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CHLOROPHYLL FLUORESCENCE AND THYLAKOID MEMBRANE ORGANISATION

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ABSTRACT

The mechanisms and structural changes involved in photosynthetic adaptation to light have been investigated using mainly room-temperature chlorophyll fluorescence techniques. A comparison made between *in vitro* alterations, brought about by either changes in cation levels or by protein-phosphorylation, with *in vivo* light-induced changes, termed State 1 - State 2 transitions, has shown that;

a) lowering the cation level leads to an increase in spillover of excitation energy from PS2 to PS1 and a small decrease in PS2 absorption cross section,

b) protein-phosphorylation leads to a decrease in PS2 absorption cross section, at high salt levels (>5mM Mg^{2+}) while at lower cation concentrations (≤2mM Mg^{2+}) an increase in spillover also arises,

c) these two phenomena are independent but interrelated processes linked by the electrostatic nature of the mechanisms,

d) State 1 - State 2 transitions reflect protein-phosphorylation at "high" background cation levels.

The effect of protein-phosphorylation on PS2 electron transfer reactions has also been investigated. Protein-phosphorylation did not alter the ability of DCMU to inhibit the Hill reaction, but did attenuate the rate of electron transfer at saturating light intensities. A comparison of the fast fluorescence transients exhibited by phosphorylated and non-phosphorylated thylakoids with those produced by atrazine-resistant and susceptible thylakoids suggest protein-phosphorylation alters the rate of electron flow from Q_A to Q_B.

A comparison of State 1 - State 2 transitions with chloroplast ultrastructure shows that this adaptive process is highly dependent upon the extent of thylakoid stacking. This could result from altered

levels of kinase and phosphatase enzymes thereby reflecting their location within the membrane.

The analysis of fluorescence induction curve parameters from DCMU-inhibited thylakoids, after various pretreatments, suggests that the Melis and Homann (1978) concept of PS2 heterogeneity ($PS2_{\alpha}$ and $PS2_{\beta}$) is not well-founded. The data presented in this thesis suggests that the observed heterogeneity is due to a population of PS2 poorly inhibited by DCMU.

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Symbols and Abbreviations:

ADP	adenosine diphosphate
ADRY reagent	PS2 inhibitor with the ability to accelerate the deactivation reactions of the oxygen evolving complex
A_{max}	area above the fluorescence rise
A_{norm}	normalised A_{max}
Ant-2p	2,(3-chloro-4-trifluoromethyl)anilino-3,5, dinitrothiophene
ATP	adenosine triphosphate
BBY	type of PS2 preparation
β_{max}	intercept of the linearly extrapolated slow, exponential phase of the area growth over the induction curve with the ordinate at time zero
BSA	bovine serum albumin
$c_{\frac{1}{2}}$	half maximal concentration
C_{550}	an absorbance shift at 550nm which acts as a linear indicator of Q_A^-
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
Chl	chlorophyll
cyt	cytochrome
ΔA	absorption change
DAD	2,3,5,6-tetramethyl-p-phenylenediamine
DBMIB	2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DCPIP	2,6-dichlorophenolindophenol
DPC	1,5-diphenylcarbazide
$E_{m7.0}$	midpoint redox potential (at pH 7.0)
EDTA	ethylenediaminetetra acetic acid
F_{685}	77K fluorescence emission at 685nm
F_{695}	77K fluorescence emission at 695nm

F_{735}	77K fluorescence emission at 735nm
Fd	ferredoxin
FeCy	potassium ferricyanide
F_m	maximum chl fluorescence yield
F_o	initial chl fluorescence yield
F_v	variable fluorescence (F_m-F_o)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HES	high energy state
HLP	high light grown plant
I_{50}	concentration to bring about 50% inhibition
k_α	initial first order rate constant for $PS2_\alpha$ area growth
k_β	first order rate constant for $PS2_\beta$ area growth
k_d	non-radiative deactivation rate constant
k_f	fluorescence rate constant
k_p	photochemical rate constant
k_t	energy transfer rate constant
LHC	light harvesting chlorophyll a/b protein
LLP	low-light grown plant
MES	2-(N-morpholino)ethanesulphonic acid
MLP	medium-light grown plant
MV	methyl viologen
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced $NADP^+$
NPhos	non-phosphorylated
OEC	oxygen evolving complex
P_{518}	electrochromic carotenoid band shift
P_{680}	reaction centre of PS2
P_{700}	reaction centre of PS1
Phos	phosphorylated

P_i	inorganic phosphate
PMS	phenazine methosulphate
PQ	plastoquinone
PS	photosystem
PSU	photosynthetic unit
Q_A	primary stable acceptor of PS2
Q_B	quinone and herbicide binding protein associated with PS2
SiMo	silicomolybdate
TEC^{3+}	tris(ethylenediamine) cobaltic (III) cation
$t_{\frac{1}{2}}$	half maximal time
Tricine	N-tris (hydroxymethyl) methylglycine
Tris	tris(hydroxymethyl) methylamine
X_{320}	absorbance change at 320nm associated with quinone reduction
Z	immediate donor to P680

CHAPTER 1

Introduction

1.1 The Overall Concept:

Photosynthesis is the process by which higher plants, algae and some species of bacteria convert light energy into chemical energy which can be used for biosynthesis and other energy-requiring processes. In eukaryotic organisms the light-driven reactions occur in and across the thylakoid membrane system located in cellular organelles called chloroplasts. This process involves the transfer of electrons from H_2O to $NADP^+$ leading to the generation of O_2 from H_2O, the reduction of $NADP^+$ to NADPH, and the synthesis of ATP from ADP and inorganic phosphate (P_i). The NADPH and ATP are utilised in the photosynthetic fixation of CO_2 and the subsequent production of carbohydrates in a series of mainly light-independent reactions, although certain key enzymes are light-activated.

The current understanding of the photosynthetic light reactions has developed from the idea of two separate photoreactions operating in series. This concept was originally proposed by Hill and Bendall (1960) to account for the participation of cytochromes f and b_{563} in the electron transfer reactions. The so called Z-scheme was based on the relationship between the various photosynthetic components in terms of their redox potentials (see Fig. 1.1). However, as a result of Mitchell's chemiosmotic hypothesis (1961) which gave a mechanism by which electron transfer was coupled to photophosphorylation, the Z-scheme was modified to account for the required spatial organisation of the photosynthetic components within the membrane (see Fig. 1.2). This meant that the protein-complexes had to be asymmetrically located across the thylakoid

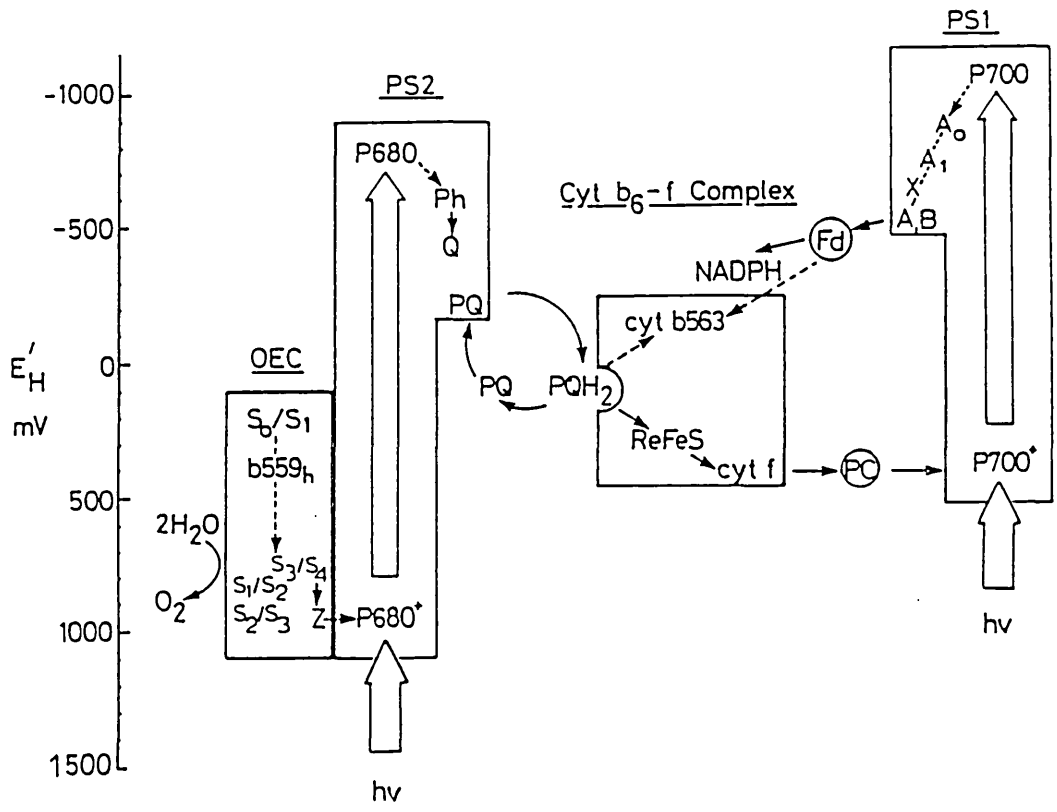


Fig. 1.1 The Z-scheme, in which the components of photosynthetic electron transport are placed on a redox scale. The major protein-complexes involved in electron transfer processes are drawn in blocks.
OEC, oxygen evolving complex; PS2, photosystem 2 complex; cyt b_6-f complex, cytochrome b_6-f complex; PS1, photosystem 1 complex; s_o, s_1, s_2, s_3, s_4, s-states for water oxidation; b_{559h}, high potential cytochrome b_{559}; Ph, pheophytin; A_o, A_1, X, postulated PS1 electron acceptors of unidentified chemical identity; A,B, iron-sulphur centres; other symbols as in symbols and abbreviations.

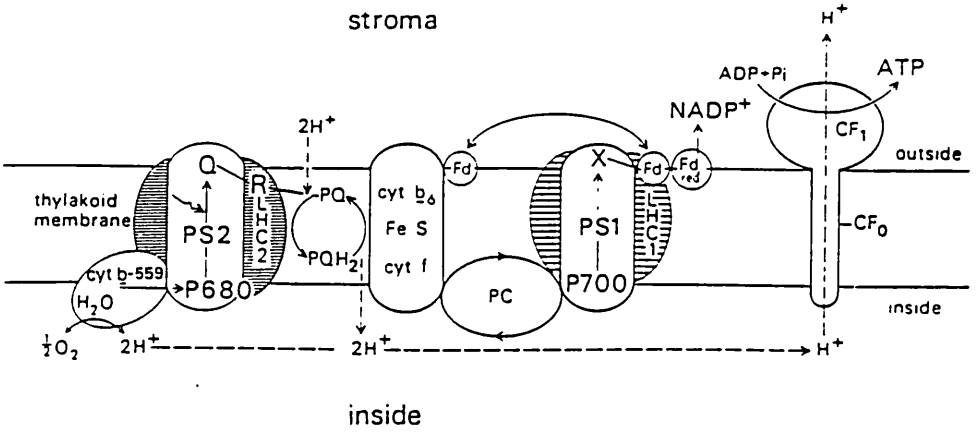


Fig. 1.2 . Schematic representation of the protein-complexes of photosynthesis, arranged as required for electron flow and proton translocation. (Taken from Anderson and Andersson, 1982).

membrane so as to bring about the vectorial transport of protons and the generation of an electrochemical potential gradient; the force required to power ATP synthesis via the ATP-synthetase complex. For these processes to occur and to maintain photosynthetic efficiency there is a requirement for a balanced energy input to both photosystem 2 (PS2) and photosystem 1 (PS1). Each photosystem contains a reaction centre consisting of special chlorophyll a (Chl a) molecules (P680 and P700 respectively) which when excited generate a charge separation across the membrane producing an oxidant and a reductant. Most of the chlorophyll molecules, however, are involved solely in the absorption and channelling of excitation energy to the reaction centres and, like the specialised Chl a molecules, are located in pigment-protein complexes. The most abundant of these, in higher plants, is the light harvesting chl a/b complex (LHC) which is usually associated with PS2.

The three chlorophyll-containing protein complexes mentioned above are believed to be laterally segregated within the thylakoid membranes, so that both PS2 and LHC are located in the appressed lamellae while PS1 is situated in the non-appressed membrane regions (see Anderson, 1982; Anderson and Andersson, 1982). As a consequence of such an arrangement, it seems that higher plants have developed a means of controlling excitation energy distribution when necessary, so as to maximise photosynthetic electron transfer reactions (see Barber, 1982a).

The work presented in this thesis is concerned with the mechanisms and structural changes involved in the control of excitation energy distribution. It concentrates on a comparison between <u>in vitro</u> induced alterations in energy distribution by cation levels and protein phosphorylation, using mainly room-temperature chlorophyll fluorescence

techniques, and relating these findings to *in vivo* light-induced changes termed State 1 - State 2 transitions.

1.2 Chlorophyll Fluorescence:

1.2.1 An intrinsic probe of photosynthesis:

A portion of the light intercepted by the higher plant is absorbed by the main photosynthetic pigments called chlorophyll a and chlorophyll b. The energy of an electronically excited chlorophyll molecule, resulting from light absorption, can be disposed of by one of several competing processes. Under optimal conditions most of this energy is used in photochemical reactions. However when this is not possible it can be transferred to neighbouring chlorophyll molecules, dissipated by non-radiative de-excitation processes like heat, cross over to another electronic state to form triplets or be re-emitted as fluorescence. The latter process constitutes a relatively large, easily detected optical signal and has, therefore, become a useful intrinsic probe of the primary photochemical reactions of photosynthesis as it instantly monitors the balance between all of the competing processes. Because fluorescence depends on the physical characteristics of the immediate environment, and on the distance and orientation between fluorescing molecules it is an ideal tool for monitoring thylakoid membrane conformational changes.

Below is outlined the basic properties of *in vivo* chlorophyll fluorescence, which has been covered in a number of review articles over the last fifteen years (see Govindjee and Papageorgiou, 1971; Papageorgiou, 1975; Barber, 1976; Lavorel and Etienne, 1977; Krause and Weis, 1984).

1.2.2 Origin of in vivo Chlorophyll Fluorescence

Chlorophyll fluorescence emission at room temperature occurs in the red region of the spectrum and shows a maximum at 685nm in the in vivo state. It is believed to originate mainly from chlorophyll a molecules associated with PS2 (Vredenburg and Slooten, 1967). Chlorophylls associated with PS1 fluoresce very little at room temperature but it has been shown that they do contribute to the small fluorescence shoulder seen at wavelengths greater than 700nm, with a possible maximum emission around 710-715nm (Kyle et al. 1983a). When thylakoids are cooled to liquid nitrogen temperatures (77K), however, the fluorescence emission spectrum becomes more complex. A large peak at 735nm is clearly visible which has been associated with PS1 chlorophylls (Satoh and Butler, 1978; Bose, 1982). The PS2-associated fluorescence emission exhibits two maxima at 685nm and 695nm. The origin of these peaks is still not fully understood. It has been postulated that F_{695} originates from the PS2 reaction centre (Breton, 1982) while F_{685} arises from either PS2 reaction centre-associated chlorophylls or LHC. The latter point is still not clearly resolved as Ryrie and co-workers (1980) have shown that isolated LHC fluoresces at 681nm at 77K while recently Lam et al. (1984) have isolated LHC which exhibits a 685nm peak. Before this latter report it was suggested that LHC was not seen to fluoresce in vivo as excitation energy was rapidly transferred to the PS2 core chlorophylls which subsequently gave rise to the fluorescence, or because of a blue-shift in the emission caused by the solubilization with detergents (Bose, 1982).

1.2.3 Fluorescence Yield:

Chlorophyll fluorescence arises as a consequence of the de-excitation of the excited chl a singlet state to the ground state. The yield

of emission is determined by all the other competing pathways for de-excitation, as mentioned above. This leads to the following equation for fluorescence yield (ϕ_F):

$$\phi_F = \frac{I_f}{I} = \alpha.\left(\frac{k_f}{k_f + k_d + k_t + k_p.f\,[Q_A^-]} \right)$$

where I_f is the intensity of fluorescence emission, I is the total intensity of absorbed light, and α is the fraction of light absorbed and transferred to PS2. k_f, k_d, k_t and k_p are first order rate constants for fluorescence emission, radiationless de-excitation, transfer of excitation to PS1, and photochemical trapping respectively. It is interesting to note that the rate of de-excitation by photochemical trapping depends on the redox state of the PS2 primary electron acceptor (Q_A) as well as on k_p. Duysens and Sweers (1963), using light which preferentially excited PS1 or PS2, were the first to notice the redox dependence of fluorescence and it was from their work that Q (nowadays called Q_A) was first used to represent a quencher of chlorophyll fluorescence. They found that Q_A reduction led to an increase in fluorescence and when Q_A was oxidised the fluorescence level was lowered. Therefore when Q_A is totally reduced, PS2 photochemical trapping will be at a minimum and fluorescence at a maximum while when Q_A is fully oxidised, PS2 photochemistry will be at a maximum and fluorescence at a minimum. The relationship between the redox state of Q_A and photochemistry at intermediate levels of Q_A reduction, however, is not always linear so the photochemical trapping rate becomes a general function of the proportion of closed PS2 reaction centres ($f[Q_A^-]$).

1.2.4 The Time-scale of Chlorophyll Fluorescence:

When considering fluorescence from a photosynthetic system there are two very different timescales involved. The microscale involves very short time lengths in the picosecond to nanosecond range while the macroscale is much longer and can extend into the minute timerange. The work presented in this thesis concerns the macroscale so that only a brief account of the microscale is outlined below (also see review by Lavorel and Etienne, 1977).

1.2.4.1 Microscale:

At the atomic and molecular level, all events in the life of an individual fluorescent molecule have durations in the 10^{-15}-10^{-8} second timescale. The order of magnitude depends on the imposed intrinsic natural lifetime (τ_o), which is the time required for an excited population of molecules to decline to $1/e$ of the initial number when the only occurring de-excitation process leads to fluorescence. The value of τ_o is related to the molecules absorption spectrum and can be calculated indirectly this way (an approximation is given by $1/\tau_o = 3x10^{-9} K_m^2 \Delta K \varepsilon_m$, where K_m and ε_m are the wave number and extinction coefficient at maximum absorption and ΔK is the band width at half maximum absorption). Since excited molecules can be deexcited by other processes, as already mentioned above, the actual lifetime (τ) is always smaller than τ_o. For in vitro chlorophylls the measured lifetimes directly agree with those computed by absorption measurements (Chl a, τ_o = 15ns while τ = 5ns) but these values will depend on the solvents used.

For a homogenous sample of identical chlorophyll molecules, it is expected that the decay of the excitated state will follow the following

equation:

$$N = N_o \, e^{-t/\tau}$$

where N_o is the initial number of excited molecules and N is the number of excited molecules at time, t. However, this will not necessarily be the case for *in vivo* chlorophyll.

Experimental work using this timescale involves the generation of an excited state and monitoring its subsequent decay kinetics over very short timescales. Such work has been widespread over the years (see brief summary by Moya and Garcia, 1983) and has been used to investigate a number of different phenomena including excitation energy transfer in intact algae (Porter et al., 1978) and isolated phycobilisomes (Searle et al., 1978) as well as the mechanisms involved in bringing about the cation-induced chlorophyll fluorescence changes (Briantais et al., 1973; Moya et al., 1977; Barber et al., 1978). More recent work, in this timescale, has been carried out by analysing picosecond fluorescence decay kinetics from isolated chloroplasts. As *in vivo* chlorophylls do not behave as a homogeneous system the decay kinetics have been arbitarily deconvoluted into three exponential components. The shortest lifetime (50-100 ps) has been attributed to radical pair recombination in PS2 of $P680^+$ and I^- (reduced pheophytin), while the longer lifetimes (400-750 ps and 1000-2000 ps) are believed to be kinetically controlled by decay processes taking place in the LHC and PS2-core chlorophylls respectively (Haehnel et al., 1982). Using this analysis and interpretation, investigations have been carried out to elucidate the excitation energy transfer processes involved in thylakoid membrane conformational changes induced by altered Mg^{2+}-levels (Nairn et al., 1982) and protein-phosphorylation (Haworth et al., 1983). However, the above interpretation of the three kinetic components has not yet been proven and has

recently been questioned by Butler et al. (1983), who suggest that PS2 heterogeneity is involved, so that $PS2_{\alpha}$ gives rise to the slow component while $PS2_{\beta}$ produces the middle component.

1.2.4.2 Macroscale:

This timescale is much longer than τ_o and therefore deals with steady-state concentrations of excited chlorophyll molecules. Here only the yield of the de-excitation processes are measured, so that the amount of detected fluorescence (F) depends on the intensity of excitation energy (I) and the ϕ_F of the samples where $F = I\phi_F$. As the actinic excitation energy remains constant in such experiments then the measured fluorescence is usually an indicator of ϕ_F. Of course the proportion of I absorbed can change if the absorption properties of the fluorescing species become altered.

Most of the fluorescence measurements on this timescale involve the kinetics of the change in ϕ_F when samples are excited. When certain dark adapted photosynthetic material is illuminated the observed changes in fluorescence show a number of complex transients which have been designated the following terminology; o i d p s m t (Lavorel, 1959; Kautsky et al., 1960). Some changes take place over a millisecond to second timescale (Fig. 1.3) while others occur over the minute range (see Fig. 1.6). The fast transients (ms-s) are believed to reflect alterations in de-excitation via photochemical trapping due to changes in the concentration of reduced Q_A. The slow transients (s-minutes) are more complicated phenomena and probably reflect changes in photochemistry and in the other rate constants (k_d and k_t) due to a number of different factors.

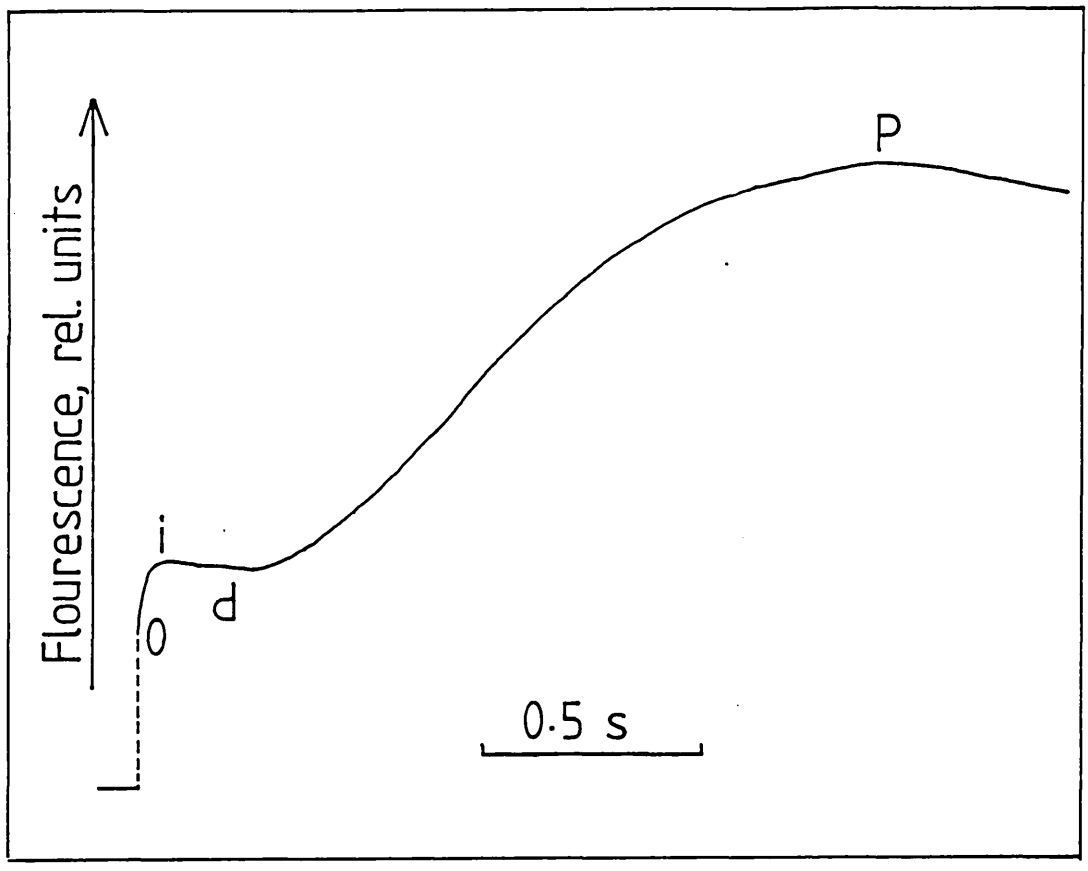


Fig. 1.3 Fast chlorophyll fluorescence transients observed at room temperature on illuminating a dark pretreated tomato leaf with blue-green light ($10 Wm^{-2}$) and showing the o,i,d and p levels of fluorescence.

The fluorescence measurements performed in this thesis deal with the macro timescale and will be discussed under the main headings of a) fast fluorescence transients, b) slow fluorescence transients and c) fast fluorescence transients in the presence of DCMU.

1.3 Fast Fluorescence Transients:

On illumination of dark adapted photosynthetic material the fluorescence yield increases in a characteristic manner as depicted in Fig. 1.3. There is a rapid rise to the o-level (often called F_o), which for detection requires the use of a fast-opening shutter and suitable recording equipment. This is believed to represent the emission from chlorophyll a molecules associated with PS2 prior to excitation energy inducing photochemical electron transport via the PS2 reaction centre (Butler and Kitajima, 1975). Therefore the quantum yield of F_o is independent of photochemical events. It was originally called the constant yield component, however, certain conditions have been shown to alter the F_o level, for example, changes induced by high temperature damage (Schreiber et al., 1976) or anaerobic conditions (Schreiber and Vidaver, 1974) both increase F_o while protein phosphorylation decreases the F_o (Horton and Black, 1983). From this initial fluorescence level there is a fast rise to the i-level followed by a slower rise to a maximum (p), which is sometimes interupted by a slight dip (d). The rise from F_o to p (termed variable fluorescence, F_v) takes place over the millisecond to second timescale but the time taken and the height of the p-level are highly dependent upon excitation energy intensity, temperature and the presence of Hill oxidants (Malkin and Kok, 1966) or on the presence of reductants like sodium dithionite (Velthuys and Amesz, 1973). Exactly

which photosynthetic processes are represented by the oidp transients is still not clear, but tentative assignments have been put forward (see Table 1.1), however, the overall rise from F_o to p mainly represents changes brought about by alterations in $k_p.[Q_A^-]$ (see Govindjee and Papageorgiou, 1971). The kinetics and extent of this rise depends on the rate of electron flow through the secondary acceptor pool of plastoquinone (PQ), the re-reduction of P680, and the degree of energy transfer from closed reaction centres to open ones. The simplified series of reactions depicted in Fig. 1.4, which omits the unstable primary electron acceptor (pheophytin), shows what events are occurring.

It is known that Z^+ is rapidly reduced and therefore the removal of electrons from Q_A via PQ usually controls the extent of open trap concentration (Malkin and Kok, 1966; Duysens and Sweers, 1963). Therefore the F_o to p rise occurs because the rate of reactions 1, 2 and 3 exceeds the rate of reaction 4 so that eventually most or all of Q_A becomes reduced. The fast fluorescence transients can, therefore, give information concerning a number of key photosynthetic reactions and has been used in a number of different ways.

1.4 Use of the Fast Fluorescence Transients:

1.4.1 Determination of Pool Sizes:

Comparison of the area over the fluorescence rise in DCMU-inhibited and non-inhibited chloroplasts allows an estimation of the electron acceptor pool size located before and after the DCMU block to be carried out (Malkin and Kok, 1966; Zankel and Kok, 1972). The area above the curve is proportional to the number of quanta required to generate the p-level (Malkin and Kok, 1966). In the presence of a PS2 inhibitor, like

Table 1.1

Tentative Assignments of the Induction Phase of Chlorophyll Fluorescence to the Photochemical and Physiological Events that intervene between a State of Dark Adaptation and a State of Light Adaptation

Fluorescence induction phase	Photochemical or physiological process[a]
Activation rise, oi	Photo conversion of the water-splitting catalyst to the S_3-state; reduction of Q_A[b-d]
Fluorescence decay, id	Reoxidation of Q_A by PS1[e]
Fluorescence rise, dp	Photoreduction of the pool of inter-system electron carriers[e-g]

a assignments refer to the predominant influencing processes

b Joliot, 1965

c Joliot, 1968

d Kok et al., 1970

e Munday and Govindjee, 1969

f Kautsky et al., 1960

g Duysens and Sweers, 1963

Taken from Papageorgiou (1975)

$$ZP_{680}\ Q_A \xrightarrow{h\nu} ZP^{*}_{680}\ Q_A \longrightarrow ZP^{+}_{680}\ Q_A^{-} \qquad (1)$$

open trap, low fluorescence — excited chlorophyll — closed trap, high fluorescence

$$ZP^{+}_{680}\ Q_A^{-} \longrightarrow Z^{+}P_{680}\ Q_A^{-} \qquad (2)$$

$$Z^{+}P_{680}\ Q_A^{-} \longrightarrow ZP_{680}\ Q_A^{-} \qquad \text{via electron flow from } H_2O \qquad (3)$$

$$ZP_{680}\ Q_A^{-} \longrightarrow ZP_{680}\ Q_A \qquad \text{via electron flow to PQ} \qquad (4)$$

open trap, low fluorescence

where Z = immediate donor to P_{680}^{+}

P_{680} = specialised PS2 reaction centre chl_a (Döring et al. 1967)

Q_A = primary stable PS2 electron acceptor which is a specialised quinone (Stiehl and Witt, 1969)

Fig. 1.4 A simplified series of PS2 electron transfer reactions.

DCMU, the area represents the number of quanta required to reduce Q_A (assuming no re-oxidation of Q_A^- by back reactions with the oxidising side of PS2 has occurred) whereas in the absence of DCMU the area represents the number of quanta required to reduce Q_A and all the inter-system electron transfer components. An estimation of the number of pool equivalents is determined by the equation

$$\text{Number of equivalents per PS2} = \frac{\text{Area over the curve (-DCMU)}}{\text{Area over the curve (+DCMU)}} = \frac{A_{max}^-}{A_{max}^+}$$

Using this technique a pool size of 20 equivalents has been estimated for isolated chloroplasts (Forbush and Kok, 1968).

A comparison of the area over the curve in the presence and absence of DBMIB has also been used to give an estimation of the PQ pool size (Schreiber et al., 1977). DBMIB, at low concentrations, inhibits photosynthetic electron transfer at the site of PQH_2 oxidation (Böhme et al., 1971) and therefore the area in the presence of this inhibitor represents the number of quanta required to reduce Q_A and PQ.

1.4.2 Dark Reoxidation of Q_A:

After illumination the fast fluorescence transients are regenerated in the presence of O_2 given a 20 minute dark period (Malkin and Kok, 1966). Recent studies have utilised this fact and by using the following equation have examined the effect of various pretreatments including the addition of cholesterol hemisuccinate (Yamamoto et al., 1981), protein phosphorylation (Kyle and Arntzen, 1983), thylakoid ageing and the effect of $MgCl_2$ (Scoufflaire and Lannoye, 1984) on the kinetics of Q_A reoxidation.

$$\frac{Q_A ox}{Q_A red} = \frac{F_\infty - F_t}{F_\infty - F_o}$$

where F_∞ = fluorescence when Q_A is fully reduced

F_t = initial fluorescence after a dark-time, t

F_o = initial fluorescence when all Q_A is oxidised

The above equation, however, is only valid when there is a linear relationship between ϕ_F and Q_A^- concentration but in many cases this is not obeyed. Therefore, it can only be used in a comparative manner as long as the original ϕ_F-Q_A^- relationship is not altered by the treatment under investigation. Furthermore, when Q_A reoxidation by the PQ pool is being examined as in Yamamoto et al. (1981) and Scoufflaire et al. (1984) care must be taken to inhibit any PS2 back reactions. This is usually accomplished by the addition of hydroxylamine, which inhibits such reactions (Bennoun, 1970).

1.4.3 A Monitor of Environmental Effects:

It has already been stated that the kinetics and extent of the fast fluorescence rise reflects P680 regeneration by the water-splitting processes and Q_A regeneration by the PQ pool, which depends on PS1-mediated electron flow. If the photosynthetic machinery becomes altered or damaged so that photosynthetic electron flow is affected then it is possible to detect this by a change in the extent and kinetics of the fluorescence rise. These measurements have become a useful tool in monitoring damage caused by adverse environmental conditions as it is quick and non evasive. Generally, if the oxidising side of PS2 is damaged it is reflected in an inhibited F_o to p rise (see Yamashita and

Butler, 1969), while if the reducing side is affected then the rise becomes much faster.

1.4.3.1 Effect of Light Intensity:

Higher plants have the ability to respond to the amount of light available during their growth. In general terms there is either a strong or a weak light response characterised by chloroplasts with different ultrastructures and photosynthetic capacities (Lichtenthaler et al., 1981; Meierand Lichtenthaler, 1981). When compared with high light grown plants, low light ones have; more granal lamellae, lower chla/b ratios, higher PS2/PS1 ratios, less PQ, cyt f, ATPase, plastocyanin and soluble proteins including ribulose 1,5 bisphosphate carboxylase (Boardman et al., 1974; Boardman, 1977; Melis and Harvey, 1981; Bjorkman, 1981; Grumbach and Lichtenthaler, 1982). Such differences in chloroplast structure and electron transfer capabilities therefore lead to different fast fluorescence transient characteristics as depicted in Fig. 1.5a. Plants grown under high light show a slower rise, after a given dark pretreatment, which results in a lower p-level when compared with plants grown under low light (Schreiber et al., 1977; Fork and Govindjee, 1980). These different transients have been used to give information concerning the number of chlorophylls associated with each PS2 reaction centre (PSU). A correlation between the time taken to reach the p-level with PSU size has been made using a number of sun and shade plant species. The shade plants showed an average PSU of 800 chl/Q_A and an average time of 825 ms to reach p, while the sun plants (PSU-390 chl/Q_A) produced on average the p-level 2400 ms after illumination (Fork and Govindjee, 1980).

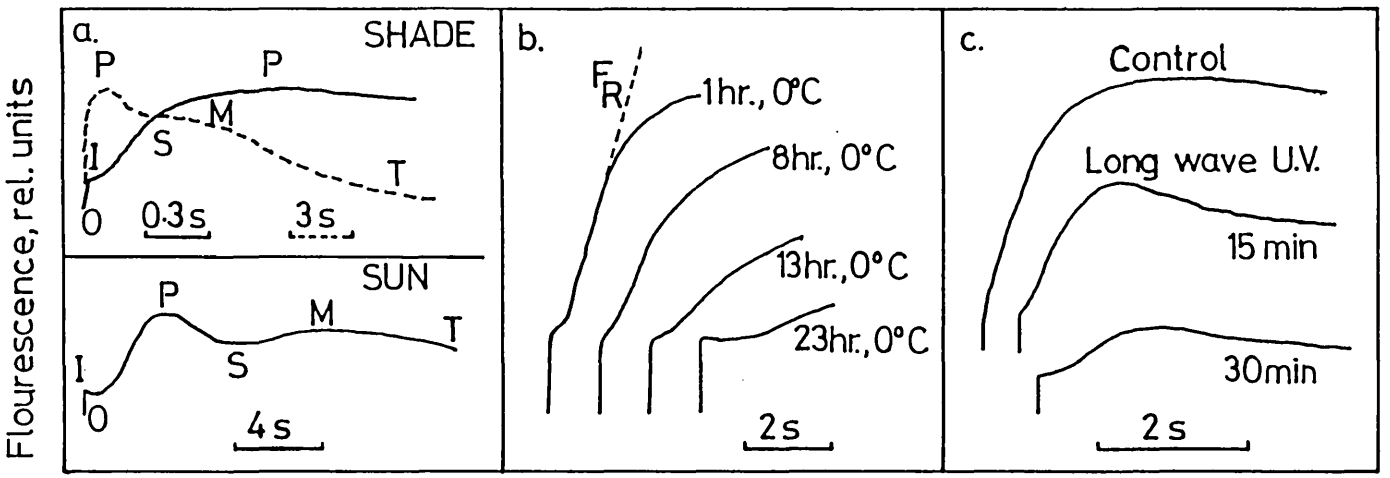


Fig. 1.5 The changes in fast fluorescence transients observed a) by comparing shade and sun grown leaves (Fork and Govindjee, 1980), b) by chilling treatments (Smillie and Hetherington, 1984) and c) by a long wave ultraviolet irradiation (Smillie, 1982).

Photoinhibition can also be detected from fast fluorescence transients as this damaging process, due to excessive light intensities, leads to chloroplasts unable to generate a normal F_v component, which is not accompanied by a change in F_o (Malkin and Jones, 1968; Powles, 1984). This inability to produce F_v cannot be restored by the addition of PS2 electron donors and therefore suggests that the PS2 reaction centre protein has been damaged leading to an increase in energy de-excitation via an increase in k_d (see review by Powles, 1984).

1.4.3.2 Water Stress:

Plants which have been subjected to differing degrees of water stress show an inability to generate F_v which is not brought about by an increase in F_o. Govindjee et al. (1981) have shown that leaves show a progressive decrease in F_v with increasing degrees of water stress. It was concluded that this form of environmental stress affected the donation of electrons to P680 via the water splitting process and that the p/F_o ratio was a good indicator of leaf water potential.

1.4.3.3 Chilling-temperature Stress:

Not all plants can tolerate growing at temperatures at or below $4^{\circ}C$. Some plants can adapt to these temperatures and one way appears to involve the lowering of the thylakoid membrane protein/lipid ratio (Chapman et al., 1983) which probably helps maintain an optimal membrane fluidity (Mitchell et al., 1984). Such plants exhibit normal fluorescence transients, however, those plant species which cannot maintain growth at $4^{\circ}C$, like H_2O-stressed plants, are incapable of generating F_v (see Fig. 1.5b), although the F_o level remains unchanged (Smillie, 1979).

A method has been devised to measure the degree of chilling-stress tolerance by calculating the slope of the fast fluorescence rise to the p-level (termed F_r). This parameter has been shown to decrease on the plant becoming injured by chilling temperatures and the rate at which F_r changes has been related to the plant's chilling tolerance (Hetherington and Smillie, 1984; Smillie and Hetherington, 1984).

1.4.3.4 Other Environmental Factors:

Plants which have been subjected to ultra-violet radiation produce fast transients with an inhibited F_v component (Katoh and Kimimura, 1974). Short U.V treatments do not affect the F_o but prolonged U.V treatments lead to a large decrease in F_o (Smillie, 1982). These changes are depicted in Fig. 1.5c. Damage caused by high ozone levels also leads to a decrease in F_v (Schreiber et al., 1978). The fluorescence technique allows damage to be predicted at least 20 hours before the appearance of any visible signs of leaf necrosis. Although ozone eventually leads to chlorophyll degradation, this is not believed to be the primary damaging event as there is no change in F_o. Therefore, ozone damage is also believed to affect the water-splitting processes (Schreiber et al., 1978). A number of other environmental stresses like mineral deficiency, H_2O logging and high salinity (Satoh et al., 1983) can also be detected by alterations in the fast fluorescence transients mainly through an inhibited F_v.

1.4.4 Herbicide Resistance:

Resistance to triazine herbicides is thought to be due to a single amino acid change in the 32 kda, herbicide binding protein associated

with PS2 which in a resistant Amaranthus sp. converts a serine to a glycine residue (Hirschberg and McIntosh, 1983). These herbicide-resistant plant species show altered fast fluorescence transients when compared with their susceptible counterparts. This difference is mainly seen in an increased F_o to i rise, in the resistant plants, followed by a slightly slower fluorescence rise from i to p (see Pfister and Arntzen, 1979). These observed changes can be explained by the equilibrium of the reaction $Q_A^- Q_B \rightleftharpoons Q_A Q_B^-$ being moved to the left in the resistant plant's chloroplasts (Vermaas and Arntzen, 1983) or by an altered ability for PQ to bind to the Q_B-protein. Although the changes involved are still not fully resolved the altered transients do give an indication that chlorophyll fluorescence can detect subtle changes in photosynthetic electron flow away from PS2. Fluorescence can also be used as a good screening technique for plant's possessing herbicide resistance. Those plants which are susceptible will not exhibit the characteristic fluorescence transients of non-inhibited plants after herbicide treatment but instead the chlorophyll fluorescence will rise rapidly to its maximum level. No such affect would occur with resistant plants.

1.5 The Slow Fluorescence Transients:

After a period of dark adaptation, photosynthetic material when illuminated undergo changes in chlorophyll fluorescence which take place over the minute timescale. These changes, which are shown in Fig. 1.6, are often designated p,s,m and t which represent the peak level(which can reflect the maximum fluorescence level when all Q_A is reduced if the actinic light intensity is strong enough), a quasi-steady state, a secondary peak and the terminal fluorescence level

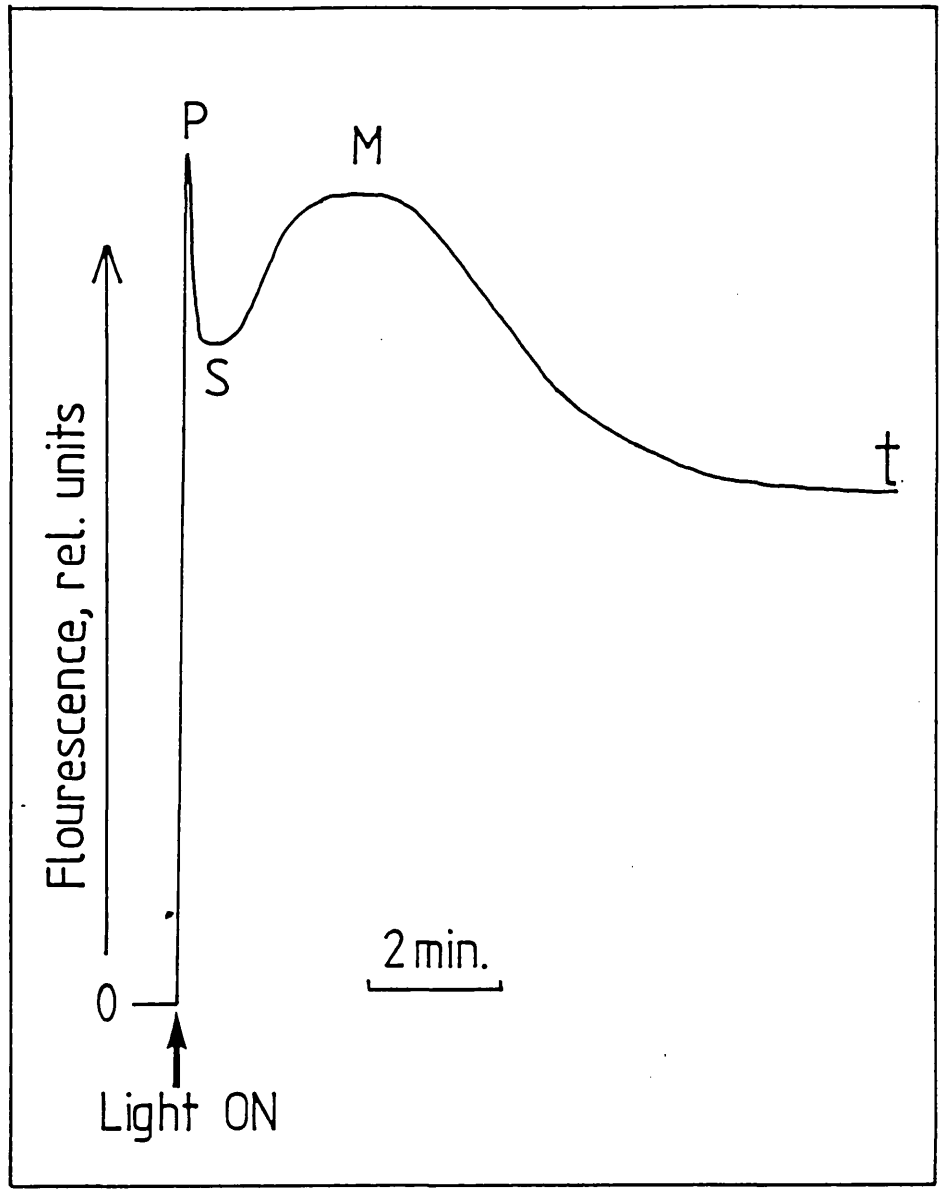


Fig. 1.6 Slow chlorophyll fluorescence transients observed at room temperature on illuminating a dark adapted pea leaf with blue-green light ($2Wm^{-2}$) and showing the p,s,m and t levels of fluorescence.

respectively. This is a simplified view of events as these transients can be altered depending on the conditions so that sometimes no secondary rise (s-m-t) is observed or any number of subsidary fluorescence oscillations can occur (see Walker, 1981; Walker et al., 1983). These changes appear to be controlled by many phenomena which arise in the photosynthetic apparatus, some of which reflect regulatory photosynthetic restraints imposed by the interaction between the so-called "light reactions" (electron transport from H_2O to $NADP^+$) and the "dark reactions" (carbon assimilation) which is inpart due to ATP and NADPH being common to both.

It was Bannister and Rice (1968) who implicated that photosynthetic electron flow, especially cyclic electron transfer, played a role in the manifestation of the slow fluorescence transients. Using a series of Chlamydomonas mutants, they showed that non-cyclic electron flow was not necessarily required for the generation of the secondary rise, while it was inhibited in those mutants which lacked cyclic electron flow. Walker and colleagues have shown that the secondary rise can be related to the onset of CO_2 fixation, for instance, Walker (1981) found that the s-m-t oscillation was inhibited by the addition of D-L-glyceraldehyde, a specific inhibitor of CO_2 assimilation (Stokes and Walker, 1972). Perhaps it is cyclic electron flow which helps to balance the ATP and NADPH levels so both the "dark" and "light" reactions can work efficiently. It has been postulated that the fluorescence oscillations arise because of the imbalance between the two ATP-consuming kinases, phosphoribulokinase and phosphoglycerate kinase, present in the reductive pentose phosphate pathway (Quick and Horton, 1984a). It is suggested that the fluorescence changes manifest themselves through alterations in Q_A redox state and pH gradient, which depend on both electron

transport rate and CO_2 assimilation. These ideas are consistent with the antiparallel relationship between fluorescence and CO_2-dependent O_2 evolution (Walker et al, 1983) and PQ redox state (Bradbury and Baker, 1981).

Using pea chloroplasts, it has been shown that changes in Q_A redox state and energy state are dependent on light intensity, electron transport rate, and the initial levels of NADPH, ADP and P_i (Horton, 1983a). These observations led Horton to suggest that these factors interact to produce a range of slow fluorescence changes. This work has been extended using broken chloroplasts reconstituted for CO_2 fixation (see Lilley and Walker, 1979) which by careful manipulation can produce similar fluorescence transients to those observed in leaves (Horton, 1983c). It was seen that changes in O_2 evolution, Δ pH, Q_A redox state and fluorescence were evoked by a) the availability of electron acceptor as determined by the addition of NADP and phosphoglycerate and b) the ratio of ATP to ADP as controlled by the addition of ribose-5-phosphate (Horton, 1983c). These observations help confirm the ideas of Walker (1981), who suggested that <u>in vivo</u> the secondary rise and slow fluorescence decline could be partially explained by alterations in adenylate status, P_i levels and phosphoglycerate reduction.

Many mechanisms are thought to be involved in bringing about the slow fluorescence changes and they can be divided up into two broad categories; 1) photochemical quenching (q_p) and 2) non-photochemical quenching (q_{np}). The photochemical quenching occurs by the oxidation of PS2 electron acceptors which lead to an increase in the rate of excitation energy dissipation via photochemistry. Non-photochemical quenching arises because of increases in the rate of chlorophyll de-

excitation by radiationless decay processes or energy transfer to non-fluorescing chlorophyll pigment-beds. This latter quenching can be brought about by a number of processes including the generation of a proton gradient across the thylakoid membrane (Barber and Telfer, 1974; Krause, 1974, 1978), the presence of reduced pheophytin (Klimov and Krasnovski, 1981) or membrane conformational changes by either alterations in cation levels (Barber, 1976), protein phosphorylation (Bennett et al., 1980) or light quality (Telfer and Barber, 1981).

1.5.1 High Energy State (HES) Quenching of Fluorescence (q_e):

Several workers have demonstrated that the slow fluorescence decline is sensitive to uncouplers (see reviews by Govindjee and Papageorgiou, 1971; Papageorgiou, 1975; Barber, 1976). Studies into this relationship, using isolated chloroplasts, were initiated by Murata and Sugahara (1969) who found a PMS-stimulated quenching of fluorescence in the presence of DCMU. This was reversed in the dark or by the addition of uncouplers like CCCP or methylamine. It was concluded that the quenching was due to a high energy state created by cyclic electron flow around PS1. Wraight and Crofts (1970), also observed similar fluorescence quenching using the cyclic electron flow co-factor, DAD. They attributed the decrease in fluorescence to the net uptake of protons into the intrathylakoid space which produced a pH gradient across the membrane. Krause (1974) proposed that the fluorescence quenching was caused by the movement of Mg^{2+} ions from the intrathylakoid space into the stroma in response to a light-induced ΔpH. The following scheme summaries the concept:

$$\text{light} \longrightarrow \Delta pH \longrightarrow \Delta Mg^{2+} \longrightarrow \Delta \text{conformation} \longrightarrow \Delta \phi_F$$

Extensions of this idea were presented in the papers of Barber et al. (1974a,b) and Telfer et al. (1974), who were the first to demonstrate

the exclusive action of Mg^{2+} in the HES phenomenon by the use of cation-specific ionophores to reverse the quenching. While the involvement of a conformational change was shown by the abolition of the HES quenching by glutaraldehyde fixation, although the proton pump was still operative (Mohanty et al., 1973).

It is believed that the HES quenching reflects an increase in thermal deactivation (k_d) and not a change in excitation energy redistribution between the two photosystems (k_t). By examining standardised, 77K fluorescence emission spectra, Krause et al. (1983) have shown that both PS1- and PS2-associated fluorescence peaks are quenched as a result of the HES. These observations would not be expected if the mechanism for the fluorescence decrease involved a change in k_t.

The relative extent of the ΔpH component involved in the slow fluorescence decline can easily be estimated. The early work of Barber and Telfer (1974) and Mills and Barber (1975) showed that the addition of DCMU to chloroplasts, which had undergone HES quenching, led to a biphasic return to a high fluorescent state. They discussed this phenomena in terms of the fast component reflecting the reduction of oxidised Q_A and the slow phase being the consequence of a slow collapsing of the proton gradient indicative of the extent of the HES quenching component involved in the total fluorescence decline.

Krause et al. (1982) have shown that HES quenching can be a predominant factor in contributing to the slow fluorescence decline exhibited by intact chloroplasts and green algae. As in the earlier studies they added DCMU to the photosynthetic material during the slow fluorescence changes and measured the fast ($t_{\frac{1}{2}}$-1s) and slow ($t_{\frac{1}{2}}$-5-15s) components.

It is believed that such a light-mediated proton gradient becomes an important factor in *in vivo* fluorescence quenching when the utilisation of the ΔpH by photophosphorylation is limited, for example, during the lag phase of CO_2 assimilation because of an increase in cyclic electron flow or in the absence of CO_2 (Walker, 1981). However, when the excitation energy is low, changes in q_e quenching have been shown not to be such an important factor (Quick and Horton, 1984b).

1.5.2 Q_A reoxidation during the Slow Fluorescence Decline:

Recent work by Bradbury and Baker (1984) has investigated the extent of q_p quenching associated with fluorescence changes observed by exciting higher plant leaves with high or low light intensities. From analysing fluorescence changes induced by an additional saturating light flash (see Bradbury and Baker, 1981) it was shown that Δq_p was of only minor importance at high actinic light intensities and during the m to t decline at low light but made a larger contribution to the p-s-m transients at low light. These contentions, however, have not been confirmed when barley protoplasts were used to examine the extent of q_p quenching during the slow decline (Quick and Horton, 1984b). They used two techniques to measure changes in q_p which involved the addition of DCMU, in a similar manner to Krause et al. (1982), and a modulated light doubling technique, which is similar in principle to that used by Bradbury and Baker (1984). Both techniques showed that significant q_p quenching took place between the p and t fluorescence levels so that at p all Q_A was reduced while at t a proportion of Q_A had become reoxidised. This was seen at both high and low light intensities and oscillated in parallel with the rate of O_2 evolution. It was also seen that the addition of DCMU at the t-fluorescence level did not lead to a complete

return to the p-level of fluorescence. This non-reversible quenching was termed q_R and it has been suggested that it might involve protein-phosphorylation-induced conformational changes, as ^{32}P has been shown to be incorporated into LHC during the time course of the fluorescence decline, or perhaps photoinhibition (see Quick and Horton, 1984b).

1.6 Conformational Changes:

It is believed that part of the slow fluorescence decline represents a redistribution of excitation energy to pigment-beds which have a lower fluorescence yield at room temperature. Such changes are thought to result from an alteration in pigment-protein organisation within the thylakoid membranes (see reviews by Barber, 1982a; 1983).

1.6.1 State 1 - State 2 Transitions:

Bonaventura and Myers (1969) and Murata (1969a) were the first to produce a model to account for the slow decline in chlorophyll fluorescence, under certain illumination conditions. Independently, they suggested that the decrease in fluorescence reflected a redistribution of trapped quanta in favour of PS1 at the expense of PS2. This was also hinted at by the work of Duysens and Talens (1969) who noticed that a slow fluorescence decline was exhibited by algae during illumination which they attributed to the generation of Q', a quencher of fluorescence which was associated with reoxidation by PS1. Bonaventura and Myers (1969) used fluorescence spectroscopy and oxygen polarography to examine the slow changes exhibited under limiting light intensities by Chlorella pyrenoidosa. This involved modulated light which preferentially excited PS2 (light 2) to investigate the effect of superimposing

non-modulated light which preferentially excited PS1 (light 1) on the quantal efficiency of modulated fluorescence and oxygen production. The addition of light 1 led to a decrease in fluorescence and an increased rate of O_2 evolution. Such an increase in quantal efficiency for O_2 emission by light 1 is an expression of the Emerson enhancement effect (Emerson et al., 1957). It was found that enhancement decreased as fluorescence decreased and if light 1 was left on for several minutes, its removal then caused both fluorescence and enhancement to increase. The high fluorescence, high enhancement situation brought about by light 1 was termed State 1, while the low fluorescence, low enhancement state was called State 2 and this was achieved by illumination with excess light 2.

Murata (1969a), using Porphyridium cruentum, arrived at the same conclusions by examining 77K fluorescence spectra. He noticed that the PS1- and PS2-associated peaks varied according to whether the algae had been illuminated prior to freezing. Illumination led to a decreased PS2 emission and an increased PS1 peak, which was reversed by prolonged light 1 treatment.

Since these pioneering observations the co called State 1-State 2 transition has been demonstrated in a wide variety of algae (Reid et al., 1977; Reid and Reinhardt, 1977 and 1980; Williams et al., 1980) while the first ever demonstration in higher plant leaves was shown by Chow et al. (1981b). Recently changes similar to those observed by Bonaventura and Myers (1969) have been demonstrated in intact higher plant leaves. This has involved the measurement of modulated oxygen evolution rates using a photoacoustic technique (Bults et al., 1982) simultaneously with the well characterised fluorescence changes (Canaari et al., 1982 and 1984).

This regulatory mechanism has been detected in a wide-range of plants but does not appear in an LHC-lacking barley mutant (Chow et al., 1981b), and has been shown to be inhibited by glutaraldehyde fixation (Vernotte et al., 1974) suggesting the involvement of a conformational change.

1.6.2 The effect of cation concentration on fluorescence yield:

At the same time as the observation and investigation of the State 1-State 2 transition in algae, Murata (1969b) noticed that both room temperature and 77K chlorophyll fluorescence could be altered in higher plant chloroplasts by manipulating the $MgCl_2$ level. Homann (1969) also noticed that broken, DCMU-inhibited chloroplasts suspended in a "low" cation medium could produce a higher fluorescence state by the addition of Mg^{2+}. Murata et al. (1970) and Murata (1971) extended this original work to demonstrate that a wide range of divalent cations as well as higher concentrations of monovalent cations could bring about similar changes in fluorescence yield as Mg^{2+}. Later, work by Barber and Searle (1978) showed that trivalent cations could also stimulate chlorophyll fluorescence after earlier observations did not show such changes (Murata et al., 1970; Mills and Barber, 1978). However a few cations like Zn^{2+}, Cd^{2+} and Pb^{2+} have been shown not to stimulate fluorescence (Murata et al., 1970; Mills and Barber, 1978).

By using 77K fluorescence spectra it was shown that salt addition increased PS2-associated fluorescence emission at the expense of PS1 (Murata, 1969b; Murata et al., 1970; Krause et al., 1982). This led Murata to postulate that the presence of cations decreased the degree of spillover of excitation energy from PS2 to PS1 giving the original

basis to the formulation of a possible mechanism for the State 1-State 2 transition which involved light-driven cation movements across the thylakoid membrane leading to changes in spillover (see Barber, 1976).

The link between spillover and salt levels has been studied intensively, not only by fluorescence measurements, but also by correlation with a number of different measurements including enhancement of full chain electron transfer (Sinclair, 1972; Sun and Sauer, 1972), PQ redox state (Marsho and Kok, 1974), and PS1 and PS2 partial reactions so that the addition of Mg^{2+} increases the quantal efficiency of PS2 reactions but decreases PS1-mediated electron transfer (Murata, 1971; Ben-Hayyim and Avron, 1971; Marsho and Kok, 1974).

Accompanying the salt-induced fluorescence changes are gross membrane conformational changes. At low cation concentrations, thylakoids are unstacked (Izawa and Good, 1966; Murakami and Packer, 1971; Gross and Prasher, 1974) and membrane proteins appear to be randomly distributed throughout the membrane (Goodenough and Staehlin, 1971). The addition of cations leads to membrane reorganisation and the differentiation of the thylakoids into stacked and unstacked regions which contain different types of protein-complex (see review by Boardman et al., 1978).

From the work of Gross and Hess (1973 and 1974), who carried out studies on salt-induced fluorescence changes and thylakoid stacking changes over a wide range of electrolyte conditions, showing an antaganism between monovalent and divalent cations, it became evident that the associated spillover changes reflected an alteration of the membrane's electrostatic properties (Barber et al., 1977; Duniec et al., 1979).

1.6.3 The Role of Surface Charge:

The electrical nature of the cation effect was suggested by three phenomena; a) the antagonism between monovalents and divalents, b) the antagonism between low levels of monovalents and high levels of monovalents and c) the dependence on the valency rather than the chemical nature of the cations. It was Barber and Mills (1976) and Barber et al. (1977), who first showed that these three effects could be explained in terms of changes in the positive space charge density adjacent to the membrane's surface by the application of the Gouy-Chapman diffuse double layer theory for mixed electrolyte conditions (see also Rubin and Barber, 1980). This theoretical approach predicted the valency dependence and antagonistic character of the cation-induced changes.

The positive space charge density immediately adjacent to the membrane's surface, is a diffusable, boundary of electrical charge screening fixed negative charges on the membrane's surface and controlling the coulombic interaction between the surfaces of exposed membrane proteins and adjacent membranes (Barber, 1980a,b and 1982a,b). Normally the surfaces of macroscopic systems experience strong long range attractive, Van der Waals forces which tend to draw them together. However, when the surfaces carry net electrical charge the attractive forces are counterbalanced by coulombic repulsive forces. The 'screening' of fixed surface charge with diffusible counter ions leads to a reduction of the repulsive forces enabling the two surfaces to approach each other, until a balance is achieved between attraction and repulsion and a "minimum energy distance" attained.

Another way of achieving this is through charge neutralisation by either cation-binding to the surface or by protonation of the surface

so that the long range coulombic forces no longer exist because of the removal of the fixed charge. This concept was advocated by Duniec et al. (1979) to explain the cation-induced changes. They proposed that protonation of the membrane was involved, however, this was later disproven by Barber and Searle (1979) who showed that the salt-induced changes still occurred at pH-values which would not allow a sufficient lowering of the pH at the membrane's surface to take place.

Charge neutralisation can be achieved by lowering the pH to 4.3 which leads to extensive membrane stacking (Barber et al., 1980; Chow et al., 1980; Scoufflaire et al., 1982). This, however, gives rise to a different type of stacking to that associated with the salt-induced phenomena, so that the thylakoid-proteins remain randomised and no large fluorescence increase is observed, as there are no changes in spillover (Barber et al., 1980). Therefore, the salt-induced changes appear to arise because of the reduction of surface potential due to 'screening' while maintaining a constant surface charge density (Barber 1980a,b) and not to charge neutralisation by either protonation as advocated by Duniec et al. (1979) or cation-binding as postulated by Sculley et al. (1980).

It has been proposed that due to the fluid nature of the thylakoid membrane (Ford and Barber, 1980) a lateral displacement of protein-complexes occurs on the addition of salt. The proteins which carry less electrical charge come together to form the appressed, granal membranes, while the highly negatively charged proteins become displaced into the non-appressed, stromal regions. This sequence of events requires the presence of LHC to take place (Vernotte et al., 1976), and leads to close membrane appression by charge displacement rather than the simpler

charge neutralization process. In the presence of "low" cation levels the electrostatic screening is low, therefore the highly charged proteins repel each other and move into the appressed regions producing an increase in coulombic repulsion between adjacent membranes leading to destacking and protein randomisation. These processes which occur under poor electrostatic screening conditions lead to a decreased spatial separation of PS2 and PS1 and thus to a low chlorophyll fluorescance yield due to increased spillover. When electrostatic screening is high (i.e. in the presence of "high" salt levels) then domains of low-charge, fluorescent pigment-protein-complexes (PS2) become segregated from domains of high-charge, quenching complexes (PS1) resulting in a high fluorescence state and low spillover (see Barber, 1982a,b).

1.6.4 The Lateral Segregation of Thylakoid Proteins:

The theoretical arguments and associated experimental results regarding surface charge distribution along with membrane fluidity studies support the contention that a salt-induced lateral diffusion and segregation of PS2 and PS1 takes place (Barber and Chow, 1979; Barber, 1980a; Chow and Barber, 1980b; Chow et al., 1981a). As a consequence PS2 and LHC become located in the appressed, granal lamellae while PS1 and the ATP synthetase complex are located in the non-appressed, stromal lamellae (Barber, 1980a). This view is shared by Andersson (1978) and Anderson (1981), who both base their arguments on the analysis of different membrane fragments. These studies have involved the mechanical shearing of thylakoid membranes by a Yeda press followed by an aqueous polymer two-phase partition technique (Andersson et al., 1981). This allows the separation of fragments according to their surface properties (Albertsson, 1978) and produce vesicles which are right-side out, a population derived from the appressed regions which are inside-out, and fragments from the stromal lamellae. By comparing the pigment-protein

complex content and photosynthetic reactions from these fragments it was concluded that most of the PS2 and LHC were located in the inside-out vesicles while the stromal-derived fragments contained PS1 and the ATPase (Andersson and Anderson, 1980; Andersson and Haehnel, 1982). These studies have been extended by the characterisation of detergent-treated thylakoids which yield membranes enriched in PS2-associated proteins (Berthold et al., 1981; Lam et al., 1983). A diagram which represents a present day understanding of membrane protein location is shown in Fig. 1.7. Such an arrangement of the pigment-protein complexes within the thylakoid membrane allows the regulation of excitation energy distribution to occur in higher plants. It is now believed that this is achieved by the phosphorylation and dephosphorylation of the LHC.

1.7 Thylakoid Membrane Protein Phosphorylation:

A significant advance in the understanding of the mechanism of the State 1-State 2 transition has been the finding that under certain conditions a number of thylakoid proteins become phosphorylated (Bennett, 1979a,b,c, 1977 and 1980). Protein-phosphorylation has now been linked to several different effects in addition to changes in excitation energy distribution (Allen et al., 1981) which include a) an enhanced affinity for DCMU binding to the Q_B-protein (Shochat et al., 1982), b) an increased charge density around Q_A (Kyle and Arntzen, 1983), c) an enhanced stability of the semiquonone species, Q_B^- (Jursinic and Kyle, 1983), d) a stimulation of a dissipative cycling of electrons around PS2 (Horton and Lee, 1983) and e) an improved protection from photo-inhibition (Horton et al., 1984). The following section will, however, concentrate solely on the role of protein-phosphorylation in bringing

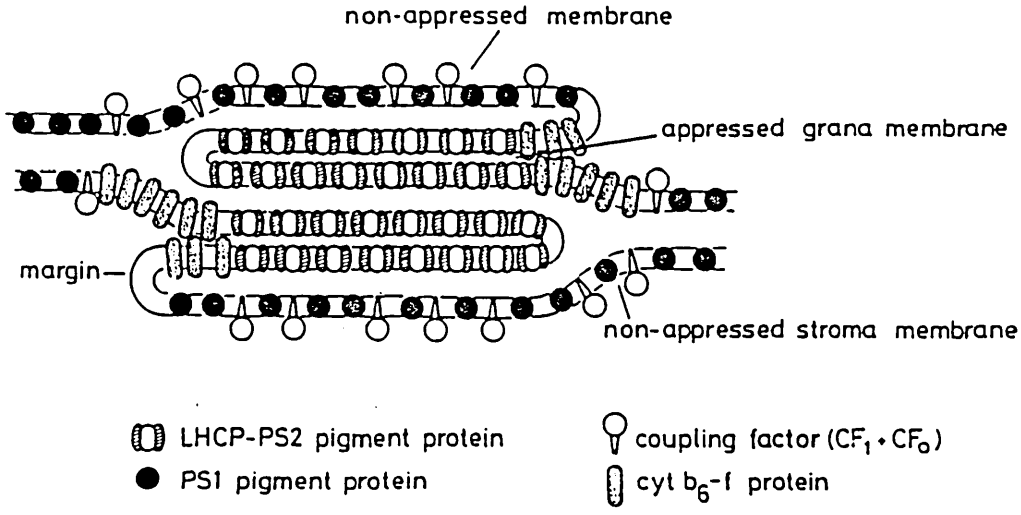


Fig. 1.7 A model for the distribution of the thylakoid complexes between appressed and stroma-exposed thylakoid membranes. Taken from Chapman and Barber (1984).

about excitation energy redistribution, thereby influencing the slow chlorophyll fluorescence decline (also see the reviews by Barber, 1983; Bennett, 1983; Allen, 1983; Staehlin and Arntzen, 1983; Horton, 1983b).

1.7.1 The Enzymes Involved:

Chloroplast thylakoid membranes possess a membrane-bound kinase (Bennett, 1979a) which can be removed by octyl-glucoside (Alfonzo et al., 1980) and a phosphatase which brings about the phosphorylation/dephosphorylation of a number of thylakoid proteins, including LHC (Bennett, 1979a, 1980). The kinase has an ATP dependence with a k_m = 90 μM (Bennett et al., 1980). The major phosphorylated species are the two LHC polypeptides (MW of 24 kda and 26 kda) which appear to be equally labelled (Bennett et al., 1980), although only 50% of the total LHC is affected (Haworth et al., 1982b). A variety of other proteins become labelled including 9 kda, 32 kda, 35 kda and 45 kda polypeptides which all appear to be associated with PS2 function (Owens and Ohad, 1982; Steinback et al., 1982). It is still not known, however, where the kinase is situated within the thylakoid membrane, in what concentration it is present, or whether the multiple forms which have been reported by Markwell et al. (1983) phosphorylate different polypeptides.

The phosphatase, which is also membrane-bound, catalyses the dephosphorylation of LHC and the other phosphorylated polypeptides, but only the LHC becomes dephosphorylated at a rate which would imply that it is involved in excitation energy distribution control as the others are dephosphorylated at slower rates (Steinback et al., 1982). The phosphatase can be inhibited by the addition of NaF or molybdate (Bennett, 1980).

1.7.2 Control of Kinase Activity:

The kinase is light-activated although it is not strictly light-dependent (Bennett, 1979b) and requires the presence of Mg^{2+} (optimal concentration for kinase activity being 3-5 mM; Bennett, 1983). It is believed that the active site contains a thiol group (Millner et al., 1982). From inhibitor and electron donor studies it was concluded that the reduction of an intersystem component of electron transfer was required for activation (Horton and Black, 1981a; Allen et al., 1981). The enzyme could be activated in the dark by reductants of PQ like dithionite (Allen et al., 1981), duroquinol (Allen and Horton, 1981) and ferredoxin (Bennett, 1979b; Chow et al., 1981b). Redox titrations of kinase activity showed the requirement of the reduction of a two electron acceptor having a midpoint potential (pH 7.8) of 0 mV which suggested PQ (Horton and Black, 1980; Horton et al., 1981). The involvement of PQ was further highlighted from single-turnover light flash titrations, the effect of DBMIB (Allen et al., 1981) and from the antagonistic effect of light 1 and light 2 (Horton and Black, 1980; Telfer and Barber, 1981; Bennett, 1983). This gives the plant a sensitive method of monitoring quantal imbalance but the direct relationship between the PQ redox state and kinase activity is still unknown.

