OPTICAL STUDIES INTO THE PRIMARY [redacted] MECHANISMS OF PHOTOSYNTHESIS

by

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ABSTRACT

Three optical techniques (measurement of delayed chlorophyll fluorescence, prompt chlorophyll fluorescence and flash-induced absorbance changes) have been used to study the primary reactions of photosynthesis in isolated intact and osmotically shocked chloroplasts from higher plants.

1. Delayed fluorescence. Temperature-jump studies on millisecond delayed fluorescence from uncoupled chloroplasts have shown that the activation energy for the back reaction leading to delayed fluorescence is significant (about 0.64eV); this emphasises the role played by the high-energy state in delayed fluorescence emission at short times. The decay of delayed fluorescence was measured in the millisecond to second time range with uncoupled chloroplasts, and a correction was made for the influence of prompt fluorescence yield on the emission. The decay kinetics are influenced by the precise fluorescence function used for the correction; it is argued that total prompt fluorescence should be used, and the resulting function is approximately linear in $J_T^{-\frac{1}{2}}$ versus time, implying a second order reaction in the mechanism leading to delayed fluorescence. The influence of temperature on the kinetics suggests an activation energy of approximately 0.56eV.

2. Prompt fluorescence. A quantitative study was undertaken on the relationship between fluorescence yield and the shape of the fluorescence induction in DCMU-blocked chloroplasts. The relationship between fluorescence and the probability of intra-PSII excitation energy transfer was found to be linear, and the activation energy for this probability was found to be small. Taken together with other data showing that the changes in fluorescence yield are accom-

panied by variations in fluorescence lifetime, it is concluded that energy transfer between the chlorophyll pigments of PSII is relatively slow, i.e. at a rate compatible with the Forster very weak coupling mechanism. In addition, it appears that the chlorophyll pigments of PSII are to some extent distributed heterogeneously.

3. Flash-induced absorbance changes. The flash-induced absorbance change at 518nm, $\Delta 518$ (which is thought to reflect the electrical potential established by primary charge separation across the thylakoid membrane) was shown to be 1.6 times larger in extent and more slowly decaying in intact chloroplasts as compared to broken chloroplasts. In addition, the onset of $\Delta 518$ in intact chloroplasts was biphasic. By considering the molecular mechanism of Δ 518 these experiments led to the concept that the thylakoid membranes of intact chloroplasts have lower passive permeability to ions than the thylakoid membranes of broken chloroplasts. This difference might be due to osmotic damage to the thylakoid membranes of broken chloroplasts.. Experiments with ionophorous antibiotics, inhibitors and other treatments generally support the concept that Δ 518 reflects the electrical potential generated across the thylakoid membranes by the primary light reactions.

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ABBREVIATIONS AND SYMBOLS

A	:	complementary area over the chlorophyll prompt fluorescence induction curve
A pool	:	pool of equivalents between PSI and PSII
ADP	:	adenosine diphosphate
ADRY	:	Acceleration of the Deactivation Reactions of the water-splitting enzyme system Y
ANT-2p	:	2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitro-thiophene
ATP	:	adenosine triphosphate
B890	:	reaction centre pigment of photosynthetic bacteria
C	:	concentration
C-550	:	absorbance change at 550nm
CCCP	:	carbonyl cyanide m-chlorophenylhydrazone
chl	:	chlorophyll
cyt b-559	:	cytochrome b-559
cyt f	:	cytochrome f
DAD	:	2,3,5,6-tetramethyl-p-phenylenediamine (diaminodurene)
DBMIB	:	2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone
DCIP	:	2,6-dichlorophenolindophenol
$DCIPH_2$	:	reduced DCIP
DCMU	:	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DNB	:	m-2,4-dinitrobenzene
E	:	activation energy
F	:	rate of intermolecular energy transfer
f_o	:	initial level of chlorophyll fluorescence
f_v	:	variable prompt chlorophyll fluorescence
f_t	:	total prompt chlorophyll fluorescence ($= f_o + f_v$)
FCCP	:	carbonyl cyanide p-trifluoromethoxyphenylhydrazone
I	:	light intensity
J_T, J_V	:	delayed chlorophyll fluorescence corrected with total and variable prompt chlorophyll fluorescence respectively

k_f	:	rate constant for fluorescence
k_h	:	rate constant for radiationless de-excitation
k_{tI}	:	rate constant for energy transfer from PSII to PSI
k_Q	:	rate constant for energy quenching
k_p	:	rate constant for photochemistry
L	:	intensity of uncorrected delayed chlorophyll fluorescence
$NADP^+$	:	nicotinamide adenine dinucleotide phosphate
NADPH	:	reduced $NADP^+$
OAA	:	oxaloacetic acid
O-D-P-S-M-T	:	stages in the long term changes in chlorophyll fluorescence yield
p	:	probability of excitation energy transfer between a PSII photosynthetic unit with a closed trap and another PSII photosynthetic unit
P680	:	reaction centre pigment of PSII
P700	:	reaction centre pigment of PSI
P_i	:	inorganic phosphate
PGA	:	phosphoglycerate
PMS	:	phenazine methosulphate
PSI	:	photosystem I
PSII	:	Photosystem II
psu	:	photosynthetic unit
Q	:	primary electron acceptor of PSII
R_o, $\bar{R}_o$	:	intermolecular distance ('Forster distance')
S	:	singlet state
S_n $(0 \leq n \leq 4)$	:	oxidation states of the oxygen evolving apparatus
S'_n $(0 \leq n \leq 4)$	:	transient light-induced state between states S_n and S_{n+1}
Signal I	:	electron paramagnetic resonance signal from PSI
Signal II	:	electron paramagnetic resonance signal from PSII
t	:	probability of excitation energy transfer out of a PSII photosynthetic unit with a closed trap
T	:	triplet state
u	:	molecular interaction energy
X-320	:	absorbance change at 320nm
Y	:	primary electron donor to PSII
Y_n	:	yield of oxygen due to the nth flash of a series
Z	:	secondary electron donor to PSII

α_n	:	fraction of misses on the nth flash of a series
$\bar{\alpha}$	:	average fraction of misses over a flash series
α_{II}	:	fraction of incoming photons directed to PSII
β	:	fraction of double hits in a flash series
Δp	:	proton motive force
ΔpH	:	transmembrane pH gradient
$\Delta\psi$	:	transmembrane electrical gradient
ϕ, ϕ_f	:	chlorophyll fluorescence yield
$\phi(o)$	:	chlorophyll fluorescence yield of a PSII psu with an open trap
ϕ_v	:	variable chlorophyll fluorescence yield
ϕ_t	:	total chlorophyll fluorescence yield
ϕ'	:	delayed chlorophyll fluorescence emission factor
κ	:	intermolecular orientation parameter
τ	:	lifetime of an excited state
τ_o	:	natural lifetime of an excited state

CHAPTER 1 : INTRODUCTION

1.1 General Introduction

Photosynthesis is the process by which chlorophyll-containing plants are able to convert solar energy into chemical energy, which is stored in the form of carbohydrates. The process is important because it is the ultimate source of all food for heterotrophic organisms, and because the products of photosynthetic activity in past eras have furnished large deposits of fossil fuels.

A detailed understanding of photosynthesis is becoming increasingly important for another reason. It has become clear over recent years that the world's supplies of fossil fuels are being rapidly exhausted, and whilst nuclear energy can to some extent provide an alternative source, a means for the efficient conversion of solar energy to a more useful form would be invaluable. The primary processes of photosynthesis are relatively efficient, so an understanding of how the photosynthetic apparatus functions may lead to the development of efficient *in vitro* solar energy conversion systems.

1.1.1 The overall process

The overall process of higher plant photosynthesis may be written as:

$$nCO_2 + nH_2O \xrightarrow{\text{light}} nO_2 + (CH_2O)_n \qquad 1$$

in which the photosynthetic apparatus uses light energy to convert the 'energy poor' compounds CO_2 and H_2O to the 'energy rich' compound carbohydrate. The reaction may be conveniently divided into those reactions which require light to operate, and

those reactions that operate in the dark. The latter 'dark reactions' are concerned with carbon fixation, the overal equation of which is :

$$6CO_2 + 12(NADPH + H^+) + 18ATP \longrightarrow (CH_2O)_6 + 12NADP^+ + 18ADP + 18P_i + 6H_2O \quad (2)$$

The fixation of each molecule of carbon dioxide thus requires three molecules of ATP and two molecules of NADPH; these are provided by the light reactions.

In higher plants photosynthesis occurs in subcellular structures called chloroplasts. In mature cells these organelles consist of an outer envelope which encloses a matrix (the stroma) and a complex network of lamellar membranes. These membranes are associated in pairs to form sac-like structures called thylakoids. In places the thylakoids are stacked together (to a greater or lesser extent) into grana. All the components associated with the light reactions (light-harvesting pigments, reaction centres, electron transport chain components and phosphorylation enzymes) are localised on or in the thylakoid membranes. The enzymes of the carbon-fixation cycle are found in the stroma. There are two general classes of chloroplasts which may be isolated from higher plants: (i) those which retain their outer envelope (whole or intact chloroplasts) and are able to fix CO_2 without the addition of cofactors, and (ii) those whose outer membranes are missing (broken chloroplasts), but which are capable of reducing $NADP^+$, and of phosphorylating ADP and P_i to ATP in the presence of an endogenous or artificial electron acceptor. In addition, if chloroplast membranes are disrupted by detergent treatment, two types of subchloroplast particle may be obtained. One type has only photosystem I activity (see section 1.1.2) and the other type has only photosystem II activity. A much more detailed classification of the different

types of chloroplasts that may be isolated has been given by Hall (1972).

1.1.2 The light reactions

The photosynthetic apparatus of higher plants contains several pigments: the chlorophylls, the carotenoids, and, in some algae, phycobilins. Chlorophyll in higher plants and green algae is present in two forms, Chl a and Chl b. Chl a is the major pigment, and has several forms which are distinguished by the wavelength of their absorption maxima in the red region of the spectrum. Evidence for these different forms of Chl a has been obtained from careful analysis of low temperature fluorescence spectra (see Papageorgiou, 1975) and from measurements of derivative absorption spectra.

A connection between these bulk pigments and the reaction centres (where primary photosynthetic energy conversion takes place) was obtained following the experiments of Emerson and Arnold in 1932. These authors used intense brief (10^{-5}s) flashes to excite photosynthesis in Chlorella. The alga was grown under varied lighting conditions to give cells containing different amounts of chlorophyll. They found that the ratio of molecules of oxygen evolved per flash to molecules of chlorophyll present in the sample was approximately 1:2500. Since it is believed that each $H^{\bullet}$ transferred from H_2O to CO_2 involves two sucessive photoacts, and that four quanta are required to liberate one molecule of oxygen, this result implies that at least eight quanta are required to liberate one molecule of oxygen and hence about 300 chl molecules may be associated with each photoact. The number of chlorophyll molecules associated with each reaction centre will depend on the precise model for charge accumulation in oxygen evolution (section 1.3.2). The association of a reaction centre and bulk pigments is known as the photosynthetic unit (psu). It has not yet been established whether the psu is a

morphological or a statistical entity

That excitation energy is not only transferred from the bulk chlorophyll molecules to the reaction centres but also from the accessory pigments (carotenoids) to chlorophyll was demonstrated by Duysens (1951) who showed that the fluorescence of chlorophyll in Chlorella could be excited by light absorbed only by carotenoids. However the mechanism of energy transfer between like and between different pigments in vivo is still not clear. In vitro measurements on energy transfer between chlorophyll molecules in liquid solution, solid solution, monolayers or lipid vesicles generally support the concept that energy transfer between these molecules is by the Forster very weak coupling mechanism (Forster, 1948: section 1.2). However, these experiments used relatively low chlorophyll concentrations, below the 0.1M that has been estimated for the concentration of chlorophyll in vivo (see Rabinowitch, 1949). Furthermore there are indications from the estimation of intermolecular coupling energies between chlorophyll molecules that the weak coupling mechanism may operate in vivo (section 1.2).

Some years ago a good deal of painstaking work was undertaken on measurement of the action spectrum of photosynthesis. Emerson and Lewis (1943) found that the plot of the quantum yield of photosynthesis (they measured oxygen evolution) versus the wavelength of the exciting light declined in the red end of the spectrum ($\lambda > 680$nm), while at the same wavelengths the absorption of light by chl a was still significant. This phenomenon is known as the 'red drop'. Emerson, Chalmers and Cederstrand (1957) discovered that the superposition of light of a wavelength absorbed by one of the accessory pigments abolished the red drop: this was called the enhancement effect.

These observations, together with the chromatic transients found by Blinks (1957) (a transient enhancement in the rate of photosynthesis on changing the wavelength of the exciting light from the red region to the green) led to the idea that in higher plant photosynthesis there were two light reactions sensitised by two forms of chl a whose absorption spectra were different (Emerson and Rabinowitch, 1960). One photoreaction, that of photosystem I (PSI, see Duysens and Amesz, 1962) is thought to contain the longer wavelength forms of chl a, the other (photosystem II; PSII) contains the shorter wavelength forms of chl a (absorbing at $\lambda <$ 680nm) together with the majority of the accessory pigment.

The reaction centre of each photosystem is composed of a charge transfer complex. This complex has a pigment molecule P, an electron acceptor A and an electron donor D. On receiving a quantum of excitation from the light-harvesting bulk pigments, the reaction centre pigment P becomes excited, followed by the reduction of the primary electron acceptor and oxidation of the primary electron donor. (see section 1.3)

$$DPA \xrightarrow{h\nu} DP^{*}A \longrightarrow DP^{+}A^{-} \longrightarrow D^{+}PA^{-}$$

The first step in the stabilisation of light energy is thus the formation of oxidised and reduced moieties. When either the electron donor or acceptor is in the wrong redox state (i.e. D^{+} or A^{-}) before excitation, charge separation cannot occur, and the reaction centre is 'closed'. The reaction centre pigment molecules are thought to be specialised chl a molecules; chl a_{II} (P680) for PSII and chl a_{I} (P700) for PSI (see section 1.3.1.3). Transient light-induced absorption changes have been identified as being due to the oxidised form of P700 (Kok, 1961) and of P680 (Doring et al., 1969; Glaser et al., 1975).

The photochemical utilisation of light energy is not the only path that can be followed by a quantum of light energy in the photosynthetic apparatus. The excited state of a bulk pigment can be dissipated through radiationless deexcitation or through fluorescence. In solution chlorophyll fluoresces strongly; about 30% of the absorbed quanta are re-emitted from the first excited singlet state. Almost all the remainder are transferred to the triplet manifold (Bowers and Porter, 1967). In vivo, however, the quantum yield of fluorescence is much lower, about 3%, since photochemical utilisation accounts for the majority of the absorbed quanta.

Only a few of the bulk pigments in photosynthetic organisms fluoresce significantly. At room temperature the fluorescence spectrum of green algae and of higher plants has a principal band centred at about 686nm, which is attributed to chl a678 of PSII. and a minor band whose maximum is at about 740nm. In PSII only chl a678 fluoresces since the other PSII pigments transfer light energy to this component with good efficiency. The chlorophyll pigments of PSI, which give rise to the 740nm band, fluoresce weakly at room temperature, but at liquid nitrogen temperatures fluoresce more strongly. This is probably due to any excitations which are not photochemically utilised being efficiently quenched by a quenching centre. At low temperatures the efficiency of this quenching centre together with that of photochemical utilisation are presumably impaired, resulting in higher fluorescence emission from PSI. The hypothetical quenching centre could be associated with the oxidised form of P700 ($P700^+$), since changing the quantum yield of photochemistry of PSI (by closing PSI traps) does not alter PSI chlorophyll fluorescence yield (Vredenberg and Slooten,1967). In photosystem II it was shown by Duysens and Sweers (1963) that

chlorophyll fluorescence is controlled by the redox state of the primary electron acceptor, Q. When in the unreduced form this component acts as a fluorescence quencher: prompt fluorescence measured at 686nm is thus a very useful intrinsic probe of PSII.

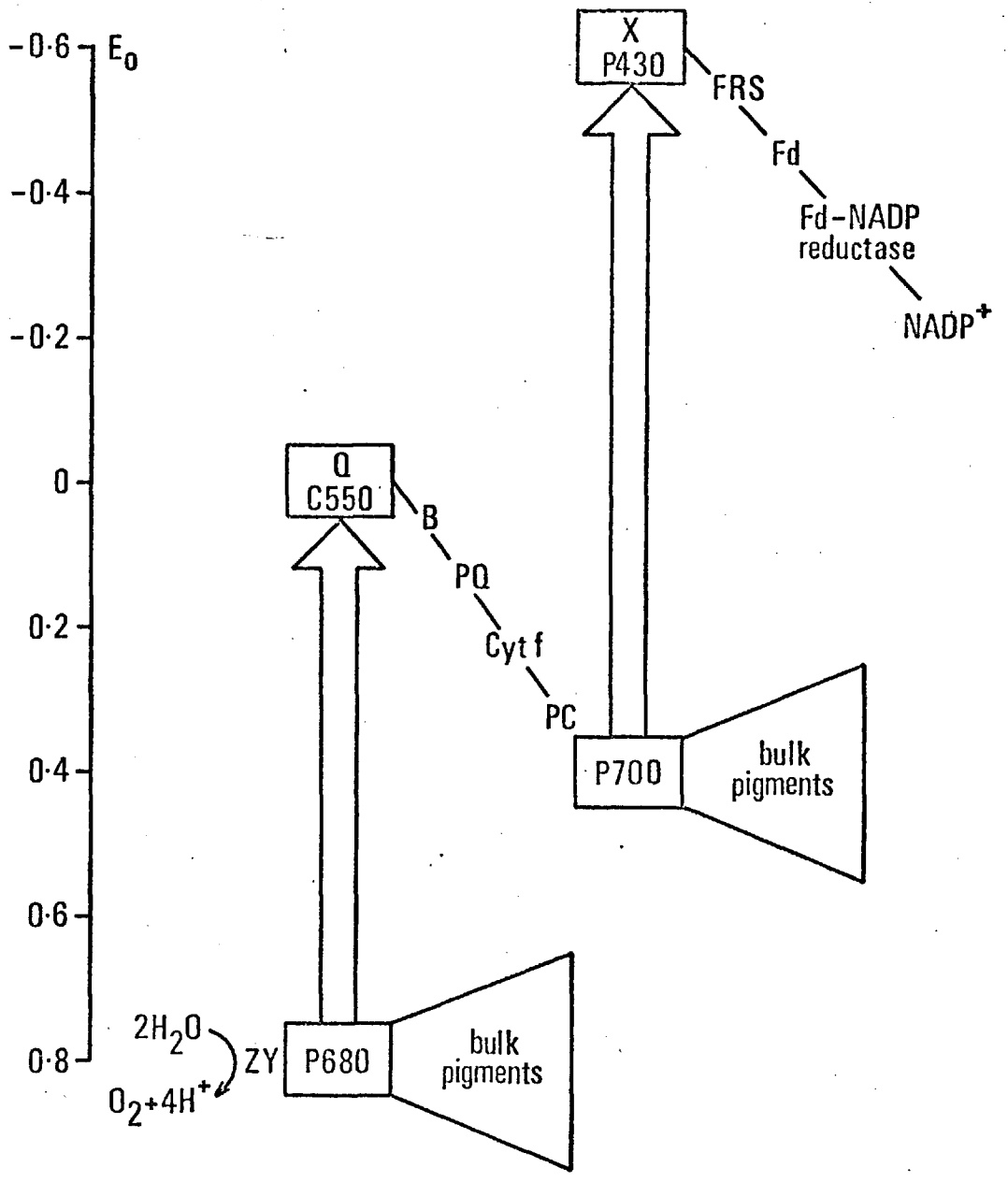
A second form of light emission from PSII occurs after the exciting light has been extinguished, and is called delayed fluorescence (delayed light emission, luminescence). This light emission, which is of very low intensity, is thought to occur as a consequence of a back reaction in PSII, and is a function of the redox state of both the primary electron donor and acceptor. These two types of light emission from PSII are described in detail in sections 1.4 and 1.5

1.1.3 Electron transport

It was originally proposed by Hill and Bendall (1960) that the two photoreactions were connected in series by intermediate electron carriers. This proposal is embodied in the Z-scheme (fig. 1) in which the electron donor for photosynthetic electron transport is water, and the ultimate electron acceptor is $NADP^+$. The scheme received support from the work of Duysens and Amesz (1962) who used sensitive absorption difference spectrophotometry to show that light absorbed by PSI caused an oxidation of cyt f while light absorbed by PSII caused a reduction of this oxidised cytochrome. Further support for the Z-scheme has come from prompt fluorescence studies on PSII. According to the series formulation of electron transport, the redox state of the primary electron acceptor of PSII (which controls the PSII fluorescence yield) may be varied by changing the rates at which PSII and PSI operate. Duysens and Sweers (1963) measured the fluorescence from PSII during illumination with light absorbed by PSII or by PSI and found that

Figure 1.

The Z-scheme for photosynthetic electron transport. Electrons are taken from water and supplied to $NADP^+$ with the intervention of two light reactions. The first is mediated by P680, a specialised chlorophyll molecule of PSII. The primary electron acceptor of PSII is Q (probably reflected by the absorbance change at 550nm, C-550) and the electron is then transferred to PSI by the chain of intermediates : B is a postulated component lying between Q and plastoquinone (PQ) since the former is a one electron acceptor and the latter is a two electron acceptor (Velthuys and Amesz, 1974); cytochrome f (cyt f) and plastocycnin (PC) may be in the reverse order. The second light reaction is mediated by P700, the specialised chlorophyll molecule of PSI. The primary electron acceptor is unknown (X) but may be indicated by the absorbance change at 430nm (P430). The components between X and $NADP^+$ are ferridoxin reducing substance (FRS), ferridoxin (Fd) and ferridoxin-NADP reductase.


-0·6
E_o
-0·4
-0·2
0
0·2
0·4
0·6
0·8
X
P430
FRS
Fd
Fd-NADP
reductase
$NADP^+$
Q
C550
B
PQ
Cyt f
PC
P700
bulk
pigments
$2H_2O$
O_2+4H^+
ZY
P680
bulk
pigments

light absorbed by PSI caused the fluorescence to decrease (as Q was oxidised by PSI via the electron transport chain) while light absorbed by PSII caused the fluorescence to increase (as Q was reduced to Q^-). This behaviour is thus consistent with the Z-scheme for electron transport. In addition, by using suitable electron donors and acceptors together with electron transport inhibitors, various sections of the electron transport chain may be made to function ('partial reactions') mediated by only one light reaction.

1.1.4 Phosphorylation

The NADPH required for the carbon-fixation cycle is provided by the reduction of $NADP^+$ acting as the terminal electron acceptor for photosynthetic electron transport. The ATP that is also required (eq.2) is phosphorylated from ADP and inorganic phosphate (P_i) in a reaction that is coupled to photosynthetic electron transport.

The mechanism of photophosphorylation has not yet been elucidated, but one of the theories that has received a good deal of support is that proposed by Mitchell (1966). The basis of Mitchell's scheme is that during photosynthetic electron transport protons are pumped from the stroma into the vesicles enclosed by the thylakoids, and that at the same time co-ions also cross the thylakoid membrane to reduce the electric potential created by the electrogenic proton pump. The result is that since the thylakoid membrane is assumed to be rather impermeable to protons, there is built up across the membrane an electrochemical gradient composed of a concentration difference of protons (Δ pH) and an electrical potential ($\Delta\psi$). The sum of these two gives the high-energy state which is thought to act as an intermediate between photosynthetic electron transport and phosphorylation. The high-energy

state, Δp (also known as the proton-motive force) is written as:

$$\Delta p = \Delta\psi + 2.303 \frac{RT}{F} \Delta pH \qquad 3$$

Phosphorylation is then thought to occur by the efflux of protons (down their electrochemical gradient) through a specific membrane-bound enzyme.

The evidence in support of the Mitchell scheme has come from various sources. Neumann and Jagendorf (1964) observed a light-induced uptake of protons by isolated chloroplasts suspended in a lightly buffered medium, and Jagendorf and Uribe (1966) found that ATP formation could be induced in chloroplasts by taking them through an acid-base transition. In addition Fowler and Kok (1974) have demonstrated, using flash illumination and a rapidly-responding pH electrode, that protons are evolved on the inside of the thylakoid membrane.

Electron transport can occur without phosphorylation when chloroplasts are treated with various compounds collectively known as uncouplers. These compounds wastefully dissipate the high-energy state by changing the permeability properties of the thylakoid membranes. The so-called 'classical' uncouplers like FCCP and CCCP make the membrane permeable to protons and directly dissipate Δ pH and $\Delta\psi$. The ionophorous antibiotics act in different ways, according to the manner in which they alter the membrane permeability. For example, nigericin facilitates a neutral exchange between K^+ and H^+ and so dissipates Δ pH whilst not affecting any electrogenic component of $\Delta\psi$. On the other hand valinomycin (which makes the membranes permeable to K^+) in the presence of K^+ will dissipate $\Delta\psi$ but not ΔpH if potassium is initially out of equilibrium. The relative magnitudes of $\Delta\psi$ and ΔpH in the equation for Δp (eq.3) will decide which of these compounds acts as an uncoupler under

given conditions. A third type of ionophorous antibiotic makes membranes permeable to a large number of ions, e.g. gramicidin makes membranes permeable to monovalent cations, and is an effective uncoupler in chloroplasts.

Witt and coworkers have shown that a light-induced absorption change at 518nm reflects the extent of the electrical potential generated across the thylakoid membrane (Junge and Witt, 1968). This sensitive intrinsic probe of membrane potential has been used to study the relationship between $\Delta\psi$ and phosphorylation when photosynthesis is excited by a flash which is of sufficiently short duration that it turns over the photosynthetic apparatus only once. The general conclusions of this work are that the amount of ATP formed in a flash is proportional to the extent of $\Delta\psi$ and that the rate of ATP formation follows the rate of decay of $\Delta\psi$ (see Witt, 1975 for review).

The action of ionophorous antibiotics on phosphorylation also supports the Mitchell scheme. Junge and Witt (1968) have shown that the addition of gramicidin to a suspension of chloroplasts inhibits phosphorylation. Quantitatively they showed that 50% inhibition occured at a concentration of gramicidin equivalent to one molecule of gramicidin per thylakoid, which gives support to the concept that intact vesicles impermeable to H^+ are necessary for efficient phosphorylation and to the idea of a delocalised high-energy state. The Mitchell scheme is however not the only one that has been proposed for the coupling of electron transport to phosphorylation. Various mechanisms have been proposed which include the concept that the intermediate between electron transport and phosphorylation is associated with conformational changes in some proteins in the membrane (see, for

example, Green and Ji, 1972). In this case the membrane potential would be on a sidepath.

There are generally thought to be two 'coupling sites' in the electron transport chain from water to $NADP^+$ (in the sense of the Mitchell scheme these would be two proton-translocating sites), and the number of molecules of ATP phosphorylated per pair of electrons transferred may be 1.8 - 2.0 (Hall et al., 1971). However, eq.2 shows that three molecules of ATP are required for the reduction of each molecule of CO_2. Any deficit may be made up by phosphorylation coupled to cyclic electron flow (see Arnon et al., 1965) which involves the circulation of electrons around PSI only or by pseudocyclic electron flow where oxygen acts as the terminal electron acceptor (Mehler reaction).

The work reported in this thesis is concerned with the light reactions of photosynthesis. Observations have been made on both types of light emission from PSII (prompt and delayed fluorescence); the results are relevant to discussions on the mechanism of energy transfer between the bulk pigment molecules, the morphological nature of the PSII photosynthetic unit and to the mechanism of delayed fluorescence. In addition the field-indicating absorption change at 518nm in intact and broken chloroplasts has been studied, and the results give indications as to the role of the light-induced membrane potential in these two types of chloroplast.

The following sections of this Introduction review the relevant literature in these fields, and indicate the questions that the experiments described in later chapters have been designed to answer.

1.2 Energy transfer in photosynthesis

1.2.1 The necessity for energy transfer and possible mechanisms

Two pieces of evidence have made it necessary to consider mechanisms for the transfer of light energy in photosynthesis. First, Duysens (1951) found that in the photosynthetic bacterium *Chromatium* the action spectrum of fluorescence of the bacteriochlorophyll B890 was similar to (although smaller than) the bacterium absorption spectrum, which implied that the accessory pigments are able to sensitise the fluorescence of B890 *in vivo*. In the red algae *Porphyra* and *Porphyridium*, Duysens observed that quanta absorbed by the phycobilin accessory pigments were more effective in exciting the fluorescence of chlorophyll *a* than quanta absorbed by chlorophyll *a* itself. This shows that energy is transferred from the phycobilins to the highly fluorescent chl *a* pigment bed with great efficiency. Similar phenomena of energy transfer from carotenoids to chl *a* were demonstrated in the green algae *Chlorella*. Second, Emerson and Arnold (1932) discovered the photosynthetic unit (section 1.6) in which about 300 chl molecules act to direct light energy to the reaction centre. This transfer of energy from the antenna chlorophyll molecules to the reaction centre must be efficient in order to maintain the overall efficiency of photosynthesis. It must also be rapid, that is within the lifetime of whatever excited state is involved. Taken together, the data on sensitised fluorescence and the evidence for the photosynthetic unit lead to the concept that light absorbed by the accessory pigments is efficiently transferred to chl *a* and is then further transferred through the light-harvesting chl molecules to the reaction centre where the energy may be trapped and photochemically utilised.

It is necessary to postulate a mechanism to account for this energy transfer. Early hypotheses included the diffusion of charges, radicals or the excited molecules themselves, but current models are based on the transfer of energy from an excited molecule to an unexcited molecule via a coupled event in which the de-excitation of the donor molecule is accompanied by a concomitant excitation of the acceptor molecule. The transfer takes place within the fluorescence lifetime of the donor molecule and is distinct from the process of reabsorption by the acceptor molecule of fluorescence emitted by the donor molecule, which is a slower and more inefficient process. Forster (1948, 1959, 1960, 1965) has made a careful study of energy transfer processes which do not involve fluorescence and reabsorption. He points out that for electronic excitation transfer to occur at all, there must be some interaction between the excited and unexcited molecules. The interaction energy can be expressed as a sum of multipole interactions between the transition charge distributions of both molecules. According to the nature of the molecules these charge distributions may have dipole moments of widely varying magnitudes or even higher moments only; depending on the character of the transition moments, the interaction energy decreases with a certain power of the reciprocal of the intermolecular distance R. At large intermolecular distances usually the dipole-dipole interaction only will be significant. The interaction energy also depends on the mutual orientation of the molecules.

1.2.2 Resonance transfer: three cases

In the transfer of electronic excitation energy from a donor molecule to an acceptor molecule energy must be conserved. Therefore the electronic energy of the acceptor state has to be the

same as that of the donor state. In resonance transfer this condition is made less strict by the molecular interaction between donor and acceptor because the interaction energy enables more than one energy level to be in resonance. Thus the interaction energy is an important parameter in determining the precise mechanism of energy transfer through its influence on the degree of resonance.

A comparison of the magnitudes of the interaction energy (u) and typical energies of the molecule provides a means of classifying the different energy transfer mechanisms. The terminology used is that proposed by Kasha (1964) not that originally used by Forster (1960). The typical energies of the molecule to be considered are (i) the total range of energy of all the vibrational levels in an electronic state $\Delta\epsilon$, which determines the Stoke's shift between the maxima of the absorption and fluorescence spectra; for aromatic molecules Forster (1960) gives a value of 3000cm^{-1} as typical, (ii) the energy of a vibrational quantum, about 1000cm^{-1}, which Forster (1960) takes as comparable with $\Delta\epsilon$ and (iii) the energy width of a single vibrational level, $\Delta\epsilon'$, typically 10cm^{-1}.

1.2.2.1 Strong coupling: $u \gg \Delta\epsilon \gg \Delta\epsilon'$. In this case the interaction energy is so large that it makes all the vibrational levels of an electronic state in the donor molecule in resonance with all the vibrational levels of the corresponding state in the acceptor. Even if the transition in the donor molecule is from a low-lying vibrational level of the excited state to a high vibrational level of the ground state there is sufficient interaction energy to supplement the energy being transferred to make possible a transition between any vibrational levels of the ground and the excited state in the acceptor. The solution of the appropriate wave functions shows that the energy cannot be described as localised

on either the donor or the acceptor but oscillates between them. Since the interaction energy is much greater than kT the process is independent of temperature. The rate of transfer is proportional to u.

1.2.2.2 Weak coupling: $\Delta\epsilon \gg u \gg \Delta\epsilon'$. Here the condition for resonance is more stringent than in the case of strong coupling. Even in molecules of the same kind the resonance is only between similar vibrational levels. The probability of transfer depends on the way the ground and excited state vibrational levels are populated, that is, an allowed de-excitation in the donor molecule must end on a vibrational level that is occupied in the acceptor molecule. The rate of transfer is expected to be dependent to some extent on the wavelength of the exciting light, since this will determine the vibrational level occupied in the excited state. The rate of transfer again depends on u.

1.2.2.3 Very weak coupling: $\Delta\epsilon \gg \Delta\epsilon' \gg u$. The condition for resonance becomes more strict as coupling decreases and in this case the finite width of each vibrational level becomes important. If there are two coinciding vibrational levels then with very weak coupling only certain regions of these levels are in resonance. The excitation stays on the donor sufficiently long for the excited molecule to settle into the lowest vibrational level of the excited state before transferring its energy. In the transfer the same amount of energy that is lost by the donor must be gained by the acceptor. The rate of transfer is proportional to u^2.

1.2.3 Chlorophyll interaction energies

Which kind of coupling applies in photosynthesis ? Let us take the

example of chl a - chl a transfer, the transfer that could be expected to occur in the photosynthetic unit. The interaction energy has to be calculated and this can be done in two ways: first from the quasi-particle nature of the exciton (Robinson,1967) and second from the dipole-dipole nature of the interaction (Forster,1960)

1.2.3.1 Robinson's calculation (Robinson,1967). In a crystalline system the momentum of an exciton may be calculated from the de Broglie relationship and conservation of momentum. Together with the expression for the kinetic energy of the particle this yields:

$$m_j^* = \frac{\hbar}{2u_j a_j^2} \qquad 4$$

where j is an index denoting the crystal axis, and a_j is the lattice spacing along the j axis. Robinson takes m_j^* to be the mass of the electron, which he considers a lower limit. Therefore, given a value of a_j, u_j may be calculated. It is perhaps easier, however, to give typical values of u_j and derive a_j, which may then be compared with values of R in the photosynthetic apparatus. Hence:

when $u_j = 10\text{cm}^{-1}$ $\quad a_j = 5.5\text{nm}$

when $u_j = 1\text{cm}^{-1}$ $\quad a_j = 17.5\text{nm}$

These values of a_j must be upper limits, but they nevertheless indicate the order of magnitude of intermolecular spacings below which the weak coupling case is applicable.

1.2.3.2 Forster's calculation (Forster,1960). As mentioned above, the interaction energy between molecules is the sum of a series of multipole terms, the largest of which is the

dipole term. Forster makes the approximation that all higher terms may be ignored; the calculation including higher terms has been made by Dexter (1953). Assuming therefore that there are only dipole-dipole interactions the interaction energy is given by

$$u = \frac{\kappa |M|^2}{n^2 R^3}$$

where M is the transition moment between ground and excited states, n is the refractive index of the medium and κ is an orientation factor. For random orientation of donor and acceptor dipoles $\kappa^2 = 2/3$. Forster related M to experimentally measurable parameters by reference to the expression for the Einstein coefficient for spontaneous emission of an excited state A_{ml}:

$$A_{ml} = \frac{64 \pi^4 \nu^3 n |M|^2}{3 h c^3}$$

Recognising that the natural fluorescence lifetime of the excited state, τ_o, is related to to A_{ml} through

$$A_{ml} = 1/\tau_o$$

then the expression for u becomes:

$$u = \frac{3 h \kappa}{\tau_o 64 \pi^4 \lambda^3 n R^3} \qquad 5$$

This expression is valid for an atomic system where the emission spectrum would be a single line, but for a molecular system Forster further approximates by taking λ to be the average of the wavelengths of the peaks of the absorption and fluorescence spectra. For chl a $\lambda \simeq 670$nm. Using 15.2ns for the chlorophyll natural lifetime (Brody and Rabinowitch, 1957), and 1.43 for n (the value for lecithin) then the values of R for given values of u are:

$u = 10 cm^{-1}$ $\quad$ $R = 3.1$nm

$u = 1 cm^{-1}$ $\quad$ $R = 6.7$nm

From these figures, if $R \leq 3$nm the weak coupling case is applicable.

The in vivo chl a - chl a intermolecular spacings have been roughly estimated by Thomas et al., (1956), Bay and Pearlstein (1963) and by Colbow (1973). Thomas et al. (1956) found R = 1.7nm by taking the ratio of the area of the lamellae divided by the total chl concentration assuming a 2-D planar array of molecules. Bay and Pearlstein (1963) based their estimate on the identity of the quantasome, a structure found by electron microscopy (see Park and Sane (1971) for review), and the photosynthetic unit. The quantasome is an oblate spheroid approximately 10nm by 20nm, and was taken to contain 400 chl molecules in either a 2-D or a 3-D array. If the 3-D model is chosen, R = 1.7nm; if the 2-D model is used, R = 1.1nm. These values are somewhat less than 3nm, so the weak coupling case would be applicable. Colbow (1973) has evaluated R as 1.5nm in a 2-D model. His calculation is based on the available thylakoid surface area per porphyrin ring being 200 to 250 A^2. This is consistent with chlorophyll composing 21% of the lipid soluble fraction of thylakoid membranes. The value of 1.5nm (giving a value for u of $58cm^{-1}$ from eq.5) also points to the weak coupling case.

Clayton (1965) has estimated u as $30cm^{-1}$, indicating a transfer mechanism intermediate between weak and very weak coupling. Robinson (1967) has criticised Clayton's estimate, and from considerations of the non-irreversibility of energy transfer supports the weak coupling case. Paillotin (1972), on the other hand, considers Robinson's criteria for very weak coupling to be too severe, and supports the very weak coupling case.

Theoretically it should be possible to determine the magnitude of coupling energy in vivo by examination of the absorption spectrum

of chloroplasts (Forster, 1965), but there are a large number of pigments involved which are not in free solution, so interpretation of the spectrum is very difficult.

In summary, it appears that in the chloroplast the energy transfer mechanism lies somewhere between the weak coupling and very weak coupling cases.

1.2.4 Weak coupling and very weak coupling

The rate of energy transfer by these two mechanisms has been calculated by Forster (1948, 1960, 1965). The general method used to estimate the overall rate of energy transfer between molecules is to calculate the transfer rate between two corresponding vibrational levels, and then to sum this rate over all vibrational levels in the electronic state weighted by their respective populations. For the weak coupling case this involves a Boltzmann distribution for the unexcited molecule and the distribution for the excited molecule is probably a function of the wavelength of exciting light. The rate of transfer may thus depend both on temperature and on the wavelength of the exciting light.

The quantitative treatment of the very weak coupling case given by Forster (1948) uses the same method: the assumptions made are (i) that thermal equilibrium has been attained before transfer takes place, so a Boltzmann distribution is used for the weighting factor in both the donor and acceptor molecules, and (ii) that only dipole-dipole interactions come into play. The overall rate of energy transfer is then given by:

$$F = \frac{9\,K^2\,\ln 10}{128\,\pi^5\,n^4\,N'\,\tau_o}\,\frac{1}{R^6}\int f_d(\tilde{\nu})\,\epsilon_a(\tilde{\nu})\,\frac{d\tilde{\nu}}{\tilde{\nu}^4} \qquad 6$$

where N' is the number of molecules per millimole, $\tilde{\nu}$ is the

wavenumber, $f_d(\tilde{\nu})$ is the normalised spectrum of donor emission, $\epsilon_a(\tilde{\nu})$ is the molar decadic extinction coefficient of the acceptor, and eq.5 has been used for u. Eq.6 may be rewritten:

$$F = \frac{1}{\tau_d}\left(\frac{\bar{R}_o}{R}\right)^6 \qquad 7$$

where τ_d is the actual donor fluorescence lifetime and $\bar{R}_o$ is the 'Forster distance' which corresponds to the molecular separation at which there is equal probability of transfer of energy from the donor and of de-excitation by all other routes. Therefore

$$\bar{R}_o^6 = \frac{9\kappa^2 . \ln 10 . \eta_d}{128\,\pi^5 n^4 N'} \int f_d(\tilde{\nu})\,\epsilon_a(\tilde{\nu})\,\frac{d\tilde{\nu}}{\tilde{\nu}^4} \qquad 8$$

where $\eta_d = \tau_d / \tau_o$, the donor fluorescence yield in the presence of the acceptor. As it stands this is not a very useful equation for determining values of $\bar{R}_o$. However, knowing the R-dependence of the rate of energy transfer, the behaviour of assemblies of molecules can be predicted. Let us take the case of molecules in a dilute solution so that only nearest-neighbour transfer need be considered. The fluorescence yield of the acceptor will be determined by energy transfer from the donor. Forster (1965) has shown that by assuming a statistical distribution of molecules in space, and fast Brownian rotation such that $\kappa^2 = 2/3$ the following expression for the quantum yield of acceptor fluorescence η_a may be derived:

$$\frac{\eta_a}{\eta_{a\,max}} = 2x\exp(x^2) \int_x^\infty \exp(-t^2)dt$$
$$= \sqrt{\pi}\; x \exp(x^2)\,(1 - \mathrm{erf}(x)) \qquad 9a$$

where erf(x) is the error function and $x = (c/c_o)$ with c as acceptor concentration. A similar expression for donor

fluorescence may be derived :

$$\frac{\eta_d}{\eta_{dmax}} = 1 - \sqrt{\pi} \times \exp(x^2)\,(1-\mathrm{erf}(x)) \qquad 9b$$

c_o is called the critical concentration, and is the concentration of acceptor at which there is, on average, one acceptor molecule within radius $\bar{R}_o$ of the donor molecule :

$$c_o = \frac{3}{2\sqrt{\pi^3}\, N' \bar{R}_o^3} \qquad 10$$

If c_o is found experimentally then $\bar{R}_o$ may be calculated. c_o is usually found by experimentally measuring a parameter that is sensitive to energy transfer (i.e. enhancement of acceptor fluorescence, quenching of donor fluorescence, or, in the case of transfer between like molecules, depolarisation of fluorescence) as a function of acceptor concentration and fitting this to eq.9. This method has led to several estimates of $\bar{R}_o$ for chlorophyll.

Knox (1973) has pointed out that there are two definitions of the 'Forster distance' in the literature. The first, defined above, makes $\bar{R}_o$ the intermolecular distance at which the probability of transfer to an acceptor molecule is equal to the probability of all other processes (radiative and non-radiative). Other definitions give

$$F = \frac{1}{\tau_o}\left(\frac{R_o}{R}\right)^6$$

in which case R_o (which is larger than $\bar{R}_o$) is the inter molecular distance at which the probability of transfer to an acceptor molecule is equal to the probability of radiative decay from the donor molecule.

Recently Kenkre and Knox (1974) have put forward a theory in

which the weak coupling and very weak coupling mechanisms are only two extremes of a unified transfer mechanism.

1.2.5 Experimental data on energy transfer from *in vitro* systems.

The study of energy transfer between chl *a* - chl *a*, chl *b* - chl *b*, and chl *b* - chl *a* has proceeded for many years. There have been four experimental systems used, and the following is a brief selective review of the results. The systems used have been (i) liquid solutions, (ii) solid solutions, (iii) monolayers and (iv) vesicles incorporating chlorophyll. The experiments have the same basis: to measure a parameter that is sensitive to energy transfer as a function of the concentration of the acceptor. In an experiment involving two pigments (heterotransfer) it is possible to measure (a) the sensitised fluorescence of the acceptor and (b) the quenching of donor fluorescence. In one pigment experiments (homotransfer) it is necessary to measure the degree of polarisation of fluorescence, using polarised exciting light. This method assumes that the molecules are not rotating but are randomly oriented so that any energy transfer will result in a decrease of polarisation. There have also been studies on concentration quenching (self-quenching) which is the decrease in fluorescence yield as the concentration of a single pigment increases.

1.2.5.1 Liquid solutions. An early study by Watson and Livingston (1950) used dilute and concentrated solutions of chl *a* and of chl *b* in ether or acetone, as well as mixtures of chl *a* and chl *b*. They established the existence of chl *a* fluorescence sensitised by chl *b*, this effect occurring at concentrations predicted by the Forster very weak coupling theory. They also

noted concentration quenching with a single pigment.

More recently Bauer, Szalay and Tombacz (1972) have made a careful analysis of the absorption, emission and fluorescence excitation spectra of chl a, chl b and equimolar mixtures of the two pigments in diethyl ether. They found that the efficiency of energy transfer from chl b to chl a was a function of the wavelength of the exciting light, a property to be expected of the weak coupling case. They also found that the calculations of energy transfer using (a) the sensitisation of the fluorescence of chl a by chl b and (b) the quenching of the fluorescence of chl b by chl a did not agree, some of the energy transferred being 'lost'. They postulated some fast transfer process (with either an R^{-3} or an R^{-6} dependence) superposed on the Forster very weak coupling process.

1.2.5.2 Solid solutions. Kelly and Porter (1970) investigated energy transfer between chl b - chl a, and pheophytin b - pheophytin a in solid solutions of lecithin. To monitor energy transfer they measured the yield of triplet states in the donor and acceptor produced by flash excitation, and in this way avoided the problem of discriminating between the fluorescence of chl b and chl a. The yields of the triplet states were measured as a function of acceptor concentration, and both the decrease of donor yield and the increase in acceptor yield due to energy transfer were adequately described by the Forster very weak coupling theory (eq.9) However, the concentration quenching data did not fit this equation, but fitted a function which included the square of the concentration of acceptor. To account for this concentration dependence, Kelly and Porter (1970) postulated that energy might be trapped by loose association of pairs of chl molecules; not dimers in the chemical sense, but pairs of chl molecules whose

intermolecular distance are at the lower end of the statistical distribution of intermolecular distances.

The concentration quenching of chl b in lecithin solid solutions was further studied by Kelly and Patterson (1971). They measured fluorescence yield, polarisation and lifetime as a function of chl b concentration. All three parameters had different concentration dependence. The fluorescence depolarisation was well described by the Forster very weak coupling theory. However, the yield and lifetime data were more complex. In particular it would be expected that for a simple mechanism these two should behave similarly, but the yield was quenched at lower concentrations of chl b than the lifetime. Kelly and Patterson suggested that the dependence on a higher power of concentration for fluorescence yield was due to a quenching process that acts very rapidly at the site of excitation. They called this phenomenon '*in situ*' quenching, and further suggested that it was due to the formation of loose dimers mentioned above. They postulated that an intermolecular distance of $\lesssim$ 3.5nm might cause quenching, and by calculating the proportion of non-fluorescent loosely associated chlorophyll as a function of the total chlorophyll concentration (from the fluorescence yield data) were able to show that the fluorescence lifetime data fitted this hypothesis.

1.2.5.3 Monolayers containing chlorophyll. The properties of chl a in lipid monolayers was studied by Trosper Park and Sauer (1968). They chose two lipids that occur in the chloroplast (monogalactolipid and sulfolipid) and two other lipids (castor oil and oleyl alcohol). They investigated the fluorescence yield and polarisation as a function of chl a concentration, and found that the Forster very weak coupling model was adequate to

describe the data.

Tweet, Ballamy and Gaines (1964) studied pure chl a monolayers and monolayers diluted with oleyl alcohol, using concentration quenching and quenching by the acceptor, copper pheophytin. They found the Forster very weak coupling model sufficient to explain the concentration quenching and copper pheophytin quenching in dilute monolayers, but in undiluted monolayers this model gave very low values of R_o.

1.2.5.4 Vesicles containing chlorophyll. Csatorday, Lehoczki and Szalay (1975) incorporated chl a into micelles of the detergent Triton X-100 and observed the depolarisation of fluorescence as a function of concentration of chl a. Their data fitted the very weak coupling theory with a value for $\bar{R}_o$ of 5.6nm. Lehoczki and Csatorday (1975) repeated these experiments with chl b and found similar behaviour with an $\bar{R}_o$ of 4.3nm. From these data it appears as though Forster's very weak coupling case is able to account for most of the *in vitro* experimental data. However, Bauer et al. (1972) have presented evidence which suggests that at least in liquid solutions the weak coupling case may have a part to play in energy transfer. Moreover, the concentration of pigments used has been rather below the $\sim$0.1M found *in vivo* (see Rabinowitch, 1949). The reason for this is that at higher concentrations the fluorescence of these pigments is reduced to a very low value by concentration quenching, and this calls into question the use of these *in vitro* systems as models for photosynthesis. In addition, the estimates of *in vivo* molecular interaction energies of chl indicate that the weak coupling case may apply. It is therefore not at all clear what energy transfer mechanism operates *in vivo*.

1.2.6 Energy migration and trapping.

The function of the photosynthetic unit is to collect light energy and deliver it to the reaction centre efficiently. If the excitation can be thought of as localised, i.e. in the very weak coupling limit, then a random walk-type analysis should be sufficient to describe the migration of the excitation through the pigment bed. However, if the weak coupling case is valid then a model of coherent excitation transfer has to be used. Robinson (1967), who believes that the weak coupling model is applicable in the photosynthetic unit, has considered this latter model. Since the excitation cannot be thought of as localised on a single molecule it has to be regarded as a property of the pigment bed as a whole. Robinson (1967) has analysed the rate of spread of the excitation over the pigment array from first being localised on a single molecule. The result of this analysis is that the width of the wavepacket at time t is:

$$\text{width} = a \left\{ 1 + \frac{u^2 t^2}{\hbar^2} \right\}^{\frac{1}{2}} \qquad 11$$

where a is the lattice constant and the wavepacket is of Gaussian shape with width a at time t = 0. Using Robinson's value for u of $100cm^{-1}$ the time to reach a width of 25a (for a photosynthetic unit of 300 chlorophyll molecules) is about 1.3ps, which approaches 10^{-3} times the fluorescence life time (section 1.2.7). For $u = 50cm^{-1}$, t = 2.6ps, and for $u = 10cm^{-1}$, t = 13ps. Therefore the excitation can be regarded as being spread over the entire pigment array in a time very much shorter than the lifetime of the excitation. This calculation assumes that the trap does not alter the excitation wavefunction. The rate of trapping is slow because the probability density of the excitation being at the trap is low. Clearly the random walk model is inapplicable to

this mechanism of energy transfer. Robinson (1967), however, has pointed out that if the rate-limiting step of energy transfer and trapping is the step of trapping, then the means of delivering excitation to the trap is immaterial. It is therefore possible to use the random walk formulation to calculate migration rates in both the weak coupling and very weak coupling cases.

