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PHOTOINHIBITION IN *CODIUM FRAGILE*

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DECLARATION

The author has not been a registered candidate nor an enrolled student for another award of the CNAA or other academic or professional institution during this research programme. Material contained in this thesis has not been used in any other submission for an academic award and is entirely the author's indivdual contribution. The author has attended appropiate lectures, seminars and conferences in partial fulfilment of the requirements of the degree.

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(Candidate)

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(Director of Studies)

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To Julie

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PUBLICATIONS ARISING FROM THIS STUDY

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CONTENTS

ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
CF_o - CF_1	ATP synthase or coupling factor
Chl	chlorophyll
Cyt b559	cytochrome b559
Cyt b_6/f	cytochrome b_6/f complex
Cyt b_{6HP}	cytochrome b_6 (or b_{563}) - high potential form
Cyt b_{6LP}	cytochrome b_6 (or b_{563}) - low potential form
D1, D2	PSII reaction centre core polypeptides
DAD	diaminodurine
DBMIB	2, 5 - dibromo - 3 - methyl - 6 - isopropyl - p - benzoquinone (dibromothymoquinone)
DCMU	3 - (3, 4 - dichlorophenyl) - 1, 1 - dimethylurea
DCPIP	2, 6 - dichlorophenolindophenol
DMBQ	2, 5 - dimethyl - p - benzoquinone
DMSO	dimethyl sulphoxide
DPC	diphenyl carbazide
DPM	disintegrations per minute
DTT	dithiothreitol
$\Delta\mu H^+$	proton electrochemical potential (proton motive force - pmf)
ΔpH	transmembrane proton concentration gradient
$\Delta\Psi$	transmembrane electrical potential gradient
EDTA	ethylene diamine tetra acetic acid
E_m	midpoint redox potential at pH 7
FAD	flavine adenine dinucleotide (oxidised form)
FNR	ferredoxin - $NADP^+$ oxido - reductase
FWt	fresh weight
HEPES	N - 2 - hydroxyethylpiperazine - N^1 - 2 - ethane sulphonic acid
LHCII	light - harvesting chlorophyll a/b protein

Met	methionine
mRNA	messenger ribonucleic acid
MW	molecular weight
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NADH	nicotinamide adenine dinucleotide (reduced form)
OEC	oxygen evolving complex
P680	PSII reaction centre chlorophyll
P700	PSI reaction centre chlorophyll
Pha	primary phaeophytin acceptor of PSII
Phb	a second phaeophytin
PPFD	photosynthetic photon flux density
PQ	plastoquinone
PS	photosynthesis
PSI	photosystem I
PSII	photosystem II
Q_A	bound primary plastoquinone acceptor
Q_B	bound secondary plastoquinone acceptor
SDS - PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SiMo	silicomolybdate (SiO_2. $12MoO_3$)
TMPD	N, N, N^1, N^1 - tetramethyl - P - phenylenediamine
UV	ultra violet
Y_D	a second tyrosine
Y_z	tyrosine primary electron donor to P680

ABSTRACT

Photoinhibition in *Codium fragile*

R. V. Sealey

Initial polarographic studies with intact fronds and isolated chloroplasts revealed an essentially stable photosynthetic response throughout the yearly life cycle. A light - induced increase in the rate of dark oxygen uptake that was unrelated to photosynthetic photon flux density (PPFD) was observed, a possible explanation being increased rates of photorespiration/ glycolate production, Mehler reaction and/ or chlororespiration, processes which may aid in the prevention of photoinhibition. Photosynthesis saturated rapidly and at low PPFD indicating efficient light harvesting and photoinhibition was not observed in intact fronds even at supraoptimal PPFD. In contrast, photoinhibition of isolated chloroplasts rapidly occurred at supraoptimal PPFD. The higher the chlorophyll concentration of the chloroplast suspension the higher the PPFD required for maximum photosynthesis (*i.e.* saturation) and the less dramatic the reduction in photosynthesis (*i.e.* photoinhibition) once the optimum irradiance was surpassed. The differences in the photosynthetic response of the intact frond and isolated chloroplasts must be due to properties of the former not shared by the latter, which enable the prevention of photoinhibition. The 'mimicking' of the frond response at high chlorophyll concentration in the chloroplast study suggests that the number and arrangement of chloroplasts within the frond may confer a vital 'self - shading' mechanism in the avoidance of photoinhibition by this alga.

Photoinhibited thylakoids exhibited an initial loss of Q_B - dependent activity (DMBQ assay) followed by a loss of Q_B - independent activity (SiMo assay) whilst photoinhibition of isolated chloroplasts caused an initial rise in F_o and a preferential quenching of F_v. Fluorography provided preliminary evidence for a loss of ^{35}S - Met from D1 during 15 - 240 min photoinhibition of chloroplasts pre - radiolabelled *in organello*. These results may suggest that under the conditions of this study, photoinhibition of isolated *C. fragile* chloroplasts initially (0 -10 min) results in damage to Q_A and that only after this primary lesion has occurred does increased D1 turnover ensue.

The adaptations of *C. fragile* that enable the apparent paradox of efficient photosynthesis at the low PPFD experienced at high tide without accompanying photoinhibition at the supraoptimal PPFD experienced at low tide, in conjunction with other features of the growth strategy of this alga, may contribute to successful colonisation, despite the extreme environment, of the intertidal zone.

CHAPTER 1. INTRODUCTION

1.1 Photoinhibition

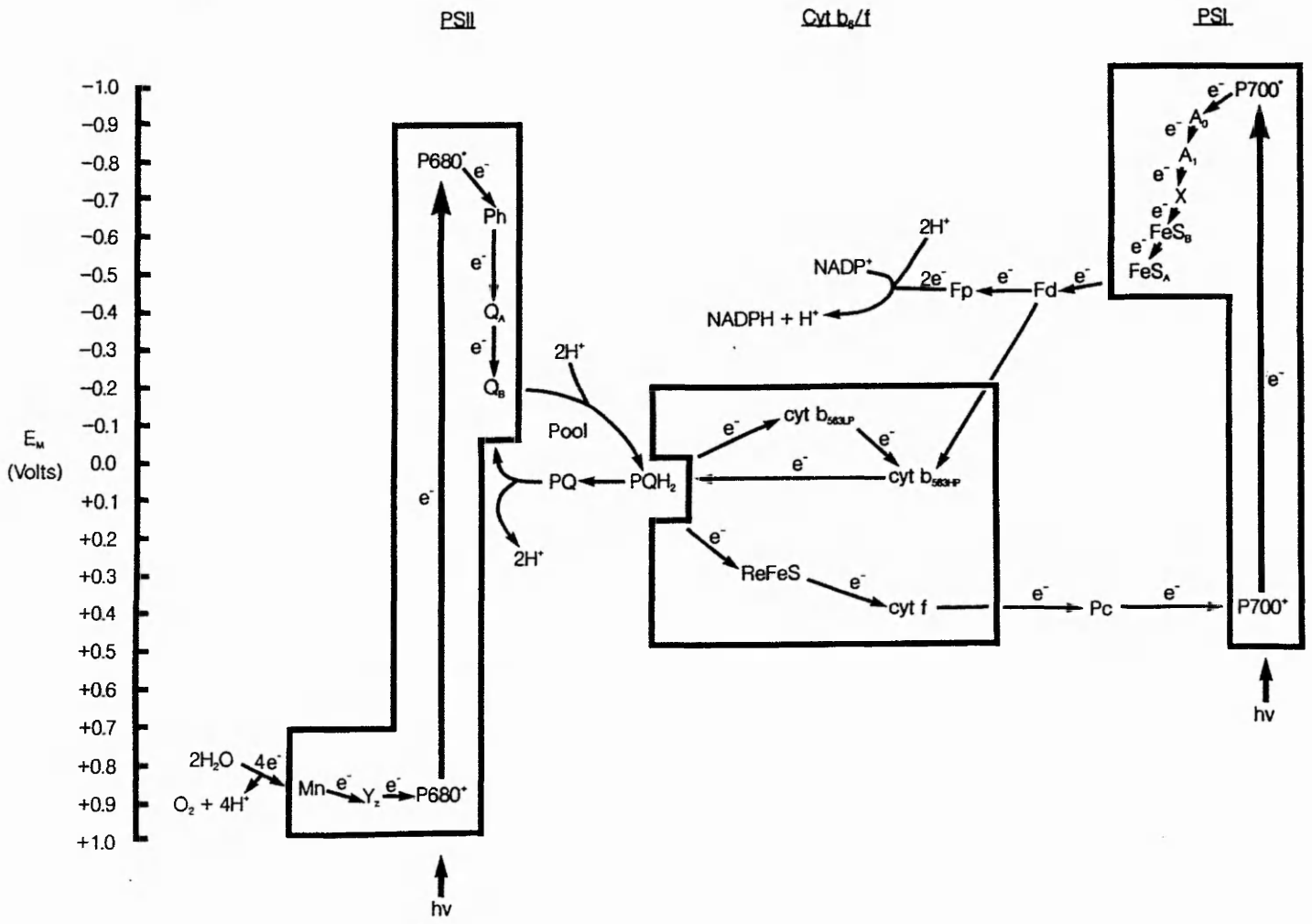
Plants grow in an ever changing light environment that can vary, even in the most open of habitats, from darkness to greater than 2000 μmol photons m^{-2} s^{-1} PPFD (photosynthetic photon flux density) on a daily, seasonal or annual basis. It is therefore not surprising that many mechanisms have evolved to enable adaptation to almost all global environments, allowing success in habitats ranging from arid sun - soaked deserts to the deeply shaded floors of dense tropical forests. It is evident that changes in the light environment have a considerable influence on the structure and function of the photosynthetic apparatus. For example, extreme forms of organisation in the photosynthetic apparatus of plants from deeply shaded and very bright habitats are apparent. There are many well established differences between such 'sun' and 'shade' plants including the following. Chloroplasts from 'sun' plants are: smaller; have larger stromal to thylakoid volumes; fewer thylakoid membranes per chloroplast; fewer thylakoids per granum; lower appressed to non - appressed membrane ratios; lower chlorophyll content per chloroplast; higher chlorophyll a / b ratios; lower xanthophylls to ß - carotene ratios; higher photosystem I (PSI) and photosystem II (PSII) activities; and higher PSII to PSI ratios, when compared with low light intensity grown 'shade' plants (Anderson *et al.*, 1988; Chow, *et al.*, 1990). Thus, due to these and other adaptive adjustments 'sun' plants have much greater photosynthetic capacities, which saturate at higher irradiance, than 'shade' plants. However, 'shade' plants use low irradiance more effectively for net photosynthetic CO_2 uptake than 'sun' plants, due to lower dark respiration rates of the former. Other mechanisms involved in the response to a changing light environment include, for example, movement of part of a plant to reduce the area for photon interception and / or increase self - shading, such as the leaf movement of *Oxalis oregano*, which minimises the incident light of direct sunflecks, preventing photoinhibition (Powles, 1984; Raven, 1989). A change of plastid shape, volume or orientation may also decrease the area for photon interception and / or increase self - shading. Thus, the photosynthetic apparatus of plants must bring about the most effective use of light intesity, duration and quality whilst withstanding short - and long - term changes in these variables. This study examines one aspect of adaptation to the light environment, namely photoinhibition in an intertidal alga *Codium*

fragile. Before considering photoinhibition of photosynthesis in detail a brief description of the relevant aspects of thylakoid membrane structure and function will now be given.

1.2 Thylakoid Structure and Function

The thylakoid membrane network of most higher plants and green algae has interconnected regions of stacked and unstacked membranes. The appressed membranes of the granal thylakoid stacks are in close contact and not directly exposed to the stroma, whilst the outer surfaces of the non-appressed membranes are in direct contact with the stroma. The latter include stroma lamellae and the end membranes and margins of the grana stacks. The inner surfaces of both appressed and non - appressed membranes enclose a continuous aqueous phase or lumen (*e.g.* Anderson, *et al.*, 1988). There are five distinct multicomponent protein complexes within the thylakoid membrane: photosystem II (PS II), photosystem I (PS I), the light - harvesting chlorophyll a/b protein (LHC II), the cytochrome b_6/f complex (cyt b_6/f) and the ATP - synthase (CF_0 - CF_1) or coupling factor (*e.g.* Gounaris, *et al.*, 1986; Hayden and Baker, 1990). It is now known that there is lateral heterogeneity in the distribution of these complexes (*e.g.* Staehelin, 1986). Most PSII complexes and their associated chlorophyll a/b - proteins (LHC II) are located in the appressed membrane regions, while PSI and CF_0 - CF_1 complexes are found exclusively in non - appressed membrane regions (Andersson and Anderson, 1980). The cyt b_6/f complexes are present in both membrane regions (Gounaris, *et al.*, 1986; Anderson, *et al.*, 1988). However, this is a very rigid picture and the membrane is in reality dynamic and fluid. For example, there is a major $PSII_\alpha$ pool, with a larger light harvesting antenna, which is indeed located in the appressed membrane, but there is also a minor $PSII_\beta$ pool, with a smaller light harvesting antenna, which is found in the non - appressed stroma lamellae (Anderson, *et al.*, 1988). It is well established that these complexes interact to achieve the transfer of electrons from water to $NADP^+$ and of protons across the thylakoid membrane, enabling the conversion of light energy into NADPH and ATP (Andreasson and Vanngard, 1988). This process of non - cyclic photophosphorylation is often presented in the form of the "Z scheme" (Hill and Bendall, 1960) a modified version of which is illustrated in Figure 1.1 (adapted from Barber, 1987; Ort and Good, 1988). Some features of the reaction sequence depicted will now be considered in greater detail.

Figure 1.1 The Z scheme for photosynthetic electron transfer based on midpoint redox potentials at pH 7, indicating how the components of the electron transport chain interact to transfer electrons and protons from water to $NADP^+$ and how, under some conditions, electrons may also cycle around PSI. Key: Mn, components involved in water splitting and oxygen evolution (the OEC); Y_z, primary electron donor to P680, the reaction centre chlorophyll of PSII; Ph, phaeophytin acceptor of PSII; Q_A, bound plastoquinone (one electron acceptor); Q_B, bound plastoquinone (two electron acceptor) able to exchange with plastoquinone (PQ) pool; ReFeS, Rieske iron - sulphur centre; Cyt, cytochrome (cyt b_{563} is equivalent to cyt b_6); Pc, plastocyanin; P_{700}, reaction centre chlorophyll of PSI; A_0, A_1, X, primary electron acceptors of PSI; FeS_A, FeS_B, bound iron sulphur acceptors A and B; Fd, soluble ferredoxin; Fp, flavoprotein with ferredoxin - $NADP^+$ oxido - reductase activity (FNR); $NADP^+$, oxidized nicotinamide adenine dinucleotide phosphate. See text for details. Adapted from Barber (1987), with reference to Ort and Good (1988).


PSII
Cyt b_6/f
PSI
E_M (Volts)
−1.0
−0.9
−0.8
−0.7
−0.6
−0.5
−0.4
−0.3
−0.2
−0.1
0.0
+0.1
+0.2
+0.3
+0.4
+0.5
+0.6
+0.7
+0.8
+0.9
+1.0
P680* e⁻
Ph
e⁻
Q_A
e⁻
Q_B
2H⁺
Pool
PQ
PQH_2
2H⁺
e⁻
$2H_2O$
4e⁻
$O_2 + 4H^+$
Mn
e⁻
Y_z
e⁻
P680*
hv
e⁻
cyt b_{563LP}
e⁻
e⁻
cyt b_{563HP}
e⁻
ReFeS
e⁻
cyt f
e⁻
Pc
e⁻
P700*
hv
e⁻
P700*
e⁻
A_0
e⁻
A_1
e⁻
X
e⁻
FeS_B
e⁻
FeS_A
e⁻
2H⁺
NADP⁺
2e⁻
Fp
e⁻
Fd
e⁻
NADPH + H⁺

1.2.1 Photosystem II Complex

In recent years the photosystem II (PSII) complex has been extensively studied and much progress has been made in the determination of its structure and in the elucidation of its function. Consequently, many diagrammatic representations of this complex have been published and general models proposed. These illustrations are generally very similar and a typical example is presented in Figure 1.2. This model is a composition adapted mainly from Hansson and Wydrzynski (1990) with reference to Sauer (1986), Babcock, *et al.* (1989), Brudvig, *et al.* (1989), Chapman and Barber (1989), Rutherford (1989), Volkov (1989), Coleman (1990), and Simpson (1990).

The PS II complex acts as a water - plastoquinone oxido - reductase (Barber, 1987) and this overall function has been summarized by Hansson and Wydrzynski (1990) as,

$$H^+_{(stroma)} + 2PQ + 2H_2O \text{ ----> } 2PQH_2 + O_2 + 4H^+_{(lumen)}$$

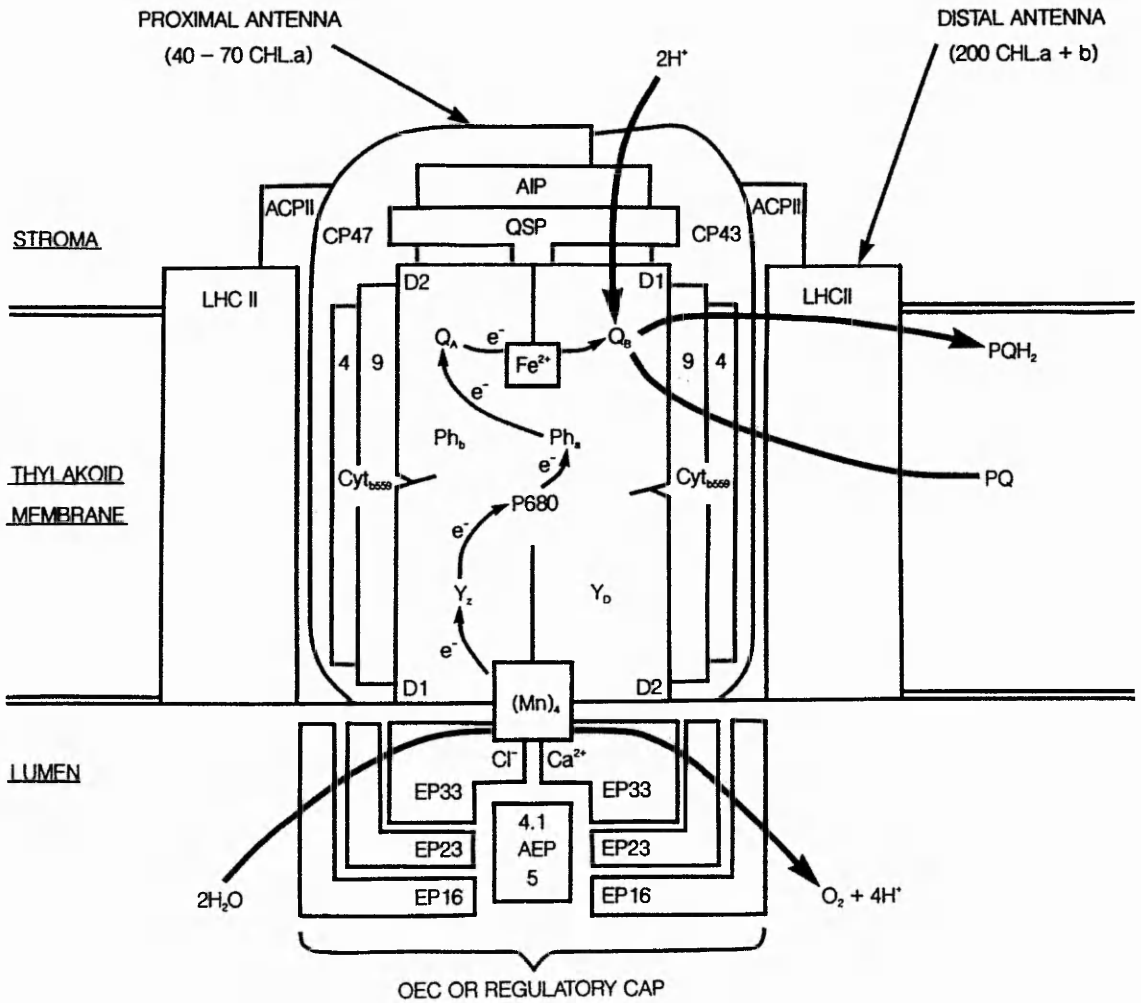
indicating the vectorial organisation of the reaction sequence across the thylakoid membrane and the associated release of protons into the lumen and uptake of protons from the stroma. However, the splitting of water is known to occur on the lumen side of the thylakoid membrane, being catalysed by a PSII region generally referred to as the oxygen evolving complex, or OEC (*e.g.* Babcock, *et al.*, 1989; Volkov, 1989; Coleman,1990), although Hansson and Wydrzynski (1990) use the term 'regulatory cap'. The light driven catalytic reaction has the stoichiometry,

$$2H_2O \text{ -----> } O_2 + 4H^+ + 4e^-$$

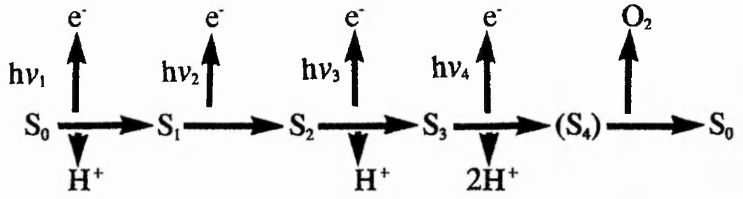
with an average E_m (midpoint redox potential at pH 7) of +0.81v per electron (Babcock, *et al.*, 1989; Volkov, 1989; Coleman, 1990). The OEC contains several extrinsic polypeptides, the three largest having molecular masses of 18, 24 and 33 KDa (Andreasson and Vanngard, 1988; Coleman, 1990; Hansson and Wydrzynski, 1990). The OEC may also contain other extrinsic polypeptides with molecular masses of less than 10 KDa and a 5 KDa protein has been identified (Coleman, 1990; Hansson and Wydryznski, 1990). In Figure 1.2 possible additional extrinsic proteins in the OEC are designated 'accessory extrinsic proteins' (AEP).

The oxidation of water is a four - electron dependent process and this must be coupled to the one - electron turnover of the reaction centre components Y_z, P680, Ph and Q_A. In order to do this the OEC must be able to store four oxidising equivalents and therefore the complete cycle for water oxidation has been represented as a series of transient states (s states) of the OEC (Coleman,1990; Hansson and

Figure 1.2 Diagrammatic representation of Photosystem II. Key: P680, the primary electron donor chlorophyll (the reaction centre chlorophyll); Ph_a, the primary electron acceptor phaeophytin; Ph_b, a second phaeophytin; Q_A and Q_B, the first and second bound quinone electron acceptors respectively; Fe^{2+}, a non - haeme iron atom; Y_Z, the tyrosine first electron donor to P680; Y_D, a second tyrosine; $(Mn)_4$, four manganese atoms involved in oxygen evolution are located near the interface of the reaction centre polypeptides and the 33 KDa extrinsic protein (chloride and calcium binding sites are also situated on the lumenal side of the membrane); Cyt $_{b559}$, cytochrome $_{b559}$ heterodimer; D1 and D2, reaction centre core polypeptides (32 and 34 KDa, respectively); QSP, quinone shielding protein (9 - 10 and / or 22 KDa proteins); AIP, accessory intrinsic proteins (2.0, 3.7, 4.8, 6.5 - 7.0, 10.0, 22.0 and 24.0 KDa proteins); EP33, EP23, EP16, extrinsic proteins of the regulatory cap or oxygen evolving complex (OEC); AEP, accessory extrinsic proteins (5 KDa protein); CP47 and CP43, chlorophyll proteins of the proximal antenna; ACPII, accessory chlorophyll proteins of the distal antenna; LHCII, the chlorophyll a / b light harvesting complex. See text for details. Adapted from Hansson and Wydrzynski (1990) with reference to Sauer (1986), Babcock, *et al.* (1989), Brudvig, *et al.* (1989), Chapman and Barber (1989), Rutherford (1989), Volkov (1989), Coleman (1990) and Simpson (1990).


PROXIMAL ANTENNA
(40 – 70 CHL.a)
2H+
DISTAL ANTENNA
(200 CHL.a + b)
AIP
ACPII
CP47
QSP
CP43
ACPII
STROMA
LHC II
D2
D1
LHCII
QA
e-
Fe2+
QB
PQH2
4
9
9
4
e-
Phb
Pha
e-
CytB559
CytB559
PQ
THYLAKOID
MEMBRANE
P680
e-
e-
Yz
YD
D1
D2
(Mn)4
Cl-
Ca2+
LUMEN
EP33
EP33
4.1
AEP
5
EP23
EP23
2H2O
EP16
EP16
O2 + 4H+
OEC OR REGULATORY CAP

Wydrzynski,1990; Vass, *et al.*, 1990). Each transition is driven by the absorption of a photon by the reaction centre chlorophyll of PS II (P680) which results in rapid charge separation, occurring in a few pico - seconds ($P680^+Ph^-$), and the generation of a powerful oxidant (the $P680^+$) that is ultimately reduced by electrons from water (Rutherford, 1989; Coleman, 1990), via the single electron carrier Y_z (Hoganson and Babcock, 1988). It is estimated that electron transfer from the OEC to Y_z takes about 50 μs to 1 ms and that electron transfer from Y_z to P680 takes about 10 ns to 10 μs (Andreasson and Vanngard, 1988). The occurrence of s states is often summarised as:



The higher s - states deactivate in the dark resulting in a stable distribution of centres having 75% S_1 and 25% S_0. Formation of the O=O bond is most likely to occur during the S_2 to S_3 or S_3 to (S_4) transition. On oxidation of water to molecular oxygen protons are released in a 1:0:1:2 pattern at the successive oxidation steps starting with the S_0 state. The removal of protons is essential to the advancement of certain s - states.

It has been confirmed that four manganese ions per PSII unit are required for full water splitting activity (Andreasson and Vanngard, 1988) and that at least some of these Mn ions are arranged in a multinuclear cluster within the OEC. It is also generally agreed that valence state changes of the Mn ions are associated with the s - state transitions (Hansson and Wydrzynski, 1990). Calcium and chloride are also thought to have a role as cofactors in OEC mediated oxygen evolution but the exact nature of the involvement of these atoms in the process is still a matter of great controversy. Some observations link manganese with the D2 protein and there are indications that manganese binding is stabilized by the 33 KDa protein of the OEC. Both proteins may contribute to the stabilization of the manganese during photoactivation (Andreasson and Vanngard, 1988). Similarly, the D1 (32 KDa) protein has been implicated in assembling the Mn cluster (Coleman, 1990) and it has been suggested that the functionally active manganese binds to the D1/D2 heterodimer. Thus, it is accepted that the Mn is situated on the lumenal side of the membrane, within a restricted compartment formed between the reaction core (D1/D2) and the extrinsic 33 KDa protein of the OEC (Hansson and Wydrzynski, 1990). It is possible that the 18, 24 and

33 KDa proteins of the OEC all have a role in the binding and regulating of Ca^{2+} and Cl^- ions, and in controlling the exchange of reactants with the manganese centre (Hansson and Wydrzynski, 1990). The 33 KDa polypeptide appears to be the most important extrinsic subunit; its presence is necessary for the binding of the 18 and 24 KDa polypeptides to the membrane, and parts of its amino acid sequence show similarities with Mn - superoxide dismutase from *E.coli* and the Ca^{2+} binding sites in calmodulin and troponin c, suggesting a role in the binding of Mn and Ca^{2+} for this polypeptide (Coleman, 1990).

There have been numerous suggestions for the arrangement of the four manganese atoms and for possible roles and interactions of Cl^- and Ca^{2+} in the reaction sequence of the OEC although it is not a proposal of this introduction to cover this area exhaustively. The models of the Mn cluster include a tetramer, two linked dimers and a trimer / monomer, in various configurations and with various valences proposed for the constituent Mn atoms to account for the speculative redox states of Mn required to explain its role in the enzyme s - state cycle. For additional details the reader is referred to Andreasson and Vanngard (1988), Homann (1988), Babcock, *et al.* (1989), Brudvig, *et al.* (1989), Rutherford (1989), Volkov (1989), Bossek, *et al.* (1990), and Coleman (1990).

It is evident that the precise mode of action of the OEC is still unclear, however the structure and function of the rest of the PSII complex is understood with, perhaps, a relatively greater degree of certainty. As is seen from Figure 1.2 the PSII complex contains a heterodimer core consisting of the D1 protein (previously referred to as the Q_B protein) and the D2 protein which have molecular masses of approximately 32 and 34 KDa respectively. The reducing - side components of PSII and the primary reactants Y_z, P680, Ph, Q_A, and Q_B are believed to be associated with these intrinsic proteins and the isolation of a D1/D2/cyt_{b559} complex (Seibert, *et al.*, 1988) containing a minimal amount of chlorophyll and phaeophytin, and with photochemical activity (Chapman *et al.*, 1988; Wasielewski, *et al.*, 1989) supports this view. The first electron donor to $P680^+$, i.e. Y_z, has been identified as the tyrosine - 161 residue of the D1 polypeptide (Debus, *et al.*, 1988b). Hoganson and Babcock (1988) propose a linear model for early electron transfer events in PSII, whereby P680 is photooxidised to the chlorophyll radical $P680^+$, which oxidises the nearby tyrosine, Y_z. The resulting Y_z^+ in turn oxidises manganese in the OEC, with Y_z being the only electron carrier between P680

and the OEC (Gerken, *et al.*, 1988). Debus, *et al.* (1988a) have also identified a second tyrosine residue, designated Y_D, in position 160 on the D2 polypeptide. This was confirmed by Vermaas, *et al.* (1988) but the function of the residue is unclear. Figure 1.2 also shows the reaction centre chlorophyll (P680), the initial electron acceptor phaeophytin a (Ph_a) and the quinone electron acceptors Q_A and Q_B, with associated non - haeme iron (Fe), to be located on the D1/D2 heterodimer within the reaction core. A second phaeophytin a molecule (Ph_b) and the second tyrosine residue, Y_D, may be located on a functionally separate part of the heterodimer. The D1/D2 heterodimer has been shown to contain ß - carotene and some forms of chlorophyll a (other than P680), which may be monomeric forms between P680 and the phaeophytins (Hansson and Wydrzynski, 1990). Both the D1 and D2 polypeptides are thought to have five transmembrane domains and two parallel helices, one exposed to the stroma, the other exposed to the lumen (Rochaix and Erickson, 1988; Mattoo, *et al.*, 1989). Trebst, *et al.* (1988) found that the conformation of the D1 polypeptide was altered if the Q_B site was occupied by either plastoquinone or a DCMU - type inhibitor and suggested that the arginine 238 residue of D1 was possibly part of the Q_B binding niche. Mattoo, *et al.* (1989) have reported that during D1 degradation and turnover the primary cleavage occurs between arginine 238 and isoleucine 248. These workers go on to describe this domain of D1, localized in the functionally active hydrophilic loop between helices D and E, as rich in glutamate residues, conserved phylogenetically and to be the region of the protein where quinone and herbicides are postulated to bind. They also suggest this region to be the genetic locale where single amino acid changes occur that confer atrazine or diuron herbicide resistance to higher plants and algae. However, most other reports agree that arginine 238 is the cleavage site, but do not implicate the region of arginine 238 to isoleucine 248 in herbicide binding (*e.g.* Chapman and Barber, 1989). It appears to be generally accepted that serine 264 is the main site associated with herbicide binding to D1 and herbicide resistance upon mutation, with other sites also possibly involved, including phenylalanine 211, valine 219, alanine 251, phenylalanine 255, glycine 256 and leucine 275 (Chapman and Barber, 1989).

The exact nature of the reaction centre chlorophyll (P680) is still uncertain. It is generally thought to consist of a chlorophyll a dimer, i.e., two 'special - pair' chlorophyll a molecules, but there are also suggestions that it may be a chlorophyll a monomer (Hansson and Wydryznski, 1990). The presence of other polypeptides has

also been suggested, designated 'quinone shielding protein' (QSP) in Figure 1.2, including two with molecular masses of 9 - 10 and 22 KDa. These do not bind any of the electron transfer components but may be implicated in optimising the conformation of the D1/D2 subunits and in the regulation of PSII reactions. The smaller of the two can be phosphorylated at a threonine residue which results in slight inhibition of oxygen evolution, possibly due to alteration of regions associated with the Q_A and Q_B sites (Hansson and Wydrzynski, 1990). It is also thought that there are two $cytb_{559}$ apoproteins per PSII unit, each of which consists of an α and ß subunit with molecular masses of 4 and 9 KDa respectively (Andreasson and Vanngard, 1988). However, the function of the $cytb_{599}$ complex is still unclear although it has been suggested that its role is to mediate cyclic electron transport around PSII, reducing photooxidised chlorophyll and thus protecting PSII against photoinhibition (Thompson and Brudvig, 1988). Other intrinsic proteins, designated as 'accessory intrinsic proteins' (AIP) in Figure 1.2, of 2.0, 3.7, 4.8, 6.5 - 7.0, 10.0, 22.0 and 24.0 KDa may also be present in PSII (Hansson and Wydrznski, 1990). The function of these polypeptides is currently unknown, but it has been suggested that they may be required as structural components, optimising the reaction core conformation. The proximal antenna has a long - wavelength absorption band similar to that of the primary donor and hence functions to couple energy transfer from the majority of the light - harvesting pigments in the distal antenna to the reaction core. The proximal antenna is tightly coupled to the reaction core and consists of two pigment - protein complexes, CP47 and CP43 (or CPa - 1 and CPa - 2) of molecular masses 45 - 51 and 40 - 45 KDa respectively (Thornber, 1986), which both bind 20 - 25 chlorophyll a and about 5 ß - carotene molecules, but no chlorophyll b or phaeophytin (Hansson and Wydrzynski, 1990). It is possible that the proximal antennae have functions in either binding or regulation of the electron transport components.

The distal antenna, as illustrated in Figure 1.2, consists of the ACP II and the LHC II. The former 'accessory chlorophyll proteins' include polypeptides of molecular masses 24, 26 and 29 KDa, and generally have a chlorophyll a/b ratio of 2 - 3. These polypeptides are not phosphorylated and are closely coupled to the proximal antenna and the reaction core. It has been suggested that they may have a role in the dissipation of excess excitation energy thereby counteracting photoinhibition (Bassi, *et al.*, 1987). The LHC II is involved in the stacking of thylakoid membranes (Staehelin, 1986) and

the distribution of energy between PSII and PSI (Allen and Holmes, 1986a; Fork and Satoh, 1986; Gounaris, *et al.*, 1986; Anderson, *et al.*, 1988). The LHC II contains several apoprotein components consisting of proteins with apparent molecular masses of between 21 and 29 KDa (Thornber, 1986; Chitnis and Thornber, 1988). In higher plants the LHC II generally has a chlorophyll a/b ratio of 1.0 to 1.4 and contains xanthophyll as the major carotenoid. However, the exact pigment composition can vary depending on the light environment and there is a great diversity of photosynthetic pigments, especially in algae. The composition of algal light - harvesting pigment - protein complexes is particularly variable (Anderson and Barrett, 1986) and this will be further discussed with respect to *Codium fragile* at a later stage.

The function of the manganese containing four - electron gate of the OEC has been dealt with and the subsequent sequence of electron transfer reactions will now be described. Using the newly developed D1/ D2/ $cytb_{559}$ preparation (Chapman, *et al.*, 1988) the time for the primary charge separation ($P680^* \dashrightarrow P680^+Ph_a^-$) has been estimated at 3 $\pm$ 0.6 ps (Schatz, *et al.*, 1988; Wasielewski, *et al.*, 1989). The charge separation is stabilised by electron transfer from Ph_a^- to Q_A (the first stable acceptor, plastoquinone). When Q_A is oxidised this electron transfer is estimated to occur within 250 - 300 ps (Andreasson and Vanngard, 1988). If Q_A is reduced or absent the electron may leave Ph_a^- and recombine with the primary donor in 2 - 30 ns to form the ground or triplet state of P680. The electron transfer from the primary electron acceptor to the first quinone acceptor was found to occur in the slightly longer time of 510 $\pm$ 50 ps in the thermophilic cyanobacterium *Synechococcus* sp. (Schatz, *et al.*, 1987). The plastoquinone Q_A is a one electron acceptor and on formation of Q_A^- the semiquinone so formed remains unprotonated. The electron is then transferred to the secondary quinone electron acceptor Q_B, which is a two electron gate (Hansson and Wydrzynski, 1990), and this occurs within 100 - 200 μs (Babcock, *et al.*, 1989). A proton uptake from the aqueous medium may occur on the formation of Q_B^-, but the protonation is thought to occur at a protein group close to the Q_B site, not on the semiquinone itself. A second electron transfer from Q_A to produce Q_B^{2-} occurs in 400 - 500 μs and protonation follows forming bound plastohydroquinone (Q_BH_2). The binding affinity for Q_BH_2 is low and therefore a plastoquinone molecule from the PQ pool can displace it. There is a non - haeme Fe^{2+} ion closely associated with Q_A and Q_B and redox titrations give a mid - point potential for the couple Fe^{2+}/ Fe^{3+} of 400 mV which correlates with

the mid - point potential of the Q_{400} component (Andreasson and Vanngard, 1988). This component may possibly be able to accept electrons from Q_A^- and the iron can be oxidized to Fe^{3+} by flash induced semiquinones of some exogenous quinone acceptors (Hansson and Wydrzynski, 1990). Bicarbonate apparently facilitates electron transport through the two electron gate of Q_B (Andreasson and Vanngard, 1988). It has recently been suggested that HCO_3^- acts as a proton donor to the protein group believed to participate in the protonation of Q_B^- and that HCO_3^- may be a ligand to the Fe_2^+ in the Q_A - Fe - Q_B complex of PSII (Eaton - Rye and Govindjee, 1988). In this model one HCO_3^- (site A) is a bidentate ligand to the Fe^{2+}, with its hydrogen group possibly involved in a hydrogen bond to a residue on D2, which is necessary to maintain the functional configuration of the reaction centre. A second high affinity HCO_3^- (site B) is proposed to be bound to arginine - 257 of the D1 protein and is involved in protonating histidine - 252 at the catalytic Q_B site. On formation of Q_B^- the HCO_3^- may protonate the histidine. The resulting CO_3^{2-} may be displaced by another HCO_3^- ensuring that the protonation is irreversible. Interaction of the new $HC0_3^-$ with the nitrogen atom of histidine may favour H^+ transfer from the histidine to Q_B^-, giving Q_B or Q_B^{2-}. A large number of low - affinity sites for HCO_3^- are suggested to act as a buffer of the intra - thylakoid HCO_3^- concentration, keeping the arginine loaded during rapid turnover of the reaction centre (see Blubaugh and Govindjee, 1988). However, although the Fe^{2+} ion may have an important role on the acceptor side of PSII, this role is by no means certain. Thus, unlike Q_A, the Q_B site is occupied by plastoquinone (PQ) or plastoquinol (PQH_2) molecules which are not firmly bound and can exchange freely with molecules in the lipid matrix of the thylakoid membrane. As previously mentioned plastoquinol has a lower binding affinity than plastoquinone and is thus displaced by PQ from the intra - thylakoid PQ pool. The PQH_2 can then diffuse through the membrane to the cyt b_6/f complex (Figure 1.3). The oxidation of PQH_2 is the rate - limiting step in non - cyclic electron flow, having a $t_{1/2}$ of 10 - 20 ms (Barber, 1987).

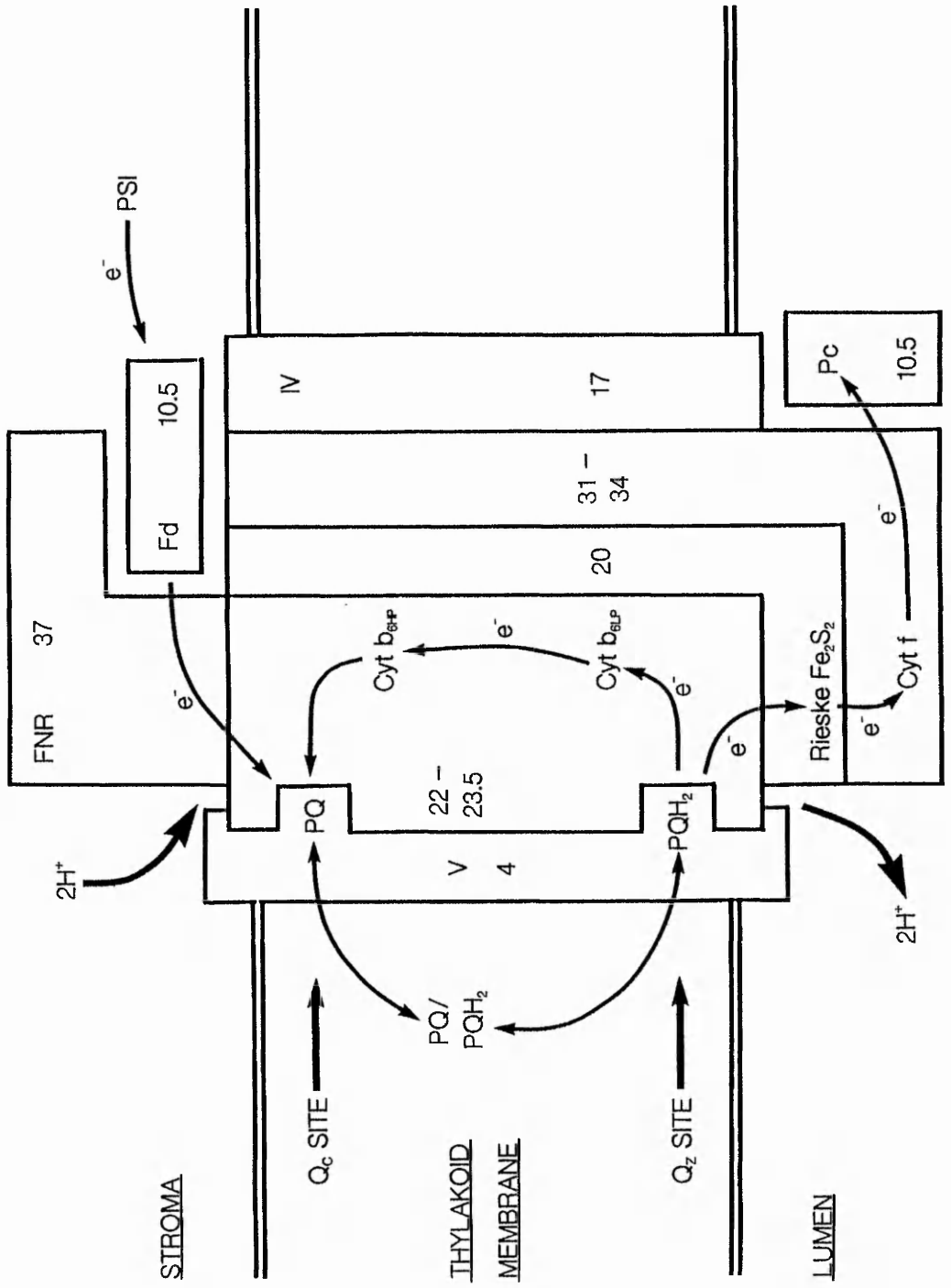
1.2.2 Cytochrome b_6/f Complex

This complex functions in higher plants and green algae as a plastoquinol - plastocyanin oxido - reductase and thus catalyses the electron / proton exchange between PSII and PSI. It can also function as a ferredoxin : plastocyanin oxido - reductase and thus allow cyclic electron flow around PSI (Gounaris, *et al.*, 1986). It

is also thought to be capable of operating a Q - cycle mechanism (Barber, 1987). The cyt b_6/f complex (Figure 1.3) consists of four major polypeptides: the cyt f apoprotein is apparently a doublet of molecular masses 34 and 31 KDa, the cyt b_6 apoprotein of molecular mass 22 KDa, the Rieske FeS protein of molecular mass 20 KDa and a 17 KDa subunit of unknown function (Barber, 1987). The redox active groups of cyt f (a haeme) and the Rieske protein (which contains two iron atoms and two sulphur atoms in the form of an Fe_2S_2 cluster) are attached to their polypeptides on the lumen side of the thylakoid membrane. Two cyt b_6 haemes are bound by a single copy of the 22 KDa protein. The stoichiometry of the subunits is 1: 1 : 1: 1 and some small polypeptides (e.g. 4 KDa) are also present. In some situations a 37 KDa FAD containing flavoprotein, ferredoxin - $NADP^+$ oxido - reductase (FNR), is associated with the complex. This is not required for plastoquinol oxidation and probably plays a part in cyclic electron flow via PSI.

The cyt b_6/f complex must couple the two electron carrier plastoquinol with reduction of the one electron carrier plastocyanin and it is generally accepted that a Q - cycle mechanism as originally proposed by Mitchell (1975) is involved (Ort, 1986). In this scheme there is a binding site for plastoquinol near the inner surface of the thylakoid membrane (Q_z) and a binding site for plastoquinone near the outer suface (Q_c). As is seen in Figures 1.1 and 1.3 the transfer of an electron to PSI via plastocyanin results in the removal of an electron from cyt f and then in turn from the Rieske Fe_2S_2 centre. The plastoquinol in the Q_z site will then pass an electron to the Rieske Fe_2S_2 centre, resulting in the production of the more reducing semiquinone species. The second electron of the semiquinone can be passed to the reoxidised Rieske centre or donated to cyt b_{6LP}, the low potential form of cytochrome b_6 (mid - point potential at pH 7, Em, about - 150 mV), as the semiquinone form is a strong reductant. If the latter occurs the electron can be donated from the cyt b_{6LP} (Em = -150 mV) to cyt b_{6HP}, the high potential form of cytochrome b_6 (Em = -50 mV). A plastoquinone at the Q_c site can be reduced to a semiquinone by receiving the electron from cyt b_{6HP} and by protonation with a H^+ from the outer stromal phase. Thus, one turnover of this sequence will result in the complete oxidation of one molecule of plastoquinol (at the Q_z site) with the release to the lumen of two H^+, and the reduction of one plastoquinone (at the Q_c site) to the level of semiquinone, accompanied by the uptake of one H^+ from the stromal phase. A second similar turnover with a plastoquinol molecule being

Figure 1.3 Diagrammatic representation of the Cytochrome b_6 / f complex illustrating a Q - cycle mechanism involving electron donation from ferredoxin (Fd) reduced by PSI. Key: FNR, ferredoxin - $NADP^+$ oxido - reductase; PQ, plastoquinone; PQH_2, plastoquinol; Cyt b_{6HP} and Cyt b_{6LP}, cytochrome b_6 (or cytochrome b_{563}) in its high potential (E_m = -50 mV) and low potential (E_m = -150 mV) forms, respectively; Q_C, binding site for plastoquinone near the outer surface of the thylakoid membrane; Q_Z, binding site for plastoquinol near the inner surface of the thylakoid membrane; Rieske Fe_2S_2, the Rieske iron sulphur protein; Cyt f, cytochrome f; Pc, plastocyanin. The molecular masses in KDa are also shown. See text for details. Adapted from Simpson (1990) with reference to Gounaris, *et al.* (1986), Ort (1986), Barber (1987), O' Keefe (1988) and Volkov (1989).


e⁻ PSI
Fd
10.5
IV
17
Pc
10.5
31 –
34
20
FNR
37
e⁻
Cyt b_{6H}
e⁻
Cyt b_{6L}
e⁻
e⁻
Rieske Fe_2S_2
e⁻
Cyt f
22 –
23.5
PQ
PQH_2
V
4
$2H^+$
$2H^+$
PQ/
PQH_2
Q_C SITE
Q_Z SITE
STROMA
THYLAKOID
MEMBRANE
LUMEN

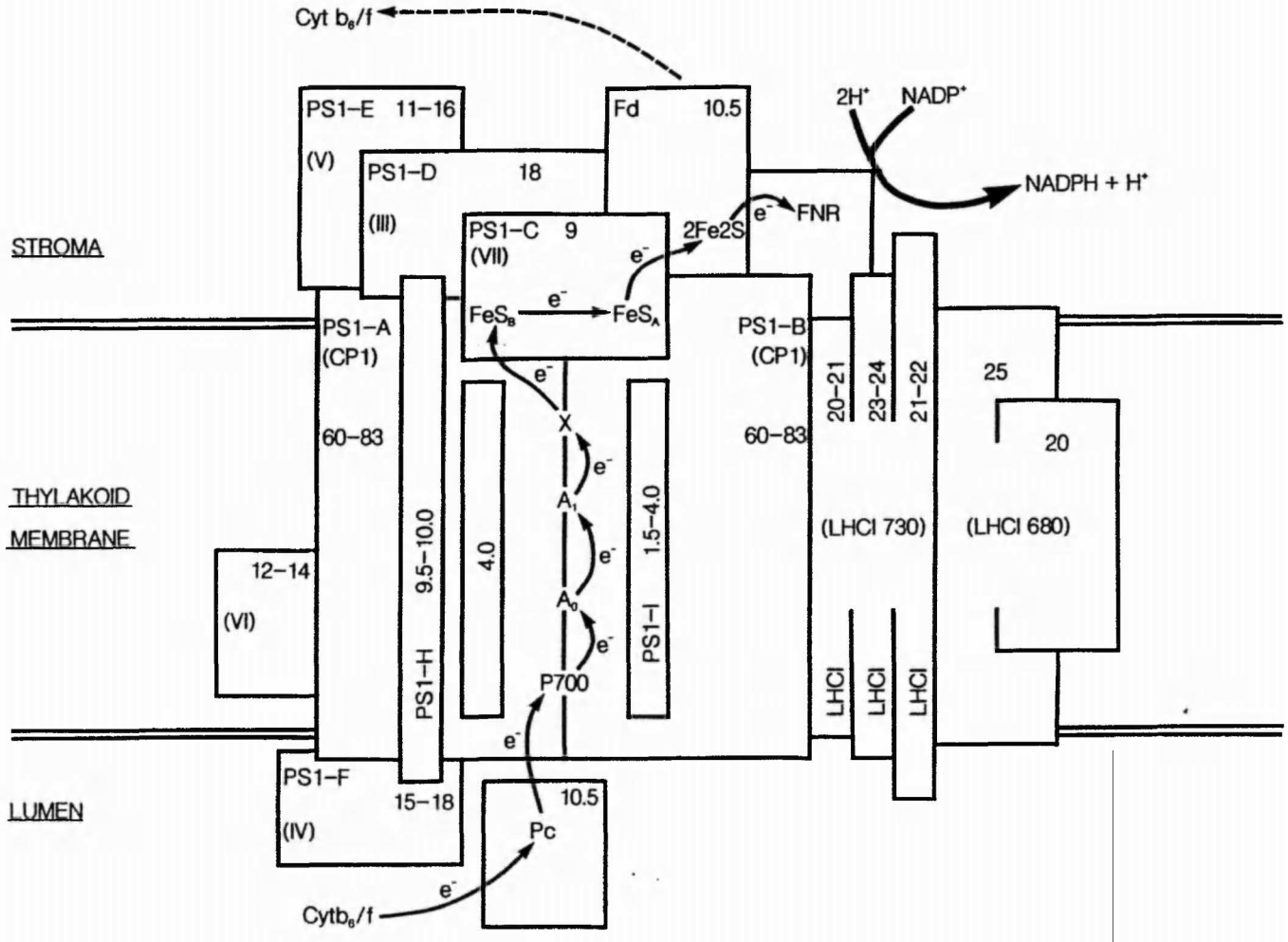
reduced at the Q_z site, may result in an identical series of events, except that at the Q_c site the semiquinone would be reduced to plastoquinol by receiving an electron from cyt b_{6HP} and uptake of a H^+ from the stromal phase. The PQH_2 will then dissociate from the Q_c site to be replaced by a plastoquinone from the membrane pool. Thus overall, the Q cycle oxidises two plastoquinols, reduces one plastoquinone (net effect is oxidation of one plastoquinol) and translocates $4H^+$ for every $2e^-$ reaching PSI (Ort, 1986). A linear scheme only achieves the movement of 2 protons per plastoquinol oxidised. That is, the Q - cycle alters the H^+/e^- stoichiometry from 1 to 2 for plastoquinol oxidation (Barber, 1987). However, this is a relatively simple model and some anomalies have been identified which are explained by modifications to this basic scheme. For example, the further reduction of the semiquinone (at the Q_c site) can apparently occur by direct electron donation from PSII or indirect electron donation from PSI via reduced ferredoxin in cyclic electron transport, as shown in Figures 1.1 and 1.3 (Barber, 1987). It is clear that reduced ferredoxin produced by PSI can either reduce $NADP^+$ via ferredoxin - NADP oxido - reductase or take part in cyclic electron transport with cyt b_6/f, although the site at which PSI - reducing equivalents are returned via soluble ferredoxin to the cyt b_6/f complex and the mechanism of ferredoxin oxidation by the complex is uncertain (Ort, 1986).

1.2.3 Photosystem I Complex

In non - cyclic photophosphorylation electrons pass from cyt f, the one electron carrier component of the cytb_6/f complex to plastocyanin, a one copper containing protein of molecular mass 10.5 KDa, on the lumen side of the thylakoid membrane (Figures 1.1 and 1.4). Plastocyanin then functions as a diffusable electron carrier between the cyt b_6/f complex and PSI (Haehnel, 1986; Knaff, 1988).

Photosystem I (PSI) acts as a light stimulated plastocyanin - ferredoxin oxido - reductase (Knaff, 1988). The number of subunits in the PSI complex and their structure and function is not yet fully understood. Five chloroplast encoded and six nuclear encoded PSI polypeptides have been identified in higher plants and the subunit composition is thought to be very similar in all species (Scheller and Moller, 1990). The reaction centre chlorophyll, P700, and the acceptors A_0, A_1 and X are all located in the pigment complex known as the P700 - chlorophyll a - protein (CP1). This complex is composed of the PSI - A and PSI - B subunits which apparently have

Figure 1.4 Diagrammatic representation of Photosystem I showing electron flow from the cytochrome b_6 / f complex, via plastocyanin, to P700, and the possibility of electron transfer, via ferredoxin, back to the cytochrome b_6 / f complex. Key: Pc, plastocyanin; Fd, soluble ferredoxin; FNR, ferredoxin - $NADP^+$ oxido - reductase; PSI - A and PSI - B (CP1), chlorophyll binding protein heterodimer; P700, the reaction centre chlorophyll; A_0, A_1, X, electron acceptors; FeS_B and FeS_A, four iron - four sulphur (4Fe - 4S) clusters (electron acceptors); LHC, light harvesting complex. The molecular masses in KDa are also shown. See text for details. Adapted mainly from Scheller and Moller (1990), who use the nomenclature for subunit labelling of Bengis and Nelson, 1977 (*e.g.* IV, V *etc.*) and Schantz and Bogorad, 1988 (*e.g.* PSI - A, PSI - D *etc.*). Reference was also made to Gounaris, *et al.* (1986), Knaff (1988), Andersen, *et al.* (1990), Moller, *et al.* (1990) and Simpson (1990).


Cyt b6/f
PS1-E 11-16
(V)
PS1-D 18
(III)
PS1-C 9
(VII)
Fd 10.5
2H+ NADP+
NADPH + H+
FNR
2Fe2S
e-
STROMA
FeSB
FeSA
PS1-A
(CP1)
60-83
PS1-B
(CP1)
60-83
X
A1
A0
P700
PS1-H 9.5-10.0
4.0
PS1-I 1.5-4.0
20-21
23-24
21-22
25
20
(LHCI 730)
(LHCI 680)
LHCI
LHCI
LHCI
THYLAKOID
MEMBRANE
12-14
(VI)
PS1-F
15-18
(IV)
LUMEN
10.5
Pc
Cytb6/f

similar molecular masses. The estimations of the molecular masses of these polypeptides reported in the literature vary depending on whether they were calculated from amino acid or nucleotide sequencing data, or determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS - PAGE). Furthermore, if the latter method is used there is apparently marked variation in the estimated molecular mass obtained with different percentage gels. There is also variation in the molecular mass estimations between workers, but generally the two polypeptides are thought to have molecular masses of between 60 and 83 KDa (Barber, 1987; Golbeck, 1987; Knaff, 1988; Margulies, 1989; Scheller and Moller, 1990; Simpson, 1990). It is believed that the two subunits each have eleven membrane spanning helices. A 9 KDa polypeptide has been identified as the location of the [4Fe - 4S] iron sulphur centres Fe_B and Fe_A (Lagoutte and Mathis, 1989). The PSI polypeptide of apparent molecular mass 1.5 KDa (calculated, 4.0 KDa) has unknown function; it may have a role in the binding of P700, A_0 and A_1 (Scheller and Moller, 1990). The peripheral PSI - D polypeptide has a molecular mass of 18 to 22 KDa, has highly positively charged areas and is known to interact with ferredoxin. It may have a function in enabling the negatively charged ferredoxin to react with PSI despite the overall negative charge of the thylakoid membrane (Scheller and Moller, 1990). A 19 KDa subunit (corresponding to the 15 KDa polypeptide PS1 - F in Figure 1.4) from spinach has been implicated in the binding of plastocyanin (Wynn and Malkin, 1988). PSI - F has many positive charges and again this may play a role in counteracting negative charge repulsion between plastocyanin and the PSI complex. Apart from providing positive charges the polypeptides PS1 - D and PSI - F may well have an important role in bringing ferredoxin and plastocyanin respectively, in to the correct position for efficient electron transfer (Scheller and Moller, 1990). The polypeptide PSI - H (9.5 KDa) may have a function in binding of the light harvesting complex I (LHCI) which is specific for PSI. However, this is unclear and similarly the functions of the 14 KDa polypeptide and the PSI - E polypeptides are currently unknown (Scheller and Moller, 1990). As is seen in Figure 1.4, apart from the polypeptides of the core complex (CP1) just described, there are also polypeptides that constitute a light harvesting complex I (LHCI) which is specific for PSI. These will not be discussed in detail. According to the model of Simpson (1990) there are two light harvesting chlorophyll proteins, LHCI - 730 and LHCI - 680. The reaction core (CP1) is thought to contain 90 chlorophyll a molecules;

the LHCI - 730, 66 chlorophyll a and 22 chlorphyll b molecules; and the LHCI - 680, 24 chlorophyll a and 8 chlorophyll b molecules.

The linear sequence of electron carriers in PSI, as indicated in Figures 1.1 and 1.4, is plastocyanin (PC), P700, A_0, A_1, X, Fe_B, Fe_A and ferredoxin (Fd). The primary donor of PSI, P700, is generally considered to be a dimer of chlorophyll a and the primary electron acceptor A_0 is believed to be a specialised monomeric chlorophyll a. However, its identity is not certain and there is still the possibility that it has a dimeric form (Andreasson and Vanngard, 1988). Similarly, the nature and properties of the secondary electron acceptor, A_1, are far from fully understood. It is thought at present that A_1 is a phylloquinone, i.e. vitamin k1 (Hauska, 1988), and although there is uncertainty all evidence so far suggests that A_1^- has the properties of a quinone radical anion (Lagoutte and Mathis, 1989). It was uncertain as to whether X was one 4Fe - 4S centre or two 2Fe - 2S centres (Knaff, 1988; Lagoutte and Mathis, 1989). However, recently evidence favouring the structure of centre X as a 4Fe - 4S cluster bridged between the two subunits of CP1 has been reported (Scheller, *et al.*, 1989). Fe_B and Fe_A are both believed to be 4Fe - 4S centres. Following excitation of an antenna pigment molecule energy transfer results in excitation of P700 in 1.5 to 90 ps. Electron transfer to A_0 occurs in 3 to 14 ps and the ensuing reduction of A_1 occurs in 32 ps, with its oxidation occurring in 15 to 200 ns (Andreasson and Vanngard,1988; Lagoutte and Mathis, 1989). The further electron transfer through X, Fe_A and Fe_B is at present completely unknown. Two pathways have been suggested: either a linear one, X - Fe_B - Fe_A or a branched one, in which X donates electrons either to Fe_B or Fe_A . Electrons are eventually passed on to reduce ferredoxin (molecular mass 10.5 KDa), a 2Fe - 2S one electron carrier, soluble in the stromal phase of the chloroplast. Ferredoxin couples electron transfer between the PSI complex and $NADP^+$ to form the NADPH necessary for CO_2 fixation. This reduction of $NADP^+$ is catalyzed by the FAD containing flavoprotein ferredoxin - $NADP^+$ oxido - reductase (FNR) of approximate molecular mass 33 KDa, that is associated with the outer surface of the thylakoid membrane (Ort, 1986). To reduce $NADP^+$ to NADPH + H^+ two electrons and two protons are necessary. This requirement can be met as the flavine adenine dinucleotide (FAD) coenzyme is a two hydrogen and two electron carrier system. The protons are provided by the aqueous stromal environment. However, the source of the electrons is the reduced soluble ferredoxin and this one electron carrier system must in some way

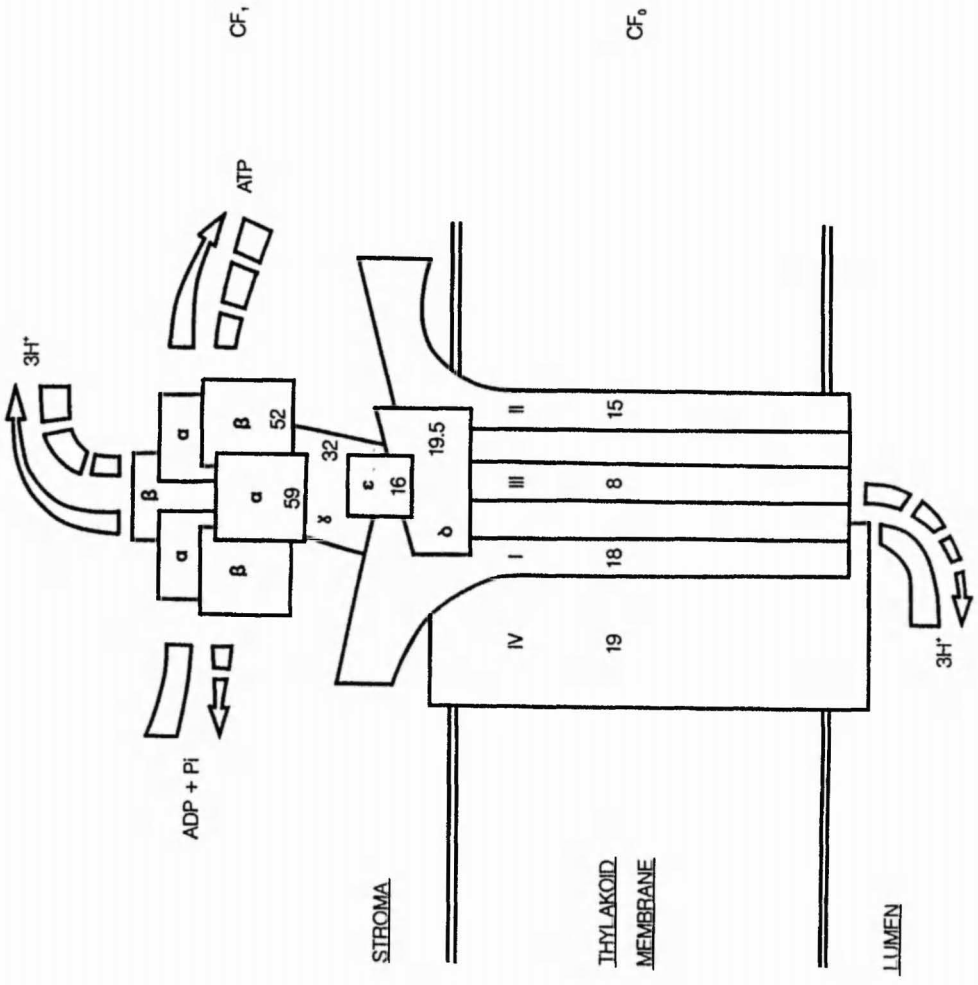
supply two electrons to the FNR complex.

1.2.4 ATP - synthase (CF_0 - CF_1)

The chemiosmotic hypothesis is now generally accepted as a good working model for the coupling of electron transfer chains to ATP synthesis by a proton electrochemical potential ($\Delta\tilde{\mu}H^+$) across the energy - transducing membrane (Nicholls, 1982). As has been seen, proton translocation arises from the reduction of $NADP^+$ at the stromal side of the membrane with the consumption of protons, and the oxidation of water taking place in the lumen, leading to the liberation of protons. Also plastoquinone is reduced at the stromal side and oxidised at the lumen side resulting in proton translocation across the membrane into the lumen. Indeed, protons may be translocated via cyclic (around PSI and $cytb_6/f$) or non - cyclic electron transport. Such translocation results in the formation of an electrochemical potential difference for protons between the stroma and lumen (proton motive force, pmf), $\Delta\tilde{\mu}H^+$, which is comprised of a proton concentration gradient (ΔpH) and an electrical potential gradient, $\Delta\Psi$ (Nicholls, 1982). As ΔpH can have a value of three or four, the proton motive force across the thylakoid membrane consists almost entirely of ΔpH (Avron, 1981; Nicholls, 1982; Foyer, *et al.*, 1990). The $\Delta\tilde{\mu}H^+$ generated by electron transfer is used to drive an ATP - hydrolysing proton pump (the ATPase) backwards (i.e in the direction of ATP synthesis), thus linking phosphorylation of ADP to the efflux of protons from the lumen to the stroma.

This mechanism and the structure and function of the CF_0 - CF_1 complex will not be described in detail. The structure depicted in Figure 1.5 was adapted from that of Simpson (1990) with reference also to Gounaris, *et al.* (1986), Barber (1987) and Volkov (1989). As illustrated, the complex consists of two parts, the CF_0 and CF_1 units. The CF_0 intrinsic component consists of four polypeptides, those denoted I, II, III and IV, having molecular masses of 15 - 18, 11 - 15, 8 - 9, and 19 KDa respectively (Ort, 1986; Barber, 1987; Simpson, 1990). The CF_0 portion probably acts as a passive proton conducting channel. The CF_1 extrinsic protein consists of five subunits, designated alpha, beta, gamma, delta and epsilon with molecular masses of 59 - 60, 52 - 56, 32 - 39, 18 - 19.5 and 13 - 16 KDa, respectively (Ort, 1986; Barber, 1987; Simpson, 1990). The CF_1 subunit is the catalytic site for ATP synthesis or hydrolysis. The substrates for CF_1 are metal - nucleotide complexes and inorganic

Figure 1.5 Diagrammatic representation of ATP synthase (CF_0 -CF_1) illustrating the association between proton translocation from the lumen to the stroma and the production of ATP from ADP and phosphate. The subunit molecular masses in KDa are shown. See text for details. Adapted from Simpson (1990) with reference to Gounaris, *et al.* (1986), Barber (1987) and Volkov (1989).


CF1
CF0
ATP
3H+
α
β
52
α
59
β
α
β
32
γ
ε
16
19.5
δ
II
15
III
8
I
18
IV
19
3H+
ADP + Pi
STROMA
THYLAKOID
MEMBRANE
LUMEN

'V* . * * ~ , > fife ** £l-

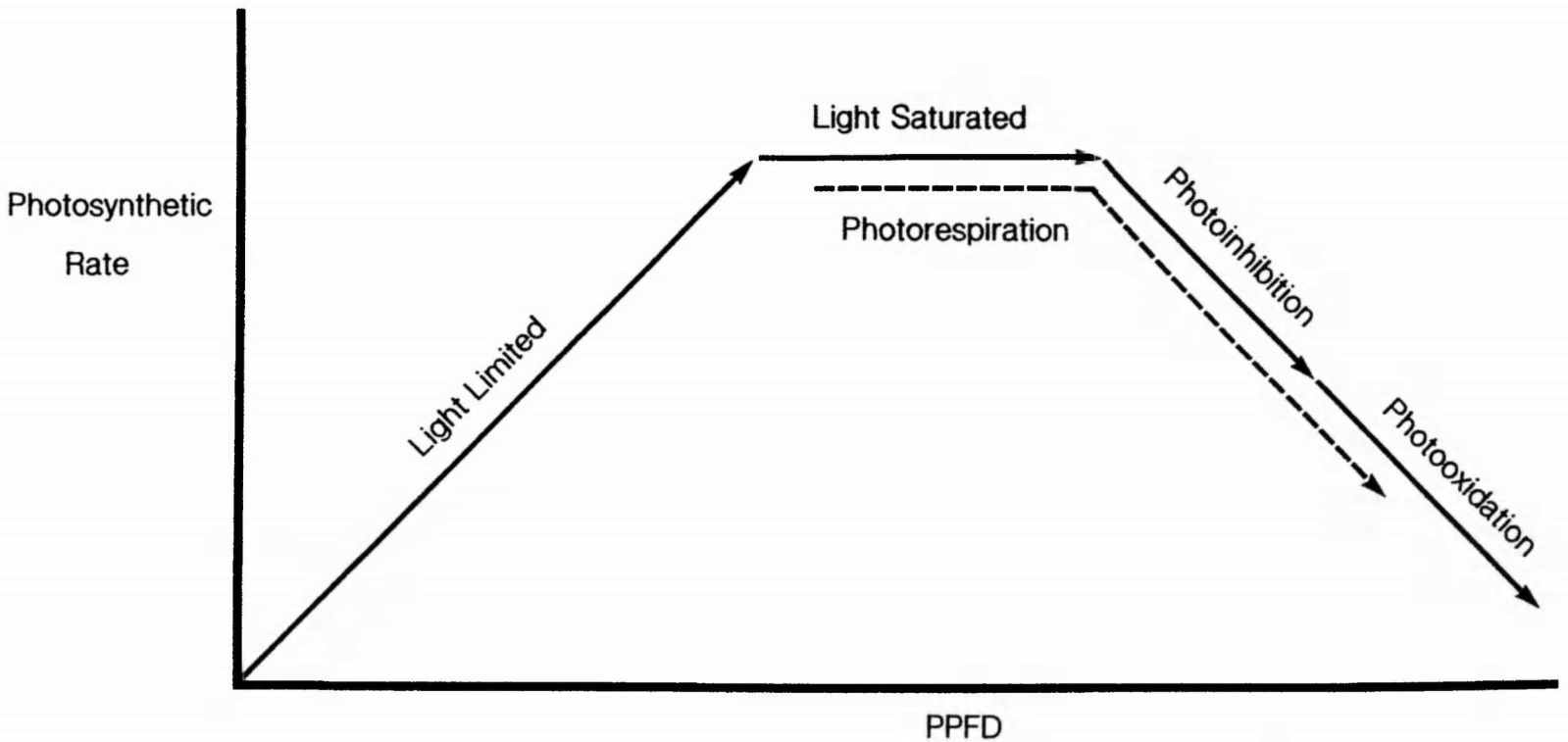
phosphate, with Mg^{2+} being the preferred cation. Three H^+ seem to be required to produce one ATP molecule although the exact stoichiometry may vary. For further details of the reaction mechanism see McCarty and Nalin (1986), Ort (1986), Strotmann (1986), and Barber (1987).

1.3 Definition of Photoinhibition

Photoinhibition can be broadly defined as a reduction in photosynthetic capacity on exposure to light (see Figure 1.6). One of the first investigations of the effect of intense light on photosynthesis was that of Emerson in 1935 and loss of photosynthetic capacity on exposure of algal cells in culture to high light intensities was observed by Myers and Burr (1940), Franck and French (1941) and Steeman - Nielsen (1952). In the following years work on photoinhibition increased and a number of descriptions and definitions of photoinhibition were put forward and refined. Kok (1956) concluded that intense light inhibited the primary photochemical process and that this process then lost its capacity for efficiently passing on light quanta, which reduced light - saturated photosynthesis. The description of the phenomenon was further embellished by Osmond (1981) who proposed that photoinhibition *in vivo* will be observed under any circumstances in which the rates of transfer of excitation energy from light - harvesting pigment assemblies (the antennae) to photochemical reaction centres are in excess of the rates of transfer from the reaction centres to the electron transfer chain (the transducers). This may occur in several ways, for example, in shade plants when transferred to full sunlight, due to relatively large concentrations of light harvesting pigments (antennae) with respect to reaction centres and electron transport intermediates (Anderson and Osmond, 1987). Environmental factors such as chilling (Oquist, *et al.*, 1987; Hayden and Baker, 1990), freezing, high temperature (Ludlow, 1987), and water stress (Boyer, *et al.*, 1987) can disrupt the organisation of the electron transport chain in the thylakoid membrane. Effective functioning of electron transfer can also be prevented when CO_2 and O_2 are not available as terminal electron acceptors of photosynthesis, so that ATP and NADPH cannot be utilised (Osmond, 1981; Krause and Cornic, 1987).

The photosynthetic apparatus may be damaged by wavelengths of light in the ultra - violet (UV), especially in the UV-B region of 280 - 320 nm (Renger, *et al.*, 1989); by light in the visible spectrum; and by interactions between visible and UV light. Thus,

Figure 1.6 Theoretical light saturation curve for photosynthesis. At low photosynthetic photon flux density (PPFD) photosynthesis is light limited and increases linearly with increasing PPFD. At optimal PPFD photosynthesis is light saturated and photosynthetic carbon reduction cycle turnover is limited mainly by CO_2 availability. However, further increase in PPFD will result in more excitation energy being transferred to the reaction centres than can be dissipated by the electron transport chain. This may result in a lesion primarily occurring within PSII which disrupts electron transport and reduces photosynthetic capacity (photoinhibition). Prolonged exposure to such supraoptimal PPFD may lead to the production of toxic free radicals from the light reactions of photosynthesis with the consequential destruction of photosynthetic pigments and thylakoid membranes (photooxidation). Although photorespiration is favoured at high PPFD, glycolate production may occur at any point along the light saturation curve depending upon the concentration of CO_2 and O_2. See text for details. From Williams (1986).


Photosynthetic
Rate
Light Saturated
Photorespiration
Light Limited
Photoinhibition
Photooxidation
PPFD

Powles (1984) further qualified photoinhibition as the reduction in photosynthetic capacity, independent of gross change in pigment composition, induced by exposure to visible light (400 - 700 nm). This definition also serves to separate the phenomena of photoinhibition and photooxidation. The latter is distinguished by Powles (1984) as the photodestruction of photosynthetic pigments that occurs on long - term exposure of plants or photosynthetic organelles to strong light, evident as oxygen and light - dependent bleaching of pigments. Prolonged treatments result in the death of the cell or organism. Usually, a time - and light - dependent decline of photosynthesis (photoinhibition) without changes in bulk pigment composition initially occurs in high light intensity, with photooxidation only ensuing if the light treatment is continued for a sufficient period of time. The developing model of photoinhibition was further expanded by Kyle and Ohad (1986), who suggested that photodamage occurs as a natural consequence of electron trasport resulting in turnover of the D1 protein of PSII and that photoinhibition is observed *in vivo* only when the rate of damage exceeds the rate of repair (removal and replacement of the D1 protein). Thus, photoinhibition may occur due to increased rates of damage, which will be enhanced by environmental stresses that reduce effective functioning of the electron transfer system (e.g. drought - induced limtitation of CO_2, O_2, $NADP^+$, ADP), or by decreased rates of repair (e.g. low nitrogen availability).

1.4 Site and Mechanism of Photoinhibition

It is now generally accepted that photosystem II is the primary site of photoinhibition and studies with a range of photoinhibited samples from leaves to isolated PSII protein complexes have indicated that the first site of damage is in the PSII reaction centre (Powles and Bjorkman, 1982; Tytler, *et al.*, 1984; Barenyi and Krause, 1985; Cleland and Critchley, 1985; Krause, *et al.*, 1985; Arntz and Trebst, 1986; Cleland, *et al.*, 1986; Theg, *et al.*, 1986; Demeter, *et al.*, 1987; Ohad, *et al.*, 1988) or close to the sites of quinone binding (Kyle, *et al.*, 1984; Kyle and Ohad, 1986; Nedbal, *et al.*, 1986; Allakhverdiev, *et al.*, 1987). All these results suggest that the primary sites of damage are localised in the D1 and D2 proteins, but until the recently successful isolation of the D1/ D2/ $cytb_{559}$ complex by Chapman, *et al.* (1989) studies of changes at these sites in PSII were hampered by the presence of more pigment molecules and extra protein components than those involved directly in the

photochemical reactions which become inhibited.

The yield and kinetics of the fluorescence emitted from the chlorophyll pigments within the thylakoid membrane can be used as an indicator of perturbations of the organisation of the membrane (*e.g.* Baker and Horton, 1987; Bjorkman, 1987; Krause and Somersalo, 1989). Due to differences in the wavelengths of fluorescence emission between the two photosystems, perturbations of PSI can be distinguished from those in PSII. Kyle (1987) reported that in cells of the green alga *Chlamydomonas reinhardtii* exposed to photoinhibitory treatments there was a marked decline in the fluorescence yield from PSII (F_{685} and F_{695}) with little effect on that of PSI (F_{715}). Similar results were reported by Bjorkman, *et al.* (1981) after exposure of *Nerium oleander* leaves to supra - optimal light levels and in photoinhibited cucumber (*Cucumis sativus*) leaves (Critchley and Smillie, 1981). Such experiments using isolated thylakoids have produced conflicting results possibly due to increased photosensitivity of these systems and variations in preparations produced by different workers. In a study using isolated spinach thylakoids Cleland and Critchley (1985) found results in agreement with those above; a decline in PSII fluorescence, but not in PSI fluorescence. However, Barenyi and Krause (1985) found a decrease in the fluorescence emissions from both PSII and PSI. Experiments involving the isolation of chloroplasts from treated leaves and the characterisation of the partial reactions of photosynthetic electron transport using assays specific to either photosysem in a variety of organisms, generally confirm the fluorescence data, indicating that the primary lesion is associated with PSII. For example, Critchley (1981) showed that PSII activity, measured polarographically as electron flow from water to potassium ferricyanide, decreased significantly after photoinhibitory treatment of cucumber (*Cucumis sativus* L.) leaves. However, PSI activity, measured as electron flow from reduced 2, 6 - dichlorophenol - indophenol / iso - ascorbate to methyl viologen in the presence of DCMU, was not significantly altered by the treatment. Similar results have been obtained using different organisms (*e.g.* Tytler, *et al.*, 1984; Nedbal, *et al.*, 1986). In *Chlamydomonas reinhardtii* cells and isolated *Pisum sativum* thylakoids overall electron transport from water to 2, 6 - dichlorophenol - indophenol was found to decline dramatically on photoinhibitory treatment, whereas Q_B - independent PSII activity (water to silicomolybdate ($SiO_2.12MoO_3$)) and PSI activity (N, N, N^1, N^1 - tetramethyl - P - phenylenediamine (TMPD) to methyl viologen) were much less affected, suggesting that the primary

lesion in electron transport is at the Q_B site of the PSII D1 protein, with little damage to either the OEC water splitting enzymes or the primary reaction centres (Kyle, *et al.*, 1984; Ohad, *et al.*, 1985). It was also apparent that the number of atrazine binding sites, and hence Q_B binding sites, was reduced by the photoinhibitory treatments, further implicating the D1 protein in photoinhibition by high light. However, in the cyanobacterium *Microcystis aeruginosa* both Q_B - dependent (water to diaminodurine) and Q_B - independent (water to silicomolybdate) PSII activities were lost on photoinhibition, suggesting damage to the reaction centre itself in this organism (Tytler, *et al.*, 1984). However, silicomolybdate physically disrupts the PSII complex, displacing the D1 protein slightly and thus exposing the Q_A site for oxidation. Such disruption necessitates care when making conclusions based on this activity alone. The reduction of Q_A can be measured directly by an optical change at 320 nm and Cleland, *et al.*(1986) found that such Q_A reduction was lost with loss of PSII activity suggesting that Q_A may be the primary site of damage. This supports the earlier finding that photoinhibition resulted in the loss of water to silicomolybdate (Q_B - independent) PSII activity in thylakoids isolated from spinach (Cleland and Critchley, 1985) and in the cyanobacterium *Microcystis aeruginosa* (Tytler, *et al.*, 1984). Electron spin resonance (ESR) or electron paramagnetic resonance (EPR) spectroscopy is an effective way of detecting molecules or atoms which have unpaired electrons (Goodwin and Mercer, 1983). The carrier Y_z^+ formed by the reduction of $P680^+$ has a characteristic ESR signal. Kyle, *et al.* (1984) found that photoinhibited *Chlamydomonas reinhardtii* cells exhibited no significant change in the Y_z^+ signal indicating that Y_z was still efficiently oxidised, suggesting that there was no initial damage to reaction centre or charge separation activity. Thus, it seemed that generally Q_B - dependent activity was lost before Q_B - independent activity, implying that the initial site of photoinhibition damage was at the Q_B binding position, but that this was rapidly followed by damage to the reaction centre itself. In more recent studies using *Chlamydomonas reinhardtii* a change in the stability of the Q_B^- binding site, without affect on the primary photochemistry of the reaction centre, as indicated by thermoluminescence and fluorescence measurements, provided further evidence that the Q_B position was the initial site of photoinhibitory damage (Ohad, *et al.*,1988, 1990). Additional changes were also apparent in a fraction of the reaction centre population, which affected the electron donor to $P680^+$, the tyr - 161 residue, Y_z. The impairment of $P680^+$ reduction

apparently correlated with an irreversible modification of D1, possibly caused by cross - linking of trans - membrane helices II and III within the polypeptide. It was further suggested that this cross - linking may be caused by interaction with free radicals, which are implicated in photoinhibition (Ohad, *et al.*, 1990).

A further means of studying the site of damage caused by photoinhibition is by investigating repair and recovery from the damage. Recovery from photoinhibition has been shown to be dependent on chloroplast directed, but not nucleus directed, protein synthesis in *C.reinhardtii* cells (Ohad, *et al.*, 1984), which further supports evidence that PSII is the primary target of photoinhibition, as all the intrinsic PSII polypeptides are chloroplast encoded. Similar results were found with *Anacystis nidulans* (Sammuelsson, *et al.*, 1985, 1987; Nedbal, *et al.*, 1986). This also shows that protein damage occurs during photoinhibition and that the damaged protein must be replaced by *de novo* protein synthesis. When *C.reinhardtii* cells were prelabelled with $^{35}SO_4$ before photoinhibitory treatment, the kinetics of the removal of the D1 protein apparently correlated with the loss of photochemical activity (Ohad, *et al.*, 1984). Similar results were observed by Edelman, *et al.* (1984). However, there was a slight lag in loss of D1 protein with respect to loss of Q_B - dependent activity, but reaction centre activity and other core proteins were lost at a significantly slower rate (Kyle, 1987). In contrast, Cleland and Critchley (1985) reported that photoinhibition was due to destruction of the PSII reaction centre with no increased destruction or turnover of the D1 protein. It was suggested that a lack of D1 turnover may be observed due to the presence of label in newly synthesized unintegrated protein, with a lag period between insertion of newly translated protein and its integration into a site where photoinhibition may result in damage and turnover (Callahan, *et al.*, 1987). Using pulse - chase labelling of *Spirodela oligorrhiza* with ^{35}S - methionine these workers found that after the pulse all of the incorporated activity was in the stroma lamellae fraction with a 33.5 KDa precursor protein as the dominant band. After 120 minutes chase virtually all of the label was associated with the 32 KDa protein within the granal fraction. The model developed to explain their results was as follows. Light - dependent synthesis of a 33.5 KDa precursor protein occurs on the 70s thylakoid bound ribosomes, attached to the stromal lamellae. Following processing, the resulting 32 KDa protein is integrated into the membrane and translocated to the granal lamellae, where it may be assembled with a PSII core complex deficient in the D1 protein. As a consequence of its function, light

- dependent damage of the D1 protein may occur and the D1 protein migrates back to the non - appressed lamellae to undergo proteolysis, whilst its place is taken with more newly synthesized protein. In more recent work a reversible conformational change of D1 causing destabilisation of Q_B^- , followed by modification of the D1 protein resulting in inactivation of Y_z, the reductant of $P680^+$, which in turn may lead to irreversible modification of D1 has been suggested as the squence of events associated with D1 turnover (Adir, *et al.*, 1990). It is proposed that the irreversible modification of D1 acts as a recognition signal for proteolysis by a highly efficient and specific membrane bound protease. Thus, there is still controversy concerning the exact site of initial damage. One view is that Q_A is the primary site of damage, its modification resulting in the characteristic changes of PSII electron transport and chlorophyll fluorescence, and that the increased D1 degradation outstripping its synthesis and thus the net loss of the protein, is the cause of photoinhibition (Kyle, *et al.*,1984; Ohad, *et al.*, 1984). Whereas Cleland and Critchley (1985) propose that photoinhibition is due to destruction of the PSII reaction centre and that D1 turnover is a consequence rather than the cause of photoinhibition.

Inactivation of the water splitting enzyme complex in leaves or isolated chloroplasts has been found to result in increased susceptibility of PSII to photoinhibition (Callahan and Cheniae, 1985; Callahan, *et al.*, 1986) indicating another distinct site of photoinhibition damage near the donor to the PSII radical pair. Also a correlation between photoinhibitory D1 degradation and manganese release from the thylakoid membrane has been reported (Virgin, *et al.*, 1988). This suggests that the D1 protein is directly or indirectly associated with the Mn ions required for oxygen evolution activity and possibly supports a role in photoinhibition of the oxidising side of PSII as suggested by Callahan and Cheniae (1985). Inhibition of electron transport through PSI by exposure of the photosynthetic apparatus to excess visible light has been reported and the site of this inhibition appears to be close to the PSI reaction centre (Powles, 1984). Photoinhibition of PSI is thought to be dioxygen dependent whilst some work indicates that photoinhibition of PSII is dioxygen independent (Asada and Takahashi, 1987). This suggests the occurrence of a different phenomenon in the two photosystems and photoinhibition of PSI can be largely prevented by DCMU blockage of PSII electron transport. However, Krause (1988) reports that although PSI is readily affected when isolated chloroplasts are subjected to high light treatments (*e.g.* Krause, *et al.*,

1988) little effect on PSI is observed *in vivo*. The reason for this discrepancy is unknown. Any inhibition of photosynthesis via PSI, and indeed via PSII, may involve the formation of reactive molecules such as hydrogen peroxide, hydroxyl radicals, superoxide and singlet oxygen.

The mechanism of photoinhibitory damage of the D1 protein is thought to be a consequence of its function. As has been discussed the secondary quinone acceptor (Q_B) is a two electron gate. An electron generated at the reaction centre by charge separation is transferred via the primary quinone acceptor, Q_A, to Q_B which is stabilised as the reduced semiquinone, Q_B^-, by the protein. A second electron results in the formation of the unstable doubly reduced quinone, Q_B^{2-}, which is rapidly protonated forming plastoquinol. The oxidised quinone is not tightly bound to the Q_B protein and may not reside on the protein for a significant length of time, whereas the reduced semiquinone anion is very tightly bound. On double reduction and protonation the binding affinity is again reduced and the plastoquinol is released from the binding site. To carry out its function the D1 protein must be successful at stabilising the reactive reduced semiquinone radical species, Q_B^-, preventing reoxidation. Thus, as with many enzymes involved with reactions concerning oxygen, this protein is at a relatively high risk of oxidative damage (Asada and Takahashi, 1987). There is some evidence to suggest that the semiquinone anion radical is implicated in the mechanism of D1 damage. For example, addition of the herbicides atrazine or diuron, which compete with the quinone for the Q_B position on the D1 protein, before a photoinhibitory treatment of *C. reinhardtii* cells partially protected the thylakoid membrane from photoinhibition (Kyle, *et al.*, 1984). However, the semiquinone is generally thought not to have the oxidation strength to cause significant damage itself (Asada and Takahashi, 1987). The process of photosynthesis results in elevated local cocentrations of molecular oxygen and autooxidation of the semiquinone could thus produce superoxide ($O_2^{\cdot-}$) molecules in the proximity of the quinone binding site (Kyle, *et al.*, 1984). This could also enable hydrogen peroxide production due to the presence of a hydrogen donor in the form of a histidine residue at the Q_B binding site, and hydroxyl radical and singlet oxygen ($^1O_2$) production, all of which are highly reactive species which are potentially very damaging (Asada and Takahashi, 1987). An alternative mechanism for D1 protein damage involving dioxygen more directly has also been suggested (Kyle, 1987). At supra - optimal light intensities there will be an excess of dioxygen and a high plastoquinol to

oxidised plastoquinone ratio. Due to the low affinity of plastoquinol the Q_B binding sites may be largely unoccupied despite electrons awaiting transfer from Q_A^-. This may allow dioxygen to act as a substrate analogue of plastoquinone, binding at the Q_B site, accepting an electron from Q_A and forming $O_2^{\cdot-}$ directly. This again could lead to production of hydrogen peroxide, hydroxide radicals and singlet oxygen, and such reactive species may readily attack proteins leading to inactivation of function. Apparently not much is known concerning such radical attack on proteins, however, Levine (1983) has reported that the oxidation of a histidine residue in glutamine synthase causes loss of activity and acts as a recognition signal for proteolysis of the enzyme. This is interesting with respect to the apparent presence of histidine at the D1 protein Q_B site.

The superoxide radical ($O_2^{\cdot-}$) can dismutate to form hydrogen peroxide either spontaneously or catalytically with superoxide dismutase (Asada and Takahashi, 1987):

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \text{-----} > H_2O_2 + O_2$$

Hydrogen peroxide can also be formed by the reduction of $O_2^{\cdot-}$ by ascorbate, thiols, ferredoxin and manganous ions. The hydroxyl radical ($HO^{\cdot}$) can be produced in cells from $O_2^{\cdot-}$ through the metal - catalysed Haber - Weiss reaction:

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \text{----} > H_2O_2 + O_2$$

$$H_2O_2 + Fe(II)(\text{or } Cu(I)) \text{---} > HO + OH + Fe(III)(\text{or } Cu(II))$$

$$O_2^{\cdot-} + Fe(III)(\text{or } Cu(II)) \text{---} > O_2 + Fe(II)(\text{or}Cu(I))$$

$$3O_2^{\cdot-} + 2H^+ \text{----} > 2O_2 + HO + OH$$

Hydroxyl radicals can also be produced when Fe(III) or Cu(II) are reduced by reductants such as paraquat, cation radicals, semiquinone radicals, thiols and ascorbate. Hydroxyl radicals may also be produced from hydrogen peroxide as follows:

$$Fe\ (III) + H_2O_2 \text{----} > Fe\ (II) + 2H^+ + O_2^{\cdot-}$$

$$Fe\ (III) + O_2^{\cdot-} \text{-----} > Fe\ (II) + O_2$$

$$Fe\ (II) + H_2O_2 \text{-----} > Fe\ (III) + HO + OH$$

Singlet oxygen ($^1O_2$) may also be formed from the other active species of oxygen. For example, it is known to be produced directly from H_2O_2 and $O_2^{\cdot-}$ (Asada and Takahashi, 1987). Singlet oxygen and the superoxide radical can also be formed when the excitation energy or the electron of excited triplet state chlorophyll ($^3chl^*$) is transferred directly to triplet oxygen ($^3O_2$). This is a type II photosensitized oxidation. Interaction

of the triplet sensitizer ($^3chl^*$) with compounds other than oxygen (e.g. cell components) is designated a type I reaction and if the substrate (S) is transformed to radicals ($S^\cdot$), these can in turn interact with $^3O_2$ to produce $O_2^{\cdot-}$ (Foote, 1976). Thus, active oxygen species can be produced in many ways and the presence of one form of reactive species can readily lead to the production of others. Therefore, if the mechanism of photoinhibition initially involves the production of $O_2^{\cdot-}$ it is quite possible that these other reactive species are also produced. Such reactive molecules can result in photooxidative damage as lipids in chloroplast membranes contain large amounts of polyunsaturated fatty acids, photooxidation of which causes fragmentation of membranes, thylakoid disruption and pigment bleaching. It is evident that there is still some uncertainty as to the exact mechanisms of photoinhibition and the number and relative importance of different sites of damage. Controversy probably results from extrapolations of results produced using isolated chloroplasts and thylakoids to explain *in vivo* phenomena, and *vice versa*. Many metabolic functions are likely to be involved and it is therefore probable that the amount and mechanism of photoinhibition may differ from the *in vivo* situation. However, there are also means by which photosynthetic organisms can prevent, reduce or tolerate photoinhbition and any associated damage.

