HETEROGENEITY OF CHLOROPLAST THYLAKOIDS IN RELATION TO INHIBITION BY HERBICIDES

by

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I dedicate this thesis to my dad whose love and support during its writing was so dearly missed.

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SUMMARY

This thesis provides data on some aspects of DCMU-induced inhibition of photosynthesis and electron transfer in relation to chloroplast thylakoid heterogeneity. The work can be viewed on the premise that: (i) inhibition of photosynthesis and eventual death of the plant require much higher concentrations of herbicide than those which prevent oxygen evolution (Ridley S. M. and Horton P. [1984]; *Z. Naturforsch* **39c**, 351-353.) and (ii) the higher plant thylakoid lamellae are known to display extreme heterogeneities, for example cytochrome *f* oxidation kinetics, lateral segregation of protein complexes, formation of regions of membrane appression and sub-populations of photosystem 2 (α and β-centres).

It was demonstrated that three phases of cytochrome *f* oxidation existed, possibly showing a spatial separation of sub-populations. By measuring cytochrome *f* redox kinetics the I_{50} for electron transfer inhibition by DCMU was found to be similar to that inhibiting water oxidation. Chlorophyll *a* fluorescence studies revealed three I_{50}'s for DCMU: 0·08-0·19, 2·95-7·84 and ~365μM. Other studies suggested an assignment of the low constant to α-centres and the rest to β-centres, indicating that a loss of oxygen evolution was seen upon inhibition of α-centres. Effects on photosystem 2_β (~5μM) was similar to a DCMU concentration which may relate to photodestruction of the plant (Ridley S. M. [1977]; *Plant Physiol.* 59, 724-732, Ridley S. M. and Horton P. [1984].)

Other studies indicated that very high DCMU concentrations affect a photocentre with a long wavelength fluorescence emission, which was predominantly located in the unappressed lamellae. These results suggest that non-cyclic electron transfer is mediated by grana-located α-centres, which have a strong affinity for DCMU. Beta-centres have a lower affinity for DCMU and may redox poise photosystem 1 mediated cyclic electron transfer. High DCMU concentrations appear to affect a stromally-located sub-population of β-centres whose function at the moment is unclear. Thus a known heterogeneity of the chloroplast lamellae appears to be expressed in the inhibition characteristics of a commonly used herbicide.

CONTENTS

ABBREVIATIONS

A	One of two secondary electron acceptors associated with P_{430}.
A_1/A_2	Primary electron acceptors of photosystem one.
ADP	Adenosine diphosphate.
ATP	Adenosine triphosphate.
B	One of two secondary electron acceptors associated with P_{430}.
BCHL	Bacteriochlorophyll.
β_{max}	Proportion of area above $F_{v\beta}$.
B-PROTEIN	Binding site of secondary electron acceptor of photosystem two (the quinone is abbreviated to B).
C_{550}	Component associated with photosystem two with absorbance at 550nm.
C_{F0}/C_{F1}	Chloroplast coupling factors zero and one.
Ches	2[N-cyclohexylamino]ethane sulphonic acid.
CYTO	Cytochrome.
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethyl urea
$DCPIPH_2$	Dichlorophenyl indophenol (reduced).
ΔpH	*P*H gradient across thylakoid lamellae.
$\Delta\Psi$	Electric field across thylakoid lamellae.
EDTA	N,N'-1,2-Ethanediylbis[N-(carboxymethyl)glycine].
E_m	Midpoint redox potential.
EPR	Electron paramagnetic resonance technique.
Hepes	N-2-hydroxyethylpiperzine-N'-2-ethane sulphonic acid.
HES	High energy state.
LHCP	Light harvesting chlorophyll protein complex.
ΦF	Flourescence yield.
F_d	Ferredoxin.
FeS	Non-haem iron sulphur protein.
F_o	Initial intensity of minimum fluorescence level from open centres.
F_m	Maximum intensity of fluorescence from closed centres.
FNR	Ferredoxin-NADP-reductase.
F_t	Terminal level of fluorescence intensity at end of a DCMU titration of chlorophyll fluorescence.
F_v	Variable intensity of fluorescence (F_m-F_o).
k_α	Rate constant for α-centre photochemistry.
k_β	————"———— β-centre photochemistry.
k_d	————"———— deactivation.
k_f	————"———— fluorescence.
k_h	————"———— thermal decay.
k_p	————"———— photochemistry.
k_t	————"———— energy transfer.
Mes	2[N-Morpholino]ethanesulphonic acid.
NADP	Nicotinamide adenine dinucleotide phosphate.
P_{518}	Light induced pigment band shift with a maximum at 518nm.
P_{430}	Absorbance change at 430nm during redox changes of photosystem 1.
P_{680}	Reaction center pigment of photosystem two.
P_{700}	Reaction center pigment of photosystem one.
PC	Plastocyanin.
PHEO	Pheophytin

Pipes	Piperazine-N,N'bis[2-ethane sulfonic acid].
PMT	Photomultiplier tube.
PQ	Plastoquinone.
PSI	Photosystem one.
PSII	Photosystem two.
Q	Primary quencher of photosystem two fluorescence ($Q_{H,L,1,2,\alpha,\beta}$).
R	Secondary stable electron acceptor of photosystem two.
$S_{0,1,2,3}$	Oxidation states of the water splitting complex.
Tricine	N-Tris[hydroxymethyl]methylglycine
X	Primary acceptor of photosystem one.
X_{320}	Pigment associated with the primary quencher of photosystem two with absorbance at 320nm.
Z_1/Z_2	Intermediate electron donors to photosystem two.

CHAPTER ONE

GENERAL INTRODUCTION

Photosynthesis, the anabolic light driven process found in all green plants is possibly one of the greatest conquests of nature. More than 10^{12}kcal. of free energy is stored annually by photosynthesis, corresponding to the assimilation of at least 10^{10} tons of carbon into carbohydrate and other classes of organic matter. Julius Robert Mayer, discoverer of the law of constant conservation of energy in 1842, stated "nature has put itself the problem of how to catch in-flight light streaming to the earth and to store the most elusive of all powers in a rigid form.... plants take one form of power, light; and produce another power, chemical difference."

If one looks at the basic equation of photosynthesis, it appears deceptively simple:

$$H_2O+CO_2 \longrightarrow (CH_2O)+O_2 \qquad (1\cdot1).$$

In this equation, (CH_2O) represents carbohydrate and a calculation reveals that higher plant photosynthesis may be over 33% efficient. The scheme is very simple in concept, two reactants, light and water are brought together to split the water (as shown by the use of radio-labelled water) producing an oxidant, oxygen, and a reductant, $NADPH_2$. The process is also manipulated to produce ATP which, in conjunction with the $NADPH_2$ is used to reduce a third reactant, carbon dioxide. This anabolic stage occurs in the absence of any light at all and is often refered to as a series of dark reactions. The carbon dioxide is said to be "fixed", (a phrase coined by Jean Senebrier in Geneva around the middle of the eighteenth century), and can be used in other biosynthetic pathways as a substrate or a driving force.

Joseph Priestley in 1780, found that plants could "restore air which had been injured by the burning of candles. The air would neither extinguish a candle, nor was it at all inconvenient to a mouse which I put into it." Six years later, Jan Ingenhousz discovered the role of light in

photosynthesis: "I discovered that plants not only have the faculty to correct bad air in six or ten days, by growing in it, as the experiments of Dr. Priestly indicate, but that they perform this important office in a complete manner in a few hours; that this wonderful operation is by no means owing to the vegetation of the plant, but to the influence of the sun upon the plant."

In 1935 Van Neil provided the absolute equation of photosynthesis:

$$H_2A+CO_2 \xrightarrow[\text{bchl}]{h\nu} (CH_2O)+A \qquad (1\cdot2).$$

For the first time, a general description of photosynthesis was offered, where H_2A is the reductant. In green plants, H_2A is water, whilst in purple sulphur bacteria the donor is inorganic sulphur (H_2S). Van Neil thus established a link between two extremes of photosynthesis. This simple view also introduced the concept of biological redox reactions, and he later rejected a notion of a "photosensitised rearrangement of water and carbon dioxide on the chlorophyll surface."

The view that plants reduce carbon dioxide with water and concommitantly evolve oxygen implies that the oxygen released originates from water and not carbon dioxide. The source of the oxygen was disputed for many years, but the work of Hill established our present understanding; broken chloroplasts, which cannot reduce carbon dioxide, are able to evolve oxygen if provided with a suitable electron acceptor: ferricyanide and ferric oxalate became known as *Hill acceptors*, allowing CO_2-independent oxygen evolution -the *Hill reaction* (Hill 1939). Furthermore, experiments with radio-labelled water and carbon dioxide supported the view that oxygen evolution was due to water splitting, although these experiments on their own were not entirely conclusive.

Emerson & Arnold (1932), using Chlorella cells illuminated with very short bright flashes of light, observed that the ratio of chlorophyll present to oxygen evolved per flash reached a peak at 2480:1. They suggested that ≈2500 molecules of chlorophyll represent 'one unit' which

was capable of evolving one molecule of oxygen upon activation by light. The size of the photosynthetic unit however, is variable from species to another and is affected by both light quantity and quality.

At actinic wavelengths ≥700nm, Emerson showed that the quantum yield of photosynthesis fell sharply. The effect was termed the *Red-drop* (Emerson & Lewis 1943), and could be avoided if shorter wavelength light was simultaneously supplemented . In fact, the rate of photosynthesis achieved was greater than the sum of the two rates independently, this became known as *Emerson enhancement*.

In 1960 Hill & Bendall proposed a sequence of reactions in which two light reactions acted in series to promote the transfer of electrons from water to $NADP^+$ via a chain of intermediate carriers, including certain cytochromes. One photoreaction oxidised cytochrome *f* and reduced $NADP^+$ whilst the second reduced cytochrome b_6 and oxidised water. Thermodynamically, ATP synthesis was envisaged upon transfer of an electron from cytochrome b_6 to cytochrome *f* (figure 1·1). At the same time, such a scheme was suggested by Emerson & Rabinowitch (1960) to explain their enhancement phenomena. Losada *et al* (1961) also showed that one light reaction oxidised water and reduced DCPIP, and the second reaction re-oxidised $DCPIPH_2$ and reduced $NADP^+$, ATP synthesis being coupled to the latter reaction. Furthermore, in the red alga *Porphyridium cruentum*; red

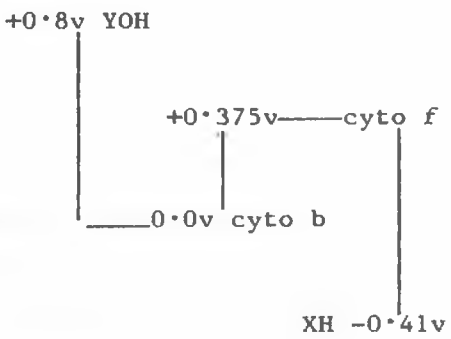


Figure 1·1: A thermodynamic representation of the energetic steps of the 'light reactions' from Hill & Bendall (1960).

light was shown to cause an oxidation of cytochrome *f* whilst green light

reduced it. The reduction, but not oxidation of cytochrome *f* was abolished by adding 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU) and other similar inhibitors (Duysens *et al* 1961 and Duysens & Amesz 1962).

The two photoreactions were assigned to separate photosystems termed photosystem 1 and photosystem 2. Longer wavelength light was absorbed preferentially by photosystem 1 and shorter wavelengths by photosystem 2. Emerson and Rabinowitch (1960) stated that two different forms of chlorophyll sensitise both photoreactions, replacing Emerson and Chalmers (1958) previous feelings that it was excitation of the accessory pigments that resulted in enhancement. Support was also provided by Duysens' earlier fluorescence studies: chlorophyll *a* was the primary photochemical sensitiser, energy being collected and transferred to this molecule from the accessory pigments.

There is now a lot of evidence in support of the Z-scheme, although there are several variations of the basic hypothesis with respect to the identity and sequence of additional electron acceptors that link the two light reactions.

Cytochrome *f*, first characterised by Hill & Scarsbrick (1951) has an E_m ≈+365 (Davenport & Hill 1952) and is reduced by photosystem 2 and oxidised by photosystem 1 (Duysens & Amesz 1962 and Whitmarsh & Cramer 1979). Haehnel (1973) showed reduction half time to be 17ms, but Dolan & Hind (1974) show a biphasic absorbance increase with half times of ≈7·5 and 85ms. Oxidation of cytochrome *f* has a half time of about 200μs (Hildreth 1968 and Dolan & Hind 1974).

Generally, the copper containing protein, plastocyanin (E_m ≈+390mv) is placed between cytochrome *f* and photosystem 1 (Katoh 1960 and Katoh *et al* 1961). Whilst plastoquinone, (first found in plant material by Kofler) is the most common of a range of very prominant non-protein electron carriers; Bishop (1959) showed its requirement for electron transfer in green plants and algae. With an E_m ≈0v, it is considered to accept a pair of electrons from photosystem 2 and reduce cytochrome *f* (Amesz 1973 and Trebst 1974). Steil & Witt (1968 and 1969) showed that photosystem 2 reduced plastoquinone with a half time of 0·6ms, oxidation by photosystem 1 taking 20ms. The abundance of plastoquinone is about 7

molecules per photosystem 2 reaction centre, ≈60% being kept reduced under steady illumination (Steil & Witt 1969). Siggel *et al* (1972) showed that ten electron transfer chains interact at the level of plastoquinone.

Cytochrome b_6 (or b_{563}) was isolated by Stuart & Wassarman (1975). Its midpoint potential is about 0v (Hill & Bendall 1960, Knaff & Arnon 1969a and Bohme & Cramer 1973) and there was possibly one molecule per chain. Kinetic studies revealed that b_6 mediated cyclic electron flow around photosystem 1 and did not participate in linear transfer (Hind & Olsen 1966, Weikard 1968 and Knaff & Arnon 1969a), although Velthuys (1979) suggested that cytochrome b_{563} may be involved in a 'Q-cycle' (Mitchell 1976) associated with a charge displacement and the slow electrochromic phase (the absorbance band shift that occurs upon thylakoide energisation). This involved plastoquinone and cytochrome *f*, and provided a means of accepting two electrons from plastoquinone prior to single electron transfers to photosystem 1.

The existence of a cytochrome *b/f* complex was established by Nelson & Neuman (1972), and was shown to have plastoquinol-1-plastocyanin oxidoreductase activity (Wood & Bendall 1976). The purified cytochrome complex of Hurt & Hauska (1981) appears to contain a molecule of cytochrome *f*, a molecule of the Rieske iron-sulphur cluster (Rieske *et al* 1964) and two molecules of *b*-type cytochrome. Rich & Bendall (1980) demonstrated the presence of cytochromes b_{563} and b_{559} (with E_m's of -90mv and +85mv respectively), whilst Hurt & Hauska (1983) suggest there are two spectral forms of cytochrome b_{563} with E_m's of -146 and -3mv. The total size of the complex is about 142kD, and SDS-polyacrylamide gel electrophoresis revealed five major polypeptides of 34, 33, 23·5, 20 and 17·5kD, the first three of which stained for haem. The largest two identified with cytochrome *f*; the presence of this doublet suggests that the particle was exposed to proteolytic action. The 23·5kD component was associated with cytochrome b_{563}, and purified Rieske protein is known to have a molecular weight of 20kD (Hurt *et al* 1981). At present, the 17·5kD polypeptide remains unidentified.

Plastoquinone, the *b/f* complex and plastocyanin thus link both light reactions as proposed by the Z-scheme. The photoreactions are associated with specific photosystem complexes, each complex consisting of

a large chlorophyll containing antenna supplying excitation to the central reaction centre (Gaffron & Wohl 1936) -as envisaged earlier by Emerson & Arnold.

The reaction centre or trap consists of both primary donor (D) and acceptor (A) molecules: P_{680}-Q and P_{700}-X (photosystem 2 and photosystem 1 respectively). Upon excitation of the donor, charge separation occurs which is stabilized across the couple (see equation 1·3). Such a proposal was supported by the observed bleaching of an absorbance band at 700nm

$$DA \xrightarrow{h\nu} D^*A \longrightarrow D^+A^- \qquad (1\cdot3).$$

Where hν could be a light quantum.

after a pulse of actinic light (Kok 1956). This was envisaged as a redox change in a chlorophyll molecule representing the primary donor of the photosystem 1 trap (P_{700}). Furthermore, the 700nm signal showed changes due to oxidation by far-red light (Kok & Gott 1960). Kok (1961) later correlated this change with one at 433nm, establishing that P_{700} was indeed a chlorophyll molecule.

Hiyama & Ke (1971) made a flash kinetic study at 430nm and assigned a spectral change to a new pigment P_{430}, possibly representing 'X'. Using EPR. techniques, Malkin & Bearden (1971) detected two non-haem iron sulphur (FeS) proteins within the photosystem 1 complex, which were termed centres A and B. At cryogenic temperatures, only one of these was photoreduced, suggesting a tight association with the primary charge separation. Further studies by Ke & Beinert (1973) provided a correlation of the P_{430} change with the FeS response. Moreover, based on half reduction potentials, P_{430} and the FeS signals were all strongly related to P_{700} (Ke *et al* 1974). Sauer *et al* (1978) demonstrated that P_{430} was probably a single protein containing both of the FeS centres (ie. P_{430}[A,B]) and also the presence of two more acceptors (termed A_1 and A_2) between P_{700} and P_{430}[A,B] (see figure 1·2). Goldbeck *et al* (1978) suggested that A_2 could be a modified FeS cluster too, but quite separate

from P_{430}[A,B]. This was enforced by Shuvalov *et al* (1979) who stated that A_2 was indeed 'X' and that A_1 was probably a chlorophyll dimer which could be reversibly reduced to an anion by donation of an electron from P_{700}.

Nugent *et al* (1981) demonstrated that at cryogenic temperatures barley chloroplasts could photoreduce centre A if centre B was pre-reduced by dithionite, (although there was still some interaction between both centres). The reverse appears to be true in spinach, indicating that these centres may act as parallel acceptors of A_2. Once reduced, P_{430}[A,B]

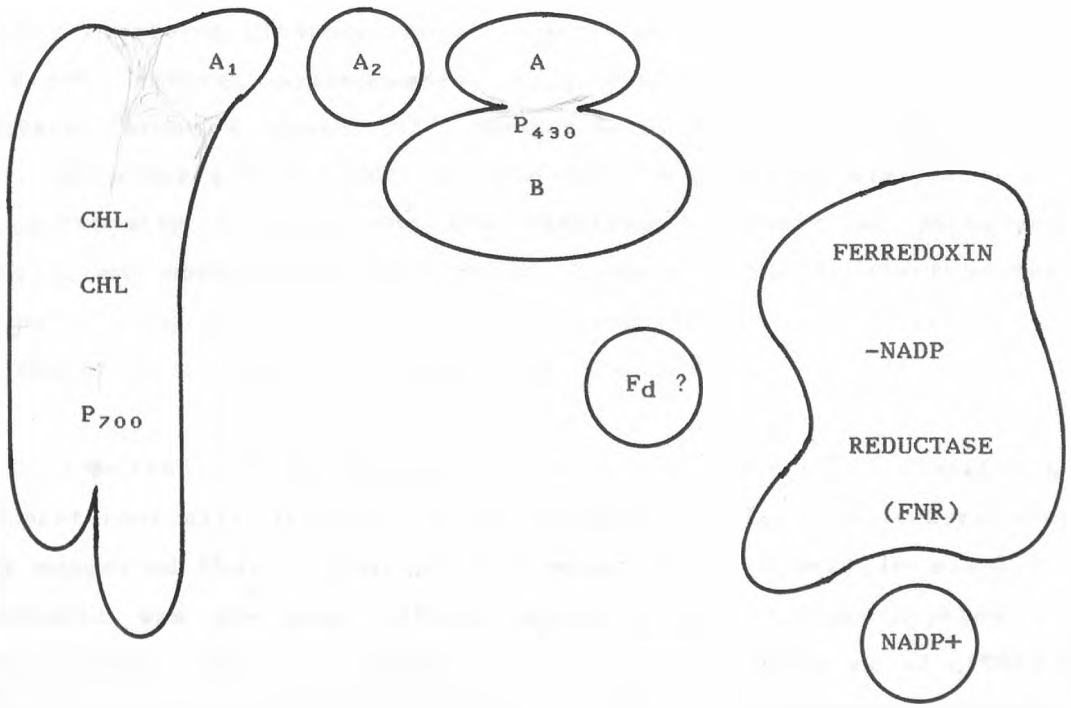


Figure 1·2: A schematic demonstration of the layout of the various Photosystem 1 components based on Sauer *et al* (1978).

passes an electron to ferredoxin (a soluble FeS protein) which in turn reduces $NADP^+$ via the enzyme ferredoxin-$NADP^+$ reductase (Buchanan & Evans 1969, Avron 1975 and Arnon *et al* 1977). This is a stepwise process, involving the semiquinone form of the flavin moiety, since $NADP^+$ is a two electron acceptor whilst ferredoxin is a one-electron carrier. However, Bouges-Bocquet (1980b) suggested that an uncharacterised component X' may mediate transfer from P_{430}[A,B] to the reductase, not ferredoxin. Although ferredoxin may complex with the enzyme.

$NADP^+$ is not the only photosystem 1 acceptor as demonstrated by the *Mehler Reaction*; in which electrons are transferred back to oxygen. Chloroplasts were shown to have have a high capacity for this mechanism by the studies of Marsho *et al* (1979) and Furbank *et al* (1982) using $^{18}O_2$.

Photosystem 1 was also shown to cycle electrons around itself (Arnon *et al* 1954): After charge separation across photosystem 1, an electron is removed from ferredoxin and restored to the 'positive hole' at the donor site of P_{700}. Consequently, electron transfer from photosystem 2 is not observed and oxygen evolution is absent. Studies on the turnover of cytochromes b_{563} and *f* (Slovacek *et al* 1979 and Olsen *et al* 1980) indicated turnover involving plastoquinone. Other studies showed the involvement of the Rieske centre, plastocyanin, P_{700}, ferredoxin and ferredoxin-$NADP^+$ reductase (Arnon & Chain 1977, Bouges-Bocquet 1978 and Malkin & Chain 1980). Crowther & Hind (1980) pointed out the potential similarity between the photosystem 1 cycle and the electron transfers of photosynthetic bacteria, and obtained evidence for a 'Q-cycle'. Cyclic electron transfer is sensitive to the 'redox poise' of the system and is inhibited by either over reduction or oxidation (Grant & Whatley 1967).

Emerson (1958), demonstrated that red light at wavelengths $\leqslant$680nm were preferentially absorbed by photosystem 2. Low temperature spectroscopy suggested that a chlorophyll *a* molecule (or dimer) in a specialised environment was the most likely candidate for the photosystem 2 trap (Murata 1968). This was supported by the work of Doring *et al* (1967) where a flash induced reversible bleaching of a species designated P_{680} (or P_{690}) was observed. A change in the blue part of the spectrum at 435nm also indicated a chlorophyll *a* molecule.

Following studies with the electron transfer inhibitor DCMU, or at cryogenic temperatures, Cramer & Butler (1969) suggested that the primary stable acceptor of photosystem 2 was a single electron carrier with an E_m of -35mv (termed 'Q' by Duysens & Sweers in 1963 -see later). Klimov *et al* (1980) showed that two atoms of iron are bound to photosystem 2 particles, these being associated with Q (or Q_A). Examination of photosystem 2 particles showed that pheophytin is an intermediate acceptor prior to Q (Klimov *et al* 1977 and 1980), see later. Studies by Knaff & Arnon (1969b) showed a broad light induced absorbance decrease at 550nm (provided

cytochrome *f* was maintained oxidised with ferricyanide). This was termed C_{550} (Knaff 1975) which is due to a band shift of a chromaphoric molecule (Okayama & Butler 1972 and Cox & Bendall 1974). C_{550} was also detected in sub-chloroplast particles containing photosystem 2 derived by digitonin fractionation (Knaff & Arnon 1969b), but not in photosystem 2 deficient mutants (Erixon & Butler 1971). C_{550} is believed to be close to the photosystem 2 trap, since the change is detected at 77K (Knaff & Arnon 1969b). Inhibition of electron donation from water abolishes the C_{550} signal, but it is re-established in the presence of artificial donors (Arnon *et al* 1971). Erixon & Butler (1971) suggested that Q and C_{550} reflect the same changes within photosystem 2, although Butler (1972) points out that the transmembrane potential indicator, P_{518} (Witt 1971), will lead to some complications. Exhaustive heptane extraction of chloroplasts abolishes the C_{550} absorbance, but it re-appears upon ß-carotene addition (Okayama & Butler 1972).

The quencher Q_A, which is a quinone itself, is re-oxidised by the plastoquinone pool via a secondary quinone acceptor (Q_B), the 'B' (or 'R') protein (Bouges-Bocquet 1973, Velthuys & Amesz 1974 and Pulles *et al* 1976). This protein shows binary oscillations in its redox state and behaves as a two electron gate (Bouges-Bocquet 1973 and Velthuys & Amesz 1974), and due to its unique properties was thought to be a 'special plastoquinone' bound at the active site. However, Goldfeld *et al* (1978) suggested that this is not the case, the quinone being a member of the bulk plastoquinone pool. Upon partial reduction to the semiquinone (PQ^-) it is strongly bound to the apoprotein. Rich (1981) and Velthuys (1981) suggested that the affinity for oxidised (PQ) or doubly reduced (PQH_2) plastoquinone would be much lower. In these two extremes of redox form, plastoquinone is believed to diffuse through the lipid matrix of the membrane, as apposed to forming a 'bucket brigade' type of chain between complexes (Salreno *et al* 1977 and Millner & Barber 1984).

The B-protein has a molecular weight of 32~34kD (Pfister *et al* 1981 and Mullet & Arntzen 1981) and is able to bind different families of herbicides at several close and overlapping sites (Pfister *et al* 1979 and Mullet & Arntzen 1981). Mild trypsin digestion of thylakoids results in a loss of sensitivity to herbicidal inhibition (Renger 1976, Renger *et al* 1976 and Regitz & Ohad 1976) and an increase in the rate of ferricyanide

mediated oxygen evolution (Renger 1976), which is thought to be due to an exposure of Q upon digestion of the herbicide binding site.

Two-four atoms of manganese are associated with the photosystem 2 reaction centre (Wydrzynski & Sauer 1980). Extraction of manganese, by Tris washing (suspension in 2-amino-2-(hydroxymethyl)propane-1,3-diol) results in a loss of oxygen evolving capacity (Cheniae & Martin 1971 and Blankenship & Sauer 1974). However, Tris washing only removes loosely bound manganese, leaving the tightly bound manganese largely undisturbed. Only removal of the latter disrupts oxygen evolution (Mansfield & Barber 1982), suggesting that water splitting is affected in a more subtle way by mild Tris washing. Other treatments, eg. hydroxylamine, high *p*H and heating also releases manganese.

Using repetitive flash conditions, Joliot *et al* (1969) observed oscillations in the yield of oxygen evolution with a periodicity of four. Kok *et al* (1970) interpreted this as an accumulation of four positive charges on the secondary donor prior to water splitting and oxygen evolution; thus, the complex must cycle through four oxidation states for every molecule of oxygen evolved. These four states are denoted S_0, S_1, S_2 and S_3, in which the subscript indicates the number of oxidising equivalents stored in the enzymatic complex. There is a fifth state, S_4, which is extremely unstable and rapidly degenerates to S_0 by evolving a molecule of oxygen. In the dark S_1 is ≈85% populated and S_2 ≈15%. The remaining S_3 (and S_0) are highly unstable also, and degeneration to S_1 usually occurs (Kok *et al* 1970).

EPR studies demonstrated the presence of two carriers within photosystem 2 called Signal $2_{very\ fast}$ and Signal 2_{fast} (Blankenship *et al* 1975 and Babcock *et al* 1976). Bouges-Bocquet (1980b) suggested that these signals represent intermediate donors to P_{680} and are termed Z_1 and Z_2. Kok's S_0 and S_1 are associated with Z_1 whilst S_2 and S_3 are related to Z_2 (see figure 1·3).

Knaff & Arnon (1969b) demonstrated that P_{680} could also oxidise another photosystem 2 component, cytochrome b_{559}. Exposure to light at temperatures approaching 77K led to oxidation of both cytochrome b_{559} and P_{680}, which correlated with Q reduction (Butler *et al* 1973 and Witt 1973).

At higher temperatures (≈-60°C), donation of electrons by S_0 and S_1 to P_{680}

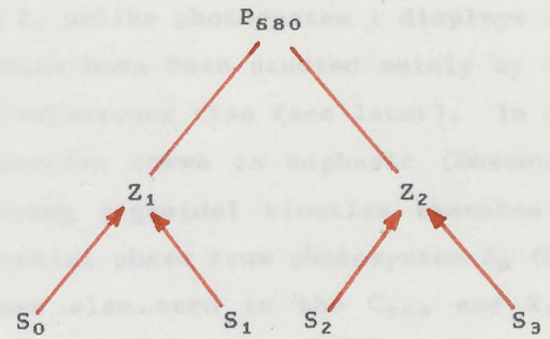


Figure 1·3: A simplified representation of the secondary donors to P_{680}, from Bouges-Bocquet (1980).

is sufficiently fast to obscure cytochrome b_{559} turnover. Based on this, Bouges-Bocquet (1980) suggested that cytochrome b_{559} may donate to P_{680} via Z_1. Oxygen evolution still occurred in the absence of this cytochrome (Cox

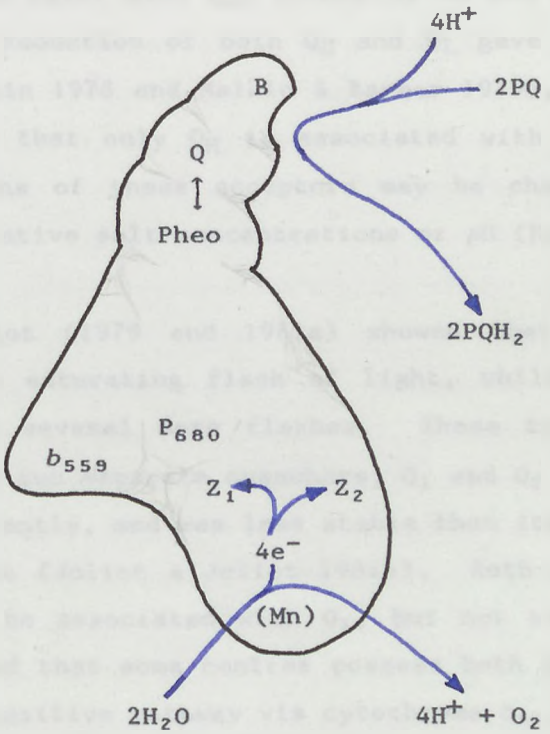


Figure 1·4: A schematic layout of the components of photosystem 2 (oriented with the outer membrane surface at the top of the diagram).

& Bendall 1972), although manganese deficient mutants lack high potential cytochrome b_{559} (Metz *et al* 1980).

Photosystem 2, unlike photosystem 1 displays a very complex series of heterogeneities which have been studied mainly by observing photosystem 2 trap closure and fluorescence rise (see later). In the presence of DCMU, the fluorescence induction curve is biphasic (Doscheck & Kok 1972); the faster phase, displaying sigmoidal kinetics emanates from photosystem 2_α and the slower exponential phase from photosystem 2_β (Melis & Homman 1975). This heterogeneity was also seen in the C_{550} and X_{320} signals (Melis & Duysens 1979 and Melis & Schreiber 1979). The α and β-centres have different excitation spectra (β being red-shifted) and the photosystem 2_α antennae is enlarged having LHCP associated with it (Thielen & Van Gorkom 1981a and Thielen *et al* 1981), in such a way that promotes energy transfer between units (see later).

Redox titration of fluorescence in chloroplasts reveals two quenching components behaving as photosystem 2 acceptors (Cramer & Butler 1969 and Horton & Croze 1979) with E_{m7} values of ≈0 and -250mv, termed Q_H and Q_L respectively. Reduction of both Q_H and Q_L gave rise to the P_{518} absorbance change (Malkin 1978 and Malkin & Barber 1979), although Diner & Delosme (1983) suggest that only Q_H is associated with the P_{518} signal. The relative proportions of these acceptors may be changed by 30% upon manipulation of the relative salt concentrations or *p*H (Horton 1981b)

Joliot & Joliot (1979 and 1981a) showed that ≈70% of F_V was eliminated by a single saturating flash of light, whilst removal of the remaining 30% required several more flashes. These two phases of trap closure were related to two separate quenchers; Q_1 and Q_2 respectively. Q_2 was reduced less efficiently, and was less stable than its Q_1^- counterpart. Q_2 may not be a quinone (Joliot & Joliot 1981a). Both the C_{550} and P_{518} signals were found to be associated with Q_1, but not with Q_2. Joliot & Joliot (1981b) suggested that some centres possess both Q's, but Q_2 may be oxidised by a DCMU insensitive pathway via cytochrome b_{563}.

The E_{m7} for Q_β is about +100mv, which is considerably more positive than either Q_H or Q_L (Horton 1981a and Thielen & Van Gorkom 1981b). Both Q_H and Q_L are therefore associated with Q_α. The fact that

the kinetics of Q_L photoreduction are altered by prior reduction of Q_H suggest that they share the same pigment bed or possibly the same centre (Horton 1981b). Moreover, fluorescence quenching associated with reduced phaeophytin in photosystem 2 is only prevalent when both Q_H and Q_L are reduced prior to illumination (Malkin & Barber 1979).

Bowes *et al* (1981) and Bowes & Horton (1982), using an oxygen evolving photosystem 2 preparation from *Phormidium laminosum* and a combination of redox-poise/flashes of light have shown that an absence of Q_L is accompanied with a loss of Q_2, thus indicating that $Q_L \equiv Q_2$ and $Q_H \equiv Q_1$, as suggested by Bouges-Bocquet (1980). Clearly, only Q_H is required for electron transfer from water to plastoquinone, allowing Q_L to be oxidised separately as described by Joliot & Joliot (1981b). This may be important for poising cyclic electron transfer or perhaps to protect photosystem 2 in stress conditions (Horton 1983).

By treating the thylakoids with detergent, Boardman & Anderson (1964) separated the two photosystems. Based on freeze fracture and electron microscopy, Arntzen *et al* (1969) suggested that photosystem 1 was externally located on the membrane surface, whilst photosystem 2 was to be found inside. Thus, photosystem 1 and photosystem 2 were thought to be in close association throughout the thylakoid lamellae and granal stacking occurred when photosystem 2 complexed with the chlorophyll *a/b* containing light harvesting protein complex (LHCP). Park & Sane (1971) obtained electron micrographs showing a granal particle ≈20nm in diameter, which they called the *Quantasome*. It was thought to be the morphological expression of the photosynthetic unit and possessed the components to oxidise water and reduce $NADP^+$. However, Goodchild & Park (1971) demonstrated that low concentrations of digitonin preferentially released the stromal membranes and showed that both detergents and mechanical fractionation act in a similar manner: the stromal lamellae are stripped off from the grana to yield a suspension of small vesicles. Thin sectioning and freeze-etching of French press derived fragments showed that the various fragments did indeed come from different thylakoid regions.