The energy migration problem in random walk terms has been stated by Montroll (1969) as: 'Given an infinite lattice of unit cells, each cell containing N points of which (N - 1) are chl molecules and one is a trap; if an arbitrary chl molecule is excited how many steps must be taken by the exciton in a random walk before it is trapped ?'. Montroll (1969) was able to show by assuming that energy transfer takes place to nearest-neighbours only, and using a purely random walk approach to the problem that the average number or steps $\langle n \rangle$ in a square lattice is given by :

$$\langle n \rangle = \pi^{-1} N \ln N + 0.1951N \qquad 12$$

plus other terms which are not important at large values of N. Alternative solutions to this same problem have been presented by Robinson (1967), Pearlstein (1967) and Knox (1968). In deriving his own result Knox (1968) showed that it was essentially the same as those of Robinson and of Pearlstein. Knox obtained

$$4F\bar{t} = 0.3184\, N \ln N + 0.2060N + 2.500 \qquad 13$$

where $\bar{t}$ is defined as the 'arrival time' for excitation at the trap. The calculation assumes (i) nearest-neighbour transfer only and (ii) the lifetime of the excitation in the photosynthetic unit is infinite, that is, the excitation is not trapped rapidly. This expression is clearly similar to that of Montroll.

Therefore it appears that provided the pairwise transfer rate

F is known, calculation of trapping times presents no difficulty. The problem lies in accurately determining F, and as has been shown, this is difficult since the photosynthetic system appears to be on the borderline between the weak coupling and the very weak coupling cases. Furthermore, Knox (1973, 1975) has pointed out that even if it could be unequivocally determined that the very weak coupling case were applicable then a number of corrections may need to be made to the rate of transfer as calculated by Forster (1948): (i) the Forster equation (eq.6) assumes that the donor and acceptor molecules are randomly oriented, leading to the value of κ^2 being 2/3. Knox (1973) notes that for an oriented system the rates of transfer in certain directions deviate from the average rate by large factors. This is particularly striking in an ensemble of oriented dipoles. By choosing some arrays Knox (1973) was able to show that the overall transfer rate can vary from 1/3 to 8 times the randomly oriented rate. (ii) When the donor and acceptor molecules approach each other closely the approximation of a pure dipole field breaks down because each molecule can 'see' the structure of the other. This effect becomes noticeable when the intermolecular distance becomes comparable with the size of the π -electron system. At 1.5nm between chl a molecules a correction of -30% to +50% (depending on mutual dipole orientation) may be necessary, and some small correction necessary for $R \leq 4.0$nm (Knox, 1975).

Bearing these points in mind, it may still be of some value to make a rough calculation of trapping times. Following Colbow (1973), let us set R = 1.5nm. Using eq.5 to calculate u, this is equal to $58cm^{-1}$. The rate of transfer (F) then becomes $0.44 \cdot 10^{12} s^{-1}$. For a photosynthetic unit of 300 chl molecules Knox's expression

(eq.13) yields

$$\bar{t} = 344\text{ps} \qquad 14$$

1.2.7 The efficiency of trapping.

In the limit of the very weak coupling case, the efficiency of trapping at the reaction centre has been assumed to be high so that the rate-limiting step in the overall process is the diffusion of the excitation to the trap. Robinson (1967), who supports the weak coupling case, points out that if there were both fast transfer and efficient trapping in the photosynthetic unit then the fluorescence lifetime would be a few tens of ps, not the hundreds of ps that is observed for PSII (see, for example, Moya, 1974). Shorter fluorescence lifetimes have recently been reported using picosecond laser pulses to excite fluorescence (Seibert and Alfano, 1974 ; Shapiro et al., 1975; Yu et al., 1975; Paschenko et al., 1975; Beddard et al., 1975), but this experimental approach has been criticised because the very high light intensities used may induce other photochemical phenomena such as singlet-singlet annihilation processes which probably distort the lifetime measurements (Campillo et al., 1976).

If there is fast transfer a low trapping efficiency is to expected since trapping will only occur when the excitation undergoes a vibrational relaxation while resident on the trap molecule. In calculating the trap efficiency in the limit of fast transfer Robinson (1967) assumes a photosynthetic unit of 300 molecules, a fluorescence lifetime of 1.5ns and a pairwise transfer time of 0.05ps, and derives a value for the efficiency of trapping of $\simeq 1\%$.

Since the mechanism of energy transfer in the photosynthetic unit appears to lie between the weak coupling and very weak coupling

limits, the only statement that can be made about trapping efficiency is that it is probably between the two values quoted above.

The experiments described in section 3.2 allow some indirect conclusions to be drawn about the nature of energy transfer between bulk pigments in photosynthesis. It appears as though the distance that an excitation may diffuse through the bulk pigments is a function of fluorescence lifetime which, together with other considerations, implies that the excitation energy transfer must be by a slow process. The implications of this are discussed in section 3.2.5.3.

1.3 Photosystem two and oxygen evolution

Photosystem II utilises light energy to produce a strong oxidant and a weak reductant. The oxidant Y^+ is used in the liberation of molecular oxygen from water, and the weak reductant Q^- acts as the first member of the electron transport chain between PSII and PSI. The oxygen evolving apparatus appears to operate in a four-step cycle (see section 1.3.2) and there is evidence for concomitant liberation of protons on the inside of the thylakoid membrane.

1.3.1 The components of PSII

The heart of PSII is the reaction centre which is thought to consist of a specialised chl a molecule, P680 (the trap) closely associated with an electron donor Y and an electron acceptor Q. (The notation for the donor is sometimes different, see section 1.3.1.2). Light energy is directed through the photosynthetic unit to the reaction centre, and excites the trap molecule to $P680^*$. This excited component quickly donates an electron to Q in a reaction with a half-time of about 20ns (Witt, 1971; Mauzerall, 1972) leaving the reaction centre in the state $Y\ P680^+\ Q^-$. $P680^+$ is a short-lived species; Glaser et al (1974) have shown that the absorbance change at 690nm which they attribute to $P680^+$ (Doring et al., 1969) decays biphasically with half-times 35μs and 200μs. (It has been suggested recently that there is an additional component of half-time 1μs (Glaser et al., 1975)). The decay of the $P680^+$ signal is due to the reduction of $P680^+$ by Y and subsequent electron donors, the reaction centre then being in the form $Y^+\ P680\ Q^-$. The scheme for changes in the reaction centre upon illumination is thus:

$$YP680Q \xrightarrow{h\nu} YP680^*Q \xrightarrow{<20ns} YP680^+Q^- \xrightarrow{1,\ 35,\ 200\mu s} Y^+P680Q^-$$

The positive change on Y^+ is passed to the oxygen-evolving apparatus and Q^- reduces the next member in the electron transport chain.

It has been argued by Doring et al. (1969) that P680 facilitates electron transfer between Y and Q by acting as a sensitiser, but more recently Doring (1975) has presented evidence which is in accord with the scheme of P680 being a mediator.

1.3.1.1 Q. The principal way in which changes in the redox state of Q have been observed is through the quenching of PSII chlorophyll fluorescence. Q quenches the fluorescence, but reduced Q does not (Duysens and Sweers, 1963) so that fluorescence yield is a measure, albeit not a simple one, of the concentration of reduced Q (see section 1.4). There are however two other ways to monitor the primary electron acceptor of PSII; they both involve absorbance changes.

In 1969 Knaff and Arnon discovered a light induced absorbance change in chloroplasts at 550nm which they called C-550 (Knaff and Arnon, 1969b). It was observed in the presence of excess ferricyanide so that absorbance changes due to reduction of the cytochromes in the electron transfer chain did not interfere. The C-550 change was induced more efficiently by PSII light than by PSI light; its decay, but not its onset, was inhibited by DCMU (a compound which blocks electron transport between PSII and plastoquinone), and it was observable at low temperatures. From these observations Knaff and Arnon (1969b) suggested that the absorbance change was connected with PSII, and that it might be indicative of an electron transport chain component.

That the species responsible for C-550 could be reduced by sodium dithionite in the dark was shown by Erixon and Butler (1971a).

They also showed that the C-550 signal was absent from PSI subchloroplast particles and mutant algae lacking PSII. At low temperatures (77°K) Erixon and Butler (1971b) found that the C-550 absorbance change and PSII chl fluorescence yield changes had very similar dependence on redox potential. They suggested that C-550 and Q might be analogous, or at least closely related. But at room temperature the correspondence between C-550 and Q is less clear. Ben Hayyim and Malkin (1972) observed that the room temperature quantum yields for the C-550 absorbance change and fluorescence yield changes were similar at short wavelengths, but differed at long wavelengths ($\lambda \simeq 715$nm). However, Butler (1972b) was apparently able to resolve this discrepancy between low temperature and room temperature measurements by showing that the light-induced absorbance changes which indicate membrane potential (see section 1.7) were contributing to the C-550 change at room temperature. More recent room temperature measurements of C-550 and fluorescence yield in subchloroplast particles prepared with deoxycholate by van Gorkom et al. (1972) support the hypothesis that C-550 and Q are related.

The second absorbance change associated with Q was characterised by Stiehl and Witt (1969), and denoted X-320 (formerly X-335) from the maximum of the change being at 320nm. The species responsible for X-320 was assumed to lie between P680 and plastoquinone since the change was sensitive to PSII light and its dark decay time (0.6ms) is that of electron transport from water to plastoquinone (Witt, 1971). This time also corresponds to the time of reoxidation of Q^- when the A pool is fully oxidised (Forbush and Kok, 1968). It was suggested (Witt, 1971) that X-320 might reflect a plastoquinone molecule. K.Witt (1973) studied this absorbance

change under flash excitation. At low temperature (140°K) the change is irreversible; at room temperature the change is made irreversible by the presence of DCMU. A second flash given to the room temperature sample in the presence of DCMU did not give any further change in absorbance. All these properties are expected of the primary electron acceptor Q. More recently van Gorkom (1974) working with a PSII subchloroplast particle prepared using the detergent deoxycholate, measured the time-course of an absorbance change with a maximum at 330nm that correlated kinetically with that of C-550. The absorbance difference spectrum is similar to the plastosemiquinone anion-minus-plastoquinone difference spectrum (Bensasson and Land, 1973), so van Gorkom suggested that in these subchloroplast particles at least the reduction of the primary electron acceptor involves the reduction of one bound plastquinone molecule per reaction centre to its semiquinone anion.

1.3.1.2 Y. The nature of primary electron donor to PSII at room temperature is unknown. At low temperature ($T < 100°K$) it has been established that cyt b-559 acts as an electron donor since it is photooxidised by PSII (Knaff and Arnon, 1969a) and has the same dependence on redox potential as C-550 and prompt fluorescence (Erixon and Butler, 1971b). At -100°C cyt b-559 is not photooxidised (Butler, Visser and Simmons, 1973); presumably another donor takes over. The properties of the water-splitting apparatus will be considered in section 1.3.2.

The nomenclature for the primary electron donor is variable. In this thesis the symbol Y will be used for the primary donor and the symbol Z for a secondary donor which may attain four redox states (the S states; section 1.3.2.). Y and Z may be the same component. Other authors have used Z for the primary electron donor.

1.3.1.3. P680. The chemical status of reaction centre pigments ought to be able to be established by electron spin resonance (esr) spectroscopy. Such studies have been made on the reaction centre pigments of PSI (P700) and of photosynthetic bacteria (B890). Norris et al. (1971) compared the esr spectrum of a chl-water adduct $(chl.H_2O)^+_n$ with that of P700 and B890, and were able to show from measurements of the linewidths of the esr spectra that both the P700 and B890 reaction centre pigments were in the form of a chl dimer containing a water molecule ($chl.H_2O.chl$). Such a conclusion has received support from electron nuclear double resonance (endor) experiments (Feher et al., 1975).

An esr signal which has been attributed to $P680^+$ has only recently been found (Malkin and Bearden, 1973; Bearden and Malkin, 1973). It appears on illumination of chloroplasts with PSII light at 77^oK provided P700 has been chemically oxidised first. The signal is enhanced in PSII subchloroplast particles, but is absent in PSI subchloroplast particles. It has a linewidth similar to that of the esr signal from $P700^+$. There is also evidence from the linewidth of the $P680^+$ esr signal from deoxycholate subchloroplast particles (PSII fraction) that P680 exists as a dimer in that system (van Gorkom et al., 1974).

1.3.2 Oxygen evolution in photosystem II

In order to produce molecular oxygen four electrons must be removed from two molecules of water. The reaction centre of PSII appears to operate with a one-electron turnover: therefore there must be some storage mechanism in which four positive charges are accumulated before the oxygen evolution step can proceed.

Early observations on oxygen evolution gave clues that a charge

storage mechanism was involved. Allen and Franck (1955) found that dark-adapted algae illuminated with a single flash did not evolve oxygen, and Joliot (1968) showed that in weak continuous light the oxygen evolution of algae or chloroplasts started after a lag period, which Joliot called 'activation'. But the most impressive experimental observations come from the measurement of oxygen evolution in a series of saturating flashes, which were made possible by the development of a sensitive rapidly-responding oxygen electrode (Joliot and Joliot, 1968).

1.3.2.1 The oxygen yield sequence. Joliot, Barbieri and Chabaud (1969) measured oxygen evolution from Chlorella and chloroplasts illuminated by a series of 10μs flashes separated by 300 ms darktime. The relative yield of oxygen per flash (Y_n for the nth flash) is shown in fig.2. The yield is zero on the first flash, small on the second and maximal on the third. The yields then perform damped oscillations of period four, the maximum yields being elicited by flashes 3,7,11.... Similar results were obtained by Kok et al. (1970) who interpreted their results by postulating that each PSII reaction centre undergoes the following sequence of reactions:

$$S_0 \xrightarrow{h\nu} S_1 \xrightarrow{h\nu} S_2 \xrightarrow{h\nu} S_3 \xrightarrow{h\nu} S_4 \rightarrow S_0 \qquad (16)$$

$$2H_2O \quad O_2 + 4H^+$$

The S states differ by the number of oxidising equivalents stored in them. The accumulation of four charges in the S_4 state leads to oxygen evolution and regeneration of the state S_0. The scheme assumes (i) each oxygen-evolving system operates independently; there is no exchange of oxidising equivalents between centres and (ii) each step $S_n \xrightarrow{h\nu} S_{n+1}$ is a one quantum process, and is

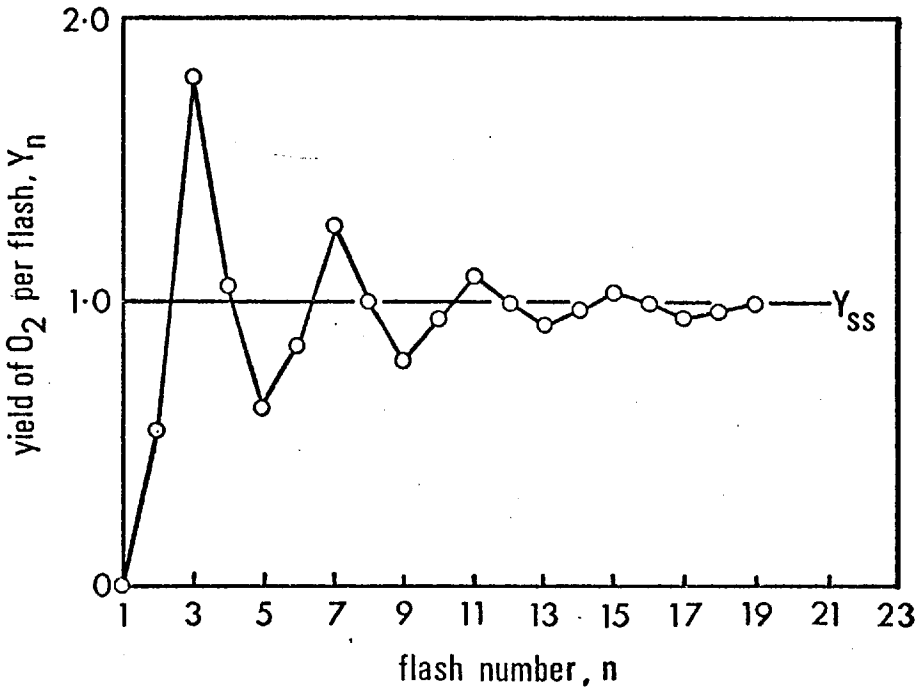


Figure 2.
The flash-yield of oxygen as a function of flash number (from Joliot et al., 1969). Dark-adapted spinach chloroplasts were illuminated with a series of brief saturating flashes and the oxygen evolution on each flash measured with a rapidly-responding oxygen electrode. The extent of the responses have been normalised to the steady-state value.

sensitised by the same pigment system. It is also assumed that both the S_1 and S_0 states are stable in the dark since if all the centres returned to the S_0 state in the dark it would be expected that the first three flashes would yield no oxygen, and the fourth would yield four times the steady-state yield.

It has been postulated that manganese is involved in the S states because (i) manganese can assume several valence states and (ii) manganese depletion leads to the inhibition of oxygen evolution (Cheniae,1970).

In theory the oxygen yield sequence should proceed undamped, but it is observed that after 25-30 flashes the oscillations become small and all flashes give the same steady-state yield of oxygen. Two factors are thought to contribute to this damping (Forbush et al., 1971) (a) Some centres undergo two photoacts during one flash (double hits). According to this hypothesis the fraction of double hits should be reduced by using exciting flashes of shorter duration. Joliot et al. (1971) have performed this experiment comparing 10μs (width at 1/3 peak) and 2μs flashes. Using the shorter flashes the oxygen yield on the second flash is reduced to zero, and the damping of the sequence reduced. (b) Some centres do not undergo a photoact during the flash (misses). The fraction of misses does not appear to be reduced by using high light intensity, and may be connected with a back-reaction in the reaction centre.

The numerical values of the fractions of centres undergoing double hits (β) and misses (α) may be estimated by comparing experimental and theoretical oxygen yield sequences. Forbush et al. (1971) found a best fit between experimental data and their theoretical model for α =0.1 and β =0.05. The model

assumes α is the same on all steps in the sequence (and denoted $\bar{\alpha}$). However, Joliot et al. (1971) have suggested that α may be different on each of the steps and have estimated values of $\alpha_1 = 0.08$, $\alpha_2 = 0.28$, $\alpha_3 = 0.25$ and $\alpha_4 = 0.20$, where the subscript refers to the flash number. Delrieu (1974) has reported that her data can be accounted for by the misses being involved only in the step $S_2 \longrightarrow S_3$.

1.3.2.2 Characteristics of the S states. (i) S_4. The $S_4 \longrightarrow S_0$ step is the slowest in the sequence, taking place in $\sim$ 1ms (Bouges-Bocquet, 1972). (ii) S_3. It has been mentioned that the S_0 and S_1 states are stable in the dark. It is assumed that the S_4 state is only present transiently, but the states S_2 and S_3 may however decay to lower states in the dark. The decay is principally by a 'one-step' mechanism (Forbush et al., 1971):

$$S_3 \longrightarrow S_2 \longrightarrow S_1$$

although a 'two-step' mechanism may also be involved (Joliot et al., 1971):

$$S_3 \longrightarrow S_1 \quad ; \quad S_2 \longrightarrow S_0$$

The deactivation rate of the S_3 state can be measured by giving a preillumination, then leaving a variable darktime, then giving a flash. The oxygen yield of this flash, Y_1, represents the concentration of S_3. Similarly S_2 can be measured from Y_2. For S_3 the deactivation, which is not first-order, is generally more rapid in algae ($t_{\frac{1}{2}} \sim$ 5-10s) than in chloroplasts ($t_{\frac{1}{2}} \sim$30-40s) (Joliot et al., 1971). (iii) S_2. The deactivation of the S_2 state is first order in both chloroplasts and Chlorella with half-times of about 40s and 20s respectively. Complete decay of the S_2 and S_3 states occurs in $\sim$200s (Chlorella) or $\sim$ 400s

(chloroplasts) at room temperature (Joliot et al., 1971).

(iv) S_1 and S_0. The states S_1 and S_0 are generally regarded as stable, but Joliot et al. (1971) have shown that an equilibrium exists between them. Bouges-Bocquet (1973) has studied this equilibrium further. She has shown that $\bar{\alpha}$ is equal to 0.11 in chloroplasts, and is unchanged by the addition of ferricyanide (FeCN). However, in the presence of FeCN the oxygen yield ratio Y_3/Y_4 is increased. Bouges- Bocquet (1973) suggested that this might be due to an increase in the concentration of S_1 at the expense of S_0. An opposite effect was found for DCIP, but Velthuys and Visser (1975) have recently reported experiments that do not agree with the equilibrium hypothesis for the action of DCIP.

1.3.2.3 The dark reactions. Each step $S_n \xrightarrow{h\nu} S_{n+1}$ is composed of a light and a dark reaction (Kok et al., 1970), so that it should be represented as

$$S_n \xrightarrow{h\nu} S_n' \longrightarrow S_{n+1}$$

The light reaction must be complete in a few μs (the duration of the flash), but the dark reactions are somewhat longer; it has already been noted that the $S_4 \rightarrow S_0$ step takes $\sim$1ms. Kok et al. (1970) measured the duration of the $S_1' \rightarrow S_2$ step to have a half-time of $\sim 200\,\mu$s and the $S_2' \rightarrow S_3$ step a half-time of $\sim 400\,\mu$s. Using similar techniques Bouges-Bocquet (1973) found a more complex situation in which steps $S_0' \rightarrow S_1$ and $S_1' \rightarrow S_2$ have time-dependent temperature coefficients and the reaction $S_2' \rightarrow S_3$ appears to consist of two reactions of similar half-times.

1.3.2.4. Induced deactivation. Some compounds, called ADRY agents (acceleration of the deactivation reactions of the water-splitting enzyme Y) appear to decrease the stability of stored

charges in the higher S states. ADRY agents include anilinothiophenes (Renger,1972), nitrophenols and indophenols (Vater,1973), CCCP and FCCP (Renger, Bouges-Bocquet and Buchel, 1973) and DCMU (Renger,1973). Their action is assumed to be by facilitating electron flow from an electron donor into the S states. The electron donor is thought to be between Q and P700 or on the reducing side of PSI (Renger, Bouges-Bocquet and Buchel,1973). This mechanism is certainly not clear, since it has been shown that in Chlorella ANT-2p (an anilinothiophene) stabilises the S states, whereas in chloroplasts it has the opposite effect (Renger, Bouges-Bocquet and Delosme,1973). Similarly, Vater (1973) has reported that in chloroplasts indophenols can accelerate or retard deactivation depending on their substituents.

1.3.2.5. Flash-induced proton liberation. Fowler and Kok (1974) have made a careful study of the evolution of protons by the photosynthetic apparatus during a series of brief ($\sim 1 \mu s$) saturating flashes. Using a specially constructed pH electrode with a response time of < 5ms they observed in untreated chloroplasts a proton uptake into the chloroplasts following each flash, and a subsequent leak of protons back into the suspending medium during the dark time between flashes. Neither the magnitude of the influx or of the leak was a function of flash number. The influx was gradually inhibited by DCMU, complete inhibition occurring after ~ 5 flashes. When the chloroplasts were uncoupled with gramicidin D or methylamine a larger proton efflux after each flash was observed and the magnitude of this efflux oscillated with with period 4. The maximal efflux was on the third flash. This oscillation, which was damped, is very similar to that of the flash-induced oxygen yield. From these observations Fowler and Kok (1974)

concluded that following the accumulation of four oxidising equivalents water is decomposed in a concerted reaction, and that the oxygen-evolving system is located on the inside of the thylakoid membrane

1.3.3 Electron spin resonance studies on PSII

PSII gives rise to two distinct esr signals. The first, from $P680^+$ (Malkin and Bearden, 1973), has already been described in section 1.3.1.3. The second was first characterised by Commoner et al. (1956) and denoted Signal II, to distinguish it from Signal I which arises from $P700^+$ (Weaver and Bishop, 1963). Signal II is present in dark-adapted chloroplasts, and is increased upon illumination. The increase is induced more efficiently by PSII light, and decays in the dark in a rather complex way. The signal has been ascribed to some quinone component on the reducing side of PSII (Blois and Weaver,1964) or to an entity on the oxidising side of PSII (Treharne et al., 1963). Esser (1974) suggested that it lay on a side path linking the oxidising and reducing sides of PSII.

Signal II has been thoroughly studied in Sauer's laboratory. It has been resolved into three components with the following characteristics. In untreated dark-adapted chloroplasts a slow phase (Signal IIs) appears on illumination of the sample, and it is thought to be associated with states S_2 and S_3 of the oxygen-evolving system (Babcock and Sauer,1973). In Tris-washed chloroplasts a rapid component is observed (Signal IIf) which has been postulated to reflect the electron donor to PSII, Y^+, when the normal electron donation processes are blocked (Babcock and Sauer,1975). In untreated chloroplasts an esr spectrometer with an improved time resolution has permitted the detection of a faster component (Signal IIvf) by Blankenship et al.(1975). This signal has been tentatively ascribed to Y^+ under normal conditions.

1.4 Light emission from PSII - prompt chlorophyll fluorescence

Light emission from the photosynthetic apparatus at room temperature comes principally from the bulk chlorophyll pigments of photosystem II. There is very little emission from the chlorophylls associated with PSI (see section 1.1.2). This light emission from PSII can be divided into two types (a) prompt chlorophyll fluorescence and (b) delayed chlorophyll fluorescence. Although they have similar spectral characteristics and arise from the same pigments, the origins of these two emissions are quite distinct. Prompt chlorophyll fluorescence represents one of the pathways for decay of excitation energy in the chlorophyll pigments of PSII; it take place in competition with photochemical trapping and occurs within the lifetime of the original excitation. Delayed fluorescence, however, represents a back-reaction occurring after the excitation has been trapped. Metastable states in the reaction centre recombine, forming a quantum of excitation which is delivered to the bulk chlorophyll pigments from where it has a certain probability of fluorescing. In this and the following section these two processes will be described.

Prompt chlorophyll fluorescence is a very useful intrinsic probe of photosynthesis. It has been used over a number of orders of magnitude of time to investigate the phenomena occurring in the light-harvesting pigments, the reaction centre, electron transport and events related to structural changes. For many years the analysis of chlorophyll fluorescence experiments relied solely on the Duysens and Sweers hypothesis (see section 1.4.1), but more recently it has become clear that this hypothesis does not apply to all of the large time range in which fluorescence measurements are now being made. This section will consider prompt chlorophyll

fluorescence from the point of view of the Duysens and Sweers hypothesis and the various modifications that have been made to it.

1.4.1 The Duysens and Sweers hypothesis

When a quantum of light is absorbed into an array of molecules it can suffer one of four fates. It can either (i) non-radiatively decay (ii) radiatively decay (iii) be transferred out of the array of (iv) be quenched by a quenching centre. If these processes can be represented by the rate constants k_h, k_f, k_t and k_Q respectively, then the quantum yield of fluorescence ϕ_f is given by

$$\phi_f = \frac{k_f}{k_f + k_h + k_t + k_Q [C]} \qquad 17$$

where [C] is the concentration of the quenching species, and the system is taken to be homogeneous.

In 1963 Duysens and Sweers performed some important experiments on the PSII chlorophyll fluorescence yield of algae and chloroplasts. They illuminated algae alternately with red (PSII) and far red (PSI) light, and discovered that with red light the fluorescence yield was high, but that it was lowered on superimposing far-red light; this action of far-red light being blocked by DCMU. From the antagonistic behaviour of PSI and PSII light and the action of DCMU, Duysens and Sweers postulated that the fluorescence yield of PSII was controlled by the redox state of the primary electron acceptor of PSII, Q. When Q was not reduced (and the trap therefore able to receive excitation) the fluorescence was quenched, but when Q was reduced (the trap unable to receive excitation) the fluorescence was not quenched. DCMU blocked the reoxidation of Q^- via PSI. This hypothesis received support from the observation that chemically reducing Q with reducing agents like sodium dithionite

increased the fluorescence yield. Eq.17 may thus be adapted by making the quencher C indentical with Q so that

$$\phi_f = \frac{k_f}{k_f + k_h + k_{tI} + k_p [Q]} \qquad 18$$

where k_p is the rate constant for photochemical trapping, $[Q]$ is the concentration of open traps and k_{tI} is the rate constant for energy transfer from PSII to PSI. It should be pointed out that this formulation assumes that the array of photosynthetic units is totally homogeneous (the uniform pigment matrix model); as will be shown in section 1.6 this is not the only assumption that can be made.

This model of chlorophyll fluorescence has been very successful in accounting for a good deal of experimental data. For example Malkin and Kok (1966) have made a useful quantitative study of the fluorescence induction in isolated chloroplasts based on the Duysens and Sweers hypothesis. The general shape of the fluorescence induction from chloroplasts without added electron acceptor is shown in fig.3. On first illuminating the chloroplasts, the fluorescence immediately attains the level f_o (see section 1.4.2.) and then rises more slowly in a rather complex way until a final level f_{max} is reached. Malkin and Kok (1966) showed that the shape of the induction of variable fluorescence (above the f_o level) as well as its dependence on temperature and light intensity could be explained by the Duysens and Sweers hypothesis of the reduction of Q causing the fluorescence to rise, bearing in mind that Q^- is transiently oxidised by a subsequent member of the electron transport chain between PSII and PSI.

The induction has also been studied by Delosme (1967) who showed

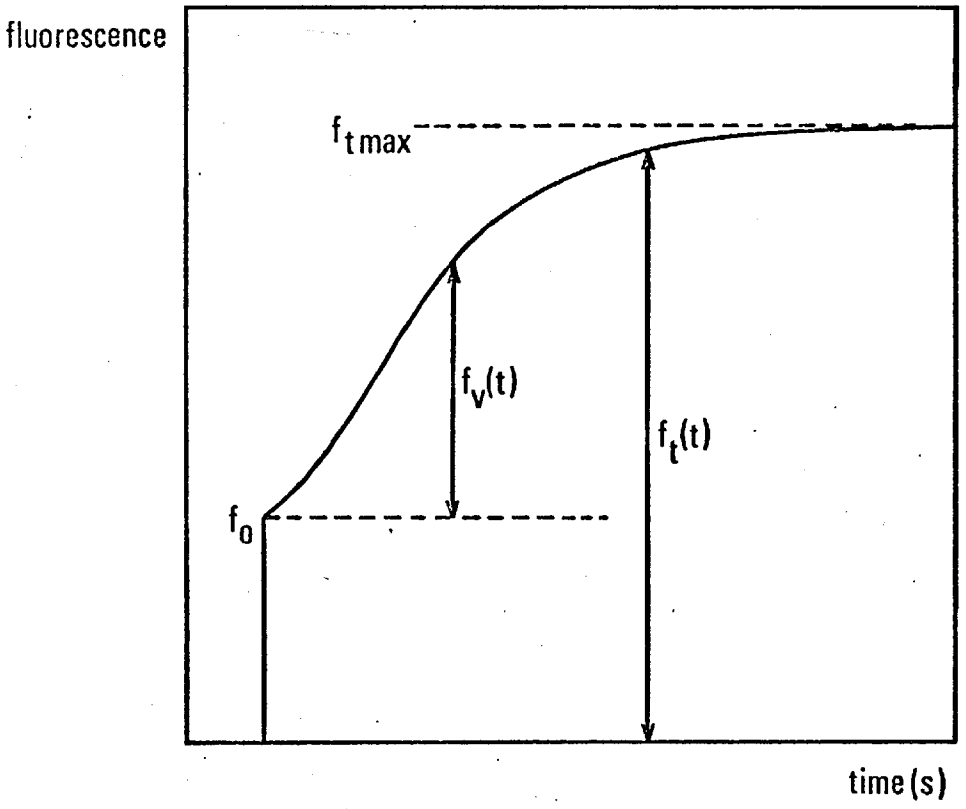


Figure 3.

Schematic chlorophyll fluorescence induction on illuminating dark-adapted chloroplasts in the absence of added electron acceptor. The fluorescence quickly reaches the f_o level and there is a rather complex induction to the final level f_{tmax}. The fluorescence above the f_o level (f_v) is known as variable fluorescence.

it to consist of an initial photochemical phase and a second thermal phase. These two phases correspond to the photoreduction of Q and the subsequent reduction of a pool of oxidising equivalents between PSII and PSI.

In general, the Duysens and Sweers hypothesis has been successful in describing chlorophyll fluorescence changes due to variations in the rate of electron transport which are reflected in the redox state of Q.

1.4.2 The f_o level of chlorophyll fluorescence

The Duysens and Sweers hypothesis appears well able to account for the variable chlorophyll fluorescence induction, but it is not able to cope with the non-variable part of PSII fluorescence. This f_o level of fluorescence appears very quickly after the actinic light is turned on. Delosme (1967) found it to be present in 5μs, and Mauzerall (1972) has shown that it is present at the end of a 10ns laser flash. Since the f_o level appears so quickly it is probable that it is not connected with any photochemical processing, but arises from pigments that are not connected with traps. This 'non-connection' could arise in two ways. Firstly the f_o level of fluorescence might emanate from pigments totally divorced from PSII traps. This was suggested by Clayton (1969) on phenomenological grounds. On the experimental side Lavorel (1962) compared the fluorescence emission spectra of the f_o and f_{tmax} levels in algae and found them different, expecially in the 720nm region. From his measurements Lavorel suggested that PSI might make a significant contribution to the f_o level of fluorescence. Etienne et al.(1974) also concluded from experiments on the quenching of fluorescence by dinitrobenzene that the zero level of PSII variable fluorescence is near the f_o level. Lavorel and

Joliot (1972) support this hypothesis by noting that the expression $(f_{tmax} - f_o)/f_{tmax}$ gives the quantum yield of PSII photochemistry and that for this to be maximised f_o should be as small as possible. If part of the f_o level came from another source (they suggest PSI) then the efficiency of photochemistry would be high.

The second kind of 'non-connection' should really be called 'virtual non-connection'. It was first proposed by Delosme (1967) that the non-variable part of PSII chlorophyll fluorescence arose from quanta emitted from the bulk pigments of PSII as the excitons migrated toward the reaction centre. The emission occurs before the excitons are aware that there is a trap (open or closed) in the pigment bed so in this sense the emission occurs from pigments unconnected to active PSII traps. This hypothesis has recently received support from Butler and Kitajima (1975a) who found that the fluorescence quenching induced by the plastoquinone antagonist DBMIB fitted a model incorporating this concept.

These two hypotheses on the origin of the f_o level of fluorescence are not easily reconciled. The latter has been more often used in the literature. Experiments described in section 3.2. of this thesis have results which indicate that at least a part of the f_o level originates from a source unconnected with PSII.

1.4.3. Prompt chlorophyll fluorescence from PSII at low temperatures.

The light-induced reactions in the reaction centre of PSII at liquid nitrogen temperature have been fairly well characterised. They can be represented as:

$$D.P.A \xrightarrow{h\nu} D.P^+.A^- \longrightarrow D^+.P.A^-$$

where the pigment molecule P is P680, the acceptor A is reflected by the absorbance change C-550 (which is thought to indicate the redox state of Q) and the donor D is cyt b-559 (although this

component is not the primary donor to PSII at low temperatures) (Erixon and Butler,1971b; Butler, 1973). The influence of the redox state of cyt b-559 on the chlorophyll fluorescence yield of PSII at low temperatures was studied by Okayama and Butler (1972). They found that when cyt b-559 was in the reduced form before freezing, the fluorescence yield at low temperature was high ($f_{tmax}/f_o \simeq 4.0\text{-}5.0$), but when cyt b-559 was oxidised by ferricyanide prior to freezing the fluorescence yield was low ($f_{tmax}/f_o \simeq 2.0\text{-}2.5$) Okayama and Butler measured f_{tmax} as a function of oxidation-reduction potential, and found that the data fitted a one-electron transition with a mid-point potential of 365mV, suggesting the involvement of cyt b-559. However, C-550 was reduced to the same extent at all redox potentials. Thus under these conditions the PSII fluorescence yield appears to be controlled by a component on the oxidising side of the reaction centre. Okayama and Butler postulated that $P680^+$ could act as a fluorescence quencher in the following way: under normal conditions cyt b-559 is initially reduced, and the low temperature light-induced reaction is

$$\text{cyt b-559. P680. C-550} \xrightarrow{h\nu} \text{cyt b-559. } P680^+\text{. } C\text{-}550^-$$

which is quickly followed by the reaction

$$\text{cyt b-559. } P680^+\text{. } C\text{-}550^- \longrightarrow \text{cyt b-}559^+\text{. P680. } C\text{-}550^-$$

giving a high fluorescence yield. If, however, cyt b-559 is oxidised before illumination, the photoreaction is then

$$\text{cyt b-}559^+\text{. P680.C-550} \xrightarrow{h\nu} \text{cyt b-}559^+\text{.}P680^+\text{. } C\text{-}550^-$$

and if $P680^+$ is stable at low temperature this could lead to fluorescence quenching. However, since cyt b-559 is not the primary electron donor at low temperature, $P680^+$ could oxidise

the primary electron donor which in its oxidised form might be a fluorescence quencher.

These studies were extended by Butler (1972a) and Butler et al. (1973). Oxidation of cyt b-559, reduction of C-550 and PSII fluorescence yield were measured simultaneously during illumination at 77°K. It was clearly shown that the kinetics of the light-induced fluorescence increase followed those of cyt b-559 oxidation, not of C-550 reduction. This suggests that the fluorescence yield is controlled by the dark reduction of $P680^+$, not the oxidation of the primary electron acceptor.

Therefore at low temperatures the Duysens and Sweers hypothesis breaks down because the oxidising side of PSII (perhaps $P680^+$) has an influence on the chlorophyll fluorescence yield.

1.4.4. Prompt chlorophyll fluorescence from PSII at short times ($\leq 100\mu s$)

The reactions of PSII at short times have only begun to be studied recently, but they have yielded some very interesting results on the functioning of the PSII reaction centre.

Duysens et al.(1972) examined the chlorophyll fluorescence yield of Chlorella during a very intense 16μs flash. It was found that at low flash intensity the fluorescence yield increased the reached a maximum of about $2.5f_o$. However, at high flash intensity the fluorescence yield reached a maximum of about 3μs after the start of the flash and then declined to a low value ($> f_o$) at about 10μs, and increased again thereafter. DCMU had no effect on these results. Duysens et al. suggested that these phenomena could be accounted for by three reactions in PSII: (i) the initial increase in chlorophyll fluorescence yield is due to the primary photoreduction of Q, (ii) the decrease of fluorescence

yield at high light intensities is caused by the light-induced transformation of a non-quenching substance or state (S) into a quenching state (T) and (iii) a subsequent dark reaction T $\rightarrow$ S takes place which relieves the quenching in a few μs. The T $\rightarrow$ S reaction was accelerated in the presence of oxygen, so it was suggested that T might be a triplet state, possible of a chlorophyll-carotenoid complex. Zankel (1973) found similar results, and gave a similar interpretation, and this model gives an explanation for the 3μs fluorescence transient observed by Mauzerall (1972) following a 10ns laser flash. An apparently different phenomenon was reported by Delosme (1972), who studied the chlorophyll fluorescence yield of Chlorella during a saturating 2μs flash as a function of flash number, and found the yield to oscillate with period 4. The maxima of fluorescence occurred on flashes 1,5,9.... and the minima on flashes 3,7,.... Since Q was fully reduced by each flash this indicated that fluorescence yield was controlled by another factor. The fluorescence oscillations were modified by preillumination or treatment with hydroxylamine, both of which modify the oxygen flash yield sequence. The similarity between the fluorescence and oxygen oscillations (although they are in the opposite sense) led Delosme (1972) to propose that the control over fluorescence yield was exercised by the oxidising side of PSII. Similar results were obtained by Zankel (1973), although from the different effects of methylamine on the fluorescence and oxygen oscillations he doubted that the oxidising side alone controls the fluorescence oscillations.

The influence of the oxidising side of PSII on chlorophyll fluorescence yield has also been studied by den Haan et al. (1974) through the effect of hydroxylamine on the fluorescence

yield at short times. Hydroxylamine does not affect triplet quenching in a saturating flash, but in a just saturating flash hydroxylamine (at 10^{-3} - 10^{-2}M) changes the biphasic fluorescence rise to a monophasic rise consisting of the slower of the two phases ($t_{\frac{1}{2}} \sim 25\mu s$)only. At this concentration of hydroxylamine oxygen evolution was inhibited. These results were interpreted as follows: in an untreated sample the increase in fluorescence is light limited, but in the presence of hydroxylamine the increase in fluorescence follows a dark reaction of half-time $25\mu s$, that is Q^- is formed but there is also a quencher formed whose dissipation has a half-time of $25\mu s$. den Haan et al. postulate that this quencher is $P680^+$ (following Okayama and Butler,1972), and that in untreated chloroplasts $P680^+$ is quickly reduced by the physiological donor to PSII. However in the presence of hydroxylamine this physiological donor is cut off and another, slower, one acts with a half-time of $25\mu s$.

Duysens et al. (1975) have to some extent linked the observations of Delosme (1972) and den Hann et al. (1974) by showing that when triplet quenching has been accounted for the fluorescence minimum found on the third flash of a sequence is due to a quencher which disappears with a half-time of $\sim 20\mu s$.

These investigations were taken further by Duysens et al. (1975) who looked at the changes in delayed chlorophyll fluorescence from PSII at short times. Delayed fluorescence (see section 1.5) arises from a back-reaction between the oxidised primary electron donor and the reduced primary electron acceptor of PSII. Duysens et al. (1975) analysed the decay of delayed fluorescence in the time range $0.6\mu s \leq t \leq 50\mu s$ after the nth laser flash of a series. The decay was found to consist of three first-order components of half-lives

0.8 μs, ~20 μs and 200 μs. The extent of the ~20 μs component oscillated as a function of flash number, but in the opposite sense to the oscillation of prompt chlorophyll fluorescence. Since Q was always maximally reduced in these experiments, an increase in delayed fluorescence indicates an increase in the concentration of the oxidised precursor of delayed fluorescence, possibly $P680^+$ or Y^+. Therefore these measurements also indicate that either $P680^+$ or Y^+ may be a quencher of fluorescence. The general picture that emerges from these results is that at short times after excitation the Q-dependence of fluorescence yield is modified by two factors (i) at high actinic light intensities the transient quenching by triplet states (decay half-time ~3 μs) and (ii) $P680^+$, or a closely related electron donor, is a fluorescence quencher, whose effect is more noticeable when electron donation to $P680^+$ is impaired.

The data of Mauzerall (1972), however, only partly fit this model. Working with Chlorella he reported that the chlorophyll fluorescence yield from dark-adapted cells following a 10ns laser flash increased from the f_o level with a half-time of 25ns, then slightly declined, then increased with a half-time of ~3 μs to a maximum at 10 μs from where the fluorescence yield decreased. After subsequent flashes of a sequence, only the 3 μs rise was seen. The 3 μs phase reported by Mauzerall (1972) is most probably the decay of triplet quenching, but the 25ns rise finds no simple explanation. It is 40 times faster than the fastest reported decay of $P680^+$ (Glaser et al., 1975), and so cannot be attributed to a decay of quenching by this species. It may reflect the photoreduction of Q: a similar kinetic has been reported for the onset of the 518nm absorbance change (see section 1.7) which Witt (1971) asscciates with primary charge separation. If this is the

case, then it must be asked why $P680^*$ has a lifetime an order of magnitude longer than that of the bulk chlorophyll pigments, and the possibility of the involvement of triplet states cannot be ruled out.

1.4.5. Prompt chlorophyll fluorescence from PSII at long time. Changes in the chlorophyll fluorescence yield of algae over long time scales (~minutes) have been known for some time (see Govindjee and Papageorgiou, 1971 for review). More recently a number of phenomena have been observed, both in broken chloroplasts and in chloroplasts retaining their outer membranes, which involve slow changes in fluorescence yield which are not connected with changes in the redox state of Q. Research in this field is progressing rapidly, but it is not clear to what extent the various phenomena reflect similar processes occurring under different conditions. The following section will outline the characteristics of the phenomena observed in broken chloroplasts, intact chloroplasts and algae, indicating possible mechanistic similarities.

1.4.5.1 Broken chloroplasts. An effect of cations on PSII chlorophyll fluorescence from broken chloroplasts was first reported by Homann (1969). Using a preparation which was blocked with DCMU (eliminating redox changes in Q) Homann showed that the addition of a few mM of Mg^{++} or a somewhat higher concentration of a monovalent cation (~100mM) caused the room temperature fluorescence to increase by up to 100%. The increase was almost all carried by the variable fluorescence, the f_o level varying only a little, if at all. This phenomenon was also studied by Murata (1969b). As well as room temperature fluorescence Murata measured the fluorescence at liquid nitrogen tamperature where PSI fluorescence becomes more significant. He found that the increase in PSII fluorescence at 695nm due to adding

cations to the reaction medium was accompanied by a decrease in PSI fluorescence (at 735nm). Moreover Mg^{++} accelerated the rate of electron transport through a PSII-mediated partial reaction ($H_2O \rightarrow$ DCIP), and inhibited the rate of a PSI-mediated partial reaction ($DCIPH_2 \rightarrow NADP^+$ in the presence of DCMU). Murata suggest that these observations indicate that Mg^{++} controls the distribution of excitation energy between the two photosystems. This control may be exercised in either of two ways (i) through the initial distribution of photons to the two light-harvesting pigment arrays or (ii) through diverting some of the excitation energy from the bulk pigments of PSII into the bulk pigment of PSI ('spillover'). By calculating the area over the PSII fluorescence induction curve and normalising the result with the variable fluorescence yield (Appendix I) Murata was able to show that the spillover hypothesis accounted for most of the change in fluorescence yield, with only a small part attributable to a change in the initial distribution of photons. The action of cations was thus interpreted as inhibiting the spillover of excitation energy from PSII to PSI (Murata, 1969b).

The spillover hypothesis has received considerable support. One piece of evidence was furnished by Briantais et al. (1973) who measured the lifetime of PSII chlorophyll fluorescence as a function of fluorescence yield in the presence and absence of Mg^{++}. The rationale behind the experiment is as follows: The fluorescence lifetime τ_f is given by

$$\tau_f = \frac{1}{k_f + k_h + k_{tI} + k_p[Q]} \qquad 19$$

which, together with the expression for fluorescence yield (eq.18) yields

$$\tau_f = \phi_f / k_f$$

Now,

$$\phi_f = I_f / \alpha_{II} I$$

where I is the incident light intensity, I_f is the intensity of fluorescence and α_{II} is the fraction of incident photons directed to PSII. Therefore

$$I_f = k_f \tau_f \alpha_{II} I \qquad 20$$

that is, I_f is proportional to τ_f as long as α_{II} is constant. Briantais et al. (1973) found that the relationship between I_f and τ_f was essentially the same in the presence or absence of Mg^{++}, and hence concluded that the spillover hypothesis was valid in their case.

Butler and Kitajima (1975b) also broadly support the spillover hypothesis. They carefully studied the changes in PSII and PSI chlorophyll fluorescence at low temperatures ($77^{\circ}K$) due to the addition of Mg^{++}. Any increases in PSI fluorescence must be due to spillover or a change in α_{II} since PSI fluorescence is not a function of the concentration of open PSI traps (Vredenberg and Slooten, 1967). Butler and Kitajima formulated a model for the fluorescence changes they might expect to see and concluded that spillover was modified (in the case of open PSII traps the quantum yield of energy transfer from PSII to PSI varied from 0.065 ($+Mg^{++}$) to 0.12 ($-Mg^{++}$); in the case of closed PSII traps from 0.23 ($+Mg^{++}$) to 0.28 ($-Mg^{++}$))and in addition there was a small change in the initial distribution of quanta to the two photosystems (α_{II} in eq.20) from 0.73 in the presence of Mg^{++} to 0.68 in its absence.

Some authors have not singled out the k_{tI} rate constant to account for these changes in fluorescence yield, but ascribe the changes to modification of the sum of the rate constants ($k_{tI} + k_h$) (Malkin and Siderer, 1974). Li (1975), however, thinks all the yield changes are due to changes in k_h.

Models for a molecular mechanism for a change in spillover have centred on structural changes which vary the distance between the light-harvesting pigments of PSII and PSI, decreasing this distance for an increase in spillover. The changes in distance need not be large. In the model of Butler and Kitajima (1975b) the changes in quantum yield of transfer from PSII to PSI are at most 50% (open traps case), and taking the Forster very weak coupling case to apply for the energy transfer process the intermolecular distance must decrease by a factor of $(2)^{1/6}$ which is roughly 12%. Clearly this need not involve gross structural changes. Alternatively the change in spillover could be effected by a change in the mutual orientation of the PSI and PSII bulk chlorophyll molecules. A detailed model of this hypothesis has been presented by Seely (1973a,b) in which an 18% change in spillover was made possible by changing the orientation of just six molecules in each photosynthetic unit of 340 chlorophyll molecules. Again gross structural changes are not necessary.

In view of the above considerations it is perhaps not surprising that changes in light scattering, which are related to gross changes in chloroplast structure like swelling, do not appear to correlate well with changes in fluorescence (Krause, 1973; Vandermeulen and Govindjee, 1974; Jennings and Forti, 1974; Telfer, Nicolson and Barber 1976), and objections to the spillover model on these grounds may not be valid (Jennings and Forti, 1974; see also Barber, 1976).

Other conformational changes have been observed by Murakami and Packer (1970a,b). These workers carefully studied changes in the thickness of the thylakoid membrane in chloroplasts that may be induced by lowering the pH of the suspending medium. The changes in membrane thickness are of the order of 25% and although there

has been no correlation between these conformational changes and variation in spillover it is not difficult to imagine that, since the photosynthetic pigments are imbedded in the thylakoid membrane, such changes could alter the distance between the PSI and PSII bulk pigments (Barber, 1976).

However, a more serious objection to the spillover model described above has been raised by Sun and Sauer (1971,1972) who have postulated that Mg^{++} does not inhibit spillover from PSII to PSI, but promotes energy transfer from PSI to PSII.

An opposite cation-induced change in chlorophyll fluorescence has been reported by Gross and Hess (1973). Broken chloroplasts were carefully washed and suspended in a medium containing only sucrose and ~0.1mM Tris base; after DCMU had been added to the chloroplasts the subsequent addition of 1 - 10mM of a monovalent cation caused the fluorescence yield to decrease. The fluorescence was restored to its original level by adding higher concentrations of monovalent cations or a few mM of a divalent cation. The divalent cation-induced fluorescence increase was not observed in the absence of low concentrations of monovalent cations. The low temperature fluorescence spectra showed an increase in PSI fluorescence at the expense of PSII fluorescence on addition of low concentrations of a monovalent cation and the reverse situation on addition of a divalent cation, suggesting that divalent cations and monovalent cations at low concentrations exert antagonistic effects on spillover. A similar molecular mechanism as that for the divalent cation-induced fluorescence changes is envisaged for this phenomenon. These two cation effects have been investigated in the experiments reported in this thesis (section 3.2.3), and the monovalent cation-induced fluorescence decrease has been used in experiments on delayed

fluorescence (section 3.1.1.3)

An apparently unrelated phenomenon of chlorophyll fluorescence quenching was reported by Murata and Sugahara (1969). They discovered that in DCMU-blocked chloroplasts the cofactor of cyclic electron flow, PMS, caused a decrease in the PSII fluorescence yield. This decrease was relieved by uncouplers of photophosphorylation like CCCP. By measuring the low temperature fluorescence emission spectrum it was shown that the PMS-induced quenching was exhibited equally by both PSII and PSI, and it was therefore assumed that the cation effects on fluorescence and this PMS-induced quenching were distinct processes.

