foliaceum Stein (synonyms: *Peridinium foliaceum* Biecheler, *Kryptoperidinium foliaceum* Lindemann) was a gift from Dr. David Sigee and was also obtained from the Culture Collection of Algae and Protozoa, Cambridge. Sigee's culture was subsequently purified for biochemical work (see Appendix) since it appeared more vigorous. However, ultrastructural observations were made on both. Other algae used during the course of this study are listed together with their source and culture media in Table 2.1.

2.1.2. Culture media and conditions.

The composition of the culture media used are outlined in Table 2.2. A totally synthetic marine medium modified from ASP_7 (Provasoli, 1964) was chosen for the culture of *Glenodinium foliaceum* since, unlike media based on natural seawater, it is completely stable to repeated autoclaving and does not favour bacterial growth. Other marine cultures were best maintained in

Table 2.1. Sources of algae and culture media used.

Organism	Source & Strain	Culture medium
Asterionella formosa Hass.	FBA L.313	not cultured
Emiliania huxleyi (Lohm.) Hay & Mohler	MBA 92d	E-S +vits
Glenodinium foliaceum Stein	CCAP 1116/3	ASP_7 or E-S
	D. Sigee	ASP_7
Gonyaulax polyedra Stein	not known	E-S
G. tamarensis Lebour	MBA 173	E-S
Gymnodinium sp.	UTEX	E-S
Gymnodinium galatheanum Tangen	K. Tangen KT76E	E-S +vits
G. micrum (Leadb. & Dodge) Loeb.III	MBA 207	E-S
Gyrodinium aureolum Hulburt	MBA Tangen's strain	E-S +vits
Prorocentrum micans Ehrenberg	MBA 97a	E-S
Scrippsiella trochoidea (Stein) Loeblich III	MBA 104	E-S
Stephanodiscus hantzchii Grun.	FBA L382/A-C	not cultured
Woloszynskia coronata (Woloszynskia) Thompson	CCAP 1117/2	Bristol's Soln.

CCAP, Culture Collection of Algae and Protozoa, Cambridge; MBA, Marine Biological Association Algal Culture Collection, Plymouth; FBA, Freshwater Biological Association Algal Culture Collection, Windermere; UTEX, University of Texas Culture Collection of Algae; E-S, Erd-Schreiber; +vits, supplemented with ASP_7 vitamins.

Table 2.2. Composition of algal growth media.

Component	Amounts per litre		
	ASP_7[a]	Erd-Schreiber[b]	Bristol's Soln.[c]
seawater[d]	-	1 l	-
NaCl	25 g	-	25 mg
$MgSO_4.7H_2O$	9 g	-	75 mg
KCl	700 mg	-	-
$CaCl_2$[e]	300 mg	-	25 mg
$NaNO_3$	50 mg	200 mg	250 mg
$Na_2HPO_4.12H_2O$	-	20 mg	-
K_2HPO_4	-	-	75 mg
KH_2PO_4	-	-	175 mg
Na_2-glycerophosphate	20 mg	-	-
nitrilotriacetic acid	70 mg	-	-
Na_2EDTA	30 mg	-	30 mg
$FeCl_3$ anhydrous	300 µg	-	300 ug
H_3BO_3	6 mg	-	6 mg
$MnCl_2.4H_2O$	1400 µg	-	1400 µg
$ZnCl_2$	110 µg	-	110 µg
$CoCl_2.6H_2O$	40 µg	-	40 µg
vitamin B_{12}	1 µg	(1 µg)	1 µg
thiamine-HCl	500 µg	(500 µg)	500 µg
biotin	1 µg	(1 µg)	1 µg
soil extract[f]	-	50 ml	-
Tris-HCl	1 g	-	-
pH	7.9	8.0	6.8

Notes:
a. modified from Provasoli (1964).
b. Föyn (1935).
c. adapted from Trelease and Trelease (1935).
d. 95 % (v/v) filtered seawater.
e. added by disolving the correct amount of $CaCO_3$ in HCl.
f. 500 $g.l^{-1}$ soil in tap water autoclaved at 5 p.s.i. for 1 h and cleared by centrifugation.

a natural seawater medium, Erd-Schreiber, as stringent aseptic precautions were not essential. Attempts were made to culture Gyrodinium aureolum and Gymnodinium galatheanum in ASP_7 but neither species could be maintained for more than one subculture in this medium.

The chemicals used in making up the culture media were of analytical grade, however, for cheapness, laboratory grade sodium chloride was substituted in 3 l and 10 l batch cultures of Glenodinium foliaceum. ASP_7 and Bristol's Solution were sterilized by autoclaving in the culture vessel whilst Erd-Schreiber was autoclaved in its constituent parts and mixed when cold so as to prevent precipitates forming. Autoclaving was performed at a pressure of 15 p.s.i. for 20 min, or 15 min per litre if the culture volume was greater than 1 litre. The medium was allowed to equilibriate at the culture temperature before subculturing was performed; at least 15 h was allowed for this to occur in the case of 10 l carboys.

All cultures, except stock cultures of G. foliaceum, were maintained at $20 \pm 2^{o}C$ under continuous fluorescent lighting (providing 6.0 $W.m^{-2}$ photosynthetically available radiation) on open culture benches. Generally the algae were grown in 75-100 ml of medium in 150 ml conical flasks stoppered with non-absorbant cotton wool. The cultures were swirled periodically to keep the algae in suspension and subculturing was performed once a month, or as necessary.

2.1.3. Maintainance and batch culturing of purified Glenodinium foliaceum.

Stock cultures of purified Glenodinium foliaceum were stored in 250 ml screw-capped Erlenmeyer flasks since the alga could not be continuously maintained in culture tubes. These were kept in

an enclosed cabinet at $18 \pm 2^{o}C$ under continuous fluorescent lighting (light intensity $5.5 \ W \ m^{-1}$). The stock culture was divided amongst five similar flasks, containing 100 ml of medium, every 2.5 - 3 weeks. Four of these cultures were used to initiate batch cultures for experimental work whilst the fifth preserved the stock line. The stock was treated with antibiotics 24 h before every other subculture by adding a filter sterilized antibiotic solution directly to the culture. This antibiotic mixture contained 50 mg penicillin, 20 mg ampicillin, 10 mg cephalosporine C and 5 mg each of streptomycin and kanamycin dissolved in about 10 ml of ASP_7.

To obtain large volume batch cultures a four stage succession was used in which each culture, having attained a sufficient cell density, was poured aseptically into four to five volumes of fresh medium to produce the next stage in the succession. Accordingly, the four subcultures of the stock were each added to 500 ml of medium in 1 l Erlenmeyer flasks which were then transferred to 2 or 3 l of ASP_7 in 5 or 6 l flasks. The succession was either harvested at this stage or continued into 10 l carboys. All the flasks were swirled every day to keep the algae in suspension and a magnetic follower was included in the 10 l carboys so as to enable these cultures to be stirred periodically. By using this 1 + 5 subculturing regime, the cultures could be maintained in exponential growth throughout the succession and greater final cell densities could be obtained in the large volume cultures than with smaller inoculum sizes. Even so, final densities in the 10 l carboys rarely exceded 10^4 cells ml^{-1} and this could not be increased by aerating the cultures with air. In fact, the final cell densities of aerated cultures were less than in cultures left standing with no agitation.

2.2. CELL COUNTING AND ANALYSIS TECHNIQUES.

2.2.1. Visual methods.

A Sedgewick Rafter counting chamber was used to determine culture densities and the following procedures were strictly adhered to (McAlice, 1971).

To obtain relative counts or estimations of cell densities, the culture was first mixed to homogeneity by ten inversions of the flask or by vigorously stirring large volume cultures. A 1 ml sample was then quickly withdrawn and pipetted into an Eppendorf tube containing a drop of 40% (v/v) formaldehyde. Samples could be stored in this condition. The Sedgewick Rafter was filled with the cover glass in position but slightly displaced so that the sample, which had been well mixed, could be quickly but steadily introduced from one corner using a Pasteur pipette. After allowing the cells to settle for about 2 min, every other row of the chamber was counted using a constant boundary convention. The row counts obtained by this procedure were acceptably homogeneous (greater than 95% probability of the variation being random) providing the number of cells per row was between 50 and 150. Consequently it was essential to adjust the density of the fixed sample to about 2×10^3 cells ml^{-1} before counting.

Other factors affecting the homogeneity of the counts were found to include the nature of the fixative used, the method of filling the Rafter and the counting technique. If iodine was employed as a fixative/stain, then the cells became so dense that it proved impossible to fill the Rafter evenly. Similarly, if 1 ml of the sample was accurately pipetted into the chamber before placing the cover glass in position, then the distribution of the cells tended to become non-random. However, even with the

fixative and filling method employed, the distribution of the cells was not entirely satisfactory since column counts were not homogeneous due to the cells settling out quickly.

The same procedure was employed to determine accurate cell densities except that larger volume samples were withdrawn so as to minimize the density change caused by the drop of fixative and more than one (usually three) samples were counted. Between sample variation was generally insignificant ($p > 0.95$) providing the culture had been mixed by inverting the flask several times rather than by swirling.

To estimate the frequency of dividing cells in a culture, the number of such cells in the first 1000 cells encountered was used. A Sedgewick Rafter was filled in the manner described above in order to do this.

2.2.2. Automatic flow methods.

(i) Electrical (Coulter) impedance monitering:

Samples of culture to be counted were adjusted to about 2×10^3 cells ml^{-1} and the number of particles in a volume of 0.5 ml was determined with a Coulter Counter Model ZB (Coulter Electronics Ltd.) using an aperture diameter (upper threshold) of 200 μm and a range of lower threshold values. The sample was counted three times at each threshold value, disregarding anomalous readings due to clogging of the aperture.

Using the number of lower threshold channels available, *Glenodinium foliaceum* cells did not constitute a size class distinctly larger than that of other particulate matter (i.e. discarded thecae) in the culture and therefore the Coulter Coun-

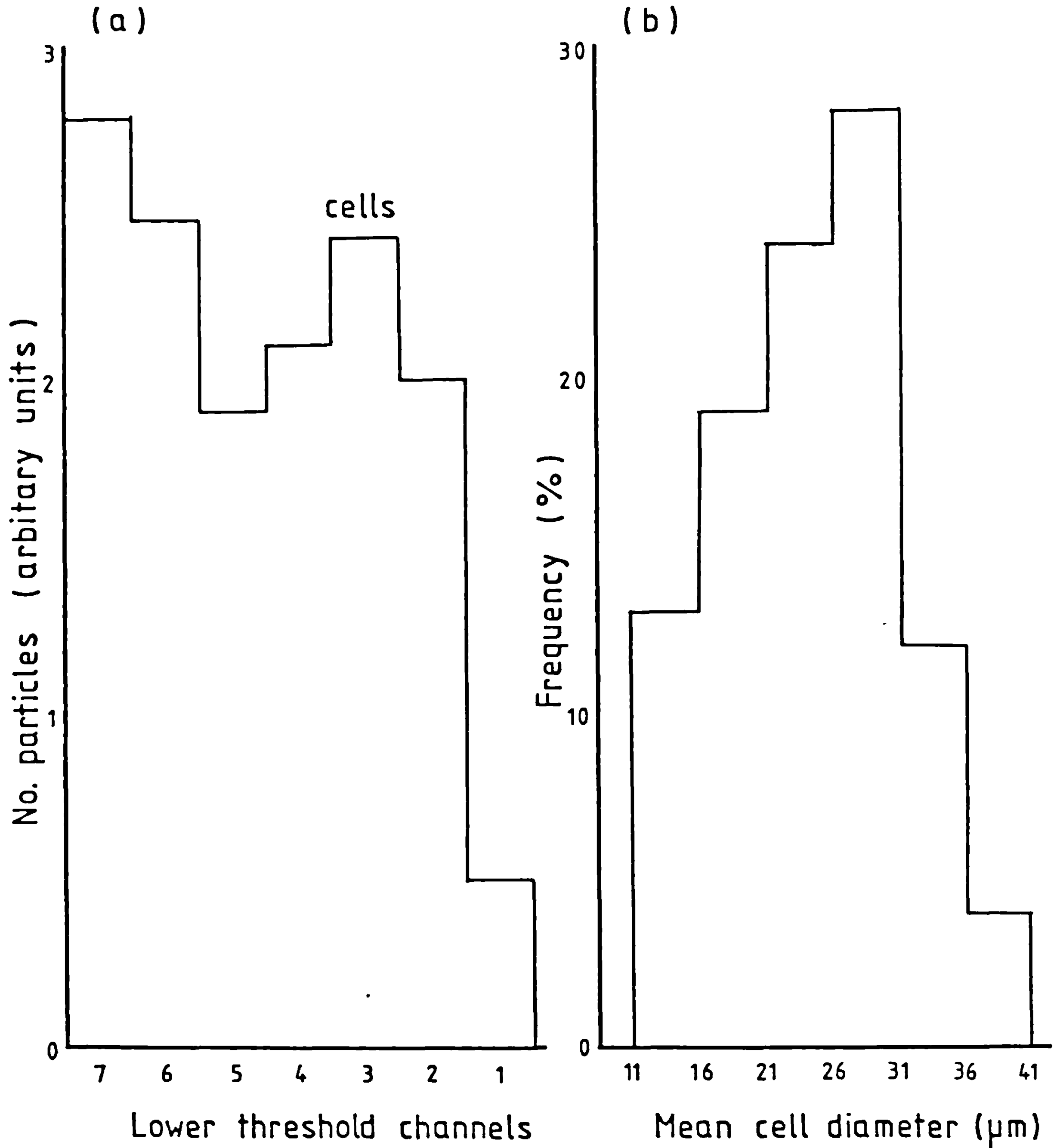


Figure 2.1. Distribution of cell sizes in a culture of _Glenodinium foliaceum_.

a. Frequency distribution of particles recorded by the Coulter Counter. The peak of larger particles (smaller channel numbers) is due to algal cells.

b. Distribution of mean cell diameters (ranked equally) measured microscopically from the same culture as in (a)

ter could not be used to provide accurate, absolute values of cell density. However, the number of cells over a given size could be estimated by setting the lower threshold value of the Coulter Counter to the peak frequency of large particles in the culture, which were due to algal cells (Figure 2.1). Thus the instrument could be used to monitor cell division by detecting changes in the number of large cells over a given period of time.

The Coulter Counter was crudely calibrated by comparing this large particle peak with the frequency distribution of mean cell diameters of the same culture, as determined from microscopic measurements of 200 cells sampled at random (Figure 2.1). No correspondance could be found with the distribution of cell volumes, assuming the cells to be spherical.

(ii) Fluorescence monitoring:

A flow cytometer (Becton Dickinson & Co., FACS 420) was used to rapidly analyse the fluorescence of individual mithramycin-stained cells. The cells were prepared and stained as described in Section 2.3.2(i) and analysed in the stain solution at a concentration of about 10^6 cells ml^{-1}. The flow cytometer was fitted with a 80 µm flow orifice which provided reliable data on cells up to 40 µm in diameter. The mithramycin-DNA complex was excited using the 458 nm band of a 200 mW argon laser and the fluorescence was detected at 90° to the laser beam by a red sensitive photomultiplyer tube using a 560 nm long pass barrier filter. Forward scattered light was also monitored to provide information on cell size. The data were collected and automatically analysed by the instrument and transferred to a computer for storage on a floppy disc. Unstained cells were also analysed and acted as controls.

2.3. LIGHT MICROSCOPY.

2.3.1. Bright field, phase contrast and Nomarski interference contrast microscopy.

An Olympus BH-2 microscope equipped with phase contrast and Nomarski interference contrast (NIC) optics was used for all non-fluorescence photomicrography. Photographs were taken on Ilford FP 4 film using either the Olympus S-Plan 0.46 N.A. x20, 0.70 N.A. x40 or 1.25 N.A. x100 (oil) objectives.

(i) Acetocarmine staining (Dodge, 1963):

Cells were collected by slow speed centrifugation and fixed in several changes of ice cold ethanol:acetic acid (3:1 v/v) for 2 - 4 h. After washing with distilled water, the cells were placed in a drop of acetocarmine and warmed gently over a spirit lamp. Excess stain was removed with 45% (v/v) acetic acid and the preparations were photographed under NIC using a green filter.

(ii) Methyl green/pyronine B staining (Taft, 1951):

The methyl green/pyronine B stain combination (Unna's stain) was used to examine nuclei in cell homogenates. In this stain DNA-containing structures (i.e. nuclei) appear blue, whilst RNA-containing ones (the cytoplasm in general) are coloured red.

The homogenate was simply mixed with a small amount of stain solution which had been prepared by dissolving 0.5 g of methyl green and 0.2 g of pyronine B in 100 ml of 0.1 M acetate buffer pH 4.4. Before adding the pyronine B the methyl green solution was repeatedly extracted with chloroform to remove contaminating methyl violet.

2.3.2. Fluorescence microscopy.

Observations on preparations stained with DNA-specific fluorochromes were made on a Leitz Dialux 20 EB microscope equipped with a Ploemopak fluorescence illuminator which provided incident light excitation of the specimen. The illuminator was fitted with a 50 W high pressure mercury vapour lamp and the Leitz filter blocks A or H2 depending on the fluorochrome used (Table 2.3). Photographs were taken on Ilford XP1 or Ektachrome 160 film using either the Leitz NPL Fluotar 0.70 N.A. x40 or 1.32 N.A. x100 (oil) objectives. By means of an England Finder (Graticules Ltd.), the area of the specimen photographed under fluorescence could be relocated on the Olympus BH-2 microscope to provide correlated fluorescence and NIC micrographs.

Table 2.3. Details of DNA-specific fluorochromes and filter blocks used in fluorescence observations (Ahrberg and Schweizer, 1984).

Flurochrome:	DAPI	mithramycin
base specificity	A-T	G-C
absorption maximum	355 nm	430 nm
fluorescence maximum	450 nm	580 nm
Leitz filter block:	A	H2
excitation filter	340-380 nm BP	390-490 nm BP
suppression filter	430 nm LP	515 nm LP

BP, band pass filter; LP, long pass filter

(i) Mithramycin staining:

Cells were given 30 min washes with ice cold ethanol:acetic acid (3:1 v/v) until most of the pigment had been extracted and then left in the cold fixative overnight. The cells were then resuspended in the stain buffer (150 mM NaCl, 15 mM $MgCl_2$, 10 mM Tris-HCl pH 7.0) and exposed to fluorescent lighting at room temperature for at least 15 h to bleach any residual chlorophyll by photo-oxidation (Yentch _et al._, 1983). The bleached cells were stained with mithramycin (Sigma) at a concentration of 50 $\mu g\ ml^{-1}$ for 1 h in the dark.

(ii) DAPI staining:

4',6-diamidino-2-phenylindole (DAPI) was purchased from Boehringer Mannheim and stored as a 1 $mg\ ml^{-1}$ stock at 4^oC.

To observe nuclei, the cells were collected by slow speed centrifugation and fixed either with ethanol:acetic acid as described above or with 2% (v/v) glutaraldehyde, 0.125 M Sörensen's phosphate buffer pH 7.8 for 30 min. The cells were then stained with 0.5 $\mu g\ ml^{-1}$ DAPI in McIlvaine's citrate-phosphate buffer pH 4.0 for 1 h in the dark, or longer if necessary. The clarity of staining could be greatly improved by slightly squashing the cells under the coverslip.

To visualize the DNA in chloroplasts, a simple squashing technique was devised. Cells were resuspended in a small volume of 0.8 M sorbitol, 5 mM $MgCl_2$, 0.1% (w/v) bovine serum albumin (BSA), 1 mM mercaptoethanol, 1 mM EDTA, 10 mM Tris-HCl pH 7.5 and

a drop of the cell suspension was placed on a slide alongside a drop of the buffer which lacked BSA but contained 4% (v/v) glutaraldehyde and 1 $\mu g \cdot ml^{-1}$ DAPI. The drops were quickly mixed and the cells were immediately squashed under a coverslip. The coverslip was then sealed and the preparation was left to stain for 30 min in the dark as before. (Note: for preparations of freshwater algae, the sorbitol concentration was reduced to 0.33 M).

Some squash preparations were subsequently digested with either DNase or RNase to confirm the specificity of DAPI for DNA. Alternatively, digestions were performed on unstained squashes prepared as described above by omitting DAPI from the drop of fixative solution. Enzymatic treatments were carried out as follows.

The coverslip was carefully removed and the slide was gently flooded with ethanol:acetic acid (3:1 v/v) for 30 min and then allowed to dry. The preparations were then washed with several changes of buffer over a period of 1.5 - 2 h to remove all traces of the fixatives. Slides to be digested with DNase were washed with 5 mM $MgCl_2$, 20 mM sodium citrate pH 6.0, whilst RNase treatments were washed with 2 mM sodium acetate pH 5.5. Digestions were carried out at $35^{\circ}C$ for 3 h using 350 units ml^{-1} bovine pancreatic DNase (Sigma, Type III) or 45 units ml^{-1} bovine pancreatic RNase (Sigma, Type XII-A) dissolved in the appropriate buffer. Controls lacking enzyme were run in parallel. The preparations were stained or restained in 0.5 $\mu g \cdot ml^{-1}$ DAPI in McIlvaine's buffer pH 4.0 after a brief rinse in buffer.

2.4. TRANSMISSION ELECTRON MICROSCOPY.

2.4.1. General methods for ultrastructural studies.

Cells to be prepared for electron microscopy were collected from about 5 ml of exponentially growing culture and resuspended in a similar volume of fixative. Details of the specific fixatives and buffers used are given in Section 2.4.2., but all fixations were performed at room temperature. After fixation, the cells were pelleted by brief, gentle centrifugation using an MSE "Minor" bench-top centrifuge, and transferred via the washing stages to 1.5 ml Eppendorf tubes. All subsequent processing was performed in these tubes using an MSE " Micro Centaur" micro-centrifuge set to "low" (unspecified RCF). Following post-fixation the cells were washed twice for 5-10 min in distilled water then dehydrated through an acetone series: 15 min in each of 20%, 50%, 75%, 90% and 100% (v/v) acetone, followed by two 30 min changes of 100% acetone. Generally the cells were then passed through two changes of propylene oxide to ensure that the material was completely dehydrated before being embedded in resin.

Spurr's resin (Spurr, 1969) was routinely used for embedding, however, for *Gyrodinium aureolum*, VCD/HXSA ultra-low viscosity resin (Oliveira *et al*., 1983) was employed. Infiltration was achieved by suspending the cells in a 1:4 (v/v) resin: propylene oxide mixture and allowing the solvent to slowly evaporate off overnight. The resin was then changed every 10 - 14 h for the next two days. With VCD/HXSA resin, acceptable and rapid infiltration could also be obtained via a resin series using two-hourly changes of 1:3, 1:1, 3:1 (v/v) resin/propylene oxide and then three changes of resin, including one overnight change. The infiltrated cells were embedded in Beem capsules (Agar Aids, size 3) such that the cells formed an even layer over

the sectioning face. This was achieved by pipetting the cell pellet into the base of the capsule and, if necessary, centrifuging the capsule in an Eppendorf tube after filling with resin. The resin was polymerized at 70°C for three days (for Spurr's) or at 60°C for 12 h (for VCD/HXSA).

Ultrathin sections (60-100 nm thick) were cut from the blocks with glass knives on a LKB ultramicrotome ("Ultrotome III") by standard methods. The sections were collected on uncoated 400 mesh, hexagonal copper grids (Gilder Grids, G400HH) and stained in 2% (w/v) aqueous uranyl acetate for 15 min at 60°C and then Reynold's lead citrate (Reynold, 1963) for 5-15 min at room temperature. Some preparations were stained _en bloc_ by incubating the fixed cells in 0.5% (w/v) aqueous uranyl acetate for 30 min before dehydration, in which case the sections were only contrasted with lead citrate

The stained sections were examined using either a Zeiss EM9a or a Zeiss EM 109 electron microscope at 60kV or 80kv respectively.

2.4.2. Specific fixation schedules.

Working strength solutions of glutaraldehyde (Agar Aids, EM grade) were always freshly prepared from ampules of 25% (v/v) stock. Crystals of osmium tetroxide (Agar Aids) were allowed to dissolve in buffer for 12 h before use and solutions were kept for up to one week. The osmolarities of fixative solutions were measured using an automatic micro-osmometer (Roebling).

(i) _Glenodinium foliaceum_:

This species has been successfully fixed by a double fixation schedule (Dodge and Crawford, 1969), however, here it was

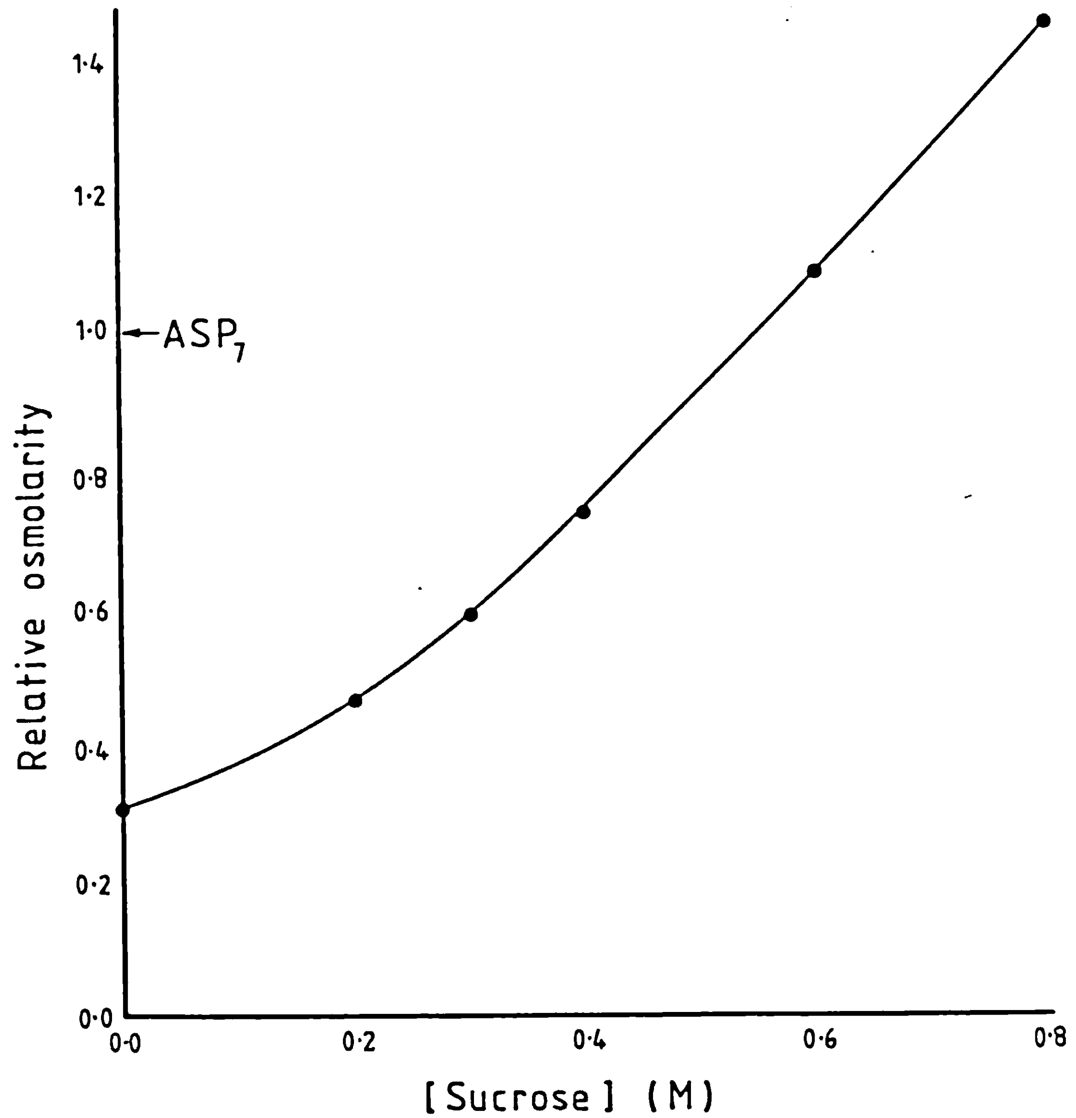


Figure 2.2. Relative osmolarity of sucrose dissolved in 0.125 M Sörensen's phosphate buffer. The culture medium ASP_7 is defined as having a relative osmolarity of 1 (880 mOs).

found that although the internal structures of the cell were well preserved by this method, the cells were always spherical and the theca and flagella were generally absent. Since cells remain osmotically active after glutaraldehyde fixation (Bullock, 1984) it was decided to see if a better general preservation could be obtained by altering the fixative osmolarity. Cells were fixed with 4% (v/v) glutaraldehyde in 0.125 M Sörensen's phosphate pH 7.8 containing 0 - 0.8 M sucrose for 1 h, and post-fixed in 2% (w/v) OsO_4 in Sörensen's buffer. The best preservation was observed with a 0.3 M sucrose treatment, which was that used by Dodge and Crawford (1969). This supports the recommendation of Bone and Denton (1971) that optimal fixation with aldehyde solutions is achieved using a fixative vehicle whose osmolarity is 60% that of the medium in which the cells are in equilibrium with in life (see Figure 2.2). However, in all treatments rounding off of the cells was evident. Cells fixed in only OsO_4 showed similar characteristics even though this fixative destroys the semi-permeable character of living cell membranes (Bone and Ryan, 1972).

A solution to this problem was suggested by concurrent experiments to purify Glenodinium foliaceum (see Appendix). It was noted that the minimum centrifugation conditions necessary to pellet the cells also caused them to become non-motile even though they remained viable, whereas cells sedimented under gravity onto a membrane filter (Nuclepore, pore size 8 μm) appeared unaltered. Using the latter method to harvest a culture and washing the cells off the filter with "isosmotic" fixative, the majority of the cells were found to retain their shape, theca and often flagella as well. Once fixed, the cells could be safely collected by centrifugation for further processing as detailed below.

Fixation schedule for <u>Glenodinium foliaceum</u>.

Cell collection:	Sedimentation onto a 8 μm Nuclepore filter
Fixation:	4% (v/v) glutaraldehyde 0.3 M sucrose 0.125 M Sörensen's phosphate pH 7.8 980 mOs 30 min
Intermediate washes:	0.3 M sucrose 0.125 M Sörensen's phosphate pH 7.8 520 mOs 15 min
	0.125 M Sörensen's phosphate pH 7.8 270 mOs 15 min
Post fixation:	2% (w/v) OsO_4 0.125 M Sörensen's phosphate pH 7.8 320 mOs 1 h

(ii) <u>Gyrodinium aureolum</u>:

There had been no published ultrastructural observations on <u>Gyrodinium aureolum</u>, even though this alga was readily available in culture, presumably because the organism is extremely difficult to fix. This proved to be the case since none of the following fixation techniques gave any cytoplasmic preservation and often the cells simply disintegrated during processing:

a. Standard double fixation as described for Glenodinium foliaceum.

b. Fixation as in (a) but varying the sucrose concentration in the buffered glutaraldehyde.

c. Double fixation using ASP_7 to buffer both the glutaraldehyde and OsO_4.

d. "In situ" fixation by adding concentrated glutaraldehyde directly to a culture and then post-fixing in OsO_4.

e. "In situ" fixation with OsO_4 only.

(Note: since Sörensen's phosphate buffer precipitates in Erd-Schreiber culture medium, the alga was either sub-cultured into ASP_7 about a week before the fixation attempt so that the cells could be collected by filtration, or the cells were pelleted by centrifugation which minimized the carry-over of medium. The former procedure was subsequently abandoned for two reasons: long term culture of G. aureulum in ASP_7 proved impossible and therefore subculturing into this medium may have been aggravating the fixation difficulties; and cultures of the alga quickly clogged the Nuclepore filters rendering this method of cell collection unsatisfactory)

Problems encountered with fixing related gymnodinoid dinoflagellates have been overcome by treating the cells simultaneously with glutaraldehyde and OsO_4 (Wilcox et al., 1982). This method gave promising results with Gyrodinium aureolum using a fixative vehicle having an osmolarity of 540 mOs, but severe osmotic disruption of the cytoplasm was still evident. This

damage could be reduced by increasing the fixative osmolarity and eventually the best preservation was obtained by using a fixative vehicle whose osmolarity equalled that of the culture medium (i.e. about 960 mOs). Infiltrating the fixed cells with VCD/HXSA resin also helped to minimize polymerization damage of the vesiculate cytoplasm (Oliveira *et al.*, 1983). Details of this fixation are given below:

Fixation schedule for *Gyrodinium aureolum*.

Cell collection:	Minimal centrifugation.
Fixation:	2% (v/v) glutaraldehyde
	1% (w/v) OsO_4
	0.38 M NaCl
	0.125 M Sörensen's phosphate pH 7.8
	1170 mOs
	15 min
Intermediate washes:	0.38 M NaCl
	0.125 M Sörensen's phosphate pH 7.8
	960 mOs
	15 min (x2)
Post fixation:	1% (w/v) OsO_4
	0.38 M NaCl
	0.125 M Sörensen's phosphate pH 7.8
	970 mOs
	1 h

(iii) *Gymnodinium galatheanum*:

This species was prepared by the standard double fixation schedule described for *Glenodinium foliaceum* and the simultaneous fixation method used for *Gyrodinium aureolum*. The alga was preserved well by both methods. Cells were collected by gentle centrifugation.

(iv) Chloroplasts:

Chloroplasts isolated from *Glenodinium foliaceum* were fixed by a standard double fixation buffered throughout with 0.125 M Sörensen's phosphate pH7.8 adjusted to an osmolarity of 1,200 mOs with sodium chloride (i.e. about 0.5 M). Fixation in 4% (v/v) glutaraldehyde (1,500 mOs) was for 20 min followed by a 1 h postfixation in 2% (w/v) OsO_4 (1,200 mOs) after a buffer wash. Preparations were stained *en bloc* with uranyl acetate.

2.4.3. Electron microscopy of chloroplast DNA.

Chloroplast DNA for electron microscopy was precipitated twice from a Sarkosyl lysate of purified chloroplasts as described in Section 2.8.2 and dissolved in 50 µl of 1 mM EDTA, 10 mM Tris-HCl pH 7.8. The DNA was spread by the basic protein monolayer technique of Kleinschmidt (1968) using the "droplet method". This procedure was very sensitive to contamination and so all glassware was cleaned with chromic acid and all solutions were prepared in laboratory distilled water that had been further purified immediately before use by passing it through a 50 ml column of "Amberlite" monobed resin MB-1 (BDH, AnalaR) followed by glass distillation.

1 µl of the chloroplast DNA preparation (assumed to have a concentration of 10 µg ml^{-1}) was mixed with 500 µl of freshly prepared droplet solution:

2.7 ml	H_2O
0.3 ml	10 mM EDTA, 2 M ammonium acetate pH 7.0
8 μl	1 mg ml^{-1} cytochrome c (Sigma, Type V from bovine heart)

50 μl drops were then placed on the surface of a sterile plastic petri dish and left covered for 45 min to allow cytochrome c denaturation and DNA adsorption to occur at the air interface.

Parts of the cytochrome c film were picked up by briefly touching the surface of the drop with a formvar coated copper grid. The grid was then immediately dipped in a solution of uranyl acetate for 30 s, washed in 90% (v/v) ethanol for 10 s and allowed to air dry. The staining solution was prepared by diluting 5 mM uranyl acetate, 50 mM HCl one hundred times with 90% (v/v) ethanol and filtering through a 0.22 μm Milipore filter prior to use.

The grids were shadowed with 15 mm of 0.2 mm diameter 80:20 platinum:paladium wire (Agar Aids) evaporated at an angle of 6.5° and a distance of 10 cm. Shadowing was then repeated at an angle of 90° to obtain even contrast of the DNA. The grids were examined with a Zeiss EM 109 electron microscope and the DNA molecules were photographed randomly.

To determine the lengths of the DNA molecules, a commercial preparation of the bacterial plasmid pBR322 (Sigma), length 4.36 kb, was spread in parallel to the sample DNA. Length measurements were made from 5x enlargements of the negatives.

2.5. PURIFICATION OF DNA FROM GLENODINIUM FOLIACEUM.

2.5.1. Preparation of deproteinization mixture.

A mixture based on phenol and chloroform was routinely used to precipitate proteins from cell or organelle extracts. The solution also contained 8-hydroxyquinoline to stabilize the phenol against oxidation and isoamyl alcohol to prevent excessive foaming of the chloroform during deproteinization.

To prepare the mixture, crystalline phenol (B.D.H., AnalaR, white crystals) was melted by gentle warming, and after adding 0.1% (w/v) 8-hydroxyquinoline, the phenol was saturated with 0.1 M Tris-HCl pH 9.0. An equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added to the organic phase which was then left to clear in a separating funnel overnight. Alternatively, the mixture was cleared by centrifugation if it was required immediately.

2.5.2. Preparation of hydroxylapatite.

Commercially prepared hydroxylapatite (HAP) (Sigma) proved unsatisfactory for purifying DNA from *Glenodinium foliaceum* because it exhibited both a low binding capacity and clogged badly under the conditions it was used. However a superior grade of HAP could be produced in the laboratory by simplifying the method described by Spencer (1978) as some of the equipment required for this method was not available.

(i) Preparation of brushite:

2 l of 0.5 M $CaCl_2$ was siphoned at a rate of 50 ml min^{-1} into the bottom of a 9 l bucket containing 2 l of 0.5 M sodium phosphate buffer pH 6.7. During the addition of $CaCl_2$ the sodium phosphate was being rapidly stirred by means of a magnetic stirrer (SEA) set at maximum speed using a 5 cm long follower. The crystals of brushite which had formed during this process were then allowed to settle and washed with 5 l of distilled water.

(ii) Conversion of brushite to hydroxylapatite:

The slurry of brushite was transferred to a well lagged 5 l beaker and 2.5 l of boiling distilled water was rapidly added whilst stirring the brushite with a glass rod. When all the water had been added the sediment was allowed to settle and the supernatant was poured off without delay. 5 ml of 0.5 M HCl was then added followed by a further 2.5 l of boiling distilled water, with stirring, and about 72 ml of 20% (w/v) NaOH. The alkali was added at 34 ml min^{-1} for the the first 30 s, 50 ml min^{-1} for the next 30 s and finally at 30 ml min^{-1} for another minute. These rates of addition are reported to keep the pH of the suspension near neutrality (Spencer, 1978) which is essential for the conversion of brushite ($CaHPO_4.2H_2O$) to HAP ($Ca_{10}H_2(PO_4)_6(OH)_2$) rather than to other forms of calcium phosphate which do not exhibit the high flow rate characteristics of HAP. After formation, the HAP was allowed to settle and was washed with 2.5 l of boiling distilled water. The HAP was finally resuspended in a further 2.5 l of boiling water before being left to cool slowly overnight in a pre-heated drying cabinet. The settled volume of the HAP was just under 1 l which is slightly greater than expected (750 ml) and may indicate that more octacalcium phosphate was produced in this simplified version than in Spencer's original method. The HAP was stored at 4°C.

2.5.3. DNA purification

(i) Solutions:

(a) Lysis buffer:

5 %	(w/v) sodium dodecyl sarcosine (Sarkosyl)
2 mM	aurintricarboxylic acid (ATA)
100 mM	NaCl
100 mM	EDTA
50 mM	Tris-HCl pH 8.0

(b) Phenol/chloroform mixture (see Section 2.5.1):

25 vol	phenol
24 vol	chloroform
1 vol	isoamyl alcohol

(c) Chloroform:

24 vol	chloroform
1 vol	isoamyl alcohol

(d) Urea/phosphate column wash:

8 M	urea
240 mM	phosphate buffer pH 7.0

(e) 2 M phosphate buffer pH 7.0 stock:

1 M	Na_2HPO_4
1 M	NaH_2PO_4

All solutions containing phosphate buffer were filtered through a 0.22 µm Milipore filter before use. The urea/phosphate was filtered whilst hot to avoid precipitation.

(ii) Glassware:

Where possible, all glassware was coated with silicone by immersing it in 2% (v/v) silicone fluid DC 1107 (Hopkin and Williams) dissolved in butanone and then baking the glassware for 2 h at 180°C.

(iii) Method:

Total cell DNA was generally prepared from 20 to 40 l of culture having a density of about 5 x 10^3 cells ml^{-1}. The cells were collected by centrifugation at 15°C and 7,600 g in a Sorvall SS-34 rotor fitted with the Sorvall KSB-R continuous flow head which allowed the continual introduction of culture into the rotor at a rate of 350 ml min^{-1}. The specifications of the system did not permit a lower relative centrifugal force to be used and the flow rate could not be increased significantly. It is likely, therefore, that any contaminating bacteria in the culture were pelleted with the algae. The cells were washed in sterile ASP_7 and pelleted in a single centrifuge tube where they were washed with ice cold lysis buffer. The volume of the cell pellet was determined at this stage (this was about 2 ml per 10 l of culture) and after re-pelleting, the cells were suspended in two volumes of lysis buffer and stored frozen at -20°C.

Cell breakage was accomplished by means of a 5 ml "tight fitting", Potter-Elvehjem homogenizer. The homogenization chamber was half filled with cell suspension and after carefully inserting the ground glass pestle, 3 ml of lysis buffer was pipetted into the funnel. One downward stroke of the pestle was all that was required to disrupt 90% of the cells, however, because of the tight-fitting nature of the homogenizer, this took about 10 min. Whilst pressure was applied to the pestle, the homogenizer chamber was rotated at about 30 rpm so as to create a greater shearing force. The lysate was poured into a glass-

stoppered measuring cylinder and mixed with an equal volume of phenol/chloroform mixture whilst the next aliquot of cells was being homogenized. The final volume of the phenolic lysate was adjusted to either 80 ml or 160 ml by adding equal parts of lysis buffer and phenol/ chloroform. The measuring cylinder was then gently rocked for 10 min to ensure complete mixing of the aqueous and organic phases and left to stand for 20 min, with intermittent shaking, to precipitate the proteins. Homogenization and deproteinization were performed at 4°C.

The precipitated proteins were concentrated at the interphase of the organic and aqueous phases by centrifugation of the mixture in two or four 50 ml glass tubes at 2,000 g for 45 min in an MSE 12 x 100 ml swing out rotor. The aqueous phase was removed from above the large protein pad with a wide bore, disposable, plastic pipette and mixed again with an equal volume of phenol/chloroform; the protein interface was rarely re-extracted. After gently mixing the solution for 20 min, the phases were separated again as before. During the second phenol extraction the temperature of the aqueous phase was allowed to rise to 15 - 20°C. Residual phenol was removed from the preparation by serial extractions with equal volumes of chloroform. The mixture was shaken for 10 min and cooled on ice before centrifuging out the phases at 2,000 g for 15 min. This process was repeated until the aqueous phase was completely clear and interphase material had been eliminated (i.e. three or four times).

To precipitate the nucleic acids from the deproteinized extract, the aqueous phases were poured into a 500 ml beaker pre-cooled to -20°C and containing 0.1 volumes of 3 M sodium acetate. The solution was then carefully overlain with 3 volumes of ethanol, also pre-cooled to -20°C, and the nucleic acids were wound out from the interphase onto a glass rod. (Note: if the

ethanol was mixed with the aqueous phase and the nucleic acids were collected by centrifugation, then the DNA preparation was badly contaminated with a gelatinous, orange-brown substance, particularly if the precipitation had been performed overnight at $-20^{o}C$. Spooling the nucleic acids from an ethanol interface substantially reduced the level of this contamination). The spool of DNA was washed twice for 10 min in ice cold 70% (v/v) ethanol and then left to air dry for 30 min before finally being dried *in vacuo*.

Further purification of the DNA was achieved by passing the preparation through a column of HAP under the binding conditions described by Britten *et al*. (1970). The spool of DNA was allowed to dissolve in 50 ml of urea/phosphate column wash for at least 5 h or preferably overnight. The volume (in ml) of HAP required to bind the DNA was estimated to be ten times the A_{260} of this solution. This conservatively assumed that the binding capacity of the HAP was 250 μg DNA ml^{-1}. Generally the column volume was about 40 ml for a starting culture volume of 20 l. The column was conveniently run in a 50 ml disposable syringe connected to a 1 m head of buffer by inserting a delivery tube through the rubber plunger seal. Under this pressure the flow rate through the column was about 90 ml h^{-1}.

The column was equilibrated with urea/phosphate buffer at room temperature before loading, and to ensure maximal binding of the DNA, the sample was passed through the column three times. The first two passages were performed at atmospheric pressure, however, the second passage of the sample was facilitated by stirring the column gently to increase the flow rate as contaminants in the preparataion caused the HAP to coagulate. The eluate from the second loading showed little reduction in 260 nm absorbance compared to the eluate of the initial loading, which

contained about 20% of the absorbance originally present in the sample. The eluate was finally washed through the column with urea/phosphate under pressure by connecting the column to the buffer head. Washing with urea/phosphate was continued until the A_{260} of the eluate had fallen below 0.01. This took 2 - 3 h. To remove the urea, the column was then washed with 0.01 M phosphate buffer pH 7.0 until the A_{260} of the eluate was again less than 0.01. During these washing stages the HAP regained its normal structure and the flow rate rose to its pre-loading value.

After washing, the depth of liquid above the column was reduced to a minimum by bleeding air into the system. This ensured that a sharp front of elution buffer passed through the HAP. The DNA was eluted with 0.5 M sodium phosphate pH 7.0 at room temperature (Goldberg, 1978). The dead volume of the column was allowed to run through and then the 260 nm absorbance of the eluate was continuously monitered (Figure 2.3) and fractions containing DNA were collected.

The DNA was dialysed against 5 l of 1 mM EDTA, 10 mM Tris-HCl pH 8.0 for 5 h at room temperature with one change of buffer and then at 4°C for a further 24 h again with one buffer change after 12 h. The volume of the preparation was then reduced to a convenient amount by repeated extractions with butanol and adjusted to 0.3 M sodium acetate before the DNA was precipitated in 3 volumes of ethanol overnight at -20°C. The DNA was pelleted at 25,000 g for 15 min at 0°C, washed in cold 70% (v/v) ethanol and stored under absolute ethanol at -20°C until required. Ethanol precipitation after dialysis into a stable buffer was found to be preferable to other methods of concentrating the DNA, such as pelleting in the ultracentrifuge, as the recovery was higher.

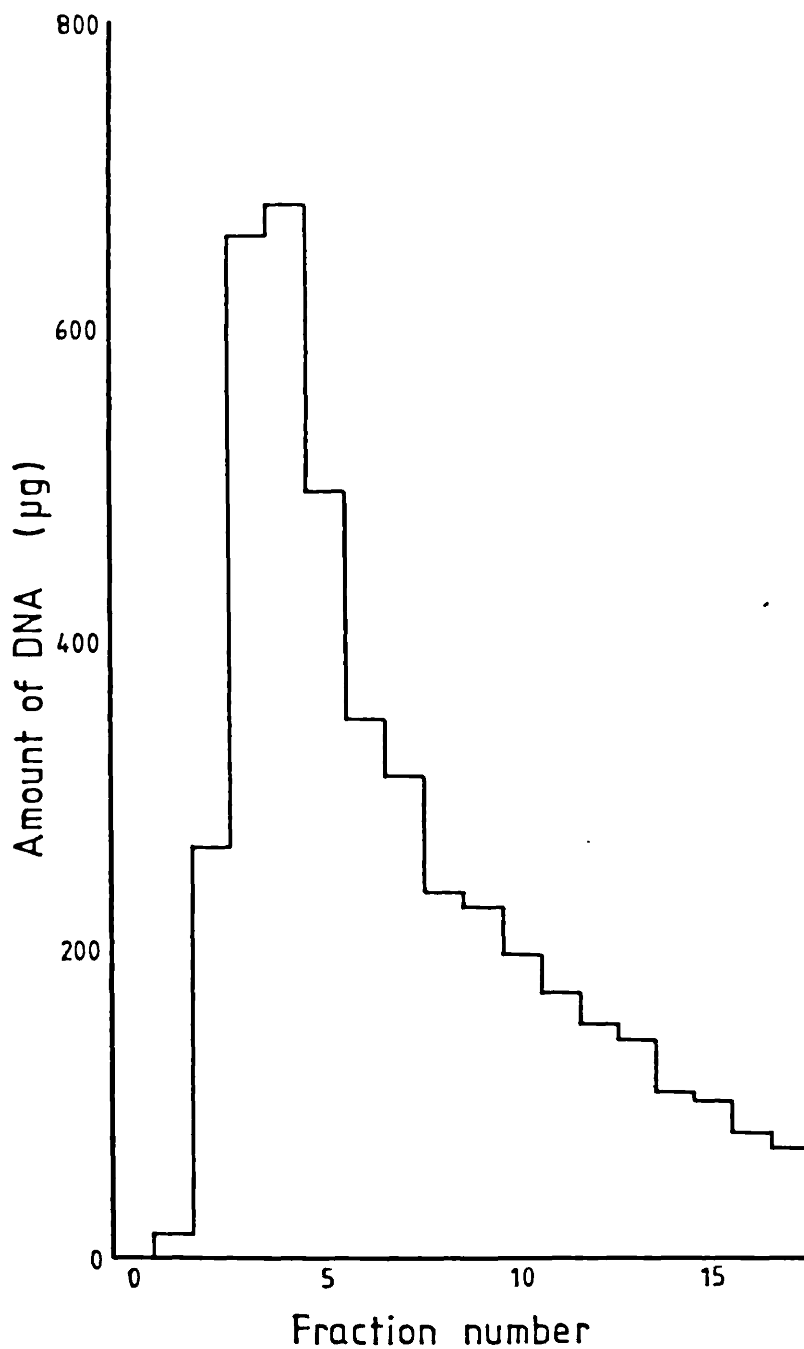


Figure 2.3. Elution profile of <u>Glenodinium foliaceum</u> DNA from laboratory made hydroxylapatite. The DNA was eluted with 0.5 M phosphate buffer from a 40 ml column of hydroxylapatite and the 260 nm absorbance of 3 ml fractions was recorded to determine the amount of DNA in each fraction.

2.6. FRACTIONATION OF WHOLE CELL DNA IN CAESIUM CHLORIDE DENSITY GRADIENTS.

2.6.1. Determination of centrifugation conditions.

The successful fractionation of *Glenodinium foliaceum* DNA in caesium chloride density gradients formed *in situ* required the proper design of the gradient experiments rather than taking a purely trial and error approach. The strategy for designing these experiments was to first 'guess' a suitable set of gradient parameters and then, from the result of this fractionation, to predict the best set of conditions which would give the type of fractionation required. These conditions were obtained by applying the few simple equations outlined below (for a discussion of the formulation of these equations using the methods of equilibrium thermodynamics see Spragg (1978)).

A MSE 8 x 14 ml fixed angle rotor was used for all density gradient centrifugation experiments and the volume of the gradient was always 10 ml. This volume sets the maximum (r_b) and minimum (r_t) rotational radii of the gradient at 7.80 cm and 5.15 cm respectively, as calculated from information suplied by the manufacturer of the rotor. Having determined r_t and r_b, the distance from the rotational axis of the isoconcentration point (r_c) can then be estimated from the following equation:

$$r_c = \left[\frac{1}{3} \left(r_t^2 + r_t . r_b + r_b^2 \right) \right]^{\frac{1}{2}}$$

r_c gives the location in the gradient of a point of known density; that is, the starting density (ρ_c). This, therefore, allows the density at other points in the gradient to be predicted:

$$\rho_2 - \rho_1 = \frac{\omega^2}{2\beta^o}\left(r_2^2 - r_1^2\right)$$

where; ρ_1 and ρ_2 are the densities at points r_1 and r_2,
ω is the angular velocity in rad s^{-1},
β^o is the density-gradient proportionality constant (see Table 2.4).

Of particular importance is the density at the bottom of the gradient since if this exceeds the density of a saturated solution of CsCl (1.92 g cm^{-3} at 25^oC), crystals of CsCl will form and the rotor will be overstressed.

The time taken for any caesium chloride gradient to form in a given rotor is constant and is simply calculated. However, this value is irrelevant in DNA fractionations since the time required for the DNA molecules to reach their equilibrium density far exceeds the centrifugation time necessary to form the gradient. For DNA molecules having a minimum size of 1×10^6

Table 2.4. Density gradient proportionality constants (β^o values) for caesium chloride in aqueous solution (from Birnie, 1978).

Density at 25^oC (g cm^{-3})	$\beta^o \times 10^{-9}$
1.4	1.33
1.5	1.22
1.6	1.17
1.7	1.14
1.8	1.12

daltons, this time, t (in hours), can be estimated from the following equation assuming the DNA bands at the isoconcentration point:

$$t = 1 \cdot 13 \times 10^{13} \frac{(\rho_c - 1)\beta^o}{N^4 r_c^2}$$

where; N is the speed of the rotor in $rev.min^{-1}$.
(see Birnie (1978) for other assumptions made in deriving this equation).

It can be seen that for native DNA, density about 1.70 g cm^{-3}, being centrifuged at 40,000 rpm, t is about 83 h.

Finally, the mass, m, of CsCl which should be dissolved to prepare the required volume, V, of a given density, ρ, is calculated by:

$$m_{CsCl} = V \rho F$$

where F is the weight fraction of CsCl in a solution of this density and:

$$F = 1 \cdot 3748 - \frac{1 \cdot 3811}{\rho}$$

for densities (in $g\,ml^{-1}$) at 25^oC. Similarly the mass of solvent required to prepare this solution is

$$m_{solvent} = V \rho (1 - F)$$

2.6.2. Caesium chloride / Hoechst dye 33258 gradients.

DNA prepared as described in Section 2.5.3, but without further purification on HAP, was used in CsCl/Hoechst dye gradient experiments. As these preparations were impure the gradients could not be run with a defined amount of DNA. However, exploratory experiments revealed that the desired fractionation could be achieved by loading the DNA obtained from 2.5 l of late exponential phase culture (i.e. about 1.25×10^7 cells) into one 10 ml gradient. Generally about 200 μg of DNA was recovered from such a gradient after fractionation.

Precipitated DNA was dissolved in 0.5 gradient volumes of 1 mM EDTA, 10 mM Tris-HCl pH8.0 (TE) overnight. The required amount of solid CsCl (Sigma, Grade 1) was then added and the solution was gently mixed by slow inversions until the CsCl had completely dissolved. The gradient solution was then made up to the correct weight and volume with distilled water and the appropriate quantities of the relevant stock solutions to give final concentrations of 200 μg·ml Hoechst dye No.33258 (Sigma) and 2% (w/v) Sarkosyl. Before centrifuging to equilibrium, the gradients were pre-spun in a MSE 6 x 16.5 ml swing out rotor at 20,000 g for 15 min to remove a 'petalloid' Hoechst precipitate. This precipitate was not fluorescent and formed a compact mat above the caesium solution after centrifugation. The clarified 10 ml gradients were loaded into 75 mm x 16 mm diameter (14 ml) polycarbonate tubes (MSE, cat.no. 34411-128), accurately balanced, and capped, with the residual volume completely occupied by light parafin oil to prevent the tubes collapsing during centrifugation. All the above operations were performed under subdued lighting to minimize possible dye photocatalysed nicking of the DNA.

The empirically-determined, absolute composition of the CsCl/Hoechst gradients and the centrifugation conditions which gave the optimal fractionation are detailed below. The parameters of these gradients are discussed in Section 3.1.2.

Quantities per 10 ml gradient:

5 ml	40 μg ml^{-1} DNA in TE
9.01 g	CsCl
1 ml	20% (w/v) Sarkosyl
200 μl	10 mg ml^{-1} Hoechst dye No.33258
adjust to 16.6 g	distilled H_2O

Centrifugation conditions:

18 h at 40,000 rpm then 70-75 h at 30,000 rpm at 25°C in a MSE 8 x 14 ml fixed angle rotor using a MSE Superspeed 65 ultracentrifuge.

After centrifugation, the rotor was allowed to come to rest unbraked and the fluorescent DNA bands were visualized under long wavelength ultraviolet light emitted from a "Black-Ray" lamp (Ultra-violet products, cat.no.B-100A). Gradients were photographed using side illumination.

2.6.3. Caesium chloride / ethidium bromide gradients.

DNA recovered from CsCl/Hoechst dye gradients, or prepared from isolated nuclei, was used in CsCl/ethidium bromide fractionations. The gradients were prepared essentially as described above and loaded with 400 μg of DNA. The optimal composition of the gradients in this case was:

Quantities per 10 ml gradient:

5 ml	80 $\mu g.ml^{-1}$ DNA in TE
7.36 g	CsCl
1 ml	5 $mg.ml^{-1}$ EtBr
adjust to 15.4 g	distilled H_2O

Centrifugation conditions:

40-48 h at 40,000 rpm and 25°C in the MSE 8 x 14 ml rotor.

All DNA solutions containing ethidium bromide were wrapped in aluminium foil and any manipulations were performed under a red safelight. The fluorescent DNA bands were visualized by long wavelength ultraviolet light, as before, but the gradients were photographed using transmitted ultraviolet light and a red filter over the camera lens. Exposure of the DNA to the ultraviolet light was kept to a minimum.

2.6.4. Recovery of DNA from caesium chloride gradients.

(i) Fractionation of gradients:

If the separation of the DNA bands was large, as in the case of the CsCl/Hoechst dye gradients, or only one band was present, then the tubes were uncapped and the DNA was withdrawn from above by means of a Pasteur pipette. The upper band was always removed first to avoid contamination of the heavier DNA species with the lighter one. When the DNA bands were not separated by a sufficiently large gradient volume to allow for the complete removal of the upper band, then the DNA was recovered by puncturing the side of the tube sub-adjacent to the band with a syringe. Parafilm "M" stretched over the tube prevented any

leakage of the gradient around the needle and a 1 ml syringe was used to draw off the DNA so as to minimize turbulence created by the suction. The syringe used to withdraw the first band was left in place whilst removing the second.

(ii) Dye removal:

It proved easier to extract the fluorescent dyes from the DNA when the latter was in a high salt (i.e. CsCl) solution, providing the necessary precautions were taken to prevent the CsCl from precipitating. The Hoechst dye was removed by serial extractions with equal volumes of CsCl-saturated isopropanol. To caesium saturate the isopropanol, first it was mixed with saturated CsCl solution and then solid CsCl was gradually added to the organic phase until no more dissolved. Dye removal from the DNA was monitored under ultraviolet light. Generally at least five extractions were required.

Ethidium bromide was removed by three extractions with butanol saturated with 1 mM EDTA, 10 mM Tris-HCl pH 8.0. Dye removal was monitored visually.

(iii) Removal of caesium chloride:

The DNA was either selectively precipitated directly from the CsCl solution using isopropanol or the CsCl was removed by dialysis. To precipitate the DNA, the sample was diluted with three volumes of 0.6 M LiCl, 1 mM EDTA, 10 mM Tris-HCl pH 8.0 (with carrier tRNA if necessary – see Section 2.8.2.) and then an equal volume of isopropanol (not saturated with CsCl) was added. After chilling for 1 - 2 h at -20°C, the DNA was collected by centrifugation. The pellets were washed five times with 70% (v/v) ethanol to remove residual CsCl and redissolved in a suitable volume of 1 mM EDTA, 10 mM Tris-HCl pH 8.0 so that the DNA could be re-precipitated as one batch with ethanol.

Dialysis of the sample was performed at $4^{o}C$ against a series of buffered solutions containing decreasing levels of salt using dialysis tubing which had been boiled in 10 mM EDTA and thoroughly washed in distilled water. Dialysis against the high salt buffer (1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 8.0) for 6 h was followed by overnight dialysis in low salt buffer (0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 8.0) and finally two 12 h changes of 1 mM EDTA, 10 mM Tris-HCl pH 8.0. The DNA was then recovered by an ethanol precipitation after first reducing the volume of the sample by serial butanol extractions.

2.7. ISOLATION OF NUCLEI AND NUCLEAR DNA.

2.7.1. Isolation of nuclei.

The following method for obtaining an almost pure preparation of dinoflagellate nuclei from *Glenodinium foliaceum* is based largely on the techniques described by Rizzo and Noodén (1973) for isolating nuclei from dinoflagellates in general. A discussion of the method, and of the possibilities of manipulating it to produce preparations of the symbiont nucleus, is given in Section 4.1.3.