It is unlikely that spectral imbalance is the only parameter controlling the extent of protein phosphorylation. It has been shown that the kinase can respond to changes in certain carbon assimilation reactions which consume ATP and NADPH. Allen and Bennett (1981), first reported that the kinase was more active during the lag period of CO_2 fixation, when non-cyclic electron flow is limited by certain carbon metabolism intermediates because of a limited ATP/ADP balance (Walker and Robinson, 1978). This situation leads to the over reduction of PQ,

the phosphorylation of LHC, a stimulation of cyclic electron flow which subsequently increases the ATP levels enabling carbon metabolism to turn over more rapidly. This inverse relationship between kinase activity and CO_2 fixation suggested that phosphorylation could regulate the balance between cyclic and non-cyclic electron flow to maintain sufficient ATP and NADPH levels for CO_2 metabolism (Allen and Bennett, 1981). It has also been proposed that the adenylate status of the chloroplast system controls the degree of phosphorylation, as the kinase is inhibited by ADP and responds to changes in energy charge (Markwell et al., 1982; Baker et al., 1983). Using a reconstituted chloroplast system, Horton and Foyer (1983), have shown that the protein kinase can be controlled by both PQ redox state and energy state which appears to provide a mechanism for adapting light harvesting to the demands of carbon assimilation. Allen (1984) has proposed, however, that under normal physiological conditions regulation by adenylate status is unlikely as such a control mechanism, whether by positive or negative feedback, would always make the situation worse. He has postulated that phosphorylation has a role to play in the redox poising of cyclic and non-cyclic electron flow, controlled by the PQ redox state, correcting imbalances in the ratio of ATP to NADPH by negative feedback control. Fernyhough et al. (1983) have been unable to correlate kinase activity with adenylate status. By using maize mesophyll chloroplasts, which contain both Calvin cycle and C_4 enzymes, the levels of ATP and NADPH were manipulated, by adding either pyruvate (consumes ATP), oxaloacetate (consumes NADPH) or 3-phosphoglycerate (consumes both), and correlated with kinase activity. This system showed that there was a clear effect of redox state on protein kinase activity and that high rates of cyclic electron flow were necessary to induce maximum kinase activity. They suggested that protein phosphorylation

functions at the interface between electron transport and carbon assimilation and not just in the State 1-State 2 transition. Recently, Fernyhough et al. (1984), using maize mesophyll chloroplasts in which linear electron transport was absent, have shown that protein kinase activity could be stimulated when the ΔpH gradient (formed by cyclic and pseudo cyclic electron flow) was diminished, by the addition of nigericin. They concluded, like Allen (1984) that protein phosphorylation could be a controlling factor in the ratio of cyclic and non-cyclic electron transfer possibly by redox poise.

1.7.3 The Consequences of Protein Phosphorylation:

An ATP-dependent decrease in room-temperature chlorophyll fluorescence in intact and shocked chloroplasts has been observed (Bennett et al., 1980; Horton and Black, 1980, 1981a, 1982; Allen and Horton, 1981; Horton et al., 1981; Telfer and Barber, 1981; Chow et al., 1981b) which has been correlated with the degree of LHC phosphorylation (Horton et al., 1981; Telfer et al., 1982). This decrease was reversible and could be affected by NaF, so that the reversal was inhibited and the decrease was faster. During this change in fluorescence an alteration in excitation energy distribution was detected from 77K fluorescence spectroscopy (Bennett et al., 1980; Horton and Black, 1980; Telfer and Barber, 1981; Steinback et al., 1982; Scoufflaire et al., 1983; Krause and Behrend, 1983). Furthermore, it led to a decreased rate of PS2 electron transfer (Steinback et al., 1982; Farchaus et al., 1982) and a subsequent increase in PS1 electron transfer rates (Horton and Black, 1982; Farchaus et al., 1982) at limiting light intensities. It has also been shown that phosphorylation could alter light-induced redox changes associated with cyt f in a way which indicated lower rates

of PS2 electron flow (Horton and Black, 1981b). However, recently Haworth and Melis (1983) have suggested that protein phosphorylation does not alter PS1 absorption cross section, as P700 reduction kinetics were unaltered by the phosphorylation treatment. These experiments were carried out at 2 mM $MgCl_2$ but the fluorescence changes observed due to phosphorylation did not show the preferential large decrease in F_v and decrease in F_o previously reported by Horton and Black (1983) which may suggest that under the conditions used by Haworth and Melis phosphorylation had not taken place. Their conclusions have since been contradicted by Telfer et al. (1984), who by measuring PS1 photo-oxidation at 820 nm did detect a change in PS1 photochemical efficiency after phosphorylation.

1.7.4 The Mechanism Involved:

The fluorescence changes suggest that phosphorylation brings about a lower extent of excitation energy going to PS2 with a subsequent increase in excitation energy arriving at PS1. This can be achieved by two mechanisms; either a change in photosystem absorption cross section or an alteration in spillover (see Butler and Kitayama, 1975). From the analysis of 77K fluorescence induction curves using the bipartite and tripartite kinetic models proposed by Butler and Kitayama (1975), Butler and Strasser (1978) and reviewed by Butler (1978) information relating to these two processes can be determined by analysing changes in F_o and F_m at both 690 nm and 730 nm. Haworth et al. (1982a) carried out such an analysis and as a result concluded that phosphorylation led to a change in both spillover and absorption cross section so that LHC-PS2 coupling was decreased and exciton transfer from PS2 to PS1 increased. However, it has been postulated that at high salt levels

phosphorylation-induced fluorescence changes are brought about solely by a change in PS2 absorption cross section (Horton and Black, 1981a and 1983). Such contentions are dealt with in more detail in section 1.8.2.

Biochemical and structural information has been obtained which suggests that the movement of phosphorylated proteins from the appressed to the non-appressed membrane regions takes place, as predicted by Barber (1982a). Freeze-fracture electron microscopy studies have shown the movement of particles from the granal to the stromal lamellae upon phosphorylation. Kyle et al. (1983b) reported the movement of a population of 8 nm particles upon phosphorylation of thylakoids which was reversed by dephosphorylation and postulated the involvement of a 'mobile pool' of LHC. Simpson (1983) also noticed the movement of particles out of the grana, but with a subsequent increase in another population of particles within the grana which he proposed may have arisen from a conformational change in PS2 or the movement of PS1 into the appressed regions. Radioactive labelling experiments followed by thylakoid fragmentation have revealed the presence of more labelled LHC in the stromal fragments (Andersson et al., 1982) while all the other phosphorylated polypeptides remained in the granal fragments (Larsson et al., 1984). Kyle et al. (1984) have recently supported the notion that phosphorylation leads to the movement of a sub-population of phosphorylated LHC from granal to stromal membranes by fragmentation studies, in which chla/b ratios and 77K fluorescence characteristics were measured, and from pulse-chase experiments. It was noted that not all of the phosphorylated LHC moved from the grana but that the LHC which did migrate out became "energetically-associated" with PS1, since the ability to excite 735 nm fluorescence at 77K by chlorophyll b was increased following phosphorylation.

Little evidence has been reported concerning the occurrence of conformational changes during the in vivo State transitions. Vernotte et al. (1975) observed no large change in chloroplast ultrastructure when leaves were preilluminated with light 1 or light 2, while Bennoun and Jupin (1974) reported a 33% decrease in stacking when Chlamydomonas reinhardi were in State 2 compared with State 1. Punnett (1970) has detected large light-induced changes in in vivo stacking which was correlated with alterations in enhancement. These observations were achieved, however, by a much longer light treatment than that required to bring about State changes. Kyle et al. (1983b) and Biggins (1982) have both reported a 10-15% decrease in membrane stacking by electron microscopy, after in vitro protein phosphorylation while Chow et al. (1981b)have also detected a small degree of unstacking using a digiton method which produced stromal lamellae with altered chl a/b ratios and chlorophyll content after in vitro phosphorylation. Further indirect measurements of conformational change, observed during the ATP-induced fluorescence decline have been made using 90° light scattering (Scoufflaire et al., 1983).

It is believed that the protein phosphorylation process is under electrostatic control (Barber 1982a but also see Barber, 1980a). The phosphorylation takes place on threonine groups located on a positively charged 2 kda, trypsin-digestable segment of the LHC (Bennett, 1980). Removal of this 2 kda segment inhibits both the phosphorylation-induced fluorescence changes and grana formation (Jennings et al., 1978, 1980; Steinback et al., 1979). The addition of phosphate groups onto the exposed surface of the LHC increases the net negative charge on the already negatively charged LHC (see Nakatani and Barber, 1980). It is postulated that the change in the protein's surface charge characteristics

alters the balance between the attractive and repulsive forces which cause the movement of phosphorylated 'mobile' LHC from granal to stromal lamellae where it then interacts with PS1.

1.8 Fast Fluorescence Transients in the Presence of DCMU:

In the presence of DCMU, or other similar PS2 inhibitors, illumination of dark pretreated thylakoids brings about a fast rise in chlorophyll fluorescence to the F_o level, followed by a rapid increase to a steady maximal level (F_m). This fluorescence rise represents the photoreduction of Q_A to Q_A^- (Duysens and Sweers, 1963). The kinetics of the induction curve was originally treated as directly reflecting the extent of reduced Q_A accumulation at any one point in time, however, this has been shown not to be the case (Malkin, 1971; Doschek and Kok, 1972). A quantitative treatment by Murata et al. (1966) suggested that the area over the fluorescence rise was proportional to the number of quanta required to reduce Q_A. This was confirmed by Bennoun and Li (1973) who observed a linear relationship between the area growth and photochemical rate.

The fluorescence rise does not follow simple first order kinetics, which would be expected for a single photochemical event. In the presence of "high" cation levels there are two distinct phases to the rise; a fast, sigmoidal component and a slower, exponential phase. This biphasic effect can easily be shown through the kinetics of the growth of the area over the fluorescence induction curve (Melis and Homann, 1975, 1976). Exponential kinetics would be expected if each PS2 reaction centre and associated antenna-chlorophylls behaved as a separate unit (see review by Barber, 1978). Sigmoidicity can be accommodated by

either a) some degree of excitation energy transfer between PS2 reaction centres (Joliot and Joliot, 1964; Lavorel and Joliot, 1972; Briantais et al., 1973; Paillotin, 1976; Hipkins, 1978) or b) the requirement for a two electron process to close each PS2 trap (Morin, 1964; Doschek and Kok, 1972).

Joliot and Joliot (1964) formulated the following equation to explain the relationship between F_v and Q_A, which related chlorophyll fluorescence to the concentration of Q_A and the probability of energy transfer from a closed PS2 reaction centre (p).

$$F_v = (1-p)(1-[Q_A])/1-p(1-[Q_A])$$

They showed that the rate of oxygen evolution (Vo_2) as a function of Q_A concentration followed the above law, with a p value of between 0.5 and 0.6 in Chlorella sp.. Their original ideas have since been criticised, modified and extended. The main criticism being that the formula assumed an equal probability of energy transfer between PS2 units. It was suggested that as Q_A became reduced the value of p would change and that the situation was further complicated by the non-random grouping of closed PS2 centres (the ilet effect) which would affect the relationship between Q_A and ϕ_F (Lavorel and Joliot, 1972). A new theory presented by Paillotin (1976) assumed that PS2 was divided into photosynthetic domains containing a certain number of reaction centres and the fate of an exciton was mainly determined by competition between the different centres for its capture which depended on the state of the centres. It is therefore believed that domains of PS2 reaction centres are randomly distributed in an array of LHC so that there is a limited interaction between PS2 and neighbouring LHC (Paillotin, 1976; Butler and Strasser, 1978). Earlier work by Joliot et al. (1973) had previously suggested that energy transfer could occur between at least three connected PS2 photosynthetic units. However, even the later

models cannot generate theoretical curves to fit the experimental fluorescence data perfectly (Hipkins, 1978; Döhnt and Renger, 1984).

In qualitative terms, the photoreduced Q_A can transfer energy to a limited number of open PS2 reaction centres and therefore a lag is observed between the level of chlorophyll fluorescence and Q_A^- accumulation. This leads to a plot of Q_A^- versus F_v being hyperbolic in nature in a similar manner to the original V_{O2} versus Q_A^- plot made by Joliot and Joliot (1964). Therefore it seems that the biphasic nature of the fluorescence rise can easily be accommodated by two forms of PS2 which differ in their degree of "connectivity ". The inability to explain the observed fluorescence curves by one type of electron acceptor and one type of pigment bed was suggested by Doschek and Kok (1972) who first noticed a slow tail in the fluorescence signal. This was later quantified by Melis and Homann (1975, 1976) and eventually Melis and Duysens (1979) showed that the biphasic reduction of Q_A, as detected by the area growth kinetics of fluorescence induction curves, correlated with ΔA_{320} changes attributed to Q_A reduction. This gave further evidence that the biphasic nature of the rise was due to two forms of PS2. The fast, sigmoidal phase representing connected PS2 centres and the slow, exponential component being due to separate PS2 centres (Melis and Homann, 1978; Melis and Ow, 1982). This idea of PS2 heterogeneity was not a novel concept, as Joliot and Joliot (1973) and Joliot et al. (1973) had already reported two forms of Q_A called Q_F and Q_S, which they believed represented a heterogeneity in either PS2 structure or antenna size. These two fluorescence quenchers were easily distinguished as Q_F (fast centres) could be reduced by a single saturating flash while Q_S (slow centres) were only reduced under continuous illumination.

1.8.1 The Consequences of Changes in Absorption Cross Section or Spillover

From theoretical assumptions it would be predicted that the two different mechanisms which can alter excitation energy distribution between the two photosystems would lead to different changes in the room-temperature chlorophyll fluorescence induction curve exhibited by DCMU-inhibited thylakoids. A decrease in PS2 absorption cross section would result in a decrease in both F_o and F_m so that the ratio F_v/F_m was left unchanged, while an increase in spillover would cause a preferential decrease in F_v with little change in F_o so F_v/F_m decreased.

Induction curves have been used frequently in the determination of the mechanism(s) involved as a result of protein phosphorylation and cation-induced phenomena. Such an approach has led to conflicting results in the literature. A large number of workers have shown that phosphorylation at "high" $MgCl_2$ levels leads to a decrease in both F_m and F_o so F_v/F_m remained unaltered, indicative of a decrease in PS2 absorption cross section (Horton and Black, 1981a, 1983; Krause and Behrend, 1983; Gerola et al., 1984). Meanwhile, Kyle et al. (1982 and 1983b) have consistently found that phosphorylation, under similar conditions as the above reports, led to a decrease in F_v/F_m and an increase in F_o. This method has also indicated that *in vitro* phosphorylation-induced fluorescence changes are dependent upon the background Mg^{2+} concentration because at lower Mg^{2+} levels (1 mM) phosphorylation produces a preferential F_v quenching leading to a lower F_v/F_m (Horton and Black, 1983).

Similar studies have been carried out using algae preadapted to State 1 or State 2 by light quality pretreatments. Bennoun (1974) showed

that compared with State 1, State 2 adapted Chlorella pyrenoidosa exhibited a lower F_o and F_v/F_m ratio. Saito et al. (1983) have shown similar differences between the two adaptive States and such observations would suggest that the in vivo State transitions occur via alterations in both absorption cross-section and spillover.

The absence of Mg^{2+} ions alters both the kinetics and the F_m level of induction curves (e.g. Homann, 1969; Murata, 1969b; Bennoun, 1974; Melis and Homann, 1976, 1978). It has also been reported by some authors that the low cation situation produces a lower F_o compared with the high salt condition (Bennoun, 1974; Butler, 1978; Hipkins, 1978). These changes have been interpreted as being due to a disruption of the statistical pigment bed (Briantais et al., 1973; Melis and Homann, 1978), thereby having a greater effect on the fast, sigmoidal component perhaps by transforming them into the slower, exponential PS2 centres (Melis and Homann, 1978). The decrease in F_m is seen to arise from an altered fluorescence emission yield from those disrupted PS2 reaction centres, as the ΔA_{320} kinetics have been shown to remain unaltered by the absence of Mg^{2+} (Melis and Ow, 1982). It has been suggested that this arises because of an increase in thermal deactivation (k_d) due to a Mg^{2+}-specific effect on the connected PS2 reaction centres (Melis and Ow, 1982), while others believe that it is a consequence of PS2 randomisation leading to an increased spillover from PS2 to PS1 (Barber, 1982a) and perhaps an uncoupling of LHC from the PS2 core complex (Nairn et al., 1983; Jennings, 1984).

1.9 PS2 Heterogeneity:

1.9.1 α and β centres:

Melis and Homann (1975) first introduced the now, well-established, kinetic analysis of the growth of the area over the room temperature chlorophyll fluorescence induction curve in DCMU-inhibited thylakoids. By plotting the natural logarithm of the normalised area growth it was shown that Q_A^- accumulation occurred in two distinct kinetic phases. Two possibilities were considered to explain the biphasic nature of the area growth; a sequential double reduction of the primary electron acceptor of PS2 or a heterogeneity of PS2 photochemical centres (Melis and Homann, 1976). However, the kinetic properties of the area growth after the firing of a single, saturating flash proved to be incompatible with the predictions of the "sequential double reduction" model. This conclusion was corroborated by results obtained from a kinetic analysis of the dark-restored area (Melis and Homann, 1976). It was therefore assumed that a heterogenous pool of PS2 reaction centres existed, with the fast sigmoidal phase termed the α-component or $PS2_\alpha$ and the slow, exponential component called the β-component or $PS2_\beta$. Further studies led Melis and Homann (1978) and Melis and Ow (1982) to propose that $PS2_\alpha$ was located in a statistical pigment bed arrangement, with energy transfer between reaction centres, in the granal lamellae, while $PS2_\beta$ were separate units, with loosely coupled LHC, a smaller absorption cross section and located in the stromal lamellae.

Other signals attributed to PS2 like the C_{550} and X_{320} absorbance changes have also been shown to exhibit a similar kinetic heterogeneity as the area growth analysis (Melis and Duysens, 1979; Melis and Schreiber, 1979). However, a C_{550} change associated with $PS2_\beta$ has

recently been disputed by Diner and Delsome (1983) which confirmed the earlier observations of Joliot and Joliot (1979) who could not show a similar relationship between F_v and C_{550} as that exhibited between F_v and Q_A^-. They concluded that the fluorescence yield was controlled by two quenchers while the C_{550} change was only a probe of one of the quenchers.

Over the past few years other differences between the two PS2 reaction centres have been investigated. Anderson and Melis (1983), using membrane fragmentation techniques, have shown via the area growth analysis that only $PS2_\beta$ is found in stromal fragments while $PS2_\alpha$ is enriched in the granal fragments. This latter point has been confirmed by analysing digitonin-produced PS2 vesicles (Lam et al., 1983) which appear only to contain $PS2_\alpha$ centres. Tobacco mutants, which apparently exhibit different proportions of the two PS2 centres, have provided evidence that $PS2_\beta$ are associated with less light harvesting antenna pigments (Thielen and van Gorkom, 1981a), which are composed of chlorophyll molecules showing different absorption characteristics to those associated with $PS2_\alpha$ in that they are far-red shifted (Thielen et al., 1981). These studies have also shown that there is a relative increase in $PS2_\beta$ correlated to a decrease in thylakoid membrane stacking (Thielen and van Gorkom, 1981b). Other differences include an absence of the herbicide binding protein (Q_B) associated with $PS2_\beta$ (Thielen and van Gorkom, 1981c) and the insensitivity of $PS2_\beta$ chlorophyll fluorescence emission to Mg^{2+} levels (Melis and Ow, 1982).

Differences in the redox properties on the acceptor side of $PS2_\alpha$ and $PS2_\beta$ (Q_α and Q_β respectively) have also been reported (Horton, 1981; Thielen and van Gorkom, 1981d). The midpoint potential (E_m) for

Q_β has been found to be approximately +120 mV (at pH 7.0) as determined by redox titrations of the removal of the slow, exponential component from induction curves (Horton, 1981). Q_β appears to be a one electron acceptor as the data fits a Nernst equation with n = 1. This observation does not fit the previous redox titrations of chlorophyll fluorescence which, as will be discussed later, indicated two quenchers with E_m values of -250 mV and 0 mV (e.g. Malkin and Barber, 1979).

In contrast to the interpretation of Melis and coworkers, other ideas have been put forward to explain the kinetic heterogeneity of the fluorescence rise. Bowes and Horton (1982), using PS2 particles prepared from Phormidium laminosium, have suggested that to obtain heterogeneity in the fluorescence induction curve does not require the presence of appressed and non-appressed membranes nor differences in pigment antenna size, as the preparation contained only 40 chlorophylls per reaction centre and were not differentiated into granal and stromal lamellae regions. Their explanation was that the presence and extent of the two kinetic components could be a consequence of differing degrees of "PS2 connectivity". Further confusion has arisen concerning what leads to the α and β components from reports that the extent of the slow phase is reflected in the degree of chloroplast intactness (Schreiber and Pfister, 1982) and that the two PS2 centres appear to show different affinities for DCMU (Brearley and Horton, 1984; Horvath et al., 1984).

A number of other types of PS2 heterogeneity have been proposed mainly from observed differences and changes in PS2 fluorescence yield as Q_A becomes reduced.

1.9.2 Q_H and Q_L:

Two components which act as 'primary' acceptors of PS2 have been determined by redox titrations of chlorophyll fluorescence yield (Cramer and Butler, 1969; Horton and Croze, 1979; Malkin and Barber, 1979; Golbeck and Kok, 1979). Based on their redox midpoint potentials they have been called Q_H ($E_{m7.0} \sim 0$ mV) and Q_L ($E_{m7.0} \sim -250$ mV). Reduction of both quenchers bring about a P_{518} absorption change, indicative of a transmembrane potential (Malkin, 1978) while it appears that only Q_H is affected by pH or low cation concentrations (Horton, 1981) and is associated with the C_{550} absorbance change (Diner and Delsome, 1983). The photoreduction of Q_L leads to a change in the reduction of Q_H and therefore it has been postulated that they share the same pigment-bed (Horton, 1981). Horton and Naylor (1979), using agranal chloroplasts, produced from intermittent light grown plants, have shown that Q_L was only observed when chloroplasts exhibited complex membrane organisation. This trait was not observed in blue-green algal derived PS2 particles which have been shown only to contain Q_H (Bowes and Horton, 1982). These particles did, however, exhibit sigmoidicity in their fluorescence induction curves which highlights the non-requirement for Q_H and Q_L to be present in $PS2_\alpha$ centres.

1.9.3 Q_1 and Q_2:

This form of PS2 heterogeneity is a kinetic difference which has emerged through the work of Joliot and Joliot (1977, 1979, 1981b). They noticed that only 70% of the variable fluorescence could be eliminated by a single saturating light flash in the presence of DCMU and

hydroxylamine (termed Q_1) while the remaining 30% of the variable fluorescence required several such flashes to remove it (termed Q_2). A number of different properties have been attributed to Q_1 and Q_2 as a result of the above mentioned work by Joliot and Joliot. The reduction of Q_1 leads to a C_{550} absorption change and the formation of a transmembrane potential. In the presence of hydroxylamine, Q_1 is photoreduced in an efficient process. Q_1 reoxidises after photoreduction, in the presence of DCMU and hydroxylamine, extremely slowly with a $t_{\frac{1}{2}}$ at room temperature of 20 minutes or more. On the other hand, Q_2 photoreduction cannot be associated with either a C_{550} change or the formation of a membrane potential. In the presence of DCMU and hydroxylamine it is reoxidised by a fast, multi-phasic process. Furthermore Q_2 appears to be DCMU-insensitive and can be associated with a secondary electron acceptor which is probably cyt b_{563}. As Q_2 does not give rise to a ΔA_{320} absorbance change it cannot be a plastoquinone molecule (Joliot and Joliot, 1981a). It is thought that Q_2 is equivalent to the PS2 electron acceptor called X_a (Eckert and Renger, 1980).

1.9.4 <u>B-type and non-B-type</u>:

The back-transfer of an electron from the semi-reduced secondary acceptor, Q_B^-, to the primary stable electron acceptor, Q_A^-, induced by mixing pre-flashed thylakoids with DCMU only affects 50% of the total variable fluorescence. The quencher involved in this process has been denoted "B-type" while the complementary part of the fluorescence is assumed to be controlled by "non-B-type" quenchers. Lavergne and Etienne (1980) proposed that the B-type acceptors were connected to the Q_B protein while the non-B-type were in some way disconnected. It has been shown that in the presence of DCMU, the B-type acceptors can only

recombine with the S_2 and S_3 states of the oxygen-evolving system while the non-B-type quenchers are totally reoxidised given any number of preillumination flashes (Lavergne, 1982a). In the presence of DCMU and hydroxylamine, the B-type quenchers like Q_1, were very stable when reduced while the non-B-type became rapidly oxidised (Lavergne, 1982b). Furthermore, the non-B-type acceptors can only become reduced after prior reduction of the B-type. Recently it has been proposed that Q_1 is equivalent to the B-type quencher while Q_2 is associated with the non-B-type (Lavergne, 1982d). The relationship between these various forms of PS2 heterogeneity is still not clear, however based on their reported differences and similarities certain links have been made (see Horton, 1982; Joliot and Joliot, 1983). For instance both Q_H and Q_L appear to be associated with $PS2_\alpha$ although their presence is not required to see fluorescence sigmoidicity (Horton, 1981; Bowes and Horton, 1982). Q_L has been linked with Q_2 while Q_H has been linked to Q_1 (see review by Bouges-Bocquet, 1980). Furthermore, the functional role of these different PS2 electron acceptors is not known at this time.

1.10 Objectives of this Thesis:

The work presented in this thesis is primarily directed towards a clearer understanding of the mechanisms involved in photosynthetic light adaptation. The approach in this study has been to use a number of chlorophyll fluorescence techniques to compare thylakoid membrane structural changes induced *in vitro* by the manipulation of cation levels and the use of protein phosphorylation with the *in vivo* changes brought about by adaptation to light quality (termed State 1 - State 2 transitions). The results being analysed in terms of distinguishing between

spillover and absorption cross section changes brought about by the various treatments and correlating the fluorescence changes with the degree of thylakoid reorganisation.

The effect of cations on fluorescence yield, thylakoid organisation and energy redistribution has been reexamined in Chapter 3 as a result of, 1) the claim that a Mg^{2+}-specific alteration in k_d at $PS2_\alpha$ reaction centres causes the fluorescence decrease observed on lowering the Mg^{2+} level and 2) the inconsistent reports in the literature concerning the cation effect on F_o. The effect of protein phosphorylation on energy redistribution has been investigated in Chapter 4 and the relationship between background Mg^{2+} levels and phosphorylation in terms of the degree of structural and fluorescence changes carried out in view of the contradictary reports concerning the mechanisms involved. By comparing the results presented in Chapter 3 with those of Chapter 4 the effect of phosphorylation in terms of changes in spillover and absorption cross section has been clarified. However, phosphorylation has been reported to alter a number of other processes which includes a stimulated cycling of electrons around PS2. This led to the work presented in Chapter 5, which has investigated the effect of phosphorylation on cyt b_{559} redox changes. This led to the placing of cyt b_{559} LP in a Q-cycle mechanism. The work carried out in Chapters 3 and 4, using room temperature fluorescence induction curves, created an interest in the PS2 kinetic heterogeneity, PS2α and PS2β. This led to the investigation presented in Chapter 6 in which various pretreatments were used to modify the fluorescence rise and to alter the extent of the two kinetic components, and in doing so the concept of two distinct forms of PS2, as suggested by Melis and Homann (1978), has been questioned.

Chapter 7 compares changes in fluorescence induction curves brought about by light quality pretreatments, indicative of the State 1 - State 2 transition, with the *in vitro* studies presented in Chapters 3 and 4 to try and elucidate if the *in vivo* changes are accomplished by alterations in spillover and/or absorption cross section.

Changes in chloroplast ultrastructure induced by growing plants under different environmental conditions have been used to investigate the effect of thylakoid structure on the plant's ability to carry out State 1 - State 2 transitions (Chapter 8). This chapter also shows the use of chlorophyll fluorescence in determining chloroplast structural changes evoked by transferring plants from growth under high light to low light. Chapter 9 shows the use of chlorophyll fluorescence transients in investigating proposed structural and functional changes brought about in plants exhibiting atrazine-resistance,

The final chapter brings the chlorophyll fluorescence and chloroplast structural changes together in a general discussion and in so doing distinguishes between the changes induced by cations, protein phosphorylation and light quality.

CHAPTER 2

Materials and Methods

2.1 Chloroplast Preparation:

The chloroplast preparation used in this study was a modification of that described by Nakatani and Barber (1977) which yielded Class I type chloroplasts. These maintain an intact outer envelope and using CO_2 as the terminal acceptor, are capable of photosynthetic electron transfer reactions. For experimentation these intact chloroplasts were subjected to a 15 second osmotic shock.

Throughout most of this study, leaves of 10-day old peas (Pisum sativum, var. Feltham First) grown from micoldusted seeds, in vermiculite, under supplementary lighting (16 hr. day), in a greenhouse, were used for the chloroplast preparation.

2.1.1 Preparation of Class I Chloroplasts:

70g. of freshly harvested pea leaves were macerated, using a Polytron (type PT 35 OD), in 200ml ice-cold grinding medium consisting of 0.33M sorbitol, 0.2mM $MgCl_2$, 20mM MES (pH 6.5, Tris). The resulting homogenate was filtered through 10 layers of muslin, of which the first two layers were separated by cotton wool. The filtrate was collected and centrifuged at 2200xg for 45s at 4°C. The supernatant was removed, and the pellet was resuspended in 0.33M sorbitol (pH 7.5, Tris), before being centrifuged at 2200xg for 20s at 4°C. The supernatant and soft pellet were removed by aspiration and the remaining hard pellet was resuspended as a concentrated stock in 0.33M sorbitol, 3mM $MgCl_2$

(pH 7.5, Tris) and stored on ice. The chloroplasts were usually 60-70% intact, as estimated by comparing the ability of potassium ferricyanide to accept electrons before and after an osmotic shock.

2.1.2 Preparation of Broken Chloroplasts:

A slightly modified chloroplast isolatiom method was used for the atrazine-susceptible and resistant plant biotypes. 10g of leaves were macerated using a polytron in a media containing 0.4M sorbitol, 3mM $MgCl_2$, 50mM sodium ascorbate, 0.5% BSA and 0.1M Tricine (pH 7.8, NaOH). The homogenate was passed through 16 layers of wet muslin and the filtrate was spun at 2200xg for 3 minutes at $4^{\circ}C$. The resulting pellet was shocked in water containing 3mM $MgCl_2$ then buffer was added to give final concentrations of 0.33M sorbitol, 2mM EDTA, 1mM $MnCl_2$, 3mM $MgCl_2$ and 0.1M HEPES (pH 7.6, NaOH). This was spun at 3000xg for 5 minutes and the pellet was resuspended as a concentrated stock in 0.33M sorbitol, 2mM EDTA, 1mM $MnCl_2$, 3mM $MgCl_2$, 0.5% BSA and 0.1M HEPES (pH 7.6, NaOH).

2.1.3 Chlorophyll Determination:

The amount of chlorophyll present in the concentrated chloroplast stock suspension was determined by the method of Arnon (1949). An aliquot of the concentrated stock, usually 50µl, was added to 80% acetone in a volumetric flask, so the final volume was 10ml. This was shaken and filtered to remove any coagulated protein particles. The absorption of the supernatant was measured using a Perkin-Elmer 554 spectrophotometer against an 80% acetone blank at 645nm, 663nm and 750nm. The latter measurement, used to determine the extent of light scattering from the sample, was subtracted from the other two readings.

The chlorophyll concentration was calculated from the following equation:

$$\text{Total chlorophyll } (\mu g/ml) = \{(8.02x\ A_{663}) + (20.2x\ A_{645})\} \times \text{dilution factor}$$

2.2 Plant Material for in vivo Measurements:

2.2.1 Light Intensity:

Most experiments were performed with pea plants grown in vermiculite in a Fisons model 600G3/TTL growth cabinet under 3 light intensities; high ($70Wm^{-2}$), medium ($50Wm^{-2}$) and low ($20Wm^{-2}$). White light was generated by 12x40W, cold-white fluorescent tubes supplemented by 4x40W, tungsten bulbs. The lower intensities were achieved by shading the plants with various layers of muslin. Seeds were planted approximately 4cm apart to minimise shading from neighbouring plants. All experiments were conducted on plants grown to the third leaf pair. All material was grown during a 16hr-8hr day-night cycle over a 14-17°C temperature range.

For some experiments, which were carried out at the Glasshouse Crops Research Institute (G.C.R.I.), Littlehampton, tomato plants, Lycopersicon esculentum (Mill.) cv Findon Cross, were grown in Hoagland no.1 solution in a controlled environment at 20°C, a v.p.d. of 0.7kPa and a 12hr photoperiod of either $80Wm^{-2}$ (high light) or $20Wm^{-2}$ (low light). The 5th leaf, used for all experiments, was maintained horizontal and unshaded. Some plants were transferred, when the 5th leaf was only 15-20% fully expanded, to the low light regime.

2.2.2 Temperature:

Pea plants were grown as described above except only the high and low light intensities were used. All material was grown over a 4-7°C temperature range.

2.2.3 Algae:

Chlorella pyrenoidosa were grown in a medium containing 5mM KNO_3, 0.5mM K_2HPO_4, 2mM $MgSO_4$, 0.5mM KH_2PO_4, 1mM KCl and 0.2% (v/v) Hutner's micronutrients, under white light emitted from 1x250W incandescent bulb ($340Wm^{-2}$) at 20°C. The growth medium was continuously aerated with air which had been filtered by passing through cotton wool and water.

Anacystis nidulans were grown under the same conditions as above except the growth medium contained 1mM $MgSO_4$, 0.6mM K_2HPO_4, 10mM KNO_3, 0.6mM sodium citrate, 10μM $Fe_2(SO_4)_3$, 0.15mM $Ca(NO_3)_2$ and was supplemented with 1% (v/v) Hughes micronutrients.

2.3 Room Temperature Chlorophyll Fluorescence:

2.3.1 Steady-state:

Chlorophyll fluorescence was induced by broad band actinic light obtained from a tungsten-halogen light source passed through Schott BG18 (2mm) and Schott BG38 (2mm) cut-off filters (intensity, $30Wm^{-2}$). The resulting fluorescence was detected at 90° by an EMI 9558B S20 photomultiplier protected by a Balzar 686nm interference filter. The photomultiplier output was displayed directly onto a pen chart recorder. All experiments were carried out at room temperature.

To investigate the cation-induced rise, intact chloroplasts were osmotically shocked at twice the final cation concentration and finally resuspended to give the following concentrations: 5μg chl/ml, 10mM Tricine (pH 8.2, KOH), 0.33M sorbitol and 10mM KCl. The effect of the addition of 5mM $MgCl_2$ on fluorescence before and 5 minutes after the addition of 1% (v/v) glutaraldehyde was measured using the apparatus detailed above.

2.3.2 Chlorophyll Fluorescence Induction Curves:

2.3.2.1 Effect of Cations:

To investigate the effect of cations, intact chloroplasts were osmotically shocked at twice the final cation concentration and resuspended to give the following final concentrations: 5μg chl/ml, 10mM Tricine (pH 8.2, KOH), 0.33M sorbitol and either 5mM $MgCl_2$, 100mM KCl, 5mM KCl or 0.1mM Tris(ethylenediamine) cobaltic (III) cation (TEC^{3+}). All samples were dark adapted for 4.5 minutes, 20μM DCMU was added and fluorescence measured after a total dark time of 5 minutes.

2.3.2.2 Effect of Glutaraldehyde:

Glutaraldehyde was used to investigate the requirement for membrane conformational change in bringing about alterations in various fluorescence parameters. Shocked chloroplasts were resuspended as in 2.3.2.1 in either 1mM $MgCl_2$ or 5mM KCl and allowed to equilibrate for 5 minutes before the addition of 1% (v/v) glutaraldehyde. Following a further 4 minute dark incubation to fix the thylakoids, 5mM $MgCl_2$ was added to some of the low cation (5mM KCl) samples. 20μM DCMU was added after another 5 minute dark incubation and fluorescence measured. Control

samples were those incubated in the absence of glutaraldehyde at different cation levels for a total dark time of 14 minutes.

2.3.2.3 Effect of Reductants and Oxidants:

To investigate the effect of the PQ pool redox state on the fluorescence rise, intact chloroplasts were shocked and resuspended as in 2.3.2.1 except only in the presence of 5mM $MgCl_2$. 20μM DCMU was introduced after 4 minutes dark incubation followed by either 2.4mM sodium dithionite (freshly prepared in buffer for each sample), or 4.2μM Spirulina maxima ferredoxin and 0.5mM NADPH, or 0.3mM methyl viologen, or 0.5mM potassium ferricyanide (before and after DCMU addition) or 4.5μM Ant-2p. Fluorescence was measured after a further 2 minute dark-time.

2.3.3 Effect of Protein Phosphorylation:

Intact chloroplasts were osmotically shocked at twice the cation concentration used and resuspended to give the following final concentrations: 30μg chl/ml, 10mM Tricine (pH 8.2, KOH), 0.33M sorbitol, 0.5mM NADPH, 4.2μM Spirulina maxima ferredoxin and 0-5mM $MgCl_2$. 10mM NaF and 0.15mM ATP were added to the samples to be phosphorylated and when ATP was present on equimolar amount of $MgCl_2$ was added to compensate for free Mg^{2+} chelation by the nucleotide.

Normally phosphorylated samples were incubated in the dark at room temperature for 60 minutes in the presence of NaF and ATP. Non-phosphorylated samples were incubated under the same conditions except in the absence of ATP. De-phosphorylated samples were incubated in the presence of ATP but in the absence of NaF for 30 minutes after which 1mM

$NADP^+$ was added to oxidise the PQ pool and after a total time of 60 minutes NaF was added.

After a 60 minute incubation all samples were diluted to 5μg chl/ml in 10mM Tricine (pH 8.2, KOH), 0.33M sorbitol, 10mM NaF, 0-5mM $MgCl_2$, 2.5μM valinomycin, 2.5μM nigericin, 1mM $NADP^+$ and 0.17mM methyl viologen for analysis. The diluted samples were dark incubated for 4.5 minutes, 20μM DCMU was added and the induction curves were measured after a 5 minute total dark time.

Protein-phosphorylation was also brought about by using sodium dithionite as the reductant. In this case, a stock suspension of thylakoids (100μg/ml chlorophyll) were suspended in a sealed reaction vessel in a medium containing 10mM Tricine (pH 8.2, KOH), 0.33mM sorbitol, 5mM $MgCl_2$, 10mM NaF and 2mM sodium dithionite. All thylakoids were incubated for 30 minutes, the phosphorylated samples contained 0.15mM ATP while the non-phosphorylated thylakoids had no ATP added. After the incubation, all thylakoids were diluted, washed and resuspended to give the following concentrations; 30μg chl/ml, 0.33mM sorbitol, 10mM Tricine (pH 8.2, KOH), 5mM $MgCl_2$, 10mM NaF and 10mM KOH. For fluorescence analysis the thylakoids were further diluted to 5μg chl/ml and suspended at various $MgCl_2$ concentrations.

2.3.4 Effect of $MgCl_2$ Concentration:

Chloroplasts were osmotically shocked and resuspended at various $MgCl_2$ levels in a reaction medium containing the following final concentrations: 5μg chl/ml, 10mM Tricine (pH 8.2, KOH), 0.33M sorbitol, 5mM KCl and 0-5mM $MgCl_2$. All samples were dark adapted for 4.5 minutes,

20μM DCMU was added and chlorophyll fluorescence measured after a 5 minute dark time.

2.3.5 Effect of State 1 and State 2 Adaptation in Algae:

Chlorella pyrenoidosa cells were diluted into water to give a maximal fluorescence level of approx. 90 relative fluorescence units at the 100% light intensity ($8Wm^{-2}$). All samples were dark-adapted for 2 minutes, 50μM DCMU was added and fluorescence measured over a range of light intensities, achieved by using various neutral density filter combinations.

Chlorella sp. cells were pretreated with either light 1 (generated by a Balzar 706nm interference filter, $8Wm^{-2}$) or light 2 (generated by Schott BG18 (2mm) and Schott BG38 (2mm) cut-off filters to give broad band blue-green light, $8Wm^{-2}$) for 10 minutes to drive them into State 1 or State 2 respectively. The algae were diluted and dark adapted for 1 minute, 50μM DCMU was added and fluorescence measured using the 100% light intensity.

2.3.6 Fluorescence Measurement:

All fluorescence induction curves were initiated by the opening of a Compur mechanical shutter (approximately 1ms opening time) and illuminating the samples with blue-green light transmitted by a 4mm Schott BG18 glass filter ($8Wm^{-2}$). The resulting fluorescence signal was detected at 90° by an EMI 9558B S20 photomultiplier protected by a Balzar 686nm interference filter (for some experiments a 731nm interference filter was used). The photocurrent generated was passed to a Datalab

transient recorder (DL905) coupled to a Datalab display module (DL403B). The fluorescence changes were recorded on a Hewlett Packard X-Y display unit (1340A) and analysed using a Datalab function module (DL417) linked to a Lear and Siegler display terminal (ADM3A) as shown in Fig. 2.1. This arrangement allowed a careful analysis of various fluorescence parameters to be conducted.

The kinetic nature of the fluorescence induction curve was analysed using the methods of Melis and Homann (1976 and 1978). This analysis was computer-aided using modified and extended software originally developed at Sheffield University. This allowed the generation of the semi-log plot of the growth of the normalised area over the induction curve and its subsequent breakdown into a fast, sigmoidal component and a slow, exponential component. This produced the initial rate constant for the fast phase (k_{α}), the rate constant for the slow phase (k_{β}), the proportion of the total area attributed to the fast (α_{max}) and slow components (β_{max}) and their area growth kinetics (see Fig. 2.2).

2.3.7 Leaf Chlorophyll Fluorescence:

Room temperature chlorophyll fluorescence at 686nm was measured from attached and detached pea leaves, grown as described in Section 2.2.1. using the apparatus shown in Fig. 2.3 (see also Chow et al., 1981b).

Leaves were placed in a temperature-controlled aluminium chamber which incorporated a three-branched optical-fibre system into its lid so that only $1cm^2$ leaf was illuminated. The procedure adopted to measure State transitions was to pre-illuminate the leaves with non-modulated light 1 from a tungsten-halogen light source passed through a Balzar 706nm interference filter at an intensity of $8Wm^2$ for 10 minutes.

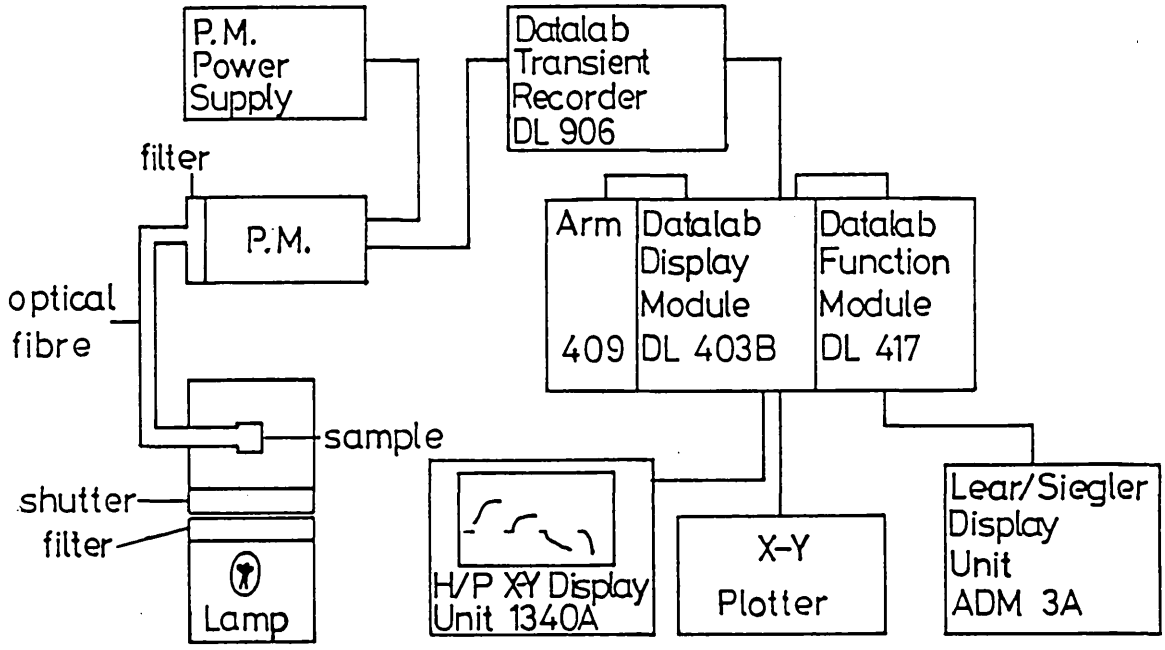


Fig. 2.1 Schematic diagram of the apparatus used to measure and analyse chlorophyll fluorescence induction curves exhibited, at room temperature, by DCMU-inhibited thylakoid membranes.

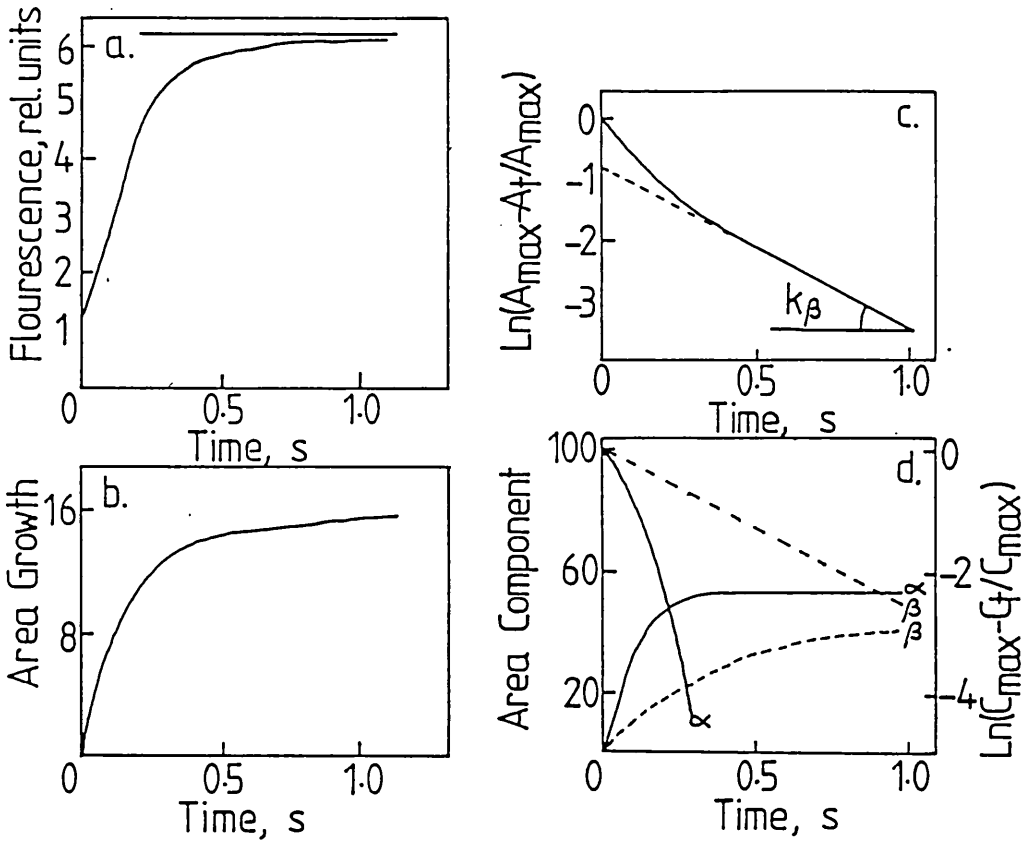


Fig. 2.2 The analysis of chlorophyll fluorescence induction curves exhibited at room temperature by DCMU-inhibited thylakoid membranes showing

a) the kinetics of the chloroplast fluorescence signal in the presence of 3mM Mg^{2+},

b) the growth of the normalised area above the fluorescence curve shown in (a),

c) the first order analysis of the kinetics of the area growth,

d) the kinetics of the fast (α) and slow (β) components of the area growth and a first order analysis of their time course.

Taken from Melis and Homann (1978).

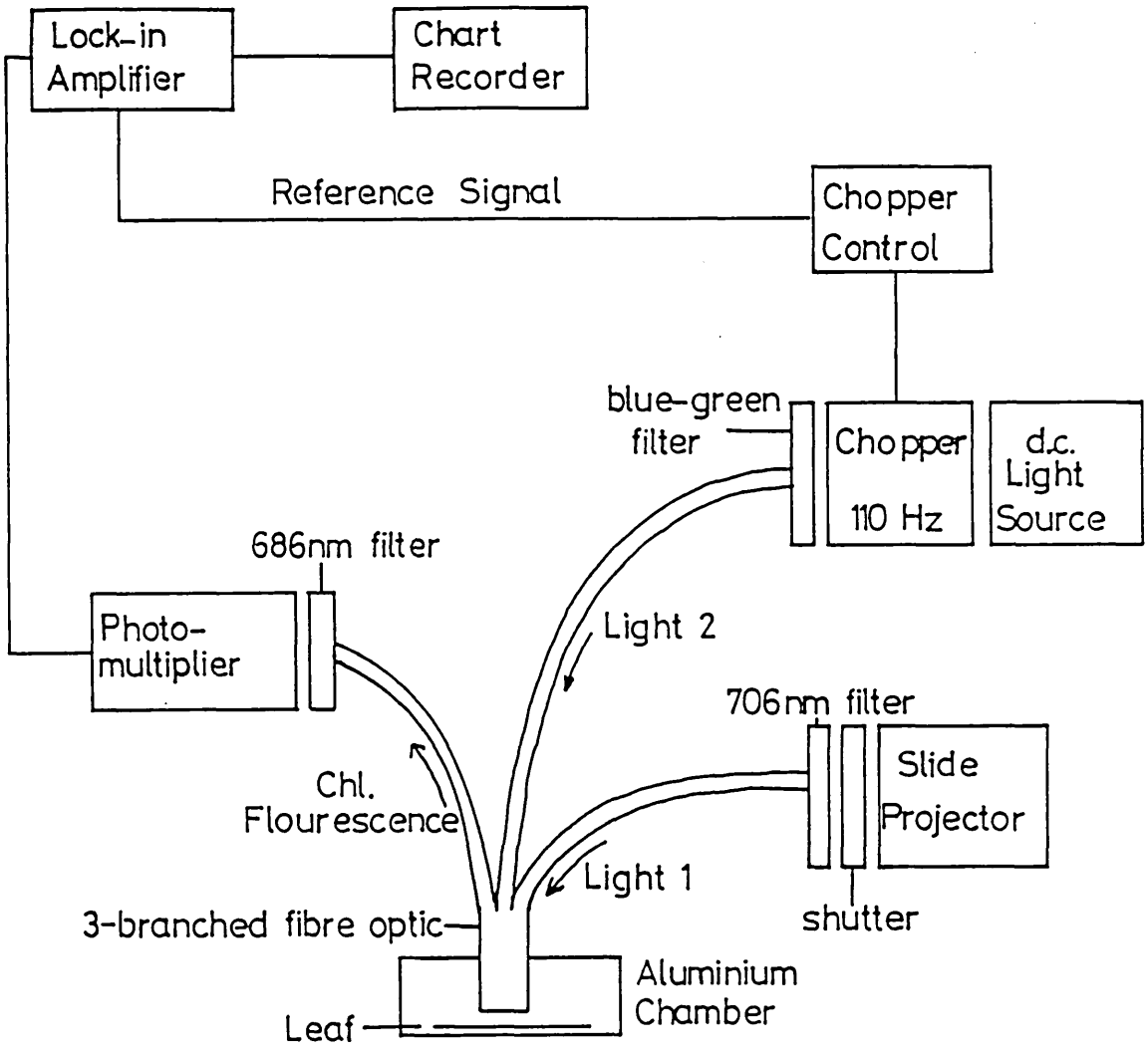


Fig. 2.3 Experimental arrangement for measuring State 1-State 2 transitions in leaves.

Modulated fluorescence measurements were initiated by turning on 110Hz modulated light 2 transmitted from a similar light source as above incorporating a 4mm Schott BG18 and a 2mm Schott BG38 cut-off filter at an intensity of $1.3 Wm^{-2}$, and removing light 1. The modulated signal was generated by a Brookdeal light chopper (model 9478) and was detected by an EMI 9558 S20 photomultiplier equipped with a Balzar 686nm interference filter. The modulated photocurrent was passed to a Brookdeal lock-in amplifier (9501) and displayed on a pen chart recorder. After 10 minutes of light 2, light 1 was reintroduced in order to probe the acceptor pool redox state and to determine the minimum fluorescence level.

For similar measurements using Chlorella sp. cells the following adjustments were carried out. Algal cells were placed in a glass cuvette and continuously stirred to avoid settling out. The cuvette was placed in a holder which incorporated the optical guide into its side.

Measurement of fluorescence induction curves from leaves in the presence and absence of DCMU was accomplished using the equipment described in Section 2.3.6 and Fig. 2.1, except the fibre optic guide and aluminium chamber were incorporated into the set-up.

2.3.8 Redox State of the PQ Pool:

The redox state of the PQ pool was estimated by the method of Bradbury and Baker (1981) which uses the fast fluorescence changes produced by the introduction of an additional higher intensity light 2. This was carried out using the apparatus described in Section 2.3.7. Attached pea leaves were given a 10 minute, light 1 pretreatment before the introduction of low intensity light 2 ($1.3 Wm^{-2}$). At various times

during the slow fluorescence decline the leaves were illuminated with additional high intensity light 2 ($20Wm^{-2}$) transmitted by a 2mm Schott BG18 cut-off filter. The fast fluorescence transients generated were monitored on a storage oscilloscope and the redox state of the PQ pool was estimated from the extent of variable fluorescence produced. This was normalised to the F_o level to remove the possibility of any changes in non-photochemical quenching of fluorescence (see Bradbury and Baker, 1981). All experiments were carried out at room temperature.

2.3.9 DBMIB-induced Fluorescence Quenching:

Chloroplasts were osmotically shocked and suspended in a buffer to give the following final concentrations; 30μg chl/ml, 10mM Tricine (pH 8.2, KOH), 0.33M sorbitol, 5mM $MgCl_2$, 0.5mM NADPH, 4.2μM ferredoxin. 10mM NaF and 0.15mM ATP were added to produce phosphorylated samples. Thylakoids were dark-incubated for 30 minutes to allow phosphorylation to occur before being diluted as previously described in Section 2.3.3 for measurement. To investigate the effect of $MgCl_2$ concentration samples were prepared as in Section 2.3.4. Chlorophyll fluorescence was measured using the apparatus described in Section 2.3.1 except the excitation light intensity was $1.5Wm^{-2}$. Titration of fluorescence with DBMIB was accomplished by the sequential addition of 0.67μM aliquots of DBMIB to continually stirred samples in the presence of 20μM DCMU.

The fluorescence quenching was analysed by the method discussed by Jennings et al. (1983). This involves plotting the total extent of quenching after n additions of DBMIB (F_m-F_n/F_n) against the degree of quenching brought about just as a result of the nth addition of DBMIB ($F_{n-1}-F_n/F_{n-1}$) where F_m = fluorescence before any DBMIB additions and F_n = fluorescence after n additions of DBMIB. From the concept that

ϕ_F is simply the ratio of k_f to the sum of the rate constants for all other de-excitations processes and from the equations set out below it is seen that the y-intercept at $F_m-F_n/F_n=0$ gives a value directly proportional to the degree of DBMIB quenching (termed interaction index).

With all PS2 traps closed

$$F_m = k_f/k_f + k_x$$

$$F_n = k_f/k_f + k_x + nDk_q$$

$$F_{n-1} = k_f/k_f + k_x + (n-1)Dk_q$$

where k_f = rate constant for fluorescence emission

k_x = rate constant for processes competing for PS2 excitons

k_q = rate constant for DBMIB-induced quenching

D = number of molecules of DBMIB per PS2 reaction centre

Thus $(F_m-F_n)/F_m = nDk_q/k_f + k_x + nDk_q$

and $(F_{n-1} - F_n)/F_{n-1} = Dk_q/k_f + k_x + nDk_q$

therefore when $(F_m - F_n)/F_m = 0$

$$(F_{n-1} - F_n)/F_{n-1} = Dk_q/k_f + k_x$$

2.4 Low Temperature (77K) Fluorescence Spectra:

Low temperature fluorescence spectra were performed on chloroplasts after various pretreatments, on various thylakoid membrane fragments, or on isolated thylakoid pigment-protein complexes (see legend of Fig. 3.3 for details).

Samples were suspended at a final concentration of 20µg chl/ml to which 1.7µM fluorescein was added as a standard. 200 µl aliquots of the samples were placed into capillary tubes and slowly frozen in liquid

nitrogen contained in a quartz dewar for analysis. The spectra were measured using a Perkin-Elmer fluorescence spectrophotometer (model MPF 44A). The excitation wavelength was 435nm (slit width, 15nm) and fluorescence emission was detected between 470 and 800nm (slit width, 3nm). The photomultiplier was protected by a 495nm cut-off filter. The spectra were not corrected.

2.5 Absorption Changes Associated with Cytochrome Redox State

Absorption changes associated with cyt b_{559} and cyt f were investigated using a Perkin-Elmer dual beam spectrophotometer (model 557).

Reduced minus oxidised difference spectra were recorded using either 2mM hydroquinone and 1mM ascorbate or 7.2μM ferredoxin and 0.5mM NADPH as reductants and 2mM potassium ferricyanide as an oxidant.

Light-induced absorption changes at 560-540nm were obtained by side-illumination of the cuvette with actinic light passed through either a 695nm cut-off filter ($55Wm^{-2}$) or a 709nm interference filter ($25Wm^{-2}$). The photomultiplier was protected by a 4mm Corning 4-96 filter and the measuring beam light path was 10mm.

Light-induced absorption spectra between 550nm and 565nm were carried out to characterise the observed changes. Cyt b_{559} high potential photooxidation was investigated in samples treated with 4.5μM Ant-2p (see Maroc and Garnier, 1979; Packham and Barber, 1984).

The effect of protein-phosphorylation on the absorption changes associated with cyt b_{559} LP, cyt b_{559}HP and cyt f was investigated.

Intact chloroplasts were osmotically shocked and suspended to give the following final concentrations: 70μg chl/ml, 10mM Tricine (pH 8.2, KOH), 0.33M sorbitol, 5mM $MgCl_2$, 10mM NaF, 0.5mM NADPH and 7.2μM ferredoxin. Phosphorylated samples had 0.5mM ATP added and all samples were incubated in the dark for 30 minutes before measurement at room temperature.

2.6 Electron Microscopy:

2.6.1 Thylakoid Membranes:

Phosphorylated and non-phosphorylated samples were prepared at different $MgCl_2$ levels using the ferredoxin/NADPH method as previously described in Section 2.3.3 except 100μg chl/ml was used. After a 30 minute dark incubation the reactions were terminated and fixation was accomplished by the addition of an equal volume of buffer containing 0.23M sorbitol, 20mM Tricine (pH 8.2, KOH), 0-5mM $MgCl_2$ and 1.66% (v/v) glutaraldehyde. The samples were incubated for 5 minutes at room temperature followed by 30 minutes on ice. Each sample was washed three times in reaction buffer for 15 minutes each time. This was accomplished by producing a sample pellet after a 1 minute, high speed spin on an MSE Microcentaur bench centrifuge. After the final wash each sample was spun down and the pellets were resuspended in 1% osmium tetroxide for 1 hr post-fixation at room temperature. Following this each sample was spun down by a 5 minute, high speed spin to form a hard pellet which was fragmented into dishes for dehydration. This was carried out through a series of changes into 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90% alcohol each for 10 minutes followed by 2x15 minute changes at 95% alcohol and finally 3x20 minute changes at absolute alcohol. The dehydrated thylakoid fragments were embedded by transfer to a 100% ethanol: Epoxypropane (1:1) mixture for 30 minutes, followed by a transfer to expoxypropane for another 30 minutes, then to a 100% epoxypropane:epon

(1:1) mixture for 60 minutes. The fragments then underwent 3-4 changes in epon resin before the resin was polymerised by a 2 day, 60°C incubation. Blocks were cut and trimmed and sectioned using a diamond knife on a Reichert microtome. Thin-sections were transferred onto formvar-coated grids and stained in 2% aqueous uranyl acetate for 30 minutes at 60°C, washed, and stained for 20 minutes at room temperature in lead citrate. The sections were examined and photographed using a Philips electron microscope (model 301).

2.6.2 Leaves:

Leaves grown under various environmental conditions were prepared in a similar way as mentioned above except fixation was conducted in 1% (v/v) glutaraldehyde at room temperature for 2 hours. This was carried out by cutting approx. $1mm^2$ leaf sections while the leaves were submerged in the fixative. All other procedures were performed as in 2.6.1 by carefully pipetting the various solutions out of holders containing the leaf segments and adding the next solution.

2.7 Photosynthetic Capacities:

2.7.1 Chloroplasts:

Steady-state rates of electron transport in shocked pea chloroplasts were measured using a Rank Brothers (Clark-type) oxygen electrode. The standard assay medium consisted of 0.33M sorbitol, 10mM Tricine (pH 8.2, KOH), 5mM $MgCl_2$ at a chlorophyll concentration of 70µg chl/ml. Full chain electron transport was measured in the presence of 5mM NH_4Cl with 1mM potassium ferricyanide as the terminal electron acceptor. PS2 electron transport was measured in the presence

of 20μM DCMU and 100μM silico molybdate (SiMo) as electron acceptor. Uncoupled electron transport rates were also measured photometrically using 0.05mM 2,6-dichlorophenol-indophenol (DCPIP) as an electron acceptor by the 560-520nm absorption change using a Perkin-Elmer spectrophotometer (model 557). The actinic light was produced by a tungsten-halogen light source equipped with a 695nm cut-off filter. The extinction coefficient used for DCPIP was 6.8 $mM^{-1}cm^{-1}$.

2.7.2 Leaves:

Photosynthetic rates exhibited by detached pea leaves were measured using a Hansatech leaf oxygen electrode (model 1) as described by Delieu and Walker (1981). The leaves were illuminated with white light of various intensities generated from an Intralux 150-H quartz-iodine lamp using neutral density filters. The O_2 electrode chamber was illuminated from above and passed through 20cm $CuSO_4$ solution and 2x Schott KG1 heat filters. The leaves were placed on a muslin disc soaked in 1M sodium bicarbonate solution to maintain the CO_2 level.

CHAPTER 3

Cations and Chlorophyll Fluorescence

It is now well established that the concentration of cations in which broken chloroplasts are resuspended can drastically alter their chlorophyll fluorescence characteristics. In the absence of "high" levels of screening cations (eg.~5mM Mg^{2+}) there is a decrease in fluorescence yield and an alteration in the kinetics of the fluorescence rise exhibited by DCMU-inhibited thylakoids. Such changes have been consistently reported, however, only some have shown a significant decrease in the F_o level when going to the "low salt" condition (eg. Bennoun, 1974; Malkin and Siderer, 1974; Kyle et al., 1982) while others have reported only a small change or none at all (eg. Homann, 1969; Hipkins, 1978; Melis and Homann, 1978; Jennings, 1984).

The fluorescence changes can result from either alterations in spillover, absorption cross section, other non-radiative decay processes or any combination of these. It has been shown, over the years, that the salt-induced phenomena reflects changes in spillover (Murata, 1969b; Briantais et al., 1973; Mohanty et al., 1973) due to membrane unstacking and protein randomisation (see review by Barber, 1982a). However, Melis and Ow (1982) have recently proposed that the decrease in fluorescence, seen on removing Mg^{2+} cations, is not brought about by changes in spillover but reflects a Mg^{2+}-dependent alteration in k_d from "connected" PS2 reaction centres associated with the fast, sigmoidal phase of the fluorescence rise ($PS2_\alpha$). Such a conclusion has arisen from the observation that the kinetics of the ΔA_{320} and ΔA_{700} signals exhibited by thylakoids suspended in the presence or absence of 5mM Mg^{2+} were identical.

The following results re-examine the mechanisms involved in bringing about the cation-induced changes in room-temperature chlorophyll fluorescence in view of the ideas mentioned above.

3.1 Mg^{2+}-Non-Specificity of the Fluorescence Changes:

Fig. 3.1 shows the changes in the room-temperature chlorophyll fluorescence induction curves of shocked DCMU-inhibited chloroplasts suspended in the presence of either 5mM Mg^{2+} and 5mM K^{+} ("high salt") or 5mM K^{+} alone ("low salt"). It can be seen that the absence of 5mM Mg^{2+} leads to a lowering of both F_o and F_m, a preferential decrease in F_v, and a change in the kinetics of the rise. Such changes are well documented in the literature (eg. Homann, 1969; Bennoun, 1974; Melis and Homann, 1978), however the change in F_o is not always as apparent as that shown in Fig. 3.1 (see Melis and Ow, 1982; Hipkins, 1978). Under the experimental conditions and using the apparatus stated in Section 2.3.4, a significant difference in F_o was always observed and could not be eliminated by using thylakoids from a broken chloroplast preparation, by varying the chlorophyll concentration between 10μg and 230μg chl/ml, by increasing the excitation energy or by decreasing the excitation path length from 10mm to 2 mm (data not shown).

Table 3.1 shows the fluorescence parameters, F_o, F_m, β_{max} and F_v/F_m for shocked chloroplasts suspended in the presence of different valency cations. It can be seen that at "high salt" there was always a higher F_o and F_m and a lower β_{max} when compared with the "low salt" condition. Furthermore, the addition of 5mM Mg^{2+} to thylakoids suspended in 0.1mM TEC^{3+} had no additional effect on the measured fluorescence parameters. Such observations would suggest that the observed changes in ϕ_F and the

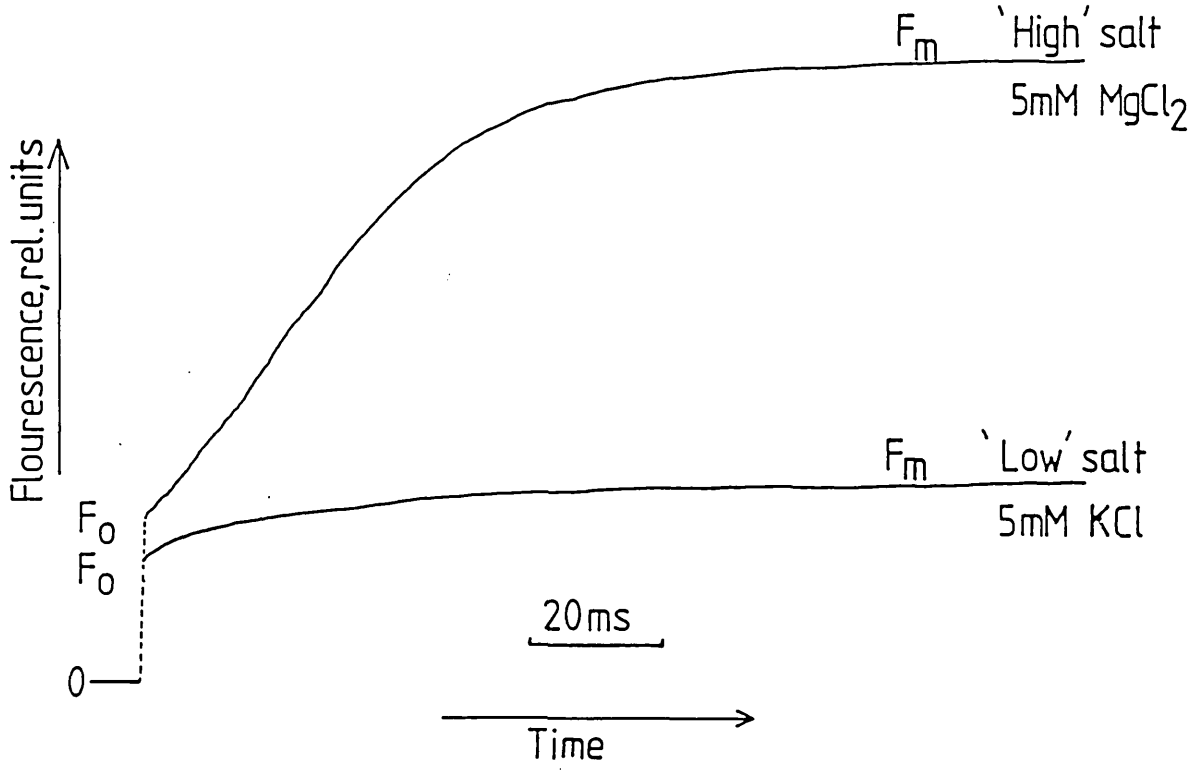


Fig. 3.1 Room temperature chlorophyll fluorescence induction curves from DCMU-inhibited pea thylakoids suspended in the presence of either 'high' salt (5mM $MgCl_2$) or 'low' salt (5mM KCl).

