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THE ANALYSIS OF FLUORESCENCE INDUCTION
TRANSIENTS FROM
DICHLOROPHENYLDIMETHYLUREA-POISONED CHLOROPLASTS

BY

SANDRA M. SPENCE, B.Sc.

A thesis submitted to the
Faculty of Graduate Studies and Research
in partial fulfillment of the requirements
for the degree of Master of Science.

Department of Biology
Carleton University
Ottawa, Ontario
March 30, 1989

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The undersigned hereby recommend to the
Faculty of Graduate Studies and Research
acceptance of this thesis, submitted by

SANDRA M. SPENCE, B.Sc.

in partial fulfillment of the requirements
for the degree Master of Science.

.....
Chairman, Dept. of Biology

.....
Thesis Supervisor

ABSTRACT

To study the effect of light and dark pre-treatments on PSII, identical chloroplast samples were prepared - one was illuminated ($6\mu\text{Em}^{-2}\text{s}^{-1}$ at 650nm) for 30 minutes while a second sample was kept in the dark for 30 minutes. The effect of such treatments on F_{max} , F_v , F_o and fluorescence induction curves was studied.

Pre-illumination of a chloroplast sample resulted in a light-induced quenching of maximum fluorescence and a reduction of both PSI and PSII partial reactions. Unlike quenching observed by Krause et al (1983), the light-induced quenching observed here was not abolished by the presence of uncouplers. A subsequent dark period did not reverse quenching and the longer the illumination the greater the quenching. The lower value of F_{max} is indicative of a light-induced leak of electrons.

Fluorescence induction transients were analysed and 4 phases were identified - α , β , τ , δ . It was demonstrated that the slowest phase was not the result of a leak of electrons past the DCMU block, unlike previously believed (Schreiber & Pfister, 1982).

Compared to control samples, a dark pre-treatment reduced the total area above the fluorescence curve as though the PSII acceptors were being chemically reduced. The rise of variable fluorescence from the light-treated samples was slower than that of the control samples although the total areas were very similar. The slow rise is indicative of a light-induced leak of electrons, perhaps the result of a conformational change within the thylakoid membranes.

The addition of ferricyanide prior to the DCMU doubled the area above the curve, consistent with the results of Ikegami & Katoh (1973). The results of a second illumination of these samples suggest that Q_{400} is only involved with δ -centres.

The results demonstrate: (1) that there exists a new type of light-induced fluorescence quenching and (2) that PSII heterogeneity is greater than previously believed.

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LIST OF SYMBOLS

SYMBOL	DESCRIPTION
α	alpha
A ₀	primary acceptor for PSI
ATP	adenosine 5'-triphosphate
β	beta
BSA	bovine serum albumin
CAD	complementary area decrease
δ	delta
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DCPIP	dichlorophenyl indophenol
EDTA	ethylenediaminetetraacetic acid
F _{max}	maximum level of fluorescence
F ₀	initial level of fluorescence
F _v	variable level of fluorescence (F _{max} - F ₀)
γ	gamma
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid
HES	high energy state
LHC	light harvesting chlorophyll complex
M(S)	oxygen evolving site
MES	2[N-morpholino] ethanesulfonic acid
MV	methyl viologen
MOPS	4-morpholine propanesulfonic acid
NADPH	nicotinamide adenine dinucleotide phosphate
OEC	oxygen evolving complex
PC	plastocyanin
PQ	plastoquinone
PS	photosystem
Q _A	primary PSII electron acceptor
Q _B	secondary PSII electron acceptor
Q ₄₀₀	electron acceptor between PSII and DCMU block
RC	reaction centre
Z	primary electron donor to PSII

CHAPTER 1: INTRODUCTION

Many living organisms respire, consuming oxygen and carbohydrates and producing carbon dioxide, water and energy. Respiration consumes 10,000 tonnes of oxygen every second and if there was not a 'recycling' process all the oxygen would be used up in 3000 years (Hall & Rao, 1987). This recycling process is known as photosynthesis and it converts carbon dioxide and water back to oxygen and carbohydrates.

In order to set the stage for the research to be presented in this thesis, a general review of photosynthesis will be followed by a more in depth look at photosystem II and photosynthetic fluorescence emission.

1.1 PHOTOSYNTHESIS

Very simply put, photosynthesis is the conversion of light energy into stable chemical energy or the conversion of inorganic raw materials into organic compounds.

All photosynthetic organisms contain pigments whose role is to absorb light. Once light is absorbed, the excitation energy of the pigment molecules permits the splitting of water molecules and the release of electrons. These electrons are then passed along an electron transport chain producing chemical energy in the form of ATP and NADPH, both of which are subsequently used to fix carbon dioxide in the Calvin-Benson cycle. Photosynthesis is divided into light-dependent (electron transport) and light-independent or dark reactions (CO₂ fixation).

The overall equation for photosynthesis is:

The many reactions which make up this overall equation all take place in the chloroplast.

1.1.1 THE CHLOROPLAST

The photosynthetic apparatus of green algae and higher plants is found in chloroplasts which are round or oval in shape and bounded by two membranes. The outer chloroplastic membrane is freely permeable to small molecules while the inner membrane forms the permeability barrier between the chloroplast matrix (stroma) and the cytoplasm (Harold, 1986).

Each chloroplast contains a number of thylakoids. A thylakoid is a flattened vesicle in which the membranes are so close together that the internal space is very small. In most higher plants the thylakoids are arranged in stacks called grana. Grana are embedded in the stroma and individual grana are interconnected by single stroma thylakoids (Hall & Rao, 1987; Staehelin, 1986).

The thylakoid membranes are composed of lipid and protein and contain the pigment molecules. The protein performs a structural role as well as acting in an enzymatic capacity. In addition, almost all the chlorophyll in the membrane is conjugated to specific hydrophobic membrane proteins, which account for 50% of the membrane protein. It is the selective permeability of the membrane which allows a separation of charge, compartmentation of chemical species and the storage of free energy in the form of electric potential or proton gradients (Harold, 1986).

The thylakoids are the site of light energy conversion, electron transport reactions and ATP synthesis. The stroma

contains mostly water soluble components. All the enzymes for the Calvin cycle are found in the stroma, with ribulose biphosphate carboxylase/oxygenase being the predominant one. Also in the stroma are starch granules, chloroplast DNA, ribosomes, mRNA, other elements for protein synthesis (Staehelin, 1986).

1.1.2 LIGHT HARVESTING

The first step in photosynthesis is the absorption of light by pigment molecules. All green photosynthetic organisms contain one or more light absorbing pigments, however, chlorophyll a is the most important and without it, all photosynthetic ability is lost.

There are three classes of pigments - chlorophylls, carotenoids and phycobilins but only chlorophylls and carotenoids are present in higher plants, phycobilins are found in algae. All these pigments have a system of conjugated double bonds which is responsible for light absorption (Hall & Rao, 1987).

A chlorophyll molecule is made up of two parts - a porphyrin 'head' and a phytol 'tail'. The head group is a cyclic tetrapyrrole with a magnesium ion complexed in the center. It is the head which contains the conjugated double bonds and is thus the light absorbing region while the tail is merely a phytol side chain which has no role in light absorption. In the membrane the porphyrin head is bound to

protein while the phytol tail extends into the lipid layer. In some chlorophyll molecules the CH_3 group (chlorophyll a) on ring II is replaced by a CHO group and this is known as chlorophyll b. Most photosynthetic organisms contain chlorophyll a, while chlorophyll b is only found in green algae and the leaves of higher plants. There are three times the number of chlorophyll a molecules as chlorophyll b (Hall & Rao, 1987).

Carotenoids are linear hydrocarbons (carotenes) or oxygenated hydrocarbons (xanthophylls) containing 40 carbons and many conjugated double bonds. Carotenoids are found in the thylakoid membranes close to chlorophyll molecules and in this way are able to transfer the energy they absorb to the nearby chlorophyll (Hall & Rao, 1987).

Of the light that falls on a plant only the wavelengths 350-750nm are photosynthetically active (Sauer, 1986) and each molecule has its own characteristic absorption band(s). For example, in organic solvents, chlorophyll a absorbs maximally at 420nm and 660nm while maximum absorption for chlorophyll b is at 435nm and 643nm and carotenoids absorb best between 420nm and 480nm (Hall & Rao, 1987).

There are more than 10,000 chlorophyll molecules in each thylakoid. When a chloroplast suspension is illuminated, with a series of saturating light flashes, only 1 molecule of oxygen is evolved for every 2500 chlorophyll molecules. The splitting of two molecules of water requires the input of 8

quanta of light to release one oxygen molecule and four electrons which pass through two photosystems - 4 electron transport steps, each of which requires two light reactions. Therefore approximately 300 (2500/8) chlorophyll molecules are involved with the processing of each quantum of light (Harold, 1986). A photosynthetic unit is composed of approximately 300 chlorophyll molecules but only one of these is involved in photochemistry - the reaction centre (RC). The rest of the chlorophyll molecules capture light and channel it to the reaction centre. The chlorophyll which forms the reaction centres is of two forms. One, P680, has its maximum absorption at 680nm (Photosystem II - PSII) while the other, P700, has its maximum at 700nm and is the reaction centre for Photosystem I (PSI). These light harvesting chlorophyll molecules also act as antenna pigments and increase the absorption cross-section of each reaction centre by several hundred fold (Sauer, 1986). Structurally, reaction centre chlorophylls are identical to other chlorophyll molecules but their properties differ due to their environment. The property that sets them apart from all other chlorophyll molecules is a lower energy level for their excited state which makes them excellent energy traps (Sauer, 1986).

Absorption of a quantum of light raises an electron from the ground state to an excited singlet level. Excited chlorophyll molecules have a lifetime of about 10^{-9} seconds

and the energy may be lost in one of several ways (Harold, 1986). Eighty-five percent of the energy is used in photosynthesis; reaction centre complexes provide an acceptor for the excited electrons and an electron donor reduces the oxidized chlorophyll very rapidly (Papageorgiou, 1975). The quantum of light may be absorbed by an antenna pigment far from the reaction centre but the energy is transferred from molecule to molecule until it reaches the centre. A second way an excited molecule can lose energy is as it is transferred from its excited singlet state to a metastable triplet state. The metastable triplet state is much longer lived and can then revert to ground state by emitting a photon at a longer wavelength. This weak emission is known as phosphorescence. A third route for the dissipation of energy involves the re-emission of light, normally at a wavelength somewhat longer than that of the exciting light. The emitted light is called fluorescence. The final mode of energy dissipation is the release of heat (Harold, 1986; Papageorgiou, 1975).

1.1.2.1 Chlorophyll-Protein Complexes

All chlorophyll and most of the carotenoids are complexed with intrinsic thylakoid membrane proteins. There may be several kinds of chlorophyll-protein complexes but they appear to have only two functions - light harvesting (LHCI and LHCII) and charge separation (RCI and RCII). The

reaction centres (RCI and RCII) contain chlorophyll a and β -carotene in a ratio of approximately 5:1. The light harvesting complexes contain both chlorophyll a and b and xanthophylls (Sato, 1986).

Using SDS-polyacrylamide gel electrophoresis (Kaplan & Arntzen, 1982) and/or mild detergent treatments combined with sucrose density gradient centrifugation and isoelectric focussing (Sato, 1986) many of the proteins associated with the reaction centre and light harvesting complexes have been separated and identified.

The Photosystem II reaction centre complex contains about 50 chlorophyll a and 10 β -carotenes and two large polypeptides of about 43 and 47kDa (figure 1) (Glazer & Melis, 1987; Sato, 1986; Kaplan & Arntzen, 1982). The accessory pigments of PSII are arranged into two light harvesting complexes, of which one is tightly bound (intrinsic) while the other is peripheral or mobile. There are about 80 chlorophyll molecules attached to the tightly bound LHCII while the peripheral complex contains about 120 chlorophyll. This means that a complete PSII unit contains a total of 250 chlorophyll molecules (Glazer & Melis, 1987). The LHCII contains as many as 3 distinct polypeptides with molecular weights of about 29, 27 and 25kDa (Glazer & Melis, 1987; Sato, 1986). It has been proposed that six 29kDa polypeptides are involved with the intrinsic complex and the 27 and 25kDa subunits are associated with the peripheral

FIGURE 1: THYLAKOID MEMBRANE PROTEINS AND THE ELECTRON TRANSPORT CHAIN

A diagrammatic representation of the electron transport chain and the proteins involved. The three complexes which comprise the electron transport chain, as well as the ATPase complex are shown, only the light harvesting complex is missing. (Taken from Govindjee & Eaton-Rye, 1986).

LHCII (Glazer & Melis, 1987).