Wraight and Crofts (1970) found essentially the same behaviour with another cyclic electron flow cofactor, DAD. The quenching was sensitive to the uncouplers nigericin and NH_4Cl, but not to valinomycin in the presence of potassium, suggesting that the quenching arises as a result of the pH gradient across the thylakoid membranes produced by proton uptake coupled to cyclic electron flow. The mechanism of high-energy state quenching is not clear, but Mills and Barber (1975) have suggested that it is connected with the cation-induced changes in fluorescence yield. They have proposed the following model: the fluorescence of chloroplasts will be a maximum when the interior of the thylakoid compartment contains a high level of Mg^{++}, principally bound to fixed negative charges on the inside of the membrane. On illumination of DCMU-blocked chloroplasts in the presence of a cofactor of cyclic electron flow electrogenic proton pumping is converted into a pH gradient across the thylakoid membrane by extrusion of Mg^{++} as a counter ion (Barber et., 1974).

Forcing Mg^{++} off the binding sites inside the thylakoid membranes in this way causes the fluorescence to decrease. Uncoupling by ionophores which dissipate the pH gradient allows the reverse process to proceed and the fluorescence to rise. This model, however, does not explain the difference in the spectral characteristics of the cation-induced and high-energy induced fluorescence changes observed by Murata and Sugahara (1969). An explanation may rest in the observation that PMS can act as an effective direct quencher of excitation in the bulk pigments (Papageorgiou, 1975; Mills and Barber, unpublished). The quenching could retain its sensitivity to the high-energy state as observed by Murata and Sugahara (1969) by a mechanism proposed by Papageorgiou (1975) in which the high-energy state causes a conformational change which makes the pigment bed more accessible to the quencher. Alternatively the pH gradient generated across the thylakoid membrane results in the accumulation of the cofactor of cyclic electron flow inside the thylakoid compartment, which leads to increased fluorescence quenching. This effect would be relieved by compounds which dissipate Δ pH (Mills and Barber,1975).

1.4.5.2 Intact chloroplasts. Chloroplasts which are isolated so as to retain their outer membrane are called intact chloroplasts. All the enzymes in the stroma are retained and the chloroplasts are able to fix CO_2 without the addition of any cofactors. The chlorophyll fluorescence characteristics of these chloroplasts have recently been studied, and they show slow fluorescence changes that are somewhat similar to those see in algae; in particular they resemble the MT phase (see section 1.4.5.3) These changes were observed by Krause (1973) and independently by Barber and Telfer (1974). On initial illumination of a dark

adapted suspension of intact chloroplasts the fluorescence yield is high; it is slowly quenched in the light ($t_{\frac{1}{2}} \simeq 1 - 3$min), and the high fluorescing state may be restored by either (i) a dark period (ii) adding an uncoupler of photophosphorylation like nigericin in the presence of K^+ of (iii) adding an inhibitor of electron transport, e.g. DCMU (Barber and Telfer,1974). That these changes are not related to changes in the redox state of Q is shown by measuring the fluorescence quenching in the presence of OAA as an electron acceptor. OAA does not require ATP for its reduction, so electron flow can occur in the presence of uncouplers. The fluorescence quenching is relieved by the uncoupler nigericin, under which circumstances electron flow to OAA is increased, suggesting that the fluorescence yield changes are not related to Q since if they were, a decrease in fluorescence yield would be expected (Barber and Telfer, 1974).

Similar changes in broken chloroplasts can be shown if they are 'reconstituted' by dark incubation for a few minutes with a few mM of a divalent cation or ~100mM of a monovalent cation (Barber and Telfer, 1974) These observations suggest that both cations and the high-energy state are involved in these fluorescence changes.

It has been proposed that these chlorophyll fluorescence changes in intact chloroplasts are controlled by light-induced redistribution of cations, particularly Mg^{++}, between the thylakoid and stromal spaces, with the chloroplast outer envelope acting as an effective permeability barrier to cations (Barber et el., 1975). This then opens the question of the relationship between light-induced quenching in intact chloroplasts and the change in spill-over induced by the addition of Mg^{++} to broken chloroplasts.

The identity of these two phenomena is an attractive unifying hypothesis, but the crucial experiments of measuring fluorescence lifetimes and low temperature fluorescence emission spectra in the high-and low-fluorescing states of intact chloroplasts have yet to be performed. The light-induced quenching in intact chloroplasts is investigated in experiments described in section 3.2.

1.4.5.3. Algae. Slow changes in the PSII chlorophyll fluorescence from algae (the Kautsky effect) are complex (see Govindjee and Papageorgiou, 1971, and Papageorgiou, 1975 for reviews). These changes can be split into three phases (a) O-P (b) P-S and (c) S-M-T (fig.4) where the nomenclature of Lavorel (1959) and Munday and Govindjee (1969) has been used. The O-P phase is connected with the closing of PSII traps, the dip D being associated with the pool of intermediates in the electron transport chain between PSII and PSI (cf section 1.4.1.1). The next phase P-S is susceptible to changes in the oxygen tension of the suspending medium, being largely eliminated by partial anaerobiosis (see Papageorgiou, 1975). The P-S phase is also eliminated by DCMU. In the S-M-T phase the fluorescence rises from the S level to a maximum M and then declines to a terminal level T, This phase has to be supported by electron transport, although in DCMU-blocked algae it is probably cyclic electron transport that comes into play.

These chlorophyll fluorescence changes in algae can be correlated to some extent with light-induced adaption within the organism which controls the distribution of electronic excitation energy between the two photosystems. In particular the M-T phase has been associated with an increase in spillover between PSII and PSI (Bonaventura and Myers,1969; Murata, 1969a). The significance

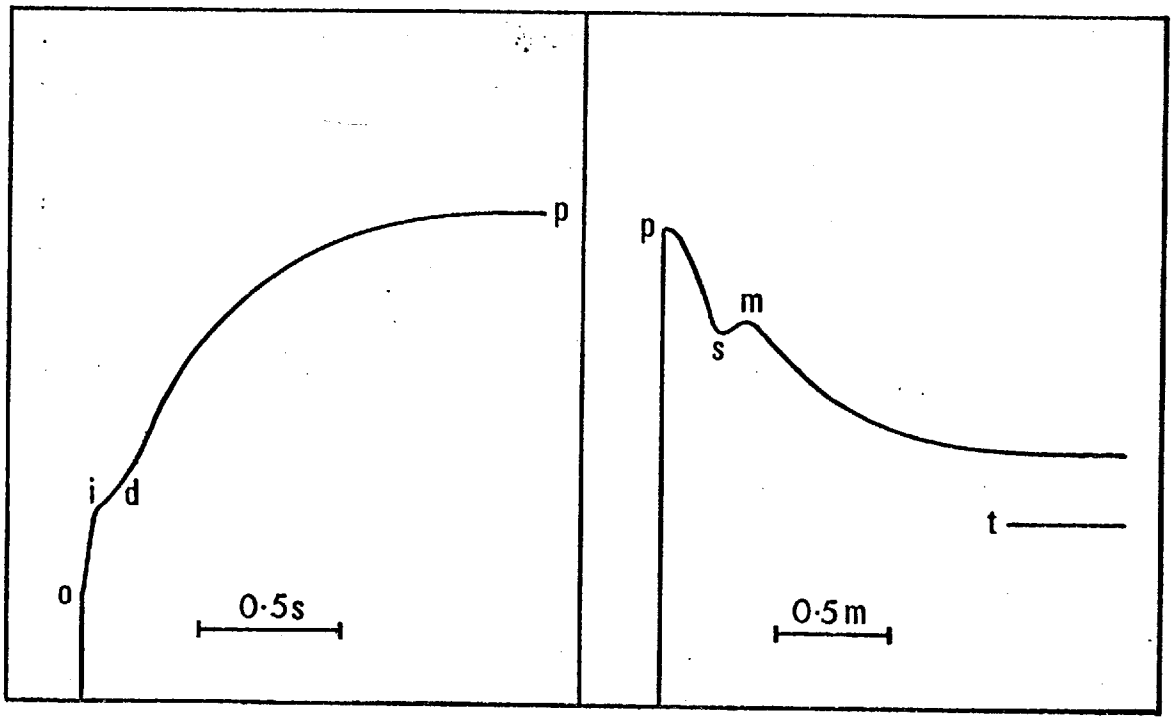


Figure 4.

Short and long term chlorophyll fluorescence changes seen on illuminating a dark-adapted spinach leaf. The final t level was recorded after 5min of illumination. Dark-adapted algae show very similar fluorescence changes.

of the P-S phase is not clear; Papageorgiou (1975) has suggested that it reflects an increase in spillover as well as an increased accessibility of the pigment bed to external quenchers (like oxygen) due to the presence of the high-energy state. This would account for the sensitivity to oxygen of the P-S phase. To what extent the mechanism of these quenching processes in algae is connected with the cation-induced and high-energy state induced changes in fluorescence yield in broken chloroplasts is not yet clear. There is some experimental evidence for mechanistic similarities (Bennoun,1974); moreover the linking of the phenomena in broken chloroplasts and algae by the observations in intact chloroplasts is a hint that the same mechanism may underlie all three experimental systems.

1.5 Light emission from PSII: delayed chlorophyll fluorescence

Delayed chlorophyll fluorescence is the second type of light emission from photosynthetic organisms. It was discovered in 1951 by Strehler and Arnold, in an experiment which was designed to measure dark ATP formation by chloroplasts after a period of illumination by monitoring the light emission from firefly extract. In a control experiment without the extract, light emission was still seen. Strehler and Arnold estimated that this very weak light emission that lasted for long times after the end of illumination (some tens of seconds) accounted for about 10^{-6} of the light absorbed by the system. They suggested that since the emission spectrum for the delayed emission was similar to that of prompt chlorophyll fluorescence it might reflect a small degree of reversibility of the photosynthetic reactions. The emission spectrum for delayed fluorescence (also called delayed light emission or luminescence) was carefully measured by Arnold and Davidson (1954) who showed it to be very close to that of prompt fluorescence, suggesting that the same pigments were involved.

Arthur and Strehler (1957) found that delayed chlorophyll fluorescence was a very general phenomenon, arising to a greater or lesser extent from all of the wide range of photosynthetic organisms they studied. Since they could not isolate a chemical precursor of delayed fluorescence they postulated that the moieties of the back-reaction must be closely linked to early products of photosynthesis.

It has now been well establised that delayed fluorescence arises from PSII of oxygen-evolving organisms. This result comes from experiments on the antagonistic effects of PSI and PSII light

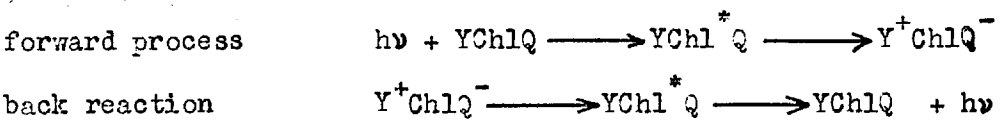
(Goedheer, 1962); from mutants blocked in PSII (Bertsch et al., 1967; Haug et al., 1972) and from studies with subchloroplast particles (Lurie et al., 1972; Vernon et al., 1972). It was suggested that since delayed and prompt chlorophyll fluorescence have such similar properties, some of the prompt fluorescence might be a fast component of delayed fluorescence (Arnold and Davidson,1963). This idea was experimentally investigated by Haug et al.(1972) using an instrument with a very rapidly closing electro-optical shutter to enable the detection of delayed fluorescence at a very short interval (darktime) after turning off the actinic light. The prompt and delayed fluorescence were shown to have very distinct kinetics in the submicrosecond range of darktime, the trailing edge of the prompt fluorescence intersecting the delayed fluorescence curve nearly at right-angles. Thus delayed fluorescence has been kinetically distinguished as a process seperate from prompt fluorescence.

Delayed fluorescence is generally measured with two types of instrument. The first type is a 'single-shot' apparatus in which the actinic light is turned off and the measuring device turned on in a darktime (t_d) that is generally mechanically limited. Delayed fluorescence can be measured from t_d onwards without limit. The second type of apparatus is repetitive. The most common form, the Becquerel phosphoroscope (see Clayton,1969) consists of two discs (on either side of the sample) which rotate on a common axis. By arranging slits in both discs with a suitable phase lag, one disc chops the actinic light and the other disc allows the measuring device to view the sample after the actinic light has been extinguished. The delayed fluorsecence may be viewed either as a series of individual decays or, after suitable

smoothing of the output signal, as the envelope of the decays. The dark time in a mechanical phosphoroscope is typically 1ms; an envelope from such an instrument is often referred to as '1ms delayed fluorescence'.

1.5.1 The back reaction in PSII

Lavorel (1968) has proposed that the back reaction in PSII that leads to delayed fluorescence is that between the reduced primary electron acceptor Q^- and the oxidised primary electron donor Y^+, in the reverse of the primary photochemical process of PSII:



21

Experimental evidence for this scheme was provided by Bennoun (1970). He found that the reoxidation of Q^- in the dark in the presence of DCMU was sensitive to low concentrations of hydroxylamine, a compound which inhibits on the oxygen-evolving side of PSII. In the presence of DCMU, hydroxylamine slowed and decreased the extent of the reoxidation of Q^-, as well as inhibiting delayed fluorescence. This result suggests that in DCMU-blocked systems Q^- can only be reoxidised by a back reaction with the oxygen-evolving side of PSII, and that such a back reaction leads to the production of delayed fluorescence. A.Joliot (1974) has also obtained evidence for a back reaction between Q^- and charges stored in the oxygen evolving apparatus.

A second back reaction in PSII at liquid nitrogen temperature has been widely reported recently (see Butler,1973 for review; also Murata et al., 1973; Knaff and Malkin, 1974; Mathis and Vermiglio, 1974; Malkin and Bearden,1975). This back reaction is between $P680^+$ and Q^-, and has a half-time of 5ms. It appears

to compete fairly efficiently with the light-induced forward reaction under these low temperature conditions. It has not yet been established whether this low temperature back reaction leads to delayed fluorescence. In addition Babcock and Sauer (1975) have reported that in Tris-washed chloroplasts the elimination of epr Signal II_f (see section 1.3.3) in the presence of DCMU takes about 10 flashes, suggesting a back reaction between the Signal II_f precursor, F, and Q^-.

More direct evidence that the back reaction leading to delayed fluorescence involves the electron donors to PSII was provided by Barbieri et al. (1970) who discovered that the delayed fluorescence measured 240ms after each of a series of flashes oscillated as a function of flash number with period 4. The delayed fluorescence sequence lagged 1 flash behind the oxygen flash sequence, which it otherwise resembled closely. This implies that the S_3 state of oxygen evolution is involved in the delayed fluorescence back reaction. In addition Zankel (1971) analysed the decay of delayed fluorescence at shorter times and found three first-order components of which the slower two (half-times 35 and 200μs) oscillated as a function of flash number in exact synchrony with the oxygen oscillations. Zankel resolved the discrepancy between his measurements and those of Barbieri et al. (1970) by showing that a 'phase inversion' occurred at a darktime of about 5ms (so that the higher intensity component at short times becomes the lower intensity component at long times; cf section 1.5.3.3). Lavorel (1975a) has offered the alternative suggestion that in the range $t_d < 100\mu$s the S_4 state may act as an efficient precursor for delayed fluorescence.

The precursors of delayed fluorescence are thus Q^- and Y^+, the

latter being dependent on the S states of the oxygen-evolving system. The intensity of delayed fluorescence may therefore be written as

$$L \propto Y^{+}ChlQ^{-} \qquad 22$$

where a first-order back reaction is assumed (Lavorel, 1975a).

What is the product of the back reaction ? Eq.21 suggests that it is the first excited singlet state of chl, but this is by no means established. Nor is the mechanism free from conceptual problems. When the reaction centre undergoes the delayed fluorescence back reaction it goes from the state of being a closed trap to being an open trap. If trapping is perfectly efficient and a singlet state is produced then delayed fluorescence should not be observed. Two hypotheses have been put forward to account for the fact that it is observed (a) exciton diffusion is very rapid and communication is possible with a large number of photosynthetic units (this is equivalent to saying that trapping is inefficient, section 1.2.7); (b) the back reaction does not produce a singlet state — a triplet state has been postulated (Lavorel, 1969). However, the existence of triplet states in delayed fluorescence has yet to find rigorous experimental verification (Stacey et al., 1971).

An alternative to these possibilities that has been discussed by Lavorel (1975b) is that the back reaction is quite efficient but that open traps retrap released singlet states fairly efficiently resulting in a low level of delayed fluorescence overall.

1.5.2 The influence of prompt chlorophyll fluorescence yield on delayed fluorescence

The emission spectra of prompt and delayed chlorophyll fluorescence

are very similar: therefore, although the two processes are fundamentally different in other respects, the final states of delayed and prompt fluorescence probably involve the same electronic state of the same pigments. If this is the case, then the intensity of delayed fluorescence should be to some extent controlled by the prompt fluorescence yield. By analogy with the expression for prompt fluorescence yield ϕ,

$$F = \phi I \qquad 23$$

where F is the intensity of fluorescence and I is the intensity of incident light, Lavorel (1968) wrote

$$L = \phi' J \qquad 24$$

where L is the intensity of delayed fluorescence, J is the rate of delivery of chlorophyll singlet states to the pigment bed via the delayed fluorescence back reaction and ϕ' is the fluorescence yield of the chlorophyll molecules through which the delayed fluorescence exciton migrates before de-excitation. A problem that still has to be resolved is the relationship between ϕ and ϕ'. The two choices that have been made for ϕ' in the literature are (i) the total fluorescence yield and (ii) the variable fluorescence yield. Lavorel (1975a) has introduced the notation of J_T and J_V for the two values of J that may be determined from eq.24 using these alternatives.

The form of ϕ' will depend to a large extent on the model adopted for the photosynthetic unit (see section 1.6). Models of the photosynthetic unit fall between two extremes (i) the separate unit model in which the photosynthetic unit consists of its light-harvesting pigments and reaction centre in a structurally discrete entity such that excitation energy in one unit cannot migrate

to another unit and (ii) the uniform pigment matrix model in which there are no morphological photosynthetic units, but just a homogeneous pigment bed with one trap per 300 chl molecules and over which excitation energy may diffuse freely. These two models will predict different yield factors for delayed fluorescence.

Assuming that the back reaction produces a first excited singlet state, then in the separate model ϕ' will be equal to the fluorescence yield for an open trap ($\phi(0)$). The magnitude of this yield will depend on the efficiency of trapping. In the uniform pigment matrix model ϕ' will be equal to the variable fluorescence yield ($\phi' = \phi_v$) provided the excitation can diffuse widely. If it cannot diffuse widely then ϕ' will be a function of the fluorescence yields of the photosynthetic units that it passes through. Lavorel (1975a) has shown that the delayed fluorescence yield factor ϕ' is then given by

$$\phi' = \sum_i \alpha(i) \sum_{d_i} \phi(i-1, d_{i-1}) \qquad 25$$

where ϕ is the fluorescence yield of a group of i photosynthetic units over which the exciton diffuses and the group of units has a shape parameter d_i. The factor $\alpha(i)$ is a term in the distribution giving the number of groups containing i units. The dependence of delayed fluorescence on prompt fluorescence should thus be twofold - through the fluorescence yield as an emission factor and through the concentration of Q^- present to act as a precursor of delayed fluorescence. An experimental study of the dependence of delayed fluorescence on prompt fluorescence yield was made by Wraight (1972). The time courses of delayed and prompt fluorescence were examined at various intensities of exciting light using uncoupled chloroplasts. It

was found that at sufficiently low light intensity the dependence of delayed fluorescence on variable prompt fluorescence was roughly linear. At higher light intensities this was no longer the case. Wraight interpreted these results as indicating that as long as the concentration of delayed fluorescence precursor on the oxygen-evolving side of PSII was constant, then delayed fluorescence was proportional to ϕ_v, but that when the concentration of both precursors was changing, this simple relationship broke down. Wraight (1972) concluded that if Q^- is a substrate for delayed fluorescence, then there was no dependence of delayed fluorescence on prompt fluorescence yield, and that therefore there was very little exciton transfer between photosystem II and photosynthetic units. In this thesis a different approach has been used to reach a similar conclusion: the monovalent cation-induced fluorescence decrease (section 1.4.5.1) was used to change the fluorescence yield factor in eq.24 independent of all other factors (section 3.1.1.3).

1.5.3. The effect of the high-energy state of phosphorylation on delayed fluorescence.

Delayed fluorescence is sensitive to the high-energy state of phosphorylation. Mayne (1967) showed that uncouplers of photophosphorylation decrease the intensity of delayed fluorescence without changing the fluorescence yield (see also Clayton,1969). The mechanism of the effect of the high-energy state is connected with the orientation of the PSII reaction centre in the thylakoid membrane, and was elucidated by experiments on the stimulation of delayed fluorescence by various ionic treatments.

1.5.3.1. Salt pulse experiments. It was shown by Miles and Jagendorf (1969), and independently by Barber and Kraan (1970)

that the sudden addition of salts to a preilluminated suspension of chloroplasts caused the delayed fluorescence to transiently increase. This increase can be seen 10s after the end of the preillumination by using a rapid mixing apparatus (Barber and Kraan, 1970) or at one millisecond after the end of the preillumination by using a phosphoroscope (Barber,1972). In either case the stimulation of delayed fluorescence is influenced by appropriate ionophorous anitbiotics. Barber and Kraan (1970) suggested that the addition of salts (e.g.KCl) to the suspending medium caused the creation of a diffusion potential across the thylakoid membranes because these membranes are more permeable to K^+ than Cl^-. This diffusion potential led to increased delayed fluorescence because it was able in some way to decrease the activation energy for the back reaction in PSII leading to delayed fluorescence, since it has been shown that the fluorescence yield is not increased by salt pulses (Kraan et al., 1970). If the diffusion potential is $\Delta\psi$ then normally

$$J \propto \exp -(E/kT) \qquad 26$$

where E is the activation energy for the back reaction. When a salt pulse is given, the equation modified to

$$J \propto \exp -\{(E - \Delta\psi)/kT\} \qquad 27$$

and leads to enhanced delayed fluorescence (Crofts, Wraight and Fleischmann,1971). The stimulation of the emission is transient, and declines in a few seconds due to the slower inward diffusion of the anion.

This phenomenon has been thoroughly studied by Barber and Varley (1972) and Barber (1972), and the general model has received support from the work of Arnold and Azzi (1971) who showed that delayed

fluorescence in the time range of $t_d \sim 30 - 40s$ could be stimulated if an electric field of $126 Vcm^{-1}$ were imposed across the sample. A quantitative calculation (Arnold and Azzi, 1971) showed that the data could be explained by the above model of the electric field lowering the activation energy for the back reaction.

It has been shown that in the thylakoid membrane K^+ is more permeable than Cl^- (Barber and Varley, 1972). This implies that the diffusion potential is positive inside the thylakoid membrane. The observation that this potential stimulates delayed fluorescence suggests that the reaction centre is oriented in the membrane with the oxygen-evolving side, Y^+, on the inside.

1.5.3.2. Acid-base transitions. Mayne and Clayton (1966) discovered that taking a suspension of chloroplasts from an acid medium to a basic medium induced a burst of delayed fluorescence. This delayed fluorescence was abolished when uncouplers were added to the chloroplasts. In contrast to the formation of ATP by an acid-base transition (Jagendorf and Uribe,1966) it was found that the acid-base light emission required the chloroplasts to be preilluminated (Mayne,1968). In addition, Mayne (1968) calculated that a pH gradient of the magnitude used in these experiments (4 - 4.5 pH units) imposed across the thylakoid membrane can provide an energy of $\sim$ 0.25eV while the energy of the light emitted was $\sim$ 1.75eV. This suggests that the energy in the pH transition does not provide the energy for the light emission but merely triggers delayed fluorescence in an analagous way to the salt pulse model described above.

The pH transition method of stimulating delayed fluorescence

has been investigated by Kraan et al. (1970) and by Kraan (1971). It was shown that the efflux of protons from the inside of the thylakoid vesicles caused a membrane potential of such a polarity that it tended to cause a 'negative salt pulse' and decrease the stimulation caused by the pH transition. The addition of KCl and valinomycin enabled this negative diffusion potential to be eliminated. The model proposed for the acid-base stimulation of delayed fluorescence included the concept that both of the precursors of delayed fluorescence Q and Y are in a pH dependent equilibrium (Kraan, 1971)

$$Q^- + H^+ \rightleftharpoons QH \qquad\qquad YH^+ \rightleftharpoons Y + H^+ \qquad 28$$

Delayed fluorescence results from the back reaction of Q^- and YH^+, which will be stimulated by a membrane potential or by a difference in proton concentration which are both positive on the inside with respect to the outside of the thylakoid membrane.

1.5.3.3 The influence of the high-energy state on the induction kinetics of ms delayed fluorescence. Wraight and Crofts (1971) studied the influence of light induced ΔpH and $\Delta\psi$ on the induction of ms delayed fluorescence from chloroplasts by using a phosphoroscope. The ΔpH component of the high energy state could be eliminated by adding nigericin and K^+ to the suspension. Nigericin facilitates a neutral exchange between K^+ and H^+ thus dissipating any pH gradient but leaving the membrane potential unchanged. Similarly the pH gradient may be retained but the membrane potential dissipated by adding valinomycin plus K^+. This antibiotic only allows K^+ to pass across the membrane. Wraight and Crofts (1971) found that the induction of the envelope of lms delayed fluorescence could be differentiated kinetically into two phases, one fast (complete in 0.1s) and one slow

(complete in 0.3s). The slow phase was eliminated by nigericin, suggesting that it was associated with the build-up of the light-induced pH gradient. A similar result was obtained with ammonium chloride. However, the fast phase was eliminated by valinomycin suggesting that it was associated with the membrane potential. Both phases were suppressed by either FCCP or a combination of valinomycin and nigericin.

Evans and Crofts (1973) extended these measurements and showed that the kinetics of the slow phase of the induction of ms delayed fluorescence was closely paralleled by the electrogenic uptake of protons, monitored by the absorbance change of the pH-sensitive dye phenol red.

The equation for the intensity of delayed fluorescence (eq.27) can thus be modified to

$$J \propto \exp - \left\{ (E - \Delta p)/kT \right\} \qquad 29$$

where Δ p is given by the expression for the high-energy state (Mitchell,1966)

$$\Delta p = \Delta\psi + 2.303 \frac{RT}{F} \Delta \text{ pH}$$

The overall expression for the intensity of delayed fluorescence becomes (from eqs 22, 24 and 29) :

$$L = \phi' \left[Y^{+} Chl Q^{-} \right] k' \nu \exp - \left\{ (E - \Delta p)/kT \right\} \qquad 30$$

where k' is a constant containing entropy terms and ν is a frequency factor. A first-order back reaction is assumed.

1.5.3.4. The effect of inhibitors and cofactors on delayed fluorescence. Delayed fluorescence is influenced by two factors (i) the prompt fluorescence yield and (ii) the high-energy state of photophosphorylation. The effect of any compound on delayed

fluorescence must be considered in the light of its effect on both these factors. For example, DCMU blocks the formation of the high-energy state (tending to lower the delayed fluorescence) but at the same time increases the concentration of Q^- and hence increases the fluorescence yield (which will tend to increase delayed fluorescence). Similarly electron acceptors like ferricyanide will have opposite effects on fluorescence and the high-energy state. The net effect of these compounds will depend on the magnitude of the two separate effects.

The overall effect of a compound will also depend on the time of observation after the preilluminating light is turned off (t_d). This is because there is a fixed amount of delayed fluorescence precursor , and if the rate of utilisation of the precursor is increased at short times compared to the control rate, then at long times the rate will be decreased compared to that of the control (see Lavorel,1975a). This can explain the somewhat confusing reports of the effect of certain compounds found in the literature. For example, Clayton (1969) reported that at t_d = 250ms DCMU stimulated delayed fluorescence whereas at t_d = 1ms DCMU inhibits it (see, for example, Wraight and Crofts, 1971). These two observations can be reconciled in the light of the above model.

The equation for the intensity of delayed fluorescence (eq.30) shows that both the delayed fluorescence emission factor ϕ' and the high-energy state Δp can have an important influence on delayed fluorescence. In particular, any measurement of the activation energy E should take into account the value of Δp under the prevailing experimental conditions, and measurements of the time course of delayed fluorescence should be corrected

for time-dependent changes both in Δp and in ϕ'.

These two factors have not always been fully taken into account in published experiments, which has led to some apparently anomolous results. In the experiments on delayed fluorescence reported in this thesis (section 3.1) I have tried to make allowances for or correct for the effects of the high-energy state and the delayed fluorescence emission factor on delayed fluorescence.

1.6 The photosynthetic unit

The classical flashing-light experiments of Emerson and Arnold (1932) on Chlorella established that about 2500 chlorophyll molecules co-operate in the photosynthetic production of one molecule of oxygen. Assuming that eight quanta are required for this process, (Emerson,1958) and that there is a one-quantum process at each reaction centre this implies that the reaction centres in both PSI and PSII are associated with roughly 300 chlorophyll molecules. This association of bulk pigments which collect and channel light energy to the reaction centre and the reaction centre itself is called the photosynthetic unit. The experiments of Emerson and Arnold (1932) do not give any clues to the structure of the photosynthetic unit, and several models have been proposed.

1.6.1. Current models for the photosynthetic unit

There are two extreme models for the photosynthetic unit (Duysens, 1967). One, the separate model, visualises the photosynthetic unit as being a structural entity independent of all other photosynthetic units. An exciton which has been absorbed in a photosynthetic unit in this model has no probability of transfer to another unit. In the second, the uniform pigment matrix model (also known as the 'statistical' or 'lake' model) there are no morphologically distinguishable units. The pigment bed has one reaction centre per 300 bulk chl molecules, but no bulk chl molecules are assigned to any particular trap. Excitons absorbed into the pigment bed are free to migrate over its entire extent, and hence can communicate with more than one trap. The relationship given for fluorescence yield in section 1.4.1 (eq. 18) is based on the second model.

These two models can be experimentally distinguished in PSII in

two ways. Firstly by simultaneous measurement of fluorescence yield and fluorescence lifetime; this approach has been used by Tumerman and Sorokin (1967), Briantais, Merkelo and Govindjee (1972) and by Moya (1974). The two models for the photosynthetic unit will predict different relations between lifetime and yield. For the separate unit model the fluorescence yield will be composed of contributions from units with open and units with closed traps (Tumerman and Sorokin, 1967):

$$\phi = \delta\phi_o + (1-\delta)\phi_c \qquad 31$$

where ϕ_o and ϕ_c are the fluorescence yields for units with open and closed traps respectively, and δ is the fraction of open traps. The relationship between yield and lifetime is thus (Tumerman and Sorokin,1967):

$$\tau = \frac{\phi_o \delta \tau_o + (1-\delta)\phi_c \tau_c}{\delta\phi_o + (1-\delta)\phi_c} \qquad 32$$

where τ_o and τ_c are the lifetimes of units with open and closed traps respectively. This expression does not give a linear relationship between ϕ and τ. On the other hand the uniform pigment matrix model predicts a linear relationship between ϕ and τ (Tumerman and Sorokin, 1967).

The yield and lifetime of PSII fluorescence were measured by phase fluorimetry during the fluorescence induction (OP) in Chlorella, and a linear relationship between ϕ and τ was found both by Tumerman and Sorokin (1967) and by Briantais et al. (1972), suggesting that the uniform pigment matrix model applies to PSII. However, Moya (1974) found that the relationship between ϕ and τ was not strictly linear, and he ascribed the deviation from linearity to restricted energy transfer between PSII photosynthetic

units.

The second method to distinguish between the two models of the PSII photosynthetic unit involves measurement of the fluorescence yield (or steady-state oxygen evolution) as a function of the fraction of closed PSII traps. In the separate units model these parameters should be a linear function of the number of open traps (for oxygen evolution) or of closed traps (for fluorescence yield). Studies of this kind were first performed by Joliot and Joliot (1964), who derived a simple relationship between the variable fluorescence yield ϕ_v, the fraction of closed traps (1 - Q), and the probability of exciton transfer from a unit with a closed trap to another unit p, for a DCMU-blocked system:

$$\phi_v = \frac{(1 - p)(1 - Q)}{1 - p(1 - Q)} \qquad 33$$

(see Appendix II). When p = 0, $\phi_v \propto (1-Q)$. Joliot and Joliot (1964) did not find the linear relationship. Neither did they find that steady-state oxygen evolution was a linear function of the concentration of open traps, implying that the separate units model was not valid in their case. From their measurements they estimated a value for p of 0.55 in Chlorella.

Other evidence has been presented to support the hypothesis of energy transfer between separate PSII units. Wang and Myers (1973) have carefully measured the relationship between the intensity of actinic flashes and the yield of oxygen elicited by them. Using Chlorella they concluded that their data could be accounted for by the model of restricted exciton transfer between PSII units proposed by Joliot and Joliot (1964) or by a model in which each photosynthetic unit contains two traps, but there is no exciton transfer between units. This is another

model of restricted exciton transfer, like the more complex one proposed by Clayton (1967). Also Joliot et al. (1973) have presented data which shows that as reaction centres are blocked in a non-quenching (closed) state the cross -section for capture of incoming photons increases by about three times, whereas the cross-section remains constant if the units are blocked in an open state. Joliot et al. (1973) take this to indicate that energy may be transferred only amongst a small number of connected PSII units, each having one reaction centre.

The model of Joliot and Joliot (1964) is not the only one that has been put forward to take account of the non-linear relationship between fluorescence yield and the state of PSII traps. Morin (1964) suggested that it was due to there being two series photoreactions in PSII: the reaction centre would thus have three possible states as opposed to two in the Duysens and Sweers hypothesis(Duysens and Sweers, 1963). This three-state model has also been suggested by Doschek and Kok (1972), and Lavorel (1972) has made an analysis of models of this type. He was able to show that not only do they predict the sigmoidal fluorescence induction, but also an assymetrical relationship between fluorescence yield and the concentration of closed PSII traps, such as is experimentally observed, but not predicted, by eq. 33. Moreover, the three-state reaction centre model predicts the 'reprise' effect, which is a difference in the shape of the fluorescence induction from a dark-adapted sample, and from one in which Q has been reduced in the light and partially oxidised by a subsequent short dark period (Lavorel and Joliot,1972). In contrast Lavergne and Lavorel (1972) have derived a model based on a simple kinetic theory for the creation and trapping of excitons which, for suitably

chosen parameters, predicts a sigmoidal fluorescence induction. More recently Paillotin (1974) and Butler and Kitajima (1975a) have proposed that the sigmoid fluorescence induction may reflect competition between photochemical excitation energy trapping and other wasteful deactivation processes in a model where excitons are free to migrate over the whole pigment bed (Appendix IV).

In the experiments to be described in section 3.2 the nature of the photosynthetic unit has been studied from analysis of fluorescence induction curves in DCMU-blocked chloroplasts. The results indicate that the degree of exciton transfer between PSII photosynthetic units is variable, and a function of fluorescence yield. The uniform pigment matrix model of the photosynthetic unit provides a basis for the description of the variable photosynthetic unit.

1.7 The absorbance change at 518nm as a probe of membrane potential

The measurement of relatively small light-induced absorbance changes in photosynthetic systems has given a good deal of information about the redox states of the intermediates in the electron transport chain between water and $NADP^+$. The absorbance changes that have been attributed to the oxidation and reduction of P680, P700, cyt b-559, X-320 and cyt f have already been mentioned. However, one of the larger light-induced absorbance changes with a maximum at about 518nm has been assigned to a different mechanism.

Reversible light-induced absorbance changes at 518nm were first observed in Chlorella by Duysens (1954). The difference spectrum showed an absorbance maximum at 515nm and minima at 478 and 420nm. These changes were investigated by Witt and Moraw (1958, a, b) using flash illumination: the changes had a very fast risetime ($< 10^{-5}$s) and a decay time of $\sim$20ms. A relationship between the absorbance change at 518nm (Δ518) in broken chloroplasts in the presence of an exogenous electron acceptor and photophosphorylation was reported by Witt et al. (1967). The inhibition of Δ 518 by both ethanol and desaspidin followed the inhibition of photophosphorylation, not the inhibition of oxygen evolution. Δ 518 might therefore reflect the high-energy state.

1.7.1. The kinetics of Δ518

The onset of Δ518 is rapid: Wolff et al.(1969) have shown that it takes place with a halftime of $\leq$ 20ns. The kinetics of the decay are rather variable. Junge and Witt (1968) have demonstrated that in the time range $0 \leq t \leq 80$ms the decay is biphasic, and can be resolved into two first-order components with halftimes

of ~40 - 200ms and ~10ms. The extent of the slow phase, which has been well correlated with the amount of ATP that is phosphorylated during the flash, varies from preparation to preparation. It has been shown, however, that the slow phase may be changed into the fast phase by suspending the chloroplasts in a hypo- or hypertonic medium, which osmotically modifies the permeability properties of the thylakoid membrane (Junge and Witt,1968).

The halftime of the decay of the slow phase can also be accelerated by the addition of ionophores (e.g. gramicidin in the presence of a monovalent cation) to the suspending medium. The acceleration can be induced by a number of cations; the cation mobility is a factor in determining which is the most effective. The two phases of the Δ 518 decay are thus interpreted as follows: the slow phase reflects the 'intrinsic diffusion' of an unidentified cation across the thylakoid membrane, which is coupled to phosphorylation, whilst the fast phase reflects rapid cation diffusion from damaged thylakoids. The cation was identified as a proton by the accelerating effect of ammonia both on the slow phase of the decay of Δ 518 and on electron transport. Gramicidin in the presence of other cations accelerates the slow phase of Δ 518 but leaves electron transport unchanged (Junge and Witt,1968)

The interpretation of Δ 518 is that it indicates changes of electrical potential across the thylakoid membrane (Junge and Witt,1968). Its very fast rise ($\leq$ 20ns) probably reflects primary charge separation (inhibiting either light reaction reduces the extent of Δ 518 by half) and the decay can reflect four processes (i) an intrinsic permeability of the thylakoid membrane to protons (ii) proton efflux from the inside of the thylakoid vesicle coupled to phosphorylation (iii) leakage of

other ions due to osmotic damage to the thylakoids and (iv) ion permeability facilitated by ionophores.

The relationship between Δ518 and phosphorylation was further investigated by Rumberg and Siggel (1968) who showed that under phosphorylating conditions the halftime of the decay of the slow phase was decreased. Joliot and Delosme (1974) have also studied the time course of Δ518 after a single flash in algae, and found that a mutant strain of Chlamydomonas which is inhibited in the terminal stages of phosphorylation lacks a fast decay phase of Δ 518 which is exhibited in wild-type algae. In addition the steady-state measurements of the absorbance change at 518nm in tightly coupled chloroplasts by Baltcheffsky and Hall (1974) confirm that under phosphorylating conditions the steady-state response is smaller than the control response. However, Neumann et al. (1970) reported that in their experiments with chloroplasts the rate of decay of flash-induced Δ518 was unrelated to ATP formation. Witt (1971) has suggested that this may be due to acceleration of the Δ 518 decay by high concentrations of phosphorylation cofactors (e.g. Mg^{++}, ADP or inorganic phosphate).

1.7.2. The molecular machanism of Δ518

The identification of the pigments involved in Δ518 has been experimentally realised by making artificial layers of the pigments thought to be involved and measuring the change in the absorption spectrum when an electric field is applied across the multi-layer (Schmidt et al., 1972). The best match between the chloroplast and multilayer absorption spectra is given by a multilayer composed of chl a, chl b and carotenoids in the ratio 1.7 : 5.2 : 1; that is, all of the bulk pigments appear to be involved (Schmidt et al., 1972). Strichartz (1971), on the

other hand, has shown that heptane-extraction of carotene and quinones from chloroplasts inhibits the 518nm change, and re-addition of β-carotene restores it.

The <u>in vitro</u> studies of Schmidt et al. (1972) raise another interesting point. The spectral changes induced by the electric field are thought to be due to 'electrochromism', that is, the absorption spectrum of the molecules is slightly shifted by an applied electric field. This response contains two terms one of which is linear in E (the applied electric field) and another that goes as E^2. The relationship (given by Witt,1971) is:

$$\Delta\nu = 1/h(\mu^* - \mu^\circ)E + \tfrac{1}{2}(\alpha^* - \alpha^\circ)E^2 \qquad 34$$

where $\Delta\nu$ is the frequency shift, μ^* and μ° are the dipole moments of the excited and ground states respectively, and α^* and α° are the polarisabilities of the two states. Since the Δ 518 response in chloroplasts is a linear function of applied field (Schliephake, Junge and Witt,1968) then it must be assumed that the pigments in the thylakoid membrane are subject to a permanent electric field of about 2.10^6 Vcm^{-1} and that the light-induced electric field of $\sim 2.10^5$ Vcm^{-1} acts as a small perturbation on the permanent field.

The events leading to the onset of Δ 518 are thought to be as follows (Witt,1971). The PSII and PSI reaction centres are arranged vectorially in the membrane such that primary charge separation leaves a negative charge at the outside of the thylakoid membrane and a positive charge at the inside. This creates an electric field across the membrane which is delocalised by the intervention of other ions whose diffusion through the membrane causes Δ 518 to decay.

This concept has been strengthened by the experiments of Witt and Zickler (1973) who have shown that when spinach chloroplasts suspended in a viscous medium are illuminated asymmetrically by a flash, an electrical potential can be measured by electrodes in the medium. This electrical potential correlates kinetically with $\Delta 518$ measured simultaneously.

Experiments to induce an electric field across the thylakoid membrane by a diffusion potential have been reported by Strichartz and Chance (1972). They added alkali cation salts to the suspending medium in the dark and detected absorbance changes that were sensitive to valinomycin, and suggested that these absorbance changes were due to the establishment of a membrane potential across the thylakoid membranes. However, Gross and Libbey (1972) have observed that the addition of cations to a chloroplast suspending medium in the dark causes volume changes (shrinkage) which superficially resemble the light-induced absorbance changes and thus cast doubt on the validity of Strichartz and Chance's conclusion.

The experiments described in section 3.3 have measured $\Delta 518$ in intact chloroplasts. The results give clues to (a) the magnitude of $\Delta\psi$ in intact chloroplasts, (b) the permeability properties of the thylakoid membranes of broken chloroplasts and (c) the role of the outer chloroplast membrane in controlling primary photosynthetic processes.

CHAPTER 2 : MATERIALS AND METHODS

2.1 Isolation of chloroplasts

2.1.1 Osmotically broken chloroplasts

Chloroplasts lacking their outer membranes were isolated from market spinach or from lettuce (var.Romaine) obtained from a local shop. Using an MSE Atomix blender, about 40g of deveined, sliced leaves were homogenised for 15s in 100 ml of a semi-frozen solution containing 0.33M sucrose, 1mM $MgCl_2$ and 10mM NaH_2PO_4 brought to pH 6.8 with KOH (for chloroplasts isolated from lettuce, 2% ascorbate was added to this grinding medium). The macerate was squeezed initially through two, and then eight layers of muslin. The crude chloroplast suspension was rapidly centrifuged at 0°C for 60s at 3000 x g using an IEC refrigerated centrifuge (Model B20). The resulting pellet was subjected to osmotic shock by resuspending in the above grinding medium diluted tenfold. After 5m of this treatment the broken chloroplasts were centrifuged at 6500 x g for 1.5m. The pellet was resuspended in an ice-cold suspending medium containing 0.33M sucrose, 50mM KCl and 20mM N-Tris (hydroxy-methyl)-methyl-2-aminoethane-sulphonic acid (TES) brought to pH 7.0 with KOH, followed by a 1.5m spin at 6500 x g. The pellet was then resuspended in the same suspending medium and stored at about 0°C in an ice-bath. For experiments an appropriate quantity of stock chloroplasts was diluted in the suspending medium.

2.1.2 Intact chloroplasts

Intact chloroplasts (that is, chloroplasts retaining their outer

membrane) were isolated from either market spinach or from peas essentially by the method of Stokes and Walker (1971). Peas (Pisum sativum, var. Feltham First) were grown in a growth chamber with a 12h light - 12h dark cycle at 20°C. Ten-day-old peas were harvested at the end of at least one hour of the light cycle. Spinach leaves, which were stored in the dark, were illuminated under cold water by white light for 30m, and rinsed in distilled water prior to maceration.

80g of sliced pea shoots or spinach leaves were macerated using a Polytron homogeniser in 200ml of a semi-frozen grinding medium containing 0.33M sorbitol, 50mM Na_2HPO_4, 50mM KH_2PO_4, 5mM $MgCl_2$, 0.1% NaCl and 0.2% sodium isoascorbate brought to pH 6.5 with HCl. The macerate was squeezed through two layers of muslin, and then filtered through an additional eight layers of muslin and a thin layer of cotton wool. The resulting suspension was rapidly centrifuged in an MSE Super Minor bench centrifuge whose rotor was precooled. The rotor was quickly accelerated to 2400 x g, kept at this force for 30s and then braked by hand. The top of the pellet was loose and was gently washed off with a few ml of the final suspending medium. The pellet was then resuspended in either of two media (i) assay medium, containing 0.33M sorbitol, 2mM EDTA, 1mM $MgCl_2$ and 50mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) brought to pH 7.6 with KOH or (ii) low-salt buffer, containing 0.33M sorbitol and 10mM HEPES brought to pH 7.6 with trishydroxymethyl amino methane (Tris) base. The chloroplasts were kept on ice in as concentrated a suspension as possible. An appropriate volume of stock chloroplasts was diluted in either of the above media just before use. Intact chloroplasts were also used to prepare osmotically shocked chloroplasts : this was done

by briefly shaking the chloroplasts in water and then adding an equal volume of double-strength suspending medium.

The percentage of the preparation with intact outer membranes was estimated using the method of Heber and Santarius (1970). The principle of this method is that the outer chloroplast membrane is impermeable to the electron acceptor potassium ferricyanide. The rate of uncoupled oxygen evolution in intact chloroplasts was measured in a Clark-type oxygen electrode in assay medium with the following additions: 10mM D-L glyceraldehyde, 1.5mM potassium ferricyanide and 5mM ammonium chloride. The D-L glyceraldehyde is present to inhibit carbon fixation in the intact chloroplasts and NH_4Cl acts as an uncoupler. The ratio of this rate to that of a suspension of osmotically shocked chloroplasts gives the fraction of broken chloroplasts in the intact preparation : hence the percentage of intact chloroplasts.

The rate of oxygen evolution using carbon dioxide as electron acceptor was also measured. Intact chloroplasts (~ 200µg chl) were suspended in assay medium with the following additions: 10mM $NaHCO_3$, 5mM $Na_4P_2O_7$ and 0.5mM K_2HPO_4, and oxygen evolution was followed using a Clark-type electrode. The rate of oxygen evolution with phosphoglyceric acid (PGA) as electron acceptor was measured by adding 1mM PGA to the medium for the carbon dioxide measurement.

Chlorophyll concentrations were estimated by the method of Arnon (1949).

2.2 Measurement of delayed and prompt chlorophyll fluorescence

2.2.1 Prompt and millisecond delayed fluorescence

The intensity of 1ms delayed fluorescence was continuously measured in a conventional rotating-sector phosphoroscope (a 'Becquerel' phosphoroscope; see Clayton, 1969). The illumination and viewing times were 1.5ms and 0.2ms respectively, with four measurements per cycle. During the illumination period the chloroplasts (at 8 - 10 μg ml^{-1}) were exposed to broad-band blue light transmitted through a filter combination consisting of a Balzer Calflex C heat filter and 4mm Schott BG38 filters giving a light intensity of 14 Wm^{-2} at the cuvette as measured with a YSI-Kettering model 65 radiometer. The delayed fluorescence was detected with an EMI 9558B photomultiplier (S20 response) through a 2mm Schott RG695 cut-off filter. Prompt fluorescence was measured with a second photomultiplier (EMI 9558B) at right-angles to the exciting light beam. The prompt fluorescence was filtered by the following combination: a Balzers B40 692nm interference filter and 4mm Schott RG695 cut-off filter . The output of both photomultipliers was chopped; the signals were passed through smoothing circuits before being recorded on a Rikadenki chart recorder.

For the temperature-jump experiments a sudden increase in the temperature of the suspension of chloroplasts was achieved by rapidly adding 1ml of hot suspending medium from a syringe to 3ml of chloroplast suspension, through a light-tight diaphragm. The temperature of the suspension was monitored with an iron-constantan thermocouple connected to a Honeywell chart recorder. Addition of other compounds to the chloroplast suspension was made by rapidly injecting 100 μl of the appropriate stock

solution through the same diaphragm.

2.2.2 Delayed and prompt chlorophyll fluorescence decays

The decay of delayed and prompt fluorescence was measured in a simple shutter apparatus (fig.5) equipped with a temperature-controlled cuvette holder. The sample was illuminated with broad-band blue light (filter combination 4mm Schott BG18 and 5mm BG38 giving a light intensity of $27 Wm^{-2}$) through a camera shutter. At the end of the preillumination time the shutter closed and the photomultiplier (EMI 9558B, S20 response), placed close behind the cuvette and protected by a 2mm Schott RG695 cut-off filter, was switched on by a high-voltage switch activated by a phototransistor. In order that the time to reach full gain on the photomultiplier be reduced (to ~3ms), the photomultiplier was switched on by the addition of the first two dynodes to the dynode chain. The signal from the photomultiplier was either fed into a storage oscilloscope (Telequipment type D53A equipped with a type K amplifier) for the time range $3ms \leq t_d \leq 800ms$, or a Honeywell chart recorder ($0.3s \leq t_d \leq 3.5s$). To monitor prompt fluorescence changes in the dark induced by the actinic beam a weak continuous blue light was used (filters: 2mm Schott BG38 and 7mm BG18), and the measurements made under the same actinic light conditions as for delayed fluorescence. The prompt fluorescence stimulated by the weak light was measured by an EMI 9558B photomultiplier (S20 response) placed at about right-angles to the weak light beam (fig.5). The intensity of the weak measuring light was adjusted such that the prompt fluorescence signal (measured through 4mm Schott RG665 cut-off filters and a Balzers B40 687nm interference filter) was not distorted by the delayed fluorescence due to

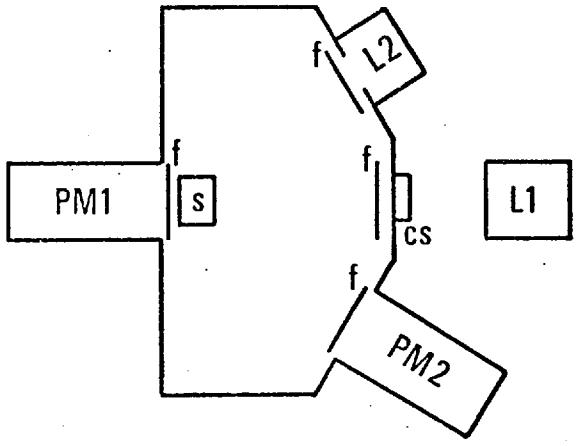


Figure 5.

The shutter apparatus used to measure delayed and prompt chlorophyll fluorescence decays and prompt fluorescence inductions. The actinic light (L1) illuminated the sample (s) through the camera shutter (cs). For delayed fluorescence decays, the photomultiplier (PM1) was switched on as the preillumination ended by means of a phototransistor located just behind the camera shutter. The optical filters (f) filtered both the actinic light and the delayed fluorescence. Prompt fluorescence decays were measured by using a weak measuring light (from lamp L2) to excite fluorescence which was detected by the photomultiplier PM2. The same actinic lighting conditions were used as for the delayed fluorescence decays. Prompt fluorescence inductions were measured by using the actinic light to excite fluorescence, and detecting this fluorescence with PM2.

the actinic beam.