TABLE 3.1

The Effect of Cations on Various Fluorescence Parameters

Condition	F_o	F_m	β_{max}	F_v/F_m
5mM Mg^{2+}	39	126	0.45	0.69
0mM Mg^{2+}	22	38	0.83	0.42
0.1mM TEC^{3+}	39	120	0.45	0.68
50mM K^{+}	36	53	0.58	0.32
100mM K^{+}	40	100	0.51	0.60
unstacked +0.1mM TEC^{3+}	34	95	0.45	0.64
unstacked +0.1mM TEC^{3+} +5mM Mg^{2+}	33	92	0.51	0.64

other fluorescence parameters were not Mg^{2+}-specific but cation non-specific and valency-dependent effects, therefore supporting the earlier steady-state fluorescence measurements carried out in the presence of different valency cations (eg. Barber and Searle, 1978).

Both Fig. 3.2a and Table 3.2 confirm the involvement of conformational changes in bringing about the alterations in the thylakoids fluorescence characteristics. Fig. 3.2a shows the inhibition of the Mg^{2+}-induced fluorescence rise by glutaraldehyde fixation (see Mohanty et al., 1973; Jennings and Forti, 1974). Furthermore, Table 3.2 shows that when thylakoids were fixed in the "low salt" condition the subsequent addition of 5mM Mg^{2+} did not lead to an increase in either F_o and F_m nor to a decrease in β_{max} to the "high salt" levels. Table 3.2 also shows that fixation at 5mM Mg^{2+} followed by the addition of EDTA resulted in the maintenance of the "high salt" characteristics. However, glutaraldehyde addition did produce a lowering in the F_m level (see Table 3.2) and this change is also seen in Fig. 3.2b, which monitors steady-state fluorescence from thylakoids suspended in the presence or absence of the fixative.

The different reports concerning the effect of salt level on F_o might be partially explained by a change in the ability of Q_A to become fully oxidised in the dark in the presence of different cation concentrations, which could vary according to chloroplast preparation. Table 3.3 shows the effect of potassium ferricyanide (an oxidant of PS2), on F_o, F_m, A_{max} and F_v/F_m. When the ferricyanide (FeCy) was added before DCMU addition there was a change in all the parameters measured with an especially large decrease in F_o at the "high salt" level, so that the difference in F_o between 5mM Mg^{2+} and minus Mg^{2+} changed from 23% to

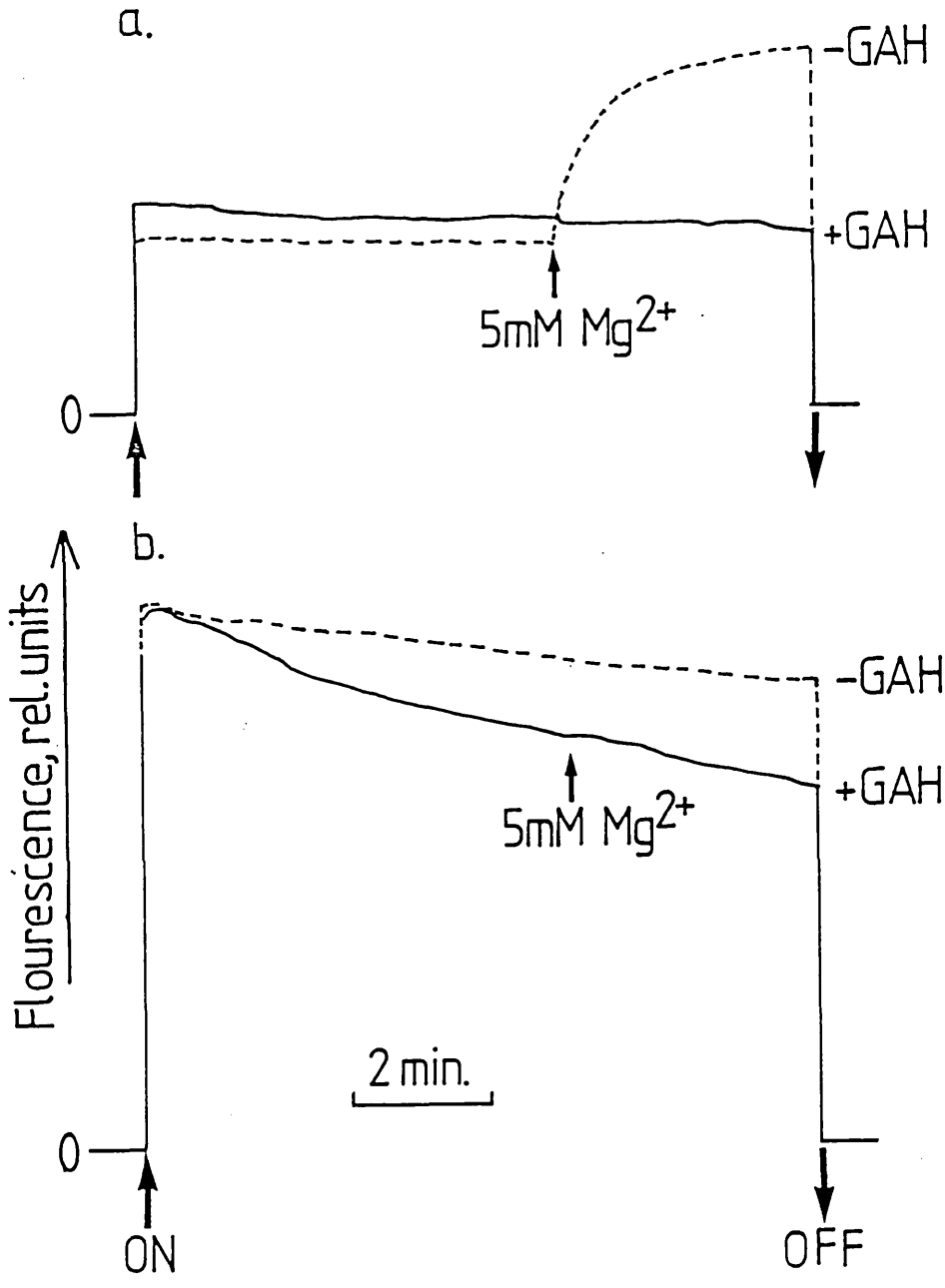


Fig. 3.2 Changes in the steady-state chlorophyll fluorescence signal exhibited by DCMU-inhibited pea thylakoids by a) the addition of 5mM Mg^{2+} to 'low' salt thylakoids suspended in the presence or absence of 1% (v/v) glutaraldehyde (GAH) and b) by the addition of 1% (v/v) glutaraldehyde to 'high' salt thylakoids.

TABLE 3.2

The Effect of Glutaraldehyde (GAH) Fixation on Mg^{2+}-induced Changes in Various Fluorescence Parameters

Treatment					
Mg^{2+} (mM)	GAH (1%v/v)	EDTA (5mM)	F_o	F_m	F_v/F_m
5	+	-	35	79.5	0.56
5	-	-	34	97.7	0.65
5	+	+	43	104.0	0.59
5	-	+	25	45.0	0.44
Mg^{2+} (mM)	GAH (1%v/v)	Mg^{2+} (5mM)	F_o	F_m	β_{max}
1	+	-	38	81.5	0.52
0	+	-	29	57.7	0.74
0	+	+	30	60.0	0.74
1	-	-	41	118.0	0.46
0	-	-	24	48.5	0.73
0	-	+	32	83.4	0.46

TABLE 3.3

The Effect of Potassium Ferricyanide (FeCy) Before (B) and After (A) DCMU-Addition on Various Fluorescence Parameters

Treatment					
Mg^{2+} (mM)	FeCy (0.5mM)	F_o	F_m	A_{max}	F_v/F_m
5	-	25.5±1.4	88.7±4.7	1090±64	0.71
0	-	19.7±1.0	48.8±3.2	765±17	0.59
5	+B	20.9±0.8	77.1±2.4	1490±147	0.73
0	+B	19.0±1.4	47.1±4.4	875±50	0.59
5	+A	23.5±1.3	78.3±4.0	920±80	0.70
0	+A	19.7±1.5	45.5±3.9	805±36	0.57

only 9% (a value similar to that observed by Melis and Ow, 1982). If the FeCy was added after the addition of DCMU then only a small decrease in F_o at 5mM Mg^{2+} was observed. Table 3.4 shows the effect of DCMU addition on F_o at both 5mM Mg^{2+} and minus Mg^{2+}. It can be seen that the addition of DCMU led to a larger increase in F_o in the "high salt" situation compared with minus Mg^{2+}, so that the difference in F_o between "high" and "low" salt thylakoids in the absence of DCMU was only 11.4% while in the presence of DCMU this difference increased to 29%. Therefore, in the thylakoids used in this work there appears to be a DCMU-stimulated increase in F_o which is most significant when the thylakoids are suspended in the presence of "high salt".

Low temperature (77K) fluorescence spectra have been used in the past to show that thylakoids suspended in the presence of "low" cation levels there is a redistribution of excitation energy in favour of PS1 (eg. Murata, 1969b; Murata et al., 1970). Until recently the changes in fluorescence were based on the normalisation of the spectra to one of the fluorescence maxima but observations have now been made using standardised spectra (Krause et al., 1983). Characterisation of the 77K fluorescence peaks has been carried out, over a number of years, by using isolated photosynthetic particles (eg. Boardman et al., 1966; Wessels et al., 1973; Thornber, 1975; Butler, 1978). Fig. 3.3a shows the spectra obtained from intact chloroplasts which exhibit fluorescence maxima at 686nm, 695nm and 735nm. Using isolated pigment-protein complexes it can be seen that LHC produces a single peak at 683nm with a shoulder at 725nm (Fig. 3.3b), a PS2 complex fluoresces at 687nm and 695nm (Fig. 3.3c) while PS1 gave rise to a main peak at 735nm (Fig. 3.3d). Therefore, in general terms F_{685} and F_{695} are PS2-associated peaks and F_{735} is associated with PS1 confirming the previously documented data mentioned above. Fig. 3.4a shows the effect of 5mM Mg^{2+}

TABLE 3.4

The Effect of 10μM DCMU on the F_o Value of Thylakoids Suspended in the Presence and Absence of 5mM Mg^{2+}

	+DCMU	-DCMU	% increase due to +DCMU
$+Mg^{2+}$	16.50	11.00	+50
$-Mg^{2+}$	11.75	9.75	+20
% decrease due to $-Mg^{2+}$	-28.8	-11.4	-

Fig. 3.3 Standardised low temperature (77K) chlorophyll fluorescence emission spectra exhibited by

a) shocked, intact chloroplasts

b) LHC (prepared by a slightly modified method of Burke et al. (1978))

c) PS2 (prepared as in Gounaris et al. (1983))

d) PS1 (derived from the pellet produced after the 15hr, 100K xg spin in the LHC method of Burke et al. (1978)).

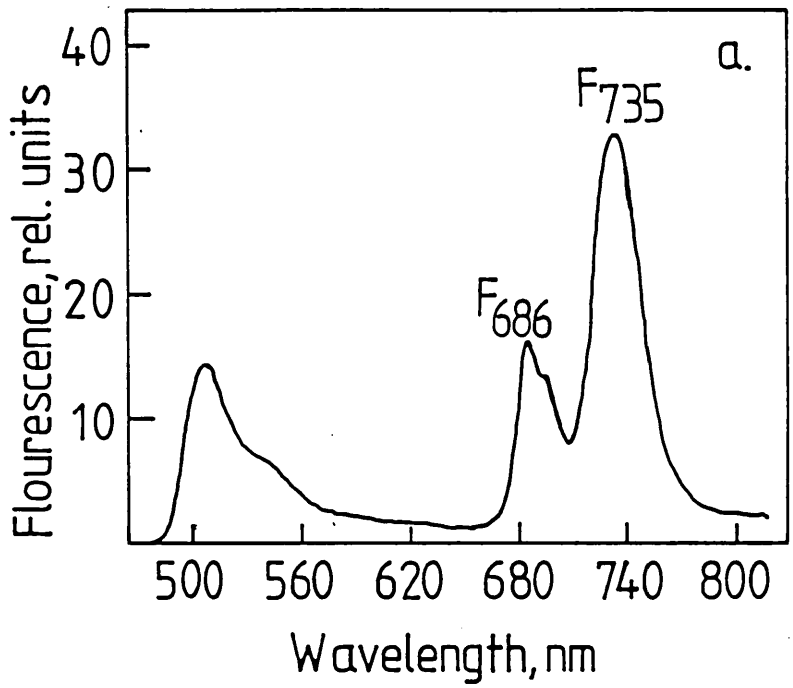


Fig. 3.3 a) shocked, intact chloroplasts

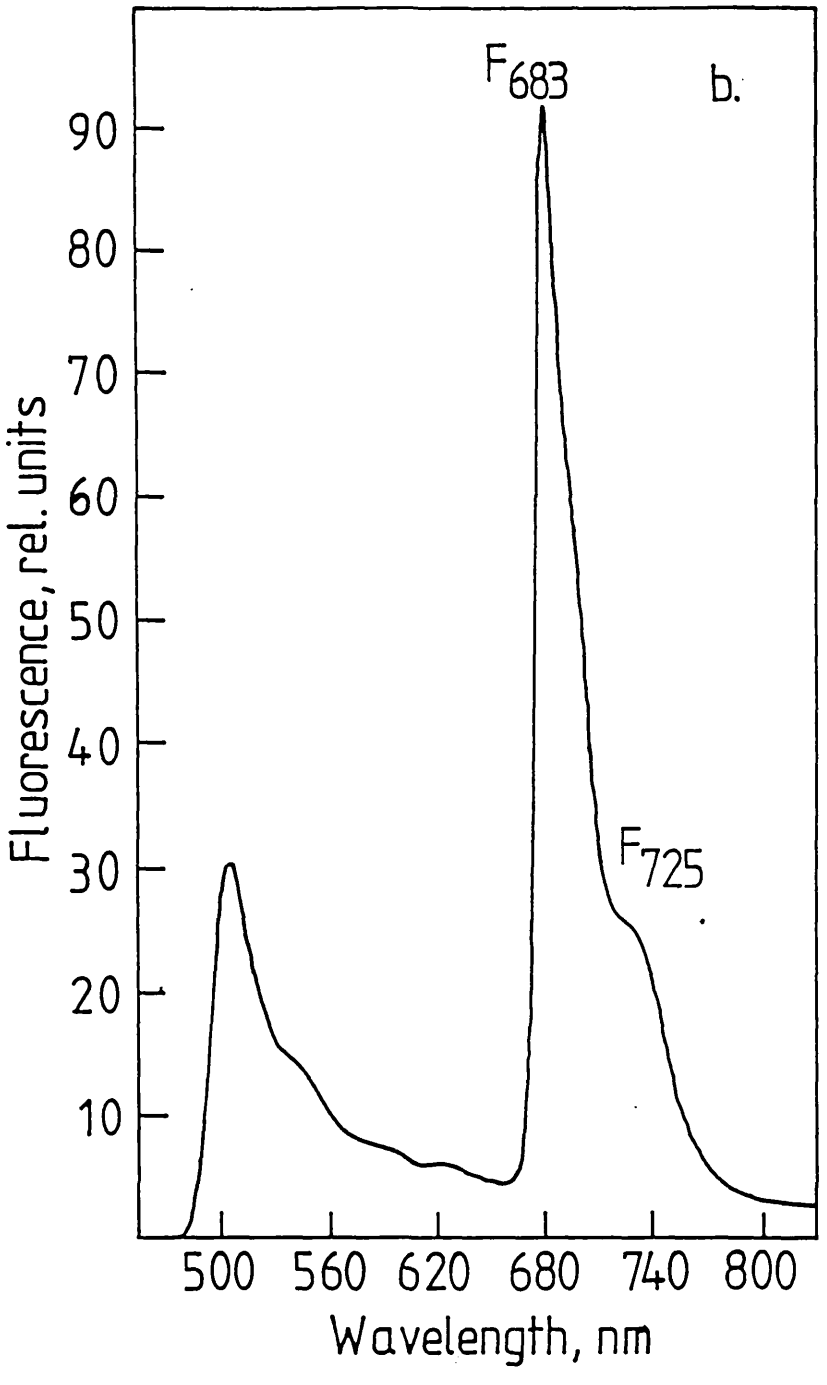


Fig. 3.3 b) LHC (prepared by a slightly modified method of Burke et al. (1978))

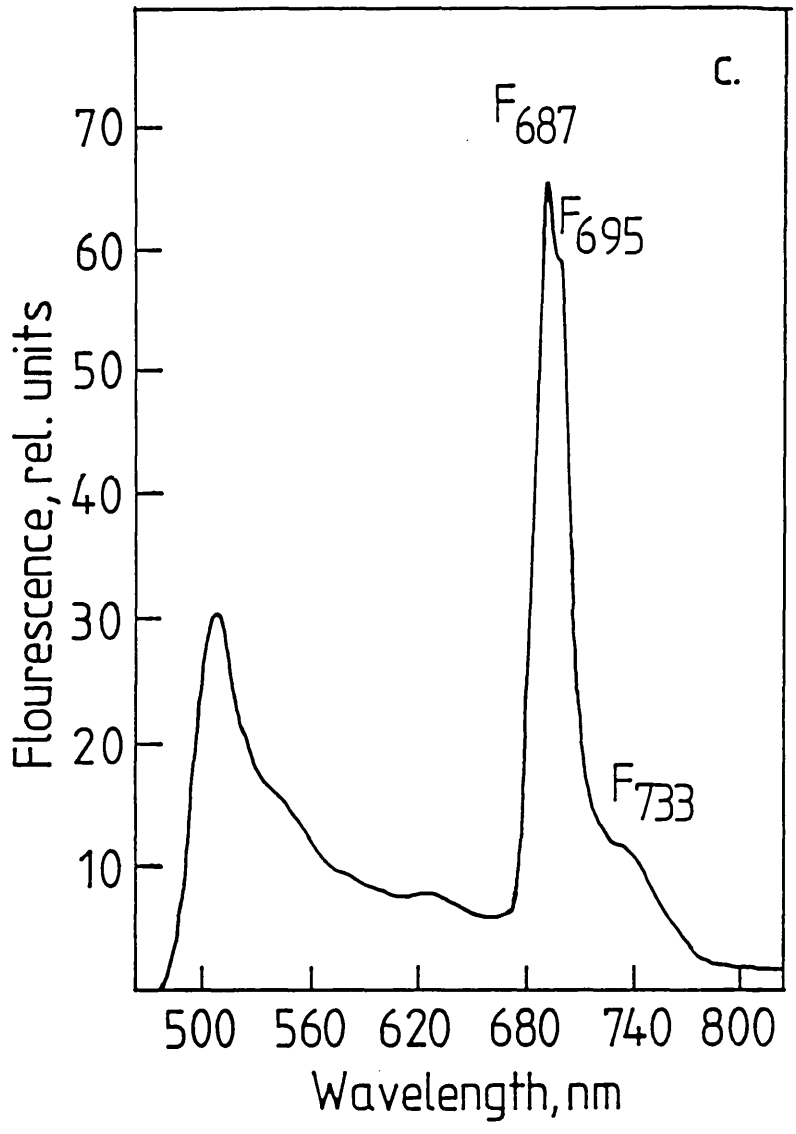


Fig. 3.3 c) PS2 (prepared as in Gounaris et al. (1983))

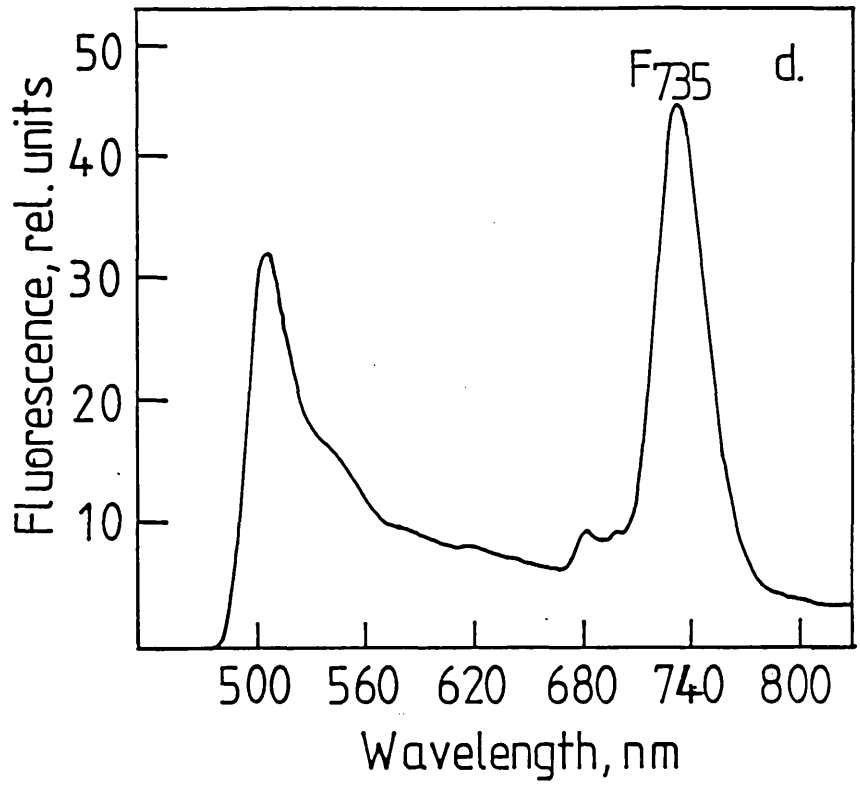


Fig. 3.3 d)PS1 (derived from the pellet produced after the 15hr, 100K xg spin in the LHC method of Burke et al. (1978))

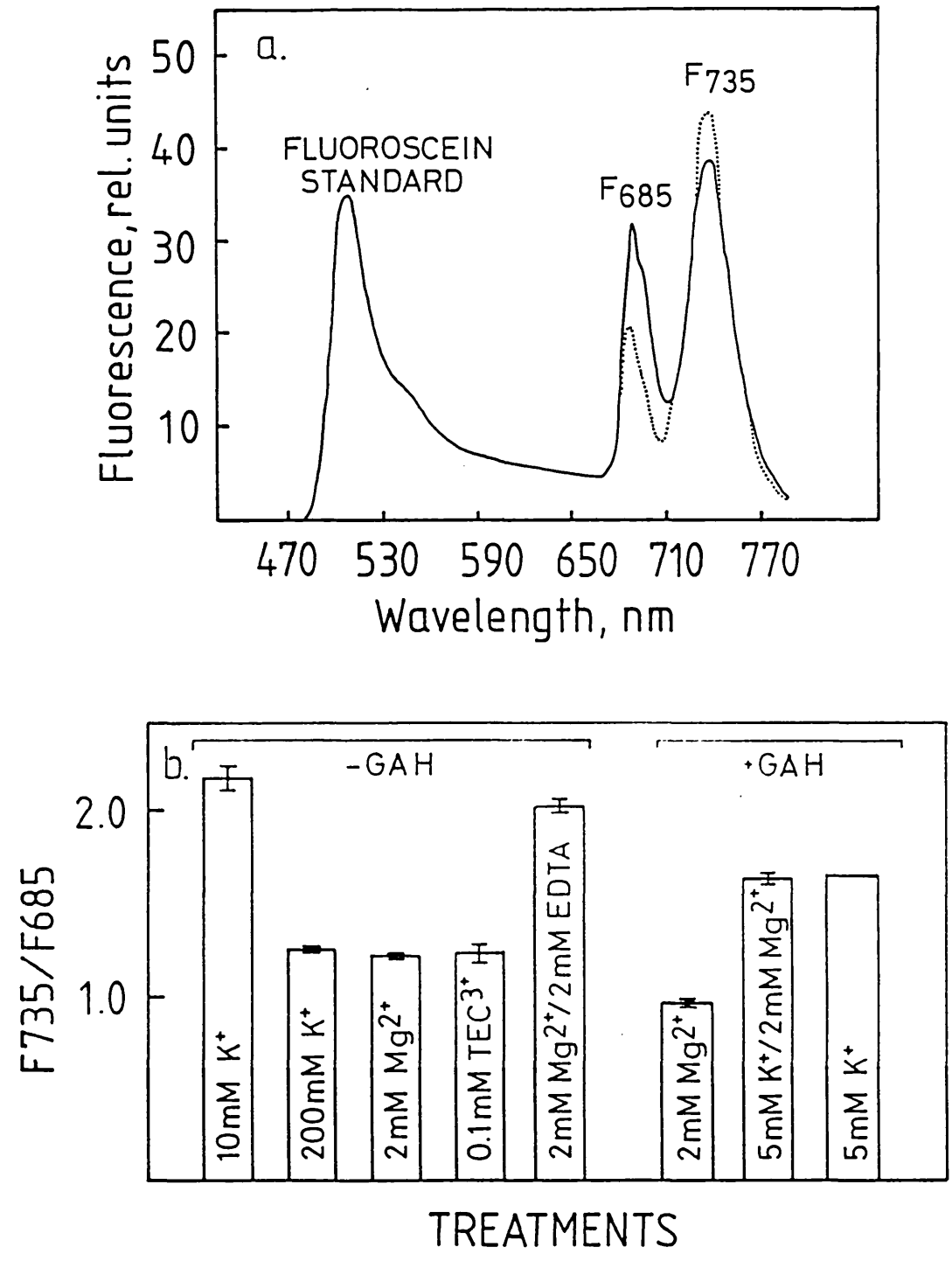


Fig. 3.4 a) Standardised 77K chlorophyll fluorescence emission spectra from thylakoid membranes suspended in "high" salt (——) and "low" salt (– – –)

b) The effect of cation valency and glutaraldehyde fixation on the ratio F_{735}/F_{685} calculated from standardised 77K chlorophyll emission spectra similar to (a)

and minus Mg^{2+} on the 77K fluorescence spectra of shocked chloroplasts in the presence of the fluorescent standard, fluorescein. This shows that the PS2-associated fluorescence decreases while the PS1-associated F_{735} peak increases when thylakoids are suspended at a "low" cation level compared with 5mM Mg^{2+}. These changes clearly indicate that excitation energy is being transferred to PS1 at the expense of PS2 but cannot distinguish between spillover and absorption cross section changes. Fig. 3.4b, in which the ratio F_{735}/F_{685} is shown for thylakoids suspended in the presence of different valency cations reaffirms the Mg^{2+}-non specificity of the changes. At all "high" salt levels the ratio is always lower than that exhibited by the "low" salt thylakoids, furthermore, when the "low" salt samples were glutaraldehyde-fixed the subsequent addition of Mg^{2+} ions did not alter F_{735}/F_{685}.

3.2 Mechanisms Involved in the Cation-Induced Fluorescence Change:

As stated in the introduction (1.8.1) it can be predicted that changes in spillover will affect the characteristics of the fluorescence rise in a different way to those brought about by alterations in absorption cross section. From Fig. 3.5 it can be seen that decreasing the excitation light intensity brought about an equal lowering in both F_m and F_o so that F_v/F_m remained unaltered (all intensities used reduced Q_A fully in the presence of DCMU). This would be expected to occur if the experimental apparatus did not over estimate F_o at the expense of F_v. This might have occurred at the higher intensities if the rise-time of a fast F_v component had come within the range of the shutter's opening time. It was also found that as F_v decreased the fluorescence rise became slower and as a consequence there was no observable difference in the absolute area over the curve (A_{max}). This expected trait occurred whether the thylakoids were suspended in 5mM

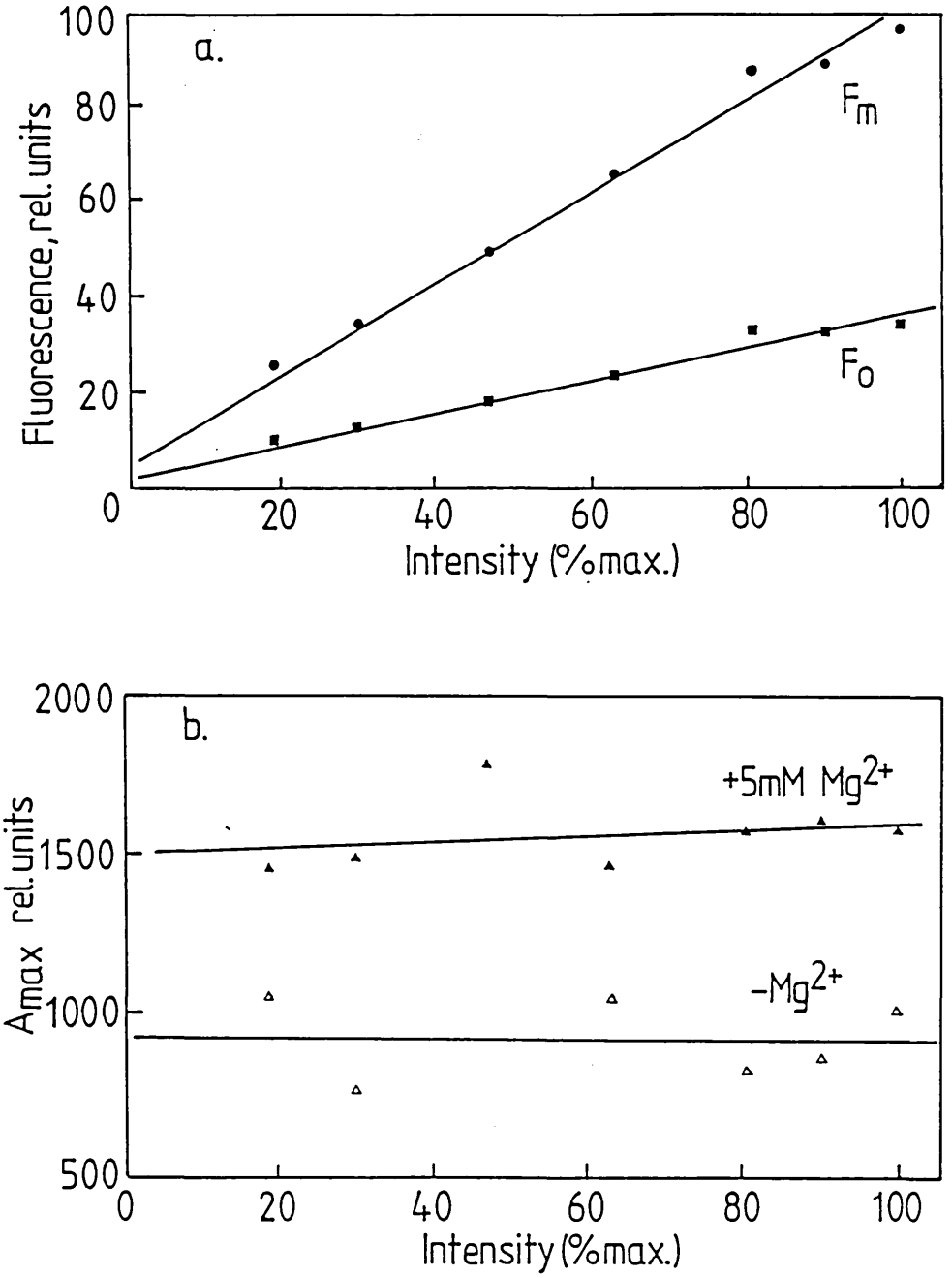


Fig. 3.5 Chlorophyll fluorescence induction curve parameters from shocked chloroplasts as a function of excitation light intensity showing the relationship between a) F_m (●) and F_o (■) levels and b) the A_{max} values for thylakoids suspended in the presence of 5mM Mg^{2+} (▲) and minus Mg^{2+} (△). All measurements were made at room temperature after a 5 minute dark adaptation and in the presence of 20μM DCMU. The 100% light intensity was $8Wm^{-2}$. The lines are the least squares fit to the data points.

Mg^{2+} or minus Mg^{2+}. Changes in fluorescence brought about by an alteration in PS2 absorption cross section would, therefore, be expected to produce similar results to those seen in Fig. 3.5.

The change in the above mentioned fluorescence parameters, as a function of Mg^{2+} level, was carried out. Fig. 3.6a and 3.6b show that lowering the Mg^{2+} concentration in which the thylakoids were suspended not only led to a decrease in F_v but also F_o, as seen in Fig. 3.1, and Tables 3.1 and 3.2. However, the Mg^{2+}-requirement for the F_o decrease ($C_{\frac{1}{2}}$ = 0.24mM) was lower than that for the decrease in F_v ($C_{\frac{1}{2}}$ = 0.66mM). In Fig. 3.6d it can be seen that lowering the Mg^{2+} level from 5mM to 0.4mM decreased only the variable fluorescence component (plotted as F_v/F_m) and this was accompanied by a similar decrease in A_{max} (Fig. 3.6c). Below 0.4mM $MgCl_2$, F_o decreased proportionally more than F_v and hence a small increase in both F_v/F_m and A_{max} was observed.

The parameter β_{max} was also measured as a function of Mg^{2+} concentration and the results are shown in Fig. 3.7. As previously noted by others (e.g Kyle et al., 1982; Melis and Homann, 1978) this parameter decreased with increasing Mg^{2+} levels whether the fluorescence changes were due to preferential alterations in F_o or F_v (see also Tables 3.1 and 3.2). Fig. 3.8 shows the half-time for the growth of the normalised area over the curve ($t_{\frac{1}{2}}$ for Q_A photoreduction). This was also altered by Mg^{2+} concentration ($C_{\frac{1}{2}}$ = 0.5mM) whether the changes were brought about by alterations in F_o or F_v. Such an effect on Q_A^- accumulation would be expected as the rate constants for the two kinetic components of the rise (k_α and k_β) are also altered by Mg^{2+} level. From Fig. 3.9 it can be seen that as F_v/F_m decreased both k_α and k_β decreased.

Fig. 3.6 The effect of $MgCl_2$ concentration on a) F_v (●), b) F_o (■), c) A_{max} (▲) and d) F_v/F_m (●) exhibited by thylakoids inhibited by 20μM DCMU. All measurements were made at room temperature, using an excitation light intensity of $8Wm^{-2}$, after a 5 minute dark adaptation.

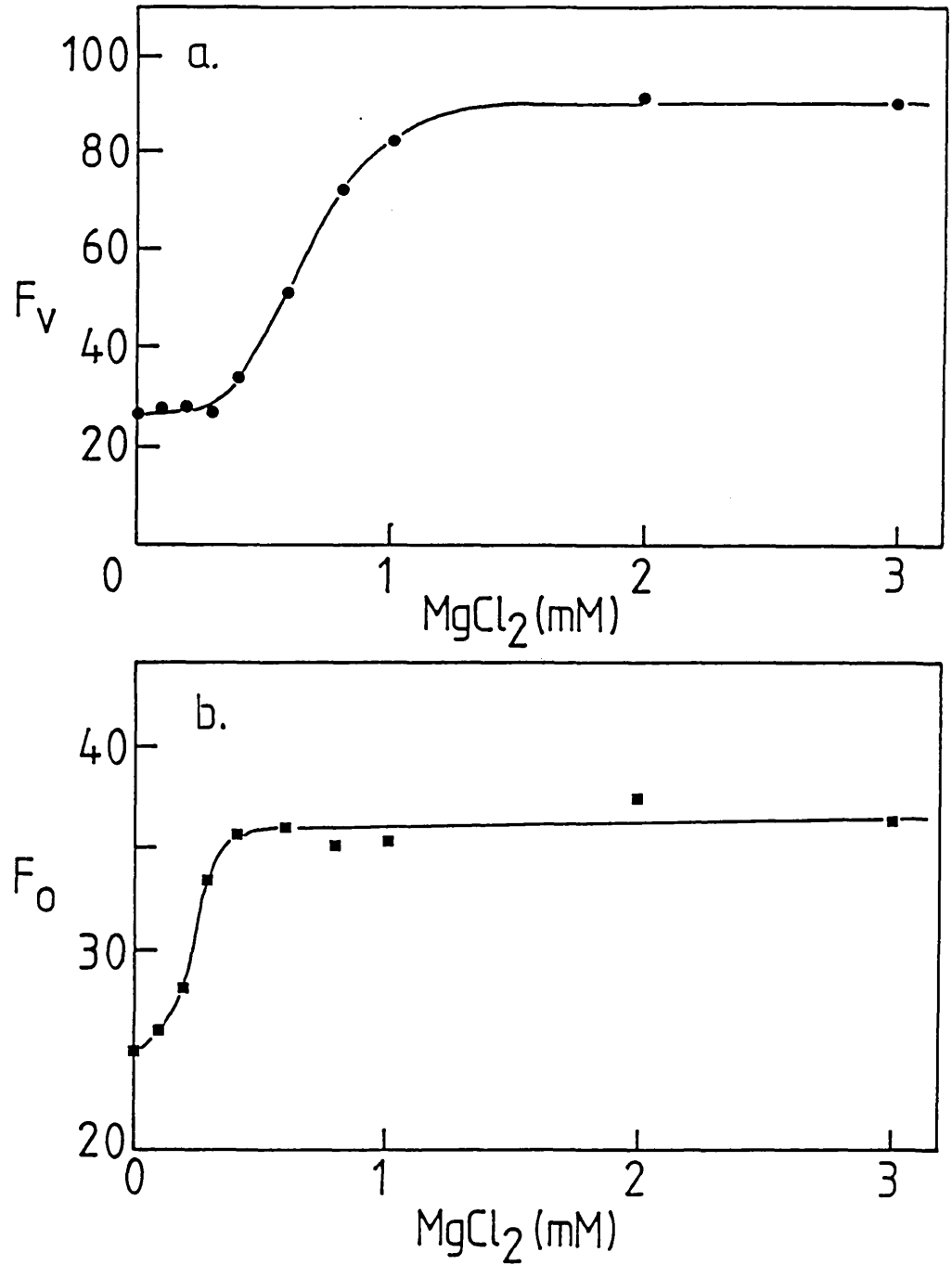


Fig. 3.6 a) F_v (●)
b) F_o (■)

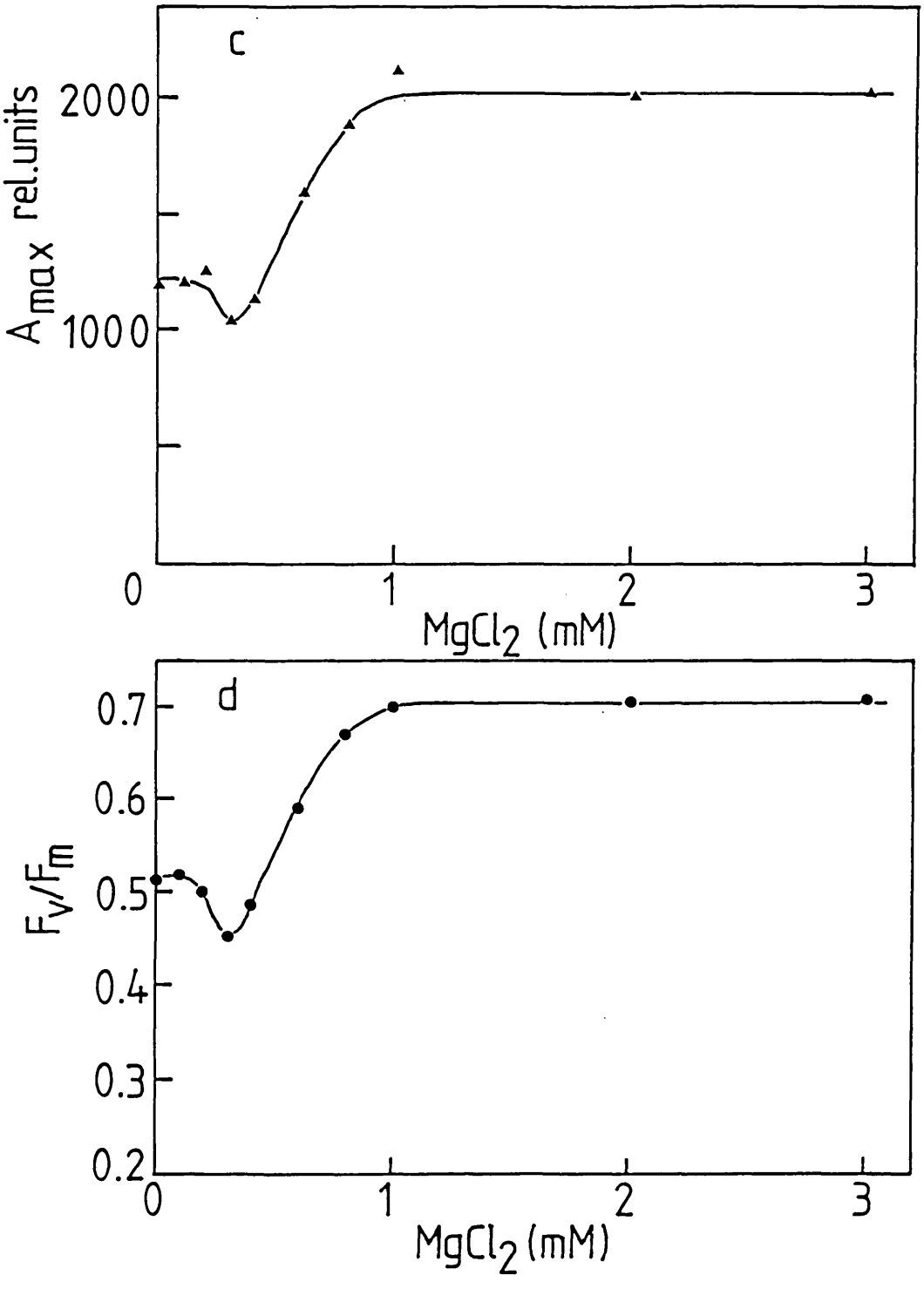


Fig. 3.6 c) A_{max} (▲)
d) F_v/F_m (●)

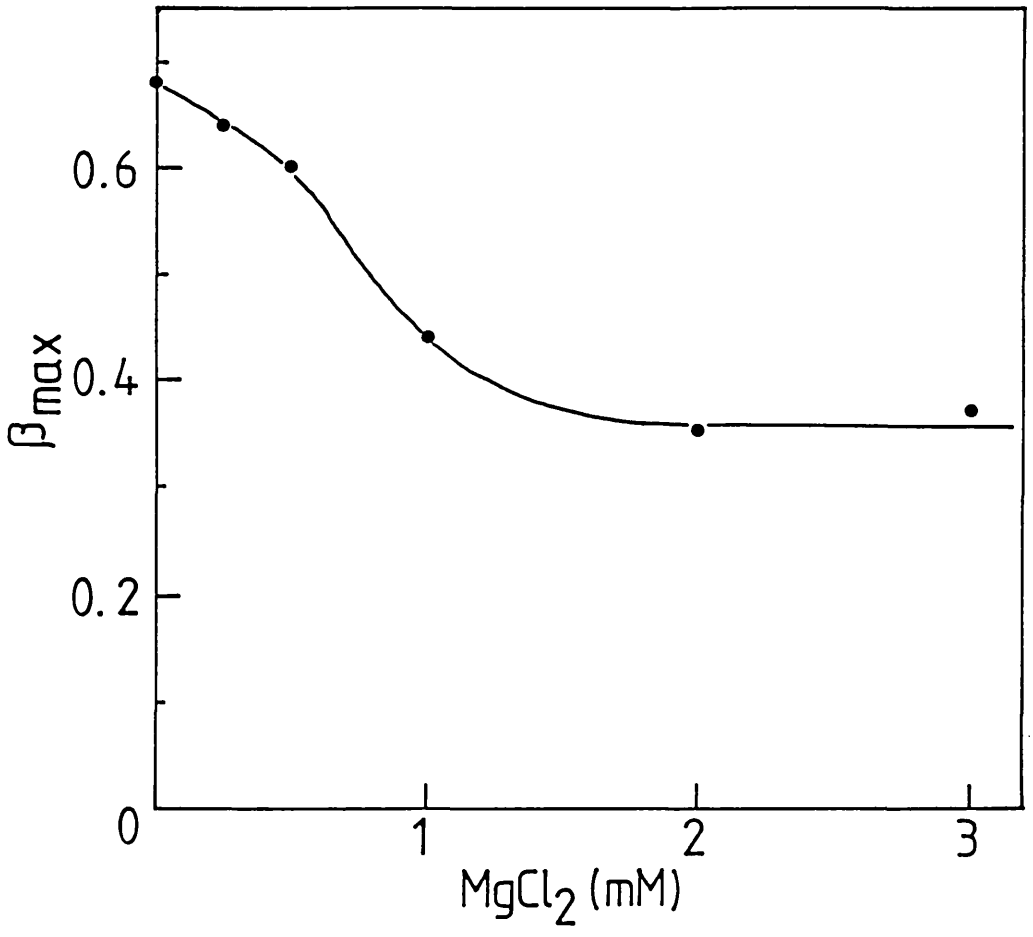


Fig. 3.7 The effect of $MgCl_2$ concentration on β_{max}, which represents the linearly extrapolated slow, exponential phase of room temperature chlorophyll fluorescence induction curves with the ordinate at time zero (see 2.3.6 and Fig. 2.2), of thylakoids inhibited with 20μM DCMU.

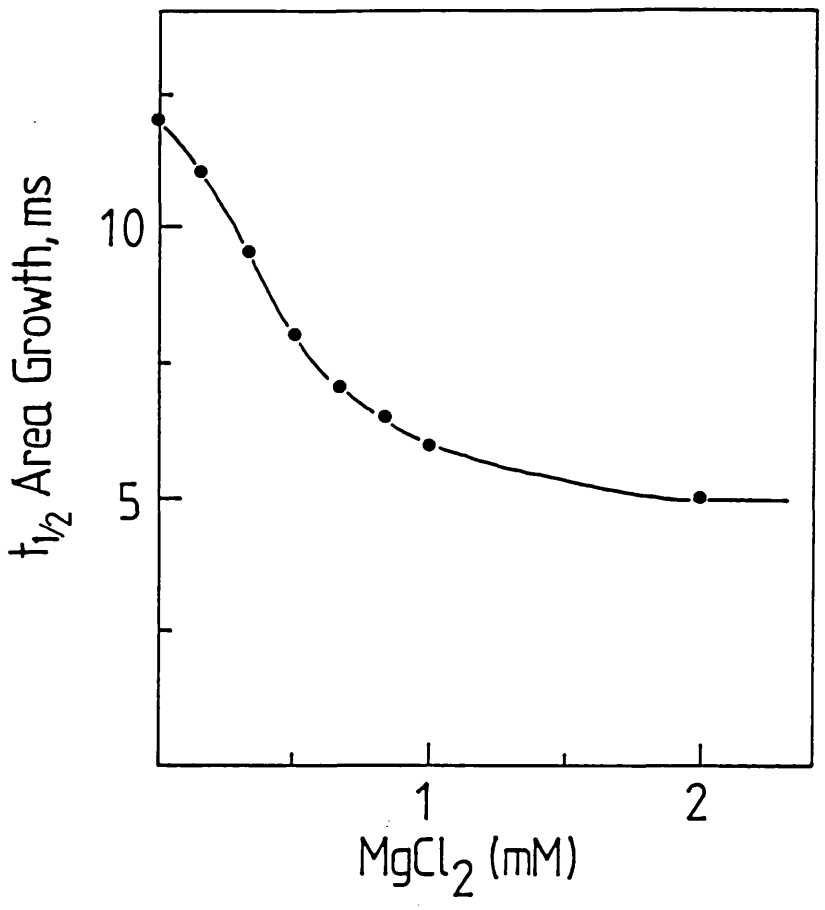


Fig. 3.8 The effect of $MgCl_2$ concentration on the $t_{\frac{1}{2}}$ for the growth of the normalised area over the room temperature chlorophyll fluorescence induction curve (indicative of Q_A^- photo accumulation) of thylakoids inhibited with 20μM DCMU.

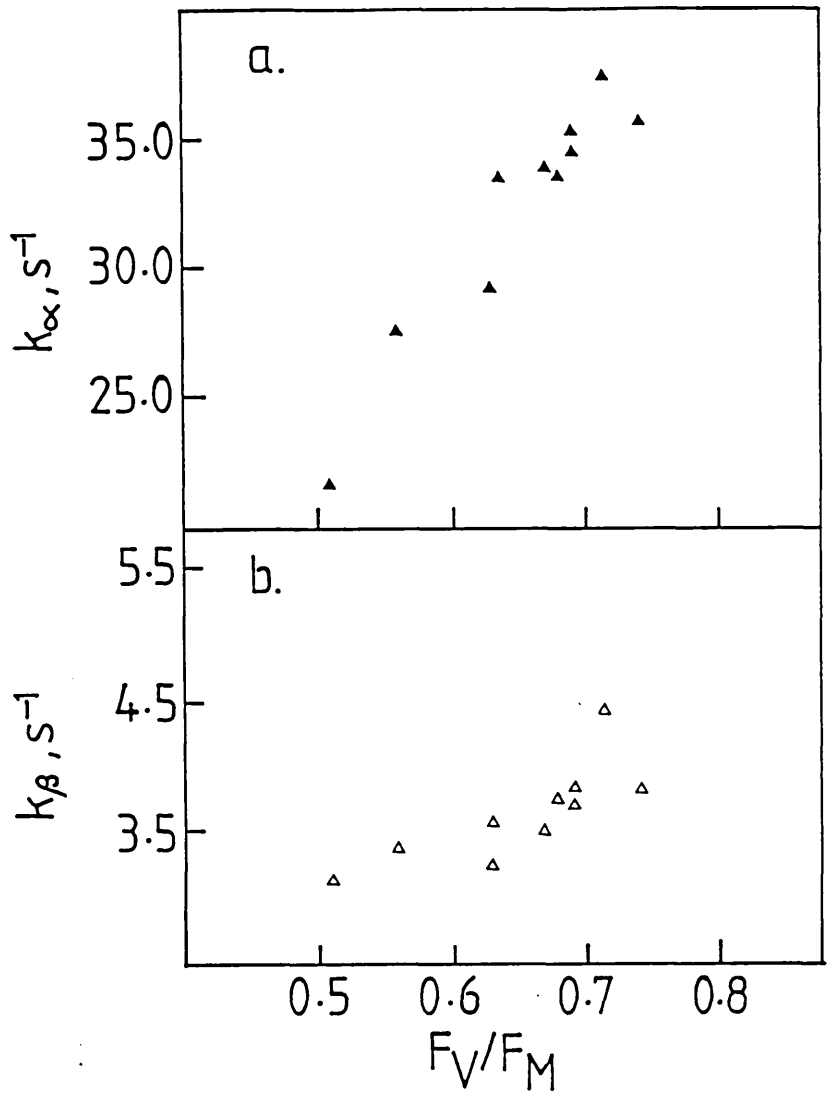


Fig. 3.9 The relationship between changes in F_v/F_m ratios, exhibited by DCMU-inhibited thylakoids, due to altered $MgCl_2$ concentrations and the rate constants for a) the initial fast, sigmoidal phase (k_α) and b) the slow, exponential phase (k_β) calculated from the analysis of room temperature chlorophyll fluorescence induction curves.

The mechanisms of the salt-induced changes have been investigated by titration of fluorescence with the quinone fluorescence-quencher, DBMIB. Such titrations by sequential additions of DBMIB have been discussed by Jennings et al. (1983) while the graphical representation used for Fig. 3.10 has been discussed in Section 2.3.9. The y-intercept at $(F_m-F_n)/F_m = 0$ gives an indication of the quenching ability of DBMIB and has been termed the interaction index. Fig. 3.10 shows that at "high" salt levels, DBMIB has a large quenching effect on fluorescence indicating a low level of other competing processes, while at "low" Mg^{2+} levels the y-intercept is low emphasising the presence of a high level of competition with other fluorescence quenching processes. Table 3.5 shows the relationship between fluorescence and interaction index over the range of Mg^{2+} concentrations used in Fig. 3.10. Using the bipartite model of Butler (1978) the ratio of the fluorescence level at 5mM Mg^{2+} (F_{max}) and at the other Mg^{2+} concentrations investigated (F_{mc}), termed F_{max}/F_{mc}, and the corresponding changes in interaction index (I_{max}/I_c) should be equal assuming homogenous quenching, unless some fluorescence quenching is occurring via non-competing processes. This relationship was respected between 5mM and 0.33mM Mg^{2+} but below this cation concentration it appeared to deviate as small fluorescence changes led to larger alterations in the interaction index.

3.3 Structural Alterations Accompanying the Fluorescence Change:

Fig. 3.11 shows representative thin section transmission electron micrographs of thylakoids suspended in the presence of 5mM, 0.5mM and minus $MgCl_2$. They clearly reemphasise the vaste change in gross membrane conformation which accompanies the salt-induced fluorescence changes, so that in the presence of 5mM Mg^{2+} there was a large degree

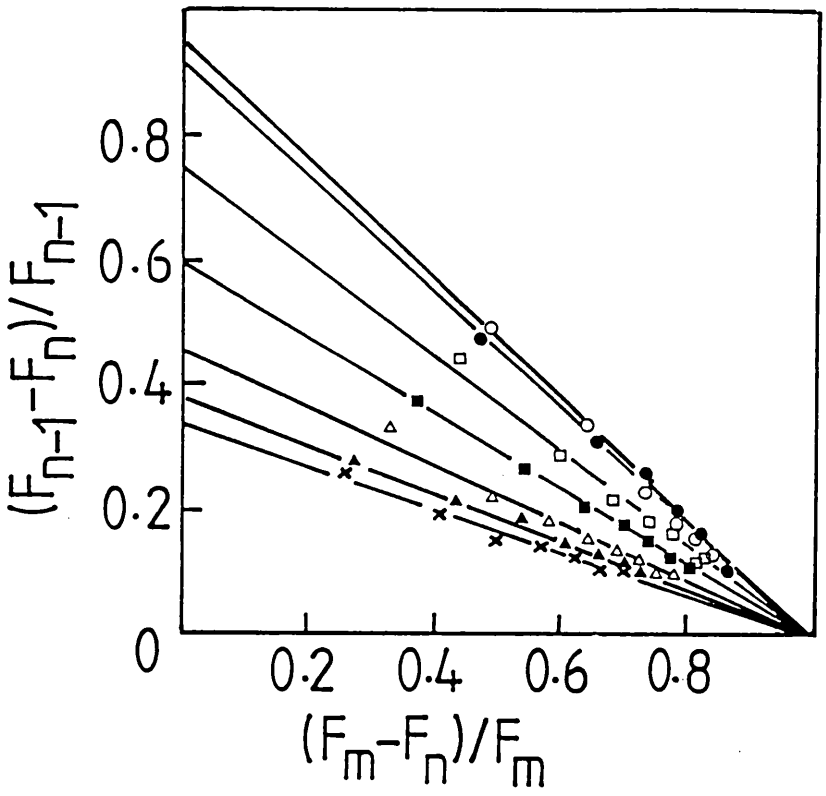


Fig. 3.10 Titration of chlorophyll fluorescence from DCMU-inhibited thylakoids suspended at different Mg^{2+} concentrations with successive additions of 0.67μM DBMIB. The Mg^{2+} levels are as follows; 5mM (o), 2mM (●), 0.67mM (□), 0.5mM (■), 0.33mM (△), 0.17mM (▲) and 0mM (X).
F_m = maximal fluorescence level before DBMIB addition
F_n = fluorescence after n additions of DBMIB
The lines are the least squares fit to all the experimental data points.

TABLE 3.5

The Effect of $MgCl_2$ Level of Maximal Chlorophyll Fluorescence (F_m) and PS2-DBMIB Interaction Index

$MgCl_2$ (mM)	F_m	Interaction Index	$\frac{I_{max}}{I_c}$ (a)	$\frac{F_{max}}{F_{mc}}$ (b)
5	64.0	0.95	1.00	1.00
2	64.0	0.92	1.03	1.00
0.67	49.5	0.75	1.27	1.29
0.5	41.1	0.60	1.58	1.56
0.33	32.8	0.46	2.06	1.99
0.17	31.8	0.38	2.50	2.01
0	28.4	0.34	2.79	2.25

(a) I_{max}/I_c represents the ratio of interaction index at 5mM $MgCl_2$ (I_{max}) with the interaction index at the other cation levels (I_c).

(b) F_{max}/F_{mc} represents the ratio of maximal fluorescence before DBMIB addition at 5mM $MgCl_2$ (F_{max}) with the maximal fluorescence at the other cation levels (F_{mc}).

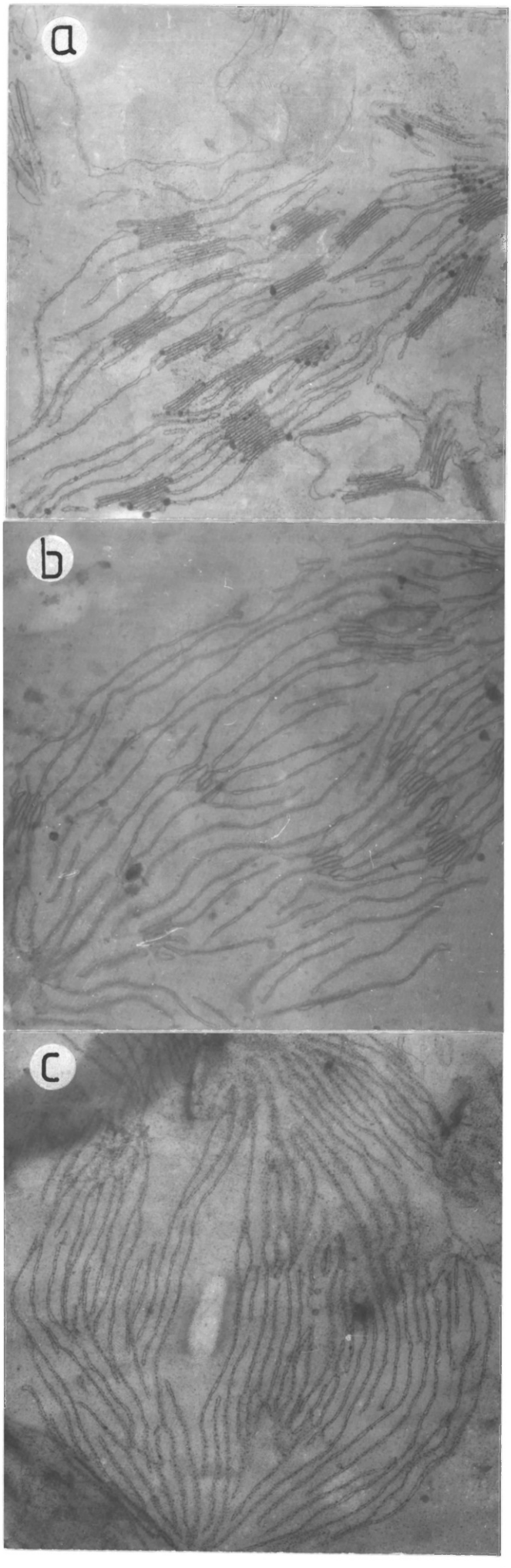
of differentiation into granal and stromal lamellae which became lost on going to minus Mg^{2+}. Similar results concerning unstacking have been shown by a number of workers in the past in response to cation levels (eg. Izawa and Good, 1966; Murakami and Packer, 1971; Gross and Prasher, 1974). Figs. 3.12a,b,c show histograms of the frequency of granal width at the Mg^{2+} levels stated above. It can be seen that as the Mg^{2+} level is lowered the grana became progressively smaller. In order to quantify the effect of Mg^{2+} concentration on membrane stacking a number of arbitary parameters have been measured from a number of micrographs, similar to those seen in Fig. 3.11, and these are presented in Table 3.6. This table also includes fluorescence and 90° light-scattering data taken from the same samples used for microscopy. This shows that substantial unstacking occurred as the salt level was lowered and that this was associated with a decrease in fluorescence and a decrease in light scattering.

3.4 Discussion:

The data presented in this chapter indicate that the cation-induced changes in room-temperature chlorophyll fluorescence are not Mg^{2+}-specific effects as stated by Melis and Ow (1982), but are, indeed brought about by a range of cations as emphasised in previous studies. The analyses presented indicate that alterations in both spillover of excitation energy from PS2 to PS1 and PS2 absorption cross section occur on the addition of cations which involves thylakoid conformational changes. However, changes in other non-radiative decay processes cannot be ruled out as a contributary factor.

From the electron micrographs presented in Fig. 3.11 and the accompanying analyses of a number of such micrographs (Fig. 3.12 and Table

Fig. 3.11 Representative electron micrographs of isolated pea thylakoids suspended in the presence of a) 5mM Mg^{2+}, b) 0.5mM Mg^{2+} and c) minus Mg^{2+} (5mM K^{+}). Magnification x20830.


a
b
c

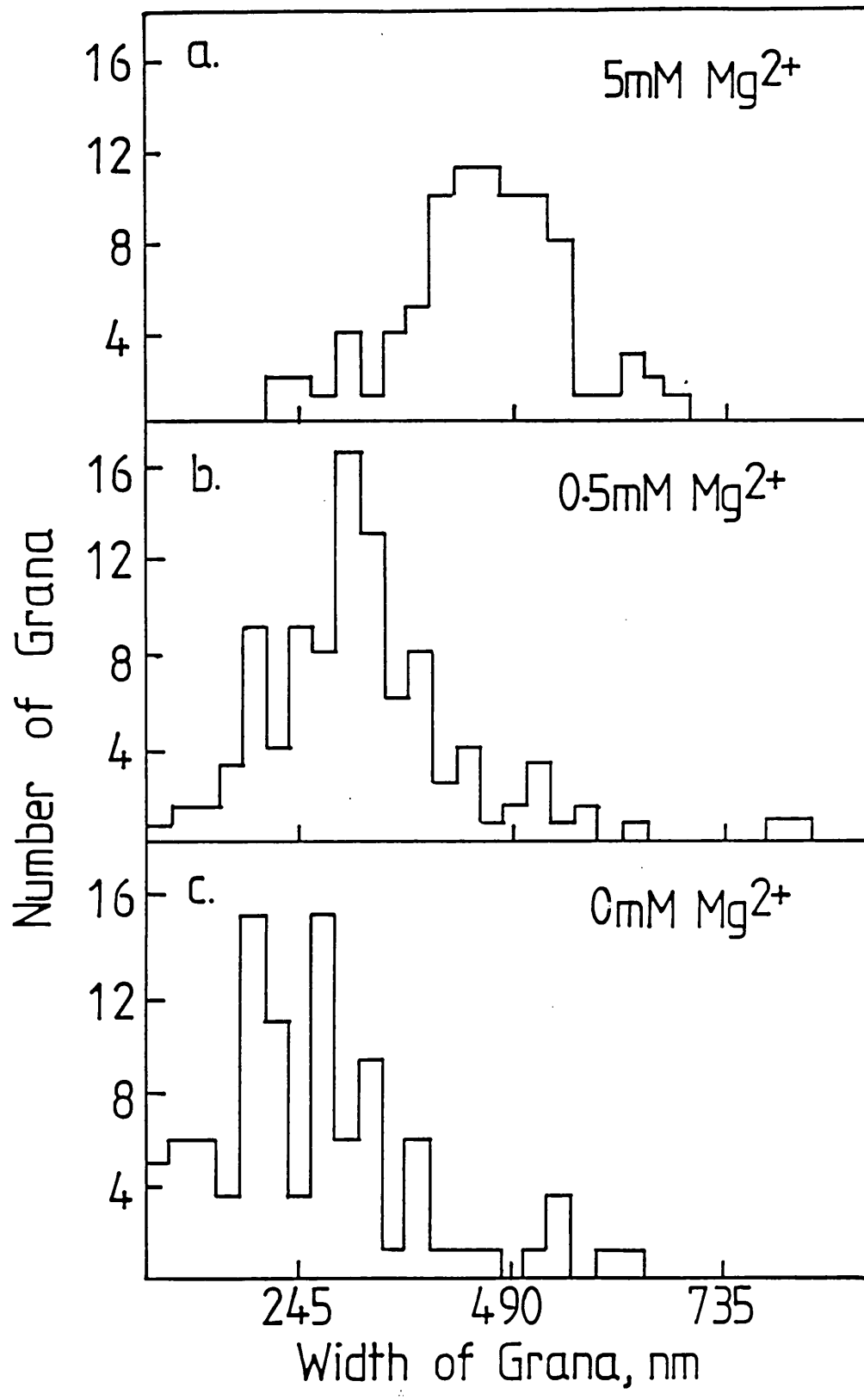


Fig. 3.12 Histograms of the frequency of grana of different widths of thylakoids suspended in the presence of a) 5mM Mg^{2+}, b) 0.5mM Mg^{2+} and c) 0mM Mg^{2+}. The values were obtained by measuring the granal widths from 12-16 different chloroplasts.

TABLE 3.6

The Effect of Mg^{2+} Concentration on Membrane Stacking, Chlorophyll Fluorescence and 90° Light Scattering

Mg^{2+} (mM)	Granal Width $(nm)_a$	Grana/ Chloroplast$_a$	% Thylakoids Stacked$_b$	%Δ Fluor$_c$	%Δ LS$_c$
5	480±120	24.5	66	100	100
0.5	312±126	16.7	18	18	29
0	261±120	8.3	4	0	0

a measured in 12-16 chloroplasts

b length of granal thylakoid/total length of thylakoids, measured in 5-8 chloroplasts

c measured after dilution to 40μg chl/ml plus 20μM DCMU.

3.6), it can be seen that alterations in thylakoid organisation are observed between "high" salt (5mM Mg^{2+}) and "low" salt (minus Mg^{2+}) conditions so that in the absence of "high" salt levels little differentiation into appressed and non-appressed membrane regions is observed. This change in thylakoid conformation appears to be an important factor in bringing about the alteration in chlorophyll fluorescence yield and other induction curve parameters as seen from the effect of glutaraldehyde fixation of "low" salt thylakoids in inhibiting the salt-induced fluorescence rise (Fig. 3.2a) and the inability of cations to alter the various fluorescence parameters measured and shown in Table 3.2. Such observations support the earlier reported conclusions which led Barber (1980a) to formulate a hypothesis concerning the mechanisms involved. It has also been shown from freeze-fracture studies that the gross membrane conformational change brings about a randomisation of proteins within the plane of the membrane (Wang and Packer, 1973; Staehelin, 1976; Boardman et al., 1978). This is indirectly confirmed by the increase in β_{max} on lowering the background Mg^{2+} level (Fig. 3.7 and Table 3.1). It is believed that β_{max} increases because of changes in the fast, sigmoidal α component of the fluorescence induction curve (Melis and Ow, 1982). The α phase represents "connected" PS2 reaction centres which can undergo a certain degree of energy transfer between centres (Melis and Homann, 1978). Therefore, the increase in β_{max} infers that a reorganisation of PS2 complexes has taken place leading to a less connected state, producing PS2 centres with an apparently smaller absorption cross section. From Fig. 3.8 it can be seen that at low cation concentrations it takes longer to photoreduce Q_A. This would be expected if PS2 reaction centres were becoming disconnected and unable to share LHC as a result of thylakoid unstacking and pigment-protein complex randomisation. Furthermore, it can be seen from Fig. 3.9 that as the F_v/F_m ratio is lowered the rate constants for the α and β components

of the rise (k_α and k_β respectively) both decrease indicating that Mg^{2+} depletion leads to a less efficient use or trapping of excitation energy which would be expected if PS2 was becoming less connected, if there was a decoupling of LHC from the reaction centres or if there was a more efficient energy transfer away from PS2.

The 77K fluorescence emission spectra (Fig. 3.3 and 3.4) provide evidence which suggests that as a consequence of the above mentioned conformational changes there is a redistribution of excitation energy from PS2 to PS1. The use of standardised fluorescence spectra means that the decrease in F_{685} and F_{695} and the subsequent increase in F_{735} on lowering the salt level is due to an increase in PS1 fluorescence at the expense of PS2-associated fluorescence. This alteration in the emission spectra would not be expected if the decrease in PS2 fluorescence was due to changes in k_d but it cannot distinguish between changes in the degree of spillover or absorption cross section.

Further evidence for a change in spillover arises from the analysis of the fluorescence induction curves from DCMU-inhibited thylakoids. From Fig. 3.5 it can be seen that if the fluorescence decrease was due to less light arriving at PS2 reaction centres then a decrease in F_o and F_m would be expected so that both F_v/F_m and A_{max} remain unaltered. Fig. 3.6 shows that between 5mM and 0.4mM Mg^{2+} this is not the case as there is a preferential quenching of F_v and a decrease in both F_v/F_m and A_{max}. These changes are expected for an increase in spillover and therefore confirm the conclusions from the altered 77K fluorescence spectra shown in Fig. 3.4 for "high" and "low" salt thylakoids. However, it appears that the cation-induced effect on fluorescence is more complex than just a change in spillover as below 0.4mM Mg^{2+} a decrease

in both F_o and F_v is observed which has very little effect on A_{max} or F_v/F_m and might be partially due to a decrease in PS2 absorption cross section by a decoupling of LHC from PS2 reaction centre proteins. Similar conclusions are arrived at from the DBMIB fluorescence titrations of Fig. 3.10 and Table 3.5. On lowering the Mg^{2+} level from 5mM to 0.33mM the decrease in fluorescence can be explained by increases in spillover and/or k_d. Below 0.33mM Mg^{2+} the changes in fluorescence and DBMIB interaction index cannot be explained in such a way and might reflect a deviation from the expected relationship between fluorescence and interaction index by LHC decoupling.