The Photosystem I reaction centre complex has approximately 130 chlorophyll and 16 β -carotene associated with it (Glazer & Melis, 1987). Each PSI reaction centre complex is believed to be composed of six polypeptides. A dimer of a 70kDa polypeptide to which the reaction centre chlorophyll is attached and one each of 25, 20, 18, 16, 8kDa polypeptides (figure 1) (Glazer & Melis, 1987; Ort, 1986; Satoh, 1986). The light harvesting antenna of PSI contains 60-80 chlorophyll molecules ($a/b=3$) therefore there is a total of 200-210 chlorophyll for every PSI unit (Glazer & Melis, 1987; Satoh, 1986). When LHCI is isolated it is found to contain 2 or 3 20-25kDa polypeptides (Satoh, 1986).

1.1.3 ELECTRON TRANSPORT

Light is absorbed by antenna chlorophyll molecules, excitation energy is transferred to the reaction centre chlorophyll a molecules of PSII (P680) and PSI (P700) forming the excited states P680* and P700*. It is P680* and P700* which initiate electron transport and within a few picoseconds an electron is transferred to an acceptor molecule (Govindjee & Eaton-Rye, 1986). This initial acceptor is pheophytin (a chlorophyll in which the Mg ion is replaced by protons) for P680 and a chlorophyll a Magnesium molecule for P700 (figure 2).

The transfer of an electron from P680* to pheophytin

FIGURE 2: ELECTRON TRANSPORT IN THYLAKOIL MEMBRANES

The Z-scheme, non-cyclic electron flow from water to NADP⁺. The mid-point redox potential of each component is indicated as well as the time required for each electron transport step. (Taken from Govindjee & Eaton-Rye, 1986).

leaves the reaction centre in an oxidized state. $P680^+$ is a strong enough oxidant that it is able to draw electrons from Z, the primary donor, which is itself reduced by M (S), the oxygen evolving complex (figure 2). On the acceptor side of PSII the initial acceptor pheophytin passes its electrons through a sequence of electron acceptors to PSI (figure 2).

Absorption of a quantum of light by P700 causes a rapid transfer of an electron to a primary acceptor which is believed to be a chlorophyll a molecule (A_0). This leaves P700 in an oxidized state and able to accept an electron from plastocyanin. The acceptor following A_0 , is thought possibly to be a quinone. Electrons are removed from A_0 by a series of iron-sulfur centres (figure 2) (Glazer & Melis, 1987; Govindjee & Eaton-Rye, 1986). The final stages of light driven electron transport involve the transfer of electrons to ferredoxin and finally to $NADP^+$ to form NADPH. The enzyme ferredoxin:NADP reductase is required for the transfer of electrons to $NADP^+$ (Hall & Rao, 1987).

The Z-scheme (figure 2) for electron transport is now believed to be organized into three separate complexes which have a specific orientation in the thylakoid membrane (figure 1) (Harold, 1986). These complexes are:

1. PSII complex: includes the enzymatic machinery for water splitting as well as the acceptors Q_A and Q_B .
2. Cytochrome b/f complex: contains cytochromes b and f and the Rieske iron-sulfur protein.
3. PSI complex: includes PSI, its primary acceptors and iron-sulfur centres.

Linking these three complexes are the mobile electron carriers: plastoquinone, plastocyanin and ferredoxin (figure 1). Other complexes present in thylakoid membranes are the light harvesting and the ATPase complexes (Barber, 1987).

1.1.4 PHOTOPHOSPHORYLATION

The function of the light reactions of photosynthesis is to produce reducing power and energy for CO_2 fixation. The reducing power is in the form of NADPH and the energy is produced as a result of proton translocation. When chloroplasts are illuminated, electron transport results in proton translocation in a number of locations.

When light is absorbed by PSII and electrons are removed from water, two protons are released in the lumen of the thylakoid (figure 1). Plastoquinone is reduced at the external surface of the thylakoid membrane and two protons are consumed from the stroma. The plastoquinone pool is oxidized at the luminal surface of the membrane and when this happens two protons are released into the lumen. The final site of proton movement is the uptake of two protons from the

matrix when NADPH is formed (figure 1). The overall sum of the four translocations is the consumption of four protons from the stroma and the release of four protons into the lumen (Harold, 1986). The movement of protons across the membrane sets up a pH gradient. The back flow of protons promotes ATP synthesis via the ATP synthase complex (Hall & Rao, 1987).

It is possible to get ATP production without the evolution of oxygen or the formation of NADPH. This happens through a cyclic flow of electrons around PSI. In this cyclic flow an electron from P700 is transported to ferredoxin via the FeS centres and then, rather than going to NADP, the electron is transported to cytochrome b_6 and then through the electron transport chain back to P700 (figure 1).

1.1.5 CARBON DIOXIDE FIXATION

In the Calvin-Benson cycle the ATP and NADPH produced in electron transport reactions are used in the formation of carbohydrates from carbon dioxide. The overall reaction of the Calvin-Benson cycle is as follows: (Hall & Rao, 1987)

1.2 THE PHOTOSYSTEM II COMPLEX

Photosystem II is the first of the photosystems involved in the light reactions of photosynthesis. It must absorb light in order to oxidize water, reduce plastoquinone and generate a transmembrane potential.

1.2.1 ELECTRON TRANSPORT

When light is absorbed by a PSII unit, P680 becomes excited (rxn 3) and this energy is then transformed to redox energy followed by a charge separation across the membrane (rxn 4) (Glazer & Melis, 1987; Govindjee et al, 1985).

Pheophytin is an intermediate electron acceptor and within 400ps (Govindjee & Eaton-Rye, 1986) the electron is transferred to the quinones (Q_A , Q_B , and PQ) on the acceptor side of PSII (figure 2). Q_A , a plastoquinone molecule tightly bound to a reaction centre polypeptide, is the primary quinone acceptor. Q_B acts as a two electron gate, meaning that it requires the transfer of two electrons from Q_A before it is fully reduced. Electron transport takes place via the following scheme (Ort, 1986; Govindjee et al, 1985)

Electrons from pheophytin reduce Q_A to the semiquinone form (Q_A^- , rxn 5) which is then oxidized by Q_B (rxn 6). Reactions 3 and 4 are repeated (rxns 7,8) and Q_B is reduced to plastoquinol (Q_BH_2) which then exchanges with a plastoquinone from the plastoquinone pool (rxn 8). At some point in this set of reactions, two protons are taken up from the stroma (Ort, 1986; Govindjee & Eaton-Rye, 1986).

Reactions 3-8 leave P680 in an oxidized state. The reduction of P680 takes place via the transfer of an electron from Z in 20-40ns (rxn 9)

Z is a one electron component which is responsible for charge transfer between the oxygen evolving complex (OEC) and the PSII reaction centre (figure 1,2) (Ort, 1986). It has been suggested that Z is a tyrosine residue in a reaction centre polypeptide (Barry & Babcock, 1987). Many researchers believe there is more than one electron donor between P680 and the OEC and they may act in series or in parallel. It has been suggested that cytochrome b_{559} may be this second donor (Bishop, 1984; Govindjee et al, 1985).

1.2.2 OXYGEN EVOLUTION

Oxygen evolution involves a four step mechanism of charge accumulation. A saturating flash of light generates a positive charge in each trapping center, while a series of brief saturating flashes causes a stepwise cycle of events. The accumulation of four charges in a center (S4) leads to oxygen evolution and a return to S₀, the ground state (Kok et al, 1970; Forbush et al, 1971).

When dark adapted chloroplasts are subjected to a series of saturating light flashes there is initially no oxygen evolved. Maximum oxygen evolution is attained after three flashes and then every following fourth flash. Kok et al (1970) interpreted this as meaning that the evolution of one molecule of oxygen requires the co-operation of four oxidizing equivalents, generated by four excitations of PSII (Kok et al, 1970; Forbush et al, 1971).

In the dark it is assumed that the system does not revert to ground state (S₀) since S₀ is only formed in the light from S₄. This means that S₁ is predominant and this is the reason that oxygen evolution is maximal after only three flashes (Forbush et al, 1971). The period four oscillation of oxygen evolution becomes damped after approximately 25 flashes due to misses and double hits. Double hits refer to centers which receive two excitations in one flash while misses refer to a certain portion of centers not converted by the flash. (Kok et al, 1970; Forbush et al, 1971).

1.2.3 THE STRUCTURE OF PHOTOSYSTEM II

In every PSII reaction centre complex there are 50 chlorophyll and 10 β -carotene molecules, 2 pheophytin, 3 plastoquinone and 1-2 cytochrome b_{559} . As already mentioned, there are also a number of proteins associated with each centre. When PSII reaction centre complexes are run through SDS-polyacrylamide gel electrophoresis, 10 major bands are visible. Two of these bands correspond to the 43 and 47kDa proteins involved with the reaction centre (figure 1). It has been suggested that it is only the 47kDa protein which is responsible for charge separation in PSII. The 43kDa protein would then bind chlorophyll and transfer excitation energy to the reaction centre (Ort, 1986).

Another 6 of the bands are produced by polypeptides on the donor side of PSII. Four of the six polypeptides are directly involved with the OEC and have weights of 18, 24, 33 and 34kDa (Govindjee et al, 1985). The actual weights of the polypeptides will vary depending on the plant used.

The 18kDa and 24kDa polypeptides are extrinsic proteins (figure 1) which can easily be removed by salt washing inside- out thylakoids. The 34kDa protein appears to be a hydrophobically attached intrinsic protein (figure 1) (Akerlund, 1983; Murato & Miyao, 1983).

There is a second protein with a molecular weight of 33kDa associated with the donor side of PSII (figure 1).

This is an extrinsic hydrophillic protein whose exact location and function are unknown (Govindjee et al, 1985).

The final polypeptide located on the donor side of PSII is Cyt b₅₅₉. This is an intrinsic polypeptide with a molecular weight of 10kDa. This protein with its two hemes almost spans the membrane (figure 1). The functions put forth for this polypeptide are numerous. It may have a redox function in water splitting or may act as a proton acceptor. It has also been implicated in the proposed cyclic flow of electrons around PSII (Govindjee et al, 1985).

On the acceptor side of PSII there is another intrinsic polypeptide with a molecular weight of 32kDa (figure 1). The removal of this polypeptide results in a loss of Q_B function and thus electron transport. This protein has been found to be the site of herbicide action, thus it is often referred to as the herbicide-binding protein. (Arntzen & Pakrasi, 1986).

1.2.4 FLUORESCENCE

When a photon is absorbed by a PSII unit, the energy is rapidly transferred to the reaction centre, P680. There it can be used for photochemistry if the PSII centre is open, which means that Q_A is in the oxidized state and able to accept electrons from pheophytin. If Q_A is reduced, this is termed closed, and the energy must be dissipated in another way. This may be as heat or the emission of light at a wavelength longer or energy shorter than was initially absorbed. This is known as fluorescence (Hall & Rao, 1987).

At physiological temperatures, the fluorescence observed from a chloroplast solution comes almost entirely from the chlorophyll a molecules associated with PSII. It is only at lower temperatures (77K) that there is measurable fluorescence from PSI chlorophyll a. At 77K there are three peaks in the emission spectra - 735nm (PSI), 695nm (PSII) and 685nm (LHC) (Lavorel & Etienne, 1977).

When speaking of fluorescence there are two distinct time scales involved. The micro-scale which refers to fluorescence at the atomic level where lifetimes of individual molecules are only 10^{-9} seconds. The other time scale, and the one of concern in this thesis, is the macro-scale which deals with steady state levels of excited molecules (Lavorel & Etienne, 1977). The steady state level of fluorescence depends not only on the number of fluorescent

pigment molecules in the excited state but also on competing dissipation processes. For example, if the rate of electron transport is high, then the level of fluorescence will be reduced and vice versa.

When isolated intact chloroplasts are illuminated a fluorescence induction curve can be observed. Each induction curve has three distinct phases - initial rise, transient phase, and a steady-state phase (Murata et al, 1966) (figure 3, curve A). The initial rise (F_0) is very rapid and is reached almost immediately after the onset of illumination. This part of the total fluorescence is not sensitive to photosynthetic electron transport and does not vary with electron transport. F_0 may arise from PSI pigments and/or from PSII pigments if the transfer of excitation energy to the reaction centre is not 100% efficient. At F_0 all electron transport intermediates are oxidized. F_0 is followed by a small rise and short lived intermediate plateau. From this plateau, fluorescence rises more slowly to its maximum level (F_{max}). This slow rise is the result of the reduction of the PQ pool and electron accumulation in Q (Melis, 1985). The difference between F_{max} and F_0 is known as variable fluorescence (F_v). F_v comes from the PSII pigments and is sensitive to electron transport through P680 as well as to changes in membrane structure (Papageorgiou, 1975).

The addition of DCMU to chloroplast samples speeds up fluorescence induction as well as increasing the final steady

FIGURE 3: FLUORESCENCE TRANSIENTS

The curves show relative levels of fluorescence from isolated intact chloroplasts. Curve B was recorded in the presence of DCMU. No DCMU was present for curve A.

state level of fluorescence (figure 3, curve B). DCMU, (3-(3',4'-dichlorophenyl)-1,1' dimethyl urea or diuron) is a herbicide which kills its target plant by interfering with photosynthesis. DCMU blocks electron transport between the primary (Q_A) and secondary (Q_B) acceptors of PSII. The blockage occurs as DCMU binds to the herbicide binding protein (Q_B protein) (Wright & Corbett, 1979). This blockage prevents the re-oxidation of Q_A , thus as time passes the proportion of closed centres becomes greater and the level of fluorescence increases.