1.5 Tolerance of Photoinhibition

Experiments using *Chlamydomonas reinhardtii* (Wettern *et al.*, 1983; Ohad, *et al.*, 1984) or *Spirodela oligorhiza* (Hoffman - Falk, *et al.*, 1982; Edelman, *et al.*, 1984) have demonstrated a light intensity - dependent turnover (removal and replacement) of the D1 protein of PSII and a correlation between photoinhibition and rate of D1 turnover has been reported (Kyle, *et al.*, 1984). Kyle and Ohad (1986) concluded that light causes damage to the D1 protein and photoinhibition may be dependent upon D1 protein turnover, only occurring if the rate of damage exceeds the rate of repair. The possible mechanisms by which the D1 protein may be damaged have already been discussed. Once damaged, the D1 protein must be either repaired, or removed and replaced. Ohad, *et al.* (1984) have shown that prelabelled D1 protein is physically removed from the membrane during photoinhibition of *Chlamydomonas reinhardtii* cells, and recovery of photoinhibited cells required chloroplast directed protein synthesis. A much more rapid and extensive loss of D1 activity occurs if

chloramphenicol is included during the photoinhibition treatment, due to the inhibition of replacement of the damaged D1 protein (Kyle and Ohad, 1986). It was suggested, therefore, that recovery from photoinhibitory damage involves removal and replacement using protein synthesized *de novo* (*i.e.* turnover) rather than a repair of the damaged protein. Indeed, it has been shown that recovery of photosynthetic activity depends not only on the ability to synthesize replacement D1 protein, but also on the removal of the damaged protein. The light - induced rapid turnover of the D1 protein requires efficient transcription and translation of the gene coding for the D1 protein (*psb* A). It is thought that regulation of D1 protein synthesis is at the translational or post - translational level, and there are very high levels of the mRNA transcripts in the chloroplast, reflecting a situation which is primed for rapid *de novo* protein synthesis when the signal (protein damage) is recognised (Kyle and Ohad, 1986). Similarly, Huse and Nilsen (1989), using *Lemna gibba,* found evidence supporting the suggestion that synthesis of the D1 protein is necessary for recovery of photosynthesis after photoinhibition, and that the balance between repair and destruction of the D1 protein is a key factor in regulating the rate of recovery from photoinhibition. The light - induced inactivation of the reaction centre of PSII, its recovery and the turnover of the D1 protein have been further studied (Adir, *et al.*, 1990; Ohad, *et al.*, 1990) and associated with a sequence of events as follows. It is proposed that there is an initial reversible conformational change of D1 causing a destabilisation of Q_B^- at low light intensities. Further exposure to high light intensities may result in the inactivation of Y_z, the reductant of $P680^+$, leading to an irreversible modification of the D1 protein. This may expose the PEST sequence, located in the hydrophilic segment connecting loops IV and V of the D1 protein, which is sensitive to proteolytic degradation, thus providing a recognition signal for the proteolysis of the modified D1 by a highly efficient, intrinsic membrane protease (Ohad, *et al.*, 1985). The modified reaction centre II may then migrate from the grana to the non - appressed thylakoid membranes where a newly synthesized D1 precursor protein is integrated in the D1 - depleted reaction centre II and processed to the mature form (D1), thus regenerating a new active reaction centre II. The functional reaction centre II can then be translocated back to the grana. Recovery after photoinhibition by means of replacement and repair of the D1 protein can in itself be looked upon as a method by which photoinhibition is tolerated. That is, the rapid light - dependent turnover of this reaction centre II component might act as a protective

mechanism against total destruction of the reaction centre II, with D1 acting as a suicide protein designed to be the target of photodamage (Schuster, *et al.*, 1988b), preventing extensive photooxidative damage to the entire photosynthetic apparatus. Wild, *et al.* (1990) have suggested that the D1 protein may have a function in thylakoids that is analagous to that of a fuse in an electric circuit. In the situation of an energy overload widespread damage that is difficult to repair is prevented by rapid destruction of the fuse component, thus switching off the system function.

In situations of excess excitation energy a limited amount may be dissipated as fluorescence and a more substantial amount by non - radiative decay, i.e. heat (Powles, 1984). Increases in the rate constants of thermal deactivation of excited pigments are regarded as controlled protective mechanisms and two such processes are described by Krause (1988). Firstly, increased thermal deactivation related to energy - dependent fluorescence quenching (q_E) which allows the photosynthetic apparatus to respond within seconds to minutes to changes in light conditions. This is related to a high trans - thylakoid proton gradient. The protection of the q_E mechanism has been observed by the increased photoinhibition of uncoupled isolated chloroplasts which have reduced ΔpH and q_E. However, the ΔpH itself is not responsible for the protection as antimycin A, which inhibits q_E without altering the ΔpH reduces the protective effect (Horton, *et al.*, 1988; Krause, *et al.*, 1988). A negative correlation between q_E and photon yield of photosynthetic CO_2 - dependent O_2 evolution was found (Krause, *et al.*, 1988) which was suggested to represent a control mechanism adjusting thermal dissipation of excess excitation energy to the photon requirement of energy consuming carbon metabolism. It was observed that the calculated photon yield of open reaction centres decreased with increasing q_E (Horton and Hague, 1988). Weis and Lechtenberg (1989) proposed a model to explain such results, in which PSII was suggested to exist in three states. A photochemically active state, $PSII_\alpha$, is converted to an inactive state with closed centres, $PSII_\alpha^-$, by reduction of Q_A. The $PSII_\alpha$ active state may accumulate during light - limited photosynthesis, but upon formation of a high ΔpH the $PSII_\alpha$ form is converted to $PSII_\epsilon$, another photochemically inactive 'quenching' state. In this latter state excitation energy is dissipated by non - radiative, non - photochemical de - excitation. Thus, changes in fluorescence quenching and in quantum yield of electron transport indicate changes of the proportion of the three forms of PSII, with the quantum efficiency being adjusted to the energetic balance of the leaf. The mechanism linking the interconversion between

different PSII states to the ΔpH of the membrane and the mechanism of energy quenching are unknown. Several quenching mechanisms may contribute to the 'high - energy quenching'; it has been proposed that excess energy can be dissipated by a 'futile' cycle of electrons around PSII (Schreiber and Neubauer, 1987; Falkowski, *et al.*, 1988; Horton, *et al.*, 1989). The pathway of such cyclic PSII electron transport is unknown but it has been suggested that the Q_B site and the cyt b_{559} of PSII are involved (McCauley, *et al.*, 1987; Schreiber and Neubauer, 1987; Falkowski, *et al.*, 1988; Horton, *et al.*, 1989). Thompson and Brudvig (1988) have proposed that photoinhibition of PSII is due to $P680^+$ oxidizing the last antenna chlorophyll, chl_z, which links the antenna to the reaction centre, and that this is involved in cyclic PSII electron transport. They suggest that when electrons are passed from P680 to the PQ pool via Ph, Q_A and Q_B in the main electron transport chain, the resulting $P680^+$ may oxidize chl_z. However, electrons may be passed from the PQ pool to cyt b_{559} (Ortega, *et al.*, 1989) and then to chl_z maintaining it in the reduced form. Chl_z may donate electrons to $P680^+$ completing the cycle. This cyt b_{559} cycle could play a role in protection against photoinhibition.

A second, slower, at least partly reversible process described by Krause (1988) is increased thermal deactivation related to the photoinhibitory fluorescence quenching (q_I) which takes place within minutes to hours. This is also associated with a decline in the ratio of variable to maximum fluorescence (Fv/Fm). The q_I mechanism appears important in preventing destruction of the photosynthetic apparatus, but the molecular mechanism of the photoinhibitory quenching and its protective function is unclear. The decline in the Fv/Fm ratio correlates with the decrease of quantum yield of oxygen evolution (Demmig and Bjorkman, 1987) and although it is related to a transient inhibition of photosynthesis it may be regarded as a protective mechanism (Somersalo and Krause, 1987). This phenomenon was further investigated and the non - photochemical fluorescence quenching resolved into three components; a rapid relaxation phase associated with q_E, a slower phase suggested to be associated with state II - state I transition, and the slowest phase associated with photoinhibition - induced fluorescence quenchinq (Horton, *et al.*, 1987; Hodges, *et al.*, 1988; Horton and Hague, 1988). It was concluded that the contribution of photoinhibition - associated quenching to the non - photochemical quenching in high light indicated that this process, like q_E, is an adaptive and useful dissipative process. Thus, several mechanisms may be

involved in q_I such as D1 protein degradation. A correlation between q_I and the formation of zeaxanthin in preference to violaxanthin and ß - carotene has led to the proposal of a protective role of the xanthophyll cycle (Demmig, *et al.*, 1987). Zeaxanthin is thought to be involved in the removal of excess excitation energy via radiationless energy dissipation in the antenna chlorophyll (Adams, *et al.*, 1989; Bilger, *et al.*, 1989; Demmig - Adams, *et al.* 1989a, 1989b, 1990; Adams, 1990). Interestingly, a high trans - thylakoid ΔpH, which is implicated in protection against photoinhibition, also promotes the formation of zeaxanthin (Demmig - Adams, *et al.*, 1989a).

State transitions (*e.g.* Fork and Satoh, 1986; Gounaris, *et al.*, 1986; Staehelin, 1986; Iordanov and Goltsev, 1987; Anderson and Andersson, 1988) are considered to have a role in the prevention of photoinhibition by enabling efficient photosynthesis to be achieved by maintaining a suitable ratio of activities of the two photosystems. Under conditions where the plastoquinone pool is over reduced (e.g. in excess PSII light - 'state 2 conditions') a membrane bound kinase is activated which catalyses phosphorylation of the LHC II complex at one or two threonine residues. When the kinase is not activated (e.g. in excess PSI light or in the dark - 'state 1 conditions') a membrane bound phosphatase dephosphorylates the LHC II. In the non - phosphorylated form the mobile LHC II is closely associated with the PSII complexes in the appressed regions ('state 1'). The phosphorylation introduces a negative charge onto the LHCII surface increasing repulsive electrostatic forces between adjacent LHCII complexes on neighbouring membranes acting in a direction perpendicular to the membrane, initiating lateral diffusion into the PSI enriched non - appressed membranes, so that the LHCII is dissociated from the PSII complex ('state II'). It has been suggested that two different complexes become phosphorylated (Allen and Holmes, 1986a): a peripheral light harvesting complex (i.e. mobile LHCII) and an intermediate light harvesting complex that is tightly bound to PSII. This would result in electrostatic repulsion of the two phosphorylated complexes in a direction parallel to the membrane, resulting in the mobile LHCII becoming dissociated from the PSII complexes. This model also suggests that thylakoid stacking and lateral heterogeneity of complexes are unnecessary for regulation of excitation energy distribution by a mechanism involving protein phosphorylation and that this mechanism as studied in higher green plants is simply a special case of a more general phenomenon. This enables an explanation of

similar mechanisms observed in other organisms that do not have heterogenous distribution of photosystems (e.g. cyanobacteria, the purple photosynthetic bacteria *Rhodospirillum rubrum*, *Rhodopseudomonas sphaeroides*).

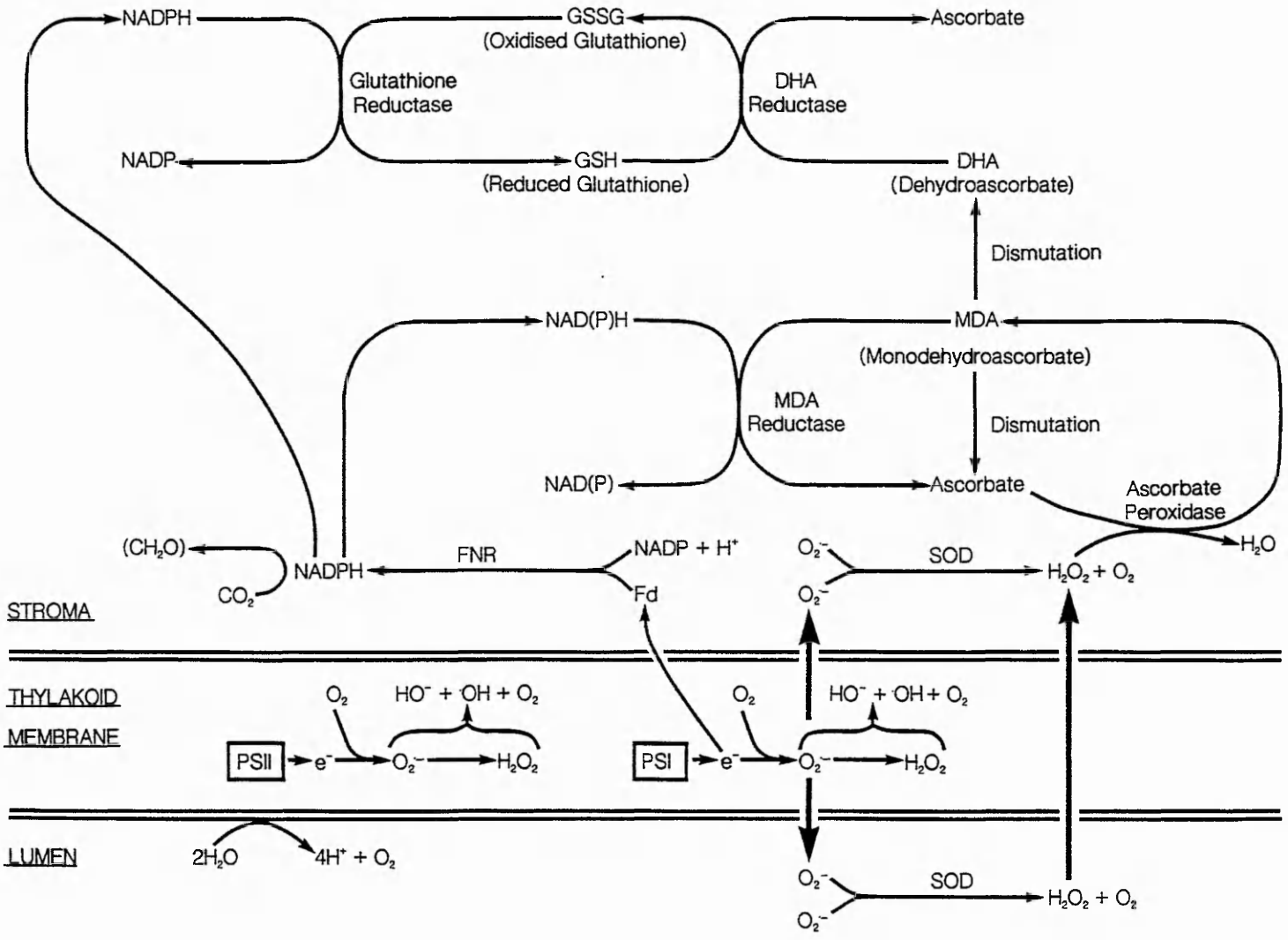
The details of this regulatory process remain unclear. It has been suggested that in state 2, excitation energy diverted away from PSII is instead utilized by PSI and *vice versa*, this redistribution of excitation energy being mediated by the lateral migration of the mobile LHCII (*e.g.* Allen and Holmes, 1986a; Fork and Satoh, 1986; Gounaris, *et al.*, 1986). An alternative view is that state 2 transitions involve a decrease in the excitation energy available to PSII reaction centres without a redirection such that the absorption cross -section of PSI is increased (Allen and Melis, 1988). That is, state 1 - state 2 transitions only regulate the excitation energy transfer from the LHCII to the PSII reaction centre. It is also unclear whether LHCII diffuses alone or whether some PSII - LHCII complexes also move into non - appressed regions. If this occurs there would be a possibility not only of changes in the absorption cross section of the two photosystems, but also in changes in energy transfer between PSII and PSI (spillover). However, the major process is thought to involve a change in absorption cross section due to migration of the LHCII alone (Barber, 1987). Spillover of energy from PSII to PSI was implicated in the avoidance of photoinhibition in the red alga *Porphyra perforata* (Oquist and Fork, 1982), whilst further studies with this alga led to the suggestion of another mechanism, termed state 2 - state 3 change, in which the measured PSII fluorescence declined without increase in PSI fluorescence (Satoh and Fork, 1983a, 1983b, 1983c). This was believed to indicate a lower energy transfer from the antennae to the PSII reaction centre, reducing the chance of photoinhibition. Thus, whatever the mechanisms involved, state transitions will enhance either the efficient distribution of excess excitation energy between PSI and PSII and / or the alleviation of over excitation of PSII, which will in turn contribute to the avoidance of photoinhibition as PSII is the site of the primary lesion.

Various systems exist that minimize photoinhibitory damage by scavenging or preventing the formation of radicals or other reactive molecular species, particularly those derived from oxygen. Carotenoids (e.g. ß - carotene) deactivate triplet chlorophyll and quench singlet oxygen to its triplet ground state (e.g. Asada and Takahashi, 1987). The superoxide dismutases, together with ascorbate peroxidase protect efficiently against $O_2^{\cdot-}$ and OH radicals and H_2O_2 (Krause, 1988). Ascorbate, glutathione and α -

tocopherol have all been implicated in the dissipation of singlet oxygen or superoxide (Powles, 1984). It is probable that α - Tocopherol is involved in trapping lipid radicals ($L^{\cdot}$, $LO^{\cdot}$, $LOO^{\cdot}$) and suppressing lipid peroxidation rather than in singlet oxygen scavenging (Asada and Takahashi, 1987). As has been mentioned the presence of one species of active oxygen can readily lead to the formation of other reactive species and the production and scavenging of superoxide and hydrogen peroxide is summarised in Figure 1.7. This scheme, which was adapted from Asada and Takahashi (1987), depicts dioxygen reduction at both PSI and PSII. The subsequent mechanism of scavenging could operate to remove active species produced at either photosystem, the pathway is shown from PSI only in the diagram for clarity. The superoxide radicals may dismutate to hydrogen peroxide and dioxygen spontaneously or via SOD catalysis. The H_2O_2 diffuses into the stroma and is reduced by ascorbate peroxidase to water, forming the ascorbate radical, monodehydroascorbate. Most of the monodehydroascorbate is directly reduced to ascorbate by MDA reductase. When NAD(P)H is limited MDA radicals may be dismuted to ascorbate and dehydroascorbate, which in turn may be reduced by DHA reductase using reduced glutathione. Glutathione reductase regenerates reduced glutathione using NADPH. MDA reductase may use both NADH and NADPH, but the rate for NADH is higher. The final electron donor is reduced ferredoxin. Under stress conditions the concentration of active oxygen is likely to increase due to increased production and / or lower capacity to scavenge, leading to photoinhibition and photooxidation. A recent study has supported the involvement of $O_2^{\cdot-}$ free radicals in the damage of the D1 protein and also found that the free radical scavengers propylgallate and uric acid inhibited the D1 protein degradation without affecting linear electron flow, showing that free radical scavengers may have a role in protection against photoinhibition (Sopory, *et al.*, 1990).

It has been suggested that photorespiratory carbon metabolism aids in protection against photoinhibition, acting as an energy consuming process enabling dissipation of excess excitation energy (Heber and Krause, 1980; Osmond, 1981; Krause and Cornic, 1987; Krause and Laasch, 1987; Krause, 1988). The photosynthetic carbon reduction cycle and its integration with the photorespiratory carbon oxidation cycle is illustrated in Figure 1.8. During C_3 photosynthesis, oxygen competes with carbon dioxide at the catalytic site of ribulose - 1, 5 - bisphosphate carboxylase / oxygenase. Oxygenation of ribulose 1, 5 - bisphosphate produces phosphoglycolate which is converted to

Figure 1.7 Production and scavenging of superoxide and hydrogen peroxide in chloroplasts. Key: Fd, ferredoxin; FNR, ferredoxin - $NADP^+$ oxido - reductase; NAD, oxidized nicotinamide adenine dinucleotide; NADP, oxidized nicotinamide adenine dinucleotide phosphate; SOD, superoxide dismutase. See text for details. Adapted from Asada and Takahashi (1987).

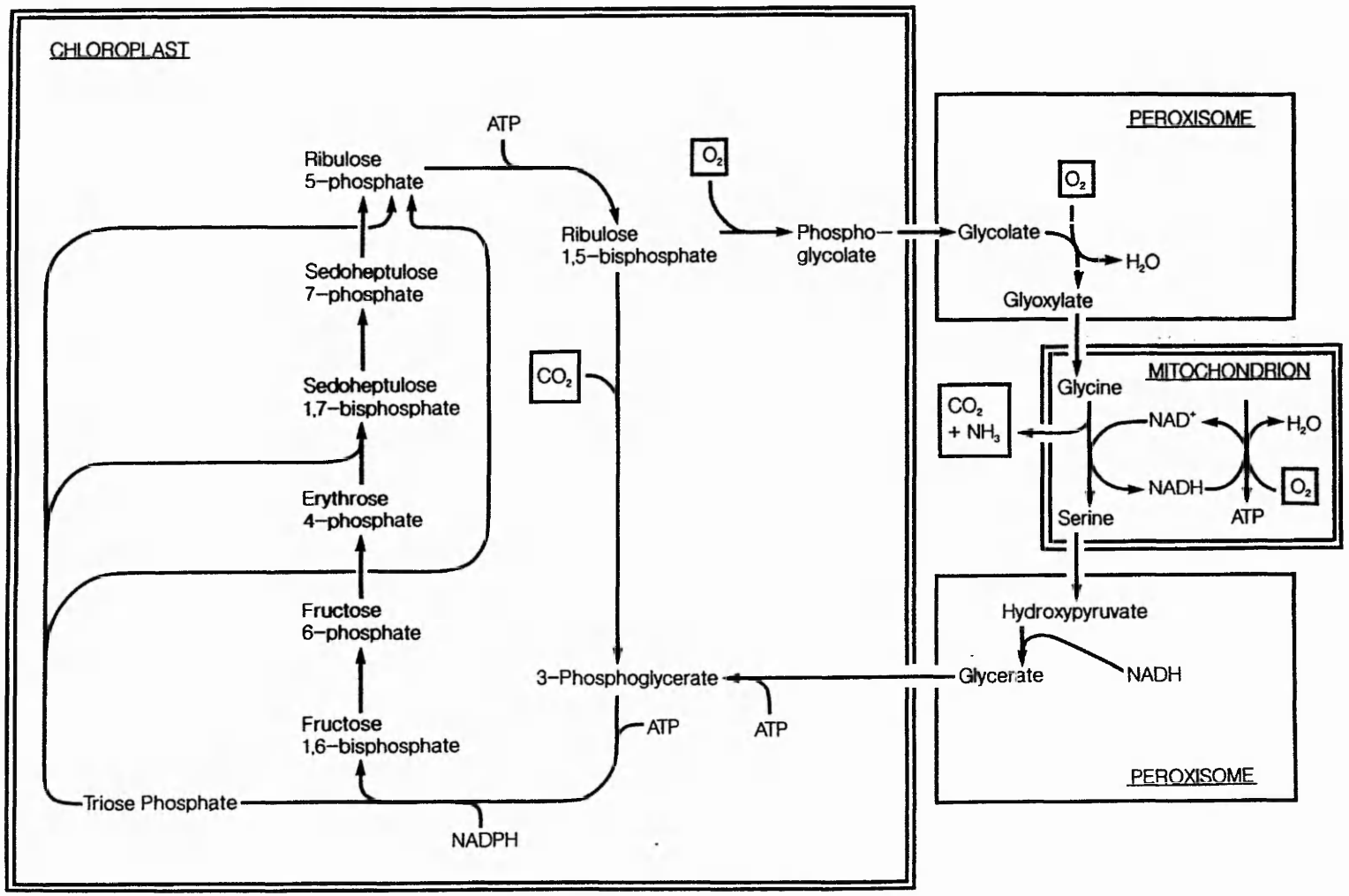

NADPH
GSSG
(Oxidised Glutathione)
Ascorbate
Glutathione
Reductase
DHA
Reductase
NADP
GSH
(Reduced Glutathione)
DHA
(Dehydroascorbate)
Dismutation
NAD(P)H
MDA
(Monodehydroascorbate)
MDA
Reductase
Dismutation
NAD(P)
Ascorbate
Ascorbate
Peroxidase
(CH_2O)
CO_2
NADPH
FNR
NADP + H^+
Fd
O_2^-
O_2^-
SOD
$H_2O_2 + O_2$
H_2O
STROMA
THYLAKOID
MEMBRANE
O_2
$HO^- + {}^{\cdot}OH + O_2$
PSII
e^-
O_2^-
H_2O_2
O_2
$HO^- + {}^{\cdot}OH + O_2$
PSI
e^-
O_2^-
H_2O_2
LUMEN
$2H_2O$
$4H^+ + O_2$
O_2^-
O_2^-
SOD
$H_2O_2 + O_2$

glycolate, the substrate of photorespiration, which is excreted from the chloroplast. The reactions of the photorespiratory carbon - oxidation pathway are concerned with the recovery of most of the glycolate (about 75 %) by returning it to the Calvin cycle. It was estimated by Osmond (1981) that in the absence of photorespiration each mol of CO_2 fixed requires 3 mol of ATP and 2 mol of NADPH, whilst at the CO_2 compensation point (when CO_2 fixation in photosynthesis equals CO_2 evolution in photorespiration) the refixation of each mol of CO_2 evolved in photorespiration requires 10 mol ATP and 6 mol NADPH. Although there is no net CO_2 fixation at the CO_2 compensation point, carbon turnover consumes 60 - 70 % more energy than net CO_2 fixation in air levels of CO_2 in the absence of photorespiration. The energy costs would normally be of intermediate value, between these two extremes, however it is clear that the photorespiratory process is essentially an energy - consuming process which may enable 'futile' dissipation of excitation energy. But Krause and Cornic (1987) concluded that although it is possible that all reactions that use photosynthetic energy may reduce photoinhibition, the protective effect of carbon metabolism cannot be explained as an energy drain from the electron transport chain in quantitative terms.

The Mehler reaction involves the reduction of oxygen by PSI producing the oxygen free radical superoxide, the hydroxyl radical and hydrogen peroxide (Powles, 1984). As previously mentioned there are efficient scavenging systems that remove these harmful species and it has been suggested that the Mehler reaction may act as an energy dissipating mechanism and thus may have a photoprotective function, oxygen reduction allowing a limited drain of photosynthetic energy by non - cyclic electron transport, partially relieving 'over - reduction' of the electron transport chain and maintaining a high trans - thylakoid proton gradient (Krause and Cornic, 1987; Krause and Laasch, 1987). It has been suggested that carbon metabolism and the Mehler reaction may only exhibit protection when working in conjunction with other protective mechanisms.

Longer - term methods of avoiding the onset of photoinhibition include changes over days or weeks in parameters such as light - harvesting, carbon metabolism and scavenger systems against active oxygen species. These acclimations are limited and genetically fixed. There are other possible mechanisms of avoidance of photoinhibition (Raven, 1989). These include the mobility of whole cells or organisms (negative phototaxis) by flagellar or gliding mobility to an environment with lower light intensity, and movement of part of a macrophyte reducing the area for photon interception /

Figure 1.8 Photosynthetic carbon reduction cycle integrated with the photorespiratory carbon oxidation cycle. See text for details. Adapted from Osmond (1981).


CHLOROPLAST
ATP
Ribulose
5–phosphate
Ribulose
1,5–bisphosphate
O_2
Phospho–
glycolate
PEROXISOME
O_2
Glycolate
H_2O
Glyoxylate
Sedoheptulose
7–phosphate
Sedoheptulose
1,7–bisphosphate
CO_2
MITOCHONDRION
Glycine
CO_2
+ NH_3
NAD^+
H_2O
NADH
O_2
Serine
ATP
Erythrose
4–phosphate
Fructose
6–phosphate
Hydroxypyruvate
3–Phosphoglycerate
Glycerate
NADH
Fructose
1,6–bisphosphate
ATP
ATP
Triose Phosphate
NADPH
PEROXISOME

increasing self shading. For example, the shade plant *Oxalis oregano* shows rapid leaf movement to minimise the incident light of direct sunflecks preventing photoinhibition (Powles, 1984; Raven, 1989). Wilting and leaflet cupping upon water loss may also have such an effect. Reorientation of plastids within cells or change of plastid shape may decrease the area for photon interception / increase self shading (Raven, 1989). Finally, exposure of cells to temperatures several degrees above those of normal growth induces the expression of genes coding for the synthesis of heat - shock proteins (HSP). Evidence has been obtained that suggests that the chloroplast HSP - 22 and / or HSP - 29 confer protection against photoinhibition at low light intensities during heat shock (Schuster, *et al.*, 1988a). The mechanism and significance of this is unclear. Other environmental conditions can affect the light intensity required for the onset of photoinhibition. For example, in species such as *Nerium oleander* drought stress may lead to tight stomatal closure reducing CO_2 concentrations to a level near to the compensation point. This could result in photosynthesis being saturated at low light intensity due to decreased production of the final electron acceptor, $NADP^+$, leading to photoinhibition at lower light intensities than required for photoinhibition when no other stresses are imposed (Kyle, 1987). Sensitivity to photoinhibition is also increased under chilling conditions (e.g. Hayden and Baker, 1990). Chilling may reduce the Calvin cycle reaction rates so that the light reactions become saturating thereby increasing the rate of damage. The mechanisms of avoidance / tolerance and repair may also be reduced by chilling temperatures, thereby decreasing recovery (Kyle, 1987). Thus, photoinhibition will occur only when the light stress is sufficient to overcome the combination of mechanisms that exist to enable the tolerance or avoidance of this phenomenon, resulting in the rate of damage to the D1 protein being greater than its rate of repair. The light intensity and duration required to achieve this is very much dependent upon the synergistic influences of other environmental factors.

1.6 Photoinhibition as a means of Photosynthetic Regulation

Recently it has been suggested that the contribution of the photoinhibition - associated quenching of chlorophyll fluorescence (q_I) to the total non - photochemical quenching in high light is, like q_E, an adaptive and useful dissipative process (Horton and Hague, 1988). It has been proposed that increases in light intensity will result in a series of responses from the chloroplast: increased photosynthesis (as observed by

increased photochemical quenching, q_Q); increased q_E - induced dissipation; protein phosphorylation; photoinhibition; and change in membrane composition. Each process may be regarded as a regulatory process with different degrees of control, different time constants and different costs in terms of lost photosynthesis when the light intensity is lowered. The extent to which each mechanism operates would be determined by the intensity and duration of the light change and would probably differ between various plant materials (*e.g.* depending upon growth light intensities and species). Thus, photoinhibition can be envisaged as one of several controlled mechanisms of dissipation of absorbed excitation energy that occur as a response to increase in light intensity. Photosynthesis (q_Q) responds in seconds to minutes to changes from darkness to photosynthetically saturating light intensities, whilst the high energy state mechanism (q_E) responds in seconds. The phosphorylation of LHCII (q_T) can respond within minutes in the range of darkness to 'low' light intensity and photoinhibition (q_I) will operate in minutes to hours in the range of saturating to supraoptimal light intensity. Finally, changes in membrane composition may occur in hours to days throughout the range of darkness to supraoptimal light intensity (Horton, *et al.*, 1988). Thus, rather than viewing photoinhibition simply as a negative phenomenon consisting of damage to the PSII complex and a subsequent loss of photosynthetic capacity, it can perhaps be interpreted as being just one of many mechanisms that are concerned with an overall process of 'photosynthetic control' (Foyer, *et al.*, 1990).

1.7 The alga *Codium fragile* (Suringar) Hariot

1.7.1 Classification, Distribution, Morphology and Reproduction

There are various systems of algal classification; three of which are presented below with respect to *Codium fragile*.

DIVISION	chlorophyta	chlorophyta	-
CLASS	-	chlorophyceae	chlorophyceae (isokontae)
ORDER	caulerpales	codiales	siphonales
FAMILY	codiaceae	codiaceae	codiaceae
GENUS	*Codium*	*Codium*	*Codium*
SPECIES	*fragile*	*fragile*	*fragile*
REF.	1	2	3

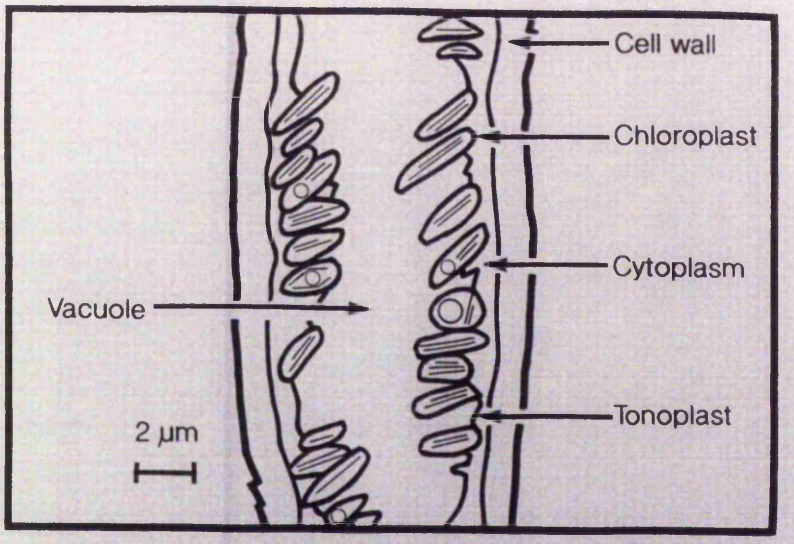
1. Bold and Wynne, 1985 2. Lee, 1989 3. Fritsch, 1977

Codium fragile is a coenocytic alga with multinucleate cells having few or no cross walls (Lee, 1989; Bold and Wynne, 1985). This has led to a morphological development to provide the necessary support of the plant body needed in the absence of septa. Thus, *C.fragile* possesses a compact, dichotomous, cylindrical thallus consisting of closely interwoven coenocytic siphons or filaments (Figure 1.9). In the medullary region of the thallus the siphons are colourless, whilst the outer layer of the thallus (cortex) is constructed of the dilated tips (utricles) of the coenocytic siphons which form a palisade - like layer, and it is only in these structures that chloroplasts are situated. The chloroplasts are smaller than those of higher plants (approximately 3 μm long - Hawes, 1979) and are arranged peripherally, in stacks, around a large central vacuole in such numbers that the thallus has an optically dense nature. The chloroplsts have unelaborate thylakoids arranged in pairs or triplets with no extensive stacking.

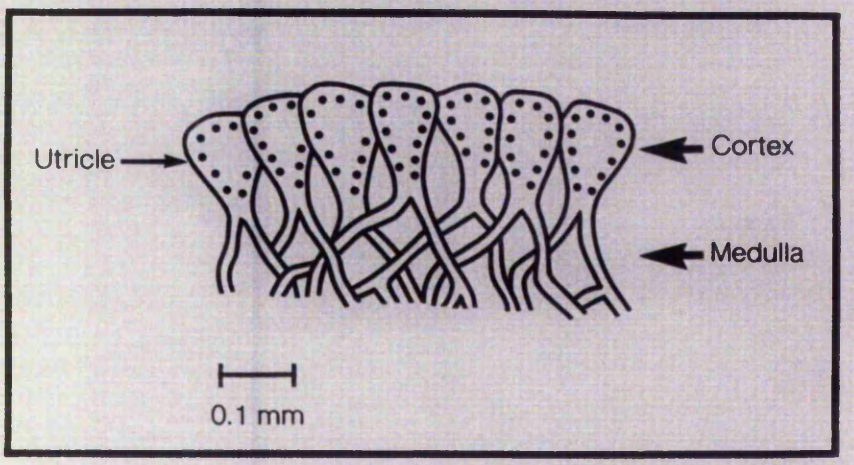
The life cycle of this alga reveals strong seasonal trends in frond morphology and chloroplast physiology, showing a growth strategy that optimises use of increased nutrient availability and reduction in algal competition (Benson, *et al.*, 1983; Williams, *et al.*, 1984). In the winter months competition from other more dominant epiphytic and epilithic algae, tidal amplitude variation and light intensity are reduced. Therefore, maximum carbon fixation, pigment content, chloroplast size and vegetative growth occurs, and nutrients are accumulated (e.g. starch, inorganic nitrogen, alkali soluble polyphosphate - as indicated by increased alkaline : acid soluble polyphosphate ratios). In the summer months competitive algal species, tidal amplitude and light intensity increase, and the nutrient status of the intertidal zone decreases. Therefore, nutrient reserves are mobilized, frond hairs develop (to enhance nutrient uptake), frond bleaching may occur, maximum photosynthetic efficiency is not maintained and *C.fragile* enters the reproductive phase (see Figure 1.10). Sexual reproduction involves the production of male and female gametangia from the utricles of the diploid thallus from which haploid biflagellate gametes are extruded (Lee, 1989; Bold and Wynne, 1985). Upon fertilization the male flagellum is lost, zygote propulsion being achieved by the female flagellum. It seems that male and female gametes from the same gametangium are incompatible and that fusion occurs only if the gametes are from different gametangia or plants (Prince, 1988). On reaching a suitable substrate the zygote germinates into a new *Codium* thallus. Dispersal may be achieved via the swimming gametes / zygote, by flotation or by fragmentation. The *Codium* genus is

Figure 1.9 Structure of *Codium fragile*. A) Diagram of the internal structure of a utricle (T.S.). After Hawes, 1979. B) Diagrammatic transverse section (T.S.) of the thallus. After Benson, 1983. C) Whole frond. See text for details.

A

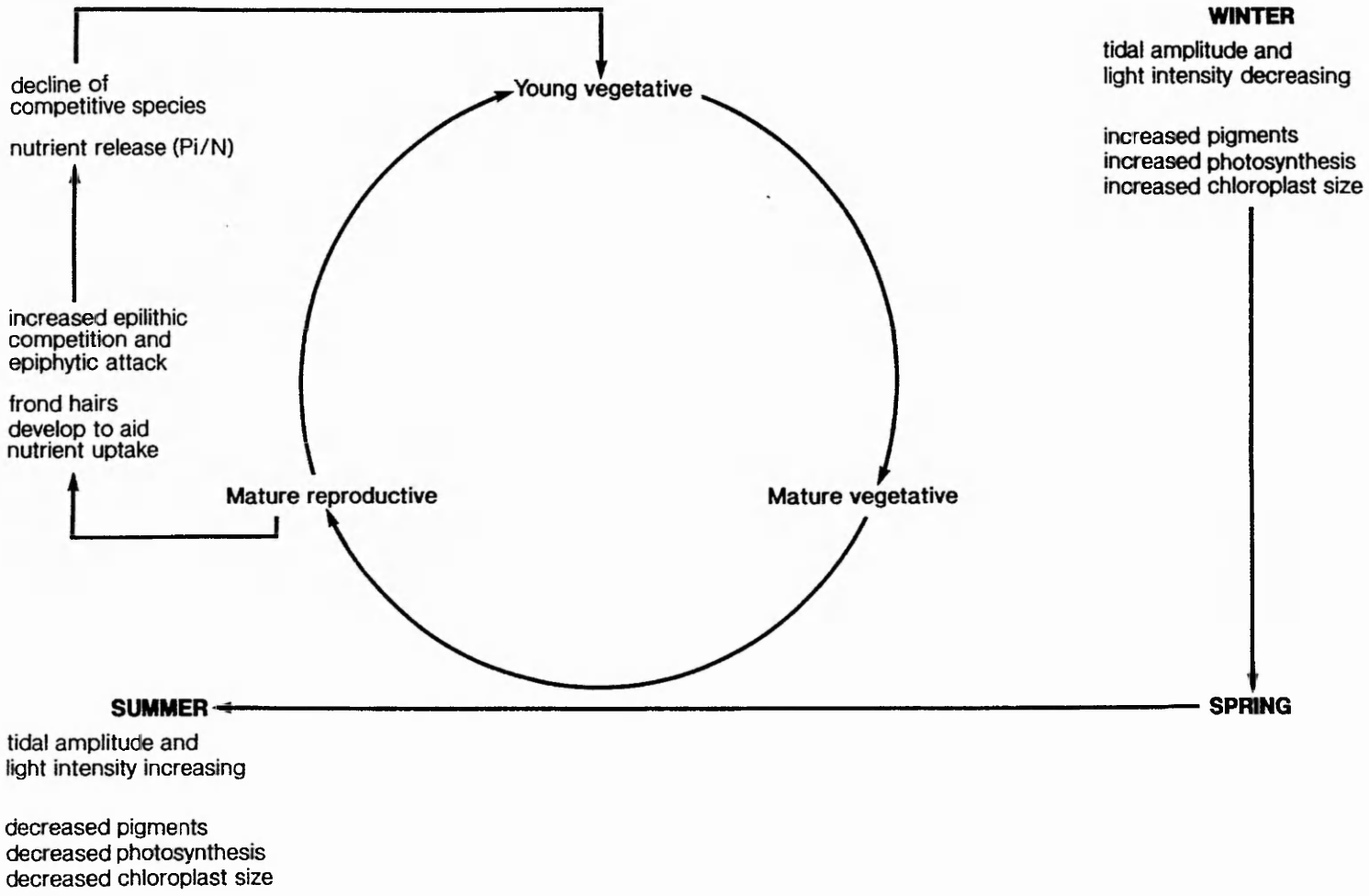

Cell wall
Chloroplast
Cytoplasm
Vacuole
Tonoplast
2 µm

B


Utricle
Cortex
Medulla
0.1 mm

C

Figure 1.10 A scheme for the seasonal photosynthetic strategy of *Codium fragile*. See text for details. Adapted from Williams, *et al.* (1984).


WINTER
tidal amplitude and
light intensity decreasing
increased pigments
increased photosynthesis
increased chloroplast size
Young vegetative
Mature vegetative
Mature reproductive
decline of
competitive species
nutrient release (Pi/N)
increased epilithic
competition and
epiphytic attack
frond hairs
develop to aid
nutrient uptake
SUMMER
tidal amplitude and
light intensity increasing
decreased pigments
decreased photosynthesis
decreased chloroplast size
SPRING

almost globally distributed occurring from the low tide mark down to 70 metres in tropical and temperate waters. Colonies of *C. fragile* around Britain prefer sheltered, moderate environments where it inhabits the intertidal zone and an abundant colony is found on the eastern coastline of the Isle of Wight at Bembridge.

1.7.2 Adaptations to the Intertidal Marine Environment

Light quality and intensity fluctuates on a daily and a seasonal basis in an intertidal habitat. An optical classification of seawater has been proposed by Jerlov (1976) as described by Dring (1981b) . There is a decrease in transparency of seawater from deep unproductive oceanic areas to shallow coastal waters due to an increase in the concentrations of inorganic biological detritus, dissolved organic compounds ('yellow substance') and phytoplankton cells. Absorption by these three components is wavelength dependent; all three absorb blue wavelenghts, whilst phytoplankton also absorbs red wavelenghts. Such variations in attenuation of the spectrum led Jerlov, 1976 (cited in Dring, 1981b) to divide coastal waters into nine types. Depending on the water type red, yellow and violet regions of the spectrum are preferentially absorbed and the light may become increasingly blue - green / green (500 to 575 nm) in temperate coastal waters as the depth of the water column increases. The absorption spectrum of *C. fragile* thylakoids shows prominent absorption in the blue region and a marked shoulder at 542 nm due to the presence of relatively large amounts of chlorophyll b, siphonoxanthin and siphonein (Benson and Cobb, 1983). Intact chloroplasts from this alga showed a similar pattern with marked absorption in the 500 to 550 nm region. Up to 60 % of the total carotenoid content of *C. fragile* is in the form of siphonoxanthin and siphonein (Benson and Cobb, 1981) and together with a high chlorophyll b content this enhances absorption in the 'green gap' of the visible spectrum. Similar absorption spectra have been noted in other algae and Kageyama, *et al.* (1977) demonstrated the transmission of excitation energy from siphonoxanthin to chlorophyll a in *Ulva japonica* and *U. pertusa*. Kageyama and Yokohama (1978) also found that siphonein could efficiently transfer excitation energy to chlorophyll a of PSII in *Dichotomosiphon tuberosus*. It was concluded that siphonein and siphonoxanthin function as light harvesting pigments in chlorophycean algae. Thus, increased amounts of these xanthophylls may assist photosynthesis in deeper waters or shaded environments. Such a presence and role of siphonein and siphonoxanthin in *C. fragile*

has also been supported by Anderson (1983 and 1985).

It should be noted that the theory of complementary chromatic adaptation as suggested by Engelmann in 1883 (cited in Dring, 1981a) in which it was proposed that the absorption spectrum of the dominant algae at different depths matches the colour of the light at these depths has found no support from more recent and detailed studies. It is now apparent that there is little correlation between pigment composition and the distribution of different algal groups. However, the pigment composition of seaweeds does seem to be associated with changes in growth irradiance and therefore ' intensity adaptation' is a more likely explanation of the distribution of different algal groups, with deep - water algae exhibiting adaptations typical of deep - shade plants (Dring, 1981a). That is, the variations in pigment composition associated with the vertical distribution of macroalgae are, in general, adaptations to low irradiance rather than spectral quality. A similar conclusion was made by Ramus, *et al.* (1976) and these workers suggested that the morphology of the macroalga was at least as important as its colour with respect to determining the vertical distribution of algae. It was concluded that two strategies were possible to enable algae to respond to changes in light intensity and wavelength: a change in pigment ratios (as in the questionable chromatic adaptation) or a simple increase in the total amount of pigment. This could occur by an increase in antenna pigments and / or an increase in photosynthetic units. Apparently *C. fragile* approaches an extreme situation where there is enough pigment to absorb the incident light almost completely (as all pigments have broad bands, absorption occurs throughout the visible spectrum). That is, further increase in pigment content will not increase the absorption as the alga is almost optically black. Dring (1981b) similarly reported that the spectral composition of incident light had little effect on the photosynthetic rate of green and brown algae with thick thalli, such as *C. fragile*, as these thalli absorb almost all wavelengths (i.e. were optically black), enabling photosynthesis in all wavelenghths. The structure of *C. fragile* results in it being optically thick and therefore the chlorophyll will be heterogenously illuminated to an extent dependent upon intensity (Ramus, *et al.*, 1976). For a useful increase in light absorption, especially at greater depths, the absorbing units must be kept near to the surface of the thallus to avoid rapid light attenuation, which is indeed the mechanism used by *Codium fragile.* Surface adapted *C. fragile* appears green whereas *C. fragile* adapted to greater depths appears black, showing that it is the surface - scattered light

being absorbed. Ramus (1981) has suggested that the structure of the utricle may enhance light absorption. The thin layer of peripheral cytoplasm surrounded by cell membranes could behave as an optically insulated wave guide similar to an optic fibre. A photon entering the cytoplasm at a low enough angle could undergo multiple internal reflections increasing the probability of photon capture as the chloroplasts are also situated in the cytoplasm. Unabsorbed photons could then pass back through the cytoplasm, reflected by an air - water interface at the base of the utricle. It is also suggested that the large central vacuole functions as an integrating sphere, dispersing light uniformly over the cytoplasm.

C. fragile exhibits several characteristics typical of terrestial shade plants. Photosynthesis in both fronds and chloroplasts saturates at low light intensities, isolated chloroplasts have low chlorophyll a : b ratios and 75 % of the pigment content may be in the light harvesting complexes (Benson, 1983), which together with high concentrations of siphonein and siphonoxanthin increases the light harvesting ability, particularly in the 'green gap' region of the spectrum. A large number of light harvesting complexes are therefore supplying excitation energy to a limited number of reaction centres. Thus, *C. fragile* is well adapted for efficient light harvesting at low light intensities, a situation that will be encountered in a submarine environment at high tide. However, these very adaptations put this alga at great risk of photoinhibition and ensuing photooxidation at the higher light intensities that will occur at low tide. It is apparent that *C. fragile* is able to withstand such conditions and thus successfully overcome the problems associated with a highly variable photosynthetic photon flux density (PPFD). For example, Arnold and Murray (1980) found that full sunlight (1405 - 1956 μmol photon m^{-2} s^{-1}) inhibited photosynthesis in fronds of *Chaetomorpha linum*, *Enteromorpha intestinalis, Ulva lobata*, and *U. rigida* but not in fronds of *Codium fragile*. Whilst Benson (1983) found that photooxidation of *C. fragile* fronds did not occur after six hours of high - intensity non - fluorescent light treatment simulating the conditions of exposure encountered at low tides. Indeed, the study of Ramus, *et al.* (1976) illustrated that although photosynthesis saturates at low PPFD in *C. fragile*, the maximum rate of photosynthesis may be maintained at increasing PPFD up to full sunlight.

Mechanisms of avoidance of photoinhibition have already been discussed and these mechanisms may operate in *C. fragile*. Indeed, superoxide dismutase has been found

in this alga (Lumsden and Hall, 1975), perhaps indicating that a system exists to scavenge or prevent the formation of reactive oxygen species. However, there may be differences in the way in which *Codium* prevents the onset of photoinhibition and subsequent photooxidation. For example, as stated earlier, it has been suggested that zeaxanthin and the xanthophyll cycle have a possible role in protection against photoinhibition (*e.g.* Demmig, *et al.*, 1987; Demmig -Adams, *et al.*, 1989a, 1989b, 1990). However, zeaxanthin has been shown to be absent from *C. fragile* chloroplasts and it has been proposed that this may also indicate the absence of the xanthophyll (epoxide) cycle in this alga (Benson and Cobb, 1983), which would obviously mean that this mechanism could not contribute to the prevention of photoinhibition. Fronds of *C. fragile* have been found to saturate at three times the PPFD of isolated chloroplasts and the former did not exhibit photoinhibition at PPFD's of 3000 μmol m^{-2} s^{-1}, whilst photoinhibition of uniformly illuminated chloroplasts did occur (Benson, 1983). This led to the suggestion that the optically dense nature of the frond confers a filtering of incident light (Benson, 1983). It has also been found that although absolute pigment concentrations in *C. fragile* vary, relative pigment composition remains constant possibly reflecting loss of entire photosynthetic units. Benson (1983) proposed that this may reduce the amount of light energy absorbed and thus be a method of preventing light - dependent damage. However, frond bleaching was observed in the summer but it was suggested that this may be a result of other environmental factors such as nutrient levels. As previously mentioned carotenoids, particularly ß - carotene, are involved in the quenching of toxic active oxygen species in higher plants (Foote, 1976). However, ß - carotene is not found in *Codium* and the chlorophylls in this alga appear more susceptible to photooxidation than those carotenoids that are present, when photooxidation is caused by illumination with a fluorescent light with a high UV emission (Benson, 1983). Therefore, the α - carotene that appears to replace ß - carotene in *C. fragile* may not function in such a photoprotective manner. In a study by Grumbach and Lichtenthaler (1982) it was found that radish seedlings grown at high light intensities showed a greater turnover rate of chlorophyll and carotenoid pigments compared to shade grown seedlings, indicating that in high light photooxidation occurs and rapid resynthesis is essential to maintain photosynthetic efficiency. Benson (1983) found constancy of pigment ratios and relative distributions and highly variable absolute pigment concentrations in *C. fragile* which was similarly interpreted as suggesting a

tightly controlled and efficient mechanism of pigment turnover, reflecting adaptation to exposure to high light intensities.

The possible role of photorespiration in the prevention of photoinhibition in *C. fragile* is of interest as light - induced glycolate excretion and carbohydrate release as hexoses has been reported (Samuel, *et al.*, 1971; Benson, 1983), especially under conditions facilitating high photorespiratory rates. Up to 40 % of assimilated carbon may be released at supra - optimal PPFD. Such extracellular carbon release may be a mechanism to excrete excess glycolate when photorespiratory rates are particularly high (Lorimer and Andrews, 1981). Perhaps photoinhibition may be partially relieved by increased energy dissipation via the photorespiratory carbon oxidation (PCO) cycle and the operation of this cycle may in turn be facilitated by carbon release as glycolate. Thus, *C. fragile* may have the ability to use the surrounding medium as an extracellular zone from or to which it may reclaim or release excessive amounts of metabolites depending on the immediate fluctuations of the light environment (Benson, 1983). However, the majority of the energy used in the PCO cycle is required in the stages that reclaim carbon lost to glycolate and thus excreting glycolate releases the very substrate required for these energy consuming reactions and this may thus reduce rather than enhance the role of photorespiration in the 'futile' dissipation of excess photosynthetic energy. But glycolate excretion may perhaps facilitate the maintenance of a certain turnover of the PCO cycle. Certainly, loss of fixed carbon as glycolate and hexoses is a process which ultimately is a waste of energy and as such may be viewed as a possible mechanism of energy dissipation, contributing to the avoidance of photoinhibition. A role for photosynthetic carbon metabolism in the regulation and dissipation of photochemical energy is supported by the observation of Hawes and Cobb (1980) that photooxidation in endosymbiotic *C. fragile* chloroplasts only occurred after the depletion of storage carbon reserves. Williams (1986) suggested that synthesis of chloroplast starch may be considered as a limited photoprotective mechanism, as a means of dissipating ATP and $NADPH_2$ which under photooxidative conditions may be in excess. For each mol of ADP - glucose produced, two molecules of $NADPH_2$ and four molecules of ATP are effectively consumed. This may also be a means of recycling phosphate within the chloroplast for photosynthesis which is essentially a phosphate consuming process. Due to anatomical differences *C. fragile* has an increased respiratory burden when compared with some other species (Ramus, 1978) and perhaps

this has an energy dissipatory role in photoprotection. For example, *Ulva lactuca* is entirely photosynthetic tissue, whereas *C. fragile* is one - third photosynthetic tissue, consequently the latter has three times the respiratory burden of the former.

1.8 Aims of the Investigation

From the preceding sections it is clear that *C. fragile* is apparently well adapted for efficient light harvesting at low light intensities. However, the intertidal habitat is regularly exposed to extremes of irradiance on a daily and a seasonal basis, the upper extreme being approximately 2000 μmole m^{-2} s^{-1} PPFD, so that these very adaptations put this alga at great risk of photoinhibition and subsequent photoxidation at the higher light intensities encountered at low tide. Although photosynthesis saturates at low light intensity maximum rates of photosynthesis may apparently be maintained at increasing irradiance up to full sunlight indicating that *C. fragile* is remarkable in its ability to avoid or tolerate the deleterious effects of photoinhibition. A study of the phenomenon of photoinhibition in this alga is, therefore, of particular interest as a growth strategy must have evolved to reduce the potentially lethal effects of excess excitation energy. Furthermore, the photosynthetic physiological ecology of marine attached macrophytes, such as seaweeds and seagrasses and floating seaweeds, such as *Sargassum spp.*, has received relatively little attention in comparison with microalgae and higher terrestial plants; in particular with respect to studies specifically devoted to photoinhibition. Thus, more work is required on the cause of high light depression of macroalgal photosynthesis. The overall aim of this investigation, therefore, is to characterise photosynthetic function and stability of *C.fragile* and to attempt to identify how it can apparently withstand the effets of photoinhibition. The approach taken will be to carry out investigations at different levels by a progression of experimentation. Initial studies will focus on the photosynthetic response of whole fronds in an attempt to identify any limitations of photosynthesis to light intensity and temperature, leading to subsequent work using isolated chloroplasts and, in turn, to investigations using thylakoid membrane systems to further elucidate photosystem function in this species, especially with respect to photoinhibitory stress. A final series of experiments will involve a study of thylakoid polypeptide turnover under photoinhibitory conditions by measuring incorporation of radiolabelled amino acids, with particular attention being paid to the D1 protein. In this way it is hoped that the underlying mechanisms of photoinhibition

will be identified which will perhaps throw light on to the matter of how this alga successfully adapts to extremes in irradiance.

CHAPTER 2. THE OXYGEN EXCHANGE OF C. FRAGILE FRONDS

2.1 Introduction

Previous studies have implied that *C. fragile* exhibits a very stable photosynthetic response in a changing environment. For example, Arnold and Murray (1980) found that full sunlight (1405 - 1956 μmol m^{-2} s^{-1} PPFD) did not inhibit photosynthesis of *Codium fragile*, whilst Benson (1983) found that photooxidation of fronds did not occur after six hours of high - intensity non - fluorescent light simulating low tide exposure conditions. Furthermore, Ramus, *et al.* (1976) found that although photosynthesis saturates at low PPFD in *C. fragile*, the maximum rate of photosynthesis was maintained at increasing PPFD up to full sunlight. The photosynthesis - temperature (P - T) response of terrestial plants has been thoroughly examined and similar studies of intertidal macroalgae have apparently found P - T responses similar to those of terrestial plants (Knoop and Bate, 1990). Photosynthesis increases exponentially from zero at the lower temperature limit or biological zero (T_{min}), reaches a maximum (P_{opt}) at the optimum temperature (T_{opt}) and declines rapidly to zero at the upper lethal limit (T_{max}). The life cycle of *C. fragile* (Figure 1.10) reveals strong seasonal trends in frond morphology and chloroplast physiology, showing a growth strategy that optimises use of increased nutrient availability and reduction in algal competition (Benson, *et al.*, 1983; Williams, *et al.*, 1984). Thus, the first set of experiments, reported in this Chapter, involved the investigation of oxygen exchange of intact fronds using polarographic methods over a range of light intensities and temperatures, with tissue sampled at three stages in the yearly life cycle of the alga. The aim was to characterise, in greater detail than previous studies, the photosynthetic response of this species, to identify any limitations of photosynthesis to light intensity or temperature, and so enable a subsequent progression to experiments with isolated chloroplasts and thylakoids.

2.2 Materials and Methods

2.2.1 Sampling and Maintenance of *C. fragile*

Fronds of *C. fragile* were harvested from intertidal rockpools at Bembridge, Isle of Wight on three occasions in the yearly life cycle as follows: 30/ 11/ 87 (winter -

early vegetative), 04/ 04/ 88 (spring - late vegetative) and 11/ 09/ 88 (summer - late reproductive). The samples were maintained in tanks each containing 25 litres of aerated seawater (Instant Ocean, Aquarium Systems Ltd.) in a 10 °C refrigerated cool room. Irradiance of approximately 40 μmol m^{-2} s^{-1} PPFD at the water surface was provided by overhead banks of Thorn 18W 'white fluorescent' tubes.

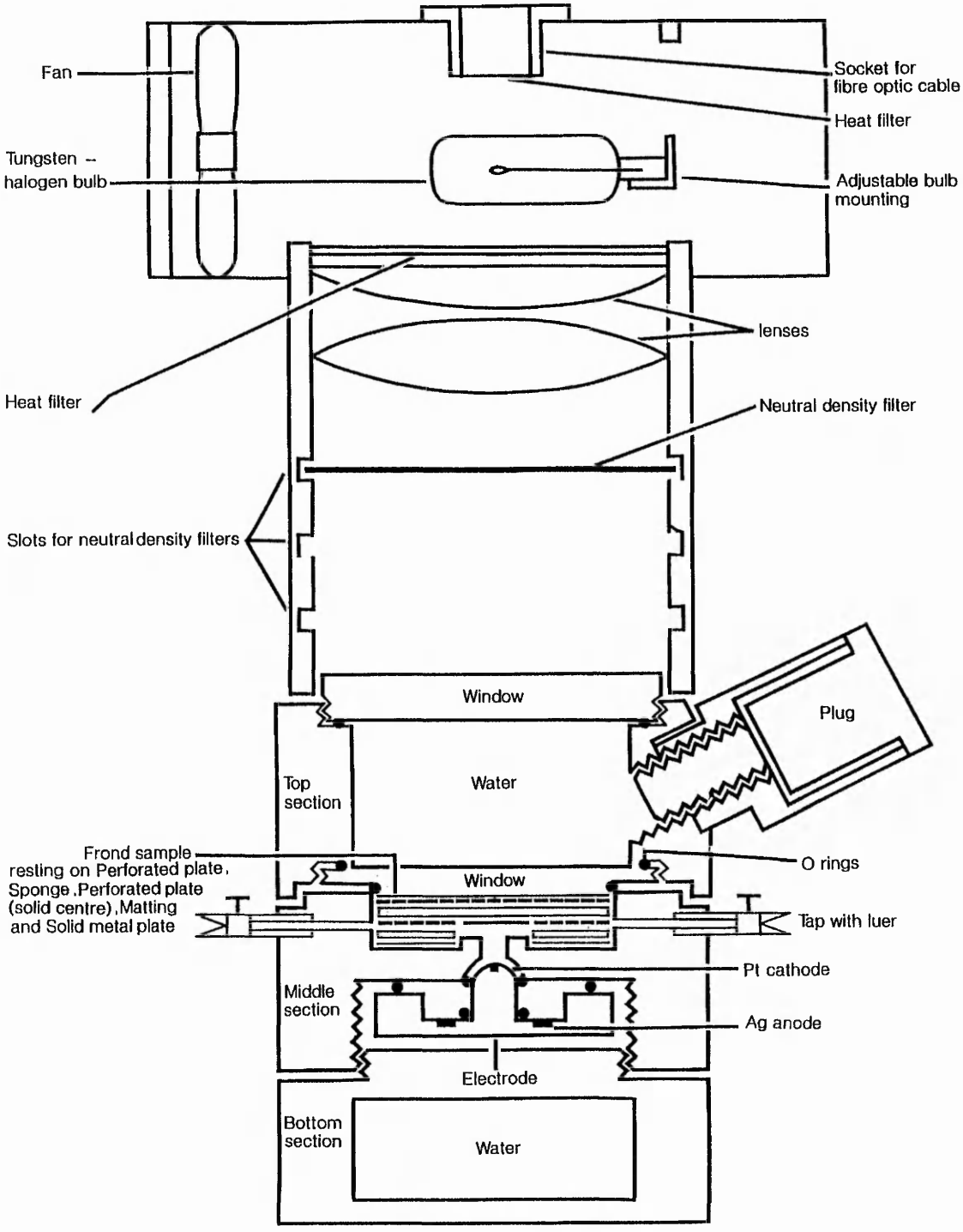
2.2.2 Measurement of Oxygen Exchange of Frond Samples

The measurement of oxygen exchange by frond tips in the gaseous phase was achieved using a leaf disc - LD2 oxygen electrode (Hansatech Ltd., King's Lynn, U.K. - see Figure 2.1) and the methods of Delieu and Walker (1981). Electrolyte (0.95 : 0.05 of 1 mol dm^{-3} $NaHCO_3$: 1 mol dm^{-3} Na_2CO_3 dissolved in half saturated KCl (w/v), at pH 9.0) was placed in the electrolyte well and a 2 cm square of tissue paper was used to cover the platinum cathode. A 2 cm square of teflon membrane was placed on top of the tissue, and these two layers were secured with a rubber 'o' ring using the membrane applicator. The electrode disc was then inserted into its position at the base of the LD2 chamber and attached to a Gould BS - 272 chart recorder and a Hansatech 1 mV/ 10 mV potentiating and zero suppress digital readout control box both set in the 10 mV range. After equilibration a 10 cm^3 syringe of nitrogen was flushed through the chamber, establishing the nitrogen line (zero oxygen). Calibration was carried out with each frond sample, with the supporting grids and matting in place. A 1 cm^3 gas tight syringe was used to introduce a 1 cm^3 volume of air and hence establish the values of R_1 and R_2 which give the electrical output (mV) corresponding to 1 cm^3 of air (of known oxygen content). This also enables the determination of the effective volume of the chamber, V:

$$V = R_1/(R_2 - R_1)$$

During experiments adequate levels of CO_2 were ensured by moistening the capillary matting with 'CO_2 generator' (0.95 : 0.05 of 1 mol dm^{-3} $NaHCO_3$: 1 mol dm^{-3} Na_2CO_3 dissolved in distilled water, adjusted to pH 9.0) and the temperature was maintained at 5.0, 7.5, 10.0, 12.5, 15.0 or 20 °C using a Techne C400 circulator in conjunction with a Techne M1000 heat exchange unit to pump water through the upper and lower water jackets. Two electrodes were set up in series, one was maintained in darkness throughout whilst the other was used with a 100 W high intensity tungsten - halogen LS2 light source (Hansatech Ltd. - see Figure 2.1) and a combination of

Figure 2.1 The Hansatech LS2 light source and LD2 gaseous phase leaf disc oxygen electrode. Adapted from Walker, 1987 and 1985, respectively.


Fan
Socket for fibre optic cable
Heat filter
Tungsten – halogen bulb
Adjustable bulb mounting
lenses
Heat filter
Neutral density filter
Slots for neutral density filters
Window
Plug
Top section
Water
Frond sample resting on Perforated plate, Sponge, Perforated plate (solid centre), Matting and Solid metal plate
O rings
Window
Tap with luer
Pt cathode
Middle section
Ag anode
Electrode
Bottom section
Water

neutral density filters to produce the seven different light intensities used in the experiments (approximately 26, 82, 196, 344, 739, 921 and 1109 μmol m^{-2} s^{-1} PPFD). Between 2 to 3g frond tip samples were placed in the two electrodes. The light treated sample was subjected to a regime of approximately five min alternate dark - light intervals starting with the lowest PPFD and working up to the final exposure at the highest PPFD. Initially the samples were equilibrated by three exposures to the lowest PPFD, before the dark oxygen uptake rate was measured prior to exposure to the initial PPFD of the actual experiment. The same frond sample was used throughout each experiment and ten such response curves were performed at each temperature investigated. On completion of each experiment the samples were weighed and placed in darkness in a -20 °C freezer to await chlorophyll determination. Results were expressed as μmoles O_2 per hour per mg chlorophyll or μmoles O_2 per hour per g fresh weight.

2.2.3 Chlorophyll Determination

The chlorophyll content of *C. fragile* frond samples was determined by using the extraction method of Jeffrey (1968) as adapted by Benson (1983), followed by the spectrophotometric method of MacKinney (1941). The samples were frozen at -20 °C for at least 30 min and dehydrated in methanol for two min. The methanol was retained on ice in the dark and the frond samples were vigorously macerated for one min in a pestle and mortar containing approximately 5 cm^3 of 100 % acetone. The resulting macerate was transferred to a 10 cm^3 centrifuge tube and centrifuged for approximately two min at 2,500 rpm (1050g) in a Denley BR401 refrigerated centrifuge. The supernatant was retained on ice in the dark and the pellet re - extracted with 100 % acetone several times until colourless, and all the supernatants were pooled. The volumes of the methanol and acetone extracts were measured and their chlorophyll content determined spectrophotometrically. The undiluted methanol extract absorbance was measured against a methanol blank at 650 nm and 665 nm in a Cecil CE 303 Series 2 Grating spectrophotometer. The chlorophyll content was determined using the equation:

$$\text{Total Chl} = (A_{650} \times 25.8) + (A_{665} \times 4)$$
$$= \text{x mg per litre.}$$

The acetone extract was diluted by a factor of 10 using 100 % acetone in triplicate and

the absorbance measured against a 100 % acetone blank at 645 nm and 663 nm. The chlorophyll content was determined using the equation:

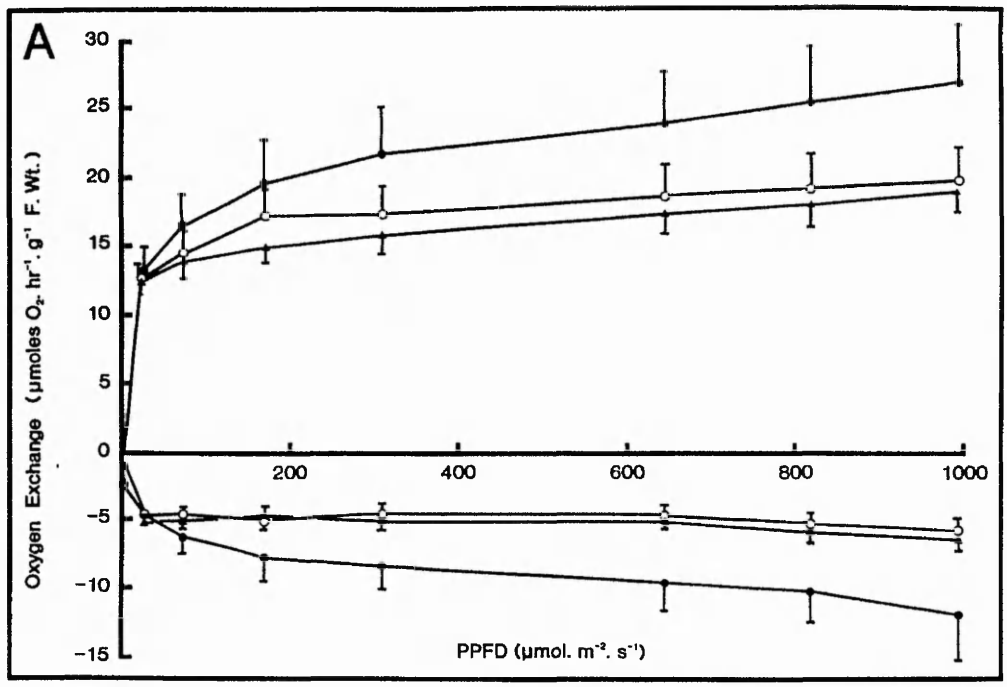
$$\text{Total Chl.} = (A_{645} \times 20.2) + (A_{663} \times 8.02) \times 10$$
$$= \text{x mg per litre.}$$

The values obtained were adjusted to take into account the volume of the supernatant and then added together to give the total chlorophyll (mg) present in the sample.

2.3 Results

The three tissue samples from different stages in the life cycle showed that both vegetative and reproductive algal frond tips exhibited a similar photosynthetic response over the temperature and light intensity ranges studied. Figure 2.2 shows some typical results. The first point of each curve, at 0 μmol m^{-2} s^{-1} PPFD, represents the dark oxygen uptake rates of fronds incubated in darkness throughout the duration of the experimental period. The other oxygen uptake values are those that were obtained with the 'light / dark' incubated fronds immediately preceding illumination at a particular light intensity. In all experiments (*e.g.* at the three temperatures 7.5, 10.0 and 20.0 °C shown in Figure 2.2) this first oxygen uptake rate (of the 'dark' incubated fronds) was lower than the dark oxygen uptake rates of the 'light / dark' incubated fronds. It was apparent that this light - dependent increase in dark oxygen uptake was unrelated to PPFD as the 'light / dark' treated fronds generally showed no relationship between light intensity and pre - illumination dark oxygen uptake at any of the temperatures. The one possible exception to this, as seen in Figure 2.2, was the response at 10 °C with the tissue harvested on 30/ 11/ 87, in which the rate of pre - illumination dark oxygen uptake appeared to increase to some extent with an increase in light intensity. As there was no change in dark oxygen uptake with light intensity the oxygen uptake rate in the dark period immediately preceding illumination at the intermediate light intensity of approximately 343 μmol m^{-2} s^{-1} PPFD was plotted against temperature to show typical oxygen uptake versus temperature responses for the 'light / dark' incubated sample and to allow comparison with the dark oxygen uptake rates of the 'dark' incubated sample (Figure 2.3). The pre - illumination dark oxygen uptake rates of the 'light - dark' incubated sample showed no clear trend with respect to temperature, although one of the lowest rates with each of the three tissue samples occurred at 20 °C (Figures 2.2 and 2.3). The oxygen uptake rate values for the 'light

Figure 2.2 Relationship between PPFD (μmol m^{-2} s^{-1}) and the oxygen exchange of *C. fragile* frond tips from tissue sampled on 30/ 11/ 87 (winter - early vegetative). A) oxygen exchange in units of μmoles O_2 hr^{-1} g^{-1} FWt. B) oxygen exchange in units of μmoles O_2 hr^{-1} mg^{-1} chl. Key: positive values are net photosynthesis; negative values are pre - illumination dark oxygen uptake; ▲, 7.5 °C; ●, 10 °C; ○, 20 °C. Each point represents the mean ± standard error of 10 observations with different frond tip samples. Standard error bars are included where greater than symbol size.


A
30
25
20
15
10
5
0
-5
-10
-15
200
400
600
800
1000
Oxygen Exchange (μmoles O2. hr-1. g-1 F. Wt.)
PPFD (μmol. m-2. s-1)

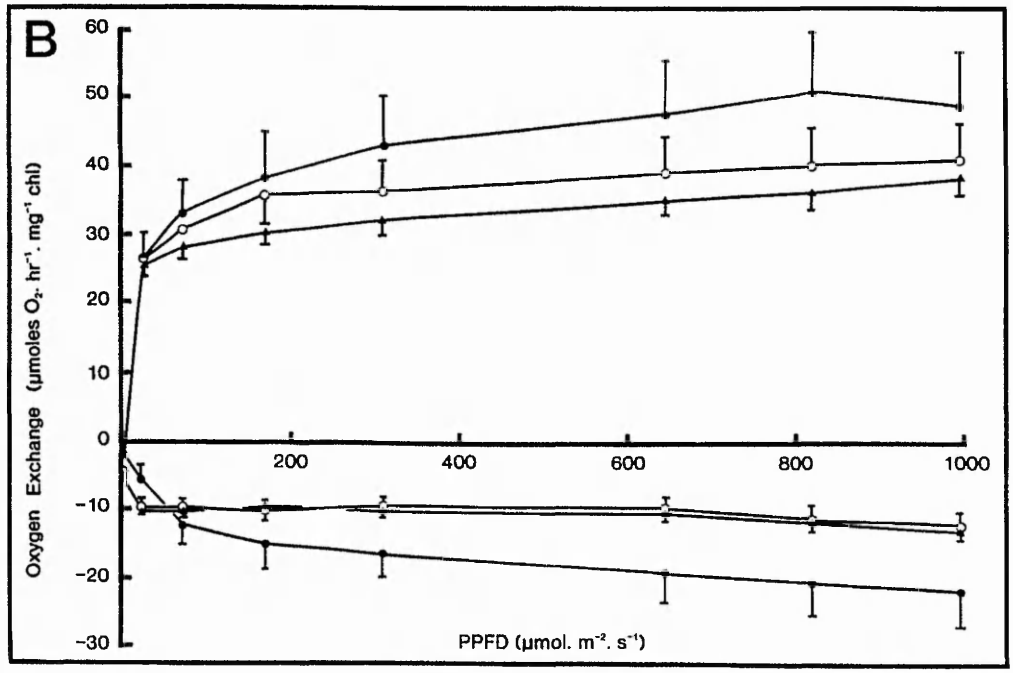

B
60
50
40
30
20
10
0
-10
-20
-30
200
400
600
800
1000
Oxygen Exchange (μmoles O2. hr-1. mg-1 chl)
PPFD (μmol. m-2. s-1)

Figure 2.3 Relationship between temperature (°C) and oxygen uptake of *C. fragile* frond tips. A) oxygen uptake in units of μmoles O_2 hr^{-1} g^{-1} FWt. B) oxygen exchange in units of μmoles O_2 hr^{-1} mg^{-1} chl.Key:

Frond tips incubated in the dark throughout the experimental period.

▲ 30/ 11/ 87 sample (winter - early vegetative)

● 04/ 04/ 88 sample (spring - late vegetative)

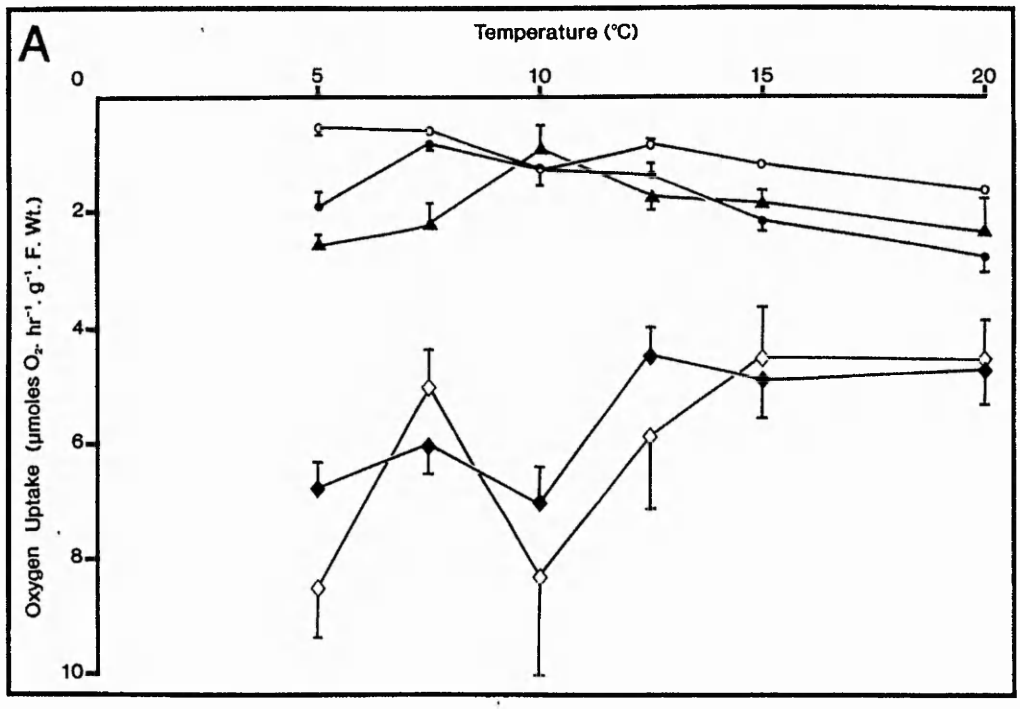
○ 11/ 09/ 88 sample (summer - late reproductive)

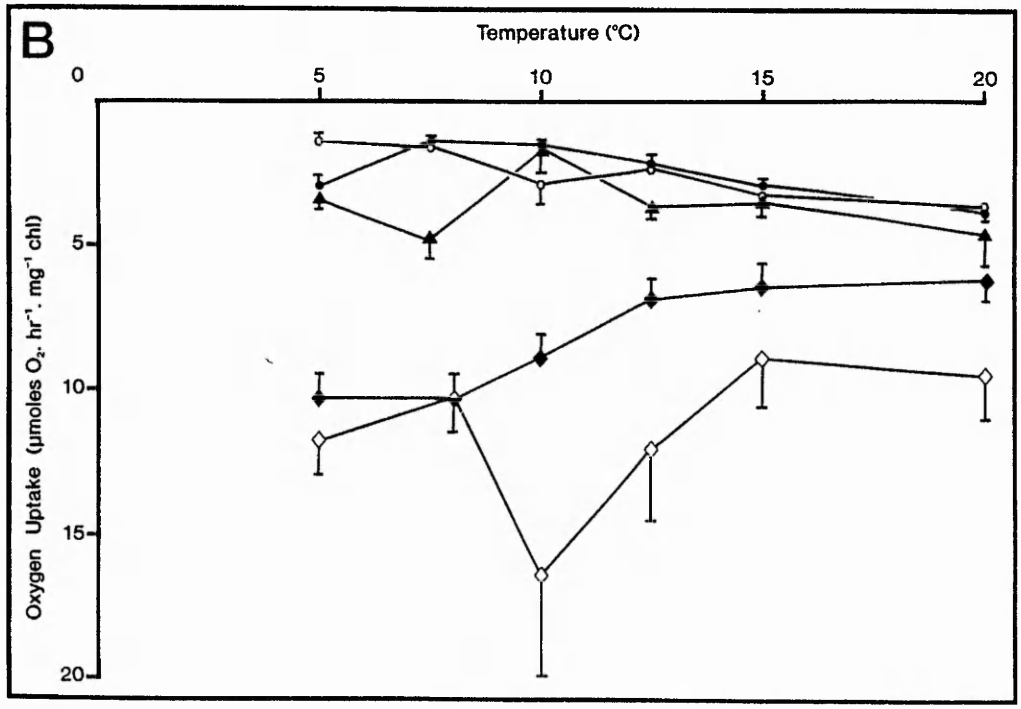
'Light / dark' incubated frond tips. Oxygen uptake in the dark period immediately preceding illumination at 343 μmol m^{-2} s^{-1} PPFD.

◇ 30/ 11/ 87 sample (winter - early vegetative)

◆ 04/ 04/ 88 sample (spring - late vegetative)

Each point represents the mean $\pm$ standard error of 10 observations with different frond tip samples. Standard error bars included where greater than symbol size.


A
Temperature (°C)
0
5
10
15
20
2
4
6
8
10
Oxygen Uptake (µmoles O_2. hr^{-1}. g^{-1}. F. Wt.)

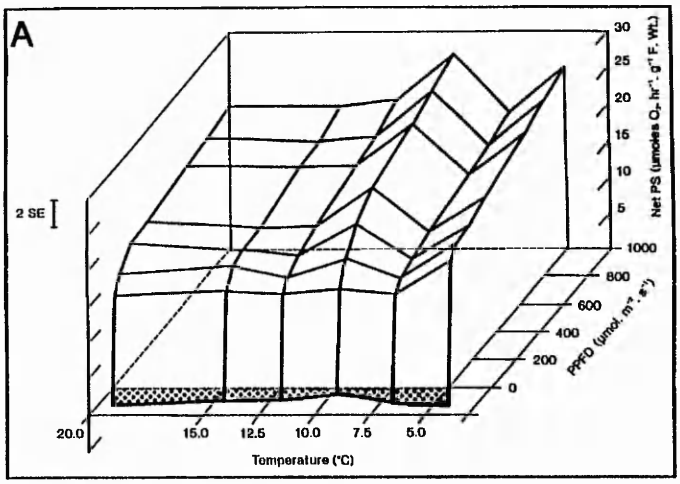

B
Temperature (°C)
0
5
10
15
20
5
10
15
20
Oxygen Uptake (µmoles O_2. hr^{-1}. mg^{-1} chl)

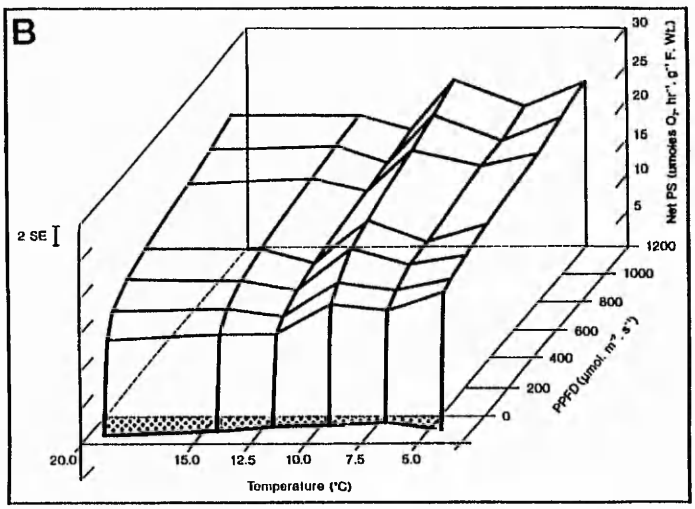
/ dark' incubated samples were always within the range of approximately 5 - 20 μmoles O_2 hr^{-1} mg^{-1} chl (2 - 12 μmoles O_2 hr^{-1} g^{-1} FWt). The dark incubated sample oxygen uptake rates appeared to show a general trend of increase with increasing temperature with the 04/ 04/ 88 and 11/ 09/ 88 tissue samples, whereas the 30/ 11/ 87 sample did not show a clear trend, exhibiting a more erratic response of O_2 uptake with change in temperature (Figure 2.3). The 'dark' incubated sample oxygen uptake rates appeared to show no difference between tissues, the rates always being within the range of approximately 1.4 - 4.7 μmoles O_2 hr^{-1} mg^{-1} chl (0.5 - 2.7 μmoles O_2 hr^{-1} g^{-1} FWt). Figure 2.2 illustrated that the rates of dark oxygen uptake in the 'dark' incubated samples were less than the dark oxygen uptake rates of the 'light / dark' incubated samples and this is clearly confirmed by Figure 2.3.

Net photosynthesis (PS) rates are obtained from the combination of gross PS rates and pre - illumination dark oxygen uptake rates. As the latter changed to a negligible extent throughout the experiments, net PS curves were approximately parallel to gross PS curves, with net PS values exceeding gross PS values. Therefore, any changes in net PS with change in PPFD were mainly due to changes in gross PS and were not complicated by changes in dark oxygen uptake. Thus, when discussing the photosynthetic response it is sufficient to consider the net PS values only. Typical results obtained with regard to net PS, PPFD and temperature are shown in Figure 2.2 for *C. fragile* fronds sampled on 30/ 11/ 87 (winter - early vegetative). These results are compared with those obtained with fronds sampled on 04/ 04/ 88 (spring - late vegetative) and 11/ 09/ 88 (summer - late reproductive) in Figure 2.4 (net PS expressed as μmoles O_2 hr^{-1} g^{-1} FWt) and in Figure 2.5 (net PS expressed as μmoles O_2 hr^{-1} mg^{-1} chl). From the relevant Figures it is evident that the response is similar with all three tissue samples and at all temperatures. Net PS increased very rapidly as the PPFD was increased from 0 to approximately 80 μmol m^{-2} s^{-1} PPFD and net PS saturated at approximately 200 μmol m^{-2} s^{-1}. Further increase in light intensity from 200 up to 1200 μmol m^{-2} s^{-1} PPFD resulted in a negligible increase in net PS rate. That is, an extended saturation plateau was observed and there was no decline in net PS rate with increase in light intensity above that required for maximum net PS.

In general, 10 °C appeared to be the optimum temperature for net PS, the highest rates occurring at this temperature at most light intensities, as illustrated in Figures 2.4 and 2.5. However, the differences in net PS rates at different temperatures were

Figure 2.4 Relationship between net photosynthesis of *C. fragile* frond tips (μmoles O_2 hr^{-1} g^{-1} FWt), temperature (°C) and PPFD (μmol m^{-2} s^{-1}). A) 30/ 11/ 87 sample (winter - early vegetative). B) 04/ 04/ 88 sample (spring - late vegetative). C) 11/ 09/ 88 sample (summer - late reproductive). Each point represents the mean $\pm$ the overall standard error of 10 observations with different frond tip samples.


A
2 SE
Net PS (umoles O2, hr-1, g-1 F. Wt.)
30
25
20
15
10
5
1000
800
600
400
200
0
PPFD (umol. m-2 . s-1)
20.0
15.0
12.5
10.0
7.5
5.0
Temperature (°C)


B
2 SE
Net PS (umoles O2, hr-1, g-1 F. Wt.)
30
25
20
15
10
5
1200
1000
800
600
400
200
0
PPFD (umol. m-2 . s-1)
20.0
15.0
12.5
10.0
7.5
5.0
Temperature (°C)

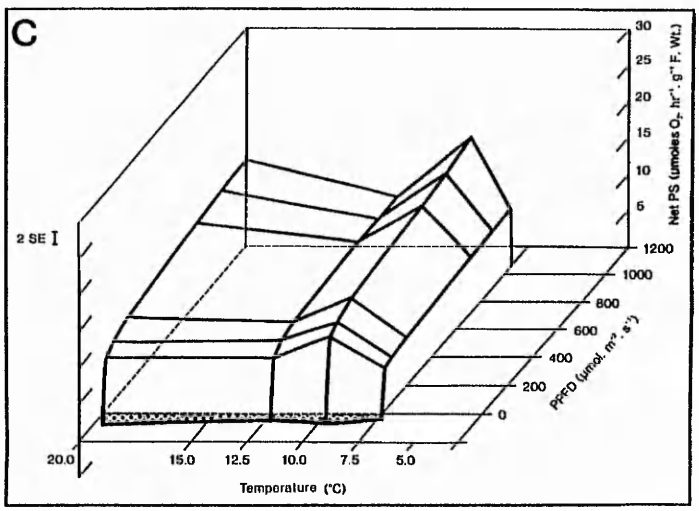
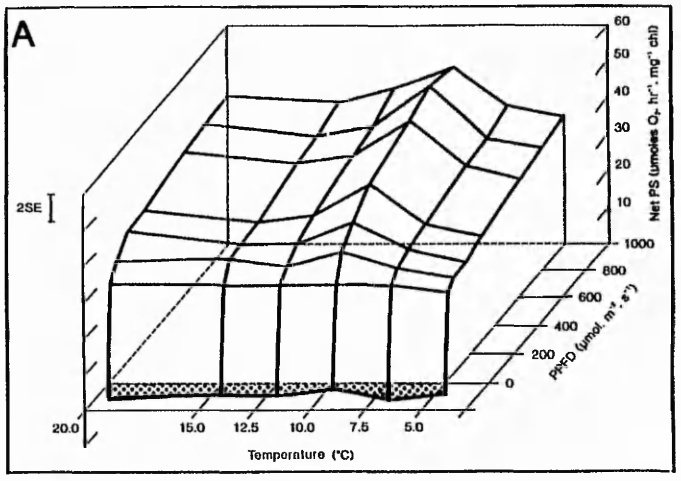
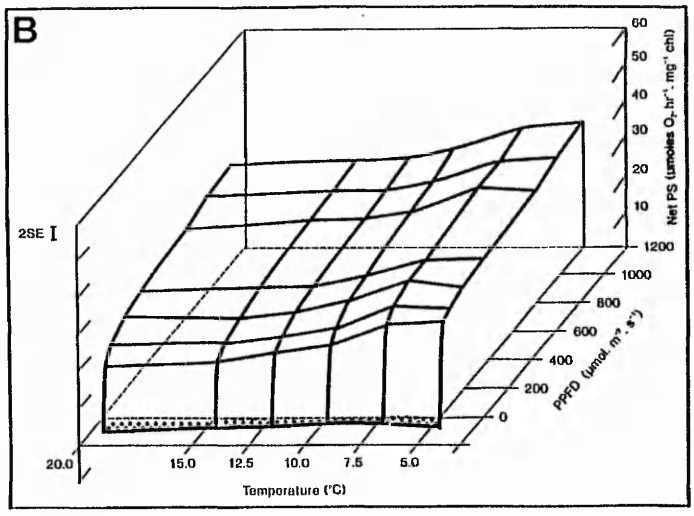

C
2 SE
Net PS (umoles O2, hr-1, g-1 F. Wt.)
30
25
20
15
10
5
1200
1000
800
600
400
200
0
PPFD (umol. m-2 . s-1)
20.0
15.0
12.5
10.0
7.5
5.0
Temperature (°C)

Figure 2.5 Relationship between net photosynthesis of *C. fragile* frond tips (μmoles O_2 hr^{-1} mg^{-1} chl), temperature (°C) and PPFD (μmol m^{-2} s^{-1}). A) 30/ 11/ 87 sample (winter - early vegetative). B) 04/ 04/ 88 sample (spring - late vegetative). C) 11/ 09/ 88 sample (summer - late reproductive). Each point represents the mean $\pm$ overall standard error of 10 observations with different frond tip samples.


A
2SE
60
50
40
30
20
10
Net PS (umoles O2. hr-1. mg-1 chl)
1000
800
600
400
200
0
PPFD (umol. m-2. s-1)
20.0
15.0
12.5
10.0
7.5
5.0
Temperature (°C)


B
2SE
60
50
40
30
20
10
Net PS (umoles O2. hr-1. mg-1 chl)
1200
1000
800
600
400
200
0
PPFD (umol. m-2. s-1)
20.0
15.0
12.5
10.0
7.5
5.0
Temperature (°C)

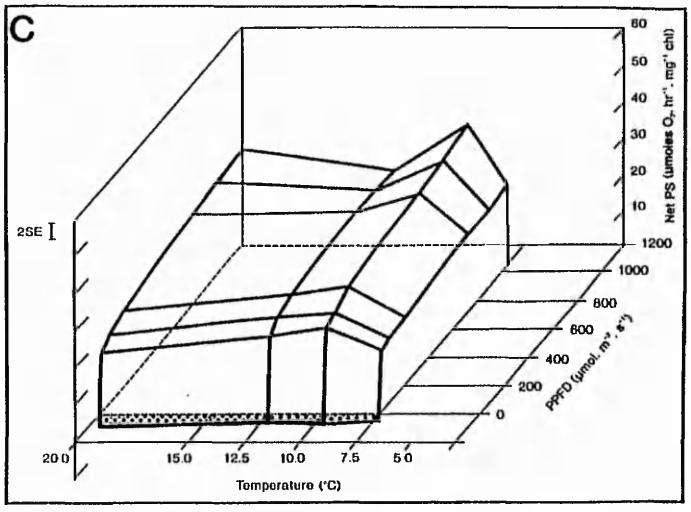

C
2SE
60
50
40
30
20
10
Net PS (umoles O2. hr-1. mg-1 chl)
1200
1000
800
600
400
200
0
PPFD (umol. m-2. s-1)
20.0
15.0
12.5
10.0
7.5
5.0
Temperature (°C)

Table 2.1 Maximum net photosynthetic rates of *C. fragile* frond tips at 1086.6 + 23.5 μmol m^{-2} s^{-1} PPFD. *Maximum photosynthetic rate with the *C. fragile* sampled on 30/ 11/ 87 was observed at 818.2 $\pm$ 5.2 μmol m^{-2} s^{-1} PPFD. Each value represents the mean $\pm$ standard error of 10 observations with different frond tip samples.

Net Photosynthetic Rate

μmoles O_2 hr^{-1} g^{-1} FWt

(μmoles O_2 hr^{-1} mg^{-1} chl)

	Harvest Date		
	30/ 11/ 87	04/ 04/ 88	11/ 09/ 88
Temp			
5.0 °C	25.4 $\pm$ 2.6	22.5 $\pm$ 1.8	21.3 $\pm$ 1.3
	(35.2 $\pm$ 4.1)	(34.2 $\pm$ 3.0)	(60.2 $\pm$ 4.0)
7.5 °C	18.9 $\pm$ 1.6	19.8 $\pm$ 1.3	7.7 $\pm$ 0.8
	(38.4 $\pm$ 2.3)	(34.0 $\pm$ 2.1)	(21.7 $\pm$ 2.1)
10.0 °C	25.5 $\pm$ 4.1*	25.4 $\pm$ 1.8	15.2 $\pm$ 1.7
	(51.1 $\pm$ 8.8)	(32.1 $\pm$ 2.2)	(33.7 $\pm$ 3.3)
12.5 °C	20.8 $\pm$ 2.6	16.5 $\pm$ 1.7	9.3 $\pm$ 0.7
	(43.2 $\pm$ 5.3)	(25.6 $\pm$ 2.8)	(26.3 $\pm$ 2.6)
15.0 °C	19.8 $\pm$ 1.3	18.2 $\pm$ 2.0	15.9 $\pm$ 0.9
	(39.2 $\pm$ 2.5)	(24.1 $\pm$ 2.8)	(43.8 $\pm$ 2.9)
20.0 °C	19.8 $\pm$ 2.3	20.5 $\pm$ 1.8	12.1 $\pm$ 1.4
	(41.1 $\pm$ 5.2)	(27.5 $\pm$ 2.4)	(26.9 $\pm$ 3.2)

Table 2.2 Chlorophyll content of *C. fragile* frond tips (μg chl g^{-1} FWt). Each value represents the mean $\pm$ standard error of 54 to 120 observations.