2.2.3 Prompt chlorophyll fluorescence inductions and long-term fluorescence changes.

Prompt fluorescence inductions were measured using (a) the phosphoroscope or (b) the shutter apparatus. Using the phosphoroscope, the rotor was kept stationary, and the photomultiplier at right-angles to the light beam was used to measure fluorescence. The filter combinations were as described above (section 2.2.1) but in this case the intensity of the actinic light was increased to $36 Wm^{-2}$. The actinic light was turned on by rapidly opening a shutter across the light beam, and the signal from the photomultiplier was fed directly into a storage oscilloscope. This apparatus was also used to measure long-term ($\sim$ minutes) fluorescence changes, in which case the photomultiplier output signal was fed into a Rikadenki chart recorder.

The shutter apparatus was used as a fluorimeter using actinic light ($14 Wm^{-2}$, filtered through 4mm Schott BG18 and 2mm BG38) to excite the fluorescence. The fluorescence was measured at approximately 40° to the actinic beam with the same photomultiplier as before (section 2.2.2) but using a Balzers B40 686nm interference filter and 4mm Schott RG695 cut-off filters as a filter combination. For fluorescence inductions the opening of the camera shutter was complete in $\sim$2ms, and the output of the photomultiplier was fed into a storage oscilloscope.

2.3 Measurement of flash-induced absorbance changes

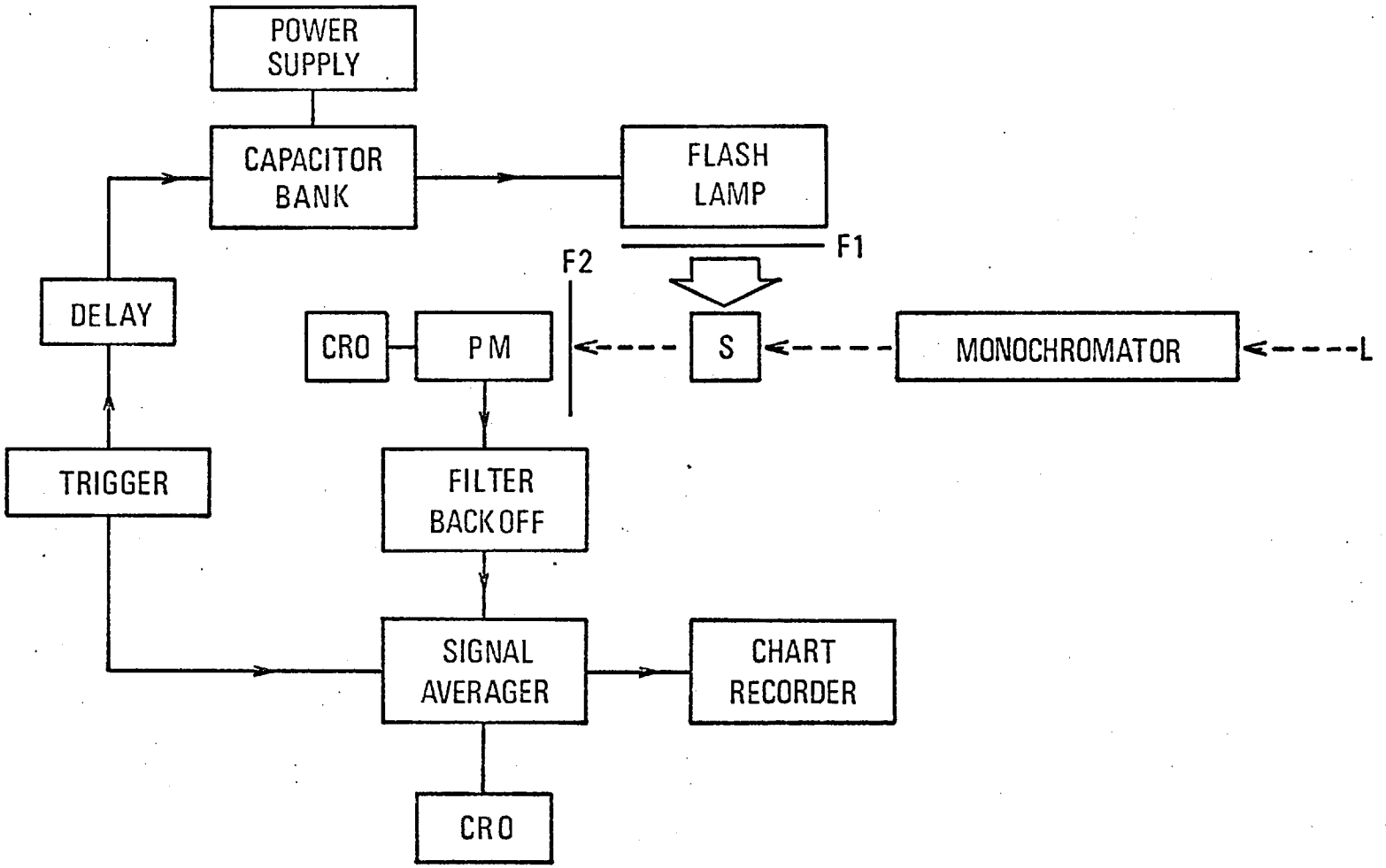
Flash-induced absorbance changes were measured in the apparatus schematically shown in fig.6 (Applied Photophysics, Ltd., London). The weak light beam monitoring the absorbance changes was passed through a monochrometer whose slits were adjusted to give a low light intensity ($\sim 0.13 Wm^{-2}$). The actinic flash was obtained from a xenon flash lamp supplied from a $2\mu F$ capacitor bank charged to 8kV. The duration of the flash was approximately $30\mu s$ (half-width, half-height). The actinic flash was filtered through a 4mm Schott RG645 cut-off filter, and to prevent flash-induced artefacts the photomultiplier (RCA 1P28,S5 response) was screened from the flash and also protected with 2mm Schott BG18 and 2mm BG38 filters.

The output of the photomultiplier was passed through an RC filtering circuit of variable RC time before being fed into an Ortec model 546 digital signal averager to improve the signal to noise ratio. By means of a variable-time delay circuit, the flash was fired a short time after the signal averager was triggered to give a baseline reference on the signal stored in the averager.

The chlorophyll concentration of the sample was $42\mu g\ ml^{-1}$; this optimised the signal size. In order to ensure uniformity of response each sample was calibrated in the following way: the output of the photomultiplier was fed straight into an oscilloscope and the magnitude of the light-dark difference was adjusted to a standard value by varying the high-voltage supply to the photomultiplier. This calibration ensured that the responses of intact and osmotically shocked chloroplasts (which scatter the weak monitoring beam to different extents) could be compared. An absolute calibration was made by comparing the absorbance

Figure 6.

Schematic diagram of the flash spectrophotometer. The weak monitoring light from lamp L was passed through the monochrometer across the sample (S) and was detected by the photomultiplier (PM). The signal was normalised by measuring the light-dark difference on a CRO linked directly to the photomultiplier, and adjusting the photomultiplier high-voltage supply to obtain a standard response. The output of the photomultiplier was filtered with an RC circuit before being fed into the signal averager. The signal averager was triggered a short time before the flash was fired by means of a delay circuit in order to have a baseline reference on the signal recorded in the averager. The output of the averager was viewed on an oscilloscope or recorded on a chart recorder. F1 and F2 are optical filters.


POWER SUPPLY
CAPACITOR BANK
FLASH LAMP
F1
F2
DELAY
CRO
PM
S
MONOCHROMATOR
L
TRIGGER
FILTER BACKOFF
SIGNAL AVERAGER
CHART RECORDER
CRO

change induced by a single flash measured on the oscilloscope with the change induced by inserting a 0.01 o.d. neutral density filter in the measuring beam.

Typically 128 signals were averaged, with the signal from the photomultiplier first being filtered by the RC circuit to such an extent that the risetime of the signal occurred in 1 address (typically 1ms) of the averager. In this way the decay of the signal was not distorted. The accumulated signal was read out onto a Rikadenki chart recorder for further analysis.

2.4 Reagents

All reagents used were of analytical grade. Valinomycin and nigericin were gifts from the Eli Lilley Corporation; gramicidin was obtained from Sigma, and CCCP from Calibiochem.

CHAPTER 3 : RESULTS AND DISCUSSION

3.1 Delayed chlorophyll fluorescence

3.1.1 The activation energy of delayed fluorescence.

Delayed fluorescence is thought to occur as a consequence of the back-reaction between metastable oxidised and reduced components in the reaction centre of PSII (see section 1.5.1). It is important to know the value of the activation energy of this back reaction since this will indicate the fraction of the energy of the absorbed quantum that has been stored by the primary photochemical reaction, and hence give an idea of the efficiency of primary photosynthetic energy conversion. However, as noted in section 1.5.3.3 (eq.30), the high-energy state of phosphorylation acts so as to reduce the activation energy for the back reaction, and hence will distort measurements of this parameter. This effect has not been fully realised in previous measurements which are reviewed in the following section.

3.1.1.1 Previous measurements of the activation energy for delayed fluorescence. There have been several attempts to estimate the activation energy of the back reaction leading to delayed fluorescence. Sweetser, Todd and Hersh (1961) measured the decay of delayed fluorescence from Chlorella in a phosphoroscope and found it to consist of three first-order components of half-life 8, 160, and 1100ms. The activation energy of the first two components was estimated as < 0.11 and < 0.03eV respectively, while the activation energy of the slowest phase was $\simeq 0.44$eV. Similarly, Tollin, Fujimori and Calvin (1958) found that temperature had little effect on the

decay of delayed fluorescence for $t_d \lesssim 10$ms. These observations suggest that the activation energy for the back reaction leading to delayed fluorescence is a function of dark time, since at short times after preillumination it is small, but at longer times it is more significant.

Activation energies for larger values of t_d have been measured by the temperature-jump (T-jump) technique. This method monitors the decay of delayed fluorescence: during the decay the temperature of the suspension is quickly increased by several degrees and the increase in the intensity of delayed fluorescence is measured. This technique was used in the time range of $1s \lesssim t_d \lesssim 10s$ by Jursinic and Govindjee (1972) who found activation energies of 0.46 to 1.12eV for DCMU-blocked Chlorella (see Malkin and Hardt, 1973), but there was a region of initial temperatures where much lower activation energies were found.

The temperature-jump technique was refined by Malkin and Hardt (1973) who were able to take into account the finite time taken to raise the temperature of the suspending medium in their calculations. For $t_d \simeq 2.5s$ they found an activation energy of 0.68 ± 0.08eV in chloroplasts.

Jursinic and Govindjee also analysed the decay of delayed fluorescence from DCMU-blocked Chlorella as a function of temperature. The plotted values of J_T (using total prompt fluorescence yield for ϕ' in eq.24) were linear in $J_T^{-\frac{1}{2}}$ versus time in the time range $1s \lesssim t_d \lesssim 10s$, and in the temperature range $2^oC \lesssim T \lesssim 20^oC$. Above 20^oC deviations from linearity were marked. Nonetheless an Arrhenius plot of the slopes of the $J_T^{-\frac{1}{2}}$ versus time plots was made which exhibited two straight portions (activation energies 0.72eV and 0.85eV, see Malkin and Hardt, 1973)

and a section of almost zero slope. Taken together these observations indicate that for longer darktimes the activation energy for delayed fluorescence is roughly 0.7eV, in contrast to the small activation energy for short darktimes.

There have also been measurements of 'glow curves'. This experimental technique consists of illuminating chloroplasts (or other photosynthetic material), taking the sample to a low temperature, typically 77°K, and then fairly rapidly heating the sample in the dark whilst observing the light emission from it. This method has been used by Arnold and Sherwood (1959), Arnold (1966) and by several Russian laboratories (see, for example, Shuvalov and Litvin,1969). The analyses of all these workers have been criticised by Malkin and Hardt (1973) on the grounds that there are two unknowns, the activation energy and a frequency factor, in one equation. It is thus necessary to assign an arbitrary value to the frequency factor before the activation energy can be estimated. This shortcoming has been overcome in the analysis of Lurie and Bertsch (1974a,b) since they were able to estimate both parameters independently. They found activation energies of 0.8eV, 0.48eV, 0.57eV for the three peaks in the glow curve which they associated with PSII. They were, however, unable to firmly associate any peak with a part of the delayed fluorescence decay.

In summary, it appears that in the $t_d \lesssim 200$ms region of delayed fluorescence the activation energy of the back reaction is small (between 0 and 0.11eV), whereas at longer times an activation energy of the order of 0.7eV has been measured. These two observations might indicate that delayed fluorescence arises from two different reactions in these time domains, or

alternatively that there is a single reaction but at short times the high-energy state might come into play to reduce the activation energy. (eq.30).

In order to try and resolve this question I have measured the activation energy of delayed fluorescence in the $t_d \simeq 1$ms time region by using the T-jump technique. Chloroplasts have been used in which the high-energy state has been completely dissipated by addition of the uncoupler gramicidin, a compound which makes the thylakoid membranes permeable to monovalent cations including protons.

3.1.1.3 Temperature-jump experiments on ms delayed fluorescence from uncoupled chloroplasts. Figure 7A shows the effect of an isothermal addition of suspending medium on prompt and ms delayed fluorescence (for details of the apparatus see section 2.2.1). The chloroplast suspension contained no electron acceptor (so that changes in the concentration of Q^- due to non-cyclic electron flow were minimised) and was preilluminated for 1m such that the maximum level of prompt fluorescence had been reached (that corresponding to the level in the presence of DCMU when all the PSII traps are closed). The shutter across the photomultiplier measuring ms delayed fluorescence was opened as indicated by o and gramicidin was injected into the cuvette. On addition of the uncoupler the intensity of delayed fluorescence dropped to a low value. The addition of isothermal suspending medium lowered both prompt and delayed fluorescence; from a number of experiments of this type it was established that the relative changes in both the recordings were the same. This was taken to indicate that the stimulation of delayed fluorescence by a T-jump should be corrected for this decrease, and that in the

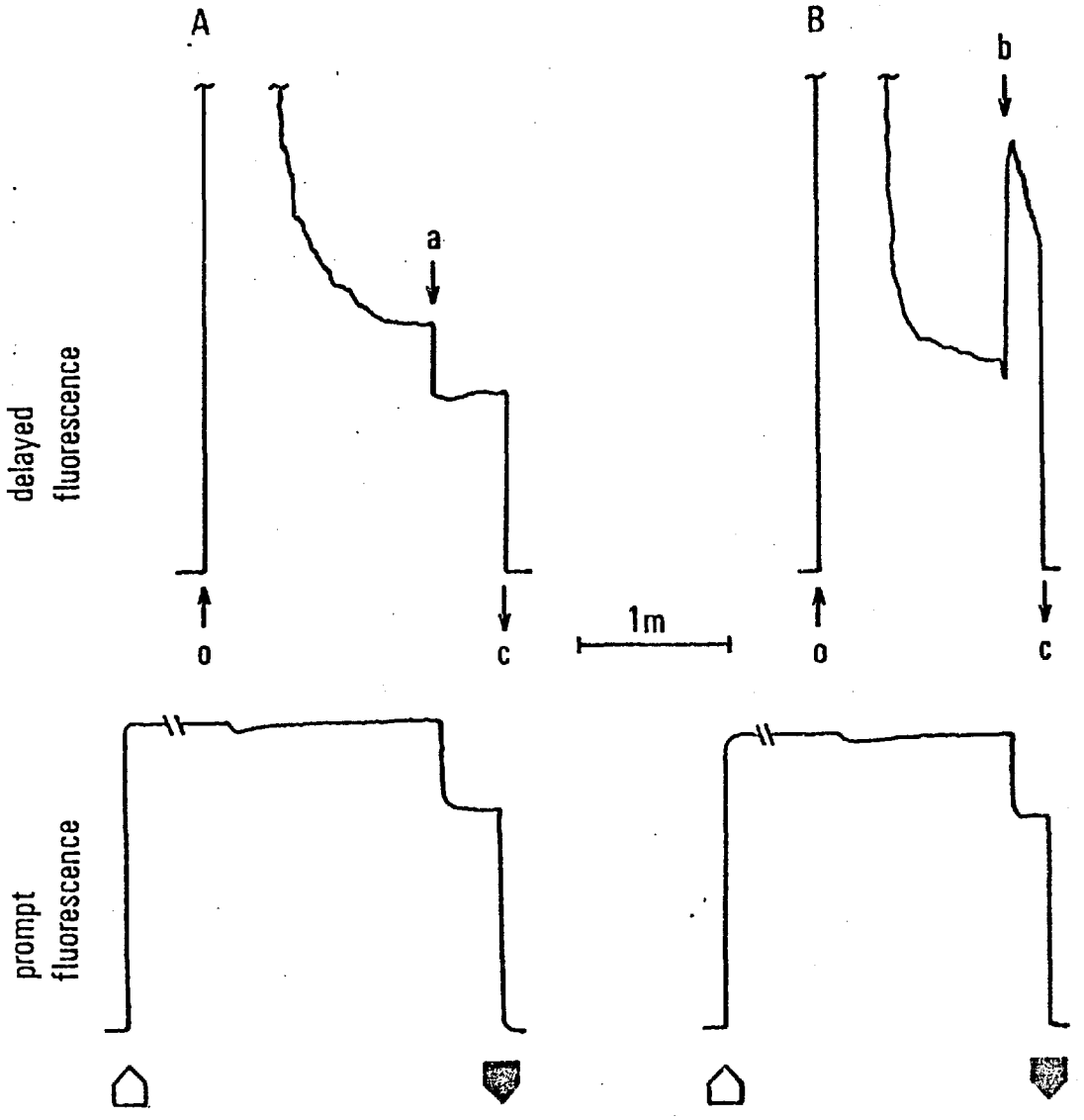


Figure 7.
The effect of an isothermal (A) and a temperature jump (B) on prompt and ms delayed chlorophyll fluorescence from uncoupled broken spinach chloroplasts. The suspension was illuminated for 1 min before the shutter across the delayed fluorescence photomultiplier was opened. The chloroplasts were then uncoupled with 10^{-7}M gramicidin (for the convenience of scale the initial level of delayed fluorescence and injection of gramicidin are not shown) and subjected to a rapid addition of isothermal (a) or hot (b) suspending medium. The temperature change due to the hot addition was from 20° to 34.5°C while the isothermal experiment was carried out at 20°C. Chlorophyll concentration 8 μg ml^{-1}. Open arrows: light on: closed arrows: light off. The opening and closing of the delayed fluorescence photomultiplier shutter are indicated by o and c respectively.

case of the isothermal addition the correction was a purely geometric one due to dilution. Figure 7B shows a typical T-jump carried out in an identical manner to the isothermal experiment except there was a change in temperature from 20° to 34.5°C at point b. Table I gives the results of a series of experiments of this type. The temperature ranges were kept fairly restricted to avoid problems of thermal denaturation and damage of the PSII reaction centres.

The activation energy may be calculated from eq.30 by taking the ratios of two equations at different temperatures, bearing in mind that $\Delta p = 0$ in an uncoupled system, and ignoring for the moment any temperature sensitivity of $[Y^+ ChlQ^-]$, ν or k'

$$E = 2.303k \frac{T_1 T_2}{T_2 - T_1} \log \frac{J_2}{J_1} \qquad 35$$

where J_2 and J_1 are the observed intensities of ms delayed fluorescence corrected for the prompt fluorescence yield changes before and after a temperature change from T_1 to T_2.

By obtaining J_2 from the peak of the T-jump signal and T_2 from a thermocouple, values of E have been calculated using eq.35, and are presented in Table I. The weighted mean of these values of E over the whole temperature range studied is 0.64eV. The analysis may be made slightly more rigorous by using transition-state theory to show that the frequency factor ν of eq.30 is a linear function of temperature, and as a consequence introduces a factor $\log (J_2T_1/J_1T_2)$ instead of $\log (J_2/J_1)$ into eq. 35. Such a correction decreases the above value of E by about 4% to 0.61eV.

The limitation of this approach is that it has assumed that all

TABLE 1

Activation energies from T-jumps

T_1 (°C)	T_2 (°C)	$T_2 - T_1$ (°C)	J_2/J_1	E (eV)
	13.6	9.6 ± 0.4	2.18 ± 0.09	0.56 ± 0.04
	15.5	11.5 ± 0.5	2.77 ± 0.06	0.61 ± 0.03
4	18.3	14.3 ± 0.2	3.41 ± 0.21	0.60 ± 0.03
	25.1	21.1 ± 1.0	4.79 ± 0.20	0.53 ± 0.03
	21.1	6.1 ± 0.3	1.56 ± 0.05	0.53 ± 0.03
	23.9	8.9 ± 0.4	2.46 ± 0.14	0.75 ± 0.03
15	28.9	13.9 ± 0.6	3.47 ± 0.13	0.69 ± 0.04
	29.2	14.2 ± 0.5	4.47 ± 0.09	0.79 ± 0.03
	27.8	6.3 ± 0.6	1.77 ± 0.04	0.69 ± 0.07
	31.7	10.2 ± 0.3	2.43 ± 0.05	0.68 ± 0.03
21.5	33.1	11.6 ± 0.7	2.63 ± 0.06	0.65 ± 0.04
	34.6	13.1 ± 1.0	3.55 ± 0.02	0.76 ± 0.07

Each J_2/J_1 ratio is the mean of six separate experiments, and the standard errors have been computed from the standard errors of the temperature increase (monitored with a thermocouple) and the standard errors of the delayed fluorescence ratios.

other processes are temperature independent. The calculations have to some extent allowed for temperature-induced changes in fluorescence yield and, since there was no net electron flow in the preparations, variations in the fluorescence yield due to changes in the redox state of PSII traps were avoided. However, it has also been assumed that the formation and non-radiative decay of the delayed fluorescence precursor $Y^{+}ChlQ^{-}$ are temperature insensitive, and that its concentration does not change during the T-jump. In a repetitive experiment like the one described here it is difficult to check whether there is any change in the rate of creation or decay of the precursor $Y^{+}ChlQ^{-}$ with temperature. Malkin and Nardt (1973) have however emphasised the necessity for such corrections which they have been able to make at longer darktimes.

3.1.2 The decay kinetics of delayed fluorescence.

Because of the possible changes in the concentration of $Y^{+}ChlQ^{-}$ with temperature, the T-jump method of estimating the activation energy for delayed fluorescence is not wholly satisfactory. A better method is to measure the temperature dependence of the reaction constant of the delayed fluorescence back reaction. This method is independent of the sensitivity of the precursor concentration to temperature, but requires an analysis of the decay kinetics and the formulation of a reaction mechanism before the activation energy can be estimated.

The analysis of the decay kinetics of delayed fluorescence is not straightforward. As shown in eq.30 the decay of delayed fluorescence would not be expected to be a simple function of time for at least two reasons. Firstly there is the delayed fluorescence emission factor ϕ' whose exact form is not known, and whose variation with time cannot accurately be predicted.

Secondly, the effect of the high-energy state of phosphorylation may well vary with time and distort the decay of delayed fluorescence. These two factors in eq.30 probably serve to make the decay more complex than it might be. These complications have not always been fully realised in the kinetic analyses performed so far.

3.1.2.1 Previous kinetic analyses of delayed fluorescence in the time range $t_d \gtrsim 1$ms. In his review of delayed fluorescence Lavorel (1975a) has made a useful distinction between two types of delayed fluorescence which he calls 'leakage' and 'deactivation'. Delayed fluorescence is proportional to the rate of decay of the delayed fluorescence precursor:

$$L = -r\left(\frac{dC}{dt}\right)_L \qquad 36$$

where C is the concentration of delayed fluorescence precursor ($[Y^+ChlQ^-]$ in eq.30) and r is a constant ≤ 1. In general delayed fluorescence will only be one of a number of pathways for the decay of the precursor, i.e.

$$-\frac{dC}{dt} = -\left(\frac{dC}{dt}\right)_L + \sum_K -\left(\frac{dC}{dt}\right)_K \qquad 37$$

where there are K other processes in competition with delayed fluorescence. Two extreme situations can occur: (i) the leakage case, where

$$-\left(\frac{dC}{dt}\right)_L \ll -\frac{dC}{dt} \qquad 38$$

and the decay of delayed fluorescence will be controlled by the decay of the precursor through other pathways, or (ii) the deactivation case, where

$$-\left(\frac{dC}{dt}\right)_L \simeq -\frac{dC}{dt} \qquad 39$$

and the precursor decays predominantly through the delayed fluorescence reaction.

The competing processes in the leakage case are probably stabilisation of the metastable charge transfer complex $ZY^{+}ChlQ^{-}$ that is thought to give rise to delayed fluorescence. From considerations of photosynthetic efficiency, leakage delayed fluorescence should predominate at short times after preillumination (Lavorel,1975a). It should thus be possible to define a critical darktime t_{dcrit} which divides these two extreme processes. The value of t_{dcrit} should be roughly the turnover time of PSII (of the order of 600μs for the reoxidation of Q^{-} when the A pool is fully oxidised, Forbush and Kok, 1968). The deactivation process should dominate not only at long times but also in systems which have their normal photochemical reactions blocked.

From the definition of t_{dcrit}, delayed fluorescence in the time range $t_d \gtrsim 1$ms is of the deactivation type. The decay kinetics of delayed fluorescence in this time range have been measured several times and various kinetic analyses have been applied to the results. However, the results are not consistent, possibly because an allowance has not been made for the variable factors in eq.30, that is ϕ' and Δp.

Analyses of the decay of delayed fluorescence in terms of a series of first-order components can always be achieved, although the physical significance of such a procedure is not always clear. This type of analysis has been performed by Tollin, Fujimori and Calvin (1958), Ruby (1968) and Bonaventura and Kindergan (1971): The general result is to obtain two first-order components in the time range $1\text{ms} \lesssim t_d \lesssim 800\text{ms}$. No attempts

were made to correct for ϕ' or for the effect of the high-energy state.

Lavorel (1975a) has taken data presented by Bennoun (1970) and shown that the deactivation type of delayed fluorescence exists in DCMU-blocked Chlorella. Bennoun found from the dark decay of variable fluorescence (and using the relationship in Appendix I) that the dark decay of Q^- was such that

$$(Q^-)^{-1} = (Q_0^-)^{-1} \quad (1 + kt_d) \qquad 40$$

where k is a constant. If the deactivation type of delayed fluorescence is applicable, then

$$L = \frac{dQ^-}{dt}$$

$$= \frac{-kQ_0^-}{(1 + kt_d)^2}$$

When $t_d = 0$, $L = L_0$, so

$$L^{-\frac{1}{2}} = L_0^{-\frac{1}{2}} \quad (1 + kt_d) \qquad 41$$

a relationship which Lavorel (1975a) found to be followed by Bennoun's data (Bennoun, 1970). Hence this supports the hypothesis that, at least in this system, the deactivation type of delayed fluorescence exists. It should be noted, however, that no change in ϕ' has been taken into account in this calculation.

A similar relationship was found by Jursinic and Govindjee (1972) who measured the decay of delayed fluorescence (corrected with total prompt fluorescence, yielding J_T) in DCMU-blocked Chlorella in the time range $1s \lesssim t_d \lesssim 10s$. They found that $J_T^{-\frac{1}{2}}$ decayed linearly with time, although there was some deviation at temperatures above 20°C.

The other data that have been presented show a different picture. Mayne (1968) and Malkin and Hardt (1972) have found that L^{-1} is linear with time; however Lavorel (1968, 1969) has shown J_T^{-1} to be linear with time, whereas Clayton (1969) has reported that J_V^{-1} (i.e. L corrected using variable fluorescence yield for ϕ') versus time is linear. In none of these experiments were efforts made to suppress the high-energy state. The significance of the two forms $L^{-\frac{1}{2}}$ and L^{-1} (and the same forms for J) will be discussed fully in section 3.1.3.

A different approach was taken by Stacey et al. (1971). They took as a model the hypothesis that delayed fluorescence singlet states are formed by the fusion of two chlorophyll triplet s tates produced by the delayed fluorescence back reaction. The model predicts a fairly complex decay, but it was found to be quite well followed in the time range $1ms \lesssim t_d \lesssim 12ms$, giving support to the triplet-triplet interaction hypothesis (see section 3.1.3)

The results reviewed here show a complex picture for the decay kinetics of delayed fluorescence. Some authors have tried to take account of changes in fluorescence yield, but none has used an uncoupled system. For this reason I have reinvestigated the decay kinetics in the time region $3ms \lesssim t_d \lesssim 3.5s$ using uncoupled chloroplasts to try and measure the decay of the precursors of delayed fluorescence free from the complications of the high-energy state, and have tried to make some correction for the delayed fluorescence emission factor.

3.1.2.2 Experiments on the decay of delayed fluorescence from uncoupled chloroplasts. The decay of delayed and prompt fluorescence from spinach chloroplasts without electron acceptor

and uncoupled with 5.10^{-7}M gramicidin was measured with the shutter apparatus described in section 2.2.2. The decays were measured in the time ranges $3ms \leq t_d \leq 800ms$ and $400ms \leq t_d \leq 3.5s$ using oscillographic recording in the former case and a chart recorder in the latter. The temperature range was $5^{\circ} \leq T \leq 30^{\circ}C$. Figures 8 and 9 show typical successive measurements of the prompt and delayed fluorescence from uncoupled chloroplasts in the two time ranges.

According to eq.24, in order to derive J the measured delayed fluorescence L has to be corrected with the delayed fluorescence emission factor ϕ'. Since the form of ϕ' is not known (see section 1.5.2) this was done with (a) total prompt fluorescence and (b) variable prompt fluorescence (i.e. that part above the f_o level, see fig.3) The decay was then analysed in terms of three decay modes (i) ln J versus time (ii) $J^{-\frac{1}{2}}$ versus time and (iii) J^{-1} versus time. Figures 10 and 11 illustrate typical plots from experimental curves (corrected using total fluorescence emission for ϕ') in the two time ranges. The linearity of each plot was estimated by calculating the correlation coefficient, and Table II summarises the results of this type of experiment carried out over the two overlapping time ranges. As can be seen from Table II, the more satisfactory straight line plots are obtained by correcting L with total prompt fluorescence, and that among the latter the $\ln J_T$ representation is less satisfactory than either the J_T^{-1} or $J_T^{-\frac{1}{2}}$ fits. In general both the J_T^{-1} and $J_T^{-\frac{1}{2}}$ plots appear to give equally good straight lines, with neither of these being consistently better than the other. If the delayed fluorescence is corrected with variable fluorescence then a different picture emerges. The J_T^{-1} plot gives a better

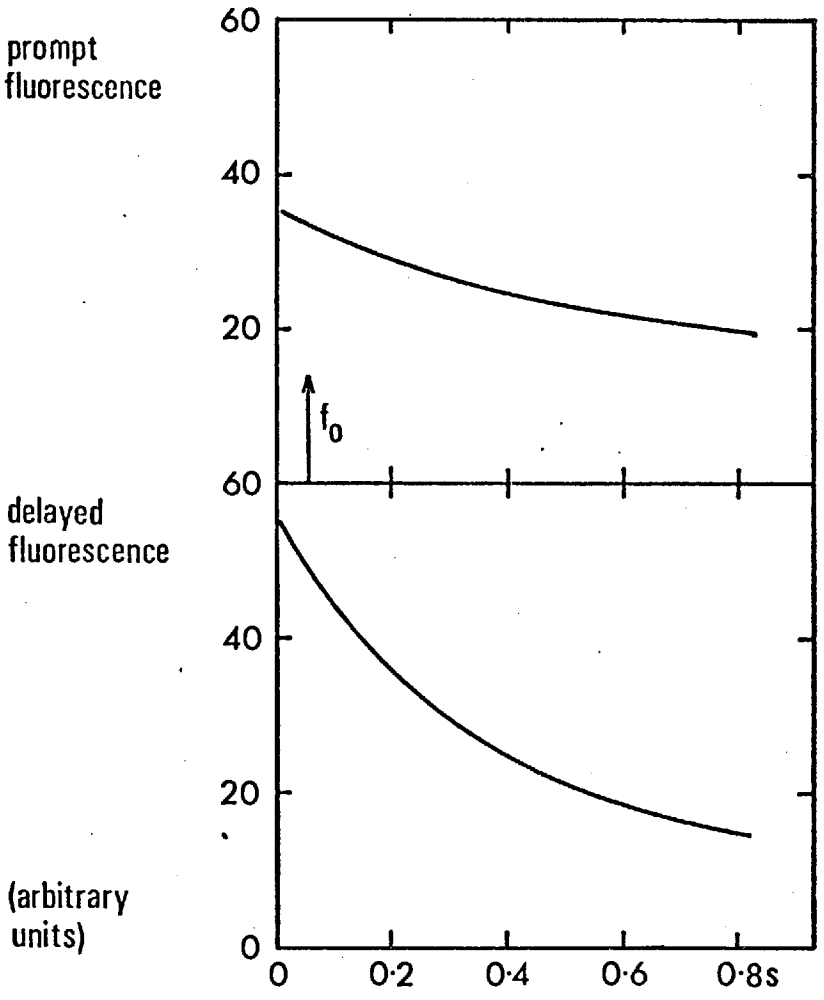


Figure 8.

The decay of prompt and delayed chlorophyll fluorescence (in arbitrary units) from broken spinach chloroplasts uncoupled with 5.10^{-7}M gramicidin in the time range 3ms$\leq t_d \leq$ 800ms, after a 25s period of continuous illumination. The two decays were measured on the same sample with a dark time of 90s between the measurements. The value of f_o was obtained from a separate experiment to measure the fluorescence induction. Traces redrawn from oscilloscope photographs. Temperature 23°C, chlorophyll concentration 12 μg ml^{-1}.

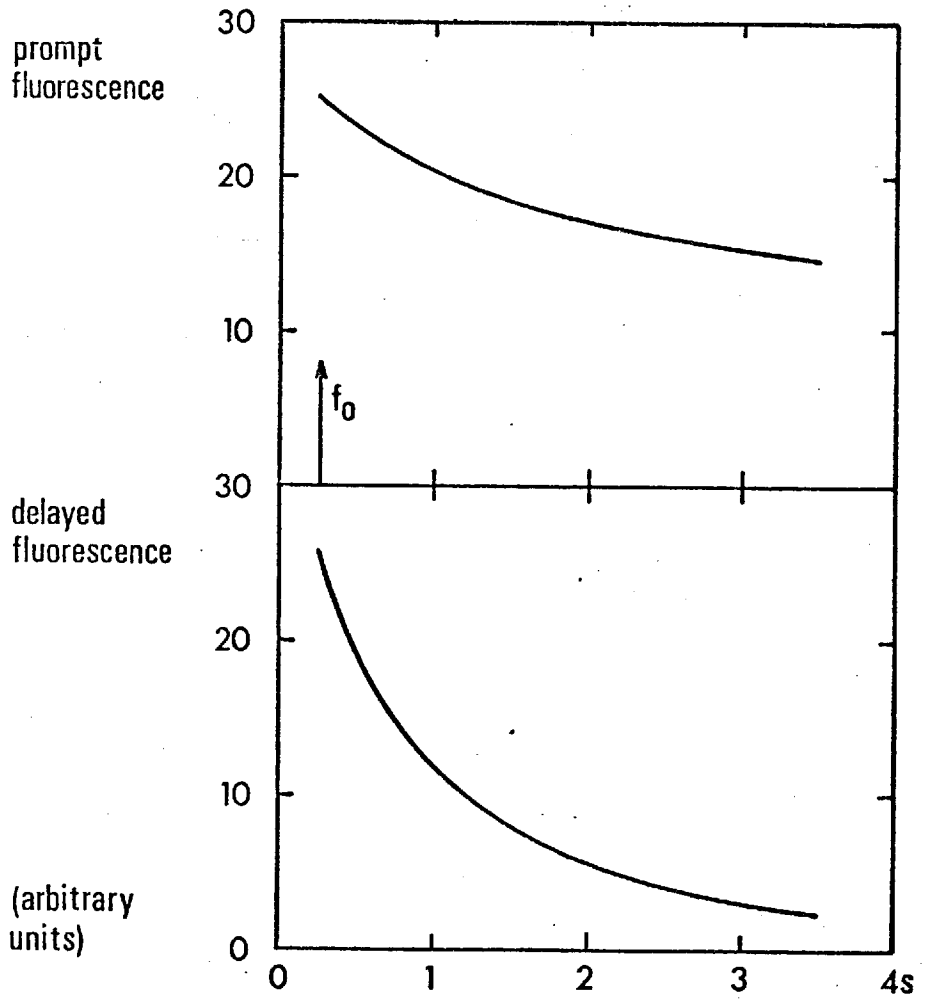


Figure 9.

The decay of prompt and delayed chlorophyll fluorescence (in arbitrary units) from broken spinach chloroplasts uncoupled with 5.10^{-7} M gramicidin in the time range $0.3s \leqslant t_d \leqslant 3.5s$, after a 25s period of continuous illumination. Sequential measurements were made as in fig.8, and the f_o level was estimated similarly. Temperature 18.3° C, chlorophyll concentration 12 µg ml^{-1}. The arbitrary units are different to those in fig. 8.

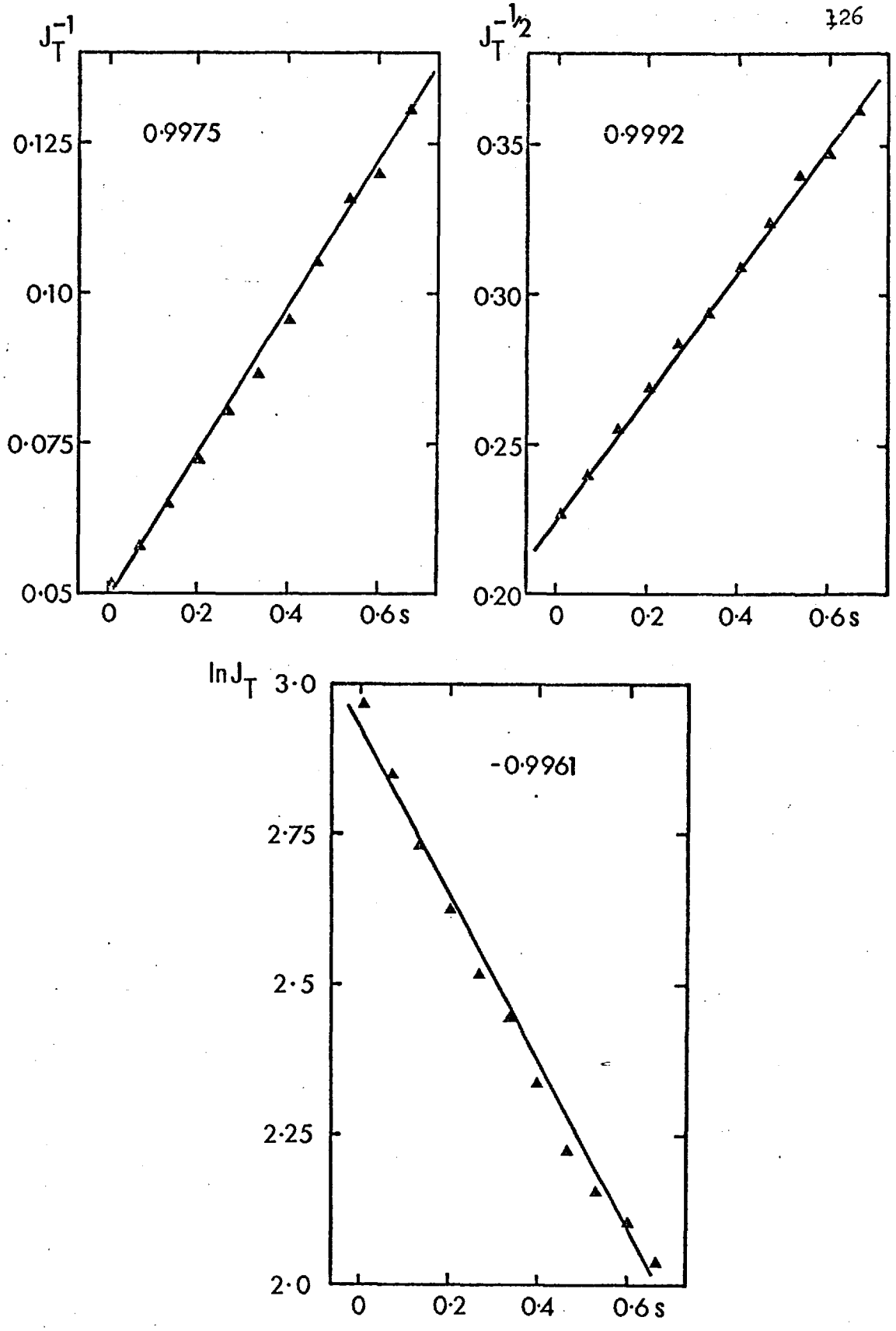


Figure 10

Plots of three representations of the data of fig. 8. The values of J_T were derived from eq. 24 using total fluorescence for ϕ'. The straight-line fits were calculated using a least-squares analysis, and the numbers on the plots denote the correlation coefficient of the particular set of points.

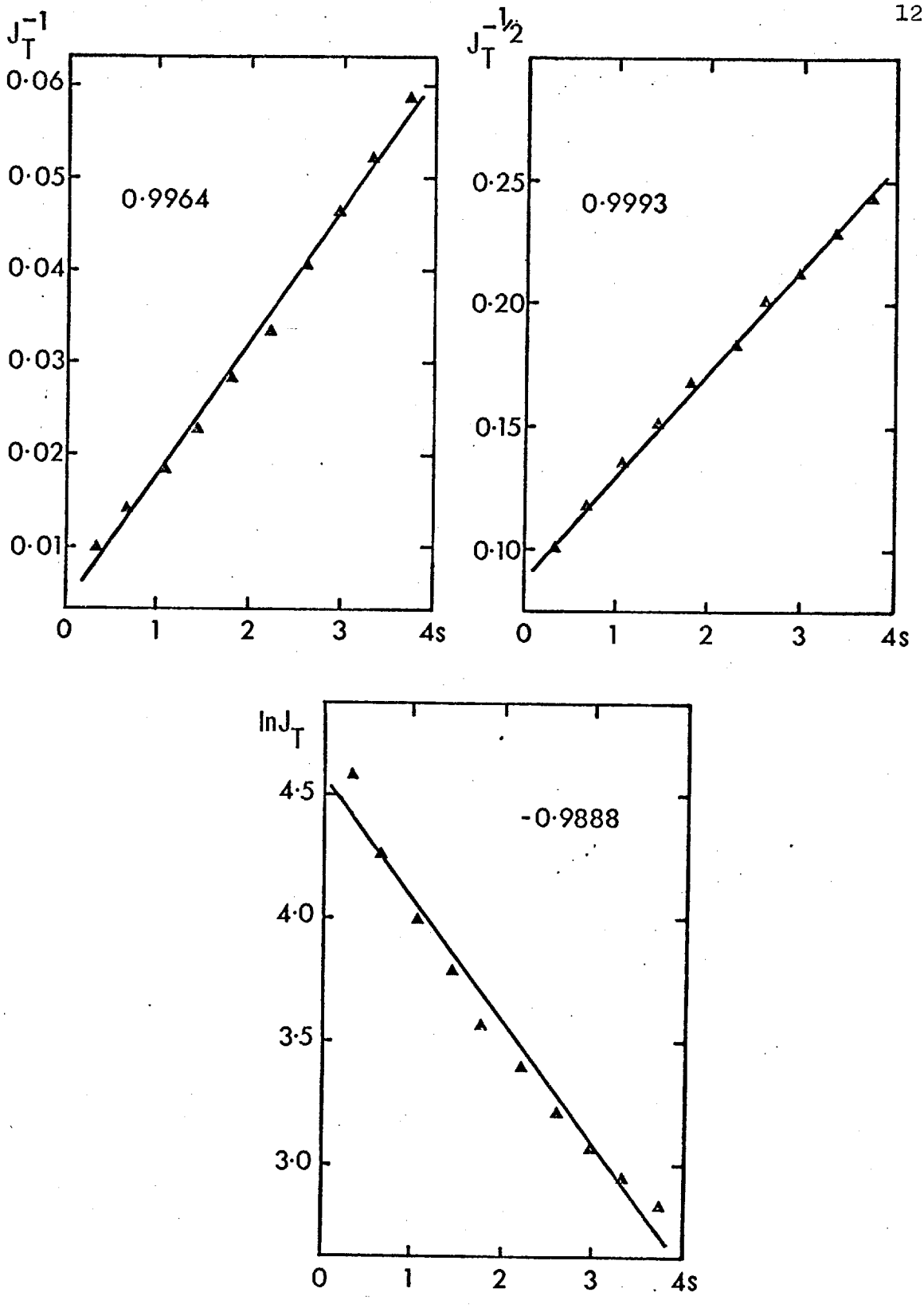


Figure 11

Plots of three representations of the data of fig. 9. The values of J_T were derived from eq. 24 using total fluorescence for ϕ'. The straight-line fits were calculated using a least-squares analysis, and the numbers on the plots denote the correlation coefficient of the particular set of points.

TABLE II

Analysis of delayed chlorophyll fluorescence from uncoupled chloroplasts expressed as correlation coefficients of the stated kinetic laws

Time range	Temperature °C	Total PF Correction			Variable PF Correction		
		ln J	J^{-1}	$J^{-\frac{1}{2}}$	ln J	J^{-1}	$J^{-\frac{1}{2}}$
	29	-0.9893	0.9934	0.9997	-0.9877	0.9983	0.9982
0.3 sec	23	-0.9823	0.9969	0.9967	-0.9779	0.9965	0.9914
-3.5 sec	18.3	-0.9888	0-9964	0.9993	-0.9890	0.9989	0.9974
	12.5	-0.9883	0.9884	0.9985	-0.9856	0.9995	0.9957
	6	-0.9819	0.9971	0.9915	-0.9609	0.9818	0.9722
	29	-0.9932	0.9971	0.9975	-0.9908	0.9961	0.9946
3 msec	23	-0.9961	0.9975	0.9992	-0.9908	0.9961	0.9946
-800 msec	18.3	-0.9906	0.9954	0.9950	-0.9861	0.9916	0.9907
	12.5	-0.9900	0.9958	0.9944	-0.9875	0.9842	0.9913
	6	-0.9872	0.9955	0.9921	-0.9844	0.9926	0.9894

fit for all times and temperatures studied, but the fit is not as good as for the data corrected with total prompt fluorescence.

From these experiments it is clear that the delayed fluorescence emission factor ϕ' is a critical parameter in evaluating the decay kinetics of delayed fluorescence, and that the form of this parameter should be established so that the delayed fluorescence decays may be unequivocally determined.

3.1.2.3 The delayed fluorescence emission factor.

The delayed fluorescence emission factor ϕ' is an important parameter in the analysis of delayed fluorescence : it would be convenient to be able to define it as a simple function of prompt fluorescence, for example of the variable or total prompt fluorescence yield. In order to evaluate the relationship between ϕ' and these macroscopic prompt fluorescence yields it is necessary to perform an experiment in which the prompt fluorescence yield may be changed but other parameters in the expression for L (eq.30), notably the concentration of reduced Q and the high-energy state, remain constant.

Variations in the concentration of Q^- may be suppressed by blocking the system with DCMU, and the high-energy state will remain constant when it is dissipated by the addition of an uncoupler. Fluorescence changes under these circumstances can be induced in several ways of which two are (i) the addition of a few mM of a divalent cation to broken chloroplasts suspended in a low-salt medium (Homann, 1969; Murata, 1969b; see section 1.4.5.1) or (ii) the addition of a few mM of a monovalent cation to chloroplasts suspended in a cation-free medium (Gross and Hess, 1973; see section 1.4.5.1). In practice it was found that the addition of divalent cations is a complicated experiment

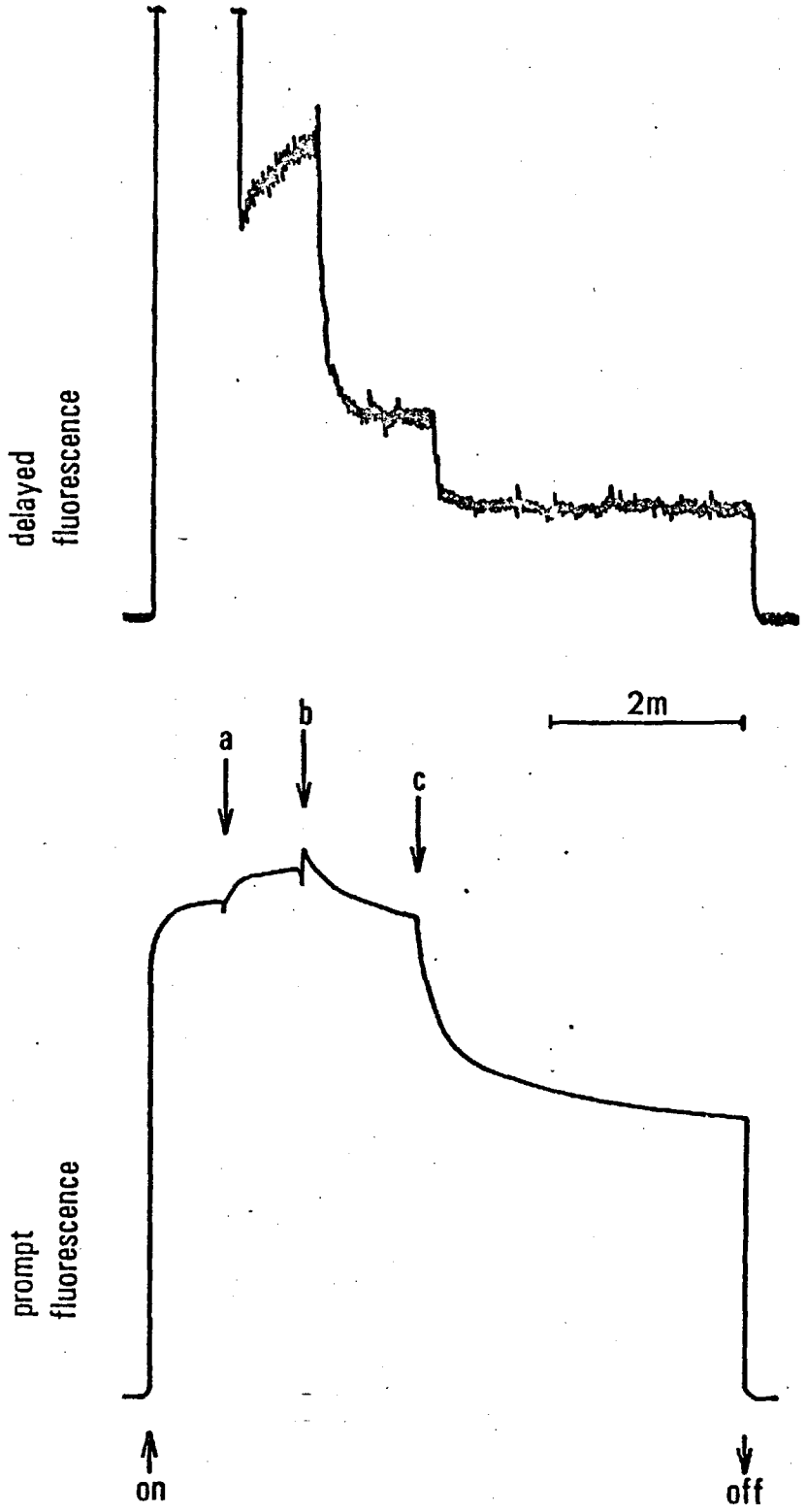
to analyse because even with uncoupled chloroplasts there is a decrease in the ms delayed fluorescence upon addition of a few mM Mg^{++} which may be attributed to a salt-pulse effect as described in section 1.5.3.1. The second method (using the monovalent cation-induced fluorescence decrease) is not troubled by this artefact since the chloroplasts can be uncoupled with an ionophore which makes the thylakoid membranes freely permeable to monovalent cations, and because the concentration of cations involved is low.