1.2.4.1 Fluorescence Quenching

The term fluorescence quenching refers to a decrease in the final level of fluorescence. The final steady state level of fluorescence is inversely proportional to the rate of electron transport. Thus anything which increases the rate of electron transport, quenches fluorescence. At room temperature, fluorescence emanates predominantly from PSII so anything which causes a re-distribution of light energy in favor of PSI will reduce fluorescence.

Phosphorylation-Induced Quenching

It has been proposed (Allen et al, 1981; Horton & Black, 1980) that the redox state of the plastoquinone pool regulates the distribution of energy between the two photosystems. When the majority of the light is transferred to PSII, PQ becomes reduced. This reduction activates a

protein kinase which then phosphorylates an exposed segment of the LHC complex. It is this phosphorylation which causes a re-distribution of light energy in favor of PSI (Bennett et al, 1980). The transition to state 2 is fully reversible and is caused by the oxidation of PQ. When more light is being transferred to PSI, the rate of electron transport away from PQ will be greater than to it. This means that PQ is oxidized, the protein becomes inactive and LHC is dephosphorylated by a thylakoid protein phosphatase (Allen et al, 1981; Horton & Black, 1980).

Cation-Induced Quenching

Murata, (1969) observed that in the presence of DCMU (all Q reduced), F_{max} was greatly increased upon the addition of 2mM $MgCl_2$ to isolated chloroplasts and suggested that Mg^{2+} in some way altered the way energy was distributed between the two photosystems. Gross & Hess (1973) found that at low salt concentrations there were no stacked thylakoids present while at higher concentrations they were in abundance. At pH 7.0 PSII and LHC have no fixed charge but PSI is negatively charged thus giving thylakoids a negative charge. Bringing thylakoids together causes an electrostatic repulsion, therefore the negative charges on PSI keep the membranes apart. The addition of cations such as Mg^{2+} shields the negative charges and allow the membranes to come together. However, the negative charges of PSI tend to push them to the non-appressed regions of the grana. In the case of stacked

thylakoids LHC is close to PSII thus most of the energy is transferred to PSII, resulting in a high level of fluorescence. When membranes become unstacked, PSI charged particles re-distribute among PSII and LHC. This means that LHC can transfer energy to the local photosystem therefore there is a greater chance of energy being transferred to PSI. This in turn reduces the level of fluorescence from PSII (Barber, 1987).

High-Energy-State (HES) Quenching

Krause et al (1983) observed a light-induced quenching of fluorescence which was not the result of a re-distribution of light energy in favor of PSI. It is believed (Briantais et al, 1979; Krause et al, 1983) that the quenching was due to the formation of a hydrogen ion gradient across the thylakoid membrane or a high energy state (HES). Illumination of chloroplasts causes an uptake of protons into the lumen of the thylakoids and the presence of electron acceptors or donors which stimulate PSI electron transport, increase ΔpH values (Briantais et al, 1979). Briantais et al (1979) showed that the light induced quenching was proportional to the proton concentration in the lumen. Krause et al (1983) suggest that the cause of the light-induced quenching is an increase in thermal de-activation of excited states.

1.2.5 The Analysis of Fluorescence Induction Curves

The addition of DCMU to chloroplasts can be an effective way of separating the two photosystems. The measurement and analysis of fluorescence induction transients in the presence of DCMU has become a useful tool in investigations of PSII (Hodges & Barber, 1986; Schreiber & Pfister, 1982; Melis & Homann, 1975).

When DCMU is present, the light-induced rise of chlorophyll fluorescence reflects the reduction of the primary PSII electron acceptor, Q . However, a linear relationship does not exist between fluorescence rise and Q reduction (Duysens & Sweers, 1963). The area between the fluorescence induction curve and F_{max} was found to be proportional to the number of quanta used by the photochemical reaction involved (Murata et al, 1966). It was then shown that the variable fluorescence was linearly proportional to the photochemical rate (Bennoun & Li, 1966). Therefore, taking these observations together, it was determined that the area above the curve is proportional to the number of PSII acceptors before the DCMU block or in other words, the photo-reduction of Q_A (Melis & Homann, 1975). At any time (t) during fluorescence induction, the accumulated area above the curve and to the left of t is a measure of the relative extent of Q reduction (Schreiber & Pfister, 1982).

When the concentration of salts is high enough to allow stacking of the grana, the fluorescence induction transients of DCMU-poisoned chloroplasts contain more than one phase (figure 4a). Earlier research identified two distinct phases - a fast non-exponential (usually sigmoidal) phase and a slow exponential one. The fast phase accounts for a large proportion of the total area above the curve but only a small percent of Fv. The reverse is true for the slow phase. This biphasic nature has been attributed to two forms of PSII centres, called α and β respectively (Melis & Homann, 1978; Melis & Homann, 1975).

It has been found that there are a number of differences between α and β -centres. α -centres have a full complement of antenna chlorophyll and are located in the grana. β centres have a reduced number of antenna chlorophyll associated with them and are located in stroma thylakoids (Anderson & Melis, 1983). β centres have been found to be lacking plastoquinone which indicates that they are not associated with a two electron gate (Melis, 1985).

More than one model has been put forth to explain the apparent heterogeneity of PSII centres. Melis and co-workers (Melis, 1985; Melis & Duysens, 1979; Melis & Homann, 1975) believe that α centres are located in 'statistical pigment beds' which allow energy transfer between units. On the other hand, β centres are separate entities with reduced light harvesting ability and are unable to transfer energy

Figure 4

A) shows a typical fluorescence transient in the presence of DCMU. The curve is multiphasic with an initial fast sigmoidal rise followed by a slower rise in the fluorescence level. Time scale changes to show the slow tail of fluorescence rise.

B) is a typical complementary area decrease (CAD) plot showing the existence of a slow exponential phase and at least one faster phase. (Adapted from Bell & Hipkins, 1985)

FIGURE 4: A TYPICAL FLUORESCENCE INDUCTION CURVE AND A CAD CURVE

between each other.

Other workers (Schreiber & Pfister, 1982; Hodges & Barber, 1983) believe that the heterogeneity is the result of differences in DCMU affinity. The β component representing PSII centres which are not easily blocked by DCMU. The slow tail of fluorescence would then be caused by a leakage of electrons past the DCMU block, which would maintain a supply of oxidized acceptor for PSII.

The biphasic nature of the fluorescence transient becomes even clearer when the log of the normalized area over the curve is plotted - complementary area decrease plot (CAD plot, figure 4b). This curve has a slow linear phase the slope of which is the rate constant for the β -phase. The y-intercept for this linear phase represents the proportion of centres in the β -phase. By subtracting this slow phase the nature of the fast phase can be determined (Melis & Homann, 1978). Under conditions where stacking of thylakoids is promoted, the CAD curve for the fast phase is downward curving which has been taken as evidence for connectivity between PSII units (Melis & Homann, 1978). In the absence of magnesium (unstacked) Melis & Homann (1978) found that the fast phase was exponential and the fluorescence transient could be described on the basis of two photochemical events with first order kinetics.

Sinclair & Spence (1988) showed that the length of the measurement period is critical to the analysis of

fluorescence transients. The length of measurement period had no effect on the initial kinetics but CAD curves were significantly different. The extent of the linear phase increased and the slope decreased as illumination time increased.

Using a theoretical fluorescence transient composed of two exponential phases, it was shown (Sinclair & Spence, 1988) that the chosen location for $F_{\max}$ has a significant effect on CAD curves. Since fluorescence curves are believed to have at least one exponential phase, choosing a value for $F_{\max}$ after a finite time means that both the area above the curve as well as the final level of fluorescence are underestimated. This had a big effect on the slopes of the two phases but the areas associated with each phase were quite close to expected. The longer the illumination period the more accurately $F_{\max}$ can be estimated and therefore the results will be more accurate (Sinclair & Spence, 1988).

Perhaps, as a result of shorter illumination times, other researchers (Melis & Homann, 1978; Schreiber & Pfister, 1982) have found a fluorescence transient to be composed of only the two phases. Sinclair & Spence (1988) were able to detect three phases which they called α , β , τ .

1.3 AIM OF THE RESEARCH

It was previously observed in this laboratory that fluorescence emission was different for samples of chloroplasts kept in the dark or light for 30 minutes. When two identical chloroplast solutions were prepared and one was placed in the light ($6\mu\text{Em}^{-2}\text{s}^{-1}$ at 650nm) for 30 minutes while the other was kept in complete darkness for 30 minutes, the fluorescence from the light-treated sample was significantly lower than that from the dark-treated sample. The goal of this research was to further investigate this phenomenon, compare it with other types of light-induced fluorescence quenching and if possible determine its cause.

Initial observations were made on the effects of light and dark treatments on the values of F_{max} , F_0 and F_v and the influence of variations in light intensity, durations of light or dark treatment and the presence of uncouplers. Subsequently, the time course of variable fluorescence rise from light and dark-treated samples was studied and fluorescence induction transients analysed in detail. Early experiments concerned the number of phases which were involved in fluorescence induction and also the appropriate concentration of DCMU to be used.

Finally, the effect of ferricyanide on fluorescence transients was studied. An earlier study (Ikegami & Katoh, 1973) showed that the addition of ferricyanide to chloroplasts in the dark, prior to the addition of DCMU, doubled the area above the fluorescence transient. This was confirmed and it was also shown that the areas associated with each phase change upon addition of ferricyanide.

CHAPTER 2: METHODS & MATERIALS

2.1 CHLOROPLAST PREPARATION

Chloroplasts were isolated from four week-old greenhouse grown spinach using two different methods - one yielding thylakoid membrane fragments, the other intact or gently broken chloroplasts.

2.1.1 BROKEN CHLOROPLASTS

Using a polytron homogenizer, approximately 15g of cold de-ribbed spinach leaves were ground for 10 seconds in 100mL of cold isolation medium (0.33M sorbitol, 30mM MOPS pH 7.8, 1mM MgCl_2 , 0.1% BSA). The large debris was removed by filtering through cheesecloth and cotton wool and the filtrate centrifuged for 90 seconds at 4000g. The resulting pellet was resuspended in a cold hypotonic medium (90mM NaCl, 30mM MOPS pH 7.8, 1mM MgCl_2) and left in the dark for 3 minutes thus breaking the outer chloroplast membranes by osmotic shock. The re-suspended pellet was centrifuged at 4000g for 90 seconds, the supernatant was discarded and the resulting pellet re-suspended in about 5mL of cold isolation medium (no BSA).

2.1.2 INTACT CHLOROPLASTS

Chloroplasts with relatively intact outer membranes were prepared using Heber's modification of the Jensen-Bassham procedure (Heber, 1973; Jensen & Bassham, 1966).

The spinach was harvested, washed, and placed in cold distilled water. Three 15g lots of the cold de-ribbed spinach leaves were each ground in 50mL of cold solution A (0.33M sorbitol, 2mM NaNO_3 , 2mM EDTA, 1mM MnCl_2 , 2mM sodium isoascorbate, 1mM MgCl_2 , 0.5mM K_2HPO_4 , 50mM MES pH 6.0, 20mM NaCl) with a polytron homogenizer for 3 seconds and then filtered through cheesecloth. The filtrate was centrifuged at 2500g for 40 seconds and the supernatant discarded. The pellet was washed carefully with a small amount of solution A so as to remove the soft pellet containing mostly broken chloroplasts. The pellets were then gently re-suspended in a total of 40mL of solution B (solution A except 50mM, HEPES pH 6.7 replacing the HEPES, pH 6.0) and centrifuged at 360g for 1 minute. The supernatant was decanted and re-centrifuged at 2500g for 30 seconds, with the resulting pellet being re-suspended in 2-3ml of cold solution B. This was the intact chloroplast suspension used for experiments.

To obtain gently broken chloroplasts, intact chloroplasts were suspended in 1mL of 50mM HEPES pH 7.5 for 10 seconds (hypotonic to rupture the outer membrane) then

1mL double strength solution C (0.33M sorbitol, 2mM NaNO_3 , 2mM EDTA, 1mM MnCl_2 , 10mM MgCl_2 , 0.5mM K_2HPO_4 , 50 mM HEPES pH 7.5) added.

To determine the proportion of functionally intact chloroplasts oxygen evolution in the presence of ferricyanide (Heber & Santarius, 1970) was measured for whole and gently broken chloroplasts. To measure the rate of oxygen evolution, enough chloroplasts were added to 3mL of solution C to give 20 μg of chlorophyll per mL. This suspension was enriched with 1mM ferricyanide. Percent intactness was generally 65-80%.