Nuclei were isolated from 10 l of late exponential phase culture (i.e. about 5×10^{7} cells) which had been kept in the dark for at least 24 h to reduce their starch content. The cells were collected using the Sorvall KSB-R continuous flow centrifugation system as described previously (Section 2.5.3) and repelleted into four SS-34 centrifuge tubes. The cell pellets were

then resuspended in 25 ml of ice cold isolation buffer; 0.25 M sucrose, 5% (w/v) Dextran 40, 2.5% (w/v) Ficoll 400, 0.5% (v/v) Nonidet P-40, 5 mM $CaCl_2$, 10 mM Tris-HCl pH 7.3. All subsequent stages were performed at 0 - 4°C. The cells were disrupted by sonication at 20 kHz using a MSE Ultrasonic Disintegrator (MkII) fitted with a 9.5 mm diameter titanium probe set to vibrate at 130 μm peak to peak. Sonication was given in 30 s bursts for a total of 2 - 3 min to achieve 95% cell breakage. The tubes were cooled on ice between periods of sonication. When most of the cells had broken, the amplitude of the probe vibration was reduced to 80 μm so as to minimize the disruption of nuclei already isolated. Cell breakage was monitored microscopically using methyl green/pyronine B staining.

After adding 10 ml of 2.4 M sucrose, 5 mM $CaCl_2$, 10 mM Tris-HCl pH 7.3 to each tube, the nuclei were pelleted at 5,800 g for 15 min in a Sorvall SS-34 rotor. The pellets were resuspended by gentle homogenization in a total of 15 ml of isolation buffer and 5 ml of homogenate was layered over each of three discontinuous sucrose gradients. The gradients were composed of 5 ml of 2.4 M sucrose + 10% (w/v) Dextran 10, 8 ml of 2.4 M sucrose and 5 ml of 2.2 M sucrose all buffered with 5 mM $CaCl_2$, 10 mM Tris-HCl pH 7.3. The interface between the 2.2 M sucrose and the homogenate was removed by stirring the upper half of the 2.2 M sucrose layer with a Pasteur pipette. The nuclei were pelleted through the gradient by centrifugation for 30 min at 22,000 g in a MSE 3 x 23 ml swing-out rotor using a MSE Superspeed 40 ultracentrifuge. Following centrifugation all of the gradient above the 2.4 M sucrose + 10% Dextran pad was discarded and the inside of the tube was carefully wiped clean with tissue. The nuclei were then resuspended by diluting the Dextran pads with isolation buffer and repelleted into one tube at 12,000 g for 10 min in the Sorvall SS-34 rotor.

At this stage the nuclear pellet was free of unbroken cells but was badly contaminated with cell wall fragments and starch grains, and also a certain amount of cytoplasmic debris trapped in the cell walls. The bulk of the wall fragments could be removed by filtration through two layers of monofilament nylon bolting cloth (Henry Simon Ltd., 20 μm pore size, 14% open space). This was essential if the preparation was to be used for estimating the amount of DNA per nucleus since the presence of the cell walls made it impossible to determine the number of nuclei in the final suspension by counting samples in a haemocytometer. However, because filtration resulted in a substantial loss in yield, non-quantitative DNA preparations were made from the crude nuclear pellet.

2.7.2. Isolation of dinoflagellate nuclear DNA.

Dinoflagellate nuclei were washed in 0.25 M sucrose, 5 mM $CaCl_2$, 10 mM Tris-HCl pH 7.3 to remove the high molecular weight polysaccharides present in the isolation buffer and pelleted into an Eppendorf tube. Lysis with 1% (w/v) SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0 for 20 min at room temperature was followed by two phenol extractions and the DNA was collected by an ethanol precipitation.

2.8. ISOLATION OF CHLOROPLASTS AND CHLOROPLAST DNA.

A full account of the development of the following procedure for isolating chloroplasts from *Glenodinium foliaceum* is presented in Section 4.2.1.

2.8.1. Isolation of chloroplasts.

Chloroplasts were isolated from 6 l of mid-exponential phase culture which had been put in the dark 24 h before use. The cells were concentrated by centrifugation in a MSE 12 x 100 ml swing out rotor, using a MSE GF-8 centrifuge, by allowing the rotor to accelerate to 2,000 g and then immediately bringing it to rest under maximum brake. The concentrated cell suspension was then pelleted and cooled to 2°C in a Sorvall RC-5B centrifuge at 12,000 g for 3 min using a SS-34 rotor. All subsequent operations were performed at 0 - 4°C.

The cells were resuspended in 20 ml of chloroplast isolation buffer (1.3 M sorbitol, 2.5% (w/v) Ficoll 400, 50 mM EDTA, 2 mM ATA, 10 mM Tris-HCl pH 7.8) and disrupted by passage through a French Pressure Cell (Aminco) at 3,500 - 4,000 psi. The needle valve of the Cell was continually adjusted so that the homogenate just dripped out whilst the pressure was maintained using a manual hydraulic press (Apex Construction Ltd.). The suspension of broken cells was then diluted with 60 ml of isolation buffer so as to fill two SS-34 centrifuge tubes and further homogenized, in two lots, in a large volume, "loose-fitting", Potter-Elvehjem homogenizer. This step released any chloroplasts that remained trapped inside partially disrupted cells and greatly increased the yield.

Unbroken cells, cell walls and nuclei were then pelleted at 2,400 g for 10 min in the SS-34 rotor. This brief, low speed centrifugation only produced a very loose pellet which ran up the side of the tube as the supernatant was decanted and so the centrifugation had to be repeated on the supernatant to reduce whole cell contamination. The pellets from the first centrifugation were resuspended, by homogenization, in isolation buffer and also repelleted to wash out sedimented chloroplasts. The cell

pellets from the second centrifugation were somewhat firmer and did not run. The chloroplasts were pelleted from the supernatants at 12,000 g for 15 min, again in the SS-34 rotor. These "crude chloroplast" pellets, contaminated to a certain extent by intact cells, accumulation bodies and mitochondria, were either used directly to obtain an enriched chloroplast DNA preparation or further purified by rate zonal centrifugation through a discontinuous sucrose gradient as follows.

The crude chloroplasts pellets were resuspended in a total of 3 ml of isolation buffer by one slow stroke of the pestle of a small volume, "loose-fitting", Potter-Elvehjem homogenizer. The chloroplast suspension was then layered over three discontinuous sucrose gradients, 1 ml per gradient. These consisted of a 5 ml 42% (w/w) sucrose pad overlain by 9 ml of 16% (w/w) sucrose and 6 ml of 8% (w/w) sucrose. All the sucrose solutions were prepared in isolation buffer. The gradients were centrifuged at 14,000 g for 10 min (including run up time but excluding run down time under brake) in a MSE 3 x 23 ml swing out rotor using an MSE Superspeed 40 centrifuge. The bulk of the chloroplasts were trapped by the upper sucrose interface, whilst most of the contaminating material passed readily through the 16% sucrose layer to band at the lower interface, or even be pelleted at the bottom of the tube. The chloroplasts were removed by means of a sterile, plastic pipette, diluted into isolation buffer, and pelleted in the Sorvall SS-34 rotor at 18,500 g for 10 min.

The pellet of purified chloroplasts, or the crude chloroplast pellets, were washed in a minimal volume (1.5 - 3 ml) of isolation buffer so that they could be repelleted in 1.5 ml Eppendorf tubes ready for lysis.

2.8.2. Isolation of chloroplast DNA.

Two methods were employed to isolate and purify DNA from the chloroplast pellets.

The crude chloroplast pellets were resuspended in about 500 µl of 2% (w/v) SDS, 8 M urea, 0.24 M sodium phosphate pH 7.0 and the chloroplasts were left to lyse for 30 min at room temperature, with intermittent rocking of the Eppendorf tube to aid lysis. The lysate was shaken with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) for 15 min and centrifuged for 10 min in a microcentrifuge for 10 min to separate out the phases. The aqueous phase was re-extracted with phenol and then extracted twice with chloroform/isoamyl alcohol (24:1 v/v) before being passed twice through a 2 ml HAP column equilibrated with 8 M urea, 0.24 M sodium phosphate pH 7.0. Subsequent washing of the HAP column and elution of the DNA was then performed essentially as described in Section 2.5.3, except that a smaller head of buffer was used. The eluate was dialysed into three 1 l changes of 1 mM EDTA, 10 mM Tris-HCl pH 8.0 and then its volume was reduced to about 300 µl by serial extractions with butanol before precipitating the DNA in ethanol. The DNA was stored in 1 mM EDTA, 10 mM Tris-HCl pH 8.0 at -20°C.

The pellets of purified chloroplasts were washed briefly, without resuspension, in ice cold 0.1 M EDTA, 0.15 M NaCl, 2 mM ATA, 50 mM Tris-HCl pH 8.0 and then resuspended in 400 µl of this buffer. The chloroplasts were lysed by adding 40 µl of 20% (w/v) Sarkosyl and incubating the tube on ice for 30 min with gentle agitation. The lysate was extracted with phenol and chloroform, as for the crude chloroplast DNA preparation, but then the DNA was precipitated directly from the deproteinized lysate by adding 2.5 volumes of ethanol. 10 µg of transfer RNA (Sigma, Type XX from Escherichia coli) was included in the lysate to act as a carrier during this precipitation and the nucleic acids were

collected after 1 h incubation at -20°C. Obvious contamination in the pellet was partially removed by repeated washing with 70% (v/v) ethanol and was reduced further by a second ethanol precipitation. After being dried, the DNA pellet was dissolved in 0.5 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.2 and loaded onto a micro-column of pre-packed NACS resin (BRL) in exactly the manner described by the manufacturer. After extensive washing of the column with loading buffer, the DNA was eluted with 2 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.2 and precipitated from the eluate with ethanol after first adding carrier tRNA. After washing with 70% (v/v) ethanol to remove precipitated NaCl, the DNA was dissolved in an appropriate buffer ready for analysis.

2.9. KINETIC ANALYSIS OF DNA.

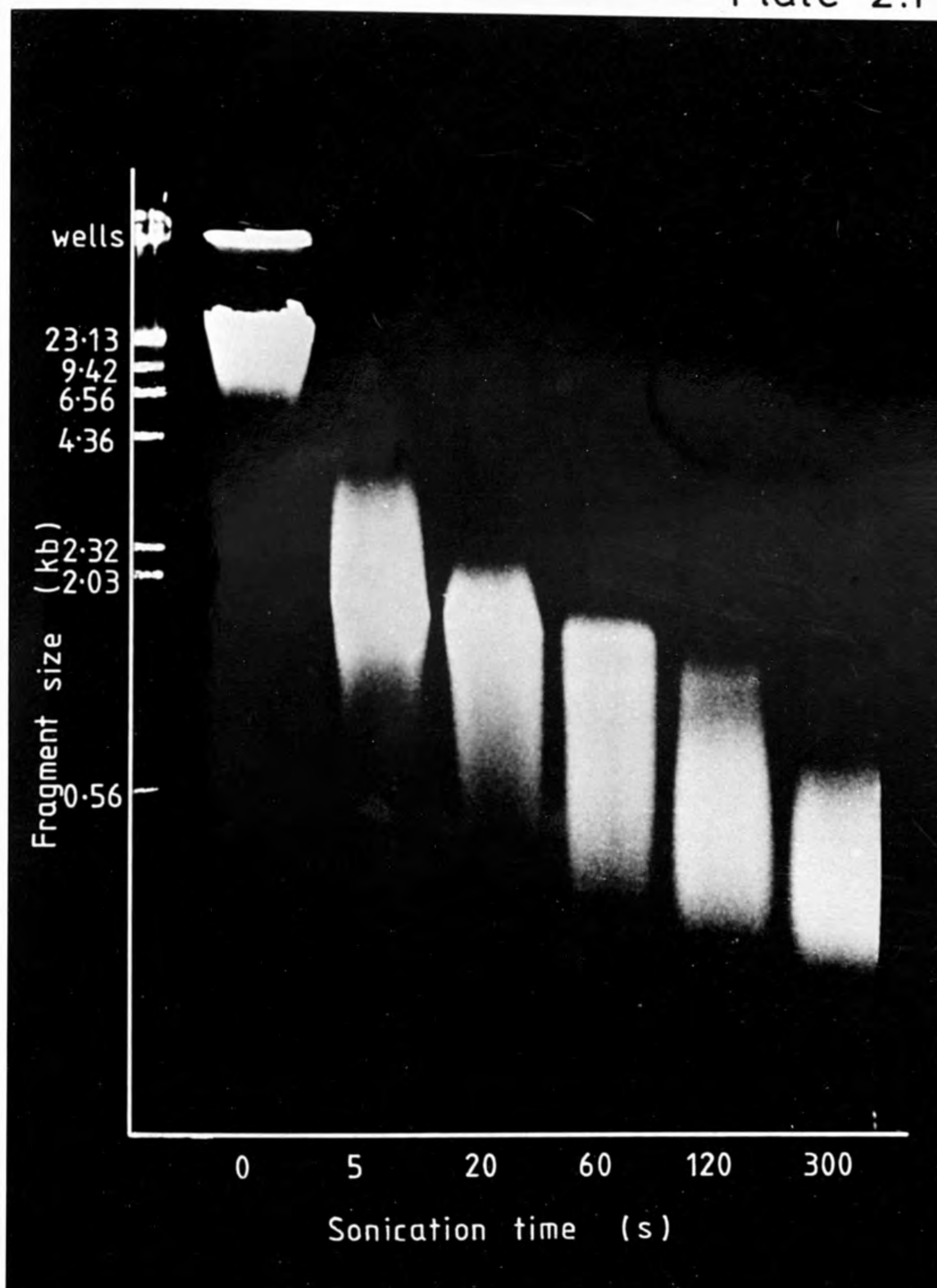
2.9.1. Preparation of DNA.

Unfractionated DNA from *Glenodinium foliaceum* was prepared by passing phenol extracted cell lysates through a column of HAP as described in Section 2.5.3. The DNA was then extensively dialysed against the appropriate phosphate buffer before analysis. The 260:230 nm and 260:280 nm absorbance ratios of this material were generally 1.4 and 2.0 respectively.

DNA for reassociation was fragmented by sonication at a frequency of 20 kHz using a MSE Ultrasonic Disintegrator (Mk II) fitted with an exponential probe (end diameter 3 mm) set to vibrate at 125 microns peak to peak. A preliminary experiment revealed that 2 min sonication given in 30 s bursts with cooling in an ethanol/ice bath, was sufficient to shear the DNA to about 500 bp (Plate 2.1).

Plate 2.1. Fragmentation of *Glenodinium foliaceum* DNA by sonication. Graphical presentation of the electrophoretic migration through a 1% agarose gel of DNA samples sonicated for various times. Fragment sizes given are those produced by digestion of λ DNA with restriction endonuclease *Hind* III.

Plate 2.1



2.9.2. Melting curves:

Samples for melting were degassed under vacuum and clarified by centrifugation. Melting was performed in a Pye Unicam SP 1800 spectrophometer equipped with an in-house produced electrically heated block which ramped up the temperature of the cuvette at 42°C per hour. 400 µl of native DNA in 120 mM sodium phosphate buffer pH 7.0 (PB) was melted in a 1 cm path length microcell. The DNA was overlain with silicone oil to prevent evaporation. The absorbance at 260 nm was recorded every 0.25°C by a microcomputer and the data were collected on a floppy disc. This equipment was developed and built by Graham King of Birkbeck College, London University.

2.9.3. Reassociation kinetics:

Sonically sheared DNA was denatured by heating to 100°C for 5 min and renatured at 60°C. Reassociation was followed by two methods:

(i) Optical monitoring:

Since double-stranded DNA absorbs less light at 260 nm than single-stranded DNA, reassociation can be followed directly in a spectrophometer.

Samples having an optical density of about 1.0 were prepared in 30 mM, 120 mM, 400 mM and 1 M PB so as to give differing relative rates of reassociation: 0.013, 1.000, 4.905 and 8.411 respectively (Britten et al., 1974). After heat denaturation, the DNA was injected directly into a 500 µl, 1 cm path length cuvette held at the incubation temperature in the spectrophotometer (Cecil CE272) by means of a circulating water bath (Braun Thermomix 1441). This technique quickly shifted the temperature

of the sample to 60°C so data could be collected within 30 s of the start time. The absorbance at 260 nm was then monitored continuously for up to 2 h and then at suitable periods for up to 150 h for the reassociations performed in 120 mM and 1 M PB. After the initial readings were recorded, the sample was overlain with silicone oil and tightly stoppered to prevent evaporation.

The decrease in absorbance was calculated relative to that of the denatured DNA (i.e. as a hypochromicity) as this is directly proportional to the degree of reassociation. From data obtained from the melting curves, fully renatured DNA was assumed to have a hypochromicity of 25%.

(ii) Hydroxylapatite chromatography:

If DNA reassociation is performed at the criterion temperature and PB concentration under which double-stranded but not single-stranded DNA binds to HAP (60°C, 120 mM PB), then the degree of reassociation can be determined directly by HAP chromatography.

Samples containing DNA at 10 - 100 μg ml^{-1} in 120 mM PB were heat denatured and reassociated at 60°C for various times. Once the desired degree of reassociation had been achieved, the sample was passed through a 2 ml water-jacketed HAP column equilibrated at the criterion conditions. So as to increase the reproducibility of the results, the sample, when feasible, always contained about 250 μg of DNA and was adjusted, when necessary, to a volume of 5 ml immediately before being loaded onto the column. The column was washed with 5 ml of 120 mM PB, preheated to 60°C, and the eluates from the loading and the washing, which contained the single-stranded DNA, were pooled. The double-stranded DNA was then eluted with 5 ml of 400 mM PB at 60°C. The column was stirred throughout all the stages to prevent channeling of the HAP.

The absorbance of the fractions at 260 nm was measured and corrected for light scatter at 260 nm, due to HAP particles, by subtracting out the absorbance at 320 nm. The absorbance data for the 120 mM PB fraction was then multiplied by a factor of 0.88 to allow for the different extinction coefficients of single- and double-stranded DNA before calculating the percentage of DNA binding to the HAP. This figure was corrected for apparently artifactual 'zero time' binding of DNA to HAP as suggested by Allen *et al*. (1975) to give the percent renatured DNA (R):

$$R = \frac{\%\text{ binding to HAP} - \%\text{ zero time binding}}{100 - \%\text{ zero time binding}}$$

The percent zero time binding was determined by passing a low concentration of denatured DNA as quickly as possible through a column of HAP after cooling to 60°C. A sample having a 260 nm absorbance of 0.225 was the lowest that could be reliably recovered and this took about 1 min to process in the above manner. The observed zero time binding for *Glenodinium foliaceum* DNA was 16.8%.

2.10. RESTRICTION ENDONUCLEASE ANALYSIS OF DNA.

2.10.1. Restriction endonuclease digestions.

Restriction endonucleases *Hind* III and *Bam* HI were purchased from Sigma whilst *Eco* RI and *Pst* I were obtained from Bethesda Research Laboratories. Except where otherwise specified, 1 μg of DNA was digested in an Eppendorf tube with 1 μl of enzyme (5 - 10 units) at 37°C for 2 - 4 h in 25 μl of the buffer recommended by the manufacturers. Digestion buffers (minus BSA) and Eppen-

dorf tubes were sterilized by autoclaving before use. Reactions were terminated by rapidly cooling the tubes on ice and adding 5 μl of gel loading buffer; 40% (w/v) sucrose, 5x electrophoresis buffer, plus just enough bromophenol blue to give adequate colouration. Digests were stored at $-20^{o}C$ if they were not to be analysed immediately. After storage, Hind III digests of λ DNA were heated to $65^{o}C$ for 5 min and quickly cooled immediately before loading onto the gel so as to dissociate the sticky ends and therefore obtain the correct fragment pattern.

2.10.2. Agarose gel electrophoresis.

DNA was size fractionated by electrophoresis through horizontal 1% (w/v) agarose slabs submerged in the running buffer.

Agarose (Sigma, Type I: Low EEO) was dissolved in distilled water in a boiling water bath for 30 min. Concentrated running buffer was then added and the agarose was allowed to cool homogeneously to $60^{o}C$ before being poured into the gel mould, which constituted part of the electrophoresis box. Two sizes of gel were used, each having 7 x 4 x 1 mm sample slots. Large gels (20 x 20 x 0.5 cm) were run in Tris-borate buffer (90 mM H_3BO_4, 2.5 mM EDTA, 90 mM Tris-HCl pH 8.0) at 30 V (20 mA) for 12 - 14 h. Small gels (8 x 8 x 0.5 cm) were run in either Tris-borate or, more usually, Tris-acetate buffer (10 mM sodium acetate, 1 mM EDTA, 40 mM Tris-HCl pH 7.8) at 40 V (50 mA) for 3.5 h, after first running the samples into the gel at 5 mA for 30 min.

The gels were stained in 0.5 $\mu g\ ml^{-1}$ ethidium bromide in the running buffer for 1 h and then photographed on a high intensity ultraviolet light box (UVP inc., TM-36) through a red filter. To determine the relative intensities of the fluorescent DNA bands, the photographic negative was scanned by a densitometer (Vitatron)

2.11. GENETIC ANALYSIS OF DNA.

2.11.1. Binding of DNA to nitrocellulose paper – dot blots.

DNA dissolved in distilled water was heated to 100°C for 5 min and cooled on ice. NaOH was then added to a concentration of 0.33 M and the alkali denaturation was allowed to continue for 10 min at room temperature. The solution was neutralized by adding HCl to 0.33 M and Tris-HCl pH 8.0 to 0.1 M and then immediately chilled on ice. The DNA was spotted directly onto nitrocellulose paper (Micro Filtration Systems, 0.45 μm pore size) in 5 μl portions using an automatic pipette. The paper was supported on creased aluminium foil whilst this was done and the heat generated by an angle poise lamp positioned overhead speeded the drying of the spots between applications. When all the DNA had been loaded onto the nitrocellulose, it was left to dry for 30 min then rinsed briefly in 2x SSC before being baked in a preheated vacuum oven at 80°C for 2 h. Standard saline citrate (1x SSC) is 0.15 M NaCl, 0.15 M trisodium citrate pH 7.0.

2.11.2. Transfer of DNA from agarose gels to nitrocellulose paper – Southern blots.

DNA was transferred from agarose gels to nitrocellulose paper essentially as described by Southern (1975). After staining and photographing the gel, any unused areas were trimmed away and the DNA was denatured by soaking the gel in two changes of 1.5 M NaCl, 0.5 M NaOH for 1 h at room temperature with constant agitation. The gel was then neutralized with 1.5 M NaCl, 1 M Tris-HCl pH 8.0 for 30 min and then equilibrated with several changes of 20x SSC over a period of 2 - 3 h, again with constant agitation.

The gel was then inverted (i.e. underside uppermost) onto its glass gel plate covered by two sheets of Whatman 17MM chromatography paper that had been soaked in 20x SSC. The gel plate was on a suitable support so that the 17MM paper formed wicks into a reservoir of 20x SSC. A nitrocellulose filter was cut to size and floated onto the surface of distilled water. When it had completely wetted from beneath it was immersed in 20x SSC for 2 - 3 min. The wet nitrocellulose paper was then placed over the area of gel to be transferred being careful to exclude any air bubbles. Exposed regions of the gel were masked with "Clingfilm" as was the area of 17MM paper immediately surrounding the gel. This ensured that no fluid by-passed the nitrocellulose during blotting. Two pieces of 17MM paper, wetted in 20x SSC and cut to the same size as the gel, were then placed over the nitrocellulose, again removing all air bubbles. Finally, a 4 - 6 cm stack of dry paper towels was placed over the 17MM paper and weighted down with a glass plate under a 500 g weight. The object of the system was to set up a uniform flow of 20x SSC through the gel so that the denatured DNA fragments were eluted from it and deposited onto the nitrocellulose with a minimal loss of resolution.

Transfer of DNA was allowed to proceed for 16 - 18 h, after which time the paper towels and pieces of 17MM paper above the gel were removed. The gel and attached nitrocellulose was then layed gel side up on a piece of filter paper and the positions of the gel slots were marked using a soft pencil. After removing the gel, the nitrocellulose was soaked briefly in 2x SSC and left to dry on filter paper. The DNA was baked onto the nitrocellulose at 80°C for 2 h in a vacuum oven.

2.11.3. Preparation of plasmid DNA from transformed *E. coli* for use as probes.

Stab cultures of *Escherichia coli* strain HB101 containing

the plasmids pSSU60 or pSSU160 were obtained from Professor R. John Ellis. These plasmids contain cDNA fragments of the gene for the small subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase of *Pisum sativum* inserted into the unique *Hind* III restriction site of the vector pBR322 (Bedbrook *et al.*, 1980). The bacterial cultures were maintained at $4^{o}C$ on slopes of L-broth solidified with 1.5% (w/v) agar. Standard aseptic precautions were observed for all manipulations.

Plasmids were prepared from the clones by a modification of the rapid, small-scale isolation method developed by Birnboim and Doly (1979). 5 ml of L-broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl), containing 25 $\mu g\ ml^{-1}$ ampicillin for antibiotic selection, was inoculated with a single colony of *E. coli* and vigorously shaken overnight at $37^{o}C$. The antibiotic was introduced from a freshly prepared stock which had been sterilized by passage through a 0.22 μm Millipore filter. 3 ml of the overnight culture was pelleted into a single 1.5 ml Eppendorf tube in two stages, centrifuging for 15 s each time in a microcentrifuge. All the medium was removed and the pellet was suspended in 100 μl of fresh 4 mg ml^{-1} lysozyme (Sigma, Grade I from chicken egg white) dissolved in 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0 by immediate vortexing. The suspension was left to stand for 5 min at room temperature to allow lysis to occur, after which time 200 μl of freshly prepared 0.2 M NaOH, 1% (w/v) SDS was added and mixed by two or three sharp inversions of the tube so as not to shear the chromosomal DNA. The mixture was incubated on ice for 5 min to denature the DNA and then 150 μl of an ice cold solution of potassium acetate (pH 4.8) was added. 100 ml of potassium acetate solution contained 60 ml of 5 M potassium acetate and 11.5 ml of glacial acetic acid. The tube was gently, and briefly, vortexed on its cap and once again stored on ice for 5 min to allow the DNA to renature. The large aggregate of chromosomal DNA which formed was then pelleted by centrifugation for 1 min in the microcentrifuge and the super-

natant, containing the plasmid DNA, was carefully transferred to a new Eppendorf tube using a Pasteur pipette. The plasmids were then preferentially precipitated from the mixture by adding 0.54 volumes of propan-2-ol at room temperature for 2 min. The DNA was collected by 1 min centrifugation and the pellet was washed with 500 μl of 70% (v/v) ethanol before being dried under vacuum.

At this stage the plasmids were substantially contaminated with RNA and other material and required further purification before they could be used as hybridization probes. Plasmids prepared from 12 ml of culture were first dissolved in 500 μl of 1 mM EDTA, 10 mM Tris-HCl pH 8.0 containing 50 μg ml^{-1} RNase (Sigma Type XII-A from bovine pancreas) and incubated at 37°C for 30 min to digest the RNA. The RNase was added from a 10 mg ml^{-1} stock solution in 15 mM NaCl, 10 mM Tris-HCl pH7.5 which had previously been heated to 100°C for 15 min to denature contaminating DNases. The digestion was terminated by two phenol extractions and the DNA was collected by an ethanol precipitation.

For further purification, the pellet was dissolved in 100 μl of 0.5 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.2 and loaded onto a microcolumn of pre-packed NACS resin (BRL). After extensive washing of the column with the binding buffer, the plasmid DNA was selectively eluted twice with 250 μl of 0.7 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH7.2. Under these salt conditions any chromosomal DNA present in the preparation remains bound to the resin (BRL NACS Applications Manual, see also Plate 4.8.1).

After purification the yield of DNA from 12 ml of bacterial culture was less than 1 μg and could not be quantified accurately at this stage. All plasmid mini-preps were analysed by agarose gel electrophoresis before use.

2.11.4. Labelling of probes with biotin by nick translation.

Biotin-11-dUTP, an analogue of dTTP, was incorporated into plasmid DNA in the presence of the deoxynucleotide triphosphates dATP, dGTP and dCTP by nick translation (Langer *et al.*, 1981).

Up to 1 μg of plasmid DNA was dissolved in 20 μl of 120 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.5 and cooled on ice. To this was added 2.5 μl of 0.4 mM biotin-11-dUTP (BRL) and 5 μl of a stock solution containing 0.2 mM each of dATP, dCTP and dGTP in 50 mM $MgCl_2$, 100 mM 2-mercaptoethanol, 100 $\mu g.ml^{-1}$ BSA, 500 mM Tris-HCl pH 7.8 (BRL Nick Translation Reagent A4). After adjusting the volume of the mixture to 45 μl with distilled water, 5 μl of a solution containing 0.4 units μl^{-1} DNA polymerase I and 40 pg μl^{-1} DNase I (BRL Nick Translation Reagent C) was added. The solution was then gently mixed and centrifuged to the bottom of the tube before being incubated at 15°C for 90 min.

The reaction was stopped by adding 5 μl of 0.3 M EDTA pH 8.0 and 1 μl of 5% (w/v) SDS. Biotin-labelled DNA was separated from unincorporated nucleotides by two ethanol precipitations and repeated 70% (v/v) ethanol washes after first adding 50 μg of sonicated, single-stranded, salmon sperm DNA to act as a carrier. The final pellet was dissolved in 100 μl of 0.1% (w/v) SDS, 0.1x SSC, 10 mM EDTA pH 7.0 and stored at -20°C.

The quantity of biotin-labelled probe was assayed by spotting a ten-fold dilution series directly onto a strip of nitrocellulose paper and visualizing the biotinylated DNA by means of the BRL DNA detection system (see Section 2.11.6). The colour intensity of the spots was compared with that of known amounts of labelled λ DNA (BRL) also spotted onto nitrocellulose and developed alongside the unknowns (see Plate 4.8.2).

2.11.5. Hybridization of biotinylated probe DNA to dot blots and Southern blots.

Hybridization of labelled probe DNA to target DNA bound to nitrocellulose paper was performed in heat-sealable polythene bags so as to minimize the volume of the hybridization solutions containing the probes. Prehybridization and hybridization buffers were slightly modified from those of Wahl *et al.*, (1979):

	Pre-hybrid. buffer	Hybrid. buffer
formamide[a] (v/v)	50 %	45 %
SSC	5 x	5 x
BSA (w/v)	0.1 %	0.1 %
polyvinylpyrrolidone[b] (w/v)	0.1 %	0.1 %
Ficoll 400 (w/v)	0.1 %	0.1 %
sodium phosphate pH 6.5	25 mM	20 mM
denatured salmon sperm DNA[c]	250 μg ml^{-1}	250 μg ml^{-1}
sodium detran sulphate[b] (w/v)	-	8 %

(Notes: a. commercial formamide (Sigma) was further purified as follows. The formamide was crudely recrystallized by initiating crystallization on the side of the bottle at -20°C for 1 h and then leaving the bottle under ice overnight. The liquid fraction was then stirred with Amberlite MB1 ion exchange resin (4 g of dried resin per 100 ml of formamide) for 4 h. The resin had been washed in ethanol then ethanol:ether (1:1 v/v) before being dried under vacuum, and was removed from the formamide by filtration. b. Average molecular weight 40,000. c. Salmon sperm DNA (Sigma, Type III) was dissolved in water at 10 $mg.ml^{-1}$ and sonicated to reduce its molecular weight as described in Section 2.9.1. The DNA was denatured by heating to 100°C for 10 min and was then quickly chilled).

The nitrocellulose was incubated with prehybridization buffer (1 ml per 10 cm^2 of paper) in a polythene bag submerged in a water bath at 42°C for 3 h. During this time the nitrocellulose paper was frequently agitated to remove air bubbles and so ensure that the binding sites on the filter paper were completely saturated with single-stranded salmon sperm DNA. After prehybridization, the buffer was removed from the bag and replaced with the same volume of pre-heated hybridization buffer. The denatured probe was then mixed with a small volume of the hybridization buffer and pipetted into the bag which was resealed, being careful to remove as much air as possible, and returned to the 42°C water bath. The probe had been denatured by heating to 100°C for 5 min and cooled quickly in an ethanol/ice bath to prevent renaturation.

Hybridization at 42°C was done to 1-4 x $C_ot_{1/2}$ by incubating the probe with the nitrocellulase filter for the number of hours estimated from the following equation (Maniatis et al., 1982):

$$\text{no. hours to achieve } 1\ C_ot_{1/2} = \frac{VX}{25.w}$$

where; V = volume of hybridization buffer (in ml),
X = complexity of probe DNA (about 5 kb for pBR322),
w = weight of probe added (in μg),

Consequently hybridization times varied from 2 - 25 h depending on the concentration of the probe used. Details of probe concentrations and hybridization times are given in the results.

After hybridization, the filter was removed from the bag and washed with 2x SSC, 0.1% (w/v) SDS then 0.2x SSC, 0.1% (w/v) SDS at room temperature. Both washes were repeated once. The filter was then washed at a high stringency with 0.16x SSC, 0.1% (w/v) SDS at 50°C for 15 min, again repeating once. These washes were those recommended by Leary _et al_. (1983) for hybridizations performed in 45% formamide. Finally, the nitrocellulose paper was briefly rinsed in 2x SSC, 0.1% (w/v) SDS at room temperature before proceeding with the assay for biotin as described below.

2.11.6. _Detection of biotinylated DNA on nitrocellulose paper._

Biotin-labelled probe DNA that had hybridized to DNA bound to the nitrocellulose was visualized by the colourimetric method developed by Leary _et al_. (1983) and marketed by Bethesda Research Laboratories (BRL DNA Detection System). All buffers were filtered through a 0.22 µm Millipore filter before use, and unless otherwise stated, all stages were performed at room temperature in suitable plastic trays.

The nitrocellulose paper was washed briefly in '7.5 buffer' (0.1 M NaCl, 2 mM $MgCl_2$, 0.05% (v/v) Triton X-100, 0.1 M Tris-HCl pH 7.5) and blocked by incubating it in 3% (w/v) BSA in 7.5 buffer for 20 min at 42°C. The blocked filter was air dried and baked _in vacuo_ at 80°C for 10 - 20 min and could be stored in this condition.

After rehydrating the nitrocellulose in the buffered BSA for 10 min, the paper was exposed to 2 µg ml^{-1} streptavidin (BRL) in 7.5 buffer for 10 min. 3 ml of streptavidin was used per 100 cm^2 of nitrocellulose and the solution was gently pipetted over the

surface of the filter throughout the incubation period. Streptavidin which had not bound to the biotin-labelled probe was removed by three 3 min washes with 7.5 buffer using at least 100 cm^3 of buffer per 100 cm^2 of nitrocellulose for each wash. The nitrocellulose was then incubated with 1 $\mu g.ml^{-1}$ biotinylated calf intestinal alkaline phosphatase polymer (poly(BAP)) (BRL) in 7.5 buffer for 10 min in exactly the manner as described for the streptavidin and washed twice with at least a 30-fold excess of 7.5 buffer as before, and then with a similar volume of '9.5 buffer' (0.1 M NaCl, 50 mM $MgCl_2$, 0.1 M Tris-HCl pH 9.5).

To detect the poly(BAP) complexed to the streptavidin linker, the filter was incubated with a mixture of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro-blue tetrazolium (NBT) in a sealed plastic bag. Sufficient dye solution to develop a 100 cm^2 filter was freshly prepared by mixing 33 μl of 75 $mg.ml^{-1}$ NBT solution (BRL) with 7.5 ml of 9.5 buffer and then adding 25 μl of 50 $mg.ml^{-1}$ BCIP solution (BRL) followed by gentle mixing. Both dye stock solutions were dissolved in 70% (v/v) dimethylformamide. The bags in which Sterilin petri dishes were packed were used for incubating the nitrocellulose paper as these did not appear to interfere with proper colour development since 5 pg of biotinylated λ DNA could readily be visualized.

Colour development was allowed to proceed in the dark for 4 h, or longer if no specific hybridization was detectable after this time. However long incubations resulted in a high, and generally uneven, non-specific background colour development, which was considerably worse if the 7.5 and 9.5 buffers had not been filtered. Development was terminated by washing the filter in 5 mM EDTA, 20 mM Tris-HCl pH 7.5. The filter was photographed and stored dry in the dark.

2.12. QUANTITATIVE EXTRACTION AND ESTIMATION OF DNA.

2.12.1. Extraction of DNA from whole cells and nuclei.

DNA was quantitatively extracted from *Glenodinium foliaceum* by combining the preliminary extractions of Ogur and Rosen (1950) with a modification of Schneider's method of extracting total nucleic acids using hot 5% perchloric acid (Schneider, 1945).

About 10^6 cells were collected from a mid-exponential phase culture by slow speed centrifugation: 2 min at 1,300 g using a MSE 12 x 100 ml swing out rotor. The cell pellets were washed three times by resuspending them in 100 ml of sterile ASP_7 and then repelleting the cells again as above. After the final wash the pellets were homogeneously resuspended in a combined volume of exactly 50 ml. Three 1 ml samples were then withdrawn and fixed by adding one drop of 40% (v/v) formaldehyde for later counting as described in Section 2.2.1. (Note: since the samples were diluted before counting the volume of fixative added was inconsequential). All the cells in the suspension were then collected by centrifugation at 12,000 g for 5 min in a Sorvall SS-34 rotor.

The pellet was homogenized in 80% (v/v) ethanol at 4^oC using a tight-fitting, Potter-Elvehjem homogenizer as described in Section 2.5.3. Homogenization was continued until microscopic examination revealed that very few unbroken cells remained. The homogenate was washed into a screw-capped test tube and pelleted at 4^oC in an MSE 'Minor' centrifuge set at maximum speed. The residue was then sequentially extracted as follows:

1st extraction: 70% (v/v) ethanol; 5 min; 4°C.
2nd extraction: 70% (v/v) ethanol, 0.1% (v/v) perchloric acid; 5 min; 4°C.
3rd extraction: 3:1 (v/v) ethanol:diethyl ether; 3 min; 65°C (i.e. gently boiling in a water bath).
4th extraction: 2% (v/v) perchloric acid; 1 min; 4°C.

All extraction were performed twice, using 5 ml of solvent for each extraction. Residues were thoroughly resuspended by vortexing and collected by centrifugation in the MSE Minor centrifuge. After the cold acid treatment, the residue was resuspended in 2 ml of 5% (v/v) perchloric acid (AR grade) and incubated at 70°C for 40 min to extract the nucleic acids.

DNA was simply extracted from isolated nuclei with the hot 5% perchloric acid. No preliminary extractions were necessary.

2.12.2. Estimation of DNA in extracts.

Burton's modification of the diphenylamine reaction was used to estimate deoxyribose in the perchloric acid extracts (Burton, 1956). Diphenylamine (Sigma, white crystals) was further purified by recrystalization from alcohol. The diphenylamine was dissolved in a minimal volume of ethanol and cooled to -20°C. The slurry of crystals was then collected on Whatman No.1 filter paper and dried under vacuum.

To assay for DNA, 2 ml of diphenylamine reagent was added to 1 ml of the perchloric acid extract which had been cleared by centrifugation. The diphenylamine reagent was prepared immediately before use by dissolving 0.75 g of diphenylamine in 50 ml of glacial acetic acid and then adding 0.75 ml of concentrated sulphuric acid and 0.25 ml of aqueous acetaldehyde solution

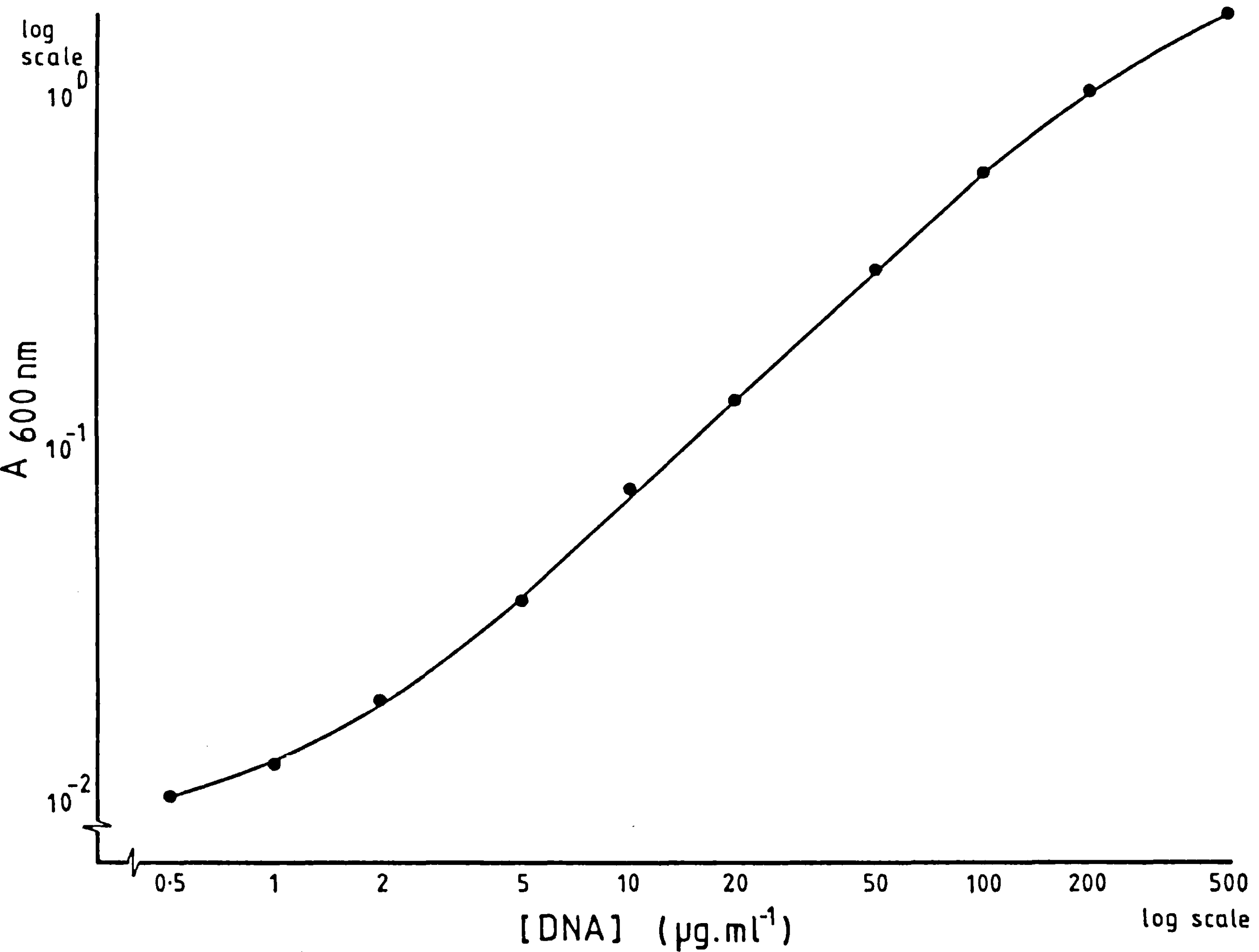


Figure 2.4. Calibration curve for the diphenylamine assay of DNA. 600 nm absorbance of standard DNA solutions incubated with 2 volumes of diphenylamine reagent at 30°C for 18 h.

perchloric acid and diphenylamine reagent was incubated at 30°C for 18 h to develop the blue colour reaction which was quantified by measuring the absorbance at 600 nm.

A series of standard DNA solutions (0.5 - 500 $\mu g.ml^{-1}$ in 5% (v/v) perchloric acid) were assayed in the same manner as described above so as to calibrate the reaction. These DNA standards were prepared by diluting a 1 $mg.ml^{-1}$ stock solution of highly polymerized calf thymus DNA (Sigma) which had been dissolved in 5% (v/v) perchloric acid at 70°C for 40 min. The absorbance at 600 nm was found to be directly proportional to the DNA content in the range of 5 - 100 $\mu g.ml^{-1}$ DNA (Figure 2.4). There was no advantage in subtracting the absorbance at 540 nm (Jensen, 1962). The number of cells or nuclei from which the sample DNA was extracted was carefully chosen so that the concentration of DNA in the extract fell within the upper part of this range. A 10^{-1} dilution of the sample could then be used to provide confirmation of the DNA assay.

CHAPTER 3

GENERAL OBSERVATIONS ON GLENODINIUM FOLIACEUM

3.1. CELL MORPHOLOGY, GROWTH AND DIVISION.

3.1.1. Cell morphology.

Viewed under the light microscope, *Glenodinium foliaceum* exhibits the external features characteristic of dinoflagellates in general (Plate 3.1.1). In culture the cells vary greatly in size, ranging from 9 - 40 μm wide and 11 - 45 μm long. The cell covering (theca or amphiesma) is divided into an epicone and a hypocone by a transverse groove (the girdle) in which is appressed the helical, transverse flagellum. The free, longitudinal flagellum emerges near to the transverse one where a longitudinal channel in the hypocone (the sulcus) joins the girdle. The detailed external structure of the cell has been described by several authors (Biecheler, 1952; Prager, 1963; Silva, 1962) but its most striking feature is its marked dorso-ventral flattening (*foliaceum* = "leaf-like"), which is unusual for a planktonic species.

Using Nomarski interference contrast enhancement, a few of the major cell organelles can be discerned. The numerous chloroplasts are peripherally located and are more or less discoid in shape in some individuals but in others they may be fairly elongated. Tenuous connections are sometimes present between two

Plate 3.1. Morphology and cell division in Glenodinium foliaceum. Nomarski interference contrast micrographs of live cells.

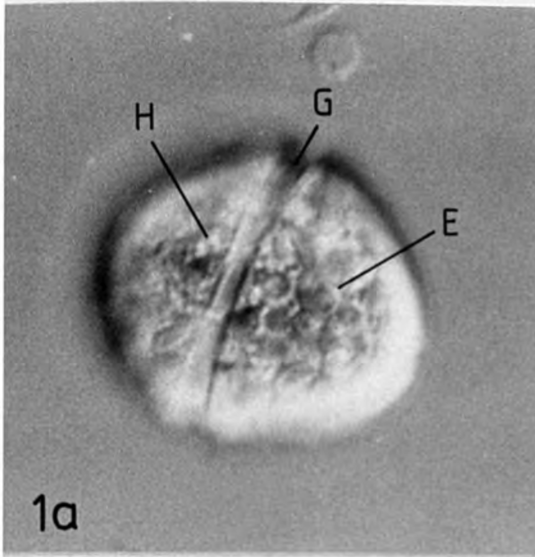
1. Two planes of focus through a motile cell:
   a. surface view showing the theca divided into the epicone (E), hypocone (H) by the girdle (G).
   b. sub-medial optical section showing the eyespot (ES).
   x 1,300

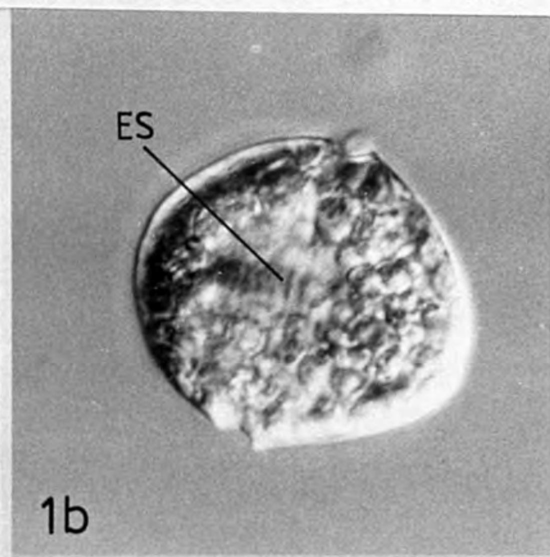
2. Cell in various stages of division removed from the bottom of a culture flask: (1), predivision cell; (2), cell undergoing cytokinesis; (3), divided cell. Note the dinoflagellate nucleus in cell 2 (Nd) and the accumulation body in cell 3 (A), and also the remains of discarded thecae (T). x 1,150.

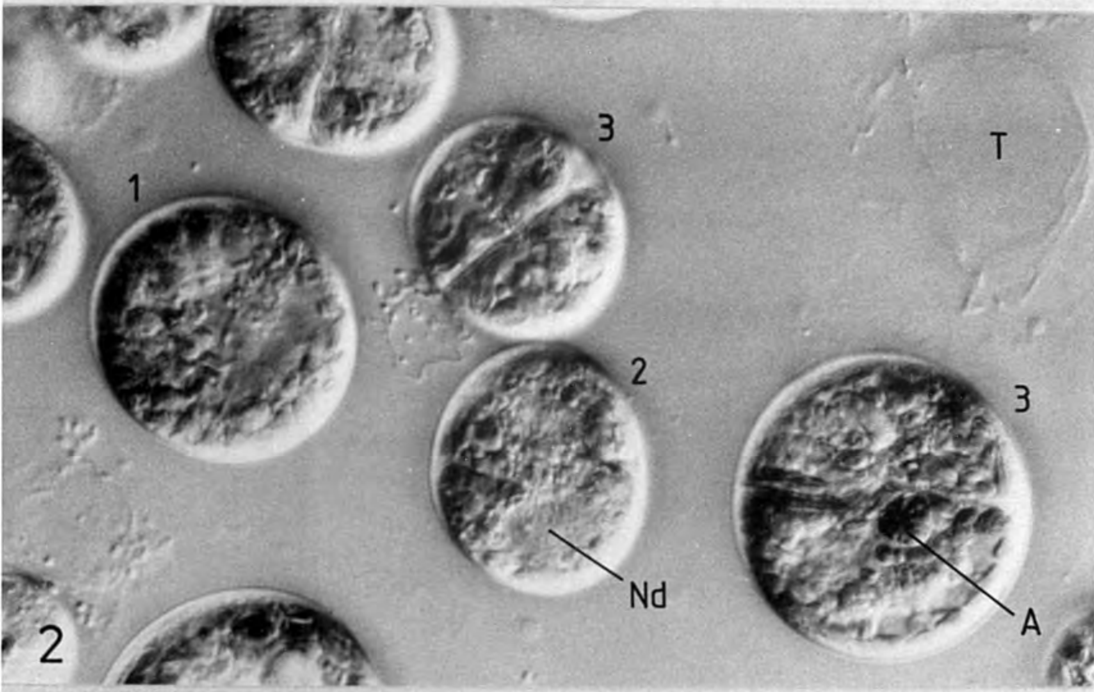
3. Four daughter cells still enclosed within the wall of the mother cell. x 1,200.

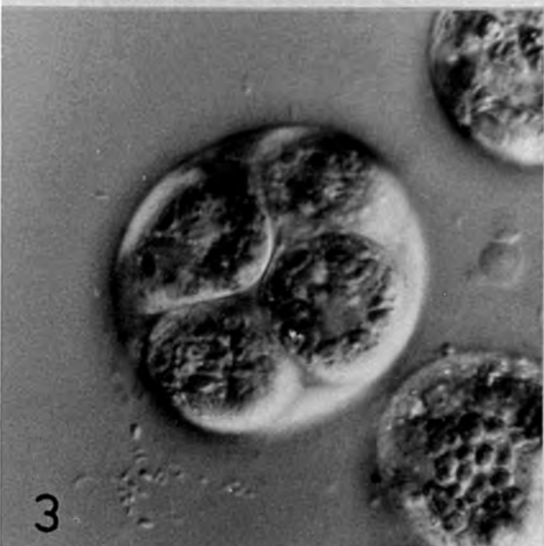
4. Doublet of normal motile cells possibly representing fusing gametes. Note the two longitudinal flagellae (LF). x 1,200.

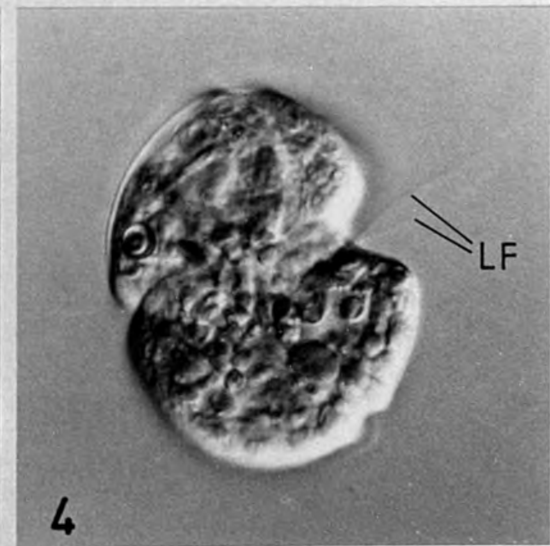
Plate 3.1











or more of the chloroplasts particularly in the larger cells. These connections are most readily seen if the autofluorescence of the chlorophyll is viewed under incident ultraviolet excitation. The overall orange-brown colour of G. foliaceum cultures is in part due to the chloroplast pigmentation but also due to the presence of one or more similarly coloured "accumulation bodies". These may become quite large in relation to the size of the cell, particularly in old cultures. The only other pigmented structure is the prominent red eyespot which lies beneath the sulcal region. A nucleus containing a disordered array of condensed chromosomes is also usually discernible towards the central region of the cell and the surrounding cytoplasm is generally packed with starch grains. In non-axenic cultures bacteria are frequently seen in senescent cells but appear to be absent from healthy individuals.

3.1.2. Growth.

The growth of an essentially bacteria-free culture of Glenodinium foliaceum in 500 ml of the synthetic medium ASP_7 is shown in Figure 3.1. This culture volume seemed to provide the most favourable conditions for growth, but even so, the doubling time during the exponential growth phase was only 4.4 days. Since all the cells do not divide by binary fission, then the actual generation time is even greater. The doubling time of G. foliaceum is compared with those of some other dinoflagellates in Table 3.1. In most species the doubling time corresponds more closely to the generation time so it is evident that under favourable conditions, G. foliaceum has a substantially longer cell cycle than other dinoflagellates, with the exception of Peridinium balticum. It is interesting to note that a slow growth rate is often characteristic of symbiotic relationships (Margulis, 1981). From a practical point of view, however, the growth characteristics of G. foliaceum makes it an unfavourable species

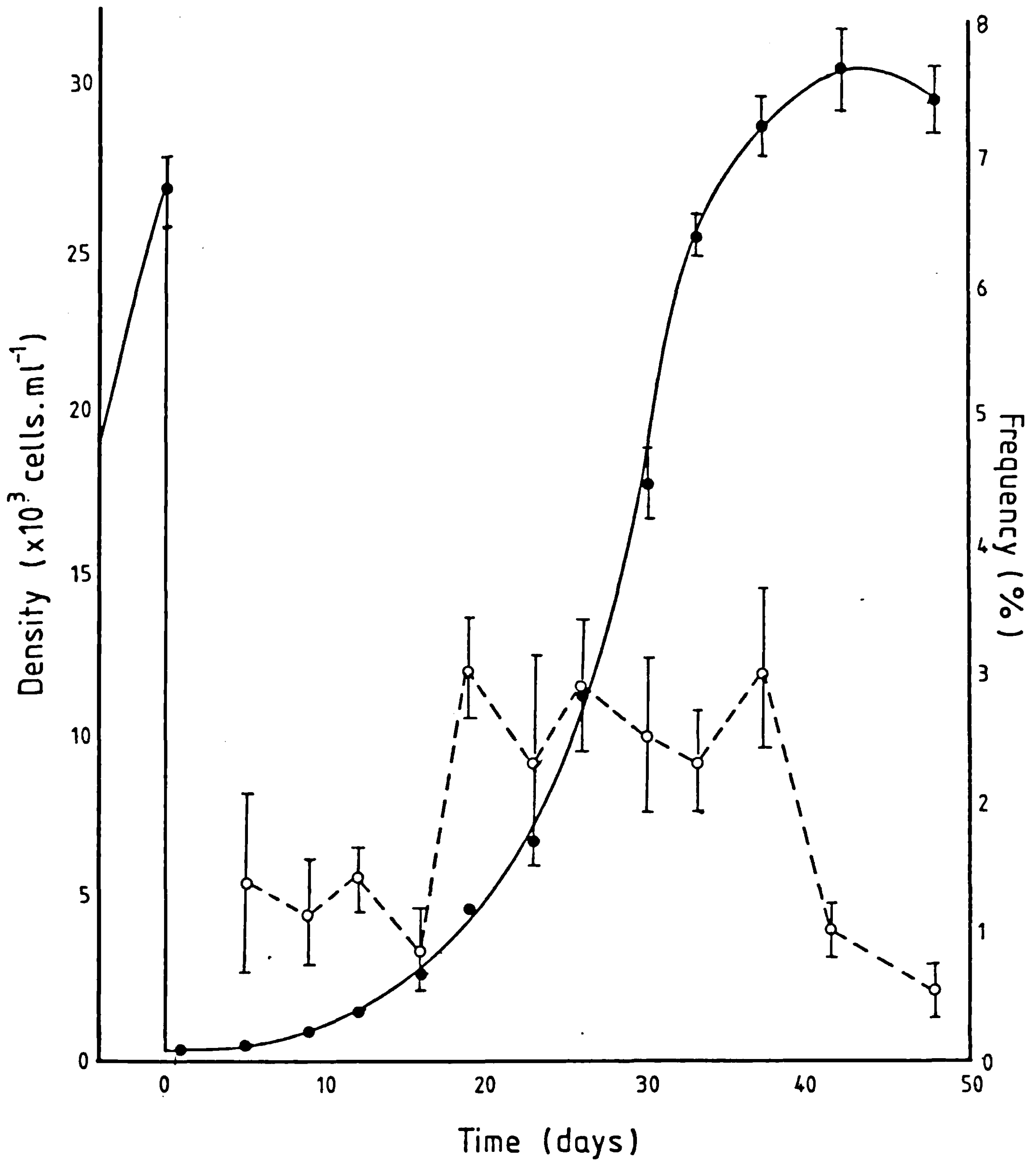


Figure 3.1. Growth characteristics of <u>Glenodinium foliaceum</u>. 500 ml of ASP_7 was inoculated with 5 ml of late exponential phase culture of low bacterial density on day 0 and incubated under continuous light at 18°C. ●——●, cell density; ○- - -○, frequency of dividing cells. Points are the means of 10 Sedgewick Rafter row counts and the bars indicate standard errors greater than the symbol size.

Table 3.1. Doubling times (in days) of photosynthetic dinoflagellates.

Species	Doubling time	Notes
Amphidinium carteri	0.8,0.7	a,b
Ceratium dens	3.3	c
C. furca	4.6	c
Dinophysis fortii	1.4	c
Glenodinium foliaceum	5.6,4.4,6.2	b,d,e
Gonyaulax tamarensis	2.4	a
Gymnodinium nelsonii	3.0	a
Heterocapsa niei	1.4	f
H. pygmaea	1.7	a
Peridinium balticum	5.0	b
Prorocentrum triestinum	1.4	a
Scrippsiella trochoidea	2.6	a
Woloszynskia coronata	3.0	g

Notes:

a. 2.8 l non-axenic cultures in a natural seawater medium on a 12L:12D photoperiod (Karentz, 1983).
b. Axenic cultures in a synthetic marine medium under continuous light (Morrill and Loeblich, 1979).
c. Natural algal populations (Weiler and Chisholm, 1976).
d. Present study, continuous light.
e. Present study, 12L:12D photoperiod.
f. Non-axenic cultures in a natural seawater medium on a 12L:12D photoperiod (Morrill and Loeblich, 1984).
g. 100 ml non-axenic cultures in an artificial freshwater medium under continuous light (P.D. Burton, personal communication).

for biochemical study.

3.1.3. Cell division.

Dividing and pre-division cells of G. foliaceum are easily distinguishable in live preparations as they are spherical and non-motile. Before division the cell discards its theca and secretes a new thickened wall. Within this wall the protoplasm is seen to divide into two (Plate 3.1.2) or, more rarely, three or four (Plate 3.1.3). On a few occasions a cell was seen to have divided to produce eight daughter cells (Plate 3.3.6). Similar multiple divisions have been reported by Silva (1962) and Blanchard-Babillot (1972). Although some of the two or three cell types may represent intermediate division stages, the majority of cells probably divide to produce only two daughters, considering the low frequency of multiple division cells in a culture (see Table 3.2). Also, many dividing cells are of an intermediate size and are seen to contain two daughter cells of approximately the same size as those in a tetrad. Therefore, if all divisions resulted in four daughters, the culture as a whole would show an eight-fold, rather than the observed four-fold, range of cell sizes.

Another possibility is that tetrads are part of the sexual cycle of G. foliaceum. Occasionally doublets of normal motile cells are seen (Plate 3.1.4). These probably represent gametes fusing rather than abnormal asexual divisions as suggested by Silva (1962). However, it does not necessarily follow that the resulting zygote will divide to produce a tetrad of meiocytes since the two meiotic divisions may be temporally separated, as in Gyrodinium uncatenum (Coats et al., 1984).