Sane *et al* (1970), using differential centrifugation techniques, showed that the heavier, faster-sedimenting grana-derived membranes had chlorophyll *a/b* ratios lower than native chloroplasts, whilst the reverse

was true for the stromal vesicles of the lighter fraction. Numerous lamellae studies have revealed that photosystem 2 is present in the appressed regions whilst photosystem 1 is located in the unstacked thylakoids. However, ≈15% of photosystem 1 activity was detected in the grana preparations (Sane *et al* 1970, Park & Sane 1971 and Anderson 1975).

Using phase separation of Yeda press disrupted membranes, Andersson & Anderson (1980) were able to obtain very pure preparations of appressed membrane vesicles containing only photosystem 2 activity, compared with the earlier preparations where photosystem 1 activity was

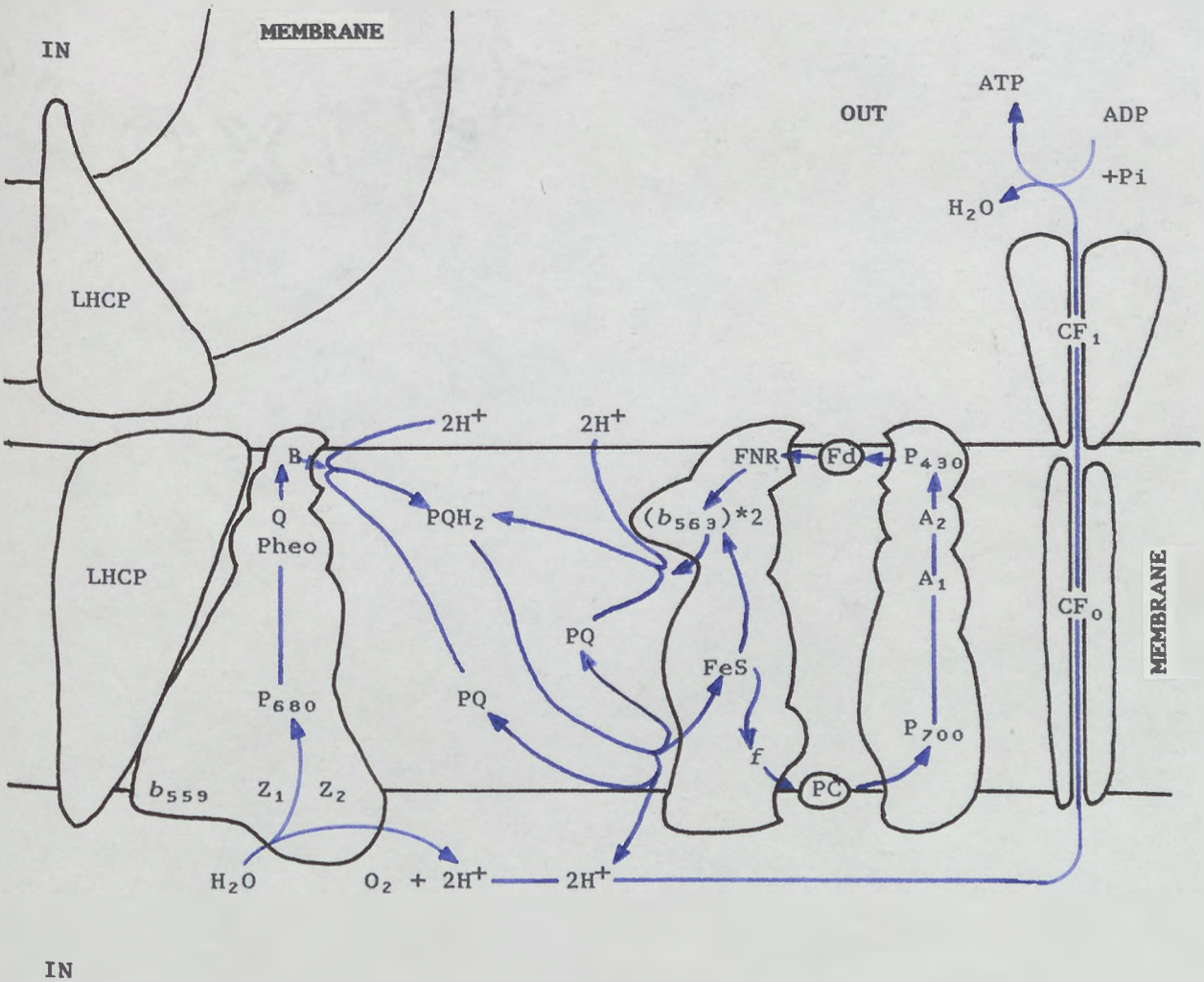


Figure 1·5: A diagramatic representation of membrane organisation of the major electron transfer components.

also detected. Thus, photosystem 1 is now believed to be exclusively

located in the un-appressed membranes (stromal thylakoids, grana end-membranes and margins), being excluded from the granal appressions. Photosystem 2 is found largely in the partitions, although ≈10→20% is now believed to reside with the photosystem 1 in the stromal lamellae.

The cytochrome *b/f* complex is uniformly distributed throughout the thylakoid system (Anderson 1982a), whilst the ATPase is only located on the stromal exposed membranes (Miller & Staehelin 1976). A simplified view of thylakoid lateral heterogeniety is shown in figure 1·5 above.

The distribution of the protein complexes and appression of the thylakoids are not fixed, as an examination of sun and shade plant chloroplasts revealed gross changes in stacking (Anderson 1982b). The plant is able to alter the relative concentrations of the two photosystems across the thylakoid, depending on the quality and quantity of incident light.

In 1969, Homann re-added Mg^{2+} to cation-free, DCMU poisioned chloroplasts. This resulted in a large increase in the fluorescence yield of the system, even though the photosystem 2 traps were closed; demonstrating that the fluorescence yield changes were not totally dependent on the redox state of the photosystem 2 trap (Duysens & Sweers 1963). Murata (1969a) and Murata *et al* (1970), using spinach chloroplasts showed that this phenomenon was not specific to Mg^{2+}, but that a range of cations could elicit the same response. However, an excess of monovalent cations were required compared to divalent cations.

The fluorescence emission spectrum of the red algae *Porphyridium crutenum* frozen to -196°C displayed an increase in the chlorophyll a_1 emission which correlated with a loss of chlorophyll a_2 fluorescence at room temperature. This effect could be reversed if the samples were pre-treated with far-red light for one minute prior to freezing (Murata 1969b).

At the same time, although independently, Bonaventura & Myers (1969) obtained similar results working with Chlorella. Using pulsed 'photosystem 2 light' (≤680nm, 'light 2'), they were able to measure modulated chlorophyll a_2 fluorescence and oxygen evolution. As the fluorescence declined from its initially high level to a steady-state level (with a concomitant increase in oxygen production), far red-light induced

enhancement became less. However, prolonged illumination with 'photosystem 1 light' resulted in an increase in chlorophyll a_2 fluorescence. They suggested that in 'light 2', some of the light initially absorbed by photosystem 2 was transferred to photosystem 1 in order to make photosynthesis more efficient. This may be due to an increase in k_t (see later) and is referred to as a *state 1→2 transition*, or often termed *spillover*. The result is a lowering of photosystem 2 fluorescence (due to an increase in the efficiency of the system and to a decrease in the exciton density within photosystem 2), and an increase in the rate of oxygen evolution -called *state 2*. It was demonstrated that the constancy of the quantum yield for oxygen evolution holds even in the spectral regions where photosystem 2 absorbs almost exclusively (Myers 1971). In 'light 1' (≥700nm), the reverse occurs; *spillover* decreases (because photosystem 1 has a sufficient exciton density) and photosystem 2 expends all of the energy absorbed by its antennae. The result, is the more fluorescent *state 1*. Under extreme cases of far-red illumination the proportion of quanta absorbed by photosystem 2 is low and the *red drop* is seen (Emerson & Lewis 1943) because energy cannot be transferred from photosystem 1 to photosystem 2 efficiently.

After further studies with Mg^{2+}, Murata (1969a) demonstrated that at 77K, a cation addition increased the fluorescence peaks at 685nm and 695nm relative to that at 735nm. This could be reversed by addition of diaminoethanetetra-acetic acid (EDTA), a chelator of divalent cations. This was also confirmed by Butler & Kitajima (1975). At low temperatures, the fluorescence emission peaks at 685, 695 and 735nm are believed to represent LHCP, photosystem 2 and photosystem 1 respectively (Butler & Kitajima 1975 and Butler 1978), although Rijgersberg *et al* (1979) demonstrated that both the 685 and 695nm peaks originate from photosystem 2. They further suggest that only at 4·7K is LHCP fluorescence emitted, and at a wavelength of 680nm (as this peak is the only one absent in a barley mutant lacking LHCP). Breton (1982) hypothesised that the 695nm emission is due to charge recombination within the photosystem 2 trap and is thus luminescence, not fluorescence. He also suggested that the 685nm signal actually emanates from the chlorophyll a_2 antennae.

Based on reports that cations are released from spinach chloroplasts during illumination (Dilley & Vernon 1965 and Nobel 1967),

Murata (1971) proposed that *spillover* was due to a reduction of the Mg^{2+} concentration within the chloroplast upon illumination; such that the physiological quenching of chlorophyll a_2 fluorescence was due to changes in k_t resulting from cation redistribution, (the reverse being true for a *state 1→2 transition*). Sun & Sauer (1971 and 1972) pointed out that a cation addition could actually increase photosystem 1 to photosystem 2 energy transfer, thus making photosystem 2 more efficient, although there is no evidence for energy transfer from photosystem 1 to photosystem 2 or LHCP (Butler 1977).

Approximately 5mM of divalent cations must be present to see *enhancement* effects (Marsho & Kok 1974 and Sun & Sauer 1972), although fixing the thylakoids with glutaraldehyde abolishes any cation induced fluorescence changes (Mohanty *et al* 1973 and Jennings & Forti 1974). Murata (1971) also demonstrated that neither photosystem 1 or photosystem 2 preparations show these changes.

The requirement of cations for most chloroplast functions is absolute; in their absence, the high energy state (HES) fails to develop (Dilley & Shavit 1968) and there is no photophosphorylation (Shavit & Avron 1967 and Gross *et al* 1969). The extent of reduction of the intermediate electron carriers is increased by a cation addition (Marsho & Kok 1974). Isolation of chloroplasts in low salt conditions causes unstacking (Izawa & Good 1966, Ohki *et al* 1971 and Barber & Chow 1979). Gross & Prasher (1974) showed that low concentrations of monovalent cations resulted in both structural and unstacking changes, whilst additions of up to 100mM monovalent cations or 10mM divalent cations reversed these changes. Further studies demonstrated that DCMU inhibited 'zero salt' thylakoids showed a fluorescence quenching if 5mM KCl was added, this being reversed with a 5mM $MgCl_2$ addition (Gross & Hess 1973 and Gross & Prasher 1974) as shown by Homann previously. Cation depletion changed the fluorescence induction curve (see later) from a sigmoid to an exponential (Briantais *et al* 1973 and Bennoun 1974). Bennoun also pointed out that the probability of energy transfer between photosystem 2 units was controlled by cations (See Marsho & Kok 1974).

Murakami & Packer (1971) correlated light scattering, stacking and cation induced changes in membrane conformational with the *state 1→2*

transition. The kinetics of fluorescence were also linked with those of salt induced stacking, possibly altering the spatial orientation of the pigments (Barber *et al* 1974, Krause 1974, Barber 1976, 1980a and b). Clearly, structural changes occured upon Mg^{2+} addition or *p*H drop, as light induced proton pumping was known to cause thylakoid shrinkage (Murakami & Packer 1969). Thus, light induced H^+ influx and protonation of fixed negative charges within the lumen, coupled to displacement of Mg^{2+} as a counter ion, was adopted as the most likely mechanism by which conformational changes of the membrane resulted in the *state 1→2 transition in vivo* (Vandermeulen & Govindjee 1974 and Vernotte *et al* 1975). Vernotte *et al* (1975) and Barber (1980a and b) pointed out however, that such gross changes in membrane adhesion and volume are unlikely to occur as a result of light induced Mg^{2+} efflux.

Using artificial electron donors in the presence of DCMU, Murata & Sugahara (1969), Wraight & Crofts (1970) and Mills & Barber (1975) studied cyclic electron flow around photosystem 1. This resulted in a chlorophyll a_2 fluorescence quenching by up to 30% (Murata & Sugahara 1969) which was independent of the redox state of Q (Duysens & Sweers 1963). Wraight & Crofts (1970) confirmed that this quenching was due to proton uptake by the lumen, which resulted in a transmembrane potential ($\Delta\psi$) and a proton gradient (ΔpH) or high energy state (HES). The quenching was reversed in the dark or in the presence of uncouplers, both of which relaxed the ΔpH. As there were no changes in the low temperature emission ratio of PSII/PSI at 77K, as observed upon manipulation of salt concentrations, these changes could not be related to the *state 1→2 transition*.

With intact chloroplasts, Krause (1973) and Barber & Telfer (1974) demonstrated that reversal of the slow fluorescence quenching upon addition of DCMU was biphasic. The fast phase being due to photosystem 2 trap closure, whilst the second slower phase ($t_{\frac{1}{2}}$ 1~2 minutes) was due to the relaxation of a ΔpH. Furthermore, the PSII/PSI fluorescence emission ratio at 77K was constant during the room temperature quenching process, and freezing actually removed the quenching (Briantais *et al* 1979).

HES quenching is thought to be manifested by increasing the thermal deactivation pathway (k_h) of the chlorophyll excited state (Briantais *et al* 1979, Krause *et al* 1982 and 1983). Such slow quenching is

quite separate from the *state 1→2 transition* (although the H^+/Mg^{2+} exchange and protonation of the membrane surface could still be involved in the *state* changes).

Protonation of unstacked thylakoids (≈*p*H 4·3) causes extensive membrane appression, without the domain formation normally associated with salt induced stacking (Barber *et al* 1980, Barber 1980a, b, 1982 and 1983). The fluorescence yield change after acidification was less than with a salt addition (Mills & Barber 1975 and 1978) and the chlorophyll *a/b* ratio of 'protonated stacks' was found to be higher than 'salt stacks' -an indication of only slight lateral segregation (Barber *et al* 1980).

What then is the difference between the addition of salt, and H^+ (a monovalent cation) ? Certain thylakoid proteins are highly charged, particularly photosystem 1, which is thought to be very electronegative, whilst the PSII/LHCP complex has a surface charge approaching zero (Barber 1980b).§ In a cation free medium, the photosystem 1 units tend to spread out over the total membrane area due to coulombic repulsion, interspersing themselves with the PSII/LHCP particles. This randomisation of photosystem 1 and PSII/LHCP tends to favour *spillover* by increasing the probability of energy transfer (k_t) from photosystem 2 to photosystem 1, thereby quenching chlorophyll a_2 fluorescence.

A cation addition screens the photosystem 1 particles and reduces coulombic repulsion, thus allowing them to approach each other. This is enforced by Van der Waals' forces pulling the electroneutral PSII/LHCP complexes together, resulting in domain formation and lateral segregation (Barber 1980a, b, 1982 and 1983).

The requirement of excess monovalent cations compared with divalent cations to elicit fluorescence changes (Murata *et al* 1970) is due to a much greater screening power of divalent cations compared to monovalent cations. Generally, it is the valency of the cation and not its

§ The other proteins within the thylakoid are dis--regarded in this discussion, although they must also obey the same energetic considerations.

chemical properties which govern the fluorescence and stacking response, where $C^{3+}>C^{2+}>C^{+}$ (Barber & Chow 1979, Barber 1980a and b).

Upon acidification however, direct chemical binding occurs, causing neutralisation of charged groups rather than screening and results in an absence of particle migration (Barber 1980a, b and Barber *et al* 1980). Charge neutralisation may also occur in the presence of higher valency cations, such as La^{3+} (Barber *et al* 1980) and polylysine (Berg *et al* 1974).

Once laterally segregated, the membranes stack with areas of PSII/LHCP forming the partitions. Since domain formation is largely absent in protonated thylakoids, photosystem 1 particles are found in the appressions. There is probably some residual charge on the photosystem 1 units after protonation as 100% stacking is never found (Barber *et al* 1980).

Rubin *et al* (1981) pointed out that even with concentrated protein domains, the Van der Waalian attraction between adjacent membranes is insufficient to overcome the repulsive hydration force associated with the structured water of the outer Helmholtz plane of the membrane plates. This was enforced by Mullet *et al* (1981) who suggest that a charged surface exposed segment of the light harvesting complex effectively cross-links the partition as evidenced by little or no stacking when the light harvesting complex was absent (Armond *et al* 1976), or modified by mild trypsination (Jennings *et al* 1978, Steinback *et al* 1979 and Mullet & Arntzen 1980).

Magnesium efflux during proton pumping affects the exterior of the thylakoids (Briantais *et al* 1979) which involves LHCP-borne acidic groups (Allen *et al* 1981). Lieberman *et al* (1978) demonstrated that LHCP is intimately involved in the *state 1→2 transition* and the salt effects. Although *in vivo* changes in salt concentration and *p*H are no longer thought to control the *state transitions*, LHCP is clearly instrumental. Cations however, are crucial to the maintenance of thylakoid topography. In 'zero salt', total loss of membrane structure does not occur, although it does lose most of its physiological function. Spillover is not controlled entirely by cations, but a precise ionic balance is essential in providing the correct environment for expression of the *state 1→2 transition*.

Certain thylakoid proteins have been shown to undergo reversible phosphorylation (Bennett 1977), the most prominent being identified as LHCP (Bennett 1977 and 1979a). The protein kinase which phosphorylates LHCP is membrane bound and activated by light, although DCMU additions showed that it is not directly stimulated by excited chlorophyll. Infact, NADPH and ferredoxin will activate the enzyme in the dark and uncoupling showed that Mg^{2+} efflux, ΔpH or ATP synthesis were not required either (Bennett 1979b and Bennett *et al* 1980). Trypsination demonstrated that the threonine-rich 2kD exposed segment of LHCP was the site of phosphorylation (Bennett 1980).

Dephosphorylation of LHCP is the most rapid of the thylakoid phosphoproteins and is accelerated in up to 10mM $MgCl_2$. The enzyme, a membrane bound phosphatase, does not require cofactors like ADP and is indifferent to light and DCMU, although it is inhibited by ≈10mM NaF (Bennett 1980).

Addition of ATP to isolated thylakoids produces a slow fluorescence quenching (Horton & Black 1981a) which is inhibited by DCMU but not nigericin. Chlorophyll fluorescence induction transients are quenched after incubation with ATP, and the PSII/PSI ratio at 77K decreases after phosphorylation (Horton & Black 1981a). Thus, reversible phosphorylation of LHCP appears to control the *state 1→2 transition* (Bennett *et al* 1980, Horton & Black 1980 and Telfer & Barber 1981), whereby phosphorylation increases excitation of photosystem 1 relative to photosystem 2. Cytochrome *f* studies also indicate that phosphorylation modulates the relative excitation of photosystem 1 and photosystem 2 (Horton & Black 1981b).

The extent of LHCP phosphorylation can be directly correlated with fluorescence lowering, as redox titrations of both of these parameters show an $n=2$ fit of a standard Nernst equation, giving an $E_{m7.8}$ of ≈0mv (Horton *et al* 1981). These data indicated that the activity of the protein kinase is modulated by the redox state of plastoquinone (Horton & Black 1980 and Horton *et al* 1981, see also Horton & Black 1981a, Allen *et al* 1981 and Allen & Horton 1981).

The above studies all suggest an explanation of the *state 1→2 transition* of Bonaventura & Myers and Murata: over excitation of

photosystem 2 causes reduction of the plastoquinone pool, which activates the kinase. LHCP (amongst other thylakoid proteins) is then phosphorylated on its exposed segment. The change in surface charge of this complex leads to lateral re-arrangement of the pigment complexes in accordance with the charge considerations of the membrane and the surrounding ionic enviroment. This results in detachment of LHCP from photosystem 2, followed by migration into the stromal lamellae (Chow *et al* 1981, Andersson *et al* 1982 and Kyle *et al* 1983b) and closer association with photosystem 1, thus increasing the absorbance cross-section of photosystem 1 -an increase in α according to Butler (1980). Physiologically, photosystem 1 excitation is increased at the expense of photosystem 2 which redresses the previous imbalance (*auto enhancement*) and partially re-oxidises plastoquinone by increasing linear electron flow. If the plastoquinone pool is over oxidised, the kinase is de-activated and the phosphatase, which is permanently active (Bennett 1980) dephosphorylates LHCP and a *state 2→1 transition* occurs.

After phosphorylation, Horton & Black (1981a, see also Horton & Black 1983 and Telfer *et al* 1983) report no loss of sigmoidicity of the remaining α-centres or alteration of the F_v/F_m ratio during a fluorescence induction measurements, suggesting that LHCP is the **only** migratory complex, thus lowering fluorescence , not quenching it. However, Kyle *et al* (1982) show losses of both the F_v/F_m ratio and sigmoidicity for similar treatments; indicating an interaction of **both** LHCP and photosystem 2 with the photosystem 1 in the stromal lamellae as found with cation depletion.

Based on the photosystem models of Butler & Strasser (1977), Strasser & Butler(1977) and Butler (1980), where the PSII-LHCP coupling is enhanced by cations, and the PSII-PSII interactions are mediated by energy transfer through LHCP, one may expect a removal of LHCP to result in some sigmoidicity changes caused by a loss of PSII-PSII connectivity, although preferential F_v quenching may not be expected. These differences in data must be reconciled as they suggest different processes for the same event. Kyle *et al* (1982) and Haworth *et al* (1982) demonstrate 'true *spillover*' (PSII→PSI energy transfer) as well as LHCP→PSI transfer, whilst Horton & Black (1981a and 1982) show only the latter. However, by lowering the Mg^{2+} concentration Horton & Black (1982) enhanced ATP quenching, which is explained as a greater cation requirement for screening phosphorylated

LHCP. Also, at low levels of Mg^{2+} F_v is preferentially quenched (Horton & Black 1983 and Telfer *et al* 1983).

The reason for these differences may be just a matter of scale. Initially, phosphorylation could cause only slight re-organisation whereby small amounts of LHCP are exported to the stromal lamellae, thus changing α, and not the F_v/F_m ratio. Greater phosphorylation causes more LHCP migration and decreases connectivity. Finally, gross movements of LHCP would favour *spillover* by isolating some of the photosystem 2 units. This may be associated with destacking as seen by Kyle *et al* (1983b). The conditions for these changes would depend on a whole spectrum of factors, for example the PSII:LHCP ratio and the background levels of cations (Telfer *et al* 1983 and Horton & Black 1983).

Vermass *et al* (1984) demonstrated that membrane phosphorylation also resulted in a lowering of the I_{50} for DCMU (this is shown in Chapter six). Pfister *et al* (1981) and Mullet & Arntzen (1981) demonstrated that herbicides like DCMU and atrazine etc. bind to the 32~34kD polypeptide. Ottmeier *et al* (1980 and 1982) show that a 41kD polypeptide is also a constituent of the herbicide binding site. Furthermore, Shochat *et al* (1982) demonstrated that a 32~35kD component of the thylakoid must have a phosphate group bound in order to witness efficient DCMU binding. Depletion of a 44~45kD polypeptide from the photosystem 2 reaction centre produces a low affinity site, indicating that a structurally complete photosystem 2 is a prerequisite for high affinity binding.

Croze *et al* (1979) demonstrated that trypsination of photosystem 2 particles resulted in a loss of DCMU sensitivity which reflected the degradation of a 32kD polypeptide. Radio-labelling studies with triazine resistant biotypes showed that only a small change in the primary sequence of the 32kD protein leads to a loss of atrazine inhibition without gross changes in DCMU binding (Pfister *et al* 1979 and Mattoo *et al* 1982).

The work of Duysens and Sweers first pointed towards the herbicidal action of DCMU, in which plastoquinone reduction and Q oxidation was prevented. This was supported by the discovery of the B-protein (Bouges-Bocquet 1973 and Velthuys & Amesz 1974). Furtheremore, Velthuys (1981) and Arntzen *et al* (1983) demonstrated that herbicide binding to B

was non-covalent and exhibits competitive binding kinetics with plastoquinone (and other similar herbicides). Thus, addition of extra plastoquinone to the thylakoids relaxes the inhibition. DCMU was thought to prevent the interaction of B with Q by lowering the E_m of B. In fact, reversed electron flow from B^- to Q may occur (Velthuys & Amesz 1974, Pfister & Arntzen 1979 and Lavergne 1982).

Inhibition of water splitting and subsequent electron transfer by DCMU and similar herbicides prevents the generation of reducing power and would eventualy lead to the death of the plant. However, this is not the case as much higher DCMU concentrations are required to effect a fatal dose (Ridley 1977 and Ridley & Horton 1984). The I_{50} that can be demonstrated to block plastoquinone reduction may be as low as 0·01μM DCMU, but a lethal dose may need to be in excess of 10μM. Ridley & Horton (1984) suggest that the curve for DCMU-induced photo-destruction follows that for inhibition of cyclic electron transfer arround photosystem 1 and has an I_{50} of 4·3μM.

By definition, cyclic involves photosystem 1, not photosystem 2 and is unlikely to bind DCMU since the 32kD polypeptide associated with herbicide binding is a constitutent of the photosystem 2 complex (Pfister *et al* 1981 and Mullet & Arntzen 1981). Thus the DCMU event at higher concentrations is completely unrelated to that associated with inhibition of linear electron transfer. How is this inhibition process achieved ? There are possibly two alternative explanations: (i) DCMU has an unknown affect on photosystem 1 or (ii) the photosystem 1 cycle is dependant on photosystem 2 activity of a different nature to 'ordinary' linear electron transfer. Clearly, the thylakoid's response to DCMU is non-homogeneous, a not too surprising fact, considering the complexities of the chloroplast thylakoid. An obvious course of research would be to examine the effects of DCMU on α and β-centres, as sub-populations of photosystem 2 might be expected to show different affinities to DCMU.

Photosystem 2_β is now believed to reside in the stroma-exposed lamellae, the stacks containing α-centres (Anderson & Melis 1984), emphasising the extreme heterogeneity of this membrane. It may be possible that β-centres provide the correct redox poise for the photosystem 1 mediated cycle and do not participate in the 'normal' electron transfer pathway of NADP reduction.

The aim of this thesis was to study the heterogeneous nature of the chloroplast thylakoid and to see if any of its structural or biochemical variability could be related to effects of DCMU binding. Chapter three commences with a study of the different populations of cytochrome *f* (Horton & Cramer 1974), and proceeds towards an examination of DCMU inhibition of electron transfer. In subsequent Chapters chlorophyll fluorescence was measured and it was found that the various photosystems displayed different degrees of DCMU binding over a very wide range of concentrations.

CHAPTER TWO

PLANT MATERIAL

Spinach (*Spinacia oleracae*, Yates hybrid 102) was greenhouse grown in plastic pots (as described by Walker 1980), under sunlight supplemented by Wotan growth lamps. Peas (*Pisum sativum*, Progress N°9) were grown in vermiculite for about fourteen days in a growth room maintained at 20°C. The plants were illuminated with four fluorescent lights, giving a surface intensity of ~30w.m^{-2}.

Intermittent light (IML) peas (Argyroudi-Akoyunoglou & Akoyunoglou 1970) were grown by storing the plants in the dark at 20°C for eight days, after which the etiolated shoots were illuminated with four 40w tungsten bulbs for two days under a regime of two minutes light every two hours. Under these conditions small quantities of chlorophyll *a* were synthesised (but not *b*) and as a result little or no LHC was formed, preventing granal appression (Argyroudi-Akoyunoglou & Akoyunoglou 1970 and Armond *et al* 1976). From these shoots agranal chloroplasts were isolated.

CHLOROPLAST PREPARATION

The same isolation procedure was used for peas and spinach, the method being essentially that described by Walker (1980). Listed below are the buffers used:

SPINACH GRINDING MEDIUM:	Sorbitol	0·33M	*p*H 6·5/HCl
	$Na_4P_2O_7$	0·01M	
	$MgCl_2$	5mM	
	Ascorbate	2mM	
PEA GRINDING MEDIUM:	Glucose	0·33M	*p*H 6·5/HCl
	Na_2HPO_4	0·05M	
	KH_2PO_4	0·05M	
	$MgCl_2$	5mM	
	Ascorbate	0·2%	
	BSA.	0·1%	

RESUSPENSION MEDIUM (RS2):	Sorbitol	0·66M	pH 7·6/KOH
	EDTA	4mM	
	$MgCl_2$	0·01M	
	Hepes	0·1M	
WASHING MEDIUM:	Glucose/Sorbitol	0·33M	pH 7·6/KOH
	$MgCl_2$	5mM	
	RS2	4%	

ESTIMATION OF CHLOROPHYLL CONCENTRATION

The chlorophyll concentration of a given sample of chloroplasts was estimated according to the method of Arnon (1949), using the formulae of Bruinsma (1963):

Chlorophyll *a*	12·7.A663-2·7.A645	(2·1)
Chlorophyll *b*	22·9.A645-4·7.A663	(2·2)
Total chlorophyll	20·2.A645+8·02.A663	(2·3)

Where: A_{645} and A_{663} are the measured extinctions at 645 and 663nm respectively.

CHLOROPLAST FRACTIONATION.

Thylakoid membranes were fractionated according to the procedures of Andersson *et al* (1975) and Andersson and Anderson (1980). A large volume Yeda press was designed and built (see Chapter three), allowing rapid preparation of large amounts of thylakoid lamellae with a simplified technique: chloroplasts were prepared from about 250g of spinach and after standing on ice for 30 minutes in resuspension buffer, the suspension was centrifuged at 1000g for 10 minutes at 2°C. The pellet, which was predominantly class II chloroplasts was washed in the same buffer and resuspended in about 100ml of pressing medium. The membranes were then

passed twice through the press at a pressure of 100 bar (obtained from a cylinder of high pressure nitrogen) and then centrifuged for 30 minutes at 10,000g.

The supernatant was decanted and centrifuged for a further 60 minutes at 140,000g. The two pellets (the first being grana and the second stromal lamellae) were then resuspended in RS2.

RESUSPENSION BUFFER:	Sucrose	50mM	*p*H 7·4
	NaCl	10mM	
	NaH_2PO_4	50mM	
	Na_2HPO_4	50mM	
PRESSING MEDIUM:	NaCl	150mM	*p*H 7·4
	NaH_2HPO_4	50mM	
	Na_2HPO_4	50mM	

All equipment was chilled prior to use.

CHAPTER THREE

CYTOCHROME STUDIES

INTRODUCTION

The cytochrome components of higher plant thylakoids are generally distinguished by their redox midpoint potentials and their absorption spectra, particularly the position of the α-peaks. Studies have shown that there are two basic cytochrome families; cytochrome *f*, an acidic c-type (Davenport & Hill 1952), and several *b*-types.

Cytochrome *f* was first described by Hill & Scarisbrick (1951) and was shown to possess an asymetric α-band at 554nm, displaying a 4-5nm red shift in comparison to cytochrome *c*. Its oxidation/reduction potential is independant of *p*H between 6 and 8, with a midpoint (E_m) of +365mv (Davenport & Hill 1952). At higher *p*H values the E_m declines by 60mv/*p*H unit.

Photosystem 1 and 2 have antagonistic effects on the redox state of cytochrome *f* during linear electron flow. This observation was paramount during the formulation of the *Z-scheme*, in which photosystem 2 driven reduction of cytochrome *f* was mediated by red light, whilst far-red light caused photo-oxidation *via* photosystem 1 (Hill & Bendall 1960, see also Duysens *et al* 1961 and Duysens & Amesz 1962). Cytochrome *f* is believed to be reduced by the Rieske protein (FeS) and oxidised *via* plastocyanin (Bendall 1977). The work of Hurt & Hauska (1981 and 1982) demonstrated that cytochrome *f* was a constituent of a supra-molecular structure, the cytochrome *b/f* complex. Purification revealed ratios of 1mol cytochrome *f* : 2mol cytochrome b_{563} : 1mol FeS.

Stuart & Wasserman (1975) purified cytochrome b_{563} as a single species of three polypeptides with a midpoint potential of -80mv, this being very similar to that reported for the whole thylakoid (Rich & Bendall 1980). The α-band of this cytochrome had a broad absorbance at temperatures around 20°C, which split into two peaks (557 and 561nm) at 77K. The peak splitting seen by Stuart and Wasserman could be indicative of contamination with another cytochrome or the presence of two haems

(Bendall 1982). Hurt & Hauska (1982) demonstrated that two haems were associated with cytochrome b_{563}, both showing one-electron transitions with redox midpoint potentials of ≈-40 and -172 at *p*H 6·5. Both E_m's were *p*H dependent

The kinetic studies of Hind & Olsen (1966) and Knaff & Arnon (1969a) suggest that cytochrome b_{563} was associated with cyclic flow around photosystem 1. Cramer & Whitmarsh (1977) discussed cytochrome b_{563} in this situation, but also presented evidence indicating photosystem 2 mediated turnover. Velthuys (1979) suggested that cytochrome b_{563} may be involved in a *Q-cycle*, but this scheme was modified to include cytochrome b_{563} as a plastoquinone dismutase.

Cytochrome b_{559} (α-band at 559nm) consists of two separate components with E_m's of +370mv and +20mv (Rich & Bendall 1980). It has been suggested that the low potential form (cytochrome b_{559LP}) is a modification of the higher potential species (cytochrome b_{559HP}) arising as a result of damage or a physiological variant (Butler 1978). However, in carefully prepared chloroplasts both redox forms can be detected. Some modified forms of cytochrome b_{559HP} have been detected in the redox range +55~+100mv, which may represent the breakdown products of the higher potential species (Rich and Bendall 1980 and Bendall 1982).

Digitonin fractionation of chloroplasts revealed that cytochrome b_{559HP} was associated with the photosystem 2 complex whilst the low potential form fractionated with cytochrome *f*, either following photosystem 1 (Boardman & Anderson 1967) or being located with the *b/f* particle (Anderson & Boardman 1973 and Wood & Bendall 1976). Cytochrome b_{559LP} was suggested to be a detergent breakdown product of cytochrome b_{563}, but this was thought unlikely since the proportions of b_{563}:*f*:b_{559LP} were the same as those obtained during the mechanical fractionation proceedure (Anderson 1982a).

As yet, no direct function for the low potential species has been shown, but Bendall (1982) discussed the possibility of an involvement with cyclic electron transfer around photosystem 1 (see Bouges-Bocquet 1980a). Cytochrome b_{559HP} is associated with photosystem 2 (Cox & Anderson 1981 and Anderson 1982a), but there is still no known function. Whitmarsh & Cramer

1977) show that photo-reduction is too slow to be a component of linear flow, but the cytochrome can be rapidly oxidised by P_{680} if oxygen evolution is impaired. There is evidence that cytochrome b_{559HP} is not required for water splitting, for example, following trypsin treatment (Cox & Bendall 1974) or in leaves that have been greened with a flashing regime; high rates of oxygen evolution were observed with only low amounts of this cytochrome (Horton *et al* 1978). However, mutants lacking cytochrome b_{559HP} are incapable of water oxidation (Bendall 1982).

