STUDIES ON THE STRUCTURE AND FUNCTION OF CODIUM FRAGILE CHLOROPLASTS

by

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DECLARATIONS

1) The observations presented in this study are, except where otherwise stated, entirely the work of the author.

2) The author has not received, and has not been registered for any other higher degree awarded by a University or the Council for National Academic Awards during the course of this work nor has any part of it been previously submitted in partial fulfilment for the award of a higher degree.

3) The author has attended conferences and programmes of study relevant to the present study.

4) Due acknowledgments have been made for the assistance given during the course of this work and in the presentation of the thesis on which it is based.

Signed .....
(Candidate)

Signed Dr. Andrew H. Cobb
(Director of Studies)

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ABSTRACT

Studies on C. fragile chloroplasts show that this alga is well adapted to its intertidal zone habitat. Photosynthesis in isolated chloroplasts saturates at low light intensities and their pigment composition confers an environmental adaptation to submarine light fields. Chlorophyll a:b ratios are low in this alga and an average of 75% of the total pigment content of the chloroplast pigment/proteins are located in the light-harvesting complexes. These complexes are enriched in chlorophyll b and siphonein and siphonoxanthin which absorb in the "green gap" region of the visible spectrum. The pigment composition of C. fragile is particularly unique as β-carotene is absent in this alga, and α - and ϵ - carotene are the only carotene isomers detected.

Although photosynthesis in C. fragile is adapted to low light intensities, photostability studies show this alga to be particularly stable when exposed to high non-fluorescent light regimes typical of those encountered at low tide. Photooxidative symptoms in C. fragile were only induced on exposure to environmentally atypical fluorescent light. These studies indicate that photooxidation in C. fragile is atypical of that in other plants as chlorophyll is preferentially degraded compared to the carotenoid pigments and ethylene is the major photooxidation product. The absence of β-carotene may suggest that photoprotective mechanisms in this alga are also different.

A study of the growth of C. fragile in the intertidal zone at Bembridge, shows that the life cycle of this alga also confers an adaptive advantage. The alga maximises nutrient and carbon assimilation during the winter and this is evidenced by increased pigment content, vegetative growth and photosynthetic rates. In the summer when competition from faster growing algae and epiphytic attack occurs C. fragile enters the reproductive phase of the life cycle which is presumably supported by the mobilization of winter-stored nutrients. Thus, the alga employs a strategic survival mechanism in which the timing and not extent of vegetative growth is the important factor.

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PUBLICATIONS

Data from Chapter 2 has been published as follows:-

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COBB, A. H. and BENSON, E. E. (1981) Photosynthetic adaptations of the intertidal zone marine alga C. fragile to light. pp. 429-433. in Photosynthesis and Productivity, Photosynthesis and Environment. Edited by G. Akoyunoglou. Proceedings of the Fifth International Photosynthesis Congress, Halkidiki, Greece (1980).

The findings of Chapters 3-7 are currently being prepared for publication.

CONTENTS

1. INTRODUCTION

Codium fragile is a siphonaceous marine green alga, noted for its stable chloroplasts and their symbiosis with the sacoglossan mollusc *Elysia viridis* (Trench, 1975). This section reviews the morphological and physiological characteristics of this alga, together with a brief account of its distribution and reproduction.

1.1 THE EVOLUTION, MORPHOLOGY AND DISTRIBUTION OF CODIUM FRAGILE (SURINGAR) HARIOT

Throughout this thesis the classification of *C. fragile* will be according to Fritsch (1977), where the siphonaceous order is the Siphonales of which the Codiaceae is a family.

The evolutionary line of the Siphonales represents a coenocytic condition probably evolved from a non-motile cell increasing in size and in nucleus and chloroplast number, with no accompanying cell division (Lee, 1980), and this is known as the siphonaceous habit. This condition has led to the development of several morphological structures to compensate for support which would have been provided by the septa. Such structures are particularly evident in the Codiaceae in which most species show a compact thallus composed of closely interwoven coenocytic threads. These are quite complex in *Codium sp* (Figure 1), and the structure formed is analogous to the palisade layer of higher plants. The coenocytic threads or siphons are vertically aligned around a medullary region, and the 'palisade' layer is composed of the dilated tips or utricles of these coenocytic filaments (Figure 2). Whilst reproduction in the Codiaceae is poorly documented, Lee (1980) presents a general account of sexual reproduction in *Codium*, in which gametangia are produced from the utricles of the diploid thallus, and this development in *C. fragile* is shown in Figure 3. The female reproductive structures are dark green and the male brown. The gametes are formed meiotically and thus constitute the only haploid structure in the life cycle. The gametes are extruded from the gametangia by means of a central canal surrounded by gelatinous material. Initially the gametes are non-motile, and lack flagellae, however, soon after release flagellae are developed in both gametes. On fertilisation the male flagellum is lost and the female flagellum is used for zygote

FIGURE 1:- THALLUS ANATOMY OF C.FRAGILE FRONDS

KEY :-

a = L.S × ~30

b = L.S × 400

▶ = Interwoven coenocytic threads

▷ = Utricles [siphons]


a

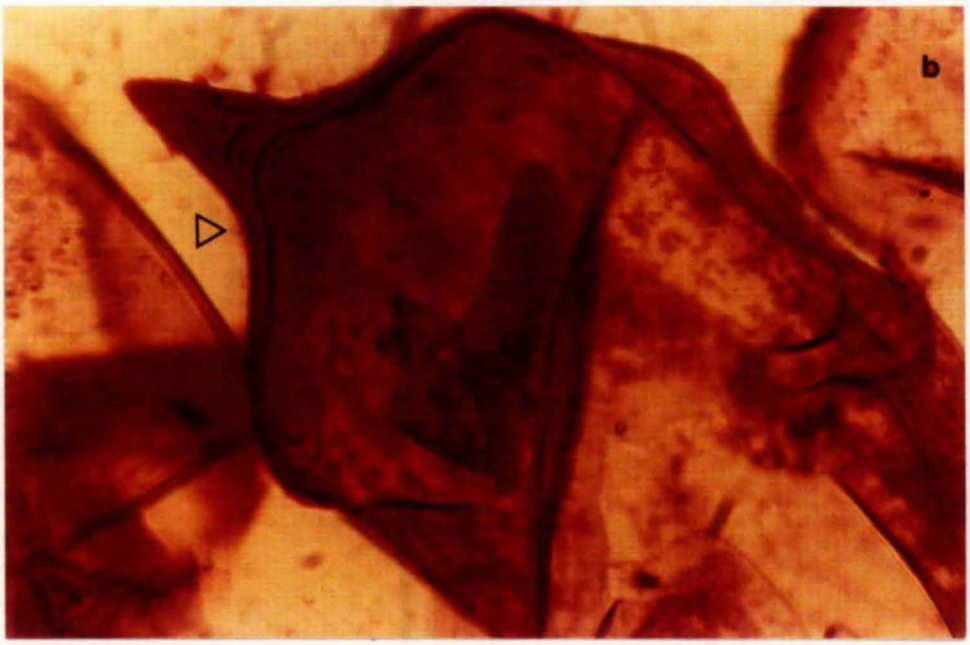

b

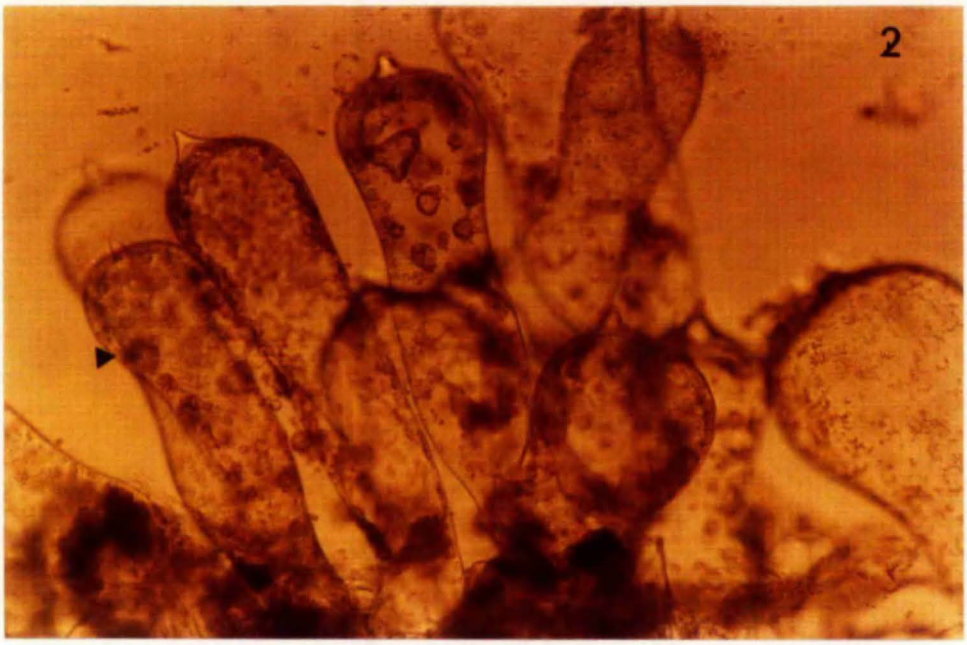
FIGURE 2 :- C.FRAGILE UTRICLES (× 100).

FIGURE 3 :- DEVELOPMENT OF FEMALE GAMETANGIA FROM C.FRAGILE UTRICLES (× 400).

KEY :-

▶ Utricles

▷ Developing gametangium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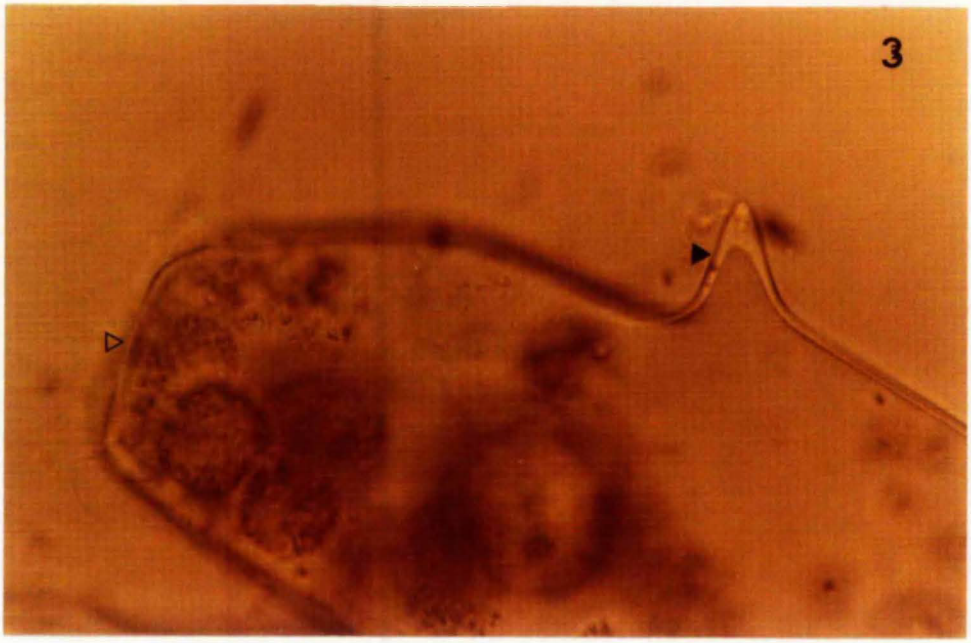
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propulsion. After settling on a suitable substrate the flagellum retracts and the zygote germinates into a new Codium thallus.

The distribution of the Codium genus is almost global, and Codium spp may occur from the marine intertidal zone to a depth of 70m in tropical waters (Lee, 1980). The only report which suggests that Codium spp is harmful to other marine life is by Ramus (1971), who describes the dense colonization of C. fragile along the N. American coast line, the alga apparently causing disruption of the extensive shellfish beds in this area. Colonies of C. fragile found in the British Isles have a preference for sheltered, moderate environments, where the alga occupies the intertidal zone. Such an environment is found along the eastern coastline of the Isle of Wight at Bembridge, where one of the few abundant English colonies of C. fragile is found.

1.2 THE CHLOROPLASTS OF CODIUM FRAGILE

1.2.1 Chloroplast structure

Algal chloroplasts can vary considerably with respect to morphology, number and distribution within cells, and because of this diversity they are important characters in algal classification (Gibbs, 1970; Dodge, 1973). A detailed ultrastructural account of C. fragile is given by Hawes (1979) who observed the chloroplasts in the cytoplasm lining the utricular walls of the fronds. They are elipsoid to ovoid in shape and approximately 3μm in length. This size is relatively small when compared to higher plant chloroplasts which can range from 4-10μm in length (Tribe and Whittaker, 1974). The photosynthetic membranes of C. fragile are less elaborate than those of higher plants and consist of bi- or trithylakoids, the stroma also contains starch grains, ribosomes and plastoglobuli (Hawes, 1979).

Hedberg, Huang and Hammersand (1981) isolated and purified chloroplast DNA from C. fragile, and found it to exist as covalently closed molecules with an average contour length of 27.3μm and a molecular size of 56×10^6 daltons. This size is 25-30% less than any yet described for chloroplast DNA, and these authors speculate that this may be due to one of two evolutionary developments. The first proposes that the genome represents an advanced condition in

which DNA has been lost or transferred to the nuclear genome. Conversely, the second suggests a primitive condition exists in the DNA in which additional sequences have not been inserted into the chloroplast genome.

1.2.2 Carbon fixation characteristics

Using a chloroplast extraction method originally designed for Acetabularia spp photosynthetic rates of 22.5μmoles CO_2.mgChl^{-1}.hr^{-1} at 21°C were obtained for C. fragile chloroplasts by Trench, Boyle and Smith (1973,a). A comparison of $^{14}CO_2$ fixation patterns between fronds and isolated chloroplasts showed abundant incorporation of label into sucrose in the fronds and glycolate in the chloroplasts. However, as these authors used relatively high light intensities in their chloroplast studies (40-80 W.M^{-2}), glycolate production may have been principally due to photorespiratory activity. Hinde (1978) refined the above extraction method and found isolated chloroplasts to release $\ll 6\%$ of their fixed carbon to the medium over a period of 24 hours and during this time glucose monophosphate and glycolate were the only labelled compounds released.

The chloroplast extraction methods of the above authors, produced impure chloroplast extracts containing tonoplast-bound cytoplasmic vesicles. Cobb (1977) devised a method by which C. fragile chloroplasts could be purified by filtration through a Sephadex G-50 (coarse) column. Using this method Cobb and Rott (1978) found that C. fragile chloroplasts achieved maximum photosynthetic rates of 40-60μm CO_2 mg.Chl^{-1}.hr^{-1} at 20°C. Subsequent investigations by these authors found the chloroplasts to possess two photosynthetic adaptations to their marine environment:- a high osmotic requirement (0.8M sucrose), and a low saturating light intensity for photosynthesis (approximately 25.W.M.$^{-2}$) when compared to higher plants (Jensen and Bassham, 1966). Cobb and Rott (1978) also found seasonal variations in extracted chloroplasts since fronds sampled in November gave maximum chloroplast photosynthetic rates of 62μM CO_2.mgChl^{-1}.hr^{-1}, whereas in June they gave rates of 21.0 ± 9.5μM CO_2.mgChl^{-1}.hr^{-1}. Regardless of these seasonal effects C. fragile

chloroplasts showed a consistent lack of lag phase at the onset of CO_2 fixation even when the algal fronds were stored in the dark for 3 days prior to chloroplast isolation. The enzyme ribulose bisphosphate carboxylase/oxygenase however, showed activation and inhibition characteristics similar to those of higher plants (Cobb and Rott, 1978).

Cobb (1978) has shown that C. fragile chloroplasts are able to store an internal resevoir of Pi in the form of long-chain inorganic polyphosphate. Since the habitat of this marine alga can be deficient in Pi this phenomenon can be regarded as an environmental adaptation. Recent studies by Rutter (1982) have shown that the import of Pi may not be an immediate pre-requisite for photosynthesis in C. fragile since internal polyphosphate is already present in the chloroplast. The availability of this phosphate varies with age, and physiological state of the tissue, and thus the affects of external phosphate on carbon fixation also varies. Recent studies on ^{32}Pi translocation in this laboratory (Rutter 1982) also suggest that C. fragile may have a unique phosphate transport system operating across the chloroplast envelope. Two translocators appear to be in operation, the first, a light sensitive Pi/triose phosphate translocator that may be inhibited by p-chloromercuriphenylsulphonate, and a novel light-independent Pi/G6P translocator insensitive to this inhibitor. In view of these developments it is feasible that C. fragile chloroplasts may differ considerably in terms of metabolite function and transport when compared to their higher plant counterparts.

1.2.3 Chloroplast stability and symbiosis

In general Siphonaceous algae display inherent chloroplast stability when compared to higher plants (Halliwell, 1978). C. fragile chloroplasts have been shown to fix CO_2 up to five days after isolation (Trench et al, 1973, a) and display 40% intactness after dark storage at $4^{\circ}C$ for 4 days (Cobb and Rott, 1978). These workers also showed that C. fragile chloroplasts were able to survive osmotic shock and still fix CO_2 at 50-70% of their original photosynthetic rate.

The most outstanding demonstration of C. fragile chloroplast stability is shown in their symbiotic relationship with the Sacoglossan mollusc E. viridis (Figure 4). This animal has a specialized feeding apparatus consisting of a radula bearing a single row of teeth which are used to puncture the algal cells and suck out the contents into their digestive diverticula (Taylor, 1968). The chloroplasts are highly persistant in E. viridis and can remain functional for at least 3 months when starved in the light and at least one month when starved in the dark, (Hinde and Smith, 1972). Rates of CO_2 fixation in the animal and algal frond are of the same order (Trench et al, 1973, b), but there is a general change in chloroplast shape from elipsoidal in the plant to oval in the animal (Hawes and Cobb, 1980). Kremer (1976) suggests that the mollusc is dependent on C. fragile for photosynthetically derived intermediates which may supplement the Krebs cycle. Experiments by Hinde (1978) show that chloroplast glycolate may be a particularly important metabolite for E. viridis and Cobb (1978) suggests that the chloroplasts may also be an important source of inorganic phosphate.

The longevity of the relationship between C. fragile chloroplasts and E. viridis raises some pertinent questions as to the survival and autonomy of these organelles in such an alien environment. Investigations into the autonomy of the chloroplasts suggests they may be able to synthesize carotenes and xanthophylls, phospholipids and probably some proteins (Trench and Smith, 1970; Trench et al, 1973, b; Trench and Ohlhorst, 1976). However, their inability to synthesize major chloroplast components such as RuBPc/o suggests their survival in the slug must be due to the longevity of the chloroplast contents already present on removal from the algal frond.

The fate of the chloroplasts after prolonged symbiosis is not resolved, and since they escape destruction on ingestion by the slug, Taylor (1968) concluded that E. viridis does not possess enzyme systems which cause chloroplast autolysis. Hawes (1979)

FIGURE 4 : - ELYSIA VIRIDIS ON C. FRAGILE FRONDS

suggests that a closely bound host phagosome membrane protects the chloroplast, and that the prolonged robustness of the chloroplasts may be due to a tough outer envelope. An ultrastructural series of experiments by Hawes and Cobb (1980) have indicated that the degradation of chloroplasts in E. viridis is primarily light dependent and no evidence could be found of digestive enzyme action.

1.3 CHLOROPLAST PIGMENTS AND PIGMENT/PROTEINS

In all photosynthetic organisms the utilization of light-energy is dependent on their ability to absorb that part of the spectrum called photosynthetically active radiation (P.A.R.) which is usually within the range 400-700nm. Light absorption is mediated by the chlorophyll, carotenoid and phycobilin, pigments which function in two main photo-events. The first involves the harvesting of light energy and its transfer to the photoreaction centres, whilst the second involves the utilization of photoreaction centre light energy to initiate a photochemical event, the products of which are utilized in the fixation of CO_2. Light-harvesting pigments are usually chlorophyll b, the carotenoids and phycobilins, and reaction centre pigments are composed of several different types of chlorophyll a. The capture and transfer of light energy is controlled by many complex factors and transfer must take place quickly before the energy is dissipated as fluorescence or heat both of which may be damaging. For two dissimilar molecules the rate of transfer is determined by the distance between the molecules, their electronic orientation, and the degree to which their emission (donor) and absorption (acceptor) bands overlap, (Clayton, 1980). It is thus clearly evident that the organization of chloroplast pigments is highly crucial to the success of the light-harvesting and photochemical functions and this is achieved by the highly organized arrangement of pigments in the chloroplast pigment/protein complexes.

The discovery that the photosynthetic light reactions take place in highly organized systems within the chloroplast has been extensively reviewed (Clayton, 1980; Halliwell, 1981). Emerson (1958) described how the photosynthetic efficiency of plants was increased by supplementing light of $\geqslant$ 685nm with light of 650nm. The total rate in the presence of

both light sources being greater than the sum of the rates for the component lights. This work led to the initial conclusion that two primary photo-events were involved in the light reactions of photosynthesis, and was later confirmed by Duysens (1965). Special types of chlorophyll a now known as P700 and P680 were characterized and these form the photochemical reaction centres of PSI and PSII (the photosystems) respectively. Hill and Bendall (1960) proposed that these two photosystems act in series and devised the first 'Z' scheme of the photosynthetic light reactions, this scheme although often updated is still in use today (Halliwell, 1981). After photoactivation electrons are ejected from P680, the reaction centre of PSII, the splitting of water then reduces $P680^{+}$. Electron transfer from P680 is not fully characterized but pheophytin is implicated (Klimov, Dolan and Ke, 1980) as the intermediate between P680 and the uncharacterised electron acceptor Q. From the latter a secondary acceptor termed 'B' or 'R' passes electrons to the plastoquinone pool (Velthuys, 1980). Transfer of electrons from the plastoquinone pool causes a phosphorylation event and ATP is produced. Electrons flow on through cytochrome f and plastocyanin, the latter being the immediate donor of P700 (Olsen, Cox, Barber, 1980). Absorption of light by P700 reaction centre chlorophyll also causes a photochemical event and ejected electrons are passed to an acceptor ($P700^{+}$ being reduced by plastocyanin). The primary electron acceptor of PSI is not fully characterized and it is now believed that several acceptors associated with iron/sulphur centres are involved (Malkin, 1982). From the primary P700 acceptor complex, electrons pass to ferredoxin which reduces $NADP^{+}$. The overall flow of electrons from water to $NADP^{+}$ is called non-cyclic electron transport. Electrons are able to bypass the production of NADPH as the acceptor complex of PSI can pass electrons to a special cytochrome (probably b563, although this has been disputed by Cox (1979). The electrons are transferred to plastoquinone, cytochrome f, plastocyanin and $P700^{+}$, this sequence of events is termed cyclic electron transport, and leads to the production of ATP but not NADPH (Arnon and Chain, 1979; Chain, 1979).

The 'Z' scheme has provided much of the background understanding of photosynthesis and its elucidation was largely dependent on the use of many varied and refined spectroscopic techniques. However, the more recent development of electrophoretic, centrifugation, and detergent-solubilization procedures has considerably aided the understanding of the photosynthetic light-reactions. This has mainly been achieved by physically separating chloroplast components using detergents and electrophoresis, and then examining their spectroscopic, protein, and pigment characteristics. These studies have led to the characterization of the so called pigment/protein complexes of thylakoid membranes.

Extraction of pigment/proteins requires the use of detergents to disrupt the chloroplast lipoprotein membranes. Of the wide variety used sodium dodecyl sulphate (SDS), Triton X-100, and digitonin are particularly important and have been used in conjunction with SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Several distinct pigment-containing bands are resolved on the gels, but unfortunately the terminology of these complexes is very varied, and in this thesis the nomenclature of Anderson (1980) is preferred. Thus, in order of least migration a typical separation of complexes using SDS-PAGE produces 6-7 bands termed:- CP_1a, CP_1, $LHCP_1$, $LHCP_2$, CPa, $LHCP_3$ and FP. CP_1a and CP_1 are proposed to contain reaction centres of PSI, $LHCP_1$, $_2$ and $_3$ are light-harvesting complexes, CPa is probably associated with PSII and FP is the free-pigment band which accumulates as pigments become dissociated from their proteins. A more detailed characterization of these complexes is now given:-

1.3.1 Chlorophyll/protein complex I (CP_1a-CP_1)

Browne, Alberte and Thornber (1975) suggest that the CP_1 protein which contains P-700, the reaction centre of PSI, is ubiquitous in eukaryotic photosynthetic organisms. The undissociated complex has variable migration patterns on SDS-PAGE and has a range of molecular weights of approximately 100KD. The number of peptides associated with CP_1 is not known, however, after chlorophyll removal the complex migrates as a single entity of molecular size 62-70KD (Hiller and Goodchild, 1981). Under extreme dissociating conditions two polypeptides of approximately 50KD are often

resolved (Boardman, Anderson, Goodchild, 1978). Typical absorbance spectra of CP_1a and CP_1 have red peaks at 678nm (Anderson, Barrett and Thorne, 1981). Chlorophyll a is the main pigment present, but β-carotene is also associated with PSI, where it appears to be an essential component of the reaction centre. Searle and Wessels (1978) have shown that removal of β-carotene from PSI causes reduction in the rate of NADPH production, and these authors suggest that β-carotene may decrease the rate of excitation energy transfer away from the reaction centre. Oquist, Samuelsson and Bishop (1980) proposed that β-carotene is important in stabilizing the far red absorbing populations of chlorophyll a in the P700 reaction centre as well as being an important photo-protectant, and excitation energy quencher, and an antenna pigment.

Although the organization of chlorophyll-proteins within the reaction centres of PSI is still rather controversial it has been established that the P700 centre is a dimeric form of chlorophyll a in which an unpaired electron is shared by two chlorophyll molecules (Katz, Shipman and Norris, 1979). However Wasielewski, et al (1981) now suggest a monomeric chlorophyll enol may be implicated as the reaction centre component. The organization of antennae chlorophyll proteins in PSI has been investigated by Mullet, Burke and Arntzen (1980), who, using mild detergent treatments, were able to isolate 'native PSI' a fraction which retained PSI in situ spectroscopic characteristics. They termed this fraction PSI-110 as the samples had a chlorophyll/P700 ratio of 110. On electrophoresis this complex was resolved as 11 bands of 16.5-24.5 KD and three low molecular weight proteins of 10.5, 11, 11.5 KD. Mullet, et al (1980) proposed a model for the organization of chlorophyll proteins derived from PSI-110:- The native complex contains a peripheral antennae of 40-45 chlorophyll/P700 (polypeptide molecular weight 21-24.5 KD), a core of 40 chlorophyll antennae/P700 (66-68 KD), and an internal antennae of 20-25 chlorophylls/P700. These authors also proposed that the peripheral antennae act as a sink for excitation energy absorbed by the chlorophyll antennae within PSI, thus promoting a concentration of excitation energy within PSI and facilitating energy

transfer to P700 chlorophyll.

1.3.2 The light-harvesting chlorophyll a/b complex (LHCPs)

The composition of LHCP complexes extracted from various plant sources can be quite different with regards to their pigment content, thus, it is usually the light-harvesting complexes of photosynthetic organisms which confer an adaptability to various light environments. Unlike CP_1 and CP_1a the LHCP complexes do not take part in the photochemical events of the reaction centres, their main function being to harvest light energy and transfer this to these reaction centres. On SDS-PAGE the LHCP complexes are usually the most intense bands resolved, however their detailed composition and subunit structure is still uncertain. The light-harvesting complexes are resolved as monomers ($LHCP_2$ or $LHCP_3$) and dimers ($LHCP_2$ or $LHCP_1$), and the lowest molecular weight protein of the monomer complex is 24-30 KD; on its dissociation two major peptides of 25 and 23 KD are sometimes resolved (Hiller and Goodchild, 1981). However subunit composition can be quite variable depending on plant species and method of isolation (Green and Camm, 1982). Hiller and Goodchild (1981) suggest the various types of peptides associated with LHCP may be modifications of a single original peptide of 29 KD, which is coded in the nucleus of most plants, this protein is thought to be a precursor of LHCP (Apel and Kloppstech, 1978,).

In most higher plants the LHCP's contain 40-60% of the total chlorophyll present in the chloroplast (Herrmann, Borner and Hagemann, 1980). The chlorophyll a:b ratio is approximately 1.3 for higher plants (Hiller and Goodchild, 1981), and large amounts of xanthophylls are usually associated with these complexes. Siefermann-Harms (1980) suggests that although traces of β-carotene are associated with the LHCPs, the xanthophyll pigments represent almost 25% of their total pigment content, and this is usually due to the presence of lutein and neoxanthin (Lichenthaler, Prenzel and Kuhn, 1981). Fluorescence excitation studies have shown that a wide range of carotenoids and chlorophyll b are able to transfer their light-harvested excitation energy to reaction centre chlorophyll a (Anderson, et al. 1981; Siefermann-Harms and Ninneman, 1982).

The latter authors have investigated the structure of LHCPs in higher plants, and suggest that the chlorophyll and carotenoid pigments are enfolded by lipophillic polypeptide chains which are located in deep crevices within the LHCP, this maintains a close structural relationship between the pigments.

1.3.3 Other pigment-protein complexes

Several bands are resolved by SDS-PAGE which as yet are not unequivocally identified, this is particularly the case with CPa. This complex is a constant component of higher plants, and has an absorbance maximum at 672nm, and a fluorescence at 685nm, the apparent molecular weight of the complex is 40-45 KD (Hiller and Goodchild, 1981). Delepaire and Chua (1979) have resolved CPa into two components and it is suggested by Machold, Simpson and Møller (1979) that one of these contains the reaction centre of PSII and the other is an inner antenna.

Using the solubilizing detergent octyl glucoside and SDS-PAGE, Camm and Green (1980) resolved a new chlorophyll a/b protein from spinach. These authors suggest this protein is present in most plant species and have characterized it as CP29 (molecular weight 29 KD). The protein is not associated with the LHCP complex and Green and Camm (1981) have proposed that CP29 is associated with PSII where it functions as an internal antenna. Oligomers of this complex have been resolved from Acetabularia cliftonii, and are particularly unusual in that they can be reformed by reassociation of the monomer during electrophoresis (Green, Camm and Van Houten, 1982).

1.3.4 Photosystem II pigment/protein (PSII)

The notable absence of PSII pigment/protein in this review, is due to the fact that this complex is not readily recovered using conventional SDS-PAGE techniques, and attempts to characterize the complex have been achieved by the use of different methods. Markwell, Miles, Boggs and Thornber (1979) isolated PSII active fractions from maize, barley and tobacco using the zwitterionic detergents Deriphat 160 and Miranol 52M-SF. Siefermann-Harms and Ninneman (1979) achieved similar success using Triton X-100 and

isoelectric focusing PAGE, which contained digitonin rather than SDS. These authors resolved 3-distinct complexes with PSII activity, all contained varying amounts of β-carotene, lutein, violaxanthin and neoxanthin. The complexes had chlorophyll a:b ratios of 2.3-4.4 and their absorption characteristics were very similar with a peak in the red region at 675nm. The high concentrations of xanthophylls associated with these PSII active complexes suggest that LHCP components were still associated with the photosystem. Larkum and Anderson (1982) using digitonin extracted PSII from spinach chloroplasts reconstituted purified complexes into artificial lipid membranes. These demonstrated PSII activity, had chlorophyll a:b ratios $>$ 20:1, and also contained β-carotene, and trace amounts of lutein. Using mild SDS-PAGE techniques these authors also resolved PSII as a green-band in the CPa position, four polypeptides of 47, 39, 31, and 6 KD were thought to be associated with the complex.

1.3.5 The photosynthetic unit (PSU)

From this review it may be concluded that the photosynthetic apparatus concerned with chloroplast light reactions is compartmentalized both structurally and functionally into three main sections i.e. PSI, PSII and the light-harvesting apparatus. Much attention has been focused on how these components are arranged *in vivo*. Clayton (1980) and Hiller and Goodchild (1981) have reviewed the various models suggested for the organization of the PSU and Model A of Figure 5 is derived from these texts. This model is based on the postulates of Thornber *et al* (1977) and four chlorophyll-protein complexes are proposed. The rectangular portion of the model representing an interconnecting LHCP for PSI and PSII. Hiller and Goodchild (1981) suggest that CPa complexes separated after this model had been postulated may correspond to the interconnecting light-harvesting area. Anderson (1980) postulated models B and C and based their composition on actual quantitative data derived from more recent studies. This data takes into account the stoichiometry of spinach thylakoid pigment/proteins in which 1 P700 and 1 P680 occur for every 400 molecules of chlorophyll a

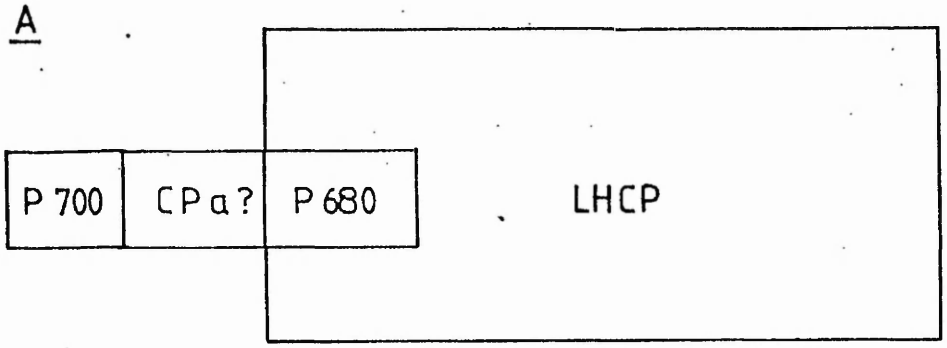
FIGURE 5 :- PROPOSED MODELS FOR THE ARRANGEMENT OF THE PHOTOSYNTHETIC UNIT

KEY :-

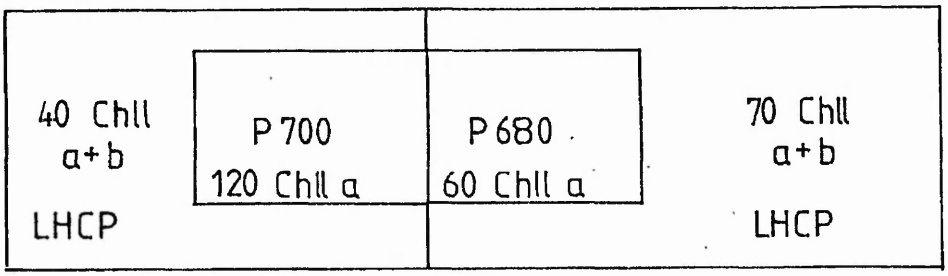
A = model after Thornber et al (1977)

B and C = models after Anderson (1980)

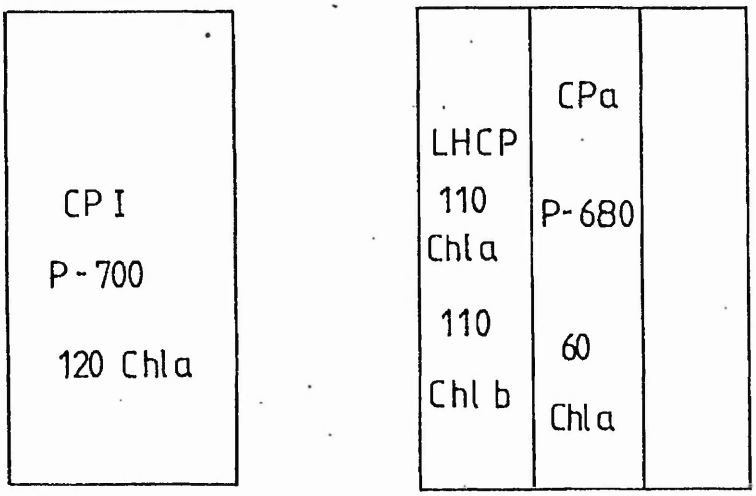
A



B



C



PHOTOSYSTEM I PHOTOSYSTEM II

and b. The PSI complex accounts for 30% of the total chlorophyll, and LHCP 53%. CPa which is presumed to be associated with P680 contains 15%. The first model of Anderson (1980) is arranged such that LHCP is in contact with both photo-systems, although primarily with PSII. The second model suggests that each photosystem has its own complement of LHCP, but the photosystems are still able to interact. Andersson and Anderson (1980) suggest that the second model may be more applicable as there is evidence for heterogeneity in the distribution of chlorophyll-protein complexes in the thylakoid membranes of spinach chloroplasts. There are two different membrane surfaces in thylakoids:- appressed membranes (or grana partitions) and non-appressed membranes which are exposed to the stroma. The above authors have shown that the partition region is depleted in the PSI reaction centre complex and enriched in PSII and LHCP. In contrast non-appressed membranes show a substantial PSI content. Using fluorescence emission techniques Steinback, Bose and Kyle (1982) have shown that phosphorylation of LHCP leads to a decrease in the amount of absorbed excitation energy distributed to PSII. These authors have interpreted this data to mean that phosphorylation of LHCP causes an alteration of the absorbed excitation energy between PSII and PSI. This is supportive of the physical evidence of a conformational relationship between the three complexes which make up the PSU.

1.4 PHOTOOXIDATION AND PHOTOPROTECTION IN CHLOROPLASTS

1.4.1 Photooxidation

When exposed to high light intensities plants show signs of chloroplast disruption, and this phenomenon is due to photo-oxidation, a process in which light, oxygen, and a photosensitizer cause the production of toxic free radicals. These are molecular species in which a single electron is removed or added to an organic molecule, which therefore, acquires an 'unpaired electron'. The formation of excited molecular species are also of importance in the process of photooxidation, and these may be termed singlet or triplet states. When a molecule is excited from its electronic ground state, it is most likely to enter a singlet excited state

in which the spin of the promoted electron remains antiparallel, and the total spin remains zero. However, if there is a magnetic interaction within the molecule the spin of the promoted electron may become reversed from its former alignment and become parallel to the spin of the ground state, this is termed a triplet excited state. Molecular oxygen is particularly interesting in that it may be classified as a free radical because when in the ground state the molecule contains two unpaired electrons with parallel spins. Thus, O_2 may also be classified as a 'triplet' ground state molecule, an unusual arrangement as most molecules exist in the more common singlet ground state with paired electrons. Using the information described above it is now possible to describe those mechanisms which effect photooxidation in plant chloroplasts.

Water is the primary donor for photosynthesis, and its cleavage is concomitant with the release of molecular oxygen which is able to diffuse freely throughout the chloroplast and cytoplasm. The fate of this O_2 is critical to the well being of the plant as its activation within the chloroplast may lead to toxic photooxidative damage. The triplet state of O_2 is particularly unreactive and the molecule requires activating before it can produce toxic species, which are singlet oxygen ($^1O_2$) and the superoxide radical ($O_2^{\cdot -}$). The process of photooxidation and radical production has been reviewed by Halliwell (1981). $^1O_2$ production involves the reaction of a photosensitizer, light and O_2. Photosensitizers have two systems of electronically excited states, the singlet (^{1}SENS) and triplet (^{3}SENS). In the chloroplast it is triplet chlorophyll which mediates the transfer of triplet-triplet energy to O_2 and so acts as the intermediate photosensitizer to produce singlet oxygen. This species is very reactive and is capable of reacting directly with polyunsaturated fatty acids. This type of reaction is designated Type II. Type I reactions result in the formation of radical species via interaction of the triplet sensitizer with compounds other than oxygen. A schematic representation of these reactions is shown in Figure 6 (Krinsky, 1979).

FIGURE 6 :- TYPE I AND TYPE II PHOTOOXIDATION (after Krinsky, 1979).

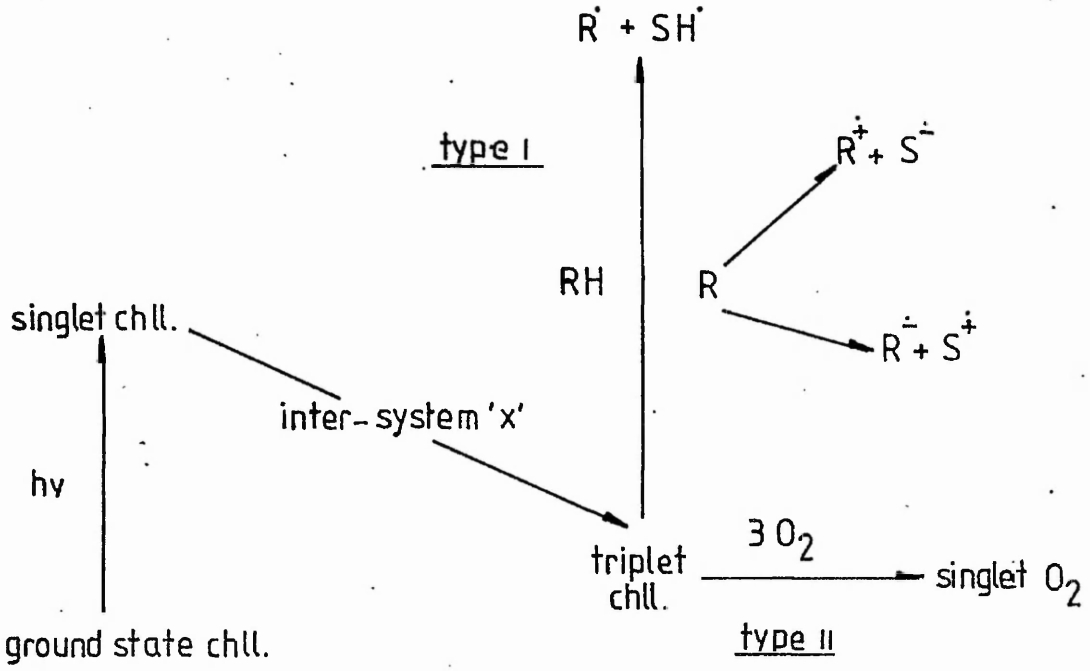
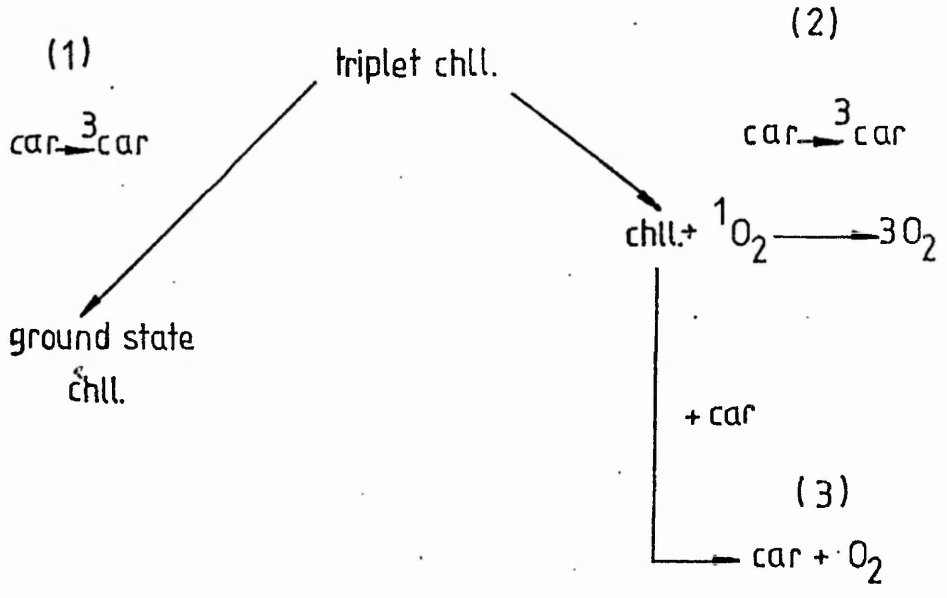


FIGURE 7 :- CAROTENOID PROTECTION (after Krinsky, 1978).



The addition of one electron to molecular oxygen produces the superoxide anion $O_2^{\bullet}-$. This type of activation is due to the photoreduction of molecular oxygen via the photosynthetic electron transport chain (Elstner, 1982). Superoxide can act as a weak oxidizing agent, becoming reduced to hydrogen peroxide (H_2O_2) and the previous author describes several components of PSI which may act as intermediates in its formation. The production of H_2O_2 may also arise from the dismutation of superoxide, mediated by superoxide dismutase. An interaction between metal ions, H_2O_2 and $O_2^{\bullet}-$ can lead to the formation of the highly reactive hydroxyl radical ($OH^{\bullet}$), and this series of reactions has been reviewed by Elstner (1982) i.e..

$$O_2^{\bullet}- + M^{n+} \longrightarrow O_2 + M^{(n-1)+}$$

$$H_2O_2 + M^{(n-1)+} \longrightarrow OH^- + OH^{\bullet} + M^{n+}$$

overall:-

$$O_2^{\bullet}- + H_2O_2 \xrightarrow[M]{} OH^- + OH^{\bullet} + O_2$$

These free radicals are extremely reactive, and because of this any damage to the chloroplast must take place very close to the site of radical generation.

Photooxidative damage is usually manifested as thylakoid disruption, and pigment bleaching. Lipids in chloroplast membranes contain large amounts of polyunsaturated fatty acids, photooxidation of which causes the formation of hydroperoxides and conjugated dienes. This results in fragmentation of membranes producing a wide variety of breakdown products of which two, malondialdehyde and ethane, can be conveniently assayed (Heath and Packer, 1968; Elstner and Youngman, 1978). Sandmann and Boger (1982) have investigated the formation of light-induced volatile hydrocarbon production in various algal species, and have found ethane, ethylene, propane, pentane and pentene to be present in detectable amounts.

Because of the extensive damage which can be caused by oxygen activation, and its byproducts, protective mechanisms in the plant have evolved to counteract such processes and these are described below.

1.4.2 Protective mechanisms

The chloroplast environment is constantly producing molecular oxygen in the presence of a photosensitizing pigment, and the organelle is faced with the possible production of photo-oxidative species. The extent of this production depends on several parameters:- the saturation level of the components of the electron transport chain, the availability of $NADP^+$, and the overall rate of CO_2 fixation. The external environment can also impose conditions which will increase photooxidation, this is particularly evident when conditions of high light intensity and low CO_2 availability prevail. The rapid removal of active oxygen derivatives and the dissipation of otherwise harmful excitation energy is therefore essential, and this is accomplished by a number of protective mechanisms.

Strategically the carotenoid pigments are the most immediate and effective defence against the production of singlet oxygen, and their protective importance has been reviewed extensively (Krinsky, 1971, 1978, 1979; Goodwin, 1980). When grown in the presence of light and O_2 carotenoid-lacking mutants of Rhodopseudomonas sphaeroides showed extensive pigment bleaching and eventually died (Sistrom, Griffiths and Stanier, 1956). The mechanism of carotenoid protection was first demonstrated by Fujimori and Livingstone (1957) who showed that the carotenoids were able to quench triplet state chlorophyll, by means of direct energy transfer:-

$$^3Chl + Car \longrightarrow Chl + {}^3Car.$$

Chessin, Livingstone, and Truscott (1966) have shown that the quenching of triplet chlorophyll a by β-carotene occurs at a very fast rate. However, as molecular oxygen is also able to quench triplet chlorophyll at a similar rate a competition affect will occur. Unless the local concentration of carotenes is very much greater than that of oxygen, quenching of triplet chlorophyll by carotenes cannot be a major protective mechanism. Foote (1976) proposes that in highly organized chloroplast photosystems, the local concentrations of carotene can be high enough to quench

triplet chlorophyll at a success rate of approximately 90%. The remaining 10% will survive and probably produce $^1O_2$, however, once this occurs a second chance of quenching is available, since local β-carotene can also quench this excited species. The type of quenching is dependent on the number of conjugated bonds in the carotenoid. Shorter carotenoid chains (< 7) are effective in protecting anaerobic photoreduction of chlorophyll by quenching its triplet state, and larger chains (> 9) are important in quenching $^1O_2$ (Foote, Chang and Denny, 1970). The overall mechanism of carotenoid protection has been summarized by Krinsky (1978) and this scheme is shown in Figure 7. A third mechanism is included whereby $^1O_2$ which is not physically quenched can be dissipated by the chemical oxidation of the carotenoids.

Of the other systems implicated with photoprotection, superoxide dismutase (SOD) has gained considerable attention. This enzyme catalyses the breakdown of the superoxide radical $O_2^{\bullet}-$ to form O_2 and H_2O_2, and has been located in chloroplasts by Asada, Urano, and Takahash.. (1973). As $O_2^{\bullet}-$ is produced by electron transport there would appear to be a functional need for SOD in the thylakoid region. Foyer and Hall (1980) purified the active component of chloroplastic SOD and found it to be dependent on bound manganese localised in the light-harvesting complex of the chlorophyll a/b protein. It is of interest to note that Lumsden and Hall (1975) have found chloroplasts from Codium fragile to contain cyanide-insensitive SOD activity in the stroma. The production of H_2O_2 by SOD, although less harmful than the superoxide radical, can also prove toxic if allowed to reach uncontrolled concentrations in the chloroplast stroma. According to Foyer and Hall (1980) chloroplasts contain significant concentrations of non-specific peroxidases, and large quantities of ascorbate which can dissipate H_2O_2.

Halliwell (1981) reviews the mechanism of enzyme protection in chloroplasts with high O_2 concentrations, as these enzymes can often contain large numbers of thiol groups which can be rapidly oxidized. Glutathione which also contains thiol groups is

preferentially oxidised by 'free' oxygen, or in certain cases glutathione can also reactivate some enzymes which have been previously inactivated on oxidation. Glutathione reacts with O_2 and forms a disulphide bridge, which may be reduced by the NADPH-dependent enzyme glutathione reductase. Ascorbate is also implicated in this protection mechanism and glutathione non-enzymically reduces dehydro ascorbate back to ascorbate under conditions of illumination in isolated spinach chloroplasts (Foyer and Halliwell, 1976). These authors have described a protective ascorbate/glutathione mechanism by which chloroplasts can maintain a high level of reduced ascorbic acid, which is an important scavenger of $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$. α-tocopherol is also thought to act as an efficient scavenger of $^1O_2$ and other free radicals present in the chloroplast (Baszynski, 1974).

All the mechanisms discussed so far are directly concerned with the products of oxygen activation or molecular oxygen itself, however photorespiration may play an important role in the regulation and prevention of photooxidative damage. Tolbert (1980) suggests that the photorespiratory pathway maintains a status quo in the internal cycling of CO_2 and O_2, thus preventing a gross lowering of the CO_2 content by the assimilatory pathway, and those conditions which can lead to excessive photooxidation. The evidence in this review certainly suggests that plants have adapted to their potentially harmful aerobic environment in many varied ways, and it is not improbable to suggest that photoprotection is one of the roles of photorespiration.

1.5 PHOTOSYNTHETIC ENVIRONMENTAL ADAPTATIONS IN MARINE ALGAE

The marine environment provides a number of habitats for diverse populations of algae, these habitats are usually classified into two main areas, the seafloor or benthic zone, and the seashore or littoral zone. The latter can be further divided into the lower shore (sublittoral), middle shore (littoral) and upper shore (littoral fringe). This type of zonation is defined by the growth of various 'marker species' of marine organisms, usually Balanus, Laminaria and Littorina spp. The marine

environment affects a diversity of marine organisms which are able to survive and adapt to the major marine parameter, tidal movement. Indeed, it is this movement which provides a second physical criterion for the classification of seashores. These are the high and low water marks in evidence along most typical shores, and a third, intertidal zone which is that part of the shore over which the margin of water usually moves between the lowest and highest levels reached during spring tides of greatest range. Tidal rhythms impose considerable daily variations in the intertidal area, but also superimposed on these daily patterns are the longer term monthly and seasonal changes which can affect tidal amplitude. It thus follows that in order to survive in the intertidal zone organisms must adapt to the rapid environmental changes which this habitat imposes upon them. This thesis contains the study of an intertidal marine alga C. fragile, and in particular its adaptation to the intertidal light environment. This section provides a general introduction to algal photosynthesis in the marine habitat and an in depth review of light adaptations in the photosynthetic unit of marine algal chloroplasts.

1.5.1 Algal photosynthesis

Light, temperature and CO_2 availability are particularly important environmental parameters which affect algal photosynthesis and growth. Temperature exerts considerable control over the geographical distribution of marine algae and seasonal adaptations in the temperature response of photosynthesis have been shown by Mathieson and Norall (1975). Darley (1982) suggests that the growth of algae in the intertidal zone is particularly influenced by extreme fluctuations in temperature especially when exposed at low tide. Inorganic carbon is available to marine algae as CO_2 or H_2CO_3, HCO_3^- and CO_3^{2-}, the absolute composition being dependent on pH, although HCO_3^- is usually the predominant form. Carbonic anhydrase has been found in a wide variety of seaweeds, and CO_2 is presumably made available for carbon fixation by means of this enzyme (Kremer 1981). The average inorganic carbon content of seawater is approximately 2.2-2.5mM L^{-1} compared to an equivalent concentration of 13 $\mu M.l^{-1}$ in the terrestrial environment. It is thus apparent that marine algae may not be limited by CO_2 concentrations under light-saturating conditions. Although the reduced diffusion of

CO_2 in aqueous solution must also be considered (Kremer, 1981). Metabolism in marine algae is also affected by nutrient availability, salinity, desiccation and tidal movements. Darley (1982) reviews the importance of these parameters, and suggests they effect photosynthetic adaptive strategies in several algal species.

There are three major pathways of photosynthetic carbon metabolism in higher plants:- the Calvin (C_3) cycle, the C_4 pathway (in which the initial products of CO_2 fixation are 4-carbon dicarboxylic acids) and photorespiration. It is generally believed that the Calvin cycle of algae operates similarly to that of higher plants. However, evidence for C_4 metabolism is debateable, and phosphoenol pyruvate carboxylase has not been detected in large concentrations in algae (Kremer, 1981). Photorespiration is a light-dependent release of CO_2 which isotopic labelling studies have shown to originate from glycolate produced by the oxygenation of ribulose bis phosphate by ribulose bisphosphate oxygenase. This pathway involves several organelles (chloroplast, peroxisome and mitochondria) and its function is still a current enigma in plant biochemistry. Various theories suggest it may be important in photoprotection and regulation, and nitrogen metabolism within the plant (Lorimer and Andrews, 1981; Halliwell, 1981). Photorespiration in algae follows generally the pathways of higher plants, however glycolate oxidase which couples the formation of glyoxylate with O_2 and the concomitant production of H_2O_2 is absent. In several algae the reaction proceeds via the enzyme glycolate dehydrogenase (Lorimer and Andrews, 1981).

One of the most unusual aspects of algal carbon metabolism is the release of large amounts of glycolate and other organic compounds into the aquatic medium. This particularly occurs under conditions associated with high photorespiratory rates, i.e. high O_2 concentrations, temperature, and light intensity (Samuel, Shah, and Fogg, 1971; Khailov & Burlakova, 1969). The importance of this extracellular carbon release is unknown, but Lorimer and Andrews (1981) suggest that it may be a mechanism to excrete excess glycolate, when photorespiratory rates are particularly high.

Mann (1973, 1977) reviews the importance of seasonal growth strategies in seaweeds and suggests that photosynthetic activity is largely responsible for these strategies in Laminaria spp. These algae demonstrated rapid growth in the winter months at a time of low light intensity and water temperature, and this growth was dependent on the use of stored carbon reserves accumulated over the summer period when photosynthetic activity was greatest. A study by Mathieson and Norall (1975) examined the combined effects of light intensity, vertical distribution and seasonal variation on Chondrus crispus. Their results indicated that optimum light conditions and temperature for photosynthesis in this alga are seasonally and spatially variable. Algae sampled during the winter displayed lower light optima for photosynthesis than spring-sampled specimens and intertidal algae exhibited higher rates than specimens sampled in deeper subtidal areas. Ramus and Rosenburg (1980) have also shown that several species of macroalgae exhibit diurnal rhythms with respect to photosynthesis. Carbon assimilation is maximum during the morning followed by an afternoon decline and a late afternoon recovery. This decline may be due to several factors:- photoinhibition, photorespiration, circadian periodicity and increased 'dark respiration', and these authors suggest the depression is particularly evident in intertidal algae.

The photosynthetic capacity of algae may also be a function of water depth as Ramus, Beale and Mauzerall (1976) have shown deep-water specimens of Ulva lactuca and C. fragile to saturate at approximately half the irradiance of surface-sampled specimens. Furthermore, when plants adapted to deep water photosynthesis (at 10m) were studied at various depths a uniform photosynthetic rate was maintained compared to the more erratic behaviour of plants adapted to photosynthesis at 0.5M.

In conclusion it appears that carbon assimilation in marine algae is generally the same as higher C_3 plants although some enzymatic differences may exist together with unexplained release of glycolate. Photosynthesis in the marine environment is particularly dependent on many complex environmental factors, and

as a consequence photosynthetic strategies in marine algae may be equally as variable.

1.5.2 Light adaptations in the photosynthetic unit of marine algal chloroplasts

The Chlorophyta have very similar PSU's when compared to higher plants (Anderson, Waldron and Thorne, 1980; Hushovd, Gulliksen and Nordby, 1982). However, the composition of PSU's in the Rhodophyta and Pheophyta are less understood and may be quite different to those already investigated. The common position of light-harvesting pigments characterizes all algae and higher plants, and it is in the former where they show the greatest diversity. The Chlorophyta have chlorophyll a and b as their major photosynthetic pigments, together with β and α-carotene, neoxanthin, violaxanthin, zeaxanthin and lutein (Appendix I). Two xanthophylls, siphonoxanthin and its ester siphonein (Appendix I) are particularly characteristic of the siphonaceous algae. Yokohama (1981) reports the presence of siphonoxanthin in deep water species of Ulvales, Cladophorales and Siphoncladales, and both siphonoxanthin and siphonein in the Codiales Derbesiales and Caulerpales. These pigments appear to be particularly important as they absorb in the 'green gap' of the P.A.R. spectrum at 540nm *in vivo*. Kageyama, Yokohama, Shimura and Ikawa (1977) report the transfer of excitation energy from siphonoxanthin to chlorophyll a in *Ulva* spp and Kageyama and Yokohama (1978) describe a similar transfer from siphonein to chlorophyll a in *Dichotomosiphon tuberosus*. Siphonoxanthin and siphonein have also been shown to be integral components of the LHCPs of *Caulerpa cactoides* (Anderson *et al*, 1980). Chlorophyll a:b ratios are characteristically low in Siphonaceous algae and this may correspond to a shade adaptation, similar to those found in higher plants (Keast and Grant, 1976). The Pheophyta possess a special light-harvesting pigment, fucoxanthin (Appendix I) which is located in the LHCP of these algae. Anderson, *et al* (1981) review the LHCP composition of brown algae and they comprise two separate complexes, one containing chlorophyll a and C_2, and fucoxanthin, and the other chlorophyll a, C_1, C_2 and violaxanthin. Fucoxanthin also absorbs in the 'green region' of the spectrum at 500-550nm (Barrett and Anderson, 1980),

and transfers excitation energy to chlorophyll C_2 (Goedheer, 1969). Barrett and Thorne (1981) have proposed a PSU for brown algal pigment/proteins in which the LHCP associated with violaxanthin is located with PSI, and the fucoxanthin containing LHCP with PSII. The Rhodophyta are considerably different to the other algal groups as their light-harvesting pigments consist of tetrapyrrole phycobilin pigments. These pigments are usually represented by phycoerythrin phycocyanin and allophycocyanin which have approximate wavelengths of maximum absorption of 570, 630 and 650 nm respectively. Phycobilins are bound to proteins and form large pigment-protein bodies called phycobilisomes which are located on the exterior of the thylakoids (Hiller and Goodchild 1981).

Light is a major factor controlling the growth and survival of marine plants, since the medium in which they grow has the ability to change the intensity and spectral composition of P.A.R. Seawater achieves these changes by preferentially absorbing the red, yellow and violet parts of the spectrum and allowing the blue and green wavelengths to filter through. Selective absorption by pure seawater has very little affect on the composition of the visible spectrum, but the large quantities of dissolved organic substances present in coastal seawater are capable of absorbing P.A.R. (Jerlov, 1977) loss of irradiance also occurs as water deepens due to the absorption and scattering caused by colloids of silts and clays. 'Yellow substances' which are highly absorptive in the ultraviolet and blue wavelengths constitute some of the greatest absorption properties of coastal seawater (Spence 1981). The physiological adaptations of marine plants to their extreme and varied light environments has long been a matter of debate. Englemann (1884) first proposed the theory of complementary adaptation and this formed a basis for light adaptive strategies in algae for many years to follow. The phenomenon of complementary chromatic adaptation is most evident in the algae of rocky shores. In these environments there is often a general gradation in algal colour complementing the quality of available light. Green algae are positioned at the high water mark, lower down are the brown algae and below the low water mark are the red algae. The gradation

in frond colour is thought to complement the presence of accessory photosynthetic pigments which harvest light energy available in their particular 'light zone'. Although the theory by Englemann (1884) is elegant in nature, more recently its importance has been questioned.

Field studies often reveal that algae do not fit into their 'colour band zone', and often green seaweeds are encountered in benthic niches (Yokohama et al, 1977), whilst red and brown seaweeds can occupy positions relatively high above the lower tide mark. Dring (1981) investigated the theory of chromatic adaptation by separating the two light variables, light quality and quantity, and showed that there was little correlation between vertical colour distribution of algae and the quality of their immediate light environment. This author suggests that light intensity may be the most important parameter governing algal growth, not light quality. Thus, deep-water plants should probably be regarded as deep-shade adapted plants rather than green-light or blue-light adaptations. A review by Ramus (1981) supports much of the above theories, and this author derides the theory of complimentary adaptation because (1) the ecological distribution of marine algae was incorrectly deduced, (2) the distribution regarded only light quality, and (3) complementary adaptation excluded the existence of other mechanisms for filling the 'green gap'. Recent studies have shown that these 'other mechanisms' may be varied, and effected by a number of physiological adaptations within the algal PSU.

If the distribution of algae is mainly dependent on higher intensity and not algal colour it thus follows that green and brown algae must both have effective means for absorbing light. Evidence for this is their possession of the light-harvesting pigments fucoxanthin and siphonoxanthin which both absorb in the 'green gap' region of the spectrum. Marine algae are also able to change their pigment and PSU composition to suit the prevailing light environment and relative proportions of chloroplast pigments vary in response to light. Steemann-Nielsen and Park (1964)

distinquished two main types of adaptation in algae. The first is a "Chlorella"-type adaptation, characterized by increases in chlorophyll content as light intensity decreases. The second a "Cyclotella"-type of adaptation, in which the chlorophyll content remains the same regardless of light intensity but the photosynthetic rate is considerably higher in cells developed at very high light intensities. This is considered to be due to an overall increase in dark metabolism to accommodate excessive photo-stimulation of the light reaction. Falkowski and Owen (1980) have suggested that there are two strategies of light-shade adaptation in marine phytoplankton. Skeletonema costatum, a diatom usually found in low light intensity niches adapts to shade by changes in size, but not number of P700 units, the P700 size increasing as light intensity decreases. However, Dunaliella tertiolecta, a chlorophyte adapted to higher light intensities has a shade adaptation in which the size of P700 decreases as the number (of P700) increases. It is of interest to note that both species respond to decreased light intensity by increasing pigment content, but the difference in their adaptation strategies is indistinquishable if based on chlorophyll and carotenoid content. From this it can be deduced that the arrangement of the PSU is of primary importance in light adaptations rather than a simple change in pigment concentration.

Ramus Beale, Mauzerall and Howard (1976) using in vivo studies found that several species of algae change their photosynthetic pigment content in response to water depth. Deep-water algae showed an increase in the ratio of phycobiliproteins and chlorophyll b:chlorophyll a with increase in water depth. Intertidal algae however, increased only their pigment concentration with increased water depth and did not alter their pigment ratios. Ramus et al, (1976) therefore concluded that the intensity adaptation observed in intertidal algae is analogous to light-shade adaptations observed in higher plants. Conversely, those species of algae growing in sub-marine environments require to modify their photosynthetic apparatus by changing both relative and absolute concentrations of pigments. It is also apparent that seasonal variation may influence the light environment and variability in

daylength and tidal amplitude can also be correlated with changes in the PSU (Jensen, 1966).