When maintained under continuous light, only about 1% of the cells are seen to be undergoing cell division during the exponen-

tial growth phase (Figure 3.1). As the culture drops out of true exponential growth after about 20 days, the frequency of dividing cells increases to 2 - 3% but falls once again to less than 1% on the approach of stationary phase. For reasons which will become apparent later, it would be interesting to study the cell cycle of G. foliaceum, particularly the process of DNA synthesis and the ultrastructural aspects of cell and nuclear division. Such studies ideally require the production of cultures in which cell division occurs more or less simultaneously in all members of the population (synchronous division), or at least in a large proportion of the cells (phased division).

The presence of phased cell division is well documented in natural assemblages of marine, photosynthetic dinoflagellates (see Weiler and Chisholm, 1976), where cell division generally occurs near dawn. The natural situation can be mimicked by growing cultures under light/dark cycles. Consequently the phased division of a number of dinoflagellates has been studied in the laboratory (Hastings and Sweeney, 1964). Again most species divide near to the onset of the light period, but exceptions have been found. For example, Gymnodinium splendens divides at the beginning of the dark period, whilst the peak of cell division occurs at the end of the light period in Prorocentrum micans.

To assess the degree of phased cell division that can be achieved with Glenodinium foliaceum, 100 ml cultures were grown under alternating 12 h light/dark cycles at a constant temperature (18°C). After one month of phasing conditions, one 24 h cycle was monitored continuously by determining, optically, the percentage of cells in division at 75 min intervals during the cycle. The number of daughter cells produced by each division was also recorded. A previously undisturbed replicate culture was used for each determination to avoid any adverse effects caused by the repeated mixing of a single culture (Karentz,

1983). The flasks were wrapped in foil at the beginning of the dark cycle to prevent exposure of the cultures to the laboratory lights whilst sampling. At various times during the 24 h cycle a culture was also counted electronically using a Coulter Counter and re-counted 3.5 to 3.75 h later to record changes that may have occurred in the cell size distribution of the population.

Under this constant temperature, 12:12 L:D cycle, the majority of cell divisions took place during the dark period (Figure 3.2). The maximum division frequency, f_{max}, was 7.5% and occurred 5 - 6 h into the night. Under continuous light only 1 - 2% of the cells were seen to be dividing at any one time. With the Coulter Counter, shifts in the frequency distribution of cell sizes could be detected. During the dark period the number of large cells (diameter greater than 26 μm) decreased and the number of small cells (diameter less than 21 μm) increased correspondingly (Figure 3.3). The reverse size shift occurred during the light period as more cells entered the large cell population than were eliminated by cell division. It is difficult to quantify the phasing of cell division from the Coulter Counter results for the reasons outlined previously (see Section 2.2.2) but a simple analysis can be performed on the division frequency data.

Taking the width of the division peak at $f_{max}/2$ in Figure 3.2 to approximate to the time interval (T) between the initiation of cytokinesis and the release of the daughter cells, then the percentage of cells dividing during one 24 h cycle (f_{tot}) is:

$$f_{tot} = \frac{\int_{t=0}^{t=24} f.dt}{T} = \frac{55}{4.5} = 12.2\%$$

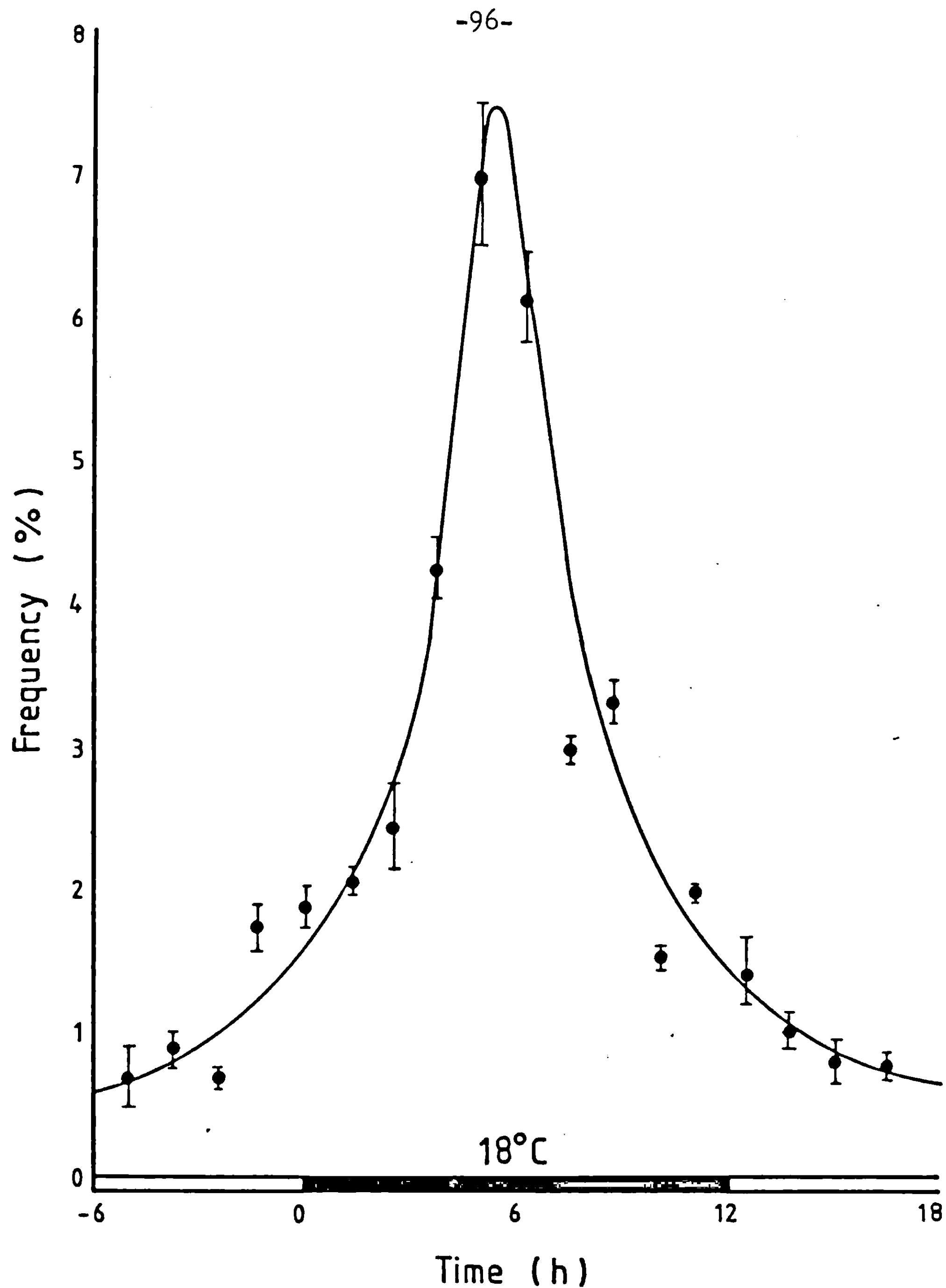


Figure 3.2. Phased cell division of Glenodinium foliaceum at constant temperature. Cultures were grown under alternating 12 h light/dark cycles at a constant 18°C and the frequency of dividing cells was determined at intervals during one 24 h cycle. Points are the means of two determinations (1,000 cells counted per determination) and the bars indicate the standard errors. The beginning of the dark period is defined as time 0.

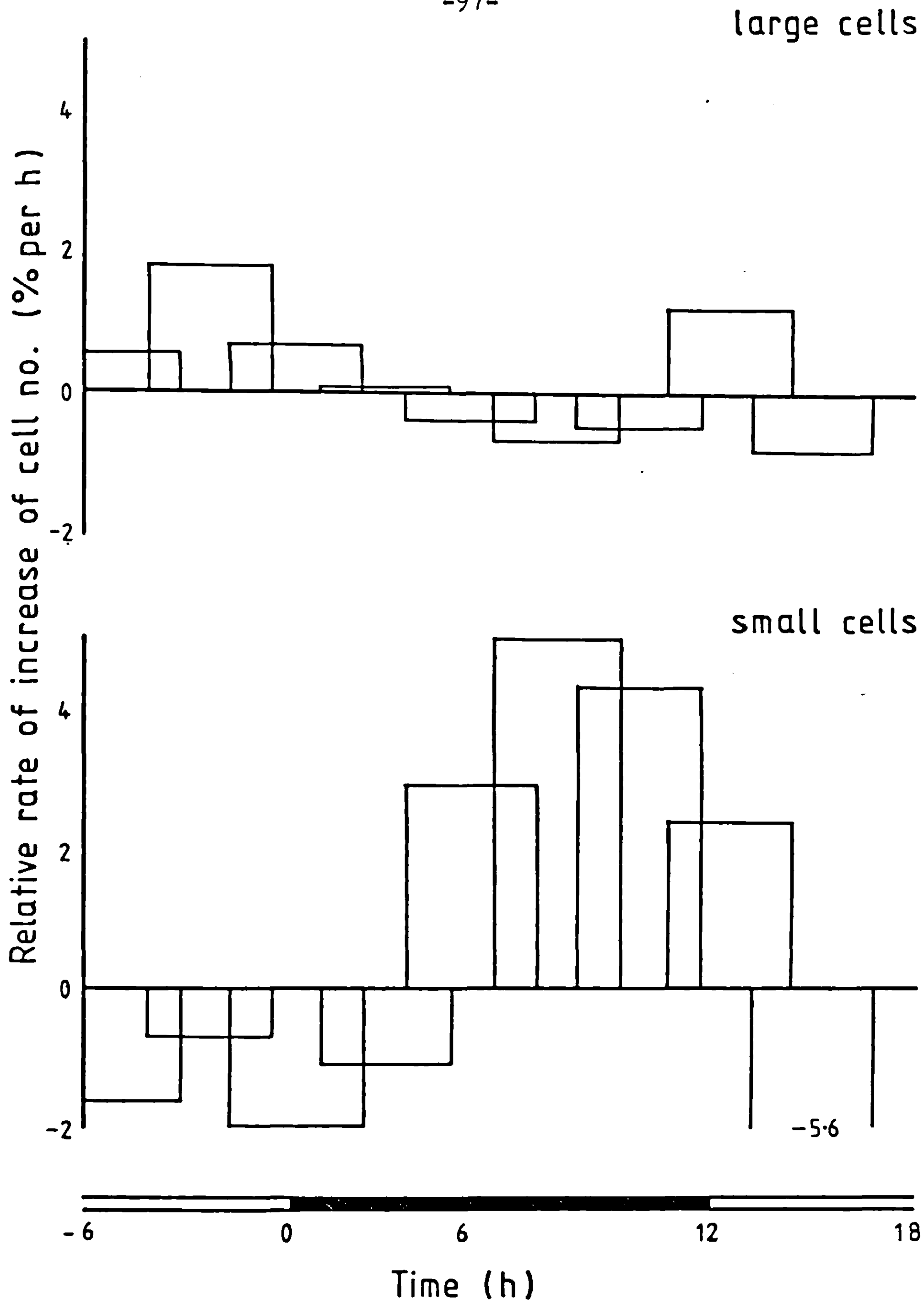


Figure 3.3. Cell size changes during the phased growth of *Glenodinium foliaceum*. Average rate of change in the number of cells greater than 26 µm in diameter ('large cells') and less than 21 µm in diameter ('small cells') during periods throughout the phased division cycle illustrated in Figure 3.2. Changes in cell number are expressed relative to the initial culture density.

Table 3.2. Average frequency of division types in a phased culture of *Glenodinium foliaceum* during a 24 h period (1,967 cells were counted).

No. of daughter cells	frequency (%)
2	78.0
3	11.5
4	10.5

This suggests a generation time of about 8 days and since *G. foliaceum* produces, on average, 2.33 daughter cells per division (Table 3.2), then f_{tot} represents a 16.2% increase in the population, i.e. a doubling time of 6.2 days. The discrepancy between f_{max} and f_{tot} gives an indication of the degree of phasing in the culture. Defining the phasing coefficient, P, as:

$$P = \frac{f_{max}}{f_{tot}}$$

then for a culture of *G. foliaceum* maintained at 18°C under a 12:12 L:D cycle, P = 0.61. This means that only 61% of the cells which divide during a single cycle actually begin to do so during the time equivalent to T/2 before f_{max}.

In an attempt to increase P, cultures were subjected to a synchronous light and temperature cycle: 12 h light, 24°C / 12 h dark, 16°C. Under these conditions the division peak occurred

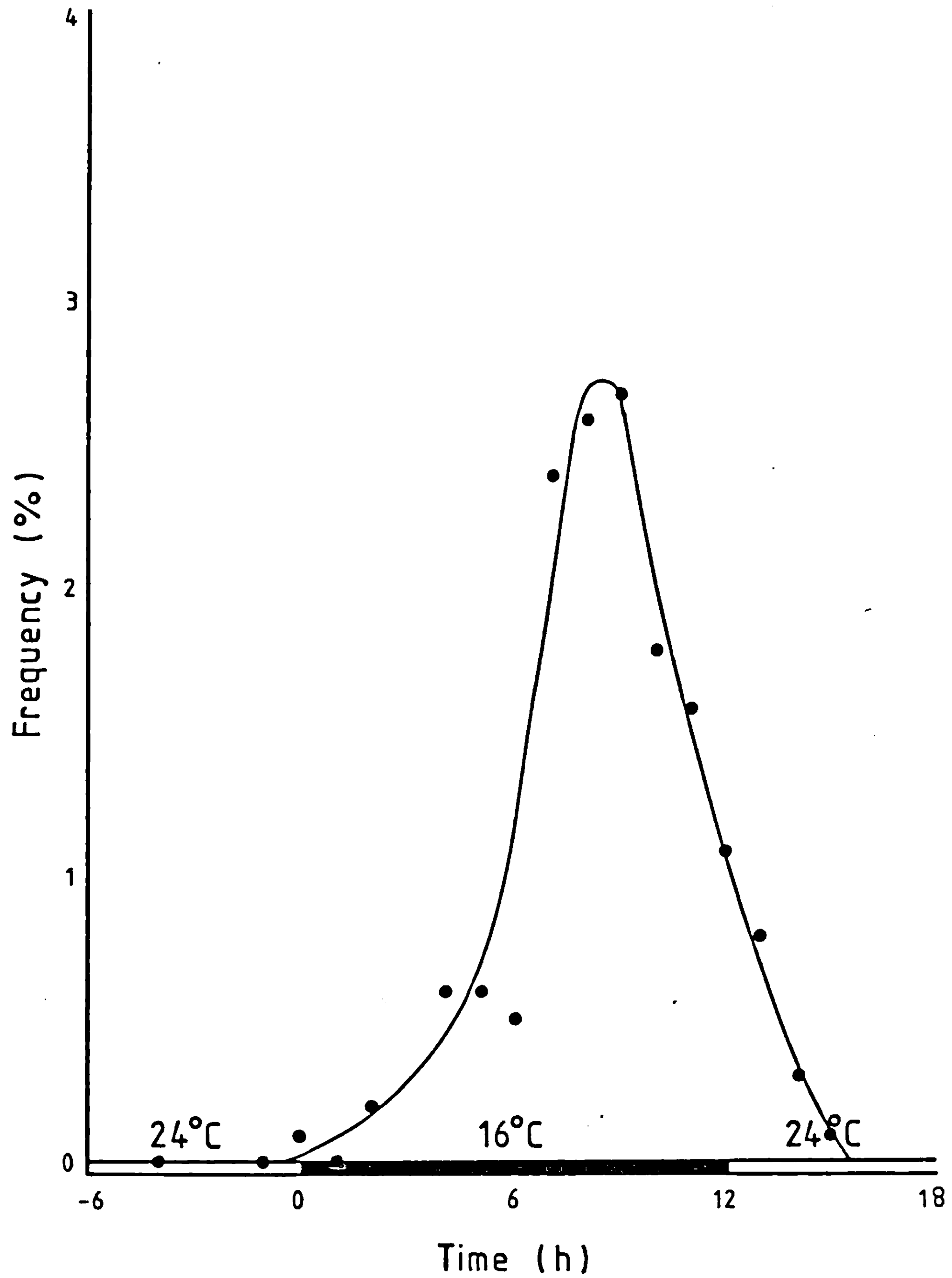


Figure 3.4. Phased cell division of Glenodinium foliaceum under varying temperatures. Details as for Figure 3.2 except that the cultures were subjected to hot days (24°C) and cold nights (16°C). Only one determination of division frequency was made.

later in the dark period (Figure 3.4) and P was 0.92. However, although the degree of phasing was greatly improved, the growth of *G. foliaceum* was extremely poor and consequently f_{max} was only 2.75%.

It seems unlikely that the value of f_{max} for *G. foliaceum* can be increased significantly by manipulating the length of the light and dark periods. Such trial and error experiments are only worthwhile for species in which the generation time is near to 24 h (Hastings and Sweeney, 1964). For example, with *Prorocentrum micans* a phased increase of 45% could be obtained by adjusting the length of the dark and light cycles (Filfilan and Sigee, 1977), whilst with *Amphidinium carteri*, one of the few dinoflagellates which divides every 24 h, a synchronous culture could be produced by similar means (Galleron, 1976). Synchrony in *Crypthecodinium cohnii* can be obtained by inducing swarmer cells to excyst in unison (Franker *et al*., 1973). This method of initiating synchrony, rather than imposing it, could be of use with *G. foliaceum*. Preliminary experiments suggested that it may be possible to initiate cultures with dividing cells isolated on the basis of their greater density. However, such cultures would have an inherent tendency to become asynchronous because of the inconsistent number of daughter cells produced at each division. Presumably a daughter cell resulting from a cell dividing into four would take twice as long to reach the critical division volume than one of two daughters.

Thus, until a higher degree of synchrony is obtained it would prove difficult to study the ultrastructure of cell and nuclear division in *G. foliaceum* and impossible to follow DNA synthesis by classical methods, such as the incorporation of labelled nucleotides into the DNA. However, techniques are now available to analyse asynchronous populations to obtain some information on cell cycle events and these are discussed in the next section.

3.2. NUCLEAR MORPHOLOGY, DIVISION AND DNA SYNTHESIS.

Classical chromosome stains such as acetocarmine have frequently been employed to study the nuclei of dinoflagellates (e.g. Dodge, 1963) since the nuclear DNA is permanently condensed into chromosomes. Using this technique, Dodge first noticed the presence of an additional acetocarmine-staining body in *Glenodinium foliaceum* (Dodge, 1971). This peripheral multilobed structure was quite distinct from the normal dinoflagellate nucleus not only in its amorphous shape but also in its lack of chromosomal condensation (Plate 3.2.1). Dodge called this nucleus-like body the "eukaryotic" nucleus to draw attention to its cytological features which differed from those of typical, "mesokaryotic" nuclei of dinoflagellates. The intermediate nature of dinoflagellate nuclear organization (hence mesokaryotic) is questionable, so here the terms supernumerary nucleus and dinoflagellate nucleus are preferred since they do not confer any evolutionary suggestions.

Whilst acetocarmine staining does give extremely effective results there are limitations associated with its use in studying the supernumerary nucleus of *G. foliaceum*. Firstly, since a mixture of ethanol and acetic acid has to be employed as the fixative, lipids are almost completely extracted and so little information can be obtained on the viability of individual cells before they were preserved. This is important because one occasionaly discovers cells which apparently lack supernumerary nuclei (Plate 3.2.2). Also ethanol/acetic acid fixation results in very poor cytoplasmic preservation which makes it difficult to confidently describe the detailed morphology of the supernumerary nucleus even though acetic acid is said to preserve nuclear structure (Jensen, 1962). Secondly, and more importantly, carmine is merely a basic dye and is not specific for DNA. Various structures may stain faintly with acetocarmine and this

fact could be responsible for the report of supernumerary nuclei in a number of dinoflagellate species (Gavrila, 1977), which appears to be erroneous (Loeblich and Loeblich, 1973).

The classical Feulgen procedure is generally considered a reliable method for the cytochemical determination of DNA. Although the supernumerary nucleus of G. foliaceum has been shown to stain positively with Feulgen (Dodge, 1971), suggesting that it does contain a substantial amount of DNA, the procedure still suffers from questionable fidelity of morphological preservation. However in reality, G. foliaceum is such an opaque cell that any pigmented stain which interacts directly with DNA, such as methyl green (Gahan, 1984), would be difficult to observe if the cell were well preserved.

This problem could be partially circumvented by using a highly fluorescent stain excited by incident illumination, since the only autofluorescent pigment in the cell is chlorophyll which fluoresces relatively weakly. Fortunately a number of fluorochromes which exhibit varying degrees of specificity for DNA have recently become available commercially. These include the fluorescent antibiotics mithramycin, chromomycin and olivomycin (Ward et al., 1965), the bisbenzimidazol Hoechst dyes, most notably Hoechst 33258 (Weisblum and Hannessler, 1974), and the trypanocide, 4',6-diamidino-2-phenylindole (DAPI) (Manzini et al., 1983). Of these, DAPI appears to be becoming the most popular cytochemical stain for DNA. The fluorescence of DAPI is enhanced 20 times when bound to DNA and is relatively stable under prolonged ultraviolet excitation. However, DAPI can bind non-specifically to cell walls and it fluoresces brightly, albeit a different colour (golden yellow), when bound to polyphosphate material (Coleman, 1978). At high stain concentrations binding to RNA may also be a problem (Coleman et al., 1981). In contrast, mithramycin suffers less with interference from intracellular compounds, although it may bind to cell walls, and

appears to be specific for the guanine in DNA even at high stain concentrations (Ward et al., 1965). The usefulness of mithramycin in simple observations is limited, though, by its lower sensitivity (4 - 8 times less sensitive than DAPI) and its rapid quenching under the exciting light.

3.2.1. Nuclear morphology.

After mithramycin staining, the supernumerary nucleus of *G. foliaceum* was found to emit a strong yellow fluorescence confirming that it does contain DNA. Mithramycin fluorescence was difficult to observe in cells fixed with glutaraldehyde because of the chlorophyll autofluorescence. However, there was no problem in visualizing the intense blue-white fluorescence of DAPI, and using this stain the supernumerary nucleus appeared to assume a variety of morphologies in different cells. For ease of description these can be arranged into an artificial series of increasing complexity.

In its simplest form, the nucleus is a slightly lobed, ovoid body similar in size to the dinoflagellate nucleus (Plate 3.2.3), i.e. about 8 - 12 μm in diameter. Usually though, the lobes are more elaborate and become constricted in places along their length. Bilobed nuclei assume a filiform appearance (Plate 3.2.3). The constrictions in the nuclear lobes can become so exaggerated that the nucleus begins to look disjointed, with sections of the nucleus appearing unconnected (Plate 3.2.4). Whether or not the supernumerary nucleus is fragmented in some cells is not clear. One would expect DAPI staining to reveal all but the most tenuous DNA links between nuclear "fragments" but, of course, these may only be connected by nuclear envelope. However, in extreme examples, sections of the nucleus occur on either side of the dinoflagellate nucleus (Plate 3.2.5) and then even this possibility seems improbable. With DAPI staining, as

Plate 3.2. Nuclear morphology in Glenodinium foliaceum. 1 & 2, Nomarski interference contrast micrographs; 3 - 6, epifluorescent micrographs.

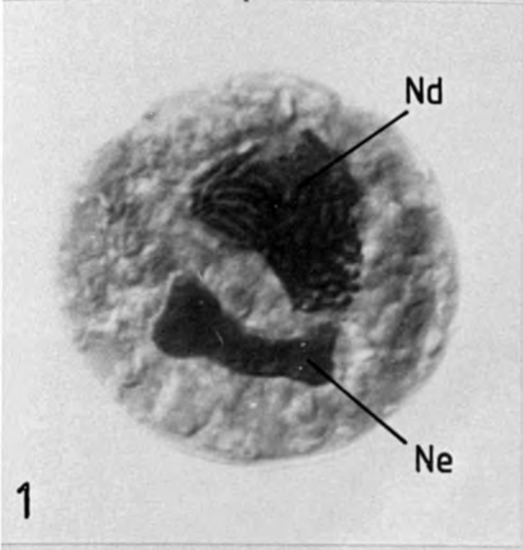
1. Cell fixed in ethanol/acetic acid and stained with acetocarmine showing the dinoflagellate nucleus (Nd) with its condensed chromosomes and the supernumerary nucleus (Ne). x 1,700.

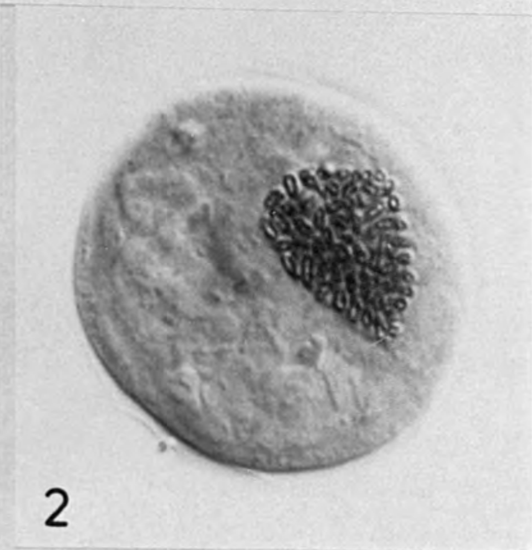
2. Acetocarmine-stained cell which lacks a supernumerary nucleus. Note that the chromosomes of the dinoflagellate nucleus appear abnormal. x 2,000.

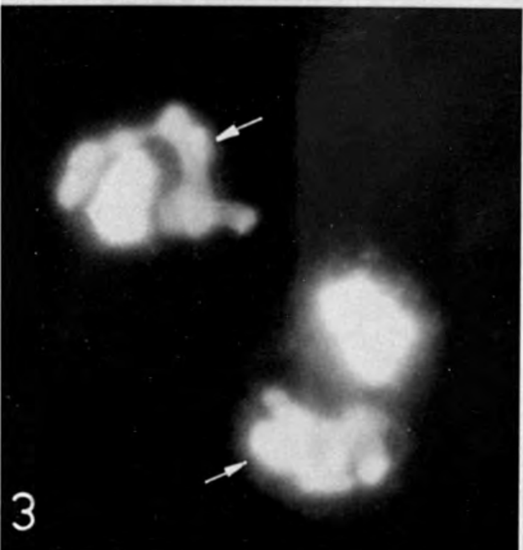
3-5. Glutaraldehyde fixed cell stained with DAPI illustrating the range of morphologies shown by the supernumerary nucleus (arrowed). All x 1,300.

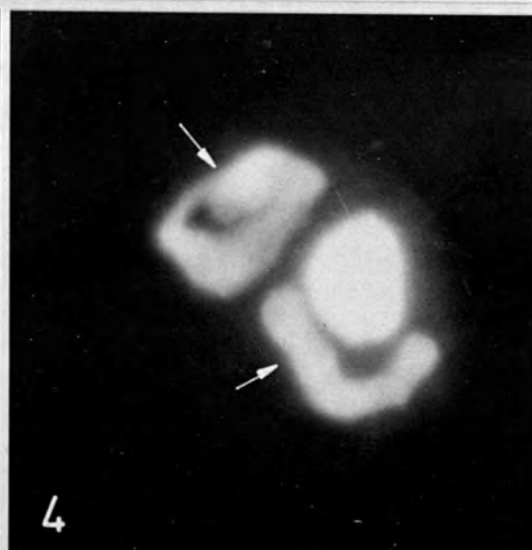
6. Cell prepared as above but squashed and photographed so as to reveal the detail of fluorescence in the two nuclei. Note the non-fluorescent nucleolar region in the dinoflagellate nucleus. x 1,600.

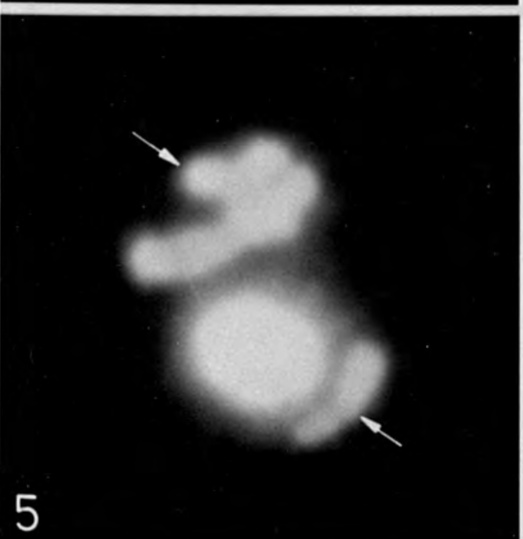
Plate 3.2

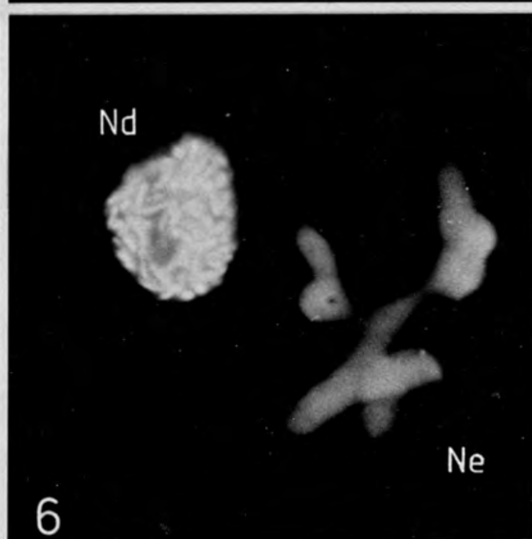












with acetocarmine staining, some cells are seen to lack a supernumerary nucleus. These cells generally show reduced chlorophyll autofluorescence and probably result from aberrant cell divisions, as described below. It is unlikely that these uninucleate cells are viable.

Other than slight variations in the intensity of DAPI fluorescence, no detectable substructure can be observed in the supernumerary nucleus. This is in contrast to the dinoflagellate nucleus where the condensed chromosomes are clearly visible (Plate 3.2.6). Most of the dinoflagellate nuclei also contain a spherical, non-fluorescent region which corresponds to the nucleolus.

3.2.2. Nuclear division.

Studying nuclear division in *G. foliaceum* is problematical; the thick walls of the dividing cells are poorly penetrated by both fixative and stain, and they also interfere with subsequent observations. Extended fixation with ice cold 3:1 ethanol/acetic acid had to be employed here in order to enhance the DAPI fluorescence and make observations possible. Even so, in unsquashed cells, the nuclei could only be distinguished on the basis of their gross morphologies since it was difficult to resolve the chromosomes of the dinoflagellate nucleus.

The dinoflagellate nucleus divides first (Plate 3.3.1) and the two daughter nuclei move to opposite ends of the cell whilst remaining equidistant from the supernumerary nucleus (Plate 3.3.2). The morphology of the supernumerary nucleus remains unchanged during this period but as cytokinesis proceeds it becomes constricted by the cleavage furrow (Plate 3.3.3). Ultimately, when cell division is completed, it is split into two (Plate 3.3.4). No chromosomal condensation was ever observed in

Plate 3.3. Nuclear division in Glenodinium foliaceum. Epifluorescent micrographs of ethanol/acetic acid fixed cells stained with DAPI. Nd, dinoflagellate nucleus; Ne, supernumerary nucleus.

1. Cell just after division of the dinoflagellate nucleus.

2. Cell in an early stage of cytokinesis containing two dinoflagellate nuclei. Note a detached lobe of the supernumerary nucleus (arrowed) near to the upper dinoflagellate nucleus.

3. Cell in a late stage of cytokinesis showing the supernumerary nucleus being split in two by the cleavage furrow.

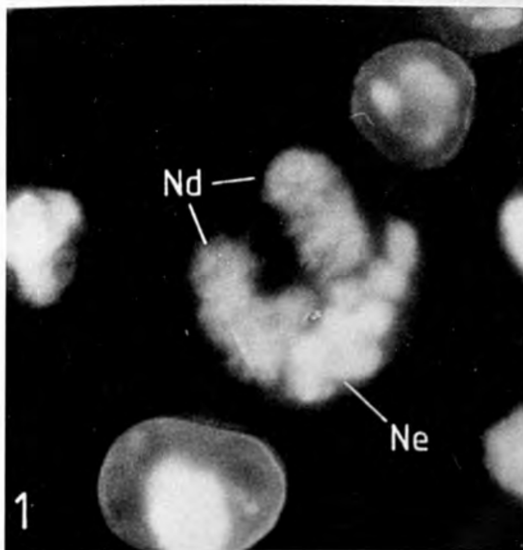
4. Divided cell with each daughter containing a portion of the supernumerary nucleus.

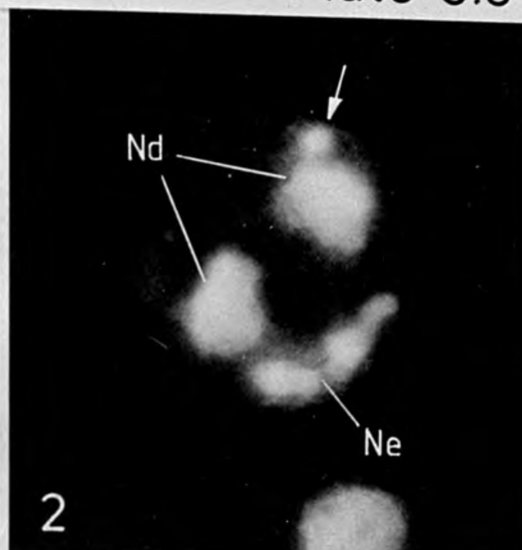
5. Nuclei of four daughter cells still unseparated.

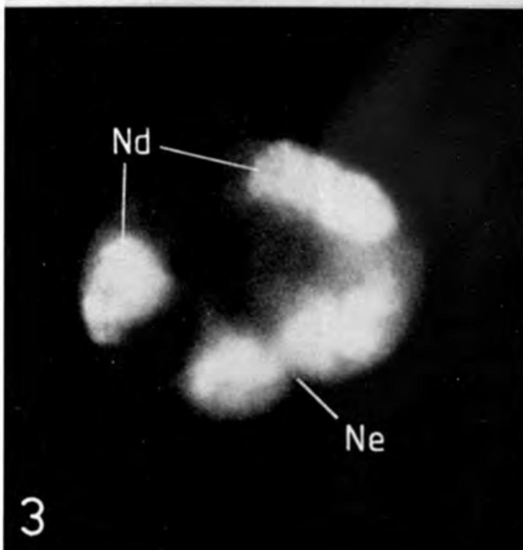
6. A complex of eight daughter cells within the wall of the mother cell. Each dinoflagellate nucleus is associated with a fragment of the supernumerary nucleus.

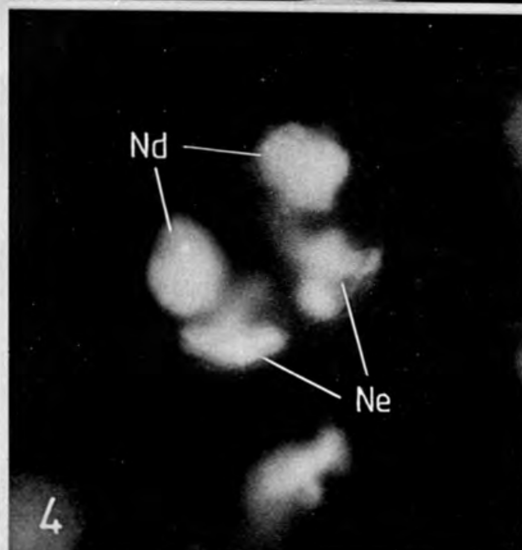
All x 1,300.

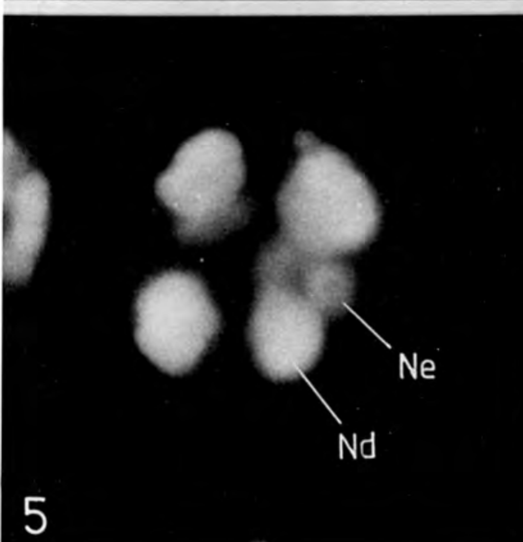
Plate 3.3

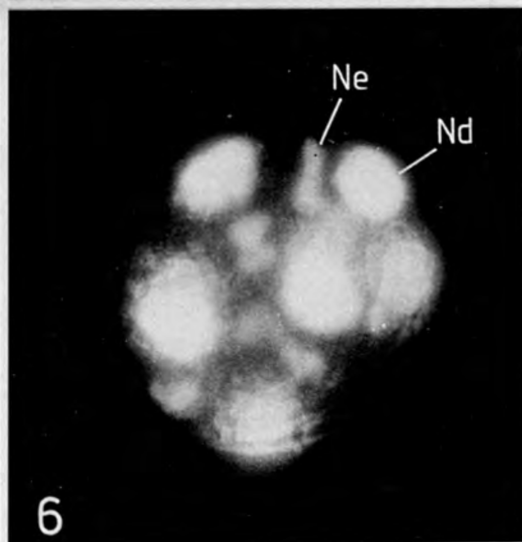












squash preparations. One gains the impression that the division of the supernumerary nucleus is not organized in any way and it is merely fortuitous that both daughter cells acquire parts of it during cell division. Examples were found in which the supernumerary nucleus was apparently missing from one of the daughter cells. Presumably such aberrant divisions arise from the asymmetric location of the nucleus before cytokinesis.

After reaching the two cell stage, nuclear and cell division may then occur again (Plate 3.3.5) but the manner in which this proceeds was not observed. A further division of the nuclei can then take place to produce a complex of eight daughter cells each containing a small fragment of the supernumerary nucleus (Plate 3.3.6). Three successive nuclear divisions must be very rare since it was only observed on a few occasions. These observations confirm those of Blanchard-Babillot (1972) who studied nuclear division in G. foliaceum using acetocarmine staining.

3.2.3. DNA synthesis.

The intensity of DAPI or mithramycin fluorescence is proportional to the amount of DNA, providing the base composition of the DNA remains constant (Ahrberg and Schweizer, 1984). So by quantifying the stain fluorescence, the relative amounts of DNA in individual cells of the same organism can be determined. This can be done either manually using a microfluorometer attached to the microscope (Coleman et al., 1981) or automatically by means of a flow cytometer (Crissman et al., 1979). The latter method is the more preferable since these instruments can analyse large numbers of cells and so produce results of greater statistical significance. Also, since with a flow cytometer the stain is only excited for about 10 milliseconds, fluorescence quenching during analysis is not a problem.

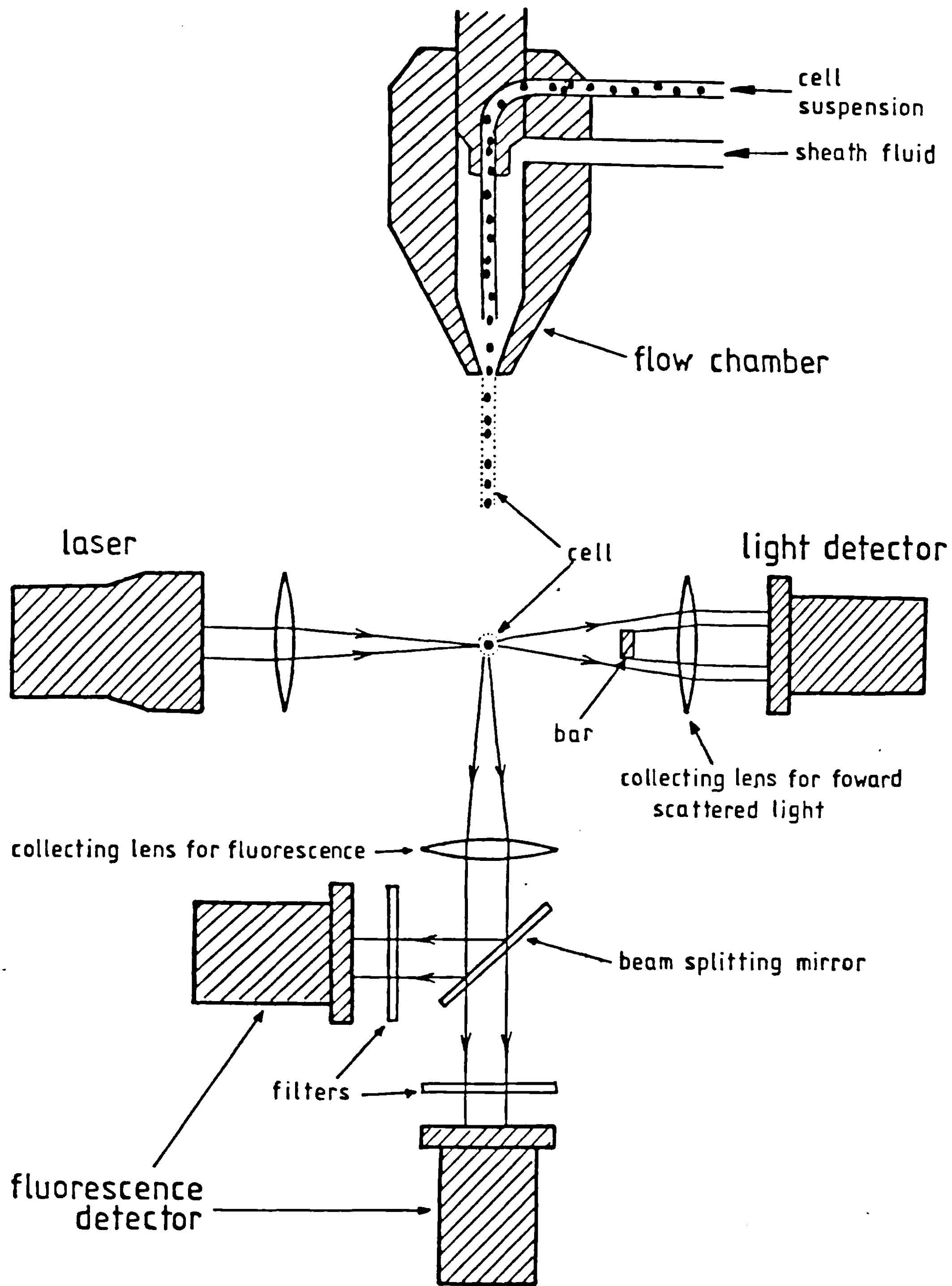


Figure 3.5. Diagrammatic representation of the FACS 420 flow cytometer. The flow chamber is shown in section above and a plan of the single cell analysis system is illustrated below. See text for explanation.

The basic operation of the flow cytometer is illustrated in Figure 3.5. The cells to be analysed are mixed with an accelerating flow of sheath fluid (buffered saline) in the flow chamber such that they emerge in single file in a jet of liquid from the nozzle. The cells then pass, one at a time, through an accurately centered laser beam. This excites the stain and the fluorescence is recorded by photodetectors positioned at right angles to the beam. A light detector placed opposite the laser, but which is prevented from seeing it directly by an obscuration bar, collects forward scattered light and provides information on cell size. The individual optical properties of up to 5,000 cells per second can be recorded in this manner and subjected to simultaneous computer analysis. Most present day flow cytometers also possess the capability of sorting out cells which conform to preset operational parameters and are hence termed fluorescence activated cell sorters (FACS) (Herzenberg *et al*., 1976; Sweet, 1979).

When *Glenodinium foliaceum* cells, stained with mithramycin, were subjected to a preliminary flow cytometric analysis, the frequency distribution of red fluorescence showed three peaks (Figure 3.6c). The weakest fluorescence peak corresponded to the residual chlorophyll autofluorescence emitted by unstained cells (Figure 3.6b). Mithramycin-DNA fluorescence was responsible for the other two peaks and was at least eight times more intense than the chlorophyll autofluorescence. This suggests that in an asynchronously dividing culture of *G. foliaceum* there are two sub-populations of cells, one sub-population containing relatively more DNA than the other. In this respect *G. foliaceum* is typical of most other eukaryotes. Cells in G2 phase of the cell cycle, which are ready to undergo mitosis (M), contain twice as much DNA as those in G1 phase, and those cells in S phase that are currently synthesizing DNA have intermediate quantities (Lloyd *et al*., 1982). However, in *G. foliaceum* the cells that are apparently in G2 exhibit only 1.5 times the intensity of

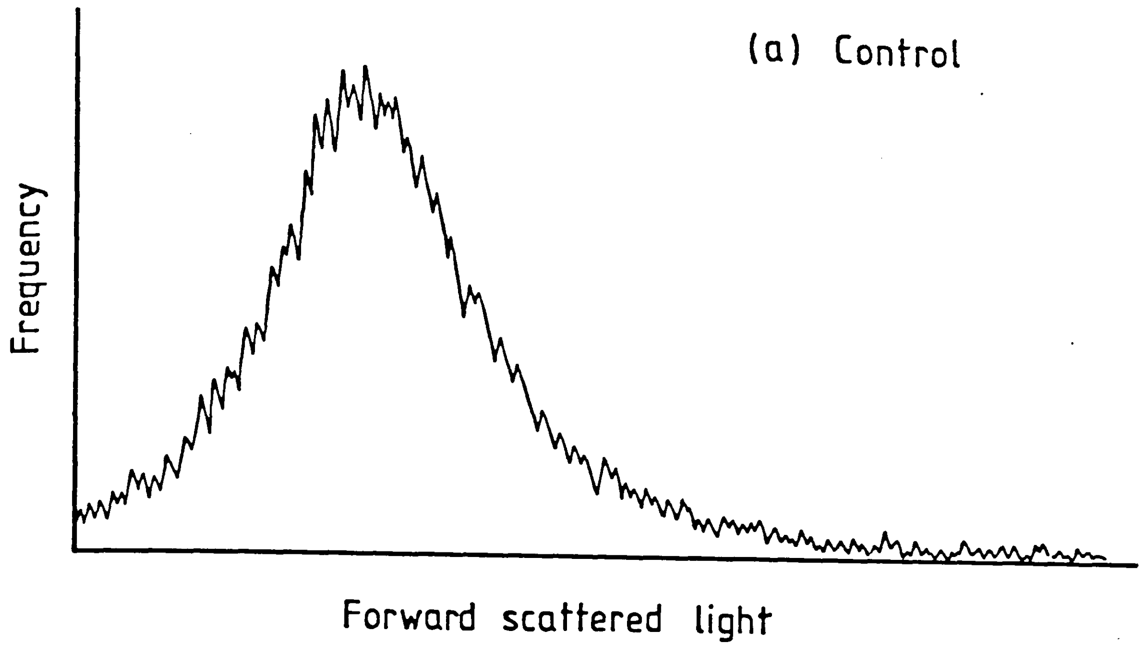
Figure 3.6. Flow cytometric analysis of _Glenodinium foliaceum_. Graphs are computer generated plots of the relative number of events recorded for each signal channel (250 channels in all).

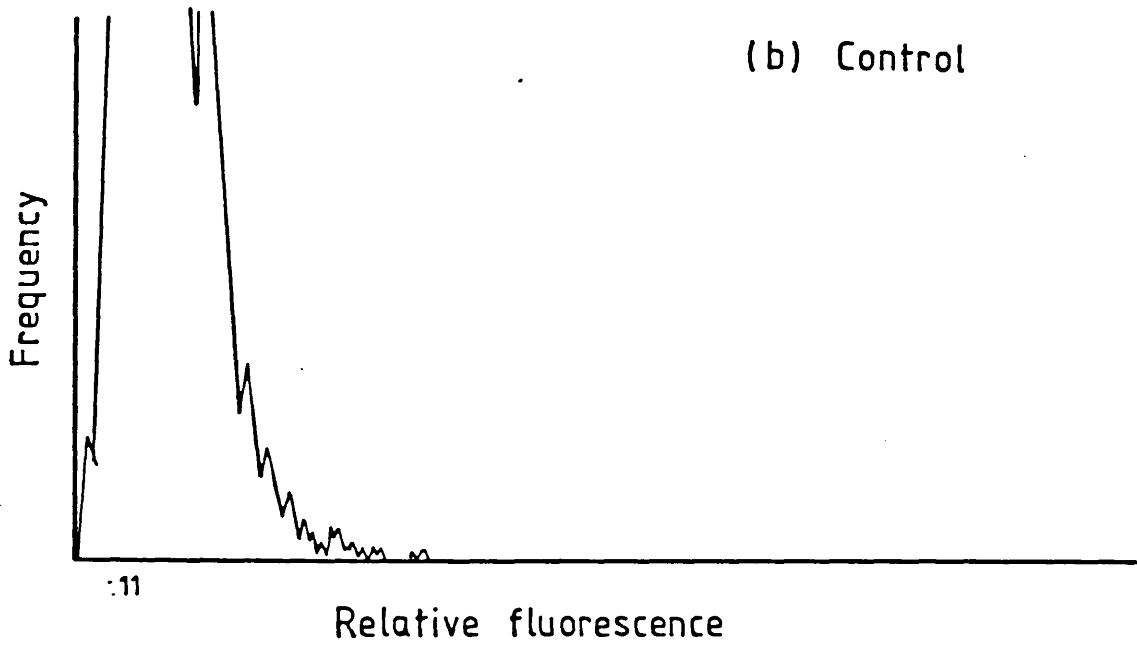
a. Distribution of cell volumes in an unstained preparation as determined from forward scattered light measurements.

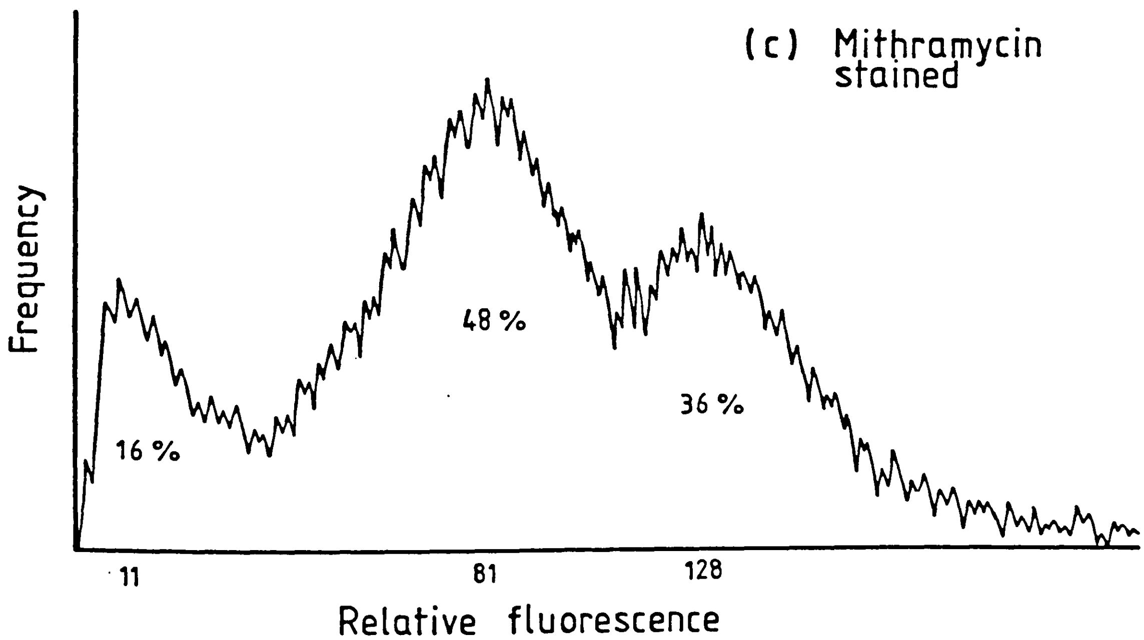
b. Red autofluorescence from the same preparation as in (a) and recorded simultaneously.

c. Distribution of red fluorescence signals in a mithramycin stained preparation. Figures on the graph refer to the relative areas under the three peaks whose relative positions are indicated on the x axis.

Figure 3.6







fluorescence as those in G1, not twice the fluorescence as expected. Also the DNA distribution cannot be uniquely partitioned into three phases since no S phase plateau separates the G1 and G2 peaks.

These features could result from a long S phase in G. foliaceum. As the duration of S phase increases relative to G1 and G2M, it becomes more difficult to resolve the two peaks and the G2M peak shifts to lower values (Gray et al., 1979). Long S phases have been found in some dinoflagellates using the standard technique of monitering labelled thymidine incorporation into DNA. In Amphidinium carterae the S phase is longer than G2M (G1 being extremely short) and residual DNA synthesis also occurs during the G2M phase (Galleron and Durrand, 1977). In Prorocentrum micans, it has been shown that DNA is synthesized continuously throughout the cell cycle (Filfilan and Sigee, 1977). However, in other dinoflagellates more discrete S phases are found (Franker et al., 1974; Loeblich, 1976; Spector et al., 1981), and a recent study of six marine dinoflagellates from different genera confirmed that a variety of patterns of DNA synthesis can occur in this group (Karentz, 1983). There is one reported flow cytometeric study of a dinoflagellate, that of Gonyaulax polyedra by Yentsch et al. (1983). They found distinct G1 and G2 peaks separated by a very shallow plateau of S phase cells. The paucity of cells synthesizing DNA was probaly because a phased culture was analysed, which was collected after cell division had occurred.

It is unlikely that a prolonged S phase in one or both of the two nuclei in G. foliaceum can completely account for the observed DNA content distribution. More probably, cytochemical or instrumental variability are confounding the analysis. For example, non-specific cell wall staining could skew the G1 peak to higher fluorescence values, cell clumping may cause an appar-

ent increase in the G2 peak, and the resolution of the instrument could be affected by the large size of some of the cells (Gray _et al._, 1979). However, the most serious complication is the presence of a substantial number of unstained cells in the sample, that is cells showing only chlorophyll autofluorescence. Microscopic observation suggested that these corresponded to cells undergoing division, as in these cells the pigments had been poorly extracted and the stain had not penetrated completely due to the presence of the thickened wall. Stain penetration appeared not to be a problem in non-dividing, motile cells, so the broadness of the mithramycin fluorescence peaks in Figure 3.6c probably reflects biological variability in DNA content rather than variability in staining.

The presence of only two peaks infers that either both nuclei in _G. foliaceum_ synthesize their DNA in synchrony, or continuous synthesis takes place in one nucleus, and a discontinuous synthesis in the other. If asynchronous, but discrete, DNA synthesis had occurred in both then one would expect to see three peaks in the distribution of DNA content. It is recognized, however, that DNA synthesis in one of the nuclei could occur immediately before division and so this peak would not be detected due to the staining difficulties. Whilst this would explain the intermediate position of the proposed G2 peak, it seems unlikely that DNA synthesis would occur at such a late stage in the cell cycle. Although some cell types begin synthesizing DNA directly after mitosis, a G2 phase is rarely absent. Interpreting the data in terms of synchronous DNA synthesis would also conform with the finding that a non-species specific, cytoplasmic factor seems to trigger DNA synthesis in mammalian cell lines (Lloyd _et al._, 1982). If such a factor occurs in _G. foliaceum_, then it might be expected to simultaneously initiate the synthesis of DNA in both nuclei.

3.3. ULTRASTRUCTURE OF GLENODINIUM FOLIACEUM.

Various fine-structural features of *Glenodinium foliaceum* have been described previously, namely the eyespot (Dodge and Crawford, 1969), the theca (Dodge and Crawford, 1970), the supernumerary nucleus (Dodge, 1971), the chloroplasts (Dodge, 1975; Jeffrey and Vesk, 1976) and the "limiting membrane" (Jeffrey and Vesk, 1976). These have been reviewed by Dodge (1983a) but are necessarily re-examined again here so as to provide a complete description of the organism. In addition, observations are made on some organelles whose presence had not been noted previously.

3.3.1. General ultrastructural organization.

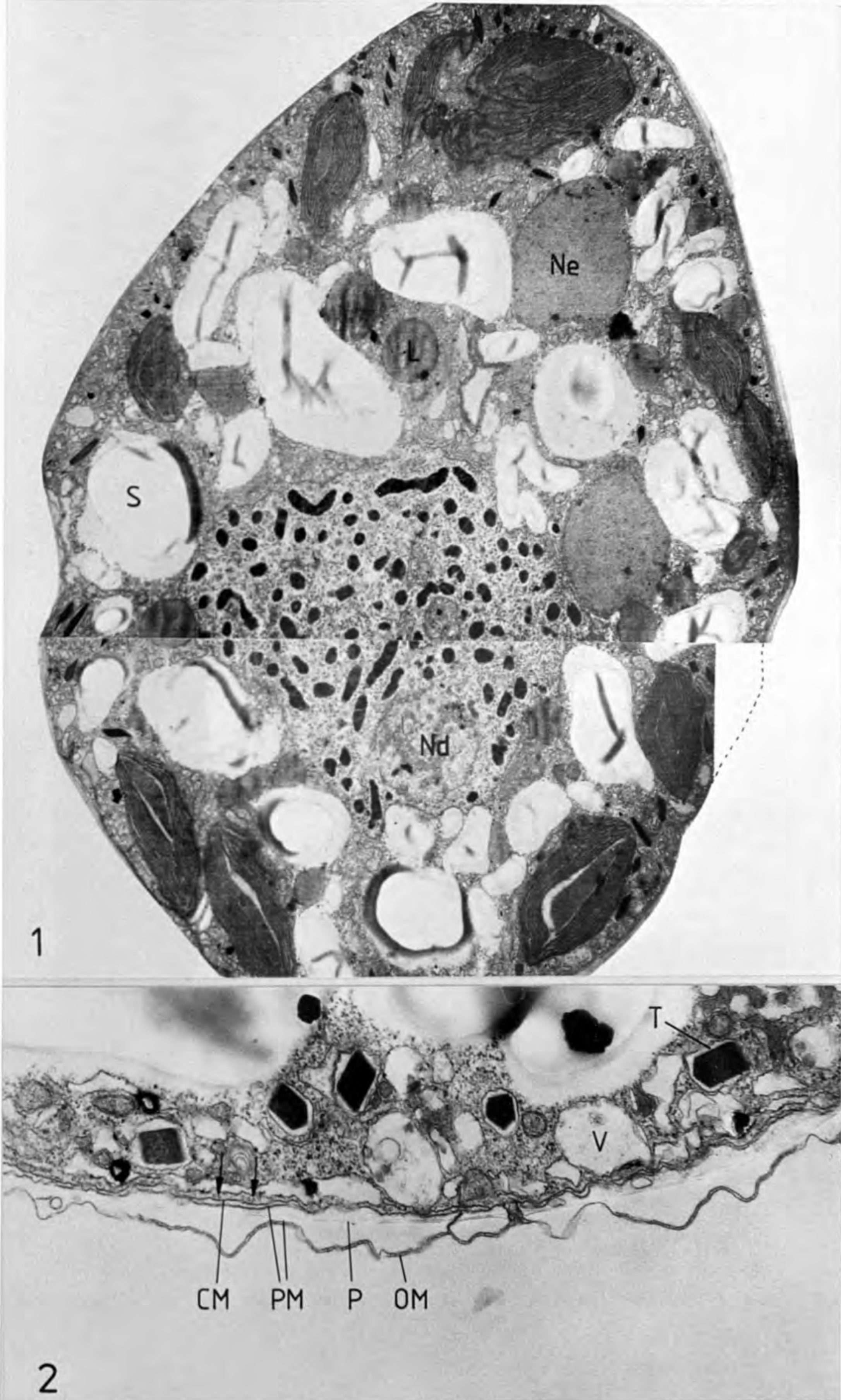
The general organization of the cell as deduced from light microscopy is confirmed under the electron microscope (Plate 3.4.1). The large dinoflagellate nucleus is situated beneath the girdle region and slightly into the hypocone. Sections through the supernumerary nucleus are generally, but not always, located towards the anterior of the cell in the epicone. The numerous elongate chloroplasts are arranged more or less tangentially around the periphery and enclose a region of cytoplasm which is packed with starch grains and lipid. The theca (or amphiesma) is usually quite well preserved and is composed of a series of vesicles containing thin cellulosic thecal plates. The vesicles lie between two membranes, the "outer membrane" and the inner, cytoplasmic membrane, which are continuous around the cell (Plate 3.4.2). No non-membranous material exterior to the cytoplasmic membrane, which could constitute a pellicle, could be seen, although a pellicle resistant to acetolysis in *G. foliaceum* has been detected by chloral hydrate-hydriodic acid-iodine staining (Morrill and Loeblich, 1981). Immediately beneath the cytoplasmic membrane is an interrupted row of micro-

Plate 3.4. General ultrastructure of <u>Glenodinium</u> <u>foliaceum</u>. Transmission electron micrographs.