At 77K very rapid photo-oxidation of cytochrome b_{559HP} is seen with a half time of 4·6ms (Floyd *et al* 1971). It is possible that this cytochrome would behave as a safety valve, preventing photosystem 2 oxidation in the event of inhibition or loss of water splitting (Bendall 1982).

Since the cytochrome *b/f* complex receives electrons from the plastoquinone pool and donates them to photosystem 1 during linear or non-cyclic electron transfer, measurement of the redox state of cytochrome *f* provides an accurate means of estimating the relative rates of oxidation and reduction. This technique will allow the efficiency of DCMU inhibition to be calculated. The first few experiments of this thesis establish a known kinetic heterogenity of the cytochrome *f* population, as shown by Horton & Cramer (1974) and also provide evidence of an additional sub-population. Can a heterogenity at this level of complexity within the thylakoid membrane be related to herbicidal effects on photosynthesis ?

METHODS

Spinach chloroplasts were isolated, prepared and stored as described in the preceeding Chapter. Immediately prior to use, a chloroplast sample equivalent to 80μg/ml of chlorophyll (see Horton & Cramer 1974) was osmotically lysed for ~45 seconds and then diluted in reaction medium (*p*H 7·6) of the following final concentrations: 0·33M sorbitol; 50mM Hepes; 5mM $MgCl_2$ and 2mM EDTA.

All experiments were performed in the absence of room light with a Bristol spectrophotometer in the dual beam mode, the beams being chopped at

200Hz and detected through a Corning CS 4-96 filter with an EMI 9558a photomultiplier tube. The instrument was equipped with actinic side illumination, which provided either red (650nm) or far-red (707nm) light of 9 and 2 $w.m^{-2}$ respectively. These emissions were defined by Balzers interference filters (B40 response, 8 and 10nm bandpass). The sample was stirred and maintained at 20°C.

The thylakoids were uncoupled with 2μM gramicidin D, and methyl viologen (100μM) added as an electron acceptor. Primarily, cytochrome *f* was studied, although extinction changes due to cytochrome b_{559} were also observed. Initially, absorbance changes could not be attributed to cytochrome b_{563} (b_6) or any other cytochrome, although during the fractionation experiments (where chloroplasts were isolated more carefully, yielding a higher percentage of intactness) extinction changes centred around 563nm were observed. Cytochrome absorbance measurements were not complicated by C_{550} (Melis & Schreiber 1979) or P_{518} (Witt 1975) contributions.

Additions to the sample were made through a port in the top of the cuvette chamber and mixing times were found to be <1 second, approaching the time resolution of the amplifier (250ms). In the experiment discussed below potassium ferricyanide (ferricyanide) was added to a dark adapted chloroplast sample.

RESULTS AND DISCUSSION

From figure 3·1 it can be seen that addition of 0·5mM ferricyanide caused a decrease in extinction at 554-540nm of ~0·0050D. The absorbance change was biphasic, in agreement with the data of Horton and Cramer (1974). The fast phase was slightly larger than the slow rise, the latter having a half time of one to two minutes. The kinetics of the fast phase however could not be time resolved with our detection system due to the length of the mixing time.

These phases could represent two separate components, both observable at 554nm, or a single species showing a heterogeneous response to the oxidant. The biphasic oxidation was further studied by adding

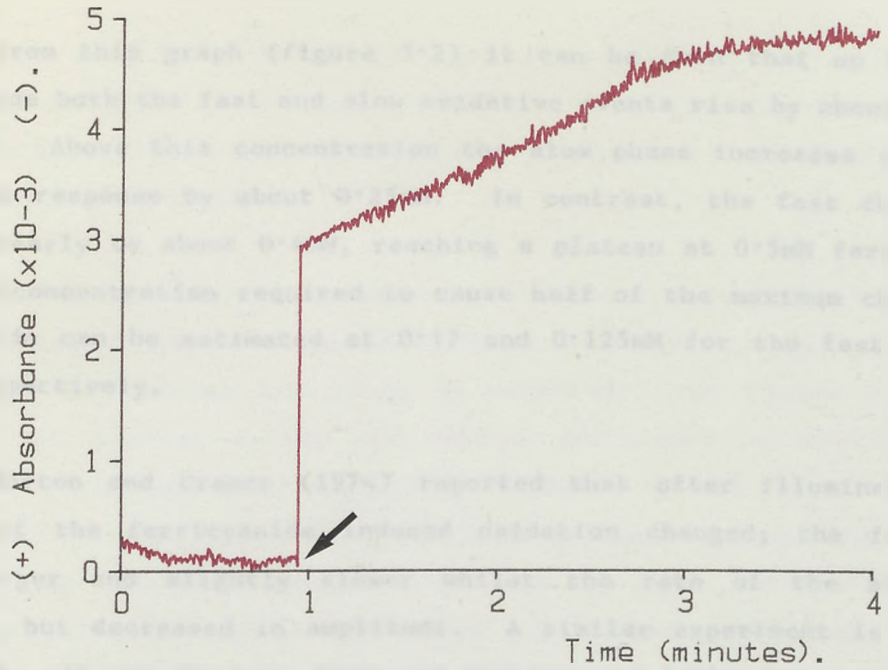


Figure 3·1: Absorbance changes (554-540nm) upon addition of 0·5mM Fecy (➘) to stirred, dark adapted spinach thylakoids at a chlorophyll concentration of 80μg/ml. Other details in methods section.

different amounts of ferricyanide, thus allowing plots of response against concentration to be drawn:

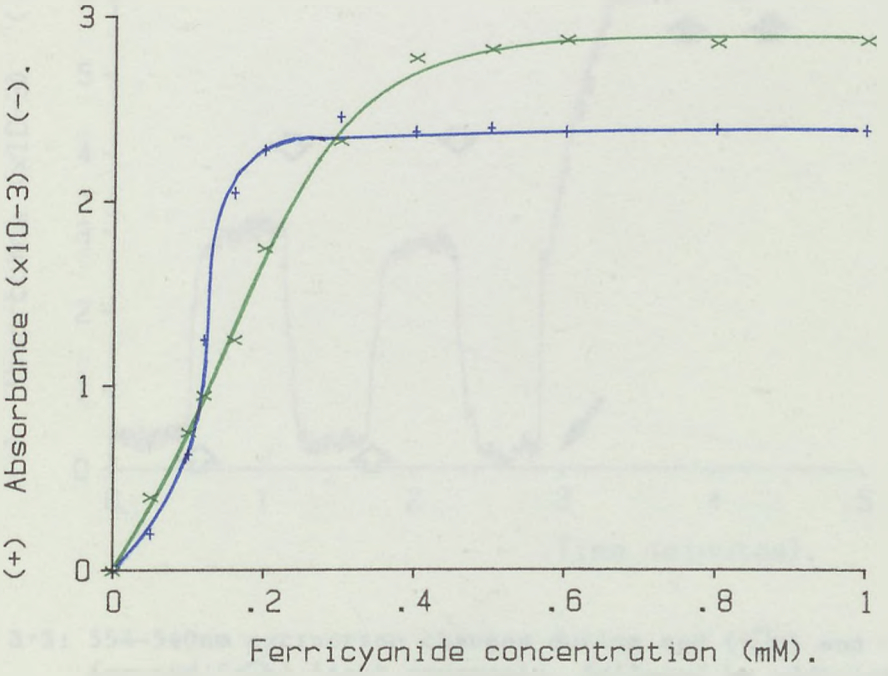


Figure 3·2: Plots of fast (——) and slow (——) changes for different Fecy concentrations. Each point is a mean of five separate experiments, details as above.

From this graph (figure 3·2) it can be seen that up to 0·1 mM ferricyanide both the fast and slow oxidative events rise by about the same amplitude. Above this concentration the slow phase increases sharply to reach peak response by about 0·25mM. In contrast, the fast change rose almost linearly to about 0·4mM, reaching a plateau at 0·5mM ferricyanide. The $S_{0.5}$ (concentration required to cause half of the maximum change) for ferricyanide can be estimated at 0·17 and 0·125mM for the fast and slow events respectively.

Horton and Cramer (1974) reported that after illumination, the kinetics of the ferricyanide induced oxidation changed; the fast phase became larger and slightly slower whilst the rate of the slow phase increased, but decreased in amplitude. A similar experiment is shown in figure 3·3. It can be seen that red and far-red light have antagonistic effects on the 554-540 absorbance. Far red light (like a ferricyanide addition) decreased the absorbance by about 0·00030D. Red light however reversed this transition, elevating the absorbance. It can also be seen that the red/far-red reversals were essentially fast monophasic changes.

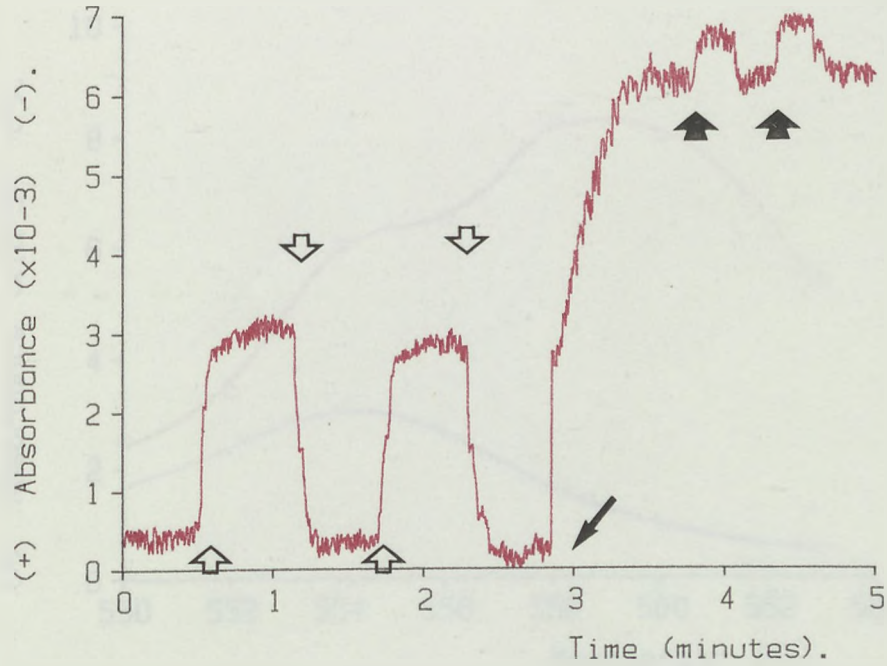


Figure 3·3: 554-540nm extinction changes during red (⇩) and far-red (⇧) light reversals, followed by addition of Fecy (➘) and further far-red/dark cycles (⇧). Red cycles gave little deflection after Fecy. See text and figure 3·1 for further details.

Upon addition of 0·5mM ferricyanide, a biphasic rise was seen, and as reported by Horton & Cramer (1974) the half time of the slow phase decreased, after two or three red/far-red reversals the half time was reduced to about 30 seconds.

The most interesting observation however was that even after oxidation with 0·5mM ferricyanide, there was still a photo-oxidisable component. This change was small in amplitude, but always detectable. After several far-red cycles the change diminished in amplitude, but returned after a short dark interval. Exposure to red light following a ferricyanide addition often resulted in an antagonistic extinction change of similar (or slightly larger) amplitude to that for far-red light, causing partial reduction of cytochrome *f*. However, red light was also seen to oxidise cytochrome *f* under identical conditions.

The wavelength dependence of these absorbance changes was also studied, the reference being maintained at 540nm. These experiments were performed according to figure 3·3 and the results (means of more than ten

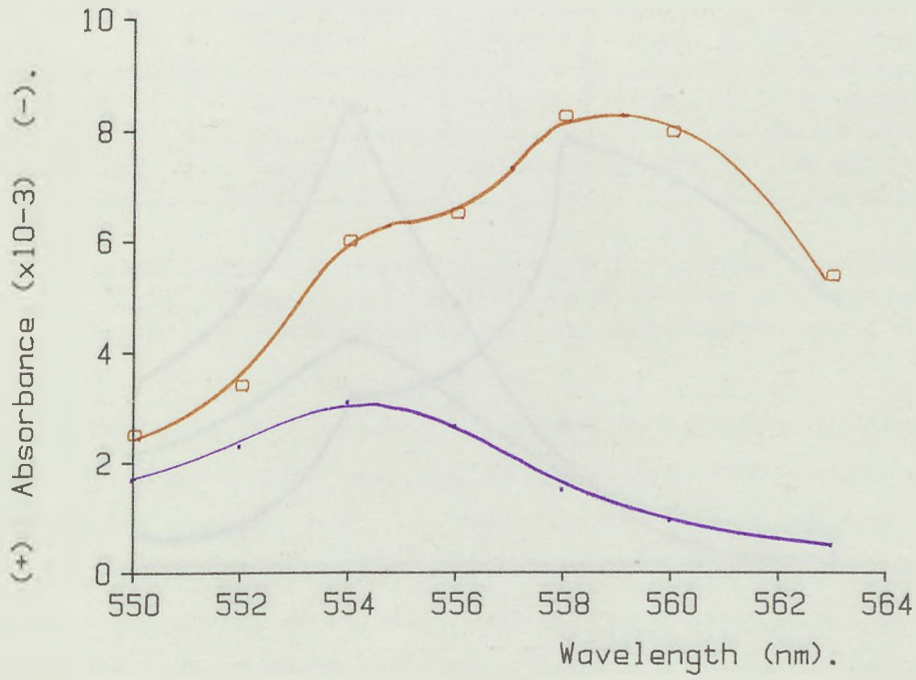


Figure 3·4: Plots of red/far-red reversals (——) and total Fecy oxidation (——) verses detection wavelength (540nm as the reference). Means of ten experiments/point, performed as shown in figure 3·3.

measurements at each point) are presented above in figure 3·4. It can be concluded that the red/far-red reversals caused changes in the redox state of cytochrome *f*, as evidenced by its difference spectrum (Hill & Scarisbrick 1951).

The spectrum of the ferricyanide-induced absorbance change (figure 3·4) has a peak around 559nm, probably due to cytochrome b_{559} and a shoulder at 554nm, indicating that cytochrome *f* is also oxidised by ferricyanide. Cytochrome b_{559} is not effected by light at room temperature (agreeing with the red/far-red reversals figure of 3·4), but is readily oxidised by aqueous oxident.

From figure 3·5 it can be seen that the slow oxidative change has a spectrum that is very similar to the red/far-red changes of figure 3·4, suggesting that this phase is due to cytochrome *f* oxidation alone. The fast absorbance change is probably composed of cytochromes *f* and b_{559} as the plot is similar to that for the total oxidative change, having a peak (~559nm) and a blue-shifted shoulder. Although very small in size, the

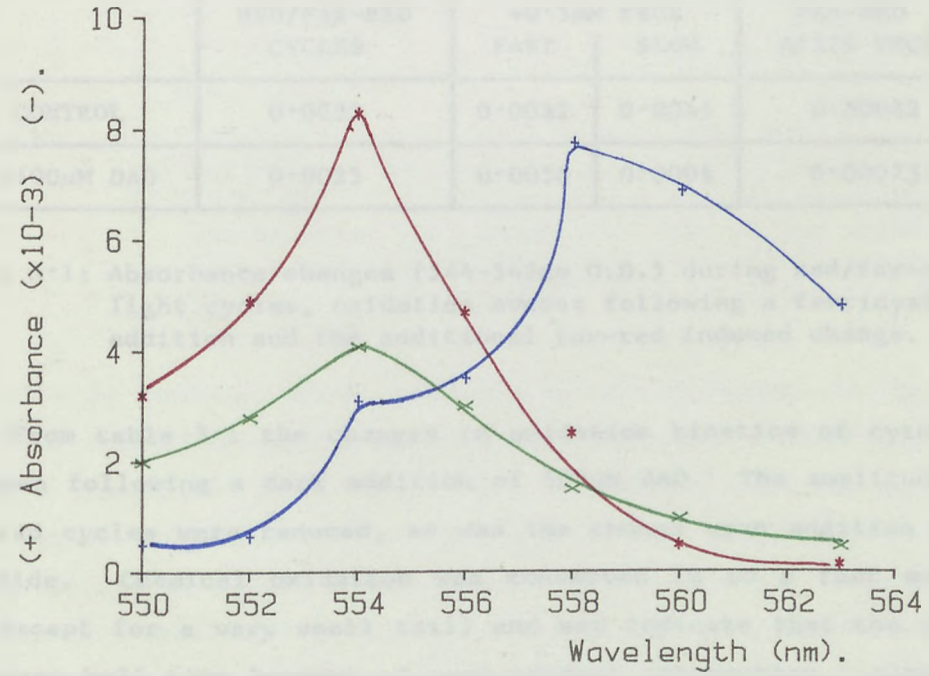


Figure 3·5: Plots of far-red (——) following fast (——) and slow (——) oxidation by ferricyanide for the data of figure 3·3. **Note far-red extinction x10.**

additional far-red induced oxidation after a ferricyanide addition also has

the spectral characteristics of cytochrome *f* (see figure 3·5). During exposure to far-red light, ferricyanide addition resulted in the same total amplitude of oxidation as above, but the spectrum was centred arround 559nm without a shoulder at 554nm.

It may be concluded that there are three sub-populations of cytochrome *f*, one reacting quickly with ferricyanide, one with slower kinetics and a partialy (or perhaps totally) unoxidised component which is photo-oxidisible.

Such a heterogeneity could be related to structural variations of individual cytochrome components, or to the ionic environments of the different bilayer regions. Oxidation events may relate to the accessibilty of the cytochrome (or associated polypeptides etc.) to the aqueous phase. The accessibility of cytochrome *f* to ferricyanide was tested by using the lipophilic electron carrier diamino durene (DAD) and by unstacking the lamellae.

	RED/FAR-RED CYCLES	+0·5mM FECY		FAR-RED AFTER FECY
		FAST	SLOW	
CONTROL	0·0032	0·0032	0·0041	0·00082
+500μM DAD	0·0025	0·0058	0·0006	0·00073

Table 3·1: Absorbance changes (544-540nm O.D.) during red/far-red light cycles, oxidation events following a ferricyanide addition and the additional far-red induced change.

From table 3·1 the changes in oxidation kinetics of cytochrome *f* can be seen following a dark addition of 500μM DAD. The amplitude of the red/far-red cycles were reduced, as was the change upon addition of 0·5mM ferricyanide. Chemical oxidation was converted in to a fast monophasic change (except for a very small tail) and may indicate that the slow rise has a longer half time because of poor oxidant interaction. Although DAD may not comunicate directly with cytochrome *f*, oxidation of the plastoquinnone pool will tend to favour cytochrome oxidation as a result of redox equilibration.

The final observation however was that the additional far-red induced oxidation after ferricyanide treatment was conserved in the presence of DAD. This suggests an explanation other than its inaccessibility to ferricyanide to account for its persistence.

To probe the relationship between the kinetics of oxidation and thylakoid organisation high valency cations were depleted from the surface layer of the membrane. To achieve this, the thylakoids were centrifuged and the pellet resuspended in a reaction medium containing 5mM KCl as a replacement for $MgCl_2$. This removed divalent cations from their screening sites on the membrane surface in exchange for K^+, preventing grana stabalisation (Barber & Chow 1979, Barber 1980a and b). The decrease in 685:730nm fluorescence ratio at 77K of 57% (see table 3·2) indicated that either *spillover* had occurred or α (according to Butler 1978) had decreased

	$F_{685/730}$	TOTAL	FAST	SLOW	Fon	F%t
-Mg	0·35	18·3	—	—	0·51	2·7
+Mg	0·82	24·6	19·0	5·6	1·62	6·5

Table 3·2: Relative changes in absorbance (554-540nm) upon addition of 5mM Fecy in the presence and absence of 10mM $MgCl_2$. F_{on} representing far-red oxidative change after Fecy oxidation. $F\%_t$ is F_{on} expressed as a percentage of the total Fecy induced change. $F_{685/730}$ is the 685:730nm fluorescence ratio at 77K.

(as reported by Murata 1969a), probably as a consequence of unstacking. After cation depletion the total absorbance decrease (554-540nm) appeared as a single exponential phase which was ~25% smaller than the control upon on ferricyanide oxidation. The amplitude of the extra far-red response was also diminished, but to a much greater extent (~60%). The reason for the decreased response to ferricyanide could be due to several factors: (1) a change in surface properties of the thylakoid as a whole, (2) a change in properties in the cytochrome *f* pool in general, (3) a change in the oxidation kinetics as a result of destacking and re-arrangement of the supra-molecular protein complexes.

Peas were also grown (and chloroplasts isolated according to Chapter two), and treated as described above. The kinetics of cytochrome *f* oxidation were examined by the manner depicted in figure 3·3. The absorbance changes of dark adapted pea chloroplasts were almost identical

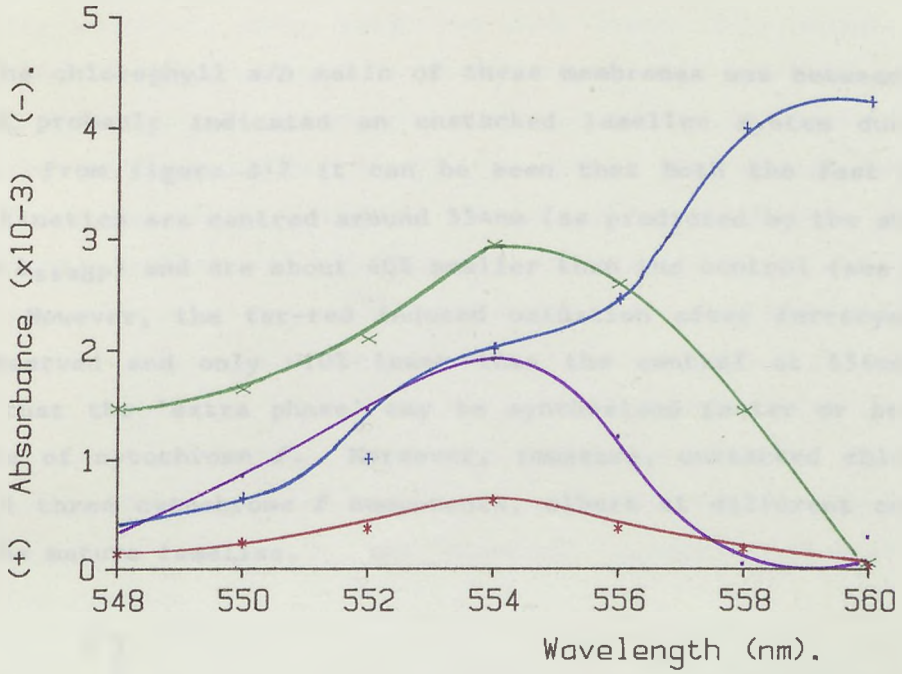


Figure 3·6: Plots of fast (——) and slow (——) extinction changes upon addition of 0·5mM Fecy following red/far-red cycles (——). Far-red change after Fecy (——). Means of ten experiments/point, as in figure 3·3, but using thylakoids from peas at a chlorophyll concentration of 80µg/ml.

to the data presented in figures 3·4 and 3·5, suggesting that the same relationships of cytochrome *f* and b_{559} exists. Furthermore, the extra far-red oxidation was preserved, providing evidence in support of a very heterogenious cytochrome *f* population.

By utilising the rapid development of plants like the pea it is possible to grow etiolated shoots and then expose them to an intermittent light (IML) regime (Argyroudi-Akoyunoglou & Akoyunoglou 1970 and Armond *et al* 1976). In the absence of light, no chlorophyll is synthesised, but the flashing regime promotes chlorophyll *a* accumulation. After continued illumination, chlorophyll *b* synthesis occurs. These biochemical changes are also accompanied with other physiological adaptations. In the absence of chlorophyll *b*, LHC formation is prevented and the resultant chloroplasts

are largely agranal (Armond *et al* 1976). Cytochrome b_{559HP} is also absent in such chloroplasts (Horton *et al* 1978). In spite of the immature appearance of these lamellae, both photochemistry and electron transfer are fully competent. The oxidation kinetics of cytochrome *f* were studied in agranal thylakoids by the proceedures described above.

The chlorophyll *a/b* ratio of these membranes was between six and eight, and probably indicated an unstacked lameller system due to LHC depletion. From figure 3·7 it can be seen that both the fast and slow oxidation kinetics are centred around 554nm (as predicted by the absence of cytochrome b_{559HP}) and are about 40% smaller than the control (see previous figures). However, the far-red induced oxidation after ferricyanide was highly conserved and only ~10% lower than the control at 554nm. This suggested that the 'extra phase' may be synthesised faster or before the other pools of cytochrome *f*. Moreover, immature, unstacked chloroplasts contain all three cytochrome *f* components, albeit at different concentrations to the mature lamellae.

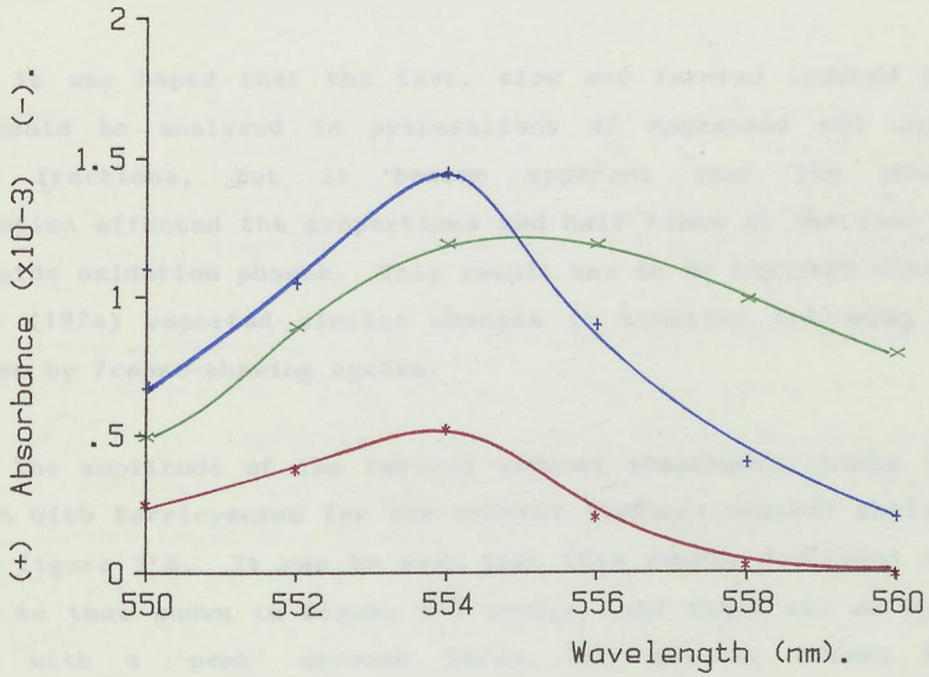


Figure 3·7: Data obtained from IML pea thylakoids at 10µg/ml. Fast (——) and slow (——) changes upon addition of Fecy. Far-red change following oxidation with Fecy (——). Details in text and as for figure 3·6.

Thylakoid fractionation techniques were also used, according to the method of (Andersson *et al* 1975 and Andersson & Anderson 1980) but simplified as detailed in Chapter two. After a pilot study, a larger volume Yeda press was desigined and built by the Engineering workshop of Sheffield University Biochemistry Department. The valve mechanism was substantially modified, which permitted much higher flow rates. It was found that the suspension could be passed through the valve at velocities approaching 200ml/minute without affecting the purity of separation. The speed adopted was ~25ml/minute as this proved to be the most convenient. Following fractionation, differential centrifugation (30 minutes at 10,000g for the 'granal' pellet and 144,000g for the stromal thylakoid vesicles) resulted in pellets with mean chlorophyll *a/b* ratios of 2·08 and 6·84 for the fast and slow sedimenting particles (compared to ~2·76 for chloroplasts), suggesting that they originated from stromal and granal membrane regions respectively (Andersson & Anderson 1980). It was also observed that prior to separation, the fractionation process often changed the chlorophyll *a/b* ratio of the membrane fragments compared to the original chloroplasts.

It was hoped that the fast, slow and far-red induced oxidative phases could be analysed in preparations of appressed and unappressed membrane fractions, but it became apparent that the process of fractionation affected the proportions and half times of the fast and slow ferricyanide oxidation phases. This result was to be expected since Horton & Cramer (1974) reported similar changes in kinetics following membrane disruption by freeze-thawing cycles.

The amplitude of the far-red induced absorbance change following oxidation with ferricyanide for the control (unfractionated) thylakoids is shown in figure 3·8. It can be seen that this sample displayed a similar spectrum to that shown in figure 3·5 except that there was an absorbance increase with a 'peak' arround 562nm. The precise reason for this discrepancy is unknown, but it may relate to the adoption of a more gentle chloroplast isolation proceedure in order to obtain a higher yield of intact chloroplasts. It was believed that the 562nm signal may represent a photosystem 1 mediated turnover of cytochrome b_{563} (which resulted in the skewed appearence of the cytochrome *f* spectrum) possibly due to retention of some of the ferredoxin that would otherwise be lost for less intact

chloroplasts.

After fractionation, the absorbance spectrum of the far-red induced change still showed the 'peak' at 562nm but the signal at 554nm is

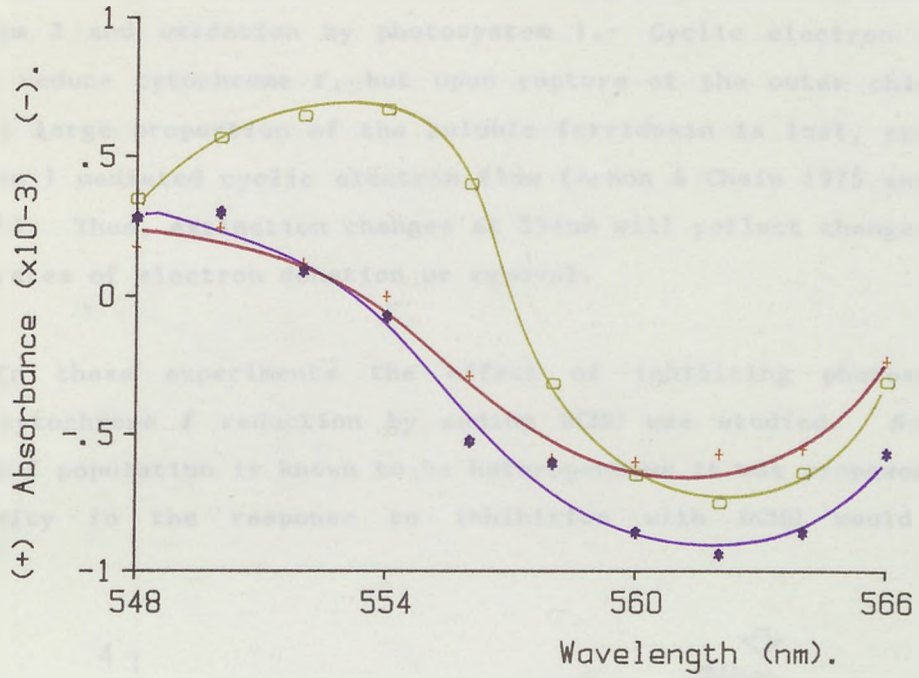


Figure 3·8: Oxidation spectra upon exposure to far-red light following addition of 0·2mM Fecy. Control (——), fractionated (but unseperated) thylakoids (——) and grana (——). Each point is a mean of five experiments. Details as for previous figures.

greatly reduced, indicating a loss of the additional pool of cytochrome *f*. The fractionation process alone may allow this species greater access to aqueous oxidants more readily. The spectrum of the granal preparation also shows these characteristics, suggesting that the pool may be located in the remaining stromal lamellae. However, this data could also interpreted as a localisation of the additional cytochrome *f* pool within the grana, since removal of the link with photosystem 1 would prevent far-red induced oxidation. Thus the apparent loss of the far-red induced change in the unseparated samples can easily be accounted for. This reasoning also rules out photosystem 1 mediated turnover of cytochrome b_{563}, which must be attributed to photosystem 2 induced electron flow, possibly as part of an electrogenic step within the cytochrome *b/f* complex.

THE EFFECTS OF DCMU ON THE REDOX STATE OF CYTOCHROME *f*

As cytochrome *f* is located centrally between the two photosystems, its redox state is determined by the relative rates of reduction by photosystem 2 and oxidation by photosystem 1. Cyclic electron transfer will also reduce cytochrome *f*, but upon rupture of the outer chloroplast envelope a large proportion of the soluble ferridoxin is lost, preventing photosystem 1 mediated cyclic electron flow (Arnon & Chain 1975 and Shahak *et al* 1981). Thus, extinction changes at 554nm will reflect changes in the relative rates of electron donation or removal.

In these experiments the effect of inhibiting photosystem 2 mediated cytochrome *f* reduction by adding DCMU was studied. Since the cytochrome *f* population is known to be heterogeneous it was proposed that a heterogeneity in the response to inhibition with DCMU would become apparent.

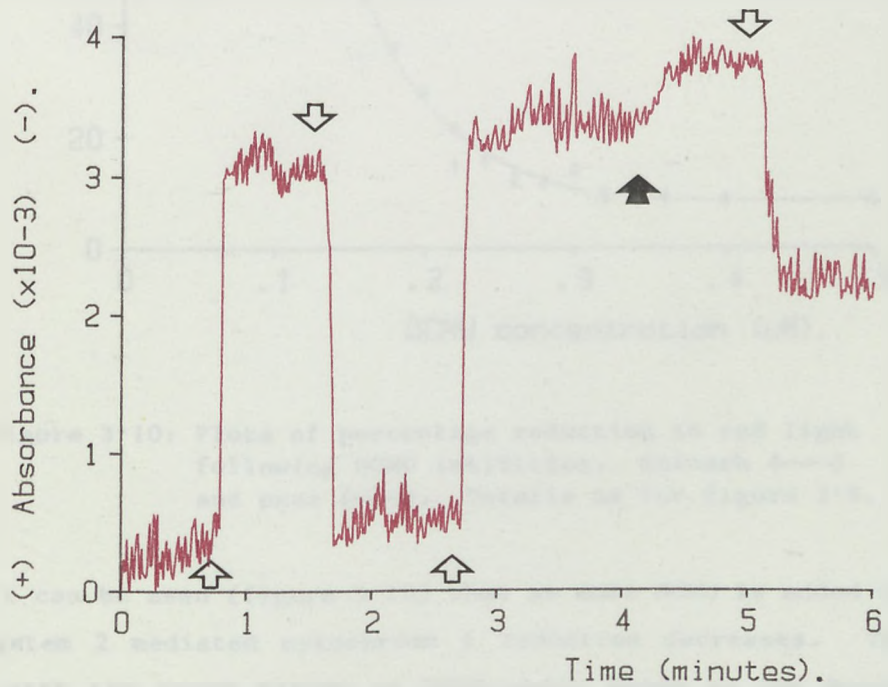


Figure 3·9: Changes in 554-540nm absorbance during red (⇩), far -red (⇧) reversals and addition of 0·16μM DCMU (▲).

Under constant photosystem 1 mediated oxidation (in the presence of methyl viologen), DCMU was added in far-red light after several

red/far-red reversals. Once the signal had stabilised red light was added.