Harvest Date	Frond Chlorophyll Content (μg g^{-1} FWt) Experimental Incubation Regime		
	'Light/dark'	'Dark'	Overall Value
30/11/87	537.4 $\pm$ 21.5	558.9 $\pm$ 28.5	547.1 $\pm$ 18.8
04/04/88	705.8 $\pm$ 18.7	702.0 $\pm$ 21.9	703.8 $\pm$ 14.5
11/09/88	392.3 $\pm$ 9.4	381.4 $\pm$ 12.0	390.8 $\pm$ 7.6

apparently not significant as illustrated in Figure 2.2 and Table 2.1. When investigating the sample harvested on 11/ 09/ 88 (summer - late reproductive), the experiments at 5 and 15 °C produced extremely high net PS rates at all light intensities when compared with the other data, as indicated by the relevant values in Table 2.1. The reason for such high net PS rates is uncertain, but the experiments at 5 and 15 °C were carried out on consecutive days and this may be significant. The photosynthetic response is similar whether calculated on a chlorophyll or fresh weight basis, an exception perhaps being the 04/ 04/ 88 sample, with which calculation on a chlorophyll basis yields a very 'flat' response, a peak at 10 °C being absent. The net PS rates of the November and April samples were generally of the same magnitude and greater than the rates of the September tissue when calculated on a fresh weight basis (Figures 2.4 and 2.5 and Table 2.1). When calculated on a chlorophyll basis the net PS rates obtained with the November tissue were greater than the rates obtained with the other two tissue samples. The average chlorophyll content of the tissue in μg chlorophyll g^{-1} FWt was calculated from the fronds used at each experimental temperature (where n = 20; 10 'dark' and 10 'light / dark' incubated samples). This calculation was repeated using data obtained at the six temperatures investigated. These results are presented in Table 2.2 where they are expressed as separate values for the 'light / dark' and 'dark' incubated samples and as an overall value with the 'light / dark' and 'dark' sample data pooled together for each tissue sample. There was no difference in the chlorophyll content of the 'light / dark' and 'dark' incubated samples and the chlorophyll content of the September sample was lowest, followed by the November tissue, with the highest chlorophyll content occurring in the April tissue.

2.4 Discussion

The experiments described in this Chapter were carried out on tissue harvested at different times in the yearly life cycle of *C. fragile* and in general all tissues, including both vegetative and reproductive fronds, exhibited similar trends in photosynthesis to varying PPFD and temperature. From Figures 2.2, 2.4, and 2.5 it may be seen that net photosynthetic rates increased rapidly as the PPFD was increased from 0 to 80 μmol m^{-2} s^{-1} and saturation occurred at approximately 200 μmol m^{-2} s^{-1}. Saturation of *C. fragile* frond photosynthesis at 346 μmol m^{-2} s^{-1} has been reported previously (Arnold and Murray, 1980). Photosynthetic saturation at such low PPFD indicates considerable

light harvesting efficiency and excitation energy transfer (Herron and Mauzerall, 1972) and is an adaptation typical of shade plants. These results support previous evidence of such adaptation in *C. fragile* as discussed in Chapter 1 . For example, *C. fragile* contains relatively large amounts of siphonein, siphonoxanthin and chlorophyll b, enhancing absorption in the 'green gap' region of the light spectrum (Benson and Cobb, 1981, 1983; Anderson, 1983, 1985), and has up to 75 % of the pigment content in the light harvesting complexes (Benson, 1983). In addition, the total pigment content may be increased to such an extent that the alga is almost optically black (Ramus, *et al.* 1976; Dring, 1981b) and the utricle structure may also enhance light absorption (Ramus, 1978). However, although net PS saturated at approximately 200 μmol m^{-2} s^{-1}, there was no decline in photosynthetic capacity of intact fronds with a further increase in PPFD up to 1200 μmol m^{-2} s^{-1}. Indeed, there was a negligible increase in net PS with this increase in PPFD. Such an extended saturation plateau suggests that *C. fragile* fronds are able to avoid photoinhibition under the conditions of the 5 min dark / 5 min light incubation regime used in this study. Similar findings have been reported. For example, Arnold and Murray (1980) found that full sunlight (1405 - 1956 μmol m^{-2} s^{-1}) did not inhibit photosynthesis in fronds of *C. fragile* and Ramus, *et al.* (1976) also found that the maximum rate of photosynthesis in this alga was maintained at full sunlight. Whilst Benson (1983) found that fronds of *C. fragile* did not exhibit photoinhibition at light intensities of 3000 μmol m^{-2} s^{-1} PPFD and that photooxidation was not induced after six hours of high intensity non - fluorescent light treatment. However, the frond anatomy may result in the photosynthetic tissue being heterogenously illuminated and unequally efficient in capturing light energy, resulting in extended photosynthetic light saturation responses (Ramus, *et al.*, 1976) and this will be exacerbated in the LD2 leaf disc electrode chamber, the design of which results in illumination from one direction, and hence of only a part of the frond surface area directly (Figure 2.1).

From Figures 2.2 and 2.3 it was evident that the rates of oxygen uptake in the 'dark' incubated frond tips were less than the dark oxygen uptake rates during the dark periods of the 'light / dark' incubated samples. That is, it appeared that the periods of light exposure used in these experiments enhanced the oxygen uptake recorded in the intervening dark periods. It was also apparent from Figure 2.2 that the dark oxygen uptake of the 'light / dark' incubated fronds showed no relationship with the light

intensity preceding the dark period, indicating that the same degree of dark oxygen uptake enhancement occurred at all light intensities used. Such a light - dependent increase in dark oxygen uptake, that is unrelated to the PPFD, may possibly be due to the operation of the photorespiratory carbon oxidation (PCO) cycle (Figure 1.8). This would involve oxygen consumption in the chloroplasts via ribulose - 1, 5 - bisphosphate oxygenase in phosphoglycolate production and in the mitochondria via cytochrome oxidase (Lorimer and Andrews, 1981). However, oxygen consumption via glycolate oxidase in the oxidation of glycolate to glyoxylate in peroxisomes, typical of many higher plants, will not occur since *Codium* possesses a glycolate dehydrogenase, which does not use molecular oxygen as an immediate electron acceptor, to carry out this reaction (Frederick, *et al.*, 1973; Reiskind, *et al.*, 1988). It has been suggested that the photorespiratory carbon oxidation cycle can give some protection against photoinhibition by contributing to the dissipation of excess excitation energy (*e.g.* Heber and Krause, 1980; Krause and Cornic, 1987; Krause and Laasch, 1987), and such a role is of particular interest in *Codium* as light induced glycolate excretion and carbohydrate release as hexoses has been reported (Samuel, *et al.* 1971; Benson, 1983), especially under conditions facilitating high photorespiratory rates. Up to 40 % of assimilated carbon may be released at supraoptimal PPFD. Such extracellular carbon release may be a mechanism to excrete excess glycolate when photorespiratory rates are particularly high (Lorimer and Andrews, 1981). Perhaps photoinhibition of the frond samples may be partially relieved by increased energy dissipation via the PCO cycle and the operation of this cycle may in turn be facilitated by carbon release as glycolate. The operation of photorespiration as indicated by such oxygen consumption would be consistent with the suggestion of Benson (1983) that *C. fragile* may have the ability to use the surrounding medium as an extracellular zone from or to which it may reclaim or release excessive amounts of metabolites as a means of adjusting to the immediate fluctuations of the light environment. However, the majority of the energy used in the PCO cycle is required in the stages that reclaim carbon lost to glycolate and thus excreting glycolate releases the very substrate required for these energy consuming reactions and this may thus reduce rather than enhance the role of photorespiration in the dissipation of excess photosynthetic energy. The loss of fixed carbon as glycolate and hexoses is a wasteful process and as such may be viewed as a possible mechanism of energy dissipation contributing to the avoidance of photoinhibition.

A second process which may result in oxygen uptake is the Mehler reaction. The Mehler reaction involves the reduction of oxygen by PSI producing the oxygen free radical superoxide, the hydroxyl radical and hydrogen peroxide (Powles, 1984). There are efficient scavenging systems that remove these harmful species and it has been suggested that the Mehler reaction may act as an energy dissipating mechanism and thus may have a photoprotective function, oxygen reduction allowing a limited drain of photosynthetic energy by non - cyclic electron transport, partially relieving 'over - reduction' of the electron transport chain and maintaining a high trans - thylakoid proton gradient (Krause and Cornic, 1987; Krause and Laasch, 1987).

Bennoun (1982) reported the existence in the thylakoid membrane of an electron transfer chain that oxidized NAD(P)H at the expense of oxygen, suggesting that such 'chlororespiration' was an oxygen uptake process distinct from photorespiration and the Mehler reaction. Further work provided evidence for a chlororespiration process in higher plant cells and isolated protoplasts and intact chloroplasts that was similar to the respiratory electron transport section operating in photosynthetic bacteria, cyanobacteria and green algae (Garab, *et al.*,1989). These workers found that inhibition of the chlororespiratory pathway with KCN increased the turnover of the Cyt b_6/f complex and suggested this was correlated with reactivation of 'silent' PSI reaction centres. In the absence of cyanide, electrons from the Cyt b_6/f complex may be diverted from photosynthetic electron transport, thus rendering a fraction of centres 'silent'. These 'silent' reaction centres can be reactivated by inhibiting the alternative, respiratory reaction route with cyanide (Garab, *et al.*, 1989). That is, the respiratory pathway competes with the photosynthetic pathway for electrons in the region of the plastoquinone pool and the Cytb_6/f complex, as in similar systems in photosynthetic prokaryotes and green algae (Bennoun, 1982). The details of this process are unclear but an increase in chlororespiration may also contribute to the light - induced increase in dark oxygen uptake observed in this study. Furthermore, if this alternative pathway competes with the photosynthetic pathway for electrons then chlororespiration may possibly have a role in protection against photoinhibition by allowing a drain of photosynthetic energy by electron transport along an alternative route.

The conditions of this study, particularly the light intensities in excess of approximately 200 μmol m^{-2} s^{-1} at which net PS was light saturated, could potentially lead to photoinhibition but there was no apparent decline in oxygen evolution at the

higher light intensities suggesting a lack of photoinhibition at supraoptimal PPFD. This, in turn, suggests that preventative / protective mechanisms were in operation. The light - dependent increase in dark oxygen uptake observed could be indicative of the operation of photorespiration and / or the Mehler reaction which may both contribute to the avoidance of photoinhibition. Whether these processes were occurring, and if so to what relative extent, is uncertain. Any such mechanisms responsible for the observed dark oxygen uptake in the 'light / dark' incubated samples appeared to be functioning to the same extent throughout the experimental period, the rate of oxygen uptake being unchanged by change in PPFD between approximately 26 and 1200 μmol m^{-2} s^{-1}. The other possible protective mechanisms, as discussed in Chapter 1, could also be responsible for the apparent lack of photoinhibition of intact frond photosynthesis as observed in this study. For example, superoxide dismutase has been found in *C. fragile* (Lumsden and Hall, 1975) indicating that a system exists to scavenge or prevent the formation of reactive oxygen species. It has been suggested that state transitions (*e.g.* Fork and Satoh, 1986; Gounaris, *et al.*, 1986; Staehelin, 1986; Iordanov and Goltsev, 1987; Anderson and Andersson, 1988) have a role in the prevention of photoinhibition by enabling efficient photosynthesis to be achieved by maintaining a suitable ratio of activities of the two photosystems. State transitions have been clearly demonstrated in fronds of *C. fragile* (Sealey, *et al.*, 1990) and this mechanism may contribute to the avoidance of photoinhibition in this alga, along with D1 repair and replacement (*e.g.* Kyle and Ohad, 1986) and the mechanisms associated with the phenomena of q_E and q_I quenchinq (*e.g.* Krause, 1988). In this study there was no difference in the chlorophyll content of the 'light / dark' and 'dark' incubated frond samples (Table 2.2) indicating that the light treatment was not severe enough to cause loss of pigment.

Maximum rates of net PS varied between 7.7 - 25.4 μmoles O_2 hr^{-1} g^{-1} FWt (21.7 - 51.0 μmoles O_2 hr^{-1} mg^{-1} chl) for September (11/ 09/ 88) tissue at 7.5 °C and December (30/ 11/ 87) tissue at 10.0 °C, respectively. On a fresh weight basis the lowest rates of net PS were obtained with the September sample (Table 2.1 and Figure 2.4) and frond chlorophyll content was highest in the April tissue, followed by the November tissue, whilst the lowest chlorophyll content occurred in the September tissue (Table 2.2). These results are highly consistent with the previous observations concerning *C. fragile* growth strategy (Benson, *et al.*, 1983; Williams, *et al.*, 1984). The pigment composition of fronds and chloroplasts is seasonally variable, with chlorophyll and

carotenoid concentrations highest in winter and spring. Chlorophyll a and b show a marked decline in the summer perhaps indicating susceptibility of these pigments to photooxidation. Carotenoids, particularly ß - carotene, are involved in the quenching of toxic active oxygen species in higher plants (Foote, 1976). However, ß - carotene is not found in *Codium* (Benson, 1983) and it has been suggested that its apparent replacement, α - carotene, may not have such a photoprotective function as the chlorophylls rather than the carotenoids present in this alga were found to be more susceptible to photooxidation (Benson, 1983). Such a susceptibility is indicated by the loss of chlorophyll in the summer months. A temperature optimum for photosynthesis of 10 °C was apparent (Figures 2.4 and 2.5), however the differences in net PS rates at the temperatures investigated appeared, generally, not to be significant. Any preference with respect to temperature is likely to reflect the temperature of the seawater of the intertidal marine habitat of *C. fragile* and the temperature of the storage tanks which were maintained in a 10 °C refrigerated cool room.

2.5 Conclusions

The experiments described in this Chapter revealed an essentially stable whole frond oxygen exchange response over the temperature and light intensity ranges investigated with the three tissue samples harvested at different stages in the yearly life cycle: 30/ 11/ 87 (winter - early vegetative), 04/ 04/ 88 (spring - late vegetative) and 11/ 09/ 88 (summer - late reproductive). A light - induced increase in the rate of dark oxygen uptake that was unrelated to PPFD was observed, a possible explanation being increased rates of photorespiration and / or Mehler reaction, both of which are thought to aid in the prevention of photoinhibition. Chlororespiration is a third process which may account for oxygen uptake. Photosynthesis saturated rapidly and at low PPFD indicating efficient light harvesting and photoinhibition was not observed, even at high PPFD. This supports previous evidence of the shade type adaptations of *C. fragile* which enable efficient light harvesting and its ability to avoid photoinhibition at high light intensities. However, the extent to which different protective mechanisms may contribute to the avoidance of photoinhibition remain to be elucidated. An insignificant temperature optimum for photosynthesis of 10 °C was observed and lowest photosynthetic rates and chlorophyll contents occurred in September, which is consistent with the growth strategy of this alga.

CHAPTER 3. THE OXYGEN EXCHANGE OF ISOLATED C. FRAGILE CHLOROPLASTS

3.1 Introduction

Studies with whole fronds, described in the previous Chapter, confirmed that *C. fragile* is highly efficient at light harvesting with photosynthesis saturating at low PPFD. There was, however, no indication of photoinhibition at supraoptimal PPFD and the frond photosynthetic response was found to be stable, with similar results being obtained with the three samples from different stages of the life cycle and at the six temperatures investigated. A temperature optimum of °10 C, although not significant, was apparent. Therefore, the experiments described in this Chapter, involved the polarographic measurement of the oxygen exchange responses of isolated chloroplasts in relation to chlorophyll concentration and PPFD, at a fixed temperature of 10 °C, with isolates from tissue sampled at three stages in the yearly life cycle of the alga. The aim was to characterise in detail the photosynthetic response of isolated chloroplasts, identifying any limitation of photosynthesis to light intensity.

3.2 Materials and Methods

3.2.1 Sampling and Maintenance of *C. fragile*

Fronds of *C. fragile* were harvested on 04/ 04/ 88 (spring - late vegetative), 11/ 09/ 88 (summer - late reproductive) and 18/ 12/ 88 (winter - vegetative) as described previously (2.2.1) and similarly maintained.

3.2.2 Chloroplast Isolation

Chloroplasts were isolated from frond tips using the method of Cobb (1977). A 90g sample of frond tips was homogenised in 200 cm^3 of semi - frozen extraction medium (0.5 mol dm^{-3} sucrose, 2 g dm^{-3} bovine serum albumin (Sigma Chemicals, Fraction V) and 0.015 mol dm^{-3} $MgCl_2$. $6H_2O$ in 0.05 mol dm^{-3} HEPES buffer, adjusted to pH 7.8 with 1 mol dm^{-3} NaOH) using a regime of 3 x 3 seconds at full speed in an Atomix blender. The homogenate was filtered through two layers of 0.3 mm width gauze ('Nytal', Henry Simon Ltd.) into 4 ice - cooled 50 cm^3 centrifuge tubes and pelleted for five min at 400g (1550 rpm) in a Denley BR401 refrigerated centrifuge. The

supernatant was carefully decanted and the crude chloroplast pellets resuspended in a total volume of 6 cm^3 of ice cold extraction medium. A 2 cm^3 amount was carefully layered onto a water - jacketed gel filtration column (Pharmacia Ltd. K series 1.6 x 20 cm) containing loosely packed Sephadex G50 coarse (2.5g Sephadex, pre - swollen for at least two h in 35 cm^3 of extraction medium at 4 °C). To prevent consolidation of the column the Sephadex was poured during the centrifugation process. Flow through the column at a flow rate of 2 cm^3 min^{-1} was achieved with a Pharmacia peristaltic pump. Chloroplasts migrated as a distinct green band and the first 3 cm^3 were collected and retained on ice. The gel - filtration was repeated for the remaining 2 cm^3 amounts of crude chloroplast suspension and the final filtrates combined. A more concentrated preparation was sometimes required and this was achieved by scaling up the above procedure. The ratio of tissue to extraction medium was maintained (*i.e.* 90g to 200 cm^3) and two gel filtration columns used. The combined filtrates from the columns were then centrifuged at 400g for approximately 5 min and then resuspended in a small volume (depending on the size of the preparation) of ice cold extraction medium, thereby producing a more concentrated chloroplast preparation.

3.2.3 Determination of Chlorophyll Content and Plastid Number

A volume of 100 μl of chloroplast preparation was added to 1900 μl of 80 % (v/v) aqueous acetone and placed on ice for 10 min in the dark in a centrifuge tube. The suspension was then pelleted for 5 min in a Denley centrifuge and the absorbance of the supernatant was recorded at 663 nm and 645 nm against an 80 % (v/v) aqueous acetone blank using a Cecil CE 303 Series 2 grating spectrophotometer. The values obtained were used in the equation of MacKinney (1941) to determine chlorophyll content:

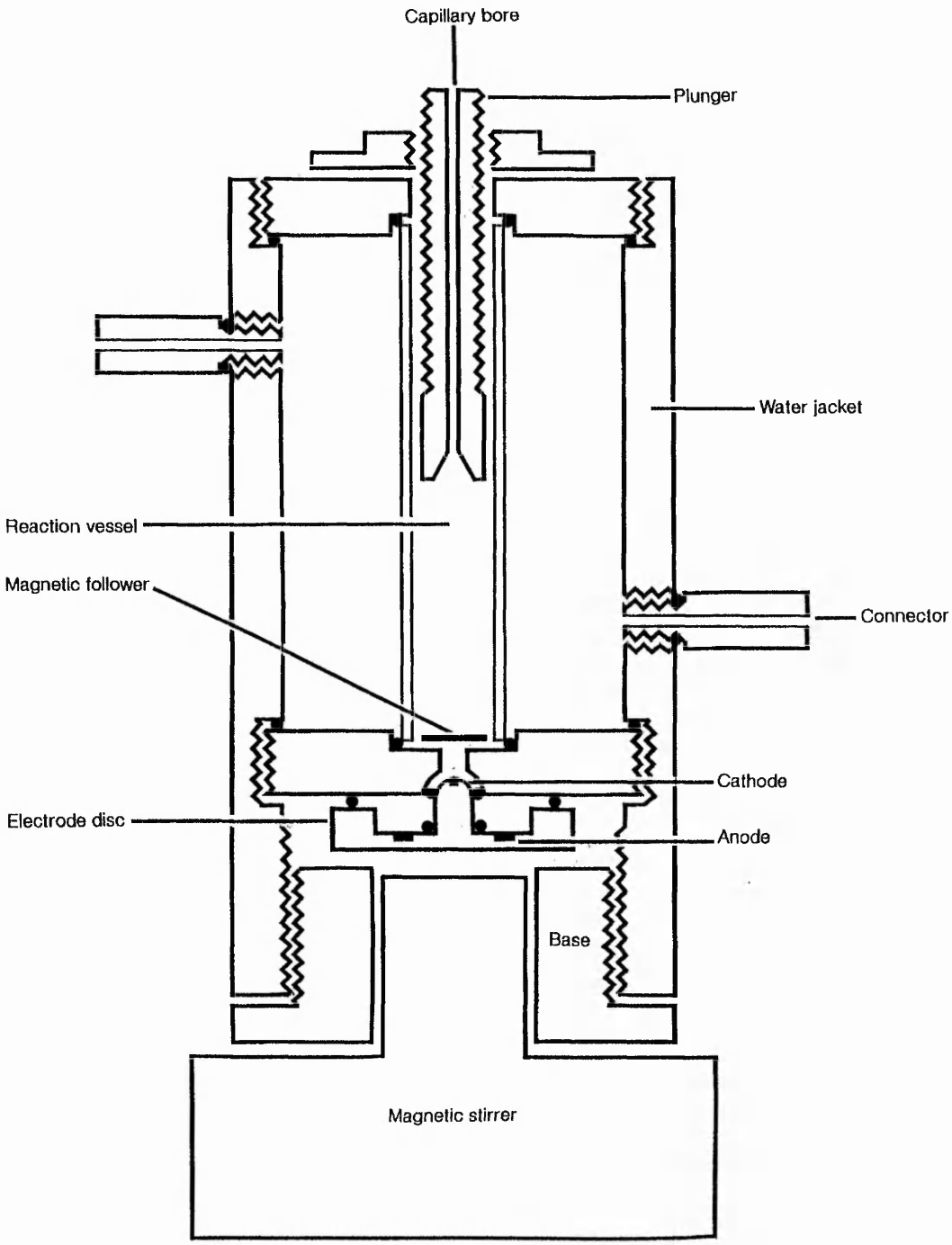
$$\text{Total Chl} = (A_{645} \times 20.2) + (A_{663} \times 8.02) \times 20$$
$$= x\ \mu g.\ cm^{-3}$$

A 20, 30 or 40 times dilution of the chloroplast preparation was made using extraction medium and the plastid number determined using a haemocyometer.

3.2.4 Measurement of Oxygen Exchange of Isolated Cloroplasts

The measurement of oxygen exchange of isolated chloroplasts in the aqueous phase was achieved using a DW1 oxygen electrode (Hansatech Ltd. - see Figure 3.1) and the

Figure 3.1 The Hansatech DW1 aqueous phase oxygen electrode. Adapted from Walker, 1985.


Capillary bore
Plunger
Water jacket
Reaction vessel
Magnetic follower
Connector
Cathode
Electrode disc
Anode
Base
Magnetic stirrer

methods of Delieu and Walker (1972). A solution of saturated KCl (electrolyte) was placed in the electrolyte well and the tissue paper and teflon membrane secured with a rubber 'o' ring using the membrane applicator as before (2.2.2). The electrode disc was then inserted into its position at the base of the DW1 chamber and attached to a magnetic stirrer, the potentiating box and a chart recorder, which were both set in the 10 mV range. The reaction chamber was maintained at 10 °C by connecting the water jacket to a Techne C400 circulator and Techne M1000 heat exchange unit. The electrode was calibrated using 1 cm^3 of air saturated distilled water (of known oxygen content) to set the upper limit of the scale and a few grains of sodium dithionite were added to remove oxygen from solution,

$$Na_2S_2O_4 + O_2 + H_2O \text{ ------} > NaHSO_4 + NaHSO_3$$

,producing the lower limit of the scale (zero oxygen). The electrode well was washed thoroughly to remove all sodium dithionite present.

Initial experiments investigated oxygen exchange and chlorophyll concentration at a fixed PPFD of 200 μmol m^{-2} s^{-1} provided by a 250 W tungsten - halogen Halight 24/ 250 projector. A 1 cm^3 reaction volume was used consisting of 100 μl of 100 mol m^{-3} $NaHCO_3$ (final concentration 10 mol m^{-3}) and an appropiate volume of chloroplast suspension (depending upon its concentration) to give a final chlorophyll concentration of 20, 50, 100, 150 or 200 μg in the 1 cm^3 reaction volume. The final volume was made up to 1 cm^3 by the addition of an appropiate volume of extraction medium. With the plunger of the DW1 electrode chamber in place and the stirrer on, the apparatus was placed in darkness by covering with a cloth and the oxygen exchange followed for 10 min. Following dark incubation the electrode was illuminated at 200 μmol m^{-2} s^{-1} PPFD for 20 min and the photosynthetic bicarbonate - dependent oxygen evolution recorded. Post light treatment dark incubation oxygen exchange was then measured for a further 10 min by replacing the dark cloth. Each chlorophyll concentration was investigated with each chloroplast preparation and each chlorophyll concentration was repeated 6 or 7 times. A second series of experiments investigated oxygen exchange and PPFD at fixed chlorophyll concentrations of 25, 50, 100, 150, and 200 μg cm^{-3}. By changing the distance between the light source and the electrode reaction chamber in conjunction with the use of suitable neutral density filters the following light intensities were achieved: 0, 12, 25, 45, 63, 100, 250 and 1000 μmol m^{-2} s^{-1}. The experiments were carried out as before. All light intensities were investigated with each chloroplast

preparation and in general each light intensity was repeated 6 or 7 times. The results were expressed as μmoles O_2 per hour per mg chlorophyll.

3.3 Results

The three tissue samples from different stages in the life cycle showed that chloroplasts isolated from both vegetative and reproductive algal frond tips exhibited a similar photosynthetic response over the chlorophyll concentration and light intensity ranges studied. Figure 3.2 illustrates a typical oxygen exchange response with increasing chlorophyll concentration of the chloroplast suspension, as obtained with chloroplasts isolated from fronds harvested on 18/ 12/ 88 (winter - vegetative). Net PS and gross PS rates increased in a linear fashion with an increase in the amount of chloroplast suspension present in the reaction volume up to the equivalent of a chlorophyll concentration of 150 μg cm^{-3}. The rate of PS increased to a lesser degree with further increase in chlorophyll concentration to 200 μg cm^{-3}. The oxygen uptake in the dark incubation before illumination appears to increase with increasing chlorophyll concentration, as does the oxygen uptake during dark incubation after the 20 min of illumination. The rate of O_2 uptake is significantly higher in the dark period after illumination than in the dark period preceding illumination, at all chlorophyll concentrations. In a second series of experiments the oxygen exchange response to different light intensities at different chlorophyll concentrations was investigated. Again, the three tissue samples behaved in a similar fashion and a typical light saturation curve is illustrated in Figure 3.3 for chloroplasts, at 25 μg cm^{-3} chlorophyll, isolated from fronds harvested on 18/ 12/ 88 (winter - vegetative). It can be seen that oxygen uptake in the dark period after 20 min illumination is always greater than in the dark period preceding illumination, at all light intensities investigated. This difference was, however, not always significant, unlike the first set of experiments. There appears to be no relationship between the rate of dark oxygen uptake and the preceding light intensity. As there is a negligible change in dark oxygen uptake preceding illumination net PS curves are approximately parallel to gross PS curves and any changes in net PS with change in light intensity are mainly due to changes in gross PS and are not complicated by changes in the dark oxygen uptake. Therefore, when studying the photosynthetic response it is sufficient to consider the net PS values only. Typical light saturation curves are illustrared in Figures 3.3 and 3.4 for chloroplasts isolated from

Figure 3.2 Relationship between oxygen exchange (μmoles O_2 hr^{-1}) at 10 °C and chlorophyll content (μg) of 1 cm^3 suspensions of *C. fragile* chloroplasts isolated from tissue sampled on 18/ 12/ 88 (winter - vegetative). Key: Net PS, net photosynthesis; Gross PS, gross photosynthesis; Pre, oxygen uptake during dark incubation before illumination at 200 μmol m^{-2} s^{-1} PPFD; Post, oxygen uptake during dark incubation after 20 min of illumination at 200 μmol m^{-2} s^{-1} PPFD. Each point represents the mean $\pm$ standard error of 6 or 7 observations. Standard error bars included where greater than symbol size.

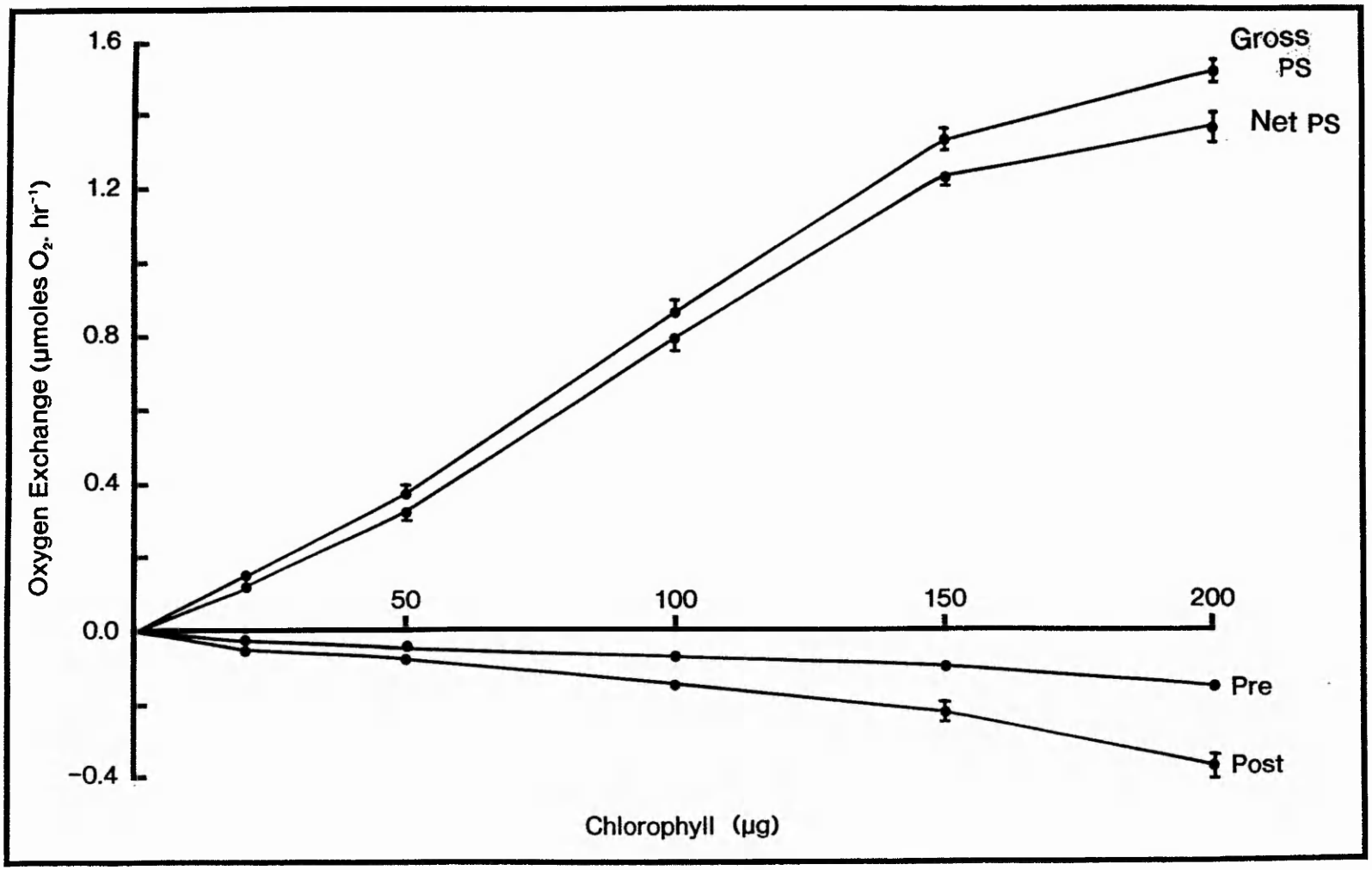
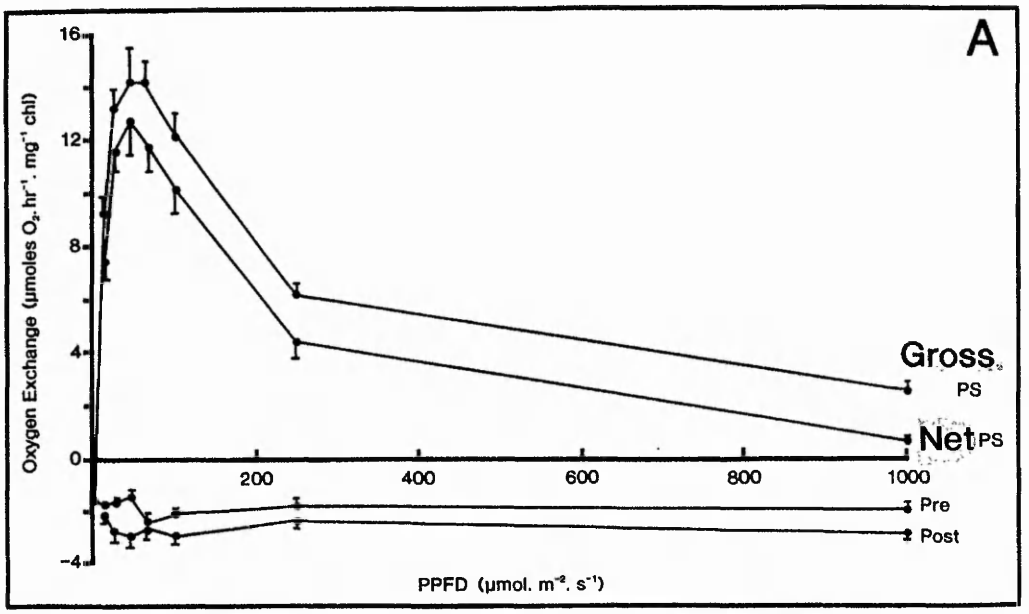

Gross PS
Net PS
Pre
Post
Oxygen Exchange (µmoles O_2. hr^{-1})
Chlorophyll (µg)
1.6
1.2
0.8
0.4
0.0
-0.4
50
100
150
200

Figure 3.3 Relationship between PPFD (μmol m^{-2} s^{-1}) and oxygen exchange (μmoles O_2 hr^{-1} mg^{-1} chl) at 10 °C of a suspension of *C. fragile* chloroplasts equivalent to a concentration of 25 μg chl cm^{-3}, isolated from tissue sampled on 18/ 12/ 88 (winter - vegetative). A) data from 0 to 1000 μmol m^{-2} s^{-1} PPFD. B) data from 0 to 250 μmol m^{-2} s^{-1} PPFD. Key: Net PS, net photosynthesis; Gross PS, gross photosynthesis; Pre, oxygen uptake during dark incubation before illumination; Post, oxygen uptake during dark incubation after 20 min illumination. Each point represents the mean $\pm$ standard error of 7 observations. Standard error bars included where greater than symbol size.


A
Oxygen Exchange (μmoles O_2.hr^{-1}. mg^{-1} chl)
16
12
8
4
0
-4
200
400
600
800
1000
Gross PS
Net PS
Pre
Post
PPFD (μmol. m^{-2}. s^{-1})

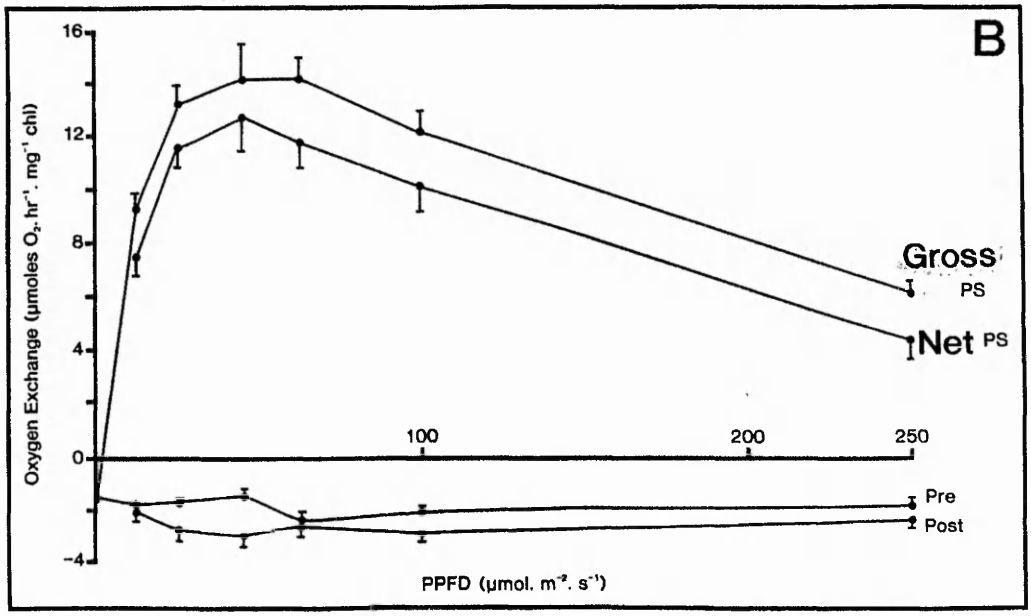
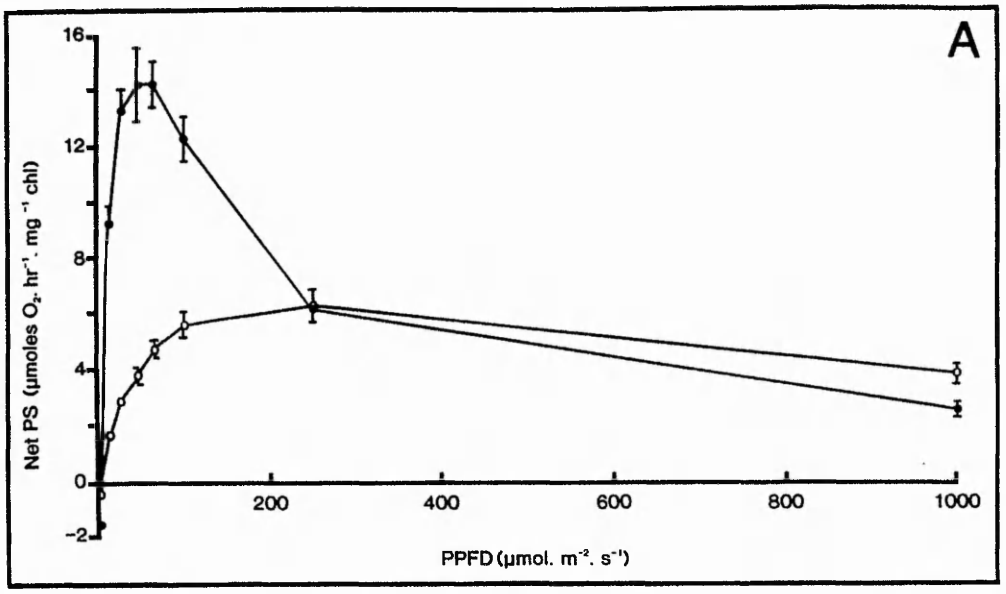
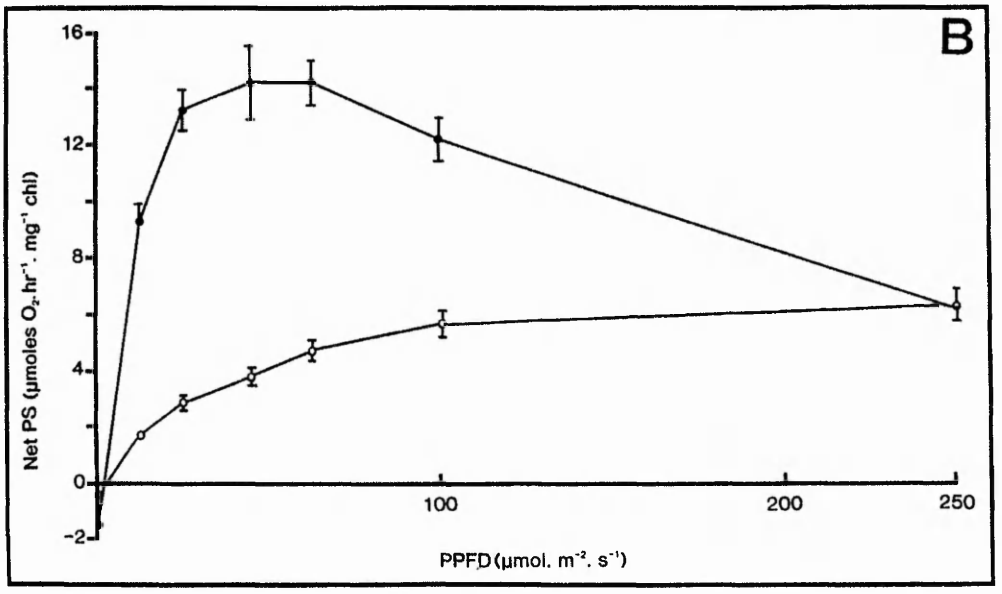

B
Oxygen Exchange (μmoles O_2.hr^{-1}. mg^{-1} chl)
16
12
8
4
0
-4
100
200
250
Gross PS
Net PS
Pre
Post
PPFD (μmol. m^{-2}. s^{-1})

Figure 3.4 Relationship between PPFD (μmol m^{-2} s^{-1}) and net photosynthesis (μmoles O_2 hr^{-1} mg^{-1} chl) at 10 °C of suspensions of *C. fragile* chloroplasts isolated from tissue sampled on 18/ 12/ 88 (winter - vegetative). A) data from 0 to 1000 μmol m^{-2} s^{-1} PPFD. B) data from 0 to 250 μmol m^{-2} s^{-1} PPFD. Key:●, chloroplast suspension equivalent to a concentration of 25 μg chl cm^{-3};○, chloroplast suspension equivalent to a concentration of 200 μg chl cm^{-3}. Each point represents the mean $\pm$ standard error of 5 - 7 observations. Standard error bars included where greater than symbol size.


A
16
12
8
4
0
-2
Net PS (µmoles O_2. hr^{-1}. mg^{-1} chl)
200
400
600
800
1000
PPFD (µmol. m^{-2}. s^{-1})


B
16
12
8
4
0
-2
Net PS (µmoles O_2. hr^{-1}. mg^{-1} chl)
100
200
250
PPFD (µmol. m^{-2}. s^{-1})

fronds harvested on 18/ 12/ 88 (winter - vegetative). The chlorophyll concentration and light intensity at which maximum net PS rates occurred are summarised in Table 3.1, with rates expressed as μmoles O_2 hr^{-1} mg^{-1} chl or μmoles O_2 hr^{-1} 10^{-9} plastids. Although a similar trend was observed, the absolute rates obtained varied markedly between tissue samples, however, these maximum rates may be designated 100 % and the rates obtained at other light intensities expressed as a per centage of these maximum rates as presented in Tables 3.2, 3.3 and 3.4, enabling graphical comparison of the three tissue samples (Figure 3.5). Apart from the 18/ 12/ 88 tissue at 200 μg cm^{-3} chlorophyll, where maximum net PS was achieved at a PPFD of 250 μmol m^{-2} s^{-1}, all other samples saturated at low irradiance; between 25 and 100 μmol m^{-2} s^{-1} PPFD (Table 3.1). It was found that beyond the optimum PPFD for each sample the rate of net PS decreased with increase in light intensity. Generally, the lower the chlorophyll concentration, the lower the light intensity required for maximum photosynthesis and the more dramatic the decline in net PS once the optimum light intensity was surpassed. This response was evident with both vegetative and reproductive tissue and is clearly illustrated in Figure 3.5 and Tables 3.1, 3.2, 3.3 and 3.4, and is highlighted by comparison of the extremes of chlorophyll concentration (Figure 3.4). At 25 μg chlorophyll net PS saturated at 45 - 63 μmol m^{-2} s^{-1} PPFD, a narrow saturation plateau was observed and supraoptimal irradiance resulted in a decline of net PS to 43.5 and 18.0 % at 250 and 1000 μmol m^{-2} s^{-1} PPFD, respectively. In contrast, at 200 μg chlorophyll net PS saturated at 250 μmol m^{-2} s^{-1} PPFD and a more extended saturation plateau was observed, but even at this concentration there was a decline in net PS to 61.5 % of the maximum rate with a further increase in intesity to 1000 μmol m^{-2} s^{-1} PPFD. From Table 3.1 it appears that the 18/ 12/ 88 tissue produced much greater maximum net PS rates than the 04/ 04/ 88 and 11/ 09/ 88 tissue. However, after harvesting of the latter two samples experiments on the whole frond were carried out prior to experiments with isolated chloroplasts. In contrast, the experiments with isolated chloroplasts were carried out immediately after harvesting the 18/ 12/ 88 tissue and therefore the storage time before commencement of the respective experiments differed markedly. As a consequence of this work results concerning the chlorophyll content of frond tip chloroplasts were accumulated and these are presented in Table 3.5. The September sample had least chlorophyll per chloroplast, followed by the April tissue, whilst the highest chlorophyll content occurred in the December sampled tissue.

Table 3.1 Relationship between chlorophyll content (μg) of 1 cm^3 chloroplast suspensions isolated from *C. fragile* and PPFD (μmol m^{-2} s^{-1}) required for maximum net photosynthesis. Each value represents the mean + standard error of between 2 to 12 observations. * First bracketed figures are the PPFD (μmol m^{-2} s^{-1}) at which maximum net photosynthesis occurred.

	Maximum Net Photosynthetic Rate μmoles O_2 hr^{-1} mg^{-1} chl (μmoles O_2 hr^{-1} 10^{-9} plastids) Harvest Date		
	04/04/88	11/09/88	18/12/88
Chl			
25	-	-	(63)*
	-	-	14.2 ± 0.9
	-	-	(10.8 ± 0.5)
50	(25)*	(45)*	(63)*
	4.5 ± 0.4	5.8 ± 0.6	14.8 ± 1.6
	(2.4 ± 0.3)	(1.8 ± 0.3)	(8.1 ± 0.6)
100	(45)*	(100)*	(100)*
	6.4 ± 0.6	5.1 ± 0.5	11.3 ± 0.5
	(3.7 ± 0.3)	(2.0 ± 0.3)	(5.7 ± 0.3)
150	(63)*	(100)*	(100)*
	2.9 ± 0.4	4.5 ± 0.6	7.4 ± 0.6
	(1.4 ± 0.2)	(1.7 ± 0.3)	(3.7 ± 0.3)
200	(100)*	-	(250)*
	3.4 ± 0.2	-	6.2 ± 0.6
	(1.6 ± 0.1)	-	(3.0 ± 0.2)

Table 3.2 Relationship between PPFD (μmol m^{-2} s^{-1}), chlorophyll concentration (μg cm^{-3}) and net photosynthesis, expressed as % maximum net photosynthesis, for chloroplasts isolated from *C. fragile* frond tips harvested on 04/ 04/ 88 (spring - late vegetative). Each value represents the mean of between 2 to 12 observations.

% Maximum Net Photosynthesis

	Chlorophyll Content (μg cm^{-3})			
	50	100	150	200
PPFD				
0	-14.0	-8.0	-19.0	-29.0
12	82.0	55.0	60.0	43.5
25	100.0	71.0	81.0	68.0
45	-	100.0	90.0	74.0
63	87.0	86.5	100.0	94.0
100	71.5	80.0	90.5	100.0
250	46.0	63.0	73.5	72.0
1000	35.0	37.0	56.5	54.5

Table 3.3 Relationship between PPFD (μmol m^{-2} s^{-1}), chlorophyll concentration (μg cm^{-3}) and net photosynthesis, expressed as % maximum net photosynthesis, for chloroplasts isolated from *C. fragile* frond tips harvested on 11/ 09/ 88 (summer - late reproductive). Each value represents the mean of 7 observations.

% Maximum Net Photosynthesis

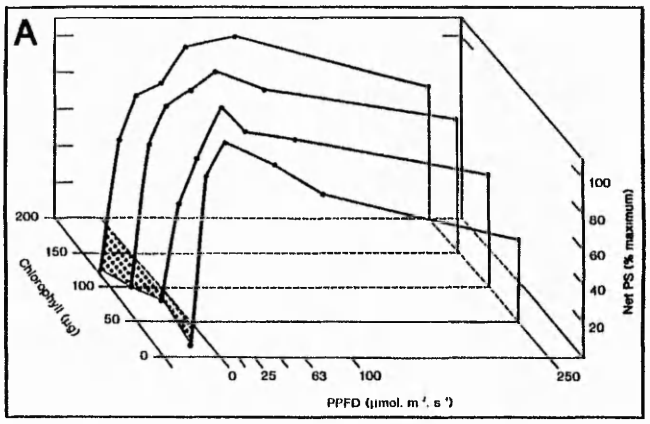
	Chlorophyll Content (μg cm^{-3})		
	50	100	150
PPFD			
0	-14.5	-15.5	-16.0
12	70.0	46.0	41.0
25	95.0	75.0	62.0
45	100.0	95.0	85.5
63	95.5	90.5	95.0
100	77.0	100.0	100.0
250	70.0	73.0	94.0
1000	29.0	33.5	51.5

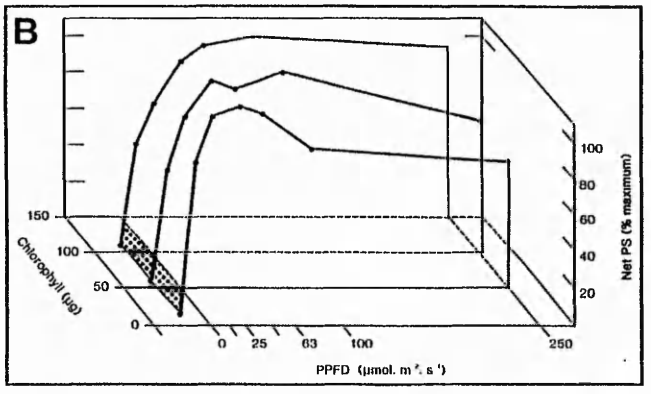
Table 3.4 Relationship between PPFD (μmol m^{-2} s^{-1}), chlorophyll concentration (μg cm^{-3}) and net photosynthesis, expressed as % maximum net photosynthesis, for chloroplasts isolated from *C. fragile* frond tips harvested on 18/ 12/ 88 (winter - vegetative). Each value represents the mean of between 5 to 7 observations.

% Maximum Net Photosynthesis

	Chlorophyll Content (μg cm^{-3})				
	25	50	100	150	200
PPFD					
0	-11.0	-6.0	-4.5	-7.0	-7.5
12	65.0	49.0	35.0	30.0	27.0
25	93.0	81.5	64.5	57.5	46.0
45	99.9	97.5	78.0	83.0	61.0
63	100.0	100.0	88.0	92.5	76.0
100	86.0	99.7	100.0	100.0	90.0
250	43.5	68.0	85.0	84.5	100.0
1000	18.0	19.0	34.0	42.0	61.5

Figure 3.5 Relationship between net photosynthesis (% maximum net photosynthesis), PPFD (μmol m^{-2} s^{-1}) and chlorophyll content (μg) at 10 °C of 1 cm^3 suspensions of chloroplasts isolated from *C. fragile* frond tips. A) 04/ 04/ 88 sample (spring - late vegetative). B) 11/ 09/ 88 sample (summer - late reproductive). C) 18/ 12/ 88 sample (winter - vegetative).


A
200
150
100
50
0
Chlorophyll (µg)
0
25
63
100
250
PPFD (µmol. m⁻². s⁻¹)
100
80
60
40
20
Net PS (% maximum)


B
150
100
50
0
Chlorophyll (µg)
0
25
63
100
250
PPFD (µmol. m⁻². s⁻¹)
100
80
60
40
20
Net PS (% maximum)

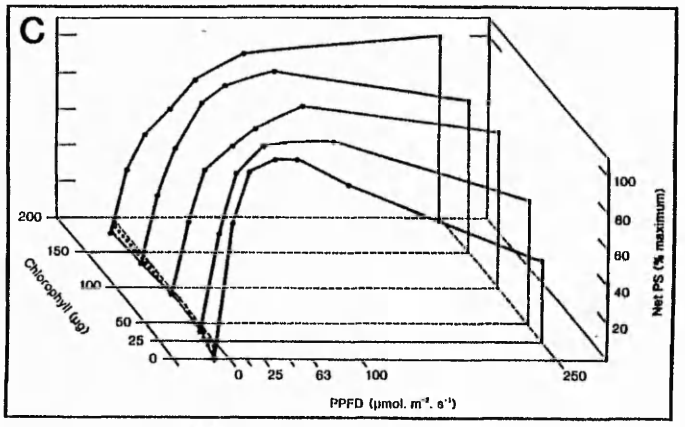

C
200
150
100
50
25
0
Chlorophyll (µg)
0
25
63
100
250
PPFD (µmol. m⁻². s⁻¹)
100
80
60
40
20
Net PS (% maximum)

Table 3.5 Chlorophyll content of *C. fragile* frond tip chloroplasts. Each point represents the mean + standard error of between 11 to 27 observations.

Harvest Date	Chlorophyll Content of Frond Tip Chloroplasts (μg chl 10^{-9} plastids)	n
04/04/88	523.0 $\pm$ 25.0	27
11/09/88	373.0 $\pm$ 28.5	11
18/12/88	579.5 $\pm$ 32.0	12
08/09/89	139.0 $\pm$ 17.5	12
19/12/89	533.0 $\pm$ 53.0	11

3.4 Discussion

The experiments described in this Chapter were carried out on chloroplasts isolated from tissue harvested at different times in the yearly life cycle of *C. fragile* and in general all tissue, including both vegetative and reproductive fronds, exhibited similar trends in photosynthesis to varying PPFD and chlorophyll concentration of isolated chloroplast suspensions. Figures 3.3, 3.4 and 3.5 showed that the rate of net PS increased rapidly with increasing light intensity and that saturation occurred at relatively low intensities. The suspension of chloroplasts, at a chlorophyll concentration of 200 $\mu g\ cm^{-3}$, isolated from tissue harvested on 18/ 12/ 88, exhibited maximum net photosynthesis at 250 $\mu mol\ m^{-2}\ s^{-1}$. In all other experiments samples at each of the chlorophyll concentrations investigated saturated at low PPFD (25 - 100 $\mu mol\ m^{-2}\ s^{-1}$) and this is comparable with the saturation intensities of isolated *C. fragile* chloroplasts of 50 - 100 $\mu mol\ m^{-2}\ s^{-1}$ (Benson, 1983) and 50 - 150 $\mu mol\ m^{-2}\ s^{-1}$ (Williams, 1986). As with the intact frond response, such rapid saturation at relatively low light intensities indicates considerable light harvesting efficiency and excitation energy transfer and is an adaptation typical of shade plants. These results support previous evidence of such adaptations in *C. fragile* (Ramus, *et al.*, 1976; Ramus, 1978; Benson and Cobb, 1981, 1983; Benson, 1983; Anderson, 1983, 1985). However, in contrast to the response of the intact frond, it was observed that at lower chlorophyll concentrations (*e.g.* 25 $\mu g\ cm^{-3}$) there was no significant saturation plateau but a marked decline in net photosynthesis was evident once the optimum PPFD was surpassed (Figures 3.3, 3.4 and 3.5 and Tables 3.2, 3.3 and 3.4). This strongly suggests the occurrence of photoinhibition at supraoptimal PPFD. Similar results have been hinted at by Benson (1983) and Williams (1986). Thus, the optimum PPFD for maximum net photosynthesis in this study was dependent upon the chlorophyll concentration of the suspension of chloroplasts used and this also appeared to influence the rate and extent of photoinhibition with increasing PPFD beyond the optimum. In general, the higher the chlorophyll concentration the higher the PPFD required to produce maximum net photosynthesis and the less dramatic the reduction in net photosynthesis due to photoinhibition with increasing PPFD beyond the optimum.

It was evident that the rate of oxygen uptake by isolated chloroplasts was greater in the 10 min dark incubation following illumination, than in the 10 min dark incubation preceding illumination (Figures 3.2 and 3.3). However, as with intact

fronds, this light dependent increase in dark oxygen uptake was not related to PPFD. This observation, as with whole fronds, may possibly be explained by the operation of chlororespiration, the Mehler reaction and photorespiration. The photorespiratory carbon oxidation pathway requires the interaction of chloroplasts, mitochondria and peroxisomes (Figure 1.8) and therefore the mechanisms behind the response observed with whole fronds may be expected to vary from those with isolated chloroplasts. However, the consumption of oxygen in the formation of phosphoglycolate from ribulose bisphosphate catalysed by ribulose bisphosphate carboxylase / oxygenase and subsequent formation of glycolate catalysed by a specific phosphatase all occurs within the chloroplast and oxygen uptake in this manner is a possibility in the isolated chloroplast experiments. Glycolate is excreted from its site of synthesis in the chloroplast in the cells of higher plants (Osmond, 1981) and therefore glycolate excretion by isolated *C. fragile* chloroplasts may occur. Similarly, the Mehler reaction may operate in isolated chloroplasts and, as with the whole frond, may contribute to the oxygen uptake observed. Both photorespiration and the Mehler reaction and possibly chlororespiration, may have a role in the prevention of photoinhibition as previously discussed (2.4). However, the majority of the energy consumed in photorespiration is concerned with the return to the Calvin cycle of carbon lost to glycolate and the reactions concerned require the interaction of mitochondria and peroxisomes and are therefore not likely to occur in a chloroplast preparation. This would reduce the usefulness of this process with regard to the prevention of photoinhibition in isolated chloroplasts. Photoinhibition was very evident, so if the enhanced oxygen uptake was due to glycolate production, Mehler reaction and chlororespiration, then these processes, and any other potentially photoprotective mechanisms that may have been operating, were clearly insufficient to prevent photoinhibition in the isolated chloroplasts in these experiments.

As previously discussed there are many mechanisms that are believed to aid in the prevention of photoinhibition which are likely to be operating in *C. fragile*. For example, superoxide dismutase has been found in this alga (Lumsden and Hall, 1975) and recently state I - state II transitions have been demonstrated in the whole frond (Sealey, *et al.*, 1990). The majority of these mechanisms involve processes that occur within the chloroplast and are therefore likely to be operative in isolated chloroplasts as well as in intact fronds. The differences revealed in photosynthetic response in this

study must be due to properties of the whole frond not shared by isolated chloroplasts, which enable the prevention of photoinhibition. For example, the cell wall may be of importance and the respiratory burden of the whole frond is greater. Fronds of *C. fragile* have been found to saturate at three times the PPFD of isolated chloroplasts and the former were found not to exhibit photoinhibition at light intensities of 3000 μmol m^{-2} s^{-1}, whilst photoinhibition of uniformly illuminated chloroplasts did occur (Benson, 1983). This led to the suggestion that the optically dense nature of the frond confers a filtering of incident light (Benson, 1983), and this is supported by the findings in this study. As the chlorophyll concentration of suspensions of isolated chloroplasts was increased (maximum investigated 200 μg cm^{-3}) and began to approach the high chlorophyll concentration of the intact frond, the photosynthetic response of the chloroplasts began to mimic that of the whole frond; with higher light intensities required for maximum net PS (*i.e.* saturation) and less dramatic reduction in net PS (*i.e.* photoinhibition) once the optimum PPFD was surpassed, *i.e*, an extended saturation plateau. As isolated chloroplasts are susceptible to photoinhibition but intact fronds are tolerant it is suggested that photoprotection may be conferred by the arrangement of the chloroplasts within the algal frond. *C. fragile* possesses a compact, dichotomous, cylindrical thallus consisting of closely interwoven coenocytic siphons or filaments (Lee, 1989; Bold and Wynne, 1985). In the medullary region of the thallus the siphons are colourless, whilst the outer layer (cortex) is constructed of the dilated tips (utricles) of the siphons which form a 'palisade - like' layer, and it is in these structures only that the chloroplasts are situated (Figure 1.9). The chloroplasts are arranged peripherally, in stacks, around a large central vacuole in such numbers that the thallus has an optically dense nature. The chloroplasts have comparatively simple thylakoids arranged in pairs or triplets with no extensive stacking. Due to the stack arrangement of chloroplasts within the utricle considerable light attenuation may operate within the utricle and a degree of 'self - shading' of the chloroplasts may occur. Hence, the number and arrangement of chloroplasts within the frond may confer a vital mechanism in the avoidance of photoinhibition by this alga.

The maximum rates of net photosynthesis of isolated chloroplasts varied from 2.9 - 14.8 μmoles O_2 hr^{-1} mg^{-1} chl (1.4 - 8.1 μmoles O_2 hr^{-1} 10^{-9} plastids) in the 04/ 04/ 88 tissue and the 18/ 12/ 88 tissue respectively (Table 3.1). This compares with rates of 8 to 25 μmoles O_2 hr^{-1} 10^{-9} plastids at 17 - 20 °C from tissue sampled in April and

November 1984 (Williams, 1986) and 30.5 and 62 μmoles CO_2 hr^{-1} mg^{-1} chl for tissue sampled in June 1983 and November 1978, respectively (Benson, *et al.*, 1983; Williams, *et al.*, 1984). From Table 3.5 it is evident that the September tissue had the lowest plastid chlorophyll content (139 - 373 μg chl 10^{-9} plastids) whilst the December tissue had the highest chlorophyll content (553 - 579.5 μg chl 10^{-9} plastids), and these values compare with those of 110 (summer / autumn fronds) and 600 (winter / spring fronds) μg chl 10^{-9} plastids reported by Benson, *et al.* (1983) and Williams, *et al.* (1984). These values are typical of the growth strategy of *C. fragile* as outlined by these workers.

3.5 Conclusions

The experiments described in this Chapter revealed a similar oxygen exchange response of chloroplasts isolated from tissue harvested at different stages in the yearly life cycle: 04/ 04/ 88 (spring - late vegetative), 11/ 09/ 88 (summer - late reproductive) and 18/ 12/ 88 (winter - vegetative). A light - induced increase in the rate of dark oxygen uptake that was unrelated to PPFD was observed, as with the intact frond experiments. Again, a possible explanation could be increased rates of glycolate production, the Mehler reaction and chlororespiration, but any protection that these and any other phenomena may provide was not sufficient to prevent photoinhibition of the isolated chloroplasts under the conditions of these experiments. Photosynthesis of isolated chloroplasts saturated at low irradiances but, unlike intact fronds, a narrow saturation plateau was observed and photoinhibition rapidly occurred at supraoptimal PPFD. The underlying mechanism of photoinhibition in *C. fragile* is not clear from these experiments, although it is likely that the processes involved will be similar to those that are thought to operate in other photosynthetic organisms, and that PSII will be the primary site of lesion. The higher the chlorophyll concentration of the chloroplast suspension the higher the PPFD required for maximum photosynthesis (*i.e.* saturation) and the less dramatic the reduction in photosynthesis (*i.e.* photoinhibition) once the optimum irradiance was surpassed. The differences in the photosynthetic response of the intact frond and isolated chloroplasts must be due to properties of the former not shared by the latter, which enable the prevention of photoinhibition. This 'mimicking' of the frond response at high chlorophyll concentration in the chloroplast study suggests that the number and arrangement of chloroplasts within the frond may

confer a vital mechanism in the avoidance of photoinhibition by this alga.

CHAPTER 4 AN INVESTIGATION OF PHOTOINHIBITION IN *C. FRAGILE* THYLAKOIDS VIA THE PARTIAL REACTIONS OF PHOTOSYNTHESIS

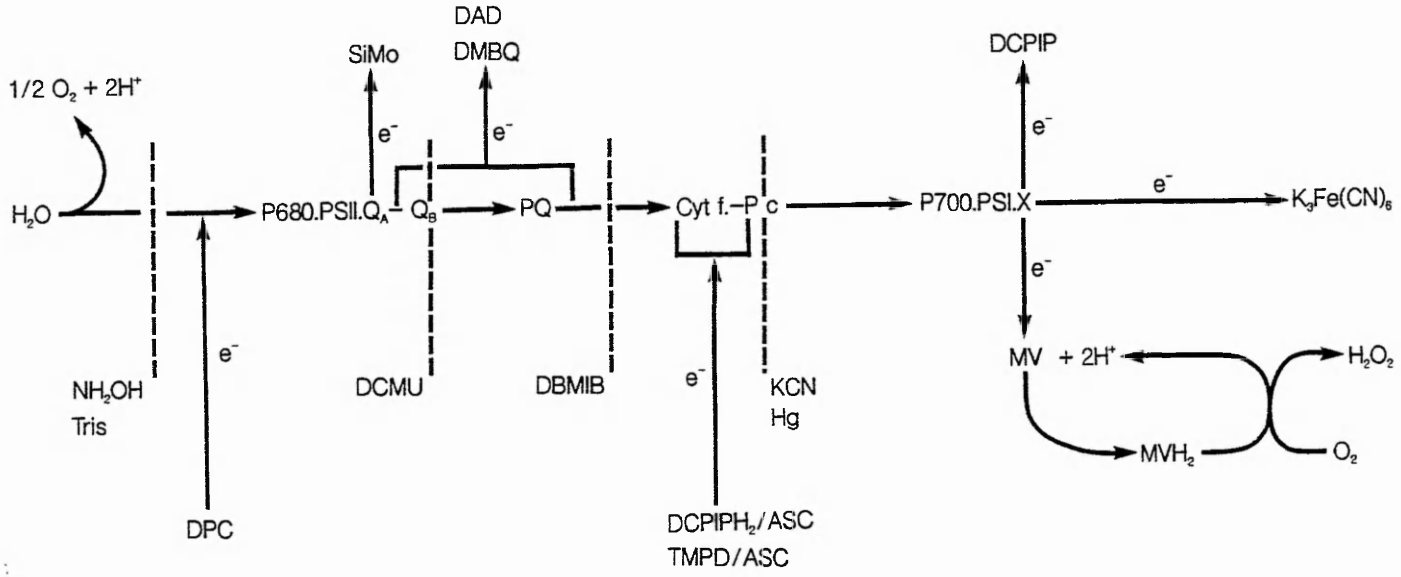
4.1 Introduction

The experiments described in Chapters 2 and 3 were carried out on tissue harvested at different times in the yearly life cycle of *C. fragile* and in general, both vegetative and reproductive fronds exhibited similar trends in photosynthesis to varying PPFD and temperature. Results obtained with whole fronds suppported previous evidence of the shade type adaptations of *C. fragile* and a paradoxical ability to avoid photoinhibition at supra - optimal PPFD. In contrast, the investigations described in Chapter 3 revealed that isolated chloroplasts were extremely susceptible to photoinhibition at high PPFD, as indicated by a decline in bicarbonate - dependent oxygen evolution. Further work is required to identify the site and mechanism of photoinhibition in *C. fragile*.

It is now generally accepted that PSII is the primary site of photoinhibition and studies with a range of photoinhibited samples from leaves to isolated PSII protein complexes have indicated that the first site of damage is in the PSII reaction centre (Critchley, 1981; Powles and Bjorkman, 1982; Tytler, *et al.*, 1984; Barenyi and Krause, 1985; Cleland and Critchley, 1985; Krause, *et al.*, 1985; Arntz and Trebst, 1986; Cleland, *et al.*, 1986; Theg, *et al.*, 1986; Demeter, *et al.*, 1987) or close to the sites of quinone binding (Kyle, *et al.*, 1984; Nedbal, *et al.*, 1986; Allakhverdiev, *et al.*, 1987; Ohad, *et al.*, 1988, 1990). All these studies suggest that the primary sites of damage are localised in the D1 and D2 proteins.

One means of studying the site of damage caused by photoinhibition involves the characterisation of the partial reactions of photosynthetic electron transport using assays specific to either photosystem. A preliminary examination of photosystem activity of thylakoids isolated from *C. fragile* tissue harvested in April and September has been carried out (Cobb, *et al.*, 1990) using assay procedures based on the techniques described by Izawa (1980), as illustrated in Figure 4.1. The activities of PSI and PSII were measured separately and together after optimum concentrations of electron donors and acceptors were determined for maximum electron transport by thylakoids, isolated from *C. fragile*, equivalent to 20 μg cm^{-3} chlorophyll, at 500 μmol m^{-2} s^{-1} PPFD. Further experiments involving photoinhibitory pretreatments (illumination at 100 and 1000 μmol m^{-2} s^{-1} PPFD for up to 100 min) clearly illustrated that PSII was the primary

Figure 4.1 A model of some measureable partial reactions of photosynthetic electron transport in isolated thylakoids showing sites of electron donation and acceptance (arrows) and sites of inhibition (broken lines). Key: P680, the reaction centre chlorophyll of photosystem II (PSII); Q_A, the primary quinone electron acceptor of PSII; Q_B, the secondary quinone electron acceptor of PSII; PQ, the plastoquinone pool; Cyt f, the cytochrome b_6/f complex; PC, plastocyanin; P700, the reaction centre chlorophyll of photosystem I (PSI); X, the primary electron acceptor of PSI; DAD, diaminodurine; DPC, diphenyl carbazide; DCMU, 3 - (3, 4 - dichlorophenyl) - 1, 1 - dimethylurea; DBMIB, 2, 5 - dibromo - 3 - methyl - 6 - isopropyl - p - benzoquinone (dibromothymoquinone); SiMo, silicomolybdate (SiO_2. $12MoO_3$); DMBQ, 2, 5 - dimethyl - p - benzoquinone; $DCPIPH_2$, 2, 6 - dichlorophenolindophenol (reduced from); Asc, ascorbate; TMPD, N, N, N^1, N^1 - tetramethyl - p - phenylenediamine; MV, methyl viologen; $K_3Fe(CN)_6$, potassium ferricyanide. Adapted from Izawa (1980).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H2O
NH2OH
Tris
DPC
e-
P680.PSII.QA- QB
SiMo
DAD
DMBQ
PQ
DCMU
DBMIB
Cyt f.–P c
DCPIPH2/ASC
TMPD/ASC
KCN
Hg
P700.PSI.X
DCPIP
K3Fe(CN)6
MV + 2H+
H2O2
MVH2
O2

site of lesion within the photosynthetic electron transport chain of isolated *C. fragile* thylakoids when incubated under conditions favouring photoinhibition, with negligible inhibition of PSI activity (Cobb, *et al.*, 1990).

As illustrated in Figure 4.1 electrons are accepted from the Q_B region of the electron transport chain by 2, 5 - dimethyl - p - benzoquinone (DMBQ), whereas silicomolybdate (SiMo) accepts electrons from the Q_A site. In *Chlamydomonas reinhardtii* cells and isolated *Pisum sativum* thylakoids, overall electron transport from water to 2, 6 - dichlorophenolindophenol (DCPIP) was found to decline dramatically on photoinhibitory treatment, whereas Q_B - independent PSII activity (water to SiMo) and PSI activity (N, N, N^1, N^1 - tetramethyl - p - phenylenediamine (TMPD) to methyl viologen) were much less effected (Ohad, *et al.*, 1985), suggesting that the primary lesion in electron transport is at the Q_B site of the D1 protein of PSII, with little damage to either the water splitting complex or the reaction centre (Kyle, *et al.*, 1984). However, other workers suggest an alternative primary target of photoinhibition; the reaction centre of PSII (Cleland and Critchley, 1985; Arntz and Trebst, 1986; Cleland, *et al.*, 1986; Cleland and Melis, 1987). Thus, the work described in this Chapter was intended to further elucidate the site of the primary lesion associated with photoinhibition in *C. fragile*. To achieve this aim stirred thylakoid suspensions were subjected to photoinhibitory treatment and a detailed study of Q_B - dependent (DMBQ assay) and Q_B - independent (DCMU insensitive SiMo assay) activities, was undertaken.

4.2 Materials and Methods

4.2.1 Sampling and Maintenance of *C. fragile*

Fronds of *C. fragile* were harvested on 08/ 09/ 89 (summer - late reproductive) as described previously (2.2.1) and similarly maintained.

4.2.2 Thylakoid Preparation

Chloroplasts were isolated from frond tips using the method of Cobb (1977) as previously described (3.2.2). *C. fragile* chloroplasts are particularly robust *in vitro* (Cobb and Rott, 1978) and so thylakoid isolation was achieved by centrifugation of the chloroplast preparation for 5 min at 1050 g (2,500 rpm) in a Denley BR401 refrigerated centrifuge, followed by resuspension in the same volume of extraction medium and

freezing for 100 min in a -20 °C freezer. Once thawed, the chlorophyll content of the thylakoid preparation was determined using the method of MacKinney (1941) as previously described (3.2.3).

4.2.3 Photosystem II Activity Measurements

Both the Q_B - dependent assay (electron flow from water to DMBQ) and the Q_B - independent assay (DCMU - insensitive electron flow from water to SiMo) were measured by following the oxygen exchange of isolated thylakoid suspensions in the aqueous phase using a DW1 oxygen electrode (Hansatech Ltd.) and the methods of Delieu and Walker (1972). The oxygen electrode was set up and calibrated, and the reaction chamber maintained at 10 °C, as previously described (3.2.4).

4.2.4 Optimisation of Silicomolybdate Concentration

The measurement of 3 - (3, 4 - dichlorophenyl) - 1, 1 - dimethylurea (DCMU) - insensitive, SiMo (SiO_2. $12MoO_3$) - dependent oxygen evolution by isolated thylakoids was adapted from the assay procedures described by Izawa (1980). A stirred reaction volume was used containing stock 1 mol m^{-3} SiMo (in 50 % v/v extraction medium and dimethyl sulphoxide (DMSO)), stock 1 mol m^{-3} DCMU (in extraction medium) and thylakoid suspension (in extraction medium). Appropiate volumes were used to achieve a final DCMU concentration of 0.05 mol m^{-3}, a chlorophyll concentration of 20 μg cm^{-3}, and SiMo concentrations of between 0.1 and 900 mmol m^{-3}, with the final volume made up to 1 cm^3 with extraction medium. The rate of oxygen exchange in the dark was followed for 5 min after which SiMo - dependent oxygen evolution at 50 μmol m^{-2} s^{-1} PPFD, provided by a Halight 24/ 250 projector, was followed for a further 5 min. The rate of oxygen evolution achieved at each SiMo concentration was investigated 1 to 5 times.

4.2.5 Photoinhibition and Q_B - dependent Activity

The measurement of DMBQ - dependent oxygen evolution by isolated thylakoids was also adapted from the assay procedures described by Izawa (1980). A stirred 1200 μl volume of thylakoid preparation (equivalent to 100 μg cm^{-3} chlorophyll) was incubated at 10 °C in a DW1 oxygen electrode at 1000 μmol m^{-2} s^{-1} PPFD provided by a Halight 24/ 250 projector for up to 100 min. At 0, 10, 25, 40, 60, 80 and 100 min

a 150 μl volume of the thylakoid suspension was removed and added to a second electrode reaction chamber, also at 10 °C, for the measurement of DMBQ - dependent oxygen evolution. The second electrode chamber contained 550 μl of extraction medium and 300 μl of 16.7 mol m^{-3} DMBQ (in extraction medium) so that on addition of the 150 μl of thylakoid suspension the total 1 cm^3 reaction volume contained a final DMBQ concentration of 5 mol m^{-3} (found to be optimal - Cobb, *et al.*, 1990) and a final chlorophyll concentration of 15 μg cm^{-3}. The oxygen exchange was followed in the dark for 5 min, after which the second electrode reaction chamber was illuminated at 1000 μmol m^{-2} s^{-1} PPFD for a further 5 min and the DMBQ - dependent oxygen evolution recorded. Each photoinhibitory exposure time was investigated 6 - 14 times.

The experiment was repeated with thylakoid suspensions of lower chlorophyll content, as follows. A stirred 1500 μl volume of thylakoid preparation (equivalent to 50 μg cm^{-3} chlorophyll) was incubated at 10 °C in the first electrode at 1000 μmol m^{-2} s^{-1} PPFD for up to 40 min. At 0, 5, 10, 15, 20, 25, 30 and 40 min, a 300 μl volume of the thylakoid suspension was removed and added to the second electrode reaction chamber for the measurement of DMBQ - dependent oxygen evolution. The second electrode chamber contained 400 μl of extraction medium and 300 μl of 16.7 mol m^{-3} DMBQ (in extraction medium), so that on addition of the 300 μl of thylakoid suspension the total 1 cm^3 reaction volume contained a final concentration of 5 mol m^{-3} DMBQ and 15 μg cm^{-3} chlorophyll. The oxygen exchange was recorded as above and each photoinhibitory exposure time was investigated 3 to 8 times.

4.2.6 Photoinhibition and Q_B - independent Activity

The measurement of DCMU - insensitive SiMo - dependent oxygen evolution by isolated thylakoids was also adapted from the assay procedures described by Izawa (1980). A stirred 1200 μl volume of thylakoid preparation (equivalent to 100 μg cm^{-3} chlorophyll) was incubated at 10 °C in a DW1 oxygen electrode at 1000 μmol m^{-2} s^{-1} PPFD provided by a Halight 24/ 250 projector for up to 100 min. At 0, 10, 25, 40, 60, 80 and 100 min a 150 μl volume of the thylakoid suspension was removed and added to a second electrode reaction chamber, also at 10 °C, for the measurement of DCMU - insensitive, SiMo - dependent oxygen evolution. The second electrode reaction chamber contained 700 μl of extraction medium, 100 μl of 2 mol m^{-3} SiMo (in 50 % (v/v) extraction medium and DMSO) and 50 μl of 1 mol m^{-3} DCMU (in

extraction medium), so that on addition of the 150 μl of thylakoid suspension the total 1 cm^3 reaction volume contained a final concentration of 200 mmol m^{-3} SiMo, 0.05 mol m^{-3} DCMU and 15 μg cm^{-3} chlorophyll. The oxygen exchange was recorded for 10 min in total as described in the previous section (4.2.5) and each photoinhibitory exposure time was investigated 6 to 11 times.

The experiment was repeated with thylakoid suspensions of lower chlorophyll content, as follows. A stirred 1500 μl volume of thylakoid preparation (equivalent to 50 μg cm^{-3} chlorophyll) was incubated at 10 °C in the first electrode reaction chamber at 1000 μmol m^{-2} s^{-1} PPFD for up to 40 min. At 0, 5, 10, 15, 20, 30 and 40 min, a 300 μl volume of the thylakoid suspension was removed and added to the second electrode reaction chamber for the measurement of DCMU - insensitive, SiMo - dependent oxygen evolution. The second electrode chamber contained 550 μl of extraction medium, 100 μl of 2 mol m^{-3} SiMo and 50 μl of 1 mol m^{-3} DCMU, so that on addition of the 300 μl volume of thylakoid suspension the total 1 cm^3 reaction volume contained a final concentration of 200 mmol m^{-3} SiMo, 0.05 mol m^{-3} DCMU and 15 μg cm^{-3} chlorophyll. The oxygen exchange was recorded as above and each photoinhibitory exposure time was investigated 3 to 8 times.

4.2.7 Photoinhibition and Chlorophyll Content

A stirred 1200 μl volume of thylakoid preparation (equivalent to 100 μg cm^{-3} chlorophyll) was incubated at 10 °C in a DW1 oxygen electrode at 1000 μmol m^{-2} s^{-1} PPFD for 100 min. At 0, 10, 25, 40, 60, 80 and 100 min, a 150 μl sample was removed (3 x 50 μl, equivalent to 3 x 5 μg chlorophyll) and added to 3 x 1950 cm^3 80 % (v/ v) acetone (a 40 x dilution) for chlorophyll determination by the method of MacKinney (1941), as previously described (3.2.3). Two such experiments were carried out giving a total of six replicates. This was repeated with thylakoid suspensions of lower chlorophyll content, as follows. A stirred 2,400 μl volume of thylakoid preparation (equivalent to 50 μg cm^{-3} chlorophyll) was incubated at 10 °C in a DW1 oxygen electrode at 1000 μmol m^{-2} s^{-1} PPFD for 40 min. At 0, 5, 10, 15, 20 and 40 min, a 300 μl sample was removed (3 x 100 μl, equivalent to 3 x 5 μg chlorophyll) and added to 3 x 1900 cm^3 80 % (v/ v) acetone (a 20 x dilution) for chlorophyll determination as before. Two such experiments were carried out giving a total of six replicates.

4.3 Results

The optimum DMBQ concentration required for Q_B - dependent activity of isolated *C. fragile* thylakoids equivalent to 20 μg cm^{-3} chlorophyll has been previously established as 5 mol m^{-3} (Cobb, *et al.*, 1990) and this concentration was used throughout this study. However, it was necessary to establish the optimum SiMo concentration required for Q_B - independent activity and these results, obtained using thylakoid suspensions equivalent to 20 μg cm^{-3} chlorophyll, are illustrated in Figure 4.2. It can be seen that as the SiMo concentration was increased from 0.1 to approximately 100 mmol m^{-3} the rate of oxygen evolution increased rapidly from 0 to approximately 9.5 μmoles O_2 hr^{-1} mg^{-1} chl. A further increase in SiMo concentration to 400 mmol m^{-3} resulted in a more modest increase in rate of oxygen evolution to approximately 10 μmoles O_2 hr^{-1} mg^{-1} chl. Further increase in SiMo concentration resulted in a decline in rates of oxygen evolution, with negligible rates occurring at 900 mmol m^{-3} SiMo. The most appropiate SiMo concentration required for Q_B - independent activity was thought to be 200 mmol m^{-3} and this concentration was used in subsequent assays.

Initial experiments involved an investigation of Q_B - dependent activity (electron flow from water to DMBQ) and Q_B - independent activity (electron flow from water to SiMo) of thylakoid suspensions equivalent to 100 μg cm^{-3} chlorophyll, subjected to a photoinhibitory pretreatment of 1000 μmol m^{-2} s^{-1} PPFD for 0 to 100 min. The results are shown in Figure 4.3. DMBQ activity, SiMO activity and chlorophyll content of the thylakoid suspension all decreased with increasing photoinhibitory pretreatment. The maximum chlorophyll content (103 $\pm$ 24 μg cm^{-3}) was observed after 10 min exposure time and the concentration was reduced to approximately 64 $\pm$ 8 and 45 $\pm$ 2 μg cm^{-3} after 40 and 100 min exposure, respectively, representing a 38 % and 57 % decrease from the maximum. The control rate of oxygen evolution at 'zero time' (*i.e.* no photoinhibitory pretreatment) may be designated 100 %, enabling the DMBQ and SiMo activities to be expressed as a percentage decrease from this maximum rate (*i.e.* % photoinhibition), as illustrated in Figure 4.4. From Figures 4.3 and 4.4 it may be seen that loss of SiMo activity was negligible from 0 to 10 min exposure, whilst DMBQ - dependent oxygen evolution declined at a rapid rate to 20 % inhibition after only 10 min. After 10 min, SiMo activity declined at a greater rate reaching approximately 40 % inhibition at 40 min exposure. Over the same exposure period (10 - 40 min) the previously rapid rate of decline of DMBQ activity was much reduced, reaching

Figure 4.2 Optimisation of silicomolybdate concentration for the Q_B - independent assay (electron flow from water to SiMo). A 1 cm^3 reaction volume containing a thylakoid suspension equivalent to 20 μg cm^{-3} chlorophyll was incubated at a PPFD of 50 μmol m^{-2} s^{-1} and at 10 °C. See text for details. Each point represents the mean $\pm$ standard error of 1 to 5 observations.

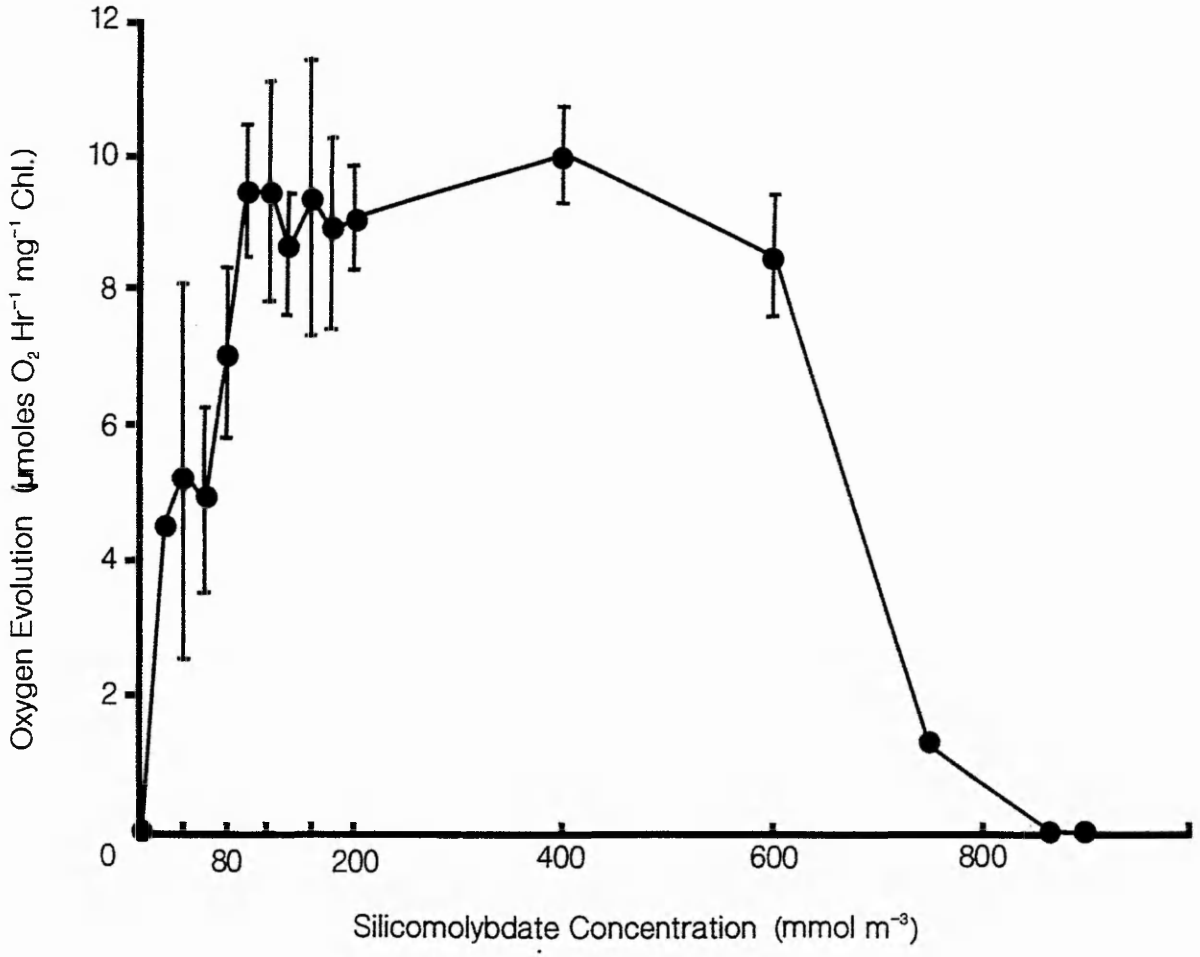
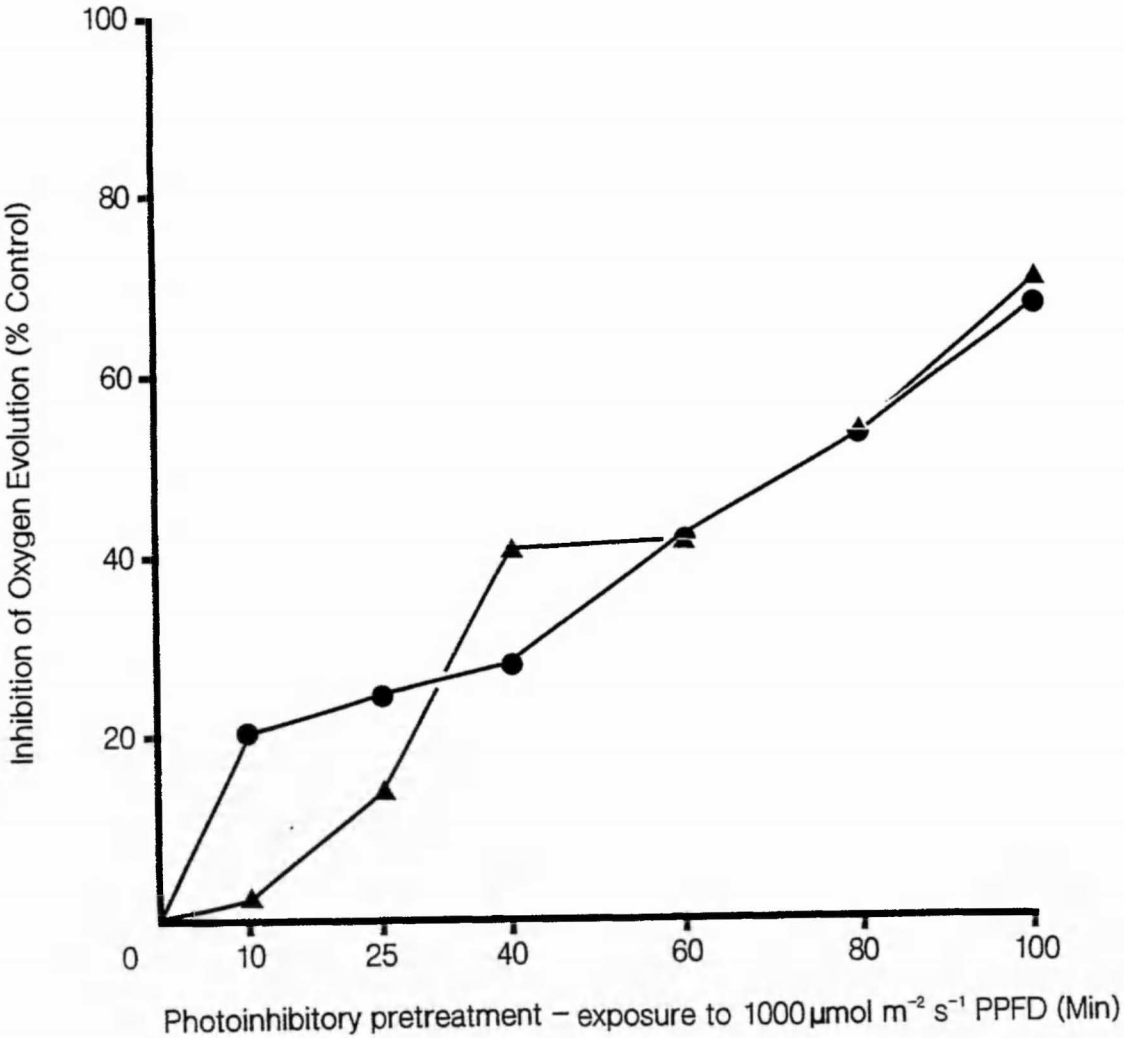

12
10
8
6
4
2
0
80
200
400
600
800
Oxygen Evolution (μmoles O_2 Hr^{-1} mg^{-1} Chl.)
Silicomolybdate Concentration (mmol m^{-3})

Figure 4.3 The relationship between time of exposure to photoinhibitory pretreatment (min), Q_B - dependent electron flow (water to DMBQ, ●), Q_B - independent electron flow (water to SiMo, ▲) and chlorophyll content (■) of the thylakoid suspension. A thylakoid preparation equivalent to 100 μg cm^{-3} chlorophyll was illuminated at 1000 μmol m^{-2} s^{-1} PPFD for up to 100 min, at 10 °C. At time intervals, 150 μl samples were removed and the rate of DMBQ - dependent oxygen evolution (n = 6 - 14) , rate of SiMo - dependent oxygen evolution (n = 6 - 11), or chlorophyll content (n = 6) determined. See text for details. Each point represents the mean ± standard error.

Oxygen Evolution (μmoles O_2 Hr^{-1} mg^{-1} Chl.)
50
40
30
20
10
0
10
25
40
60
80
100
Photoinhibitory Pretreatment – exposure to 1000 μmol m^{-2} s^{-1} PPFD (Min)
120
100
80
60
40
20
Chlorophyll content (μg cm^{-3})

Figure 4.4 Photoinhibition of Q_B - dependent electron flow (water to DMBQ, ●) and Q_B - independent electron flow (water to SiMo, ▲). The rates are expressed as a percentage decrease from the maximum rates of oxygen evolution obtained with the control sample (before photoinhibition). A thylakoid preparation equivalent to 100 μg cm^{-3} chlorophyll was illuminated at 1000 μmol m^{-2} s^{-1} PPFD for up to 100 min, at 10 °C. At time intervals 150 μl samples were removed and the rate of DMBQ - dependent (n = 6 - 14) or SiMo - dependent (n = 6 - 11) oxygen evolution determined. See text for details.