2.1.3 CHLOROPHYLL CONCENTRATION

The chlorophyll concentration was measured by Vernon's procedure (Vernon, 1960). A small aliquot of the stock chloroplast suspension was diluted 1:100 with 80% acetone in water and centrifuged at 733g for 5 minutes to remove chloroplast debris. Using a Bausch & Lomb Spectronic 70 the absorbance of the chlorophyll solution was read at 649nm and 665nm and the chlorophyll concentration determined as follows:

$$[\text{Chl}] (\mu\text{g/ml}) = [17.72(A_{649}) + 6.45(A_{665})] \times 100$$

2.2 FLUORESCENCE EXPERIMENTS

2.2.1 INITIAL EXPERIMENTS TO MEASURE F_o , F_{max} AND F_v

The activating light in this apparatus was a tungsten halogen lamp with the intensity ($130\mu\text{Em}^{-2}\text{s}^{-1}$) modulated at a frequency of 100Hz by a Princeton Applied Research (PAR) light chopper type 222. The light was then passed through a blue glass filter with a cut-off wavelength of 550nm, and directed through one wall of the quartz cuvette containing the chloroplast suspension. The activating light in this apparatus was later replaced by four light emitting diodes (Hewlett Packard, HLMP 3750) with maximum emission at 590nm (yellow). A specially designed (Science Technology Centre, Carleton University) power supply for the photodiodes produced a square wave form modulated at 20kHz. In this apparatus illumination took place through all four walls of the quartz cuvette (1LED/wall) and fluorescence was observed through the bottom of the cuvette.

Fluorescence was observed using an optic light-guide and a photomultiplier tube (EMI, type 9659B), protected by a 690nm interference filter (Baird Atomic, type B1), situated perpendicular to the activating light. A lock-in amplifier (PAR, type HR8) connected to a chart recorder (Kipp and Zonen, type BD41) and/or a storage oscilloscope (Tektronic, type R5103N) was used to measure the output of the photomultiplier tube.

2.2.2 EXPERIMENTS DESIGNED TO OBSERVE FLUORESCENCE TRANSIENTS IN DETAIL

The second apparatus used for fluorescence experiments enabled detailed analysis of the fluorescence induction curves. The activating light was the light emitting diode set-up but this time the maximum emission was at 635nm and the intensity was $12\mu\text{Em}^{-2}\text{s}^{-1}$. The output of the photomultiplier tube was measured by a lock-in amplifier (Ithaco, type 3962) with a time constant of 1ms. Through the use of an A/D board (Data Translation, type DT2801), the lock-in amplifier output was digitized and fed into a micro-computer (Raven/8, Science Technology Centre, Carleton University) where it was processed and stored on disk. The processing as well as the analysis of the data was done using the Asyst software package (MacMillan Software Co. version 1.56).

2.2.2.1 Analysis of Fluorescence Transients

Initial fluorescence (F_0) was taken as the level of fluorescence attained after 12ms of illumination. The maximum level of fluorescence (F_{max}) was taken as the average value for the last 10% of the fluorescence transient curve. Using numerical integration, the area between the fluorescence curve and F_{max} and between F_0 and any point later was determined and normalized with respect to the total area (Sinclair & Spence, 1988). This area will be referred

to as AREA normal. In the results section the natural logarithm of (1 - AREA normal) is plotted against time and this is termed a complementary area decrease plot (CAD). A CAD plot represents the reduction of PSII electron acceptors and allows the evaluation of rate constants. Most CAD plots had an initial curved part followed by a slower linear section. This biphasic nature was taken to represent the sum of two exponentials. Using the Method of Least Squares the best fit straight line was determined for the linear portion of the CAD curve. From this straight line the rate constant (slope) and the size (y-intercept) of the exponential phase were determined. The slower linear phase was then subtracted from the rest of the data and another CAD curve produced, again the rate constant and size were determined. This procedure was repeated until the CAD curve was no longer biphasic (Sinclair & Spence, 1988).

2.2.3 THE STANDARD EXPERIMENT

To study the effect of light and dark pre-treatments on fluorescence two identical chloroplast samples were prepared - one was placed in the dark for 30 minutes while the other was illuminated at $6\mu\text{Em}^{-2}\text{s}^{-1}$ (650nm) for 30 minutes.

Broken chloroplasts were used for the early experiments in which only F_o , F_v and F_{max} were measured. In this case the incubation medium contained 0.33M sorbitol, 30mM MOPS pH 7.8, 10mM MgCl_2 , 50mM NaCl. For the work with fluorescence

transients, gently broken chloroplasts were employed and the incubation medium was solution C. Methyl Viologen (0.1mM) was added to all samples. Chloroplasts were added to 2mL of the appropriate incubation medium to give a final chlorophyll concentration in the range 5-8 μ g/mL and the sample incubated for 30 minutes in the light or the dark. Light treated samples were placed in a box which permitted illumination from a microscope lamp filtered by a 7cm thickness of water and a 650nm interference filter (Baird Atomic, type B1). The illuminating light had an intensity of 6 μ E m⁻².s⁻¹ and a duration of 30 minutes. Dark treated samples were placed in complete darkness for 30 minutes. After light or dark pre-treatment, the sample was transferred to a quartz cuvette, placed in the fluorescence apparatus and left in the dark for five minutes. DCMU (DuPont deNemours & Co.) was added to give a final concentration of 20 μ M and the sample left in the dark for one minute prior to the measurement of fluorescence. For most of the transient analysis work, 50 μ M DCMU was used.

Fluorescence tended to drop through the day, so rather than calculating a daily average for the light and dark samples, experiments were designed in such a way that a dark sample followed a light sample or vice versa. This made it possible to express the fluorescence intensity from a light sample as a percentage of the fluorescence from the corresponding dark sample.

The same set of experiments was repeated a number of times each day for 2-3 days. This made it possible to average the light percentages and calculate a standard deviation. The Student's t-test at a 95% level of confidence was used to determine if results were significantly different.

The Effect of Uncouplers

Three uncouplers with different modes of action were added to the incubation medium prior to the 30 minute pre-incubation in the light or the dark. The uncouplers were:

1. 10mM Ammonium Chloride
2. 1 μ M Nigericin in ethanol
3. 1 μ M Valinomycin in water/ethanol

These concentrations were chosen as they produced maximal oxygen evolution and therefore the greatest amount of uncoupling.

Nigericin requires hydrogen and potassium ion exchange in order to uncouple, therefore, for experiments with Nigericin 50mM KCl replaced the 50mM NaCl in the incubation medium (Shavit et al, 1968).

2.3 PARTIAL REACTION EXPERIMENTS

Photosystems I and II can be biochemically isolated through the use of appropriate inhibitors and redox compounds. The effect of light and dark pre-treatments on the functioning of the two photosystems in isolation was studied using the methods described below.

2.3.1 APPARATUS

The apparatus used for both of these experiments was a biological oxygen monitor (Yellow Springs Instrument Co., Model 53) connected to a chart recorder (Perkin-Elmer, model 165). The samples were placed in the bath-stirrer assembly (YSI, model 5301) which was kept at 21°C through the use of a constant temperature circulator (Lauda, model K-2). The light for the experiments was provided by a 300W floodlamp (Westinghouse) with a 7cm waterbath placed between it and the sample, to absorb heat.

Broken chloroplasts were used for these experiments and were suspended in a high salts incubation medium (0.33M sorbitol, 30mM MOPS pH 7.8, 10mM MgCl₂, 50mM NaCl).

2.3.2 PSI PARTIAL REACTION

For all oxygen consumption experiments enough broken chloroplasts were added to 3mL of incubation medium enriched with 0.1mM methyl viologen to give a final chlorophyll concentration of 50 μ g/mL. The samples were incubated in the dark or light for thirty minutes as described earlier, after which 5 μ M DCMU, 0.1mM DCPIP and 3mM ascorbate were added.

DCMU blocks electron transport (no detectable oxygen evolution) and does not allow the reduction of plastoquinone (PQ) by PSII (Wright & Corbett, 1979). This leaves the reduced form of DCPIP to donate electrons in front of PSI, with the ascorbate assuring a constant supply of DCPIPH₂ (rxn 10). Methyl Viologen is a low potential artificial electron acceptor, the reduction of which can be followed by monitoring oxygen consumption (Ort & Izawa, 1974).

In the presence of chloroplasts and light there is a transfer of electrons from DCPIP H_2 to methyl viologen (MV^{2+}) (rxn 11). Aerobic oxidation then ultimately leads to the formation of H_2O_2 and the consumption of oxygen (rxn 12&13). This consumption of oxygen is detected by the oxygen electrode (Ort & Izawa, 1974).

Once the DCMU, DCPIP and ascorbate were added, the chloroplast sample was placed in the reaction vial and the electrode inserted. The vial and stir-bath were covered with a light-proof cloth and O_2 consumption recorded in the dark until a flat or steady trace was attained. At this point the light was switched on and the sample uncovered. The light intensity was $83\mu\text{Em}^{-2}\text{s}^{-1}$ (within light limiting region) after the light was passed through two 665nm filters (Baird Atomic, type RG665). Following 3-4 minutes of illumination the light was switched off, the sample covered and O_2 consumption recorded in the dark for one minute.

Rates of O_2 consumption were expressed as $\mu\text{moles } O_2/(\text{mg chl}) \cdot \text{hr}$ (see appendix A for sample calculation). If there was O_2 consumption in the dark then this was subtracted from the rate in the light, thus giving a rate for light induced O_2 consumption.

2.3.3 PSII PARTIAL REACTION

When a leaf is illuminated and photosynthesis proceeds, oxygen is evolved. This oxygen comes from the splitting of water at PSII and the rate of oxygen evolution is proportional to electron transport through PSII. When isolated chloroplasts are illuminated, very little if any oxygen is evolved unless an artificial electron acceptor is added. The acceptor used was DCPIP (dichlorophenyl indophenol).

Due to its hydrophilic nature DCPIP will only accept electrons from PSI in intact chloroplasts but in broken chloroplasts, as were used, DCPIP comes into play before PSI (Hall & Rao, 1987).

As was the case with oxygen consumption experiments enough chloroplasts were added to 3ml of the high salts

incubation medium to give a chlorophyll concentration of $50\mu\text{g/mL}$. For the oxygen evolution experiments 2mM ferricyanide (PSI acceptor) was added and then the samples incubated for 30 minutes in the light or the dark. Following the 30 minute incubation period 0.1mM DCPIP was added and the samples placed in the reaction vial. The sample was covered and oxygen evolution recorded in the dark until the trace was flat. The light was switched on, the sample uncovered and oxygen evolution in the light measured for 4-6 minutes. The light intensity was $688\mu\text{Em}^{-2}\text{s}^{-1}$ which was below light saturating levels. Following the 4-6 minutes of illumination the sample was again covered and oxygen evolution in the dark measured for approximately one minute. Rates of oxygen evolution were expressed as $\mu\text{moles O}_2/(\text{mg chl})\cdot\text{hr}$ (see appendix A).

2.4 ABSORPTION SPECTRA

The effect of light and dark pre-treatments on the absorption spectrum of chloroplasts was studied and compared to those from samples receiving no pre-treatment. In order to reduce the variability due to pipetting, samples containing 10mL of incubation medium (0.33M sorbitol, 30mM MOPS, 10mM $MgCl_2$, 50mM NaCl, 0.1mM methyl viologen) and enough chloroplasts to give a final chlorophyll concentration of 8 μ g/mL were prepared. From this 10mL sample, three 2.5mL aliquots were removed, two were placed in small screw top sample vials - one in the dark for 30 minutes, the other in the light for 30 minutes. The third aliquot was placed in a glass cuvette and 5 μ M DCMU added. An absorption spectrum from 350-750nm (Varian, Cary 219 spectrophotometer) was then run on this sample. Following the 30 minute incubation period, 5 μ M DCMU was added to the light and dark treated samples and absorption spectra run.

As a control for variability, experiments were performed where three identical (three 2.5mL aliquots from a 10mL sample) non-incubated samples were run one right after the other.

CHAPTER 3: RESULTS

FLUORESCENCE EXPERIMENTS

3.1.1 INITIAL OBSERVATIONS OF F_o , F_m , AND F_v

The maximum level of fluorescence (F_m) from a sample pre-treated with light for 30 minutes was on average only $86 \pm 6\%$ that from an identical sample kept in the dark for 30 minutes (figure 5). Initial fluorescence (F_o) was greater from the light-treated sample thus making variable fluorescence ($F_m - F_o = F_v$) much lower from the light samples (figure 5). Prior to the addition of DCMU there was no difference in fluorescence from light and dark samples (data not shown).

3.1.1.1 The Effect of Methyl Viologen & Hydroxylamine on F_o , F_v and F_m

Normally there was 0.1mM Methyl Viologen present in each sample during the 30 minute incubation. Leaving the methyl viologen out of the incubation medium did not have a significant effect on the light-induced quenching. The difference in F_{max} , F_v and F_o between light and dark-treated samples remained the same as when methyl viologen was present during the incubation (table 1).