From figure 3·9 it can be seen that addition of DCMU in far-red light actually resulted in a small oxidation of cytochrome *f* (methanol additions did not result in any extinction changes), which was probably due to a slight absorbance of 707nm light by the photosystem 2 antennae. Upon exposure to red light cytochrome *f* underwent partial reduction. The size of this change was expressed as a percentage of the red/far-red reversals prior to DCMU addition. This data is shown in figure 3·10 for various DCMU concentrations:

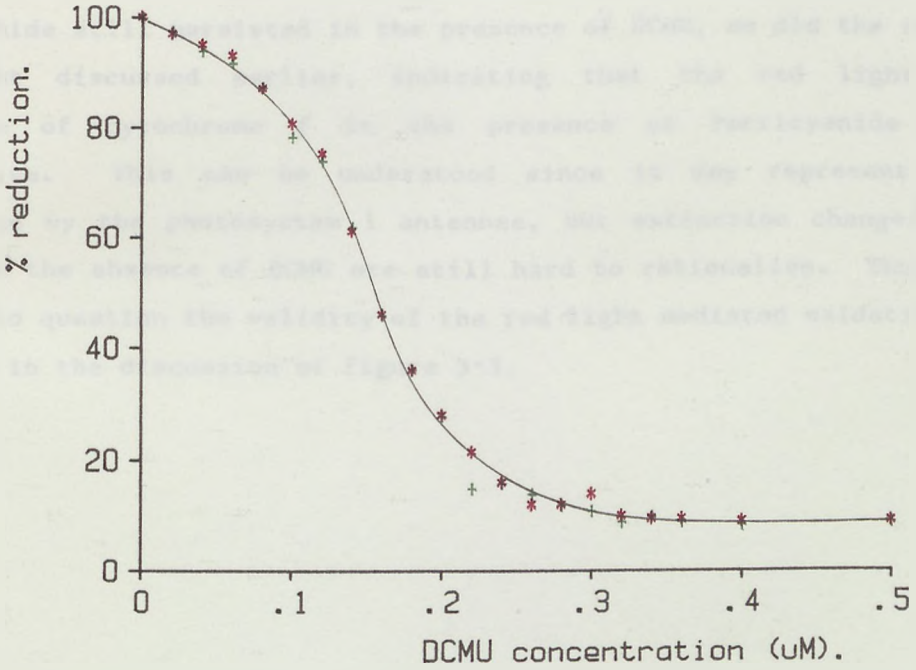


Figure 3·10: Plots of percentage reduction in red light following DCMU inhibition. Spinach (——) and peas (——). Details as for figure 3·9.

It can be seen (figure 3·10) that as more DCMU is added the extent of photosystem 2 mediated cytochrome *f* reduction decreases. This is in agreement with the known action of DCMU which binds to the B-protein and prevents plastoquinone reduction (Bouges-Bocquet 1973 and Velthuys & Amesz 1974). From this figure an I_{50} for DCMU inhibition was calculated of 0·125μM. Only one DCMU inhibitory event was observed as no further inhibition could be seen even in the presence of 10μM DCMU (approximatly 10% of the cytochrome *f* population remained photo-reducible with red

light). This may be expected since photo-oxidation of cytochrome *f* appeared monophasic (see figures 3·3 and 3·9), even though chemical oxidation with ferricyanide displayed biphasic kinetics (figures 3·1 and 3·3). Thus the observed heterogenity of the cytochrome *f* population was not reflected by a heterogeneous response to DCMU binding, although the photo-reducable component remaining at a DCMU concentration of 10μM may relate to a seperate pool of cytochrome *f*. Alternatively, this observation may now relate to the DCMU insensitive electron transfering pathway described by Joliot & Joliot (1981b and 1983)

The far-red induced absorbance following oxidation with ferricyanide still persisted in the presence of DCMU, as did the respone to red light discussed earlier, indicating that the red light induced reduction of cytochrome *f* in the presence of ferricyanide is DCMU insensitive. This can be understood since it may represent a small absorbance by the photosystem 1 antennae, but extinction changes of this nature in the absence of DCMU are still hard to rationalise. Thus, one is tempted to question the validity of the red light mediated oxidative change reported in the discussion of figure 3·3.

CHAPTER FOUR

AN INTRODUCTION TO MEASUREMENTS OF CHLOROPHYLL *a* FLUORESCENCE AS AN ASSAY OF HERBICIDE BINDING

Fluorescence is the emission of a light quantum (a photon) upon de-excitation of an electron from one electronic level to another. Usually, fluorescence is associated with the downward transition from the first singlet excited state to the ground state.

Providing the electronic and magnetic vectors within an atom or molecule are in almost perfect alignment with those of an incoming photon, the time taken to absorb quanta is about 10^{-15} seconds. The resultant excited state remains stable for only 10^{-9} seconds (called the excited state lifetime) and de-excitation occurs at a similar speed to the absorption act. Within this era there is sufficient time for migration of the excited electron through the various vibrational and rotational substates of the molecule towards a lower electronic level (see fig 4·1). This is termed internal conversion or radiationless de-excitation and is associated with a heating of the system, not wave propagation.

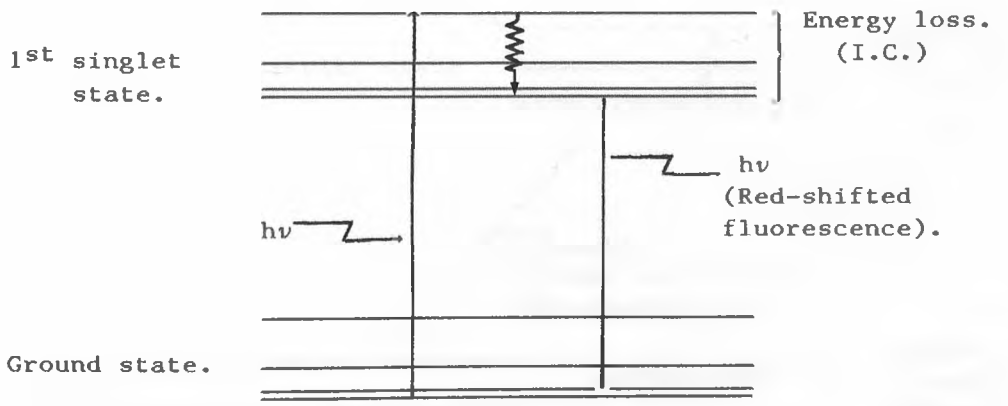


Figure 4·1: An atomic representation of electronic absorption and deactivating events accompanying changes in energy state.

Figure 4·1 shows that the photon emitted upon electronic de-excitation is less energetic than the one first absorbed. This gives rise to *Stokes shift*, whereby fluorescence is red shifted with respect to absorption. The extent of shifting is dependant both on the dipole and the immediate electronic environment.

If another de-activating process competes for the exciton during the excited state lifetime, fluorescence will not occur due to energy dissipation elsewhere (as in the drop from higher excited states by non radiative decay); for example, in the formation of the first triplet excited state (see figure 4·2). This may take about 10^{-12} seconds, and although significantly longer than the fluorescence lifetime, a singlet to triplet conversion is only favoured in certain circumstances (for example, during the excitation of oxygen). The transition is slow because the excited electron undergoes spin reversal, giving two electrons with unpaired spin. When a triplet electron falls back to ground, a second spin reversal must occur to regenerate the original spin pair, fulfilling the rules of the Pauli Exclusion Principle whereby two electrons occupying the same orbital cannot have identical spins. Emissions associated with triplet state decay are termed phosphorescence because they occur very late after the absorption event and are extremely red-shifted.

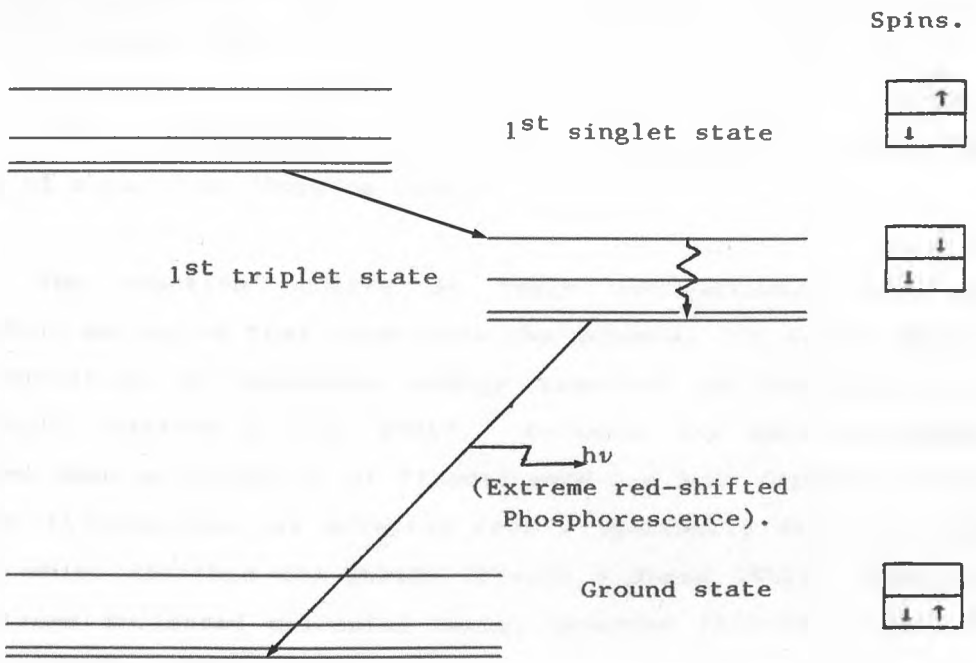


Figure 4·2: A diagrammatic representation of electronic spin configurations during changes in energy state.

A further route for excitation decay is by transfer to another molecule, referred to as a quencher or acceptor: as the exciton falls back to the ground state, the emitted energy is instantaneously re-absorbed by the quencher. This process is so efficient that the energy is essentially

transferred from one excited state to another by resonance transfer, but is only favoured if the electronic and magnetic vectors are perfectly aligned and ≤7nm apart. At 2·5nm spacing, resonance transfer may be almost 100% efficient (for example in a chlorophyll antennae).

In the higher plant thylakoids, chlorophyll is the molecule associated with light capture, of which there are two basic types; chlorophyll *a* and chlorophyll *b*. The absorption spectrum of chlorophyll is typified by strong extinction in both the blue and red regions. Absorbance of a blue photon promotes an electron to the second singlet excited state (equivalent to about 50Kcal/mole of quanta). This decays to the first excited state, which is populated by red photons of about 40Kcal/ mole.

The position of the absorbance bands of chlorophyll *a* and *b* ultimately result in energy transfer to the first singlet excited state of chlorophyll *a*, and it is from this species that photochemistry occurs. The transfer of energy from one chlorophyll to the next is believed to occur by inductive resonance or resonance transfer (Duysens 1964) as described by Förster (1948). Measurement of the light harvesting chlorophylls reveals a spacing of about 2 nm (Duysens 1964).

The reaction centres or traps are situated in 'lake' of chlorophyll molecules that constitute the antenna. It is the latter which pass excitation by resonance energy transfer to the reaction centre chlorophyll (Gaffron & Whol 1936). Evidence for this arrangement was furnished when polarisation of fluorescence was lost (Arnold & Meck 1956) and when fluorescence was detected from a spectrally distinct pigment to the one which absorbed the photon (French & Young 1952). Both of these observations indicated molecular energy transfer (Arnold & Meck 1956 and Wong & Govindjee 1981).

Once in the reaction centre, the exciton is trapped during charge separation by donating the excited electron to the primary acceptor, oxidising the donor and reducing the recipient. The primary donor of photosystem 2 is P_{680} and the acceptor is Q, the quencher of chlorophyll fluorescence (Duysens & Sweers 1963). This a specialized plastoquinone molecule (often referred to as Q_A), complexed with iron (Klimov *et al* 1980). Following the establishment of a photochemical dipole (see equation

1·3), further electron transfers occur that reduce P_{680}^{+} and oxidise Q^{-}, regenerating the original couple. Studies with photosystem 2 preparations demonstrate that there is an intermediate 'metastable' acceptor between P_{680} and Q (Klimov *et al* 1977 and 1980). This is a pheophytin molecule which upon excitation by P_{680} develops the characteristics of a pheophytin anion radical. See equations 4·4 and 4·5.

Once reduced, Q^{-} is unable to accept further electrons from P_{680} as the trap is said to be *closed*. Subsequent excitons approaching a closed trap must de-excit by another route. Thermal decay (radiationless de-excitation, k_h), although possible has a low probability except under certain conditions of high ΔpH (Briantais *et al* 1979, Krause *et al* 1982 and 1983). Transfer of energy out of the closed centre to another open centre is believed to occur (Joliot & Joliot 1964), as shown by the sigmoidicity of the fluorescence induction curve (see below). Finally, the excited electron can fall back to the ground state by emitting a photon. Thus, deactivation of the first singlet excited state of *in vivo* chlorophyll can be expressed as the sum of the competing terms as follows:

$$k_d = k_h + k_t + k_f + k_p.[Q] \qquad (4·1).$$

Where: k_d = rate constant for deactivation.
k_h = ————————‖———————— thermal decay.
k_t = ————————‖———————— energy transfer.
k_f = ————————‖———————— fluorescence.
k_p = ————————‖———————— photochemistry.
$[Q]$ = concentration of quencher (1→0).

Where excitation results in formation of Q^{-} and closes the trap, the product term for photochemistry becomes zero. To maintain the rate of de-activation, the remaining components of equation 4·1 must increase. The observer can easily verify this statement as the fluorescence emission increases upon trap closure (Duysens & Sweers 1963), but the yield of fluorescence is not directly related to the number of closed centres. The intensity of fluorescence can be expressed as a function of the incident light absorbed and is termed fluorescence yield (ΦF):

$$\Phi F = \frac{I_f}{I_o} \qquad (4\cdot2).$$

Where: I_f = intensity of fluorescence.
I_o = ———"——— absorbed light.

Measurement of the total extinction of a sample is problematic, but since absorption energy must populate the excited state, then measurement of the latter relates directly to the true extinction of the sample. This energy (absorption energy) can be represented by k_d in equation 4·1; whereby the rate of deactivation is proportional to the rate of population of the excited states. The yield of fluorescence can be represented thus:

$$\Phi F = \frac{k_f}{k_f + k_h + k_t + k_p.[Q]} \qquad (4\cdot3).$$

As stated previously, reduction of Q increases the fluorescence yield, providing an estimate of the number of closed centres. Thus some of the changes in fluorescence can now be rationalized: as the traps close [Q] approaches zero, and the fluorescence measured is said to represent the maximum fluorescence yield (F_{max} or F_m). Fully open centres produce the smallest yield of fluorescence because [Q] becomes unity, quenching fluorescence to its lowest level (F_o). In the intermediate situation, where $0 < [Q] < 1$, the fluorescence yield varies between F_o and F_m and is referred to as the variable yield of fluorescence (F_v).

Although fluorescence is generally considered to result from the release of energy due to trap closure upon Q reduction, it is probably fast luminescence due to charge recombination (Klimov *et al* 1977 and Klimov & Dolan 1980):

$$P_{680}\ \text{Pheo} \xrightarrow{h\nu} P_{680}^{*}\text{Pheo} \qquad (4\cdot4).$$

$$P_{680}{}^{*}Pheo \longrightarrow P_{680}{}^{+}Pheo^{-} \quad (4\cdot5).$$
(metastable)

$$P_{680}{}^{+}Pheo^{-} \longrightarrow P_{680}{}^{*}Pheo \quad (4\cdot6).$$

$$P_{680}{}^{*}Pheo \longrightarrow P_{680}\ Pheo + h\nu \quad (4\cdot7).$$
(luminescence)

Luminescence can not be directly related to the redox state of Q since it represents an interaction of P_{680} and pheophytin (pheo), but because the Q/pheophytin interaction is so rapid, the couple may essentially be regarded as a single redox component. Following charge separation, the dipole can only be considered stable once the electron is resident on the plastoquinone moiety. However the transfer of an electron from pheophytin is determined by the redox status of Q, if Q is reduced the the transfer is not permitted and charge recombination occurs regenerating the excited state of P_{680} (equation 4·6). Deactivation must now proceed by another route.

The fluorescence yield at F_o is often called dead fluorescence because it emanates from open traps. F_o could originate from detached chlorophyll (Clayton 1969), be partly due to a photosystem 1 emission (Lavorel & Joliot 1972), or to inefficient trapping processes within the LHCP antennae. Kitajima & Butler (1975) demonstrated however that F_o and F_v are the same type of fluorescence, both emanating from the bulk chlorophylls of photosystem 2. A closer examination of equation 4·3 (where the denominator sums to unity), reveals that even when k_h, k_t and k_p are maximum, there is still a finite probability of fluorescence, thus F_o can be considered as a statistical representation of the minimum number of excited states that decay by fluorescence emission, as a probability function of all of the competing pathways.

Exposure of DCMU poisoned, dark adapted chloroplasts to light allows a fluorescence induction curve to be recorded, showing the transients of figure 4·3. The F_v portion of the curve represents the progressive closure of the photosystem 2 traps. At F_o all of the traps are open, ie Q is fully oxidised and the fluorescence is quenched to its lowest

state, whilst at F_m, the quencher is totally reduced to Q^- and there is no

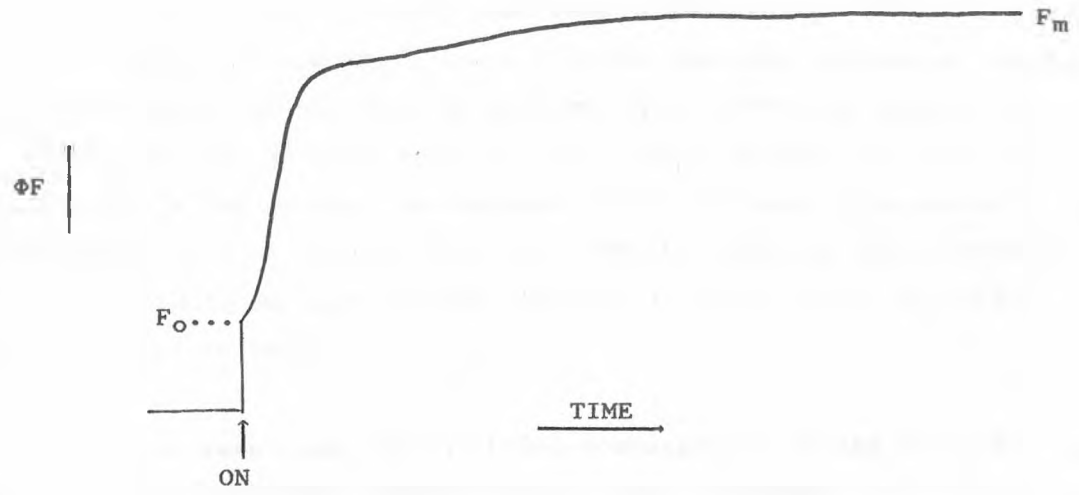


Figure 4·3: A fluorescence induction curve recorded after dark incubation in the presence of DCMU and an uncoupler.

quenching of fluorescence. Thus, propagation of the fluorescence rise represents photochemical closure of photosystem 2 upon reduction of Q to Q^-. The quenching that does occur at any point in time reflects the amount of oxidised quencher remaining (Duysens & Sweers 1963).

Integration of the area above the F_v portion of the induction curve provides an estimate of the size of the quencher. In the absence of DCMU, an induction curve approaches F_m very slowly producing a large area; indicating a large oxidised acceptor pool. Clearly, Q is re-oxidised by the plastoquinone pool, and only when the latter is significantly reduced does the fluorescence yield approach F_m.

The induction curve of figure 4·3 reveals two kinetic phases; a fast phase and a slower phase (Morin 1964 and Doschek & Kok 1972). Melis & Homman (1975) measured the areas above these two portions of the curve and showed that the later phase was exponential and had a slower rate constant than the non-first order fast phase. The complex nature of the fluorescence rise was related to a heterogeneous photosystem 2 population, the fast phase emanating from photosystem 2α and the tail phase from photosystem 2β. Butler (1980) indicated that the slower centres are

isolated photosystem 2 units, whilst the faster kinetics may simply be an expression of communicatory reaction centers.

Joliot & Joliot (1964) ascribed sigmoidicity of the α-phase to exciton transfer between photosystem 2 units, whereby excitation reaching a closed trap could be donated to another open centre by energy transfer. This quenched the fluorescence in the early stages of the induction (resulting in a lag in the fluorescence rise), followed by a rapid increase as the number of open centres declined, thereby reducing the probability of an exciton finding an open centre (Lavorel & Joliot 1972, Briantais *et al* 1973 and Paillotin 1976).

At the same time, Morin (1964) postulated a series of double light reactions with differing photochemical rate constants for Q reduction. This concept was re-stated by Doschek & Kok (1972) as two possible alternatives, one of which required Q to accept a pair of electrons, and another which considers two very fast interacting acceptors:

$$Q \xrightarrow{h\nu} Q^{-} \xrightarrow{h\nu} Q^{2-} \qquad (4\cdot8).$$

$$QR \xrightarrow{h\nu} Q^{-}R \xrightarrow{h\nu} Q^{-}R^{-} \qquad (4\cdot9).$$

Thus, trap closure would depend on the relative reduction rates, where by the second photo-reduction was only favoured after the first. Such co-operativity could generate non-first order kinetics as seen in the sigmoidal rise.

The observation of a redox heterogeneity of Q (Cramer & Butler 1969) is compatible with equation 4·9 as suggested by Horton (1981b), where the two quenchers Q_H ($E_m \approx 0v$) and Q_L ($E_m \approx -250mv$) are shown to exist within the same photosynthetic unit. It was suggested that exposure to light could result in a sequential reduction sequence where by sigmoidicity would be generated by the constraints of equation 4·9 above; one photo-act depending on, and being favoured by the other (equation 4·10).

$$Q_H Q_L \xrightarrow{h\nu / k_1} Q_H^- Q_L \xrightarrow{h\nu / k_2} Q_H^- Q_L^- \quad (4{\cdot}10).$$

Bowes and Horton (1982) demonstrated that photosystem 2 particles from *Phormidium laminosum* are deficient in Q_L, but still show a biphasic fluorescence induction curve with both sigmoidal and exponential components, proving that 'double hitting' of the photosystem 2 trap in accordance with equation 4·10 is not a prerequisite for sigmoidicity. Furthermore, the isolation procedure reduces the size of the photosynthetic unit, which is accompanied with a slight increase in the energy transfer constant between photosystem 2 units, suggesting that a closer photosystem packing could increase sigmoidicity.

Cation depletion of thylakoids (Briantais *et al* 1973, Bennoun 1974 and Melis & Homman 1978) yielded a fluorescence induction curve consisting of two exponential phases with unaltered rate constants. Thus, destacking grana preferentially affected the α-centres, implying that localisation in the partition is a prerequisite for interactivity. Beta-centres are considered to represent a sub-population of photosystem 2 that are independent structures from photosystem 2_α (Thielen & Van Gorkom 1981a) and reside in the unstacked lamellar regions (Melis & Thielen 1980) in isolation from the α-centres. The exponentiality associated with the fluorescence rise from β-centres suggests that they are entirely non-interacting. However, because Q_β is homogeneous when compared to Q_H and Q_L of Q_α (Horton 1981) it could be argued that linearity is to be expected without having to invoke zero interactivity.

Analysis of the antennae composition of the photosystems revealed that chlorophyll *b* is almost exclusively associated with photosystem 2_α (Thielen *et al* 1981). This may account for the faster rate constant for photochemistry compared with the β-centres, since the quantum efficiency of both systems is close to unity (Thielen & Van Gorkom 1981a), a larger antenna would account for the faster rate of reaction. The interactivity, and therefore the sigmoidal induction kinetics of the α-centres is also believed to be mediated via the chlorophyll *a/b* light harvesting complex (Armond *et al* 1976, Butler & Strasser 1977, Strasser & Butler 1977 and Butler 1980) -supporting Joliot & Joliot's view that sigmoidicity is in

fact due to the energy transfer between photosystem 2 centres.

In experiments where herbicides such as DCMU are present, the fluorescence yield changes little once F_m is reached unless additional processes occur, for example photo-inhibition or photo-destruction. In the absence of DCMU, the kinetics of the rise reflect different events and proceed beyond F_m whereupon fluorescence quenching is seen, often followed by further secondary kinetics until a terminal 'steady-state' develops Walker *et al* (1983). Since emission of fluorescence is largely due to trap closure (Q reduction), then the steady-state fluorescence yield is dependent on the rate of electron flow through the system to an electron acceptor. On top of this consideration, the other fluorescence quenching (fluorescence lowering) phenomena described in figures 4·1 and 4·2 must be superimposed. The steady-state fluorescence yield is therefore a development of all these factors.

Measurement of fluorescence from a system in steady-state must be studied with care due to the complexity of the signal. However several steps can be taken to deconvolute the quenching process. For example, addition of an uncoupler should collapse the ΔpH and remove any associated HES quenching whilst addition of a terminal electron acceptor should oxidise the electron transfer chain *via* photosystem 1 and hence, the redox state of Q should reflect the ratio of electron flux from P_{680} (input rate) versus the oxidation or output rate by photosystem 1. An oxidised chain also favours de-phosphorylation to a state of 'minimal *spillover*' such that the fluorescence yield reflects the redox state of Q under steady-state conditions. Thus, by changing the intensity of incident light, it is possible to examine the capacity of the thylakoids to transfer electrons by studying chlorophyll fluorescence.

An 'induction' recorded in low light ($\sim 1 w.m^{-2}$) from a dark adapted, oxidised sample produces a yield of fluorescence that is only slightly above F_o (see figure 4·4), which is referred to as F_i. As mentioned above, adding more light elevates the fluorescence as Q becomes reduced. However, it is possible to progressively close photosystem 2 traps by preventing Q oxidation (as in figure 4·3 where DCMU is added in the dark) by adding small quantities of this herbicide in a stepwise manner, thus titrating the fluorescence as a function of the DCMU

concentration (see Chapter six). Under these conditions the fluorescence yield remained fairly constant for a given concentration of DCMU for ~20 minutes.

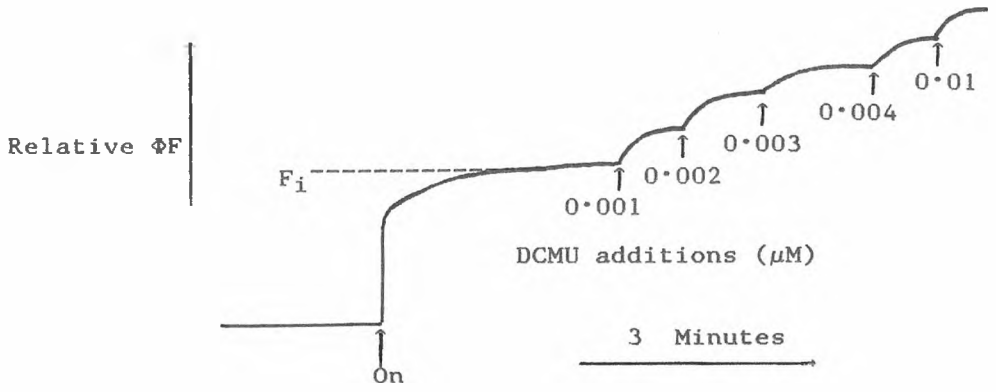


Figure 4·4: An 'induction curve' followed by a DCMU titration recorded through a Schott RG 665 filter in low light ($1 w.m^{-2}$, defined by a Corning CS 4-96 filter) following dark adaptation in the presence of 100μM methyl viologen and 1μM gramicidin D at a concentration of 80μg/ml of chlorophyll. For further details see following discussions on induction measurements.

In this way a specific concentration of DCMU can be shown to elevate the fluorescence by a percentage of the fluorescence yield at F_m (when all the traps are considered closed). It should be noted that although this relates to a specific proportion of the photosystem 2 traps, it is not possible to directly quantify trap closure by this technique because the yield of fluorescence is not a linear indicator of the concentration of Q^-, but Paillotin (1976) demonstrated a mathematical relationship between the intensity of fluorescence and the concentration of Q^-.

MEASUREMENT OF FLUORESCENCE INDUCTION CURVES

After a period of complete darkness, exposure of a chloroplast sample to light results in a fluorescence induction curve similar to the one shown in figure 4·3. To secure such measurements, one must have the facility to dark adapt the sample (to ensure total oxidation of Q) and expose it in a controlled manner. This basic requirement must be precisely timed in terms of exposure, data acquisition/storage and eventually data handling. Such a system commonly consists of the following components: a dark sample chamber, a stable actinic light source, an electronic shutter, a timer, a detector, a transient recorder and a data handling facility. This hardware was assembled as shown in figure 4·5 below and was used in a specific sequence. The chloroplasts were dark incubated in the sample

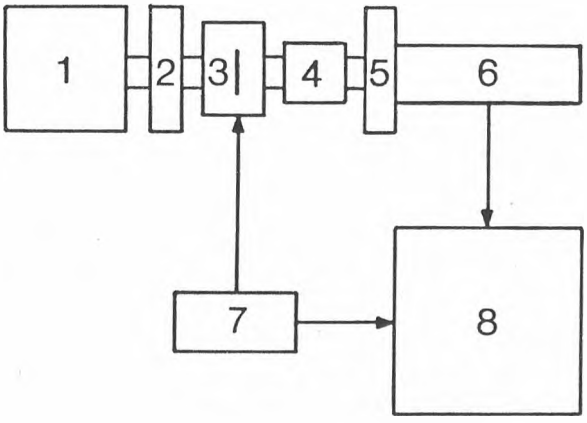


Figure 4·5: Diagram showing assembly of equipment for recording of fluorescence inductions; 1 light source, 2 heat/ actinic filters, 3 shutter, 4 cuvette, 5 emission filter, 6 detector, 7 trigger, 8 transient recorder.

chamber. The timer (a pulse generator) was fired, activating the transient recorder which commences data acquisition. After a specified delay a second pulse was produced, opening the electronic shutter for a predetermined period. This allowed actinic light to pass through an exciting filter and into the sample. Photochemistry occurred and the fluorescence emitted was measured by the detector through the emission filter. The electrical signal from the detector was fed into the transient

recorder and the induction curve recorded. Once the process is finished (ie the shutter closed and data collection terminated), the data may then be processed.

The exciting and detection filters are selected to prevent actinic light from striking the detector, but allowing fluorescence (which is red-shifted due to *Stokes Shift*) to be measured.

The fluorescence induction data in this work was recorded on a Datalab 40000B system and was processed on-line by the in board microprocessor. The system was subsequently up graded by adding a second micro computer, allowing data storage onto floppy diskettes.

The pulse generator used to trigger the shutter and transient recorder was designed, developed and built specifically for the project. The essential elements were three cascaded monostable timers of variable duration:

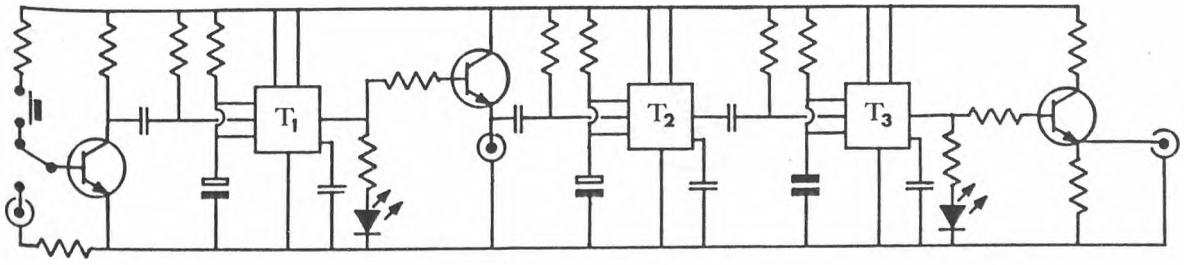


Figure 4·6: Circuit diagram of the timer designed and built for induction measurements.

Operation commences with a trigger input (externally synchronized or manual) firing the first timer (T_1), the output of which is instantaneous and is used to trigger the transient recorder. The same signal is fed into the second timer (T_2) which applies a delay (programmable) before pulsing the third timer (T_3). Once activated, the final timer is used to open the electronic shutter, but is programmed to keep the shutter open for a set period of time.

STEADY-STATE FLUORESCENCE MEASUREMENTS

The rigors of precise timing and data acquisition in fluorescence induction detection are not required for measurement of steady-state fluorescence. The transient recorder is often replaced with a chart recorder, for example in Chapter six where DCMU titration experiments are discussed. Also in Chapter six, spectra of steady-state fluorescence are measured by defining the emission through a monochromator, the data was then fed directly into the DL 4000B system.

One major improvement to the steady-state measurements was the utilisation of a *lock-in* amplifier. This has two major advantages; firstly, the signal size is increased and is therefore less prone to interference and noise; secondly, (and most important), the *lock-in* technique is one in its own right and by definition, the amplifier 'locks into' a modulating signal, rapidly sampling the signal at regular intervals. It is thus possible to measure tiny signals from otherwise unsuitable samples with high levels of background noise.

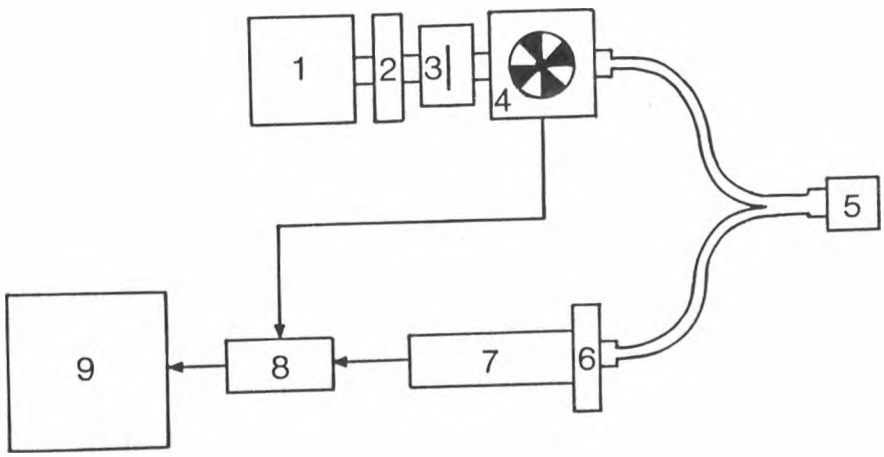


Figure 4·7: Optical and electronic arrangement for detection of steady state fluorescence; 1 light source, 2 heat/ actinic filters, 3 shutter, 4 chopper, 5 cuvette, 6 emission filter, 7 detector, 8 *lock-in* amplifier, 9 chart or transient recorder.

To detect modulated fluorescence, a modulated actinic source must be used. This can be achieved by passing the exciting light through a frequency programmed chopper (a rotating slotted disk). The amplifier is

then synchronized with the chopper. In practice, one is able to use very low intensity measuring beams, that are unaffected by stray light and sample noise.