100
80
60
40
20
0
10
25
40
60
80
100
Inhibition of Oxygen Evolution (% Control)
Photoinhibitory pretreatment – exposure to 1000 µmol m⁻² s⁻¹ PPFD (Min)

approximately 28 % inhibition at 40 min exposure time. From 40 to 60 min the rate of inhibition of DMBQ increased, whilst that of SiMo was constant, with both rates exhibiting approximately 44 % inhibition after 60 min. From 60 to 100 min exposure the rates of inhibition of SiMo and DMBQ activities were similar, reaching 70 % and 65 % inhibition respectively at 100 min exposure. At 40 min, SiMo activity was inhibited more than DMBQ activity. At higher exposure times the degree of inhibition of the two activities was essentially the same, whilst at shorter exposure times the SiMo activity was inhibited less than the DMBQ activity.

In a second series of experiments the Q_B - dependent and Q_B - independent activities were investigated in greater detail. The sensitivity of the experimental system was increased by reducing the concentration of the thylakoid suspension to 50 μg cm^{-3} chlorophyll and attention was focused on the initial stages of photoinhibition by using a reduced pretreatment exposure time of 40 min at 1000 μmol m^{-2} s^{-1} PPFD, with activity determinations at 5 min intervals. The results are shown in Figure 4.5. The chlorophyll content of the thylakoid suspension seemed somewhat erratic, although marginal decrease with increasing photoinhibitory pretreatment was apparent. The maximum chlorophyll content (52 $\pm$ 3 μg cm^{-3}) was again obseved after 10 min exposure, and the concentration was reduced to 36 $\pm$ 2 μg cm^{-3} after 40 min, represnting a 30 % decrease from the maximum. The rate of DMBQ - dependent oxygen evolution clearly declined with increasing photoinhibitory pretreatment, whereas the SiMo activity declined to a lesser extent. Again, the decline in DMBQ and SiMo activities may be expressed as percentage inhibition from the maximum rate (after no photoinhibitory pretreatment), as illustrated in Figure 4.6. From Figures 4.5 and 4.6 it may be seen that, as in the previous set of experiments, loss of SiMo activity was negligible after up to 10 min exposure, whilst DMBQ - dependent oxygen evolution again declined to 20 % inhibition at 10 min. From 10 to 30 min exposure both activities declined, with DMBQ - and SiMo - dependent oxygen evolution rates exhibiting approximately 55 and 22 % inhibition, respectively, at 30 min. From 30 to 40 min exposure the rate of inhibition of SiMo activity increased, whilst the rate of decline of DMBQ activity was much reduced, the respones appearing to begin to plateau. The DMBQ and SiMo activities exhibited approximately 56 and 49 % inhibition, respectively, after 40 min photoinhibitory pretreatment. At all exposure times the DMBQ activity was always more inhibited than the SiMo activity, although

Figure 4.5 The relationship between time of exposure to photoinhibitory pretreatment (minutes), Q_B - dependent electron flow (water to DMBQ, ●), Q_B - independent electron flow (water to SiMo, ▲) and chlorophyll content (■) of the thylakoid suspension. A thylakoid preparation equivalent to 50 μg cm^{-3} chlorophyll was illuminated at 1000 μmol m^{-2} s^{-1} PPFD for up to 40 min, at 10 °C. At time intervals 300 μl samples were removed and the rate of DMBQ - dependent oxygen evolution (n = 3 - 8), rate of SiMo - dependent oxygen evolution (n = 3 - 8), or chlorophyll content (n = 6) determined. See text for details. Each point represents the mean ± standard error.

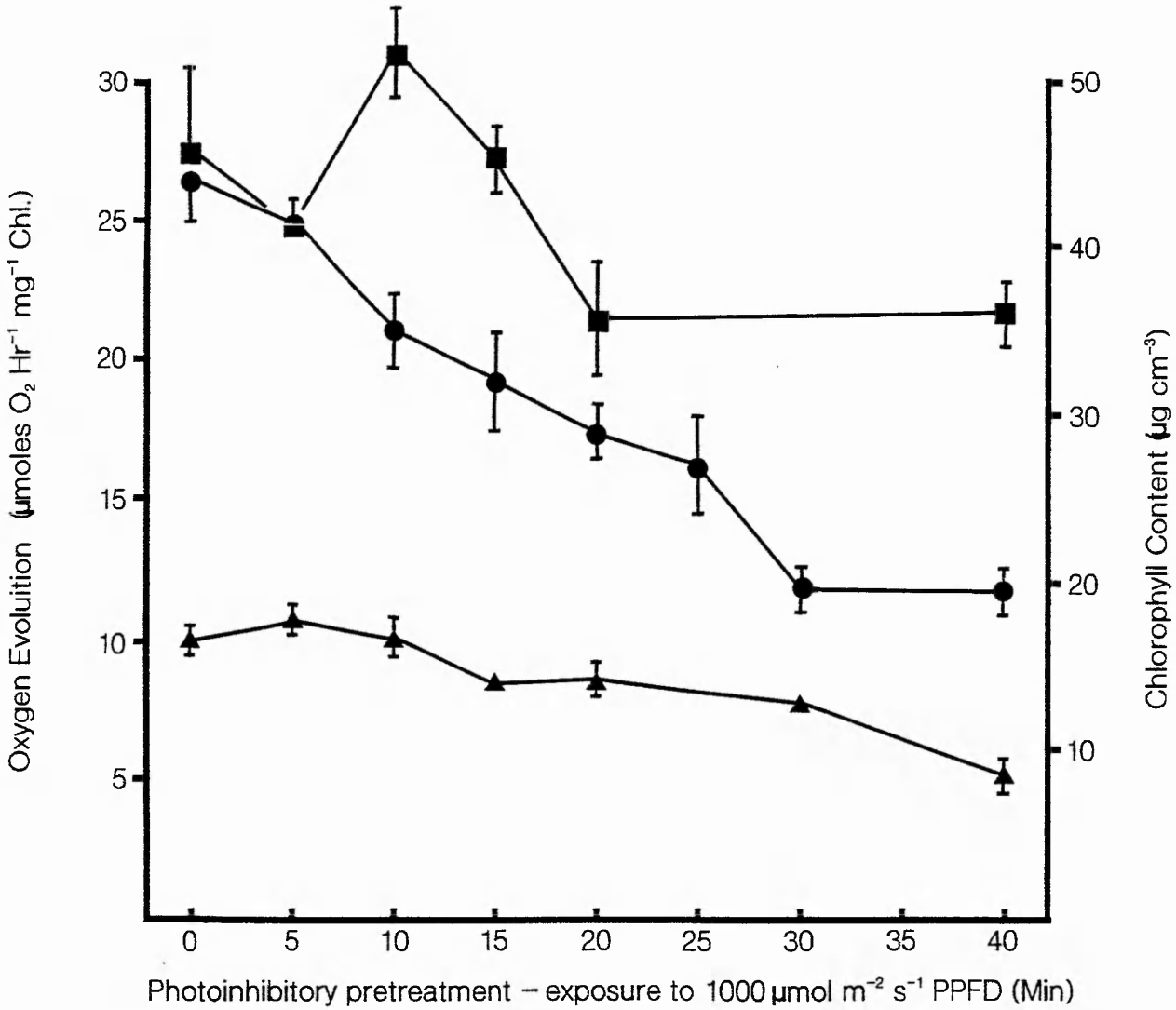

Oxygen Evoluition (µmoles O_2 Hr^{-1} mg^{-1} Chl.)
Chlorophyll Content (µg cm^{-3})
30
25
20
15
10
5
50
40
30
20
10
0
5
10
15
20
25
30
35
40
Photoinhibitory pretreatment – exposure to 1000 µmol m^{-2} s^{-1} PPFD (Min)

Figure 4.6 Photoinhibition of Q_B - dependent electron flow (water to DMBQ, ●) and Q_B - independent electron flow (water to SiMo, ▲). The rates are expressed as a percentage decrease from the maximum rates of oxygen evolution obtained with the control sample (before photoinhibition). A thylakoid preparation equivalent to 50 μg cm^{-3} chlorophyll was illuminated at 1000 μmol m^{-2} s^{-1} PPFD for up to 40 min, at 10 °C. At time intervals 300 μl samples were removed and the rate of DMBQ - dependent (n = 3 - 8) or SiMo - dependent (n = 3 - 8) oxygen evolution determined. See text for details.

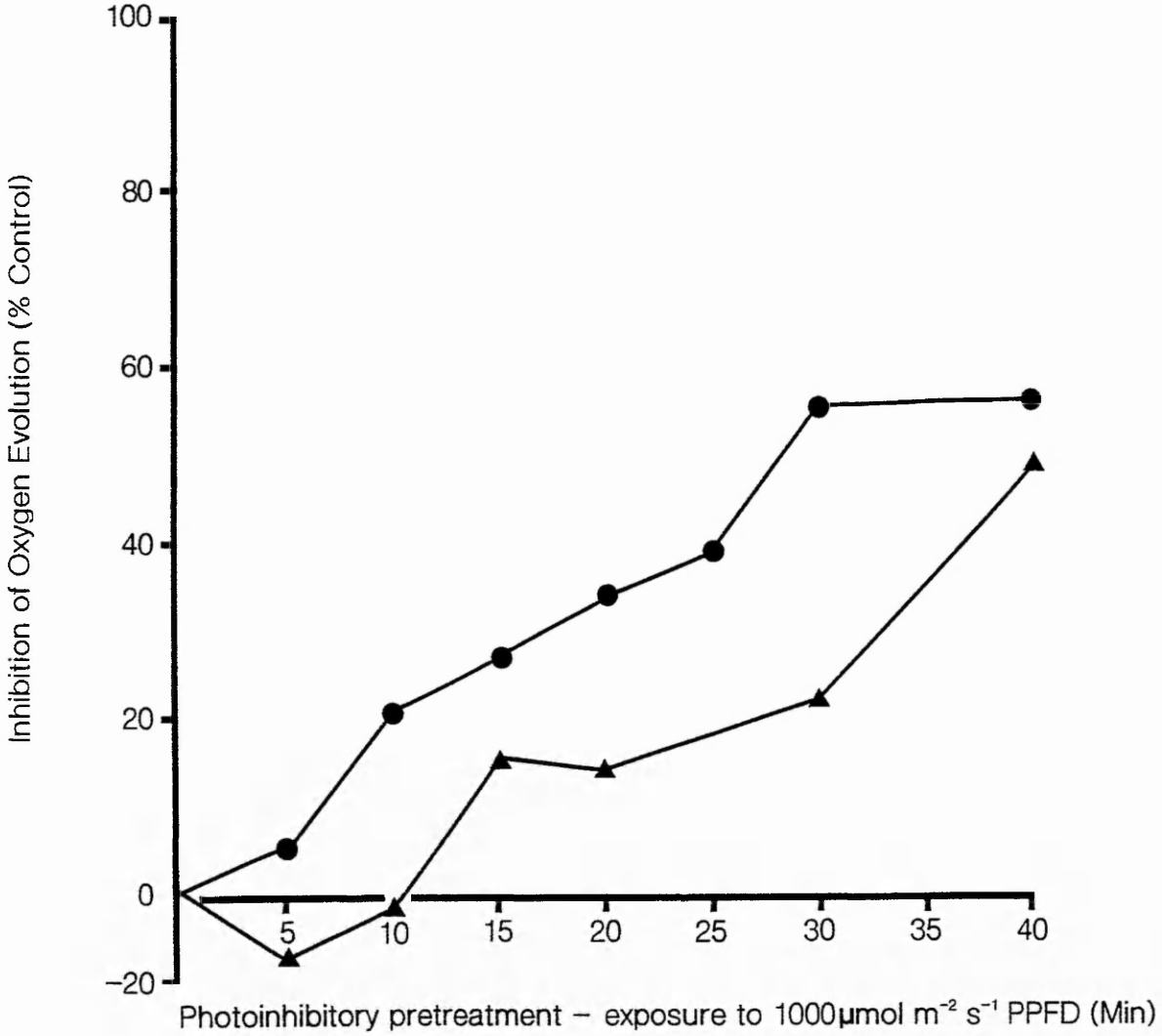

Inhibition of Oxygen Evolution (% Control)
100
80
60
40
20
0
-20
5
10
15
20
25
30
35
40
Photoinhibitory pretreatment – exposure to 1000µmol m-2 s-1 PPFD (Min)

Figure 4.7 Photoinhibition of Q_B - dependent (water to DMBQ) and Q_B - independent (water to SiMo) photosystem II activities with increasing exposure time to photoinhibitory pretreatment of 1000 μmol m^{-2} s^{-1} PPFD (min) at 10 °C. The rates are expressed as a percentage decrease from the maximum rates of oxygen evolution obtained with the control sample (before photoinhibition). Key: ● , DMBQ (Q_B - dependent) activity of pretreated thylakoid suspensions equivalent to 100 μg cm^{-3} chlorophyll (n = 6 - 14); ○ , DMBQ (Q_B - dependent) activity of pretreated thylakoid suspensions equivalent to 50 μg cm^{-3} chlorophyll (n = 3 - 8); ▲ , SiMo (Q_B - independent) activity of pretreated thylakoid suspensions equivalent to 100 μg cm^{-3} chlorophyll (n = 6 - 11); △ , SiMo (Q_B - independent) activity of pretreated thylakoid suspensions equivalent to 50 μg cm^{-3} chlorophyll (n = 3 - 8). See text for details.

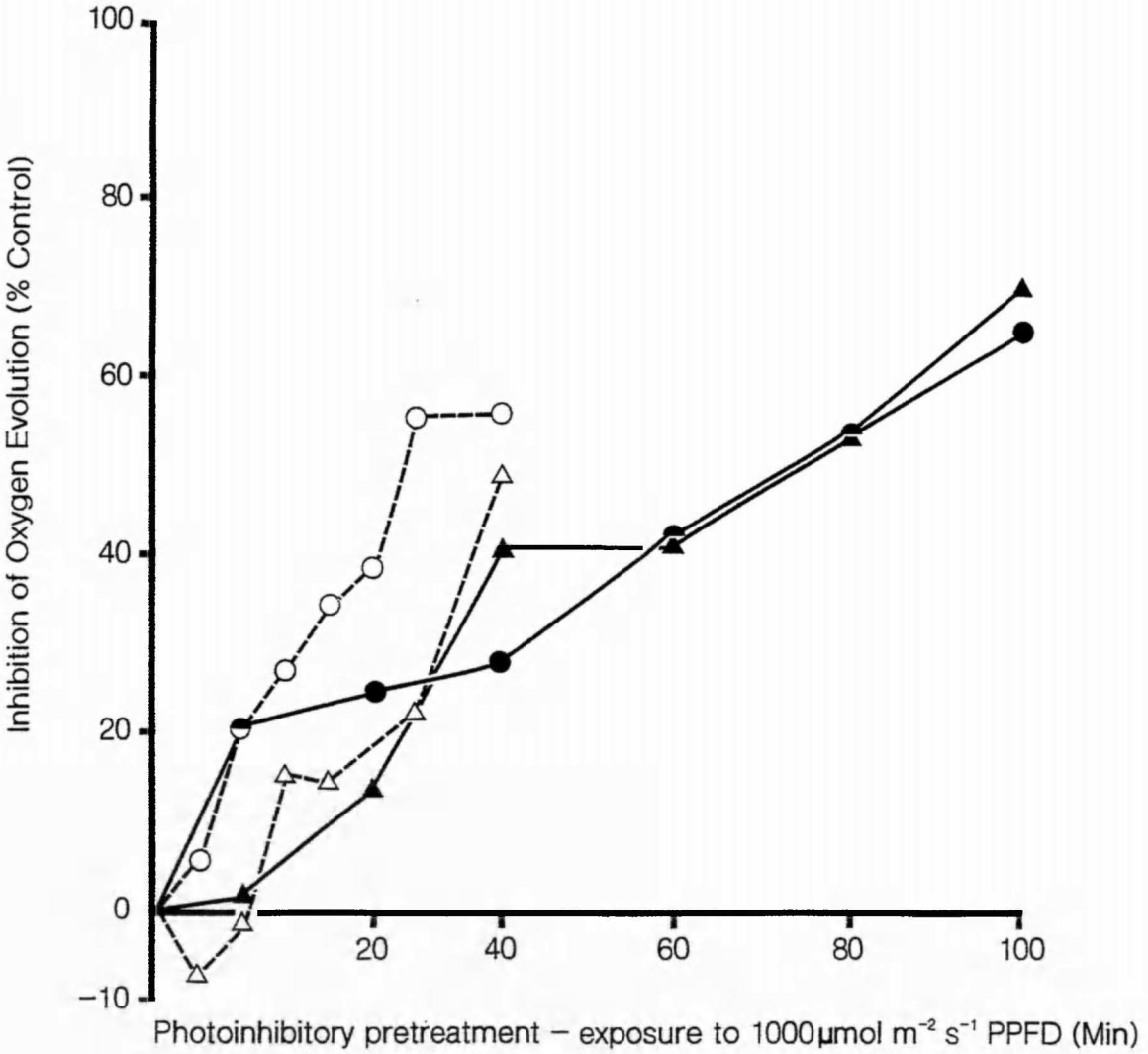
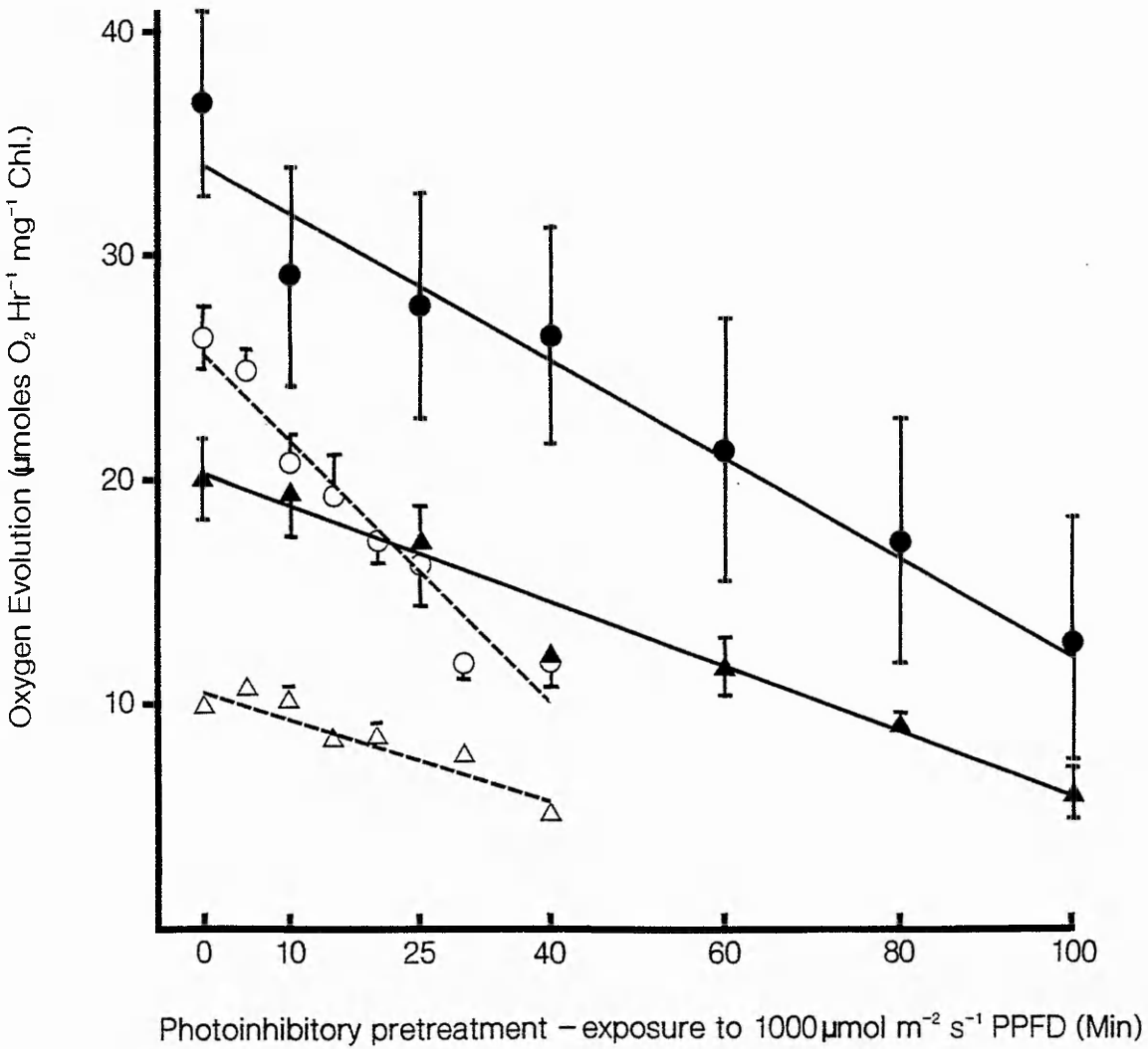

Inhibition of Oxygen Evolution (% Control)
100
80
60
40
20
0
−10
20
40
60
80
100
Photoinhibitory pretreatment – exposure to 1000 µmol m⁻² s⁻¹ PPFD (Min)

Figure 4.8 Decline in Q_B - dependent (water to DMBQ) and Q_B - independent (water to SiMo) photosystem II activities (oxygen evolution - μmoles O_2 hr^{-1} mg^{-1} chl) with increasing exposure time to photoinhibitory pretreatment of 1000 μmol m^{-2} s^{-1} PPFD (min), at 10 °C. Key: ● , DMBQ (Q_B - dependent) activity of pretreated thylakoid suspensions equivalent to 100 μg cm^{-3} chlorophyll (n = 6 - 14); ○ , DMBQ (Q_B - dependent) activity of pretreated thylakoid suspensions equivalent to 50 μg cm^{-3} chlorophyll (n = 3 - 8); ▲ , SiMo (Q_B - independent) activity of pretreated thylakoid suspensions equivalent to 100 μg cm^{-3} chlorophyll (n = 6 - 11); △ , SiMo (Q_B - independent) activity of pretreated thylakoid suspensions equivalent to 50 μg cm^{-3} chlorophyll (n = 3 - 8). See text for details. Each point represents the mean ± standard error.


Oxygen Evolution (μmoles O_2 Hr^{-1} mg^{-1} Chl.)
40
30
20
10
0
10
25
40
60
80
100
Photoinhibitory pretreatment – exposure to 1000 μmol m^{-2} s^{-1} PPFD (Min)

the amount of inhibition of both activities began to approach a similar level after 40 min exposure.

Figure 4.7 summarises the results of both experiments expressed as percent inhibition. Up to 10 min exposure the rate of inhibition of DMBQ activity and SiMo activity was similar, whether the thylakoid concentration was equivalent to 50 or 100 μg cm^{-3} chlorophyll, with approximately 20 % and negligible photoinhibition, respectively, apparent at 10 min. From 10 to 40 min exposure the rate of inhibition of DMBQ activity was much greater at 50 μg cm^{-3} than at 100 μg cm^{-3} chlorophyll. In contrast, there was much less of a difference between the photoinhibition of SiMo activity at 50 μg cm^{-3} and 100 μg cm^{-3} chlorophyll over this exposure period (10 to 40 min). It seems clear that DMBQ activity was inhibited before SiMo activity, and generally to a greater extent than SiMo activity, over the first 40 min of photoinhibitory pretreatment, at thylakoid concentrations of both 50 and 100 μg cm^{-3} chlorophyll. When expressed as oxygen evolution (μmoles O_2 hr^{-1} mg^{-1} chl) all four relationships appear to be linear and all four regression analyses are significant (Figure 4.8). Over 40 minutes photoinhibitory pretreatment of thylakoid suspensions equivalent to 50 μg cm^{-3} chlorophyll, the DMBQ activity clearly declined at a much more rapid rate (gradient = -0.390) than the SiMo activity (gradient = - 0.127). In the less sensitive experimental system using thylakoid suspensions equivalent to 100 μg cm^{-3} chlorophyll, the decline of DMBQ and SiMo activities with increasing photoinhibitory pretreatment were similar. However, the rate of decline of DMBQ activity (gradient = -0.219) was greater than that of SiMo activity (gradient = - 0.142).

4.4 Discussion

Although 400 mmol m^{-3} SiMo appeared to give the highest rates of oxygen evolution (Figure 4.2) it was considered that the optimum concentration for Q_B - independent activity was 200 mmol m^{-3} , as this concentration was sufficient to support rates of oxygen evolution comparable to those obtained at 400 mmol m^{-3} and use of the lower concentration ensured that the inhibition of activity observed at higher concentrations (*i.e.* 600 mmol m^{-3} and above) was avoided. Therefore, a concentration of 200 mmol m^{-3} SiMo was used in Q_B - independent activity assays. This is a higher concentration than that reportedly used with higher plant thylakoid systems. For example, Allen and Holmes (1986b) recommend a concentration of 1 mmol m^{-3}, whilst

Cleland and Critchley (1985) used 85 mmol m^{-3} SiMo. However, Tytler, *et al.* (1984) used 0.25 mol m^{-3} SiMo, a higher concentration than that used in this study, when studying the unicellular cyanobacterium *Microcystis aeruginosa*. The relatively high SiMo concentration required for optimum Q_B - independent activity is consistent with earlier work investigating other partial reactions of isolated *C. fragile* thylakoids, which found that the concentrations of donor and acceptor molecules required for maximum photosynthetic rates in *Codium* were much greater than those reported for higher plants (Cobb, *et al.*, 1990). It was suggested that entry into the fractured chloroplast may be restricted, thus diluting the acceptor and donor molecules. Figure 4.2 illustrates that at supra - optimal SiMo concentrations (*e.g.* 900 mmol m^{-3}) the rate of oxygen evolution rapidly decreased to almost zero. This is in agreement with the findings of Izawa (1980) who reported that excessive SiMo concentrations strongly uncouple and quickly abolish all electron transport activity. The concentration required for this to occur depends upon the extent of protection of the thylakoid membrane (*e.g.* via BSA in the extraction medium) and other as yet unclarified conditions. SiMo physically disrupts the PSII complex, slightly displacing the D1 protein and exposing the Q_A site to be oxidized. Such substantial perturbation of the PSII complex may lead to secondary effects and therefore results obtained using this technique must be treated with caution (Allen and Holmes, 1986b; Kyle, 1987).

Figures 4.3 and 4.5 show that the chlorophyll content of the thylakoid suspensions generally appeared to decrease with increasing photoinhibitory pretreatment. The decline in chlorophyll content observed in this study supports previous evidence of the loss of photosynthetic pigment of thylakoids isolated from *C. fragile* on prolonged exposure to supra - optimal PPFD. Under such conditions Cobb, *et al.* (1990) found increased malondialdehyde (MDA) production which, after 180 min, was approximately proportional to the square root of the PPFD, as reported in a previous study (Heath and Packer, 1968). It was also apparent that the higher the chlorophyll concentration of the thylakoid suspension the lower the MDA production and it was suggested that these results indicated the onset of photooxidation, resulting in the destruction of chlorophyll observed and of the unsaturated fatty acids of the thylakoid membranes (Cobb, *et al.*, 1990). In contrast to photoinhibition, photooxidation can result in irreversible damage (Powles, 1984). The degree of photooxidation of isolated *C. fragile* thylakoids was apparently dependent upon the amount of chlorophyll present, a higher concentration

giving some protection (Cobb, *et al.*, 1990). It has been shown that chloroplasts and fronds of *C. fragile* do not exhibit photooxidative symptoms (bleaching and hydrocarbon gas release) after illumination for 4 - 6 h at 600 - 1000 μmol m^{-2} s^{-1} PPFD using a non - fluorescent tungsten - halogen source (Benson, 1983), even though photoinhibition of chloroplasts occurs at such intensities. Indeed, chloroplasts exposed to 2000 μmol m^{-2} s^{-1} PPFD for 3 h did not produce MDA (Benson, 1983). It was suggested that MDA release from within the intact, robust (Trench, *et al.*, 1973; Cobb and Rott, 1978) chloroplast envelope is reduced to undetectable amounts or that thylakoid preparations are lacking in properties possessed by intact isolated chloroplasts that effect protection against photooxidation. For example, the chloroplast envelope itself may have a role in the prevention of photooxidation. Systems that scavenge or prevent the formation of radicals or other reactive molecular species, particularly those derived from oxygen, are known to operate in the prevention of photoinhibition and subsequent photooxidation in other photosynthetic organisms (Asada and Takahashi, 1987) and similar systems are likely to operate in *C. fragile*; for example superoxide dismutase has been found in this alga (Lumsden and Hall, 1975). Perhaps the superoxide dismutases and ascorbate peroxidase which protect against $O_2^{\cdot-}$ and $OH^{\cdot}$ radicals and H_2O_2 (Krause, 1988) and ascorbate, glutathione and α - tocopherol that are implicated in dissipation of singlet oxygen or superoxide, are present in the chloroplast but absent from the thylakoid preparation. Carotenoids, particularly ß - carotene, are involved in the quenching of toxic active oxygen species in higher plants (Foote, 1976). However, ß - carotene is absent in *Codium* (Benson, 1983) and its apparent replacement, α - carotene, may not have such a photoprotective function, as the chlorophylls rather than the carotenoids present in this alga have been observed to be more susceptible to photooxidation (Benson, 1983). Such a susceptibility has been clearly indicated in previous studies (Cobb, *et al.*, 1990) in which the chlorophyll concentration of thylakoid suspensions were seen to decrease with increasing PPFD and exposure time, and is also supported in the present study (Figures 4.3 and 4.5).

The pigment composition of fronds and chloroplasts is seasonally variable, with chlorophyll and carotenoid concentrations highest in winter and spring. Chlorophyll a and b show a marked decline in summer (Benson, *et al.*, 1983; Williams, *et al.*, 1984; this study) further suggesting susceptibility of these pigments to photooxidation. Thus, the decline in rates of DMBQ - and SiMo - dependent oxygen evolution observed in

this study may be partly due to loss of photosynthetic pigment. However, in previous studies of MDA production and chlorophyll content at a PPFD of 1000 μmol m^{-2} s^{-1} the exposure times investigated were 90 and 180 min, using a chlorophyll concentration of 20 μg cm^{-3}. In the present study higher chlorophyll concentrations (50 and 100 μg cm^{-3}) and generally lower exposure times (0 - 40 and 0 - 100 min) were investigated. In the previous study the reduction in chlorophyll content and the onset of MDA production after 90 min were not particularly marked (especially at the higher chlorophyll concentrations investigated: 50 and 100 μg cm^{-3}) and therefore it is suggested that little photooxidation will occur over the time course and conditions of the present study. However, there was a general loss of chlorophyll with increasing photoinhibitory pretreatments in this study (Figures 4.3 and 4.5), but this decline was somewhat erratic and difficult to relate to the loss of DMBQ - and SiMo - dependent oxygen evolution activities. Also, it is suggested that any general loss of photosynthetic pigment will essentially influence both the DMBQ - and SiMo - dependent activities in a similar fashion, allowing both to be compared directly.

It has been established that PSII activity (measured by electron transport from water to DMBQ) is the primary target of photoinhibition in thylakoids isolated from *C. fragile* and that inhibition of PSI activity (measured by electron transport from $DCPIPH_2$/ Ascorbate to methyl viologen, in the presence of DCMU) is negligible (Cobb, *et al.*, 1990). These workers found that at both 100 and 1000 μmol m^{-2} s^{-1} PPFD, and at a chlorophyll concentration of 20 μg cm^{-3}, photoinhibition of PSII was rapid and reached a maximum of 60 % after approximately 40 and 5 min respectively. The more detailed investigations of this study are consistant with this preliminary work, with a maximum inhibition of DMBQ activity of 65 % after 100 min exposure to 1000 μmol m^{-2} s^{-1} PPFD, at 100 μg cm^{-3} chlorophyll (Figure 4.4) and 56 % after 40 min exposure to 1000 μmol m^{-2} s^{-1} PPFD, at 50 μg cm^{-3} chlorophyll (Figure 4.6). From Figure 4.4 it is observed that at a chlorophyll concentration of 100 μg cm^{-3} Q_B - independent (SiMo) activity was inhibited to a negligible extent after 10 min photoinhibitory pretreatment, whilst the Q_B - dependent (DMBQ) activity had rapidly declined to 20 % inhibition, and a similar observation was made at a chlorophyll concentration of 50 μg cm^{-3} (Figure 4.6). However, it was evident that after 10 min, inhibition of the SiMo activity began to occur, whilst the previously rapid rate of inhibition of the DMBQ activity began to decline. At the lower photoinhibitory pretreatment times of thylakoids equivalent to 100

μg cm^{-3} the DMBQ activity was inhibited more than the SiMo activity (Figure 4.4), whilst at 50 μg cm^{-3} chlorophyll the DMBQ activity was more inhibited than the SiMo activity at all pretreatment exposure times (Figure 4.6). Thus from Figures 4.4, 4.6 and 4.7 it is apparent that the primary target of photoinhibition was the Q_B - dependent (DMBQ) activity and that only after this initial damage to the Q_B site had occurred did inhibition of the Q_B - independent (SiMo) activity proceed. Inhibition of SiMo activity suggests that a secondary lesion had occurred in the electron transport chain between water and SiMo (which accepts electrons from Q_A). From Figure 4.4 it is observed that after 60 min photoinhibition the DMBQ - and SiMo - dependent activities were inhibited essentially to the same extent. This inhibition, between 60 and 100 min, may be governed mainly by the secondary lesion between water and SiMo, as any damage in this part of the electron transport chain would also be expected to influence the DMBQ activity, as DMBQ accepts electrons from further along the electron transport chain. A similar situation was approached when the thylakoid suspension was equivalent to 50 μg cm^{-3} chlorophyll (Figure 4.6). At 40 min pretreatment the DMBQ and SiMo activities approached a similar level of inhibition. If the pretreatment time had been continued beyond 40 min it is suggested that the inhibition of both DMBQ and SiMo activities would have been essentially the same, reflecting the damage nearest the beginning of the electron transport chain, between water and SiMo. If the results are not converted to % inhibition and are treated as linear relationships essentially the same conclusions may be drawn (Figure 4.8). The Q_B - dependent (DMBQ) activity is clearly inhibited to a greater extent than the Q_B - independent (SiMo) activity, particularly with thylakoid suspensions equivalent to 50 μg cm^{-3} chlorophyll. Again this suggests initial damage at the Q_B site, followed by further damage of the electron transport chain between water and SiMo (*i.e.* Q_A).

These results are in agreement with those of other workers. Allakhverdiev, *et al.* (1987) found that PSII particles which were nearly completely photoinactivated, as determined by decline in electron transport from water to DMBQ and in variable fluorescence, were fully functional in the primary separation of charges between P680 and phaeophytin, as determined by light induced absorbance changes at 685 nm. The EPR signal II_f also remained unchanged, demonstrating that functioning of Y_z was not impaired. They concluded that the target of PSII inhibition must lie beyond phaeophytin, with an increase in F_o fluorescence under reducing and anaerobic

conditions suggesting impairment of electron transport in the Q_A - Q_B region. It was also suggested that the disappearance of the D1 protein from the thylakoid membrane was a consequence of photoinhibition and not part of the mechanism of photoinhibition. Kyle, *et al.* (1984) used fluorescence, photochemical assays and herbicide binding studies and concluded that quinone anions, which may interact with molecular oxygen to produce oxygen radicals, selectively damage the Q_B site of the D1 protein, rendering it inactive. In this study, using *Chlamydomonas reinhardtii* cells, electron transfer activities from water to DCPIP, from DPC to DCPIP, both of which require PSI and PSII (Figure 4.1), and from water to DAD, which requires PSII only, were all significantly reduced by photoinhibitory treatment. Whereas, the electron transfer activities from TMPD/ Ascorbate to methyl viologen, which requires PSI only, from water to SiMo, and from DPC to SiMo, which are PSII assays that do not require Q_B, were all unaffected by photoinhibitory treatment. These results confirmed that the principal effect of photoinhibition is at PSII with little effect on PSI. Furthermore, by using assays specific to the PSII reaction centre and not involving the secondary quinone acceptor Q_B (*i.e.* SiMo reduction) it was apparent that little damage had occurred to either the water splitting enzymes or the reaction centre. Similar findings were made when the partial reactions of thylakoids isolated from *Pisum sativum* were investigated (Ohad, *et al.*, 1985), with initial damage to the Q_B region being followed by damage to the reaction centre. The rate of loss of Q_B - dependent, followed by Q_B - independent, electron flow during photoinhibition was faster in the *in vitro* thylakoid system. Ohad, *et al.* (1988) carried out thermoluminescence, fluorescence kinetics and oxygen flash yield measurements and suggested that a series of events occurred during photoinhibition. Firstly, a change in the whole population of reaction centre II destabilising the S_2 - Q_B^- state, indicating a change in the Q_B^- binding site, accompanied by a corresponding rise in the F_o fluorescence value, with only a slight loss of the S_2 - Q_A^- thermoluminescence signal. The primary photochemistry of reaction centre II was not affected, as indicated by the extent of $P680^+$ formation and generation of S_2 - Q_A^- detected by the thermoluminescence measurements. These results are in agreement with previous reports showing the persistance of electron donation in photoinhibited *C. reinhardtii* cells (Kyle, *et al.*, 1984) to SiMo, an acceptor of electrons at a site prior to Q_B. Additional changes were also apparent in a fraction of the reaction centre II population which affected Y_z. This was followed by a complete loss of the S_2 - Q_B^-

signal and loss of the S_2 - Q_A^- signal which could be related to a total loss of reaction centre II activity. This two stage process of photoinhibition may correspond to that seen in this study, where Q_B - dependent activity was inhibited initially, followed by further inhibition between water and SiMo, which accepts electrons from Q_A. Ohad, *et al.* (1988) suggested that these initial changes in the reaction centre II expressed as destabilisation of the S_2 - Q_B^- state can be interpreted as being the result of a change in the D1 polypeptide conformation, perhaps being the trigger for, or the result of, initiation of the D1 degradation process, which is light dependent. Furthermore, the correlation between the initial changes in reaction centre II thermoluminescence properties and the increase in the rate of D1 synthesis and initiation of the 'recovery' response led to the conclusion that the two phenomena are related. More recent work has essentially confirmed these findings (Ohad, *et al.*, 1990). The initial light - induced modification has been found to be reversible at low light intensities, whilst prolonged exposure at higher PPFD resulted in irreversible modification of the D1 protein. It was suggested that the modification of D1 could involve an internal cross - link between transmembrane helices II and III, perhaps via the action of free radicals, which have been proposed as a possible cause of photoinhibition. The light - induced change in conformation may facilitate damage to D1 by radicals formed within the reaction centre and confined to the D1 protein (*e.g.* Q_B^-, $P680^+$, Y_z^+) either directly or mediated via an oxygen or hydroxyl radical.

Kirilovsky, *et al.* (1988) studied photoinhibition of *Synechocystis* 6714 using partial reaction, fluorescence and flash - induced Q_A reduction measurements. The decrease of F_v (variable fluorescence) and electron flow through Q_B (water to dichlorobenzoquinone) were found to be similar and the fastest phenomena during photoinhibition. The loss of Q_B - independent electron transfer (water to SiMo) was much slower. It was apparent that the loss of water to dichlorobenzoquinone activity correlated with the photoreduction of Q_A^- (measured by the complementary area over the fluorescence induction curve). In contrast, the SiMo activity measurements exhibited no such relationship and it was suggested that there was a lack of correlation between the decrease of variable fluorescence and the damage to the reaction centre itself (primary photochemistry). These workers concluded that the decrease of F_v is related to damage of the Q_B site, which they proposed as the primary target of photoinhibition. This damage could be reversed by *de novo* synthesis of the D1 and D2 proteins. The

reversible inhibition was followed by a more extensive inactivation of the PSII reaction centre as demonstrated by decrease in SiMo - dependent oxygen evolution, which occurred in parallel with a decrease in Q_A photoreduction. The secondary process was irreversible and resulted in decreased energy transfer from the phycobilisomes to PSII and an inability to perform charge separation. Again, this two step process appears similar to the response observed, with thylakoids isolated from *C. fragile*, in the present study. A more recent investigation found that photoinhibited *S. oleracea* thylakoids exhibited strong inhibition of 1, 4 - benzoquinone mediated electron flow rates compared to only slightly decreased rates of SiMo electron transport, implicating the Q_B site of the D1 protein as the initial site of damage (Richter, *et al.*, 1990a). It was suggested that this inactivation of the Q_B site, possibly caused by a conformational modification, was followed by a second process involving turnover of the damaged protein. This second process was accompanied by the loss of PSII reaction centre activity. Thus, an initial inactivation step at the Q_B site was distinguished from D1 protein degradation. Further work identified a major contribution of activated oxygen species to this loss of Q_B function and D1 degradation process and the related phenomena of photoinhibition (Richter, *et al.*, 1990b). The presence of exogenous superoxide dismutase and catalase and the antioxidants glutathione and ascorbic acid gave protection, albeit not complete, of D1 protein, photochemistry and fluorescence from photoinhibition. Interestingly, a two step process of photoihibition is again suggested.

However, other investigations have indicated that the first site of photoinhibitory damage is in the PSII reaction centre. In the unicellular cyanobacterium *Microcystis aeruginosa* both Q_B - dependent (water to DAD) and Q_B - independent (water to SiMo) PSII activities were lost on photoinhibition (Tytler, *et al.*, 1984). Similar inhibition was observed if DPC was used as electron donor. Thus, the damage to PSII appeared to be between the site where DPC donates electrons and the site where SiMo accepts electrons. It was suggested that the initial photoinhibitory damage was at, or very close to, the reaction centre itself in this organism. Similarly, Cleland and Critchley (1985) found loss of SiMo reduction activity of thylakoids isolated from *Spinacia oleracea* and concluded that photoinhibition was due to destruction of the PSII reaction centre. Theg, *et al.* (1986) studied chloride depleted chloroplasts isolated from *S. oleracea* by the use of fluorescence techniques and partial reaction assays. These workers believed that their

results indicated that the irreversible inhibition produced in the photosynthetic electron transport chain by illuminating Cl^- free or Tris - washed chloroplasts is mechanistically the same as *in vivo* photoinhibition, the former occurring on an accelerated time scale. They suggested that the initial event in photoinhibition *in vivo* is a reduction of water splitting activity, followed by development of a lesion close to the reaction centre, P680, or its immediate electron donor Y_z, as a result of reduced electron flow into $P680^+$. The possibility that the primary photoinduced lesion is at the second stable PSII electron acceptor, Q_B, was excluded. Callahan, *et al.* (1986) concluded that weak light photoinhibition of hydroxylamine (NH_2OH) extracted wheat (*Triticum aestivum*, var. Oasis) leaves and NH_2OH or Tris extracted chloroplasts affected principally the secondary donor, Y_z, to $P680^+$ and that the Q_B site was not involved. They found that photoreduction of SiMo by DPC was diminished by photoinhibition and that the number of atrazine specific binding sites for the Q_B position was unaffected. Cleland, *et al.* (1986) investigated photoinhibition of thylakoids and chloroplasts isolated from *S. oleracea* using a variety of methods including measurements of variable and non - variable fluorescence yield and of the absorbance changes of Q_A (ΔA_{320}) and C550 (ΔA_{540} - A_{550}). The former absorbance gives a measure of Q_A reduction state whilst the latter absorbance difference is associated with the reaction centre phaeophytin molecule, hence also giving a measure of Q_A photoreduction. It was concluded that the primary site of photoinhibition was the reaction centre of PSII. In further work, Cleland and Melis (1987) treated thylakoid membranes with NH_2OH, which inactivates the OEC, blocking electron donation to $P680^+$, thus maintaining Q_A and Q_B in the oxidized state. It was found that NH_2OH treatment prior to high - light exposure intensified photoinhibition as shown by decline in the amplitude of ΔA_{320} and suppression of variable fluorescence yield. It was concluded that semiquinone anion formation at Q_B and / or electron accumulation at Q_A is not a prerequisite to photoinhibition, as suggested by Kyle, *et al.* (1984), as photoinhibition occurs even when Q_A and Q_B are in the oxidized state. Their results implicated the reaction centre of PSII as the primary site of photoinhibition. Demeter, *et al.* (1987) investigated the primary site of photoinhibitory damage in *C. reinhardtii* cells and *S. oleracea* chloroplasts by measuring the phaeophytin a (ΔA_{685}) and Q_A (ΔA_{320}) photoreduction signals. They found that both parameters displayed identical kinetics, suggesting that the reduction of phaeophytin is adversely affected and that inhibition of Q_A photoreduction is only a

consequence of inhibition in the primary charge separation, between P680 and phaeophytin. That is, the primary lesion of photoinhibition results in the prevention of the primary charge separation between P680 and phaeophytin, due to damage at, or close to, P680.

The techniques of delayed luminescence and thermoluminescence were used by Vass, *et al.* (1988) in a study of photoinhibition of thylakoids isolated from *S. oleracea*. These workers concluded that the Q_B binding site was not the primary target of photoinhibition in isolated thylakoids. They suggested that electron transfer is impaired at a site in the Y_z - P680 - Ph - Q_A region, with damage at Q_A or between Ph and Q_A most probable. A minor possibility that Q_A and Q_B undergo simultaneous damage was also considered. It has been suggested that the primary cause of photoinhibition is the light - induced accumulation of the reactive quinone species Q_B^{2-} which damages the Q_B binding site (Kyle, *et al.*, 1984). Vass, *et al.* (1988) have proposed that accumulation of the reduced form of the primary quinone acceptor (Q_A^-) may cause similar damage to the Q_A binding site. It was argued that this enabled an explanation of the primary photodamage to the Q_B site in algal cells (*e.g.* Kyle, *et al.*, 1984; Ohad, *et al.*, 1988) and the parallel impairment of Q_A and Q_B in isolated thylakoids as observed in their own studies (Vass, *et al.*, 1988). In isolated thylakoids and PSII preparations which do not have CO_2 fixation capability high PPFD will fully reduce all acceptor pools. Due to the equal amount of permanently reduced Q_B and Q_A both the Q_B and Q_A binding sites will be damaged to the same extent. In intact algal cells electrons will be continuously drained off the plastoquinone pool towards PSI resulting in a partially reduced electron transport chain and hence a smaller population of reduced Q_A than reduced Q_B. It is suggested that this would result in slower photodamage of the Q_A site compared to that of Q_B, as observed in algal cells (Ohad, *et al.*, 1988). Although this arguement may be of interest when considering thermoluminescence experiments (Ohad, *et al.*, 1988; Vass, *et al.*, 1988), its relevance when considering partial reaction experiments that involve electron acceptors from PSII (*e.g.* DMBQ and SiMo assays) is uncertain. Jegerschold, *et al.* (1990) studied the light - dependent inhibition of electron transport activities in *S. oleracea* thylakoid membranes in which the oxygen evolution had been reversibly inhibited by Cl^- depletion. The results indicated that the accumulation of $P680^+$ and / or Y_z^+, both of which are highly oxidizing, occurs initially and that this triggers D1 protein

degradation. Setlik, *et al.* (1990) investigated photoinactivation of oxygen evolving PSII particles isolated from *Pisum sativum* and identified three photoinactivation processes; fast ($t_{1/2}$ = 1 - 12 min), slow ($t_{1/2}$ = 15 - 40 min), and very slow ($t_{1/2}$ > 100 min). The fast process resulted in a decline of Fv and of Hill reaction rate (electron transport through PSII measured by benzoquinone - or ferricyanide - dependent oxygen evolution), accompanied by an antiparallel increase of constant fluorescence (F_o). It was suggested that this was due to trapping of Q_A in a negatively charged state, Q_A^-. The slow process was associated with a decline in maximal fluorescence (F_m) and inhibition of the Hill reaction and it was proposed that this process represented the neutralisation of the negative charge on Q_A rendering it non - functional. The very slow photoinactivation process was associated with loss of charge separation ability of the PSII reaction centre (Nedbal, *et al.*, 1990). Thus, these workers believe that Q_A is the initial site of lesion upon photoinhibition. A similar conclusion was made by Styring, *et al.* (1990) after a study of photoinhibition in thylakoid membranes isolated from *S. oleracea* using electron paramagnetic resonance (EPR) spectroscopy. Their results indicated that photoinhibition inhibits the electron transfer between phaeophytin and Q_A probably by impairing the function of Q_A. The primary charge separation was still operational. It was suggested that photoinhibition of the electron transport chain was caused by the double reduction of Q_A, which then leaves its site. Photoinhibition also resulted in rapid oxidation of cytochrome b_{559} and a change of cytochrome b_{559} from its high potential form to its low potential form. This is of interest when considering the proposal that cytochrome b_{559} may protect the reaction centre from damaging photooxidation reactions caused by the action of $P680^+$ (Thompson and Brudvig, 1988). The data of Styring, *et al.* (1990) indicate that cytochrome b_{559} is oxidized early during photoinhibition before the degradation of the reaction centre proteins, leading these workers to suggest that it can not exert its putative protective function when needed most. The details of any such process are not yet fully understood. Klimov, *et al.* (1990) suggest that a common feature found in studies of photoinhibition is a loss of ability of the reaction centre of PSII to have a functional interaction with the electron transport chain while the primary photoreactions of charge separation remain.

It is apparent that there is still controversy concerning the site of the primary lesion of photoinhibition. There is substantial experimental evidence to support all of the many alternative sites proposed, which include Q_B, Q_A, the reaction centre (P680) and Y_z. The

complexity of this particular area of interest is exacerbated by the great variety of experimental systems and conditions used. These have included the study of whole cells (*e.g.* unicellular algae), chloroplasts, Cl^- depleted chloroplasts, NH_2OH or Tris extracted chloroplasts, thylakoid membranes and PSII particles, in aerobic, anaerobic or strongly reducing incubation conditions. Caution is needed when comparing the data obtained by different workers. However, the results obtained in this study provide preliminary evidence that photoinhibition of thylakoids isolated from *C. fragile* occurs in a two stage process. It appears that the first stage involves damage to the Q_B site, whilst further damage, in the second stage of the process, results in inhibition of electron transport between water and Q_A. This suggests that the mechanism of photoinhibition in *C. fragile* initially involves the Q_B site and is similar to that suggested by Kyle, *et al.* (1984), Ohad, *et al.* (1985, 1988, 1990), Kirilovsky, *et al.* (1988) and Richter, *et al.* (1990a). However, alternative mechanisms involving initial damage to the electron transport chain at sites other than Q_B (*e.g.* the reaction centre) can not be categorically excluded on the basis of the experiments described in this Chapter alone. It is not possible to be more specific about the site of the secondary damage from the partial reactions investigated in this study, however this may be further elucidated, and indeed the initial damage to Q_B confirmed or questioned, by using some of the many other techniques available for the investigation of the initial events of photoinhibition. These methods include room temperature and 77 k fluorescence analysis, herbicide binding assays, thermoluminescence and delayed luminescence, oxygen flash yield measurements and flash induced Q_A reduction measurements. Light induced absorbance changes at 685 nm, 320 nm and 540 - 550 nm, may be used to monitor P680 - phaeophytin charge separation, Q_A reduction and reaction centre phaeophytin, respectively, whilst Y_z can be studied via its characteristic EPR signal.

4.5 Conclusions

Suspensions of thylakoids isolated from *C. fragile*, equivalent to 50 and 100 μg cm^{-3} chlorophyll, and exposed to 1000 μmol m^{-2} s^{-1} PPFD over a time course of 40 and 100 min, respectively, exhibited an initial loss of Q_B - dependent activity as indicated by inhibition of electron transfer from water to DMBQ. This was followed by a loss of Q_B - independent activity as indicated by inhibition of electron transfer from water to SiMo. It is suggested that this provides preliminary evidence that photoinhibition in

C. fragile occurs in a two stage process. The first stage involves inactivation of the Q_B site, whilst the second stage in the process involves further damage to the electron transport chain between water and the primary quinone acceptor, Q_A. Such supra - optimal PPFD also resulted in a decline in chlorophyll content of isolated thylakoid suspensions and prolonged exposure may result in destruction of photosynthetic pigment indicating the onset of photooxidation.

CHAPTER 5 THE CHARACTERISATION OF ROOM TEMPERATURE CHLOROPHYLL FLUORESCENCE OF CODIUM FRAGILE CHLOROPLASTS.

5.1 Introduction

The experiments described in Chapter 3 revealed that chloroplasts isolated from *C. fragile* were extremely susceptible to photoinhibition at supra - optimal PPFD, as indicated by a decline in bicarbonate - dependent oxygen evolution. Further investigations suggested that this photoinhibition occurred in two stages (Chapter 4). The first stage involved inactivation of the Q_B site, whilst the second stage in the process involved further damage to the electron transport chain between water and the primary quinone acceptor, Q_A. Photoinhibition is accompanied by modifications of the chlorophyll fluorescence emission characteristics, including large changes in the kinetics of chlorophyll fluorescence induction and a decrease in the steady state fluorescence yield, which may be used to identify photoinhibitory stress (*e.g.* Critchley, 1981; Critchley and Smillie, 1981; Kyle, *et al.*, 1984; Barenyi and Krause, 1985). This technique thus offers another means by which photoinhibition in *C. fragile* may be further examined.

However, fluorescence induction kinetics are complex and closely associated not only with the photochemical activities of thylakoids, but also with the metabolic processes of carbon assimilation (*e.g.* Krause and Weis, 1984; Sivak and Walker, 1985). Consequently, there has been an increasing amount of work in this area over the last decade in an attempt to clarify such relationships (*e.g.* Walker, 1981; Sivak and Walker, 1983; Sivak, *et al.*, 1983; Walker, *et al.*, 1983a, 1983b; Ireland, *et al.*, 1984; Sivak, *et al.*, 1985; Walker and Sivak, 1985; Laisk and Walker, 1986; Sivak and Walker, 1986; Walker and Osmond, 1986; Seaton and Walker, 1990), but uncertainties still remain. For example, there is still some controversy concerning the origin of chlorophyll fluorescence (Barber, *et al.*, 1989). It is neither possible nor appropiate to exhaustively discuss this subject in this introduction. However, it is necessary to briefly describe some generally accepted principles regarding chlorophyll fluorescence and the associated terminology. Many articles may be referred to for further details (*e.g.* Lavorel and Etienne, 1977; Hipkins and Baker, 1986; Baker and Horton, 1987; Bjorkman, 1987; Walker, 1987).

At room temperature almost all of the fluorescence emission arises from

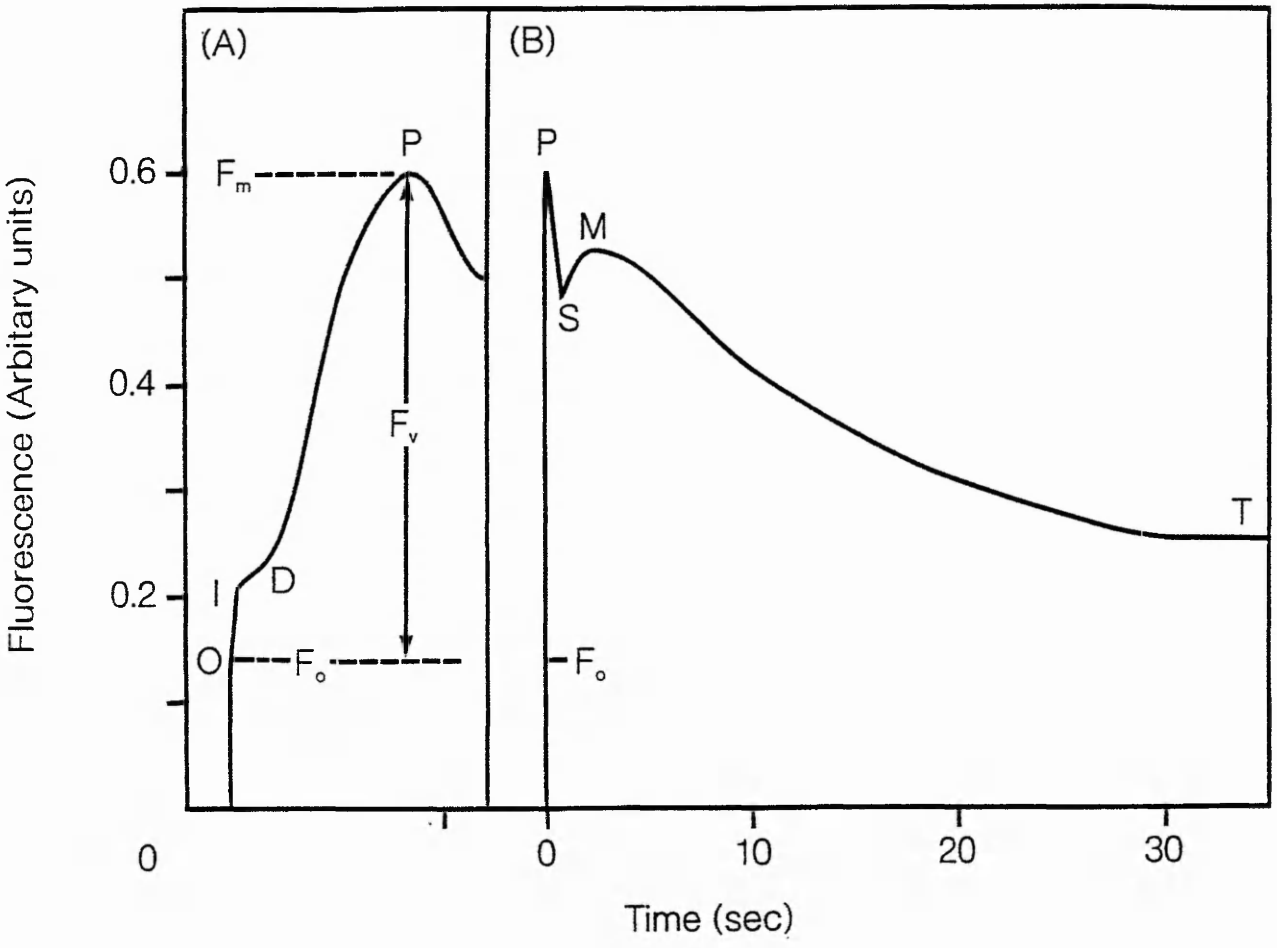
chlorophylls associated with PSII (Baker and Horton, 1987). Minor components of the emission spectrum associated with PSI are present at wavelengths greater than 710 nm and it is possible to use a ratio of fluorescence emission at 695 nm to that at 720 nm to compare PSII and PSI kinetics. However, for the purpose of this introduction the fluorescence induction curve may be considered to be dominated by changes in PSII fluorescence, which has a peak at approximately 685 nm (Walker, 1987). Fluorescence may be emitted directly from the LHCII or from the 40 - 50 antenna chlorophylls closely associated with the PSII reaction centre, and thus be in competition with photochemical trapping (prompt fluorescence). Alternatively, it may be derived from a recombination reaction between oxidized P680 and reduced phaeophytin within the PSII reaction centre, that is, delayed fluorescence (Baker and Horton, 1987; Barber, *et al.*, 1989).

Fluorescence is just one way in which excited chlorophyll electrons may regain the ground state. De - excitation via fluorescence is in competition with de - excitation by other mechanisms of both photochemical and non - photochemical nature, so that fluorescence yield, ϕ_f, is given by,

$$\phi_f = K_f / (K_f + K_h + K_{ic} + K_s + K_p [P]),$$

where K_f, K_h, K_{ic}, K_s and K_p are the rate constants for de - excitation of chlorophyll via fluorescence, radiationless decay to heat (vibrational relaxation), internal conversion to triplet (does not occur to any significance *in vivo*), energy transfer to non - fluorescing species such as PSI (spillover) and photochemistry. [P] is the fraction of open PSII reaction centres with the primary donor P680 reduced and primary quinone acceptor Q_A oxidized. When all the reaction centres are open, [P] = 1, and the minimal flourescence level, F_o, is obtained (Figure 5.1). When all the reaction centres are closed (*i.e.* all Q_A is fully reduced), [P] = 0, and the maximum yield of fluorescence, F_m, occurs (Baker and Horton, 1987; Barber, *et al.*, 1989). The difference between F_m and F_o (*i.e.*, F_m - F_o) is the variable fluorescence, F_v, and the ratio F_v/F_m is proportional to the quantum yield of photochemistry and generally has a value between 0.6 and 0.8 (Baker and Horton, 1987; Bjorkman, 1987). The area over the curve between F_o and F_m is proportional to the pool size of the electron acceptors on the reducing side of PSII (Bolhar - Nordenkampf, *et al.*, 1989). Measurement of the area over the curve is complex, but a simple indicator is given by $t_{1/2}$, one half of the time required for the rise in fluorescence from O to P (Figure 5.1). The quenching of fluorescence by

Figure 5.1 Characteristic fluorescence induction kinetics or Kautsky curve illustrating the lettering convention OIDPSMT. Key: A, fast kinetics; B, slow kinetics; F_m, maximum fluorescence; F_o, minimal fluorescence; F_v, variable fluorescence ($F_v = F_m - F_o$). See text for details. Adapted from Bolhar - Nordenkampf, *et al.* (1989).


(A)
(B)
P
P
F_m
M
S
0.6
0.4
0.2
F_v
T
I
D
O
F_o
F_o
0
0
10
20
30
Fluorescence (Arbitary units)
Time (sec)

photochemistry is termed q_Q (or sometimes q_P). However, quenching of PSII fluorescence can also occur which is independent of the redox state of Q_A and this is termed non - photochemical quenching (q_{NP} or sometimes q_N). A number of mechanisms may contribute to non - photochemical fluorescence quenching, including the following. In the presence of a trans - thylakoid ΔpH, an increase in non - radiative decay of excitation occurs. This fluorescence quenching is referred to as energy - dependent or high energy state quenching (q_E). This is generally thought to be the major non - photochemical mechanism under physiological conditions, however, the molecular mechanism of such quenching is not understood. Quenching may occur that is related to state transitions (q_T quenching). The transition from unphosphorylated to phosphorylated LHCII can cause a 20 - 25 % drop in fluorescence (Baker and Horton, 1987). Finally, quenching in closed reaction centres may occur under some conditions, either due to the maintenance of oxidized P680 or reduced phaeophytin. Such PSII reaction centre states are not likely to be maintained when non - cyclic electron transport is operating normally, but are more likely to occur under stress conditions and may be a primary cause of the decreased fluorescence emission upon photoinhibition, sometimes referred to as, q_I, or photoinhibition associated quenching (Baker and Horton, 1987; Walker, 1987).

When illuminated, photosynthetic plant samples fluoresce continuously but this provides little information in itself due to the many physico - chemical and metabolic factors that can determine the absolute fluorescence level (Bolhar - Nordenkampf, *et al.*, 1989). Far more information is obtained by studying the time - course of fluorescence following a perturbation in light level. Therefore, studies of chlorophyll fluorescence normally concentrate on the fluorescence response when plant material is suddenly illuminated, following several minutes or hours of darkness. Upon illumination of a dark - adapted leaf or suspension of chloroplasts a characteristic fluorescence induction occurs that reflects changes in photochemical and non - photochemical quenching associated with the events of the induction period of photosynthesis when metabolite levels are increasing from the low 'dark' levels (*e.g.* Walker, 1981). The kinetics of this fluorescence response are termed the Kautsky curve and, as illustrated in Figure 5.1, phases of the curve are labelled OIDPSMT (Papageorgiou, 1975; Lavorel and Etienne, 1977; Walker, 1987; Bolhar - Nordenkampf, *et al.*, 1989). On illumination, fluorescence rises immediately (in

picoseconds but limited by the opening time of the shutter) to the F_o level, followed by an initial rise of variable fluorescence to a level (I), followed by a dip (D), a peak (P), and a fall, via a quasi - steady state (S) to a terminal value (T). Sometimes a second maximum (M) is seen between S and T. The pause at I and the dip at D are thought to reflect imbalances between the rate of reduction and reoxidation of Q_A. Thus, the fluorescence rise from the F_o level to the peak level, P (*i.e.* F_p - that occurs in one or two seconds), is a process that mainly reflects the reduction of PSII centres (Q_A) which are oxidized in the dark (Bradbury and Baker, 1984). The fluorescence level F_p tends towards the F_m level, but does not usually reach it especially if the onset of electron transfer to $NADP^+$ and beyond is rapid, if there is electron transfer to O_2 or if the light intensity is low. After F_p the fluorescence is quenched to a terminal steady - state level at T over several seconds or minutes. The kinetics of P to T quenching and the underlying causes are variable and complex, but some generalisations have been agreed upon, as follows. The P to T quenching involves both Q oxidation (q_Q) and high energy state quenching, q_E (Bradbury and Baker, 1981; Krause, *et al.*, 1982; Quick and Horton, 1984a, 1984b). In low light intensity, q_Q predominates, whilst in high light intensity q_E is the main quenching mechanism. The M peak, as observed in isolated chloroplasts and leaves, is associated with changes in q_Q and q_E (Bradbury and Baker, 1981). After the M peak, one or more additional transients are observed under some conditions. Such oscillatory behaviour is lost during photoinhibition (Walker and Osmond, 1986).

The main problem in the measurement of chlorophyll fluoresence is to prevent the photodiode from detecting light that is not fluorescence (Walker, 1987). For example, a large fraction of the actinic light (the light used to drive photosynthesis) may be reflected by the sample into the detector. Thus, fluorescence (emitted at wavelengths between 660 nm and 800nm, with a peak at 685 nm) can be detected with a photodiode protected by a filter which transmits only selected wavelengths between 660 nm and 800 nm. The radiation between 660 nm and 800 nm contained in reflected white light is much greater than the emitted chlorophyll fluorescence. Since such red and far - red radiation cannot be distinguished from chlorophyll fluorescence by the detector, it is normally not possible to measure chlorophyll fluorescence emission accurately when white light is used to excite the sample. Therefore a red actinic light is often used (*e.g.* the 660 nm centre frequency Hansatech LS1 light source) in conjunction with a

photodiode detector protected by an interference filter with a centre frequency of 740 nm (there is a second smaller fluorescence maximum at this wavelength). Alternatively, blue actinic light (*e.g.* white light filtered through a Corning 4 - 96 filter) may be used in conjunction with a photodiode protected by a Wrattan 88A filter which passes long wavelength red light. The fluorescence signals obtained by these two methods are usually comparable (Walker, 1987). However, these arrangements limit the range of experiments that can be undertaken, for example, precluding measurements in white light. This problem can be remedied by the use of modulated fluorescence techniques. By using a modulated light source to excite fluorescence in conjunction with a fluorescence detection system which monitors only fluorescence emitted at the frequency of the modulated exciting light it is possible to excite the sample with actinic d.c. light containing the same wavelengths as the monitored flourescence and yet monitor only the fluorescence generated by the modulated light source. The d.c. fluorescence signal produced by excitation of the sample with the actinic d.c. light source is not monitored by the detection system. The detection system can be a photodiode connected to a lock - in amplifier, which is 'tuned' to the frequency of the modulated excitation light. If the modulated light intensity used is low enough not to generate any variable fluorescence from the sample, the level of fluorescence generated by the modulated light is analagous to the F_o level that is generated immediately on exposure to continuous light (Ogren and Baker, 1985; Hipkins and Baker, 1986). Exposure of the sample, whilst being irradiated with weak modulated light, to actinic light will produce a variable fluorescence emission, which is observed as a variable component of the modulated fluorescence signal. That is, the chlorophyll fluorescence caused by the white actinic light is not measured directly, but the reactions generated within the sample by the white actinic light affect the chlorophyll fluorescence generated by the weak modulated light. It is these reactions and therefore the changes in the modulated fluorescence signal that they generate that are of interest. The Hansatech modulated fluorescence measurement system (MFMS) is based on these principles.

The fluorescence measurement systems outlined above and the associated experimental methods have been developed to enable analysis of the two main forms of fluorescence quenching: photochemical (q_Q) and non - photochemical (q_{NP}). The action of the two quenching components q_Q and q_E can be resolved in experiments with

isolated thylakoids, chloroplasts, protoplasts or unicellular algae by the addition of DCMU during the induction curve (Krause, *et al.*, 1982), which results in a biphasic reversion of fluorescence quenching (see Figure 5.9A in Results section 5.3). The rapid phase of the DCMU -induced fluorescence rise, R_f, has a half rise time of approximately 1 sec and is a result of rapid reduction of Q_A, and represents the fluorescence level when Q_A is maximally reduced. It thus reflects the amount of photochemical quenching (q_Q). The slow phase of the DCMU - induced fluorescence rise, R_s, has a half rise time of approximately 5 to 15 sec and is a result of the relaxation of the proton electrochemical gradient across the thylakoid membranes. The fluorescence level after this rise thus reflects the amount of the high - energy state quenching, q_E (Hipkins and Baker, 1986). Unfortunately, this DCMU addition technique can only be used with photosynthetic systems in suspension, and not with leaf tissue due to the slow rate of DCMU penetration. Also, if the contributions to fluorescence quenching made by q_Q and q_E are to be resolved throughout the induction curve, measurements must be made on many samples (20 or more) that must behave similarly (Horton, 1983; Quick and Horton, 1984b). An alternative approach is 'light - doubling' (Bradbury and Baker, 1981, 1984). The fast phases of fluorescence induction (OIDP) occur within fractions of a second and exclusively reflect changes in q_Q, as changes in q_{NP} require a longer time (approximately 10 sec to several min). This difference is the basis for the 'light - doubling' method for separating q_Q and q_{NP} (Bolhar - Nordenkampf, *et al.*, 1989). At any point in time along the fluorescence induction curve, application of sufficiently strong light will result in full reduction of PSII reaction centres, rapidly suppressing q_Q (Figures 5.9 A and B in Results section 5.3). The remaining quenching is q_{NP}. Quick and Horton (1984b) used modulated light in experiments with protoplasts and demonstrated an acceptable correlation between q_Q determination by the 'light - doubling' method and the DCMU addition method (Figure 5.9A). The pulse - saturation technique (Schreiber, *et al.*, 1986), as illustrated in Figure 5.9B, involves the generation of modulated fluorescence by a low intensity modulated light source that is non - actinic (*i.e.* produces fluorescence analagous to F_o). The fluorescence induction curve is generated by a white actinic light source of the desired PPFD and short pulses (*e.g.* 300 msec) of saturating light are superimposed at appropiate intervals (*e.g.* every 10 to 20 sec). F_m is the maximal fluorescence obtained with a dark - adapted sample on addition of a saturating light pulse. Photochemical and

non - photochemical quenching are then determined by addition of a saturation pulse at any given time in the induction curve. The increase in fluorescence level on addition of a saturation pulse reflects q_Q, whilst the difference between this level and F_m reflects q_{NP}. This technique can be used to determine q_Q and q_{NP} in leaf samples. However, q_E cannot be resolved unambiguously from other non - photochemical quenching mechanisms. Whereas in thylakoids, chloroplasts, protoplasts or unicellular algae the q_{NP} can be resolved further, as shown in Figure 5.9A, as q_E can be estimated by the slow DCMU - induced rise (Krause, *et al.*, 1982; Horton, 1983; Quick and Horton, 1984b). A proportion of fluorescence quenching has been observed that is neither q_Q nor q_E and this was termed remaining quenching, q_R (Quick and Horton, 1984b). Recently, Horton and Hague (1988) used pulse saturation in conjunction with DCMU addition in experiments with barley (*Hordeum vulgare* L. cv Marko) protoplasts (Figure 5.9A). The q_R component was further resolved into state transition - dependent quenching (q_T) that was sodium fluoride (NaF) sensitive, and photoinhibition - dependent quenching (q_I), which increased as PPFD was increased. Thus, it is now possible to resolve q_Q, q_E, q_T and q_I components of fluorescence quenching.

The work described in this Chapter used some of the available fluorescence techniques, as outlined above, for an initial study and characterisation of the fluorescence response in chloroplasts isolated from *C. fragile*. This study investigated the early events of chlorophyll fluorescence induction in the msec range and this necessitated the use of the Hansatech DW2 oxygen electrode in conjunction with the LH7 red LED light source (centre frequency 660 nm; spectral line half width 30 nm) and TR1 transient recorder. The main aim was to measure F_o, F_m and F_v/F_m, and to establish whether these parameters were comparable to those determined in other photosynthetic organisms. It was hoped that this would enable progression to a second series of experiments using the Hansatech modulated fluorescence measurement system and white actinic light in conjunction with the DCMU addition technique to further characterise the fluorescence response by resolving some of the quenching mechanisms identified by other workers. A final series of experiments was carried out with the objective of investigating the affect of pretreatment at 1000 μmol m^{-2} s^{-1} PPFD for up to 25 min on the various fluorescence parameters, thus obtaining further information concerning the mechanism of photoinhibition.

5.2 Materials and Methods

5.2.1 Sampling and Maintenance of *C. fragile*

Fronds of *C. fragile* were harvested on 19/ 12/ 89 (winter - vegetative) as described previously (2.2.1) and similarly maintained.

5.2.2 Chloroplast and Thylakoid Preparation

Chloroplasts and thylakoids were isolated from frond tips as previously described (3.2.2 and 4.2.2, respectively) and the chlorophyll content determined using the method of MacKinney, 1941 (3.2.3).

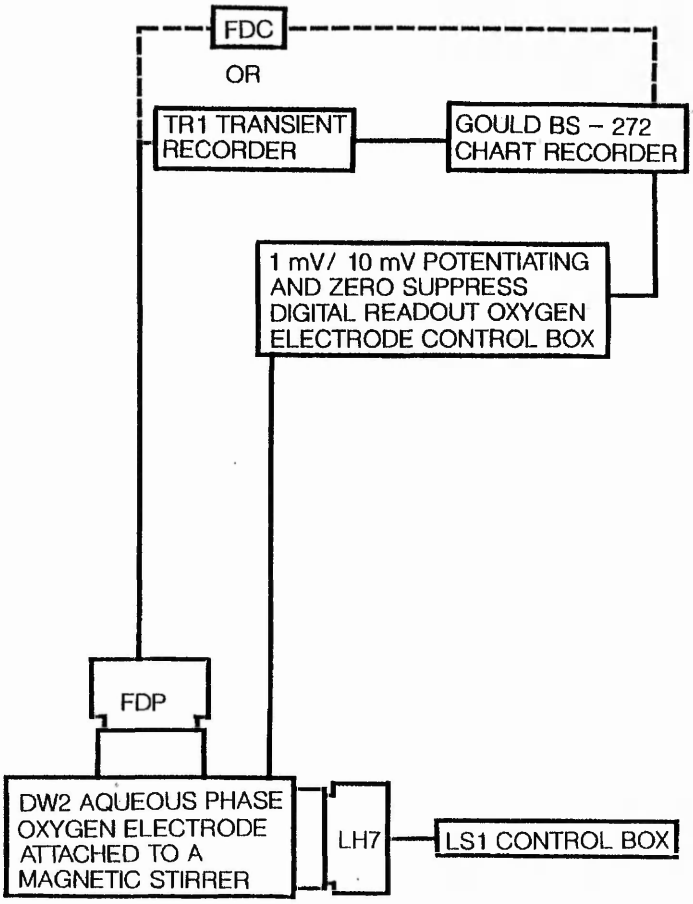
5.2.3 Measurement of Oxygen Exchange of isolated Chloroplasts and Thylakoids.

The DW2 is a modified version of the Hansatech Ltd (King's Lynn, UK) DW1 oxygen electrode (see Figure 3.1), consisting of a black light - tight reaction chamber and water jacket, which has four transparent 'ports' that accept light sources or photodetectors, enabling the simultaneous measurement of oxygen exchange and fluorescence in the aqueous phase. The measurement of oxygen exchange of isolated chloroplast and thylakoid suspensions was achieved using a DW2 oxygen electrode and the methods of Delieu and Walker (1972). The oxygen electrode was set up, calibrated, connected to the first channel of a two pen chart recorder, and the reaction chamber maintained at 10 °C, as previously described (3.2.4). The activity of PSI and PSII together in isolated thylakoids was measured polarographically by electron transport from water to 7.5 mol m^{-3} $K_3Fe(CN)_6$ (found to be the optimum concentration by Cobb, *et al.*, 1990), using an assay procedure based on that described by Izawa, 1980 (see Figure 4.1).

5.2.4 Experimental Apparatus used for the Study of the Early Events of Chlorophyll Fluorescence Induction

The DW2 oxygen electrode was set up to enable oxygen exchange measurements (5.2.3) as required and the remaining apparatus was assembled to allow the measurement of the early events of fluorescence induction in the msec range, as illustrated in Figure 5.2. A 'probe' type light housing (LH7) containing 7 high intensity red LEDs (centre frequency 660 nm; spectral line half width 30 nm), with low infra -

Figure 5.2 Diagrammatic representation of the experimental system used in the study of the early events of chlorophyll fluorescence induction. Key: LS1, light source control box which provides very fast 'turn on' of the light source, eliminating the need for electronic/ mechanical shutters; LH7, 'probe' type light housing containing 7 high intensity red LEDs (centre frequency 660 nm, spectral line half width 30 nm); DW2, modified version of the DW1 oxygen electrode, enabling the simultaneous measurement of oxygen exchange and fluorescence in the aqueous phase; FDP, fluorescence detector probe; TR1, transient recorder; FDC, fluorescence detector control box (an alternative to TR1). See text for details.


FDC
OR
TR1 TRANSIENT RECORDER
GOULD BS – 272 CHART RECORDER
1 mV/ 10 mV POTENTIATING AND ZERO SUPPRESS DIGITAL READOUT OXYGEN ELECTRODE CONTROL BOX
FDP
DW2 AQUEOUS PHASE OXYGEN ELECTRODE ATTACHED TO A MAGNETIC STIRRER
LH7
LS1 CONTROL BOX

red content, and low heat generation thus making it unnecessary to use optical filtering, was placed in one port of the DW2 oxygen electrode to provide actinic light. The LH7 was connected to the 'LED housing socket' of the LS1 control box, which provided very fast 'turn on' of the light source, eliminating the need for electronic/ mechanical shutters. The LH7 can be set to provide accurate single pulses of light (0.1, 1.0, 10, 100 or 1000 msec), or continuous, or modulated light. It is highly suitable for the study of the early events of chlorophyll fluorescence induction in the msec range. The LS1 was set to maximum intensity (approximately 400 μmol m^{-2} s^{-1}) and the 'pulse selector switch' set to provide a continuous light source. The FDP fluorescence detector probe was placed in a second port of the DW2 chamber. The FDP consists of a photodiode and preamplifier which detect chlorophyll fluorescence and convert it into an electrical signal proportional to the light received. To prevent reflected actinic light (660 nm) from reaching the photodiode, a 740 nm interference filter was placed in the FDP, which allows light in the fluorescence range to pass. The two remaining DW2 ports remained 'plugged' with black 'stoppers' designed to provide dark conditions within the chamber. The FDP was connected to a Hansatech TR1 transient recorder via the 7 pin DIN socket on the front panel. The TR1 transient recorder can read up to 2048 data points at up to 34,000 points/ sec. The data points may be replayed at a slower speed, enabling a chart recorder to display very fast transients. The data, once recorded, remains in memory until either a new recording is made or the TR1 is switched off. A trace may be replayed as many times as required and over different time intervals. The record times possible are 60 msec, 0.6 sec, 6 sec and 60 sec, whilst the replay times possible are 0.6 sec, 6 sec, 1 min and 10 min. The second channel of a two pen chart recorder (set on the 2 volt scale for the fluorescence signal) was connected to the BNC 'output' socket of the TR1 recorder to enable replay of the recorded signals to the chart recorder. The 'mode' switch on the front panel of the TR1 was set to 'recorder'. Alternatively, the FDP was connected to the FDC fluorescence detector control box via the 7 pin DIN socket. The FDC amplifies the signal from the FDP to a level suitable for connection to a chart recorder. The output signal is between 0 - 8 volts, but depends on experimental conditions, type and number of filters in the probe housing and position of the 'output' control. Filters, especially narrow band interference filters, can give a marked attenuation of light input to the photodiode, reducing the output signal. The maximum record time with the TR1 is 60 sec, the FDC enables the fluorescence

induction to be measured for longer time periods (*e.g.* 10 min), although resolution of F_o is not possible. The second channel of a two pen chart recorder (set on the 500 mV scale, which was found to be optimal for the fluorescence signal) was connected to the BNC 'output' socket of the FDC control box.

5.2.5 Recording and Replaying Signals with the TR1 Transient Recorder

To record and replay a signal with the TR1 transient recorder the manufacturer's instructions were followed. The 'back - off' switch was set to 'cancel' and the 'gain' control was turned to a 'mid - way' setting. The trigger level was set to a value such that the increase in signal level caused by switching the actinic light on caused the TR1 to trigger and start recording, as follows. The 'trigger' switch on the rear panel was set to 'internal'. The 'on/ off' switch on the rear panel was switched 'on', causing the yellow 'stop' light to illuminate. The 'trigger level' control was set 'mid - way'. The 'record' button was pressed, illuminating both the 'record' light and 'stop' light, indicating that the TR1 was primed but not triggered. The 'trigger level' was slowly turned anti - clockwise until the 'stop' light went out, indicating that the TR1 had triggered and the record cycle was initiated. The trigger level was increased slightly (clockwise) so that the trigger level was slightly greater than the dark - condition input signal. On pressing 'record', the 'stop' light and 'record' light were both illuminated: any increase in input signal resulted in the input signal being greater than the trigger level, causing the TR1 to record. As a transient was detected, the record cycle began and the 'stop' light went out. On completion of the record cycle (of 60 msec, 0.6sec, 6 sec or 60 sec duration) the 'record' light went out and the 'stop' light came on. The signal was now recorded and ready to be replayed to the chart recorder.

The replay switch was set to the required time (0.6 sec, 6 sec, 60sec or 10 min) and the chart recorder speed was also set to the required time/ distance setting (10 cm/ min). The chart recorder was switched on and the 'replay' button of the TR1 recorder pressed: the 'stop' light went out and the green 'replay' light was illuminated. At the end of the replay time the 'replay' light went out and the 'stop' light came on.

5.2.6 Recording a Signal with the FDC Fluorescence Detector Control Box

The maximum record time of the TR1 transient recorder was 60 sec. It was possible to follow the fluorescence induction curve for longer periods of time by using

the FDC fluorescence detector control box. However, resolution of F_o was not possible. Fluorescence signals were recorded with the FDC according to the manufacturer's instructions, as follows. The fluorescence detector gave a small unwanted positive signal in addition to that produced by chlorophyll fluorescence. This was caused mainly by photodiode 'dark current' and reflected actinic light not completely eliminated by the filters. The 'back - off' control was used to compensate for the unwanted positive signal by providing a stable d.c. voltage which was subtracted from the total photodiode signal voltage, as follows. The apparatus was set up as for an experiment with the chloroplast suspension omitted. With the 'back - off' switch turned 'on', the fluorescence detector and light source were switched on. The 'back - off' control was then adjusted to give zero on the recorder. The light was switched off, the chloroplast suspension inserted and the experiment carried out. The output control was used to adjust the reading on the recorder without disturbing the back - off.

5.2.7 Measurement of the Early Events of Chlorophyll Fluorescence Induction in Chloroplasts isolated from *C. fragile*

A stirred reaction volume was used consisting of 100 μl of 100 mol m^{-3} $NaHCO_3$ in extraction medium (final concentration 10 mol m^{-3}), a volume of chloroplast suspension to give a final chlorophyll concentration of 25 μg cm^{-3}, with the final volume made up to 1 cm^3 with extraction medium. To measure fluorescent transients in the presence of DCMU, the reaction volume contained 50 μl of stock 1 mol m^{-3} DCMU in extraction medium (final concentration 0.05 mol m^{-3}), with an appropiate adjustment in the volume of extraction medium to maintain the 1 cm^3 final reaction volume. The chloroplast suspensions were preincubated in the dark for 5 min at 10 °C in the presence or absence of DCMU, prior to exposure to the LH7 red (660 nm) actinic light of 400 μmol m^{-2} s^{-1} intensity (which triggered the record cycle of the TR1 transient recorder).

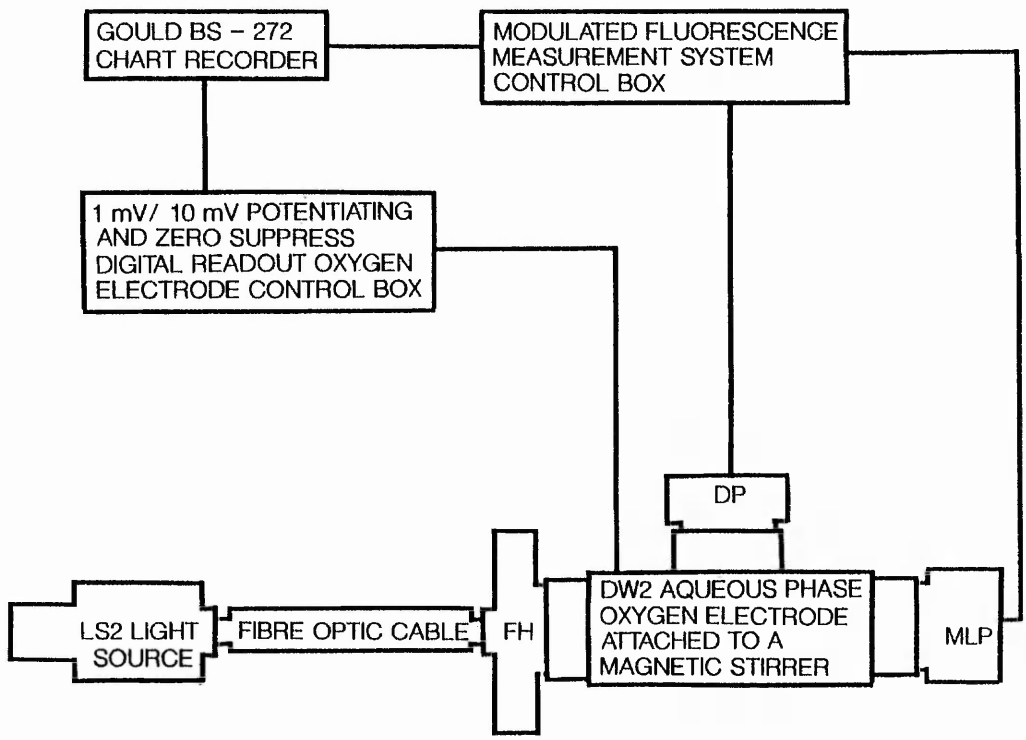
Initially, the TR1 was set to give a record time of 60 msec (highest record speed) and the recorded fluorescence signal was replayed with a replay time of 10 min (whole signal not recorded), enabling measurement of F_o (Figure 5.4A in Results section 5.3). The same fluorescence induction signal was also replayed with a replay time of 1 min and the whole signal recorded, enabling the measurement of the fluorescence level reached after 60 msec. However, F_p was not obtained in 60 msec. This was repeated

in the presence of DCMU and under these conditions F_m was obtained within 60 msec, enabling the measurement of F_o, F_m, F_v/F_m and $t_{1/2}$ (Figure 5.4B). To further charactrerise the fluorescence induction curve the procedure described above was repeated with the longer TR1 record times of 6 sec and 60 sec (Figures 5.5 and 5.6, respectively, in Results section 5.3). The fluorescence signals recorded at each record time were again replayed with replay times of 1 and 10 min. It was not possible to determine F_o at these record speeds. However, F_p (or F_m in the presence of DCMU) could be determined (Figures 5.5 and 5.6). The mean F_o values determined at the previous record speed (60 msec) were used to estimate F_v/F_m and $t_{1/2}$. Finally, the fluorescence induction was followed for 10 min (Figures 5.7 A and B in Results section 5.3) using the FDC (as the maximum record time with the TR1 is only 1 min), enabling the measurement of F_p (or F_m in the presence of DCMU) and oxygen exchange (traces not shown).

5.2.8 Experimental Apparatus used for the Study of the Slow Chlorophyll Fluorescence Induction Kinetics

The DW2 oxygen electrode was set up to enable oxygen exchange measurements (5.2.3) as required and the remaining apparatus was assembled, according to the manufacturer's instructions, to allow measurement of the slow chlorophyll fluorescence induction kinetics, as illustrated in Figure 5.3. A white actinic light was provided by the LS2 tungsten - halogen light source via a fibre optic cable (500 x 13 mm active diameter) and a 50 x 50 mm filter holder, which was inserted into one port of the DW2 reaction chamber. The filter holder enabled the use of a combination of neutral density filters allowing control of the PPFD. The modulated light probe (MLP) of the Hansatech modulated fluorescence measurement system (MFMS) contains an array of 7 high intensity yellow LEDs (centre wavelength 583 nm, half power bandwidth 36 nm) of squarewave modulation frequency 870 Hz, fitted with a low pass 620 nm cut - off optical filter (Ealing Beck 35 - 5404). The detector probe consists of a photodiode with a frequency selective amplifier housed in an aluminium casing to provide screening from electrical interference, protected by a 700 nm interference filter. The control box contains the modulation and drive circuits for the light probe and the amplifier and demodulator circuits for the photodetector, which provide an output to the digital display and the chart - recorder socket. The modulated light probe housing was placed

Figure 5.3 Diagrammatic representation of the experimental system used in the study of the slow chlorophyll fluorescence induction kinetics. Key: LS2, light source providing white actinic light via a fibre optic cable; FH, filter holder for 50 x 50 mm filters; DW2, modified version of the DW1 oxygen electrode, enabling the simultaneous measurement of oxygen exchange and fluorescence in the aqueous phase; MLP, modulated light probe containing 7 high intensity yellow LEDs (centre wavelength 583 nm, half - power bandwidth 36 nm) of squarewave modulation frequency 870 Hz, fitted with a low - pass 620 nm cut - off optical filter (Ealing Beck 35 - 5404); DP, detector probe consisting of a photodiode with a frequency selective amplifier housed in an aluminium casing to provide screening from electrical interference, protected by a 700 nm interference filter. The control box contains the modulation and drive circuits for the light probe and the amplifier and demodulator circuits for the photodetector, which provide an output to a digital display and a chart recorder. See text for details.


GOULD BS – 272
CHART RECORDER
MODULATED FLUORESCENCE
MEASUREMENT SYSTEM
CONTROL BOX
1 mV/ 10 mV POTENTIATING
AND ZERO SUPPRESS
DIGITAL READOUT OXYGEN
ELECTRODE CONTROL BOX
DP
LS2 LIGHT
SOURCE
FIBRE OPTIC CABLE
FH
DW2 AQUEOUS PHASE
OXYGEN ELECTRODE
ATTACHED TO A
MAGNETIC STIRRER
MLP

in a second port of the DW2 chamber and was plugged into the three pin socket on the front panel of the MFMS. The rotary 'intensity' control was used to set the modulated beam to 0.5 μmol m^{-2} s^{-1}, which was found to be optimum in initial experiments with respect to F_o to F_p levels. The detector probe was placed in a third port of the DW2 electrode reaction chamber and was plugged into the five pin socket on the front panel of the MFMS. One channel of a two pen chart recorder was connected to the BNC socket 'rec o/p' on the front panel of the MFMS, to enable the fluorescence signal to be recorded on the chart recorder (set on the 500 mV scale for the fluorescence signal and generally at a chart speed of 30 cm/ hr or 30 cm/ min). The MFMS 'response' switch was set to 'slow' and the 'reference' switch was set to 'internal'. The fourth port of the DW2 reaction chamber remained plugged.

5.2.9 Measurement of the Slow Chlorophyll Fluorescence Induction Kinetics in Chloroplasts Isolated from *C. fragile*

The slow chlorophyll fluorescence induction kinetics in isolated chloroplasts were investigated using the DCMU addition technique of Krause, *et al.* (1982), to resolve q_Q and q_E quenching mechanisms throughout the induction curve. A stirred reaction volume was used containing 100 μl of 100 mol m^{-3} $NaHCO_3$ in extraction medium (final concentration 10 mol m^{-3}), a volume of chloroplast suspension to give a final chlorophyll concentration of 50 μg cm^{-3}, with the final volume made up to 1 cm^3 with extraction medium. The stirred chloroplast suspensions were preincubated for 5 min in the 'dark' (modulated beam on) at 10 °C prior to exposure to white actinic light of 11.5, 28.5, 45.0, 63.0, 100.0, 250.0 or 1000.0 μmol m^{-2} s^{-1} PPFD. The fluorescence transient and oxygen evolution were followed for up to 6 min at a chart speed of 30 cm hr^{-1}. At 0, 40, 80, 120, 160, 200, 240, 280, 320 or 360 sec after the actinic light was switched on 10 μl of 5 mol m^{-3} DCMU (final concentration approximately 0.05 mol m^{-3}) was added through the central hole of the DW2 'plunger'. Just before addition of the DCMU the chart speed was changed to 30 cm min^{-1} (Figures 5.8 A, B and 5.9C in Results section 5.3). Addition of DCMU before the actinic light was switched on (*i.e.* time = 0 sec) enabled determination of F_m. In the absence of DCMU the fluorescence level obtained immediately upon illumination of a dark adapted sample was designated F_p. Where possible, the levels of fluorescence after the DCMU - induced rise (FQ and FE) and the remaining quenching, FR, were also determined (Figure

5.9C).