However, the decrease in F_o (attributed above solely to a decrease in PS2 absorption cross section) may also arise as a consequence of other processes which change as a result of either alterations in thylakoid conformation or cation concentration. Table 3.3, showing the effect of FeCy on various fluorescence parameters including F_o, gives further information concerning the large F_o difference (23%) between "high" and "low" salt conditions. The addition of FeCy before DCMU poisoning led to there being only a 9% difference in F_o level between the two cation conditions (a value similar to that observed by Melis and Ow, 1982), while the addition of FeCy after DCMU inhibition had only a small effect on F_o. Table 3.4 would suggest that the large difference in F_o between "high" salt and "low" salt thylakoids arises after the addition of DCMU, because in the absence of DCMU only an 11% difference in F_o between the two salt conditions was observed while in the presence of DCMU this difference increased to 29%. This situation arises because of a larger DCMU-induced increase in F_o when thylakoids are suspended in the presence of "high" salt levels (see also Table 3.1). One possible explanation is that when the thylakoids are stacked there is a larger number of PS2 centres in the $Q_A Q_B^-$ state, perhaps due to an

altered $Q_A^- Q_B \rightleftharpoons Q_A Q_B^-$ equilibrium, so when on the addition of DCMU the reaction $Q_A Q_B^- \rightarrow Q_A^- Q_B$ takes place more PS2 centres are closed before illumination in the "high" salt situation thereby leading to a higher F_o.

It therefore appears that initially, as the Mg^{2+} level is lowered from 5mM, PS2-LHC and PS1 complexes randomise due to alterations in the screening of the overall surface charge on the membranes leading to membrane unstacking and an increase in excitation energy spillover from PS2 to PS1 (see Barber, 1980a; Barber, 1982a). At very low Mg^{2+} concentrations, it is postulated that there is a decoupling of LHC from PS2 perhaps as a result of increased coulombic repulsion between complexes due to poor local electrostatic screening conditions leading to a decrease in PS2 absorption cross section and also perhaps to an altered stabilisation of Q_B^-. Similar conclusions concerning the complexity of the cation-induced changes have been put forward from experiments involving a number of different fluorescence techniques. Henkin and Sauer (1977), using a modulated fluorescence technique, reported very similar results to those shown in Fig. 3.6. Nairn et al. (1983), using picosecond fluorescence decay kinetic analyses, and Jennings (1984) who used a number of methods including preferential excitation of PS2 and LHC to measure induction curve parameters and DBMIB titration of chlorophyll fluorescence came to similar conclusions concerning the presence of at least two different salt-induced phenomena with different cation sensitivities.

Furthermore, no Mg^{2+}-specific effect on fluorescence has been detected (see Table 3.1 and Fig. 3.4b) as proposed by Melis and Ow (1982). It is seen that K^+ and TEC^{3+} can replace Mg^{2+}, as long as the concentration is high enough to maintain membrane appression, in generating similar fluorescence induction curve parameters thus confirming and

extending earlier work (eg. Barber and Searle, 1978). This concentration and valency dependence highlights the proposed electrostatic screening mechanism put forward by Barber and co-workers (see Barber et al., 1977; Barber, 1980a)to explain the ultrastructural changes. It is therefore very unlikely that the Mg^{2+}-dependent change in k_d recently put forward by Melis and Ow (1982) is a very complete explanation for the observed changes in fluorescence.

CHAPTER 4

Protein-Phosphorylation and Chlorophyll Fluorescence

For many years the mechanism for the State 1 - State 2 transition was poorly understood and initially it was postulated that it might be controlled by light-induced ionic movements across the thylakoid membranes (see Barber, 1976). This was concluded, mainly as a result of the observed cation-induced changes in fluorescence exhibited by isolated thylakoids which, as seen in Chapter 3, can produce high or low fluorescence levels with concomitant changes in energy distribution between PS2 and PS1, and the high energy state-induced fluorescence changes. Over the past few years it has been reported that the in vivo State transitions are related to the reversible phosphorylation of thylakoid proteins and especially LHC (Bennett et al., 1980). This has led to a confusion in the literature concerning the relationship between the cation- and protein phosphorylation-induced changes in chlorophyll fluorescence from isolated thylakoids. It is believed that both mechanisms lead to an alteration in excitation energy redistribution between the two photosystems but whether this is achieved by changes in spillover, absorption cross section, thermal deactivation or any combination of these three processes is still unclear.

This chapter involves an investigation of protein-phosphorylation-induced fluorescence changes as a function of background Mg^{2+} levels in order to analyse the mechanism(s) involved.

4.1 The Effect of Protein-Phosphorylation on Fluorescence Induction-Curves in the Presence of DCMU:

Fig. 4.1 shows examples of the chlorophyll fluorescence induction

curves for DCMU-inhibited pea thylakoids suspended at 5mM, 2mM or 1mM Mg^{2+} in either the phosphorylated or non-phosphorylated state. The larger proportional decrease in F_v is clearly seen at the lower salt levels (2mM and 1mM Mg^{2+}) while a relatively consistent decrease in F_o is present at all cation concentrations after phosphorylation. It is the careful computer-aided analysis of such curves which has been used to obtain information concerning the mechanisms involved in bringing about the phosphorylation-induced changes in excitation energy distribution.

Using the ferredoxin/NADPH method of reducing the PQ pool and activating the protein kinase to phosphorylate thylakoids in the dark (see 2.3.3), it can be seen from Fig. 4.2a that phosphorylation at either 5mM or 2mM Mg^{2+} led to a decrease in F_o and F_v so that the F_o was comparable with the F_o exhibited by non-phosphorylated thylakoids at minus Mg^{2+}. At 5mM Mg^{2+}, phosphorylation brought about changes in F_v and F_o which left F_v/F_m unaltered (see also Table 4.1) as previously reported by Horton and Black (1981a and 1983). Moreover, at this "high" salt level, A_{max} also remained relatively constant when comparing the non-phosphorylated and phosphorylated samples as seen in Fig. 4.2b. However, at 2mM $MgCl_2$, phosphorylation induced a preferential quenching in F_v relative to F_o so that F_v/F_m was lowered. At this Mg^{2+} concentration, phosphorylation also led to a decrease in A_{max} when compared with the non-phosphorylated samples. These changes are more like those seen at low Mg^{2+} levels under non-phosphorylating conditions (see Fig. 3.6, Chapter 3).

To elaborate on the experiments shown in Fig. 4.2, the reversal of the fluorescence changes by dephosphorylation was undertaken. This was

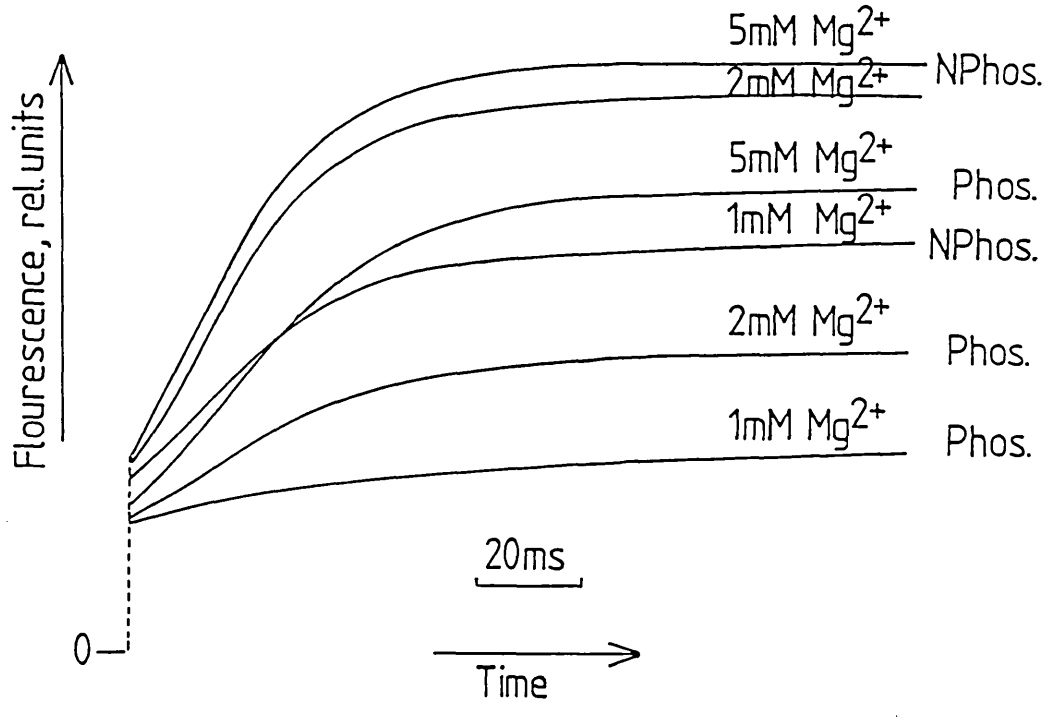
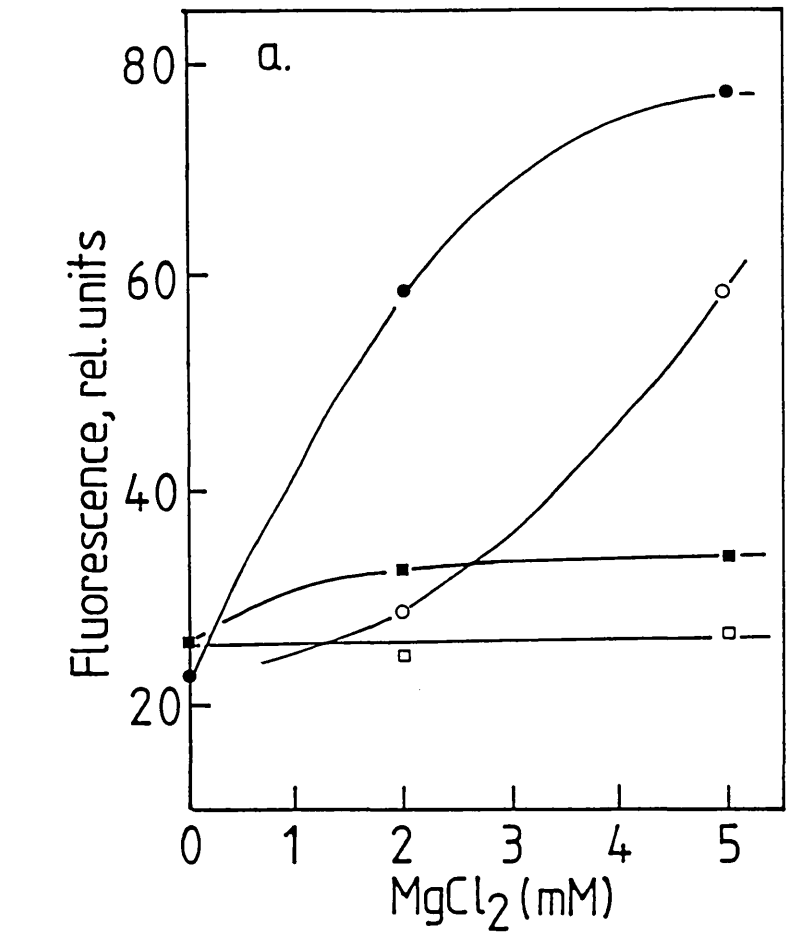
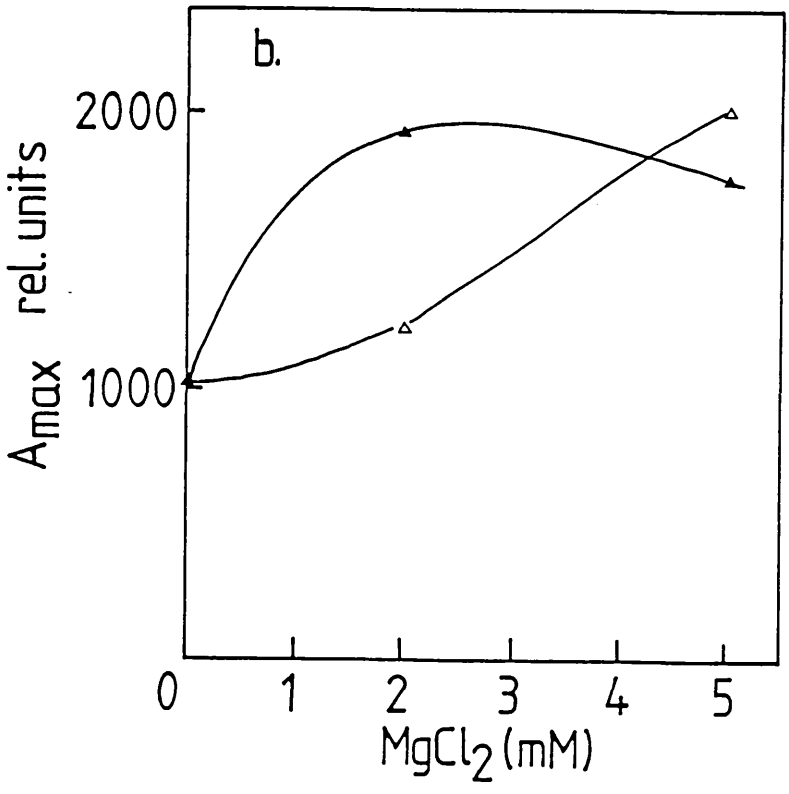


Fig. 4.1 Room temperature chlorophyll fluorescence induction curves from DCMU-inhibited pea thylakoids suspended at 5mM, 2mM and 1mM Mg^{2+} and which had been either phosphorylated (Phos) or non-phosphorylated (NPhos). Protein-phosphorylation was carried out using the Fd/NADPH method described in 2.3.3.

Fig. 4.2 a) The effect of protein-phosphorylation on the room temperature chlorophyll fluorescence parameters F_v (o) and F_o (□) exhibited by DCMU-inhibited thylakoids suspended in the presence of 5mM and 2mM $MgCl_2$, compared with the non-phosphorylated values of F_v (●) and F_o (■) carried out at 5mM, 2mM and 0mM $MgCl_2$.

b) The A_{max} values obtained from phosphorylated (▲) and non-phosphorylated (△) thylakoids for the conditions mentioned in (a).


a.
80
60
40
20
Fluorescence, rel. units
0
1
2
3
4
5
MgCl2 (mM)


b.
2000
1000
Amax rel. units
0
1
2
3
4
5
MgCl2 (mM)

accomplished by the introduction of excess $NADP^+$ to oxidise PQ and thereby inhibiting kinase activity. Dephosphorylation occurred as indicated by the return of the fluorescence characteristics to those exhibited by the non-phosphorylated controls. Table 4.1 confirms the observations shown in Fig. 4.1 and 4.2 and also gives the β_{max} values for the various treatments. It can be seen that β_{max} increased after phosphorylation at both cation levels but a greater change occurred at 2mM $MgCl_2$. At 5mM Mg^{2+}, dephosphorylation led to an increase in F_o and F_m so F_v/F_m and β_{max} both returned to the non-phosphorylated control values. However, at 2mM Mg^{2+} dephosphorylation did not lead to an increase in F_o or a full return to the control F_m level, while β_{max} and F_v/F_m did return to the 2mM Mg^{2+} non-phosphorylated values.

Fig. 3.6 and 4.2 show that the absence of Mg^{2+} and protein-phosphorylation both led to a decrease in F_o. In order to determine whether these changes were the same or independent effects, induction curves were measured in the absence of Mg^{2+} after thylakoids had been phosphorylated or non-phosphorylated at 5mM Mg^{2+}. Table 4.2 shows that on phosphorylation there was a decrease in the minus Mg^{2+} F_o level which was lower than that exhibited by the non-phosphorylated thylakoids. This additional change was accompanied by a change in F_v/F_m to a level similar to that seen in the minus $MgCl_2$ control. These results suggest the change due to phosphorylation is different from the minus Mg^{2+} effect. This independence is emphasised by the fact that the percentage decrease in F_o, due to phosphorylation, was similar plus or minus $MgCl_2$ and the percentage decrease, due to minus $MgCl_2$ was similar plus or minus phosphorylation.

Fig. 4.3 shows that the kinetics of the area growth, equivalent to Q_A photoreduction, was altered by both changes in Mg^{2+} level (Fig. 4.3a)

TABLE 4.1

The Effect of Phosphorylation and Dephosphorylation of Thylakoid Membranes on Various Fluorescence Parameters

$MgCl_2$ (mM)	Treatment		F_o	F_v	β_{max}	F_v/F_m
5	NPhos	(a)	36.5	70.8	0.305	0.66
	NPhos	(b)	41.5	86.0	0.21	0.67
	Phos	(i)	31.0	67.3	0.45	0.68
	Phos	(ii)	31.0	62.3	0.47	0.67
	DPhos		38.8	75.5	0.29	0.66
2	NPhos	(a)	34.0	75.5	0.46	0.69
	NPhos	(b)	37.3	79.3	0.47	0.68
	Phos	(i)	29.0	24.0	0.73	0.46
	Phos	(ii)	30.0	26.0	0.74	0.46
	DPhos		30.3	65.0	0.46	0.68
0	NPhos		29.0	19.0	0.81	0.40

Comparison of the fluorescence parameters F_o, F_v, β_{max} and F_v/F_m for pea thylakoid membranes which were phosphorylated (Phos), non-phosphorylated (NPhos) and de-phosphorylated (DPhos) at 5mM and 2mM $MgCl_2$ with membranes suspended in the absence of Mg^{2+}. NPhos (b) and Phos (ii) was achieved by the procedures outlined in the MATERIALS AND METHODS. NPhos (a) represents samples which were incubated for 60 min in the presence of ATP in the presence of ferredoxin and $NADP^+$. After 60 min of this treatment NADPH and NaF were added to balance the overall content of the media relative to those of the other experiments. Phos (i) represents samples which were incubated in the presence of ATP for 30 min but in the absence of NaF. After 30 min incubation, NaF and $NADP^+$ was added to inhibit phosphatase and kinase activity respectively.

TABLE 4.2

Comparison of the Effect of the Absence of Mg^{2+} on Fluorescence of Phosphorylated and Non-Phosphorylated Thylakoid Membranes

	F_v/F_m		F_o		Decrease in F_o due to $-Mg^{2+}$ (%)
	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	
NPhos	0.64	0.39	46.5	27.3	41
Phos	0.64	0.40	36.0	22.0	39
Decrease in F_o due to Phos (%)	-	-	23	19	

Pea thylakoid membranes were phosphorylated (Phos) or non-phosphorylated (NPhos) at 5mM $MgCl_2$ for 60 min as described in the MATERIALS AND METHODS. Fluorescence induction was then measured as previously described either in the presence of 5mM $MgCl_2$ ($+Mg^{2+}$) or after addition of sufficient EDTA to chelate the Mg^{2+} present in the diluted sample ($-Mg^{2+}$). Fluorescence induction of thylakoids initially incubated in the absence of Mg^{2+} was essentially the same as that seen in the NPhos samples in which Mg^{2+} had been chelated by EDTA.

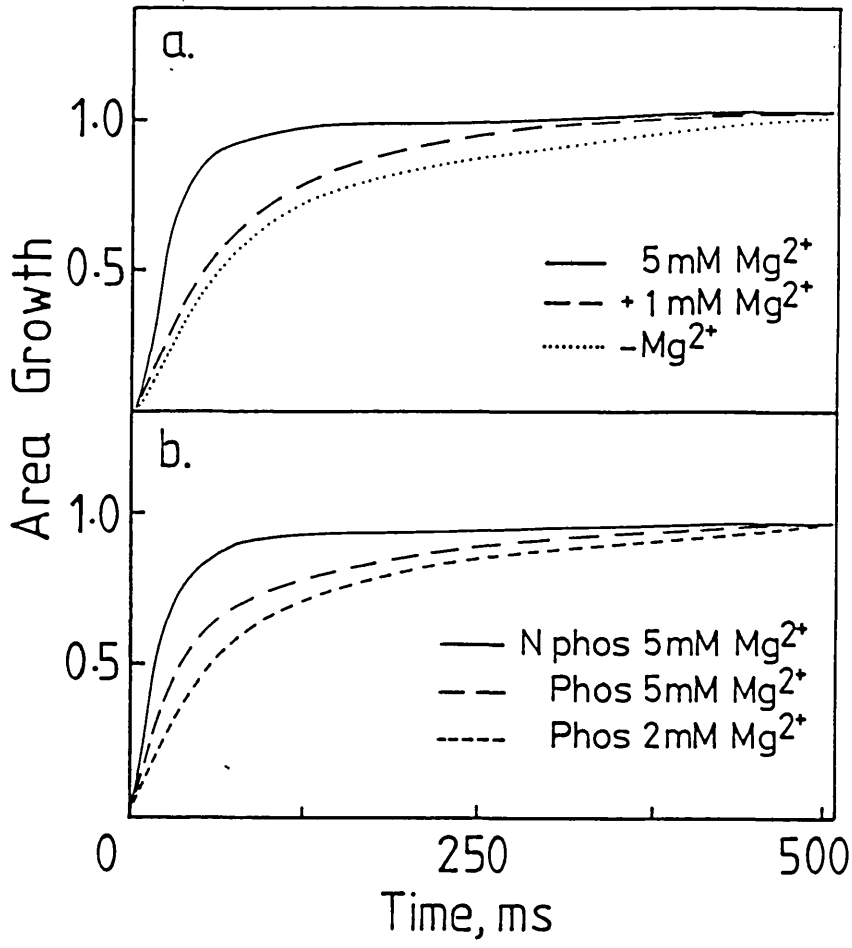


Fig. 4.3 The growth of the normalised area above room temperature chlorophyll fluorescence induction curves (indicative of Q_A^- accumulation) exhibited by DCMU-inhibited thylakoids suspended at a) 5mM, 1mM and minus Mg^{2+} and b) phosphorylated at 5mM and 2mM Mg^{2+} and non-phosphorylated at 5mM Mg^{2+}.

and by protein-phosphorylation (Fig. 4.3b). Both effects brought about a slower rate of Q_A reduction but phosphorylation allowed similar rates to those seen at "low" salt levels to occur at higher Mg^{2+} levels. By comparing the $t_{\frac{1}{2}}$ values for Q_A accumulation it is seen that 2mM Mg^{2+}, phosphorylated samples produced a similar half-time to that observed for the minus Mg^{2+}, non-phosphorylated thylakoids.

Phosphorylation was also carried out using sodium dithionite as the reductant to activate the kinase. Fig. 4.4 shows the effect of phosphorylation on F_m and F_o as a function of background Mg^{2+} concentration. Using this method the effect of cation level is not as drastic as that seen in Fig. 4.2. This may reflect the different methods of obtaining phosphorylated membranes. The use of dithionite appears to affect the thylakoids ability to generate high F_v/F_m ratios probably because of higher F_o values as a result of the strong reducing conditions produced. However, as both phosphorylated and non-phosphorylated thylakoids underwent similar treatments a comparison is still justified. By comparing Fig. 4.4a with Fig. 4.4b it can be seen that the $c_{\frac{1}{2}}$ values for F_m and F_o are increased by protein-phosphorylation. This becomes clearer from Fig. 4.4c, which shows the percentage decrease in F_o and F_m due to phosphorylation as a function of Mg^{2+} level. This effect would be expected for a mechanism under electrostatic control since a higher level of cations is now required to "screen" the additional surface charge produced by phosphorylation.

4.2 The Effect of Protein Phosphorylation on DBMIB-Induced Fluorescence Quenching:

Protein phosphorylation has been investigated by the titration of chlorophyll fluorescence with DBMIB. The ability of the quinone to

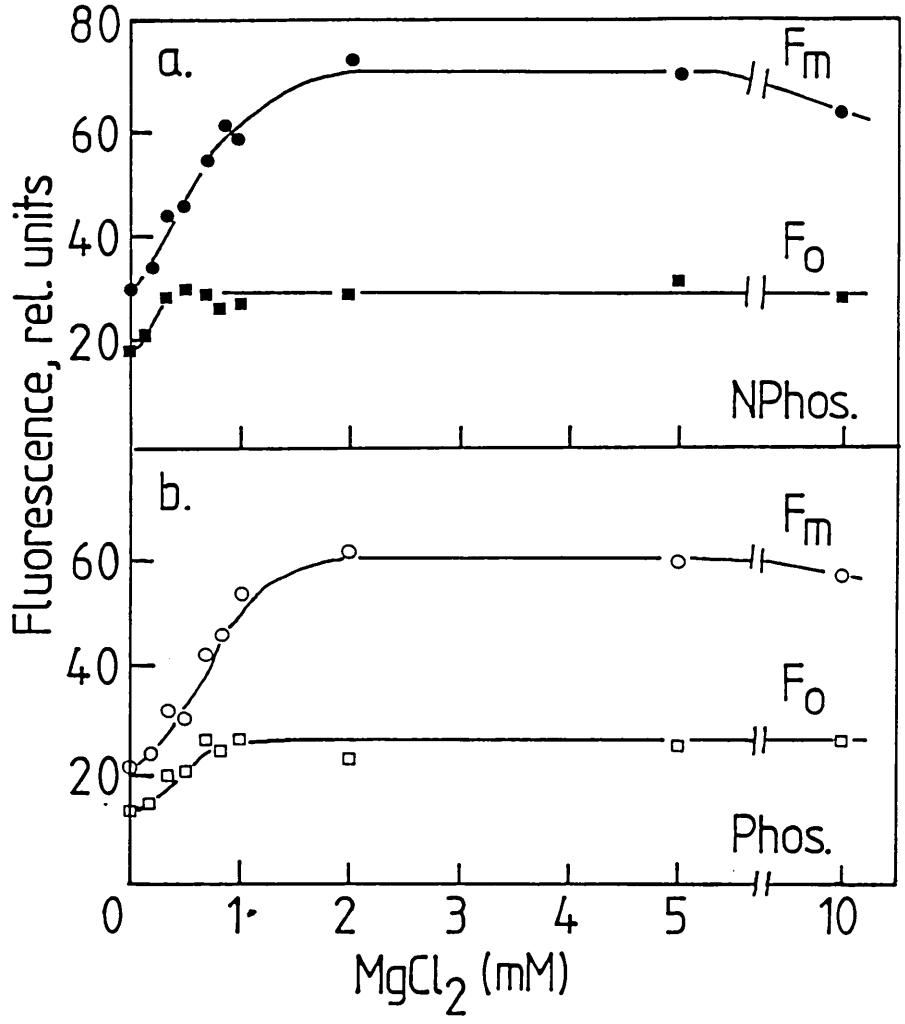


Fig. 4.4 The effect of $MgCl_2$ concentration on the F_m and F_o levels of room temperature chlorophyll fluorescence exhibited by a) non-phosphorylated (closed cymbols) and b) phosphorylated (open symbols) DCMU-inhibited thylakoids.

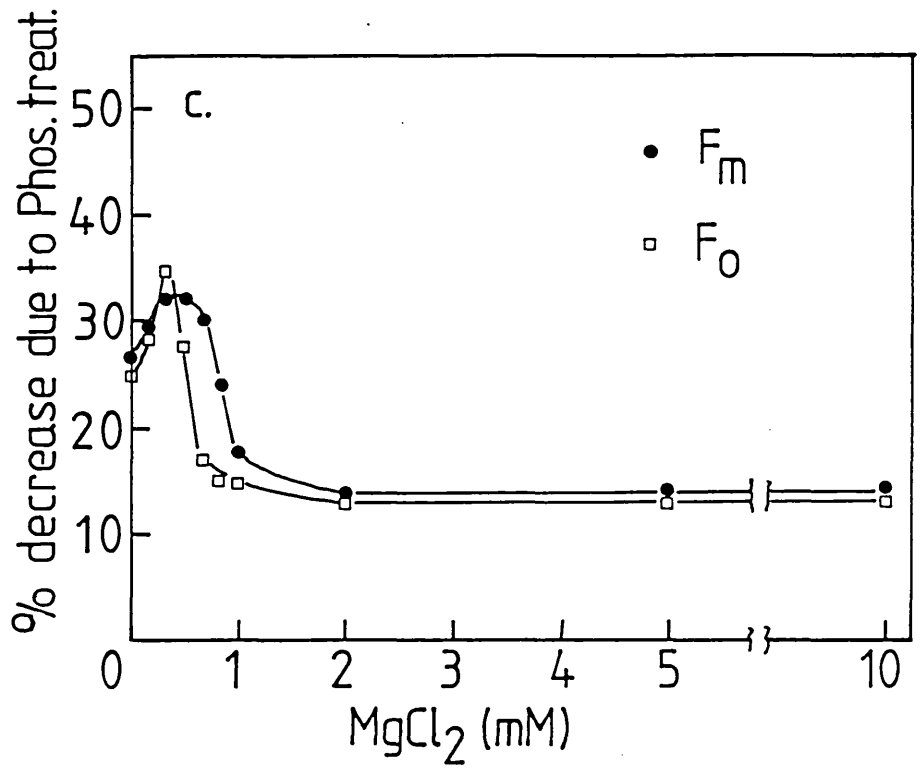


Fig. 4.4 c) The percentage change in F_m (●) and F_o (□) due to protein-phosphorylation as a function of background $MgCl_2$ concentration.
Protein-phosphorylation was carried out using the dithionite method described in 2.3.3.

quench fluorescence in thylakoids phosphorylated at 10mM Mg^{2+} and then resuspended at either 10mM or minus $MgCl_2$ is shown in Fig. 4.5. In both cases, it can be seen that, there was no change in the quenching capacity of DBMIB between the phosphorylated and non-phosphorylated membranes, although the F_m had been attenuated by 15% in both cases by phosphorylation (see Table 4.3). However, it can be seen from Fig. 4.6 that, phosphorylation can lead to a change in the quenching capabilities of DBMIB, and that the extent is cation-concentration dependent. Fig. 4.6a shows that at 5mM Mg^{2+} there was a small decrease in interaction index suggesting that even at this cation-level phosphorylation led to a small increase in either spillover or k_d. At the lower Mg^{2+} levels (2mM and 1mM) the large decreases in fluorescence brought about by phosphorylation were mirrored by large decreases in DBMIB-PS2 interaction (Fig. 4.6b and c). Table 4.3 shows that the change in F_m before DBMIB addition as a result of phosphorylation was not equal to the change in interaction-index, as seen in Table 3.5 for the cation-effect, indicating that a proportion of the fluorescence decline was via a process not in competition with the DBMIB-induced quenching of fluorescence. Fig. 4.7 shows the relationship between interaction index and Mg^{2+} level in phosphorylated and non-phosphorylated samples. It can be seen that, like in Fig. 4.4, the Mg^{2+}-requirement to attain the same degree of DBMIB-PS2 interaction is greater after phosphorylation ($c_{\frac{1}{2}}$ = 1.7mM $MgCl_2$) than in the absence of phosphorylation ($c_{\frac{1}{2}}$ = 0.8mM $MgCl_2$).

4.3 The Effect of Protein Phosphorylation on Thylakoid Organisation:

The effect of phosphorylation on thylakoid membrane organisation at different background Mg^{2+}-levels has been investigated by analysing electron micrographs. Fig. 4.8 shows representative micrographs of

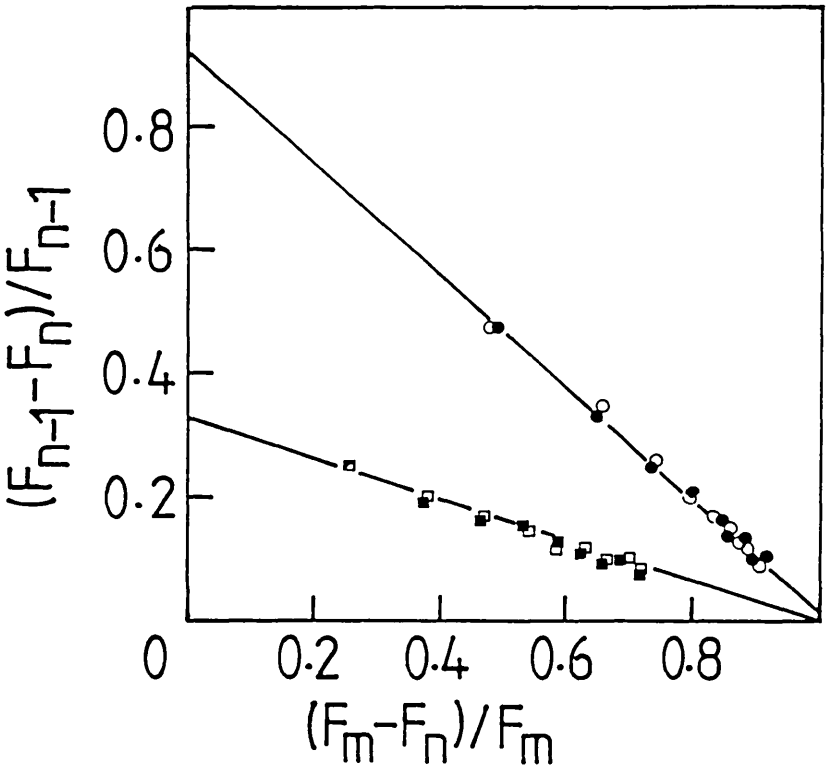


Fig. 4.5 Titration of chlorophyll fluorescence from DCMU-inhibited thylakoids at 10mM Mg^{2+} under phosphorylated (●) and non-phosphorylated (○) conditions and at 0mM Mg^{2+} under phosphorylated (■) and non-phosphorylated (□) conditions with successive additions of 0.67μM DBMIB.
For 0mM Mg^{2+} the thylakoids were phosphorylated at 10mM Mg^{2+}, diluted and resuspended in 1mM EDTA to remove all Mg^{2+}. The lines are the least squares fit to all the experimental data points.

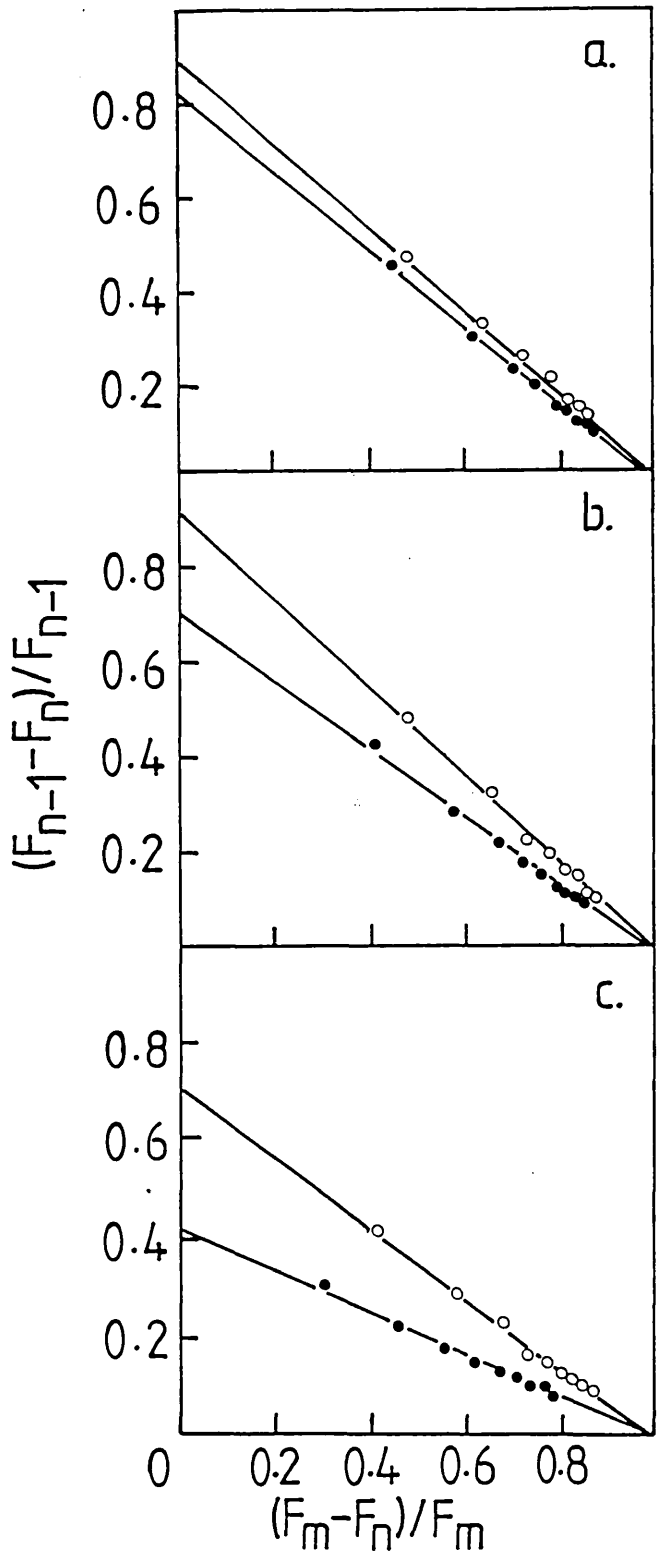


Fig. 4.6 Titration of chlorophyll fluorescence from DCMU-inhibited thylakoids either under phosphorylation (●) or non-phosphorylation (○) conditions with successive additions of 0.67μM DBMIB suspended at a) 5mM Mg^{2+}, b) 2mM Mg^{2+} and c) 1mM Mg^{2+}.

The lines are the least squares fit to all the experimental data points.

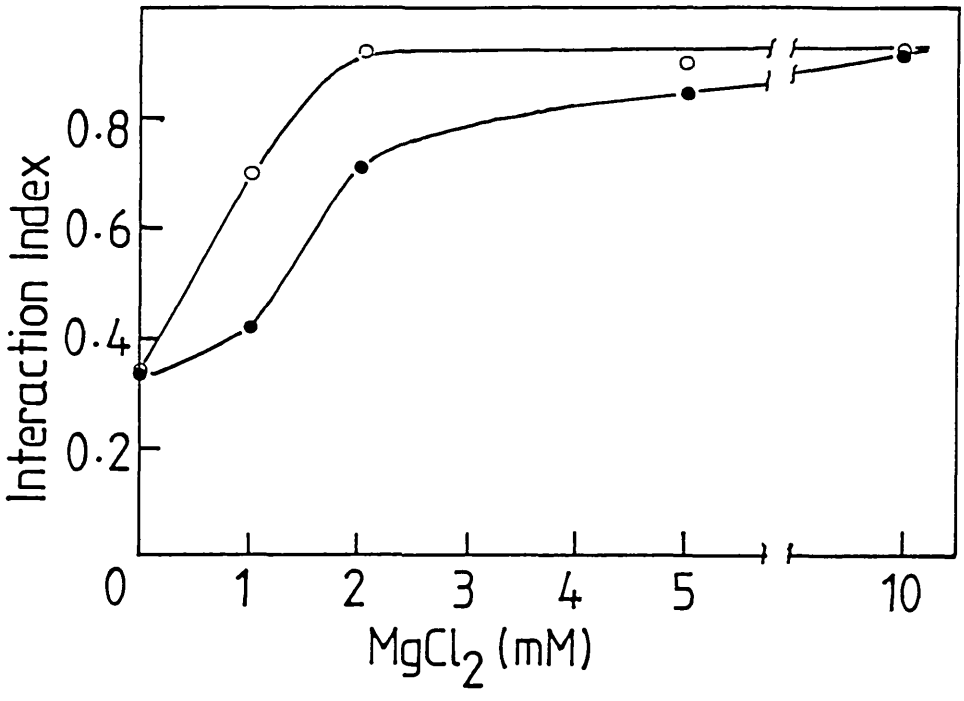


Fig. 4.7 The DBMIB-PS2 interaction index of phosphorylated (●) and non-phosphorylated (○) thylakoids as a function of background $MgCl_2$ concentration.

TABLE 4.3

The Effect of Phosphorylation of Thylakoid Membranes on Maximal Chlorophyll Fluorescence (F_m) and PS2-DBMIB Interaction Index

$MgCl_2$ (mM)	Treatment	F_m	Interaction Index	$\frac{I_{NPhos}}{I_{Phos}}$ (a)	$\frac{F_{NPhos}}{F_{Phos}}$ (b)
10	NPhos	72.0	0.92	1.00	1.16
	Phos	62.2	0.92		
5	NPhos	72.5	0.90	1.07	1.25
	Phos	57.8	0.84		
2	NPhos	73.0	0.92	1.30	1.54
	Phos	47.2	0.71		
1	NPhos	55.0	0.70	1.67	1.67
	Phos	32.9	0.42		
0	NPhos	30.5	0.33	1.00	1.18
	Phos	25.8	0.33		

(a) I_{NPhos}/I_{Phos} represents the ratio of interaction index of non-phosphorylated (NPhos) and phosphorylated (Phos) samples at the same $MgCl_2$ concentration.

(b) F_{NPhos}/F_{Phos} represents the ratio of maximum fluorescence yield of NPhos and Phos samples at the same $MgCl_2$ concentration.

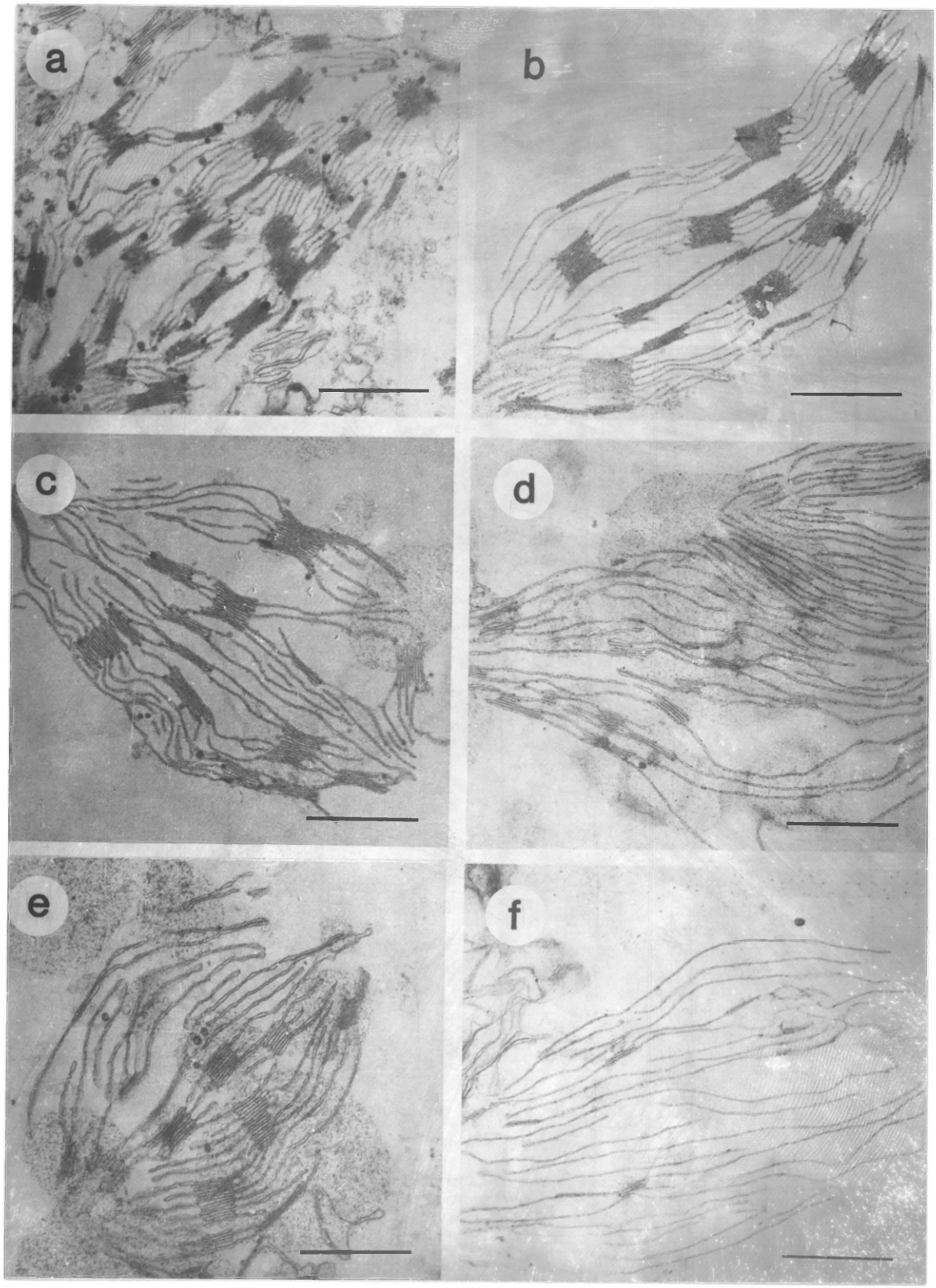
thylakoids phosphorylated at 5mM, 2mM and 1mM $MgCl_2$ and their controls. It can be seen that at the lower cation levels the phosphorylated samples showed extensive unstacking while at 5mM Mg^{2+} no obvious organisational change could be seen between the phosphorylated and non-phosphorylated membranes.

In order to quantify the effect of phosphorylation on stacking, a number of parameters were measured. Fig. 4.9 shows histograms of the frequency of grana of different widths. It emphasises the differences in thylakoid organisation between the phosphorylated and non-phosphorylated membranes at the lower salt levels and suggests that a small degree of unstacking might have occurred even in the 5mM Mg^{2+}, phosphorylated samples. Further parameters are shown in Table 4.4 including fluorescence and 90° light scattering changes for the thylakoid samples used in the electron microscopy. It can be seen that not only did the average granal width decrease upon phosphorylation at intermediate Mg^{2+} concentrations, but so did the number of grana per chloroplast. Table 4.4 also shows that the percentage of stacked thylakoids was substantially decreased in 2mM and 1mM Mg^{2+}, phosphorylated samples when compared with the relevant controls so that the 1mM Mg^{2+}, phosphorylated membranes were essentially unstacked.

4.4 Discussion:

It is now widely believed that thylakoid protein phosphorylation is one mechanism by which higher plants can regulate excitation energy distribution between the two photosystems. There appears to be two ways in which light redistribution can be achieved; either by energy transfer from PS2 to neighbouring PS1 complexes via spillover or by changes in the absorption cross section of PS2 and PS1 by LHC movement.

Fig. 4.8 Representative electron micrographs of pea thylakoids suspended at various Mg^{2+} concentrations and either phosphorylated (b,d,f) or non-phosphorylated (a,c,e) using the Fd/NADPH method. $MgCl_2$ concentrations were as follows; a,b, 5mM; c,d, 2mM; e,f, 1mM. The bar is 1μM.


a
b
c
d
e
f

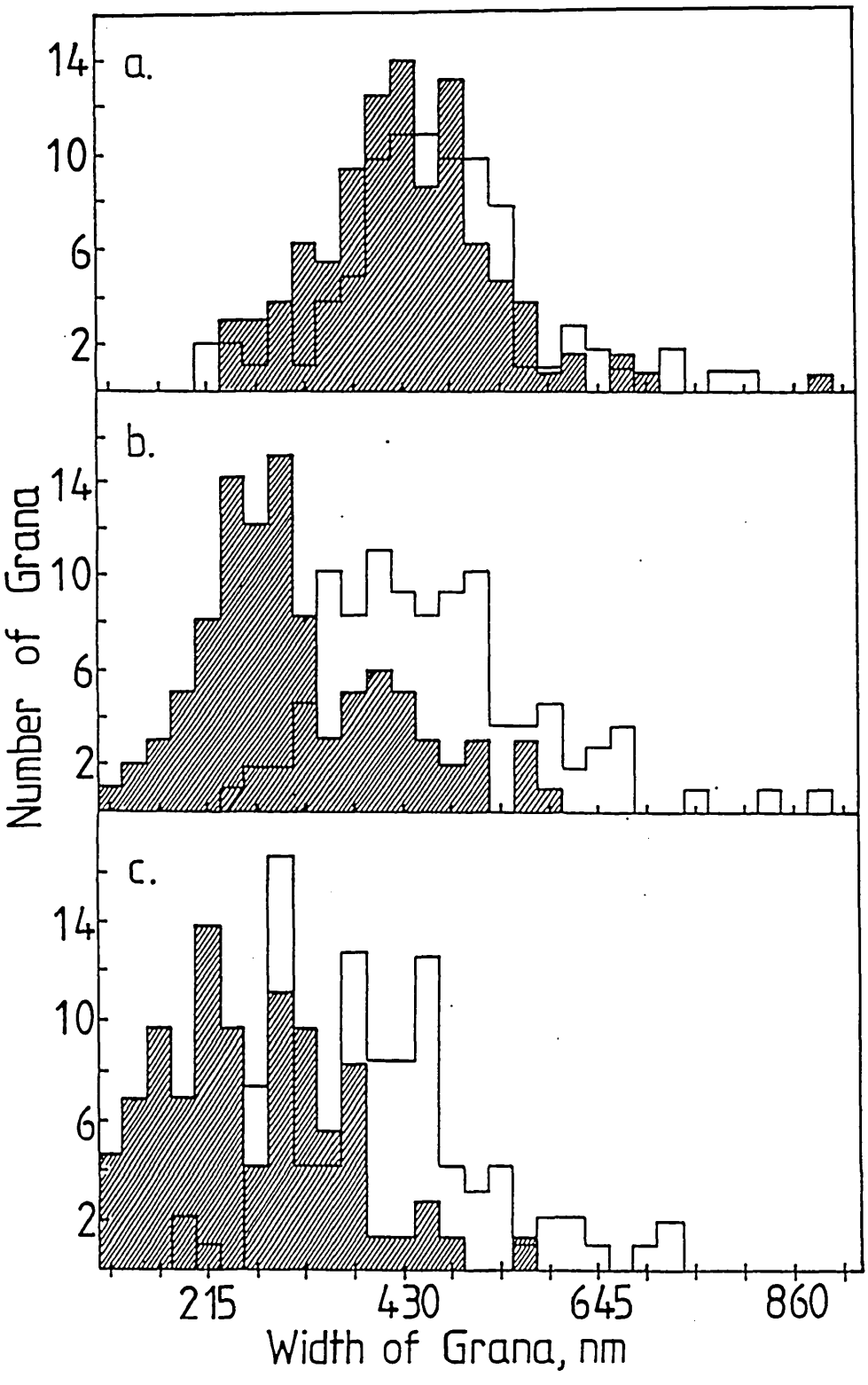


Fig. 4.9 Histograms of the frequency of grana of different widths as a function of Mg^{2+} concentration and protein-phosphorylation. The values were obtained from a series of electron micrographs of which representative pictures are seen in Fig. 4.8. The average width of a proportion of grana was measured from 12-16 chloroplasts. $MgCl_2$ concentrations were as follows; a) 5mM; b) 2mM and c) 1mM.

□ NPhos.; ▨ Phos.

TABLE 4.4

The Effect of Mg^{2+} Concentration and LHC-Phosphorylation on Membrane Stacking, Chlorophyll Fluorescence and 90° Light Scattering

Experimental Conditions mM Mg^{2+}	Phos	Granal Width (nm)$_a$	Grana/ Chloro-plast$_a$	% Thylakoids Stacked$_b$	%Δ Fluor$_c$	%Δ LS$_c$
5	-	480±120	24.5	66	100	100
5	+	430±99	23.5	58	64	90
2	-	451±95	23.0	63	89	96
2	+	332±101	14.8	26	24	46
1	-	403±114	21.0	49	47	52
1	+	281±97	12.8	10	5	4
0.5	-	312±126	16.7	18	18	29
0	-	261±120	8.3	4	0	0

a measured in 12-16 chloroplasts.

b length of granal thylakoids/total length of thylakoids x 100, measured in 5-8 chloroplasts.

c measured after dilution to 40μg chlorophyll/ml with basic medium plus 20μM DCMU and the Mg^{2+} concentrations indicated.

In Chapter 3 it was re-emphasised that the cation-induced changes were mainly brought about by alterations in spillover. The results in this chapter indicate that protein-phosphorylation leads to a decrease in PS2 absorption cross section at "high" salt levels but as a consequence of the electrostatic nature of the process, at lower cation concentrations an increase in spillover is also observed. By comparing the salt-induced data of chapter 3 with the phosphorylation-induced results it is seen that they are two independent but interrelated processes.

From the analysis of fluorescence induction curves (see Figs. 4.2, 4.4 and Table 4.1) it was seen that at 5mM Mg^{2+}, phosphorylation brought about an equal decrease in F_v and F_o so F_v/F_m remained unchanged, confirming the results of Horton and Black (1981a) but disagreeing with the changes reported by Kyle et al. (1982) who noticed a decrease in F_v/F_m because of an increase in F_o. Moreover, it is found that A_{max} remained more or less constant (Fig. 4.3). From the changes seen in the fluorescence parameters on decreasing the excitation light intensity (Fig. 3.5) the observations mentioned above would support the contention that at "high" salt levels the sole effect of phosphorylation is to decrease PS2 absorption cross section. At lower Mg^{2+} levels ($\leq$2mM), however, phosphorylation led to a preferential quenching of F_v relative to F_o (see Fig. 4.2 and Table 4.1). This is similar to the changes seen for the salt-induced phenomena and would infer an alteration in the extent of spillover. From these results it can be seen that both mechanisms for altering the balance of excitation energy between PS2 and PS1 maybe operative when thylakoid proteins are phosphorylated depending on the background Mg^{2+}-level.

However, this may not wholly be the case, when the DBMIB titration

results of Fig. 4.5 and 4.6 are considered. They confirm that the effect of protein-phosphorylation can be partially explained by a change in PS2 absorption cross section which according to the analysis of the DBMIB-induced quenching probably occurs at all the cation levels investigated. It is seen that at 10mM $MgCl_2$ the decrease in fluorescence yield can be fully accounted for by a change in absorption cross section because the ability of DBMIB to quench fluorescence is unaltered (Fig. 4.5). Contrary to the induction curve interpretation, at 5mM Mg^{2+} only 65% of the total fluorescence decrease due to phosphorylation can be attributed to such a change while an additional quenching component is observed which competes with DBMIB to lower chlorophyll fluorescence and therefore reflects an alteration in spillover or k_d. This small change due to spillover, or k_d, is probably not detected from the induction curves as it would only be expected to produce very small changes. At the lower Mg^{2+} levels investigated the additional component became more pronounced, as indicated by the significant lowering of the y-intercept seen in Figs. 4.6b and 4.6c. It is at these lower Mg^{2+} levels where large decreases in membrane appression occur as a result of phosphorylation (Fig. 4.8) and therefore the large fluorescence decrease probably reflects an increase in spillover in a manner similar to that proposed in Chapter 3 for the salt-induced phenomena.

The cation- and phosphorylation-induced effects appear to be independent processes, this is seen in both the induction curve and DBMIB-titration experiments. From Table 4.2 it can be seen that phosphorylation led to a further decrease in both F_o and F_m at minus $MgCl_2$, confirming and extending the work of Horton and Black (1982), and emphasising the additive nature of the two phenomena.

Similarly, when phosphorylated thylakoids were resuspended at minus

Mg^{2+}, a 15% attenuation in fluorescence yield was still observed when compared with the non-phosphorylated membranes and no difference was seen in the ability of DBMIB to quench fluorescence. Therefore, the fluorescence decrease due to phosphorylation cannot be attributed to a change in relative lifetimes and probably reflects the same change seen after phosphorylation at 10mM $MgCl_2$ which is still present at minus $MgCl_2$.

Such results would imply that the change in F_o due to salt-depletion is not via the same process which brings about the phosphorylation-induced F_o decrease. However, because in one case the LHC carries more negative surface electrical charge, differences in LHC-photosystem interaction may still occur even when the protein-complexes are completely randomised, although the additive effect could partially reflect the proposed thylakoid conformation-dependent DCMU-induced F_o increase discussed in Chapter 3 which is more prominent under "high" salt conditions.

The fluorescence data of Fig. 4.4 and 4.7 highlight the electrostatic nature of the phosphorylation-induced phenomena as proposed by Barber (1980a and 1982a). It is shown that to obtain similar F_o and F_m levels and the same PS2-DBMIB interaction index in phosphorylated thylakoids compared with the non-phosphorylated membranes, there is a requirement for higher $MgCl_2$ levels (approximately double). This suggests that as a consequence of the additional phosphate groups placed on the proteins by the phosphorylation process more salt is required to maintain a state of membrane organisation similar to that exhibited by the non-phosphorylated thylakoids. This would be expected for a process involving alterations in electrical surface charge, and indirectly agrees with Kyle and Arntzen (1983) who have shown a difference in local membrane

charge near Q_A when thylakoids are phosphorylated. Therefore, at high salt ($\geqslant$5mM $MgCl_2$), when electrostatic screening is high, the phosphorylation-induced change is less extensive than at the lower salt levels when screening is low. The electrical properties of the membrane surface become altered by the introduction of additional negative charges onto the exposed surfaces of PS2-associated proteins. This alters the balance between coulombic repulsive and van der Waals attractive forces which exist between the exposed surfaces of intrinsic proteins and between adjacent membrane surfaces. As a consequence of this phosphorylated membranes require more screening cations to maintain the same degree of thylakoid organisation.

It is well established that for the phosphorylation-induced phenomena to take place there is a requirement for conformational change (e.g. Scoufflaire et al., 1983). Direct evidence that such changes occur are seen from the electron micrographs of Fig. 4.8 and their subsequent analyses (Fig. 4.9 and Table 4.4). At "low" cation levels the changes in fluorescence due to phosphorylation correlate well with thylakoid unstacking, which is expected to occur when electrostatic screening is sub-optimal. At 5mM Mg^{2+} the relationship between fluorescence and stacking is less clear. However, a small change in the degree of stacking can be quite significant in terms of LHC displacement from appressed to non-appressed lamellae. According to Table 4.4 there is a 50nm decrease in granal width after phosphorylation at 5mM Mg^{2+}. If it is assumed that grana are circular and there is no change in the number of grana per chloroplast, such a change represents a 20% decrease in appressed area. Viewed in this way, this change in area is consistent with the observed fluorescence decrease. The histograms of Fig. 4.9 and the data in Table 4.4 suggest that phosphorylation leads to an "unzipping"

of the appressed regions consistent with the movement of proteins into the appressed lamellae. Such protein movements have been observed using freeze-fracture microscopy (Staehelin et al., 1982; Simpson, 1983), radioactive labelling followed by thylakoid fragmentation studies (Larsson et al., 1984) and pulse-chase experiments (Kyle et al., 1984).

Indirect evidence of alterations in membrane organisation as a result of phosphorylation arise from the change in the kinetics of Q_A photoreduction (Fig. 4.3), the parameter β_{max} (Table 4.1) and a decrease in k_α (not shown). All of these observations would suggest that PS2 organisation is being altered by protein-phosphorylation so PS2 centres become less "connected" and their absorption cross section diminished.

It is concluded, therefore, that protein-phosphorylation at "high" salt levels leads to a decrease in PS2 absorption cross section probably as a result of the movement of a "mobile" pool of phosphorylated LHC from the appressed to the non-appressed regions of the thylakoid membrane (see Kyle et al., 1983b). Such a conclusion has been made by other workers including Horton and Black (1981a and 1983), Tsala and Strasser (1984) and Gerola et al. (1984). As the background cation level decreases then protein-phosphorylation leads to a gradual unstacking and randomisation of protein-complexes which facilitates PS2-PS1 interaction and increases spillover. The results presented also suggest that the cation- and phosphorylation-induced changes are independent processes which are interrelated because they both depend upon the negative surface charge density of the thylakoid membrane. The different nature of these two processes has also been shown by Tsala and Strasser (1984) from the analysis of 77K chlorophyll fluorescence transients measured at 735nm and 685nm.

CHAPTER 5

The Effect of Protein-Phosphorylation on Other Photosynthetic Processes

Protein-phosphorylation can alter the distribution of excitation energy between PS2 and PS1, as discussed in Chapter 4. Such a change might also be expected to alter the kinetics of the redox reactions of the electron transfer components operating between the two photosystems. Horton and Black (1981b) have previously demonstrated that the re-reduction of photooxidised cyt f was impaired after phosphorylation. They interpreted their results as indicating a decreased PS2 absorption cross section without altering that of PS1. This is inconsistent with the reports of increased PS1 activity under limiting light conditions after phosphorylation (Farchaus et al., 1982; Telfer et al., 1984). An explanation could be that as Horton and Black used light 1 to investigate cyt f photooxidation after phosphorylation this may not have excited the phosphorylated 'mobile' LHC thereby leading to no observable change in PS1 antenna size.

Other changes in photosynthetic processes due to protein phosphorylation have also been reported (see Introduction, 1.7), including an increased affinity for DCMU binding (Shochat et al., 1982) and a decreased rate of PS2 electron transfer reactions which have recently been shown to occur even at saturating light intensities (Horton et al., 1984). This decrease in electron transfer rate would not be expected to occur at high light intensities if phosphorylation only altered photosystem absorption cross section and therefore it has been discussed as being a result of an increased dissipative pathway of electrons around PS2 (see Horton and Lee, 1983; Horton et al., 1984).

This chapter investigates (1) the effect of protein phosphorylation on a light-induced absorption decrease at 560-540nm, in coupled chloroplasts, attributed to cyt b_{559LP} and an absorption decrease at 560-540nm, in the presence of Ant 2p (an ADRY reagent), attributed to cyt b_{559HP} and (2) phosphorylation-induced changes in PS2 electron transfer reactions and their inhibition by DCMU.

5.1 The Effect of Protein-Phosphorylation on Cytochrome b_{559} Photooxidation:

Fig. 5.1 shows the effect of ambient redox potentials on the light-induced absorption decrease at 560-540nm in coupled chloroplasts. In the presence of hydroquinone and ascorbate a fast photooxidation was observed which was quickly reversed in the dark (Fig. 5.1a). Under more reducing conditions, as provided by ferredoxin and NADPH, a slow absorption decrease ($t_{\frac{1}{2}} \sim 10s$) was also detected (Fig. 5.1b). The components involved in this absorption change were identified from the light-induced absorption spectra shown in Fig. 5.2. By measuring the absorption change during the first 2s of illumination and the total change minus the 2s component as a function of wavelength it became apparent that the fast absorption decrease was due to cyt f photooxidation (λ_{max}, 554nm), whereas the slow component appeared to reflect the photooxidation of approximately 1 cyt b_{559}/600chl (λ_{max}, 559nm). From the differential effect of hydroquinone/ascorbate and Fd/NADPH, it was assumed that the slow component arose from the oxidation of cyt b_{559} low potential (E_m = +20mV) and not from the high potential from (E_m = +350mV) (see review by Cramer and Whitmarsh, 1977). This conclusion was supported by the oxidation-reduction difference spectra shown in Fig. 5.3. The difference in the absorption at 560nm between Fig. 5.3a and Fig. 5.3b indicated that the hydroquinone-reducible cyt b_{559HP} was

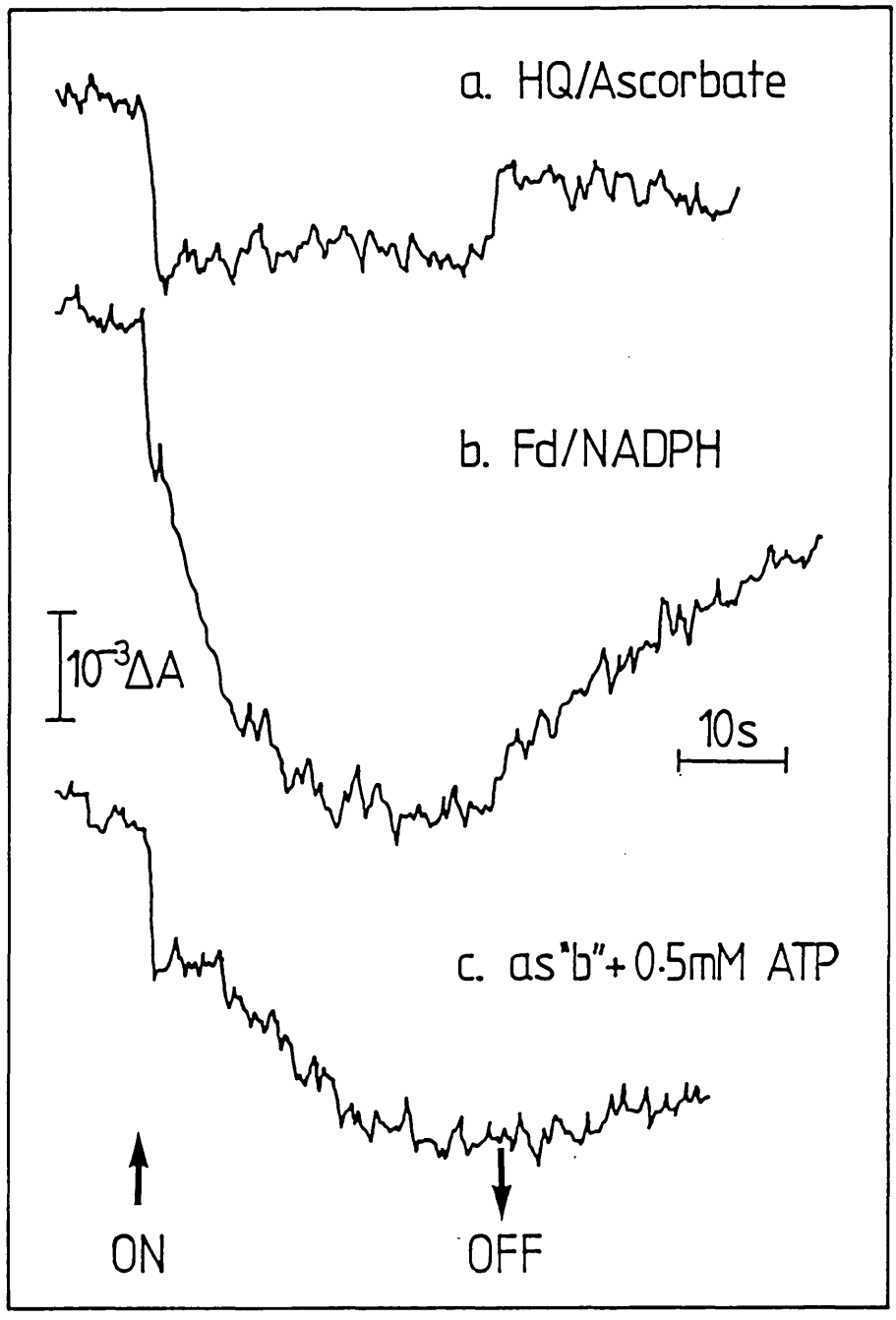


Fig. 5.1 The light-induced absorption changes at 560-540nM in coupled chloroplasts (70μg chl/ml) incubated in the dark for 30 minutes a) in the presence of 1mM hydroquinone (HQ) and 1mM ascorbate, b) in the presence of 7.2 μM ferredoxin (Fd) and 0.5mM NADPH, c) as (b) except under ATP-dependent protein-phosphorylation conditions. Actinic light was from a 695nm cut-off filter (intensity 55μWm^{-2}).

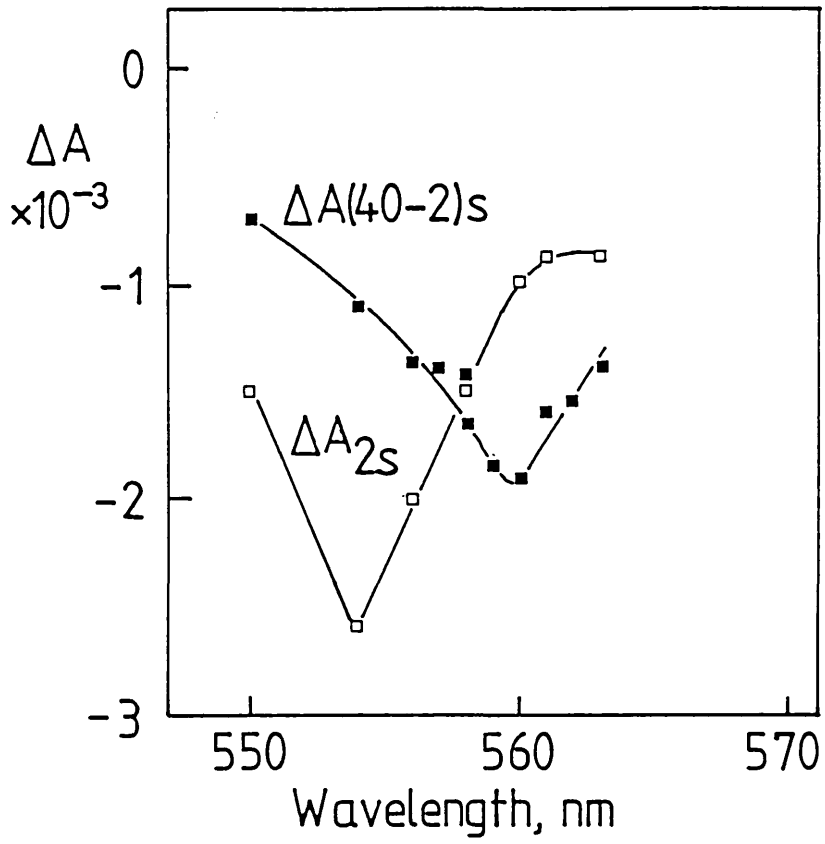


Fig. 5.2 The light-induced absorption spectra of the initial (ΔA_{2s}) and slow ($\Delta A_{(40-2s)}$ components. Conditions were as in Fig. 5.1b.