The final point concerning fluorescence measurements is the optical arrangement of the detector and the actinic light. Fluorescence is often measured at 90° to the actinic light beam. However, by using optical fibres, 'front face' detection is also possible (see figure 4·7). Fluorescence recorded at 90° to the actinic source must traverse the bulk of the sample, whilst a *front face* measurement may only detect the fluorescence originating near the sample surface. These differences in optics probably affect the fluorescence perceived as large differences can be seen (figure 4·8). By detecting at 90° some of the fluorescence may be re-absorbed by the chlorophyll present and thus the spectrum may differ to that detected *front face* for this reason.

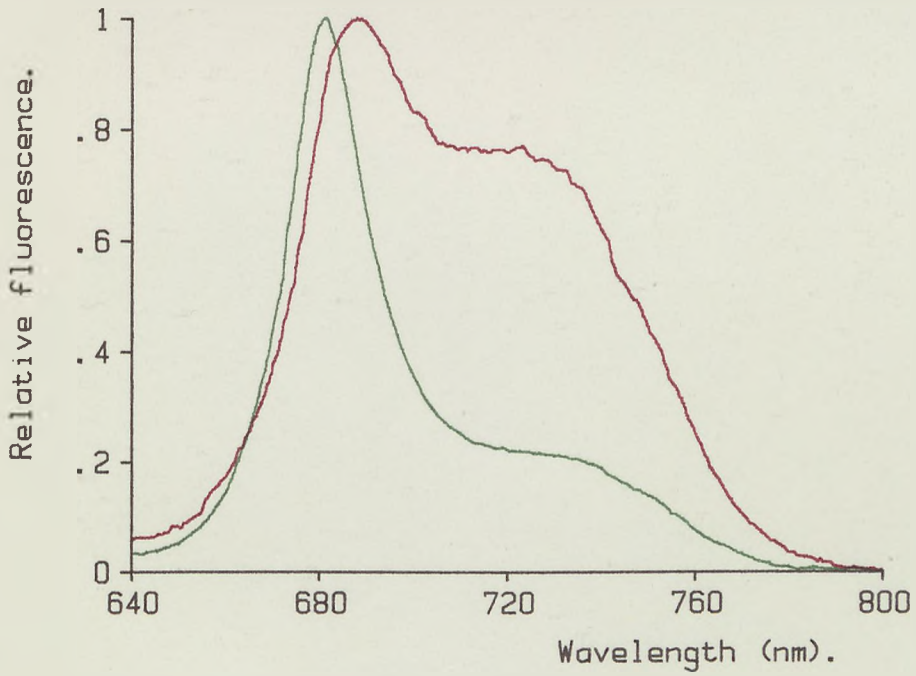


Figure 4·8: Scans of fluorescence at 80μg/ml of chlorophyll in 10μM DCMU. Data recorded *front face* (⟶) and 90° to the actinic beam (⟵). Details in text.

The data of this experiment were averages of several scans of different chloroplast samples. It can be seen that a change in optics resulted in large distortions in the fluorescence spectrum. It might be predicted from this data that fluorescence scans at different chlorophyll

concentrations would also reveal differences in the spectral yield. In Chapter six the apparent elevation of the long wavelength fluorescence is utilised.

CHAPTER FIVE

MEASUREMENTS OF FLUORESCENCE INDUCTIONS CURVES

INTRODUCTION

The observation of a biphasic fluorescence rise by Doschek & Kok (1972) and the subsequent kinetic interpretation by Melis & Homman (1975), suggesting a non homogeneous photosystem 2 population has prompted many workers to ask the question "is this apparent heterogenity related to two independent structures, or differential expression of the basic unit ?" It may be argued that β-centres can be considered as non-comunicatory photosystem 2 according to a statistical probability function, compared with the bulk photosystem 2. Conceivably, these centres might be localised on the periphery of the concentrated photosystem 2 domains.

Many authors take the view that β-centres are indeed separate physiological photosystem 2 units, which have been shown to reside solely in the stromal exposed thylakoids (Anderson & Melis 1984). Whilst α-centres reside in a bed of antenna chlorophyll, β-centres are believed to be solitary, behaving according to the 'separate package' model (Butler & Kitajima 1975 and Butler 1980). Alpha-centres however are readily described by the 'connected package' model (Butler 1980), which can be considered as a hybrid of the 'matrix or statistical pigment bed' and the 'separate package' models (Lavorel & Joliot 1972, Butler & Kitajima 1975 and Butler & Strasser 1977).

Thielen *et al* (1981) demonstrated that β-centres have a smaller antenna than α-centres, which may account for the slower rate constant of photochemistry. Hodges & Barber (1983) suggest that photosystem 2 heterogenity could be related to a weak inhibition by DCMU of some centres, possibly producing a slower rate constant. Such a proposal is supported by the observations that chloroplast integrity affected DCMU inihibition and β_{max}, the area above the F_v portion of the fluorescence induction curve emanating from photosystem 2_β (Schreiber & Pfister 1982). However, Thielen & Van Gorkom (1981c) pointed out that in wild type and mutant chloroplasts DCMU does inhibit the re-oxidation of both Q_α and Q_β when measured by DCPIP reduction, even though the B-protein of β-centres does not show binary oscillations in redox state. Furthermore, Thielen & Van Gorkom (1981a) demonstrate that both photosystem 2_α and β have quantum yields approaching

unity in the presence of DCMU; thus, DCMU must inhibit Q→PQ electron transfer in photosystem 2_β otherwise this value would decrease. However, this does not preclude DCMU binding with lower affinity.

The hetrogeneous nature of photosystem 2 was studied by recording fluorescence induction curves in the presence of known amounts of DCMU. The basic question asked was "Is photosystem 2 heterogenity reflected in its properties of herbicide binding ?"

METHODS

Spinach chloroplasts (prepared according to the methods of Chapter two) were stored on ice in the dark at a known chlorophyll concentration. Immediately prior to use, a sample of chloroplasts were transferred into the cuvette to give a final concentration of 80μg chlorophyll/ml and osmotically lysed for ~45 seconds to rupture their outer envelopes, this chlorophyll concentration was selected in preference to the lower concentrations used by other workers to allow direct comparison with the inhibition data of Chapter three. Reaction medium (*p*H 7·6) was added, containing the following final concentrations: 0·33M sorbitol; 50mM Hepes; 5mM $MgCl_2$ and 2mM EDTA. To this mixture 100μM methyl viologen was provided as an electron acceptor and 2μM gramicidin D was added to uncouple the thylakoids. The sample was stirred in the dark for five minutes and then a known concentration of DCMU in methanol added. After a further 30 seconds the stirrer was turned off and the chlorophyll fluorescence induction recorded.

The optical and electronic equipment was assembled as in figures 4·5 and 4·7 using a combination of heat absorbing glass and a Corning CS 4-96 filter to define the exciting light (of about 90w.m^{-2}). A Balzers 695nm interference filter (10nm bandpass B40 response) was placed in front of the photomultiplier. The transient recorder of the DL 4000B system sampled the detector output between 0·4 and 1ms intervals, resulting in a total recording time of ~0·3 second. The resolution of the ordinate was 12 bits.

RESULTS

Figure 5·1 shows two fluorescence induction curves recorded in the presence of low (0·2μM) and high (6·0μM) DCMU. With 0·2μM DCMU, the fluorescence rise was slow, F_m being attained after approximately 90% of the total time had expired. At higher DCMU concentrations the rise was more rapid, indicating a faster rate of trap closure. This is attributed to an inhibition of plastoquinone mediated Q oxidation by DCMU (Duysens & Sweers 1963).

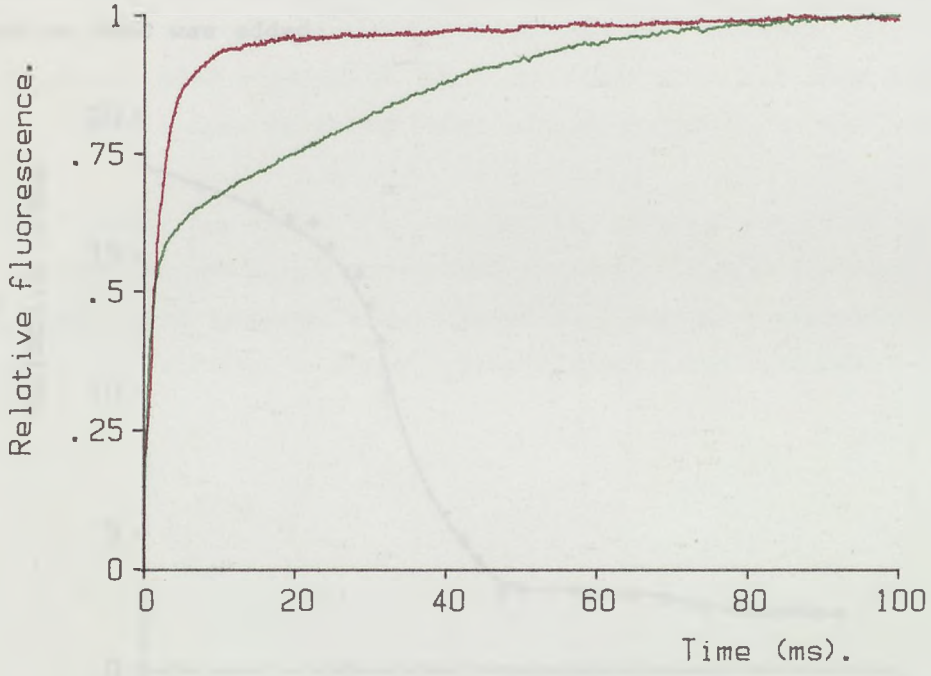


Figure 5·1: Chlorophyll *a* fluorescence induction curves recorded in the presence of [a] 0·2 (——) and [b] 6·0μM (——) DCMU. F_v/F_m ratios were 0·827 and 0·784 respectively (details in text).

The area bounded by F_o and F_m above curves [a] and [b] were calculated on-line by the DL 417, these had the values $8{\cdot}8\times10^{42}$ and $2{\cdot}44\times10^{42}$ respectively. This experiment was repeated for varying DCMU concentrations and areas above F_v calculated. The resultant values are plotted in figure 5·2. This data shows several features. Initially the area decreases rapidly with increasing DCMU concentrations, but it begins to level out in the 1→2μM region. At around 5μM DCMU a second wave of area loss becomes apparent. This second phase levels out at around 10μM DCMU.

DISCUSSION

The calculated area over the fluorescence induction curve provides a measure of the associated acceptor pool, and the rate at which it is filled upon trap closure (Malkin & Kok 1966). In the absence of DCMU the pool is large (consisting of plastoquinone) and the fluorescence rise is consequently slow. DCMU isolates Q from the plastoquinone pool (Duysens & Sweers 1963) and the rise reflects the rate of Q reduction. The data in figure 5·2 shows that it is possible to titrate DCMU inhibition of electron flow by measuring the area above F_v. Initially the area was large, but it diminished as DCMU was added:

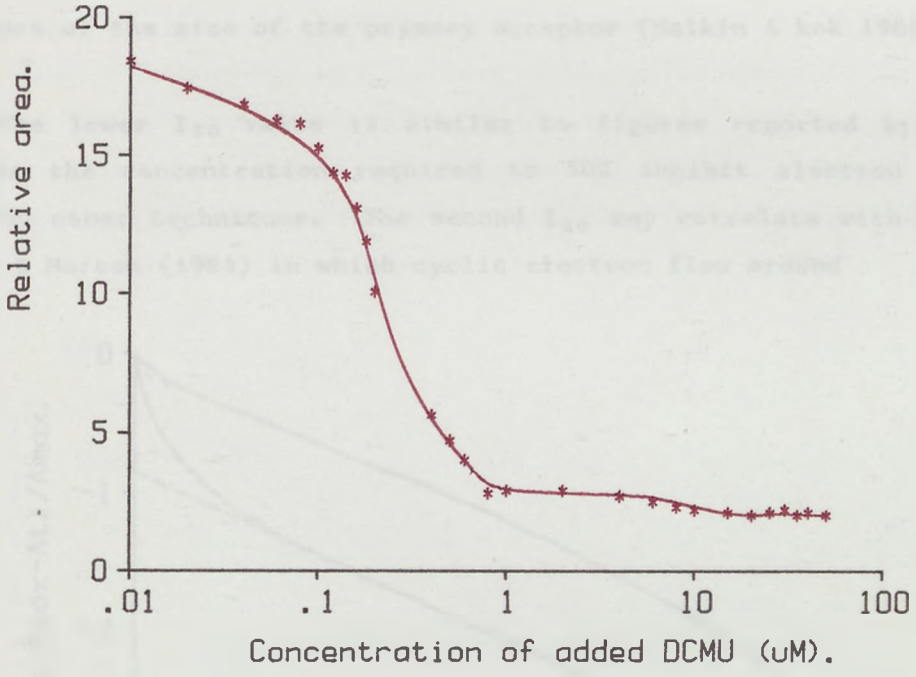


Figure 5·2: Plot of area bounded by F_m above F_v (F_m-F_o) at varying DCMU concentrations, data similar to figure 5·1. Note $\log_{10}$ scale on abscissa. Each datum point is a mean of five measurements. See previous figure for details.

It is important to stress that the rate of electron donation to Q by P_{680}, described by the complex function (f) is assumed to be unaffected by variation of the DCMU concentration; thus only the oxidation rate of Q is changed such that the redox state of Q at a given point in time is dependent on the relative reduction and oxidation rates of the system. A second assumption, is that the function (f) was non-variant for the duration of each experiment, ie. the input rate, although complex, is not

changing during an induction and is unaffected by DCMU. It is generally believed that DCMU does indeed bind between Q and plastoquinone, thus validating the first assumption, although Rosenberg *et al* (1972) suggested that DCMU binding may also inhibit the oxidative side of photosystem 2.

Figure 5·2 also shows that DCMU appears to have two phases of inhibition, the first phase has an I_{50} of 0·19μM and the second around 5·16μM DCMU. As the latter accounts for only 5% of the total DCMU induced area loss it is possibly an expression of DCMU binding to B^-, although this component was reported to be 30% populated in the dark (Joliot & Joliot 1983). The area above F_v that remains in the presence of saturating concentrations of DCMU represents (f), the time taken for trap closure and is a measure of the size of the primary acceptor (Malkin & kok 1966).

The lower I_{50} value is similar to figures reported by several authors as the concentration required to 50% inhibit electron flow as measured by other techniques. The second I_{50} may correlate with the data of Ridley & Horton (1984) in which cyclic electron flow around

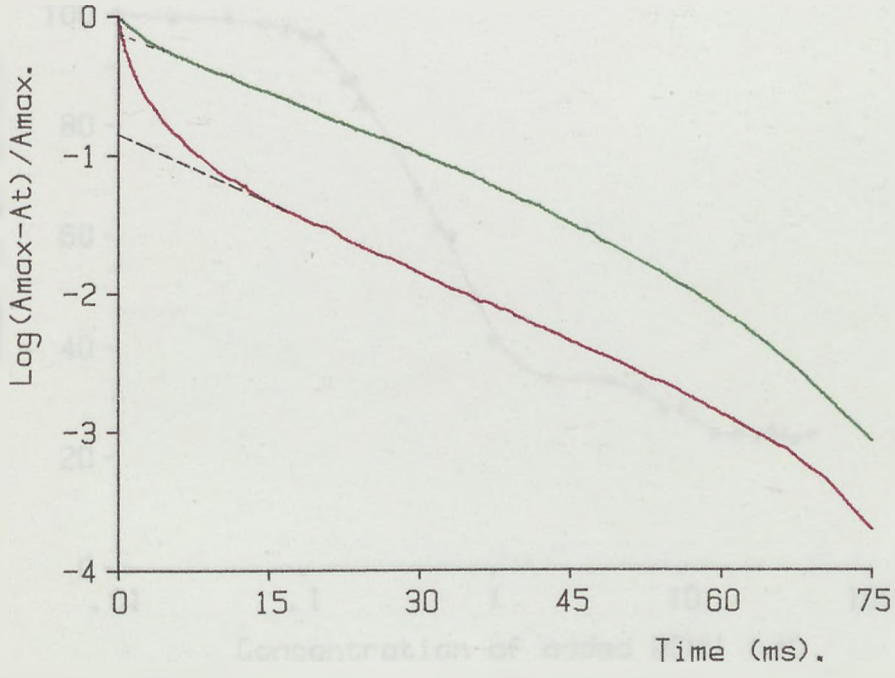


Figure 5·3: Semilogarithmic plots of area accumulation with time from data in figure 5·1, (——) 0·2 and (——) 6·0μM DCMU. A_t and A_{max} represent timed and maximal area accumulation. Extrapolation lines provide estimate of β_{max}.

photosystem 1 was inhibited and photodestruction commenced. The area decrease associated with the I_{50} of about 5μM DCMU may be related to a loss of cyclic because it is known that the electron carriers must be at the correct redox poise before cyclic occurs (Grant & Whatley 1967). It is possible that photosystem 2_β performs this role as it is believed to reside in the unapressed lamellae (Anderson & Melis 1984) and may even relate to Q_z mediated reduction of the cytochrome *b/f* complex (Joliot & Joliot 1981b and 1983). Thus, inhibition of a pathway that performs this poising function may cause a loss of cyclic around photosystem 1.

The data from these experiments were analysed further and rates of logarithmic area growth were calculated by the procedures of Melis & Homman (1976) to produce the plots of figure 5·3 above. By extrapolating the linear portion of the plots back to zero time, it is possible to estimate the relative proportions contributed by the α and β-centres to the total area. The upper curve gives a β contribution (β_{max}) of 88·3%, for the 6·0μM DCMU curve this value diminishes to 30·6%. The data from figure 5·2 was treated in a similar manner and the calculated β_{max}'s plotted against

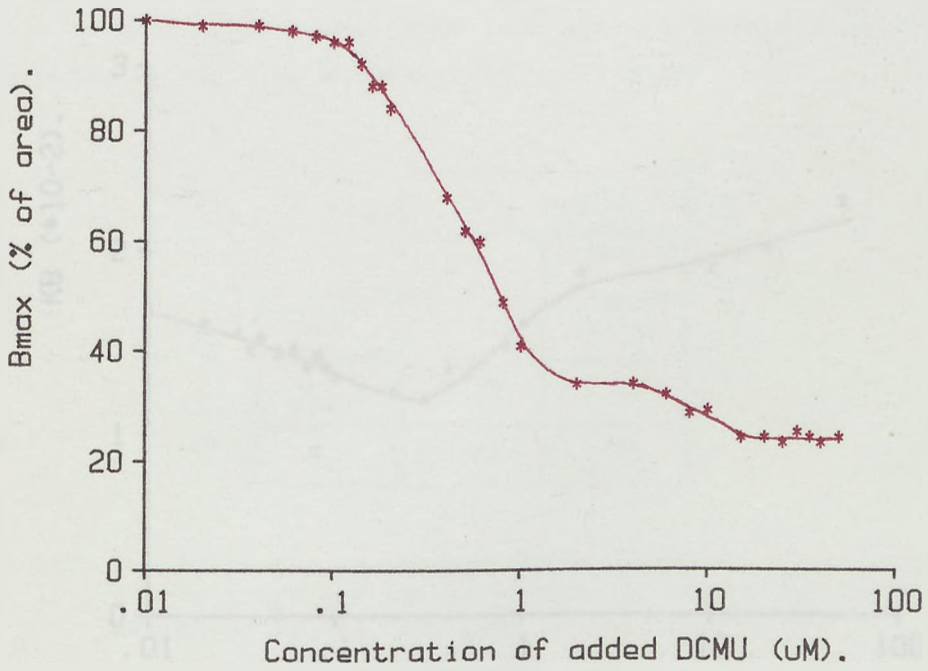


Figure 5·4: A plot of β_{max} verse DCMU concentration for the induction data of figure 5·2. Details in text.

the DCMU concentration used (figure 5·4). The results of this treatment

show that increasing the DCMU concentration reduces the β_{max} value in a similar manner to the area loss, with I_{50}'s of 0·43 and 7·84μM DCMU.

As well as reflecting figure 5·2, the data presented in figure 5·4 suggests that the proportion of β-centres is reduced with increasing DCMU. Presumably at low DCMU concentrations the induction is slow because Q is re-oxidised by plastoquinone and as a result the α-centres appear kinetically similar to β-centres. As the concentration of DCMU is increased, Q becomes isolated from B and the photochemical rate constant of photosystem 2_α (k_α) increases, allowing k_β to be seen, and β_{max} to be estimated more accurately in the absence of a contribution from α-centres with similar kinetics to the β-centres.

Another feature of figure 5·4 is the persistence of the 'second phase'. Clearly the curve represents the gradual inhibition of photosystem 2, but it may also indicate that α-centres could be inhibited before the β-centres. Thus the latter phase could be inhibition of photosystem 2_β whilst the early phase probably represents the appearance of α-centres that show a large kinetic difference to the β-centres. However, the second wave

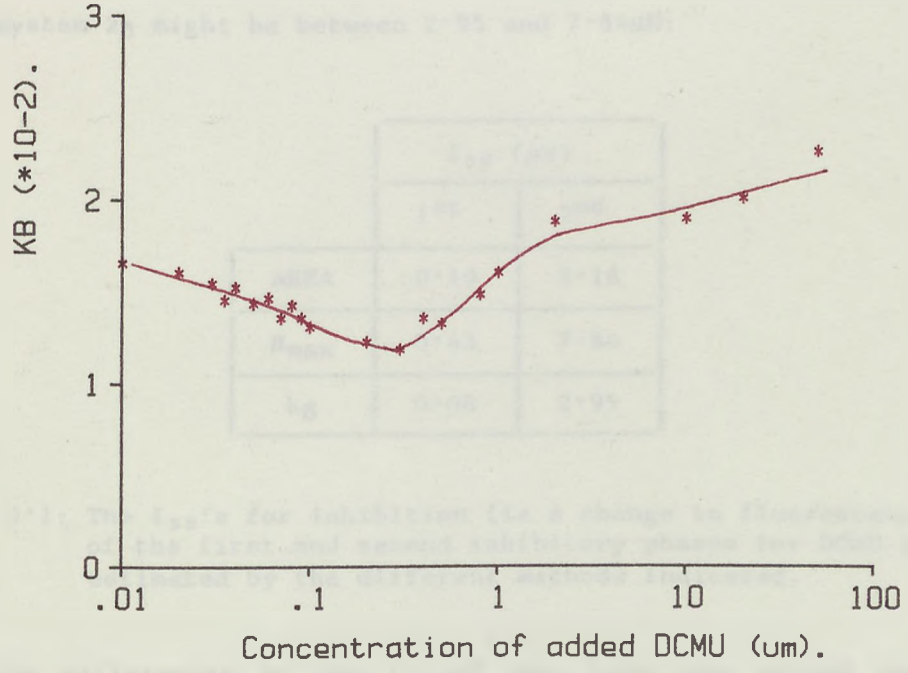


Figure 5·5: A plot of k_β verse DCMU concentration from the induction data of figure 5·2. See text for further details.

could be due to binding of DCMU to B^- or even related to inhibition of Q_z (Joliot & Joliot 1983). To further this study, a third analysis of the induction data was performed in which the rate constant of the β area (k_β) was calculated for the different DCMU concentrations (figure 5·5). This plot shows an unusually complex transition: at first the rate constant falls to a fairly low level after which a larger secondary change is seen, eventually reaching a plateau. The I_{50}'s for these two changes are 0·07and 2·95μM DCMU. Presumably, the reduction in k_β over the lower DCMU range is due to the 'speeding up' of the α-centres. The rate constant for largely uninhibited β-centres is seen at ~0·2μM DCMU as the signal has the majority of the α contribution removed (although some β-centres will be closed). At DCMU concentrations above this value, β-centres probably 'speed up', becoming inhibited and thus the function (f) is seen between 20~50μM DCMU, where no further changes are witnessed.

From figure 5·5 the I_{50} for photosystem 2_α inhibition is 0·07μM DCMU whilst β-centres appear to be half inhibited at 2·95μM DCMU. From figure 5·2 and 5·4 these constants are 0·19, 0·43 and 5·16, 7·84μM respectively. Thus from these experiments photosystem 2_α may have an I_{50} which falls in the range 0·07→0·43μM (see table 5·1 below) whilst the I_{50} for photosystem 2_β might be between 2·95 and 7·84μM:

	I_{50} (μM)	
	1st	2nd
AREA	0·19	5·16
β_{max}	0·43	7·84
k_β	0·08	2·95

Table 5·1: The I_{50}'s for inhibition (ie a change in fluorescence) of the first and second inhibitory phases for DCMU as estimated by the different methods indicated.

The differences in the I_{50} of the first and second phases are approximately twenty five-fold, which is similar to that reported by Joliot & Joliot (1983) for the difference in affinity of B and B^- for DCMU. However the second phase only accounts for 10% of the total fluorescence

and not 30%. Q_2 may also be a candidate for the second phase component as the I_{50} (~3μM) is similar to that reported here.

Although these experiments permit two separate changes in fluorescence to be observed, the DCMU concentration ranges over which these changes occur are not very well conserved. This suggests that either these results express different events, or the shape of the titration curves for the various analyses are being affected in different ways. However, the trends presented here are very similar and indicate a common source. It should be noted that the area and β_{max} measurements are both affected by k_β, thus compounding subsequent changes.

CHAPTER SIX

TITRATIONS OF CHLOROPHYLL FLUORESCENCE

In Chapter five it was shown that the amount of oxidised photosystem 2 acceptor can be estimated by recording fluorescence induction curves in the presence of known amounts of DCMU. In this chapter the same parameter will be measured by means of the steady-state fluorescence yield, as a continuation of the herbicide binding study to see if the additional binding event observed in the proceeding Chapter at higher concentrations of DCMU can be expressed as a heterogenity of photosynthetic electron flow. Although in Chapter three only a single DCMU-induced change in the redox state of cytochrome *f* was perceived. Steady-state fluorescence was continually monitored at 90° to the measuring beam and recorded as a function of the DCMU present.

METHODS

Chloroplasts were prepared as described in Chapter two and dark stored on ice prior to use. Aliquots equivalent to 80μg chlorophyll/ml were osmotically lysed for ~45 seconds and diluted into reaction medium (*p*H 7·6), providing the following final concentrations: 0·33M sorbitol; 50mM Hepes; 5mM $MgCl_2$ and 2mM EDTA. The thylakoids were uncoupled with 2μM gramicidin D and methyl viologen (100μM) was added to accept electrons from photosystem 1. The reaction medium was continually stirred and maintained at 20°C. Fluorescence was titrated with DCMU (in methanol), allowing about 20 seconds for the signal to stablise between additions. Methanol additions (100μl) alone caused very little change in the fluorescence signal.

The arrangement of the optical and electronic equipment was similar to that shown in figure 4·7. Actinic light was provided by a stabilized tungsten-halogen source filtered through a combination of heat absorbing glass and Corning CS 4-96 filters. The fluorescence emission was defined by a Schott RG 665 cut-off filter (50% transmission at 665nm) and detected with an EMI 9558B photomultiplier tube.

RESULTS AND DISCUSSION

The yield of fluorescence is generally considered to reflect the redox state of the primary acceptor Q (Duysens & Sweers 1963). Clearly, if the ratio of the rates of trap closure:trap oxidation increase then the fluorescence will rise. This balance can be altered by either changing

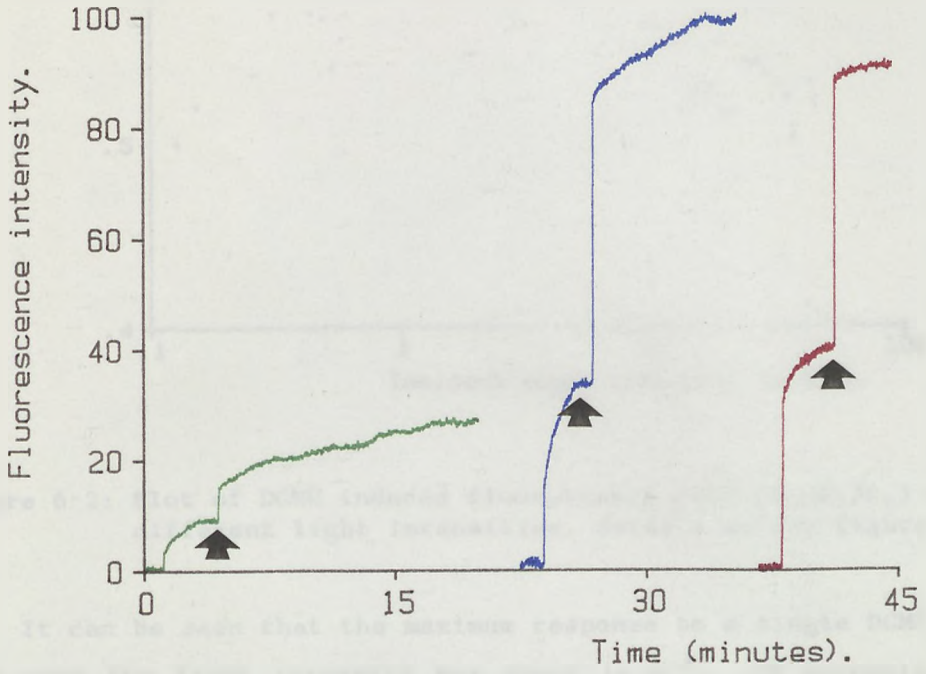


Figure 6·1: Terminal fluorescence level (F_t) upon addition of 100μM DCMU (▲) after establishment of F_i for [a] 0·4 (——), [b] 1·0 (——) and [c] 10w.m^{-2} (——) of incident light. Note that for curve [c] ΦF+10. See text for other details.

the rate of photon delivery or the rate at which Q is re-oxidised. To obtain the maximum response to DCMU, the actinic light must be poised at exactly the correct intensity; too high and a large proportion of the traps close in the absence of DCMU, too low and the measured fluorescence may be underestimated because the rate of charge recombination due to back reactions will compete with k_f and significantly lower the yield (see equation 4·3). In figure 6·1, single DCMU additions were made at different incident light intensities (once F_i had been attained, see figure 4·4), for samples of thylakoids at 80μg/ml of chlorophyll. It is obvious that trace [b] gives the highest F_t-F_i:F_t ratio, suggesting that the largest response to DCMU is found at a specific light intensity. In total, the experiment

was repeated more than 100 times over a range of light intensities, the results of which are plotted in figure 6·2:

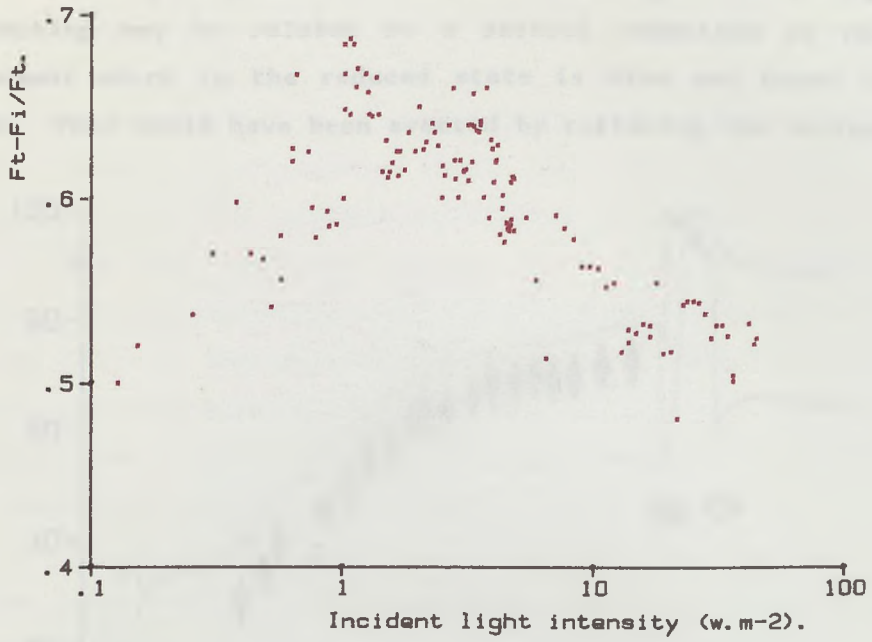


Figure 6·2: Plot of DCMU induced fluorescence rise (F_t-F_i/F_t) at different light intensities, details as for figure 6·1.

It can be seen that the maximum response to a single DCMU addition occurred when the light intensity was about $1w.m^{-2}$. At intensities above or below this value the relative fluorescence rise due to DCMU decreases.

A titration of fluorescence with DCMU is shown in figure 6·3[a]. At the end of the titration (i.e., at F_t where no further fluorescence increase could be obtained) 300μM sodium dithionite was added to close any centres remaining open. Even in the presence of high concentrations of DCMU, some traps remained open and consequently had a low fluorescence yield. For instance, a particular centre may not (or only poorly) bind DCMU, or at lower intensities the effect of back reactions etc. are greater, diminishing the fluorescence intensity. Addition of dithionite reduced the number of open traps and elevated the fluorescence to the maximum level. Some of this fluorescence increase may be related to the proportion of back reactions or oxidised plastoquinone.

The amount of DCMU required to saturate the fluorescence rise (see figure 6·3[a]) was 560μM. Upon dithionite addition there was a transient fluorescence lowering followed by a rise to a higher level (F_m). The initial quenching may be related to a partial reduction of the methyl viologen present which in the reduced state is blue and known to absorb fluorescence. This could have been averted by replacing the viologen with

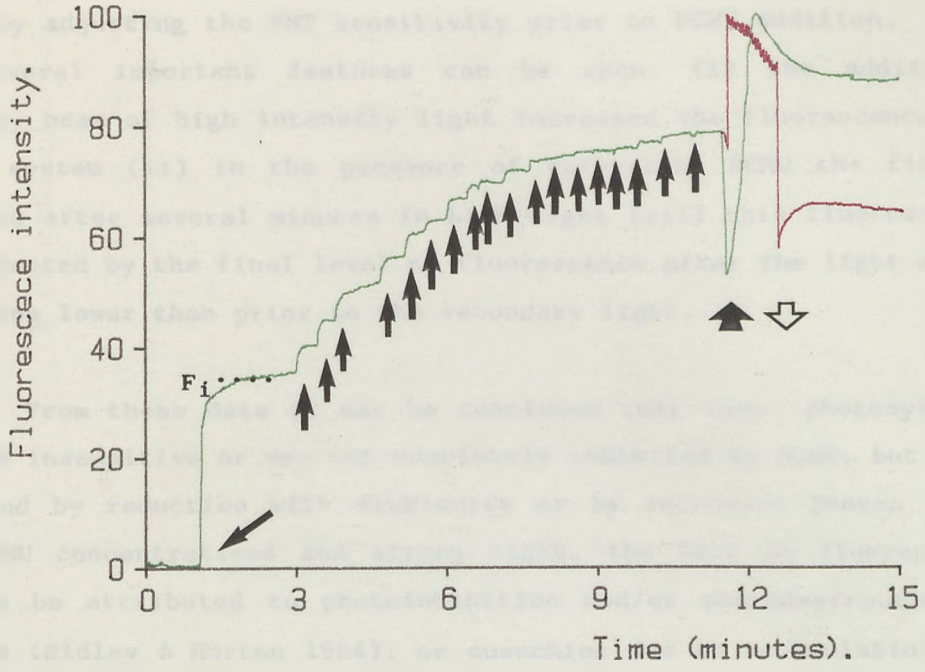


Figure 6·3: Fluorescence titrations with upto 560μM DCMU (↑). Measuring beam on (◢), establishing F_i. Addition (⬆) of [a] dithionite (——) or [b] secondary light (——), secondary light off (⇩). Details as above.

another oxidant, for example ferricyanide, but it was found that ferricyanide lowered the I_{50} for DCMU. The reason for this is unknown, but the ferricyanide could be maintaining the plastoquinone pool at a higher redox potential, impeding the formation and stabilization of the semiquinone. As the affinity of oxidised and doubly reduced quinone for the binding site is lower (Lavergne 1982), the I_{50} will be less as DCMU is able to compete more strongly with plastoquinone for the active site.