1. Whole cell showing the centrally located dinoflagellate nucleus (Nd) and sections through two lobes of the supernumerary nucleus (Ne) towards the anterior of the cell. The cytoplasm is packed with starch grains (S) and lipid (L). Various profiles through the chloroplasts can be seen around the periphery of the cell. x 6,400.

2. Detail of the theca and sub-adjacent cytoplasm. The thin thecal plates (P) are located in vesicles bound by a single membrane (PM) between the cytoplasmic membrane (CM) and an outer membrane (OM). Note the microtubules beneath the cytoplasmic membrane (arrowed), the sub-thecal vesicles (V) and the trichocysts (T). x 23,000.

Plate 3.4



tubules and in the subadjacent cytoplasm are normal dinoflagellate trichocysts. Membrane bound vesicles, 0.7 -1.5 μm in diameter, are also characteristic of the peripheral cytoplasm. These vesicles are usually totally extracted during fixation but when their contents have been partially preserved they appear to be lipid in character (see Plate 3.8.2).

3.3.2. The dinoflagellate nucleus.

At the fine-structural level, the dinoflagellate nucleus again shows the characteristics typical of those of normal uninucleate species (Plate 3.5.1). The most striking feature of this nucleus is, of course, the condensed chromosomes. These can assume various appearences depending on the fixation and staining conditions and the state of the cell. In apparently healthy motile cells which have been fixed in an isotonic solution, the chromosomes are generally 500 nm - 700 nm in diameter and are composed of fibrils compactly arranged in such a manner that the chromosome assumes a banded appearance in longitudinal section. The ultrastructure of dinoflagellate chromosomes has been studied in some detail (Herzog and Soyer, 1983; Dodge, 1985). The chromosomes are set in a granular matrix which is enclosed by a double membrane envelope perforated by pores. Some chromosomes are seen to be in close association with, and are possibly attached to the nuclear envelope. No connections between the nuclear membrane and endoplasmic reticulum can be observed. The prominent nucleolus of the dinoflagellate nucleus, which was readily observed by light microscopy (Plate 3.2.6), appears to be composed entirely of granular material, i.e. ribosomal ribonucleoprotein particles. Whilst this is frequently invaded by the nuclear ground matrix, a distinct fibrillar component, which is generally characteristic of nucleoli, is not obvious. However, Dodge (1971) has noted small fibrillar patches in his sections. Located near to the dinoflagellate nucleus are one or more dictyosomes (Plate 3.5.1).

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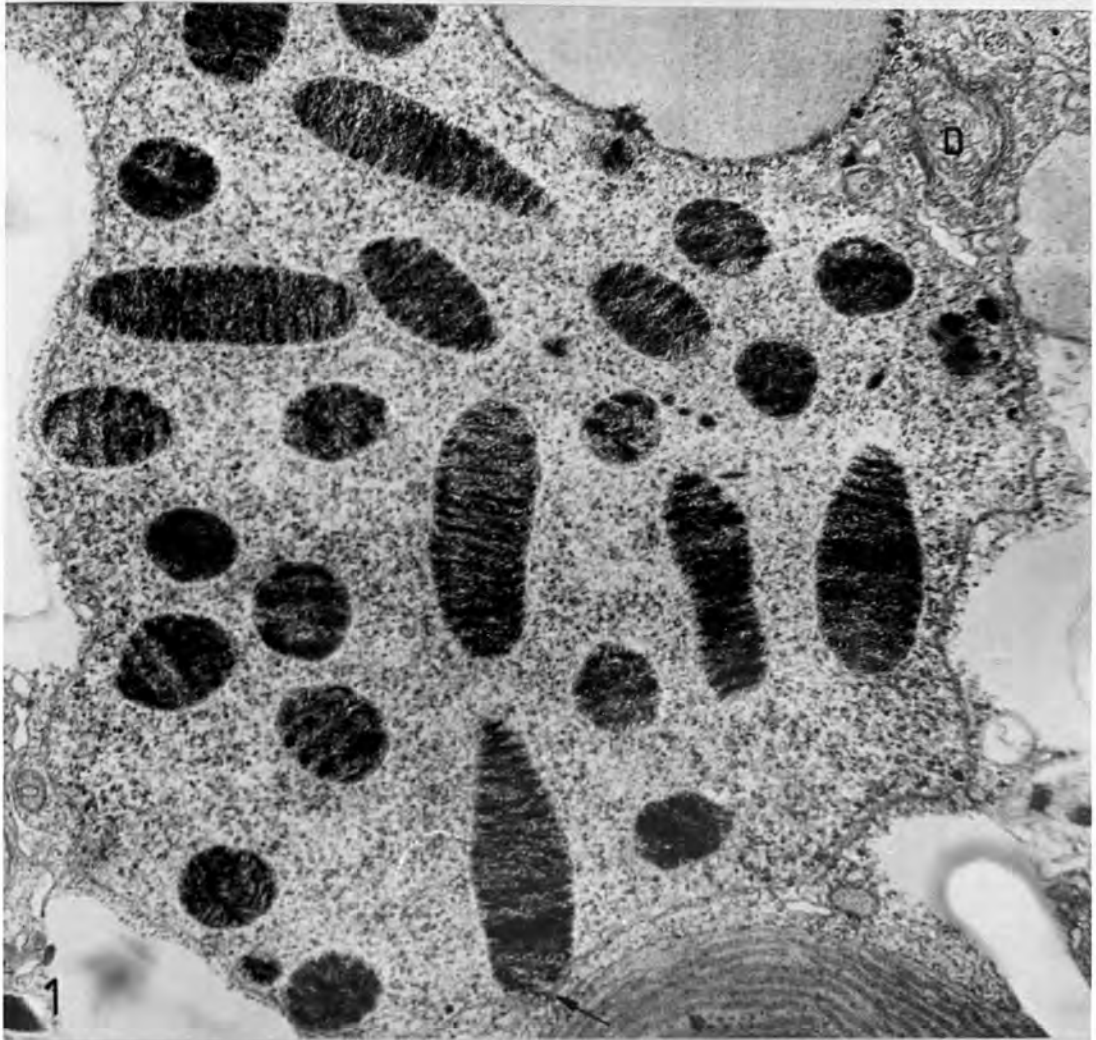
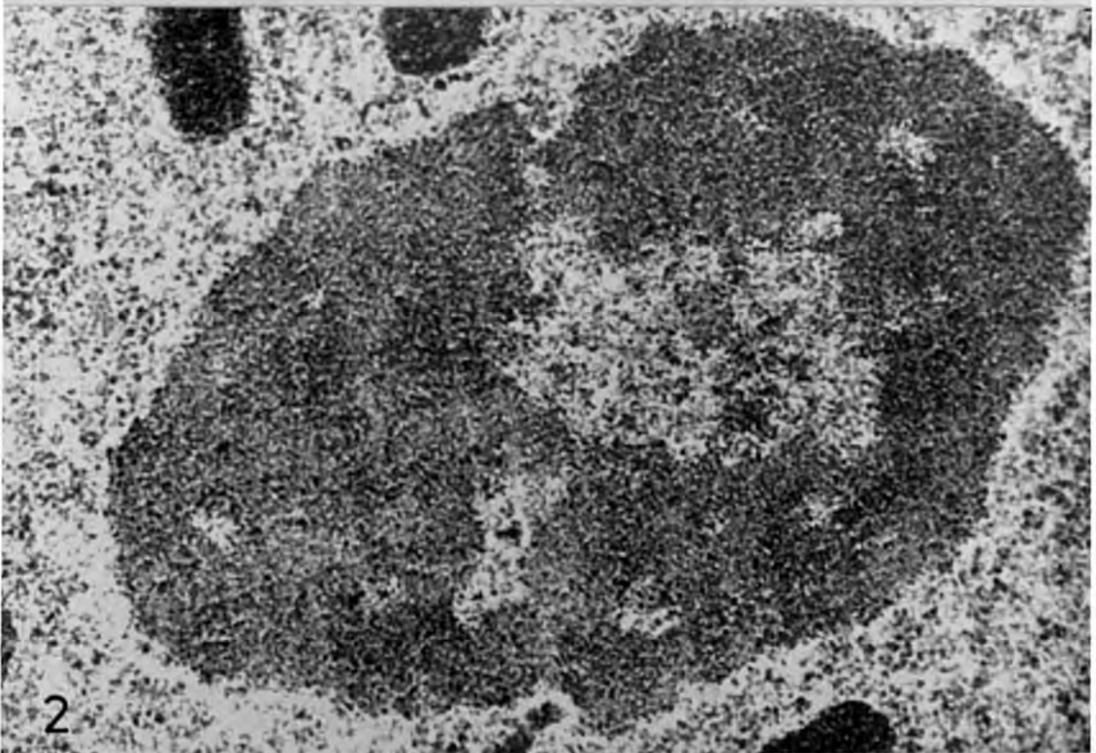
Plate 3.5. Ultrastructure of the dinoflagellate nucleus of *Glenodinium foliaceum*. Transmission electron micrographs

1. A non-median section through the dinoflagellate nucleus showing the condensed chromosomes cut in various planes. The chromosome sectioned longitudinally at the bottom of the nucleus appears to be in close association with the nuclear envelope (arrow). D, dictyosome. x 19,500.

2. The nucleolus of the dinoflagellate nucleus composed of granular component dissected with nuclear matrix. x 32,300.

Plate 3.5





The position of the dictyosomes appears not to be related to that of the supernumerary nucleus, as has been suggested in *Peridinium balticum*, another binucleate species (Tomas and Cox. 1973)

3.3.3. The supernumerary nucleus.

The supernumerary nucleus has entirely different ultrastructural characteristics (Plate 3.6.1). As in typical interphase nuclei of the vast majority of eukaryotes, the chromosomes are dispersed into fibres of chromatin but the supernumerary nucleus lacks the large regions of densely staining heterochromatin which occur in the nuclei of animal and plant cells. Instead, the chromatin fibres are randomly aggregated into small areas of heterochromatin scattered throughout the nucleoplasm. However, such an arrangement of chromatin is not unusual amongst protist nuclei (Dodge, 1973) and is, moreover, found in three dinoflagellate genera. During the vegetative cycle of *Noctiluca* (Zingmark, 1970), the feeding cycles of *Oodinium* (Cachon and Cachon, 1977) and *Stylodinium* (Timpano and Pfiester, 1985) the chromosomes are completely dispersed and the chromatin is randomly arranged. This type of dinoflagellate nucleus was described as "nocticaryotic" by Zingmark (1970). The supernumary nucleus of *G. foliaceum* differs from these nuclei in that the chromosomes do not condense during division. Furthermore, the nocticaryotic nuclei of *Noctiluca* and *Oodinium* become transformed into typical "dinocaryotic" nuclei (i.e. having permenantly condensed chromosomes) during sporogenesis (Soyer, 1972; Cachon and Cachon, 1977).

The lobes of the supernumerary nucleus in *G. foliaceum* are themselves frequently dissected by finer invaginations of the cytoplasm (Plate 3.6.1). Such is the contorted nature of the two membrane nuclear envelope that glancing sections through the envelope are often obtained and reveal a dense array of pores (Plate 3.6.3, see also Plate 3.8.2). The outer nuclear membrane

Plate 3.6. Ultrastructure of the supernumerary nucleus of *Glenodinium foliaceum*. Transmission electron micrographs.

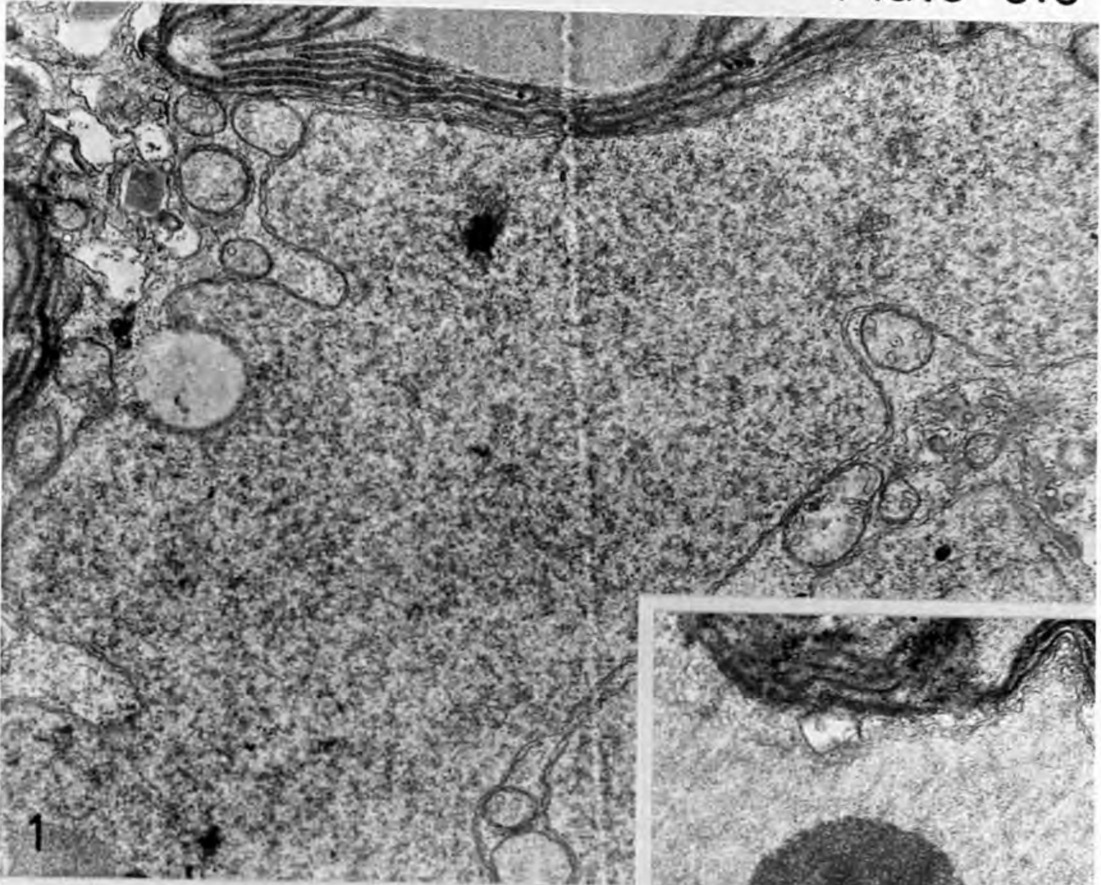
1. Longitudinal section through a lobe of the supernumerary nucleus showing the invaginations of the cytoplasm and associated mitochondria. Note the random aggregation of chromatin fibres into small areas of heterochromatin distributed throughout the nucleus. x 18,500.

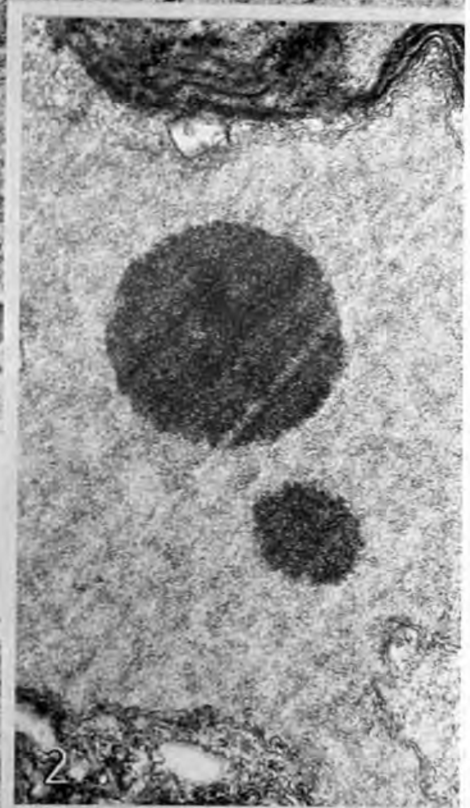
2. Nucleoli in the supernumerary nucleus. This material was fixed for 1 h with 2% (w/v) OsO_4 in 0.125 M Sörensen's phosphate buffer pH 7.8 and subsequently processed as described in Section 2.4.1. The arrangement of chromatin in the nucleus is similar to that in material prepared by the standard double fixation so is probably not artifactual. x 17,600.

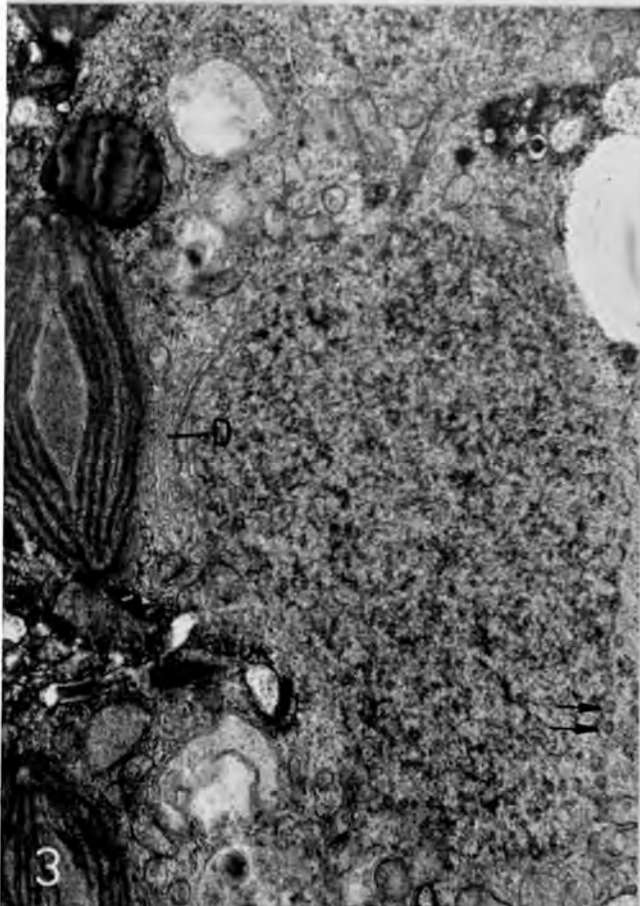
3. Transverse section through a lobe of the supernumerary nucleus showing the nuclear pores in glancing section (arrowed) and the position of an associated dictyosome (D). x 16,500.

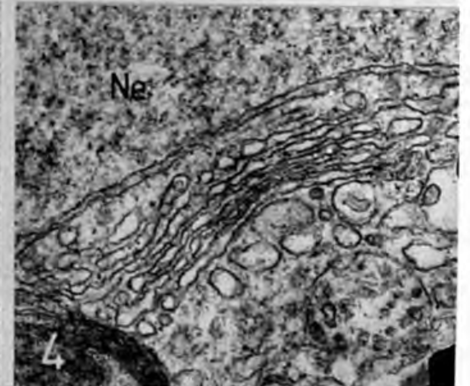
4. Detail of a dictyosome closely adjacent to the supernumerary nucleus (Ne) and a chloroplast. x 43,400.

Plate 3.6









is continuous in places with endoplasmic reticulum (see Plate 3.8.2). Nucleoli are present in the supernumerary nucleus and in this study they were best observed after using OsO_4 as the primary fixative (Plate 3.6.2). Like the nucleolus of the dinoflagellate nucleus, these nucleoli seemed to be only composed of granular material, but again, nucleoli with less dense centres have been seen by Dodge (1971). Dictyosomes associated with the supernumerary nucleus had not been noted previously, but in this re-examination they were observed on two seperate occasions (Plates 3.6.3 & 4). Both dictyosomes were compressed in the narrow region of cytoplasm between a lobe of the nucleus and the chloroplasts and so could easily be overlooked (Plate 3.6.3)

3.3.4. Chloroplasts.

The chloroplasts generally appear spindle-shaped in longitudinal section and contain an orderly array of lamellae which run alongside and around the central pyrenoid (Plate 3.7.1). A girdle lamella is present which lies just inside the chloroplast envelope and surrounds the internal lamellae. The lamellae are composed of three loosely stacked thylakoids which occasionally separate but invariably rejoin into the same stack, thylakoid connections between lamellae being rare (Plate 3.7.2). Beneath the girdle lamella at the ends of the chloroplast, and scattered elsewhere in the organelle, are small electron translucent regions of stroma probably corresponding to areas of DNA (Plate 3.7.1). Lipid globules are also common in the chloroplasts.

The chloroplast envelope consists of two membranes surrounded by a sac of endoplasmic reticulum (chloroplast ER) (Plates 3.7.2 & 3). Connections between the outer membrane of the chloropast ER and the envelope of the supernumerary nucleus have been demonstrated (Dodge, 1983b). Whether or not all the chloro-

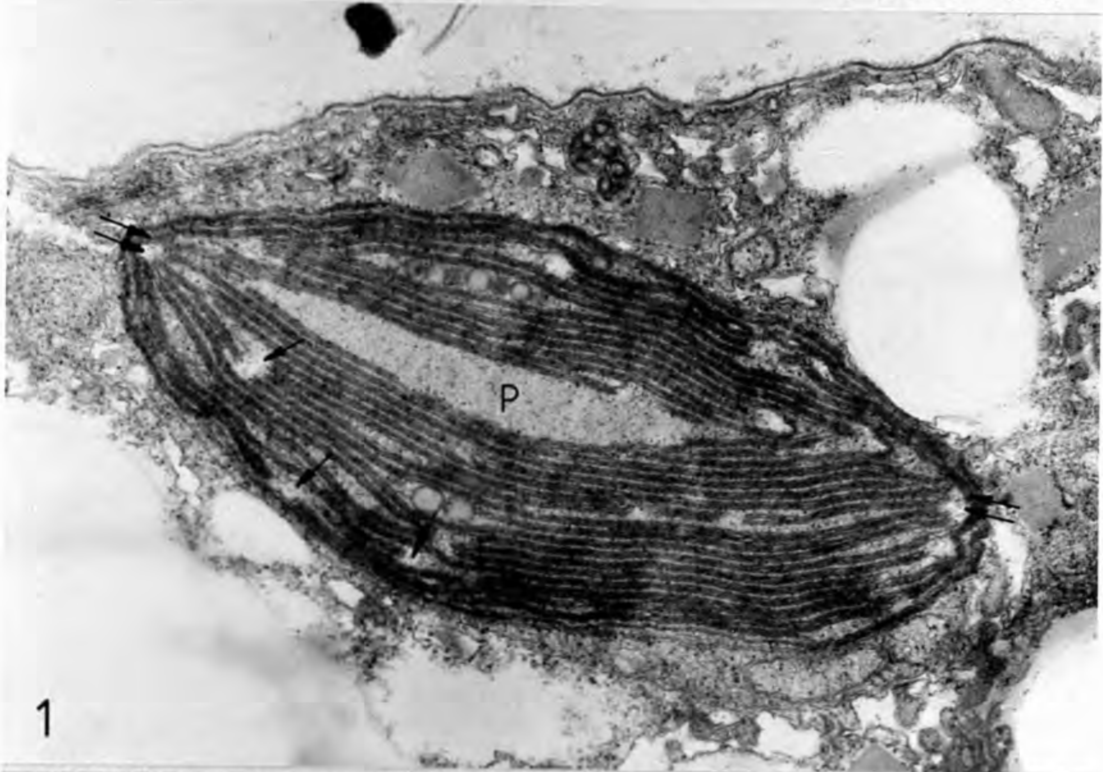
Plate 3.7. Ultrastructure of the chloroplasts of *Glenodinium foliaceum*. Transmission electron micrographs.

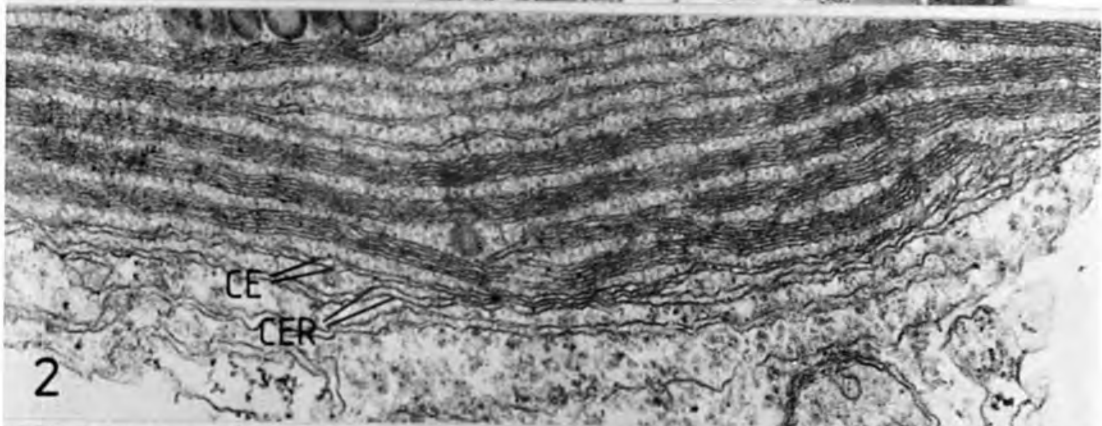
1. Longitudinal section through a chloroplast showing an orderly array of photosynthetic lamellae surrounding the internal, spindle-shaped pyrenoid (P). Scattered amongst the lamellae are lipid globules and lightly staining regions of stroma probably corresponding to areas of chloroplast DNA (single arrows). Areas of DNA also occur beneath the girdle lamella at each end of the chloroplast (double arrows). x 23,100.

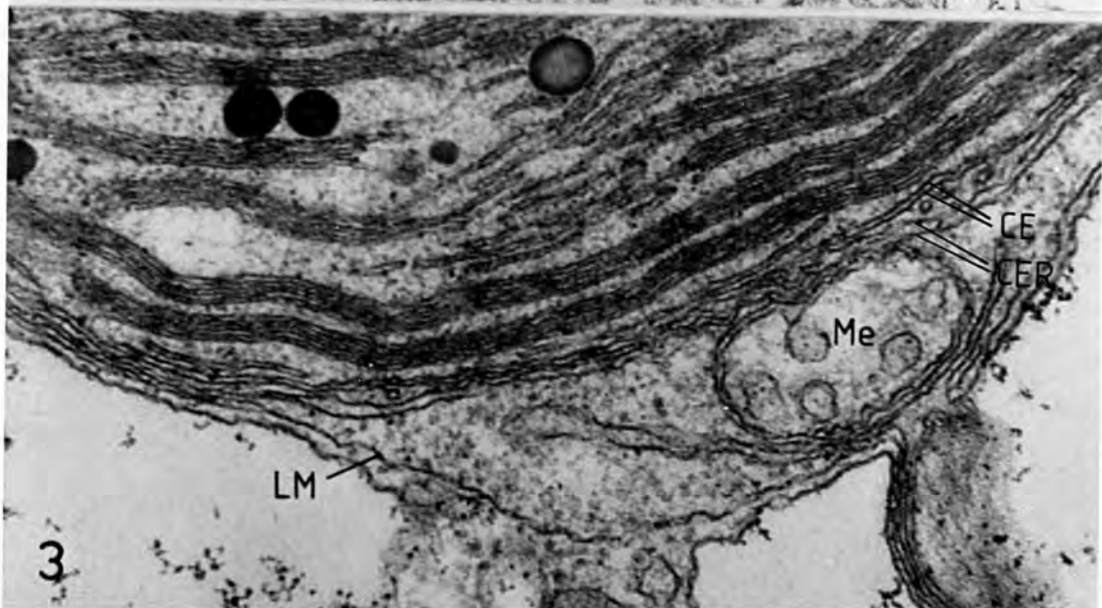
2. Detail of the chloroplast membranes. The chloroplast is surrounded by four membranes; two membranes of the chloroplast envelope (CE) and two membranes of the chloroplast endoplasmic reticulum (CER). The chloroplast lamellae are composed of three thylakoids which are clearly seen where they separate. x 64,700.

3. Detail of the "limiting membrane" (LM) enclosing a region of cytoplasm, containing mitochondria (Me), between it and the chloroplast membranes (abbreviations as in 2). x 76,000.

Plate 3.7







plasts are linked to the supernumerary nucleus in this manner is not known; no obvious connection were observed in this study (but see Plate 3.8.2). Chloroplast ER is a characteristic of chromophyte algae (Gibbs, 1970) but not dinoflagellates where the chloroplast envelope consists of three often closely appresed membranes (Dodge, 1975). Although individual anomalous chloroplast features have been reported in some dinoflagellates; such as a two membrane envelope in some sections of *Prorocentrum micans* (Dodge, 1983), false girdle lamellae (see Dodge, 1975), and connections between chloroplast and nuclear membranes in *Gymnodinium microadriaticum* (Taylor, 1969); the combination of chloroplast characteristics shown by *Glenodinium foliaceum* is absent from all other dinoflagellates with the exception of *Peridinium balticum* (Tomas and Cox, 1973).

3.3.5. The "limiting membrane" and the concept of an endosymbiont.

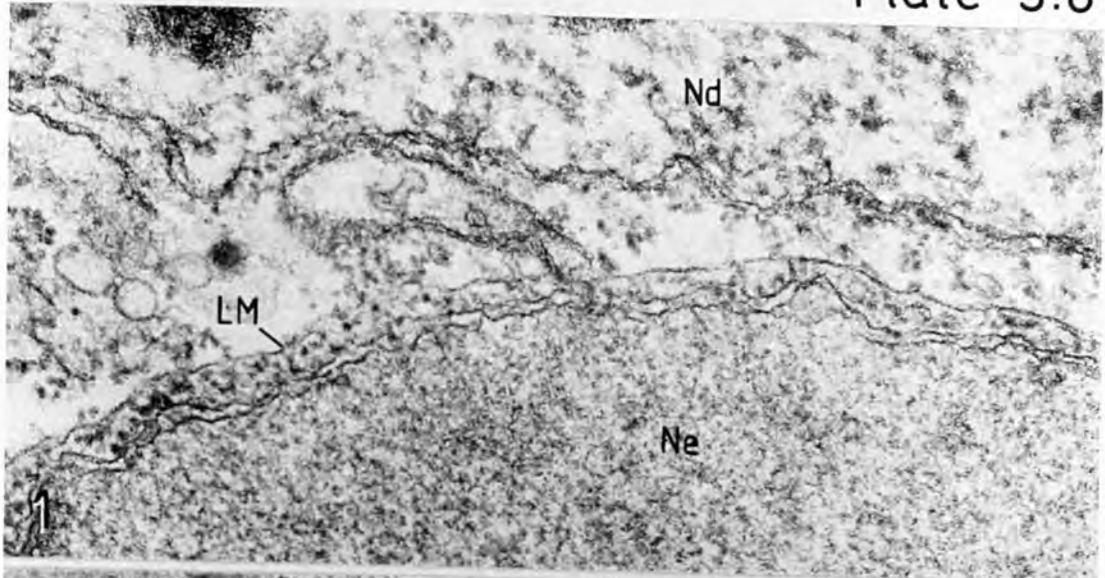
In addition to the two membranes of the chloroplast envelope and the two membranes of the chloroplast ER, a fifth membrane is often seen lying outside the other four chloroplast membranes in *G. foliaceum* (Plate 3.7.3). This "limiting membrane" separates off an area of cytoplasm between it and the chloroplast ER but unlike these membranes it does not normally completely surround the chloroplast. Mitochondria are frequently positioned close to the chloroplast in this area of cytoplasm (Plate 3.7.3). A similar limiting membrane can be seen where the dinoflagellate nucleus and supernumerary nucleus are closely adjacent to one another. Here it runs parallel to the nuclear envelope of the supernumerary nucleus, again enclosing a thin band of cytoplasm (Plate 3.8.1). In a favourable section this membrane can be traced out into the cytoplasm and can be shown to be continuous with the limiting membrane of the chloroplasts. Consequently this membrane encloses an area of ribosome-rich cytoplasm which

Plate 3.8. General ultrastructural features of the endosymbiont in *Glenodinium foliaceum*. Transmission electron micrographs.

1. Detail of the limiting membrane (LM) separating the dinoflagellate nucleus (Nd) from the supernumerary nucleus (Ne). x 71,300.

2. General features of the cytoplasmic compartment enclosed by the limiting membrane (small arrows). This compartment contains the chloroplasts (C), some mitochondria (Me) and the supernumerary nucleus (Ne) and is almost certainly derived from an endosymbiotic alga. Note the difference in size between the endosymbiont mitochondria (Me) and the mitochondria in the dinoflagellate cytoplasm (Md), and also the continuity of the outer membrane surrounding the endosymbiont nucleus with the endoplasmic reticulum and possibly chloroplast ER (large arrows). Pores in the nuclear envelope are also obvious in glancing section. Starch grains (S) and lipid (L) both lie outside the limiting membrane in the host cytoplasm. x 25,000.

Plate 3.8



contains the supernumerary nucleus and its associated dictyosome, all the chloroplasts and numerous mitochondria (Plate 3.8.2).

In evolutionary terms, the most conservative hypothesis for this origin of the subcellular compartment is that it was derived from an endosymbiotic alga which has now lost its morphological identity. If one assumes that the endosymbiont was a chromophytic alga, then this simultaneously accounts for the ultrastructural characteristics of the chloroplasts and supernumerary nucleus which are typical of some members of that group. Such a hypothesis was first forwarded by Tomas *et al.* (1973), and later elaborated on by Tomas and Cox (1973) to explain the fine structural features of *Peridinium balticum*, which are almost identical to those of *G. foliaceum*. The idea was subsequently applied to *G. foliaceum* (Jeffrey and Vesk, 1976; Dodge, 1983a) and it is on this interpretation the this thesis is based. Henceforth, therefore, the membrane bound cytoplasmic compartment in *G. foliaceum* will be termed "the endosymbiont" and the supernumerary nucleus will be referred to as the endosymbiont nucleus or more simply the symbiont nucleus. Similarly, the remainder of the cell will sometimes be called "the host". These terms are by no means satisfactory as they do not convey the high degree of morphological, presumably functional and possibly genetic integration that exists between the two partners. However, introducing a more suitable terminology at this stage seems an unnecessary complication.

3.3.6. Mitochondria.

The presence of mitochondria in the endosymbiont has already been noted. These have tubular cristae and from their general structure would appear to be metabolically active. Mitochondria also occur in the host cytoplasm but have relatively few tubular

cristae and somewhat dense contents. They are also smaller than the endosymbiont mitochondria being only half the diameter and much shorter. Consequently the host mitochondria have a slightly redundant appearance (examples can be seen in Plates 3.4.2, 3.5.1 and 3.8.2). However, occasionally giant mitochondria are encountered which can measure up to 10 μm across (Plates 3.9.1 & 2). The tubular cristae of these giant mitochondria stem relatively infrequently from the inner membrane but ramify throughout the matrix, branching repeatedly. By virtue of their size, they have been observed close to the dinoflagellate nucleus and no membrane separates the two organelles, so presumably they belong to the host. A chromosome-like structure can be seen in some of the giant mitochondria (Plate 3.9.1). This consists of tightly condensed fibrils surrounded by a clear region and is presumably the location of the mitochondrial DNA.

Giant mitochondria very similar to those of G. foliaceum, and containing "mitochondrial chromosomes", have been found in the dinoflagellate Gonyaulax polyedra (Herman and Sweeney, 1979). Interestingly, giant mitochondria have also been reported in the host cytoplasm of Peridinium balticum (Tomas and Cox, 1973) but had not until now been seen in G. foliaceum. Giant mitochondria are formed temporarily during the cell cycle of Euglena gracilis and Chlamydomonas reinhardii, probably by the fusion of smaller mitochondria (Osafune, 1973). These mitochondria differ from those seen in G. foliaceum in that the cristae do not extend into the middle of the matrix. If the giant mitochondria of G. foliaceum are formed in a similar manner then further development of the cristae must occur. The significance of giant mitochondria in both Glenodinium foliaceum and Peridinium balticum is unclear.

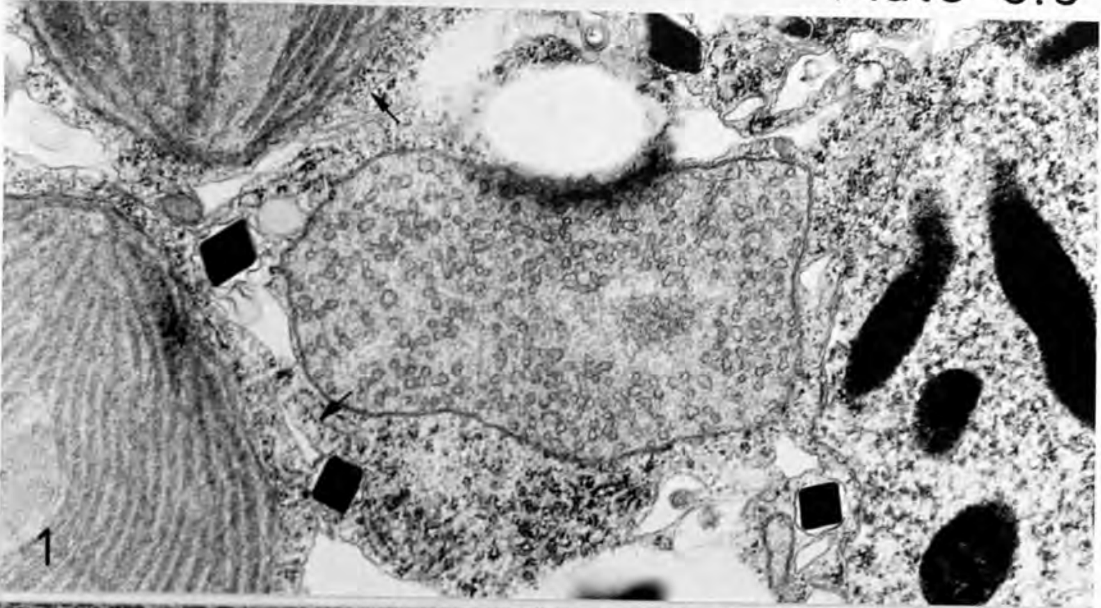
Plate 3.9. Some ultrastructural features of the host in Glenodinium foliaceum. Transmission electron micrographs.

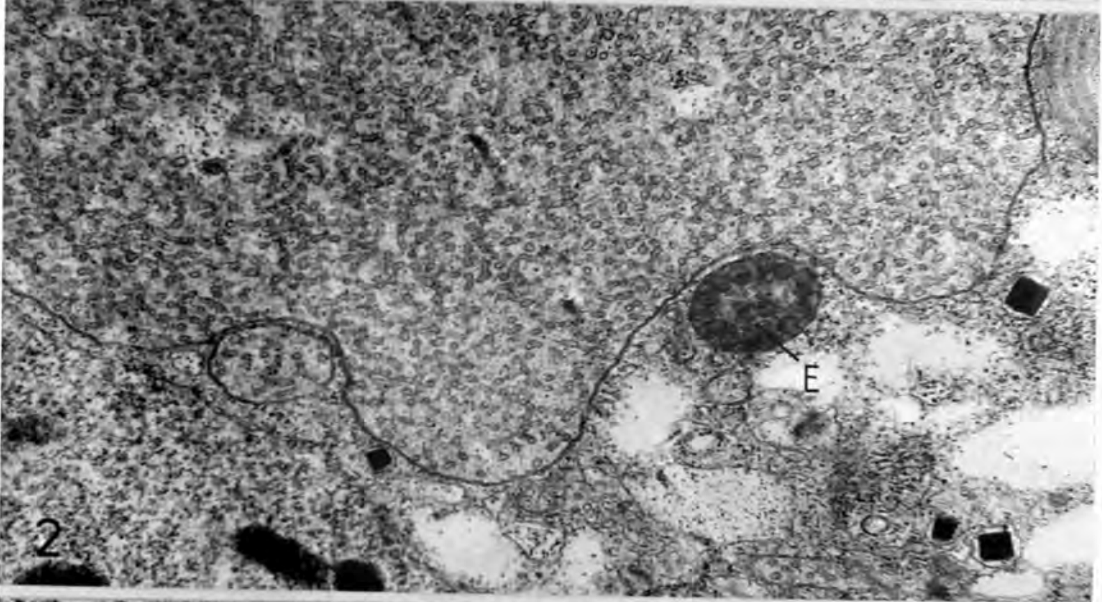
1. Giant mitochondrion with a prominent mitochondrial "chromosome". The mitochondrion is clearly in the host cytoplasm since the limiting membrane (arrowed) separates it from the chloroplasts and not from the dinoflagellate nucleus. x 23,000.

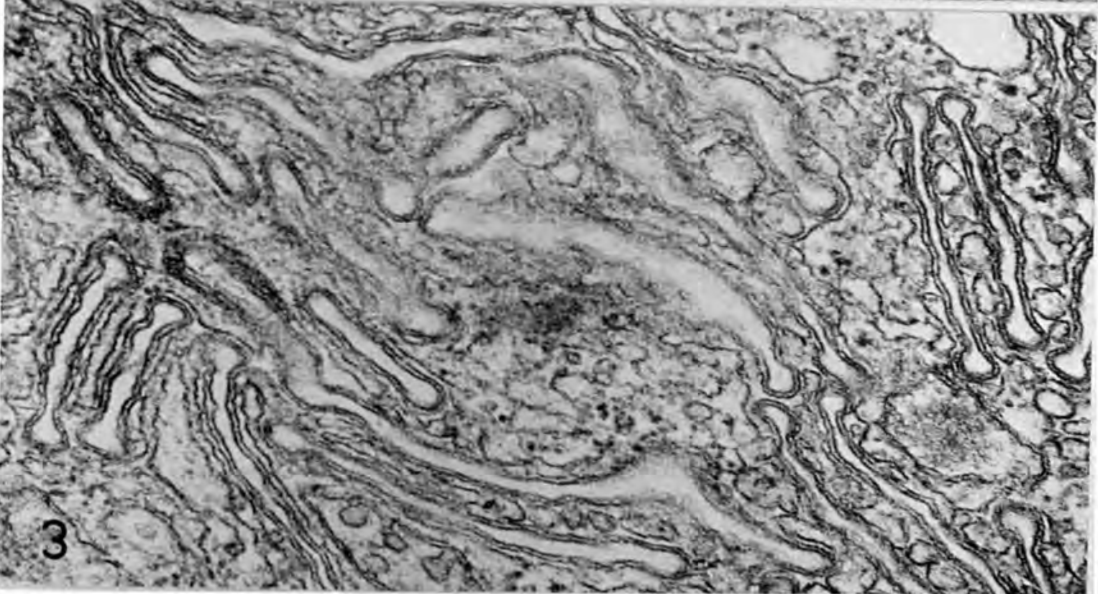
2. Section through part of a giant mitochondrion lying near to the eyespot (E). Note that the tubular mitochondrial cristae completely fill the matrix. x 20,800.

3. Part of the pusule of G. foliaceum. x 62,400.

Plate 3.9







3.3.7. Other organelles.

To complete the general description of *Glenodinium foliaceum* the presence of two other organelles in the host cytoplasm needs to be noted. The eyespot (Plate 3.9.2) basically consists of a number of carotenoid-containing lipid globules bound by a three membrane envelope and is therefore analagous to, and perhaps homologous with, a typical dinoflagellate plastid. Its structure has been described in some detail by Dodge and Crawford (1969) so will not be elaborated on here. The pusule in *G. foliaceum* is a complex of elongated vesicles and smooth endoplasmic reticulum (Plate 3.9.3). The vesicles appear to be bound by two membranes. The pusule is a characteristic organelle in dinoflagellates whose precise function is uncertain but is probably associated with either osmoregulation or excretion (Dodge, 1972). Finally, it is worth emphasizing that the host cytoplasm lacks chloroplasts whilst the endosymbiont is devoid of food storage products so there is an obvious nutritional relationship between the two (Dodge, 1983a).

CHAPTER 4

ANALYSIS OF THE DNA OF GLENODINIUM FOLIACEUM

4.1. ISOLATION AND CHARACTERIZATION OF NUCLEAR DNA.

4.1.1. Problems associated with the purification of DNA from Glenodinium foliaceum.

Although Glenodinium foliaceum is not a heavily armoured species, the theca, nevertheless, makes it resistant to gentle lysis techniques such as osmotic shock, repeated freezing and thawing and treatment with detergents. Ecdysis can be induced by resuspending cells in 25% Ficoll as described by Rizzo et al. (1984) for Crypthecodinium cohnii. However, this procedure was not very reliable when applied to Glenodinium foliaceum and often failed to have any effect. Other factors such as the age of the culture and the centrifugal force with which the cells were collected probably influence the success of the Ficoll treatment. Even if sphaeroplasts are produced they are still resistant to digestion with 1 $mg.ml^{-1}$ pronase, 1% SDS at $50^{o}C$ for 4 h. Pronase digestion solubilizes a cell wall deficient mutant of Chlamydomonas reinhardii (Rochaix, 1982), so this would suggest that Glenodinium sphaeroplasts are not simply surrounded by a protein/lipid membrane. The reported presence of a pellicle in G. foliaceum (Morrill and Loeblich, 1981) could explain the cell's resistance to digestion, but a pellicle was not detected in the ultrastructural observations (Section 3.3.1).

Fortunately, G. foliaceum is quite a large cell which makes it reasonably susceptible to mechanical breakage. Here it was found that the use of a finely ground, Potter-Elvehjem homogenizer produced higher yields of DNA of greater average fragment size than either grinding the cells to a powder in liquid nitrogen, passage through a French Pressure Cell at 12,000 psi or shearing with a Wareing Blender.

Another problem associated with obtaining total DNA preparations from Glenodinium foliaceum is the co-precipitation in ethanol of large amounts of an orange gelatinous substance, following phenol extraction of the homogenate. This contaminating substance could be removed by passing the preparation through a column of laboratory made hydroxylapatite equilibriated with 8 M urea, 0.24 M sodium phosphate buffer (Figure 4.1). Under these condition, double-stranded DNA binds to the hydroxylapatite whilst RNA, most denatured proteins, polysaccharides and other possible contaminants do not (Britten et al., 1970). The only minor drawback with this technique was that the contaminated DNA preparation caused the hydroxylapatite to clog badly which hindered the loading and washing of the column. The one step purification of DNA using hydroxylapatite, described by Britten et al. (1970), could not be applied to G. foliaceum because the cells collapse in 8 M urea and then become extremely difficult to disrupt mechanically.

A third problem is that of yield with respect to culture volume. Between 3 - 4 mg of DNA was routinely obtained from 20 l of G. foliaceum culture (cell density approximately 5×10^3 cells.ml^{-1}). From estimates of the average DNA content of a cell (see Section 4.1.4) this represents 30 - 40% of the total recoverable DNA. However, milligram quantities are required to study the reassociation kinetics of DNA using hydroxylapatite chromatography, so such yields were barely adequate for this type of work.

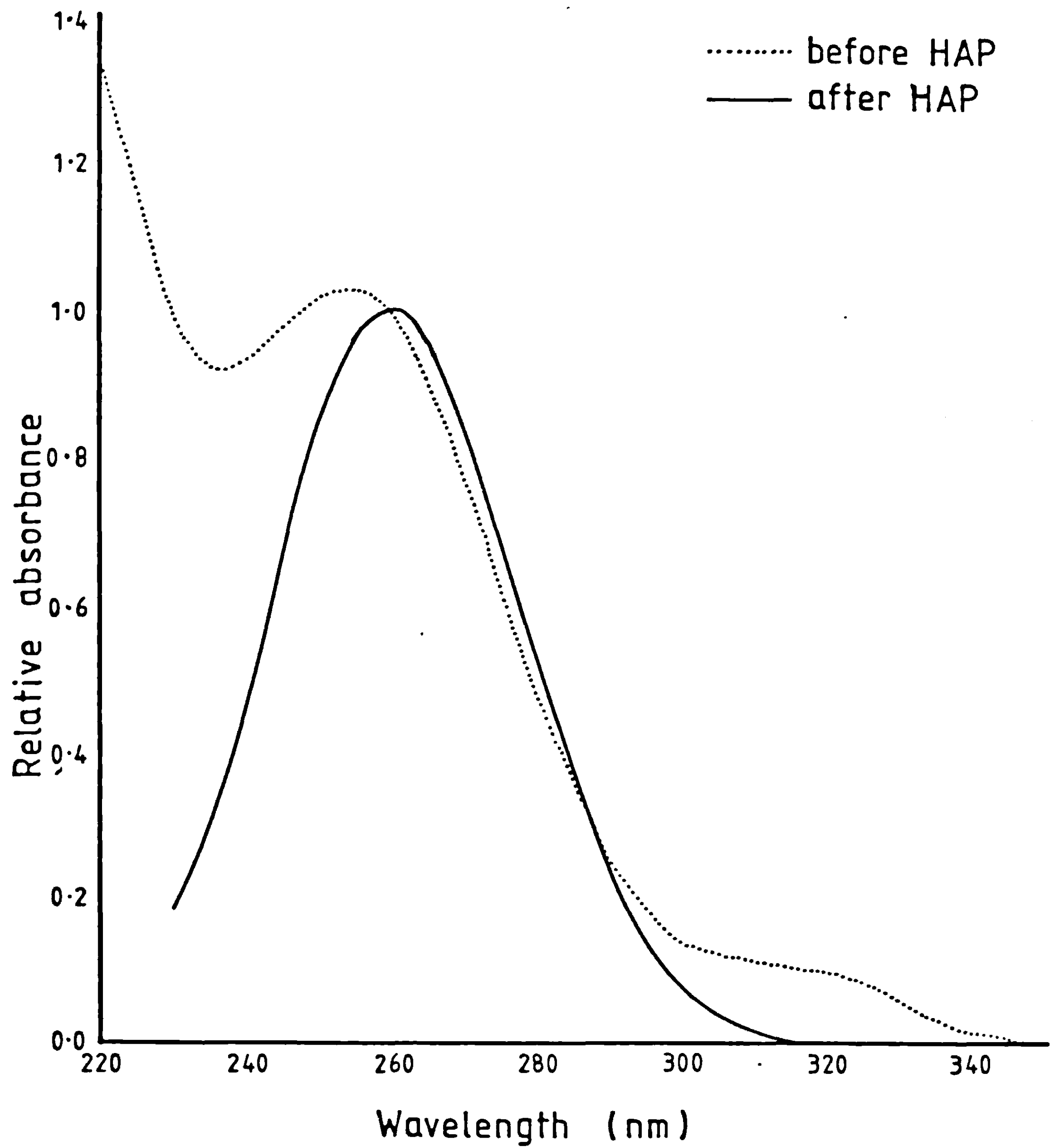


Figure 4.1. Purification of Glenodinium foliaceum DNA by hydroxylapatite chromatography. Absorption spectra of whole cell DNA extracts before and after passing through a column of hydroxylapatite (HAP).

4.1.2. Fractionation of DNA in caesium chloride density gradients.

Total cell DNA extract of *Glenodinium foliaceum* are, of course, composed largely of nuclear DNA. Since the cell has two nuclei and the dinoflagellate nucleus is likely to contain a substantial amount of DNA, the level of cytoplasmic DNA contamination is expected to be low. Even so, small amounts of chloroplast DNA would have confused any results obtained from the proposed genetic analysis and so had to be removed from the preparations.

The chloroplast DNA from many, but not all, of the unicellular algae studied to date is separable from the nuclear DNA on the basis of its differing physical characteristics. Unlike in higher plants (Kolodner and Tewari, 1975; Herrmann *et al.*, 1975), the chloroplast DNA of algae is relatively rich in A-T base pairs so it runs as a low density satellite in caesium chloride density gradients. This is true for the green algae *Chlamydomonas reinhardii* and *Chlorella ellipsoidea* (Howell and Walker, 1976; Yamada, 1982); *Euglena gracilis* (Manning *et al.*, 1971); two species of the red alga *Porphyridium* (Charles, 1977; Cragie, 1982); the chromophytes *Ochromonas danica*, *Polyedriella helvetica* and *Olisthodiscus luteus* (Charles, 1977; Aldrich and Cattolico, 1981); and possibly also the dinoflagellate *Amphidinium carterae* (Galleron and Durrand, 1978). The bouyant densities of chloroplast DNA from *Dunaliella bioculata* and *Cyanidium caldarium* are not distinctly different from those of the nuclear DNA (Marano, 1979; Cragie, 1982). However, if the fluorescent dye Hoechst 33258 is included in the CsCl gradients, the chloroplast DNA of *Cyanidium* can be resolved as a satellite band (Kite, unpublished observation). This dye has also been used to accentuate the separation of the satellite DNAs of *Olisthodiscus luteus* (Aldrich and Cattolico, 1981).

Glenodinium foliaceum DNA was not subjected to analytical ultracentrifugation in CsCl so it is not known whether any of the DNA is naturally satellitic, but after fractionation of total DNA in preparative CsCl gradients containing Hoechst dye, a single satellite species was present (Plate 4.1.1). There was no evidence of a second satellite as in Olistodiscus luteus (Aldrich and Cattolico, 1981) and the main band seemed to be composed of two major sub-bands which were not resolved by these gradients. By manipulating the gradient parameters, the satellite could be drawn well away from the main band DNA allowing it to be recovered without the need for re-centrifugation.

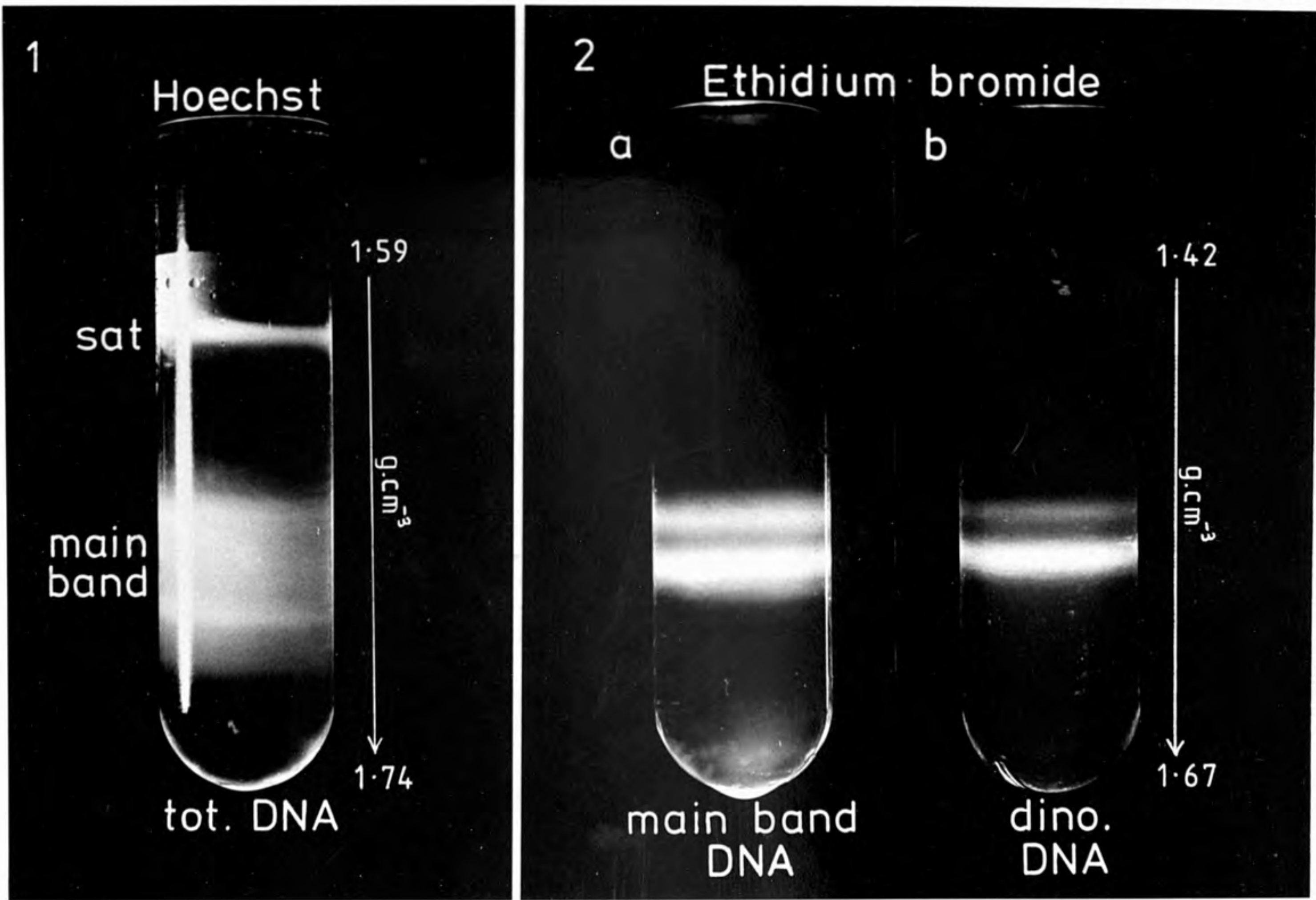
Optimal fractionation was obtained in a shallow CsCl gradient produced from a starting density of 1.66 g cm^{-3} by centrifugation at 80,000 g. The densities at equilibrium in a 10 ml gradient ranged from 1.59 to 1.74 g cm^{-3}. So as to reduce the time taken for the DNA molecules to reach their equilibrium density under these conditions, the gradients they were first centrifuged at a higher relative centrifugal force and then the steep gradient so formed was allowed to "relax" at the equilibrium speed (Birnie, 1978). To obtain a one step fractionation it was important not to overload the gradients with DNA. The maximum amount of DNA that could be run in one 10 ml gradient was 200 μg with a dye to DNA ratio of 10:1 (w/w). The satellite DNA constituted about 1% of the cellular DNA and was identified as pure chloroplast DNA on the basis of a restriction endonuclease analysis (see Section 4.2.3). Any mitochondrial DNA presumably banded in the broad main band, that is, with the nuclear DNA. However the level of contamination of the nuclear DNA with mitochondrial DNA is likely to be extremely low and should not interfere with subsequent experiments.

Plate 4.1. Fractionation of *Glenodinium foliaceum* DNA in caesium chloride density gradients. Photographs of gradients taken under ultraviolet light to visualize the DNA bands.

1. Separation of satellite DNA in a CsCl gradient containing Hoechst 33258.

2. Centrifugation of DNA in CsCl/ethidium bromide gradients:
   a. fractionation of the main band DNA from (1) into its two component sub-bands.
   b. analysis of DNA extracted from a preparation of the dinoflagellate nucleus.

Plate 4.1



that the denser of the two nuclear DNA sub-bands of G. foliaceum corresponds to DNA from the dinoflagellate nucleus, and the upper band DNA is symbiont nuclear DNA.

This assertion was partially confirmed by subjecting DNA extracted from an enriched preparation of dinoflagellate nuclei to a density gradient analysis. This DNA formed two bands as with total nuclear DNA, but most of the DNA was present in the lower band as estimated from the relative intensities of ethidium bromide fluorescence (Plate 4.1.2b). Thus, although the lower band DNA will be referred to as dinoflagellate nuclear DNA and the upper band as endosymbiont nuclear DNA, this assignment is not definitive.

4.1.3. Isolation of nuclei from Glenodinium foliaceum.

Nuclei have only been isolated from a few species of unicellular algae which possess rigid cell walls. Fortunately, though, and largely through the work of Rizzo, nuclei isolation protocols have been developed for seven species of dinoflagellates; Gymnodinium nelsonii (Mendolia et al., 1966; Rizzo and Noodén, 1973), Crypthecodinium cohnii, Peridinium cinctum, Scrippsiella trochoidea (Rizzo and Noodén, 1973), Peridinium balticum (Rizzo and Cox, 1977), Prorocentrum micans (Herzog and Soyer, 1981) and Gymnodinium breve (Rizzo et al., 1982). A reasonably pure preparation of symbiont nuclei has been obtained from Peridinium balticum (Rizzo and Burghardt, 1980; Rizzo, 1982) but even though the dinoflagellate nuclei outnumbered the eukaryotic nuclei in certain homogenates (Rizzo, 1982), the former nucleus has not been purified. Little success has been had with isolating either nuclei from Glenodinium foliaceum (Rizzo, personal communication).

The symbiont nucleus of G. foliaceum is possibly naturally fragmented and in any case would be likely to become so during isolation. As one of the reasons for wanting to isolate nuclei from G. foliaceum was to estimate the amount of DNA in the symbiont nucleus, it would be futile trying to obtain a pure preparation of fragments of this nucleus. Also, exploratory experiments suggested that more success would be had with isolating the dinoflagellate nucleus. As with P. balticum, the dinoflagellate nucleus of G. foliaceum was more frequent in most cell homogenates, so if this fact could be manipulated further to produce homogenates which only contained this nucleus, then the subsequent purification would be greatly simplified.