This account has detailed the capture of light energy by the PSU directly, however Ramus (1978) suggests that seaweed anatomy is a further critical factor in the absorption of light by marine plants. Using Ulva lactuca and C. fragile, Chlorophytes with similar photophysiology but different anatomies, this author investigated those parameters governing light absorptance (i.e. fraction of incident light absorbed). Absorptance by U. lactuca was dependent on pigment concentration, but in C. fragile absorptance was independant of pigment content and the thallus of this alga always absorbed 97-95% of the incident light. The author concludes that light absorption by algae may be more than a function of incident light intensity and pigment content alone. Indeed, this may be the case as the capture of light by the PSU is only a function of light absorption by the entire thallus.

1.6 Aims of the Investigation

The aim of this thesis is to study the marine alga C. fragile in relation to its intertidal niche and particularly its response to large environmental fluctuations in light energy. This will be studied using three major areas of research. The first involves the separation, identification and quantitative determination of photopigments in C. fragile chloroplasts, together with an investigation into the localization and function of these pigments in the pigment/protein complexes of the alga. The second objective of this thesis is to survey the growth of C. fragile in its intertidal environment at Bembridge and this forms the basis of the succeeding experimental chapters. These determine the effects of controlled laboratory light regimes on pigment content, photosynthesis, photooxidation and glycolate production in isolated chloroplasts and intact C. fragile fronds.

2.0 THE SEPARATION, IDENTIFICATION AND QUANTITATIVE DETERMINATION OF PHOTOPIGMENTS FROM CODIUM FRAGILE

2.1 INTRODUCTION

Members of the order Siphonales show unusual pigment characteristics when compared to other algal groups. The presence of the xanthophyll siphonoxanthin, and its ester siphonein is indicative of this family, as is the relative abundance of α-carotene compared to β-carotene (Strain, 1965; Goodwin, 1971). ε-Carotene has also been identified in Bryopsis corticulans (Strain, 1951), although its presence in other Siphonales has not been investigated. The aim of this investigation is to develop techniques for the separation and identification of pigments in C. fragile, and to quantitatively determine the concentration and distribution of these pigments in this alga.

2.2 MATERIALS AND METHODS

2.2.1 Sampling and Maintenance of C. fragile

Young, (up to five dichotomies) and old, (up to eight dichotomies) specimens of C. fragile were collected from intertidal rock pools at Bembridge, Isle of Wight during November, 1979. The alga was maintained in aerated seawater at approximately $10^{o}C$ with a light intensity of 10 $W.m^{-2}$ at the water surface.

2.2.2 Extraction of Pigments from C. fragile

(1) Fronds

C. fragile pigments were extracted according to the method of Jeffrey (1968). Samples of 50g were frozen at $-20^{o}C$ for 30 minutes and dehydrated in methanol for 2 minutes. Diced fronds were macerated for 1 minute in a homogenizer containing approximately 50 ml of distilled water, filtered, and macerated further using a pestle and mortar, containing 100% acetone, and 1-2g of Na_2CO_3 (anhydrous) used to neutralize excess acidity. The macerate was filtered and re-extracted with acetone several times, until colourless. The filtrate was then

transferred to diethyl ether (AnalaR) in a separatory funnel, and washed with NaCl (20-40% aqueous) until all traces of acetone were removed from the ether layer. Further concentration to exactly 5 or 10 ml was achieved by the passage of a stream of nitrogen over the ether extract. Care was taken to ensure a rapid extraction and to prevent excessive exposure to both heat and light. Pigment extracts were stored in the dark under nitrogen at $0^{\circ}C$.

(2) Chloroplasts

Chloroplasts were extracted from 120g of fronds using the method of Cobb (1977), below:-

Extraction Medium

Sucrose 0.8M
Bovine serum albumin (Sigma type v) 2.0g. L^{-1}
$MgCl_2 . 6H_2O$ 3.05g. L^{-1}
HEPES buffer, (N-2 hydroxyethyl piperazine N^1-2-ethane), 0.05M.

adjusted to a pH of 7.8 with 1 N NaOH.

50-150g of C. fragile fronds were placed in a chilled homogenizer containing 70-100 ml of the (semi-frozen) above extraction medium. The fronds were macerated for 2 x 5 second bursts of a homogenizer, filtered through two layers of muslin into centrifuge tubes, and centrifuged for 5 minutes at 500g using a B.T.L. bench centrifuge. The supernatant was discarded and the pellet resuspended in 2-3 ml of chilled extraction media, the final extract was filtered a second time through two layers of muslin, and stored on ice in the dark, whilst the preparation of the purification step took place.

Chloroplast Purification

2.5g of Sephadex G.50 coarse (Pharmacia, particle size 100-300 μ , bed volume g. dry gel = 9-11 ml) was swollen

in 35 ml of extraction medium. After 3-8 hours of storage in refrigerated conditions, the sephadex was poured into a water-cooled Pharmacia (klb) column. The column was left for several minutes to equilibriate, and the flow rate through the column adjusted to 2-3ml/minute using a Watson-Marlow peristaltic pump, the chloroplast suspension was then carefully layered onto the column head. As the leading edge of the chloroplast fraction on the column contains less cytoplasmic contamination (Cobb, 1977) only this fraction (2ml) was used to represent pure chloroplasts. Chloroplast numbers in the final preparation were calculated using a haemocytometer.

A chloroplast suspension of known number was then macerated using a pestle and mortar and the pigments extracted as in the method described for whole frond tissue.

2.2.3 The Separation and Identification of Pigments in C. fragile

(1) Xanthophyll Separation

Various recommended TLC methods for the separation of xanthophylls were investigated (e.g. Jeffrey, 1968; Davies, 1976). However in each case the resolution of xanthophylls in pigment extracts from C. fragile proved unsatisfactory. In most systems the chlorophylls during the latter stages of development formed a diffuse band which masked violaxanthin. However, the major problem was the achievement of complete resolution of neoxanthin and siphonoxanthin, as these pigments formed a composite band in all the TLC systems investigated. It was found that oven-activated silica gel (G60) plates achieved the best resolution of xanthophyll pigments, however, recommendations on the unsuitability of silica gel in the resolution of acid-labile carotenoids (e.g. violaxanthin) proved a point of concern. Personal communication with Merck, resulted in the finding that their pre-prepared

Silica gel G.60 plates, were neutral and suitable for the separation of acid-labile components. Thus, by using the above plates, and the subsequent development of a suitable solvent system, resolution of xanthophyll pigments in C. fragile was achieved as follows:-

20 µl of concentrated diethyl ether extract was applied to the origin of plastic strips (7 x 2.5 cm) pre-coated with neutral silica gel G.60 of 0.2 mm thickness (Merck) and developed in 6% ($^{v}/v$) acetone in diethyl ether for 2-3 minutes in total darkness (Figure 8). For the further separation of neoxanthin from siphonoxanthin the sample was developed on larger strips (12 x 3 cm) for 1 hour in the same solvent system (Figure 9). All solvents used were of AnalaR grade.

(2) Carotene Separation

Various standard methods for the separation of carotenes were investigated (Davies, 1976). However, the technique described below was found to be the most successful in terms of resolution and speed of development:-

Glass plates (7 x 2.5 cm) were coated with a slurry of MgO (light) and anhydrous $CaSO_4$ (ratio 1:4) and dried overnight at room temperature to prevent cracking. Concentrated diethyl ether extracts (20 µl) were applied to the origin and the plates developed in 4% ($^{v}/v$) n-propanol in petroleum ether (b.r. 60-80°C) for 3-5 minutes in total darkness (Figure 10).

(3) Elution of Carotenoids

Carotenes and adsorbant were scraped from the plates and rapidly eluted into 2 ml of 100% acetone with the aid of a vortex mixer, and transferred to 3 ml of petroleum ether (b.r. 60-80°C) by washing with 2 ml of 20-40% ($^{w}/v$) aq. NaCl. Xanthophylls were directly eluted from the silica gel plates into 3 ml of absolute ethanol.

FIGURE 8 : - TLC SEPARATION OF SIPHONEIN AND VIOLAXANTHIN

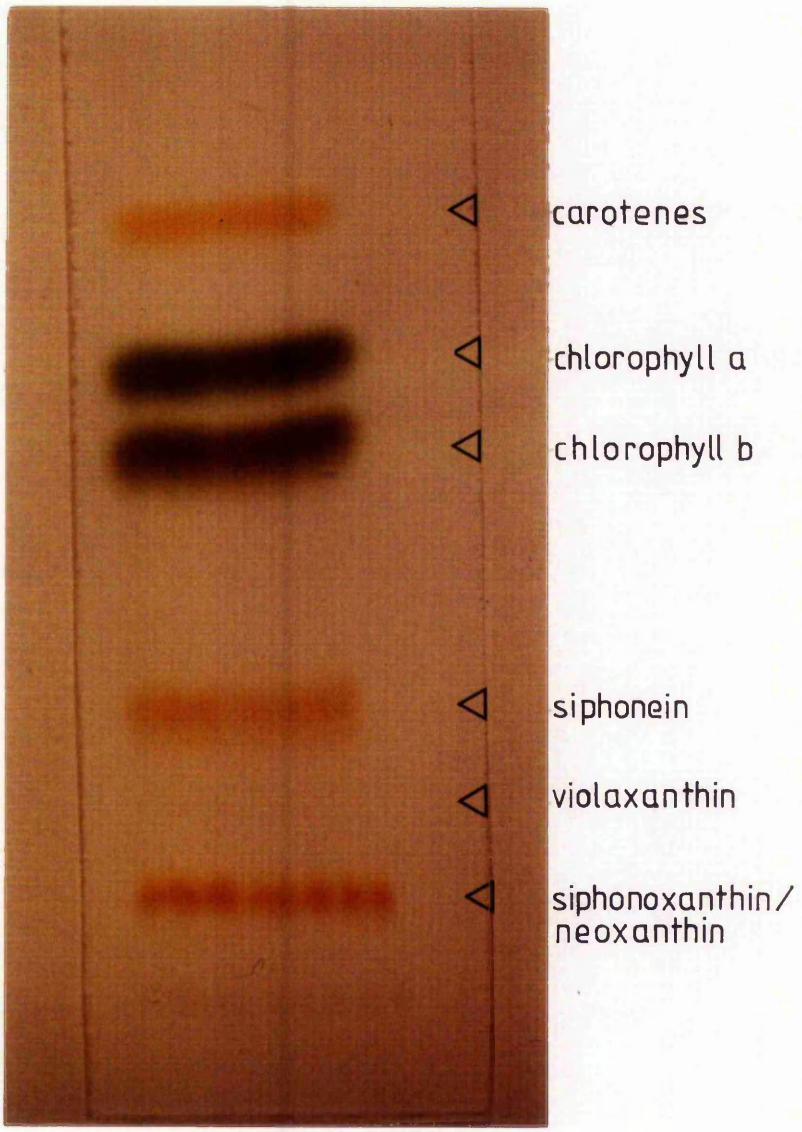


FIGURE 9 :- TLC SEPARATION OF SIPHONOXANTHIN AND NEOXANTHIN

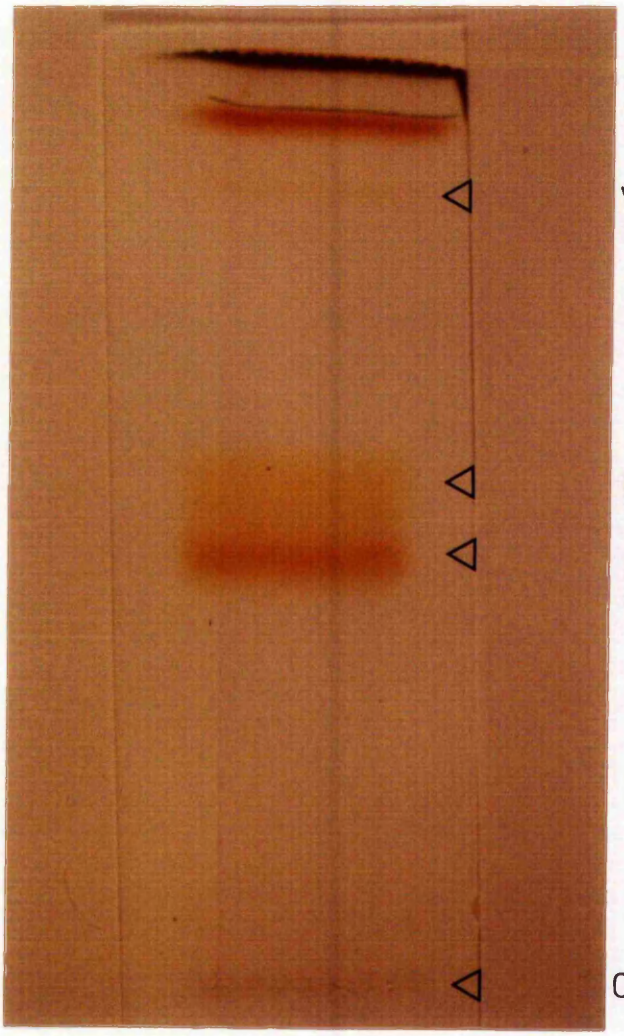
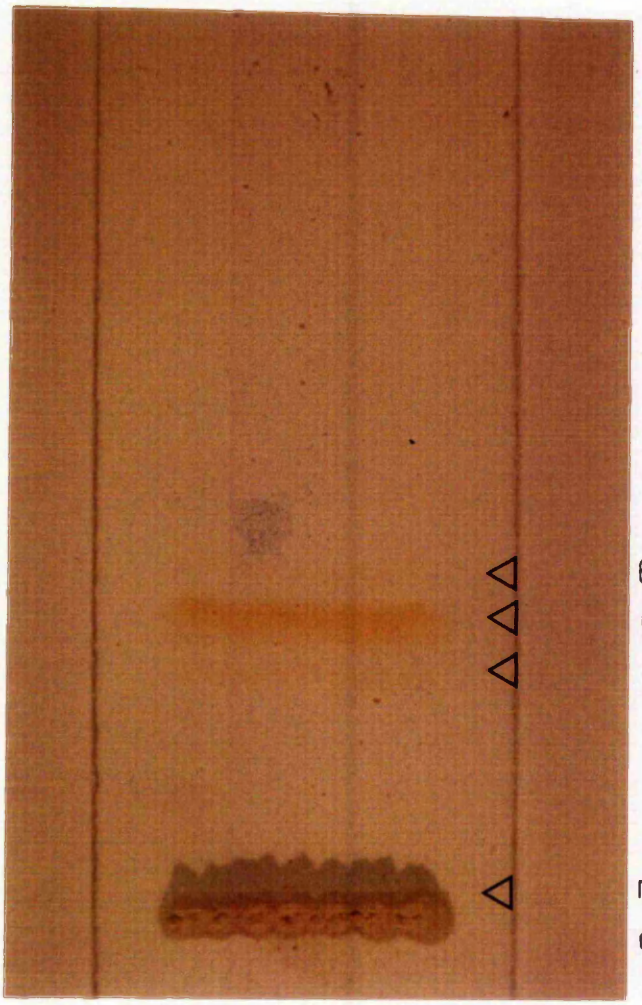


FIGURE 10 : - TLC SEPARATION OF CAROTENE PIGMENTS



Centrifugation at 700g for 6 minutes removed the adsorbant from the solvent.

(4) Pigment Identification

Pigments were identified by their relative positions on the TLC plates and by their characteristic absorption spectra in various solvents, the criteria used for this are shown in Table 1, absorption spectra were recorded using a Perkin Elmer 550S spectrophotometer.

Table 1 CRITERIA USED FOR THE IDENTIFICATION OF PIGMENTS FROM CODIUM FRAGILE

PIGMENT	SOLVENT	Rf	ABSORPTION MAXIMA (nm)
α-Carotene	Pet.ether	0.47	422, 444, 473.
ε-Carotene	"	0.70	418, 440, 470.
'Unknown' carotene	"	0.15	418, 438, 466.
Siphonoxanthin	Ethanol	0.50	447.
Neoxanthin	"	0.57	415, 438, 467.
Violaxanthin	"	0.30	417, 441, 469.
Siphonein	"	0.40	450.

2.2.4 The Quantitative Determination of Pigments in C. fragile

(1) Carotenoids

The quantitative determination of individual pigments was achieved by the use of specific extinction coefficients ($E^{1\%}_{1cm}$) as described by the method of Britton and Goodwin, 1971. $E^{1\%}_{1cm}$ is the extinction at a given wavelength and in a stated solvent of a 1% carotenoid solution in a 1 cm light path. According to the above authors, if x g of a carotenoid in y ml of solution gives an extinction of E at a given wavelength of maximum absorption then:-

$$x = \frac{EY}{E^{1\%}_{1cm} \times 100}$$

From this equation, the quantitative determination of carotenoids was achieved by using the extinction values at wavelengths of maximum absorption for pigments recovered in the eluted 3 ml volumes. Ethanol and petroleum ether (60-80°C) were used as the respective blanks for xanthophylls and carotenes. $E^{1\%}_{1cm}$ values are shown below.

Table 2 THE $E^{1\%}_{1cm}$ FOR CAROTENOIDS ISOLATED IN C. FRAGILE (after Davies, 1976)

PIGMENT	$E^{1\%}_{1cm}$	REFERENCE
α-Carotene	2800 in Pet. ether	Schwieter, et al. 1965
ε-Carotene	3120 " "	" " "
Siphonoxanthin	1160* in ethanol	Ricketts, 1971.
Neoxanthin	2243 "	Cholnoky, et al. 1966.
Violaxanthin	2550 "	Karrer & Jucker, 1943.
Siphonein	2500** "	Ricketts, 1971.

* This value is probably low due to occluded solvent (Ricketts, 1971).

** This value is likely to be higher than the true extinction coefficient since many ketonic xanthophylls have extinction coefficients < 2500 (Ricketts, 1971).

(2) Chlorophylls

The chlorophyll content of fronds from C. fragile was determined by the method of MacKinney (1941). 10-50 µl aliquots of pigment extract or chloroplast suspension were extracted with 80% (v/v) aqueous acetone in a total volume of 5 ml. The extraction procedure was performed

in the dark for 10 minutes, after which the solution was centrifuged at 2, 500g for 3 minutes. The supernatant was read against an 80% ($^{v}/v$) acetone blank at wavelengths 645 nm and 663 nm. It was possible to determine the chlorophyll concentration by using the equations of MacKinney (1941), derived from the use of specific absorption characteristics for chlorophyll a and b and simultaneous equations, viz:-

$$\text{TOTAL CHL } mg.L^{-1} = 20.2 \times A645 + 8.02 \times A663$$

$$\text{CHL a} \quad mg.L^{-1} = 12.7 \times A663 - 2.69 \times A645$$

$$\text{CHL b} \quad mg.L^{-1} = 22.9 \times A645 - 4.68 \times A663$$

The quantitative determination of carotenoid and chlorophyll pigments was carried out using a Perkin Elmer 550S spectrophotometer.

2.2.5 Recovery and Reproducibility Tests on TLC Methods used to Separate Pigments in C. fragile

(1) Recovery Tests

Since β-carotene is apparently absent in C. fragile (see Table 1), it was used to determine the recovery of pigments in TLC systems used in this study. A freshly prepared solution of β-carotene (Sigma, prepared from carrot type IV), in AnalaR petroleum ether (b.r. 60-80°C) was investigated for spectral characteristics using a Perkin Elmer 550S spectrophotometer. The exact concentration of β-carotene in this stock solution was determined using λ max = 453 nm and $E_{1\%}^{1cm}$ = 2592 (Davies, 1976). 10 µl of this stock solution were made up to 2 ml and aliquots used for spotting on the TLC plates (both $MgO/CaSO_4$ and silica gel G.60 Merck). After elution from the developed plates, the absorbance at λ max. was determined and the percentage recovery calculated. As the pet. ether/ β-carotene solution was very volatile, the concentration of β-carotene in the stock solution was monitored throughout the experiment.

Recovery on $MgO/CaSO_4$ Plates

As carotene separation in this method was carried out on TLC plates of 7 x 2.5 cm dimensions, and the development performed in Coplin jars, development time was very rapid (3-5 minutes). Within this time the recovery of β-carotene was 100%, however if the time was increased to 10 minutes, the recovery was reduced to 70%. Since components in a total pigment extract from C. fragile may inhibit the movement of carotenes on the MgO plates, a mixture of β-carotene and extract was run on the TLC plates. Recovery of β-carotene under these conditions was 90% for a 5 minute run and 70% for a 10 minute run. From this investigation it was concluded that Codium extract does not appear to significantly impair the recovery of β-carotene but an increase in development time does.

Recovery on Silica Gel G.60 Plates

Since this system is not able to separate β-carotene from other carotene isomers, it was not possible to investigate the recovery of β-carotene in the presence of a total pigment extract from C. fragile. The recovery of β-carotene on Silica Gel G.60 plates of 7 x 2.5 cm dimensions and a running time of 5 minutes was 72%, at a running time greater than 10 minutes the recovery dropped to 70%. The recovery of β-carotene can only be regarded as a guide-line to the stability of xanthophyll separation in this TLC system. However, since violaxanthin, which is probably the least stable of the pigments found in C. fragile, can be separated by a particularly quick technique, pigment loss is minimised. The separation of neoxanthin and siphonoxanthin takes considerably longer (45 minutes-1 hour), thus increasing the possibility of pigment loss, however, this potential loss must be compromised with the fact that the resolution of two otherwise

poorly resolved pigments is greatly increased. A further indication of the recovery and stability of C. fragile pigment extracts during TLC was achieved by reproducibility studies.

(2) Reproducibility Tests

A pigment extract was prepared from a 50g sample of C. fragile fronds (after Section 2.2.2) and TLC separations with ten replicates for each pigment were performed using the above systems. The results obtained in Table 3 show an acceptable degree of reproducibility, the speed of elution and conditions of development (running time and light exclusion) are of particular importance in maintaining good replication.

Table 3 REPRODUCIBILITY TESTS FOR TLC SEPARATION

PIGMENT	MEAN PIGMENT CONCENTRATION ($\mu g.g^{-1}$ ± s.d.)
α-Carotene	7.4 ± 0.68
ε-Carotene	0.62 ± 0.18
Siphonoxanthin	19.0 ± 3.0
Siphonein	6.6 ± 0.71
Violaxanthin	3.1 ± 0.8
Neoxanthin	8.4 ± 1.9

2.3 RESULTS

2.3.1 Pigments Identified in C. fragile

Figures 11, 12 and 13 show those pigments which were present in C. fragile, i.e. α, and ε-carotene, siphonoxanthin, siphonein, neoxanthin, violaxanthin and chlorophylls a and b. Figure 14 shows the absorption spectrum of intact C. fragile chloroplasts in extraction buffer for comparison. The inability

FIGURE 11 : - ABSORPTION SPECTRA OF TOTAL PIGMENT AND CHLOROPHYLL EXTRACTS (IN ETHANOL) FROM C. FRAGILE

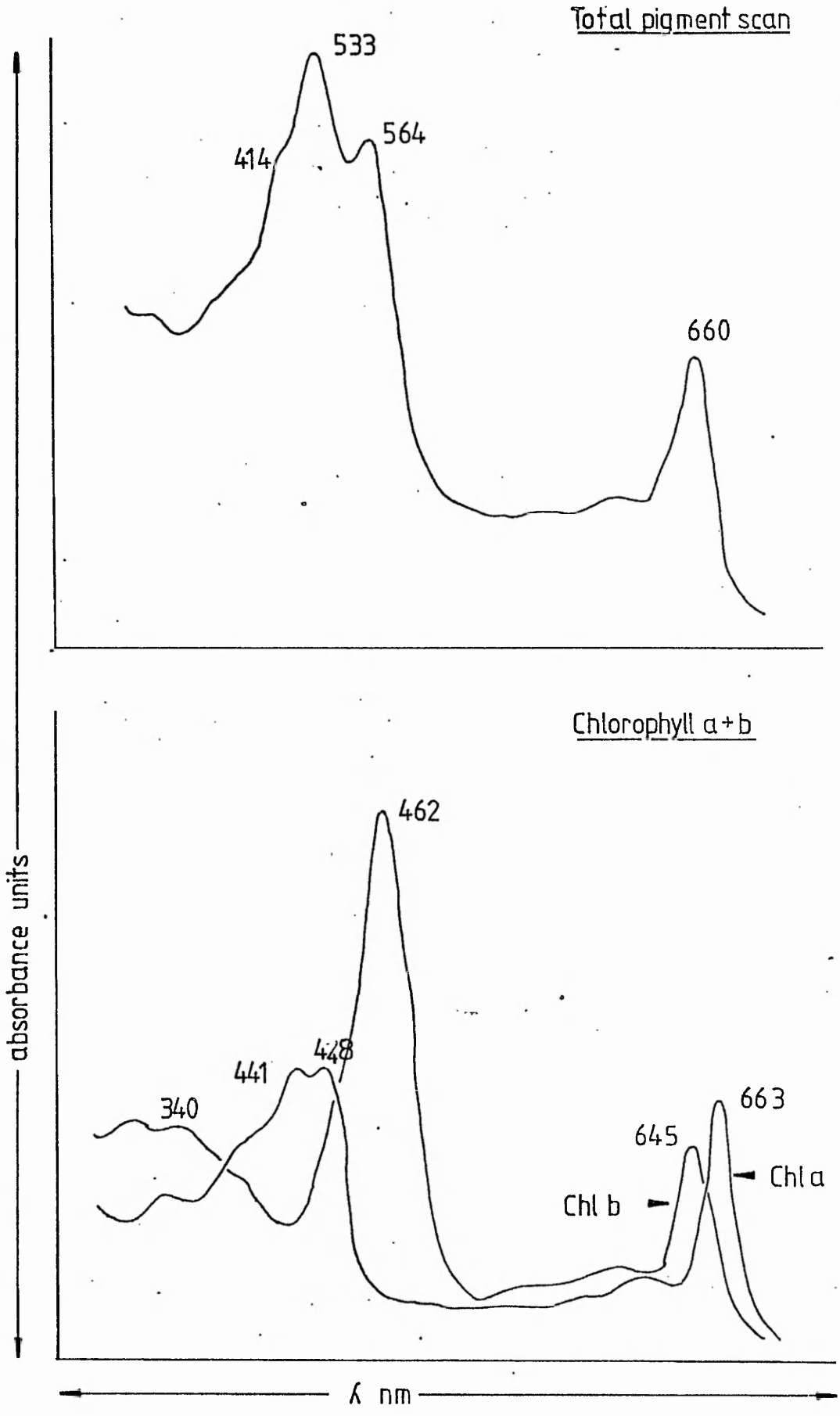

Total pigment scan
533
564
414
660
Chlorophyll a+b
462
448
441
340
663
645
Chl a
Chl b
absorbance units
λ nm

FIGURE 12 :- ABSORPTION SPECTRA OF CAROTENE EXTRACTS (IN PET.ETHER) FROM C.FRAGILE AND ZEA MAYS

KEY :-

a = β - Carotene from Z. mays

b = α - Carotene from C. fragile

c = ε - Carotene " "

d = unidentified carotene "

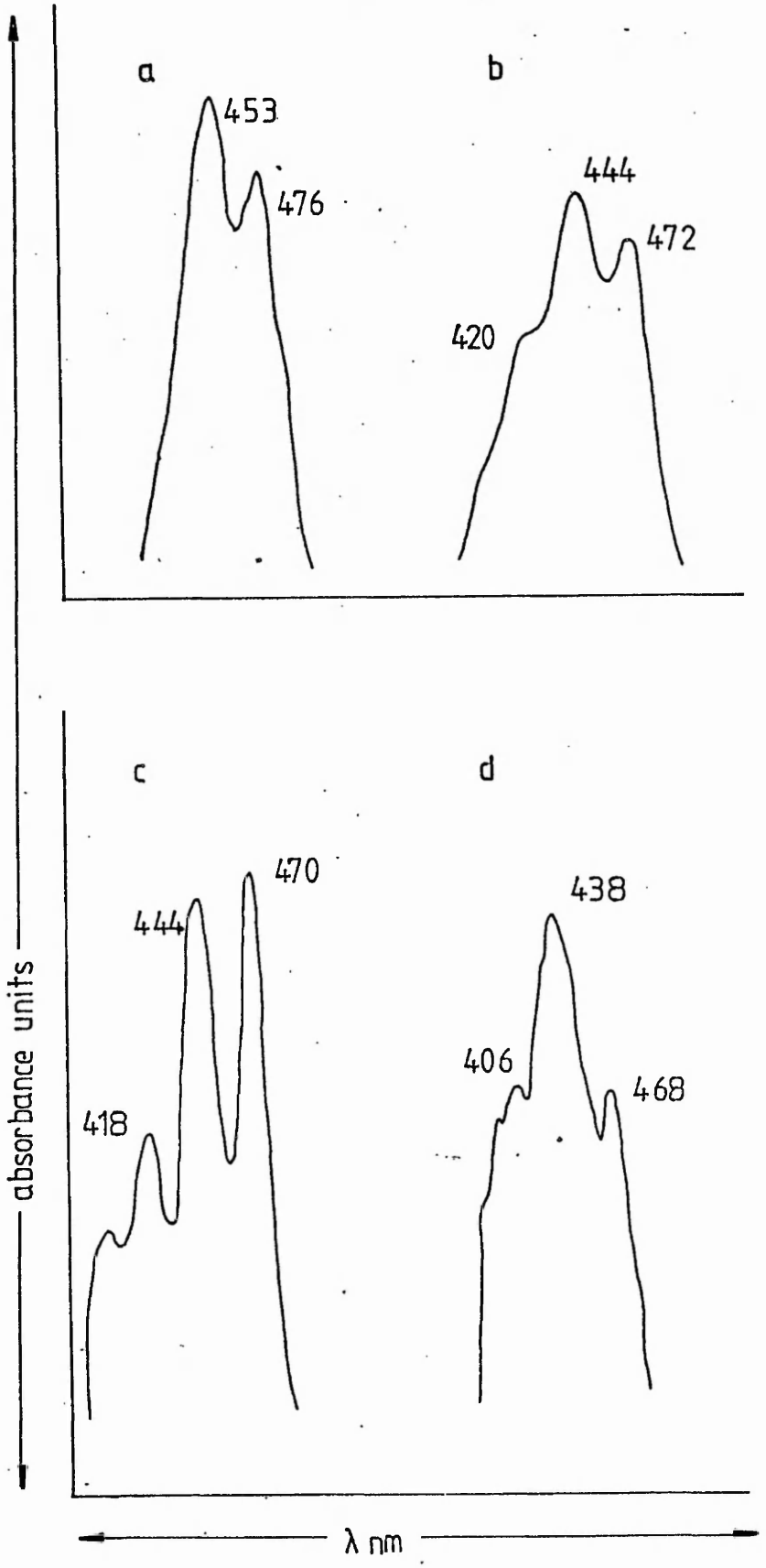

a
453
476
b
444
472
420
c
470
444
418
d
438
406
468
absorbance units
λ nm

FIGURE 13:- ABSORPTION SPECTRA OF XANTHOPHYLL EXTRACTS (IN ETHANOL) FROM C.FRAGILE

KEY

a = Siphonein

b = Siphonoxanthin

c = Violaxanthin

d = Neoxanthin

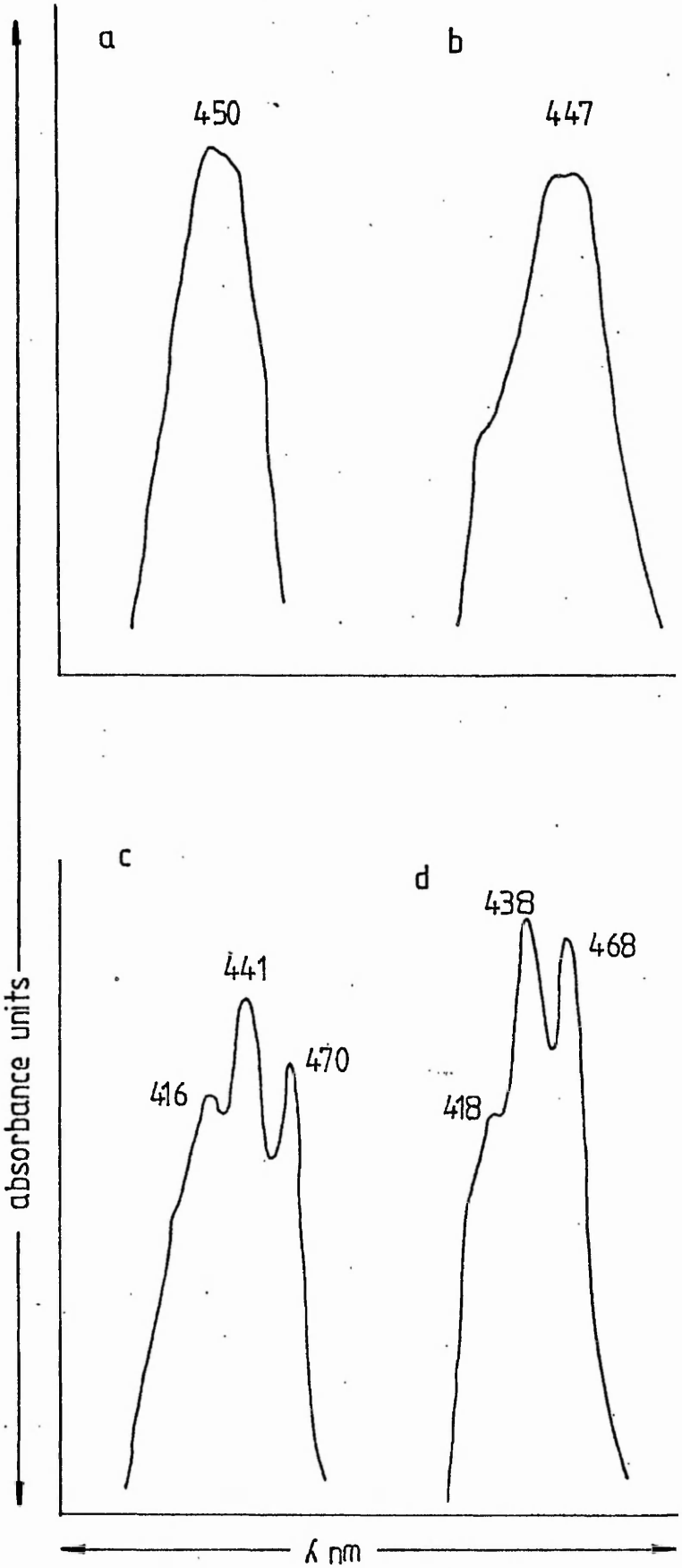
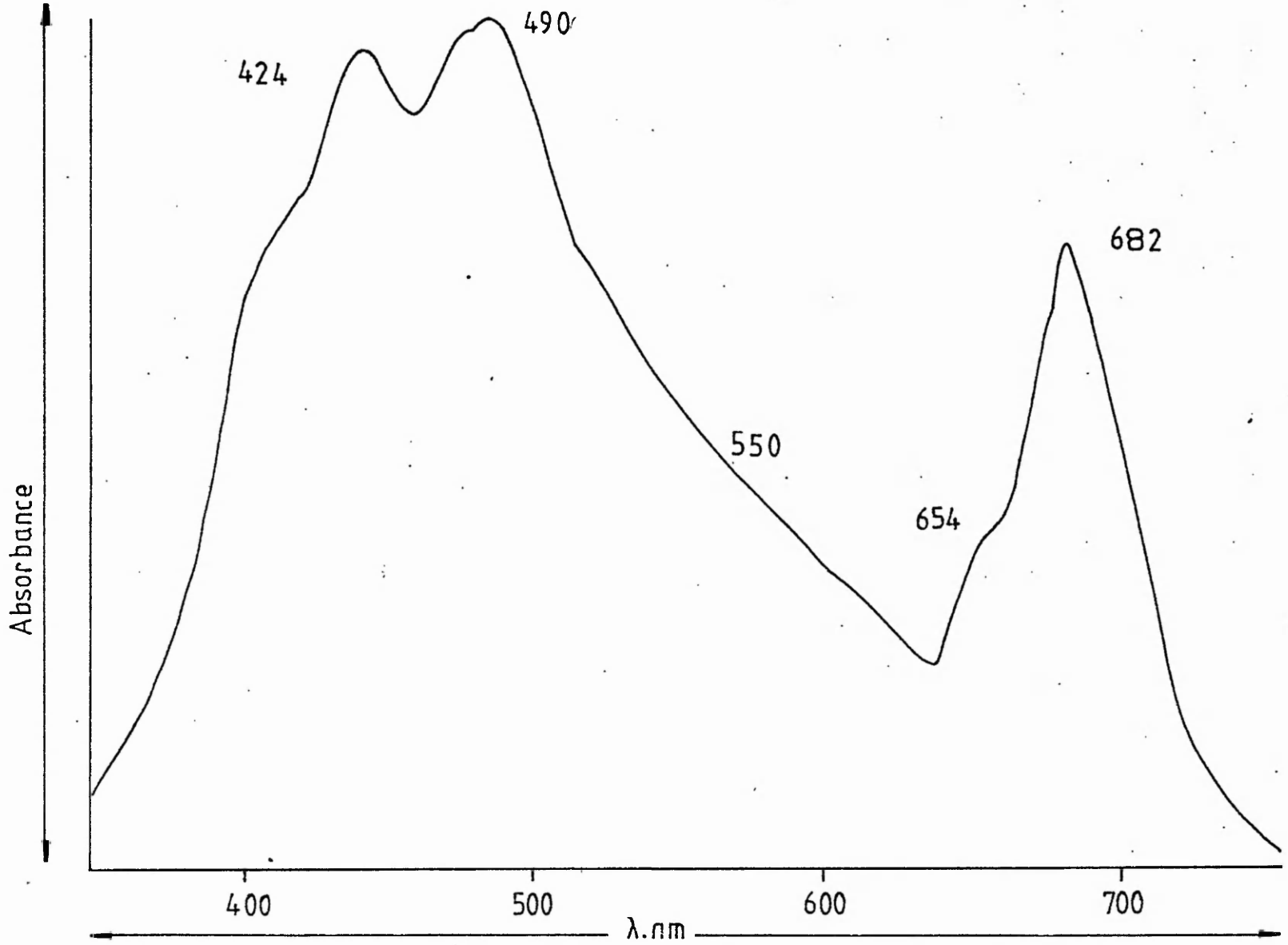

a
b
450
447
c
d
441
438
468
470
416
418
absorbance units
λ nm

FIGURE 14 :- ABSORPTION SPECTRUM OF ISOLATED C.FRAGILE CHLOROPLASTS IN EXTRACTION MEDIUM


424
490
682
550
654
Absorbance
400
500
600
700
λ.nm

to separate and identify β-carotene in C. fragile using both standard TLC systems (Davies, 1976), and the system described in Section 2.2.3 (2) initiated a further investigation of these methods. β-Carotene extracts from Zea mays and purified β-carotene from Sigma were separated in the above systems giving Rf values and absorption characteristics typical of the β-isomer. This evidence together with the recovery tests described in Section 2.2.5 (1) suggests that the inability to detect β-carotene in C. fragile was not due to a fault in the TLC system used. The possibility that very low concentrations of this isomer in C. fragile prevented its detection was also remote, since concentrations of ε-carotene equal to 0.5 $\mu g.g^{-1}$ fresh wt. were detected both on the TLC plate and spectroscopically. Furthermore, the extraction of large samples of C. fragile fronds ($\geqslant$ 200g) also failed to detect its presence.

The identity of the 'unknown' carotene proved elusive, its position on the $MgO/CaSO_4$ plates suggested it may be β-carotene, however the colour of this pigment on TLC was yellow, whereas β-carotene was red, also its absorption characteristics were atypical of the β-Isomer (Fig. 12). The observation that the spectral characteristics of this pigment are very similar to neoxanthin was noted. However, the $MgO/CaSO_4$ plates were unable to separate xanthophylls which were retained at the origin together with the chlorophylls. The intermittant occurrence of this pigment on TLC plates, and its very low concentration make identification very difficult, thus its identity is tentatively attributed to a non-cyclic carotenoid precursor, as suggested by its relative position on the TLC plates.

2.3.2 The Quantitative Distribution of Pigments in C. fragile

Table 4 describes the pigment content ($\mu g.g^{-1}$ fresh wt) of whole fronds of C. fragile. For this analytical work between five and eight 50g samples of C. fragile were used and four or five separate TLC determinations were performed for each pigment.

Table 5 compared the distribution of C. fragile carotenoid pigments in intact fronds to that in isolated chloroplasts, and clearly indicates a chloroplastic location. The isolated chloroplast data was derived from seven 120g extractions of C. fragile fronds, where the mean total carotenoid concentration was 22.7 ± 14.3 x 10^8 µg per chloroplast, TLC replications were as described above.

Table 4 PIGMENT CONTENT (µg.g^{-1} FRESH WT.) OF WHOLE FRONDS OF CODIUM FRAGILE

PIGMENT	FROND SIZE			
	Up to 5 dichotomies µg.g^{-1} ± s.d.		Up to 8 dichotomies µg.g^{-1} ± s.d.	
α-Carotene	6.4	1.0	4.0	0.92
ε-Carotene	0.76	0.34	0.5	0.06
Siphonoxanthin	28.3	5.6	16.2	3.4
Siphonein	11.7	1.5	6.0	1.2
Violaxanthin	4.9	0.9	3.6	0.5
Neoxanthin	14.5	3.3	7.4	3.0
Total carotenoids	66.0	10.0	38.6	6.8
Chlorophyll a	180.0	30.0	91.0	15.8
Chlorophyll b	123.0	21.0	57.0	23.8
Total chlorophyll	306.0	51.0	129.0	15.8
Ratio of total chlorophyll: carotenoid	4.9	1.5	5.0	2.3
Ratio of chlorophyll a:b	1.5	0.13	1.5	0.28

Table 5 PERCENTAGE DISTRIBUTION OF CAROTENOID PIGMENTS IN ISOLATED CHLOROPLAST AND WHOLE FROND EXTRACTS

PIGMENT	Isolated chloroplasts %	± s.d.	Fronds up to 5 dichotomies %	± s.d.	Fronds up to 8 dichotomies %	± s.d.
α-Carotene	12.8	2.9	10.0	3.2	10.6	4.3
ε-Carotene	1.5	0.014	1.2	0.5	1.2	0.2
Siphonoxanthin	37.6	3.6	43.0	3.0	42.0	2.6
Siphonein	19.6	3.5	16.5	1.5	15.6	1.3
Violaxanthin	8.6	3.4	7.6	0.5	9.4	0.8
Neoxanthin	20.6	4.5	22.0	1.9	2 1	3.5

2.4 DISCUSSION

The composition of pigments in C. fragile is quite unique as β-carotene is absent and α and ε-carotenes have been detected together with the xanthophylls siphonoxanthin, siphonein, neoxanthin and violaxanthin. The absence of β-carotene in this study is particularly interesting since it has been previously reported in C. fragile, (Trench and Smith, 1970; Trench, Boyle and Smith, 1973b; and Trench, 1975). However, the detection of β-carotene by the above authors can be disputed since their method of separation using Silica Gel G TLC (Trench, Green and Bystrom, 1969) is unable to separate carotene isomers (Britton and Goodwin, 1971). Jeffrey (1968) detected β-carotene in Codium spp. using an MgO adsorbant which should allow the separation of the isomers, however the use of the above authors solvent system in this study did not show resolution of carotenes in extracts of C. fragile and purified β-carotene. In conclusion it would appear that the techniques described above may account for the previous assumption that β-carotene was present in C. fragile or, alternatively the alga used in these studies is a different sub-species to those used by other workers. Thus, despite the use of extensive detection methods, β-carotene has been shown to be either totally absent in C. fragile or is so rapidly metabolized as to be undetected.

β-Carotene is of major importance in higher plant chloroplasts in which it has two primary functions as a photoprotectant and an accessory pigment. The absence of this isomer in C. fragile suggests that these roles may be achieved by other pigments in the alga. However, to suggest the total absence of β-carotene in C. fragile does pose another problem since the alga is able to synthesize xanthophylls which contain the β-Ionone ring (e.g. Siphonoxanthin, Appendix I). The biosynthetic origins of the α and β-Ionone rings in C. fragile may therefore be particularly interesting and the work of Grumbach (1979) provides several important clues as to their syntheses. This author found evidence for the presence of two separate pathways of carotenoid biosynthesis in both higher plant and algal chloroplasts. The first pathway is thought to be responsible for the biosynthesis of β-Ionone containing xanthophylls and is supplied by the normal carotenoid pathway (Appendix II). β-carotene is rapidly metabolized in this pathway and is represented as a relatively small pool of biosynthetic importance. The second pathway is independent of the one previously described and is controlled at neurosporene (Appendix II), this pathway supplies β-carotene associated with antennae and photochemical functions. If this scheme is correct it may be possible that a small β-carotene pool in C. fragile is metabolized at such a rate as to be undetectable, but at the same time β-carotene shows its "biosynthetic existence" by the presence of xanthophylls which contain the β-Ionone ring. The absence of a large β-carotene pool associated with photoevents in C. fragile suggests the existence of different photoprotective and light-harvesting mechanisms in this alga. The unusual carotene composition of C. fragile has been described for other members of the Siphonales in that α-carotene is relatively more abundant than β-carotene (Goodwin, 1971). Furthermore, the presence of ε-carotene in these alga has also been reported (Strain, 1951). Chapman and Haxo (1963) suggest that species showing a preference for the synthesis of α-carotene also produce the ε-Isomer. It would therefore appear that the general distribution of carotenes in the Siphonales may be of taxonomic, evolutionary and biosynthetic significance,

the absence of β-carotene, and presence of α-carotene in C. fragile providing yet another point of interest.

A further characteristic of the Siphonaceous algae is their possession of the pigment siphonoxanthin and its ester siphonein (Appendix I). Originally both pigments were thought to be specific to the Siphonales however they have also been found in members of the Prasinophyceae (Ricketts, 1971); Microthamnion knetzingianum (Weber and Czygan 1972); and Ulva spp (Yokohama, et al. 1977). Yokohama (1981) investigated the distribution of these pigments in fifty different species of marine algae and concludes that the distribution of siphonoxanthin is ecologically significant in the Ulvales, Cladophorales and Siphoncladales in which it absorbs in the "greengap" in deepwater species. Siphonoxanthin and siphonein were however present in the Codiales, and Caulerpales regardless of their habitats and this author suggests that the possession of green light absorbing pigments in the siphonaceous algae indicates an evolutionary origin in deep waters.

Siphonoxanthin and siphonein (Appendix I) are products of a particularly sophisticated xanthophyll biosynthetic pathway, as the majority of algal xanthophylls have unmodified conjugated chains (e.g. violaxanthin, neoxanthin, zeaxanthin, Appendix I). The structure of siphonoxanthin is basically that of lutein, with a ketone and primary hydroxyl group in the conjugated side chain linking the two ring systems, (Walton et al. 1970; Ricketts, 1971). Saponification of siphonein produces siphonoxanthin and a fatty acid which in Caulerpa prolifera has been identified as lauric acid (Kleinig and Egger, 1967). Siphonoxanthin is esterified by a wide range of unidentified fatty acids, three of which may be unsaturated (Kleinig and Egger, 1967). Esterification of chloroplastic xanthophylls is rather unusual and its significance seemingly unknown, however, it may be tentatively suggested that the relative distribution of siphonoxanthin and its ester may be of importance to chloroplast function in the Siphonales.

Tables 4 and 5 show the quantitative distribution of the pigments identified in C. fragile. The first table shows absolute pigment concentrations are dependent on frond size, and younger fronds generally have higher pigment concentrations. The percentage distribution of

carotenoid pigments remains very constant, regardless of frond size, and similar distributions observed in isolated chloroplasts show that all the pigments isolated in C. fragile are chloroplastic in location. The carotenes constitute approximately 10% by weight of the total carotenoids present, whereas the xanthophyll siphonoxanthin and its ester siphonein comprise approximately 60%. Neoxanthin is also present in relatively high concentrations, however violaxanthin is only a minor constituent of the carotenoid content. This xanthophyll is usually associated with its photoconversion to zeaxanthin (Appendix I) in the chloroplast membranes but the absence of zeaxanthin in C. fragile suggests this alga lacks the epoxide cycle which is often found in higher plants (Krinsky, 1978).

Chlorophyll distribution in C. fragile confirms the work of Keast and Grant (1976) who report that members of the Siphonales have chlorophyll a:b ratios considerably below those of other plant groups. These ratios are particularly stable in C. fragile and although frond age affects pigment concentration the ratio of chlorophyll a:b and total chlorophyll:carotenoid remain remarkably constant (Tables 4 and 5). This suggests that although older fronds contain less pigment, control over pigment distribution in the chloroplasts is still maintained (Table 4).

Absorption spectra of pigments extracted from C. fragile are shown in Figures 11, 12 and 13, however these scans show pigment characteristics in vitro with solvent extracts. Although important for pigment identification, these scans do not relate the in vivo characteristics of the pigments when bound to their pigment/protein complexes within the chloroplast membranes. An indication of their in vivo characteristics is shown in Figure 14 the absorption spectrum of intact chloroplasts. From this spectrum it is evident that C. fragile absorbs strongly in the region 500-550nm, this is probably due to the pigments siphonoxanthin and siphonein which absorb at 540-542nm when protein bound (Kageyama et al, 1977; Kageyama and Yokohama, 1978). These authors have also shown that both pigments are able to transfer excitation energy to chlorophyll a thus suggesting a light-harvesting role. The relative abundance of both pigments in C. fragile may be indicative of a similar function in this alga.

2.5 CONCLUSIONS

Techniques for the separation, identification and quantitative determination of photopigments in *C. fragile* have been developed. Using these techniques it has been found that *C. fragile* chloroplasts contain a unique spectrum of carotenoids, notable by the absence of β-carotene and the presence of α and ε-carotene, siphonoxanthin, siphonein, neoxanthin and violaxanthin. The carotenes are present in low concentrations whereas siphonoxanthin and siphonein account for as much as 60% of the total carotenoids present by weight. Frond age determines the absolute concentrations of these pigments ($\mu g.g^{-1}$ fresh wt.) there being less pigment in mature tissue. However, pigment distribution and ratios remain constant despite the size of the frond.

3. PIGMENT/PROTEIN COMPLEXES IN C. FRAGILE CHLOROPLASTS

3.1 INTRODUCTION

C. fragile chloroplasts have a unique carotenoid and chlorophyll composition. β-Carotene is absent in this alga and α-carotene is the major carotene isomer together with traces of ε-carotene. Siphonoxanthin and siphonein are particularly abundant and chlorophyll a:b ratios in C. fragile chloroplasts are low compared to higher plants and non-siphonaceous algae.

The aim of this chapter is to investigate the functions of these pigments in C. fragile chloroplasts by determining their location in pigment/protein complexes as defined by sodium dodecyl sulphate/ polyacrylamide gel electrophoresis (SDS/PAGE).

3.2 MATERIALS AND METHODS

3.2.1 Isolation and solubilization of C. fragile thylakoids

3.2.1.1 Thylakoid isolation: C. fragile thylakoids were extracted using a modified method of Pallett and Dodge (1980) all extraction procedures were performed at 4°C, an MSE Chilspin was used for all centrifugation steps.

Extraction medium:-

0.05M Tris-HCL pH 8.0
0.8M Sucrose
0.02M Ethylene diamine tetra acetic acid (EDTA)

100-150g of C. fragile frond tips were placed in a chilled homogenizer containing 70-100ml of the above semi-frozen extraction medium. The fronds were macerated for 2 x 5 second bursts on the homogenizer and the macerate filtered through two layers of muslin into centrifuge tubes. After centrifugation at 1,000g for 30 seconds to remove frond debris the supernatant was removed and re-centrifuged at 3,700g. The supernatant

was discarded and the chloroplast pellet washed several times in the following washing medium, using 5 minute spin speeds of 3,700g.

Washing medium

0.0025M Tris-HCl pH 8.0
0.004M Glycine

The final pellet was re-suspended in 1-2ml of washing medium. An indication of free pigment content and pigment/protein disruption was the presence of chlorophyll in the final supernatant which was largely removed after 3 washes.

3.2.1.2 Thylakoid solubilization:- The chlorophyll content of the final thylakoid extract was determined by the method of MacKinney (1941) as described previously (Chapter 2). Chloroplast membranes were solubilized using a known ratio of sodium dodecyl sulphate (SDS): chlorophyll ($^{W}/_{w}$). Anderson, Waldron and Thorne (1980) used a ratio of 40:1 SDS:chlorophyll for thylakoid disruption in Caulerpa cactoides. A similar ratio of 50:1 SDS:chlorophyll was found to be optimum for C. fragile thylakoid disruption. Higher SDS ratios often caused a precipitation in the preparation and lower ratios sometimes produced 'streaking effects' on electrophoresis. Immediately after the addition of SDS the thylakoid preparation was mixed and centrifuged at 3,700g for 10 minutes to remove unsolubilized material. After centrifugation the supernatant appeared clear green and any opacity due to unsolubilized membranes was removed by a second centrifugation step at 3,700g. After successful solubilization the supernatant was carefully removed from the pellet and stored at 4°C in the dark. Electrophoresis was immediately performed on freshly prepared extracts.

3.2.2 The separation of pigment/protein complexes by preparative rod-SDS-PAGE

3.2.2.1 Preparation of 10% polyacrylamide gels:- Preparative rod-PAGE was performed on C. fragile thylakoid extracts using the method of Wild, Krebs and Ruhle (1980). Electrophoresis solutions were prepared as follows:-

Running buffer:-

0.05M Tris-HCl pH 8.0
0.025% (W/v) SDS

Gel buffer:-

0.01M Tris-HCl pH 8.0
0.5% (W/v) SDS
0.1% (V/v) N,N,N',N'-tetramethylethylene diamine (TEMED)

Acrylamide stock:-

30% (W/v) Acrylamide
0.8% (W/v) N,N' -methylene bis acrylamide

Ammonium persulphate:-

1% (W/v) ammonium persulphate (freshly prepared)

To prepare 10% acrylamide gels the above solutions were mixed as follows:-

Gel buffer	20.0ml
Distilled water	7.4ml
Acrylamide stock	30.0ml
Ammonium persulphate	2.6ml

From this polymerization mixture 25 gel rods (4.0mm internal diameter x 70 mm length) were prepared. The gels were cast to a filling height of 60mm and overlaid with approximately 10mm of distilled water to ensure oxygen exclusion and an even surface for sample application. The mixture usually polymerized within 30

minutes of preparation. However, gels were stored at 4°C for approximately 3 hours before sample application to ensure complete polymerization. Gels were pre-electrophoresed for 1 hour under standard running conditions to remove impurities and free acrylamide or persulphate.

3.2.2.2 Sample application and running conditions:- Approximately 20% (w/v) sucrose was added to the final thylakoid extract and 40-100µl were applied to the surface of the rod gels. Electrophoresis was performed using a Pharmacia gel electrophoresis (vertical) apparatus (GE-2/4) with a buffer circulator to prevent buffer and pH gradient formation. 3mA/gel were supplied using a Pharmacia power supply (EPS 500/400) for 5-10 minutes to concentrate the applied sample. The current was then increased to 6mA/gel to separate the protein complexes. The whole electrophoresis was completed after 30-40 minutes, corresponding to a migration distance of 3-5cm. During the time the apparatus was maintained in a dark incubator at 4°C.

3.2.2.3 Isolation and spectrophotometric characterisation of pigment/protein complexes:-

The aim of preparative PAGE was to rapidly isolate C. fragile pigment/protein complexes and spectroscopically identify them, the following technique proved a rapid means of extracting the complexes from their resolving gels. Rod tubes were filled to a height of 2cm with standard polymerisation mixture and allowed to set, after which the gel plug was overlaid to 4cm with running buffer. Pigment/protein complexes were extracted from the separating gel by carefully cutting out pigment containing bands. These bands were placed over the top of the running buffer in the 'plugged' tubes, care was taken to ensure continuity of buffer and gel surface.

The rods were placed in the electrophoresis unit and 6mA/gel applied; thus the complexes electrophoretically extracted from the separating gel and collected in the running buffer resevoir. The entire extraction procedure took 2-5 minutes depending on the volume of the pigment/protein plug. Pigment/proteins could be concentrated into a volume of approximately 0.5ml depending on the plug size and volume of resevoir buffer. After separation and recovery procedures the complexes were scanned using a Perkin Elmer 550S spectrophotometer. Running buffer was used as a blank and the complexes were scanned at $4^{o}C$ using a Perkin Elmer cooling unit.

3.2.3 The separation of pigment/protein complexes by analytical slab-SDS-PAGE:-

The aim of this investigation was to determine the location and, where possible, the relative concentration of chlorophyll and carotenoid pigments in the pigment/protein complexes of C. fragile.

3.2.3.1 Preparation of 10% polyacrylamide slab gels:- Exactly the same isolation and electrophoretic techniques were used as described in section 3.2.2. However, the following volumes of gel components were mixed together as a larger volume of polymerization mixture was required.

Gel buffer	90.0ml
Distilled water	22.2ml
Acrylamide stock	60.0ml
Ammonium persulphate	7.8ml

The gels were prepared using Pharmacia gel slab casting apparatus (GSC-8), gel dimensions were 2.7mm internal diameter and 80 x 80mm height and width. These gels required 1 hour to polymerize but were usually stored overnight at $4^{o}C$ to ensure complete polymerization. Before use the gels were pre-electrophoresed for 1-2 hours under normal running conditions.

3.2.3.2 Sample application and running conditions:- The sample was prepared as described in section 3.2.2.2 and approximately 200µg of chlorophyll were added to each slab gel. This corresponded to a final loading volume of 350-500µl depending on the chlorophyll content of the thylakoid extract. The gels were electrophoresed (using Pharmacia equipment) for 20-25 minutes at 12mA/4 gels to concentrate the applied sample then 120mA/4 gels for 35-40 minutes to complete the separation.

3.2.3.3 Extraction of pigments from pigment/protein complexes:- Due to practical difficulties it was not possible to electrophoretically extract pigment/protein complexes from slab gels. However as pigment recovery was more important than retaining the integrity of the whole complex the slower extraction technique of Wild, et al (1980) was used as follows:-

Elution medium:-

0.05M Tris-HCl pH 8.2
0.02% ($^{W}/_{v}$) SDS

Bands containing the pigment complexes were carefully removed from eight gels, corresponding bands were pooled and macerated using a pestle and mortar. The gel macerate was then suspended in 10-15ml of the above elution medium and placed in a dark incubator at 4^{o}C for overnight extraction of pigments. The eluate was then separated from the gel macerate by filtration through a Buchner funnel. To achieve complete extraction of the pigments the residue was washed several times with eluting medium. Scans of these filtrates using a Perkin Elmer 550S spectrophotometer were almost identical to those obtained from preparative rod gel SDS-PAGE. This suggests that the complexes were stable in their absorption characteristics even after several hours in elution buffer.

The pigment/protein eluate was diluted 50% ($^{v}/v$) with 100% acetone and allowed to stand for 15 minutes in the dark at 4°C. This mixture was then transferred to a 50ml separatory funnel and approximately 5ml of diethyl ether (AnalaR) added. The pigments were concentrated in the diethyl ether layer by frequent washings with 20% aqueous NaCl. The concentrated ether extract was then evaporated to 0.1-0.5ml under nitrogen prior to pigment separation and identification.

3.2.3.4 <u>The separation and quantitative determination of chlorophylls and carotenoids in pigment/protein complexes</u>:- Chlorophyll determination was performed according to the method of MacKinney (1941) as described in Chapter 2. For carotenoid determination 100µl of diethyl ether extract were spotted onto 7 x 3cm strips of silica gel G60 (Merck) and developed in 4% acetone in diethyl ether ($^{v}/v$). This solvent mixture retarded the migration of the chlorophylls thus preventing their overlapping of the carotene band. Carotene isomer separation was not attempted on MgO plates as concentrations of total carotene were limiting. After 5-10 minutes of development the pigment/silica gel strips were removed and the pigments eluted as described in Chapter 2. Pigment zones from two strips were pooled, thus increasing the final concentration of pigments to aid quantitative analysis. The pigment concentrations were very low and direct measurement on the spectrophotometer at the corresponding absorption maxima often gave readings < 0.1 absorbance units. Thus, to minimise errors concentrations were only determined from pigment scans. Pigments were quantified as described in Chapter 2, and the concentrations expressed as $\mu g.ml^{-1}$ diethyl ether. As absolute pigment concentrations varied as a consequence of the efficiency of the thylakoid extraction pigment distribution between and within the

complexes was quantified on a percentage basis.

i.e.

% distribution of pigment X between complexes a, b and c.

$$= \frac{(X) \text{ in a}}{(X)_a + (X)_b + (X)_c} \times 100$$

and % distribution of pigments X, Y, Z, within band a

$$= \frac{(X) \text{ in a}}{(X)_a + (Y)_a + (Z)_a} \times 100$$

Molar ratios were determined for pigments isolated from the pigment/protein complexes, formula weights for the respective pigments were calculated and the ratios based on the molarity of each pigment in the diethyl ether extracts. The formula weight of siphonein was estimated as the fatty acid associated with this pigment is unknown in C. fragile. Kleinig and Egger (1967) suggest that lauric acid is associated with siphonein in Caulerpa prolifera and this acid was used in the calculation of siphonein molar ratios in the pigment/protein complexes of C. fragile. Molar ratios represent means of 3 separate determinations the error term was calculated as standard deviation. Due to considerable variability in many of the ratios only molar ratios with s.ds of $< 20\%$ of the mean value were recorded. Formula weights of those pigments shown in section 3.3.2.2 are as follows:-

Pigment	f.weight
Chlorophyll a	892.35
Chlorophyll b	906.35
α-carotene	536.9
Siphonoxanthin	600.9
Siphonein	658.9

3.2.4 The separation and molecular characterisation of thylakoid proteins using rod-gel electrophoresis

The aim of this study was to use PAGE techniques for the molecular characterisation of proteins associated with C. fragile pigment/protein complexes.

3.2.4.1 Preparation of protein extracts:-

Calibration proteins A Pharmacia low molecular weight electrophoresis calibration kit containing lyophilized protein (section 3.2.4.6) was prepared for gel electrophoresis using a buffer containing:-

0.05M Tris-HCl, pH8.0
2.5% ($^{W}/_{v}$) SDS
5% ($^{V}/_{v}$) mercaptoethanol

The mixture was dissolved in 100µl of the above buffer and heated at 100°C for 5-10 minutes.

Sample proteins Total thylakoid extracts were prepared and solubilized as described in section 3.2.1.1 and 3.2.1.2. A third of the extract was retained for direct application and the remainder denatured using the following methods adapted from Mendiola-Morganthaler and Morganthaler (1974):-

Heat denaturation was achieved by adding 5% ($^{V}/_{v}$) mercaptoethanol to the thylakoid preparation, and heating the mixture at 100°C for two minutes. On cooling the extract was stored at -10°C. Non-heat denaturation was achieved by adding 5% ($^{V}/_{v}$) mercaptoethanol to the extract which was then transferred to an incubator at 4°C for 6 hours, after this period the extract was stored at -10°C.

Pigment/protein extracts were prepared as described in sections 3.2.3.1-3.2.3.3, and the complexes concentrated by dialysis against 1M sucrose using Visking tubing as the dialysis membrane. The pigment/protein extracts were denatured as described above.