By using a lock-in amplifier it was possible to supplement additional (D.C.) actinic light as a reductant. As discussed before, the system only detects the events relating to increased photochemistry. The second light was defined by a Corning CS 4-96 filter and added at 90° to

the measuring beam from a stabilized light source *via* an optical fibre. The intensity of this light was varied for each experiment to attain the absolute maximum yield of fluorescence at F_m. Generally the intensity was between 100~120w.m^{-2}.

The data represented in figure 6·3[b] were extremely reproducible providing the initial fluorescence level (F_i) was normalised for each sample by adjusting the PMT sensitivity prior to DCMU addition. From this data several important features can be seen: (i) the addition of a secondary beam of high intensity light increased the fluorescence measured by the system (ii) in the presence of saturating DCMU the fluorescence decreased after several minutes in high light (iii) this fluorescence loss was reflected by the final level of fluorescence after the light was turned off, being lower than prior to the secondary light.

From these data it may be concluded that some photosystem units are DCMU insensitive or are not completely inhibited by DCMU, but these may be closed by reduction with dithionite or by increased photon flux. At high DCMU concentrations and strong light, the fall in fluorescence may possibly be attributed to photoinhibition and/or photodestruction of the pigments (Ridley & Horton 1984), or quenching due to accumulation of P_{680}^+ or the pheophytin anion (Klimov *et al* 1977 and Renger *et al* 1983).

The cumulative fluorescence change during the DCMU titration, expressed as a percentage of the fluorescence level observed at saturating concentrations of DCMU and reductant ie. the 'true F_m', is shown in figure 6·4. It can be seen that both data yield very similar titration curves. At a DCMU concentration of 2mM no further increase in fluorescence was observed, this final level being only about 70% of 'true F_m'. The fluorescence level rose very sharply over the lower DCMU concentrations, appearing strongly sigmoid. Over the range 0·2 to 10μM DCMU a shallow gradient was seen and beyond this region there was a second major elevation in fluorescence which began to level out at 1.5 mM DCMU. From these data three I_{50}'s for DCMU can be calculated of 0·044, 1·2 and 365μM DCMU for the sigmoidal, secondary and final phases respectively.

In conclusion, by titrating the steady state fluorescence against DCMU it was possible to identify three DCMU-associated phenomena, the first

two corresponding with the previous data obtained by other techniques (see table 5·1). If phases one and two are assumed to a representation of α and

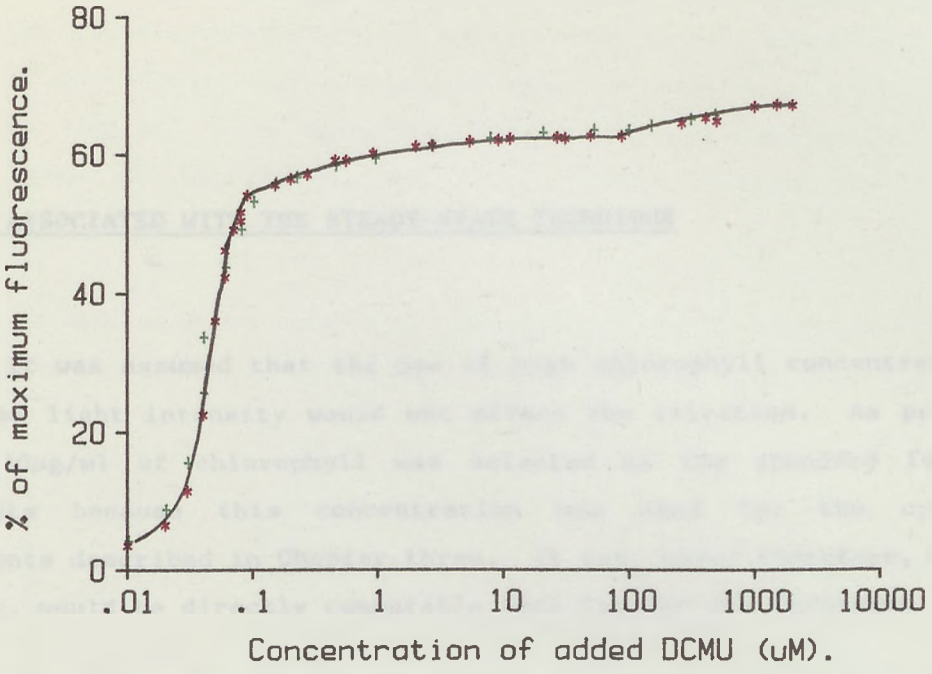


Figure 6·4: Fluorescence expressed as a percentage of F_m verses DCMU concentration. Data from figure 6·3[a] (⟵) and mean of 20 experiments similar to [b] (⟶).

β-centres respectively, such that the latter require a higher concentration of DCMU for inhibition (Chapter five), as indicated by Hodges and Barber (1983) and supporting the data of Ridley & Horton (1984); then the identity of the third phase has still to be resolved. One other possibility is that the second phase is due to inhibition of centres in the B^- state, or it may relate to binding of DCMU to Q_2.

Schreiber and Pfister (1982) suggested that a binding event requiring high concentrations of DCMU was related to chloroplast damage, but previously Tischer and Strotmann (1977) demonstrated a non-specific binding of DCMU at elevated concentrations. The data presented here suggests that a DCMU binding event with an I_{50} of 365μM does indeed occur, and moreover, produced a precise change in the fluorescence yield of the system. This binding event may relate to non-B-type centres (Lavergne 1982), in which the B-protein was suggested to be modified because binary oscillations in its redox state were not evident during flash reduction. Therefore, low concentrations of DCMU may bind weakly to the non-B protein,

but binding is more effective at higher concentrations. The non-B acceptor may show binary oscillation at elevated DCMU concentrations.

PROBLEMS ASSOCIATED WITH THE STEADY-STATE TECHNIQUE

It was assumed that the use of high chlorophyll concentrations at a very low light intensity would not affect the titration. As previously stated, 80μg/ml of chlorophyll was selected as the standard for these experiments because this concentration was used for the cytochrome measurements described in Chapter three. It was, hoped therefore, that the I_{50}'s etc. would be directly comparable (see Tischer and Strotmann 1977).

The increased yield of fluorescence at high DCMU concentrations may be attributed to high chlorophyll or low light. Blue-green actinic light at an intensity of $1w.m^{-2}$ will not evenly illuminate 200μg of chlorophyll in a cuvette of $1cm^2$ area. Thus, fluorescence may be preferentially detected from chlorophyll molecules that are closer to the cuvette surface. This type of optical arrangement could result in artefacts in the yield of fluorescence. This may indeed be the case since the technique may selectively detect fluorescence from centres that under certain conditions (higher light etc.) may be masked by other, more fluorescent pigments.

To study this, a simple experiment was performed in which the chlorophyll concentration was halved and the titration repeated. From the normalised data, expressed as a percentage of F_m (figure 6·5), it can be seen that the final DCMU-induced fluorescence level falls by about 5% upon halving the chlorophyll concentration. Such a small fluorescence change indicates that the actinic light was perfectly poised during these titration experiments, such that a lower intensity would have elevated the final fluorescence level at 40μg/ml. However the 5% fluorescence loss upon decreasing the chlorophyll was to be expected (figure 6·1), since the effective photon density is increased at lower chlorophyll and the ratio of

F_m-F_i:F_i predictably declines.

A calculation of the high binding phase contribution as a proportion of the total response to DCMU reveals that these phases are 12 and 15% for the 80 and 40μg/ml curves respectively. By decreasing the chlorophyll present to 40μg/ml, it is demonstrated that the amplitude of the high DCMU binding phase is unaffected, and is therefore unlikely to reflect an artefactual response to DCMU.

It can also be seen that the gradient of the intermediate phase was constant during these titrations, supporting the view that a discrete

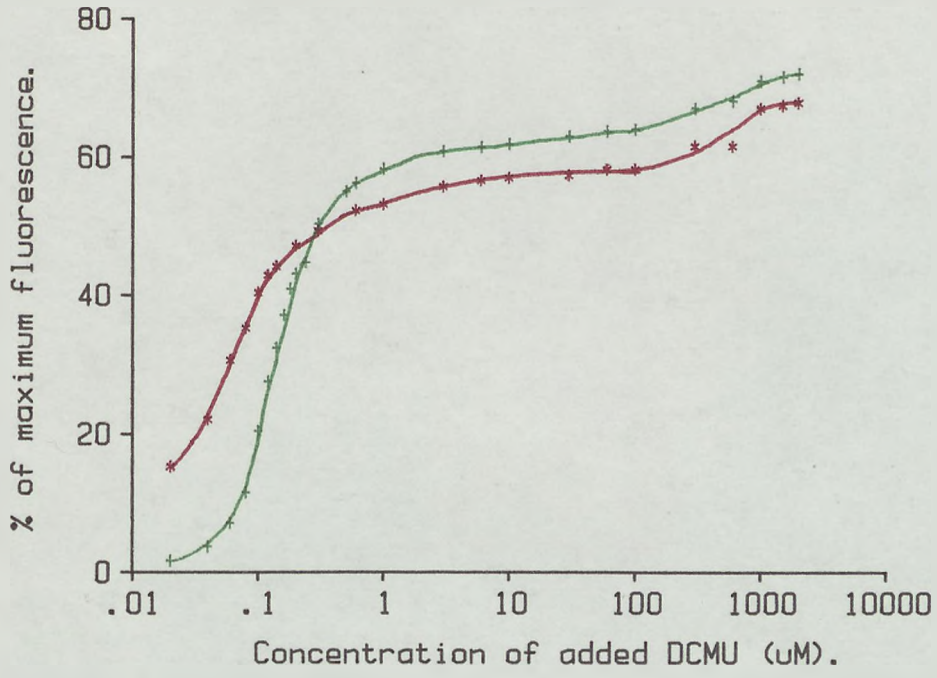


Figure 6·5: DCMU titrations at 80 (——) and 40μg/ml (——) of chlorophyll. Details in text.

binding event occurs over this range of DCMU concentration. The I_{50}'s that are associated with this portion of the curves are 2·51 and 3·55μM DCMU (for 40 and 80μg/ml of chlorophyll respectively). Although the value at 80μg/ml of chlorophyll is higher than reported earlier in this Chapter (see figure 6·4), the trends presented in figure 6·5 are in agreement with the finding of Tischer and Strotmann (1977) where the I_{50} declines with decreasing chlorophyll. This is probably due to a combination of two factors. The first is that the chlorophyll:DCMU ratio is decreased,

reflecting the partition of DCMU into the lipophilic phase, elevating the effective DCMU concentration. Secondly, the rate of Q reduction will be raised due to a slightly higher photon density:trap ratio.

FLUORESCENCE TITRATIONS AT DIFFERENT *p*H

The majority of the experiments for this thesis were performed in a reaction medium of *p*H 7·6. Melis and Homann (1975) demonstrated that the F_m level of an induction curve increased with decreasing acidity over the range *p*H 5·5 to 8·0, whilst the fluorescence yield from the β-centres fell. These changes were related to alterations in the stability of the S-states first described by Kok *et al* (1970) under different external conditions (Melis & Homann 1975). The four states posses varying degrees of stability, probably as a result of the midpoint potential of the couples. S_1 and S_2 are believed to remain stable longer than S_0 and S_3 (Bouges-Bocquet 1980b) as they are approximately 85% and 15% populated in the dark. The *p*H of the suspending medium will therefore affect the relative stabilities as the potential of the couples vary by -60mv/*p*H unit. The proposed relationships between the various I_{50}'s for DCMU and photosystem 2 heterogeneity could therefore be further explored by a study of fluorescence at different *p*H.

Chloroplasts were osmotically ruptured and then diluted into an osmoticum of the required *p*H. The *p*H of the sample after the titration was usually ±0·15 of a *p*H unit compared with the stock buffer. To cover the *p*H range 5·5 to 9·0 the following buffers were used in the reaction media at a concentration of 50mM:

5·5, 6·0 } Mes/HCl

6·5, 7·0 } Pipes/HCl

7·6 }
8·0 } Hepes/KOH

8·5 }
9·0 } Tricine/KOH

Raising the *p*H of the medium from 5·5 to 8·0 (figure 6·6) increased the maximum level of fluorescence attained for each titration, in agreement with the data of Melis and Homann (1975). This may reflect a *p*H effect on the rate of back reactions (Wraight *et al* 1972), such that at higher *p*H fewer charge recombinations occurred.

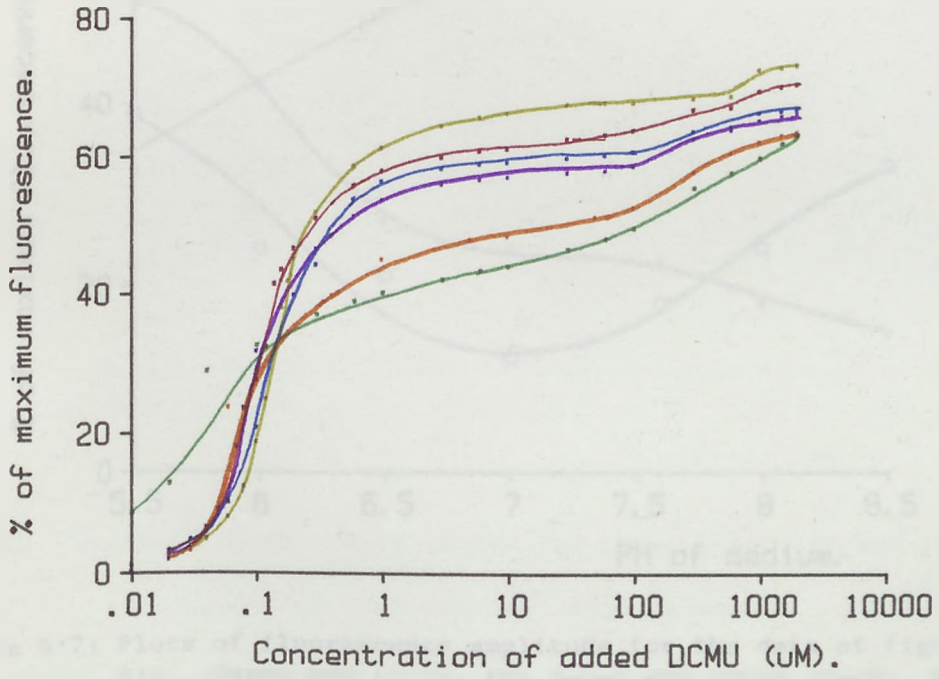


Figure 6·6: DCMU fluorescence data measured at the following *p*H: 5·5 (——), 6·0 (——), 6·5 (——), 7·0 (——), 7·6 (——) and 8·0 (——). Data not shown for *p*H 8·5 and 9·0 for simplicity, see text for details.

The terminal fluorescence level fell from *p*H 8·0 to 9·0 (data not shown to avoid confusion), and may be due to an inhibition of the water splitting enzyme of the photosystem 2 donor (Melis & Homann 1975). At low *p*H, DCMU inhibition appears to be incomplete even at 2mM DCMU.

It can be seen from figure 6·7 that the relative proportions of the various phases depend upon the *p*H of the suspending medium. The maximum amplitude of phase one (60%) was seen at *p*H 8·0, whilst the minimum

(4%) of the second phase was detected at *p*H 7·0. Away from these critical *p*H values the first phase declined whilst the second increased. The third phase did not display a distinct *p*H optimum, but was maximal (17%) at low *p*H and minimal (4%) at *p*H 8·5.

The loss of fluorescence from the first and third phases could be due to an alkaline induced inhibition of the water splitting system and may be prevented by using NH_2OH as an electron donor (Cheniae & Martin 1971).

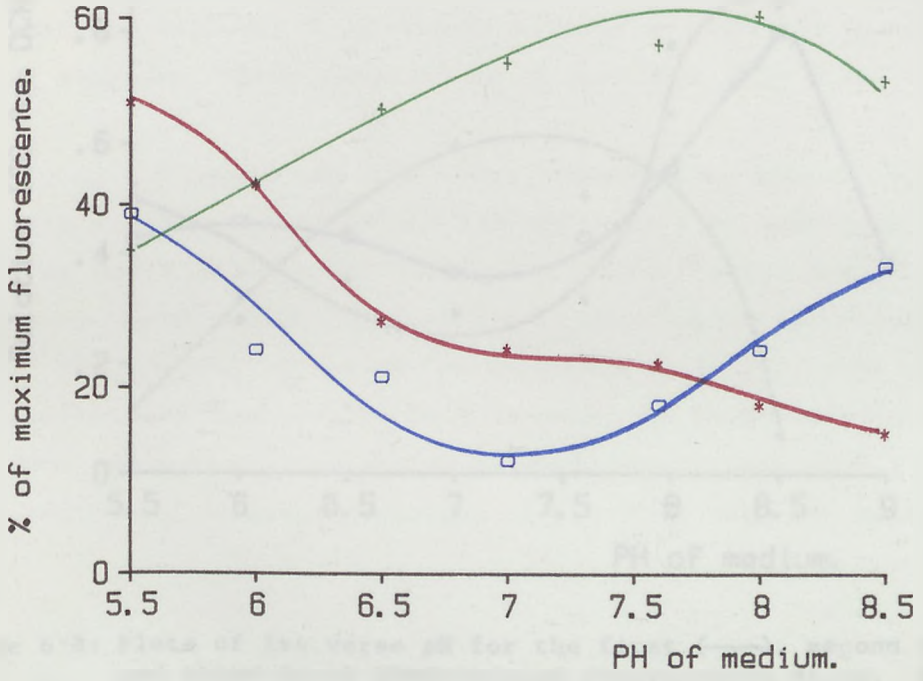


Figure 6·7: Plots of fluorescence amplitude for the data of figure 6·6. Phase one (——), two (——) and three (——). **Note ΦFx3 for second and final phase.**

This data shows a gradual loss of fluorescence from the third phase and strongly resembled the trend of β_{max} loss at high *p*H described by Melis & Homann (1975), suggesting that this may also relate to photosystem 2_β. Fluorescence from the first phase rose in an almost antiparallel manner to the second phase.

Figure 6·8 shows plots of I_{50} verses *p*H, and demonstrates that at low *p*H the first I_{50} is 0·023μM DCMU. As the *p*H is increased to 7·6 the I_{50} rises to about 0·13μM DCMU but above *p*H 7·6 the affinity is increased to 0·014μM at *p*H 8·5. The data for the intermediate phase shows quite a different trend. Between *p*H 5·5 and 7·6 there is almost no deviation of

the I_{50} value of ~2·13μM, but from *p*H 7·6 to 9·0 a sharp *p*H optimum appears, during which the I_{50} for DCMU almost doubles to 3·98μM at *p*H 8·5, before falling to 1·91μM at *p*H 9·0. The final binding phase shows a trend which is generally similar to the second, as the I_{50} decreases from *p*H 5·5 to 6·0 (524 to 300μM) and then sharply increases to 847μM between *p*H 7·5 and 8·5.

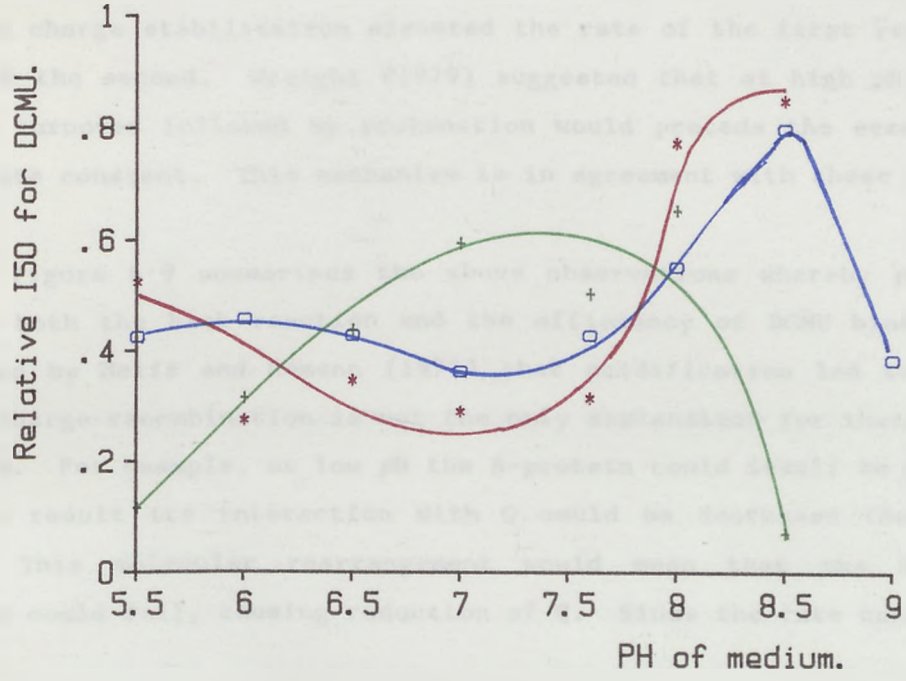


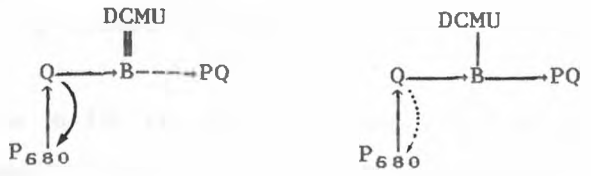
Figure 6·8: Plots of I_{50} verse *p*H for the first (——), second (——) and third (——) DCMU-induced fluorescence rises. Scaled zero to 0·2μM, 5μM and 1mM respectively.

Melis and Homann (1975) demonstrated that the rate constants k_α and k_β showed *p*H profiles similar to those described here, which were explained as a *p*H dependence of the back reaction (due to charge recombination). Thus, at alkaline *p*H the primary charge separation (see figure 1·3) was stabilized more efficiently, giving a faster area growth. In the experiments described here the rate of Q oxidation has been titrated with DCMU, the resultant *p*H profiles probably reflecting similar changes in photochemistry and molecular organisation as observed by Melis and Homann. Increasing the *p*H increases the I_{50} for DCMU, but can alterations in the rate of back reactions also be expressed through changes in the I_{50}? Possibly, the increased charge stability (across P_{680}-Q) could result in conformational changes of the B-protein, or the oxidised quinone could be more strongly attracted to the active site thus competing with DCMU.

Either of these processes could elevate the I_{50} for DCMU.

Velthuys (1981) suggested that plastosemiquinone competed more strongly than either PQ or PQH_2 for the binding site on the B-protein. The I_{50} would be higher therefore if the singularly reduced form of plastoquinone was prevalent due to a stronger competition with DCMU for the active site (Lavergne 1982). Such a situation could develop if the increased charge stabilisation elevated the rate of the first reduction of B but not the second. Wraight (1979) suggested that at high *p*H the first electron turnover followed by protonation would precede the second with a slower rate constant. This mechanism is in agreement with these results.

Figure 6·9 summarises the above observations whereby protonation elevates both the back reaction and the efficiency of DCMU binding. The suggestion by Melis and Homann (1975) that acidification led to a higher rate of charge recombination is not the only explanation for their data, or this data. For example, at low *p*H the B-protein could itself be protonated and as a result its interaction with Q could be decreased (Bowes *et al* 1983). This molecular rearrangement would mean that the rate of B reduction could fall, causing reduction of Q. Since the rate constant for



Low *p*H High *p*H

Figure 6·9: A model representing changes in the pathways of electron movements following alterations in *p*H. Horizontal arrows indicate electron flow, vertical arrows represent charge seperation and recombination. DCMU binding is shown by straight lines. Darker lines are indicative of stronger or prefered events.

excited state decay must equal that for its formation (figure 4·1 and discussion), k_h may be expected to increase. As the B-protein becomes more oxidised the I_{50} for DCMU inhibition would decrease. Thus, DCMU inhibition

is affected by the *p*H, and probably other factors like the ionic balance.

The *p*H dependency of the first and second DCMU binding components shown in figure 6·8 are similar to the rate constant plots (k_α and k_β respectively) of Melis & Homann (1975), suggesting that the Iso for DCMU of β-centres may be 10~20 fold larger than that of α-centres. Furthermore, the *p*H profile of the low affinity binding phase may also be related to β-centres also.

SINGLE ADDITION OF 2mM DCMU IN THE STEADY-STATE

These experiments were performed in a manner similar to the steady -state titrations, except that 2mM DCMU was introduced as a single addition. After the fluorescence yield had remained stable for several minutes, the second light was added, fully closing all of the centres and attaining the 'true F_m' level.

From figure 6·10 it can be seen that a single DCMU addition resulted in a biphasic rise to the terminal level of fluorescence. The first phase was very rapid and accounted for about 44·5% of F_m. The second phase, lagging after the first and contributed 17·5%, and had a half time of 30 seconds. During the lag between the phases a fluorescence transient was sometimes seen.

As only two and not three phases were resolved it might be possible that the intermediate phase was around the transient or masked by the initial rise, although kinetic resolution of three phases may not be expected. It was found that an addition of 50μM DCMU elevated the fluorescence in a single fast rise, whilst further additions (up to 2mM) slowly raised the signal to the terminal level. In the following experiments the amplitude of the first phase was treated as the sum of the

first and second phases discussed previously.

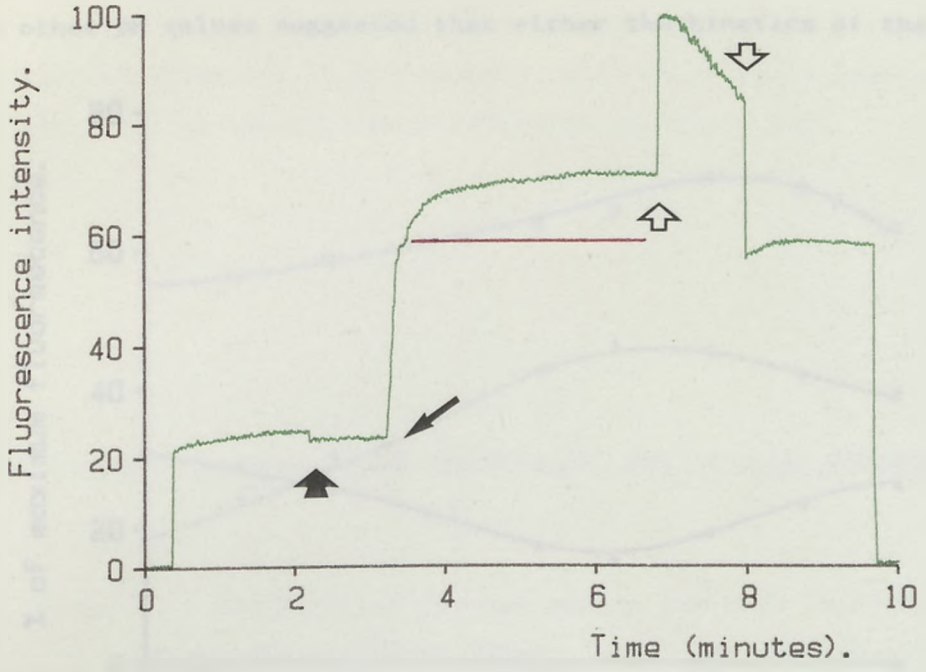


Figure 6·10: Fluorescence change upon addition ( ) of 50μM (——) or 2mM DCMU (——). Adjustment of PMT voltage to normalise F_i ( ). Additional light on ( ) and off ( ). See figure 6·3 and text for details.

The effects of *p*H on the various fluorescence transients was studied by adjusting the *p*H of the medium prior to chloroplast rupture as detailed for the steady state titrations. The *p*H range was extended to *p*H 9·5 by using 50mM Ches/KOH in addition to the buffers listed previously, other conditions were identical.

The relative amplitude of the two phases and the total rise were calculated and are expressed as the percentage of F_m. These values are plotted in figure 6·11 against the *p*H of the medium used. It can be seen that the largest total rise in fluorescence upon DCMU addition was 70% and optimally detected at *p*H 8·5. The fast phase showed an optimum at *p*H 8·0 with a 46% rise. Away from either of the optimum *p*H values resulted in a loss of fluorescence. The slower phase however showed a different trend, from *p*H 5·5 to 8·0 the fluorescence fell from 31 to 15% of F_m and then rose to 26% at *p*H 10·0. Thus, the fast and slow phases generally behave in an antiparallel manner, but show different *p*H optima.

Around *p*H 8·0 the amplitude of the fast fluorescence rise was at a maximum, the slow phase being at a minimum. The diminution of the fast phase at other *p*H values suggested that either the kinetics of the DCMU

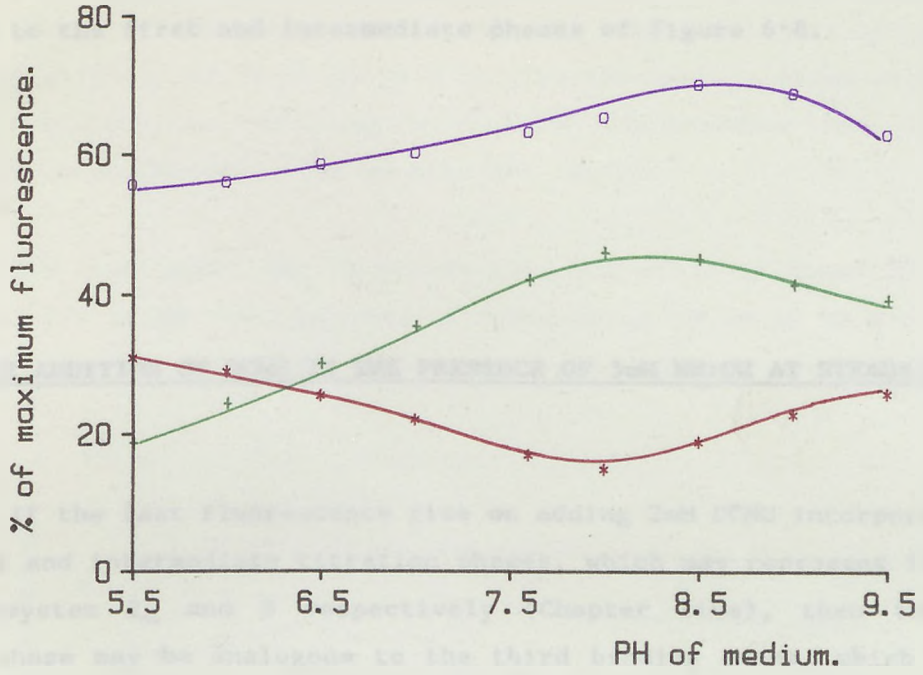


Figure 6·11: Plots of amplitude of the fluorescence rise up on DCMU addition verse *p*H. First (—·—), final (——) and total (——) changes. Details as for figure 6·10.

response have changed (some centres may show a slower fluorescence rise, possibly due to a lower DCMU affinity and therefore a weaker binding event), which would support the elevation of the slow phase, or *p*H induced quenching processes may be occurring. At other than ~*p*H 8·0, the size of the slow phase is increased which could be due to a slowing down of a proportion of the faster responding centres.

These *p*H profiles are similar to those presented in figure 6·7. The third DCMU binding phase detected by the titration showed decreased response over the *p*H range 5·0→8·0, as did the slow phase for the single addition. The *p*H optimum of the fast phase for the experiments of figures 6·10 and 6·11 are very similar to the first phase plot of figure 6·9. Furthermore, the slewed appearance of the *p*H profile was conserved. From these two experiments it may be concluded that the fast fluorescence rise upon addition of DCMU could be the same as the first and intermediate DCMU binding phases (as determined by the titration experiments). The slow

responding phase to a 2mM DCMU addition being the third kinetic phase.

It might be predicted that if the fast fluorescence rise could be resolved into two distinct kinetic phases, then these would have similar *p*H profiles to the first and intermediate phases of figure 6·8.

SINGLE ADDITION OF DCMU IN THE PRESENCE OF 5mM NH_2OH AT STEADY-STATE

If the fast fluorescence rise on adding 2mM DCMU incorporates both the first and intermediate titration phases, which may represent inhibition of photosystem 2_α and β respectively (Chapter five), then the slower kinetic phase may be analogous to the third binding event, which based on its I_{50} *p*H profile may also be a type of β-centre. Changes in the back reaction rates of these membrane components might be expressed in terms of their *p*H profiles of DCMU response. In order to examine this possibility, hydroxylamine (NH_2OH) was added to thylakoids in the dark. A high concentration of NH_2OH is known to be an inhibitor of photosystem 2, but at lower concentrations it behaves as an electron donor, diminishing the back reaction by rapidly reducing the oxidised equivalents stored on the secondary donor (Bennoun 1970, Homann 1971 and Cheniae & Martin 1971). It has a pronounced effect on β-centres as β_{max} is diminished in its presence (Melis and Homann 1975). The *p*H titration of the fluorescence response to a single DCMU addition may be affected by NH_2OH treatment.

The relative amplitudes of the two phases were calculated at each *p*H and expressed as a percentage of F_m. These results are plotted in figure 6·12. By comparing the figures 6·12 and 6·11 it can be seen that addition of 5mM NH_2OH reduces the protonation effects over the range *p*H 5·5 to 9·5, resulting in a diminished response for all three plots. For the total rise, optimal response (68·5%) was detected at *p*H 7·0, this being 2% lower than the control. At *p*H 5·5 and 9·5 both fluorescence values were 64%, the former being about 8% larger than the control whilst the latter

was the same. Thus addition of NH_2OH results in an increase of the fluorescence rise and a slight shift in the *p*H optimum.

After NH_2OH addition the slow phase shows almost no response to *p*H changes. At *p*H 5·5 the fluorescence value is 16%, falling to 10% at *p*H 8·5 and rising slightly to 10·2% by *p*H 9·5. For the control these were: 31, 15 and 26% respectively, resulting in a total fluorescence loss at all *p*H values but an exaggerated loss at high and low *p*H.