5.2.10 Photoinhibitory Pretreatment and the Slow Chlorophyll Fluorescence Induction Kinetics in Chloroplasts and Thylakoids Isolated from *C. fragile*

A stirred 2.5 cm^3 volume of chloroplast or thylakoid preparation (equivalent to 50 μg cm^{-3} chlorophyll) containing 250 μl of 100 mol m^{-3} $NaHCO_3$ or 250 μl of 75 mol m^{-3} $K_3Fe(CN)_6$ (see 5.2.3) in extraction medium (final concentrations 10 or 7.5 mol m^{-3}), was incubated at 10 °C in a DW1 oxygen electrode reaction chamber at 1000 μmol m^{-2} s^{-1} PPFD provided by a Halight 24/ 250 tungsten - halogen projector for up to 25 min. At 0, 5, 10, 15, 20 or 25 min a 1 cm^3 volume of the chloroplast or thylakoid suspension was removed and transferred to a DW2 oxygen electrode for simultaneous measurement of bicarbonate - or ferricyanide - dependent oxygen exchange and fluorescence transients. In the DW2 electrode the oxygen exchange was followed in the 'dark' (modulated beam on) for 5 min, after which the electrode chamber was illuminated at 100 μmol m^{-2} s^{-1} PPFD, provided by the LS2 tungsten - halogen light source via a fibre optic, and the fluorescence and bicarbonate - or ferricyanide - dependent oxygen evolution recorded for 6 min. After 6 min 10 μl of 5 mol m^{-3} DCMU in extraction medium (final concentration approximately 0.05 mol m^{-3}) was added through the central hole of the DW2 'plunger'. Over the first 6 min the chart recorder was set at 30 cm hr^{-1}; just before addition of the DCMU the chart speed was changed to 30 cm min^{-1} (Figure 5.14 in Results section 5.3). The fluorescence level obtained immediately upon illumination of a dark adapted sample was designated F_p. Where possible, the levels of fluorescence after the DCMU - induced rise (FQ and FE) and the remaining quenching, q_R, were also determined (Figure 5.9C).

5.3 Results

As there is no published information concerning fluorescence induction in chloroplasts isolated from *C. fragile* it was necessary to characterise such transients in this alga. An initial study investigated the early events of chlorophyll fluorescence induction using chloroplast suspensions equivalent to 25 μg cm^{-3} chlorophyll and facsimiles of typical fluorescence traces are illustrated in Figures 5.4 to 5.7. With a TR1 record time of 60 msec and a replay time of 10 min the F_o level could be determined (Figure 5.4). However, the peak fluorescence level, F_p, did not appear to

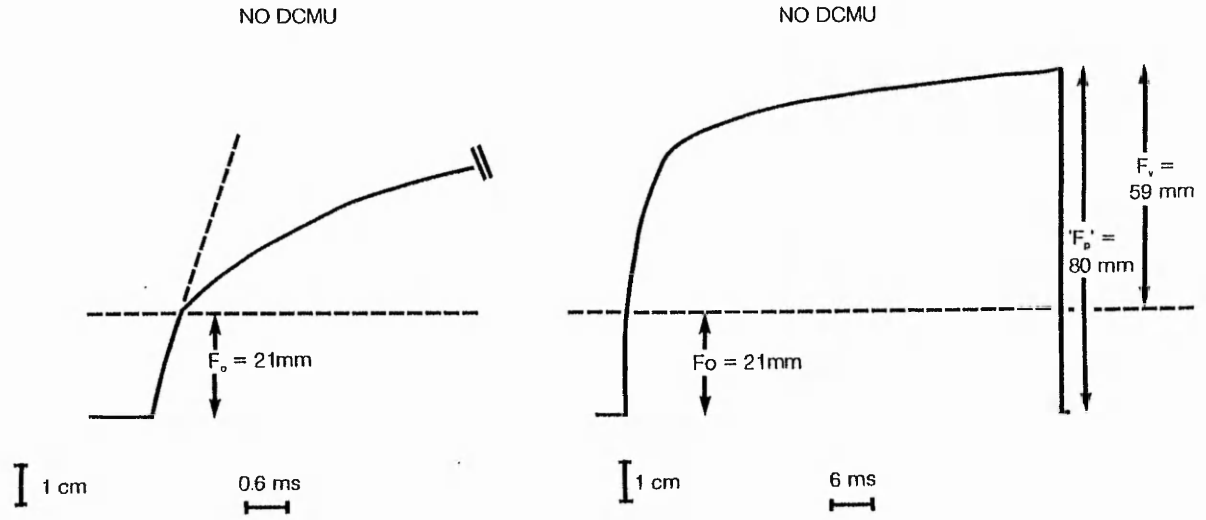
Figure 5.4 Typical traces obtained when measuring the early events of chlorophyll fluorescence induction in the absence (A) and presence (B) of 0.05 mol m^{-3} DCMU. Chloroplast suspensions equivalent to 25 μg cm^{-3} chlorophyll in a reaction mixture containing 10 mol m^{-3} $NaHCO_3$ were preincubated in the dark for 5 min at 10 °C, prior to exposure to red actinic light (660 nm) of 400 μmol m^{-2} s^{-1} intensity. The TR1 transient recorder was set to record the first 60 msec of the fluorescence signal which was replayed with a TR1 replay time of 1 or 10 min. Key: F_o, fluorescence intensity of dark adapted sample (when all PSII reaction centres are open); F_p, peak fluorescence intensity obtained with dark adapted sample upon application of actinic light (not obtained within 60 msec); F_m, maximum fluorescence intensity obtained with dark adapted sample in the presence of DCMU upon application of actinic light (when all PSII reaction centres are closed); F_v, variable fluorescence; A_{DCMU}, area over the fluorescence induction curve in the presence of DCMU; $t_{1/2}$, one half of the time required for the rise in fluorescence from F_o to P (the minimal and peak fluorescence levels respectively). See text for details (section 5.2.7 for experimental details).

A

Chart recorder set at 10 cm/ min (2 V scale)

TR1 Transient recorder settings:
Record time = 60 ms
Replay time = 10 min

TR1 Transient recorder settings:
Record time = 60 ms
Replay time = 1 min



B

Chart recorder set at 10 cm/ min (2 V scale)

TR1 Transient Recorder settings:
Record time = 60 ms
Replay time = 10 min

TR1 Transient recorder settings:
Record time = 60 ms
Replay time = 1 min

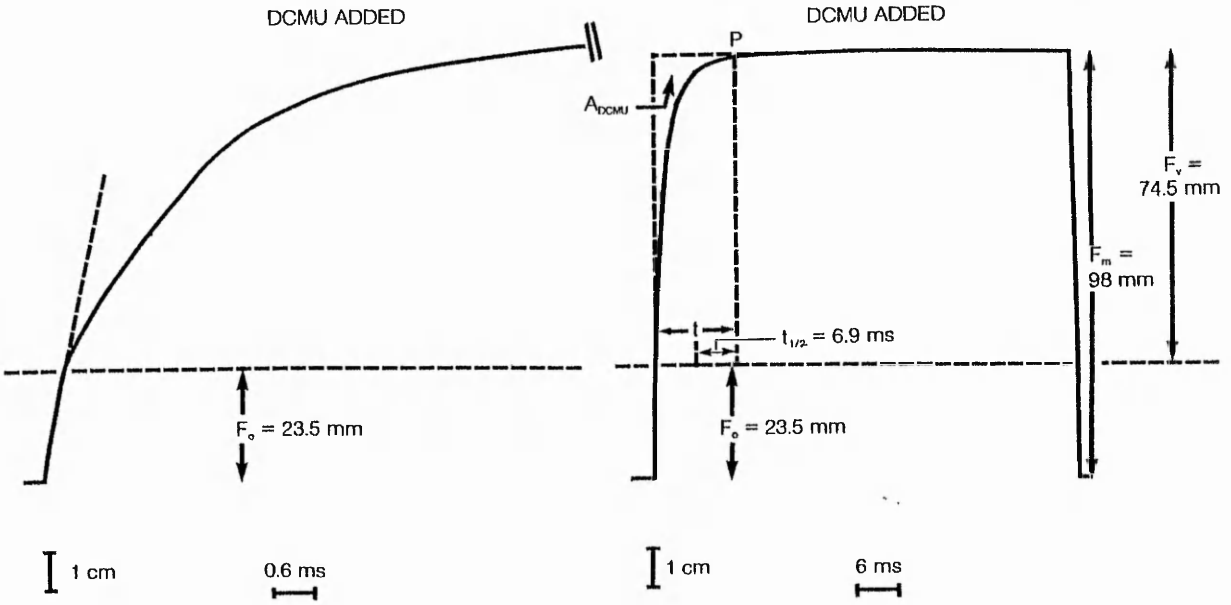


Figure 5.5 Typical traces obtained when measuring the early events of chlorophyll fluorescence induction in the absence (A) and presence (B) of 0.05 mol m^{-3} DCMU. Chloroplast suspensions equivalent to 25 μg cm^{-3} chlorophyll in a reaction mixture containing 10 mol m^{-3} $NaHCO_3$ were preincubated in the dark for 5 min at 10 °C, prior to exposure to red actinic light (660 nm) of 400 μmol m^{-2} s^{-1} intensity. The TR1 transient recorder was set to record the first 6 sec of the fluorescence signal which was replayed with a TR1 replay time of 1 or 10 min. The mean F_o value measured with a record time of 60 msec (Figure 5.4) is shown. The peak fluorescence level, F_p, was obtained within the 6 sec record time. Key: F_o, fluorescence intensity of dark adapted sample (when all PSII reaction centres are open); F_p, peak fluorescence intensity obtained with dark adapted sample upon application of actinic light ; F_m, maximum fluorescence intensity obtained with dark adapted sample in the presence of DCMU upon application of actinic light (when all PSII reaction centres are closed); F_v, variable fluorescence. See text for details (section 5.2.7 for experimental details).

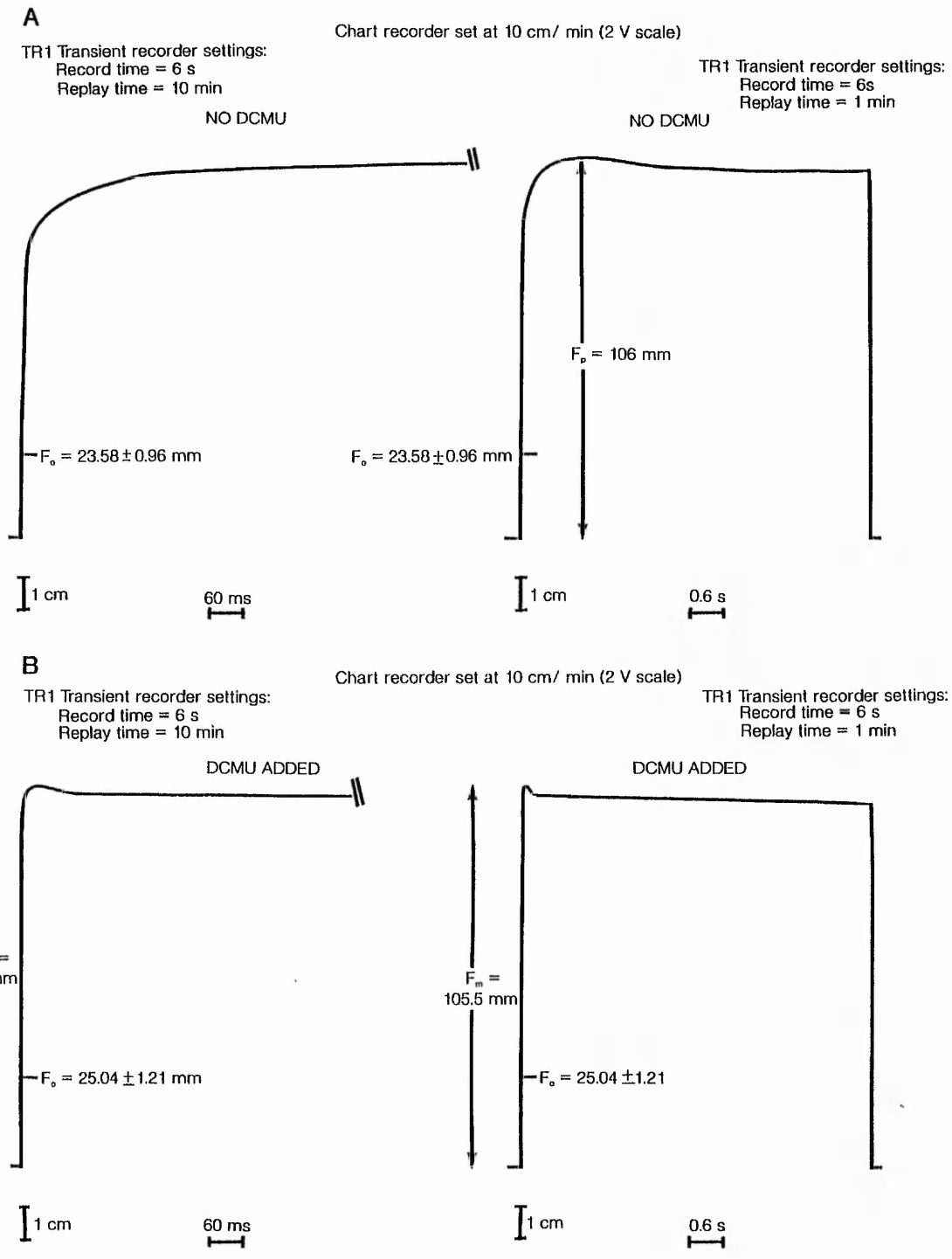

A
Chart recorder set at 10 cm/ min (2 V scale)
TR1 Transient recorder settings:
Record time = 6 s
Replay time = 10 min
NO DCMU
F_o = 23.58 ± 0.96 mm
1 cm
60 ms
TR1 Transient recorder settings:
Record time = 6s
Replay time = 1 min
NO DCMU
F_p = 106 mm
F_o = 23.58 ± 0.96 mm
1 cm
0.6 s
B
Chart recorder set at 10 cm/ min (2 V scale)
TR1 Transient recorder settings:
Record time = 6 s
Replay time = 10 min
DCMU ADDED
F_m = 105.5 mm
F_o = 25.04 ± 1.21 mm
1 cm
60 ms
TR1 Transient recorder settings:
Record time = 6 s
Replay time = 1 min
DCMU ADDED
F_m = 105.5 mm
F_o = 25.04 ± 1.21
1 cm
0.6 s

Figure 5.6 Typical traces obtained when measuring the early events of chlorophyll fluorescence induction in the absence (A) and presence (B) of 0.05 mol m^{-3} DCMU. Chloroplast suspensions equivalent to 25 μg cm^{-3} chlorophyll in a reaction mixture containing 10 mol m^{-3} $NaHCO_3$ were preincubated in the dark for 5 min at 10 °C, prior to exposure to red actinic light (660 nm) of 400 μmol m^{-2} s^{-1} intensity. The TR1 transient recorder was set to record the first 60 sec of the fluorescence signal which was replayed with a TR1 replay time of 1 or 10 min. The mean F_o value measured with a record time of 60 msec (Figure 5.4) is shown. The peak fluorescence level, F_p, was obtained within the 6 sec record time. Key: F_o, fluorescence intensity of dark adapted sample (when all PSII reaction centres are open); F_p, peak fluorescence intensity obtained with dark adapted sample upon application of actinic light ; F_m, maximum fluorescence intensity obtained with dark adapted sample in the presence of DCMU upon application of actinic light (when all PSII reaction centres are closed); F_v, variable fluorescence. See text for details (section 5.2.7 for experimental details).

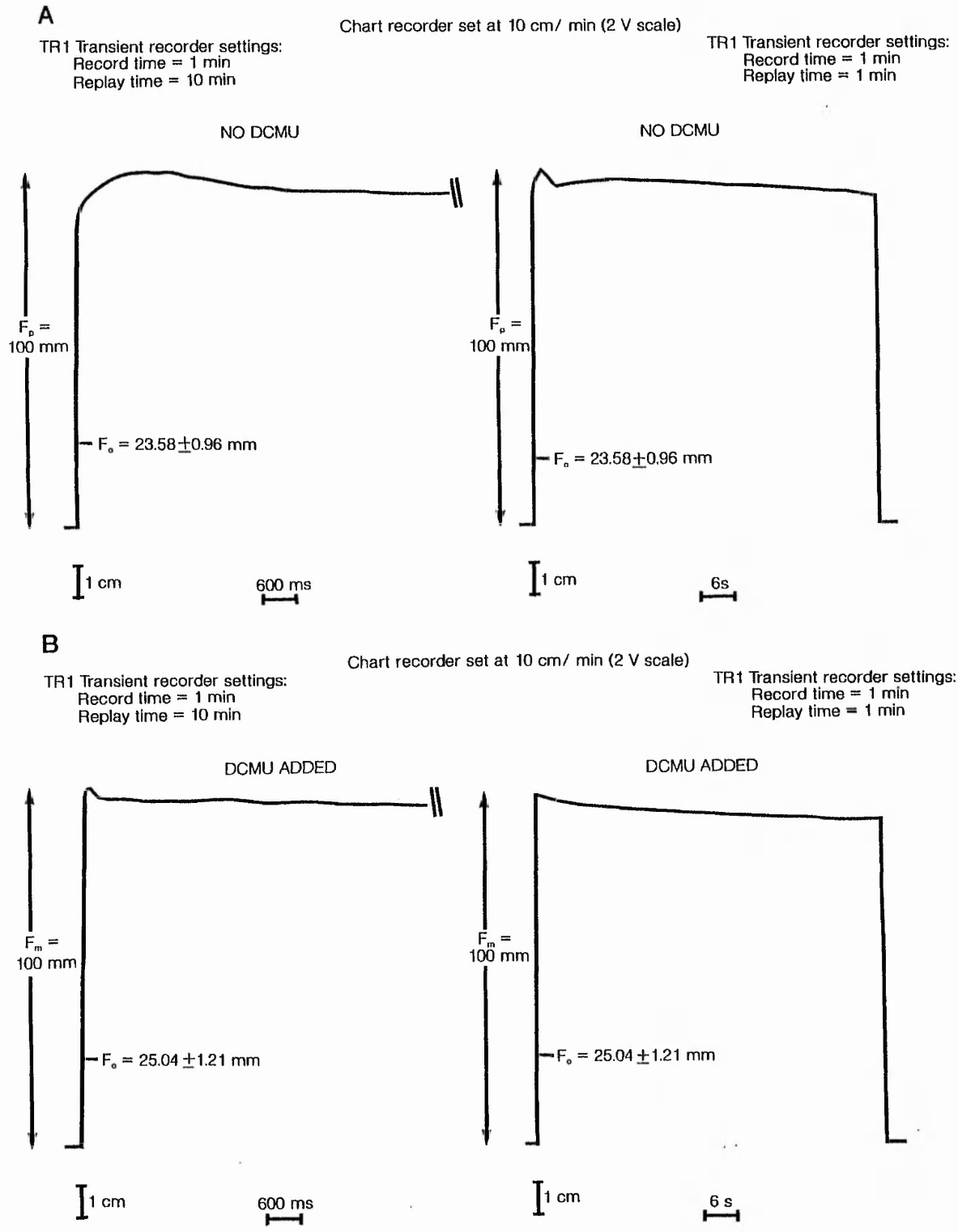
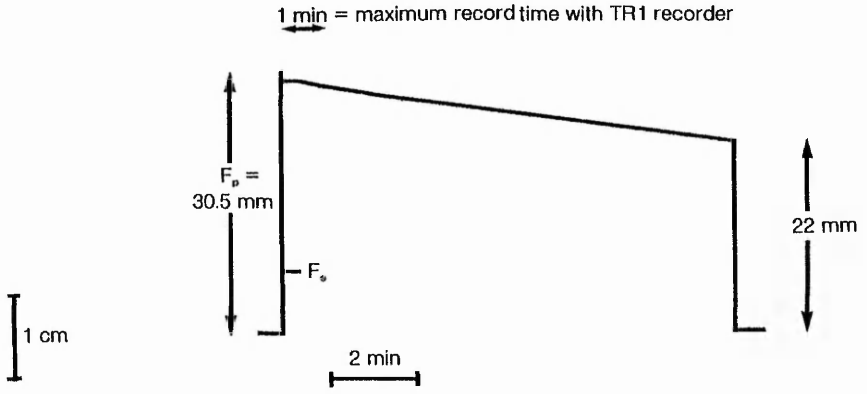

A
Chart recorder set at 10 cm/ min (2 V scale)
TR1 Transient recorder settings:
Record time = 1 min
Replay time = 10 min
TR1 Transient recorder settings:
Record time = 1 min
Replay time = 1 min
NO DCMU
NO DCMU
F_o = 100 mm
F_o = 23.58±0.96 mm
F_o = 100 mm
F_o = 23.58±0.96 mm
1 cm
600 ms
1 cm
6s
B
Chart recorder set at 10 cm/ min (2 V scale)
TR1 Transient recorder settings:
Record time = 1 min
Replay time = 10 min
TR1 Transient recorder settings:
Record time = 1 min
Replay time = 1 min
DCMU ADDED
DCMU ADDED
F_m = 100 mm
F_o = 25.04±1.21 mm
F_m = 100 mm
F_o = 25.04±1.21 mm
1 cm
600 ms
1 cm
6 s

Figure 5.7 Typical traces obtained when measuring chlorophyll fluorescence induction in the absence (A) and presence (B) of 0.05 mol m^{-3} DCMU. Chloroplast suspensions equivalent to 25 μg cm^{-3} chlorophyll in a reaction mixture containing 10 mol m^{-3} $NaHCO_3$ were preincubated in the dark for 5 min at 10 °C, prior to exposure to red actinic light (660 nm) of 400 μmol m^{-2} s^{-1} intensity. The FDC was used to record the first 10 min of the fluorescence signal, enabling simultaneous measurement of O_2 evolution. The mean F_o measured with the TR1 with a record time of 60 msec (Figure 5.4) was used to estimate the F_o level of the fluorescence signal obtained with the FDC. (C) The fluorescence trace of (A) adjusted to enable comparison with a typical induction curve obtained with *Pisum sativum* chloroplasts dark adapted for 5 min and then exposed to 100 μmol m^{-2} s^{-1} broad band blue irradiation (from Baker and Horton, 1987). Key: F_o, fluorescence intensity of dark adapted sample (when all PSII reaction centres are open); F_p, peak fluorescence intensity obtained with dark adapted sample upon application of actinic light; F_m, maximum fluorescence intensity obtained with dark adapted sample in the presence of DCMU upon application of actinic light (when all PSII reaction centres are closed); F_v, variable fluorescence. See text for details (section 5.2.7 for experimental details).

A

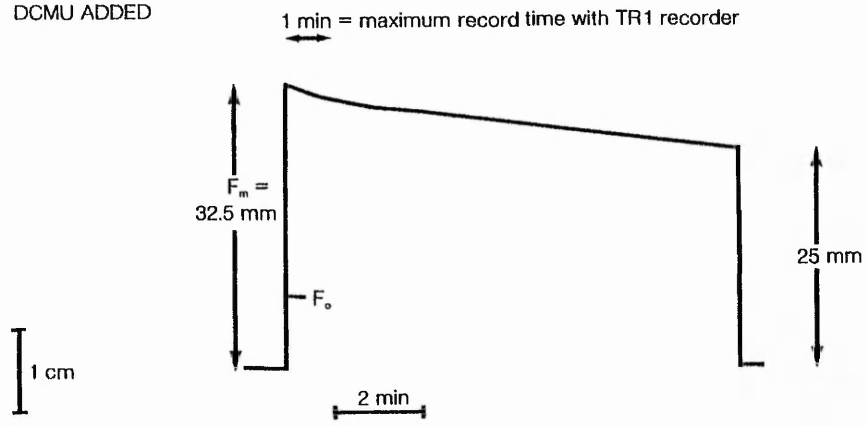
NO DCMU

FDC fluorescence detector used
Chart rcorder set at 30 cm/ hr
Fluorescence – 500 mV scale
Oxygen – 10 mV scale

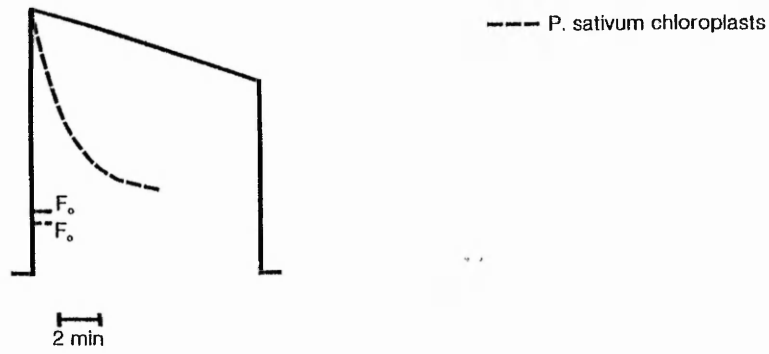


B

DCMU ADDED



C



be obtained within 60 msec, as the fluorescence signal was still increasing at the end of this record time (Figure 5.4A). In the presence of DCMU (Figure 5.4B) the peak fluorescence level, F_m, did appear to be obtained, as the fluorescence signal had finished rising after 60 msec, enabling the determination of $t_{1/2}$ and F_v (F_m - F_o). With a record time of 6 sec F_p (Figure 5.5A) and F_m (Figure 5.5B) could be determined and it was evident that there was little quenching from the peak fluorescence level within this time period. It was also possible to measure F_p and F_m using a record time of one min (Figure 5.6) and again it was apparent that there was little quenching from the peak fluorescence level over the first minute of the induction curve. The TR1 has a maximum record time of 1 min. To record the fluorescence induction for longer time periods it was thus necessary to use the FDC (Figure 5.7). It was found that a chart recorder setting of 500 mV was optimal, but the fluorescence signal observed was still much reduced when compared to that obtained with the TR1, making it difficult to compare the fluorescence transients obtained with the two different recorders. There was some quenching of fluorescence from the peak value over the first 10 min of induction. However, the amount of quenching was much less and slower than that typically observed in other species (Figure 5.7C). Following the fluorescence induction for 10 min using the FDC enabled the measurement of chloroplast oxygen evolution under the red (660 nm) actinic light of 400 μmol m^{-2} s^{-1} intensity. Thus, using a record time of 60 msec the F_o level was determined, whilst a record time of 6 sec or 60 sec enabled the determination of F_p and F_m. Using the mean F_o level measured at 60 msec in conjuction with the F_p and F_m values measured at 6 or 60 sec the values of F_v/F_p (F_p - F_o/ F_p), F_v/F_m (F_m - F_o/F_m) and $t_{1/2}$ were determined. The results obtained in this initial investigation are summarised in Table 5.1. It is clear that the F_o level is the same in the presence or absence of DCMU, as is the peak fluorescence level (*i.e.* F_p is equal to F_m) under the conditions of this study, and hence F_v/F_p (0.7687) is essentially equal to F_v/F_m (0.7513). The value of $t_{1/2}$ is much greater in the absence of DCMU (495 msec) than in the presence of DCMU (7 msec).

In further experiments the slow fluorescence induction kinetics were investigated using the MFMS, enabling the use of white actinic light and the simultaneous measurement of oxygen exchange. Initially, the conditions required to produce the highest F_v/F_p ratios were established. Chloroplast suspensions equivalent to 10, 25, 50, 100, 150 and 200 μg cm^{-3} chlorophyll were used with white actinic light of 25, 50, 65,

Table 5.1 Summary of the values of the fluorescence parameters F_o, F_p, F_m, F_v/F_p (F_p - F_o/ F_p), F_v/F_m (F_m - F_o/ F_m) and $t_{1/2}$ (msec) and net photosynthesis (μmoles O_2 hr^{-1} mg^{-1} chl) of chloroplasts isolated from *C. fragile* frond tips, determined with red actinic light (660 nm) of 400 μmol m^{-2} s^{-1} intensity. See Figures 5.4 to 5.7 and text for details (section 5.2.7 for experimental details).

Parameter	Without DCMU (n)	With DCMU (n)
F_o (mm)	23.58 ± 0.96 (12)	25.04 ± 1.21 (12)
F_p (mm)	101.96 ± 1.39 (14)	_
F_m (mm)	_	100.70 ± 1.21 (25)
F_v/F_p	0.7687	_
F_v/F_m	_	0.7513
$t_{1/2}$ (msec)	495.00 ± 24.88 (13)	7.00 ± 0.49 (19)
Net PS	2.42 ± 0.58 (7)	0.00 ± 0.00 (7)

100, 250, 700 and 1000 μmol m^{-2} s^{-1} PPFD, and modulated beam intensities of 0.5, 2.0, 4.0, 6.0 and 8.5 μmol m^{-2} s^{-1}. The voltage scale of the channel of the chart recorder receiving the fluorescence signal was set at 0.5, 1.0, 2.0, 5.0 or 10 volts. It was found that the highest F_v/F_p ratios were obtained with a chloroplast suspension equivalent to 50 μg cm^{-3} chlorophyll, a modulated beam intensity of 0.5 μmol m^{-2} s^{-1} and a chart recorder voltage scale of 500 mV. These three variables were maintained at these values throughout the work carried out using the MFMS. Facsimiles of typical fluorescence and oxygen exchange traces obtained with white actinic light of 100 and 1000 μmol m^{-2} s^{-1} PPFD are illustrated in Figures 5.8A and 5.8B, respectively. The level of fluorescence in the presence of the modulated beam is analagous to F_o. It is clear that, despite the optimisation of conditions for the highest F_v/F_p ratios, the amount of variable fluorescence (F_p - F_o) relative to the minimal fluorescence level F_o is low. The F_o level (22 mm) is greater than F_v (16 mm) at 100 μmol m^{-2} s^{-1} PPFD (Figure 5.8A) and only slightly lower (23 mm) than F_v (24 mm) at 1000 μmol m^{-2} s^{-1} PPFD (Figure 5.8B). This represents F_v/F_p ratios of 0.4211 and 0.5106 at 100 and 1000 μmol m^{-2} s^{-1} PPFD actinic light, respectively. There was no quenching from F_p at light intensities of 100 μmol m^{-2} s^{-1} PPFD or lower. Some quenching from F_p was sometimes observed at light intensities of 250 and 1000 μmol m^{-2} s^{-1} PPFD. Addition of DCMU at the steady state (6 min) caused an increase in the fluorescence signal that appeared to be biphasic, at most light intensities. However, at 1000 μmol m^{-2} s^{-1} PPFD, DCMU addition often caused no increase in fluorescence signal. A typical fluorescence trace obtained using the MFMS in conjunction with an LD2 leaf disc electrode (Figure 2.1) and whole frond tips is illustrated in Figure 5.8C for comparison. The F_p level (60 mm) is more than three times the F_o level (17 mm) and represents an F_v/F_p ratio 0.7167. Furthermore, significant quenching from F_p occurred within one min of the induction curve.

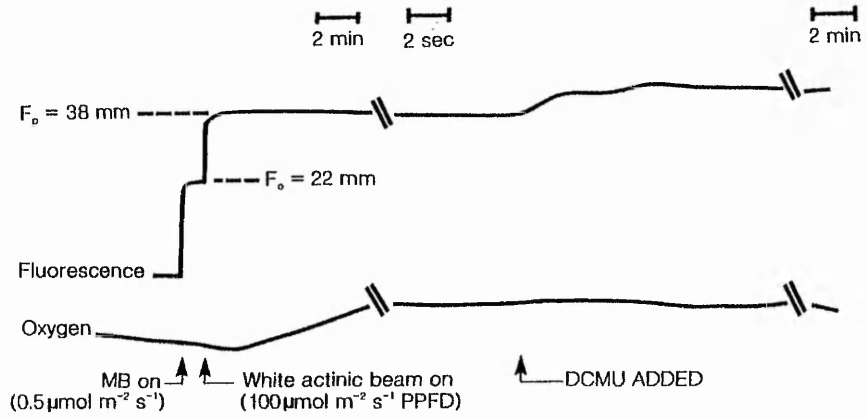
The fluorescence response obtained with isolated *C. fragile* chloroplasts using the MFMS (Figure 5.8) is compared with that obtained with *Hordeum vulgare* protoplasts and *Phaseolus vulgaris* leaves, using similar techniques, via diagrammatic representation of the induction curves, in Figure 5.9. The amount of variable fluorescence, that is, the rise from F_o to the peak fluorescence level is much lower in *C. fragile* than in other species studied. That is, the F_o fluorescence level is a much greater proportion of the total fluorescence yield in *C. fragile*. It is clear that there is

Figure 5.8 Typical traces obtained when measuring the slow chlorophyll fluorescence induction kinetics with the MFMS, with an actinic light of 100 (A) and 1000 (B) μmol m^{-2} s^{-1} PPFD. Chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll in a reaction mixture containing 10 mol m^{-3} $NaHCO_3$ were preincubated in the 'dark' (in the presence of the modulated beam (MB) of 0.5 μmol m^{-2} s^{-1} intensity) for 5 min at 10 °C prior to exposure to white actinic light of 11.5 to 1000 μmol m^{-2} s^{-1} PPFD. The fluorescence transient and oxygen evolution were followed for up to 6 min (steady state). DCMU (0.05 mol m^{-3}) was added at various times after application of the actinic beam, AB (generally after 6 min). See Figure 5.9 and text for details of the definition of the fluorescence parameters and section 5.2.9 for experimental details. A typical trace obtained with whole fronds in an LD2 leaf disc electrode (Figure 2.1) is also shown for comparison (C).

A

Chart recorder set at 10 mV scale (oxygen evolution) and 500 mV scale (fluorescence)

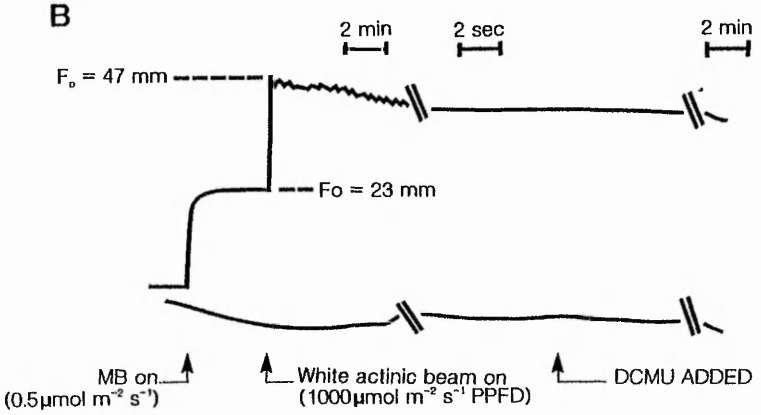
$F_v/F_p = F_p - F_o / F_p = 0.4211$



0·5 mV - OXYGEN
2·5 mV - FLUORESCENCE

B

$F_v/F_p = F_p - F_o/F_p = 0.5106$

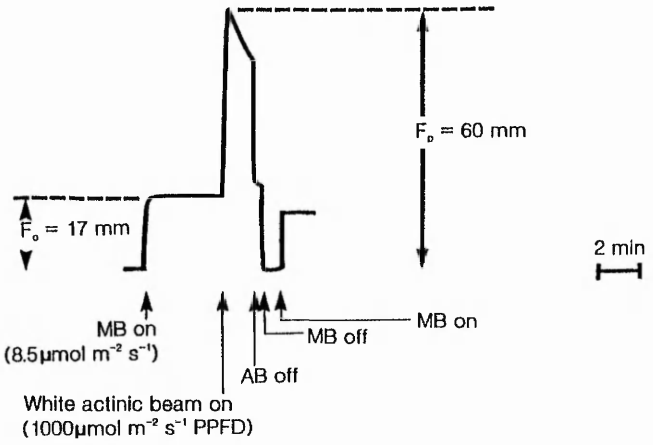


0·5 mV - OXYGEN
2·5 mV - FLUORESCENCE

C

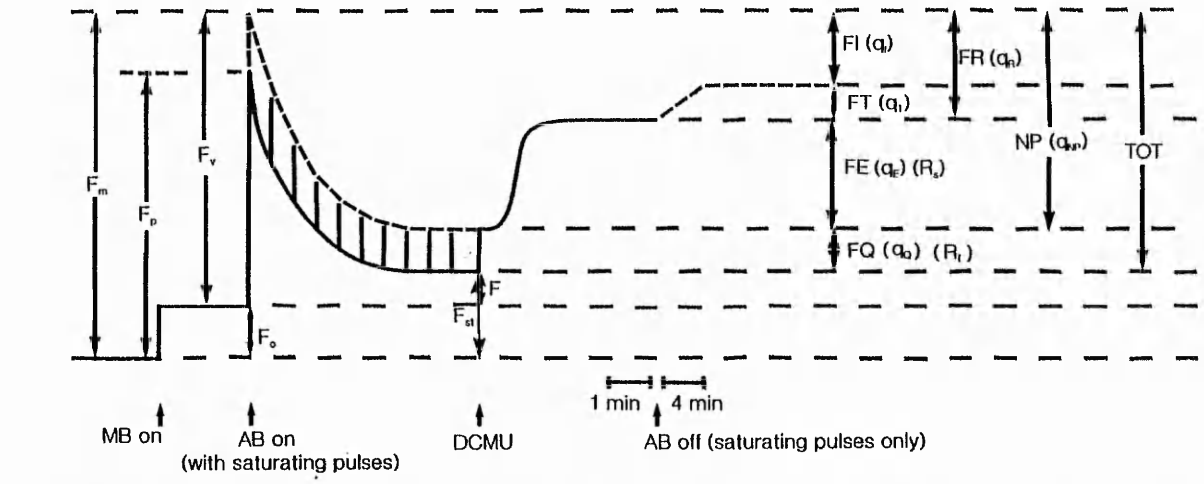
Chart recorder set at 1 V scale for fluorescence

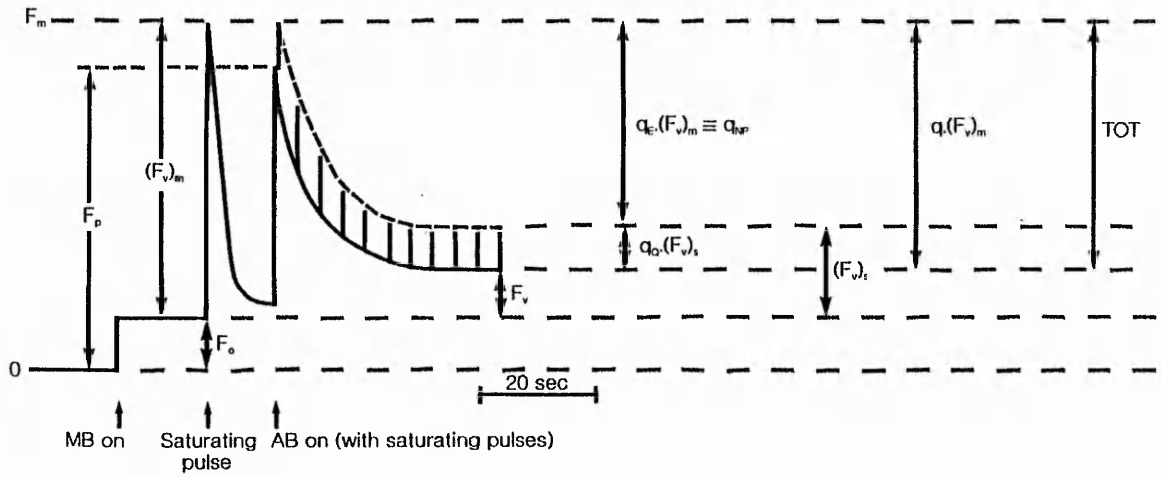
$F_v/F_p = F_p - F_o/F_p = 0.7167$

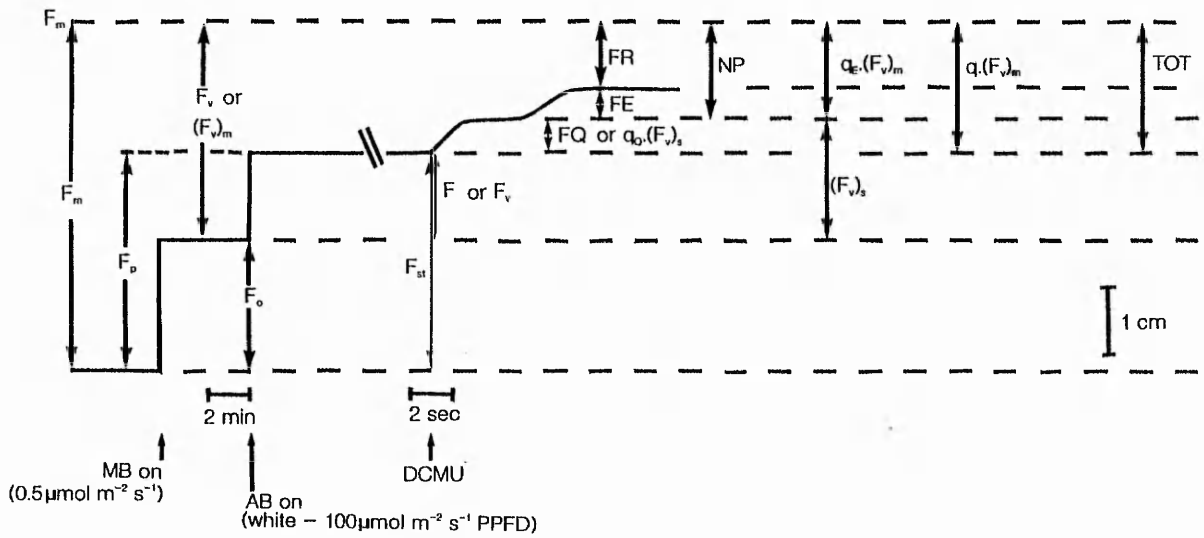


0·5mV - OXYGEN
2·5mV - FLUORESCENCE

Figure 5.9 Definition of quenching coefficients and terminology of characteristic fluorescence parameters; comparison of fluorescence transients obtained in this study and those typically reported by other workers. (A) Typical chlorophyll fluorescence yield in barley (*Hordeum vulgare* L. cv Marko) protoplasts illuminated with an actinic beam (AB) of approximately 1400 μmol m^{-2} s^{-1} PPFD. At 7 sec intervals saturating pulses of 1 sec duration (-——) of 1800 μmol m^{-2} s^{-1} PPFD were given and at steady state 10 mmol m^{-3} DCMU added. After 4 min, the constant beam was turned off and pulsing continued for a further 15 min. Adapted from Horton and Hague, 1988. (B) Typical chlorophyll fluorescence yield in *Phaseolus vulgaris* leaves illuminated with an actinic beam (AB) of 20 Wm^{-2}, with saturating light pulses of 2000 Wm^{-2} of 300 msec duration. Adapted from Schreiber, *et al.*, 1986. (C) Typical fluorescence yield in *C. fragile* chloroplasts illuminated with an actinic beam (AB) of 100 μmol m^{-2} s^{-1} PPFD, using the MFMS. At steady state (6 min after application of the AB) 0.05 mol m^{-3} DCMU was added. See Figure 5.8 for a facsimile of a typical fluorescence trace obtained with *C. fragile* chloroplasts and text for details of the definition of the fluorescence parameters shown.


Fm
Fp
Fv
Fo
F
Fst
FI (qI)
FR (qR)
FT (qT)
FE (qE) (Rd)
NP (qNP)
TOT
FQ (qQ) (Rt)
1 min
4 min
MB on
AB on
(with saturating pulses)
DCMU
AB off (saturating pulses only)


Fm
Fp
(Fv)m
Fo
0
Fv
qE.(Fv)m ≡ qNP
qt.(Fv)m
TOT
qQ.(Fv)s
(Fv)s
20 sec
MB on
Saturating
pulse
AB on (with saturating pulses)


Fm
Fv or
(Fv)m
Fp
Fo
Fst
F or Fv
FR
FE
FQ or qQ.(Fv)s
NP
qE.(Fv)m
qt.(Fv)m
TOT
(Fv)s
1 cm
2 min
2 sec
MB on
(0.5 µmol m-2 s-1)
AB on
(white – 100 µmol m-2 s-1 PPFD)
DCMU

little, if any, quenching from F_p after 6 min of illumination. In contrast, isolated chloroplasts, protoplasts and leaves of other species exhibit marked quenching from the F_p level. The time required for such quenching varies, but is often less than a minute and certainly less than 6 min. Thus, the fluorescence level observed in this study is different from that generally described. It was hoped that DCMU addition would enable a study of the fast and slow relaxation of fluorescence quenching, indicating the contribution of the q_Q and q_E mechanisms respectively and hence the amount of remaining quenching, q_R (Figure 5.9A). This would appear to be somewhat difficult in the absence of fluorescence quenching. However, it was found that despite the lack of quenching, the fluorescence level did increase in an apparently biphasic manner, on addition of DCMU. It is uncertain whether this increase in fluorescence level shares the underlying mechanisms of that observed by other workers upon the relaxation of marked fluorescence quenching. Nonetheless, the fluorescence increase upon DCMU addition repeatedly observed in this study may be measured as a means of further characterising the induction curve. The definition of quenching coefficients and terminology of characteristic fluorescence parameters, according to Horton and Hague (1988) and Schreiber, *et al.* (1986) can be seen in Figures 5.9A and 5.9B, respectively. Although the DCMU induced rise in chlorophyll fluorescence observed with *C. fragile* may not be due to the same underlying mechanisms, it is convenient and less confusing to adopt the same nomenclature as previous workers. It is necessary to define some of the terms used in Figure 5.9. F_o is the fluorescence intensity of a dark adapted sample in the presence of the modulated beam (MB) of negligible actinic intensity (fluorescence level when all PSII reaction centres are open). F_m is the maximum fluorescence intensity obtained with a dark adapted sample upon application of a saturating light pulse or, the fluorescence intensity obtained upon application of actinic light to a chloroplast dark adapted in the presence of DCMU (fluorescence level when all PSII reaction centres are closed). F_p is the peak fluorescence intensity obtained with a dark adapted sample upon application of the actinic beam (AB). The above terms are shared by Figures 5.9A, B and C.

The additional terms of Schreiber, *et al.* (1986) included in Figure 5.9B are defined as follows. $(F_v)_m$ is the maximal variable fluorescence of a dark adapted sample, F_v is the variable fluorescence at any given time during induction, whilst $(F_v)_s$ is the maximal variable fluorescence at any given time during induction. The measuring beam (MB)

is sufficiently weak not to induce a fluorescence increase beyond F_o. With a dark adapted sample, in which the energy dependent quenching is assumed to be zero, a saturating light pulse induces a maximal increase of variable fluorescence, $(F_v)_m$, corresponding to $q_Q = 0$ (100 % Q_A^-). In the following dark period the fluorescence decay reflects the reoxidation of Q_A^-. Upon application of continuous actinic light there is the typical change of variable fluorescence, F_v, which is suppressed relative to the maximal variable fluorescence, $(F_v)_m$, by the given amount of fluorescence quenching:

$$F_v = (F_v)_m - q(F_v)_m \qquad (1)$$

where q is the quenching coefficient which may vary between $q = 0$ (100% Q_A^-, $q_E = 0$) and $q = 1$ (full suppression of F_v). Following determination of saturated variable fluorescence, $(F_v)_s$, by saturating light pulses, it is possible to differentiate between the photochemical and non - photochemical parts of the total quenching:

$$F_v = (Fv)_s - q_Q(F_v)_s \qquad (2)$$

$$(F_v)_s = (F_v)_m - q_E(F_v)_m \qquad (3)$$

$$F_v = (F_v)_m - q_E(F_v)_m - q_Q(F_v)_s \qquad (4)$$

with $0 \leq q_Q \leq 1$, $0 \leq q_E \leq 1$.

By substituting $(F_v)_s$ in (4) by (3) it is apparently possible to obtain:

$$F_v = (1 - q_E)\,(1 - q_Q)(F_v)_m$$

It is then evident that the fluorescence yield is influenced by two independent factors. The following expressions may also be derived:

$$(1 - q_Q) = F_v / (F_v)_s \qquad (1 - q_E) = (F_v)_s / (F_v)_m$$

$$q_Q = (F_v)_s - F_v / (F_v)_s \qquad q_E = (F_v)_m - (F_v)_s / (F_v)_m$$

The nomenclature of Horton and Hague (1988) included in Figure 5.9A requires the following definition. F_v is the variable fluorescence and F is the observed steady state variable fluorescence. FQ is the fluorescence level reached after a saturaring light pulse or the fast DCMU -induced rise (sometimes referred to as the fast relaxation of fluorescence quenching, R_f) associated with photochemical quenching (q_Q). FE is the fluorescence level reached after the first slow DCMU - induced rise (sometimes referred to as the slow relaxation of fluorescence quenching, R_s) associated with energy - dependent quenching (q_E). FR is the difference between the fluorescence level F_m and the fluorescence level FE, which is neither q_Q nor q_E and is termed remaining quenching (q_R). FT is the fluorescence level reached after the second slow, NaF - sensitive, DCMU - induced rise, associated with a decrease in the proportion of excitation

transferred to PSII, a process determined by protein phosphorylation (q_T quenching or state transition dependent quenching). FI is the difference between the fluorescence level F_m and the fluorescence level FT, which is not q_Q, q_E or q_T and is termed irreversible quenching (q_I) and is associated with photoinhibition (*i.e.* photoinhibition - dependent quenching). NP is the difference between the fluorescence level F_m and the fluorescence level FQ, associated with non - photochemical quenching (q_{NP}) and TOT is the total quenching. Horton and Hague (1988) normalised the quenching amplitudes with respect to 'available' variable fluorescence, according to the principle described by Schreiber, *et al.* (1986). The sequential relaxation of quenching upon DCMU addition allows normalisation according to the formula, $q_X = \Delta FX/ (\Delta FX + FX')$, where X refers to the quenching process, ΔFX is the increase in amplitude of fluorescence due to the induced relaxation of quenching mechanism X, and FX' is the level of fluorescence reached after relaxation of the previous quencher minus F_o in the order F, FQ, FE and FT. Thus,

$$(1 - q_Q) = F/ (FQ + F)$$

$$(1 - q_E) = (FQ + F)/ (FE + FQ + F)$$

$$q_Q = FQ/ (FQ + F)$$

$$q_E = FE/ (FE + FQ + F)$$

Figure 5.9C illustrates the adaptation of the nomenclature of Schreiber, *et al.* (1986) and Horton and Hague (1988) to the typical fluorescence yield obtained with chloroplasts isolated from *C. fragile*. An additional term F_{st}, is the fluorescence level at steady state (F_o + F). The fluorescence yield obtained with *C. fragile* chloroplasts was normalised according to Horton and Hague (1988) as described above. Other terms were also calculated:

$$(1 - q_R) = (FQ + FE + F)/ (FR + FQ + FE + F)$$

$$(1 - q_{NP}) = (FQ + F)/ (FE + FR + FQ + F)$$

$$q_R = FR/ (FR + FQ + FE + F)$$

$$q_{NP} = (FE + FR)/ (FE + FR + FQ + F)$$

The relationship between actinic light PPFD and the fluorescence levels F_o, F_p, F_{st} and the ratio of F_v/F_p, together with net photosynthesis, may be seen in Figure 5.10. From the initial study using red (660 nm) actinic light of 400 μmol m^{-2} s^{-1} intensity it was found that F_p was equal to F_m and that F_v/F_p was equal to F_v/F_m. When using the MFMS, application of actinic light to a sample dark adapted in the presence of DCMU,

Figure 5.10 The relationship between the actinic PPFD (μmol m^{-2} s^{-1}), net photosynthesis, ● (μmoles O_2 hr^{-1} mg^{-1} chl) and the fluorescence parameters F_o (▲), F_p (■), F_v (○) and F_v/F_p (□) in relative fluorescence units, measured using the MFMS. The ratio F_v/F_p is equivalent to F_p - F_o/ F_p. Chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll in a reaction mixture containing 10 mol m^{-3} $NaHCO_3$ were preincubated in the 'dark' (in the presence of the modulated beam (MB) of 0.5 μmol m^{-2} s^{-1} intensity) for 5 min at 10 °C prior to exposure to white actinic light of 11.5 to 1000 μmol m^{-2} s^{-1} PPFD. After 6 min (steady state) 0.05 mol m^{-3} DCMU was added to give the fast and slow relaxation of fluorescence quenching (FQ and FE, respectively - see Figure 5.11). If DCMU is added to a dark adapted sample the fluorescence rise upon illumination with actinic light is F_m. This value, which enables an estimation of remaining quenching (q_R - see Figure 5.11), is also shown (×). See Figure 5.9 and text for details of the estimation of the fluorescence parameters and section 5.2.9 for experimental details. Each point represents the mean ± standard error of 4 - 8 observations. Standard error bars included where greater than symbol size.

NET PHOTOSYNTHESIS (µmoles O2 Hr-1 mg-1 Chl)
5
4
3
2
1
0
−1
0
11
45
100
200
250
1000
Actinic light PPFD (µmol m-2 s-1)
0.5
0.4
0.3
0.2
0.1
Fluorescence parameter (arbitary units)
Fo, Fp, Fsl, Fv/Fp

resulted in a fluorescence peak designated F_m. This F_m value was determined with 11 different chloroplast samples and the mean value is shown in Figure 5.10. Application of actinic light to a dark adapted sample with no DCMU present resulted in a fluorescence level designated F_p. The F_m level was determined in order to estimate any fluorescence associated with remaining quenching after the increase in fluorescence yield resulting from addition of DCMU to samples at the steady state (6 min). The mean F_m level was slightly greater than the F_p levels obtained at lower light intensities. However, at 100 μmol m^{-2} s^{-1} PPFD and above, the F_p level obtained approached the F_m level and in some individual experiments the fluorescence level obtained on addition of DCMU after 6 min resulted in fluorescence levels equal to or greater than the F_m level. In such situations the fluorescence associated with remaining quenching was assigned the value zero. Thus, when using the DCMU addition technique, rather than pulse saturation, the F_m value must be estimated in different chloroplast samples from the other fluorescence parameters F_p, F_{st}, FQ and FE. As the difference between F_p and F_m was small (the maximum difference was at 11.5 μmol m^{-2} s^{-1} PPFD when F_p was approximately 20 % less than F_m) and F_o and F_p can be determined in the same sample along with F_{st}, FQ and FE the, parameter F_v/F_p (F_p - F_o/ F_p) was preferred to F_v/F_m (F_m - F_o/ F_m).

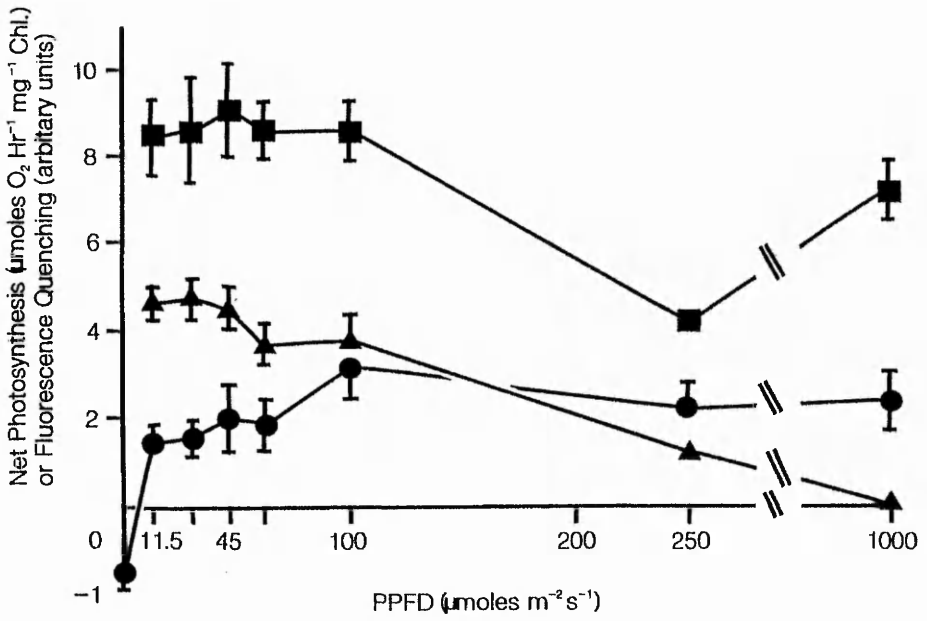
As would be expected the F_o level remains essentially constant, whilst F_p increases slightly with increasing actinic PPFD (Figure 5.10). The F_v/F_p ratio will increase if either F_p increases or F_o decreases and it is observed that the F_v/F_p ratio increases gradually with increasing actinic PPFD, mainly reflecting an increase in F_p, as F_o is essentially unchanged. The value of F_v/F_p ranged from 0.3384 to 0.4747 at 11.5 and 1000 μmol m^{-2} s^{-1} PPFD, respectively. This is lower than the F_v/F_p ratio measured with red (660 nm) actinic light of 400 μmol m^{-2} s^{-1} and chloroplast suspensions equivalent to 25 μg cm^{-3} chlorophyll, using the TR1 (F_v/F_p = 0.7687), further indicating the low amount of variable fluorescence relative to F_o fluorescence when using the MFMS (as seen in Figures 5.8 and 5.9). The fluorescence level at steady state, F_{st}, was very similar to F_p, particularly at the lower light intensities, indicating little, if any, quenching from F_p over the first 6 min of induction. A small amount of quenching from F_p was evident at light intensities of 100 and 1000 μmol m^{-2} s^{-1} PPFD. A light intensity of 100 μmol m^{-2} s^{-1} PPFD was optimum for net photosynthesis, supporting a rate of 3.22 $\pm$ 0.767 μmoles O_2 hr^{-1} mg^{-1} chlorophyll. Any further increase in PPFD resulted

in little change in the rate of net photosynthesis. The relationship between the actinic PPFD and the other fluorescence amplitudes measured may be seen in Figure 5.11. FQ became a smaller proportion of the fluorescence yield as the PPFD increased, whereas the NP component generally increased with increasing PPFD. The FE component increased as the PPFD increased to approximately 45 μmol m^{-2} s^{-1}, but further increase in PPFD resulted in a decline in FE. The FR component generally increased with increasing PPFD.

The fluorescence amplitudes were normalised with respect to 'available' variable fluorescence as described and the effect of PPFD on the various fluorescence quenching parameters is illustrated in Figure 5.12. As the PPFD increases from 0 to approximately 100 μmol m^{-2} s^{-1}, q_Q decreases whilst q_E increases. At higher light intensities both q_Q and q_E decrease. The q_R and q_{NP} parameters generally increase with increasing PPFD, with the exception of a comparitively low value at 250 μmol m^{-2} s^{-1} PPFD. A further means of characterising the fluorescence response is by DCMU addition throughout the induction curve and such a preliminary investigation of the FQ and FE fluorescence levels is illustrated in Figure 5.13. If DCMU is added to a dark adapted sample (*i.e.* at time = 0 sec) the fluorescence rise upon illumination is F_m. This value enables the estimation of the fluorescence associated with remaining quenching (FR) throughout the induction curve. With an actinic PPFD of 100 μmol m^{-2} s^{-1} an apparently biphasic DCMU - induced fluorescence rise was obtained throughout the first 6 min of the induction curve, allowing measurement of FQ and FE fluorescence levels. At 250 μmol m^{-2} s^{-1} PPFD actinic light it was not always possible to distinguish two phases in the fluorescence increase induced by DCMU, and therefore any fluorescence increase obtained could not be resolved into FQ and FE components. At 1000 μmol m^{-2} s^{-1} PPFD actinic light there was no increase in fluorescence level upon addition of DCMU at any time other than t = 0 sec.

The influence of photoinhibition on the fluorescence induction curve and parameters defined so far, was also investigated. Photoinhibitory pretreatment involved exposure to 1000 μmol m^{-2} s^{-1} PPFD for up to 25 min and facsimiles of typical fluorescence and oxygen exchange traces are illustrated in Figure 5.14. It is clear that photoinhibition induced changes in the induction curve. The F_p level, and hence the F_s level, is very much reduced in the photoinhibited sample. That is, the variable fluorescence yield is reduced. Also apparent from Figure 5.14 is a slight decrease in the F_o level in the

Figure 5.11 The relationship between the actinic PPFD (μmol m^{-2} s^{-1}), net photosynthesis, ● (μmoles O_2 hr^{-1} mg^{-1} chl) and the fluorescence parameters FQ (▲), FE (△) and FR (○) in relative fluorescence units, measured using the MFMS. The non - photochemical parameter, □, (NP, a combination of FE and FR) and the total quenching (■) are also shown. Chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll in a reaction mixture containing 10 mol m^{-3} $NaHCO_3$ were preincubated in the 'dark' (in the presence of the modulated beam (MB) of 0.5 μmol m^{-2} s^{-1} intensity) for 5 min at 10 °C prior to exposure to white actinic light of 11.5 to 1000 μmol m^{-2} s^{-1} PPFD. After 6 min (steady state) 0.05 mol m^{-3} DCMU was added to give the fast and slow relaxation of fluorescence quenching (FQ and FE, respectively). If DCMU is added to a dark adapted sample the fluorescence rise upon illumination with actinic light is F_m. This value enables the estimation of the fluorescence level associated with remaining quenching (FR). See Figure 5.9 and text for details of estimation of the fluorescence parameters and section 5.2.9 for experimental details. Each point represents the mean ± standard error of 4 to 8 observations. Standard error bars included where greater than symbol size.


Net Photosynthesis (μmoles O_2 Hr^{-1} mg^{-1} Chl.)
or Fluorescence Quenching (arbitary units)
10
8
6
4
2
0
−1
11.5
45
100
200
250
1000
PPFD (μmoles m^{-2} s^{-1})

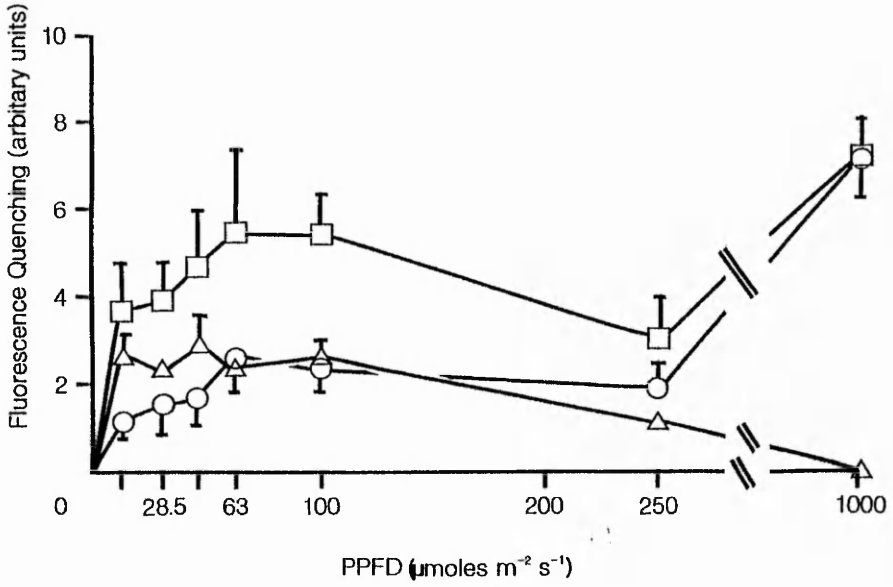
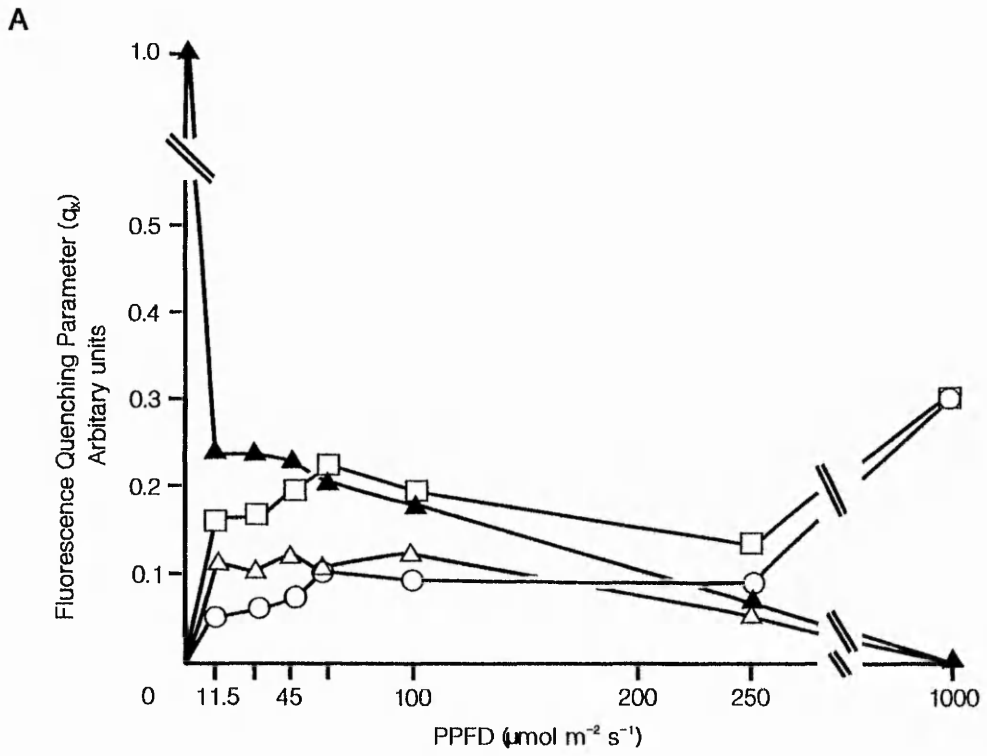

Fluorescence Quenching (arbitary units)
10
8
6
4
2
0
28.5
63
100
200
250
1000
PPFD (μmoles m^{-2} s^{-1})

Figure 5.12 (A) The relationship between the actinic PPFD (μmol m^{-2} s^{-1}) and the normalised fluorescence parameters q_Q (▲), q_E (△), q_R (○) and q_{NP} (□), and (B) the same parameters expressed as a percentage of the total quenching, measured using the MFMS. Chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll in a reaction mixture containing 10 mol m^{-3} $NaHCO_3$ were preincubated in the 'dark' (in the presence of the modulated beam (MB) of 0.5 μmol m^{-2} s^{-1} intensity) for 5 min at 10 °C prior to exposure to white actinic light of 11.5 to 1000 μmol m^{-2} s^{-1} PPFD. After 6 min (steady state) 0.05 mol m^{-3} DCMU was added to give the fast and slow relaxation of fluorescence quenching (FQ and FE, respectively). If DCMU is added to a dark adapted sample the fluorescence rise upon illumination with actinic light is F_m. This value enables the estimation of the fluorescence level associated with remaining quenching (FR). The q_Q, q_E, q_R and q_{NP} fluorescence parameters were normalised with respect to 'available' variable fluorescence according Schreiber, *et al.* (1986) and Horton and Hague (1988). The total quenching is $q_Q + q_E + q_R$ and the percentage contribution of, for example, q_Q, is $q_Q / (q_Q + q_E + q_R) \times 100$. See Figure 5.9 and text for details of estimation of the fluorescence parameters and the normalisation routine and section 5.2.9 for experimental details. Each point represents the mean of 4 to 8 observations.


A
1.0
0.5
0.4
0.3
0.2
0.1
0
11.5
45
100
200
250
1000
Fluorescence Quenching Parameter (q_x)
Arbitary units
PPFD (μmol m^{-2} s^{-1})

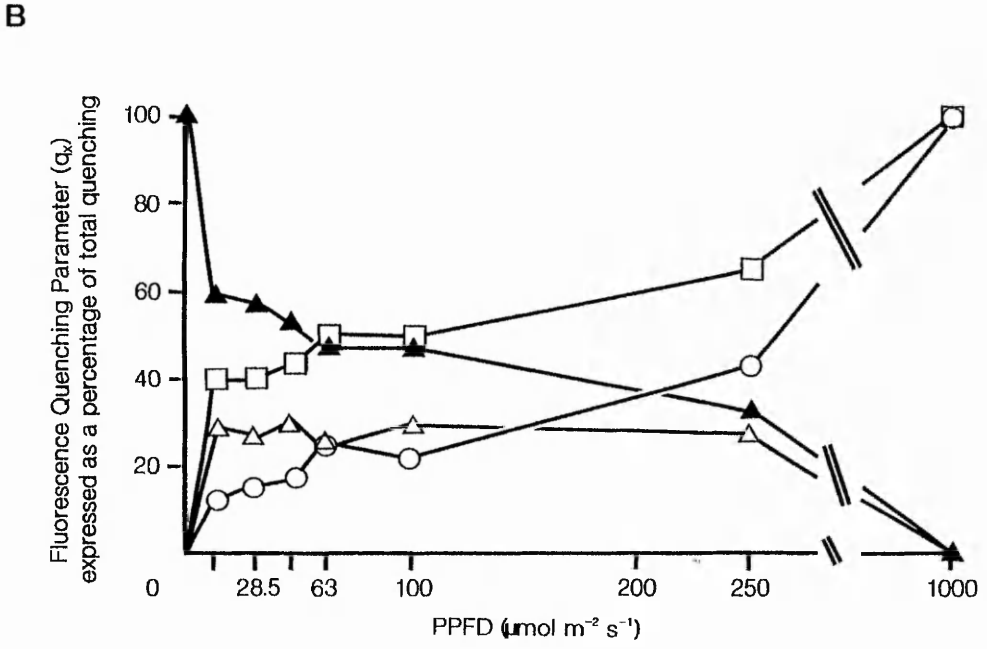
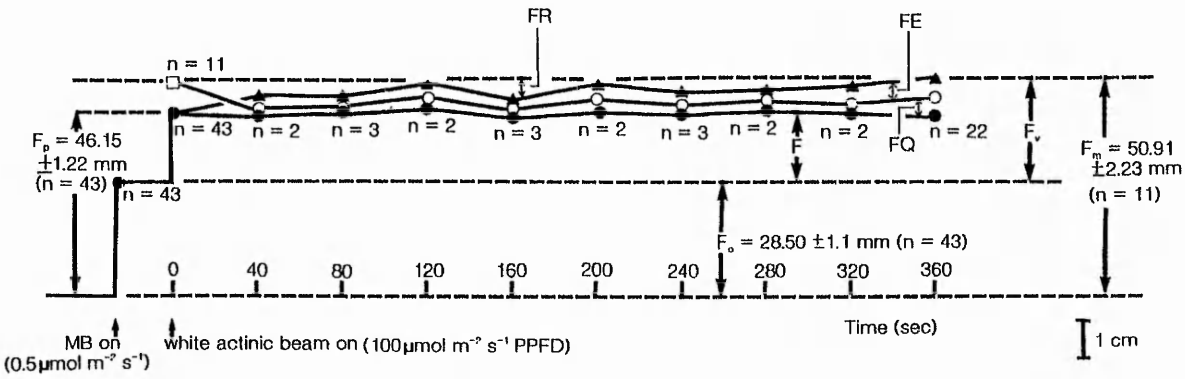
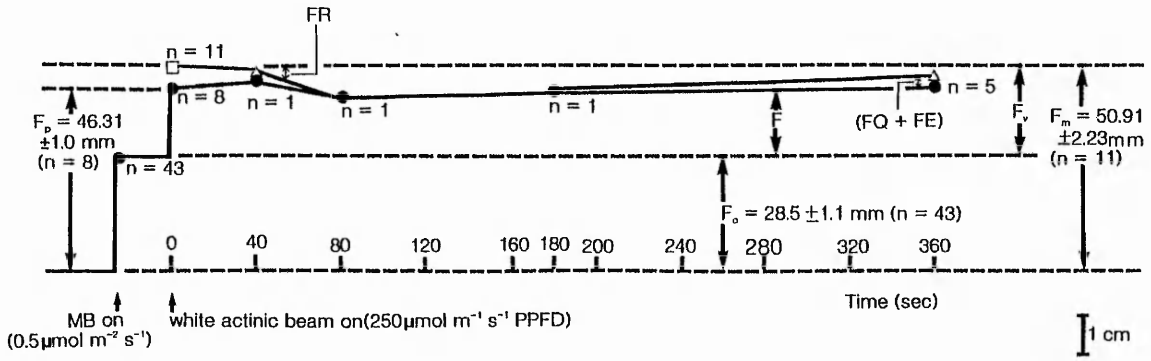

B
100
80
60
40
20
0
28.5
63
100
200
250
1000
Fluorescence Quenching Parameter (q_x)
expressed as a percentage of total quenching
PPFD (μmol m^{-2} s^{-1})

Figure 5.13 Diagrammatic representation of the fluorescence quenching throughout the induction curve with an actinic light of (A) 100, (B) 250, and (C) 1000 μmol m^{-2} s^{-1} PPFD, measured using the MFMS. Chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll in a reaction mixture containing 10 mol m^{-3} $NaHCO_3$ were preincubated in the 'dark' (in the presence of the modulated beam (MB) of 0.5 μmol m^{-2} s^{-1} intensity) for 5 min at 10 °C prior to exposure to white actinic light. At 0, 40, 80, 120, 240, 280, 320 or 360 seconds after the application of actinic light 0.05 mol m^{-3} DCMU was added to give the fast and slow relaxation of fluorescence quenching (FQ and FE, respectively). If DCMU is added to a dark adapted sample (*i.e.* time = 0 sec) the fluorescence upon illumination is F_m. This value enables an estimation of the fluorescence associated with remaining quenching (FR). See Figure 5.9 and text for details of estimation of the fluorescence parameters and section 5.2.9 for experimental details.

A


FR
FE
n = 11
n = 43
n = 2
n = 3
n = 2
n = 3
n = 2
n = 3
n = 2
n = 2
n = 22
FQ
F
F_v
F_m = 50.91 ±2.23 mm (n = 11)
F_o = 46.15 ±1.22 mm (n = 43)
n = 43
F_o = 28.50 ±1.1 mm (n = 43)
0 40 80 120 160 200 240 280 320 360
Time (sec)
1 cm
MB on (0.5µmol m^{-2} s^{-1})
white actinic beam on (100µmol m^{-2} s^{-1} PPFD)

B


FR
n = 11
n = 8
n = 1
n = 1
n = 1
n = 5
(FQ + FE)
F
F_v
F_m = 50.91 ±2.23mm (n = 11)
F_o = 46.31 ±1.0 mm (n = 8)
n = 43
F_o = 28.5 ±1.1 mm (n = 43)
0 40 80 120 160 180 200 240 280 320 360
Time (sec)
1 cm
MB on (0.5µmol m^{-2} s^{-1})
white actinic beam on(250µmol m^{-2} s^{-1} PPFD)

C

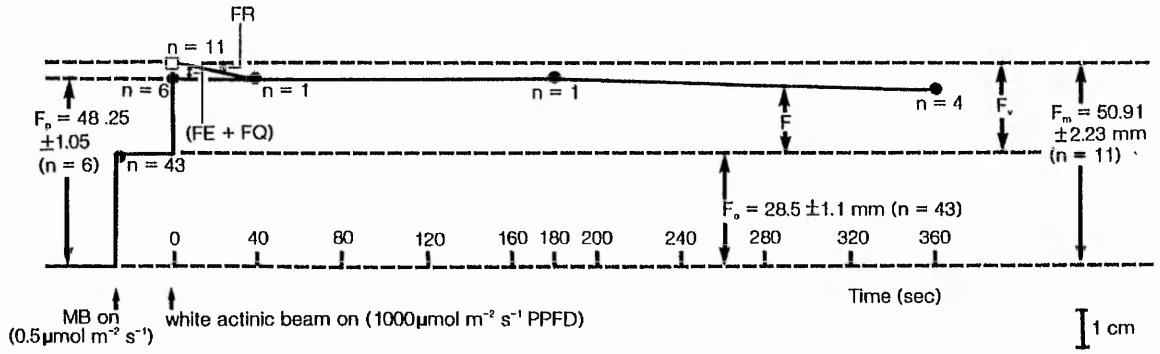
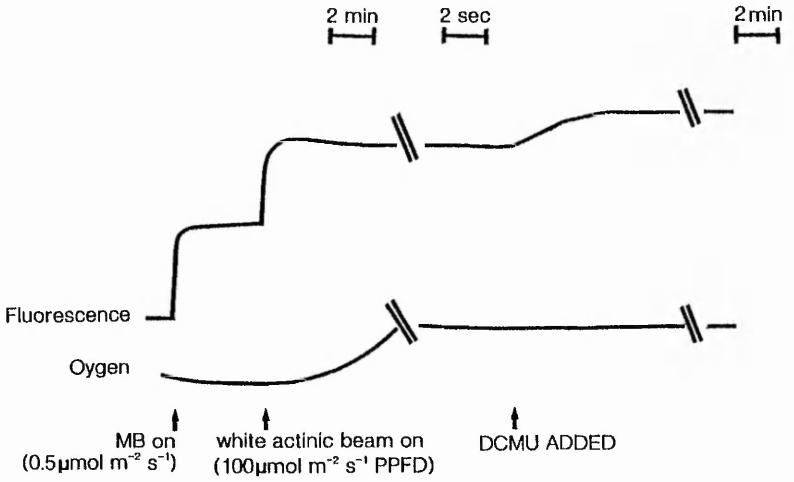

FR
n = 11
n = 6
n = 1
n = 1
n = 4
(FE + FQ)
F
F_v
F_m = 50.91 ±2.23 mm (n = 11)
F_o = 48 .25 ±1.05 (n = 6)
n = 43
F_o = 28.5 ±1.1 mm (n = 43)
0 40 80 120 160 180 200 240 280 320 360
Time (sec)
1 cm
MB on (0.5µmol m^{-2} s^{-1})
white actinic beam on (1000µmol m^{-2} s^{-1} PPFD)

Figure 5.14 Typical fluorescence traces obtained when measuring the slow chlorophyll fluorescence induction kinetics with the MFMS with (A) untreated chloroplasts and (B) chloroplasts photoinhibited by 25 min exposure to 1000 μmol m^{-2} s^{-1} PPFD, at 10°C. Chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll in a reaction mixture containing 10 mol m^{-3} $NaHCO_3$ were subjected to a photoinhibitory pretreatment of 1000 μmol m^{-2} s^{-1} PPFD for up to 25 min. After pretreatment the chloroplast suspension was incubated in the 'dark' (in the presence of the modulated beam (MB) of 0.5 μmol m^{-2} s^{-1} intensity) for 5 min, followed by the generation of the fluorescence induction with white actinic light of 100 μmol m^{-2} s^{-1} PPFD. After 6 min (steady state) 0.05 mol m^{-3} DCMU was added to give the fast and slow relaxation of fluorescence quenching (FQ and FE, respectively). See Figure 5.9 and text for details of the estimation of the fluorescence parameters and section 5.2.10 for experimental details.

A

Chart recorder set at 10 mV scale (oxygen) and 500 mV scale (fluorescence)

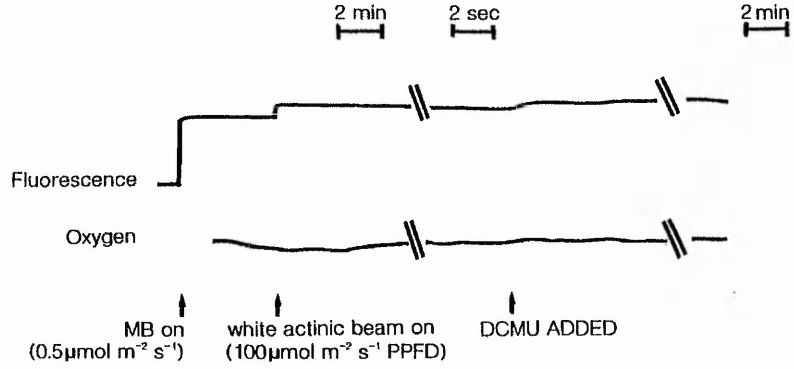
t = 0 – No pretreatment at 1000µmol m^{-2} s^{-1} PPFD



0·5mV - OXYGEN
25mV - FLUORESCENCE

B

t = 25 minutes pretreatment at 1000µmol m^{-2} s^{-1} PPFD

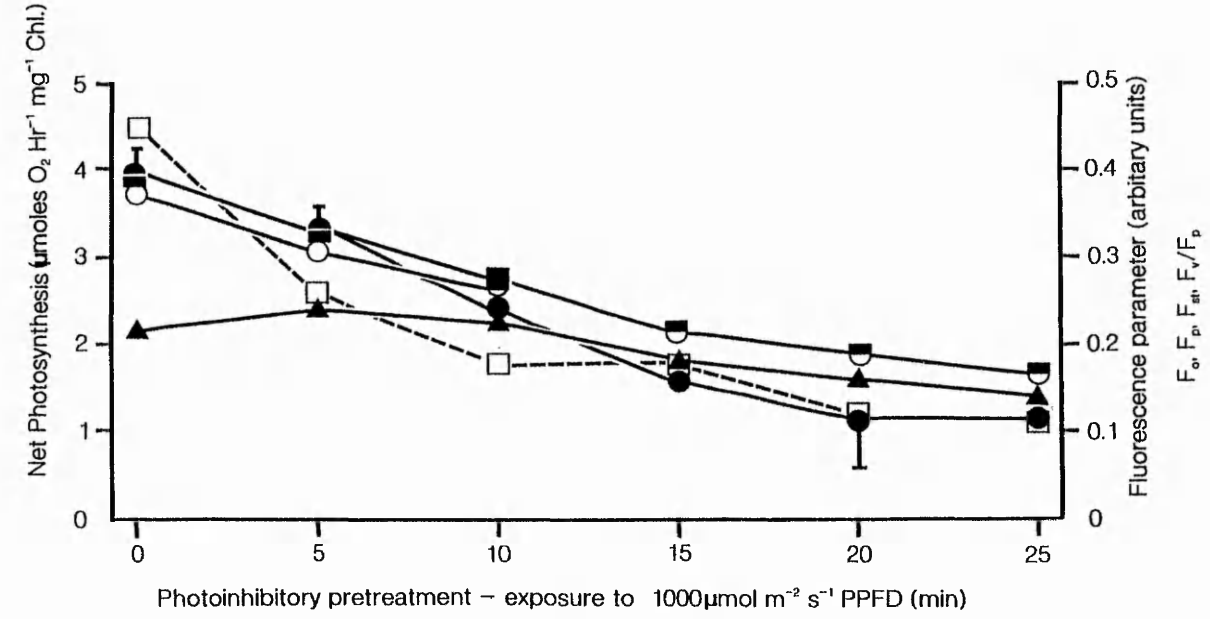


0.5mV - OXYGEN
25mV - FLUORESCENCE

photoinhibited sample. Any increase in fluorescence level upon addition of DCMU at the steady state (6 min after illumination with the actinic light) is also reduced in the photoinhibited sample and a marked decrease in the rate of net photosynthesis is also observed. The relationship between photoinhibition, net photosynthesis and the fluorescence parameters F_o, F_p, F_{st} and F_v/F_p is shown in Figure 5.15. The F_o level showed a slight increase after 5 min photoinhibition but then decreased with increasing pretreatment time. The other parameters all decreased markedly with increasing photoinhibition. The F_v/F_p ratio was particularly sensitive and exhibited the highest rate of inhibition, reaching approximately 65 % after 10 min. Further pretreatment resulted in less dramatic inhibition, approximately 76 % occurring after 25 min. The rate of decline of net photosynthesis was lower than that of the F_v/F_p ratio and was approximately linear up to 15 min pretreatment, reaching approximately 61 % inhibition. Interestingly, from 15 to 25 min the degree of inhibition of net photosynthesis was very similar to the degree of inhibition of F_v/F_p, the former reaching approximately 71 %. The rapid decrease in F_v/F_p over the first 5 min of stress was due to an increase in F_o (approximately 10 %) and a decrease in F_p (approximately 17 %). The rate of inhibition of F_p was approximately linear up to 15 min, reaching approximately 47 %. Therefore the decline in rate of inhibition of F_v/F_p appears to reflect an increasing rate of F_o inhibition from 5 to 10 min (6 % inhibition) and, in particular, from 10 to 15 min (22 % inhibition), pretreatment. From 15 to 25 min pretreatment the rates of inhibition of F_o and F_p were very similar. The F_{st} level was very similar to the F_p level, indicating little, if any, quenching from F_p over the first 6 min of induction, at all pretreatment times. The relationship between photoinhibitory pretreatment time and the other fluorescence parameters measured may be seen in Figure 5.16. The FR component increased dramatically with increasing time of pretreatment, mainly reflecting the loss of variable fluorescence, that is, the difference in fluorescence levels obtained with photoinhibited samples and the F_m level estimated in non - photoinhibited samples. FQ and FE decreased with increasing pretreatment and beyond 15 min these parameters could not be resolved. As the NP component is the combination of FE and FR and the total quenching is the combination of FE, FQ and FR, the parameters NP and TOT increase with increasing photoinhibition, mainly reflecting the increase in the FR component. The fluorescence parameters were normalised with respect to 'available' variable fluorescence and the effect of

Figure 5.15 (A) The relationship between photoinhibitory pretreatment (min), net photosynthesis, ● (μmoles O_2 hr^{-1} mg^{-1} chl) and the fluorescence parameters F_o (▲), F_p (■), F_v (○) and F_v/F_p (□) in relative fluorescence units, measured using the MFMS and (B) the same parameters expressed as a percentage decrease from the control (no photoinhibition). The ratio of F_v/F_p is equivalent to $F_p - F_o / F_p$. Chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll in a reaction mixture containing 10 mol m^{-3} $NaHCO_3$ were subjected to a photoinhibitory pretreatment of 1000 μmol m^{-2} s^{-1} PPFD for up to 25 min. After pretreatment the chloroplast suspension was incubated in the 'dark' (in the presence of the modulated beam (MB) of 0.5 μmol m^{-2} s^{-1} intensity) for 5 min, followed by the generation of the fluorescence induction with white actinic light of 100 μmol m^{-2} s^{-1} PPFD. After 6 min (steady state) 0.05 mol m^{-3} DCMU was added to give the fast and slow relaxation of fluorescence quenching (FQ and FE, respectively). See Figure 5.9 and text for details of the estimation of the fluorescence parameters and section 5.2.10 for experimental details. Each point represents the mean $\pm$ standard error of 3 to 7 observations. Standard error bars included where greater than symbol size.

A


Net Photosynthesis (μmoles O_2 Hr^{-1} mg^{-1} Chl.)
Fluorescence parameter (arbitary units)
F_o, F_p, F_{st}, F_v/F_p
Photoinhibitory pretreatment – exposure to 1000μmol m^{-2} s^{-1} PPFD (min)

B

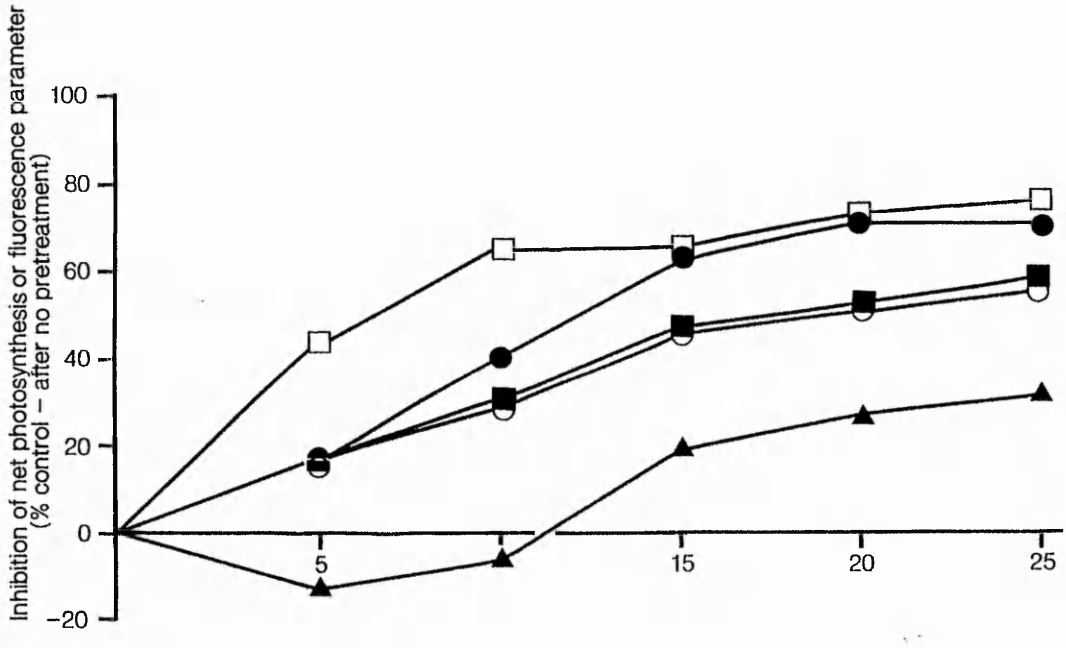
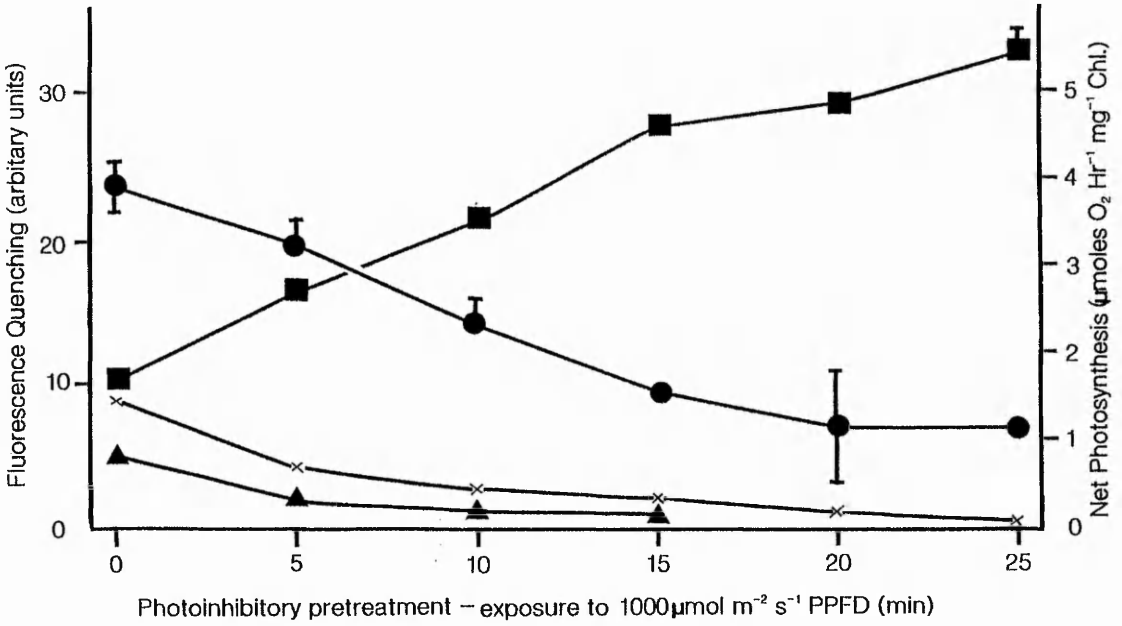

Inhibition of net photosynthesis or fluorescence parameter
(% control – after no pretreatment)
Photoinhibitory pretreatment – exposure to 1000μmol m^{-2} s^{-1} PPFD (min)

Figure 5.16 The relationship between the photoinhibitory pretreatment (min), net photosynthesis, ● (μmoles O_2 hr^{-1} mg^{-1} chl) and the fluorescence parameters FQ (▲), FE (△) and FR (○) in relative fluorescence units, measured using the MFMS. The non - photochemical parameter, □, (a combination of FE and FR) and the total quenching (■) are also shown. Chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll in a reaction mixture containing 10 mol m^{-3} $NaHCO_3$ were subjected to a photoinhibitory pretreatment of 1000 μmol m^{-2} s^{-1} PPFD for up to 25 min. After pretreatment the chloroplast suspension was incubated in the 'dark' (in the presence of the modulated beam (MB) of 0.5 μmol m^{-2} s^{-1} intensity) for 5 min, followed by the generation of the fluorescence induction with white actinic light of 100 μmol m^{-2} s^{-1} PPFD. After 6 min (steady state) 0.05 mol m^{-3} DCMU was added to give the fast and slow relaxation of fluorescence quenching (FQ and FE, respectively). If DCMU is added to a dark adapted sample the fluorescence rise upon illumination with actinic light is F_m. This value enables an estimation of FR. FQ and FE could not be resolved at pretreatments greater than 15 min, therefore FQ and FE combined is also shown (×). See Figure 5.9 and text for details of the estimation of the fluorescence parameters and section 5.2.10 for experimental details. Each point represents the mean ± standard error of 3 to 7 observations. Standard error bars included where greater than symbol size.