When 1mM hydroxylamine was added to the samples just prior to the measurement of fluorescence, the difference in fluorescence between the light and dark-treated samples was

FIGURE 5: THE VALUES OF F_m , F_v & F_o FROM LIGHT PRE-TREATED SAMPLES AS PERCENTAGES OF CORRESPONDING VALUES FOR DARK-TREATED SAMPLES.

The 'Light vs Dark Effect': Two identical chloroplast samples containing methyl viologen were prepared - one was placed in the dark for 30 minutes while the other was illuminated at $6\mu\text{Em}^{-2}\text{s}^{-1}$ (650nm) for 30 minutes. Samples were placed in the fluorescence apparatus and left in the dark for 5 minutes prior to the addition of $20\mu\text{M}$ DCMU. Samples were left in the dark for another minute and then fluorescence measured. Experiments were done in pairs (one light & one dark) with the light value being expressed as a percent of the dark value (relative fluorescence). A decrease in relative fluorescence indicates an increase in the difference between light and dark. The error bars represent the standard deviation on the average of 30 trials.

TABLE 1: THE EFFECT OF METHYL VIOLOGEN AND
HYDROXYLAMINE ON THE LIGHT VS DARK EFFECT

TRMT	Fm	Fv	Fo
CONTROL	88±6	78±7	108±6
- METHYL VIOLOGEN	87±4	75±6	102±13
+HYDROXYLAMINE	87±6	78±8	105±5

Experiments as described in figure 5, except that, either the 0.1mM methyl viologen was left out, or 1mM NH_2OH was added following the 30' incubation. Light results expressed as a percent of the dark values, plus or minus the standard deviation (n=15).

still present (table 1). In fact, the difference between light and dark-treated samples remained the same for maximum and variable fluorescence.

3.1.1.2 The Effect of Uncouplers on F_o , F_v and F_m

When 10mM ammonium chloride was added to samples before pre-treatment the difference between the fluorescence emitted by light and dark-treated samples was increased (table 2).

In terms of absolute values, the addition of ammonium chloride decreased the maximum level of fluorescence from both light and dark treated samples as compared to samples without ammonium chloride (data not shown) but the decrease was much greater for the light samples (25% as compared to 5%, table 2). This made the difference in F_m between light and dark-treated samples larger (32%). The difference between light and dark-treated samples for F_v was even greater (41%). In the absence of NH_4Cl , F_o was 12% larger for the light samples but when NH_4Cl was present there was no longer any difference between light and dark (table 2).

When nigericin replaced ammonium chloride there was a slight increase in the difference between light and dark-treated samples (as compared to samples without nigericin) for F_m , F_v and F_o but it was not significant (table 2).

The third and final uncoupler used was valinomycin and the results were very similar to those for samples containing nigericin. With DCMU present there was not a significant

TABLE 2: THE EFFECT OF UNCOUPLERS ON THE
LIGHT VS DARK EFFECT

TRMT	Fm	Fv	Fo
CONTROL	88±6	78±7	108±6
AMMONIUM CHLORIDE	68±11	55±6	97±12
NIGERICIN	85±6	74±7	106±5
VALINOMYCIN	89±3	77±4	109±6

Experiments as described in figure 5, except that 10mM ammonium chloride or 1mM nigericin or 1mM valinomycin was added to half the samples prior to the 30 minute incubation. Light results expressed as a percent of dark values plus or minus the standard deviation with and without uncouplers (n=20 for control, NH₄Cl and nigericin, n=10 for valinomycin).

difference for F_m , F_o and F_v . The inclusion of both nigericin and valinomycin in the medium prior to the pre-treatment was unable to reverse the light-induced quenching.

3.1.1.3 The Effect of Pre-Illumination Conditions on F_o , F_v and F_m

As it seemed that it was the light samples rather than the dark samples which were different from non-incubated controls (table 6), a number of variations of the standard illumination conditions used above were tried.

The normal pre-illumination intensity was $6\mu\text{Em}^{-2}\text{s}^{-1}$. Lowering this to $3\mu\text{Em}^{-2}\text{s}^{-1}$ had no effect on the difference between light and dark for F_m , F_o or F_v (table 3). Doubling the light intensity to $12\mu\text{Em}^{-2}\text{s}^{-1}$ further increased the difference between light and dark - 8% to 17% for F_m and 16% to 25% for F_v . At $12\mu\text{Em}^{-2}\text{s}^{-1}$ the difference between light and dark for F_o was not significantly different from that at $6\mu\text{Em}^{-2}\text{s}^{-1}$ (table 3).

A dark period following the 30 minute pre-illumination did not reverse the light-induced quenching of fluorescence (figure 6a). A dark period further decreased maximum fluorescence from the light samples rather than restoring it to the level of the dark samples (figure 6b). The maximum fluorescence from the dark samples also tended to decrease with time, more or less in parallel with light samples (figure 6b). The difference between light and dark increased

TABLE 3: THE EFFECT OF PRE-ILLUMINATION INTENSITY
ON THE LIGHT VS DARK EFFECT

TRMT	Fm	Fv	Fo
CONTROL ($6\mu\text{Em}^{-2}\text{s}^{-1}$)	88 ± 6	78 ± 7	108 ± 6
$3\mu\text{Em}^{-2}\text{s}^{-1}$	87 ± 7	75 ± 6	107 ± 12
$12\mu\text{Em}^{-2}\text{s}^{-1}$	79 ± 2	69 ± 3	105 ± 7

Experiments as in figure 5 except that the pre-illumination light intensity was varied. In addition to the normal intensity ($6\mu\text{Em}^{-2}\text{s}^{-1}$) a lower intensity ($3\mu\text{Em}^{-2}\text{s}^{-1}$) and a higher intensity ($12\mu\text{Em}^{-2}\text{s}^{-1}$) were used. Light results expressed as a percentage of dark values plus or minus the standard deviation (n=10)

Figure 6

Two identical samples were pre-treated as in figure 5. Following the 30' light period the sample was transferred to the dark for the periods of time indicated, while the dark samples was kept in the dark the entire time. The rest of the procedure was as described in figure 5. The fluorescence from the light/dark samples was expressed as a percent of the fluorescence from the corresponding dark samples plus or minus the standard deviation (n=8).

FIGURE 6: REVERSIBILITY OF THE LIGHT VS DARK EFFECT

after a 30 minute dark period but was reduced after a 45 minute dark period and after 60 minutes was at the same level as with no dark period (figure 6a). The difference in F_v between light and dark-treated samples also varied with the length of the dark period. However, the variability of the results for 45 and 60 minutes was quite large thus making it difficult to determine a definite trend (figure 6a). The difference in F_o changed very little in response to a dark period (figure 6a).

3.1.2 THE ANALYSIS OF FLUORESCENCE TRANSIENTS

It was previously shown (Sinclair & Spence, 1988) that the length of the measurement period was critical to an accurate analysis of fluorescence transients. Using the longer illumination times, a fluorescence transient composed of three phases was found. Subsequent to this, a fourth phase has been detected.

The fluorescence induction transient in Figure 7a was found to be composed of four phases which are being called, from fastest to slowest, α , β , τ , δ . Figure 7b is the first CAD plot for the transient in 7a. This curve is biphasic and the linear portion represents the slowest phase (δ) with a slope of -0.156s^{-1} . This δ -phase makes up 46% of the total area above the curve. Subtracting this slow phase yields another biphasic CAD curve (figure 7c) with a slope of -1.30s^{-1} and corresponding to 25% of the total area (τ -phase). Again subtracting the slowest phase leads to a biphasic CAD curve (figure 7d). This time the slope of the linear part is -7.20s^{-1} and the β -phase represents 14% of the total area. The remaining 15% percent of the total area is accounted for by the non-exponential α -phase (figure 7e).

Figure 7a

The first 200ms of a fluorescence induction transient for a non-incubated control sample. The sample was prepared, dark adapted for 5 minutes prior to the addition of 50 μ M DCMU. A further 1 minute dark period was allowed then the sample was illuminated and the fluorescence induction transient recorded. F_{max} was taken as the average of the last 500 points, F_o taken after 12ms of illumination and $F_v = F_{max} - F_o$. This fluorescence transient is from one experiment but is typical of others obtained using the same procedure.

FIGURE 7a: A FLUORESCENCE INDUCTION TRANSIENT

Figures 7b-e

Figures 7b-e are complementary area decrease (CAD) curves for the fluorescence transient in Figure 7a. Using numerical integration, the area between the fluorescence curve and F_{max} and between F_0 and any point later was determined and normalized with respect to the total area (area normal). The linear (slowest) portion of the CAD curve is subtracted from the rest of the data and another CAD curve produced. This is repeated until the CAD curve is no longer biphasic.

FIGURE 7b: THE FIRST COMPLIMENTARY AREA DECREASE (CAD) CURVE

FIGURE 7c: THE SECOND CAD CURVE

FIGURE 7d: THE THIRD CAD CURVE

FIGURE 7e: THE FOURTH CAD CURVE

3.1.2.1 DCMU Sensitivity

Increasing the DCMU concentration from $1\mu\text{M}$ to $50\mu\text{M}$ had a major effect on fluorescence induction transients (figure 8). The fluorescence rise was more rapid in the presence of $50\mu\text{M}$ DCMU and the F_{max} values for the $1\mu\text{M}$ DCMU samples were lower (table 4)

When the area above the fluorescence curves was analysed into the four phases, the differences were quite significant (table 4). Other than the fastest phase (α), the CAD curves were quite different. The total area above the curve for $1\mu\text{M}$ samples was twice the value in the presence of $50\mu\text{M}$ DCMU (table 4). The larger area was mostly accounted for by an increase in the slowest two phases. The β -phase was unchanged while the α -phase was larger in the presence of $50\mu\text{M}$ DCMU.

Increasing the DCMU concentration to $500\mu\text{M}$ had little effect on the appearance of the fluorescence transient (figure 8). The same was true for the CAD plots (not shown) and likewise the total area and the areas associated with each phase (table 5).

Based on these results, $50\mu\text{M}$ DCMU was used for all other experiments involving the analysis of fluorescence transients.

Figure 8

Non-incubated samples were dark-adapted for 5 minutes before the DCMU was added. 100uL of DCMU was added to give final concentrations of 1, 50 and 500 μ M DCMU. A 1 minute dark period followed the addition of DCMU then the sample was illuminated and the fluorescence transient recorded. Analysis of these curves produced the numbers in Tables 4 and 5.

FIGURE 8: THE EFFECT OF DCMU CONCENTRATION ON FLUORESCENCE
INDUCTION TRANSIENTS

TABLE 4: $1\mu\text{M}$ DCMU COMPARED TO $50\mu\text{M}$ DCMU

Initial, Variable and Maximum Fluorescence			
TRMT	Fo	Fv	Fm
$1\mu\text{M}$ DCMU	1.20	2.02	3.22
$50\mu\text{M}$ DCMU	1.34	2.20	3.54

Transient Analysis					
TRMT	TOTAL AREA	α	β (fastest to slowest) (relative units)	τ	δ
$1\mu\text{M}$ DCMU	271	13	17	89	152
$50\mu\text{M}$ DCMU	108	19	16	28	45

Experiments as described for figure 8. Analysis of the curves in figure 8 produced the results in table 4.

TABLE 5: 50 μ M DCMU COMPARED TO 500 μ M DCMU

Initial, Variable and Maximum Fluorescence			
TRMT	F _o	F _v	F _m
50 μ M DCMU	1.28	2.26	3.54
500 μ M DCMU	1.35	2.27	3.62

Transient Analysis					
TRMT	TOTAL AREA	α	β (fastest to slowest) (relative units)	τ	δ
50 μ M DCMU	111	18	14	26	53
500 μ M DCMU	119	20	13	26	61

Experiments as described for figure 8. Analysis of the curves in figure 8 produced the results in table 5.

3.1.2.2 The Effect of Light & Dark Pre-treatment on Fluorescence Induction Transients

The difference in fluorescence produced by light and dark-treated samples was thought to be a light effect, meaning that it was the samples illuminated for 30 minutes which were different from non-incubated controls. This was based upon values for maximum and variable fluorescence (table 6) where the difference between light-treated and non-incubated control samples was on average 28% but only 9% between dark-treated and control samples.