The fast phase was flattened too, but still retained an elevated optimum of 57% at *p*H 7·0 (compared with 64% minus NH_2OH at *p*H 8·0). At *p*H 5·5 the fluorescence value was almost 20% higher than the control, and the

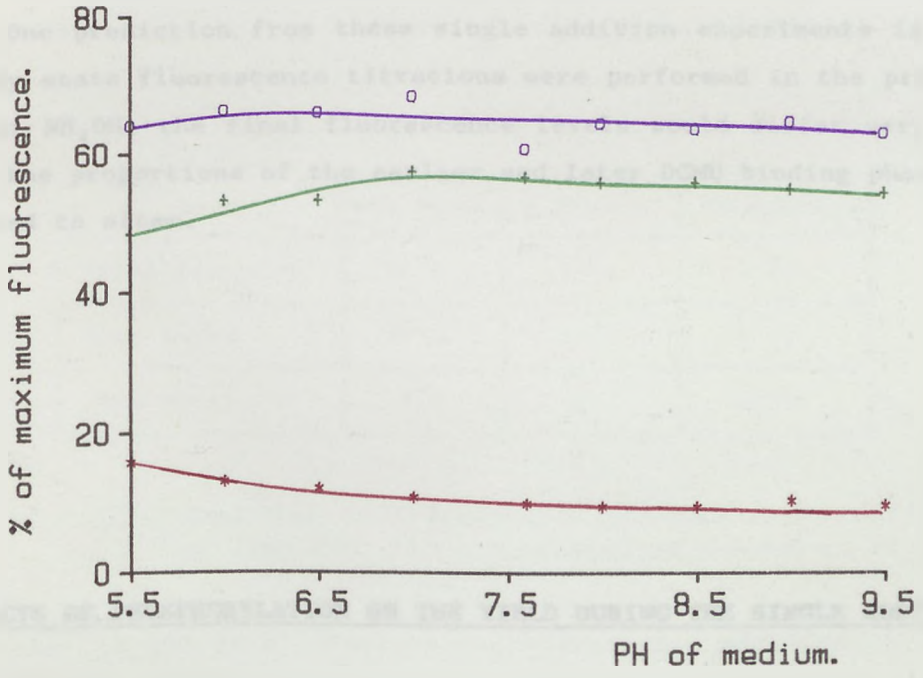


Figure 6·12: Plots of fluorescence response to DCMU additions at various *p*H in the presence of 5mM NH_2OH. First (——), final (– – –) and total (—·—) rise events.

value at *p*H 9·5 also increased by 10% to 55% of F_m. The profile of the fast phase is similar to the slow and total changes, but to a lesser extent; the fast phase increased in amplitude but retained some of the original *p*H profile.

The similarities of the increase in the fast phase component and reduction of the slow phase for the DCMU addition at extremes of *p*H may

indicate a gain of the faster responding centres at the expense of the slower ones. Such an interchange of kinetics may suggest that NH_2OH interacts very strongly with the slower phase components. The mechanism by which this occurs is unknown, but NH_2OH may preferentially donate electrons to the kinetically slower centres, stabilizing the charge separation more efficiently, this could apparantly increase the rate of trapping (by decreasing the rate constant for back reactions) and alter the proportions of fast and slow phases, the effect being less pronounced at neutral *p*H. However these results can be interpreted simply as a change of only one phase (perhaps the slower one) which, due to the normalisation procedure used causes an apparent change in more than one component. However un-normalised data also shows these trends.

One prediction from these single addition experiments is that if the steady state fluorescence titrations were performed in the presence or absence of NH_2OH, the final fluorescence levels would differ very little, although the proportions of the earlier and later DCMU binding phases might be expected to alter.

EFFECTS OF PHOSPHORYLATION ON THE YIELD DURING THE SINGLE ADDITION

Samples of chloroplasts were incubated ±ATP under protein kinase (thylakoid-located) activating conditions (Bennett 1977). The effects on DCMU binding were studied initially by the single addition method and then by the titration technique.

The conditions used to stimulate membrane phosphorylation were as follows: 3ml of ruptured uncoupled chloroplasts (with 2μM gramicidin D) were dark adapted for 20 minutes. The phosphatase was inhibited with 10mM NaF (Bennett 1980), and 0·2mM ATP added to the samples but omitted from the controls. Both the treated and untreated thylakoids were placed in a water

bath at 20°C and exposed to saturating white light to activate the protein kinase (Bennett 1977). After ten minutes illumination 10mM NaF was then added to the control and both samples were dark adapted on ice for ten minutes. To demonstrate that phosphorylation had occurred 0·5ml of each sample was inhibited with 50μM DCMU and F_m measured. This provided a measure of the extent of phosphorylation; after phosphorylation, LHCP is believed to detach from photosystem 2, and as a result the fluorescence yield falls. Generally fluorescence from the ATP treated samples was 8 to 14% lower than the controls. To the remaining 2·5ml of each treatment 100μM methyl viologen was added as an electron acceptor for the addition experiments (see figure 6·10).

The averaged results of single DCMU addition experiments at *p*H 7·6 are tabulated below. It can be seen that after phosphorylation there was

	Non phos.	Phos.	Non/phos.
Total response	56·2	54·1	1·04
Fast phase	39·6	34·5	1·15
Slow phase	15·8	21·1	0·81

Table 6·1: Amplitude of fluorescence increase upon addition of 2mM DCMU. Data expressed as percentage of F_m.

little change in the total fluorescence change upon addition of 2mM DCMU. This result was expected because the level of fluorescence at F_i was adjusted to the same point for each experiment. Thus, had a large total DCMU change been observed, it could have been concluded that *spillover* had altered in addition to a change in the portion of energy reaching photosystem 2 (β).

The fast phase however exhibited a 5% fluorescence quenching, as indicated by the ratio of non/phos (table 6·1), whilst the level of the slow phase fluorescence is increased by about 5%. This data suggests that phosphorylation, like the *p*H effect (see figure 6·11) could decrease the amplitude of the fast phase by increasing the deactivating processes

competing for the dipole (i.e., charge recombination), the reverse being true for the slow phase. Alternative explanations would be a migration of LHCP away from the faster responding centres or the conversion of the fast DCMU responding phase into the slower one. The mechanism by which this could be achieved is unknown but addition of a phosphate group or a proton to a polypeptide probably causes gross alterations in the surface charge density of that protein. Such a change could influence the spatial relationships and interactivity of the various components of the photochemical apparatus, which in turn may effect processes like dipole stabilization and back reactions.

Melis and Homann (1975) demonstrated that low *p*H did indeed elevate the rate of back reactions and as a result both k_α and k_β were reduced. The response of k_α was linear with *p*H. A *p*H titration would decrease k_α and apparently increase β_{max}. Thus the earlier suggestion that low *p*H and phosphorylation convert the fast DCMU responding phase into the slower one could be modified by suggesting that a proportion of the fast phase is modified and is apparently converted to the slower one.

Under conditions of high light intensity the plastoquinone pool could be heavily reduced and a large ΔpH might develop. These situations could be reversed by reducing the antennae size of the trap or if the effective rate of charge separation decreased. This could also be achieved if the dipoles (P_{680}^+/Q^-) were destabilized, causing energy dissipation by back reactions. Protonation of membrane components might cause direct de-activation of the traps by increasing k_h of equation 4·1 as previously proposed by Briantais *et al* (1979) and Krause *et al* (1982 and 1983). The reduced plastoquinone pool would activate the protein kinase and membrane phosphorylation would occur. Apart from phosphorylating LHCP, and thus lowering the initial proportion of light harvested by photosystem 2; thylakoid protein phosphorylation could specifically promote back reactions within photosystem 2, thus opening the traps and quenching fluorescence. An additional role for membrane phosphorylation is thus identified as a protective mechanism for photosystem 2. This suggestion is in agreement with Horton & Lee (1983 and 1984).

Equation 6·1 is an unconventional modification of the basic equation for fluorescence (equation 4·3), allowing full intergration of the

constants in relation to the extinction cross-section.

$$\Phi F = I_o . \Sigma . \frac{k_f}{k_f + k_t + k_p + k_h} \qquad (6 \cdot 1).$$

Where: ΦF= Fluorescence yield
I_o= Incident light intensity
Σ = Photosystem extinction
k_f= Rate constant of fluorescence
k_t= ————‖———— energy transfer
k_p= ————‖———— photochemistry
k_h= ————‖———— de-excitation

Upon phosphorylation of the membrane, the fluorescence yield would be lowered by detaching LHCP from photosystem 2 (Chow *et al* 1981, Horton & Black 1981a and 1982, Andersson *et al* 1982 and Kyle *et al* 1983b) thus effectively lowering the extinction or absorption cross-section of the trap, possibly altering spillover by increasing k_t (Haworth *et al* 1982 and Kyle *et al* 1982), and elevating k_h.

Protonation resulting from a high ΔpH would elevate k_h, but it may also alter the absorbance cross-section of photosystem 2 by affecting the coupling of LHCP to the core, which is known to be controlled by cations (Butler 1978a, Strasser and Butler 1977 and Butler 1980).

These differences in response to DCMU additions for ATP treated and control samples suggested that the appearance of the steady state titrations would be different. The steady state fluorescence yield of phosphorylated thylakoids was titrated (see figure 6·3) at *p*H 7·6.

Averages of several experiments are expressed as a percentage of F_m verses DCMU concentration (figure 6·13). The upper trace represents an untreated sample, the lower traces are the non-phosphorylated control and the ATP treated samples respectively. The first striking feature is that during these experiments, ten minutes illumination reduced the overall fluorescence response to DCMU by about 7% when compared to the data presented in figure 6·4. Since the F_i level of each sample was normalised, specific fluorescence lowering due to LHCP migration can be accounted for

(after phosphorylation F_i was 10→15% lower than the control, suggesting

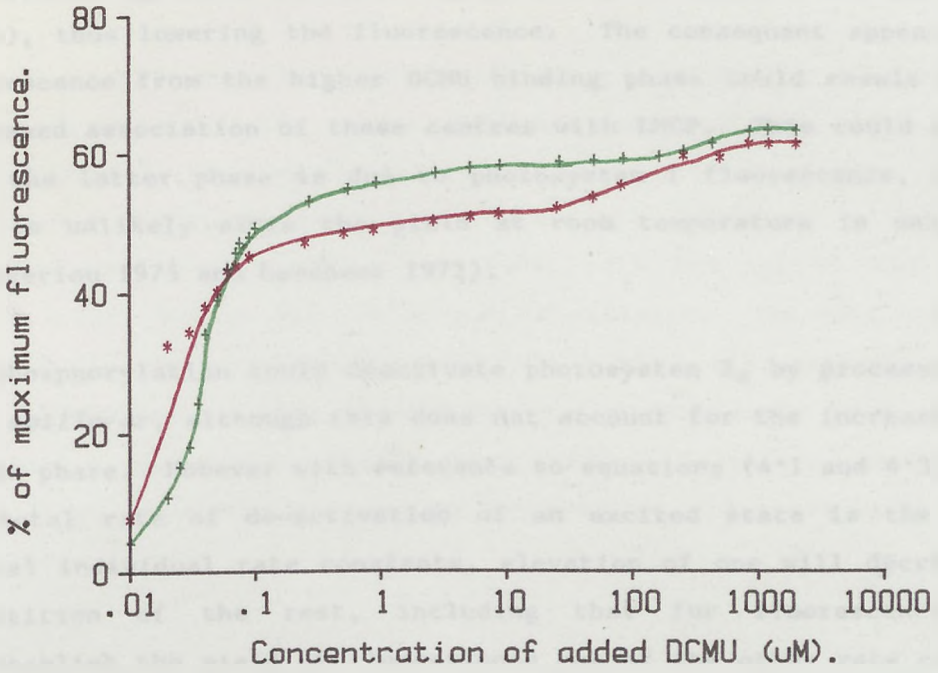


Figure 6·13: DCMU fluorescence titrations of phosphorylated (——) and non-phosphorylated (——) thylakoid samples. Performed according to figure 6·3. See text for details.

that the kinase had been activated). At F_t there was a slight loss of fluorescence which indicated a change in the effective F_v/F_m ratio. This may be an indication of a photo-inhibitory effect during the ten minute incubation to activate the protein kinase.

When comparing the phosphorylated and control thylakoids it can be seen that the first and second DCMU inhibitory phases are lowered from about 57 to 50%. However the final level of fluorescence remained fairly constant at about 60%. It may be concluded that the decrease of fluorescence during the first phase of the titration is accounted for at higher DCMU concentrations, as the yield of fluorescence increased. This may be explained as follows:

(1) phosphorylation increases fluorescence from the 'high DCMU component' by elevating re-absorption or quenching of fluorescence from the 'lower DCMU component' (for example by an energy transfer type of mechanism).

(2) loss of the early phase could be due to migration of LHCP away from photosystem 2_α (Chow *et al* 1981, Horton & Black 1982 and Kyle *et al* 1983b), thus lowering the fluorescence. The consequent appearance of fluorescence from the higher DCMU binding phase could result from an increased association of these centres with LHCP. This could indicate that the latter phase is due to photosystem 1 fluorescence, although this is unlikely since the yield at room temperature is only small (Papageriou 1975 and Goedheer 1972).

(3) phosphorylation could deactivate photosystem 2_α by processes other than *spillover*, although this does not account for the increase in the slower phase. However with reference to equations (4·1 and 4·3), where the total rate of de-activation of an excited state is the sum of several individual rate constants, elevation of one will decrease the competition of the rest, including that for fluorescence. To re-establish the yield of fluorescence one of the other rate constants must be decreased.

(4) the higher affinity DCMU component was 'converted' into the lower affinity form. It has been shown, for example, that phosphorylation quenches fluorescence and may convert α-centres into β-centres (Kyle *et al* 1982). From a closer examination of figure 6·13 it might be concluded that the amplitude of the intermediate phase was conserved during the phosphorylation process. Thus fluorescence from the first phase (possibly associated with photosystem 2_α) is lost, but not that from the intermediate phase (which may emanate from photosystem 2_β).

Another feature of the titration of phosphorylated thylakoids was the shift of the sigmoidal phase towards lower DCMU concentrations. Such a phenomenon may be predicted from the work of Shochat *et al* (1982) and was reported by Vermaas *et al* (1984). Here the I_{50} was decreased by about 59%. The gradient of the sigmoidal region of the titration decreased after phosphorylation, indicating a loss of sigmoidicity. This is supported by the observation of LHCP migration from the photosystem 2 rich stacks upon phosphorylation (Andersson *et al* 1982 and Kyle *et al* 1983b) and is associated with a decreased sigmoidicity of the fluorescence induction curve (Kyle *et al* 1982 and Haworth *et al* 1982), as LHCP is believed to

mediate energy transfer between photosystem 2 units (Armond *et al* 1976, Strasser & Butler 1977, Butler 1980 and Haworth *et al* 1982).

In conclusion, fluorescence titration with DCMU of phosphorylated thylakoids revealed that the I_{50} for DCMU was decreased from 0·036→0·015μM. Some photosystem 2_α associated sigmoidicity may be lost, and the amplitude of fluorescence from these centres was decreased. It cannot be stated that the β-centre emission (possibly associated with the second DCMU binding phase) increased, but it did not appear to diminish. The increase in the amplitude of fluorescence from the high DCMU binding site reflects the loss from the lower phase. This data supports the previously held view that phosphorylation may help to protect photosystem 2_α by slowing down the rate of trap closure, possibly by creating centres that are kinetically similar to photosystem 2_β.

CHAPTER SEVEN

SPECTRAL STUDIES OF CHLOROPHYLL FLUORESCENCE

According to *Stoke's Law* chlorophyll fluorescence is red-shifted with respect to absorption, and the wavelength range of emission is generally narrower than the absorbance bands (see Chapter four and figure 4·1). Measurement of fluorescence spectra of known chlorophyll containing pigment complexes allows classification of the various thylakoid components (Butler & Kitajima 1975 and Butler 1978). Spectral interpretation however is very complex.

In this chapter chlorophyll fluorescence was recorded as a function of wavelength. Since different photocentres exist, variations may be seen in the spectral properties of the pigment systems. Indeed, Thielen *et al* (1981) demonstrated that the absorbance spectrum of photosystem 2_β is red-shifted by about 10nm with respect to photosystem 2_α, and based on *Stokes Shift*, the fluorescence spectra could be expected to show similar trends. Moreover, if differences in DCMU binding are postulated, spectral changes associated with specified concentrations of DCMU may become apparent.

METHODS

Chloroplasts were prepared daily (see Chapter two) and dark stored on ice at a concentration of about 3mg/ml of chlorophyll. The chloroplast sample (equivalent to 200μg of chlorophyll) was osmotically shocked and diluted in reaction medium; providing the following final concentrations: 0·33M sorbitol; 50mM Hepes; 5mM $MgCl_2$ and 2mM EDTA, buffered to *p*H 7·6 with KOH. Before measurement 100μM methyl viologen and 2μM gramicidin D were added. The reaction medium was stirred, maintained at 20°C, and illuminated with actinic light of $1w.m^{-2}$ (defined by heat and Corning CS 4-96 filters) for about five minutes before recording the fluorescence spectrum.

The electronic and optical equipment was assembled similar to that depicted by figure 4·7. However, fluorescence was detected at a right angle to the actinic beam by a photomultiplier tube *via* an optical fibre

and an Applied Photophysics f3·4 grating monochromator. The data was amplified by a lock-in amplifier (300ms time constant) and recorded on the DL 4000B system. Abscissa resolution was 1024 points sampled at 80ms intervals, and the fluorescence scanned from 640→804nm at a speed of 2nm/second. This combination of scan rate, time constant and sweep interval was found to be optimal. Individual spectra were averages of four scans, and the data presented here are a mean of several of these. Curve smoothing routines were applied to difference data following curve subtraction.

RESULTS AND DISCUSSIONS

Figure 7·1 represents the results of a experiment where the fluorescence was scanned with zero and 50μM DCMU present. The spectra were normalised to the maximum fluorescence level. It can be seen that the fluorescence is greatest at about 687nm, but there is a large shoulder

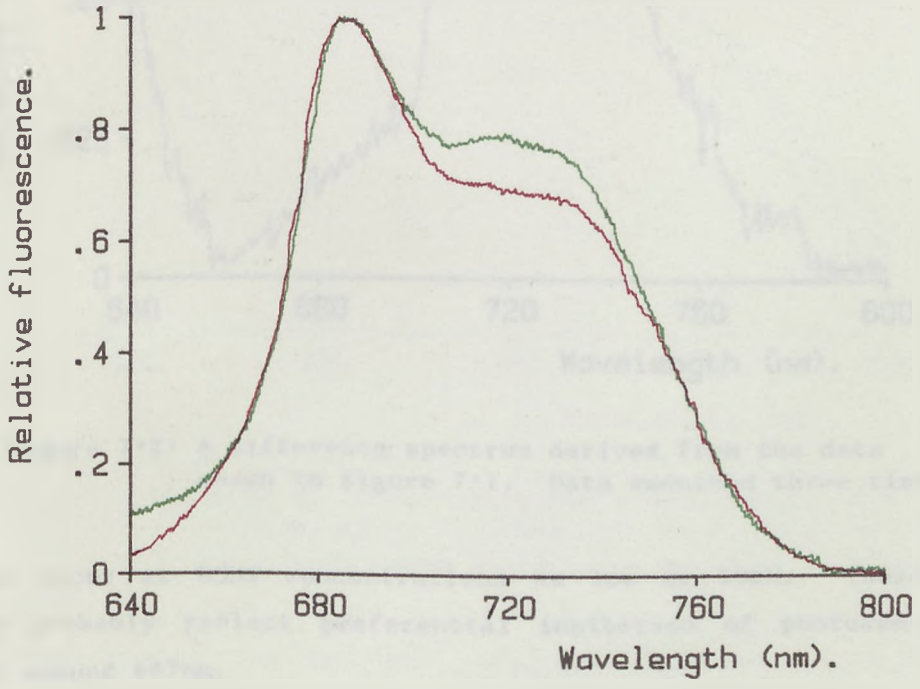


Figure 7·1: Fluorescence scans from 640→800nm in the presence (——) and absence (– – –) of 50μM DCMU.

around 725nm. In the absence of DCMU (a situation similar to F_i) the height of the shoulder was constantly found to be ~0·8, whilst in the

presence of 50μM DCMU it fell to about ~0·7. The apparent loss of fluorescence from this shoulder probably reflects the normalisation procedure of the whole curve to the 680nm peak, as an elevation of the peak would be perceived as a loss of fluorescence from the shoulder. In figure 7·2 below, a difference spectrum is shown for these two curves. The data shows a maximum at 725nm and the spectral difference is believed to arise from an uninhibited photocentre.

Horvath *et al* (1984) report an F_v quenching at elevated DCMU concentrations (300μM), which could result in loss of fluorescence at longer wavelengths. However, it is felt that such a chaotropic effect

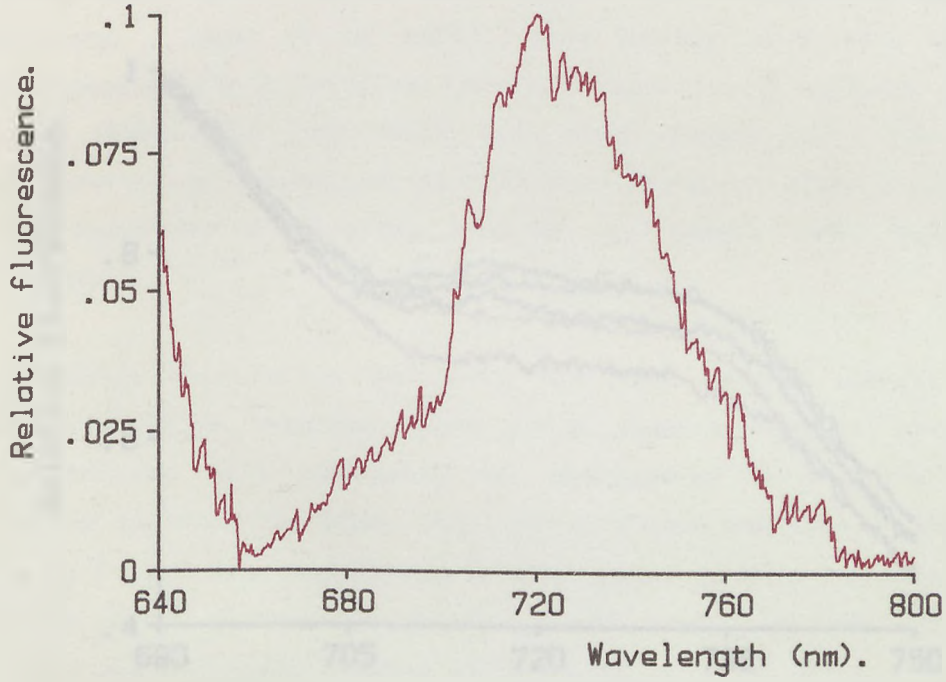


Figure 7·2: A difference spectrum derived from the data shown in figure 7·1. Data smoothed three times.

would not occur at DCMU concentrations as low as 50μM. These changes therefore probably reflect preferential inhibition of photocentres that fluoresce around 687nm.

Figure 7·3 shows the changes in fluorescence from 700→750nm upon addition of varying DCMU concentrations. It can be seen that addition of 10μM DCMU lowers the fluorescence at 725nm to 0·725 and that the presence of 50μM DCMU results in a level of 0·688. Further additions however

reverse this trend, 500μM produced a level of 0·725 whilst 2mM DCMU resulted in a plot that was very similar to the F_i curve (0·765 compared with 0·835).

As suggested above, the fluorescence loss incurred at DCMU levels up to 50μM could have resulted from a specific quenching process at longer wavelengths. However the reversibility of this transition at higher concentrations indicates that this may not be the case, unless specific quenching of the 687nm components only occur at DCMU concentrations ≫50μM . Another explanation could be inhibition of the 680nm fluorescent centres by lower concentrations of DCMU (a binding event with high affinity) whilst maximum inhibition of the 725nm fluorescent centres only occurs at

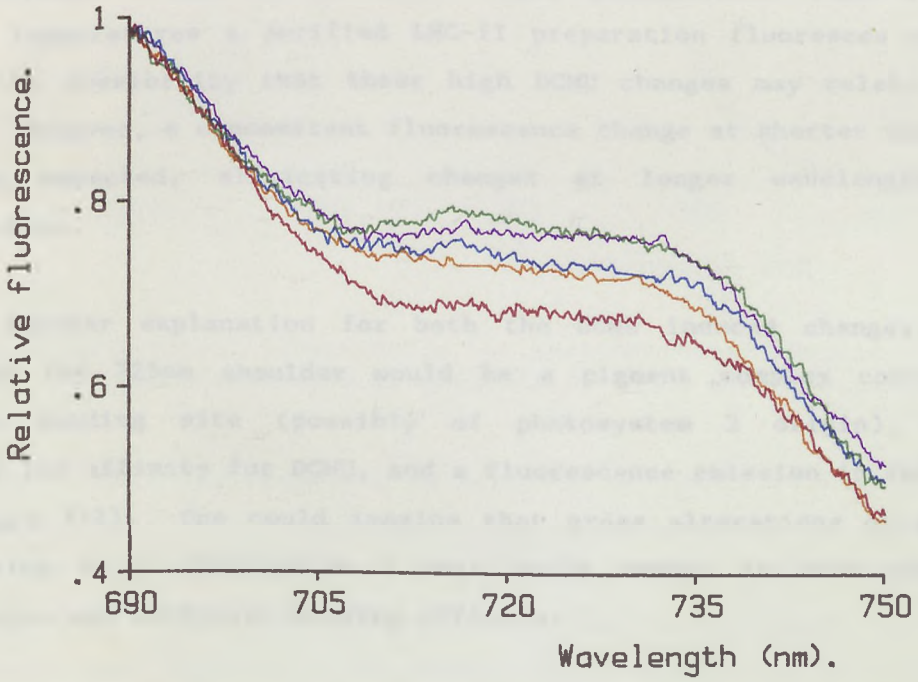


Figure 7·3: Scans of fluorescence for the following DCMU concentrations: 0 (——), 10 (——), 50 (——), 500 (——) and 2000μM (——). See text for details.

concentrations approaching 2mM DCMU, thus elevating the longer wavelength region and re-establishing a spectrum similar to the uninhibited one.

The identity of the fluorescent species at 725nm is unknown. It could be photosystem 1, but the yield of photosystem 1 fluorescence at room temperature is extremely small (Goedheer 1972, Papageriou 1975 and Kyle *et al* 1983a). Vrendenberg & Slooten (1967) showed that the yield of

fluorescence of isolated photosystem 1 particles remains constant under conditions in which electrons are transferred from P_{700} to $NADP^+$. At liquid nitrogen temperature photosystem 1 fluorescence can readily be detected (Butler & Kitajima 1975 and Butler & Strasser 1977), the yield often being larger than that of photosystem 2. However, Kyle *et al* (1983a) demonstrated that after phosphorylation longer wavelength fluorescence at room temperature increased, and was attributed to photosystem 1.

An increase in fluorescence from photosystem 1 upon DCMU addition is unlikely, as the herbicide binding protein has been shown to be part of the photosystem 2 complex (Pfister *et al* 1981 and Arntzen *et al* 1983), unless chaotropic effects specific to photosystem 1 occur at higher DCMU concentrations. Fuad *et al* (1983) have demonstrated that at liquid nitrogen temperatures a purified LHC-II preparation fluoresces at 736nm, raising the possibility that these high DCMU changes may relate to this species. However, a concomitant fluorescence change at shorter wavelengths might be expected, eliminating changes at longer wavelengths after normalisation.

Another explanation for both the DCMU induced changes and the origin of the 725nm shoulder would be a pigment complex containing a herbicide binding site (possibly of photosystem 2 origin), with an unusually low affinity for DCMU, and a fluorescence emission in the far-red (see figure 7·2). One could imagine that gross alterations of molecular organisation of a photosystem 2 unit could result in both changes of fluorescence and herbicide binding affinity.

The data presented previously suggests that high concentrations of DCMU predominantly affect a photocentre similar to photosystem 2_β. Moreover, low *p*H and phosphorylation were shown to increase the amount of this component. It might be expected that phosphorylation could affect the fluorescence spectrum. To study this further, the membranes were either phosphorylated in the presence of 0·2mM ATP or simply illuminated (see Chapter six), providing the non-phosphorylated control. During these experiments the 'phosphorylation test' showed that phosphorylation had indeed occurred, as the fluorescence quenching was in the order of 15→25% of the control samples.

From figure 7·4 the effect of membrane phosphorylation on the spectral properties of chlorophyll fluorescence can be seen. Phosphorylation results in an elevation of the 725nm shoulder by ~0·05 units relative to the 687nm peak:

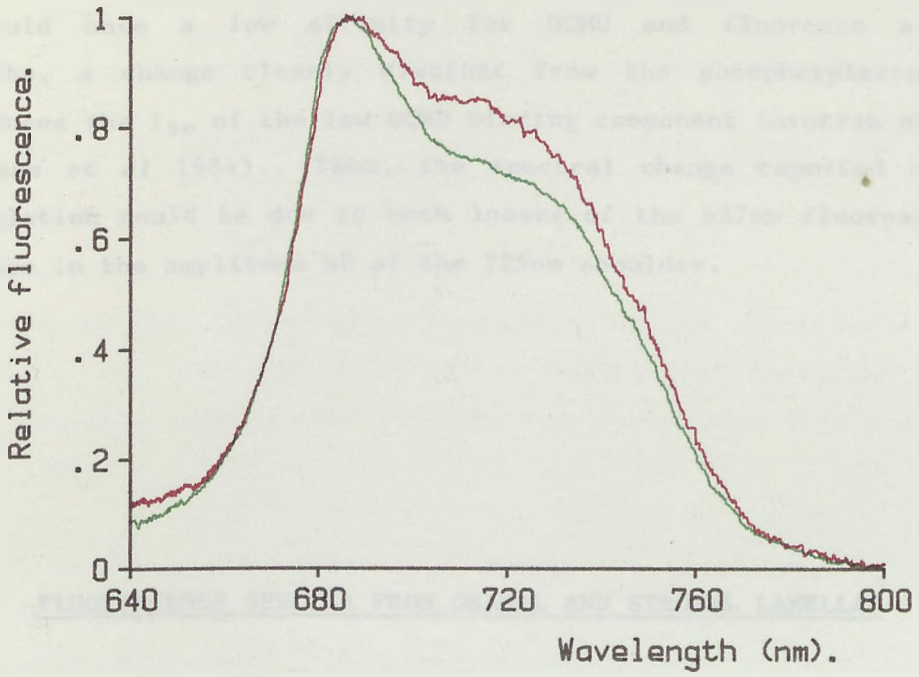


Figure 7·4: Scans of fluorescence for phosphorylated samples (——) and non-phosphorylated controls (——) in the absence of DCMU.

Membrane phosphorylation is believed to result in LHCP detachment from photosystem 2_α and consequential migration into the stromal lamellae (Chow et al 1981, Anderson *et al* 1982 and Kyle *et al* 1983b) where association with photosystem 1 may occur. After such a change, photosystem 2 fluorescence was shown to be decreased (Horton & Black 1981a & 1983, Kyle *et al* 1983b and Telfer *et al* 1983). These results may be readily explained if the long wavelength shoulder is attributed to photosystem 1 emission at this temperature. If the shoulder is not attributed photosystem 1, then the **relative** loss of the 687nm peak could also be explained by the *state 1→2 transition*, as suggested by Kyle, Baker & Arntzen (1983a). The proposal that LHC-II fluoresces at ~730nm, albeit at low temperatures (Fuad *et al* 1983) would also provide a possible explanation of these results.

Other explanations would include an elevation of the 725nm shoulder as a result of improved trapping efficiency or a larger effective

antenna, or possibly an increased rate of back reaction (k_h) within the trap of the P_{680} component. This would increase the apparent height of the shoulder by quenching the fluorescence yield of the peak. An increase in charge recombinations could be achieved by converting normal photosystem 2_α centres into the centres described in Chapter six (unusual β-centres?). These would have a low affinity for DCMU and fluoresce at longer wavelengths, a change clearly distinct from the phosphorylation effect which reduces the I_{50} of the low DCMU binding component (Arntzen *et al* 1983 and Vermaas *et al* 1984). Thus, the spectral change reported here upon phosphorylation could be due to both losses of the 687nm fluorescence and an increase in the amplitude of of the 725nm shoulder.

FLUORESCENCE SPECTRA FROM GRANAL AND STROMAL LAMELLAE

The lateral heterogeneity of the thylakoid lamellae is largely due to the diffusion properties of the various protein complexes of the membrane and their environment. Their relative positions are determined by electronic interactions with membrane lipids, other complexes, the structured layer of water surrounding the bilayer and themselves (Barber 1980 a and b).

A general description of the Z-scheme components and thylakoid topography can be found in Chapter one. Membrane fractionation (see Chapters two and three) followed by scanning of fluorescence emission provides a very simple technique to study pigment distribution following various treatments.

The chlorophyll *a/b* ratios of the unfractionated thylakoids, and the granal and stromal lamellae were determined by the methods of Arnon (1949), are tabulated below. From table 7·1 it can be seen that after separation, the granal pellets had chlorophyll *a/b* ratios lower than the unfractionated samples, whilst the stromal pellets had much higher ratios;

as reported by Chow *et al* (1981). This suggests a preferential

	Unfractionated	Granal	Stromal
CHL *a/b*	3·42	2·04	5·84

Table 7·1: Chlorophyll *a/b* ratio for control, granal and stromal lamellae. Means of ten experiments.

accumulation of LHCP in the grana compared with the unappressed lamellae (Andersson *et al* 1982 and Kyle *et al* 1983b) and indicates that the separation largely removed the LHCP depleted photosystem 1 rich thylakoids (Anderson 1981).

It was generally found that during the preparation of thylakoid fragments prior to differential centrifugation changes in the chlorophyll *a/b* ratio were observed as reported in Chapter three.

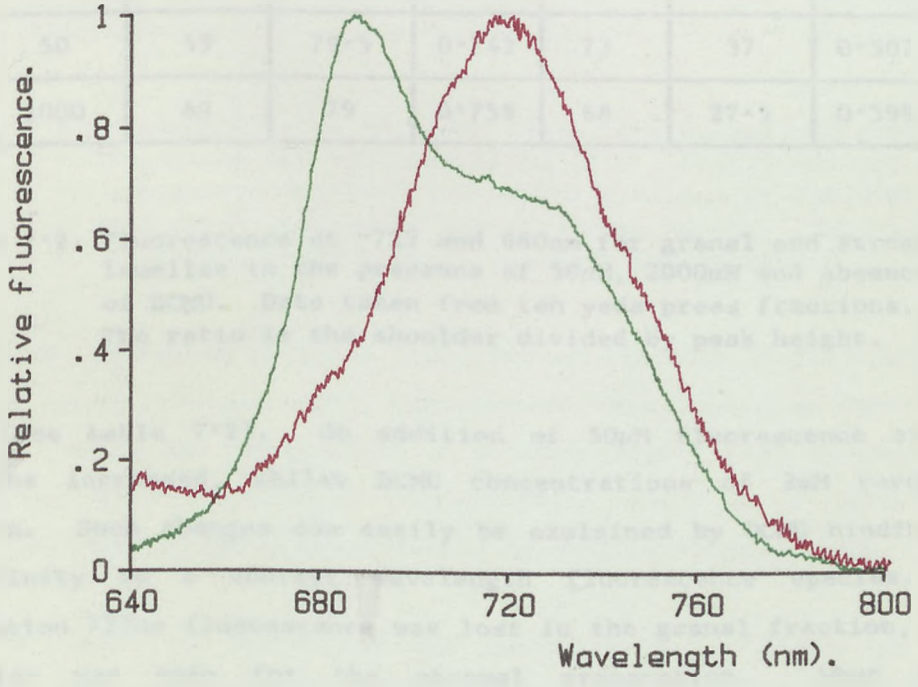


Figure 7·5: Fluorescence scans of granal (——) and stromal (——) lamellae in the absence of DCMU. Details as for figure 7·4.