Two factors which could conceivably affect nuclear stability were investigated. These were the composition of the isolation buffer and the method of cell disruption. Cells were broken in a standard osmoticum (0.25 M sucrose, 5 mM $CaCl_2$, 10 mM Tris-HCl pH 7.3) containing either 50% (v/v) glycerol or 7.5% (w/v) high molecular weight polysaccharides (5% Ficoll 400, 2.5% Dextran 40; i.e. a modified Honda medium) as nucleus stabilizing agents. Cell disruption was accomplished by homogenization in a Potter-Elvehjem hand homogenizer or by sonication (see legend to Figure 4.2 for further details).

The results showed that the symbiont nucleus was more susceptible to sonic disruption than the dinoflagellate nucleus (Figure 4.2). The stability of the symbiont nucleus, and to a lesser extent the dinoflagellate nucleus, was enhanced in 50% glycerol, as with Peridinium balticum (Rizzo, 1982). However, although the symbiont nucleus was quite frequent in the modified Honda medium after homogenization, it seemed to be completely absent after sonication. Conversely the symbiont nucleus, or nuclear fragments, outnumbered the host nuclei after homogenization in the glycerol medium.

Figure 4.2. Stability of <u>Glenodinium</u> <u>foliaceum</u> nuclei during isolation. A known concentration of cells was disrupted by sonication or homogenization in 0.25 M sucrose, 5 mM $CaCl_2$, 10 mM Tris-HCl pH 7.3 containing either 50% (v/v) glycerol or 5% (w/v) Dextran 40 plus 2.5% (w/v) Ficoll 400. Sonication was carried out as described in Section 2.7.1 but at a constant probe vibration of 150 μm peak to peak. Homogenization was performed in a 1 ml "tight-fitting", Potter-Elvehjem hand homogenizer. After various times of sonication or number of strokes in the homogenizer, 20 μl samples were withdrawn and mixed with 5 μl of methyl green/pyronine B stain solution to determine the yield of isolated nuclei by counting in a haemocytometer. Points represent the means of two determinations. ●——●, dinoflagellate nuclei; O----O, symbiont nuclei or nuclear fragments.

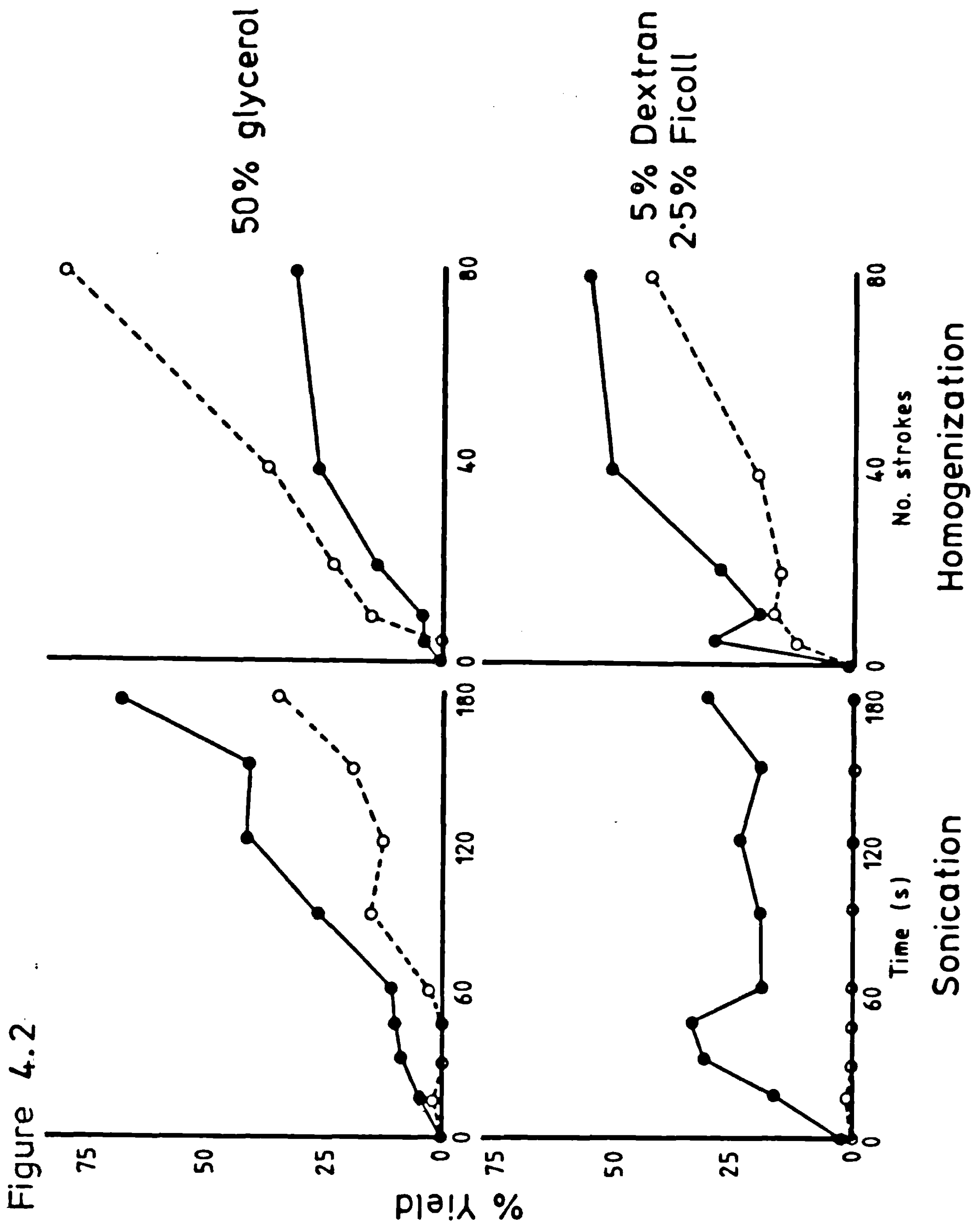


Figure 4.2

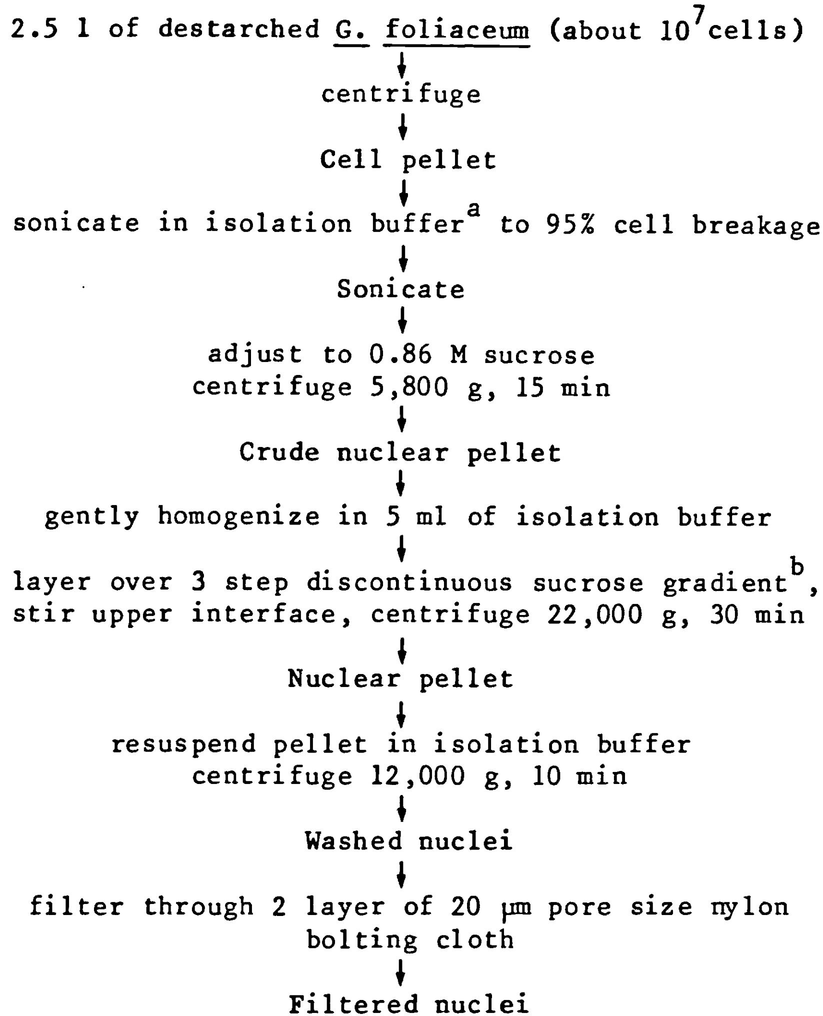
Although the yield of dinoflagellate nuclei was poorest in the Honda sonicates, the 'quality' of these nuclei was the highest. Homogenization produced many dinoflagellate nuclei which had a ragged appearance, whilst in glycerol, these nuclei were swollen and stained faintly with methyl green/pyronine B. The yield of dinoflagellate nuclei in Honda sonicates could be improved by reducing the amplitude of the probe vibration. However, longer sonication times had to be employed to disrupt 95% of the cells and some symbiont nuclei were also present in the preparations. The sonication conditions detailed in Section 2.7.1 were found to be the best compromise between yield and purity. The yields of _Crypthecodinium cohnii_ nuclei have been greatly improved by including hexylene glycol in the isolation buffer (Rizzo _et al._, 1984) but since this compound also preferentially stabilizes the symbiont nucleus of _P. balticum_ (Rizzo, 1982) its effect was not tested here.

The major problem encountered with the subsequent concentration and purification of dinoflagellate nuclei from the Honda sonicates was the removal of the cell wall fragments. These readily pelleted with the nuclei through the 2.4 M sucrose + 10% Dextran 10 pad of the discontinuous sucrose gradients used by Rizzo and Burghardt (1980) for _Peridinium balticum_. The cell wall contamination could not be completely removed by repeating the centrifugation through the 2.4 M sucrose/10% Dextran 10 from an overlay of the isolation buffer at lower speed (18,000 g) (Rizzo and Nooden, 1973). Although most of the nuclei were trapped at the interface, whilst the pellet was enriched in cell wall fragments, the loss in yield would have been too severe if this process were to be repeated to obtain a pure preparation. A viscosity gradient of 20 - 40% (w/v) Ficoll 400 in isolation buffer also failed to fractionate the sonicate.

The simplest and most effective means of removing the cell walls was by filtration. The dinoflagellate nuclei of G. foliaceum were rarely greater than 12 μm in diameter whilst the cell wall fragments were of similar size to the cells (Plate 4.2.1). Therefore two crossed layers of a 20 μm pore size nylon mesh removed all but the smallest wall fragments from the preparation. Filtration was most effective when performed on the pelleted nuclei after the sucrose/dextran density gradient rather than on the unfractionated sonicate. Even so, this filtration step resulted in a substantial loss of nuclei because the nylon mesh soon became clogged with wall fragments.

A flow chart of the final isolation procedure is presented in Figure 4.3 and a light micrograph of the nuclear pellet after filtration and stained with methyl green/pyronine B is shown in Plate 4.2.2. The nuclei seem to be intact and reasonably free of cytoplasmic 'tabs'. A nucleolus, which stains pink, was visible in most nuclei. Although the nuclei appear clumped, these could be dispersed by gentle homogenization to allow the nuclei to be counted in a haemocytometer. The final yield of nuclei was about 5%.

It may be possible to obtain a preparation of symbiont nuclear fragments by fractionating homogenates obtained in 50% glycerol, in which these nuclei predominate. The symbiont nuclei seem to pass more freely than the dinoflagellate nuclei through the interface between the 0.24 M sucrose layer and the 0.24 M sucrose/10% Dextran 10 pad in the standard sucrose gradient. This fact has been exploited by Rizzo and Burghardt (1980) to obtain enriched preparations of the symbiont nucleus of Peridinium balticum. Probably by manipulating the density gradient further the complete separation of symbiont nuclei from contaminating dinoflagellate nuclei in G. foliaceum could be achieved. The use of hexylene glycol to enhance the stability of the symbiont nucleus could also be investigated (Rizzo, 1982).



a. 0.25 M sucrose
5 % Dextran 40
2.5 % Ficoll 400
0.5 % Nonidet P-40
5 mM $CaCl_2$
10 mM Tris-HCl pH 7.3

b. 5 ml 2.2 M sucrose
8 ml 2.4 M sucrose
5 ml 2.4 M sucrose +
10 % Dextran 10

all in isolation buffer

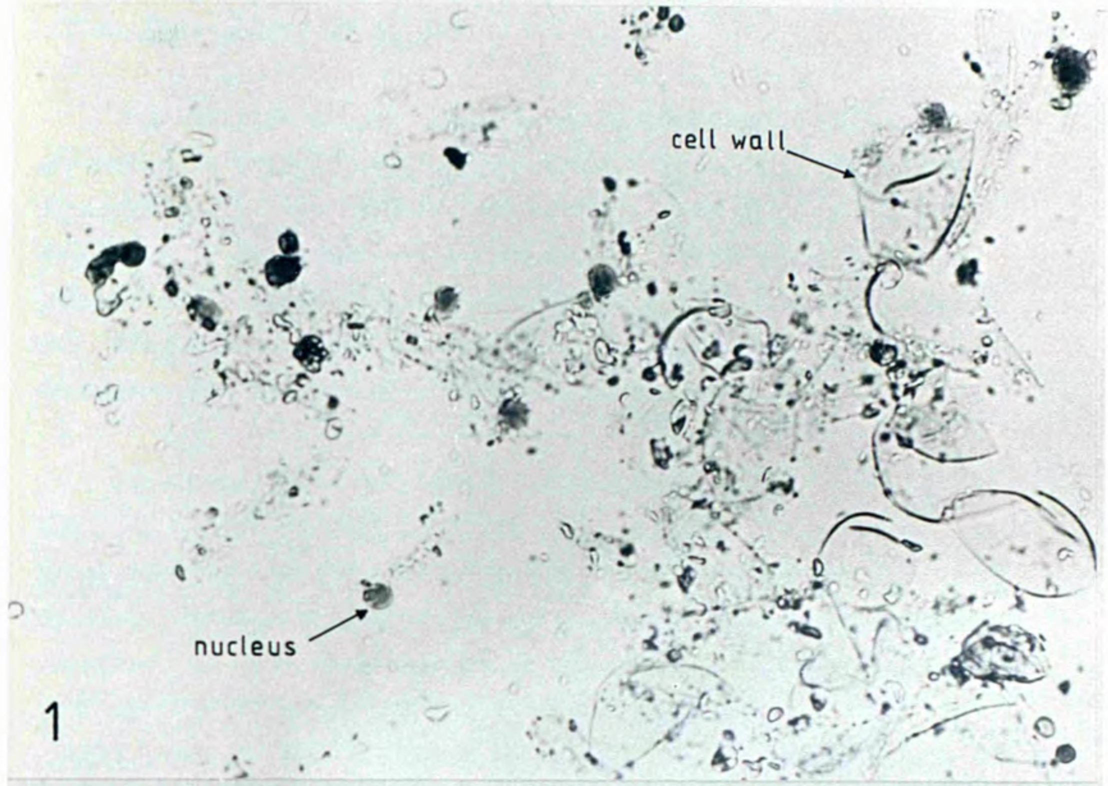
Figure 4.3. Flow chart for the isolation of the dinoflagellate nucleus from *Glenodinium foliaceum*.

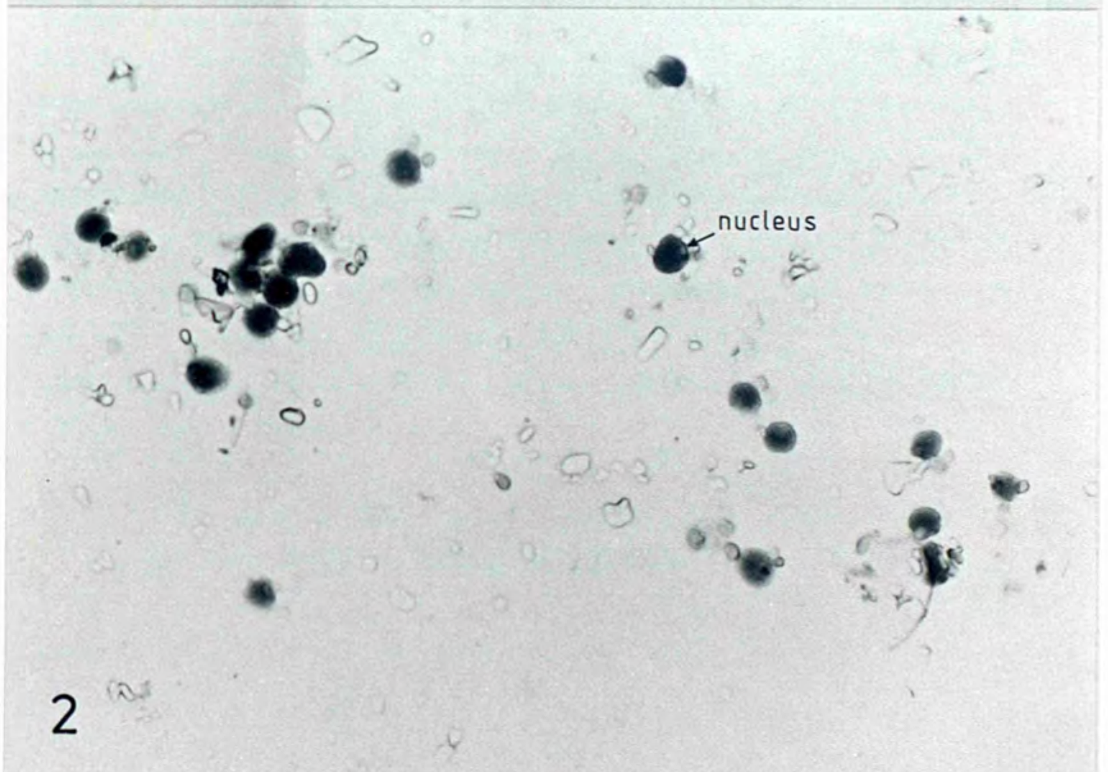
Plate 4.2. Isolated dinoflagellate nuclei. Light micrographs of methyl green/pyronine B stained preparations.

1. Unfiltered nuclear pellet from a sucrose density gradient showing the contamination with cell wall fragments. x 400

2. Preparation after filtration to remove the wall fragments. x 450

Plate 4.2





4.1.4. DNA content of nuclei

The dinoflagellate nucleus of *Glenodinium foliaceum* was estimated to contain, on average, 40 ± 8 pg ($p = 0.95$) of DNA, as determined by the diphenylamine assay of DNA quantitatively extracted from a nuclear preparation. This figure probably represents a slight overestimate due to possible contamination of the preparations with symbiont nuclear fragments, although this contamination was not detectable microscopically.

Dinoflagellates contain a large amount of DNA (Table 4.1) but it is not definitely known whether these values represent true haploid genome sizes or that dinoflagellate nuclei show various degrees of polyploidy or polyteny. Polyploidy has been reported in some dinoflagellates such as *Crypthecodinium cohnii* and species of *Heterocapsa* (see Beam and Himes, 1984) but in *C. cohnii* data on the chromatid size suggests the chromosomes are highly polytene (Haapala and Soyer, 1974) whilst data on the mutation frequency of this species (Roberts *et al.*, 1974) and the reassociation kinetics of its DNA (Allen *et al.*, 1975) are consistent with a haploid condition. *Crypthecodinium cohnii* contains relatively little DNA compared to other dinoflagellates but it is unlikely that higher values can simply be accounted for by extreme polyploidy or polyteny. Presumably dinoflagellates contain a large proportion of genetically inactive DNA which may perform some structural or skeletal function (Cavalier-Smith, 1982a; Sigee, 1984).

Whole cells of *Glenodinium foliaceum* were found to contain 75 ± 4 pg ($p = 0.95$) of DNA by the diphenylamine method. Since about 1% of this is due to chloroplast and mitochondrial DNA (see Section 4.1.2) then, by subtraction, the endosymbiont nucleus must contain about 34 ± 12 pg ($p = 0.95$) of DNA. This supports

Table 4.1. DNA contents (in pg) of dinoflagellate nuclei from log phase cells.

Species	DNA	Reference	Notes
Amphidinium carterae	3.2	Holm-Hansen (1969)	b,c
Crypthecodinium cohnii	6.9	Rizzo & Nooden (1973)	a,c
	7.3	Allen *et al*. (1975)	b,d
Cachonina niei	10	Holm-Hansen (1969)	b,c
	10	Loeblich (1976)	b,d
Gymnodinium sp.	15	Allen *et al*. (1975)	b,d
Woloszynskia coronata	20	Blikstad & Nordby (unpublished)	e
Scrippsiella trochoidea	34	Rizzo & Nooden (1973)	a,c
Glenodinium foliaceum	40	present study	a,c
Prorocentrum micans	42	Haapala & Soyer (1974)	b,c
Polykrikos kofoidii	60	Gaines (1985)	b,f
Gyrodinium resplendens	66	Allen *et al*. (1975)	b,d
Gymnodinium breve	113	Rizzo *et al*. (1982)	a,c
Peridinium volzii	142	Hayhome *et al*.(1985)	a,g
Gymnodinium nelsonii	143	Rizzo & Nooden (1973)	a,c
Gonyaulax polyedra	200	Holm-Hansen (1969)	b,c

Notes: a, isolated nuclei analysed; b, whole cells analysed; c, diphenylamine method; d, diaminobenzoic acid method; e, quoted in Spector (1984); f, from relative DAPI fluorescence; g, from relative mithramycin fluorescence.

the previous observation that the cell contains approximately equal amounts of dinoflagellate and symbiont DNA. It should be stressed that estimates of whole cell and dinoflagellate nuclear DNA were both made from log phase cultures since these values may alter as the culture approaches stationary phase (Allen *et al.*, 1975).

Table 4.2 lists the haploid DNA contents of various algae and higher plants. For comparison, fungi contain 0.005 - 0.19 pg of DNA per haploid nucleus and all multicellular animals (excluding amphibians) have between 0.1 and 23 pg with the lower values being more typical (Cavalier-Smith, 1978). The ploidy of the symbiont nucleus of *G. foliaceum* is not known, so direct comparisons cannot be made. However, it would appear to contain substantially more DNA than the nuclei of its nearest probable free-living relatives; namely the chromophytes in general and diatoms in particular. The highest figure quoted for a chromophyte is that for the diatom *Ditylum brightwellii* which contains about 14 pg of DNA per cell (Holm-Hansen, 1969) Greater quantities of DNA have been reported in other unicellular algae (e.g. *Eudorina elegans* and dinoflagellates) and larger genome sizes also appear to be common amongst multicellular plants. The amoeba *Chaos chaos* is reported to contain 1,400 pg of DNA per cell (Friz, 1968)!

Consequently, from the data on DNA content alone, no definite conclusions can be made about whether or not the endosymbiont nucleus shows any amplification of its genetic material or merely a large haploid genome size.

Table 4.2. Haploid DNA contents (in pg) of photosynthetic cells.

Organism	DNA content
Cyanobacteria	0.0026 - 0.013
Rhodophyceae:	
Porphyridium cruentum	0.1
Chrysophyceae:	
Monochrysis lutheri	0.1
Ochromonas danica	0.2
Olisthodiscus luteus	1.7
Haptophyceae:	
Syracosphaera elongata	4.0
Eustigmatophyceae:	
Polydriella helvetica	0.1
Bacillariophyceae:	
Navicula pelliulosa	0.05
Skeletonema costatum	1.3
Thalassiosira fluviatilis	2.8
Ditylum brightwellii	7.0
Dinoflagellates	3.2 - 200
Euglenophyceae:	
Euglena gracilis	3.0
Chlorophyceae:	
Chlorella sp.	0.066
Scenedesmus obliquus	0.4
Dunaliella tertiolecta	2.5
Chlamydomonas reinhardii	12.4
Volvox carteri	11.4, 26
Pandorina morum	20, 25
Eudorina elegans	17, 50
Bryophytes	0.6 - 4.3
Pteridophytes	6.0 - 310
Gymnosperms	4.2 - 50
Angiosperms	1.0 - 89

Data from Holm-Hansen (1969), Charles (1977), Cavalier-Smith (1978), Coleman (1979a), Rizzo and Burghardt (1983).

4.1.5. Thermal denaturation of Glenodinium foliaceum DNA.

Heating a solution of DNA disrupts the hydrogen bonds which hold the two strands of the double helix together. As the strands separate the bases are released from the electrostatic attractions that had maintained their stacked arrangement in the double helix and this can be detected as an increase in absorbance (hyperchromicity) in the ultraviolet. The thermal denaturation or "melting" of DNA, can therefore be followed by monitering its 260 nm absorption peak in the spectrophotometer.

The transition from from single- to double-stranded DNA usually occurs over a relatively small temperature range and the temperature at which half the maximum hyperchromicity is reached is known as the melting temperature (T_m). The value of T_m shows a direct relationship with the base composition of the DNA since G-C base pairs are more stable than A-T ones as they are held together by three hydrogen bonds rather than two. Thus, the G + C content of DNA can be calculated from its T_m value by using the following equation:

$$\% G + C = 2.44 (T_m - 81.5 - 16.6 \log M)$$

where M is the molarity of the counterion (usually sodium) (Mandel and Marmur, 1968).

In recent years melting curves of DNA have been determined more precisely by using equipment which simultaneously records sample temperature and optical density with great accuracy (e.g. Guttmann et al., 1977; Blake and Lefoley, 1978; Szécsi and Dobrovolszky, 1980). This has permitted the construction of high resolution differential melting curves with respect to temperature. These derivative curves usually reveal some heterogeneity in the melting of the DNA, with regions which denature above and

below the T_m. On the differential plot, T_m is taken to be the temperature corresponding to the principle point of inflection (Pivec et al., 1970) and so is not necessarily equivalent to the temperature of half hyperchromicity.

The high resolution derivative melting curve of native, unfractionated Glenodinium foliaceum DNA in 120 mM phosphate buffer is shown in Figure 4.4. Its general form, being composed of a major and a minor peak, is almost identical to that of the DNA from another dinoflagellate Prorocentrum micans (Herzog and Soyer, 1982). The major component of G. foliaceum DNA denatures fairly homogeneously and has a T_m of 85.9^{o}C, which corresponds to a G + C content of 40.9%. The minor component denatures more heterogeneously between 66 and 68°C, which is below that expected for DNA at this salt concentration! Herzog and Soyer (1982) could not eliminate this component from P. micans DNA by caesium chloride density gradient fractionation or 4B-sepharose chromatography, but these authors did not comment on its identity.

Dinoflagellate DNA has a low thermal stability due to the presence of hydroxymethyluracil and so the G + C value obtained from the melting temperature is an underestimate of the actual base composition (Rae and Steele, 1978). It has been demonstrated that hydroxymethyluracil is not distributed evenly throughout dinoflagellate DNA (Steele and Rae, 1980). Therefore, the anomalous melting component could actually represent regions of DNA with a low G + C content and possibly a high replacement of thymine with hydroxymethlyuracil. More support for this argument would come from the study of the denaturation of fractionated G. foliaceum DNA, since the thermal labile component should be absent from the symbiont nuclear DNA if it is not an artifact due to a contaminant.

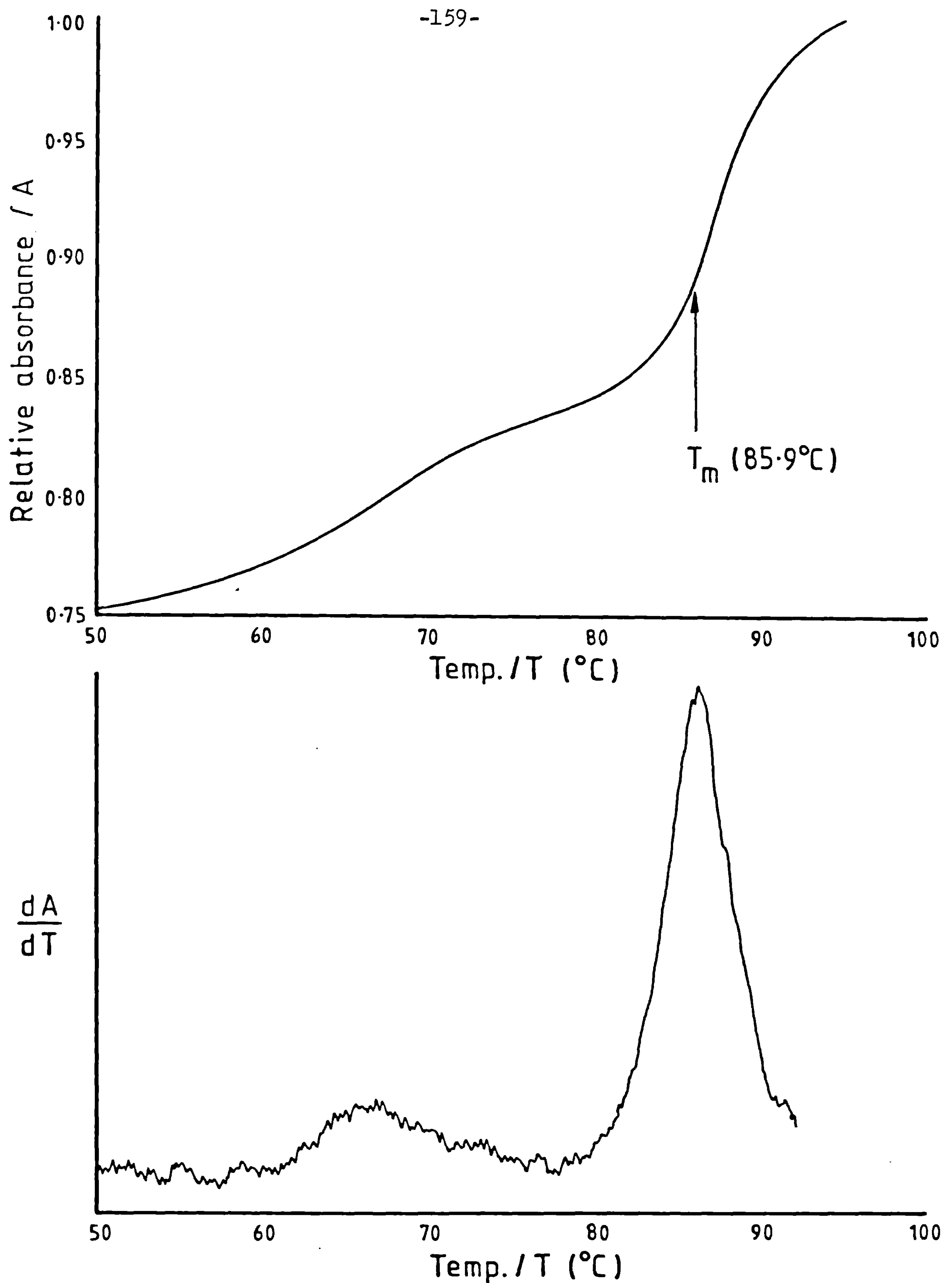


Figure 4.4. Melting curves of native Glenodinium foliaceum DNA in 120 mM phosphate buffer. Thermal denaturation profile of the DNA measured at 260 nm and its unsmoothed, computer-generated, differential plot. T_m, temperature at which the maximum rate of strand dissociation occurs.

4.1.6. Reassociation kinetics of Glenodinium foliaceum DNA.

When incubated at a temperature below its T_m, the complementary strands of denatured DNA will reassociate. The maximum rate of reassociation occurs at about $T_m - 25^{o}C$. By following the renaturation kinetics of DNA that has been sheared into small fragments, information can be gained about the organization of sequences in the DNA. However, the interpretation of DNA reassociation kinetics can become complex if one takes into consideration all of the factors, both experimental and theoretical, that affect the analysis of the data (see Britten *et al.*, 1974). Consequently, only a simple analysis of some preliminary results obtained for *Glenodinium foliaceum* DNA is attempted here, and the following account is not intended to be an exhaustive or definitive description of the sequence organization in this species.

DNA reassociates as a bimolecular second-order reaction, so the fraction (F) of single-stranded DNA remaining after time (t) is given by:

$$F = \frac{1}{1 + k.C_o.t} \qquad (1)$$

where C_o is the initial concentration of single-stranded DNA and k (in litres (moles of nucleotide)$^{-1}$.second^{-1}) is the second-order rate constant. k is related to the complexity of a sheared DNA preparation. The complexity (X) is the length (in nucleotides) of the longest, non-repeating sequence that could be produced by splicing together fragments in the population. For organisms whose DNA contains no repeated sequences, X is simply the genome size. Providing that the concentration of DNA remains the same, the larger the genome the slower its rate of reassociation, because the individual complementary sequences are present at a lower concentration. Implicit in this is that if any sequences are repeated in a given genome, then in a sheared

preparation of the DNA these sequences will reassociate faster.

From the value of k obtained for Escherichia coli DNA, the relationship between X and k under standard reassociation conditions ($60^{o}C$, 180 mM Na^{+}) can be approximately stated as

$$X = \frac{10^6}{k} \qquad (2)$$

assuming that the complexity of E. coli is equivalent to the genome size (Lewin, 1980). It can be seen from equation (1) that k is the reciprocal of the function C_ot when F = 0.5. This C_ot value is defined as the $C_ot_{1/2}$ and can be simply obtained from a plot of F against C_ot (a so-called "Cot" curve).

The reassociation of sheared Glenodinium foliaceum DNA is presented as a Cot curve in Figure 4.5. The DNA is clearly composed of more than one kinetic component since it reassociates over at least six orders of magnitude of C_ot values. From equation (1) a single kinetic component would proceed from 10 - 90% renaturation in no more than two orders of magnitude of C_ot. In this analysis, three kinetic components (A,B and C) were assumed to be present in G. foliaceum DNA which comprise 32%, 12% and 56% respectively of the total genome. In order to fit a curve to the data points, the $C_ot_{1/2}$ values of these three components in the mixture were estimated to be 4.5×10^{-3}, 5 and 2,500 mole l^{-1} s, from which their complexities can be calculated as: A, 1.44 kb; B, 600 kb; and C, 1.4×10^6 kb (Note: to calculate X it is necessary to know the $C_ot_{1/2}$ that each component would exhibit alone. This is obtained by multiplying the observed $C_ot_{1/2}$ value by the fractional contribution of the component to the genome). As the $C_ot_{1/2}$ of B is 500 times the $C_ot_{1/2}$ of C, then the former sequences must be 500 times more numerous in the genome than the latter ones. Similarly, component A is at least 500,000 times more frequent than the most slowly reassociating component.

Figure 4.5. Reassociation kinetics of sheared *Glenodinium foliaceum* DNA. The degree of reassociation was determined either optically (open symbols) or with hydroxylapatite (closed symbols). For optical monitoring of reassociation the DNA concentration was about 50 μg ml^{-1} and the sodium phosphate buffer concentrations were (in mM); 30 (△), 120 (○), 400 (▽), and 1000 (□). For hydroxylapatite determinations reassociation was always performed in 120 mM sodium phosphate buffer and the DNA concentrations were (in μg ml^{-1}); 11 (■), 170 (●), 207 (▼) and 710 (▲). All reassociations were carried out at 60°C and the C_ot values have been corrected where necessary to be relative to reassociations performed in 180 mM Na^+ (120 mM PB) (Britten *et al*., 1974). The curve was fitted by a trial and error method. Three components were assumed to be present (A,B, and C) and the second-order rate constants (k) for each of these were predicted (see text) so that their Cot curves could be generated mathematically (dashed lines). The k values were then adjusted appropriately so that the summed Cot curves fitted the data points, as judged by eye.

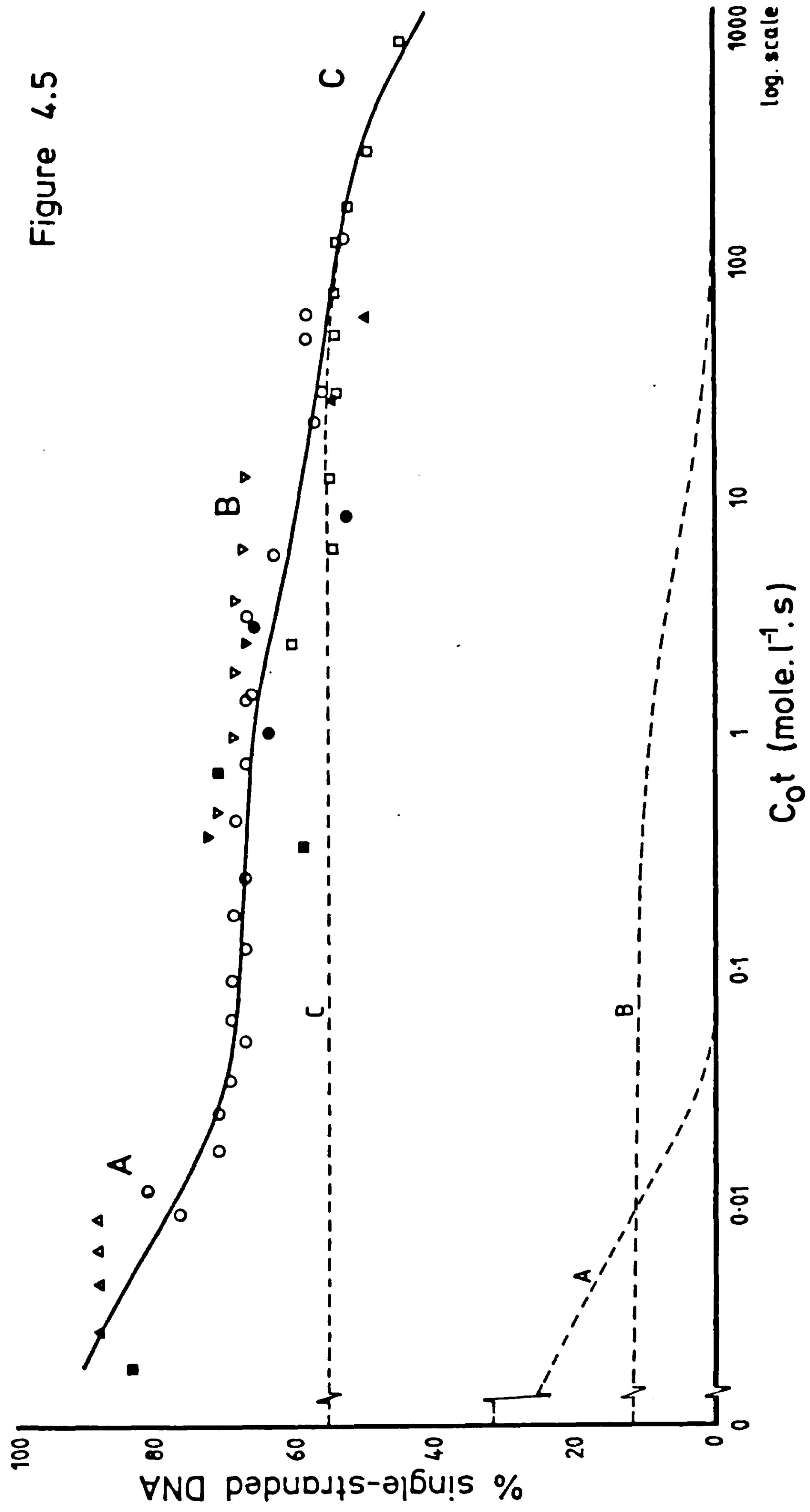


Figure 4.5
% single-stranded DNA
100
80
60
40
20
0
0
0·01
0·1
1
10
100
1000
log. scale
C_ot (mole. l^{-1}. s)
A
B
C

In containing two families of repetitive DNA, G. foliaceum is remarkably similar to most other eukaryotes (Britten and Kohne, 1968; Straus, 1976) in which both short tandem repeats and higher complexity dispersed repeats occur (Southern, 1984). For example, human DNA contains a 770 kb component repeated 500 times and a 4.5 kb component repeated 50,000 times. The proportion of the genome occupied by these repeated sequences varies widely, in plants they may constitute up to 80% of the genome (Flavell, 1982). Glenodinium foliaceum appears to contain rather more repetitive DNA than other lower eukaryotes but this probably reflects its large genome size.

The reassociation kinetics of two species of dinoflagellates have been examined. About half of the DNA of Prorocentrum cassubicum (Steele, 1981) and Crypthecodinium cohnii (Roberts et al., 1974) is composed of highly repeated sequences. In C. cohnii this repeated class has a complexity of 500 kb and a repitition frequency of 3,600 (Allen et al., 1975; Hinnebusch et al., 1980). The remaining 40 - 45% of C. cohnii DNA is composed of a highly complex class (1.5 x 10^6 kb) which has been shown to be unique. The most slowly reassociating component of G. foliaceum DNA (component C) has a similar complexity but it is not clear whether it represents unique sequences. From the kinetic data the haploid genome size of G. foliaceum would appear to be about 2.4 x 10^6 kb (the sum of the complexities of the components multiplied by their relative repetition frequencies). Taking 1 pg to equal 0.965 x 10^6 kb (Lewin, 1980), this represents a DNA content of 2.3 pg which is more than 30 times less than the DNA content of the cell (74 pg) as determined chemically (see Section 4.1.4).

It is posible that G. foliaceum is highly polyploid but it should be stressed that the $C_o t$ value of component C was only predicted on the basis of a few data points and a more slowly reassociating class of DNA could be present. Due to the limited

amount of DNA that was available, the degree of reassociation at C_0t values greater than 100 could not be measured using hydroxylapatite. A C_0t of 700 could be achieved by performing the reassociation in 1 M phosphate buffer and monitoring hypochromicity. However these conditions probably result in a greater number of mismatched duplexes forming which leads to an under estimation of the true $C_0t_{1/2}$ value (Flavell, 1982).

As indicated at the beginning of this Section, numerous factors can complicate the analysis of reassociation data. For example, the degree of renaturation recorded by hydroxylapatite chromatography and by spectrophotometry are not equivalent, as has been assumed here. Hypochromicity measurements give an accurate estimate of the proportion of renatured DNA whilst hydroxylapatite will retain DNA fragments in which only 10% of the fragment has renatured (Flavell, 1982). Hydroxylapatite chromatography therefore leads to an overestimate of the degree of renaturation. Also the relationship between complexity and k is highly dependent on the reassociation conditions and ideally radioactively-labelled E. coli DNA should be included in all reassociations to determine this the under the actual experimental conditions. It was not possible to use a radioactive control in this study.

Bearing these limitations in mind, it can only reasonably be concluded from the results of this preliminary study that the DNA sequence organization in Glenodinium foliaceum is similar to that of other eukaryotes. Two repeated components are present which do not show any features which might be suggestive of an unusual organization of DNA sequences in the symbiont nucleus. However, as might be expected, the reassociation kinetics of G. foliaceum DNA appear to differ slightly from those of Prorocentrum cassubicum and Crypthecodinium cohnii, neither of which contain endosymbionts.

4.1.7. Restriction endonuclease analysis of nuclear DNA.

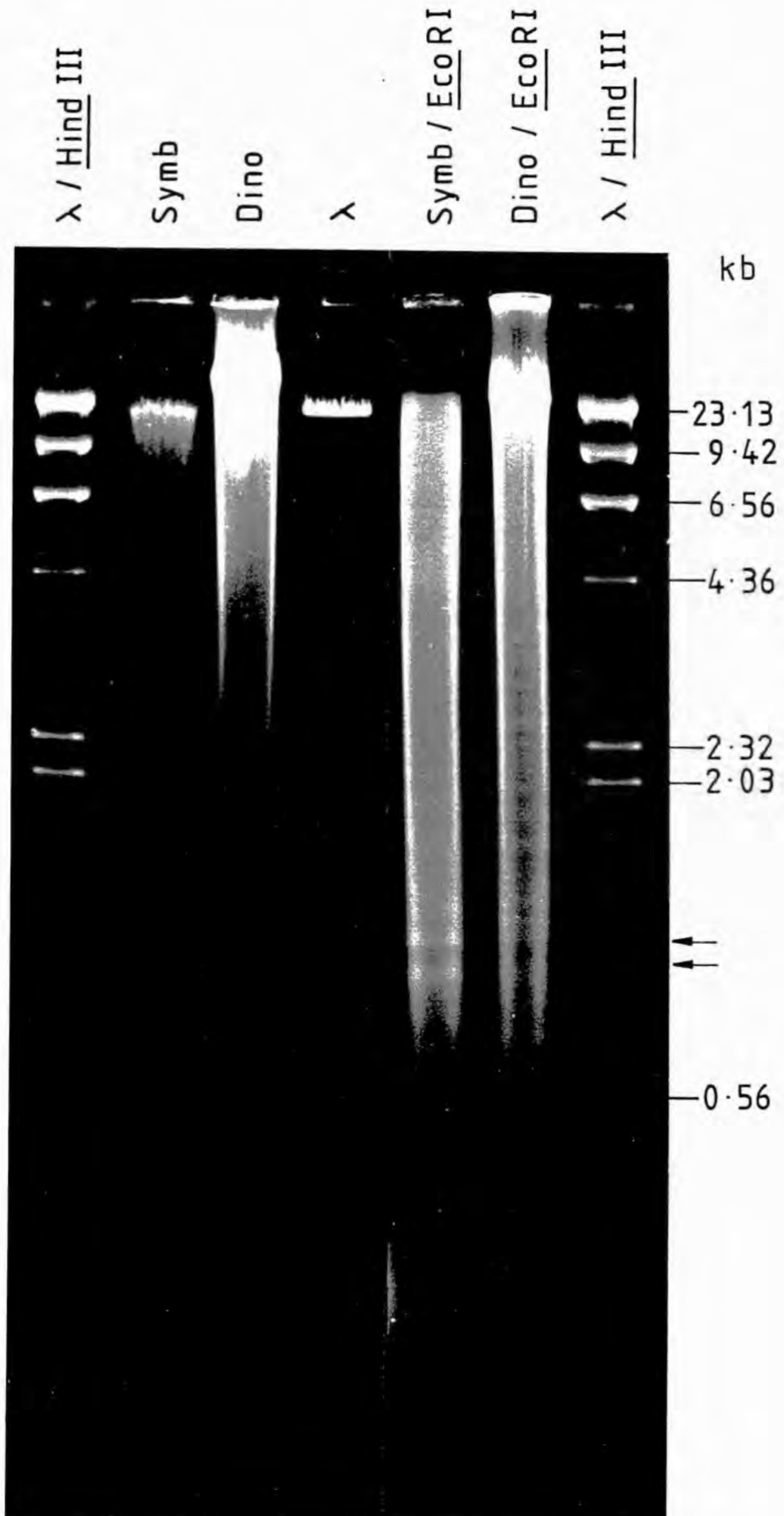
Repeated DNA sequences have a low complexity and so contain relatively few specific endonuclease recognition sites. The restriction fragments of repeated DNA will therefore be of a limited number of sizes, whilst the fragments of unique DNA will show a random array of lengths. If a genome contains a high proportion of repetitive DNA, as in higher plants, then the restriction fragments of the repeated sequences will be detectable, after agarose gel electrophoresis, as more intense bands against a background smear of unique fragments (Flavell, 1982).

The isolated nuclear DNAs of *Glenodinium foliaceum* contained only high molecular weight molecules and neither showed any obvious banding pattern after digestion with *Eco* RI (Plate 4.3). Only a very faint and complex ladder of bands could be seen in the digested dinoflagellate nuclear DNA but not in the symbiont nuclear DNA. However, the symbiont DNA did show two prominent, rapidly migrating bands corresponding to *Eco* RI fragments having lengths of about 0.9 and 1.0 kb. The total complexity of these two fragments is in approximate agreement with the complexity of the highly repeated sequences detected in the kinetic analysis of total *G. foliaceum* DNA (see previous Section). Failure to observe other repeated restriction fragments is probably because any sequences of intermediate repetitiveness that might be present in the genome, did not constitute a large enough proportion of the DNA to be clearly visible.

Thus, highly repeated DNA sequences could be a feature of the endosymbiont, which would explain why they were not detected in the kinetic analysis of *C. cohnii* DNA (Allen *et al.*, 1975).

Plate 4.3. Restriction endonuclease analysis of the nuclear DNA of *Glenodinium foliaceum*. Electrophoresis of undigested and *Eco* RI digested endosymbiont (Symb.) and dinoflagellate (Dino.) nuclear DNA. *Hind* III cleaved λ DNA and native λ DNA (48.5 kb) act as molecular size markers. Neither of the nuclear DNAs show any obvious highly repeated *Eco* RI fragments with the possible exception of two low molecular weight fragments in the symbiont nuclear DNA digest (positions arrowed at the side).

Plate 4.3



4.2. ISOLATION AND CHARACTERIZATION OF CHLOROPLAST DNA.

4.2.1. Isolation of chloroplasts.

Purifying chloroplast DNA from *Glenodinium foliaceum* presented two major problems. Firstly, there were no published procedures for the isolation of chloroplasts from dinoflagellates, and secondly, the growth characteristics of the alga created difficulties in obtaining sufficient yields of DNA.

There are now well established protocols for obtaining bulk preparations of chloroplast DNA from *Chlamydomonas reinhardii* (Rochaix, 1982) and *Euglena gracilis* (Hallick *et al*., 1982). These illustrate the two alternative strategies that can be adopted for dealing with unicellular algae. In *Chlamydomonas*, total DNA is extracted and fractionated by centrifugation in caesium chloride where the chloroplast DNA separates out as a low density satellite. This method does, of course, require prior knowledge about the satellitic nature of the chloroplast DNA and can suffer from contamination with other satellite DNA species. For example, nuclear ribosomal DNA and mitochondrial DNA have been found to be present in *Chlamydomonas* chloroplast DNA fractionated from total DNA extracts in CsCl (Rochaix, 1978).

In *Euglena*, chloroplasts are first isolated and then the DNA is extracted from the organelle preparation. This strategy is preferable because there is no doubt as to the identity of the DNA and the purity of the chloroplast preparation determines the extent of any contamination with other DNA species. Also, since chloroplasts can be lysed gently by chemical means, unlike most algal cells with cell walls, there is the possibility of isolating high molecular weight, or even intact, chloroplast DNA molecules (e.g. Manning *et al*., 1971). Consequently this is the method of choice in higher plants where isolating chloroplasts

presents few difficulties (Kolodner and Tewari, 1975). However, in many unicellular algae the often single chloroplast may have an elaborate shape and be of a similar size to the cell, and so isolating it may be impracticable if not impossible. Fortunately Glenodinium foliaceum has discoid chloroplasts which are sufficiently smaller than the cell for it to be feasible to break open the cell wall without damaging the chloroplasts too severely.

For a routine preparation of about 100 µg of chloroplast DNA from Euglena gracilis, chloroplasts are isolated from 2×10^{10} cells which corresponds to culture volume of 14 l (Hallick et al., 1982). As the density of Glenodinium foliaceum rarely exceeds 1×10^{4} cells ml^{-1} in large volume cultures, it would clearly be difficult to grow a similar number of Glenodinium cells (this would require 2,000 l of culture!). However, only a few micrograms of DNA are required to perform a restriction endonuclease or genetic analysis, so it was predicted that enough chloroplast DNA should be obtained from a harvestable culture volume providing a suitable chloroplast isolation protocol could be developed. Such a protocol would have to be reasonably simple and fairly rapid to permit the repeated re-isolation of the DNA.

There is a dearth of information on techniques for isolating intact chloroplasts in bulk from marine algae for the purposes of DNA isolation. Apparently methods have only been developed for two unicellular species, the chrysophyte Olisthodiscus luteus (Aldrich and Cattolico, 1981) and the diatom Odontella sinensis (Linne von Berg et al., 1982); three filamentous species, the brown algae Pilayella littoralis and a Sphacelaria species (Dalmon and Loiseaux, 1981), and the green alga Derbesia marina (Linne von Berg et al., 1982); and one thalloid brown alga, Dictyota dichotoma (Kuhsel and Kowallik, 1985). Details of the methods used by Linne von Berg et al. (1982) have not been published and the chloroplasts of Glenodinium foliaceum were unstable in the isolation buffers employed by Aldrich and Catto-

lico (1981) and Dalmon and Loiseaux (1981). Sugar alcohols such as sorbitol are generally used to provide the correct osmotic environment for isolated chloroplasts. In plants and freshwater algae these are added to 0.33 M whilst for two of the marine brown algae mentioned above, 0.8 M sorbitol has been suggested (Dalmon and Loiseaux, 1981). By gently squashing cells in various concentrations of buffered sorbitol, it was discovered that at least 1.2 M sorbitol was required to prevent the chloroplasts of G. foliaceum from swelling and eventually bursting. Thus 1.3 M sorbitol plus 2.5% Ficoll was employed as the osmoticum in the isolation buffer. At higher sorbitol concentrations the cells collapsed making them difficult to disrupt. Ficoll was included as it is reported to improve chloroplast stability (see Dunham and Bryant, 1983)

The high density of the isolation buffer allowed cell homogenates to be simply fractionated by differential sedimentation. The efficiency with which a brief low speed centrifugation removed intact cells, cell walls and nuclei from the homogenates meant that cell breakage could be performed reasonably gently, and consequently incompletely, which increased the possibility of releasing intact chloroplasts. Unfortunately, mitochondria cosedimented with the chloroplasts at this density and always contaminated the chloroplast pellet. However the most obvious contamination of the chloroplasts at this stage was with accumulation bodies. Whilst these do not contain DNA, their presence did interfere with the subsequent purification of the chloroplast DNA and so they had to be removed.

Equilibrium centrifugation in continuous or discontinuous Percoll density gradients is probably the most effective method for further purifying chloroplast preparations (Höinghaus and Feierabend, 1983). The cost of Percoll prohibited its use here but it can be substituted with sucrose. The chloroplasts of G. foliaceum appeared to be unstable at their equilibrium density

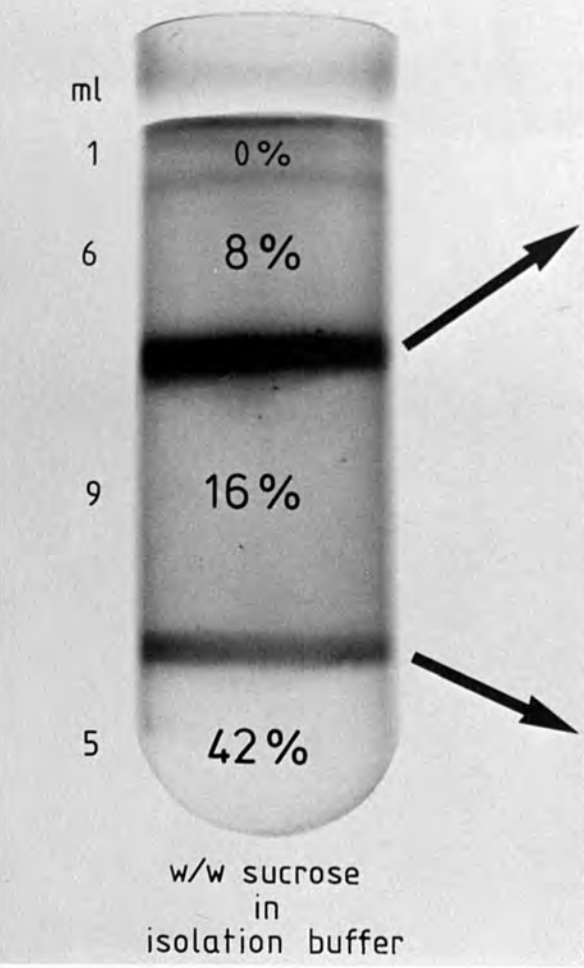
in the discontinuous sucrose gradients used by Sager and Ishida (1963) for purifying Chlamydomonas chloroplasts. Osmotic lysis of the chloroplasts also occurred during rate zonal centrifugation through the discontinuous sucrose gradients proposed by Milfin and Beevers (1974) to separate intact higher plant chloroplasts from broken chloroplasts and mitochondria. Consequently these gradients had to be redesigned for G. foliaceum using the chloroplast isolation buffer as the 'ground buffer' of the gradient so as to ensure chloroplast stability, rather than simply buffering the sucrose solutions with Tris/EDTA.

It was decided to use rate zonal centrifugation to increase the speed of the preparation and eventually the gradient illustrated in Plate 4.4 was found to give a reasonable separation of seemingly intact chloroplasts from obviously broken ones and accumulation bodies. In contrast to the separation achieved with Chlamydomonas and higher plant chloroplasts in sucrose density gradients, the 'intact' chloroplasts of Glenodinium passed less readily through the denser sucrose solutions than the broken ones, and so banded above rather than below them. The reason for this is not clear. It is not suggested that these gradient and centrifugation conditions (detailed in Section 2.8.1) represent the best fractionation that can be obtained. Probably minor adjustments in the sucrose concentrations, centrifugation speed and duration would result in some improvement but such experiments would be time consuming. The major factor affecting the degree of the contamination with accumulation bodies was the age of the culture. Since accumulation bodies became larger and more frequent in cells from late exponential phase and stationary phase cultures, it was important to harvest cultures before mid-exponential phase. This of course further aggravated yield problems.

The non-refractile appearance of most of the purified chloroplasts (see Plate 4.4) suggested that they had lost their

Plate 4.4. Purification of Glenodinium foliaceum chloroplasts by sucrose density gradient centrifugation. Nomarski interference contrast (middle) and phase contrast (right) light micrographs of the upper and lower bands formed at the interfaces of a discontinuous sucrose gradient (left) after rate zonal centrifugation of a crude chloroplast preparation. The upper interface traps the intact (arrow) and reasonably refractile (double arrow) chloroplasts, whilst the broken chloroplasts, most of the accumulation bodies (A) and any remaining whole cell debris band at the lower interface. Micrographs x 1000.

Plate 4.4



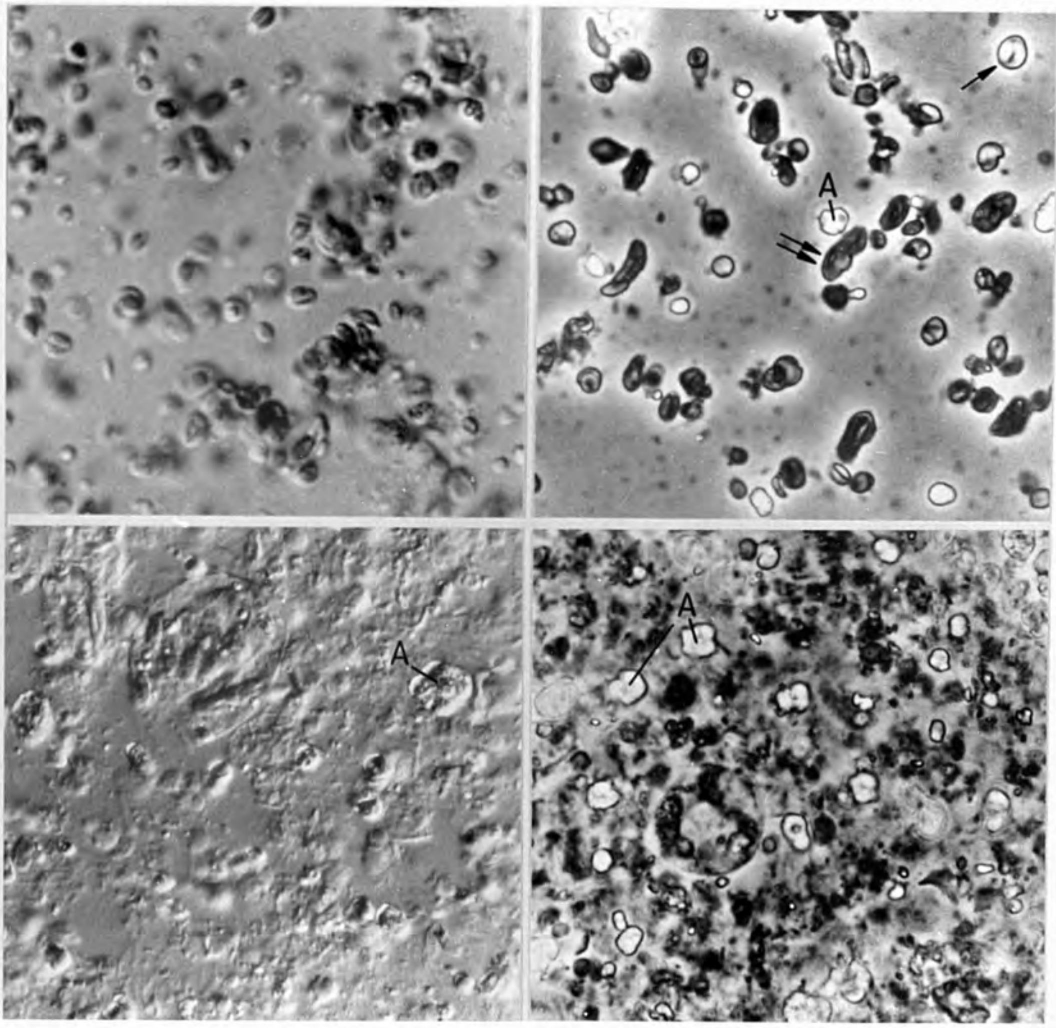


Plate 4.5. Ultrastructure of isolated chloroplasts from *Glenodinium foliaceum*. Transmission electron micrographs.