When the first 200ms of a fluorescence transient are examined (figure 9a) it is obvious that the curves for non-incubated controls and the dark-treated samples were quite similar (almost identical up to 70ms) except that in the end the control sample attained a slightly higher value for F_{max} (table 6). It can be seen in figure 9a that the shape of the transient from the light-treated sample is significantly different from the other two samples. After a 30 minute pre-illumination period, not only was maximum fluorescence reduced but also the fluorescence transient appears to lose much of the sigmoidicity which is present in control and dark curves in the first 60ms (figure 9b). The straight lines associated with the fluorescence curves in figure 9b are the best-fitting straight lines for the first 20ms of the variable fluorescence curves. The curve for the non-incubated sample is clearly sigmoidal as the best-fit line

**TABLE 6: THE EFFECT OF LIGHT & DARK
PRE-TREATMENTS**

Initial, Variable and Maximum Fluorescence			
TRMT	F _o	F _v	F _m
CONTROL	1.28	2.36	3.64
LIGHT	1.27	1.64	2.91
DARK	1.26	2.05	3.31

Transient Analysis					
TRMT	TOTAL AREA	α	β (fastest to slowest) (relative units)	τ	δ
CONTROL	238	8	28	38	165
LIGHT	232	7	18	40	167
DARK	135	16	17	28	74

Experiments performed as in figure 9A. Fluorescence induction transients corresponding to these values are those of figure 9a.

Figure 9

Samples prepared and pre-treated as in Figure 5. Non-incubated samples received no pre-treatment. The fluorescence transients were recorded in the presence of 50 μ M DCMU and the analysis of these transients yielded the results presented in Table 6. A) shows the first 200ms of the fluorescence induction curve. In B) the straight lines represent the best-fit lines for the first 20ms of the variable fluorescence curves. These lines are an indicator of sigmoidicity of the initial part of the curve. In C) the levels of fluorescence for 1 second intervals of illumination were calculated and plotted as a percent of the final level of fluorescence. The results presented in figure 9 are the results from only one set of experiments (control, light, dark) but are representative of other results obtained.

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FIGURE 9b: CHANGES IN SIGMOIDICITY

deviates first below then above the transient. After a 30 minute illumination all sigmoidicity is gone and the straight line is tangential to the curve (figure 9b) while the 30 minute dark period had very little effect on sigmoidicity.

After looking at the first 200ms of the fluorescence transient and seeing that the curve for the light-treated sample was so much different from the other two (dark & control), it was surprising to find that as far as transient analysis was concerned, it was the light-treated samples which were most like the controls (table 6). At most there was a 6% difference between total areas for non-incubated control samples and light-treated samples while the difference between dark-treated and control samples was at least 40%. This large decrease in area was due in large part to a decrease in the size of the slowest phase (δ -phase). The size of the δ -phase was reduced by 50-70% while the τ -phase was reduced by 10-25% relative to control samples. For both of these phases the results from light-treated samples were identical to the controls. For the β -phase it was the areas for the light and dark-treated samples which were identical and 30-40% less than areas from controls. The fastest phase (α) was small and tended to be variable but in many cases it was twice as large from the dark-treated samples as from controls and the area from the light-treated samples was even smaller (table 6).

If fluorescence induction kinetics are fast, this means

that the level of fluorescence rises more quickly and levels off sooner. The faster rise should be reflected by a reduction in the area above the curve. The area above the curve for dark-treated samples is much smaller than for light-treated samples, and as figure 9c shows, the fluorescence level rose much more quickly for the dark-treated samples. After 1 second of illumination, the fluorescence from the dark samples is 98.19% of F_{max} while it is only 97.0 and 95.5% for control and light-treated samples respectively. After 6 seconds the values are 99.6%, 98.9% and 98.7% (dark, control, light).

3.1.2.3 Development of the Light Versus Dark Effect

When fluorescence was measured after 0, 5, 15, 30 minutes illumination, it was found that both maximum and variable fluorescence decreased with illumination time in a more or less linear fashion while initial fluorescence remained fairly constant (figure 10a). There was an average 27% drop in F_v and 16% drop in F_{max} over the 30 minute incubation period. Fluorescence levels from dark-treated samples also tended to decrease as pre-incubation time increased, but the decrease was only half that for the light-treated samples (figure 10b). Again, the decrease in F_o was minimal (figure 10b). Since the decrease in absolute fluorescence levels for light-treated samples was twice as large as for dark-treated samples, the differences in F_m and

FIGURE 9C: AVERAGE FLUORESCENCE AS A
PERCENT OF F_{max}

Figure 10

Samples treated as in figure 5 except that the length of the incubation period varied between 0 and 30 minutes. DCMU (50 μ M) was added, followed by a minute of darkness, then the fluorescence transient recorded. F_m was taken as the average of the last 500 points, F_o taken after 12ms of illumination and $F_v = F_m - F_o$. The data presented in figure 10 correspond to the transients in figures 12a & 13 and are the results of one set (0, 5, 15, 30) of light or dark experiments. However, another 10 sets fit these trends.

FIGURE 10: THE EFFECT THAT VARYING THE LENGTH OF PRE-TREATMENT HAS ON MAXIMUM, VARIABLE AND INITIAL FLUORESCENCE

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Fv between light and dark-treated samples increased with time. For Fm the difference went from 5% after five minutes to 13% after thirty minutes (figure 11) and the drop for Fv was from 7% to 13% (figure 11). As with Fm and Fv, the difference in Fo increased with illumination time except that light values were higher than dark (figure 11). Lengthening the pre-incubation period to 60 minutes further increased the difference between light and dark.

Examining the first 200ms of the fluorescence transients from light-treated samples (figure 12a), it is apparent that shape changes with time. As previously shown, a light pre-treatment reduced sigmoidicity. The sigmoidicity is gradually reduced as the length of the illumination period is increased. This is evidenced by the best-fitting lines and the transients coming closer together in figure 12b.

The fluorescence transients from dark-treated samples exposed to dark periods of varying lengths were almost identical up to 40ms (figure 13). Five minutes in the dark seemed to have little effect on the fluorescence transient as it was very similar to the non-incubated control. However, a 15 minute dark period did have an effect and the 30 minute dark period had an even bigger effect. The great similarity between curves in the first 40ms means that the dark pre-treatment had little effect on the sigmoidicity of the curves.

FIGURE 11: DEVELOPMENT OF THE LIGHT VS DARK EFFECT

Experiments as outlined for figure 5, except incubation periods varied between 0 and 60 minutes. Values from light-treated samples are expressed as a percent of values from dark-treated samples, plus or minus the standard deviation (n=10).

Figure 12

Samples prepared, and pre-treated as in figure 5 except that the length of the light period was varied - 0, 5, 15 and 30 minutes. Figure 12a shows how the initial fluorescence induction kinetics (first 200ms) change with the length of pre-illumination. In figure 12b the best-fitting straight lines were fit to the first 20ms of the variable fluorescence curve to show the changes in sigmoidicity. Figure 10a plots fluorescence levels (F_m , F_o , F_v) from these transients while figure 14a shows how the area above the curve changes with illumination. The results presented here are the results from only one set of experiments but are representative of other results obtained.

FIGURE 12a: THE EFFECT OF VARYING THE LENGTH OF
PRE-ILLUMINATION

FIGURE 12b: THE GRADUAL LOSS OF SIGMOIDICITY

Figure 13

Experiments carried out as explained in figure 12, except that it was the length of the dark period that was varied. These transients correspond to the data in figures 10b and 14b. The results presented here are the results from only one set of experiments (0, 5 , 15 , 30' dark) but are representative of other results obtained.

FIGURE 13: THE EFFECT OF VARYING THE LENGTH
OF THE DARK PERIOD

TABLE 7: THE EFFECT OF FERRICYANIDE

Initial, Variable and Maximum Fluorescence			
TRMT	F _o	F _v	F _m
- FeCN	2.23	2.25	4.48
+ FeCN	1.77	2.33	4.10

Transient Analysis					
TRMT	TOTAL AREA	α	β (fastest to slowest) (relative units)	τ	δ
- FeCN	120	17	20	31	52
+ FeCN	262	10	31	58	162

Experiments as in Figure 15. The first 200ms of the fluorescence induction curves which produced these results are pictured in figure 15.

Figure 14

Procedure as described in figure 5 except using various length of pre-incubation and 50 μ M DCMU.

Detailed analysis of the transients in figures 12a (light) and 13 (dark), using the procedure outlined in the methods and materials, separated four phases from each transient. Figures 14a & b show how the areas associated with each phase change in response to the length of pre-incubation. Again the data is from one set of experiments but it is representative of the other sets.

A) is for pre-illumination B) for dark pre-incubation.

FIGURE 14: THE EFFECT THAT VARYING THE LENGTH OF PRE-TREATMENT HAS ON FLUORESCENCE INDUCTION TRANSIENT ANALYSIS

3.1.2.4 The Effect of Ferricyanide

The addition of ferricyanide five minutes prior to the addition of DCMU had a dramatic effect on fluorescence transients (figure 15). Ferricyanide greatly slowed the induction kinetics thus doubling the area above the curve (table 7). The δ -phase was the most affected by the addition of ferricyanide, increasing 60% over samples without ferricyanide. There was also a significant increase in the area associated with the τ (40%) and β -phase (30%). The size of the α -phase was reduced by ferricyanide (table 7). F_o was an average of 24% higher and F_{max} approximately 9% higher in the absence of ferricyanide while F_v was relatively unaffected by ferricyanide (table 7).

3.1.2.5 The Effect of a Second Illumination

After a second five minute dark period, the chloroplasts were illuminated and a second transient recorded. This second illumination caused a considerable increase in F_o and a decrease in the extent of the variable fluorescence. The decrease in F_v was more than overcome by the increase in F_o thus F_{max} was greater following the second illumination. The total area was reduced to half following the second illumination and upon analysis it became apparent that the decrease in the δ -phase was almost entirely responsible (table 8). Calculation of the ratios of the sizes of the four phases for the two illuminations (2nd/1st) confirms that

Figure 15

The samples were prepared, placed in the fluorescence apparatus and 0.5mM ferricyanide added in the darkness. The samples was dark adapted for 5 minutes prior to the addition of 50 μ M DCMU and 1 minute after the DCMU. Then the light was turned on and the fluorescence induction transient recorded. Figure 15 shows the first 200ms of the fluorescence induction curves for a typical ferricyanide experiment.

FIGURE 15: THE EFFECT OF FERRICYANIDE ON FLUORESCENCE
INDUCTION KINETICS

TABLE 7: THE EFFECT OF FERRICYANIDE

Initial, Variable and Maximum Fluorescence			
TRMT	Fo	Fv	Fm
- FeCN	2.23	2.25	4.48
+ FeCN	1.77	2.33	4.10

Transient Analysis					
TRMT	TOTAL AREA	α	β (fastest to slowest) (relative units)	τ	δ
- FeCN	120	17	20	31	52
+ FeCN	262	10	31	58	162

Experiments as in Figure 15. The first 200ms of the fluorescence induction curves which produced these results are pictured in figure 15.

Table 8

Samples were prepared as in figure 5, except no pre-treatment given. The sample was illuminated and fluorescence measured twice with an intervening 5 minute dark period. This was done in the presence and absence of ferricyanide. These results are representative of other results obtained.

TABLE 8: THE EFFECT OF A SECOND ILLUMINATION

Initial, Variable and Maximum Fluorescence			
TRMT	Fo	Fv	Fm
<u>- FeCN</u>			
1ST LIGHT	1.70	2.50	4.20
2ND LIGHT	2.13	2.27	4.40
<u>+ FeCN</u>			
1ST LIGHT	1.42	2.27	3.69
2ND LIGHT	1.57	2.17	3.74

Transient Analysis					
TRMT	TOTAL AREA	α	β (fastest to slowest) (relative units)	τ	δ
<u>- FeCN</u>					
1ST LIGHT	187	21	21	32	114
2ND LIGHT	92	18	16	32	26
<u>+ FeCN</u>					
1ST LIGHT	259	13	34	57	155
2ND LIGHT	156	16	31	50	59

it is the slowest phase which shows the greatest change while the others were reduced a small amount (α : 0.84 ± 0.13 β : 0.88 ± 0.13 τ : 0.94 ± 0.09 δ : 0.30 ± 0.1).

Illumination of a sample for a second time greatly reduced the sigmoidicity of the induction curve (figure 17). Drawing best-fit lines for the first 20ms of the variable fluorescence curve illustrates the loss of sigmoidicity quite well. The transient from the first illumination was nicely sigmoidal while the best-fit line for the second transient is almost tangential which indicates that the transient is completely exponential. This is supported by the shape of the final CAD curve which corresponds to the α -phase (figure 17a). For the second illumination the α -phase CAD curve is linear (exponential) while that from the first illumination is downward curving (sigmoidal).

A second illumination of samples containing ferricyanide increased F_o , but an equivalent decrease in F_v meant that F_{max} remained unchanged. Calculation of the ratios between first and second illuminations showed that the large decrease in total area which was observed was accounted for almost entirely by a decrease in the slowest phase (α : 1.0 ± 0.25 β : 1.0 ± 0.1 τ : 0.94 ± 0.07 δ : 0.42 ± 0.06). Sigmoidicity was not affected when ferricyanide was present (figure 16).

Figure 16

Samples containing 0.5mM ferricyanide were illuminated and a fluorescence transient recorded as described in figure 15. Following measurement of fluorescence the samples were left in the dark for another 5 minutes before being illuminated for the second time. The results of figure 16 are from one set of experiments but are typical of all others. These transients correspond to the numbers in table 8.