3.2.4.2 Protein determination using the Folin Ciocalteu assay:- Protein determinations were performed on non-denatured total thylakoid and pigment/protein complex extracts using the Folin Ciocalteu assay (Lowry, Roseburgh, Farr and Randall, 1951) as follows:-

Reagent A

Folin Ciocalteu reagent (BDH) was diluted 1:1 ($^{v}/_{v}$) with distilled water.

Reagent B

50ml of 2% ($^{w}/_{v}$) Na_2CO_3 in 0.1N NaOH were added to 1ml of 0.5% ($^{w}/_{v}$) $CuSO_4.5H_2O$ in 1% ($^{w}/_{v}$) sodium potassium tartrate.

Protein standards were prepared from bovine serum albumin (BSA) Sigma type V over a calibration range 20-200$\mu g.ml^{-1}$ and assayed as follows:-

5ml of solution B was added to 1ml of protein standard or prepared extract. The solutions were mixed thoroughly and allowed to stand for 10 minutes at room temperature. After this period of time 0.5ml of 1:1 ($^{v}/_{v}$) Folin Ciocalteu reagent was added and after immediate mixing the samples were maintained at room temperature for 30 minutes. The absorbance of the samples at 750 nm was determined using a Perkin Elmer 550S spectrophotometer. Standard calibration plots were used to determine the protein content of the sample extracts. Protein determinations were not performed on mercaptoethanol containing extracts, as this reagent interferes with the assay.

3.2.4.3 Preparation of polyacrylamide rod gels:- Resolving gels were prepared as described in section 3.2.2.1, incorporating a stacking gel prepared according to Laemmli (1970) as follows:-

Stacking gel buffer:-

0.1M Tris-HCl, pH6.8
0.5% ($^{W}/_{v}$) SDS

To prepare a 3% polyacrylamide stacking gel the following solutions were mixed:-

Stacking gel buffer	10ml
Acrylamide stock as in 3.2.2.1	2ml
TEMED	5µl
1% Ammonium persulphate	0.5ml
Distilled water	7.5ml

The surface of the resolving gel was dried and the stacking gel carefully applied to a height of 10mm above the resolving gel. The gel was allowed to polymerize (approximately 30 minutes) and pre-electrophoresed for 1-2 hours under normal running conditions.

3.2.4.4 Sample application and running procedures:- 20% ($^{W}/_{v}$) sucrose was added to the thylakoid protein extracts and 20-100µg of protein were applied per sample gel. 3µl of calibration protein extract were added per calibration gel and 1µl of 0.5% ($^{W}/_{v}$) bromophenol blue in 20% ($^{W}/_{v}$) sucrose solution was added as a tracking dye to each gel. The gels were electrophoresed as described in section 3.2.2.2 until the marker had travelled the complete distance of the gel, (approximately 3 hours).

3.2.4.5 Protein staining and de-staining procedures:- Before staining the gels were fixed for $\frac{1}{2}$ hour in a 10% ($^{W}/_{v}$) sulphosalicylic acid solution. The staining solution was composed of 0.1% ($^{W}/_{v}$) Cooma ssie blue G in 25% methanol, 10% acetic acid and water (v/v/v) and was filtered before use. After removal from the glass rods, gels were immersed in the fixing solution, removed and stained overnight. The destaining solution consisted of 25% methanol, 10% acetic acid, 2.5% glycerol and water (v/v/v/v). After staining the gels were immersed in the

destainer which was changed every hour up to six hours and then after 24 hours until the stain had been removed.

3.2.4.6 Molecular weight determination of thylakoid proteins:- The Pharmacia low molecular weight calibration kit used in this study consisted of the following proteins which on denaturation produced the corresponding polypeptide subunits used as molecular weight markers:-

Protein	Mol.wt.of subunit (KD)
Phosphorylase b	94.0
Albumin	67.0
Ovalbumin	43.0
Carbonic anhydrase	30.0
Trypsin inhibitor	20.1
α-lactalbumin	14.4

protein mobility was expressed as a relative value to the most mobile protein α-lactalbumin, i.e.

$$R_f = \frac{\text{d moved by protein}}{\text{d moved by } \alpha\text{-lactalbumin}}$$

Where d was measured from the start of the resolving gel. Calibration graphs were drawn as Log_{10} molecular weight (ordinate) and R_f (abscissa). Typical regression analyses of the standard curves obtained produced the following characteristics:-

Intercept = 5.033 Correlation = 0.9576
Slope = -(0.881)

The characterisation of molecular size was determined on 5-8 replicates for total thylakoid extracts and 2-3 for pigment/protein extracts. Error values for these determinations refer to s.d.s of the mean. Total thylakoid and pigment/protein extracts were examined under ultra violet light (Handovia fluorescent lamp) for chlorophyll fluorescence and fluorescent bands are indicated 'f' on the appropriate figures.

3.3 RESULTS

3.3.1 The isolation and spectrophotometric characterization of pigment/protein complexes

Figure 15 shows a typical electrophoretic separation of pigment/protein complexes isolated from C. fragile fronds sampled during November 1981 and March 1982. The band nearest the origin was minor, pale green in colouration and difficult to extract due to its low pigment content whereas the second band was bright green in colouration suggesting a relatively high chlorophyll a content. These bands were identified by their electrophoretic behaviour as CPla and CP_1 respectively, the P700 chlorophyll a proteins described by Anderson, Waldron and Thorne (1980). The third and fifth bands were the most intense zones separated on the gel, and were dark green in colour suggesting that considerable amounts of chlorophyll b were associated with them. These bands were identified as the light-harvesting pigment/protein complexes (LHCPS). Occasionally a faint band was observed between the LHCP's, although this band contained pigment it was highly unstable and not identified spectroscopically. Anderson et al (1980) suggest that this band (CPa) may be associated with PSII which is normally labile using SDS-PAGE (Siefermann-Harms and Ninneman, 1979; Markwell, Miles, Boggs and Thornber, 1979). The sixth and most mobile band on the gel corresponds to the free pigment zone. Figure 16 shows the absorption spectra of thylakoids and pigment/protein complexes extracted from C. fragile chloroplasts. Of particular note is the shoulder at 540-542nm present in total thylakoid extract and the light-harvesting complexes. This corresponds to the in vivo peak of siphonoxanthin and siphonein as described by Kageyama, Yokohama Shimura and Ikawa (1977) and Kageyama and Yokohama (1978). A 540nm shoulder was not present in CP_1a CP_1 or FP (Figure 16). The presence of chlorophyll b in the light-harvesting complexes was indicated by absorbance at 652nm and a particularly strong peak in the blue region of the absorption spectra at 470-475nm.

FIGURE 15 : - THE SEPARATION OF PIGMENT/PROTEIN COMPLEXES FROM C.FRAGILE USING SDS/PAGE.

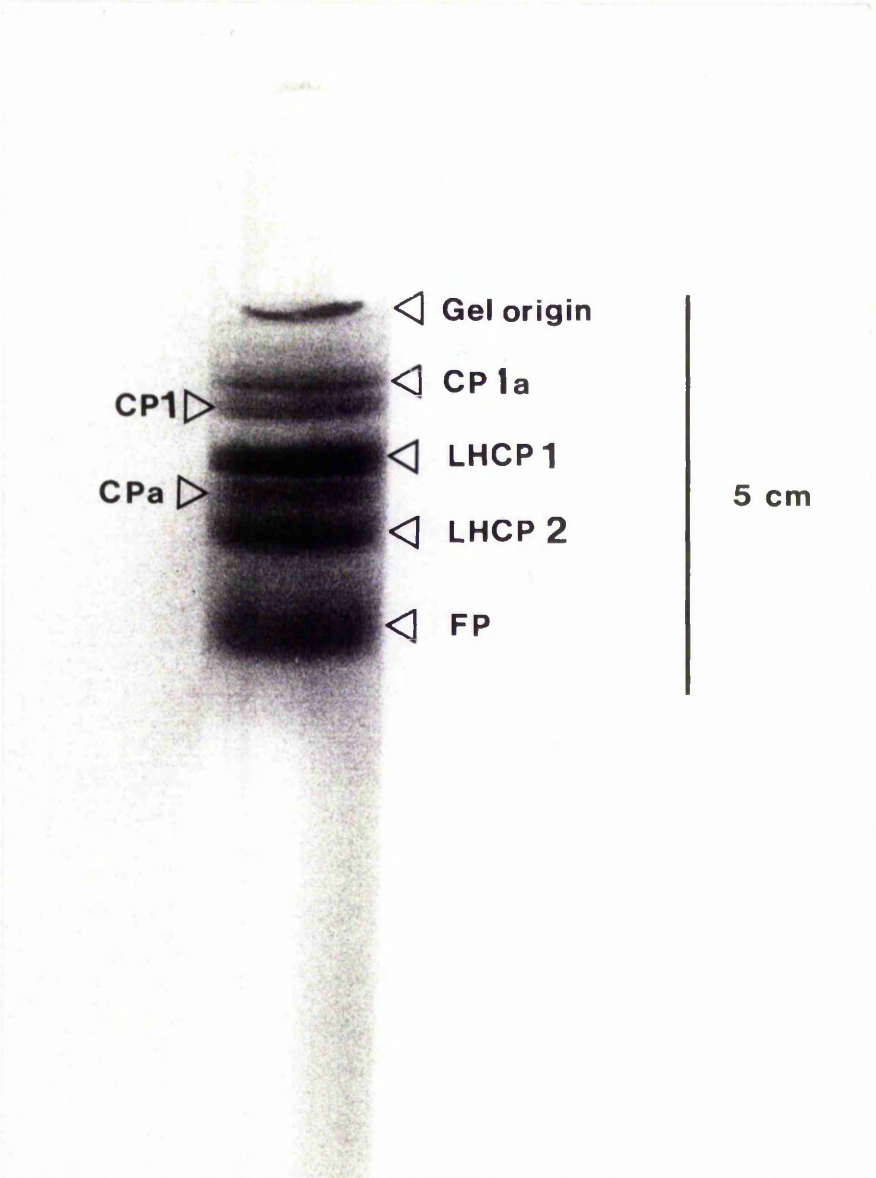
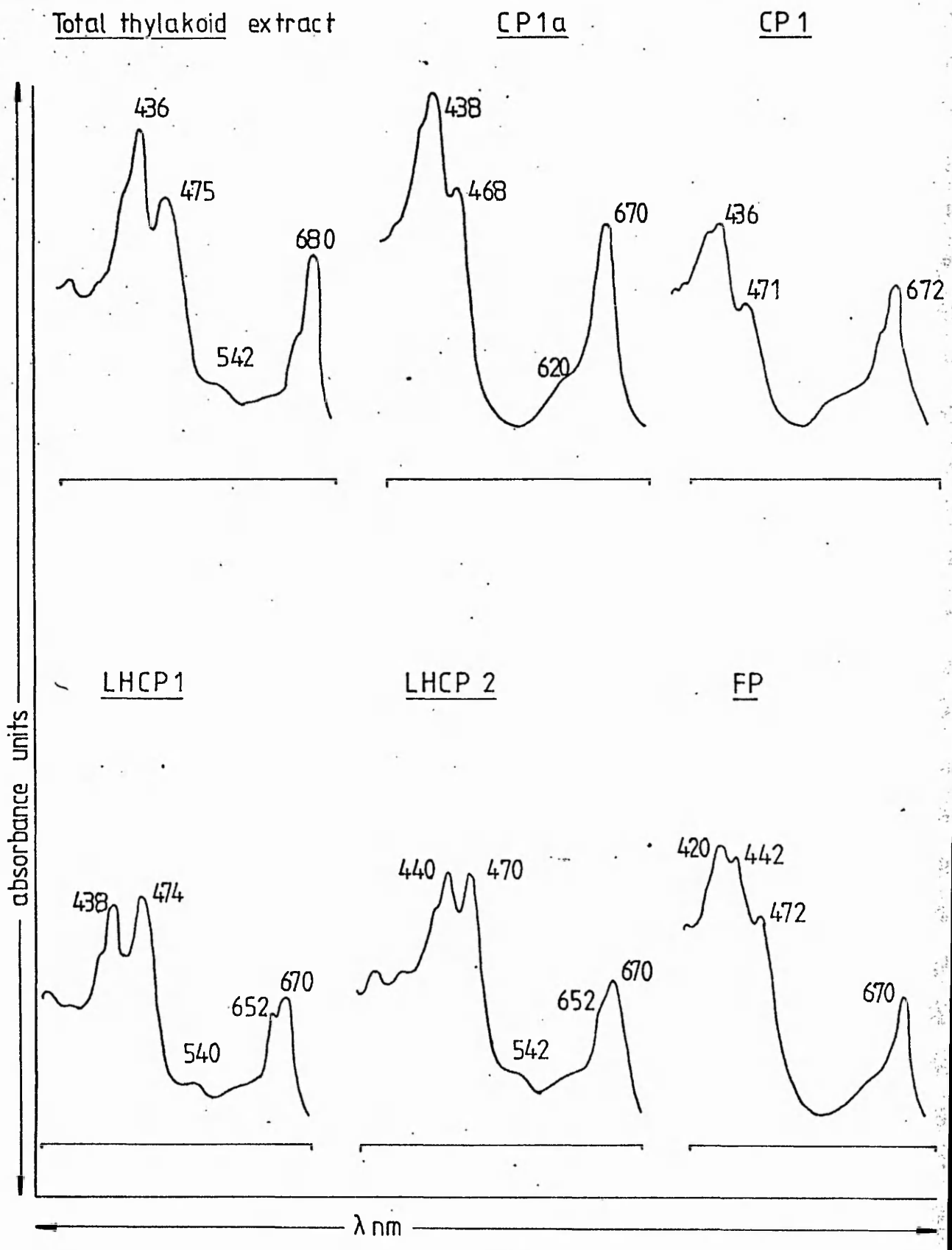


FIGURE 16 :- ABSORPTION SPECTRA OF C.FRAGILE

THYLAKOIDS AND PIGMENT/PROTEIN COMPLEXES

Scanning medium = SDS / Tris - HCL buffer pH 8·0


Total thylakoid extract
436
475
542
680
CP1a
438
468
620
670
CP1
436
471
672
LHCP1
438
474
540
652
670
LHCP 2
440
470
542
652
670
FP
420
442
472
670
absorbance units
λ nm

This absorption may also be due to the presence of protein bound carotenoids (Ogawa, Nakamura and Shibata, 1975). Scans of CP_1a and CP_1 show relatively small peaks for chlorophyll b in both red and blue regions of the absorption spectra.

3.3.2 The quantitative determination of pigments in pigment/protein complexes

3.3.2.1 Composition of the free pigment zone:- Using protein staining procedures the free pigment zone (FP) was shown to be a composite band of chlorophyll and carotenoid pigments which were not protein bound. Thus the composition of this band reflects the stability of the pigment/proteins and/or the degree of association of pigments in the complexes. Table 6 shows the proportion of total gel pigment associated with F.P. and has been calculated as % distribution (calculated as described in section 3.2.3.4). The error term in the following tables refers to ± s.d. of far different thylakoid extracts from separate C. fragile plants.

Table 6 THE PROPORTION OF TOTAL GEL PIGMENT ASSOCIATED WITH THE FREE PIGMENT ZONE

PIGMENT	% DISTRIBUTION ± s.d.
Chlorophyll a	15.8 ± 4.8
Chlorophyll b	11.5 ± 6.8
α-Carotene	26.2 ± 8.7
Siphonoxanthin	50.5 ± 24.3
Siphonein	25.6 ± 13.1
Neoxanthin	35.4 ± 19.5
Violaxanthin	81.0 ± 20.8
Total chlorophyll	13.6 ± 5.5
Total carotenoid	29.0 ± 21.0

The FP zone contained proportionally more carotenoid pigments than chlorophylls and pigment composition was very variable as reflected by the high s.d. values. The overall chlorophyll a:b ratio of the total chlorophyll recovered from the gels was very constant at 0.89 ± 0.06 and shows chlorophyll b enrichment. This suggests that chlorophyll a may be lost before PAGE as chlorophyll a:b ratios of fronds and isolated chloroplasts were 1.5 (Chapter 2). The FP zone was enriched in violaxanthin compared to the pigment complexes.

Table 7 shows the % distribution of the pigments within the FP zone, pigment concentration is expressed as either total chlorophyll content or total carotenoid content of the FP.

Table 7 DISTRIBUTION OF PIGMENTS WITHIN THE FREE PIGMENT ZONE

PIGMENT	% DISTRIBUTION ± s.d.
Chlorophyll a	59.0 ± 15.0
Chlorophyll b	41.2 ± 15.0
α-carotene	14.0 ± 2.5
Siphonoxanthin	57.0 ± 13.0
Siphonein	11.0 ± 3.2
Violaxanthin	6.6 ± 3.8
Neoxanthin	20.0 ± 2.9

The distribution of chlorophyll a and b in the FP is almost equal, however carotenoid distribution within the zone can vary considerably. Siphonoxanthin accounts for almost 60% of the total carotenoid content, and α-carotene neoxanthin and siphonein almost all the remainder. Violaxanthin contributes only 6.6 ± 3.8% reflecting the low concentration of this pigment on a frond fresh weight basis (Chapter 2).

3.3.2.2 Composition of the pigment/protein complexes:-
Table 8 shows the distribution of pigments between the complexes CPIa, CP_1, $LHCP_1$ and $LHCP_2$, calculated on a % basis. The comment 'trace amounts' in this table refers to pigments detected on TLC plates but undetectable on eluate scans. The variability of siphonoxanthin, siphonein and neoxanthin in CP_1 refers to variable detection of these pigments in other than trace amounts. In two out of the four thylakoid extracts all three xanthophylls were measurable in CP_1, however in the remaining extracts only trace amounts were detected.

α-Carotene was the only pigment in CP_1a and CP_1 to show a consistant and measurable presence, violaxanthin was not detected in these complexes. It is possible that the variability in xanthophyll content of CP_1a and CP_1 was due to contamination from $LHCP_1$ when the bands were removed from the gels. The light-harvesting complexes contain 75% of the total chlorophyll and approximately 75% of the total carotenoids (dependent on the variability of xanthophyll distribution in CP_1). All major carotenoid pigments detected in C. fragile are present in the light-harvesting complexes in measurable amounts.

Table 8 DISTRIBUTION OF PIGMENTS BETWEEN PIGMENT/PROTEIN COMPLEXES

PIGMENT	% DISTRIBUTION BETWEEN PIGMENT/PROTEIN ± s.d.			
	CP_1a	CP_1	$LHCP_1$	$LHCP_2$
Chlorophyll a	10.5 ± 4.0	23.0 ± 4.0	38.0 ± 7.8	29.0 ± 8.9
Chlorophyll b	9.3 ± 5.1	14.6 ± 5.7	47.0 ± 11.0	30.0 ± 11.0
α-Carotene	25.0 ± 18.0	32.0 ± 17.0	21.2 ± 13.0	22.0 ± 13.0
Siphonoxanthin	TRACE	VARIABLE	50.0 ± 9.7	37.0 ± 3.0
Siphonein	"	"	60.0 ± 15.0	21.0 ± 9.0
Neoxanthin	"	"	56.7 ± 7.0	32.0 ± 11.0
Violaxanthin	NOT PRESENT	NOT PRESENT	TRACE	TRACE
Total chlorophyll	10.0 ± 4.7	18.7 ± 4.7	43.0 ± 9.2	29.0 ± 10.0
Total carotenoid	4.7 ± 5.6	15.5 ± 5.3	43.2 ± 10.0	30.1 ± 9.8

Table 9 shows the distribution of pigments within the pigment/protein complexes when calculated on a percentage basis. Total pigment refers to either total carotenoid or total chlorophyll content.

Table 9 DISTRIBUTION OF PIGMENTS WITHIN PIGMENT/PROTEINS COMPLEXES

PIGMENT	% DISTRIBUTION WITHIN PIGMENT/PROTEINS ± s.d.			
	CP_1a	CP_1	$LHCP_1$	$LHCP_2$
Chlorophyll a	50.0 ± 5.9	58.0 ± 8.3	41.0 ± 2.6	47.0 ± 2.6
Chlorophyll b	50.0 ± 5.4	42.0 ± 7.9	59.0 ± 2.0	53.0 ± 2.5
Chlorophyll a:b	1.0 ± 0.2	1.4 ± 0.5	0.69 ± 0.08	0.84 ± 0.05
α-Carotene	100.0 ± 0	50.0 ± 38.0	15.0 ± 11.0	15.0 ± 7.0
Siphonoxanthin	TRACE	VARIABLE	41.3 ± 10.0	40.0 ± 7.4
Siphonein	"	"	23.0 ± 8.0	16.0 ± 3.3
Neoxanthin	"	"	26.6 ± 6.0	29.0 ± 7.0
Violaxanthin	NOT PRESENT	NOT PRESENT	TRACE	TRACE

The data in Table 9 is supportive of that in Table 8. α-Carotene is the major carotenoid in CP_1a and CP_1 although the variable xanthophyll content (Trace - 50%) of CP_1 effects the % distribution of α-carotene in this complex. Pigment distribution in the light-harvesting complexes is very similar, siphonoxanthin is the major carotenoid present, followed by neoxanthin. Chlorophyll a:b ratios show an enrichment in chlorophyll a in CP_1a and CP_1 and chlorophyll b in $LHCP_1$ and $LHCP_2$.

A further comparison of pigment distribution within pigment/proteins was attempted using molar ratios. However, these proved to be extremely variable and only those ratios showing the greatest consistency are quoted in Table 10.

Table 10 MOLAR RATIOS OF PIGMENTS WITHIN PIGMENT/PROTEIN COMPLEXES

COMPLEX	PIGMENT:PIGMENT	RATIO ± s.d.
CP_1	Chlorophyll a: α-Carotene	90.0 ± 16.6
$LHCP_1$	Chlorophyll a:Siphonoxanthin	44.0 ± 7.0
	Chlorophyll b:Siphonoxanthin	59.0 ± 10.0
	Chlorophyll a:b	0.7 ± 0.08
$LHCP_2$	Chlorophyll a:b	0.86 ± 0.047
	Siphonoxanthin:Siphonein	5.2 ± 0.46

C. fragile plants sampled from the same harvest as used in pigment/protein studies were used for whole frond pigment analyses. Table 11 compares the distribution of pigments in whole fronds (based on μg pigment.g^{-1} fresh weight) with distributions in total gel extracts (based on μg pigment.gel $extract^{-1}$). Carotenoid distributions based on percentages of total carotenoid in gel or frond extracts are very similar as are xanthophyll:carotene ratios. However chlorophyll a:b ratios and chlorophyll:carotenoid ratios show considerable differences.

Table 11 A COMPARISON OF PIGMENT DISTRIBUTION IN GEL AND FROND EXTRACTS

PIGMENT	PIGMENT/GEL EXTRACT ± s.d. (n=4)	PIGMENT/FROND EXTRACT ± s.d. (n=4)
% DISTRIBUTION OF CAROTENOIDS		
α-carotene	16.1 ± 1.3	9.7 ± 1.3
Siphonein	17.3 ± 5.2	19.0 ± 2.1
Siphonoxanthin	43.8 ± 2.3	41.1 ± 2.6
Neoxanthin	22.4 ± 4.8	26.1 ± 4.1
PIGMENT RATIOS		
Chlorophyll a:b	0.89 ± 0.06	1.5 ± 0
Xanthophyll:carotene	5.2 ± 0.6	9.1 ± 1.7
Total chlorophyll:carotenoid	85.0 ± 2.5	13.5 ± 1.5
Total chlorophyll: α-carotene	388.3 ± 140.0	142.0 ± 36.0
Total chlorophyll:siphonein	423.5 ± 140.0	71.0 ± 14.0
Total chlorophyll:siphonoxanthin	290.0 ± 73.0	52.0 ± 10.0
Total chlorophyll:neoxanthin	160.0 ± 20.0	32.5 ± 3.1

3.3.3 The characterisation of thylakoid proteins using rod-SDS PAGE

3.3.3.1 Non-denatured extracts:- The characterisation of proteins from non-denatured extracts is shown in Figure 17. Three major bands were resolved in the total thylakoid extract (molecular weights, 93.4 ± 2.8, 68.2 ± 1.0 and 60.3 ± 1.1 KD) and several intermediately stained bands with molecular weights of 15-55 KD were also present. Using ultraviolet light, four chlorophyll bands (f on Figures 17-19) were detected with molecular weights of 15.0 ± 0.66, 23 ± 0.5, 68.2 ± 1.0 and 93.4 ± 2.8 KD. The resolution of protein bands from CP_1a and CP_1 extracts was inconclusive as the applied samples were retained at the gel origin. A high molecular weight protein

(100 ± 3.0 KD) was detected on two CP_1 gels. Two major protein bands (molecular weights 71.4 ± 4 and 56. ± 2.4 KD) were observed in $LHCP_1$ and both showed chlorophyll fluorescence, together with a less intense non-fluorescent band of molecular weight 32 ± 2.8 KD. $LHCP_2$ protein bands were slightly different to those of $LHCP_1$ with two fluorescent bands of 36 and 18 KD. No protein bands were observed on CP_a and FP extracts this may have been due to low loading of the gel.

3.3.3.2 Denatured extracts:- Thylakoid extracts denatured with 5% (v/v) mercaptoethanol in the absence of heat produced one band of intermediate stain at 52 KD and several minor bands of higher molecular size (100-55 KD) one of which (67.1 KD) fluoresced under ultraviolet light (Figure 18). Two bands were resolved with molecular weights of 36.8 ± 1.3 and 27.2 ± 0.32 KD together with a third fluorescent band of 15.2 KD. A low molecular weight polypeptide (21.2 ± 0.8 KD) was also observed.

Denatured CP_1a and CP_1 extracts were totally resolved on the gels (Figures 18 and 19) and no retention at the origin was apparent. Non-heat denatured CP_1a displayed one band of 53.1 ± 1.7 KD and CP_1 showed two bands of 62.3 ± 7.7 and 32.6 KD. Denatured $LHCP_1$ extracts consisted of several minor components one of which (57 KD) fluoresced, an intermediate band of 30.5 KD was also observed. $LHCP_2$ consisted of two minor components of which one (57 KD) fluoresced and an intermediate non-fluorescent band of 30.5 KD as present in $LHCP_1$. The profile of denatured CP_a extracts was very similar to those of the LHCP's with protein components of 57, 54 and 30.5 KD. The FP band was composed of two minor bands which were probably contaminants from the light-harvesting complexes, and a major low molecular weight polypeptide of 18 KD.

KEY FOR FIGURES 17—19

r = retention at gel origin

f = chlorophyll fluorescent band

intensity of stain:-

—— = major band

___ = intermediate band

---- = minor band

npr = no protein resolved

FIGURE 17:- NON-DENATURED PROTEIN PROFILES OF THYLAKOID AND PIGMENT/PROTEIN COMPLEX EXTRACTS FROM C.FRAGILE.

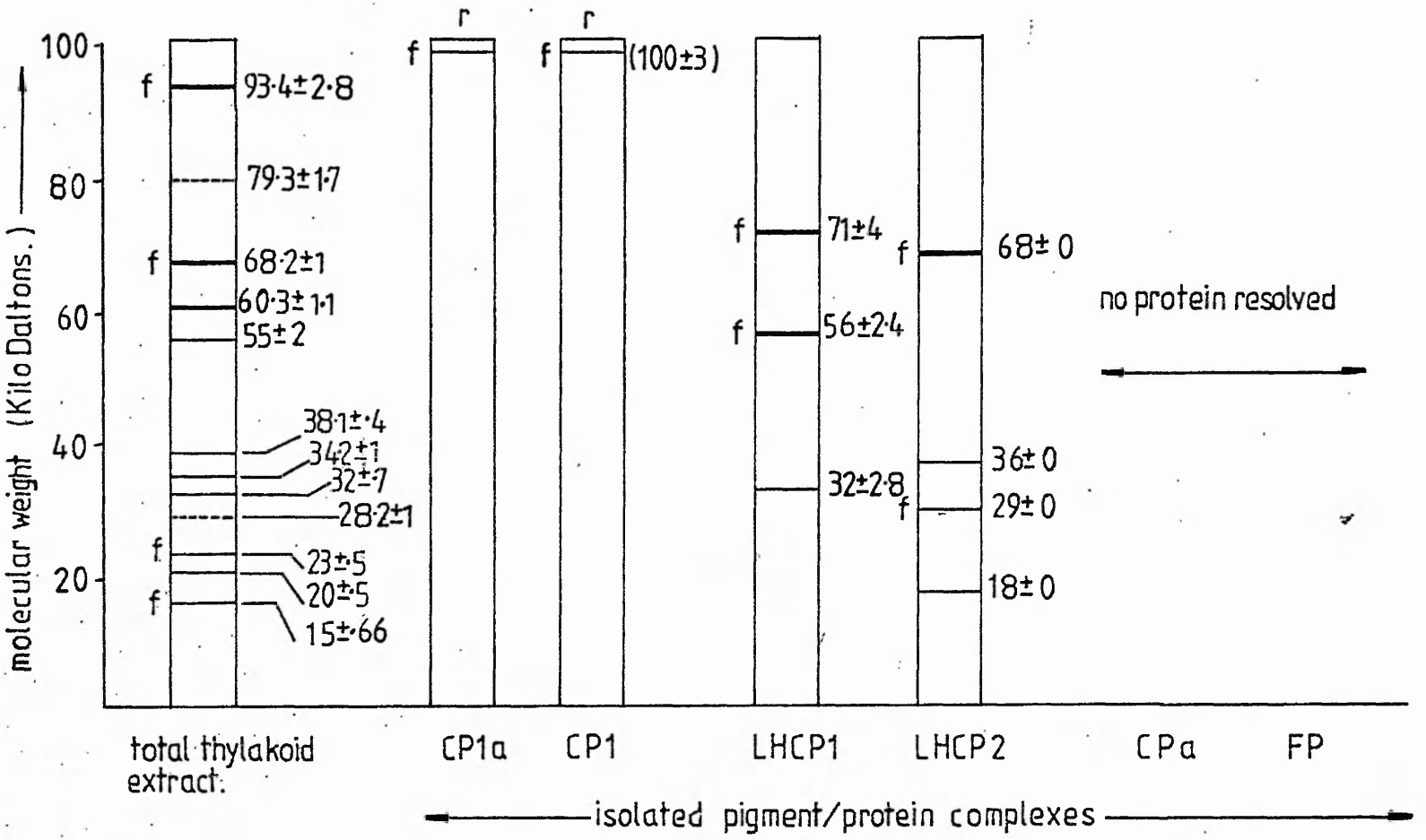


FIGURE 18: – DENATURED PROTEIN PROFILES OF THYLAKOID AND PIGMENT/PROTEIN COMPLEX EXTRACTS FROM C. FRAGILE

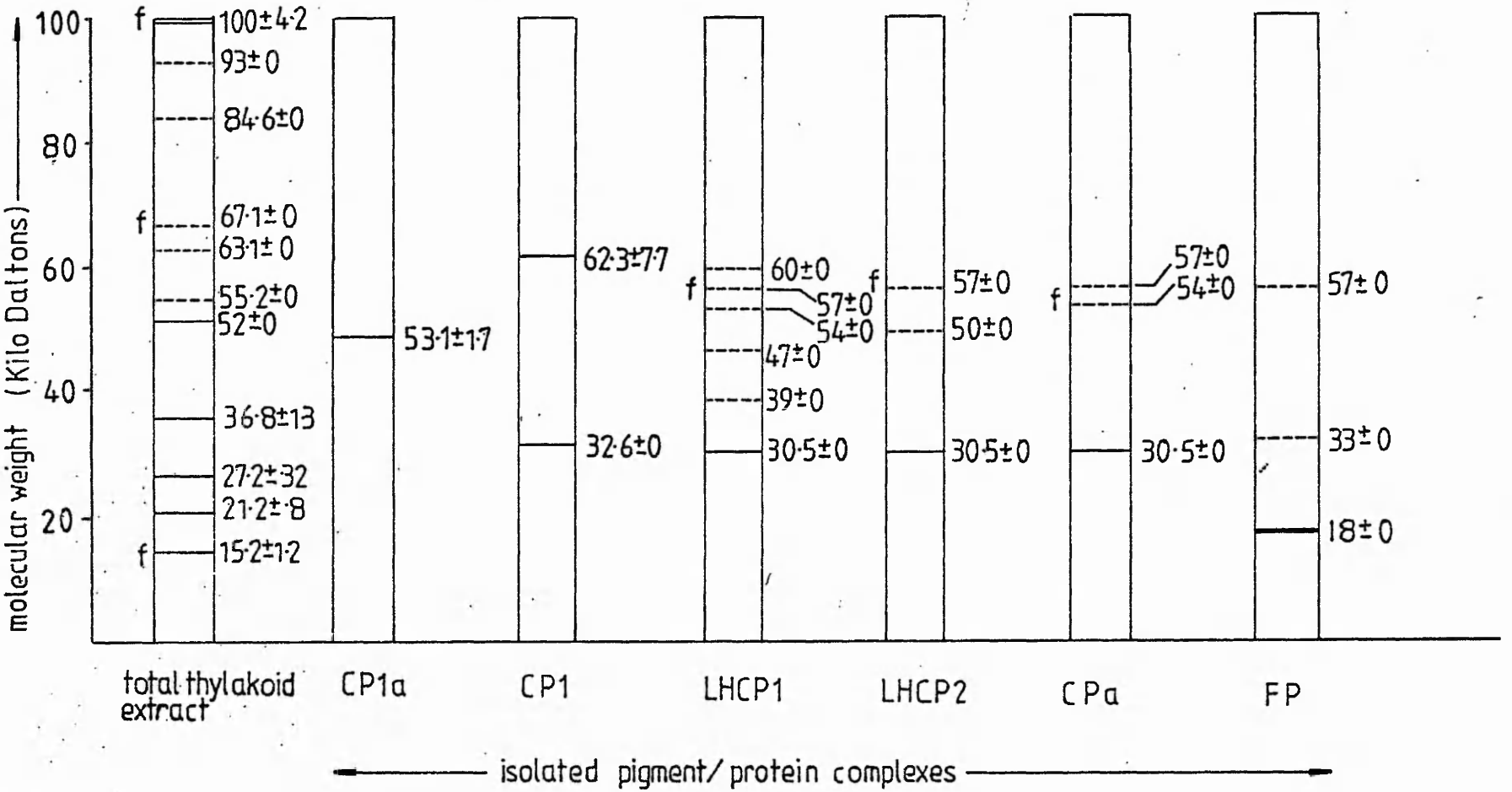
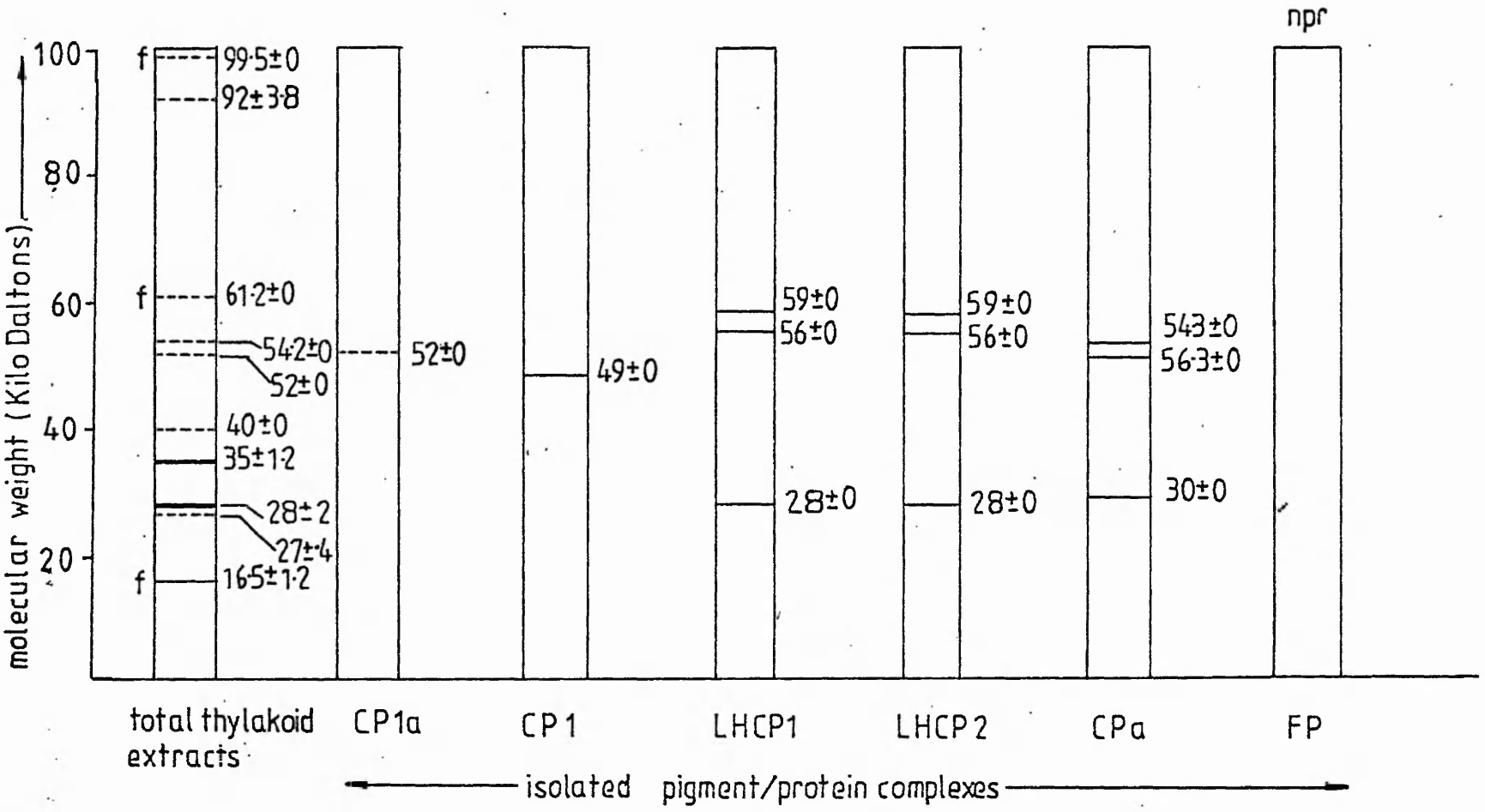


FIGURE 19: - HEAT—DENATURED PROTEIN PROFILES OF THYLAKOID AND PIGMENT/PROTEIN COMPLEX EXTRACTS FROM C.FRAGILE.



Thylakoid extracts which were heat denatured in the presence of mercaptoethanol (Figure 19) produced two major bands at 35 ± 1.2 and 28 ± 2.0 KD and three minor chlorophyll fluorescent bands (99.5, 61.2 and 16.5 KD). Single bands were observed in CP_1a and CP_1 extracts with molecular weights of 52 and 49 KD respectively. $LHCP_1$ and $LHCP_2$ produced identical profiles of 59, 56, and 28 KD, a similar pattern was observed for CP_a with molecular weights of 54.3, 56.3 and 30 KD. The free pigment extract was protein-free and none of the pigment/protein extracts which were heat treated fluoresced under ultraviolet light.

3.4 DISCUSSION

3.4.1 Experimental procedures

The successful characterisation of chloroplast pigment/proteins is largely dependant on the solubilization and electrophoretic techniques employed (Chapter 1). The methods used in this study were based directly on the electrophoretic system of Wild *et al* (1980) and the separation of pigment/proteins from *C. fragile* was comparable with the electrophoretic pattern observed for *Sinapsis alba* by these authors. Using the nomenclature described in section 1.3 the pigment/protein complexes extracted from *C. fragile* thylakoids were named as follows:- CPa CP_1a, CP_1, $LHCP_1$ and $LHCP_2$. The least mobile bands were identified as pigments/proteins associated with PSI (P-700 complexes) and $LHCP_1$ and $LHCP_2$ were identified as chlorophyll a/b light-harvesting complexes. CP_a was tentatively identified as a chlorophyll-protein associated with PSII, however, this complex was never successfully characterised in any gel preparation. FP refers to the free-pigment zone which becomes dissociated from the protein complexes and forms the most mobile band resolved on the gel.

The use of SDS-PAGE as a technique for characterising pigment/protein complexes has been considerably exploited (e.g. Thornber and Alberte, 1977, Chua 1980). However, one of the inherent problems with this technique is the inevitable release of pigments from the complexes together with alterations in complex structure which often accompany the PAGE and solubilization procedures. Direct evidence for these effects is shown in Table 11. This data compares the distribution of carotenoids and chlorophylls in whole fronds (μg pigment.g^{-1} frond f.wt) extracts and gel eluates (μg. pigment.gel $extract^{-1}$) for tissue sampled during March 1982. Carotenoid distributions in both systems are comparable, however, chlorophyll a:b ratios indicate a loss of chlorophyll during solubilization. The ratio normally obtained from intact chloroplasts is 1.5 (Chapter 2) whereas in gel extracts the ratio was 0.89. This is reflected further in the high and variable chlorophyll:carotenoid ratios in gel extracts (Table 11). Thus, despite the apparent stability of carotenoid distributions within gel extracts it is also evident that considerable amounts of carotenoid pigments are also lost on solubilization.

The resolution of pigment/protein complexes on SDS-PAGE is dependent in part on the complete solubilization of the thylakoid membrane (Thornber and Alberte, 1977). It is apparent that particularly high SDS:chlorophyll ($^{w}/w$) treatments are required for total membrane solubilization in siphonaceous algae. *Caulerpa cactoides* requires 40:1 ($^{w}/w$) SDS:chlorophyll (Anderson *et al*, 1980) and *C. fragile* 50:1 ($^{w}/w$) in contrast to *Sinapsis alba* which required 10:1 ($^{w}/w$), (Wild *et al* 1980). An indication of pigment/protein complex stability after detergent solubilization is given by the composition of the FP zone. The inevitable release of pigments from the complexes results in their migration to the free pigment band typical of SDS-PAGE. Therefore, before any quantitative assessment of pigment distribution in the complexes is attempted the nature and composition of FP must be investigated.

Table 6 shows the relative distribution of pigments in the FP band. Of the total chlorophyll present in the gels 13.6 ± 5.5% occurred as FP, and this was composed of almost equal proportions of chlorophyll a and b. Using a similar SDS-PAGE system Anderson et al. (1980) reported a yield of 10% free chlorophyll and these results suggest that the vigorous solubilization procedures required for siphonaceous chloroplasts do not produce large quantities of free chlorophyll on electrophoresis. Wild et al (1980) suggest that most of the chlorophyll in FP extracts from S. alba was derived from the light-harvesting complexes, CP_1 proteins being quite stable on PAGE. The presence of very high concentrations of light-harvesting xanthophylls in the FP of C. fragile extracts may support these observations. The carotenoid composition of FP appeared to be particularly unstable as demonstrated by large s.d. values in Tables 6 and 7. Similarly there are proportionally more carotenoids (29 ± 21%) than chlorophylls (13.6 ± 5.5%) in the FP zone. These results indicate that either carotenoid pigments were easily detached from the proteins (as suggested by Siefermann-Harms, 1980) or, a certain proportion of them exist in a free form, or bound to areas of the chloroplast other than the pigment/protein complexes. This is particularly evident for violaxanthin, 81 ± 20.8% of which occurred as FP in C. fragile thylakoid extracts. This distribution may be due in part to the different binding properties of this pigment (Siefermann-Harms, 1980). Violaxanthin is particularly enriched in the envelopes of higher plant chloroplasts (Siefermann-Harms, 1980; Lichtenthaler, Prenzel and Kuhn, 1982) where it functions in the epoxide cycle (Siefermann-Harms, Joyard and Douce, 1978). The absence of zeaxanthin in C. fragile chloroplasts also suggests the absence of the epoxide cycle in the chloroplast envelope of this alga. However, since almost all the pigment is present as FP violaxanthin is probably not bound to the pigment/protein complexes either, except perhaps in trace amounts.

Table 7 demonstrates the distribution of pigments within the FP band. The chlorophylls do not show a preferential accumulation, but it is significant that siphonoxanthin comprises almost 60% of the total free carotenoid content, followed by neoxanthin (20%). It therefore appears that on electrophoresis C. fragile thylakoid extracts yield large amounts of non-protein bound siphonoxanthin. This phenomenon may merely reflect the relatively high concentrations of siphonoxanthin present in C. fragile chloroplasts or that this pigment is easily dissociated from the complexes.

3.4.2 Pigment characterization of pigment/protein complexes isolated from C. fragile

Figure 16 shows the absorption spectrum of isolated C. fragile thylakoids in SDS-Tris-HCL buffer (pH 8.0). This scan has a prominent absorption in the blue region and a marked shoulder at 542nm, these peaks being due to the presence of large amounts of chlorophyll b and siphonoxanthin and siphonein respectively. Kageyama et al (1977) and Kageyama and Yokohama (1978) have shown that the characteristic in vivo absorbance at 540nm in the algae Dichotomosiphon tuberosus, Ulva japonica and Ulva pertusa was due to siphonein and siphonoxanthin. Ogawa, Nakamura and Shibata (1975) also describe this characteristic shoulder in Bryopsis maxima and Anderson, Barret and Thorne (1981) report siphonoxanthin in vivo absorbance at 540nm in Caulerpa cactoides. Using fluorescence emission techniques Kageyama et al (1977) have demonstrated the transmission of excitation energy from siphonoxanthin to chlorophyll a in U. japonica and U. pertusa. Kageyama and Yokohama (1978) have also shown that siphonein has the same function in Dichotomosiphon tuberosus and these authors concluded that siphonein and siphonoxanthin function as light-harvesting pigments in Chlorophycean algae. Further evidence for this proposal is inferred by comparing the absorbance spectra of CP_1a, CP_1 and FP with those of the LHCPs (Figure 16). Both light-harvesting complexes have characteristic shoulders at 540nm, however the P-700 complexes and FP lack this shoulder despite the fact that measurable amounts of siphonein and

siphonoxanthin were detected in them. It therefore appears that these pigments function in a light-harvesting capacity in C. fragile and this is directly inferred by their enriched location in $LHCP_1$ and $LHCP_2$ together with their characteristic absorbance at 540nm which only occurs in these complexes.

The absorption spectra of CP_1a and CP_1 complexes isolated from C. fragile are comparable to those described for other Chlorophycean algae (Ogawa et al 1975). Figure 16 shows the absorbance spectrum of the FP zone, however, this is a representative scan as variability in pigment composition was also reflected by variability of absorption spectra.

One of the major difficulties in this investigation was the quantitative assessment of pigment distribution in the isolated complexes. Frond pigment concentrations were extremely limiting in summer-sampled tissue and successful quantitative analyses were only performed on fronds sampled during March 1982 when the pigment content of fronds was very high. Only the major carotenoids present in C. fragile could be quantitatively assessed and those pigments present in low concentrations (ε-carotene and violaxanthin) were not assayed with success.

Tables 8 and 9 show the distribution of individual pigments both within and between the pigment/protein complexes separated from C. fragile and demonstrates the considerable differences in pigment distribution. The pigment composition of P700 complexes is debatable, Alberte and Thornber (1978) state that these chlorophyll complexes contain chlorophyll a only. The presence of chlorophyll b in C. fragile CP_1a and CP_1 complexes may be questionable as Anderson et al (1980) were able to extract chlorophyll b-free CP_1 complexes from C. cactoides. This may indicate that although siphonaceous algae have characteristically low chlorophyll a:b ratios their P700 complexes are the same as those of other plants in that they contain chlorophyll a only. Braumann, Weber and Grimme (1982) found considerable quantities of chlorophyll b and xanthophylls associated with CP_1a and CP_1

complexes in Spinacia chloroplasts. Using a very sensitive high pressure liquid chromatography technique these authors demonstrated the presence of xanthophylls and chlorophyll b in CP_1a and CP_1 and conclude that the pigment composition of these complexes is dependent on their purity on isolation. Anderson et al (1981) suggest that CP_1a and CP_1 may still associate with the light-harvesting apparatus and total resolution of purified P700 is critically dependent on further purification steps. It is thus possible that CP_1a and CP_1 complexes from C. fragile were contaminated or in association with neighbouring LHCP complexes particularly as the xanthophyll content of CP_1 was so variable and siphonoxanthin/siphonein absent in the 540nm absorbing form. For a complete characterization of P-700 complexes in C. fragile it is evident that a revision or separation techniques and the use of several purification steps may be required.

The carotenoid composition of CP_1a and CP_1 in other plant species has been extensively investigated (Thornber and Alberte 1977; Siefermann-Harms, 1980; Lichtenthaler et al 1982), and β-carotene found to be the major carotenoid associated with these complexes. Anderson et al (1981) comment that β-carotene is located in PSI and PSII and is the only carotenoid present in all eukaryotic and prokaryotic plants. However, β-carotene has been shown to be consistently absent in C. fragile fronds and chloroplasts (Chapter 2), and only α and ε-carotene have been detected in this alga throughout this three-year period of study. α-Carotene was the only carotenoid associated with CP_1a in measurable amounts, and the major and most consistent carotenoid present in CP_1. This suggests that CP700 complexes in C. fragile may be different to their β-carotene containing counterparts in other plant groups. β-Carotene is intimately associated with PSI and PSII in these plants where it functions in photo-protective (Foote, 1976; Krinsky, 1978) and antennae roles, (Oquist, Samuelsson, and Bishop, 1980; Searle and Wessels, 1978).

In C. fragile chloroplasts it appears that not only is α-carotene the major carotene isomer, but its location in isolated PSI P-700 complexes suggests it replaces the β-isomer in function. Furthermore, the photoprotective and antennae functions of α-carotene may be different to those encountered in β-carotene containing complexes. The Siphonales are particularly noted for their unusual carotene composition and although β-carotene is reported in these algae, the α-isomer is the major carotene present (Strain, 1965; Goodwin, 1971). In view of these investigations with C. fragile it would be of considerable interest to determine the location of α and β-carotene in chloroplasts from other siphonaceous species. The relatively high concentrations of α-carotene in these algae may reflect the preferential importance of this isomer in the photoprotection and function of P-700 complexes, as these studies with C. fragile chloroplasts already suggest.

Tables 8 and 9 demonstrate the distribution of pigments between and within the light-harvesting complexes of C. fragile. Although $LHCP_1$ contains more pigment than $LHCP_2$ the distribution of carotenoids within these complexes is very similar (Table 9). α-Carotene comprises only 15% of the total carotenoid content and it appears that siphonoxanthin, siphonein and neoxanthin are the major light-harvesting pigments. The location of high concentrations of siphonein and siphonoxanthin in the LHCPs therefore supports the previous spectroscopic data (Figure 16) and the proposal that the major function of these pigments is one of light-harvesting. Chlorophyll a:b ratios in $LHCP_1$ and $LHCP_2$ of C. fragile are particularly low (0.69 ± 0.08, 0.84 ± 0.05, respectively) when compared to higher plants. Braumann et al (1982) report ratios of 1.52 and 1.54 for their corresponding LHCP complexes in Spinacia, chlorophyll a being the predominent chlorophyll. However, the LHCPs of C. fragile are comparable to those of another siphonaceous alga Caulerpa cactoides. Three LHCPs have been isolated from this alga, all contain siphonoxanthin and have chlorophyll a:b ratios of 0.6, 0.72 and 0.74 respectively

(Anderson et al, 1980). Siphonaceous algae are particularly noted for their low chlorophyll a:b ratios (Keast and Grant 1976) and the above findings may suggest an important light-harvesting role for the relatively high chlorophyll b content of these algae.

An additional means of evaluating the relationship between pigments in isolated complexes is to use molar pigment ratios. This data was calculated for pigments in C. fragile complexes, unfortunately the ratios proved extremely variable. This may reflect the electrophoretic instability of the complexes and incomplete pigment recovery, or it may indicate the extent of association between individual pigments. Table 10 shows the least variable molar ratios calculated for pigments within the pigment/proteins. Variability is based on a % of the s.d., the results in Table 10 have $\leq$ 20% variability (s.d.), whereas those ratios excluded had $>$75% variability as s.d. The ratio between chlorophyll a and α-carotene is relatively stable at 90 $\pm$ 16.6, Thornber and Alberte (1977) report a ratio of chlorophyll a:β carotene in higher plants of 20:30 suggesting that less carotene occurs in the CP_1 complexes of C. fragile. Chlorophyll: siphonoxanthin ratios in the light-harvesting complexes of C. fragile are relatively constant despite the presence of large amounts of siphonoxanthin in the FP zone. Siphonoxanthin:siphonein in $LHCP_2$ is also very constant (5.2 $\pm$ 0.46), however this is not the case in $LHCP_1$. The factors contributing to the variability of these pigment ratios is evidently complex. However the most salient feature of the data in Table 10 is the stoichiometry between the major light-harvesting pigments siphonoxanthin, siphonein, and the chlorophylls. This appears to occur regardless of the large accumulation of free siphonoxanthin in the FP band on electrophoresis. Braumann et al (1982) show a similar phenomenon in that 30% of the carotenoids separated from Spinacia pigment/protein complexes were complexed to SDS in the FP zone. However the carotenoids bound to protein complexes were reproducible in their quantitative distribution and these authors concluded that the carotenoids exist in two different pools within the

chloroplast. The above authors also deduce that since little chlorophyll is liberated during SDS-PAGE it may also be assumed that the carotenoid pool bound to the complexes is also shielded against solubilization. However, a second pool of carotenoids may be present in the chloroplast which easily complexes with SDS and migrates as FP. The findings of Braumann et al (1982) are comparable to those demonstrated in C. fragile particularly the stoichiometry of siphonoxanthin when protein bound, and its relatively high concentration in the FP zone. This may suggest that siphonoxanthin has two functions in the chloroplast, the first in light-harvesting as demonstrated by its presence in the LHCPs, and a second as yet undetermined function in which it participates as a free pigment. A purely speculative approach as to the second function of siphonoxanthin may be its importance in the biosynthesis of its ester siphonein. This pigment is presumably derived directly from the parent xanthophyll and esterification may require siphonoxanthin to exist in the unbound form. The relative importance and indeed significance of siphonoxanthin and siphonein which both absorb at 540-542nm and both transfer excitation energy to chlorophyll a is as yet an enigma. The presence of xanthophyll esters in chloroplasts is highly unusual (Goodwin 1971), however the fatty acid group of siphonein may be of structural importance in the arrangement of chloroplast LHCPs. Lutein is thought to have a structural role in the LHCPs of higher plants. The hydroxyl groups of lutein (Appendix I) are involved in the formation of a complex between carotenoid and chlorophyll (usually b) and give rise to a permanent stable electric field, (Sewe and Reich, 1977). Siphonoxanthin and siphonein also possess the same OH groups as lutein (Appendix I) with an additional OH group within the isoprenoid chain in siphonoxanthin. It is thus possible that these siphonaceous xanthophylls have a similar role in algae as that demonstrated by lutein in higher plant chloroplasts.

It is apparent that little detailed information on the localization and function of carotenoids in the thylakoid membranes

is available. This is partially due to difficulties in extracting and quantitatively assessing the low pigment concentrations of the complexes. However, the more recent use of sensitive HPLC methods (e.g. Braumann et al, 1982) may lead to the more complete elucidation of carotenoid location and function within the photosynthetic apparatus.

One of the most salient features of pigment distribution in isolated C. fragile complexes is that approximately 75% of the photosynthetic pigment content is located in the light-harvesting apparatus (Table 8). This value is probably underestimated if purified CP_1a and CP_1 are considered to contain chlorophyll a and α-carotene only. It therefore appears that most of the photosynthetic apparatus of C. fragile chloroplasts is devoted to the harvesting of light energy. This is comparable with the findings of Anderson (1981) who reports that 'sun' plants have approximately 30% of total chlorophyll associated with PSI whereas 'shade' plants have reduced PSI complexes containing only 15% total chlorophyll. C. fragile pigment/protein complexes therefore show similarities with the 'shade' adapted chloroplasts of higher plants. The above author also suggests that low rates of electron transport in shade plants may be due to the reduction of PSI and PSII, and it is of interest to note that C. fragile chloroplasts also exhibit low rates of photosynthesis (Chapter 5). However, in depth studies based on reaction centre activities would be required before this proposal could be substantiated.

The light-harvesting apparatus is the variable component of the PSU (as reviewed in Chapter 1) and these variations permit photosynthetic organisms to adapt to their light environments. C. fragile grows in a constantly changing light environment where 'shade' conditions occur at high tide and 'sun' conditions at low tide. The ability of this alga to adapt its photosynthetic apparatus to both high and low light intensities is therefore crucial. From these investigations it appears that C. fragile may be well adapted to the low light intensities encountered in its intertidal niche. The possession of high concentrations of

light-harvesting units, siphonein and siphonoxanthin which harvest at 540-542nm and narrow the green-gap and large amounts of light-harvesting chlorophyll b all appear to contribute to the successful utilisation of light in an environment where 'shade' conditions are constantly encountered. Anderson et al (1981) suggest that similar adaptations in higher plants lead to extensive granal stacking within the chloroplast which accomodates large numbers of light-harvesting units. Although an attractive theory in higher plant chloroplasts this type of adaptation would appear of little consequence in C. fragile chloroplasts which are at the most bi- or tri-thylakoid(Hawes 1978). Rutter (1982) has shown that C. fragile chloroplasts increase their volume during the winter when shade conditions are particularly evident in the intertidal zone. C. fragile chloroplasts may, therefore, accommodate large concentrations of LHCPs by increasing chloroplast volume in preference to increasing the internal 'surface area' of the chloroplast membranes by thylakoid stacking.

3.4.3 Protein characterisation of pigment/protein complexes isolated from C. fragile

As a natural conclusion to the preceding investigation an attempt was made to characterise the proteins associated with the complexes of C. fragile chloroplasts. Herrmann, Borner and Hagemann (1980) review the difficulties in determining the exact molecular weights of chlorophyll proteins in SDS-systems and comment that several complexes (especially those associated with PSI) have abnormal migration rates when SDS is used. These authors suggest that the best use of SDS-PAGE in complex, protein characterisation is to use thylakoid extracts which have not been lipid extracted, and perform re-electrophoresis of isolated complexes after denaturation and/or mercaptoethanol treatment. Following these suggestions Figures 17, 18 and 19 show the protein profiles of intact thylakoid extracts and re-electrophoresed pigment/protein complexes. These figures also demonstrate changes in profiles after successive denaturing treatments with mercaptoethanol and/or heat. The gel patterns obtained from intact

thylakoids are referred to as protein profiles in this text and those from denatured extracts polypeptide profiles. However as the discussion develops it is apparent that denatured extracts may in some cases contain trace amounts of both proteins and their constituent polypeptide subunits.

The reports on molecular weight and polypeptide composition of CP_1 are still very controversial (Hermann et al 1980). However, the protein profiles obtained from total thylakoid extracts in particular suggest that the PSI complexes of C. fragile may be similar to those reported in other plant species. High molecular weight proteins were resolved from denatured and intact thylakoid extracts, corresponding to molecular weights of 93.4 $\pm$ 2.8 - 100 $\pm$ 4.2 KD. These proteins showed chlorophyll fluorescence in all but the heat-denatured extracts and it is suggested that they are associated with CP_1 and CP_1a complexes resolved previously (section 1.3.1). Møller, Høyer-Hansen and Hiller (1981) separated a similar protein of molecular weight 110 KD from Hordeum vulgare thylakoid extracts which they identified as containing the reaction centre of P700 of PSI. Additionally these authors resolved two low molecular weight proteins of 18.3 and 15.2 KD which they suggest were related to PSI. A chlorophyll fluorescent bound of molecular weight 15.2 $\pm$ 1.2 - 16.5 $\pm$ 1.2 KD was also resolved from C. fragile extracts with the occasional appearance of an 18 KD protein. It is therefore proposed that these bands may also be associated with PSI complexes of this alga. The characterisation of PSI reaction centres in higher plants has been partially resolved by Mullet, Burke and Arntzen (1980). These authors separated a purified form of PSI from Pisum sativum and designated the complex native PSI-110, since the complex contained 110 chlorophylls/ P700 and exhibited high rates of PSI-mediated electron transport. On solubilization with SDS this complex was resolved into several bands comprising of doublets of 66 and 68 KD, together with several individual bands ranging in size from 16.5 to 24.5 KD.

On considering these findings it is interesting to note that a second chlorophyll fluorescent band with a molecular weight of 68.2 ± 1.0 KD was also resolved from C. fragile intact thylakoid extracts and a 67.1 KD fluorescent band in denatured extracts. Denatured CP_1 extracts also resolved a 62.3 ± 7.7 KD band on electrophoresis. Examination of total thylakoid extracts (Figures 18 and 19) indicates the disappearance of two major protein bands (93.4 and 68.2 KD) and the formation of many minor ones. It is therefore proposed these protein/polypeptide profiles indicate a progressive breakdown of the large molecular weight. PSI proteins and the formation of several lower molecular weight polypeptide subunits. These are tentatively identified as being similar to the denatured products of PSI-110 isolated by Mullet et al (1980).

Re-electrophoresed non-denatured CP_1a and CP_1 extracts were unresolved on electrophoresis and the retention of chlorophyll fluorescent material at the origin suggests that high molecular weight or insolubilized material was unable to penetrate the 10% gel. However on denaturation these extracts entered the resolving gel and denatured CP_1 produced two bands of 62.3 ± 7.7 KD and 32.6 KD, and CP_1a produced a single band of 53.1 ± 1.7 KD. On heat-denaturation the polypeptide profile of CP_1a remained the same but a 49 KD polypeptide appeared in CP_1 gels. Unfortunately recovery of total protein extracts from CP_1a and CP_1 was extremely low and the appearance of high molecular weight proteins on LHCP gels suggests these bands may have been contaminated with PSI proteins, thus contributing to their low recovery in CP_1a and CP_1. However it does appear that on heat-denaturation polypeptide subunits of molecular weights 49 and 53 KD may also be associated with CP_1a and CP_1 complexes from C. fragile.

The resolution of two light-harvesting complexes from C. fragile thylakoid extracts was noted in section 3.3.1 and these were designated $LHCP_1$ and $LHCP_2$. These complexes appear to correspond to the oligomeric and monomeric LHCP complexes reported by Hiller, Genge and Pilger (1974). Evidence for this

phenomenon occurring in C. fragile LHCPs is apparent from the similarities in protein profiles, pigment distributions, and absorption characteristics of these complexes (Table 8, Figure 16). Thus, the least mobile $LHCP_1$ complex isolated from C. fragile is identified as an oligomeric or dimeric form of the light-harvesting monomer $LHCP_2$. The characterisation of proteins associated with these complexes was deduced from the profiles of thylakoid extracts before and after denaturation (Figures 17, 18 and 19). It is therefore suggested that the denaturation and heat-denaturation of the dimeric $LHCP_1$ (associated proteins 60.3 $\pm$ 1.1 to 52.0 KD) leads to the progressive accumulation of the monomeric light-harvesting component of molecular weight 27-28.2 KD. Re-electrophoresis of intact $LHCP_1$ and $LHCP_2$ extracts confirms this interpretation as $LHCP_1$ was resolved as a major chlorophyll fluorescent band of molecular weight 56 $\pm$ 2.4KD (the dimer) and $LHCP_2$ as a chlorophyll fluorescent band of 29 KD (the monomer). The detailed composition of LHCP complexes in higher plants has not been resolved, the lowest molecular weight of the monomer complex is reported in the range 24-30KD (Hiller and Goodchild, 1981) and from the above account C. fragile LHCP monomers appear to agree with this evaluation. Two other chlorophyll fluorescent proteins were resolved from re-electrophoresed intact LHCP extracts, however these proteins were very similar to those of CP_1a and CP_1 and as previously discussed were probably contaminants.