1. Section through pelleted upper band material (see Plate 4.4) showing it to be composed of chloroplasts with an intact (single arrow) or broken (double arrow) envelopes, a few accumulation bodies (A) and possibly some mitochondria (M). x 6,250.

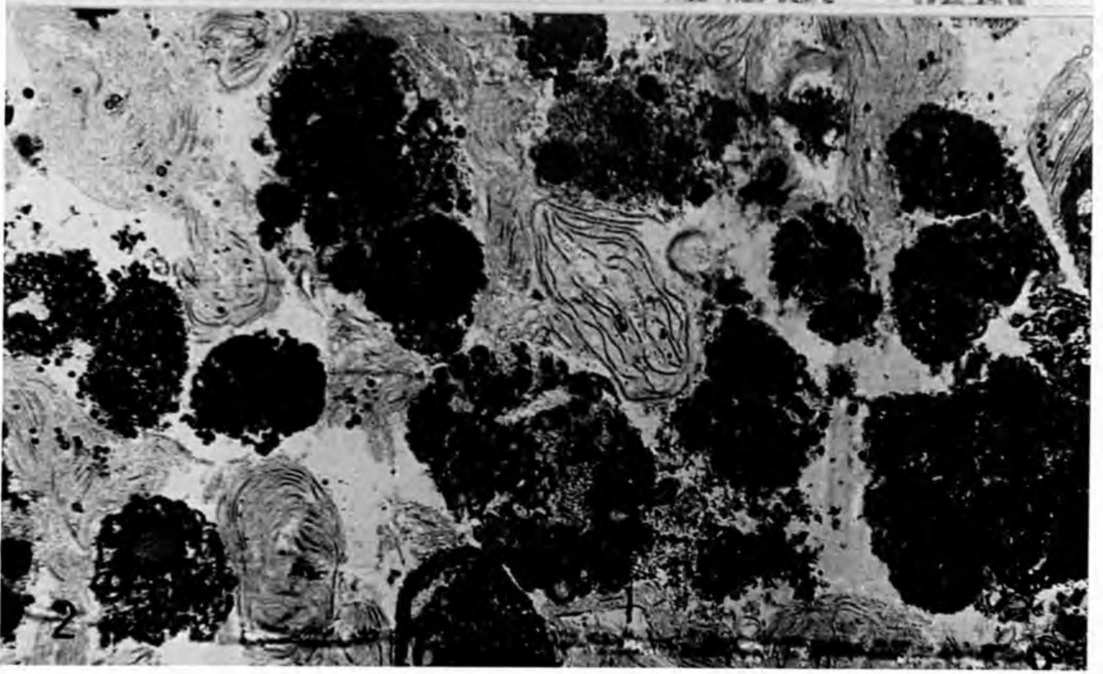
2. Section through pelleted lower band material composed mainly of electron dense accumulation bodies and chloroplasts stripped of enveloping membranes. x 6,500.

Plate 4.5

1

M

A

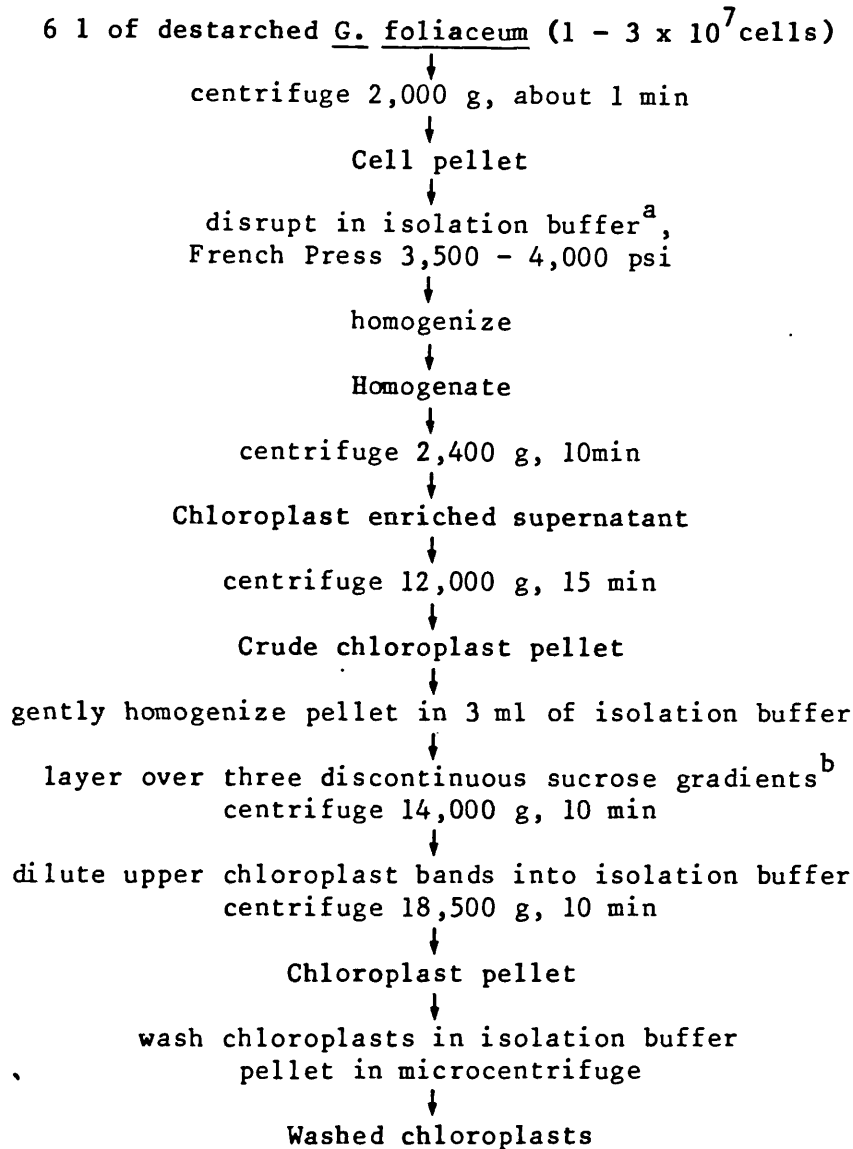


structural integrity either through osmotic dehydration or mechanical breakage. Two lines of evidence support the latter cause. Firstly, electron microscopic examination of the chloroplasts revealed that the outer envelope was often broken (Plate 4.5.1), and secondly, the DNA in the chloroplasts was sensitive to enzymatic digestion. If the chloroplasts were digested with 50 $\mu g \cdot ml^{-1}$ DNase I for 1 h at 0^oC before being fractionated in the sucrose gradient, the DNA isolated from them was found to be degraded (see Plate 4.7. lane c). DNase is unable to enter chloroplasts with an intact membrane otherwise it would not be a standard procedure, when preparing higher plant chloroplast DNA, to treat the chloroplasts with DNase so as to remove adhering strands of chromatin (Kolodner and Tewari, 1975). Thus, to protect the chloroplast DNA against enzymatic degradation, the general nuclease inhibitor aurintricarboxylic acid (ATA) (Blumenthal and Landers, 1973; Bina-Stein and Tritton, 1976) was included in the isolation buffers. The presence of this compound has been found to increase the number of intact molecules in isolates of chloroplast DNA from _Olisthodiscus luteus_ (Aldrich and Cattolico, 1981)

A simplified flow chart of the procedure developed for the isolation of chloroplasts from _Glenodinium foliaceum_ is presented in Figure 4.6. This small scale method ("mini prep.") yields about 50 μl of purified chloroplasts from 6 l of mid-exponential phase culture ($1 - 3 \times 10^7$ cells). The preparation can readily be completed in three hours.

4.2.2. Electron microscopy of chloroplast DNA.

The circularity of chloroplast DNA has readily been demonstrated by electron microscopic visualization of isolated molecules spread on a protein monolayer. In higher plants the chloroplast DNA molecule has a contour length of 37 - 46 μm,



a. 1.3 M sorbitol
2.5 % Ficoll 400
50 mM EDTA
2 mM ATA
10 mM Tris-HCl pH 7.8

b. 6 ml 8% (w/w) sucrose
9 ml 16% (w/w) sucrose
5 ml 42% (w/w) sucrose
all in isolation buffer

Figure 4.6. Flow chart for the isolation of the chloroplasts from *Glenodinium foliaceum*.

depending on the species, and high yields of intact molecules (up to 80% of the total chloroplast DNA isolated) have been obtained (Kolodner and Tewari, 1975; Herrmann *et al*., 1975). Extracting unbroken chloroplast DNA molecules from algae has generally been less successful but circular molecules have been isolated, if only in small yields, from a number of species. These range in length from 18 μm to 62 μm (Table 4.3). In *Acetabularia cliftonii* only linear molecules up to 200 μm long and minicircles have been observed (Green, 1976). However it is doubtful whether even these long linear molecules represent the entire genome and it is likely that all chloroplast DNA is circular.

Table 4.3. Contour lengths of circular algal chloroplast DNA molecules.

Organism	length (μm)	Reference
Pilayella littoralis	18	Dalmon *et al*. (1983)
Codium fragile	27	Hedberg *et al*. (1981)
Sphacelaria sp.	36	Dalmon *et al*. (1983)
Vaucheria sessilis	37	Hennig and Kowallik[a]
Euglena gracilis	40	Manning *et al*. (1971)
Dictyota dichotoma	40	Kuhsel and Kowallik (1985)
Cyanophora paradoxa	42[b]	Bohnert *et al*. (1983)
Olisthodiscus luteus	46	Aldrich and Cattolico (1981)
Chlorella ellipsoidea	56	Yamada (1982)
Chlamydomonas reinhardii	62	Behn and Herrmann (1977)

Notes: a. unpublished observation, cited in Behn and Herrmann (1977),

b. personal analysis of data.

Thus, the contour length of chloroplast DNA molecules demonstrated to be circular by electron microscopy provides a direct measure of the chloroplast genome size. The technique only requires nanogram quantities of DNA so is useful in situations where isolating microgram quantities of DNA for restriction endonuclease analysis is a problem. Unfortunately no circular DNA molecules could be found in lysates of Glenodinium foliaceum chloroplasts when spread for electron microscopy. Only linear molecules with contour lengths of up to 37 μm were observed (Plate 4.6). The average length of the fragments was 17.1 μm which corresponds to a molecular size of 55 kb by comparison with the length of the plasmid pBR322 spread in the same manner, assuming the same mass per unit length. If the largest fragments observed represent entire molecules (i.e. a circular molecule broken once) then this sets an approximate lower limit to the chloroplast genome size of G. foliaceum at 114.5 kb.

4.2.3. Restriction endonuclease analysis of chloroplast DNA.

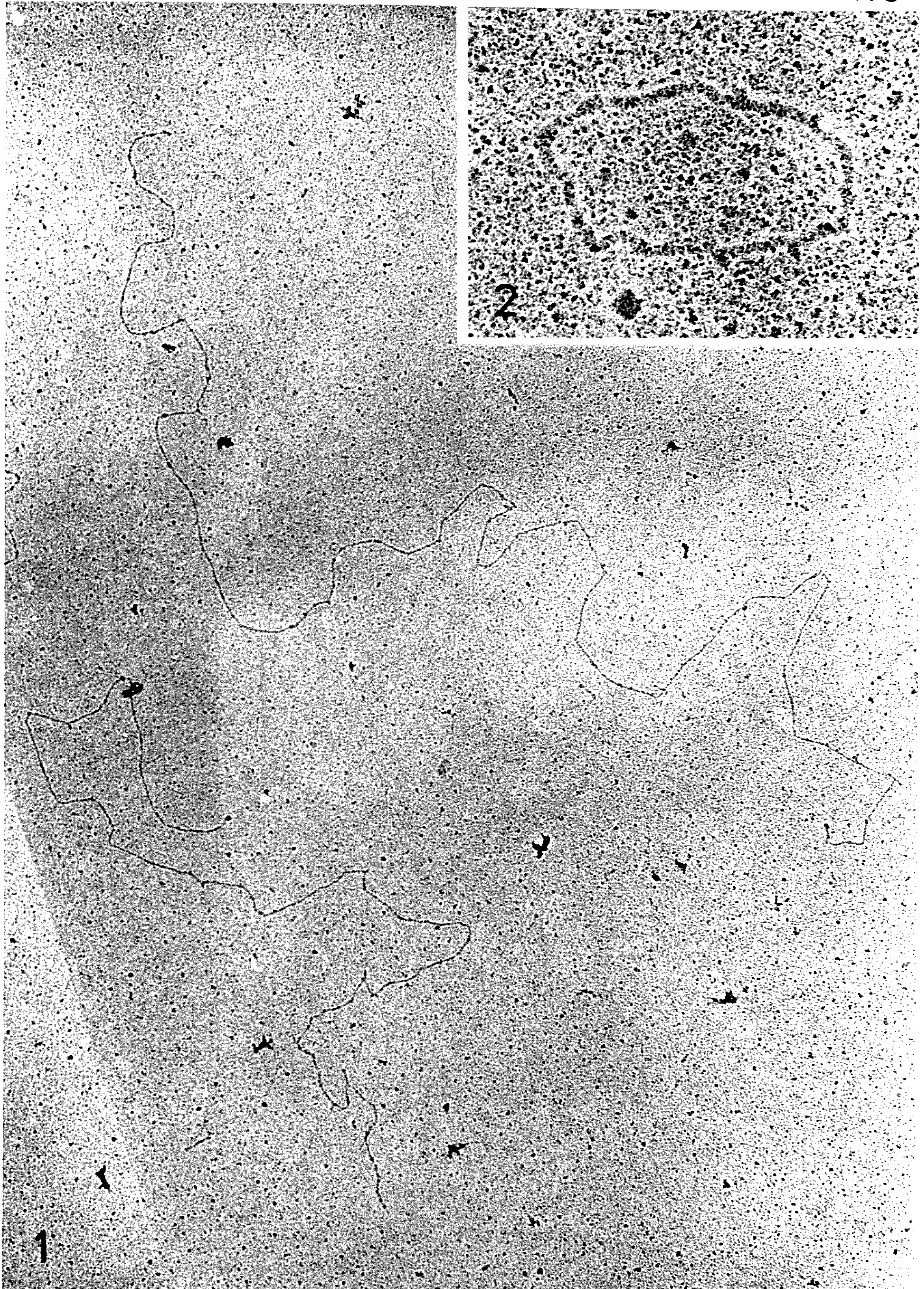
The molecular weight of a reasonably small DNA molecule that has been randomly fragmented during isolation can be determined by digesting these fragments with restriction endonucleases. These enzymes cleave double-stranded DNA where specific sequences of bases occur. Thus, providing that the isolated fragments are, on average, larger than the greatest distance between two of the enzyme's recognition sequences, the restriction endonuclease digest of the fragmented molecule will be the same as that of the intact one, except that a population of small randomly sized end fragments will be present in the former. The molecular weights of the restriction fragments can be determined from their relative migration through an agarose gel under the influence of an electric field (Southern, 1979), and by summing these, the molecular weight of the intact molecule can be predicted.

Plate 4.6. Electron microscopic visualization of isolated chloroplast DNA molecules.

1. Two fragments of chloroplast DNA molecules measuring approximately 96 kb and 64 kb. x 21,150.

2. The circular 4.36 kb plasmid pBR322 used as the size standard. x 145,800.

Plate 4.6



DNA extracted from isolated Glenodinium chloroplasts ran as a sharp band alongside λ DNA (molecular size 48.5 kb) during agarose gel electrophoresis (Plate 4.7, lanes a & b). The average size of the isolated fragments was therefore at least 50 kb which is in agreement with the electron microscopic observations. These fragments are large enough to perform a restriction endonuclease analysis, however, the yield of DNA from 6 l of culture was estimated to be only 0.25 μg from the intensity of the band fluorescence. Ideally at least a microgram is required to visualize low molecular weight restriction fragments.

Glenodinium chloroplast DNA which had been crudely purified by phenol deproteinization and ethanol precipitation was resistant to digestion with the restriction endonucleases Eco RI, Bam HI and Hind III. As λ DNA co-incubated with the chloroplast DNA also failed to show any site specific cleavage, this resistance to digestion was probably not due to any inherent features of the chloroplast DNA (such as base modification or absence of restriction sites) but rather due to the presence of an inhibitor (Lambert and Carr, 1984). Since this inhibitor protected the λ DNA it seemed unlikely that it was tightly bound to the chloroplast DNA, nevertheless it was not removed by hydroxylapatite chromatography. However it was discovered that the chloroplast DNA became susceptible to Pst I digestion after purification through NACS resin, but, as predicted, the yield of purified DNA from a single preparation was not sufficient to visualize the entire restriction fragment pattern (Plate 4.7, lane f).

Crude Glenodinium chloroplast DNA, that is DNA extracted from chloroplasts purified only by differential sedimentation, can be obtained in higher yields, however it is contaminated with nuclear and mitochondrial DNA. Pst I cleaves nuclear DNA much less frequently than expected but this is not because it is susceptible to the methylation of cytosine in its recognition

Plate 4.7. Restriction endonuclease analysis of the chloroplast DNA of Glenodinium foliaceum. Samples subjected to electrophoresis through 1% agarose gels.

lane a. 0.25 μg of λ DNA (molecular weight marker).

lane b. All the DNA obtained from a single chloroplast mini prep., undigested.

lane c. DNA extracted from chloroplasts that had been digested with DNase I prior to isolating the DNA.

lane d. Total cell DNA digested with Pst I.

lane e. DNA from crudely purified chloroplasts digested with Pst I.

lane f. All the DNA from a single chloroplast mini prep., subsequently purified with NACS resin, and digested with Pst I.

lane g. Satellite DNA digested with Pst I.

lane h. Satellite DNA digested with Eco RI.

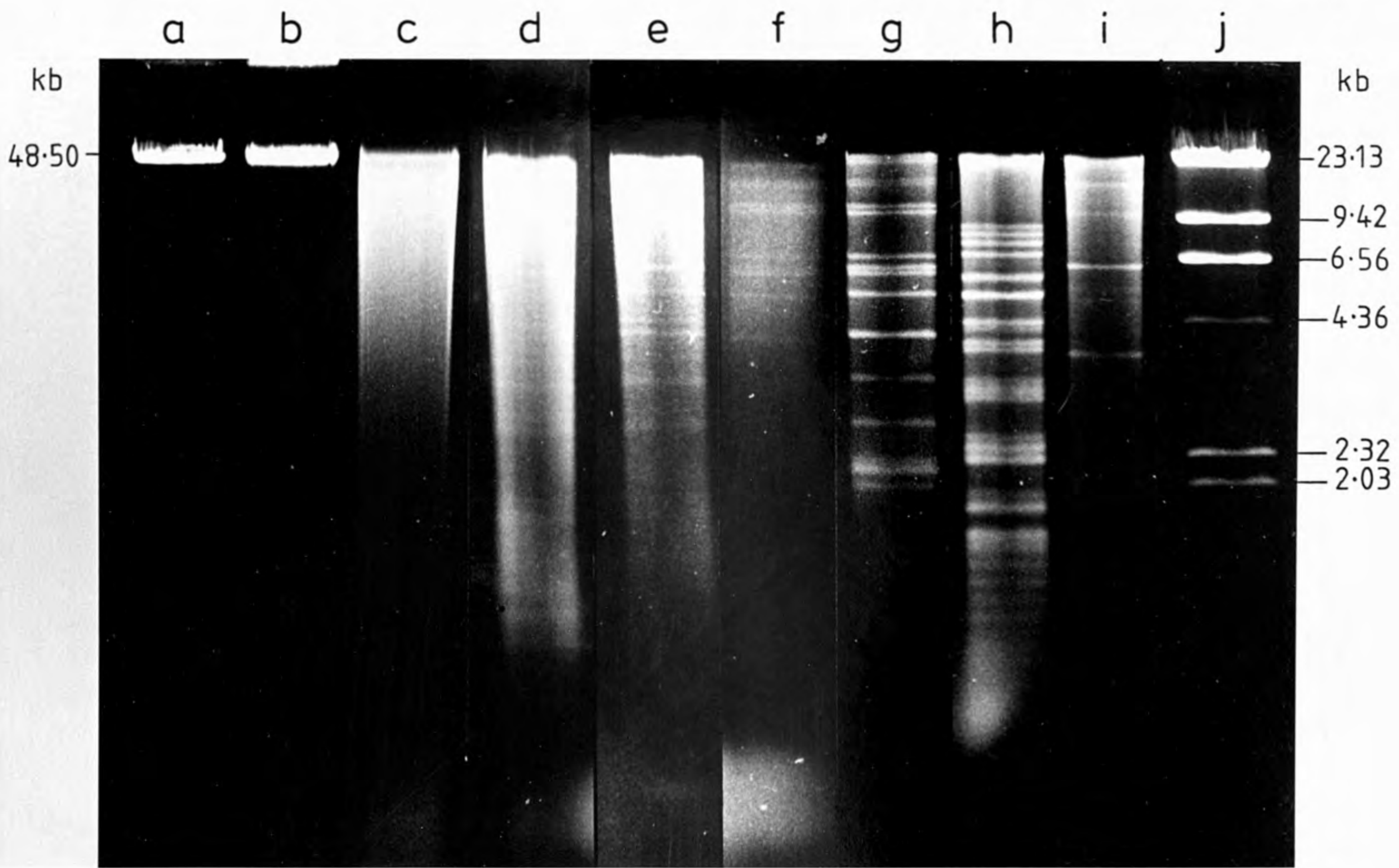
lane i. Satellite DNA digested with Bam HI.

lane j. Hind III digest of λ DNA (molecular weight marker).

Note: The samples were not all analysed together. Lanes forming part of the same gels were: a & b; d, e, f & j; and h & i. Lanes c and g were run separately. Comparisons of actual migration distances between these groups is therefore not strictly valid, however all the lanes are reproduced at approximately the same scale as judged from Hind III digested λ DNA included in all the gels.

See text for further details.

Plate 4.7



sequence (Flavell, 1982). Consequently Pst I digests of total cell DNA extracts from plants often reveals the Pst I restriction fragment pattern of the chloroplast DNA. Similarly, crude chloroplast DNA from G. foliaceum could be digested with Pst I to visualize some of the lower molecular weight chloroplast restriction fragments (Plate 4.7, lane e). Note that a Pst I fragment running parallel with the 4.36 kb Hind III fragment of λ DNA is present in the crude chloroplast DNA digest but not in the digest of pure chloroplast DNA. This is probably mitochondrial DNA and provides a useful marker for chloroplast DNA purity. Due to the large amount of nuclear DNA in G. foliaceum, digestion of total cell DNA with Pst I did not satisfactorily reveal the chloroplast DNA restriction fragments (Plate 4.7, lane d).

The DNA from G. foliaceum which formed a satellite in caesium chloride density gradients containing Hoechst dye (see Section 4.1.2) was also found to be resistant to restriction endonuclease digestion before NACS resin purification. A similar problem has been found with satellite DNAs from Cyanidium caldarium (Kite, unpublished observation) and other algae (Cattolico, personal communication). All these DNA extractions had been performed with ATA in the isolation buffers and it could be that carry over of this compound is responsible for the enzyme inhibition. ATA completely inhibits restriction endonucleases at concentrations as low as 100 μM (Hallick et al., 1977) and so the relative merit of using ATA when isolating DNA for restriction endonuclease analysis needs further consideration.

When digested with Pst I the satellite DNA of G. foliaceum produced a restriction endonuclease fragment pattern which was identical to that of the DNA extracted from isolated chloroplasts (Plate 4.7, lane g). Note the absence of the 4.36 kb Pst I fragment which shows that the satellite DNA consists only of chloroplast DNA. Yields of chloroplast DNA obtained by fractionating whole cell lysates were much higher than from isolated

Table 4.4. Sizes of restriction endonuclease fragments of _Glenodinium foliaceum_ chloroplast DNA. Satellite DNA was cleaved with _Eco_ RI, _Pst_ I and _Bam_ HI and fractionated on a 1% agarose gel. Fragment sizes (in kb) were estimated relative to those of λ DNA cut with _Hind_ III.

Fragment	Restriction endonuclease		
	Eco RI	_Pst_ I	_Bam_ HI
1	8.61	17.78	15.14
2	7.94	12.30	10.00
3	7.41	11.22	6.31
4	6.76	7.59	3.43
5	+ 5.62	+ 6.92	1.82
6	+ 5.00	+ 6.53	
7	4.07	5.50	
8	4.00	+ 4.17	
9	3.55	3.31	
10	3.47	2.63	
11	2.95	+ 2.19	
12	2.79	+ 2.09	
13	2.66	1.95	
14	2.24		
15	+ 2.14		
16	+ 2.00		
17	1.70		
18	+ 1.62		
19	+ 1.41		
20	1.33		
21	1.29		
22	1.15		
23	1.07		
24	0.98		
Total (kb)	99.55	106.08	-

+ Stochiometric value of 2 assigned (see Figure 4.7)

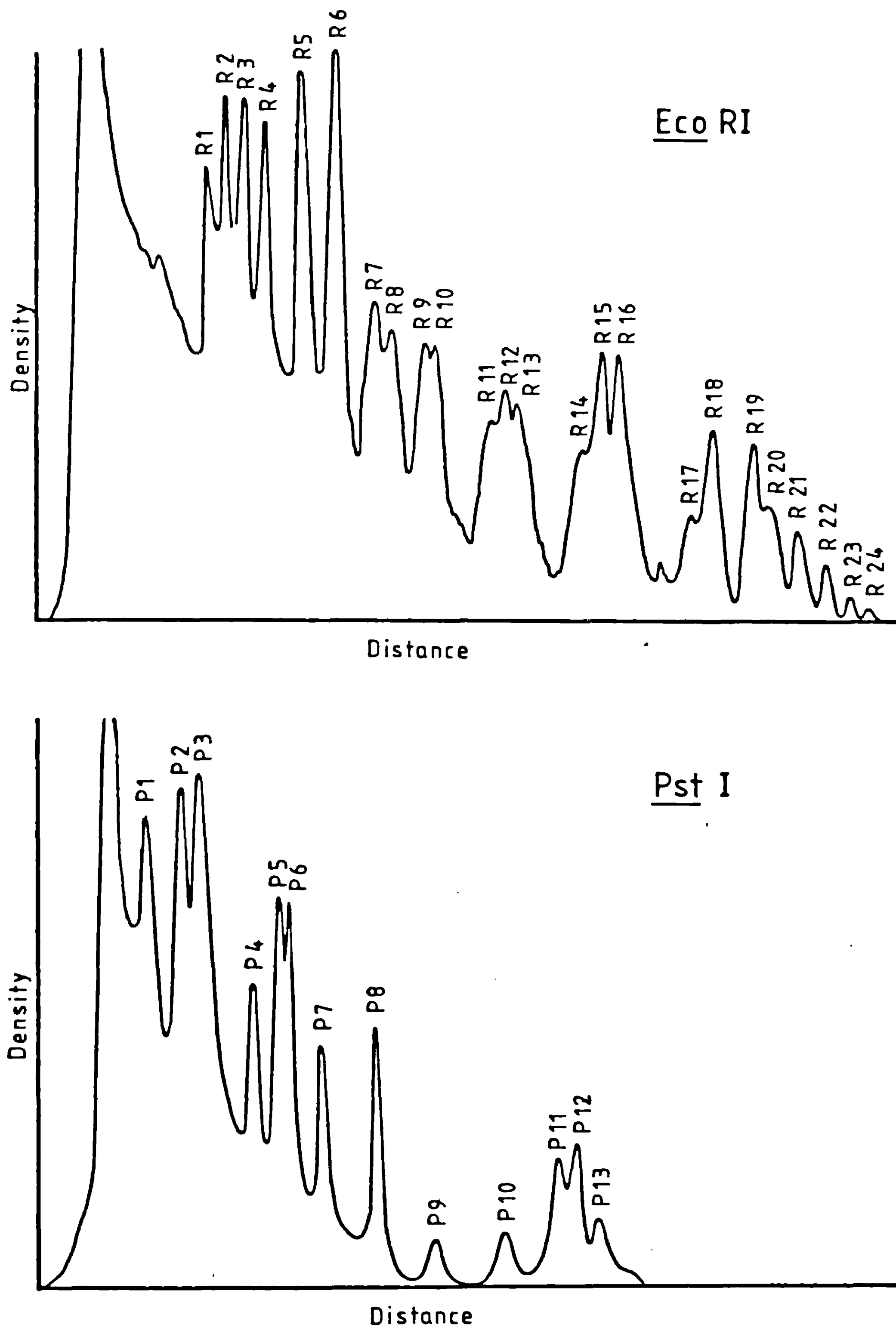


Figure 4.7. Relative stochiometries of Glenodinium foliaceum chloroplast DNA restriction fragments. Scanning densitometer traces of the Pst I and Eco RI restriction fragment patterns of chloroplast DNA shown in Plate 4.7 (lanes g and h). Fragment numbers correspond to those given in Table 4.4.

chloroplasts (up to 10 μg from 20 l of culture) and were probably nearer to the theoretical maximum. Therefore, for work on *Glenodinium* chloroplast DNA, this method offers a great advantage.

The size of the restriction fragments produced by the complete digestion of chloroplast (satellite) DNA with *Eco* RI, *Pst* I and *Bam* HI are listed in Table 4.4. Some of these fragments were obviously more frequent in the digests than others (Plate 4.7, lanes g - i) and so these were assigned a stochiometric value of two from analysis of densitometer scans of the gels (Figure 4.7). Accurate stochiometric assignment was made difficult by the decreasing frequency of high molecular weight fragments due to the increasing probability of the isolated DNA molecules not containing both restriction sites. This problem confounded the analysis of the chloroplast DNA with *Bam* HI as the molecule clearly only contains a few *Bam* HI restriction sites. Since some of the *Bam* HI fragments are likely to be larger than the majority of the DNA fragments being isolated, the *Bam* HI digest cannot be used to determine the total size of the chloroplast DNA.

Summing the lengths of the *Eco* RI and *Pst* I restriction fragments yields two estimates of the chloroplast genome size which are 100 kb and 106 kb respectively. These estimates agree reasonably well with the minimum genome size predicted from the electron microscopic examination of isolated chloroplast DNA fragments. The errors involved in determining accurate contour lengths due to variations in the degree of spreading, and instrumental magnification inaccuracies between the sample and standard preparations, probably account for the slight discrepancy.

All published estimates of the molecular weights of algal chloroplast DNAs are listed in Table 4.5. These have either been determined by restriction endonuclease analysis, contour length measurements or kinetic analysis. In compiling this table it has

Table 4.5. Genome sizes (in kb) of algal chloroplast DNAs.

Organism	Genome size	Reference
Glaucophyceae:		
Cyanophora paradoxa		
Pringsheim strain	127	Löffelhardt *et al.* (1983)
Kies strain	138	Löffelhardt *et al.* (1983)
Chrysophyceae:		
Olisthodiscus luteus	148	Aldrich *et al.*(1982)
Xanthophyceae:		
Vaucheria (8 species)	108-116	Linne von Berg *et al.* (1982)
Botrydium granulatum	120	Linne von Berg *et al.* (1982)
Tribonema viride	129	Linne von Berg *et al.* (1982)
Phaeophyceae:		
Pilayella littoralis	56	Dalmon *et al.* (1983)
Sphacelaria sp.	112	Dalmon *et al.* (1983)
Dictyota dichotoma	123	Kuhsel *and* Kowallik (1985)
Bacillariophyceae:		
Odontella sinensis	110	Linne von Berg *et al.* (1982)
Euglenophyceae:		
Euglena gracilis	130	Gray & Hallick (1978)
Chlorophyceae:		
Codium fragile	84	Hedberg *et al.* (1981)
Derbesia marina	97	Linne von Berg *et al.* (1982)
Eremosphaera viridis	144	Linne von Berg *et al.* (1982)
Chlorella ellipsoidea	174	Yamada (1982)
Chlamydomonas reinhardii	189	Rochaix (1978)
C. eugametos	243	Lemieux *et al.* (1985)
Acetabularia cliftonii	22,800	Padmanabhan & Green (1978)
Charophyceae:		
Chara hispida	168	Linne von Berg *et al.* (1982)

been assumed that 1 Md is equivalent to 1.5 kb and that 1 μm of spread DNA has a molecular weight of 3.1 kb. All higher plants have chloroplast genomes which fall within the range of 121 - 182 kb with most dicotyledonous angiosperms having a chloroplast DNA molecule of about 150 - 155 kb (Groot, 1984; Curtis and Clegg, 1984). There appears to be little relationship between the size of the chloroplast genome and the phylogenetic position of the alga, although a chloroplast DNA phylogeny based on size has been constructed on the hypothesis that the major evolutionary trend is through genome reduction (Wallace, 1982; 1983). The variation in genome size probably reflects the great evolutionary diversification of the algae. A more detailed analysis of the arrangement of sequences in the chloroplast DNA molecule is required to follow any evolutionary trends but only the chloroplast genomes of Chlamydomonas reinhardii and Euglena gracilis have been studied in detail (Stutz et al, 1984). Such a sequence analysis of higher plant chloroplast DNA has enabled the evolution of the molecule to be traced and accounted for in terms of a series of sequence inversions and deletions (Palmer and Thompson, 1982). Bearing this in mind, the similarity in size between the chloroplast genomes of Glenodinium foliaceum and the diatom Odontella sinensis is interesting, but not particularly significant.

With the exception of Euglena gracilis, all chloroplast DNA molecules that have been examined contain two inverted repeat regions (Palmer, 1983). Together these constitute 17% of the chloroplast genome of Cyanophora paradoxa (Löffelhardt et al., 1983), 20% in Chlamydomonas reinhardii (Rochaix, 1978), 26% in Chlorella ellipsoidea (Yamada, 1983) and about 32% of the chloroplast DNA molecule in higher plants (Kolodner and Tewari, 1979). Depending on the strain, the Euglena chloroplast DNA molecule has two, three or five tandemly repeated sequences (Gray and Hallick, 1978; Flamant et al., 1984; Ravel-Chapuis et al., 1984).

Glenodinium foliaceum chloroplast DNA also appears to show repeated sequences. The repeated Eco RI restriction fragments have a total length of 17.8 kb and those in the Pst I digest have a total length of 21.9 kb. The repeated sequences may therefore account for between 37% and 42% of the chloroplast DNA molecule, accepting that some of the restriction fragments that have a stochiometry of two may be two unique sequences which are fortuitously the same size. Nothing can be stated about the orientation of these repeated regions.

4.3. GENETIC ANALYSIS OF GLENODINIUM FOLIACEUM DNA:

The enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) is the most abundant protein in nature and is involved in the primary reactions of both photosynthetic carbon dioxide fixation and photorespiration within the chloroplast (Lorimer, 1981). RuBisCO is commonly composed of eight catalytic large subunits and eight small subunits of unknown function (Miziorko and Lorimer, 1983).

The large subunit appears to be coded, transcribed and translated in the chloroplast (Whitfeld and Bottomley, 1983) and the exact location of the gene (rbc L) on the chloroplast DNA molecule has been determined for many higher plants (see Gallagher et al, 1984), Chlamydomonas reinhardii (Dron et al., 1982), Euglena gracilis (Stiegler et al., 1982) and also Cyanophora paradoxa (Heinhorst and Shively, 1983). In plants the gene for the small subunit (rbc S) is encoded in the nuclear DNA (Kawashima and Wildman, 1972; Tobin and Silverthorne, 1985) where it is present as a small multigene family (Cashmore, 1979; Berry-Lowe et al, 1982; Broglie et al, 1983). Since the rbc S gene of

Chlamydomonas reinhardii had also been localized in the nucleus (Dobberstein *et al*, 1977), it did seem that the segregation of the large and small subunit genes into the chloroplast and nuclear genomes was going to be a universal feature of photosynthetic eukaryotes. However the chloroplast (cyanelle) genome of *Cyanophora paradoxa* has since been shown to encode the small subunit of RuBisCO as well as the large subunit (Heinhorst and Shively, 1983). Although *C. paradoxa* might be expected to be unusual, considering the cyanobacterial features of its chloroplasts (Trench, 1982), there is indirect evidence that *rbc* S is also chloroplast located in two red algae, *Cyanidium caldarium* and *Porphyridium aerugineum* (Steinmüller *et al*., 1893; Zetsche *et al*., 1983) and the chromophyte, *Olisthodiscus luteus* (Reith and Cattolico, 1985). Thus it was of interest to localize the *rbc* S gene in *Glenodinium foliaceum*.

4.3.1. *Attempted localization of the rbc S gene in* Glenodinium foliaceum.

Specific genes in single-stranded target DNA immobilized on nitrocellulose, can be localized by incubating it with single-stranded probe DNA which is known to encode the gene under investigation. The probe DNA hybridizes to complementary sequences in the target DNA and this can be detected providing that the probe has been labelled in some way. Localization studies can be performed crudely by spotting the target DNA directly onto a nitrocellulose filter. More usually, though, a restriction endonuclease digest of the target DNA is first fractionated by agarose gel electrophoresis and then the restriction fragments are transferred onto nitrocellulose by the method developed by Southern (1976). The probe should then only hybridize to a few of the restriction fragments whose positions on the genome may, or may not, be known.

Probe DNAs are invariably sections of genes that have been cloned into bacterial plasmid vectors so as to amplify the DNA. The only probes for the *rbc* S gene that were available were the plasmids pSSU60 and pSSU160. These contain fragments of the nuclear *rbc* S gene from *Pisum sativum* inserted into the unique *Hind* III restriction site of the pBR322 vector (Bedbrook *et al.*, 1980). pSSU160 contains a 330 bp fragment encoding about 290 bp into the 5' terminus of the gene, whilst pSSU60 contains a 380 bp fragment encoding about 80 bp of the 3' terminus and the transcribed flanking sequence. Together both fragments cover the entire 368 bp coding sequence of the *rbc* S gene.

These plasmids were purified from the host bacterium by coupling the small scale plasmid isolation procedure of Birnboim and Doly (1979) with NACS resin chromatography to remove contaminating chromosomal DNA (Plate 4.8.1). However, the probes failed to hybridize strongly to either Southern transfers or dot blots of *Glenodinium foliaceum* DNA. After incubating a Southern transfer of 10 μg each of *Eco* RI restricted dinoflagellate and endosymbiont nuclear DNA with 10 $ng \cdot ml^{-1}$ of labelled probe DNA (equal quantities of pSSU60 and pSSU160) for 25 h, no specific hybridization could be detected. This time corresponds to at least a hybridization to 1 x $C_ot_{1/2}$ under the incubation conditions used (see Section 2.11.5) without accounting for the accelerating effect of the dextran sulphate in the hybridization buffer (Wahl *et al.*, 1979). Therefore the amount of probe available for hybridization to the filter at the end of the experiment was negligible (see Maniatis *et al.*, 1982). Failure to observe any hybridization was not due to inadequacies in the system used to detect the biotin label of the probe. Using the BRL DNA Detection System, 5 pg of biotinylated λ DNA could readily be visualized, as could the labelled probe DNA showing that biotin-11-dUTP had been efficiently incorporated into the plasmid by nick translation (Plate 4.8.2a & 2b).

Plate 4.8. Genetic analysis of *Glenodinium foliaceum* DNA.

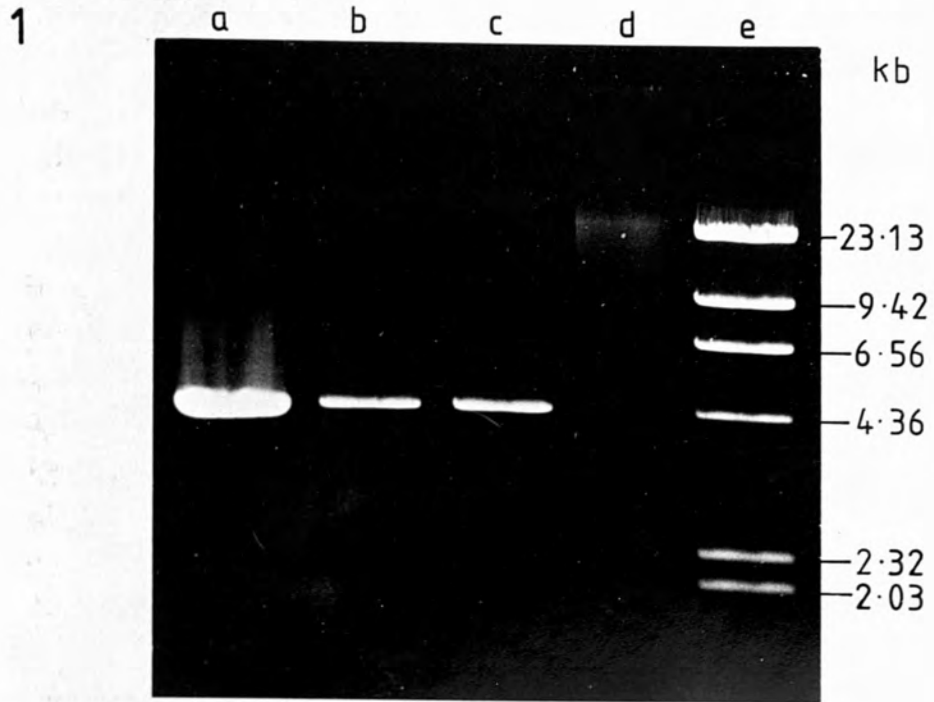
1. NACS resin purification of chimaeric plasmid DNA isolated from *Escherichia coli*. Electrophoresis of column eluates through a 1% agarose gel showing the separation of plasmid DNA (eluted in 0.7 M NaCl) from contaminating high molecular weight chromosomal DNA (eluted in 2 M NaCl). Plasmids were linearized by digestion with *Hind* III.
   lane a. 0.25 μg of plasmid pBR322.
   lane b. 0.7 M eluate of a pSSU60 plasmid mini prep.
   lane c. 0.7 M eluate of a pSSU160 plasmid mini prep.
   lane d. 2 M eluate of the pSSU160 plasmid mini prep.
   lane e. *Hind* III digest of λ DNA (size marker).

2. Hybridization of *G. foliaceum* DNA with biotin-labelled probe DNA (pSSU60 and pSSU160) encoding the small subunit of RuBisCO from pea.
   a. Example of an assay of labelled probe concentration after nick translation. 5 μl aliquots of a ten times dilution series were spotted onto nitrocellulose paper and the biotinylated DNA was visualized by the BRL DNA Detection System.
   b. Known amounts of commercially prepared biotinylated λ DNA spotted onto nitrocellulose and processed with the sample being analysed to act as a colour intensity standard. In the example in (a) the labelled probe concentration was estimated to be between 10 and 20 μg ml^{-1}.
   c. Visualization of biotin-labelled probe DNA which hybridized to a nitrocellulose strip loaded with the stated quantities of total cell DNA from *G. foliaceum*, DNA from the symbiont nucleus, DNA extracted from a crude preparation of chloroplasts (chloroplast enriched DNA), and dinoflagellate nuclear DNA.

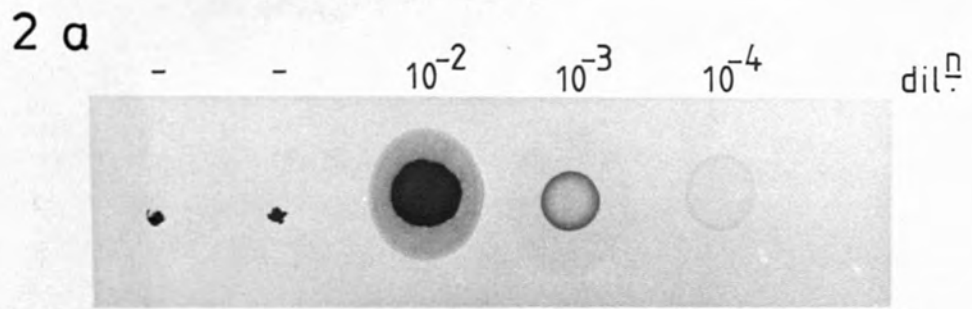
   See text for further details.

Plate 4.8

1



2 a



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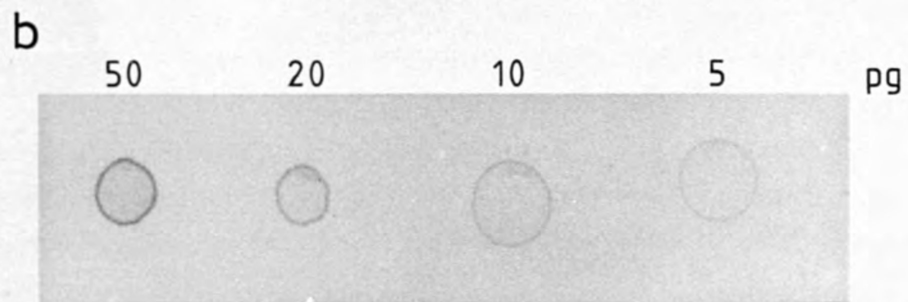
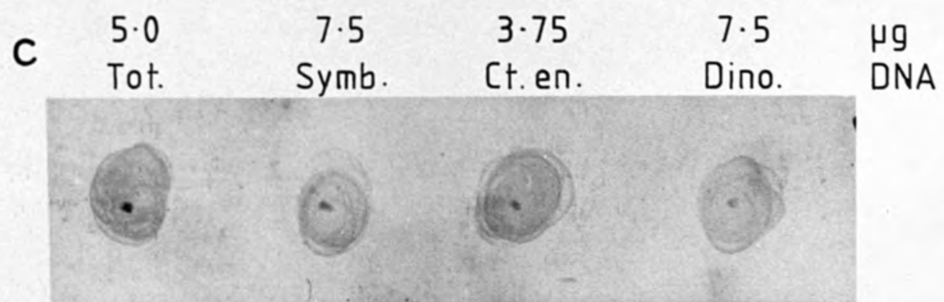


c



At the time these experiments were performed the identity of G. foliaceum satellite DNA had not been established. Consequently Southern blots of restricted chloroplast DNA were not probed with the pea rbc S gene as not enough DNA could be obtained from isolated chloroplasts to drive the hybridization reaction. However a dot blot of enriched chloroplast DNA (i.e. DNA extracted from a crude chloroplast preparation) was examined. This hybridization was performed for 4.5 h using 100 ng ml^{-1} of the labelled probe plasmid mixture which corresponded to at least a 4 x $C_o t_{1/2}$. The nuclear DNAs were also re-examined at the same time. Using this higher probe concentration, some hybridization was detected but the probe appeared to cross react at a low level with all the DNAs (Plate 4.8.2c). There did appear to be slightly more hybridization to the chloroplast enriched DNA compared to the pure nuclear DNAs even though only half as much DNA was initially loaded into the nitrocellulose. This difference is not significant enough to suggest that the rbc S gene is definitely chloroplast encoded in G. foliaceum.

The pSSU160 probe has been successfuly used to localize the rbc S gene in Cyanophora paradoxa (Heinhorst and Shively, 1983). However in order to detect any cross reaction, the nitrocellulose filter was only washed to a low stringency after hybridization of the probe. This was to compensate for the evolutionary distance between Cyanophora and Pisum. In the present study high stringency washes were employed which would have removed any probe DNA that had hybridized only weakly as a result of frequent base pair mismatches. Nevertheless it should be noted that apparently non-specific probe hybridization was still evident even after these high stringency washes, so decreasing the hybridization stringency would possibly only have resulted in a higher background signal.

High stringency washes were used here because biotin and not ^{32}P was used to label the probe DNA. Biotin-labelling is a

relatively new and unused technique which was developed for laboratories that were not equipped to work with radioactively labelled DNA (Leary *et al*., 1983). Consequently a complete set of stringency washes has not been formulated, other than those originally proposed by Leary *et al*. (1983) which were specified to give a fairly high stringency. Unfortunately the method of detecting biotin-labelled DNA suffers from a major disadvantage in that the colourimetric visualization of the biotin can only be performed once. In contrast, using a radioactive label the degree of probe hybridization can be assayed after each wash of increasing stringency by simply exposing the filter to an X ray plate.

Despite the problems created by using biotin-labelled DNA, the probable divergence in the nucleotide sequences between the pea *rbc* S gene and that of chromophyte algae may render it inadequate for gene localization studies in this group. Using more standard techniques, this probe also showed no detectible hybridization to the nuclear or chloroplast DNAs of *Olisthodiscus luteus* (Cattolico, personal communication). An *Olisthodiscus rbc* S probe, which is currently being developed, should be of more use in the future.

CHAPTER 5

GENERAL OBSERVATIONS ON ANOMALOUSLY PIGMENTED DINOFLAGELLATES.

5.1. INTRODUCTION.

The major carotenoid in most photosynthetic dinoflagellates is thought to be peridinin (Strain *et al*, 1971). However, a few species contain an atypical complement of carotenoids from which peridinin is absent (Jeffrey *et al*, 1975). These dinoflagellates possess major carotenoids, such as fucoxanthin, which are more typical of other groups of algae (Table 5.1).

The chloroplast carotenoids of *Glenodinium foliaceum* clearly belong to the chromophyte-like endosymbiont and the anomalous pigmentation of *Peridinium balticum* and *Gymnodinium eucyaneum* can also be ascribed to the presence of endosymbionts (Loeblich, 1984). The ultrastructure of *P. balticum* has been described in some detail and the fine structural features of the endosymbiont are almost identical to those of the symbiont in *G. foliaceum* endosymbiont (Tomas *et al*, 1973; Tomas and Cox, 1973). *Gymnodinium eucyaneum* also contains a supernumerary nucleus (Li *et al*, 1979) but this species has cryptophyte-like chloroplasts which are possibly associated with nucleomorphs (Hu and West, unpublished observations). This suggests that it contains an endosymbiotic cryptophyte rather than a chromophyte. An

Table 5.1 Anomalously pigmented dinoflagellates. Species in which pigment analysis has shown that the major chloroplast carotenoid is not peridinin.

Species	major carotenoid	Reference
Glenodinium foliaceum	fucoxanthin	Mandelli (1968) Withers & Haxo (1975)
Gymnodinium breve	unidentified	Haxo (1975) (cited by Jeffrey *et al*. (1975)
Gymnodinium eucyaneum	phycocyanin	Hu *et al*. (1980) Zhang *et al*. (1982)
Gymnodinium galatheanum	unidentified (carotenoid 3)	Bjørnland & Tangen (1979)
Gymnodinium micrum	fucoxanthin	Whittle & Cassleton (1968)
Gymnodinium veneficum	fucoxanthin	Riley & Wilson (1967)
Gyrodinium aureolum	19'-hexanoyloxy-fucoxanthin	Tangen & Bjørnland (1981)
Peridinium balticum	fucoxanthin	Withers *et al*. (1977)

ultrastructural study has conclusively demonstrated the presence of an endosymbiotic cryptophyte in a similar blue-green dinoflagellate, Gymnodinium acidotum (Wilcox and Wedemayer, 1984), but there is no pigment analysis data for this species.

However, not all the species listed in Table 5.1 contain recognizable endosymbionts. No supernumerary nuclei, or other obviously foreign organelles, were observed in fine structural studies of Gymnodinium micrum (Leadbeater and Dodge, 1966), G. veneficum (Dodge, 1975) or G. breve (Steidinger et al., 1978) and acetocarmine staining showed that a supernumerary nucleus was also lacking in Gyrodinium aureolum (Tangen and Björnland, 1981). Recently another blue-green dinoflagellate, Amphidinium wigrense, has been the subject of an ultrastructural examination (Wilcox and Wedemayer, 1985). Once again no evidence could be found of a nucleus which might belong to an endosymbiont, but the structural data strongly suggested that the chloroplasts were cryptophycean in origin, although this morphological interpretation was not supported by a pigment analysis.

Electron microscopic observations on Gyrodinium aureolum and Gymnodinium galatheanum have not been reported and so these species were studied here in order to complete the ultrastructural data on dinoflagellate species currently known to possess anomalous pigmentation. The organization of the chloroplast DNA in these two species, Glenodinium foliaceum and Gymnodinium micrum was also examined by DAPI staining to provide an additional morphological character which might be of phylogenetic significance.

5.2. ULTRASTRUCTURE OF GYRODINIUM AUREOLUM AND GYMNODINIUM GALATHEANUM.

5.2.1. Gyrodinium aureolum.

The general morphology of Tangen's isolate of *Gyrodinium aureolum* has been described previously (Tangen and Bjørnland, 1981). The cell is relatively broad and flattened (24 -40 μm long and 17 - 32 μm wide) and fairly translucent so that the large dinoflagellate nucleus can clearly be seen positioned towards one side (see Plate 5.8.1). The few elongated chloroplasts are also obvious under the light microscope. The species can be responsible for the mortality of marine farmed fishes when it occurs in red tides (Jones *et al*., 1982; Roberts *et al*., 1983).

A brief discussion of the problems encountered with preparing this organism for electron microscopy was given in Section 2.4.2(ii). The ultrastructural preservation that was finally achieved was still far from satisfactory. The nucleus, for example, frequently showed symptoms associated with osmotic shrinkage, but fixations performed in lower osmolarity buffers completely failed to preserve cytoplasmic detail. Consequently this makes a detailed description of this species not only difficult but also subject to misinterpretation due to fixation artifacts

The most obvious fine structural feature of the cell is the reticulum of vesicles which extends throughout the cytoplasm (Plate 5.1). The diameter of these vesicles is probably not truely representative of their real size in a live cell. Within the dissected channels of cytoplasm are to be found mitochondria with tubular cristae, numerous dictyosomes and other characteristic dinoflagellate organelles such as a pusule and trichocysts

Plate 5.1. General ultrastructure of *Gyrodinium aureolum*. Longitudinal section through a whole cell showing the vesicular reticulum (V) and the large dinoflagellate nucleus (N). C, chloroplast; D, dictyosome. Note that the transverse flagellum (TF) has been retained during fixation. Transmission electron micrograph. x 6,900.

Plate 5.1

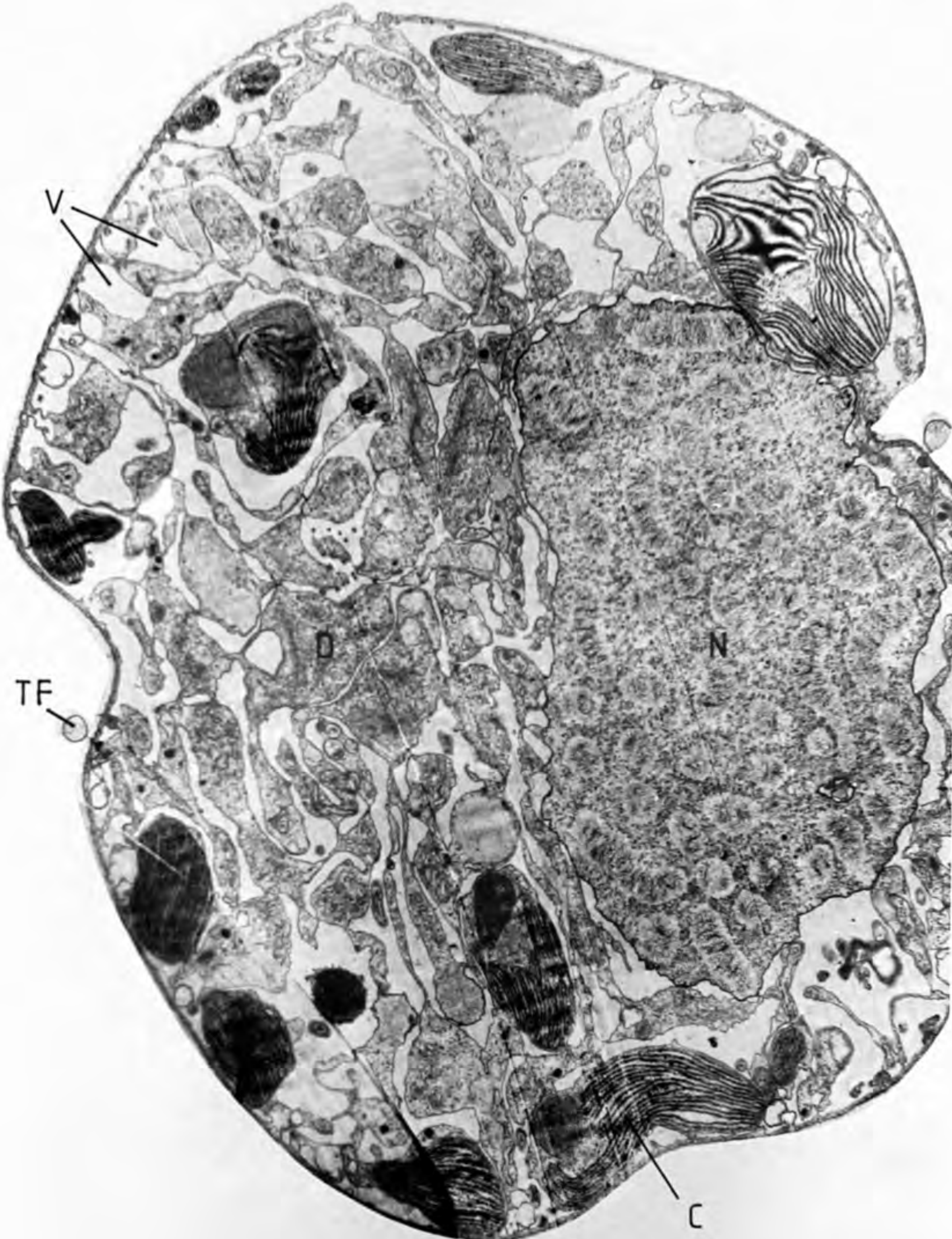


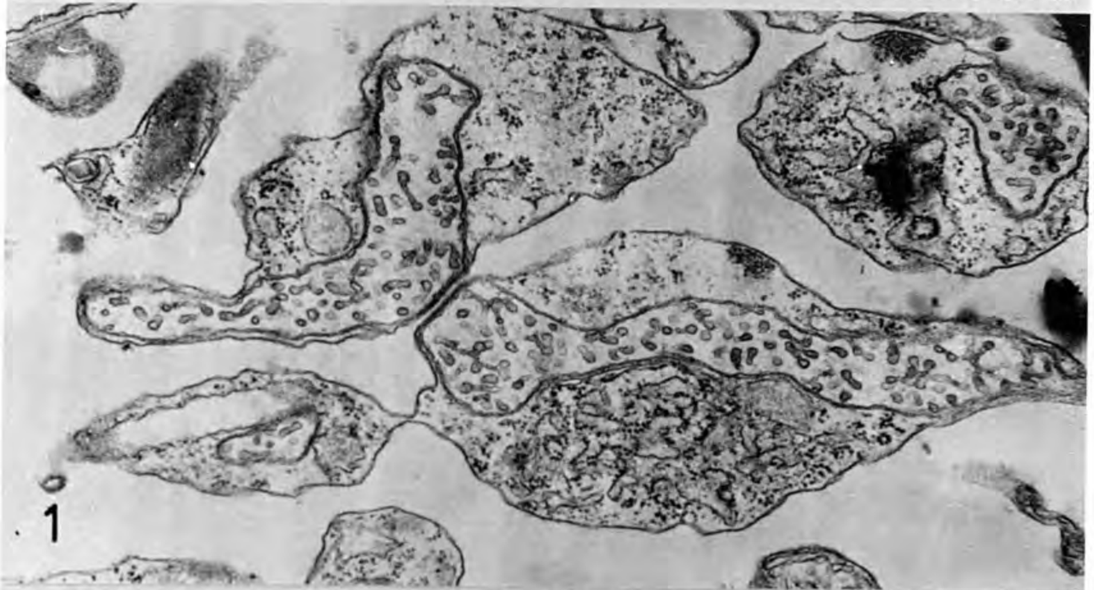
Plate 5.2. Ultrastructural features of the vesiculated cytoplasm of *Gyrodinium aureolum*. Transmission electron micrographs.