FIGURE 16: THE EFFECT OF A SECOND ILLUMINATION
IN THE PRESENCE OF FERRICYANIDE

Figure 17

Experiments as in figure 16 except no ferricyanide was added. The straight lines are the best-fitting straight lines for the first 20ms of the variable fluorescence curve and are an indicator of sigmoidicity. Detailed analysis of the transients in figure 17 yield the numbers in table 8. Figure 17a shows the CAD curves for a first and second illumination.

FIGURE 17a: CAD CURVES FOR A SECOND ILLUMINATION

3.2 PARTIAL REACTIONS

When it was found that the 30 minute light pre-treatment caused a decrease in maximum fluorescence, partial reaction experiments were performed to see if the light treatment resulted in a change in the way that absorbed light energy was distributed between the two photosystems. A decrease in energy going to PSII would cause a decrease in F_{max} , but should also cause a decrease in the PSII partial reaction rate under light limiting conditions. There should also be an increase in the PSI partial reaction rate.

3.2.1 PSI PARTIAL REACTION

To measure PSI reaction rate, electron transport from PSII is blocked through the use of DCMU. DCPIP is employed as an electron donor, methyl viologen as the acceptor, and oxygen consumption is measured. Absolute rates of oxygen consumption tended to be somewhat variable but if treated as fluorescence values and light samples expressed as a percentage of dark samples, then variability was reduced.

The difference in the PSI reaction rate between light and dark-treated samples was insignificant (table 9).

TABLE 9: THE EFFECT OF A LIGHT PRE-TREATMENT ON
ON PSI AND PSII PARTIAL REACTIONS

PARTIAL REACTION	LIGHT RELATIVE TO DARK
PSI	99±13
PSII	63±6

For PSI partial reaction experiments samples containing 0.1mM methyl viologen were pre-treated as in figure 5. Then 5 μ M DCMU, 0.1mM DCPIP and 3mM ascorbate were added and oxygen consumption measured. For PSII partial reaction experiments samples containing 2mM ferricyanide were pre-treated as in figure 5. Then 0.1mM DCPIP was added and oxygen evolution measured. Rates of oxygen evolution or consumption were calculated as in Appendix A then the rates from light samples expressed as a percent of the rate from dark samples (n=20).

3.2.2 PSII PARTIAL REACTION

The activity of PSII was followed by measuring oxygen evolution in the presence of DCPIP. As with oxygen consumption, absolute rates of oxygen evolution were variable but when a pair of samples (1 light, 1 dark) are compared, results are more consistent.

The PSII reaction rate from light-treated samples was only 63% that from the dark-treated samples (table 9).

3.3 ABSORPTION SPECTRA

The lower level of fluorescence emitted from light samples could be caused by a photo-induced destruction of light absorbing chlorophyll molecules. Absorption at 649nm was routinely checked for each sample and it did not vary between light and dark treatments (data not shown). It is possible that if there was a change in the absorption properties after the 30 minute light period it was not noticable at 649nm. However, a complete absorption spectra (400 - 700nm) would show such a change.

As can be seen in figures 18 a & b, light pre-treatments did not reduce the amount of light absorbed by a sample. In fact, for 70% of the trials the absorption spectrum from the light-treated sample was higher than that of the dark-treated sample. The difference between light and dark-treated samples was not significant, particularly when the variation between control samples was taken into account (figure 18c). After a 30 minute incubation (light or dark) the absorbance was less than from non-incubated samples which correlates well with the drop in fluorescence observed after light or dark pre-treatment (table 6).

Figure 18

A 10mL sample containing enough chloroplasts to give a final chlorophyll concentration of 8ug/mL was prepared. A 2.5mL aliquot of this sample was put in the dark for 30 minutes, another in the light for 30 minutes. DCMU was added to the third aliquot and an absorption spectrum run. After the 30 minute incubation DCMU was added to the light and dark samples and absorption spectra run. A) shows the effect on the entire spectrum (360-750nm) while B) is only a partial spectrum (610-740nm). In C) spectra were run on three identical non-incubated control samples to show the variation which exists in the procedure. O - non-incubated sample, L - sample illuminated for 30 minutes, D - sample kept in the dark for 30 minutes.

FIGURE 18: THE EFFECT OF LIGHT AND DARK PRE-TREATMENT ON THE ABSORPTION SPECTRUM OF CHLOROPLASTS

CHAPTER 4: DISCUSSION

4.1 THE EFFECT OF LIGHT & DARK PRE-TREATMENTS

Light pre-treatment of chloroplasts samples was found to reduce the maximum level of fluorescence by 14% (figure 5) but this in itself is not a new finding. Light-induced quenching has been observed by a number of researchers (Krause et al, 1983; Eriantais et al, 1979; Krause, 1974). When Krause et al (1983) pre-illuminated a sample of chloroplasts they also found that the maximum level of fluorescence was reduced compared to a dark incubated sample. They noted that this light-induced quenching was sensitive to the presence of both uncouplers and methyl viologen. Uncouplers completely reversed quenching, while methyl viologen increased it. The quenching which they observed was also found to be reversible - a one minute dark period following a three minute illumination was sufficient to completely reverse the quenching. In addition, Krause et al (1983) found that fluorescence quenching increased with the length of the pre-illumination period up to 3 minutes, and decreased at a lower light intensity. They proposed that the quenching resulted from the development of a high-energy-state - the more electron transport which takes place the greater is the pH gradient and thus quenching increases (Krause, 1974).

There are reasons for believing that the quenching of Fm which was observed here is not due to HES formation. For example, the quenching observed in figure 5 was still present when methyl viologen was absent (table 1) and was not abolished by the presence of uncouplers (table 2). Both of these treatments actually increased quenching slightly. Reversibility was not a characteristic of the quenching in figure 5 (figure 6). Figures 10 and 11 are in agreement with the finding of Krause et al (1983) that the quenching increases with illumination time. However their results indicate that quenching is maximal after 3 minutes, and it is clear from figure 11 that the quenching increased up to 30 minutes and continued even after that.

The results of the above experiments are different and so are a number of the experimental procedures. Krause et al (1983) illuminated for only three minutes with light of maximum emission at 480nm, compared to 650nm for 30 minutes. Perhaps the most significant difference in procedure is the absence of DCMU in Krause's experiments. When the experiments in figure 5 were carried out in the absence of DCMU, the levels of fluorescence were the same from light and dark incubated samples.

The differences in results as well as the differences in experimental procedure thus make it fairly safe to conclude that the quenching seen in figure 5 is not the same as that seen by Krause et al (1983) and was not caused by the

development of a high-energy-state. Other possible explanations for the light-induced quenching are a re-distribution of light energy in favor of PSI or a cyclic flow of electrons around PSII.

A re-distribution of light energy can be caused by the phosphorylation of LHC. The reduction of plastoquinone activates a kinase which then phosphorylates a portion of the LHC (Allen et al, 1981). In intact chloroplasts, the phosphorylation of ADP and electron transport are coupled. The presence of ATP means that LHC may be phosphorylated. Uncouplers remove this phosphorylation potential thus speeding up electron transport and abolishing phosphorylation induced quenching. The addition of ammonium chloride or nigericin, both of which collapse the pH gradient (Jagendorf, 1975), did not reverse quenching, rather they seemed to stimulate it (table 2). The same was also true of valinomycin which collapses the membrane potential difference (Jagendorf, 1975). These results with uncouplers make it quite clear that if there is a re-distribution of light energy, it is not phosphorylation-induced.

The PSI and PSII partial reactions were measured to see if there was a re-distribution of light energy. The rate of oxygen evolution (PSII) for light-treated samples was reduced as it would be if proportionally more light energy was going to PSI. When there is a re-distribution there should be an equivalent increase in the PSI reaction rate. However, this

was not observed; the rate of oxygen consumption (PSI) was the same for light and dark pre-treated samples. These results are consistent with the uncoupler results and together suggest that the light-induced quenching is likely not caused by a re-distribution of light energy, phosphorylation-induced or otherwise. To confirm that there is not a re-distribution of light energy in favor of PSI, 77K fluorescence spectra could be run on the light and dark-treated samples. At 77K there is a peak for PSII (695nm) and one for PSI (735nm). A re-distribution of light energy in favor of PSI would show up as an increase in the size of the PSI peak and a reduction of the PSII peak. When Krause et al (1983) measured 77K fluorescence from their light and dark-treated samples they found that fluorescence was lower from both photosystems after the pre-illumination.

A cyclic flow of electrons around PSII could also cause a decrease in F_{max} since it would produce an oxidation of Q_A thus increasing the proportion of open centres. The more open centres the lower the level of fluorescence. It is possible that the 30 minute illumination stimulated this leakage of electrons from the reducing side of PSII to the oxidizing side. Hydroxylamine is an analog of water and competes with water for the active site of the oxygen evolving enzyme (Radmer & Ollinger, 1982). This keeps the electron transport components on the oxidizing side of PSII reduced, thus preventing the cyclic flow of electrons around

PSII. When 5mM hydroxylamine was added to samples at the same time as the DCMU, the difference between light and dark samples remained unchanged (table 2). Therefore, the light-induced quenching of fluorescence does not appear to be the result of a stimulated leakage of electrons around PSII.

Another possible explanation for the light-induced quenching is a photo-destruction of pigment molecules. This would be expected to result in a reduced level of absorption by a light-treated sample. Figure 18 shows that this was not the case. The difference between the level of absorbance from light and dark-treated samples was not significant and in many cases the absorption was higher from the light-treated samples, which would relate to a higher level of fluorescence rather than the reduced level which was seen. The lower level of absorbance from the pre-incubated samples compared to non-incubated samples may be due to the lability of pigments at room temperature. An alternative to photo-destruction of pigments is a light-induced re-organization of pigments and proteins. This might show up as a change in the shape of the absorption spectra. No change in shape was observed but this does not mean there were not some small (undetectable with a spectrophotometer) but significant re-organizations.

When a chloroplast sample is illuminated in the presence of an electron acceptor (methyl viologen) there will normally be electron transport. Leaving methyl viologen out of the

incubation medium did not remove the difference between light and dark. However, this does not mean that electron transport is not required for the light-induced quenching, as oxygen may be acting as the electron acceptor. The addition of an uncoupler during pre-illumination should stimulate electron transport as should increasing the light intensity. Both of these treatments also increased the extent of the light-induced quenching so it is possible that the quenching is in some way related to electron transport.

The light-induced quenching observed in figure 5 is not due to HES quenching, a re-distribution of light energy in favor of PSI, cyclic flow of electrons around PSII or to a change in the absorption characteristics of the chloroplasts. It can therefore be concluded that the quenching observed is due to a new mechanism, rather than to one that has already been established. A possible explanation for the light-induced quenching is a light-induced decrease in the affinity of DCMU for its binding site. The reduced affinity means a leak of electrons which will keep a proportion of Q_A oxidized and the maximum level of fluorescence will be reduced.

4.2 THE ANALYSIS OF FLUORESCENCE INDUCTION TRANSIENTS

In order to gain deeper insight into the effect of light and dark pre-incubation on fluorescence, a detailed analysis of the fluorescence induction transients was performed. Although this is a frequently performed analysis, two aspects were considered in detail: (1) the concentration of DCMU used and (2) the duration of the illumination period.

4.2.1 DCMU SENSITIVITY

There does not seem to be any question that DCMU can block electron transport between Q_A and Q_B (the first and second quinone acceptors) but what is uncertain is the concentration necessary to do so completely. If electron transport is not completely blocked there will be open PSII centres and thus a slow rise of fluorescence. When looking at fluorescence induction curves, researchers have used DCMU concentrations ranging between $5\mu\text{M}$ and $50\mu\text{M}$. With their work with sodium dithionite, Hodges & Barber (1983) called into question the ability of these concentrations of DCMU to block electron transport completely. Using $20\mu\text{M}$ DCMU, they found that the addition of sodium dithionite abolished the slow β -phase. Their interpretation was that the β -phase is the result of a leak of electrons past the DCMU block to the PQ pool. When PQ is fully reduced by sodium dithionite it is

unable to accept electrons and hence to oxidize Q_A . Thus the leak is stopped and Q_A reduction is faster. They proposed that the leak of electrons gives rise to the slow β -phase.

The leakage proposal (Hodges & Barber, 1983) was consistent with earlier work (Schreiber & Pfister, 1982) which showed that as DCMU concentration was raised to $3 \times 10^{-4} M$ both the area above the curve and the extent of the β -phase were reduced. They suggested that there were PSII centres which only bind DCMU at high concentration because they are in the form $Q_A Q_B^-$ which do not readily bind the inhibitor. Again it was concluded that the β -phase was largely the result of a leakage of electrons past the DCMU site of inhibition.