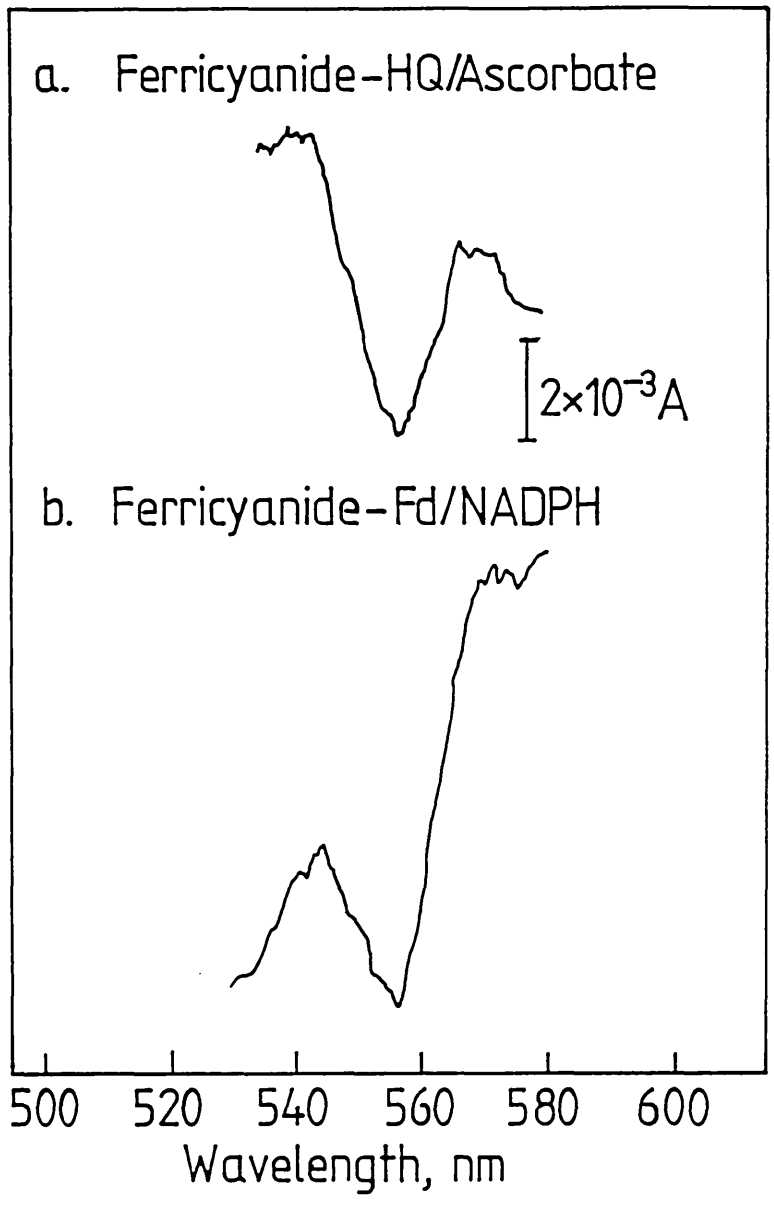


Fig. 5.3 The oxidation-reduction difference spectra of coupled thylakoids (104µg chl/ml) incubated in the dark for 30 minutes under the following conditions: a) 2mM Fe $(CN)_6^{-3}$ minus 2mM hydroquinone/1mM ascorbate and b) 2mM $Fe(CN)_6^{-3}$ minus 7.2µM ferredoxin/0.5mM NADPH.

present at an approximately 2-fold higher concentration than the cyt b_{559LP}, observed by the additional absorption change in the Fd/NADPH samples. From Fig. 5.3a the 560nm absorption was $3.5x10^{-3}$ equivalent to 2.3 cyt b_{559}/600 chl whereas from Fig. 5.3b the absorption at 560nm was $5.0x10^{-3}$ equivalent to 3.3 cyt b_{559}/600 chl (assuming an extinction coefficient of $10mM^{-1}cm^{-1}$).

Somewhat surprisingly, it was found that the slow absorption change attributed to cyt $b_{559\ LP}$ was inhibited by DCMU, DBMIB, methyl viologen (MV), ammonium chloride and minus $MgCl_2$ (Figs. 5.4 a-e). From the oxidation-reduction difference spectra (not shown) the inhibition of the photooxidation at minus $MgCl_2$ was seen not to be due to an ineffective reduction of the cyt b_{559LP} by Fd/NADPH. These results would suggest that the detection of the cyt b_{559LP} oxidation was, in some way, sensitive to electron flux between PS2 and PS1, as well as to the poising of the ambient redox potential. Further support for the contention that the observed slow absorption change was not due to cyt b_{559HP} photooxidation can be seen from Fig. 5.5 which shows the photooxidation of cyt b_{559HP} seen when thylakoids are treated with 4.5μM Ant 2p (see Packham and Barber, 1984), as this absorption change was relatively insensitive to the background Mg^{2+} level.

In thylakoids treated with 500μM ATP, under conditions which brought about a 20% decrease in room-temperature chlorophyll fluorescence (indicative of protein-phosphorylation), the cyt b_{559LP} photooxidation was partially inhibited both in kinetics and amplitude (Fig. 5.1c). Moreover, the dark re-reduction of the oxidised cytochrome became slower. The effect of light 1 (λ709nm) at a low light intensity on cyt b_{559LP} photooxidation before and after phosphorylation is shown in Fig. 5.6.

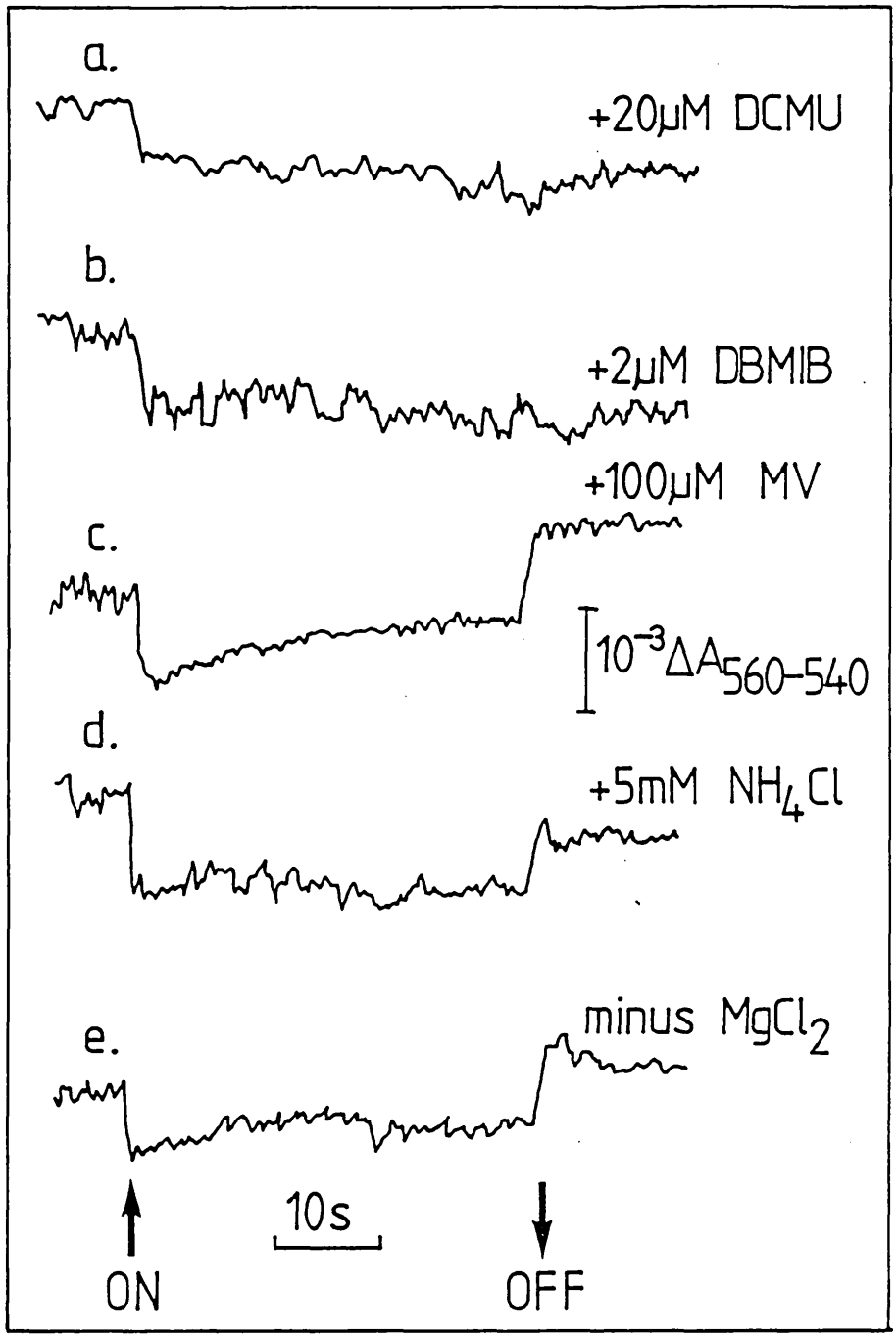


Fig. 5.4 The light-induced absorption change at 560-540nm in the presence of; a) 20μM DCMU; b) 2μM DBMIB; c) 100μM MV; d) 5mM NH_4Cl; e) minus $MgCl_2$. All other conditions were as for Fig. 5.1b.

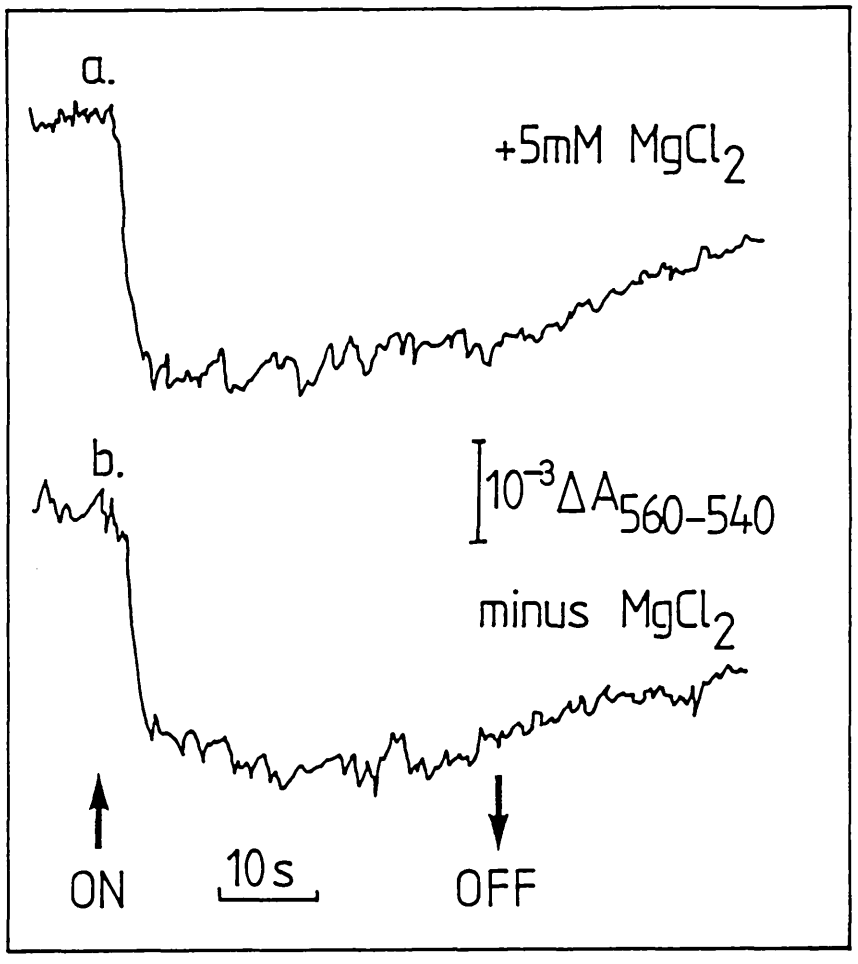


Fig. 5.5 The light-induced absorption changes at 560-540nm in thylakoids treated with 4.5μM Ant 2p and incubated a) in the presence of 5mM $MgCl_2$, and b) in the absence of $MgCl_2$. The signal represents cyt b_{559}HP photooxidation. Other conditions were as for Fig. 5.1b.

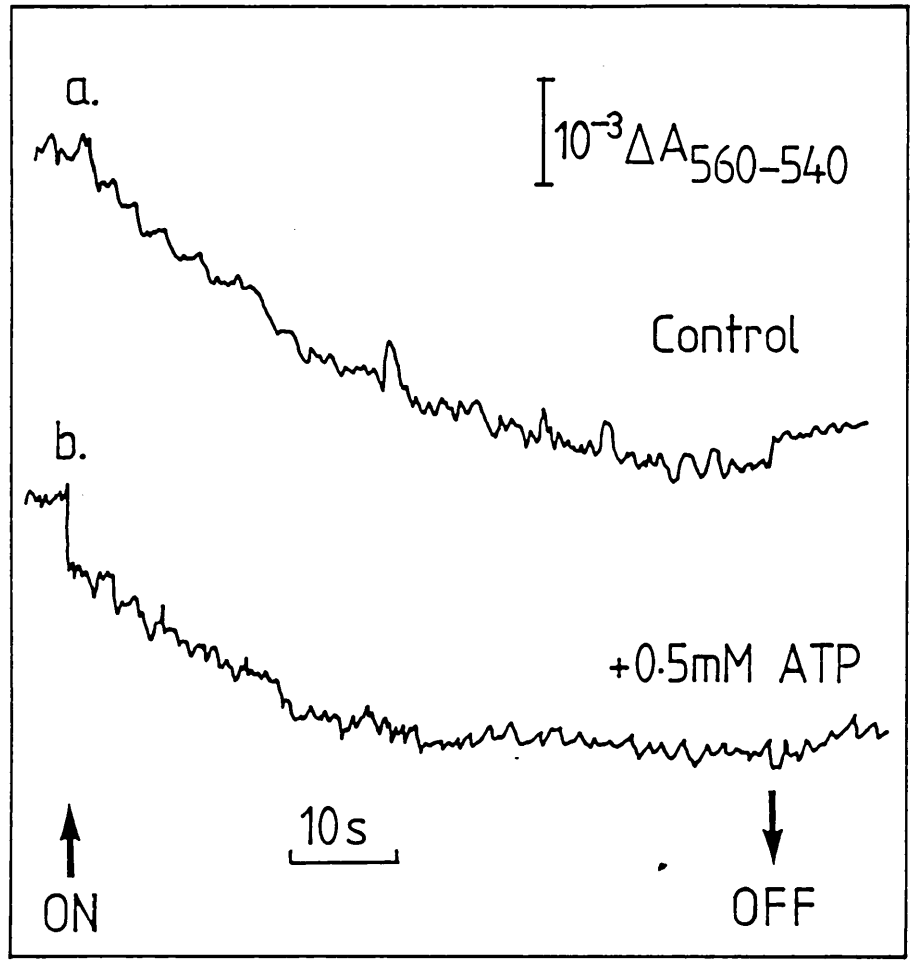


Fig. 5.6 The effect of protein-phosphorylation on the absorption change at 560-540nm, using far-red actinic light. Conditions were as for Fig. 5.1b, except actinic light was generated using a 709nm interference filter (intensity $25 Wm^{-2}$).

It can be seen that protein-phosphorylation caused an inhibition in the extent of the signal similar to that observed with broad band red light (Fig. 5.1c) and the cyt f oxidation became more clearly detected. This indicates that light below 700nm transmitted by the 709nm interference filter may have become redirected from PS2 to PS1 as a consequence of phosphorylation or that less light was going to PS2.

Fig. 5.7 shows the effect of phosphorylation on the photooxidation of cyt b_{559HP}, detected in the presence of Ant 2p and DCMU. Fig. 5.7a shows that in the presence of DCMU no slow photooxidation was observed (see also Fig. 5.4a), but on the addition of Ant 2p a photooxidation became apparent (Fig. 5.7b). Protein-phosphorylation effected the cyt b_{559HP} photooxidation so that the extent remained the same as in the non-phosphorylated thylakoids but the kinetics were made faster.

5.2 The Effect of Protein-Phosphorylation on PS2 Electron Transfer Rate:

To investigate if protein-phosphorylation brought about an inhibited rate of PS2 electron transfer, as would be expected to happen at low excitation intensities by a decrease in PS2 absorption cross section, PS2 rates of electron transfer from H_2O to DCPIP were measured as a function of light intensity using phosphorylated and non-phosphorylated thylakoids (Figs. 5.8a and 5.8b). It can be seen that at all the light intensities investigated, except perhaps at very low intensities below 5% maximum, phosphorylation led to a decrease in PS2 electron transfer rate to DCPIP. The observed inhibition at saturating light, as a consequence of phosphorylation, is not expected and cannot be explained by the proposed change in PS2 antenna size. To see if the extent of PS2 inhibition at saturating light was related to the degree of protein-phosphorylation,

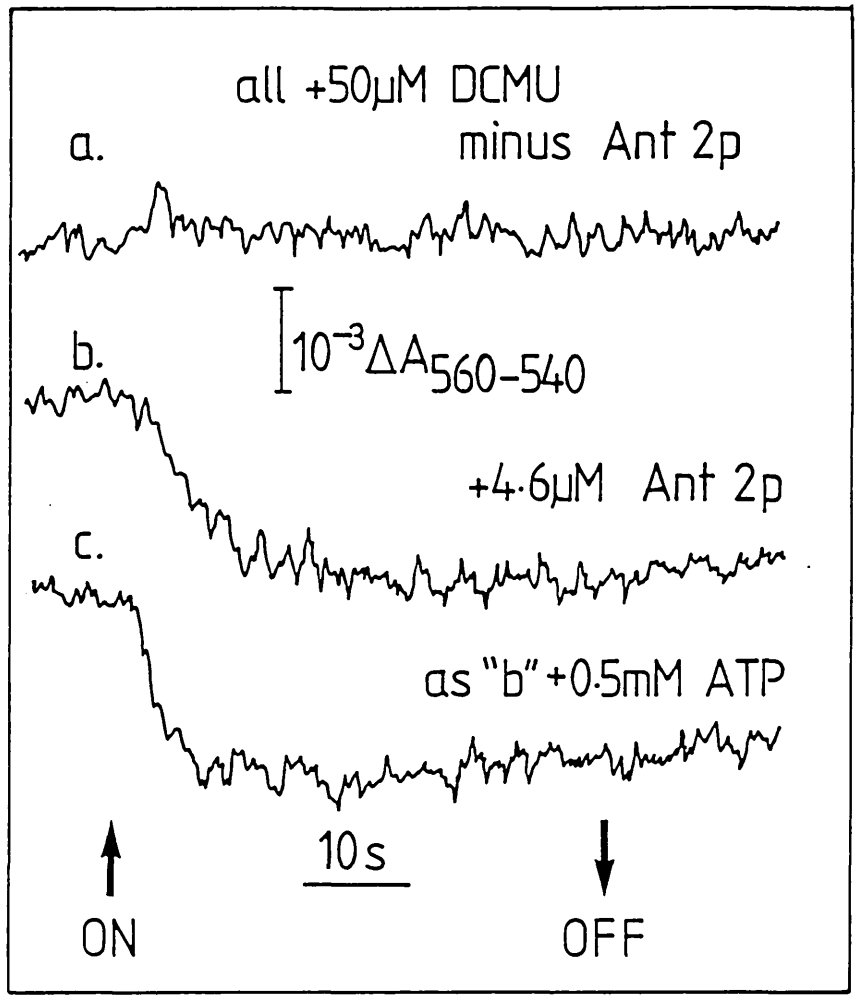


Fig. 5.7 The light-induced absorption change at 560-540nm exhibited by thylakoids inhibited with 50μM DCMU and suspended in a) the absence of Ant 2p; b) the presence of 4.6μM Ant 2p and c) as in b) except under ATP-dependent protein phosphorylation conditions. All other conditions were as in Fig. 5.1b.

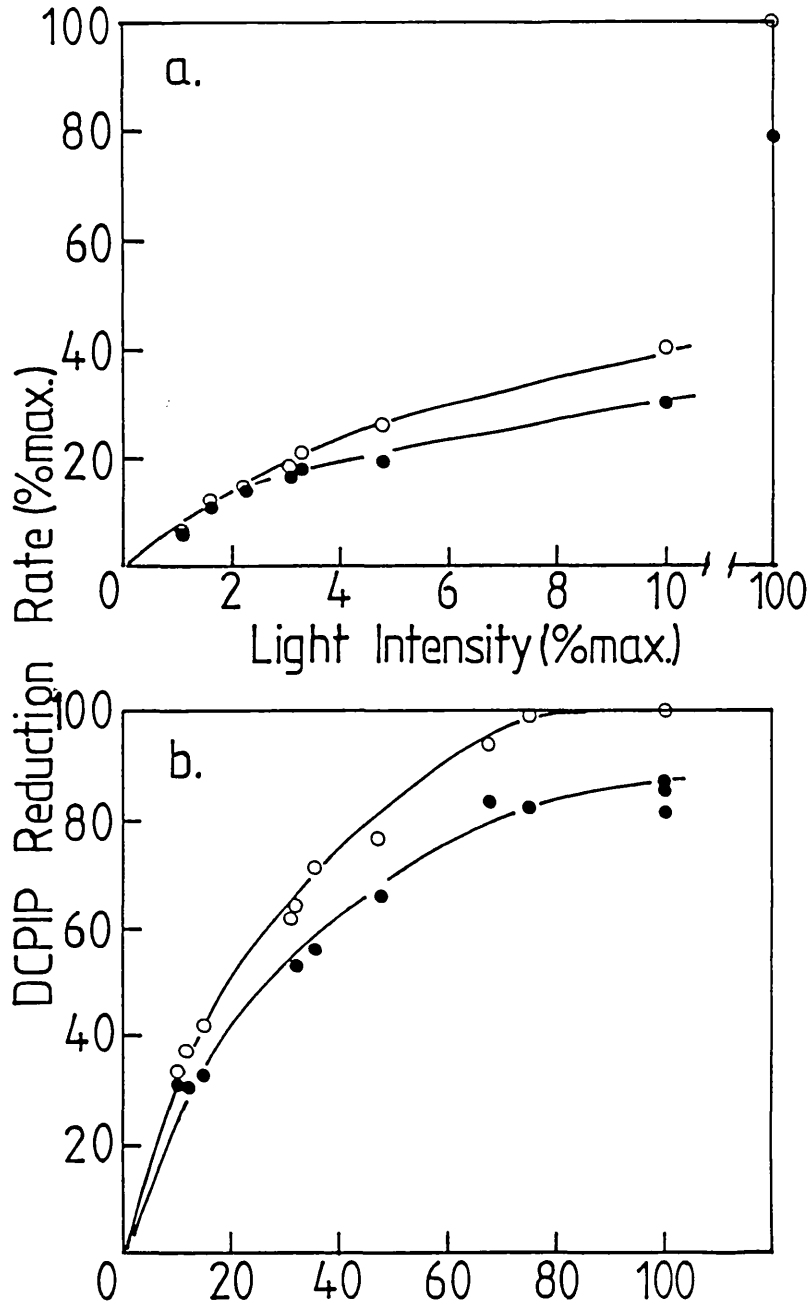


Fig. 5.8 The effect of protein-phosphorylation on the PS2 electron transfer rate, measured using DCPIP as the electron acceptor and H_2O as the electron donor, at a) low light intensities (0-10% max) and b) high light intensities (0-100% max). The 100% light intensity used was $55 Wm^{-2}$.

thylakoids were phosphorylated to differing extents (as measured by the change in room-temperature chlorophyll fluorescence in DCMU-treated thylakoids). Fig. 5.9 shows the relationship between the percentage change in fluorescence and the percentage inhibition of H_2O to DCPIP electron transfer rates, which indicates that the PS2 inhibition is indeed dependent on the degree of protein-phosphorylation.

By using electron donors and acceptors of PS2 the site of the inhibition was investigated. It was seen that the addition of DPC, a donor to Z^+, could not remove the phosphorylation-induced inhibition (data not shown). However, the different electron transfer rates exhibited by phosphorylated and non-phosphorylated membranes could be eliminated by using the PS2 acceptor silico molybdate, which can accept electrons directly from Q_A in a DCMU insensitive reaction (see Zilinskas and Govindjee, 1975). The non-phosphorylated thylakoids gave H_2O to SiMo rates of 36μmoles O_2/mg chl/hr while the phosphorylated samples produced 37.5μmoles O_2/mg chl/hr.

5.3 The Effect of Protein Phosphorylation on the Acceptor Side of PS2:

It has been shown that protein-dephosphorylation leads to a decrease in DCMU-binding affinity and a reduction in the number of DCMU-binding sites (Shochat et al., 1982). Recently, Vermaas et al. (1984) have reported that light-induced phosphorylation decreases the I_{50} (concentration for 50% inhibition) for the Hill reaction, H_2O to DCPIP. A titration of H_2O to DCPIP electron transfer rate with DCMU is shown in Fig. 5.10 for both phosphorylated and non-phosphorylated thylakoids. It can be seen, by comparing Fig. 5.10a with Fig. 5.10b, that chemically-induced phosphorylation appeared to have no effect on the ability of DCMU to inhibit PS2 electron flow (this was also seen for atrazine, data not

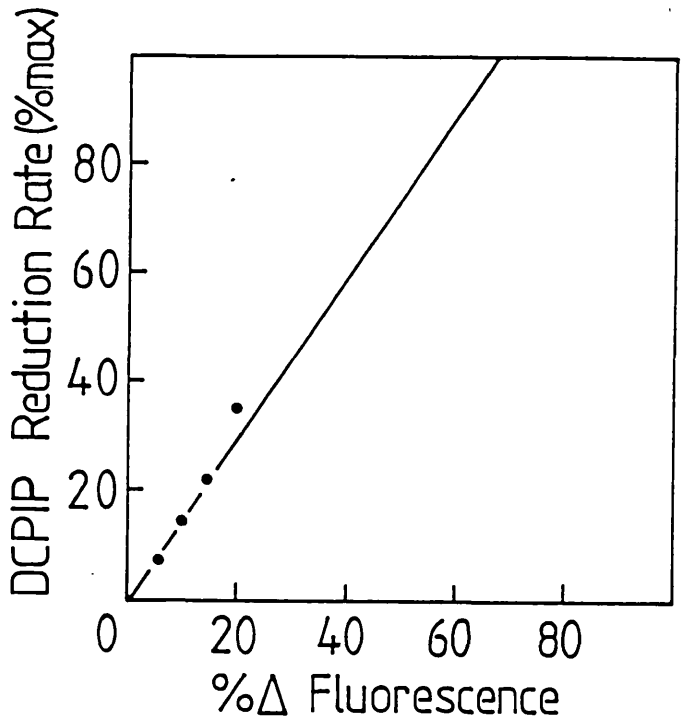


Fig.5.9 The relationship between the percentage change in maximal chlorophyll fluorescence due to phosphorylation and the percentage change in H_2O to DCPIP electron transfer rate at saturating light conditions. The different degrees of protein-phosphorylation were achieved by varying the Fd/NADPH levels present and the reaction time.

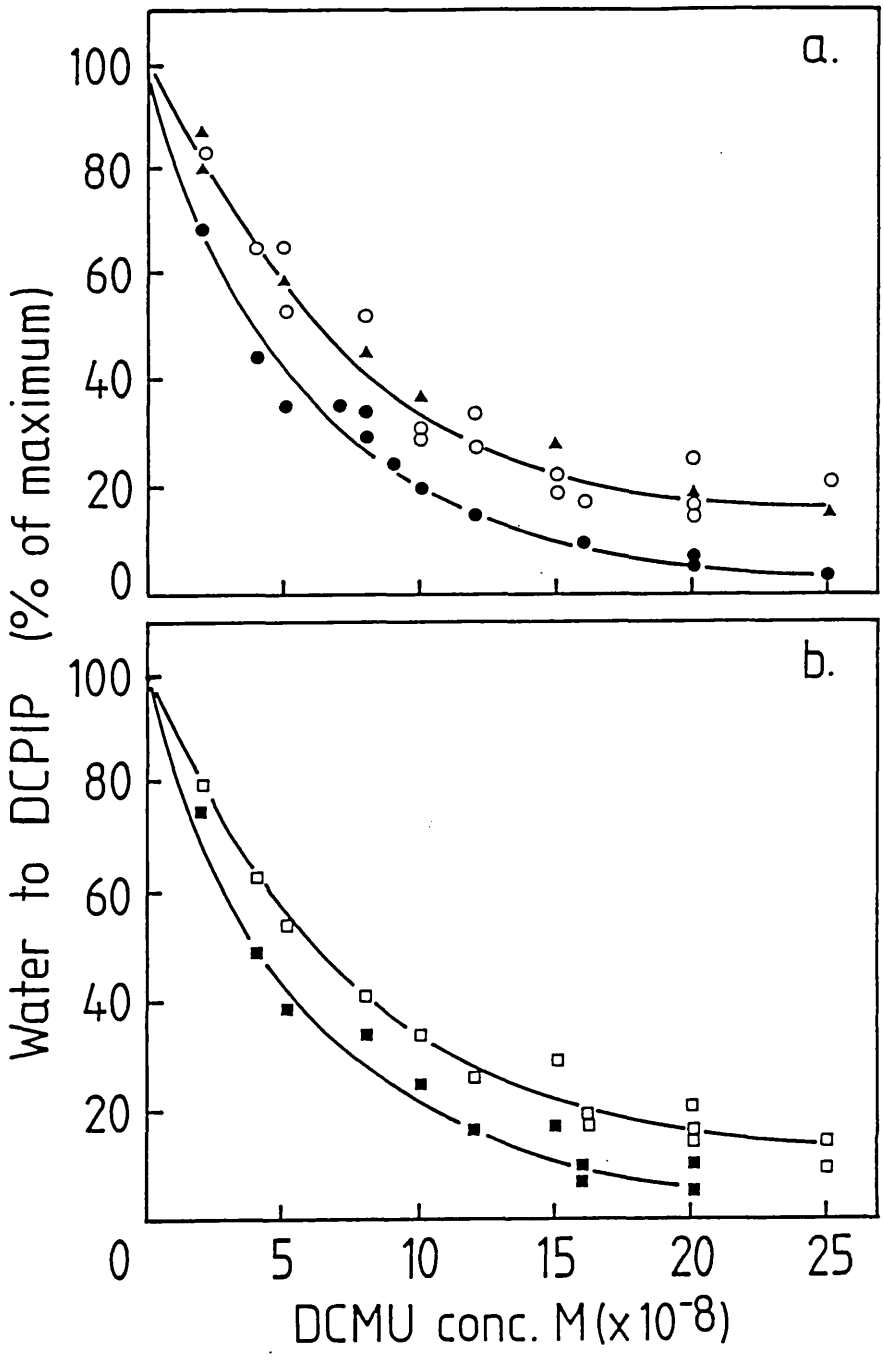


Fig. 5.10 The effect of DCMU concentration on the inhibition of PS2 electron transfer from H_2O to DCPIP in a) non-phosphorylated thylakoids suspended in the absence of excess $NADP^+$ (●), the presence of $NADP^+$ (○) and dark-adapted in the absence of Fd/NADPH (▲) and b) phosphorylated thylakoids suspended in the absence (■) and presence (□) of excess $NADP^+$. Protein-phosphorylation was carried out using the Fd/NADPH method. The average control rates (zero DCMU) were as follows; non-phosphorylated, 228μmoles DCPIP/mg chl/hr and phosphorylated, 186μmoles DCPIP/mg chl/hr.

shown). Furthermore, light-induced phosphorylation also caused no change in the I_{50} values (data not shown) and therefore the difference in observations between Fig. 5.10 and the above mentioned reports do not reflect different methods in bringing about phosphorylation. Fig. 5.10, however, shows that the I_{50} value could be altered by changing the redox level of the PQ pool, as brought about by the addition of excess $NADP^+$ or a dark pretreatment under non-reducing conditions. This will be discussed later in terms of the results of Vermaas et al. (1984).

If phosphorylation affects the 32kda herbicide-binding protein (Q_B) it might be expected that differences in the uninhibited fast fluorescence transients exhibited by phosphorylated and non-phosphorylated thylakoids may be apparent. Fig. 5.11 shows normalised fluorescence curves for phosphorylated and non-phosphorylated membranes while Table 5.1 gives various unnormalised measurable fluorescence parameters. It was seen that the i-level of the fluorescence transient could be increased in the phosphorylated membranes from 43% total F_v, in the control, to 50% total F_v, while the A_{max} decreased. It can also be seen from Table 5.1 that, even in the absence of DCMU phosphorylation led to a decrease in F_o and F_m so F_v/F_m remained unchanged, indicating that in both cases all of the quinones (Q_A and PQ) were equally reduced.

3.4 Discussion:

There has been much confusion in the literature, over the past 10 years or so, concerning cyt b_{559} (see reviews by Cramer and Horton, 1975; Cramer, 1977; Bendall, 1982). It is now believed that there are two distinct forms of cyt b_{559} present *in vivo* which can be distinguished through their different midpoint redox potentials (cyt b_{559LP} $E_m \sim +20mV$; cyt b_{559HP} $E_m \sim +370mV$) although it is thought that a damaged or altered

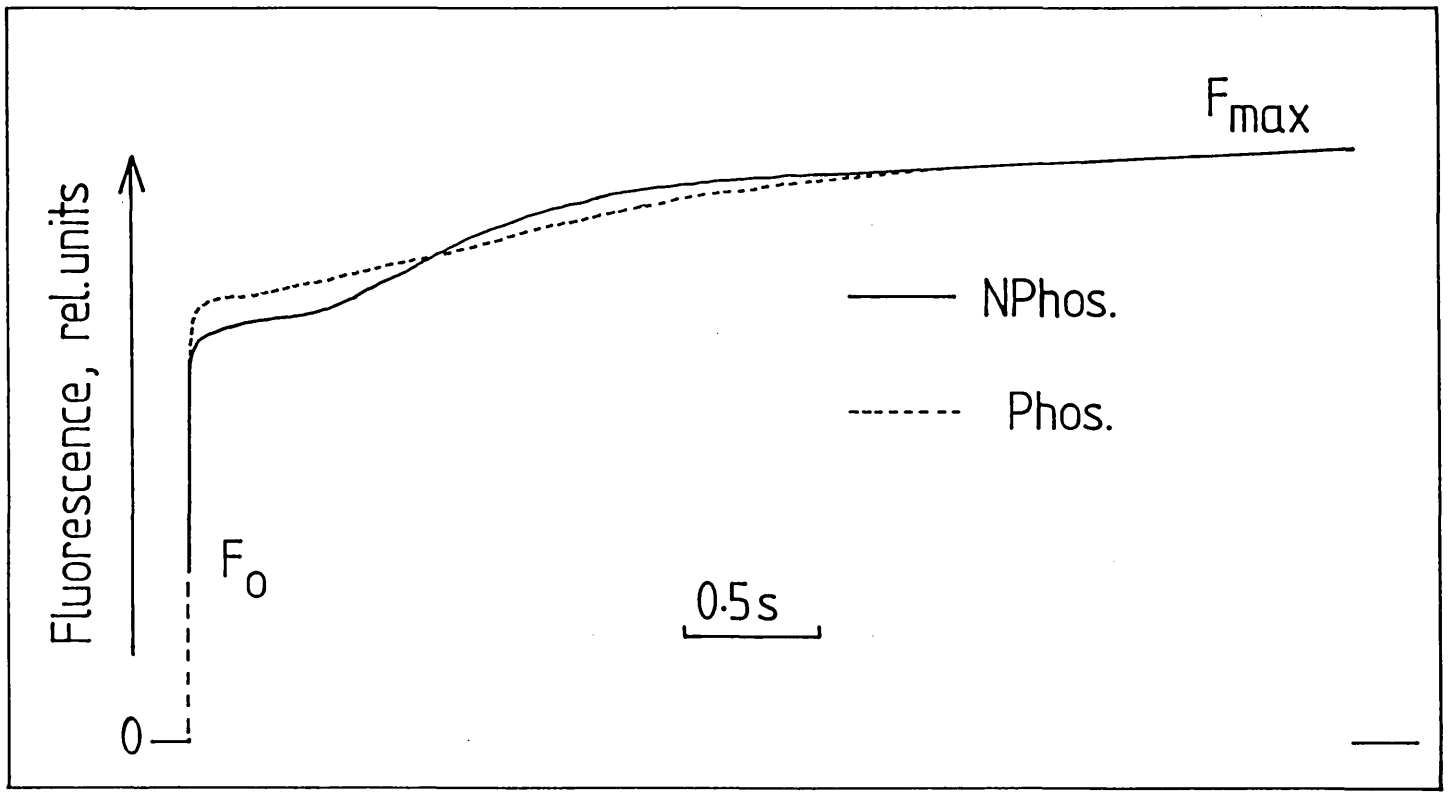


Fig. 5.11 Normalised fast chlorophyll fluorescence transients exhibited by phosphorylated (Phos) and non-phosphorylated (NPhos) thylakoids at room temperature.

TABLE 5.1

Fluorescence Parameters from Fast Fluorescence Transients Exhibited by Non-phosphorylated (NPhos) and Phosphorylated (Phos) Thylakoids

Condition	F_o	F_i	F_m	A_{max}	F_v/F_m
NPhos	21.0	52.0	75.6	4475	0.72
Phos	17.3	45.2	61.8	3800	0.72

cyt b_{559HP} can be found which has an $E_m \sim +110mV$. From fragmentation studies it has been proposed that the cyt b_{559HP} is associated with PS2 while the cyt b_{559LP} is loosely coupled to the cyt b/f complex and appears in PS1 particles (see Bendall, 1982). The exact role for these two cytochromes is still not clear and over the years it has been suggested that cyt b_{559} is associated with a) H_2O splitting processes (Bendall and Sofrova, 1971), b) cycling of electrons around PS2 (Cramer and Böhme, 1972; Mende and Weisnner, 1984), c) intersystem electron transfer (Horton and Cramer, 1975) and d) cyclic electron flow around PS1 (Peters et al., 1984).

The data presented in this chapter suggest that a slow light-dependent absorption change at 560-540nm can be attributed to the photooxidation of cyt b_{559LP}. In addition to this, a fast decrease was observed which was apparently due to cyt f oxidation. The slow signal was very sensitive to experimental conditions and only appeared in coupled thylakoids possessing low rates of net electron flow (as indicated from the fluorescence signal for Fd/NADPH treated thylakoids shown in Fig. 6.4). The absorption change was inhibited by DBMIB indicating that PS1 does not directly oxidise cyt b_{559LP} in a manner similar to the oxidation of cyt f, which is resistant to the quinone's action (Fig. 5.4b). The effect of DCMU on the signal, however, shows that the reaction appears to be dependent on PS2-mediated electron transfer. The sensitivity of the cyt b_{559LP} oxidation to electron flow rate is unclear but one possible explanation is that it is determined by the balance of oxidation and rereduction kinetics. An increased electron flow supported by either uncouplers or MV might attenuate the oxidation by selectively increasing the reduction rate or decreasing the oxidation rate. An increase in reduction rate maybe reflected in the decreased

cyt f contribution seen under these conditions when compared with Fig. 5.1b. The MV effect (Fig. 5.4) also seems to indicate that the reduction of the photooxidised cyt b_{559LP} is not mediated directly by the reducing side of PS1 in cyclic electron flow but may indicate that this process is required for cyt b_{559LP} oxidation.

In order to explain these observations it may be necessary to propose that cyt b_{559LP} is involved in a Q-cycle type electron transfer (see Mitchell, 1976). The cytochrome can be envisaged as receiving electrons from quinol-oxidation by the Rieske iron-sulphur centre, possibly via cyt b_{563}, and donating them to an anionic semiquinone which is perhaps generated by Fd-mediated cyclic electron flow around PS1 (see Fig. 5.12). Under conditions where electron flow through both photosystems is slow net photooxidation of cyt b_{559LP} might be expected to occur. When electron flow is stimulated, the Q-cycle turnover might either be increased and cyt b_{559LP} kept in a reduced state or perhaps switched off. The latter case might be more plausible as cyt b_{563} reduction has been shown to rely on the cyt f oxidation kinetics (see Bendall, 1982). This proposal of cyt b_{559LP} being involved in a Q-cycle mechanism can explain the inhibitory action of DBMIB since it will inhibit the formation of the oxidant for cyt b_{559LP} while the inhibition by DCMU is not so easily explained.

A role for cyt b_{559LP} in a Q-cycle can also account for the effect of cations and phosphorylation on the extent of the photooxidation. It has been argued that PS1 activity is increased at the expense of PS2 as a result of these two different treatments (eg. Ben-Hayyim and Avron, 1971; Farchaus et al., 1982; Krause and Behrend, 1983). Changes in the balance of PS1 and PS2 activity appear to be reflected in the extent of

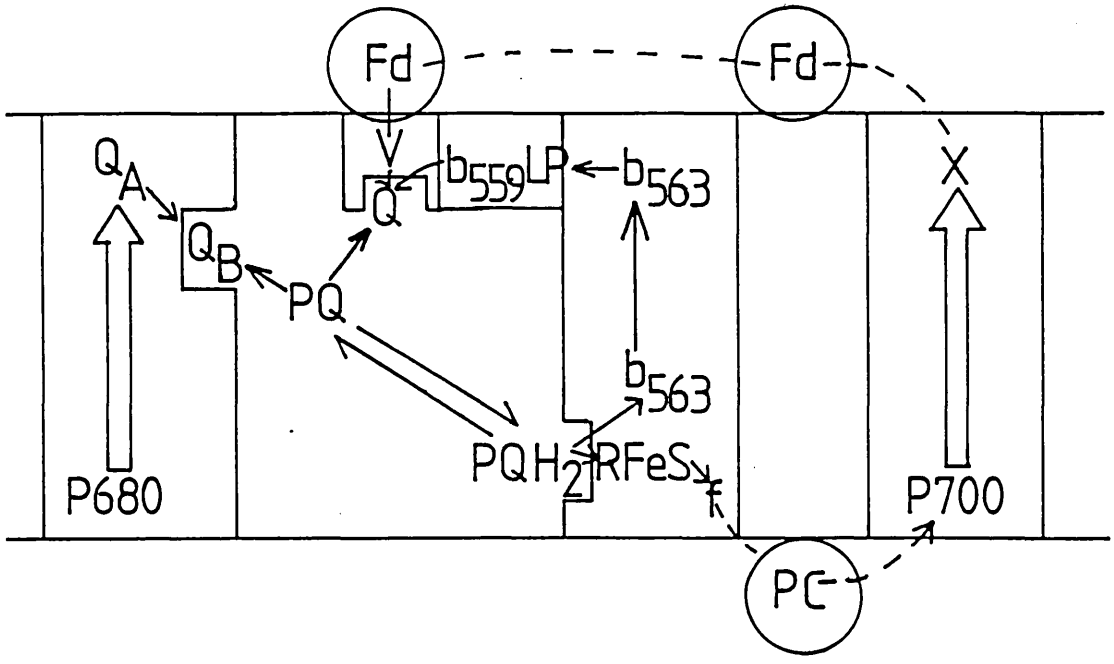


Fig. 5.12 A modified Q-cycle type scheme proposed to account for the role of cyt b_{559}LP in intersystem electron transfer reactions. The cytochrome receives electrons from cyt b_{563} and donates them to a bound anionic semiquinone generated by Fd-mediated cyclic electron flow around PS1. Symbols as in Fig. 1.1.

cyt b_{559LP} photooxidation (Figs. 5.1 and 5.6) so that an increase in PS1 activity relative to PS2 inhibits the photooxidation perhaps because the Q-cycle is inhibited.

An inhibition in PS2 electron transfer from H_2O to DCPIP, as a result of protein-phosphorylation, can be seen in Fig. 5.8. Such a decrease would be expected at limiting light intensities (Fig. 5.8a) because of the proposed decrease in PS2 antenna size (see Chapter 4) but not at saturating light intensities (Fig. 5.8b), where a 20% inhibition is still observed. This inhibition at high-light intensities was not removed by the addition of DPC, which donates electrons to Z^+, and therefore suggests that a phosphorylation-induced alteration of the oxygen evolving processes is unlikely. The extent of the inhibition appears to be related to the degree of phosphorylation, as determined by the attenuation in fluorescence exhibited by DCMU-treated thylakoids (Fig. 5.9). Horton and Lee (1983) have postulated that phosphorylation stimulates a dissipative cycling of electrons around PS2. This was concluded from the proportionally larger stimulation of fluorescence on hydroxylamine addition by the phosphorylated thylakoids. As hydroxylamine is believed to inhibit a PS2 back reaction (Bennoun, 1970), the larger effect on phosphorylated membranes indicated a stimulated back transfer of electrons. However, it has been shown that hydroxylamine possibly effects the reducing-side of PS2, as it appears to modify atrazine binding (Jursinic and Stemler, 1983). It has been suggested that the dissipative pathway could explain the PS2 inhibition at saturating light intensities, although the greatest effect of hydroxylamine occurred at limiting light intensities and was greatly reduced by DCMU, when a cycling of electrons might have been expected to be more evident.

This back reaction may involve cyt b_{559HP} in a mechanism similar to that proposed for the green algae, Chlamydobotrys stellata, in which electrons are passed from Q_A to the cytochrome (5μs) followed by donation to P680 (60μs) (Mende and Wiesnner, 1983). The results presented in this chapter cannot be interpreted using such a scheme, if phosphorylation stimulates such a cycling involving cyt b_{559HP}. It is seen that electron transfer to SiMo was unaffected by phosphorylation at saturating light intensities and therefore if a dissipative cycling of electrons was more prominent in the phosphorylated thylakoids, it might be expected to compete with SiMo for electrons leading to an inhibited H_2O to SiMo rate. Furthermore, if the cycling involved cyt b_{559HP} then the observed changes in cyt b_{559HP} photooxidation (Fig. 5.7) upon phosphorylation would not be expected if such a mechanism was stimulated. Such an increase in cyclic electron flow around PS2 might be expected to inhibit or produce a slower cyt b_{559HP} photooxidation, however, the absorption change became faster with no change in its extent after phosphorylation. This observation, concerning cyt b_{559HP} photooxidation, can be simply explained by a decrease in the amount of excitation energy arriving at PS2 (see Packham and Barber, 1984).

Other proteins are known to become phosphorylated including a 32kda and a 47kda species. It has been shown that protein de-phosphorylation decreases the affinity for DCMU to bind to the Q_B-protein (Shochat et al., 1982) and that phosphorylation alters the I_{50} for herbicide inhibition of H_2O to DCPIP electron transfer rates (Vermaas et al., 1984). It has also been shown that light-induced phosphorylation leads to a stabilisation of the bound semiquinone species, Q_B^- (Jursinic and Kyle, 1983). This latter observation has been interpreted as being the consequence of either a stimulated cyclic flow of electrons around PS2 or to an inhibition of an endogenous reductant to interact with Q_B.

Fig. 5.10 shows that chemically-induced protein-phosphorylation did not lead to an enhanced ability of DCMU to inhibit PS2 electron flow to DCPIP and this was also observed for light-induced thylakoid phosphorylation (not shown), contrary to what might be expected from the results mentioned above. This probably reflects the poor non-phosphorylated control used by Vermaas et al. (1984), who compared light-induced phosphorylated thylakoids with dark-adapted non-phosphorylated membranes. From Fig. 5.10, it can be seen that such a comparison would indeed produce a difference in I_{50} values between phosphorylated and non-phosphorylated thylakoids. However, when membranes are given the same pretreatment, except for the addition of ATP, which generated the same redox conditions then no such difference is observed.

Reducing conditions are expected to fully reduce the semiquinone species, Q_B^-, thereby increasing the affinity of DCMU to bind, by removing the low affinity state, $Q_A Q_B^-$ (see Lavergne, 1982d), while oxidised PQ would be expected to compete with DCMU for the Q_B binding site. These observations may suggest that phosphorylation does not stabilise the $Q_A Q_B^-$ state but in fact stabilises the state $Q_A^- Q_B$ in the light, perhaps through altering the equilibrium between $Q_A^- Q_B \rightleftharpoons Q_A Q_B^-$ to the left in a manner similar to that proposed for atrazine-resistant plant species (see Vermaas and Arntzen, 1983).

It is interesting to note that the changes seen in Fig. 5.11 and Table 5.1 after protein-phosphorylation are similar, although smaller, to those seen when comparing atrazine resistant with atrazine-susceptible plant species (Vermaas and Arntzen, 1983 and Fig. 9.2). It is believed that herbicide resistance arises from a single amino acid change in the Q_B protein (Erickson et al., 1984). Therefore, the addition of negatively charged phosphate

groups onto the same protein or other PS2 associated proteins might alter PS2 functioning in some way so as to affect PS2 electron flow to PQ. It is seen from Fig. 5.11 and Table 5.1 that there is a larger o-i rise in the phosphorylated thylakoids, indicative of a faster initial accumulation of Q_A^-. This may suggest an inhibited flow of electrons from Q_A to Q_B, which at high light intensities might become quite significant. The decrease in A_{max} (Table 5.1) suggests that less quanta are now required to reduce all of the quinone pool in the phosphorylated membranes. This decrease in A_{max} is again similar to that observed for herbicide-resistant species (see Chapter 9).

Therefore, perhaps at low light intensities protein phosphorylation decreases PS2 reactions by a decrease in PS2 antenna-size while at higher light intensities PS2-inhibition arises because of a slower Q_A to Q_B electron transfer rate.

CHAPTER 6

PS2 Heterogeneity as Determined from Chlorophyll Fluorescence Induction Curves

Room-temperature chlorophyll fluorescence induction curves measured using DCMU-inhibited thylakoids reflect the photoreduction of Q_A and produces a curve which is not indicative of a single first order photochemical event. The biphasic nature can be readily detected, when thylakoids are suspended in "high" salt, by analysing the growth of the normalised area over the curve (Melis and Homann, 1976). This shows two kinetically different phases, one being fast and non-exponential while the other is slower and rises exponentially. These two kinetic components have been attributed to two distinct forms of PS2 termed α and β centres (Melis and Homann, 1978). Many differences have been attributed to these centres, as has already been discussed in the introduction (1.9.1). In contrast to the above interpretation it has also been suggested that the biphasic nature of the fluorescence rise reflects different levels of PS2 "connectivity" (Bowes and Horton, 1982) or different degrees of chloroplast integrity linked to alterations in DCMU affinity (Schreiber and Pfister, 1982).

This chapter investigates these concepts by measuring and analysing room-temperature chlorophyll fluorescence induction curves from DCMU-poisoned pea thylakoids with and without various pretreatments.

6.1 $PS2_\alpha$ and $PS2_\beta$

Fig. 6.1 shows that β_{max} (the y-intercept of the semi-log plot of the area growth, see 2.3.6 and Fig. 2.2) obtained from thylakoids which had undergone cation- or protein-phosphorylation-induced membrane

conformational changes could be correlated with the normalised area over the fluorescence curve (A_{norm}). This area is believed to represent the number of quanta required to reduce all Q_A and it gives an overall picture of Q_A photoreduction kinetics assuming the total number of Q_A remains constant.

Melis (1978) showed that altering the redox conditions under which the thylakoids were suspended changed the kinetics of the overall fluorescence rise so that at lower redox potentials (-80mV compared with +380mV) the slow phase was selectively eliminated. Fig. 6.2 shows that the biphasic nature of the fluorescence rise can be drastically altered by the addition of sodium dithionite, which reduces the PQ pool prior to illumination. This can be seen in Fig. 6.2a by the change in the semi-log plot of the area growth and in Fig. 6.2b which shows the alteration in the growth of the normalised area when thylakoids were incubated with and without reductant. In the presence of this strong reductant, Q_A appeared to be photoreduced more rapidly (this was also seen in the absence of Mg^{2+}, not shown) while the slow kinetic component was either removed or became faster.

Another way of analysing the fluorescence rise is by plotting F_v against the normalised area. If the rise is exponential then such a plot yields a straight line, however this is rarely the case as fluorescence is not usually a linear indicator of Q_A^- accumulation. Fig. 6.3 shows the effect of dithionite on the relationship between F_v and Area. By comparing Fig. 6.3a with Fig. 6.3b it can be seen that dithionite altered the plot especially at the tail end of the rise where a non-linear deviation was changed to a simple linear relationship.

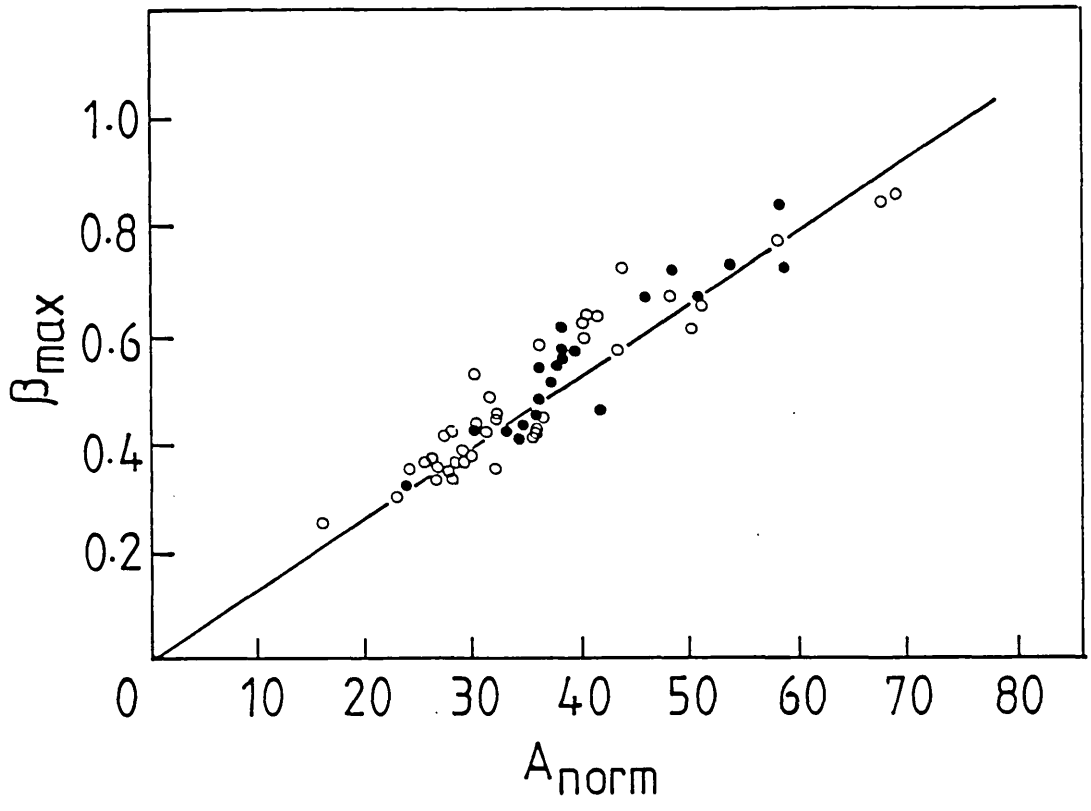


Fig. 6.1 The correlation of the slow phase (β_{max}) with the normalised area over the fluorescence induction curve (A_{norm}). The data were obtained by either cation-induced (o) or protein-phosphorylation induced (●) conformational changes. The line is the least squares fit to the experimental points.

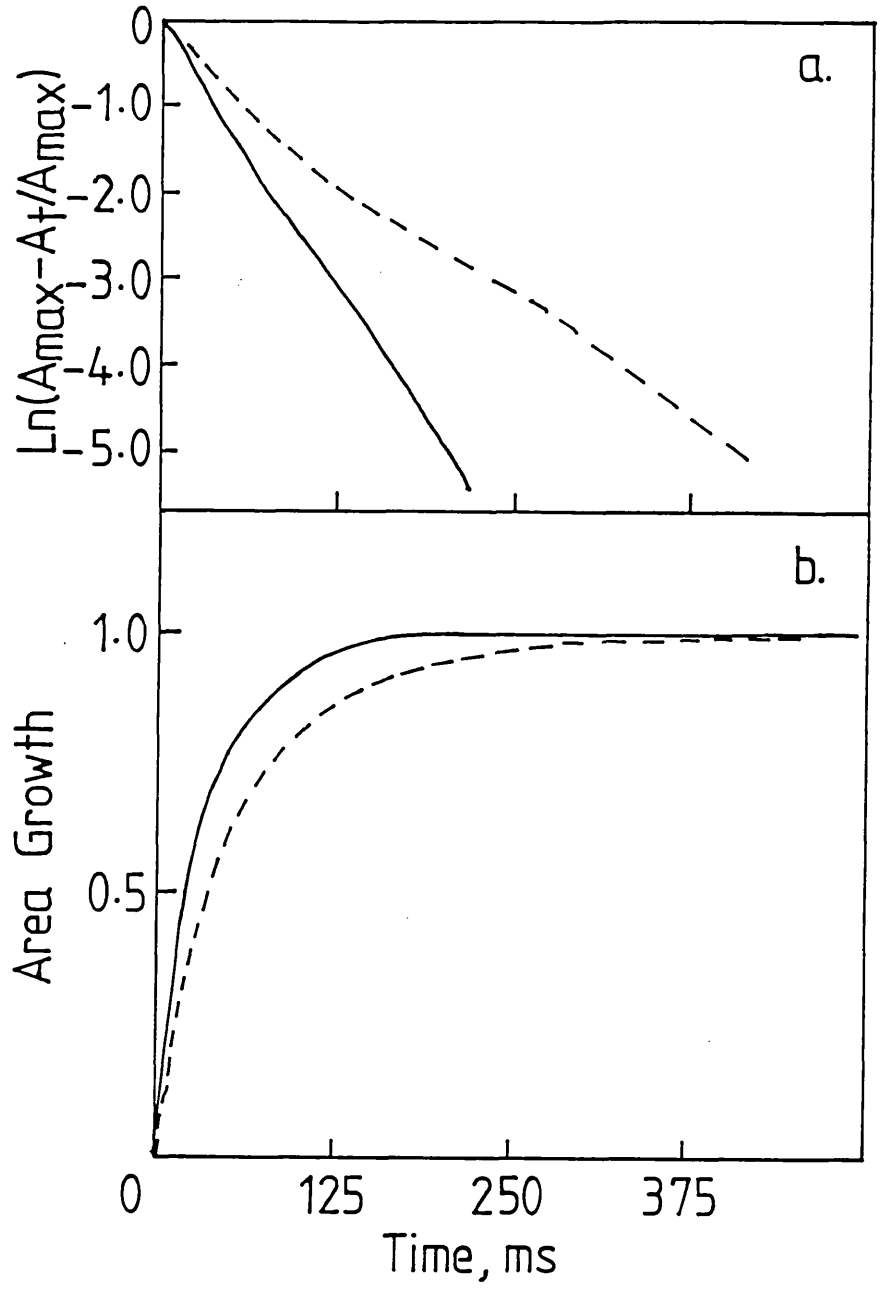


Fig. 6.2 The effect of the presence (——) and absence (— —) of sodium dithionite on; a) the semi-log plot, Ln (A_{max}-$A_{(t)}/A_{max}$) and b) the growth of the normalised area over the room-temperature fluorescence induction curve (indicative of Q_A^- accumulation) exhibited by DCMU-inhibited thylakoids.

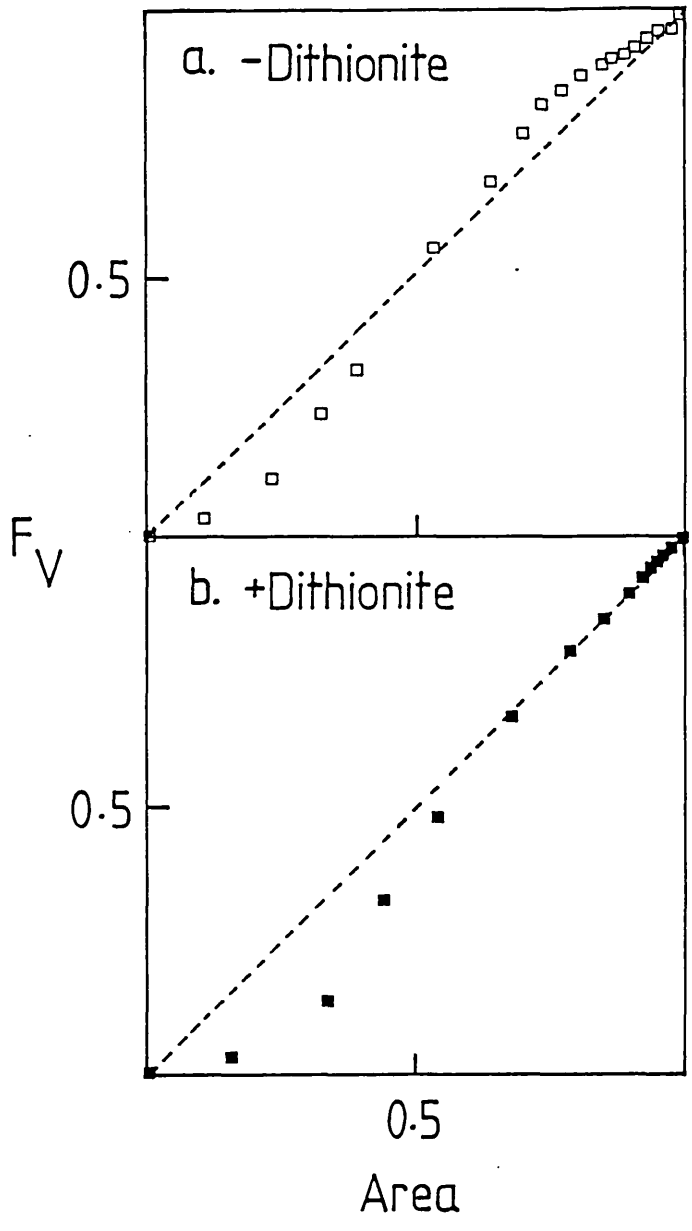


Fig. 6.3 Fraction of closed PS2 centres (Area) versus the F_v for DCMU-inhibited thylakoids suspended in (a) the absence (□) and b) the presence (■) of dithionite. The theoretical prediction for zero PS2 connectivity is given by the dotted line.

Table 6.1 indicates that various induction curve parameters are changed by having the PQ pool reduced prior to illumination. Under these reducing conditions there was an increase in F_o and F_m so F_v/F_m remained relatively unaltered. However, A_{max} became smaller after PQ reduction, indicating less quanta were now required to photoreduce Q_A perhaps reflecting the removal of electron acceptors associated with $PS2_\beta$, as the slow phase giving rise to β_{max} was removed.

The ability of various reagents to alter photosynthetic electron transfer reactions is evident from the fluorescence signals shown in Fig. 6.4. It can be seen that in the presence of reductants, like Fd/NADPH, or PS2 inhibitors, like DCMU, electron flow away from PS2 was inhibited as there was a fast rise to the maximum fluorescence level. However, the F_m level was higher in the presence of Fd/NADPH probably because of the removal of some fluorescence quenching by oxidised PQ (see Vernotte et al., 1979). In the absence of reductant or DCMU, the fluorescence rose slowly due to competition between PS2-induced reduction of Q_A and its reoxidation by PS1-mediated electron flow. This conclusion is supported by the observation that addition of MV led to an even slower fluorescence rise presumably due to a stimulated PS1 turnover. The addition of FeCy to thylakoids has also been shown to have a similar effect on fluorescence as MV, when no DCMU is present (see Melis and Anderson, 1983).

To see if the slow, fluorescence rise exhibited by DCMU-inhibited thylakoids could be due to a leak of electrons through the DCMU block the effect of various electron acceptors and inhibitors of electron transfer on the fluorescence signal was investigated. Fig. 6.5A compares the semi-log plots and area growth curves for thylakoids suspended

TABLE 6.1

The Effect of PQ Reduction on Induction Curve Fluorescence Parameters

Condition	F_o	F_m	A_{max}	F_v/F_m	β phase
+ dithionite	33	98.4	1795	0.67	absent
- dithionite	27	84.4	2750	0.68	present

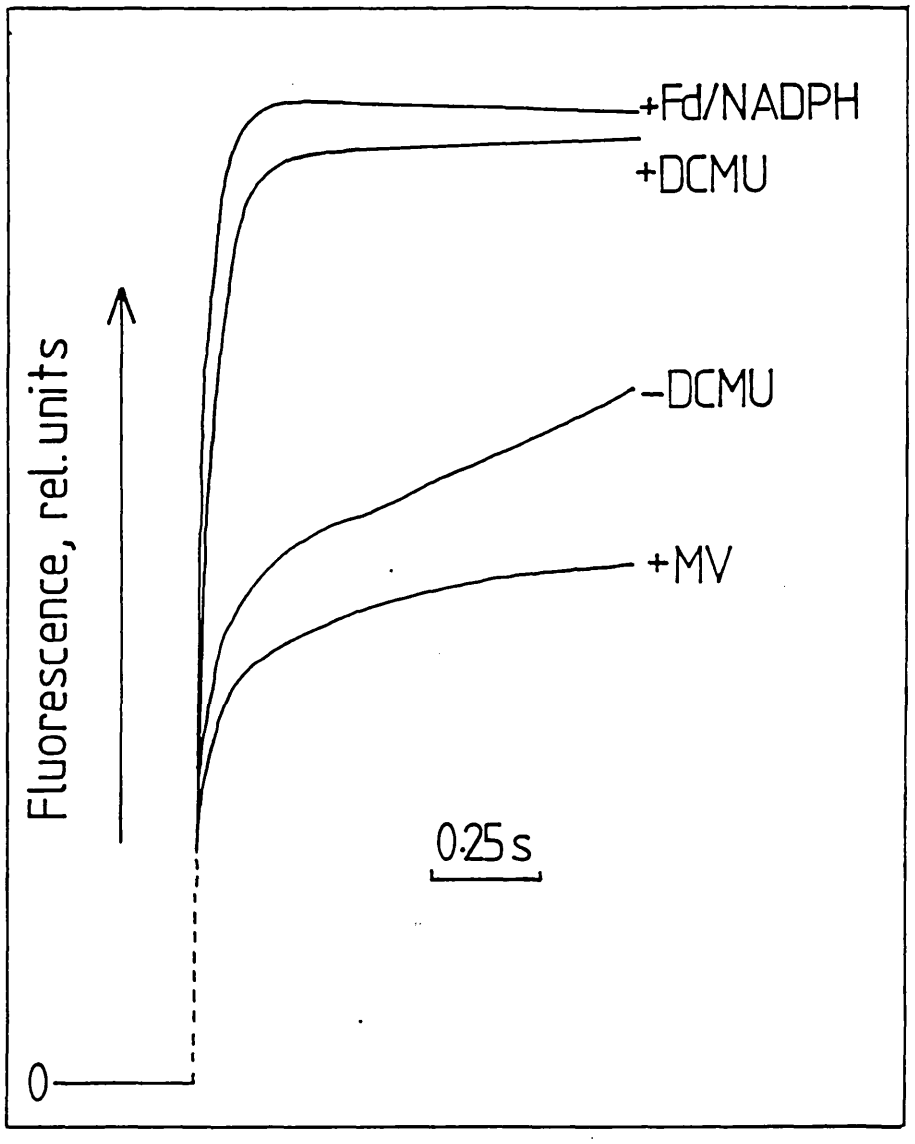


Fig. 6.4 The room temperature chlorophyll fluorescence signals exhibited by thylakoids suspended in the presence of 5mM Mg^{2+} and a) 7.2μM ferredoxin and 0.5mM NADPH, b) 20μM DCMU, c) minus DCMU and d) 100μM methyl viologen.

in the presence of o-phenanthroline (a PS2 inhibitor) or DCMU. Fig. 6.5B gives similar plots for thylakoids suspended in the presence of FeCy added before or after DCMU inhibition. The presence of o-phenanthroline or FeCy (after DCMU) both led to a faster rate of Q_A reduction due to an increase in k_β (approximately doubled, not shown). The addition of FeCy (before DCMU), however, led to a slower rate of Q_A^- photoaccumulation mainly as a result of a decrease in k_α (not shown). Fig. 6.5C shows that the addition of Fd/NADPH led to a faster reduction of Q_A, in a manner similar to that shown in Fig. 6.2 for dithionite. Fig. 6.6 shows the effect of MV on the semi-log plot and area growth curves in the presence of 20μM DCMU. The addition of MV, again surprisingly, led to a faster photoreduction of Q_A. Table 6.2 gives the various fluorescence parameters measured from the induction curves of DCMU treated thylakoids in the presence or absence of MV. It can be seen that MV had no effect on the F_o but did lead to lower F_m and A_{max} values. Furthermore, there was a small increase in k_α and approximately a doubling of k_β which led to a decrease in β_{max}. When the area due to each component was calculated it was seen that the addition of MV produced a 48% decrease in $Area_\beta$ with only a 17% decrease in $Area_\alpha$.