Figures 18 and 19 show the protein profiles or re-electrophoresed denatured and heat-denatured LHCPs from C. fragile. The light-harvesting complexes have very similar profiles and proteins of 57-55KD, 56-50KD and 28.7-30.5KD were resolved. This gel pattern strongly suggests that denatured LHCP extracts re-electrophorese as both monomeric and dimeric forms, rather than the monomeric component only as would be expected. Furthermore it appears that CP_a is also composed the same or very similar proteins as the LHCPs. The behaviour of these complexes may be accounted for by incomplete denaturation procedures i.e. the dimeric proteins were not totally denatured to their component

monomers, or polypeptide subunits. However as heat treatment with 5% mercaptoethanol was used, this explanation seems unlikely. It therefore appears that re-electrophoresed denatured LHCP extracts reassociate to form both monomeric and dimeric components. This unusual behaviour has been noted in only one other system which, coincidently was derived from the thylakoid extracts of another siphonaceous alga Acetabularia cliftonii. Green, Camm and Van Houten (1982) isolated a minor pigment-containing band from this alga which they characterised as CP29 (Chapter 1). Green and Camm (1981) suggest this protein is present in most plant species where it may function as an internal antenna in PSII. CP29 in A. cliftonii is particularly unusual in that it is able to reform dimerson re-electrophoresis of dissociated CP29 monomers. The re-association of LHCP monomers in C. fragile together with similarities in the pigment profiles of denatured CP_a and $LHCP_1$ and $_2$ of this alga is rather an enigma. This phenomenon may merely be a physical consequence of detergent, and electrophoretic treatments, however, as a similar event has been described in the siphonaceous alga A. cliftonii possible biological implications cannot be dismissed. Using proteolytic digestion techniques, and further electrophoretic analyses the relationship between CP29, CP_a and LHCP may be investigated further and therefore elucidate the nature of the re-associating LHCP complexes of C. fragile.

3.5 CONCLUSIONS

Six pigment containing bands were isolated from C. fragile thylakoid extracts. Two (CP_1a and CP_1) were associated with PSI and were enriched in chlorophyll a and α-carotene. Two light-harvesting complexes $LHCP_1$ and $LHCP_2$ were also resolved and these bands comprised 75% of the total pigment content of the complexes. The light-harvesting apparatus was enriched in chlorophyll b and the xanthophyll pigments, especially siphonein and siphonoxanthin which absorbed at their in vivo light-harvesting wavelength of 542nm. The CP_a band was originally considered to be associated with PSII, however this band was never successfully characterised and its pigment composition is uncertain.

The last and most mobile band (FP) was composed of free pigment and contained almost all the violaxanthin recovered from the gel eluates.

The pigment composition of the pigment/protein complexes isolated from C. fragile was somewhat different to those reported for higher plants. β-Carotene appears to be replaced by α-carotene in the PSI P-700 complexes and relatively high concentrations of light-harvesting pigments occurred in $LHCP_1$ and $LHCP_2$. This suggests that a large proportion of the photosynthetic pigments present in C. fragile chloroplasts function in a light-harvesting role and this may be an adaptation to accommodate submarine photosynthesis.

Protein profiles obtained from C. fragile thylakoid extracts suggest that the PSI complexes of this alga may be similar to those reported in other plant species. Several high molecular weight proteins (93.4 ± 2.8 - 100 ± 4.2 KD, 68.2 ± 1.0 KD) are thought to be associated with CP_1a and CP_1 together with two low molecular weight proteins of 15.2 ± 1.2 - 16.5 ± 1.2 and 18.0 KD. $LHCP_1$ and $LHCP_2$ have been identified as dimeric and monomeric forms of the light-harvesting complexes. $LHCP_1$ corresponds to the dimer of molecular weight, 56 ± 2.4 KD and $LHCP_2$ the monomer of molecular weight 29 KD. The behaviour of denatured $LHCP_1$ and $LHCP_2$ extracts is particularly unusual in that on re-electrophoresis the denatured monomer reassociates to form two dimers of similar molecular weights. Reassociation of monomeric complexes has only been reported in one other species, Acetabularia cliftonii. In this siphonaceous alga unusual complexes corresponding to the CP29 protein reassociate to form dimers. These complexes appear to correspond in molecular size to the LHCP and CP_a complexes or C. fragile. Similarities in the protein profiles of CP_a, $LHCP_1$, $LHCP_2$ together with the ability of both monomeric CP29 and light-harvesting complexes of C. fragile to reassociate suggests these algae may both have unique protein compositions. This is particularly interesting as both algae are members of the Siphonales.

4. THE GROWTH OF C. FRAGILE IN INTERTIDAL AND STORAGE ENVIRONMENTS

4.1 INTRODUCTION

The major theme of this thesis has been to investigate light adaptations in C. fragile and relate these adaptations to the growth of this alga in its intertidal niche. Thus, the consideration of the growth strategy of C. fragile in its natural environment is a necessary prerequisite to subsequent laboratory investigations. On regular visits to Bembridge considerable differences in frond morphology and pigmentation have been routinely observed and have produced an accumulation of seasonal information relating to the period November 1979-June 1982. This chapter describes the seasonal growth, reproduction, and pigment content of C. fragile and forms a basis for succeeding chapters.

The intertidal zone from which C. fragile is sampled is influenced by a number of interacting environmental parameters however, following collection for experimental purposes the alga is maintained in a relatively constant storage environment. A further aim of this chapter is therefore, to monitor the behaviour of C. fragile in storage by recording changes in frond morphology and pigmentation.

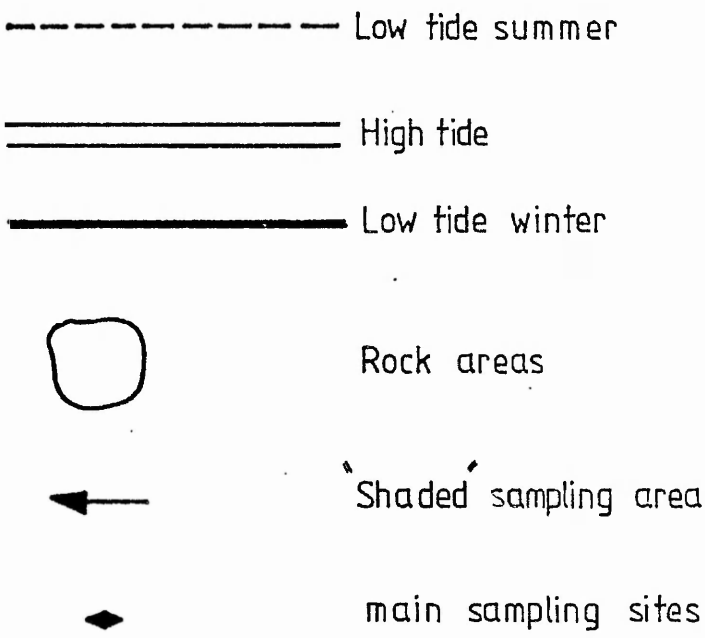
4.2 MATERIALS AND METHODS

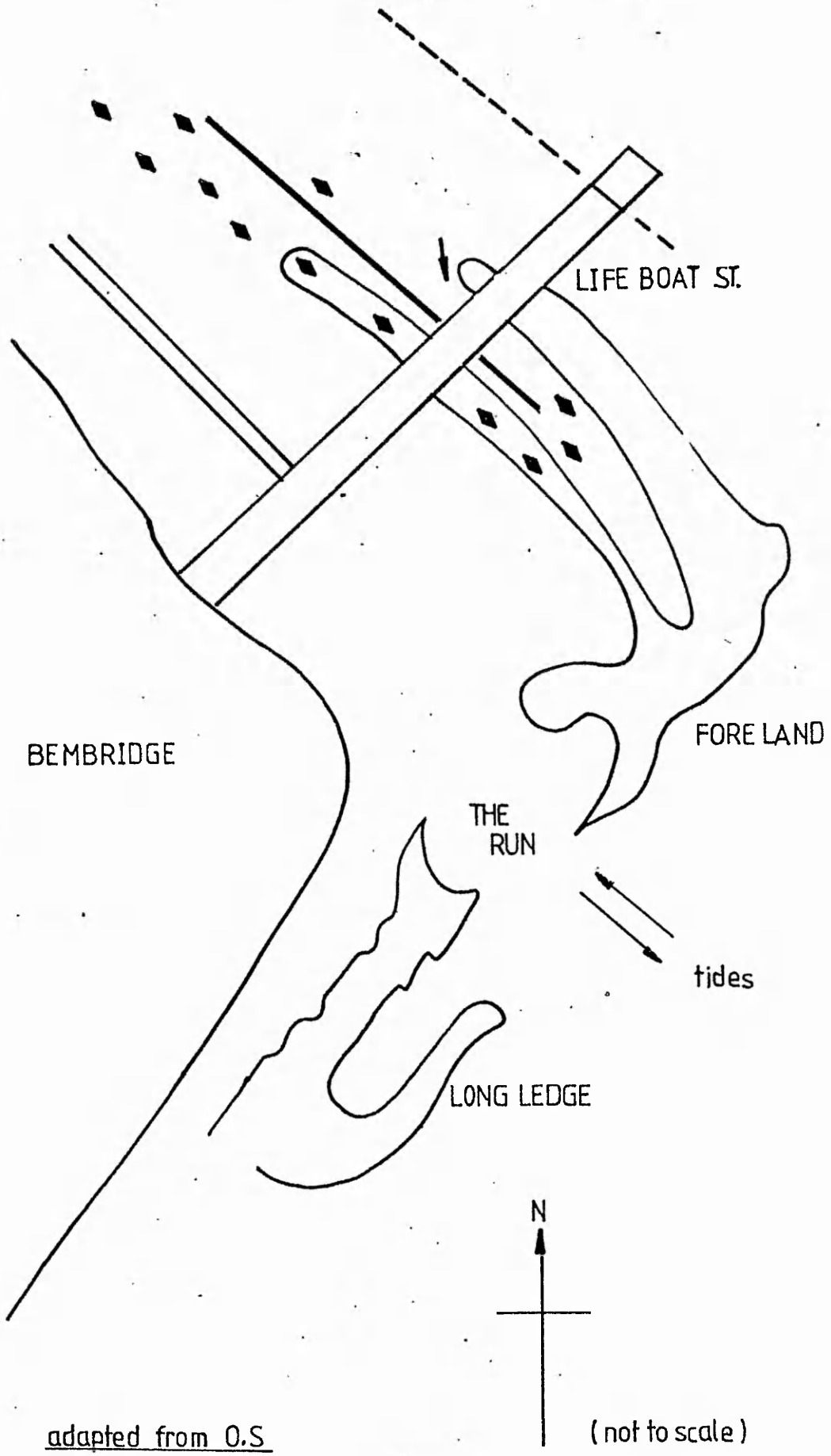
4.2.1 Seasonal investigations

C. fragile was sampled from Bembridge, the Isle of Wight, during the period November 1979-June 1982 and Figures 20 and 21 show the main sampling areas on Bembridge beach. Morphological characteristics (i.e. number of frond dichotomies, presence and nature of epiphytic growth, hair development and reproductive structures) were recorded at the time of sampling. Microscopic examinations were performed on fronds sampled June 21st, 1982 using a Leitz Dialux light microscope. Pigment analyses were performed on fronds within seven days of sampling using methods described in Chapter 2.

FIGURE 20 :- SAMPLING LOCATIONS AT BEMBRIDGE BEACH

KEY :-





adapted from O.S sheet, 196

FIGURE 21 : - THE INTERTIDAL ZONE AT BEMBRIDGE BEACH

4.2.2 Storage investigations

Epiphytes were removed from C. fragile after sampling and the fronds were stored at 10-15°C with a constant light intensity of 60-80μE.M.$^{-2}$.S^{-1} P.A.R. at the water surface using Thorne 20W "White 3500" fluorescent tubes. Bembridge seawater was used when possible however, during the winter months the beach was often contaminated by domestic water flooding and at such times Gerrards 2GV-110-+ seawater was used. The tanks were aerated, and cleaned every 4-6 weeks. Pigment analyses were performed over 0-150 days during the storage periods of fronds sampled 29.11.1980 and 17.6.1980. Carotenoid and chlorophyll determinations were performed as described in Chapter 2. Changes in frond morphology were noted throughout the storage period particular attention was given to the development of reproductive structures.

4.3 RESULTS

4.3.1 Seasonal changes in frond morphology of C. fragile

Table 12 shows variation in frond morphology of C. fragile sampled November 1979-June 1982.

Table 12 VARIATION IN FROND MORPHOLOGY OF C. FRAGILE SAMPLED 29.11.1979-21.6.1980

SAMPLE DATE	NUMBER OF DICHOTOMIES	PRESENCE OF REPRODUCTIVE STRUCTURES	PRESENCE OF HAIRS	EPIPHYTIC GROWTH	FROND BLEACHING
NOV. 1979	3-4				
JUNE 1980	7-9	✓	✓	✓	✓
NOV. 1980	3-4				
MARCH 1981	4-6				
JULY 1981	6-8	✓	✓	✓	✓
NOV. 1981	3-4				
MARCH 1982	4-6				
JUNE 1982	7-8	✓	✓	✓	✓

Variation in frond morphology can be divided into two distinct seasonal phases, namely the winter/spring period (November-April) and the summer/autumn period (May-October). Figures 22 and 23 show typical frond appearance for these growth stages. Fronds sampled in late November were entirely vegetative and consisted of young colonies with 3-4 dichotomies, (approximate plant size 5-15 cm). Growth of other algae was minimal at this time of year and epiphytic growth was absent on C. fragile fronds. The frond tissue was uniformly dark green in colour with no apparent signs of bleaching, frond hairs were also absent. Fronds sampled in March showed similar characteristics, however, the number of dichotomies had increased to 4-7 (with a maximum plant size of 15-20 cm). Recolonization of the intertidal zone by other algal species was also noted.

C. fragile sampled during the summer period comprised mature plants, dichotomy number 6-8 and frond size 20-25 cms. Green reproductive structures corresponding to the female gametangia were present (Figure 24). Summer sampled fronds showed considerable bleaching and extensive hair development (Figures 23 and 27). Of particular significance were the extensive epiphytic growths on the holdfast which mainly consisted of Rhodymenia palmata (Lamour). Grev. and Ulva lactuca (Thuret), and on the fronds (Lomentaria articulata (Huds)., Ascophyllum nodosum (L.). Lejois. Chondrus crispus (Stakh.). and Enteromorpha sp.). At this time of year algal growth was quite prolific, often shading C. fragile, and Sargassum spp. in particular dominated the intertidal zone (Figure 25).

Tidal movements at Bembridge follow a semi-daily pattern of two high and two low tides every 24 hours, but superimposed on these daily movements is a seasonal effect. During winter sampling trips the range between high and low water was observed to be particularly small. Furthermore, high winds typical of this area during the winter months resulted in the intertidal zone being only briefly exposed between high and low tides.

FIGURE 22 :- WINTER-SAMPLED VEGETATIVE C. FRAGILE FRONDS

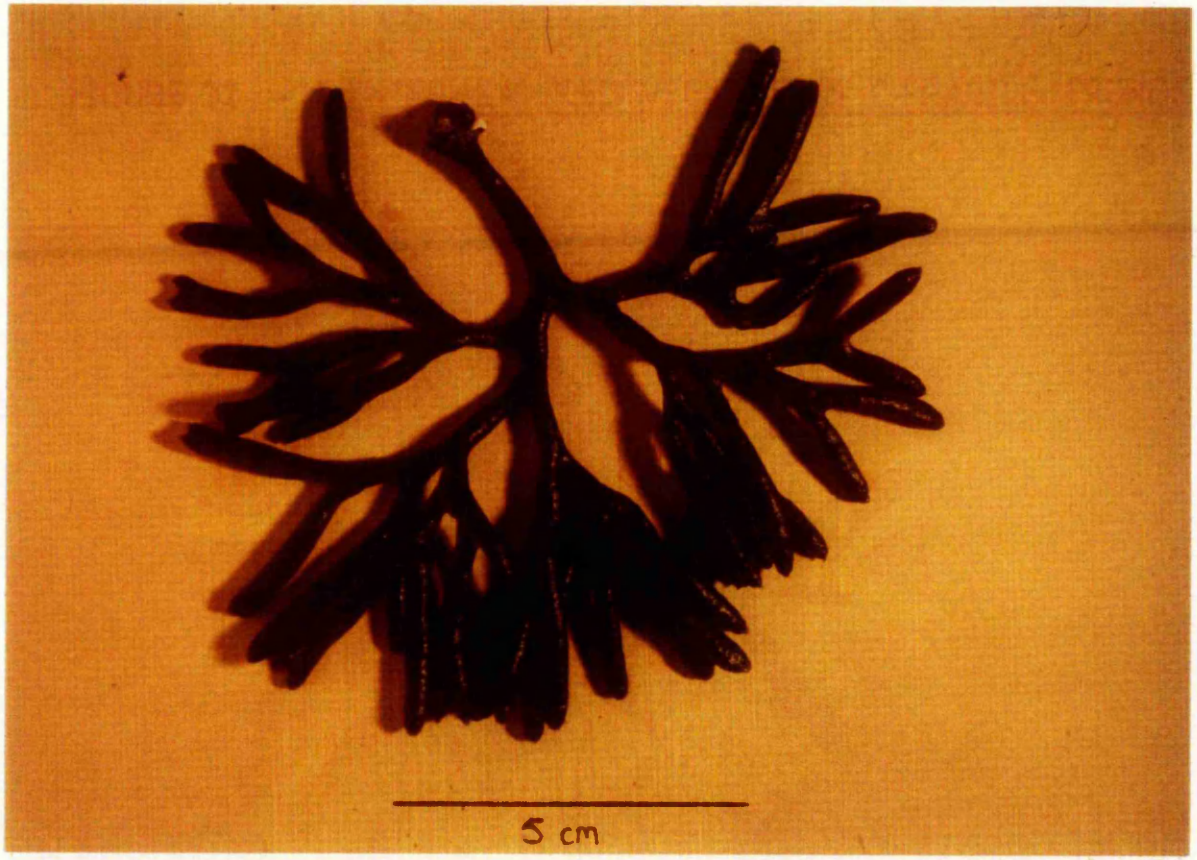


FIGURE 23 : - SUMMER-SAMPLED REPRODUCTIVE C. FRAGILE FRONDS

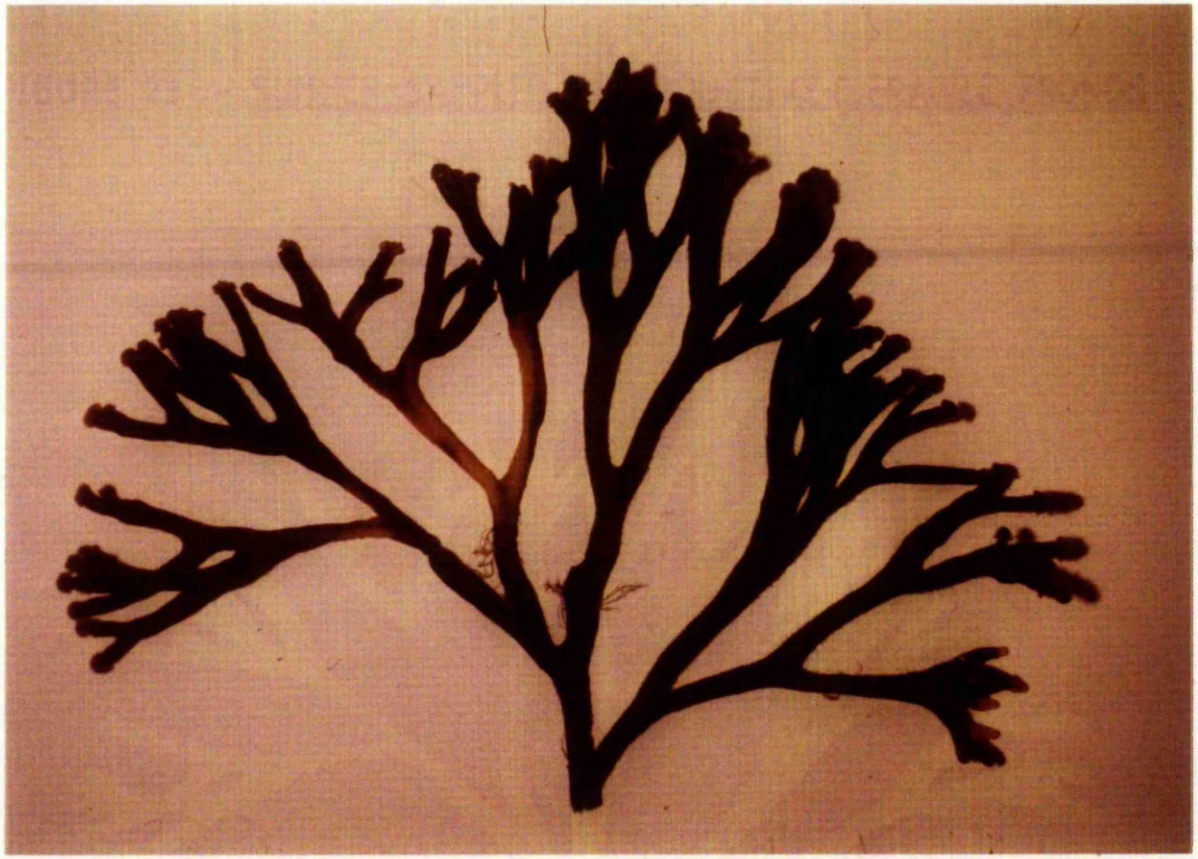
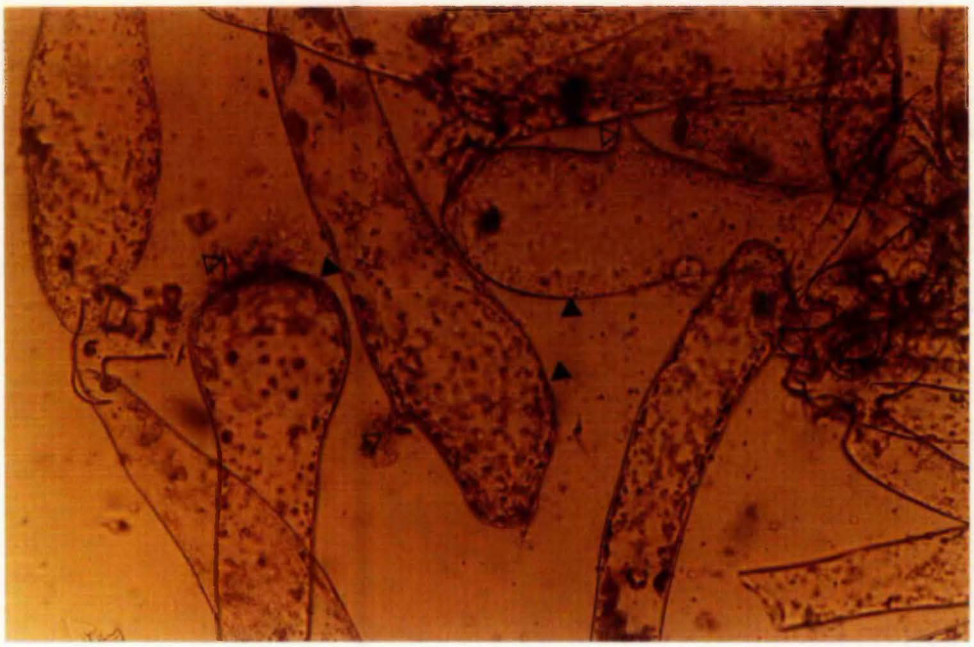


FIGURE 24:- FEMALE GAMETANGIA FROM SUMMER SAMPLED C.FRAGILE

KEY :-

▶ Gametangia

▷ Utricles



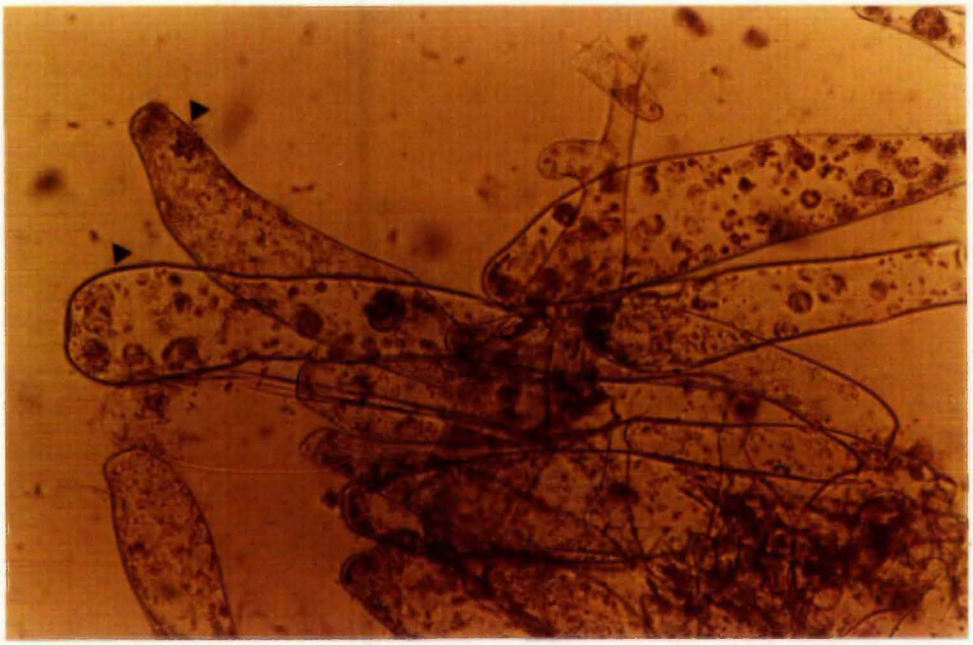


FIGURE 25: - SUMMER GROWTH OF C.FRAGILE IN THE INTERTIDAL ZONE

FIGURE 26: - SUMMER HAIR DEVELOPMENT IN C.FRAGILE SAMPLED FROM A SHADED SUB-INTERTIDAL LOCATION AT BEMBRIDGE

KEY: -

▶ Hair development (× 100)

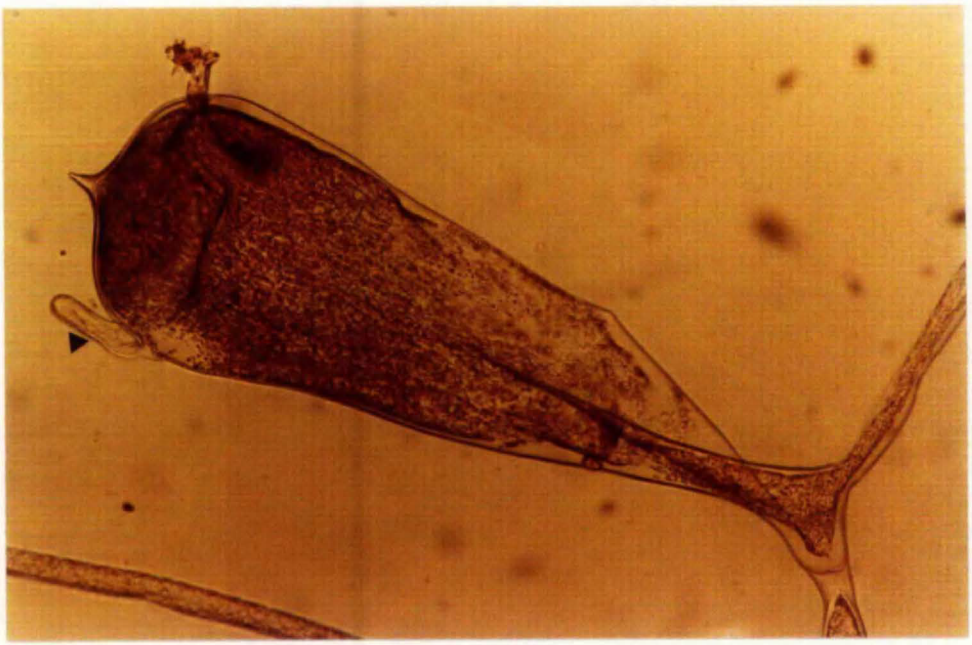
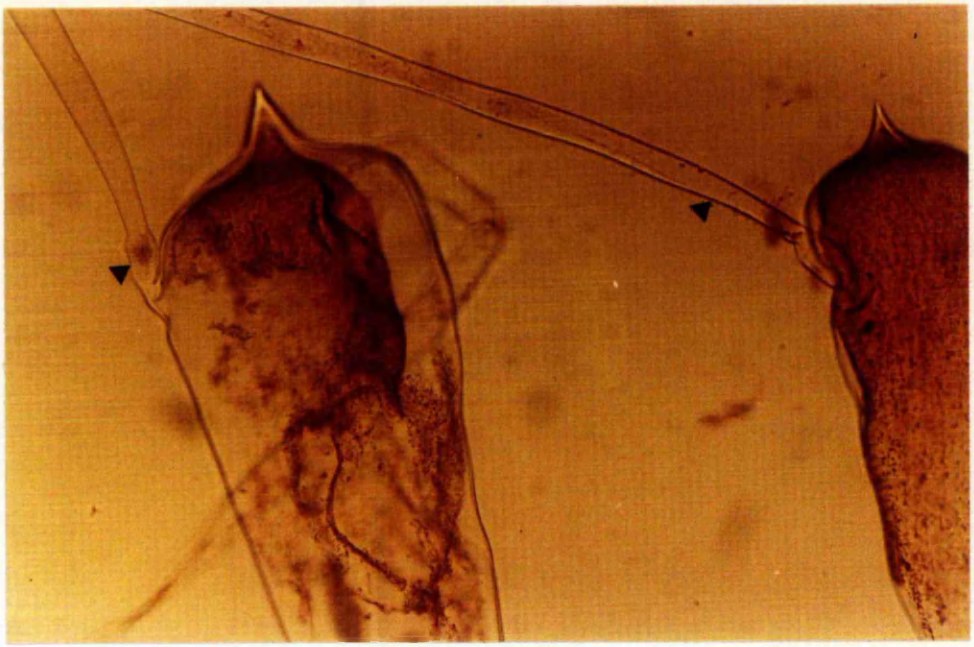
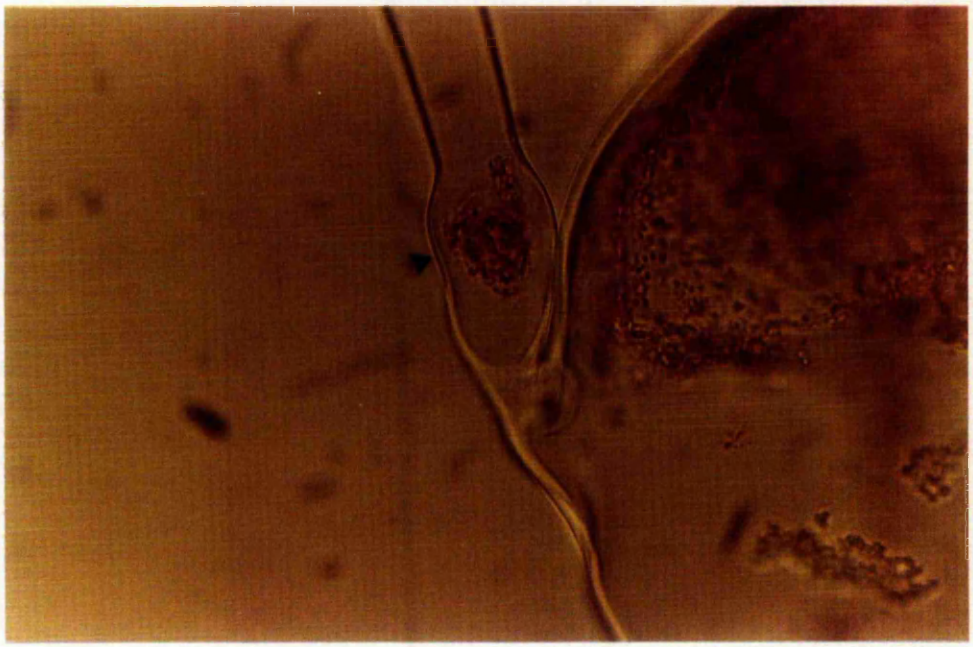


FIGURE 27:- SUMMER HAIR DEVELOPMENT IN C.FRAGILE SAMPLED FROM A TYPICAL INTERTIDAL ZONE LOCATION

KEY :-

▶ Hair development (×100, × 400)





However, during the mid summer sampling trips, extremely low mid-day tides were often observed, and the intertidal zone was exposed for a longer period of time at low tide. On one such occasion (June 1982) a small colony of C. fragile was found growing just below the intertidal zone, in a shaded area beneath the Bembridge life boat pier (Figure 20). This colony, despite being reproductive and the same size as the intertidal colonies was atypical of the summer-sampled C. fragile in that the frond lacked hairs and epiphytes, and were not bleached. Microscopic examination of these fronds showed very early development of frond hairs (Figure 26) their greatest length being 1.6mm compared to 4.1mm on the intertidal sampled algae (Figure 27).

4.3.2 Seasonal changes in the pigment content of C. fragile

Figures 28-31 show considerable seasonal variations in both carotenoid and chlorophyll concentrations in C. fragile. Maximum pigment concentrations coincide with the vegetative stages of the life cycle, and the lowest pigment concentration when the alga is in the reproductive phase. However, despite such seasonal variation, the percentage distribution of individual carotenoids remained constant (Figure 31). Figure 30 shows seasonal variation in pigment ratios in which the ratio of total chlorophyll to carotenoids shows considerable fluctuation due to a large synthesis of chlorophyll during the vegetative phase although chlorophyll a:b ratios remain constant at 1.5-1.8:1 throughout. Total chlorophyll: ε-carotene and violaxanthin ratios have not been included in Figure 30 since both these carotenoids are present in very low concentrations. However, total chlorophyll: ε-carotene was in the range 337 $\pm$ 221 (summer)-637 $\pm$ 165 (winter) and total chlorophyll:violaxanthin from 28 $\pm$ 2.9 (summer)-605 $\pm$ 96 (winter).

TABLE 13 PIGMENT CHARACTERISTICS OF C. FRAGILE FRONDS SAMPLED FROM THE INTERTIDAL ZONE, AND A SUBTIDAL SHADED AREA IN JUNE 1982

PARAMETER	"SHADED"	INTERTIDAL
α-carotene $\mu g.g^{-1}$.FWt	4.9 ± 0.2 (13.6 ± 2.9)	2.6 ± 0.15 (13.5 ± 1.05)
ε-carotene "	0.77 ± 0.12 (3.0 ± 1.2)	0.38 ± 0.1 (1.9 ± 0.5)
Siphonoxanthin "	22.1 ± 4.7 (51.1 ± 2.9)	10.2 ± 2.4 (52.2 ± 8.3)
Siphonein "	7.4 ± 1.8 (20 ± 1.2)	2.9 ± 0.05 (15.1 ± 1.6)
Violaxanthin "	0.1 ± 0.04 (0.23 ± 0.1)	0.8 ± 0.8 (3.9 ± 3.6)
Neoxanthin "	4.9 ± 0.75 (13.2 ± 2.3)	2.6 ± 0.45 (13.3 ± 2.8)
Total carotenoids "	37.3 ± 9.0	19.5 ± 1.9
Total chlorophylls "	186 ± 23	117 ± 4.8
Chlorophyll a "	114 ± 22.3	69 ± 28
Chlorophyll b "	74.2 ± 1.2	48 ± 20.1
Chlorophyll a:b	1.5 ± 0.23	1.44 ± 0.02
Total chlorophyll:Total carotenoids	5.1 ± 0.7	5.9 ± 1.9
Total xanthophylls:Total carotenes	5.6 ± 0.41	5.4 ± 0.6
Total chlorophyll: α-carotene	38 ± 5.0	44.5 ± 18
" " : ε-carotene	1784 ± 1509	337 ± 221
" " :siphonoxanthin	10 ± 2.2	11.1 ± 1.8
" " :siphonein	25.4 ± 2.9	39.8 ± 16
" " :violaxanthin	2385 ± 1107	605 ± 963
" " :neoxanthin	38.4 ± 6.7	48.5 ± 2.8

NB Values in parentheses represent carotenoid distribution as a percentage of total carotenoid content. Values represent means of 4-5 50g frond samples ± s.d.

FIGURE 28 :- SEASONAL VARIATION IN THE CHLOROPHYLL CONTENT OF C.FRAGILE FRONDS

KEY :-

▲--------- Total chlorophyll

▼——— Chlorophyll a

▽——— " b

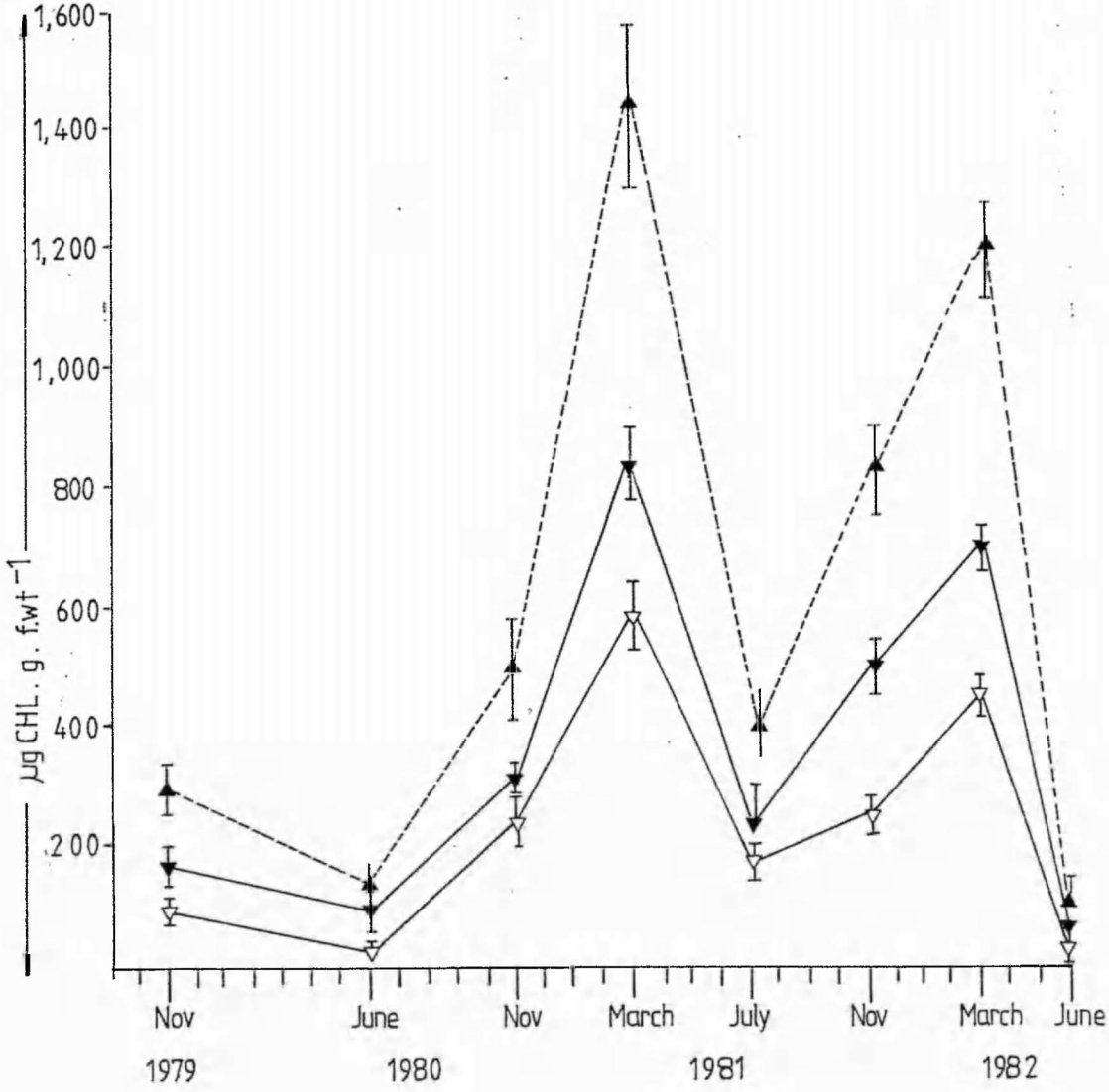

1,600
1,400
1,200
1,000
800
600
400
200
µg CHL. g. f.wt⁻¹
Nov
June
Nov
March
July
Nov
March
June
1979
1980
1981
1982

FIGURE 29:- SEASONAL VARIATION IN THE CAROTENOID CONTENT OF C.FRAGILE FRONDS.

KEY:-

- ●——— Siphonoxanthin
- ◆——— Neoxanthin
- ○——— Siphonein
- □——— α-Carotene
- ◇——— Violaxanthin
- ▲——— ε - Carotene

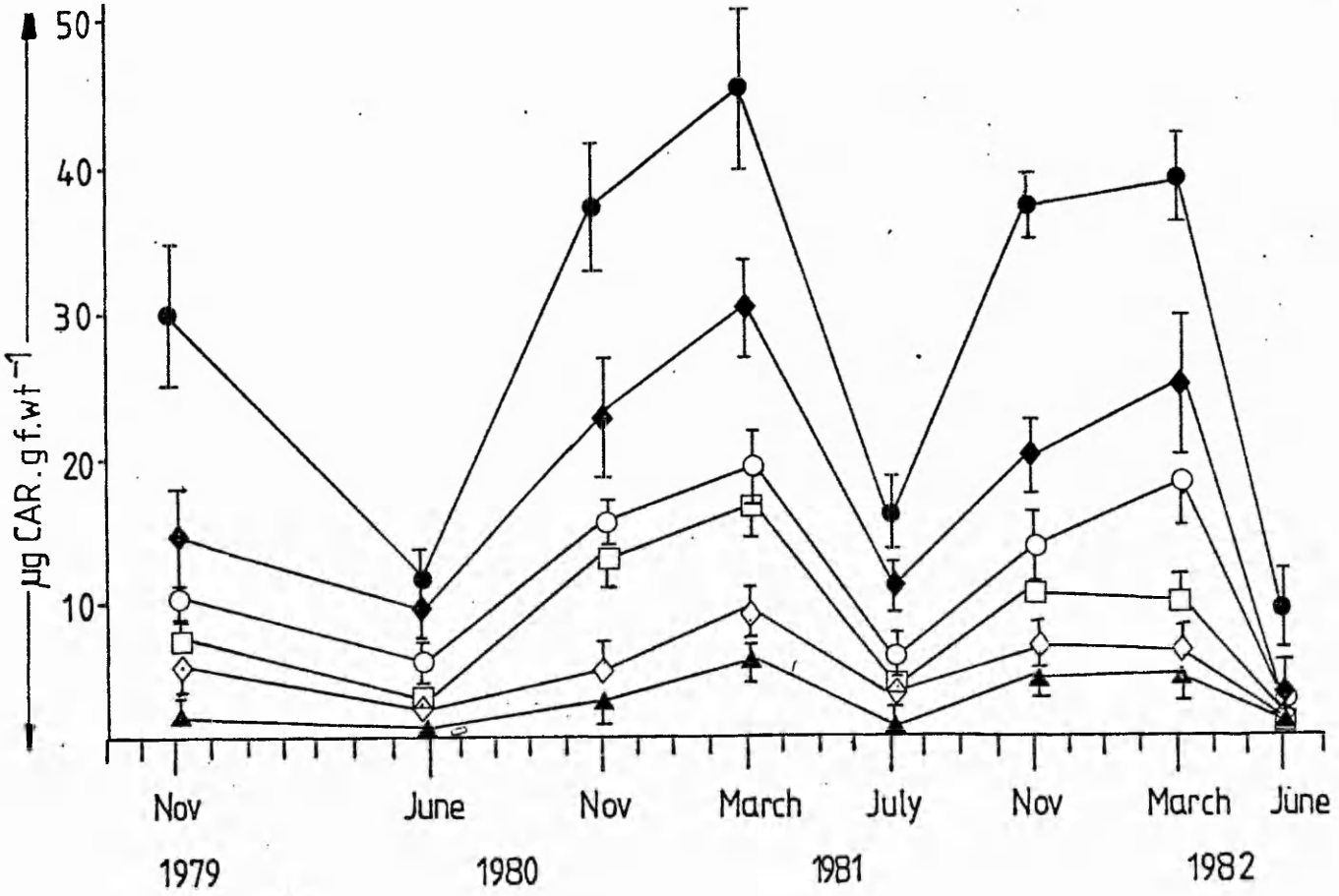

50
40
30
20
10
µg CAR. g f.wt⁻¹
Nov
June
Nov
March
July
Nov
March
June
1979
1980
1981
1982

FIGURE 30 : - SEASONAL VARIATION IN THE PIGMENT RATIO

DISTRIBUTION OF C.FRAGILE FRONDS

KEY : -

◆-------- Total chlorophyll : α - carotene

◆——— " " : siphonein

▼-------- " " : neoxanthin

▼——— " " : siphonoxanthin

▲——— " " : total carotenoids

▽——— Chlorophyll a : b

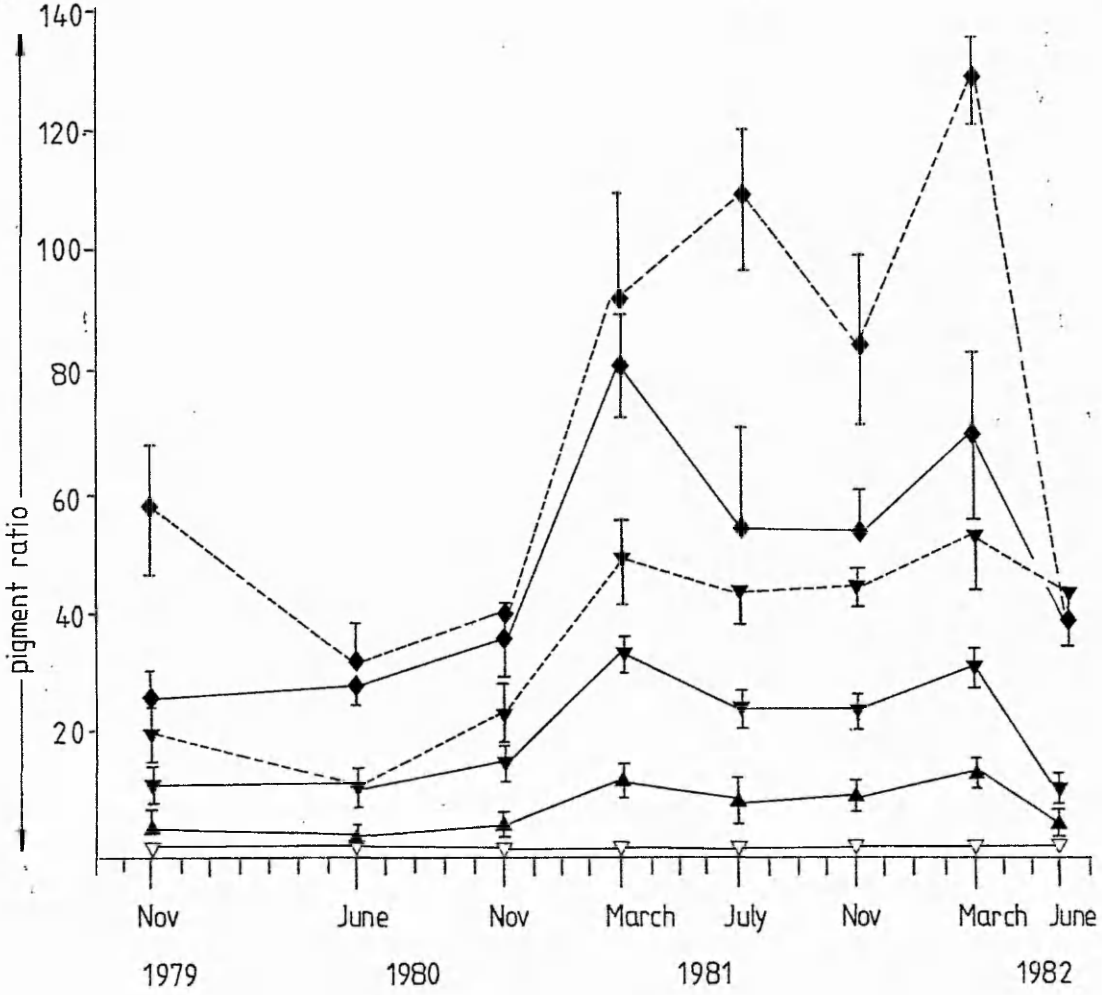
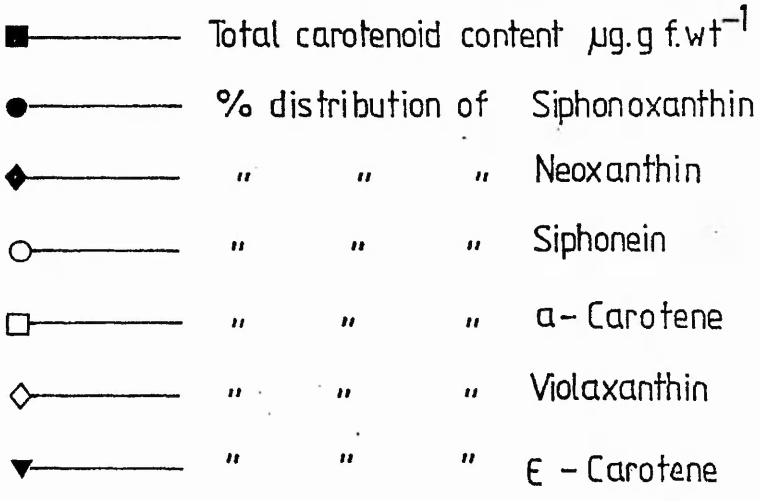
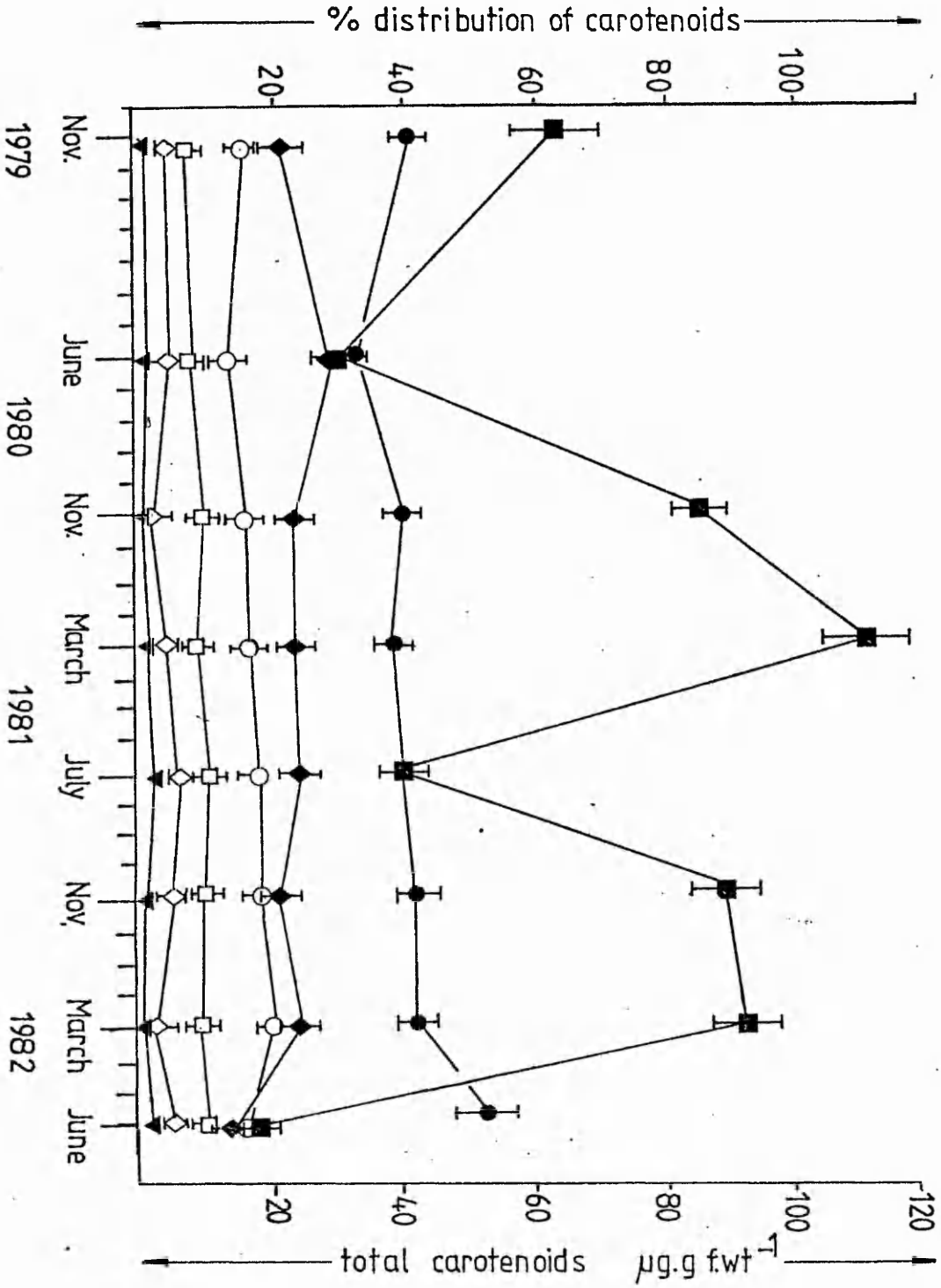

140
120
100
80
60
40
20
pigment ratio
Nov
June
Nov
March
July
Nov
March
June
1979
1980
1981
1982

FIGURE 31:- SEASONAL VARIATION IN THE % AND TOTAL DISTRIBUTIONS OF CAROTENOID PIGMENTS IN C. FRAGILE FRONDS

KEY :-




% distribution of carotenoids
20
40
60
80
100
1979
Nov.
1980
June
Nov.
1981
March
July
Nov.
1982
March
June
20
40
60
80
100
120
total carotenoids µg.g f.wt⁻¹

The November 1979, pigment content was probably under estimated as the fronds were in storage for several weeks, before the TLC methodology and pigment identification procedures had been devised. Table 13 shows a comparison in pigment content between C. fragile fronds sampled from the intertidal zone during June 1982, and the atypical summer-sampled fronds from the shaded area. Overall there is almost twice the amount of pigment in the shaded fronds compared to the intertidal fronds, yet the relative distribution of pigments in both types of algae is almost identical.

4.3.3 Storage changes in the frond morphology of C. fragile

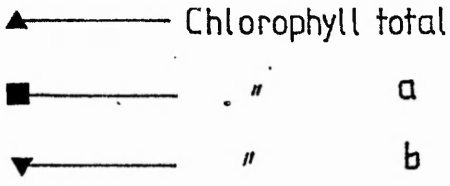
Frond morphology changes in November-sampled fronds as female gametangia developed after prolonged storage (> 3 months). Reproductive structures in summer-sampled fronds degenerated after 3-4 weeks in storage and loss of frond hairs was also apparent 1-2 weeks after sampling.

4.3.4 Storage changes in the pigment of C. fragile fronds

Figures 32 and 33 show changes in the pigment composition of vegetative and reproductive C. fragile. The individual carotenoid content of fronds sampled in November was relatively stable, however, net changes occur for the period of 54-88 days. These changes are reflected in the total carotenoid content which varies between 75 and 92 $\mu g.g^{-1}$ F.Wt. over the period of study. A net increase in chlorophyll concentration occurred in stored vegetative fronds although a decline in chlorophyll content was apparent after 53 days of storage. Fronds sampled during the reproductive phase of the life cycle show similar behaviour in both chlorophyll and carotenoid pigments. Long term storage produces more erratic behaviour as the pigment content of both vegetative and reproductive shows. However despite this finding the relative proportions of the pigments remained constant and chlorophyll a:b ratios were maintained at 1.5:1 throughout storage.

FIGURE 32:- CHANGES IN THE CHLOROPHYLL CONTENT OF C.FRAGILE FRONDS DURING STORAGE

KEY :-



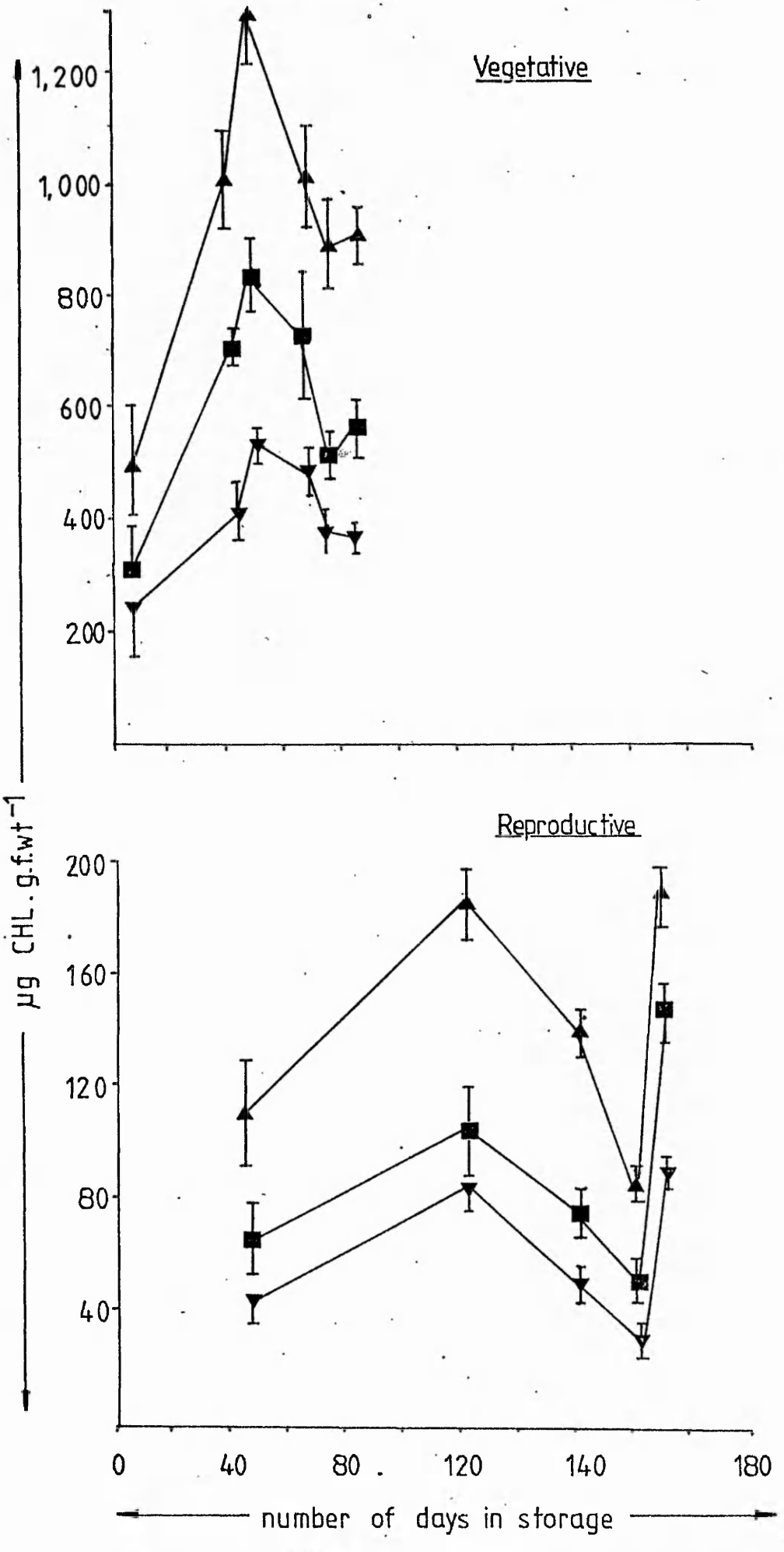
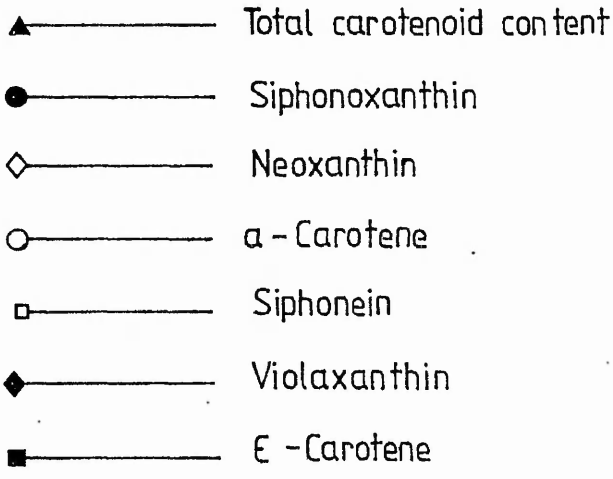
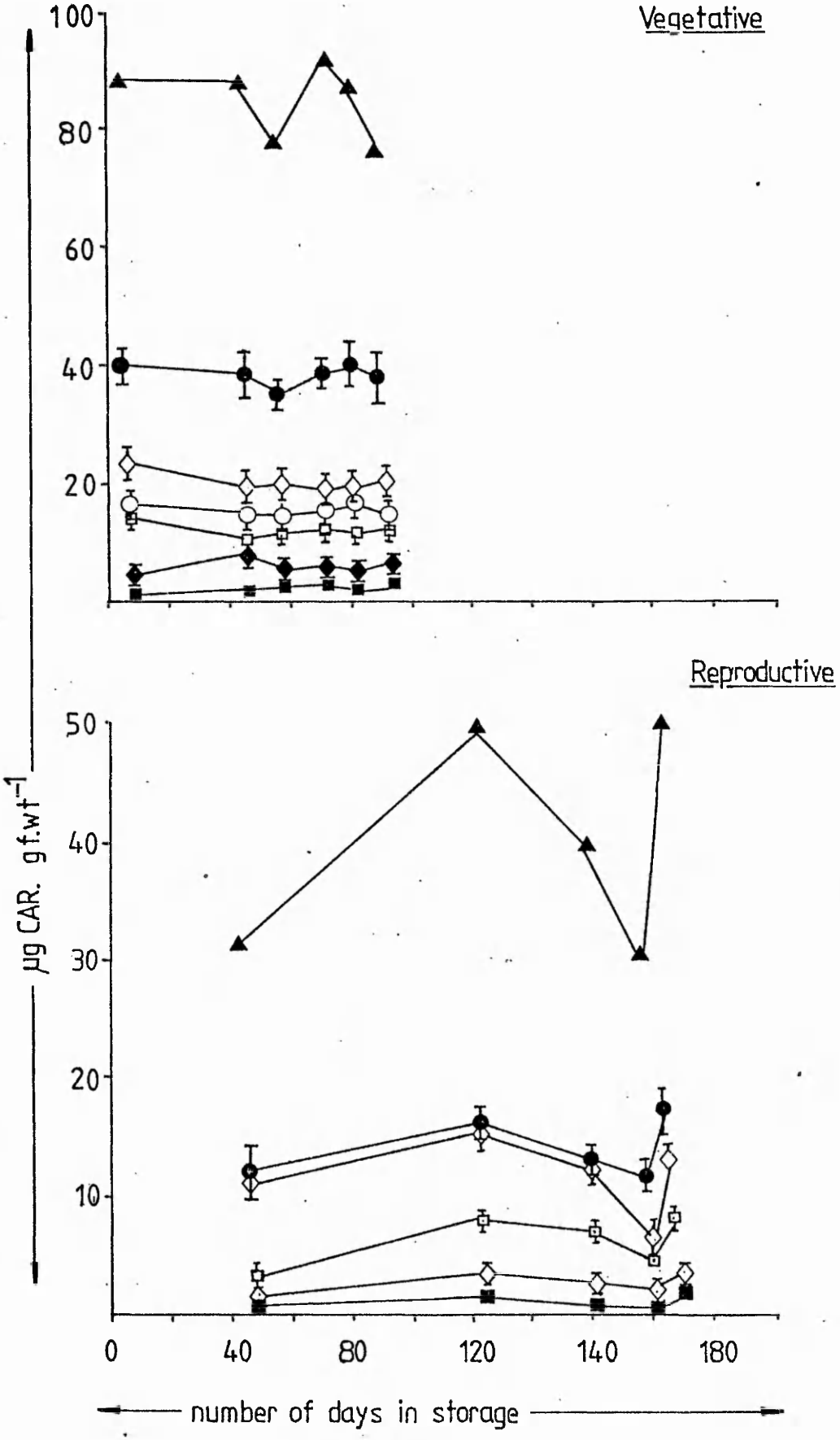

Vegetative
1,200
1,000
800
600
400
200
Reproductive
200
160
120
80
40
µg CHL. g.f.wt⁻¹
0
40
80
120
140
180
number of days in storage

FIGURE 33 :- CHANGES IN THE CAROTENOID CONTENT OF C.FRAGILE FRONDS DURING STORAGE

KEY :-




Vegetative
Reproductive
100
80
60
40
20
50
40
30
20
10
0
40
80
120
140
180
µg CAR. g f.wt^{-1}
number of days in storage

4.4 DISCUSSION

The Bembridge intertidal zone supports a diverse population of algae. The survival and success of C. fragile in this habitat is largely dependent on the ability of the alga to compete with other algal species and maintain growth in a constantly changing physical environment.