Fluorescence Quenching (arbitary units)
Net Photosynthesis (μmoles O_2 Hr^{-1} mg^{-1} Chl.)
Photoinhibitory pretreatment – exposure to 1000μmol m^{-2} s^{-1} PPFD (min)

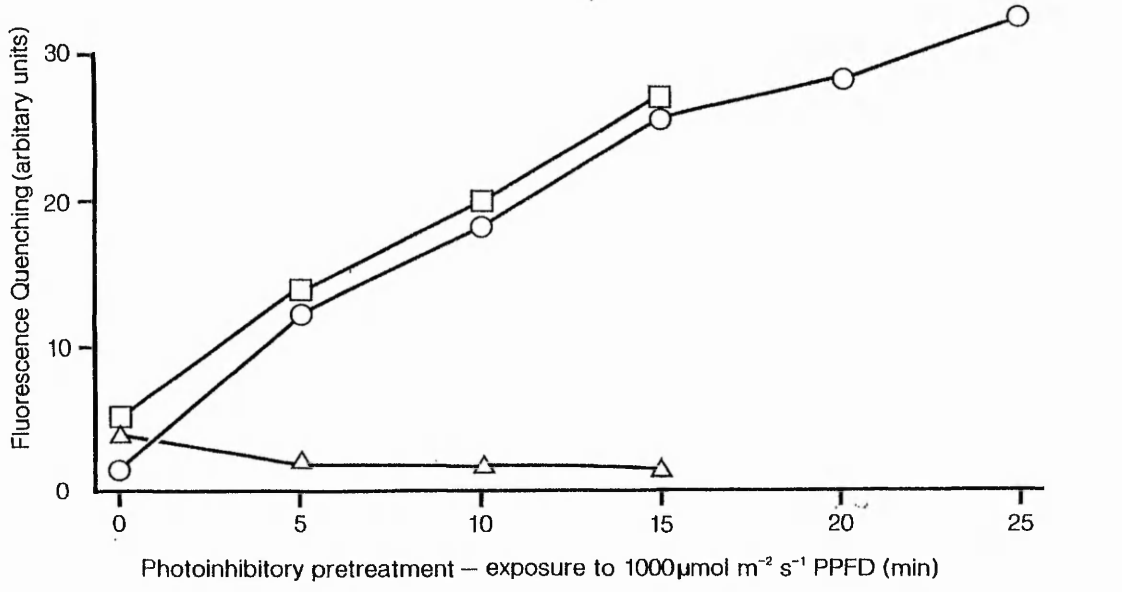
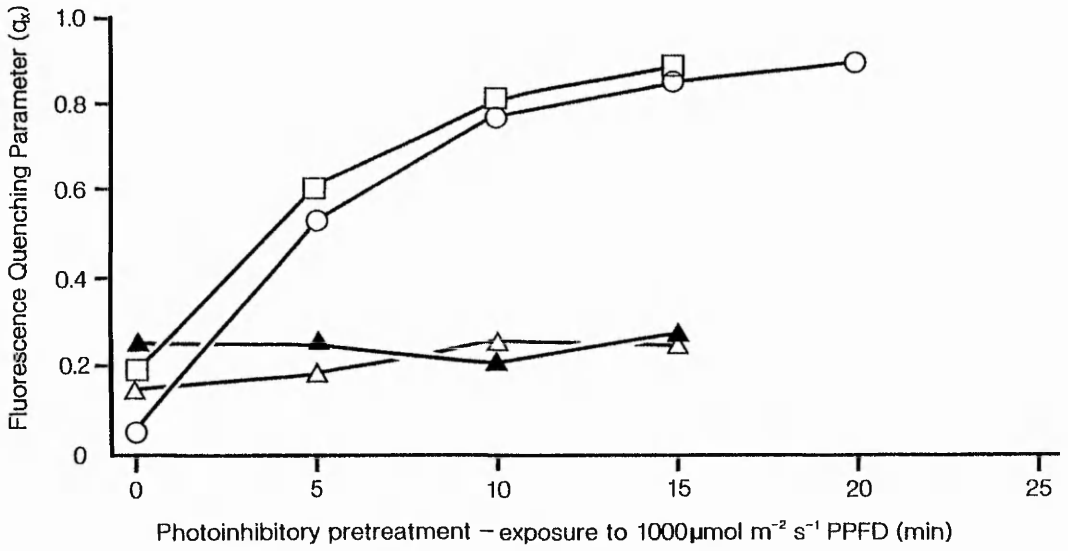

Fluorescence Quenching (arbitary units)
Photoinhibitory pretreatment – exposure to 1000μmol m^{-2} s^{-1} PPFD (min)

Figure 5.17 (A) The relationship between photoinhibitory pretreatment (min) and the normalised fluorescence quenching parameters q_Q, (▲), q_E (△), q_R (○) and q_{NP} (□), and (B) the same parameters expressed as a percentage of the total quenching, measured using the MFMS. Chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll in a reaction mixture containing 10 mol m^{-3} $NaHCO_3$ were subjected to a photoinhibitory pretreatment of 1000 μmol m^{-2} s^{-1} PPFD for up to 25 min. After pretreatment the chloroplast suspension was incubated in the 'dark' (in the presence of the modulated beam (MB) of 0.5 μmol m^{-2} s^{-1} intensity) for 5 min, followed by the generation of the fluorescence induction with white actinic light of 100 μmol m^{-2} s^{-1} PPFD. After 6 min (steady state) 0.05 mol m^{-3} DCMU was added to give FQ and FE. If DCMU is added to a dark adapted sample the fluorescence rise upon illumination with actinic light is F_m. This value enables an estimation of FR. The q_Q, q_E, q_R and q_{NP} fluorescence parameters were normalised with respect to 'available' variable fluorescence according to Schreiber, *et al.* (1986) and Horton and Hague (1988). The total quenching is $q_Q + q_E + q_R$ and the percentage contribution of for example, q_Q, is $q_Q / (q_Q + q_E + q_R) \times 100$. See Figure 5.9 and text for details of the estimation of the fluorescence parameters and the normalisation routine, and section 5.2.10 for experimental details. Each point represents the mean of 3 to 7 observations.

A


Fluorescence Quenching Parameter (q_x)
1.0
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
Photoinhibitory pretreatment – exposure to 1000µmol m^{-2} s^{-1} PPFD (min)

B

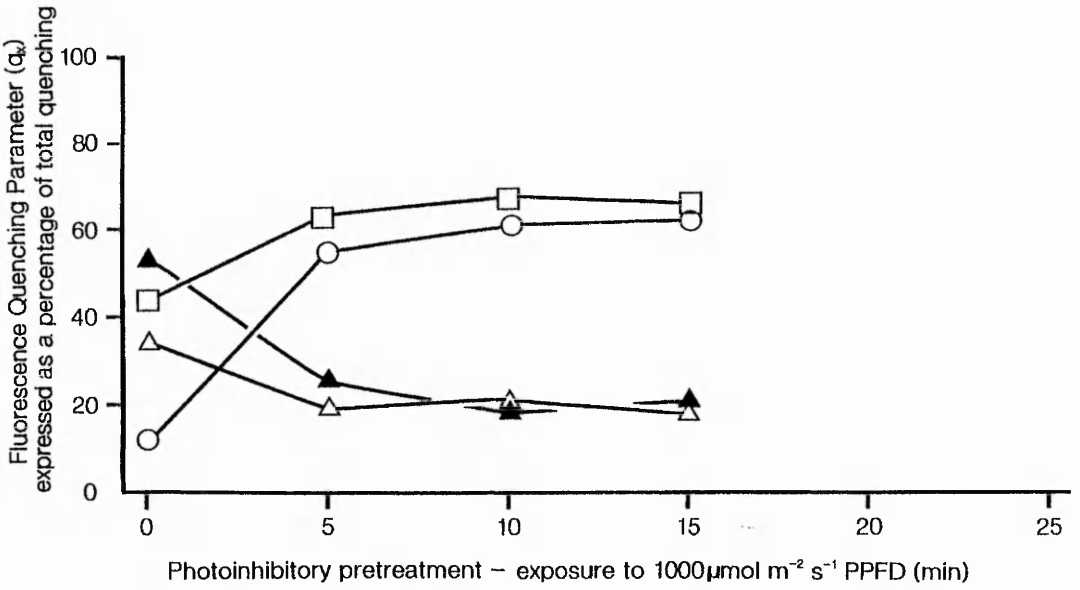
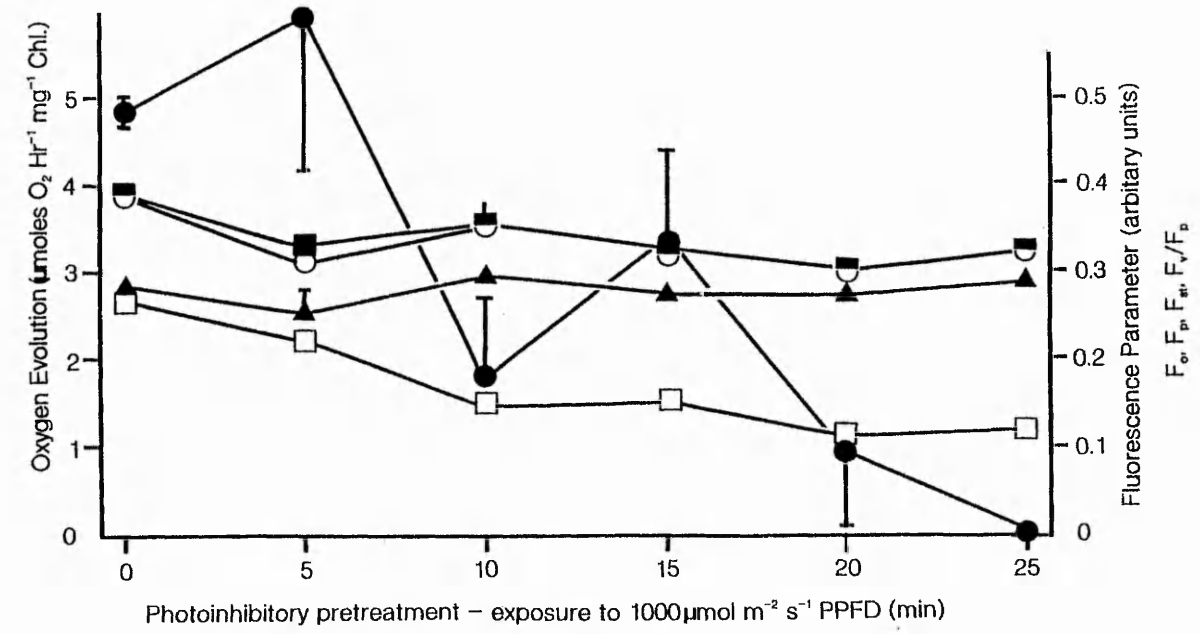
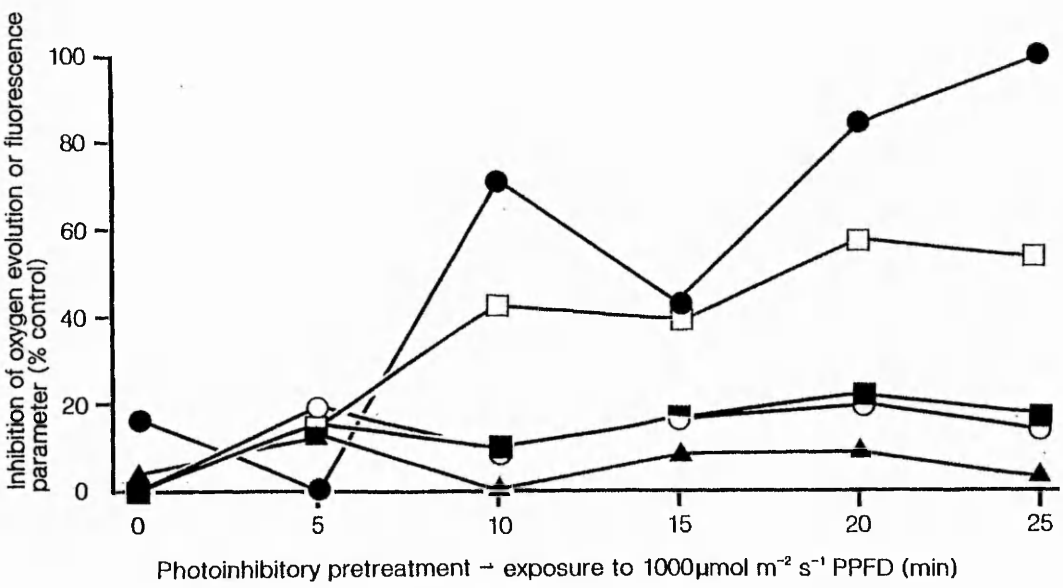

Fluorescence Quenching Parameter (q_x) expressed as a percentage of total quenching
100
80
60
40
20
0
0
5
10
15
20
25
Photoinhibitory pretreatment – exposure to 1000µmol m^{-2} s^{-1} PPFD (min)

Figure 5.18 (A) The relationship between photoinhibitory pretreatment (min), potassium ferricyanide - dependent oxygen evolution, ● (μmoles O_2 hr^{-1} mg^{-1} chl) and the fluorescence parameters F_o (▲), F_p (■), F_v (○) and F_v/F_p (□) in relative fluorescence units, measured using the MFMS and (B) the same parameters expressed as a percentage decrease from the control (no photoinhibition). The ratio of F_v/F_p is equivalent to $F_p - F_o / F_p$. Thylakoid suspensions equivalent to 50 μg cm^{-3} chlorophyll in a reaction mixture containing 7.5 mol m^{-3} $K_3Fe(CN)_6$ were subjected to a photoinhibitory pretreatment of 1000 μmol m^{-2} s^{-1} PPFD for up to 25 min. After pretreatment the thylakoid suspension was incubated in the 'dark' (in the presence of the modulated beam (MB) of 0.5 μmol m^{-2} s^{-1} intensity) for 5 min, followed by the generation of the fluorescence induction with white actinic light of 100 μmol m^{-2} s^{-1} PPFD. After 6 min (steady state) 0.05 mol m^{-3} DCMU was added. See Figure 5.9 and text for details of the estimation of the fluorescence parameters and section 5.2.10 for experimental details. Each point represents the mean $\pm$ standard error of 2 observations. Standard error bars included where greater than symbol size.

A


Oxygen Evolution (μmoles O_2 Hr^{-1} mg^{-1} Chl.)
Fluorescence Parameter (arbitary units)
F_o, F_p, F_{st}, F_v/F_p
0
1
2
3
4
5
0.1
0.2
0.3
0.4
0.5
0
5
10
15
20
25
Photoinhibitory pretreatment – exposure to 1000μmol m^{-2} s^{-1} PPFD (min)

B


Inhibition of oxygen evolution or fluorescence parameter (% control)
0
20
40
60
80
100
0
5
10
15
20
25
Photoinhibitory pretreatment → exposure to 1000μmol m^{-2} s^{-1} PPFD (min)

photoinhibtion on the various parameters is illustrated in Figure 5.17. The q_Q component remained little changed with increasing pretreatment time, whilst q_E exhibited a slight increase. The q_{NP} and q_R components both increased dramatically with increasing photoinhibitory pretreatment, therefore the percentage contribution to total quenching of q_Q and q_E declined markedly with increasing pretreatment. This again mainly reflects the loss of variable fluorescence, that is, the difference in fluorescence levels obtained with photoinhibited samples and the F_m level estimated in non - photoinhibited samples.

A preliminary investigation of the influence of photoinhibition on the fluorescence induction curve and parameters defined so far was also carried out using thylakoid preparations equivalent to 50 μg cm^{-3} chlorophyll. The activity of PSI and PSII together was measured polarographically by electron transport from water to potassium ferricyanide (Figure 5.18). The fluorescence traces obtained were similar to those observed with chloroplast preparations (Figure 5.14) except that no increase in fluorescence level was apparent upon addition of DCMU. Therefore parameters FQ, FE and FR were not determined. The relationship between photoinhibition, oxygen evolution and the fluorescence parameters F_o, F_p, F_{st} and F_v/F_p is shown in Figure 5.18. The ferricyanide - dependent oxygen evolution exhibited an erratic response, but generally decreased with increasing photoinhibition. F_o was essentially unchanged over the 25 min pretreatment period, whilst F_p showed a gradual decrease. This was reflected by a clear decrease in the F_v/F_p ratio with increasing photoinhibition. The F_{st} fluorescence level was very similar to F_p, indicating little quenching from F_p over the first 6 min of the induction curve, at all pretreatment times.

5.4 Discussion

The initial study of this Chapter investigated the early events of chlorophyll fluorescence induction using chloroplast suspensions equivalent to 25 μg cm^{-3} chlorophyll and red (660 nm) actinic light of 400 μmol m^{-2} s^{-1} intensity. The results, which are summarised in Table 5.1, clearly demonstrate that the F_o level was the same in the presence or absence of DCMU, as was the peak fluorescence (*i.e.* F_p was equal to F_m), under the conditions of this study. Thus, F_v/F_p (0.7687) was essentially equal to F_v/F_m (0.7513). This suggests that the light intensity of 400 μmol m^{-2} s^{-1} was sufficiently high to achieve saturation, that is, to fully reduce Q_A, closing all PSII

reaction centres, thus producing a fluorescence peak analagous to F_m in the absence of DCMU. This is supported by the results of the study of chloroplast photosynthetic response in Chapter 3, which revealed that even chloroplast suspensions of 200 μg cm^{-3} chlorophyll were photosynthetically saturated at 100 - 250 μmol m^{-2} s^{-1} PPFD, whilst chloroplast suspensions equivalent to 25 μg cm^{-3} were photosynthetically saturated at 63 μmol m^{-2} s^{-1} PPFD (Table 3.1). The value of F_v/F_m (or F_v/F_p) compares quite well with values reported in the literature for all other species so far investigated. For example, an extensive study of 151 species, including ferns, coniferous and deciduous trees, dicotyledon, monocotyledon, C3, C4 and CAM plants found a range of F_v/F_m values of 0.8000 $\pm$ 0.0170 to 0.8530 $\pm$ 0.0040 (Bjorkman, 1987), whilst other workers state that the F_v/F_m ratio is typically in the range of 0.75 - 0.85 (Bolhar - Nordenkampf, *et al.*, 1989) and 0.6 to 0.8 (Baker and Horton, 1987). Thus, the fluorescence response of isolated *C. fragile* chloroplasts appears to be the same as that of other organisms with regard to the F_v/F_m parameter. The area over the induction curve between F_o and the maximum fluorescence level is proportional to the pool size of the electron acceptors on the reducing side of PSII (Bolhar - Nordenkampf, *et al.*, 1989). If transfer from the reaction centres to the quinone pool is blocked by DCMU this area will be dramatically reduced. Measurement of the area over the curve is complex but a simple indicator is given by $t_{1/2}$, one half of the time required for the rise in fluorescence from O to P. This effect was clearly seen, as indicated by $t_{1/2}$ values of 495 msec and 7 msec in the absence and presence of DCMU, respectively (Table 5.1). Again, these values are comparable with typical values reported for wheat (*Triticum aestivum* cv. Artus) leaves of approximately 300 and 10 msec in the absence and presence of atrazine, respectively (Bolhar - Nordenkampf, *et al.*, 1989). A $t_{1/2}$ value of approximately 7 msec in the presence of DCMU for fronds of *Chondrus crispus* and *Ulva sp.* has also been reported (Bates and Craigie, 1988).

There were marked differences between the fluorescence induction kinetics of *C. fragile* chloroplasts observed in this study and the response typically observed with other species. From Figures 5.4 to 5.6 it was apparent that, there was little quenching from F_p over the first min of the induction curve. The time required for fluorescence quenching varies, but substantial quenching often occurs in less than one min. A 10 min fluorescence transient obtained using the FDC is shown in Figure 5.7 and some quenching from F_p is evident. This could suggest that quenching is occurring, but over

several minutes, resulting in little quenching being observed over the first min of fluorescence induction, as recorded with the TR1. However, when the scale of the fluorescence transient is altered to enable comparison with a typical induction curve obtained with *Pisum sativum* chloroplasts (Baker and Horton, 1987) it is quite obvious that the degree of quenching from F_p is indeed much reduced compared with other systems studied (Figure 5.7C). In the particular examples shown in Figure 5.7 the fluorescence level is reduced from 30.5 to 22 mm (approximately a 28 % reduction) in the absence of DCMU and from 32.5 to 25 mm (approximately a 23 % reduction) in the presence of DCMU. That is, the amount of quenching from F_p is very similar in the presence or absence of DCMU. Fluorescence quenching in the presence of DCMU has not been previously reported and the occurrence of quenching may suggest that electron transport was not completely blocked by the DCMU. However, the dramatic influence of DCMU on the value of $t_{1/2}$ suggests that the DCMU is indeed blocking electron transport and maintaining Q_A in the reduced form. Thus, as with other organisms the F_p level was approximately 4 times the F_o level giving F_v/F_m ratios of approximately 0.76; but, unlike other organisms, there was little quenching from F_p over the first 10 min of the induction curve.

Following the initial study of fast fluorescence induction kinetics the MFMS was used to study the slow fluorescence induction kinetics using white actinic light and chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll. The conditions were optimised to produce the highest F_v/F_p ratios but the amount of variable fluorescence (F_p - F_o) relative to the F_o fluorescence level (generated by the modulated beam) was very low (Figures 5.8 and 5.9). That is, the F_o fluorescence level was a much greater proportion of the total fluorescence yield in *C. fragile* chloroplasts than in other species studied using similar techniques. The F_p level was, on average, only 1.63 times the F_o level, representing a range of F_v/F_p ratios from 0.3384 $\pm$ 0.0218 to 0.4747 $\pm$ 0.0504 at actinic light intensities of 11.5 and 1000 μmol m^{-2} s^{-1} PPFD, respectively (Figure 5.10). In a range of species and samples, including isolated chloroplasts, protoplasts and intact leaves it has been found that the peak fluorescence level upon application of a saturating light pulse is typically 4 to 5.5 times the F_o level produced by the weak modulated light measuring beam (Dietz, *et al.*, 1985; Schreiber, *et al.*, 1986; Havaux, 1987; Horton and Hague, 1988; Horton, *et al.*, 1988; Genty, *et al.*, 1989; Quick and Stitt, 1989), representing F_v/F_m ratios of 0.781 to 0.821. Lower F_v/F_p ratios may be

expected at the lower light intensities used in this study, as a smaller proportion of PSII reaction centres would be closed by Q_A reduction and thus the peak fluorescence yield would be reduced. However, the work of Chapter 3 demonstrated that chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll photosynthetically saturated at 25 - 63 μmol m^{-2} s^{-1} PPFD (Table 3.1). Therefore at light intensities greater than 63 μmol m^{-2} s^{-1} PPFD it would be expected that the degree of Q_A reduction upon application of the actinic light should result in a substantial fluorescence peak and at intensities of 250 and 1000 μmol m^{-2} s^{-1} PPFD it would be expected that the initial fluorescence peak observed would approach F_m. It was also clear that there was little, if any quenching from F_p over the first 6 min of the induction curve of *C. fragile* chloroplasts when using the MFMS (Figures 5.8, 5.9 and 5.10). Again, this is in contrast to the substantial quenching typically observed using similar techniques (Dietz, *et al.*, 1985; Schreiber, *et al.*, 1986; Havaux, 1987; Horton and Hague, 1988; Horton, *et al.*, 1988; Genty, *et al.*, 1989; Quick and Stitt, 1989).

Thus, when using the red (660 nm) actinic light and the TR1 the F_o, F_p (or F_m) and hence the F_v/F_m parameters were typical of those reported elsewhere, but there was very little quenching from F_p. In contrast, when using the MFMS the F_p level was vey low, relative to the F_o level, resulting in very low F_v/F_p ratios and, again, there was little, if any, quenching from F_p. This fluorescence study was concerned with isolated chloroplasts. A preliminary investigation has been made which clearly illustrated that the fluorescence response of whole fronds using the MFMS was very similar to that obtained with other species and a facsimile of a typical fluorescence trace is shown in Figure 5.8C. The F_p level was often 3.5 to 4 times greater than the F_o level generated by the modulated beam and F_v/F_p ratios in the region of 0.7 were repeatedly obtained. Furthermore, rapid and substantial quenching from F_p, again similar in nature to that reported in other species, was also apparent. This suggests that the reduced quenching observed when using both of the fluorescence measurement systems of this study, and the reduced variable fluorescence level observed when using the MFMS, are due to properties of the isolated chloroplasts that are not shared by the whole frond. Furthermore, the reduced quenching was obtained with both measurement systems suggesting that it was a genuine property of the isolated chloroplasts, whereas reduced variable fluorescence was not observed with the TR1 system, suggesting that the MFMS had a role in the suppressed level of F_p.

The reasons for the reduced quenching from F_p are uncertain and very few reports of such transients appear in the literature. Very similar fluorescence transients to those obtained with the MFMS and *C. fragile* chloroplasts have been obtained using osmotically shocked *Spinacia oleracea* L. chloroplasts (Krause, 1973, 1974). In this work it was found that intact chloroplasts displayed the typical fluorescence peak immediately upon illumination, followed by rapid quenching from the peak. In contrast, chloroplasts that were exposed to osmotic shock demonstrated a much reduced initial fluorescence peak, with little subsequent quenching from this initial level. It was concluded that substances were retained within the envelope of intact chloroplasts, which were responsible for fluorescence changes. It was also apparent that addition of $MgCl_2$ to 'broken' chloroplasts, particularly if added before rupture, restored the fluorescence kinetics characteristic of intact chloroplasts. The fluorescence yield of intact chloroplasts was not influenced by addition of $MgCl_2$, probably due to high concentrations of monovalent and divalent cations within the chloroplasts. The Mg^{2+} could only affect the thylakoids in the dark state or in a state of low energy, for example, in the presence of uncouplers of photophosphorylation or inhibitors of electron transport. Addition of DCMU, at the steady state, to 'broken' chloroplasts in the absence of exogenous $MgCl_2$ produced only a slight increase in fluorescence level, very similar in nature to that observed in the MFMS study of this Chapter (Figure 5.8A). To achieve maximum fluorescence at the steady state the combination of Mg^{2+} plus uncoupler (or inhibitor) was required in 'broken' chloroplasts, whilst the uncoupler (or inhibitor) alone was required in intact chloroplasts, due to their cation content. It was suggested that the energy - dependent fluorescence quenching reflects movement of Mg^{2+} and other cations from the thylakoids to the stroma. Cation efflux processes are associated with light dependent proton uptake by the thylakoids and as cations strongly increase the fluorescence yield, their efflux lowers the fluorescence, apparently via structural changes of the membrane system. However, although the MFMS fluorescence transients are highly similar to those of osmotically shocked chloroplasts (Krause, 1973, 1974), the fluorescence yield obtained with the TR1 exhibited no suppression of the initial fluorescence peak. As previously mentioned, this suggests that the genuine property of chloroplast fluorescence observed in this study is reduced quenching from F_p and that the low variable fluorescence obtained with the MFMS is associoated with the measuring system. Furthermore, *C. fragile* chloroplasts are notoriously robust

(Cobb and Rott, 1978) with preparation of thylakoids only possible using a freeze - thaw regime and therefore it is unlikely that the preparations used in this study contained significant proportions of 'broken' chloroplasts. For these reasons it is unlikely that the phenomena described by Krause (1973, 1974) explain the reduced quenching observed in this study.

The reduced quenching from F_p suggests that the processes responsible for such quenching were occurring to a much reduced extent in the isolated *C. fragile* chloroplasts. These processes include photochemical quenching (*i.e.* reoxidation of Q_A) and the non - photochemical mechanisms of: energy - dependent quenching, high - light quenching resulting from prolonged illumination and correlated with zeaxanthin formation (Demmig, *et al.*, 1987), quenching that results from damage to the PSII reaction centre upon photoinhibition (Cleland, *et al.*, 1986), and changes in fluorescence due to the phosphorylation of the LHCII during state transitions (Horton and Hague, 1988). It is thought that photochemical quenching (Q_A reoxidation) and energy - dependent quenching (q_E) make the major contributions to fluorescence quenching from F_p under many conditions (Baker and Horton, 1987; Walker, 1987), however, under low light, state transition associated quenching may be significant, whilst at high light photoinhibition associated quenching may become increasingly important (Horton and Hague, 1988; Lee, *et al.*, 1990). An inability to reoxidize Q_A reflects an inability to pass electrons along the electron transport chain to $NADP^+$ and ultimately to CO_2. This could occur if the electron transport chain becomes 'over - reduced', for example, due to inadequate supplies of carbon in the reaction medium. A concentration of 10 mol m^{-3} $NaHCO_3$ was used as a carbon source and there is no reason to suspect that this concentration is not adequate as reasonable rates of bicarbonate - dependent oxygen evolution were observed in the studies of chloroplast oxygen exchange (Chapter 3). At 50 μg cm^{-3} chlorophyll a range of maximum photosynthetic rates from 4.5 $\pm$ 0.4 to 14.8 $\pm$ 1.6 μmoles O_2 hr^{-1} mg^{-1} chl was obtained, with tissue harvested on 04/ 04/ 88 (spring - late vegetative) and 18/ 12/ 88 (winter - vegetative), respectively (Table 3.1). During the fluorescence work, which was all carried out on chloroplasts isolated from tissue harvested on 19/ 12/ 89 (winter - vegetative), maximum net photosynthesis was recorded using the MFMS and white actinic light of 100 μmol m^{-2} s^{-1} PPFD, which supported a rate of 3.22 $\pm$ 0.767 μmoles O_2 hr^{-1} mg^{-1} chl (Figures 5.8A, 5.10 and 5.11). This is a much lower rate than that

obtained with the corresponding tissue from the previous year. Rates of net photosynthesis of whole fronds at 10 °C ranged from 32.1 ± 2.2 to 51.1 ± 8.8 μmoles O_2 hr^{-1} mg^{-1} chl, with tissue harvested on 04/ 04/ 88 (spring - late vegetative) and 30/ 11/ 87 (winter - early vegetative), respectively (Table 2.1). Thus, the rate of net photosynthesis, calculated on a chlorophyll basis, is much lower in isolated chloroplasts than in whole fronds. A lower rate of net photosynthesis may reflect a lower rate of electron transport and hence a lower rate of reoxidation of Q_A in the isolated chloroplasts, which may contribute to the reduced quenching from F_p. A lower rate of electron transport, *i.e.* reoxidation of Q_A, would not affect the initial fluorescence peak when Q_A is fully reduced. This may explain the occurrence of a typical fluorescence peak immediately upon illumination in both isolated chloroplasts and intact fronds and partly account for the reduced quenching observed in chloroplasts but not in fronds. The net photosynthetic rates referred to above were all maximum rates obtained at optimum PPFD and therefore it is expected that little photoinhibition would have occurred, and thus any reduction in rates of electron transfer in chloroplasts relative to whole fronds would be uncomplicated by photoinhibitory damage, and in turn, there should be little photoinhibition associated fluorescence quenching to complicate the fluorescence response. However, the lower net photosynthetic rates may indicate that CO_2 assimilation was limited, which will influence the energy - dependent fluorescence quenching (q_E), as follows. If CO_2 assimilation is limited, ATP consumption will be limited and therefore ADP levels will be low (Walker, 1987). ADP and inorganic phosphate are required to discharge the proton gradient across the thylakoid membrane, bringing about ATP synthesis. Thus, if ADP is in short supply the proton gradient will increase and as it does so, there will be a shift from energy dissipation as fluorescence, to energy dissipation as heat (q_E). Thus, low carbon assimilation may reduce the rate of Q_A reoxidation, reducing q_Q, but would be expected to result in an increased trans - thylakoid proton gradient and hence increased q_E, resulting in quenching of fluorescence from F_p.

It is difficult to account for the apparent reduction in fluorescence quenching observed in isolated chloroplasts, as conditions that result in a decrease in one quenching mechanism often result in a complementary increase in another quenching mechanism. The only obvious situation that could result in the maintenance of Q_A in a reduced state and at the same time not result in an increase in the trans - thylakoid

proton gradient and hence q_E, would appear to be a block in electron transport close to the reducing side of Q_A, such as that caused by various herbicides (*e.g.* DCMU). However, in this event the net photosynthesis observed in this study would not have occurred. Any explanation must be based on properties of the chloroplasts not shared by the intact fronds. The most obvious difference between fronds and chloroplasts is perhaps associated with photorespiration. The photorespiratory carbon oxidation pathway requires the interaction of chloroplasts, mitochondria and peroxisomes (Figure 1.8) and is essentially an energy consuming process. Osmond (1981) estimated that in an atmosphere that allowed no photorespiration 3ATP and 2NADPH are theoretically required for the fixation of one CO_2 molecule during C3 photosynthesis, whilst at the CO_2 compensation point (when CO_2 fixation in photosynthesis equals CO_2 evolution in photorespiration) 10ATP and 6NADPH are required. It has been suggested that photorespiration may give protection against photoinhibition by contributing to the dissipation of excess excitation energy (*e.g.* Heber and Krause, 1980; Krause and Cornic, 1987; Krause and Laasch, 1987) and this, in turn, may contribute to fluorescence quenching in whole fronds. The energy requirements of photorespiration may maintain rates of electron transfer that enhance Q_A reoxidation, and thus also enhance a build up of the proton gradient and hence q_E. Phosphoglycolate is formed via the oxidation of ribulose bisphosphate and subsequently converted to glycolate within the chloroplast. Glycolate is excreted from its site of synthesis in the cells of higher plants (Osmond, 1981) and glycolate excretion by isolated *C. fragile* chloroplasts may occur. However, in the absence of peroxisomes and mitochondria there is no means by which the glycolate may be converted to glycerate for return to the chloroplast (Figure 1.8). The majority of the energy consumed in photorespiration is concerned with the return to the Calvin cycle of carbon lost to glycolate, but it is unlikely that these reactions will occur in a chloroplast preparation. This may reduce the energy requirements associated with photorespiration, perhaps reducing the rate of electron transport, thus reducing fluorescence quenching. However, loss of carbon to glycolate is possible in isolated chloroplasts and this process could act as a means of preventing 'over reduction' of the electron transport chain. Thus, any contribution that might be made to fluorescence quenching by photorespiration is unlikely to explain the difference in the fluorescence response of fronds and isolated chloroplasts. Furthermore, the lack of a complete photorespiratory pathway does not have such an influence on the

fluorescence response typically observed with chloroplasts isolated from other species, which always exhibit marked quenching from F_p.

It has been suggested that zeaxanthin and the xanthophyll cycle have a possible role in protection against photoinhibition (*e.g.* Demmig - Adams, *et al.*, 1989b, 1990) and that there may be high light quenching resulting from prolonged illumination that is correlated with zeaxanthin formation (Demmig, *et al.*, 1987). Zeaxanthin has been shown to be absent from *C. fragile* and it has been proposed that this may indicate the absence of the xanthophyll (epoxide) cycle in this alga (Benson and Cobb, 1983), which would obviously mean that zeaxanthin formation could not contribute to fluorescence quenching. Again, this does not explain the difference in the fluorescence response of fronds and isolated chloroplasts. Another mechanism that may make a minor contribution to fluorescence quenching is associated with the re - distribution of excitation energy due to the phosphorylation of the LHCII during state transitions (*e.g.* Horton and Hague, 1988; Lee, *et al.*, 1990). Interestingly, the ability of chloroplasts within the *C. fragile* frond to re - distribute excitation energy has been clearly shown, whilst such state transitions could not be demonstrated in isolated chloroplast preparations (Sealey, *et al.*, 1990). Thus, state transition - dependent fluorescence quenching may operate in the frond but not in the isolated chloroplast and this may partly explain the difference between the fluorescence response of the former and the latter. The role of state transitions in fluorescence quenching is generally regarded as a minor one and, although it has been suggested that it may be of more significance at lower light intensities (Horton and Hague, 1988; Lee, *et al.*, 1990), it is improbable that the absence of this mechanism alone can account for the reduced fluorescence quenching observed in isolated chloroplasts in this study.

It is also difficult to explain why there was reduced variable fluorescence relative to F_o fluorescence in isolated chloroplasts when using the MFMS, whilst typical yields of variable fluorescence relative to F_o fluorescence were recorded in isolated chloroplasts when using the TR1 system. Furthermore, the variable fluorescence yield relative to F_o fluorescence was similar to that typically observed in other organisms when whole fronds were investigated with the MFMS, exhibiting none of the suppression of variable fluorescence observed in isolated *C. fragile* chloroplasts (Figure 5.8C). From the above, it may be concluded that the suppressed yield of variable fluorescence must not only be associated with properties of the isolated chloroplast not

shared by the intact frond, but also with properties of the MFMS not shared by the TR1/ red (660 nm) light measuring system. *C. fragile* shows prominent absorption in the 'green gap' region of the spectrum (500 to 550 nm) due to the presence of relatively large amounts of chlorophyll b, siphonoxanthin and siphonein (Benson and Cobb, 1981, 1983). Similar absorption spectra have been noted in other algae and Kageyama, *et al.* (1977) demonstrated the transmission of excitation energy from siphonoxanthin to chlorophyll a in *Ulva japonica* and *U. pertusa*, whilst Kageyama and Yokohama (1978) found that siphonein could efficiently transfer excitation energy to chlorophyll a of PSII in *Dichotomosiphon tuberosus*. It was concluded that siphonein and siphonoxanthin function as light harvesting pigments in chlorophycean algae. Such a presence and role of these xanthophylls in *C. fragile* has also been reported (Anderson, 1983, 1985; Anderson and Barret, 1986). The modulated light measuring beam of the MFMS has a centre frequency of 583 nm (half band width 36 nm) and is used in conjunction with a 620 nm low pass filter. Due to the accessory pigments of *C. fragile* this alga does appear to exhibit enhanced absorption at 583 nm, relative to other higher plant species, such as *Spinacia oleracea* (Anderson, 1983, 1985). The modulated beam is intended to be of such a low intensity (0.5 μmol m^{-2} s^{-1}, in this study) that to all intents and purposes the sample is in the dark, and hence the fluorescence level generated is analagous to F_o. It could be suggested that the accessory pigments of *C. fragile* and its excellent adaptation to low light intensities enable enhanced absorption at 583 nm, making it difficult to achieve conditions analagous to darkness, no matter how low the intensity of the modulated beam. That is, the modulated beam could be driving photosynthesis to some extent, hence generating some variable fluorescence. The fluorescence increase upon application of the actinic beam would reflect increased reduction of Q_A by the higher light intensity, but the apparent variable fluoresence yield would be reduced by an amount equivalent to the variable fluorescence caused by the modulated beam. If the modulated beam was causing variable fluorescence it would be expected that the level of fluorescence generated would not remain constant, but would reveal some fluorescence quenching from an initial peak obtained immediately upon application of the modulated light. As seen earlier a characteristic of the fluorescence response of the isolated chloroplasts was reduced or lack of quenching from the initial peak and therefore it is perhaps not possible to use this criterion to determine whether the modulated beam is indeed generating any variable fluorescence. However, the

accessory pigments and associated enhanced absorption in the relevant region of the spectrum are characteristics shared by the fronds and the isolated chloroplasts and therefore this arguement is unlikely to account for the reduced variable fluorescence yield that is observed in isolated chloroplasts but not in intact fronds. On the other hand, isolated chloroplasts equivalent to 50 μg cm^{-3} chlorophyll exhibit photosynthetic saturation at 25 - 63 μmol m^{-2} s^{-1} PPFD (Table 3.1), whilst whole fronds exhibited photosynthetic saturation at approximately 200 μmol m^{-2} s^{-1} PPFD (Figures 2.2, 2.4 and 2.5) and this difference may increase the possibility that the weak modulated light induced a level of variable fluorescence that is a significant proportion of the variable fluorescence observed upon application of the actinic light, in isolated chloroplasts but not in intact fronds.

Differential penetration of the modulated light and the actinic light into highly concentrated chloroplast populations in leaves can result in the modulated light and the actinic light illuminating different populations of chloroplasts within a leaf (Ogren and Baker, 1985) and this is exacerbated by higher actinic light intensities. This can result in differences between the fluorescence response obtained using continuous actinic light, shutters and transient recorders, and that obtained using the MFMS. The significance of this differential penetration will depend on many factors including species, leaf/ frond thickness and the intensity and quality of both the modulated and actinic light. It is possible that the dense pigmentation of *C. fragile* fronds relative to that of a continuously stirred chloroplast suspension equivalent to 50 μg cm^{-3} chlorophyll, will influence the possibility of significant variable fluorescence generation by the modulated light and result in much greater differential penetration in fronds compared to chloroplasts, perhaps partially explaining the differences between the response of whole fronds and isolated chloroplasts, when using the MFMS.

The fluorescence response of isolated chloroplasts measured with the MFMS (Figures 5.8 and 5.9C), as discussed above, was very similar over a range of actinic light intensities from 11.5 to 1000 μmol m^{-2} s^{-1} PPFD (Figure 5.10). The F_o level remained essentially constant as would be expected with a constant modulated light intensity of 0.5 μmol m^{-2} s^{-1} PPFD. The F_p level increased slightly with increasing actinic PPFD, probably reflecting a slight increase in Q_A reduction. As chloroplasts equivalent to 50 μg cm^{-3} chlorophyll exhibit photosynthetic saturation at 25 - 63 μmol m^{-2} s^{-1} PPFD (Table 3.1) it would be expected that greater light intensities would be

almost saturating with regard to full Q_A reduction, certainly at an intensity of 1000 μmol m^{-2} s^{-1} PPFD. This is supported by the increase in F_p with increasing actinic PPFD to 100 μmol m^{-2} s^{-1}, above which increase in F_p with increase in PPFD was negligible. The F_v/F_p ratio increases gradually with increasing actinic PPFD, mainly reflecting the increase in F_p, but the ratio never reaches a value comparable to that obtained in other species, even at 1000 μmol m^{-2} s^{-1} PPFD, as previously discussed. The similarity between F_p and F_s again indicates the reduced quenching over the first 6 min of induction.

It was envisaged that the DCMU addition technique (Krause, *et al.*, 1982) would enable a study of the contribution of q_Q and q_E to fluorescence quenching at steady state (6 min), with the range of actinic light intensities 11.5 to 1000 μmol m^{-2} s^{-1} PPFD. Secondly, the addition of DCMU throughout the induction curve at constant actinic PPFD has been used to demonstrate the contribution of q_Q and q_E, and hence estimate q_R, throughout fluorescence induction (Krause, *et al.*, 1982; Quick and Horton, 1984b; attempted in this study as illustrated in Figure 5.13). The virtual absence of fluorescence quenching over the first 6 min suggested that such further analysis of the induction curve would be very difficult, as a relaxation of non - existent quenching by the addition of DCMU appeared to be impossible. However, it was found that, despite the reduced quenching, the fluorescence level did increase in an apparently biphasic manner on addition of DCMU (Figures 5.8A and 5.9C). This fluorescence increase may represent a further increase in the degree of Q_A reduction, relative to that at steady state (*i.e.* a decrease in q_Q) and a decrease in the trans - thylakoid proton gradient and hence q_E, as described by other workers (*e.g.* Krause, *et al.*, 1982; Horton, 1983; Quick and Horton, 1984b; Hipkins and Baker, 1986; Baker and Horton, 1987; Horton and Hague, 1988). However, due to the considerable differences between the fluorescence response of the isolated chloroplasts in this study and responses typically observed by other workers it is uncertain whether the DCMU - induced fluorescence increase observed in both cases share the same underlying mechanisms and, if they do, whether they are occurring to the same extent. Indeed, as shown in Figure 5.9, the fluorescence increase upon DCMU addition at steady state observed with *C. fragile* chloroplasts was very much smaller than the fluorescence increase obtained with other species (*e.g.* Krause, *et al.*, 1982; Horton, 1983; Quick and Horton, 1984b; Hipkins and Baker, 1986; Horton and Hague, 1988), as might be expected in the relative

absence of fluorescence quenching at the steady state with *Codium* chloroplasts. Therefore, although the various fluorescence levels can be normalised with respect to 'available' variable fluorescence, according to the principles of Schreiber, *et al.* (1986) as described by Horton and Hague (1988), the resulting parameters termed q_Q, q_E, q_R and q_{NP} and the total quenching, TOT (Figures 5.11, 5.12, 5.13, 5.16 and 5.17), are not necessarily equivalent to these parameters, as measured by other workers. That is, an estimation of these quenching parameters, along with q_I and q_T quenching, as defined by other workers, was not possible in isolated *C. fragile* chloroplasts. Thus, the relationship between these and other parameters often reported, such as the apparent quantum yield of oxygen evolution (ϕ = net PS/ PPFD) and the quantum yield of open reaction centres (ϕ/q_Q), and the resulting information that they provide concerning the photosynthetic response to changing conditions, such as light intensity, are not available from the measurements made with *C. fragile* chloroplasts in this study (*e.g.* Dietz, *et al.*, 1985; Schreiber, *et al.*, 1986; Havaux, 1987; Horton and Hague, 1988; Horton, *et al.*, 1988; Genty, *et al.*, 1989; Horton, *et al.*, 1989; Quick and Stitt, 1989; Weis and Lechtenberg, 1989). Thus, the parameters q_Q, q_E, q_R, q_{NP} and TOT may be used simply as a means of further characterising the fluorescence response observed in this study as described in the results section, but a comparison of these parameters with those reported elsewhere requires extreme caution. The q_Q parameter declines with increasing PPFD perhaps suggesting that dissipation of excitation energy by photosynthesis became a smaller proportion as the light intensity increased (Figures 5.11 and 5.12). The q_{NP} component increased complimentarily, the q_R component increased as PPFD increased, whilst q_E first rose and then fell. These results are very similar to the results of Horton and Hague (1988) obtained in a similar study of barley (*Hordeum vulgare* L. cv Marko) protoplasts using saturation pulse and DCMU addition at the steady state over a range of actinic PPFD up to approximately 3,250 μmol m^{-2} s^{-1}. Certainly, the changes of q_Q, q_E, q_{NP} and q_R with increasing PPFD, up to 250 μmol m^{-2} s^{-1}, superficially resemble those described by Horton and Hague (1988). However, the DCMU - induced rise and hence q_Q and q_E, if they are associated with this rise, were completely suppressed at 1000 μmol m^{-2} s^{-1} PPFD (Figures 5.11 and 5.12). In the study of Horton and Hague (1988) q_E rose to a maximum value at approximately 1500 μmol m^{-2} s^{-1} and remained unchanged with increasing actinic PPFD up to approximately 3,250 μmol m^{-2} s^{-1}, whilst q_Q was not fully suppressed even at this intensity. This

further suggests that the q_Q, q_E, q_R and q_{NP} parameters measured in this study are not comparable to those determined in other investigations.

A final series of experiments explored the influence of photoinhibitory pretreatment on the fluorescence response characteristics identified in the preceding work. A preliminary investigation of isolated thylakoids showed the fluorescence response (Figure 5.18) to be similar to that of isolated chloroplasts. The F_o level obtained with isolated chloroplasts showed a slight increase after 5 min photoinhibition but then decreased with increasing pretreatment time. The net photosynthesis, F_p, F_{ss} and F_v/F_p parameters and the DCMU induced fluorescence rise at steady state all decreased with increasing photoinhibition (Figures 5.14 and 5.15). The general changes in the fluorescence induction curve are similar to those reported elsewhere. For example, Bradbury and Baker (1986) found that exposure of intact pea chloroplasts to 3000 μmol m^{-2} s^{-1} of broad band blue irradiation for 10 min reduced the peak fluorescence, F_p. Over a 20 min time course of photoinhibition these workers found that F_m (analagous to F_p in this study) decreased and they suggested that this indicated the development of a stress - induced, non - photochemical quenching process, which operated independently of thylakoid photochemical activity. F_o increased initially with the onset of the stress and then decreased, whilst F_v/F_m (F_v/F_p in this study) decreased throughout. The rapid decrease in F_v/F_m over the first 2 min of the stress was mainly due to a large increase in F_o, whilst a more gradual decrease in F_v/F_m after 2 min was due to a greater decrease in F_m than F_o. In the present study the decrease in F_v/F_p was most rapid over the first 5 min of stress and was clearly enhanced by an increase in F_o (Figure 5.15). The decline in F_v/F_p began to decrease between 5 and 15 min mainly due to the decrease in F_o over this time period. Bradbury and Baker (1986) suggested that such data indicates the involvement of at least two distinct phenomena in producing the changes in the fluorescence response upon photoinhibition. One process results in an increase in F_o which is characteristic of PSII inactivation, possibly due to a decrease in the photochemical efficiency of PSII reaction centres, whilst the second is responsible for the quenching of both F_o and F_m (F_p). The quenching of F_m (F_p) was proportionally greater than that of F_o, as in this study (Figure 5.15), indicating a preferential quenching of F_v, which may indicate an increase in a non - photochemical quenching process at or close to the reaction centre. That is, it has been suggested that a decrease in F_v may possibly be due to damage to pigments in close association with the PSII

reaction centre since the contribution to F_v is greater from antenna pigments closer to the reaction centres than those which are more remote. Alternatively, there is evidence that variable fluorescence is recombination luminescence from the reaction centre and therefore preferential quenching of F_v could indicate extensive damage to the reaction centres, such that charge recombination is prevented (Baker and Horton, 1987). The parameter F_v/F_m (F_v/F_p) is proportional to the quantum yield of photochemistry (*e.g.* Bjorkman, 1987; Krause and Somersalo, 1989) and a decline in this ratio is symptomatic of the effect of photoinhibitory stress and such changes indicate a loss of photochemical efficiency, however, it is important to distinguish changes in F_o from changes in F_v, as outlined above. A decrease in the variable fluorescence which is not readily reversible (*i.e.* not q_E quenching) as a consequence of photoinhibition has been reported in intact leaves (Powles and Bjorkman, 1982), algal cells (Kyle, *et al.*, 1984; Nedbal, *et al.*, 1986), isolated chloroplasts (Critchley, 1981) and in isolated thylakoids (Cleland and Critchley, 1985). Thus, the pretreatment induced changes in the fluorescence parameters F_o, F_p, F_v and F_v/F_p observed in this study are typical of those reported by other workers and clearly indicate the occurrence of photoinhibition.

The F_v/F_p ratio was very sensitive exhibiting the highest rate of inhibition over the first 10 min (Figure 5.15), whilst the decline in net photosynthesis was less dramatic and approximately linear up to 15 min. Interestingly, from 15 to 25 min, the degree of inhibition of net photosynthesis and F_v/F_p were very similar. An initial rapid inhibition of the F_v/F_p ratio indicates rapid reduction in the quantum yield of photochemistry, predominantly due to an increase in F_o which, in turn, may indicate changes in the photochemical efficiency of PSII reaction centres. The net photosynthesis is inhibited to a much lesser extent. This suggests a greater inhibition of quantum yield than maximum photosynthetic capacity and this has been previously reported (Baker and Horton, 1987). From 15 to 25 min pretreatment the F_v/F_p ratio and net photosynthesis are inhibited to the same extent suggesting that quantum yield and maximum photosynthetic capacity are equally affected.

The increase in F_o has been observed by a number of workers (Powles and Bjorkman, 1982; Bradbury and Baker, 1986; Cleland, 1988; Nedbal, *et al.*, 1990; Setlik, *et al.*, 1990) and Demmig and Bjorkman (1987) suggested that under conditions of excess excitation an increase in K_D, the rate constant for non - radiative decay in the antenna, caused the observed quenching in F_v and F_o and hence the decline in F_v/F_m,

whilst a decrease in K_p, the rate constant for photochemistry, was suggested to explain the rise of F_o. General loss of variable fluorescence has been suggested to be associated with the transformation of PSII reaction centres to photochemically inactive fluorescence quenchers (Cleland and Critchley, 1985; Cleland, *et al.*, 1986; Cleland and Melis, 1987). Krause, *et al.* (1990) similarly proposed that photoinhibition is based on transformation of active reaction centres to photochemically inactive fluorescence quenchers, which convert excitation energy to heat. These workers found no evidence for increased thermal deactivation in the antenna system of PSII. Kyle (1987) reported that the variable fluorescence is quenched during photoinhibition but that the reaction centre remains intact and suggested several mechanisms by which damage and removal of the D1 protein may alter rate constants for alternative mechanisms of energy dissipation, including the following: establishment of a futile cycle around PSII via cyt b_{559}, or Q_A donation to $P680^+$, increasing the rate constant of photochemistry (K_p); an enhancement of energy transfer to PSI; accumulation of the quenched reaction centre state P680 - $Phaeo^-$ by a low quantum yield electron donation to $P680^+$; and, an increase in the rate constant for thermal deactivation. It has been shown that characteristics of quenching of the 77K fluorescence of photoinhibited leaves are consistent with, and can only be explained by, an increase in the rate constant for thermal deactivation (Bjorkman, 1987; Demmig and Bjorkman, 1987). It has also been suggested that an increased K_D may represent a protective mechanism to prevent futher photoinhibitory damage to PSII (Krause and Cornic, 1987).

Cleland (1988) also suggested that an initial rise in F_o and decrease in F_m, and a subsequent decrease in F_o may reflect the operation of two processes during photoinhibition. It was further suggested that the initial process was due to a decrease in K_p, resulting in the rise in F_o, and perhaps being associated with the actual event of damage. A second process, perhaps occurring virtually simultaneously, responsible for the quenching of F_v and F_o, was suggested to be caused by an increase in either K_d, the rate constant for non - radiative decay from the reaction centre, and/ or K_D, the rate constant for non - radiative decay in the antenna. Cleland (1988) believes that the most likely site of primary damage during photoinhibition is P680 and that associated alterations in either the forward or back reaction of charge separation may possibly account for the fluorescence changes. If the energy required for the back reaction to proceed is decreased, then the rate of charge separation would also decrease as the

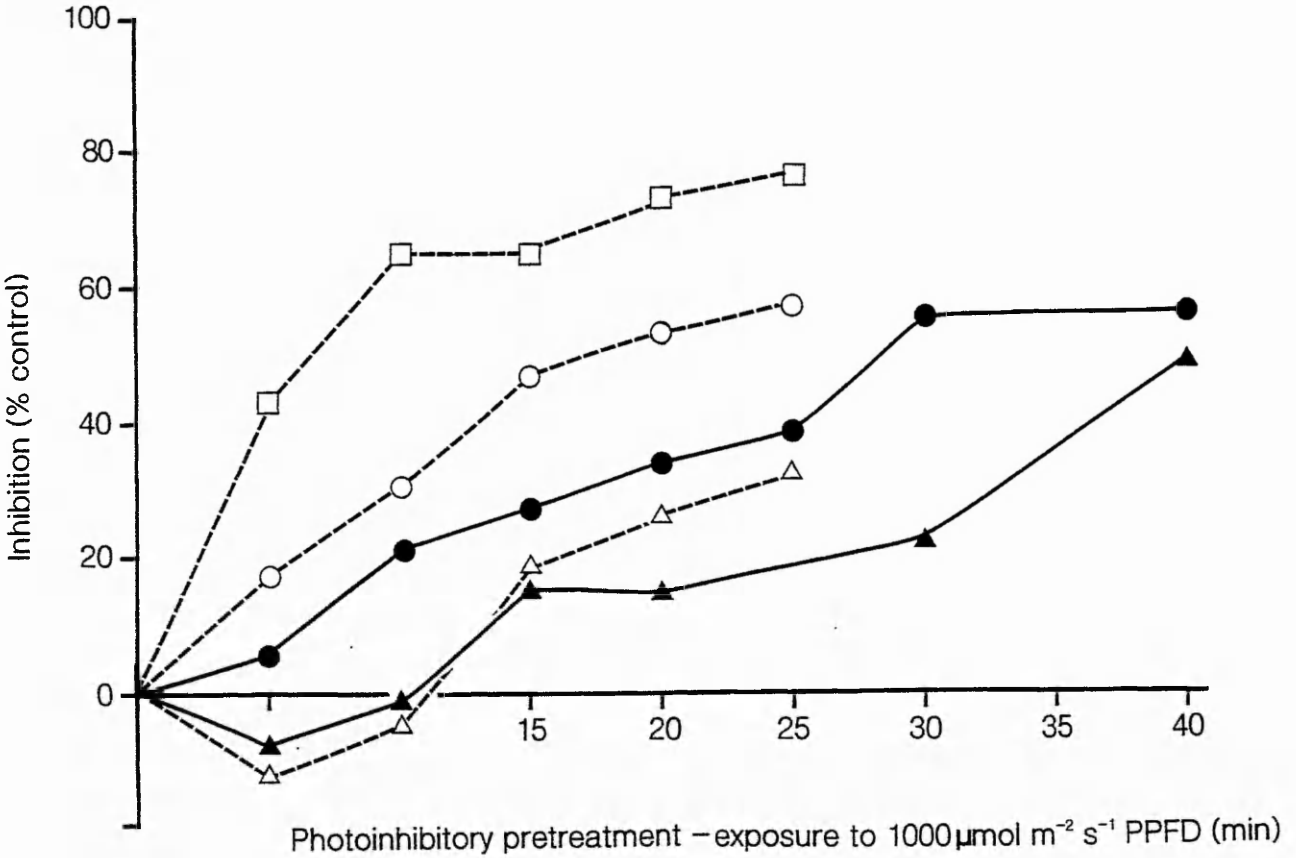
probability that the electron would fall back to the ground state would be greater than that of its proceeding to the electron acceptor. This may be associated with an increase in K_d which may contribute to fluorescence quenching, due to an increase in non - radiative decay from the reaction centre. Thus, the primary event is damage to P680, which decreases K_p and therefore increases F_o. The result of this primary event is an increase in the probability of the back reaction, which dissipates any further captured energy (increase in K_d) and effectively quenches both F_o and F_m.

Setlik, *et al.* (1990) identified three types of photoinactivation process. A fast process that occurred under strongly reducing and anaerobic conditions and resulted in a decline of F_v and Hill reaction rate (electron transport through PSII using benzoquinone or ferricyanide as electron acceptors), accompanied by an increase in F_o, was suggested to be due to trapping of Q_A in a negatively charged stable state (Q_A^-). A slow process was characterised by a decline of F_v and Hill reaction rate and was suggested to consist of the neutralisation of the negative charge in the Q_A domain in a manner that rendered Q_A non - functional; charge separation in the reaction centre was suggested to be still possible, but with the energy of excitation being dissipated thermally. A very slow process was linked to loss of charge separation ability of the reaction centre. Thus, these workers favour the view that Q_A is the primary site of damage. Kirilovsky, *et al.* (1988) reported a lack of correlation between the decrease of variable fluorescence and damage to the reaction centre (primary photochemistry) and concluded that a decrease in F_v is related to the damage of the Q_B site, which they suggested was the primary target of photoinhibition. Similarly, *Spirodela oligorhiza* depleted *in vivo* of a large proportion of D1 protein exhibited reduction in variable fluorescence (Edelman, *et al.*, 1984). Ohad, *et al.* (1988) reported a series of gradual changes induced in the reaction centre of PSII by photoinhibitory conditions. Firstly, a change in the whole population of reaction centre II affecting the $S_2Q_B^-$ stability and accompanied by a corresponding rise in the F_o value, with only a slight loss of the $S_2Q_A^-$ thermoluminescence signal, occurred. This was followed by a complete loss of the $S_2Q_B^-$ signal and loss of the $S_2Q_A^-$ signal, which paralleled the reduction of the maximal variable fluorescence in the presence of DCMU, F_m. This second stage was related to a total loss of reaction centre II activity. Subsequent work found that initial reversible modification of reaction centre II, involving modification of the stability of the Q_B^- binding site was correlated with a proportional loss of variable fluorescence and partial

loss of oxygen evolution (Ohad, *et al.*, 1990), similar to the results observed in this study. The primary photochemistry was not affected. It was further suggested that the change in stability of Q_B^- could be considered as a lowering of its redox potential leading to equilibration of the charge with Q_A resulting in the formation of Q_A^-, thus causing the rise in F_o. This would also result in the reduction of the rate of electron flow to the plastoquinone pool. It was reported that in photoinhibited cells the kinase responsible for phosphorylation of LHCII is inactivated resulting in complete dephosphorylation and dissociation of PSII from the antenna complex. This may be a reason for the reduction of F_m upon photoinhibition.

Thus, it seems generally accepted that the fluorescence response upon photoinhibition indicates that at least two processes are occurring. As observed in this study of *C. fragile* chloroplasts, there is a process that causes an initial rise in F_o and superimposed on this is a second process responsible for the preferential quenching of F_v. These fluorescence changes have been interpreted in many different ways by different workers. A comparison of the percentage inhibition of DMBQ - and SiMo - dependent oxygen evolution (Figure 4.6) and the percentage inhibition of F_o, F_p and F_v/F_p is interesting (Figure 5.19). It was concluded in Chapter 4 that photoinhibition in *C. fragile* occurs in a two stage process. The first stage involves inactivation of the Q_B site (as shown by inhibition of DMBQ - dependent O_2 evolution), whilst the second stage in the process involves further damage to the electron transport chain between water and the primary quinone acceptor, Q_A (as shown by inhibition of SiMo - dependent O_2 evolution). There is a similarity between the inhibition of F_o and that of SiMo - dependent O_2 evolution over the first 15 min of photoinhibitory pretreatment (Figure 5.19). This perhaps suggests that the proposals of Setlik, *et al.* (1990) and Ohad, *et al.* (1988, 1990) best explain the results of this study. Both groups of workers suggest that an initial event in photoinhibition involves the generation of Q_A^- and that this may cause the increase in F_o. The variable fluorescence was also reduced but the primary photochemistry was not affected and Setlik, *et al.* (1990) reported reduction in electron flow from water to p - benzoquinone, whilst Ohad, *et al.* (1988, 1990) reported a reduction in electron flow from water to 2, 3 - dichloro - 1, 4 - benzoquinone, but the persistence of electron transfer from water to SiMo (Kyle, *et al.*, 1984). This appears to be similar to the first stage of photoinhibition in *C. fragile* chloroplasts which revealed an initial increase in F_o accompanied by a reduction in

Figure 5.19 Photoinhibition of Q_B - dependent electron flow (water to DMBQ, ●), Q_B - independent electron flow (water to SiMo, ▲), F_o (△), F_p (○) and F_v/F_p (□). The parameters are expressed as a percentage decrease from the values obtained with the control sample (before photoinhibition). The thylakoid partial reactions were carried out according to the legend of Figure 4.6 and the fluorescence parameters measured according to the legend of Figure 5.15. Thylakoid or chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll were illuminated at 1000 μmol m^{-2} s^{-1} PPFD for up to 40 or 25 min, respectively. Each point represents the mean of 3 to 8 observations. See text for details.


100
80
60
40
20
0
Inhibition (% control)
15
20
25
30
35
40
Photoinhibitory pretreatment – exposure to 1000 µmol m⁻² s⁻¹ PPFD (min)

variable fluorescence, a reduction in electron flow from water to DMBQ, but persistence of electron transfer from water to SiMo, over the first 10 min of photoinhibitory pretreatment (Figure 5.19). Setlik, *et al.* (1990) described a following stage (with a $t_{1/2}$ of 15 - 40 min) characterised by a constant F_o level and a parallel decline in F_v and electron transfer from water to p - benzoquinone. This was associated with the neutralisation of the negative charge on Q_A^-, rendering Q_A non - functional. It is suggested that had SiMo - dependent O_2 evolution been measured that inhibition of this activity would have occurred at this stage. Reaction centre charge separation was still operational but the excitation energy was dissipated thermally. A final very slow process ($t_{1/2}$ > 100 min) was linked to loss of reaction centre charge separation ability. Ohad, *et al.* (1988, 1990) only reported two stages, the second of which was associated with the complete loss of the thermoluminescence signal $S_2Q_B^-$ and a loss of the $S_2Q_A^-$ signal in parallel with a loss in F_m (representing loss of F_v) and a loss of reaction centre II activity. The loss of the $S_2Q_A^-$ signal may represent the in - activation of Q_A as described by Setlik, *et al.* (1990). If Q_A^- is inititially trapped in the negative state electron transfer from water to DMBQ will be blocked but SiMo may sill be able to accept electrons from Q_A^-, allowing this activity to continue. These partial reaction studies therefore seem to suggest that Q_B is the primary site of photoinhibition, as discussed in Chapter 4. However, the development of Q_A^- (as suggested by the increase in F_o) may eventually lead to the complete inactivation of Q_A. If Q_A is non - functional then the rate of electron transport from water to DMBQ and, more pertinently, from water to SiMo would be inhibited and this appears to be the case once 10 min photoinhibitory pretreatment is surpassed (Figure 5.19). Thus the results of Chapter 4 in conjunction with the results of Chapter 5 may be interpreted as suggesting that the site of primary lesion of photoinhibition in *C. fragile* is the primary quinone acceptor, Q_A. It is evident from the previous discussion that changes in the kinetics of chlorophyll fluorescence induced by photoinhibitory treatments can be used in the study of the nature of photoinhibitory damage to photosynthetic systems. There were marked differences between the fluorescence response of isolated chloroplasts from *C. fragile* and that typical of other organisms that could not be explained adequately and it is suggested that further characterisation of the fluorescence response in the chloroplasts and fronds of this alga is desirable. It cannot be over - emphasised that many factors influence the fluorescence - emission characteristics of thylakoids, chloroplasts,

protoplasts and leaves and therefore care must be taken in interpreting the underlying reasons for such fluorescence changes. Similar changes in fluorescence characteristics may be observed from a given system for different reasons and the need for caution is increased due to the differences in the fluorescence induction curve observed in this study. Where possible, measurement of other photosynthetic parameters should be made to support conclusions based on analysis of the characteristics of chlorophyll fluorescence.

5.5 Conclusions

The parameters F_v/F_m and F_v/F_p were essentially equal, having values of 0.7513 and 0.7687, respectively, when measured with red actinic light (660 nm) of 400 μmol m^{-2} s^{-1} intensity and the TR1 measuring system. These values are within the range typically reported in all species so far investigated. In contrast, the F_v/F_p ratios obtained when using white actinic light and the MFMS were very low, ranging from 0.3384 to 0.4747 at light intensities of 11.5 and 1000 μmol m^{-2} s^{-1} PPFD, respectively. Thus, the F_o fluorescence was a much greater proportion of the total fluorescence yield in *C. fragile* chloroplasts than in other species studied using similar techniques. Furthermore, fluorescence quenching from the initial peak was highly reduced in comparison to the substantial quenching typically observed with other species, when using both the red light/ TR1 or MFMS. The reason for this reduced quenching (and suppressed F_v when using the MFMS), is uncertain, especially as intact fronds of *C. fragile* are known to exhibit fluorescence induction characteristics similar to those typical of other species, when using the MFMS. Despite the reduced quenching, the fluorescence level increased in an apparently biphasic manner, on addition of DCMU. It is uncertain whether the DCMU induced fluorescence increase shares the same underlying mechanisms as the DCMU induced fluorescence increases described by other workers and therefore the parameters designated q_Q, q_E, q_R, q_{NP} and TOT may not be comparable to those defined in the literature. Photoinhibitory pretreatment of isolated chloroplasts induced changes in the fluorescence inductiion kinetics which suggested that at least two processes were occurring. There was initial rise in F_o and a preferential quenching of F_v. The increase in F_o has been associated with the generation of Q_A^-. The results of this Chapter in conjunction with those of Chapter 4 and the proposals of Setlik, *et al.*(1990) and Ohad, *et al.* (1988, 1990), provide preliminary evidence that may be interpreted as suggesting

that the site of primary lesion of photoinhibition in *C. fragile* is the primary quinone acceptor, Q_A.

CHAPTER 6. ^{35}S - METHIONINE INCORPORATION INTO *C. FRAGILE* THYLAKOID POLYPEPTIDES UNDER CONTROL AND PHOTOINHIBITORY REGIMES.

6.1 Introduction

Early studies revealed that isolated intact *Pisum sativum* chloroplasts use both light energy and added ATP to incorporate ^{35}S - Methionine (^{35}S - Met) into membrane bound proteins, and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS - PAGE) revealed that one of the major products of such protein synthesis was of 32 KDa molecular weight (MW), now generally referred to as the D1 protein (*e.g.* Eaglesham and Ellis, 1974). Ellis and Hartley (1982) provide a detailed description of the methods used for *in organello* radiolabelling of thylakoid proteins with ^{35}S - Met. They found that the most active chloroplasts were those prepared from pea (*Pisum sativum*), whilst those isolated from spinach (*Spinacia oleracea*), tobacco (*Nicotiana tabacum*), maize (*Zea mays*) and barley (*Hordeum vulgare*) were less active. The preparation of chloroplasts active in light - driven protein synthesis from bean (*Phaseolus vulgaris*) and wheat (*Triticum vulgare*) was not possible. They further suggested that the most convenient method for analysing the products of incorporation of labelled amino acids is SDS - PAGE, followed by fluorography or scintillation counting of gel slices. There are many detailed accounts of such methods (*e.g.* Chua, 1980; Hames, 1981; Piccioni, *et al.*, 1982). Further work led to improvements in the incubation conditions of isolated chloroplasts that enabled efficient translation of endogenous messenger RNA, using light as an energy source, for up to one hour (Fish and Jagendorf, 1982; Nivison and Jagendorf, 1984; Nivison, *et al.*, 1986).

A major problem encountered when studying the D1 protein is its low steady - state concentration compared to other chloroplast proteins. This can make visualisation via protein staining techniques difficult. Therefore, Marder, *et al.* (1986) suggested that identification and characterisation of the D1 protein is best achieved by radiolabelling, gel electrophoresis and fluorography, and these workers provide further details of these methods, along with details concerning the preparation of labelled samples for SDS - PAGE. Using similar techniques rapid synthesis of a 32 KDa thylakoid membrane protein was demonstrated in *Spirodela oligorrhiza*, *Chlamydomonas reinhardtii*, *Pisum sativum*, *Zea mays*, *Nicotiana tabacum*, *Solanum nigrum*, *Bromus tectorum* and *Lactuca*

sativa (Mattoo, *et al.*, 1981; Hoffman - Falk, *et al.*, 1982). These workers found significant sequence homologies among the various 32 KDa polypeptides from the species studied. Preferential labelling of 32 - 34 KDa polypeptide bands was also observed by Wettern, *et al.* (1983) in a sudy of *Chlamydomonas reinhardtii* and the turnover of a 32.5 KDa polypeptide was found to be light dependent and inhibited by DCMU, indicating that its proteolysis was induced by or followed changes occurring during active electron flow via PSII. The rapid, light - dependent metabolism of a 32 KDa protein and its broad species distribution and phylogenetic similarity was also reported by Edelman, *et al.* (1984). It was also suggested that the protein may become denatured as a consequence of its function, making it necessary for replacement at a rate attuned to the rate of degradation. The D1 protein, pulse - labelled with $^{35}SO_4$ prior to photoinhibitory treatment, has been shown to be lost during photoinhibition (Ohad, *et al.*, 1984) and it was suggested that the turnover of this protein was a normal consequence of its function *in vivo* and is a physiological process that is necessary to maintain the photosynthetic integrity of the thylakoid membrane. It was proposed that photoinhibition occurs when the rate of light - dependent inactivation and subsequent removal exceeds the rate of resynthesis of D1 (Ohad, *et al.*, 1985; Kyle and Ohad, 1986). However, other workers have provided evidence that the primary damage causing photoinhibition involves inactivation of the reaction centre function and that degradation of D1 may be a consequence, but is not the cause, of photoinhibition (*e.g.* Cleland and Critchley, 1985; Cleland, *et al.*, 1986; Cleland and Melis, 1987; Cleland, 1988; Critchley, 1988; Cleland, *et al.*, 1990).

Thus, the objectives of the work described in this Chapter were to prepare chloroplasts from *C. fragile* frond tips active in light - driven protein synthesis, enabling the *in organello* radiolabelling of thylakoid proteins with ^{35}S - Met. An initial investigation was intended to determine the optimum conditions for radiolabelling and for the preparation of radiolabelled samples for subsequent analysis by SDS - PAGE, fluorography and scintillation counting of gel slices, allowing the identification and characterisation of radiolabelled polypeptides. Further study of the time course of radiolabel incorporation under optimal conditions was intended to lead to experiments involving the pre - radiolabelling of thylakoid proteins prior to photoinhibitory treatment. It was envisaged that such an approach would yield information concerning the turnover of thylakoid polypeptides during photoinhibition, particularly the D1

protein, perhaps further elucidating the role of this PSII component in high - light stress of *C. fragile* chloroplasts.

6.2 Materials and Methods

6.2.1 Sampling and Maintenance of C. fragile

Fronds of *C. fragile* were harvested on 19/ 12/ 89 (winter - vegetative) and 23/ 06/ 90 (early summer - reproductive) as described previously (2.2.1) and similarly maintained.

6.2.2 Protein Estimation

For protein determination the method of Lowry, *et al.* (1951) was used. The assay mixture contained a final concentration of 0.15 % (w/v) SDS in order to solubilise all proteins for assay. Stock reagent solutions were 0.4 % (w/v) NaOH, 2 % (w/v) Na_2CO_3 and 0.02 % (w/v) sodium potassium tartrate (solution A) and 0.5 % (w/v) $CuSO_4.5H_2O$ (solution B) in distilled water. A 100 μl volume of protein sample was added to 100 μl of 2 % (w/v) SDS and vortex mixed. Stock solutions were then mixed in a ratio of 49 parts A : 1 part B and 1 cm^3 of this solution added to each solubilised protein sample. Samples were vortex mixed and incubated for 20 min at room temperature. To each sample, 100 μl of Folin - Ciocalteau reagent (diluted 1:1 with distilled water) was added. Samples were vortex mixed and incubated for a further 20 min at room temperature, when the absorbance at 750 nm was measured using a Cecil CE 292 Series 2 Digital Ultra Violet spectrophotometer. Bovine serum albumin, Sigma Fraction V (0 - 1 mg cm^{-3}) was used to construct a standard curve (Figure 6.1 in Results section 6.3).

6.2.3 SDS - PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out using the method of Laemmli (1970). The following solutions were made up in distilled water:

Tris - glycine electrode buffer	15.15 g Tris base
(25 mol m^{-3} Tris, 192 mol m^{-3}	72.00 g glycine
glycine) pH 8.3	5.00 g SDS
	pH 8.3 in 5000 cm^3

Tris - SDS stock solution	30.30 g Tris base
(0.25 mol dm^{-3} Tris) pH 6.8	2.00 g SDS
	pH 6.8 in 1000 cm^3
Tris - SDS stock solution	90.80 g Tris base
(0.75 mol dm^{-3} Tris) pH 8.8	2.00 g SDS
	pH 8.8 in 1000 cm^3
Sample buffer	25 cm^3 Tris - SDS stock, pH 6.8
(62.5 mol m^{-3} Tris)	2.00 g SDS
	10 cm^3 glycerol
	5 cm^3 2 - mercaptoethanol
	0.1 cm^3 1 % (w/v) bromophenol blue
	in 100 cm^3
Acrylamide stock	30.00 g acrylamide
	0.80 g N' - N methylene - bisacrylamide
	in 100 cm^3
Ammonium persulphate	0.10 g in 10 cm^3
TEMED	(N, N, N', N' - tetramethylenediamine)

The gel mould apparatus used for vertical slab gel electrophoresis (L.K.B., Bromma, Sweden) was assembled according to the manufaturer's instructions after the glass plates had been cleaned with detergent, followed by distilled water and ethanol. Gel spacers were used to produce 1.5 mm thick gels. The resolving gel was prepared by mixing 25 cm^3 of acrylamide stock solution, 30 cm^3 of Tris - SDS stock solution (pH 8.8) and 5 cm^3 of distilled water in a Buchner flask, and was degassed for 10 min. Immediately before the gels were poured, 1.5 cm^3 of freshly made 1 % (w/v) ammonium persulphate and 15 μl of TEMED were added. This solution was mixed avoiding the introduction of air and then poured into the gel mould to a height of 12 cm. The gel solution was carefully overlaid with 2 - butanol to exclude air and produce a flat top to the gel. Polymerisation was allowed to proceed for two hours at room temperature. Before applying the stacking gel, the 2 - butanol was removed and the

surface of the gel washed with 50 % (v/v) Tris - SDS stock solution (pH 8.8) and any excess buffer absorbed with filter paper. The stacking gel solution was prepared by mixing 2 cm^3 of acrylamide stock, 10 cm^3 of Tris - SDS stock solution (pH 6.8) and 8 cm^3 of distilled water. Following degassing for 10 min, 0.5 cm^3 of freshly made 1 % (w/v) ammonium persulphate and 10 μl of TEMED were added and the mixed solution poured on top of the resolving gel to the top of the gel mould which contained a comb for the formation of sample wells. Polymerisation was allowed to proceed for two hours at room temperature before sample application. The gels prepared contained acrylamide concentrations of 12.5 % (w/v) and 3 % (w/v) in the resolving gel and stacking gel, respectively. The sample wells were washed and overlaid with electrode buffer and samples applied to the base of the wells beneath the buffer using a microlitre syringe. The remaining electrophoresis apparatus was assembled and electrophoresis carried out at a constant current of 30 mA per gel until the tracking dye was approximately 1 cm from the gel base. The cooling coil was connected to a Techne C400 circulator and Techne M1000 heat exchange unit, which were adjusted to maintain the circulating water at 5 °C.

Molecular weight protein standards (Sigma) were electrophoresed adjacent to the samples to allow estimation of sample protein molecular weights. In an initial experiment the protein standards were: myosin from rabbit muscle (205 KDa); ß - galactosidase from Escherichia coli (116); phosphorylase b from rabbit muscle (97); bovine albumin (66); egg albumin (45);and carbonic anhydrase from bovine erythrocytes (29). In all further experiments different standards were used, as follows. An SDS - 7 Dalton Mark VII - L molecular weight marker kit containing 3.5 mg of a lyophilised mixture of 7 protein standards (Sigma) was allowed to solubilise overnight at 4 °C in 2 cm^3 of sample buffer, giving 35 μg of protein in 20 μl (*i.e.* 5 μg per protein in 20 μl) for loading in sample wells. This preparation gave approximately 50 x 40 μl volumes of protein molecular weight markers solubilised in sample buffer, which were stored in Eppendorf tubes in a -20 °C refrigerator. The seven protein standards were: bovine albumin (66 KDa); egg albumin (45); glceraldehyde - 3 - phosphate dehydrogenase from rabbit muscle (36); carbonic anhydrase from bovine erythrocytes (29); PMSF treated trypsinogen (24); soybean trypsin inhibitor (20.1); and α - lactalbumin (14.2). Prior to electrophoresis a 40 μl volume of molecular weight markers in sample buffer were immersed in boiling water for four min to ensure that

the proteins were dissolved and fully denatured.

6.2.4 Fixing, Staining and Destaining of Gels

Following electrophoresis, gels were carefully removed from the glass mould and fixed for 30 min in fixative solution containing 500 cm^3 methanol, 500 cm^3 distilled water and 100 cm^3 glacial acetic acid. The gel was then stained for two hours in 1000 cm^3 of 0.1 % (w/v) Coomassie Brilliant Blue G (Sigma) dissolved in fixative and filtered. Gels were destained in a solution containing 5 % (v/v) methanol and 7 % (v/v) glacial acetic acid in distilled water. Once destained, the gels were photographed and placed on a glass plate over a light box to facilitate measurement of protein band migration and hence the determination of R_f values, the construction of a standard molecular weight curve (Figure 6.2 in Results section 6.3) and the estimation of molecular weights of the protein bands separated in the sample lanes. Gels used to separate radiolabelled proteins were further analysed by scintillation counting of excised, solubilised gel slices (6.2.5) or by fluorography (6.2.6).

6.2.5 Determination of Radioactivity in Acrylamide Gel Sections

Following SDS - PAGE (6.2.3), fixing, staining and destaining (6.2.4), the radioactivity in acrylamide gel slices was determined by liquid scintillation counting using the method described by Hames (1981). Gels were placed on a glass plate on a light box and a sharp razor blade used to separate individual lanes and carefully excise the protein bands that had been separated and visualised by Coomassie Blue staining (6.2.4) in each sample lane. Each gel slice was placed in a clean glass scintillation vial and allowed to dry at 55 °C for three hours. A 1 cm^3 volume of 30 % (w/v) H_2O_2 (100 volumes) was added to each vial so that the gel slices were completely immersed and the vials were tightly capped and incubated at 55 °C to allow solubilisation of the acrylamide. The vials were allowed to cool to room temperature and 10 cm^3 of OptiPhase 'Hisafe' II (L.K.B.) scintillation cocktail added for liquid scintillation counting. To detect the ^{35}S - Met radioisotope the scintillation vials were placed in racks and loaded into a Packard Tri - Carb 300c Liquid Scintillation Counter with channels A and B set in the ranges 0 - 156 and 4 - 156 Kv, respectively. Radiolabelled samples were generally counted for 10 min.

6.2.6 Fluorography

Following SDS - PAGE (6.2.3), fixing, staining and destaining (6.2.4), the ^{35}S - Met radiolabelled proteins were detected by fluorography (Hames, 1981), as follows. Gels were immersed in 'Entensify - Part A, universal autoradiography enhancer: precipitating reagent' (Du Pont, Boston, USA) and gently agitated for 45 min. The gels were then immersed in 'Entensify - Part B, universal autoradiography enhancer: aqueous fluor solution' for a further 45 min. The gels were then placed on Whatman 3 mm paper, covered with cling film and dried at 80 °C under vacuum for eighty min in a BioRad Model 543 gel dryer. Dried gels were exposed to pre - flashed Fuji - RX medical X - ray film at -70 °C in a light tight x - ray film cassette for time periods between 7 1/4 hours and 17 days. If the film is pre - sensitised by exposure to an instantaneous flash of light ($\leq$ 1 msec) the absorbance of the fluorographic image becomes proportional to the sample radioactivity and the sensitivity of the method is increased. The intensity of the flash was adjusted to increase the absorbance of the developed film to 0.15 (A_{540} nm) above that of the unexposed film (Laskey and Mills, 1975). After a suitable period of exposure, the film was developed by gentle agitation in Kodak D19 developer for two min, followed by a one min immersion in Ilford Hypam fixative. Developed films were rinsed for 10 min in running tap water and were air dried at room temperature.

6.2.7 Optimisation of Incubation Conditions for the *In Organello* Radiolabelling of Thylakoid Proteins with ^{35}S -Met and Subsequent Sample Preparation for SDS - PAGE.

Chloroplasts were isolated from frond tips of *C. fragile* harvested on 19/ 12/ 89 (winter - vegetative) or 23/ 06/ 90 (early summer - reproductive) as previously described (3.2.3), except that 'modified extraction medium' (designated incubation solution A) was used (0.5 mol dm^{-3} sucrose, 2 g dm^{-3} BSA, 0.015 mol dm^{-3} $MgCl_2.6H_2O$ in 0.05 mol dm^{-3} HEPES buffer, adjusted to pH 7.8 with 1 mol dm^{-3} KOH), *i.e.*, adjusted to pH 7.8 with 1 mol dm^{-3} KOH not with 1 mol dm^{-3} NaOH, as in the original extraction medium. The chloroplast preparation was resuspended in a final volume of 1 cm^3 of solution A and the chlorophyll concentration was determined by the method of MacKinney, 1941 (3.2.3). Chloroplast suspensions were radiolabelled with ^{35}S - Met using incubation solutions and methods based on those described by Ellis and Hartley (1982), Hoffman - Falk, *et al.* (1982), Nivison and Jagendorf (1984) and

Nivison, *et al.* (1986). The radiolabelled samples were prepared for SDS - PAGE using a method based on that described by Marder, *et al.* (1986).

Four DW1 oxygen electrode water jackets were secured to magnetic stirrers and connected in series to a Techne C400 circulator and Techne M1000 heat exchange unit, enabling the incubation temperature to be maintained at 10 °C. Translucent plastic inserts, each containing a magnetic flea, were placed in each oxygen electrode water jacket, providing temperature controlled reaction chambers for light - driven *in organello* radiolabelling. The four reaction chambers were illuminated with four Halight 24/250 projectors at 50 μmol m^{-2} s^{-1} PPFD (at the electrode water jacket surface).

Chloroplast suspensions (final volume 0.5 cm^3) equivalent to 50 μg cm^{-3} chlorophyll were radiolabelled for one hour, under 21 different incubation conditions (summarised in Table 6.1). The amount of ^{35}S - Met (38.5, 50.0 or 100.0 μCi cm^{-3}) and non - radioactive 'cold' Met (0 to 50 μmol dm^{-3}) was varied, giving specific activities of approximately 1 to 785 Ci $mmol^{-1}$, and five incubation solutions were investigated:

Extraction medium: 0.5 mol dm^{-3} sucrose, 2 g dm^{-3} BSA, 0.015 mol dm^{-3} $MgCl_2.6H_2O$ in 0.05 mol dm^{-3} HEPES buffer, adjusted to pH 7.8 with 1 mol dm^{-3} NaOH.

Solution A - modified extraction medium: 0.5 mol dm^{-3} sucrose, 2 g dm^{-3} BSA, 0.015 mol dm^{-3} $MgCl_2.6H_2O$ in 0.05 mol dm^{-3} HEPES buffer, adjusted to pH 7.8 with 1 mol dm^{-3} KOH.

Solution B (Ellis and Hartley, 1982): 0.2 mol dm^{-3} KCl, 66 mol m^{-3} Tricine - KOH (pH 8.3), 6.6 mol m^{-3} $MgCl_2$. $6H_2O$.

Solution C (Ellis and Hartley, 1982): 0.33 mol dm^{-3} sorbitol, 50 mol m^{-3} Tricine - KOH (pH 8.4), 6.6 mol m^{-3} $MgCl_2.6H_2O$.

Solution D (Nivison and Jagendorf, 1984; Nivison, *et al.*, 1986): 1.87 mol m^{-3} EDTA, 200 mmol m^{-3} $MgCl_2.6H_2O$, 200 mmol m^{-3} leucine, 200 mmol m^{-3} isoleucine, 200 mmol m^{-3} threonine, in 350 mol m^{-3} sorbitol, 33 mol m^{-3} HEPES -KOH (pH 8.3), 0.9 mol m^{-3} DTT, 10 mol m^{-3} $NaH_2PO_4.2H_2O$.

A 'cold' control non - radiolabelled sample was provided by incubation of a

Table 6.1 Incubation conditions used for the *in organello* radiolabelling of thylakoid proteins with ^{35}S - Met. Incubation solutions were: E.M. or extraction medium (0.5 mol dm^{-3} sucrose, 2 g dm^{-3} BSA, 0.015 mol dm^{-3} $MgCl_2.6H_2O$ in 0.05 mol dm^{-3} HEPES buffer, adjusted to pH 7.8 with 1 mol dm^{-3} NaOH); A, modified extraction medium (0.5 mol dm^{-3} sucrose, 2 g dm^{-3} BSA, 0.015 mol dm^{-3} $MgCl_2.6H_2O$ in 0.05 mol dm^{-3} HEPES buffer, adjusted to pH 7.8 with 1 mol dm^{-3} KOH); B, 0.2 mol dm^{-3} KCl, 66 mol m^{-3} Tricine - KOH (pH 8.3), 6.6 mol m^{-3} $MgCl_2.6H_2O$; C, 0.33 mol dm^{-3} sorbitol, 50 mol m^{-3} Tricine - KOH (pH 8.4), 6.6 mol m^{-3} $MgCl_2.6H_2O$; D, 1.87 mol m^{-3} EDTA, 200 mmol m^{-3} $MgCl_2.6H_2O$, 200 mmol m^{-3} leucine, 200 mmol m^{-3} isoleucine, 200 mmol m^{-3} threonine, in 350 mol m^{-3} sorbitol, 33 mol m^{-3} HEPES - KOH (pH 8.3), 0.9 mol m^{-3} DTT, 10 mol m^{-3} $NaH_2PO_4.2H_2O$. The four solutions A, B, C and D were used with different combinations of 'cold' non - radioactive Met and ^{35}S - Met, giving specific activities of approximately 1.990, 0.998, 3.980, 89.000 and 785.000 Ci $mmol^{-1}$ and total radioactivity of 100.0, 50.0, 100.0, 100.0 and 100.0 μCi cm^{-3} (II, III, IV, V and VI, respectively). In an initial experiment extraction medium was used with ^{35}S - Met at 38.5 μCi cm^{-3} and 0.860 Ci $mmol^{-1}$ (I). See section 6.2.7 for experimental details. A total of 21 incubation conditions were investigated.

No.	Incubation solution		^{35}Smet[a]	Cold Met[a]	Total Met[a]	Total Act.[b]	Sp. Act.[c]
1	E.M.	I	0.1381	44.56	44.70	38.5	0.860
2	A	II	0.1060	50.00	50.11	100.0	1.990
3	B	II	0.1060	50.00	50.11	100.0	1.990
4	C	II	0.1060	50.00	50.11	100.0	1.990
5	D	II	0.1060	50.00	50.11	100.0	1.990
6	A	III	0.0530	50.00	50.05	50.0	0.998
7	B	III	0.0530	50.00	50.05	50.0	0.998
8	C	III	0.0530	50.00	50.05	50.0	0.998
9	D	III	0.0530	50.00	50.05	50.0	0.988
10	A	IV	0.1060	25.00	25.11	100.0	3.980
11	B	IV	0.1060	25.00	25.11	100.0	3.980
12	C	IV	0.1060	25.00	25.11	100.0	3.980
13	D	IV	0.1060	25.00	25.11	100.0	3.980
14	A	V	0.1300	1.00	1.13	100.0	89.000
15	B	V	0.1300	1.00	1.13	100.0	89.000
16	C	V	0.1300	1.00	1.13	100.0	89.000
17	D	V	0.1300	1.00	1.13	100.0	89.000
18	A	VI	0.1300	0.00	0.13	100.0	785.000
19	B	VI	0.1300	0.00	0.13	100.0	785.000
20	C	VI	0.1300	0.00	0.13	100.0	785.000
21	D	VI	0.1300	0.00	0.13	100.0	785.000

a, mmol m^{-3} b, μCi cm^{-3} c, Ci $mmol^{-1}$

chloroplast suspension equivalent to 50 μg cm^{-3} chlorophyll, in the absence of ^{35}S - Met.

After one hour of radiolabelling, 400 μl samples were removed from each insert and added to 320 μl of 'stopping solution' (50 mol m^{-3} 'cold' non - radioactive Met in 2.5 mol m^{-3} Tris - glycine pH 8.5, 0.15 mol dm^{-3} NaCl) in Eppendorf microcentrifuge tubes. The samples were vortex mixed and centrifuged for two min at low speed (3352 g) in an MSE microcentaur minicentrifuge. The supernatants were carefully decanted into scintillation vials for storage and washings from the following stages were combined with the supernatants. The pellets were each resuspended in 1 cm^3 of 2.5 mol m^{-3} Tris - glycine, pH 8.5, 0.15 mol dm^{-3} NaCl, frozen for 100 min at -70 °C and then thawed to yield thylakoid preparations as previously described (4.2.2). The thylakoid preparations were vortex mixed and washed once in 1 cm^3 of 2.5 mol m^{-3} Tris - glycine pH 8.5 (no NaCl). The resulting pellets were each finally resuspended in 40 μl of 2.5 mol m^{-3} Tris - glycine pH 8.5. Two 40 μl volumes of 'cold' control sample were each mixed with 160 μl of distilled water (to mimic dilution with sample buffer) giving 2 x 200 μl samples, which were further diluted by 1/5, 1/10, 1/40 and 1/80 with distilled water for protein estimation by the method of Lowry, *et al.*, 1951 (6.2.2). Remaining 40 μl volumes of 'cold' control sample plus the radiolabelled 40 μl samples were each mixed with sample buffer (6.2.3) in a 1:4 ratio (*i.e.* 40 μl sample plus 160 μl sample buffer) and left overnight at 4 °C to allow detergent solubilisation. The samples were clarified by a 5 min centrifugation in an MSE microcentaur minicentrifuge at 'high' speed (13000 rpm or 13400 g) prior to separation by SDS - PAGE. For a complex mixture of polypeptides a volume equivalent to 50 - 100 μg protein is usually sufficient for optimal results (Hames, 1981). Thus, depending upon the sample protein concentration, volumes of 40, 60 or 80 μl of each sample were loaded in the sample wells of the stacking gel. A 40 μl volume of SDS - 7 Dalton Mark VII - L molecular weight markers in sample buffer (6.2.3) was immersed in boiling water for four min and two lanes were loaded with the standards, one containing a 10 μl volume and a second containing a 15 μl volume. Electrophoresis was carried out at a constant current of 30 mA per gel until the tracking dye was approximately 1 cm from the gel base (5 to 7 hours). The gels were fixed, stained, destained and photographed (6.2.4), and the ^{35}S -Met radiolabel incorporation into protein bands detected by scintillation counting (6.2.5) or fluorography (6.2.6).