Schreiber and Pfister (1982) reported that the slow component could be removed by raising the concentration of DCMU, such an observation perhaps indicates that some PS2 centres have an exceptionally low affinity for the binding of DCMU. However, Horvath et al. (1984) have suggested that such high levels of inhibitor preferentially damages $PS2_\beta$ centres so that they are unavailable for photoreduction. DCMU titration curves on various fluorescence parameters are shown in Figs. 6.7a,b,c and d. It can be seen from Fig. 6.7a that on raising the DCMU level from 0.2μM (DCMU/Chl = 0.05) to 100μM (DCMU/Chl = 27.0) there was

Fig. 6.5 The semi-log plots, Ln $(A_{max}-A_{(t)}/A_{max})$, and the growth of the normalised area over room temperature fluorescence induction curves for thylakoids suspended at 5mM Mg^{2+} and in A) the presence of either 2mM O-phenanthroline (——) and 20μM DCMU (– – –); B) the presence of 0.5mM FeCy before (·····) or after (——) DCMU addition and in the absence of FeCy (– – –) and C) the presence of 20μM DCMU with (——) and without (– – –) the addition of 4.2μM Fd and 0.5mM NADPH.

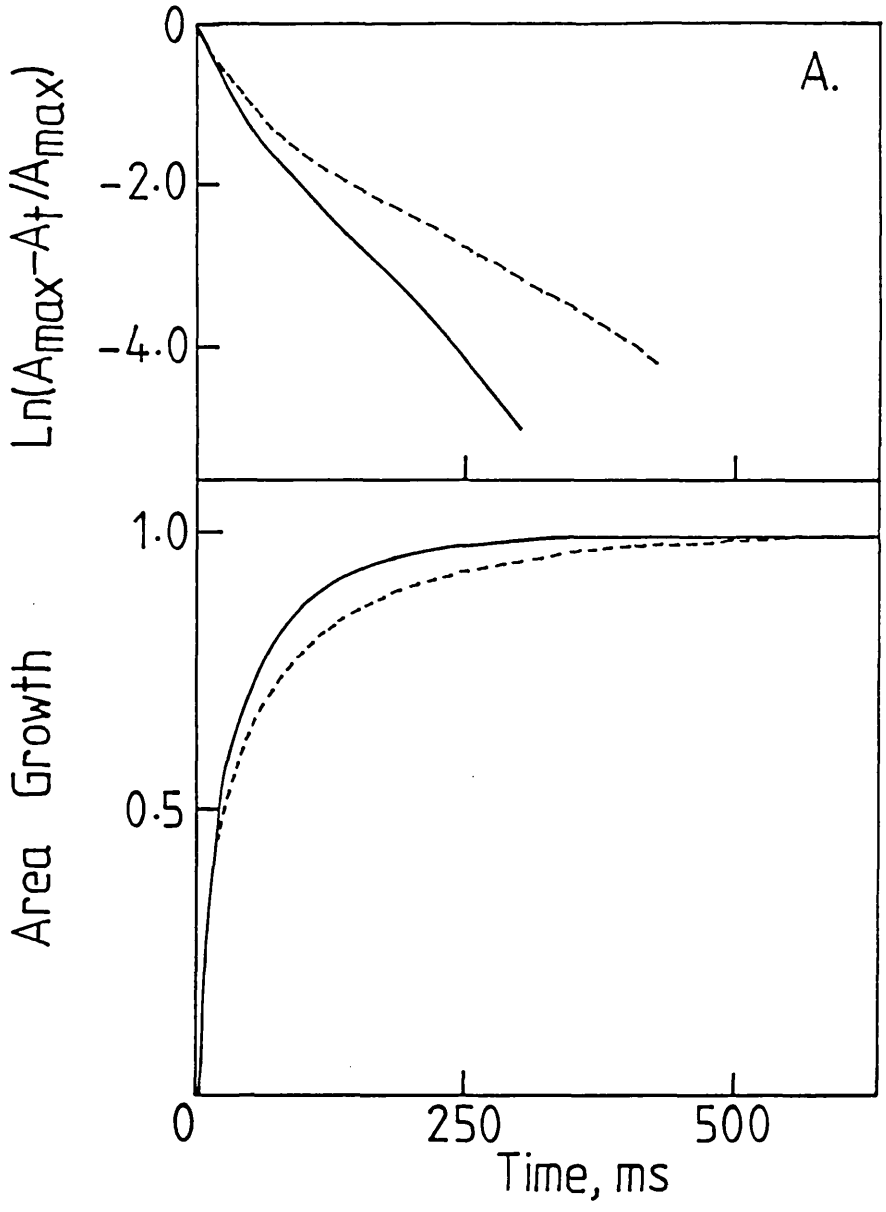


Fig. 6.5 A) the presence of either 2mM O-phenanthroline (——) and 20μM DCMU (– – –).

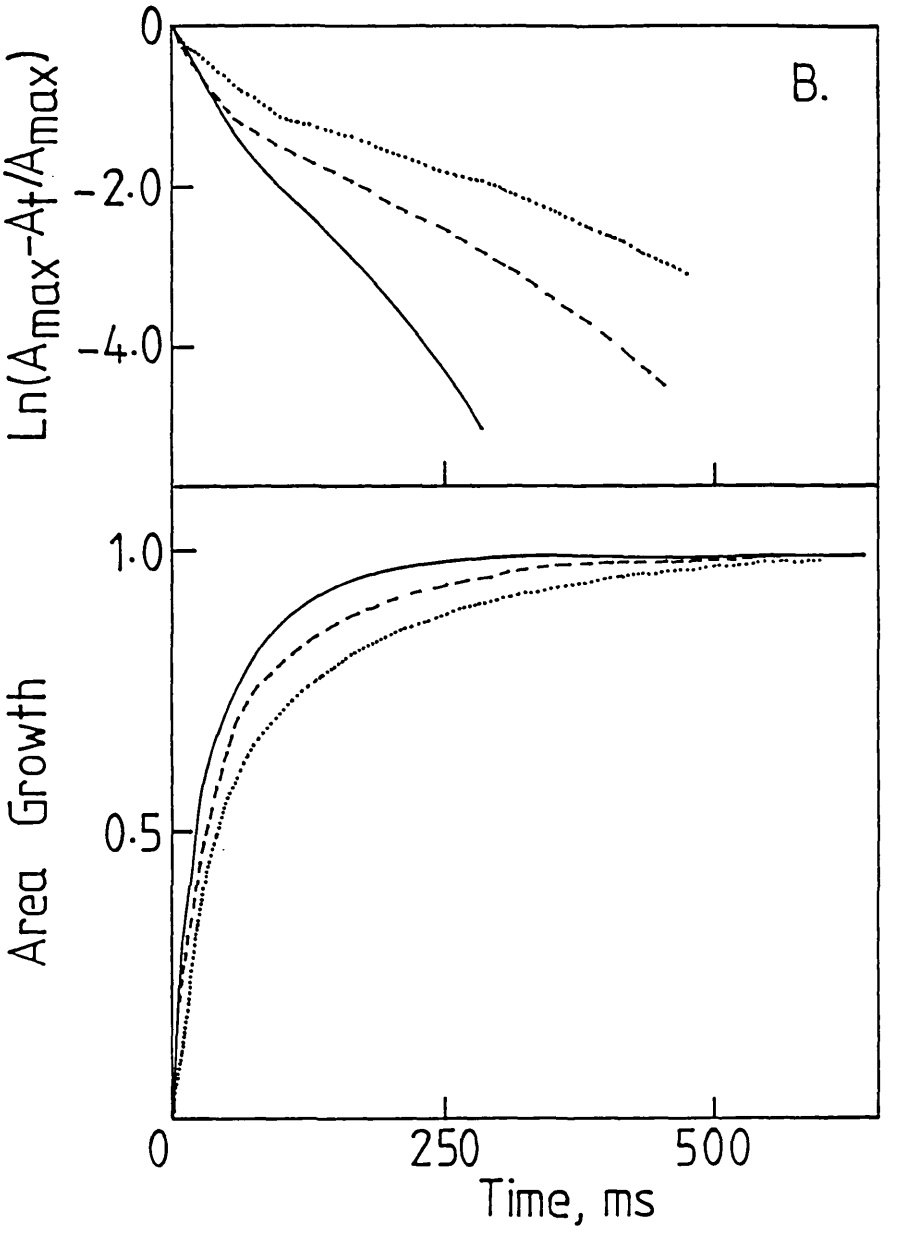


Fig. 6.5 B) the presence of 0.5mM FeCy before (·····) or after (——) DCMU addition and in the absence of FeCy (---).

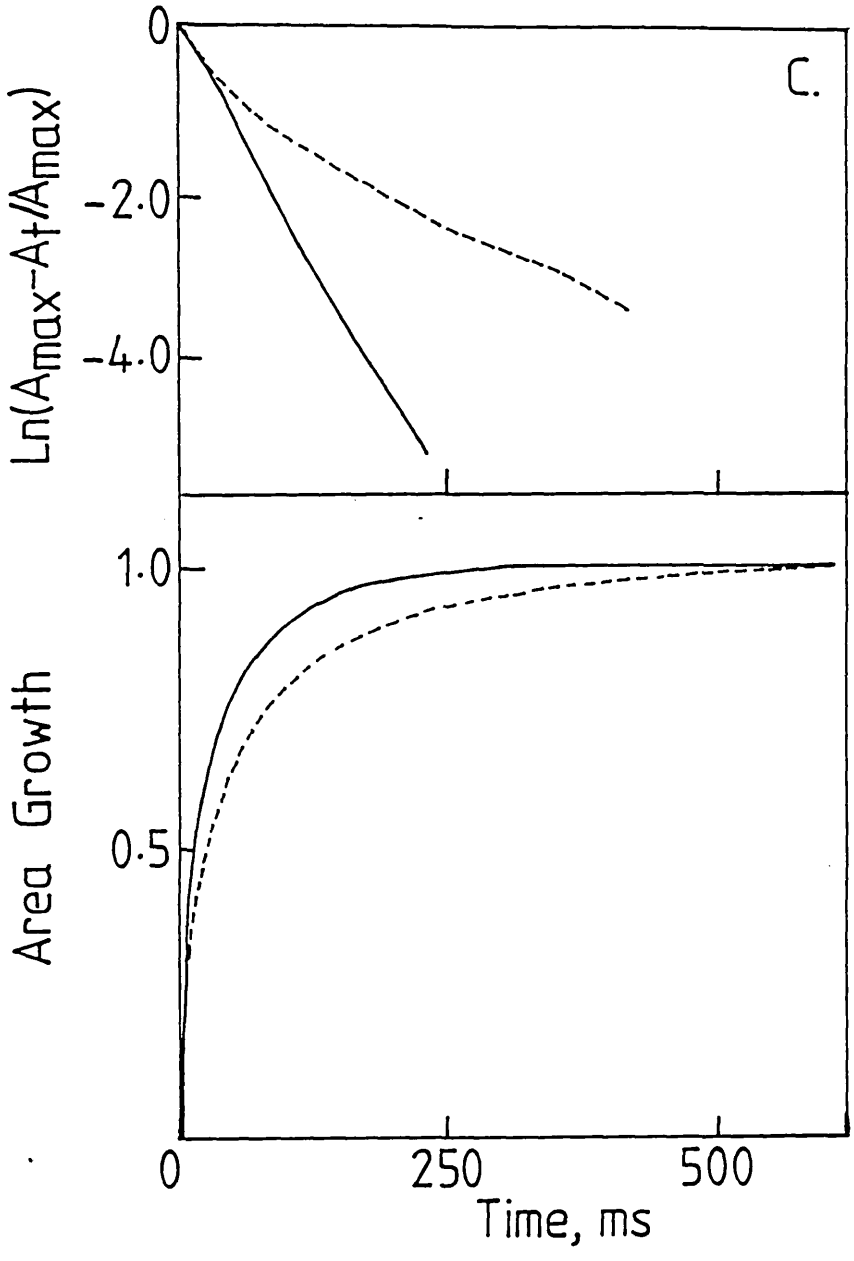


Fig. 6.5 C) the presence of 20μM DCMU with (——) and without (– – –) the addition of 4.2μM Fd and 0.5mM NADPH.

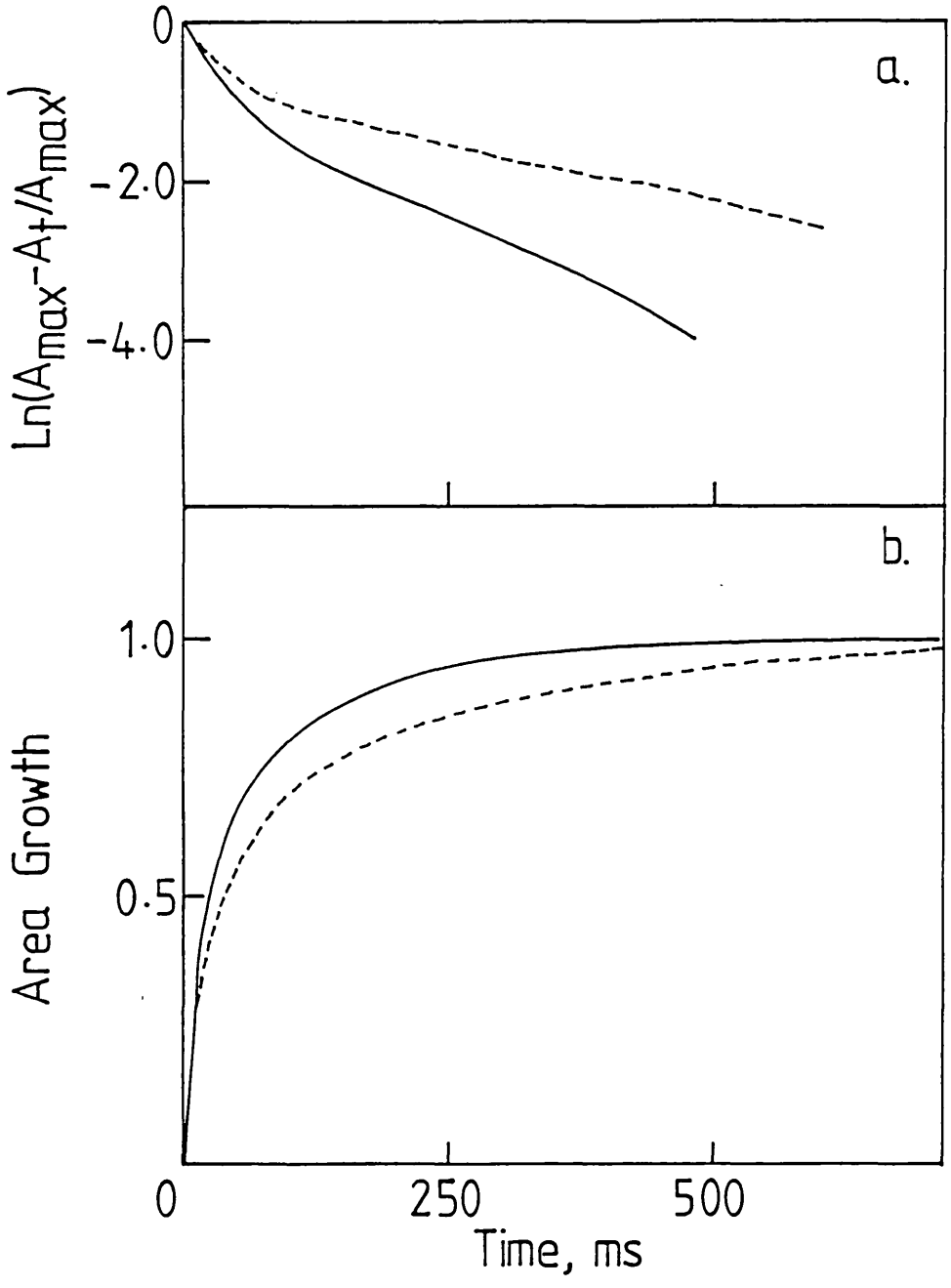


Fig. 6.6 The effect of the presence (——) and absence (– – –) of 100μM methyl viologen on a) the semi-log plot, Ln $(A_{max}-A_{(t)}/A_{max})$, and b) the growth of the normalised area over the room temperature fluorescence induction curve exhibited by DCMU-inhibited thylakoids suspended in 5mM Mg^{2+}.

TABLE 6.2

The Effect of Methyl Viologen (MV) on Various Fluorescence Parameters

Condition	F_o	F_m	A_{max}	k_α (s^{-1})	k_β (s^{-1})	β_{max}	$Area_\alpha$	$Area_\beta$
-MV	22	86.9	1251	42.7	3.8	0.31	870	381
+MV	22	80.1	918	44.5	6.8	0.21	720	197

little change in F_m, F_o and F_v, while the A_{max} value decreased by 43% with no significant change in F_v/F_m (Fig. 6.7b). Parameters from the kinetic analyses of the fluorescence rise as a function of DCMU concentration are presented in Fig. 6.7c. It is seen that on increasing the DCMU level there was a preferential increase in k_β (68% change) with only a 7% alteration in k_α. From the various β_{max} values generated, the areas due to the α and β components were calculated in response to changing DCMU levels (Fig. 6.7d). This indicated that increasing the DCMU concentration preferentially effects the PS2β centres, leading to a 68% decrease in $Area_\beta$ over the concentration range investigated, while only decreasing $Area_\alpha$ by 17%.

After a saturating light pretreatment to reduce Q_A, even in the presence of DCMU and an inhibitor of PS2 backreactions with the oxidising side of PS2, Q_A slowly becomes partially reoxidised in the dark. Thielen and van Gorkom (1981a) have previously reported that the reoxidised Q_A could be attributed to $PS2_\alpha$ centres, this observation would not be expected if $PS2_\beta$ resulted from poorly inhibited reaction centres. Fig. 6.8 shows the semi-log plots and area growth curves for control thylakoids (no light pretreatment) and those thylakoids given 15s dark reoxidation after a light pretreatment. It is seen from Fig. 6.8 that the reoxidised PS2 centres gave rise to a Q_A component which was subsequently rereduced with mainly, slow exponential kinetics. Table 6.3 gives the various fluorescence parameters associated with the two situations mentioned above. After the light treatment followed by 15s dark reoxidation the F_o remained high while the F_m was slightly lowered compared with the control which had no light pretreatment. Most of the area over the curve which was regenerated by the reoxidation could be attributed to the slow, β-component.

Fig. 6.7 The effect of DCMU concentration on the room temperature fluorescence induction curve parameters a) F_m (▲), F_v (●) and F_o (■); b) A_{max} (△) and F_v/F_m (○); c) k_α (▲) and k_β (△) and d) $Area_\alpha$ (■) and $Area_\beta$ (□) exhibited by thylakoids suspended in the presence of 5mM $MgCl_2$.

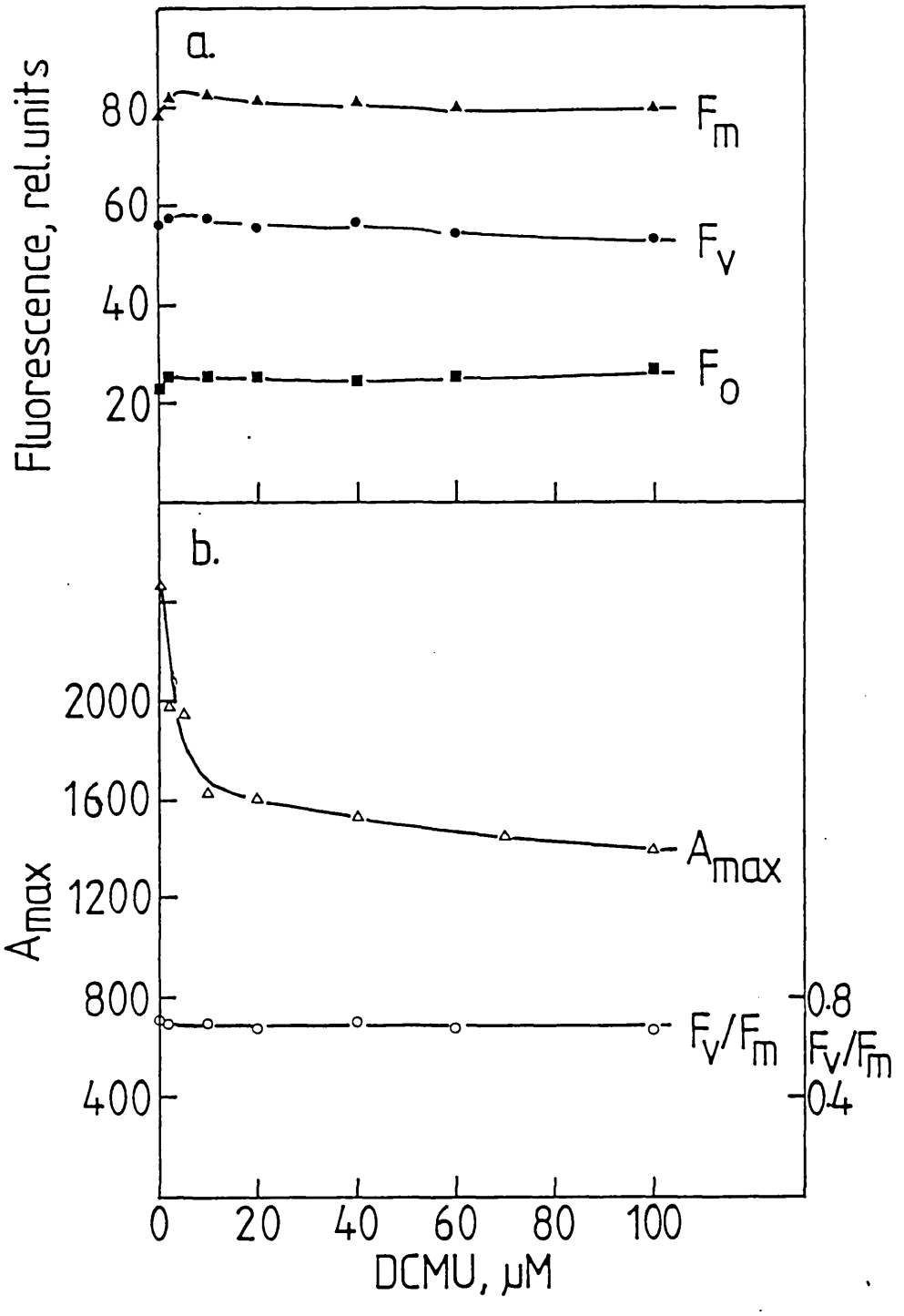


Fig. 6.7 a) F_m (▲), F_v (●) and F_o (■).

b) A_{max} (△) and F_v/F_m (○).

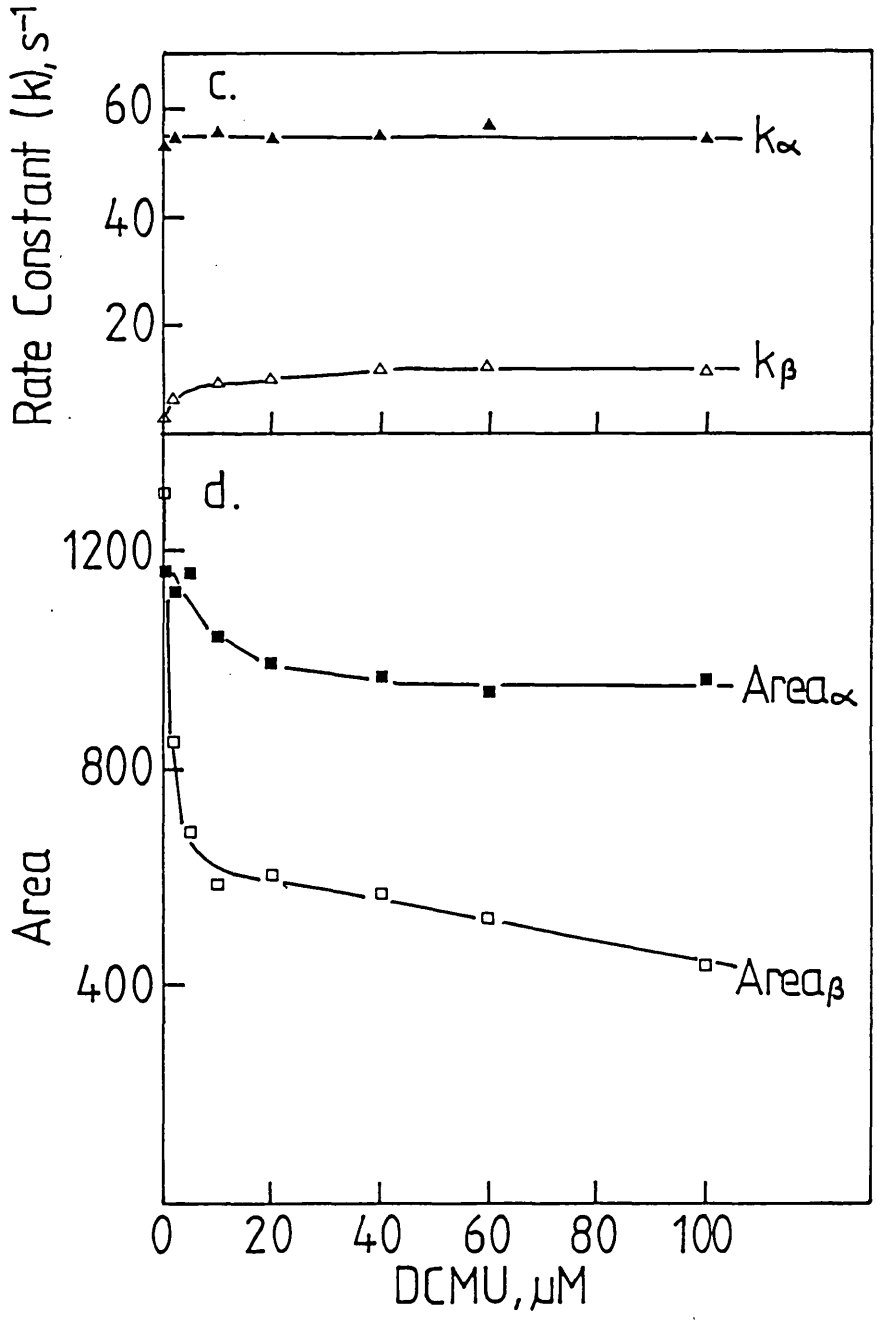


Fig. 6.7 c) k_α (▲) and k_β (△).

d) $Area_\alpha$ (■) and $Area_\beta$ (□).

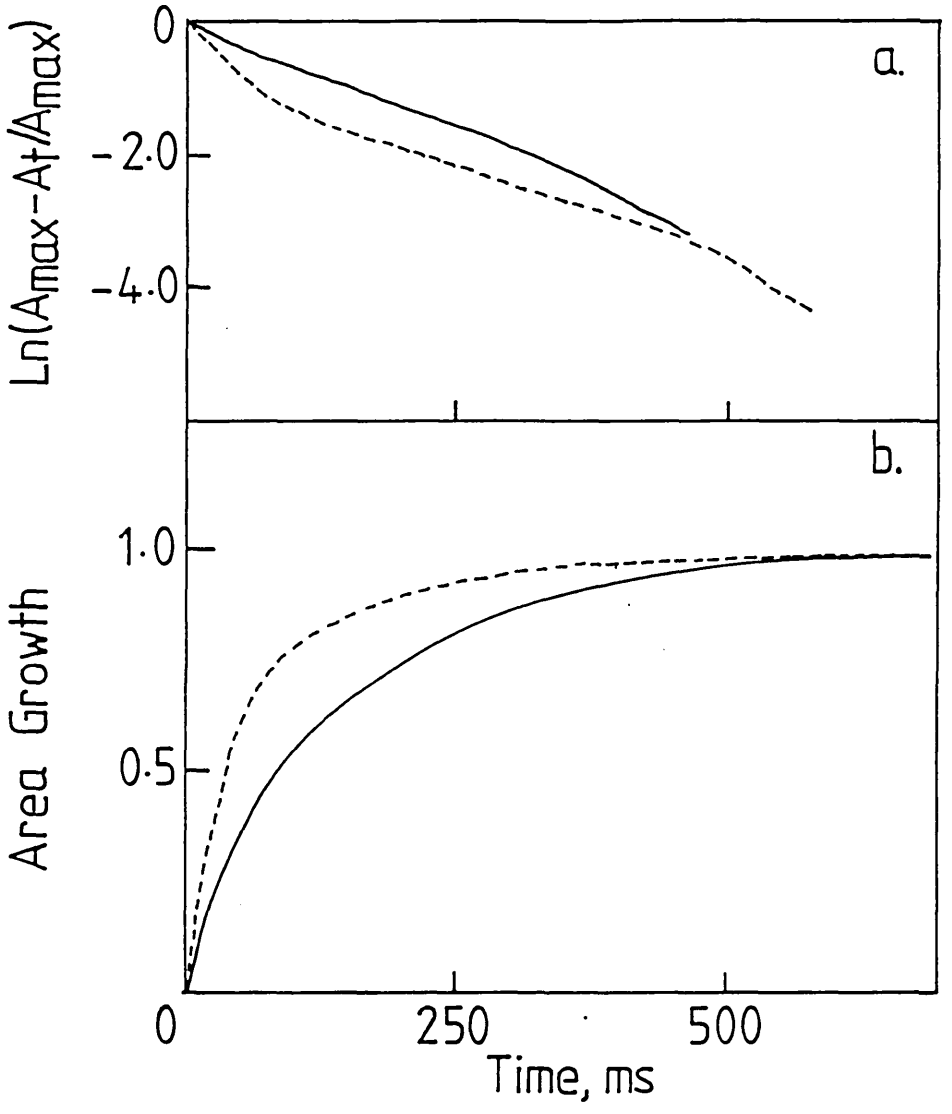


Fig. 6.8 a) The semi-log plot, Ln $(A_{max}-A_{(t)}/A_{max})$, and b) the area growth curves exhibited by control thylakoids given no preillumination treatment (---) and light-pretreated thylakoids given a 15s dark reoxidation time after preillumination (——). All experiments were carried out at room temperature in the presence of 20μM DCMU, 4.5μM Ant 2p and 5mM Mg^{2+}.

TABLE 6.3

The Effect of a Preillumination Light Treatment on Various Fluorescence Induction Curve Parameters

Condition	F_o	F_m	A_{max}	k_α (s^{-1})	k_β (s^{-1})	β_{max}	$Area_\alpha$	$Area_\beta$
Control (no light treatment)	25	75.4	1470	30	5.4	0.35	956	515
15s reoxidation (after light treatment)	61	72.7	398	64	5.9	0.81	757	322

Recently, it has been reported that $PS2_\alpha$ and $PS2_\beta$ have different fluorescence emission spectra so that $PS2_\beta$ fluoresces with a maximum at 715-730nm compared with 685nm for $PS2_\alpha$ (Brearley and Horton, 1984). Table 6.4 gives various fluorescence parameters from fluorescence induction curves detected at either 685nm or 731nm. Although F_o, F_m and A_{max} levels cannot be compared because of the different transmission properties of the filters and altered fluorescence yields at the two wavelengths the kinetic data can be compared due to the normalisation involved in the analyses. It was seen that there was no observable difference between k_α, k_β and β_{max} when the fluorescence signal was monitored at either 685nm or 731nm. This observation disagrees with the contention that $PS2_\alpha$ and $PS2_\beta$ have different fluorescence emission characteristics as might be predicted from the reported shift in the absorption properties of the two types of centre (Thielen and van Gorkom, 1981b).

It was originally proposed by Melis and Homann (1978) that $PS2_\alpha$ was located in the granal lamellae while $PS2_\beta$ was situated out in the stromal membranes. The fluorescence analysis of membrane fragments has so far confirmed the above contention (Anderson and Melis, 1983; Lam et al., 1983). Table 6.5 compares various fluorescence parameters obtained from induction curves generated from O_2-evolving PS2 preparations (BBY) with intact thylakoid membranes, both at pH 6.0 and pH 8.2. A slow component perhaps corresponding to β centres was observed in the PS2 particles which are believed to originate from the appressed, granal lamellae. From the k_α and F_v/F_m values it would seem that these preparations are not as efficient at trapping quanta as shocked chloroplasts, indicating that some degree of PS2 damage or reorganisation had occurred. This is highlighted by the high F_o levels and low F_v/F_m

TABLE 6.4

Various Fluorescence Induction Curve Parameters Measured at 685nm and 731nm

Condition	F_o	F_m	A_{max}	k_α (s^{-1})	k_β (s^{-1})	β_{max}	F_v/F_m	$Area_\alpha$	$Area_\beta$
F_{685}	25	90.5	1340	41.9	4.6	0.37	0.72	840	500
F_{731}	20.2	58.5	777	44.5	4.9	0.40	0.65	467	310

TABLE 6.5

Various Fluorescence Induction Curve Parameters Measured from Intact Pea Chloroplasts and a PS2 Preparation (BBY)

Condition	F_o	F_m	A_{max}	k_α (s^{-1})	k_β (s^{-1})	β_{max}	F_v/F_m
pH 8.0							
BBY	40	68.5	1170	27.0	4.0	0.57	0.42
Intact	34	104.5	1600	38.4	4.1	0.34	0.68
pH 6.0							
BBY	40	88.5	3800	20.0	2.1	0.70	0.55
Intact	30	93.0	2400	31.0	2.7	0.52	0.68

ratios indicative of an inability to generate oxidised Q_A before illumination in the BBY's.

All of the work which has so far been carried out to investigate the $PS2_\alpha$, $PS2_\beta$ type of heterogeneity, as well as the original experiments by Melis and Homann, were carried out *in vitro*. Fig. 6.9 shows the semi-log plots and area growth curves for DCMU-inhibited intact pea leaves and isolated pea thylakoids. It can be seen that *in vivo* the semi-log plot was not easily differentiated into two kinetic components, although the k_α values and F_v/F_m ratios (not shown) exhibited by leaves were comparable to that obtained *in vitro* when each had been given the same excitation light intensity.

6.2 Discussion:

The data presented in this chapter suggests that the biphasic nature of room temperature fluorescence induction curves from DCMU-inhibited thylakoids may not be due to two distinct forms of PS2 centre located in different thylakoid regions as previously proposed by Melis and co-workers.

It has already been shown in Chapter 3 that the relative extent of β_{max} is altered when thylakoid membranes undergo conformational changes (Table 3.1 and 3.2). However, this change in β_{max} does not necessarily reflect a conversion of the $PS2_\alpha$ centres into $PS2_\beta$ centres. From absorption difference spectroscopy in the ultra violet (320nm), Melis and Ow (1982), have suggested that the absolute number of the two centres does not change when comparing thylakoids suspended in the presence and absence of $MgCl_2$. They proposed that the discrepancy between the ΔA_{320}

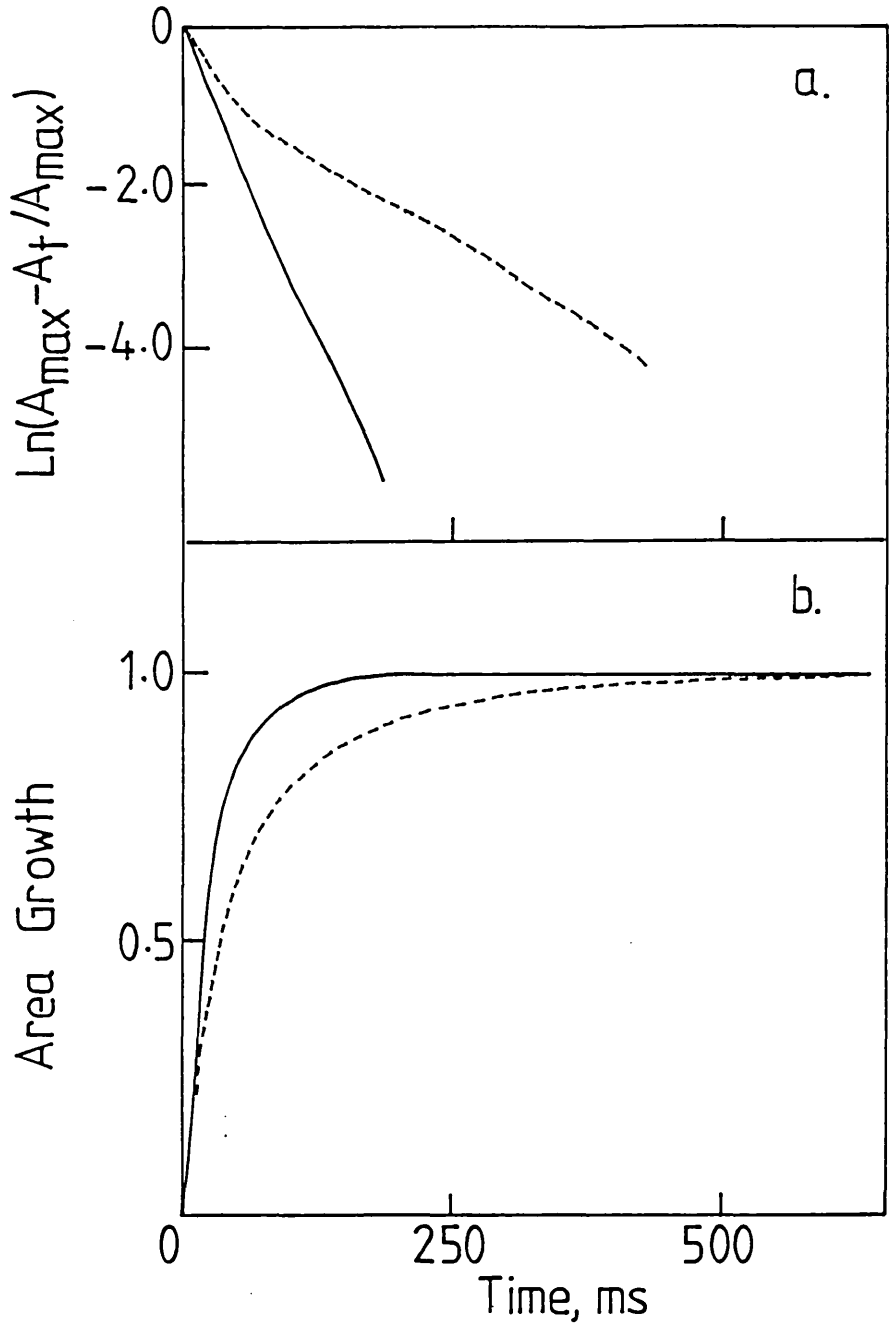


Fig. 6.9 a) The semi-log plot, Ln $(A_{max}-A_{(t)}/A_{max})$ and b) the area growth curves exhibited by intact pea leaves (——) and isolated pea thylakoids (---) suspended in the presence of 5mM Mg^{2+}, and inhibited with DCMU.

measurements and fluorescence analysis was due to a Mg^{2+}-specific effect on $PS2_{\alpha}$ centres which altered their fluorescence yield. Table 3.1 and 3.2, however, indicated that no Mg^{2+}-specific effect was apparent and that the change in fluorescence induction profile probably reflected a cation-specific alteration in membrane and protein organisation which altered PS2-PS2 interactions as well as PS2-PS1 interactions (see Barber, 1982a). If the number of the two centres remains the same, as argued by Melis and Ow (1982), then the observed changes in β_{max} brought about by various conditions (such as cation levels and protein phosphorylation) can be explained by a change in the fast, sigmoidal α component due to differences in PS2-PS2 interaction. Such changes in PS2-PS2 interactions could be brought about by either pigment-protein randomisation or LHC detachment. By the nature of the analysis such a change in the α-phase means β_{max} becomes altered. This can be seen in Fig. 6.1, where β_{max} is correlated to A_{norm}. This area gives an indication of the overall kinetics of Q_A photoreduction, taking into account any changes in fluorescence emission yield. Not surprisingly, the extent of β_{max} brought about by either salt- or phosphorylation-induced changes led to a similar change in A_{norm}. As the two processes are believed to alter PS2 organisation in different ways (see Chapters 3 and 4) it is difficult to reconcile changes in β_{max} with the conversion of $PS2_{\alpha}$ into $PS2_{\beta}$ or vice-versa, therefore the change in β_{max} probably reflects alterations in the α-component which brings about an overall slower photoreduction of Q_A.

Vernotte et al. (1979) have shown that the redox state of the PQ pool can alter F_m levels and Table 6.1 shows that the F_o can also be affected. These changes in F_o and F_m, due to dithionite addition, meant that F_v/F_m stayed unaltered. This was not observed by Melis (1978) who

under reducing conditions found a lower F_v/F_m ratio. Another consequence of the dithionite treatment was that A_{max} decreased, indicating less quanta were now required to photoreduce Q_A, perhaps because of the removal of a pool of electron acceptors. This treatment also led to a removal of the biphasic nature of the fluorescence rise and an accelerated rate of Q_A reduction (Fig. 6.2 and Table 6.1). The prior reduction of the PQ pool, by dithionite, also meant that the relationship between F_v and Q_A^- accumulation was altered so that the deviation attributed to $PS2_\beta$ (see Melis and Schreiber, 1979) became linear. These results are very similar to those reported by Melis (1978) who discussed his data in terms of different midpoint redox potentials for $PS2_\alpha$ and $PS2_\beta$. However, such observations could also suggest that the slow phase may, inpart, reflect PS2 centres not fully inhibited by DCMU so that when PQ is over-reduced and electron transfer is inhibited (see Fig. 6.4) the reoxidation of a certain pool of Q_A, associated with the slow phase, via a DCMU-insensitive reaction cannot take place.

The involvement of PQ in the manifestation of the slow phase is consistent, in part, with the reported midpoint potential for Q_β. Redox titrations of the β-component has produced an $E_{m7.0}$ = +120mV with an n=1 fit to the Nernst equation (Horton, 1981). Although the midpoint potential is similar to that for PQ, the n=1 fit would imply a 1 electron acceptor which is therefore inconsistent with PQ, as this is a 2 electron acceptor. Perhaps, however, this may reflect the involvement of the bound, semi-quinone form of PQ in the generation of a slow component. It has been reported that when the bound semi-quinone form ($Q_AQ_B^-$) is present the DCMU binding affinity at these sites is lowered by a factor of 20 (Lavergne, 1982c). Brearley and Horton (1984) have suggested from DCMU titrations of F_m, that α and β centres show different

affinities for DCMU so that the I_{50} for $PS2_{\alpha}$ was 0.05μM while the I_{50} for $PS2_{\beta}$ was 365μM. Joliot and Joliot (1983) have also noticed that DCMU levels preferentially effect Q_{β} in that the $t_{\frac{1}{2}}$ for the reduction of Q_A associated with the slow phase was inversely proportional to DCMU concentration. Furthermore, Schreiber and Pfister (1982) have reported that chloroplast intactness and membrane integrity influence the extent of the slow component and they have also suggested that β centres reflect those PS2 units with a low DCMU affinity. Such reports are, therefore, consistent with the concept that an altered DCMU affinity may partly explain the slow rise. However, Horvath et al. (1984) have shown that high DCMU/Chl ratios preferentially alter $PS2_{\beta}$ so as to inhibit their photoreduction which leads to a decrease in F_v with little change in F_o. They concluded that fluorescence measurements were only meaningful if low DCMU/Chl ratios were used because with a DCMU/Chl = 3.75 no $PS2_{\beta}$ photoreduction was apparently observed, due to some destructive conformational change taking place at the $PS2_{\beta}$ reaction centre. Fig. 6.7 shows that even at a DCMU/Chl ratio of greater than 10 very little change was seen in either F_o, F_m or F_v but there was a preferential DCMU effect on the slow component so k_{β} was increased and $Area_{\beta}$ drastically diminished. However, even in the presence of 100μM DCMU a slow component still existed and this may reflect those PS2 centres which produce the I_{50} of 365μM. Furthermore, Diner and Delsome (1983), while investigating PS2 electron acceptors found that, contrary to Melis and Schreiber (1979), Q_{β} reduction did not give rise to a C_{550} absorption change. They also found that Q_{β} did not generate a transmembrane potential on reduction , which is consistent with a report by Meiburg et al. (1984). An explanation put forward by Diner and Delsome was that if Q_{β} had a midpoint potential of +120mV then it was not a PS2 primary electron acceptor and therefore may be a secondary

electron acceptor or related to PS2 centres with modified DCMU-binding. This latter conclusion is again consistent with the reports already mentioned above and with the work of Thielen and van Gorkom (1981c) who suggested that β centres were not associated with the two electron gate (Q_B) because $PS2_\beta$ did not generate fluorescence on DCMU addition in response to saturating preflash number. They also showed that tris-washing led to the removal of the slow rise. This treatment inhibits electron donation to $P680^+$ and could imply the requirement for more than one PS2 turnover to generate the slow phase, again consistent with the involvement of uninhibited PS2 centres in the manifestation of the slow phase.

It can be seen from Fig. 6.5a that the PS2-inhibitor o-phenanthroline altered the kinetics of the slow rise. Thielen and van Gorkom (1981c) also reported this effect and suggested it was due to an increase in k_d from $PS2_\beta$ centres as a result of an altered PS2 organisation. Vermaas et al. (1984) have shown that o-phenanthroline exhibits rapid exchange kinetics with the herbicide-binding protein (Q_B) compared with DCMU. Therefore, if part of the slow rise was due to $Q_AQ_B^-$ then after a single PS2 turnover in these centres o-phenanthroline could quickly bind and inhibit Q_A reoxidation before the regeneration of $Q_AQ_B^-$ leading to a faster k_β, or perhaps o-phenanthroline is capable of binding to those PS2 centres which show a very low affinity for DCMU.

The slow rise kinetics can also be altered by the addition of PS1 electron acceptors. It is seen from Fig. 6.4 that the addition of MV to unpoisoned thylakoids meant that Q_A was kept more reoxidised than in the absence of MV. This is also seen for FeCy addition (Anderson and Melis, 1983). In the presence of MV (Fig. 6.6) or FeCy (Fig. 6.5B) it

can be seen that the slow phase was affected even in the presence of saturating levels of DCMU which are supposed to totally inhibit O_2 evolution. Joliot and Joliot (1983) have also observed these changes due to MV and FeCy and explained their results as being due to the removal of the $Q_A Q_B^-$ state which is still present in the dark. They concluded that the slow fluorescence rise was partly due to those PS2 centres in the $Q_A Q_B^-$ state and partly due to $PS2_\beta$ centres. The removal of the $Q_A Q_B^-$ state is consistent with the results shown in Table 3.3, however, another possible explanation is that k_β is increased and β_{max} decreased due to a poor evaluation of A_{max}. Bell and Hipkins (1984) have shown that only a 1% underestimation of F_m can lead to a 17% lowering of A_{max}, which in turn produces an apparent faster reduction of Q_A. If a number of PS2 reaction centres are not inhibited by DCMU then the presence of MV or FeCy would mean that these centres might be expected to be photoreduced more slowly (see Fig. 6.4) producing an extended, very slow fluorescence tail which might lead to an underestimation of A_{max}. Table 6.2 shows that MV addition, even in the presence of DCMU, led to a decrease in F_m, implying the generation of either a fluorescence quencher or the inability to remove one. This may reflect the presence of reduced PQ in the dark before illumination which is removed efficiently in the light or the inhibited reduction of PQ in the light by MV or FeCy.

After a saturating light pretreatment, in the presence of DCMU and hydroxylamine, a portion of Q_A still becomes reoxidised in the dark (see Bowes et al., 1981). Fig. 6.8 and Table 6.3 show that the major component which becomes re-oxidised can be attributed to $PS2_\beta$ centres, suggesting again that a permanently DCMU-insensitive PS2 reaction centre might be responsible for the slow phase. The slow component which is

regenerated by the dark pretreatment probably does not reflect those PS2 centres in the $Q_AQ_B^-$ state because the 15s light pretreatment used would have produced several PS2 turnovers and should have removed $Q_AQ_B^-$. These observations are in contrast to Thielen and van Gorkom (1981a) who showed that the major reoxidisable Q_A was associated with $PS2_\alpha$ centres.

All work relating to this form of PS2 heterogeneity has been carried out on isolated thylakoids. It has, however, been mentioned in the literature that the slow phase associated with $PS2_\beta$ is not seen in intact algae (see Duysens, 1983). Fig. 6.9 shows that in intact leaf tissue, a slow component similar to that exhibited by isolated thylakoids is not present, although the α-phase is comparable with that of thylakoids (as determined from k_α values produced by similar excitation energies). Therefore, perhaps the slow component is dependent upon membrane integrity as suggested by Schreiber and Pfister (1982), and only arises because of some change(s) brought about by chloroplast isolation.

It has been reported that $PS2_\beta$ centres have a smaller antenna size consisting of red-shifted chlorophylls ($\lambda_{max} \sim 683$nm) compared with $PS2_\alpha$ (Thielen et al., 1981). This led Brearley and Horton (1984) to investigate the fluorescence emission characteristics of the 2 centres. In the presence of MV and at 80μg chl/ml they showed that β-centres had a maximum fluorescence emission at 725-735nm. Table 6.4 shows that in the absence of MV and with 3.33μg chl/ml present no differences were seen in the level of β_{max} when fluorescence was measured at 685nm or 731nm. These results would suggest that no such difference in fluorescence emission characteristics is seen between $PS2_\alpha$ and $PS2_\beta$, and might indicate that the chlorophylls associated with the two types of PS2 are also similar.

It has also been shown, using membrane fragments from appressed and non-appressed membrane regions, that α-centres are associated only with appressed, granal lamellae while β-centres are located only in the stromal lamellae (Anderson and Melis, 1983; Lam et al., 1983). However, from the results shown in Table 6.5, which gives various fluorescence parameters for a PS2 preparation derived from appressed lamellae by detergent treatment (BBY), the above contention is not borne out. These membrane fragments show a similar slow component to that observed in unfractionated thylakoids but produce a higher β_{max} value because of an altered fast component (k_{α} became lower). This type of preparation produced PS2 fragments which gave a low F_v/F_m ratio and a high F_o level when compared with unfractionated chloroplasts, suggesting some degree of altered PS2-interaction and an inability to generate oxidised Q_A. These observations do not support the concept of the 2 types of PS2 being located in different membrane regions.

Furthermore, if $PS2_{\beta}$ was located in the stromal lamellae then it would be predicted that they would spillover excitation energy to PS1, thereby lowering their fluorescence yield (see Chapter 3). Such an effect would mean that the fluorescence analysis would underestimate the proportion of $PS2_{\beta}$ with respect to $PS2_{\alpha}$. However, the fluorescence analysis produces similar α/β ratios to those obtained from the kinetic analysis of ΔA_{320} absorption changes (Melis and Ow, 1982). Such an observation would not be expected unless either $PS2_{\beta}$ centres do not spillover excitation energy to PS1 or they are not located in the stromal lamellae. The observed change in k_{β}, on lowering the Mg^{2+} level (Fig. 3.9) would also not be expected.

Much of the original work on $PS2_{\alpha}$ and $PS2_{\beta}$ has been carried out on tobacco mutants lacking LHC (see Thielen and van Gorkom, 1981a,b;

Thielen et al., 1981). Such mutants showed an increase in β_{max} correlated to a decrease in membrane stacking, which is very similar to the observations seen with thylakoids on lowering the background cation level. Therefore, these mutants may not contain more $PS2_{\beta}$ centres. The apparent increase may just be a reflection of an altered degree of PS2 connectivity due to a lack of membrane appression as a result of the low levels or absence of LHC, which changes the kinetics of the fast, α-component leading to a larger β_{max} value. Furthermore, if these two types of PS2 centres have different pigment-antenna sizes, as suggested, then it is surprising that in one of the tobacco mutants used by Thielen and van Gorkom (1981b) the k_{α}/k_{β} ratio was only 1.02.

The literature concerning this form of PS2 heterogeneity is very confused. This is partly a consequence of workers using different types of photosynthetic material, like mutants and thylakoid fragments, while always determining the apparent number of α and β centres from the fluorescence analysis of Melis and Homann (1976). This kinetic analysis is very sensitive to thylakoid membrane conformation and can be drastically altered by underestimating the area over the curve. The fluorescence rise is probably far more complex than just the two phases it is usually shown to exhibit. It could be made up from components representing "connected" PS2 centres, separate PS2 centres brought about by the ilet effect, PS2 centres which are initially in the $Q_A Q_B^-$ state as well as poorly inhibited PS2 centres. The results in this chapter do not appear to agree with the concept of the slow, exponential component being representative of PS2 centres with a small antenna-size consisting of red-shifted chlorophylls, located in the stromal lamellae, with different fluorescence emission characteristics and a primary electron acceptor (Q_{β}) with an E_m = +120mV. Many of the observations

presented in this chapter can be explained by a certain number of PS2 centres which have a very low affinity to bind DCMU. This area of research, obviously requires further *in vitro* and *in vivo* investigation preferably using different techniques to quantify and examine the PS2 heterogeneity phenomenon.

CHAPTER 7

State 1 - State 2 Transitions in a Unicellular Green Algae

To maintain maximum rates of photosynthesis at limiting light intensities during changes in light quality, plants have evolved a mechanism which enables them to optimise the balance of incoming quanta between PS1 and PS2 (see Myers, 1971). This readjustment in excitation energy distribution has been termed State 1 - State 2 transitions, and is now a well-established physiological mechanism found in a wide range of photosynthetic systems including higher plants (see Chow et al., 1981b). What is not yet fully known are the details of the mechanism(s) involved, although this adaptive process has been linked with the reversible phosphorylation of LHC (Saito et al., 1983; Bennett, 1983; Carraari et al., 1984), which is controlled by the redox state of the PQ pool (eg. Horton et al., 1981).

From the results of Chapter 4 it can be seen that the mechanisms involved in, and the degree of thylakoid reorganisation brought about by, *in vitro* protein-phosphorylation are highly dependent upon the background level of Mg^{2+} cations. As the *in vivo* levels of stromal cations are relatively high (see Portis, 1981) it might be expected that the State 1 - State 2 transition represents changes similar to those seen to occur *in vitro* upon protein-phosphorylation at "high" Mg^{2+} concentrations.

This chapter analyses the fluorescence characteristics of the *in vivo* State 1 - State 2 change in order to relate them to the *in vitro* protein-phosphorylation studies of Chapter 4, carried out using pea thylakoids. This has involved the careful analysis of chlorophyll

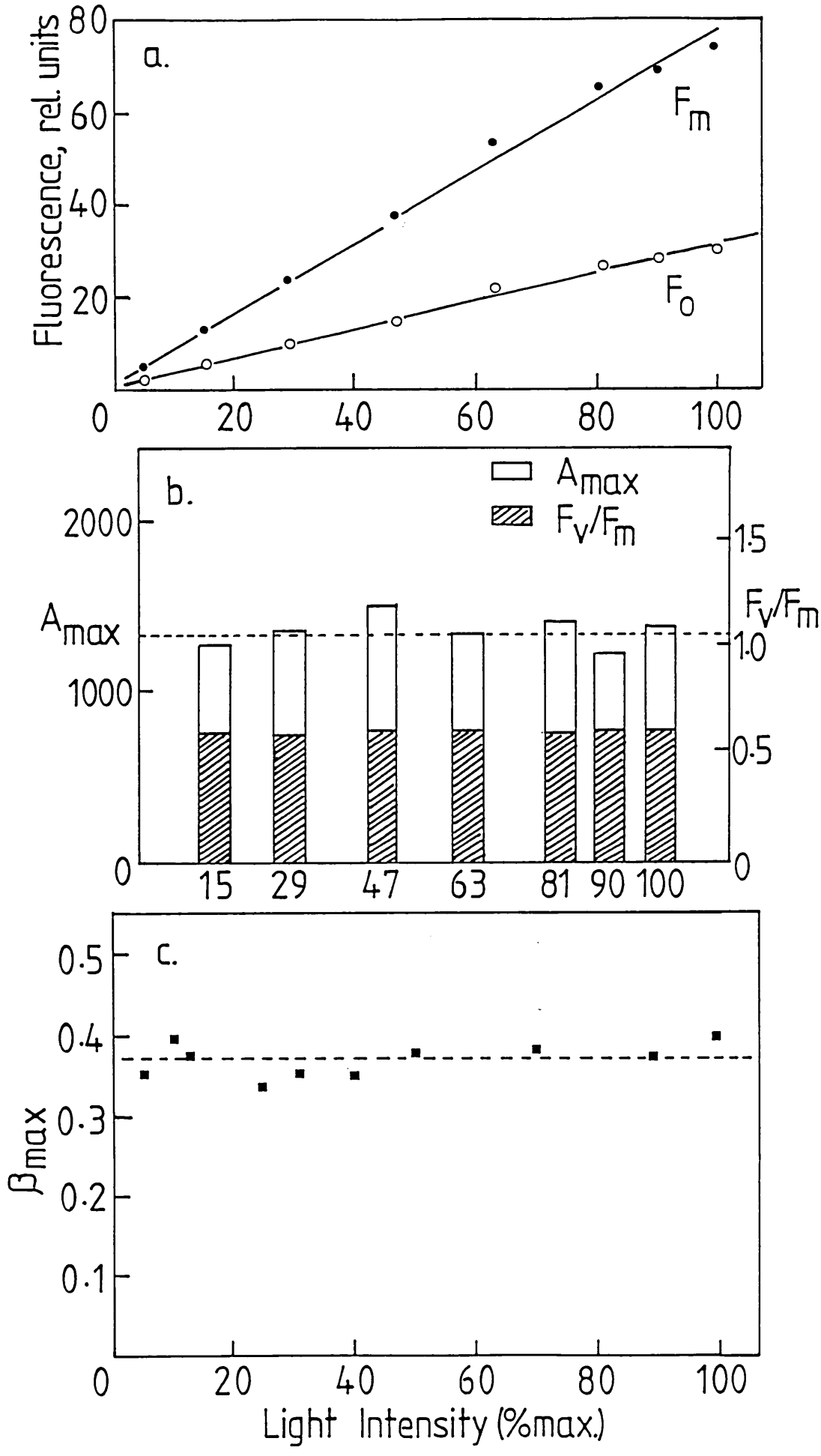
fluorescence induction curves from DCMU-poisoned Chlorella pyrenoidosa cells adapted to either State 1 or State 2.

7.1 Analysis of in vivo Chlorophyll Fluorescence Induction Curves:

Fig. 7.1a shows the F_o and F_m levels obtained from fluorescence induction curves of dark-adapted Chlorella pyrenoidosa cells poisoned with DCMU. As the light intensity was reduced from 100% ($8Wm^{-2}$) to 5% there was a linear decrease in both F_o and F_m so that F_v/F_m and A_{max} remained unchanged (Fig. 7.1b). These results are consistent with those observed using isolated pea thylakoids (Fig. 3.5) showing that the in vivo and in vitro systems behaved in a similar manner to changes in light intensity. The significance of these results has already been discussed in terms of a "true" F_o measurement and the expected fluorescence changes associated with an altered PS2 absorption cross section, in Chapter 3. Fig. 7.1c, extends the previous observations, and shows that the fluorescence changes brought about by alterations in excitation energy intensity were not accompanied by changes in β_{max}.

Fig. 7.2a shows a typical State 1 - State 2 chlorophyll fluorescence trace for the particular illumination conditions used. The algal suspension was pretreated with light 1 for 10 minutes to induce State 1. The introduction of modulated light 2 and the simultaneous removal of light 1 gave the initial fluorescence yield indicative of State 1, where an imbalance of energy distribution of light 2 between the two photosystems leads to a high fluorescence level due to the overreduction of Q_A and PQ. As light 2 was left on the fluorescence was seen to decrease to a lower level. After several minutes of light 2, the algae had adapted to State 2, since superimposing light 1 had only a small quenching effect on the modulated fluorescence yield. Thus at this

Fig. 7.1 Chlorophyll fluorescence properties of Chlorella cells as a function of actinic light intensity showing the changes in a) F_m (●) and F_o (○); b) A_{max} (□) and F_v/F_m (▨) and c) β_{max} (■). All measurements were made at room temperature in the presence of 50μM DCMU after a 2 minute dark adaptation. 100% intensity was $8Wm^{-2}$ and the lines are the least squares fit to the data points.


a.
Fluorescence, rel. units
80
60
40
20
0
20
40
60
80
100
F_m
F_o
b.
A_{max}
F_v/F_m
2000
1000
0
1.5
1.0
0.5
0
15
29
47
63
81
90
100
c.
β_{max}
0.5
0.4
0.3
0.2
0.1
Light Intensity (%max.)

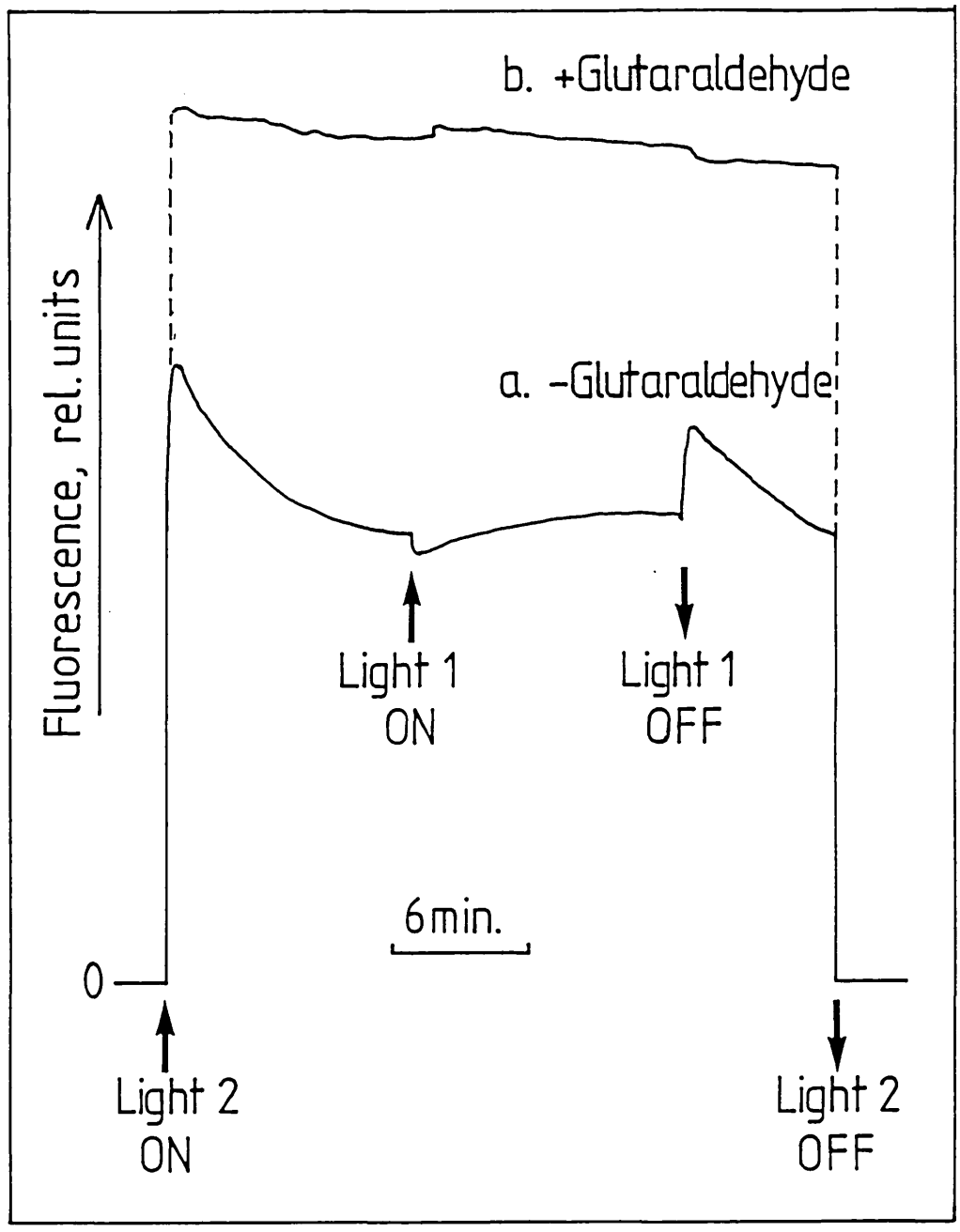


Fig. 7.2 Relative changes in the yield of modulated chlorophyll fluorescence from Chlorella cells suspended a) in the absence of glutaraldehyde and b) in the presence of glutaraldehyde. The algae were pretreated for 10 minutes with light 1 and the following sequence of light treatments used were as described in 2.3.7.

stage electron transport away from PS2 was not significantly limited by PS1 turnover and therefore the algae had achieved a better balance of PS2 and PS1 activities with light 2.

Fig. 7.2b shows the inhibition of these fluorescence changes when the algae had been prefixed with glutaraldehyde, suggesting that the fluorescence fluctuations were accomplished by some structural reorganisation within the thylakoid membranes.

Fig. 7.3 shows typical chlorophyll fluorescence induction curves measured using DCMU-inhibited Chlorella cells which had previously been adapted to State 1 or State 2, using the illumination conditions already described for Fig. 7.2. These fluorescence transients were subjected to a careful analysis to obtain the various characteristic fluorescence parameters presented in Fig. 7.4. From Fig. 7.3, it can be seen that adaptation from State 1 to State 2 led to a decrease in both F_o and F_m and a slower rise to F_m in State 2 ($t_{\frac{1}{2}} \sim 20$ms) compared with State 1 ($t_{\frac{1}{2}} \sim 10$ms). Values of F_m, F_v and F_o for both State 1 and State 2 are given in Fig. 7.4, where it can be seen that all three parameters were lower in the State 2 condition compared with State 1. Fig. 7.5a shows that the changes seen in Figs. 7.3 and 7.4 did not lead to an alteration in F_v/F_m or A_{max} (Fig. 7.5b). When the curves were analysed to determine the extent of the two kinetic components it was seen that β_{max} had become significantly larger in the State 2 adapted Chlorella compared with those cells in State 1 (Fig. 7.5c). It is interesting to note that the relative amount of the slow kinetic phase was considerably smaller in vivo to that seen in isolated pea thylakoids. Furthermore, the slow phase exhibited faster in vivo kinetics compared with the in vitro situation, as determined by the rate constant, k_β (data not shown).

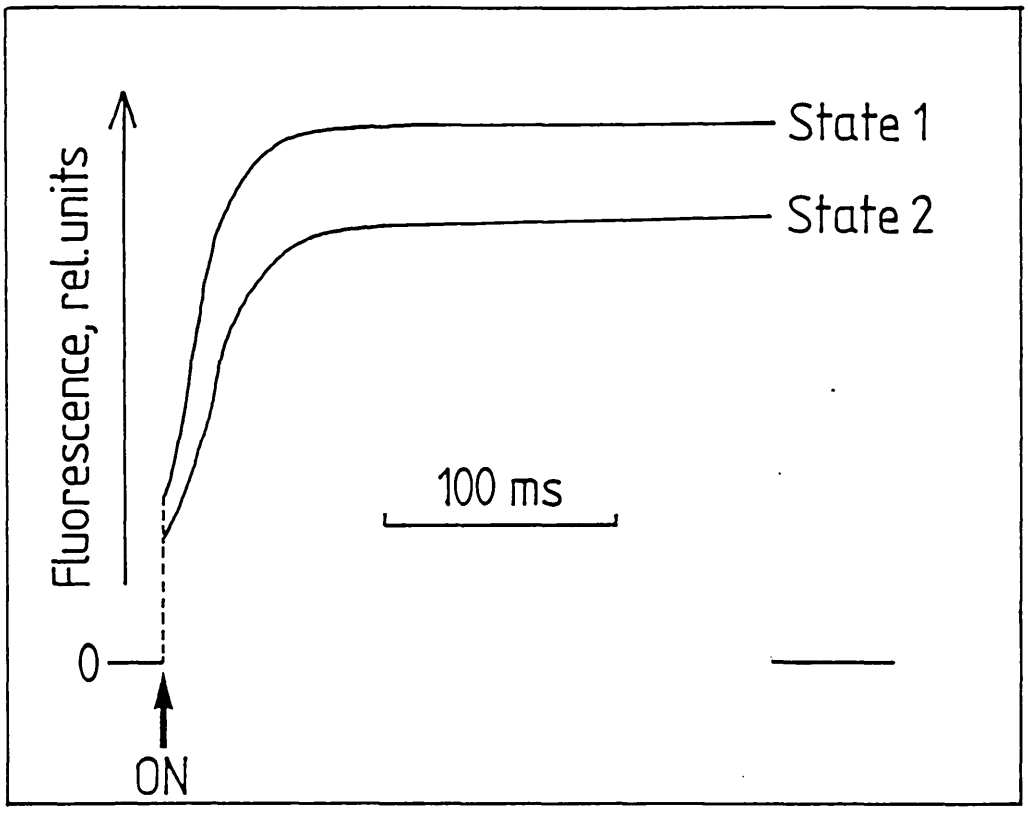


Fig. 7.3 Room temperature chlorophyll fluorescence induction curves in DCMU-inhibited Chlorella cells previously adapted to either State 1 or State 2 with 10 minute pretreatments with light 1 and light 2 respectively. The fluorescence signal was initiated after a 1 minute dark incubation.