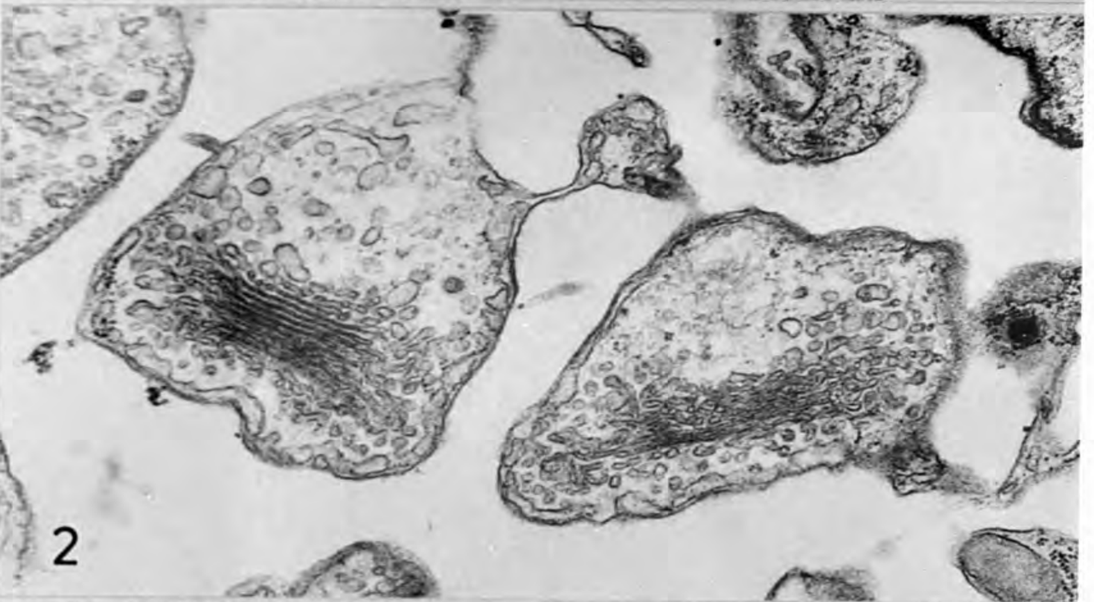
1. Mitochondria and rough endoplasmic reticulum. x 27,000.

2. Dictyosomes located near to the nucleus. x 27,700.

3. Pusule. x 23,100.

Plate 5.2





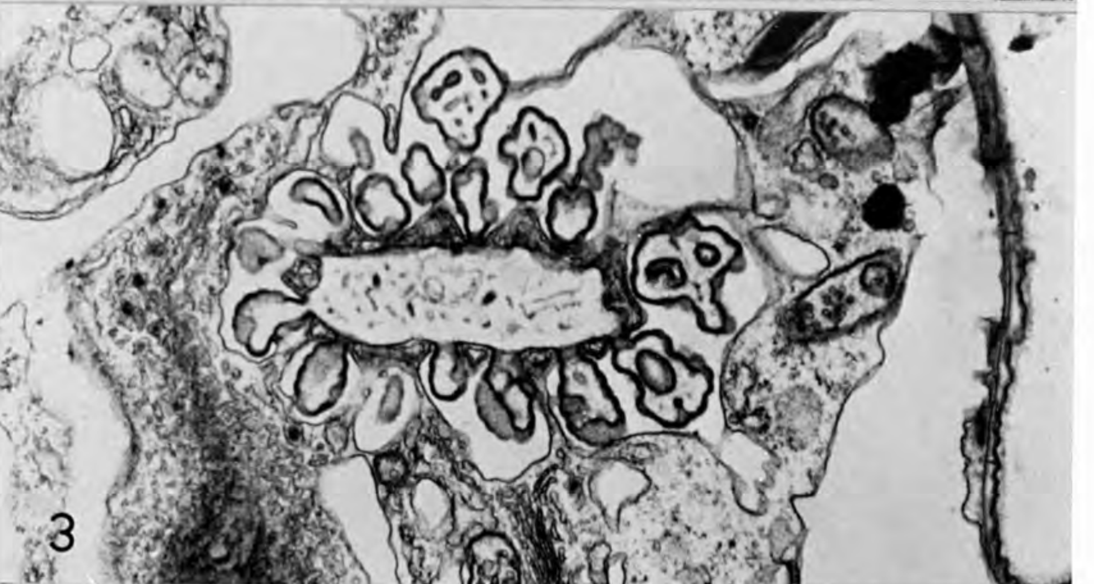


Plate 5.3. Ultrastructure of the chloroplast and pyrenoid of *Gyrodinium aureolum*. Transmission electron micrographs.

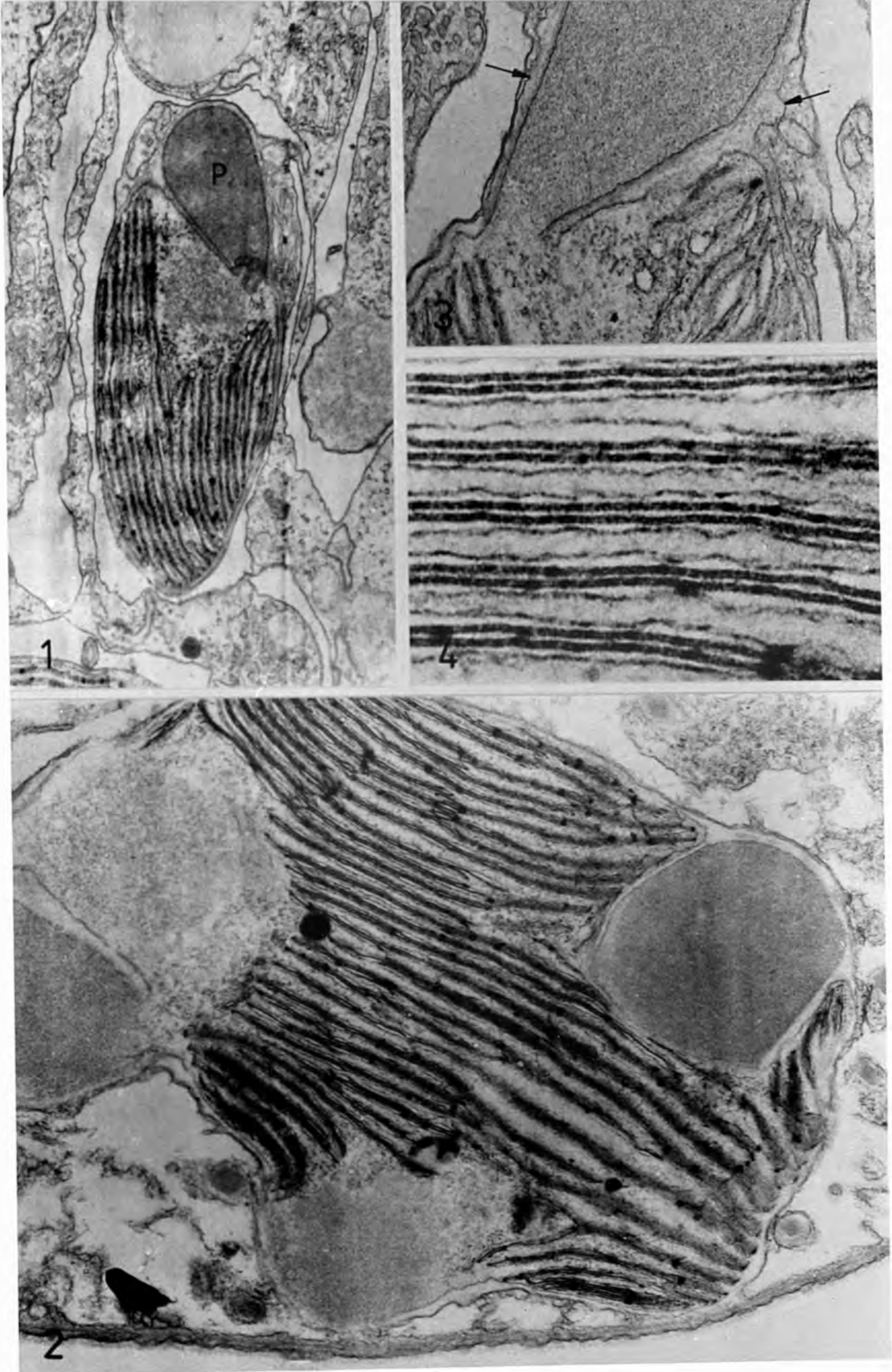
1. Transverse section through a chloroplast showing the stalked pyrenoid (P) and the thylakoid-free area in the subadjacent chloroplast stroma. x 18,000.

2. Chloroplast with three pyrenoids. x 28,000.

3. Detail of the pyrenoid where it connects with the main body of the chloroplast. Note the membrane which surrounds the pyrenoid (arrowed) which remains distinct from the chloroplast envelope and which merges with the membrane delimiting the surrounding vesicles. x 40,000.

4. Detail of the chloroplast lamellae composed of three thylakoids. x 147,200.

Plate 5.3



(Plate 5.2). No structure exists which might possibly be a supernumerary nucleus, the only nucleus in the cell being the large dinoflagellate one containing typically condensed chromosomes (Plate 5.1).

The chloroplasts of G. aureolum possess one or more prominent stalked pyrenoids whose position is associated with a thylakoid free area of the chloroplast stroma (Plate 5.3.1 & 2). The external nature of the pyrenoid conflicts with Tangen and Bjørnland's tentative interpretation of their light microscopic observations, in which they suggest that the pyrenoid of G. aureolum is internal (Tangen and Bjørnland, 1981). No starch sheath or cap surrounds the pyrenoid as in other dinoflagellates that possess stalked pyrenoids (Dodge and Crawford, 1971), however, a single membrane does lie exterior to it (Plate 5.3.3). This membrane may be one of the chloroplast envelope membranes but it does not closely follow the contour of the chloroplast in the region of the pyrenoid stalk and invariably appears to "fuse" with the membrane which delimits the surrounding vesicular reticulum. The chloroplast envelope proper always remains separate from the vesicle membrane and its structure has not been resolved. Within the chloroplast the lamellae are arranged in parallel ranks and are composed of three appressed thylakoids as is typical of most dinoflagellates. No girdle lamellae is present.

The ultrastructural features of Gyrodinium aureolum show certain similarities with those of Gymnodinium breve, another species with anomalous pigmentation. Like G. aureolum it also has a highly vesiculated cytoplasm, which incidentally creates similar fixation difficulties (Steidinger et al., 1978). More significantly its chloroplasts possess stalked pyrenoids which are not associated with a starch cap. The micrographs of Steidinger et al. (1981) also suggest that, as in G. aureolum, a thylakoid-free region of the chloroplast stroma lies beneath the

pyrenoid and that the arrangement of membranes around the pyrenoid is similar.

Thus, there is no direct morphological evidence that the chloroplasts of *Gyrodinium aureolum* belong to an endosymbiont. The chloroplasts do not possess any major and uniquely different ultrastructural features that distinguishes them from other dinoflagellate chloroplasts and which might suggest that they had a secondary endosymbiotic origin. Many algal groups, including dinoflagellates, have stalked pyrenoids and lack a girdle lamella (Dodge, 1973). Of these, only haptophytes and eustigmatophytes characteristically contain the carotenoid fucoxanthin or a fucoxanthin derivative. If, therefore, one makes the *a priori* assumption that fucoxanthin was acquired in dinoflagellates via endosymbiosis, then the chloroplasts of *G. aureolum* would appear to be related to the chloroplasts of one of these two groups. Interestingly, the fucoxanthin derivative, 19'-hexanoyloxyfucoxanthin, which is the major carotenoid in *G. aureolum* (Tangen and Björnland, 1981) has only been reported in one other alga, *Emiliania huxleyi* (Arpin *et al*., 1976), which is a haptophyte.

5.2.2. Gymnodinium galatheanum.

The morphology and pigmentation of Tangen's isolate KT76D of *Gymnodinium galatheanum* has been described under the name of *Gyrodinium* sp.-A (Björnland and Tangen, 1979). The culture used here, KT76E, was isolated two weeks after KT76D but from the same locality and is assumed to be the same species (Tangen, personal communication).

The cells are about 11 - 14 µm long and slightly less wide. Their general ultrastructural organization is shown in Plate 5.4.1. Once again one of the most prominent features is the large dinoflagellate nucleus and, as with *Gyrodinium aureo-*

lum, there is no evidence of a supernumerary nucleus. As the general ultrastructure of the cytoplasm is typical of most gymnodinoid dinoflagellates, it will not be described here. However, one feature worth mentioning is the array of plug-like structures beneath the thecal membranes (Plate 5.4.2) since these have also been described in Gymnodinium micrum (Leadbeater and Dodge, 1966).

The chloroplasts of Gymnodinium galatheanum contain a simple, internal pyrenoid and lamellae which are more or less arranged parallel to one another (Plate 5.5.1). There is no girdle lamella and although the outermost lamella may frequently follow the chloroplast envelope, it is interrupted at various points where the internal lamellae converge on the chloroplast envelope and apparently attach to it. The lamellae themselves are composed of three thylakoids and are occasionally interconnected by thylakoid bridges (Plate 5.5.2). The chloroplast envelope consists of three membranes which are usually closely appressed to one another after fixation and so are difficult to discern (Plate 5.5.3).

The arrangement of chloroplast lamellae in G. galatheanum is similar to that seen in Gymnodinium micrum, G. veneficum and G. vitiligo, all of which also contain simple, internal pyrenoids that are usually lenticular in shape (Dodge, 1975). The only other dinoflagellates in which internal, lenticular pyrenoids have been found are Glenodinium foliaceum and Peridinium balticum, where, of course, they belong to the endosymbiont. Gymnodinium micrum and G. veneficum are reported to possess fucoxanthin rather than an unidentified major carotenoid as in G. galatheanum (see Table 5.1). However, Bjørnland and Tangen (1979) have pointed out that the method of pigment identification applied in these species is not adequate to distinguish fucoxanthin from the major carotenoid in G. galatheanum. The pigments of G. vitiligo have not been examined.

Plate 5.4. General ultrastructure of Gymnodinium galatheanum. Transmission electron micrographs.

1. Longitudinal section through a whole cell. C, chloroplast; N, nucleus; M, mitochondrion. x 10,500.

2. Detail of the plug-like structures (arrowed) beneath the thecal membranes. x 33,000.

Plate 5.4

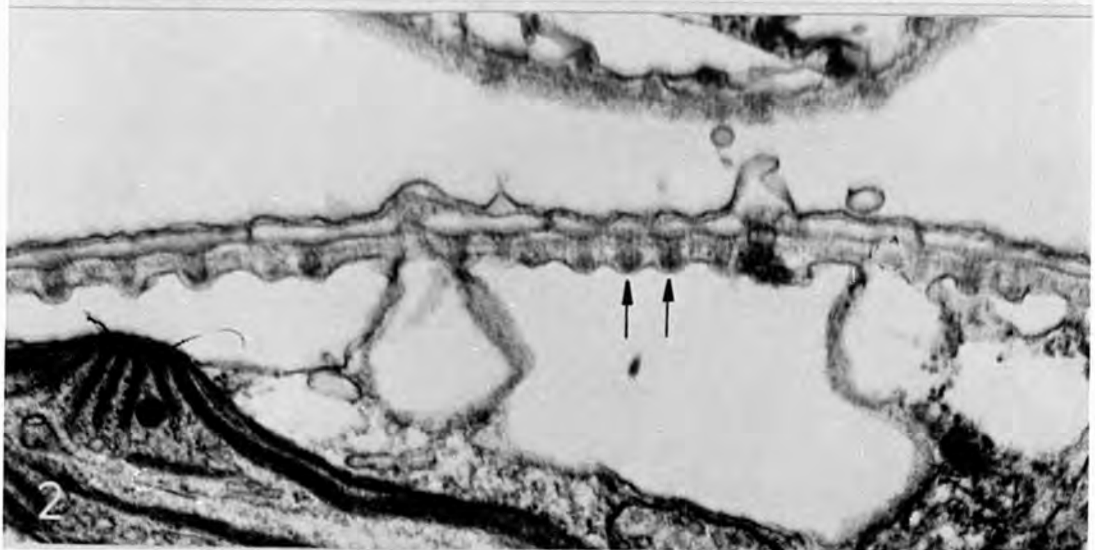


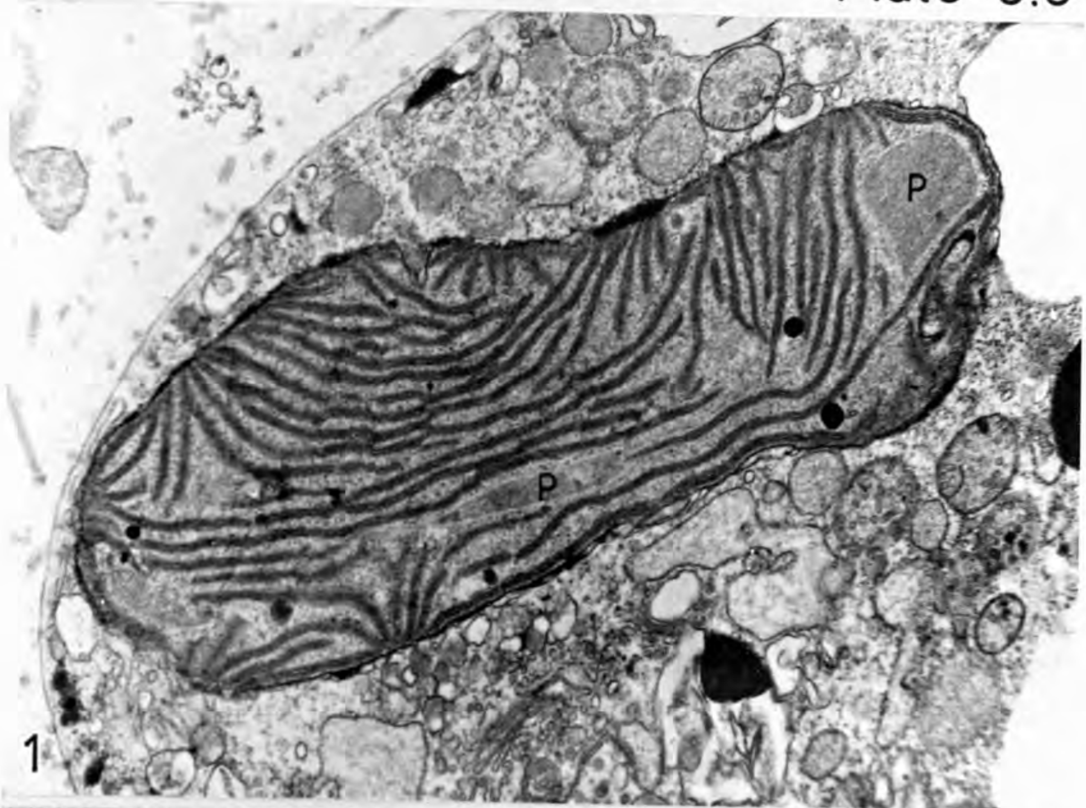
Plate 5.5. Ultrastructure of the chloroplasts of *Gymnodinium galatheanum*. Transmission electron micrographs.

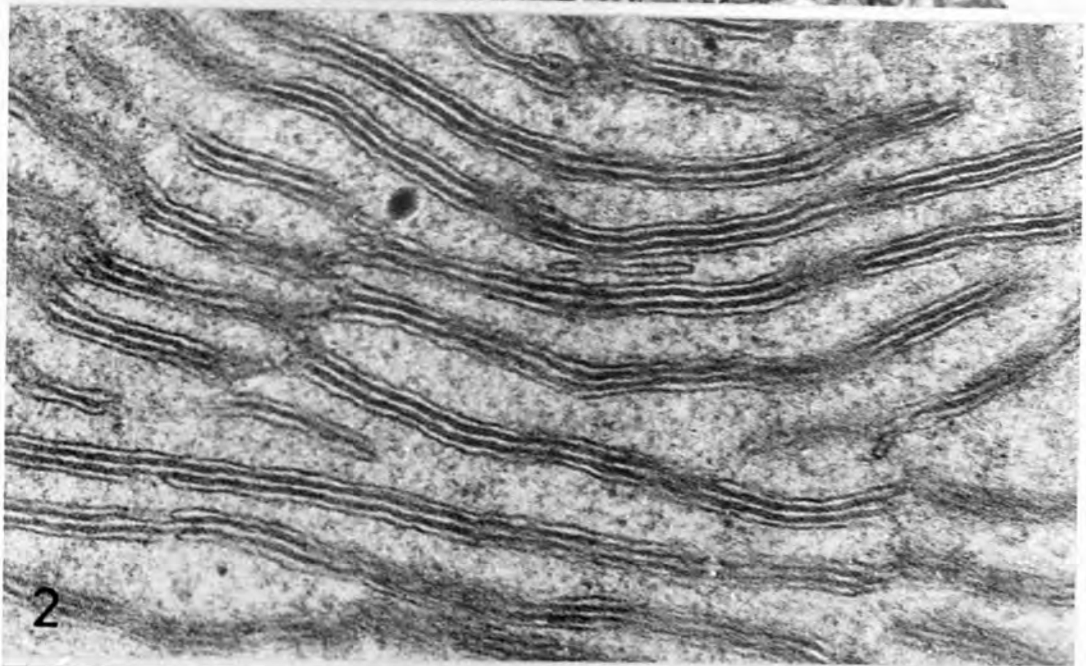
1. Longitudinal section through a chloroplast showing the simple internal pyrenoid (P) sectioned in places along its length and the lamellae converging onto the chloroplast envelope. x 18,800.

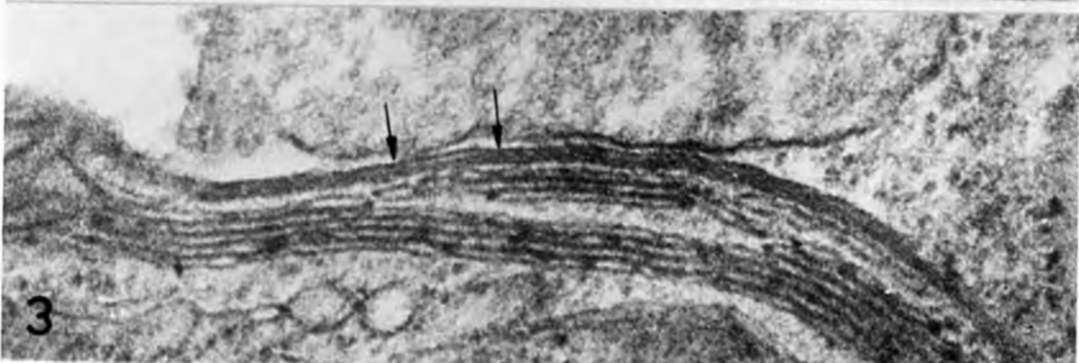
2. Detail of the lamellae which are composed of three thylakoids. x 100,000.

3. Detail of the triple membrane chloroplast envelope. The individual membranes are closely appressed but are just discernible in the arrowed section. x 99,200.

Plate 5.5







These gymnodinoids could therefore constitute a group of dinoflagellates in which anomalous pigmentation is correlated with an anomalous chloroplast structure. This would strongly favour an endosymbiotic origin for the chloroplasts. Only members of the Haptophyceae possess chloroplasts with simple, internal pyrenoids but not girdle lamellae.

5.3. CHLOROPLAST DNA ORGANIZATION IN ANOMALOUSLY PIGMENTED DINOFLAGELLATES.

Under optimal staining conditions the fluorescence emitted by DAPI when complexed with DNA is intense enough to visualize as little as 0.00015 pg of DNA (Coleman *et al.*, 1981), which is about the mass of a single chloroplast DNA molecule. Consequently, this fluorochrome has been used to study the supermolecular organization of the DNA in chloroplasts.

Two basic patterns of chloroplast DNA organization have been found which appear to be correlated with the presence or absence in the chloroplast of a girdle lamella. When a girdle lamella is present the DNA is organized into a peripheral ring, as in brown algae, diatoms and xanthophytes (Coleman 1979b; Kuroiwa *et al.*, 1981; Kuroiwa and Suzuki, 1981). In green algae, red algae, cryptophytes, haptophytes, eustigmatophytes, dinoflagellates and higher plants (James and Jope, 1978; Kuroiwa *et al.*, 1981; Kuroiwa *et al.*, 1982; Coleman and Lewin, 1983; Possingham *et al.*, 1983), all of which lack a girdle lamella, the chloroplast DNA is localized as scattered nucleoids. The diversity of algal chloroplast DNA organization has recently been reviewed experimentally (Coleman, 1985). Although this study confirmed that ring and point nucleoids were the two major types of arrangement,

exceptions were found. The chloroplasts of euglenophytes, colonial freshwater chrysophytes, xanthophytes which lack girdle lamellae and Pseudochariopsis (Eustigmatophyceae) all had an interconnected network of DNA. Although minor variations occur between each of these exceptional types, they can all be considered as elaborations on the scattered nucleoid arrangement, where connections between individual nucleoids probably occur (Possingham et al., 1983; Coleman, 1985).

Coleman (1985) comments on the difficulty in preparing dinoflagellates for fluorescence cytology because of the large amount of nuclear DNA and the highly refractile wall of non-naked forms. In the present study these problems were largely overcome by squashing the cells in fixative rather than squashing fixed cells (Kuriowa and Suzuki, 1980) or squashing cells and then fixing them (Coleman, 1979b). The latter technique invariably spreads the dinoflagellate nuclear DNA which then interferes with subsequent observations on the DAPI fluorescence from the chloroplasts. The simple squashing technique described in Section 2.3.2 enabled the chloroplast DNA organization in four species of anomalously pigmented dinoflagellates to be determined.

5.3.1. Glenodinium foliaceum.

The majority of Glenodinium foliaceum cells contain chloroplasts in which the DNA is arranged as a beaded, peripheral ring enclosing scattered point nucleoids (Plate 5.6.1 - 3). The number of these point nucleoids is variable, generally being less than 10 per chloroplast but sometimes as many as 20 or more may be present (Plate 5.6.2). Some chloroplasts appear to lack point nucleoids altogether (Plate 5.6.4) and conversely, in a few cells the ring nucleoid is absent from all the chloroplasts (Plate 5.6.5). The beaded DNA ring of typical chloroplasts is sometimes fragmented and follows a random path around the

Plate 5.6. Organization of chloroplast DNA in _Glenodinium foliaceum_. Epifluorescence micrographs of DAPI stained preparations correlated where appropriate with NIC micrographs.

1. Squashed but still intact cell in which the faint rings of chloroplast DNA fluorescence (arrow) can be seen around the periphery compared to the the intense nuclear fluorescence. x 2,500.

2. Chloroplast squashed out of a cell showing that the DNA is organized into a beaded peripheral ring and numerous internal and apparently isolated point nucleoids. x 6,000.

3. Three planes of focus (a - c) through a typical chloroplast having rather fewer internal DNA nucleoids than in (2). Note how the peripheral DNA ring and the point nucleoids are not visible when out of the plane of focus. The same plastid is shown under NIC in (d). All x 4,400.

4. Correlated fluorescence and NIC micrographs of a chloroplast lacking internal DNA nucleoids. x 4,400.

5. Similar correlated micrographs of a chloroplast lacking a peripheral ring of DNA. x 3,300.

6. Isolated chloroplasts digested with RNase. The chloroplasts have become distorted during processing but the pattern of DAPI fluorescence appears to be unaffected by this enzyme (compare with Plate 5.7). x 4,200.

Plate 5.6

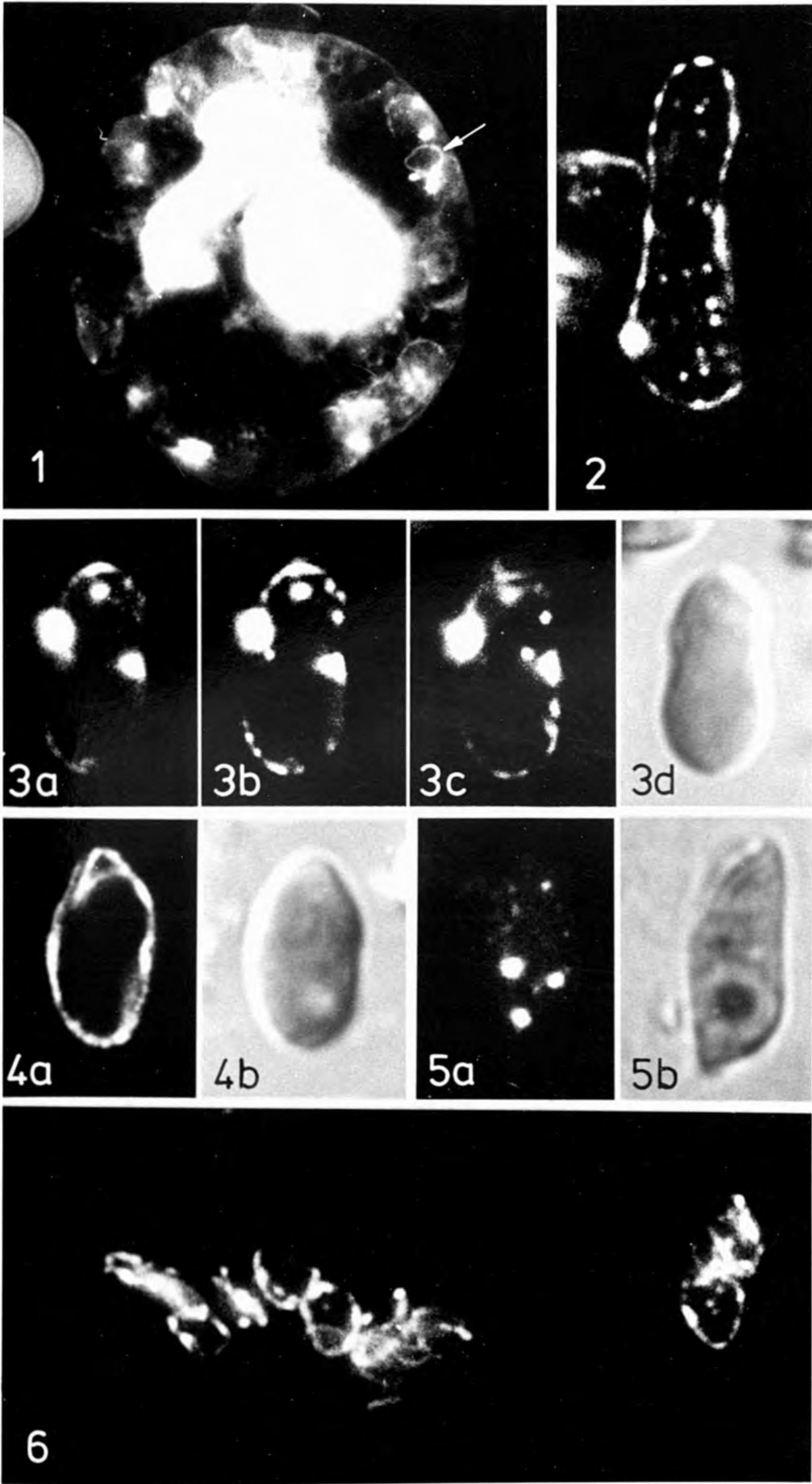
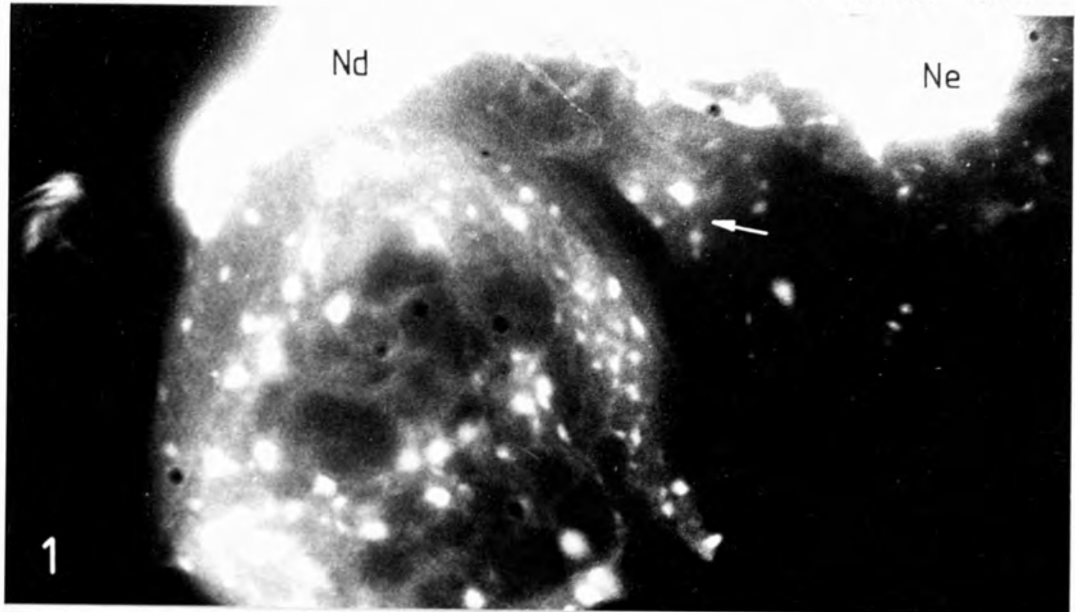


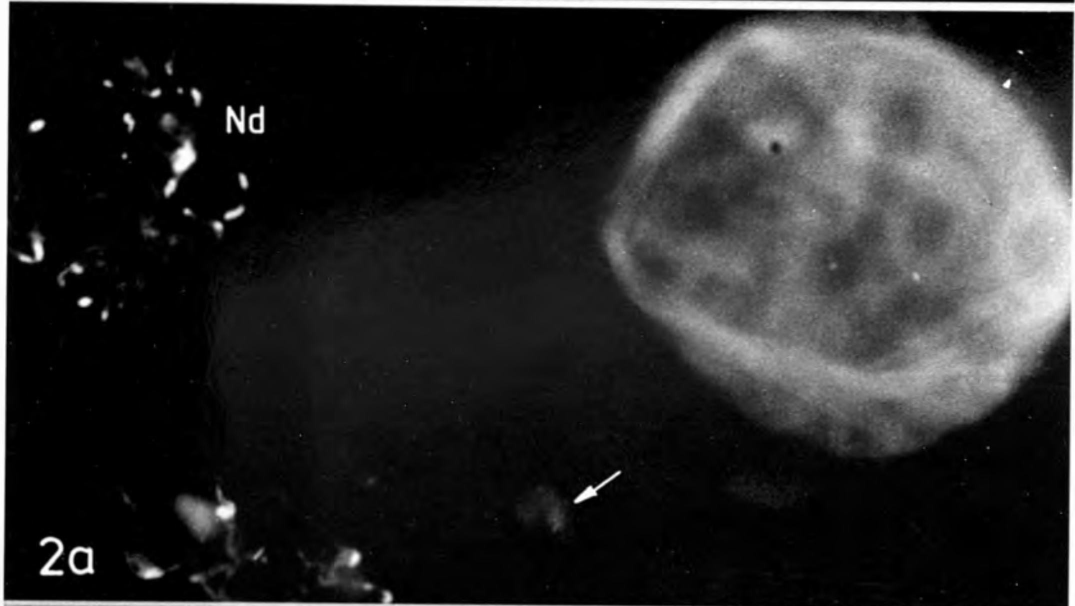
Plate 5.7. Sensitivity of DAPI fluorescence in *Glenodinium foliaceum* chloroplasts to DNase. Epifluorescence and NIC micrographs of squashed cells.

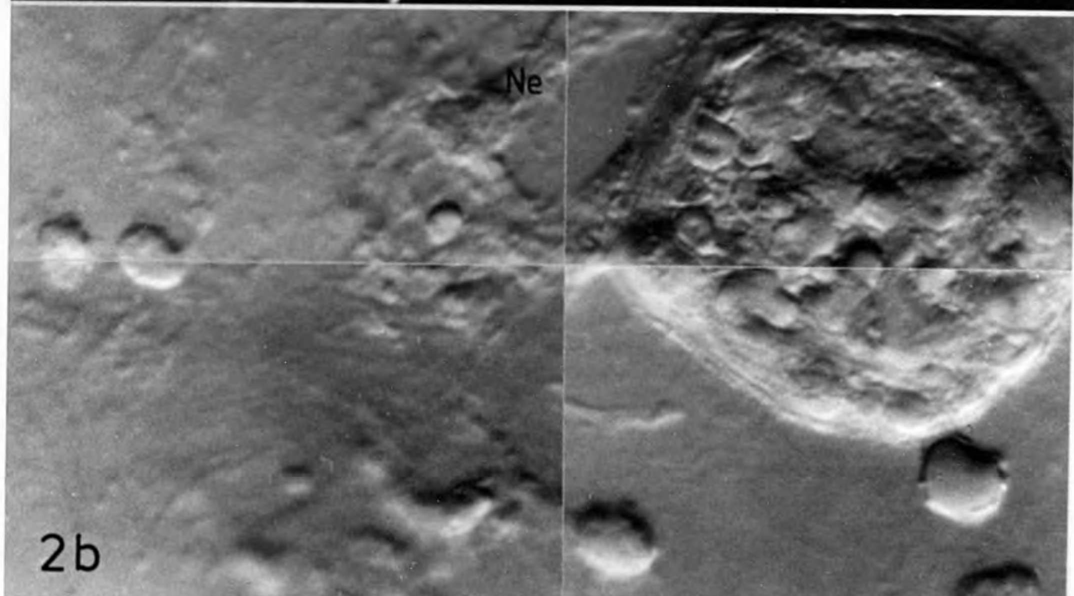
1. Control preparation incubated under the conditions used for enzymatic digestion but without the enzyme showing the DAPI fluorescence from the spread dinoflagellate (Nd) and symbiont (Ne) nuclei, and from the chloroplasts both remaining inside the theca and isolated from it (arrow). x 2,300.

2. Preparation digested with DNase showing that the DAPI fluorescence is much reduced from the dinoflagellate nucleus and has been eliminated from the symbiont nucleus and chloroplasts (symbols as in (1)). The correlated NIC micrograph reveals that there are numerous chloroplasts inside the thecal remains. Note that the non-specific DAPI fluorescence from the theca is more intense after DNase treatment probably because of the absence of competitive binding sites. x 2,500.

Plate 5.7







periphery of the organelle. DAPI fluorescence from the plastids was unaffected by digestion with RNase (Plate 5.6.6) but was eliminated by DNAse treatment (Plate 5.7) showing that it is entirely due to the interaction of the stain with DNA.

5.3.2. Gyrodinium aureolum.

Gyrodinium aureolum contains a relatively large amount of chloroplast DNA as estimated from the intensity of the DAPI fluorescence. This DNA is consistently organized into one or two beaded bands located to one side of the chloroplast (Plate 5.8.1 & 2). The coalescence of two bands occasionally gives the DNA area a plate-like appearance in surface view. As with *Glenodinium foliaceum*, the DAPI fluorescence from *Gyrodinium aureolum* chloroplasts was sensitive to DNase treatment (Plate 5.8.3 & 4).

5.3.3. Gymnodinium galatheanum, G. micrum and other dinoflagellates.

The organization of chloroplast DNA in *Gymnodinium galatheanum* and *G. micrum* is similar. In both species only isolated points of DAPI fluorescence can be seen (Plate 5.9.4 & 5). All peridinin containing dinoflagellates examined so far also contain only point chloroplast nucleoids which vary in size, shape and number (six species from five genera, listed in Table 2.1, were looked at here and Coleman (1985) has studied a further two species). *Scrippsiella trochoidea* with only a few relatively large and spherical nucleoids (Plate 5.9.1) and an unidentified *Gymnodinium* species with numerous small and irregularly shaped nucleoids (Plate 5.9.3) probably represent the extremes of the variation that can occur. *Gonyaulax polyedra* is perhaps a typical example of how dinoflagellate chloroplast DNA is arranged (Plate 5.9.2).

Plate 5.8. Organization of chloroplast DNA in *Gyrodinium aureolum*. Epifluorescence micrographs of DAPI stained preparations correlated where appropriate with NIC micrographs.

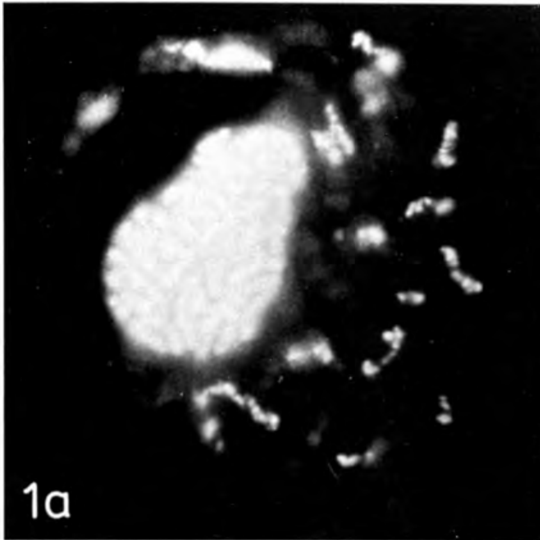
1. Squashed cell showing numerous beaded bands of DAPI fluorescence in the cytoplasm correlated with the positions of the chloroplasts. x 1,700.

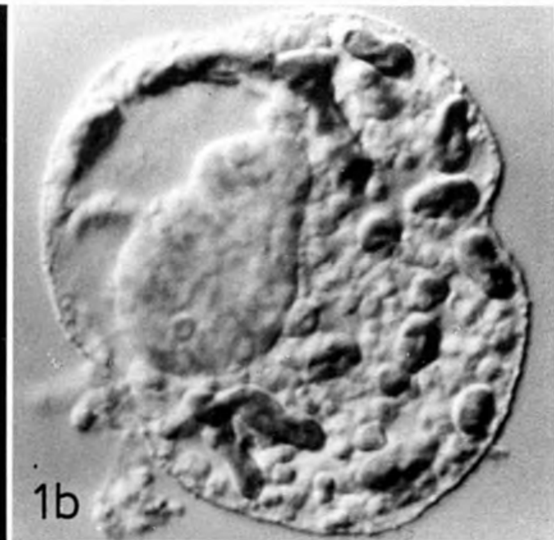
2. Isolated chloroplast containing a single beaded band of DNA. x 5,000.

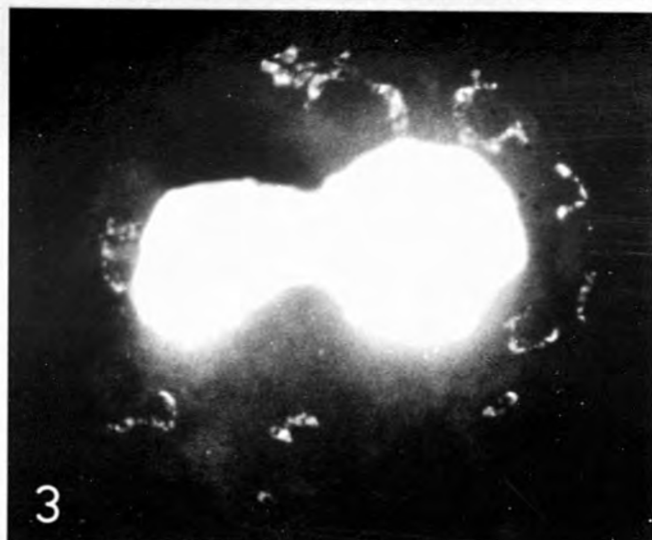
3. Control preparation of a whole cell incubated under DNase digestion conditions but without any enzyme showing bands of DAPI fluorescence in the cytoplasm. x 1,700.

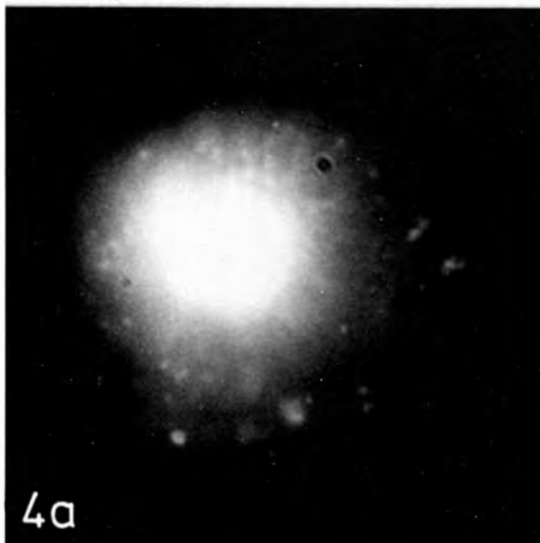
4. Whole cell digested with DNase showing only faint DAPI fluorescence from the cytoplasm which appears not to be correlated with the positions of the chloroplasts. The DAPI fluorescence from the nucleus has been reduced but not eliminated. x 2,000.

Plate 5.8









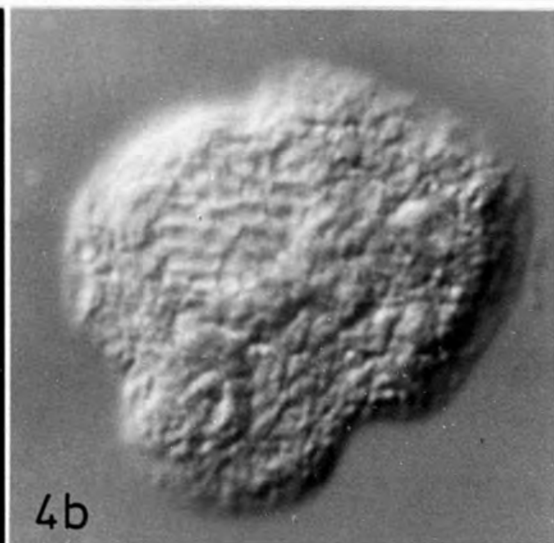


Plate 5.9. Patterns of extranuclear DAPI fluorescence in various algae. Epifluorescence micrographs of squashed cells.

1. *Scrippsiella trochoidea* (Dinophyceae). x 2,300.

2. *Gonyaulax polyedra* (Dinophyceae). x 1,700.

3. *Gymnodinium* sp. (Dinophyceae). x 1,600.

4. *Gymnodinium galatheanum* (Dinophyceae). x 3,400.

5. *Gymnodinium micrum* (Dinophyceae). x 2,100.

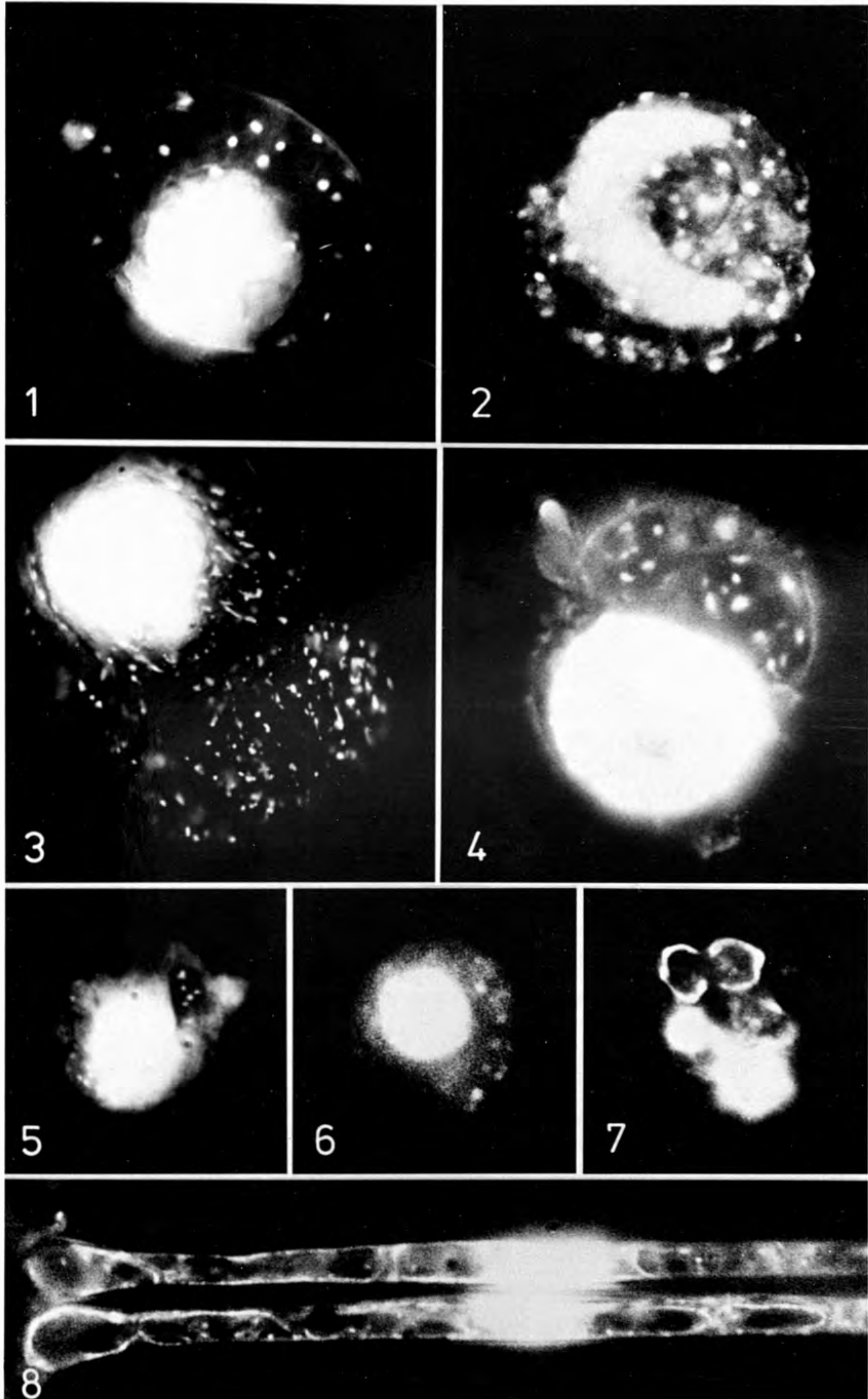
6. *Emilliania huxleyi* (Haptophyceae). x 5,000.

7. *Stephanodiscus hantzchii* (Bacillariophyceae). x 3,200.

8. *Asterionella formosa* (Bacillariophyceae). x 2,500.

See text for interpretation.

Plate 5.9



5.3.4. Chloroplast DNA organization as a phylogenetic marker.

The four anomalously pigmented dinoflagellates exhibit three different types of chloroplast DNA organization. The two Gymnodinium species fall into the scattered nucleoid catagory together with other dinoflagellates, whereas Glenodinium foliaceum could equally be classified as having ring nucleoids or point nucleoids. Gyrodinium aureolum appears to be entirely different from all other algae and the beaded band of DNA in its chloroplasts most closely resembles the central DNA nucleoid of cyanobacteria (Tschermak-Woess and Schöller, 1982; Coleman and Lewin, 1983).

The DAPI fluorescence data supports a classification of anomalously pigmented dinoflagellates (excluding those containing phycobilins) into three types based on the ultrastructural features shown by their chloroplasts. Namely those possessing chloroplasts with an internal pyrenoid and a girdle lamella (e.g. Glenodinium foliaceum), those having an internal pyrenoid but no girdle lamella (e.g. Gymnodinium galatheanum) and those whose chloroplast lack both an internal pyrenoid and a girdle lamella (e.g. Gyrodinium aureolum). Further support for this classification would come from a study of the arrangement of chloroplast DNA in Gymnodinium breve. The unusual chloroplast nucleoid of Gyrodinium aureolum probably resides in the thylakoid-free area of stroma observed under the electron microscope (see Plate 5.3.1) and this also seems to be an ultrastructural feature of the G. breve chloroplast (Steidinger et al., 1978).

Does the configuration of the chloroplast DNA in these anomalous species provide any independent evidence favouring an endosymbiotic origin of their chloroplasts? Clearly not in the case of Gymnodinium galatheanum and G. micrum since the chloroplast DNA organization exhibited by these two species does not

provide an unusual character on which to base a judgement. The chloroplast DNA arrangement in Gyrodinium aureolum is, however, different from that of typical dinoflagellates, but it is equally dissimilar from that of other algae and in particular the haptophyte Emilliania huxleyi (Plate 5.9.6; Kite and Dodge, 1985) which possesses the same major carotenoid (Tangen and Bjørnland, 1981). This either means that the chloroplasts of G. aureolum and E. huxleyi are unrelated, or that the organization of chloroplast DNA has become altered as a consequence of endosymbiosis. The arrangement of chloroplast DNA in Glenodinium foliaceum provides some information on this latter possibility.

On the basis of ultrastructural observations and pigment analysis data, Dodge (1983a) proposed that the endosymbiont of G. foliaceum is a diatom. Only diatoms have chloroplasts which possess fucoxanthin, internal lenticular pyrenoids and girdle lamellae. The organization of chloroplast DNA in G. foliaceum and diatoms should therefore be similar. Although it has been reported that the chloroplast DNA of diatoms is localized exclusively into a circular nucleoid (Kuriowa et al, 1981; Coleman, 1985), the chloroplasts of the pennate diatom Asterionella formosa do appear to contain both ring and point nucleoids (Plate 5.9.8). Faint points of DAPI fluorescence can also be seen within the circular chloroplast genophores of the centric diatom Stephanodiscus hantzchii (Plate 5.9.7). Coleman has suggested that the point nucleoids which were observed in another centric diatom, Ditylum brightwellii, (Coleman, 1979), originated from mitochondrial DNA. This possibility cannot be excluded here because the diatom chloroplasts were not viewed in isolation from the cytoplasm since they could not be successfully squashed out of the frustule. Therefore the existence of point nucleoids in diatom chloroplasts remains unconfirmed, but it should be noted that mitochondrial DNA fluorescence has rarely been observed in algae and the electron micrographs of Manton and von Stosch (1966) appear to show scattered, faintly staining regions of

stroma (usually regarded as areas of DNA) between the chloroplast lamellae of the diatom *Lithodesmium undulatum*.

Despite this confusion, the presence of a ring nucleoid in *Glenodinium foliaceum* shows that the chloroplasts have retained a stable DNA conformation in an endosymbiotic situation. This does not necessarily imply that chloroplast DNA organization is an autonomous chloroplast character since it might be under the control of genes in the endosymbiont nucleus. Without further information on the limitations involved with using the organization of chloroplast DNA as an indicator of phylogenetic relationships, is difficult to comment on the significance of the unique chloroplast DNA nucleoid found in *Gyrodinium aureolum*.

In conclusion then, the chloroplasts of *Gyrodinium aureolum* show anomalous cytological characteristics whilst those of *Gymnodinium galatheanum* possess anomalous ultrastructural features. Thus, there is some evidence that both may have had a different ancestry from the chloroplasts of typical dinoflagellates.

CHAPTER 6

SUMMARY AND CONCLUSIONS

The cytological and ultrastructural features of *Glenodinium foliaceum* are consistent with the proposal that the organism represents an endosymbiotic association between two phylogenetically unrelated, unicellular protists (Jeffrey and Vesk, 1976). The host has retained its external morphological characteristics and is clearly a dinoflagellate. In contrast, the symbiont has evolved in its intracellular environment into an amorphous cytoplasmic compartment delimited by a single membrane. Nevertheless, its chloroplasts exhibit a carotenoid complement (Withers and Haxo, 1975) and a fine structure which one normally associates with free-living members of the Bacillariophyceae (Dodge, 1983a), although the immunological cross-reactivity of RuBisCO from *G. foliaceum* appears to be different from that of diatoms (Rothschild, 1985).

6.1. FUNCTIONAL ASPECTS OF GLENODINIUM FOLIACEUM.

The symbiont nucleus shows a high degree of morphological and physiological integration with the host. Functionally it exists as a "surrogate" chloroplast, and as the host only shows

limited heterotrophic capabilities (Morrill and Loeblich, 1979), an exchange of nutrients must occur between the two (Dodge, 1983a). There also appears to be a co-ordination of cell cycle events, with DNA synthesis probably occurring in synchrony and the symbiont nucleus dividing just prior to cytokinesis. However, this division is not organized in any way, the nucleus is simply split in two by the cleavage furrow in the host cytoplasm and it never shows any obvious chromosomal condensation. This suggests that the symbiont nucleus divides amitotically which could have serious implications on the functioning of the endosymbiont as its genetic material would be partitioned randomly between the daughter cells.

Amitosis in the endosymbiont nucleus of *Peridinium balticum* has been demonstrated more conclusively by electron microscopy (Tippit and Pickett-Heaps, 1976) and the nucleomorph of cryptophytes also divides in this manner (McKerracher and Gibbs, 1982). In *P. balticum* the chromatin of the nucleus does not condense nor are there any microtubules associated with its division: microtubules being a universal feature of mitotic mechanisms (Margulis, 1981). Thus, amitosis in the endosymbiont nucleus of *Glenodinium foliaceum* also seems likely, particularly as the nucleus fails to even round off before division, as it does in *P. balticum*. In addition, the symbiont nucleus of *G. foliaceum* looks fragmented in some interphase cells, so it is important to consider whether these apparently degenerate features reflect a functional redundancy. Some information on this point can be gained from a comparison of the symbiont nucleus with the macronucleus of ciliates in which amitosis occurs regularly (Corliss, 1975).

6.1.1. DNA organization in the symbiont nucleus of *Glenodinium foliaceum* and the macronucleus of ciliates.

The macronuclear genes of ciliates are amplified so that each daughter nucleus is reasonably assured of a copy of every gene (Prescott *et al*., 1973). Due to this genetic amplification, the macronucleus of *Oxytricha nova* contains 58 pg of DNA, whilst the haploid DNA content of the micronucleus, from which the macronucleus is generated, is only 0.66 pg (Lauth *et al*., 1976). The symbiont nucleus of *Glenodinium foliaceum* contains about 34 pg of DNA, which is between 2 and 340 times the amount of DNA that is found in the nuclei of its probable free-living relatives, i.e. chromophyte algae (see Table 4.2). If this 'extra' DNA in the symbiont nucleus is due to amplified DNA analagous to that found in the ciliate macronucleus, then it should be detectable by other means.

In hypotrich ciliates, the process of macronuclear development from the micronucleus first involves a number of rounds of DNA replication which occur in the absence of cell division so that polytene chromosomes are formed (Spear and Lauth, 1976). Massive DNA degradation then follows due to the elimination of all the repetitive DNA and approximately 95% of the unique sequences that were originally present in the genome of the micronucleus (Lauth *et al*., 1976). Internal sequences may also be spliced out of some of the conserved genes (Klobutcher *et al*., 1984). This removal of the spacer sequences between the chromosomal genes results in the production of numerous gene-sized DNA molecules (Prescott, 1984). These "macronuclear genes" range in size from 0.4 to 20 kb with an average size of 2.2 kb (Swanton *et al*., 1980) and constitute individual replicons with replication occuring at one or both ends of the molecule (Murti and Prescott, 1984). There are approximatley 24,000 different macronuclear genes each repeated on average 1,000 times per nucleus, and consequently the macronuclear DNA reassociates as a

single kinetic component of complexity 5.4×10^4 kb (Lauth *et al.*, 1976).

It was not possible to fractionate enough *Glenodinium foliaceum* DNA to study to renaturation kinetics of the symbiont nuclear DNA in isolation. However, total *G. foliaceum* DNA does not seem to contain a kinetic component with a complexity and repetition frequency equivalent to that of the ciliate macronuclear genes. The only highly repeated sequences in the genome have a low complexity, as is typical for eukaryotes in general. Furthermore, no amplified gene-sized (i.e. 1 - 3 kb) fragments were detected by agarose gel electrophoresis of unrestricted, symbiont nuclear DNA. Ciliate macronuclear DNA produces a distinct banding pattern after such an analysis (Swanton *et al.*, 1980).

Thus, the symbiont nucleus of *G. foliaceum* does not contain individual amplified genes, but the possibility that it may be highly polyploid cannot be excluded. If we suppose that the average chromosome contains 0.15 pg of DNA (Lewin, 1980) and that diatoms have an average haploid DNA content of 2.8 pg (see Table 4.2), then, assuming that the *G. foliaceum* endosymbiont is a diatom, it could be argued that its haploid genome would be expected to be divided amongst about 20 chromosomes (see for example Manton *et al*, 1970), and that the nucleus contains enough DNA for these to be repeated 23 times. According to the equations proposed by Gabriel (1960), this degree of polyploidy would ensure that each daughter cell receives at least one copy of every chromosome in over 99% of amitotic divisions. Therefore, the morphological degeneration of the endosymbiont nucleus cannot yet be forwarded as an argument for its functional redundancy.