The growth of C. fragile ssp tomentosoides along the Eastern North Atlantic coastline has been well documented and the alga is considered a highly successful colonizer in this environment, (Malinowski and Ramus, 1973; Ramus, 1971; Bouck and Morgan, 1957). Colonies of C. fragile at Bembridge, although firmly established are by no means the major species in terms of growth and population size. Thus, while maintaining a successful niche it is not a dominant colonizer unlike its North American counterpart. However, similarities between the two different types of C. fragile do occur, in that they both reproduce by means of parthenogenesis. Male gametes as seen by Borden and Stein (1969) were not observed in populations of C. fragile sampled from Bembridge.

The most pertinent feature in the natural life cycle of C. fragile at Bembridge is that the period of maximum pigment concentration and vegetative growth occurs during the winter and early spring when conditions of short daylength, low light intensity and low water temperature prevail. This data is supported by Malinowski and Ramus (1973) and the work of Cobb and Rott (1978) who showed that maximum rates of photosynthesis from C. fragile chloroplasts were obtained when young fronds were collected in November and March. Older tissue collected during the summer months gave reduced rates for chloroplast photosynthesis. Thus, a reduction in pigment content and photosynthetic rate coincides with maximum reproductive activity in C. fragile.

Maximum growth in reproductive fronds occurred during June and July and ceased after the development of reproductive structures. Subsequent winter sampling trips yielded young small fronds. It may be possible that the size of larger reproductive fronds is reduced by winter fragmentation, a process by which the entire frond divides into small fragments that separate from the parent plant by means of basal

constrictions on the dichotomies. Fralick and Mathieson (1972) considered this to be a means of vegetative propagation. However, Hanisak (1979) suggests the process to be an important means of regulating frond size, ensuring frond resistance to high levels of wave action and a lower requirement for light and nutrients. This may also be important for C. fragile at Bembridge. A further consideration is that winter fragmentation may be a means of removing redundant photosynthetic tissue from reproductive fronds which have suffered the 'summer bleaching' phenomenon. On early autumn sampling trips a small number of C. fragile colonies were observed to be atypical of the young plants usually found at that time of year. These colonies were dark green in colour and their large size (7-8 dichotomies) suggested they could have been reproductive during the summer. However, the position of these plants in the alga's life cycle is debatable. They may have reached maturity late in the season, or alternatively recovered from the typical summer bleaching phenomenon by means of winter fragmentation or frond tip recovery.

Although quarterly monitoring of C. fragile at Bembridge was achieved during this study a more detailed investigation of the alga's life cycle is necessary. The marking of C. fragile colonies in the sampling areas using frond "ringing" techniques would be particularly advantageous in life cycle studies. Similarly, the importance of competition effects from other algal species on the growth and development of C. fragile would also be enlightening.

The overall seasonal growth strategy for C. fragile is typical for a number of algal species. In Pelvetia canaliculata maximum photosynthetic activity and pigment concentration coincide with the non-reproductive part of the life cycle (Jensen, 1966). Mann (1973) has shown that Laminaria spp. also have a period of maximum carbon fixation and pigment content in their life cycle, and concludes that such fluctuations in algal growth patterns afford an environmental advantage. Using a similar premise for a growth strategy in C. fragile an explanation for seasonal adaptation can also be proposed. Four major interacting factors contribute to the success of C. fragile in its

intertidal niche:- the physical environment, seasonal aspects of tidal movement, competition from other algae and nutrient availability. A strategy for growth in C. fragile is best outlined by following the life cycle of the alga and relating this to the prevailing environmental conditions listed above.

During the summer months the environmental conditions in the intertidal zone are unfavourable for maximum assimilation in C. fragile. Lower tides cause prolonged periods of desiccation and photooxidation may account for a substantial reduction in pigment content as evidenced by frond bleaching. Considerable competition from other faster growing algae for space, light and nutrient is observed during the summer months, and at this time C. fragile suffers extensive epiphytic attack. That nutrient availability may be greatly reduced is indicated by the work of Tait (1972) and Hanisak (1979) who recorded seasonal variation in seawater content, where phosphate and nitrate concentrations were minimal in the spring-autumn period. This seasonal variation in nutrient availability is particularly pronounced in coastal waters where it is dependent on the growth and decline of the algal species present. Evidence to suggest C. fragile is sensitive to a lowered nutrient status during the summer months is provided by the development of hairs on the surface of summer sampled fronds (Figure 27). Head and Carpenter (1975) found that colonies of C. fragile maintained in low-nutrient culture developed whitish hairs which dissappeared if returned to high nutrient status. Schonbeck and Norton (1979) have also shown a similar phenomenon in Fucus spiralis germlings. These authors suggest that hair increases the available surface area for nutrient uptake when nutrient status is low. It is of interest to note that summer sampled C. fragile from Bembridge soon loses its hairs when placed in fresh seawater during storage (section 4.3.3).

Two distinctly different types of C. fragile sampled from Bembridge in June 1982 (section 4.3.1 and 4.3.2) provided some interesting environmental information. One of the major differences between the two colonies was the lack of epiphytic growth and hair development on the fronds sampled from the shaded subtidal area. Figure 26 shows very early hair development on these shaded fronds

Figure 27 demonstrates fully developed hairs on fronds sampled from the intertidal zone. It is possible that the nutrient status in shaded subtidal C. fragile colonies was considerably better than that of the intertidal zone, as evidenced by lack of epiphytic attack and less competition from other algal species. Thus, hair development in C. fragile may reflect the nutrient status of its immediate environment, especially in the summer when nutrient availability is low.

Head and Carpenter (1975) also suggest that frond bleaching may be a symptom of low nutrient status since bleached fronds cultured in nitrogen rich medium soon became green. Furthermore, Rosenburg and Ramus (1982) found that levels of photosynthetic pigments in Ulva spp increased following peaks in nutrient availability. These increases were not related to incident photon flux density, and increases in available nitrogen appeared to correspond to an increase in nitrogen associated with the chlorophyll/protein complexes of the alga. The pigment content of C. fragile fronds sampled from two different locations in June 1982 are shown in Table 13. It is particularly interesting that those fronds sampled from the shaded area have approximately double the pigment concentration of those sampled in the intertidal zone. However, the major pigment distribution in both colonies is almost identical (as shown by pigment ratios), and the shaded fronds have chlorophyll:carotenoid ratios typical of summer sampled fronds. It is most probable that the differences in pigment content shown in Table 13 are environmentally induced. These results also suggest that the appearance of bleached midsummer sampled C. fragile from the intertidal zone may be due to a chlorotic condition induced by lowered nutrient status in conjunction with photooxidative damage caused by prolonged exposure during low midsummer tides. It is therefore important to note that the increased pigment content of shaded fronds may have been a response to light and/or nutrient status. Ramus, Beale, Mauzerall and Howard (1976) have shown that C. fragile sampled from Woods Hole Massachusetts showed greater photosynthetic pigment content when sampled at increased depths. Also, C. fragile together with other algae usually located in the intertidal zone, were found to change only their pigment concentration but not their pigment ratio.

Ramus et al (1976) suggest this type of adaptation to be analogous to the higher plant, "sun and shade response". These findings suggest that such a light response may also be implicated as inferred from the results in Table 13. However, the environmental factors initiating these effects are extremely complex, and an indepth study of C. fragile in relation to its environment is required. Particular attention should be given to the importance and interaction of nutrient status and light.

With the onset of winter many changes take place in the intertidal zone. The amplitude of the tide is reduced and there is a reduction in daylength and temperature. These changes mark the decline in growth of several summer dominant algae, especially Sargassum sp together with a number of algae which were epiphytic on C. fragile. This decline is particularly important to C. fragile since competition is almost eliminated and there is an input of nutrients, particularly nitrogen and phosphates coincident with the degradation of the declining species. During the winter period following this autumnal decline, C. fragile shows a massive increase in pigment content, based on a fresh weight basis, Figures 28 and 29. Similarly Rutter (1982) has shown the highest levels of total chlorophyll per chloroplast also occur in the winter and early spring, together with a concomitant increase in chloroplast size. As daylength increases in early spring an increase in chlorophyll content in C. fragile also occurs, Figure 28. It is possible that this alga is able to adjust its photosynthetic capacity to suit seasonal environmental conditions. The accumulation of storage reserves during intensive winter assimilation may also provide additional support for the reproductive phase the following summer when environmental conditions are less favourable. Evidence for nutrient accumulation is given by Hanisak (1979) who found that populations of C. fragile from Rhode Island showed seasonal variations in the nitrogen status of their fronds. Internal nitrogen was minimal in the summer and maximal in the winter, indicating nitrogen limitation during the summer and nitrogen storage in the winter. Hanisak (1979) also found that C. fragile can utilize NO_3^-, NO_2^-, NH_4^+, and urea, a substantial competitive advantage over other algae which may not be able to use all these nitrogen sources. A similar nutrient storage

response was found in C. fragile sampled at Bembridge by Rutter (1982) who observed lower ratios of acid polyphosphate:alkali polyphosphate in chloroplasts extracted from November-March sampled fronds. Since alkali soluble polyphosphate is less easily mobilized in the chloroplast, this forms a storage resevoir of phosphate, which presumably accumulates when environmental phosphate is high during the winter. During the summer, chloroplasts have an increased ratio of acid:alkali soluble polyphosphate, corresponding to an increase in the more easily mobilized acid form. This coincides with a period when environmental phosphate is low, due to summer competition effects. It is thus possible that C. fragile, is able to regulate its own nutrient stores as an adaptive response to the limiting conditions in its immediate environment.

Stein (1973) reports that Siphonaceous algae may be maintained for short periods of time in aquaria supplied with artificial light provided the seawater is changed frequently and the temperature maintained at $< 23^{o}C$. Codium fragile can grow in a wide salinity range and requires a minimal temperature of 10-13^{o}C for growth (Hanisak, 1979). Thus the conditions described in Chapter 2 were used for the maintenance of C. fragile in short-term storage. The maintenance of actively growing cultures of C. fragile was not attempted, frequent sampling trips to Bembridge being preferred. However, the development of gametangia in vegetative fronds which had been stored for long periods of time (> 3 months) may suggest a certain amount of stability under storage conditions. The dissappearance of frond hairs in reproductive fronds after short periods of storage also suggests an adaptation in the fronds to increased nutrient status after removal from the intertidal zone. The effects of storage on the pigment content of November sampled fronds appears to parallel the winter accumulation of chlorophyll described in the natural life cycle of the alga. Alternatively this may be an adaptive response to increased light-harvesting ability in the low light intensity conditions of storage. Although, if this were the full explanation, increased carotenoid synthesis would be expected also. Carotenoid content remains relatively stable with a maximum accumulation of 91 $\mu g.g^{-1}$ and a minimum content of 75 $\mu g.g^{-1}$ (total carotenoids) after 84 days of storage. Summer-sampled fronds exhibited similar behaviour for both

chlorophyll and carotenoid pigments which after long term storage demonstrated erratic changes in concentration. This probably reflects the original status of the frond on summer-sampling as photooxidative symptoms were apparent, and a low nutrient status in the intertidal zone may also have contributed to frond bleaching. The initial increase in pigment content of summer-sampled tissue may indeed reflect increased nutrient status on storage. Similarly, this may also be a factor in the loss of frond hairs after several days in storage conditions.

Interpretation of the effects of storage on C. fragile is particularly complex since changes in frond physiology and morphology may be effected by the storage conditions and/or natural developmental changes in the life cycle of the alga. Indeed, the overall problem with maintaining cultures of macro algae is that their growth and development cannot be strictly controlled as is possible in higher plant studies. This is especially the case for intertidal algae as storage conditions simulating tidal effects are particularly impractical. For these reasons constant storage conditions were considered to be the best means of maintaining C. fragile and this appears to be effective for most studies with macro algae (Stein, 1973). Rutter (1982) has shown that chloroplast photosynthesis is maintained during short periods of storage (2-3 months) and the affects of storage on pigment content and frond morphology largely parallels the seasonal growth of the alga. Thus, with the use of storage 'controls' experimental investigations using stored fronds should prove satisfactory.

4.5 CONCLUSIONS

The life cycle of C. fragile shows strong seasonal trends. The period of maximum carbon fixation, pigment content, and chloroplast size occurs during the winter when competition from other algae is reduced and C. fragile is able to maintain a high nutrient status. At this time the alga may be able to accumulate storage reserves of nitrogen and phosphates

In the summer months when the physical environment is more extreme (due to prolonged tidal exposure) and competition from other algae greater, photosynthetic efficiency is reduced and the alga enters a reproductive phase. This part of the life cycle may be aided by the development of frond hairs to increase nutrient uptake and the mobilization of stored nutrients, and possibly assimilates which were sequestered during the winter vegetative phase.

The effects of storage on frond morphology and pigment content have been monitored and results suggest that short term storage of C. fragile does not have adverse affects of either parameter. However, the use of "storage controls" is incorporated in subsequent experiments.

5.0 THE EFFECT OF LIGHT INTENSITY AND QUALITY ON PHOTOSYNTHESIS IN C. FRAGILE FRONDS AND CHLOROPLASTS

5.1 INTRODUCTION

The remaining chapters in this thesis describe the effects of controlled light environments on C. fragile fronds and chloroplasts with particular emphasis on pigment stability at high light intensities, and photooxidation in this alga. This chapter provides a preliminary investigation into the effects of light intensity and quality on photosynthesis in C. fragile fronds and chloroplasts.

5.2 MATERIALS AND METHODS

5.2.1 Sampling of C. fragile fronds

Chloroplast studies were performed on fronds sampled 3.3.1982 and whole frond photosynthesis was investigated using C. fragile sampled 22.6.1982.

5.2.2 Chloroplast isolation

Chloroplast preparation was performed as described in Chapter 2. Extracts were maintained on ice in the dark and appropriate chloroplast samples were removed as required throughout the photosynthetic experiments. The results in these studies represent four different chloroplast preparations from different C. fragile plants. Chloroplast number and chlorophyll content were in the range 16.0-45 x 10^8 CPS.ml^{-1}. and 89-193µg Chl.ml^{-1}. respectively. Samples containing 20.6-48.2 µg Chl.Ml^{-1} were used for each photosynthetic determination.

5.2.3 Measurement of photosynthesis in isolated chloroplasts

Photosynthesis was measured as photosynthetic oxygen evolution using a Clarke-type oxygen electrode and the methods of Delieu and Walker (1972):-

The platinum cathode was soaked with saturated KCL solution and a small Teflon membrane was fitted over its surface with an '0' ring. The electrode jacket was fitted over the well

and the apparatus connected to a magnetic stirrer, potentiating unit, and a Bryans 2800 chart recorder. The electrode was maintained at 14°C using a cooling jacket connected to a mains water supply. 2ml of chloroplast extraction medium (Chapter 2) was placed in the electrode well and the magnetic stirrer was switched on. The voltage on the chart recorder was set to 10 mV and the potentiating unit adjusted to give a voltage of 1v. The electrode was allowed to stabilize for usually 15-30 minutes, and the sensitivity dial on the potentiating unit adjusted to give a deflection of approximately 100% on the chart recorder (after a chart-recorder baseline check). Once a steady chart recorder trace was achieved for the O_2 saturated extraction media, 2 grains of sodium dithionite were added, to remove the O_2 from solution, i.e.:-

$$Na_2S_2O_4 + O_2 + H_2O \longrightarrow NaHSO_4 + NaHSO_3$$

This caused a rapid deflection on the chart recorder and thus calibrated the distance between the air-line at saturated O_2 condition, and the zero O_2 condition of the electrode. The O_2 content of air saturated water is dependent on temperature, and using the data of Delieu and Walker (1972), the O_2 content of 14°C was 0.29 $\mu M.Ml^{-1}$. Thus the distance d, on the chart recorder corresponds to 0.29 μm O_2. After this procedure the well was washed thoroughly with distilled water to remove all traces of dithionite. A 1 ml reaction volume consisting of 20.6 - 48.2 μg chlorophyll in extraction medium and 100 μl of 100 mM $NaHCO_3$ was used to measure photosynthetic rate. This mixture was purged of O_2 using a stream of N_2 and placed immediately into the electrode chamber, which was then fitted with the plunger and the stirrer switched on. The entire apparatus was placed in the dark until a steady baseline trace was achieved on the chart-recorder, after which the chloroplasts were illuminated with a suitable light source and photosynthetic O_2 evolution recorded. From the calibration procedure a 1cm distance on the chart recorder was equivalent to x μM O_2 at 14°C,

thus from the recorder trace:-

$$\frac{\text{A cm x X}}{\text{B minutes}} = \mu \text{ moles } O_2.\text{min}^{-1}$$

Final photosynthetic rates were calculated as $\mu M\ O_2.\mu g\ Chl^{-1}.hr^{-1}$.

5.2.4 The effect of light intensity on C. fragile chloroplast photosynthesis

This was investigated using a wide range of light intensities provided by a tungsten/halogen Halight projection lamp. Intensities were within the range 20-600 $\mu E.M^{-2}.S^{-1}$ P.A.R. which is equivalent to 38-972 $W.M^{-2}$. total radiation. For this lamp the P.A.R.:total radiation calibration was 1 $\mu E.M^{-2}.S^{-1}$ = 1.92 $W.M^{-2}$. This was measured using a Crump system 550 quantum and photometric sensor. At least twelve different light intensities were used in the above light range for each chloroplast preparation.

5.2.5 Measurement of photosynthesis in C. fragile fronds

Photosynthesis was measured as photosynthetic O_2 evolution using a Hansatech leaf disc electrode unit and the methodology of Delieu and Walker (1981):- The electrolyte consisted of 1.0 M $Na_2CO_3/NaHCO_3$ in 50% saturated KCL at pH 9.0. A 1 inch square of tissue paper was placed over the platinum cathode of the electrode disc and moistened with electrolyte. The same solution was used to flood the well housing the silver anode. A 1 inch square of Teflon membrane was placed over the tissue paper and an '0' ring applied to secure the membrane over the electrode. The electrode unit was then inserted into the base of the chamber and attached to the chart recorder and potentiating unit. The electrode apparatus was supplied with a calibrating head fitted with apertures for gas-tight syringes. This was fitted to the electrode base and the system allowed to equilibrate in the 1mV recorder range using a 1mV/10mV Hansatech. potentiating unit. After equilibration a 10ml syringe of N_2 was plunged into the calibrating head, this caused a deflection

on the chart recorder which equilibrated at zero O_2. Thus 1mV was equivalent to fully saturated O_2, and the x mV reading to zero (N_2). This reading was never 0 mV as a small residual current was always maintained across the electrodes even after O_2 removal.

When using leaf-disc electrodes it is essential to calibrate the volume of the electrode chamber when the leaf disc is in place. A 1ml air filled, gas tight syringe was inserted into the calibrating head and the controls set to give a steady chart-recorder reading of R_1 (mV) which should be approximately $\frac{2}{3}$ full scale deflection. The plunger was depressed to give an increased reading R_2. At a given temperature and pressure the signal from the electrode is proportional to the activity of O_2 in the chamber, so if the above procedure is followed with a leaf disc in the chamber the effective volume will be:-

$$V = \frac{R_1}{R_2 - R_1}$$

1M solutions of $NaHCO_3$ and Na_2CO_3 were prepared and 0.95 and 0.05 ml respectively were placed in a petri dish. A section of capillary matting was moistened with this solution and then transferred to the base of the electrode chamber. Care was taken not to cover the electrode with the matting. A small piece of nylon gauze was placed over the electrode and supported by the 'O' ring groove this protected the electrode from direct contact with plant material. 2-3g samples of C. fragile frond tips were placed on the gauze and the electrode was calibrated as described above. Due to the high moisture content of C. fragile fronds a water cooling unit was not applied to the electrode jacket. Condensation of water vapour on the illuminated surface interferred with the incident light source, thus all experiments were performed at ambient temperatures which were in the range 25-27°C. The entire apparatus was placed in the dark until a steady reading on the chart recorder was achieved after which the fronds were illuminated with a suitable light source.

To calculate photosynthetic rate:-

The O_2 activity in the air is given as K

$$K = \frac{9.375 \times 273}{273 + T^{o}C.}$$

Since at S.T.P. the activity of O_2 in air containing 21% (by volume) of O_2 $= \frac{0.21}{22.4} = 9.375\ \mu m.Ml^{-1}$.

If K was calculated to be x

then the amount of O_2 in a fully saturated chamber with a leaf disc

$$= x \left\{ \frac{R_1}{(R_2 - R_1)} \right\} = y\ \mu m\ O_2$$

from the O_2, N_2 calibration.

$$O_2 - N_2 = Z\ mV$$

$$\therefore\ Z\ mV = y\ \mu M\ O_2$$

This was corrected to standardize the readings to 1 mV and the chart recorder readings were read as 1 mV or 1 cm equivalents directly from the chart paper. Photosynthetic rates were calculated as described in section 5.2.3 and expressed as $\mu M\ O_2\ g.fwt^{-1}\ hr^{-1}$.

5.2.6 The effect of light intensity on C. fragile frond photosynthesis

This was investigated using a light beam reflected from a projector lamp onto the surface of the electrode unit by means of a series of mirrors so that the frond tips were illuminated from above. Intensities were within the range 20-2,700 $\mu E.M^{-2}.sec^{-1}$ P.A.R. which is equivalent to 38-5,184 $W.M^{-2}$. total radiation. Six light dose response curves were performed on fronds sampled in June 1982, and at least 12 separate light intensities were used for each light curve.

5.2.7 The effects of light quality on photosynthesis in C. fragile fronds

Six Agfa colour filters were used to modify the tungsten/halogen light source illuminating C. fragile fronds in the leaf-disc electrode unit, Table 14 shows the characteristics of filters used in these experiments. The light source was arranged to supply the same light intensity (300 $\mu E.M^{-2}s^{-1}$ P.A.R.) of filtered light to the surface of the fronds. Thus, light quality was modified by the filters but not light intensity. This could not be achieved for the blue and Cyan filters as their absorption range corresponded to a large proportion of the P.A.R. For these filters maximum light intensities attained were 120 $\mu E.M^{-2}.sec^{-1}$ P.A.R.

Table 14 AGFA-COLOUR FILTER CHARACTERISTICS

Colour of filter	λ of light absorbed (nm)	Light intensity with filter in place $\mu E.M^{-2}s^{-1}$ P.A.R.
Yellow	360-380	300
Orange	340-500	300
Light Pink	500-580	300
Dark Pink	500-610	300
Cyan	300-310, 580-680	120
Light Blue	315-340, 580-654	120

The filters were placed over the surface of the electrode unit and the light beam directed through them. All other parts of the electrode were sealed with silver foil to ensure no other light but that modified by the filter was available to the fronds.

The measurement of photosynthetic rate was performed as described in section 5.2.5. The effects of each filter treatment was determined for 3-4 different samples of C. fragile frond tips.

Control samples were illuminated with 300 $\mu E.M^{-2}s^{-1}$ or 120 $\mu E.M^{-2}s^{-1}$ P.A.R. white light from the projector lamp. Photosynthetic rates were determined as described previously before and after filter treatments. Care was taken to remove the filter before photosynthetic saturation had occurred.

5.3 RESULTS

5.3.1 The effects of light intensity on chloroplast photosynthesis

Figure 34 shows the effects of light intensity on chloroplast photosynthesis using four different chloroplast extracts. Although chloroplasts were extracted from different C. fragile plants the general light saturation characteristics are similar, however, absolute photosynthetic rates do vary within the range 8 to 21 $\mu M\ O_2\ \mu g\ Chl^{-1}.hr^{-1}$. The chloroplasts saturate at low light intensities (50-100 $\mu E.M^{-2}s^{-1}$ P.A.R.), have a very narrow saturation plateau (50-100 $\mu E.M^{-2}s^{-1}$ P.A.R.) and photo-inhibition occurs as light intensity increases beyond this plateau. Photosynthetic studies with C. fragile chloroplasts showed a consistent absence of a lag phase at the onset of light incubation.

5.3.2 The effects of light intensity on frond photosynthesis

Photosynthetic rates were determined the second day after sampling from Bembridge, and subsequent replicated experiments showed the tissue to decline in response to light intensity after a short storage period (Figure 35). Day 2 shows a light dose response curve with a sharp increase in photosynthetic rate after 700 $\mu E.M^{-2}s^{-1}$ P.A.R. A short saturation plateau was maintained for 250 $\mu E.M^{-2}s^{-1}$ P.A.R. after which inhibition appeared to occur. Analysis after 3 storage days showed a similar response however, photosynthetic rate was reduced and no saturation plateau was evident. After 4 days in storage the fronds did not respond to increased light intensities and this response was maintained after approximately 1 month in storage. Maximum photosynthetic rates for fronds were 3.4 $\mu M\ O_2\ g\ fwt^{-1}.hr^{-1}$ at day 2. A sample of frond tissue was analysed for pigment content and estimates of frond photosynthetic rates on a chlorophyll basis were 2.5-17.5 $\mu M\ O_2.\mu g\ Chl^{-1}.hr^{-1}$. A lag phase was not apparent for C. fragile

FIGURE 34 THE EFFECTS OF LIGHT INTENSITY ON PHOTOSYNTHESIS IN ISOLATED C.FRAGILE CHLOROPLASTS

KEY: -

figures 1-4 = replicates of chloroplast extracts from four separate C.fragile plants.

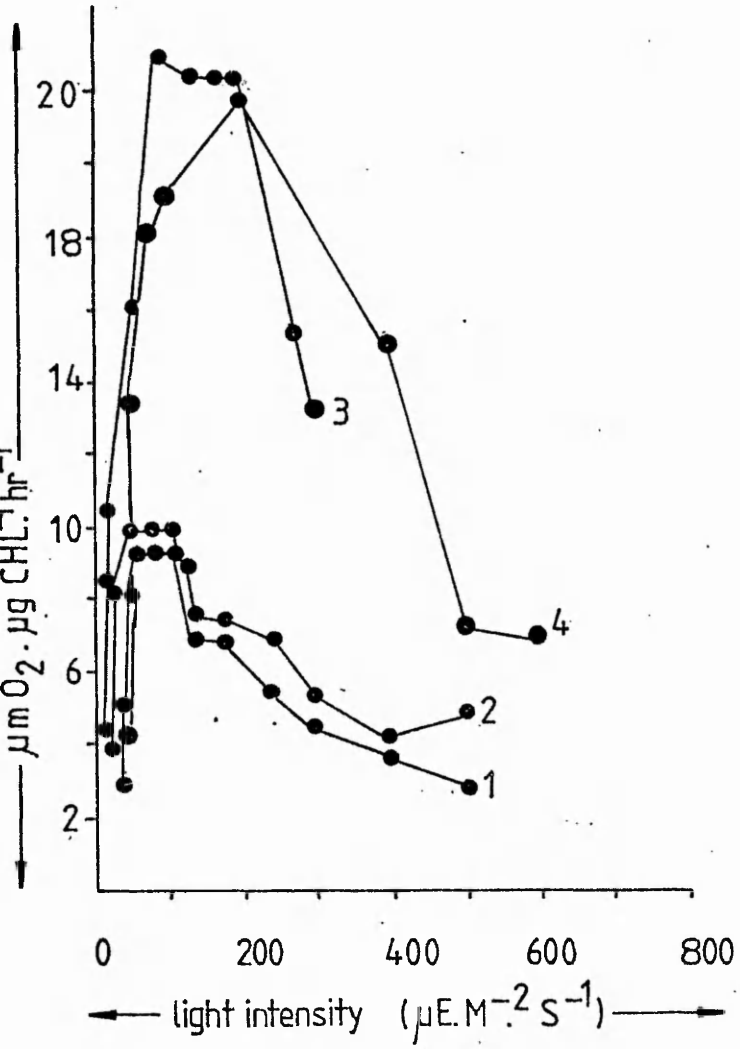
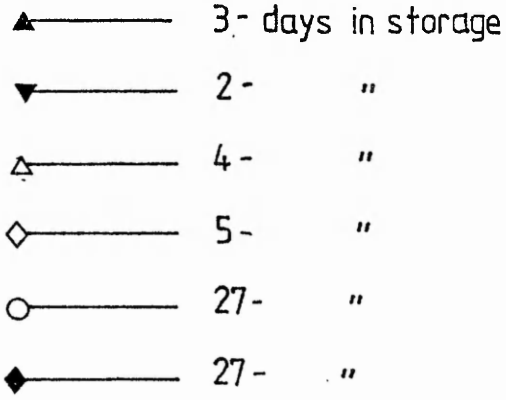

20
18
14
10
6
2
μm O2 . μg CHL-1 hr-1
3
4
2
1
0
200
400
600
800
light intensity (μE.M-2 S-1)

FIGURE 35:- THE EFFECTS OF LIGHT INTENSITY ON PHOTO-SYNTHESIS IN STORED C.FRAGILE FRONDS

KEY :-



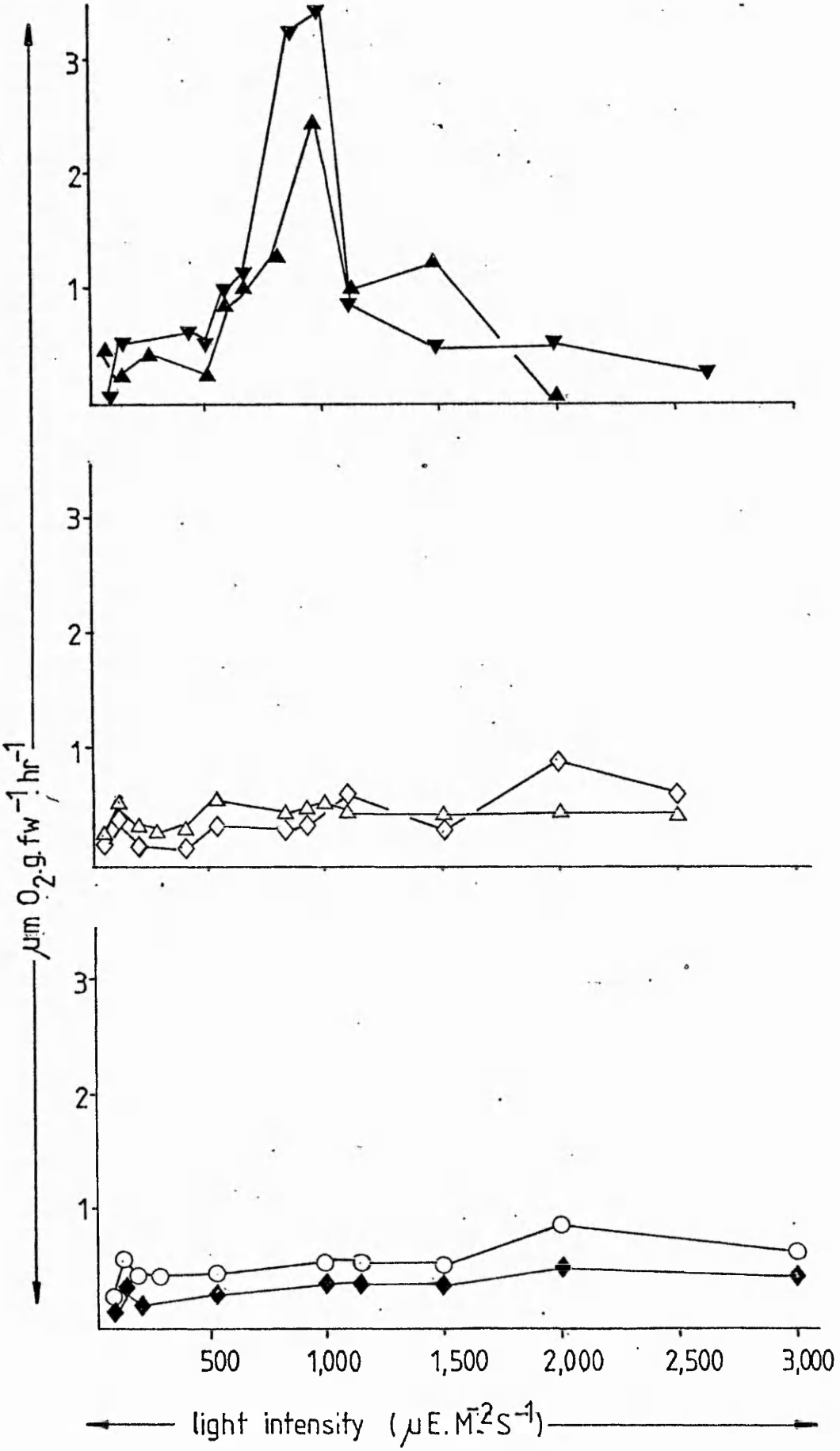

3
2
1
3
2
1
3
2
1
μm O_2.g.fw^{-1}.hr^{-1}
500
1,000
1,500
2,000
2,500
3,000
light intensity (μE.M^{-2}S^{-1})

FIGURE 36:- THE EFFECT OF LIGHT QUALITY ON PHOTO-SYNTHESIS IN C.FRAGILE FRONDS

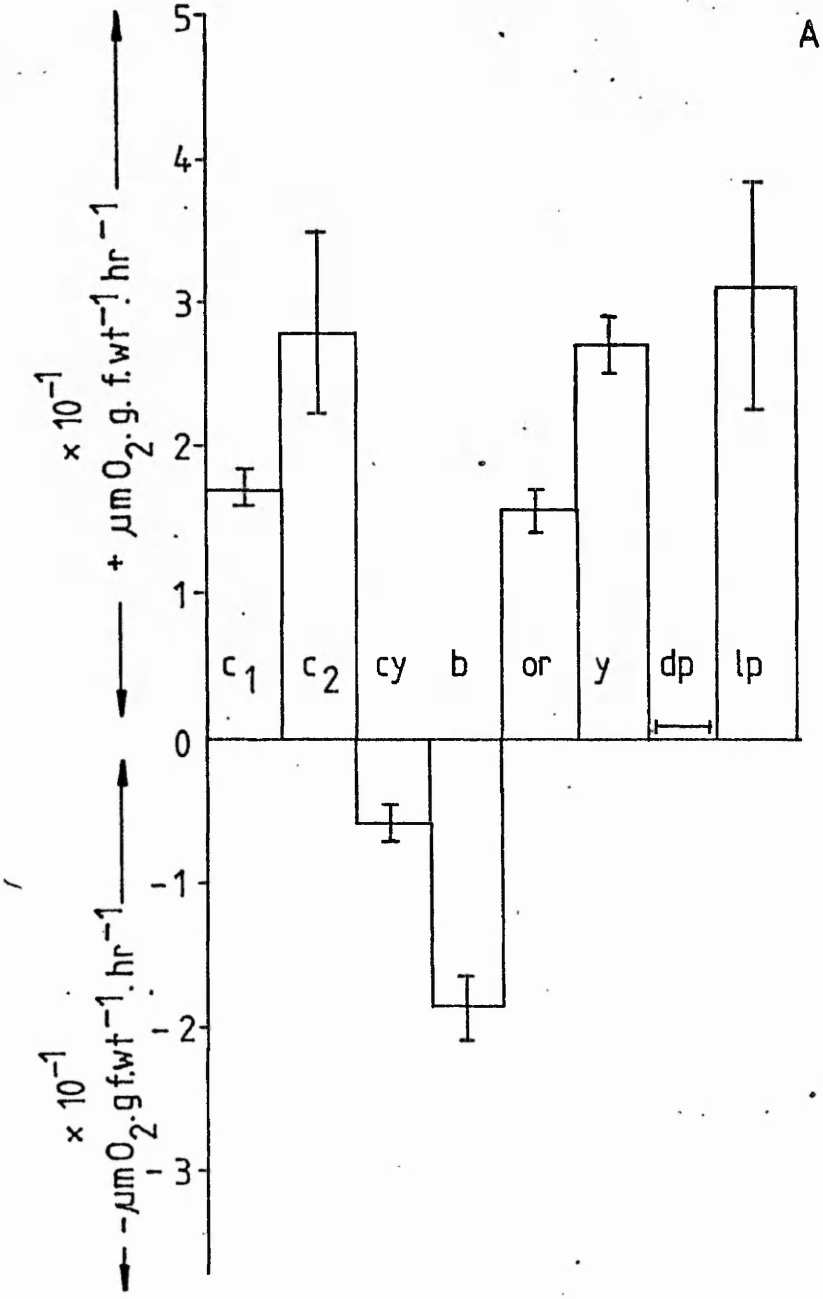
A. Photosynthesis during filter treatment

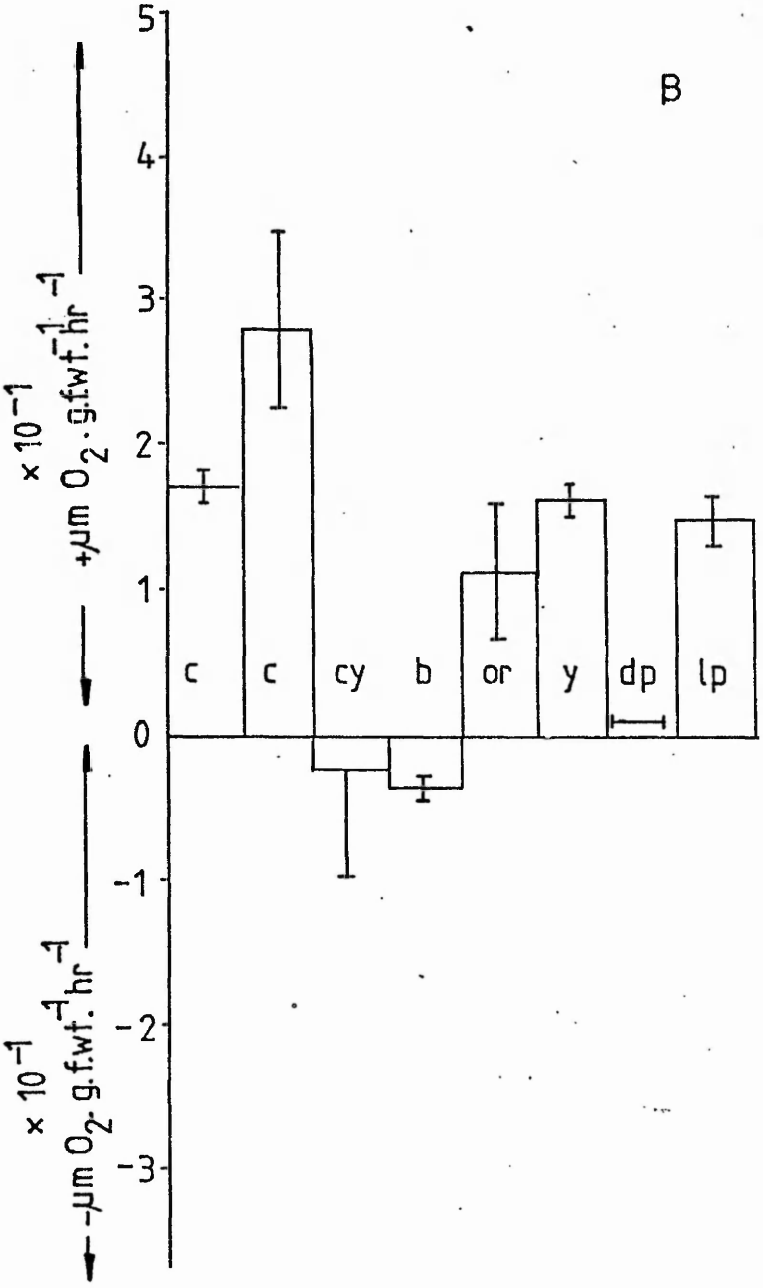
B. Photosynthesis after filter treatment

KEY :-

c_1 = Control at 300 $\mu E.M^{-2}.S^{-1}$
or = Orange filter " "
y = Yellow " "
dp = Dark pink " "
lp = Light pink " "

c_2 = Control at 120 "
cy = Cyan filter " "
b = blue " "


A
5
4
3
2
1
0
-1
-2
-3
$\times 10^{-1}$
$+ \mu m O_2 . g . f.wt^{-1} . hr^{-1}$
$- \mu m O_2 . g f.wt^{-1} . hr^{-1}$
c_1
c_2
cy
b
or
y
dp
lp


B
5
4
3
2
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0
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-2
-3
c
c
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or
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× 10⁻¹ +µm O₂. g.f.wt.⁻¹. hr⁻¹
× 10⁻¹ -µm O₂. g.f.wt.⁻¹. hr⁻¹

frond photosynthesis however, reduced electrode response and sensitivity limits on the chart recorder made interpretation of the electrode scans rather difficult at the onset of the reaction curve.

5.3.3 The effects of light quality on C. fragile frond photosynthesis

Figures 36a and 36b show the effects of light qualities on the photosynthetic rates of C. fragile fronds during and after filter treatments. The fronds had been in storage for 30 days. Blue filters stimulated O_2 uptake at the rate of 0.06-0.19 μM O_2. $g.fwt^{-1}.hr^{-1}$. Pink filters produced different photosynthetic responses depending on the amount of light they absorbed. The light pink filter reduced P.A.R. by approximately 40% and enhanced the photosynthetic rate of the fronds. However, the dark pink filter which absorbed approximately twice the incident light, reducing P.A.R. by 70% effectively inhibited net photosynthesis as follows:- A very slight increase in O_2 evolution was observed after 8 minutes exposure, this was followed by a sharp decrease, on removal of the filter O_2 evolution increased slightly but then immediately decreased. Four different samples of C. fragile fronds responded similarly.

5.4 DISCUSSION

Isolated higher plant chloroplasts usually exhibit an initial lag phase at the onset of carbon fixation but this response is absent in C. fragile and confirms the previous findings of Cobb and Rott (1978) and Rutter (1982). Frond photosynthesis also appears to show a lack of an induction period, however leaf disc electrode studies were limited by chart recorder sensitivity and definite conclusions on the lag phase cannot be made for intact fronds.

Ramus (1981) describes the general features of the classic response curve of light intensity versus photosynthetic rate. Light intensity is linearly related to rate until a saturation plateau is reached when other factors such as CO_2 availability and activity of dark reaction enzymes becomes limiting. After this plateau photosynthesis

declines presumably due to photoinhibition. C. fragile chloroplasts appear to reach a maximum rate of photosynthesis over a very narrow range of low light intensities (Figure 34), and similar results have been shown by Cobb and Rott (1978) and Rutter (1982). Higher plants saturate at higher light intensities and attain saturation over a greater range (Jensen and Bassham, 1966). Herron and Mauzerall (1972) suggest that the gradient of the linear region of the light intensity curve is related in part to the efficiency of the photosynthetic apparatus. Using greening studies in Chlorella vulgaris these authors show the initial slope of the light saturating curve to be proportional to reaction centre number and their cross sectional area. It presumably follows that this area of the curve is also a function of light-harvesting efficiency and excitation energy transfer to the reaction centres. If this deduction is valid the particularly inclined gradients observed for C. fragile (Figure 34) indicate an ability of this alga to make highly efficient use of low light intensities. This may also reflect the particularly high light-harvesting capacity of C. fragile as demonstrated by the presence of large quantities of LHCP units in isolated thylakoid extracts (Chapter 3). Once photosynthetic saturation occurs C. fragile chloroplasts exhibit a narrow saturation plateau (Figure 34) and this supports the findings of Cobb and Rott (1978). Furthermore, C. fragile is unable to maintain this light-saturating plateau over an extended range of light-intensities and photoinhibition occurs once supra-optimum light intensities for photosynthesis are reached. This may be a consequence of low light intensity adaptation in the alga. Thus, large numbers of light-harvesting units transfer excitation energy to a comparatively small number of reaction centres, which at supra-optimum light-intensities become rapidly saturated. The turnover of photochemical events becomes out of step with the amount of light energy being harvested and the condition soon leads to photoinhibition, and presumably photooxidative damage. The exact mechanism of photoinhibition is apparently varied and complex and the turnover rates of the photosynthetic dark reactions and photorespiration are also important. Bjorkman (1981) suggests that the reaction centre of PSII is a primary target of inactivation when photoinhibition occurs and this may reflect the close association of

PSII with the light-harvesting components of plant chloroplasts (Chapter 1). It is evident that isolated C. fragile chloroplasts are susceptible to photoinhibition and summer-sampled fronds often show signs of pigment bleaching. In order to investigate the effects of light intensity. On intact C. fragile plants, light response curves were performed on fronds sampled during June 1982. Tissue without extensive bleaching symptoms was preferentially used.

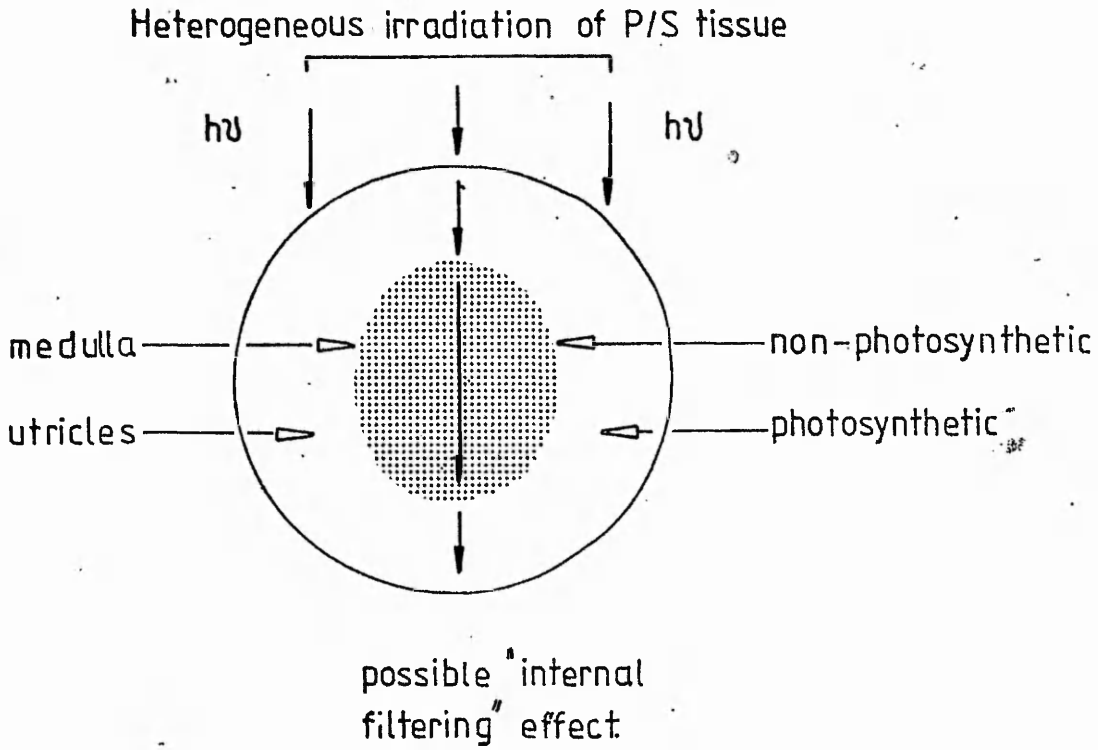
Figure 35 shows the effects of light intensity on frond photosynthesis and rates are given as $\mu M\ O_2\ g.fwt^{-1}.hr^{-1}$. These values are probably underestimated as the cylindrical nature of the algal frond did not present an entire illuminating surface. Initial analyses (2-3 days after sampling) did not show a typical light response curve as described for higher plants and indeed C. fragile chloroplasts (Figure 34). A linear response to increased light intensity and a light saturation plateau for maximum photosynthesis were not observed. Photoinhibition at light intensities $> 1{,}000\ \mu E.M^{-2}s^{-1}$ P.A.R. was apparent during storage day 2, but a similar response was not defined on subsequent analyses. Four days after sampling the fronds showed no response to light intensity and this continued up to 1 month after storage. This could indicate that the alga has suffered some type of damage during storage, however, the fronds appeared morphologically healthy and photosynthetic O_2 evolution in the leaf-disc electrode was comparable to higher plant leaf-disc studies. Furthermore, when frond data was based on pigment content rates of maximum photosynthesis were of a similar order ($17.5\ \mu M\ O_2.\mu g.Chl^{-1}.hr^{-2}$) to those in chloroplast studies (Figure 34). Storage does not appear to affect chloroplast photosynthesis and the data presented in the previous figure was based on tissue which had been in storage for several weeks. Rutter (1982) has also shown that short term storage is not deleterious to chloroplast photosynthesis in C. fragile.

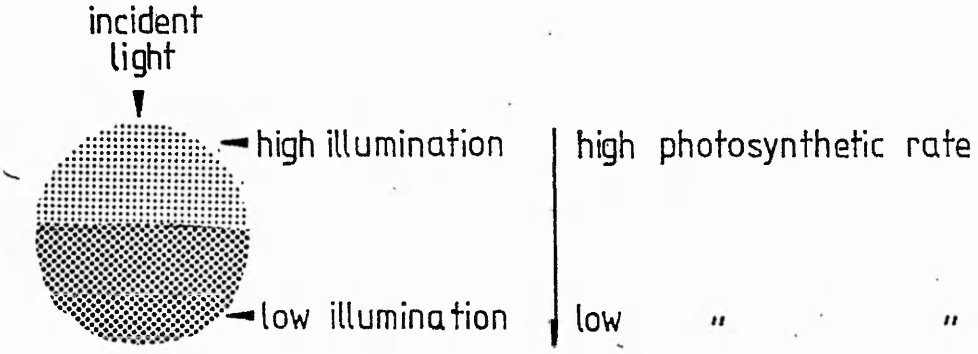
Various workers have placed particular emphasis on the importance of frond morphology in the capture of light energy and suggest that frond structure is an important determinant of photosynthetic response. Ramus (1978) found that C. fragile fronds saturated at low light intensities, and that the fronds were highly efficient at absorbing

incident low intensity light. However, this author suggests that C. fragile should show little response in photosynthetic rate if light intensity increases, and this phenomenon is based on the anatomy of the frond. Ramus (1978) argues that because C. fragile fronds are cylindrical in shape and that the chloroplast-containing utricles are arranged around the medullary centre the photosynthetic tissue is unequally efficient in capturing light energy. Hence, this frond morphology may produce prolonged and extended photosynthetic light saturation responses (Ramus et al. 1976), this hypothesis is explained schematically in Figure 37. This behaviour may be particularly evident in leaf disc chambers where uniformity of illumination is difficult for other than flat leaf surfaces. However, when C. fragile chloroplasts are isolated and uniformly illuminated a more typical light saturation curve is obtained. Although frond anatomy may be responsible in part for atypical frond photosynthesis other factors are evidently involved as a decline in light sensitivity occurs in stored C. fragile (Figure 35). Ramus and Rosenburg (1980) have shown that photosynthetic light response curves are variable in time and space, and this is particularly true for marine algae. Tidal, and diurnal rhythms, fluctuations in light and nutrient environment, desiccation etc., are important parameters in a marine environment. It is thus possible that the removal of C. fragile from an intertidal environment may be responsible in part for the decline in photosynthetic response to light intensity in stored C. fragile fronds. Ramus et al (1976) expected little change in photosynthesis when in situ. C. fragile plants were exposed to natural variations in light intensity, as in accordance with their frond morphology hypothesis. However, this was not the case, and the alga displayed optimum photosynthetic capacities over a range of light intensities. Figure 35, shows a residual response to light intensity in freshly sampled fronds which was eventually eroded in storage. This may suggest that frond architecture may only become an important factor in frond photosynthesis when all other environmental parameters are constant, as in a stable storage environment.

A comparison of chloroplast and frond (day 2-3) light saturation curves shows that fronds 'saturate' at approximately 3 times the light

FIGURE 37 :- THE EFFECT OF FROND ANATOMY ON LIGHT ATTENUATION AND PHOTOSYNTHESIS IN C.FRAGILE





Photosynthetic rates of heterogeneously irradiated C.fragile fronds are not proportional to :-

1. the total f.wt of the frond
2. the total chloroplast and chlorophyll content of the frond.

intensity of chloroplasts and this suggests a filtering affect by the intact fronds. A "relative" photoinhibiting effect was not observed in fronds stored after 3 days even at light intensities of 3000 $\mu E.M^{-2}sec^{-1}$ P.A.R. This intensity can be typical during the midsummer when frond bleaching in C. fragile occurs.

Ramus (1981) considers absorptance (that fraction of incident light absorbed) to be the primary measurement of light for photosynthetic events, and has measured the absorptance properties of various algal thalli. U. lactuca was shown to uniformly absorb in the P.A.R. spectral region but not in the 'green window'. C. fragile however gave a uniformly high absorptance throughout the 380-700 nm region including the green wavelengths. Ramus (1978) suggests this non-specific absorption in C. fragile is due to the ability of the utricles to enhance the capture of incident light energy. This enhancement being independent of wavelength and is described by the author as "low specific chlorophyll absorption". This is a rather misleading term as chlorophyll absorption is wavelength specific in all plants, as indicated by their chloroplast and pigment absorption spectra. This is also typical in C. fragile as shown in Chapters 2 and 3.

Using various filters the affects of different light quality treatments on photosynthesis in C. fragile fronds was investigated as the absorptance of light by intact thalli was not thought to reflect the utilization of absorbed light in photochemical processes (Ramus 1981). Figures 36a and 36b show photosynthetic rates during and after filter treatments. On removal of filters no fronds showed recovery to control photosynthetic rates. This suggests that filtered light is in some way deleterious to the photosynthetic apparatus. This may be due to excessive excitation of one or more components of the light-absorbing apparatus when light intensity was adjusted (to 300 or 120 $\mu E.M^{-2}S^{-1}$ P.A.R.) to compensate for reduction in P.A.R. when specific wavelengths of light were removed by filter treatments.

When fronds were exposed to blue light O_2 uptake occurred for both types of filter treatment. Oxygen consumption activated by blue light has been reported by Voskrensenskaya (1972, 1979). This uptake was not thought to be due to photorespiration but respiration as blue

light was observed to increase the rate of glycolysis and TCA cycle reactions and also enhance protein synthesis. The above author also suggests that O_2 uptake in blue light causes the reoxidation of reduced intermediates in photosynthetic electron transport. This may account in part for the continued but reduced O_2 uptake on removal of the blue filter (Figure 36b). Thus, reduction in O_2 uptake may indicate a slight recovery after treatment, but positive O_2 output was not achieved due to a possible impairment of photosynthetic electron transport reactions. Yellow filter treatment increased photosynthetic O_2 evolution in fronds and this may reflect increased absorption at red wavelengths when the light intensity was adjusted to give 300 $\mu E.M^{-2}.S^{-1}$. P.A.R. The orange filter appeared to have no significant affect on photosynthetic rate. The pink filters removed light in the 500-550 nm region where siphonoxanthin and siphonein absorb, (540-542 nm). When approximately 40% of this light was removed (estimated as % decrease in P.A.R.) the fronds showed increased rates of photosynthesis. Presumably this was due to an increased absorption of red light when intensities were adjusted to 300 $\mu E.M^{-2}S^{-1}$. P.A.R. However, removal of 70% of the available light in the 500-550 nm region resulted in a net loss of photosynthesis even after filter removal. As all other wavelengths of the visible spectrum were available to the fronds, including chlorophyll and other carotenoid specific absorption wavelengths one may conclude that light energy in the region 500-550 nm is crucial to the optimal photosynthetic functions of *C. fragile* fronds. Indeed, this concurs the data in Chapter 3 confirming the importance of siphonein and siphonoxanthin in light harvesting. Ramus *et al* (1978) comments on the importance of siphonoxanthin in *C. fragile* suggesting that "low specific chlorophyll absorption" in this alga already fills the "green window". Furthermore, the above authors consider siphonoxanthin to be of greater advantage to *U. lactuca* as the fronds of this alga do not absorb in the "green window". A similar explanation is given by Dring (1981) who states that the spectral composition of incident light is irrelevant to the photosynthesis of algae with thick thalli, and this may presumably refer to *C. fragile*.

A more correct explanation may be that although the thalli of these algae show non-specific light absorption (i.e. absorb all visible wavelengths uniformly), only certain wavelengths of this light will be utilized in photosynthesis by highly specific light absorbing pigments. However non specific light absorbtion by algal thalli may still confer an environmental advantage. Ramus (1978) suggests this may be an important factor in an alga's ability to grow in the weakest of submarine light fields. Thus, thallus anatomy confers an overall 'light-harvesting' advantage at all wavelengths throughout the visible spectrum. The photosynthetic pigments then harvest that light specific to the photochemical reactions. In C. fragile siphonoxanthin and siphonein appear to be particularly important in this respect, and furthermore Yokohama (1981) also suggest that these pigments correlate strongly with the morphological character of C. fragile. Because this alga has a thick and cylindrical thallus, the chloroplasts in the inner parts of the utricles may receive unusually green light which has been filtered by the chloroplasts in the outer parts. These authors propose siphonoxanthin and siphonein to be important in 'internal' frond chromatic adaptation. This is an interesting theory particularly as light microscope studies demonstrate the occurrence of chloroplasts throughout the length of the utricle, although the highest concentration is often at the tip (Figure 2).

5.5 CONCLUSIONS

Chloroplast photosynthesis in C. fragile demonstrates a consistent lack of an induction period at the onset of illumination and the light saturation characteristics of this alga suggests the chloroplasts are adapted to low light intensities. Fronds however, were insensitive to increasing light intensity four days after sampling. The removal of C. fragile from its intertidal environment may be responsible in part for the decline in photosynthetic response to light intensity in the stored alga. Frond anatomy may also contribute to this atypical response especially when environmental parameters are constant as in a stable storage environment. Photosynthesis in C. fragile fronds is sensitive to light quality, even after storage. Blue light promoted

O_2 uptake in the alga and green light appears to be essential for optimum photosynthesis in this alga, confirming in part the light-harvesting roles of siphonein and siphonoxanthin.

6. THE EFFECTS OF CONTROLLED LIGHT EXPOSURES ON INTACT FROND PIGMENT CONTENT IN C. FRAGILE

6.1 INTRODUCTION

Previous studies indicate that C. fragile has several light adaptations to a shade environment. Chloroplast photosynthesis in this alga saturates at low light intensities (Chapter 5) and plastids contain large concentrations of light-harvesting complexes (Chapter 3). Chlorophyll a:b ratios are particularly low in C. fragile (Chapter 2) and this may indicate a chlorophyll adaptation similar to that found in shade-adapted higher plants. Ramus (1978) has also shown C. fragile fronds to be highly efficient at absorbing incident light energy. These adaptations may be particularly important in submarine environments when light intensities may be substantially reduced by the water column. However, when the tide recedes C. fragile may be daily exposed to high light intensities, particularly in the summer months when tidal amplitude is increased. Frond bleaching during the summer indicates that C. fragile is susceptible to photooxidative damage. Thus, in order to survive in its intertidal niche C. fragile must possess photoprotective mechanisms. The aim of the following experiments is to investigate the effects of short time exposure to relatively high controlled light intensities on the pigment content of C. fragile fronds. Exposure times were chosen to simulate various 'natural' exposure times when fronds in the intertidal zone are exposed to daylight intensities as the tide recedes. Since seasonal fluctuations occur in the pigment content of C. fragile both vegetative and reproductive fronds were examined.

6.2 MATERIALS AND METHODS

C. fragile was sampled from the Isle of Wight during June and November 1980. Frond samples were exposed to controlled light intensities of 500, 750, 1,000, 1,500 and 2,000 $\mu E.M^{-2}.sec^{-1}$ P.A.R. for $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2, 3, 5 and 7 hour periods. The light source was an Osram 1000W solar colour SON/T clear tubular lamp which did not transmit long wave ultra-violet radiation. A Crump system 550 quantum/photometric

sensor was used to measure the light intensities required, and using this system 1 $\mu E.M^{-2}.sec^{-1}$ P.A.R. was equivalent to 2.2 $W.M.^{-2}$ total radiation. Temperature affects were reduced using a water cooling system which maintained the frond temperature at the highest light intensity at approximately 27°C. Fronds were kept moist with Gerrard's seawater to prevent desiccation. After irradiation frond samples were immediately frozen at -20°C and analysed for changes in pigment content using the methods described in Chapter 2. A non-irradiated control sampled directly from the storage tanks was included in the analyses for each light regime. Statistical analysis of the data from this experiment was performed as described in Appendix III.

6.3 RESULTS

This section is accompanied by a statistical analysis, the procedure of which is shown in Appendix III. ε-Carotene has been omitted from the figures in this section since its occurrence at low concentrations ($\leq$ 0.5 $\mu g.g^{-1}$ F.Wt: $<$ 1% total carotenoid), particularly in reproductive tissue, makes graphical representation difficult. ε-carotene concentrations have been included in the statistical analysis (Appendix III), however the limitations of the assay procedure at such low pigment concentrations must be noted.

Figures 38 and 39 show changes in chlorophyll and carotenoid content ($\mu g.g^{-1}$ F.Wt) in C. fragile fronds exposed to high controlled light intensities, (500-2,000 μ $E.M^{-2}S^{-1}$) for controlled periods of time ($\frac{1}{2}$ - 7 hours). This data initially confirms the findings in Chapter 4, in that large differences occur in pigment content between reproductive and vegetative tissue. Furthermore, the 4-way ANOVA (Appendix III), performed on this data shows growth phase to contribute the greatest variability in the analysis. Using the data in Figures 38 and 39 changes in pigment content have been calculated as percentages of the controls used in each light regime (Figure 40). This data demonstrates a greater fluctuation in pigment content in the reproductive tissue compared to the vegetative tissue. Furthermore, the fluctuation in pigment content appears to increase as light intensity increases, although the affect is more pronounced in the reproductive fronds.

The above findings are supported by the 3-way ANOVA in Appendix III, however this analysis also shows a difference in the effects of exposure time between vegetative and reproductive tissue. In reproductive fronds both exposure time and light intensity affect variability in pigment concentration, but light intensity only, has a significant effect on pigment content in vegetative tissue. A 2-way ANOVA (Appendix III) on individual pigments in the different growth phases of C. fragile has shown significant light affects for both carotenoid and chlorophyll content in the reproductive tissue. However, only the chlorophylls and ε-carotene are significantly effected by light in the vegetative fronds (Table 15).