Figure 12 shows a typical experiment in which ms delayed fluorescence and prompt fluorescence were continuously measured in a phosphoroscope. The chloroplasts were prepared intact, then osmotically shocked, carefully washed and resuspended in a medium contining only 100mM sorbitol and 0.05mM Tris base to bring the pH to 7.2. The chloroplasts were therefore in an essentially cation-free medium. Upon illumination the chloroplasts were in a 'high fluorescing' state, that is the fluorescence yield had a value comparable to that of chloroplasts suspended in a medium contining ~ 5mM Mg^{++} (section 1.4.5.1). The addition of 10^{-7}M gramicidin increased the prompt fluorescence slightly (the reason for this is not known) and greatly decreased the delayed fluorescence (section 1.5.3). Subsequent addition of 6.10^{-6}M DCMU caused a transient increase in prompt fluorescence and a further decrease in delayed fluorescence to a low level. The addition of 4mM KCl to the chloroplast suspension caused concomitant decreases in prompt and delayed fluorescence.

The correlation between changes in prompt and delayed fluorescence is not good. It can be seen from fig.12 that after the addition of DCMU (but before the addition of KCl) the prompt

Figure 12

Concomitant changes in prompt and ms delayed chlorpphyll fluorescence induced by the addition of a low concentration of monovalent cations to broken chloroplasts suspended in a medium containing 100mM sorbitol and 0.05mM Tris base, and treated with DCMU and gramicidin. Additions (final concentrations) a: 10^{-7}M gramicidin, b: 6.10^{-6}M DCMU, c: 4.10^{-3}M KCl. For the convenience of scale the level of delayed fluorescence before the addition of gramicidin is not shown. Chlorophyll concentration 12 μg ml^{-1}. On and off indicate turning the actinic light on and off.


delayed
fluorescence
2m
a
b
c
prompt
fluorescence
on
off

fluorescence slowly decreases, but the level of ms delayed fluorescence stays roughly constant. More importantly, the kinetics of the decrease of prompt and ms delayed fluorescence induced by the addition of KCl do not match.

This observation seems to imply that since at all times J (eq.24) was constant there is no simple linear relationship between macroscopic prompt fluorescence yield and the delayed fluorescence emission factor ϕ'. However, a more complex relationship might be involved. The decrease in prompt fluorescence can be resolved into two essentially first-order decays of approximate halftimes 4s and 55s. The extent of the fast phase is a function of the concentration of added KCl. From the kinetics of the change it might be supposed that the decrease in delayed fluorescence was correlated with the fast phase of the prompt fluorescence decrease. However, an analysis of the percentage decrease in prompt fluorescence represented by the fast phase compared to the percentage decrease in delayed fluorescence at various concentrations of added KCl shows that this is not strictly so: whilst the relationship is approximately linear it does not extrapolate back through the origin as would be expected. Therefore this experiment indicates that the ms delayed fluorescence emission factor ϕ' is not simply related to the macroscopic prompt fluorescence yield.

3.1.2.4 The decay of Q^- in the dark. Since Q^- is intimately connected with the delayed fluorescence precursor Y^+ChlQ^- a knowledge of the kinetics of its reoxidation in the dark should help in the determination of the reaction mechanism of delayed fluorescence.

The reoxidation of Q^- in the dark was measured in the following

way: a dark-adapted chloroplast suspension was illuminated with continuous light for 25s to fully reduce Q then given a dark period of variable duration, then the fluorescence induction upon reillumination was recorded on an oscilloscope. The value of Q^- as a fraction of the total pool of Q was estimated by the procedure of Murata et al. (1966) and Malkin and Kok (1966) (see Appendix I).

The experiment was performed under two conditions (a) with chloroplasts blocked with DCMU and (b) with chloroplasts uncoupled with gramicidin. The results of the experiment performed with DCMU-blocked chloroplasts is shown in fig.13 where the quantity of Q remaining reduced after the darktime is plotted as a first- or a second-order reaction. Neither of these representations is a very good description of the kinetics over the whole time range, but over short times ($t_d \lesssim 8s$) the second order plot appears slightly better. This result is in agreement with that of Bennoun (1970), although using DCMU-blocked Chlorella he found the reoxidation of Q^- to occur rather faster.

The second experiment was performed on chloroplasts not blocked with DCMU, but uncoupled with gramicidin; these conditions parallel those of the experiments to measure prompt and delayed fluorescence decays (section 3.1.1.2). The results are shown in fig. 14. It can be seen from these plots that over the much shorter time period used in these experiments the linearity of these plots is less good than those of chloroplasts blocked with DCMU. The results do however show that the rate of reoxidation of Q^- is sensitive to temperature.

An important result from these experiments is that the reoxidation

Figure 13.

The kinetics of the reoxidation of Q^- in the dark following 25s of continuous illumination. Osmotically shocked spinach chloroplasts in low salt buffer, in the presence of 13 μM DCMU. Chlorophyll concentration 16 μg ml^{-1}. Temperature 25°C. The value of $[Q^-]$ was estimated from the area over the fluorescence induction curve (Appendix I).

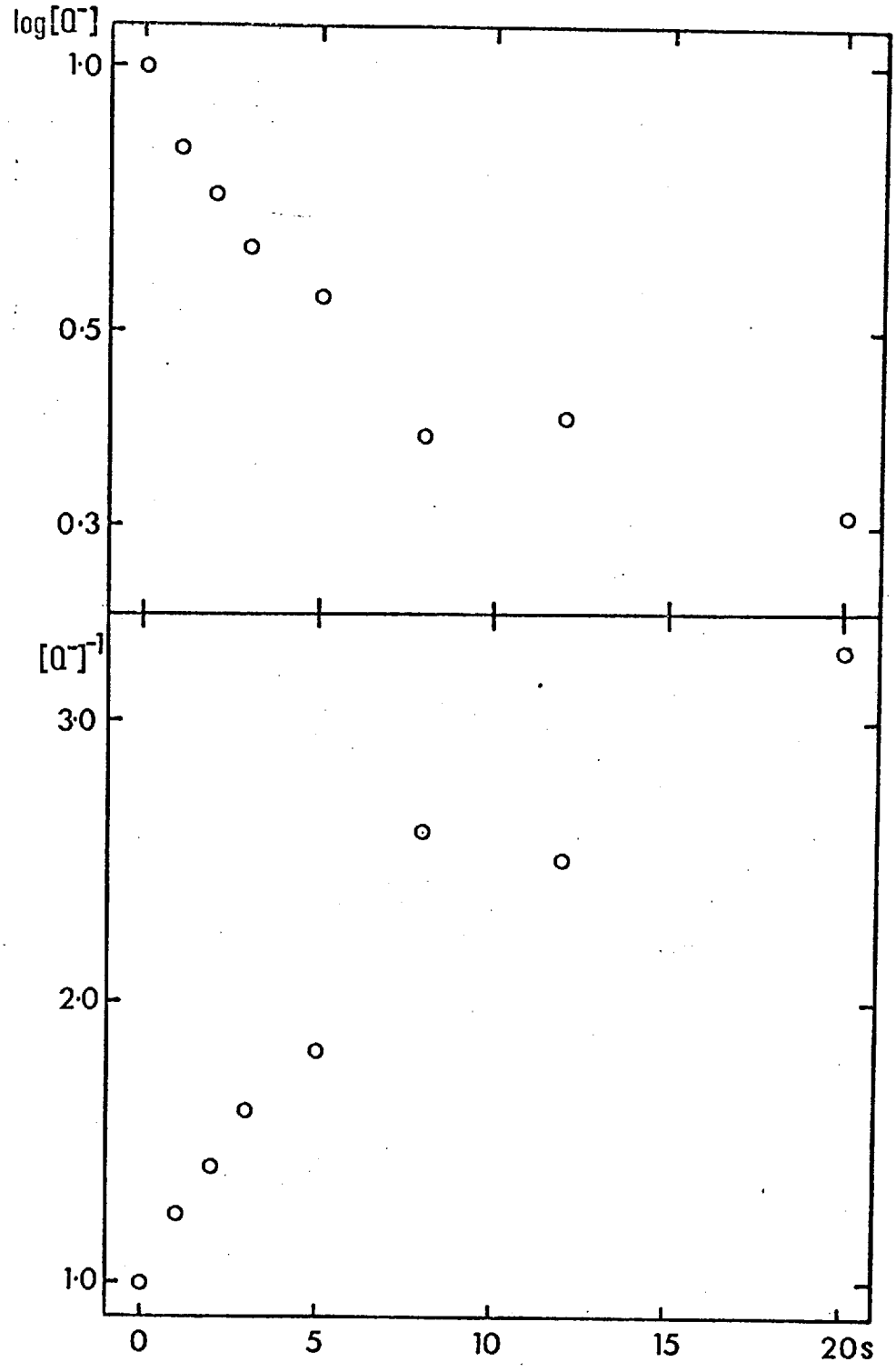
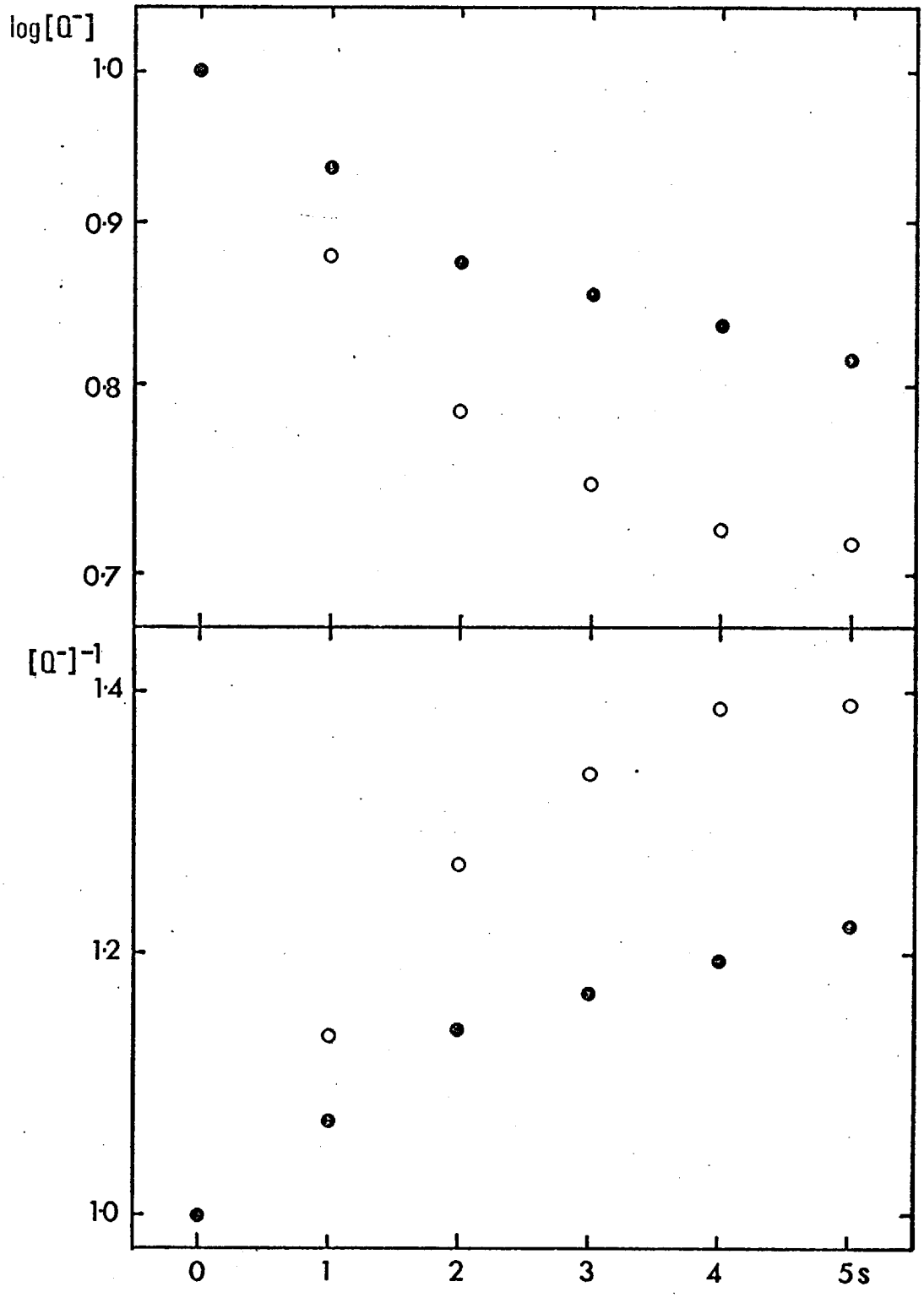

log[Q⁻]
1·0
0·5
0·3
[Q⁻]⁻¹
3·0
2·0
1·0
0
5
10
15
20s

Figure 14.

The kinetics of the reoxidation of Q^- in the dark following 25s of continuous illumination. Broken lettuce chloroplasts in assay medium in the presence of $1.3 .10^{-7}$ M gramicidin. Chlorophyll concentration 20μg ml^{-1}. Closed symbols: 16°C, open symbols: 23°C. The value of Q^- was estimated from the area over the fluorescence induction curve (Appendix I).


log[Q⁻]
1·0
0·9
0·8
0·7
[Q⁻]⁻¹
1·4
1·2
1·0
0
1
2
3
4
5s

of Q^- in the dark does not follow simple first-order kinetics as might be expected from the primary nature of the back reaction between Q^- and Y^+ (see also Lavorel,1975a).

3.1.3 Discussion

The measurements of the activation energy of delayed fluorescence by the T-jump technique from uncoupled chloroplasts in the $t_d \simeq 1$ms region has removed the discrepancy between previous activation energy measurements at short and long dark times (see section 3.1.1.1) and also emphasises the important role that the high-energy state of photophosphorylation plays in the emission of ms delayed fluorescence. In addition, accepting that more than one oxidation state of the oxygen-evolving system is involved in delayed fluorescence (Barbieri et al., 1970; Zankel, 1971) the value of $E \simeq 0.61$eV indicates that about 1.2eV can be extracted from the 1.8eV available from the first excited singlet state of chlorophyll for driving the primary reactions, a value which is consistent with the theoretical arguments of Knox (1969).

The significance of the high-energy state on the emission process can now be considered. For a coupled system eq.29 simplifies to

$$J_c \propto \exp - \left\{ (E - \Delta p)/kT \right\}$$

while for an uncoupled system

$$J_u \propto \exp - (E/kT)$$

Taking the ratio gives

$$J_c/J_u \propto \exp(\Delta p/kT) \qquad 42$$

If Δp is of the order of 200mV or more as expected (Mitchell, 1966) then the ratio J_c/J_u would exceed 2.10^3. In fact such a

large ratio is not observed. Usually it has been found that in the absence of an electron acceptor the ratio is at most 15, corresponding to a value for Δp of about 70mV, while in the presence of an electron acceptor the ratio never exceeds 100, indicating a maximum Δp of 120mV, These simple considerations suggest either that only one component of Δp (ΔpH or $\Delta\psi$) acts on the back reaction or that the influence of the total proton motive force is reduced because of the positioning or orientation of the precursor complex Y^+ChlQ^- in the thylakoid membrane.

The second approach used to measure the activation energy for the back reaction leading to delayed fluorescence involves analysing the decay of delayed fluorescence and measuring the temperature dependence of the reaction constant. The results presented in section 3.1.2.2 indicate that either $J_T^{-\frac{1}{2}}$ or J_V^{-1} versus time may be a good representation of the data, but the $\ln J_T$ and $\ln J_V$ versus time plots do not seem to fit the data. This might seem surprising, since the argument that delayed fluorescence results from a back reaction of Y^+ChlQ^- as a single substrate implies first-order behaviour (Lavorel, 1975a) However, the following considerations show that the notion of a first-order reaction can be included in a reaction mechanism leading to a $J^{-\frac{1}{2}}$ or a J^{-1} description.

If the reaction leading to the decay of the delayed fluorescence precursor is first-order, then

$$J = -\frac{dC_{df}}{dT}$$

where C_{df} is that part of the precursor Y^+ChlQ^- that decays via the delayed fluorescence back reaction. In the deactivation case (section 3.1.2.1):

$$J = -\frac{dC_{total}}{dt}$$

Under these circumstances the second order $J^{-\frac{1}{2}}$ plot gives support to a mechanism already suggested by others (Lavorel, 1969; Jursinic and Govindjee, 1972) in which delayed fluorescence results from the fusion of two chlorophyll triplet states (T) to give a chlorophyll singlet state (S) in a second order reaction so that

$$J = \frac{dS}{dt} = -k_2 C_T^2$$

where C_T is the concentration of triplet states and k_2 is a second-order rate constant. The triplet states are produced in a first-order back reaction from the precursor Y^+ChlQ^- :

$$\frac{dC_T}{dt} = -k_1 C_{total}$$

If this reaction is rate-limiting, then

$$\frac{dS}{dt} = k_1 C^2_{total}$$

and hence

$$J^{-\frac{1}{2}} = J_o^{-\frac{1}{2}} + k_1^{\frac{1}{2}} t \qquad 43$$

(see Jursinic and Govindjee, 1972) where J_o is the value of J at time t = 0. The involvement of triplet states in bringing about delayed fluorescence has yet to find rigorous experimental verification (Stacey et al., 1971). Nevertheless, this triplet model is attractive in view of the problem of understanding how delayed fluorescence excitons are able to diffuse into the bulk chlorophyll molecules away from the open trap from which they originate, although recent considerations of Lavorel (1973) and Wraight (1972) do not rule out non-perfect trapping within a

single photoactive unit.

The non-first-order decay of delayed fluorescence might also arise because other simultaneous processes compete with the first-order back reaction for the delayed fluorescence precursor in a 'leakage' reaction. These simultaneous processes could include the non-radiative decay of the precursor both on the oxidising and on the reducing side of the charge transfer complex $Y^{+}ChlQ^{-}$. In this case the decay will reflect the complexity of the reactions consuming the precursor. Consider a simple case where the competing reaction is second order, then

$$J = -\frac{dC}{dt} = k_1 C \qquad 44$$

and

$$C^{-1} = C_o^{-1} + k_2 t \qquad 45$$

Assuming that $k_2 \gg k_1$, the combination of eqs. 44 and 45 yields

$$J^{-1} = -(k_1 C_o)^{-1} + (k_2/k_1)t \qquad 46$$

Such a model could explain the J^{-1} versus time relationship, but in the experiments described in section 3.1.2.2 it is likely that $k_2 \not\gg k_1$ since there was no net electron flow. Another explanation for the linear J^{-1} versus time plot has been presented by Lavorel (1968, 1975a) who has suggested that the Elovich-Cope law (Cope, 1964, 1975) could be suitable for describing the decay of delayed fluorescence (see below).

If the deactivation process was operative under the experimental conditions described here then the possibility should be considered that the relatively good fit of the data to a $J^{-\frac{1}{2}}$ law reflects a second-order back reaction leading to delayed fluorescence (see Malkin and Hardt, 1973).

For example

$$J \propto k\ [Y^+]\ [Q^-]$$

in which case eq. 43 would apply for the decay. The concept of a second order reaction receives from support from the work of Bennoun (1970) in which he showed that the decay of Q^- in DCMU-blocked Chlorella (that is, under apparently deactivation conditions) was linear in $(Q^-)^{-1}$. This observation is given some support by the experiments described in section 3.1.2.4. Overall, the suggestion that the decay is due to a second-order process means that at least one of the charged precursors of delayed fluorescence is diffusible, and could give support to the general model of electron-hole interactions along the lines of that suggested by Bertsch et al. (1971). On the other hand it has been proposed by Mar and Roy (1974) that the $(Q^-)^{-1}$ decay observed by Bennoun (1970) is not a second-order reaction. They postulate that in DCMU-blocked systems the reoxidation of Q^- in the dark depends on the rate of diffusion of Q^- toward Y^+ within the Y^+ChlQ^- complex, and calculate an expression for the average jump frequency. The resulting expression is complex but approximates to a $(Q^-)^{-1}$ decay at short times. The decay of Q^- in uncoupled chloroplasts not blocked with DCMU is not very well described by second-order kinetics (fig.13). It might be thought that in this case the deviation from second-order kinetics could be accounted for by the model of Mar and Roy (1974) mentioned above, but the deviation from linearity in their model is in the opposite sense to that seen here.

In trying to clarify the mechanism of delayed fluorescence there is the serious problem of choosing between the two kinetic laws $J^{-\frac{1}{2}}$ and J^{-1}. As has been shown, a crucial factor in determining

the shape of the delayed fluorescence decay curve is the emission factor ϕ' in eq. 24. The functions that have been used for ϕ' in the literature have generally been the variable prompt fluorescence (Clayton, 1969) and total prompt fluorescence yield (Lavorel, 1968,1969; Jursinic and Govindjee, 1972). However, the experiments described in section 3.1.2.3, where care has been taken to measure changes in ms delayed fluorescence which are supposed to be due only to changes in ϕ', indicate that there is no linear relationship between ϕ' and macroscopic fluorescence yield, be it variable or total. Nonetheless, the delayed and prompt fluorescence did both change in the same sense and at the same time in this experiment (fig. 12) so some correction for changes in fluorescence yield is necessary.

The form of ϕ' will depend on the model chosen for the photosynthetic unit. In the separate model the delayed fluorescence emission factor will be equal to the prompt fluorescence yield of a unit with an open trap (assumed to be non-zero), and will thus be independent of time and of the macroscopic fluorescence yield , which arises from the other photosynthetic units (Lavorel and Joliot, 1972). In the uniform pigment matrix model it is not possible in theory to distinguish between a prompt and a delayed fluorescence exciton and therefore ϕ' will be equal to the variable fluorescence yield ϕ_v (Lavorel, 1975a). The delayed fluorescence emission factor is thus dependent on the degree of connection between photosynthetic units. Although models have been proposed (Joliot and Joliot, 1964; Lavorel and Joliot, 1972) the extent of connection between units, and thus the measure of ϕ' is not clear (see section 3.2.6). Lavorel and Joliot (1972) have proposed that the model for the photosynthetic unit is intermediate between

the two extreme cases presented above, so that changes in ϕ' ought to depend on changes in prompt fluorescence to some extent, but not be as strong a function of these changes as variable fluorescence itself. Similarly, the experiments described in section 3.1.2.3 indicate that not all of the prompt fluorescence has an influence on delayed fluorescence. It is therefore clear that some intermediate fluorescence function is required for ϕ'. Such a function could be the total prompt fluorescence ϕ_t. In using this function for ϕ' no meaning is intended for the f_o level of fluorescence: it is merely a convenient way to reduce the dependence of ϕ' on changes in fluorescence. Moreover, in a computer simulation of the photosynthetic unit Lavorel (1973) has shown that in a two-dimensional model an exciton would only visit 2 to 3 photosynthetic units on the average. This could suggest that an even less strong function of changes in fluorescence than total fluorescence should be used for ϕ'.

The delayed fluorescence decays can be better represented by a $J^{-\frac{1}{2}}$ plot than a J^{-1} plot as the delayed fluorescence emission factor is changed from variable fluorescence to total prompt fluorescence yield. In the light of the necessarily qualitative considerations given above it might be expected that as the delayed fluorescence emission factor were made an even less strong function of variable fluorescence the data would fit a $J^{-\frac{1}{2}}$ representation better. The $J^{-\frac{1}{2}}$ law is thus favoured as a better description of the kinetics, as has been done by others (Jursinic and Govindjee, 1972).

For the $J^{-\frac{1}{2}}$ plot eq.43 may be used to obtain k at various temperatures and an Arrhenius plot constructed. Figure 15 shows such plots in the two time ranges measured. The activation energy

was found from the slope of the plots in the usual way, and the error was taken as the error of the least-square fit of the slope to the experimental points. The results of several experiments are given in Table III; the weighted mean of these values is 0.56 ± 0.03eV.

This value of the activation energy is derived by making the following assumptions: (i) the total prompt fluorescence is the better measure of the delayed fluorescence emission factor, (ii) the $J^{-\frac{1}{2}}$ representation is the correct description of the decay of delayed fluorescence and (iii) the deactivation type of delayed fluorescence exists under the described experimental conditions. The value for the activation energy of 0.56eV is in fairly good agreement with the value of 0.61eV found from the millisecond T-jump studies, and also with the estimates of E made by others at larger values of t_d (Jursinic and Govindjee, 1972; Malkin and Hardt, 1973). Moreover, the relatively constant nature of the activation energy measured over the time range 3ms t_d 3.5s, together with the indication that there is no gross change with time in the decay mode suggests that there may be a single mechanism leading to delayed fluorescence over the whole time range studied.

As discussed above the second-order plot probably reflects a second-order process somewhere in the back reaction leading to delayed fluorescence. The most likely candidates for this second-order process are : (i) triplet-triplet annhilation following a first-order back reaction leading to triplet production or (ii) a second-order back reaction itself. The dependence of the intensity of delayed fluorescence on the square of the exciting-light intensity (Jones, 1967) lends support to the former hypothesis. The suggestion of Lavorel

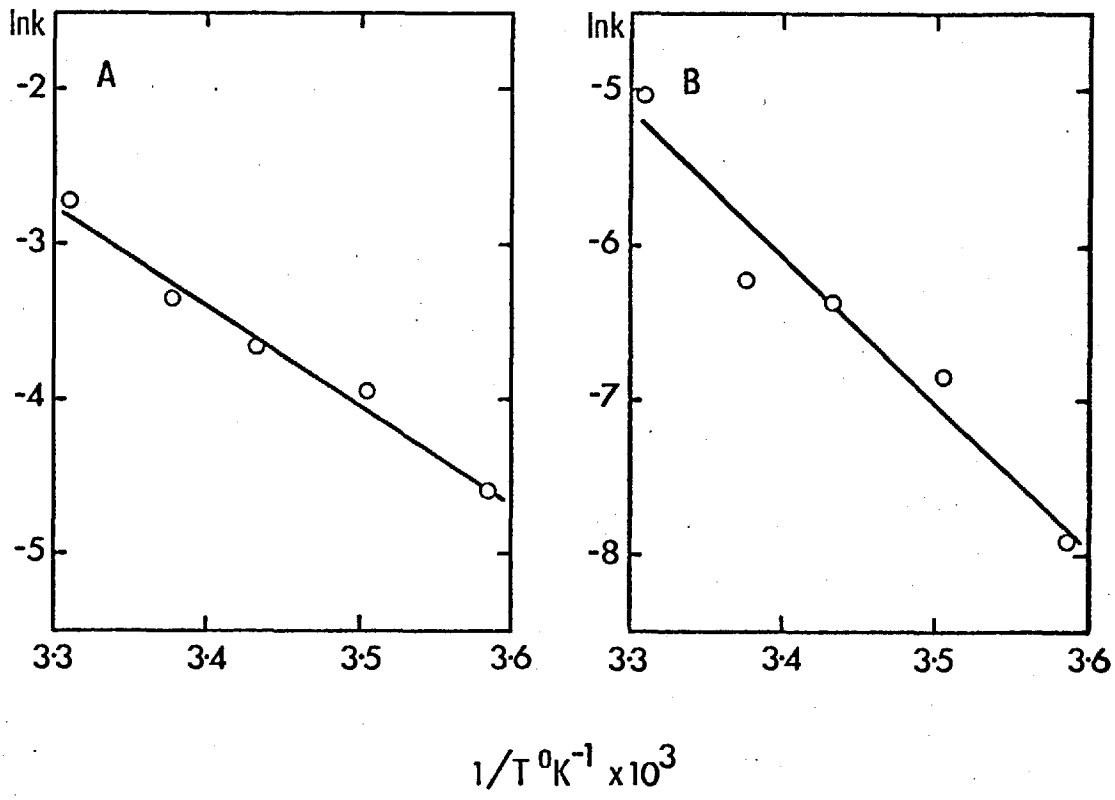


Figure 15.

Arrhenius plots of the reaction constants derived from the slopes of the $J_T^{-\frac{1}{2}}$ versus time plots. A: time range $3ms \leqslant t_d \leqslant 800ms$; B: time range $0.3s \leqslant t_d \leqslant 3.5s$. The activation energies calculated from the slopes of the Arrhenius plots are A: $0.55 \pm 0.04eV$, B: $0.82 \pm 0.10eV$, where the errors are derived from the uncertainty in the least-squares fit to the data.

TABLE III

Activation energies in eV of the delayed chlorophyll fluorescence back reaction from Arrhenius plots of the decay constant of the $J_T^{-\frac{1}{2}}$ representation.

Expt	Time Range	
	3 - 800ms	0.4 - 3.5s
A	0.47 ± 0.12	-
B	0.31 ± 0.14	0.55 ± 0.13
C	0.46 ± 0.09	0.76 ± 0.11
D	0.55 ± 0.04	0.82 ± 0.11

(1975a) that the $J^{-\frac{1}{2}}$ kinetic reflects a process obeying the Elovich Cope law (Cope 1964, 1975) is not tenable under the uncoupled experimental conditions used here since the model (Lavorel, 1975a) envisages that there is a pH gradient across the thylakoid membrane which sets pH-dependent equilibria on both sides of the membrane.

3.1.4 Summary

1. The activation energy for the delayed fluorescence back reaction in uncoupled chloroplasts is roughly constant in the time range $3ms \leqslant t_d \leqslant 3.5s$, and is equal to about 0.6eV.
2. The high-energy state of phosphorylation has an important role in delayed fluorescence emission at short times.
3. Not all of the high-energy state exerts an influence on ms delayed fluorescence.
4. Changes in prompt fluorescence yield in uncoupled, DCMU-blocked chloroplasts do not correlate with changes in delayed fluorescence, indicating that the delayed fluorescence emission factor is not a simple function of fluorescence yield.
5. Total prompt fluorescence yield is probably a better function to use for the delayed fluorescence emission factor, but it is likely that an even less strong function of fluorescence yield changes should be used.
6. The reoxidation of Q^- in the dark in uncoupled chloroplasts is quasi-second-order.
7. The decay of delayed fluorescence (corrected with total prompt fluorescence yield) may be described by a $J_T^{-\frac{1}{2}}$ representation, and this indicates a second-order process somewhere in the delayed fluorescence reaction.
8. This second-order process might be triplet-triplet interaction, or a second-order back reaction.

3.2 Prompt chlorophyll fluorescence and the photosynthetic unit

3.2.1 Analysis of prompt chlorophyll fluorescence inductions

The experiments described in this section are principally concerned with the analysis of prompt chlorophyll fluorescence inductions in DCMU-blocked chloroplasts. In recent years there has been some striking evidence that the shape of the prompt fluorescence induction (which, if interpreted in the model of Joliot and Joliot (1964), is linked to the probability of exciton transfer between PSII photosynthetic units) is variable. These reports come from work both on broken chloroplasts and on algae.

3.2.1.1 Broken chloroplast studies. Homann (1969) showed that monovalent or divalent cations added to a suspension of DCMU-blocked chloroplasts in a low-salt medium increased the chlorophyll fluorescence yield (see section 1.4.5.1). In subsequent experiments Murata (1969b) showed that as well as a change in yield, there was a change in the shape of the Q-dependent fluorescence induction curve. Increasing the fluorescence yield changed the induction from being roughly exponential to being more sigmoid. The change in fluorescence induction can be interpreted as either (i) a change in the degree of energy transfer between PSII units (Joliot and Joliot, 1964) or (ii) a change in one or more of the states in the three-state reaction centre model (Lavorel, 1972). This change has been noted by Briantais et al. (1973) and Bennoun (1974) and the increase of sigmoidicity has been ascribed to a change in inter-PSII transfer (Briantais et al., 1973), the transfer being more probable in the presence of cations. Marsho and Kok (1974) reported a similar effect of cations on the yield of oxygen as a function of closed PSII traps, finding the parameter p of eq.33 to be 0.55 when

cations were present in the suspending medium, and about zero when cations were absent. Malkin (1974) has made a careful study of the flash yield for DCIP reduction as a function of flash intensity, and found that in low-salt media exciton transfer between PSII units is limited, but that in high-salt media it is enhanced.

3.2.1.2 Algae. In algae the experimental results are not quite as clear cut as compared to the cation-induced effects in broken chloroplasts. Delrieu (1972) and Wang and Myers (1974) have measured steady-state oxygen yield as a function of open traps for algae in State I (corresponding to the M phase of the fluorescence induction) and in State II (corresponding to the T phase), and in both cases found no change in p. On the other hand Bennoun (1974) and Vernotte et al. (1975) have measured the relationship between chlorophyll fluorescence yield and the fraction of closed PSII traps and found significant changes in p in the high fluorescing state (State I) and in the low fluorescing state (State II). It is probably worth noting that the experiments of Delrieu (1972) and of Wang and Myers (1974) were over a fairly restricted range of values of $(1-Q)$, whereas the experiments of Bennoun (1974) and of Vernotte et al. (1975) were over the complete range of $(1 - Q)$ values.

Vernotte et al. (1975) also found changes in p in chloroplasts isolated, using a medium containing the membrane-fixative gluteraldehyde, from leaves kept in State I or State II by appropriate illumination. p was higher in chloroplasts isolated from leaves in State I (high fluorescing) than in chloroplasts isolated from leaves in State II (low fluorescing).

The general picture that emerges from these experiments is that the shape of the prompt fluorescence induction is variable. The experiments described in this section have analysed the changes in fluorescence induction shape and fluorescence yield more closely, and the results give indications as to the nature of the PSII photosynthetic unit and exciton transfer *in vivo*.

3.2.2 The characteristics of prompt chlorophyll fluorescence inductions from DCMU-blocked chloroplasts in the high and low fluorescing states.

3.2.2.1 The effect of salts on prompt chlorophyll fluorescence inductions in DCMU-blocked broken chloroplasts. Figure 16 illustrates the changes in the shape of the prompt fluorescence induction that can be induced by the addition of cations to the suspending medium. The inductions are taken from DCMU-blocked chloroplasts suspended in a medium containing 7mM K^+ ('low-salt') or 50mM K^+ ('high-salt'). The two principal differences between the fluorescence inductions from the chloroplasts in low and high salt media are (i) the variable fluorescence yield in high salt chloroplasts is approximately twice that of the low salt chloroplasts and (ii) the variable fluorescence induction in the low salt chloroplasts is approximately exponential, while that of the high salt chloroplasts is markedly sigmoid (fig 17). It is worth emphasising that since these chloroplasts were DCMU-blocked these changes are dependent only on the light-induced closing of PSII traps (reduction of Q) and are independent of the redox state of other components of the electron transport chain.

3.2.2.2 Analysis of the chlorophyll fluorescence inductions in the high and low fluorescing states. The results presented above and in the introduction to this section suggest that the

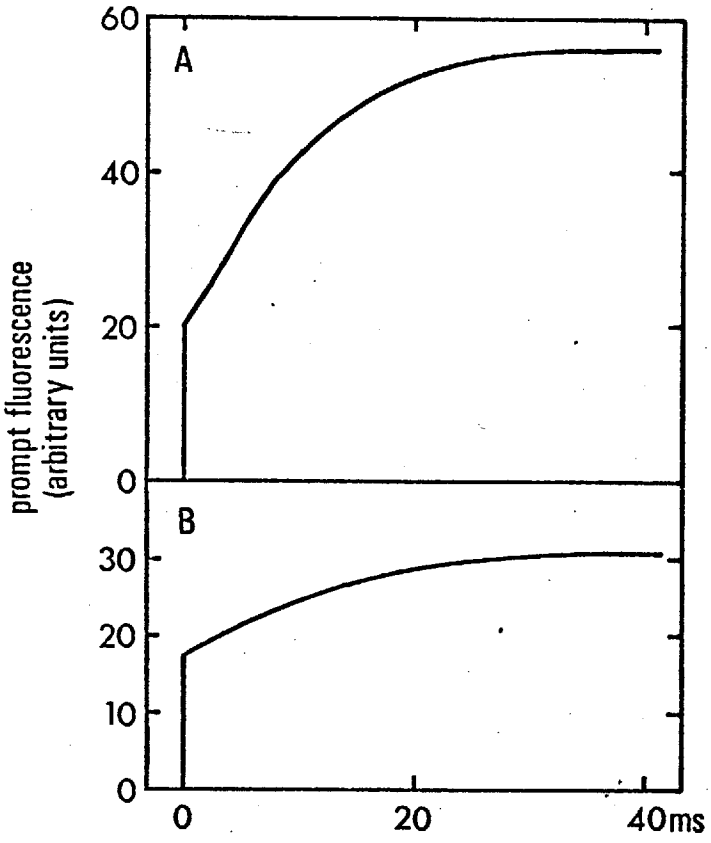


Figure 16

The chlorophyll fluorescence induction of broken spinach chloroplasts blocked with 20μM DCMU. A: chloroplasts suspended in low salt medium (~7mM K^+). B: chloroplasts suspended in high salt medium (~50mM K^+). The chloroplasts were dark-adapted, and DCMU was added to the suspension in the dark. The inductions were measured using the phosphoroscope as a fluorimeter; the traces are redrawn from photographs of the oscilloscope screen.

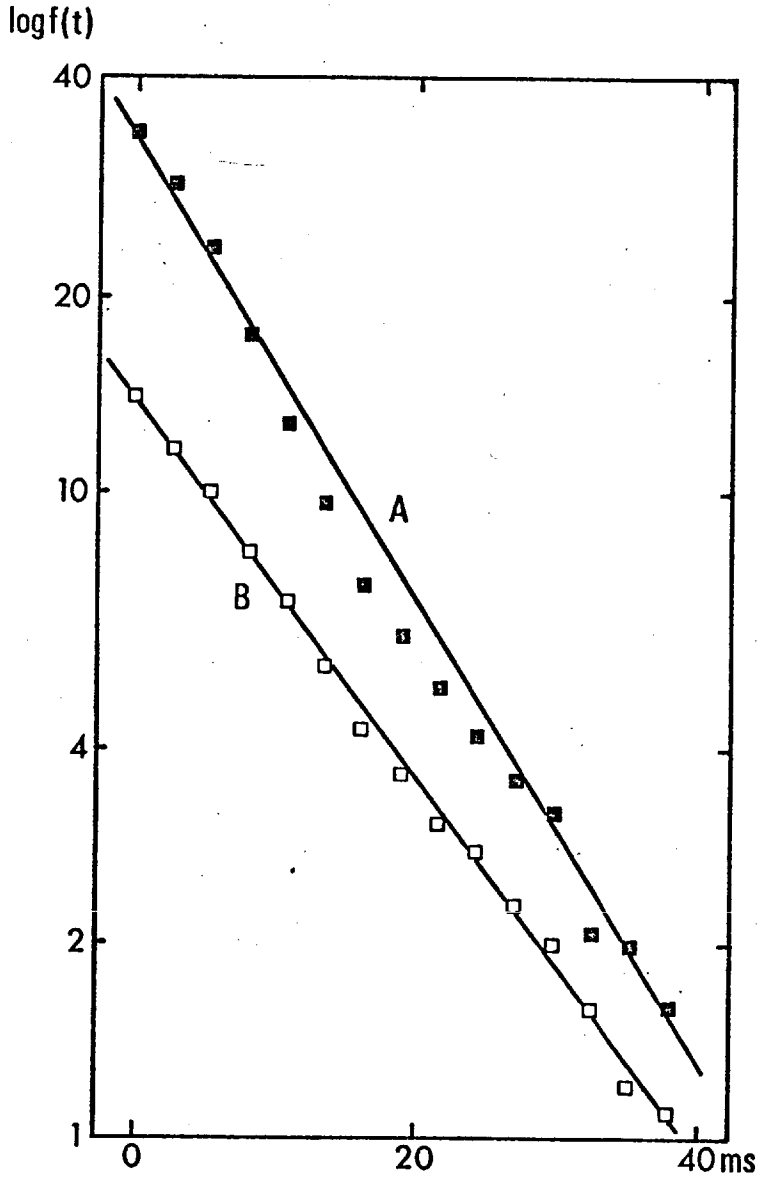


Figure 17.

Log-plots of the variable chlorophyll fluorescence inductions of fig 16.

addition of salts to DCMU-blocked chloroplasts can induce a change in variable fluorescence yield and in the shape of the variable fluorescence induction. The question arises as to whether this is a unique property of the fluorescence changes induced by the addition of cations to chloroplasts in a low salt medium, or a more general phenomenon. To examine the variability of the kinetics of the fluorescence rise more closely, four methods were used by which fluorescence yield may be altered without modifying the concentration of open PSII traps. The four methods were :

(i) the addition of a high concentration of a monovalent cation or a low concentration of a divalent cation to broken chloroplasts suspended in a low-cation medium (see section 1.4.5.1). This treatment increases the fluorescence yield and is thought to decrease the magnitude of k_{tI} ('spillover').

(ii) the addition of a low concentration of a monovalent cation to broken chloroplasts suspended in an essentially cation-free medium (section 1.4.5.1). Conversely to (i) this treatment lowers the fluorescence yield and is thought to increase the magnitude of k_{tI}.

(iii) The addition of an external quencher of chlorophyll fluorescence (e.g. m-dinitrobenzene (DNB)) to broken chloroplasts. This treatment adds a parasitic quenching site to the bulk pigments, and adds an extra term to the denominator of eq. 18.

(iv) the light-induced fluorescence quenching of intact chloroplasts (section 1.4.5.2). This is again thought to be connected with a decrease in spillover (k_{tI}), but could also involve a change in the value of k_h.

The high and low fluorescing states attained by these four methods were characterised by the following parameters (a) the f_o level of fluorescence, (b) the variable fluorescence (c) the area over the variable fluorescence induction and (d) the degree of sigmoidicity of the fluorescence induction. In order to quantify this last parameter it was decided to use the probability of exciton transfer between PSII photosynthetic units, which is derived from the shape of the fluorescence induction and is calculated from the equation given by Joliot and Joliot (1964) :

$$\phi_v = \frac{(1 - p)(1 - Q)}{1 - p(1 - Q)} \qquad 33$$

where ϕ_v is the variable fluorescence yield, $(1 - Q)$ is the fraction of PSII traps in the closed state and p is the probability of transfer of an exciton from a PSII photosynthetic unit with a closed trap to another PSII unit (Appendix II). The value of ϕ_v was obtained from photographs of the oscillographic display of the induction and the value of $(1 - Q)$ was estimated from the integral relation whose derivation is given in Appendix I:

$$\left[1 - Q\right](t) = \frac{\int_0^t f(t)dt}{\int_0^\infty f(t)dt} \qquad 47$$

where f(t) is the difference between the variable fluorescence at time t and the maximum (asymptotic) variable fluorescence (fig.A1).

Equation 33 is, however, not totally adequate to deal with the variable fluorescence inductions. It predicts a non-linear but symmetrical relationship between ϕ_v and $(1 - Q)$. In practice the relationship is asymmetrical, the values of p apparently decreasing as $(1 - Q)$ increases. The problem with eq. 33 is that

it assumes that the system remains homogeneous at all values of (1 - Q). But as Lavorel and Joliot (1972) have pointed out, this assumption may not be valid since restricted exciton transfer from photosynthetic units with closed traps will tend to produce groups of units with closed traps ('islets'), thus creating inhomogeneity within the system. This will result in making the relationship between ϕ_v and (1 - Q) asymmetrical, since in an islet the migrating exciton will 'see' a higher local value of (1 - Q) than the mean value for the whole system. This islet effect will be minimal at low values of (1 - Q), so calculation of p from eq. 33 bas been restricted to low values of (1 - Q).

A second problem that presents itself in the evaluation of p is illustrated in fig. 18, where fluorescence inductions of osmotically shocked spinach chloroplasts in low salt buffer ± 5mM Mg^{++} are presented. Even in the presence of 100 μM DCMU, the fast fluorescence increase which accompanies the light-induced closing of PSII traps is followed by a much slower phase of fluorescence increase. This slow increase in the presence of DCMU has been observed by others (see, for example, Joliot and Joliot, 1971); whatever its cause it makes the estimation of the area over the fast induction curve subject to errors. In the experiments described here the slow phase has been ignored since the hypothesis that it is not connected with the initial closing of PSII traps seem quite reasonable.

Typical fluorescence inductions in the high and low fluorescing states obtained by the four different methods outlined above are shown in fig.19 (experimental details will be found in the legend). It can be seen that in all cases high fluorescence is associated with a more sigmoid induction and low fluorescence

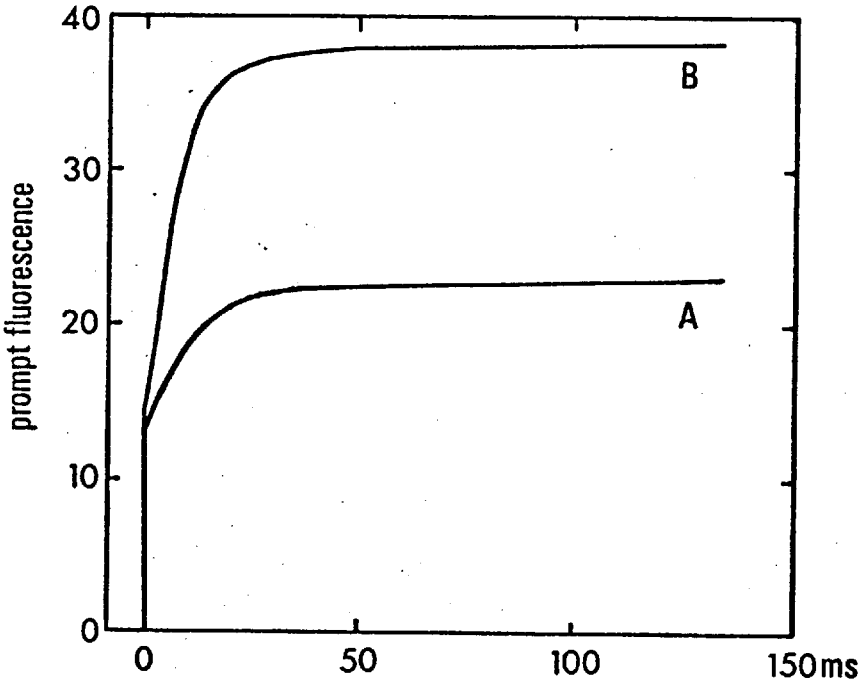


Figure 18.

Chlorophyll fluorescence inductions from osmotically shocked spinach chloroplasts resuspended in low salt buffer in the presence (A) or absence (B) of 5mM $MgCl_2$, and blocked with 100μM DCMU. The slow phase following the fast fluorescence increase can be seen in both the high and low fluorescing states.

Figure 19.(i)

Q-dependent chlorophyll fluorescence inductions from DCMU-blocked chloroplasts in high and low fluorescing states. Each figure represents two measurements made on different samples.

A: fluorescence quenching with DNB: dark adapted osmotically shocked pea chloroplasts were suspended in assay medium to which 5mM $MgCl_2$ had been added. The chloroplasts were dark incubated for 5 min and then the required concentration of DNB was added (10^{-4}M for the low fluorescing state, none for the high fluorescing state). The suspension was dark incubated for a further 30s, then 100μM DCMU (final concentration) was added, and the induction taken 10s later.

B: fluorescence quenching induced by a low concentration of monovalent cations: dark adapted osmotically shocked pea chloroplasts were suspended in an essentially cation-free medium (100 mM sorbitol with sufficient Tris base to bring the medium to pH 7.2, typically 0.05mM). 10mM KCl was added to one sample which brought the fluorescence to a relatively low level; no addition was made to the other sample. The chloroplasts were dark incubated for 5 min, then 10s before the induction was taken 100μM (final concentration) DCMU was added.