From figure 7·5 it can be seen that granal and stromal membrane preparations have very different normalised fluorescence spectra both to each other and to the unfractionated membranes (see figure 7·1 for comparison). The granal spectrum displays a peak at 689nm with a reduced shoulder (0·72). In contrast, the stromal lamellae have a peak at 722nm and only a slight shoulder at ~680nm. Such observations suggest that the longer wavelength fluorescence species is largely localised within the stromal lamellae; but because the granal preparation consists of both partitions (appressed lamellar regions), and end membranes and margins (unstacked lamellae), it was not possible to quantitate the spectral distribution precisely. The underlying trends however do suggest a lateral segregation.

The fluorescence scans of figure 7·5 were measured in the absence of DCMU (ie at F_i), although herbicide addition produced predictable

	GRANAL LAMELLAE			STROMAL LAMELLAE		
DCMU	'722'	'680'	RATIO	'722'	'680'	RATIO
0	60·5	79	0·766	74	27·5	0·372
50	59	79·5	0·742	73	37	0·507
2000	60	79	0·759	68	27·5	0·399

Table 7·2: Fluorescence at ~722 and 680nm for granal and stromal lamellae in the presence of 50μM, 2000μM and absence of DCMU. Data taken from ten yeda press fractions. The ratio is the shoulder divided by peak height.

results (see table 7·2). On addition of 50μM fluorescence at shorter wavelengths increased, whilst DCMU concentrations of 2mM reversed the transition. Such changes can easily be explained by DCMU binding with a high affinity to a shorter wavelength fluorescence species. After normalisation 722nm fluorescence was lost in the granal fraction, whilst a 689nm rise was seen for the stromal preparation. When the DCMU concentration was increased to 2mM, an elevation in the 722nm shoulder for the grana was seen and a loss of 680nm fluorescence in the stromal membranes was observed.

The 680nm shoulder seen with the stromal lamellae could be derived from photosystem 2 contamination, or it may be a unique stromal photosystem 2 population. This view would be supported by the report that photosystem 2_β is located in the unappressed lamellae (Anderson & Melis 1984). Stromally located LHCP would provide an alternative explanation for this observation. Under the ionic conditions used here, a very low yield of fluorescence would be expected from LHCP directly, due to strong coupling and very efficient energy transfer to either photosystem 1 or photosystem 2 (Butler & Strasser 1977 and Rijgersberg *et al* 1979). It is possible however, that 100% energy transfer is not very probable in fractionated lamellae, and could result in a low yield of 680nm fluorescence in these stromal membrane preparations. This explanation does not account for a 680nm rise upon addition of low DCMU concentrations, -unless 0→50μM causes a specific 722nm fluorescence quenching.

	NON-PHOSPHORYLATED			PHOSPHORYLATED		
	'722'	'680'	RATIO	'722'	'680'	RATIO
GRANAL	56	79	0·709	64	79	0·810
STROMAL	75	18	0·240	75	25	0·333

Table 7·3: Fluorescence at ~722 and 680nm from granal and stromal fractions for phosphorylated (+ 0·2mM ATP) and non-phosphorylated lamellae in the presence of 2mM DCMU. The ratio represents shoulder/peak height. Data taken from four experiments. See text for details.

The separation of grana from stromal lamellae could remove chlorophyll that would otherwise mask fluorescence emissions from centres with a low yield. Hence the observation of a 680nm signal from stromal lamellae may indicate that chlorophyll concentration could be a very important factor, complicating the interpretation of a fluorescence study.

A sample of thylakoids was phosphorylated and the two membrane components were separated (see table 7·3). After phosphorylation the fluorescence of the granal lamellae increased from the shoulder around 722nm, as indicated by the ratio change of 0·709 to 0·801. The height of the 680nm shoulder of the stromal preparation also increased by a similar

proportion (a ratio change of 0·240 to 0·333). The shift of the stromal spectra could indicate an influx of LHCP from the granal stacks due to a change in surface charge of LHCP upon phosphorylation (Barber 1980a, b, 1982 and 1983), and is accompanied with a loss of '680nm' fluorescence from the granal lamellae.

The appearance of a long wavelength shoulder around 722nm during the above experiments has not been reported previously, and prompted further study to discover whether the phenomenon was unique to either the chloroplasts or the measuring techniques. The use of 80μg/ml of chlorophyll is unusual for a fluorescence study, and the right angled optical arrangement is not the most commonly used. Usually a 'front-face' approach is employed. The spectral properties of the thylakoids may have been affected by alteration in both chlorophyll concentration and optics. The optical geometry of the system were altered, such that both actinic illumination and fluorescence were transmitted *via* a bifurcated optical fibre to the same side of the chloroplast sample (*front-face* detection).

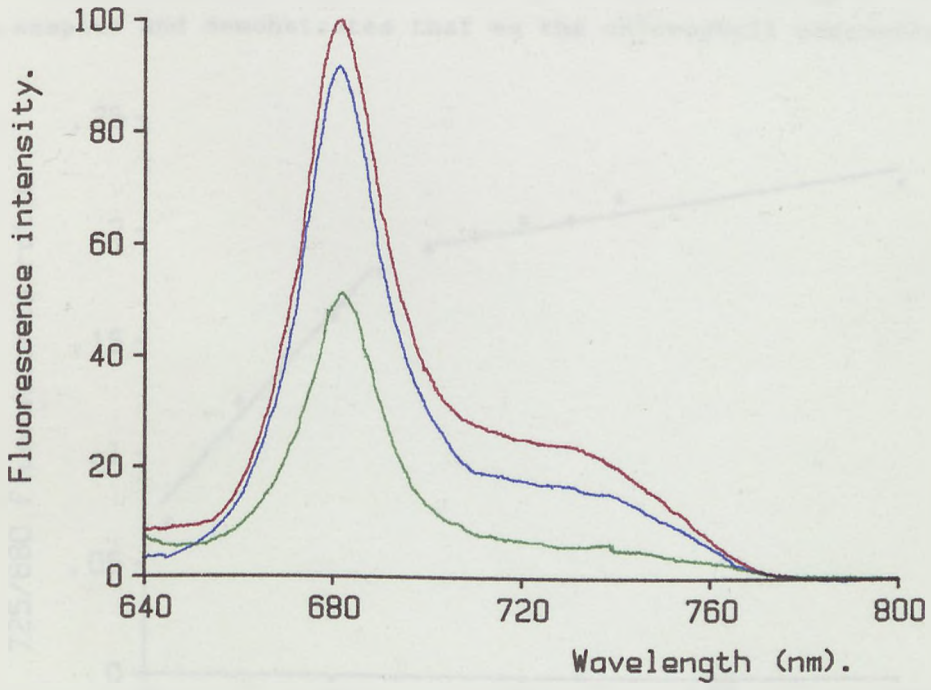


Figure 7·6: Scans of fluorescence as a function of wavelength at the following chlorophyll concentrations: 5 (——), 40 (——) and 160μg/ml (——). Details in text.

After passing through a chopper, the intensity of the actinic light was adjusted to about $1w.m^{-2}$ at the inner cuvette surface. The fluorescence

emission was transmitted by the second arm of the fibre through the monochromator and into the detector. The photomultiplier output was amplified and recorded directly onto chart paper. Each sample was scanned at 0·5nm/second with an amplifier rise time of 300ms.

From figure 7·6 it can be seen that varying the chlorophyll concentration from 5μg/ml to 160μg/ml increases the amplitude of the emitted fluorescence and results in a shift of the main fluorescence peak from 680·5 to 683nm. It can also be seen that the size of the 725nm shoulder at 80μg/ml is proportionately smaller than that shown in figure 7·1, where fluorescence was detected at right angles to the actinic beam. This suggests that by detecting fluorescence through the volume of the sample (as opposed to from the surface) alters the fluorescence spectrum, preferentially selecting longer wavelength emissions. The size of the 725nm shoulder can be expressed as a percentage of the '680nm peak', effectively normalising the ratio of the two components. Such a manipulation was performed on the above data, and at other chlorophyll concentrations. Each point of figure 7·7 is a mean of at least four separate samples and demonstrates that as the chlorophyll concentration is

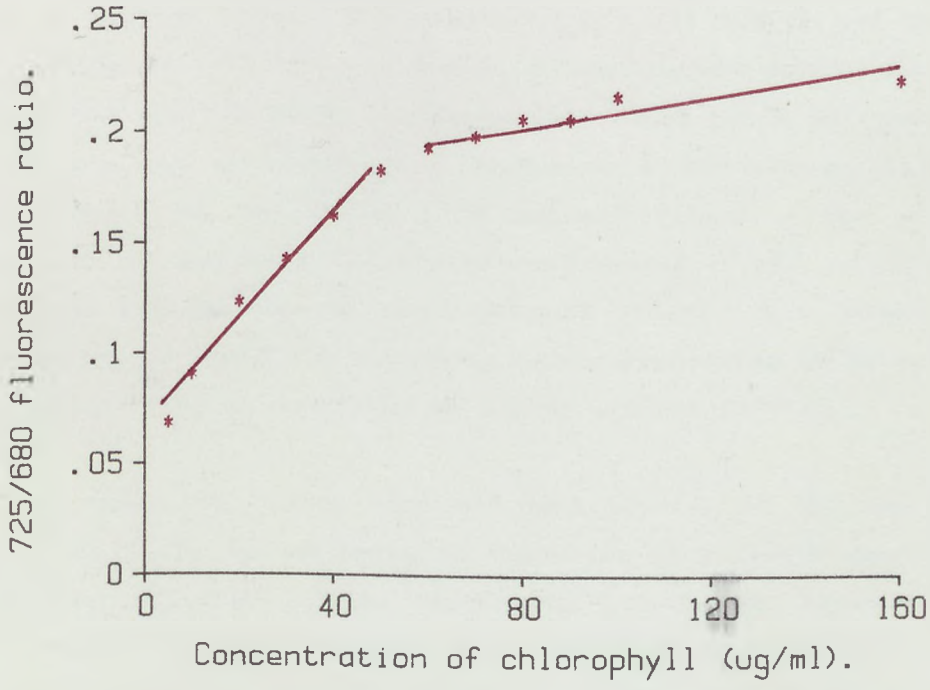


Figure 7·7: Plots of shoulder:peak fluorescence ratio as a function of chlorophyll concentration. See text for details.

increased the 725nm:680nm ratio increases. At chlorophyll concentrations

between 5 and 60μg/ml the ratio increases from 6·9 to 19·25 in an almost linear fashion. Above 60μg/ml of chlorophyll the ratio increases were much smaller. At 160μg the shoulder was 22·4.

From this data it can be concluded that by increasing the chlorophyll concentration it is possible to study longer wavelength fluorescence, whilst shorter wavelength emissions are readily detected at lower chlorophyll concentrations. It should be noted that detecting fluorescence at 90° to the measuring beam compared to 'front face' also increases the ratio of long:short wavelength fluorescence. The reasons for these apparent artefacts may be related to fluorescence re-absorption within the fluorescence/absorption overlap integral of the system. This enhances longer wavelength emissions relative to short wavelengths by self absorption of the shorter wavelength components of the emission spectrum. This effect is exaggerated at higher chlorophyll concentrations or when the fluorescence is detected through the bulk of the sample. Bradbury & Baker (1981) have also reported such changes at wavelengths >690nm for a chlorophyll concentration of 100μg/ml.

FLUORESCENCE INDUCTION CURVES AT DIFFERENT CHLOROPHYLL CONCENTRATIONS

Since changes in chlorophyll concentration alter the relative spectral distribution of fluorescence, the origin of fluorescence at the various wavelengths must be examined. Fuad *et al* (1983) suggest that at low temperatures LHC-II has an emission band centred around 736nm, which may have a spectrum similar to photosystem 1. By measuring the fluorescence emission from photosystem 2 at different chlorophyll concentrations, it might be expected that the proportion of β_{max} would vary since the ratio of shoulder to peak height was shown to change in figure 7·7. Such a proposal might also be accompanied with spectral changes at different DCMU concentrations.

Chloroplast samples were maintained at 20°C and stirred in the dark for ten minutes in the presence of 100μM methyl viologen and 2μM gramicidin D prior to DCMU addition (10μM). The amount of chlorophyll was adjusted for each experiment and stirring turned off during the measurement. Inductions were recorded on the equipment described in Chapter four (as depicted by figure 4·5) in accordance with the methods described in Chapter five. The actinic light ($112 w.m^{-2}$) was defined by heat and Corning CS 4-96 filters, and introduced to the sample *via* one limb of a bifurcated optical fibre. Chlorophyll fluorescence was observed *via* the second arm and detected by a Hamamatsu R 928 photmultiplier tube through a Schott RG 665 broad band cut-off filter. The signal was amplified with an inverting 741 operational amplifier and recorded by the DL4000B system (512 points and sweep time of 300μs). The transients were analysed on-line by the DL417 according to the procedures of Melis & Homann (1975, 1976 and 1978) as described by Horton & Black (1981a).

The induction curves recorded were similar to the one shown in figure 4·1, the F_v/F_m ratios being in the order of 0·77→0·8 over the whole chlorophyll concentration range, indicating that F_m was attained even at higher chlorophyll concentrations. Area analyses were performed, and the contribution of the slow area component (β_{max}) as a proportion of the total calculated. These values are plotted in figure 7·8:

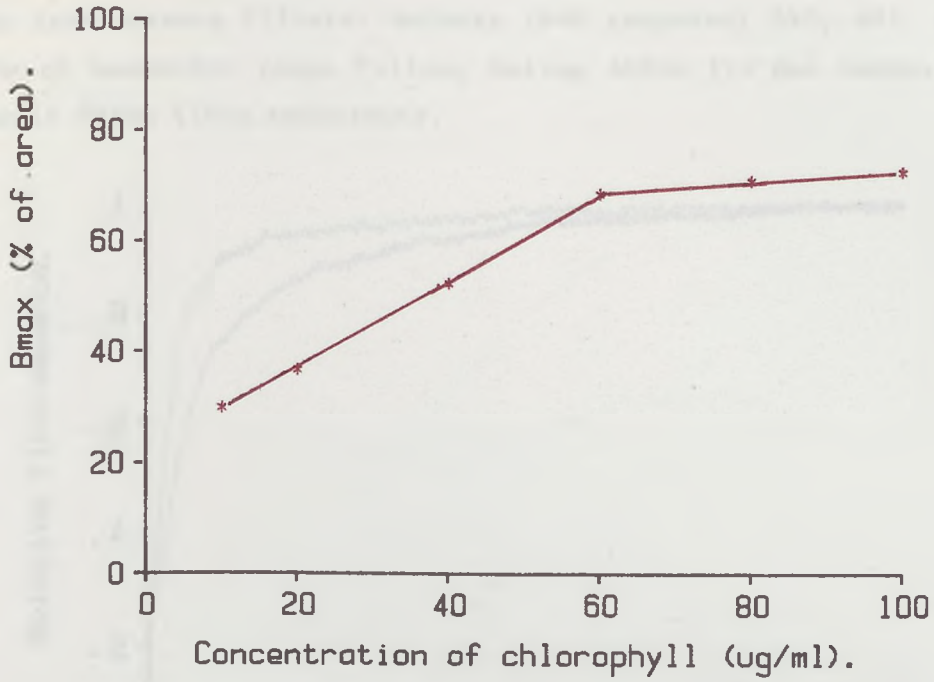


Figure 7·8: Plot of β_{max} versus chlorophyll concentration.

As the chlorophyll concentration is increased, the β_{max} proportion also increases (figure 7·8). Up to about 60μg/ml of chlorophyll, there is a linear relationship between β_{max} and chlorophyll concentration. Above this value a second linear response with a much smaller gradient was observed. Thus, at 10μg/ml of chlorophyll a value of 30% is obtained, whilst at 60μg/ml the β_{max} is elevated to almost 70%.

These results suggest that care must be taken when comparing measurements made over a range of chlorophyll concentrations. Clearly, ambiguity could arise from data recorded over the 10-60μg/ml range.

It can also be concluded that as the amount of chlorophyll in the cuvette is increased then spectral selection could occur in favour of slow area accumulation rather than the faster kinetics. Thus, at elevated chlorophyll concentrations spectral distortion occurs, possibly due to the overlap integral discussed earlier.

From this data, and that of the previous section it may be predicted that the β_{max} would vary with wavelength. The induction experiments were repeated at two fixed chlorophyll concentrations (20 and 80μg/ml), and this time the fluorescence emission was defined by the

following interference filters: Balzers (B40 response) 650, 685, 694, 706 and 740nm of bandwidth range 7→11nm; Ealing 660nm (11·6nm bandwidth) and Baird Atomic 728nm (10nm bandwidth).

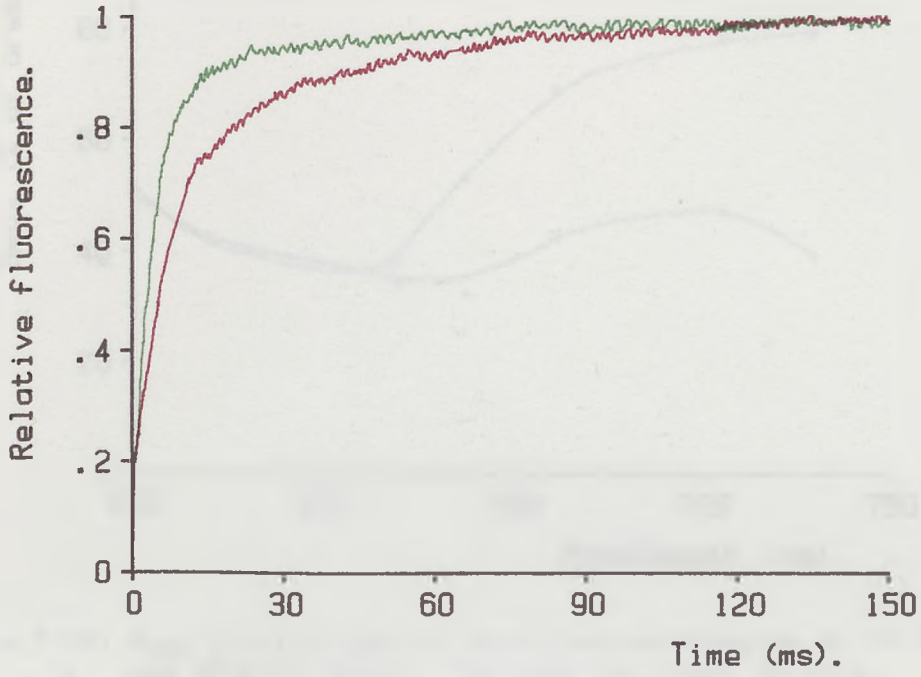


Figure 7·9: Fluorescence induction curves recorded at 694 (——) and 728nm (——). Chlorophyll concentration 80μg/ml.

From figure 7·9 it can be seen that the rise of the fluorescence induction curve recorded at 728nm was very slow compared with 694nm, having an extended exponential phase. Semi-logarithmic area manipulations were performed and from these β_{max} values were calculated. For the 694nm curve the value was only 50% of the total area, whilst at 728nm it may be as high as 80%. The β_{max} proportions were measured at all of the wavelengths listed above (four or five measurements at each wavelength) and these are plotted in figure 7·10. It can be seen that proportion of the β_{max} varies with the detection wavelength at both of the chlorophyll concentrations used. Clearly there is a stronger dependence at the higher concentration, but the trend is present at 20μg/ml of chlorophyll. A peak of β_{max} proportion is found at 725→730nm and a trough at 680→685nm. This data suggests that the area above the fluorescence induction curve derived from photosystem 2_β is maximal at 725→730nm, and this may indicate photosystem 2_β fluoresces at longer wavelengths compared to photosystem 2_α. It should be pointed out that these spectra are not absolute emission spectra, but

plots of relative area. The estimation of β_{max} (and α_{max}) is a

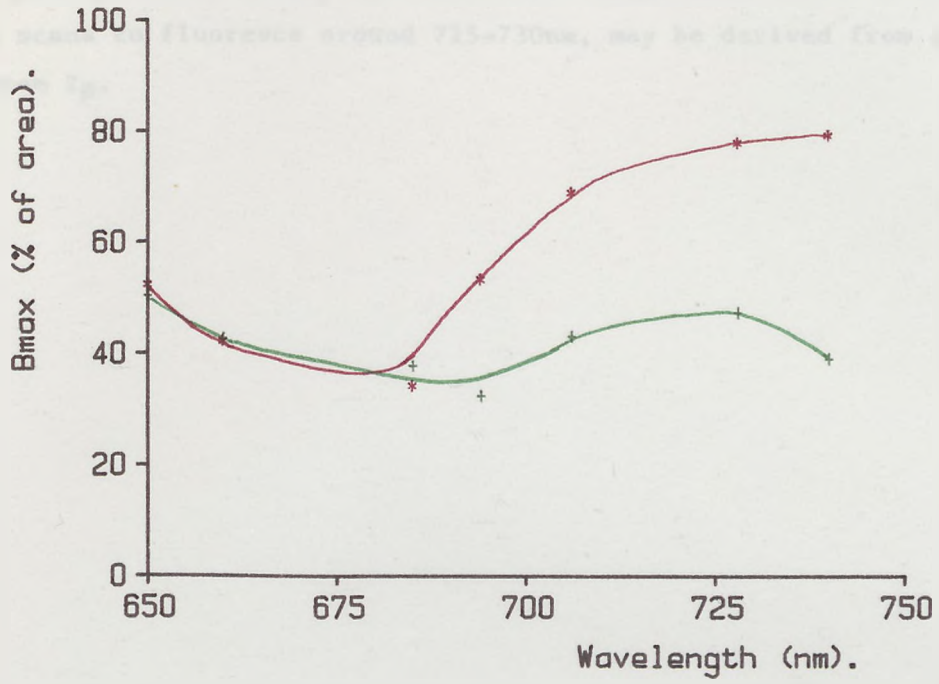


Figure 7·10: β_{max} plotted against detection wavelengths at 20 (——) and 80μg/ml (——). See text for other details.

proportional calculation and implies that as one area increases the other must decrease, thus slight changes are exaggerated. This does not alter the fact that photosystem 2_β is optimally detected (and probably fluoresces therefore) at longer wavelengths than photosystem 2_α. This result was to be expected, since the absorbance spectrum of the two systems are different (Thielen *et al* 1981). An alternative interpretation of this data could be that the red-shift of the β-emission is due to energy transfer, and consequential emission from photosystem 1, which may fluoresce at wavelengths greater than 700nm (Kyle *et al* 1983a). This view is further supported by the probable location of β-centres in the unappressed lamellae along with photosystem 1 (Anderson 1981 and Anderson & Melis 1984).

The observation of a loss of pronounced spectral profile at lower chlorophyll concentrations indicates that the measurements are very prone to distortion. This is possibly due to re-absorption at higher chlorophyll concentrations, which tends to favour longer wavelength emissions in preference to those at shorter wavelengths. Thus there is an apparent red-shift of the total fluorescence emission. The fluorescence emission of

β-centres at longer wavelengths also supports the view that the high DCMU binding phase, found during the titration experiments and shown by the spectral scans to fluoresce around 725→730nm, may be derived from a form of photosystem 2_β.

CHAPTER EIGHT

From the work of Chapter three two oxidisable forms of cytochrome *f* were shown to exist in both spinach and pea thylakoid membranes (see figures 3·3 and 3·6), in agreement with Horton & Cramer (1974). Cytochrome b_{559} was also shown to be chemically, but not photo oxidisable. After cycling in red and far-red there was a decrease in the half time of the slow phase of ferricyanide oxidation by about 50% (see figures 3·1 and 3·3), and in addition to the well documented sub-populations of cytochrome *f*, a third component was identified, which was photo-oxidisable following treatment with 0·5mM ferricyanide. This species was shown to be related to the degree of thylakoid appression (figure 3·8) and a 'granal lamellae' fraction prepared from yeda press treated membranes (Andersson & Anderson 1980) was shown to contain little of this pool of cytochrome *f*. This data suggested that this pool may reside within the stromal lamellae. Inclusion of up to 500μM DAD did not remove the photo-oxidative redox change (although the slow ferricyanide oxidation kinetics were significantly accelerated).

DCMU was shown to inhibit photosystem 2 mediated reduction of cytochrome *f* (see figure 3·9), but as predicted by the Z-scheme (Hill & Bendall 1960) not photosystem 1 driven oxidation. The I_{50} for inhibition (figure 3·10) was found to be around 0·125μM DCMU, and only one inhibitory event was witnessed. This constant probably reflects the inhibition of water splitting and whole chain electron transfer, but not that shown to cause photo-destruction and ultimately death of the plant (Ridley 1977).

DCMU was shown to reduce the time taken for a chlorophyll *a* fluorescence induction curve to rise from F_o to F_m (figures 5·1 and 5·2), and was accompanied by a decrease in the size of the area above the F_v portion of the curve (figure 5·3). The effect of DCMU on the area was found to be biphasic, the first phase accounted for about 90% of the total area and allowed an I_{50} for DCMU to be calculated at ~0·2μM. The second component had an I_{50} of about 5μM DCMU.

Further examination of the fluorescence induction data revealed that as the DCMU concentration was increased β_{max} (the area above F_v contributed by photosystem 2_β) decreased with similar kinetics to the area change, allowing two I_{50}'s to be calculated at 0·4 and ~7μM DCMU. These results can be interpreted as a loss of $F_{v\beta}$ (and area) because of the acceleration of the fluorescence rise upon isolation of Q from its physiological oxidant plastoquinone. This is expressed in figure 5·5 as a decrease in the rate constant for the β-centres (k_β) as the rate constant for the α-centers (k_α) increases and are subsequently excluded from the calculations of β_{max} and k_β. This apparent change in k_α may occur because photosystem 2_α has a larger antenna than photosystem 2_β (Theilen & Van Gorkom 1981a). As the DCMU concentration was increased further, photosystem 2_β was inhibited, increasing k_β and diminishing $F_{v\beta}$.

This data suggests that α and β-centres have different affinities for DCMU, the latter being an order of magnitude less. Such a result is supported by Joliot & Joliot (1981a and 1983) who suggested a DCMU insensitive photosystem 2 mediated turnover of cytochrome b_{563} and Hodges & Barber (1983) who relate a slow rate constant to poor inhibition of electron flow by DCMU through β-centres. However, Joliot & Joliot (1983) also present evidence indicating that B^- has a lower affinity for DCMU than B. The I_{50} found in these experiments at this concentration range was ~5μM DCMU and may relate to this phenomenon, although the size of the associated fluorescence differed. The appearance of a second DCMU-induced effect reflects the heterogeneous complexity of the chloroplast thylakoid, and if these different inhibition constants do relate to a sub-populations of photosystem 2, then their precise roles in photosynthesis must be questioned. Beta-centres might not function in main chain electron transfer, but may provide the correct redox poise to support a photosystem 1 cycle. Inhibition of this cycle is known to be the lethal step in the killing of plants by certain herbicides as photo-destruction follows very shortly (Ridley & Horton 1984).

Figures 6·1 and 6·2 demonstrate that the rise in fluorescence from F_i in steady-state is related to the incident light intensity. The rise may be reduced at higher light intensities because of trap closure in the absence of DCMU, and at less than $1 w.m^{-2}$ the yield of fluorescence may be grossly underestimated as a result of back reactions etc.

DCMU titrations of fluorescence performed at actinic light intensities of about $1 w.m^{-2}$ reveal two and possibly three separate DCMU mediated fluorescence increase events with I_{50}'s of around 0·05, 2 and 365μM DCMU. The first two may compare with those found during observations of fluorescence induction, and the former with the single constant estimated during the cytochrome measurements (see table 8·1). These three phases are not lost by halving the chlorophyll concentration and probably reflect true DCMU binding phenomena.

	I_{50} for DCMU (μM)		
MEASUREMENT	FIRST	SECOND	THIRD
CYTOCHROME *f*	0·125	——	——
AREA	0·19	5·16	——
β_{max}	0·43	7·84	——
k_β	0·08	2·95	——
TITRATION	0·044	1·20	365

Table 8·1: Listed in this table are the various I_{50}'s for DCMU that have been obtained from the work performed during this thesis for 'normal' thylakoids.

DCMU affinity increased at higher *p*H, which would be manifested as increased rate constants during measurements of fluorescence induction curves due to an effectively stronger inhibition by DCMU (and therefore a slower oxidation of Q by plastoquinone). This apparent increase in rate constant would decrease β_{max} by appearing to convert photosystem 2_β into photosystem 2_α. Similar reasoning also explains an increase of the high affinity component at the expense of the lower ones (see figure 6·8) and the *p*H profiles also suggest association of the various phases with specific photo-centres. The first phase (high affinity binding) has a *p*H profile similar to that reported by Melis & Homann (1975) for photosystem 2_α, the second and third phase *p*H profiles resemble those presented for photosystem 2_β.

Single additions of DCMU (figures 6·11 and 6·12) revealed two phases of fluorescence rise, a very fast time-unresolved fluorescence elevation and a slow rise with a half time of about 30 seconds. These two phases may relate to the three found during the titrations and as their *p*H profiles are similar to those drawn in figure 6·8. However, the changes in proportion of the fast phase were reflected by the proportion changes of the slow phase. Addition of hydroxylamine depressed the *p*H profile of the slow phase and increased the amplitude of the fast phase (figure 6·13). This data may indicate a strong interaction of hydroxylamine with the slower phase component, which is thought to be related to β-centres. Melis & Homann (1975) demonstrated a similar decrease in β_{max} in the presence of hydroxylamine.

A titration of phosphorylated thylakoids showed that the first phase decreased in amplitude, whilst the third phase increased (the fluorescence intensity at F_t was fairly similar, and the fluorescence induction measurements suggested significant alterations of energy distribution). These results indicate an interchange of the components of the high and low affinity DCMU binding events. The I_{50} for the high affinity portion of the DCMU fluorescence titration curve was lowered after phosphorylation, in agreement with the data of Vermaas *et al* (1984) and consistant with evidence that phosphorylation inhibits photosystem 2 electron transfer in the Q to plastoquinone region (Horton & Lee 1984).

Spectra of chlorophyll *a* fluorescence detected at 90° to the measuring light and *front face* revealed large spectral differences (figures 7·1 and 7·6). There was a very pronounced long wavelength (~725nm) shoulder when the fluorescence was detected through the volume of the sample. The size of the shoulder was shown to vary with the concentration of chlorophyll in the cuvette (see figures 7·6 and 7·7) in agreement with Bradbury & Baker (1981). Measurements of β_{max} (figure 7·8) varied in an almost identical manner to figures 7·6 and 7·7. At two chlorophyll concentrations (20 and 80 μg/ml) β_{max} also varied with respect to detection wavelength, figure 7·10 suggest that $F_{v\beta}$ is optimally detected at about 722nm.

Fluorescence spectra recorded in the presence of up to 50μM DCMU typically exhibit a ~10% decrease in the amplitude of the long wavelength

shoulder compared to the main peak of DCMU free thylakoids (see figures 7·1, 7·2 and 7·3). As the concentration of added DCMU is increased the amplitude of the fluorescence shoulder approaches the un-inhibited curve once again. This data suggests that a population of β-centres fluoresce at about 722nm and are maximally inhibited by 365μM DCMU. Figure 7·5 indicates that the 722nm fluorescent species is probably located in the stroma exposed thylakoids, whilst the '680nm fluorescent' photo-centres are resident in the granal lamellae.

In conclusion, studies of the redox behaviour of cytochrome *f* suggested that the I_{50} for inhibition of 'electron flow' by DCMU is in the region of 0·125μM. This is supported by chlorophyll *a* fluorescence studies and furthermore suggest that 'inhibition of photosynthesis' occurs in this herbicide concentration range and is related to inhibition of photosystem 2_{α}. Fluorescence studies also revealed additional DCMU binding events which probably relate to photosystem 2_{β}. An I_{50} is identified around 5μM DCMU which may relate to Ridley & Horton's (1984) constant that was shown to inhibit photosystem 1 mediated cyclic electron flow. This indicates that the β-centres with 'medium' affinity for DCMU may poise this series of reactions (Grant & Whatley 1967) and could possibly function through cytochrome b_{563} as Joliot & Joliot (1981a and 1983) suggested such a pathway in the presence of about 10μM DCMU. A third phase of DCMU induced fluorescence rise was identified with an I_{50} of about 365μM. This may be considered insensitive in terms of inhibition of photosynthesis. However, it is possible that this event may also relate to a type of β-centre and might be an expression of a non-B type of reaction centre (Lavergne 1982). It is hard to assign a precise role for such a type of reaction centre, but if it is located in the photosystem 1 rich domains of the stroma exposed thylakoids (Anderson & Melis 1984) it might conceivably behave as an additional antenna for photosystem 1 or provide electrons to directly activate membrane bound components.

The effects of DCMU on photosynthesis are far from simple; the extremely heterogeneous environment of the chloroplast thylakoid is born out in a non-homogeneous response to the herbicide DCMU. This data supports the notion that the I_{50} to partially inhibit oxygen evolution and electron transfer is significantly less than that which proves fatal to the plant. After all, inhibition of water splitting will only starve the plant

to death, it does not prove lethal in terms of photo-destructive effects that are witnessed at (and required by) higher concentrations of DCMU. Moreover, Ridley & Horton (1984) demonstrated that 5μM DCMU achieved the desired effect. Data is presented here which confirms this fact and identifies an additional component that may be responsible for these observations. This thesis demonstrates that DCMU has several physiological effects as may be expected upon its addition to a non-homogenous environment.

THE NEXT STEP ?

Many experiments could be imagined to continue this study, for example a flash kinetic study similar to the Q_1/Q_2 measurements of Joliot & Joliot (1981b), the measurement of β_{max}, k_β and k_α over a much wider concentration range of DCMU than used in Chapter five. This could also be done at different chlorophyll concentrations and emission wavelengths. These studies also need to be made on different membrane fractions in conjunction with techniques like polyacrylamide gel electrophoresis, allowing identification of the different constitutents of the total fluorescence yield at different DCMU concentrations. The precise involvement of other components like cytochrome b_{563} could also be examined, and the studies might even move towards isolation of chlorophyll protein complexes and reconstitution experiments into liposomes to allow specific interactions to be studied

The key question in relation to the data presented in this thesis is "What factor(s) determine sensitivity to DCMU ?" Experiments could examine the B-protein to see if any modifications are evident in the basic structure or even if a totaly different protein exists at this locus in the various photosystem 2 complexes. There is a distinct possibility that this is indeed the case, since the B-protein of β-centres do not show binary oscillations in redox state. If this suggestion is accepted, how is it

modified in β-centres such that the affinity for DCMU is affected. Morover could molecular biological studies and site-directed mutagenesis be used to develope new and very different biotypes which possess extreme herbicidal tolerance or resistance, thus allowing greater freedom of crop spraying and weeding ?

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