Table 15 SIGNIFICANT LIGHT EFFECTS ON PIGMENTS IN REPRODUCTIVE AND VEGETATIVE FRONDS OF C. FRAGILE

(See Appendix III for full statistical analysis)

PIGMENT	GROWTH PHASE	
	REPRODUCTIVE	VEGETATIVE
α-Carotene	Signficant	
ε-Carotene	"	Significant
Siphonoxanthin		
Siphonein	Significant	
Violaxanthin	"	
Neoxanthin	"	
Chlorophyll a	"	Significant
Chlorophyll b	"	"

Exposure time is not significant for both reproductive and vegetative fronds in the 2-way ANOVA, and this causes a disparity with the findings of the 3-way ANOVA. An explanation of these findings must take into account differences in the number of data points between the two analyses, i.e. the total degrees of freedom for the 2-way ANOVA is 32, but in the 3-way ANOVA is 266 (Appendix II). Thus, the significant

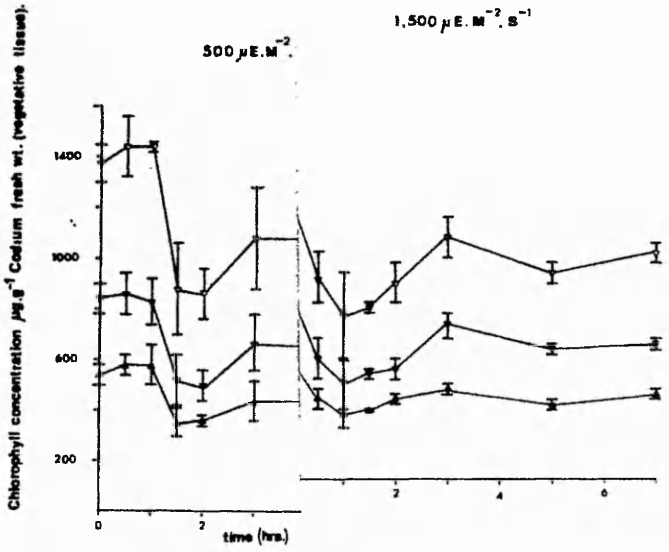
FIGURE 38:- THE EFFECT OF LIGHT INTENSITY AND EXPOSURE TIME ON CHLOROPHYLL CONTENT IN VEGETATIVE AND REPRODUCTIVE C.FRAGILE

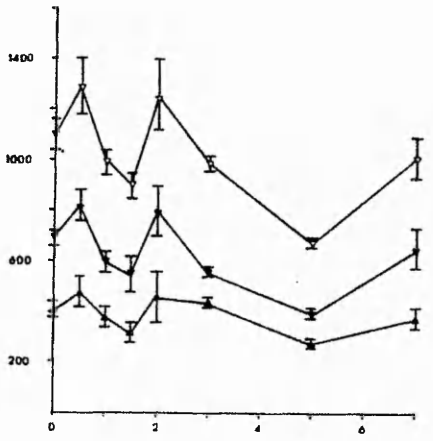
KEY:-

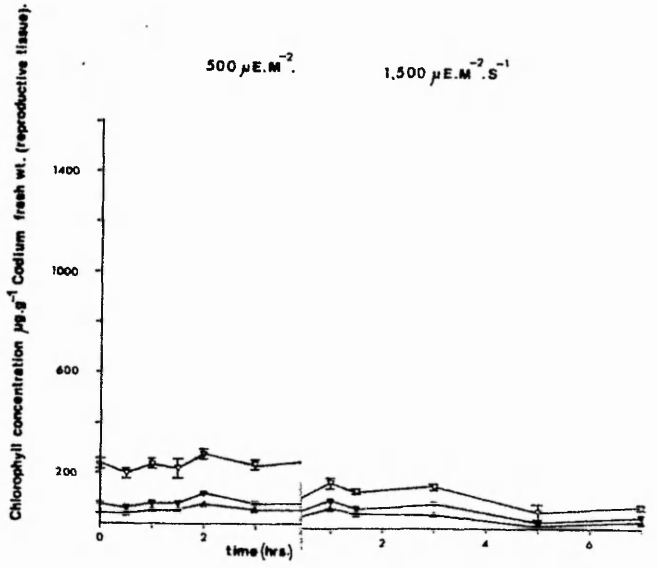
▽——— Total chlorophyll (T)

▼——— Chlorophyll a (A)

▲——— Chlorophyll b (B)


500 μE.M⁻².
1,500 μE.M⁻².s⁻¹
Chlorophyll concentration μg.g⁻¹ Codium fresh wt. (vegetative tissue).
1400
1000
600
200
0
2
4
6
time (hrs.)


2,000 μE.M⁻².s⁻¹
1400
1000
600
200
0
2
4
6


500 μE.M⁻².
1,500 μE.M⁻².s⁻¹
Chlorophyll concentration μg.g⁻¹ Codium fresh wt. (reproductive tissue).
1400
1000
600
200
0
2
4
6
time (hrs.)

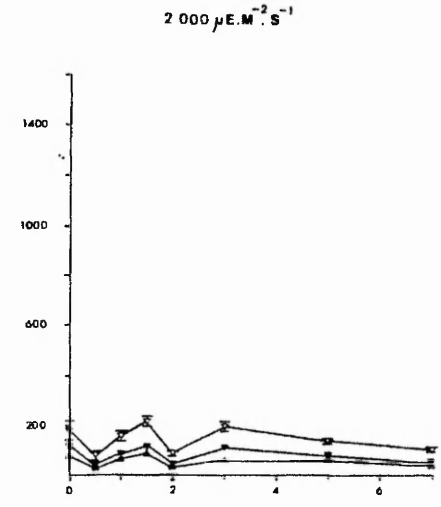
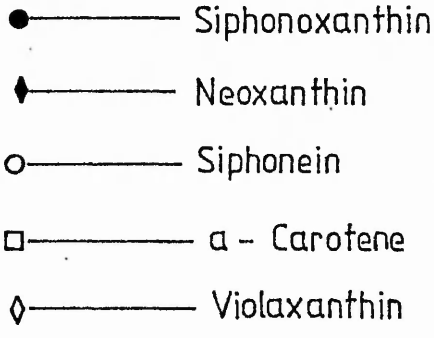
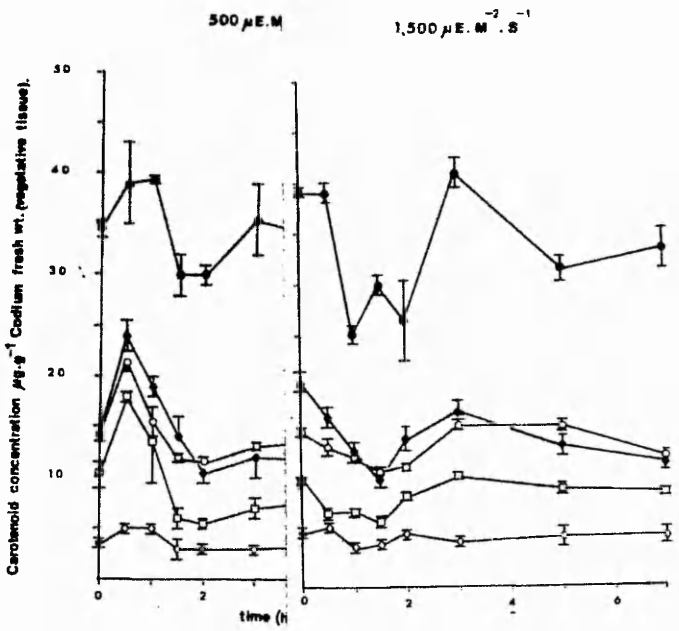

2 000 μE.M⁻².S⁻¹
1400
1000
600
200
0
2
4
6

FIGURE 39 :- THE EFFECT OF LIGHT INTENSITY AND EXPOSURE TIME ON CAROTENOID CONTENT IN VEGETATIVE AND REPRODUCTIVE C.FRAGILE

KEY :-




500 µE.M
1,500 µE.M⁻².S⁻¹
2,000 µE.M⁻².S⁻¹
Carotenoid concentration µg.g⁻¹ Codium fresh wt. (vegetative tissue).
time (h

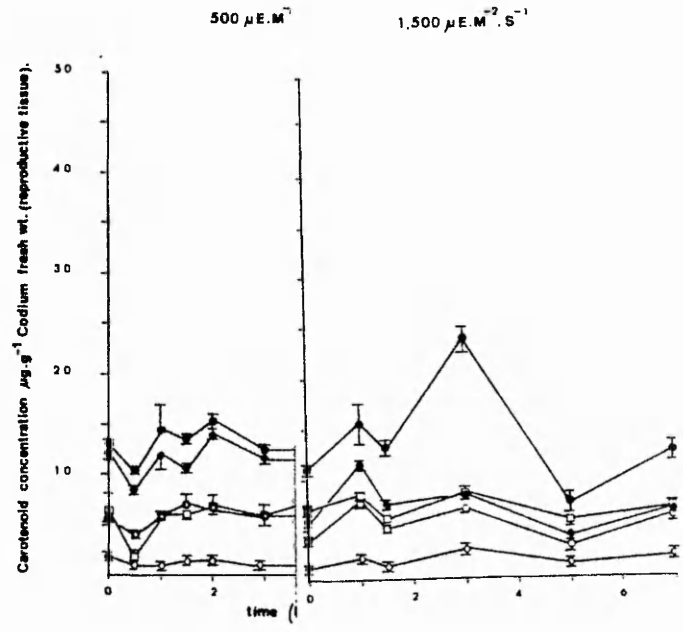
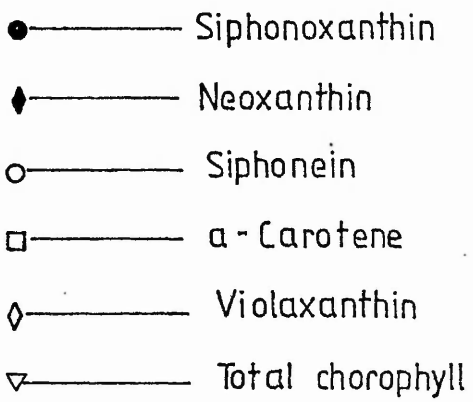
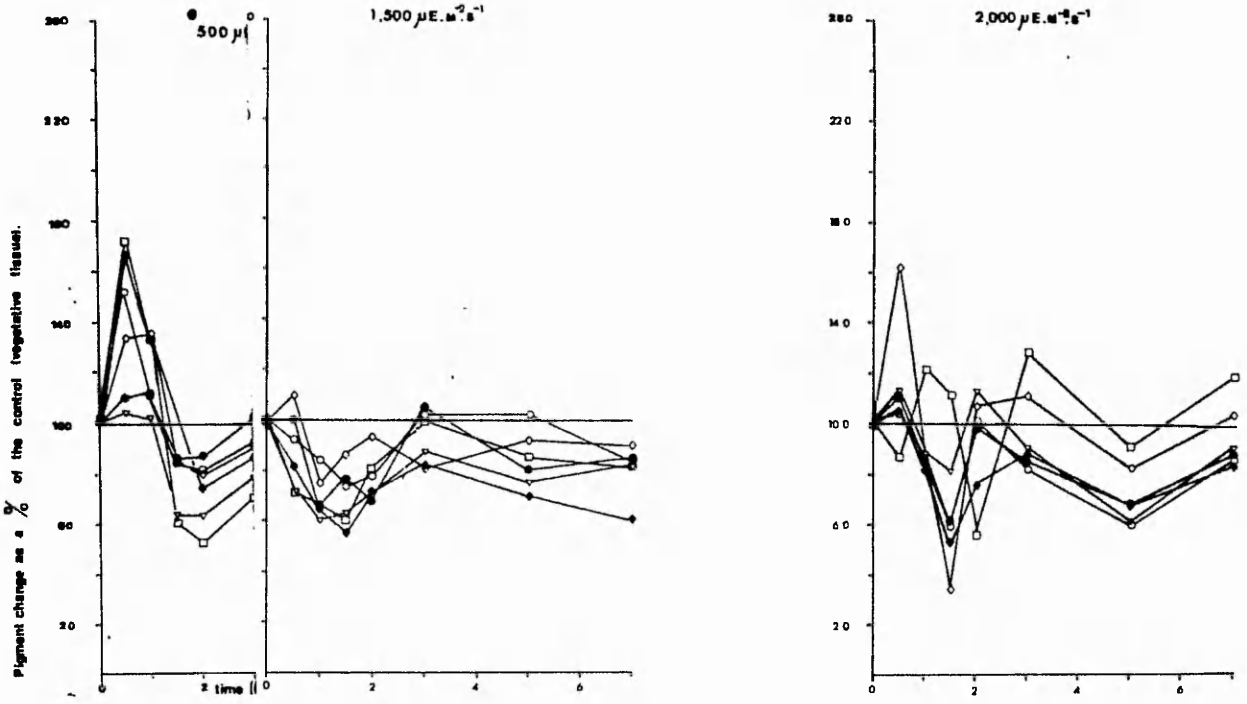

500 µE.M⁻²
1,500 µE.M⁻².S⁻¹
2,000 µE.M⁻².S⁻¹
Carotenoid concentration µg.g⁻¹ Codium fresh wt. (reproductive tissue).
time (

FIGURE 40 : - THE EFFECT OF LIGHT INTENSITY AND EXPOSURE TIME ON PIGMENT DISTRIBUTION (AS A % OF THE CONTROL) IN VEGETATIVE AND REPRODUCTIVE C. FRAGILE

KEY : -




500 μE
1,500 μE.m⁻².s⁻¹
2,000 μE.m⁻².s⁻¹
Pigment change as a % of the control (vegetative tissue).
Time (hr)

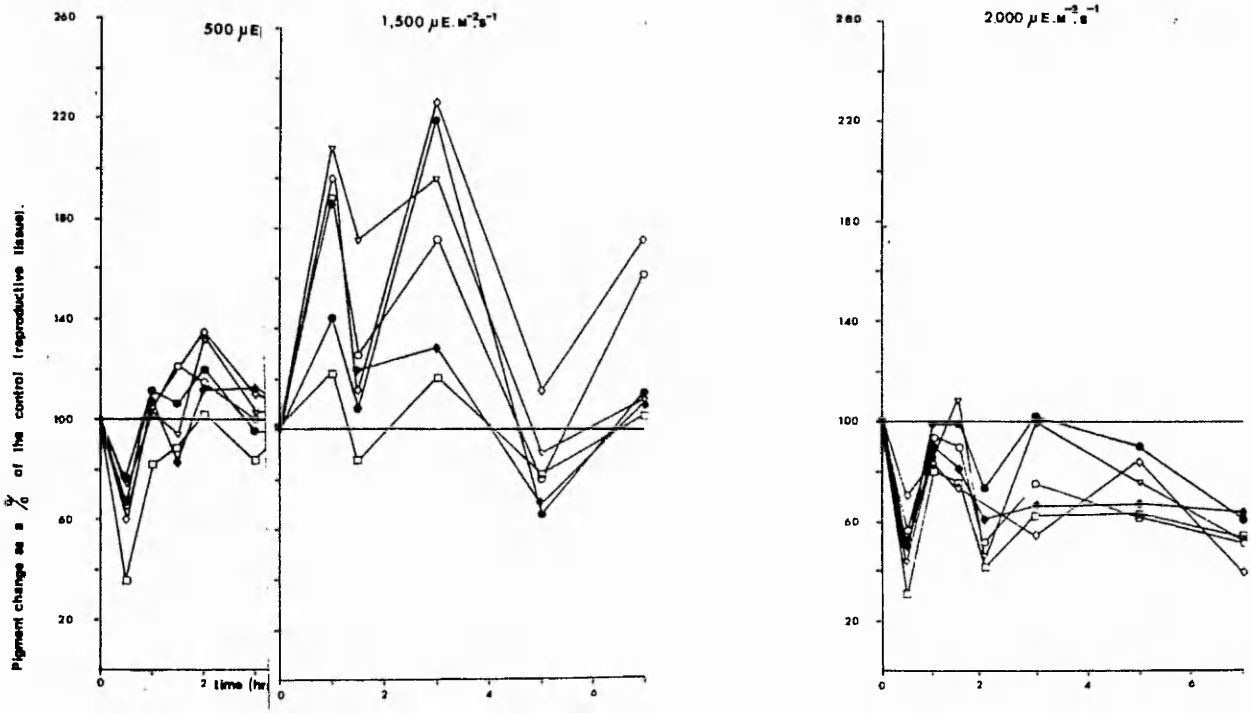
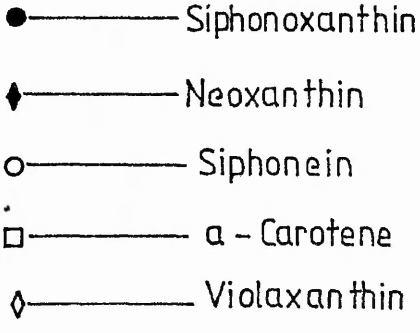
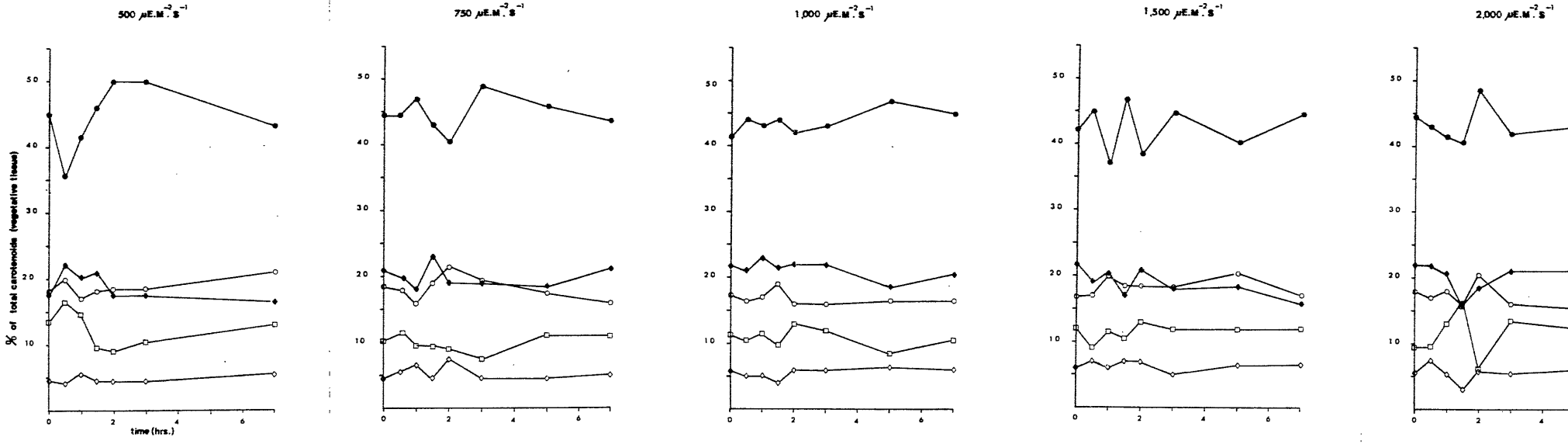

500 μE
1,500 μE.m⁻².s⁻¹
2,000 μE.m⁻².s⁻¹
Pigment change as a % of the control (reproductive tissue).
Time (hr)

FIGURE 41 : - THE EFFECT OF LIGHT INTENSITY AND EXPOSURE TIME ON % DISTRIBUTION OF CAROTENOIDS IN VEGETATIVE AND REPRODUCTIVE C. FRAGILE

KEY : -




500 μE.M⁻².S⁻¹
750 μE.M⁻².S⁻¹
1,000 μE.M⁻².S⁻¹
1,500 μE.M⁻².S⁻¹
2,000 μE.M⁻².S⁻¹
% of total carotenoids (vegetative tissue)
time (hrs.)

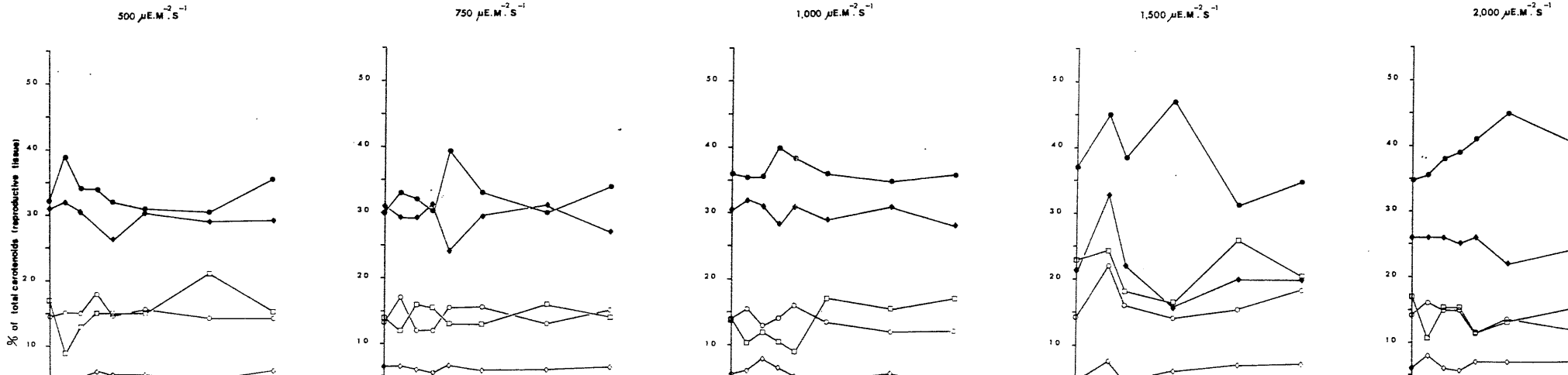
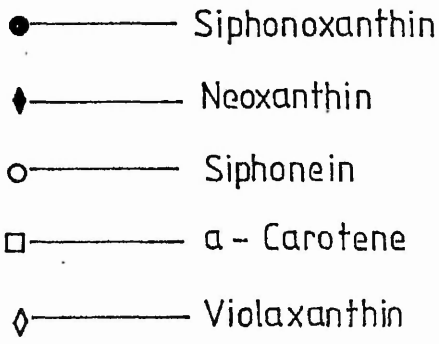

500 μE.M⁻².S⁻¹
750 μE.M⁻².S⁻¹
1,000 μE.M⁻².S⁻¹
1,500 μE.M⁻².S⁻¹
2,000 μE.M⁻².S⁻¹
% of total carotenoids (reproductive tissue)

FIGURE 42 : - THE EFFECTS OF LIGHT INTENSITY AND EXPOSURE TIME ON PIGMENT RATIOS (CHL:CAR) IN VEGETATIVE AND REPRODUCTIVE C.FRAGILE

KEY : -



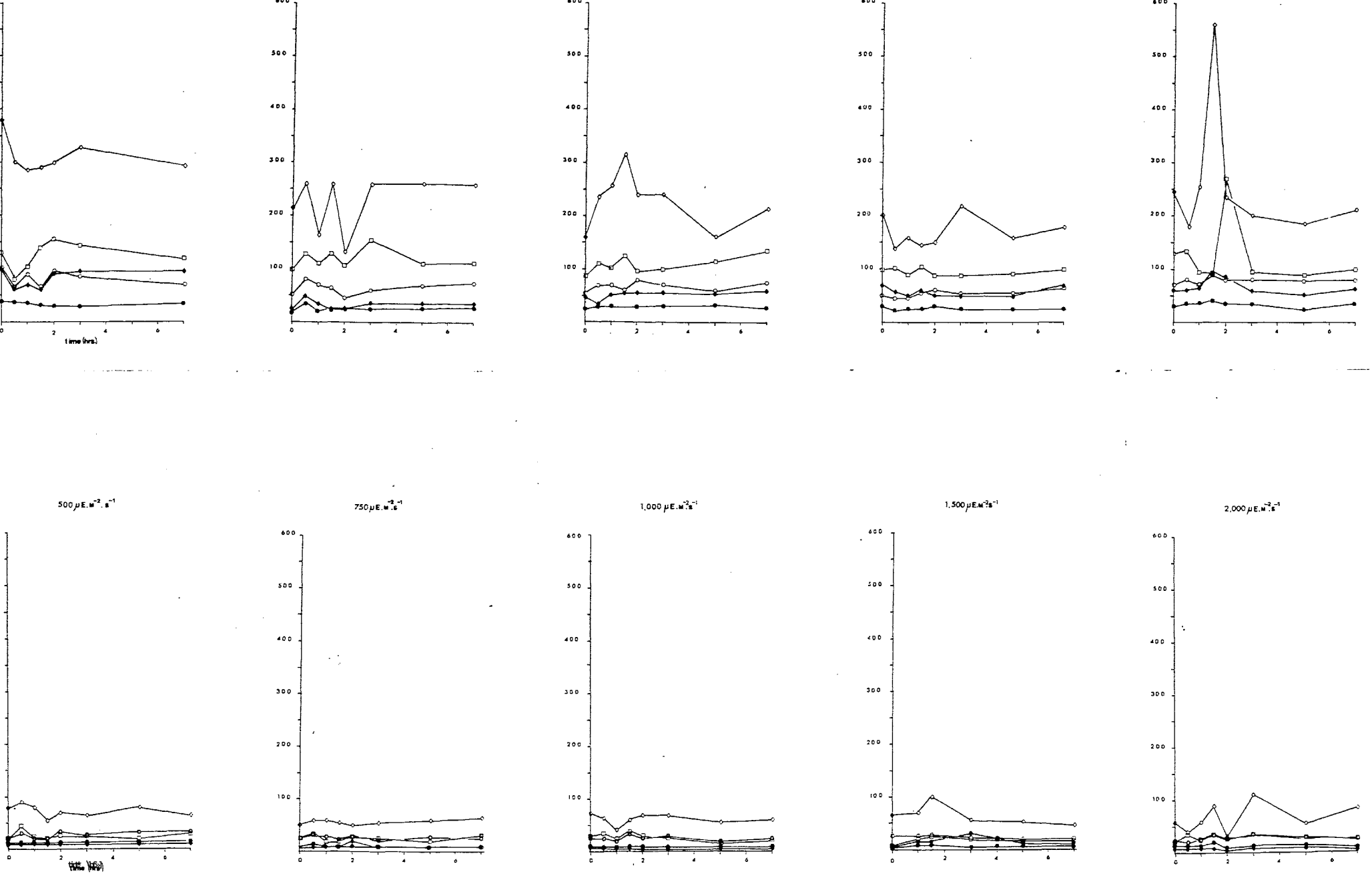
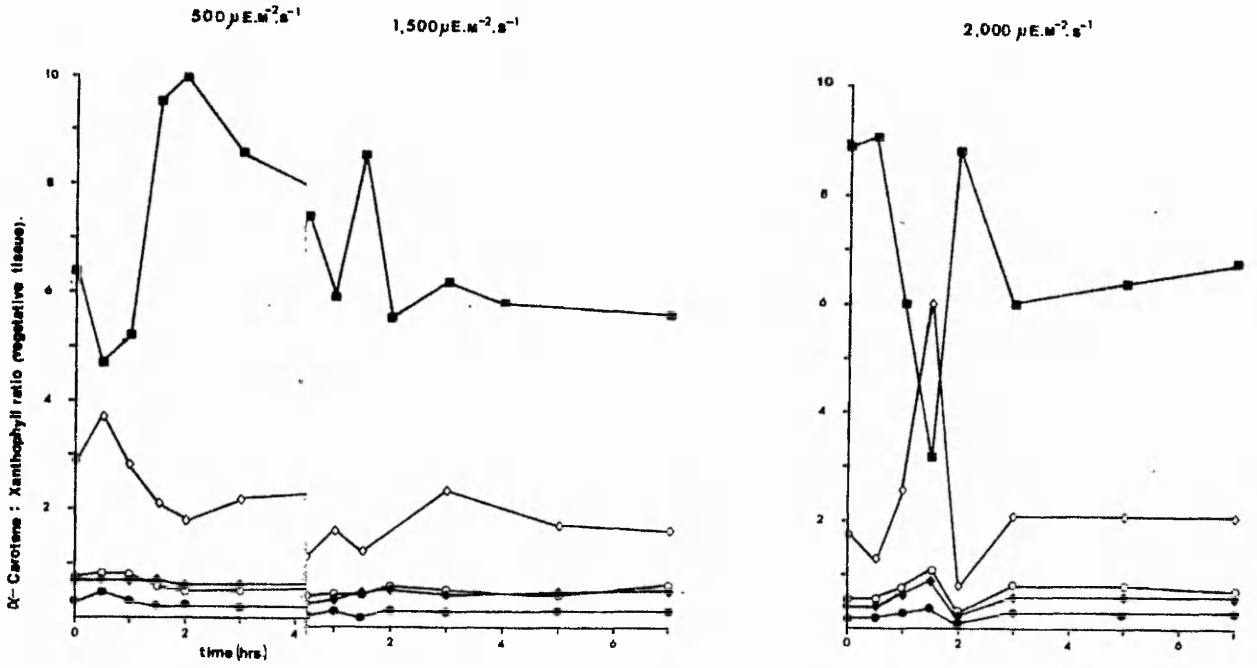
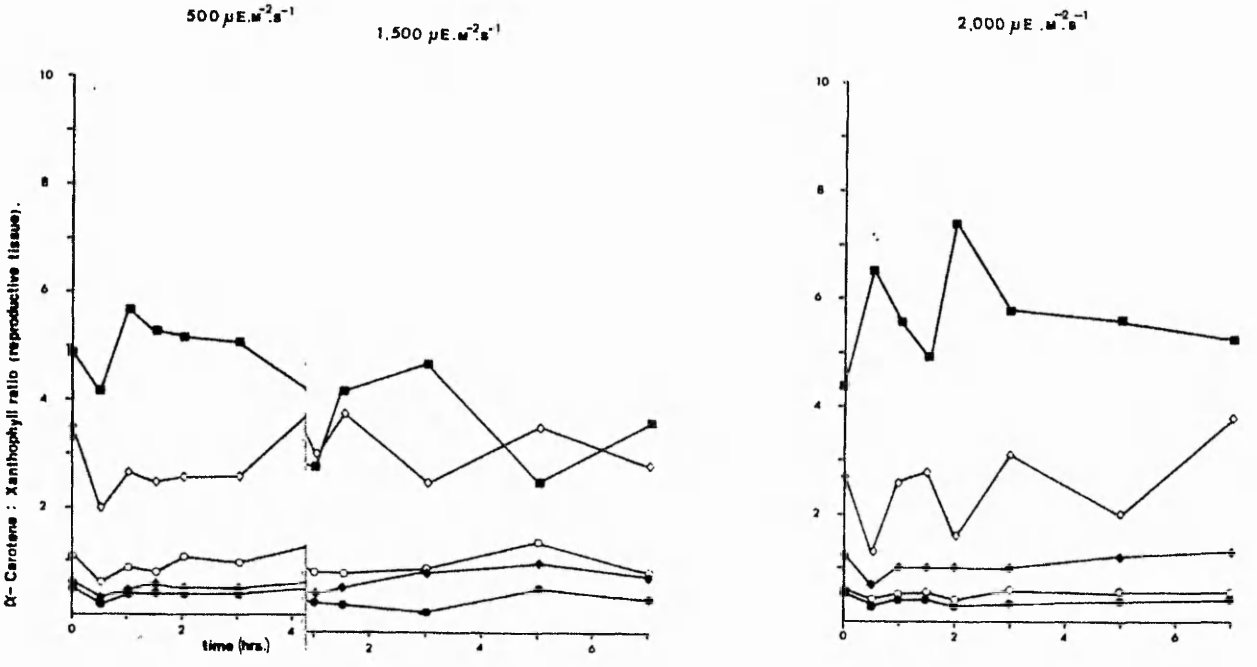

500 μE.m⁻².s⁻¹
750 μE.m⁻².s⁻¹
1,000 μE.m⁻².s⁻¹
1,500 μE.m⁻².s⁻¹
2,000 μE.m⁻².s⁻¹
time (hrs)

FIGURE 43 :- THE EFFECTS OF LIGHT INTENSITY AND EXPOSURE TIME ON α-CAROTENE:XANTHOPHYLL RATIOS IN VEGETATIVE AND REPRODUCTIVE C. FRAGILE

KEY :-

- α - Carotene : Siphonoxanthin
- " : Neoxanthin
- " : Siphonein
- " : Violaxanthin
- Total xanthophyll : α-Carotene


500 μE.m⁻².s⁻¹
1,500 μE.m⁻².s⁻¹
2,000 μE.m⁻².s⁻¹
α- Carotene : Xanthophyll ratio (vegetative tissue).
time (hrs.)


500 μE.m⁻².s⁻¹
1,500 μE.m⁻².s⁻¹
2,000 μE .m⁻².s⁻¹
α- Carotene : Xanthophyll ratio (reproductive tissue).
time (hrs.)

effect of exposure time on total pigment content in the 3-way ANOVA may be lost when individual pigments are examined in a 2-way analysis, which, as a consequence reduces the number of data points.

Figure 41 shows the distribution of carotenoids in vegetative and reproductive fronds expressed as a percentage of the total carotenoid content. Some differences are apparent between the growth phases for example, neoxanthin is more abundant in reproductive tissue compared to vegetative tissue. There is also a greater fluctuation in the distribution of carotenoids in the reproductive fronds, but the relative concentrations of the pigments maintain a high degree of constancy in all the light regimes. Similarly total chlorophyll: carotenoid ratios in Figure 42 show remarkably constant behaviour, with the exception of violaxanthin. Since α-carotene is the major carotene in C. fragile, α-carotene:xanthophyll ratios were calculated for possible biosynthetic relationships. However, despite considerable variations in total carotene:total xanthophyll ratios, α-carotene maintains very constant ratios with the individual xanthophylls, with the exception of violaxanthin (Figure 43).

6.4 DISCUSSION

Figures 38 and 39, together with the statistical evidence in the 4-way ANOVA (Appendix III) show the considerable quantitative differences in pigment concentrations in the growth phases of C. fragile. Figure 40, indicates the different behaviour of pigments in reproductive and vegetative fronds when exposed to high light intensities and expressed as % of control values. The most prominent feature of these results is that the effects of light intensity and exposure time do not produce a uniform trend (e.g. as a decrease in pigment content), rather, responses are manifest as an overall trend towards increasingly erratic behaviour in pigment content. Examination of the 3-way ANOVA (Appendix III) and Figure 40, show greater variations in the pigment content of reproductive tissue compared to the vegetative tissue. Further evidence for increased erratic behaviour in the reproductive tissue is demonstrated by the main parameters of the 3- and 2-way ANOVAS i.e. light intensity and exposure time. The 3-way ANOVA shows

both parameters to be significant in reproductive tissue but light intensity only, is significant in vegetative fronds. The effect of time in the 2-way analysis has been lost, but again reproductive tissue proves to be less able to accommodate high light intensities since carotenoid and chlorophyll pigments both show significant variation with light. Whereas, the chlorophylls and α-carotene only are affected in the vegetative fronds. This may indicate differences in the stability of carotenoids and chlorophylls, i.e. chlorophylls are more sensitive to higher light intensities.

Changes in the life cycle of C. fragile may be responsible for the different behaviour of frond pigments. Older, reproductive fronds have already been subjected to higher light intensities in their natural environment before the commencement of this experiment. Furthermore, reproductive tissue has a reduced pigment content and photosynthetic rate (Cobb and Rott, 1978). Thus, as a consequence of the environmental conditions and/or natural life cycle, reproductive C. fragile no longer achieves or indeed requires stringent mechanisms of control or protection for its photopigments. Vegetative tissue however, is photosynthetically active and in order to maintain this activity must possess fully operational mechanisms for the control, synthesis and protection of its photosynthetic pigments.

Using light/shade studies with radish chloroplasts, Grumbach and Lichtenthaler (1982) have shown differences in chlorophyll and carotenoid metabolism. Radish seedlings grown at high light intensities exhibit a much greater turnover rate of chlorophyll and carotenoid synthesis compared to seedlings grown in shade conditions. This suggests that in high intensity light fields pigments are continuously degraded by photooxidation and rapid resynthesis is essential to maintain photosynthetic efficiency. The constancy of pigment ratios and relative distributions together with highly variable absolute pigment concentrations in both reproductive and vegetative light-treated C. fragile suggests a tightly controlled mechanism of pigment synthesis in this alga. It is thus possible that highly efficient processes in the pigment metabolism of C. fragile may reflect an adaptation to the high light intensities to which it is daily exposed in the intertidal zone.

The absence of β-carotene in this alga may indeed reflect such a process as this isomer may be so rapidly metabolized as to go undetected by TLC. The "presence" of this carotene is only indicated by the occurence of the β-Ionone ring containing xanthophylls found in this alga (Appendix I). Pigment ratios are also relatively stable in reproductive fronds even though the absolute pigment content of these light-treated fronds is more variable. This suggests that photoprotective mechanisms may still be operational during the reproductive part of the life cycle of C. fragile. Indeed, this would be an environmental advantage as photooxidation within the chloroplast may also result in more widespread tissue damage and injury to the gametangia.

Photosynthetic pigments are not randomly arranged within the chloroplast membranes but are organized within the constraints of the PSU (Chapters 1 and 3). Thus, an examination of the relative concentrations of pigments in this experiment is particularly important and is achieved by the calculation of pigment ratios (Figures 42 and 43). An indication that chlorophyll distribution is stable is given by the constant chlorophyll a:b ratios (1.5-1.8:1), noted for both growth phases and each light regime. However, the most striking feature of these results is the constancy of total chlorophyll:siphonoxanthin, siphonein and neoxanthin. This constancy occurs in both reproductive and vegetative C. fragile fronds even at the highest light intensity. These carotenoids are localized in the light-harvesting apparatus of C. fragile and constant total chlorophyll:siphonoxanthin and siphonein ratios in light exposed fronds may reflect their corresponding stoichiometric stability on electrophoresis (Chapter 3). Conversely the erratic behaviour of chlorophyll:violaxanthin ratios may be indicative of the spatial separation of this xanthophyll which is predominently found in the chloroplast envelope (Siefferman-Harms, 1980). SDS-PAGE studies indicate that violaxanthin is not a component of the thylakoid pigment/protein complexes isolated in this alga, and mainly accumulates as free-pigment on the gels. A further conclusion involving the constancy of pigment ratios in C. fragile is that decreases in pigment concentration are not individually sequential and also appear 'controlled' as ratios still remain constant. Presumably pigment

degradation is due to photooxidative damage, and thus the above observations indicate that entire PSUs are being lost, rather than individual pigments.

Since α-carotene is the major carotene in C. fragile its importance in the turnover of xanthophyll pigments was investigated by calculating α-carotene:xanthophyll ratios. These ratios show a strong correlation with the chlorophyll:carotenoid content (Figure 43). Thus, although total carotene:xanthophyll and α-carotene:violaxanthin ratios vary, α-carotene:siphonoxanthin, siphonein and neoxanthin remain remarkably constant in both vegetative and reproductive fronds. The constancy of these ratios are not reflected in pigment/protein studies, and α-carotene is not a major component of the LHCP complex in C. fragile. However, a detailed examination of α-carotene: xanthophyll ratios in this study shows a close relationship in the distribution of α-carotene and particularly siphonoxanthin. Decreases in α-carotene are concomittant with increases in siphonoxanthin and vice versa and this relationship is particularly prominant in reproductive tissue. The absence of β-carotene in C. fragile (Chapter 2) may suggest α-carotene plays a particularly important role in the regulation and biosynthesis of xanthophylls in this alga.

6.5 CONCLUSIONS

Despite exposure to high light intensities for up to 7 hours, the apparent disruption of pigment stability in C. fragile masks an inherent control of pigment biosynthesis demonstrated as stability of pigment ratios and carotenoid distributions. Furthermore, although reproductive tissue shows a greater display of erratic behaviour it too demonstrates a high degree of pigment ratio control. This behaviour may constitute an adaptive mechanism to high light intensities in this alga.

7. PHOTOOXIDATION AND GLYCOLATE PRODUCTION IN C. FRAGILE FRONDS AND ISOLATED CHLOROPLASTS

7.1 INTRODUCTION

C. fragile was able to control the relative proportions of its photosynthetic pigments when illuminated at high light intensities for short term exposure periods (Chapter 6). Frond bleaching was not experimentally observed in these fronds despite the fact that fronds sampled during mid-summer months showed symptoms of photooxidation. The aim of this chapter was to determine if frond bleaching in C. fragile is due to photooxidation by exposing fronds and chloroplasts to controlled light regimes and measuring the photooxidative breakdown products ethane, ethylene and malondialdehyde (MDA), and by monitoring changes in pigment content.

A number of algae are also noted for their excretion of glycolate into the aquatic medium when exposed to high light intensities and this phenomenon was also investigated in this chapter.

7.2 MATERIALS AND METHODS

7.2.1 Preparation of plant material

7.2.1.1 Preparation of fronds for ethane, ethylene, glycolate and pigment analyses:- Tissue sampled in March 1982 was removed from the storage tanks and washed several times in freshly prepared Gerrard sea salt solution (ZGV-110-T). 5g samples of frond tips were placed into individual glass bottles, covered with 10ml of the above salt water containing 2.5mM.L^{-1} $NaHCO_3$ and sealed with air tight rubber serum bungs. Before experimental use the exact volume of head space covering the frond/seawater solution was determined using known volumes of water. Dark-controlled fronds were covered with aluminium foil to exclude any incident light.

7.2.1.2 Preparation of chloroplasts for ethane, ethylene and pigment analyses:- Chloroplasts were extracted from

fronds sampled in March 1982 using the method described in section 2.2.2 with 10mM.ml^{-1} $NaHCO_3$ added to the preparation. Chloroplast experiments were performed in oxygen electrode wells and each vessel contained a final concentration of 6.5 x 10^9 plastids. After standardization of the air space a 1ml chloroplast suspension was placed in separate electrode wells which were then sealed with gas-tight serum bungs. The electrode unit was connected to a microstirrer to ensure complete mixing of the chloroplast suspension throughout the experiment. Dark controls were prepared in electrode wells and covered with aluminium foil as described above.

7.2.1.3 Preparation of chloroplasts for malondialdehyde analysis:- Due to the interference of various organic osmotica with the reagents used in the analysis of MDA, C. fragile chloroplasts were prepared in a modified extraction medium using NaCl as the osmoticum and the extraction method of Schonfeld, Rahatt and Neumann (1973). These authors used this method for the isolation of siphonaceous chloroplasts:-

Extraction media

500 mM NaCl
50 mM H.E.P.E.S. buffer (as free acid)
pH was adjusted to 7.8 using 1 N NaOH.

Using the above extraction medium chloroplasts were prepared from C. fragile fronds sampled in March 1981 using the procedure described in section 2.2.2. Chloroplast experiments were performed in Oxygen electrode wells as described in section 7.2.1.2. Each vessel contained a final concentration of 4.4 - 7.0 x 10^8 chloroplasts. Ml^{-1} and 10 mM Ml^{-1} $NaHCO_3$ was added to the preparation.

7.2.2 The determination of ethane and ethylene by gas-liquid chromatography (after Elstner and Youngman, 1978)

Hydrocarbons were detected using a Perkin Elmer F33 GLC unit fitted with an Alltech 2m (8-100 mesh) activated alumina column. The unit was programmed with an injection temperature of 100°C and an oven temperature of 80°C. Flow rates of input gases were:-

N_2 = 40 ml. min^{-1}

H_2 = 14 ml. min^{-1}

air = 24 ml. min^{-1}

N_2 was the carrier gas and the gas peaks were detected by ionization in an H_2 flame. An attenuation range of 2-16 was used to accommodate the gas concentrations analysed using a Perkin Elmer chart recorder.

Preparation of standard curves:- Purified calibration gases of ethane and ethylene were used throughout. Conical flasks were filled with either gas dispensed from pressurized gas canisters. The process was performed under water and the flasks sealed with airtight serum-bungs. Standard curves were prepared by injecting appropriate volumes of standard gas into the GLC column using gas-tight syringes. Each standard curve was prepared from at least 8 calibration points in the range 5-80 µg ethane and ethylene three replicates for each gas concentration were used. Calibration curves were performed for each daily determination of test gases to compensate for discrepancies in the performance of the GLC unit.

Typical regression plots for ethane and ethylene standard curves were:-

Ethane:- $y = 2.59x + (-0.65)$

where y = Peak height 2.59 = gradient

x = µg ethane -0.65 = intercept

correlation coefficient = 0.99

Ethylene:- $y = 1.9x + (-0.52)$

where y = Peak height 1.9 = gradient
x = µg ethylene -0.52 = intercept

correlation coefficient = 0.99

Analysis of test gases:- 0.25-1.0 ml samples of head space gas was sampled from either frond or chloroplast containing vessels and injected into the GLC column. At least three separate injections were performed for each head space. Volatile hydrocarbons from the plant material were identified by their retention times in the column and compared with the retention times of the standard gases. Hydrocarbon gas production by plant material was calculated as follows:-

$$\mu g\ C_2H_4 \text{ or } C_2H_6 . g\ \text{f.weight}^{-1} \text{ or } 10^8 \text{ chloroplasts}^{-1} = \frac{\text{GLC response (Peak Height) x Calibration factor x Volume factor}}{\text{fresh weight or chloroplast number}}$$

7.2.3 The Extraction of glycolate from seawater (after Shah and Fogg 1973)

Seawater was first treated with Brockmann Grade 1 neutral alumina (100-250 mesh) which absorbed glycolate and various other organic compounds. The alumina was then filtered and stripped of glycolate using 2N H_2SO_4. The following recovery test was performed using standard glycolic acid solutions and the Calkin's method of glycolate determination:-

200-1000 $\mu g.ml^{-1}$ standards of glycolic acid were prepared and treated with alumina at a rate of 200 $g.L^{-1}$ for 6 hours under constant stirring conditions. The above recovery procedure using $2NH_2SO_4$ was performed for a further 6 hours (with constant stirring of the mixture) and the percentage recovery of glycolate determined.

Frond studies:- Fronds were washed several times with freshly prepared Gerrard's seawater, which was then treated with Brockmann Grade 1 alumina at a rate of 0.1g,ml^{-1}. The seawater/alumina mixture was stirred overnight to ensure complete absorption of

glycolate by the alumina, which was then filtered and the seawater discarded. The alumina was stripped of glycolate using a 2N H_2SO_4 treatment and the mixture stirred for a minimum of 6 hours.

A precaution was taken to ensure that glycolate production in seawater containing C. fragile fronds was produced by the alga and not microbial activity. 200g of C. fragile from the storage tanks were washed in 100ml of Gerrard's seawater thus removing any microbial flora which may have been present on the fronds. Samples of this extract were placed in flasks, stoppered, and the seawater analysed for glycolate at 4½, 24, 48 and 72 hourly intervals.

Seawater studies:- Bembridge seawater was collected from the intertidal zone during June 1982 when algal growth was particularly prolific. Glycolate was stripped from 4 x 500ml samples of seawater using alumina at a rate of $100g.l^{-1}$. The alumina was filtered and treated with 50ml of 2N H_2SO_4 as described previously.

7.2.4 The determination of glycolic acid (after Calkins, 1943)

The detection of glycolate is dependent on the condensation of formaldehyde derived from glycolic acid with 2,7 dihydroxy napthalene. The reaction only proceeds in concentrated H_2SO_4 and at a temperature of 100°C. The solution to be analysed must be 2N with H_2SO_4 and should not contain > 100 µg of glycolate ml^{-1}. In these experiments concentrated solutions of glycolate were diluted accordingly (using $2NH_2SO_4$) to maintain the sensitivity of the assay. The presence of >5% ($^v/_v$) of water in the reaction mixture interferes with colour development as do certain glycols and aldehydes (Takahashi, 1972). For this reason glycolate determinations in chloroplast preparations could not be attempted as the organic osmotica and indeed the chloroplasts themselves interfered with 2,7 dihydroxy napthalene producing a large number of coloured complexes.

Assay procedure:- 0.2ml of the 2N H_2SO_4 sample solution was placed into a boiling tube and 2.0ml of a 0.01% ($^w/_v$) solution of 2,7 dihydroxy napthalene in concentrated H_2SO_4 added. The tube

was kept on ice during the addition of the reagent. The tube was then placed in a boiling water bath for 20 minutes and a red/violet complex developed. The tube was removed, cooled on ice and diluted with 4ml of 2N H_2SO_4 and mixed thoroughly. After cooling the absorbance of the complex was measured at 530nm on a Perkin Elmer 550s spectrophotometer using a Gerrards seawater/reagent blank.

A standard curve was prepared using a stock solution of pure glycolate in 2N H_2SO_4. The assay colibration range was 2.5-200µg and a typical glycolate calibration curve regression was:-

$$y = 0.015x + 0.15$$

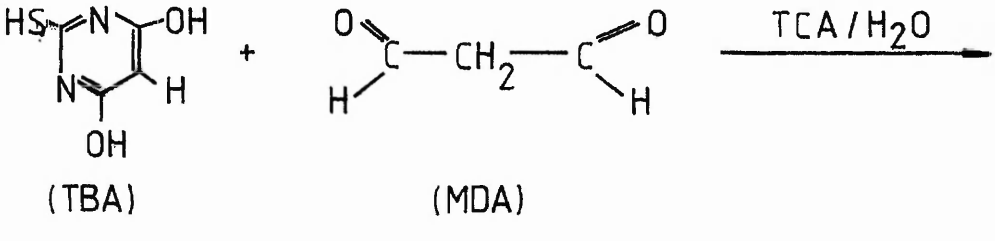
where y = absorbance 0.015 = gradient
x = glycolate standard 0.15 = intercept

correlation coefficient = 0.99

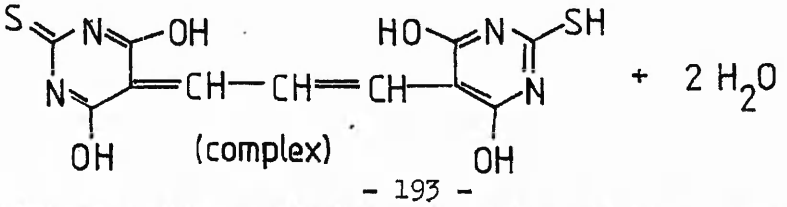
Glycolate analyses were usually performed in triplicate and absorbance scans were performed to check for interference in the assay.

7.2.5 <u>The determination of malondialdehyde in chloroplasts (after Heath and Packer. 1968)</u>

The basis of this assay is the reaction of thiobarbituric acid (TBA) with fatty acid oxidation products to form a red complex absorbing at 532nm. Although various peroxidation products can react with TBA, malondialdehyde (MDA) is one of the major TBA-reactive compounds. The complex produced is formed by the condensation of two molecules of TBA with one of MDA:-



(TBA) (MDA)



(complex)

Assay procedure:- 1.5ml of 0.5% TBA (in 20% ($^{w}/_{v}$) trichloroacetic acid) was added to an equal volume of reaction sample containing the chloroplast suspension. This mixture was incubated in a boiling water bath for 25 minutes, after which the mixture was cooled on ice and the chloroplast debris removed on centrifugation at 2,500g for 10 minutes using an MSE bench centrifuge. The absorbance of the supernatant was determined at 532 and 600nm using a Perkin Elmer 550S spectrophotometer and reagent blanks. The concentration of MDA in the chloroplast extract was calculated using an extinction coefficient of 155mM ml^{-1} at 532nm. This value was corrected for non-specific absorbance at 600nm by subtracting the absorbance readings at 600 from those of 532nm. MDA production was calculated as nm MDA. μg $chlorophyll^{-1}$.

7.2.6 Pigment analysis of fronds and chloroplasts

Total pigment analyses were performed on fronds using methods described in Chapter 2. To ensure a rapid analysis of pigment content and reduce the affects of storage degradation in the pigment content of the fronds TLC replicates for carotenoid determinations were not performed. However, analytical errors for carotenoid content determination have proved constant throughout this thesis and an estimate of these errors is given in Chapter 2. The chlorophyll content of chloroplast extracts assayed using the methods shown in Chapter 2. Pigment analyses were performed on fronds sampled from the storage tanks at the onset of each experiment and these represent the frond pigment controls.

7.2.7 The effects of light on pigment content and ethane, ethylene and glycolate production in C. fragile fronds and chloroplasts

Preliminary investigations:-

Ethane and ethylene production by C. fragile fronds and chloroplasts were investigated using plant material prepared in triplicate and described in section 7.2.1.1 and 7.2.1.2, three dark controls were also incorporated. Plant preparations were exposed to light intensities of 600 $\mu E.M^{-2}.sec^{-1}$ P.A.R. (equivalent to 1152 $W.M^{-2}$) and 1000 $\mu E.M^{-2}.sec^{-1}$ P.A.R.

(equivalent to 1920 $W.M^{-2}$) for 4-6 hours. A tungsten/halogen Halight projection lamp was used as the light source and light intensities were measured using a Crump system 550 fitted with quantum ($\mu E.M^{-2}S^{-1}$) and photometric ($W.M^{-2}$) sensors. Ethane and ethylene production was measured as described in section 7.2.2.

To determine glycolate production 54g of C. fragile fronds were covered with freshly prepared Gerrard's seawater and exposed to 1000 $\mu E.M^{-2}.Sec^{-1}$ P.A.R. (equivalent to 2,200 $W.M^{-2}$) for 8 hours. An Osram Solar colour lamp was used as the light source and light intensity was measured as described above. Glycolate production was determined as described in section 7.2.3.

Long term investigations

Four flasks containing 5g of C. fragile frond tips were labelled for the following exposure times:- $4\frac{1}{2}$, 24, 48 and 72 hours. Four flasks were also prepared as dark controls. Chloroplasts were maintained in light and dark controlled electrode wells from which air samples were taken at $4\frac{1}{2}$, 24, 48 and 72 hours. Frond and chloroplast preparations were in duplicate and were exposed to a light intensity of 250 $\mu E.M^{-2}.Sec^{-1}$. P.A.R. (equivalent to 280 $W.M^{-2}$) in a light cabinet. Samples were continuously illuminated with 4 x 40W Thorne white light tubes and maintained at a constant temperature of 25°C. Dark-controlled plant material was also incubated at this temperature in the cabinet, light intensity measurements were recorded using a Crump 550 meter. Frond preparations were assayed for hydrocarbon gas production at the specified time intervals. Similarly frond tips were filtered and washed with Gerrard's seawater and immediately frozen at -18°C in preparation for pigment determinations. The seawater filtrate was retained for glycolate analysis. The same chloroplast preparation had to be used for the entire time course with sequential removal of gas head space at $4\frac{1}{2}$, 24, 48 and 72 hourly intervals. The volume of head space removed for GLC analysis was always replaced by an equivalent amount of

air. Thus, hydrocarbon determinations were based on corrected cumulative volumes over the 72 hour exposure period. Chloroplast number and chlorophyll concentration were determined before and after exposure at 72 hours.

7.2.8 The effects of light on malodialdehyde production in C. fragile chloroplasts

Chloroplast preparations were prepared as described in section 7.2.1.3 and 5ml preparations were placed in light and dark controlled oxygen electrode wells. Using a Halight halogen/tungsten projection lamp the chloroplast suspension was exposed to 500, 750, 1000, 1500 and 2000 $\mu E.M^{-2}.sec^{-1}$ P.A.R. (equivalent to 960-3840 $W.M^{-2}$ total radiation) for three hours. Aliquots of the light and dark controlled chloroplast suspensions were removed at 15 minute intervals and MDA production was measured as described in section 7.2.5.

7.3 RESULTS

7.3.1 The effects of light on pigment content and ethane, ethylene and glycolate production in C. fragile fronds and chloroplasts

Preliminary investigations:- Chloroplasts and fronds exposed for 4-6 hours to 600 and 1000 $\mu E.M^{-2}.sec^{-1}$ P.A.R. using a Halight halogen/tungsten projection lamp did not produce measurable amounts of ethane or ethylene and these hydrocarbons were not detected by GLC even after 6 hours exposure time at 1000 $\mu E.M^{-2}.sec.^{-1}$ P.A.R. Pigment bleaching was not evident in chloroplasts or fronds. C. fragile fronds exposed to a light regime of 1000 $\mu E.M^{-2}.sec^{-1}$ P.A.R. for 8 hours using an Osram Solar colour lamp produced 0.23 μg glycolate. g^{-1} Codium f.Wt. hr^{-1}. A spectrophotometric scan of the 2,7 dihydroxynapthalene complex produce together with the complex formed by the glycolate standard is shown in Figure 44.

Long-term investigations:- Figure 45 shows a representative GLC trace for a head gas sample from vessels containing C. fragile fronds and chloroplasts. Ethane and ethylene were conclusively identified after $4\frac{1}{2}$ hours exposure to 250 $\mu E.M^{-2}.sec^{-1}$ P.A.R.

FIGURE 44 :- ABSORPTION SPECTRA OF 2,7 DIHDROXY-NAPTHALENE COMPLEXES DERIVED FROM STANDARD GLYCOLATE AND SEAWATER CONTAINING C.FRAGILE FRONDS EXPOSED TO NON-FLUORESCENT LIGHT (1,000 μE.M.$^{-2}$S^{-1})

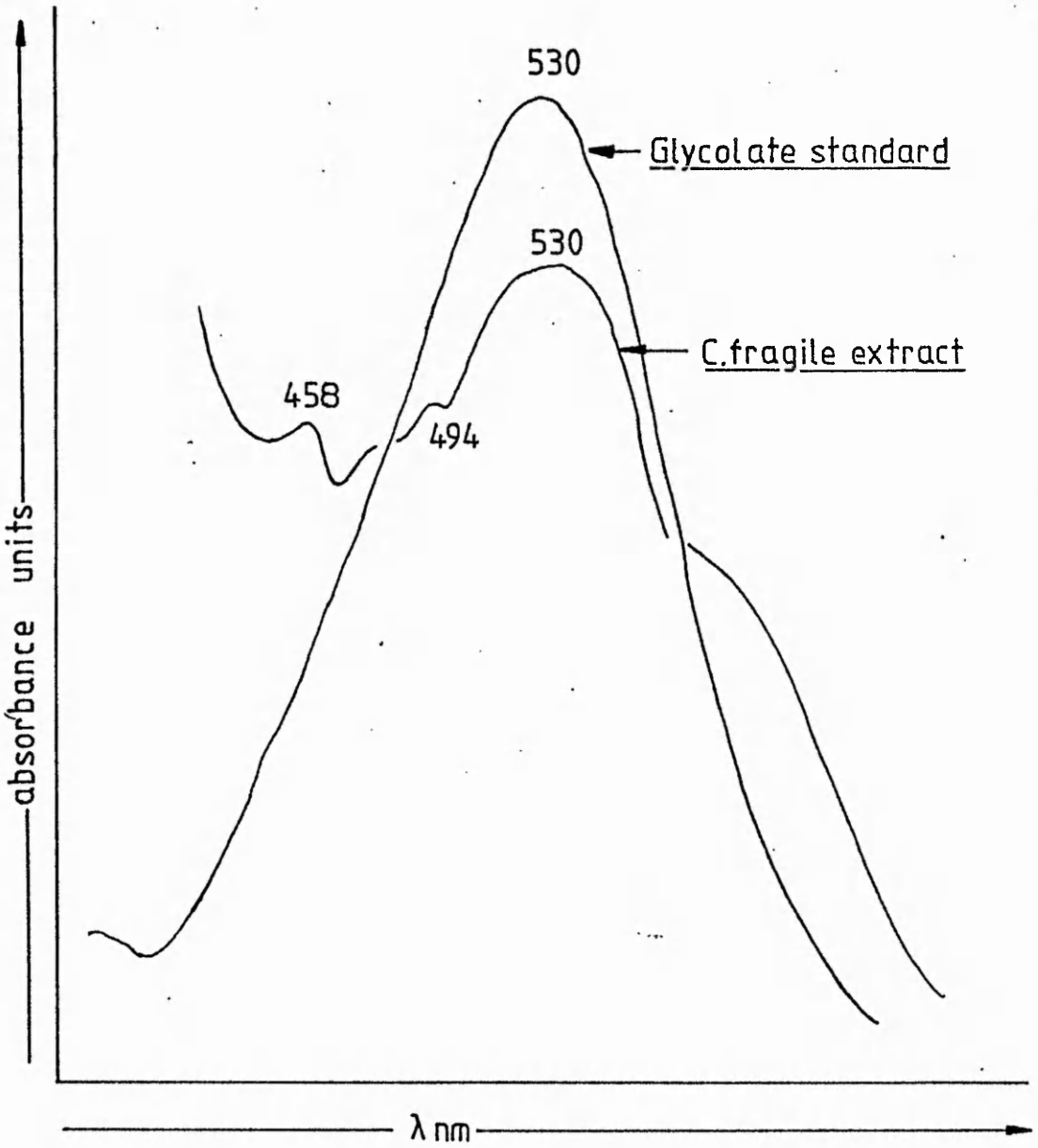
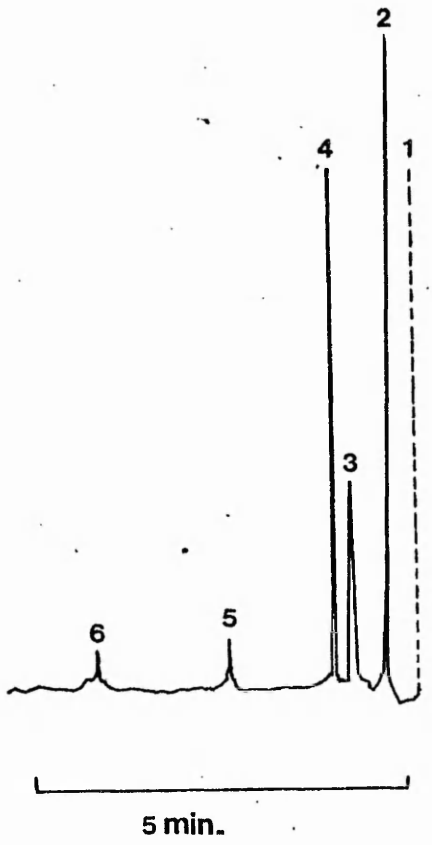

530
Glycolate standard
530
C.fragile extract
458
494
absorbance units
λ nm

FIGURE 45:- A TYPICAL GLC SCAN OF HEAD SPACE GAS SAMPLED FROM C.FRAGILE FROND AND CHLOROPLAST PREPARATIONS



1 = Injection peak

2 = Air peak (also methane)

3 = Ethane

4 = Ethylene

5 = Propane

6 = Butane

using 40W Thorne fluorescent tubes. After prolonged exposure to this light source two minor peaks were observed in the GLC trace which were tentatively identified as propane and butane. An air peak was observed soon after the injection peak and this was probably largely composed of methane (K. E. Pallett, personal communication), this peak increased in height with exposure time. Unfortunately methane had a very short retention time on the column and this made its routine analysis with ethane and ethylene very difficult. Retention times for hydrocarbon gases resolved from air samples corresponded with standard gases and are given as follows:-

Air peak	=	0.5 minutes
Ethane	=	0.9 minutes
Ethylene	=	1.2 minutes
Propane	=	2.6 minutes
Butane	=	4.3 minutes

This experiment was performed in duplicate and the following sections represent one set of results from frond or chloroplast samples. Each replicate showed the same trends, although absolute differences in the concentrations of the measured parameters occurred. Where incorporated, error values represent s.d.'s of 3-5 analytical replicates. Figure 46 shows the production of ethane and ethylene by fronds and chloroplasts exposed to light and dark regimes for controlled time intervals. Only ethane and ethylene were detected in chloroplast extracts where propane and butane were detected in light exposed fronds after 24 hours, propane was observed in dark controlled fronds after 72 hours.