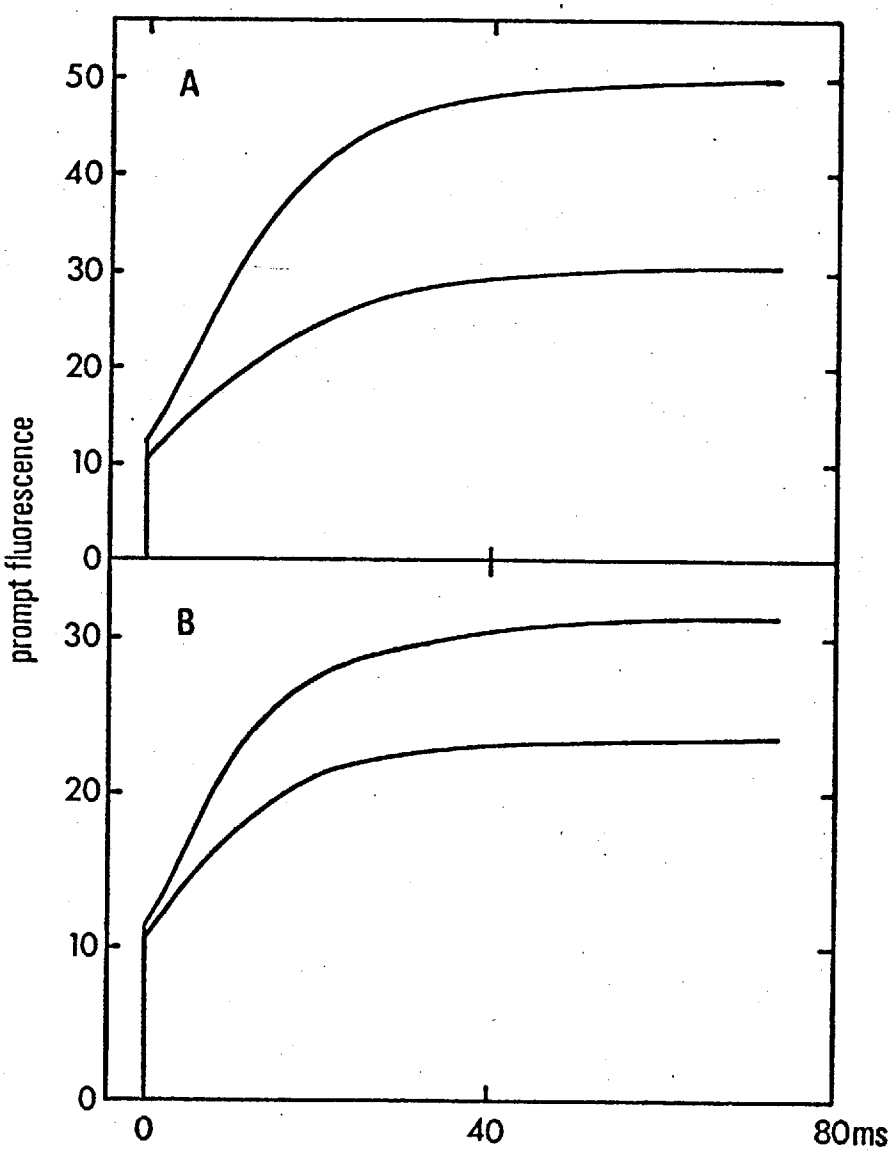
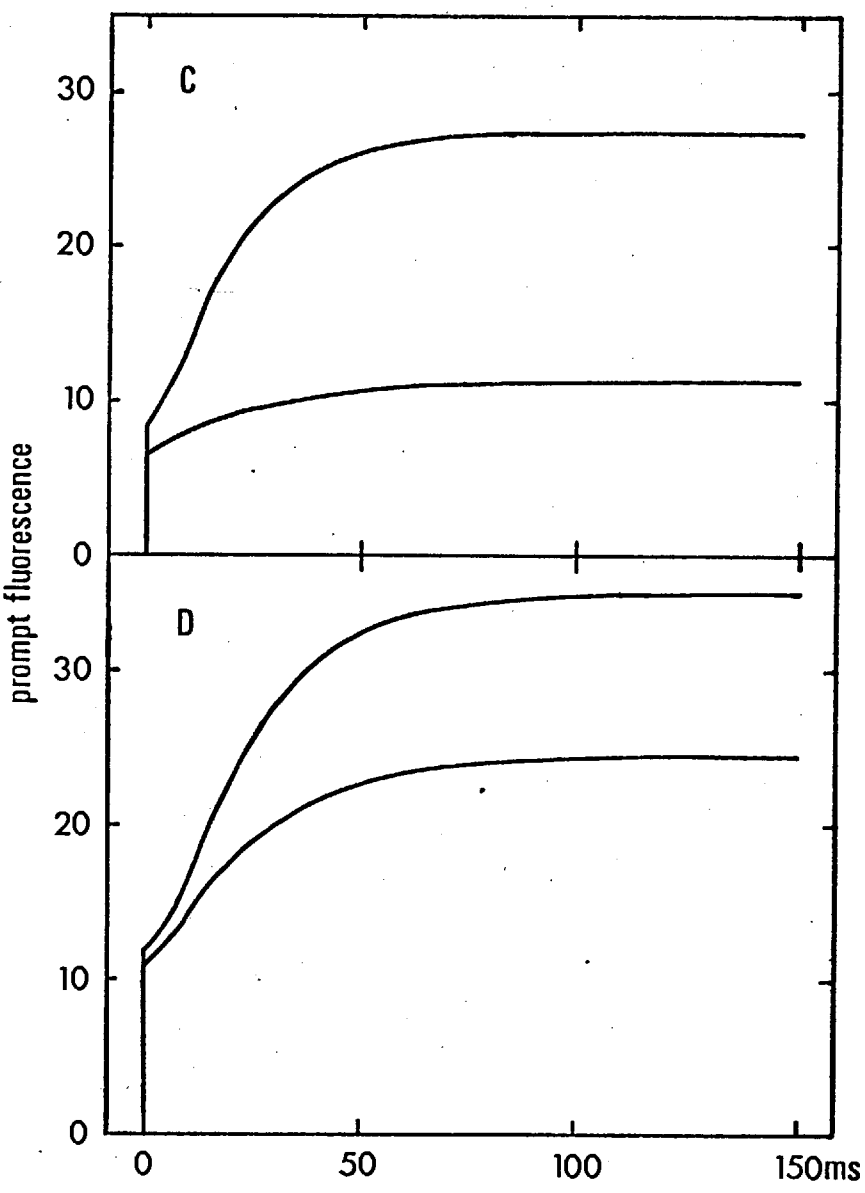

A
B
50
40
30
20
10
0
30
20
10
0
prompt fluorescence
0
40
80ms

Figure 19. (ii)

C: fluorescence increase induced by a divalent cation: dark adapted osmotically shocked chloroplasts were suspended in low salt buffer. 5mM $MgCl_2$ was added to one sample, which increased the fluorescence yield markedly; no addition was made to the other sample. The chloroplasts were dark adapted for 5 min. 10s before the induction was measured 100 μM DCMU was added to the suspension.

D: light-induced quenching of fluorescence in intact chloroplasts. The high fluorescing state was measured by adding 100 μM (final concentration) DCMU to dark adapted intact chloroplasts 10s before taking the fluorescence induction. For the low fluorescing state the chloroplasts were exposed to broad-band blue (PSII) light during which exposure the prompt fluorescence was quenched. The quenching was monitored on a chart recorder or oscilloscope: when the fluorescence had reached its minimum value (this took 8 - 10 min) the actinic light was extinguished. 10s dark time was then given to allow Q^- to be reoxidised, 100 μM DCMU was then added and 10s later the induction was taken. The fluorescence yield measured in this way did not correspond to the minimum level because the chloroplasts tend to revert to the high fluorescing state in the dark (Barber and Telfer,1974), but such a dark period is necessary to reoxidise Q^-.


C
D
30
20
10
0
30
20
10
0
prompt fluorescence
0
50
100
150ms

with a more exponential induction. Figure 20 shows plots of ϕ_v against $(1 - Q)$ (with ϕ_{vmax} normalised to unity in each case) for the high and low fluorescing states of fig. 19 D: in the high fluorescing state the experimental points deviate from linearity to a greater extent than in the low fluorescing state. These plots enable the probability of exciton transfer between PSII units, p, to be calculated from eq. 33. This was done by taking about four pairs of values of ϕ_v and $(1 - Q)$ for small values of $(1 - Q)$ (to avoid the islet effect) for both the high and low fluorescing states and deriving a weighted mean of p. For fig. 20 this resulted in a value for p of 0.63 ± 0.05 in the high fluorescing state and of 0.38 ± 0.07 in the low fluorescing state. Theoretical plots of these two values of p (from eq. 33) are shown in fig 20: it can be seen that in the high fluorescing state there are distinct deviations from the theoretical plot for high values of $(1 - Q)$, whereas in the low fluorescing case this is not so. This is qualitatively in accord with assigning the deviation to being due to the islet effect, since this effect would be expected to be less marked for small values of p. The remainder of the traces in fig.19 have been analysed similarly; the results are presented in Table IV. It can be seen that when the variable fluorescence f_v is quenched, the value of p decreases, irrespective of which of the four methods is used to effect the quenching. At the same time the f_o level is quenched slightly and the area over the fluorescence curve is decreased, but the quotient A/f_{vmax} is changed much less.

Figure 20.

Plots of the fraction of variable chlorophyll fluorescence yield ϕ_v against the fraction of closed PSII traps (1 - Q) for the high (A) and low (B) fluorescing states of DCMU-blocked intact pea chloroplasts. Data of fig.19D. The maximum variable fluorescence has been normalised to unity in each case. The solid lines are theoretical plots from eq.33, with p = 0.63 (A) and p = 0.38 (B); the dotted line in each case is for p = 0.

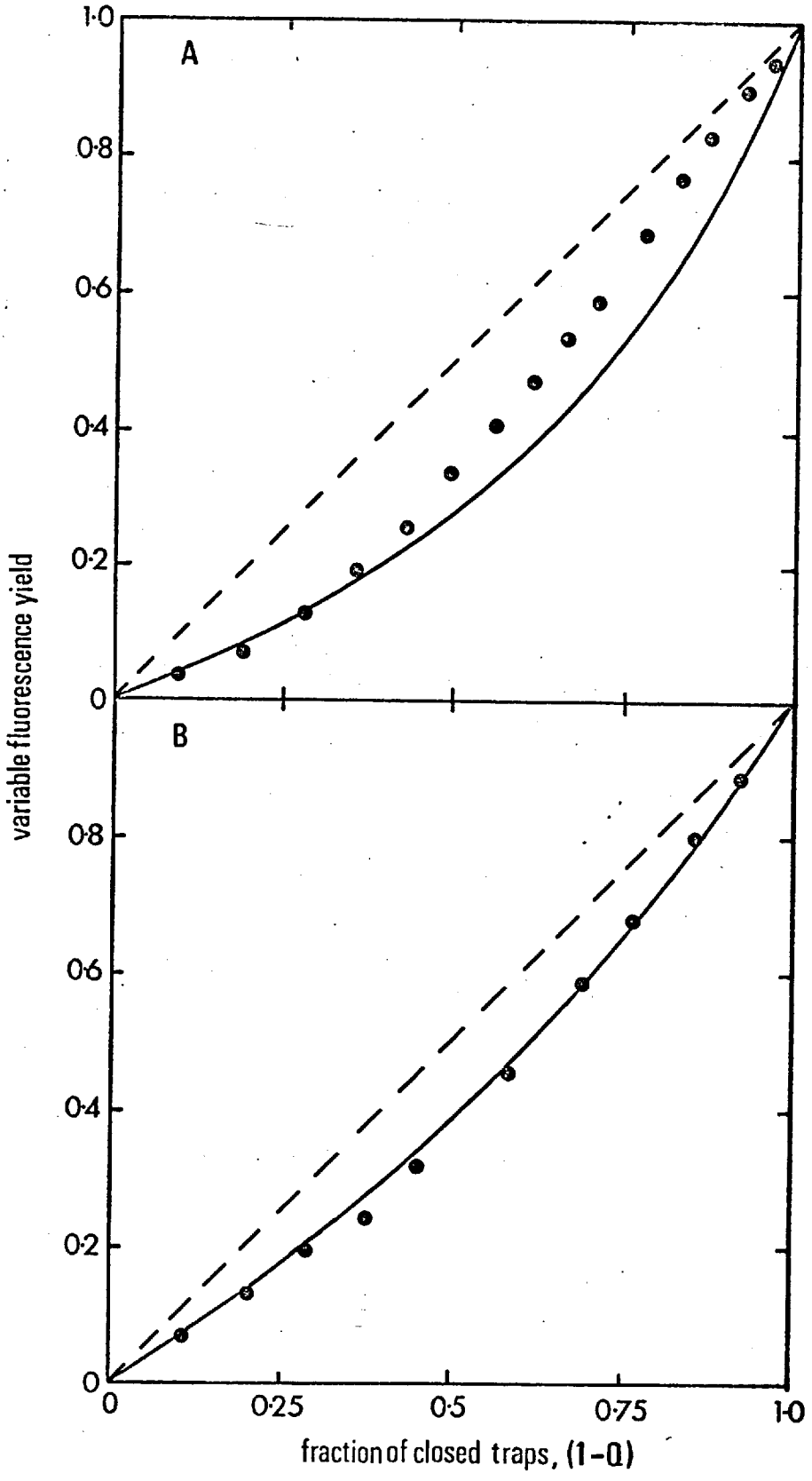

A
B
1·0
0·8
0·6
0·4
0·2
0
0·25
0·5
0·75
1·0
variable fluorescence yield
fraction of closed traps, (1-Q)

TABLE IV

The parameters of the prompt chlorophyll fluorescence inductions of DCMU-blocked pea chloroplasts in the high and low fluorescing states

	Experiment	Addition	f_o	f_v	A	A/f_v	p	t
A	Osmotically shocked chloroplasts in low salt buffer	+5mM $MgCl_2$	8.5	19.5	168.5	8.64	0.56 ± 0.05	-
		no addition	6.5	5.3	57.5	10.95	0.29 ± 0.08	.59
B	Osmotically shocked chloroplasts in cation free medium	no additions	8.5	22.5	122.5	5.44	0.61 ± 0.06	-
		+ 1mM Kcl	8.0	16.0	96	6.0	0.25 ± 0.10	.47
C	Osmotically shocked chloroplasts in assay medium plus 5mM $MgCl_2$	no additions	12.2	38.0	222.5	11.7	0.34 ± 0.03	-
		$+10^{-4}$M DNB	10.2	21.2	133.4	12.6	0.21 ± 0.03	.42
D	Intact pea chloroplasts in assay medium	dark adapted	12.0	23.5	241.5	10.3	0.63 ± 0.05	-
		light quenched	11.2	13.5	124.5	9.2	0.38 ± 0.07	.47

The values of fluorescence and area are in arbitrary units. The measurements of experiments A to D are not comparable since they were performed on different days using different chloroplast preparations. $A = \int_0^\infty f(t)dt$ = area over the fluorescence induction curve (Appendix I). p is derived from eq.33 (Appendix II); the value presented is the weighted mean of four values with $(1 - q) \leq 0.35$. t is assumed to be zero in the high fluorescing state, and is calculated from eq.55 in the low fluorescing state.

3.2.3 The relationship between variable prompt chlorophyll fluorescence and the probability of exciton transfer between PSII photosynthetic units

It appears from the results presented in section 3.2.2.2 that changes in variable fluorescence and changes in the exponentiality of the fluorescence induction are correlated. This correlation was further investigated using DNB-induced quenching of osmotically shocked pea chloroplasts suspended in assay medium with 5mM Mg^{++} added. This means of fluorescence quenching was employed because it is the easiest to handle experimentally. However, it is possible to quench not only the variable fluorescence but also the f_o level of fluorescence if the concentration of DNB is sufficiently high (Etienne, Lemasson and Lavorel, 1974). Care was thus taken to restrict the range of DNB concentrations to those which quenched the f_o level negligibly. In order to obtain one measurement in the region of low variable fluorescence (where DNB would have quenched both f_v and f_o) an induction was taken from a sample containing neither Mg^{++} nor DNB. The results of a typical experiment are given in Table V. The relationships between p and variable fluorescence (f_{vmax}) total fluorescence ($f_{tmax} = f_{vmax} + f_o$), and the ratio f_{tmax}/f_o are shown in fig.21. These plots show a roughly linear relationship between p and all three parameters chosen. In particular, p is linear with f_t and f_v extrapolating back approximately to the f_o level and the origin respectively. The p versus f_t/f_o plot does not extrapolate back through $f_t/f_o = 1$ probably because this method or presenting the data is sensitive to small changes in f_o.

TABLE V

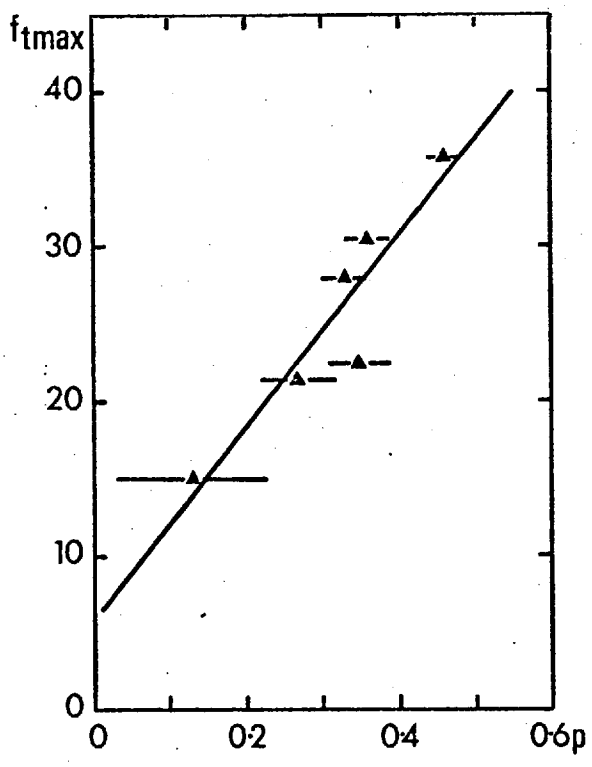
Parameters of prompt chlorophyll fluorescence inductions in DCMU-blocked osmotically shocked pea chloroplasts quenched with dinitrobenzene

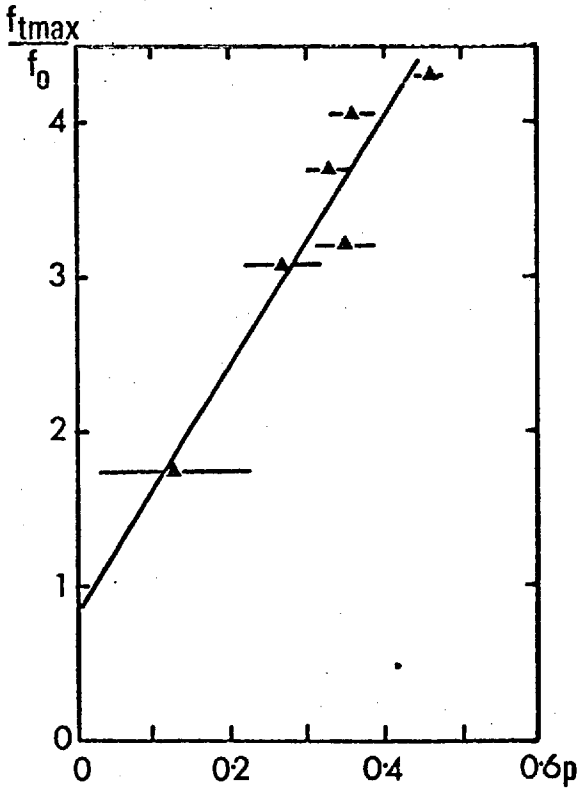
Addition	f_o	f_{vmax}	f_{tmax}	A/f_{vmax}	p	$P^{(b)}$	f_{vmax}/f_{tmax}
0	8.3	27.5	35.8	11.1	0.46 ± 0.02	0.60	0.77
2.10^{-5}M	7.5	23.0	30.5	11.8	0.36 ± 0.03	0.48	0.75
5.10^{-5}M	7.5	20.5	28.0	11.9	0.33 ± 0.03	0.45	0.73
10^{-4}M	7.0	15.5	22.5	11.4	0.35 ± 0.04	0.51	0.69
$1.3.10^{-4}$M	7.0	14.5	21.5	12.5	0.27 ± 0.05	0.40	0.67
$0,-Mg^{++}$ (a)	8.5	6.5	15.0	13.2	0.13 ± 0.10	0.30	0.43

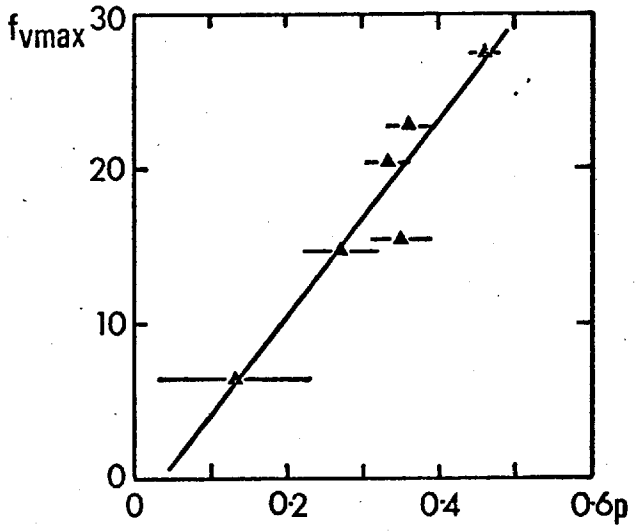
(a): the suspending medium contained neither Mg^{++} nor DNB. (b): the value of P was calculated from $P = p / (1 - (f_o/f_{tmax}))$. The chloroplasts were suspended in assay medium with 5mM $MgCl_2$ added. The values of fluorescence and area are in arbitrary units. $A = \int_o^\infty f(t)dt$ = area over the fluorescence induction curve (Appendix I). p is a weighted mean of four values calculated from eq. 33 with $(1 - Q) \leq 0.35$

Figure 21.

Plots of p against (A) total chlorophyll fluorescence yield f_{tmax} $(= f_o + f_{vmax})$; (B) variable fluorescence f_{vmax} and (C) the ratio f_{tmax}/f_o. Data of Table V. The changes in fluorescence were made by addition of various concentrations of DNB to a suspension of DCMU-blocked osmotically shocked pea chloroplasts in assay medium with 5mM $MgCl_2$ added. The exception is the point showing lowest fluorescence which was obtained from DCMU-blocked chloroplasts in assay medium with neither DNB nor $MgCl_2$ added. The straight lines are least-square fits. Chlorophyll concentration 15 $\mu g\ ml^{-1}$.


f_{tmax}
40
30
20
10
0
0
0·2
0·4
0·6p


$\frac{f_{tmax}}{f_0}$
4
3
2
1
0
0
0·2
0·4
0·6p


f_{vmax}
30
20
10
0
0
0·2
0·4
0·6p

3.2.4 The relationship between the probability of exciton transfer between PSII photosynthetic units and temperature

It has been demonstrated above that there is a linear relationship between the level of variable fluorescence and the degree of sigmoidicity of the fluorescence induction as measured by p. The experiments described in this section were designed to establish whether the degree of sigmoidicity of the fluorescence inductions is a function of temperature. The range of temperatures used was restricted to physiological temperatures ($6^{\circ} \leq T \leq 35^{\circ}C$) to avoid thermal denaturation of PSII. The experiments were performed with a thermostatically controlled cuvette holder. Figure 22 illustrates typical inductions from osmotically shocked pea chloroplasts blocked with DCMU at $6.8^{\circ}C$ and $34.2^{\circ}C$. The difficulties in the analysis of the experiment are shown by the fact that the yield of variable fluorescence is a function of temperature. This means that values of p at different temperatures will have to be corrected for changes in variable fluorescence. Ideally this should be done by making a plot of f_v versus p for each temperature used, but in the experiments reported here it has been assumed that the relationship between f_v and p at 19° (fig.21) also holds at other temperatures. That is, the intercept of the f_v versus p plot is the same at all temperatures. The value of p at various levels of f_v can thus be normalised to a single value of f_v (fig.23). In the experiment whose data is shown in Table VI, all values were normalised to the yield found at $24.8^{\circ}C$. Figure 24 shows an Arrhenius plot of the data of Table VI: the error bars are those for the uncorrected values of p. The slope of this Arrhenius plot gives a value of 0.022 ± 0.03eV, where the error is given by the error

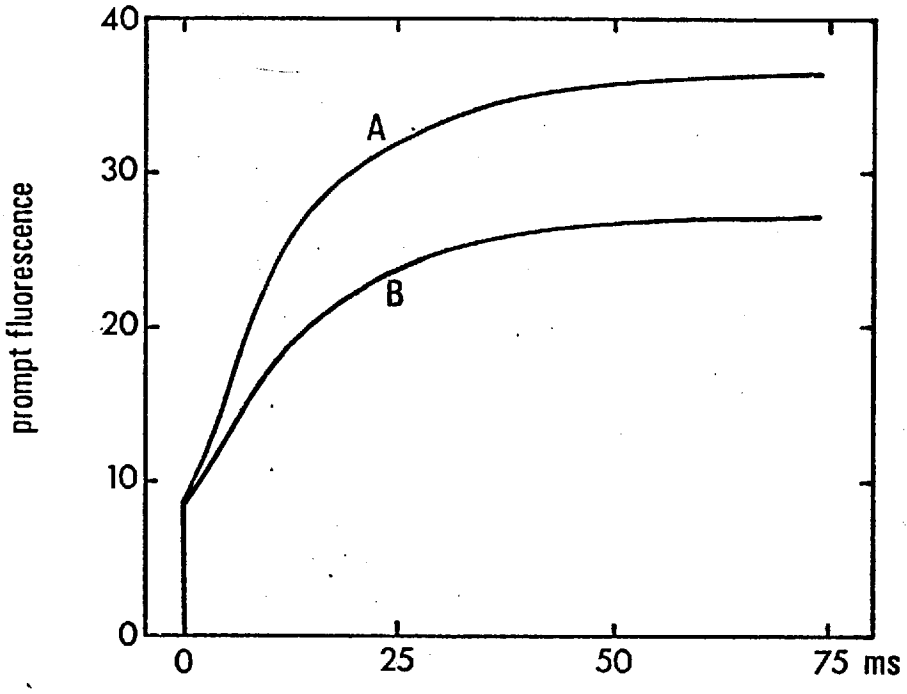


Figure 22.

Chlorophyll fluorescence inductions from osmotically shocked pea chloroplasts suspended in assay medium plus 5mM $MgCl_2$ and blocked with 100μM DCMU. A: 34.2°C; B: 6.8°C. Chlorophyll concentration 15μg ml^{-1}.

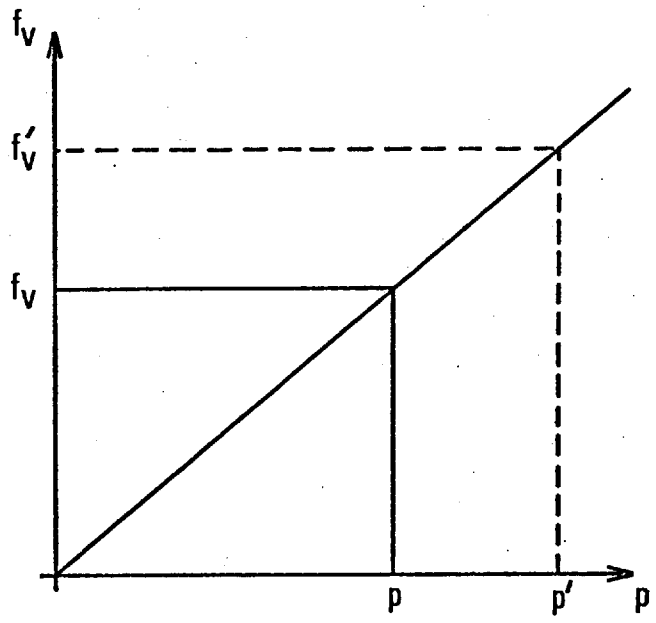


Figure 23.

The method used to correct values of p for changes in f_v. It is assumed that at all temperatures the plot of f_v versus p goes through the origin. If the experimental point found is (f_v, p) and if the 'standard' value of variable fluorescence is f'_v, then it can be seen that the value of p has to be corrected to p'.

TABLE VI

Data from chlorophyll fluorescence inductions from DCMU-blocked osmotically shocked pea chloroplasts at different temperatures.

Temperature	f_o	f_v	A	A/f_v	p	p'
6.8	8.6	27.5	252	9.16	0.50 ± 0.02	0.45
6.9	7.8	27	248	9.2	0.42 ± 0.02	0.38
12	8.3	21.5	258	9.4	0.51 ± 0.03	0.58
12.1	8.8	26	245	9.4	0.46 ± 0.02	0.43
19.1	8.7	24	254.5	10.6	0.47 ± 0.02	0.48
19.3	8.6	25	261.5	10.5	0.43 ± 0.02	0.42
24.8	8.0	24.5	249.5	10.2	0.43 ± 0.02	0.43
24.8	8.1	25	273.5	10.9	0.43 ± 0.02	0.42
29.8	8.0	21.5	240.5	11.9	0.51 ± 0.03	0.58
29.9	8.1	21	224.5	10.7	0.42 ± 0.03	0.49
34.0	8.9	20.5	226	11.0	0.36 ± 0.03	0.43
34.2	8.7	18.5	195.5	10.6	0.35 ± 0.03	0.46

The chloroplasts were suspended in assay medium with 5mM $MgCl_2$ added. The values of fluorescence and area are arbitrary units. $A = \int_0^\infty f(t)dt$ = area over the fluorescence induction curve (Appendix I). p is a weighted mean of four values calculated from eq.33 with $(1-Q) \leq 0.35$. The values of p' were derived as outlined in the legend of fig.23.

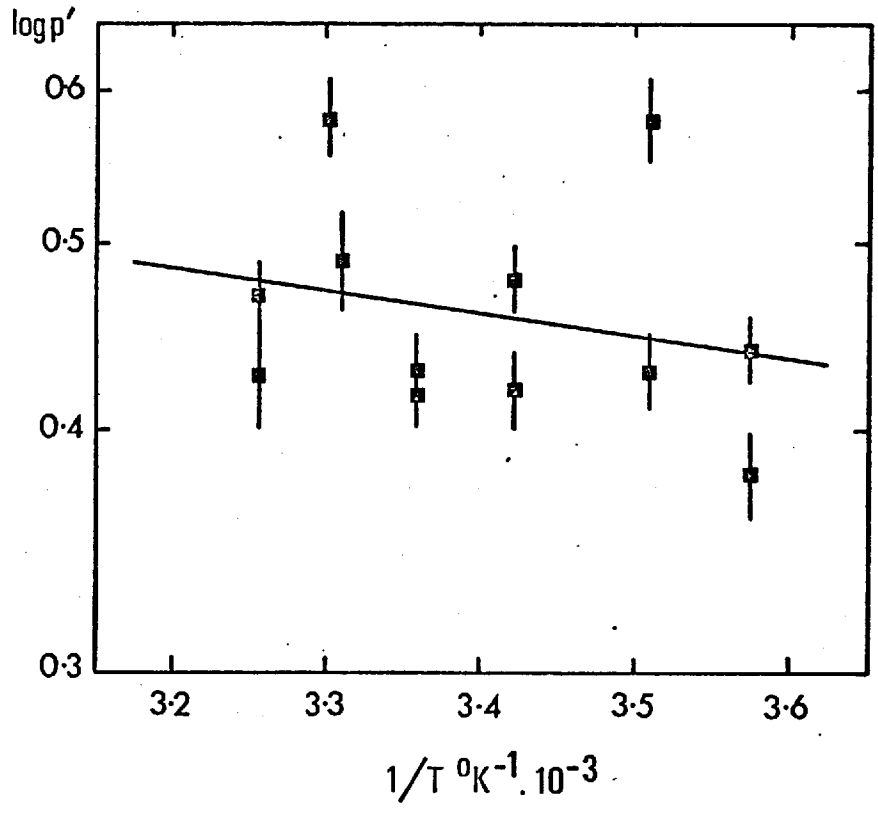


Figure 24

Arrhenius plot of values of p' from Table V. An allowance has been made for changes in f_v with temperature by assuming that the plot of f_v versus p goes through the origin at all temperatures, and normalising all the values of f_v to that found at 24.8°. The error bars are those of the uncorrected parameter p. The straight line is a least-squares fit whose slope gives an activation energy of 0.022 ± 0.03eV.

of the least-squares fit. A weighted mean of this and two other similar experiments yields an apparent activation energy for exciton transfer between PSII photosynthetic units of 0.027 ± 0.02eV. The significance of this apparent activation energy is discussed in section 3.2.5.3.

3.2.5 Discussion

The experiments described in this section have shown that when the variable PSII fluorescence yield of a photosynthetic system is altered by some means independent of the redox state of the PSII primary electron acceptor there is an accompanying change in the shape of the fluorescence induction. These two changes appear to be closely linked, since all four methods which have been used to alter variable fluorescence yield also change the shape of the variable fluorescence induction. It is therefore necessary to think of a mechanism that may induce both changes simultaneously. When a DCMU-blocked photosynthetic system is in the high fluorescing state the PSII variable fluorescence induction is sigmoid (fig.19). Three different mechanisms have been suggested to account for the sigmoidicity of the fluorescence induction. These are (i) energy transfer between PSII photosynthetic units (Joliot and Joliot, 1964) (ii) deactivation processes competing with photochemical trapping when excitation energy transfer through the pigment bed is free (Paillotin, 1974; Butler and Kitajima, 1975a; Appendix IV) and (iii) the PSII reaction centre having three redox states, that is, requiring two quanta for complete reduction (Morin, 1964, Lavorel, 1972)

$$L \xrightarrow{h\nu} M \xrightarrow{h\nu} N$$

where N is the fully reduced form of L, as compared to the two state reaction centre in the Duysens and Sweers hypothesis (Duysens

and Sweers, 1963):

$$Q \xrightarrow{h\nu} Q^-$$

However, when the system is in the low fluorescing state the induction tends to be exponential (fig.19) which, in terms of the three models presented above will entail either (i) a decrease in the probability of intra- PSII exciton transfer, (ii) a change in the ratio of PSII deactivation rate constants (Appendix IV) or (iii) a change from a three to a two state reaction centre.
A variation in the probability of intra-PSII energy transfer, p, can be related to changes in fluorescence in either of two ways, depending on the model chosen for the photosynthetic unit. In the uniform pigment matrix model, if energy transfer between bulk pigment molecules is relatively slow (for instance by the Forster very weak coupling mechanism, see section 1.2.4) then the distance that the exciton may migrate through the pigment bed will be limited by the fluorescence lifetime. If the fluorescence lifetime is increased, then the exciton diffusion distance will also increase and the probability of the exciton being able to migrate far enough to encounter more than one trap will be enhanced. On the other hand, it may be imagined that the photosynthetic units are described by a modified independent units model (Paillotin,1972) and that the independent nature of the units arises because each unit is composed of a heterogeneous array of pigment molecules, as in the model put forward by Seely (1973a,b). In this model the chlorophyll molecules whose absorption maxima are at short wavelengths form the periphery of the photosynthetic unit whilst those which absorb at longer wavelengths, together with the reaction centre, form the centre of the photosynthetic unit. Free exciton migration between photosynthetic units is then inhibited because this heterogeneous

arrangement of pigment molecules decreases the overlap integral for energy transfer in the direction (long wavelength pigments → short wavelength pigments) that would take the exciton out of the photosynthetic unit and into a neighbouring one. In this model the probability p will also be a function of fluorescence lifetime insofar as the probability of surmounting the activation energy barrier will be a function of the number of attempts to do so.

The model of free excitation energy transfer, which links changes in p to alteration in the ratio of the rate constants for photochemical capture and for deactivation, will clearly also lead to p being a function of fluorescence lifetime.

However, the third model for changing the shape of the fluorescence induction entails a change from a three-state to a two-state reaction centre. Suppose that under normal (high-fluorescing) conditions the PSII reaction centres are a mixture of the two-state and three-state types. These reaction centres will give rise to a sigmoid fluorescence induction. Suppose also that the various treatments applied in the experiments shown in fig.19 have the effect of blocking the three-state reaction centres in a non-fluorescent state. This would give both a decrease in fluorescence yield and an increase in the exponentiality of the fluorescence induction. It would not, however, give a change in fluorescence lifetime unless the units with three-state reaction centres had a longer fluorescence lifetime than those with a two-state reaction centre. Such a model of a mixed population of units with different fluorescence lifetimes has, however, been ruled out by the experiments on the simultaneous measurement of fluorescence yield and lifetime of Tumerman and Sorokin (1967) and

Briantais et al. (1973) (see section 1.6.1).

To distinguish between the first two and the third models presented above it is therefore necessary to establish whether the changes in fluorescence yield are associated with a change in fluorescence lifetime.

3.2.5.2 Changes in fluorescence yield. The intensity of fluorescence, f, observed from PSII is given by

$$f = \phi_f \alpha_{II} I \qquad 48$$

where ϕ_f is the fluorescence yield, I is the incident light intensity and α_{II} is the fraction of incoming photons that are directed to PSII. Clearly, changes in fluorescence can be due to changes in either ϕ_f or α_{II}. Because of the relationship between fluorescence yield and fluorescence lifetime, the changes in fluorescence due to variation of the value of α_{II} will be independent of fluorescence lifetime.

Concomitant changes in fluorescence yield and lifetime can be detected in the following way: the integral relation for determining the amount of oxidant associated with PSII is (Appendix I)

$$[Q] = \frac{\alpha_{II} I}{f_{vmax}} \int_0^\infty \left[f_{vmax} - f_v(t) \right] dt \qquad 49$$

where f_{vmax} is the maximum value of the variable fluorescence, and $f_v(t)$ is the variable fluorescence at time t. For a DCMU-blocked system $[Q]$ will be equal to the amount of oxidised primary electron acceptor available to be reduced. The derivation of this equation takes into account changes in any of the rate constants for the decay of an exciton in PSII. Equation 49 is useful since it predicts that the ratio

$$\frac{\int_0^\infty \left[f_{vmax} - f_v(t) \right] dt}{f_{vmax}} = \frac{A}{f_{vmax}} = \frac{\text{area over the fluorescence induction curve}}{\text{maximum variable fluorescence}} \qquad 50$$

is invariant when [Q] is constant, except for changes in α_{II}. In particular if the flourescence yield ϕ_f is changed due to alteration of any of the rate constants in eq. 18, eq.49 predicts that there will be no change in A/f_{max}. Comparison of the values of A/f_{vmax} in the high and low fluorescing states will therefore give a means of distinguishing between changes in fluorescence due to ϕ_f or α_{II}.

However, the experimental determination of A is subject to errors from two sources. The smaller error is due to the slow increase in fluorescence in DCMU-blocked systems shown in fig. 18. By estimating the uncertainty of the final level of fluorescence this error is found to be in the region of 8%. A more serious source of error arises because eq.49 assumes that the real zero of variable fluorescence may be located accurately. That this is so may be tested by examining the data on DNB-induced quenching in Table V. At low concentrations DNB is thought to act as a trapping centre in the bulk chlorophyll molecules (Etienne et al., 1974). If so, then the action of DNB can be thought of as adding another term to the denominator or the expression for fluorescence yield (eq.18):

$$\phi_f = \frac{k_f}{k_f + k_h + k_{tI} + k_p[Q] + k_Q[C]} \qquad 51$$

where k_Q is the rate constant for DNB-induced quenching and [C] is the concentration of the quencher. DNB thus acts by changing ϕ_f, and should also change the fluorescence lifetime: this hypothesis has been confirmed by Moya (1973, cited by Etienne et al., 1974) in an experiment measuring fluorescence lifetime and fluorescence yield simultaneously. However, Etienne et al. (1974) have observed that the ratio A/f_{vmax} decreases when f_{vmax} increases, and this effect is confirmed by the data presented in Table V. Etienne et al. (1974)

postulated that this was due to the true zero of variable PSII fluorescence being located below the f_o level, a suggestion supported by the fact that the values of A/f_{vmax} in Table V may be made much more consistent by assuming that 30 - 50% of the f_o level belongs to f_v. Unfortunately, it appears difficult to make a quantitative correction for the real zero level of variable fluorescence since it changes with chloroplast preparation.

Bearing in mind that the measurement of A/f_{vmax} is subject to these two sources of error, the data in Table IV may be examined. Experiments A, B and C show a decrease in A/f_{vmax} in going from the low to the high fluorescing state. In experiment B (monovalent cation-induced fluorescence decrease) this decrease is 9.5%, and in experiment C (DNB-induced fluorescence decrease) it is 7%: both these differences can easily be accomodated by the sources of error in A/f_{vmax} mentioned above. In experiment A (divalent cation-induced fluorescence increase) the change in A/f_{vmax} is greater, 21%, but since the change in f_t is large this difference can still be accounted for by including 25 - 30% of the f_o level in the variable fluorescence used to normalise A.

In contrast to these experiments, the data of experiment D show that when the fluorescence is quenched by PSII light in intact chloroplasts, A/f_{vmax} also decreases by 10%. This can be explained by either (a) an increase in α_{II} on going from the high to the low fluorescing state or (b) a small part of the primary electron acceptor not being reoxidised in the 10s dark period between the preillumination and the addition of DCMU prior to the fluorescence induction. The first possibility is unlikely, since an increase in α_{II} predicts an increase in fluorescence yield, and a decrease is seen. The incomplete

reoxidation of Q^- is therefore offered as an explanation for the decrease in A/f_{vmax} seen in experiment D.

In these four experiments the largest change in A/f_{vmax} was 21%, and this was accompanied by a fluorescence decrease of 73%. If the change in fluorescence was due to a change in α_{II}, an increase of 73% in A/f_{vmax} would be expected. While the date of Table IV do not rule out changes in α_{II} as a contributory factor to changes in fluorescence, they clearly show that the main part of the fluorescence changes in all the experiments shown in fig.19 must be due to variations in true fluorescence yield of the PSII chlorophyll molecules.

For the divalent cation-induced increase in PSII fluorescence this conclusion is in broad agreement with the work of several authors. Murata (1969b), Briantais et al (1973) and Mohanty et al. (1973) have all suggested that the increase in fluorescence is due to a decrease in k_{tI} ('spillover'); that is, due to an increase in fluorescence yield. Similarly, Malkin and Siderer (1974) have suggested that the change in fluorescence is due to a decrease in k_{tI} or k_h. The experiments described here do not allow a distinction to be made between changes in k_{tI} and k_h so none of the above hypotheses can be preferred. Very recently Butler and Kitajima (1975b) have proposed that the fluorescence increase on adding divalent cations (measured at liquid nitrogen temperature) is due to a decrease in k_{tI} together with a small increase in α_{II}. As shown above, this conclusion is also compatible with the results presented here.

The data given here present new evidence that the addition of a low concentration of a monovalent cation to chloroplasts suspended

in a cation-free medium increases k_{tI} or k_h: this conclusion is in overall agreement with that of Gross and Hess (1973) although from low-temperature fluorescence data they were able to postulate that the change was in k_{tI} alone.

In addition the result on the light-induced fluorescence quenching of intact chloroplasts implies that this fluorescence change is due to an increase in k_{tI} or k_h, or both (see also Telfer, Nicolson and Barber, 1976). This conclusion fits in well with other data presented by Bennoun (1974), Barber et al (1975) and Vernotte et al. (1975) on the similarities between the light-induced fluorescence changes in intact chloroplasts and algae and the changes in spillover in broken chloroplasts induced by cations.

3.2.5.3 Transfer of energy within the photosynthetic unit. It has been established that the changes in fluorescence illustrated in fig.19 are probably due to changes in fluorescence yield, which involve a change in fluorescence lifetime. The two state to three state reaction centre model of variability in the shape of fluorescence induction is therefore not tenable. The observed fluorescence changes may then be due to either of the two other models put forward in section 3.2.5.1, and which can be classed broadly as models of free or of restricted excitation energy transfer.

The model for free excitation transfer predicts that the value of p is given by (Appendix IV):

$$p = \phi_{vmax}/\phi_{tmax} \qquad 52$$

However, as shown in Table V the measured values of p and the ratios ϕ_{vmax}/ϕ_{tmax} at various concentrations of added DNB are not in good agreement. If it is assumed that a part of the f_o

level arises from a source outside PSII, the agreement becomes even less good. For this reason it appears that, at least for the experiments described here, the free energy transfer model is also untenable. The concept of restricted energy transfer, with changes in the degree of connection between PSII photosynthetic units is thus preferred to account for the variability of the prompt fluorescence induction. Two possibilities for this model have been suggested in section 3.2.5.1: one based on the uniform pigment matrix model of the photosynthetic unit, and one based on the separate model. The latter model includes barriers to free exciton diffusion between photosynthetic units due to the pigments which compose the photosynthetic unit having an inhomogeneous spatial distribution. By considering the exciton as a particle executing Brownian motion within the apparent potential energy well that such a photosynthetic unit constitues, it can be seen that p should be a function of temperature in the separate units model (Paillotin,1972).

The results of experiments designed to measure the sensitivity of p to temperature are presented in section 3.2.4. The Arrhenius plot of log p versus 1/T, where the values of p have been corrected for changes in fluorescence yield (fig.24), show that the apparent activation energy barrier to intra-PSII exciton transfer is small: 0.027 ± 0.02eV. At room temperature this value is comparable with kT so, again regarding the exciton as a Brownian particle, free exciton migration would be expected.

A result which supports this model is that obtained by Okayama and Butler (1972). These authors quenched fluorescence from spinach chloroplasts at -196°C by adding potassium ferricyanide to the chloroplast suspending medium. They observed that the

induction was fairly exponential in the high fluorescing state and that there was apparently no change in the induction shape when the fluorescence had been quenched. This can be accounted for by considering that kT at -196°C is one quarter of that at room temperature, and the apparent activation energy for exciton transfer between PSII photosynthetic units now becomes significant. If in the high fluorescing state p = 0.5 at room temperature, then at -196°C p = 0.125 assuming E = 0.027eV. When the variable fluorescence is quenched by 50% at low temperature, p will decrease to about 0.06 (fig.21), and the difference between these two values of p expected at low temperature will be very small when reflected on the fluorescence inductions.

If the uniform pigment matrix model is suitable to describe the photosynthetic unit at room temperature, then the observation that an increase in fluorescence (lifetime) of about 50% can increase the probability of an exciton migrating far enough through the bulk pigments to encounter more than one trap implies that energy transfer through the bulk pigments must be relatively slow. The linear relationship between p and fluorescence can then be accounted for by the following model: from elementary two-dimensional random walk theory the average distance, R, walked from the starting point is given by

$$R \propto n^{\frac{1}{2}}$$

where n is the number of steps taken. In a two-dimensional model, if the traps are randomly distributed through the pigment bed, then the number of traps encountered will be proportional to R^2, so

$$\text{number of traps encountered} \propto n$$

and since n is a linear function of fluorescence lifetime (and hence yield)

$$\text{number of traps encountered} \propto \phi_f$$

and hence

$$p \propto \phi_f$$

as found in fig.21.

This analysis is, however, a little simplistic since it assumes that trapping by open PSII reaction centres is perfect. This may not be so, and Paillotin (1974, 1976) has made a careful theoretical study of the more general case where the probability of exciton transfer from a PSII photosynthetic unit with a closed trap to another PSII photosynthetic unit (here defined as P) is a function of two parameters. These parameters are (i) the probability p introduced by Joliot and Joliot (1964) whose significance now is as a measure of the competition between different reaction centres for capturing the excitation, and (ii) a parameter L' which is defined as the probability for the exciton to be transferred from one PSII photosynthetic unit to another. L' will be a function of the redox state of the photosynthetic unit from which the exciton is transferred, and its temperature dependence will depend on the heterogeneity of the pigments making up each photosynthetic unit. By assuming that all fluorescence ($f_o + f_v$) arises from PSII, then it can be shown that p and P are related through

$$p = P(1 - \phi_o/\phi_{tmax}) \qquad 53$$

(Paillotin, 1974; 1976), where p is measured from the ϕ_v versus (1 - Q) plots. The data presented in Table V show that P is a function of fluorescence, and that therefore (using similar arguments as earlier) excitation energy migration is restricted

in this model.

If the energy migration is restricted then, again, it could be due to either (i) slow energy transfer or (ii) activation energy barriers to exciton diffusion due to pigment inhomogeneity within the photosynthetic unit. Using the relation

$$P = \frac{L'}{(k_f/\ell_{tmax})+L'} \qquad 54$$

(Paillotin,1976) L' can be derived from P. Using eqs.53 and 54 values of L' at different temperatures have been derived from the date presented in Table VI, and from other similar experiments. Plotting these values of L' on Arrhenius plots yields a weighted mean value for the activation energy of 0.042 ± 0.05eV. This activation energy is not very much larger than kT at room temperature, so free excitation energy migration would be expected. Provided, therefore, that the means used to change fluorescence yield in the experiments illustrated in fig.19 do not alter L' itself, this model of the photosynthetic unit also implies that energy transfer in the pigment network of photosynthesis is slow. These experiments therefore give support to the model of slow transfer of excitons through the bulk chlorophyll pigments of PSII.

This result is important because there is very little <u>in vivo</u> data on the mechanism of excitation energy transfer. The evidence that exists comes from work on low-temperature spectroscopy of algae (Cho and Govindjee,1970a,b) and from work on the change in polarisation of fluorescence on addition of DCMU to algae (Mar and Govindjee, 1972; Whitmarsh and Levine, 1974).

Cho and Govindjee (1970a, b) carefully studied the absorption

spectra and the excitation spectra of chl a fluorescence between 4°K and 77°K in Chlorella and Anacystis and found that in Chlorella the efficiency of energy transfer from chl b to chl a and from chl a670 to chl a678 were independent of temperature although the efficiency of energy transfer from chl a to the reaction centre was probably temperature dependent. In Anacystis energy transfer from chl a670 to chl a678 was temperature independent whilst the energy transfer from phycocyanin to chl a was temperature dependent, being 20% lower at 4°K than at 77°K. The Forster very weak coupling mechanism of energy transfer (section 1.2.4) is expected to be a weak function of temperature, since the magnitude of the overlap integral will decrease as the absorption and fluorescence spectra of the pigments sharpen and shift with decreasing temperature. Although this temperature dependence is not large, it is probably this that is reflected in the results of Cho and Govindjee (1970a, b), which can be interpreted as showing a mixture of the weak and very weak coupling transfer processes depending on the pigments involved.

The polarisation of fluorescence from Chlorella and Porphyridium was measured by Mar and Govindjee (1972). These workers found that when DCMU was added to the algal suspension the degree of polarisation decreased and ascribed this, in the absence of an 'unconnected' chlorophyll pigment with highly polarised fluorescence, to there being a greater probability of energy transfer between pigment molecules when the fluorescence lifetime was increased on addition of DCMU. They thus postulated that energy was transferred by the Forster very weak coupling mechanism (section 1.2.4). Whitmarsh and Levine (1974) measured polarisation at various wavelengths using Chlamydomonas Reinhardi and found similar results which they also interpreted as indicating that the Forster very weak coupling

mechanism operates *in vivo* for those pigments absorbing below 660nm, although they could not rule out the possibility of the weak coupling mechanism playing a role.

The results of Mar and Govindjee (1972) and Whitmarsh and Levine (1974), taken together with those presented in this thesis, indicate that the Forster very weak coupling mechanism of energy transfer is probably involved in the transfer of excitation energy in the fluorescing chlorophyll pigments of PSII. This result is somewhat surprising in view of the theoretical arguments reviewed in section 1.2. There it was shown that the chlorophyll intermolecular spacing is of the order of 1.5nm and that as a result the molecular interaction energy is of the order of $60cm^{-1}$ (eq.5). This value of molecular interaction energy implies that the weak coupling mechanism is applicable. However, as noted in section 1.2.6, Knox (1973, 1975) has pointed out that there are at least two factors that may come into play to change the rate of energy transfer from the expected rate as calculated by Forster (Forster, 1948; eq.6). The first of these factors is involved when the intermolecular spacing decreases to such an extent that it becomes comparable with the size of the π-electron system. Knox (1975) estimates that at a spacing of 1.5nm between chl *a* molecules a correction of +50% to -30% (depending on mutual dipole orientation) may have to be made to the rate given by eq. 6.

The second factor that may change the transfer rate is mutual dipole orientation itself. The Forster equation (eq.6) assumes that the donor and acceptor molecules are randomly oriented, leading to the value of κ^2 being 2/3. Knox (1973) has pointed out that for an oriented system the rates of transfer in certain directions deviate from the average rate by large factors. The

range of corrections necessary for certain oriented arrays of dipoles is from 1/3 to 8 times the average rate ($\kappa^2 = 2/3$). This point is especially interesting in view of the recent work of Cotton et al. (1974) on *in vitro* analogues of the bulk chlorophyll pigments. These workers have found that in aliphatic hydrocarbon solvents chl *a* tends to form an oligomer $(\text{chl } a_2)_n$ where n can be up to 10 for concentrated solutions (10^{-1}M chl *a*) because the central Mg atom of the chlorophyll molecule has a co-ordination number of four, and one of its axial positions can be filled by the keto carbonyl oxygen function of ring V of another chlorophyll *a* molecule. At the same time the absorption spectra of the solutions have been carefully deconvoluted as a series of Gaussian components. On comparison of the absorption spectrum of a green alga (*Tribonema aequale*) containing only chl *a* and no other pigments, Cotton et al. (1974) found it to closely resemble that of the chlorophyll oligomer with a value for n of ~10. This suggests that the bulk chlorophyll molecules may be in an oriented array. Whilst it is premature to identify the chl *a* oligomer with the *in vivo* arrangement of chlorophyll molecules, it is conceivable that the arrangement might be such that excitation energy transfer is slowed because of the mutual dipole orientation of the donor and acceptor molecules. The concept of slow energy transfer is thus not incompatible with the data on high intermolecular coupling energies.