In a following experiment a time - course of ^{35}S - Met incorporation was

investigated, essentially using the methods described above, as follows. A 3.25 cm^3 volume of chloroplast suspension (in incubation solution A), in the presence of 100 μCi cm^3 ^{35}S - Met (specific activity aprroximately 680 Ci $mmol^{-1}$) and no 'cold' non - radioactive Met, was incubated for four hours. At 0, 5, 10, 15, 20, 30, 40, 50, 60, 120, 180 and 240 min after initiation of the radiolabelling 250 μl samples were removed and added to 1200 μl of stopping solution in Eppendorf microcentrifuge tubes. In a second reaction vessel 2 cm^3 of chloroplast suspension was similarly incubated for 240 min in the absence of ^{35}S - Met, providing a cold control sample. The samples were prepared for SDS - PAGE essentially as described above.

6.2.8 ^{35}S - Met Content of Thylakoid Proteins During Four Hours of Photoinhibition, Following Two Hours Pre - Radiolabelling.

Chloroplasts were isolated from frond tips of *C. fragile* harvested on 23/ 06/ 90 (early summer - reproductive) as previously described (3.2.2) using 'modified extraction medium' (6.2.7). The chlorophyll concentration of the 1 cm^3 of chloroplast preparation was determined by the method of MacKinney, 1941 (3.2.3). Three DW1 oxygen electrodes/ translucent plastic insert reaction vessels were set up as in section 6.2.7 and *in organello* radiolabelling was carried out in one of these vessels in a similar fashion. A total reaction volume of 3.5 cm^3 (in incubation solution A) was used, containing chloroplasts equivalent to 50 μg cm^{-3} chlorophyll, no exogenous 'cold' non - radioactive Met, and 128 μCi cm^{-3} of ^{35}S - Met at a final concentration of approximately 0.236 mmol m^{-3} and a specific activity of approximately 650 Ci $mmol^{-1}$. A 2 cm^3 volume of 'cold' control chloroplast suspension in solution A was incubated in a second reaction vessel, as before (6.2.7). After two hours incubation at 50 μmol m^{-2} s^{-1} PPFD, 2 x 1.75 cm^3 of the 'pre - radiolabelling' chloroplast reaction mixture were added to 2 x 1.4 cm^3 of 'stopping solution' (0.5 mol dm^{-3} sucrose, 2 g dm^{-3} BSA, 0.015 mol dm^{-3} $MgCl_2.6H_2O$ and 50 mol m^{-3} 'cold' non - radioactive Met in 0.05 mol dm^{-3} HEPES buffer, adjusted to pH 7.8 with 1 mol dm^{-3} KOH) in two 4 cm^3 capped centrifuge tubes. The 2 cm^3 of 'cold' control non - radiolabelling chloroplast reaction mixture was added to 1.6 cm^3 of 'stopping solution', as above. The samples were gently mixed, centrifuged for ten min at 1550 rpm (400 g) and the supernatants carefully decanted into scintillation vials for storage. The two chloroplast pellets from the 'pre - radiolabelling' reaction mixture were resuspended in a total volume of 3.5 cm^3 of non -

radiolabelling incubation medium (solution A, with 1 mmol m^{-3} 'cold' non - radioactive Met), providing the 'pre - radiolabelled' sample for photoinhibitory treatment. The chloroplast pellet from the non - radiolabelling 'cold' control reaction mixture was resuspended in 2 cm^3 of non - radiolabelling incubation medium (solution A, with 1 mmol m^{-3} 'cold' non - radioactive Met), providing a 'cold' control sample.

The photoinhibitory treatment was carried out as follows. A clean transparent plastic insert, containing a clean magnetic flea, was placed in each of three oxygen electrode water jackets, enabling the incubation of three stirred reaction volumes of up to 4 cm^3. A 3 cm^3 volume of the pre - radiolabelled chloroplast suspension was placed in one of the reaction vessels, and the 2 cm^3 volume of 'cold' control non - radiolabelled chloroplast suspension placed in a second reaction vessel. These samples were incubated at 1000 μmol m^{-2} s^{-1} PPFD and 250 μl volumes were removed from the pre - radiolabelled chloroplast suspension at 0, 5, 10, 15, 20, 30, 40, 60, 120, 180 and 240 min after initiation of the photoinhibitory light treatment, and transfered to 11 Eppendorf microcentrifuge tubes. After 240 min, 8 x 250 μl samples were removed from the 'cold' control non - radiolabelled chloroplast suspension and were similarly transferred to a further 8 Eppendorf tubes. The remaining 0.5 cm^3 of the pre - radiolabelled chloroplast suspension was placed in the third reaction vessel and was simultaneously incubated in complete darkness for 240 min, after which 2 x 250 μl volumes were transferred to 2 Eppendorf tubes, providing dark incubated control samples of the pre - radiolabelled chloroplast suspension ('dark' control).

The samples were centrifuged for two min at low speed (6500 rpm or 3352 g) in an MSE microcentaur minicentrifuge, the pellets resuspended in 1 cm^3 of 2.5 mol m^{-3} Tris - glycine, pH 8.5, 0.15 mol dm^{-3} NaCl, frozen, thawed and the thylakoid preparations finally resuspended in 25 μl of 2.5 mol m^{-3} Tris - glycine, pH 8.5, as described in section 6.2.7. Four of the 25 μl volumes of 'cold' control non - radiolabelled sample were used for protein estimation, the remaining 4 x 25 μl of 'cold' control sample, along with the 11 x 25 μl of pre - radiolabelled sample subjected to photoinhibition and the 2 x 25 μl of dark incubated pre - radiolabelled sample were mixed with sample buffer in a 1:4 ratio and used for SDS - PAGE (as in 6.2.7). A 60 μl volume (equivalent to 89 μg protein) of each sample was loaded in the sample wells and two wells were loaded with molecular weight markers. Electrophoresis was carried out at a constant current of 30 mA per gel until the tracking dye was approximately 1

cm from the gel base (5 1/2 hours).

6.3 Results

To determine the protein content of a sample the method of Lowry, *et al.* (1951) was used and a calibration curve was constructed using BSA. A typical standard curve is illustrated in Figure 6.1. Similarly, each time SDS - PAGE was carried out molecular weight standards were used and their relative mobilities, R_f, calculated as follows:

R_f = distance of protein migration/ distance of tracking dye migration.

The R_f values were plotted against log molecular weight to produce a calibration curve, enabling the determination of the molecular weights of sample polypeptides. A typical standard curve is illustrated in Figure 6.2. In an initial experiment a thylakoid membrane preparation was produced and subjected to SDS - PAGE. A 1:4 and 1:2 thylakoid preparation: sample buffer ratio was used and the samples were either allowed to solubilise overnight at 4 °C or immersed in boiling water for four min and 10, 40 or 80 μl volumes were loaded into the sample wells (Figure 6.3, Table 6.2). The resolution of the separation was clearly strongly influenced by the amount of protein added, whereas the ratio of sample to sample buffer and the method of solubilisation (4 °C overnight or 4 min in boiling water) appeared to have little effect. Therefore, in further experiments a 1:4 sample: sample buffer ratio was always employed to ensure adequate protein denaturation, and solubilisation was carried out at 4 °C overnight as a precaution; avoiding boiling which can apparently cause aggregation of certain membrane polypeptides (Marder, *et al.*, 1986). Up to 30 polypeptides were separated in each lane, depending on the amount of sample loaded (Figure 6.3, Table 6.2). A heavily stained protein of 24.0 $\pm$ 0.2 KDa (band number 23) was observed and serves as a useful reference protein, as its identity is unmistakeable and it would thus be difficult to confuse this protein with another when comparing different gels. Two doublets were evident a small distance above protein band 23 in lanes 2, 3, 8 and 9. These represent protein band numbers 19, 20, 21 and 22 and have molecular weights of 30.0 $\pm$ 0.2, 29.1 $\pm$ 0.1, 26.8 $\pm$ 0.3 and 25.7 $\pm$ 0.3 KDa. It will become clearer from following gels that these protein bands are within the molecular weight range of interest (the D1 protein has a molecular weight of 32 KDa) and therefore attention will be focussed on this region. In other lanes the loading was such that each doublet was

Figure 6.1 A typical standard curve for protein estimation by the method of Lowry, *et al.*, 1951. See section 6.2.2 for experimental details.

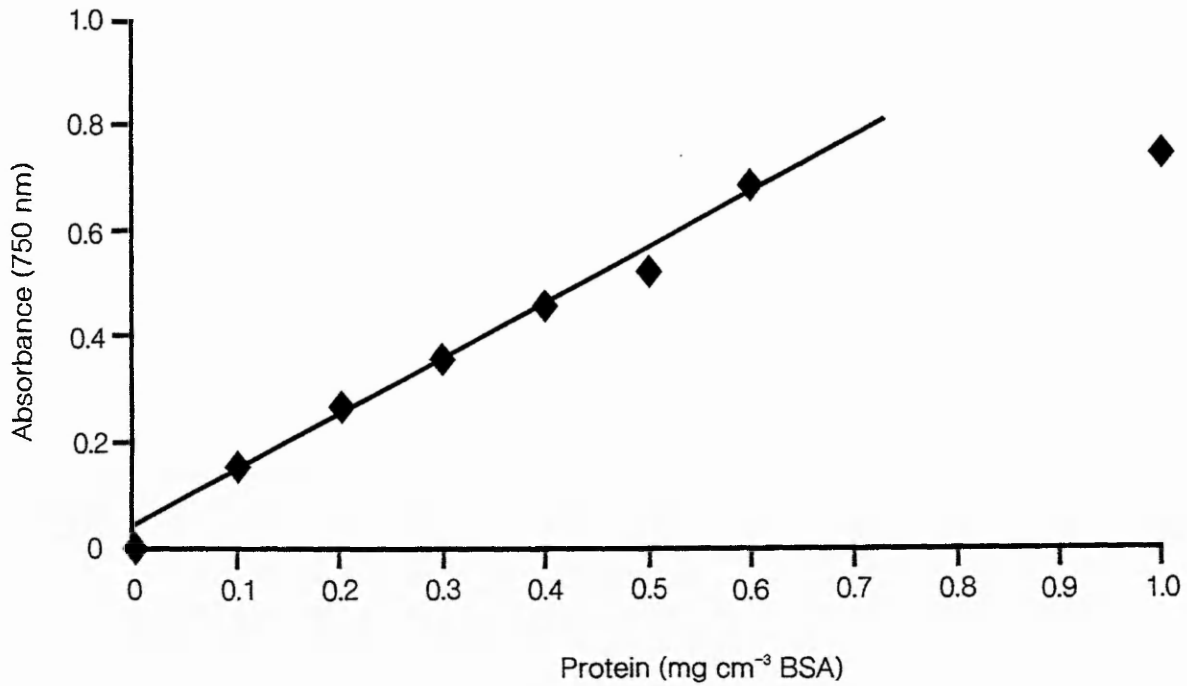

1.0
0.8
0.6
0.4
0.2
0
Absorbance (750 nm)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Protein (mg cm^{-3} BSA)

Figure 6.2 A typical standard curve for the estimation of polypeptide molecular weight after SDS - PAGE. The seven protein standards were: bovine albumin (66 KDa); egg albumin (45); glyceraldehyde - 3 - phosphate dehydrogenase from rabbit muscle (36); carbonic anhydrase from bovine erythrocytes (29); PMSF treated trypsinogen (24); soybean trypsin inhibitor (20.1); and α - lactalbumin (14.2). See sections 6.2.3 and 6.2.4 for experimental details.

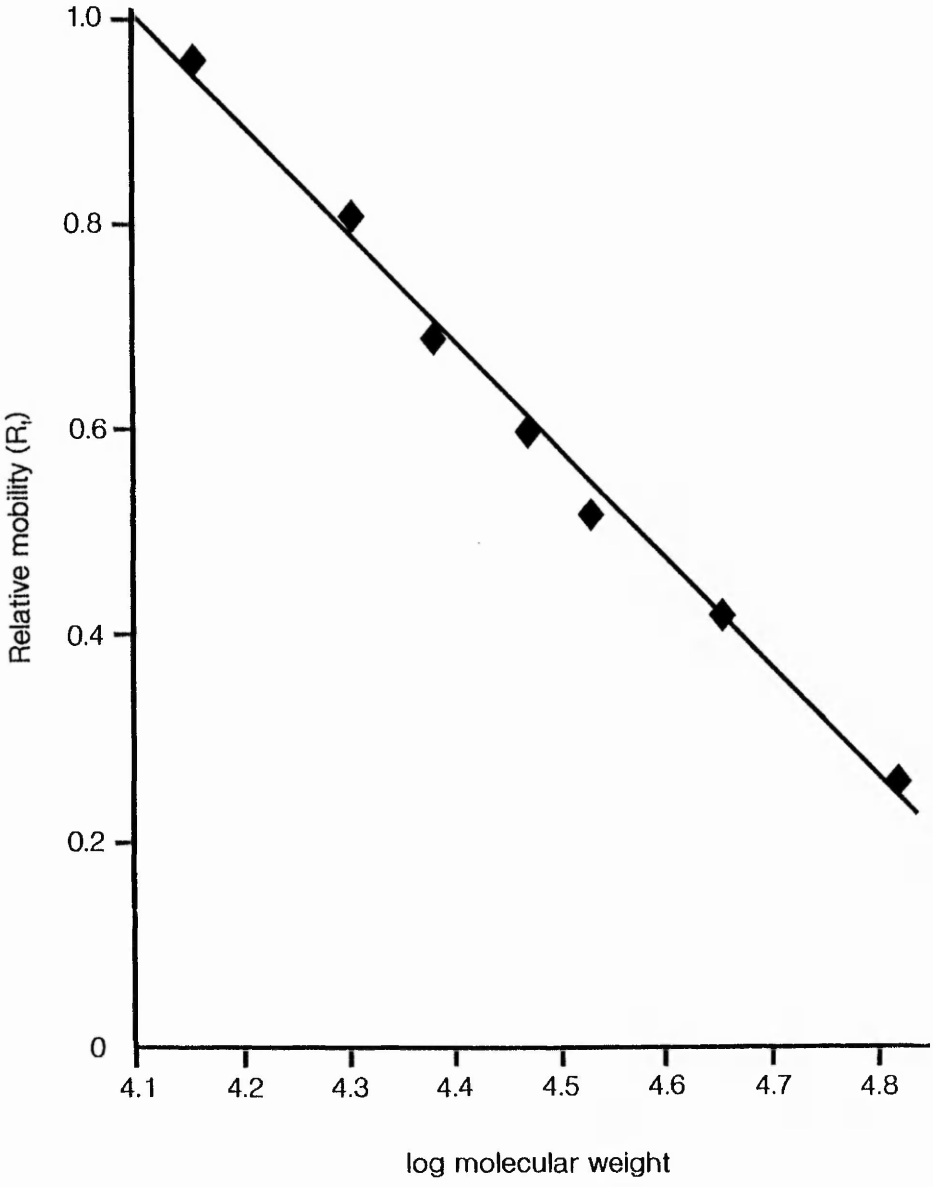
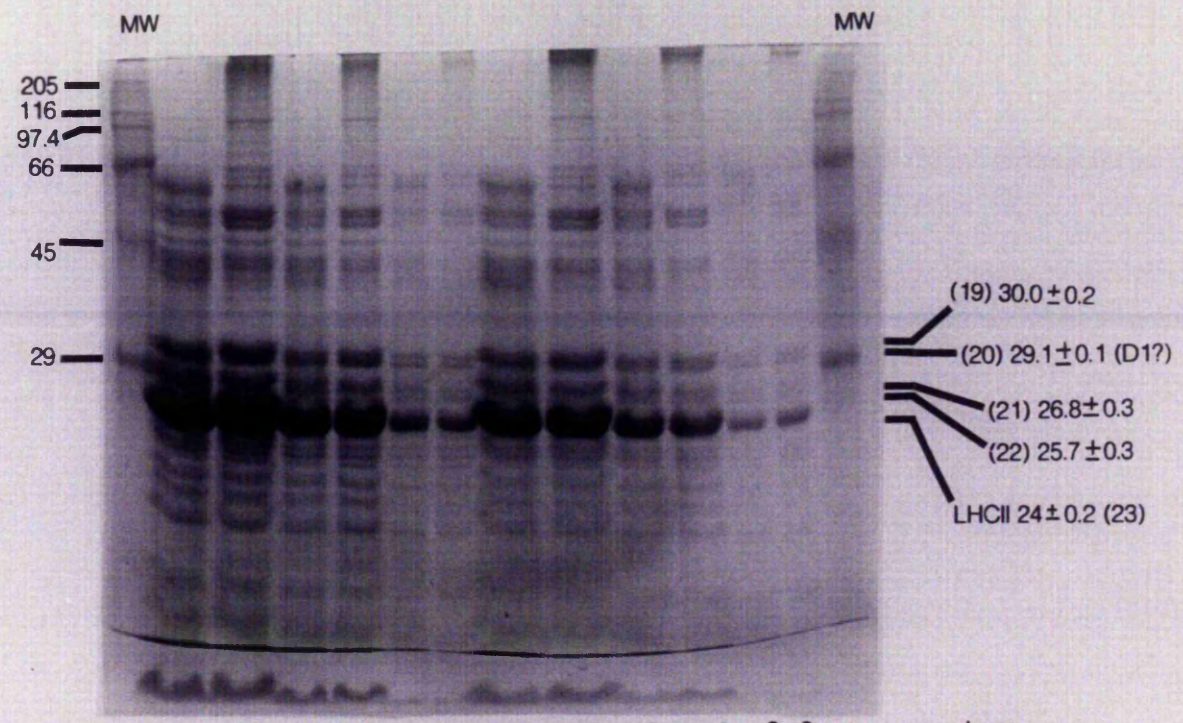

1.0
0.8
0.6
0.4
0.2
0
Relative mobility (R_f)
4.1
4.2
4.3
4.4
4.5
4.6
4.7
4.8
log molecular weight

Figure 6.3 Typical separation of thylakoid proteins by SDS - PAGE. The molecular weight standards were: myosin from rabbit muscle (205 KDa); ß - galactosidase (116); phosphorylase b from rabbit muscle (97); bovine albumin (66); egg albumin (45); carbonic anhydrase from bovine erythrocytes (29). Key: treatment B, sample plus sample buffer boiled for 4 min; treatment C, sample plus sample buffer left at 4 °C overnight; S:SB, ratio of sample to sample buffer. Electrophoresis was carried out for 7 hr 35 min at a constant current of 30 mA. Up to 30 polypeptides were separated in each lane. A heavily stained band of 24.0 KDa (band 23 - LHCII) is a useful reference protein and above this two doublets were separated in some lanes: bands 19, 20, 21 and 22 of 30.0, 29.1, 26.8 and 25.7 KDa, respectively. Band 20 (29.1 KDa) is possibly the D1 protein. See Table 6.2 and section 6.2.7 for experimental details.

MW standards stds



Lane	14	13	12	11	10	9	8	7	6	5	4	3	2	1
Treatment	B	C	B	C	B	C	B	C	B	C	B	C	B	B
Vol. (µl)	10	80	80	40	40	10	10	80	80	40	40	10	10	10
S:SB	1:4	1:2	1:2	1:2	1:2	1:2	1:2	1:4	1:4	1:4	1:4	1:4	1:4	1:4
Protein (µg)	–	178	178	89	89	22	22	107	107	54	54	13	13	–

Table 6.2 Molecular weights (KDa) of the thylakoid proteins typically separated by SDS - PAGE. Key: T, treatment of sample, involving either B, sample plus sample buffer boiled for four min, or C, sample plus sample buffer left at 4 °C overnight; S: SB, ratio of sample to sample buffer; μl, volume of sample loaded; μg, amount of protein loaded. Electrophoresis was carried out for 7 h 35 min at a constant current of 30 mA. Up to 30 polypeptides were separated in each lane. A heavily stained band of 24 KDa (band number 23) was present, representing the LHCII, and served as a useful reference protein. Above this two doublets were separated in lanes 2, 3, 8 and 9 designated band numbers 19, 20, 21 and 22 of molecular weights 29.9, 29.0, 26.8 and 25.8 KDa, respectively. Band number 20 (29 KDa) is possibly the D1 protein. In other lanes the loading was such that each doublet was not resolved into two distinct bands. The single band representing bands 19 and 20 was designated band 19.5 and the single band representing bands 21 and 22 was designated band 21.5. See Figure 6.3 and section 6.2.7 for experimental details.

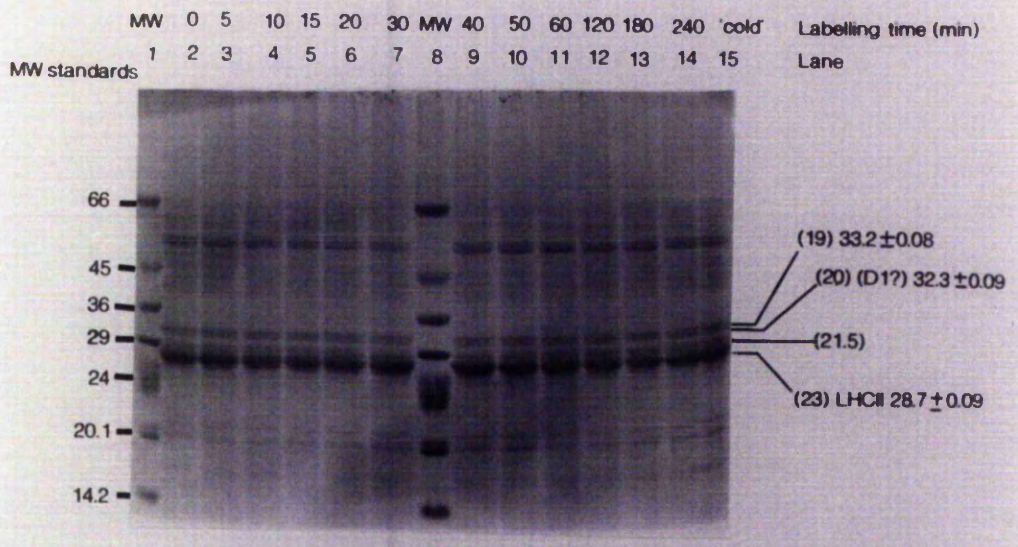
	Lane of gel											
	2	3	4	5	6	7	8	9	10	11	12	13
Band No.												
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	112.2	-	-	-	117.5	-	117.5	117.5
3	104.7	102.3	104.7	104.7	104.7	104.7	104.7	104.7	104.7	109.7	104.7	-
4	-	-	-	-	100.0	-	-	-	97.7	-	97.7	-
5	-	-	-	-	87.1	-	-	-	89.1	-	89.1	-
6	87.1	83.2	83.2	83.2	83.2	83.2	83.2	85.1	85.1	87.1	85.1	-
7	-	-	-	-	81.3	-	-	-	83.2	-	83.2	-
8	-	-	72.4	-	75.9	-	-	-	75.9	-	75.9	-
9	-	-	-	-	-	-	-	-	-	-	74.1	-
10	-	-	66.0	-	66.1	66.1	66.1	-	67.6	-	67.6	-
11	63.1	61.7	61.7	-	61.7	-	63.1	-	64.6	-	63.1	-
12	-	58.9	57.5	57.5	58.9	57.5	58.9	58.9	60.3	58.9	58.9	60.3
13	52.5	52.5	52.5	51.3	53.7	52.5	53.7	53.7	53.7	53.7	-	53.7
14	-	-	50.1	50.1	50.1	50.1	52.5	52.5	51.3	51.3	52.5	53.1
15	49.0	49.0	49.0	47.9	49.0	49.0	50.1	50.1	50.1	50.1	50.1	51.3
16	45.0	45.7	45.7	45.0	45.7	45.7	45.7	45.7	46.8	46.8	46.8	46.8
17	-	-	-	38.9	-	-	-	-	39.8	-	-	-
18	-	-	33.5	33.9	34.7	34.7	-	-	34.7	34.7	35.5	35.5
19	29.5	29.5	29.5	30.2	29.5	29.5	30.2	30.9	30.2	30.2	30.9	30.9
20	28.8	28.8	28.8	28.8	-	-	29.5	29.5	-	-	-	-
21	26.3	26.3	-	26.9	26.9	26.9	26.9	27.5	27.5	27.5	27.5	28.2
22	25.2	25.2	26.3	-	-	-	26.3	26.3	-	-	-	-
23	22.9	23.4	23.4	23.4	24.6	24.6	24.0	24.0	24.0	24.0	24.6	24.6
24	21.4	21.4	21.4	21.9	21.9	21.9	21.9	21.9	21.9	21.9	21.9	22.4
25	20.4	20.4	20.9	20.9	21.4	21.4	21.4	21.4	21.4	21.4	21.4	21.9
26	19.5	20.0	20.0	20.4	20.4	20.4	20.4	20.9	20.4	20.9	20.9	20.9
27	18.2	18.6	18.6	19.5	19.5	19.5	19.1	19.1	19.5	19.9	20.0	20.0
28	17.0	17.6	17.8	20.0	18.2	18.2	17.8	17.8	18.2	18.2	18.2	18.6
29	15.5	15.9	15.9	16.2	16.6	16.2	16.2	15.9	16.2	16.2	16.6	15.9
30	12.3	12.9	12.9	12.9	-	-	12.9	12.9	12.6	12.9	13.2	13.5
T	B	C	B	C	B	C	B	C	B	C	B	C
μl	10	10	40	40	80	80	10	10	40	40	80	80
S:SB	1:4	1:4	1:4	1:4	1:4	1:4	1:2	1:2	1:2	1:2	1:2	1:2
μg	13.4	13.4	53.6	53.6	107.2	107.2	22.3	22.3	89.2	89.2	178.4	178.4

not resolved into two distinct bands. The single band representing bands 19 and 20 may be designated band 19.5 and the single band representing bands 21 and 22 may be designated band 21.5. A very large molecular weight band was apparent at the very top of each lane (band number 1 in Table 6.2) that was beyond the range of the R_f versus log MW calibration curve.

In a second series of experiments the incubation conditions required for the *in organello* radiolabelling of thylakoid proteins with ^{35}S - Met was investigated. Chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll were radiolabelled with ^{35}S - Met under 50 μmol m^{-2} s^{-1} PPFD. A total of 21 incubation conditions were studied, using extraction medium, four other incubation solutions (A, B, C and D) and various concentrations of ^{35}S - Met and 'cold' non - radioactive Met to give 6 (I - VI) values of total radioactivity (μCi cm^{-3}) and specific activity (Ci $mmol^{-1}$), as summarised in Table 6.1. After *in organello* radiolabelling the chloroplasts were prepared to provide thylakoid membrane samples for SDS - PAGE (a typical separation is illustrated in Figure 6.4) and the resulting gels were used for fluorography or liquid scintillation counting. However, the ^{35}S - Met incorporation into the thylakoid proteins during initial experiments was not sufficient for the successful use of fluorography, as this technique is less sensitive than scintillation counting of re - solubilised gel slices. It was concluded that, of the incubation conditions investigated, that which supported the highest incorporation was number 18 (Table 6.1), *i.e.*, solution A (modified extraction medium - 0.5 mol dm^{-3} sucrose, 2 g dm^{-3} BSA, 0.015 mol dm^{-3} $MgCl_2.6H_2O$ in 0.05 mol dm^{-3} HEPES buffer, adjusted to pH 7.8 with 1 mol dm^{-3} KOH) containing 0.13 mmol m^{-3} ^{35}S - Met at 100 μCi cm^{-3} and 785 Ci $mmol^{-1}$, with no added 'cold' non - radioactive Met (data not shown).

A subsequent investigation of the time - course of ^{35}S - Met incorporation under similar conditions revealed that the optimum incubation time appeared to be two hours (Figures 6.4, 6.5 and 6.6). Chloroplast suspensions were radiolabelled from 0 to 4 hours using incubation solution A containing 0.17 mmol m^{-3} ^{35}S - Met (final concentration) at 100 μCi cm^{-3} and 680 Ci $mmol^{-1}$, with no added 'cold' non - radioactive Met. After *in organello* radiolabelling thylakoid membrane samples were separated by SDS - PAGE and the resulting gels were used for liquid scintillation counting or fluorography. In the gel used for scintillation counting (Figure 6.4A) thirteen major bands were visualised by protein staining, including the reference protein

Figure 6.4 SDS-PAGE separation of thylakoid proteins after a time - course of *in organello* radiolabelling with ^{35}S-Met under optimal conditions. The MW standards were: bovine albumin (66 KDa); egg albumin (45); glyceraldehyde - 3 - phosphate dehydrogenase (36); carbonic anhydrase (29); trypsinogen (24); soybean trypsin inhibitor (20.1); and α - lactalbumin (14.2). Samples were prepared from chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll, radiolabelled under optimal conditions for up to four hours (6.2.7). MW standards were loaded in lanes 1 and 8 (A) and 4 and 15 (B), and samples prepared from chloroplasts radiolabelled for 0 to 240 min loaded in lanes 2-7 and 9 -14 (A) and lanes 1-3 and 5-13 (B), whilst a 'cold' control sample was loaded in lane 15 (A) and 14 (B). In the gel (A) used for scintillation counting (see Fig 6.5), 13 major bands were visualised by protein staining, including those of: 33.2 $\pm$ 0.08 (band 19); 32.3 $\pm$ 0.09 (band 20 - D1 ?); and 28.7 $\pm$ 0.09 (band 23 - LHCII) KDa (n = 13). The doublet designated 21.5 was not resolved but a darker background stain in this area was apparent. In the gel (B) used for fluorography (see Fig 6.6), 7 major bands were visualised by protein staining, including those of: 33.2 $\pm$ 0.2 (band 19); 32.5 $\pm$ 0.2 (band 20 - D1 ?); and 28.6 $\pm$ 0.2 (band 23 - LHCII) KDa (n = 13). The doublet designated 21.5 was not resolved, but a darker background stain in this area was apparent.


MW 0 5 10 15 20 30 MW 40 50 60 120 180 240 'cold' Labelling time (min)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 Lane
MW standards
66
45
36
29
24
20.1
14.2
(19) 33.2±0.08
(20) (D1?) 32.3±0.09
(21.5)
(23) LHCII 28.7±0.09

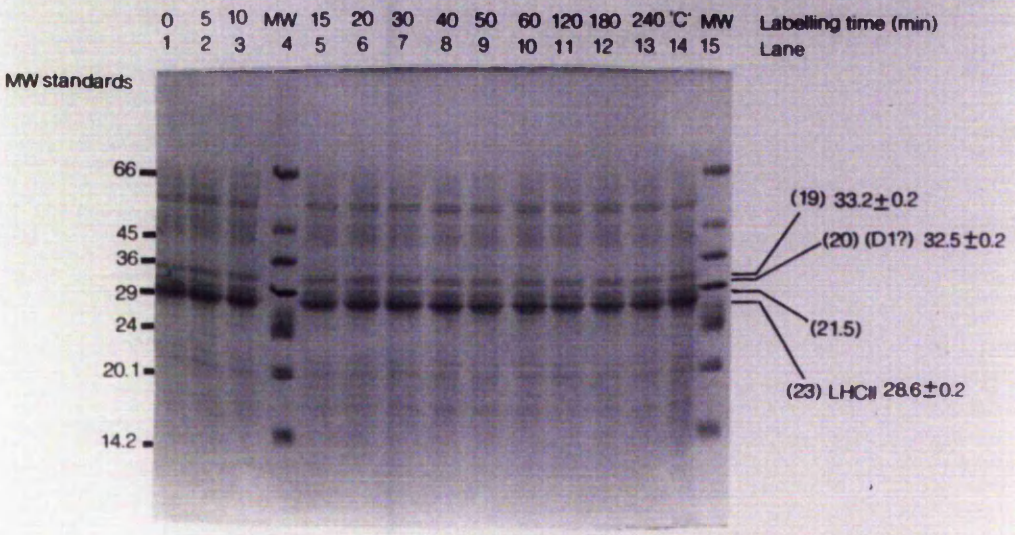

0 5 10 MW 15 20 30 40 50 60 120 180 240 'C' MW Labelling time (min)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 Lane
MW standards
66
45
36
29
24
20.1
14.2
(19) 33.2±0.2
(20) (D1?) 32.5±0.2
(21.5)
(23) LHCII 28.6±0.2

Figure 6.5 Time - course of ^{35}S - Met incorporation under optimal conditions for the *in organello* radiolabelling of thylakoid proteins. Chloroplast suspensions equivalent to 50 $\mu g\ cm^{-3}$ chlorophyll were radiolabelled under optimal conditions for up to four hours (6.2.7). The radiolabelled thylakoid proteins were then separated by SDS - PAGE. The results presented in this Figure were obtained from the gel of Fig 6.4A and are the DPM of gel slices containing bands visualised by protein staining (determined by scintillation counting). A 'cold' control sample which had not been radiolabelled was loaded in lane 15 (Fig 6.4A). The DPM of each of the protein bands separated in the 'cold' control sample were subtracted from the DPM of the corresponding bands separated in the other, radiolabelled samples. Protein staining revealed 13 major bands, including those of: 33.2 $\pm$ 0.08 (band 19); 32.3 $\pm$ 0.09 (band 20 - D1 ?); and 28.7 $\pm$ 0.09 (band 23 - LHCII) KDa (n=13).

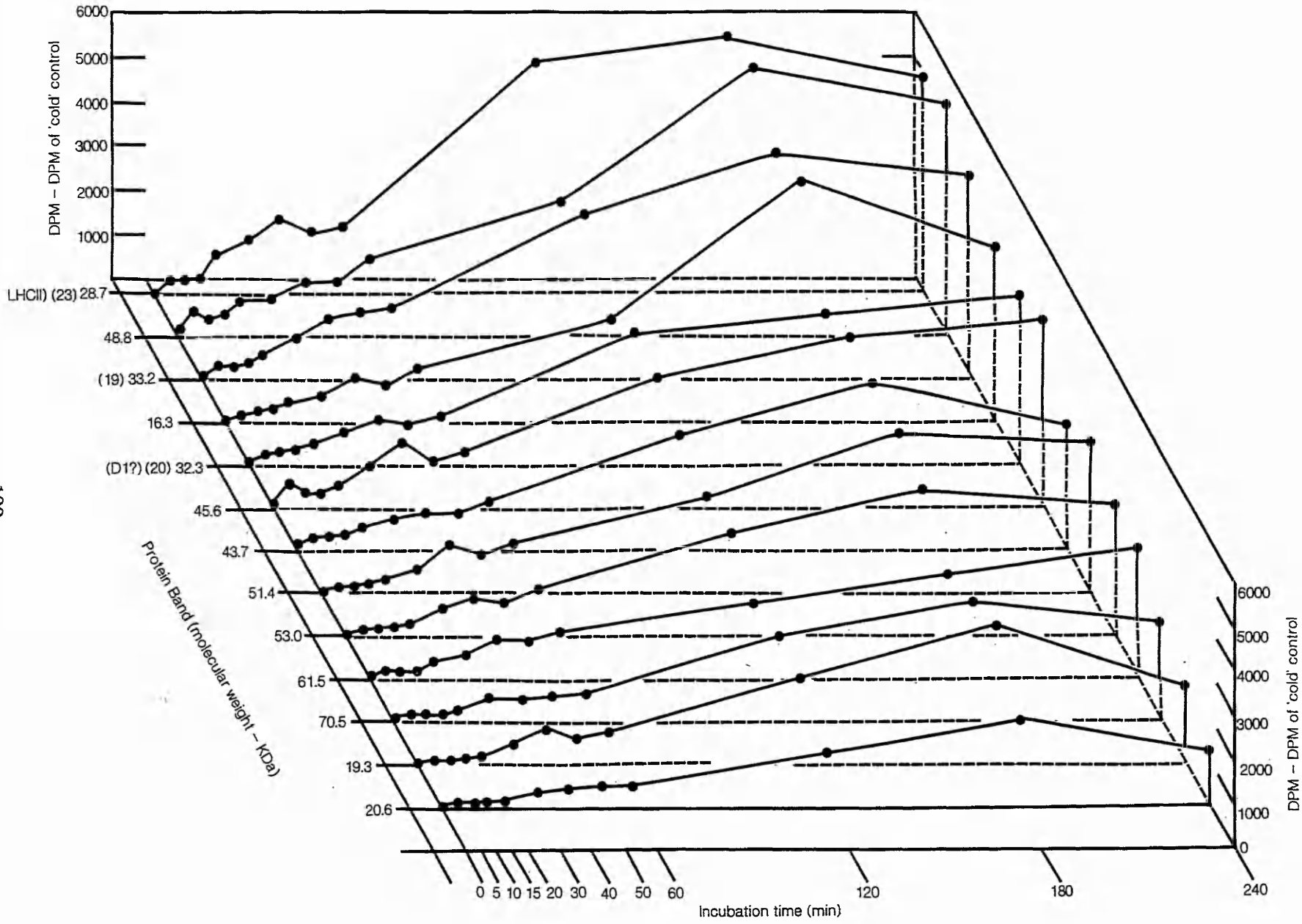
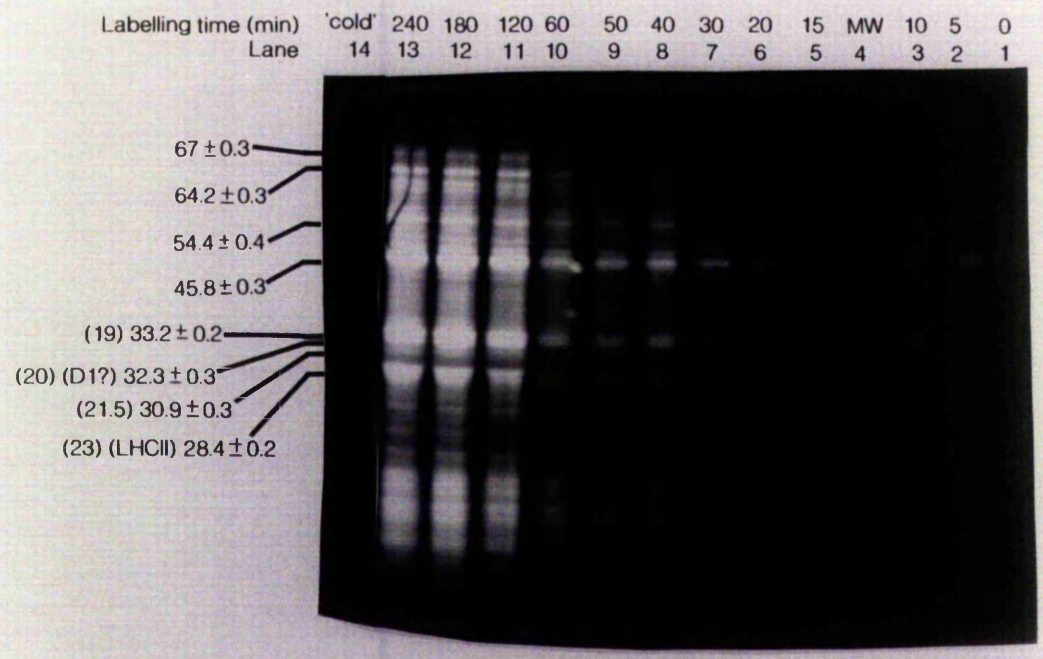
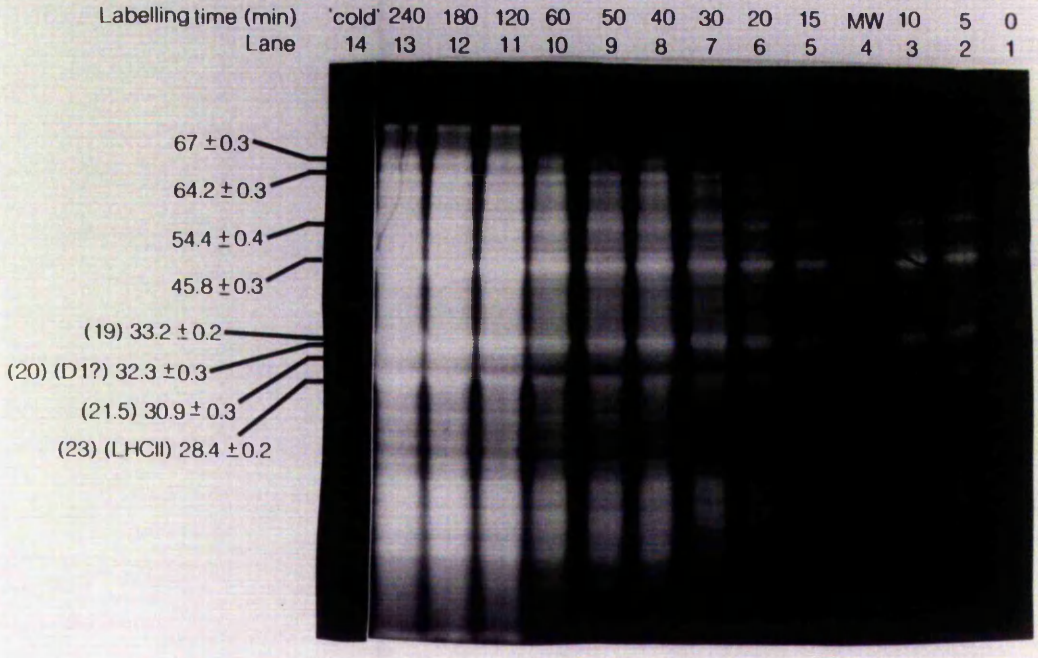

DPM – DPM of 'cold' control
6000
5000
4000
3000
2000
1000
LHCII) (23) 28.7
48.8
(19) 33.2
16.3
(D1?) (20) 32.3
45.6
43.7
51.4
53.0
61.5
70.5
19.3
20.6
Protein Band (molecular weight – KDa)
0 5 10 15 20 30 40 50 60
120
180
240
Incubation time (min)
6000
5000
4000
3000
2000
1000
0
DPM – DPM of 'cold' control

Figure 6.6 SDS-PAGE of thylakoid proteins after a time course of *in organello* radiolabelling under optimal conditions: fluorograph of gel of Fig 6.4B. Samples were prepared from chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll, radiolabelled under optimal conditions for up to four hours (6.2.7). MW standards were loaded in lanes 4 and 15 and samples prepared from chloroplasts radiolabelled for 0 to 240 min loaded in lanes 1-3 and 5-13, whilst a 'cold' control sample was loaded in lane 14. The gel was used for fluorography and exposure times of 7 (A) and 17 (B) days are shown. Fluorography revealed 17 major bands including those of: 33.2 $\pm$ 0.2 (band 19); 32.3 $\pm$ 0.3 (band 20 - D1 ?); 30.9 $\pm$ 0.3 (band 21.5); and 28.4 $\pm$ 0.2 (band 23 - LHCII) KDa (n = 12).

(A)


Labelling time (min) 'cold' 240 180 120 60 50 40 30 20 15 MW 10 5 0
Lane 14 13 12 11 10 9 8 7 6 5 4 3 2 1
67 ± 0.3
64.2 ± 0.3
54.4 ± 0.4
45.8 ± 0.3
(19) 33.2 ± 0.2
(20) (D1?) 32.3 ± 0.3
(21.5) 30.9 ± 0.3
(23) (LHCII) 28.4 ± 0.2

(B)


Labelling time (min) 'cold' 240 180 120 60 50 40 30 20 15 MW 10 5 0
Lane 14 13 12 11 10 9 8 7 6 5 4 3 2 1
67 ± 0.3
64.2 ± 0.3
54.4 ± 0.4
45.8 ± 0.3
(19) 33.2 ± 0.2
(20) (D1?) 32.3 ± 0.3
(21.5) 30.9 ± 0.3
(23) (LHCII) 28.4 ± 0.2

(band 23) of 28.7 ± 0.09 KDa. Two bands were separated in the area of interest of 33.2 ± 0.08 and 32.3 ± 0.09 KDa. These may represent bands 19 and 20, respectively, as identified in the initial separation (Figure 6.3, Table 6.2). The doublet designated 21.5 was not resolved, but a darker background stain in this area was apparent. The gel used for fluorography (Figure 6.4B) was similar, seven major bands being visualised by protein staining, including the reference protein (band 23) of 28.6 ± 0.2 KDa. Again, two bands were separated in the area of interest of 33.2 ± 0.2 and 32.5 ± 0.2 KDa, possibly representing bands 19 and 20, respectively. The doublet designated 21.5 was again not resolved, but a darker background stain in this area was apparent.

The thirteen bands observed in each lane of the gel in Figure 6.4A were excised and the gel slices solubilised in 1 cm^3 30 % H_2O_2 and the ^{35}S - Met incorporated into the protein bands determined by liquid scintillation counting. The DPM of each of the protein bands separated in a non - radiolabelled 'cold' control sample (which varied from 71 to 289 DPM) were subtracted from the DPM of the corresponding bands separated in the other radiolabelled samples, to correct for background radiation. The results are presented in Figure 6.5. The bands of particular interest are of 32.3 and 33.2 KDa, which exhibited ^{35}S - Met incorporation of 2 960 and 3 690 DPM at 2 hours, with increase to 3 735 and 4 500 DPM after 4 hours incubation, respectively. A compromise has to be made between total amount of radiolabel incorporated and the length of time required for this incorporation and it seems that a radiolabelling time of two hours is favourable. The reference protein (band 23) exhibited incorporation of 5 160 DPM after 2 hours. The high DPM incorporated into band 23 is probably mainly a reflection of the abundance of this protein compared with the other proteins separated. The gel slices containing band 23 used for liquid scintillation counting were typically approximately 3 or 4 mm wide, whilst the gel slices containing all other protein bands were typically approximately 1 - 2 mm wide.

The gel in Figure 6.4B was used for fluorography and prints of the fluorographs obtained after 7 days and 17 days exposure are shown in Figure 6.6A and 6.6B, respectively. By studying the fluorographs over a light box it was possible to visualise 17 major bands by radiolabelling (more than the 7 major bands visualised by protein staining of the same gel - Figure 6.4B). It is clear from Figure 6.6 that as the incubation time increases the amount of radiolabel incorporation increases, as was

shown by the scintillation counting of gel slices obtained from the second gel that was run simultaneously (Figures 6.4A, 6.5). Fluorography revealed initial preferential radiolabelling (*i.e.* over approximately the first 15 to 20 min of incubation) of six proteins, of: 67.0 $\pm$ 0.3; 64.2 $\pm$ 0.3; 54.4 $\pm$ 0.4; 45.8 $\pm$ 0.3; 33.2 $\pm$ 0.2; and 32.3 $\pm$ 0.3 KDa (n = 12). The latter two proteins represent band numbers 19 and 20, respectively. Just below bands 19 and 20, a further band was faintly visible, of 30.9 $\pm$ 0.3 KDa (n=12), and this may represent the doublet designated 21.5 (Figure 6.3, Table 6.2). It is interesting that, despite the abundance of the reference band 23, of 28.4 $\pm$ 0.2 KDa, as revealed by protein staining (and the high incorporation revealed by scintillation counting of the second gel that was run simultaneously - Figure 6.5), the amount of incorporation revealed by fluorography is initially relatively low, when compared with the six proteins mentioned above. The bands designated 19, 20 and 23, were visualised by both protein staining and radiolabelling. In contrast, whether or not the 4 other bands initially preferentially radiolabelled, referred to above, correspond to any of the protein bands visualised by protein staining is uncertain (compare Figure 6.4B and Figure 6.6). That is, the radiolabelling may have visualised different proteins than those revealed by protein staining. Therefore, it is suggested that it may be important to use both fluorography and scintillation counting of gel slices containing bands identified by protein staining, to ensure that proteins present in amounts insufficient to be stained are not overlooked.

A final experiment investigated a time course of ^{35}S - Met content of thylakoid proteins during four hours of photoinhibition, following two hours of pre - radiolabelling under optimal conditions. Chloroplast suspensions (isolated from tissue harvested on 23/ 06/ 90: early summer - reproductive) equivalent to 50 μg cm^{-3} chlorophyll were radiolabelled, under 50 μmol m^{-2} s^{-1} PPFD, for two hours, using incubation solution A containing ^{35}S - Met at 128 μCi cm^{-3}, 650 Ci $mmol^{-1}$ and 0.236 mmol m^{-3} final concentration, with no added 'cold' non - radioactive Met. Following pre - radiolabelling, the chloroplast suspension was subjected to a photoinhibitory treatment of 1000 μmol m^{-2} s^{-1} PPFD for up to 4 hours. A pre - radiolabelled control sample was kept in the dark for four hours ('dark' control). A 'cold' control sample, which had not been pre - radiolabelled was simultaneously photoinhibited for four hours. The chloroplasts were prepared to provide thylakoid membrane samples for separation by SDS - PAGE and the resulting gels were used for liquid scintillation

counting or fluorography. Unfortunately, the gels were not successfully photographed and therefore there is no such visual record of the gels stained for protein. The separation obtained was similar to that illustrated in Figure 6.4 and the results are summarised in Table 6.3.

In the gel used for scintillation counting (Table 6.3 - column A) six major protein bands were visualised by protein staining, including the reference protein (band 23) of 29.3 $\pm$ 0.1 KDa. Two bands were separated in the area of interest of 33.9 $\pm$ 0.2 and 31.5 $\pm$ 0.2 KDa. These may represent bands 19 (or 19 and 20 in an unresolved state, *i.e.*, 19.5) and 21.5 (*i.e.* bands 21 and 22 unresolved), respectively, as identified in the initial separation (Figure 6.3, Table 6.2). The gel used for fluorography (Table 6.3 - column B) was similar, with eight major bands being visualised by protein staining, including the reference protein (band 23) of 29.0 $\pm$ 0.2 KDa. Again, two bands were separated in the area of interest of 33.7 $\pm$ 0.2 and 31.3 $\pm$ 0.2 KDa, possibly representing bands 19 (or 19.5) and 21.5, respectively.

The six bands observed in each lane of the gel of Table 6.3 - column A were excised and the gel slices solubilised in 1 cm^3 of 30 % H_2O_2 and the ^{35}S - Met (DPM) incorporated into the protein bands determined by liquid scintillation counting. The results are presented in Table 6.4. After two hours of pre - radiolabelling and no photoinhibition (*i.e.* time = 0 min) the reference protein (band 23) exhibited incorporation of 56 586 DPM, whilst the bands of 33.9 $\pm$ 0.2 and 31.5 $\pm$ 0.2 KDa exhibited incorporation of 68 267 and 49 769 DPM, respectively. The reference band 23 was by far the most abundant protein, as revealed by protein staining: the excised gel slice containing band 23 was approximately 3 - 4 mm wide, whilst the gel slices excised containing all other protein bands were typically 1 - 2 mm wide. The other proteins observed (present in similar amounts, as revealed by protein staining, to the two proteins of interest, of 33.9 and 31.5 KDa) exhibited lower incorporation of 27 062 and 30 649 DPM after two hours of pre - radiolabelling. For these reasons it is suggested that the comparitively high radiolabel levels (68 267 and 49 769 DPM) in the two proteins of interest represents quite considerable preferential radiolabelling of these two polypeptides.

The DPM of each of the protein bands separated in a 'cold' control sample (which varied from 309 to 1268 DPM - Table 6.4), which had not been radiolabelled, were subtracted from the DPM of the corresponding bands separated in the other

Table 6.3 SDS - PAGE of thylakoid proteins after up to 4 hours of photoinhibition, following 2 hours of *in organello* pre - radiolabelling with ^{35}S - Met under optimal conditions. Chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll were radiolabelled for two hours under optimal conditions. Following pre - radiolabelling the chloroplast suspension was subjected to a photoinhibitory treatment of 1000 μmol m^{-2} s^{-1} PPFD for up to 4 hours. A pre - radiolabelled control sample was kept in the dark for 4 hours ('dark' control). A 'cold' control sample which had not been pre - radiolabelled was simultaneously photoinhibited for 4 hours (section 6.2.8). The thylakoid proteins were then separated by SDS - PAGE. The gels were used for scintillation counting (A) or fluorography (B). The gels were not successfully photographed and therefore there is no visual record of the gels stained for protein, hence the tabular presentation of the results. In the gel used for scintillation counting, A (see Table 6.4, Figures 6.7 and 6.8), 6 major bands were visualised by protein staining, whilst in the gel used for fluorography, B (see Figure 6.9), 8 major protein bands were visualised by protein staining. Subsequent fluorography revealed up to 21 major proteins (Figure 6.9), including an extra band (number 20 - D1 ?) in the region of interest, of 32.2 $\pm$ 0.08 KDa (n = 13), C.

	Scintillation counting	Fluorography	
Band No.	(A)	(B)	(C)
-	51.6 ± 0.2	51.7 ± 0.3	-
-	50.0 ± 0.3	50.1 ± 0.2	-
-	-	45.3 ± 0.2	-
-	-	42.7 ± 0.2	-
19 or 19.5	33.9 ± 0.2	33.7 ± 0.2	33.7 ± 0.08
20 (D1?)	-	-	32.2 ± 0.08
21.5	31.5 ± 0.2	31.3 ± 0.2	31.2 ± 0.1
23 (LHCII)	29.3 ± 0.1	29.0 ± 0.2	28.6 ± 0.08
-	20.0 ± 0.2	19.7 ± 0.2	-

Table 6.4 ^{35}S - Met incorporation (DPM) into the 6 major protein bands separated by SDS - PAGE, from chloroplast suspensions photoinhibited for 0 to 240 min. Chloroplast suspensions equivalent to 50 $\mu g\ cm^{-3}$ chlorophyll were radiolabelled for two hours, under optimal conditions. Following pre - radiolabelling the chloroplast suspension was subjected to a photoinhibitory treatment of 1000 $\mu mol\ m^{-2}\ s^{-1}$ PPFD for up to 4 hours. A pre - radiolabelled control sample was kept in the dark for 4 hours ('dark' control). A 'cold' control sample which had not been pre - radiolabelled was simultaneously photoinhibited for 4 hours (section 6.2.8). The thylakoid proteins were then separated by SDS - PAGE and the gels used for fluorography or scintillation counting. The results presented in this Table are the DPM of gel slices excised from lanes 2 - 15, containing bands visualised by protein staining (Table 6.3 - column A). See Figure 6.3, Table 6.2 for band numbering. The molecular weights (KDa) are also shown. The values in Figures 6.7 and 6.8 are derived from this Table.

				DPM			
				Band 19	Band 21.5	Band 23	
		51.6±0.2 KDa	50.0±0.3 KDa	33.9±0.2 KDa	31.5±0.2 KDa	29.3±0.1 KDa	200±02 KDa
Lane	Min						
2	0	30649	287	68267	49769	56586	27062
3	5	23156	28625	59189	54845	66181	27264
4	10	26006	23518	69761	44043	63706	27022
5	15	24478	29210	59086	36954	50368	25757
7	20	23433	24951	60026	38946	52876	23951
8	30	19335	20781	50793	26080	34196	18670
9	40	24454	20462	58352	45651	47841	24324
10	60	24703	22607	66190	36934	52009	25003
11	120	23272	27538	62101	31151	50364	15484
12	180	17959	22874	54120	30652	39769	22111
13	240	17323	19112	41965	27349	34973	18940
14	Dark	23020	28032	64633	43321	65706	19974
15	Cold	309	385	616	649	1268	828

radiolabelled samples, to correct for background radiation. The corrected DPM determined in a particular band before photoinhibition (*i.e.* time = 0 min) was designated 100 % and the other values were expressed as a percentage of this initial value. The relationship between the ^{35}S - Met content of the protein bands, expressed in this way, and the time of photoinhibition (min) is shown in Figure 6.7. The ^{35}S -Met content of a pre - radiolabelled sample maintained in darkness for four hours ('dark' control) is also shown. In general, the ^{35}S - Met content of all the proteins decreased with increasing time of photoinhibition. The most pronounced responses over the fist 15 min of photoinhibition were apparent with the proteins of 51.6, 29.3 and 31.5 KDa, which showed decreases in radiolabel content to approximately 80, 89 and 74 % of the level determined at 0 min photoinhibition, respectively. From 15 to 240 min photoinhibition the radiolabel content of these proteins generally continued to decrease at a more gradual rate, reaching levels of 56.1, 61.0 and 54.4 %, respectively. The radiolabel content of these proteins in the 'dark' control sample (4 hours incubation in the dark) exhibited values of 75, 117 and 87 % of the level determined at 0 min photoinhibition, respectively.

Alternatively, the DPM (corrected for background radiation) determined in a particular protein band before photoinhibition (*i.e.* time = 0 min - Table 6.4) can be designated 100 % (or 0 % loss of DPM) and the other values expressed as a percentage reduction of ^{35}S - Met content from this initial value. The relationship between the percentage reduction of the ^{35}S - Met content of the protein bands and the time of photoinhibition (min) is shown in Figure 6.8. The most marked initial response is that of the protein of 31.5 KDa, which exhibits a 26 % reduction of ^{35}S - Met after 15 min of photoinhibition. Over the same time period the proteins of 51.6, 33.9 and 29.3 KDa exhibited reductions of ^{35}S - Met of 20, 14 and 11 %. After four hours photoinhibition the proteins of 31.5, 51.6, 29.3 and 33.9 KDa exhibited 46, 44, 39 and 39 % reductions of ^{35}S - Met, whilst the corresponding proteins of the 'dark' control sample showed 13, 25, -17 and 5.4 % reductions of ^{35}S - Met content. Thus, the significance of the 44 % decrease of the ^{35}S - Met content of the 51.6 KDa protein under photoinhibitory light is reduced by the fact that there was a substantial (25 %) loss of ^{35}S - Met in the dark.

The gel of Table 6.3 - column B was used for fluorography and prints of the fluorographs obtained after 7 hours 15 min and 20 hours 25 min exposure are shown

Figure 6.7 Time course of ^{35}S-Met incorporation into thylakoid proteins during 4 hours of photoinhibition, following 2 hours of *in organello* pre - radiolabelling. Chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll were radiolabelled under optimal conditions for two hours. Following pre - radiolabelling the chloroplast suspension was subjected to a photoinhibitory treatment of 1000 μmol m^{-2} s^{-1} PPFD for up to 4 hours. A pre - radiolabelled sample was kept in the dark for 4 hours ('dark' control). A 'cold' control sample which had not been pre - radiolabelled was simultaneously photoinhibited for 4 hours (6.2.8). The thylakoid proteins were then separated by SDS - PAGE. The results presented in this Figure were obtained from the gel of Table 6.3 - column A and are derived from the DPM of gel slices (Table 6.4) containing bands visualised by protein staining (determined by scintillation counting). The corrected DPM (DPM - DPM 'cold' control) determined in a particular band before photoinhibition (*i.e.* time = 0 min) was designated 100 % and the other values were expressed as a percentage of this initial value. The 'dark' control value is also shown, ■. Protein staining revealed 6 major protein bands, including those of: 33.9 $\pm$ 0.2 (band 19 or 19 and 20 unresolved, *i.e.*, 19.5); 31.5 $\pm$ 0.2 (doublet designated 21.5); and 29.3 $\pm$ 0.1 (band 23 - LHCII) KDa (n=13).

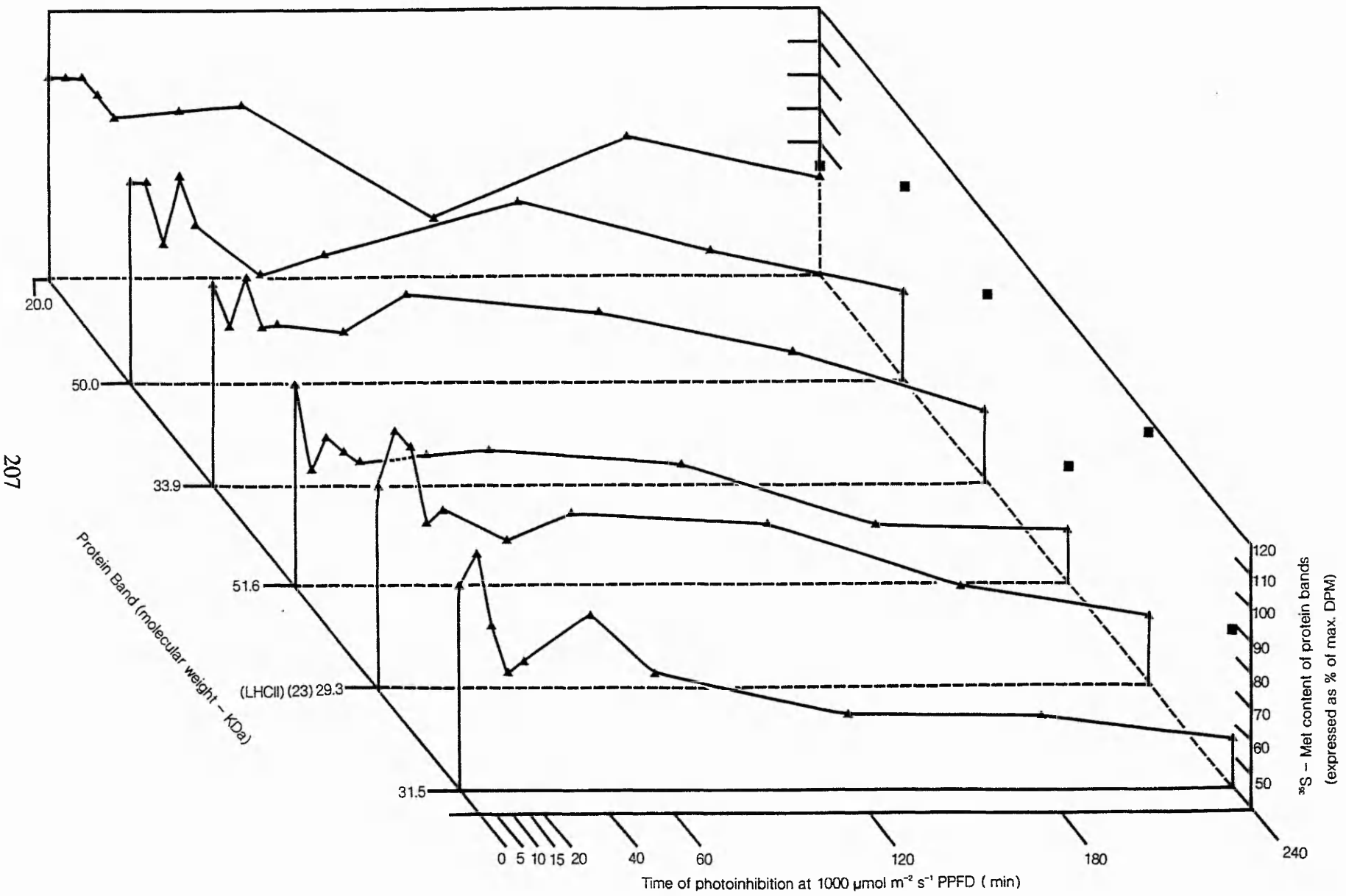
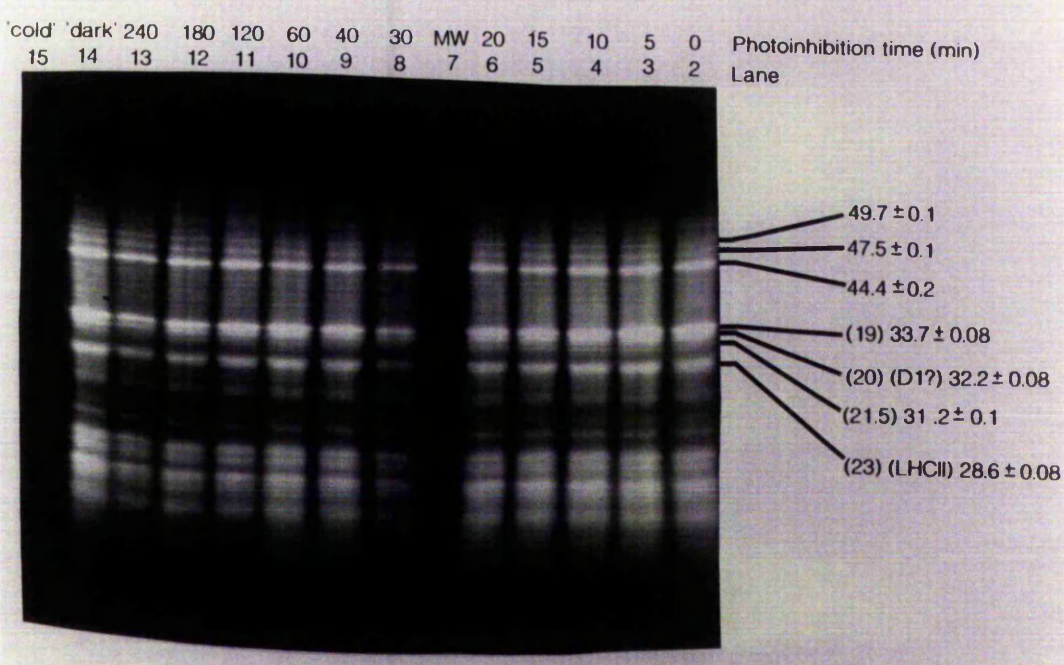

20.0
50.0
33.9
51.6
(LHCII) (23) 29.3
31.5
Protein Band (molecular weight – KDa)
0 5 10 15 20 40 60 120 180 240
Time of photoinhibition at 1000 µmol m⁻² s⁻¹ PPFD (min)
120 110 100 90 80 70 60 50
³⁵S – Met content of protein bands
(expressed as % of max. DPM)

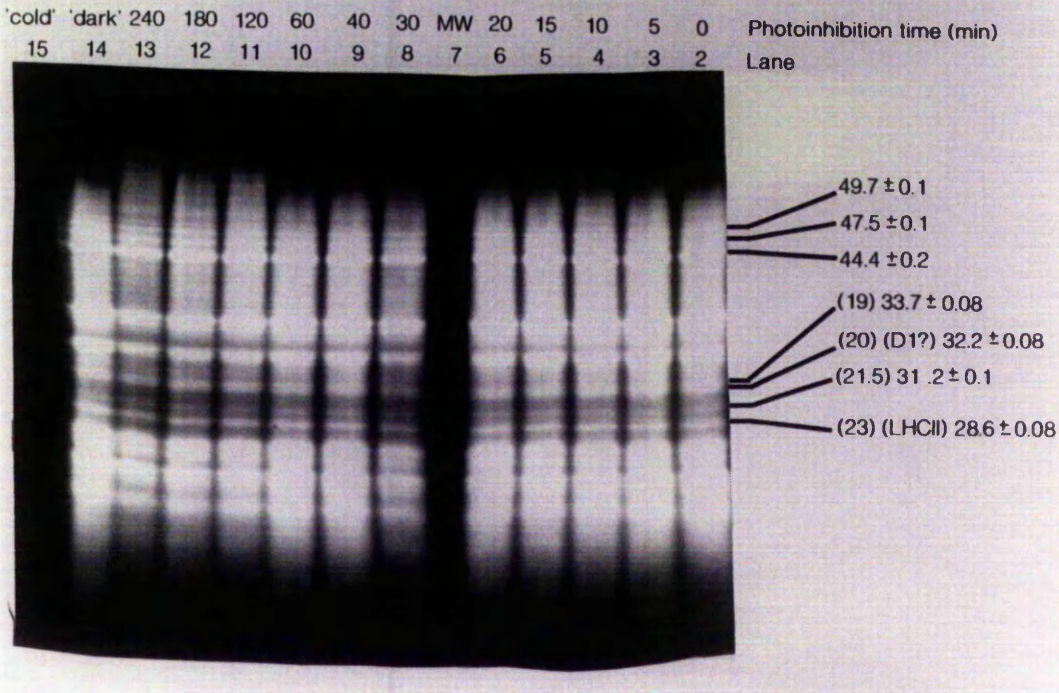
Figure 6.8 Time course of ^{35}S-Met incorporation into thylakoid proteins during 4 hours of photoinhibition, following 2 hours of *in organello* pre - radiolabelling. Chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll were radiolabelled under optimal conditions for two hours. Following pre - radiolabelling the chloroplast suspension was subjected to a photoinhibitory treatment of 1000 μmol m^{-2} s^{-1} PPFD for up to 4 hours. A pre - radiolabelled sample was kept in the dark for 4 hours ('dark' control). A 'cold' control sample was simultaneously photoinhibited for 4 hours (6.2.8). The thylakoid proteins were then separated by SDS-PAGE. The results presented in this Figure were obtained from the gel of Table 6.3 - column A and are derived from the DPM of gel slices (Table 6.4) containing bands visualised by protein staining (determined by scintillation counting). The corrected DPM (DPM - DPM 'cold' control) determined in a particular band before photoinhibition (*i.e.* time = 0 min) was designated 100 % (or 0 % loss of DPM) and the other values were expressed as a percentage reduction of ^{35}S-Met content from this initial value, with time of photoinhibition. The 'dark' control value is also shown, ■. Protein staining revealed 6 major protein bands including those of: 33.9 $\pm$ 0.2 (band 19 or 19 and 20 unresolved, *i.e.*, 19.5); 31.5 $\pm$ 0.2 (doublet designated 21.5); and 29.3 $\pm$ 0.1 (band 23 - LHCII) KDa (n=13). See Figure 6.10.

Reduction of ^{35}S – Met content
of protein bands (expressed as
% decrease from max. DPM)
50
40
30
20
10
0
240
180
120
60
40
20
15
10
5
0
Time of photoinhibition at 1000 µmol m^{-2} s^{-1} PPFD (min)
20.0
50.0
33.9
31.5
(LHCII) (23)
29.3
51.6
Protein Bands (molecular weight – KDa)

in Figure 6.9A and 6.9B, respectively. By studying the fluorographs over a light box it was possible to visualise 21 major bands by radiolabelling (more than the eight major bands visualised by protein staining of the same gel: Table 6.3 - column B). It was possible to detect, by eye, from the fluorograph that the amount of radiolabel generally incorporated decreased with increasing time of photoinhibition, especially if the last two lanes (13 and 14) were compared, which contained the sample photoinhibited for the longest time period (240 min) and the sample that had been incubated in the dark for 240 min ('dark' control), respectively. This reduction in ^{35}S - Met content with increasing time of photoinhibition is in agreement with that clearly demonstrated by scintillation counting of gel slices obtained from the second gel that was run simultaneously (Table 6.3 - column A, Table 6.4, Figures 6.7 and 6.8). Six proteins were particularly conspicuous when observed by fluorography (Figure 6.9), of: 49.7 $\pm$ 0.1; 47.5 $\pm$ 0.1); 44.4 $\pm$ 0.2; 33.7 $\pm$ 0.08 (possibly band 19); 32.2 $\pm$ 0.08 (possibly band 20); and 28.6 $\pm$ 0.08 (band 23) KDa ($n = 12$). A seventh band, although labelled less heavily, of 31.2 $\pm$ 0.1 KDa (possibly the doublet designated 21.5), was also quite distinct. Thus, fluorography revealed an extra band in the region of interest compared with protein staining (Table 6.3 - columns B and C). The latter method revealed two bands of molecular weights 33.7 and 31.3 KDa, which were approximately equally stained by Coomassie Blue. These correspond to bands of 33.7 and 31.2 KDa, as revealed by fluorography. Although these bands were equally stained by Coomassie Blue, the radiolabel incorporation was quite different. The radiolabelling of the larger protein was considerably greater, the smaller protein appearing as a faint but distinct band, as previously described. The extra protein of 32.2 KDa revealed by fluorography was radiolabelled to an intermediate extent, approaching the density of radiolabelling of the 33.7 KDa protein. In contrast, the extra protein of 32.2 KDa was not visible at all after protein staining with Coomassie Blue. Thus, a reasonable interpretation seems to be that the protein bands of approximately 33.7, 32.2 and 31.2 KDa represent the bands designated 19, 20 and 21.5, respectively, as identified in the initial separation (Figure 6.3, Table 6.2). This is of importance when considering the second gel, run simultaneously under identical conditions, and used for scintillation counting. A very similar separation was achieved, particularly in the region of interest, with bands of 33.9 $\pm$ 0.2 and 31.5 $\pm$ 0.2, revealed by protein staining. It is not known whether the third protein of approximate molecular weight 32.2 KDa was also

Figure 6.9 SDS-PAGE of thylakoid proteins after up to 4 hours of photoinhibition, following 2 hours of *in organello* pre - radiolabelling: fluorograph of gel of Table 6.3, column B. Chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll were radiolabelled under optimal conditions for two hours. Following pre - radiolabelling the chloroplast suspension was subjected to a photoinhibitory treatment of 1000 μmol m^{-2} s^{-1} PPFD for up to 4 hours. A pre - radiolabelled sample was kept in the dark for 4 hours ('dark' control). A 'cold' control sample which had not been pre - radiolabelled was simultaneously photoinhibited for 4 hours (6.2.8). MW standards were loaded in lanes 1 and 7 and samples prepared from pre - radiolabelled chloroplasts photoinhibited for 0 to 240 min were loaded in lanes 2-6 and 8-13, whilst the 'dark' and 'cold' controls were loaded in lanes 14 and 15, respectively. The gel was used for fluorography and exposure times of 7 1/4 (A) and 20 1/2 (B) hours are shown. Fluorography revealed 21 major bands including those of: 33.7 $\pm$ 0.08 (band 19); 32.2 $\pm$ 0.08 (band 20 - D1 ?); 31.2 $\pm$ 0.1 (the doublet designated 21.5); and 28.6 $\pm$ 0.08 (band 23 - LHCII) KDa (n = 12).


'cold' 'dark' 240 180 120 60 40 30 MW 20 15 10 5 0
15 14 13 12 11 10 9 8 7 6 5 4 3 2
Photoinhibition time (min)
Lane
49.7 ± 0.1
47.5 ± 0.1
44.4 ± 0.2
(19) 33.7 ± 0.08
(20) (D1?) 32.2 ± 0.08
(21.5) 31 .2 ± 0.1
(23) (LHCII) 28.6 ± 0.08


'cold' 'dark' 240 180 120 60 40 30 MW 20 15 10 5 0
15 14 13 12 11 10 9 8 7 6 5 4 3 2
Photoinhibition time (min)
Lane
49.7 ± 0.1
47.5 ± 0.1
44.4 ± 0.2
(19) 33.7 ± 0.08
(20) (D1?) 32.2 ± 0.08
(21.5) 31 .2 ± 0.1
(23) (LHCII) 28.6 ± 0.08

separated, but not detected by Coomassie Blue staining, as in the gel used for fluorography. If this was the case then the previous designation (*i.e.* 19, 20 and 21.5) would seem appropiate. This would mean that only bands 19 and 21.5, and not band 20, were excised and used for scintillation counting (Table 6.3 - column A, Table 6.4, Figures 6.7 and 6.8). However, the separation achieved in two gels, even when running simultaneously under apparently identical conditions, is not always exactly the same, as often observed in this study. Therefore, the band of 33.9 KDa (Table 6.3 - column A) could alternatively represent bands 19 and 20 unresolved (*i.e.* 19.5). In preceding experiments the incorporation of ^{35}S - Met was not sufficient to permit successful fluorography (data not shown), which is less sensitive than scintillation counting. It is possible that important information, especially concerning the region of the gel of interest, may have been provided in these experiments, had the fluorography been successful. It is apparent from the above that the bands revealed by protein staining in different gels were not necessarily consistent and therefore the identification of the same protein in different gels may not necessarily be unambiguous, particularly when the proteins concerned are of such similar molecular weight. This emphasises the usefulness of using more than one method of protein visualisation, and of the reference protein (band 23), which was unmistakeable when identified by either protein staining or fluorography.

As in the previous experiment (Figure 6.6) it was apparent that despite the abundance of the reference band 23, as revealed by protein staining and the high incorporation revealed by scintillation counting of the second gel that was run simultaneously (Table 6.4), the amount of incorporation revealed by fluorography is relatively low, when compared with the other five conspicuous proteins, as mentioned above. In contrast to the bands 19, 20, 21.5 and 23 referred to in detail above, the five other bands preferentially radiolabelled do not appear to definitely correspond to any of the bands visualised by protein staining (compare Table 6.3 -column B and Figure 6.9). The proteins identified by fluorography, rather than by protein staining can be observed by studying Figures 6.6 and 6.9. In all, 17 (Figure 6.6) and 21 (Figure 6.9) major bands were visualised by fluorography. The proteins designated 19, 20, 21.5 and 23 were observed in both experiments having molecular weights of 33.2, 32.3, 30.9 and 28.4 KDa (Figure 6.6) and 33.7, 32.2, 31.2 and 28.6 KDa (Figure 6.9), respectively. Apart from these four bands only one other is easily identified as the same

Figure 6.10 Photoinhibition of Q_B - dependent electron flow (water to DMBQ,●), Q_B - independent electron flow (water to SiMo,▲), F_o (△), F_p (○), F_v/F_p (□) and ^{35}S-Met content of the protein bands designated 21.5 and 19.5 of 31.5 + 0.2 KDa,■, and 33.9 + 0.2 KDa, ◆, respectively. The parameters are expressed as a percentage decrease from the values obtained with the control sample (before photoinhibition). The thylakoid partial reactions were carried out according to the legend of Fig 4.6 and the fluorescence parameters measured according to the legend of Fig 5.15, whilst the thylakoid protein ^{35}S-Met content was determined according to the legends of Figs 6.7 and 6.8. Thylakoid or chloroplast suspensions equivalent to 50 μg cm^{-3} chlorophyll were photoinhibited at 1000 μmol m^{-2} s^{-1} PPFD. Each point represents the mean of 1 to 8 observations. See text for details.

Inhibition of F_v/F_p, F_p, F_o, DMBQ activity, SlMo activity and reduction in ^{35}S – Met content of 31.5 and 33.9 KDa proteins (expressed as % of these values in non – photoinhibited control)
100
80
60
40
20
0
−20
0
1
15
20
25
30
35
40
240
Photoinhibitory pretreatment – exposure to 1000 µmol m^{-2} s^{-1} PPFD (min)

protein in the two fluorographs: a heavily radiolabelled band of molecular weight 45.8 $\pm$ 0.3 KDa (Figure 6.6) and 44.4 $\pm$ 0.2 (n = 12) KDa (Figure 6.9).

The two bands in the molecular weight range of interest, that were separated and identified by protein staining and subsequently used for scintillation counting are of 31.5 and 33.9 KDa (Figure 6.8). The percent reduction of ^{35}S - Met radiolabel content of these proteins, with increasing time of photoinhibition, is compared with the changes in the fluorescence parameters F_o, F_p, F_v/F_p and the Q_B - dependent and the Q_B - independent electron flow rates, induced by such light stress, as determined in Chapters 4 and 5 (Figure 6.10).

6.4 Discussion

In an initial experiment a thylakoid membrane sample was subjected to SDS - PAGE and up to 30 polypeptides were separated in each lane depending on the amount of sample loaded (Figure 6.3, Table 6.2), of between 12.9 and 116.6 KDa. Other workers have reported similar results (Eaglesham and Ellis, 1974; Chua, 1980; Fish and Jagendorf, 1982; Hoffman - Falk, *et al.*, 1982; Piccioni, *et al.*, 1982; Wettern, *et al.*, 1983; Nivison and Jagendorf, 1984; Ohad, *et al.*, 1984, 1985; Marder, *et al.*, 1986; Nivison, *et al.*, 1986; Covello, *et al.*, 1988; Cleland, *et al.*, 1990; Kuhn and Boger, 1990; Nedbal, *et al.*, 1990). A very heavily Coomasie Blue stained protein of 25 - 29 KDa was identified as the major apoprotein of the LHCII complex by these workers. This band was so heavily stained, compared with all other bands, that it was unmistakeably and rapidly identifiable. The same can be said of the reference band 23 (23.0 - 30.2 KDa) of this study. When comparing the gels of other workers and the gels of this Chapter it is apparent that band 23 corresponds to that identified as the LHCII by these workers. It is suggested that the reference band 23 represents either the major apoprotein of *C. fragile* LHCII or several different components of the LHCII (*e.g.* Ohad, *et al.*, 1984, 1985; Nedbal, *et al.*, 1990) in an unresolved state.

Identification of the other bands within the molecular weight range of interest, by comparison of the gels from this and other studies, such as those referred to above, is less certain. It seems that of the two doublets separated above the LHCII, the upper consisting of the bands designated 19 and 20, is of the most interest to this study (Figure 6.3, Table 6.2). By comparison with previous work, referred to above, it seems likely that either band 19 or 20 represents the D1 protein of *C. fragile*. The initial

preferential radiolabelling (over the first 10 - 15 min of incubation) of bands 19 and 20 (Figure 6.6) and the heavy radiolabelling of these bands observed in Figure 6.9, in contrast to the faint/ lack of staining by Coomassie Blue, support the proposal that one of these bands represents D1. In the latter experiment it was apparent that protein staining failed to reveal band 20 (Table 6.3 - column B) whilst fluorography of the very same gel clearly showed a significantly radiolabelled band 20 (Table 6.3 - column C, Figure 6.9) of 32.2 KDa. This strongly suggests that band 20 is D1. The two bands 19 and 20 were often resolved in this investigation (Figures 6.3, 6.4, 6.6 and 6.9), with molecular weights of 30.0 - 34.1 and 29.1 - 33.4 KDa, respectively.

The gel profiles of Chow, *et al.* (1989), Cleland, *et al.* (1990) and Nedbal, *et al.* (1990) were complicated by the presence of the OEC components of 16 KDa, 23 KDa, and, in particular, 34 KDa. It is unlikely that the components of the OEC will be present in significant amounts in the gels of this study, as the extensive washing of the thylakoid membranes in 2.5 mol m^{-3} Tris -glycine, pH 8.5, 0.15 mol dm^{-3} NaCl washing buffer during preparation for SDS - PAGE will remove peripheral proteins from the membrane and these proteins will remain in the supernatant upon centrifugation (Marder, *et al.*, 1986; Bassi, *et al.*, 1989). In the absence of the largest OEC component (33 - 34 KDa) it could be suggested that the smallest protein of the doublet of interest (band 20) represents D1, whilst the larger protein (band 19) may represent the precusor of D1 (33.5 - 34.8 KDa) as identified by Fish and Jagendorf (1982) and Nivison and Jagendorf (1984). A further possibility is provided by the fact that the D2 protein, generally attributed the molecular weight of 34 KDa, is also diffusely stained by Coomassie Blue (Critchley, 1988) and could thus also be represented by band 19. A further alternative is provided by Callahan, *et al.* (1990). These workers subjected thylakoid membranes to SDS - PAGE and resolved a doublet in the molecular weight range of interest in granal lamellae preparations. The smaller protein was identified as D1 and the larger, less mobile band was suggested to be a novel, modified form of this protein, designated 32*. Thus, bands 19 and 20 could represent the 32* and 32 KDa - D1 proteins separated by Callahan, *et al.* (1990). The identification of D1 may be aided by the use of ^{14}C -azidoatrazine (*e.g.* Ohad, *et al.*, 1984; Chow, *et al.*, 1989; Kuhn and Boger, 1990) or antibodies raised against D1 (*e.g.* Aro, *et al.*, 1990; Kuhn and Boger, 1990; Virgin, *et al.*, 1990). If such approaches are used in further work the identity of D1 may be determined with greater certainty. The

doublet consisting of bands 21 and 22 (Figure 6.3, Table 6.2) was often represented by a single band (Figures 6.3, 6.4, 6.6 and 6.9) or was not visualised at all (gels not shown). It is possible that this doublet, in the molecular weight range of 25.7 - 31.5 KDa as estimated in the gels of this study, may represent a minor apoprotein of the LHCII (Ohad, *et al.*, 1984, 1985) or possibly a 31 KDa polypeptide (that is not D1) that accumulates at low temperature (5 °C) and high light and is thought to be the unprocessed precursor of apoprotein CP29, a minor chlorophyll protein complex intimately associated with LHCII (Covello, *et al.*, 1988; Hayden and Baker, 1990). The most abundant stroma ribosome product is the large subunit of ribulose bisphosphate carboxylase/ oxygenase of 54 KDa molecular weight and the absence of heavy Coomassie Blue staining in this region of the gel is an indication that there is little contamination of the thylakoid preparation with soluble stroma proteins due to inadequate washing (Fish and Jagendorf, 1982). In future experiments it might be interesting to subject the pooled supernatants produced during the preparation of thylakoid membranes to SDS - PAGE. This may yield pertinent information concerning the stroma proteins and also extrinsic membrane proteins, such as the components of the OEC.

From Figures 6.5, 6.6 and 6.9, and Table 6.4 (Figures 6.7 and 6.8) it is evident that the *in organello* radiolabelling of *C. fragile* thylakoid membranes with ^{35}S - Met was successfully achieved. Not all species studied have been successfully used for such *in organello* radiolabelling. For example, Ellis and Hartley (1982) found that the most active chloroplasts for protein synthesis were prepared from the leaves of peas (*Pisum sativum*), whilst chloroplasts from spinach (*Spinacia oleracea*), tobacco (*Nicotiana tabacum*), maize (*Zea mays*), and barley (*Hordeum vulgare*) were less active. These workers were unable to prepare chloroplasts active in light - driven protein synthesis from either bean (*Phaseolus vulgaris*) or wheat (*Triticum vulgare*). Thus, the successful adaptation of this technique to chloroplasts isolated from *C. fragile*, which has not been previously reported in the literature, as developed in this Chapter, was not at the outset assured, and can therefore in itself be regarded as an achievement.

Codium fragile chloroplasts are highly stable *in vitro* and remain largely intact for many hours (even days) of storage in the dark at 4 °C (Cobb and Rott, 1978). When stored in the dark at 5 °C in a simple mineral medium containing mannitol as an osmoticum and no added cofactors, isolated *C. fragile* chloroplasts continued to fix

carbon photosynthetically for at least five days after isolation, although at a progressively declining rate (Trench, *et al.*, 1973). It was also noticed during the course of this work that bicarbonate - dependent oxygen evolution of isolated chloroplasts and the DMBQ - , SiMo - and $K_3Fe(CN)_6$ - dependent oxygen evolution activities of isolated thylakoids were as high after up to approximately eight hours storage on ice in the dark as the rates measured immediately after isolation. The stability of isolated *C. fragile* chloroplasts may be a factor contributing to the successful *in organello* radiolabelling of thylakoid membrane proteins with ^{35}S - Met. Furthermore, the 'longevity' of the isolated chloroplasts may enhance the potential usefulness of this *in organello* radiolabelling technique, enabling the performance of experiments requiring a length of time that would preclude the use of the majority of isolated higher plant chloroplasts, which are generally active for a much shorter time period.

In order to study the functioning of the chloroplast translation system and its interaction with the nuclear - cytoplasmic and mitochondrial translation systems, it is important to be able to isolate and reconstitute these processes (Nivison, *et al.*, 1986). To achieve this, fully functional isolated chloroplasts are essential. For many years translation activity in isolated chloroplasts was very short lived, with the most rapid rates declining after 5 min and stopping completely after 15 to 30 min (*e.g.* Ellis and Hartley, 1982). Thus, much work was carried out with rapidly decaying systems whose similarity to the *in vivo* state was uncertain. More recently procedural improvements have been made allowing active translation by isolated pea chloroplasts for almost one hour (Nivison, *et al.*, 1986). The radiolabelling achieved in this study compares favourably, with increasing incorporation for up to two hours, and even up to four hours, albeit at a lower rate. Such levels of incorporation are likely to be sufficient to enable a variety of future experiments to be carried out with regard to the turnover of thylakoid proteins, including D1, during photoinhibition.

There are advantages to using light, rather than exogenous ATP, as the energy source. It is characteristic of intact isolated chloroplasts that they can use light to synthesise ATP in the absence of added cofactors (Ellis and Hartley, 1982). Broken chloroplasts cannot do this, because of the dilution of ferredoxin that occurs on breakage. Thus, it is not necessary to purify intact chloroplasts free from broken chloroplasts if light is used as the energy source to drive protein synthesis. It is thought

better to use intact chloroplasts as conditions around the polysomes and polymerase complexes are more likely to be normal than in lysed chloroplasts. Therefore correct elongation, termination and release of macromolecular chains is more likely, and these processes must occur correctly if discrete, identifiable products are to be obtained. Light - dependence is a good criterion for synthesis occurring only in intact chloroplasts, especially in crude preparations that may be contaminated with nuclei, mitochondria, cytoplasmic polysomes and microorganisms. The protein synthesis observed in this study was carried out under 50 μmol m^{-2} s^{-1} PPFD, without added ATP. Although it is believed that there is little doubt that protein synthesis was light driven, a control consisting of a chloroplast suspension in the presence of ^{35}S - Met incubated in the dark for the same duration as the radiolabelling period, in which little incorporation would be expected, was not included in these experiments and perhaps this should be carried out in a future experiment to unequivocally demonstrate light - dependence.

The vital component in incubation solutions for *in organello* radiolabelling was found to be the presence of K^+ (Ellis and Hartley, 1982). These workers found that if K^+ were replaced with Na^+ in such solutions, no light - driven protein synthesis occurred. It was also discovered that ATP - driven protein synthesis in lysed chloroplasts similarly required K^+ and it was concluded that chloroplast ribosomes have a specific requirement for these ions, as do bacterial and animal ribosomes. It was therefore interesting that incubation condition 1 which used extraction medium (Table 6.1) appeared to support a certain amount of incorporation in *C. fragile* chloroplasts (data not shown), despite the use of NaOH rather than KOH in pH adjustment. The incorporation under incubation condition 1 appeared to be equal to, or greater than, that under incubation conditions 2, 5, 6, 7, 8 and 9, all of which included solutions containing K^+ rather than Na^+ (data not shown). It should be emphasised that these experiments were carried out only once and repetition would be desirable for conclusions to be made with more certainty. These initial experiments may suggest that Na^+, as well as K^+, can support ^{35}S - Met incorporation in *C. fragile* chloroplasts. However, the levels of endogenous Na^+ and K^+ within the chloroplasts was unknown and perhaps K^+ were present in sufficient amounts to support the relatively small amounts of incorporation observed in these initial experiments (data not shown).

Incubation solution B was devised by Ramirez, *et al.* (1968) and contains KCl as

osmoticum as well as buffer and Mg^{2+}. Solution C was devised by Bottomley, *et al.* (1974) and contains sorbitol as osmoticum. Ellis and Hartley (1982) found several important differences between the results obtained with these two incubation solutions. For example, chloroplasts from all species tested exhibited light - driven protein synthesis in solution B, but those from maize and barley showed no such synthesis in solution C, unless Mg^{2+} ions were added to 6.6 mol m^{-3}. Secondly, the rates and products of light - driven protein synthesis by pea and spinach chloroplasts were the same in solutions B and C but in solution C the large subunit of ribulose bisphosphate carboxylase produced also assembled into the holoenzyme. These workers therefore recommended that both solutions B and C should be investigated, as in this study, in experiments with chloroplasts from untested species. From the results of various experiments Ellis and Hartley (1982) also concluded that light - driven protein synthesis was proceeding only in intact chloroplasts, that it was driven by photophosphorylation, and that it was using mRNA synthesised before the chloroplasts were isolated. It is possible that this was also the situation during the radiolabelling of *C. fragile* chloroplasts carried out in this study.

Various parameters were found to extend the time course of protein synthesis by isolated chloroplasts (Fish and Jagendorf, 1982; Nivison and Jagendorf, 1984; Nivison, *et al.*, 1986). These parameters included: Mg^{2+} in the medium at optimal concentrations; additional amino acids, especially leucine, isoleucine and threonine; inorganic phosphate at 100 to 300 mmol m^{-3}; lower rather than higher light intensity; and ATP addition, even in the light. The improvements did not increase initial rates of incorporation (*i.e.* over the first 10 min), probably because these rates were close to *in vivo* rates of translation, but the time courses were extended to 60 min and consequently the total amount of incorporation was increased by approximately 85 %. The improvement in incorporation varied depending upon the concentrations of the various additions, but optimal radiolabelling was found to occur, by these workers, when the incubation medium contained 9.5 mol m^{-3} ATP, 12.5 mol m^{-3} $MgCl_2$ and 200 mmol m^{-3} of leucine, isoleucine and threonine. If ATP was omitted and the reaction driven by light only, the optimum $MgCl_2$ concentration was 200 mmol m^{-3}. Thus, considering the advantages of using light, rather than ATP, as the energy source (Ellis and Hartley, 1982) a third incubation solution (D) was investigated in this study, which was based on that of Nivison and Jagendorf (1984) and Nivison, *et al.* (1986). Solution D

consisted of 1.87 mol m^{-3} EDTA, 200 mmol m^{-3} $MgCl_2.6H_2O$, 200 mmol m^{-3} leucine, 200 mmol m^{-3} isoleucine, 200 mmol m^{-3} threonine, in 350 mol m^{-3} sorbitol, 33 mol m^{-3} HEPES -KOH (pH 8.3), 0.9 mol m^{-3} DTT, 10 mol m^{-3} $NaH_2PO_4.2H_2O$. Increasing ^{35}S - Met incorporation with increasing Met concentration was also demonstrated, saturating at about 50 mmol m^{-3} Met (Nivison and Jagendorf, 1984; Nivison, *et al.*, 1986) and therefore this factor was investigated in this study by varying the amount of Met included in the incubation solutions (Table 6.1). A fourth incubation solution (A) was also investigated which consisted of modified extraction medium (0.5 mol dm^{-3} sucrose, 2 g dm^{-3} BSA, 0.015 mol dm^{-3} $MgCl_2.6H_2O$ in 0.05 mol dm^{-3} HEPES buffer, adjusted to pH 7.8 with 1 mol dm^{-3} KOH), the pH of which was adjusted using KOH, rather than NaOH.