Figure 47 shows the recovery of standard glycolate from alumina-treated seawater samples. Percentage recovery is dependent on the concentration of glycolate in the original solution. At concentrations $> 100\ \mu g.ml^{-1}$ recovery was $\sim 90\%$, whereas at $250\ \mu g.ml^{-1}$ only 40% was recovered. This may be due to alumina saturation in the presence of increased concentrations of glycolate. However as glycolate extracts from C. fragile seawater

FIGURE 46:- THE EFFECTS OF FLUORESCENT LIGHT ($250\ \mu E.M^{-2}S^{-1}$) ON ETHANE AND ETHYLENE RELEASE FROM C.FRAGILE FRONDS AND CHLOROPLASTS

KEY:-

■——— C_2H_4 Light

■- - - - - - - C_2H_4 Dark-controlled

□——— C_2H_6 Light

□---------C_2H_6 Dark-controlled

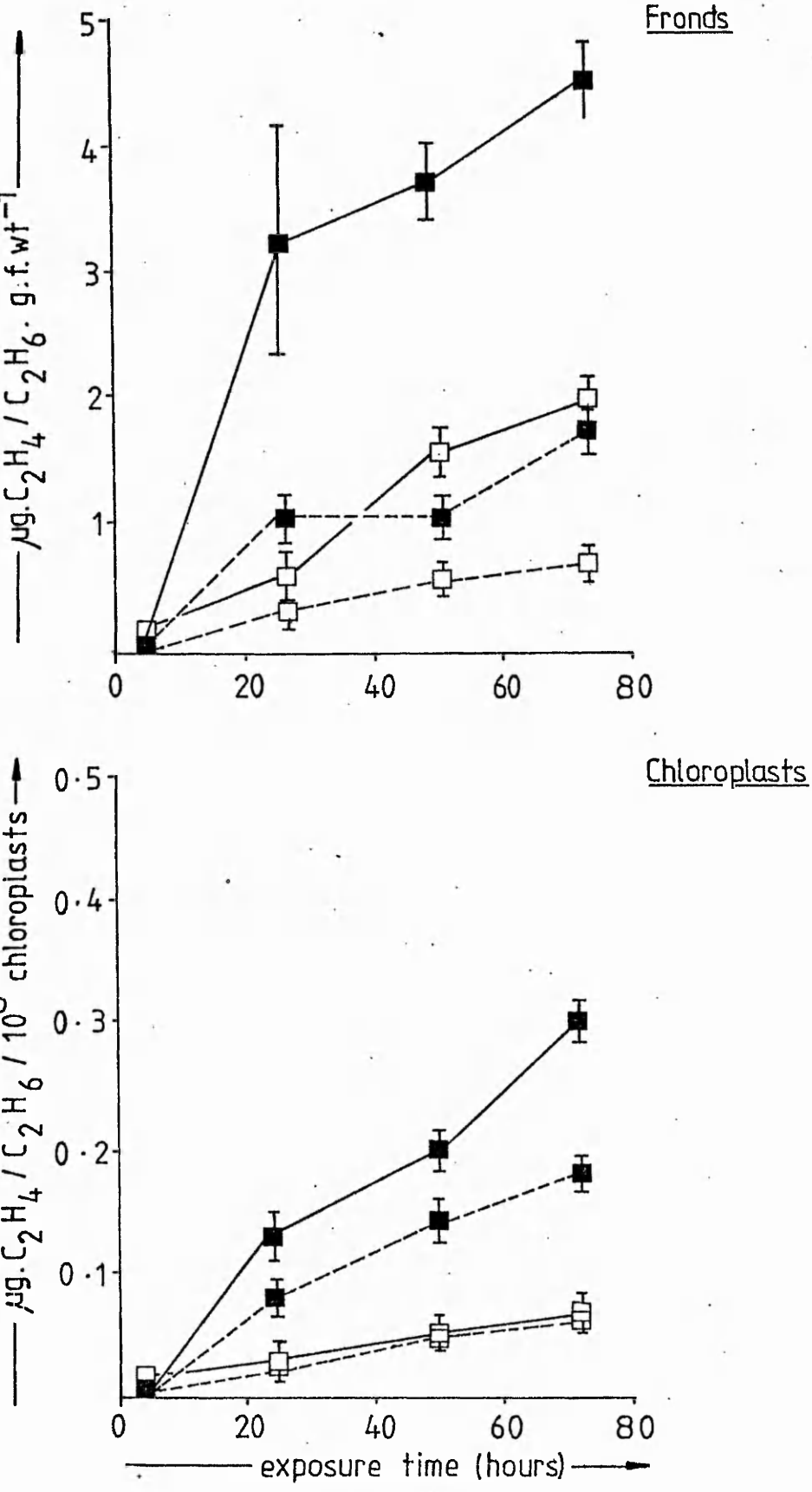
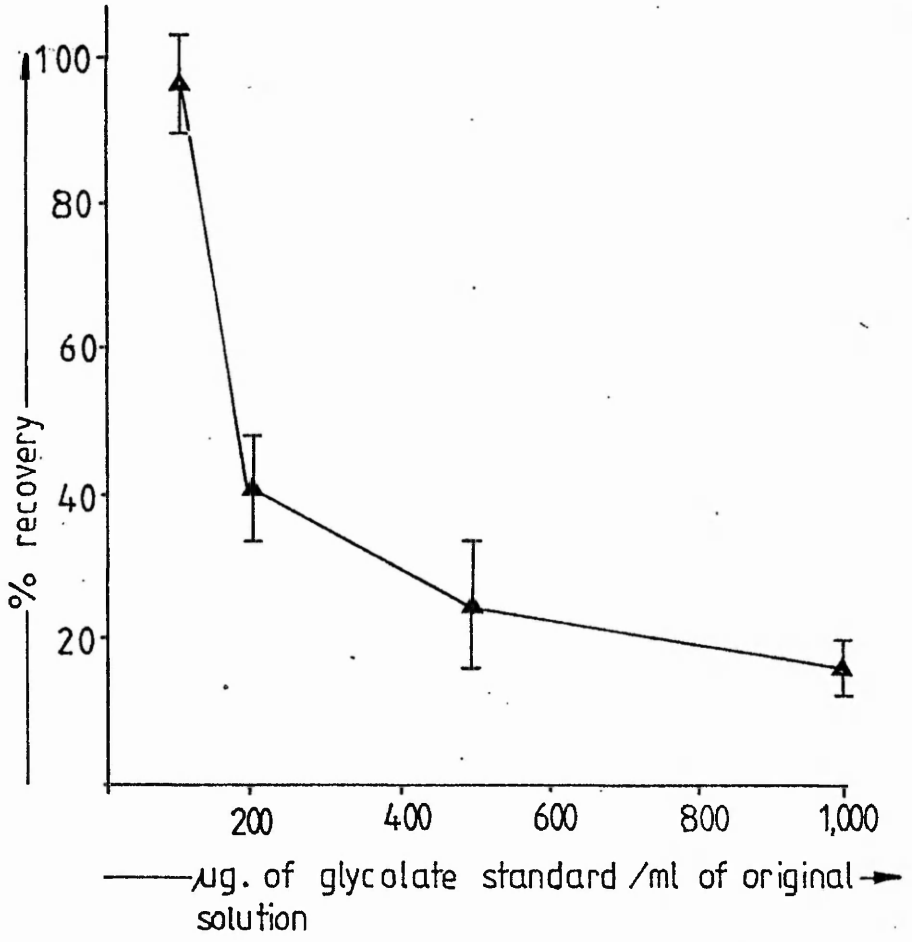

Fronds
Chloroplasts
μg. C2H4 / C2H6 . g.f.wt-1
μg. C2H4 / C2H6 / 10^8 chloroplasts
exposure time (hours)
0
20
40
60
80
1
2
3
4
5
0·1
0·2
0·3
0·4
0·5

FIGURE 47:- THE RECOVERY EFFICIENCY OF GLYCOLATE FROM SEAWATER


100
80
60
40
20
% recovery
200
400
600
800
1,000
µg. of glycolate standard /ml of original solution

samples did not exceed 4.0 µg glycolate in the original 2N H_2SO_4/alumina extract this assay may presumably be used with confidence. Figures 48a and 48b show absorption spectra of the glycolate assay from light exposed and dark controlled. A 530 nm peak is present in all the light exposed frond samples, however, shoulders at 456-460 and 491-3 nm indicate that other 2,7 dihydroxynapthalene reactive substances may be present. Absorption specta for the dark controlled fronds suggest that glycolate is absent although the presence of several different peaks suggests that some compounds are present in the seawater which are able to react with the assay reagents. Figure 49 shows the production of glycolate in light-exposed fronds as µg. glycolate.g^{-1} fwt. The absorbance at 530nm was recorded for dark controlled seawater, however these results (Figure 49) do not correspond to the glycolic acid/2,7 dihydroxynapthalene reaction complex. Glycolate was not present in seawater used to extract microbial flora from the surface of C. fragile and this confirms the glycolate to be algal in origin. Glycolate was not present in seawater sampled from Bembridge, although various coloured complexes were formed, but these had absorption values of < 0.05.

Figures 50a and 50b show the effects of light on the pigment content of C. fragile fronds over a 72 hour period of exposure. Chlorophyll content decreases with exposure time by approximately 81.7% of its original concentration. Chlorophyll a:b ratios also change indicating that chlorophyll a is more susceptible to photooxidation than chlorophyll b. Although the total carotenoid content shows an overall decline (Figure 50b) this is less apparent than that of the chlorophylls. After 72 hours carotenoids show an approximate decrease of only 31%. This relative stability in carotenoid content is also demonstrated in Figure 51a, the % distribution of violaxanthin and α-carotene is very stable and siphonein shows the greatest decrease. Neoxanthin shows little overall change and siphonoxanthin appears to increase during the $4\frac{1}{2}$-48 hr exposure period. The different

FIGURE 48:- ABSORPTION SPECTRA OF THE 2,7-DIHYDROXYNAPTHALENE COMPLEXES DERIVED FROM SEAWATER CONTAINING C.FRAGILE EXPOSED TO FLUORESCENT LIGHT ($250 \mu E.M.^{-2}S^{-1}$)

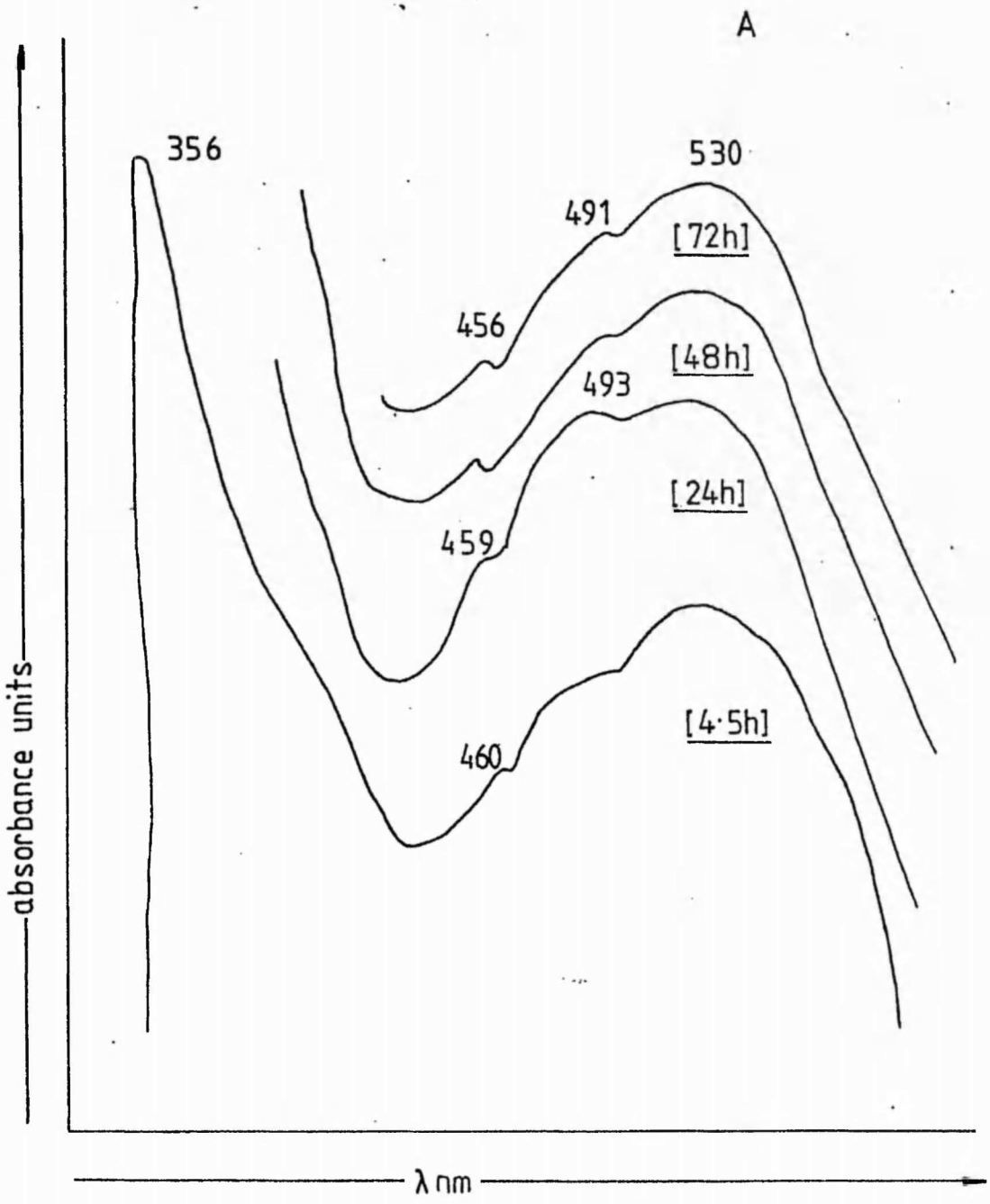
A:- Light-exposed fronds

B:- Dark-controlled fronds

KEY:-

Figures not in parentheses are major absorbing - wavelengths

Figures in parentheses are exposure times.


A
356
530
491
[72h]
456
[48h]
493
[24h]
459
[4·5h]
460
absorbance units
λ nm

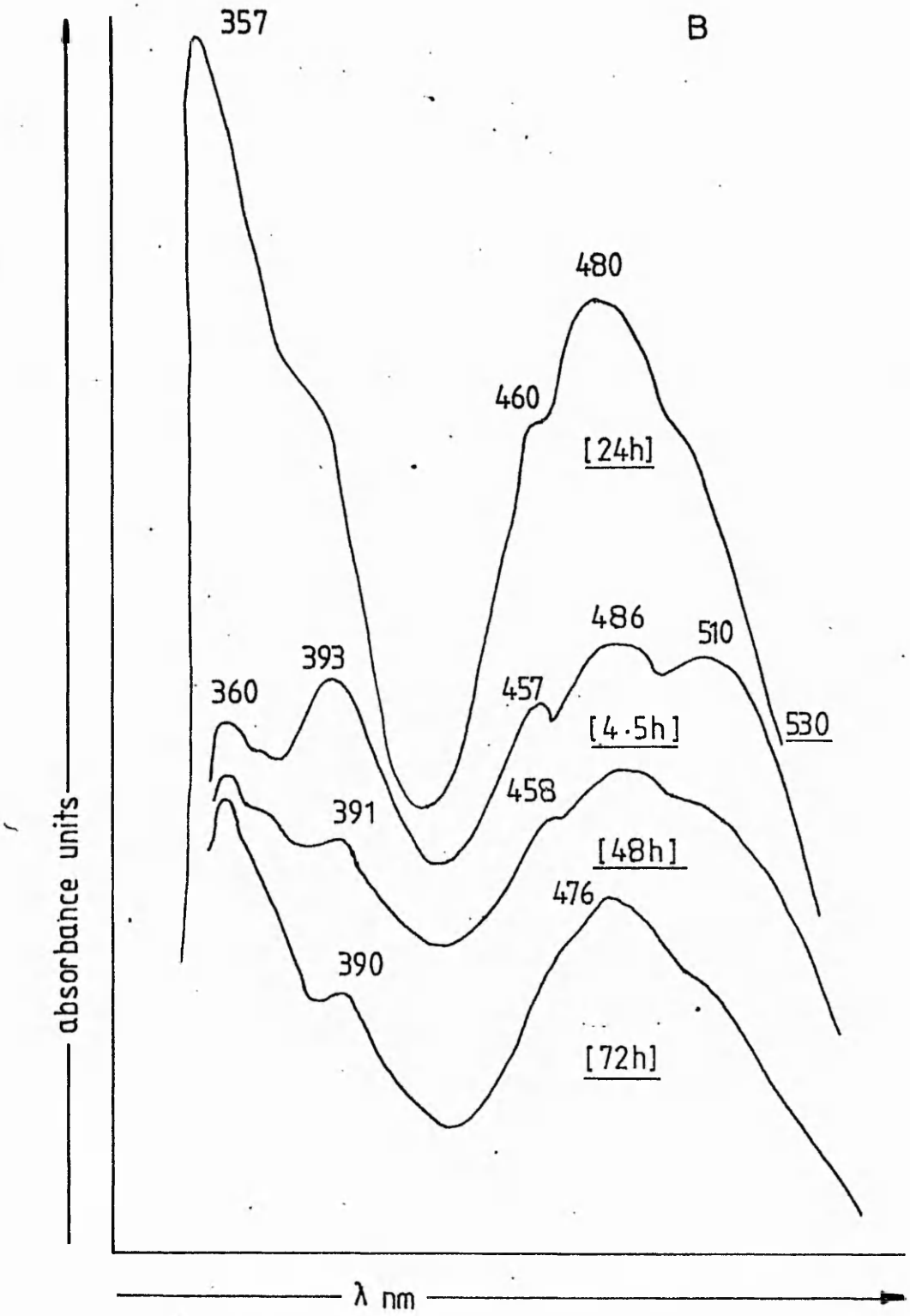

357
B
480
460
[24h]
486
510
393
360
457
[4·5h]
530
458
391
[48h]
476
390
[72h]
absorbance units
λ nm

FIGURE 49 : - THE EFFECTS OF FLUORESCENT LIGHT ($250 \mu E.M^{-2}S^{-1}$) ON GLYCOLATE EXCRETION BY C.FRAGILE

KEY

□——— Light-induced glycolate excretion

■——— Dark [2,7 di hydroxynapthalene-reactive] products, measured at λ= 530 nm

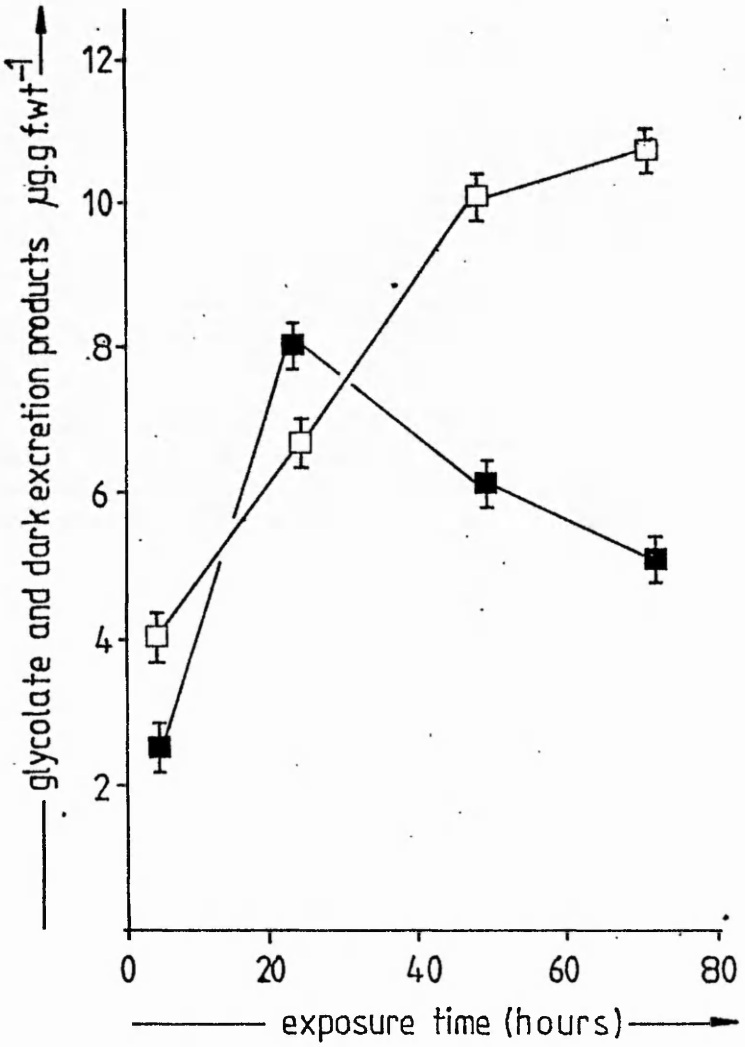

glycolate and dark excretion products µg.g f.wt⁻¹
12
10
8
6
4
2
0
20
40
60
80
exposure time (hours)

FIGURE 50 :- THE EFFECTS OF FLUORESCENT LIGHT (250 $\mu E.M.^{-2}S^{-1}$) ON THE ABSOLUTE DISTRIBUTION OF PIGMENTS IN C.FRAGILE

A :- Variation in carotenoid content

KEY :-

- ●——— Siphonoxanthin
- □——— Neoxanthin
- ■——— Siphonein
- ○——— α-Carotene
- ◇——— Violaxanthin
- ●-------- Total carotenoid content

B :- Variation in chlorophyll content

KEY :-

- ●——— Total chlorophyll
- ○——— Chlorophyll a
- ●------- Chlorophyll b

[Figures in parentheses = CHL a:b ratios]

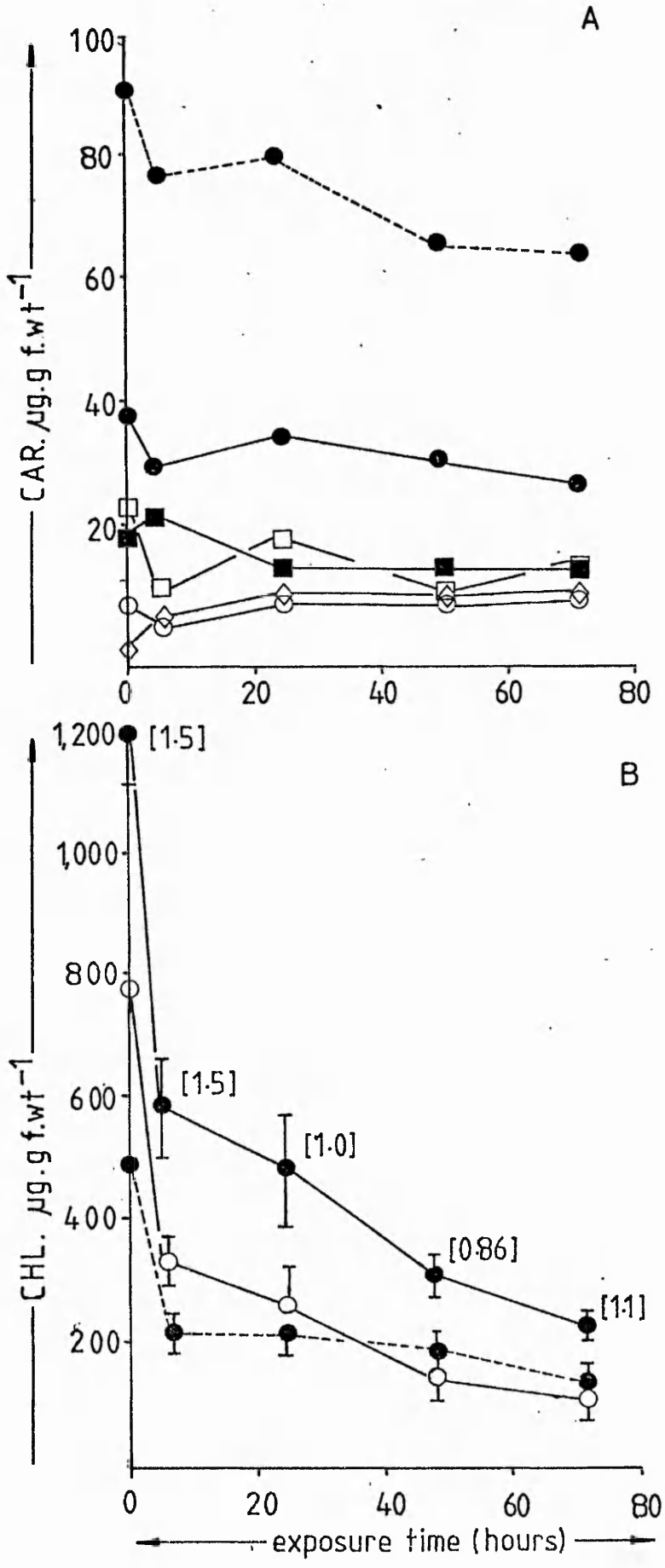

A
100
80
60
40
20
CAR. µg.g f.wt⁻¹
0
20
40
60
80
B
1,200
1,000
800
600
400
200
CHL. µg.g f.wt⁻¹
[1·5]
[1·5]
[1·0]
[0·86]
[1·1]
0
20
40
60
80
exposure time (hours)

FIGURE 51:- THE EFFECTS OF FLUORESCENT LIGHT (250 $\mu E.M^{-2} S^{-1}$) ON THE RELATIVE DISTRIBUTION OF PIGMENTS IN C.FRAGILE.

A :- % Distribution of carotenoids

KEY

- ●——— Siphonoxanthin
- ■——— Siphonein
- □——— Neoxanthin
- ◇——— Violaxanthin
- ○——— α-Carotene

B :- Total chlorophyll : individual carotenoid ratios

KEY

- ○——— α-Carotene
- ◇——— Violaxanthin
- □——— Siphonein
- ■——— Neoxanthin
- ●——— Siphonoxanthin

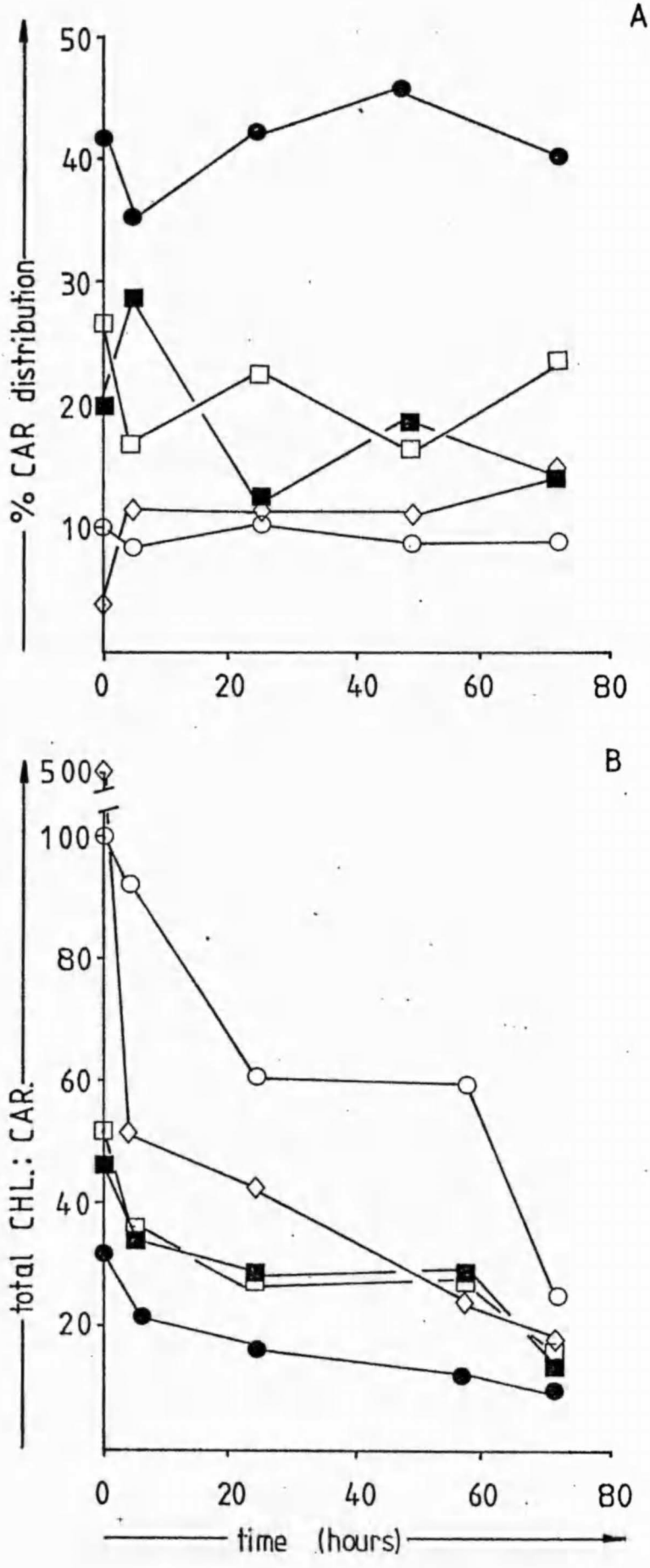

A
50
40
30
20
10
% CAR distribution
0
20
40
60
80
B
500
100
80
60
40
20
total CHL.: CAR.
0
20
40
60
80
time (hours)

FIGURE 52 :- LIGHT-INDUCED ETHANE AND ETHYLENE PRODUCTION (as ng.μg pigment^{-1}) BY C.FRAGILE FRONDS

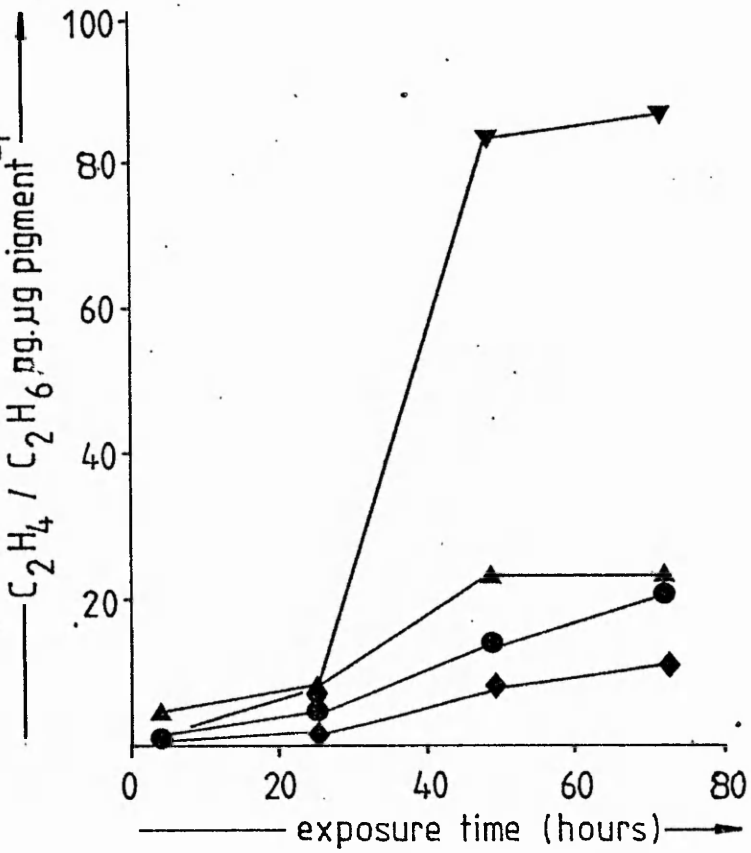
KEY :-

▼——— Ethylene / total carotenoid

▲——— Ethane / " "

●——— Ethylene / total chlorophyll

◆——— Ethane / " "


100
80
60
40
20
0
20
40
60
80
C_2H_4 / C_2H_6, pg.µg pigment^{-1}
exposure time (hours)

behaviour of carotenoids and chlorophylls is also demonstrated in Figure 51b in which a gradual decrease in ratios occurs. When ethylene and ethane production is based on pigment concentration (Figure 52) a further difference in the behaviour of chlorophylls and carotenoids is indicated. Hydrocarbon release appears proportional to chlorophyll concentration but not carotenoid content.

7.3.2 The effects of light on malondialdehyde production in C. fragile chloroplasts

Absorbance spectra of chloroplast suspensions assayed for MDA production showed very low non-specific absorption and there were no significant differences in the absorption spectra of light and dark treated chloroplasts. These results suggest that the characteristic MDA/TBA complex did not develop on the assay treatment of C. fragile chloroplasts. Considerable variations in absorption specta and non-specific absorption readings at 600nm suggests that other substances may have interferred with the assay. It therefore appears that there is little evidence for the formation of light induced MDA in C. fragile chloroplasts using the assay method described in section 7.2.8.

7.4 DISCUSSION

7.4.1 Photooxidation and photostability in C. fragile

C. fragile fronds and chloroplasts showed symptoms of photooxidation (bleaching and hydrocarbon gas production) after 4½ hours exposure to a light intensity of 250 μ E.$M^{-2}Sec^{-1}$. P.A.R. using a fluorescent white light source (Figures 46 and 50-52). In contrast, a tungsten/halogen projection lamp used to illuminate chloroplasts and fronds for 4-6 hours at light intensities of 600-1000 μ E.M^{-2}.sec^{-1} P.A.R. did not produce photooxidation symptoms even though photoinhibition of chloroplast photosynthesis was known to occur at these intensities (Chapter 5). Chloroplasts exposed to the latter light source did not produce MDA after 3 hours exposure to a light intensity of 2000 μE.M^{-2}. $Sec.^{-1}$ P.A.R., however this result may have been due to assay

interference. Studies using whole fronds illuminated for 7 hours with an Osram Solar colour lamp at intensities of 500-2000 $\mu E.M^{-2}$. Sec^{-1} P.A.R. also showed pigment stability within the fronds (Chapter 6). It is therefore apparent that under certain light regimes C. fragile is particularly stable at high light intensities. However, if fluorescent light is used C. fragile fronds and chloroplasts rapidly show signs of photooxidation at relatively low light intensities. The disparity in these studies suggests that the spectral composition of the light source used may be responsible for producing these different responses in the alga.

The use of artificial light in plant studies is not without complications and it is difficult to ensure that the light regimes used approximate the daylight spectrum. This is uniform between 400 and 800nm but aquatic studies are complicated further as water attenuates the 700-800nm zone relative to the rest of the visible spectrum (Smith and Morgan, 1981). Throughout the studies in this thesis efforts have been made to utilize light sources which correspond to in situ light characteristics. Sources which emit incandescent light are usually recommended of which tungsten/halogen and quartz/iodine projector lamps are common examples. These are particularly convenient in O_2-electrode studies which require intense, narrow beams of light to illuminate a small area (Delieu and Walker, 1972). The experiments using whole fronds necessitated the use of a larger light source for illuminating large quantities of tissue, and an Osram Solar colour lamp was preferentially used for this purpose. This lamp had the advantage of irradiating a large surface area, and being incandescent presumably approximated the emission spectrum of the slide projection lamp, used in the previous experiments. For long term photooxidation studies a light cabinet provided the most convenient light source. However, the rapid development of photooxidation symptoms in C. fragile on exposure to the 40W fluorescent white light lamps requires careful interpretation. Commercially available fluorescent lamps are noted for their high emission in the far-red region of the spectrum (700-800nm)

and although often used in plant studies are criticised for their "physiologically abnormal" emission spectra (Austin, 1972). This may be particularly significant when used to study marine algae as the water column can selectively attenuate the red region of the visible spectrum (Chapter 1). These algae may therefore be adapted to light regimes in which certain parts of the spectrum have been chromatically changed. If this is the case the finding that fluorescent light was the only light source able to induce photooxidation symptoms in C. fragile may be particularly significant. The incandescent light sources used are probably better approximations of in situ light regimes and the inability of these light sources to produce photooxidation in C. fragile may suggest that this alga is protected against photooxidation in its natural environment. Investigations into the effects of long term exposure to fluorescent light on C. fragile may however, be important in elucidating these protective mechanisms and these effects are now discussed.

Ethylene is considered a plant hormone associated with major developmental processes, however the hormonal source of ethylene is different to that produced by photooxidative damage. Methionine is probably the physiological precursor of hormonal ethylene and gas production is enzyme controlled (Lieberman, 1979). In contrast, peroxidized fatty acid systems produce a variety of hydrocarbons (including ethane and ethylene) when treated with high light intensities and/or metal ions (Sandmann and Boger, 1982). Figure 46 shows this type of production of ethylene and ethane in C. fragile fronds and chloroplasts. Volatile hydrocarbon production in both systems is evidently light induced, although the dark controls do exhibit a gradual increase in the gases over the 72 hour period of treatment. As ethane and ethylene production is also associated with tissue injury, dark production of these gases may be due to physical damage to membranes and tissues (Liebermann, 1979; Kimmerer and Kozlowski, 1982). After 4½ hours of incubation in the light and dark C. fragile fronds produce greater concentrations of ethane,

as compared to ethylene, but after 24 hours ethylene is the predominant hydrocarbon formed. A similar lag phase for ethylene production has been observed in wounded tissue although this phenomenon is usually associated with enzymatic production of the gas (Leibermann, 1979).

In general ethane appears to be the major hydrocarbon produced in association with fatty acid, oxidation in higher plants and algae (John and Curtis, 1977; Sandmann and Boger, 1982; Elstner and Pils, 1979). The preferential production of ethylene compared to ethane in C. fragile may be indicative of differences in photooxidative processes. Sandmann and Boger (1980) demonstrated enhanced production of ethylene in photosynthetic membranes subjected to copper mediated light oxidation. Copper blocks electron transport at the oxidizing side of PSII and the reducing side of PSI, and this causes the production of several free radicals which attack α-linolenic acid ($CH_3(CH_2\ CH = CH)_3(CH_2)_7\ COOH$) which then releases the ethyl radical ($CH_3-CH_2\cdot$). The preferential release of ethane or ethylene from this radical is dependent on its reaction with Cu^{2+} or Cu^{+}. Reaction with the former producing C_2H_4 and with Cu^{+}, C_2H_6. Although C. fragile was not treated with Cu^{+}/Cu^{2+}, lipid oxidation in this alga may follow analogous free radical processes which lead to the preferential release of the unsaturated hydrocarbon ethylene. A further indication that photooxidation in C. fragile may be different to that observed in other plants is that MDA was not produced when the alga was exposed to high light intensities. In contrast MDA has been reported as a photooxidation product in several higher plant studies (Heath and Packer, 1968; Benson unpublished (1979)). Unfortunately, the detection of MDA using TBA is not a very satisfactory indication of photooxidation as many compounds interfere with the assay reagent and any interpretation of MDA studies must consider these limitations.

The products and nature of lipid photooxidation in plant membranes is dependent on their fatty acid composition. Sandmann

and Boger (1982) showed that fatty acids with two or more double bonds yield short chain volatile hydrocarbons. The detection of ethane and ethylene in C. fragile indicates the presence of poly-unsaturated lipids in the membranes of this alga.. Unfortunately however, their constituent fatty acids cannot be determined from these studies, although it may be tentatively suggested that linoleic ($CH_3(CH_2)_3(CH_2CH = CH)_2(CH_2)_7COOH$) and linolenic acids are present. These are major precursors of ethane and ethylene in a number of photooxidised systems (Sandmann and Boger, 1982). On prolonged exposure to light C. fragile fronds form low concentrations of hydrocarbons tentatively identified as propane and butane (Pallett, 1982, personal communication). Dark controls also show the production of these gases after 72 hours. This gradation in the type of volatile hydrocarbon formed suggests a parallel gradation in tissue damage after prolonged incubation. This may be due in part to extensive damage of membrane lipids which were probably less accessible to photooxidation at the onset of the experiment.

Figures 50-52 show the affects of light intensity on pigment stability in C. fragile fronds. After $4\frac{1}{2}$ hours both carotenoids and chlorophylls show a drop in concentration with the exception of siphonein and violaxanthin. Chlorophylls only show a major decrease after $4\frac{1}{2}$ hours, and after 72 hours chlorophylls decreased by 81.7%, in contrast to the carotenoids which decreased by only 31%. This suggests that the chlorophyll pigments are more susceptible to photooxidation compared to the carotenoids. This is further substantiated by Figure 52 in which ethylene and ethane production is calculated as ng.μg Pigment^{-1}. Chlorophylls show a linear relationship with ethane and ethylene production but carotenoids do not. Enhanced photosensitivity of chlorophyll pigments has also been observed in previous studies. 2-way ANOVA data in Chapter 6 shows chlorophyll a and b and ϵ-carotene to be the only pigments which significantly vary with light intensity when vegetative tissue is exposed to high light intensities. Furthermore seasonal data

(Chapter 4) shows reduced chlorophyll:carotenoid ratios for fronds sampled during the summer months.

The photooxidation of C. fragile pigments is considerably different to the proposed mechanism of pigment degradation in other plant systems. Strategically the carotenoid pigments are the most effective and immediate defence against photooxidation and as a consequence are preferentially photooxidised with respect to the chlorophylls (Pallett, 1978). The degradation of carotenoid pigments may occur via three types of protective interactions (Figure 6):- (i) the quenching of triplet chlorophyll, (ii) the quenching of singlet O_2 and (iii) their direct oxidation by O_2 and its excited species. As it is the chlorophyll and not carotenoid pigments which are preferentially photooxidised in C. fragile this suggests that different photooxidative and photoprotective mechanisms are operational in the alga. The extensive breakdown of chlorophyll a may suggest that the excited $^{T}Chl.$ is not being quenched by either molecular O_2 or indeed the carotenoid pigments which is the usual occurrence in higher plant systems (Chessin et al, 1966; Foote, 1976). Thus the involvement of O_2 and the carotenoid pigments in photooxidative processes may be quite different in C. fragile. Indeed the carotenoid pigments may be unable to quench excessive chlorophyll excitation energy in the chloroplasts of this alga. The source of this excitation energy is usually reaction centre singlet chlorophyll a which undergoes inter-system crossing to produce the potentially harmful triplet species (Figure 6). If triplet chlorophyll is not converted to ground state chlorophyll the chain of events leading to photooxidation will occur. It is interesting to note that chlorophyll a appears to be more susceptible to photooxidation in C. fragile compared to chlorophyll b (Figure 50a) and this probably reflects the location and function of chlorophyll a in the PSI and PSII reaction centres. In higher plants reaction centres ^{3}Chl is usually quenched by β-carotene

which either directly interacts with ^{3}Chl to form $^3\beta$-carotene or quenches singlet O_2 (Figure 7). C. fragile chloroplasts however do not contain β-carotene (Chapter 2) and pigment/proteins associated with P-700 reaction centres are enriched in α-carotene. The photooxidative stability of α-carotene (Figures 51a and 51b) in C. fragile suggests that this pigment may not be involved in the photoprotection of reaction centre chlorophyll a. Furthermore, this indicates that α-carotene does not replace the primary functions of β-carotene in higher plants. The quenching efficiency of β-carotene is largely dependent on its localised concentration within the reaction centres (Foote, 1976). Higher plants have reaction centre chlorophyll a: β-carotene ratios of 20:30 (Thornber and Alberte, 1977)., however in contrast the chlorophyll a: α-carotene ratio of the CP1 complex isolated from C. fragile was 90 $\pm$ 16.6. Thus the localised α-carotene concentrations may not be high enough to quench ^{3}CHL in C. fragile reaction centres. The ability of other carotenoid pigments to quench excitation energy in C. fragile is debatable as violaxanthin, neoxanthin, siphonein and siphonoxanthin all remain relatively stable on exposure to the light regime used in this experiment (Figure 50b). The stability of violaxanthin may reflect the location of this pigment in regions of the chloroplast other than the PSU and neoxanthin and siphonein are located largely in the LHCP complexes (Chapter 3). However, it is of interest to note that siphonoxanthin may exist in a form not associated with the light-harvesting apparatus (Chapter 3) and together with its ester, this 'free-form' siphonoxanthin may be of photoprotective importance. A fatty acid associated with a carotenoid may provide a preferential target for photooxidation and if the fatty acid is lost leaving the isoprenoid/ionone unit intact the parent compound siphonoxanthin may still be functionally and structually important. Unfortunately there is little evidence to indicate the relationship between siphonein and siphonoxanthin in this thesis and their function other than light-harvesting must still remain speculative.

The marked photooxidation of chlorophyll and the relatively stable carotenoid content of C. fragile fronds throughout the duration of this experiment may also provide an explanation for the atypical photooxidation of chlorophyll and carotenoid pigments in C. fragile. Fluorescent light is particularly noted for high emission in the far red (Austin, 1972). As chlorophyll absorbs at "in vivo" wavelengths of 670 and 682nm in C. fragile pigment/proteins (Chapter 3) the chlorophylls may be excessively photosensitised by this light source. Results in Chapter 6 indicate that pigment stability in C. fragile may be due in part to a high turnover of pigment biosynthesis. Red light may be particularly important in the metabolism of chlorophyll which incorporates a phytochrome response (Sundquists, Bjorn and Virgin, 1980). Thus a degradation of chlorophyll in C. fragile fronds illuminated with fluorescent light emitting in the far red region of the spectrum may reflect a breakdown in the biosynthetic maintenance of chlorophyll. Although phytochrome is also implicated in carotenoid biosynthesis 'blue light' photoreceptors may also be important and thus the pathways of chlorophyll and carotenoid biosyntheses are probably very different in their light activation requirements. (Harding and Shropshire, 1980; Rau, 1976). The disparity in carotenoid and chlorophyll photooxidation may therefore indicate that their biosynthetic pathways are altered by the fluorescent light source. Photooxidation of C. fragile chloroplasts exposed to fluorescent light has also been observed by Hawes and Cobb (1980). Using Atlas grow lux fluorescent lights (at 6.4 $W.M^{-2}$) these authors demonstrated the photooxidative degeneration of chloroplast membranes in C. fragile chloroplasts endosymbiotic in Elysia viridis. However membrane disruption was only significant after 27 days of light starvation of the animal and these results may also reflect an inherent photo-stability of C. fragile chloroplasts.

Photostability in isolated chloroplasts is unlikely to be solely due to rapid biosynthetic turnover of chloroplast

pigments and other photo-protective mechanisms must be operational in the alga. Furthermore, fronds and chloroplasts exposed to high non-fluorescent light sources although showing net stability do exhibit fluctuations in pigment content which may suggest pigment breakdown is taking place. However, this 'breakdown' is not accompanied by ethane, ethylene or MDA production suggesting that free radical scavenging mechanisms and indeed some carotenoid quenching may be operational. Superoxide dismutase has been detected in C. fragile chloroplasts (Lumsden and Hall, 1975) and it is highly probable that the other photo-protective mechanisms common to most chloroplasts are also present (e.g. α-tocopherol, glutathione, ascorbate). These mechanisms may be impaired or overcome when fluorescent light is used to induce photooxidation.

An indication that C. fragile chloroplasts may be further adapted to high light intensities is evidenced by glycolate excretion when fronds were exposed to light irrespective of its spectral composition. This phenomenon will be discussed as follows

7.4.2 Glycolate excretion by C. fragile fronds

Hawes and Cobb (1980) noted that photooxidation in endosymbiotic C. fragile chloroplasts only occurred after depletion of storage carbon reserves. This finding is consistent with the recognised importance of photosynthetic carbon metabolism in the regulation and dissipation of photochemical energy (Chapter 1). The photorespiratory pathway is considered an important means of controlling carbon metabolism when plants are exposed to high light intensities and reduced CO_2 concentrations (Chapter 1).

Figure 44 shows a characteristic absorption spectrum of the 2,7 dihydroxynapthalene/glycolate complex using standard glycolate. Figures 44 and 48 show the absorption spectra of this complex for seawater used to bathe C. fragile fronds exposed to light regimes of 1000 $\mu E.M^{-2}.S^{-1}$ P.A.R. for 8 hours using an Osram solar colour lamp and 250 $\mu E.M^{-2}.S^{-1}$ P.A.R. for 72 hours

using Thorne 40W fluorescent tubes. Both light regimes appear to induce glycolate excretion by C. fragile fronds as evidenced by the 2,7 dihydroxynapthalene complex formed with absorption peak at 530nm. This assay reagent also formed other complexes as indicated by additional absorption peaks e.g. 456, 491nm, Fogg (1976) also reports similar interference phenomena. Absorbance spectra for complexes formed from dark controlled fronds did not show a characteristic absorbance at 530nm, thus indicating that glycolate is not produced in the dark. This confirms the findings of Fogg (1966) who demonstrated the liberation of extracellular products in marine algae but concluded that dark excretion was different to that occurring in the light. Glycolate was not produced in seawater used to wash C. fragile fronds thus glycolate originates from algal and not bacterial metabolism. Using the solar colour lamp 0.23µg.glycolate.g. $fwt^{-1}.hr^{-1}$ were produced after 8 hours of exposure at 1000µE.$M.^{-2}$. Sec^{-1} P.A.R. After 8 hours exposure to 250µE.$M^{-2}.Sec^{-1}$ P.A.R. using Thorne 40W fluorescent tubes approximately 0.57µg glycolate. $g^{-1}.hr^{-1}$. were produced. This indicates that the fluorescent light regime may enhance the production of glycolate by C. fragile. Production of glycolate using this light regime increased with exposure time, and a maximum of 11µg glycolate.g^{-1} fwt was produced after 72 hours exposure (Figure 49). Dark production of 2,7 dihydroxynapthalene-reactive substances are indicated in Figure 49 (using absorbance at 530nm). However, this cannot be correlated to glycolate release and merely indicates the production of other compounds by the alga. Glycolate excretion by C. fragile may also be accompanied by carbohydrate release as hexoses, as this alga has been found to excrete 40% of CO_2 assimilated during photosynthesis when exposed to supra-optimal light saturation intensities (Brinkhuis and Churchill, 1972).

The extracellular release of carbon compounds by algae appears to be a normal function of healthy cells (Mague, Friberg, Hughes and Morris, 1980). Coastal waters have been reported to contain up to 80µg.glycolate L^{-1}. (Shahand Wright, 1974),

although Bembridge seawater did not contain measurable amounts of 2,7 dihydroxynapthalene-reactive substances. The release of glycolate is not species specific but has been observed in many types of marine and freshwater algae and angiosperms (Khailov and Burlakova, 1969; Samuel, Shah and Fogg, 1971; Fogg, 1976). Thus the widespread occurence of this phenomenon is particularly intriguing and leads to the question of why do aquatic plants release large quantities of important photosynthetic metabolites from the cell? Photorespiration has been associated with metabolite excretion in algae as glycolate has been identified as a major excretary product. Additionally glycolate release has been documented at high light intensities and supersaturation with O_2 (Fogg, 1976). Photorespiration has been demonstrated in many different algal species using various enzymatic and carbon fixation studies (Kremer, 1981). However, its importance in the overall productivity of these algae is uncertain as is the ecological significance of glycolate excretion. The light-induced excretion of glycolate by C. fragile fronds together with the importance of starch turnover in the development of photo-oxidative symptoms in isolated chloroplasts (Hawes and Cobb, 1980) may suggest that carbon flow is an important means of regulating photooxidation in this alga and other aquatic plants. As reviewed in Chapter 1, photorespiration may be a means of regulating excessive excitation energy production by coordinating the 'light' and 'dark' reactions of photosynthesis and this may be a plausible explanation for aquatic plants also. However, since the production of light-induced glycolate also results in its release to the surrounding aquatic medium, these plants may vary somewhat to their terrestrial counterparts. Algae may have the ability to use their surrounding medium as extracellular zone from which they may reclaim or release excessive amounts of metabolites in accordance with the immediate fluctuations in their light environment.

A summary of the mechanisms of photoprotection and photo-oxidation thought to be operational in C. fragile is given in Figure 53.

7.5 CONCLUSIONS

These studies indicate that photooxidation can be induced in C. fragile fronds and chloroplasts and this may account in part for summer-frond bleaching in the alga. However, these symptoms were only manifested when fluorescent light regimes were used and the alga appears very photo-stable when exposed to high non-fluorescent light intensities. This may infer that C. fragile is quite stable on exposure to wide ranging intertidal zone light intensities. If this is the case then summer-frond bleaching may be largely due to other environmental factors e.g. low nutrient status.

Photostability in this alga may be due in part to a high turnover of chlorophyll and carotenoid pigments, but other mechanisms may also be operational. Glycolate excretion may implicate the photorespiratory pathway and superoxide dismutase has been isolated in this alga (Lumsden and Hall, 1975). Studies using fluorescent light suggest that carotenoid quenching (particularly α-carotene) may not be involved in dissipating excessive chlorophyll excitation energy. However, it is not possible to exclude this means of photoprotection in C. fragile fronds exposed to non-fluorescent and in situ light regimes.

FIGURE :- 53 PHOTOOXIDATION AND PHOTO-PROTECTION IN C.FRAGILE

KEY :-

P/S = Photosynthesis

ISC = Inter-system crossing

CHL^1 = Singlet chlorophyll

CHL^3 = Triplet chlorophyll

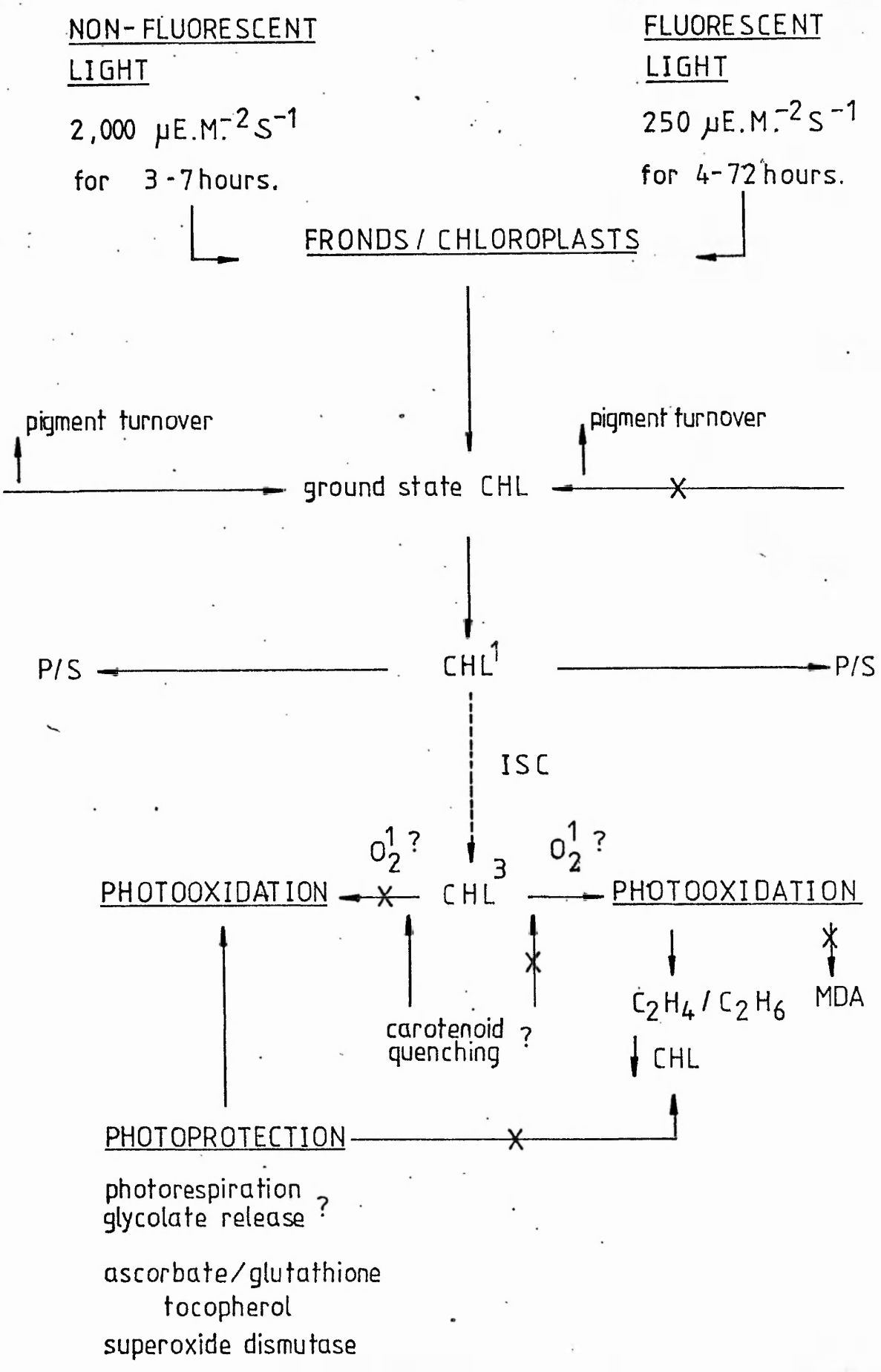
O_2^1 = Singlet oxygen

MDA = Malondialdehyde

↑ = Increase

↓ = Decrease

—X— = Inhibited event


NON-FLUORESCENT LIGHT
2,000 µE.M.-2S-1
for 3-7 hours.
FLUORESCENT LIGHT
250 µE.M.-2S-1
for 4-72 hours.
FRONDS / CHLOROPLASTS
pigment turnover
pigment turnover
ground state CHL
P/S
CHL1
P/S
ISC
O21 ?
O21 ?
PHOTOOXIDATION
CHL3
PHOTOOXIDATION
C2H4 / C2H6
MDA
carotenoid quenching ?
CHL
PHOTOPROTECTION
photorespiration
glycolate release ?
ascorbate/glutathione
tocopherol
superoxide dismutase

8. GENERAL DISCUSSION

In order to survive and successfully grow in an intertidal habitat marine algae must withstand the extreme fluctuations in growth parameters which their environment imposes upon them. This thesis contains a study of one such alga and this section attempts to conclude the investigation by formulating a physiological basis for the intertidal zone adaptations of *C. fragile*.

One of the greatest problems with any ecological study is to simulate *in situ* environmental parameters under laboratory conditions. In higher plant studies this has been largely overcome by the use of growth cabinets which are able to maintain controlled environmental conditions. Similarly, in the study of unicellular algae micribiological culture techniques have been exploited in which the algal cells are sustained in batch or continuous culture. The latter especially, provides a convenient and controlled approach to simulating natural conditions in the laboratory. This is probably a major factor for the preponderance of phytoplankton studies in phycological research. The growth and maintenance of macroalgae under laboratory conditions is however, more complex. In unicellular algae growth and reproduction is largely synonymous, but in macroalgae the environmental factors affecting growth and reproduction are usually very different (Darley, 1982). For this reason the maintenance of macroalgae cultured in the laboratory is particularly difficult and would require extensive study before stable colonies may be exploited for further research. These limitations have resulted in two major approaches to the study of macroalgae:-

(a) Removing the alga from the environment and sustaining growth under constant laboratory conditions

(b) *In situ* studies of the alga.

Undoubtedly (b) is the ideal method but unfortunately *in situ* experiments are often inconvenient or impractical and this is particularly

the case for the study undertaken in this thesis. Thus, method (a) is probably the most standard means of investigating macroalgae and as such was used in this study of C. fragile. The limitations of this method may be reduced considerably by indepth observations at the time of sampling which supply background information on in situ changes in environmental parameters. This provides supplementary detail to most types of ecological investigations and may indeed support subsequent laboratory findings. In this study of C. fragile an inventory of seasonal information was compiled. This included an assessment of physical environmental parameters (e.g. tidal amplitude), morphological characteristics, growth of other algae in the intertidal zone, and a physiological assessment of C. fragile chloroplasts immediately after sampling. The measurement of these parameters provided an empirical basis to the understanding of adaptations in C. fragile. Unfortunately, an indepth ecological study of this alga in the intertidal zone was not within the scope of this thesis. However, it is the opinion of this author that an extensive ecological survey of the intertidal zone at Bembridge may provide a greater understanding of adaptations in not only C. fragile but other algal members of the intertidal community. This may be achieved by frequent quantitative assessments of the seasonal distribution of algal populations and the succession of individual species together with an indepth assessment of the physical environment. Such parameters as nutrient availability, temperature and submarine light intensities may be particularly important in monitoring seasonal changes in the intertidal zone. Within the constraints of this study the less meticulous observations of environmental parameters at Bembridge still showed significant seasonal changes in the intertidal zone. When these changes were assessed in relation to the life cycle of C. fragile an ecologically advantageous growth strategy in this alga soon become apparent. This growth strategy provides an important overall understanding of intertidal adaptations in C. fragile and will now be discussed.

Figure 54 shows a scheme for the growth of C. fragile in the intertidal zone in relation to seasonal succession, nutrient availability, tidal amplitude and light intensity. Maximum vegetative growth in

FIGURE 54 : - SEASONAL ADAPTATIONS IN C.FRAGILE

KEY : -

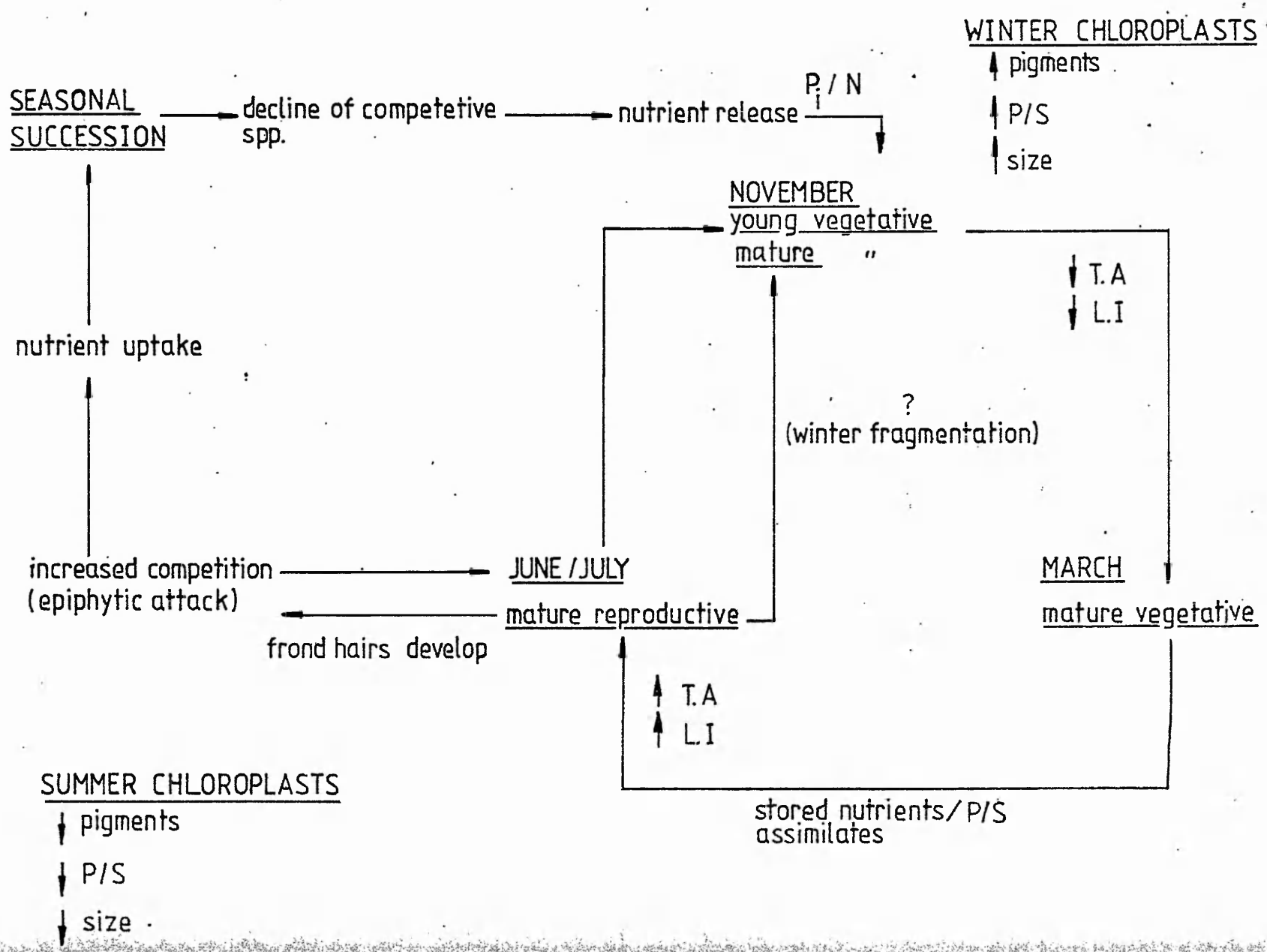
P/S = Photosynthesis

T.A = Tidal amplitude

L.I = Light intensity

↑ = Increase

↓ = Decrease


WINTER CHLOROPLASTS
pigments
P/S
size
SEASONAL SUCCESSION
decline of competetive spp.
nutrient release
P_i / N
NOVEMBER
young vegetative
mature "
T.A
L.I
nutrient uptake
?
(winter fragmentation)
increased competition
(epiphytic attack)
JUNE / JULY
mature reproductive
MARCH
mature vegetative
frond hairs develop
T.A
L.I
SUMMER CHLOROPLASTS
pigments
P/S
size
stored nutrients / P/S assimilates

C. fragile occurs during the winter and early spring when young colonies of the alga are able to develop in a physical environment which is less extreme due to reduced tidal amplitude, and less competition from more dominant annual species. Similarly, older colonies of C. fragile which were subject to frond bleaching and epiphytic attack may recover, probably by means of winter fragmentation. Winter-sampled fronds sometimes showed symptoms of this phenomenon and "frond ringing" techniques would elucidate further the role of winter-fragmentation in this alga. Winter vegetative growth also coincides with maximum chlorophyll and carotenoid content, carbon fixation (Cobb and Rott, 1978) and chloroplast size (Rutter, 1982). There is also evidence to suggest that during this period of its life cycle C. fragile is able to accumulate storage reserves and nutrients such as Pi (Rutter, 1982). This winter accumulation probably reflects minimal competition from other algae and indeed their decline will aid nutrient release into the intertidal zone (Figure 54). More recent observations in this laboratory (Williams, 1983 unpublished) has shown that vegetative fronds may be able to store photosynthetic assimilates as starch, which are then mobilized later in the reproductive part of the life cycle. This is an important observation as it suggests that in the winter months both photosynthetic competence and light-harvesting pigment accumulation increases. In contrast, shade adaptations in higher plants are often typified by low photosynthetic electron transport rates and high pigment content (Anderson et al, 1981). These differences suggest that shade and growth strategy adaptations are operational in C. fragile chloroplasts as compared to higher plant plastids which in most cases may be shade adapted only.