On the other hand this concept does not mean that the pigment bed is only composed of an array of chl *a*. It has been shown (section 3.2.4) that there is a small, but not negligible, energy barrier for excitation transfer between PSII photosynthetic units. The magnitude of this barrier (~0.027eV) is rather larger than the apparent activation energy for the Forster very weak coupling

mechanism which will be of the order of 6 meV (Paillotin, personal communication). However, it may indicate some pigment heterogeneity along the lines of that suggested by Seely (1973a, b) which will tend to restrict free excitation energy movement, especially at low temperatures, if the exciton is viewed as a Brownian particle.

It is also interesting to note that if excitation energy transfer in the chlorophyll pigments of PSII is slow, then it may be limited to such an extent that only two reaction centres are connected (i.e. there are photosynthetic 'domains' (Clayton, 1967) each containing two reaction centres) and the three-state hypothesis for the reaction centre of PSII (Lavorel, 1972) may be modified to include photosynthetic domains with three fluorescent states (both traps closed, one closed, both closed). Exponentialisation of the induction would then be a conequence of a decrease in fluorescence lifetime which limited excitation energy migration within the photosynthetic domain and give domains containing only one reaction centre. This type of model is attractive since, as noted before (section 1.6.1; Lavorel, 1972) it is able to predict the 'reprise' effect, a property not found in models incorporating two-state reaction centres (Lavorel, 1973).

The conclusion that energy transfer within the photosynthetic unit of PSII is relatively show also has consequences for delayed fluorescence. It has been discussed in section 1.5.1 that delayed fluorescence will occur with non-zero probability of emission if excitation transfer away from the trap is rapid (i.e. trapping is inefficient) or if energy transfer is slow (i.e. trapping must be efficient) but a triplet state is produced by the delayed fluorescence back reaction. Since the excitation transfer is not rapid, this gives support to the concept previously suggested that the

$J_T^{-\frac{1}{2}}$ decay of delayed fluorescence (section 3.1.2.2) may reflect a second-order triplet-triplet annhilation mechanism leading to delayed fluorescence singlet states.

In addition, the concept of relatively slow excitation energy transfer adds support to the assumption (section 3.1.3) that the delayed fluorescence emission factor is not a very strong function of fluorescence yield because the delayed fluorescence excitation will not be able to migrate over the whole pigment bed.

The intercept of the plot of f_v versus p will also give information about the zero level of PSII fluorescence. When p = 0 (i.e. there is zero probability of exciton transfer between PSII photosynthetic units) the exciton will still take a finite time to migrate from the place in the pigment bed where it is absorbed to the trap. Since this finite time implies a non-zero fluorescence yield, it would be expected that the zero level of PSII fluorescence lies below the intercept of the f_v versus p plot, that is below the f_o level (see following section).

3.2.5.4 An overall model for the fluorescence changes. The model of Joliot and Joliot (1964) which has been used extensively in this discussion is well able to account for the changes in the shape of the fluorescence induction by postulating changes in the probability for exciton transfer between PSII photosynthetic units. It has to be used with care, since the islet effect comes into play when more than a few traps have been closed. However, the results in fig.20 show that the islet effect is qualitatively a reasonable explanation for deviations from the prediction of eq.33 since these deviations are minimal at low values of p, as would be expected.

Nevertheless the Joliot and Joliot model cannot handle concomitant

changes in fluorescence and p. It may be modified to do so by extending the derivation in Appendix II to include a probability for exciton loss from PSII via either spillover or radiationless de-excitation. Calling this probability t, eq.33 modifies to (Appendix III)

$$\phi_v = \frac{(1 - t - p)(1 - Q)}{1 - p(1 - Q)}$$

The fluorescence yield at any value of $(1 - Q)$ is now dependent both on p and t. In particular, the maximum yield (all traps closed) is given by

$$\phi_{vmax} = \frac{1 - p - t}{1 - p} \qquad 55$$

as compared to $\phi_{vmax} = 1$ when eq. 33 is used.

Simultaneous changes in fluorescence yield and p can now be handled by changing both p and t. For example, the analysis of fig.20 shows that the values of p for the high and low fluorescing traces of fig.19 D are p = 0.63 and p = 0.38 respectively. By noting that in going from the high to the low fluorescing states the variable fluorescence has been quenched to 57.5% of the high fluorescing level, eq. 55 may be used to show that by assuming t = 0 in the high fluorescing state, the value of t in the low fluorescing state is 0.36. Modifying the Joliot and Joliot model in this way enables changes both in fluorescence yield and p to be taken into account, but involves altering the values of two parameters. These parameters may be linked by the experimental result (fig.21) which shows that the dependence of f_v on p is linear i.e.

$$f_v = kp + i \qquad 56$$

where k and i are constants. This equation makes no assumptions about the zero level of PSII fluorescence, it merely recognises

the linear relationship between f_v and p. Substituting from eq. 55,

$$\frac{1 - p - t}{1 - p} = kp + i \qquad 57$$

and hence

$$t = (1 - p)(1 - kp - i) \qquad 58$$

which links the probability for exciton transfer between PSII photosynthetic units to the probability for transfer out of PSII either by spillover or radiationless de-excitation. The values of p and t are known in the high and low fluorescing states in each pair of observations in Table IV, so eq. 58 may be solved simultaneously for k and i. The units are such that $f_{vmax} = 1$ in each case. The significance of the parameter i is that it indicates the zero level of PSII fluorescence. Analysing the results given in Table IV in this manner broadly indicates that the zero level of PSII fluorescence lies at or slightly above the zero level of the fluorescence emission measured at 692nm. This assignment of the zero level of fluorescence is not very accurate because (a) it has been arbitrarily assumed that t = 0 in the high fluorescing state, which is very probably not so, and (b) the calculation relies on the values of p which are not very accurate, and the values of t which have been derived from them. However, locating the zero level of PSII fluorescence at this value is in good agreement with the work of other authors. The experiments on fluorescence lifetimes (Moya, 1974) and the discussion presented in section 3.2.5.2 (see also Etienne et al., 1974) also imply that a part of the f_o level is associated with variable PSII fluorescence, although the fraction of the f_o level involved may be a function of the chloroplasts preparation (section 3.2.5.2; Malkin and

Siderer, 1974). The experiments of Kitajima and Butler (1975b) go further, and suggest that all of the f_o level is associated with photoactive chlorophyll, thus giving support to the hypothesis originally put forward by Delosme (1967) in which the f_o level of fluorescence originates from radiative decay of excitons migrating toward the PSII traps

3.2.6 Summary

1. The concept that the PSII photosynthetic unit is an invariant entity is no longer valid
2. Variations of fluorescence in DCMU-blocked chloroplasts shown in fig.19 all appear to be due to changes in fluorescence yield.
3. Variation in the probability of exciton transfer between PSII photosynthetic units is a function of fluorescence lifetime, which in turn is a function of the probability for exciton loss from PSII.
4. There is a small apparent activation energy (0.027 ± 0.02eV) for exciton transfer between PSII photosynthetic units.
5. A part of the f_o level is attributable to PSII variable fluorescence, but the precise location of the zero level of variable fluorescence cannot be accurately made, possibly because the fraction of f_o involved is a function of the chloroplast preparation.
6. Energy transfer between fluorescent bulk pigment molecules of PSII is relatively slow, which implies that since the interaction energy is quite high the mutual orientation of bulk pigment molecules does not favour rapid energy transfer.
7. The PSII photosynthetic units may be described at room temperature by the uniform pigment matrix model modified by the inclusion of some degree of pigment inhomogeneity, and exciton diffusion path lengths being limited by fluorescence lifetime.

3.3 The 518nm flash-induced absorbance change in intact chloroplasts

In broken chloroplasts the flash-induced absorbance change at 518nm (Δ518) has been suggested to be a measure of an electrical potential across the thylakoid membrane generated by the primary light-reactions (Junge and Witt, 1968; Witt,1975). This absorbance change in intact chloroplasts has been studied by using steady-state methods (Larkum and Bonner, 1972; Larkum and Boardman, 1974) but to my knowledge no studies have been undertaken on the flash-induced 518nm absorbance change in intact chloroplasts. Study of this absorbance change is worthwhile because the chloroplast outer membrane plays an important role in controlling the ionic environment of the thylakoid membranes (Gimmler, Schafer and Heber, 1975) which in turn has marked effects on the primary photochemical events of photosynthesis (Barber et al., 1975; section 3.2.5). Comparison of the extent of Δ518 in intact and broken chloroplasts should also indicate the relative magnitude of the light-induced membrane potential in these two systems. This comparison can easily be made since broken chloroplasts are prepared by osmotically shocking intact chloroplasts just before making measurements. In addition the rate of decay of Δ518 in the two systems should give a measure of the change in the passive permeability to ions of the thylakoid membranes after removal of the stromal phase.

The interpretation of flash-induced absorbance changes is simpler than that of steady-state changes. The latter are susceptible to artefacts due to chloroplast swelling and shrinkage associated with energy conversion processes. Indeed, the degree of light scattering at 520nm is often used as a measure of gross conformational changes of chloroplasts. With the flash method, however, these gross

structural changes are negligible because the actinic flash is so brief that no significant electron flow occurs during the flash sequence of measurement.

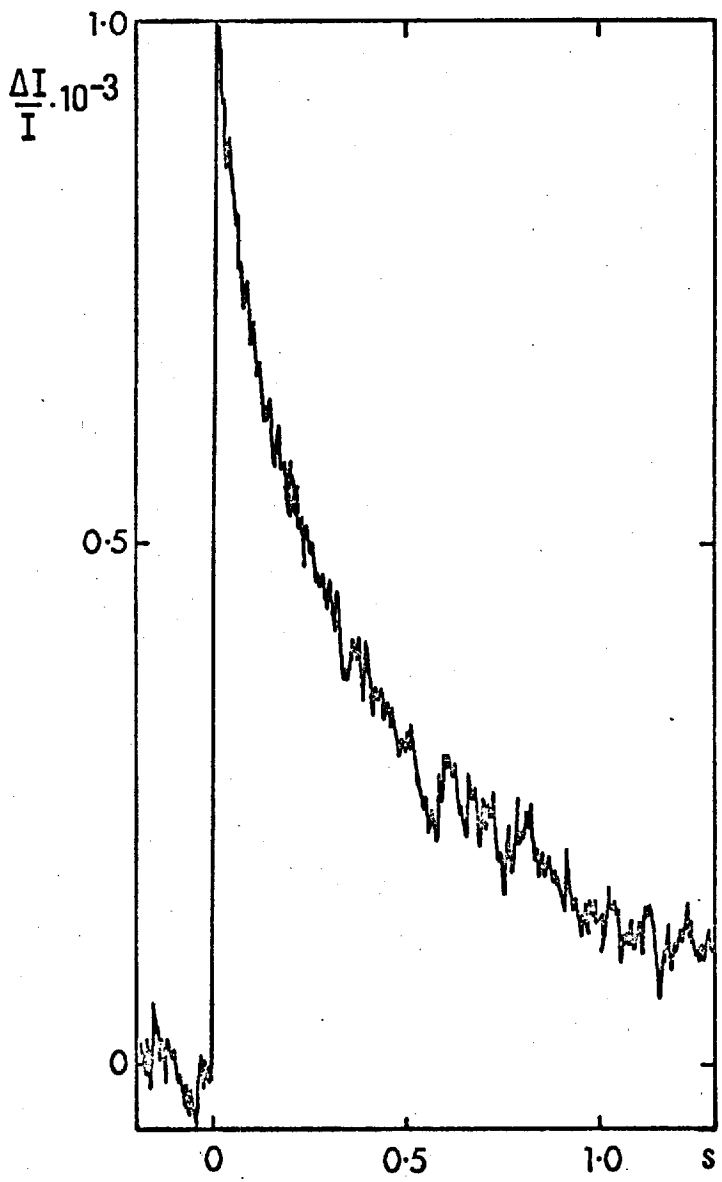
3.3.1. The flash-induced 518nm absorbance change in intact and broken chloroplasts, and the light-minus-dark difference spectra

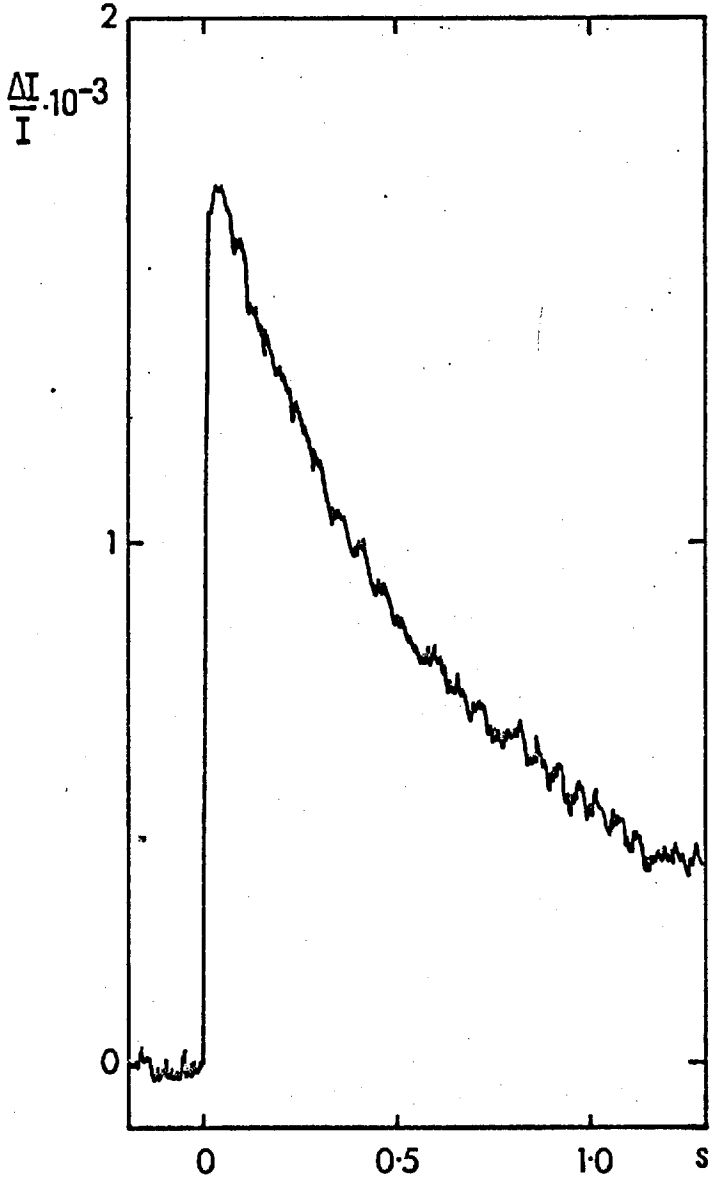
Figure 25 shows Δ 518 in intact and osmotically shocked pea chloroplasts measured with the flash spectrophotometer described in section 2.3. The two samples of chloroplasts were from the same batch. Intact chloroplasts were resuspended in assay medium; broken chloroplasts were obtained by shaking intact chloroplasts in water and adding an equal volume of double-strength assay medium. It can be seen in fig.25 that the overall extent of the absorbance change of intact chloroplasts is 1.6 times that of the osmotically shocked chloroplasts even though the chlorophyll concentration was identical for both measurements. The rise-time of Δ 518 from osmotically shocked chloroplasts is instrument limited (to 1.6ms, the address time of the signal averager). However, in the case of intact chloroplasts the rise appears to be biphasic. Figure 26 shows that for intact chloroplasts about 90% of the signal appears within the time resolution of the instrument (here 50μs) whilst the remaining 10% takes about 10ms to grow in. The biphasic nature of the rise was not always so clearly seen, however.

The decay of the absorption changes is shown in fig 27 where the traces of fig. 25 are presented as semilogarithmic plots. Both decays could be resolved into slow and fast first-order phases. The slow and fast phases of the intact chloroplast signal were found to have longer halftimes than those of the osmotically shocked chloroplast signal. While the absolute values of the

Figure 25

The time course of the flash-induced absorbance change at 518nm in osmotically shocked (A) and intact (B) pea chloroplasts suspended in assay medium. No additions were made to either sample. The intact chloroplasts were estimated to be 76% intact by the ferricyanide test (section 2.1.2). The signals are the result of averaging 128 decays in a signal averager. The signal into the averager was electrically filtered by a filter with an RC time of 27μs. Chlorophyll concentration 42μg ml^{-1}.


1·0
$\frac{\Delta I}{I}.10^{-3}$
0·5
0
0
0·5
1·0
s


2
$\frac{\Delta I}{I}.10^{-3}$
1
0
0
0·5
1·0
s

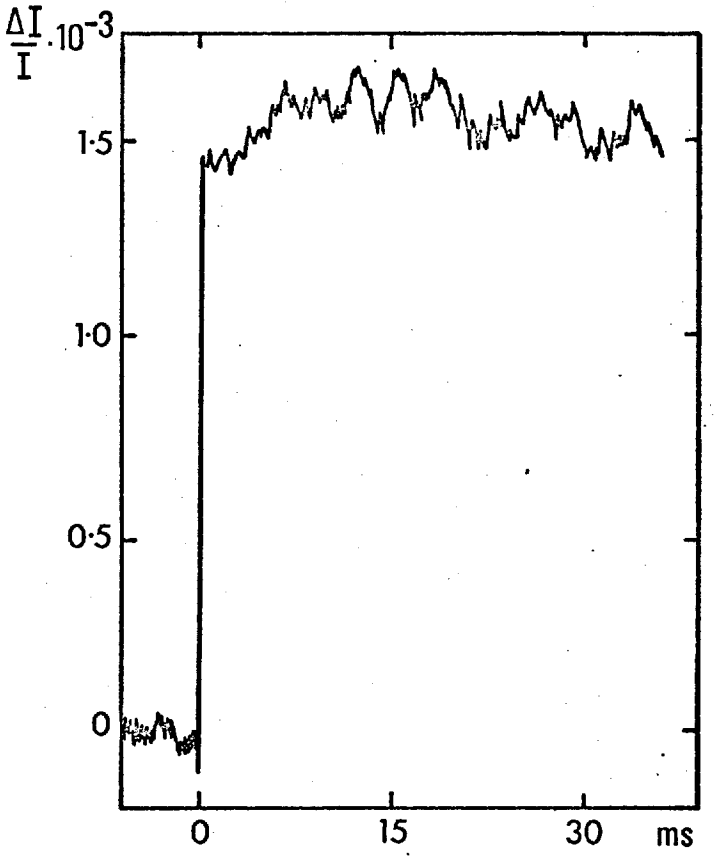


Figure 26.

Time course of the initial part of the flash-induced absorbance change at 518nm in intact pea chloroplasts suspended in assay medium with no additions. Chloroplasts estimated to be 76% intact. Chlorophyll concentration 42 μg ml^{-1}. Average of 200 decays, with no electrical filtering.

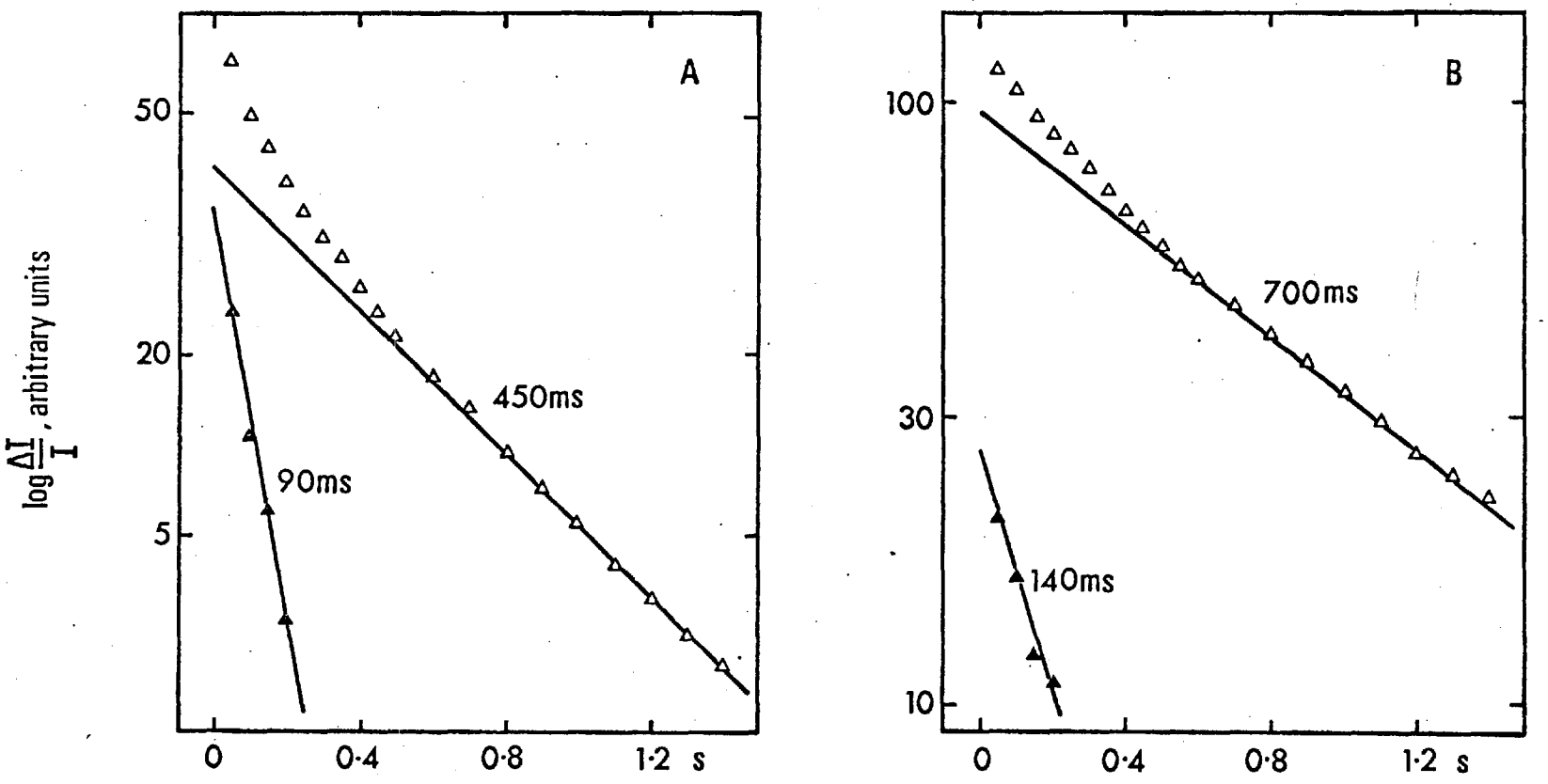


Figure 27

Semi-log plots of the time course of the flash-induced absorbance change at 518nm in osmotically shocked (A) and intact (B) pea chloroplasts. Data of fig. 25.

halftimes were quite variable, in all cases the Δ 518 measured with intact chloroplasts was larger and more slowly decaying than that of the osmotically shocked chloroplasts.

The difference in extent between Δ 518 measured with intact and shocked chloroplasts might be due to a shift in the light-minus-dark difference spectrum. However, as can be seen in fig.28 the uncoupler-sensitive light-minus-dark difference spectra for the peak of the change in intact and osmotically shocked chloroplasts are fairly similar. Therefore it must be concluded that Δ 518 is intrinsically larger in intact chloroplasts.

3.3.2. The effect of uncouplers of phosphorylation on the flash-induced absorbance change at 518nm.

Junge and Witt (1968) showed that ionophores which catalyse electrogenic ion movement across membranes, and other uncouplers of photophosphorylation accelerate the decay of the slow phase of Δ 518 in broken chloroplasts. The mode of action of these compounds is thought to be by allowing cations to diffuse across the thylakoid membrane down their electrochemical gradient, and hence to dissipate the light-induced membrane potential. In the absence of ionophores the membrane potential will decay more slowly because only the normal passive leakage of ions through the thylakoid membrane, governed by its intrinsic permeability, can occur.

Figure 29 shows the results of an experiment to measure the decay of Δ 518 in intact chloroplasts as a function of the concentration of valinomycin added to the chloroplast suspension. Valinomycin is an ionophorous antibiotic that makes artificial lipid membranes permeable to potassium (Greville, 1969). The addition of valinomycin appears to have two effects (i) it accelerates both the slow and

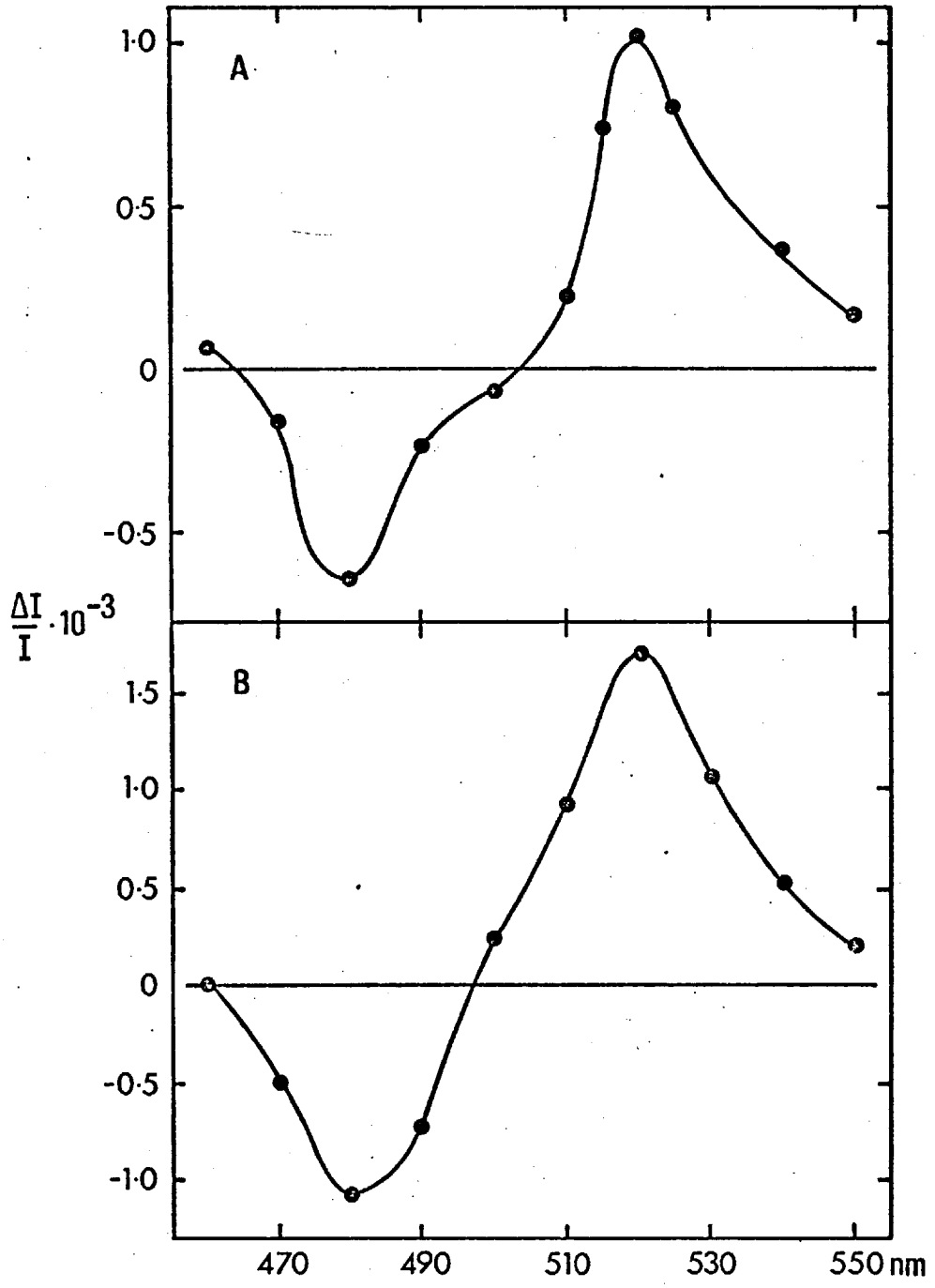


Figure 28.
The uncoupler-sensitive light-minus-dark difference spectrum of intact (A) and osmotically shocked (B) pea chloroplasts in assay medium. The spectra recorded in the presence of uncoupler (10^{-7}M gramicidin in A, 7.5. 10^{-7}M valinomycin in B) were subtracted from a control spectrum in each case. Intact chloroplasts estimated to be 76% intact.

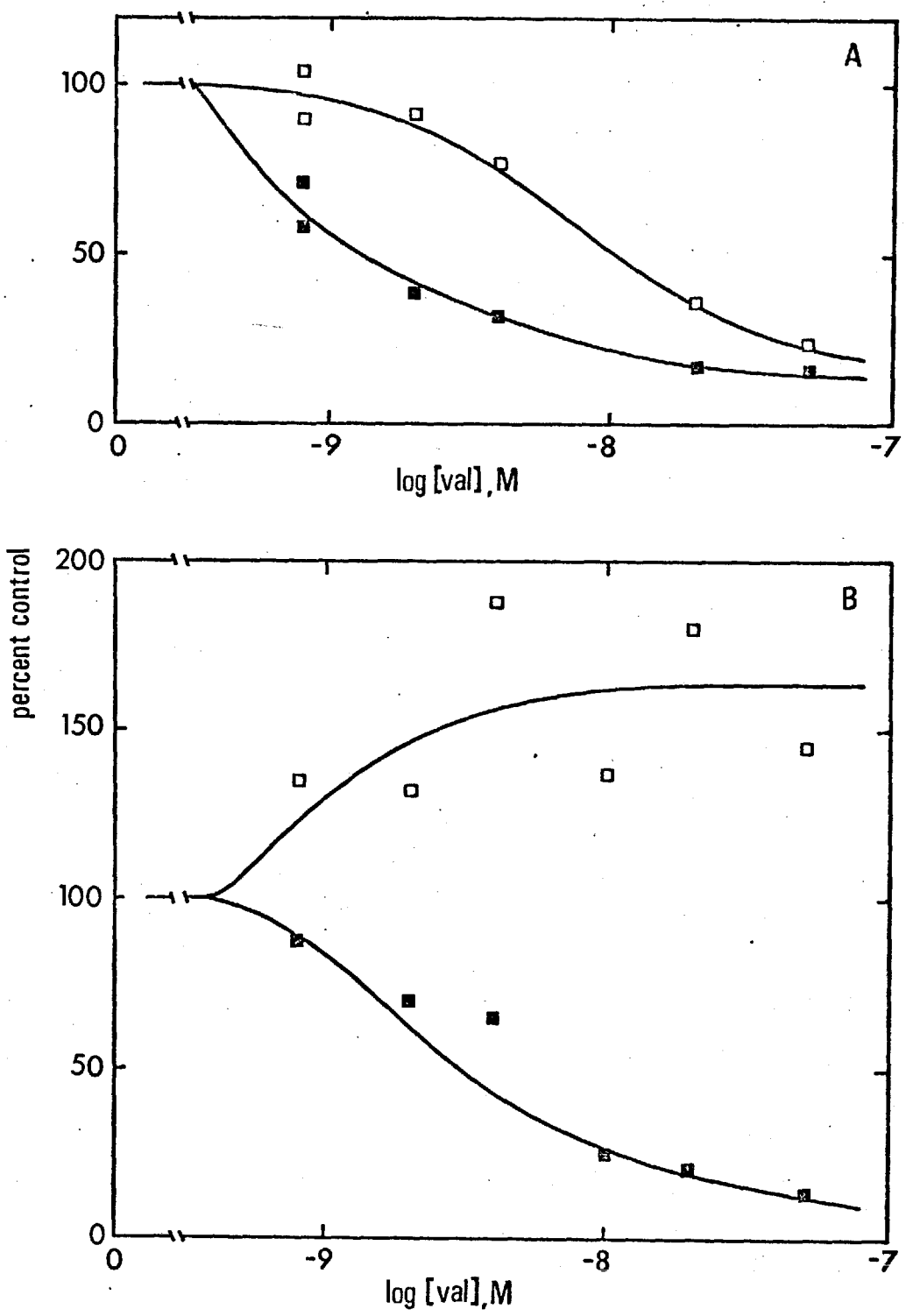


Figure 29

The effect of valinomycin on the flash-induced absorbance change at 518nm in intact pea chloroplasts suspended in assay medium. The decays were analysed as in fig. 27 and characterised by the extent and halftime of each of the two first-order phases into which the overall decay could be decomposed. A: slow phase, B: fast phase. Open symbols: extent; closed symbols: halftime. Chloroplasts estimated to be 60% intact.

fast phases of the decay and (ii) at the same time it converts the slow phase into the fast phase. This latter effect is shown by the decrease of the extent of the slow phase being accompanied by an increase in the extent of the fast phase.

A broadly similar effect is seen on adding CCCP, a compound which makes membranes permeable to protons (Greville, 1969), to osmotically shocked chloroplasts. Figure 30 shows that CCCP at concentrations less than 10^{-10} M gradually decreases the halftime of the decay of both the fast and slow phases and increases the extent of the fast phase at the expense of the slow phase. At concentrations above 10^{-10} M however, CCCP appears to inhibit both the fast and slow phases by decreasing their extents and halftimes.

The effect of CCCP on intact chloroplasts is not so clear (fig.31). As with broken chloroplasts increasing concentrations of the uncoupler gradually decrease the halftimes of both the fast and slow phases, but in the case of intact chloroplasts a decrease in the extent of the slow phase is not accompanied by an increase in the extent of the fast phase. In addition, at low concentrations of CCCP the Δ 518 of intact chloroplasts appears to be less sensitive to the uncoupler than broken chloroplasts. The ionophorous antibiotic nigericin is an uncoupler of a different type; it catalyses an electroneutral exchange of a proton for a potassium ion across membranes (Greville, 1969). In principle it would not be expected to change the potential gradient $\Delta\psi$, and hence not affect Δ 518. However, as fig. 32 shows, nigericin inhibits the extent of both the fast and slow phases of the Δ 518 decay in osmotically shocked chloroplasts and at the same time accelerates these decays (fig 32 C.D). In contrast, the extent of both phases of Δ 518 in intact chloroplasts appears to be unaffected by nigericin, but the halftimes of both phases are

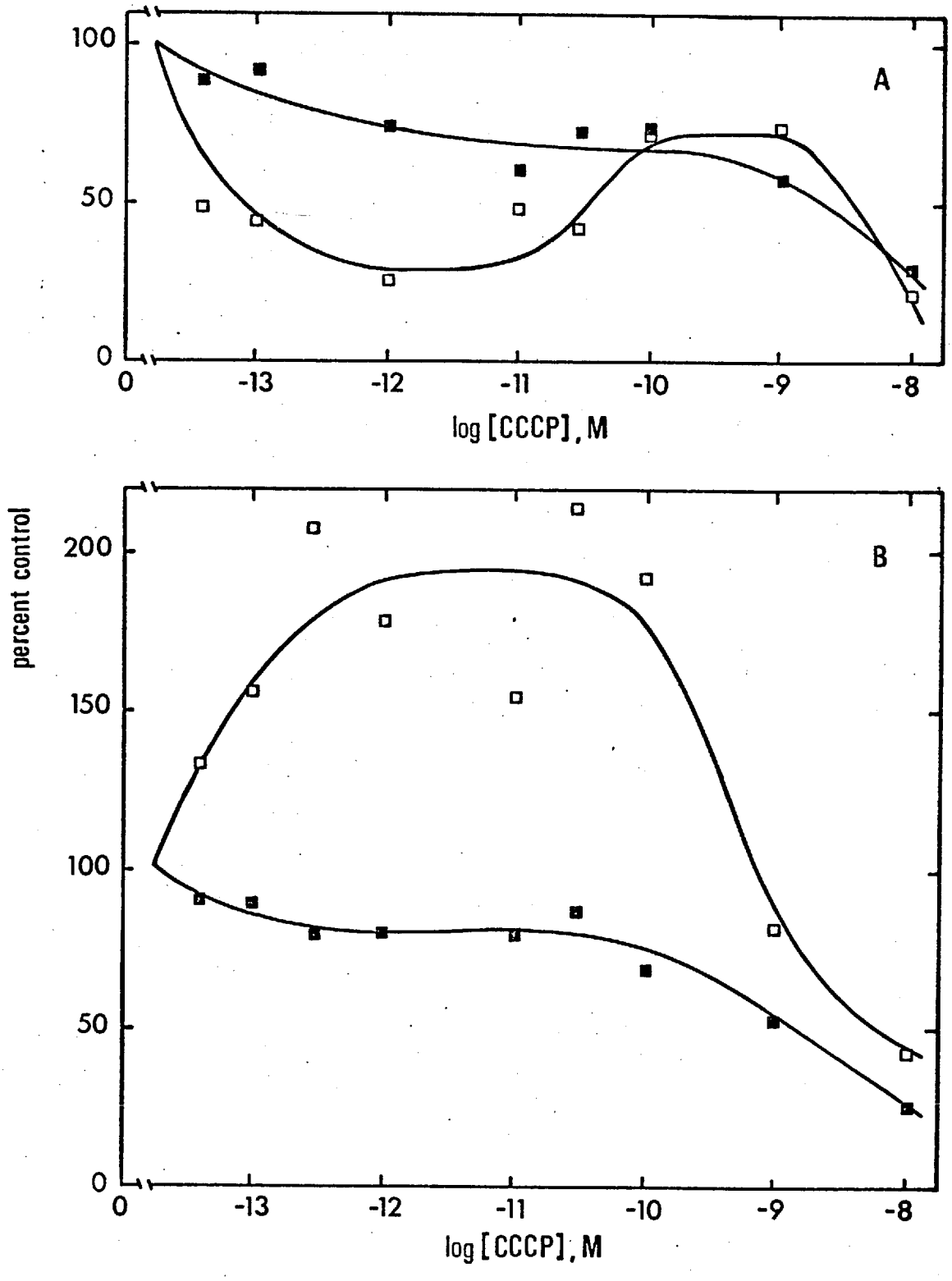


Figure 30

The effect of CCCP on the flash-induced absorbance change at 518nm in osmotically shocked pea chloroplasts suspended in assay medium. Analysis as in fig. 29. A: slow phase; B: fast phase. Open symbols: extent; closed symbols: halftime.

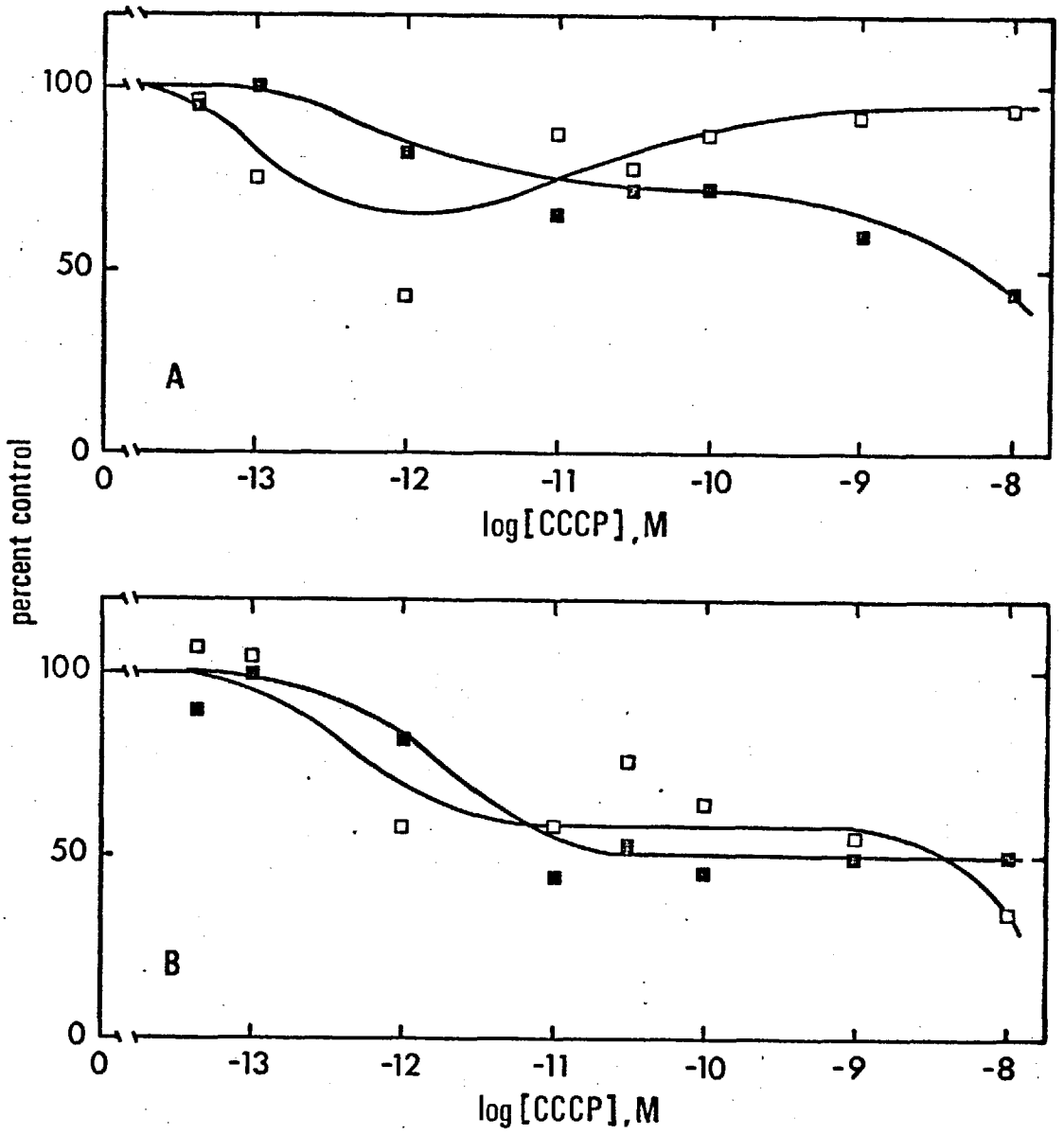
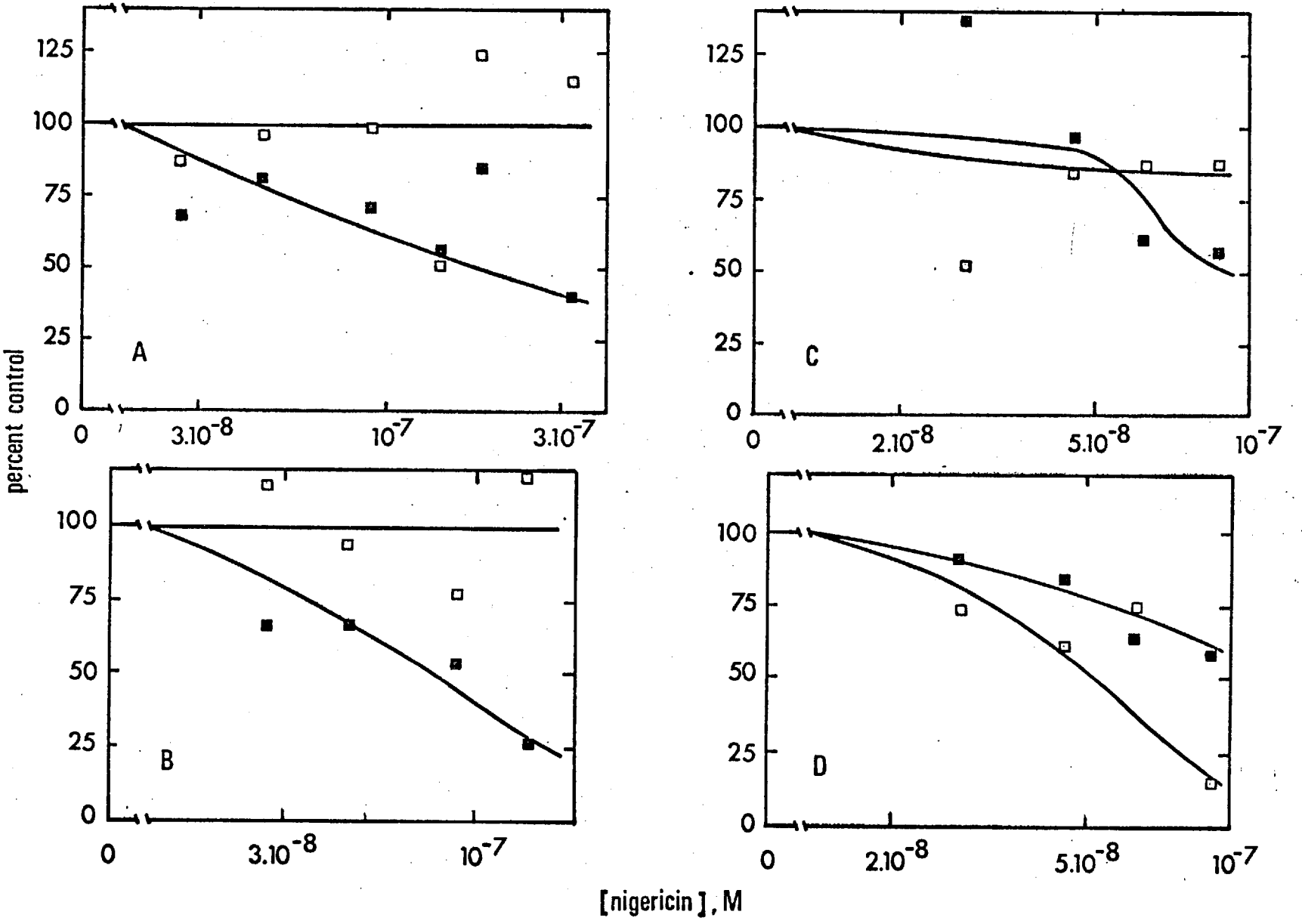


Figure 31.

The effect of CCCP on the flash-induced absorbance change at 518nm in intact pea chloroplasts suspended in assay medium. Analysis as in fig.29. A: slow phase, B: fast phase. Open symbols: extent, closed symbols: halftime. Chloroplasts estimated to be 64% intact.

Figure 32.

The effect of nigericin on the flash-induced absorbance change at 518nm in intact and osmotically shocked chloroplasts suspended in assay medium. Analysis as in fig.29. A: intact chloroplasts, fast phase; B: intact chloroplasts, slow phase; C: osmotically shocked chloroplasts, fast phase; D: osmotically shocked chloroplasts, slow phase. Open symbols: extent; closed symbols: halftime. Intact chloroplasts estimated to be 71% intact.


percent control
125
100
75
50
25
0
A
0
3.10-8
10-7
3.10-7
C
2.10-8
5.10-8
B
D
[nigericin], M

decreased about equally (fig. 32, A,B.).

3.3.3 The effect of DCMU on the flash-induced absorbance change at 518nm

In the presence of DCMU photosynthetic electron transport is blocked between the primary electron acceptor of PSII, Q, and plastoquinone, and Q^- can thus only be reoxidised by a back reaction within the PSII reaction centre. When Q remains in the form Q^- the PSII reaction centre is closed, and light-induced primary charge separation cannot occur. Therefore if the rate of flashing is sufficiently fast that in the presence of DCMU only a small fraction of Q^- can back react with the oxidising side of PSII, the action of this inhibitor should be to decrease the extent of Δ 518 without having any effect on the kinetics of the decay.

The data presented in fig.33 show that this prediction is essentially borne out in an experiment using intact chloroplasts where the flashing frequency was 0.5Hz. The extents of the slow and fast phases of Δ518 are inhibited with similar concentration dependence while the half times of these phases are affected much less.

3.3.4 The effect of pH on the flash-induced absorbance change at 518nm.

Thorne et al. (1975) have recently shown that the extent of the steady-state light-induced absorbance change at 518nm in broken chloroplasts is a function of the pH of the chloroplast suspending medium. However, the interpretation of their results was complicated by pH-induced changes in the structure of the chloroplasts. It was thus decided to investigate the dependence of Δ 518 on pH since measurements using the flash illumination technique are not subject to such difficulties.

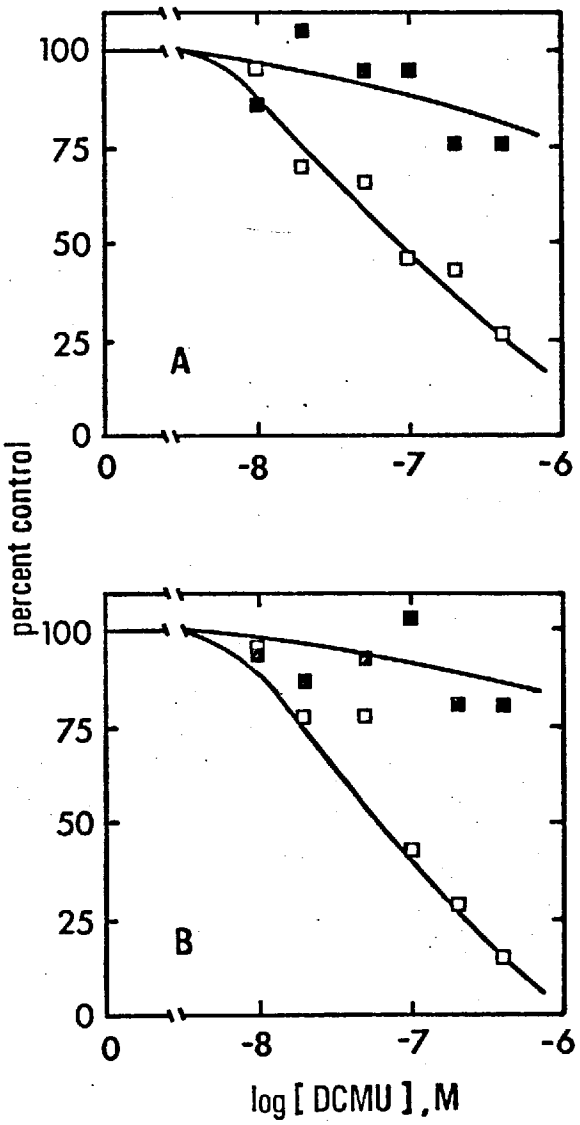


Figure 33.

The effect of DCMU on the flash-induced absorbance change at 518nm in intact pea chloroplasts suspended in assay medium. Analysis as in fig.29. A: fast phase; B: slow phase. Open symbols: extent, closed symbols: halftime. Chloroplasts estimated to be 68% intact.

Figure 34 shows the effect of changing the pH of the suspending medium on Δ 518 in intact and osmotically shocked chloroplasts. The extent of Δ 518 in both types of chloroplasts was maximal at pH7 - 7.5 (as compared to pH6.3 for the maximal extent of the steady-state response; Thorne et al., 1975). The pH dependence of the halftimes of the decay of Δ 518 was much more variable, however. In osmotically shocked chloroplasts the halftimes of both the fast and the slow phases peaked at about pH7.0. In intact chloroplasts the halftime of the fast phase gradually decreased as the pH of the suspending medium was raised while that for the slow phase showed a broad minimum at pH 6.5 and a maximum at pH 8.0.