More recently it was shown in this laboratory (Sinclair, unpublished results) that $250 \mu M$ DCMU was necessary to reduce oxygen evolution to an undetectable level (as measured with a modulated oxygen electrode). At $50 \mu M$ DCMU the rate of oxygen evolution was 0.2% of the uninhibited rate. Similar results were obtained by Hodges et al (1985) except they used lower concentrations of DCMU. This means that a number of transient analysis experiments have been performed under conditions which allowed some electron transport between the two photosystems. However, the relevant question to ask is how important is this leak of electrons for the analysis of fluorescence induction curves? If increasing the DCMU concentration and reducing electron transport has no effect

time of more than 50 minutes (Wraight, 1985) therefore after a second illumination, Q₄₀₀ is reduced and any phase which is affected by Q₄₀₀ should be smaller. As seen in table 8, there was a significant decrease in the total area after the second illumination. This decrease was almost completely (93%) accounted for by a decrease in the slowest phase. It is therefore suggested that Q₄₀₀ is not present in PSII centres which give rise to the α , β , and γ phases but only with the PSII δ -centres. Even after the second illumination 30% of the δ -phase was still present. The fact that the second illumination does not completely eliminate the slowest phase therefore indicates a heterogeneity among δ centres with respect to the presence of Q₄₀₀.

The α -phase was relatively unaffected by the addition of ferricyanide and two explanations are possible. These centres may not be accessible to ferricyanide or more likely, they may be completely oxidized during the dark period. The β and γ -phases were sensitive to the presence of ferricyanide (table 7), which could mean that they are located where they are accessible to ferricyanide and are not completely re-oxidized during the dark period or possibly that these centres are not completely blocked.

Only part of the four phases were affected by Q₄₀₀ thus not all PSII centres contain Q₄₀₀. This suggests that a single model will not accurately describe PSII centres.

effect on the analysis of areas (table 5). This means that neither the β -phase nor any other phase is entirely the result of electron leakage as has been proposed (Hodges & Barber, 1983; Schreiber & Pfister, 1982). The fact that there was no difference in fluorescence levels or areas for 50 μ M and 500 μ M DCMU would suggest that 50 μ M DCMU is sufficient to block electron transport as far as fluorescence transients are concerned.

4.2.2 DURATION OF THE ILLUMINATION PERIOD

Quite critical to the analysis of fluorescence induction transients is an accurate value for F_{max} . Fluorescence transients are made up of several exponential phases and the problem is therefore to estimate F_{max} . Both Sinclair & Spence (1988) and Bell & Hipkins (1985) showed quite clearly that ending experiments too early results in erroneous analysis of fluorescence induction curves. However, longer periods of illumination result in an improved estimate of F_{max} and therefore of the area above the curve.

Using longer illumination times, three phases were seen in the CAD plots (Sinclair & Spence, 1988). However a fourth phase is now routinely isolated (figure 7). The four phases are α , β , τ , and δ (fastest to slowest). Schreiber & Pfister (1982) and Bell & Hipkins (1985) both discussed their results in terms of only two phases - α and β . Examination of their results suggests that if they had analysed the initial curved

part of their CAD curves they may have found one and perhaps two more phases. When only two phases are distinguished, the α -phase accounts for about half the PSII centres but the finding of two more phases reduced the relative size of the α -phase quite considerably (figure 7e).

The area above the fluorescence induction curve for a particular phase is related to the total number of electron acceptors available to PSII before the DCMU block (Melis & Homann, 1975). However, the kinetics of area development are also dependent on the number of pigment molecules associated with each reaction centre in the phase since this will determine the speed at which photochemistry will take place at that reaction centre. The more pigment molecules, the greater the chance that a photon will be absorbed and the faster the centre will become closed. A number of researchers (Melis, 1985, Thielen & Van Gorkum, 1981; Melis & Duysens, 1979) believe that α and β -centres are the result of two distinct forms of PSII reaction centres. They contend that α -centres are connected PSII units which allow transfer of energy between reaction centres. When excitation energy reaches a closed centre it cannot be used for photochemistry but with connected PSII units there is then the possibility that it can be transferred until it reaches an open centre. As more centres become closed, the effective size of the photosynthetic unit associated with an open centre will get larger and the rate constant should increase. It is this

transfer of energy which would cause the lag in, or sigmoidal rise, of the fluorescence transient (Horton, 1981). The β -phase is thought to be due to a group of separate PSII reaction centres in PSII units where energy transfer is not possible, therefore they exhibit slower kinetics (Horton, 1981).

Schreiber & Pfister (1982) as well as Hodges & Barber (1983) are of the opinion that the two types of reaction centres are the result of different DCMU binding affinities. β -centres weakly bind DCMU thus allowing a leak of electrons and a slower rise of fluorescence. α -centres would be easily blocked by DCMU and thus be responsible for the fast kinetics.

As discussed above, a fast, non-exponential phase in the area above the fluorescence induction curve and the sigmoidal shape of the fluorescence induction transient has been taken to indicate connectivity or energy transfer between PSII units (Horton, 1981). It has been shown (Melis & Homann, 1978) that a low concentration of cations produces a linear fast phase while the β -phase remains unchanged. This was interpreted to mean that in the absence of magnesium the connectivity of the α -centres is lost. Figures 9b and 12b show that sigmoidicity is lost when a sample is pre-treated with light. A loss of sigmoidicity was also seen when samples were illuminated for a second time (figure 17) and this corresponded to a linear final CAD curve (figure 17a).

According to Melis & Homann (1978) both light pre-treatment and a second illumination would reflect a loss of connectivity between PSII units. The loss of sigmoidicity in both these cases was accompanied by a decrease in the size of the α -phase and an increase in F_{max} . This suggests that both the pre-illumination and the first illumination cause structural changes in the thylakoid membrane which prevents the transfer of energy between α -centres.

4.2.3 THE EFFECT OF LIGHT AND DARK PRE-TREATMENTS

In the previous section, fluorescence level indicated that there was a light-induced quenching of fluorescence taking place. When the area above fluorescence induction transients from light and dark pre-treated samples were analysed into their four phases it became evident that there were more differences between such samples than previously appreciated.

There is a reduction in the total area after a dark pre-treatment (compared to control samples) and the slowest phase is absent in many cases. These results may suggest that the slowest phase is associated with a small number of light harvesting pigments and that the dark period in some way inactivates these centres. This could be the result of an inactivation of the water-splitting system or of the reaction centres themselves. Alternatively and perhaps the more likely explanation is a chemical reduction of the PSII

acceptors. This is consistent with the finding that a thirty second exposure of a dark-treated sample to far-red light following dark adaptation greatly increased the area above the curve. The far-red light is principally absorbed by PSI and would serve to oxidize the acceptors which had been reduced by the dark pre-treatment thus increasing the availability of PSII acceptors. This theory would explain why the fluorescence induction transients in figure 9a are so similar for the dark-treated and control samples.

Although the total areas for the control and light-treated samples are quite similar the shapes of the initial 200ms of the fluorescence transient are quite different (figure 9a). The slower rise for the light-treated samples could be the result of a light-induced leak of electrons. If these results are compared with those from control samples containing $1\mu\text{M}$ DCMU (table 4) it can be seen that the trends are similar. As was the case with the samples with $1\mu\text{M}$ DCMU the leak of electrons means that there are a greater proportion of open PSII centres. This light-induced leak could be the result of a conformational change within the thylakoid membranes.

4.3 THE EFFECT OF FERRICYANIDE

The addition of ferricyanide in the dark prior to DCMU slowed fluorescence induction kinetics quite considerably (figure 15) and doubled the area above the curve (table 7). This doubling of the area above the curve is consistent with earlier studies (Wraight, 1985; Ikegami & Katoh, 1973). Based on their results Ikegami and Katoh (1973) proposed the involvement of a redox component (Q_{400}) which is readily oxidized by ferricyanide thus providing more electron acceptors for PSII. They also showed that if DCMU was added before the ferricyanide, Q_{400} was not oxidized. It has been shown (Petrouleas & Diner, 1986) that Q_{400} is an iron atom in the quinone-iron complex of PSII and it is believed that electrons first reach Q_A then Q_{400} (Petrouleas & Diner, 1985).

Wraight (1985) and Ikegami and Katoh (1973) believed that the increase in area was due to a single redox component but they did not investigate the effect of ferricyanide on the individual area phases. Table 7 shows that the four phases were not equally affected by ferricyanide. By illuminating a sample twice it was determined which of the increases in area were caused by Q_{400} . A sample was illuminated twice with an intervening five minute dark period which is long enough to allow re-oxidation of Q_A but not of Q_{400} . Q_{400} was found to be re-oxidized with a half-

time of more than 50 minutes (Wraight, 1985) therefore after a second illumination, Q₄₀₀ is reduced and any phase which is affected by Q₄₀₀ should be smaller. As seen in table 8, there was a significant decrease in the total area after the second illumination. This decrease was almost completely (93%) accounted for by a decrease in the slowest phase. It is therefore suggested that Q₄₀₀ is not present in PSII centres which give rise to the α , β , and τ phases but only with the PSII δ -centres. Even after the second illumination 30% of the δ -phase was still present. The fact that the second illumination does not completely eliminate the slowest phase therefore indicates a heterogeneity among δ centres with respect to the presence of Q₄₀₀.

The α -phase was relatively unaffected by the addition of ferricyanide and two explanations are possible. These centres may not be accessible to ferricyanide or more likely, they may be completely oxidized during the dark period. The β and τ -phases were sensitive to the presence of ferricyanide (table 7), which could mean that they are located where they are accessible to ferricyanide and are not completely re-oxidized during the dark period or possibly that these centres are not completely blocked.

Only part of the four phases were affected by Q₄₀₀ thus not all PSII centres contain Q₄₀₀. This suggests that a single model will not accurately describe PSII centres.

CHAPTER 5: CONCLUSIONS

CONCLUSIONS

The light-induced quenching of fluorescence seen here appears to be different from the forms of light-induced quenching previously observed by other workers. Light-induced quenching caused by either the formation of a high-energy-state or the phosphorylation of LHC does not take place in the presence of an uncoupler. However, the quenching seen here was even greater in the presence of uncouplers. The reduction in F_{max} could be caused by a light-induced conformational change in the thylakoid membranes thus allowing a leakage of electrons.

Most researchers (Melis, 1985; Hodges & Barber, 1983; Schreiber & Pfister, 1982) have found two types of PSII reaction centres which have been called α and β . Using the transient analysis procedure outlined, and taking the necessary precautions (Sinclair & Spence, 1988), four different phases were found in CAD curves - α , β , τ , δ . Three of these phases were exponential while the fourth and fastest phase was non-exponential. Thus the results presented indicate that the heterogeneity of PSII is perhaps greater than previously suspected.

Evidence has also been presented which supports the idea that none of the four phases are entirely due to an electron leak. Increasing the DCMU concentration from $1\mu\text{M}$ to $50\mu\text{M}$ showed that it was the γ and δ -centres which were not completely blocked by $1\mu\text{M}$ DCMU while the α and β -centres were unaffected.

The addition of ferricyanide did not effect all centres equally and a second illumination showed that Q_{400} was only involved with the δ -phase but that even some of these centres lack Q_{400} .

Finally, light and dark pre-treatments had different effects on different phases. The transients from dark-treated samples suggest that there is a chemical reduction of the PSII acceptors while there seems to be a light-induced leak of electrons in the light-treated samples.

APPENDIX

APPENDIX 1

- Air saturated water contains
0.29mM oxygen

- Air saturated water set at
90 on the chart recorder

$$\begin{aligned} 90 \text{ units} &= 0.29\text{mM O}_2 \\ 25 \text{ units} &= x \text{ mM O}_2 \\ x &= 0.08055 \text{ mM} \end{aligned}$$

That is 0.08055mmoles in 1000mL
but there only were 3mL

$$\begin{aligned} 0.08055\text{mmoles in } 1000\text{mL} \\ x \text{ mmoles in } 3\text{mL} \\ x &= 0.0002417\text{mmoles} \\ &= 0.2417\mu\text{moles} \end{aligned}$$

That means 0.2417 μ moles were evolved/consumed in 2.5 min

$$\begin{aligned} \text{RATE} &= 0.2417\mu\text{moles} / 2.5\text{min} \\ &= 0.0967\mu\text{moles} / \text{min} \end{aligned}$$

The common units for oxygen evolution/consumption rates
are: $\mu\text{moles O}_2/(\text{mg chl})/\text{hr}$

Enough chloroplasts were added to give a final
concentration of 50 $\mu\text{g/mL}$, therefore in 3mL there
are 150 μg in the sample

$$\begin{aligned} \text{RATE} &= 0.0967\mu\text{moles O}_2/(0.150\text{mg chl})/0.0167\text{hr} \\ &= 38.6 \mu\text{moles O}_2/(\text{mg chl})/\text{hr} \end{aligned}$$

Therefore the rate of oxygen evolution/consumption is
38.6 $\mu\text{moles O}_2/(\text{mg chl})/\text{hr}$

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