It was clear from the results that incubation solution A consistantly supported higher ^{35}S - Met incorporation than the other three solutions (data not shown). It was also evident that, in contrast to other workers findings (*e.g.* Nivison, *et al.*, 1986), the highest incorporation was achieved with very low Met concentrations (*i.e.* 0.17 and 0.236 mmol m^{-3} in the experiments of Figure 6.5 and Table 6.4, respectively), with no added 'cold' non - radioactive Met. The optimum time for radiolabel incorporation was two hours (Figure 6.5) and incorporation continued to increase, albeit at a reduced rate, for up to 4 hours. This time course of radiolabelling compares favourably with that of 60 min, which is the most extended time course previously reported (Nivison and Jagendorf, 1984; Nivison, *et al.*, 1986). Thus, of the conditions investigated, that which supported the highest incorporation consisted of incubation solution A, in the presence of no 'cold' non -radioactive Met. Solution D included the improvements suggested by Nivison and Jagendorf (1984) and Nivison, *et al.* (1986) and the various additions were at concentrations found to be optimum for radiolabelling pea chloroplasts, which may not be optimum for *C. fragile* chloroplasts. The components of the solutions investigated in this study include sucrose, BSA, $MgCl_2.6H_2O$, HEPES, NaOH, KOH, KCl, Tricine - KOH, sorbitol, EDTA, leucine, isoleucine, threonine, methionine, ^{35}S - Met, DTT and $NaH_2PO_4.2H_2O$ and the pH of the solutions were 7.8, 8.3 or 8.4. The effect of inclusion of all 20 amino acids, as studied by Nivison and Jagendorf (1984), was not investigated. It is obvious that by using different combinations of these components and by adjusting the pH to different levels an almost limitless number of different incubation solutions could be produced. Thus, it is

possible that further investigation could result in the discovery of an incubation solution that supports greater ^{35}S - Met incorporation by isolated *C. fragile* chloroplasts, than that observed under the conditions examined so far.

A final experiment investigated the ^{35}S - Met content of thylakoid proteins during four hours of photoinhibition following two hours of *in organello* pre -radiolabelling. Chloroplast suspensions equivalent to 50 $\mu g\ cm^{-3}$ chlorophyll were radiolabelled for two hours using incubation solution A, containing ^{35}S - Met at 128 $\mu Ci\ cm^{-3}$, 650 Ci $mmol^{-1}$ and 0.236 mmol m^{-3} final concentration, with no added 'cold' Met. Following pre - radiolabelling the chloroplast suspension was subjected to a photoinhibitory treatment of 1000 $\mu mol\ m^{-2}\ s^{-1}$ PPFD for up to four hours. The thylakoid proteins were separated by SDS - PAGE and the gels used for fluorography (Table 6.3 - columns B and C, Figure 6.9) or scintillation counting (Table 6.3 - column A, Table 6.4, Figures 6.7 and 6.8).

Protein staining revealed two bands in the molecular weight range of interest of 33.9 and 31.5 KDa in the gel used for scintillation counting (Table 6.3 - column A) and 33.7 and 31.3 KDa in the gel used for fluorography (Table 6.3 - column B). However, fluorography clearly revealed three bands in the molecular weight range of interest of 33.7, 32.2 and 31.2 KDa (Table 6.3 - column C). The larger two proteins possibly represent the bands designated 19 and 20, whilst the smaller protein may represent the band designated 21.5. As previously discussed it is thought that the band most likely to represent the D1 protein is that designated number 20, although band 19 is also a possibility.

The second gel that was run simultaneously and then used for scintillation counting exhibited a similar Coomassie Blue staining pattern in the region of interest, suggesting that a similar separation was achieved in the two gels. Thus, it is possible that band 20 (D1 ?) was present but not stained (as in the gel used for fluorography) and hence not visible. If this was so, then the only bands in the region of interest to be subjected to scintillation counting would be those designated 19 and 21.5, with the unstained band 20 being unfortunately overlooked. It is thus possible that the turnover of the protein of most interest (D1) under photoinhibitory conditions was only monitored by fluorography (Figure 6.9) and was not also followed by scintillation counting (Table 6.4, Figures 6.7 and 6.8).

It was possible to detect, 'by eye', from the fluorograph (Figure 6.9) that the

amount of radiolabel generally incorporated decreased with increasing time of photoinhibition, especially if the last two lanes (13 and 14) were compared, which contained the sample photoinhibited for the longest time period (240 min) and the sample that had been incubated in the dark for 240 min ('dark' control), respectively. This rather subjective means of differentiation of the radiolabel content of polypeptide bands is less than satisfactory. An initial (over the first 0 - 10 min) preferential reduction of radiolabel content in D1, which would be expected if this protein was turning over particularly rapidly, is not obvious from the fluorograph of Figure 6.9. However, from 15 to 240 min of photoinhibition the radiolabel content of D1 does appear to decrease. This preliminary evidence for decline in the D1 radiolabel content only after 15 min of photoinhibition may be interpreted as being consistent with the results concerning the events of photoinhibition as discussed in Chapters 4 and 5, in which some of the proposals of Setlik, *et al.* (1990) and Ohad, *et al.* (1988, 1990) appeared particularly similar to the results of the present study.

Briefly, both groups of workers suggested that an initial event in photoinhibition may involve the generation of Q_A^- and that this may increase F_o. The variable fluorescence was also reduced but the primary photochemistry was not affected and Setlik, *et al.* (1990) reported reduction in electron flow from water to p - benzoquinone, whilst Ohad, *et al.* (1988, 1990) reported a reduction in electron flow from water to 2, 3 - dichloro - 1, 4 - benzoquinone, but the persistence of electron transfer from water to SiMo (Kyle, *et al.*, 1984). This is similar to the first stage of photoinhibition in *C. fragile* chloroplasts which revealed an initial increase in F_o accompanied by a reduction in variable fluorescence (Chapter 5), a reduction in electron flow from water to DMBQ, but persistence of electron transfer from water to SiMo (Chapter 4), over the first 10 min of photoinhibitory treatment (Figure 5.19). Thus, following the proposals of these workers it was suggested that these findings may indicate that the first stage of photoinhibition in *C. fragile* may involve the development of Q_A^- (blocking electron transfer to DMBQ due to trapping of Q_A in the negative state), eventually (after 10 min) leading to complete inactivation of Q_A (blocking electron transfer to DMBQ and SiMo).

Following this initial stage, Setlik, *et al.* (1990) reported constant F_o, declining F_v and electron flow from water to p - benzoquinone and eventually loss of reaction centre charge separation ability. Ohad, *et al.* (1988, 1990) also reported ensuing loss of F_v

and, eventually, reaction centre II activity. Perhaps in *C. fragile* similar processes are occurring, resulting in inactivation of Q_A (after approximately 10 min under the conditions of this study) and only once this initial damage has occurred does increased D1 turnover ensue (becoming apparent after approximately 15 min of photoinhibition when monitored by fluorography - Figure 6.9). This would also be consistent with the suggestion that initial damage to D1 could act as a recognition signal for proteolysis of the modified protein by a highly efficient intrinsic membrane protease (Ohad, *et al.*, 1985, 1990). The fluorograph of Figure 6.9 represents one experiment only and repetition is obviously required to provide confirmation of any results observed and the conclusions drawn from them. However, this initial experiment is encouraging and suggests that further use of this approach may enable a detailed study of polypeptide turnover during photoinhibition of *C. fragile* chloroplasts, especially if more quantitative analyses of the fluorographs are made.

Fluorographs can be analysed densitometrically by using a spectrophotometer fitted with a scanning attachment, enabling quantitation by measurement of the peak area, although the resolution of closely migrating bands is poor (Hames, 1981). The use of this technique to carry out such analysis of the fluorograph may have yielded more information about the level of ^{35}S - Met incorporation into the various proteins, particularly D1, over the photoinhibitory time course. Alternatively, each lane of the slab gel could have been sliced into 1 mm sections, solubilised in H_2O_2 and subjected to scintillation counting, thereby determining the radiolabel level throughout the entire length of each lane (*e.g.* Eaglesham and Ellis, 1974; Hames, 1981; Callahan, *et al.*, 1986). This would also prevent radiolabelled proteins, present in amounts too small for detection by Coomassie Blue staining, from being overlooked. A third option is the use of silver staining for proteins, which can be up to 100 times more sensitive than Coomassie Blue (Hames, 1981), in conjunction with scintillation counting of visualised bands. However, a compromise has to be made between the extra time, expense and effort required for different techniques and the advantages that they provide. The doublet designated 19 and 20 was often successfully visualised by Coomassie Blue staining (Table 6.2, Figures 6.3, 6.4A and 6.4B) and it is suggested that this comparatively simple and rapid method of visualisation is adequate, but that if more time is available fluorography, densitometry and/ or scintillation counting of the entire gel (in 1 mm slices) to provide a more detailed record of the radiolabelling of all the

thylakoid proteins separated, is desirable. The number of bands visualised by radiolabelling and subsequent fluorography in this study (17 major bands in Figure 6.6 and 21 major bands in Figure 6.9) approaches that described by other workers (*e.g.* Eaglesham and Ellis, 1974; Fish and Jagendorf, 1982; Hoffman - Falk, *et al.*, 1982; Nivison and Jagendorf, 1984; Nivison, *et al.*, 1986).

The scintillation counting revealed that the ^{35}S - Met content of all the proteins decreased with increasing time of photoinhibition (Figures 6.7 and 6.8), supporting the general decrease that was discernable in the fluorograph of Figure 6.9. The most pronounced responses over the first 15 min of photoinhibition were apparent with the proteins of 51.6, 29.3 and 31.5 KDa, which showed decreases in radiolabel content to approximately 80, 89 and 74 % of the level determined at zero min photoinhibition, respectively (Figure 6.7). From 15 min to 240 min photoinhibition the radiolabel content of these proteins generally continued to decrease at a more gradual rate, reaching levels of 56.1, 61.0 and 54.4 %, respectively. The radiolabel content of these proteins in the 'dark' control sample (4 hours incubation in the dark) exhibited values of 75, 117, and 87 % of the level determined at 0 min photoinhibition, respectively. These results may suggest that these proteins were turning over more rapidly than the others analysed, especially over the first 15 min of photoinhibition.

To aid comparison with results obtained in other Chapters the results of this experiment were also expressed as percentage reduction of ^{35}S - Met content from the level of incorporation at 0 min photoinhibition (Figures 6.8, 6.10). The most marked initial response was that of the protein of 31.5 KDa which exhibits a 26 % reduction of ^{35}S - Met after 15 min of photoinhibition. Over the same time period the proteins of 51.6, 33.9 and 29.3 KDa exhibited reductions of ^{35}S - Met content of 20, 14 and 11 %, respectively. After four hours photoinhibition the proteins of 31.5, 51.6, 29.3 and 33.9 KDa exhibited 46, 44, 39 and 39 % reductions of ^{35}S - Met, whilst the corresponding proteins of the 'dark' control sample showed 13, 25, -17 and 5.4 % reductions of ^{35}S - Met content, respectively. Thus, the significance of the 44 % decrease of the ^{35}S - Met content of the 51.6 KDa protein under photoinhibitory light was reduced by the fact that there was a substantial (25 %) loss of ^{35}S - Met in the dark. These results may suggest that initially (first 15 min) the 31.5 KDa protein turns over most rapidly, whilst that of 33.9 KDa turns over less rapidly, followed by the rate of turnover of the 29.3 KDa protein. As discussed there is some uncertainty regarding whether either of the

proteins in the relevant molecular weight range, that is, those of 31.5 and 33.9 KDa, represents the D1 protein. It is believed that it is very unlikely that the smaller protein is D1 (this is more likely to represent the doublet designated 21.5 - Table 6.4, column A). Although it is perhaps more reasonable to suggest that the larger protein could represent band 19 or bands 19 and 20 in an unresolved state and could thus possibly represent D1, it is more likely that the larger protein is band 19 and that band 20 was not visualised by protein staining, and thus not subjected to scintillation counting. Therefore, it is not possible to draw conclusions about D1 turnover during photoinhibition from this experiment, with any certainty. It should also be emphasised that this experiment was performed only once and repetition is obviously desirable. It was unfortunate that bands 19 and 20 were not successfully visualised by Coomassie Blue staining in this experiment. However, these bands were often identified in the other gels of this study and thus there is great potential for repetitions of such experiments to yield information about protein turnover, particularly D1, which may further elucidate the processes that occur during photoinhibition in *C. fragile*.

The percentage reduction of radiolabel in the bands of 31.5 and 33.9 KDa are compared to the other parameters measured in previous Chapters (Figure 6.10). The larger of these proteins is perhaps most relevant, possibly representing D1 or the precursor to D1, as previously discussed. After 5 min photoinhibition there is a 14 % increase in the radiolabel content of the 33.9 KDa protein, which coincides with a 5 % decrease in DMBQ activity and a 7 % and 12 % increase in SiMo activity and F_o, respectively. It is possible that a loss of label from D1 could indicate degradation of this protein and thus explain a loss of DMBQ activity as Q_B, the donor of electrons to DMBQ, is situated on D1. If loss of label from D1 indicates a reduction in the amount of this protein and this is indeed the cause of reduced DMBQ activity, this in turn suggests that degradation of the protein is exceeding the rate of repair and replacement with funtional D1. The exact location of Q_A, the donor of electrons to SiMo, is less certain but it is thought that the Q_A binding site could be on the D2 protein, with P680 and phaeophytin a in a domain shared by both the D1 and D2 subunits (Kyle, 1987). If this is so then removal of D1 could initially expose an otherwise hidden Q_A site assisting the penetration of SiMo, thus enhancing this electron transport activity and perhaps explaining the 7% increase observed after 5 min photoinhibition. It has been suggested that if the D1 and D2 proteins are both required for the stabilisation of P680

and phaeophytin a then removal of D1 would render the reaction centre non - functional (Kyle, 1987). If this were so then SiMo activity would also be reduced due to the breakdown of the electron transfer chain prior to Q_A. However, several groups have reported that the D1 protein is rapidly turned over in the light without a concurrent loss of PSII capacity if an electron acceptor from Q_A is used (Mattoo, *et al.*, 1981; Ohad, *et al.*, 1985; Nedbal, *et al.*, 1986; Arntz and Trebst, 1986). The ^{35}S - Met content of the 33.9 KDa protein is a bit erratic, as a slight increase over the level at 0 min photoinhibition is observed after 10 min incubation (Figure 6.10). The percentage increase was very similar to that observed in F_o and the SiMo activity after 10 min treatment, whilst the DMBQ activity had been further reduced to 21 %. From 10 to 15 min the radiolabel content decreased to 13 % and remained virtually constant up to 40 min photoinhibition.

It is not possible to make valid conclusions about possible connections between the turnover of the 33.9 KDa protein and the other parameters such as F_o, DMBQ and SiMo activities, because the radiolabel experiment was carried out once only. Thus, it is quite reasonable to suggest that any one of the points, at any photoinhibitory time, is not typical. For example, the 14 % decrease after 5 min could be an extreme value. A negligible loss of radiolabel, or even a slight increase in radiolabel, after 5 min would have suggested little D1 turnover after up to 10 min photoinhibition (which is in agreement with the fluorograph of Figure 6.9 in which increased D1 turnover only became noticeable after 15 min of photoinhibition), after which loss of radiolabel and hence increased turnover would have been apparent. In contrast, the 3 % increase in radiolabel after 10 min could be an extreme value. Had this value been in the region of a 12 - 14 % decrease, then a very different interpretation would have ensued: that D1 degradation occurred immediately between 0 and 5 min, after which the amount of turnover remained essentially constant up to 40 min photoinhibition, as reflected by the essentially constant ^{35}S - Met content. Thus, an erroneous value of incorporation at either one of two points could lead to quite different conclusions about the order of events during photoinhibition of *C. fragile*. That is, is D1 degradation the cause or a consequence of photoinhibition? The problem is, of course, exacerbated by the uncertain identity of the 33.9 KDa protein. Again, this indicates the need for repetition.

There have been many reports supporting the proposal that damage to the Q_B site and hence degradation of the D1 protein is the cause of photoinhibition (*e.g.* Kyle, *et*

al., 1984; Ohad, *et al.*, 1984, 1985, 1988, 1990; Nedbal, *et al.* 1986; Kyle, 1987; Kirilovsky, *et al.*, 1988; Schuster, *et al.*, 1988b; Vonshak, *et al.*, 1988; Shyam and Sane, 1989; Hundal, *et al.*, 1990; Kuhn and Boger, 1990; Shochat, *et al.*, 1990; Virgin, *et al.*, 1990). An alternative view is that the primary lesion involves another component such as P680, phaeophytin a or Q_A, and that D1 degradation is simply a consequence of photoinhibition (*e.g.* Cleland and Critchley, 1985; Arntz and Trebst, 1986; Allakhverdiev, *et al.*, 1987; Cleland, 1988; Critchley, 1988; Aro, *et al.*, 1990; Cleland, *et al.*, 1990; Jegerschold, *et al.*, 1990; Kirilovsky, *et al.* 1990). The more recent literature appears to reveal a general consensus that the second suggestion is more likely, that is, that D1 degradation is a consequence of photoinhibition. Much of the work supporting this suggestion, including some of the reports referred to above, illustrate that light - dependent D1 degradation does occur, it is simply the order of events that remains controversial. The results of Chapters 4 and 5 in conjunction with the preliminary results of this Chapter also appear to suggest that the second proposal is more likely. That is, that initial damage to isolated *C. fragile* chloroplasts upon photoinhibition is at Q_A and that only after this does increased D1 turnover occur. There has also been an increasing amount of work further associating photoinhibition and D1 turnover (regardless of whether it is the cause or a consequence) by clearly demonstrating that *de novo* protein synthesis is required in order to allow repair of the damaged D1 protein and hence recovery from photoinhibition (*e.g.* Samuelsson, *et al.*, 1985; Gong and Nilsen, 1989; Huse and Nilsen, 1989; Falk, *et al.*, 1990; Krupa, *et al.*, 1990).

6.5 Conclusions

Thylakoid membranes were subjected to SDS - PAGE and a band designated number 23, and unmistakeably characterised by extremely heavy Coomassie Blue staining, of 23.9 - 30.2 KDa, was identified as either the major apoprotein of *C. fragile* LHCII, or several different components of the LHCII in an unresolved state. A diffusely stained doublet consisting of bands designated 19 and 20, of molecular weights 30.0 - 34.1 and 29.1 - 33.4 KDa, respectively was also observed. It is believed that either of these bands could represent D1, although it is thought most likely that the smaller of the two (band 20) corresponds to this PSII component. The identity of the larger band (19) is uncertain; it is possible that it could represent the D1 precursor, D2

or a novel, less mobile form of D1, designated 32*. A doublet designated 21 and 22 (often represented as a single unresolved band) in the molecular weight range of 25.7 to 31.5 KDa, was often diffusely stained. This may represent a minor component of the LHCII and/ or the 31 KDa polypeptide, that is thought to be the unprocessed precursor of CP29. Light - driven *in organello* radiolabelling of *C. fragile* thylakoid membrane proteins with ^{35}S - Met, which has not been previously reported, was successfully achieved. Furthermore, the time course of radiolabelling (incorporation continued to increase for two hours, and even up to four hours, albeit at a reduced rate) compared favourably with that of 60 min: the most extended time course previously reported (for pea chloroplasts). The levels of incorporation, together with the longevity of isolated *C. fragile* chloroplasts, may provide a potentially very useful experimental system not only for the study of the role of protein turnover (in particular D1) in the photoinhibition of *C. fragile*, but also for more general investigations of the functioning of the chloroplast translation system and its interaction with the nuclear - cytoplasmic and mitochondrial translation systems. Incubation solution A (modified extraction medium - 0.5 mol dm^{-3} sucrose, 2 g dm^{-3} BSA, 0.015 mol dm^{-3} $MgCl_2.6H_2O$ in 0.05 mol dm^{-3} HEPES buffer, adjusted to pH 7.8 with 1 mol dm^{-3} KOH) containing no 'cold' non - radioactive methionine and ^{35}S - Met at approximately 100 μCi cm^{-3} resulted in the greatest radiolabel incorporation. In contrast to other reports the presence of Na^+ in the medium, rather than K^+, supported some incorporation. An initial (over the first 0 - 10 min) preferential reduction of the ^{35}S - Met content of D1, which would indicate preferential turnover of this protein, during photoinhibition of a pre - radiolabelled chloroplast preparation was not obvious when followed by fluorography. However, from 15 to 240 min of photoinhibition the radiolabel content of D1 did appear to decrease. In conjunction with the results of Chapters 4 and 5 this could be interpreted as suggesting that, under the conditions of this study, photoinhibition of isolated *C. fragile* chloroplasts initially (0 - 10 min) results in damage to Q_A and that only after this primary lesion has occurred does inreased D1 turnover ensue (*i.e.* after 15 min of photoinhibition). The initial damage to D1 could act as a recognition signal for proteolysis of the protein. Scintillation counting revealed a general loss of radiolabel from the majority of the proteins separated during the photoinhibitory time period. After the photoinhibitory treatment the bands 19 and 20 were not successfully separated by SDS - PAGE and stained with Coomassie Blue, therefore it was not possible to

make conclusions about D1 turnover from the results of the scintillation counting with any certainty. However, there is potential for repetitions of such experiments to provide information about protein turnover, particularly of D1, perhaps further elucidating the processes that occur during photoinhibition in *C. fragile*.

CHAPTER 7 DISCUSSION

7.1 Introduction

It is the intention of this final discussion to briefly consider the conclusions that can be drawn from the results of this study concerning, firstly, the possible mechanism of photoinhibition in isolated *C. fragile* chloroplasts and thylakoids and, secondly, the apparent ability of the frond to avoid or tolerate photoinhibition. The limitations of the methods used and the associated criticisms of the results will also be deliberated, along with suggestions regarding further work that may provide a greater understanding of the occurrence and significance of photoinhibition of photosynthesis in *C. fragile*.

7.2 Criticism of Techniques

Detailed criticisms of idividual experiments were extensively considered in the discussion sections of each Chapter and it is not the purpose of the present Chapter to reiterate these very specific experimental limitations. An obvious limitation of the work described in this study is the nature of the tissue investigated. *C. fragile* inhabits the intertidal zone where it experiences regular changes in environmental conditions on both a seasonal and a daily basis (*e.g.* light quality, light intensity, depth of submersion and hence degree of desiccation). For obvious reasons it was not possible to carry out work of the nature described in this thesis on *C. fragile* in its intertidal habitat. This necessitated sampling and maintenance of *C. fragile* tissue in artificial conditions quite different from those of the intertidal zone consisting of tanks of aerated sea water (instant ocean, Aquarium Systems Ltd) in a 10 °C refrigerated cool room under 40 μmol m^{-2} s^{-1} PPFD (at the water surface) provided by banks of 18 W 'white fluorescent' tubes. Furthermore, although the triannual samples were collected at approximately the same time each year, the length of time that a *C. fragile* frond sample had been in storage before it was used for a particular experiment varied due to the different time periods required for different investigations and it is possible that the condition of the tissue may vary with time in storage, resulting in variation in the tissue used during different experiments.

Frond photosynthesis was measured in the LD2 leaf disc oxygen electrode using frond tips freshly cut from the *C. fragile* in storage, whilst other work involved the use of aqueous phase oxygen electrodes for the study of photosynthesis, fluorescence and

in organello radiolabelling of isolated chloroplasts and the partial reactions of photosynthesis in isolated thylakoids. The frond samples were illuminated on one surface only (necessitated by the LD2 electrode) and were subjected to alternate 5 min dark/ 5 min light intervals starting with the lowest PPFD investigated (the maximum was approximately 1200 μmol m^{-2} s^{-1}). The isolated chloroplast suspensions, of 25 - 200 μg cm^{-3} chlorophyll concetration, were subjected to 0 - 1000 μmol m^{-2} s^{-1} PPFD for 20 min in aqueous phase oxygen electrodes, whilst thylakoid preparations were subjected to 1000 μmol m^{-2} s^{-1} PPFD for 0 - 100 min (at 100 μg cm^{-3} chlorophyll) or 0 - 40 min (at 50 μg cm^{-3} chlorophyll). Thus, the different experimental approaches used required the use of different tissue samples and different experimental conditions. It is evident from the literature that there is still some controversy concerning the site of the primary lesion within PSII upon photoinhibition and the mechanism of this phenomenon. The complexity of this particular area of interest is exacerbated by the great variety of experimental systems and conditions used, as demonstrated to some extent in this study. These have included the study of leaves, whole cells (*e.g.* unicellular algae), protoplasts, chloroplasts, chloride depleted chloroplasts, NH_2OH or Tris extracted chloroplasts, thylakoid membranes and PSII particles in aerobic, anaerobic and strongly reducing conditions. Caution is required when comparing the data obtained by different workers. Uncertainty as to the exact mechanism of photoinhibition and the number and relative importance of different sites probably results from extrapolation of results produced using these different systems and conditions to explain *in vivo* phenomena and *vice versa*. Many metabolic functions are likely to be involved and it is therefore probable that the amount and mechanisms of photoinhibition may differ from the *in vivo* situation. Thus, care must be taken when comparing the results obtained using the different experimental systems described in previous Chapters and when considering the relevance of these results, obtained under the artificial conditions of the experiments of this study, to *C. fragile* in the intertidal zone environment.

SiMo has been suggested to disrupt the PSII complex physically, exposing Q_A to be oxidised by the polyanion (Allen and Holmes, 1986b; Kyle, 1987). Recent results (Graan, 1986), however, have indicated that SiMo accepts electrons from Q_B as well as from Q_A and that it displaces DCMU from the herbicide - binding site (Boger, 1982), thereby rendering the assay apparently DCMU insensitive. Therefore, caution is required when interpreting results obtained using this method.

There were marked differences between the fluorescence response of isolated chloroplasts from *C. fragile* and that typical of other organisms that could not be adequately explained. It cannot be over - emphasised that many factors influence the fluorescence emission characteristics of thylakoids, chloroplasts, protoplasts and leaves and therefore care must be taken in interpreting the underlying reasons for such fluorescence changes. Similar changes in fluorescence characteristics may be observed from a given system for different reasons and the need for extreme caution is increased due to the differences in the fluorescence induction curve observed in this study. Differential penetration of the modulated light and the actinic light into highly concentrated chloroplast populations in leaves can result in the modulated light illuminating different populations of chloroplasts within a leaf (Ogren and Baker, 1985) and this is exacerbated by higher light intensities. This can result in differences between the fluorescence response obtained using continuous actinic light, shutters and transient recorders, and that obtained using the MFMS. The significance of this differential penetration will depend on many factors including species, leaf/ frond thickness and the intensity and quality of both the modulated and actinic light.

Successful *in organello* radiolabelling of isolated chloroplasts was apparent (Chapter 6), however, the similarity of the translation process and polypeptide turnover in the isolated chloroplast to the situation in the intact frond, particularly in the intertidal zone, is uncertain. There was strong evidence, in the form of preferential radiolabelling, that band 20 was D1. However, unequivocal identification of D1 was not achieved and this protein was seemingly overlooked, with regard to scintillation counting, during the final experiment, due to lack of Coomassie Blue staining, suggesting that this technique was not completely satisfactory when study of the D1 protein is intended. Fluorography did appear to reveal band 20, putatively identified as D1, but did not provide a fully quantitative analysis of radiolabel incorporation. Furthermore, the optimisation of radiolabelling experiments (involving 21 different incubation conditions), the time - course under optimal conditions and the measurement of radiolabel incorporation during photoinhibition were all carried out once only and repetition is obviously desirable to produce more reliable results.

7.3 Interpretation of Results

The work described in Chapter 2 revealed an essentially stable whole frond oxygen

exchange response over the temperature (5 - 20 °C) and irradiance (approximately 0 - 1200 μmol m^{-2} s^{-1} PPFD) ranges investigated with tissue samples harvested at different stages in the yearly life cycle (vegetative and reproductive). A subsequent investigation revealed that chloroplasts isolated from tissue harvested at different stages in the yearly life cycle also demonstrated an essentially consistent oxygen exchange response, at 10 °C, under the irradiance range studied (Chapter 3). In both fronds and isolated chloroplasts, photosynthesis saturated rapidly and at low PPFD indicating efficient light harvesting and excitaion energy transfer (an adaptation typical of shade plants), supporting previous evidence of such adaptation in *C. fragile* (*e.g.* Ramus, *et al.*, 1976; Ramus, 1978; Dring, 1981b; Benson and Cobb, 1981, 1983; Anderson, 1983, 1985; Benson, 1983). However, under the experimental conditions of this study no photoinhibition of frond photosynthesis was apparent even at the maximum PPFD investigated (1200 μmol m^{-2} s^{-1}), which is consistent with previous work that has alluded to the apparent absence of photoinhibition in this alga (Ramus, *et al.*, 1976; Arnold and Murray, 1980; Benson, 1983). In contrast, the onset of photoinhibition in isolated chloroplasts was rapid at supraoptimal PPFD and this is in agreement with similar observations that have suggested the occurrence of this phenomenon in isolated *C. fragile* chloroplasts (Benson, 1983; Williams, 1986).

It is generally accepted that PSII is the primary site of photoinhibitory damage. A preliminary examination of photosystem activity of thylakoids isolated from *C. fragile* clearly demonstrated that PSII was the primary site of lesion within the photosynthetic electron transport chain under photoinhibitory conditions, with negligible inhibition of PSI activity (Cobb, *et al.*, 1990). Therefore, the current investigation concentrated on the response of PSII activity during photoinhibition. There is still controversy concerning the site of the primary lesion within PSII upon photoinhibition and the mechanism of this phenomenon. There is substantial experimental evidence to support all of the many sites proposed. There have been many suggestions that damage to the Q_B site and hence degradation of the D1 protein is the cause of photoinhibition (*e.g.* Kyle, *et al.*, 1984; Ohad, *et al.*, 1984, 1985; Kyle, 1987; Schuster, *et al.*, 1988b; Vonshak, *et al.*, 1988; Shyam and Sane, 1989; Hundal, *et al.*, 1990; Kuhn and Boger, 1990; Richter, *et al.*, 1990a, 1990b; Shochat, *et al.*, 1990). An alternative view is that the primary lesion involves another component such as phaeophytin a, Q_A (*e.g.* Vass, *et al.*, 1988; Setlik, *et al.*, 1990; Styring, *et al.*, 1990), Y_z (Callahan, *et al.*, 1986;

Theg, *et al.*, 1986; Jegerschold, *et al.*, 1990) or the reaction centre P680 (Tytler, *et al.*, 1984; Cleland and Critchley, 1985; Arntz and Trebst, 1986; Cleland, *et al.*, 1986, 1990; Cleland and Melis, 1987; Demeter, *et al.*, 1987; Cleland, 1988; Critchley, 1988; Aro, *et al.*, 1990) and that D1 degradation is simply a consequence of photoinhibition rather than the cause.

The more recent literature appears to reveal a general consensus that the second suggestion is more likely, that is, that D1 degradation is a consequence of photoinhibition. Much of the work supporting this suggestion, including some of the reports referred to above, illustrate that light - dependent D1 degradation does occur, it is simply the order of events that remains controversial. There has been an increasing amount of work further associating photoinhibition and D1 turnover (regardless of whether it is the cause or a consequence) by clearly demonstrating that *de novo* protein synthesis is required in order to allow repair of the damaged D1 protein and hence recovery from photoinhibition (*e.g.* Samuelsson, *et al.*, 1985; Gong and Nilsen, 1989; Huse and Nilsen, 1989; Falk, *et al.*, 1990; Krupa, *et al.*, 1990).

The results of this study appear to suggest that the second proposal is more likely. That is, that initial photoinhibitory damage to isolated *C. fragile* chloroplasts upon photoinhibition is at Q_A and that only after this does increased D1 turnover ensue. Some of the observations of Setlik, *et al.* (1990) and Ohad, *et al.* (1988, 1990), in particular, seemed to resemble those of this investigation (Table 7.1). Both groups of workers suggest that an initial event in photoinhibition may involve the generation of Q_A^- and that this may cause an increase in F_o. The variable fluorescence was reduced but the primary photochemistry was not affected and Setlik, *et al.* (1990) reported reduction in electron flow from water to p - benzoquinone, whilst Ohad, *et al.* (1988, 1990) reported a reduction in electron flow from water to 2,3 - dichloro - 1, 4 - benzoquinone, but persistence of electron transfer from water to SiMo (Kyle, *et al.*, 1984). This is similar to the first stage of photoinhibition in *C. fragile* chloroplasts which revealed an initial increase in F_o accompanied by a reduction in variable fluorescence (Chapter 5), a reduction in electron flow from water to DMBQ, but persistence of electron transfer from water to SiMo (Chapter 4), over the fist 10 min of photoinhibitory treatment (Figure 5.19).

Setlik, *et al.* (1990) described a following stage (with a $t_{1/2}$ of 15 - 40 min) characterised by a constant F_o level and a parallel decline in F_v and electron transfer

Table 7.1 Putative order of events during photoinhibition of *C. fragile*. Key: Q_A, primary quinone acceptor; F_o, minimal fluorescence; F_v, variable fluorescence; BQ, p - benzoquinone; DCBQ, 2,3 - dichloro - 1,4 - benzoquinone; SiMo, silicomolybdate; Y_z, primary electron donor to P680, the reaction centre chlorophyll of PSII. See text for details.

	1	2	3
Setlik, *et al.* (1990)	$t_{1/2}$ 1 – 12 min Q_A^- generated F_o increased. F_v decreased. Reaction centre primary charge separation functional. BQ – dependent oxygen evolution decreased.	$t_{1/2}$ 15 – 40 min Q_A non – funtional. F_o constant. F_v decreased. Reaction centre primary charge separation funtional – but energy dissipated thermally. BQ – dependent oxygen evolution decreased.	$t_{1/2}$ 100 min Reaction centre primary charge separation non – functional.
Ohad, *et al.*(1988, 1990)	Reversible change. Q_A^- generated F_o increased. F_v decreased. Reaction centre primary charge separation functional. DCBQ – dependent oxygen evolution decreased. SiMo – dependent oxygen evolution unaltered. S_2–Q_B^- signal lost. S_2–Q_A^- signal slightly lost. Electron transfer from Y_z to P680$^+$ slightly impaired in a fraction of reaction centre II.	Irreversible change (i.e. requires D1 replacement) F_v decreased. Reaction centre primary charge separation non – functional. S_2–Q_B^- signal lost completely. S_2–Q_A^- signal lost.	
This study	0 – 10 min Q_A^- generated F_o increased. F_v decreased. Reaction centre primary charge separation functional. DMBQ – dependent oxygen evolution decreased. SiMo – dependent oxygen evolution unaltered.	10 – 15 min Q_A non – functional. F_o decreased slightly. F_v decreased further. Reaction centre primary charge separation non – functional. DMBQ – dependent oxygen evolution decreased. SiMo – dependent oxygen evolution decreased.	15 – 240 min. Increased D1 turnover?

from water to p - benzoquinone. This was associated with the neutralisation of the negative charge on Q_A^-, rendering Q_A non - functional. Reaction centre charge separation was still operational but the excitation energy was dissipated thermally. A final slow process ($t_{1/2}$ > 100 min) was linked to loss of reaction centre charge separation ability. Ohad, *et al.* (1988, 1990) only reported two stages, the second of which was associated with the complete loss of the thermoluminescence signal $S_2Q_B^-$ and a loss of the $S_2Q_A^-$ signal in parallel with a loss of F_m (representing loss of F_v) and a loss of reaction centre activity.

If Q_A^- is initially trapped in the negative state (Setlik, *et al.*, 1990) electron transfer from water to DMBQ may be blocked but SiMo may still be able to accept electrons from Q_A^- (Chapter 4), allowing this activity to continue. These partial reaction studies, therefore, may seem to suggest that Q_B is the primary site of photoinhibition. However, the development of Q_A^- (as suggested by the increase in F_o) may eventually lead to the complete inactivation of Q_A (Chapter 5). If Q_A is non - functional then the rate of electron transport from water to DMBQ and, more pertinently, from water to SiMo would be inhibited and this appears to be the case once 10 min photoinhibitory treatment under the conditions of this study is surpassed (Figure 5.19). The results of Chapters 4 and 5 may be interpreted as suggesting that the primary site of lesion of photoinhibition in *C. fragile* is the primary quinone acceptor, Q_A.

An initial (over the first 0 - 10 min of photoinhibition) preferential reduction of radiolabel content in D1, which would be expected if this protein was turning over particularly rapidly, was not obvious when studied by fluorography (Figure 6.9 - Chapter 6). However, from 15 to 240 min of photoinhibition the radiolabel content of D1 did appear to decrease. This preliminary evidence for decline in the D1 radiolabel content, only after 15 min of photoinhibition may be interpreted as being consistent with the initial events of photoinhibitory damage described above. It is possible that Q_A is inactivated and that only once this initial damage has occurred does increased D1 turnover ensue. This would also be consistent with the suggestion that initial damage to D1 could act as a recognition signal for proteolysis of the modified protein by a highly efficient intrinsic membrane protease (Ohad, *et al.*, 1985, 1990). However, alternative mechanisms involving initial damage to the electron transport chain at sites other than Q_A (*e.g.* Y_z, P680) can not be categorically excluded on the basis of these experiments alone.

The exact nature of the initial damage at the Q_A site is uncertain and it is quite possible that further damage occurs, perhaps at other sites, following Q_A inactivation. There seems to be considerable evidence that reactive radical species, particularly those derived from oxygen, have a role in photoinhibitory damage (Foote, 1976; Levine, 1983; Powles, 1984; Asada and Takahashi, 1987; Kyle, 1987; Krause, 1988; Richter, *et al.*, 1990b; Soporoy, *et al.*, 1990). It is likely that this is the situation in *C. fragile*, but the work of this study did not concentrate on the role of such active species in photoinhibtion and their involvement can not be supported or challenged on the basis of the results of this investigation.

F_v/F_m (F_v/F_p in this study) is proportional to the quantum yield of photochemistry (*e.g.* Bjorkman, 1987; Krause and Somersalo, 1989) and a decline in this ratio is symptomatic of the effect of photoinhibitory stress and such changes indicate a loss of photochemical efficiency, however, it is important to distinguish between changes in F_o from changes in F_v. It is generally accepted that the fluorescence response upon photoinhibition indicates that at least two processes are occurring (*e.g.* Bradbury and Baker, 1986; Demmig and Bjorkman, 1987; Cleland, 1988). As observed in this study, there is a process that causes an initial rise in F_o and superimposed on this is a second process responsible for the preferential quenching of F_v. These fluoresecence changes have been interpreted in different ways by different workers. The interpretation of the change in F_o which is consistent with the other data of this study has been discussed above. Preferential quenching of Fv may indicate damage to antenna pigments in close association with the reaction centre of PSII or damage to the reaction centres preventing charge recombination (Baker and Horton, 1987).

Many processes have been attributed a potential role in protection against photoinhibition (Table 7.2), as discussed extensively in previous Chapters, including several that are reflected by changes in the fluorescence response upon photoinhibition. Decline in F_v/F_m (F_v/F_p in this study) correlates with a decrease of quantum yield of oxygen evolution (Demmig and Bjorkman, 1987) and although related to a transient inhibition of photosynthesis it may be regarded as a protective mechanism (Somersalo and Krause, 1987). It has been suggested that preferential quenching of F_v, as observed in this investigation, indicates an increase in K_D, the rate constant for non - radiative decay in the antenna (Demmig and Bjorkman, 1987) and/ or K_d, the rate constant for non - radiative decay from the reaction centre (Cleland, 1988), or transformation of

Table 7.2 Mechanisms putatively contributing to the avoidance or tolerance of photoinhibition. Key: F_v, variable fluorescence; F_m, maximum fluorescence. See text for details.

Decline in F_v/F_m / preferential quenching of F_v

Increase in K_n – rate constant for non – radiative decay in the antenna
Increase in K_d – rate constant for non – radiative decay from the reaction centre
Transformation of PSII reaction centres to photochemically inactive quenchers

High energy state mechanism (q_E)

State transitions (q_T)

Photoinhibition associated fluorescence quenching (q_I)

Scavenging systems

Photorespiration/ carbohydrate release

Mehler reaction

Chlororespiration

D1 repair and replacement

Orientation of chloroplasts within the frond to effect 'self – shading'

PSII reaction centres to photochemically inactive quenchers (Cleland and Critchley, 1985; Cleland, *et al.*, 1986; Cleland and Melis, 1987; Weis and Lechtenberg, 1989) which convert excitation energy to heat (Weis and Lechtenberg, 1989; Krause, *et al.*, 1990). Increases in the rate constants of thermal dissipation of excitation energy are regarded as controlled protective mechanisms (Krause, 1988), as they are in competition with photosynthetic dissipation and therefore are believed to contribute to the prevention of photoinhibition. Such mechanisms may thus operate in *C. fragile*.

It is possible to resolve non - photochemical quenching of variable fluorescence into q_E (energy dependent), q_T (state transition associated) and q_I (photoinhibition associated) quenching (Horton, *et al.*, 1987; Hodges, *et al.*, 1988; Horton and Hague, 1988), although this was not achieved in this investigation. Thus, although it is possible that such mechanisms also operate in *C. fragile*, the contribution that they may make to the avoidance of photoinhibition is uncertain. Several quenching mechanisms may contribute to q_E including the dissipation of excess excitation energy via a 'futile' cycle of electrons around PSII (Schreiber and Neubauer, 1987; Falkowski, *et al.*, 1988; Thompson and Brudvig, 1988; Horton, *et al.*, 1989). Similarly, several mechanisms may be involved in q_I. A correlation between q_I and the formation of zeaxanthin in preference to violaxanthin and ß - carotene has led to the proposal of a protective role of the xanthophyll (epoxide) cycle (Demmig, *et al.*, 1987). Zeaxanthin was suggested to be involved in the removal of excess excitation energy via radiationless energy dissipation in the antenna chlorophyll (Adams, *et al.*, 1989; Bilger, *et al.*, 1989; Demmig - Adams, *et al.*, 1989a, 1990; Adams, *et al.*, 1990). However, zeaxanthin was absent from *C. fragile* chloroplasts and it has been proposed that this may indicate the absence of the xanthophyll cycle in this alga (Benson and Cobb, 1983), which suggests that such a mechanism would not contribute significantly to the prevention of photoinhibition.

State transitions (*e.g.* Fork and Satoh, 1986; Gounaris, *et al.*, 1986; Staehelin, 1986; Iordanov and Goltsev, 1987; Anderson and Andersson, 1988) are thought to have a role in the prevention of photoinhibition by enabling the efficient distribution of excess excitation energy between PSI and PSII (*e.g.* Allen and Holmes, 1986a; Fork and Satoh, 1986; Gounaris, *et al.*, 1986) and/ or the alleviation of over excitation of PSII (Allen and Melis, 1988). State transitions have been clearly demonstrated in fronds of *C. fragile* (Sealey, *et al.*, 1990) and this mechanism may contribute to the avoidance

of photoinhibition.

Systems exist that minimise photoinhibitory damage by scavenging or preventing the formation of radicals or other reactive molecular species, particularly those derived from oxygen (e.g. Asada and Takahashi, 1987; Krause, 1988; Richter, *et al.*,1990b; Soporoy, *et al.*, 1990) and superoxide dismutase has been found in *C. fragile* (Lumsden and Hall, 1975), perhaps indicating the presence of such systems. Carotenoids (especially ß - carotene) are involved in quenching of toxic active oxygen species in higher plants (Foote, 1976). ß - carotene is not found in *Codium*, however, and the chlorophylls in this alga appear more susceptible to photooxidation than those carotenoids that are present (Benson, 1983). Therefore, the α - carotene that appears to replace ß - carotene in *C. fragile* may not function in such a photoprotective manner.

The possible role of photorespiration in the prevention of photoinhibition in *C. fragile* is of interest as light - induced glycolate excretion and carbohydrate release as hexoses has been reported (Samuel, *et al.*, 1971; Benson, 1983). Up to 40 % of assimilated carbon may be released at supra - optimal PPFD. Such extracellular carbon release may be a mechanism to excrete excess glycolate when photorespiratory rates are particularly high (Lorimer and Andrews, 1981). Perhaps photoinhibition may be partially relieved by increased energy dissipation via the photorespiratory carbon oxidation (PCO) cycle and the operation of this cycle may in turn be facilitated by carbon release as glycolate. Thus, *C. fragile* may have the ability to use the surrounding medium as an extracellular zone from or to which it may reclaim or release excessive amounts of metabolites depending on the immediate fluctuations of the light environment (Benson, 1983). The Mehler reaction (Powles 1984; Krause and Cornic, 1987; Krause and Laasch, 1987) and chlororespiration (Bennoun, 1982; Garab, *et al.*, 1989) may act as alternative dissipative routes for excitation energy, perhaps contributing to the avoidance of photoinhibition in *C. fragile*. Light - induced increase in dark oxygen uptake was observed in fronds (Chapter 2) and chloroplasts (Chapter 3), possibly supporting the activity of such oxygen consuming processes. Williams (1986) suggested that the synthesis of chloroplast starch may be considered as a limited photoprotective mechanism, as a means of dissipating ATP and $NADPH_2$. *C. fragile* has an increased respiratory burden when compared with some other species (Ramus, 1978) and perhaps this has a minor energy dissipatory role in photoprotection. For example, *Ulva lactuca* is entirely photosynthetic tissue, whereas *C. fragile* is one - third

photosynthetic tissue, consequently the latter has three times the respiratory burden of the former.

Recovery after photoinhibition by means of replacement and repair of D1 can itself be regarded as a method by which photoinhibition is tolerated. Rapid light - dependent turnover of D1 may act as a protective mechanism against total destruction of reaction centre II, with D1 acting as a suicide protein designed to be the target of photodamage (Schuster, *et al.*, 1988b), preventing extensive damage to the entire photosynthetic apparatus. Wild, *et al.* (1990) have suggested that D1 may have a function in thylakoids analagous to that of a fuse in an electric circuit. In the situation of an energy overload widespread damage that is difficult to repair is prevented by rapid destruction of the fuse component, thus switching off the system function. There was preliminary evidence of increased D1 turnover during 15 - 240 min of photoinhibition of isolated *C. fragile* chloroplasts under the conditions of this investigation (Chapter 6).

Photoinhibition was very evident in isolated chloroplasts, but not in fronds, of *C. fragile*. Thus, any of the potentially photoprotective mechanisms discussed that may operate were clearly insufficient to prevent photoinhibition of the isolated chloroplasts. Differences revealed in this study in the photosynthetic response must be due to properties of the whole frond not shared by isolated chloroplasts which enable the prevention of photoinhibition. However, the majority of the photoprotective mechanisms mentioned above, including preferential F_v quenching (increase in rate constants of thermal dissipation of excess excitation energy), q_E, q_I, state transitions, scavenging systems, glycolate production, Mehler reaction and chlororespiration are associated with structures and processes within the chloroplast and may operate in isolated chloroplasts as well as in intact fronds. The cell wall (absent in chloroplast suspensions) may be of importance and the respiratory burden of the whole frond is greater. Furthermore, the ability of chloroplasts within the *C. fragile* frond to re - distribute excitation energy has been clearly shown, whilst such state transitions could apparently not be demonstated in isolated chloroplast preparations (Sealey, *et al.*, 1990). However, it is considered that any contribution that these factors may make to the avoidance of photoinhibition is not likely to be of a sufficient magnitude to explain the dramatic difference, regarding photoinhibition, between fronds and chloroplasts observed in this study.

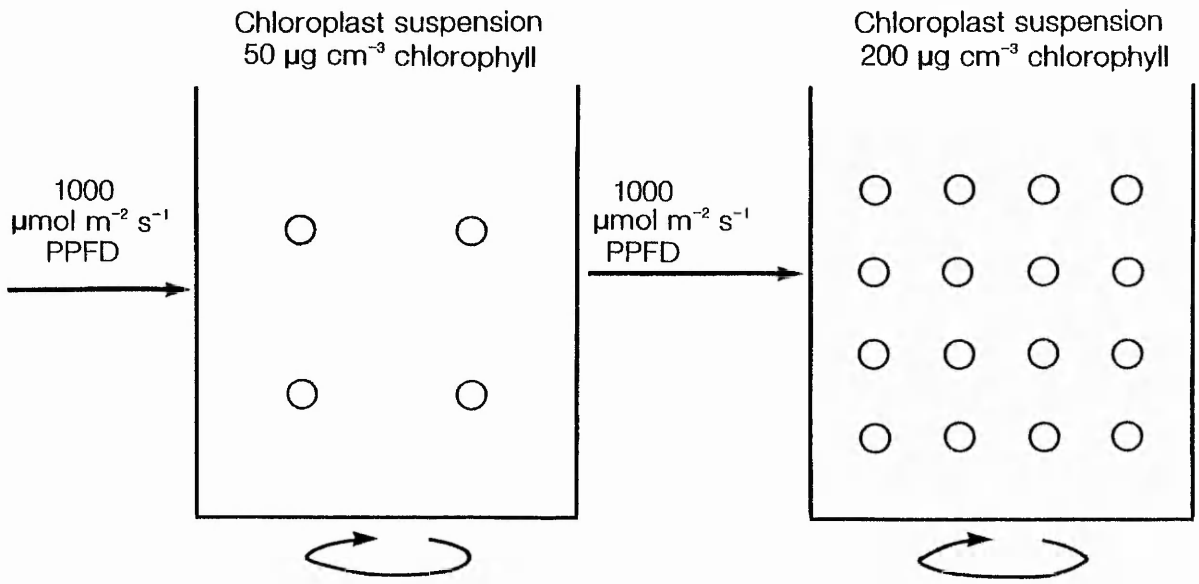
As the chlorophyll concentration of suspensions of chloroplasts was increased

(maximum investigated was 200 μg cm^{-3}) and began to approach the high chlorophyll concentration of the intact frond, the photosynthetic response of the chloroplasts began to mimic that of the whole frond; with higher light intensities required for maximum net photosynthesis (*i.e.* saturation) and less dramatic reduction in net photosynthesis (*i.e.* photoinhibition) once the optimum PPFD was surpassed, *i.e.*, an extended saturation plateau. As isolated chloroplasts are susceptible to photoinhibition but intact fronds are tolerant it is suggested that photoprotection may be conferred by the arrangement of the chloroplasts within the algal frond. *C. fragile* possesses a compact, dichotomous, cylindrical thallus consisting of closely interwoven coenocytic siphons or filaments (Lee, 1989; Bold and Wynne, 1985). In the medullary region of the thallus the siphons are colourless, whilst the outer layer (cortex) is constructed of the dilated tips (utricles) of the siphons which form a 'palisade - like' layer and it is in these structures only that the chloroplasts are situated (Figure 1.9). The chloroplasts are arranged peripherally, in stacks, around a large central vacuole in such numbers that the thallus has an optically dense nature. The chloroplasts have unelaborate thylakoids arranged in pairs or triplets with no extensive stacking. Due to the stack arrangement of the chloroplasts considerable light attenuation may operate within the utricle and a degree of 'self - shading' of the chloroplasts may occur. Hence, the number and arrangement of the chloroplasts within the frond may confer a vital mechanism in the avoidance of photoinhibition by this alga.

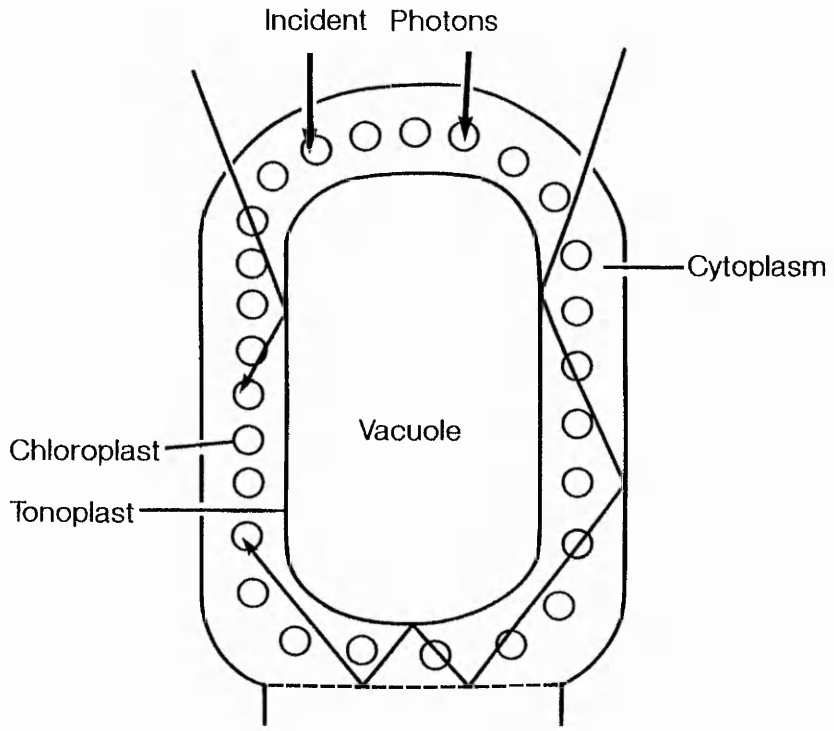
The photoinhibition of continuously stirred isolated chloroplasts under unidirectional irradiance was far more dramatic at 50 μg cm^{-3} chlorophyll than at 200 μg cm^{-3} chlorophyll. At the higher concentration the chances that a particular chloroplast will be at the 'light side' of the reaction vessel may be reduced and when chloroplasts are not at the 'light side' they will be 'shaded' by a greater number of other chloroplasts, *i.e.*, there will be a greater number between them and the light source (Figure 7.1). Therefore, the PPFD at the 'dark side' of the reaction vessel may be lower than the PPFD at the 'dark side' of the reaction vessel when the chlorophyll concentration is only 50 μg cm^{-3}. This will enhance the avoidance of photoinhibition as the time spent at maximum PPFD may be reduced and when at the 'dark side', the PPFD will be reduced, perhaps preventing further photoinhibition or even enabling 'recovery' from any initial photoinhibitory processes caused by the brief time at the 'light side' of the reaction vessel. That is, a particular chloroplast may be subjected to a relatively rapid

Figure 7.1 Comparison of photoinhibition (A) in isolated chloroplasts in suspension and (B) in a utricle within the frond of *C. fragile*, showing enhanced light absorption as proposed by Ramus (1981). See text for details.

A


Chloroplast suspension
50 µg cm⁻³ chlorophyll
1000
µmol m⁻² s⁻¹
PPFD
1000
µmol m⁻² s⁻¹
PPFD
Chloroplast suspension
200 µg cm⁻³ chlorophyll

B


Incident Photons
Cytoplasm
Vacuole
Chloroplast
Tonoplast

alternation between photoinhibitory and non - photoinhibitory conditions. Looked at in another way, the total amount of pigment present (μg cm^{-3}) is greater and continual movement of the chloroplasts in suspension enables the incident PPFD to be more evenly distributed to this greater amount of pigment. At lower chlorophyll concentrations the PPFD is similarly distributed to the lower amount of total pigment present and there may, therefore, be a greater possibility of photoinhibition.

The chloroplasts within the frond are in a comparatively fixed position, although reorientation of plastids within cells and change of plastid shape has been suggested as a means of decreasing the area for photon - interception and increasing 'self - shading', thus contributing to the avoidance of photoinhibition (Raven, 1989). Furthermore, the chlorophyll concentration within the frond is exceptionally high due to high chloroplast density. It is suggested that 'self - shading' could perhaps protect the chloroplasts deeper within the utricle from photoinhibition, whereas the outer most chloroplasts may be more prone to damage. Ramus (1981) suggested that the structure of the utricle may enhance light absorption. The thin layer of peripheral cytoplasm surrounded by cell membranes was proposed to behave as an optically insulated waveguide similar to an optic fibre. A photon entering the cytoplasm at low enough angle could undergo multiple internal reflections increasing the probability of photon capture as the chloroplasts are also situated in the cytoplasm. Unabsorbed photons could then pass back through the cytoplasm, reflected by an air - water interface at the base of the utricle. It was also suggested that the large central vacuole functions as an integrating sphere, dispersing light uniformly over the cytoplasm. Perhaps this is a mechanism that enables the incident PPFD to be more evenly distributed to the large amount of total chlorophyll pigment contained within the dense number of chloroplasts present within the frond, similar to the situation observed in a continuously stirred chloroplast suspension of 200 μg cm^{-3} chlorophyll. On the other hand, up to 75 % of the pigment content of *C. fragile* may be in the LHCII (Benson, 1983), idicating that a large number of light harvesting complexes are supplying excitation energy to a limited number of reaction centres, a situation that will increase the likelihood that photoinhibition will occur at supra - optimal PPFD, perhaps suggesting that any such mechanisms that may increase the distribution of incident PPFD uniformly over the cytoplasm, will also be distributing the potential for photoinhibition uniformly over the cytoplasm.

Thus, there is some uncertainty concerning the mechanism by which *C. fragile* fronds avoid photoinhibition and further work is required to confirm or challenge the importance of 'self - shading'. It has been found that the intertidal species *Porphyra perforata* shows a greater resistance to photoinhibition than the subtidal species *P. nereocystis* (Herbert and Waaland, 1988; Bose, *et al.*, 1988). Photoinhibition resistance in *P. perforata* appeared to be due to a reduced rate of photoinhibition damage rather than due to an accelerated rate of photoinhibition repair (Herbert, 1990). It was suggested that reduction of damage may be by means of biophysical processes which increase the radiationless decay of excitation to heat in PSII, slight structural alterations to sensitive proteins that enhance their stability, or greater protection by enzyme systems that quench radicals formed by over excitation of PSII. It was also proposed that reduction of damage is a reasonable way to limit photoinhibition in *P. perforata* during the severe desiccation and exposure to full sun that occur simultaneously during daily low tides, conditions under which the protein synthesis required for photoinhibition repair may not be possible (Herbert, 1990).

Resistance to photoinhibition may be a common attribute of high intertidal algal species. It was suggested that in combination with desiccation tolerance and other physiological and biochemical adaptations, photoinhibition resistance may contribute to the persistence of these species in the extreme environment of the high intertidal zone where they escape the greater herbivore grazing pressure and competition for space present in the low intertidal and subtidal zones (Bose, *et al.*, 1988; Herbert and Waaland, 1988). This may also be so for *C. fragile*. Indeed, the life cycle of *C. fragile* reveals strong seasonal trends in frond morphology and chloroplast physiology, showing a growth strategy that optimises use of increased nutrient availability and reduction in algal competiton (Benson, *et al.*, 1983; Williams, *et al.*, 1984; see Figure 1.10). In the winter months competition, tidal amplitude variation and PPFD are reduced. Therefore, maximum carbon fixation, pigment content, chloroplast size and vegetative growth occurs, and nutrients are accumulated. In the summer months competitive algal species, tidal amplitude and PPFD increase and the nutrient status of the intertidal zone decreases. Therefore, nutrient reserves are mobilised, frond hairs develop (to enhance nutrient uptake), maximum photosynthetic efficiency is not maintained and *C. fragile* enters the reproductive phase.

Such a growth strategy may be a contributing factor to the almost global

distribution of *Codium* from the low tide mark down to 70 metres in tropical and temperate waters (Lee, 1989). It is suggested that the adaptations of *C. fragile* that enable the apparent paradox of efficient photosynthesis at the low PPFD experienced at high tide (Ramus, *et al.*, 1976; Ramus, 1978; Benson and Cobb, 1981, 1983; Anderson, 1983, 1985; Benson, 1983) without accompanying photoinhibition and photooxidation at the supra - optimal PPFD experienced at low tide, especially in the summer months, are necessary for *C. fragile* to fully benefit from its adaptive growth strategy, and in conjunction with this growth strategy enable successful colonisation, despite the extreme environment, of the intertidal zone.

7.4 Topics of Further Study

There is a need for a range of future experimental investigation to provide further understanding of photoinhibition in *C. fragile* and its importance, particularly relative to that of the other adaptations that constitute the growth strategy, to the success of this organism. The potential extent of relevant further work undertaken and the approaches adopted would seem to be limited only by the imagination. It is suggested that confirmation of the primary site of lesion and of the sequence of events during photoinhibition could provide an initial area for further study. Due to differences in wavelenghts of fluorescence emission between the two photosystems, perturbations in PSI can be distinguished from those in PSII (*e.g.* Baker and Horton, 1987; Bjorkman, 1987; Kyle, 1987; Krause and Somersalo, 1989). The observation of such fluorescence emissions in *C. fragile* fronds and chloroplasts during photoinhibition may indicate if PSI is also damaged, confirming the results of the initial partial reaction work.

It was concluded from the results presented in this thesis that the primary site of damage of photoinhibition may be Q_A and this may be confirmed or challenged by using some of the many other techniques available. Due to the doubt concerning the mode of action of SiMo it may be prudent to carry out partial reaction experiments similar to those described in Chapter 4 using the technique of mild trypsinisation (Kyle, 1987; Critchley, 1988) to confirm the SiMo results. Mild trypsinisation is also thought to abolish the Q_B binding site (Critchley, 1988) and disrupt the PSII complex, exposing the Q_A site and thus enabling its oxidation by other soluble acceptors such as ferricyanide. However, mild trypsinisation also causes substantial perturbation of PSII and therefore care is again required in interpretation of such results (Kyle, 1987).

Electron spin resonance (ESR) or electron paramagnetic resonance (EPR) spectroscopy is an effective way of detecting molecules or atoms that have unpaired electrons (Goodwin and Mercer, 1983). EPR spectroscopy permits direct observation of most of the redox components in PSII using illumination at various temperatures (Styring, *et al.*, 1990). For example, EPR has been used to monitor the functioning of Y_z (Kyle, *et al.*, 1984; Allakhverdiev, *et al.*, 1987) and Q_A (Styring, *et al.*, 1990) during photoinhibition. Primary charge separation between P680 and phaeophytin a may be measured via the light induced absorbance change at 685 nm (*e.g.* Allakhverdiev, *et al.*, 1987), whilst absorbance changes of Q_A (A_{320}) and C550 (A_{540} - A_{550}) also yield useful information. The former absorbance gives a measure of the Q_A reduction state whilst the latter absorbance difference is associated with the reaction centre phaeophytin a molecule, hence also giving a measure of Q_A photoreduction (Cleland, *et al.*, 1986). Measurement of the phaeophytin a (A_{685}) photoreduction signal is also possible (Demeter, *et al.*, 1989). Thermoluminescence enables the study of the early events in photoinhibition *in vivo* by allowing detection and quantitation of the $S_2Q_A^-$ and $S_2Q_B^-$ states and their stability under various conditions (Ohad, *et al.*, 1988, 1990). Once the initial site of photoinhibition is established it would perhaps be logical to investigate the exact nature of the damage. For example, the putative role of reactive radical species, particularly those derived from oxygen, could provide a further area of study.

There were marked differences between the fluorescence response of chloroplasts isolated from *C. fragile* and that typically observed in other organisms and intact fronds, that could not be explained adequately and it is suggested that further characterisation of the fluorescence response of the chloroplasts and fronds of this alga is desirable. An alternative approach to the DCMU addition technique for the analysis of q_Q and q_E quenching is light - doubling or the saturation pulse method (Bradbury and Baker, 1981, 1984; Bolhar - Nordenkampf, *et al.*, 1989; Schreiber, *et al.*, 1986), which can be used to analyse chloroplast fluorescence and whole frond fluorescence, which would be particularly useful.

Photoinihibition is increasingly being regarded as a means of photosynthetic regulation. It has been suggested that the contribution of photoinhibition - associated quenching of chlorophyll fluorescence (q_I) to the total non - photochemical quenching in high light is, like q_E, an adaptive and useful dissipative process (Horton and Hague, 1988). It has been proposed that photoinhibition can be envisaged as one of several

controlled mechanisms of dissipation of absorbed excitation energy that occur as a response to increase in PPFD. Increase in light intensity may result in a series of responses from the chloroplast. Photosynthesis (q_Q) responds in seconds to minutes to changes from darkness to photosynthetically saturating PPFD, whilst the high energy state mechanism (q_E) responds in seconds. The phosphorylation of LHCII (q_T) can respond within minutes in the range of darkness to 'low' PPFD and photoinhibition (q_I) may operate in minutes to hours in the range of saturating to supra - optimal PPFD. Finally, changes in membrane composition may occur in hours to days throughout the range of darkness to supra - optimal PPFD (Horton, *et al.*, 1988). Thus, rather than viewing photoinhibition simply as a negative phenomenon consisting of damage to PSII and subsequent loss of photosynthetic capacity, it may be interpreted as being just one of many mechanisms that are concerned with an overall process of 'photosynthetic control' (Foyer, *et al.*, 1990). It is suggested that the investigation of this alluring hypothesis and the ratification or otherwise of such a photoregulatory role for photoinhibition in *C. fragile* and its contribution to the success of this alga in the intertidal zone is an area that may demand substantial further work. However, it appears from the literature that the use of fluorescence techniques and the routine measurement of such parameters as q_E, q_T and q_I are crucial to such studies, verifying the need for further characterisation of the fluorescence response of *C. fragile* fronds and chloroplasts.

The unequivocal identification of D1 was not achieved in this study (Chapter 6) and this may be aided in future experiments by the use of ^{14}C - azidoatrazine (*e.g.* Ohad, *et al.*, 1984; Chow, *et al.*, 1989; Kuhn and Boger, 1990) or antibodies raised against D1 (*e.g.* Aro, *et al.*, 1990; Kuhn and Boger, 1990; Virgin, *et al.*, 1990). Although it is believed that there is little doubt that the protein synthesis (Chapter 6) was light driven, a control consisting of a chloroplast suspension in the presence of ^{35}S - Met incubated in the dark for the same duration as the radiolabelling period, in which little incorporation would be expected, was not included in these experiments and perhaps this should be carried out in a future experiment to unequivocally demonstrate light dependence.

Many components were included and 3 pH levels were used in the incubation solutions investigated for their ability to support *in organello* radiolabelling. Use of different combinations of these components and adjustment of the pH to different levels

and the use of different PPFDs to drive protein synthesis could provide an almost limitless number of alternative incubation conditions. Further investigation could thus result in the discovery of incubation conditions that support improved ^{35}S - Met incorporation by isolated *C. fragile* chloroplasts, compared to that observed under the conditions so far examined. In future work it may be advantageous to use densitometry of gels and fluorographs, scintillation counting of the entire gel (lanes sliced into 1 mm sections) and, perhaps, the more sensitive silver staining method, in order that a more quantitative analysis of the thylakoid proteins is achieved and to ensure that important polypeptides (*e.g.* D1) are not overlooked. The experiments involved in the investigation of radiolabelling were all carried out once only and repetition is obviously desirable to produce more reliable results. The levels of incorporation achieved are believed to be sufficient to enable a variety of future experiments to be undertaken with regard to the turnover of thylakoid proteins, particularly D1, during photoinhibition of *C. fragile*.

A further experimental approach has been to study the 'life cycle' of the D1 protein during photoinhibition (*e.g.* Callahan, *et al.*, 1987; Kyle, 1987; Ohad, *et al.*, 1990), which has revealed that a 33.5 KDa precursor protein is associated exclusively with the stroma lamellae and that following processing the D1 (32 KDa) protein is translocated to the stacked, granal lamellae, where it is integrated with the PSII complex and where, in turn, photoinhibition may occur. Once damaged, D1 migrates back to the non - appressed lamellae for proteolysis by a specific protease. The successful development of *in organello* radiolabelling achieved in the present work may make it possible to carry out similar studies on the behaviour of D1 in *C. fragile* during photoinhibition, including investigation of repair and recovery from photoinhibition and the control of this process, perhaps leading to possible identification and characterisation of the protease responsible for, and associated mechanisms involved in, proteolysis. This would enable the study of the importance to photoinhibition resistance in *C. fragile*, and hence to the success of this alga, of reduced rate of photoinhibition damage relative to accelerated rate of repair. Reduction of photoinhibition damage was suggested to be more important to photoinhibition limitation in the intertidal *P. perforata* during desiccation and exposure to full sun at low tide (Herbert, 1990).

The method of avoidance of photoinhibition by *C. fragile* fronds is still a moot - point. The 'self - shading' concept described does not at present seem completely

satisfactory and further investigation is required, however, it is difficult to conceive how this phenomenon may be further tested experimentally. The extent to which different protective mechanisms may contribute to the avoidance of photoinhibition remain to be elucidated and future study of the operation and importance of these mechanisms to the avoidance of photoinhibition in *C. fragile* may indirectly indicate the importance of other mechanisms such as 'self - shading'. Therefore it is suggested that further work on preferential variable fluorescence quenching (q_E, q_T and q_I), state transitions, scavenging systems, photorespiration, extracellular carbon release, Mehler reaction, chlororespiration and D1 replacement and repair in fronds and isolated chloroplasts, and a determination of the relative importance of these processes to *C.fragile* in the intertidal zone may be useful.

Finally, other environmental factors such as chilling (Oquist, *et al.*, 1987; Hayden and Baker, 1990), freezing, high temperature (Ludlow, 1987) and water stress (Boyer, *et al.*, 1987) can disrupt the organisation of the electron transport chain in the thylakoid membrane and can affect the PPFD required for the onset of photoinhibition. Photoinhibition will occur when the light stress is sufficient to overcome the combination of mechanisms that exist to enable the tolerance or avoidance of this phenomenon. The PPFD and duration required to achieve this is very much dependent upon the synergistic influences of other environmental factors, for example, *C. fragile* may be prone to desiccation at low tide, particularly in the summer. This provides a further area that may possibly warrant investigation in future work.

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PHOTOINHIBITION IN THYLAKOIDS AND INTACT CHLOROPLASTS OF CODIUM FRAGILE (SURINGAR) HARIOT.

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1. INTRODUCTION

There is a general acceptance in the literature that photosystem II (PSII) is the primary site of photoinhibition and that prolonged exposure to excessive photosynthetic photon flux density (PPFD) results in pigment loss and lipid breakdown due to photooxidation (as reviewed in 1). However, growth of the marine alga C. fragile is adapted to low PPFD (2,3,4) even though its intertidal habitat is regularly exposed to wide extremes of PPFD on both a seasonal and daily basis. Therefore, this alga is especially at risk of photoinhibition and photooxidation at the higher PPFD's occurring at low tide. Experiments described in this paper illustrate that chloroplasts and thylakoids isolated from this alga photoinhibit at irradiances supra-optimal for photosynthesis (PS). As with other photosynthetic organisms, the primary lesion appears to be associated with PSII and photooxidative damage occurs with prolonged exposure to light.

2. MATERIALS AND METHODS

Vegetative and reproductive algal fronds were sampled from intertidal rockpools at Bembridge, Isle of Wight (UK) and maintained as previously described (5). Due to the notoriously robust nature of C. fragile chloroplasts in vitro (6), thylakoid isolation was only achieved after freezing isolated chloroplasts for 100 min. Oxygen exchange by isolated chloroplasts (7) was measured at 0 to 1000 µmol m^{-2} s^{-1} (PPFD) at 10°C (8) using a DW1 oxygen electrode (Hansatech Ltd, UK). Chloroplast density within the electrode vessel was varied from 25 to 200 µg chlorophyll cm^{-3}. Time course studies followed oxygen exchange over a 10 min dark, 20 min light, 10 min dark incubation regime with illumination provided by a 250 w tungsten-halogen, Halight 24/250 projector.

Stirred thylakoid suspensions (3 to 4 cm^3) were illuminated at

100 and 1000 µmol m^{-2} s^{-1} (PPFD) for up to 100 min with 200 mm^3 samples being removed for oxygen exchange studies. The following photosystem activities were determined in 1 cm^3 reaction volumes at 11 to 16°C; PSI and PSII combined, measured by electron transport from water to 7.5 mol m^{-3} ferricyanide; PSII, measured by electron transport from water to 5 mol m^{-3} dimethylbenzoquinone (DMBQ); PSI, measured by electron transport from the reducing couple 5 mol m^{-3} dichlorophenolindophenol ($DCPIPH_2$) /5 mol m^{-3} ascorbate to 0.2 mol m^{-3} methyl violgen (MV), in the presence of 0.05 mol m^{-3} 1-(3,4-dichlorophenyl)-1, 1-dimethyl urea (DCMU) (9). Photooxidation in isolated thylakoids incubated for 3h at 50 to 1000 µmol m^{-2} s^{-1} (PPFD) was assessed by the spectrophotometric determination of fatty acid peroxidation as malondialdehyde production (10).

3. RESULTS AND DISCUSSION

Chloroplasts isolated from both vegetative and reproductive fronds exhibited similar trends in PS to varying PPFD and chlorophyll content within the electrode vessel. Typical light saturation curves are illustrated in Figure 1. Maximum net PS occurred at 60 µmol m^{-2} s^{-1} (PPFD) for a chlorophyll content of 25 µg cm^{-3} as opposed to 250 µmol m^{-2} s^{-1} (PPFD) for a chlorophyll content of 200 µg cm^{-3}. This illustrates considerable light harvesting efficiency and excitation energy transfer at low PPFD and supports previously published data on the dynamics of PS within this alga (2,3,4,5 and 12). However, no significant saturation plateau was observed at the lower chlorophyll concentration. Indeed, a marked decline in net PS indicative of photoinhibition occurred once the optimum PPFD was surpassed. In general, the higher the chlorophyll concentration within the electrode vessel, the higher the PPFD required to produce maximum PS and the less dramatic the reduction in net PS once irradiance was increased beyond the optimum.

Further studies examined photosystem activity of isolated thylakoids at varying PPFD (Figures 2A and 2B). At both 100 and 1000 µmol m^{-2} s^{-1} the decline in PSII activity was rapid and reached a maximum of 60% after approx. 40 and 5 min respectively. As the chlorophyll content within the electrode vessel was maintained at 20 µg, these results suggest a constant potential for PSII activity and inhibition within the experimental system. It is clear from the results obtained that PSII is the primary site of lesion within the photosynthetic electron transport chain of C. fragile chloroplasts when incubated under conditions favouring photoinhibition. Furthermore, prolonged thylakoid exposure to such conditions produced clear evidence of photooxidation as measured by the production of malondialdehyde (MDA) (Figure 3) and chlorophyll degradation (Figure 4). These data illustrate the

susceptibility of isolated choroplasts and thylakoids of C. fragile to these photodestructive processes even at the relatively low PPFD of 100 µmol m^{-2} s^{-1}. This raises the question of how this alga is adapted to survive in the intertidal habitat at varying PPFD. The intact frond must presumably possess some photoprotective features that are absent in the isolated chloroplasts or thylakoids, and these are discussed in the following paper (13).

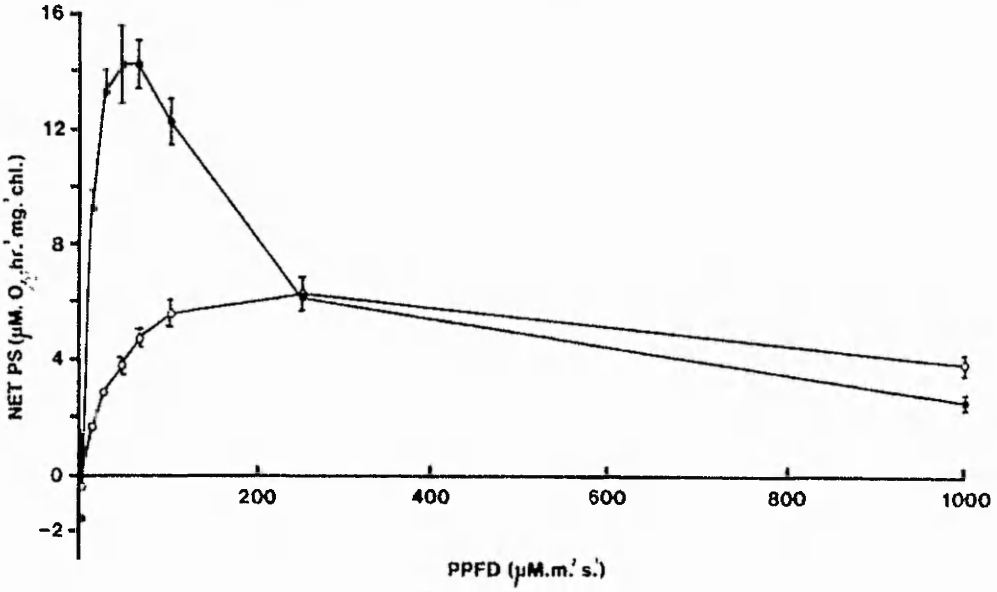


FIG 1: Effect of PPFD on net photosynthesis at 10°C by chloroplast suspensions equivalent to 25 µg cm^{-3} (●) and 200 µg cm^{-3} (○). Each point = $\bar{x}$ ± S.E, where n = 5-7.

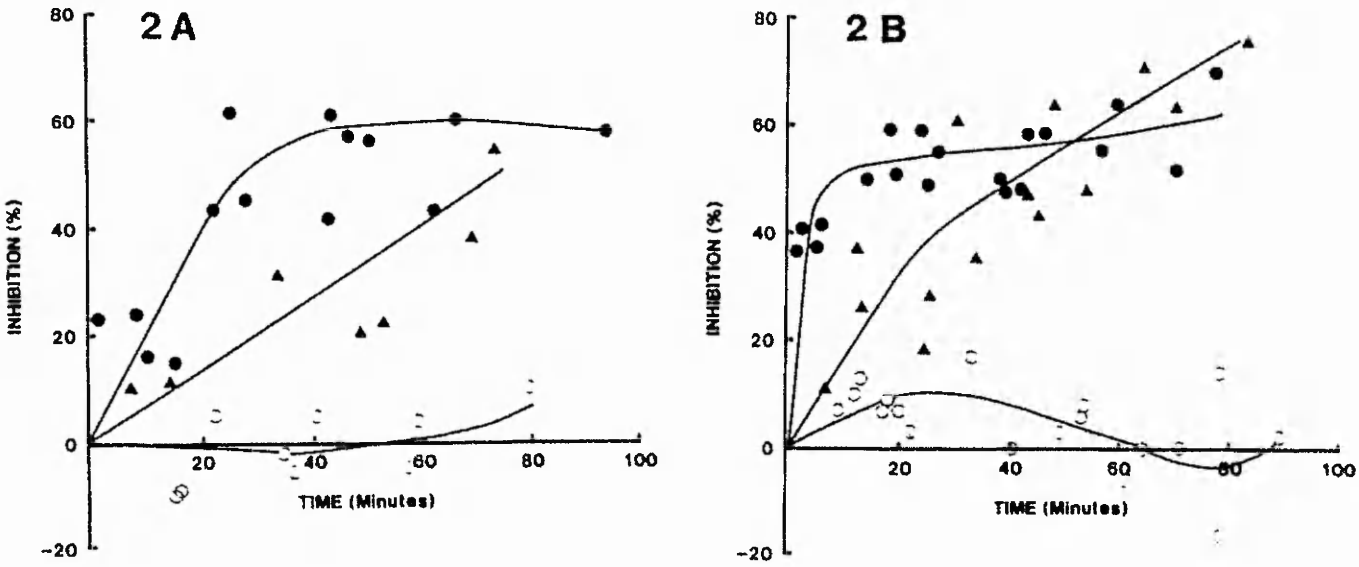
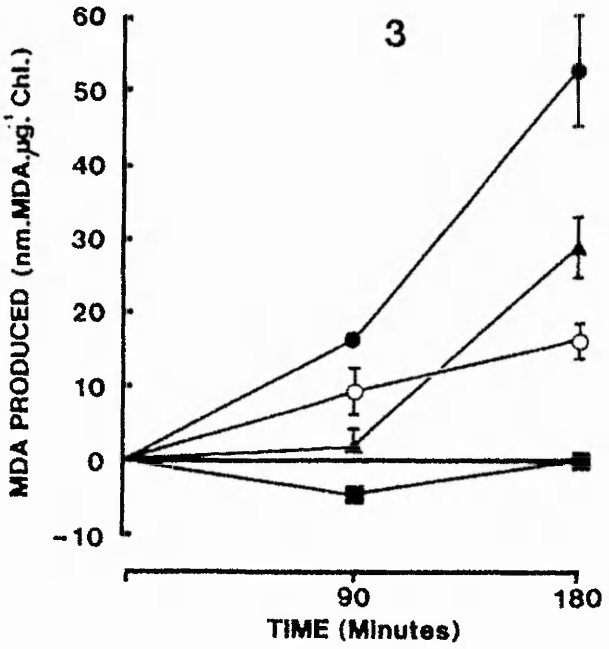
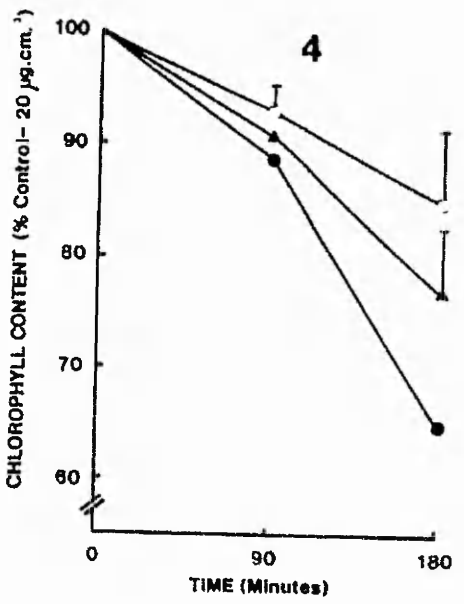


FIG 2: Time-course of inhibition of electron transport in isolated thylakoids (equivalent to 20 µg cm^{-3} chlorophyll) at (A) 100, and (B) 1000 µmol m^{-2} s^{-1} PPFD. PSI (○); PSII (●); PSI and PSII (▲). Each point is a single observation.





FIGS 3 and 4: Time-course of MDA production from (3), and chlorophyll content of (4), isolated thylakoids exposed to 50 (■), 100 (○), 500 (▲) and 1000 (●) µmol m^{-2} s^{-1} PPFD.
Each point = $\bar{x}$ ± S.E, where n = 5.

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ADAPTATIONS OF *CODIUM FRAGILE* (SURINGAR) HARIOT FRONDS TO PHOTOSYNTHESIS AT VARYING FLUX DENSITY

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1. INTRODUCTION

The intertidal alga *Codium fragile* exhibits shade adaptations enabling efficient photosynthesis (PS) at the low photosynthetic photon flux densities (PPFD) experienced at high tide. Thus, chloroplasts contain high levels of siphonoxanthin, siphonein and chlorophyll b (1,2,3), reveal both low chlorophyll a:b and high photosystem II: photosystem I ratios (1,4,5) and possess 75% of the total pigment content within the light harvesting complexes (1). However, the intertidal habitat is subjected to regular extremes of PPFD on a daily and a seasonal basis so that this alga is at great risk of photoinhibition and subsequent photooxidation at the higher PPFD's encountered at low tide. Indeed, isolated chloroplasts and thylakoids are particularly vulnerable to these photodestructive processes (6). In contrast, this paper provides evidence that intact fronds of *C. fragile* are able to avoid or tolerate photoinhibition and hence photooxidative damage and possess the ability to re-distribute excitation energy between the photosystems (4,5).

2. MATERIALS AND METHODS

Vegetative and reproductive fronds were sampled from intertidal rockpools at Bembridge, Isle of Wight (UK) and maintained as described previously (7). Oxygen exchange by frond tips was measured polarographically at 0 to 20°C using a gaseous phase, LD2 oxygen electrode (Hansatech Ltd, UK) (8). Incident light was varied from 0 to 1109 μmol m^{-2} s^{-1} (PPFD) using an LS2, tungsten-halogen light source in conjunction with a series of neutral density filters.

Photosystem II (PSII) fluorescence was continually monitored at 678 nm over a series of light treatments using a Perkin Elmer LS-5 Luminescence Spectrometer. Frond tips (3 cm) of vegetative tissue were preincubated (dry) with light of 709 nm (photosystem I (PSI) light) for 10 min then excited with light of 435 nm (PSII light) only. After approx. 15 min the frond tips were illuminated with both PSI and PSII light combined and then PSII

light only. Excitation and emission optima were taken from Anderson (1983; 1985).

3. RESULTS AND DISCUSSION

Both vegetative and reproductive algal frond tips exhibited a similar photosynthetic response over the temperature and light ranges studied (Figure 1). Net rates increased rapidly as PPFD increased from 0 to 80 $\mu mol\ m^{-2}\ s^{-1}$ with saturation of PS occurring at 200 $\mu mol\ m^{-2}\ s^{-1}$. Saturation of PS at such low PPFD's suggests considerable light harvesting efficiency and excitation energy transfer (9) and is typical of C. fragile (1,2,3,10). Furthermore, increased PPFD up to 1,200 $\mu mol\ m^{-2}\ s^{-1}$ did not result in a decline in PS. Such an extended PS saturation plateau indicates that C. fragile fronds, unlike isolated chloroplasts and thylakoids (6), are able to avoid photoinhibition under conditions of prolonged exposure to light. Maintenance of a suitable ratio of activities of the two photosystems may be seen as important in preventing the onset of photoinhibition, especially considering the high PSII: PSI ratios present within C. fragile chloroplasts (2,3,11,12). Figure 2 clearly illustrates the ability of chloroplasts within the algal frond to re-distribute excitation energy over a range of light treatments. When light exciting PSII only is provided to frond tips previously adapted to PSI light, PSII fluorescence increases to a maximum, then falls over a period of 10 min to a lower constant value which is relatively unaffected by re-provision of PSI light. This is indicative of a re-distribution of excitation energy from PSII to PSI (4,5,11,12) or an alleviation of the over excitation of PSII. Such "state transitions" could not be induced in isolated chloroplast preparations.

It is apparent that whilst isolated chloroplasts and thylakoids of C. fragile are most susceptible to photoinhibition and photooxidative damage, intact fronds are surprisingly tolerant. Photoprotective mechanisms must therefore exist in situ that are absent in organello. We believe that the answer involves the arrangement of the chloroplasts within the algal frond.
C. fragile possesses a compact, dichotomous thallus consisting of closely interwoven coenocytic siphons or filaments (13, 14). The outer layer of the thallus is constructed of the dilated tips (utricles) of the siphons and it is in these structures only that the chloroplasts are situated (Figure 3). The chloroplasts are arranged peripherally, in stacks, around a large central vacuole in such numbers as to confer an optically dense nature to the thallus. It has been suggested (15) that the presence of the large vacuole serves to reflect incident light around the thin column of cytoplasm thus increasing the chance of chloroplast interaction. As considerable light

attenuation would operate within the utricle a degree of 'self-shading' of chloroplasts would presumably occur. Hence, it would seem that the number and arrangement of chloroplasts within the frond confers a vital mechanism in the avoidance of photoinhibition by this alga.

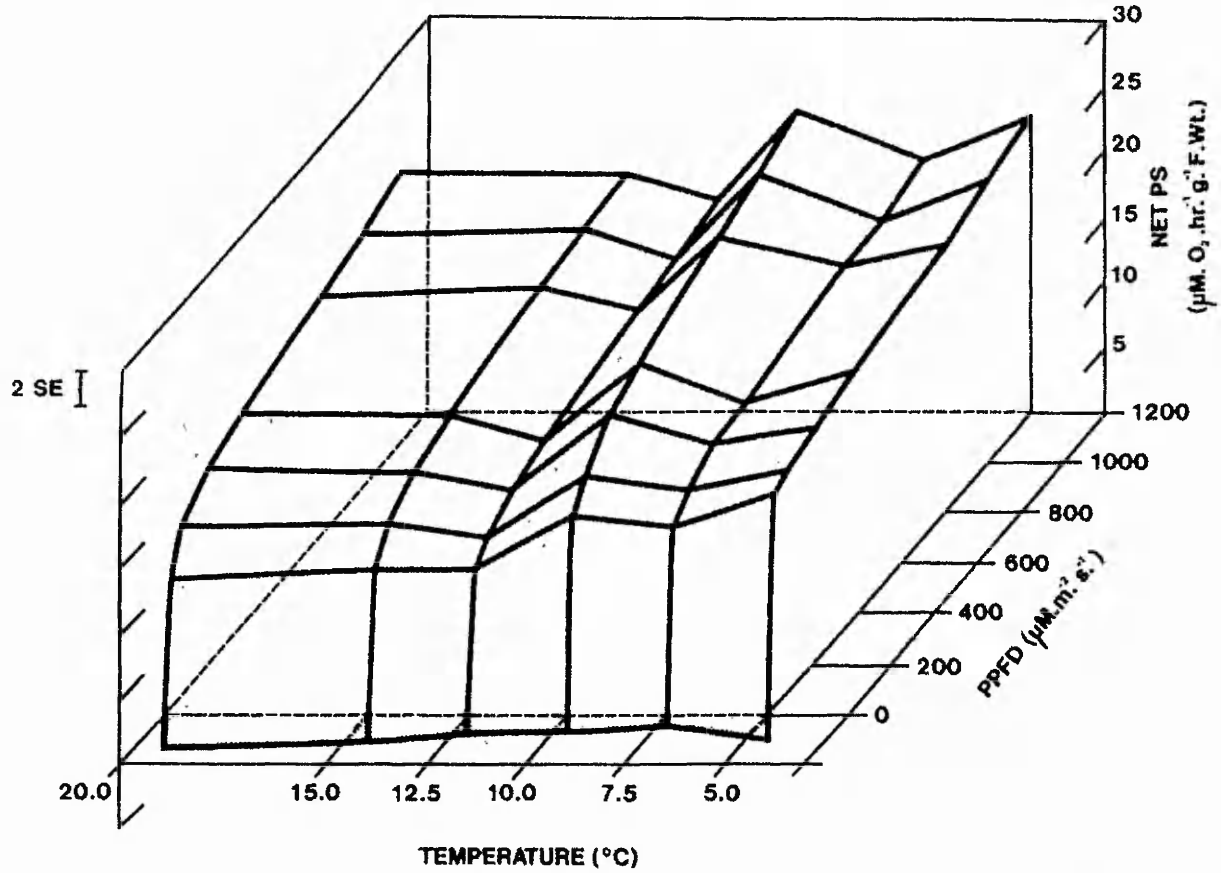

FIG 1: Relationship between light intensity, temperature and net photosynthesis by frond tips. Each point = mean of 10 observations ± overall S.E of the 48 observations.

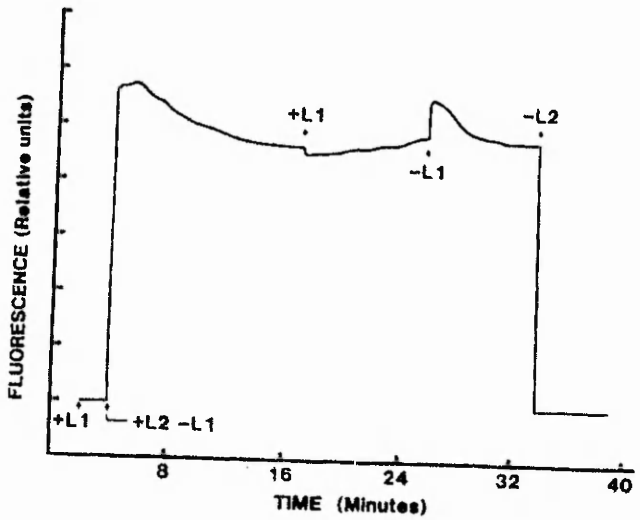

FIG 2: Time-course of PSII fluorescence emission at 687 nm from frond tips pre-incubated for 10 min at 10°C in PSI light (709 nm - L1) and then subjected to a regime of PSI and/or PSII (435 nm - L2) light as indicated.

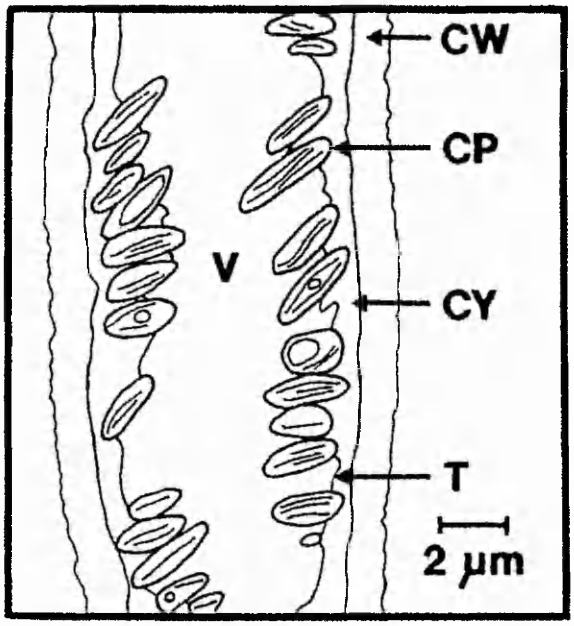

FIG 3: Diagram of internal structure of a utricle (T.S.). CW, utricle cell wall; CP, chloroplasts; CY, cytoplasm T, tonoplast; V, vacuole. After Hawes, 1979 (16).

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