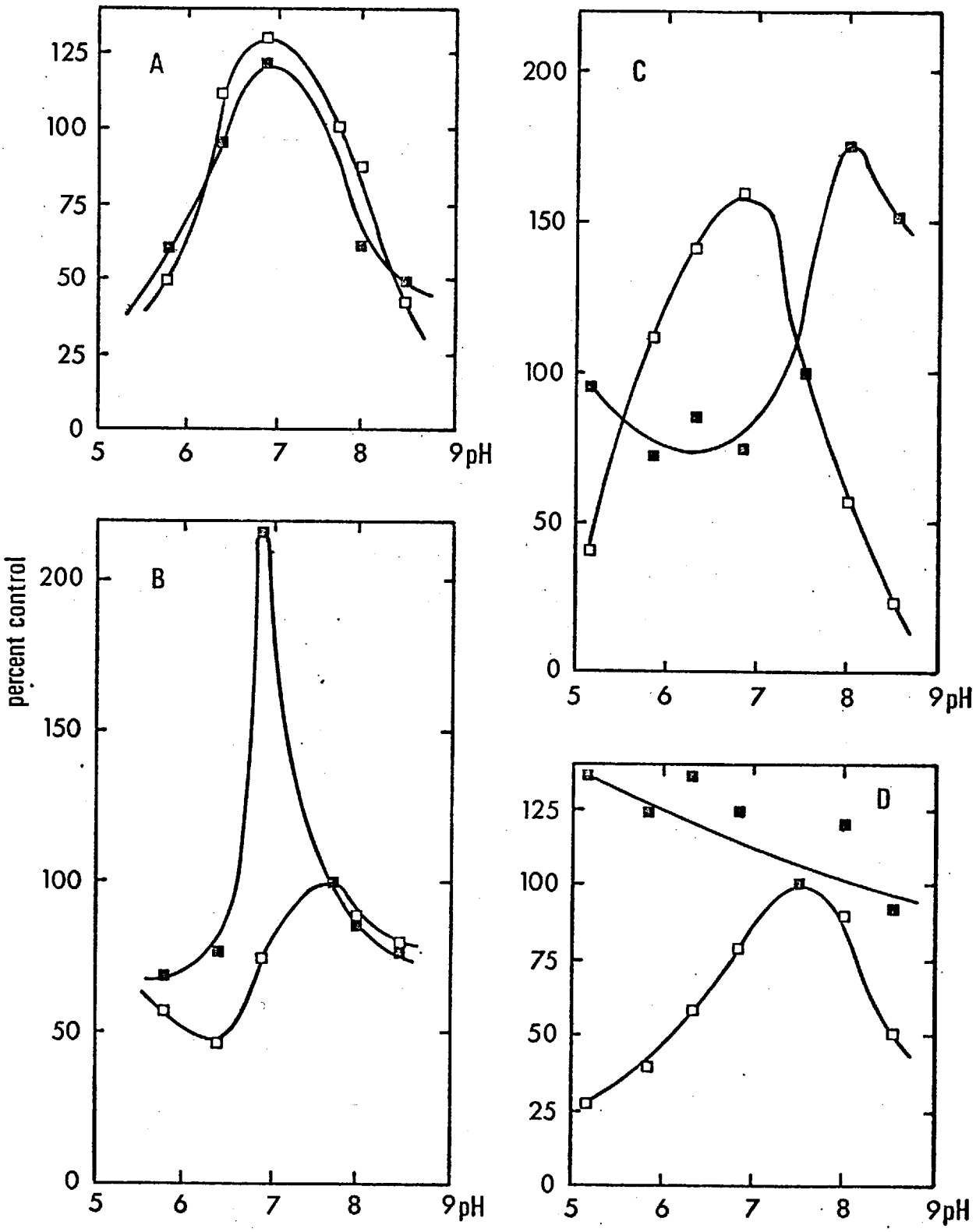
3.3.5 The effect of salts on the flash-induced absorbance change at 518nm in osmotically shocked chloroplasts suspended in a low-salt medium.

Barber et al. (1975) have shown that osmotically shocked chloroplasts may be 'reconstituted' to show fluorescence phenomena similar to those exhibited by intact chloroplasts by adding a few mM of a divalent cation or a rather higher concentration of a monovalent cation to the chloroplast suspending medium. In view of the differences between the extent and decay characteristics of Δ 518 in osmotically shocked and intact shloroplasts (section 3.3.1) it was of interest to see if Δ 518 of osmotically shocked chloroplasts could be made more like that seen in intact chloroplasts by the addition of KCl to the suspending medium.

Δ 518 in intact chloroplasts is both larger in extent and more slowly decaying than the corresponding change in osmotically shocked chloroplasts. The results presented in fig. 35 show that as the concentration of added KCl is increased there is an

Figure 34.

The effect of pH on the flash-induced absorbance change at 518nm in intact and osmotically shocked pea chloroplasts suspended in assay medium. Analysis as in fig.29. A: osmotically shocked chloroplasts, slow phase; B: osmotically shocked chloroplasts, fast phase; C: intact chloroplasts, slow phase; D: intact chloroplasts, fast phase. Open symbols: extent; closed symbols: halftime. The control was taken as pH 7.6, the standard pH for assay medium. Each sample was brought to the required pH by the addition of KOH or KCl. Intact chloroplasts estimated to be 53% intact.


A
B
C
D
percent control
125
100
75
50
25
0
200
150
100
50
0
5
6
7
8
9pH

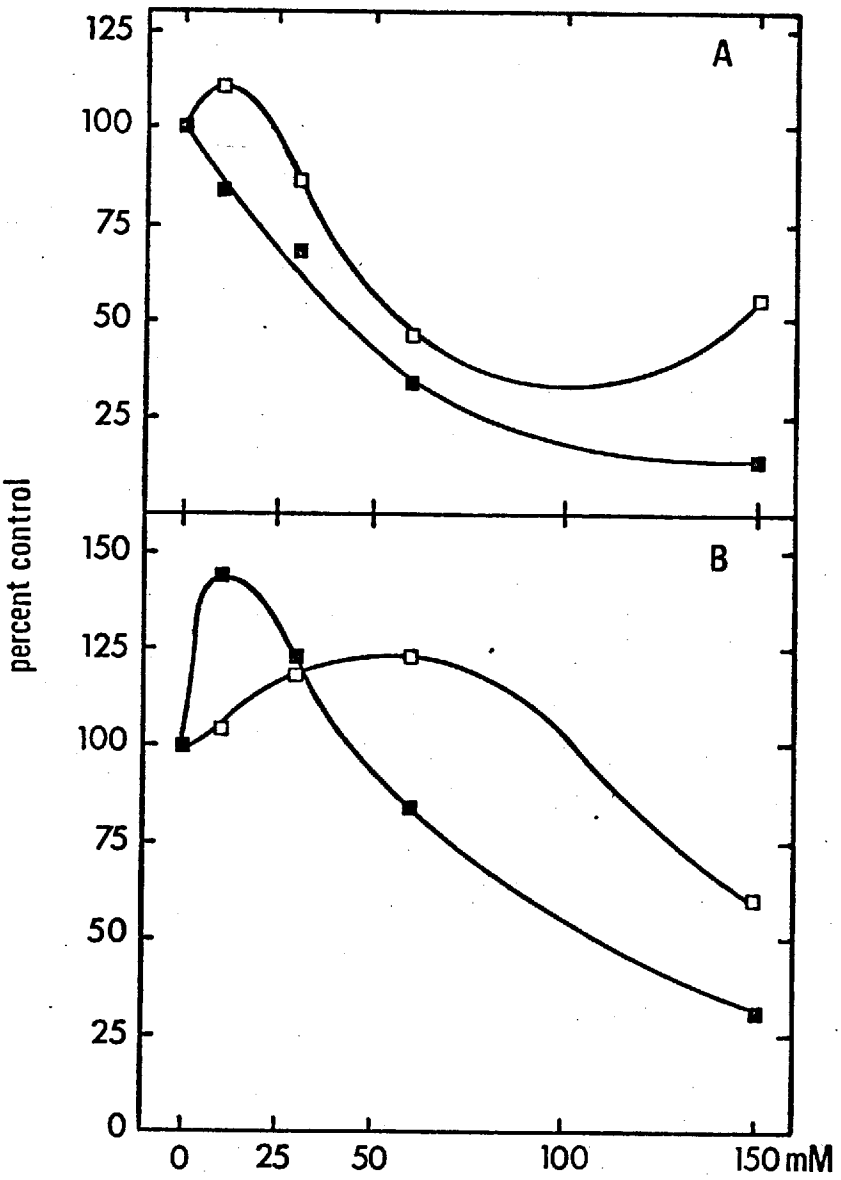


Figure 35

The effect of potassium chloride on the flash-induced absorbance change at 518nm in osmotically shocked pea chloroplasts suspended in low-salt buffer. Analysis as in fig. 29. A: fast phase; B: slow phase. Open symbols: extent; closed symbols: halftime.

increase in the extent of both phases of Δ 518 followed at higher concentration by a decline. The halftime of the fast phase also goes through a transient increase followed by a decline while the halftime of the slow phase decreases monotonically.

3.3.6 Discussion

The hypothesis of Junge and Witt (1968) that the flash-induced absorbance change at 518nm reflects the electrical potential generated across the thylakoid membrane by the primary light reactions now has relatively good experimental support. For example the in vitro studies of Schmidt et al.(1972) have demonstrated that the imposition of an electrical field across an artificial lipid multilayer containing chl a, chl b and carotenoids can well reproduce the light-minus-dark difference spectrum of Δ 518. In addition the onset of Δ 518 has a rise-time of $\leq$ 20ns (Wolff et al. 1969), a value which is compatible with Δ 518 being associated with primary charge separation in the reaction centres. The experiments of Witt and Zickler (1973), which showed that electrode measurements of an electrical potential imposed across a viscous suspension of broken chloroplasts correlated kinetically with measurements of Δ 518 add further support to this concept.

Given that Δ 518 does reflect the light-induced membrane potential then the in vivo results of Witt and co-workers may be examined. It has been well established that making the thylakoid membranes permeable to cations by, for example, the addition of an ionophorous antibiotic, accelerates the decay of Δ 518 (Junge and Witt, 1968). This result, together with the observation noted above that Δ 518 involves all the chloroplast bulk pigments, implies that the electric field initially formed by vectorial charge separation in

the reaction centre must be converted rapidly into a delocalised separation of charge across the thylakoid membrane. Witt (1971) suggests that this occurs by a proton binding to the negative charge at the outside of the membrane and a hydroxyl ion binding to the positive charge on the inside of the membrane (which amounts to net proton influx) leaving an H^+ and an OH^- to diffuse freely in the inner and outer phases respectively and to form a delocalised charge separation across the membrane (fig.36). It is this delocalised charge separation that may be abolished by proton carrying compounds like CCCP, or by compounds which allow other cation species to equilibrate across the membrane. The decay of Δ 518 should therefore be controlled by the rate at which ions are able to cross the membrane either through passive leaks, ionophore-catalysed diffusion or through the ATPase (Witt, 1971).

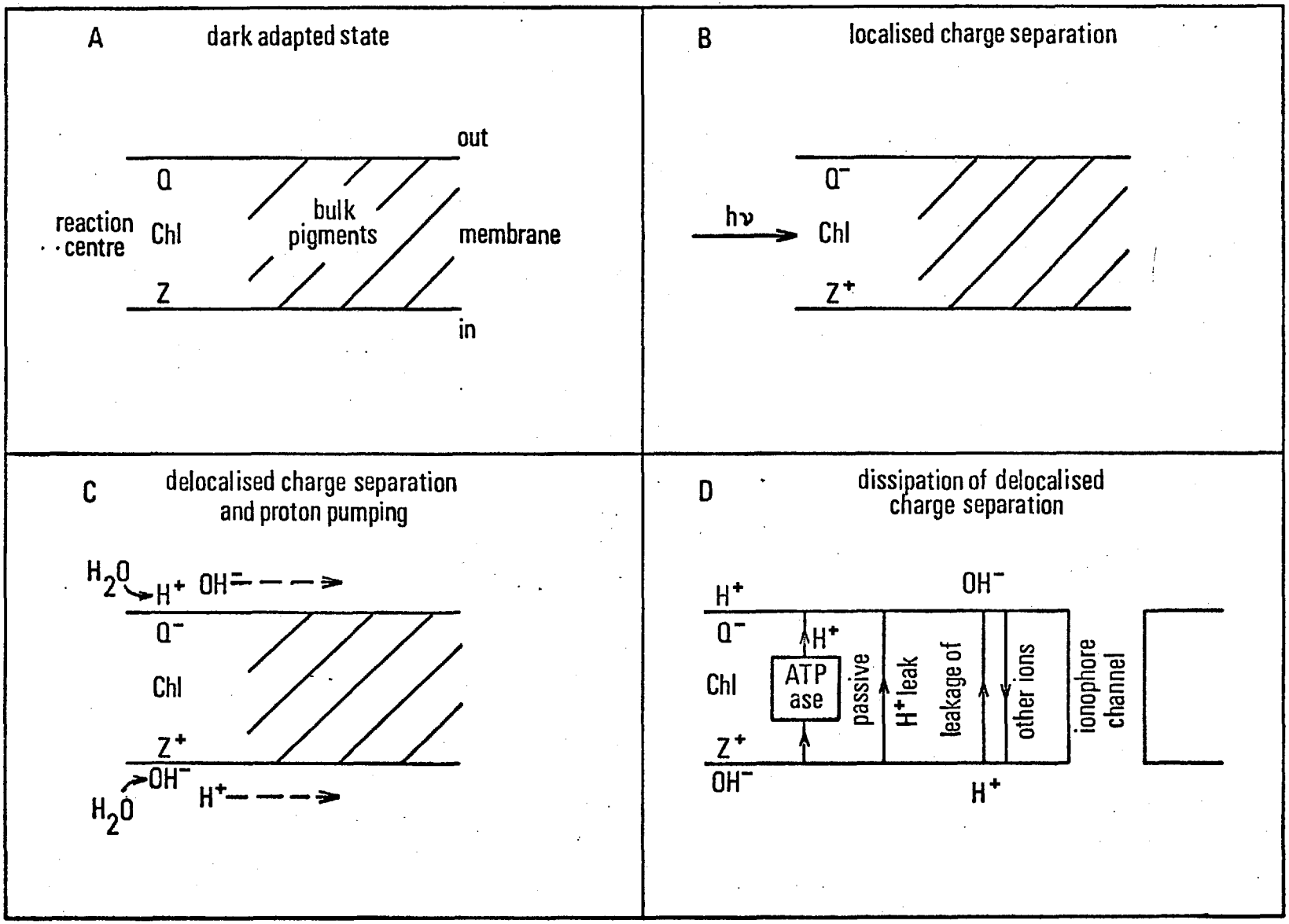
In broken chloroplasts the extent of Δ 518 has been related to the magnitude of the light-induced membrane potential by Witt (1971) who made some simple assumptions about the order of magnitude of the parameters of the thylakoid membrane. Given that the absorbance change ΔA is a linear function of the potential change $\Delta\psi$ (Shliephake et al., 1968), then

$$\Delta\psi = \frac{Q}{C} = a\,\Delta A \qquad 59$$

where Q is the charge translocated across the membrane whose capacity is C and a is a constant. If both photoreactions operate once only during the flash, the amount of charge translocated will be $2e^-$. In addition the area of the thylakoid membrane covered by one electron transport chain, S, has been estimated to be 10^5 $(\text{Å})^2$, and the thickness of the lipid layer of the membrane, l, is taken by Witt (1971) to be 30Å. Using an effective membrane dielectric

Figure 36

Schematic diagram showing the steps leading to the build-up and decay of the flash-induced absorbance change at 518nm. A: in the dark-adapted state the components of the reaction centre are in their resting state. It is postulated that the reaction centre is arranged in the membrane with the reducing half on the outside and the oxidising half on the inside (Witt, 1971). B: When a quantum of light is received by the reaction centre charge separation occurs rapidly, but is localised to the reaction centre itself. C: The charge separation is delocalised by the binding of hydroxyl ions to the positively charged site on the inside of the membrane and the binding of protons to the negatively charged site at the outside of the membrane. This leaves protons and hydroxyl ions free to diffuse in the thylakoid space and the stroma respectively, and at the same time constitutes the translocation of a proton into the thylakoid space. The delocalised charge separation now creates an electric field across the bulk pigments and the 518nm shift is caused by the electrochromic effect of this field on the pigments. D: The field may be dissipated, and hence the 518nm shift decreased, by four possible processes: (i) the leakage of protons from the thylakoid space through the ATPase, (ii) the passive leakage of H^+ through the thylakoid membrane, (iii) the passive leakage of other ions through the thylakoid membrane or (iv) the leakage of ions through ionophore-created channels through the membrane.


A
dark adapted state
out
Q
reaction centre
Chl
bulk pigments
membrane
Z
in
B
localised charge separation
Q⁻
hν
Chl
Z⁺
C
delocalised charge separation and proton pumping
H2O
H+
OH⁻
Q⁻
Chl
Z⁺
OH⁻
H2O
H+
D
dissipation of delocalised charge separation
H+
OH⁻
Q⁻
Chl
Z⁺
OH⁻
H+
ATP ase
passive
H+ leak
leakage of
other ions
ionophore channel
H+

constant of 2, eq. 51 yields:

$$\Delta\psi = a\Delta A = Q/C = \frac{2e}{2\epsilon\epsilon_o/l} = 2e/l\ \mu F = 50mV \qquad 60$$

for a single turnover flash. This equation predicts that the extent of Δ 518 should always be the same except when (i) the exciting flash is long, and the reaction centres turn over more than once (Shliephake et al., 1968); (ii) either or both of the photoreactions is inhibited (Shliephake et al., 1968); (iii) the thylakoid membrane is made very leaky by the addition of a high concentration of an ionophore such that the equilibration of cations through the transmembrane ionophore-channels occurs faster than the charge separation can be delocalised (Witt, 1975) or (iv) there is a change in the value of one or more of the parameters in eq. 60. Another mechanism which has been postulated to be responsible for changes in the extent of the steady-state absorbance change at 518nm, gross structural changes in the chloroplasts (Gross and Libbey, 1972; Thorne et al., 1975) is usually assumed to be insignificant using the flash-excitation technique.

Bearing these considerations in mind, the data presented in fig. 25 may be examined. The extent of Δ 518 in intact chloroplasts is about 1.6 times that of broken chloroplasts when the same experimental conditions and chlorophyll concentrations are used for the measurements. The change in extent is therefore not due to a change in flash duration, and the primary photoreactions are not likely to be inhibited by the removal of the chloroplast outer membrane. The smaller Δ 518 in broken chloroplasts could then be due to the thylakoids having been made very leaky on osmotically shocking intact chloroplasts.

This is shown to some extent by the faster decay of Δ 518 in broken chloroplasts (fig.27). Alternatively, since the area covered by one electron transfer chain is not likely to alter, variation in the membrane thickness or dielectric constant could cause a change in the extent of Δ 518.

The hypothesis that the thylakoid membrane is made leaky by osmotically shocking the outer chloroplast membrane is an attractive one, since not only could it reflect damage sustained by the thylakoid membrane on osmotic shock, but it could also indicate an intrinsic permeability of the thylakoid membrane to, say, potassium which is not exhibited in the intact chloroplast because the activity of that particular ion in the stroma is low. In fact, Telfer et al. (1975) have shown that potassium does not seem to be able to act as a counter ion for proton pumping in the stroma of the intact chloroplast under steady-state illumination. As for a change in membrane thickness, such changes have been observed and appear to be associated in steady-state light with proton pumping, or in the dark with a change in the pH of the chloroplast suspending medium (see Murakami et al., 1975, for review). Any change in thylakoid membrane thickness on osmotically shocking intact chloroplasts is probably rather small since the pH of the stroma ($\simeq$ pH8) is quite close to that of the assay medium (pH 7.6). Nonetheless, these two possible mechanisms are not mutually exclusive, and both may contribute to the smaller extent of Δ 518 seen in broken chloroplasts.

The above considerations cast some doubt on the validity of the calculation made by Witt (1971) using eq. 60. The assumptions used for the calculation on broken chloroplasts could just as easily be applied to the intact chloroplast which would also

result in a value for $\Delta\psi$ of 50mV despite the extent of Δ 518 being larger. The difficulty lies firstly in assigning arbitrary values to l and ϵ, and then postulating that these apply to the broken chloroplast, and secondly in assuming that the method of isolation of broken chloroplasts does not damage the thylakoid membranes. From the results presented here I would suggest that since the thylakoid membranes within the intact chloroplast (a) are more likely to be undamaged and (b) are in their physiological medium (the stroma) the calculated membrane potential of 50mV should apply more closely to the intact chloroplast and that Δ 518 in broken chloroplasts appears to indicate a smaller potential because, possibly as a result of damage sustained during isolation, a part of the potential across the membrane is dissipated as fast as it is delocalised.

The difference in the kinetics of the onset of Δ 518 in intact and broken chloroplasts is also in accord with this hypothesis. In intact chloroplasts the onset is biphasic (fig.26) whereas in broken chloroplasts the rise is limited by the time response of the signal averager. This result suggests that in broken chloroplasts the rapid initial decrease in the extent of Δ 518 masks the final slow increase. The observation that the half time of the decay of Δ 518 is less fast in intact as compared to broken chloroplasts (fig.27) can also be accomodated within the above hypothesis. The thylakoid membranes of broken chloroplasts may be damaged in such a way that as well as a very quickly decaying fraction of Δ 518, the slowly decaying phases are also accelerated to some extent. In addition the ionic composition of the stroma might contribute to the slow decay of Δ 518 since, as mentioned above, the activity of one of the major ions in the stroma appears to be low.

The physical basis of the biphasic rise of Δ 518 in intact chloroplasts could be similar to that proposed by Jackson and Dutton (1973) to account for the different phases of the onset of the field-indicating absorbance change in bacterial chromatophores. These workers suggested that the separation of charge across the membrane might take place in more than one electrogenic step because the primary charge separation might occur over, for example, half of the width of the membrane, and the charge separation would be completed by electron transport processes involving redox components that completed the span of the membrane. In this hypothesis the slow phases of the onset of the absorbance change reflect these secondary electron transport processes.

The effect of uncouplers and ionophores on intact chloroplasts can also be interpreted in terms of the acceleration of the decay of various phases of the decay of Δ 518. The action of valinomycin (fig.29) is to transform the slow phase of the Δ 518 decay into the fast phase, and at the same time to accelerate the decay of both phases. However, the analysis of these results is complicated by the sample being composed mainly of intact chloroplasts, but also including a significant fraction of broken chloroplasts (typically 25 - 30%). The effect of valinomycin on both these components must therefore be considered. In general the effect of valinomycin on broken chloroplasts is to accelerate the rate of decay of Δ 518 (Junge and Witt,1968),and this effect is seen in fig.29. In addition, however, there appears to be a similar phenomenon to that seen by Junge and Witt (1968) where very low concentrations ($\lesssim 10^{-10}$M) of gramicidin (i.e. at a concentration corresponding to one gramicidin molecule per thylakoid vesicle) caused the slow phase of the decay of Δ 518 in broken chloroplasts to be changed into the fast

phase. The results shown in fig.29 may thus be interpreted as the sum of these two effects: valinomycin increases the rate of decay of Δ 518 in broken chloroplasts and changes the membrane properties of the intact chloroplast thylakoid membranes in such a way that the slow phase of Δ 518 is transformed into the fast phase. The latter effect occurs at a somewhat higher concentration than that found by Junge and Witt (1968) for gramicidin, which suggests that the chloroplast outer membrane may act as a permeability barrier to valinomycin.

CCCP does not appear to have the same effect. When added to broken chloroplasts (fig.30) it does not decrease the halftime of either phase significantly at concentrations less than 10^{-10}M (i.e. at a concentration that corresponds to about one molecule of CCCP per thylakoid vesicle) but causes an increase in the extent of the fast phase at the expense of the slow phase. The effect of this compound on broken chloroplasts is therefore similar to the effect of valinomycin on intact chloroplasts. At concentrations above 10^{-10}M (i.e. where there is more than one CCCP molecule per thylakoid vesicle) the halftimes of both the fast and slow phases are increased, presumably because the thylakoid membranes become quite leaky, and at the same time the extent of both phases is decreased since delocalisation of the charge separation across the membrane is inhibited. In intact chloroplasts, however, the situation is somewhat different (fig.31). The halftime and extent of the fast phase is more sensitive to CCCP than that of the slow phase. This differential behaviour may again reflect the inhomogeneous nature of the intact chloroplast sample, but the results do show that the extent of the slow phase of Δ518 is less affected by CCCP than in the case of broken chloroplasts. It seems rather unlikely that this is due to CCCP not being

completely able to penetrate the chloroplast envelope, since it is one of the few compounds that is able to penetrate intact algae. The results of Krause (1974) and Barber et al. (1974) on the reversal of light-induced fluorescence quenching (see section 1.4.5.2) also indicate that at high concentrations (2.10^{-7}M) CCCP and the closely related compound FCCP are able to exert an effect at the thylakoid membrane of intact chloroplasts. It appears, then, that the less marked effect of CCCP (as compared to broken chloroplasts) could be due to the outer chloroplast membrane acting as a permeability barrier to low concentrations of CCCP and that the effect of this compound on the fast phase reflects its action on the broken chloroplasts present in the sample. Alternatively, the lack of effect could be due to the specific ionic composition of the stroma which is maintained by the chloroplast outer membrane. Since the outer membrane is to some extent permeable to protons, the presence of CCCP in the outer membrane should not modify the ionic composition of the stroma.

The effect of nigericin on Δ 518 in broken chloroplasts shown in fig. 32, which has been observed before (Neumann et al., 1970), appears surprising since this ionophore is thought to catalyse an electroneutral H^+/K^+ exchange across the thylakoid membrane (Shavit et al., 1968). However the acceleration of Δ 518 in broken chloroplasts may be accounted for in the following model. If chloroplasts are subjected to regular flash excitation a proton gradient will be built up across the thylakoid membrane if the rate of flashing is such that the decay of the proton gradient induced by each flash is not allowed to go on to completion (Boeck and Witt, 1972). The rate of flashing used in the experiment shown in fig. 32 was fairly slow (0.5Hz), but nevertheless could cause the build-up of a small

pH gradient (Boeck and Witt, 1972). This gradient is not normally dissipated since the thylakoid membranes must have low passive permeability to protons in order to maintain a pH gradient under steady-state illumination. If, however, the thylakoid membranes are more permeable to potassium than protons (as suggested by salt-pulse induced delayed fluorescence experiments; Barber and Warley, 1972), then the nigericin-facilitated exchange of K^+ for H^+ inside the thylakoid vesicle will lead to the leak of K^+ out of the thylakoid down its electrochemical gradient, and hence accelerate the decay of Δ 518. In the experiments shown in fig 32 this mechanism is probably quite effective, since the extent of Δ 518 is also decreased, presumably by the inhibition of charge delocaisation. In the intact chloroplast, however, the effect of nigericin is similar but not so marked on the extent of Δ 518. This could be due to the concentration of available K^+ in the stroma being limited by the amount that can be translocated by nigericin acting in the outer chloroplast membrane. In addition the more marked effect of nigericin on the decay of Δ 518 in intact chloroplasts could also indicate that the thylakoid membranes of intact chloroplasts have lower passive permeability to protons than the thylakoid membranes of broken chloroplasts.

DCMU modifies Δ 518 in intact chloroplasts in a manner that is in good agreement with the suggestion that this absorbance change is due to an electrical potential across the thylakoid membrane generated by primary charge separation (Junge and Witt, 1968). In the presence of DCMU the primary light reaction can occur only once, since Q is blocked in the state Q^- and the PSII trap is then closed. Similarly, the turnover of PSI will be inhibited once the pool of equivalents between PSII and PSI becomes exhausted. Therefore increasing concentrations of DCMU should progressively

inhibit the extent of Δ518, but not the halftimes of the decays, and this is essentially what is observed (fig. 33). Although the rate of flashing in this experiment was fairly slow ($\simeq$0.5Hz) it was presumably too fast to allow significant reoxidation of Q^- by the back reaction within PSII. Such reoxidation of Q^- in the presence of DCMU has recently been observed by Renger and Wolff (1975) who allowed sufficient dark time (30s) to elapse between actinic flashes. The extent of Δ518 they measured was not significantly inhibited by the presence of DCMU at this flashing frequency.

The effect of pH on Δ518 from broken chloroplasts shown in figure 34 is a relatively difficult experiment to interpret because of the large number of effects that changes in pH have on the functioning of the photosynthetic apparatus. In the following discussion three of these effects will be mentioned: the effect of pH on the rate of basal electron flow, loss of manganese from PSII and changes in the thickness of the thylakoid membrane.

According to the chemiosmotic hypothesis (Mitchell, 1966) the rate of electron flow in chloroplasts is controlled by the magnitude of the high-energy state of phosphorylation. When, under any given circumstances, the maximal high-energy state has been built up, this exerts a 'back pressure' which tends to inhibit electron flow. Under ideal conditions, the back pressure should completely halt electron flow, but in practice it is only slowed down, and the residual rate of flow is thus a measure of the passive dissipation of the high-energy state. Under conditions where there are no co-factors of phosphorylation present the residual rate of electron flow is called the basal rate, and reflects the passive permeability of the thylakoid membranes to protons. Basal electron flow is a function of the pH of the chloroplast suspending medium (see Good

et al., 1966 for review), being maximal at about pH 8 and minimal at pH 7. Since this means that the passive proton leak is maximal at pH 8 it would be expected that the rate of decay of Δ 518 should be fastest at this pH, and this is seen in fig.34. In addition the decay of Δ 518 should be slowest at pH 7, and this is also seen. A recent publication by Portis et al. (1975) suggests that the increase in basal electron flow is due to a pH-dependent conformational change in the ATPase which makes it more permeable to protons at high pH. The decay of Δ 518 also accelerates at low pH, and in the absence of data in the literature on basal electron flow below pH 6 one may speculate that it increases at these low pH's.

The pH dependent acceleration of the rate of decay of Δ 518 in broken chloroplasts could to some degree account for the changes in the extent of Δ 518 which show a somewhat similar pH profile (fig.34). However other effects may come into play. One of these is the effect of pH on the loss of manganese from PSII found by Cheniae and Martin (1967). These workers measured the loss of manganese from chloroplasts suspended at different pH's at 5°C for 21h. They showed that the loss was small at pH 6.5 and much larger at acid and alkaline pH. Although these experiments used a very long incubation time they do indicate that since manganese is an essential component of the oxygen-evolving apparatus of PSII, inhibition of PSII would be expected at pH's other than pH 6.5. This effect is probably reflected in the decreased extent of Δ 518 at the extreme pH values used in the experiment shown in fig. 34.

Another factor which may contribute to changes in the extent of Δ 518 at different pH values is the thickness of the thylakoid membrane (Murakami and Packer, 1970a). The thickness of this

membrane was observed to be a function of pH, and was decreased by 25% as the pH of the suspending medium was reduced from pH 7.8 to pH 4.0. According to eq. 60, $\Delta\psi$ is a linear function of membrane thickness l, so that the observed decrease in the extent of Δ 518 as the pH of the suspending medium is lowered from pH 7 (fig. 34) may be partially accounted for by this mechanism.

The changes induced in Δ 518 in intact chloroplasts (fig. 34) are more complex than those in broken chloroplasts. The chloroplast outer membrane is permeable to protons (Gimmler et al., 1975) and changes in the pH of the suspending medium therefore affect the pH of the stroma. The pH profiles of the extent of Δ 518 in intact chloroplasts are broadly similar to those of broken chloroplasts, and thus these changes are probably due to the same mechanism of pH-dependence of the efficiency of the primary photoreactions and membrane thickness. The data on the halftimes of the decay of Δ 518 are, however, quite different for the two types of chloroplast. Based on the same model presented above for broken chloroplasts the data on the slow phase of the decay of Δ 518 (fig. 34) suggest that basal electron flow in intact chloroplasts is maximal at about pH 6.5 and minimal at pH 8.2. The difference seen here between intact and broken chloroplasts may again be a reflection of the damage sustained by the thylakoid membranes on osmotic shocking. The data on the halftime of the fast phase of Δ 518, where the halftime decreases monotonically with increasing pH, do not agree with the data on the slow phase, and suggest that in intact chloroplasts the two phases of the decay of Δ 518 may reflect different processes.

Figure 35 shows the effect of the addition of salts on Δ 518 in broken chloroplasts suspended in low-salt buffer. The effect

of salts on this absorbance change has been studied before using steady-state methods by Strichartz and Chance (1972) and by Gross and Libbey (1972). The latter authors found either ~100mM of a monovalent cation or a few mM of a divalent cation inhibits the absorbance change and suggested from their results that the addition of salts had two effects (i) to change the turbidity of the chloroplast suspension and increase the scattering at 518nm, and (ii) to inhibit the light-induced absorbance change at 518nm. The data presented in fig.35 show that the extent of Δ 518 is decreased by the addition of ~ 100mM K^+, and also that the decay is accelerated. This is in agreement with the observations of Neumann et al. (1970). The acceleration of the decay is probably due to increasing the number of ions available to equilibrate across the membrane (see Junge and Witt, 1968). The decrease in the extent of Δ 518 could be due to inhibition of the delocalisation of charge separation, but the observation that the concentration dependence of the decrease of the extent of Δ 518 is similar to that for the cation-induced increase of fluorescence yield (~70mM) indicates that these two phenomena may be linked in some way. Changes in spillover (section 1.4.5.1) are unlikely to play a part in changing the extent of Δ 518, since both photosystems are thought to contribute to Δ 518 equally (Shliephake, Junge and Witt, 1968). However, since variation in spillover is thought to involve microscopic structural changes in the thylakoid membrane, particularly in membrane thickness (section 1.4.5.1), these changes could be reflected in the extent of Δ 518.

At low concentrations of added K^+, the extent of both phases of Δ 518 and the lifetime of the fast phases are stimulated (fig.35). This is probably connected with the observations of Jagendorf and

Smith (1962) who found that it was necessary to add ~10mM of a monovalent cation or ~0.1mM of a divalent cation to chloroplasts in a salt-free medium in order to achieve good coupling. The stimulation of Δ 518 by 10mM K^+ suggests that at least the fast phase of Δ 518 in broken chloroplasts reflects the rate of leak of ions from the thylakoid.

In the experiments discussed above it is quite difficult to determine whether the two phases of the decay of Δ 518 in intact chloroplasts reflect distinct processes, or whether they reflect the same process. As mentioned earlier the analysis of the decay curves is complex because the preparations of intact chloroplasts contain a mixture of intact and broken chloroplasts. Nevertheless, the experiments with valinomycin (fig.29) indicate that it is the slow phase of Δ 518 that is sensitive to the action of this compound. Similarly the experiment of fig. 31 indicates that the slow and fast phases have different responses to CCCP, and the effect of pH on the two phases is again different (fig.34). However, the action of DCMU and nigericin on the two phases is rather similar (figs. 33 and 32) and while it could be argued that the inhibitory effect of DCMU only indicates that both phases of Δ 518 are connected with the light-reactions, the effect of nigericin suggests that the two phases reflect the same process.

3.3.7 Summary

1. The <u>in vitro</u> evidence to support the concept that Δ 518 reflects an electrical potential generated across the thylakoid membrane is good.

2. The magnitude of Δ 518 measured with intact chloroplasts is 1.6 times the magnitude of Δ 518 measured with broken chloroplasts

under comparable conditions. This could be due to either (a) the thylakoid membranes of broken chloroplasts having been made leaky on osmotically shocking intact shloroplasts or (b) the thylakoid membranes of intact chloroplasts having an intrinsic permeability to an ion which has a low activity in the stroma

3. The slower decay of $\Delta 518$ in intact chloroplasts as compared to broken chloroplasts also indicates that the equilibrium of ions across the thylakoid membrane occurs more slowly in intact chloroplasts for either of the reasons mentioned in 2.

4. The calculation made by Witt (1971) (eq. 60) which gives a value of 50mV to the membrane potential in broken chloroplasts is probably in appropriate. This membrane potential may more closely be applied to the intact chloroplast and consequently the membrane potential in broken chloroplasts may be assigned a value of about 30mV.

5. The biphasic induction of $\Delta 518$ in intact chloroplasts may reflect a second electrogenic process associated with secondary electron flow.

6. The chloroplast outer envelope appears to act as a permeability barrier to both valinomycin and CCCP.

7. The experiments with uncouplers generally indicate that $\Delta\psi$ is associated with phosphorylation.

8. The experiments with nigericin also suggest that the thylakoid membranes are more permeable to K^+ than H^+, and that the difference in permeabilities is more marked in intact chloroplasts.

9. The effect of pH on $\Delta 518$ in broken chloroplasts can be accounted for by the pH sensitivity of the rate of basal electron flow, thylakoid membrane thickness and inhibition of PSII by loss of manganese. The pH dependence of basal electron flow in intact chloroplasts is probably different compared to that of broken

chloroplasts.

10. Adding K^+ to broken chloroplasts in a low salt medium accelerates the decay of Δ 518 and decreases its extent; the latter effect may be due to a change in membrane thickness. A transient increase in the halftime of the decay of Δ 518 at low concentrations of added K^+ may be accounted for by the necessity of a low concentration of cations for good coupling.

11. These experiments generally support the concept that $\Delta\psi$ is linked to phosphorylation, although there is uncertainty as to whether both phases of the decay of Δ 518 reflect the decay of $\Delta\psi$.

Appendix I: The determination of the concentration of reduced Q from the prompt chlorophyll fluorescence induction from DCMU-blocked chloroplasts

The following theory was first given by Murata, Nishimura and Takamiya (1966), and subsequently by Lavorel (1973) and Lavergne (1973). A somewhat similar derivation was presented by Malkin and Kok (1966).

The rate of reduction of Q to Q^- in DCMU-blocked chloroplasts is given by :

$$\frac{dQ^-}{dt} = \alpha_{II} I \phi_p \qquad A1$$

Where α_{II} is the fraction of incoming photons directed to PSII, I is the incident light intensity, ϕ_p is the quantum yield of photochemistry of PSII and Q^- is the fractional concentration of reduced Q.

Now, by definition

$$\phi_p + \phi_f + \phi_{tI} + \phi_h = 1 \qquad A2$$

Where ϕ_p, ϕ_f, ϕ_{tI} and ϕ_h are the quantum yields of photochemistry, variable fluorescence, energy transfer to PSI and radiationless de-excitation respectively.

Letting $\phi_f + \phi_{tI} + \phi_h = \beta \phi_f \qquad \beta > 1$

then $\phi_p = 1 - \beta\phi_f \qquad A3$

Substituting eq.A3 into A1:

$$\frac{dQ^-}{dt} = \alpha_{II} I (1 - \beta\phi_f) \qquad A4$$

What is the value of β? From eq. A3

$$\beta = \frac{1 - \phi_p}{\phi_f}$$

Now:

$$\phi_f = \frac{k_f}{k_f + k_{tI} + k_h + k_p} \qquad \text{and} \qquad \phi_p = \frac{k_p}{k_f + k_{tI} + k_h + k_p}$$

(These expressions assume the separate model for the PSII photosynthetic unit: the same result is obtained using the uniform pigment matrix model).

$$\therefore \beta = \frac{k_f + k_{tI} + k_h}{k_f} \qquad \text{A5}$$

Thus $1/\beta$ is equal to the fluorescence yield when there is no photochemistry, i.e. when all the PSII traps are closed.

$$\therefore \frac{1}{\beta} = \phi_{vmax} \qquad \text{A6}$$

Substituting eq. A6 into eq. A4 and rearranging:

$$\frac{dQ^-}{dt} = \frac{\alpha_{II} I}{\phi_{vmax}} \left(\phi_{vmax} - \phi_v(t) \right)$$

$$\therefore [Q^-] = \frac{\alpha_{II} I}{\phi_{vmax}} \int_0^\infty f(t)\, dt \qquad \text{A7}$$

where f(t) is defined in figure A1, and $[Q^-]$ is the total amount of Q available to be photoreduced.

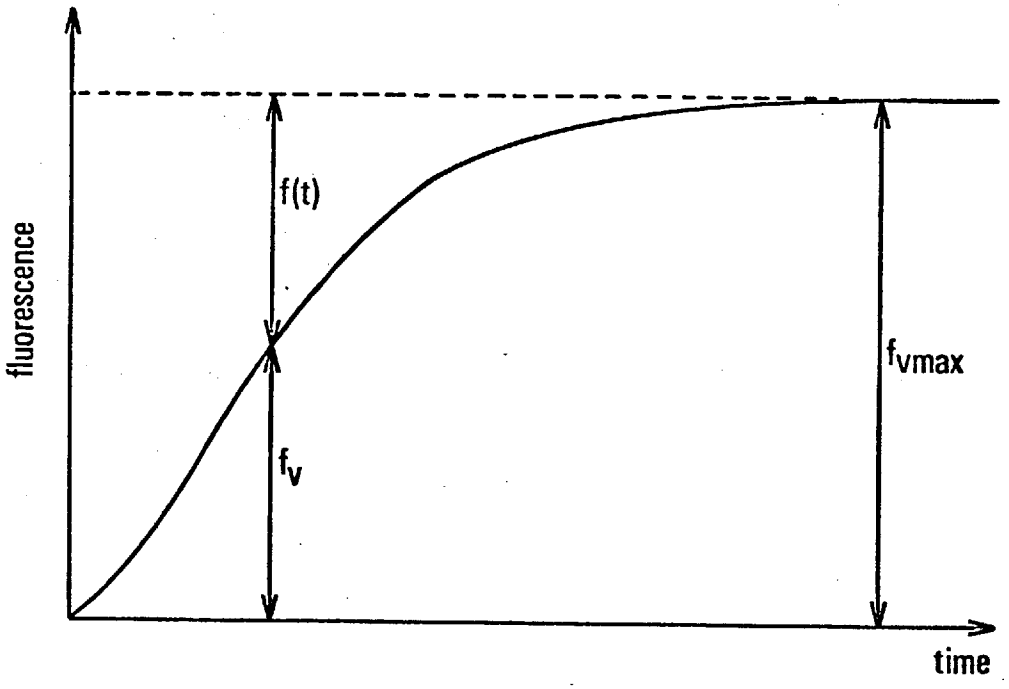


Figure A1
Schematic representation of the induction of variable fluorescence from DCMU-blocked chloroplasts showing the definition of f(t).

Appendix II: Derivation of the relationship between variable chlorophyll fluorescence yield, the concentration of closed PSII traps and the probability of exciton transfer between PSII photosynthetic units.

The following derivation is due to Joliot and Joliot (1964)

Consider that each photosynthetic unit contains one trap, and that at any time a fraction Q of all the traps are open and 1 - Q are closed. If a photon is absorbed by a unit with an open trap then it is trapped with 100% efficiency ('perfect trapping' assumption). If a photon is absorbed by a unit with a closed trap then it can either (a) be transferred to a neighbouring unit with probability p or (b) be emitted as fluorescence with probability (1 - p). The fate of a single photon may be followed schematically in fig. A2, where the probabilities that the photon will follow any particular path are given. The fluorescence yield is the total probability of fluorescence from all units, i.e.:

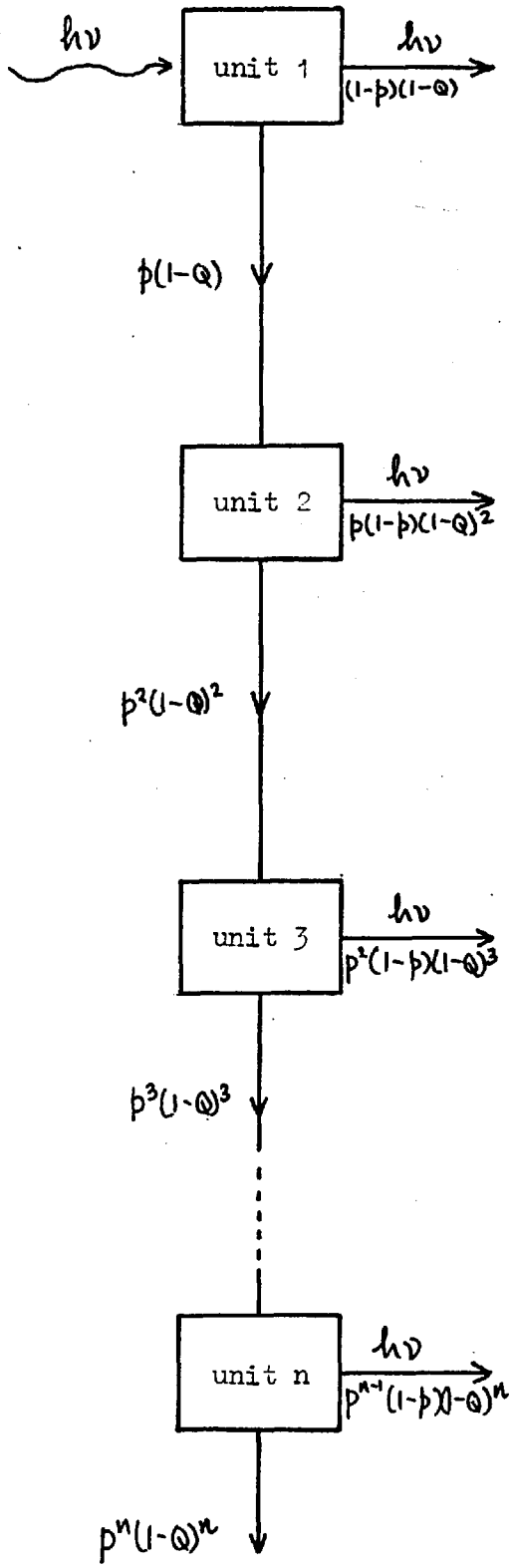
$$\phi_v = (1-p)(1-Q) + p(1-Q)(1-p)(1-Q) + p^2(1-Q)^2(1-p)(1-Q)... \quad A8$$

$$= (1-p)(1-Q) \sum_{n=1}^{\infty} p^n(1-Q)^n$$

$$\phi_v = \frac{(1-p)(1-Q)}{1-p(1-Q)} \quad A9$$

This expression gives a relationship between the variable fluorescence yield ϕ_v, the fraction of closed PSII traps (1-Q) and the probability of energy transfer between PSII units p. The fraction of closed traps at any time may be calculated from the fluorescence induction by the method given in Appendix I.

Eq. A9 is most conveniently represented by a plot of ϕ_v vs (1-Q). When p = 0 this plot is linear; when $p > 0$ it is curved, and the degree of curveature allows an estimate of p.



EVENT	PROBABILITY
unit 1 closed	$(1 - Q)$
transfer to unit 2	$p(1 - Q)$
fluorescence from unit 1	$(1 - p)(1 - Q)$
unit 2 closed	$(1 - Q)$
transfer to unit 3	$p^2(1 - Q)^2$
fluorescence from unit 2	$p(1 - p)(1 - Q)^2$
unit 3 closed	$(1 - Q)$
transfer to unit 4	$p^3(1 - Q)^3$
fluorescence from unit 3	$p^2(1 - p)(1 - Q)^3$
⋮	⋮
unit n closed	$(1 - Q)$
transfer to unit n+1	$p^n(1 - Q)^n$
fluorescence from unit n	$p^{n-1}(1 - p)(1 - Q)^n$

Figure A2

Schematic diagram representing the probabilities of each of the pathways that are available to a single photon. The photosynthetic units are assumed to have one perfectly efficient trap each

Appendix III: Derivation of the relationship between variable chlorophyll fluorescence yield, the concentration of closed PSII traps, the probability of exciton transfer between PSII photosynthetic units and the probability of exciton transfer out of PSII

The following derivation is similar to, and includes the same assumptions as, that of Joliot and Joliot (1964) given in Appendix II, but includes another parameter, the probability that while in a PSII photosynthetic unit with a closed trap an exciton will be transferred out of PSII. This could occur by transfer to PSI ('spillover') or through radiationless de-excitation, but in either case the transfer out of PSII is in competition with fluorescence and transfer to another PSII unit. The probability of transfer out of PSII will be denoted t.

In the same way as done in Appendix II, the fluorescence yield is derived from the sum of the probabilities of fluorescence from each unit, i.e.

$$\phi_v = (1-Q)(1-p-t) + p(1-Q)(1-Q)(1-p-t) + p^2(1-Q)^2(1-Q)(1-p-t)....$$

$$= (1-Q)(1-p-t) \sum_{n=0}^{\infty} p^n(1-Q)^n$$

$$\phi_v = \frac{(1-p-t)(1-Q)}{1 - p(1-Q)} \qquad \text{A10}$$

Since t is a constant throughout the induction, eq. A10 predicts (for a given value of p) the same shape of ϕ_v vs (1 - Q) plot as eq. A9: however, if $t \neq 0$, the fluorescence yield will be depressed to the same extent at all values of (1 - Q).

Appendix IV: The relationship between the sigmoidicity of the PSII chlorophyll fluorescence induction in a DCMU-blocked system and the ratio $\phi_{vmax} / \phi_{tmax}$

The following derivation is due to Paillotin (1974); the result has also been obtained independently by Butler and Kitajima (1975a).

Consider a group of photosynthetic units containing a total of r traps of which a fraction i are closed, and over which excitation energy may migrate freely. Then assuming that both the f_o level of fluorescence and variable fluorescence come from PSII only, eq. 18 gives:

$$\phi_o = \frac{k_p}{K}$$

where $K = k_f + k_h + k_{EI}$

$$\phi_{tmax} = \frac{k_p}{K + rk_p}$$

and $$\phi_i = \frac{k_p}{K + ik_p}$$

The expression for ϕ_i may be rewritten as

$$\phi_i = \frac{k_f}{K+rk_p} \cdot \frac{r(K+rk_p)(K+rk_p)}{rK(K+rk_p) + irk_p(K+rk_p)}$$

$$= \frac{k_f / K+rk_p}{\frac{K}{K+rk_p} - \frac{i}{r}\left[\frac{-rk_p}{K+rk_p}\right]}$$

$$= \frac{\frac{K}{K+rk_p} \cdot \frac{k_f}{K}}{\frac{K}{K+rk_p} - \frac{i}{r}\left[\frac{K}{K+rk_p} - 1\right]}$$

$$\therefore \quad \phi_i = \frac{R_p \phi_o}{R_p - \frac{i}{r}(R_p - 1)} \qquad \text{where } R_p = \frac{\phi_{tmax}}{\phi_o}$$

Therefore the expression for variable fluorescence becomes:

$$\frac{\Phi_i - \Phi_o}{\Phi_{tmax} - \Phi_o} = \frac{\dfrac{R_p \Phi_o}{R_p - \frac{i}{r}(R_p - 1)} - \Phi_o}{R_p \Phi_o - \Phi_o}$$

$$= \frac{(1-p)(1-Q)}{1 - p(1-Q)}$$

where $(1 - Q) = i/r$ and $p = 1 - \frac{1}{R_p}$.

This expression is identical to the one derived by Joliot and Joliot (1964) (Appendix II, eq. A9) except that now

$$p = 1 - \frac{1}{R_p} \qquad \text{A11}$$

$$\text{i.e.} \quad p = \phi_{vmax} / \phi_{tmax} \qquad \text{A12}$$

p will thus be a function of the relative magnitudes of the rate constants in the expressions for ϕ_{vmax} and ϕ_{tmax}, and may be increased by, for example, decreasing k_{tI}. In this model of free excitation migration the value of p, and hence the sigmoidicity of the fluorescence induction reflects the competition for excitation capture by the reaction centre, and deactivation by other processes. The relation between ϕ_v and $(1 - Q)$ will become more linear when $p \rightarrow 0$, i.e. when the probability of photochemical excitation capture becomes small compared to the probability of deactivation by other processes.

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