In the summer months competition from other algal species can lead to nutrient deficiency and the epiphytic attack of C. fragile fronds. The development of frond hairs in reproductive C. fragile is suggestive of low environmental nutrient status, and this is further substantiated by their degeneration when the alga is placed in storage conditions of increased nutrient availability. Summer-frond bleaching may be caused by nutrient effected chlorosis and/or photooxidation.

Although the direct cause of these symptoms requires further investigation it is known that efficient photosynthesis in summer sampled C. fragile is reduced (Cobb and Rott, 1978) and the alga enters the reproductive phase of its life cycle. Male gametangia were never observed on fronds sampled from Bembridge, and C. fragile most probably reproduces by parthenogenesis in this habitat. It is most likely that the development of reproductive structures in C. fragile is supported by the stored assimilates and nutrients sequestered during the vegetative winter/spring period of growth.

Thus, the growth strategy of C. fragile maximises nutrient and carbon assimilation in the winter and employs a strategic survival mechanism in which the timing and not the extent or rate of vegetative growth in the important factor. In the summer, when competition from faster growing algae is greater, C. fragile enters the reproductive phase of the life cycle which is supported by the mobilization of winter-stored nutrients.

The growth of C. fragile in the intertidal zone is also largely dependent on the alga's ability to accommodate the extremes of light intensity and quality which it encounters daily. Higher plants are often categorized into "shade" and "sun" adapted species which display a number of corresponding photosynthetic adaptations. However, C. fragile grows in a constantly changing light environment in which, relatively speaking, "shade" conditions occur at high tide and "sun" conditions at low tide. Seasonal variation in tidal amplitude may also enhance these tidal changes in light intensity. Coastal waters are also noted for their large organic matter content and this together with large concentrations of particulate silts and clays can often change the quality as well as intensity of light penetrating the water column (Jerlov, 1977).

The major aim of this thesis was to investigate light adaptations in C. fragile and although a seasonal study provided an important background much of the experimental investigation had to be performed under laboratory conditions using controlled light regimes. This necessitated the use of C. fragile which had been in storage for several weeks. The behaviour of C. fragile in storage suggests that

the alga may parallel *in situ* development as vegetative fronds showed increases in chlorophyll concentration and developed reproductive structures. The alga was maintained under constant storage conditions as it would be particularly difficult to simulate tidal movement, nutrient flow and competition from other species. Whilst storage conditions were atypical of the natural environment an important means of standardizing the fronds before experimental investigation was provided. This procedure has been adapted for many phycological studies (Darley 1982) and the appropriate 'storage controls' were always incorporated into each experiment. However, *in situ* environmental monitoring of *C. fragile* would be particularly worthwhile in further research, especially as storage conditions appeared to affect photosynthetic light saturation characteristics of intact fronds (Chapter 5). One of the most difficult parameters to simulate under laboratory conditions was the natural daylight spectrum. Osram Solar Colour and Halight tungsten/halogen lights were preferentially used in this study. However, it is appreciated that the simulation of submarine light fields is particularly difficult. The water column of natural coastal waters is known to reduce the available light reaching submerged algae and also change the spectral quality of this incident light (Jerlov, 1977). These changes can be transient in the intertidal zone where there is a daily flux in the mixing of dissolved light-attenuating particulate matter. Where possible laboratory light experiments were performed on both the vegetative and reproductive stages of the alga's life cycle. Unfortunately, because of the seasonal and environmental limitations of this investigation, it was inevitable that constraints were placed on the experimental study of *C. fragile*. Thus, many of the experimental investigations were designed to produce supportive evidence if the absolute measurement of a specific aspect of *C. fragile* was limited by the nature of the sampled fronds. A major example of this approach was the inability to characterize the pigment/protein complexes of reproductive *C. fragile*. The pigment content of these complexes was very low and successful analysis was beyond the limitations of the TLC assay. However, an indepth survey of reproductive frond pigmentation was performed over a wide range of light intensities, and this information yielded some indication of the differences and similarities in the vegetative and

reproductive PSUs of C. fragile. By using such methods of investigation this thesis has shown that C. fragile is particularly well adapted to the variable light regimes of the intertidal zone and this is evidenced by the 'sun and shade' type adaptations displayed by both fronds and isolated chloroplasts. These adaptations are summarized in Figure 55 and are discussed as follows.

Variations in light quality and intensity in the intertidal zone are largely controlled by tidal movement and seasonal factors. Thus, at high tide, algae in the intertidal zone are subjected to a decrease in the incident light field and this effect is enhanced in the winter months when tidal amplitude is decreased. At low tide the algae are exposed to increased light intensities, this is particularly apparent in the summer months when tidal amplitude is increased. In response to these environmental fluctuations C. fragile has developed shade and sun adaptations respectively.

Shade adaptations have been previously reported in C. fragile by Ramus (1978). This author suggests that frond structure in the alga may be of importance in the capture of incident light when the alga is subjected to low intensity light fields. Using absorption studies Ramus (1978) demonstrated uniformly high non-specific absorptance by C. fragile fronds throughout the 400-700nm region of the visible spectrum. This type of attenuation was termed "low specific chlorophyll absorption" and increases the alga's ability to utilize the "green gap" wavelengths. It is evident that the thallus anatomy of C. fragile is able to maximise the absorption of incident light and in doing so confers an overall "light-harvesting" advantage at all wavelengths in the visible spectrum. However, Ramus (1978) is incorrect in the supposition that this is due to "non-specific chlorophyll absorption" as this suggests that all wavelengths absorbed by the fronds may be utilized in photosynthesis. High non-specific absorption by the fronds may increase the ability of C. fragile to grow in weak light fields by conferring an overall light-harvesting advantage. However, the benefits of this are only reflected by the utilization of this light in photosynthetic processes which are not independent of wavelength. Thus, the utilization of frond-harvested light is directly dependent on the pigment composition and arrangement of the PSU in the chloroplast.

A detailed examination of the pigment content and pigment/protein composition of C. fragile formed a main part of this thesis and this study provides an insight into the ability of C. fragile to grow at low light intensities.

The pigment composition of C. fragile chloroplasts indicates that this alga is adapted to submarine photosynthesis. Chlorophyll a:b ratios are low (1.5:1) and typical of those found in shade adapted higher plants (Anderson et al, 1973). Intact chloroplasts absorb in the 'green-gap' region of the visible spectrum and this is due to the specific absorption of light at 540-542nm by siphonoxanthin and siphonein. These pigments comprise approximately 60% of the total carotenoid content of C. fragile chloroplasts and are located in the light-harvesting pigment/protein complexes ($LHCP_1$ and $LHCP_2$). Siphonoxanthin and siphonein have been shown to transfer excitation energy to chlorophyll a in a number of algae (Yokohama, 1981) and are therefore also thought to be of importance in the harvesting of light energy in C. fragile. This may be especially important in coastal waters where particulate and organic matter may selectively attenuate blue and red regions of the spectrum in preference to green. Dring (1981) suggests that the spectral composition of light is irrelevant to the photosynthesis of algae with thick thalli such as C. fragile. However, in this study, when 70% of green light available to C. fragile fronds was reduced, the remainder of the visible spectrum was unable to support phohotosynthesis. Further investigations into the importance of siphonein and siphonoxanthin in energy transfer to chlorophyll a are therefore recommended in the continued study of C. fragile.

The quantitative distribution of pigments in the pigment/protein complexes of C. fragile chloroplasts is a further indication that the PSU of this alga is adapted to low light intensities. $LHCP_1$ and $LHCP_2$ comprised approximately 75% of the total pigment content of the pigment/proteins resolved by SDS-PAGE, and the light-harvesting apparatus was enriched in chlorophyll b, siphonoxanthin and siphonein. This data is representative of fronds sampled during the November-March period when "shade" conditions in the intertidal zone are prevalent. Unfortunately, the low pigment content of summer-sampled fronds made successful pigment/protein characterization difficult and a comparison of summer- and

winter-adapted C. fragile pigment/proteins was not possible. Such a study may have revealed a reduction in the light-harvesting apparatus of the alga when sampled during the summer. However, the seasonal and light stable chlorophyll a:b ratios and % carotenoid distributions in reproductive and vegetative fronds may indicate otherwise. Furthermore, summer-sampled fronds from a "shaded" region of the sub-intertidal zone had increased pigment content, yet pigment distribution was the same as fronds sampled from the more exposed sampling site. These findings may suggest that the arrangement of the chloroplast PSU in C. fragile remains the same throughout the life cycle of the alga, the alga being constantly adapted to low light intensities by virtue of the relatively high light-harvesting pigment content of both summer and winter sampled fronds.

Photosynthetic light saturation characteristics of isolated C. fragile chloroplasts also indicate that this alga is adapted to low light intensities and the studies in this thesis confirm the findings of Cobb and Rott (1978) and Rutter (1982). C. fragile chloroplasts reach a maximum rate of photosynthesis over a very narrow range of low light-intensities and this may reflect the enhanced light-harvesting efficiency of the PSU. Also, C. fragile chloroplasts are unable to maintain the photosynthetic light-saturation plateaux over an extended range of light intensities and photoinhibition occurs once supra-optimum intensities are attained. It is possible that large numbers of light-harvesting units supply only a small number of reaction centres, and the turnover of photochemical events soon becomes out of step with the amount of light energy being harvested. Thus, shade adapted chloroplasts in C. fragile are unable to maintain the extended light saturation plateaus characteristic of many higher plants. The light saturation characteristics of photosynthesis in C. fragile fronds are considerably different to those of isolated chloroplasts, and indeed atypical of the curves usually obtained from intact higher plants. From the investigations performed in Chapter 5 it is evident that storage conditions and the interpretation of photosynthetic rate on total chlorophyll or fresh weight bases may have strongly influenced the data obtained.

As photoinhibition in isolated C. fragile chloroplasts occurs at relatively low light intensities and frond bleaching was often observed in the summer-sampled alga, C. fragile appears to be susceptible to photooxidative and photoinhibitory damage. Thus, although C. fragile is adapted to low light intensities, exposure to increased intensities occurs during the summer at low tide when light intensities in excess of 2,500 $\mu E.M^{-2}S^{-1}$ P.A.R. are often encountered. Bjorkman (1981) suggests that obligate higher plant shade species have a very limited potential to adjust to increased light levels and prolonged exposure at even moderate light intensities soon results in photoinhibition. C. fragile cannot be classed as an obligate shade species as it has to withstand wide fluctuations in light intensities. The possibility that this alga is adapted to both low and high light intensities has therefore been examined. When C. fragile fronds and chloroplasts were exposed to high intensity non-fluorescent light both appeared to be remarkably stable when exposed for periods corresponding to tidal exposure. Both vegetative and reproductive fronds maintained stable pigment ratios on exposure to light intensities $\leq$ 2,000 $\mu E.M^{-2}.S^{-1}$, and vegetative fronds and chloroplasts exposed to light intensities twenty times greater than that causing inhibition of chloroplastic photosynthesis failed to produce photooxidation by-products. Similarly, pigment breakdown was not evident in these fronds or chloroplasts. Using fluorescent light Hawes and Cobb (1980) were able to demonstrate photooxidative membrane damage in C. fragile chloroplasts which were endosymbiotic in E. viridis. However, these symptoms only became significant after 27 days of light-starvation of the mollusc. These findings suggest that C. fragile chloroplasts may be adapted to high as well as low light intensities. Photostability may be due to a number of factors as suggested in Figure 55. On exposure to high non-fluorescent light intensities both vegetative and bleached reproductive fronds were able to maintain stable pigment ratios despite large fluctuations in absolute pigment concentration. This suggests that C. fragile is highly efficient in maintaining pigment composition and arrangement of the PSU and this maybe an important means of photoprotection. An indication that this alga has a high pigment turnover is also evidenced by the inability to detect β-carotene. Thus,

FIGURE 55 :- LIGHT ADAPTATIONS IN C.FRAGILE.

KEY :-

LHCP = Light-harvesting pigment/protein complex

PSU = Photosynthetic unit

P/S = Photosynthesis

P/R = Photorespiration

CHL^1 = Singlet chlorophyll

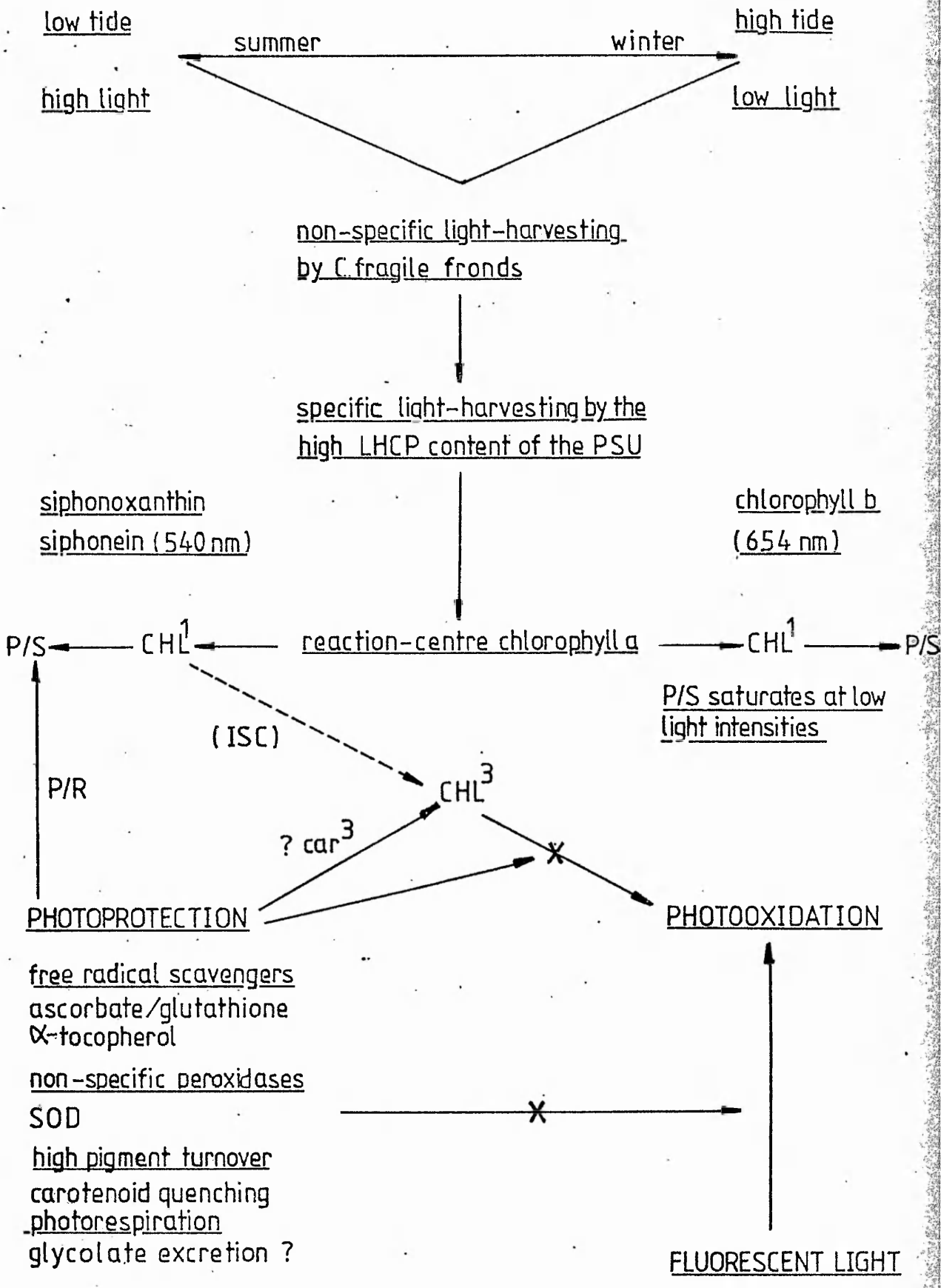
CHL^3 = Triplet chlorophyll

car^3 = Triplet carotenoid

ISC = Inter-system crossing

SOD = Superoxide dismutase

—X— = Inhibited event


low tide
high tide
summer
winter
high light
low light
non-specific light-harvesting by C. fragile fronds
specific light-harvesting by the high LHCP content of the PSU
siphonoxanthin
siphonein (540 nm)
chlorophyll b
(654 nm)
P/S
CHL1
reaction-centre chlorophyll a
CHL1
P/S
P/S saturates at low light intensities
(ISC)
P/R
CHL3
? car3
PHOTOPROTECTION
PHOTOOXIDATION
free radical scavengers
ascorbate/glutathione
α-tocopherol
non-specific peroxidases
SOD
high pigment turnover
carotenoid quenching
photorespiration
glycolate excretion ?
FLUORESCENT LIGHT

β-carotene may be so rapidly metabolized as to be undetected in fronds or chloroplasts. The 'presence' of this pigment can only be inferred from the biosynthetic origins of xanthophylls which contain the β-Ionone ring. Although fronds exposed to high non-fluorescent light exhibit net pigment stability some pigment breakdown is evidenced. However, as this is not accompanied by the release of photooxidation products (e.g. MDA, ethane and ethylene) other photoprotective mechanisms may be operational in the alga. Superoxide dismutase has been isolated from C. fragile chloroplasts (Lumsden and Hall, 1975) and this, together with free radical scavengers such as ascorbate, glutathione and α-tocopherol, may be important in the photoprotection of the alga (Figure 55). When C. fragile fronds were exposed to high non-fluorescent and fluorescent light glycolate excretion occurred. As this process is associated with photorespiratory activity in aquatic plants (Samuel, Shah and Fogg, 1971) this may be another means of photoprotection in C. fragile. Fronds and chloroplasts isolated from C. fragile were photostable if exposed to light regimes approximating those of the intertidal zone. However, if exposed to relatively low intensity fluorescent light, photooxidation as ethane and ethylene evolution and pigment breakdown was apparent after only $4\frac{1}{2}$ hours exposure. Although these findings primarily demonstrated that C. fragile was sensitive to 'environmentally' abnormal fluorescent light they also provided a means whereby the mechanism of photooxidation and indeed photoprotection in the alga could be studied further. Photo-oxidation in C. fragile was evidenced by the lipid oxidation products ethane, ethylene, propane and butane, but unlike the majority of other plant systems investigated (Elstner, 1982) ethylene was the primary breakdown product and MDA was not detected. Furthermore, the chloro-phyll pigments were more susceptible to photooxidation than the carotenoids. These findings suggest that photooxidation and photo-protection in C. fragile may be considerably different to higher plants. The use of fluorescent lighting may explain in part these atypical results. The physiologically abnormal RED:FAR RED ratios of this lighting may impair pigment synthesis (especially chlorophyll) and enhanced emission of red light may excessively excite reaction centre

chlorophyll a. However, the quenching of reaction centre chlorophyll does not appear to take place as α-carotene is extremely stable throughout the entire exposure period of 72 hours. Indeed, the carotenoid pigments of C. fragile are stable in fronds exposed to both fluorescent and non-fluorescent light regimes. This suggests that the carotenoid pigments of this alga do not function in photoprotective capacity, but are themselves photoprotected. In higher plants the major means of dissipating excessive excitation energy in reaction centres is by the following transfer:-

$$^{T}\text{Chlorophyll} + \beta\text{-carotene} \longrightarrow {}^{T}\beta\text{-carotene} + \text{Chlorophyll}$$

The absence of β-carotene in C. fragile together with the stability of α-carotene may suggest that the functions of the α-isomer are dissimilar to those of the β-isomer. As a consequence carotenoid protective mechanisms in C. fragile may be different to those described for other plants. Because of these findings an indepth study of carotenoid quenching mechanisms in C. fragile may be especially important in elucidating further the relative roles of α- and β-carotenes in both C. fragile and higher plant chloroplasts, particularly as the absence of β-carotene has not been reported in any other photosynthetic eukaryotic or prokaryotic plant (Anderson et al, 1981). Although carotenoid quenching in fluorescent-light induced photooxidation does not appear to occur, the apparent stability of chloroplasts and fronds exposed to non-fluorescent light does suggest that photoprotective mechanisms are highly efficient in C. fragile chloroplasts. Thus, although the PSU of C. fragile is adapted to reduced light intensities at low tide, the alga is also able to prevent photooxidative damage for the duration of exposure to high light intensities at high tide. Unfortunately the effect of fluorescent light on summer-sampled fronds was not investigated and this is a suggested area for further research. The relative importance of fluorescent light induced photooxidation in both reproductive and vegetative tissue may be particularly important in determining the relative stability of C. fragile to summer and winter light intensities. As the pigment content of reproductive C. fragile fronds was 'stable' as demonstrated by the constancy of pigment ratios when exposed to high

light intensities, mechanisms may still be operational in protecting the reproductive alga against increased mid-summer light intensities. Thus, other factors such as nutrient status may be more significant in promoting the bleaching of summer-sampled fronds. Indeed, this summer-frond bleaching may be an important adaptation by which the alga reduces the number of its PSU's. The distribution of pigments still remains the same as reflected by seasonally stable chlorophyll a:b ratios and % distributions of carotenoid thus, entire PSU's may be lost rather than LHCPs only. The net effect is to reduce the absorption of light energy at times when the excessive excitation of the PSU may occur. However, the alga is still able to retain an efficient light-harvesting mechanism for when the light field is reduced at high tide. In conclusion, it therefore appears that C. fragile is able to survive the wide fluctuations in light intensities which occur in the intertidal zone by virtue of a number of complex adaptations which are operational at the whole plant and cellular level.

Although the survival of C. fragile in the intertidal zone is due to a wide range of interacting adaptations, an inspection of the order Siphonales reveals a number of characteristics of these algae which are independent of habitat. Chlorophyll a:b ratios are typically low in the Siphonales (Keast and Grant, 1976) and the presence of siphonein and siphonoxanthin in all the eusiphonean orders appears to be of taxonomic significance. In contrast the distribution of these pigments in the Ulvales, Cladaphorales and Siphonocladales is confined to species growing in deep or shaded coastal waters (Yokohama, 1981). This thesis describes an investigation into the environmental adaptations of a member of the Siphonales, C. fragile, and as the above account suggests these adaptations must also be considered in terms of their taxonomic and evolutionary significance. Many siphonaceous algae show similar characteristics to C. fragile despite their occurence in different habitats and an evolutionary pattern may be of particular importance in understanding further the adaptations of C. fragile. Yokohama (1981) suggests that members of the Siphonales which possess siphonein and siphonoxanthin may have originated in deep waters and

their descendents, which grow in habitats where the light field is considerably greater, still retain these pigments as evolutionary relics. Bjorkman (1981) also suggests that many plant characteristics reflect the previous evolutionary history of the species and may not be an adaptive response to current environmental conditions. Furthermore, an adaptation may be directly induced by the environment but only within the constraints determined by the genotype. If this premise is correct low chlorophyll a:b ratios and the presence of siphonoxanthin and siphonein in C. fragile may reflect the low light intensity adaptations of this alga's evolutionary ancestors. Adaptations to high light intensities are probably derived from a more recent evolutionary history which is also conferred on other members of the Siphonales. Adaptability is probably the most important factor controlling the evolution and survival of different species. Thus, in addition to the species specific adaptations described in C. fragile, one must also include the evolutionary legacy of the Siphonales which applies little constraint to the growth and survival of its descendents in many different and variable habitats.

Investigations of light adaptations in marine alga span a considerable number of years. Englemann (1884) first proposed the theory of complementary chromatic adaptation which for sometime provided the main hypothesis. To date Ramus (1981) and Dring (1981) have challenged the suggestion that light quality is the most important factor influencing algal distribution and propose that light-intensity is largely responsible for the light adaptations in marine algae. Throughout this three year study it has become clearly apparent that there is no unifying theory as to how marine algae adapt to their marine light environment. Perhaps this truly reflects the range and diversity of both algae and their adaptations and more importantly that a number of parameters (e.g. both light quality and intensity) contribute to a major physiological adaptation. Indeed, one of the major criticisms of the many research approaches used to study environmental adaptations is that investigations usually incorporate only one or two levels of examination, i.e. the environmental, whole plant or cellular level. Because of the limitations in this type of approach it has been the aim of this thesis to examine and consider light adaptations in

C. fragile at all levels. Thus, although the chloroplastic studies of this alga provided a central theme the perspectives of whole plant and environmental investigation were also incorporated. It is therefore, the opinion of the author that any further study of adaptations in the intertidal alga *C. fragile*, and indeed other algal species, must proceed at both environmental and physiological levels. Only by using this unified approach will a full understanding of environmental light adaptations in marine algae be achieved.

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APPENDIX I

CAROTENOID STRUCTURE AND NOMENCLATURE

I. β-carotene

β, β -carotene

II. α-carotene

β, ε -carotene

III. ε-carotene

ε, ε -carotene

IV. Violaxanthin

5, 6, 5'6' - Diepoxy - 5, 6, 5'6' tetra hydro β - β-carotene - 3, 3' diol.

V. Neoxanthin

5', 6',-epoxy 6-7, dihydro, 5,6, tetrahydro β, β-carotene, 3, 4, 3' triol.

VI. Lutein

β- ε-carotene-3-3', diol.

VII. Zeaxanthin

β, β,-carotene 3,3' diol.

VIII. Siphonoxanthin

3,19,3' trihydroxy-7-8-dihydro, β, ε -carotene-8-one

* Esterification = Siphonein (IX)

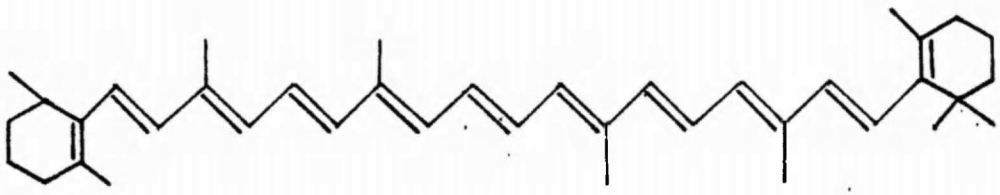
X. Fucoxanthin

5,6-Epoxy-3,3',5'-trihydroxy,6',7' dihydrog,5,6,7,8 5' hexahydro, β, β, careten-8-one 3' acetate.

XI. Antheraxanthin

5,6,epoxy, 5,6,dihydro- β, β -carotene-3,3'-diol.

I



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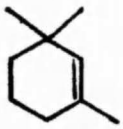

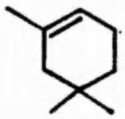

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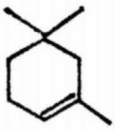

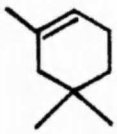

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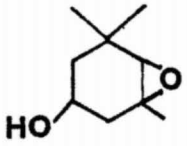

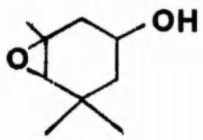

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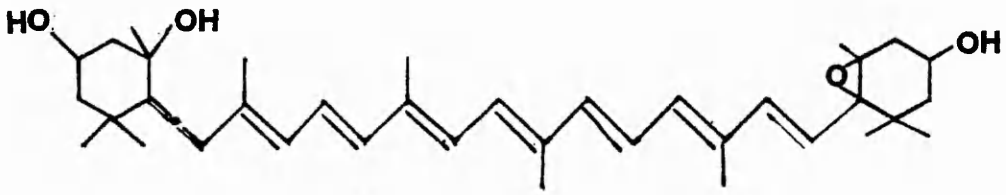
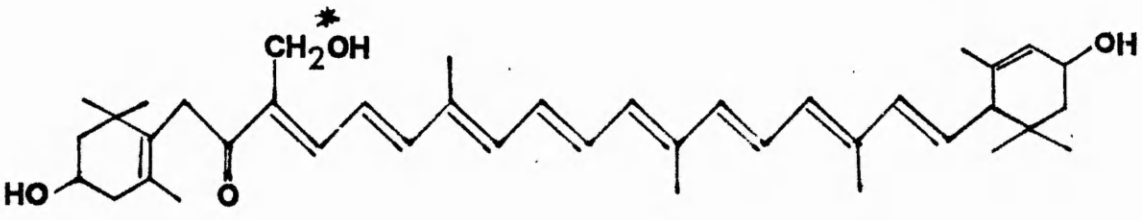


VI

VII

VIII



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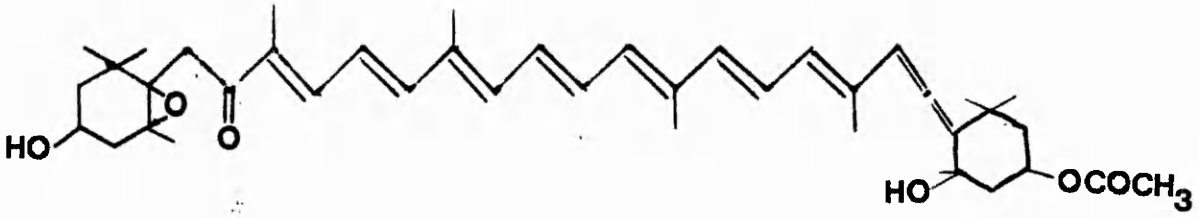
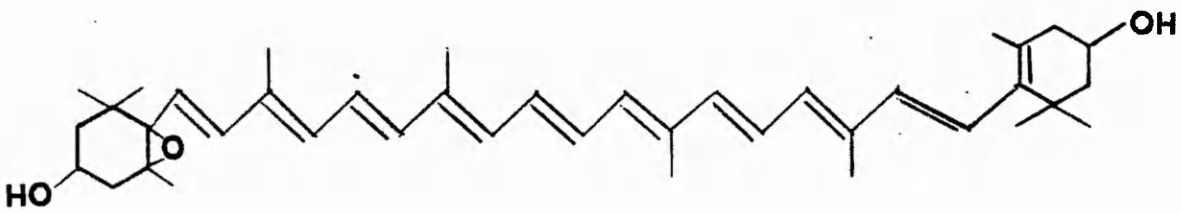


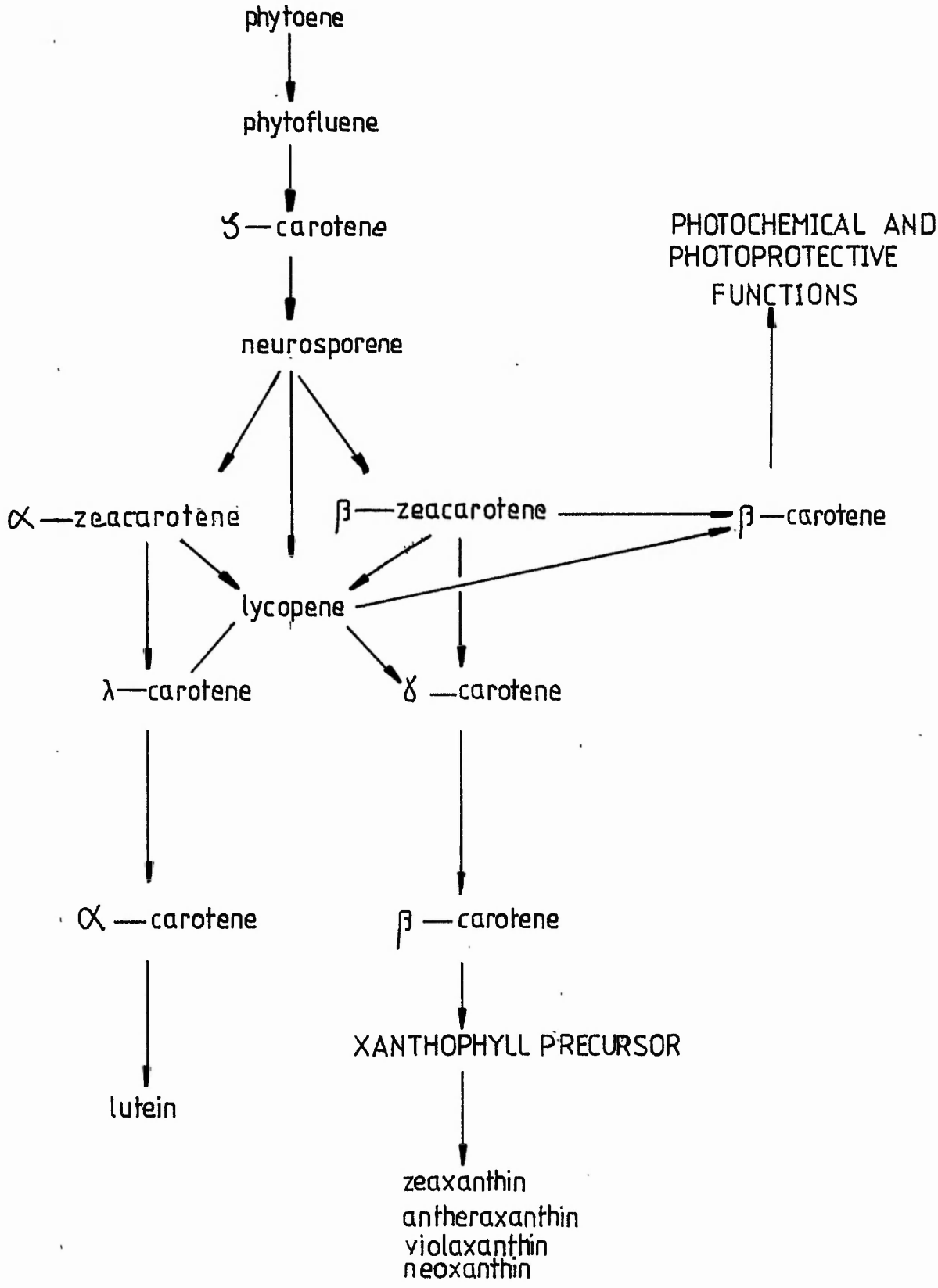
XI



APPENDIX II

CAROTENOID BIOSYNTHESIS

(Adapted from Grumbach, 1979)



APPENDIX III

STATISTICAL ANALYSIS OF DATA FROM SECTION 6

Using the University of Pittsburg SPSS-10 statistical programme, data from the experiment in section 6 was investigated using an analysis of variance (ANOVA). The statistics used in this analysis comprised the absolute pigment concentrations ($\mu g.g^{-1}$ Fresh wt.) shown in Figures 38 and 39 standardised with respect to the control, i.e.

($\mu g.g^{-1}$ pigment @, T=x, L.I=y)-($\mu g.g^{-1}$ pigment @ T=0, L.I=0)

where T = time of exposure (hrs), and L.I = light intensity ($\mu E.M^{-2}S^{-1}$).

An initial 4-way ANOVA was performed on the data with growth phase, time of exposure, light intensity and pigment concentration as the four variables. This analysis showed that the greatest source of variation in the data is derived from the growth phase and pigment concentration, Table I.

Table I. 4-WAY ANALYSIS OF VARIANCE

SOURCE OF VARIATION	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	F	SIGNIFICANCE OF F
MAIN EFFECTS	8908906	14	636350	95.37	0 *
GROWTH PHASE	1248756	1	1248756	187.1	0 *
TIME OF EXPOSURE	77761	6	12960	1.94	0.073
LIGHT INTENSITY	17597	4	4399	0.65	0.621
** PIGMENT CONTENT	751948	3	2506496	375	0 *
EXPLAINED	13660946	81	168653	25.2	0 *
RESIDUAL	2942411	441	6672		
*** TOTAL	16603357	522	31807		

* Significant sources of variation, p = < 0.001.

** Due to lack of work space on the program used, pigment content was represented as pigment pairs, i.e. Chlorophyll a + b, α-carotene + ε-carotene, siphonoxanthin + siphonein, and neoxanthin + violaxanthin.

*** Interactions are omitted from this table since the use of pigment pairing and large differences between growth phases would question the validity of any 'biological' interpretation.

Table II. 3-WAY ANOVA FOR VEGETATIVE TISSUE

(A)

SOURCE OF VARIATION	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	F VALUE	SIGNIFI-CANCE OF F
MAIN EFFECTS	325773	13	25059	13.7	0 *
TIME	22759	6	3793	2.08	0.056
LIGHT	151512	4	37878	20.8	0 *
PIGMENT PAIRS	154746	3	51582	28.3	0 *
RESIDUAL	347052	191	1817		
TOTAL	1270602	255	4982		

(B) 3-WAY ANOVA FOR REPRODUCTIVE TISSUE

SOURCE OF VARIATION	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	F VALUE	SIGNIFI-CANCE OF F
MAIN EFFECTS	403290	13	31022	387	0 *
EXPOSURE TIME	157172	6	26195	327	0 *
LIGHT INTENSITY	280564	4	70141	875	0 *
PIGMENT PAIRS	18643	3	6214	77	0 *
RESIDUAL	15955	199	80		
TOTAL	2827261	266	10628		

* Significant sources of variation, $p = < 0.001$.

Table III. 2-WAY ANOVA FOR α-CAROTENE

b. VEGETATIVE TISSUE

SOURCE OF VARIATION	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	F	SIGNIFICANCE OF F
MAIN EFFECT	66.3	10	6.6	0.83	0.600
EXPOSURE TIME	28.8	6	4.8	0.606	0.723
LIGHT INTENSITY	37.0	4	9.2	1.16	0.353
EXPLAINED	66.3	10	6.6	0.83	0.600
RESIDUAL	166.3	21	7.9		
TOTAL	232.6	31	7.5		

a. REPRODUCTIVE TISSUE

SOURCE OF VARIATION	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	F	SIGNIFICANCE OF F
MAIN EFFECT	101	10	10.1	6.4	0 *
EXPOSURE TIME	17.1	6	2.8	1.8	0.139
LIGHT INTENSITY	78.5	4	19.6	12.5	0 *
EXPLAINED	101	10	10.1	6.4	0 *
RESIDUAL	34.4	22	1.5		
TOTAL	136	32	4.2		

* Significant sources of variation, $p = < 0.001$.

An interaction analysis was not included because a reduction in the number of data points used in the 2-way ANOVAS is statistically limiting.

In the 4-way ANOVA (Table I), growth phase contributes the greatest source of variability, reflecting the large differences in pigment content between vegetative and reproductive tissue as previously described in Section 4. In order to eliminate the affects of growth phase on the ANOVA, a 3-way analysis of variance was performed using pigment pairs, and light intensity and exposure time as variables for the separate investigation of vegetative and reproductive data. Table II shows all sources of variation to be significant for this data, with the exception of exposure time for vegetative tissue. However a high degree of similarity between the two growth phases is disputed on examination of the mean square values in the above Table. These mean square values (M.S.) indicate the variance of the parameters under investigation, thus a comparison of these values may show differences between reproductive and vegetative tissue. Examination of the M.S. values for pigment pairs shows a higher value for vegetative tissue. This is a reflection of the higher pigment concentration in the vegetative growth phase. However, of particular importance is the differences in M.S. values for exposure time and light intensity for the different growth phases. The finding that these values are higher in the reproductive tissue suggests that the response of this tissue (as changes in pigment concentration) to exposure time and light intensity is more erratic than observed in the vegetative tissue. Furthermore light intensity and exposure time both appear to be important in the variability of pigment content in the reproductive tissue, but light intensity only, is significant in the vegetative phase.

The results of these analyses confirm the laboratory observation that pigment content varies considerably between reproductive and vegetative tissue. Differences between pigment pairs contribute considerably to the sources of variation in these analyses. Since this variability is accounted for (i.e. differences between chlorophyll and carotenoid concentrations are expected), together with differences between growth phases the data was reanalysed without

growth phase and pigment content as variables. Thus a 2-way ANOVA was performed for each individual pigment in separate growth phases, with exposure time and light intensity as the two variables under examination. Table III shows a worked example of a 2-way ANOVA for α-carotene in the reproductive and vegetative growth phases. Table IV shows the results of 2-way ANOVAs performed on all the carotenoid and chlorophyll pigments in C. fragile. Exposure time did not have a significant affect on individual pigment content in reproductive or vegetative tissue, thus Table IV only shows the affects of light intensity.

Table IV. SIGNIFICANT LIGHT EFFECTS ON INDIVIDUAL PIGMENTS IN REPRODUCTIVE AND VEGETATIVE TISSUE FROM C. FRAGILE
(Using a 2-way Analysis of Variance)

PIGMENT	GROWTH PHASE	
	REPRODUCTIVE	VEGETATIVE
α-Carotene	√ *	
ε-Carotene	√	√
Siphonoxanthin	**	
Siphonein	√	
Violaxanthin	√	
Neoxanthin	√	
Chlorophyll a	√	√
Chlorophyll b	√	√

*√ Significant F. value, $p = < 0.001$.

** A missing value for siphonoxanthin disqualified this pigment for 2-way ANOVA.

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THE SEPARATION, IDENTIFICATION AND QUANTITATIVE DETERMINATION OF PHOTOPIGMENTS FROM THE SIPHONACEOUS MARINE ALGA *CODIUM FRAGILE*

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(*Accepted 15 December 1980*)

SUMMARY

The photopigments of the Siphonaceous marine alga *Codium fragile* have been quantitatively determined using thin layer chromatography (TLC). *Codium fragile* fronds contain a unique spectrum of carotenoids, notable by the absence of β-carotene, and the presence of α- and ϵ-carotenes, siphonoxanthin, siphonein, neoxanthin and violaxanthin. The carotenes are present in low concentrations whereas siphonoxanthin and its ester siphonein account for as much as 60% of the total carotenoids present. The significance of these findings is discussed in relation to the habitat of this alga.

INTRODUCTION

Members of the order Siphonales show peculiar pigment characteristics when compared to other algal groups. The presence of the xanthophyll siphonoxanthin and its ester siphonein is indicative of this family as is the relative abundance of α-carotene compared to β-carotene (Strain, 1965; Goodwin, 1971). ϵ-Carotene has also been identified in *Bryopsis corticulans* (Strain, 1951), although its presence in other Siphonales has not been investigated. Indeed, a paucity of information exists regarding the quantitative determination of pigments throughout the Siphonales. Furthermore the functions of these pigments are largely unknown. The aim of this report is to determine quantitatively the pigments of the siphonaceous marine alga *Codium fragile*.

MATERIALS AND METHODS

Collection and maintenance of plant material

Young (up to five dichotomies) and old (up to eight dichotomies) specimens of *C. fragile* were collected from intertidal rock pools at Bembridge, Isle of Wight in November 1979. The alga was maintained in aerated sea water at approximately 10 °C with a light intensity of 10 W m^{-2} at the water surface.

Pigment extraction

Fronds. C. fragile pigments were extracted according to the method of Jeffrey (1968). Samples of 50 g were frozen at minus 20 °C for 30 min and dehydrated in methanol for 2 min. Diced fronds were macerated with 100% acetone in a pestle and mortar and 1 to 2 g of Na_2CO_3 (anhydrous) added to neutralize excess aciditity. The macerate was filtered and re-extracted two or three times until colourless. The

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filtrate was then transferred to diethyl ether (AnalaR) in a separatory funnel and washed with NaCl (20 to 40% aq.) until all traces of acetone were removed from the ether layer. Further concentration to exactly 5 ml was achieved by the passage of a stream of nitrogen over the ether extract. Care was taken to ensure a rapid extraction and to prevent excessive exposure to both heat and light. Pigment extracts were stored in the dark under nitrogen at 0 °C.

Chloroplasts. Chloroplasts were isolated from 120 g of fronds as previously described (Cobb, 1977), with 0·05 M Hepes, pH 7·8, as buffer. This method involved the purification of a crude chloroplast pellet through a loosely packed column of Sephadex G50 coarse. Post-column chloroplasts were further purified by centrifugation (4 min at 100 *g*), and resuspended in extraction medium for the determination of chloroplast number using a haemocytometer. A chloroplast suspension of known number was then extracted in 100% acetone as described above.

Separation of carotenoids by thin layer chromatography

Carotenes. Glass plates (7 × 2·5 cm) were coated with a slurry of MgO (light) and anhydrous $CaSO_4$ (ratio 1:4) and dried overnight at room temperature to prevent cracking. Concentrated diethyl ether extract (20 μl) was applied to the origin and the plates developed in 4% (v/v) *n*-propanol in petroleum ether (b.r 60 to 80 °C) for 3 to 5 min in total darkness.

Xanthophylls. Twenty microlitres of concentrated diethyl ether extract was applied to the origin of plastic strips (7 × 2·5 cm) pre-coated with neutral silica gel G60 of 0·2 mm thickness (Merck), and developed in 6% (v/v) acetone in diethyl ether for 2 to 3 min in total darkness. For the further separation of neoxanthin from siphonoxanthin the sample was developed on larger strips (12 × 3 cm) for 1 h in the same solvent system. All the solvents used were of AnalaR grade.

Pigment elution, identification and determination

Carotenes and adsorbant were scraped from the plates and rapidly eluted into 2 ml of 100% acetone with the aid of a vortex mixer, and transferred to 3 ml of petroleum ether (b.r. 60 to 80 °C) by washing with 2 ml of 20 to 40% aq. NaCl. Xanthophylls were directly eluted from the silica gel plates into 3 ml of absolute ethanol. Centrifugation at 700 *g* for 6 min removed the adsorbant from the solvent. Absorption spectra were accurately obtained using a Perkin Elmer 550S scanning spectrophotometer over a range of 350 to 550 nm. Pigments were identified by their relative positions on the TLC plates and by their characteristic absorption spectra in various solvents. Quantitative determination of individual pigments was achieved by the use of specific extinction coefficients ($E^{1\%}_{1cm}$), as described by Britton and Goodwin (1971).

Chlorophylls *a* and *b* were routinely determined by the methods of MacKinney (1941). For analytical work between five and eight, 50 g samples of *C. fragile* were used, and four or five separate TLC determinations were performed, for each pigment.

Criteria used for the identification and quantitative determination of pigments from Codium fragile. (*Modified from Davies, 1976*)

Pigment	R_f* value	Solvent	Absorption maxima (nm)	$E^{1\%}_{1cm}$	
α-Carotene	0·47	Petroleum ether	422,444,473	2800	(Schwieter *et al.*, 1965)
ε-Carotene	0·70	Petroleum ether	418,440,470	3120	(Schwieter *et al.*, 1965)
'Unknown' carotene	0·15	Petroleum ether	418,438,466	—	
Siphonoxanthin	0·50	Ethanol	447	1160†	(Ricketts, 1971)
Neoxanthin	0·57	Ethanol	415,438,467	2243	(Cholnoky *et al.*, 1966)
Violaxanthin	0·30	Ethanol	417,441,469	2550	(Karrer and Jucker, 1943)
Siphonein	0·40	Ethanol	450	2500‡	(Ricketts, 1971)

* Determined as described above.

† This value is probably low due to occluded solvent (Ricketts, 1971).

‡ This value is likely to be higher than the true extinction, since many ketonic xanthophylls have extinction coefficients which are less than 2500 (Ricketts, 1971).

Using the Perkin Elmer 550S Spectrophotometer, pigment concentrations of 0·5 μg pigment g^{-1} fresh wt could be accurately determined.

Results

Table 1 shows the pigment content in intact fronds of *C. fragile* of different age. Carotenoid pigments present were α and ε-carotene, siphonoxanthin, siphonein, neoxanthin and violaxanthin. An unidentified pigment present in low concentrations was also separated on the $MgO/CaSO_4$ plates. The spectral characteristics and behaviour of this pigment on TLC suggest that it may be a non-cyclic carotene precursor. Pigment concentrations are lower in older tissue, although ratios are consistently similar. Low chlorophyll *a*:*b* ratios support previous data with isolated chloroplasts (Cobb and Rott, 1978).

Table 2 compares the distribution of *C. fragile* pigments in intact fronds to that in isolated chloroplasts and clearly indicates a chloroplastic location.

Table 1. *Pigment content (μg g^{-1} fresh wt) of whole fronds of* Codium fragile

Pigment	Frond size			
	Up to 5 dichotomies μg g^{-1} ± s.d.		Up to 8 dichotomies μg g^{-1} ± s.d.	
α-Carotene	6·4	1·0	4·0	0·92
ε-Carotene	0·76	0·34	0·5	0·06
Siphonoxanthin	28·3	5·6	16·2	3·4
Siphonein	11·7	1·5	6·0	1·2
Violaxanthin	4·9	0·9	3·6	0·5
Neoxanthin	14·5	3·3	7·4	3·0
Total carotenoids	66·0	10·0	38·6	6·8
Chlorophyll *a*	180	30	91	15·8
Chlorophyll *b*	123	21	57	23·8
Total chlorophyll	306	51	129	15·8
Ratio of total chlorophyll: carotenoid	4·9	1·5	5·0	2·3
Ratio of chlorophyll *a*:*b*	1·5	0·13	1·5	0·28

Table 2. *Percentage distribution of carotenoid pigments in isolated chloroplast and whole frond extracts*

			Fronds			
	Isolated chloroplasts		Up to 5 dichotomies		Up to 8 dichotomies	
Pigment	%	±s.d.	%	±s.d.	%	±s.d.
α-Carotene	12·8	2·9	10	3·2	10·6	4·3
ε-Carotene	1·5	0·014	1·2	0·5	1·2	0·2
Siphonoxanthin	37·6	3·6	43	3·0	42	2·6
Siphonein	19·6	3·5	16·5	1·5	15·6	1·3
Violaxanthin	8·6	3·4	7·6	0·5	9·4	0·8
Neoxanthin	20·6	4·5	22	1·9	21	3·5

The isolated chloroplast data is derived from seven 120 g extractions of *C. fragile* fronds, where the mean total carotenoid concentration was $22{\cdot}7 \pm 14{\cdot}3 \times 10^8$ μg per chloroplast.

DISCUSSION

C. fragile fronds contain a unique spectrum of carotenoids notable by the absence of β-carotene and the presence of α- and ε-carotenes, siphonoxanthin, siphonein, neoxanthin and violaxanthin (Table 1). The pigments are chloroplastic in location (Table 2), and are present in high concentrations relative to chlorophyll (Table 1). The carotenes constitute approximately 10% of the total carotenoids present whereas the xanthophyll siphonoxanthin and its ester siphonein comprise approximately 60%. Although β-carotene was reported in *C. fragile* (Trench and Smith, 1970; Trench, Boyle and Smith, 1973; Trench, 1975), it has not been detected in this study, even in extracts concentrated from 200 g of fronds. Several TLC techniques (Britton and Goodwin, 1971; Davies, 1976) were employed to test this observation, and cochromatography using pure β-carotene (Sigma) and β-carotene extracted from *Zea mays*, consistently proved its absence. Thus, we conclude that β-carotene is either totally absent in this species, or is so rapidly metabolized as to be undetected. To suggest the total absence of β-carotene in *C. fragile* does, however, pose a problem, since the alga is able to synthesize xanthophylls which contain the β-ionone ring, e.g. siphonoxanthin and siphonein. The inability to detect β-carotene in this study may be of considerable interest regarding carotenoid synthesis in the algae in general, and in particular the biosynthetic origin of the β-ionone rings in α-carotene and the xanthophyll derivatives.

This study also demonstrates the presence of ε-carotene, a carotene isomer not previously detected in *C. fragile* but present in *Bryopsis corticulans* (Strain, 1951). This observation supports the suggestion of Chapman and Haxo (1963) that species which show a preference for the synthesis of α-carotene compared to β-carotene also produce the ε-isomer, and may therefore show similar biosynthetic pathways.

The roles of carotenoid pigments found in *C. fragile* are currently undefined, although siphonoxanthin has been shown to transfer excitation energy to chlorophyll *a* in *Ulva japonica*, inferring a light harvesting function (Kageyama *et al.*, 1977). Carotenoids in higher plants are thought to function as accessory agents in

photosynthesis (e.g. Cogdell, 1978), colouring agents, and as agents protective against potentially harmful radiation (Krinsky, 1971, 1978). Data shown in Table 2 show that the pigments of *C. fragile* are chloroplastic in location and hence may function in chloroplast metabolism. Furthermore, the relative abundance of siphonoxanthin and siphonein suggests some specific function(s) currently unknown in this alga.

C. fragile, as a result of its intertidal zone habitat, is subjected to wide daily and seasonal variations in light quality and quantity. The pigment spectrum outlined above may therefore be adapted for optimum light harvesting in this environment.

Similarly, whereas photosynthesis in isolated chloroplasts of this species is adapted to submarine light intensities of approximately 25 W m^{-2} (Cobb and Rott, 1978), the fronds are regularly exposed to at least 10 times this intensity at low tides. Indeed, during the summer months, fronds *in situ* show considerable bleaching indicative of photoxidation. Hence the possession of a unique range of pigments may provide the alga with some ability to accommodate extreme fluctuations in light energy.

It is of interest to note that the taxonomic separation of the Siphonales from the rest of the green algae is largely influenced by their carotenoid pigments, namely the presence of siphonoxanthin and siphonein, and the relative abundance of α- compared to β-carotene, (Goodwin, 1971). However, siphonoxanthin has been reported in members of the Prasinophyceae (Ricketts, 1971), and more recently in *Ulva japonica*, *Ulva pertusa*, *Cladophora wrightiana*, *Valonia macrophysa* (Yokohama *et al.*, 1977), and *Microthamnion kuetzingianum* (Weber and Czygan, 1972). A review of the literature indicates, therefore, that siphonein may be more specific to the Siphonales.

Keast and Grant (1976) report that members of the Siphonales have chlorophyll *a*:*b* ratios considerably below those found in other species, (supported in Table 1 of this study), and suggest that the ratio observed may be a genus characteristic and need not be the result of an adaptation to growth under particular environmental conditions. The problem arises, however, that whilst the Siphonales possess a unique complement of pigments, the individual members of the group show wide diversities in their morphology and type of habitat. Thus, as well as suggesting that the production of unique pigments in *C. fragile* is purely an adaptation to a specific type of environment, one must also consider the taxonomic and evolutionary significance of these pigments in relation to the Siphonales and the rest of the Chlorophyta.

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Photosynthesis VI. Photosynthesis and Productivity, Photosynthesis and Environment
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PHOTOSYNTHETIC ADAPTATIONS OF THE INTERTIDAL ZONE MARINE ALGA CODIUM FRAGILE TO LIGHT

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ABSTRACT

The siphonaceous marine alga Codium fragile exhibits several photosynthetic adaptations to its intertidal zone habitat, and appears able to tolerate a wide range of light intensities. This species possesses a unique carotenoid spectrum that may impart some photoprotective function. The possible role of these carotenoids as an adaptive mechanism to fluctuating light intensities is discussed.

INTRODUCTION

Chloroplast function throughout the plant kingdom is physiologically adapted to ensure that photosynthesis may efficiently function in a variety of environmental conditions. One example is the siphonaceous marine alga Codium fragile, which exhibits several photosynthetic adaptations to its intertidal zone habitat. In this species photosynthesis appears to: (1) follow a tidal rhythm (Cobb, unpublished observations), (2) saturate at low light intensities (approximately 25 $W.m^{-2}$. Cobb and Rott, 1978), (3) require high osmotic support (Cobb and Rott, 1978), and, (4) is aided by an internal supply of inorganic phosphate in the form of long chained polyphosphate, to overcome the low levels of inorganic phosphate in sea water, (Cobb, 1978).

Although this alga is adapted to low light saturation for submarine photosynthesis, the fronds are regularly exposed to the atmosphere and relatively high light intensities at low tide. Furthermore, the observation that ageing fronds of C. fragile, exposed at low tide become bleached by mid-summer light intensities suggests that this marine alga is susceptible to photooxidation. Indeed, the photochemical disruption of C. fragile chloroplasts appears to be the major cause of chloroplast breakdown when in symbiosis with the saccoglossan mollusc Elysia viridis,

(Hawes and Cobb, 1980; for a general review see Trench, 1975). However, since this process may take more than 14 days in constant light to become apparent (Hawes and Cobb, 1980), C. fragile chloroplasts may be less susceptible to photooxidation than their higher plant counterparts. As saturating light intensities for photosynthesis in this species are only 1-10% of the intensities endured by the exposed fronds at low tide, protective mechanisms must be operational to counteract the lethal effects of free-radical attack, as described by Halliwell, 1978.

Carotenoids are thought to play a unique role as photoprotectants and can function in at least three ways involving (1), the quenching of the chlorophyll triplet state, (2), inactivating singlet oxygen and (3), serving as an oxidisable substrate to protect other molecules and processes from photodestruction (Krinsky, 1971 and 1978). This poster-paper attempts to relate the changes in carotenoid content of C. fragile fronds to the wide variation of light intensities encountered by this alga in its intertidal zone habitat.

MATERIALS AND METHODS

Specimens of C. fragile were collected from intertidal rock pools at Bembridge, Isle of Wight, U.K. in November 1979 and June 1980, and maintained in aerated seawater at 10°C with a light intensity of 10 $W.m^{-2}$ at the water surface.

The techniques used to extract, separate and quantitatively determine the chloroplastic pigments of C. fragile fronds will be described elsewhere (Benson and Cobb, 1981). Pigment extraction was essentially as reported by Jeffrey, 1968, and separation achieved by T.L.C. Carotenes were separated using plates coated with MgO and $CaSO_4$ (ratio 1:4), with 4% (v/v) n-propanol in petroleum ether (b.r. 40-60°C) as developing solvent. Xanthophylls were separated using pre-coated silica gel G.60 (0.2mm thickness, Merck), and developed in 6% (v/v) acetone in diethyl ether. Carotenoids were then quantitatively determined by the method of Britton and Goodwin (1971), and chlorophylls determined according to Mackinney (1941). In this study between 5 and 8 50g samples of C. fragile fronds were used, and 4-5 separate T.L.C. determinations performed for each pigment, for each column of data in Table 1.

TABLE 1

PIGMENT CONTENT OF C. FRAGILE FRONDS (μg. g. fresh wt.$^{-1}$) OF DIFFERENT AGE AND PHYSIOLOGICAL STATE

DATE SAMPLED	November 1979		June 1980
FROND CONDITION	Vegetative		Reproductive
NO. DICHOTOMIES'	3 - 4	5 - 8	7 - 8
PIGMENT	PIGMENT CONTENT AS μg.g. fresh wt.$^{-1}$ ± S.D. (figures in parentheses represent % of total carotenoids)		
α-carotene	6.4 ± 1.0 (10.0 ± 3.2)	4.0 ± 0.9 (10.6 ± 4.3)	3.7 ± 0.9 (12.0 ± 2.5)
ε-carotene	0.8 ± 0.3 (1.2 ± 0.5)	0.5 ± 0.06 (1.2 ± 0.2)	Trace (< 1.0)
siphonoxanthin	28.3 ± 5.6 (43.0 ± 3.0)	16.2 ± 3.4 (42.0 ± 2.6)	10.6 ± 1.5 (34.6 ± 5.6)
siphonein	11.7 ± 1.5 (16.8 ± 1.5)	6.0 ± 1.2 (15.6 ± 1.3)	4.5 ± 0.8 (13.4 ± 1.1)
violaxanthin	4.9 ± 0.9 (7.6 ± 0.5)	3.6 ± 0.5 (9.4 ± 0.8)	1.9 ± 0.6 (6.6 ± 1.8)
neoxanthin	14.5 ± 3.3 (22.0 ± 1.9)	7.4 ± 3.0 (21.0 ± 3.5)	10.3 ± 2.5 (33.0 ± 3.7)
TOTAL	66.0 ± 10.0	38.6 ± 6.8	31.0 ± 4.7
chlorophyll a	180 ± 30	91 ± 16	65 ± 16
chlorophyll b	123 ± 21	57 ± 24	48 ± 12
TOTAL	306 ± 51	129 ± 16	116 ± 30
Ratio of total chlorophyll to carotenoid	4.9 ± 1.5	5.0 ± 2.3	3.7 ± 0.5

RESULTS AND DISCUSSION

Codium fragile fronds contain a unique spectrum of carotenoids, as shown in Table 1, and described in detail elsewhere (Benson and Cobb, 1981). β-carotene is absent in this species, although both α- and ε-carotenes are present. C. fragile xanthophylls are notable for the presence, and relative abundance, of siphonoxanthin and its ester siphonein, which together constitute up to 60% of the total carotenoids present. These pigments are chloroplastic in location (Benson and Cobb, 1981), and are present in relatively high concentrations with respect to chlorophyll, although their precise functions remain to be determined. Table 1 presents a quantitative pigment analysis of fronds at different developmental stages, harvested in November 1979 (young vegetative tissue) and June 1980, (mature reproductive tissue). Hence comparisons may be made of relative pigment composition at different growth stages. In the younger tissue (Table 1, columns 1 and 2), the percentage carotenoid distribution appears consistent irrespective of plant size, whereas the older reproductive fronds (Table 1, column 3) show a significant increase in neoxanthin and a slight decrease in siphonoxanthin when compared to younger fronds of the same size. Table 1 also shows that both chlorophyll and carotenoid content g.fresh wt.$^{-1}$ appear to decrease with increasing plant size.

Differences in the relative pigment concentration of fronds harvested in June 1980, compared to November 1979, may represent either a senescent response following a physiological change from vegetative to reproductive growth, or may be evidence for an increased relative level of "filtering" carotenoids to protect the photo-sensitive lamellae, as supported by a decreased chlorophyll to total carotenoid ratio (Table 1, column 3). Some increased photoprotection may be considered necessary since, during midsummer months some frond tip bleaching is apparent in situ, when incident light intensities are at least ten times the level needed to saturate photosynthesis in this species.

That carotenoids may be implicated in a photoprotective role in C. fragile may be inferred from short term exposures of fronds to high light intensities. Preliminary studies in this laboratory have shown that exposure to 2000μES.sec.$^{-1}$cm.$^{-2}$ (P.A.R.) causes rapid and large fluctuations in pigment concentrations within the frond, that return to their original values within a few hours of continuous exposure (Benson, unpublished observations). Experiments are now in progress to determine whether these carotenoid fluctuations correspond to chloroplast lipid

peroxidation at high light intensities.

Observations that both algae and higher plants may be able to adjust their pigment concentrations to accommodate variations in light intensities have been reported in the literature (Halldal 1970, and 1972), although not regarding the Siphonales. Continuing studies with C. fragile may elucidate further the roles of carotenoids in this intertidal zone alga.

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