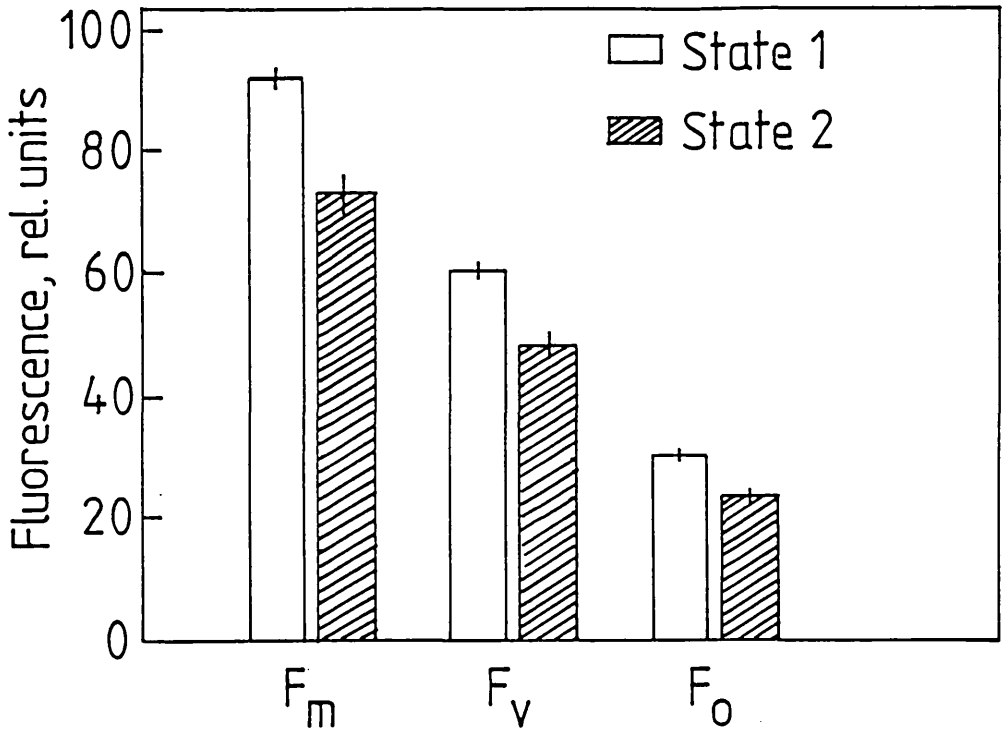


Fig. 7.4 The effect of State 1 and State 2 adaptation of Chlorella cells on the room temperature fluorescence induction curve parameters F_m, F_v and F_o exhibited by DCMU-inhibited algae.

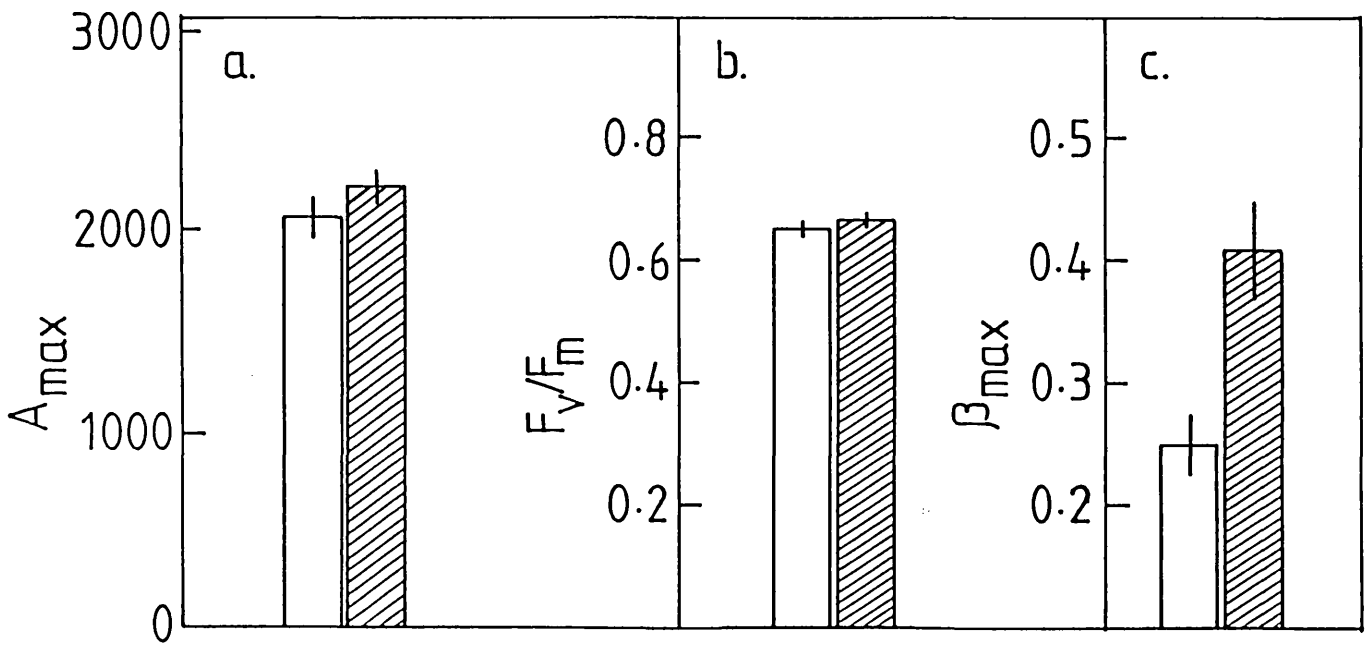


Fig. 7.5 The effect of State 1 and State 2 adaptation of Chlorella cells on the room temperature fluorescence induction curve parameters a) A_{max}, b) F_v/F_m and c) β_{max} exhibited by DCMU-inhibited algae. Statistical tests of the data showed that only the changes in β_{max} were significant (at the 1% level).

7.2 Discussion:

There is good evidence to suggest that protein-phosphorylation brings about the *in vivo* State 1 - State 2 transitions (Telfer et al., 1982; Saito et al., 1983; Bennett, 1983). What is not clear is whether the mechanism giving rise to this physiological control process involves changes in photosystem absorption cross section or excitation energy spillover or both. From Chapter 4, it can be seen that the manifestation of these two different methods of altering light distribution between the two photosystems will be highly dependent on the background cation levels present in the stroma. Bennoun (1974) suggested, from fluorescence studies, that the State transitions exhibited by *Chlamydomonas reinhardti* showed similar alterations in induction curve characteristics to the observed *in vitro* cation-induced changes shown by isolated algal thylakoids. He saw a preferential quenching of F_v, a decrease in F_o and an altered F_v/F_m ratio and A_{max} value on going from State 1 to State 2, indicative of a spillover mechanism and a change in absorption cross section both being involved. These changes were also accompanied by a 33% decrease in membrane stacking (Bennoun and Jupin, 1974). Saito et al. (1983), using *Chlorella vulgaris*, have also reported a decrease in F_o and F_m with a small lowering in the F_v/F_m ratio on going from State 1 to State 2. However, Sane et al. (1982), again using Chlorella cells, investigated the effect of uncouplers on the State transition and concluded that redox changes may be wholly responsible for the development of State 2.

The results presented in this chapter indicate that the State 1 - State 2 transition reflects changes in PS2 absorption cross section. Measurements of various fluorescence parameters from isolated thylakoids

poisoned with DCMU have suggested that it is possible to distinguish between the two mechanisms which can operate to alter excitation energy distribution (see Chapters 3 and 4). It can be seen that a decrease in the amount of light absorbed by PS2 brought about an attenuation in F_o and F_m so both F_v/F_m and A_{max} remained unchanged (Fig. 7.1a and b). Similar changes are observed in Figs. 7.3, 7.4 and 7.5 for Chlorella which have undergone a State 1 - State 2 transition, indicating that less quanta are now arriving at PS2 reaction centres. These changes are identical to those exhibited by isolated thylakoids from higher plants which have undergone chemically-induced protein-phosphorylation at "high" Mg^{2+} levels, and would indicate that this might be the mechanism involved in bringing about the State transition within intact tissue. However, as seen in Chapter 4 from the DBMIB fluorescence titration results, at 5mM Mg^{2+}, such changes in the fluorescence induction curve characteristics cannot rule out small increases in spillover taking place.

An increase in β_{max} (Fig. 7.5c) was seen when Chlorella were adapted to the State 2 condition. This would be observed whether fluorescence was being quenched by spillover or absorption cross section changes. The β_{max} value appears to give an indication of the degree of PS2-PS2 interaction (see Chapter 6). The increase in β_{max} would therefore indicate that a decrease in PS2 connectivity was taking place. This change in β_{max} along with the inhibition of the State transitions by glutaraldehyde fixation (Fig. 7.2b) show that conformational changes are involved in producing the in vivo change which leads to an alteration in PS2 organisation. As expected, the β_{max} value did not change in the experiments presented in Fig.7.1, where F_m and F_o were decreased by lowering the light intensity and not by conformational changes.

It is concluded that *in vivo* State 1 - State 2 transitions, in *Chlorella*, show fluorescence changes comparable with those observed when thylakoids are phosphorylated at "high" background cation levels. This would be consistent with the estimated "high" Mg^{2+} levels present in intact chloroplasts (Portis, 1981). It, therefore, seems likely that protein-phosphorylation induces sufficient electrostatic repulsion to allow the lateral movement of a "mobile" pool of phosphorylated LHC from the appressed to the non-appressed membrane regions where it can then act as PS1 antenna pigments (see Chapter 4 and section 1.7.4). This process may also lead to a small increase in spillover from PS2 to PS1, as seen after *in vitro* protein-phosphorylation at 5mM $MgCl_2$ (Fig. 4.6a), but such a change cannot be clearly distinguished from the results presented above.

CHAPTER 8

Photosynthetic Adaptation to Growth Conditions in Relation to Chloroplast Ultrastructure

Higher plants have the ability to respond to environmental growth conditions. The effect of light intensity on chloroplast ultrastructure and photosynthetic capacity is now well established. In general terms there appears to be either a strong or weak light response which produces characteristic chloroplasts differing in the degree of thylakoid appression and the levels of enzymes and pigment-containing protein complexes (see Introduction, 1.4.3.1). The interplay between light intensity and temperature on structural adaptation, however, is still poorly understood. It is believed that part of the plant's low temperature adaptive strategy is to alter thylakoid membrane fluidity by synthesising thylakoids with lower protein/lipid ratios (Chapman et al., 1983). The first part of this chapter correlates observed structural differences induced by different growth conditions with the State 1 - State 2 transitions exhibited by pea leaves at room temperature. Because of the proposed mechanisms involved in bringing about this short-term adaptive mechanism to light quality, which requires the phosphorylation and dephosphorylation of LHC (see section 1.7; Chapters 4 and 7), it may be expected to depend on a number of structural factors which 1) control the balance of kinase and phosphatase activities and 2) affect the rate of intrinsic protein movement.

The second part of this chapter presents chlorophyll fluorescence studies which have been used to investigate structural changes induced by transferring tomato plants from high light to low light growth conditions, and which were carried out at the Glasshouse Crops Research Institute (G.C.R.I.), Littlehampton.

8.1 The Effect of Light Intensity on State 1 - State 2 Transitions and Associated Fluorescence Changes:

Fig. 8.1a shows a State 1 - State 2 transition, as measured by chlorophyll fluorescence, from an attached pea leaf for the particular illumination conditions described in the Materials and Methods (Section 2.2), which involved a 10 minute, light 1 preillumination to drive the leaf into State 1 before the signal was recorded. The slow fluorescence decline observed from intact pea leaves is very similar to that exhibited by the green algae, Chlorella pyrenoidosa (Fig. 7.2). Fig. 8.1b shows the fluorescence signal from a barley mutant which lacks LHC in response to the same changes in light quality which led to the fluorescence trace shown in Fig. 8.1a. This observation reaffirms the LHC-requirement, in higher plants, for the State 1 - State 2 transition to take place as previously shown by Chow et al. (1981b).

Normalisation of fluorescence signals, similar to those shown in Fig. 8.1a, to the State 1 (maximum fluorescence) level for pea leaves grown at different light intensities are shown in Fig. 8.2. It can be seen that the extent and kinetics of the State 1 - State 2 transition are altered by growing the plants under different light regimes, so that low light grown plants (LLP) have the ability to respond more quickly to changes in light quality than those plants grown under high light (HLP).

The change from State 1 to State 2 is expected to be accompanied by an alteration in the redox state of the PQ pool. As the input of quanta to the two photosystems becomes better balanced then the PQ pool should become more oxidised, as a result of an overall increase in electron transfer rates. Using the method described in the Materials and

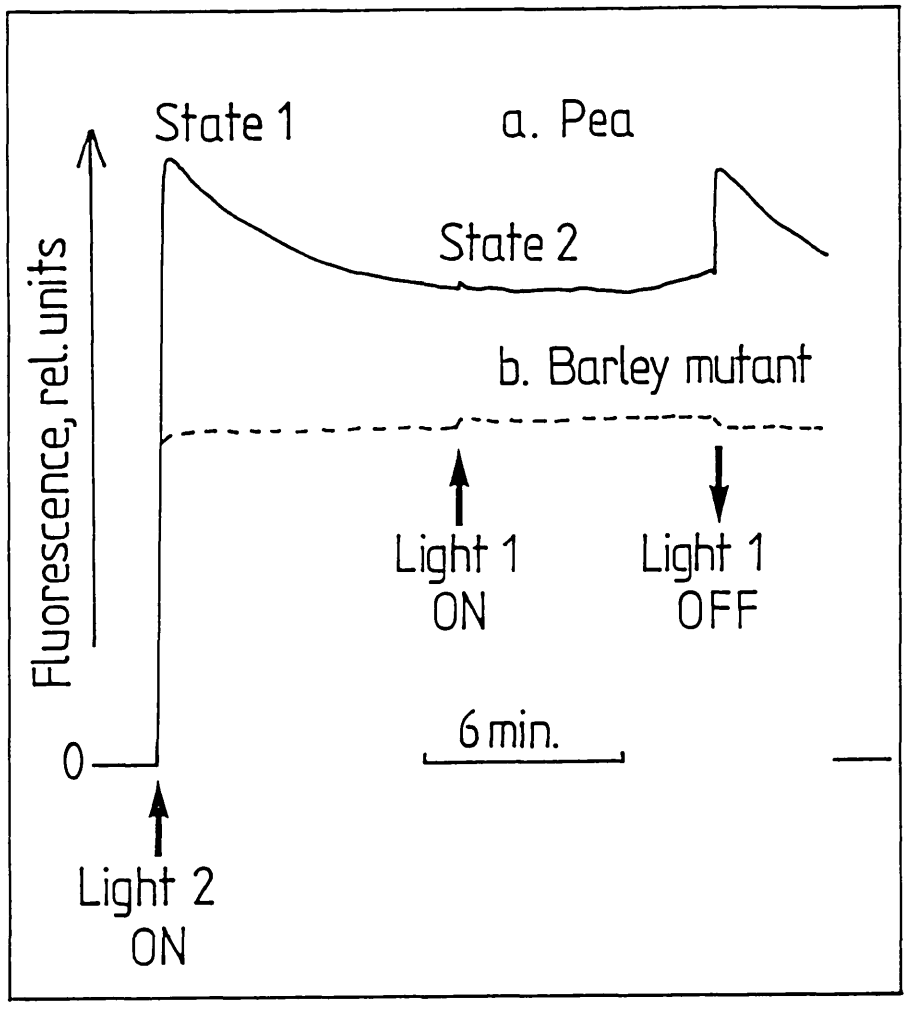


Fig. 8.1 Relative changes in the yield of room temperature modulated chlorophyll fluorescence yield from a) attached pea leaves (——) and b) detached leaves from an LHC-lacking barley mutant (---). Illumination conditions used were as described in 2.3.7.

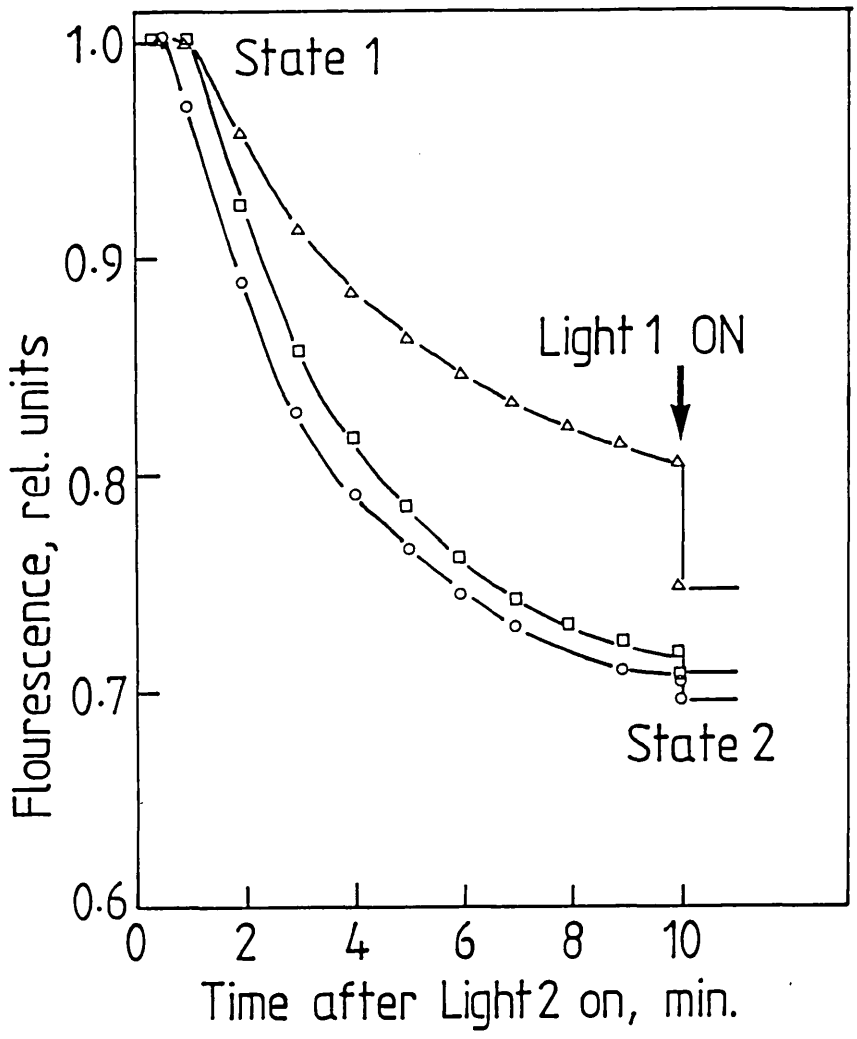


Fig. 8.2 Relative changes in the yield of room temperature modulated chlorophyll fluorescence, indicative of a State 1 - State 2 transition, from attached pea leaves from plants grown at 17°C and under light intensities of 70Wm^{-2} (△); 50Wm^{-2} (□) and 20Wm^{-2} (○). The maximum fluorescence yield at State 1 has been normalised to unity. Each curve is an average of 10 replicates.

Methods (Section 2.3.8), the redox state of the PQ pool was estimated during the State transitions of attached pea leaves of plants grown under three different light regimes. Fig. 8.3 shows the anticipated relationship between PQ redox state and the State 1 - State 2 transition, indicating that LLP were capable of reoxidising their PQ pool, at limiting light intensities, faster than either the HLP or medium light grown plants (MLP).

Fig. 8.4 shows the fast change in fluorescence exhibited by leaves from pea plants grown under each light intensity investigated, which had undergone adaptation to State 1 by the 10 minute, light 1 preillumination used before measuring the State transitions shown in Fig. 8.1 and 8.2. The kinetics of these fast signals indicate the rate of intersystem electron flow through the PQ pool and will depend on the rate of PQ reduction and oxidation. The fluorescence signals presented in Fig. 8.4, which have been normalised to the maximum level, show that the LLP are able to reduce their PQ pool faster than the MLP and HLP indicating possible differences in electron transfer capacity.

Fig. 8.5 shows fast fluorescence transients from pea leaves located at different heights down a HLP canopy. The leaves were dark-adapted before illumination and the fluorescence signal generated was detected via a transient recorder using the equipment detailed in section 2.3.6 and Fig. 2.1. It can be seen that leaves situated at the bottom of the canopy (Bottom), which would have received lower light intensities enriched in far red wavelengths, produced the fastest and highest fluorescence transients when compared with leaves located at the top of the plant canopy (Top), which would be "normal" HLP leaves. These differences are very similar to the characteristics shown in Fig. 8.4 for the

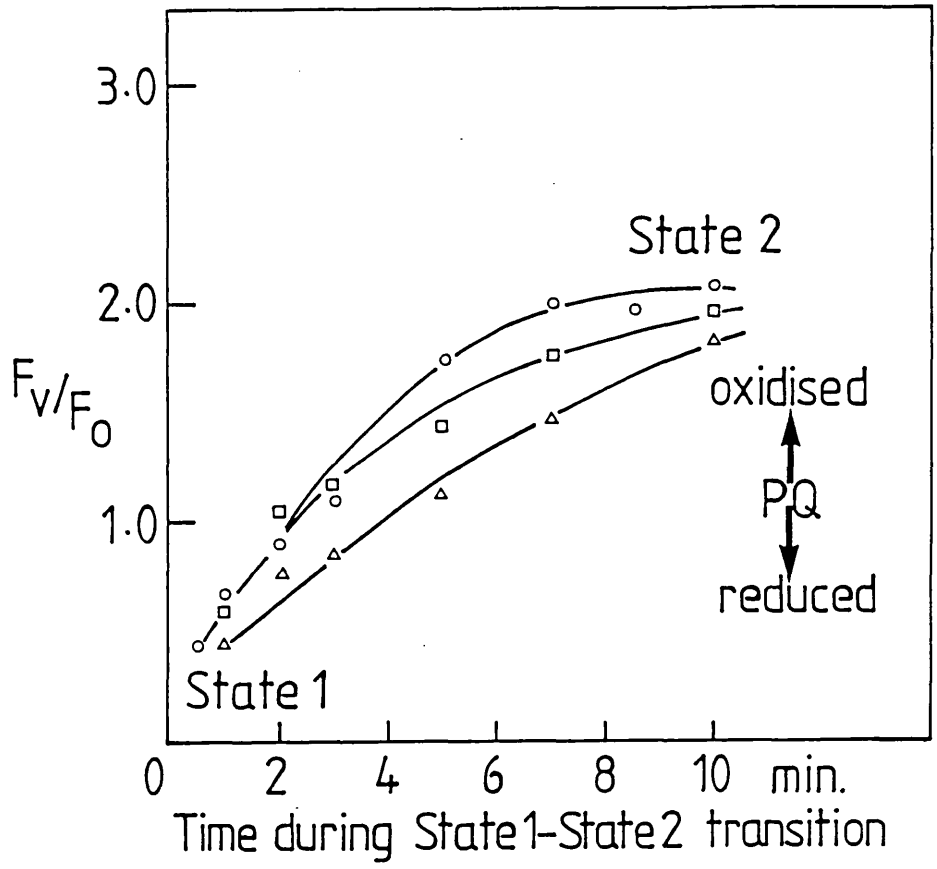


Fig. 8.3 Changes in the redox state of the PQ pool, as estimated by the F_v/F_o ratio, during the State 1 - State 2 transitions exhibited at room temperature by attached pea leaves grown at 17°C under high (△), medium (□) and low (○) light intensities. The method used to determine PQ redox state was as described in 2.3.8.

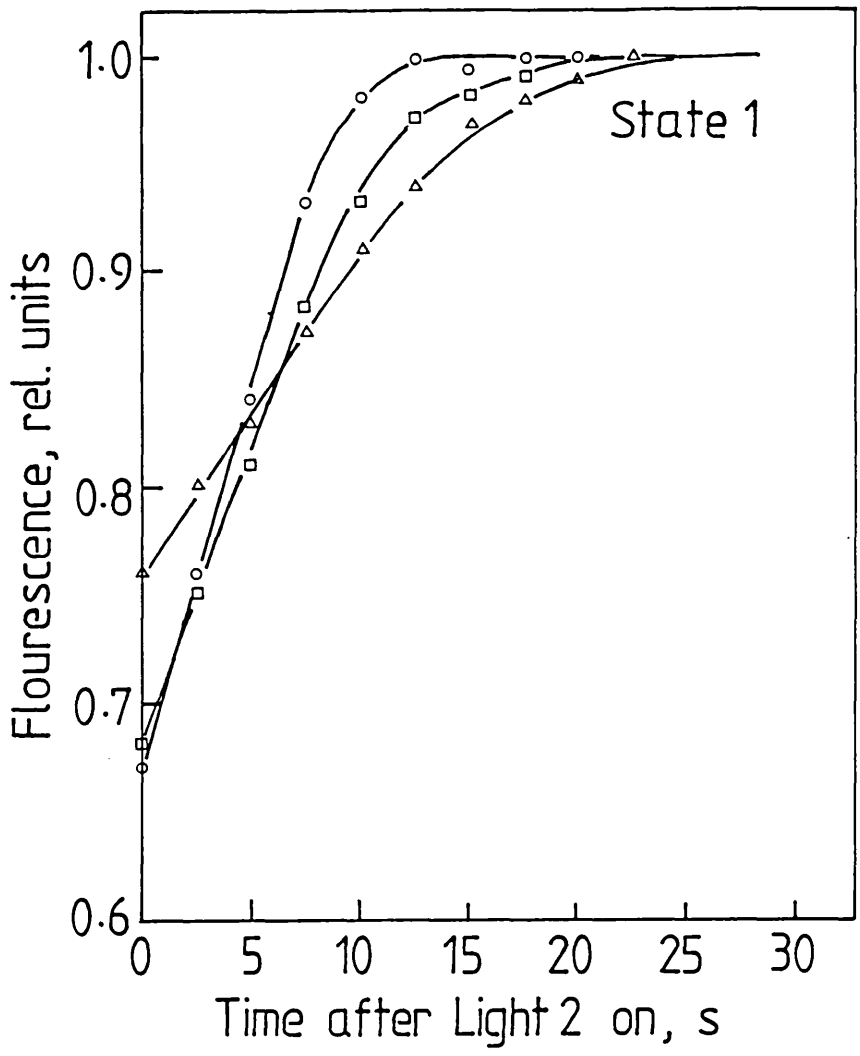


Fig.8.4 The fast chlorophyll fluorescence rise exhibited at room temperature by attached pea leaves grown at 17°C and under high (△), medium (□) and low (○) light intensities, after a 10 minute light 1 pretreatment. The maximum fluorescence yield has been normalised to unity. The time taken to reach the State 1 fluorescence level in low-, medium- and high-light grown plants was 17s, 21s and 27s respectively.

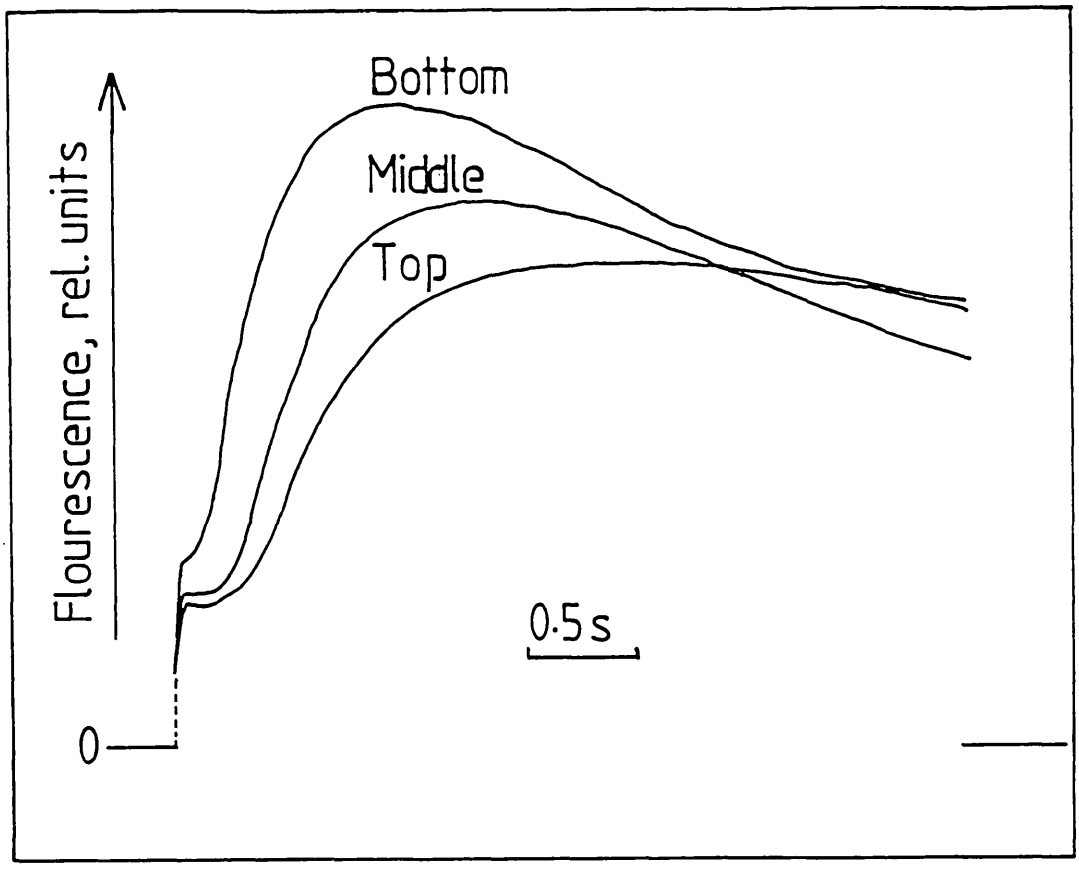


Fig. 8.5 Room temperature fast chlorophyll fluorescence transients exhibited by detached pea leaves grown at 17°C under high light. The three traces show the fluorescence signals from the 3rd (Top), 2nd (Middle) and 1st (Bottom) leaf pairs down the pea canopy. All leaves were dark adapted for 5 minutes before measurement.

top leaf pair of plants grown at different light intensities and are indicative of faster rates of PQ reduction in the shade leaves.

The photosynthetic rates, based on leaf area, exhibited by HLP and LLP were measured as a function of excitation light intensity using a leaf oxygen electrode. Fig. 8.6 shows that at high excitation energies the HLP produced faster rates of photosynthetic O_2 evolution when compared with LLP, while at limiting light intensities HLP and LLP exhibited similar rates.

The effect of light intensity during growth on chloroplast ultrastructure was investigated by analysing thin section electron micrographs. Fig. 8.7 shows representative micrographs of *in vivo* chloroplasts from HLP and LLP plants. It can be seen that no starch was found in the LLP chloroplasts, while the HLP chloroplasts apparently contained less thylakoid membrane. In order to quantify any differences in thylakoid organisation between the two growth conditions a number of parameters were measured (see Table 8.1) including the frequency of grana of different widths (Fig. 8.8), which emphasise the different arrangement of thylakoid membranes between HLP and LLP.

8.2 The Effect of Temperature on the State 1 - State 2 Transition:

The interplay between light intensity and growth temperature has also been investigated in respect to the plant's ability to undergo State 1 - State 2 transitions. Fig. 8.9 shows normalised State 1 - State 2 transitions, measured at room temperature, from attached pea leaves of plants grown at either high or low light intensities at low temperatures (4-7°C). It can be seen that very little difference is

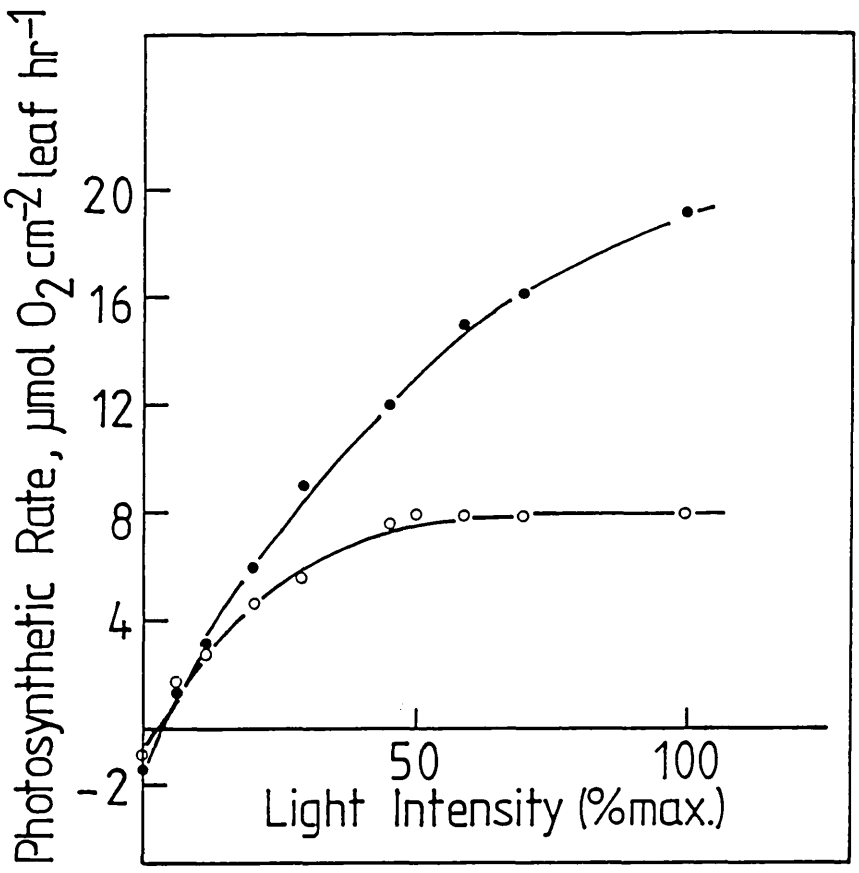
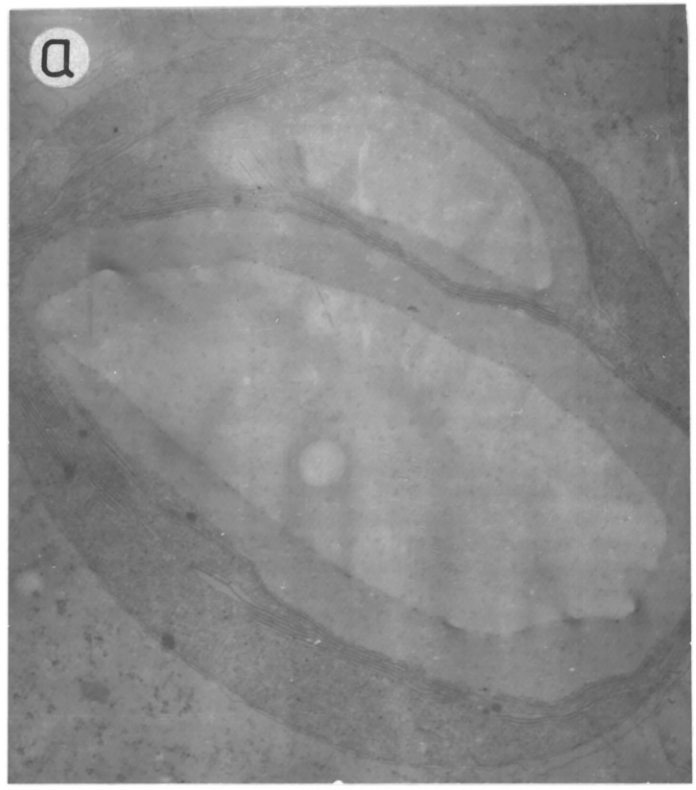
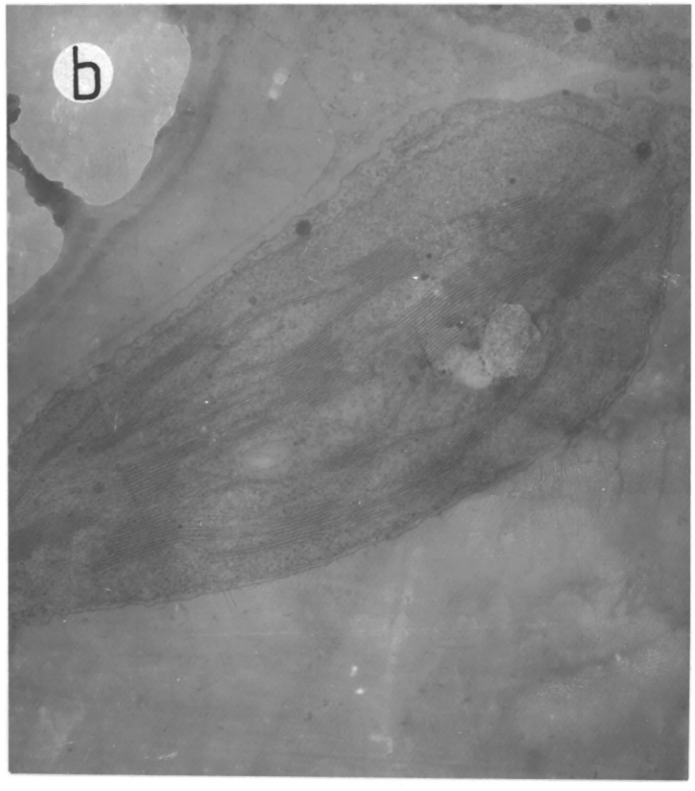


Fig. 8.6 Light saturation curves of photosynthetic O_2 evolution from detached pea leaves of plants grown at 17°C and under high (●) and low (○) light intensities. The leaves were illuminated with white light of different intensities at 21°C and O_2 was detected using a Hansatech leaf oxygen electrode. 100% intensity was 200Wm^{-2}.

Fig. 8.7 Representative electron micrographs of intact pea chloroplasts situated in the leaves of plants grown at 17°C and under a) high and b) low light intensities. Magnification x20830.


a


b

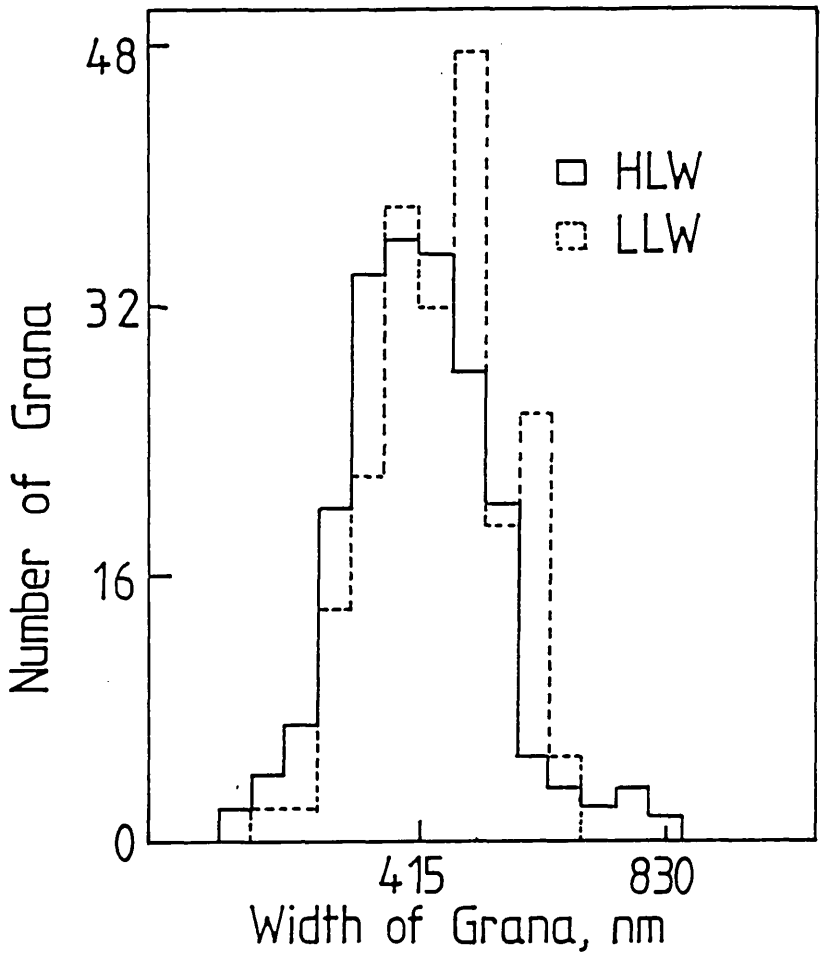


Fig. 8.8 Histogram of the frequency of grana of different widths from high light(HLW) and low light (LLW) pea chloroplasts grown at 17°C. The values were obtained by measuring the granal widths from 12-16 different chloroplasts.

found in their ability to carry out a State 1 - State 2 transition, with both exhibiting similar fluorescence changes to those produced by LLP grown at 14-17°C (Fig. 8.2). When similar experiments were carried out on the cold-grown pea plants at 4°C, instead of room temperature, only a very small fluorescence decline took place and the leaves could only produce a low, State 2 fluorescence signal after the 10 minute, light 1 pretreatment (data not shown). This observation tends to suggest that the chloroplasts are usually in State 2 under normal lighting conditions and that at 4°C the State 1 - State 2 transition becomes inoperative or slowed down.

Fig. 8.10 shows representative electron micrographs of HLP and LLP chloroplasts from pea plants grown at 4-7°C. Only the HLP (cold) chloroplasts contained starch, similar to that seen for the HLP (warm) plants, but there was no obvious large differences in the amount of thylakoid membrane present or the degree of membrane stacking between the high and low light cold-grown plants (see Table 8.1 and Fig. 8.11).

A comparison of the State 1 - State 2 fluorescence changes with structural parameters associated with the chloroplasts present in leaves from plants grown under the different environmental growth conditions are shown in Table 8.1. The major differences in both State transitions and chloroplast ultrastructure can be seen in the warm grown, HLP when compared with the other three growth conditions. It can be seen that the slowest and smallest State 1 - State 2 transitions can be associated with those chloroplasts which contained the smallest degree of thylakoid stacking.

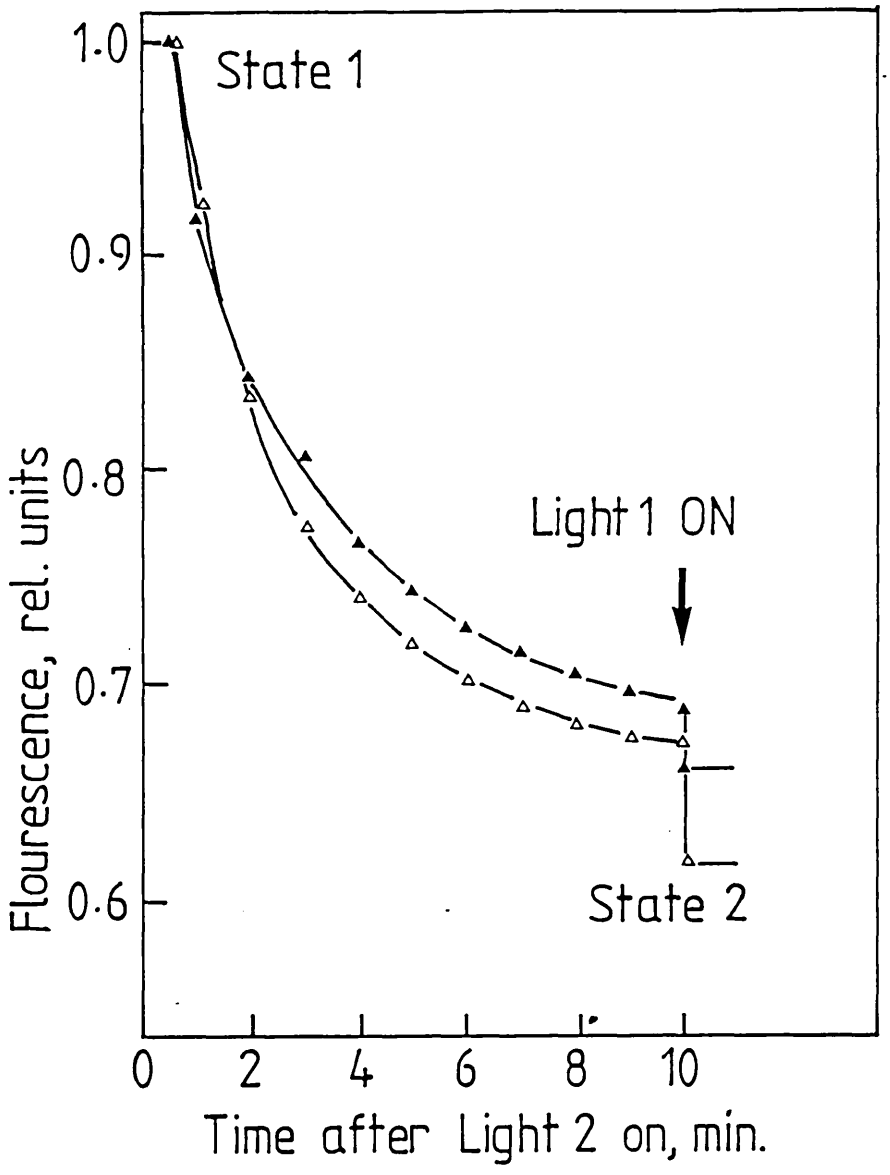
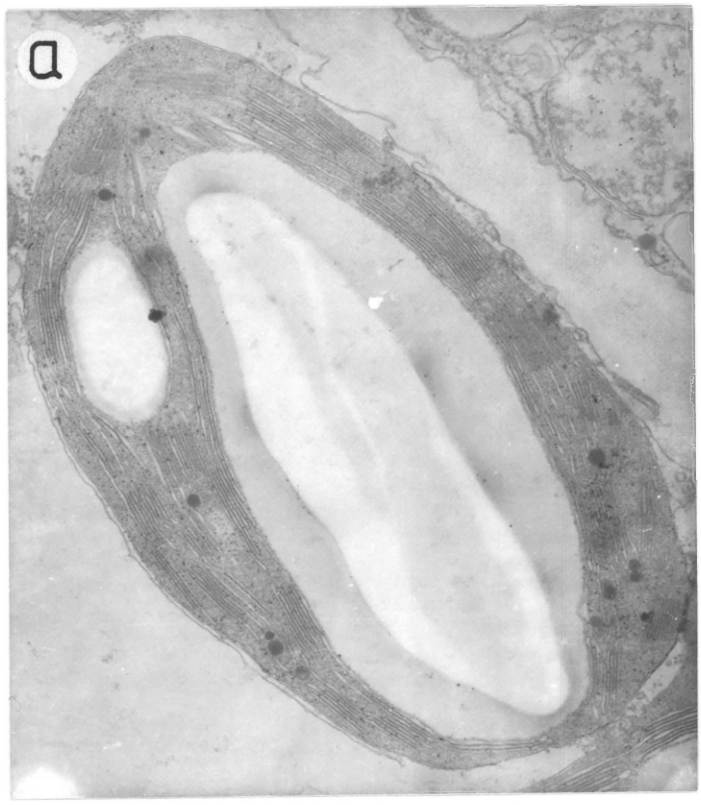
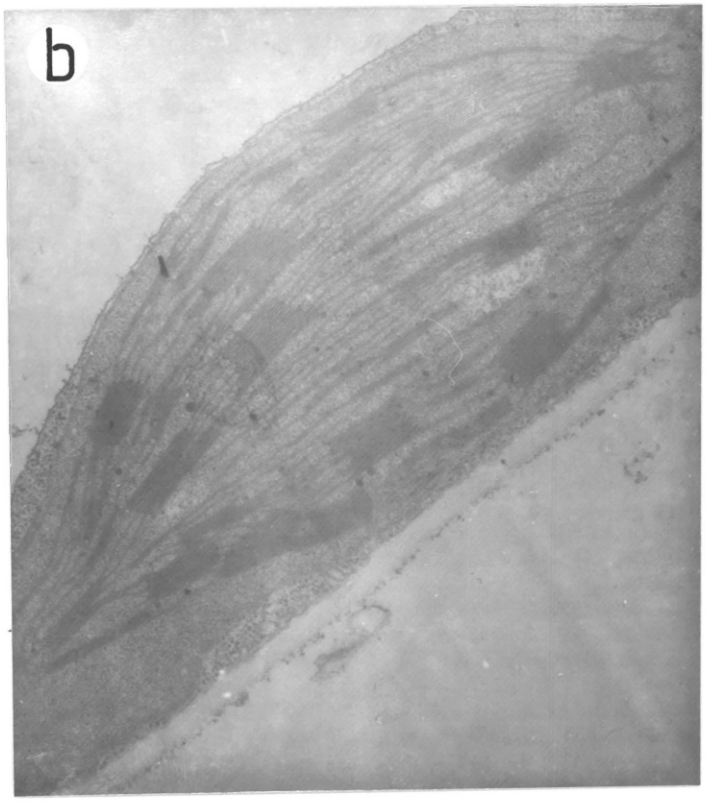


Fig. 8.9 Relative changes in the yield of room temperature modulated chlorophyll fluorescence, indicative of a State 1 - State 2 transition, from attached pea leaves grown at 4°C under either high (△) or low (▲) light intensities. The maximum fluorescence yield at State 1 has been normalised to unity.

Fig. 8.10 Representative electron micrographs of intact pea chloroplasts situated in the leaves of plants grown at 4°C and under a) high and b) low light intensities. Magnification x20830.


a


b

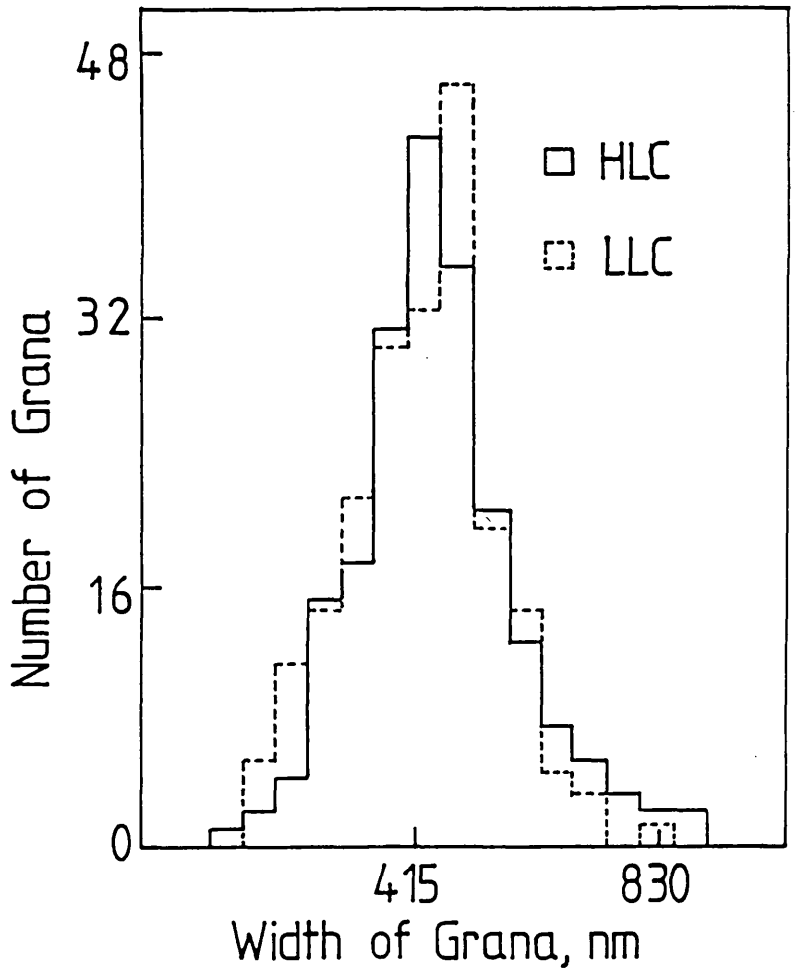


Fig. 8.11 Histogram of the frequency of grana of different widths from high light (HLC) and low light (LLC) pea chloroplasts grown at 4°C. The values were obtained by measuring granal widths from 12-16 different chloroplasts.

TABLE 8.1

Comparison of State 1 - State 2 Fluorescence Changes with Structural Parameters Associated with Different Environmental Growth Conditions

State 1-State 2 Transitions			Chloroplast Ultrastructure			
Growth Conditions	$t_{\frac{1}{2}}$ (min)	% Change	Chla/b ratio	Thylakoid/ Grana	Granal Length (nm)	% Thylakoid Stacked
High light 17°C/14°C	6.2	27	3.5	4.5	447	60
High light 7°C/4°C	2.8	38	3.2	6.4	488	87
Low light 17°C/14°C	2.9	32	3.2	6.7	489	84
Low light 7°C/4°C	2.3	34	3.0	6.5	475	78

8.3 Light Adaptation in Tomato Plants:

The response of tomato leaves following transfer from high to low light regimes suggest an adaptive change which alters the "transferred" plants light-saturated photosynthetic capacity and efficiency as measured by CO_2 exchange (Withers et al., 1984). Such a change only occurred, however, when the leaves were young and still developing (~15% final leaf area) and was not brought about by an altered ribulose 1,5 bisphosphate carboxylase (R5PC) activity (Withers et al., 1984). It was found, however, that the fast fluorescence characteristics of the leaves changed after the low light transfer (Chow et al., 1984).

The fluorescence characteristics of DCMU-inhibited leaves have been measured in both control (maintained under high light) and high to low transferred plants as a function of the time after the transfer to investigate if changes in PS2 organisation were involved in the adaptive process. Fig. 8.12 shows the F_m, F_o and F_v/F_m values for detached, intact tomato leaves from control and "transferred"plants. It can be seen that the main change was in the F_m level exhibited by the "transferred" and control leaves, while the other parameters remained relatively unaltered. Another major difference was found to occur in the rate constant, k_α, which is believed to be determined by the absorption cross section of "connected" PS2 reaction centres (see Chapter 6). Fig. 8.13 shows that, within a day after transfer, this kinetic parameter started to increase in the "transferred" plants reaching a maximum value three to four days after the transfer, indicating that PS2 is indeed involved in the changes taking place during the low light adaptation.

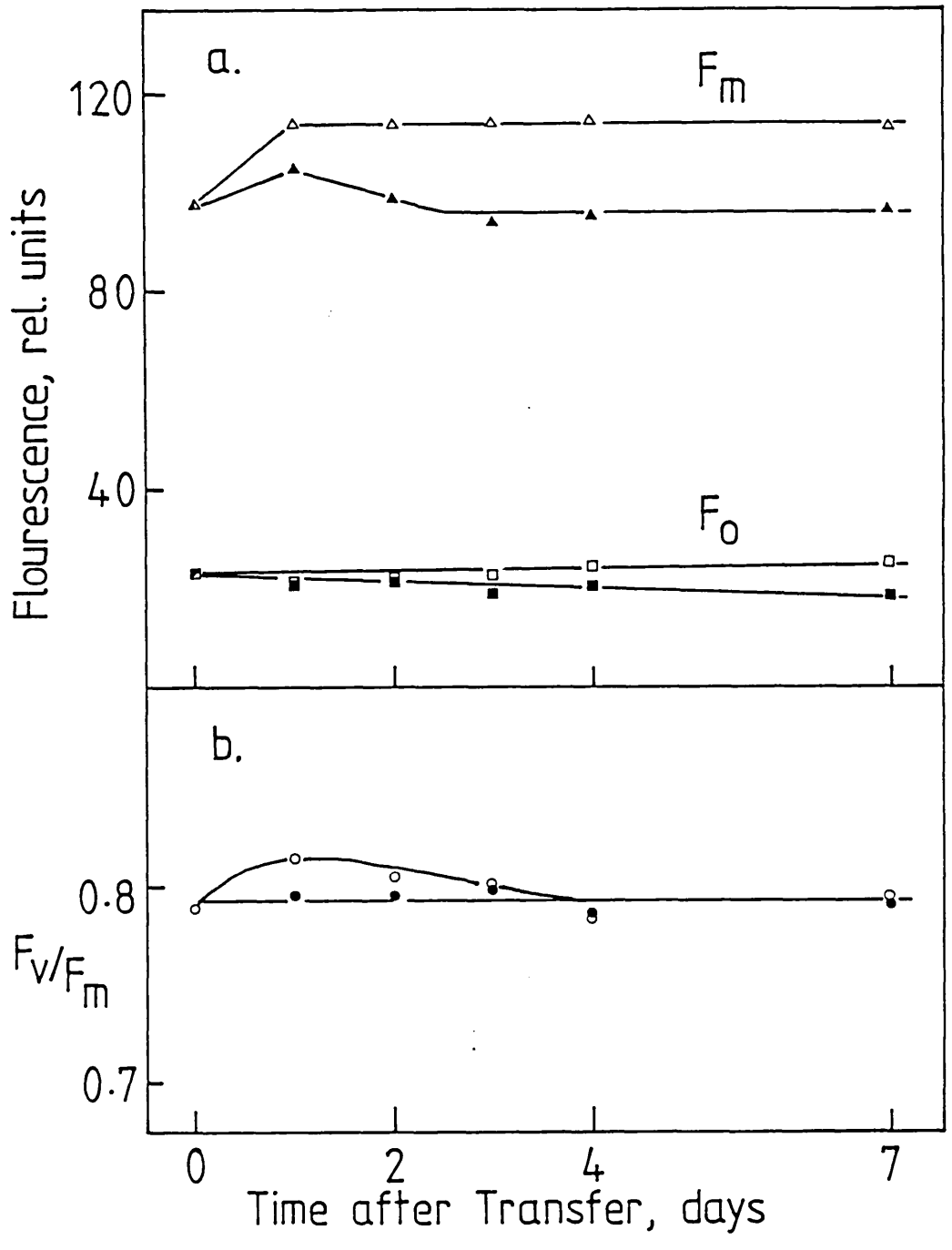


Fig. 8.12 The effect of transfer of tomato plants from growth under high light ($80Wm^{-2}$) to low light ($20Wm^{-2}$) on the room temperature chlorophyll fluorescence induction curve parameters a). F_m and F_o and b) F_v/F_m ratio, exhibited by DCMU-inhibited, detached leaves as a function of time after transfer.
Transferred plants (open symbols); Control plants (closed symbols).

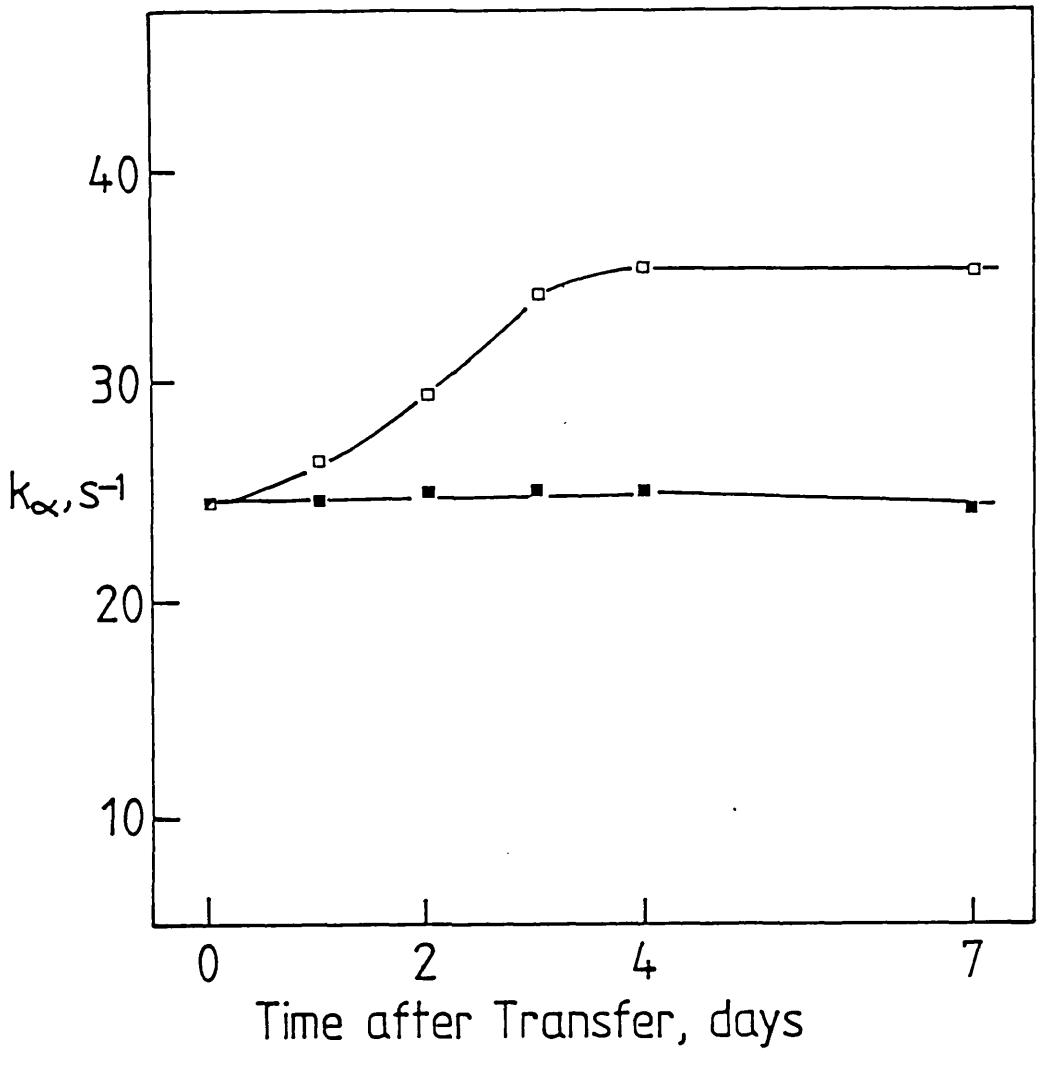


Fig. 8.13 The effect of transfer of tomato plants from growth under high light ($80 Wm^{-2}$) to low light ($20 Wm^{-2}$) on the initial rate constant of the fast kinetic component of room temperature fluorescence induction curves (k_α) exhibited by DCMU-inhibited, detached leaves of transferred (□) and control (■) plants.

Similar experiments were carried out using isolated tomato chloroplasts from both control and "transferred" plants. These chloroplasts were subject to a rapid deterioration after isolation and therefore yielded poor and unreliable fluorescence signals. Table 8.2 shows the chl a/b ratios and various fluorescence parameters obtained from these tomato chloroplast preparations. It can be seen that the F_v/F_m ratios exhibited by the "transferred" chloroplasts were quite low perhaps indicating an inability to fully oxidise Q_A in the dark-time given before illumination, leading to an incorrect F_o value. However, it is interesting to note the lower chl a/b ratios and F_m levels which are produced by the "transferred" plant's chloroplasts, again indicative of an alteration in PS2 taking place after the transfer to low light.

8.4 Discussion:

The chlorophyll fluorescence changes exhibited by intact pea leaves induced by light 1 and light 2 (Fig. 8.1a) are similar to those shown by Chlorella pyrenoidosa in Chapter 7 and therefore probably reflect the same mechanism involving the phosphorylation of LHC and changes in photosystem absorption cross section (see 1.7 and Chapters 4 and 7). The requirement for LHC, in higher plants, in allowing this adaptive process to take place is evident from Fig. 8.1b (see also Chow et al., 1981b). Fig. 8.2 shows that as a consequence of photosynthetic adaptation to light intensity during plant growth, the leaves exhibit different State 1 - State 2 transitions both in extent and kinetics. Furthermore, it is seen from Fig. 8.3 that the expected change in PQ redox state followed the observed kinetics of the State 1 - State 2 transitions (Fig. 8.2). This reoxidation of the PQ pool indicates that on going from State 1 to State 2, light 2 becomes more effective in

TABLE 8.2

The Effect of High to Low Light Transfer on Various Fluorescence Parameters and chl a/b Ratios

	day after transfer	F_o	F_m	A_{max}	k_α (s^{-1})	F_v/F_m	chl a/b
control	0	21.8±0.3	73.5±0.8	1980	22.6±.5	0.70	4.07
transfer	1	22.5±1.0	56.4±1.6	1260	25.2±.5	0.60	3.69
control	2	24.6±0.4	75.6±2.2	1920	21.5±.8	0.67	4.18
transfer	3	19.8±0.4	44.2±0.8	1160	23.6±.3	0.55	3.54

driving electron transfer between the two photosystems.

The LLP not only exhibited the fastest and largest State transitions but from Fig. 8.4 it can be seen that they also produced the quickest rise in chlorophyll fluorescence after the 10 minute, light 1 pretreatment. This difference indicates that LLP are capable of photoreducing their intersystem electron transfer components more readily than HLP. This would depend on a number of structural factors which influence the balance of electron flow into and out of the PQ pool, and is therefore highly dependent upon PS2- and PS1-mediated electron flow. The observed differences between HLP and LLP could be brought about by different electron transfer capacities at limiting light, different sized pigment-beds associated with the two photosystems, altered PS2/PS1 ratios or altered PS2/PQ ratios. The first possibility does not appear to be the case, because little difference in photosynthetic oxygen evolution can be seen from Fig. 8.6, between HLP and LLP, at limiting light. Only at the higher light intensities was the characteristic difference in photosynthetic capacity between HLP and LLP observed.

The data obtained from the electron micrographs (Figs. 8.7, 8.8 and Table 8.1), however, indicate that the LLP contain more thylakoid membranes which exhibit a greater degree of stacking compared with the HLP. Such differences imply that the LLP contain either a higher PS2/PS1 ratio or perhaps more chlorophylls associated with PS2 reaction centres. These differences have been reported in the literature along with less intersystem electron transfer components like PQ and cyt f being located in the LLP (see reviews by Boardman, 1977 and Bjorkman, 1981). Therefore it seems likely that these structural changes may help explain the differences seen in Fig. 8.4 and Fig. 8.5, in that the

LLP are capable of trapping more light 2 which leads to an over reduction of PQ because of a greater imbalance between PS2 electron input to PQ and PS1-mediated electron removal from PQ.

Pea plants grown at 4-7°C, however, do not exhibit such large differences in their ability to undergo a State 1 - State 2 transition at room temperature when grown under different light intensities (see Fig. 8.9). This may suggest that any low temperature-induced changes in chloroplast structure may override the differences usually imposed by high and low light regimes. It is not surprising, therefore that the chloroplasts from the cold grown HLP and ^{L}LP showed very little ultrastructural differences (Figs. 8.10, 8.11 and Table 8.1). The chloroplasts were, in fact, similar to those found in the warm grown LLP in terms of the degree of thylakoid stacking, granal width and number of thylakoids per granum.

It therefore seems that the in vivo mechanism for adaptation to changes in light quality is highly dependent upon chloroplast ultrastructure. In general terms, pea plants grown under conditions which limit their photosynthetic capacity appear to synthesise chloroplasts containing high levels of thylakoid stacking. Such chloroplasts are then capable of undergoing larger and faster State 1 - State 2 transitions compared with chloroplasts found in pea leaves which do not show low photosynthetic capacities at saturating light and which are associated with less stacking and less thylakoid membranes. This relationship between State transitions and chloroplast ultrastructure could be due to a number of reasons which include 1) altered PS2, PS1 and PQ levels, 2) differing amounts of "mobile" LHC, 3) different amounts of kinase and phosphatase enzymes with perhaps altered specific activities

and 4) changes in thylakoid membrane fluidity. The exact reason(s) are still unresolved and require further experimentation.

The transfer of young, developing tomato leaves from high light to low light growth conditions has been previously shown to lead to a decrease in the leaves net photosynthetic capacity after only a few days after the transfer (Withers et al., 1984). Using the analysis of chlorophyll fluorescence induction curves from DCMU-inhibited leaves and thylakoids, information concerning the possible adaptive changes associated with PS2 has been determined.

Fig. 8.12 shows that DCMU-inhibited leaves from "transferred" plants produce higher F_m levels per leaf area with only small changes in F_o and F_v/F_m. This suggests that the level of the fluorescing species has increased, on a leaf area basis, soon after the transfer. These observations are contrary to the work presented by Chow et al. (1984) who found a large increase in F_o with a subsequent decrease in F_v/F_o ratio in unpoisoned "transferred" leaves. This discrepancy arose because of an altered rate or ability of the "transferred" leaves to fully reoxidise Q_A in the short darktime given by Chow et al. (1984) after they had preilluminated the leaves for 4 minutes with light 2. When the "transferred" plants were given a longer dark-time before measuring the fast fluorescence transients the F_o was seen to decrease and no change in F_v/F_o was detected between the high light control leaves and those which had been "transferred".