6.2. GLENODINIUM FOLIACEUM AND THE ORIGIN OF CHLOROPLASTS FROM EUKARYOTIC ENDOSYMBIONTS.

It has been suggested that *Glenodinium foliaceum*, together with *Peridinium balticum* and *Mesodinium rubrum*, illustrate possible intermediate stages in the evolution of certain chloroplasts from eukaryotic endosymbionts (Tomas and Cox, 1973; Loeblich, 1976; Gibbs, 1978; 1981a; Taylor, 1979; Dodge, 1979, 1983b; Whatley and Whatley, 1981). However, whether or not these organisms actually represent true intermediates, in which there is the potential for the endosymbionts to develop into *de facto* photosynthetic organelles, requires more careful consideration (Kite and Dodge, 1984). In particular, one must examine the genetic aspects of these associations since any further structural degeneration of the endosymbiont, particularly its nucleus, must be preceded by integration at the genetic level.

6.2.1. Coding capacity of the chloroplast genome in Glenodinium foliaceum.

The chloroplast genome of *Glenodinium foliaceum* appears to have a size of approximately 100 - 106 kb. This represents enough DNA to code for about 130 polypeptides with an average molecular weight of 30,000 daltons, assuming asymmetric transcription of the DNA and no overlapping genes. At least 200 polypeptides can be revealed by two-dimensional gel analysis of higher plant chloroplast protein (Ellis, 1983a) and probably many more are actually present. Thus, the chloroplast genome of *G. foliaceum* cannot encode all of the proteins that occur in the organelle.

In plants the majority of chloroplast proteins are coded for by the nucleus and are synthesized on cytoplasmic ribosomes

(Ellis, 1984). Amongst these are the enzymes which catalyse chlorophyll synthesis, several kinds of photosynthetic membrane proteins, numerous chloroplast isoenzymes and the small subunit of RuBisCO (Weeden, 1981; Ishida, 1983; Hooper, 1984; Kloppstech et al., 1985). Consequently chloroplasts are unable to survive indefinitely in a foreign cellular environment (Giles and Sarafis, 1971), and organisms which retain chloroplasts as temporary 'endosymbionts', such as certain marine molluscs (Hinde and Smith, 1972; Trench, 1979), ciliates (Blackbourn et al., 1973) and foraminifera (Lanners, 1983), have to acquire fresh organelles to maintain a functional population.

The evidence that many chloroplast genes are nuclear encoded has come from the study of the inheritance of these genes (Kirk and Tilney-Basset, 1978); from the use of inhibitors specific for chloroplast or cytoplasmic ribosomes (Ellis, 1981); and, more recently, by employing cloned DNA probes to directly locate the gene in question (Groot, 1984). Unfortunately in the present study, such a molecular analysis of DNA failed to satisfactorily locate the rbc S gene in Glenodinium foliaceum. Therefore, although it is clear from the size of their genome that the chloroplasts of G. foliaceum are reliant on genes located elsewhere in the cell, no information is available on the identity of these genes or, more importantly, in which of the two nuclei they are to be found. One would assume that the symbiont nucleus encodes the genes that are essential for the maintainance of the chloroplasts, since these organelles are, after all, a part of the endosymbiont.

6.2.2. Glenodinium foliaceum as an intermediate in the evolution of chloroplasts from eukaryotic endosymbionts.

What then are the possibilities that Glenodinium foliaceum could evolve into a uninucleate, photosynthetic cell? Other

anomalously pigmented dinoflagellates such as Gyrodinium aureolum and Gymnodinium galatheanum show only atypical cytological or ultrastructural chloroplast features, and in fact there are reports of uninucleate populations of both Glenodinium foliaceum and Peridinium balticum (Biechler, 1952; Taylor, 1979) but these remain unconfirmed and uninvestigated. Divergent organelle characteristics in host cells which can be shown to be related is classed as "evidence which is proof" of an endosymbiotic origin of the organelle (Gray and Doolittle, 1982). Certainly it would seem improbable that Amphidinium wigrense could have evolved cryptophyte-like chloroplasts autogeneously. So it is tempting to draw an evolutionary series leading from binucleate to uninucleate anomalously pigmented dinoflagellates via the gradual reduction and eventual loss of the endosymbiont cytoplasm and nucleus (Tomas and Cox, 1973; Dodge, 1983b; Wilcox, 1984; Wilcox and Wedemayer, 1985). However, these uninucleate types could have acquired their chloroplasts directly from the same source as the endosymbionts in their binucleate counterparts and so they cannot be forwarded as unequivocal evidence that present day endosymbionts can undergo a similar morphological reduction. Indeed, since nucleomorphs are absent from A. wigrense (Wilcox and Wedemayer, 1985), there is the possible senario that this organism is related to the ancestors of cryptophyte chloroplasts: "My theory on the origin of dinozoan and cryptophyte plastids predicts that a dinozoan with phycobilins and chlorophyll c_2 once existed. Perhaps some still do" (Cavalier-Smith, 1982b).

If essential chloroplast genes are located in the symbiont nucleus of Glenodinium foliaceum, then clearly they must be transferred elsewhere before this nucleus could be made redundant. The endosymbiont hypothesis for the origin of organelles necessarily invokes a model to explain the subcellular distribution of organellar genes (Weeden, 1981), but until recently the genetic integration of symbionts was not widely discussed (but see Margulis, 1976), except by opponents of the hypothesis

(Uzzell and Spolsky, 1974). Perhaps this was because that, in the absence of conclusive evidence that genes could be transferred between organelle (or pro-organelle) and nuclear genomes, a rational analysis of the genetic data favoured alternative hypotheses for their origin (Bogorad, 1982). However, due to the discovery of promiscuous DNA (Lewin, 1984) and the Agrobacterium transformation system (Nester et al., 1984), the existance of gene transfer cannot be doubted any longer.

The term "promiscuous DNA" was originally proposed by Ellis (1982) to describe nucleotide sequences that occurred in both chloroplasts and mitochondrial genomes of maize (Stern and Lonsdale, 1982). Since then, other examples of promiscuous DNA have been found (Ellis, 1983b), such as homologous sequences in the mitochondrial and nuclear DNAs of yeast (Farrelly and Butow, 1983), locusts (Gillisen et al., 1983) and the fungus Podospora anserina (Wright and Cummings, 1983) and in the chloroplast and nuclear DNAs of spinach (Timmis and Scott, 1983). Furthermore, every cloned chloroplast DNA sequence from spinach has shown homologies with discrete restriction fragments of both the mitochondrial and nuclear DNAs (Stern and Palmer, 1984; Scott and Timmis, 1984). Some of these homologous sequences are known to encode chloroplast proteins such as the large subunit of RuBisCO, so, clearly, the direction of gene transfer has been from chloroplast to mitochondrion; translocation could have occurred in either direction to produce chloroplast and nuclear DNA homologies.

The widespread occurrence of promiscuous DNA indicates that interorganellar DNA transfer is an ongoing process and therefore could also take place in Glenodinium foliaceum. However, the inadequate size of the Glenodinium chloroplast genome suggests that mass translocation of chloroplast genes back to the chloroplast has not yet occurred, and perhaps could not occur. In general, chloroplast genomes show little size or sequence varia-

tion compared with those of mitochondria where there is often a great difference in size even between quite closely related species (Wallace, 1982). The variability of the mitochondrial genome could be partly due to the presence of chloroplast DNA sequences, which infers that the chloroplast genome is much more suscepible to structural modifications, such as the incorporation of foreign DNA (Lewin, 1984). Furthermore, the mechanism of gene transfer from the endosymbiont nucleus to the chloroplast would have to show some specificity for the chloroplast genes otherwise the process would appear to be statistically unlikely.

Stern and Palmer (1984) suggest three possible mechanisms for the interorganellar transfer of genetic information: via direct physical contact and membrane continuity between organelles; by the transformation of one organelle with DNA from another that had broken or lysed; or through the action of a vector such as a transducing phage or transposable element. Only the last mechanism would have the required specificity, and at present, there is no evidence for the operation of a specialized vector system, whilst there are numerous observations of membrane continuities between different organelles (e.g. Osafune et al., 1985).

If the mass transfer of genes back to the chloroplast of Glenodinium foliaceum seems unlikely, then there remains the alternative possibility that genetic exchange could occur between the endosymbiont and host nuclei. This seems more probable since the dinoflagellate nuclear DNA would be more accomodating to foreign nuclear encoded genes. An interesting corollary to this possibility is that gene transfer need not occur at all! Glenodinium foliaceum possesses an eyespot in the host cytoplasm which is perhaps analagous to a dinoflagellate plastid (Dodge, 1984). Therefore the view has been expressed that the host cell was itself once photosynthetic (Dodge, 1979; Taylor, 1983); the suggestion being that the endosymbiont chloroplasts outcompeted

those of the host. If this is true, then the host nuclear DNA could already encode for chloroplast proteins. These proteins might not be functional in the endosymbiont chloroplasts, nor might they have the correct transit peptide which would enable them to pass through the chloroplast membranes (van den Broeck *et al*., 1985) but they would constitute phenotypes on which natural selection could operate.

In conclusion, rather than representing a possible evolutionary intermediate, *Glenodinium foliaceum* tends to illustrate the problems involved in the secondary acquisition of chloroplasts from eukaryotic endosymbionts. If certain chloroplasts had such an origin, then the process of symbiont degeneration would have been altogether more straight-forward if the eukaryotic endosymbiont had itself only recently acquired its pro-chloroplasts via a primary endosymbiosis with a prokaryote and gene transfer had not yet occurred. Therefore, the origin of chloroplasts from eukaryotic endosymbionts may well have been a unique event in the evolution of photosynthetic cells.

APPENDIX

PURIFICATION OF GLENODINIUM FOLIACEUM CULTURES

A.1. INTRODUCTION.

The relative dearth of information on the diversity of algal chloroplast DNA and the scarcity of suitable axenic algal cultures are not unrelated factors. Although bacterial contamination is tolerated in work on higher plant DNA, the level of contamination is orders of magnitude higher in algal cultures where the bacteria frequently outnumber the algae. Starter cultures of *Glenodinium foliaceum* were neither unialgal or axenic, therefore a reasonably pure culture had to be produced before work on DNA characterization could begin.

Provasoli *et al* (1951) and Spencer (1952) were amongst the first workers to use antibiotics to purify algal cultures. Since then, the technique seems to have become the method of choice in many laboratories largely as a result of the ease with which it can be applied. However, antibiotic treatment should really be considered the method of last resort because one cannot be sure that the algae are not adversly affected by prokaryotic-specific growth inhibitors. In this study the decision to employ antibiotics was only taken after it had become clear that "cell washing" procedures were not going to be successful.

These manipulative procedures are not described here in

detail, although much time was spent in investigating their potential. They proved to be mostly ineffective and are described adequately elsewhere (Stein, 1973). However, protocols for antibiotic treatments are rarely elaborated on and so it seems pertinent to include a discussion of the techniques employed although they are not directly of relevance to the present thesis.

A.2. GENERAL METHODS.

Standard aseptic techniques were observed during all purification attempts which were performed in an inoculating room sterilized by ultraviolet irradiation. Antibiotic solutions were prepared in sterile distilled water, or medium, as appropriate and then passed through a 0.22 μm Millipore filter using a syringe filter apparatus. All other media and glassware were sterilized by autoclaving.

A.2.1. Bacterial growth media and contamination tests.

The following bacterial growth media and incubation conditions were used:

a. Liquid media:

(i) Marine Nutrient Broth.

Nutrient Broth (lab m No.2) adjusted to 25 g l^{-1} with respect to sodium chloride.
$35^{o}C$ / 1-2 days.

(ii) ZoBell's Medium (ZoBell 1941).

proteose peptone	5 g
ferric sulphate	0.1 g
seawater	1 l

$20^{o}C$ / 1 month in the dark.

b. Solid media:

(i) Seawater Agar.

glucose	1 g
proteose peptone	1 g
Erd-Schreiber	1 l
Agar	15 g

$20^{o}C$ / 1 week

(ii) Marine Nutrient Agar.

Nutrient Agar (lab m) adjusted to 25 g l^{-1} sodium chloride.
$20^{o}C$ / 1 day.

(iii) ZoBell's Agar.

ZoBell's Medium solidified with 12 g l^{-1} agar.
$20^{o}C$ / 2 weeks.

Solid media were used for contamination tests as follows. 90 mm diameter plates were spread with 0.1 ml of the culture under examination and incubated together with control plates spread with sterile ASP_7 or untreated culture. If, after incubation, contamination was not visible with the naked eye, then the surface of the plate was examined microscopically for small bacterial colonies. Seawater agar was used as a relatively quick general purpose medium for testing the effectiveness of cell washing procedures. After antibiotic treatments, however, marine

nutrient agar was employed as a rapid test so that contaminated cultures could quickly be discarded. Remaining cultures were subsequently plated onto ZoBell's agar to detect contamination with marine heterotrophic bacteria.

Routine contamination checks on purified stock cultures were made by direct epifluorescent microscopic observations on samples stained with 0.5 $\mu g\ ml^{-1}$ of the DNA specific fluorochrome DAPI as described in Section 2.3.2. This technique has detected bacterial contamination in cultures deemed axenic by other means (Coleman, 1980).

A.2.2. Determination of the minimum inhibitory concentration of various antibiotics on selected culture bacteria.

In order to assess the relative effectiveness of several common antibiotics against those bacteria which were able to grow in nutrient broth, the minimum inhibitory concentration of each antibiotic was first determined.

10 ml of marine nutrient broth was inoculated with 400 μl of a mid-exponential phase culture of *Glenodinium foliaceum* and shaken overnight at 35°C. The approximate concentration of bacteria was then determined by optical counting and adjusted to about 5×10^7 bacteria ml^{-1}. Generally only one x10 dilution was required to achieve this. 0.1 ml of this dilution was used to inoculate 2 ml aliquots of marine nutrient broth in capped test tubes containing various levels of the antibiotic under examination. The antibiotic had been added from 10^0, 10^{-1}, 10^{-2}, 10^{-3}, or 10^{-4} dilutions of a 2 $mg\ ml^{-1}$ stock dissolved in distilled water and subsequently filter sterilized. Either 50, 100 or 200 μl of each dilution was dispensed into the tubes to give a final approximate x2 dilution series ranging from 200 $\mu g\ ml^{-1}$ to 5 $ng\ ml^{-1}$. Appropriate controls lacking antibiotics were also

prepared.

After 7 days incubation at 35°C the tubes were scored for bacterial growth by comparing their turbidity with a control tube that had been held at 4°C. This was done either visually or by monitoring the degree of light scatter at 520 nm where the turbidity seemed similar to the control. The minimum inhibitory concentration (MIC) was defined as the lowest level of the antibiotic which allowed no increase in culture turbidity. The following antibiotics were tested: penicillin G (potassium salt), ampicillin, cephalosporin C (potassium salt), bacitracin, streptomycin sulphate, neomycin sulphate and kanamycin sulphate (all were purchased from Sigma except penicillin G which was obtained from BDH).

A.2.3. Treatment of *Glenodinium foliaceum* with single antibiotics.

Stock solutions of each antibiotic were made up at x1000 their determined MIC in sterile ASP_7 from which 10^0, 10^{-1}, and 10^{-2} dilutions were prepared. The antibiotics were not filter sterilized. 0.5 ml of each dilution was added to 4.5 ml of *Glenodinium foliaceum* culture in a screw-capped culture tube to give final concentrations of x1, x10 and x100 the antibiotics MIC (bacitracin was not tested at the x100 level). After 5 days the cultures were examined microscopically.

A.2.4. Treatment of cultures with mixtures of antibiotics.

Stock solutions of antibiotic mixtures, which were formulated from the results of the above experiments, were prepared in ASP_7 and filter sterilized. Generally each mixture was tested at three concentration levels which corresponded to 10^0, 10^{-1} and

10^{-2} dilutions of the stock. 5 ml aliquots of each level of antibiotic mixture were then pipetted into screw-capped culture tubes and inoculated with 250 μl of concentrated cell suspension. The algal concentrates were produced by pouring a late exponential phase culture into tapered-ended glass centrifuge tubes and then pipetting up the aggregate of cells which formed at the bottom of the tubes after about 5 min. To stimulate bacterial growth, 100 μl of either marine nutrient broth or ZoBell's medium, or 50 μl of 2 mg ml^{-1} glucose, 2 mg ml^{-1} proteose peptone, were then added to the culture tubes. All treatments were prepared in duplicate together with controls lacking antibiotics or inoculated with sterile ASP_7 instead of algae. The culture tubes were placed under culture conditions for 1, 2, 6 or 16 days after which time the algal cells, if still alive, were colected by aggregation and transferred to 5 ml of fresh ASP_7. For treatments in which no motile cells were visible, 250 μl of "culture" was inoculated into fresh medium for the purposes of comparative contamination assays. Two weeks after the last transfers were made the cultures were tested for the presence of bacteria by plating onto marine nutrient agar and then ZoBell's agar.

A.3. PURIFICATION OF GLENODINIUM FOLIACEUM.

A.3.1. Production of unialgal cultures.

To eliminate a small, unidentified, green flagellate that was present at low levels in the cultures, use was made of the chance observation that *Glenodinium foliaceum* collects at the bottom of tapered-ended glass tubes. Within about 2 min of a culture being poured into such tubes a dense aggregate of cells

forms which does not disperse even after a period of 24 h, despite the cells in the aggregate being motile. Since most of the aggregate could be withdrawn by means of a Pasteur pipette in a volume of about 250 µl, it was possible to inoculate subcultures to a high initial cell density whilst minimizing the transfer of the old medium. The contaminating flagellate did not appear to behave in this manner since after repeatedly initiating small volume cultures with cell aggregates, it was eventually diluted out of existence. This method of collecting cells by "aggregation" was also employed in treating cultures with antibiotics.

A.3.2. Production of bacteria-free cultures.

All attempts to remove bacteria from cultures concentrated by slow speed centrifugation, aggregation or filtration, by repeated washing of the concentrate in sterile medium, failed. *Glenodinium foliaceum* did not generally survive more than four rounds of centrifugation and washed cultures still showed a high level of contamination on seawater agar, suggesting that the bacteria were being co-sedimented with the algae since four washings should have eliminated them from the pellet. The viability of washed aggregates was better, however it was estimated that the carry-over of old medium was at least 50 times greater than with cell pellets and the number of washings would have had to have been increased accordingly. A similar problem was encountered when attempts were made to repeatedly wash cells sedimented onto an 8 µm Nuclepore filter, also the bacteria did not appear to pass freely through the filter. *Glenodinium foliaceum* did not exhibit any phototactic response when placed in a phototactic chamber similar to that described by Stein (1973), so the algae could not be induced to migrate into sterile medium by this means. A similar observation was made by Withers and Haxo (1978).

Washing single cells by micropipetting them through a series of cavity slides containing sterile medium removed culture bacteria more effectively than the bulk washing procedures. However the technique proved very tedious as the success rate of maintaining a viable cell through four aseptic transfers (the minimum number required to reliably dilute out the bacteria) was less than 20%. The quality of the micropipette was critical in this respect, and although 52 cells were successfully washed in this manner, none ever survived to produce clonal cultures. In an attempt to reduce the number of washings required to eliminate the bacteria, the culture from which the cells were being isolated was first treated with antibiotics. An antibiotic mixture suggested by Stein (1973) was used which contained 10 mg ml^{-1} penicillin, 5 mg ml^{-1} streptomycin and 1 mg ml^{-1} chloramphenicol and was included in the culture at three levels such that the final concentration of penicillin was either 30, 200 or 600 μg ml^{-1}. *Glenodinium foliaceum* seemed particularly susceptible to all concentrations of this antibiotic and cells that were still motile after 2 days incubation did not respond well to micropipetting. Once again, no clonal cultures were obtained but the cells were generally bacteria free after just three washings.

Although a mixture of penicillin, streptomycin and chloramphenicol has often been recommended as a standard antibiotic treatment (eg. Droop, 1967; Stein, 1973), the logic of combining two bacteriocidal drugs (penicillin and streptomycin) with a bacteriostatic one (chloramphenicol) has rightly been questioned (Tuttle and Loeblich, 1974). Chloramphenicol has also been shown to inhibit the growth of four species of *Gonyaulax* (Divan and Schnoes, 1982) and has well documented effects on chloroplast ultrastructure and function (e.g. Margulies and Brubaker, 1970; Goodenough, 1971; Smith-Johannsen and Gibbs, 1972; Gibbs, 1979). For these reasons, and because of the apparent susceptibility of *G. foliaceum* to the standard antibiotic mixture, chloramphenicol was not used in subsequent antibiotic tests.

It is helpful, perhaps, to consider that there are two populations of bacteria in an algal culture: one population having been derived from cells present in the original isolate, and the other originating from bacteria that have subsequently found there way into the culture as a result of asterile culturing techniques. The former sub-population represent the true "marine bacteria", whilst the latter are conveniently referred to as "culture bacteria". For practical purposes, however, the culture bacteria are better defined as those micro-organisms capable of rapid growth on nutrient agar at elevated temperatures (35°C), and the marine bacteria as those slow growing types requiring more exacting conditions (ZoBell, 1941). The planned strategy for purifying G. foliaceum was to determine the relative effectiveness of various antibiotics against the culture bacteria and then to design a suitable mixture which would hopefully be effective against the marine bacteria as well.

The determined minimum inhibitory concentrations (MIC's) of seven antibiotics tested on culture bacteria growing in marine nutrient broth are listed in Table A.1. Those antibiotics causing mistranslation of mRNA on 70S ribosomes (streptomycin, kanamycin and neomycin) all appeared equally effective whilst a range of MICs were shown by the inhibitors of peptidoglycan synthesis. Of these, bacitracin was substantially less effective than the three β-lactams, of which cephalosporine C was noticeably the more potent since it induced cell lysis, that is the clearing of the culture, at its MIC. Generally lysis only occurred at higher concentrations of the other antibiotics.

None of the antibiotics had any obvious deleterious short term effects on G. foliaceum when included in the culture medium at concentrations up to one hundred times their MIC. In fact the cultures treated with the β-lactam antibiotics appeared to show an increased division rate compared to the controls lacking antibiotics. This may have been due to bacterial lysis providing

Table A.1. Effectiveness of individual antibiotics against culture bacteria. Minimum inhibitory concentration (MIC) and mode of action of various antibiotics on bacteria present in *Glenodinium foliaceum* cultures which were able to grow in marine nutrient broth.

Antibiotic	MIC ($\mu g\ ml^{-1}$)	mode of action
penicillin G	5	CW
ampicillin	2	CW
cephalosporine C	1	CW
bacitracin	50	R
streptomycin	5	R
neomycin	5	R
kanamycin	5	R

(CW, inhibits cell wall synthesis; R, inhibits ribosome function)

additional nutrients rather than a direct effect of the antibiotics themselves (Ukeles and Bishop, 1975). However bacterial contamination was still detected by plating onto nutrient agar in all the cultures except the x100 penicillin treatment. The difference in effectiveness (at least two orders of magnitude) of the antibiotics against bacteria in culture media and the same bacteria in nutrient broth, amply demonstrates the need to include a bacterial growth stimulant along with the antibiotics when attempting to purify cultures. Bacteriocidal concentrations of antibiotics will not kill bacteria which do not happen to be growing during the active life of the antibiotic in solution. Consequently the nature of the growth stimulant could well be as

important as the level and types of antibiotics used when attempting to purify cultures.

On the basis of the above results, a basic antibiotic mixture (BAM) was formulated. In the x1 BAM, penicillin, ampicillin and cephalosporine C were included at ten times their MIC whilst streptomycin and kanamycin were present at their MIC. Emphasis was placed on the peptidoglycan synthesis inhibitors as these would be least likely to affect the algae. Bacitracin and neomycin were omitted from this basic mixture because the former was relatively ineffective against culture bacteria and the latter is reported to affect eukaryotic ribosomes (Gale *et al.*, 1981). The composition of the x1 BAM was therefore:

penicillin	50 $\mu g\ ml^{-1}$
ampicillin	20 $\mu g\ ml^{-1}$
cephalosporine C	10 $\mu g\ ml^{-1}$
streptomycin	5 $\mu g\ ml^{-1}$
kanamycin	5 $\mu g\ ml^{-1}$

The effectiveness of this mixture was investigated at three levels using various periods of incubation and two bacterial growth stimulants, marine nutrient broth and ZoBell's medium. The results of the preliminary contamination tests using marine nutrient agar are summarized in Table A.2. The x10 BAM proved sufficient to eliminate the culture bacteria when nutrient broth was included as the growth stimulant. This level of antibiotics was less effective when co-incubated with ZoBell's medium, which probably reflected the lower bacterial growth rate in these treatments. At the x100 concentration, the antibiotic mixture was always lethal to both bacteria and algae. Generally the concentration of antibiotics appeared to be more important than the duration of incubation and it is probable that the antibiotics became inactive after about two days in solution. Plating onto ZoBell's agar revealed that all the viable cultures which

Table A.2. Effectiveness of mixtures of antibiotics against culture bacteria. Effect of various levels of the basic antibiotic mixture (BAM) on culture bacteria and *Glenodinium foliaceum*.

growth stimulant	level of BAM	no. of days incubation			
		1	2	6	16
nutrient	x1	+	+	+	+X
broth	x10	·	·	(+)	·
(NB)	x100	X	X	X	X
ZoBell's	x1	+	+	+	+
medium	x10	+	(+)	·	X
(Z)	x100	X	X	X	X

Explanation of symbols: +, high level of bacterial contamination on marine nutrient agar; (+), low level of bacterial contamination (less than 50 colonies per plate); X, treatments which were algicidal or from which no viable cultures were produced (note: the algae in the x1.NB.16 treatment were killed by excessive bacterial growth). Absence of a symbol indicates treatments which produced viable cultures that were free from bacteria detectable on nutrient agar.

showed negative on marine nutrient agar were contaminated with marine bacteria, although the level of contamination was quite low and was often only detectable after microscopic examination of the plates. Repeating the antibiotic treatment on these cultures using the x10 BAM, but this time substituting glucose plus proteose peptone as the bacterial growth stimulant, further reduced the level of marine bacterial contamination to less than 500 bacteria ml^{-1}.

In an effort to increase the growth rate of the marine bacteria during antibiotic treatments, an attempt was made to elevate the culture temperature during incubation. *Glenodinium foliaceum* could survive, in the dark, for two days at $30^{o}C$ but higher temperatures were lethal. However, after one of the purified cultures was treated with the x10 BAM at $30^{o}C$, using nutrient broth growth stimulation, the level of contamination on ZoBell's agar was found to have increased substantially. This suggested that the marine bacteria remaining in the purified cultures were largely resistant to the basic antibiotic mixture and that a more complex mixture would have to be employed.

Accordingly, bacitracin and neomycin were included in the x10 BAM at once and five times their MIC's respectively. These levels were chosen so as to make the antibiotic concentrations similar to those of a mixture used by Morrill and Loeblich (1979). These workers were apparently successful in purifying *Glenodinium foliaceum* but their method of testing for bacterial contamination was questionable. The composition of the Morrill and Loeblich mixture is compared with the modified x10 BAM in Table A.3. When applied to a culture in the usual manner, using glucose and proteose peptone to stimulate bacterial growth, this mixture reduced the number of bacteria present to about 350 cells ml^{-1} after 2 days incubation, as estimated from the number of microscopic colonies detected on ZoBell's agar. This corresponded to a bacteria:algae ratio of approximately 1:100.

Table A.3. Composition of antibiotic mixtures. Concentrations of antibiotics used by Morrill and Loeblich (1979) and in the present study to purify _Glenodinium foliaceum_ cultures.

antibiotic	concentration ($\mu g\ ml^{-1}$)	
	Morrill & Loeblich	modified x10 BAM
penicillin G	125	500
ampicillin	73	200
cephalosporine C	50	100
streptomycin	25	50
kanamycin	25	50
bacitracin	40	50
neomycin	25	25

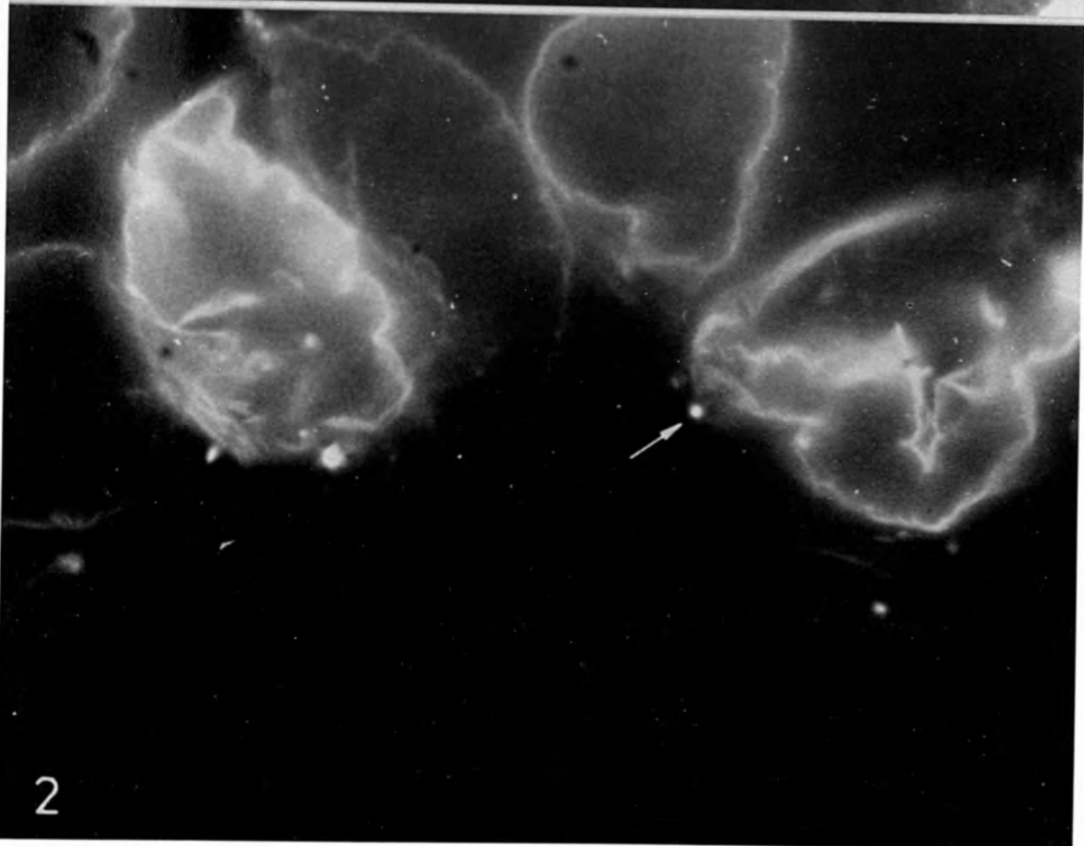
The appearance of this culture when stained with DAPI, before and after treatment with the antibiotics, is illustrated in Plate A.1. Most of the contamination in the original culture was due to a large rod-shaped bacterium but this was completely eliminated by the antibiotic treatment. The only non-algal DAPI-DNA fluorescence observable in the purified culture were small isolated points of fluorescence, usually associated with thecal debris, which presumably corresponded to small coccoid bacteria. This culture was used in all biochemical work.

Plate A.1. Comparison of the degree of bacterial contamination in purified and unpurified Glenodinium foliaceum cultures. Epifluorescence micrographs of cultures stained directly with 0.5 $\mu g\ ml^{-1}$ DAPI.

1. Numerous large rod-shaped bacteria associated with thecal debris from the original untreated culture. x 3,000.

2. Thecal debris from a culture treated with the modified x10 BAM showing only a few points of DAPI-DNA fluorescence (arrowed) probably corresponding to coccoid bacteria. Note the non-specific DAPI fluorescence from the thecae. x 3,000.

Plate A.1



A.3.3. Further considerations on the production of pure cultures of *Glenodinium foliaceum*

The short term viability of *G. foliaceum* cultures that showed very low levels of bacterial contamination after antibiotic treatment was consistently noted during the course of numerous purification attempts. This tends to suggest that there are deficiencies in the culture media employed and that the algae might be dependent on essential growth substances produced by the bacteria. Consequently it would be impossible to produce axenic cultures until these factors are artificially substituted.

The occurrence of intracellular bacteria in *Glenodinium foliaceum* and other dinoflagellates has been reported (Gold and Pollinger, 1971; Silva, 1978; Silva, unpublished observations). One might postulate, therefore, that there could be some interdependence between the two symbionts, so explaining the correlated death of algae and bacteria. However the strain of *G. foliaceum* used in this study seemed to lack any observable bacterial symbionts, as evidenced by DAPI staining and electron microscopy, so this possibility must be disregarded.

Considering our current ignorance on dinoflagellate nutrition, it could prove as difficult to get most autotrophic species into axenic culture as it is to grow heterotrophic types in laboratory culture media.

A.3.4. Isolation of DNA from non-axenic cultures.

In order to minimize contamination of *Glenodinium foliaceum* DNA preparations with bacterial DNA, the following precautions were observed:

a. Stock cultures were treated with the x10 BAM every other subculture.

b. When possible, cultures were harvested by a very brief, slow speed centrifugation. Well over half the bacteria present could be removed in this manner.

c. If appropriate, cell breakage techniques were employed which were unlikely to lyse the bacteria.

d. DNA preparations for genetic analysis were either obtained from isolated organelles or from DNA fractionated on CsCl gradients.

It is not known whether the repeated antibiotic treatments had any long term effects on the alga, but for the purposes of this study such considerations are largely irrelevant. The organism remained a viable biological entity and so, even if structural alteration to the chloroplast DNA had occurred (Heizmann *et al*., 1982), its ability to function was unimpared.

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Few institutions can afford all the equipment for modern research and although I was generously alloted more than my fair share of departmental resources, some work had to be performed elsewhere. Thus, I am grateful to Dr. Ed Munn for making available the Agricultural and Food Research Council's flow cytometer at the Institute of Animal Physiology, Babraham, which was expertly operated by Nigel Miller. Also, Graham King of Birkbeck College, London, unselfishly let me use the equipment that he constructed for generating high resolution DNA melting curves. Just as essential as equipment is the experimental material and various people or institutions kindly provided cultures during the course of my research. These sources have already been acknowledged in the text but I would especially like to thank again Dr. David Sigee and Dr. Karl Tangen for the cultures of *Glenodinium foliaceum* and *Gymnodinium galatheanum* and Prof. John Ellis for the *rbc* S probe. I am also grateful for the advice

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STRUCTURAL ORGANIZATION OF PLASTID DNA IN TWO ANOMALOUSLY PIGMENTED DINOFLAGELLATES[1]

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ABSTRACT

The structural organization of DNA in the plastids of two anomalously pigmented dinoflagellates, Glenodinium foliaceum *Stein and* Gyrodinium aureolum *Hulburt, was determined using the DNA-specific fluorochrome DAPI and correlated with TEM observations. The plastids of* G. foliaceum *were found to possess both a peripheral DNA ring and isolated point nucleoids. This arrangement was shown to be similar to that of the diatom* Asterionella formosa *Hass. and may be characteristic of the Bacillariophyceae.* G. aureolum *exhibited a novel distribution of plastid DNA as one or two beaded bands, whereas the plastids of the similarly pigmented haptophyte,* Emiliania huxleyi *(Lohm.) Hay & Mohler, possessed scattered point nucleoids. These findings support the idea that* G. foliaceum *harbours an endosymbiotic diatom, but suggest that the plastids of* G. aureolum *and* E. huxleyi *are unrelated. The use of plastid DNA configuration as a phylogenetic marker is considered.*

Key index words: Glenodinium foliaceum; Gyrodinium aureolum; *plastids; DNA organization; DAPI; TEM*

A new generation of DNA-specific fluorochromes has enabled plastid DNA (plDNA) to be visualized with the light microscope (Coleman 1978, James and Jope 1978). Hitherto the three-dimensional configuration of DNA in this organelle could only be reconstructed from serial electron micrographs (Kowallik and Haberkorn 1971, Gibbs et al. 1974). Studies using one of these fluorochromes, 4′-6-diamidino-2-phenylindole (DAPI), have revealed two basic patterns of organization. A peripheral ring of

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DNA is present in the plastids of phaeophytes, diatoms (Coleman 1979, Kuroiwa et al. 1981) and xanthophytes (see Kuroiwa and Suzuki 1981) whilst in chlorophytes (Kuroiwa et al. 1982), haptophytes, eustigmatophytes, dinoflagellates (Coleman and Lewin 1983) and higher plants (James and Jope 1978, Kuroiwa et al. 1981, Possingham et al. 1983) the plDNA is localized as scattered nucleoids. Although the former organizational strategy may have arisen from the fusion of isolated nucleoids situated around the plastid periphery, an arrangement reported to occur in red algae (Kuroiwa et al. 1981), an intermediate organization of plDNA, in which both ring and point nucleoids are present, has not been observed.

The characteristic plastid carotenoid of most dinoflagellates is peridinin; however, in a few species this is replaced by fucoxanthin (Jeffrey et al. 1975). It is possible that the plastids of these species may have had a different evolutionary ancestry (Dodge 1983a) and are probably more closely related to the plastids of chrysophytes than to those of other dinoflagellates. The observation that amongst the algae containing fucoxanthin is to be found each of the two major patterns of plDNA organization suggested that it would be interesting to study similarly pigmented dinoflagellates. Here we examine the use of plDNA configuration as a criterion for assessing plastid relationships in anomalously pigmented dinoflagellates by determining the DAPI-plDNA fluorescence patterns in *Glenodinium foliaceum* and *Gyrodinium aureolum*. These two species were selected for study because the plastids of the former are known to belong to a chrysophyte-like endocytobiont (Jeffrey and Vesk 1976) whilst the pigmentation of *G. aureolum* closely resembles the haptophyte *Emiliania huxleyi* (Tangen and Björnland 1981). This knowledge allows suitable comparisons of plDNA configurations to be made.

MATERIALS AND METHODS

Algae and culture conditions. Gyrodinium aureolum and *Emiliania huxleyi* were obtained from the Marine Biological Association, Plymouth, and grown in a natural seawater medium (Erd-Schreiber). *Glenodinium foliaceum* was a gift from Dr. David Sigee and was cultured in modified ASP_7 (Morrill and Loeblich 1979). All cultures were maintained under continuous fluorescent light at 20° C. A sample of the diatom *Asterionella formosa* was provided by the Freshwater Biological Association, Windermere, and examined directly.

DAPI staining. Cells were collected by gentle centrifugation and resuspended in a small volume of Buffer S which contained 0.8 M sorbitol, 5 mM $MgCl_2$, 0.1% (w/v) bovine serum albumin (BSA), 1 mM mercaptoethanol, 1 mM EDTA, 10 mM Tris pH 7.3 (for *Asterionella* preparations the sorbitol concentration was reduced to 0.33 M). A small drop of the cell suspension was placed on a slide, mixed with a drop of Buffer S lacking BSA but containing 4% (v/v) glutaraldehyde and 1 $\mu g \cdot ml^{-1}$ DAPI (Boehringer Mannheim) and immediately squashed under a coverslip. The coverslip was ringed with clear nail varnish and the preparation left to stain in the dark for 30 min.

Fluorescence microscopy. Observations were made on a Leitz Dialux 20EB fluorescence microscope equipped with a 50 W high pressure mercury vapour lamp which provided incident light excitation via a Ploemopak fluorescence illuminator. The illuminator was fitted with a Leitz filter block A consisting of a 340–380 nm band pass excitation filter, a 400 nm short pass reflection filter (beam splitting mirror) and a 430 nm long pass suppression filter. With this filter combination, the red autofluorescence of chlorophyll could be observed as well as the blue-white DAPI fluorescence, which permitted the rapid location of isolated plastids and allowed an assessment of their intactness to be made. Damaged plastids were ignored. A supplementary suppression filter was not employed to eliminate autofluorescence in detailed observations because this would have reduced the overall light transmission and would not have resulted in a significant increase in resolution (E. Leitz Ltd., personal communication). By means of an England Finder (Graticules Ltd.), specimens were relocated under an Olympus BH-2 Nomarski Interference Contrast (NIC) microscope to provide correlated fluorescence and NIC micrographs. Photographs of fluorescence were taken on Ilford XP1 film.

Nuclease digestion. Some preparations of *Glenodinium foliaceum* and *Gyrodinium aureolum* were subsequently digested with either DNase or RNase. Alternatively, digestions were performed on unstained squashes prepared exactly as described above by omitting DAPI from the fixative solution. After carefully removing the coverslip the slides were flooded with ethanol/acetic acid (3:1 v/v) for 30 min and allowed to dry. The slides were then washed extensively over a period of 1.5–2 h in 5 mM $NgCl_2$, 20 mM sodium citrate pH 6 or 2 mM sodium acetate pH 5.5 respectively, depending on whether DNase or RNase digestions were to follow. Digestions were carried out for 3 h using 350 units$\cdot ml^{-1}$ bovine pancreatic DNase (Sigma, Type III) or 45 units$\cdot ml^{-1}$ bovine pancreatic RNase (Sigma, Type XII-A) dissolved in the appropriate buffer. Controls lacking enzyme were run in parallel. Preparations were restained for 15 min with 0.5 $\mu g \cdot ml^{-1}$ DAPI in McIlvaine's buffer pH 4 after a brief rinse in buffer.

Transmission electron microscopy (TEM). For ultrastructural observations on *Glenodinium foliaceum*, cells were collected by sedimentation onto a Nuclepore Filter (pore size 8 μm) and fixed for 1 h with 4% (v/v) glutaraldehyde in 0.125 M Sörensen's phosphate buffer pH 7.7 made to an osmolarity of 1000 mOs with sucrose, and post fixed for 1 h with 2% (w/v) OsO_4 in Sörensen's buffer. After dehydrating through an acetone series, the cells were embedded in Spurr's low viscosity resin. *Gyrodinium aureolum* was collected by slow speed centrifugation and fixed for 15 min with a mixture of 2% glutaraldehyde and 1% OsO_4 buffered at pH 7.5 with 0.125 M Sörensen's phosphate which had been made isosmotic with the culture medium (i.e. 960 mOs) by the addition of sodium chloride. The final osmolarity of the fixative mixture was 1200 mOs. Post fixation was for 1 h with 1% OsO_4 in the high osmolarity buffer. Dehydrated cells were embedded in VCD/HXSA ultra-low viscosity resin (Oliveira et al. 1983) to give better preservation of the vacuolate cytoplasm. Ultrathin sections of both preparations were stained for various periods with 0.5% (w/v) aqueous uranyl acetate at 65° C and then Reynold's lead citrate before examination with a Zeiss EM9A electron microscope.

RESULTS

Under incident ultra-violet illumination, areas of intense blue-white DAPI fluorescence could be observed within all the plastids of squashed cells of *Glenodinium foliaceum*. The simple squashing technique described here was found to be the most acceptable method for observing plDNA in this alga. With more elaborate means of preparation in which fresh squashes were frozen in liquid nitrogen and then fixed in ethanol/acetic acid (3:1) after removal

of the coverslip (Coleman 1979), problems were encountered with spread strands of nuclear DNA adhering to the plastids. By mixing cells with glutaraldehyde immediately before squashing, a far superior degree of subcellular integrity and preservation was obtained. Whilst, as a result, the emission of chlorophyll autofluorescence from the plastids was high and may have obscured finer detail in the DAPI fluorescence (e.g. internucleoid connections), the major pattern of plDNA organization was readily apparent.

The majority of *Glenodinium foliaceum* cells possessed plastids in which the DNA was arranged as a beaded peripheral ring enclosing scattered unconnected nucleoids (Fig. 1). The number of these point nucleoids was variable, generally being less than 10 per plastid, but frequently as many as 20 or more were present (Fig. 2). In some plastids point nucleoids were lacking and just the DNA ring was present (Fig. 3). Conversely, a few cells were observed in which all the plastids contained only point nucleoids (Fig. 4). The beaded DNA ring of typical plastids, although sometimes fragmented, was observed in all orientations and often had one more intense point of fluorescence along its length. DAPI fluorescence from the plastids was sensitive to DNase but remained after treatment with RNase and in the buffer controls.

With the electron microscope, possible DNA-containing regions of the plastid stroma could be distinguished as electron translucent areas scattered amongst the lamellae particularly where these looped back upon themselves (Fig. 5). Despite searching through a number of plastid profiles a complete peripheral ring of DNA was never seen, although DNA areas beneath the girdle lamella at each end of the plastid were frequently observed. The plastids of *G. foliaceum* were found to be in close association with numerous mitochondria (Fig. 6).

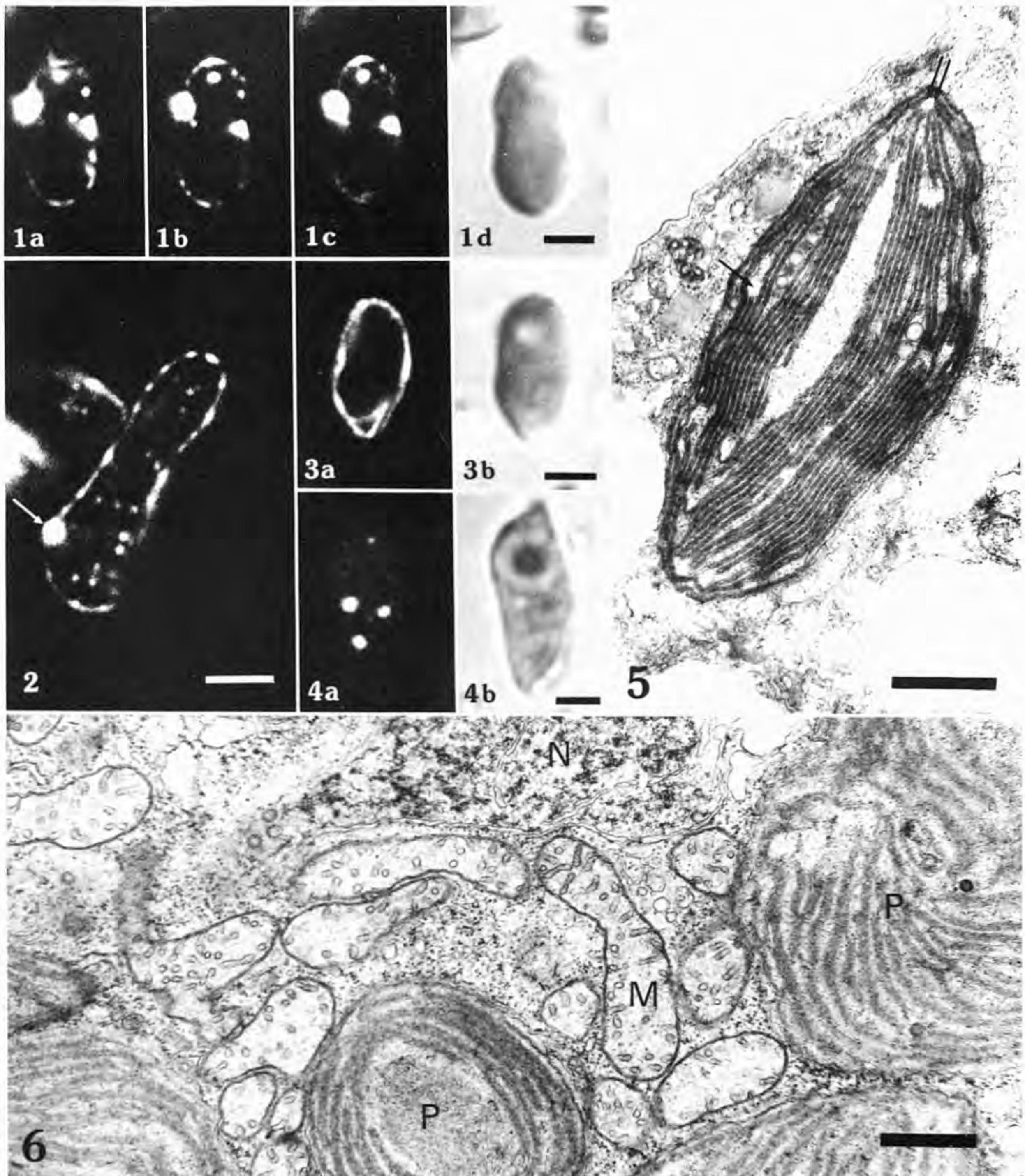
The plastids of *Asterionella formosa* could not be released from the frustule by squashing without causing severe damage to them. However, in slightly flattened colonies rings of DAPI fluorescence corresponding to the positions of the plastids were visible within the cells (Fig. 7). Points of fluorescence could also be resolved although the exact location of these was not clear. Whilst they generally occurred inside the bounds of the DNA rings, a few were seen in other parts of the cell.

Gyrodinium aureolum contained a relatively large amount of plDNA as estimated from the intensity of DAPI fluorescence. This was reduced by subsequent DNase digestion of the preparations and no DAPI fluorescence was emitted from the plastids after digestion of previously unstained squashes, although it was present in controls, showing it to be entirely due to DNA. The DNA was consistently organized as one or more beaded bands confined to one side of the plastid (Figs. 8, 9). The coalescence of two bands occasionally gave the DNA area a plate-like appearance in surface view. This asymmetrical distribution was partly confirmed by ultrastructural observations where a thylakoid-free region of the plastid was always found to be situated adjacent to the external stalked pyrenoid (Fig. 10) and was probably the location of the plastid nucleoid. The arrangement of plDNA in *G. aureolum* contrasted with that of *Emiliania huxleyi* where DAPI staining revealed scattered point nucleoids in the plastid lobes surrounding the nucleus (Figs. 11, 12). The small size of this species made detailed recording of the plDNA configuration difficult.

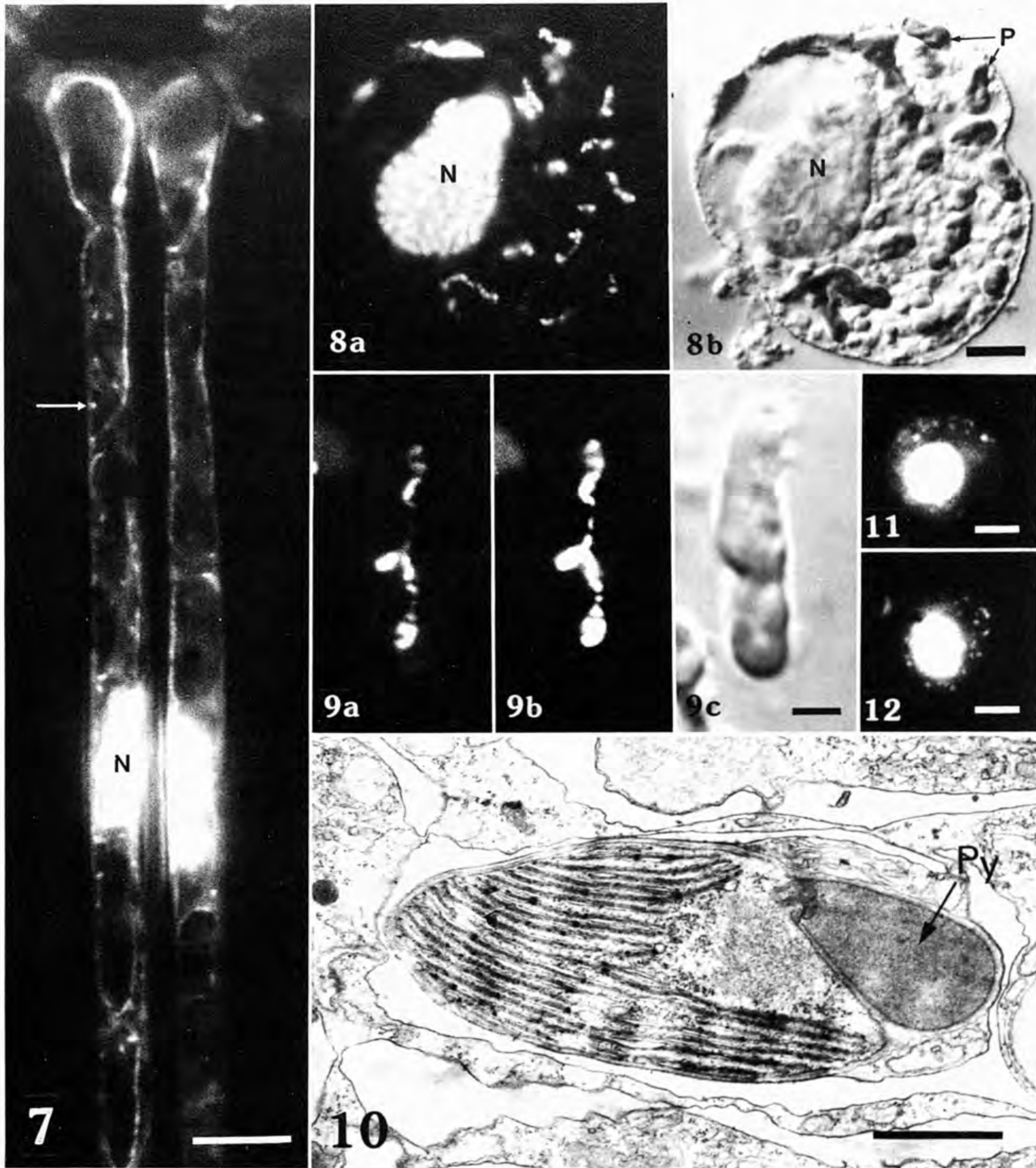
DISCUSSION

The presence of peripheral rings of DNA within the plastids of *Glenodinium foliaceum* provides further evidence that they belong to an endocytobiont of chrysophyte affinities (Jeffrey and Vesk 1976) since such an arrangement is lacking from typical dinoflagellates whose plastids contain only scattered nucleoids (Kowallik and Haberkorn 1971, Coleman and Lewin 1983, and personal observations on eight species covering six genera). However, with the possible exception of the diatom *Ditylum* (Coleman 1979), internal point nucleoids have not previously been detected by DAPI staining in algae which show circular plastid genophores (Coleman 1979, Kuroiwa et al. 1981, Kuroiwa and Suzuki 1981). The plastids of *G. foliaceum* are in close association with mitochondria (Fig. 6) within a membrane-bound cytoplasmic compartment—the endocytobiont—also containing the "eukaryotic nucleus" and dictyosomes (Kite and Dodge 1984). Therefore it might be argued that the point nucleoids observed originated from mitochondrial DNA as suggested for *Ditylum* (Coleman 1979), but since some nucleoids were observed in the same plane of focus as the peripheral DNA ring and were also detectable in electron micrographs of sectioned plastids (Fig. 5), this seems unlikely.

On the basis of ultrastructural observations (Jeffrey and Vesk 1976) and pigment analysis (Mandelli 1968, Withers and Haxo 1975), Dodge proposed that the symbiont of *G. foliaceum* was a diatom (Dodge 1983b). It therefore seemed appropriate to re-examine the plDNA configuration of diatoms. Plastids of the pennate diatom *Asterionella formosa* appeared to exhibit a similar pattern of DAPI fluorescence to those of *G. foliaceum*, although the possibility of mitochondrial DNA interference cannot be eliminated here because of the failure to isolate intact plastids from the cells. The centric diatom *Stephanodiscus* may also possess point plDNA nucleoids as well as the DNA ring (Kite, personal observations) and in the electron micrographs of Manton and Von Stosch (1966) scattered areas of DNA can clearly be seen within the plastids of the male gametes of *Lithodesmium*. A wider range of species would have to be examined before any definite conclusions can be drawn, but it does seem that the endocytobiont of



FIGS. 1–6. *Glenodinium foliaceum.* FIGS. 1–4. Epifluorescent and Nomarski Interference Contrast (NIC) photomicrographs of isolated plastids stained in DAPI. FIG. 1. a–c, Three planes of focus through the DAPI fluorescence of a typical plastid showing both a peripheral ring of DNA and isolated point nucleoids; d, the same plastid viewed under NIC. FIG. 2. Epifluorescent micrograph of a plastid with numerous internal point nucleoids; note the beaded ring of DNA shows one more intense point of fluorescence (arrowed). FIG. 3. Epifluorescent (a) and NIC (b) micrographs of a plastid lacking point nucleoids. FIG. 4a, b. Similar correlated micrographs of a plastid lacking a DNA ring. All scale bars = 2 μm. Correlated micrographs are reproduced at approximately the same magnification. FIG. 5. TEM of a plastid with lightly staining regions characteristic of DNA scattered amongst the lamellae (single arrow) and situated beneath the girdle lamella at each end of the plastid (double arrow). Scale bar = 1 μm. FIG. 6. Ultrastructure of part of the endocytobiont showing the "eukaryotic" nucleus (N) and plastids (P) closely associated with mitochondria (M). Scale bar = 1 μm.



FIGS. 7–12. FIG. 7. Two cells of *Asterionella formosa* stained with DAPI and viewed under epillumination showing the fluorescent rings of plDNA either side of the nuclei (N). Note also the isolated points of fluorescence (arrowed). Scale bar = 5 μm. FIGS. 8–10. *Gyrodinium aureolum*. FIG. 8. Whole cell slightly squashed and stained in DAPI showing numerous bands of fluorescence in the cytoplasm (a) correlated with the location of the plastids (P) in the NIC micrograph (b). Nucleus (N). Scale bar = 5 μm (the magnification is the same for both micrographs). FIG. 9. Two planes of focus (a & b) through the single beaded-band of DAPI-DNA fluorescence in an isolated plastid, also shown at the same magnification under NIC (c). Scale bar = 2 μm. FIG. 10. TEM of a plastid sectioned transversely showing the unusual stalked pyrenoid (Py) adjacent to an area of plastid stroma which lacks thylakoids. Note the vesiculate cytoplasm. Scale bar = 1 μm. FIGS. 11 & 12. Epifluorescent micrographs of whole cells of *Emiliania huxleyi* stained in DAPI showing scattered point nucleoids of plDNA surrounding the nucleus. Scale bar = 2 μm.

G. foliaceum and diatoms show a similar organization of plDNA which is distinct from that of other algal groups. Using the terminology of Kuroiwa et al. (1981), their plastids show a combination of both SN- and CL-type distributional patterns of DNA, having both small scattered nucleoids (SN) and a circular nucleoid associated with a girdle lamella (CL).

The major carotenoid of *Gyrodinium aureolum* is 19'-hexanoyloxyfucoxanthin (Tangen and Björnland 1981); a fucoxanthin-related pigment which has only been reported in one other alga, the haptophyte *Emiliania huxleyi* (Arpin et al. 1976). Other haptophytes possess fucoxanthin as their major carotenoid (Berger et al. 1977). In acetocarmine stained *G. aureolum* only one nucleus was detectable (Tangen and Björnland 1981) whereas two nuclei are seen in *Glenodinium foliaceum* (Dodge 1971). The presence of only a single nucleus in *Gyrodinium aureolum* is confirmed here with DAPI staining (Fig. 8) thereby excluding the possibility of the cell harbouring a recognizable endocytobiont. Nevertheless it has been proposed that the plastids of *G. aureolum* and *E. huxleyi* may be related (Tangen and Björnland 1981), presumably by some past endosymbiotic event (Dodge 1979, Whatley and Whatley 1981). However the present work shows that the plDNA configuration of these two organisms is entirely different. *Emiliania huxleyi* resembles other haptophytes in having scattered point nucleoids of DNA whilst *G. aureolum*, with its beaded bands of plDNA, exhibits an arrangement which is possibly unique.

It is debatable whether the organization of DNA in plastids would remain unaltered during their evolution. In *Glenodinium foliaceum* one observes a variation in the arrangement of plDNA between plastids, such as the fragmentation or the complete absence of the DNA ring. This may reflect ultrastructural changes during the cell cycle, or a drift in the organization of plDNA from that found in the plastids of free-living diatoms as a consequence of endosymbiosis. The latter possibility makes it difficult to draw any firm phylogenetic conclusions from the different plDNA configurations shown by *Gyrodinium aureolum* and *Emiliania huxleyi*. More information about the factors controlling plDNA organization is required in order to do this. It is pertinent though to note that the distribution of DNA in the cyanelles of *Cyanophora paradoxa* (Bohnert et al. 1983) resembles the central nucleoid of cyanobacteria (Tschermak-Woess and Schöller 1982, Coleman and Lewin 1983) in being restricted to a similar region in the organelle. Cyanelles and cyanobacteria are thought to be related (Trench 1979) and the organization of cyanelle DNA does not seem to have changed during its integration with the host cell (Jaynes and Vernon 1982).

Further information on the usefulness of plDNA organization as a phylogenetic marker should come from the study of other anomalously pigmented dinoflagellates (Jeffrey et al. 1975, Björnland and Tangen 1979). Some of these are morphologically related to species possessing peridinin as their main carotenoid (Tangen and Björnland 1981) and so comparisons of DAPI-plDNA fluorescence patterns could be made both with algae having the same pigmentation as the anomalous species and a closely related, but typical, dinoflagellate.

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