The most significant change observed in the "transferred" leaves was in k_α, which increased in the "transferred" plants compared with the control tomatos, indicating the synthesis of more PS2 antenna pigments or perhaps a greater PS2 "connectivity" (Fig. 8.13). This

proposed increase is borne-out by the observed decrease in chl a/b ratio and perhaps by the lower F_m levels exhibited by the transferred tomato chloroplasts (see Table 8.2). The decrease in F_m might be expected if the fluorescence signal arises from a back reaction in the PS2 reaction centre between reduced pheophytin and P680 (Breton, 1982). If the chloroplasts synthesise more LHC than the number of PS2 reaction centres per 10μg chlorophyll would be expected to decrease leading to a lower fluorescence signal. However, these changes could also reflect an increase in the level of PS2 compared with PS1 with no alteration in PS2 antenna size. Such observations do show that a relatively rapid adaptive change in PS2 can take place in response to changes in light intensity and from Fig. 8.13 it appears that this process begins almost immediately after the high to low light transfer. Perhaps, the plant attempts to synthesise low light-type chloroplasts (see 1.4.3.1) but this might be complicated by the chloroplasts being able to maintain certain "long-turnover" proteins which have already been synthesised under the high light growth conditions at "high-light" response levels. This could lead to the "transferred" plants containing chloroplasts which are similar in some respects to low light chloroplasts while maintaining certain "high-light" characteristics. Such chloroplasts would, however, be expected to show lower photosynthetic capacities at saturating light intensities.

CHAPTER 9

Atrazine-Resistance

Resistance to triazine herbicides has been shown for a number of different weed biotypes (Pfister and Arntzen, 1979; Arntzen et al., 1982). It is now believed that this can arise because of a single amino acid change in the 32kda herbicide-binding protein associated with PS2, so that in an Amaranthus resistant species a serine becomes converted to a glycine residue (Hirschberg and McIntosh, 1983). It has been proposed that this mutation leads to an alteration in the equilibrium between $Q_A^-Q_B$ and $Q_AQ_B^-$ which slows down electron flow from Q_A to PQ in resistant plants (Bowes et al., 1980; Vermaas and Arntzen, 1983). The consequences of this change appears to be two-fold, in that chloroplast structure and photosynthetic capacity can be affected. Burke et al. (1982) have reported that an atrazine-resistant Brassica sp. produced low-light type chloroplasts exhibiting a large degree of membrane stacking and lower H_2O to DCPIP rates of electron transfer compared with the susceptible chloroplasts. Ort et al., (1983) have also shown inhibited PS2 partial reactions in resistant chloroplasts of Amaranthus hybridus but found no difference in full chain electron transfer rates, however the intact leaves of these plants did show lower rates of CO_2 reduction. In this chapter room-temperature chlorophyll fluorescence has been used to investigate changes in PS2 organisation and function in four different species of atrazine-resistant plants.

9.1 Chlorophyll Fluorescence Changes Associated with Atrazine-Resistance:

Fig. 9.1 shows room-temperature chlorophyll fluorescence fast

transients from susceptible and resistant Amaranthus retroflexus thylakoids suspended in the presence and absence of 10μm atrazine. It can be seen that the addition of atrazine to the resistant thylakoids caused only a small change in the fast fluorescence rise while in the susceptible thylakoids the rise was completely altered to the expected fast fluorescence rise characteristics exhibited by PS2-inhibited thylakoids. Similar observations were made using thylakoids isolated from Poa annua, Chenopodium album and Senecio vulgaris, although differing extents in the change in fluorescence were observed in the resistant thylakoids when atrazine was added, indicating perhaps different degrees of herbicide resistance (data not shown).

Fig. 9.2 shows chlorophyll fluorescence transients exhibited by thylakoids of resistant and susceptible Poa annua in the absence of any PS2 inhibitor. The curves have been normalised to the F_m level of the resistant thylakoids, which was found to be slightly higher than the F_m level of susceptible thylakoids. This figure shows the now well-documented larger extent of the o-i rise in fluorescence exhibited by resistant thylakoids followed by a slightly slower rise to F_m (Pfister and Arntzen, 1979). The unnormalised fluorescence data from these curves are given in Table 9.1. It can be seen that the F_m and F_o levels were higher in the resistant membranes, while the A_{max} was lower. Furthermore, the percentage of the total F_v due to the o-i rise is given for the two types of thylakoid showing the smaller extent of this fast rise in the atrazine-susceptible Poa annua.

Table 9.2 gives various fluorescence parameters obtained by analysing fluorescence induction curves from DCMU-inhibited thylakoids prepared from the four species of atrazine-resistant and susceptible plants

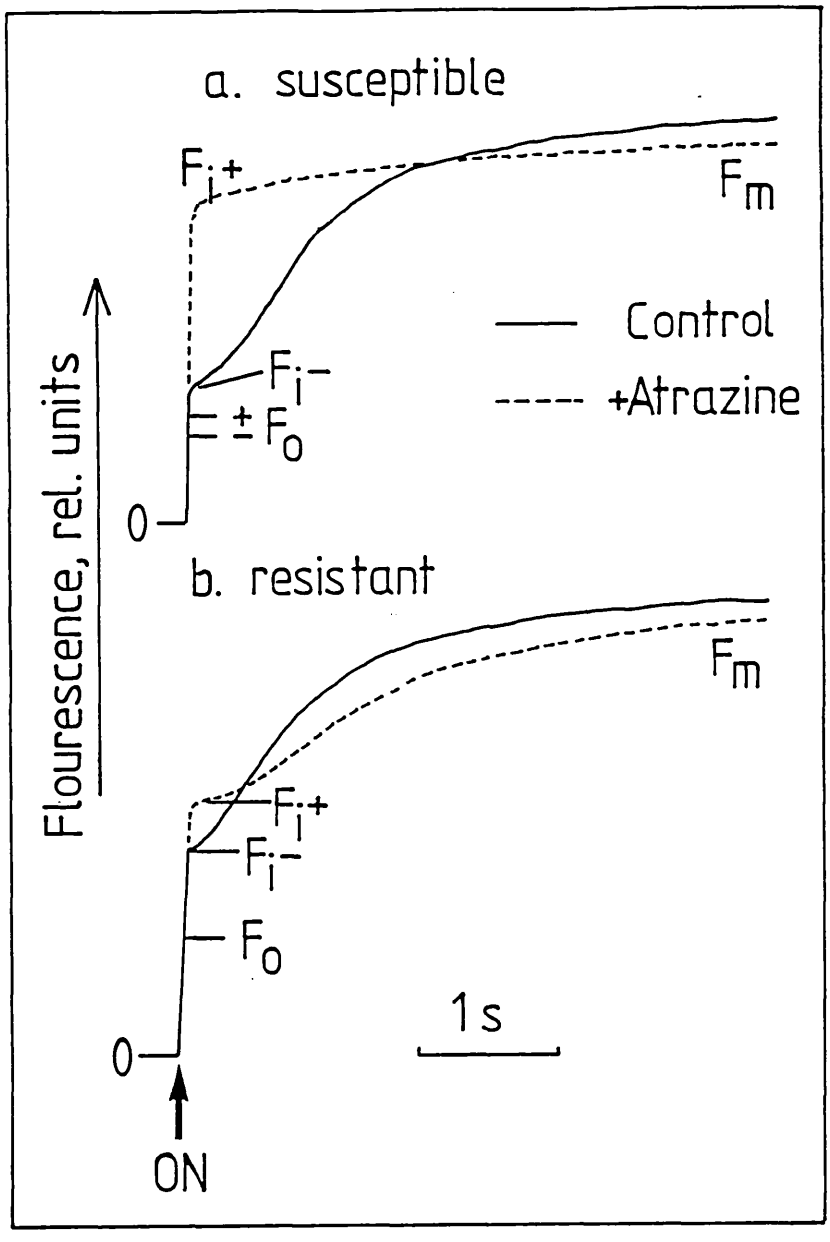


Fig. 9.1 Room temperature fast chlorophyll fluorescence transients exhibited by a) atrazine susceptible and b) atrazine resistant Amaranthus retroflexus thylakoids in 5mM Mg^{2+} and either in the presence (---) or absence (——) of 10μM atrazine.

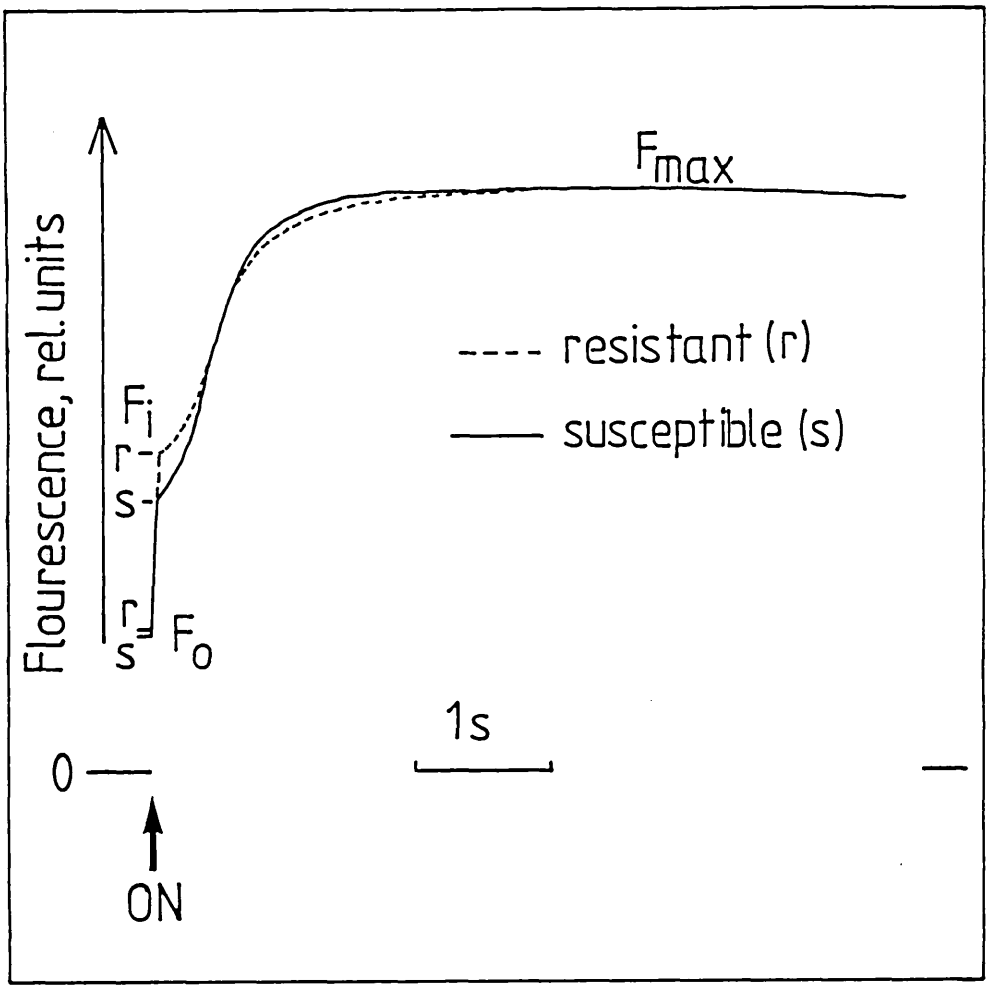


Fig. 9.2 Room temperature fast chlorophyll fluorescence transients of Poa annua thylakoids, suspended in 5mM Mg^{2+}, from atrazine resistant (r) and susceptible (s) plants. The curves have been normalised to an equal F_{max} level, since it was slightly higher in the resistant thylakoids.

TABLE 9.1

Fast Fluorescence Transient Parameters Exhibited by Atrazine-Susceptible (Sus.) and Atrazine-Resistant (Res.) Poa Annua

	F_o	F_i	F_m	A_{max}	F_v/F_m	$\%F_{vi}$
Susc.	19.0	37.7	80.1	4570	0.76	30.6
Res.	20.5	46.6	85.1	3750	0.76	40.5

$\%F_{vi}$ represents the percentage of the F_o to F_i component in the total F_v rise.

already mentioned above. It can be seen that all four species show common differences when a comparison is made between the resistant and susceptible membranes. The resistant plants always produced higher F_m and F_o levels, larger A_{max} and smaller k_α values with very little change in the F_v/F_m ratio. No common observable differences were seen in the parameters k_β and β_{max}, both associated with the slow kinetic component of the fluorescence rise.

Table 9.3 shows the A_{max} values obtained from fast fluorescence transients in the absence of DCMU (A_{max}-) for all four plant biotypes and the ratio of the A_{max} values produced in the absence and presence of DCMU (A_{max}-/A_{max}+). This ratio can be used to estimate the size of the PQ pool and it always gave a lower value for the resistant thylakoids when compared with the susceptible membranes. However, such a comparison only becomes meaningful if no differences occur in the kinetics of PQ reduction and oxidation, therefore any conclusion concerning the PQ pool size might be invalid.

Fig. 9.3 gives the chl a/b ratios for the four species of resistant and susceptible plants investigated. It was always seen that the resistant plants showed a lower chl a/b ratio when compared with the equivalent susceptible plants.

The effect of herbicide resistance on the plant's ability to undergo State 1 - State 2 transitions has been investigated for Chenopodium sp. Fig. 9.4 shows unnormalised fluorescence changes associated with State transitions exhibited by resistant and susceptible leaves. The only obvious difference arises in the fluorescence levels at State 1 and State 2. Table 9.4 gives the average values of various measurable

TABLE 9.2

Various Fluorescence Parameters Exhibited by DCMU-Inhibited Atrazine-Susceptible (Susc.) and Resistant (Res.) Plant Species

Species		F_o	F_m	A_{max}	k_α (s^{-1})	k_β (s^{-1})	β_{max}	F_v/F_m
Poa annua	Susc.	24.0	79.8	1340	48.0	4.1	0.52	0.70
	Res.	26.3	87.9	1730	44.0	3.8	0.55	0.70
Senescio vulgaris	Susc.	17.0	61.1	1280	35.0	4.1	0.49	0.72
	Res.	17.3	66.2	1550	32.0	4.3	0.50	0.74
Chenopodium album	Susc.	22.8	70.7	1070	45.5	3.9	0.66	0.68
	Res.	25.3	77.7	1150	40.0	4.4	0.56	0.67
Amaranthus retroflexus	Susc.	19.0	72.8	1070	48.5	5.3	0.57	0.75
	Res.	21.0	84.6	1540	41.5	5.0	0.55	0.74

TABLE 9.3

The Area Above the Fluorescence Curve in the Absence of DCMU (A_{max}-) and the Ratio of the Area in the Absence and Presence of DCMU (Area-/Area+) in Atrazine Susceptible (Susc.) and Resistant (Res.) Plant Species

Species		A_{max}-	Area-/Area+
Poa annua	Susc.	22835	17.0
	Res.	18735	11.0
Senecio vulgaris	Susc.	24500	19.0
	Res.	18700	12.0
Chenopodium album	Susc.	21500	20.0
	Res.	16500	14.0
Amaranthus retroflexus	Susc.	25180	24.0
	Res.	21055	14.0

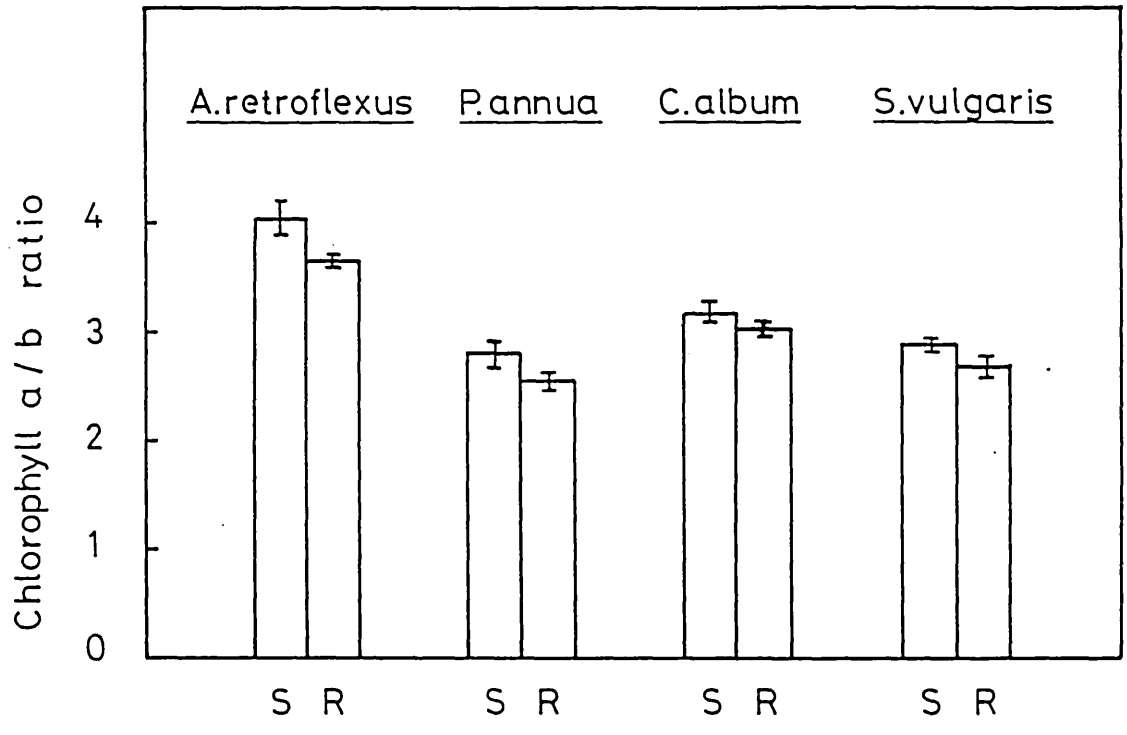


Fig. 9.3 Chlorophyll a/b ratios of atrazine-susceptible (S) and resistant (R) plant biotypes.

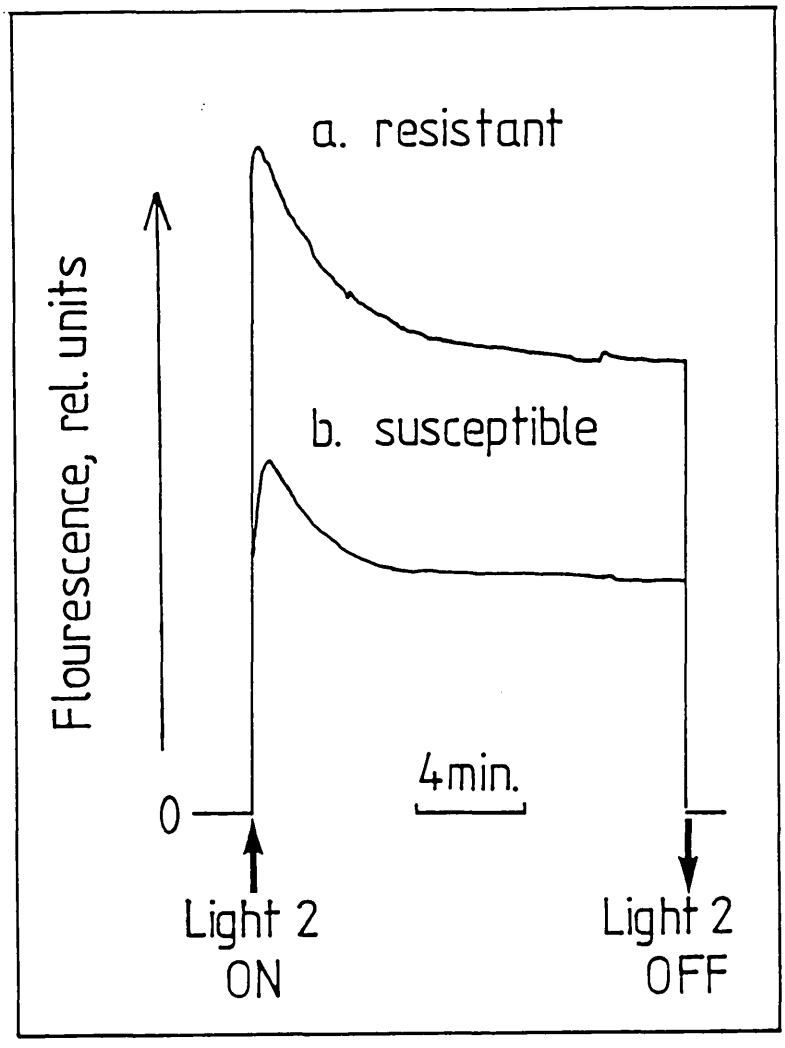


Fig. 9.4 Room temperature chlorophyll fluorescence signals indicative of State 1 - State transitions exhibited by a) atrazine resistant and b) atrazine susceptible Chenopodium album. The illumination conditions used were as described in 2.3.7.

TABLE 9.4

Various State 1 - State 2 Fluorescence Parameters Exhibited by Atrazine Susceptible (Susc.) and Resistant (Res.) Chenopodium album

	Fluorescence Level		% fluorescence change from State 1 to State 2	$t_{\frac{1}{2}}$ (min)
	State 1	State 2		
Susc.	77	47.7	34	2.2
Res.	109	79	28	2.4

parameters associated with fluorescence changes similar to those seen in Fig. 9.4 which shows little difference in extent and kinetics of the State changes between resistant and susceptible Chenopodium album.

9.2 Discussion:

An easy method of screening for resistance to PS2 herbicides in plants is to measure room-temperature chlorophyll fluorescence transients in the presence and absence of the inhibitor. Fig. 9.1 shows that the addition of high levels of atrazine to resistant thylakoids only slightly altered the characteristic fast fluorescence transients observed with uninhibited chloroplasts compared with the large differences seen when similar treatments were carried out on susceptible plant material. The addition of atrazine to susceptible chloroplasts also led to a lower F_m and a higher F_o when compared with those thylakoids which had no herbicide added. The lower F_m arises because in the presence of atrazine the PQ pool becomes oxidised, when Q_A is reduced, which leads to a lowering of fluorescence as a result of PQ quenching (see Vernotte et al., 1979) while in the absence of the herbicide PQ becomes reduced so no such quenching is observed at the F_m level. The higher F_o probably indicates that atrazine is binding to the Q_B-protein which causes a back flow of electrons from Q_B^- to Q_A so Q_A becomes slightly reduced before illumination. These changes are not seen in the atrazine-resistant species suggesting that atrazine is not binding to the Q_B-protein to any great degree even at the high concentrations used. However, atrazine addition did lead to a further increase in the o-i rise and an even slower rise to F_m which might reflect either a small amount of atrazine-binding which becomes easily displaced by PQ or perhaps an inhibition of PQ binding through a competitive binding process (see Velthuys, 1978).

It has been suggested that the mutation leading to atrazine resistance alters the equilibrium $Q_A^- Q_B \rightleftharpoons Q_A Q_B^-$ to the left (Vermaas and Arntzen, 1983). Such a change predicts a higher o-i rise and a slower reduction of the PQ pool indicative of a decreased rate of electron transfer from Q_A to Q_B. These changes are seen in Fig. 9.2 and Table 9.1 in which the larger o-i component and slower rise is presented for Poa annua. Table 9.1 also shows that A_{max} becomes decreased in the resistant chloroplasts infering that less quanta are now required to fully reduce Q_A and PQ. This could arise because of the restricted flow of electrons from Q_A to Q_B, a higher PS2/PQ ratio or an altered PS2 absorption cross section. The lower F_m and F_o levels observed for a given concentration of chlorophyll indeed indicates that in the susceptible-chloroplasts there are more chlorophylls per PS2 reaction centres than in the atrazine resistant thylakoids.

The fluorescence data obtained from the analysis of DCMU-inhibited thylakoids from resistant- and susceptible-plants gives further information concerning small differences in PS2 functioning between the two biotypes. It is again seen from Table 9.2 that in all four plant species investigated the F_m and F_o levels were higher in the resistant species while the F_v/F_m ratio was relatively unaltered, confirming that there appears to be less chlorophyll per PS2 reaction centre in the resistant plants. Accompanying these changes was a decrease in k_α associated with the resistant-thylakoids, again suggesting that there is less chlorophyll attached to "connected" PS2 reaction centres when compared with susceptible plants. These differences might imply that as a result of atrazine-resistance there has been a change in the PS2/PS1 ratio so that the resistant-chloroplasts contain more PS2 to PS1. This is confirmed by the larger A_{max} values obtained with the resistant-

thylakoids suggesting that either more PS2 reaction centres are present or they are associated with less chlorophyll. The lower chl a/b ratios shown in Fig. 9.3 would imply that more LHC is present in the resistant chloroplasts. It might be that an increase in the number of PS2 reaction centres and a small decrease in PS2 absorption cross section have occurred as a result of herbicide-resistance. Such changes could help to compensate for the proposed slower rates of electron flow between Q_A and Q_B, by increasing the amount of Q_A to donate electrons to the PQ pool and by decreasing the rate of exciton capture so as to reduce photoinhibitory processes and to help maintain a similar rate of electron transfer to PS1. However these proposed changes in pigment-protein complex levels did not appear to bring about a noticeable alteration in the extent of thylakoid stacking (data not shown) as reported by Burke et al. (1982).

The difference seen in the ratio $A_{max}-/A_{max}+$ might reflect an increased PS2/PQ ratio in resistant-thylakoids indicating another structural change, however, such a conclusion is probably ill-founded because of the proposed decrease in electron transfer rate from Q_A to Q_B in the atrazine-resistant species.

Atrazine-resistance appears to have only a little effect on the plant's ability to undergo State 1 - State 2 transitions, as measured by room-temperature chlorophyll fluorescence. Both resistant and susceptible plants show quite normal fluorescence changes (see Fig. 9.4) which might infer that there is very little difference in chloroplast ultrastructure between the two cases. However, differences in State transitions due to structural alterations could be masked by the

different rates of electron transfer from Q_A to Q_B which keeps PQ more oxidised and so slow down the State changes because of an altered kinase/phosphatase balance.

CHAPTER 10

Summary and Final Discussion

The aim of this study has been directed towards a clearer understanding of the mechanisms involved in chloroplast adaptation to light. In this final chapter the main conclusions from Chapters 3 to 9 are summarized and interrelated. As a consequence of this questions are raised concerning important unresolved points which require further investigation. This chapter has been divided into four main sections reflecting differing degrees and levels of chloroplast, thylakoid membrane and thylakoid protein structural and organisational change.

10.1 Overall Chloroplast Thylakoid Ultrastructure:

It is well-established that plants are capable of adapting to the various environmental conditions under which they are grown, and that one way this is achieved is by being able to assemble chloroplasts which contain different levels of photosynthetic enzymes and pigment-protein complexes (see Section 1.4.3.1). As a result of these differences, the organisation of the thylakoid membrane system can vary in the extent of membrane appression. This can be easily observed when comparing sun and shade grown chloroplasts (see Boardman, 1977; Lichtenthaler et al., 1981) and to a lesser degree between the high light and low light grown plants used in this study (see Fig. 8.7). It is not surprising that these chloroplasts, with different thylakoid composition and organisation, exhibit different photosynthetic capacities (Fig. 8.6). Furthermore, it is possible to detect such differences, in a non-evasive manner, by measuring the fast chlorophyll fluorescence transients (see Sections 1.3 and 1.4) from dark pretreated leaves

(Fig. 8.5). This fluorescence signal varies according to the rate of electron flow through the electron transfer chain and therefore depends on the levels of PS2, PQ and PS1 present and the pigment-antenna size associated with the two photosystems. Leaves containing highly-stacked chloroplasts (eg. low light grown), contain a greater PS2 to PS1 imbalance and a larger PS2 antenna size. For these reasons they exhibit fast transients, consisting of a large F_v component, because the chloroplast ultrastructure favours Q_A photoreduction. Meanwhile, leaves with chloroplasts containing low amounts of stacking (eg. high light grown) produce slower transients with a smaller F_v component because Q_A reoxidation via PS1-mediated electron flow is more prominent.

Gross chloroplast ultrastructure also appears to be reflected in a plant's ability to carry out short-term adaptive changes in response to alterations in light quality at limiting light intensities as measured by a slow fluorescence decline (termed a State 1 - State 2 transition). From some of the work presented in Chapter 8, which compared the effect of chloroplast ultrastructural change induced by growing pea plants under different light and temperature regimes with the plant's ability to carry out State 1 - State 2 transitions given the same light 1 pretreatment, it can be seen that plants containing chloroplasts with the greater degree of thylakoid stacking exhibit the larger and faster State transitions (see Figs. 8.2, 8.9 and Table 8.1). There are a number of possible reasons why this should be the case, based on the proposed mechanism for the State 1 - State 2 transition (see Section 1.7) and the observed structural differences:

1) Altered levels of PS2, PQ and PS1 between chloroplasts with differing amounts of thylakoid stacking could lead to differing degrees of PQ over-reduction and hence to different kinase activities.

2) Those chloroplasts which contain more thylakoid stacking may

have larger quantities of 'mobile' LHC, leading to a greater capacity for excitation energy redistribution.

3) The differences in chloroplast ultrastructure may lead to thylakoids with altered membrane fluidity, which could affect the rate of LHC movement.

4) The differing degrees of stacked and unstacked thylakoid membranes may reflect different concentrations of the protein-kinase and phosphatase enzymes. Such a distribution may occur if the kinase is located in the granal lamellae and the phosphatase is situated in the stromal lamellae, as might be expected considering the proposed mechanism (see Bennett, 1983). Therefore, highly-stacked chloroplasts would show a greater imbalance between the two enzymatic activities which would lead to an apparently faster State transition.

The observed differences in State 1 - State 2 transition could therefore be due to any one or any combination of the above reasons. Further work is required to locate the enzymes responsible, to determine their activities and to narrow down the important structural factors which could affect this adaptive mechanism. However, the work presented does highlight the fact that environmental conditions can alter a plant's ability to undergo State transitions, and therefore care must be taken when comparing results from different research groups concerning this phenomena.

The State 1 - State 2 transition is a short-term mechanism by which plants can adapt to those environmental conditions which lead to an over-reduction of the PQ pool and hence inefficient use of trapped light energy. This can be brought about by changes in light quality and may be an important adaptive process in leaves situated in the lower parts of a vegetation canopy, where there is not only a reduction in light

intensity but a change in spectral quality in favour of PS1 light. Such shade-type leaves are subject to alterations in lighting conditions during their lifetime, for example by sunflecks, and when this occurs they would require a rapid method of changing energy distribution to maximise photosynthetic efficiency and minimise photoinhibitory processes. Furthermore, such a process is probably required to help chloroplasts located at different depths within a single leaf to adapt to light quality and quantity changes throughout the day.

It also appears, however, that plants are able to adapt their photosynthetic machinery to more permanent changes in environmental light conditions by altering their chloroplast structure and organisation over a period of a few days after the change. This ability only appears to be present in those leaves which are still developing (see Withers et al., 1984) and would suggest the requirement for active protein synthesis. It has been shown that transfer from low to high light leads to an increased photosynthetic capacity while the opposite transfer leads to a decrease in photosynthetic capacity (Withers et al., 1984). Chlorophyll fluorescence techniques have been used in this thesis to investigate the structural and functional changes which might be taking place within the chloroplast in response to the high to low light transfer. Changes in the fast fluorescence transients suggest that the transferred plants begin to synthesise low-light type chloroplasts (data not given), while by analysing fluorescence induction curves in DCMU-poisoned leaves and isolated chloroplasts an idea concerning PS2 organisational changes are obtained. The observed changes in F_m per total chlorophyll (Table 8.2) and k_α (Fig. 8.13) suggest that the transferred plants are rapidly synthesising more LHC, such a conclusion being confirmed by the altered chl a/b ratios (Table 8.2). It appears, therefore, that plants

do not maintain a rigid chloroplast organisation but they are able to synthesise those enzymatic and pigment-containing components which are necessary to survive under either low or high light intensities so as to maximise their photosynthetic efficiency and limit energy wastage.

10.2 Thylakoid Protein Reorganisation:

The State 1 - State 2 transition was initially proposed to be brought about by light-induced ionic movements across the thylakoid membrane (see Barber, 1976), but it has now become evident that this adaptive process reflects the reversible phosphorylation of LHC (see Allen et al., 1981; Telfer et al., 1983; Bennett, 1983; Canaari et al., 1984). This has led to a number of reports and hypotheses comparing the cation-induced and protein-phosphorylation-induced changes (Barber, 1982, 1983; Horton and Black, 1982, 1983; Kyle et al., 1982).

Since the original work by Murata (1969b) and Homann (1969) concerning the salt-induced changes in chlorophyll fluorescence, a number of important observations have been made which has led to the elucidation of the mechanisms involved. These observations include:

a) cation-induced changes in thylakoid organisation and protein arrangement (eg. Izawa and Good, 1966; Murakami and Packer, 1971; Gross and Prasher, 1974; Boardman et al., 1978),

b) a cation-induced change in the distribution of excitation energy between the two photosystems as detected by changes in 77K fluorescence spectra (Murata, 1969b; Murata et al., 1970; Krause et al., 1983) and electron transfer reactions (Murata, 1971; Sinclair, 1972; Sun and Sauer, 1972; Marsho and Kok, 1974),

c) non specificity of cations for inducing the above mentioned fluorescence changes (eg. Mills and Barber, 1978),

d) valency dependence of the cation effect for the above changes (Murata, 1971; Barber and Searle, 1978; Barber and Chow, 1979),

e) the antagonistic effect between monovalent and divalent cations in bringing about the changes in chlorophyll fluorescence and membrane stacking (Gross and Hess, 1973, 1974).

Such observations led to the proposal that the cation-induced changes reflected alterations in the spillover of excitation energy from PS2 to PS1, due to alterations in membrane conformation. It was argued that these conformational changes reflected alterations in the electrostatic properties of the thylakoids in response to alterations in the space charge density adjacent to the membrane (see Barber et al., 1977; Barber, 1980a;Barber, 1982). Recently, Melis and Ow (1982) proposed that the cation-induced decrease in fluorescence yield represented a Mg^{2+}-specific effect on connected $PS2_{\alpha}$ centres which led to an increase in thermal de-activation (k_d). Results presented in Chapter 3 do not support this contention but in many ways confirm the earlier reports and postulations concerning thylakoid organisation (Fig. 3.2 and 3.11), PS2 organisation (Fig. 3.7 and Table 3.2), cation non-specificity (Table 3.1 and Fig. 3.4) and excitation energy redistribution (Fig. 3.4).

Room-temperature chlorophyll fluorescence induction curves from DCMU-poisoned thylakoids have also been shown to be cation-sensitive (eg. Homann, 1969; Melis and Homann, 1976, 1978; Bennoun, 1974; Hipkins, 1978; Melis and Ow, 1982). It is agreed that the absence of "high" background cation levels leads to an altered kinetic profile of the fluorescence rise and a large decrease in fluorescence yield. A significant decrease in F_o is not, however, always reported, although it was observed in this present work (see Fig. 3.1 and Table 3.1). Chapter 3 produces several interesting conclusions concerning the salt-induced changes as a result

of a careful analysis of the $MgCl_2$ titration of fluorescence induction curves. Firstly, the large difference in F_o between thylakoids suspended in the presence of either "high" or "low" cation concentrations appears to be due in part to a conformation-induced difference in a DCMU-stimulated reduction of Q_A before measurement probably via the reaction $Q_A Q_B^- + DCMU \rightarrow Q_A^- Q_B DCMU$, this process being more prominent when thylakoids are stacked (Table 3.3 and 3.4). Secondly, the mechanism involved in bringing about the Mg^{2+}-induced changes is probably more complicated than originally proposed by Barber (1980a). For instance, the difference in sensitivity of F_v and F_o to Mg^{2+} concentration indicates that two independent control mechanisms might be involved (Fig. 3.6). A possible model to explain the effect of low Mg^{2+} levels (<0.5mM) on fluorescence is one in which PS2-LHC coupling is reduced due to increased coulombic repulsion controlled by the local surface charge characteristics of the neighbouring protein-complexes. In contrast the changes caused by lowering the Mg^{2+} concentration from a saturating level are probably brought about by an enhanced PS2-LHC-PS1 interaction as a result of protein randomisation which is governed by the overall surface charge characteristics of the thylakoid membrane. This interpretation of events is partially confirmed by the DBMIB-titration of fluorescence results shown in Fig. 3.10 and Table 3.5, in which it was observed that between 5mM and 0.33mM $MgCl_2$ the changes could be explained by alterations in spillover while below 0.33mM $MgCl_2$ other events take place which disrupt the predicted relationship between fluorescence and PS2-DBMIB quenching.

Henkin and Sauer (1977), using a modulated fluorescence technique, found very similar results to those shown in Fig. 3.6a and 3.6b, in that F_B ($\equiv F_o$) saturated at 0.5mM Mg^{2+} while F_v saturated at 2.5mM Mg^{2+}. They

interpreted their results solely in terms of changes in PS2 absorption cross-section. Bennoun (1974) and Butler (1978), from fluorescence analyses, and Jennings (1984), using DBMIB-titration and preferential chl b excitation studies, have all proposed that the cation-induced changes in energy distribution reflect alterations in both spillover and PS2 absorption cross section, as proposed in this work. On the other hand, Nairn et al. (1982) have suggested, as a result of the changes in picosecond fluorescence decay kinetics, that there is an increase in spillover on lowering the Mg^{2+} level but at very low cation concentrations (0-0.75mM $MgCl_2$) there is a decreased PS2-PS1 interaction. Such results all confirm, however, that the cation-induced changes are more complicated than first suggested.

Protein-phosphorylation-induced changes are also believed to be under electrostatic control. The additional negatively charged phosphate groups attached to protein surfaces are expected to alter the electrostatic balance between adjacent surfaces by increasing the surface charge density of exposed segments of protein. This series of events would increase the coulombic repulsion between these surfaces and neighbouring proteins causing perhaps the lateral displacement of the phosphorylated species (see Barber, 1980a,1982a,1983). However, very little evidence has been produced which shows this proposed increase in surface charge density. The technique of 9 amino acridine fluorescence quenching to probe the change in surface charge density (see Searle et al., 1977; Chow and Barber, 1980a) has produced conflicting reports in that Scoufflaire et al. (1983) observed differences after phosphorylation while Kyle and Arntzen (1983) and Telfer (unpublished observations) did not. Recent work by Telfer, using laser doppler cytopherometry, has also been unable to show any significant changes in surface charge density between phosphorylated and non-phosphorylated thylakoids. On the other

hand, Kyle and Arntzen (1983) concluded from studies utilising ferricyanide-induced Q_A reoxidation that surface charge density changes do occur in a region localised around PS2, in response to protein-phosphorylation. It has been argued that the change in surface charge density brought about by phosphorylation may be too small to be detected by the techniques available, even though a large number of thylakoid proteins appear to be phosphorylated (see Telfer et al., 1983; Steinback et al., 1982). However, the requirement for higher background cation levels to be present when thylakoids are phosphorylated to maintain similar degrees of thylakoid organisation (Fig. 4.8), similar fluorescence levels (Fig. 4.2 and 4.4) and similar PS2-DBMIB interactions (Fig. 4.7) to those exhibited by non-phosphorylated thylakoids, indeed suggests that the surface charge density of phosphorylated membranes is greater than that of non-phosphorylated membranes.

There has also been confusion in the literature concerning whether the phosphorylation induced changes in excitation energy distribution are brought about by alterations in spillover (as originally assumed by Barber, 1982a), PS2 absorption cross section (as proposed by Horton and Black, 1983), non-radiative decay processes (Haworth and Melis, 1983) or any combination of these processes (Kyle et al., 1982; Haworth et al., 1982a). Work by Kyle et al. (1982), Haworth et al. (1982) and Haworth et al. (1983), using both room temperature and 77K fluorescence induction techniques and analyses, suggested that both changes in spillover and absorption cross section were involved when phosphorylation was carried out at 5mM $MgCl_2$. Horton and Black (1982, 1983), on the other hand, proposed that at 5mM Mg^{2+}, phosphorylation led to a decrease in PS2 absorption cross section while at 1mM Mg^{2+}, it also led to changes in spillover. While Haworth and Melis (1983) were unable to show any increase in PS1 absorption cross section after phosphorylation and proposed

that the rate constant for thermal deactivation was altered. This latter point, however, has been contradicted by reports suggesting an increase in PS1 absorption cross section after phosphorylation as measured by increased PS1 electron flow rates (Horton and Black, 1982; Farchaus et al., 1982) and from flash-induced ΔA_{820} measurements indicative of P700 oxidation (Telfer et al., 1984). The results shown in Chapter 4 confirm that at 5mM Mg^{2+}, phosphorylation leads to changes in the fluorescence induction curve parameters indicative of a change in absorption cross section (Fig. 4.2 and 4.4) while phosphorylation at lower Mg^{2+} levels ($\leq$ 2mM) leads to changes which suggest an increase in spillover and a change in absorption cross section. The DBMIB-titration data, however, (see Fig. 4.5 and 4.6) indicates that even at 5mM $MgCl_2$, phosphorylation produces a small increase in spillover which is not observed after phosphorylation at 10mM $MgCl_2$ (Fig. 4.5). It is therefore concluded that the extent of the protein-phosphorylation-induced changes in gross thylakoid organisation (Figs. 4.8, 4.9 and Table 4.4) and chlorophyll fluorescence (see Fig. 4.2 and 4.4) depends on the background level of 'screening' cations. At "high" cation concentrations, when screening is high, the phosphorylation-induced changes are less extensive and probably represent the proposed lateral migration of a "mobile" pool of phosphorylated LHC from granal to stromal lamellae (see Kyle et al., 1983$_b$,1984). As the background cation-level is lowered, the capacity to screen the additional surface charge diminishes which allows a greater randomisation of pigment-protein complexes to take place, thylakoids destack, and subsequently leads to an increase in spillover.

The results of Chapters 3 and 4 also emphasise that the cation and protein-phosphorylation induced changes in excitation energy distribution and fluorescence yield are independent but interrelated processes linked

by the common electrostatic nature of their mechanisms. Such conclusions showing that the two processes reflect different ways in bringing about energy redistribution have been put forward recently by Tsala and Strasser (1984) and Gerola et al. (1984). However, Horton and Black (1982), first showed the additive nature of the two phenomena by demonstrating that phosphorylation induced a similar decrease in F_m whether the thylakoids were suspended at 5mM or 0mM Mg^{2+}. This observation has been extended, in this thesis, to show the effect on F_o and PS2-DBMIB interaction when phosphorylated thylakoids are resuspended in the absence of $MgCl_2$. These results infer that an additional absorption cross section component is still present in the phosphorylated membranes even at minus Mg^{2+}. One explanation for such observations would be that even when the thylakoid proteins are totally randomised within the plane of the membrane, phosphorylated LHC is unable to interact with PS2. However, it may be that under minus $MgCl_2$ conditions PS2 is still fully coupled to LHC and that the apparent small change in PS2 absorption cross section detected from the decrease in F_o, after the removal of the DCMU-effect, is solely a consequence of the disconnection of PS2-LHC complexes from neighbouring PS2-LHC complexes.

The important question of how does the *in vitro* phosphorylation experiments relate to the *in vivo* light-quality-induced changes arises. Previous reports in the literature have shown a preferential F_v quenching with a small change in F_o on going from State 1 to State 2 (Bennoun, 1974; Saito et al., 1983). However, the data shown in Chapter 7 would imply that the State transitions are a consequence of protein-phosphorylation at "high" background cation levels and therefore *in vivo* excitation energy redistribution is probably achieved solely by an alteration in the two photosystems absorption cross section, although a small change in spillover cannot be ruled out. The reported Mg^{2+} levels present in

intact chloroplasts (see Portis, 1978) would support the above mechanism in which the *in vivo* changes reflect protein-phosphorylation at "high" salt concentrations. This would be expected to bring about no obvious change in the extent of thylakoid appression, as seen from the representative electron micrographs and structural analyses presented in Fig. 4.8, 4.9 and Table 4.4. Such observations are similar to previously reported changes in membrane stacking of between 5 and 20% upon *in vitro* protein-phosphorylation (Simpson, 1983; Kyle et al., 1983). If, on the other hand, the *in vivo* State changes reflect changes in spillover and absorption cross section then it would be expected to lead to thylakoid unstacking, as seen after *in vitro* phosphorylation at "low" Mg^{2+}-levels (Fig. 4.8 and Table 4.4). The reported changes in thylakoid organisation in response to light quality have been varied. Punnett (1970) and Bennoun and Jupin (1974) have shown extensive alterations in the degree of stacked thylakoids in response to light 2 treatments. It is interesting that the 33% decrease in stacking reported by Bennoun and Jupin (1974) was seen using the same photosynthetic material and under the same conditions in which Bennoun (1974) had shown a preferential F_v quenching on going to State 2 and therefore, in this case, a change in thylakoid organisation might have been expected. Vernotte et al. (1975), on the other hand, did not find any significant difference in thylakoid organisation when leaves were pretreated with light 1 or light 2. Preliminary experiments carried out during this work have also suggested that no large alteration in thylakoid stacking is seen between State 1 and State 2 (data not shown). Such diverse observations may simply reflect different growth conditions under which the experimental material was grown, which might alter the mechanisms involved in bringing about the State 1 - State 2 transition. This can be seen to occur by the *in vitro* phosphorylation of intermittent light grown (IML) plants which exhibit a preferential F_v quenching (Black et al., 1984).

The proposed inter-relationship between the *in vitro* cation- and protein phosphorylation-induced changes and *in vivo* light quality-induced changes in thylakoid organisation and excitation energy distribution are summarised in Fig. 10.1.

However, it must be remembered that the activity of the protein kinase which brings about phosphorylation is not only altered by the imbalance of energy input to the two photosystems (see 1.7.2). It can also be affected by the ATP/ADP ratio (Markwell et al., 1982), the NADPH/NADP ratio (Fernyhough et al., 1983), and changes in the pH gradient (Fernyhough et al., 1984). Furthermore, it appears that the kinase can be affected by the CO_2 fixation reactions (Allen and Bennett, 1981).

Protein kinase activity not only leads to the phosphorylation of LHC but also to the phosphorylation of a number of other PS2-associated polypeptides (see Steinback et al., 1982; Telfer et al., 1983). In this context it has been shown that protein-phosphorylation can also affect other photosynthetic processes, including an enhanced affinity for DCMU-binding (Shochat et al., 1982), although this is not borne out from Fig. 5.10; and a stimulated dissipative cycling of electrons around PS2 (Horton and Lee, 1983) which could help explain the reported increase in the stability of the semiquinone species, Q_B^- (Jursinic and Kyle, 1983). It is interesting to point out that the increased Q_B^- stability and the enhanced DCMU-affinity appear to be incompatible, if the work of Lavergne (1982c,d) concerning DCMU-affinity and the Q_B^- state is considered. He reported that those PS2 reaction centres which were in the so-called "odd-state" ($Q_A Q_B^-$) exhibited a 20 fold lower affinity to bind DCMU. It has also been shown that protein-phosphorylation can cause the inhibition of PS2 electron transfer capacity even at saturating light intensities

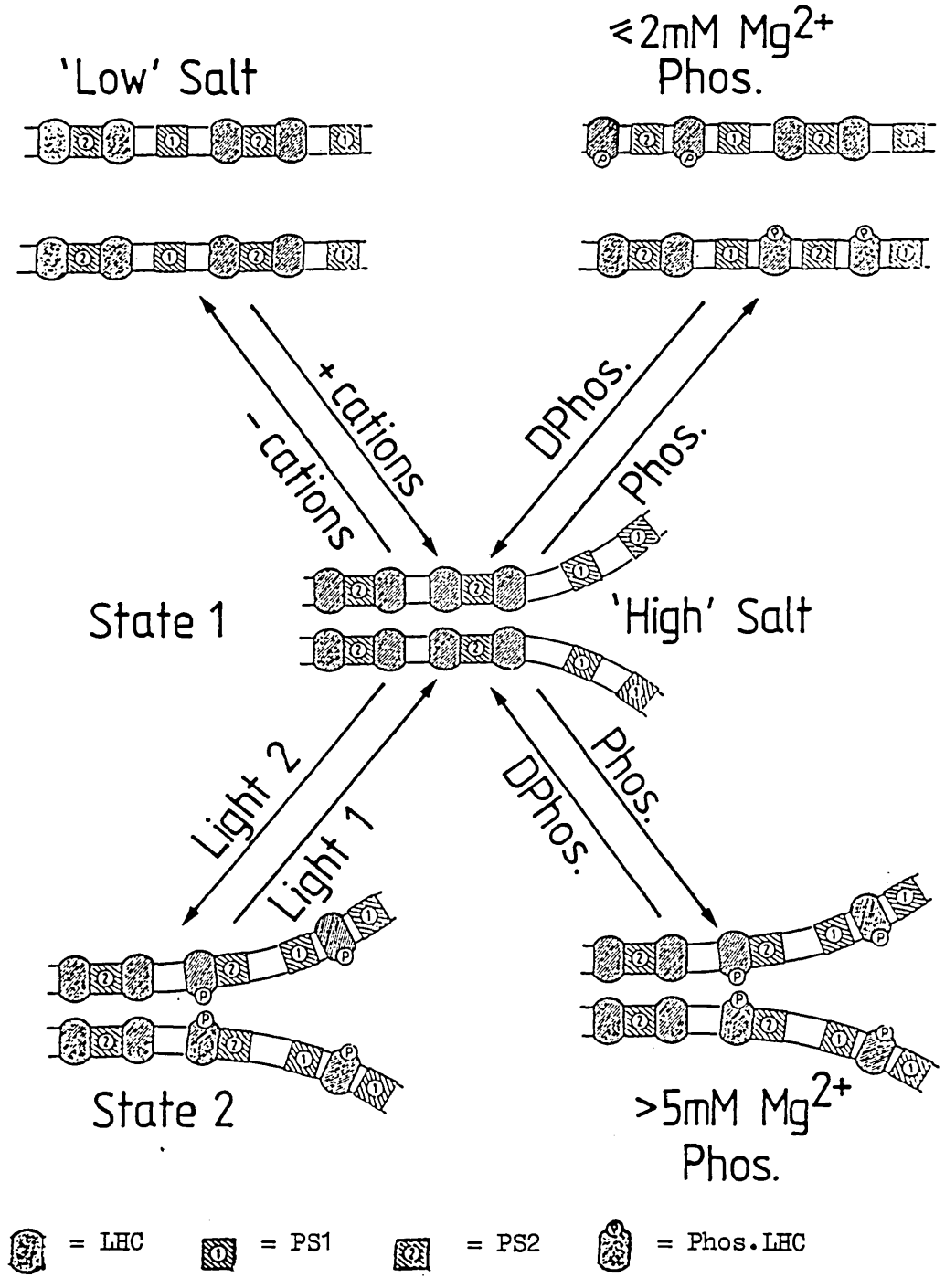


Fig. 10.1 A scheme showing how the spatial relationships between intrinsic pigment-protein-complexes of the thylakoid membrane and the extent of membrane appression can be altered <u>in vitro</u> by cation-levels and protein-phosphorylation and <u>in vivo</u> by light quality. At 'low' cation concentrations, poor electrostatic screening leads to unstacking as does phosphorylation at Mg^{2+} levels at or below 2mM Mg^{2+}. Phosphorylation at 'high' Mg^{2+} concentrations (>5mM) introduces sufficient negative charge onto a 'mobile' pool of LHC, and like State 2-adapted leaves, brings about only a very small decrease in membrane stacking.

(Horton et al., 1984; Fig. 5.8). A number of important questions are raised by these observations. What brings about the inhibition of PS2 electron transfer at saturating light? Are the other phosphorylated proteins involved in the in vitro manifestation of these reported effects? What is the physiological significance of the other phosphorylated proteins and the reported in vitro changes?

The fact that a 9kDa polypeptide, possibly cyt b_{559} (Metz et al., 1983) is one of the proteins phosphorylated, led in part to the work presented in Chapter 5. The data shown in Fig. 5.1 to 5.6 suggests that the cyt b_{559} under initial investigation was a low potential form, which appeared to be an intersystem electron transfer component, and not the high potential form associated with the oxidising-side of PS2 (see review by Bendall, 1982). In order to explain the effect of DCMU and DBMIB (Fig. 5.4) on the photooxidation of the cyt b_{559LP}, it was necessary to propose that it was involved in a Q-cycle type electron transfer mechanism (Mitchell, 1975). Cyt b_{559LP} is envisaged as receiving electrons from the oxidation of the quinol by the Rieske-iron sulphur centre, possibly via cyt b_{563}, and donating them to an anionic semiquinone. The effect of protein phosphorylation on the extent of cyt b_{559LP} photooxidation can be explained by the proposed redistribution of light energy in favour of PS1, which could enhance the reduction kinetics via an increased PS1 electron flow, inhibit the oxidation kinetics of the cytochrome or modify the Q cycle leading to an observed partial inhibition of the photooxidation (Fig. 5.1). However, the decreased photooxidation could also simply reflect the inhibition of PS2 electron transfer (Fig. 5.9) which would lead to a reduced reoxidation of cyt b_{559LP}. This later proposal is in agreement with Horton and Black (1981) who were unable to explain changes in cyt f redox reactions after phosphorylation in terms of an increase in PS1-associated reactions but only by a decreased

electron flux from PS2.

Is the decrease in PS2-mediated electron flow a consequence of the proposed stimulation of electrons cycling around PS2 (Horton and Lee, 1983)? The phosphorylation-induced changes in cyt b_{559HP} photooxidation, shown in Fig. 5.7, cannot be explained by such a mechanism if this redox component is involved in such a cycling mechanism as implicated in algae by Mende and Wiesnner (1983). In fact, the observed increased kinetics of the photooxidation after phosphorylation can be explained by a decrease in PS2 absorption cross section (see Packham and Barber, 1984). However, the conditions required to see cyt b_{559HP} photooxidation (eg. the presence of Ant 2p) may inhibit the postulated back-flow of electrons from PS2.

The inability of DPC to restore the PS2 electron transfer rate in phosphorylated thylakoids suggests that the site of inhibition is not the oxygen-evolving complex (OEC). While the similar H_2O to SiMo rates seen before and after phosphorylation would not be expected if a stimulated cycling of electrons was responsible for the inhibited PS2 electron transfer rates. It appears, therefore, that the electron flow is being inhibited after Q_A. Such a proposal could explain the reported increase in the stability of Q_B^- in the dark due to an alteration in the accessibility of Q_A to interact with Q_B. The observed differences in the fast fluorescence transients (Fig. 5.11 and Table 5.1), in which phosphorylated thylakoids exhibit a greater F_o to i rise followed by a slower increase to the p-level, is consistent with a reduced electron flow from Q_A to Q_B (see Vermaas and Arntzen, 1983). These changes are similar to those seen when comparing atrazine-resistant and susceptible plant species, which can also show inhibited PS2 electron transfer reactions

(see Burke et al., 1982; Ort et al., 1983). Further work needs to be carried out in this area of protein-phosphorylation to determine the cause and in vivo significance of the reported in vitro observations. One proposal put forward by Horton and colleagues is that protein-phosphorylation, by changes in photosynthetic capacity and PS2 absorption cross section, could exert regulatory and protecting effects. This could be achieved by altering the balance between cyclic and non-cyclic electron transfer reactions in response to changes in CO_2-assimilation demand and lead to the protection of PS2 from photoinhibitionary effects.

10.3 Protein Heterogeneity:

All the types of PS2 heterogeneity reported in the literature (see Section 1.9) have been as a consequence of chlorophyll fluorescence measured in isolated thylakoids. This is an example where chlorophyll fluorescence measurements have given rise to the proposed structural and functional PS2 heterogeneities instead of known differences in PS2 properties being investigated using fluorescence techniques. The work presented in Chapter 6 of this thesis investigates the $PS2_\alpha$, $PS2_\beta$ type of PS2 heterogeneity proposed by Melis and Homann (1976 and 1978) to explain the observed biphasic kinetic nature of chlorophyll fluorescence induction curves exhibited by DCMU-poisoned thylakoids. A number of differences between these two forms of PS2 centre have since been reported (see Introduction, 1.9.1). It has been shown, by analysis of the fluorescence induction curve, that $PS2_\alpha$ is located in the granal lamellae while $PS2_\beta$ is found only in the stromal thylakoids (Anderson and Melis, 1983). However this contention is not borne out by the data shown in Table 6.5 in which both types of PS2 are detected in PS2-enriched particles derived from appressed membranes. The difference may

be due to the methods of analysing the curves, in that Anderson and Melis (1983) calculated the area growth by hand while it was carried out by a computer in this present work. Thielen and van Gorkom (1981b,d) have shown, using tobacco mutants apparently exhibiting different $PS2_\alpha$/$PS2_\beta$ ratios, that $PS2_\alpha$ centres are associated with a larger antenna-size than the $PS2_\beta$. Moreover they found that the $PS2_\beta$ associated chlorophylls have red-shifted absorption properties compared with the chlorophyll of $PS2_\alpha$. However, the technique of measuring the amounts of the two PS2 centres, via fluorescence-induction curve analysis, might mean that there was only an apparent increase in the number of $PS2_\beta$ in the mutants because of a limited degree of PS2-PS2 interaction, as seen in the Mg^{2+}-level experiments of Chapter 3 where an apparent increase in $PS2_\beta$ centres was observed in response to a decrease in thylakoid stacking. This might mean that in the mutants, which lack normal quantities of LHC, only a small proportion of PS2 centres are "connected" and located in appressed membranes. The majority of the PS2, not being associated with LHC, is therefore situated in the stromal lamellae along with PS1. Such PS2 centres would naturally contain less antenna pigments, give rise to an altered fluorescence signal both in size and kinetics due to spillover of excitation energy to PS1 and the smaller antenna size. Such a complex situation suggests that using such material to investigate $PS2_\alpha$ and $PS2_\beta$ is not a very good idea.

Brearley and Horton (1984) have investigated the fluorescence emission characteristics of $PS2_\alpha$ and $PS2_\beta$ and have shown that $PS2_\beta$ gave rise to a fluorescence emission with a maximum at around 730nm. This observation could not be repeated, using the experimental conditions and set-up described in this thesis, as seen from Table 6.4 in which equal proportions of $PS2_\alpha$ and $PS2_\beta$ were detected when chlorophyll fluorescence was measured either at 685nm or 731nm.

Redox titrations of the two PS2 centres in the presence (Horton, 1981) and absence (Thielen and van Gorkom, 1981c) of redox mediators have shown that the primary electron acceptors of $PS2_\alpha$ and $PS2_\beta$ (Q_α and Q_β respectively) have different midpoint potentials. The reported midpoint potential (at pH 7.0) of Q_β of +120mV might suggest that PQ is involved in the manifestation of the slower, kinetic component. It has been suggested that the extent of the $PS2_\beta$-attributed slow phase is highly dependent upon chloroplast integrity and perhaps DCMU-binding affinity (Schreiber and Pfister, 1982). Furthermore, Diner and Delsome (1983) while investigating PS2 electron acceptors, found that Q_β did not show a C_{550} absorbance signal, nor did it participate in a transmembrane potential. An explanation put forward was that Q_β did not represent a primary electron acceptor but that it could arise from another source like a secondary acceptor or a modification in DCMU-binding. Recent work by Bréarley and Horton (1984) has suggested that there are two populations of PS2 with very different affinities for DCMU (I_{50} values of 0.05μM and 365μM). Lavergne (1982d) has also proposed that PS2 centres have different DCMU-binding affinities according to whether they are in the $Q_AQ_B^-$ state (which exhibit a 20 fold lower affinity for DCMU) or the Q_AQ_B state. It therefore may be possible to explain the biphasic nature of the fluorescence rise in terms of PS2 centres with different DCMU-binding properties. It is difficult to understand the alteration in the kinetics of the fluorescence rise, in the presence of 20μM DCMU, by the addition of FeCy or MV (Fig. 6.5b and 6.6) if PS2 is fully inhibited. Such observations have also been reported by Joliot and Joliot (1983) who explained them in terms of the removal of the $Q_AQ_B^-$ state which is present in dark pretreated thylakoids. They concluded that the slow phase was due to $PS2_\beta$ centres and those PS2 centres in the $Q_AQ_B^-$ state, and that PS2 centres include several sites with different DCMU sensitivities. They postulated that more than 20μM DCMU was required to surpress the $Q_AQ_B^-$

dependent slow component. Perhaps more than one factor gives rise to the slow fluorescence component, as MV and FeCy do not fully remove this phenomenon. The addition of reductants, like dithionite, do however, fully remove the slow phase (see Figs. 6.2 and 6.3). This can be explained either by the removal of the $Q_AQ_B^-$ state which would increase the DCMU-affinity at those PS2 centres, or it might reflect the reduction of PQ which stops Q_A reoxidation taking place in PS2 centres which are permanently uninhibited by DCMU, either through structural damage during chloroplast isolation or through non-association with the Q_B-protein. This later point might help explain why, even in the presence of 100μM DCMU, a slow kinetic component was still observed (Fig. 6.7); a concentration expected to remove any $Q_AQ_B^-$ component (see Joliot and Joliot, 1983). In fact the data of Fig. 6.7 is surprising as Horvath et al. (1984) have shown that in the presence of high herbicide concentrations $PS2_\beta$ centres do not fluoresce but appear to be damaged in some way so as not to undergo photoreduction. They proposed that this explained the observations of Schreiber and Pfister (1982). It has been shown by Thielen and van Gorkom (1981c), that $PS2_\beta$ centres did not appear to be associated with the Q_B-protein, and that the slow phase attributed to such centres could be removed following tris-treatment. Both these points might suggest that some PS2 centres may not be blocked by DCMU and that the manifestation of the slow phase requires more than one PS2 turnover. This is also suggested by the dark reoxidation of a slow fluorescence component after complete PS2 photoreduction in the presence of DCMU and back reaction inhibitors (Fig. 6.8).

This type of PS2 heterogeneity obviously requires further study, preferably using non-fluorescence techniques. If such PS2 heterogeneity exists *in vivo* then work is required to elucidate its significance and physiological role. Fluorescence data compiled using intact leaves

(Fig. 6.9), however, suggests that $PS2_{\alpha}$ and $PS2_{\beta}$ may not exist *in vivo*. Meanwhile, it has been postulated that such an heterogeneity would enable plants to make more efficient use of a wide range of light conditions (Thielen and van Gorkom, 1981) while Anderson and Melis (1983) have proposed that $PS2_{\alpha}$ is involved in non-cyclic reactions while $PS2_{\beta}$ is useful in controlling cyclic photophosphorylation.

10.4 Protein Structure and Function:

It appears that a single base change which leads to the alteration of a single amino acid residue in the Q_B-protein associated with PS2 enables those plants which have this mutation to be resistant to atrazine herbicides (see Hirschberg and McIntosch, 1983; Erickson et al., 1984). This small change appears to have a large effect on the functioning of the Q_B-protein, which can lead to an inhibited photosynthetic capacity as well as to large alterations in thylakoid organisation (Burke et al., 1982). The exact mechanism which brings about these changes is still not fully understood. It has been suggested that the altered Q_B-protein leads to a change in the redox potential of Q_B/Q_B^- (Bowes et al., 1980) so that the equilibrium $Q_A Q_B^- \rightleftharpoons Q_A^- Q_B$ is shifted to the left in the resistant species which produces a slower flow of electrons from Q_A to Q_B and perhaps a partial inhibition of the interaction between the Q_B-protein with PQ (Vermaas and Arntzen, 1983). These proposals explain the different fast fluorescence transients exhibited by resistant and susceptible biotypes (Fig. 9.1 and 9.2). Such a change may also explain the reported inhibition of PS2 electron transfer rates (Burke et al., 1982; Ort et al., 1983), although it has been reported that atrazine-resistance also leads to a greater cycling of electrons around PS2 (Holt et al., 1983). The reduced photosynthetic capacity may cause secondary effects on chloroplast ultrastructure so that herbicide resistant plant species synthesise

the type of chloroplast seen when plants are grown under environmental conditions which limit their photosynthetic ability (see Fig. 8.7 and 8.10). However it appears that atrazine resistance can occur without this type of structural alteration taking place, as the four species investigated in Chapter 9 did not show lower photosynthetic electron transfer rates nor large changes in the extent of thylakoid stacking when comparing resistant plants with their susceptible equivalents (data not given; see Chapman et al., 1984). There was, however, a consistent decrease in chl a/b ratio associated with the resistant plants indicative of altered PS2/PS1 levels or larger PS2 antenna-size (see Fig. 9.3), and from chlorophyll fluorescence analyses, a small change in PS2 organisation within the resistant plants was hinted. The consistently lower k_{α} values and higher F_m per total chlorophyll levels (Table 9.2) exhibited by the thylakoids from resistant plants indicates that perhaps these membranes contained more PS2 reaction centres per total chlorophyll than the susceptible biotypes. The secondary effects of atrazine resistance is still under active research. It might be that the reason why in some cases, herbicide resistance leads to large changes in chloroplast structure and photosynthetic capacity while in others there appears to be no adverse effects depends on the environmental conditions under which the plants are grown.

Preliminary experiments have shown that the change in the Q_B-protein does not stop the atrazine-resistant plants from carrying out State 1 - State 2 transitions (see Fig. 9.4), although it may, in some instances, alter the kinetics and extent of this adaptive process to some degree.

It thus becomes apparent that the higher plant has developed an ability to adapt to the everchanging environmental conditions under

which it has to live and propagate. Such changes are evident throughout the whole of the plant system and, of course, not only involves the photosynthetic machinery and its functioning but also other important metabolic processes and anatomical structures. The photosynthetic adaptation can be seen to take place over a wide range of timescales from months to days to minutes and seconds. Changes occur throughout the leaf and encompasses macroscopic, anatomical alterations to microscopic structural reorganisation involving revised levels of enzymatic proteins and subtle rearrangements of pigment-protein complexes within the chloroplast, thylakoid membranes. However, this apparent adaptive flexibility is probably restrained by the inherent, genetic constraints imposed on the plant.

Chlorophyll fluorescence has, over the years, become a useful tool for monitoring the adaptive processes which lead to an altered chloroplast architecture thereby determining the rate at which certain photosynthetic reactions can proceed. It can be seen from the work presented in this thesis that fluorescence can give vital information concerning various levels of reorganisation within the chloroplast. This has only been possible because of our ever increasing knowledge concerning the relationship between chlorophyll fluorescence and various photosynthetic phenomena which has taken place over the past twenty years or so. It was Duysens and Sweers in 1963 who first showed that the fluorescence yield reflected the redox state of the PS2 primary stable acceptor, Q_A. Their early pioneering work, using light of different spectral quality to change fluorescence levels, hinted at the presence of two photosystems acting in series giving support to the earlier proposals of Hill and Bendall (1960). Later in 1966, Malkin and co-workers analysed room-temperature fluorescence signals to give information concerning the

transfer of electrons within the pool of intersystem carriers. Then in the early 1970's it was discovered that chlorophyll fluorescence was dependent upon the structural organisation of the thylakoid membranes. This led Butler and colleagues to formulate kinetic models to represent the energy distribution in the photochemical apparatus of photosynthesis. Using their kinetic analyses it became possible to quantify the change in excitation energy distribution via spillover and absorption cross section brought about by thylakoid conformational changes. Meanwhile, Barber and his research group showed, mainly through fluorescence studies, that the structural changes were related to the electrostatic properties of the membranes. Today, chlorophyll fluorescence is being used to monitor photosynthetic damage caused by adverse environmental conditions which produce lesions in the electron transfer chain, before any visible signs of damage are seen. This is because the fluorescence signal is sensitive to both electron donation to PS2 by the water-splitting processes and electron removal from PS2 via PS1-mediated electron transfer reactions.

Chlorophyll fluorescence is, however, a very complex phenomena and it can be influenced by numerous events. The interpretation of the measured signal is often extremely difficult, especially the slow, steady-state changes monitored from intact leaves. Today much research time and money is devoted to attempting to elucidate the in vivo changes which contribute to the complex fluorescence signals. So far it has been shown that both the so-called "dark" and "light" reactions of photosynthesis influence the slow fluorescence changes, probably as a result of the common nature of certain metabolite pools. More valid information concerning photosynthetic processes can be obtained when several fluorescence approaches are used and combined. For instance, by bringing together room temperature fluorescence induction curve data with 77K

fluorescence spectra and kinetic analyses a more thorough understanding concerning the exact mechanisms for energy redistribution as a consequence of membrane reorganisation has been achieved. Although more attention is now required concerning the in vivo alterations in photosynthetic processes brought about, for instance, by protein-phosphorylation.

More progress will only be made when the fluorescence studies are supplemented by complementary signals, like A_{320} and C_{550} absorbance changes. Such an approach is necessary to further our understanding concerning any PS2 heterogeneity present within the thylakoid system. Furthermore, fluorescence measurements must also be carried out along with other types of measurement which can directly probe phenomena like cytochrome redox changes, electron transfer reactions and structural alterations. This multiple cross checking approach is necessary to fully elucidate the complexity of the chlorophyll fluorescence signal as well as to provide a far greater understanding of the photosynthetic processes which are vital to the survival of mankind.

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